ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2023 May 14; 29(18): 2704-2887

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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The *WJG* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports, Index Medicus, MEDLINE, PubMed, PubMed Central, Scopus, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJG* as 5.374; IF without journal self cites: 5.187; 5-year IF: 5.715; Journal Citation Indicator: 0.84; Ranking: 31 among 93 journals in gastroenterology and hepatology; and Quartile category: Q2. The *WJG*'s CiteScore for 2021 is 8.1 and Scopus CiteScore rank 2021: Gastroenterology is 18/149.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Hua-Ge Yu*; **Production Department Director:** *Xu Guo*; **Editorial Office Director:** *Jia-Ru Fan.*

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

PUBLICATION DATE
May 14, 2023

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https://www.wjgnet.com/bpg/gerinfo/242

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World J Gastroenterol 2023 May 14; 29(18): 2704-2716

DOI: 10.3748/wjg.v29.i18.2704

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Key elements determining the intestinal region-specific environment of enteric neurons in type 1 diabetes

Mária Bagyánszki, Nikolett Bódi

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Baccari MC, Italy; Castelucci P, Brazil; Sahin Y, Turkey

Received: December 15, 2022
Peer-review started: December 15, 2022
First decision: March 15, 2023
Revised: March 28, 2023
Accepted: April 17, 2023
Article in press: April 17, 2023
Published online: May 14, 2023

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Abstract

Diabetes, as a metabolic disorder, is accompanied with several gastrointestinal (GI) symptoms, like abdominal pain, gastroparesis, diarrhoea or constipation. Serious and complex enteric nervous system damage is confirmed in the background of these diabetic motility complaints. The anatomical length of the GI tract, as well as genetic, developmental, structural and functional differences between its segments contribute to the distinct, intestinal region-specific effects of hyperglycemia. These observations support and highlight the importance of a regional approach in diabetes-related enteric neuropathy. Intestinal large and microvessels are essential for the blood supply of enteric ganglia. Bidirectional morpho-functional linkage exists between enteric neurons and enteroglia, however, there is also a reciprocal communication between enteric neurons and immune cells on which intestinal microbial composition has crucial influence. From this point of view, it is more appropriate to say that enteric neurons partake in multidirectional communication and interact with these key players of the intestinal wall. These interplays may differ from segment to segment, thus, the microenvironment of enteric neurons could be considered strictly regional. The goal of this review is to summarize the main tissue components and molecular factors, such as enteric glia cells, interstitial cells of Cajal, gut vasculature, intestinal epithelium, gut microbiota, immune cells, enteroendocrine cells, pro-oxidants, antioxidant molecules and extracellular matrix, which create and determine a gut region-dependent neuronal environment in diabetes.

Key Words: Enteric neurons; Neuronal environment; Gut region specificity; Type 1 diabetes, Hyperglycemia; Microbiota-gut interactions

Core Tip: Diabetes-related intestinal motility disturbances result from multifactorial damage to the enteric nervous system. However, the diversity of the neuronal environment in different gut segments basically determines the regionality of diabetic enteric neuropathy. Therefore, in this review, we highlight the role of enteric glial cells, gut circulation, intestinal epithelium, gut microbiota, immune and enteroendocrine cells, pro-oxidants, antioxidant defence and extracellular matrix, which have great impact on the formation and maintenance of a region-specific enteric neuronal environment in diabetes.

Citation: Bagyánszki M, Bódi N. Key elements determining the intestinal region-specific environment of enteric neurons in type 1 diabetes. *World J Gastroenterol* 2023; 29(18): 2704-2716
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2704.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2704

INTRODUCTION

In the middle of last century, the general belief was that the neurons within the intestinal wall are parasympathetic neurons[1]. In recent decades, it has become evident that the neurons and glia cells of the gastrointestinal (GI) tract form a third, unique division of the nervous system besides the sympathetic and parasympathetic divisions[2]. The enteric nervous system (ENS) can function independently from the rest of the nervous system and at the same time are in a close, bidirectional connection with it [2,3]. The types and the proportion of enteric neurons were characterized by their morphology, neurochemical code, function and intestinal location in different species[1,4,5]. Nowadays specialized high-throughput "omics" technologies like single cell RNA sequencing even combined with spatially barcoded RNA sequencing can confirm and supplement previous data[6,7].

There is a large body of data on the structure and function of the ENS in physiological state, and it is clear that many pathological conditions strongly affect the enteric plexuses. Since the enteric plexuses are embedded in the histological layers of the intestinal wall (Figure 1), the projections of neurons and glia cells weave through the cross-section of the entire intestine, and because of the lack of blood-brain barrier in the periphery, the role of the environment surrounding the enteric ganglia and neuronal projections is increasingly evident in both health and diseases[2,8].

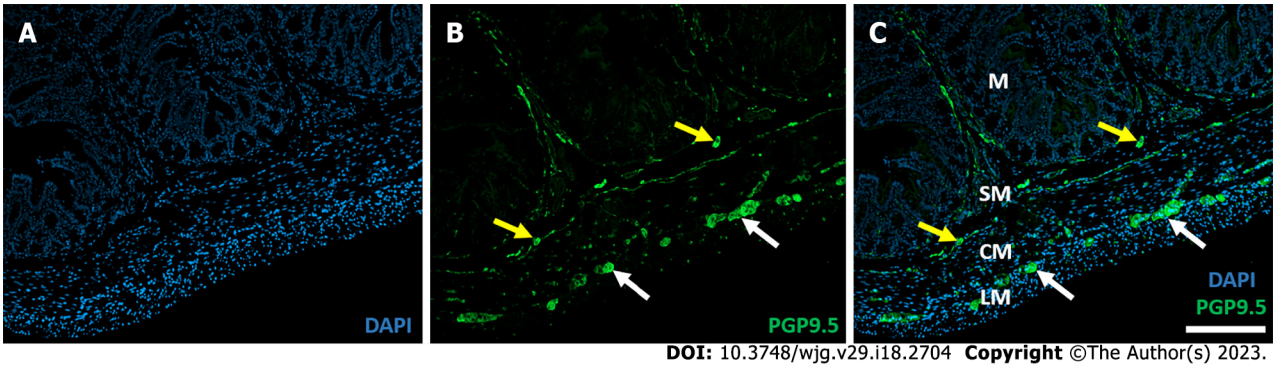
In this review, we provide a brief overview of the effects of type 1 diabetes (T1D) on the intestinal region-specific enteric neuronal environment. Unfortunately, the incidence of T1D is increasing and this incurable disease causes severe GI symptoms[8]. Chronic hyperglycemia influences the structural and functional features of the enteric neurons[9,10], it could change neurochemical code or even can cause neuronal cell death and thus lead to enteric neuropathy described by others and in our former review [11-13]. Hyperglycemia-related enteric neuropathy shows gut-region specific alterations. Therefore, the aim of this paper is to review the main environmental factors (Figure 2) in the intestinal tube from enteric glia cells (EGCs) to the luminal microbiota, which can play a crucial role in the region-specific damage of enteric neurons in the diabetic state. Some key factors like gut microbiota[14-16], GI immune [17-19] or epithelial cells[20-24] are highly emphasized in several papers, so here these are briefly summarized, while other, also critical components of the neuronal environment [*e.g.* EGCs, intestinal vasculature, pro-oxidant/antioxidant balance and extracellular matrix (ECM) molecules], are discussed in more detail.

EGCs AND INTERSTITIAL CELLS OF CAJAL

EGCs are not only supporting their neighboring neurons, but also actively regulate GI barrier function, immune homeostasis, or gut motility[25,26]. They directly interact with numerous cells in the gut wall, like enterocytes, immune cells, muscle cells, enteric neurons and vasculature, and these cross-talks influence their survival and functions in different intestinal layers and gut segments in health and disease[27].

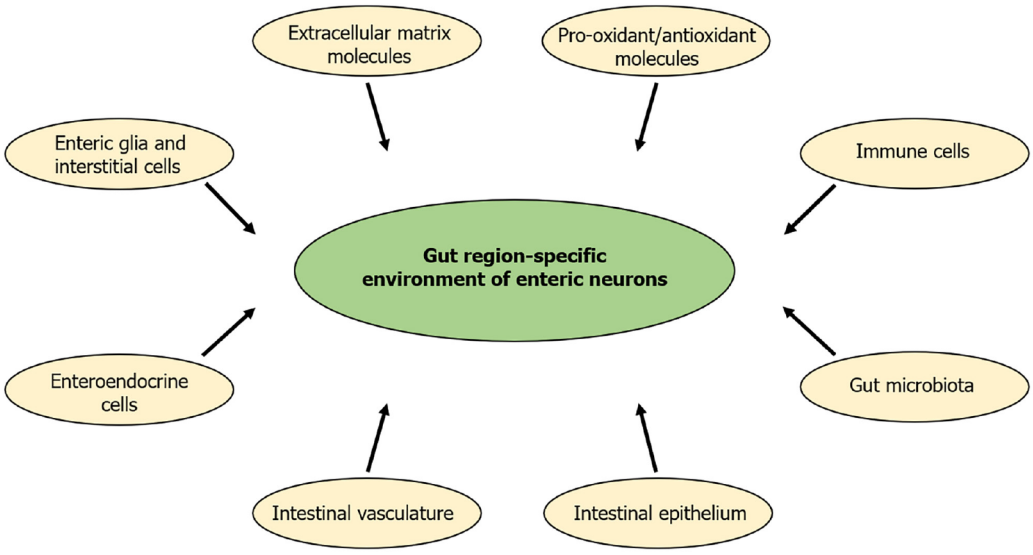
Based on cellular morphology and location within different anatomical layers, EGC subtypes are classified into mucosal, intramuscular, submucosal and myenteric glia cells[28,29]. Glial fibrillary acidic protein (GFAP), S100ß and Sox10 are among the main glial markers, but expression patterns of different EGCs inside and outside the ganglia can be varied and reflect dynamic gene regulation[30]. In summary, the variety of EGCs, as functional heterogeneity and phenotypic plasticity, fundamentally determine the cellular microenvironment within the gut wall[31,32].

Besides gut layer-dependent diversity, intestinal regional heterogeneity of EGCs is also demonstrated among GI segments. Unique developmental patterns derived from different enteric precursors and specialized functions of EGCs are associated with different GI regions (*e.g.* esophagus, stomach or intestine) and result in local environmental properties[32,33]. Different protein expression and transcrip-



DOI: 10.3748/wjg.v29.i18.2704

Figure 1 Representative fluorescent micrograph of a paraffin section of intestinal wall from control rat colon after Protein gene product 9.5 immunohistochemistry. Protein gene product 9.5 (PGP9.5) (green) was used as a pan-neuronal marker to label neuronal elements and 4',6-diamidino-2-phenylindole (DAPI) (blue) labelled nuclei. A: DAPI; B: PGP9.5; C: DAPI + PGP9.5. M: mucosal layer, SM: Submucosal layer; CM: Circular muscle; LM: Longitudinal muscle; White arrows: Myenteric ganglia, Yellow arrows: Submucous ganglia; PGP9.5: Protein gene product 9.5; DAPI: 4',6-diamidino-2-phenylindole; Scale bar: 200 μm.



DOI: 10.3748/wjg.v29.i18.2704

Figure 2 Main elements determining the gut region-specific neuronal microenvironment. Enteric glia cells, interstitial cells of Cajal, intestinal vasculature and epithelium, enteroendocrine cells, intestinal microbiota and immune cells, balance of pro-oxidants and antioxidants and extracellular matrix molecules create and determine a strictly regional environment of enteric neurons in diabetes.

tional profiles of myenteric glia cells have observed in mouse ileum and colon[31,34]. EGCs of myenteric ganglia displayed region-dependent responses to neuromodulators and glial regulation of gut contractility was also region- and pathway-specific in the duodenum and colon[35].

Development and function of EGCs and enteric neurons are in close interdependence[36]. Different neurotransmitters can activate EGCs and glial derived neurotrophic factors are crucial for neuronal survival and maintenance[27,37]. EGCs could also act as a critical link in the communication of enteric nervous and immune systems through the modulation of macrophages[38]. Because of the close neuron-glia relationship, it would be beneficial to investigate the involvement of EGCs in diabetes in addition to gut region-specific diabetic neuronal damage[39].

In the duodenum of type 2 diabetic mice with high-fat diet, a decline in the mucosa-associated glial network density was observed, however, neither the glial density and ultrastructure nor the expression of S100ß, Sox10 and GFAP markers were changed in the EGCs of myenteric ganglia[40]. Meanwhile, in a distal direction, an intense reduction in the number of both the enteric neurons and S100-immunoreactive glia cells was seen in diabetic rat jejunum[41]. Expression of GFAP and neurotrophins, like glia cell-derived neurotrophic factor (GDNF) and neurotrophin-3 were decreased in the colon of diabetic rats [42]. Loss of enteric neurons and progressive decrease in GDNF expression was demonstrated with the course of diabetic state both in proximal and distal colon of Sprague-Dawley diabetic rats. Moreover, reduced Akt phosphorylation also accompanied these changes[43]. Also, hyperglycemia stimulated EGC apoptosis in culture by repressing the PI3K/Akt molecular pathway[44]. The down-regulation of

the PI3K/Akt pathway, an important mediator of neuronal survival, is heavily involved in the diabetic damage of enteric neurons[45,46].

Interstitial cells of Cajal (ICCs) are pacemaker cells in GI motility that generate spontaneous and rhythmic slow waves to promote the spontaneous contractions of smooth muscles[47]. The delayed gastric emptying both in diabetic patients and diabetic animal models is associated with ICC depletion [10,48]. Furthermore, damage of ICCs contributes to impaired motility in other GI regions causing constipation[49,50].

INTESTINAL VASCULATURE

Blood vessels enmeshing the small and large intestine are important in nutrient transport and also responsible in supplying enteric cells. Macro- and microvascular anatomy of the GI tract fundamentally determine its regionality. Extramural circulation of the duodenum arises from the coeliac trunk, the jejunum and ileum are supplied by branches of superior mesenteric artery, while different parts of large intestine are supplied by the superior or inferior mesenteric arteries[51]. The impairment of large mesenteric vessels has been described in T1D[52], and substantial heterogeneity of endothelial dysfunction of different large arteries has been observed in a type 2 diabetic animal model[53]. Besides the variability of diabetic macroangiopathy, the impact of diabetes on the intestinal microvasculature can also be region-dependent[54-56].

Investigation of small capillaries in the close vicinity of myenteric ganglia revealed their different susceptibility to diabetic damage along the duodenum-ileum-colon axis[54]. Structural changes such as thickening of the endothelial basement membrane, caveolar hypertrophy and tight junction opening were confirmed in the ileum and colon, whereas only junctional alterations were visible in the duodenal capillaries. In addition, a severely impaired regulation of vascular permeability was shown in ileal and colonic capillaries, while an accelerated, but well-balanced albumin transport was indicated in the duodenum. Immediate insulin treatment prevented most of the diabetes-related changes of the capillary endothelium in the ileum, but not in the colon[54]. Increased thickening of arteriolar wall representing microangiopathy in colonic submucosal vessels was also shown in diabetic patients[56].

Naturally, distinct degrees of capillary damage in different gut segments strongly determine a segment-specific cellular environment and contribute to the diabetic fate of the cells they supply[8]. Close interaction between the capillary endothelial cells and migrating neural crest-derived cells has already been observed in intestinal neurovascular development and has an important role in creating a favorable neuronal microenvironment[57]. Therefore, the diabetes-related regional capillary impairments may greatly contribute to region-dependent enteric neuropathy in T1D[8].

However, not only the structural complications in the vascular system, but also the circulating microparticles can impair the endothelial function in diabetes[58]. Different gut segments feature their distinct microbial compositions and metabolites. Imbalance in the microbial composition accompanying diabetes results in changes of metabolites production, like short-chain fatty acids, bile acids, or tryptophan catabolites[59,60]. Enhancement of gut permeability related to dysbiosis may allow not only the release of different metabolites and endotoxin but also bacterial translocation from the gut to the venous circulation. These elements as integral mediators significantly contribute to vascular inflammation and immune activation[59-62].

INTESTINAL EPITHELIUM

The lining of the GI tract is directly exposed to an ever-changing environment. This single layer of epithelial cells is crucial for preserving gut homeostasis and functions both as barrier and channel for the crosstalk between the GI immune cells and microbiota[23,63].

Epithelial tight junctions are the key components of the physical intestinal barrier along the GI tract [20]. Altered barrier function of enterocytes and colonocytes leads to several pathologic conditions, including obesity or diabetes[20,21,23]. The chronic hyperglycemia-related breakdown of barrier integrity leads to the systemic influx of microbial products and an enhanced incidence of enteric infection[64].

The intestinal epithelium also has an immunological role, as it contains pattern recognition receptors, such as the Toll-like receptors (TLRs)[20]. Recently, several studies demonstrated the expression of TLR4 in metabolic diseases[65-67]. When sensing microbial lipopolysaccharides of Gram-negative bacteria, TLR4 can activate pro-inflammatory pathways in the GI tract[16,22], thus TLRs may play a crucial role in diabetic enteropathy[65]. TLR4 not only affects ENS function, but also modulates neuro-immune interactions by mediating the effects of the intestinal microbiota[65].

GUT MICROBIOTA

In the last two decades it has become clear that the imbalance of microbial species due to a reduction in microbial diversity, known as dysbiosis, is associated with several pathological conditions like autoimmune diseases[68], cancers[69], arteriosclerosis[70] depression[71,72], neurodegenerative diseases[73], obesity or diabetes[74-76]. Dysbiosis contribute to the formation of a proinflammatory milieu and gut leakiness[77,78].

Decreased microbiota diversity has been observed in T1D. At the phyla level, the proportion of *Firmicutes* decreased in patients compared to the healthy individual group, while *Bacteroidetes* abundance increased[16,79].

It is also obvious that the composition of microbiota and the number of microbes is different along the GI tract and each segment contains unique microorganism communities[80,81]. Unfortunately, only a very few studies performed longitudinal comparisons, but results showed that the mode and severity of dysbiosis has also been distinct in different gut segments[82-85]. Besides a longitudinal variability, a horizontal gradient also exists in the gut, with oxygen, redox and mucus gradients from the mucosal surface to the lumen[80] and these variables can contribute to the differences of luminal and mucosal microbiota both in health and disease[83,84,86].

By now, a sufficient amount of evidence has been gathered which show that probiotics have a beneficial influence on diabetes-related dysbiosis[78], and a plethora of studies investigate the effects of prebiotics, synbiotics and fecal microbiota transplantation on hyperglycemia and other diabetes-associated symptoms. Among others, *Roseburia intestinalis, Lactobacillus casei, Akkermansia muciniphila* and *Bacteroides fragilis* have been shown to ameliorate glucose metabolism and insulin sensitivity[75]. In the last 20 years, considerable progress has been made and the intestinal microbiota most certainly represents a promising target for T1D prevention and therapy; however, numerous unresolved concerns require further in-depth investigation.

GI IMMUNITY

The GI tract is the largest immune organ in vertebrates, where the intestinal homeostasis is determined by the gut microbiota, intestinal epithelium and host immunity[21,87].

The complex and enormous amount of information available about the GI immune system is summarized in other reviews[17-19], here we would like to highlight only one aspect. In earlier studies, the GI immune system has been examined as small *vs* large intestine, based on obvious differences in structure and function. Recently it has become apparent that the immunological niches of the GI tract differ between more refined functional compartments, making it necessary to study them separately to understand the consequences on intestinal immune homeostasis[88].

Based on the review of Brown and Esterházy[88], the GI tube can be divided into the following five main parts: Proximal small intestine, gut-draining lymph nodes, distal small intestine, large intestine and mesentery. Each intestinal niche is influenced by a combination of intrinsic tissue properties, extrinsic environmental signals, and immune cell composition.

It would be beneficial if therapies would take into account the regionally different susceptibility of the GI tract to infections and diseases.

ENTEROENDOCRINE CELLS

Enteroendocrine cells (EECs) not only play a role in humoral processes but also act as sensory cells in the GI mucosa next to the neurons and immune cells[89]. Therefore, microbial metabolites could stimulate or suppress hormone secretion by EECs, while endocrine and paracrine factors regulate GI functions and affect several metabolic processes in the body[90]. The diversity of EECs prompts the introduction of a new classification scheme. Earlier, EECs were classified based on producing a single hormone, but in the last decade it was shown that most EECs contain multiple hormones. Several hormones, like secretin and serotonin, are in separate storage vesicles at subcellular level[91]. The hormones produced by EECs might have a big potential in the future as novel microbiota-based therapies to alter metabolically active hormone levels, similarly to the use of the anorectic gut hormone, glucagon-like peptide 1, in the treatment of obesity and type 2 diabetes[90].

It is well established that EEC composition and proportion is different along the GI tract[92,93]. A recently published paper by Martin *et al*[93] has indicated that regional differences in nutrient sensing capability exist in mouse EECs. Colonic EECs has been shown to be more sensitive to glucose, while duodenal EECs to fructose and sucrose.

PRO-OXIDANTS AND ANTIOXIDANTS

The intestinal redox state is critical in maintaining gut homeostasis and functional regulation. The maintenance of this delicate balance in redox state is influenced by the gut microbiota, immune cells and epithelium, which can all produce and respond to redox signals[94]. Numerous genera of bacteria has been identified as biomarkers for gut redox state[95]. Reactive sulfur species-producing bacterial families enhance the host's antioxidant capacity[96], however, sulfur metabolism can be distracted by opportunistic pathogens[94]. Large differences can be observed in different GI segments regarding the quantity and composition of microbiota, intestinal pH or partial pressure of oxygen within the luminal-facing epithelium[80]. There is a richer and more diverse microbial community and deeper anaerobic state from proximal to distal parts of the gut, therefore, it is not surprising that oxidant and antioxidant mechanisms have also been linked to strict region-dependency in health and disease[97]. Foods containing numerous antioxidants, such as vitamins, carotenoids, flavonoids, polyphenols, bioactive peptides or others, however, also include a lot of pro-oxidant molecules[98-100] and all of these can modulate the composition of microbial communities[101]. Consumed and endogenously produced antioxidants have varied strategies at different levels to maintain optimal redox balance[102,103]. Still, the accumulation of reactive oxygen species and/or decrease in antioxidant defence contribute to serious imbalance of intestinal pro-oxidant/antioxidant milieu[104].

Higher mucosal vitamin E and carotenoid concentration, higher total antioxidant activity, superoxide dismutase and catalase activity, as well as glutathione level were observed in the duodenum compared to the ileum and colon of different animal species[105-107]. The presence of probiotic *Lactobacillus* species also reflects a highly beneficial cellular environment in the duodenum[108]. Moreover, in diabetic rats, an increased abundance of the genus *Lactobacillus* has been observed relative to controls [83], which can result in enhanced antioxidant capacity[109]. While no significant changes in peroxynitrite production has been observed, a robust increase of metallothionein 2 and elevated glutathione level has been found in the duodenum of diabetic rats[110], which may contribute to cell survival in this particular gut segments.

In contrast to the duodenum, diabetes increased lipid peroxidation and catalase activity, as well as the percentage of nitrotyrosine-immunoreactive myenteric neurons in the jejunum[111]. Decreased superoxide dismutase and increased myeloperoxidase enzyme concentrations were also demonstrated in the diabetic jejunum[112]. Enhanced lipid peroxidation and protein oxidation accompanied with significantly lower superoxide dismutase levels, catalase and glutathione levels were also observed in the diabetic ileum[113,114]. However, a great increase in the activity of the endogenous heme oxygenase system was shown in myenteric neurons of diabetic ileum[115], maybe as an effect of microbial changes [116]. In the colon of diabetic rats, the doubled peroxynitrite level, reduced superoxide dismutase activity and the induction of the endogenous heme oxigenase system emphasizes the observation that distal gut segments have greater susceptibility to the diabetic oxidative environment, which is in correlation with diabetic neuronal cell loss[97,110,115].

ECM

ECM structures composed of various proteins and polysaccharides are essential in the maintenance and well-regulated remodeling of tissues and have a key role in regulating different cellular events, like cell proliferation, differentiation or migration[117-119]. In the gut, numerous cells (*e.g.* epithelial, mesenchymal, stem cells) participate in the production of matrix molecules, and their precise composition is indispensable for the optimal cellular environment and normal intestinal function. Sensing the stiffness or the porosity of the ECM through specific receptors such as integrins, intestinal cells can change their intracellular state or dynamics[120,121].

Diabetes-related alterations of ECM is demonstrated in all parts of the gut, but with different extent in different regions and intestinal layers. In streptozotocin-induced diabetic rats, a significant increase in the amount of laminin-1 and fibronectin was observed in the small intestine by Western blotting and immunohistochemistry, and the strong labelling was restricted mainly to the intestinal smooth muscle and serous layers[122]. These hyperglycemia-mediated ECM accumulation was reversed by insulin treatment[122]. Additionally, in the distal colon, a marked increase of type 1 collagen was detected with no changes in type 3 and 4 collagen expression[123]. Besides of the well-marked pockets of collagen among the smooth muscle cells, formation of advanced glycation end-products was also observed in diabetic rats. Type 1 collagen deposits and glycation increase stiffness of the diabetic colon muscle, which contribute to limited colonic function[123]. There is a strict association between collagen content and mechanical properties, however, this varied in different parts of the small intestine [124]. Increased ECM deposition, as well as high levels of type 1 and 3 collagen and fibronectin mRNAs were also detected in diabetic colon mucosa[125]. The accumulation of ECM in the mucosa of the diabetic colon was associated with the deregulation of the transforming growth factor (TGF)-β1/Smad signaling pathway[125]. However, TGF-β can also influence deposition of matrix molecules by upregulating several ECM receptors[126].

Structural alterations of basement membranes as specialized ECM structures have been characterized in diabetes mellitus. Thickening of capillary basement membrane is among the first histological hallmarks of the disease. Capillaries located in gut smooth muscle in different gut segments displayed region-specific thickening of their basement membranes in T1D[54]. Additionally, significant increase in mRNA levels of different matrix scaffold proteins, like fibronectin or procollagen type 1, was observed in the aorta and mesenteric artery of type 2 diabetic Goto-Kakizaki rats[127]. Moreover, gene expression was restored in the mesenteric bed but not in the aorta using an endothelin-1 antagonist[127]. Basement membrane thickening of smooth muscle cells was also demonstrated in the small intestine[122] and colon[123]. Moreover, the basement membrane surrounding the myenteric ganglia was also thickened in diabetic rats with strict regionality in different gut segments[128].

ECM accumulation can be due to the enhanced synthesis of matrix components, but also their decreased degradation, which in turn leads to the imbalance of ECM dynamics[129]. Matrix metalloproteinases (MMPs) and their tissue specific inhibitors (TIMPs) mainly produced by macrophages, neutrophils or epithelial cells have an essential role in tissue remodeling as a response to intestinal inflammation[130,131]. Growing molecular evidence support that these proteolytic enzymes are also targets of diabetic damage. In the diabetic ileum, MMP9 expression decreased in myenteric ganglia, capillary endothelial cells and intestinal smooth muscle cells, while these values did not change in the duodenum, which is in perfect agreement with the regionally distinct thickening of the ganglionic basement membrane. However, a specific, but great induction was revealed in MMP9 and TIMP1 at the mRNA level both in duodenum and ileum homogenates of diabetics[128]. Increased early expression of MMP2 and MMP9 mRNAs and MMP1 later on was also demonstrated in the diabetic colon mucosa. On the other hand, increased TIMP1 and TIMP2 expression could be the result of decreased MMPs degrading activities here[125].

CONCLUSION

Because of its various functions, as food intake, mechanical and chemical breakdown, motility, absorption, regulation of blood flow, secretion, water reabsorption and immune functions, the GI tract has unique features. It has three detecting systems, which are more extensive than those of any other organ: (1) The ENS contains as many neurons as the spinal cord and different subpopulations of the EGCs cover all histological layers of the intestinal wall. Intrinsic primary neurons, interneurons and motoneurons can form local reflex circuits in the gut wall[1,2]; (2) There are more than 20 hormones produced by several types of EECs[89]; and (3) The GI tract is the largest immune organ with the cc. 70-80% of the body's immune cells[87,132].

In addition to the three systems listed above, other essential factors such as intestinal epithelial barrier, microcirculation of the gut wall, pro-oxidant/antioxidant milieu, ECM components and gut microbiota also play a crucial role in the formation of the enteric neuronal environment in both physiological and pathological states. Results demonstrated a clear relationship between intestinal microorganisms and the occurrence of T1D, but the correlation or causality remains an important question for several reasons. Altered gut microbiota-mediated redox imbalance and changes in cellular cross-talks may contribute to enteric neuropathy and also influence the function of gut-brain axis[15].

Considering these properties and the size of the GI tract, it is not surprising that it shows profound functional and structural differences along its length. When planning experiments, the gut should be regarded as a multiple organ, and in the case of illness, the applied therapies should take into account the intestinal segment-specific effects[88].

ACKNOWLEDGEMENTS

The authors sincerely thank to their PhD students, Abigél Egyed-Kolumbán, Afnan AL Doghmi, Bence Pál Barta and Benita Onhausz for useful comments on draft of this manuscript.

FOOTNOTES

Author contributions: Bagyánszki M and Bódi N wrote the original draft, they also wrote the review and editing, performed the visualization; all the authors have read and approve the final manuscript.

Supported by Hungarian NKFIH Fund Project (N.B.), No. FK131789; János Bolyai Research Scholarship of The Hungarian Academy of Sciences (N.B.); and New National Excellence Program of The Ministry for Innovation and Technology from The Source of The National Research, Development and Innovation Fund (N.B.); No. ÚNKP-22-5.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: Hungary

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S-Editor: Fan JR
L-Editor: A
P-Editor: Yu HG

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 May 14; 29(18): 2717-2732

DOI: 10.3748/wjg.v29.i18.2717

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Paediatric gastrointestinal endoscopy in the Asian-Pacific region: Recent advances in diagnostic and therapeutic techniques

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Cabezuelo AS, Spain; Maldonado KE, Guatemala

Received: December 29, 2022
Peer-review started: December 29, 2022
First decision: February 1, 2023
Revised: February 12, 2023
Accepted: April 14, 2023
Article in press: April 14, 2023
Published online: May 14, 2023

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Abstract

There has been a rapid expansion in the knowledge of paediatric gastroenterology over the recent decade, with a fast-growing repertoire of diagnostic techniques and management strategies for a wide spectrum of childhood gastrointestinal (GI) diseases. Paediatric GI endoscopy is a core competency every paediatric gastroenterologist should possess, and represents one of the most common procedures performed in children for both diagnostic and therapeutic purposes. Yet there remains a dearth of literature on the utility and outcomes of paediatric GI endoscopy in the Asia-Pacific region. Data on the diagnostic value of paediatric GI endoscopy would be an important aspect of discussion, with the emergence of inflammatory bowel disease (IBD) and eosinophilic GI disease as increasingly common endoscopic diagnoses. Time-based trends in paediatric GI endoscopy do point towards more IBD and gastroesophageal reflux disease-related complications being diagnosed, with a declining incidence of GI bleeding. However, the real-world diagnostic value of endoscopy in Asia must be contextualised to the region-specific prevalence of paediatric GI diseases. *Helicobacter pylori* infection, particularly that of multidrug-resistant strains, remains a highly prevalent problem in specific regions. Paediatric functional GI disorders still account for the majority of childhood GI complaints in most centres, hence the diagnostic yield of endoscopy should be critically evaluated in the absence of alarm symptoms. GI therapeutic endoscopy is also occasionally required for children with ingested foreign bodies, intestinal polyposis or oesophageal strictures requiring dilation.

Endoscopic haemostasis is a potentially life-saving skill in cases of massive GI bleeding typically from varices or peptic ulcers. Advanced endoscopic techniques such as capsule endoscopy and balloon-assisted enteroscopy have found traction, particularly in East Asian centres, as invaluable diagnostic and therapeutic tools in the management of IBD, obscure GI bleeding and intestinal polyposis. State of the art endoscopic diagnostics and therapeutics, including the use of artificial intelligence-aided endoscopy algorithms, real-time confocal laser endomicroscopy and peroral endoscopic myotomy, are expected to gain more utility in paediatrics. As paediatric gastroenterology matures as a subspecialty in Asia, it is essential current paediatric endoscopists and future trainees adhere to minimum practice standards, and keep abreast of the evolving trends in the diagnostic and therapeutic value of endoscopy. This review discusses the available published literature on the utility of paediatric GI endoscopy in Asia Pacific, with the relevant clinical outcomes.

Key Words: Endoscopy; Paediatric; Asia; Children; Gastroscopy; Colonoscopy

Core Tip: Paediatric gastrointestinal (GI) endoscopy has gained traction in Asia as an invaluable tool in the diagnosis and management of chronic GI diseases of current and emerging epidemiological importance. Yet the lack of consensus guidelines and heterogeneity in clinical practice, variability in the referral patterns, healthcare access and prevalence of diseases across the Asian continent, inevitably leads to a wide variance in outcomes for different endoscopic modalities. There is a need for comprehensive and accreditable paediatric endoscopy training in Asia, so that endoscopists adhere to a minimum practice standard and are adequately trained to apply diagnostic and therapeutic endoscopic techniques appropriately and competently.

Citation: Huang JG, Tanpowpong P. Paediatric gastrointestinal endoscopy in the Asian-Pacific region: Recent advances in diagnostic and therapeutic techniques. *World J Gastroenterol* 2023; 29(18): 2717-2732
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2717.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2717

INTRODUCTION

Gastrointestinal (GI) symptoms remain a common presenting feature of various ailments in childhood, and the advent of modern GI diagnostic and therapeutic techniques have allowed centres across Asia to utilise GI endoscopy early in the evaluation of a child with suspected GI disease. There remains a lack of consensus statements and guidelines on the utility of paediatric GI endoscopy in the Asian context, leading to a lack of consistency in clinical practice and highly variable clinical outcomes between centres [1]. The epidemiological situation in Asia is unique as rapidly evolving disease trends occur amidst changing lifestyle and environmental factors: Paediatric inflammatory bowel disease (IBD) is of rapidly emerging importance[2], yet its early diagnosis is often complicated by the relatively high prevalence of GI infections such as intestinal tuberculosis[3] and other infectious diseases in certain endemic regions. *Helicobacter pylori* (*H. pylori*) has fallen in prevalence in developed regions of Asia but remains a highly prevalent pathogen in association with peptic ulcer disease and gastric cancer in other regions[4]. There is increasing awareness and knowledge of the role of the gut-brain-axis in disorders of gut-brain interaction or functional GI disorders[5], prompting interest in the actual diagnostic yield of GI endoscopy in childhood abdominal pain syndromes. While Asia remains vastly heterogeneous in socioeconomic status, access to early endoscopy and advanced endoscopy techniques has greatly improved, and has enhanced both the diagnostics and therapeutics in various chronic paediatric GI ailments such as IBD, intestinal polyposis syndromes, varices in portal hypertension and GI strictures.

DIAGNOSTIC ENDOSCOPY

Time-based trends in paediatric endoscopy in Asia: More IBD and gastroesophageal reflux disease-related complications

Improved healthcare access and the maturation of paediatric gastroenterology as a specialty has contributed to the rapid rise in endoscopies performed in children[6]. There are few paediatric

endoscopy consortiums within Asia, with most of the published experience coming from East Asian centres. Data from the Japan Paediatric Endoscopy Society demonstrated a five-fold increase in the number of paediatric endoscopies performed in the latest survey in 2011-2016 *vs* 2000-2004, an increase in advanced endoscopies such as endoscopic retrograde cholangioscopies and balloon-assisted enteroscopies, with a slight rise in adverse events (0.25% from 0.03%) inevitably so from increased procedural complexity[7]. A retrospective review of children undergoing upper GI endoscopy at the Children's Hospital of Philadelphia (CHOP) demonstrated a rapid rise of first-time endoscopes from 107 in 1985 to 1294 in 2005[8]. Interestingly this same study also showed a decline in the proportion of GI bleeding (34% to 5%), a decline in both the overall clinical severity of cases and corresponding endoscopic and histological abnormalities, and yet a rise in the proportion of upper GI endoscopies done for abdominal pain (23% to 43%)[8]. It begets the question if centres are performing more endoscopies in otherwise healthy children, hence the need to examine the diagnostic yield of paediatric GI endoscopies and the impact on clinical management.

There are very limited publications comparing trends in paediatric endoscopy within Asia, to contrast with the aforementioned North American data. In contrast to the significant fall in oesophageal histological abnormalities detected in the CHOP study, a Japanese paediatric study demonstrated a significant rise in the proportion of erosive oesophagitis (9.8% to 18.1%) or endoscopic Barrett's oesophagus (2.5% to 9.6%) between eras 2005-2012 to 2013-2019[9]. It is postulated by the authors that this trend is related to dietary changes and the decreasing prevalence of *H. pylori* amongst the Japanese population. Published adult data from Japan[10] and Malaysia[11] also clearly demonstrates the decline in peptic ulcers and associated upper GI bleeding, with rates of gastroesophageal reflux disease (GERD) increasing on the contrary.

A single centre in Beijing shows a statistically significant rise in the number of colonoscopies performed amongst the 0-3 year age group, from the era 2005-2011 to 2012-2017 (3.0% to 14.1%, $P < 0.001$) but no significant change in diagnostic yield rates between the two eras (36.3% to 38.2%)[12]. The proportions of IBD and colonic polyps detected did not differ between the two eras. A paediatric South Korean cohort study in Busan, however did show a substantial rise in colonoscopies from 2001-2005 to 2011-2015 (200 to 746)[13], with trends in indications somewhat mirroring those in the CHOP study. The number of colonoscopies performed for abdominal pain increased from 27.5% to 43.7%, while those performed for haematochezia fell from 56.0% to 42.5%. There was an actual rise in diagnostic yield with the proportion of Crohn's disease diagnosed doubling from 13.5% (2001-2005) to 26.8% (2011-2015). This is consistent with the rapid rise in paediatric IBD incidence observed in South Korea[14] and the rest of Asia[2].

Diagnostic yield varies widely according to referral indication and regional disease prevalence

Tables 1 and 2 describe the overall diagnostic yield in upper and lower endoscopies (follow-up endoscopies excluded unless stated otherwise). The positive endoscopic yield varies substantially between centres: Between 45%-93% for upper endoscopies (Table 1) and correspondingly 43%-85% (Table 2) for lower endoscopies. The large variability in the diagnostic yields is clearly multifactorial, from varying referral patterns and referral indications, regional differences in disease prevalence and healthcare resource allocation between centres. For instance, in single-centre studies out of Malaysia[15] and India[16], abdominal pain/dyspepsia were indications for just 13.4% and 17.4% of upper GI endoscopies respectively, while upper GI bleeding and variceal surveillance were more common indications. This is in contrast to other cohorts *e.g.*, the North American CHOP[8] and the South Korean cohort[17], where abdominal pain/dyspepsia were the most common indications (43%-64% of upper GI endoscopies) and upper GI bleeding being far less prevalent. These differing trends in GI bleeding rates could be attributed to varying prevalence in *H. pylori*-associated gastroduodenal ulcer disease and upper GI haemorrhage[10], variability in healthcare access, timeliness of referrals for biliary atresia and other childhood liver diseases. Resource-scarce centres also likely prioritise allocation of endoscopy resources for GI emergencies, typically acute GI bleeding, rather than uncomplicated abdominal pain.

Childhood H. pylori infection remains a highly prevalent problem in Southeast Asia and South/West Asia

It must be emphasised that the vast Asian continent is both economically and culturally heterogenous: Peptic ulcerations and *H. pylori* still remain fairly common in certain regions. Jordanian data from 2014-2020 showed *H. pylori* accounted for 66.1% of all abnormal upper GI endoscopies in children[18]; an earlier Israeli paediatric study showed 22.5% of all upper GI endoscopies had peptic ulcerations, of which 66.3% of these were *H. pylo*ri positive[19]. The global trend of increasing antimicrobial resistance in *H. pylori*[20] hinders the implementation of eradication strategies in these regions with high *H. pylori* prevalence. Vietnam has a very high *H. pylori* prevalence rate (> 75%) associated with the highest prevalence of gastric cancer in Southeast Asia[4]. A study of 237 symptomatic Vietnamese children undergoing upper GI endoscopy for suspected *H. pylori*-associated gastroduodenal disease showed 80.6% and 71.7% of *H. pylori* isolates were resistant to clarithromycin and amoxicillin respectively[21]. These figures place Vietnam as one of the regions with the highest rates of *H. pylori* antimicrobial resistance, and emphasises the added importance of upper GI endoscopy to obtain biopsies for a culture

Table 1 Diagnostic yield of oesophagogastroduodenoscopies

Country	Year	Cohort size (*n*)	Indications (top three) (%)	Findings (%)	Ref.
China	2018-2019	2268	Abdominal pain (86.2). Vomiting (31.1). Weight loss (15.1)	62.5% abnormal. Highest yield in dysphagia	[105]
Nepal	2013-2016	270	Abdominal pain (77.3). Vomiting/reflux (8.4). Failure to thrive (7.0)	92.5% abnormal. Gastroduodenitis (28.1). Antral gastritis (18.5). Erosive gastritis (15.9)	[106]
India	2013-2016	822	Variceal surveillance (19.1). Dyspepsia (17.4). Upper GI bleed (16.5)	45.8% abnormal. Duodenal ulcers/varices most common	[16]
Israel	2014	407	Suspected coeliac disease (28.2). Abdominal pain (15.0). Persistent *H. pylori* (10.3)	59.2% abnormal. Coeliac disease (28), *H. pylori* (16.5), Crohn's disease (5.4)	[107]
Jordan	2014-2020	778	Abdominal pain (45.1). Vomiting (21.1). Weight loss (10.3)	47.2% abnormal. *H. pylori* (66.1). Coeliac disease (30.4). Eosinophilic GI disease (3.6)	[18]
Malaysia	2008-2011	231 OGD. 44 OGD and Colonoscopies	Variceal surveillance (50.0). Upper GI bleed (26.0). Abdominal pain (13.4)	79.0% abnormal	[15]
South Korea	2008-2013	554	Abdominal pain (64.1). Dysphagia (9.0). Vomiting (9.0)	88.1% abnormal. Gastritis (53.1). Esophagitis (17.7)	[17]
Thailand	2000-2002	38	Recurrent abdominal pain	45% abnormal. *H. pylori* (26.3)	[108]
United States	2002-2005	454	Recurrent abdominal pain	38.1% abnormal. Reflux esophagitis (23.0). *H. pylori* (5.0). Peptic ulcers (3.0)	[37]

OGD: Oesophagogastroduodenoscopies; *H. pylori*: *Helicobacter pylori*; GI: Gastrointestinal.

and antimicrobial sensitivity-based eradication strategy. This has implications on the feasibility of healthcare access and early endoscopy, as *H. pylori* is typically most prevalent in regions of lower socioeconomic status.

Paediatric IBD: A rapid rise in prevalence with possibly many more undiagnosed cases

While IBD was once considered uncommon in Asia, an emerging number of paediatric publications have documented its meteoric rise in prevalence[2,14] as alluded to earlier. Table 2 describes IBD detection rates between 14%-40% of all abnormal paediatric colonoscopies performed in Asia, emphasising the importance of IBD as an endoscopic diagnosis. Of note, 'non-specific colitis' accounts for a substantial 8%-27% of all abnormal colonoscopic findings across most published studies. It is highly plausible that these unspecified cases may evolve into a more definite diagnosis of IBD on follow-up investigations: These published IBD rates underestimate the true burden of IBD in Asia and may just represent the tip of the iceberg. Paediatric-onset IBD typically has less classical endoscopic features and more subtle histologic findings[22] compared to adult-onset disease, often prompting a diagnosis of 'indeterminate colitis'[23] or 'IBD-unclassified'.

Intestinal tuberculosis, while often considered as an important differential to IBD, was far less common a colonoscopic diagnosis than IBD, even in regions with high tuberculosis burden. Intestinal tuberculosis accounted for 1.5% cases *vs* 14.1% IBD and 27.0% non-specific colitis in a Mainland Chinese paediatric cohort[12]; a Kuwaiti study showed Intestinal tuberculosis in 1.2% *vs* IBD in 21.3% of children undergoing colonoscopy[24]. There is a paucity of epidemiologic data from the Indian subcontinent, where the world's tuberculosis burden is the highest. An Indian publication in 1991 described a cohort of 72 Indian children undergoing colonoscopy: Tuberculous colitis was seen in 2.7%, ulcerative colitis in 5.5% and amoebic colitis in 1.3% of cases[25]. A more recent Bangladeshi study of 332 children undergoing colonoscopy showed intestinal tuberculosis in 1.5% *vs* IBD in 6% and nonspecific colitis in 13.6%[26]. It is not detailed in the aforementioned studies how intestinal tuberculosis is reliably distinguished from IBD, and this distinction remains a diagnostic challenge especially in India. A therapeutic trial of empirical anti-tuberculous therapy is still often practised in cases of diagnostic uncertainty[3].

Paediatric eosinophilic GI disease

There is little published data on the actual prevalence of eosinophilic GI diseases (EGIDs) in Asia, although it is believed the incidence of EGIDs will rise in tandem with the rise of allergic disorders[27]. Symptoms may mimic GERD especially in infants and young children, to recurrent dyspepsia, dysphagia and/or food impaction in the older child. The diagnosis of an EGID hinges greatly on histological findings of significant tissue eosinophilia (> 15 eosinophils per high power field) in the absence of other attributable causes. The increasingly widespread empirical use of proton-pump inhibitors in children[28,29] may reverse the tissue eosinophilia in a subset of patients with acid

Table 2 Diagnostic yield of ileocolonoscopies

Country	Year	Cohort size (*n*)	Indications (top three) (%)	Findings (%)	Ref.
Australia	2001-2010	999 colonoscopies (15.0% done as follow-up)	Suspected IBD (45.0). Haematochezia (20.0). Abdominal pain (5.0)	61.0% abnormal. IBD (28.2). Polyp (3.9)	[109]
China	2005-2017	326	-	62.6% abnormal. IBD (14.1). Nonspecific colitis (27.0). Polyp (12.0)	[12]
China	2013-2016	229	Abdominal pain (35.4). Haematochezia (27.9). Crissum abscess/anal fistula (17.5)	64.2% abnormal. IBD (38.8). Polyp (27.2). Nonspecific colitis (26.5)	[110]
Hong Kong	2003-2008	79	Haematochezia (58.0). Suspected IBD (29.1)	50.6% abnormal. IBD (16.5). Polyp (29.1)	[111]
Japan	2011-2016	275	Haematochezia (75.0). Diarrhoea (13.0). Abdominal pain (2.2)	77.1% abnormal. IBD (18.5). Eosinophilic GI disease (23.0). Polyp (14.0)	[33]
Japan	2007-2015	274	Haematochezia (42.7). Abdominal pain (30.7). Diarrhoea (15.3)	66.8% abnormal. IBD (43.4). Eosinophilic GI disease (2.2). Polyp (5.9). Nonspecific colitis (8.4)	[112]
Malaysia	2010-2015	121	Suspected IBD (30.0). Haematochezia (21.0). Change in bowel habits (17.0)	85.0% abnormal. IBD (42.0). Polyp (7.0). Nonspecific/infective colitis (25.0)	[113]
Saudi Arabia	1993-2002	183	-	44.0% abnormal. Nonspecific colitis or rectal ulcer (71.0). Polyp (20.0)	[114]
South Korea	2008-2013	168	Abdominal pain (37.5). Diarrhoea (28.0). Haematochezia (27.4)	43.5% abnormal. IBD (19.6). Polyp (1.8). Nonspecific inflammation (14.3)	[17]
South Korea	2011-2015	746	Abdominal pain (43.7). Haematochezia (42.5). Diarrhoea (29.1)	72.2% abnormal. IBD (33.9). Polyp (11.5)	[13]
Taiwan, China	1998-2010	192	Haematochezia (53.5). Abdominal pain (20.6). Iron deficiency anaemia (11.8)	75% abnormal. IBD (8.3). Polyp (20.4). Nonspecific colitis (23.4)	[115]

IBD: Inflammatory bowel disease; GI: Gastrointestinal.

suppression-responsive EGID[30], further complicating the diagnostic process. Nevertheless, a short finite trial of acid-suppression is still deemed reasonable, with upper GI endoscopy and/or pH-impedance testing reserved for those refractory to empirical treatment or for those who cannot be weaned off acid-suppressive therapy[31].

In a fairly large study of 910 South Korean children presenting with symptoms of oesophageal dysfunction (vomiting, dysphagia, persistent reflux), 1.5% was diagnosed with eosinophilic oesophagitis (EoE) and 1.3% with eosinophilic gastroenteritis. 30.8% of patients with EGID had normal macroscopic findings, stating the importance of performing biopsies on even normal-appearing segments. The authors commented that the incidence of EoE was similar to a previous Japanese study but much lower than 10%-15% incidence in Western cohorts[32]. A multi-centre study in Japanese children undergoing colonoscopies by Nambu *et al*[33] showed EGIDs accounted for a substantially high proportion (23.0%) of the diagnoses followed by IBD (19.0%), but the authors had included food allergies and food protein-induced proctocolitis within the spectrum of EGIDs. Future studies are required to see if the incidence of EGIDs in Asia would mimic the rising trend seen with IBD.

Low diagnostic yield of endoscopy in uncomplicated abdominal pain

While paediatric endoscopy has high value in the diagnosis and management of IBD, *H. pylori* and EGIDs, most children with GI complaints have a non-organic aetiology yet the prevalence of functional GI disorders in Asian children is largely unknown and seldom described. Functional constipation was the most common condition identified in otherwise healthy Vietnamese (5.6%) and Mainland Chinese (7.0%) children between 7-48 mo of age[34,35]. Recurrent abdominal pain and dyspepsia in children is far more likely to be of a functional aetiology than in adults, prompting one to question the necessity and cost-effectiveness of invasive investigations. El-Matary *et al*[36] evaluated a cohort of 103 British children fulfilling Apley's original criteria of recurrent abdominal pain, *via* a series of blood, stool, imaging investigations with endoscopy done as clinically indicated. Approximately 70% of these children had a non-organic aetiology to their abdominal symptoms, with irritable bowel syndrome being the most common diagnosis. This study was conducted within a hospital setting, and it is likely that the actual prevalence of paediatric functional GI disorders would be much higher if the study were to be repeated in a community setting.

The diagnostic yield of paediatric GI endoscopy in childhood abdominal pain varies widely between published cohorts (Table 1), depending on the referral indication, presence of alarm symptoms[37] and/or index of suspicion guided by abnormal biochemistry (*e.g.*, coeliac serology) or imaging pre-

endoscopy. Moreover, the definition of a 'positive diagnostic yield' can be debatable as positive endoscopic pathology does not equate causality of symptoms *e.g.*, *H. pylori* infection is often asymptomatic and may be an 'innocent bystander' in children with functional abdominal pain[38], especially in the high-prevalence areas. An Israeli study of 329 children undergoing endoscopy for various indications (abdominal pain, diarrhoea, failure to thrive, short stature and iron deficiency anaemia) showed only 36% of children with abdominal pain had a diagnostic finding on endoscopy; if the child had abdominal pain in association with an objective test *e.g.*, positive coeliac serology and/or iron deficiency, the diagnostic yield would be more than 50% while those with subjective symptoms of nausea and constipation would have a positive yield in less than 25%[39]. A Hong Kong-based cohort of 80 children, fulfilling the Rome III criteria of functional dyspepsia and undergoing upper GI endoscopy, showed only 6.3% had ulcerations or erosions. There was a strongly positive correlation between alarm features *e.g.*, nocturnal pain and endoscopic findings[40]. These findings suggest that a risk stratification strategy, combining a clinical assessment for alarm features and the use of non-invasive objective tests, would be potentially helpful in discerning those who would benefit most from diagnostic endoscopy. An example would be the stool calprotectin assay, which is now commonly used globally as a highly sensitive stool biomarker for gut inflammation, both in the diagnosis and monitoring of IBD[41,42].

Advanced paediatric GI endoscopy in Asia

Advanced endoscopy techniques such as video capsule endoscopy (VCE) and balloon-assisted enteroscopy (BAE) can enhance diagnostics especially for obscure small bowel pathology, which would otherwise be inaccessible with standard endoscopy. VCE has been approved in children as young as 2 years old since 2009[43] and remains one of the commonly used modalities to complement standard endoscopy findings or when standard endoscopy has been non-diagnostic. Much of the published Asian experience with VCE has been from large East Asian (China[44], Japan[45] and South Korea[46]) adult and paediatric cohorts. A large Mainland Chinese cohort of 825 children in a single paediatric IBD centre underwent VCE for the main indication of abdominal pain (61.2%) followed by anaemia (17.0%) [44]. The authors noted a much higher diagnostic yield (55.6%) primarily of Crohn's disease-related small bowel pathology, compared to previous studies for similar indications quoting 20%-28%[47,48]; this was ascribed to a higher referral load of suspected IBD patients, further emphasising the strong influence of referral indications on published yields. Other typical VCE findings included idiopathic small bowel ulcers, intestinal polyps, lymphangiectasia and vascular malformations.

While the experience of VCE in adult cohorts has been for obscure GI bleeding, VCE has gained increasing utility for the pan-enteric evaluation of IBD[49]. VCE can be utilised as a non-invasive first-line modality in suspected IBD cases after a patency capsule test, followed by confirmatory endoscopy. VCE may also be advantageous in detecting early mucosal healing in IBD disease monitoring. A Korean paediatric study of Crohn's disease found VCE to be more sensitive than magnetic resonance enterography in detecting mucosal healing and early therapeutic response in the first year post diagnosis[50]. Other indications for VCE include the surveillance of intestinal polyposis, particularly in Peutz-Jeghers' syndrome where small bowel polyps have been known to form a lead point for small bowel intussusception. The use of VCE facilitates the planning of BAE for definitive small bowel polyp clearance[51].

As observed with studies of VCE, much of the published Asian paediatric experience with BAE are from East Asian cohorts[52-54]. Of note, these cohorts may include a varying number of patients undergoing balloon-assisted endoscopic retrograde cholangioscopes primarily for therapy of biliary stenoses. BAE complements the diagnostic value of VCE by providing the means to obtain histological samples and provide therapeutic intervention. Hagiwara *et al*[54] described a multi-centre study of 96 BAEs (both antegrade and retrograde) in 79 paediatric patients. The main indications were for follow-up of IBD, obscure GI bleeding, abdominal pain, and therapy of hereditary polyposis syndromes. The positive diagnostic yield in obscure GI bleeding and abdominal pain was 48%. There were higher reported diagnostic yields of approximately 77% for similar indications in the mainland Chinese paediatric cohorts[53,55], but this variation can be explained by differing referral indications as aforementioned.

State of the art advanced endoscopic diagnostic techniques

While most of these techniques are not in mainstream use at most Asian-Pacific paediatric gastroenterology centres, they are worth discussing in brevity as recent publications have discussed their utility in enhancing current paediatric GI diagnostics.

Transnasal endoscopy: This technique would be useful especially in children with EoE as they commonly require multiple upper GI endoscopies for mucosal surveillance[56]. Transnasal endoscopy may be done as an unsedated office procedure with topical pharyngeal anaesthesia, and significantly save time, costs and endoscopy resources.

Mucosal impedance: This technique measures transmucosal conductivity and thus the mucosal integrity of the oesophageal mucosa, *via* a catheter containing very closely spaced impedance sensors resting close to the mucosa wall. This catheter is inserted through the working channel of a standard upper GI scope, such that the sensors rest directly on the oesophageal mucosa. This has advantages over

standard 24-h pH impedance studies, by directly measuring the barrier function of the oesophageal mucosa in a matter of seconds while the child is sedated[57]. Mucosal impedance can be used to discern between GERD, EoE and non-GERD conditions, as well as to monitor treatment response/disease activity in GERD and EoE[58,59].

Endoluminal functional lumen imaging probe: Endoluminal functional lumen imaging probe (EndoFLIP) is another adjunct technique to measure oesophageal luminal dimensions, distensibility, pressure changes and motility[60], typically while the child is sedated for standard upper GI endoscopy. It involves insertion of a sensor balloon-mounted catheter transorally, and inflating the balloons within the oesophageal and gastric lumens. While high resolution oesophageal manometry is the current gold standard in the assessment of oesophageal function, it is potentially uncomfortable and requires the child to cooperate with swallowing during the manometry. EndoFLIP has utility in the assessment of patients with GERD, EoE[61] and achalasia, as well as patients experiencing persistent symptoms post fundoplication.

Confocal laser endomicroscopy: Confocal laser endomicroscopy is an endoscopic technique that allows for high-resolution histological examination of the GI mucosa at the cellular level. Besides its obvious utility in real-time detection of neoplastic lesions in adults, the group at Sheffield Children's Hospital, United Kingdom has described its usefulness as a biopsy-free method of assessing mucosal pathology in enteropathies, EoE, ileo-colitis and polyposis[62]. This enhances the diagnostic accuracy of GI endoscopy, and reduces the time, risks and costs associated with multiple GI mucosal biopsies.

Artificial intelligence and machine learning in endoscopic diagnostics: Patel *et al*[63] recently reviewed the potential application of artificial intelligence (AI) in paediatric GI pathologies. The aim of utilising machine learning, a form of AI, is to at least semi-automate the process of macroscopic pathology and pattern recognition, which would otherwise be subject to the endoscopist's individual expertise and experience. In paediatrics, AI could potentially automate the process of accurately classifying macroscopic disease severity and extent in IBD and celiac disease, as current endoscopic scoring systems are often time-consuming and subjective. Current AI research in adults is driven by the need for accurate polyp detection: Early published data of the computer-aided detection EYE system demonstrates its AI driven algorithm detects and classifies polyps with excellent sensitivity and specificity[64]. AI would play a major role in standardising endoscopy outcomes across different endoscopists of varying experience levels, especially in units with rotating trainees.

THERAPEUTIC ENDOSCOPY

Foreign body removal

Foreign body ingestion is a common presentation in the ambulatory and emergency department settings. The exploratory behaviour and normal development of young infants and toddlers lead these kids to accidentally grasp and ingest foreign objects. The common age group usually ranges between 6 mo to 6 years[65]. The initial manifestation can range from asymptomatic to significant GI symptoms (drooling, dysphagia, vomiting, obstructive symptoms), coughing, choking, severe respiratory distress, or even death[66,67]. One of the largest cohorts from China reported 1265 children (aged 6 mo to 16 years) admitted with a history of foreign body ingestion, of which 552 (43%) children had detected foreign bodies from endoscopies. The two most common objects were coins (49%) and non-metallic sharp objects (31%)[66]. A systematic review also noted that the coin was the most frequently ingested object in the paediatric population[68]. In contrast to most reports, a medical record review in 105 Iranian children, the button battery was the most commonly found object in 41%[69]. The types of foreign body vary between different countries and geographic locations, that may be likely due to diverse sociocultural and dietary factors[70]. Another study from Khorana *et al*[67] from northern Thailand included 194 patients aged < 15 years (median age of 44 mo) found that most were symptomatic (56%), with vomiting as the commonest complaint. The most common location of impacted foreign bodies was in the oesophagus (37%), similar to most previous reports[68]. Plain radiography can usually confirm the location, size and shape of most radiopaque objects (such as coin, magnets, safety pin, *etc.*), but has little diagnostic value for radiolucent objects such as plastic toys, fishbone, or woods. Contrast studies or computed tomography scans may be needed to locate the radiolucent foreign bodies before deciding on the further management.

Timing of foreign body removal: As most of the ingested objects were coins, which were mostly considered as small and blunt objects, spontaneous passage usually occurred[67,71]. However, endoscopic intervention or even surgical exploration may be needed in some cases especially individuals with symptoms or complications. The timing of endoscopic intervention in paediatric foreign body ingestion has been proposed in various guidelines published from various scientific societies, namely the European Society for Paediatric Gastroenterology Hepatology and Nutrition

(ESPGHAN)/European Society of Gastrointestinal Endoscopy (ESGE)[71], and the North American Society of Paediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) Endoscopy Committee[72]. The proper timing of endoscopy is mainly based on 3 key factors: The type of ingested objects, the location, and the presence of symptoms. Types of common foreign bodies are button batteries, magnets, sharp objects, long objects, absorptive objects, drug packets, impacted food boluses, and coins.

Button batteries: During the past few decades, major concerns with button battery ingestion have been raised as the integrity of the strong alkali-containing battery can be degraded and cause severe caustic injury to the GI mucosa, especially the oesophagus (*i.e.*, a hollow organ with small lumen). A cohort from the United States reviewing 8648 cases of button and cylindrical battery ingestions, occurring between 1990-2008, found 73 cases with major adverse outcomes (0.8%), including prolonged compromise of feeding and/or breathing that required surgical procedures, tube feedings, tracheostomies. There were 13 deaths (0.15%) related to damage to the oesophagus, major vessels, and/or the airway[73]. Huang *et al*[74] from China reported children with inhaled/ingested button batteries and found that 13 of 116 (11%) cases had button batteries either in the oesophagus or stomach (n = 6 and 7, respectively). One child developed an oesophageal stricture and another one died from sudden cardiac arrest during the perioperative period. A recent Position Paper from ESPGHAN proposed 2 major strategies in the diagnosis and management of button battery ingestion in children. This includes: (1) Computed tomography scan to evaluate injuries to the adjacent organs and blood vessels before endoscopic removal if ingested > 12 h even in asymptomatic children; and (2) Honey (in children > 1 year of age) and sucralfate can be considered in ingestions < 12 h while waiting for endoscopic removal [75].

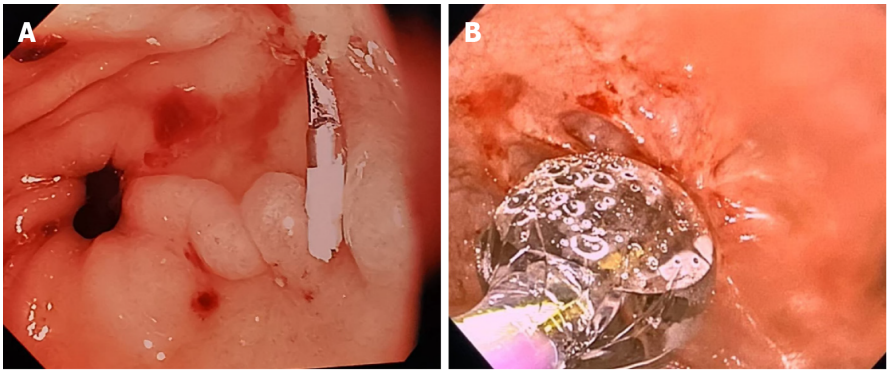
Endoscopic techniques of foreign body removal: Conventional flexible endoscopy is a safe and effective tool for removing most foreign bodies from the GI tract. A high success rate is found when using retrieval nets, polypectomy snares, and the rat-tooth forceps[71]. Opasanon *et al*[76] reported 34 Thai patients with upper GI tract foreign bodies and found that removal was successfully performed in all cases with either rat-tooth forceps, snare, dormia basket or tripods with no procedure-related complications.

Device-assisted enteroscopy for foreign body removal: The challenging cases in paediatric foreign body ingestion are typically the ones with objects beyond the reach of the conventional endoscope, *i.e.*, the depths of the small bowel in the jejunoileal area. Adult-based ESGE guidelines on small bowel endoscopy and device-assisted enteroscopy strongly recommend enteroscopy as an alternative to surgery for retrieving foreign bodies retained in the small bowel in patients without acute intestinal obstruction[77]. Device-assisted enteroscopy refers to any adjunct device used to assist endoscopic advancement into the small bowel (balloon, overtube, stiffening device). As with the use of BAE as an advanced diagnostic tool discussed earlier[54], age- and weight-appropriate device-assisted enteroscopy may be used for foreign body removal and other therapeutic applications within the small bowel.

Haemostasis of GI bleeding

GI bleeding can be divided into upper GI and lower GI bleeding. Upper GI bleeding is defined as bleeding from the GI tract proximal to the ligament of Treitz, while lower GI bleeding is bleeding occurring distal to the aforementioned ligament. Upper GI bleeding can also be further divided into non-variceal bleeding and variceal bleeding which may (or usually) need haemostatic intervention (Figure 1A). On the other hand, for the paediatric population, lower GI bleeding rarely needs endoscopic intervention in the colon or distal small bowel mainly because of the 3 following reasons: (1) Most of the severe GI bleeding occurs up in the upper GI tract; (2) Acute colonic bleeding in children usually stops spontaneously; and (3) Various common aetiologies such as Meckel's diverticulum, intussusception, colitis from infection, inflammation or allergy, or anal fissures rarely need endoscopic intervention to stop bleeding. One of the few exceptions being endoscopic polypectomy for juvenile (colonic) polyps which usually present in a non-urgent setting. After initial haemodynamic stabilization consisting of judicious fluid resuscitation and, if necessary, blood product replacement, endoscopic haemostatic intervention for the lesions causing GI bleeding would need to be justified based on the site and type of the lesion, patient's underlying disease and resource availability/procedural feasibility in conjunction with the potential contraindications.

Haemostasis for acute upper GI bleeding: Non-variceal and variceal bleeding: ESPGHAN/ESGE guidelines suggest early oesophago-gastroduodenoscopy within 12 h in cases presenting with acute upper GI bleeding that require ongoing circulatory support, or those presenting with large volume haematemesis or melena (weak recommendation and low quality of evidence, but a strong recommendation and moderate quality of evidence in cases with a known history of oesophageal varices) [71]. Most recommendations with regards to upper GI bleeding have been adopted from previous adult-dominated studies. An oesophago-gastroduodenoscopy should also be performed in cases with bleeding that require packed red blood cell transfusion due to a haemoglobin value below 8 g/dL and



DOI: 10.3748/wjg.v29.i18.2717

Figure 1 Images. A: Haemostatic clip applied to a bleeding gastric antral ulcer; B: Balloon dilation in oesophagus with a mucosal tear.

an acute drop of at least 2 g/dL, or a bleeding clinical severity index score above a defined validated threshold such as the Sheffield Scoring System[78]. Furthermore, endoscopy should be performed before discharge from hospital, in children with pre-existing liver disease or portal hypertension.

Endoscopic haemostatic techniques for non-variceal lesions: Overall, lesions causing non-variceal upper GI bleeding such as bleeding ulcers or Dieulafoy lesion should use either thermal techniques such as heater probe, bipolar probe or mechanical techniques such as haemostatic clips, with/without epinephrine injection for controlling bleeding[71,79]. Yabe *et al*[80] reported 36 Japanese children with upper GI bleeding from gastroduodenal ulcers (50%) or gastritis (26%), and 14/36 (39%) underwent haemostatic intervention [clips (n = 12), hypersaline and epinephrine injection and coagulation therapy (n = 1), pure ethanol injections (n = 1)] with 100% initial success rate. Rebleeding occurred only in one patient who was initially treated with ethanol injections.

Endoscopic haemostatic techniques for variceal bleeding: For oesophageal varices, Zargar *et al*[81] demonstrated that endoscopic variceal band ligation is more effective than sclerotherapy in 49 Indian children with extrahepatic portal vein obstruction and variceal bleeding. Band ligation required fewer endoscopic sessions [3.9 (SD 1.1) *vs* 6.1 (SD 1.7) times for sclerotherapy] and had lower rates of rebleeding (4% *vs* 25%)[81]. Since then, studies comparing the efficacy of banding with sclerotherapy in children are sparse. A recent Cochrane Review was initially planned to analyse randomized controlled trials (RCTs) but was eventually unable to find any RCTs comparing band ligation *vs* sclerotherapy as primary variceal prophylaxis (*i.e.*, preventing the first variceal bleeding episode in children with oesophageal varices) in children with chronic liver disease or portal vein thrombosis[82].

Polypectomy

The technique of polypectomy is based on the location, morphology, and size. The ESPGHAN guideline suggests using cold biopsy forceps for small polyps (< 3 mm), hot or cold snaring in polyps diameter 3-8 mm, and hot snaring in the larger polyps[71]. However, hot biopsy forceps induce larger histopathological lesions, increased necrotic depth and submucosal inflammation in a pig's colon model[83], and more cytological artefacts[84]. A retrospective study in 91 Korean children, who underwent endoscopy to find polyps, found that polyp size was the one single factor associated with the presence of any polyps located proximal to the splenic flexure [odds ratio = 2.3, 95% confidence interval (CI): 1.3-4.3]. Polyps proximal to the splenic flexure and sessile morphology were associated with the presence of any adenomatous polyp. Therefore, the authors concluded that a full colonoscopy remains crucial before the occurrence of complications[85]. Another study from Thailand investigated 32 patients with symptoms of colorectal polyps such as haematochezia, rectal mass, or diarrhoea. Most (20/32, 63%) had a single polyp, 6/32 had 2-4 polyps, and a minority (6/32) was diagnosed with polyposis coli. Most had polyps in the rectosigmoid region and only 6 cases had polyps proximal to the splenic flexure. All had pathologically confirmed juvenile polyps without adenomatous changes, which demonstrated an absence of malignant potential[86].

Oesophageal dilation

Dilation of the oesophagus is indicated when symptoms of oesophageal stricture/stenosis occur. Various causes include congenital anomalies, post caustic ingestion, EoE, and GERD[87]. Symptoms of oesophageal stricture include dysphagia, odynophagia, food bolus impaction, vomiting and poor oral intake. Nowadays, the 2 main options for oesophageal dilatation in oesophageal stricture are balloon dilation and bougie dilation.

Balloon dilation (Figure 1B) can be safely performed under both the direct endoscopic and/or fluoroscopic examinations, while most of the bougie dilations are Savary-Gilliard bougies that could dilate up to 12 mm in children age < 5 years and 15 mm in older children. The "rule of 3" has been widely used as dilation to not more than 3 times the stricture diameter with a minimal period of 3 wk between dilation sessions and an average of 3 sessions in total[88,89]. Lan *et al*[90] reported 75 children from Hong Kong with oesophageal strictures [post-oesophageal atresia repair (n = 63), reflux esophagitis (n = 7), caustic ingestion (n = 3) and post-fundoplication (n = 2)], who underwent a total of 260 balloon dilations (mean number of 3.4 sessions per patient). Four oesophageal perforations (1.5%) were noted, with one child required surgical repair; all other patients were asymptomatic after the dilation sessions. Balloon dilation has been reported to be more effective and less traumatic than the bougie dilation, but a study from India reported comparable complications (perforations of 0.9%)[89], and a recent study from China reported a high perforation rate of 4.4% in children undergoing oesophageal balloon dilation[91]. The aforementioned study also reported only a 60% success rate, and found that stricture length was the main determining factor of treatment outcome. Therefore, a universally-agreed dilation choice remains controversial. Furthermore, oesophageal stent placement, intralesional mitomycin C or steroid injections have also been used in refractory oesophageal strictures in children[92].

Bowel preparation of ileocolonoscopy

Low volume bowel preparations preferred: Ileocolonoscopy is an established diagnostic and therapeutic tool in a variety of GI disorders. The optimal bowel preparation is an important key success factor in paediatric ileocolonoscopy[93]. While standardised bowel preparation protocols remain unavailable[94], more recent recommendations propose low-volume preparations using either polyethylene glycol (PEG)[95] along with ascorbate or sodium picosulfate magnesium citrate (SPMC)[71]. The preferable regimen in standard clinical practice should provide optimal colonic cleansing with small volumes of laxatives, acceptable palatability and drinkability and minimal side effects. In 2017, 15 RCTs (n = 1435) from 2124 studies with heterogeneity/bias risk compared PEG with other medications (sodium phosphate enema (n = 2 studies, relative risk = 1.27 with 95%CI: 0.66-2.44), SPMC (n = 3 studies, relative risk = 0.99 with 95%CI: 0.89-1.11), sennasoids (n = 3 studies, relative risk = 0.73 with 95%CI: 0.31-1.76) which showed no difference in the bowel preparation quality. Noninferior efficacy was also noted when comparing low volume PEG with SPMC *vs* standard volume PEG. Children who received PEG regimen also needed nasogastric tube insertions more often than those receiving the SPMC regimen (38% *vs* 1.6%)[96]. Later in 2022, four good quality RCTs (n = 390) showed higher tolerability and acceptability in the SPMC group when compared to the PEG group with comparable efficacy[97].

Split bowel preparation regimens: The newer studies also implement a 'split regimen' of the laxatives at different timepoints prior to GI endoscopy[98-100]. Sriphongphankul *et al*[98] performed an RCT in 45 children aged 2-18 years. The split dose group was given PEG in 2 split doses for 8-12 h apart and at least 6 h before the procedure, and the full single dose was given once the night before scope. Successful preparation (defined as Boston Bowel Preparation Scale ≥ 6) was superior in the split group (95% *vs* 72% in the standard high-volume PEG regimen). Willingness to repeat the same protocol was also much higher (83% *vs* 36%, P = 0.002), but nasogastric tube insertion rates were comparable (57% *vs* 68%). A later meta-analysis, including 4 paediatric studies, also found a trend of significantly higher efficacy in the split dose group (P = 0.07) but significant heterogeneity was noted among studies[99]. Therefore, further high-quality RCTs with low risk of bias are required. With regards to the recommended diet before the procedure, Jiao *et al*[101] studied 321 Chinese children and found that either 1-d or 2-d low residue diet had similar efficacy in bowel preparation but the 1-d low residue diet group had higher acceptability.

State-of-the-art interventions for therapeutic endoscopy

The endoscopic interventions for (esophageal) achalasia, a rare condition with incomplete or lack of normal lower esophageal sphincter relaxation, include pneumatic dilation and botulinum toxin injection. Recently, peroral endoscopic myotomy (POEM) has become another therapeutic intervention in both adults and children that demonstrates satisfactory success rate. The NASPGHAN Endoscopy Committee recently reviewed various aspects of POEM[102]. In brief, the myotomy is made starting from 8-10 cm above to 2-3 cm below the gastroesophageal junction along the cardia. Submucosal injection of the posterior wall is then performed to create a mucosotomy and submucosal tunnel, which would reveal the circular muscle fibers of the lower oesophagus. Complete myotomy of the circular muscle layer is made with longitudinal muscle layer underneath and the clip is finally deployed to close the mucosotomy. However, studies reporting efficacy and complications of POEM in children remain limited. Furthermore, appropriate training and adequate number of the performed procedures would also be required before implementing POEM as a standard of care in paediatric achalasia.

Future training opportunities in paediatric endoscopy in the Asia-Pacific region

ESPGHAN launched a Position Paper on paediatric endoscopy training in 2020[103]. The main content on achievement of training milestones, with regards to competency and procedural numbers including

'Train the trainers' courses, have been mentioned. Educational material such as e-learning, simulator training would also be needed to train the trainees in paediatric endoscopy.

Different scientific societies recommend varying competency thresholds for lower and upper GI endoscopies. NASPGHAN, the Joint Advisory Group in GI Endoscopy Paediatric Certification from the United Kingdom, the Conjoint Committee for Recognition of Training in Gastrointestinal Endoscopy from Australia proposed a minimum of 100-120 lower GI endoscopies with a caecal intubation rate of ≥ 90% (ranges from 15-30 min). NASPGHAN and the Joint Advisory Group from the United Kingdom proposed a minimum of 100 upper GI endoscopies and the Australian Committee proposed a minimum of 200 upper GI endoscopies (≥ 100 in children). Interestingly, ESPGHAN did not specifically define any numbers for endoscopies. The number of therapeutic endoscopies such as foreign body removal, haemostatic intervention, and polypectomy vary greatly across different societies. One of the preprocedure objective outcomes is the rate of adequate bowel preparation before ileocolonoscopy, with a minimum standard of 90% and a target of 95%.

Another important point is that the group suggests endoscopic procedures in children be performed by endoscopists trained in paediatric gastroenterology with established procedure-specific competency. Special consideration must be made to a child requiring GI endoscopy. Physicians need to consider the size of the patient, indications and contraindication of the procedure, proper equipment, bowel preparation, anaesthesia and sedation as well as the psycho-emotional factors of the children and their caregivers throughout the process[104]. Therefore, before implementing training in paediatric endoscopy in the Asia-Pacific region, aforesaid aspects should be carefully considered and implemented in the formal standardized curriculum. The Asian Pan-Pacific Society for Paediatric Gastroenterology, Hepatology and Nutrition conducted its first Paediatric Endoscopy Masterclass on June 2 to 3, 2022 in Bangkok with the purpose of addressing gaps in paediatric endoscopy training within the Asia-Pacific region. The subsequent way forward would be a regionalised set of guidelines and consensus statements, to facilitate standardisation of indications and endoscopic terminology across different paediatric GI centres.

CONCLUSION

Paediatric GI endoscopy has undoubtedly gained utility in the Asia-Pacific region as an invaluable tool in the diagnostics and management of GI diseases of current and emerging epidemiological importance. This is the first article to comprehensively review the evolving epidemiologic trends in paediatric GI endoscopy within Asia-Pacific, and delve into the future directions for paediatric endoscopy training and the advent of state of the art endoscopic techniques which are increasingly applied in the adult population. Yet the lack of consensus guidelines and heterogeneity in clinical practice, variablity in the referral patterns, healthcare access and disease prevalence across the Asian continent, inevitably leads to a wide variance in outcomes for different endoscopic modalities. While it is crucial that early endoscopy is done for a prompt diagnosis and treatment, it must be balanced with avoiding un-necessarily invasive investigations in otherwise benign functional GI conditions. The maturation of paediatric gastroenterology as a subspecialty hence necessitates comprehensive and accreditable endoscopy training, so that paediatric endoscopists in Asia adhere to a minimum practice standard and are adequately trained to apply diagnostic and therapeutic endoscopic techniques appropriately and competently. The inauguration of regular endoscopy masterclasses and workshops by the regional society Asian Pan-Pacific Society for Paediatric Gastroenterology, Hepatology and Nutrition sets the stage for more uniformity in endoscopic practices and outcomes, as well as future inter-regional collaborative efforts in paediatric endoscopic research.

FOOTNOTES

Author contributions: Huang JG and Tanpowpong P involved in the conception of the work, data collection, drafting of the article, critical revision of the article and final approval of the published version.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Chen YX

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World Journal of Gastroenterology

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World J Gastroenterol 2023 May 14; 29(18): 2733-2746

DOI: 10.3748/wjg.v29.i18.2733

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Study of tumor necrosis factor receptor in the inflammatory bowel disease

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Specialty type: Anatomy and morphology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Xu B, China; Yoo JH, South Korea

Received: December 30, 2022
Peer-review started: December 30, 2022
First decision: January 22, 2023
Revised: February 14, 2023
Accepted: April 4, 2023
Article in press: April 4, 2023
Published online: May 14, 2023

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Abstract

Ulcerative colitis (UC) and Crohn's disease (CD) are part of Inflammatory Bowel Diseases (IBD) and have pathophysiological processes such as bowel necrosis and enteric neurons and enteric glial cells. In addition, the main inflammatory mediator is related to the tumor necrosis factor-alpha (TNF-α). TNF-α is a mediator of the intestinal inflammatory processes, thus being one of the main cytokines involved in the pathogenesis of IBD, however, its levels, when measured, are present in the serum of patients with IBD. In addition, TNF-α plays an important role in promoting inflammation, such as the production of interleukins (IL), for instance IL-1β and IL-6. There are two receptors for TNF as following: The tumor necrosis factor 1 receptor (TNFR1); and the tumor necrosis factor 2 receptor (TNFR2). They are involved in the pathogenesis of IBD and their receptors have been detected in IBD and their expression is correlated with disease activity. The soluble TNF form binds to the TNFR1 receptor with, and its activation results in a signaling cascade effects such as apoptosis, cell proliferation and cytokine secretion. In contrast, the transmembrane TNF form can bind both to TNFR1 and TNFR2. Recent studies have suggested that TNF-α is one of the main pro-inflammatory cytokines involved in the pathogenesis of IBD, since TNF levels are present in the serum of both patients with UC and CD. Intravenous and subcutaneous biologics targeting TNF-α have revolutionized the treatment of IBD, thus becoming the best available agents to induce and maintain IBD remission. The application of antibodies aimed at neutralizing TNF-α in patients with IBD that induce a satisfactory clinical response in up to 60% of patients, and also induced long-term maintenance of disease remission in most patients. It has been suggested that anti-TNF-α agents inactivate the pro-inflammatory cytokine TNF-α

by direct neutralization, *i.e.*, resulting in suppression of inflammation. However, anti-TNF-α antibodies perform more complex functions than a simple blockade.

Key Words: Tumor necrosis factor 1 receptor; Tumor necrosis factor 2 receptor; Inflammatory bowel diseases; Enteric nervous system; tumor necrosis factor-alpha

Core Tip: This review summarizes the role of Tumor Necrosis Factor-alpha (TNF-α) in promoting inflammation. Studies have suggested that TNF-α is one of the main pro-inflammatory cytokines involved in the pathogenesis of Inflammatory Bowel Diseases (IBD). In addition, the enteric nervous system is affected by IBD. There are two receptors for TNF: The tumor necrosis factor 1 receptor; and the tumor necrosis factor 2 receptor. They are involved in the pathogenesis of IBD. The application of antibodies aimed at neutralizing TNF-α in patients with IBD induce a satisfactory clinical recovery. This review addresses the main aspects of TNF-α in IBD and anti-TNF therapies.

Citation: Souza RF, Caetano MAF, Magalhães HIR, Castelucci P. Study of tumor necrosis factor receptor in the inflammatory bowel disease. *World J Gastroenterol* 2023; 29(18): 2733-2746
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2733.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2733

INTRODUCTION

Inflammatory bowel diseases (IBD) comprise ulcerative colitis (UC) and Crohn's disease (CD), which are chronical and recurrent disorders that affect the gastrointestinal (GI) tract[1-4]. The etiology of IBD is not yet fully understood, but there are reports of a complex relationship between genetic[5,6], immunological and environmental factors[7-10], as well as gut microbiota[11-15]. There is an imbalance between anti- and pro-inflammatory cytokines that cause an exacerbated and inadequate immune response.

The enteric nervous system (ENS) is composed by intrinsic neurons, their axons and enteric glial cells, which constitute a complex of structures responsible for controlling motility of the GI tract, secretion of gastric acid, regulation of fluid movement through the epithelium, local blood flow control, and interactions with the endocrine and immune systems of the gut[16-18]. The ENS is affected by IBD which causes necrosis, apoptosis, degeneration of enteric ganglia and alterations on motility patterns [19-23].

Tumor necrosis factor-alpha (TNF-α) is a mediator in intestinal inflammatory processes, thus being one of the main cytokines involved in pathogenesis of IBD, with high levels in patients with IBD[24,25]. There are two receptors for TNF-α as following: The tumor necrosis factor receptor 1 (TNFR1); and the tumor necrosis factor receptor 2 (TNFR2). After TNF-α binding to these receptors, it activates signaling pathways for cell survival, death and differentiation[26]. An increase in TNF-α expression can lead to mucosal barrier defects in patients with IBD, which increases inflammation and worsens the prognosis [10].

Due to the TNF-α relation with IBD, biological drugs have been used to treat CD and UC with neutralizing monoclonal antibodies used for TNF-α target and blockade, which reduce the development of the inflammatory process and the activation of immune system cells[27]. Anti-TNF-α agents are indicated by various guidelines for the treatment of IBD[28,29] and the use of these agents induces a satisfactory clinical response, and long-term maintenance of disease remission in most patients[24,30]. This review aims to provide the main aspects of IBD and TNF-α relationship, elucidating the role of the TNF-α receptors, anti TNF-α therapy and some perspectives related to the involvement of the ENS.

THE ENS AND IBD

The ENS is a complex of structures responsible for controlling motility of the GI tract, secretion of gastric acid, regulation of fluid movement through the epithelium, changes in local blood flow, and interactions with the endocrine and immune systems of the gut[16-18]. The ENS is located in the intestinal wall and is organized into two ganglionated plexuses: The myenteric plexus; and the submucosal plexus[16-18].

The myenteric plexus (Auerbach's plexus) is situated between the longitudinal muscle layer and the circular muscle layer throughout the entire GI tract, from the esophagus to the rectum. It has three

components as following: A primary plexus; a secondary plexus; and a tertiary plexus. The myenteric plexus is involved with reflex regulation of the contractile activities of the muscle layer[16,17]. The submucosal plexus (Meissner's plexus) is predominantly found in the small and large intestines, with a smaller ganglion and finer interconnected fibers when compared to the myenteric plexus[16,17]. It has a direct role in controlling secretion and absorption through motor neurons that regulate the secretomotor and vasomotor activity of the mucosal layer[16,17].

Enteric neurons can be classified according to their function into motor neurons, interneurons, and Intrinsic Primary Afferent Neurons[16]. Motor neurons are further divided according to their chemical code into excitatory neurons (labeled by the enzyme choline acetyltransferase or by calretinin), inhibitory neurons (labeled by the enzyme neuronal nitric oxide synthase), and secretomotor/vasodilator neurons. Excitatory and inhibitory motor neurons are observed in the myenteric plexus and are involved in motility control, while secretomotor/vasodilator neurons are observed in the submucosal plexus and are responsible for innervating the mucosa and regulating secretion, absorption, and local blood flow[16,17].

Enteric glial cells are small, consisting of numerous processes that do not synthesize myelin, and are also essential for the organization and function of the ENS[31]. These cells have a star shape with numerous branches that surround the neurons. They also exhibit a series of voltage-dependent ion channels that are connected by gap junctions, giving the impression of an intimate and complex intertwining of neurons and glial cells. In addition to their role in supporting neurons, enteric glia cells regulate synaptic transmission, release cytokines, and mediate communication with the immune system [32-34].

Enteric glial cells are identified by immunohistochemical methods such as labeling of cells expressing glial fibrillary acidic protein (GFAP) and/or calcium-binding protein S100/S100β since mature enteric glial cells express these protein types[35,36]. GFAP expression is modulated by enteric glial cell proliferation, differentiation, and inflammation[22]. S100 is a calcium-binding protein that can be found in the nucleus or cytoplasm and acts to regulate the cytoskeleton structure and function as well as the calcium homeostasis in the cytoplasm[35,37,38]. Under inflammatory conditions, enteric glial cells can acquire new functional properties, and in patients with IBD there was an increase in GFAP expression in mucosal inflammation compared to non-inflamed areas[39].

The enteric plexuses are formation of cells that spread throughout the entire GI tract, however, differences in the density and size of enteric neurons and glial cells, as well as in ganglion morphology, may occur in the same segment of the GI tract in different species, or in different experimental models such as undernourishment protein and renutrition[40,41], obesity[42,43], ischemia and reperfusion[44-47], and intestinal inflammation[23,48-52].

About IBD specifically, they are classically classified into UC and CD, disorders that chronically and recurrently affect the GI tract[1-4]. Inflammatory reactions in the intestinal mucosa cause epithelial damage that compromises the intestinal barrier[53]. Although the etiology of IBD is not yet fully understood, they are usually triggered by a complex relationship between genetic[5,6], immunological and environmental factors[7-10], and gut microbiota itself[11-15]. There is, then, an imbalance between anti- and pro-inflammatory cytokines that cause an exacerbated and inadequate immune response. It is emphasized that, in colitis, there are alterations in the neuronal density such as imbalances of contractile and secretory functions associated with diarrhea due to intestinal inflammation[20,49,50,52].

CD is characterized by a segmental and transmural disorder that can affect the entire GI tract, and it could be noted there is also involvement of T-auxiliary cells (TCD4) in the pathogenesis of the disease [54,55]. In contrast, UC is characterized by continuous inflammation of the distal colon and a modification of the cytokine profile coming from Th2 cells, involved in humoral immune response patterns[2,56]. The main clinical manifestations of IBD are abdominal pain, diarrhea, vomiting, weight loss, and the presence of blood in the stool[2,50] and, worryingly, it has been demonstrated that this occurrence has increased worldwide over the years[57-60].

The literature has shown that the ENS may be affected by the IBD, thus presenting necrosis, apoptosis and degeneration of enteric ganglia[19-23]. Similarly, alterations in neurotransmitters and neuropeptides of enteric neurons have been noted in these pathologies[19,48]. Furthermore, injuries in the ENS result in activation of enteric glial cells[61,62], and the onset and/or progression of IBD can be attributed to an immune-mediated damage of enteric glial cells[32]. This activation is pointed to as a signaling mechanism that results in subsequent neuronal death[63].

The diversity of neurotransmitters and receptors found in the ENS makes the intestine one of the main tissue choices for studies of neurotransmitter receptors in pharmacological tests. In turn, several ENS-related treatment strategies have been explored to alter gut function in an effort to improve symptoms, which include drugs targeting opioid, serotoninergic, dopaminergic, and cholinergic receptors[64].

Induced colitis models in the laboratory has been widely used to study intestinal inflammation, especially through dextran sulfate sodium and 2,4,6-trinitrobenzene sulfonic acid[65-69]. Despite this, the cell signaling mechanisms underlying neuronal and tissue damage are poorly understood[49,70], and new therapeutic approaches for IBD are emerging[71-74].

As expected, experimental animal models of colitis, enteric neuronal hyperplasia and hypoplasia may be associated with increased and reduced levels of TNFα production, respectively[75,76]. In addition,

hypertrophy and hyperplasia of enteric neurons have been reported in IBD[77]. Although, the mechanism responsible for neuronal modulation of inflammation severity is still unclear, due to modulations of neuroimmune interactions, it is speculated that enteric neurons could produce and regulate cytokines involved in IBD[76].

TNFR

Specific markers have been identified in GI tract when tissues are affected by injury or disease such as IBD. They are responsible for mucosal injury and tissue damage and consequently may trigger autoimmune disease - specific immune responses in UC[10]. The TNF-α, inducible nitric oxide synthase, heme oxygenase 1, arginase-1 or CD206 in higher levels can lead to massive tissue damage in the intestine[25,54,78].

TNF-α is a mediator in intestinal inflammatory processes, being one of the main cytokines involved in pathogenesis of IBD, since their levels, are frequently high in patients with IBD[24,25]. TNF-α was first described in 1975 by a group in Sloan-Kettering who identified TNF as a promising serum soluble activity[79]. Thus, TNF-α plays roles in promoting inflammation such as the production of interleukins (IL) such as IL-1β and IL-6[10,80]. In the central nervous system (CNS), TNF-α plays homeostatic roles by regulating crucial physiological processes, such as synaptic plasticity, learning and memory, sleep and food and water intake[81]. However, some findings demonstrated that overexpression of TNF in the CNS has negative effects on cognitive functions and TNF deficiency is related to learning processes and poor memory[82]. In addition, it may be involved in gliosis and tissue remodeling and in various processes about mechanisms subserving cognition, such as synaptic scaling, change in neurotransmitter metabolism, and the process of neurodevelopment[82,83]. This observation may pave the way for understand mechanisms of innate immunity and the pathogenesis of infectious diseases which are driven by the same cascade of pro-inflammatory cytokines[79].

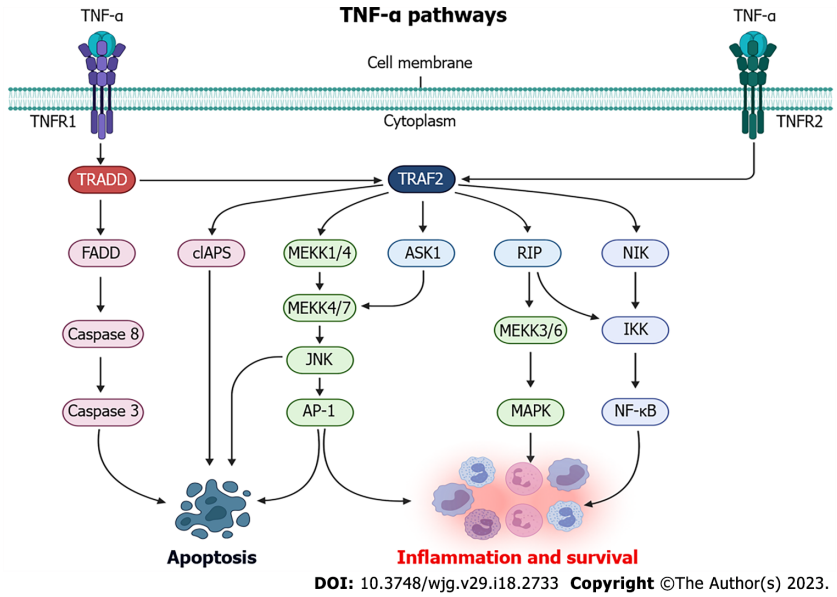
TNF-α is mainly generated by macrophages and monocytes. However, other cells such as some subsets of T cells, NK cells, dendritic cells, B cells, cardiomyocytes, fibroblasts, and astrocytes are also low-level producers of this cytokine[84]. In pathological conditions, astrocytes and microglia release large amounts of TNF-α, seeing that the production of this cytokine is an important component of the neuroinflammatory response that is associated with various neurological disorders such as Alzheimer's, Parkinson's, multiple sclerosis and amyotrophic lateral sclerosis[81].

TNF-α operates significantly in the apoptotic phase increasing the expression of the TNF receptor-associated factor 2 (TRAF2)[53]. Furthermore, TNF-α activates mitogen-activated protein kinase and nuclear factor which contribute to cell differentiation and proliferation and increased expression of pro-inflammatory cytokines[10]. Two distinct forms of TNF-α were identified: 26kDa homotrimeric transmembrane precursor form (mTNF), which is cleaved by the TNF-α-converting enzyme, and matrix metalloproteinase (TACE/Adam17) to its next 17kDa TNF soluble form (sTNF) of monomers[85,86]. Both forms of TNF are biologically active and are signaled through two distinct receptors discussed below.

There are two receptors for TNF being: The 75 kDa TNFR1, ubiquitously expressed and has a cellular death domain; and the 55 kDa TNFR2 has been found in lymphocytes and endothelial cells. Interactions of TNF-α with its receptor activate signaling pathways for cell survival, death and differentiation that control immune function and disease, and various receptors pairs of ligands within TNF family molecules expressed by immune cells that play important roles in T cell Immunity[26].

When TNF-α binds to TNFR1 and TNFR2, several intracellular pathways are activated, thus mediating cell death and/or survival response (Figure 1). When TNF-α binds to TNFR1, TNF receptor associated death domain protein (TRADD) is activated which, in turn, can induce the activation of three signaling pathways. In the first one, after TNFR1 activation, TRADD binds to FAS-associated death domain protein, which recruits caspase 8 proteins, culminating in the activation and cleavage of caspase 3, as well as leading to cell death by apoptosis[87]. The second TNFR1 pathway is related to the recruitment of TRAF2 and receptor-interacting protein (RIP) kinase *via* TRADD. TRAF2, in turn, recruits the IκB kinase (IKK) protein, which will be activated by RIP and will result in the phosphorylation of nuclear factor κB (NF-κB), which will mediate the transcription of proteins involved in the inflammation response and cell survival[87,88]. The third pathway resulting from TNFR1 activation is connected with activation of mitogen-activated protein kinase (MAPK) pathways *via* TRAF2, which activate MAPK kinase kinase 1/4 (MEKK1/4) and, upon phosphorylation, MEKK4/7 leads to activation of c-Jun N-terminal kinase, which is translocated to the nucleus and activate transcription factors such as activator protein 1 (AP-1), that can converge to activate the apoptotic and survival responses[89-91].

Although the pathways behind TNFR2 activation remains poorly understood, when the TNF-α binds to TNFR2, its activation is mediated by TRAF2, and TNFR2 has been widely known as a mediator of the activation of genes related to cell survival and proliferation[91-93]. After TNF-α binds to TNFR2, TRAF2 is activated which, through common signaling pathways to TNFR1 activation, can activate NF-κB through IKK, and AP-1 *via* MEKK, which can also be activated *via* apoptosis signal-regulating kinase 1 [94]. Furthermore, activation of TRAF2 *via* TNFR2 can lead to the recruitment of cellular inhibitor of



DOI: 10.3748/wjg.v29.i18.2733

Figure 1 Tumor necrosis factor-alpha signaling pathways. TNF-α: Tumor necrosis factor-alpha; TNFR1: Tumor necrosis factor receptor 1; TNFR2: Tumor necrosis factor receptor 2; TRADD: TNF receptor associated protein with death domain; FADD: FAS-associated death domain protein; cIAPS: Cellular inhibitor of apoptosis; MEKK: MAPK kinase kinas; JNK: N-terminal jun kinase; ASK1: Apoptosis signal-regulating kinase 1; IKK: IκB kinase; NF-κB: Nuclear factor κB; RIP: Receptor-interacting protein kinase. Created with BioRender.com.

apoptosis (cIAPS), which will partially inhibit caspase activation and, for this reason, reduce apoptosis response[95]. When both TNFR1 and TNFR2 are activated together, cIAPS recruitment is reduced and the caspase activity, mainly mediated by TNFR1, is activated[91].

These receptors are involved in the pathogenesis of IBD and their expression is correlated with disease activity[96]. The sTNf binds selectively to the TNFR1 receptor, and its activation results in a signaling cascade with effects such as apoptosis, cell proliferation and cytokine secretion[24,97]. In contrast, the mTNF can bind both to TNFR1 and TNFR2[91]. TNFR1 signaling pathways deserve attention, due to cytotoxic effects triggered by activation of TNFR1 *via* sTNF binding and it could be noted that some aspects regarding TNFR2 function are still unclear[91].

Some studies pointed to the presence of a functional cross-talk between TNFR1 and TNFR2, whichever TNFR2 would act as an complement-dependent cytotoxic effect of TNFR1, thus being responsible for the inhibition of anti-apoptotic pathways and for the increase in the cytotoxicity triggered by TNFR1 when both receptors are co-expressed and activated[91,98,99]. This finding could be seen as a distinct situation of the classic phenomenon in which the balance between apoptotic and anti-apoptotic signals triggered by TNF-α determines the accuracy in cell signaling[91].

The TNFR2 pathway does not contain death domains and its stimulation can result in proliferation, migration and production of cytokines such as IL-1 and IL-6. This receptor not only activate an intracellular signaling pathway, but can also induce reverse signaling within the cell expressing TNF-α [100].

TNFR2 is also involved in several autoimmune diseases, playing a protective role or being involved in its development. Thus, TNFR2 is known to be involved in rheumatoid arthritis, CD, erythematosus systemic lupus, UC, scleroderma, among other diseases[10]. It was recognized that the expression of TNFR2 is more limited than that in TNFR1, *i.e.*, this finding suggests that the sTNF-mediating signaling pathway *via* TNFR1 drives a predominantly pro-inflammatory program, whereas mTNF binding to TNFR2 primarily initiates immune modulation and tissue regeneration[84]. Thus, an increase in TNF-α expression can cause mucosal barrier defects in patients with IBD, exacerbating inflammation[10]. However, only a part of the role of TNF-α receptors in the pathogenesis of IBD is understood. It is known that it can be indicated the presence of TNFR2 in the CNS, *i.e.*, in neurons of the cerebral cortex [83], and no data in the literature could identify the presence of TNFR2 in enteric neurons.

ANTI-TNF-α TREATMENT

The immune response plays an important role in the initiation and maintenance of UC[101]. Cytokines play key roles in inflammatory processes, such as targeting cell signaling molecules during inflammation and UC pathogenesis through different roles, such as the production of inflammatory mediators and activation of inflammatory pathways[5,57,102,103]. They are also involved in several biological processes, such as cell activation, differentiation and central factors in the process of developing the

inflammatory and immune response. In addition, studies provide evidence of their involvement in epithelial injury, and consequently, intestinal barrier injury and tissue damage[5,104,105].

The treatment approach in the control of inflamed GI tissue in IBD, including clinical, laboratory conditions, endoscopic and histological remission for a prolonged period, play an important role in its evolution and possibly modifying the natural course of the disease[9,106]. Recent studies have suggested that TNF-α is one of the main pro-inflammatory cytokines involved in the pathogenesis of IBD, as higher TNF-α levels are present in the serum of both patients with UC and CD[25].

For the treatment of IBD, different classes of drugs can be used, respecting the particularities of CD and UC, with the aim of alleviating the symptomatic crisis of patients, as well as inducing and maintaining remission of the disease[107,108]. Generally, the first treatment option for mild to moderate cases of UC and CD involves aminosalicylates, or 5-ASAs, and other treatments include the use of corticosteroids, immunosuppressants, and biological drugs[109,110].

Biological drugs used to treat IBD are monoclonal antibodies that neutralize/block key targets in the development of intestinal[27]. There are biological drugs that target, for example, integrins, interleukins, and cytokines, such as TNF-α, which are related to the development of the inflammatory process and the activation of immune system cells (Figure 2)[111,112].

Currently, four anti-TNF-α agents are indicated by various guidelines for the treatment of IBD: Infliximab; adalimumab; certolizumab pegol; and golimumab[28,29]. Infliximab and adalimumab are indicated for the treatment of both UC and CD, while golimumab is indicated only for the treatment of UC and certolizumab pegol only for CD. American Gastroenterological Association (AGA) and American College of Gastroenterology (ACG) guidelines recommend the use of infliximab, adalimumab, and certolizumab pegol to induce and maintain CD remission in moderate to severe patients unresponsive to corticosteroid or methotrexate treatment, and regarding UC, it could be noted that AGA and ACG recommend the use of infliximab, adalimumab and golimumab in patients who have not responded to conventional therapy[28,29]. These anti-TNF-α antibodies bind to TNF-α, blocking its harmful effects, such as NF-κB activation and increase of pro-inflammatory cytokines, that mediate intestinal inflammation[113,114].

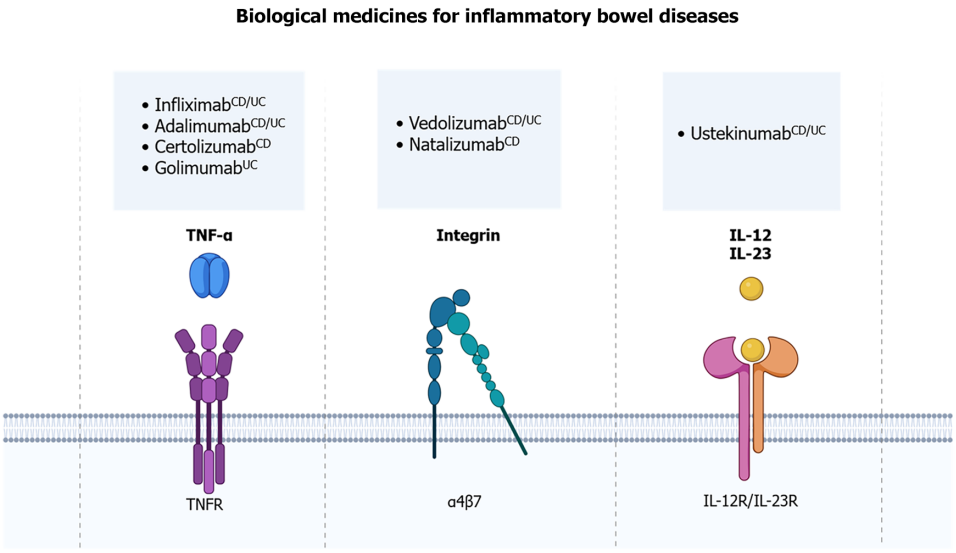
Intravenous and subcutaneous biologics targeting TNF-α have revolutionized the treatment of IBD, becoming the best available agents, when conventional therapy does not work, to induce and maintain IBD remission[115-117]. The application of this type of antibodies in patients with IBD induces a satisfactory clinical response in up to 60% of patients, and induced long-term maintenance of disease remission in most patients[24,30] and also reduces colorectal cancer risks[118]. Despite this, some patients do not improve after the use of these antibodies, some relapses within the first year of treatment and, one alternative used for the management of these cases has been to change the anti-TNF-α used for another one, or even to increase the dosage used[119]. Other alternative option for anti-TNF-α non responsiveness is the use other biological medicines like anti-integrin or anti-interleukin therapies.

Anti-integrin therapy, such as vedolizumab, which can be used for UC and CD treatment, block the integrin α4β7, which is expressed in B and T lymphocytes and interact with mucosal addressin-cell adhesion molecule-1 on intestinal vessels[120-122]. So, vedolizumab blocks lymphocyte trafficking only in the gut, preventing a systemic immunosuppression[123]. Otherwise, natalizumab blocks the integrin α4β7 and α1β7 and blocks lymphocyte trafficking in other organs, for example the brain, which can lead to infections such as progressive multifocal leukoencephalopathy[124]. For this reason, the use of natalizumab is very carefully, considering risk-benefits factors, and it is not available in some countries. Other biological medicine is ustekinumab, an anti-interleukin agent for subunit p40 of IL-12 and IL-23, used for CD and UC. This biological medicine attenuates the immune cell activation by these interleukins and consequently reduces inflammatory response and pro-inflammatory signaling[125-127].

Limitations of therapy with biological drugs include the high cost of adherence to therapy, differences in legislation and availability of these drugs in different countries, and the meticulous risk-benefit analysis related to the choice over treatment alternatives. However, new strategies and medicines have constantly been studied at pre-clinical and clinical instances for treatment of IBD.

ANTI-TNF-α TREATMENT AND APPROACH IN THE ENS

Although the involvement of TNF-α in the ENS is poorly described in the literature, it is reported that the ENS has TNF-α receptors and responds to the inflammatory stimulus, can lead to changes in motility patterns and fluid and electrolyte balance, a condition often found in patients with IBD[128]. When comparing the intestine, under normal conditions, and the intestine in IBD, some aspects must be considered (Figure 3). Under physiological conditions, large populations of microorganisms (bacteria, virus and fungi) inhabit the gut, which constitute the gut microbiota[129]. This microbiota establishes a symbiosis with the host[130]. The intestinal barrier, composed mainly by mucus layer and epithelial, is an intact and functional structure[131]. There is a balance between the levels of pro-inflammatory cytokines TNF-α, IL-12 and IL-23, and anti-inflammatory cytokines such as transforming growth factor-beta and IL-10 by innate immune cells, which leads to a balance between regulatory and effector T cells,



DOI: 10.3748/wjg.v29.i18.2733

Figure 2 Biological medicines for inflammatory bowel diseases. Biological medicines used for the treatment of inflammatory bowel diseases (IBD) can be classified into anti- Tumor necrosis factor (TNF)-α, anti-integrin and anti-interleukin therapies. Anti-TNF-α binds to TNF-α and inhibits TNF receptor activation. Infliximab, adalimumab, certolizumab pegol and golimumab are examples of anti-TNF-α used in the treatment of IBD. Vedolizumab and natalizumab are anti-integrin agents, which bind to the α4β7 integrin and prevent the migration of inflammatory cells into the intestinal tissue. Ustekinumab is an anti-interleukin that blocks interleukin (IL)-12 and IL-23, which cannot bind to IL-12 receptor and IL-23 receptor on T and B lymphocytes, thus reducing the inflammatory response in the gut. ^{CD}indication only for Crohn's Disease; ^{UC}indication only for Ulcerative Colitis; $^{CD/UC}$indication both for Crohn's Disease and Ulcerative Colitis. TNF-α: Tumor necrosis factor-alpha; IL: Interleukin. Created with BioRender.com.

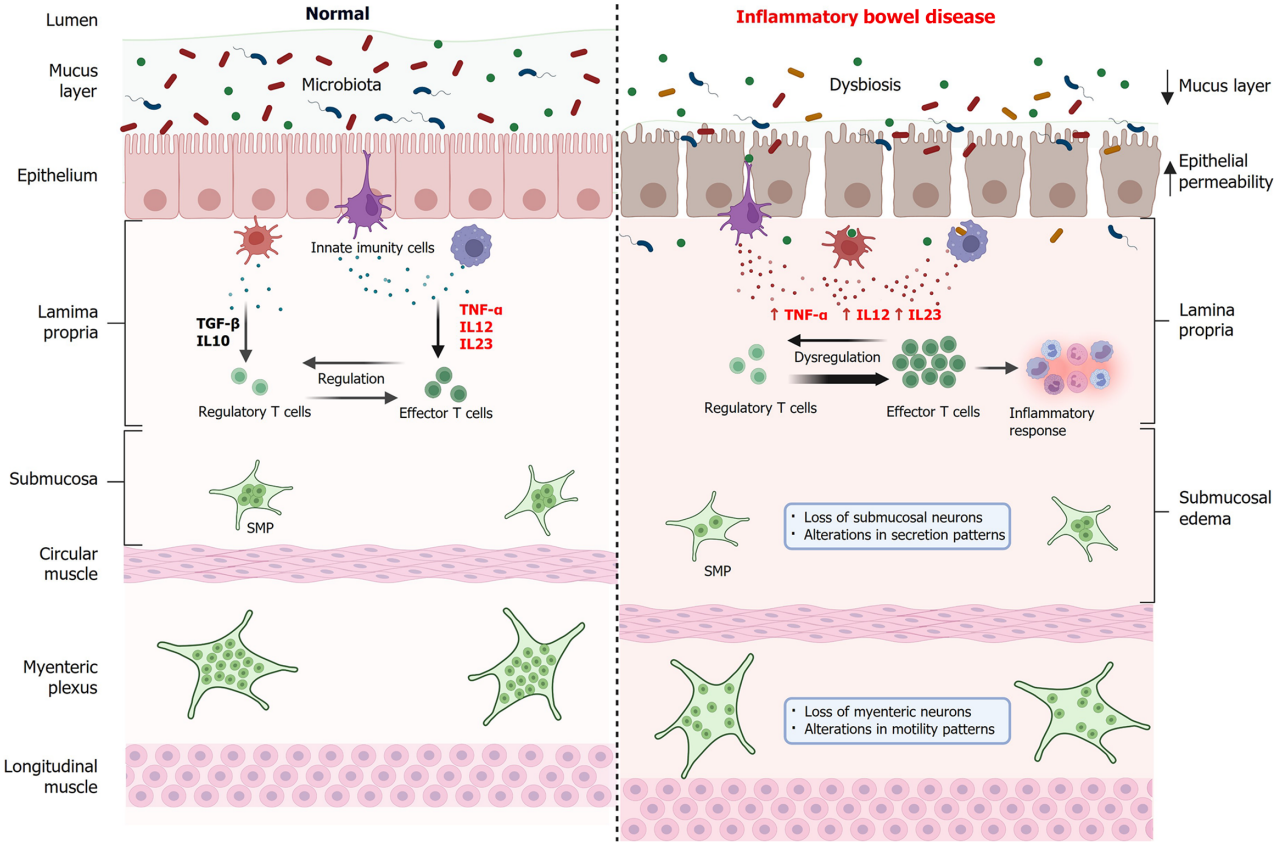
inducing tolerance to microorganisms from the gut microbiota[130,132]. In physiological conditions, both submucosal plexus and myenteric plexus are functional and controls, respectively, fluid secretion and intestinal motility.

In IBD, there is an imbalance in the gut microbiota (dysbiosis), the intestinal barrier is compromised, with a reduction in the mucus layer, weakening of the intercellular junctions and consequently increased epithelial permeability and entry of microorganisms in the lamina propria[15,133]. Innate immune cells increase the secretion of pro-inflammatory cytokines such as TNF-α, IL-12 and IL-23, which leads to a dysregulation of the immune system, which increases the activity of effector T cells which, in turn, recruit cells for the inflammatory response[134]. Morphological findings include submucosal edema, as well as a reduction in the number of neurons in the SMP, causing changes in secretion patterns and loss of neurons in the myenteric plexus, thus changing the motility patterns[51,52,135].

Experimental UC has been shown to affect enteric neurons, causing changes in the number of enteric neurons and glia, as well as changes in intestinal motility and secretion patterns[50-52,136]. As the presence of TNFR1 and TNFR2 have been reported in the ENS, the use of anti-TNF-α agents can exert a series of beneficial effects on ENS levels, improving the inflammatory response, peristalsis and intestinal secretion patterns, relieving symptoms such as diarrhea, fecal bleeding and colic. However, despite the important approach to the ENS and its relationship with IBD, literature data on anti-TNF treatment with analysis focused on the ENS are scarce. Therefore, further studies on the role of mechanisms/signaling pathways of sTNF, mTNF, TNF-α and their receptors in enteric neurons in IBD are needed, since this approach can guide the choice of a more adequate, effective anti-TNF-α agent with lower chances of failure responsiveness.

CONCLUSION

This review provided main details about TNF-α relationship with IBD. The role of TNF receptors in the development of IBD is an issue that deserves attention and may be a key to the treatment of UC and CD. In addition, anti- TNF-α treatments have been very promising in the treatment of IBD unresponsive to conventional therapies. The relationship of the ENS with TNF-α and its response to anti- TNF-α treatment are important aspects to be addressed, as they may direct new therapies and reduce non-responsiveness to specific anti- TNF-α agents.



DOI: 10.3748/wjg.v29.i18.2733

Figure 3 Comparison of the intestine in normal conditions and in the bowel with inflammatory bowel disease. Under normal conditions (left), the intestinal microbiota establishes a symbiotic condition with the host, the intestinal barrier is intact and functional, and there is a balance between the levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-12 and IL-23 and anti-inflammatory cytokines such as transforming growth factor beta and IL-10 by innate immune cells. In this way, there is a regulation of the immune response through a balance between regulatory T cells and effector T cells. Submucosal plexus (SMP) and myenteric plexus neurons are functional and controlling, respectively, fluid secretion and intestinal motility. In inflammatory bowel diseases (IBD) (right), there is an imbalance in the intestinal microbiota, the intestinal barrier is compromised, with a reduction in the mucous layer, increased epithelial permeability and consequent passage of microorganisms to the lamina propria. Innate immune cells increase the secretion of pro-inflammatory cytokines such as TNF-α, IL-12 and IL-23, which leads to a dysregulation of the immune system mediated by T cells, increasing the activity of effector T cells which, in turn, recruit cells for the inflammatory response. In IBD, submucosal edema is observed, as well as a reduction in the number of neurons in the SMP, causing changes in secretion patterns and loss of neurons in the myenteric plexus, resulting in changes in motility patterns. TNF-α: Tumor necrosis factor-alpha; IL: Interleukin. Created with BioRender.com.

FOOTNOTES

Author contributions: Souza RF, Caetano MAF and Magalhães HIR were responsible for the literature review, and analysis and written; Castelucci P performed the critical interpretation and revised the manuscript for intellectual content; all authors have read and approved the final manuscript.

Supported by Research Support Foundation of the State of São Paulo (FAPESP, Brazil), No. 2014/25927-2, No. 2018/07862-1; National Council for Scientific and Technological Development (CNPq, Brazil); and Higher Education Personnel Improvement Coordination (CAPES, Brazil).

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Country/Territory of origin: Brazil

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S-Editor: Chang KL

L-Editor: A
P-Editor: Chen YX

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World Journal of Gastroenterology

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World J Gastroenterol 2023 May 14; 29(18): 2747-2763

DOI: 10.3748/wjg.v29.i18.2747

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Current trends in acute pancreatitis: Diagnostic and therapeutic challenges

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Matsuo Y, Japan; Tellez-Avila F, United States

Received: January 9, 2023
Peer-review started: January 9, 2023
First decision: February 1, 2023
Revised: February 7, 2023
Accepted: April 18, 2023
Article in press: April 18, 2023
Published online: May 14, 2023

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Abstract

Acute pancreatitis (AP) is an inflammatory disease of the pancreas, which can progress to severe AP, with a high risk of death. It is one of the most complicated and clinically challenging of all disorders affecting the abdomen. The main causes of AP are gallstone migration and alcohol abuse. Other causes are uncommon, controversial and insufficiently explained. The disease is primarily characterized by inappropriate activation of trypsinogen, infiltration of inflammatory cells, and destruction of secretory cells. According to the revised Atlanta classification, severity of the disease is categorized into three levels: Mild, moderately severe and severe, depending upon organ failure and local as well as systemic complications. Various methods have been used for predicting the severity of AP and its outcome, such as clinical evaluation, imaging evaluation and testing of various biochemical markers. However, AP is a very complex disease and despite the fact that there are of several clinical, biochemical and imaging criteria for assessment of severity of AP, it is not an easy task to predict its subsequent course. Therefore, there are existing controversies regarding diagnostic and therapeutic modalities, their effectiveness and complications in the treatment of AP. The main reason being the fact, that the pathophysiologic mechanisms of AP have not been fully elucidated and need to be studied further. In this editorial article, we discuss the efficacy of the existing diagnostic and therapeutic modalities, complications and treatment failure in the management of AP.

Key Words: Pancreatitis; Etiology and pathogenesis; Diagnostic criteria; Nutrition; Antibiotics; Management of complications

Core Tip: Acute pancreatitis (AP) is an inflammatory disease of the pancreas, with abnormal trypsinogen activation as the primary pathogenesis and varies from clinically mild to fulminant form. Severe forms of AP are a relatively common cause of death. Progress in the establishment of biochemical, imaging and clinical criteria for the severity and prognosis of the disease has markedly influenced the therapeutic approach and the outcome of the disease. This article presents the diagnostic and therapeutic modalities with regard to their effectiveness, complications and treatment failure, as well as discussing some of the controversial issues in the treatment of AP.

Citation: Zerem E, Kurtcehajic A, Kunosić S, Zerem Malkočević D, Zerem O. Current trends in acute pancreatitis: Diagnostic and therapeutic challenges. *World J Gastroenterol* 2023; 29(18): 2747-2763
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2747.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2747

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory pancreatic disease affecting all ages, with an annual incidence of 10-50 cases per 100000 persons[1]. The main causes of AP are gallstone migration and alcohol abuse. Among these, predominance of one cause over the other depends on socioeconomic, ethnic, and cultural differences[2-5].

Most patients with AP present with a mild and self-limited disease. Conversely, 15%-20% patients with AP develop local and/or systemic complications, frequently leading in multiple (respiratory, cardiovascular, renal, and hepatic) organ failure (MOF) and death. According to the revised Atlantic classification, the severity of AP is divided into three levels: Mild, moderately severe, and severe, based on organ failure as well as local and systemic complications[3]. However, the pathophysiologic mechanism of AP has not been fully elucidated and there are several controversies regarding the diagnostic and therapeutic modalities due to their effectiveness and complications in the treatment of the disease. These controversies primarily relate to the therapeutic treatment at the early stage of the disease, which includes fluid resuscitation, including the most appropriate type of fluid to use, as well as the time, volume, and rate of administration. Other controversies include the timing of restart and the importance of nutritional support, the role of prophylactic antibiotics, the timing of application of more aggressive methods including surgery, as well as the treatment of complications which can negatively impact the patient's prognosis and quality of life[4-6].

This editorial article presents the most important diagnostic and therapeutic modalities with respect to their efficacy, complications and treatment failure in the treatment of AP.

ETIOLOGY OF AP

There are multiple causes and pathological conditions potentially associated with AP (Table 1). It is generally accepted that gallstones and alcohol abuse are responsible for about 90% of all cases of AP. The role of gallstones is very important in the etiopathogenesis of AP and any finding indicating the presence of gallstones in the gallbladder or biliary tract in patients with AP can be classified as the cause of the disease. Therefore, all patients with AP should be screened by ultrasound for the presence of cholecystolithiasis, common bile duct stones, or diagnose signs of biliary obstruction[2,7,8]. Alcohol-induced pancreatitis is more common in young and middle-aged people in whom an idiosyncratic sensitivity to alcohol may exist at levels of alcohol exceeding 80 g/dL. Other predisposing factors may be the level of alcohol dehydrogenase activity in the gastric mucosa and the liver[2,7,8].

Hypertriglyceridemia (triglycerides > 600 mg) is well-known cause of AP. One of the predominant causes of serum triglycerides elevation is alcohol intake. Therefore, it is sometimes challenging to assess whether the cause of AP is alcohol consumption or hypertriglyceridemia[9-11].

A large variety of drugs have been related to AP. Although, some drugs such as diuretics, azathioprine, sulfonamides, drugs used in the treatment of acquired immunodeficiency syndrome such as didanosine and zalcitabine, and steroids can cause AP through a direct toxic effect, most cases of drug-related pancreatitis are probably triggered by individual sensitivity. In large epidemiological

Table 1 Etiology of acute pancreatitis

Causes of acute pancreatitis
Toxic and metabolic
Alcohol
Hyperlipidemia (triglycerides > 600 mg)
Hypercalcemia (hyperparathyroidism)
Diabetes mellitus
Hypothyroidism
Uremia
Drugs (medicaments)
Scorpion venom
Mechanical
Gallstones, biliary sludge
Ampullary obstruction (Crohn's disease, villous tumors of the ampulla)
Pancreatic obstruction (pancreatic tumor, chronic pancreatitis)
Sphincter of Oddi dysfunction
Pancreas divisium
Post ERCP-pancreatitis
Congenital malformation
Trauma
Others
Ischemia
Organ transplantation (bone marrow transplantation)
Iatrogenic injury
Infection
Hereditary
Autoimmune
Cystic fibrosis
Tropical (Ascaris lumbricoides)
Idiopathic pancreatitis

ERCP: Endoscopic retrograde cholangiopancreatography.

studies, it has been proven that potentially pancreatotoxic drugs are not independent risk factors for the development of AP. The interval from the beginning of drug intake to the development of AP is highly variable and ranges from a few weeks, in a drug-induced immunologic reaction, to several months, when accumulation of toxic metabolites (*e.g.* valporic acid, pentamidine, didanosine) is required. The mechanism of AP for many of these medications is obscure[12-16].

PATHOGENESIS OF AP

AP is predominantly acute inflammatory process which involves the parenchyma of the pancreas, with involvement of other regional tissues or distant organ systems in severe forms of the disease. The pathogenic mechanism of AP is presented by inappropriate activation of trypsinogen and destruction of secretory cells followed by systemic release of cytokines and inflammatory mediators, causing the activation of inflammatory cells, fever and MOF. Calcium overload, mitochondrial dysfunction, impaired autophagy, endoplasmic reticulum stress, and exosomes are other factors in pathogenesis of the disease[16]. Edema of pancreatic and peripancreatic tissues and fat necrosis are common in all forms

of AP (mild, moderately severe and severe) however, in the severe AP (SAP), there is a possibility of hemorrhage within the pancreas[4,7,17,18].

Cellular necrosis elements (acinar cells, duct cell, and islet cells) are considerable in SAP but necrosis is usually absent in mild and moderate forms of the disease. During SAP, pancreatic necrosis (PN) develops due to impairment in pancreatic microcirculation and its complete development takes usually several (approximately 4-7) day after the beginning of the disease but, the development of PN is not strictly fixed in time and may progress during the first 2 wk[4,16-18]. During that period, PN is usual sterile and its infection is extremely rare. After the first 1-2 wk, the development of secondary infection in PN, due to translocation of intestinal flora, is associated with increased morbidity and mortality[4].

Early in the course of AP, acute pancreatic fluid collections (APFC) can occur as amylase-rich and protein-rich pancreatic juice collections and they usually resolve spontaneously. Pancreatic fluid collections, which present for more than 4 wk, are usually caused by disruption of pancreatic duct (PD) with extravasation of pancreatic juice and they are termed as pancreatic pseudocysts (PPC) or pancreatic walled-off necrosis (WON). Extra-pancreatic manifestations of SAP include systemic inflammatory response syndrome (SIRS) following by systemic MOF or exacerbation of serious pre-existing illness related to AP[4,16-18].

DIAGNOSIS OF AP

Diagnosis of AP is based on clinical presentation, laboratory tests, and imaging findings and requires two out of the following three criteria to be present: Clinical (acute pain attack in the upper abdomen spreading to the back), laboratory (serum lipase and/or amylase levels are three or more times higher than normal values) and typical imaging [computed tomography (CT), magnetic resonance imaging, ultrasonography] findings that are characteristic for AP[5].

The most common presenting symptoms of the disease are abdominal pain (80%–95%) followed by nausea and vomiting (40%–80%), rebound tenderness, breathlessness, impaired consciousness with pyrexia, distension and reduced bowel sounds[4,16].

The diagnosis of AP can be supported by serum and urinary laboratory tests to clarify its origin. They usually reflect organ dysfunction and metabolic disturbances. Amylase is traditionally the laboratory test of choice but, given its higher sensitivity and specificity, lipase (serum level greater than three times normal appears) is more valuable test for diagnosing of AP. The elevation of these enzymes in the serum occurs due to the leakage of pancreatic acinar cells into the interstitial space followed by their subsequent absorption into the circulation[19-21]. Some authors consider that the lipase/amylase ratio can be a crucial parameter in establishing alcohol as the etiology of AP. Transaminases are mostly used in differentiation biliary from other causes of AP[22]. The negative predictive value of urinary trypsinogen-2 level is 99%. Therefore, urinary trypsinogen-2 levels accurately diagnose AP and may be considered as useful markers to determine extra-pancreatic inflammation in AP[19,23,24]. Serum immunoreactive trypsin, chymotrypsin, elastase, phospholipase A2, alfa2-macroglobulin, methem-albumin, and carboxipeptides levels have been suggested in diagnosing AP. However, these tests are not in routine use and are not commercially available[25,26].

When the clinical presentation is typical but the laboratory parameters are ambiguous or inconclusive, imaging techniques are necessary. Contrast-enhanced CT (CECT) is the most performed imaging test and the modality of choice for the diagnosis of PN, the determination of its extent, and the diagnosis of local complications[4,27-29]. However, the complete development of PN takes usually several (approximately 4-7) days from the beginning of the disease and CECT cannot be applied to reliably assess the presence and extent of necrosis before that time[1,4].

Magnetic resonance imaging is a good alternative to CECT due to its superiority soft tissue contrast resolution and better evaluation of the biliary tree and PD. Also, this method can be used as a substitute for endoscopic retrograde cholangiopancreatography (ERCP) in the diagnostic evaluation of the PD[4, 30-32].

Ultrasound, endoscopic ultrasound and ERCP are adjuncts to CECT and they are used for the diagnosis of cholelithiasis, disconnected PD, evaluation of the collection contents, and follow-up imaging. Imaging examinations are usually not necessary in an emergency when the clinical presentation and laboratory tests are consistent with features of AP[1,4,28,33,34].

PREDICTION OF SEVERITY AND OUTCOME OF AP

AP varies from clinically mild to fulminating disease and has been recorded as a cause of sudden death. The outcome of acute attack depends in part on the etiology and in part on the severity of the attack. It is generally accepted that death from AP has a bimodal temporal distribution: Early mortality is the consequence of SIRS followed by MOF, while late mortality is caused by superinfection of PNs and peripancreatic fluid collections resulting in sepsis[4,7,34,35].

Advances made in establishing diagnostic criteria for the severity and prognosis of an attack have markedly influenced therapeutic approach. Several multifactorial scoring systems (Ranson, APACHE II, Glasgow-Imrie, SOFA, Balthazar, BISOP, *etc*.) have been extensively applied with the goal of predicting which patients might have a severe clinical course and which of them will recover without major complications[7,20,21,36-41].

A commonly accepted definition of SAP was not established until 1993, when the first version of the Atlanta classification for AP was published. The classification emphasizes the difference between milder interstitial form of AP from SAP related to 'local complications', but do not contain a clear definition of pancreatic and peripancreatic collections that was standardized worldwide[42]. The classification was revised in 2012[1,3], defining early and late AP which can be either edematous interstitial or necrotizing one. According to the severity of the disease, AP is divided into mild, moderately severe, and severe form, based on organ failure as well as local and systemic complications. AP collections are differentiated into APFC, PPC, acute necrotic collection, and pancreatic WON, according to the type of AP[1,3,7] (Figure 1).

However, the value of early prognostic evaluation of AP remains uncertain due to the fact that patients who have the same initial predicting scores often have very different subsequent clinical courses of the disease. Also, the relevance of the prognostic evaluation is markedly affected by the lack of widely accepted definition of severity and outcome of the disease[1,4,7].

INITIAL TREATMENT OF AP

AP is a dynamic disease process in which most attacks are mild (mild and moderately severe forms), AP is a dynamic disease process in which most attacks are mild (mild and moderately severe forms), with ongoing recovery after a few days of conservative supportive therapy. However, some patients with SAP can develop local and/or systemic complications and MOF. There are two treatment periods in AP: Early treatment of the acute attack should be applied in both mild and severe forms of AP, while late management includes treatment of SAP complications. The most important measures of intensive monitoring and supportive therapeutic procedures in AP are presented in Table 2.

In early stage of AP, all patients require appropriate conservative treatment and sufficient nutritional support. Most patients with mild or moderately SAP will recover with conservative treatment that includes correction of hypovolemia and hypoxemia, as well as pain relief. For a long time, correction of hypovolemia, even with mild AP, was carried out by early aggressive hydration with monitoring vital constants and urine output[3,5,6,42-44]. However, there is conflicting evidence regarding the fluid management strategy both in terms of fluid type, optimal volume and rate of administration, as well as severity of AP. Several recent randomized trials showed that early aggressive fluid resuscitation, in patients with AP, resulted in a higher incidence of fluid overload (with potentially increasing risk for acute kidney injury and pulmonary edema) without improvement in clinical outcomes[45-48]. Other, also randomized controlled trials, reported that early aggressive intravenous hydration hastens clinical improvement in patients with AP and that aggressive fluid strategy is beneficial especially for certain subsets of patients and some types of AP[48-51]. Most authors agree that these discrepancies are not fully elucidated and that future studies are needed to investigate which fluid management strategy is optimal for majority of the patients and which subsets of patients with AP may benefit from different management of fluid replacement[6,47].

Pain control is a very important therapeutic measure in the early stage of AP and can be provided by appropriate intravenous administration of a non-opiate analgesic. Opiates (*e.g*., meperidine) may also be given as required[52-54]. Some recent systematic reviews and meta-analysis suggest that epidural anesthesia is safe and effective in reducing pain severity, improving pancreatic perfusion, and decreasing mortality, within the first 24 h of AP onset. However, there is paucity of evidence to guide pain management in AP with small datasets per study[55,56] Hypoxemia is a rare event in mild and moderately SAP but, in severe forms of the disease, respiratory insufficiency with hypoxemia is often present as single organ failure. Hypoxemia could be avoided by ensuring airway patency and supplemental application of humidified oxygen, which would allow maintenance of arterial oxygen saturation above 95%. In case of development of respiratory insufficiency, mechanical ventilation with positive expiratory pressure is mandatory[4-6,43,44].

Conversely, patients with SAP are at high risk of developing PN, MOF, and septic complications. The most important therapeutic goals in the initial treatment of SAP are the provision of supportive therapy and the treatment of specific complications that may occur at the beginning of the disease[4].

Nutritional support

The nutritional management strategy for patients with AP has generated intense debate over the past few decades. Oral nutrition should be restarted immediately in patients with preserved gastrointestinal peristalsis, without abdominal pain, nausea, vomiting, or evidence for intestinal obstruction or ileus[5,18]. Most patients with SAP have increased basal energy needs, pronounced protein catabolism and endogenous gluconeogenesis.

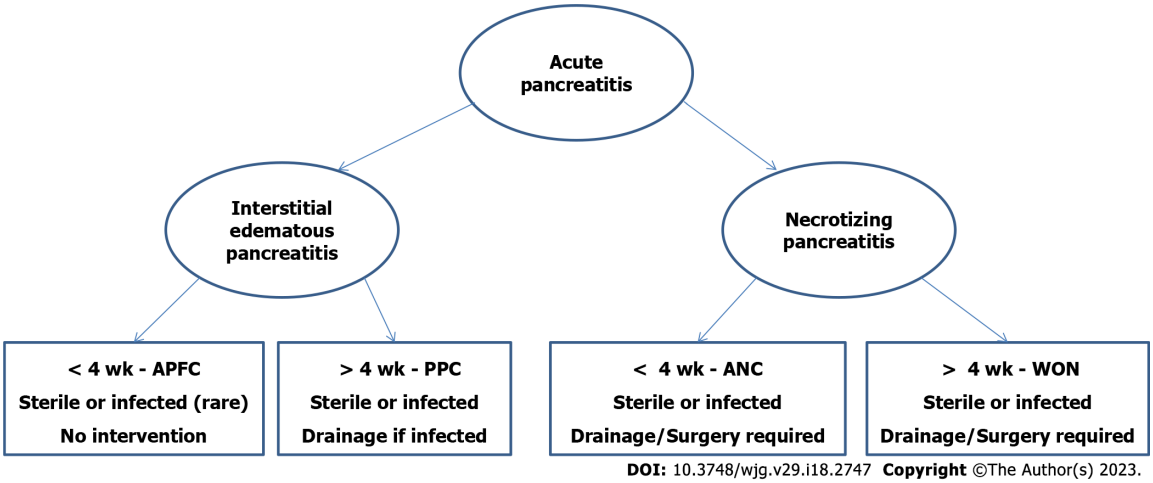
Table 2 Management of acute pancreatitis

Cornerstone treatment measures
Intensive monitoring and support of cardiac, pulmonary, renal, and hepatobiliary functions
Fluid resuscitation with monitoring of vital constants and urine output
Electrolyte solutions and Plasma expanders
Humidified oxygen administration
Catecholamine (dopamine, dobutamine) to prevent renal failure
Appropriate nutritional support
Early treatment of systemic complications
Mechanical ventilation with positive end-expiratory pressure
Catecholamine (epinephrine) if shock develops
Hemofiltration, dialysis
Insulin and calcium substitution to treat metabolic complications
Prevention of infectious complications
Biliary tract management
Management of necrotizing pancreatitis
Conservative, Imaging, Endoscopic, Surgical management
Management of late complications
Pancreatic pseudocysts
Walled-off pancreatic necrosis
Disconnected pancreatic duct syndrome/pancreatic fistula
Acute non-infectious complications in acute pancreatitis
Intra-abdominal hypertension
Pseudoaneurysm
Venous thrombosis
Bowel fistula
Management of special types of acute pancreatitis
Pediatric
Hyperparathyroidism
Hypertriglyceridemia
Post-ERCP pancreatitis
Trauma
Pregnancy
Long term complications and long-term care

ERCP: Endoscopic retrograde cholangiopancreatography.

The purpose of nutritional support is the reduction of wasting, to support the structure and function of organs, and to have a positive influence on the clinical course of the disease. Also, if patients with SAP develop paralytic ileus as a complication of the disease, keeping the pancreas at rest is necessary. Since they require nutritional support to achieve a positive nitrogen balance, parenteral nutrition should be started as soon as possible with the aim to achieve a positive nitrogen balance within the first 72 h after the onset of the disease[4].

However, SAP represents a typical model of septic syndrome due to the gastrointestinal barrier failure, which reduces gastrointestinal motility and damages mucosal integrity with subsequent increases in its permeability. This leads to an increased risk of bacterial overgrowth and their translocation from the intestinal tract to peripancreatic necrosis. Therefore, one of the main therapeutic goals in



DOI: 10.3748/wjg.v29.i18.2747

Figure 1 The revised Atlanta Classification: Key terms diagram. APFC: Acute pancreatic fluid collection; PPC: Pancreatic pseudo cyst; ANC: Acute necrotic collection; WON: Walled-off necrosis.

SAP is to maintain intestinal integrity to prevent bacterial and endotoxin translocation and improve the immune system of the gastrointestinal tract. Administration of enteral feeding, with or without immunonutrition can maintain mucosal integrity and prevent or decrease bacterial translocation. Therefore, early nutritional support should be prioritized as soon as possible (optimally, within the first 24–72 h)[4,18,57-59].

Role of antibiotics

The presence of infected PN is the most important negative indicator and is the main cause responsible for morbidity and mortality in SAP. The infection organisms that are responsible for PN infection are mostly Gram-negative bacteria of intestinal origin, and they can reach PN through a previously damaged intestinal mucosal barrier[4,18].

Broad-spectrum antibiotics with ability to pass into PN (*e.g.*, carbapenems, quinolones, and metronidazole), should be prescribed only when infected necrosis is confirmed or strongly suspected [18]. However, the administration of antibiotic prophylaxis in order to prevent infection of sterile necrosis is controversial. Some authors advocate the use of prophylactic antibiotics in SAP considering that they can prevent the development of superinfection in necrotic tissues, which is the only measure of initial treatment of PNs, since their development are not preventable[4,18,60-65]. Some authors consider that there is a reduction in pancreatic infection in the subgroup of patients who received broad-spectrum antibiotics, concluding that more evidence is needed[60,61].

However, multiple prospective, randomized, placebo-controlled trials and most important guidelines have demonstrated that the routine use of prophylactic broad-spectrum antibiotics, in patients with SAP, has no influence on the development of infected necrosis, systemic complications, need for surgery, or mortality[18,62-65]. Besides, prolonged antibiotic therapy increases the prevalence of fungal infections[4,18,61]. Evidence supporting the prophylactic use of antifungal agents in patients with PN is lacking. Also, the use of probiotic prophylaxis is not suggested for the prevention of infectious complications in AP[18,62,63].

MANAGEMENT OF NECROTIZING PANCREATITIS

The development of extensive PN is a main cause of complications and mortality in patients with SAP. Therefore, it is very important to apply the best methods to identify patients with PN who require more aggressive interventions than those who could be treated with less aggressive measures. Different clinical entities, such as persistent pancreatic fluid collections, pancreatic fistula, persistent SIRS, obstructive jaundice, and ongoing symptoms can be detected during SAP and they can predict the severity of clinical course of the disease. Their management vary depending on the severity and the type of complication, since different complications require different treatment modalities. The revised Atlanta classification offers useful recommendations to determine the strategy for management of the SAP complications[1,3-5,18,66].

Step-up approach

The step-up approach is used, in the treatment of infected PN, as a less invasive alternative approach compared to early surgical necrosectomy. This approach is based on the statement that surgical

debridement should be delayed until demarcation of necrotic from normal tissue is established, which would reduce the risk of bleeding into the necrotic tissue during or after the surgical intervention. Several studies that have conducted long-term follow-up of clinical outcomes in patients with AP have shown that the step-up approach leads to a reduction in morbidity and mortality and should be preferred over the classic surgical approach if both methods are technically feasible[4,8,18,67-71].

However, there are still certain disagreements regarding the comparison of the endoscopic and surgical step-up approach. In a randomized trial, Bang *et al*[72], compared outcomes of the surgical *vs* endoscopic step-up approach and they reported that the endoscopic was superior to the surgical step-up approach in reducing major complications, lowered costs, and increased quality of life in patients with infected PN[72]. Dutch Pancreatitis Study Group, in the TENSION trial, reported that the endoscopic approach may be more suitable than the surgical step-up approach, in the treatment of infected PN, based on favorable short-term outcomes. However, they presented that the endoscopic was not superior to the surgical step-up approach in reducing death or major complications in patients with infected PN, while the rate of pancreatic fistulas and length of hospital stay were lower in the endoscopy group[73-75]. Most experts in the discussion of this issue point out that the endoscopic approach is the best management for patients with infected PN, but that it is necessary to develop common protocols for endoscopic approach, based on all the observations and suggestions[76-79]. Finally, it can be concluded that, some segments of the step-up approach can be changed and improved, but its basic concept (delay, drain, and debride) remains as the reference standard intervention for PN[71].

The role of abdominal paracentesis drainage in necrotizing pancreatitis

If necrotizing pancreatitis is associated with liquefied necrotic debris in the pancreatic and peripancreatic regions, with the presence of abdominal and/or pelvic fluid several authors[80-85] have advocated the concept of removing the peritoneal fluid. The rationale for removing the peritoneal fluid is to reduce inflammation and disease severity since the intra-abdominal fluid, accumulated during the disease, may contain factors that trigger and increase the severity of AP, including proinflammatory mediators and infection.

In general, abdominal paracentesis drainage (APD) has the role of a preparatory procedure before the application of PCD with the intent to achieve better result than through conventional step-up approach. Therefore, the integration of APD into a step-up approach is beneficial for patients, due to the fact that this procedure removes great number of inflammatory factors from the seroperitoneum[82].

Management of sterile necrosis and acute fluid collections

Early in the course of AP, pancreatic inflammation, manifesting as partial or total PN, causes more liquefied areas and leads to extravasations of enzyme-rich pancreatic juice into the peripancreatic regions, with the consequent development of sterile necrosis and ANC. They usually resolve spontaneously and the vast majority of patients with sterile PN can be treated conservatively and without interventional procedures (*i.e.* catheter drainage or necrosectomy). However, if unresolved spontaneously, they can lead to a poor clinical course and allow active pancreatic enzymes to initiate physiologic pathways leading to MOF and sepsis[4,17].

Therefore, PCD or endoscopic drainage may be required in symptomatic patients with sterile PN and persistent malaise characterized by abdominal pain, nausea, vomiting, and nutritional failure or with associated complications, including gastrointestinal and/or biliary obstruction, persistent SIRS, or fistulas[5]. Continuous PCD may justify this approach based on the concept that elimination of cytokines and inflammatory mediators from initially sterile pancreatic juice collections may avoid or prevent systemic complications in severe forms of AP[4,17,86-90]. Besides, in cases whereby a high output of amylase-rich fluid continues to drain through the catheter, continued prolonged drainage is necessary despite the sterility of the collection[91].

Treatment of infected PN

The development of secondary infection in PN is associated with increased morbidity and mortality (15%–20%) and there is consensus that infected necrotic tissue should be removed in order to prevent the sepsis[4,8,33,92,93]. A small proportion of patients with documented infected PN who remain clinically stable can be managed with only conservative treatment and close monitoring, without intervention[5]. The traditional method of treating patients with infected PN by laparotomy retains its role in the treatment of the disease. However, surgical intervention is performed under general anesthesia and causes significantly greater trauma compared to minimally invasive methods, with possible consequent worsening of organ dysfunction, profuse bleeding and sepsis as well as postoperative mortality[8,94,95]. Therefore, laparotomy should be delayed for as long as possible or avoided in order to decrease mortality and morbidity rates[96]. Although there is no globally accepted treatment modality and it should be tailored to each individual patient, the step-up approach starting with monitoring and conservative measures, followed by PCD or endoscopic drainage and minimally invasive VARD has been shown to produce superior outcomes to the traditional open necrosectomy in the treatment of infected PN[4,8,18,66-72,97-99].

Disconnected PD syndrome

Disconnected PD syndrome (DPDS) is anatomic condition which is usually seen in PN. Early in the course of necrotizing pancreatitis, pancreatic inflammation causes extravasation of pancreatic secretion into the pancreatic and perpancreatic tissues with the consequent development of sterile PN which may lead to the PD disruption and interruption of continuity between the duct in the left sided pancreas and the luminal gastrointestinal tract. In such clinical setting, when the PD has no continuity with the viable tissue of the pancreas, this part no longer drains its contents into the duodenum, but produces a persistent pancreatic fistula, there is a high probability of consequent peripancreatic collection formation [1,18,100-102].

There are three types of DPDS: Concurrent (there is necrosis of the neck and body, but there is perfusion of the tail of the pancreas), delayed (PPC or WON occupy the middle part of the gland but with the perfused left-sided remnant of the pancreas) and DPDS associated with chronic pancreatitis (PD is blocked by a stricture or calculus in the proximal part, leading to atrophy of the distal segment of the duct and resulting in the formation of PPC)[1,102].

Standard treatment for DPDS is operative resection of the disconnected pancreas[103,104]. PN causing DPDS could be initially treated with percutaneous[105], endoscopic, or minimally invasive surgical techniques as temporary measures. However, elective distal pancreatectomy is mandatory in most patients as definitive treatment for DPDS[106-108].

Pancreatic fistula

Pancreatic fistulas are the consequence of pancreatic autodigestion or necrosis that results in a persistent PD disruption. Disruption of the PD secondary to PN causes the lack of continuity between PD and the viable pancreatic tissue, so that this segment of the pancreas no longer drains into the duodenum, but into the surrounding regions (usually in the pancreatic tail area) leading to its accumulation and PPC formation[1,4,18,100]. However, pancreatic secretions can also reach distant sites, causing pancreatic ascites, pleural effusion, distant PPC, or pancreatocutaneous fistula. According to that, pancreatic fistulas can be divided into two groups: (1) Internal, in which the PD communicates with the peritoneal or pleural cavity or some other hollow viscus; and (2) external, where the PD communicates with the skin[1,4,100,105,109].

Treatment of pancreatic fistulas depends on both the site of duct disruption and presence or absence of downstream ductal obstruction or DPDS. In the beginning of the disease, the management is usually conservative including total parenteral nutrition and the administration of pancreatic secretory inhibitor octreotide. However, if this management fails interventional procedures (Figure 2) and surgery are the following options. Surgical intervention, for fistula treatment, is technically challenging and could be followed with major complications[100-102,106-112].

Pancreatic WON

Pancreatic WON is a located WON of pus resulting from liquefaction of necrotic areas or secondary infection of acute PPC, with or without communication with main PD. WON occurs at a relatively late stage, most commonly three to five weeks after of onset of AP. APFCs tend to be poorly walled-off and can leak into retroperitoneum, the peritoneal cavity, the mediastinum, the pleura or the soft tissues. Pancreatic WON is a heterogeneous, low-density collection in a defined cavity containing gas bubbles (Figure 3). CECT scanning is the diagnostic test of choice. Diagnosis may be confirmed by percutaneous or endoscopic aspiration[113]. Asymptomatic WON does not mandate intervention and may resolve spontaneously over a period of time[29]. Symptomatic WON generally requires intervention. The most common treatment modality is PCD (Figure 2) or endoscopic drainage. Surgical drainage is done rarely, only when percutaneous drainage is not successful[114-116].

PPC

A PPC is a fluid collection usually found near the pancreas that is formed by the secretion of pancreatic juice from the inflamed parenchyma or from a disrupted duct. The PPC wall consists of fibrous non-epithelialized tissue[42]. PPC can sometimes appear at a great distance from the pancreas (*e.g.* thorax, groin) when the fluid dissects through tissue planes. At early stage of AP, APFC are common, but majority of them regress spontaneously and need no treatment. About 5% of patients with APFC develop PPCs, which are defined by their ellipsoidal shape and well-formed wall. Treatment for PPCs varies depending on their size and the presence of symptoms. Asymptomatic PPCs, less than 50 mm in diameter, should only be monitored by ultrasound. However, some of them may persist and progress to produce complications such as pain, infection, gastric outlet, intestinal or biliary obstruction. These PPCs are symptomatic and require treatment. The most common treatment modalities for symptomatic PPCs are minimally invasive approaches, such as PCD or endoscopic drainage. Surgical drainage is done rarely, only when percutaneous drainage is not successful[4,97,105,110,115-119].

Hemorrhage

Upper gastrointestinal bleeding is common in AP and usually results from stress ulcers, peptic ulcer disease, or hemorrhagic gastroduodenitis. Massive hemorrhage occurs rare in AP, most commonly into

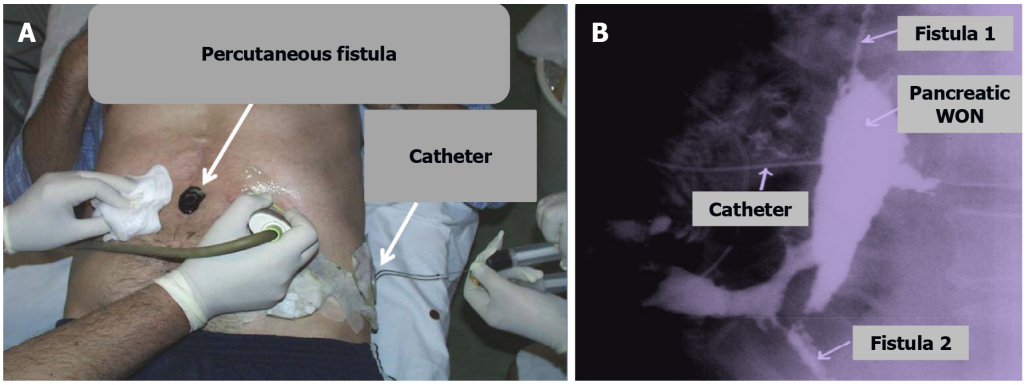


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Figure 2 Treatment of pancreatic walled-off necrosis and two fistulas by percutaneous drainage. A: Appearance of iodine *via* cutaneous fistula after its instillation into the walled-off necrosis (WON); B: Retropancreatic WON with two fistulas. Fistula 1: Retropancreatic-percutaneous fistula; Fistula 2: Retropancreatic-sigmoidal fistula. WON: Walled-off necrosis.

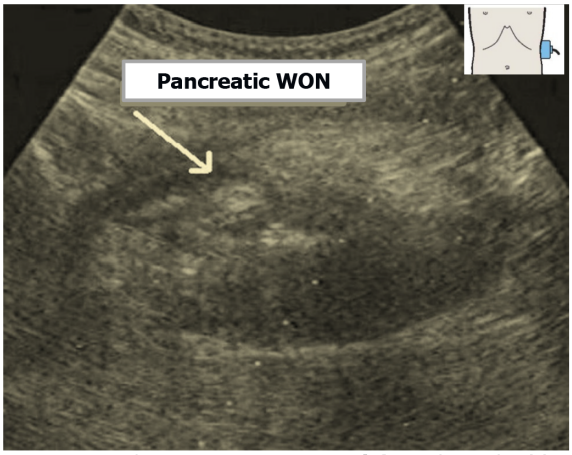

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Figure 3 Ultrasound appearance of pancreatic walled-off necrosis after acute necrotizing pancreatitis. WON: Walled-off necrosis.

the gastrointestinal tract, the abdominal cavity or into the PD. Erosion of the great pancreatic or peripancreatic vessel leads to rupture or formation of a pseudoaneurysm. The splenic artery is most often affected vessel, followed by the pancreaticoduodenal and gastroduodenal arteries[42,63]. Pseudoaneurysm formation should be suspected if there are repeated episodes of gastrointestinal bleeding, an increasing pulsatile abdominal mass and in patients with bloating and increasing abdominal pain. Unfortunately, aneurysm rupture in these arteries mostly results in severe and life-threatening bleeding. The diagnosis can be made by angiography or angio-CT. Sometimes, arteriography with embolization of bleeding vessel in order to stop the bleeding can be performed as a temporary measure. Otherwise, some patients will require urgent surgery to stop the bleeding[33,63,120].

How to prevent relapses of AP?

The prevention of relapses of AP starts with the current episode treatment. Potential etiologic causes should be determined and adequately treated in order to prevent future relapses of the disease. This implies an adequate and prompt diagnostic and therapeutic approach to patients with biliary stones and sludge, as well as to patients with hyperlipidemia and hypercalcemia[1,5,7,16].

AP caused by hyperlipidemia tend to develop more severe forms of the disease and up to 50% of them develop SAP. Therefore, appropriate diet and drug management of the lipoprotein metabolic disorders as well as alcohol abstinence are crucial in preventing relapses of pancreatitis[9-11]. Morphologic abnormalities and tumors also have to be excluded.

After discharge from the hospital, patients with AP should follow a diet without alcohol and high fat food consumption, eating frequent small meals four to six times per day. A few months after the acute phase of AP, patients can try to introduce a diet with a slightly increased fat content and grilled meat. Patients should be warned that the next attack of AP could be more dangerous than the previous one, so lifestyle changes are necessary to reduce the risk of disease relapse[16,121,122].

CONCLUSION

AP is an inflammatory pancreatic disease that is characterized by inappropriate activation of trypsinogen and destruction of secretory cells which leads to activation of inflammatory cells, fever, and MOF. Diagnosis of AP is based on clinical, laboratory and imaging parameters, which are included into prognostic scoring system, developed with the aim of predicting the severity of the disease. Advances made in establishing diagnostic criteria for the severity and prognosis of AP have markedly influenced therapeutic approach and reduced the mortality rate of the disease.

There are two treatment periods in AP: Initial management remains supportive, consisting of conservative treatment which should be applied in both mild and severe forms of AP, while late management incorporates the treatment of the SAP complications. Currently, the treatment of SAP complications has shifted from early surgical approach to minimally invasive step-up strategy as the reference standard intervention. However, AP is a complex disease and despite the existence of numerous criteria, it is difficult to predict its clinical course. Additional researches, preferably randomized trials or prospective collaborative studies, are required to increase understanding of the pathophysiology of the disease and enable adequate responses to the diagnostic and therapeutic challenges, in order to improve the management of particularly severe forms of AP.

FOOTNOTES

Author contributions: Zerem E contributed to conception and design of the paper, writing of the paper and final revision; Kurtcehajic A, Kunosić S, Zerem Malkočević D and Zerem O contributed to the literature search, writing of the paper and final revision of the paper.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Zhao S

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World Journal of Gastroenterology

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World J Gastroenterol 2023 May 14; 29(18): 2764-2783

DOI: 10.3748/wjg.v29.i18.2764

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Wingless/It/β-catenin signaling in liver metastasis from colorectal cancer: A focus on biological mechanisms and therapeutic opportunities

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Hashimoto K, Japan; Takemura N, Japan

Received: January 23, 2023
Peer-review started: January 23, 2023
First decision: February 7, 2023
Revised: February 28, 2023
Accepted: April 17, 2023
Article in press: April 17, 2023
Published online: May 14, 2023

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Abstract

The liver is the most common site of metastases in patients with colorectal cancer. Colorectal liver metastases (CRLMs) are the result of molecular mechanisms that involve different cells of the liver microenvironment. The aberrant activation of Wingless/It (Wnt)/β-catenin signals downstream of Wnt ligands initially drives the oncogenic transformation of the colon epithelium, but also the progression of metastatization through the epithelial-mesenchymal transition/mesenchymal-epithelial transition interactions. In liver microenvironment, metastatic cells can also survive and adapt through dormancy, which makes them less susceptible to pro-apoptotic signals and therapies. Treatment of CRLMs is challenging due to its variability and heterogeneity. Advances in surgery and oncology have been made in the last decade and a pivotal role for Wnt/β-catenin pathway has been recognized in chemoresistance. At the state of art, there is a lack of clear understanding of why and how this occurs and thus where exactly the opportunities for developing anti-CRLMs therapies may lie. In this review, current knowledge on

the involvement of Wnt signaling in the development of CRLMs was considered. In addition, an overview of useful biomarkers with a revision of surgical and non-surgical therapies currently accepted in the clinical practice for colorectal liver metastasis patients were provided.

Key Words: Wingless/It/β-catenin signaling; Colorectal cancer; Epithelial-mesenchymal transition/mesenchymal-epithelial transition; Liver metastasis; Markers; Surgical and non-surgical therapies

Core Tip: The liver is the most common site of metastasis in patients with colorectal cancer. Wingless/It (Wnt)/β-catenin signals can drive progression and metastatization by epithelial-mesenchymal transition/mesenchymal-epithelial transition. In the hepatic microenvironment, metastatic cells can survive through dormancy and become refractory to therapy. Further studies are needed to elucidate involvement of Wnt signaling in the development of colorectal liver metastases and to improve current surgical and non-surgical therapeutic approaches.

Citation: Selvaggi F, Catalano T, Lattanzio R, Cotellese R, Aceto GM. Wingless/It/β-catenin signaling in liver metastasis from colorectal cancer: A focus on biological mechanisms and therapeutic opportunities. *World J Gastroenterol* 2023; 29(18): 2764-2783
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2764.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2764

INTRODUCTION

In patients with colorectal cancer (CRC), the liver and the peritoneum are the most common sites of visceral metastases[1]. Especially in Western countries, CRC appears to be a primary cancer with a predominant ability to metastasize to the liver[2-4]. Although CRC represents the third most common cancer worldwide, it appears to be the leading cause of death in both sexes[1,5,6]. Colorectal liver metastases (CRLMs) develop in 15%-60% of CRC patients[7-9]; at the time of diagnosis, approximately 20%-34% of CRC patients have synchronous liver metastases and more than 50% develop distant metastases within 5 years of primary tumor diagnosis[2,10]. If untreated, median survival of patients with CRC and unresectable CRLM is 5-10 mo[11]. CRLMs are the consequence of sequential molecular events. CRCs are caused by an aberrant Wingless/It (Wnt)/β-catenin pathway, which in 70%-80% of cases is rooted in mutational inactivation of the tumor-suppressor gene adenomatous polyposis coli (*APC*)[12,13]. Aberrant activation of both canonical and non-canonical Wnt/β-catenin signalings, downstream of Wnt ligands, initially drives the process of colon epithelial oncogenic transformation [14]. Particularly in CRC cells, activation of the canonical pathway induces transcriptional regulation of molecules that control cell division, apoptotic evasion, and metabolic demand of the microenvironment, which favor tissue growth. In contrast, in the non-canonical pathway, β-catenin-independent signal transduction controls cytoskeleton activation and invasiveness[14]. Increased nuclear β-catenin has been documented in the invasive front of primary CRC and in the liver metastases; furthermore, this increased expression has been correlated with increased invasive capacity and synchronous CRLM formation[15-17]. Wnt signaling pathway, initially deregulated in CRC tumorigenesis, may crosstalk with RAS-extracellular signal-regulated kinase (ERK)[18], epidermal growth factor receptor (EGFR) cascade[19] and also with vascular endothelial growth factor (VEGF)[19,20]. CRLM differs in *WNT* and *EGFR* gene expression compared with normal liver tissue, showing a high degree of heterogeneity[21]. Treatment of metastatic CRC is challenging because of its variable and heterogeneous characteristics. Advances in the field of oncology have been made in the last decade, and a central role of Wnt/β-catenin pathway has been recognized in CRC chemoresistance[17]. The molecular features of intrahepatic metastatic tissue, including the mutational status of *EGFR* or *VEGF*, may be a therapeutic target to increase the efficacy of neoadjuvant chemotherapy[22]. Current treatments, including radical surgery as well as systemic and localized therapy, achieve clinical results in only a minority of patients with CRLM, who have also a high recurrence rate. Surgical resection appears to be the unique treatment that can offer long-term survival and a better chance of cure, although it applies to only one-third of the patients with CRLM[23]. Unfortunately, about 80% of patients have unresectable metastatic lesions at the time of diagnosis, and for this, 5-year overall survival is low, with a rate of around 48%[24]. For these reasons, development of new integrated treatments has to be advocated to improve CRLM patient clinical outcome. According to these concepts, it is important to remember that early CRC diagnosis remains paramount to achieve a better prognosis[25,26].

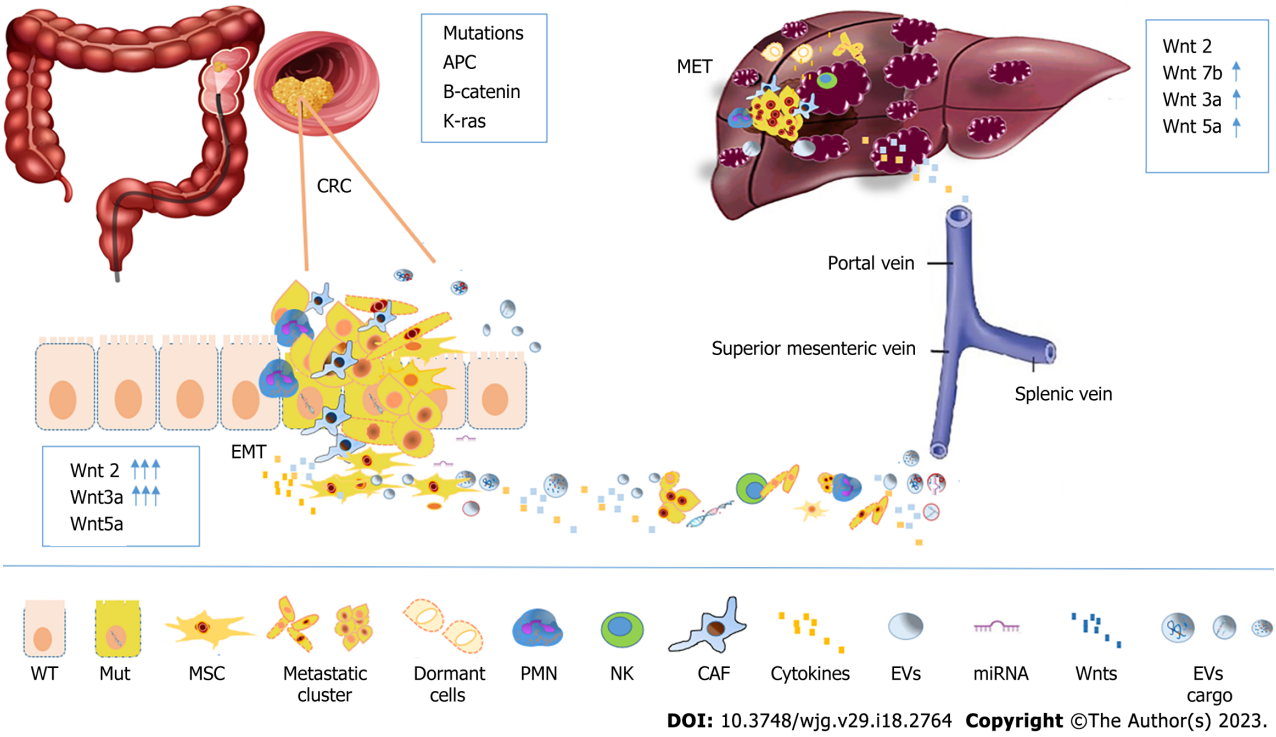
A better understanding of the mechanisms that regulate CRLM holds great potential for both adapting conventional therapies and developing new diagnostic methodologies. In this review, current knowledge on Wnt signaling in the process of CRLM was considered. In addition, an overview of valuable biomarkers with a revision of surgical and non-surgical therapies accepted in the clinical practice for CRLM patients were provided.

WNT SIGNALING: A DRIVER OF LIVER METASTASES FROM CRC

Cancer metastases result from complex selective processes depending on hepatic tissue anatomical, biological and microenvironmental factors. Evidence of metastatic propensity and organ-specific tropism of metastatic tumor cells mirror the concepts of "seed-soil", pre-niche and crosstalk between tumor cells and immune cells. These events allow tumor cells dislocated from the primary site to express their anchoring features on the tissue to be colonized as a site of implantation. Understanding of the interdependence of these biological mechanisms can provide valuable insights into treatment of CRLM[24]. During the metastatic event, tumor cells go through several stages, which include: Epithelial-mesenchymal transition (EMT) process, local tissue and vascular invasion, transition into the vascular system, extravasation process and seeding into the niche of the hepatic tissue. At the end of this multistep and dynamic model, metastatic cells finally have to be able to survive and grow by integrating into the cell community of the metastatic site[27]. Wnts are secreted glycoproteins that regulate multiple signaling pathways through both β-catenin-dependent and -independent mechanisms[28]. Aberrant activation of cellular pathways by Wnt ligands and β-catenin-dependent signaling promotes tumor progression and regulates EMT in CRC[1]. Wnt pathway somatic mutations are present in approximately 80%-90% of CRC patients. Both tumor cells and the surrounding microenvironment can express and release specific hyperactive Wnt ligands that can drive metastases even in cells with APC-inactivating mutations. However, several follow-up studies have revealed that the most aggressive subtypes of CRC do not show the highest levels of Wnt signaling[29]. RNA sequencing has shown that interference with Wnt signaling leads to up-regulation of gene programs that promote cell migration, invasion and downregulation of inflammation signatures in the tumor microenvironment (TME)[29]. Furthermore, it is believed that multiple ligands of Wnts may trigger numerous signaling pathways in addition to the β-catenin-mediated one; some of these alternative pathways have not yet been adequately studied in liver metastases[30]. Alterations in gene and protein expression allow CRC cells to make EMT/mesenchymal-epithelial transition (MET). EMT/MET program generates migrating tumor cells with intermediate phenotypic characteristics (Figure 1). CRC cells with a hybrid EMT/MET program migrate individually or in clusters through local or systemic spread[31]. Wnt signaling dysregulation activates downstream EMT by promoting emergence of migrating cancer stem cells (mCSCs) at the invasive front of the primary lesion. MCSCs invade locally through the driving force of the local TME and form distant metastases (Figure 1)[32].

Tumor progression results from interaction and cooperation between tumor and stromal cells of the hepatic microenvironment. In particular, inflammatory and metabolic signals can significantly influence rooting of metastatic cells in tissue niche, which can lead to reversion from EMT to MET and to their integrated adhesion with the new tissue site (Figure 1). The microenvironmental signals then induce EMT reversal (also called MET) to establish secondary micrometastases (colonization)[33-35].

Recent studies have revealed that cancer cells can follow an intermediate metastatic transition, with a mixture of cells showing features of either epithelial, mesenchymal phenotype or both at the molecular and morphological levels[36,37]. In intermediate stages, "quasi-mesenchymal" cells, which are mobile, more challenging to kill, and aggressive, express the *CDH1* gene (coding for E-cadherin protein) at the transcriptional level without displaying E-cadherin protein on the cell surface[38]. At this intermediate stage the cells have characteristics similar to stem cells and may co-express genes typical of both the epithelial and mesenchymal phenotype[39]. EMT/MET hybrid intermediates can promote metastatic tendency. It is unclear whether EMT/MET hybrid intermediate subpopulations may be responsible for treatment failure (immunotherapy, radiotherapy and/or chemotherapy). EMT can be regulated by different transcription factors and their gene regulatory networks that drive multiple levels of molecular changes. Some key transcription factors, such as Snail family transcriptional repressor 1 (Snail1)/Slug and zinc-finger E-box binding homeobox 1/2 (Zeb1/2), and myocyte enhancer factor 2A, promote the mesenchymal phenotype, while other transcription factors, such as ovol-like zinc finger 1/2 and grainyhead like transcription factor 2, suppress it by promoting the epithelial phenotype[40-43]. These transcription factors can act as either activator or repressor of downstream target gene expression, depending on Wnt signaling pathway activation level. One well-studied example involves the transcriptional activities of zinc finger E-box binding homeobox 1 (ZEB1) and Wnt/β-catenin signaling that mutually modulate each other, as ZEB1 potentiates transcription factor 4/β-catenin-mediated transcription, which in turn transforms ZEB1 from repressor to activator[44]. In addition to modulating its functions as either activator or repressor, Wnt/β-catenin signaling regulates ZEB1 protein expression [44].



DOI: 10.3748/wjg.v29.i18.2764

Figure 1 Metastatic liver microenvironment in colorectal cancer. Genetic changes such as adenomatous polyposis coli, β-catenin mutations or K-RAS mutations can generate epithelial-mesenchymal transition/mesenchymal-epithelial transition; aberrant activation of Wingless/It (Wnt)2, Wnt7b, Wnt3a and Wnt5a ligands promotes local invasion of mesenchymal stem cell and cell migration from primary lesion to form distant colorectal liver metastases; tumor microenvironment, including extracellular matrix, blood vessels, extracellular vesicles, different types of cells, such as cancer and immune cells, proinflammatory cytokines, chemoattractants, and angiogenic factors, influences the metastatic cell colonization in tissue niches. EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; APC: Adenomatous polyposis coli; Wnt: Wingless/It; CRC: Colorectal cancer; MSC: Mesenchymal stem cell; CRLMs: Colorectal liver metastases; WT: Wild type; MUT: Mutant; PMNs: Polymorphonuclear neutrophils; CAF: Cancer-associated fibroblast; NK: Natural killer; EVs: Extracellular vesicles; miRNA: MicroRNA.

EMT regulation is also influenced by the action of microRNAs and long noncoding RNAs, as well as chromatid and post-translational modulations[43,45,46]. In the liver, Wnt/β-catenin signaling has physiological functions related to hepatocellular growth, metabolic liver zonation and regeneration[47]. Signal transduction of the canonical pathway mainly involves β-catenin and its ability to modulate T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF)-dependent nuclear transcription factors, together with the activation of genes involved in cell proliferation, survival, differentiation, and migration, such as matrix metalloproteinases and c-Myc[48]. The non-canonical Wnt signals are many and involve calcium-dependent and independent pathways, as well as pathways mediated by c-Jun N-terminal kinase (JNK), protein kinase C, Ca^{2+}, Rho-type GTPases, mitogen-activated protein kinases (MAPK), and nuclear factors such as JUN/FOS and nuclear factor of activated T-cells[28]. While the canonical pathway drives tumor cells towards undifferentiation and growth, the non-canonical pathways appear to be involved in remodeling tissue architecture and mesenchymal differentiation.

Wnt family proteins transduce signals from tissue microenvironment through Frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP) 5/6 receptors to the Wnt/β-catenin signaling as the canonical Wnt pathway and through FZD and/or tyrosine kinases ROR1/ROR2/RYK receptors to the Wnt/planar cell polarity cascade, while Wnt/receptor tyrosine kinase transduces Wnt signals to Wnt/Ca^{2+} signaling called non-canonical pathway[14,28].

Wnt signaling inhibits glycogen synthase kinase-3β (GSK3β) to stabilize β-catenin, which translocates into the nucleus to recruit transcription factors, LEF and TCF, and to promote the expression of SNAIL1 and SLUG, which modulate EMT. Loss of E-cadherin has long been believed to be a hallmark of EMT [49]. Its suppression is mainly attributed to the functions of SNAIL and SLUG expression, which directly bind to the E-box of the promoter region and downregulate its expression[49].

In adult tissue, renewal cell fate decisions appear to be distinct between the opposing Notch/Wnt responses. Crosstalk between Notch and Wnt allows signaling across the two pathways to be resolved into Notch-ON/Wnt-OFF[50]. Notch signaling indirectly activates β-catenin to promote and regulate EMT. Indeed, activation of Notch results in cleavage of Notch intracellular domain, which undergoes nuclear translocation of transcriptional factor, binds to SNAIL promoter, and regulates the mRNA level of SNAIL1/2 and ZEB1/2[35].

Interestingly, Wnt2 mRNA is frequently up-regulated in colorectal polyps, CRC, and CRLM[51]. Wnt2 contributes to CRC-derived cell invasive and metastatic ability by generating genetic changes in fibroblasts. This process seems to occur through extracellular vesicles (EVs) that play a key role in CRC genesis by activating Wnt signaling[17]. Wnt2 protein released from cancer-associated fibroblasts (CAFs) enhances invasion and migration of CRC cells[52]. Activation of Wnt7b may trigger EMT process through Wnt/β-catenin signaling and promote CRLM. Overexpression of the Wnt3a ligand can stimulate Wnt/β-catenin pathway in such a way as to modify cell morphology, regulate EMT and thus favor invasive capacity of tumor cells (Figure 1)[52]. In CRC, Wnt3a and Wnt5a are highly expressed at both primary and metastatic sites. In particular, Wnt3a expression increases at the primary site with a concordance rate higher than 70%. Wnt5a shows no correlation with pathological features or the expression of invasion-related proteins[13]. The expression profile suggests that Wnts might be involved initially when CRC develops and during tumor progression[13]. Recently, several Wnt/β-catenin target genes, including *S100A4*, *p16INK4a* and *BAMBI*, have been identified, showing the ability to promote cell migration *in vitro* and metastases *in vivo*[53]. Interestingly, three Wnt/β-catenin target genes, *5 BOP1* , *CKS2* and *NFIL3* have been found to be correlated with experimental metastases[53].

Increased expression of miR-92a-3p activates the Wnt/β-catenin pathway and promotes stemness, EMT, and metastases from CRC cells[52]. Nuclear β-catenin expression at the invasive front and in CRC tissue vasculature predicts metachronous liver metastases[51]. Both canonical and non-canonical Wnt signaling cascades play a key role in the development and evolution of CSCs. In addition to the classical reversible EMT/MET-driven transport pathway (hybrid-EMT), an alternative cell death process-driven transport pathway [blebbishield metastatic-witch (BMW)] involving a reversible cell death process has been identified[54]. Knowledge of EMT and BMW pathways is important for metastatic tumor therapy, as these pathways confer drug resistance and immune evasion/suppression in the context of coordinated oncogenic, metabolic, immunological, and cell biological events that drive metastases[54]. Specifically, in the tissue microenvironment, cell death signals such as apoptosis, ferroptosis, necroptosis, and neutrophil extracellular traps formation (NETosis) related to BMW or EMT pathways recruit immune cells that may despite themselves promote migration of cancer cells to distant sites to establish metastases[54]. Proinflammatory molecules of the TME may modulate CRC progression. In TME, stromal cells secrete multiple factors, such as chemokines that attract inflammatory cells producing soluble cytokines that promote tumor cell survival. Indeed, high levels of tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β and chemokines, such as CXC ligand of chemokines 1 (CXCL1), CXCL2 and CXCL12, counteract host defense mechanisms. Increased levels of IL-6 expression are associated with advanced-stage CRC, since liver metastasis formation is supported by CAFs involved with the creation of a prometastatic microenvironment through IL-6 and monocyte chemo-attractant protein-1 activation. On the other hand, IL-1β activates inflammasome and induces angiogenesis in both the primary colon cancer and metastases[55]. High expression of chemokine receptor type 4 (CXCR4) is observed in patients with liver metastases from CRC, while its ligand CXCL12 is highly expressed in the most frequent metastatic sites of CRC, such as liver, lymph nodes, and lungs, and is a chemoattractant for CXCR4-positive cancer cells[56,57]. Some of these inflammatory mediators, such as TNF-α, may also fulfil the role of tumor suppressors, reconstitute TME by increasing cytotoxic T-cell activity, mature dendritic cells and prevent neoangiogenesis[55].

Immune components of TME may modulate tumor progression and represent interesting therapeutic targets in liver metastases. Indeed, CRLM is also promoted by activating TDO2-kinurenin-AHR pathway, which facilitates programmed cell death protein-1 (PD-1)-mediated immune evasion and maintenance of stemness through Wnt signaling[58]. Recently, in a single-cell analysis on intratumor mutational diversification of CRC cells, a highly heterogeneous tumor immune microenvironment has been found to be enriched with the granulocyte component in CRLMs. Therefore, it was proposed that activation of Wnt signaling coupled with ferroptosis death may promote granulocyte migration into the tumor and metastatic microenvironment (MME)[59].

Metastatic organotropism is believed to be a process that relies on the intrinsic properties of tumor cells and their interactions with molecules and cells in the microenvironment. Even before tumor cells spread, hepatocytes secrete multiple factors to recruit or activate immune cells and stromal cells in the liver to form a favorable premetastatic niche. Liver-resident cells, including Kupffer cells, hepatic stellate cells (HSCs), and hepato-sinusoidal endothelial cells, are co-opted by recruited cells, such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages to establish an immunosuppressive hepatic microenvironment suitable for tumor cell colonization and growth. For these reasons, understanding of the mechanisms that regulate metastasis-prone hepatic immune microenvironment could facilitate immuno-oncology interventions for treating CRLM[27,59,60].

The spread of tumor cells and their ability to survive and grow in a secondary site require intercellular communication pathways with other cells residing in the tissue microenvironment. In recent years, several signaling cascades have been found to use EVs in tumor-stroma interaction. Indeed, modulation of Wnt signaling may also be associated with EVs formation. Tumor cell-derived EVs exert their protumorigenic effects through direct interactions among biologically active surface molecules, transfer of proteins and nucleic acids into recipient cells, or transfer of metabolites that can be used as energy source by the recipient cell; these events induce physiological and phenotypic alterations in tissue environment[61]. Secretion of Wnt proteins through endosomal compartments on

exosomes plays an evolutionarily conserved functional role in extracellular vesicular transport[62]. CRC cells promote angiogenesis through Wnt/β-catenin signaling mediated by Wnt4-enriched exosomes in endothelial cells under hypoxic conditions, which could represent a novel mechanism in the development of CRC and its progression towards CRLM[63]. Moreover, constitutively active mutant β-catenin can be transported through EVs, activate the Wnt pathway in recipient cells and promote cancer progression[64].

Accumulating evidence indicates that EVs have roles in pre-metastatic niche formation and organotropic metastasis. EVs modify the microenvironment to recruit distinct supporting stromal cells, upregulate pro-inflammatory genes, and activate an immunosuppressive state[65]. These signals may mediate the awakening of dormant niches of cancer cells. A minority of disseminated tumor cells (DTCs), surviving as latent entities may take root in the new microenvironment of the recipient organ even years after the removal of a primary CRC. Possibly, over time, the conditions of the recipient organ may change and favor awakening and engrafting of latent tumor cells into the premetastatic niche[66]. Recently, the gut microbiome has also been documented in controlling the metastatic process and premetastatic niche formation[67]. Various bacteria have been implicated in CRC progression by modulating β-catenin pathway[17]. In the liver, CRC premetastatic niche is induced by bacteria dissemination from the primary tumor[10]. Moreover, in CRC patients concomitant chronic hepatitis B virus infection significantly increases the risk of CRLM[68]. Furthermore, in some patients, the same therapeutic programs could induce macro- and micro-environmental changes in the receiving organ, to favor a greater engraftment capacity of cancer cells[69]. Autocrine inhibition of Wnt could promote metastatic latency and immune evasion by deregulating the expression of Dickkopf's Wnt signaling pathway inhibitor 1 (DKK1), in which case the cells arriving to the recipient organ would undergo a slow cell cycle state which would allow them to evade natural killer cell-mediated clearance. By expressing a stem state but actively silencing Wnt signaling, cells could enter quiescence and evade innate immunity to remain dormant for prolonged periods[70]. Finally, cancer cells can actively emit large amounts of EVs with onco-functionality in a variety of contexts such as stromal crosstalk, immune evasion, metastatic site priming and drug resistance. In other cases, tumor cells may remain as latent entities at low cycles and sometimes reactivate following changes in the microenvironment. Currently, there is a lack of clear understanding of why and how this occurs and thus, where exactly the opportunities for developing anti-CRLM therapies may lie.

DORMANT CELLS IN LIVER METASTASES

Cancer cells can enter dormancy, which is a state characterized by either a reversible arrest or a slow cycling[71]. Dormant cells arise from DTCs derived from primary lesion[72]. They acquire new features, becoming non- or slower proliferating cancer cells and resistant to chemo- or targeted therapy, while tumor progression is not clinically visible. A dormant cancer mass (indolent small clusters) includes equilibrium between cell division and apoptosis. Dormant cells can be activated to re-enter cell cycle under particular conditions[72-75]. Micrometastases of CRC can enter a dormant state for years before recurring as metastatic disease[76]. CRC is characterized by two different types of recurrence, the first of which results from reactivation of dormant tumor cells, possibly in distal organs. In contrast, the second type is a relapse after surgical remission due to micrometastatic lesions present in apparently normal tissue adjacent to the tumor. Recurrence implicates changes in TME or immune escape[77]. Indeed, a successful metastasis depends on the dynamic interactions between cancer cells and the host MME[7]. During dissemination and tumor progression, dormant cells show a reduced metabolism associated with constrained growth and can survive under hypoxia and nutritional deprivation[73]. Dormant cells reduce E-cadherin expression and up-regulate activity of the survival pathway through unfolded protein response. This results in down-regulation of major histocompatibility complex class I molecules and immune evasion through undetectability by CD8+ T lymphocytes[78-80]. E-cadherin blocks cell cycle to reduce fraction and velocity of cell proliferation, limiting the effectiveness of therapeutic agents targeting cell cycle. Dormancy-independent resistance stems from E-cadherin signaling pathway and survival kinases, such as AKT, ERK and Janus kinase (JAK)[78]. A high ratio of p38 MAPK/ERK MAPK can induce DTCs into dormancy, while a high ratio of ERK MAPK/p38 MAPK can induce dormant cell awakening and proliferation. These pathways prevent cMET activation, while their downregulation induced by hepatocytes in the MME induces E-cadherin re-expression[73]. Primary CRCs can promote the formation of hepatic pre-niches through microRNA (miRNA)-containing exosomes[24]. Moreover, proinflammatory cytokines, chemoattractants, and angiogenic factors produced by primary tumor induce pre-niche formation by the involvement of bone marrow-derived cells, and marrow-derived granulocytic MDSCs[24]. Activation of HSCs into proliferative myofibroblasts is a significant cause of recurrence of CRLM and hepatic fibrosis[78]. The liver microenvironment offers beneficial conditions for cancer cell dormancy[72].

Surgical procedures can lead to increased levels of pro-angiogenic growth factors, such as VEGF, which could activate dormancy tumor cells by altering their equilibrium through the involvement of the immune system; inhibition of angiogenesis limits tumor growth through increased cancer cell apoptosis

[71,81]. Detection of single-nucleotide polymorphisms in cellular and angiogenic dormancy-related (*NOTCH3* and *NME1*) and dormant CSC related genes (*CD44*) has been associated with treatment response, recurrence and clinical outcome in patients with resected CRLMs[82]. In human CRLMs, quiescence induced by 5-fluorouracil (5-FU) is linked to activation of Yes tyrosine kinase (YES1) and to nuclear depletion of Yes-associated protein (YAP). Moreover, *YES1* silencing decreases nuclear YAP accumulation and induces cell quiescence in 5-FU-free conditions. Increased *YES1* and *YAP* transcript levels in residual CRLMs treated with adjuvant chemotherapy are related to the risk for CRC recurrence and reduced survival[76,83].

The number of resident hepatic $CD25^{-}$ TCR^{+} cells is critical for tumor dormancy in the presence of immunosuppressant cyclosporin A in a rat model. Transformation of growth factor-beta 1 is involved with the acquisition of tumor invasiveness and metastatic spread[84]. Cancer dormancy is poorly understood in its complexity[79]. In disease recurrence and metastases, reactivation of chemotherapy-resistant quiescent cancer cells is the key mechanism that needs to be fully understood. Interestingly, itraconazole-derived subsequent inhibition on suppressor of fused activation in Wnt epithelial tumor cells prevents nuclear localization of β-catenin causing Wnt inhibition. Itraconazole perturbs dormancy by signaling effects on Wnt pathway[85]. Dormant cells are also re-activated through JAK/STAT3 pathway regulation induced by chemokine (C-C motif) ligand 7 (CCL7), which is secreted by monocytic MDSCs[75].

It is of clinical importance to effectively identify and target dormant CRC cells as potential drivers of CRLM with emphasis on Wnt/β-catenin deregulation. Induction of Wnt signaling has been involved with activation of cell cycling of quiescent cells and regulation of stem cell self-renewal[86]. Specifically, Wnt3a alters cell fate program of primitive hematopoietic stem and progenitor cells[86]. Moreover, Wnt pathway is implicated in reactivation of dormant cancer cells induced by extracellular matrix (ECM)[87]. It has been hypothesized that ECM constituents derived from metastasis-initiating cancer cells (stem cell-like properties) and stromal cells may create a suitable microenvironment that activates signaling pathways useful for metastatic cell proliferation and colonization[88]. On the other hand, inhibition of Wnt signaling by DKK1 is a mechanism used by cancer cells to enter quiescence[87]. Several markers can be used to assess CRC cell dormancy state in the liver, such as CK, E-cadherin, Sox-2 and CD133 and cell awakening state such as vimentin, cyclin-D1, Ki-67, c-Myc and VEGF[89]. Some studies have found that tumor cell quiescence can also be induced by metabolic modulation and reactive oxidative species (ROS) *via* miRNAs and peroxisome proliferator-activated receptor γ coactivator 1α, which is a pivotal factor in lipid and metabolic regulation[90,91]. It has recently been suggested that redox mechanisms can control dormant or low-activity tumor cell life cycle, including long-term dormancy and metastatic recurrence. Indeed, quiescent tumor cells overexpressing antioxidant enzymes may survive at low levels of ROS. These cells are strongly involved with cancer recurrence and are able to escape chemotherapy-induced death[92]. On the other hand, increased redox levels associated with oxidative stress may be responsible for a reprogramming process leading to reactivation of dormant cancer cells. Moreover, activation of p38 MAPK signaling is redox-mediated and has priority over ERK1/2 under oxidative stress. In this context, endoplasmic reticulum-stress signaling can induce a dormant state in DTCs[92]. Cooperation between antioxidant enzyme nuclear factor erythroid 2-related factor 2 (Nrf2) and β-catenin has been found in hepatocellular cancer. Nrf2 activation plays a role in oxidative stress as transcriptional regulator of many genes with effects on carcinogenesis suppression[93]. Under acute oxidative distress, CRC cells subjected to growth factor deprivation that mimics cell dormancy show differential gene expression in Wnt/β-catenin-dependent and independent pathways, and cytoplasmic APC modulation[94]. Furthermore, ROS regulate relationships between β-catenin and forkhead box O in JNK signaling activation and cell quiescence[95]. These findings demonstrate that Wnt pathway is a redox-dependent signaling in cancer cellular dormancy and can play an important role in CRLM development and progression.

TUMOR BIOMARKERS AND TARGET THERAPIES IN CRLM

In CRC patients, biomarkers are increasingly needed to improve tumor stratification, detection and prognosis[96]. Biomarkers are both clinical and biochemical, such as the ECOG Performance Status Scale, white blood cell count, alkaline phosphatase, lactate dehydrogenase, CRC staging according to the tumor-nodes-metastasis (TNM) system from the American Joint Committee on Cancer[97].

It is recommended that CRLM patients, especially those considered in a third-line/salvage-therapy setting, should be stratified according to whether their tumors are *RAS* wild-type or *RAS* mutant[98]. Indeed, in metastatic disease setting, the presence of activating *RAS* (*KRAS*/*NRAS*) mutations represents a negative predictive biomarker for cancer cell resistance to monoclonal antibodies directed to EGFR[99], which may detrimentally affect patient health status, specifically when combined with an oxaliplatin-based cytotoxic backbone.

As a result, the European Medicines Agency has restricted chemotherapy and EGFR-directed antibodies, such as cetuximab and panitumumab, only to patients with *RAS* wild-type metastatic CRC. In CRC tumorigenesis, the most frequent mutations are *APC* and *KRAS*[18]. Dysregulation of Wnt/β-

catenin signaling plays a pivotal role in the development and progression of several human cancers, including CRC[17]. A synergistic cooperation between Wnt/β-catenin and RAS-ERK pathways has been observed in CRC with *APC* and *KRAS* mutational status, which has led to stabilization of both β-catenin and RAS[18].

For prognostic assessment of CRLM patients, both the European Society for Medical Oncology and the National Comprehensive Cancer Network guidelines recommend assessing *RAS* and *BRAF* mutation status simultaneously[100,101]. Double mutations in *APC* and *BRAF* are associated with poorer prognosis as compared to single mutations[102]. It has been reported that two-thirds of *BRAF*-mutant primary tumors are located on the right side of the colon and associated with an increased incidence of peritoneal and distant lymph node metastases[103]. *BRAF* gene mutations (most commonly V600E substitution) are present in 8%-12% of patients with CRLM and are considered unfavorable prognostic biomarkers. Indeed, the presence of mutated BRAF correlates with a median survival of 10.4 mo as compared to 34.7 mo in wild-type BRAF[103]. Notably, $BRAF^{V600E}$ mutation, when present, is almost exclusively non-overlapping with *RAS* mutations. $BRAF^{V600E}$ mutation is a negative predictive biomarker for EGFR antibody therapy for CRLM patients, making response to panitumumab or cetuximab treatment highly unlikely. $BRAF^{V600E}$ mutations are present in nearly one-third of CRC patients with microsatellite instability (MSI). Among patients with CRLM, MSI has been documented in less than 10%[104].

Predictive data from treated patients with CRLMs have recently shown that the mismatch repair status can predict an objective response of a tumor to blockade of PD-1[105]. Thus, MSI testing in patients with CRLM has become strongly recommended for its predictive value in treating CRC patients with immune checkpoint inhibitors (with pembrolizumab or nivolumab ± ipilimumab). In terms of response to adjuvant treatment, for CRC patients with *RAS* and *BRAF* wild-type tumors, chemotherapy associated with anti-EGFR antibodies has demonstrated to be significantly more beneficial in the presence of primary left-sided tumors. In contrast, for right-sided tumors, greater survival is associated with chemotherapy combined with bevacizumab[106].

Interactions between the Wnt/β-catenin and RAS-ERK pathways have already been reported in CRC [18]. Combined anti-CSC therapy with drugs targeting both Wnt signaling and tyrosine kinases could be a useful anti-cancer rationale[107]. Tyrosine kinases are aberrantly activated in cancer cells due to genetic alterations. Tyrosine kinase inhibitors can improve CRC prognosis; however, unavoidable drug resistance and cancer relapse are serious problems in clinical practice[107].

Resistance to targeted therapies in CRLM patients exists even among patients whose tumors are wild-type in *KRAS*/*NRAS* and *BRAF* genes, underlining the need to find and better characterize additional resistance biomarkers. A list of biomarkers beyond *RAS*, *BRAF*, and MSI molecular testing is emerging, which may impact the next clinical decisions in targeted therapies. Though they are not recommended for routine patient management outside a clinical trial setting, some of the emerging biomarkers are: *ERBB2* and *MET* gene amplifications, ligands such as amphiregulin (*AREG*), epiregulin (*EREG*), alterations/mutations in phosphoinositide-3-kinase (*PI3KCA*), phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) and *HER3*.

In CRLM, *MET* amplification is reported to be a potential mechanism of patient acquired resistance to anti-EGFR therapy[108]. However, multiple trials with different types of MET inhibitions have been unsuccessful in proving a predictive value for MET inhibition in CRLM patients previously treated with chemotherapy and EGFR-directed antibodies[109].

EGFR activation is involved with cell survival and proliferation through MAPK and the PI3K pathways[110]. Approximately 20% of *PIK3CA* mutations have been reported in exon 20 of its gene locus on chromosome 3q26.32. The presence of these mutations in tumor tissue has been associated with resistance to anti-EGFR therapies in chemorefractory CRC[111]. However, at the moment, there are no clinical recommendations for detecting mutations in *PIK3CA* exon 20 outside the CRC research field.

Other biomarkers associated with sensitivity to anti-EGFR therapies are gene expression status of *EREG* and *AREG*, which encode for EGFR ligands epiregulin and amphiregulin[112]. *EREG* and *AREG* are strongly regulated by methylation, and their expression is associated with CpG island methylator phenotype status and primary tumor site[113]. It is well documented that high *EREG* and *AREG* expression and left-sided primary colon tumors are associated with efficacy of anti-EGFR therapy[114]. Therefore, assessment of methylation status of *EREG* and *AREG* could be rationally used to predict resistance or susceptibility to anti-EGFR therapies in CRLM patients.

Recently, attention has been placed on the role that HER3 plays in resistance to anti-EGFR therapy. HER3 is overexpressed in several human cancers, including CRC[115]. Somatic mutations in *HER3* have been found in approximately 10% of CRC patients[116], and patients harboring CRC overexpressing HER3 display a worse clinical outcome as compared to those with low expression levels[117]. HER3-targeted therapies based on the use in clinical trials of either monoclonal antibodies, such as patritumab [118] and seribantumab[119], or bispecific monoclonal antibodies against EGFR/HER3, such as duligotuzumab[120], failed to have clinical benefits in CRLM patients.

We need to establish predictive biomarkers that are reliable indicators of activated Wnt signaling. Biomarkers on Wnt activity that show diagnostic, prognostic and therapeutic importance are strongly advocated. Plasmalemma vesicle-associated protein-1, which is a marker of intestine-vascular barrier impairment, is a prognostic marker for CRC recurrence, leading to CRLM[10]. High Wnt6 expression in

CRC indicates an unfavorable prognosis for patients with CRLM after hepatectomy, suggesting that Wnt6 expression may be a valuable biomarker[51]. Loss of Wnt5a expression is correlated with recurrence reduction and decreased survival in node-negative CRC patients[121]. Serum Wnt4 level may represent a potential biomarker for CRC patients[52]. COX-2 overexpression may have prognostic/diagnostic implications regarding Wnt signaling[121]. In addition, the presence of β-catenin in the nucleus is the only irrefutable proof of Wnt pathway activation. Nuclear β-catenin expression in metastatic lymph node is associated with age, tumor differentiation, TNM stage and liver metastases. Nuclear β-catenin expression may represent a clinically useful marker in differentiating highly metastatic and less invasive CRC[16]. Developing more sensitive antibodies that detect activated (dephosphorylated) β-catenin may represent valid prognostic markers.

THERAPEUTIC STRATEGIES IN CRLMS

Recamier introduced the concept of metastatic cancer cells in 1829 and only in 1952, the first liver metastasis was removed by Lortat-Jacob[81]. The incidence of CRLM is around 12.8%-15% during five years of follow-up after primary CRC diagnosis[7,122]. About 14%-25% of patients have synchronous CRLM at diagnosis, defined as CRLM detected concurrently or before primary CRC, while 7%-40% develop metastatic lesions during the follow-up[6,9,11,122-124]. The occurrence of metachronous CRLMs is related to histopathology and serum-based biomarkers, TME and liquid biopsy[122].

Surgical approaches for CRLM patients

Unfortunately, only 20%-30% of patients are eligible for hepatectomy[9,125,126]. However, after resection, around 65% of patients develop recurrence[127,128]. For patients with only CRLM, surgical resection is still the treatment for curative intent (Figure 2)[6,8,9]. Liver resection has perioperative mortality and morbidity rates of 1%-3% and 30%, respectively[128]. Specifically, major hepatectomy for CRLM has a mortality and major morbidity of 1%-5% and 20%, respectively[9]. In resectability assessment, CRLM patients are considered "resectable", "borderline" or "unresectable" (Figure 3)[6]. The definition of borderline patients concerns technical and biological difficulties that reduce the possibility of achieving an R0 resection. Based on the European Society of Medical Oncology guidelines and multidisciplinary evaluation, three approaches are currently defined: Upfront liver surgery followed by adjuvant chemotherapy (FOLFOX or CAPOX schemes), perioperative chemotherapy (chemotherapy before and after surgery based on FOLFOX or CAPOX scheme), and upfront systemic chemotherapy plus biological agents followed by liver surgery[128]. Surgery planning is based on a "classic" approach with primary CRC resection and a "reverse" approach, where liver metastases are resected first[9,124,128]. The liver-first approach or reversed strategy is more appropriate for either asymptomatic CRC or in locally advanced CRC[6,9]. Classic and reverse surgery showed similar outcomes. In common clinical practice, patients have symptomatic primary tumors with bleeding, obstruction, and a high risk of perforation. For this population of patients, the classic procedure is more often indicated (Figure 3). The reverse approach is performed when the primary tumor is asymptomatic with the aim of reducing the risk of CRLM progression[6,128]. A better prognosis is documented in patients treated with liver resection, which includes a resection margin > 1 mm from the tumor border [128,129]. R1 resection is associated with a higher rate of intra-hepatic local recurrence. Combined colorectal and hepatic resection in one setting is reserved to patients with easy-to-resect primary cancers and limited hepatic disease[9]. Currently, no differences in surgical outcome or survival have been reported when comparing classic, synchronous and liver-first approaches[9,81]. Liver resection combined with chemotherapy offers the best chance of cure with a reported 5-year survival and 10-year survival of 33%-58% and 23%-39%, respectively[123]. Routine chemotherapy improves progression-free survival by 8% at 3 years[130]. Metastatic cancer cells can also remain dormant after obtaining R0 surgical resection[131]. In literature, the percentage of disappearing CRLM ranges from 2.7% to 37%[9]. Although disappearing CRLM cells are of great interest, no consensus on their management has been reached[132]. Disappearing CRLM should be resected whenever feasible because a conservative management of leaving disappearing hepatic lesions results in a local recurrence of 19%[9]. This is a complex topic and the incidence of disappearing CRLM is likely to increase with advances in chemotherapy. The current management of patients with CRLM is multidisciplinary. Current guidelines for major hepatectomy recommend a future liver remnant (FLR) of > 20%-25% in healthy patients, > 30% in patients treated with chemotherapy, and > 40% in cirrhotic patients[6,130]. If FLR is inadequate, a variety of techniques are indicated to induce hepatic hypertrophy, *i.e.,* portal vein embolization, two-stage hepatectomy, association of liver partition and portal vein ligation for staged hepatectomy (ALPPS). Technically, ALPPS consists in liver transection and ligation of the portal vein (first operative approach) followed by resection of metastatic liver segments[9]. Surprisingly, ALPPS causes great hepatic hypertrophy as compared to portal vein ligation with low perioperative risk and satisfactory survival in large hospitals[9]. Laparoscopy has become the gold standard for minor hepatectomies[133]. With implementation of surgical education and minimally invasive techniques, such as laparoscopy and robotic systems, major and extended hepatectomies have been progressively reported. Laparoscopic

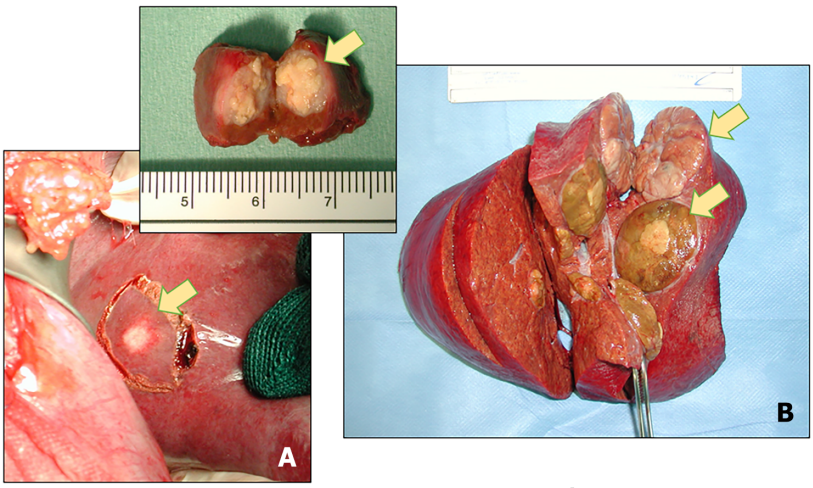


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Figure 2 Intraoperative images. A: Minor liver resection for colorectal liver metastasis (CRLM); B: Major hepatectomy for multiple CRLMs. Courtesy of Professor Paolo Innocenti, from his personal archive.

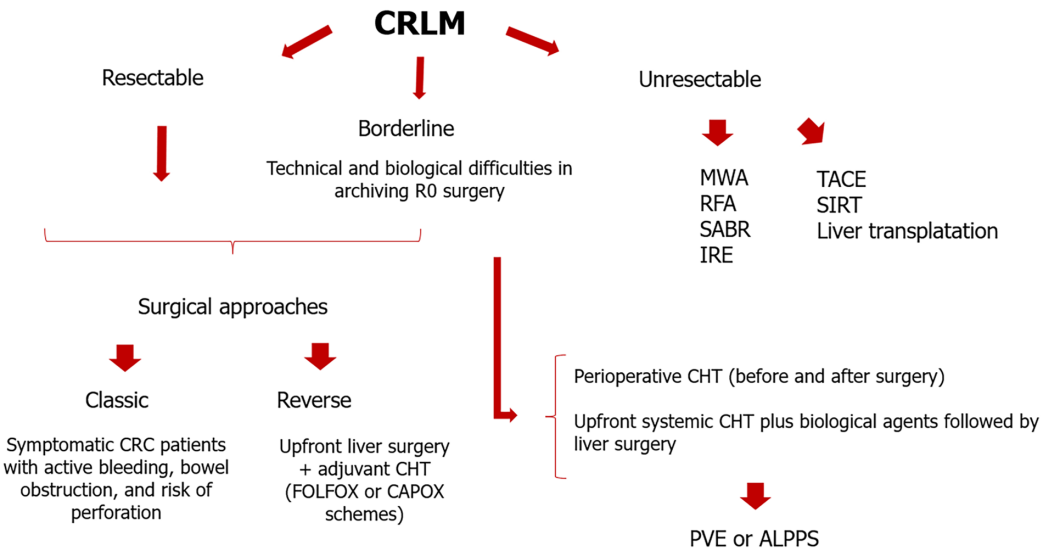

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Figure 3 Guidelines in colorectal liver metastasis treatments. CHT: Chemotherapy; CRC: Colorectal carcinoma; MWA: Microwave ablation; RFA: Radiofrequency ablation; SABR: Stereotactic ablative body radiotherapy; IRE: Irreversible electroporation; TACE: Transarterial chemoembolization; SIRT: Selective internal radiation therapy; PVE: Portal vein embolization; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; CRLM: Colorectal liver metastasis.

liver resection (LLR) improves hospitalization and decreases complications as compared to open surgery[123]. LLR is commonly indicated for tumors located along the liver periphery (segment II, III, IVb, V and VI)[134]. For larger tumors located in superior or posterior hepatic segments, either robotic or hybrid approach (hand-assisted or laparoscopic-assisted open approach) has potential advantages and has overcome the limitations of laparoscopic procedures. After LLR, the reported overall survival at 1-, 3-, and 5-years is 88%, 69%, and 50%, respectively. For disease-free survival, the reported results at 1-, 3-, and 5-years are 65%, 43% and 43%, respectively[134]. Recent data confirm that simultaneous laparoscopic resection of CRC and liver metastases are safe and feasible with the same benefits in terms of oncological outcome compared with open approach[11,124]. Robotic surgery is considered one of the options for CRLM resection to ameliorate the technical limitations of LLR[135]. Robotic systems overcome the disadvantages of laparoscopic approach and facilitate complex surgical procedures such as right hepatectomy, left hepatectomy, central bisegmentectomy, and posterior sectionectomy[136]. Current evidence documents a longer operative time for robotic surgery for CRLM compared with open approach, but a significantly shorter hospital stay[137]. Robotic surgery for CRLM might achieve R0 resection with similar results in terms of overall and disease-free survival compared with open surgery. A multi-institutional analysis of ultrasound-guided robotic surgery for CRLM reported a curative resection rate of 92% in patients with a median tumor size of 2.7 cm (range 0.4-13 cm)[135]. In patients

with CRLM treated with major hepatectomy using robotic systems, 1-, 3-, and 5- year overall survival rates are 87%, 87% and 87%, while disease-free survival rates are 85%, 85% and 85%, respectively[136]. In a multi-center Italian experience, the reported 1- and 3-year disease-free survival is 83.5% and 41.9%, while the 1- and 3-year overall survival is 90.4% and 66.1%, respectively[135]. The open approach has still a role in treating CRLM, especially in patients with a previous history of abdominal open surgery, with synchronous colonic and liver disease and with large liver metastases (Figure 2). Current guidelines consider thermal ablation the gold standard to eliminate small unresectable CRLM[137]. Microwave ablation (MWA), radiofrequency ablation (RFA), irreversible electroporation (IRE) and stereotactic ablative body radiotherapy (SABR) are valid local treatment options for patients with CRLM (Figure 3)[137]. RFA uses alternating electrical current at the frequency of 400 MHz to generate thermal energy, while MWA uses electromagnetic waves with frequencies greater than 900 MHz. Ablative therapies are indicated for patients with unresectable CRLM in combination therapy with hepatectomy, and for patients with comorbidity that are unfit to undergo major surgery. Ablation procedure could be performed using open, laparoscopic or percutaneous approaches[137,138]. The final objective is to obtain complete ablation with 10 mm margins in all directions[9]. While RFA is susceptible to the heat-sink effect with its limits in treating hepatic lesions in close proximity of large vessels, MWA has demonstrated effective in reaching higher tissue temperatures with a more homogeneous tissue heating [138]. Ablative treatments are relatively safe and less invasive methods. The reported morbidity rate is 4%-9% and the mortality rate is 0%-2.0%. Common complications include postoperative bleeding, infections, pneumothorax, hepatic abscess, and biliary tract injury[138,125]. Regarding local tumor control, recurrence after RFA ranges from 4%-40%, while after MWA the reported range is from 6%-10%. Tumor size > 3 cm and ablation margin < 0.5 cm represent predictor factors of local recurrence. Patients with unresectable CRLM have shown a median disease-free survival of 6 mo and median overall survival of 24 mo after laparoscopic RFA[138]. Other studies have reported a median survival of 24-39 mo. 1-, 2-, 3-, and 5-year overall survival ranged from 73%-92%, 41%-72% and 20%-40% in patients with CRLM of 3-5 cm[125]. Median disease-free survival is around 12 mo in patients with CRLM of 4-5 cm. For CRLM > 3 cm, 1-, 2-, 3- and 5-year overall survival ranged from 74%-93%, 30%-70%, and 8%-31%. The reported median disease-free survival in CRLM > 3 cm is 12.4 mo[125]. In patients with difficult-to-reach anatomical lesions, some authors have suggested SABR as an alternative procedure to treat large and unresectable CRLMs[125]. An Italian study reported a 1-, 2-, and 3-year overall survival of 68%, 40% and 17%, respectively, in patients with CRLM > 3 cm. SABR seems to have benefits in local control of larger hepatic lesions compared with MWA. The reported pooled 1- and 2- year control rates are 67% and 59.3%, while 1- and 2-year overall survival are 67.2% and 56.5%, respectively[9,137]. Guidelines have defined SABR as a reasonable therapy for CRLM patients unsuitable for surgery or ablative therapies, but a definitive validation in a large randomized analysis is required[9]. A relatively new non-thermal ablative method for unresectable CRLM is represented by IRE[8]. It is a nonthermal ablation modality using high-voltage electric pulses that induce permanent cell membrane disruption by sparing ECM and preserving critical structures such as blood vessels and biliary ducts[8,9,137]. After IRE treatment, the reported median overall survival ranges from 19.7-32.4 mo[125]. The reported hepatic IRE efficacy varies from 45.5%-100%[8]. A phase II trial (COLDFIRE-2) has reported that IRE is an effective relatively safe treatment for CRLM of 5.0 cm or smaller, with an overall complication rate of 40% (infected biloma, portal vein thrombosis, embolic event, cardiac arrhythmias and acute myocardial infarction). Around 68% of patients are alive one year after IRE treatment, with a median overall survival of 2.7 years after first IRE, and 4.8 years after CRC resection[8]. IRE might be indicated for liver recurrence after previous percutaneous treatment. After repeated IRE, local tumor control is reached in 74% of patients[8]. IRE represents a new and attractive research field and should be considered for patients with oligometastatic CRLM of 5.0 cm or smaller, anatomically unsuitable for surgical procedure or thermal ablation. Although IRE is a promising technique, evidence of IRE in CRLM treatment is still under validation[137]. Liver transplantation is a rare procedure for CRLM patients but mounting evidence suggests survival benefits in selected instances[6]. CRLM prognosis strongly depends on node-positive primary CRC, disease-free interval from primary tumor resection and metastasis detection < 12 mo, hepatic lesion > 5 cm, carcinoembryonic antigen levels > 200 ng/mL and > 1 hepatic lesion. More than two reported prognostic determinants are related to poor prognosis[128]. Surgical resection remains the gold standard for CRLM patients. CRLM distribution, size and number may have prognostic value[6]. CRLM outcome might be improved only if a personalized treatment approach is taken into account, and this has to consider tumor biology, disease staging and patient condition[9].

Chemotherapy for CRLMs

Neoadjuvant chemotherapy has the advantage of down-staging metastatic disease to facilitate curative hepatic resection. It is indicated for patients with borderline resectable or unresectable CRLM with high surgery risk (Figure 3)[129]. First-line schemes consist in fluorouracil-based regimens containing oxaliplatin and/or irinotecan. Neoadjuvant therapy offers no survival advantage in resectable synchronous CRLM with a 5-year overall survival of 42%, which is similar to patients treated with upfront surgery[9,129]. Systemic oxaliplatin- or irinotecan-based chemotherapy constitutes the standard for CRLM patients in many countries[129,139]. Systemic treatments for CRLM consist in a combination of fluorouracil (plus leucovorin) and either irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) plus

bevacizumab, or XELOX (capecitabine and oxaliplatin) and fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI)[7,129,140]. The choice of a primary line therapy is based on the physician choice and not on drugs, because irinotecan or oxaliplatin lead to similar results. Induction therapy with FOLFOXIRI plus bevacizumab improve CRLM outcome and increase incidence of some adverse events compared with FOLFIRI plus bevacizumab[140]. Several trials have shown that using anti-angiogenic bevacizumab in addition to systemic chemotherapy improves overall survival and progression-free survival[128]. Chemotherapy might downsize CRLM and 12%-54% of unresectable patients become resectable[7,129]. Adjuvant hepatic arterial infusion pump (HAIP) chemotherapy demonstrates promising results. In a phase II study based on the results of two high-volume centers in the Netherlands, the authors have demonstrated that adjuvant HAIP chemotherapy is safe and feasible in resectable CRLM patients[139]. Trans-arterial chemoembolization (TACE) is based on the infusion of high concentration of cytotoxic agents in liver metastases[9]. Usually, mytomycin C and cisplatin/doxorubicin are the conventional drugs for TACE[137]. This selective infusion of tumoricidal agents enhances the effect on liver tumor and minimizes the damage to normal liver. Hepatic arterial infusion chemotherapy (HAIC) represents an attractive strategy to expand resectability and tumor progression. Administration of chemotherapy into the hepatic artery is a selective procedure that allows drug delivery to the tumor with sparing of normal liver parenchyma[126,127]. Systemic side effects of HAIC are limited due to the high first-pass effect in the liver[140]. Most protocols no longer include the use of HAIC alone in favor of a combination therapy with HAIC plus systemic chemotherapy, for patients with initially unresectable CRLM[126]. Floxuridine has been the primary HAIC agent used in several studies by inserting a catheter in the gastroduodenal artery with the tip at the hepatic artery or by subcutaneous port[139]. Floxuridine improves overall survival of HAIC patients treated with concurrent systemic chemotherapy[6,139]. Floxuridine has been approved since 1971, but it is still not registered in Europe[140]. Systemic chemotherapy plus HAIC are associated with a response rate of 85% in patients with previous chemotherapy, and around 100% in chemotherapy naïve patients[6]. The combination of systemic chemotherapy and HAIC induces conversion to resectability in around 52% of patients.

Similar to TACE, selective internal radiation therapy (SIRT) is based on infusion of radiolabeled microspheres (Yttrium-90) in the branches of the hepatic artery (Figure 3)[9]. SIRT associated with systemic 5-FU prolonged progression-free survival in chemo-refractory patients[9]. Currently, data on the efficacy of Yttrium-90 radiotherapy are limited and should be interpreted with caution[137]. The use of irinotecan-loaded drug-eluting beads represents an emergent technique for administration of TACE in liver disease[129]. In addition, immunotherapy represents an alternative option to chemotherapy, for patients with CRLM derived from CRC with high MSI (MSI-H) or mismatch repair deficiency (dMMR)[129]. Pembrolizumab, a monoclonal antibody targeting PD-1 has been found of great interest in patients who had tumors with MSI-H and dMMR. Several trials are needed to evaluate the safety and efficacy of combined immune- and chemotherapy for CRLM patients with dMMR[129].

Wnt/β-catenin signaling pathway as a pharmacological target in CRLM patients

There are currently no approved drugs targeting Wnt/β-catenin pathway available for clinical use in CRLM patients, although several compounds capable of inhibiting Wnt/β-catenin signaling in advanced CRCs have been developed[52]. Inhibitory signaling molecules have been targeted towards the ability to form and secrete Wnt ligands (*e.g.*, PORCN) or towards their receptors and coreceptors (*e.g.*, FZD and LRP5/LRP6), or cytoplasmic proteins (*e.g.*, tankyrase and CK1alpha)[141].

Currently, some drugs that have an inhibitory effect on Wnt signaling are already used for CRC treatment[52]. They include indomethacin, pyrvinium, sulindac, aspirin, celecoxib and rofecoxid. Clinical trials are investigating the role of aspirin as an adjunctive drug for CRLM prevention[52,142,143]. As vitamin D deficiency is a common feature of patients with metastatic rectal cancer, its supplementation could be of great interest. Recently, a pilot study has examined the effect of vitamin D supplementation in patients with stage II-III CRC undergoing chemotherapy. Interestingly, the active form of vitamin D can promote binding of β-catenin to vitamin D receptor and increase expression of E-cadherin. The result is reduction of available β-catenin molecules that can bind to TCF/LEF transcription factors[52].

A phase I/II clinical trial has indicated that genistein combined with chemotherapy is an effective treatment for metastatic CRC. Genistein is a soy-derived isoflavone and phytoestrogen that inactivates Wnt signaling by regulating GSK3β and E-cadherin expression[52,143,144]. Other plant compounds such as curcumin and (-)-epigallocatechin-3-gallate can inhibit Wnt signaling by increasing β-catenin degradation[143,145]. A phase I trial has initially explored the efficacy and safety of curcumin in combination with 5FU in metastatic colon cancer and in combination with irinotecan for metastatic CRC patients[143].

The role of PORCN inhibitor LGK974 is under consideration in a phase II trial in a population of BRAF V600-mutated metastatic CRC patients, since in *vivo* studies have demonstrated that LGK974 inhibits tumor invasion and metastases[143,144]. Vantictumab (OMP-18R5) is a novel monoclonal antibody that interacts with FZD receptors[144]. To date, two phase I/II clinical trials are ongoing with OMP-18R5 modulating ligand/FZD-receptor interfaces, and with PRI-724 molecule interfering with β-catenin transcription[146].

OMP-18R5 inhibits CRC growth by synergizing with irinotecan[143]. OMP-54F28 is a recombinant protein that competes for binding with Fz8 receptor through sequestering Wnt ligands and inhibiting tumor growth[143,144,147,148]. Foxy-5, a Wnt5a peptide mimic, is evaluated in phase I-II clinical trials of metastatic CRC[147]. Secreted R-spondins (RSPO1-3) and their receptors RNF43/ZNRF3 are required to potentiate Wnt signaling in various conditions[144]. Rosmantuzumab (OMP-131R10), a monoclonal antibody against RSPO3 has been evaluated in phase I trial for metastatic CRC, but no results have been published[144]. Currently, phase I studies with anti-LGR5 and anti-RSPO3 therapies are under evaluation for patients with metastatic CRC. The tankyrase inhibitor IWR-1 has the potential to prevent tumor metastases by blocking Wnt/β-catenin pathway[140]. Besides developing new antagonistic molecules, pharmacological research could be directed towards repurposing non-oncology drugs, which are already active for other diseases, and evaluating natural compounds that may have an anti-inflammatory effect on TME. This theoretical concept might be valid for primary and secondary liver cancers. New drugs should be evaluated individually or in combination.

CONCLUSION

Wnt/β-catenin signaling pathway is an emerging target for cancer research and regulates liver metastasis through a complex network of interactions modulated by TME. Immune components of TME can modulate progression and metastatic capacity by promoting CRC cell survival. Proinflammatory molecules and maintenance of stemness through Wnt pathway may be considered potential therapeutic targets. Wnt signaling dysregulation activates downstream EMT by promoting cancer cell migration at the invasive front of the primary lesion. These biological mechanisms are not fully defined. Some evidence of invasive propensity and organ-specific tropism of metastatic tumor cells mirrors the concepts of "seed-soil", pre-niche and crosstalk between tumor and immune cells. Understanding the interdependence of these biological mechanisms can provide useful insight into CRLM treatment. Different cells participate to metastatization, and dormant cells show a leading role. Cancer dormancy is poorly understood in its complexity. It is of clinical importance to effectively identify and target dormant cells as potential drivers of CRLM with emphasis on Wnt/β-catenin deregulation and with the aim to reach a consensus in clinical management. Advances in the field of oncology have been made in the last decade and a central role for Wnt/β-catenin pathway has been recognized in CRC chemoresistance. At the current state of research, there is a lack of clear understanding of why and how CRC chemoresistance occurs, and thus, where exactly the opportunities for developing anti-CRLM therapies may lie. Although several compounds have been developed that inhibit Wnt/β-catenin signaling in advanced CRCs, there are currently no approved drugs targeting Wnt/β-catenin pathway and available for clinical use in CRLM patients. In this review, we considered current knowledge on clinical implication of Wnt signaling in CRLM process, provided the state of the art concerning potential biomarkers with a revision of surgical and non-surgical therapeutic guidelines for CRLM patients. Further efforts in translational medicine are needed to develop and validate novel therapies that antagonize both CRC cell metastatic capacity and their ability to be harbored in liver tissue.

ACKNOWLEDGEMENTS

The authors would like to thank Professor Paolo Innocenti (Fondazione Villa Serena per la Ricerca, Città Sant'Angelo - PE) for the courtesy of the intraoperative images.

FOOTNOTES

Author contributions: Aceto GM conceptualized and designed the study; Selvaggi F and Aceto GM developed the first draft, refined, edited, and revised the manuscript; Catalano T, Lattanzio R and Cotellese R contributed to the content materials and final editing; Aceto GM and Selvaggi F designed the images.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Chen YX

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 May 14; 29(18): 2784-2797

DOI: 10.3748/wjg.v29.i18.2784

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Infliximab *vs* adalimumab: Points to consider when selecting anti-tumor necrosis factor agents in pediatric patients with Crohn's disease

Eun Sil Kim, Ben Kang

Specialty type: Pediatrics

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Govindarajan KK, India; Gravina AG, Italy; Moshref RH, Saudi Arabia

Received: December 28, 2022
Peer-review started: December 28, 2022
First decision: March 8, 2023
Revised: March 20, 2023
Accepted: April 17, 2023
Article in press: April 17, 2023
Published online: May 14, 2023

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Abstract

Biologic agents with various mechanisms against Crohn's disease (CD) have been released and are widely used in clinical practice. However, two anti-tumor necrosis factor (TNF) agents, infliximab (IFX) and adalimumab (ADL), are the only biologic agents approved by the Food and Drug Administration for pediatric CD currently. Therefore, in pediatric CD, the choice of biologic agents should be made more carefully to achieve the therapeutic goal. There are currently no head-to-head trials of biologic agents in pediatric or adult CD. There is a lack of accumulated data for pediatric CD, which requires the extrapolation of adult data for the positioning of biologics in pediatric CD. From a pharmacokinetic point of view, IFX is more advantageous than ADL when the inflammatory burden is high, and ADL is expected to be advantageous over IFX in sustaining remission in the maintenance phase. Additionally, we reviewed the safety profile, immunogenicity, preference, and compliance between IFX and ADL and provide practical insights into the choice of anti-TNF therapy in pediatric CD. Careful evaluation of clinical indications and disease behavior is essential when prescribing anti-TNF agents. In addition, factors such as the efficacy of induction and maintenance of remission, safety profile, immunogenicity, patient preference, and compliance play an important role in evaluating and selecting treatment options.

Key Words: Anti-tumor necrosis factor; Infliximab; Adalimumab; Crohn's disease; Pediatric

Core Tip: In pediatric Crohn's disease (CD), the choice of biologic agents should be made more carefully to achieve the therapeutic goal. This review article focuses on comparing the efficacy of induction and maintenance of remission, safety profile, immunogenicity, preference, and compliance between infliximab and adalimumab in pediatric CD.

Citation: Kim ES, Kang B. Infliximab *vs* adalimumab: Points to consider when selecting anti-tumor necrosis factor agents in pediatric patients with Crohn's disease. *World J Gastroenterol* 2023; 29(18): 2784-2797
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2784.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2784

INTRODUCTION

Crohn's disease (CD) has become an important concern of clinicians owing to its rapidly increasing prevalence and incidence worldwide, including in emerging industrial countries[1]. Even though the incidence rate in Western countries has stabilised, most studies have revealed a statistically significant increase in the incidence of pediatric CD[2,3]. Pediatric patients with CD are more likely to have complications such as growth impairment, delayed puberty, psychosocial problems, aggressive disease course, and extensive gastrointestinal involvement than adult patients[4].

After infliximab (IFX) was approved by the Food and Drug Administration (FDA) in 1998[5], biologic agents with various mechanisms have been released and are widely used in clinical practice[6]. Among this broad spectrum of biologics, anti-tumor necrosis factor-α (TNF-α) agents have been classically used as first-line biologics for the treatment of moderate-to-severe CD refractory to conventional therapy[7]. Anti-TNF agents modulate the inflammatory response by binding to the TNF receptor on the cell membrane. IFX is a purified, recombinant DNA-derived chimeric human-mouse immunoglobulin G monoclonal antibody. Adalimumab (ADL) is a human monoclonal antibody that binds specifically to TNF-α[8,9]. Anti-TNF agents, such as IFX and ADL, are the only biologic agents that are currently approved by the FDA for pediatric patients with CD (Table 1)[10,11]. Therefore, the initial biologic agents to modify the disease course of CD and to achieve the therapeutic goal should be chosen more carefully in pediatric patients with CD.

Although head-to-head trials are the gold standard method for determining which treatment option is more effective, to date, no head-to-head trials have directly compared biologic agents. Until direct comparative studies of which biologic agents should be used first are performed, several points must be considered when selecting the initial biologic agent. In this study, we provide practical insights into the choice of anti-TNF therapy in pediatric CD. We reviewed the comparative efficacy, safety profile, immunogenicity, preference, and compliance between IFX and ADL.

MAIN STUDIES IN PEDIATRIC CD ASSESSING THE EFFICACY OF ANTI-TNFα THERAPIES

Anti-TNFα therapies have been well studied in adults and have showed efficacy in both the induction and maintenance of remission[12,13]. Targan *et al*[14] found that after a single 5 mg/kg IFX infusion, more than 80% of patients had a clinical response after four weeks. In the ACCENT-I study in which 58% of 573 patients with CD who had a response after the first dose of IFX were randomised, the IFX 5 mg/kg and 10 mg/kg groups were more effective in achieving clinical remission at week 54 than the placebo group[15].

After IFX and ADL were approved for use in the treatment of pediatric CD in 2006 and 2012, respectively, more than 20 years of data, including those from clinical trials, have been accumulated. There is evidence from randomised controlled trials (RCT) involving open-label induction and randomised dose-ranging maintenance therapies (Table 2). Four RCTs conducted on pediatric CD treated with anti-TNF agents showed the clinical remission rate in both the induction and maintenance periods[16-19].

The first RCT with IFX in pediatric patients with CD showed clinical response and remission in the induction and maintenance phases[16]. Among 112 patients, 99 patients (88.4%) responded to IFX, and 66 patients (58.9%) showed clinical remission at week 10. Patients responding to IFX were randomly assigned to receive IFX 5 mg/kg every 8 or 12 wk. By week 54, 63.5% of patients receiving IFX every eight weeks had a clinical response, and 55.8% achieved clinical remission, which is significantly higher than the clinical remission rate of 23.5% in those who received IFX every 12 wk. Ruemmele *et al*[17] also demonstrated the efficacy of IFX in pediatric patients with CD. Forty patients received IFX according to the induction regimen (weeks 0, 2, and 6) and were then randomly assigned to maintenance therapy of

Table 1 Biologic agents currently used or under study for the treatment of pediatric Crohn's disease

Class	Biologics	FDA approval for CD	Pediatric CD indications
Anti-TNF	Infliximab	Adult: 1998; Pediatric: 2006	Moderate to severe diseases refractory to conventional therapy[10]
	Adalimumab	Adult: 2007; Pediatric: 2012	First-line therapy for patients with CD who are at risk for progressive disease or for whom corticosteroids may exacerbate underlying conditions[10]; Prophylactic therapy for preventing postoperative recurrence in high-risk patients[10]
Anti-α4β7 integrin	Vedolizumab	Adult: 2014; Pediatric: N/A	Guideline recommendations for this pediatric indication are not yet available
IL-12/23 p40 inhibitor	Ustekinumab	Adult: 2016; Pediatric: N/A	Second-line biologic therapy after anti-TNF agent failure[11]

FDA: Food and Drug Administration; CD: Crohn's disease; TNF: Tumor necrosis factor; IL: Interleukin; N/A: Not applicable.

Table 2 Studies evaluating infliximab efficacy in pediatric Crohn's disease in the induction and maintenance phases

Ref.	Study group	Anti-TNF-α	Participants	Study design and aims	Definition of the outcome	Number of patients (*n*)	Age at diagnosis (yr)	Time	Clinical response	Clinical remission
Hyams *et al* [16], 2007	REACH	IFX	CD with a PCDAI > 30	Comparison of IFX maintenance intervals; every 8 *vs* 12 wk. Primary responders were randomised at week 10	Response: ΔPCDAI = -15. Remission: PCDAI ≤ 10	Total: 103. Every 8 wk: 52. Every 10 wk: 51	13.3	Week 10. Week 54	88.4%. Every 8 wk: 63.5%. Every 12 wk: 33.3% (P = 0.002)	58.9%. Every 8 wk: 55.8%. Every 12 wk: 23.5% (P < 0.001)
Ruemmele *et al*[17], 2009	GFHGNP	IFX	CD	Comparison of IFX infusion every 8 wk at maintenance *vs* IFX on demand. Primary responders were randomised at week 10	Remission: Harvey Bradshaw index < 5	Total: 40. Every 8 wk: 18. On demand: 13	13.9	Week 10. Week 60	N/A	85%. Every 8 wk: 83%. On demand: 61% (P = 0.001)
Hyams *et al* [18], 2012	IMAgINE	ADL	Moderate-to-severe CD	Comparison of ADL dose; HD (40 mg or 20 mg for body weight ≥ 40 kg or < 40 kg) *vs* LD (20 mg or 10 mg for body weight ≥ 40 kg or < 40 kg). Primary responders were randomised at week 4	Response: ΔPCDAI = -15. Remission: PCDAI ≤ 10	Total: 188. HD: 93. LD: 95	HD: 13.7 ± 2.52. LD: 13.5 ± 2.47	Week 26. Week 52	HD: 59.1%, LD: 48.4%. HD: 41.9%, LD: 28.4%	HD: 38.7%; LD: 28.4%. HD: 33.3%; LD: 23.2%
Assa *et al* [19], 2019	PAILOT	ADL	Biologic-naïve CD	Comparison of proactive TDM *vs* reactive TDM. Primary responders were randomised at week 4	Remission: PCDAI ≤ 10	Total: 78. Proactive: 38. Reactive: 40	Proactive: 12.9 ± 2.6. Reactive: 13.5 ± 2.7	Week 4. Week 72	NA	NA. Proactive TDM: 82%; Reactive TDM: 48%

CD: Crohn's disease; RCT: Randomised controlled trial; IFX: Infliximab; PCDAI: Pediatric Crohn's disease activity index; NA: Not applicable; ADL: Adalimumab; HD: High dose; LD: Low dose; TDM: Therapeutic drug monitoring.

IFX infusion every two months or an on-demand regimen. Around 85.0% of patients achieved clinical remission during IFX induction therapy. After the induction phase, the relapse rate was significantly higher in the on-demand group (91.7%) than in the IFX-maintenance group (23.1%).

A double-blind RCT evaluating the efficacy and safety of a dose-dependent maintenance regimen with ADL following open-label, weight-adjusted induction therapy (IMAgINE-1) was conducted on both IFX-naïve patients and patients who did not respond to IFX therapy[18]. In patients who had a clinical response in the induction phase, 38.7% and 33.5% of clinical remission was observed at week 26 and week 52, respectively. In addition, there was no statistically significant difference between the high- and low-dose groups. In a recently published RCT conducted in anti-TNF-naïve pediatric patients with CD, the clinical remission rate after the induction phase was much higher than that in the IMAgINE-1 study (48%-82% *vs* 38.7%)[19]. These results are in line with findings from previous adult studies[12,13] and highlight the importance of the choice of initial biologic agents according to risk stratification.

ANTI-TNFα AND ITS INDICATIONS FOR PEDIATRIC CD

The indications for the use of biologic agents have changed over the last two decades since the introduction of anti-TNF agents for the treatment of pediatric CD. Previously, anti-TNF agents were considered when disease activity was not controlled despite conventional therapies such as immunomodulators (IMMs), the so-called step-up strategy[16]. However, the guidelines recently published by the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) recommended early anti-TNF treatment within < 3 mo after diagnosis for the induction of remission in moderate-to-severe pediatric CD with a high risk of complications such as extensive disease, deep colonic ulcers, perianal disease, stricturing (B2), or penetrating disease (B3), growth impairment, the so-called top-down strategy[20]. The RISK study demonstrated that early induction therapy with anti-TNF agents was associated with higher corticosteroid- and surgery-free remission rated at 1 year compared to induction with exclusive enteral nutrition (EEN) and corticosteroids[21]. Kugathasan *et al*[22] also reported that early induction therapy with anti-TNF agents significantly lowered the risk of penetrating (B3) complications, however did not seems to reduce the risk of stricturing (B2) complications. In addition, even in patients with low risk of poor outcome, anti-TNF therapy should be considered in patients with severe growth impairment or who have not achieved clinical (pediatric CD activity index < 10) and biochemical remission (fecal calprotectin < 250 μg/g) despite induction therapy with EEN or corticosteroids[20].

Walters *et al*[21] reported that early anti-TNF therapy was more effective at maintaining remission than IMM monotherapy [relative risk (RR), 1.41; 95%CI: 1.14-1.75; P = 0.0017]. In addition, a prospective study in 76 pediatric patients with CD compared the step-up group and the top-down group in terms of endoscopic healing[23]. Compared with that in the step-up strategy, the rate of achieving endoscopic healing at week 54 was higher in the top-down group of pediatric patients with CD (42% *vs* 72%, P = 0.007), which means that seizing the therapeutic window of opportunity in pediatric CD should be considered earlier than generally accepted[24]. Based on these results, guidelines suggest that either IFX or ADL can be provided to pediatric patients with CD who have not previously received anti-TNF therapy, taking into account the efficacy, route of administration, and preference.

Standard dosing of IFX is weight-based at 5 mg/kg at weeks 0, 2, and 6, followed by maintenance treatment every 8 wk. In the case of ADL, patients weighing < 40 kg received 80/40 mg, and those weighing ≥ 40 kg received 160/80 mg in the first 2 wk. Thereafter, patients weighing < 40 kg were administered 20 mg, and patients weighing ≥ 40 kg were administered 40 mg every 2 wk. Dose escalation is considered in patients who lose response to standard anti-TNF treatment; adjustment of the infusion interval to 4 or 6 wk or an increment in the dose of 10 mg/kg for IFX; and adjustment of the administration interval to every week for ADL. Especially, children at risk for accelerated IFX clearance during induction [*i.e.*, patients < 30 kg, those with extensive disease, and those with low serum albumin] require dose escalation to achieve target trough levels (TLs) or their first proactive therapeutic drug monitoring (TDM) at the second or third anti-TNF infusion[25].

EFFICACY OF ANTI-TNF THERAPY IN PEDIATRIC CD

Comparative efficacy of anti-TNF agents for induction of remission in CD

Head-to-head trials, in which each drug or treatment strategy is compared formally, are the gold standard method for comparing distinct therapies[26]. However, there are currently no head-to-head trials of biologic agents in pediatric or adult CD. Owing to the absence of results, the choice of IFX or ADL relied on expert opinion, real-world data, or indirect comparison of biologic agents. Unfortunately, for pediatric patients with CD, there is a lack of accumulated data, which requires the extrapolation of adult data for the positioning of biologics in pediatric CD.

In 2018, the results of a network meta-analysis that indirectly compared the efficacy of FDA-approved biologic agents in the treatment of CD, which included IFX, ADL, vedolizumab, ustekinumab, and certolizumab pegol, were published[27,28]. When IFX and ADL were compared with respect to the efficacy of remission induction, IFX was ranked higher than ADL [surface under the cumulative ranking (SUCRA) 0.93 *vs* 0.75] for inducing clinical remission in biologic-naïve patients with moderate-to-severe CD. Additionally, IFX was predicted to be more effective in induction therapy than ADL; the rates of achieving clinical remission with induction therapy were 59.6% and 48.7% for IFX and ADL, respectively.

These findings are partly explained by differences in the pharmacokinetics or tissue penetration of IFX and ADL[29]. Drug levels of IFX and ADL show completely different patterns over time after administration. Intravenous (IV) formulations, such as IFX, show the highest concentrations with administration, and the concentrations gradually decrease over time, dropping to the lowest level just before the next administration, that is, to the TLs. In the case of subcutaneous (SC) formulations, including ADL, the concentrations at the time of administration, at the peak point, and at the lowest point are similar[30]. Because IFX has relatively large fluctuations in drug levels according to the drug infusion type, it is necessary to increase drug levels during the induction phase to maintain TLs,

whereas ADL maintains relatively constant drug concentrations.

Post-induction TLs, which can modulate inflammation in patients with high disease and inflammatory burdens, differ according to the type of anti-TNF agent used. In Figure 1A[31], we assumed the threshold of drug levels to control the inflammatory burden in the induction phase as the purple dotted line. IFX exhibits higher TLs than ADL during the induction phase, which is beneficial for maintaining post-induction TLs above the threshold required for treatment in patients with severe inflammation. Therefore, IFX might be more advantageous than ADL in patients with a high inflammatory burden owing to differences in the pharmacokinetics of the two anti-TNF agents during the induction period.

Specifically, a post-hoc analysis of the ACCENT-I study found that high IFX TLs after induction therapy were a key factor in maintaining response after one year of treatment[32]. The study revealed that more than 3.5 μg/mL of post-induction TLs of IFX was associated with a durable, sustained response to maintenance therapy. Feng *et al*[33] reported that post-induction TLs of IFX were correlated with endoscopic healing, and the median TLs in patients who achieved endoscopic healing after the induction of IFX were 7.5 μg/mL.

Similar to adult inflammatory bowel disease (IBD), higher post-induction IFX TLs were the only independent factor that predicted clinical or biochemical remission and durable sustained response during the first year of treatment in pediatric IBD[34]. Singh *et al*[35] reported that cut-off levels of > 3, > 4, > 7 μg/mL of IFX TLs had positive predictive values of 64%, 76%, and 100%, respectively, for predicting persistent remission in pediatric IBD. Recently, El-Matary *et al*[36] showed that higher post-induction IFX TLs had a strong relationship with the healing of the fistula in pediatric perianal CD. The post-induction IFX TLs in the clinical responder group were higher than those in the non-responder group (12.7 μg/mL *vs* 5.4 μg/mL, $P = 0.002$).

Likewise, post-induction TLs of ADL correlated with clinical and biochemical remission[37,38]. Zittan *et al*[37] reported that ADL TLs at week 4 were higher in the biological remission group than in the non-responder group of adult patients with CD (19.8 μg/mL *vs* 10.2 μg/mL, $P = 0.001$). After induction therapy, it was shown that similar to adult CD, there was a positive relationship between ADL TLs and clinical outcomes in pediatric CD[38]. The cut-off values of ADL TLs at weeks 4 and 8 to predict clinical and biological remission at week 24 were 22.5 μg/mL and 12.5 μg/mL, respectively.

Although the cut-off values of post-induction TLs for regulating the inflammatory burden at anti-TNF initiation are different for IFX and ADL, it is anticipated that the higher the post-induction TLs, the higher the clinical and endoscopic remission rate. Considering the pharmacokinetics of the route of administration, IFX can reach drug levels above the threshold in a shorter period of time than ADL and exhibits a rapid response of induction. Therefore, predictions based on the pharmacokinetics of anti-TNF agents and the difference in remission according to post-induction TLs show that IFX is more advantageous than ADL when the inflammatory burden is high.

Comparative efficacy of anti-TNF agents for maintenance of remission in CD

As with the selection of anti-TNF agents for the induction of remission, there are no head-to-head trials comparing the efficacy of maintenance therapy between IFX and ADL. According to a network meta-analysis study conducted in adults, ADL was superior to IFX in the maintenance phase, in contrast to the induction phase. In biologic-naïve adult patients with moderate-to-severe CD, the SUCRA of maintaining remission over one year was 0.97 and 0.68 for ADL and IFX, respectively[27,28].

These results can also be explained by the differences in the pharmacokinetics of IFX and ADL. For IV drugs, a clear distinction can be made among the peak, intermediate, and trough concentrations available for TDM. However, for SC drugs, there is no clear distinction among the peak, intermediate, and trough concentrations. In the case of SC drugs, the sampling time for TDM is less important because the TLs of SC drugs are kept relatively constant because not only is frequent administration required but also the absorption rate is relatively low[29].

Figure 1B shows the concentration changes in IFX and ADL during the maintenance phase, and the purple dotted line indicates the threshold for controlling the inflammatory burden during the maintenance phase[31]. The drug level of IFX tends to be lower than the threshold as it approaches the trough time, whereas the drug level of ADL is continuously maintained above the threshold because of the relatively constant levels of ADL. In the maintenance phase, it is important to keep the drug concentrations above the threshold to not only inhibit the formation of anti-drug antibodies (ADAs) but also to suppress the occurrence of loss of response and increase the durability of anti-TNF agents. Owing to the differences in the pharmacokinetics of the two anti-TNF agents, ADL might be more advantageous than IFX in the maintenance phase.

The association between IFX TLs in the maintenance phase and clinical outcomes has been demonstrated in many studies conducted on adults. One meta-analysis indicated that patients who achieved clinical remission had significantly higher IFX TLs than those who did not achieve remission during the maintenance phase (3.1 μg/mL *vs* 0.9 μg/mL)[39]. In addition, it has been shown in several studies that IFX TLs in the maintenance phase are an important prognostic factor in achieving endoscopic healing. Another study revealed that the only factor associated with endoscopic healing was an increase in IFX TLs > 0.5 μg/mL (likelihood ratio, 2.02; 95%CI: 1.01-4.08; $P = 0.048$) in patients with IBD[40]. Additionally, Yarur *et al*[41] demonstrated a correlation between IFX TLs and fistula healing [area under the curve (AUC), 0.82; $P < 0.0001$]. Likewise, higher maintenance IFX TLs were associated

with clinical and biochemical remission in pediatric patients with CD[42]. Recently, it has been reported that IFX TLs during maintenance treatment are important determinants of endoscopic healing as well as clinical remission in pediatric patients with CD. According to this study, IFX TLs to achieve endoscopic remission with 80% specificity were ≥ 5 μg/mL[43].

Similar to IFX, maintenance TLs of ADL were associated with clinical and laboratory responses in adult patients with CD[44]. The study showed that ADL TLs were associated with clinical remission (AUC, 0.748; $P < 0.001$), with an optimal cut-off value for predicting clinical remission of 5.85 μg/mL (sensitivity, 68%; specificity, 70.6%). In addition, Zittan *et al*[45] conducted a large, homogenous CD cohort study which revealed that patients with endoscopic healing have higher ADL TLs during the maintenance phase than those without endoscopic healing (14.7 μg/mL *vs* 3.4 μg/mL, $P < 0.001$). Similar results were found in studies conducted on pediatric patients with CD. The IMAgINE-1 study showed that patients with clinical remission at week 26 had slightly higher ADL TLs than those without remission (11.3 μg/mL *vs* 10.5 μg/mL, $P = 0.028$)[46]. Choi *et al*[47] reported that pediatric patients with endoscopic healing had significantly higher ADL TLs at week 16 than those without endoscopic healing (13.0 μg/mL *vs* 6.2 μg/mL, $P = 0.023$).

As can be inferred from the above studies, clinical remission and endoscopic healing can be achieved when the drug concentrations are sustained above the threshold despite the difference in the cut-off values for withstanding the inflammatory burden in the maintenance phase between IFX and ADL. Considering the pharmacokinetics of the maintenance phase, ADL maintains drug levels more constantly than IFX; therefore, it is expected that ADL is more advantageous in sustaining remission than IFX in the maintenance phase.

IMMUNOGENICITY OF ANTI-TNF AGENTS

Although anti-TNF agents are effective in patients with CD refractory to conventional therapy, loss of response increases over time, and approximately, half of patients among primary responders require dose escalation[48]. Among patients receiving anti-TNF agents, 60%-87% of patients show clinical remission or partial response in the induction phase, and less than 40% of patients maintain clinical remission at one year[49]. Immunogenicity due to the formation of ADAs to anti-TNF agents as the main reason for the loss of response.

Immunogenicity to anti-TNF agents develops when the immune system of patients recognises drugs as antigens and triggers the formation of ADAs. ADAs accelerate drug clearance by the reticuloendothelial system and neutralise drugs by binding to anti-TNF agents[50]. Additionally, suboptimal TLs of anti-TNF agents are associated with a more immunogenic state, which leads to lower efficacy and greater loss of response[37,51,52]. Higher body weight, the development of ADAs to anti-TNF agents, a low albumin level, and an elevated C-reactive protein level are the covariates that accelerate the clearance of anti-TNF agents[53-56].

Vermeire *et al*[57] reported that the rate of ADA formation in IBD patients receiving IFX was up to 65.3% and that in patients receiving ADL was 38.0%. Theoretically, as ADL is a humanised monoclonal antibody, it is thought that the incidence of immunogenicity in the human body is lower than that for IFX, which is a monoclonal chimeric anti-TNF antibody (partly murine, partly human). Therefore, ADL was superior to IFX in terms of immunogenicity.

ANTI-TNF AGENTS FOR GROWTH IMPROVEMENT

In Selecting Therapeutic Targets in IBD-II, restoration of normal growth was established as an intermediate target for pediatric patients[58]. Therefore, a very important goal in treatment for pediatric patients with CD is to normalise the linear growth.

To date, no study has compared the effects of IFX and ADL on the restoration of linear growth. Studies have shown that each of the two anti-TNF agents has a positive effect on the recovery of normal growth. In the case of IFX, there is a study published on the restoration of growth as well as clinical response and endoscopic healing in 195 pediatric patients with CD[59]. The effect on the recovery of linear growth was greater when IFX was administered at the Tanner 1-2 stage with growth potential than at the Tanner 4-5 stage. Another study showed that early administration of IFX within one month after diagnosis was more effective for linear growth than the conventional step-up therapy ($P = 0.026$) [60]. For Tanner stage 4-5 patients receiving IFX, there was no statistically significant difference in height z-score between patients with early IFX administration and those with the conventional step-up therapy ($P = 0.438$). However, in patients with Tanner 1-2, the restoration of growth was significantly improved in patients with early IFX administration ($P = 0.016$).

Similarly, it was reported that ADL was effective in restoring linear growth at weeks 26 and 52 compared with baseline in patients with growth impairment at diagnosis (median height z-score, baseline, -3.25; 26 wk, -0.34; 52 wk, 0.21, $P < 0.001$)[61]. Additionally, Matar *et al*[62] showed that ADL improves weight as body mass index as well as linear growth after 72 wk of treatment.

SAFETY AND ADVERSE EVENTS DURING ANTI-TNF THERAPY

From an immunological point of view, as TNFα is a cytokine responsible for macrophage activation, neutrophil recruitment, and granuloma formation, anti-TNF agents are associated with an increased risk of infection, especially granulomatous infection[63]. Dulai *et al*[64] reported that the rate of serious infectious disease in pediatric patients with IBD who were treated with anti-TNF agents was similar to that of pediatric patients who received IMMs [352/10000 *vs* 33/10000 patient-years of follow-up evaluation (PYF); 95%CI: 0.83-1.36] but significantly lower than that of adult patients (654/10000 PYF; 95%CI: 0.43-0.67). In addition, the risk of infection is higher when anti-TNF agents are administered in combination with IMMs than with anti-TNF monotherapy (RR, 1.19; 95%CI: 1.03-1.37)[65]. According to a network meta-analysis of adult studies that indirectly compared IFX and ADL, IFX had a lower risk of any infection (SUCRA, 0.83) than ADL (SUCRA, 0.22)[27].

Previous studies have shown the risk of malignancy and lymphoproliferative disorders with IBD treatment, particularly with thiopurine and anti-TNF agents. Based on a meta-analysis of 49 randomised placebo-controlled studies comprising 14590 adult patients, there was no evidence related to an increased risk of malignancy with the use of biologic agents including IFX or ADL (odds ratio, 0.90; 95%CI: 0.54-1.50)[66]. Studies with pediatric patients also showed similar results to those of adult studies. In a study conducted using the DEVELOP registry including 5776 pediatric patients with IBD treated with anti-TNF agents, malignancy occurred in 15 patients[67]. An increased risk of malignancy was found in patients treated with thiopurine when a stratified analysis of thiopurine exposure was performed regardless of biologic agents. Even though the standardised incidence of malignancy for thiopurine exposure was 2.43 when compared to the prevalence in healthy children, no significant increase in the incidence of malignancy was observed in children who were only exposed to IFX.

Recent studies showed that the most common complications in patients with IBD treated with anti-TNF agents were dermatologic complications such as psoriasis, eczema, and skin infection[68]. Similarly, the frequency of skin problems appears to be high in pediatric patients with CD on anti-TNF. When comparing patients treated with IFX and ADL, the rate was much higher in IFX-treated patients than in ADL-treated patients. In a pediatric retrospective, large cohort study comprising 409 patients, 11.5% of patients showed at least one dermatologic complication. Among them, 35 were treated with IFX and 12 with ADL. In particular, among patients who developed psoriasis, the proportion of patients treated with IFX was significantly higher than that of those treated with ADL (84.8% *vs* 15.2%, $P = 0.05$) [69]. Additionally, Hradsky *et al*[70] reported that the only predictive factor for any dermatologic complication in pediatric CD was IFX therapy (*vs* ADL, hazard ratio, 2.07; 95%CI: 1.03-4.17).

EFFECTS OF CONCOMITANT IMM TREATMENT

For patients starting on IFX, combination therapy with IMM including azathioprine (AZA) and methotrexate (MTX) is recommended. As the first RCTs regarding the comparison of combination therapy of IFX and AZA with monotherapy of IFX or AZA, the SONIC trials showed the superiority of combination therapy to monotherapy regarding clinical remission, endoscopic healing, pharmacokinetics, and immunogenicity in adult patients with CD[71]. At week 30, ADAs developed in only 0.9% of patients receiving combination therapy, whereas these were produced in 14.6% of patients receiving IFX monotherapy, leading to higher IFX TLs in the combination therapy group than in the IFX monotherapy group (3.5 μg/mL *vs* 1.6 μg/mL, $P < 0.001$). Additionally, the combination therapy group was more likely than the IFX or AZA monotherapy group to achieve corticosteroid-free clinical remission and endoscopic healing. Likewise, it was revealed that the combination of IFX plus MTX had a lower ADA development (4% *vs* 20%, $P = 0.01$) and higher IFX TLs (6.35 μg/mL *vs* 3.75 μg/mL, $P = 0.08$) than IFX monotherapy in the COMMIT trial conducted in adult[72].

No RCT has compared the effects of combination therapy with IFX and IMM and IFX monotherapy in pediatric CD. A retrospective study conducted on 229 pediatric patients with CD confirmed that combination therapy with IFX and AZA reduced the formation of ADAs and loss of response compared to IFX monotherapy[73]. Moreover, pediatric patients who were treated with IFX monotherapy had a lower probability of remaining ADA than patients with combination therapy at 12, 24, and 36 mo after induction of IFX (72.6% *vs* 93.4%, 57.7% *vs* 91.0%, and 48.1% *vs* 91.0%, respectively). Similarly, pediatric studies comparing combination of IFX plus IMM (including AZA and MTX) and IFX monotherapy reported results similar to those in adult studies[59,74,75]. Therefore, up-front anti-TNF agents in combination with IMMs should be considered in patients with high risk of poor outcomes such as perianal disease, structuring (B2) or penetrating (B3) disease behaviour or severe growth impairment.

A meta-analysis comparing the efficacy of combination therapy of ADL and IMMs and ADL monotherapy in adult CD revealed that the induction of remission rate of ADL monotherapy was lower than that of combination therapy with IMMs, although the maintenance of remission was comparable [76]. In contrast to the results of studies on adults, a post-hoc analysis of the IMAgINE-1 study found that combination therapy of ADL and IMMs is not superior to ADL monotherapy in terms of pharmacokinetics, efficacy, and safety in pediatric patients with CD[77]. Clinical response and remission rates

were comparable in patients treated with combination therapy and ADL monotherapy at weeks 4, 26, and 52. Regarding pharmacokinetics, there were no significant differences in the mean TLs between the two groups. These results are in line with the findings of other studies showing that combination therapy with ADL and IMMs was not more effective than ADL monotherapy in pediatric CD[48,78].

Therefore, the recently updated ESPGHAN guidelines for the medical treatment of CD in children and adolescents recommend combination therapy with IFX and IMMs, whereas ADL monotherapy can be an alternative to combination therapy with IMMs[20].

PREFERENCES OF PATIENTS AND PARENTS

There are several differences between IFX and ADL. However, the primary difference is the mode of administration. The IV delivery of biotherapeutics has the advantage of being able to elicit a relatively rapid induction of response and is suitable for administering a large volume of drugs. On the other hand, SC formulations have the advantage of requiring fewer frequent visits to the clinic and being less invasive than IV administration[79]. Because of these differences in the route of administration, not only the efficacy of anti-TNF agents but also the preference of patients and caregivers for the delivery of drugs should be considered.

In a study conducted on rheumatoid arthritis patients treated with anti-TNF agents, patients under the age of 61 years showed a tendency to prefer SC preparations to IV preparations owing to the convenience of administration[80]. Similarly, a study conducted on adult patients with CD in Switzerland also showed the same results. The patient's choice of a specific anti-TNF agent was influenced by the convenience of use (69%), time required for treatment (34%), frequency of drug administration (31%), scientific evidence for efficacy (19%), and fear of syringes (10%). For these reasons, most patients prefer SC rather than IV injection when choosing anti-TNF agents[81].

However, a recent study conducted on anti-TNF selection in Korea reported the opposite result. Among 189 anti-TNF naïve patients with CD, 63.5% of patients preferred IFX, and 36.5% of patients preferred ADL[82]. In contrast to Western studies, the reason for choosing the IV route of administration over the SC route was the reassurance from the presence of doctors (68.3%).

The differences in results of these studies seem to show differences between Western and Eastern countries in terms of culture and medical environments. Unlike Western countries, Asia has a cultural context in which patients have relative interdependence in the decision-making process during treatment[83]. Therefore, characteristics, daily life, preferences, and cultural differences between patients and caregivers should be considered when selecting biologic agents for the treatment of pediatric patients with CD. Clinicians should discuss the route of administration of biologic agents with patients and their caregivers before prescribing anti-TNF therapy.

ADHERENCE TO ANTI-TNF AGENTS

Low compliance and delayed administration of anti-TNF agents are highly related to the formation of ADAs, which can lead to adverse events and loss of response due to low TLs[84,85]. In the treatment of patients with CD, adherence to anti-TNF agents plays an important role in improving treatment efficacy and patient outcomes. The rate of adherence to anti-TNF agents is known to be approximately 70% in patients with CD[86]. When the adherence rates of IFX and ADL were compared, the adherence rate of IFX was 66%-85%, and that of ADL was 55[87-89], with an RR of 0.76 (95%CI: 0.64-0.91)[86].

The difference in adherence between IFX and ADL is thought to be caused by the route of administration, intervals of injection, and supervision of clinicians during the injection. Adherence could be controlled in favour of IFX because the administration of IV drugs requires patient visits to an outpatient clinic.

However, special circumstances, such as coronavirus disease 2019 (COVID-19), may lead to different results. In 2020, the Pediatric IBD Porto Group of ESPGHAN published a society paper[90]. While investigating and reporting the experience of pediatric IBD management during the COVID-19 situation in China and South Korea, it has been recommended that standard treatment be not stopped or delayed. During the COVID-19 pandemic period, anti-TNF infusion delays were reported in 28% of cases in China and 5% in Korea, and exacerbation of disease among delayers was reported in 21% and 23%, respectively. The difference in infusion delay between the two countries may have been contributed to some extent by social factors such as social distancing or lockdown. However, it can be assumed that the main reason is that self-injectable ADL is not available in China, and only IFX, which requires an outpatient visit and IV infusion, can be administered. Therefore, when contagious diseases such as COVID-19 are spreading, ADL might have an advantage in terms of adherence to IFX.

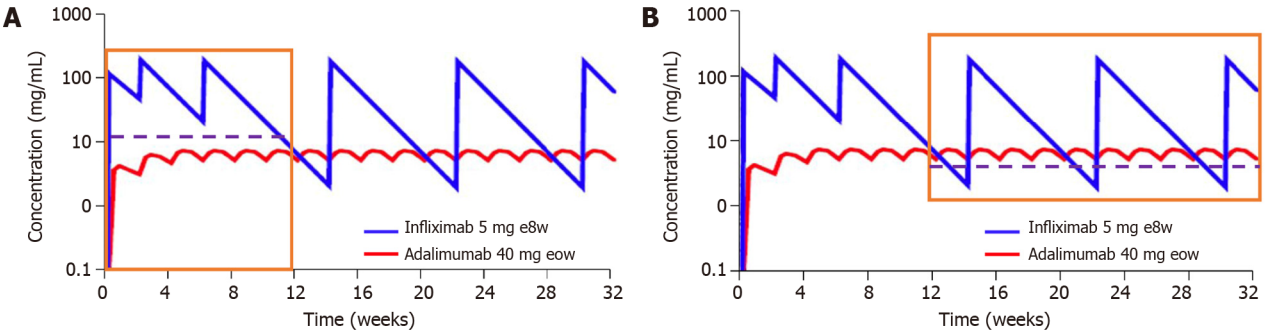
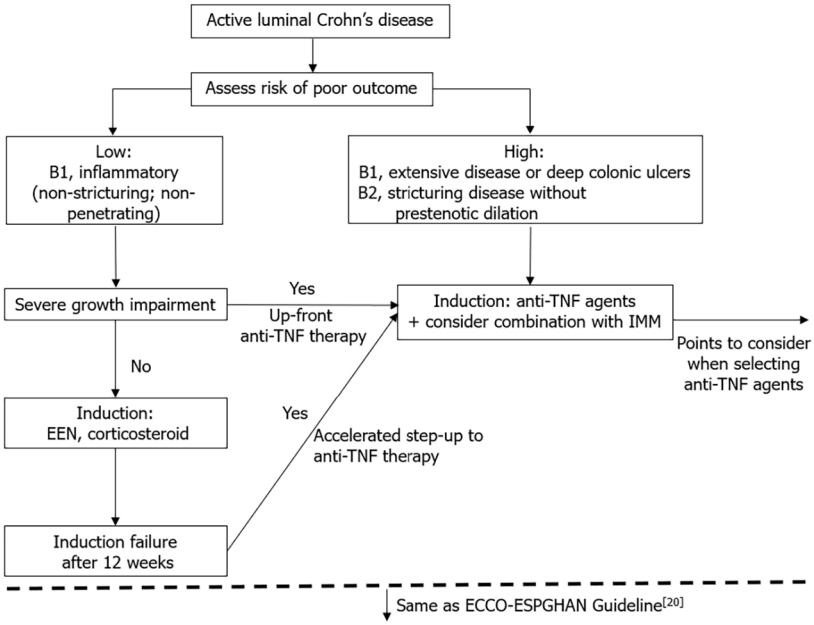


Figure 1 The pharmacokinetic profile of an intravenously or subcutaneously administered anti-tumor necrosis factor agent. A: According to a theoretical induction dosing regimen; B: According to a theoretical maintenance dosing regimen. TNF: tumor necrosis factor. Citation: Gibson DJ, Ward MG, Rentsch C, Friedman AB, Taylor KM, Sparrow MP, Gibson PR. Review article: determination of the therapeutic range for therapeutic drug monitoring of adalimumab and infliximab in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2020; 51: 612-628. Copyright ©John Wiley & Sons Ltd. 2020. Published by John Wiley & Sons[31].



Factors	Infliximab	Adalimumab
Inflammatory burden	Superior	Inferior
Rapid response to induction	Superior	Inferior
Any infection	Superior	Inferior
Adherence	Superior	Inferior
Growth improvement	Equivalent	Equivalent
Malignancy	Equivalent	Equivalent
Sustaining remission in maintenance phase	Inferior	Superior
Immunogenicity	Inferior	Superior
Dermatologic complications	Inferior	Superior
Preference	Depend on culture and medical environment	

Figure 2 Summary flowchart of medical management of pediatric luminal Crohn's disease and points to consider when selecting anti-tumor necrosis factor agents. TNF: Tumor necrosis factor; EEN: Exclusive enteral nutrition; IMM: Immunomodulators. Citation: van Rheenen PF, Aloi M, Assa A, Bronsky J, Escher JC, Fagerberg UL, Gasparetto M, Gerasimidis K, Griffiths A, Henderson P, Koletzko S, Kolho KL, Levine A, van Limbergen J, Martin de Carpi FJ, Navas-López VM, Oliva S, de Ridder L, Russell RK, Shouval D, Spinelli A, Turner D, Wilson D, Wine E, Ruemmele FM. The Medical Management of Paediatric Crohn's Disease: an ECCO-ESPGHAN Guideline Update. *J Crohns Colitis* 2020. Copyright ©Oxford University Press 2020. Published by Oxford University Press[20].

CONCLUSION

Anti-TNF agents have proven to be effective in endoscopic, clinical, and biochemical remission in pediatric patients with moderate-to-severe CD. However, careful anti-TNF therapy is required because of the limitations of biologics approved for pediatric patients. Careful evaluation of clinical indications and disease behavior is essential when prescribing anti-TNF agents. In addition, factors such as the efficacy of induction and maintenance of remission, safety profile, immunogenicity, patient preference, and compliance play an important role in evaluating and selecting treatment options (Figure 2)[20]. Larger cohorts and clinical trials comparing groups based on risk stratification are needed to provide more effective and personalised treatment strategies for pediatric patients.

ACKNOWLEDGEMENTS

There are no additional acknowledgments associated with this article. All authors approved the final version of the article.

FOOTNOTES

Author contributions: Kim ES contributed to the literature search, evidence procurement, writing and editing manuscript, revision; Kang B was the guarantor of the article, contributed to the literature search, evidence procurement, writing and editing manuscript, critical review of manuscript, revision and editing of final manuscript. All authors contributed to the article and approved the submitted version.

Conflict-of-interest statement: All authors have no conflicts of interest to declare.

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S-Editor: Zhang H
L-Editor: A
P-Editor: Yu HG

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 May 14; 29(18): 2798-2817

DOI: 10.3748/wjg.v29.i18.2798

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Calcitriol attenuates liver fibrosis through hepatitis C virus nonstructural protein 3-transactivated protein 1-mediated TGF β 1/Smad3 and NF-κB signaling pathways

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Andersen JB, Denmark; Heij LR, Germany; Mohamed GA, Egypt

Received: December 13, 2022
Peer-review started: December 13, 2022
First decision: February 23, 2023
Revised: March 8, 2023
Accepted: April 10, 2023
Article in press: April 10, 2023
Published online: May 14, 2023

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Abstract

BACKGROUND

Hepatic fibrosis is a serious condition, and the development of hepatic fibrosis can lead to a series of complications. However, the pathogenesis of hepatic fibrosis remains unclear, and effective therapy options are still lacking. Our group identified hepatitis C virus nonstructural protein 3-transactivated protein 1 (NS3TP1) by suppressive subtractive hybridization and bioinformatics analysis, but its role in diseases including hepatic fibrosis remains undefined. Therefore, additional studies on the function of NS3TP1 in hepatic fibrosis are urgently needed to provide new targets for treatment.

AIM
To elucidate the mechanism of NS3TP1 in hepatic fibrosis and the regulatory effects of calcitriol on NS3TP1.

METHODS
Twenty-four male C57BL/6 mice were randomized and separated into three groups, comprising the normal, fibrosis, and calcitriol treatment groups, and liver fibrosis was modeled by carbon tetrachloride (CCl_4). To evaluate the level of hepatic fibrosis in every group, serological and pathological examinations of the liver were conducted. TGF-β1 was administered to boost the *in vitro* cultivation of LX-2 cells. NS3TP1, α-smooth muscle actin (α-SMA), collagen I, and collagen III in every group were examined using a Western blot and real-time quantitative polymerase chain reaction. The activity of the transforming growth factor beta 1 (TGFβ1)/Smad3 and NF-κB signaling pathways in each group of cells transfected with pcDNA-NS3TP1 or siRNA-NS3TP1 was detected. The statistical analysis of the data was performed using the Student's *t* test.

RESULTS
NS3TP1 promoted the activation, proliferation, and differentiation of hepatic stellate cells (HSCs) and enhanced hepatic fibrosis *via* the TGFβ1/Smad3 and NF-κB signaling pathways, as evidenced by the presence of α-SMA, collagen I, collagen III, p-smad3, and p-p65 in LX-2 cells, which were upregulated after NS3TP1 overexpression and downregulated after NS3TP1 interference. The proliferation of HSCs was lowered after NS3TP1 interference and elevated after NS3TP1 overexpression, as shown by the luciferase assay. NS3TP1 inhibited the apoptosis of HSCs. Moreover, both Smad3 and p65 could bind to NS3TP1, and p65 increased the promoter activity of NS3TP1, while NS3TP1 increased the promoter activity of TGFβ1 receptor I, as indicated by coimmunoprecipitation and luciferase assay results. Both *in vivo* and *in vitro*, treatment with calcitriol dramatically reduced the expression of NS3TP1. Calcitriol therapy-controlled HSCs activation, proliferation, and differentiation and substantially suppressed CCl_4-induced hepatic fibrosis in mice. Furthermore, calcitriol modulated the activities of the above signaling pathways *via* downregulation of NS3TP1.

CONCLUSION
Our results suggest that calcitriol may be employed as an adjuvant therapy for hepatic fibrosis and that NS3TP1 is a unique, prospective therapeutic target in hepatic fibrosis.

Key Words: Nonstructural protein 3-transactivated protein 1; Calcitriol; Liver fibrosis; Hepatic stellate cells; Mouse model; TGFβ1/Smad3; NF-κB; Signaling pathway

Core Tip: We proved that hepatitis C virus nonstructural protein 3-transactivated protein 1 (NS3TP1) promoted hepatic fibrosis *via* the transforming growth factor beta 1/Smad3 and NF-κB signaling pathways. Calcitriol attenuates liver fibrosis through NS3TP1-mediated above both signaling pathways. These novel findings profoundly expand our knowledge about the mechanisms underlying the role and function of NS3TP1 in hepatic fibrosis. The relationship between NS3TP1 and liver fibrosis was discussed for the first time and provided a foundation for research related to liver fibrosis by targeting NS3TP1. We first showed that calcitriol alleviated hepatic fibrosis through the above signaling pathways *via* NS3TP1.

Citation: Shi L, Zhou L, Han M, Zhang Y, Zhang Y, Yuan XX, Lu HP, Wang Y, Yang XL, Liu C, Wang J, Liang P, Liu SA, Liu XJ, Cheng J, Lin SM. Calcitriol attenuates liver fibrosis through hepatitis C virus nonstructural protein 3-transactivated protein 1-mediated TGF β1/Smad3 and NF-κB signaling pathways. *World J Gastroenterol* 2023; 29(18): 2798-2817
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2798.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2798

INTRODUCTION

Hepatic fibrosis is a dynamic process that occurs in any type of chronic liver damage that causes a net increase in the extracellular matrix. Further development of liver fibrosis can lead to liver cirrhosis and a series of complications[1,2]. Early intervention for liver fibrosis is crucial, but currently, there is no

effective treatment. Therefore, additional studies on the mechanism of liver fibrosis are urgently needed to provide new targets for treatment.

The open reading framework region of the hepatitis C virus (HCV) genome consists of a core protein region, envelope protein region and a nonstructural protein region. The NS3 gene is located in the nonstructural protein region of the HCV genome and it has serine protease activity and RNA helicase activity. HCV NS3 is crucial for the maturation and replication of the HCV RNA protein[3]. Moreover, this gene contributes to the occurrence of hepatic fibrosis through interaction with host cell components [4]. In this study, we screened and cloned HCV nonstructural protein 3-transactivated protein 1 (NS3TP1) by suppressive subtractive hybridization and bioinformatics analysis. This protein is also referred to asparagine synthetase domain containing 1 (ASNSD1) and registered in GenBank as NBLA00058. The registration number is AY11696. This gene is located on human chromosome 2q32.2. The total length of the gene coding sequence was 1932 nucleotides, and the coding product was 643 amino acid residues. NS3TP1 is widely distributed in the human body[5-8]. NS3 regulates the occurrence of liver fibrosis by interacting with host cells. Whether NS3TP1, as a trans-activator of NS3, can also affect liver fibrosis should be further investigated. Meienberg *et al*[9] demonstrated that NS3TP1 may interact with type 3 collagen alpha 1 chain (COL3A1). In our studies, we found that the NS3TP1 protein increased the expression levels of transforming growth factor beta receptor I (TGFβRI) by gene chip technology. Moreover, TGFβRI is one of the key molecules involved in the activation of the classic transforming growth factor beta 1 (TGFβ1)/Smad3 pathway in liver fibrosis[10]. Therefore, we speculate that NS3TP1 is related to the occurrence of liver fibrosis.

Furthermore, calcitriol was shown to downregulate the expression of NS3TP1 at the mRNA level in the Comparative Toxicogenomics Database (CTD)[11]. Thus, a correlation was established between calcitriol, NS3TP1, and liver fibrosis. As a conventional drug for treating vitamin D deficiency-related rickets, calcitriol regulates biological effects by binding to the vitamin D receptor (VDR). Pop *et al*[12] discovered that VDR was substantially generated in HSCs and Kupffer cells but weakly expressed in hepatic cells. Moreover, VDR is fully functional in HSCs and Kupffer cells. The role of vitamin D in the emergence of chronic hepatic fibrosis was highlighted by the identification of VDR as a crucial endocrine checkpoint for the fibrogenic activity of HSCs[13,14]. This finding indicated that the active component of vitamin D is a vital regulator of hepatic fibrosis. The activating of VDR in HSCs inhibits liver inflammation and fibrosis through the TGFβ1/Smad3 signaling pathway[15]. Moreover, activation of VDR reduces hepatic fibrosis by alleviating inflammation[16,17]. The activation of hepatic stellate cells (HSCs) has been proven to be a main driving factor of liver fibrosis[18]. The TGFβ1/Smad3 signaling pathway is the classical pathway of liver fibrosis activation[19,20]. In addition, NF-κB regulates cell death, inflammation, and wound healing and is therefore a crucial regulator of the progression of hepatic fibrosis[21,22].

The mechanism and mutual regulation between NS3TP1 and calcitriol in liver fibrosis have not been elucidated. However, based on previous studies, it was speculated that NS3TP1 increases hepatic fibrosis by triggering the TGFβ1/Smad3 and NF-κB signaling pathways. Moreover, calcitriol can regulate NS3TP1, but further studies are necessary for verification.

MATERIALS AND METHODS

Materials

Calcitriol was purchased from MedChemExpress (United States).

Animals

Liver fibrosis was induced by carbon tetrachloride (CCl_4) solubilized in corn oil (Sigma-Aldrich, Germany). Male C57BL/6 mice aged 8 wk were bought from Beijing Weitonglihua Laboratory Corporation (China). They experienced a week in a clean animal room at 24 °C with unrestricted access to food and water prior to the trials. The average weight of mice before modeling was 23-25 g, and mice were given an intraperitoneal (*i.p.*) injection with CCl_4 three times/wk (0.5 μL/g) for 4 wk to induce fibrosis[21,23,24]. Mice with liver fibrosis were successfully established and randomly separated into calcitriol and negative control (NC) groups. Mice in the control group were administered normal saline (10 μL/g/d, 5 times/wk) intragastrically for 4 wk and injected (*i.p.*) with corn oil or CCl_4, while mice in the calcitriol group were intragastrically administered calcitriol (1 μg/kg/d, 5 times/wk) and intraperitoneally injected with CCl_4 for 4 wk. Finally, 48 h after being injected with CCl_4, the mice were sacrificed. Normal saline was used to dilute 100% avertin to a 2.5% solution, and the anesthesia dose for mice was an *i.p.* injection of 100-200 μL/10 g. The mice were euthanized by deep anesthesia, followed by cervical dislocation. The Xi'an Jiaotong University Medical Science Center's specific pathogen free Animal Laboratory Center served as the site for all investigations. The Research Ethics Council of the Xi'an Jiaotong University Medical Science Center (Xi'an, China) gave its approval to all animal trials. All animals were treated humanely, and the experimental protocols were carried out in line with the regulations of the institution.

Cell culture

LX-2 cells are hepatogenic mesenchymal human cells and were obtained from Xiang Ya Central Laboratory (Xiangya Medical College, China). The cells were transfected using jetPRIME reagent (PolyPlus Transfection SA, NY, United States) and treated with calcitriol. Recombinant human TGFβ1 (BioLegend, CA, United States) was administered to the cell cultivation medium at 2.5 ng/mL or 5.0 ng/mL for 24 h[18,25]. All procedures were executed in accordance with the manufacturer's guidelines.

Plasmids and shortinterfering RNA (siRNA) oligonucleotides

PcDNA3.1/mycHis(-)NS3TP1, pGL4.10-NS3TP1 promoter, pGL4.10-p65 promoter, and pGL4.10-TGFβ 1R promoter were constructed by Beijing Genomics Institute (BGI, China). NS3TP1-siRNA was purchased from Gene Pharma (Hong Xun, Suzhou, Jiangsu Province, China).

Real-time quantitative polymerase chain reaction

Total RNA (Total RNA Kit, Omega, United States) was extracted from LX-2 cells and reverse-transcribed into single-stranded cDNA (Prime Script RT Reagent Kit, TaKaRa, China). Real-time quantitative polymerase chain reaction (RT-qPCR) was used to amplify the genes using specific primers (Hong Xun, Suzhou, Jiangsu Province, China), and β-actin was used as the internal control gene. The level of the target gene was estimated using the ΔΔCT method and normalized to that of the control. Supplementary Table 1 includes a list of the primer sequences.

Immunoblot

Proteins were extracted from mouse livers or LX-2 cells and isolated using 10% BIS-Tris gel/MOPS (Invitrogen, NY, United States) in MOPS SDS-PAGE (Thermo Fisher, United States). Transferring the separated proteins to a membrane made of polyvinylidene fluoride (PVDF) (Millipore, United States), which was incubated with secondary antibodies for 1.5 h after being exposed to primary antibodies (Supplementary Table 2) for 12 h at 4 °C. The immunoreactive bands were created using intensified chemiluminescence (Thermo Fisher Scientific) and visualized using the ChemiDoc™ touch imaging system (Bio-Rad, Hercules, CA, United States). ImageJ was used to assess the pictures.

Coimmunoprecipitation

Ice-cold M-PERTM mammalian protein extraction reagent was used to lyse LX-2 cells, and it also contained a mixture of protease and phosphatase inhibitors. Whole cell lysates (500 μL) were incubated with 50 μL protein magnetic beads for 2 h at room temperature and centrifuged, and the supernatant was transferred to a new 1.5 mL centrifuge tube. After that, the beads were mixed for 2 h at room temperature with whole-cell lysates containing 2 μL of anti-HIS antibody and 3 μL of regular mouse IgG antibody. The beads were eluted in DTT-free Laemmli buffer and then washed three times with PBST before being subjected to western blotting analysis.

Proliferation assay

On a 96-well plate, LX-2 cells were exposed to various calcitriol concentrations for 24 h. PBS was used to wash the cells twice after the initial medium was removed. Each well received a 1:100 addition of CCK-8 (Dojindo, Kumamoto, Japan) reagent before being cultured for 1 h at 37 °C. At 450 nm, the optical density was calculated.

Flow cytometry

LX-2 cells were treated with various concentrations of calcitriol for 48 h. The original medium was washed twice with PBS. The cells were collected by flow cytometry, treated with the reagents from an Annexin V-FITC/7-AAD apoptosis detection kit (BioLegend, CA, United States), and measured by a FACSCalibur flow cytometer (Beckman Coulter, CA, United States).

Hematoxylin-eosin staining, Masson staining, Sirius red staining, and oil red O staining

Hematoxylin was obtained from Yili Reagent Company (Beijing, China). Eosin was obtained from Zhongshan Jinqiao Biotechnology Company (Beijing, China). A Masson Tricolor staining kit was bought from Bogoo Corporation (Shanghai, China). A Pico Sirius Red Staining Kit was bought from Ruisai Biologicals (Shanghai, China). Oil red dye was obtained from Sigma-Aldrich (MO, United States). The manufacturer's instructions were strictly adhered to the conduct of every experiment.

Immunohistochemistry

Liver slices underwent an overnight incubation at 4 °C with a primary antibody and a 40-minute incubation at room temperature with an enzyme-labeled anti-rabbit antibody (PV-6001, ZSGB-BIO, Beijing, China). The slices were then developed using DAB substrate (Gene Science and Technology Company, China) after 30 min at room temperature incubation with avidin-biotin complex (PK-6100, Vectastain Elite ABC Standard kit, Vector Laboratories, Burlingame, CA, United States).

Assay of luciferase activity

HepG2 cells were transiently transfected with different promoter plasmids. A renal cell luciferase vector plasmid was used as a control. Dual-luciferase reporter gene activity was evaluated using a dual-luciferase reporter gene detection kit (Promega, United States).

Statistical analysis

Statistical analyses were performed using SPSS 24.0. The Student's *t* test was used to statistically examine all data. A P value < 0.05 was regarded as statistically significant. The data is shown as the mean ± SE.

RESULTS

Upregulated expression of NS3TP1 in HSCs and CCl_4-treated mouse livers

Herein, we confirmed the *in vivo* and *in vitro* roles of NS3TP1 in hepatic fibrosis. The level of NS3TP1 was measured in mice with CCl_4-induced hepatic fibrosis and TGF β1-activated LX-2 cells. Hematoxylin-eosin staining, Masson staining, Sirius red staining, and immunohistochemical staining for α-SMA demonstrated the successful establishment of CCl_4-induced fibrosis models (Supplementary Figure 1). The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were elevated in mouse plasma (Supplementary Figure 2), suggesting hepatocyte injury. Hepatic fibrosis scores confirmed the disease, and the Ishak score was higher in the CCl_4 group (Supplementary Figure 3).

In vivo, COL1A1, COL3A1, α-SMA, and NS3TP1 were considerably elevated at the mRNA level in CCl_4-treated mouse livers (Figure 1A). The level of α-SMA in the CCl_4-treated mouse livers was upregulated, as indicated by immunofluorescence (Figure 1B). NS3TP1 in CCl_4-handled mouse livers was upregulated, as indicated by immunohistochemistry (Figure 1C and D) and immunofluorescence (Figure 1E).

In vitro, Western blotting was performed to monitor the level of NS3TP1 in various cell lines, such as L02, LX-2, Huh7, and HepG2, and NS3TP1 was significantly raised in LX-2 cells (Figure 1F). In addition, LX-2 cells were administered with recombinant human TGFβ1 protein for 24 h. Then, α-SMA, collagen I, collagen III, and NS3TP1 were found to be significantly upregulated at the protein and mRNA levels (Figure 1G and H).

Therefore, *in vivo* and *in vitro* investigations validated the elevation of NS3TP1 in hepatic fibrosis.

Influence of NS3TP1 overexpression or interference on the progression of hepatic fibrosis in vitro

The pcDNA-NS3TP1 plasmid was constructed and transiently transfected into LX-2 cells. The expression levels of fibrosis-related proteins were detected by Western blotting after 48 h. Compared to those in the negative control group, the levels of collagen I and α-SMA in the NS3TP1 overexpression group were increased (Figure 2A). The mRNA levels of COL1A1, COL2A1, COL3A1, COL4A2, and -SMA were monitored using RT-qPCR, and the results matched those of the Western blotting (Figure 2B). The levels of collagen I, collagen III, collagen IV, and α-SMA in NS3TP1 knockdown LX-2 cells were measured by Western blotting and RT-qPCR after 48 h. The results showed that in the NS3TP1 gene interference group, the expression of collagen I, collagen III, collagen IV, and α-SMA at the protein and mRNA levels was significantly downregulated (Figure 2C and D). This finding was the opposite of that of the NS3TP1 overexpression group.

NS3TP1 was interfered or overexpressed in LX-2 cells, the impact of NS3TP1 on HSC growth was then examined after 24 h, 48 h, and 72 h using a Cell Counting Kit-8 (CCK-8) cell proliferation and activity detection kit. Compared to the control group's results, the proliferation of HSCs in the NS3TP1 interference group decreased, while it increased after NS3TP1 overexpression, indicating that NS3TP1 increased the proliferation of HSCs (Figure 2E and F).

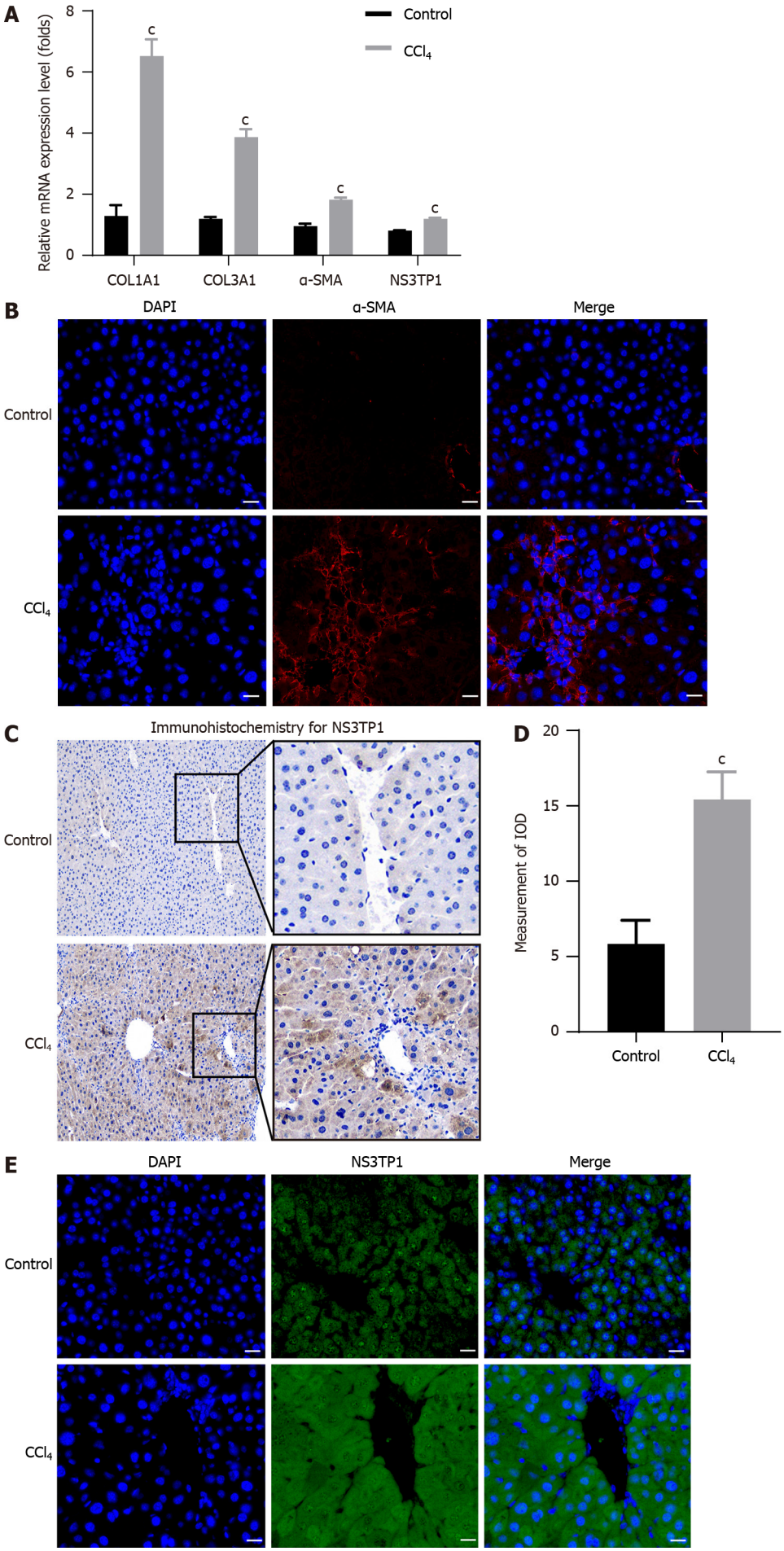
In LX-2 cells, after NS3TP1 was knocked down, the level of Bcl-2 was downregulated, the level of Bax was upregulated, apoptosis was enhanced, and the effects induced by overexpression were reversed. These results indicated that NS3TP1 inhibited the apoptosis of HSCs (Figure 2G and H).

Consequently, *in vitro* research supported NS3TP1's significance in fostering hepatic fibrosis.

Effect of NS3TP1 overexpression or interference on the TGFβ1/Smad3 and NF-kB signaling pathways

After transient transfection of LX-2 cells with the NS3TP1-overexpressing plasmid or siRNA-NS3TP1 for 48 h, the activity of the TGFβ1/Smad3 and NF-κB signaling pathways was evaluated. In comparison to that of the control group, the activity of both signaling pathways was elevated in the NS3TP1 overexpression group and decreased in the gene interference group. These results suggested that NS3TP1 promoted hepatic fibrosis *via* both signaling pathways (Figure 3A and B).

We applied 3 μmol/L Smad3-specific inhibitor (SIS3) and 2 μmol/L licochalcone D (LD, inhibition of the phosphorylation of NF-κB at serine 276) to inhibit the above pathways and then added TGFβ1 (5 ng/mL) or LPS (1 μg/mL). NS3TP1 and downstream collagen I, α-SMA, p-smad3, and p-p65 Levels were decreased, indicating that NS3TP1 was localized downstream of Smad3 and p65 (Figure 3C and


A
Relative mRNA expression level (folds)
Control
CCl4
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COL3A1
α-SMA
NS3TP1
B
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α-SMA
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Control
CCl4
C
Immunohistochemistry for NS3TP1
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CCl4
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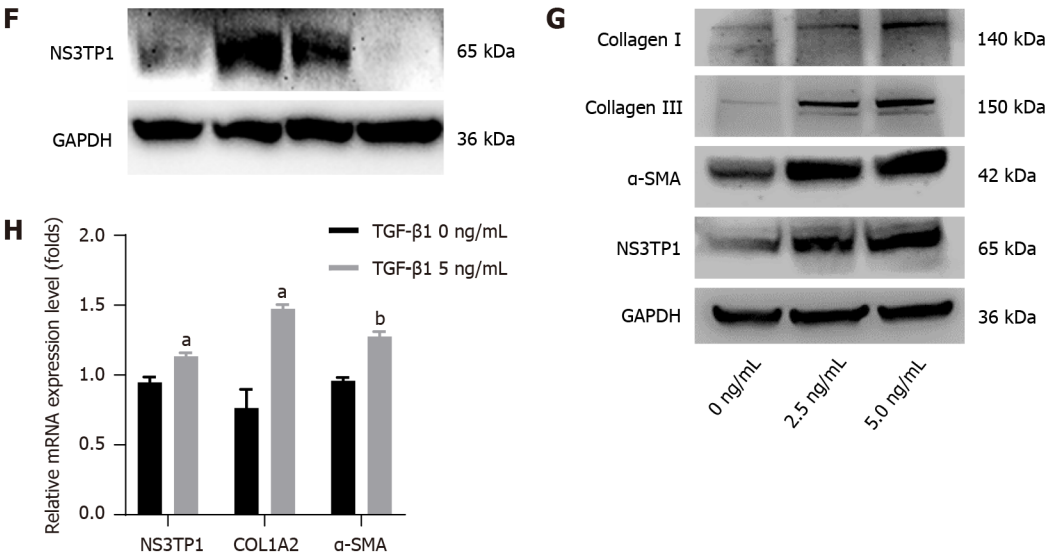

DOI: 10.3748/wjg.v29.i18.2798

Figure 1 Evaluation of nonstructural protein 3-transactivated protein 1 expression in carbon tetrachloride-induced hepatic fibrosis and Transforming growth factor 1 beta 1-stimulated LX-2 cells. A: Real-time quantitative polymerase chain reaction analysis of fibrosis-related genes in liver tissues (n = 6); B: Immunofluorescence staining for alpha smooth muscle actin (α-SMA); red: α-SMA (n = 3), scale bar = 20 μm; C: Immunohistochemical staining for α-SMA, scale bar = 100 μm; D: Image J analysis of immunohistochemical staining for α-SMA (n = 3); E: Immunofluorescence staining for nonstructural protein 3-transactivated protein 1 (NS3TP1); green: NS3TP1 (n = 3), scale bar = 20 μm; F: Western blot analysis of NS3TP1 in L02, LX-2, Huh7, and G2 cells; G and H: NS3TP1 was overexpressed in LX-2 cells treated with transforming growth factor 1 beta 1 (TGFβ1) for 24 h (n = 3). The data was presented as mean ± SE. ^{a}P < 0.05, ^{b}P < 0.01 *vs* TGFβ1 (0 ng/mL) group; ^{c}P < 0.01 *vs* corn oil control group. NS3TP1: Nonstructural protein 3-transactivated protein 1; TGFβ1: Transforming growth factor 1 beta 1; CCl_4: Carbon tetrachloride; RT-qPCR: Real-time quantitative polymerase chain reaction; α-SMA: Alpha smooth muscle actin.

D).

Using coimmunoprecipitation (Co-IP), we found that NS3TP1 could bind to Smad3 and p65 (Figure 3E). The dual luciferase assay revealed that NS3TP1 enhanced TGFβRI promoter activity (Figure 3F), while TGFβ1 had no effect on NS3TP1 promoter activity (Figure 3G). Moreover, NS3TP1 had no effect on p65 promoter activity (Figure 3H), while p65 increased NS3TP1 promoter activity (Figure 3I). Therefore, NS3TP1 may inhibit the activity of both signaling pathways by suppressing the phosphorylation of Smad3 or p65 at the protein level. In addition, NS3TP1 may regulate the TGFβ1/Smad3 signaling pathway at the mRNA level by upregulating the TGFβRI promoter, and the regulation of the NS3TP1 and NF-κB signaling pathways at the mRNA level may be realized through the upregulation of the NS3TP1 promoter by p65.

Calcitriol alleviates liver fibrosis

The CCK-8 kit was used to detect LX-2 cells stimulated with various concentrations of calcitriol. After incubation for 48 h with the CCK-8 reagent, the absorbance value decreased with increasing calcitriol concentration, which could be attributed to calcitriol-inhibited cell proliferation (Supplementary Figure 4). Calcitriol reduced the activation of HSCs in a concentration- and time-dependent manner. The optimum concentration of calcitriol was 16 μmol/L (Figure 4A). The optimum time for calcitriol treatment was 48 h (Figure 4B). Calcitriol promoted the return of TGFβ1-activated HSCs to quiescent HSCs (qHSCs) (Figure 4C). Following calcitriol administration, a Western blot analysis revealed concentration-dependently elevated Bax and diminished Bcl-2 Levels (Figure 4D). Flow cytometry showed that LX-2 cell apoptosis increased with increasing calcitriol concentration (Figure 4E and F). Oil red O staining showed an increased number of fat droplets in LX-2 cells (Supplementary Figure 5). Calcitriol reduced the migration of activated HSCs (Figure 4G and H).

In vivo, the dose and the protocol for this study were chosen based on the results of previous studies [26-28]. The optimal dosage of calcitriol was identified as 1 μg/kg/d, 5 times/wk according to the preliminary experimental results, and subsequent experiments were carried out with this dosage.

Calcitriol treatment reduced CCl_4-induced collagen accumulation in the mouse liver, as shown by hematoxylin-eosin staining, Masson staining, Sirius red staining, immunohistochemistry for α-SMA (Figure 4I), and immunofluorescence for α-SMA (Figure 4J). Western blot results further support these conclusions (Figure 4K and L). Liver fibrosis was improved by calcitriol treatment, according to the Ishak scoring system (Figure 4M). Plasma ALT and AST levels also demonstrated that calcitriol decreased CCl_4-induced inflammation in the mouse liver (Figure 4N). These findings showed that calcitriol prevented hepatic fibrosis both *in vivo* and in a lab setting.

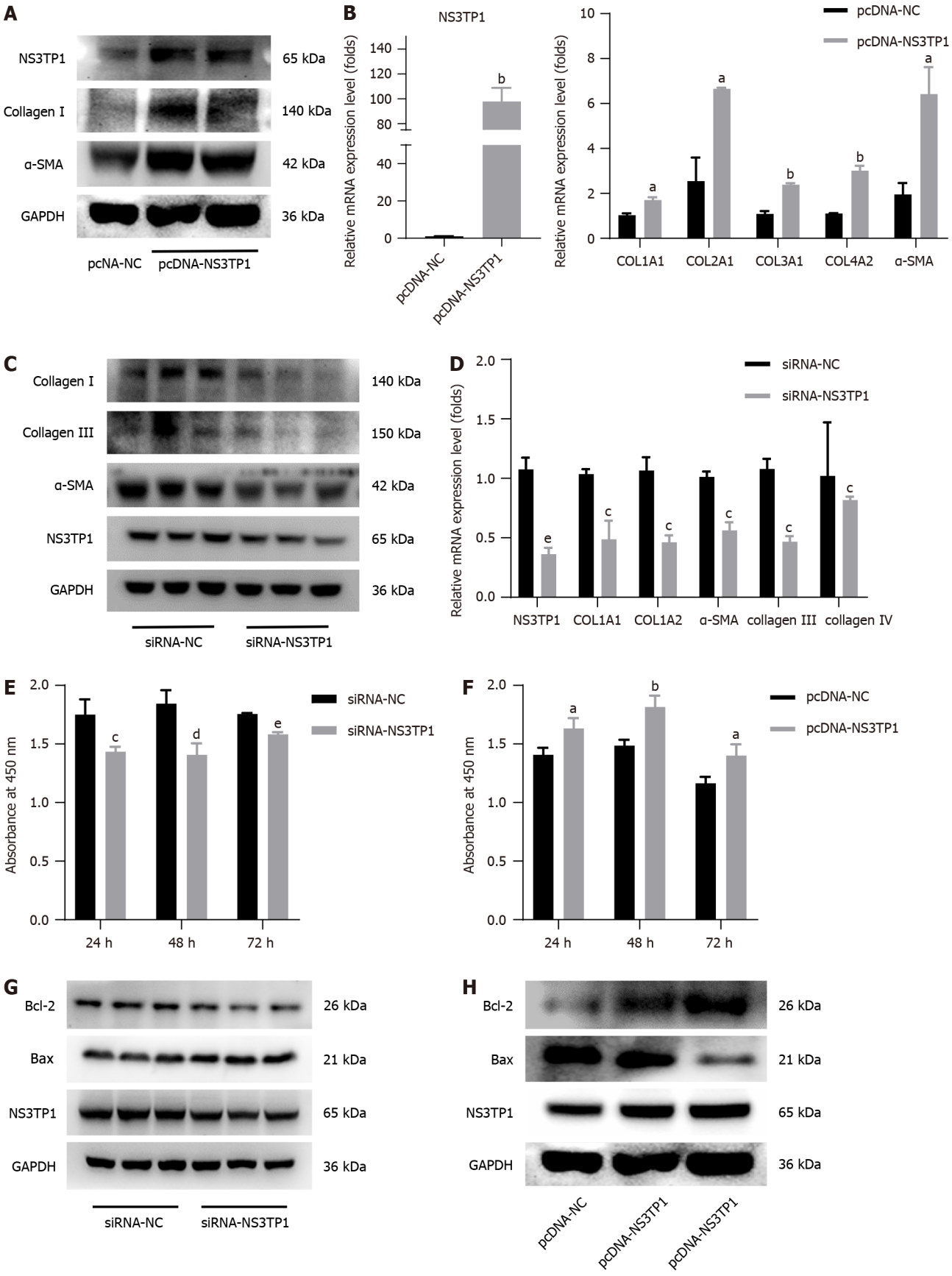

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Figure 2 Influence of nonstructural protein 3-transactivated protein 1 on liver fibrosis *in vitro*. A: Western blot analysis of fibrosis-related genes after Nonstructural protein 3-transactivated protein 1 (NS3TP1) overexpression (n = 3); B: Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of fibrosis-related genes after NS3TP1 overexpression (n = 3); C: Western blot analysis of the fibrosis-related genes after NS3TP1 interference (n = 3); D: RT-qPCR analysis of the fibrosis-related genes after NS3TP1 interference (n = 3); E and F: Cell proliferation was measured by cell counting kit-8 assays after interference or

overexpression of NS3TP1 (n = 3); G and H: Western blot analysis of Bcl-2 and Bax after interference or overexpression of NS3TP1 (n = 3). The data was represented as mean ± SE. [a]P < 0.05, [b]P < 0.01 *vs* pcDNA-NS3TP1 group; [c]P < 0.05, [d]P < 0.01, [e]P < 0.001 *vs* siRNA-NS3TP1 group. NS3TP1: Nonstructural protein 3-transactivated protein 1; CCK-8: Cell counting kit-8; COL1A1: Type 1 collagen alpha 1 chain; COL1A2: Type 1 collagen alpha 2 chain; COL3A1: Type 3 collagen alpha 1 chain; COL4A2: Type 4 collagen alpha 2 chain.

Prevention of calcitriol on liver fibrosis through the TGFβ1/Smad3 and NFkB signaling pathways via NS3TP1 in vivo and in vitro

Calcitriol prevented the expression of NS3TP1 at the mRNA level in the CCl_4-treated mouse liver, as shown by RT-qPCR (Figure 5A) and by immunohistochemistry (Figure 5B and C). Moreover, calcitriol inhibited the expression of NS3TP1 at the protein level, as shown by immunofluorescence staining for NS3TP1 (Figure 5D). In LX-2 cells, the inhibition of NS3TP1 by calcitriol at the protein level was time- and concentration dependent, with an optimal concentration of 16 μmol/L and an optimal treatment time of 48 h (Figure 5E and F). In addition, the activity of the NS3TP1 promoter was inhibited by 32 μmol/L calcitriol in HepG2 cells, as validated by dual-luciferase reporter gene assay (Figure 5G).

Both signaling pathways were inhibited in a dose- and time-dependent manner after 12 h of TGFβ1 treatment and 48 h of calcitriol treatment in LX-2 cells (Figure 5H). Calcitriol significantly inhibited the LPS-activated NF-κB signaling pathway in HSCs (Figure 5I). PcDNA 3.1/myC-His(-)-NS3TP1 was transfected into LX-2 cells for 24 h, and then the cells were stimulated with calcitriol for 48 h. Calcitriol inhibited LX-2 cell activation following NS3TP1 overexpression (Figure 5J).

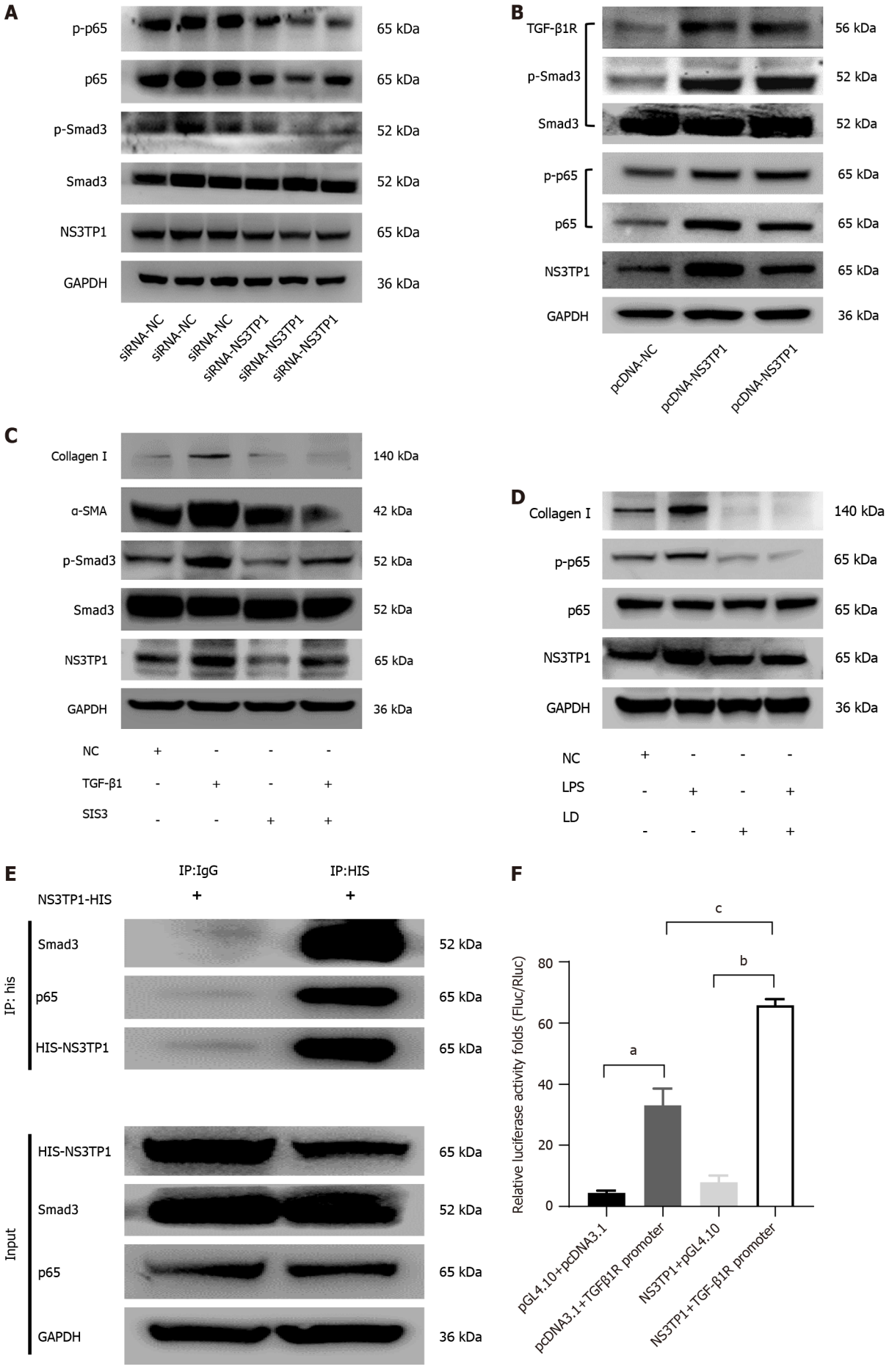
In conclusion, these results confirm that calcitriol prevented hepatic fibrosis through the above signaling pathways *via* NS3TP1.

DISCUSSION

The present study first showed that NS3TP1 promoted hepatic fibrosis by enhancing the TGFβ1/Smad3 and NF-κB signaling pathways. NS3TP1 controlled HSC activation, proliferation, and differentiation. These results provide solid proof for the role of NS3TP1 contributing to hepatic fibrosis.

Both *in vivo* and *in vitro* experiments confirmed that NS3TP1 was elevated in liver fibrosis (Figure 1), which was consistent with our group's previous results. TGF-β1 activates hepatic stellate cells effectively[29], and the CCl_4-induced liver fibrosis model is one of the classical models of liver fibrosis [30]. There was an increase in NS3TP1 expression levels in the treated cells and tissues, indicating that NS3TP1 plays a role in liver fibrosis development. Moreover, NS3TP1 promoted the activation and proliferation but inhibited the apoptosis of LX-2 cells (Figure 2), Meienberg *et al*[9] demonstrated that NS3TP1 may interact with COL3A1, which further supports that NS3TP1 regulates the occurrence of hepatic fibrosis. The TGFβ1/Smad3 and NF-κB signaling pathways have been well established as two classical pathways for hepatic stellate cell activation[20,22,31]. To confirm the correlation between NS3TP1 and the activity of both signaling pathways, we overexpressed or interfered with NS3TP1 in LX-2 cells. We found that the activities of both signaling pathways were enhanced in the NS3TP1 overexpression group. However, the activities of both signaling pathways were decreased in the gene interference group, suggesting that NS3TP1 regulated hepatic fibrosis through both the above signaling pathways. To further elucidate the specific mechanisms between NS3TP1 and both signaling pathways, we used Co-IP and dual-luciferase assays. Co-IP analysis demonstrated that Smad3 and p65 could bind to NS3TP1. Therefore, NS3TP1 may decrease the activity of both signaling pathways by inhibiting the phosphorylation of Smad3 or p65. It is necessary to examine the possible colocalization of NS3TP1 and Smad3 or p65 in LX-2 cells by confocal microscopy. The dual-luciferase assay revealed that NS3TP1 increased the activity of the TGFβRI promoter and that p65 increased the activity of the NS3TP1 promoter. Altogether, NS3TP1 regulated both signaling pathways at protein and mRNA levels (Figure 3). The key targets of both signaling pathways are p-smad3 and p65 respectively, and p65 is the classic regulatory factor of the inflammatory pathway[32,33], which further supports our research.

We confirmed that calcitriol inhibited hepatic fibrosis *in vitro* and *in vivo* (Figure 4). This finding is consistent with previous research[26,34,35]. *In vivo* experiments were conducted using CCl_4-induced model mice, wherein calcitriol was found to attenuate liver fibrosis. The optimal dosage of calcitriol to treat liver fibrosis was 1 μg/kg/d, administered five times per week, which also reduced CCl_4-induced inflammation in the mouse liver. Calcitriol decreased the deposition of extracellular matrix following HSC activation, prevented the proliferation, activation, and migration of HSCs, and promoted cell apoptosis, and these *in vitro* experiments were primarily conducted using LX-2 cells. In addition, HSCs were also able to accumulate lipid droplets when exposed to calcitriol, which may be related to the dedifferentiation of activated HSCs into qHSCs, suggesting that calcitriol reversed hepatic fibrosis as described previously[25,36]. In conclusion, calcitriol inhibited liver fibrosis, however, the precise mechanism was uncertain. Therefore, we investigated the mechanisms by which calcitriol prevented hepatic fibrosis.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LD - - + +
E
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IP:HIS
NS3TP1-HIS + +
IP: his
Smad3
p65
HIS-NS3TP1
52 kDa
65 kDa
65 kDa
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Smad3
p65
GAPDH
65 kDa
52 kDa
65 kDa
36 kDa
F
Relative luciferase activity folds (Fluc/Rluc)
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20
40
60
80
a
b
c
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pcDNA3.1+TGFβ1R promoter
NS3TP1+pGL4.10
NS3TP1+TGF-β1R promoter

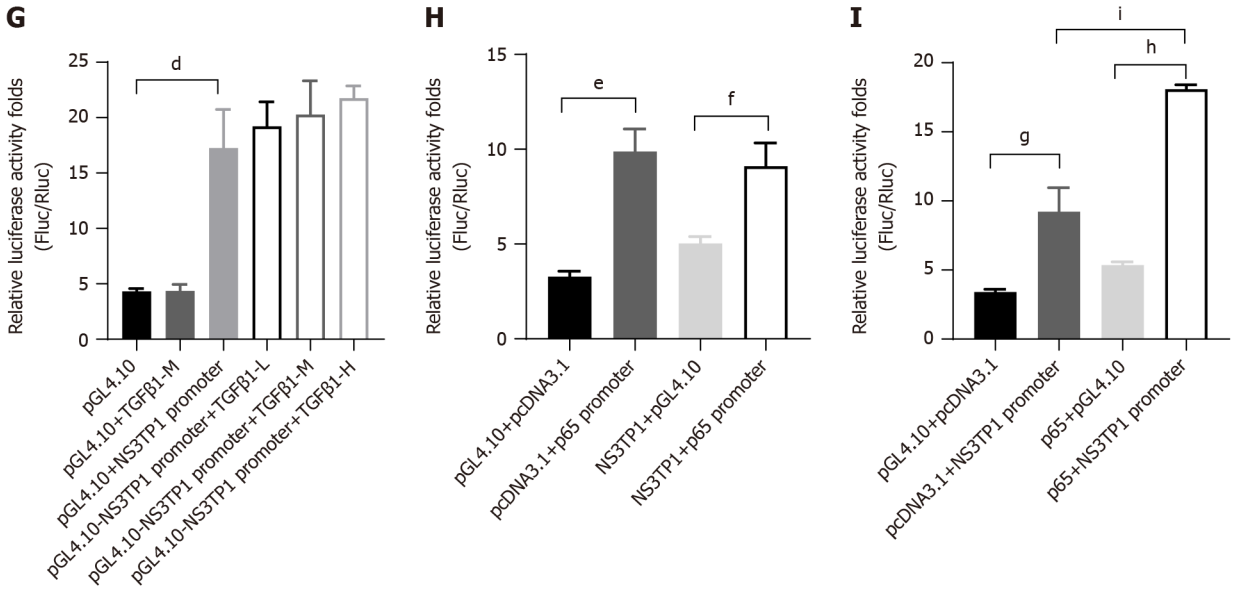

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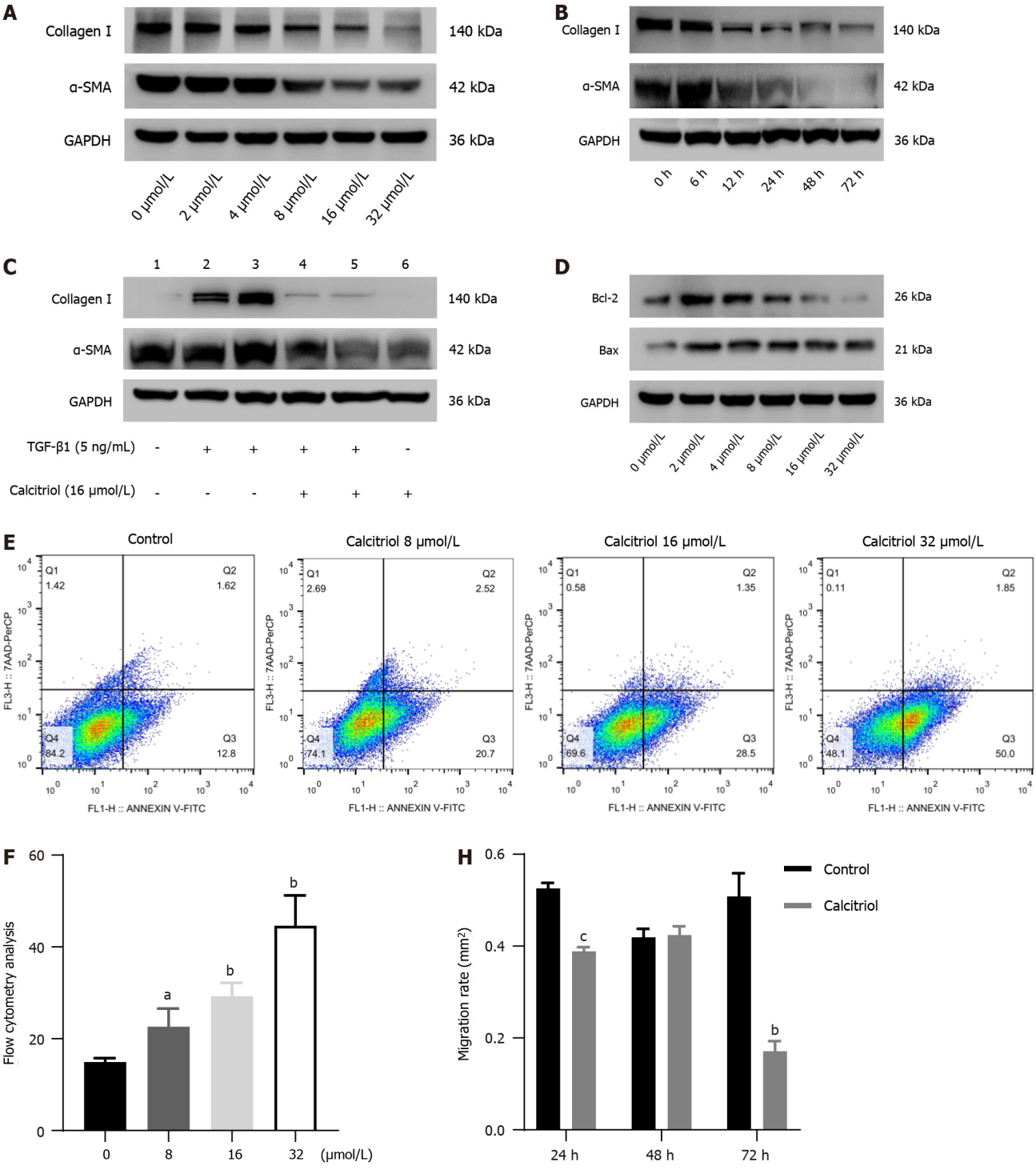
Figure 3 Promotion of nonstructural protein 3-transactivated protein 1 in hepatic fibrosis *via* transforming growth factor beta 1 receptor/Smad3 and NF-kB signaling pathways. A and B: The protein levels of molecules in the transforming growth factor beta 1 (TGFβ1)/Smad3 and NF-kB signaling pathways after nonstructural protein 3-transactivated protein 1 (NS3TP1) interference or overexpression in LX-2 cells were analyzed by Western blot (n = 3); C and D: Western blot analysis of the above signaling pathway molecules in LX-2 cells after Smad3-specific inhibitor or licochalcone D treatment (n = 3); E: Coimmunoprecipitation analysis between NS3TP1 and Smad3 or p65; F: Luciferase activity analysis between NS3TP1 and the TGFβ1 receptor 1 (TGFβRI) promoter (n = 3); G: Luciferase activity analysis between TGFβ1 and the NS3TP1 promoter; TGFβ1 (L: 2.5 ng/mL, M: 5 ng/mL, H: 10 ng/mL) (n = 3); H: Luciferase activity analysis between NS3TP1 and the p65 promoter (n = 3); I: Luciferase activity analysis between p65 and the NS3TP1 promoter (n = 3). The data was represented as mean ± SE. $^{a}P < 0.001$ pcDNA3.1 + TGFβ1R promoter *vs* pGL4.10 + pcDNA3.1, $^{b}P < 0.0001$ NS3TP1 + TGFβ1R promoter *vs* NS3TP1 + pGL4.10; $^{c}P < 0.001$; NS3TP1 + TGFβ1R promoter *vs* pcDNA3.1 + TGFβ1R promoter; $^{d}P < 0.01$ pGL4.10 + NS3TP1 promoter *vs* pGL4.10; $^{e}P < 0.001$ pcDNA3.1 + p65 promoter *vs* pGL4.10 + pcDNA3.1, $^{f}P < 0.01$ NS3TP1 + p65 promoter *vs* NS3TP1 + pGL4.10; $^{g}P < 0.01$ pcDNA3.1 + NS3TP1 promoter *vs* pGL4.10 + pcDNA3.1, $^{h}P < 0.0001$ p65 + NS3TP1 promoter *vs* p65 + pGL4.10; $^{i}P < 0.001$ p65 + NS3TP1 promoter *vs* pcDNA3.1 + NS3TP1 promoter. NS3TP1: Nonstructural protein 3-transactivated protein 1; TGFβ1R: Transforming growth factor beta 1 receptor; p-smad3: Phosphorylated sekelsky mothers against decapentaplegic homolog 3; SIS3: Smad3-specific inhibitor; LD: Licochalcone D; Co-IP: Coimmunoprecipitation.

We demonstrated that calcitriol downregulated NS3TP1 at the mRNA level in CTD[11]. Moreover, we first proved that calcitriol inhibited NS3TP1 *in vivo* and *in vitro* (Figure 5) in a dose-dependent manner, and the increased NS3TP1 expression levels were found to be suppressed by calcitriol in mouse liver fibrotic tissues. The dual-luciferase assay showed that calcitriol blocked the promoter activity of NS3TP1, which conforms with the research of Wang *et al*[11]. Finally, we first demonstrated that calcitriol prevented hepatic fibrosis through the above signaling pathways *via* NS3TP1. Calcitriol inhibited liver fibrosis by binding to VDR, and NS3TP1 may be one of the targets. Whether other molecules that can bind to VDR can also reduce the expression of NS3TP1 will be the focus of further research. The results by Hah *et al*[15] supported this phenomenon. It was found that activating VDR in HSCs prevented liver inflammation and fibrosis through the TGFβ1/Smad3 signaling pathway. Activation of the TGFβ1 signaling pathway leads to genome-wide reallocation of VDR binding through TGFβ1-dependent chromatin remodeling in the presence of VDR ligands. By attaching to Smad3, VDR decreases Smad3 occupancy at these locations and inhibits fibrosis[15]. We proved that calcitriol decreased liver fibrosis through the NF-κB signaling pathway *via* NS3TP1. This finding is consistent with previous studies. Calcitriol suppresses the NF-κB signaling pathway, which confirms its anti-inflammatory effect, and activated HSCs are involved in inflammation[37].

Regarding the limitations of this study, animal experiments were not completed on NS3TP1-KO mice. In our research, we discovered that NS3TP1-KO mice could not give birth normally. Combined with the results of GeneCards database retrieval, we found that the NS3TP1 content in the reproductive system is quite rich. We therefore speculated that knocking down NS3TP1 would also have an impact on reproductive functions. To learn more about how the protein contributes to liver fibrosis, we will concentrate on NS3TP1 knockouts that target the liver.

CONCLUSION

In conclusion, NS3TP1 regulates TGFβ1/Smad3 and NF-κB signaling pathways to induce liver fibrosis. These results contribute to NS3TP1 as a novel, prospective therapeutic target for the treatment of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GAPDH
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Collagen I
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GAPDH
140 kDa
42 kDa
36 kDa
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1 2 3 4 5 6
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α-SMA
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E
Control
Calcitriol 8 μmol/L
Calcitriol 16 μmol/L
Calcitriol 32 μmol/L
FL3-H :: 7AAD-PerCP
FL1-H :: ANNEXIN V-FITC
Q1 1.42
Q2 1.62
Q4 84.2
Q3 12.8
Q1 2.69
Q2 2.52
Q4 74.1
Q3 20.7
Q1 0.58
Q2 1.35
Q4 69.6
Q3 28.5
Q1 0.11
Q2 1.85
Q4 48.1
Q3 50.0
F
Flow cytometry analysis
0
20
40
60
a
b
b
0
8
16
32
(μmol/L)
H
Migration rate (mm²)
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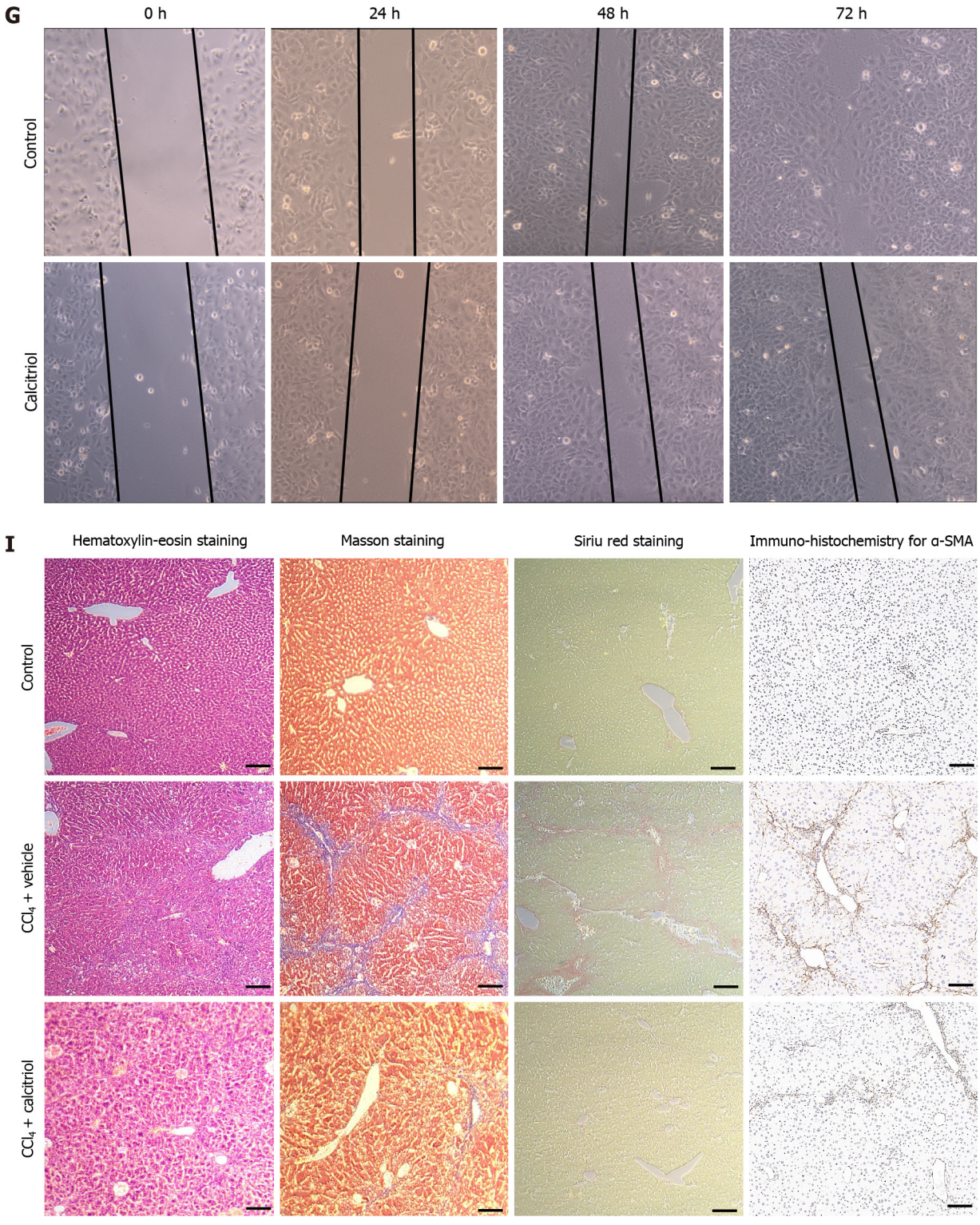
0.2
0.4
0.6
c
b
Control
Calcitriol
24 h
48 h
72 h


G
0 h
24 h
48 h
72 h
Control
Calcitriol
I
Hematoxylin-eosin staining
Masson staining
Siriu red staining
Immuno-histochemistry for α-SMA
Control
CCl4 + vehicle
CCl4 + calcitriol

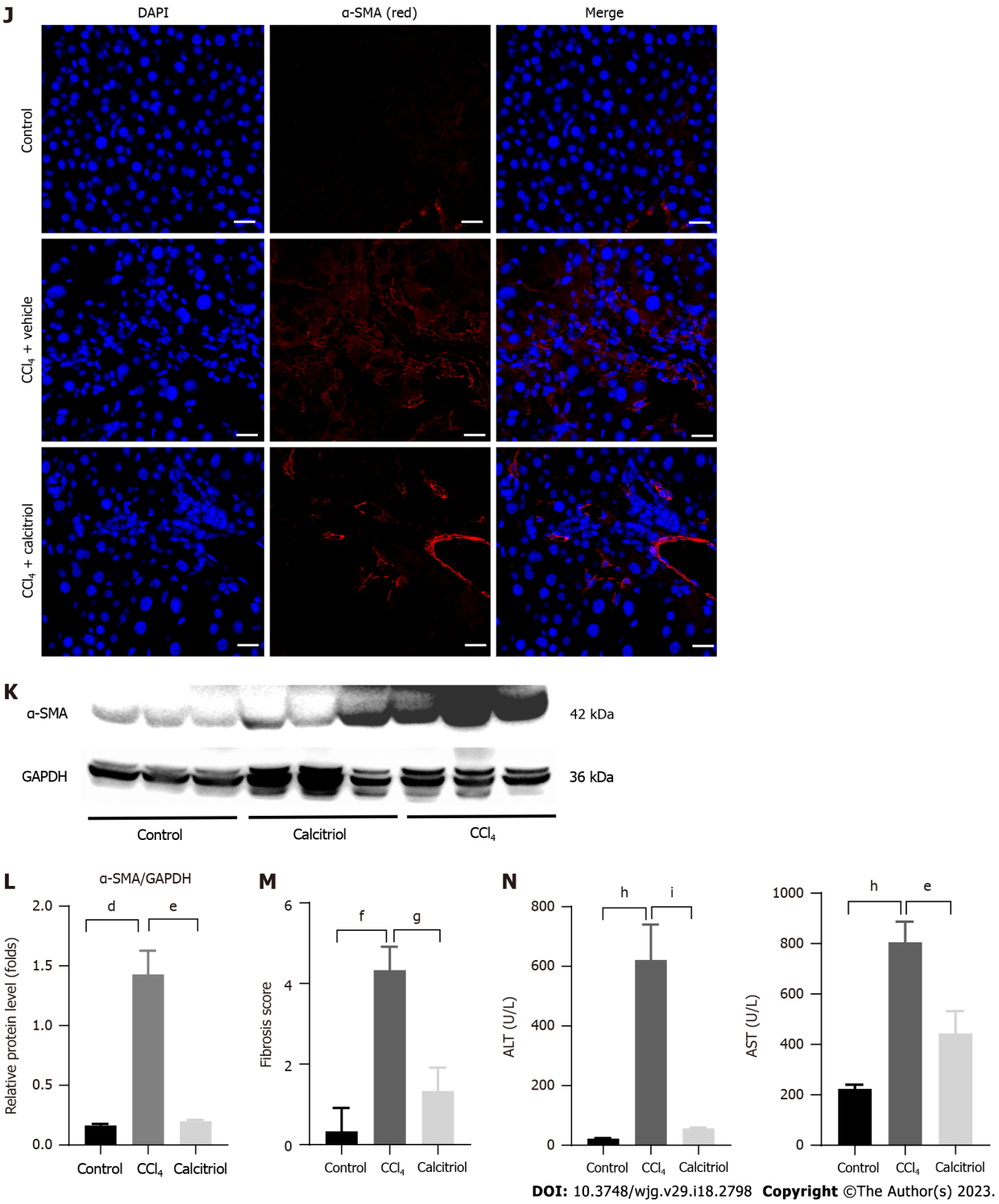
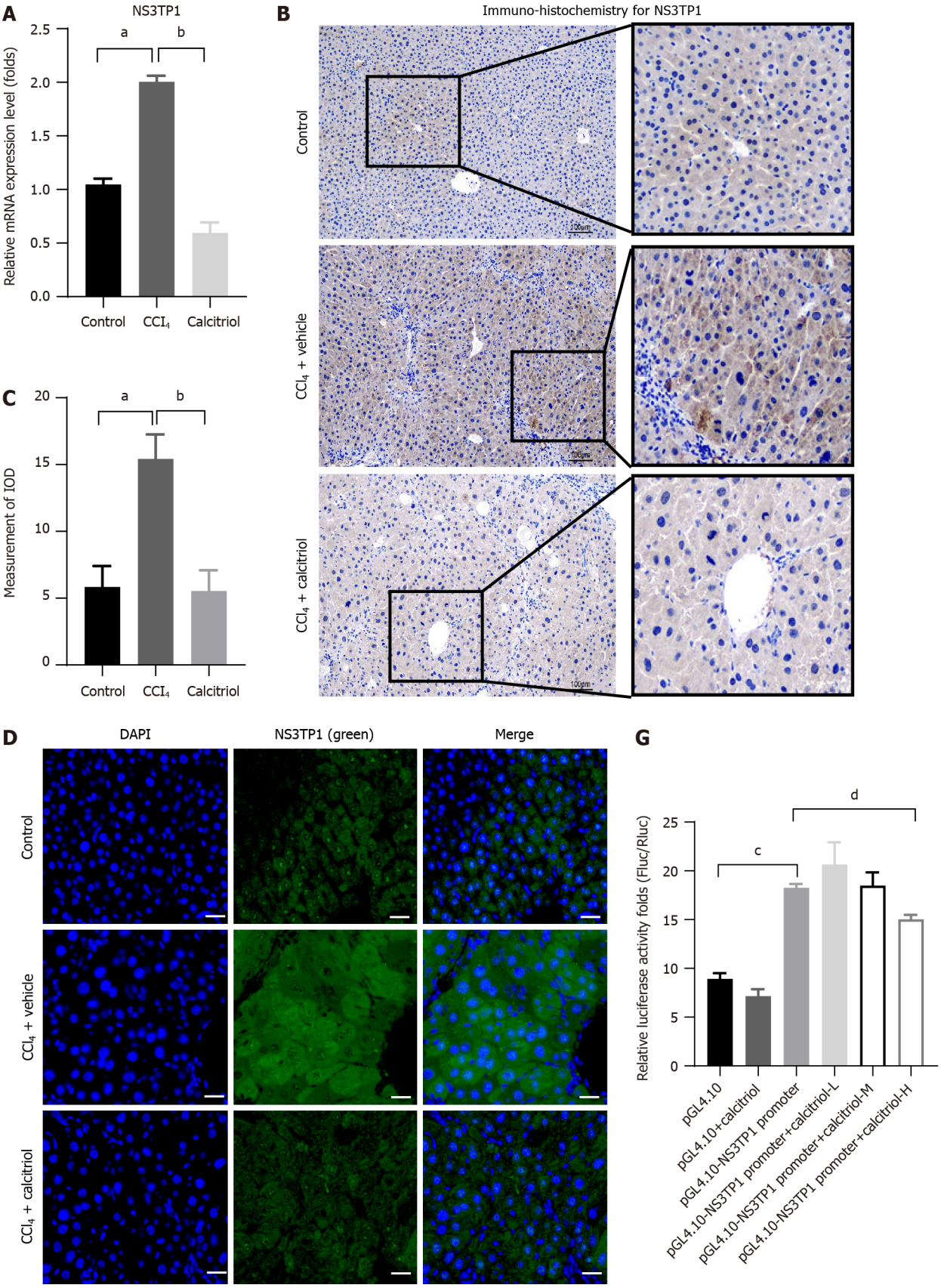


Figure 4 The role of calcitriol in hepatic fibrosis. A: Western blot analysis of collagen I and α-smooth muscle actin (α-SMA) in LX-2 cells treated with different concentrations of calcitriol; B: The protein levels of collagen I and α-SMA after calcitriol treatment at various time points were analyzed by Western blot; C: Calcitriol reduced the transforming growth factor beta 1 (TGFβ1)-induced elevation of collagen I and α-SMA at the protein level (n = 3); D: Western blot analysis of the calcitriol-induced level of Bcl-2 and Bax; E and F: An Annexin V-FITC/7-AAD kit was utilized to measure cellular apoptosis by flow cytometry; G and H: LX-2 cell migration was measured using the wound-healing test at 0 h, 24 h, 48 h, and 72 h (× 100) (n = 3); I: Hematoxylin-eosin, Masson staining, Sirius red staining, and immunohistochemical staining for α-SMA in hepatic tissue, scale bar = 100 μm; J: Immunofluorescence staining for α-SMA in hepatic tissue, red: α-SMA (n = 3), scale bar = 20 μm; K and L: Protein expression of α-SMA in the hepatic tissues was evaluated by Western blot (n = 3); M: The fibrosis score was analyzed according to the Ishak scoring system (n = 6); N: Levels of alanine aminotransferase and aspartate aminotransferase in plasma for the three groups. The carbon tetrachloride (CCl_4) group was contrasted with the corn oil group, while the calcitriol group was contrasted with the CCl_4 group (n = 6). The data was presented as mean ± SE. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.0001$ *vs* without calcitriol control group; $^dP < 0.001$ CCl_4 group *vs* corn oil control group, $^eP < 0.001$ calcitriol group *vs* CCl_4 group; $^fP < 0.01$ CCl_4 group *vs* corn oil control group, $^gP < 0.01$ calcitriol group *vs* CCl_4 group; $^hP < 0.0001$ CCl_4 group *vs* corn oil control group, $^iP < 0.0001$ calcitriol group *vs* CCl_4 group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CCl_4: Carbon tetrachloride; TGFβ1: Transforming growth factor beta 1.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b
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Calcitriol
B
Immuno-histochemistry for NS3TP1
Control
CCl4 + vehicle
CCl4 + calcitriol
100μm
C
Measurement of IOD
20
15
10
5
0
a
b
Control
CCl4
Calcitriol
D
DAPI
NS3TP1 (green)
Merge
Control
CCl4 + vehicle
CCl4 + calcitriol
G
Relative luciferase activity folds (Fluc/Rluc)
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20
15
10
5
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c
d
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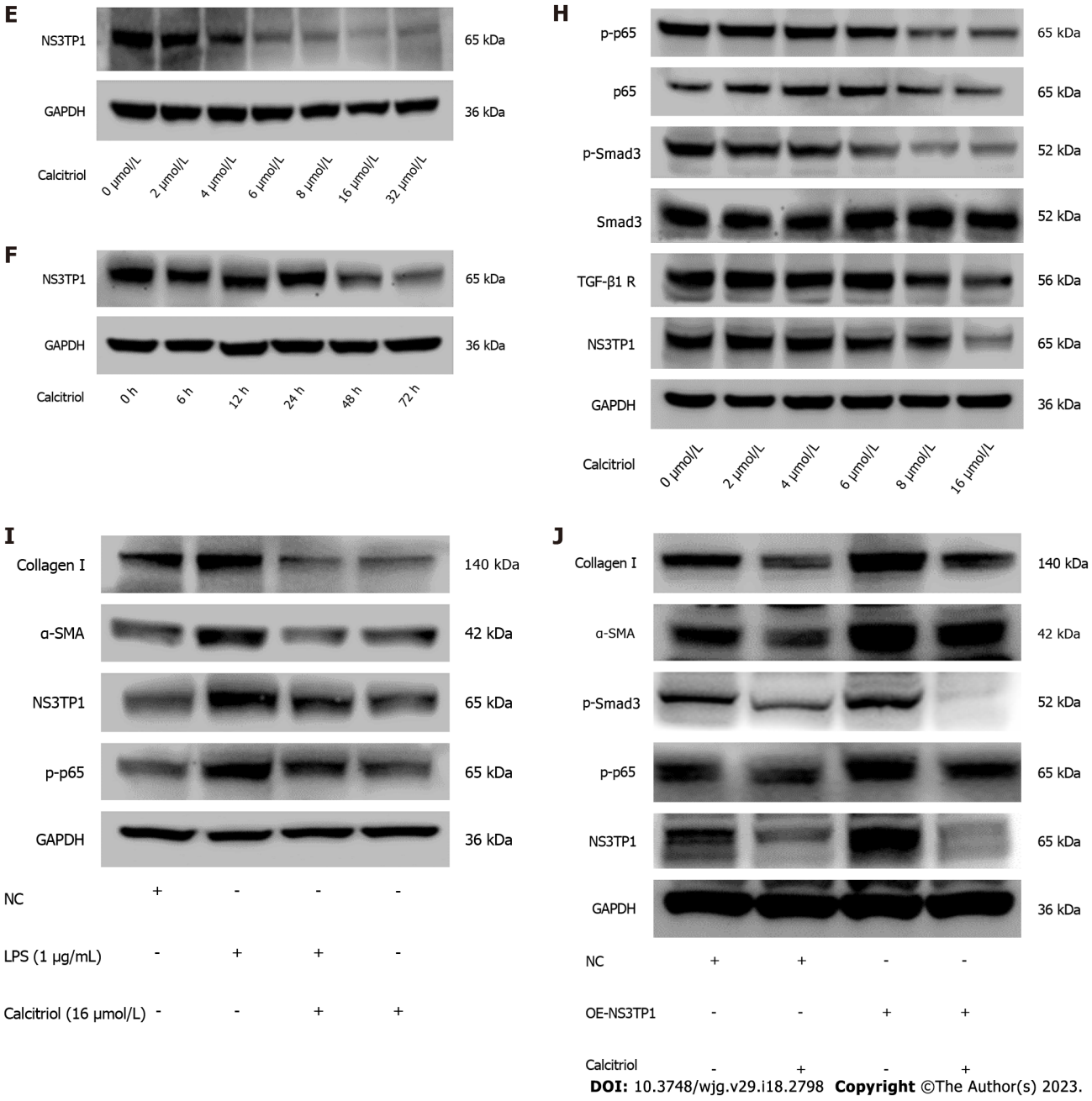

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Figure 5 The relationship between calcitriol and nonstructural protein 3-transactivated protein 1. A: RT-qPCR analysis of nonstructural protein 3-transactivated protein 1 (NS3TP1) in mice treated with calcitriol (n = 6); B: Immunohistochemical staining for α-smooth muscle actin (α-SMA) in hepatic tissue, scale bar = 100 μm; C: Image J analysis of immunohistochemical staining for α-SMA, (n = 3); D: Immunofluorescence staining for NS3TP1 in liver tissue, green: NS3TP1 (n = 3), scale bar = 20 μm; E and F: Western blot analysis of NS3TP1 in LX-2 cells stimulated with various concentrations of calcitriol or with 16 μmol/L calcitriol for various durations (n = 3); G: Analysis of luciferase activity between the NS3TP1 promoter and calcitriol in HepG2 cells, calcitriol (L: 8 μmol/L, M: 16 μmol/L, H: 32 μmol/L); H: Western blot analysis of the activity of both above signaling pathways in LX-2 cells treated with different concentrations of calcitriol; I: Collagen I, α-SMA, NS3TP1, and p-p65 in calcitriol-treated LX-2 cells were measured by Western blot and compared to LPS-treated cells; J: Extracellular matrix accumulation was evaluated by Western blot in LX-2 cells stimulated with calcitriol after NS3TP1 overexpression. The data was represented as mean ± SE. $^{a}P < 0.01$ CCl_4 group *vs* corn oil control group, $^{b}P < 0.01$ calcitriol group *vs* CCl_4 group; $^{c}P < 0.0001$ pGL4.10-NS3TP1 promoter *vs* pGL4.10, $^{d}P < 0.0001$ pGL4.10-NS3TP1 promoter + calcitriol (32 μmol/L) *vs* pGL4.10-NS3TP1 promoter. ECM: Extracellular matrix; LPS: Lipopolysaccharide; NS3TP1: Nonstructural protein 3-transactivated protein 1; TGFβ1: Transforming growth factor 1 beta 1; CCl_4: Carbon tetrachloride.

hepatic fibrosis, and calcitriol may be employed as an adjuvant therapy.

ARTICLE HIGHLIGHTS

Research background

Despite the lack of a specific therapeutic medicine, liver fibrosis still constitutes a serious hazard to human health, hence it is critical to identify novel targets for its therapy. After screening using suppressive subtractive hybridization and bioinformatics analysis, our research team discovered that hepatitis C virus nonstructural protein 3-transactivated protein 1 (NS3TP1) may be involved in the occurrence of liver fibrosis. As a result, the role of NS3TP1 in liver fibrosis was investigated to provide a new target for the treatment of liver fibrosis.

Research motivation

A potential new target for the treatment of hepatic fibrosis is provided by this work.

Research objectives

To determine whether NS3TP1 can promote liver fibrosis and whether calcitriol can inhibit the occurrence of liver fibrosis through NS3TP1.

Research methods

In vitro experiments were performed on carbon tetrachloride mouse liver, and NS3TP1 and fibrosis-related indexes were studied through serological and pathological tests. *In vivo* experiments were performed on LX-2 cells, and siRNA-NS3TP1 and pcDNA-NS3TP1 were constructed and transfected into LX-2 cells, respectively. Collagen I, collagen III, α-smooth muscle actin (α-SMA), TGFβ1/Smad3 and NF-κB signaling pathways were detected by western blot, RT-PCR, Co-imunoprecipitation and luciferase assays.

Research results

Collagen I, collagen III, α-SMA, transforming growth factor beta (TGFβ1)/Smad3, and NF-κB signaling pathways were found to be up-regulated following overexpression of NS3TP1, whereas the aforementioned indices were shown to be down-regulated after NS3TP1 interference *in vitro*. Results from Co-IP and Luciferase assays confirmed that Smad3 and p65 could both bind to NS3TP1, and that p65 boosted NS3TP1's promoter activity while NS3TP1 increased the promoter activity of TGFβ receptor I (TGFβ-RI). NS3TP1 and fibrosis-related indicators decreased after calcitriol therapy both *in vitro* and *in vivo*, and calcitriol restrained the expression of TGFβ1/Smad3 and NF-κB signaling pathways *via* NS3TP1.

Research conclusions

NS3TP1 promotes hepatic fibrosis through TGFβ1/Smad3 and NF-κB signaling pathways. Calcitriol further inhibits TGFβ1/Smad3 and NF-κB signaling pathways to reduce liver fibrosis by down-regulating NS3TP1.

Research perspectives

NS3TP1 provides a novel target for the treatment of liver fibrosis and a direction for the research of potential drug targets. Calcitriol is endowed with new functions as an adjunct therapeutic drug for liver fibrosis.

ACKNOWLEDGEMENTS

We would like to thank all the coauthors who participated in this study: Feng Ye, Lei Shi, Yun-Ru Chen, Xi Zhang, Jian-Zhou Li, and Yi-Fan Xu. Thank you to all the teachers at the Institute of Infectious Diseases at Beijing Ditan Hospital.

FOOTNOTES

Author contributions: Shi L, Zhou L, Han M, Zhang Y, Zhang Y, Yuan XX, Lu HP, Wang Y, Yang XL, Liu C, Wang J, Liang P, Liu SA, Liu XJ, Cheng J, and Lin SM contributed to the study conception and design; Liu S, Li Z, Ming H, Yu Z, Yang Z, Xue YX, Ping LH, Yun W, Liang YX, Chen L, Jun W, and Jing LX participated in the design and completion of the experiment; Liu S wrote the original draft; Liu S, Jun C, and Mei LS reviewed and edited the manuscript; Pu L and Ai LS finished the project administration; Jun C and Mei LS have the same contribution to the article; all authors approved the final version of the article.

Supported by the National Key Research and Development Program of China, No. 2017YFC0908104; and National Science and Technology Projects, No. 2017ZX10203201, No. 2017ZX10201201, and No. 2017ZX10202202.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Xi'an Jiaotong University Medical Science Center (approval No. 2020-1739).

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: No additional data are available.

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

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: A
P-Editor: Yuan YY

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 May 14; 29(18): 2818-2835

DOI: 10.3748/wjg.v29.i18.2818

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

BanXiaXieXin decoction treating gastritis mice with drug-resistant *Helicobacter pylori* and its mechanism

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Specialty type: Biochemistry and molecular biology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Lee SC, Korea; Razek AAKA, Japan; Sakamoto S, Egypt

Received: February 28, 2023
Peer-review started: February 28, 2023
First decision: March 14, 2023
Revised: March 16, 2023
Accepted: April 13, 2023
Article in press: April 13, 2023
Published online: May 14, 2023

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is the main pathogen that causes a variety of upper digestive diseases. The drug resistance rate of *H. pylori* is increasingly higher, and the eradication rate is increasingly lower. The antimicrobial resistance of *H. pylori* is an urgent global problem. It has been confirmed that Banxia Xiexin decoction (BXXXT) demonstrates the effects of treating gastrointestinal diseases, inhibiting *H. pylori* and protecting gastric mucosa. The purpose of the present study is to further explore the therapeutic effects of BXXXT on drug-resistant *H. pylori*.

AIM

To confirm that BXXXT demonstrates therapeutical effects *in vivo* and *in vitro* on gastritis mice with drug-resistant *H. pylori* and explain its mechanism to provide an experimental basis for promoting the application of BXXXT.

METHODS

The aqueous extract of BXXXT was gained by water decocting method. The inhibitory effect of the aqueous extract on *H. pylori* was detected by dilution *in vitro*; drug-resistant *H. pylori* cells were used to build an acute gastritis model *in*

vivo. Thereafter, the model mice were treated with the aqueous extract of BXXXT. The amount of *H. pylori* colonization, the repair of gastric mucosal damage, changes of inflammatory factors, apoptosis, *etc*., were assessed. In terms of mechanism exploration, the main medicinal compositions of BXXXT aqueous extract and the synergistic bacteriostatic effects they had demonstrated were analyzed using mass spectrometry; the immune function of peripheral blood cells such as $CD3^+$ T and $CD4^+$ T of mice with gastritis before and after treatment with BXXXT aqueous extract was detected using a flow cytometry; the *H. pylori* transcriptome and proteome after treatment with BXXXT aqueous extract were detected. Differently expressed genes were screened and verification was performed thereon with knockout expression.

RESULTS

The minimum inhibitory concentration of BXXXT aqueous extract against *H. pylori* was 256-512 μg/mL. A dose of 28 mg/kg BXXXT aqueous extract treatment produced better therapeutical effects than the standard triple therapy did; the BXXXT aqueous extract have at least 11 ingredients inhibiting *H. pylori*, including berberine, quercetin, baicalin, luteolin, gallic acid, rosmarinic acid, aloe emodin, *etc*., of which berberine, aloe emodin, luteolin and gallic acid have a synergistic effect; BXXXT aqueous extract was found to stimulate the expressions of $CD3^+$ T and $CD4^+$ T and increase the number of $CD4^+$ T/$CD8^+$ T in gastritis mice; the detection of transcriptome and proteome, quantitative polymerase chain reaction, Western blotting and knockout verification revealed that the main targets of BXXXT aqueous extract are *CFAs* related to urea enzymes, and *CagA*, *VacA*, *etc*.

CONCLUSION

BXXXT aqueous extract could demonstrate good therapeutic effects on drug-resistance *H. pylori in vitro* and *in vivo* and its mechanism comes down to the synergistic or additional antibacterial effects of berberine, emodin and luteolin, the main components of the extract; the extract could activate the immune function and enhance bactericidal effects; BXXXT aqueous extract, with main targets of BXXXT aqueous extract related to urease, virulence factors, *etc*., could reduce the urease and virulence of *H. pylori*, weaken its colonization, and reduce its inflammatory damage to the gastric mucosa.

Key Words: Banxia Xiexin decoction; *Helicobacter pylori*; Drug resistance; Therapeutic effects; Mechanism

Core Tip: The failure rate of treating *Helicobacter pylori* (*H. pylori*) infectious diseases is increasing, leading to an urgent need to study and develop anti-*H. pylori* drugs. Banxia Xiexin decoction (BXXX) has a good effect on Hp infection and Hp-infection-related diseases. However, its pharmacological mechanism remains unclear, and whether it has an effect on drug-resistant *H. pylori* infection has not been confirmed by animal experiments. Our study confirms that BXXX decoction (BXXXT) has good therapeutic effects on drug-resistant *H. pylori* infection through *in vivo* and *in vitro* experiments in mice, then the composition of BXXXT and effective components, the immunomodulatory effect, the main target were verified. We preliminarily explain why BXXXT has a good effects.

Citation: Li XH, Xu JY, Wang X, Liao LJ, Huang L, Huang YQ, Zhang ZF. BanXiaXieXin decoction treating gastritis mice with drug-resistant *Helicobacter pylori* and its mechanism. *World J Gastroenterol* 2023; 29(18): 2818-2835

URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2818.htm

DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2818

INTRODUCTION

Helicobacter pylori (*H. pylori*) is the main pathogen that causes a variety of upper digestive diseases[1], such as chronic gastritis, peptic ulcer, and gastric cancer[2]. At present, the treatment options for *H. pylori* infections include standard triple therapy, bismuth-containing quadruple therapy, and sequential therapy. The drug resistance rate of *H. pylori* is increasingly higher, and the eradication rate is increasingly lower. Clarithromycin-resistant *H. pylori* has been listed as a focus for the research and development of antibiotics by the World Health Organization in 2017. The antimicrobial resistance of *H. pylori* is an urgent global problem. Multiple antibiotic resistance existed in 16847 *H. pylori* strains

isolated in Wenzhou from 2013-2020. Separate statistics on the resistance rates of *H. pylori* to the six commonly used antibiotics revealed that the resistance rates to levofloxacin, clarithromycin and metronidazole were high in the region, respectively 32.81%, 26.02%, and 95.67%. Therefore, how to avoid, overcome and eradicate *H. pylori* and *H. pylori*' s drug resistance brings challenges to the current clinical work. In China, traditional Chinese medicine could be used to treat a variety of refractory diseases, reducing drug resistance and improving the eradication rate of *H. pylori*[3]. "BXXXT" comes from Zhang Zhongjing' s Treatise on Febrile Diseases, which consists of 15 g *Pinellia ternate*, 9 g *Radix scutellariae*, 9 g *Dried ginger*, 9 g *Ginseng*, 9 g *Roasted licorice*, 3 g *Coptis chinensis*, and 4 *Jujubes*. At present, experimental studies and clinical efficacy of this prescription have been reported for gastrointestinal dysfunction, peptic ulcer, chronic gastritis, atrophic gastritis and other digestive system diseases[4-6]. There are numerous studies at home and abroad on the efficacy of this prescription[7-9], and it has been confirmed that BXXXT demonstrates the effects of treating gastrointestinal diseases, inhibiting *H. pylori* and protecting gastric mucosa[10-12]. However, whether it has the same effect on the refractory gastritis caused by drug-resistant *H. pylori* remains unreported.

Therefore, the purpose of the present study is to further explore the therapeutic effects of BXXXT on drug-resistant *H. pylori* through establishing mouse models, and to study the molecular mechanism of BXXXT applied in the treatment of the refractory gastritis caused by drug-resistant *H. pylori* through ingredient analysis, immune regulation, identification of therapeutical targets, *etc*., so as to provide an experimental basis for improving the application efficiency and of this prescription worldwide.

MATERIALS AND METHODS

Strains and culture conditions

H. pylori strains (standard 26695, G27, NSH57, and multi-drug-resistant BHKS159 all provided by Professor Bi Hongkai of Nanjing Medical University) containing the preservation solution and stored at -80 °C, clinical strains HPBS001-HPBS016 that had been isolated by Huang Yanqiang Laboratory, Youjiang Medical College for Nationalities were used. The *H. pylori* strains were cultured in a Columbia (OXOID, United Kingdom) medium with 10% serum or a BHI (OXOID, United Kingdom) medium in a microaerobic environment (85% nitrogen, 5% oxygen, and 10% carbon dioxide) at 37 °C.

Experimental animals

SPF C57BL/6 mice aged six to eight weeks were purchased from Changsha Tianqin Biological Co., Ltd.; the number of SPF animal license: SYXK Gui 2017-0004; animal experiment ethics number: No. 2019112501.

Preparation of BXXXT aqueous extract

The prescription consists of 15 g *Pinellia ternate*, 9 g *Radix scutellariae*, 9 g *Dried ginger*, 9 g *Ginseng*, 9 g *Roasted licorice*, 3 g *Coptis chinensis*, and 4 g *Jujubes*. In the first round, the medical materials used to prepare BXXXT were crashed to crude powder that was placed in a beaker with sterile distilled water at a ratio of 1:10, soaked for 4 h, treated with diluted and boiled for 0.5 h with the liquid filtered out. In the second round, the filtered powder was treated with distilled water in a ratio of 1:5 and boiled for 20 min with the filtrate filtered out; in the third round, the filtered powder was treated following the same steps described in the second round but boiled for 5 min with the liquid filtered out. The liquids prepared during the three rounds were combined, concentrated to crude drug (1 g/mL), evaporated under atmospheric pressure, sterilized, and stored for later use.

Evaluation of MIC of BXXXT against H. pylori in vitro

A medium of BXXXT aqueous extract that had been diluted two-fold was prepared as the treatment group: BXXXT aqueous extract was mixed with BHI and diluted two-fold with berberine set as the positive drug control group. *H. pylori* cells were adjusted at OD^{600} = 0.03 (equivalent to 1.0 × 10^7 CFU/mL). Bacterial solution (100 μL, equivalent to 1.0 × 10^6 CFU/mL) was inoculated into a 96-well plate that contained medicated BHI and incubated at 37 °C for 48 h to determine the results with the lowest concentration of drugs that could inhibit *H. pylori* growth being minimum inhibitory concentration (MIC).

Detection of therapeutic effects of BXXXT aqueous extract on mice with drug-resistant H. pylori gastritis in vivo

BXXXT aqueous extract, amoxicillin (Sigma-Aldrich, Germany), clarithromycin (Sigma-Aldrich, Germany) and omeprazole (Sigma-Aldrich, Germany) were all dissolved and diluted to 10 mg/mL. C57BL/6 model mice (BHKS159) were divided into four groups: The omeprazole + amoxicillin + clarithromycin group (the dose was 138.2 mg/kg of omeprazole, 28.5 mg/kg of amoxicillin, and 14.3 mg/kg of clarithromycin), the omeprazole + BXXXT aqueous extract (28 mg/kg), the omeprazole + BXXXT aqueous extract (7 mg/kg) and the phosphate-buffered saline (PBS) group, with six mice in each group.

Mice were given administration once a day for three consecutive times. Two days after drug withdrawal, the blood was collected from the eyeballs of the mice. The mice were then sacrificed through cervical dislocation with tissues taken from their stomach and broken to acquire *H. pylori* that was then isolated, cultured, and identified with the amount of colonization calculated. Part of the stomach tissues was made into paraffin sections with HE staining, TUNEL immunohistochemistry and fluorescence immunoassay performed thereon.

Main pharmacodynamic components of BXXXT aqueous extract

This analysis was performed by Beijing Bio-Tech Pack Technology Company Ltd using a TripleTOF5600 + and an AB SCIEX™ with the ion source being ESI. The chromatographic column was SHIMADZU InerSustain C18 (100.0 mm × 2.1 mm, 2 μm) with the column temperature being 35 °C and flow rate of 0.300 (mL/min). The mobile phase: (1) Equate = "acetonitrile"; and (2) equate = "0.1% CH3COOH-H2O". The chromatographic conditions were shown in Table 1. The scanning range of mass spectrometry conditions was m/z 100-1500. The scanning mode: DIA. Capillary voltage: 5000 V (positive) and 4500 V (negative). Capillary Temp: 500 °C, DP 60 V, CE 35 V, and CES 15 V.

Synergistic antimicrobial effects of main pharmacodynamic components of BXXXT aqueous extract

According to the protocols of the checkerboard method, drug A in the first row and drug B in the first column were diluted two-fold respectively. Then the transverse and longitudinal drugs were also cross-diluted two-fold and treated with 100 μL bacterial suspension, with the optimal combination effect selected to calculate the antimicrobial concentration index (FICI) after culture for 48 h. FICI= MIC of A drugs used in combination/MIC of A drugs used alone + MIC of B drugs used in combination/MIC of B drugs used alone. Criteria: synergistic effects (FICI ≤ 0.5); additive effect (0.5 < FICI ≤ 1.0); no effect (1.0 < FICI ≤ 2.0); antagonistic effects (FICI > 2.0).

Immunobactericidal effects of BXXXT aqueous extract

During the process of testing the efficacy of BXXXT aqueous extract described previously in section 1.5, the peripheral blood of mice before and after administration was collected and cells thereof were treated with anticoagulant and then with 50 μL antibody mixtures: CD3, CD4, and CD8, *etc*. After the membranes were fixed and broken, the cells were treated with 100 μL antibody mixtures: IFN and IL-4. After filtration, the expressions of immune cells and cytokines were detected using a flow cytometry.

Effect mechanism of BXXXT aqueous extract on H. pylori

BHKS159 bacteria were cultured on a Columbia plate overnight. Thereafter, the single colony was selected and diluted to 0.5 Mcfarland standard (MCF) with 2 μL taken and added to 5 mL BXXXT aqueous extract (1/2 MIC, prepared by being treated with BHI). The negative control group was induced with PBS, shaken at 37 °C and centrifuged to collect the bacteria solutions after it had been treated with the aqueous extract for 4 h and 8 h. The bacteria solutions were delivered to Nanjing Medical University where they were observed under a transmissive electron microscope. *H. pylori* cells were treated with BXXXT aqueous extract at the half inhibitory concentration that had been detected before for 8 h, after which the samples were collected and frozen in liquid nitrogen for 10 min, frozen with dry ice and delivered to Beijing Allwegene Technology Co., Ltd. for detection and analysis of transcriptome and proteome. The transcriptome analysis was performed using the Illumina PE150 sequencing strategy; the length of RNA fragments was detected using Agilent 2100; the alignment and transcript assembly analysis were performed using Boetie2 and the Rockhhoper software; quantitative protein analysis was performed using the ITRAQ labeling quantitative strategy, ITRAQ/TMT labeling performed using isoheavy isotope labeling, and the quantitative detection of target genes performed using a fluorescent polymerase chain reaction (PCR) instrument and Western blotting. Strains with low expressions of related target genes were used to verify MIC changes and mutant strains were constructed with reference to the protocols described in the previous studies[13]. The relevant mRNA amplification primers are shown in Table 2, and the relevant antibody information is displayed in Table 3.

Statistical analysis

Statistical analysis and mapping were performed using the Graphpad Prism software, version 8.0. Continuous data were expressed as mean ± SD. Differences between groups were analyzed using the one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

The MIC of BXXXT aqueous extract on H. pylori detected in vitro

The MICs of BXXXT aqueous extract on three sensitive *H. pylori* strains and 11 drug-resistant *H. pylori* strains were detected by applying the solid plate method. BXXXT aqueous extract was found to have

Table 1 Chromatographic conditions

Time (min)	Parameter
0	A: 0%, B: 100%
10	A: 50%, B: 50%
13	A: 95%, B: 5%
14	A: 0%, B: 100%
15	A: 0%, B: 100%

Table 2 Primer information

No.	Primer	Sequence (5' to 3')	Company
1	UREA F	GCCAATGGTAAATTAGTT	Shanghai Invitrogen Biotech Co., Lt
2	UREA R	CTCCTTAATTGTTTTTAC	
3	UREB F	TCTATCCCTACCCCACAACC	
4	UREB R	CCATCCACGAACACATGGTA	
5	CagA F	ACCCCTAGTCGGTAATG	
6	CagA R	GCTTTAGCTTCTGATACTGC	
7	VacA F	GTCAGCATCACACCGCAAC	
8	VacA R	CTGCTTGAATGCGCCAAAC	
9	16sRNA F	CTGGAGAGACTAAGCCCTCC	
10	16sRNA R	AGGATCAAGGTTTAAGGATT	

Table 3 Antibody information

Name	Art. No.	Company
CagA (A-10)	sc-32746	Santa Cruz
VacA	sc-28368	Santa Cruz
m-IgGk BP-HRP	sc-516102	Santa Cruz
GAPDH Ab	AF7021	Affinity Biosciences
Goat anti-rabbit IgG (H+L) HPR	S0001	Affinity Biosciences

antibacterial effects on strains, both resistant and sensitive (the MIC was 256-512 μg/mL). The antibacterial effect of BXXXT aqueous extract was compared with that of berberine (the MIC was 512-2048 μg/mL), as shown in Table 4. The results suggested that there was a two-to-four-fold difference between them and that BXXXT aqueous extract produced better antibacterial effects than 98% pure berberine. The reason might be that although berberine accounts for a small portion of the prescription, there might be other antibacterial components, or there might be synergistic or additive effects among these components.

Therapeutic effects of BXXXT aqueous extract were detected in vivo on mice with H. pylori-resistant acute gastritis

Model mice with acute gastritis caused by the drug-resistant strain BHKS159 were constructed and treated with PBS, omeprazole (OPZ) + amoxicillin clarithromycin (AC), OPZ + BXXXT (28 mg/kg) and OPZ + BXXXT (7 mg/kg), respectively. Although *H. pylori* colonization, inflammatory factors IL-1β, IL-6 and tumor necrosis factor-alpha (TNF-α), inflammatory damage, and apoptosis factors Bcl-2 and Bax were improved in OPZ + AC treatment group, there were still significant differences compared with OPZ + BXXXT (28 mg/kg) treatment group. After OPZ + BXXXT (28 mg/kg) treatment, the mice could basically recover to the normal level, implying therapeutical effects significantly better than that of the triple group (Figure 1). However, amoxicillin which does not develop drug resistance, produced therapeutical effects that contributed to the improvement in the OPZ + AC treatment group when

Table 4 Minimum inhibitory concentration of Banxia Xiexin decoction aqueous extract against *Helicobacter pylori* (μg/mL)

Strain	Drug-resistanct strain	BXXXT aqueous extract	Berberine
26695	Sensitive	512	1024
G27	Sensitive	512	1024
NSH57	Sensitive	256	512
BHKS159	Resistant to levofloxacin, clarithromycin and metronidazole	512	1024
HPBS001	Resistant to levofloxacin, clarithromycin and metronidazole	512	1024
HPBS002	Resistant to metronidazole	512	1024
HPBS003	Resistant to clarithromycin	512	1024
HPBS004	Resistant to levofloxacin	512	1024
HPBS005	Resistant to levofloxacin and metronidazole	256	1024
HPBS006	Resistant to clarithromycin and metronidazole	256	1024
HPBS007	Resistant to clarithromycin	512	1024
HPBS010	Resistant to metronidazole, clarithromycin and levofloxacin	512	2048
HPBS011	Resistant to metronidazole and clarithromycin	512	1024
HPBS013	Resistant to metronidazole, clarithromycin and levofloxacin	512	1024
HPBS014	Resistant to metronidazole, clarithromycin, amoxicillin and levofloxacin	512	1024

BXXXT: Banxia Xiexin decoction. The minimum inhibitory concentrations of the drugs for sensitive and drug-resistant strains are amoxicillin ≥ 0.5 μg/mL, clarithromycin ≥ 1.0 μg/mL, levofloxacin ≥ 2.0 μg/mL, and metronidazole ≥ 8.0 μg/mL.

combined with omeprazole. BXXXT, which did not demonstrate good effects *in vitro*, produced obvious therapeutical effects *in vivo*, which might be related to the synergistic and immunomodulatory effects of BXXXT aqueous extract.

Main pharmacodynamic components of BXXXT aqueous extract

MS-DIAL 3.70 (MS-DIAL: Data independent MS/MS deconvolution for comprehensive metabolome analysis) (Nature Methods, 12, 523-526, 2015).The original LC-MS data of BXXXT aqueous extract were imported into MS-DIAL, version 3.70 for preprocessing (MS-DIAL: Data independent MS/MS deconvolution for comprehensive metabolome analysis) (Nature Methods, 12, 523-526, 2015), including peak value extraction, noise-removal, deconvolution and peak alignment, and thereafter the three-dimensional data matrix in comma-separated values format was derived (original data matrix). The peak information extracted was compared with the database, with the full database search of MassBank, Respect and GNPS (14951 records in total). About 428 monomer components were identified, among which, as the related literature suggests, there were a total of 78 major components related to *H. pylori* resistance. Eleven species including berberine, emodin, baicalin, quercetin have been widely reported and demonstrate good antibacterial effects. Their ion additions and molecular structure are displayed in Table 5. It could be suggested that among the components of BXXXT aqueous extract, in addition to berberine, other components such as emodin also have inhibitory effects, which provides experimental basis for the better antibacterial effects *in vitro* BXXXT aqueous extract could produce compared with berberine.

Synergistic antimicrobial effects of main pharmacodynamic components of BXXXT aqueous extract

Six groups of berberine and emodin, berberine and luteolin, luteolin and gallic acid, luteolin and rosmarinic acid, catechuic acid and quercetin, catechuic acid and emodin were selected from 12 main anti-HP components of water extract of BXXXT aqueous extract for combined drug sensitivity detection. The results suggested that the six groups demonstrated additive or synergistic effects on *H. pylori* (Table 6), berberine and emodin, luteolin and gallic acid in particular producing better synergistic effects. Similar effects might also be found in other component combinations that had not been verified, which provides further experimental evidence that BXXXT aqueous extract could produce better antibacterial effects *in vitro* than berberine.

Immunobactericidal effects of BXXXT aqueous extract on mice

The *t*-test was used to analyze the proportion of $CD3^+$ T, $CD4^+$ T, and $CD8^+$ T cells in total lymphocytes

Table 5 Information of main pharmacodynamic components of Banxia Xiexin decoction aqueous extract

Name	Molecular structural formula
Berberine	COC1=C(OC)C2=C[N+]3=C(C=C2C=C1)C1=CC2=C(OCO2)C=C1CC3
Baicalin	C1=CC=C(C=C1)C2=CC(=O)C3=C(C(=C(C=C3O2)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)C(=O)O)O)O)O)O)O
Luteolin	OC1=CC(O)=C2C(=O)C=C(OC2=C1)C1=CC(O)=C(O)C=C1
Gallic acid	OC(=O)C1=CC(O)=C(O)C(O)=C1
Gingerol	CCCCCC(O)CC(=O)CCC1=CC(OC)=C(O)C=C1
Wogonoside	COC1=C(O)C=C(O)C2=C1OC(=CC2=O)C1=CC=CC=C1
Rosmarinic acid	OC(=O)[C@H](CC1=CC(O)=C(O)C=C1)OC(=O)\C=C\C1=CC(O)=C(O)C=C1
Aloe-emodin	OCC1=CC2=C(C(O)=C1)C(=O)C1=C(C=CC=C1O)C2=O
Catechin	CCCCCC(CC(=O)CCC1=CC(=C(C=C1)O)OC)O
Naringenin	OC1=CC=C(C=C1)[C@@H]1CC(=O)C2=C(O)C=C(O)C=C2O1
Quercetin	OC1=CC(O)=C2C(OC(=C(O)C2=O)C2=CC(O)=C(O)C=C2)=C1

before and after administration. After administration, the proportion of $CD3^+$ T and $CD4^+$ T in total lymphocytes increased ($P1 = 0.0009$, $P2 = 0.0115$), as shown in Figure 2A-F; no significant difference was found between $CD8^+$ T cells and TH1 cells ($P3 = 0.1937$, $P4 = 0.8061$), Figure 2A, C, D, and G, and the ratio of $CD4^+$ T/$CD8^+$ T cells was increased ($P5 = 0.0280$) as displayed in Figure 2H. These results indicated that BXXXT aqueous extract could improve the ratio of lymphocytes $CD3^+$ T and $CD4^+$ T to the total number of lymphocytes and the ratio of $CD4^+$ T/$CD8^+$ T cells. However, BXXXT aqueous extract could enhance immune functions, thus helping improve the immunity and antibacterial ability of the body, which might explain why BXXXT could produce better treatment effects *in vivo*.

Mechanism of action of BXXXT aqueous extract on H. pylori

No significant changes were found at × 10000 magnification on the morphological structures of *H. pylori* after 4 h and 8 h of BXXXT treatment (Figure 3A), indicating that BXXXT did not produce antibacterial effects by directly destroying morphological structures. In the samples treated with BXXXT for 8 h, a total of 357 differentially expressed genes were detected after transcriptome analysis, among which 133 genes were up-regulated and 224 genes down-regulated (Figure 3B), mainly concentrating in five metabolic pathways including metabolic pathways, the epithelial cell signaling in *H. pylori* infection and the microbial metabolism in diverse environments (Figure 3C). The epithelial cell signaling in *H. pylori* infection pathway suggested that it is closely related to urease genes and virulence genes, as shown in Figure 3D. The proteome detection found 86 differentially expressed genes, among which 44 were up-regulated and 42 down-regulated (Figure 3E), mainly concentrating in oxidoreductases and transferases pathways (Figure 3F). Among the related genes and proteins found in transcriptome and proteome, respectively, and concentrating in the main pathways, five possible proteins of BXXXT were screened, among which four were urease-related and one was related to the virulence gene *CagA* (Table 7). The quantitative PCR (Q-PCR) and Western blot detection were performed to confirm the correlation between BXXXT action and virulence genes and urease genes. The results suggested that the mRNAs and protein expressions of *CagA* and *VacA* after BXXXT treatment were significantly decreased (Figure 4A-D), providing additional evidence for the obvious effects of BXXXT *in vivo* from the point that BXXXT could reduce virulence of *H. pylori*. The gene *CFAs* related to environmental regulation, urease and drug resistance was mutated (Figure 4E), after which the MIC of the mutant strains increased 2-4 times (Figure 4F), further proving that the urease-related gene *CFAs* might be one of the main targets of BXXXT. However, the decrease of urease could affect the adaptive regulation of stomach acid for which *H. pylori*' s ability to colonize would be significantly reduced.

DISCUSSION

There are about 4.4 billion people with *H. pylori* infections worldwide, with an average infection rate of 62.8%. Southeast Asia could be considered as high-incidence areas, mainly China, Japan, and South Korea[14,15]. The eradication of *H. pylori* has proven to prevent gastric cancer. However, the overuse of antibiotics leads to serious drug resistance. The drug resistance rate varies in different countries and regions and will change over time, indicated by those of clarithromycin, metronidazole, and levloxacin, all increasing over time. For example, the drug resistance rate of clarithromycin rose to 21% between 2012 and 2016[16-18]. Therefore, the failure rate of treating *H. pylori* infectious diseases is increasing,

Table 6 Minimum inhibitory concentrations of main antibacterial components in Banxia Xiexin decoction aqueous extract (μg/mL)

Strain	MIC of drugs used alone		MIC of drugs used in combinations		FIC	Effect
	Berberine	Emodin	Berberine	Emodin		
G27	1024	512	256	256	0.75	Addictive
26695	1024	512	256	128	0.50	Synergistic
BHKS159	1024	512	256	128	0.50	Synergistic
	Berberine	Luteolin	Berberine	Berberine		
G27	1024	1024	512	512	1.00	Addictive
26695	1024	1024	512	256	0.75	Addictive
BHKS159	1024	1024	512	512	1.00	Addictive
	Luteolin	Gallic acid	Luteolin	Gallic acid		
G27	1024	1024	256	256	0.50	Synergistic
26695	1024	1024	256	256	0.50	Synergistic
BHKS159	1024	1024	256	256	0.50	Synergistic
	Luteolin	Rosmarinic acid	Luteolin	Rosmarinic acid		
G27	1024	1024	512	256	0.75	Addictive
26695	1024	1024	512	512	1.00	Addictive
BHKS159	1024	1024	512	256	0.75	Addictive
	Catechuic acid	Quercetin	Catechuic acid	Quercetin		
G27	1024	1024	512	256	0.75	Addictive
26695	1024	1024	512	256	0.75	Addictive
BHKS159	1024	1024	512	256	0.75	Addictive
	Catechuic acid	Emodin	Catechuic acid	Emodin		
G27	1024	512	512	256	1.00	Addictive
26695	1024	512	512	256	1.00	Addictive
BHKS159	1024	512	256	256	0.75	Addictive

MIC: Minimum inhibitory concentration.

leading to an urgent need to study and develop anti-*H. pylori* drugs. In 2018, Hu *et al*[19] from Peking University proposed that non-antibiotic drugs such as traditional Chinese medicine, mucosal protective agents and probiotics could be used to treat *H. pylori* infection. Traditional Chinese medicine, including BXXXT, has a good effect on *H. pylori* infection and *H. pylori*-infection-related diseases[20,21]. However, its pharmacological mechanism remains unclear, and whether it has an effect on drug-resistant *H. pylori* infection or not has not been confirmed by animal experiments.

This study confirms that BXXXT has good therapeutic effects on drug-resistant *H. pylori* infection through *in vivo* and *in vitro* experiments in mice, which provides an experimental basis for elaborating that BXXXT could treat refractory gastritis caused by drug-resistant bacteria. While the efficacy of BXXXT is well established, explaining its mechanism is difficult. Traditional Chinese medicine, especially compound prescriptions, has complex components and a very complex mechanism of action in the body, which might be affected by multiple factors, especially those in stomach. Besides, it produces effects that are multi-target. As the content of the main components of the prescription is not high, the effects on the target may not always appear[22-24], which makes it difficult to elaborate on the mechanism of action. In the present study, the composition of BXXXT was analyzed, the effective anti-HP components were screened out with reference to the related literature and reports, the material basis of the efficacy was identified, and the synergistic effects among some of the effective components was verified. It was found that most of the components had additive or synergistic effects, such as berberine and emodin, luteolin and gallic acid. This indicated that though only accounting for a small portion, the active components of the Chinese medicine prescription, which could produce synergistic or additive effects demonstrated better antibacterial effects. The MIC of BXXXT against *H. pylori* is 256-512 μg/mL, much worse than that of clinical antibiotics but producing better therapeutical effects *in vivo*, especially

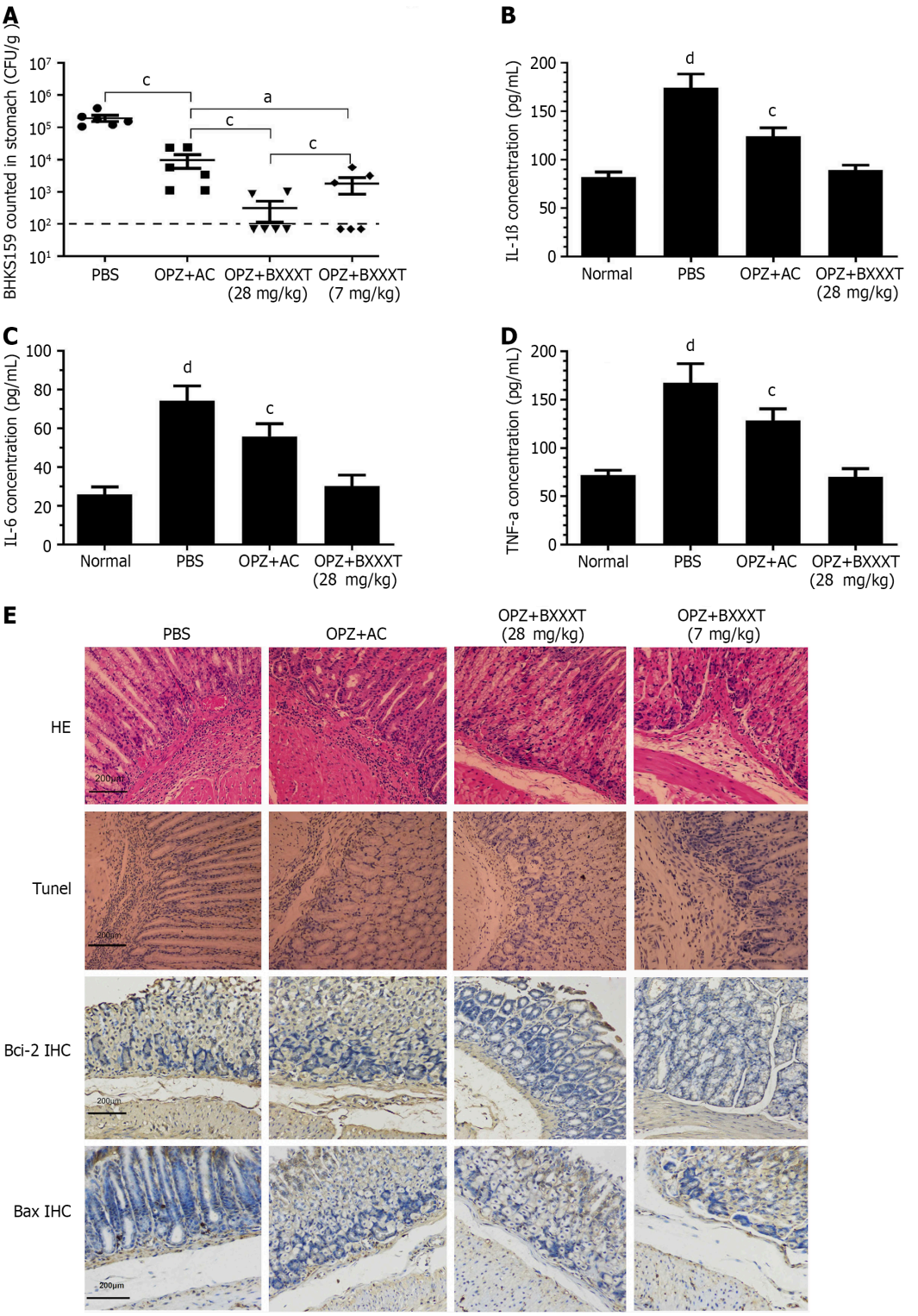
Table 7 Information of related proteins identified by transcriptome analysis after Banxia Xiexin decoction treatment

Uniprot Accession Number	Protein name	Protein amino acid sequence	*P* value	Reliability
E6NPU2	Urease subunit alpha OS = *Helicobacter pylori* (strain F57), OX = 866346, GN = urea, PE = 3, SV = 1	MKLTPKELDKLMLHYAGELARKRKEKGIKLNYVEAVALISAHIMEEARAGKKSAAEL MQEGRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTPIESNGKLVPGELFLK NEDITINEGKKAVSVKVKNVGDRPVQIGSHFHFFEVNRCLDFDREKTFGKRLDIASGT AVRFEPGEEKSVELIDIGGNRRIFGFNALVDRQADNESKKIALHRAKERGFHGAKSDD NYVKTIKE	0.001211988	High
A0A2A6SFH9	Urease (fragment) OS = *Helicobacter pylori*, OX = 210, GN = BB479_08100, PE = 4, SV = 1	MKLTPKELDKLMLHYAGELARKRKEKGIKLNYVEAVALIXAHIMEEARAGKKTAAEL MQEGRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTXIEANGKLVPGELFLKN EDITINEGKKAVSVKVKNVGDRPVQIGSH	0.033815784	High
A0A0L0QH58	Urease subunit alpha OS = *Helicobacter pylori*, OX = 210, GN = urea, PE = 3, SV = 1	MKLTPKELDKLMLHYAGELAKKRKEKGIKLNYVEAVALISAHIMEEARAGKKSAAEL MQEGRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTPIEANGKLVPGELFLKN EDITINEGKKAVSVKVKNVGDRPVQIGSHFHFFEVNRCLDFDREKTFGKRLDIASGTAV RFEPGEEKSVELIDIGGNRRIFGFNALVDRQADNESKKIALHRAKERGFHGAKSDDNY VKTIKE	0.033744196	High
N4TND2	Urease accessory protein UreG OS = *Helicobacter pylori* Hp A-11, OX = 992035, GN = ureG, PE = 3, SV = 1	MVKIGVCGPVGSGKTALIEALTRHMSKDYDMAVITNDIYTKEDAEFMCKNSVMPRER IIGVETGGCPHTAIREDASMNLEAVEEMHGRFPNLELLLIESGGDNLSATFNPELADFTIF VIDVAEGDKIPRKGGPGITRSDLLVINKIDLAPYVGADLKVMERDSKKMRGEKPFIFTNIR AKEGLNDVIAWIKRNALLED	0.016839824	High
Q8RRP6	Cytotoxin associated protein CagA (Fragment), OS = *Helicobacter pylori*, OX = 210, GN = cagA, PE = 4, SV = 1	ALADLKNFSKEQLAQQAQKNESFNAGKKFEFSQSVRNGVNGTLVGNGFSQAEATTL SKNFSDIKKELNAKLGNFNNNNINGLKNSTEPIYAKVNKKETGQAASPEEPIYTQVAKKVN AKIDRLNQIASGLGVVGQAAGFPLKRHDKVDDLSKVGRSVSPEPIYATIDDLGGPFPLKRH DKVDNLSKVGRSVSPEPIYATIDDLGGPFPLKRHDKVDNLSKVGLSRNQELTQKIDNLSQA VSEAKAGFFGNLEQTIDKLKDSTKHNVVNLWAESAKKVPASLSAKLDNYA	0.03652953	High

for drug-resistant *H. pylori*, why?

H. pylori can adhere to the gastrointestinal mucosa, produce virulence factors, damage gastric epithelial cells, and induce, control and regulate inflammatory responses. CagA encodes variety of proteins, including CagA and VacA, *ect*[25]. Karbalaei *et al*[26] found that *CagA* and *VacA* genes were potentially associated with resistance to clarithromycin, metronidazole, amoxicillin, tetracycline and levofloxacin. The VacA can not only destroy mitochondria[27,28], but also reduce the proliferation of T cells, B cells, and the other immune cells, and affect the immune response[29-31]. Among the components of BXXXT, Gingerol, a crude extract containing gingerol, is able to can inhibit the growth of *H. pylori* strains (MIC range is 0.78 μg/mL to 12.5 μg/mL) and has significant activity against $CagA^+$ strains[32]. Kaempferol is able to reduce the transcription of subunit protein A by the type IV secretory system, reduce the expression of pro-inflammatory cytokines (TNF-α, IL-1β) and IL-8 production in cells [33]. Urease can increase the pH value of the stomach and provide a suitable environment for *H. pylori* colonization. Palmatine in Coptis coptitis can act on sulfhydryl at the active site of urease and inhibit the conformational change of the urease molecules, and reduce urease activity[34]. Hesperidin can reduce the expression of UreA and UreB[35].

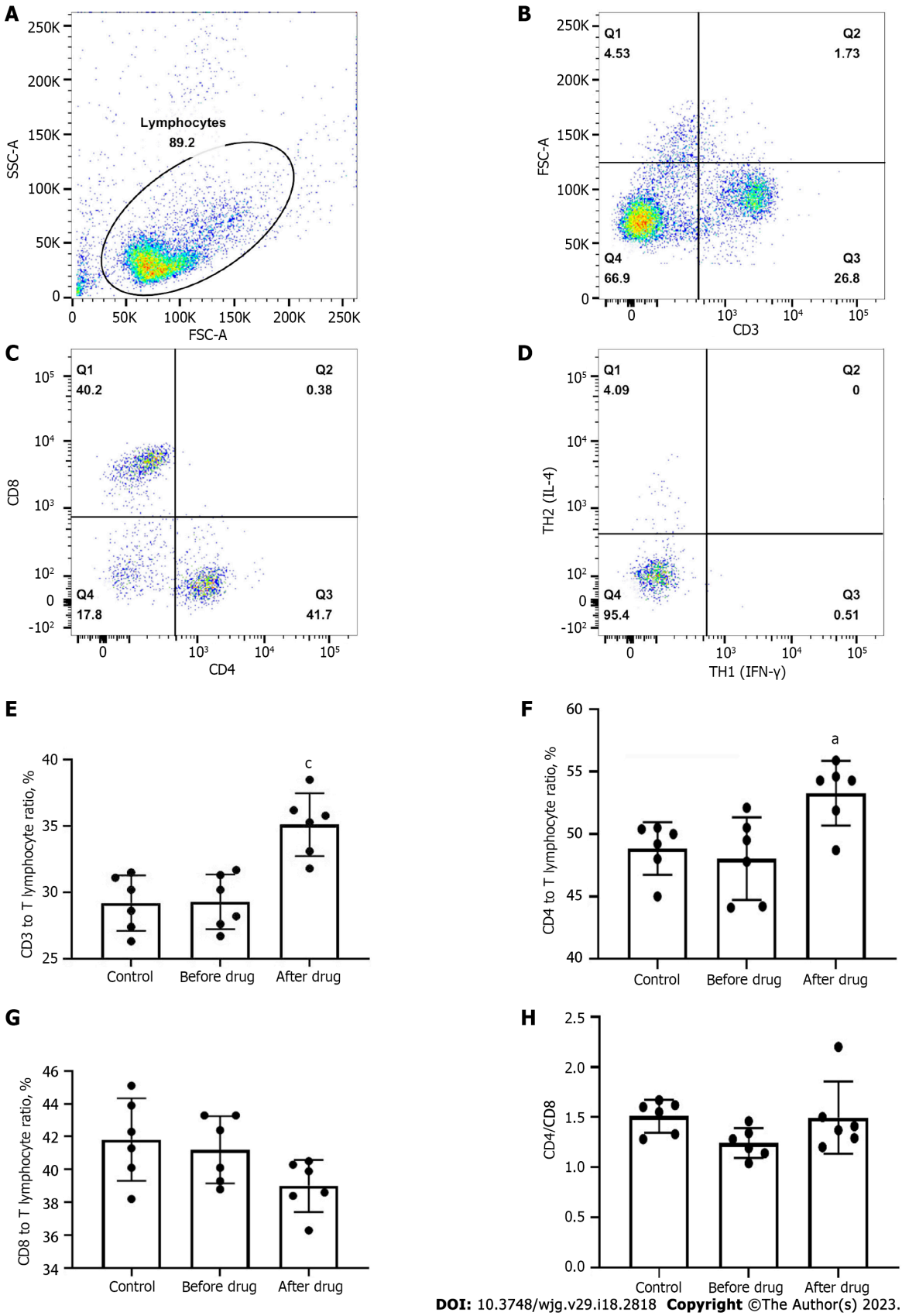
In the present paper, the immunomodulatory effect of BXXXT was analyzed, and it was found that it could up-regulate the expressions of immune factors such as $CD4^+$ T and enhance immunity and the ability of sterilization; the main target of BXXXT was urease-related gene *CFAs*, which was related to the virulence factors *CagA* and *VacA*. When urease was destroyed, *H. pylori* cells could not survive in the gastric acid environment, and its colonization ability would be significantly weakened. With low expressions of *H. pylori* virulence factors, the inflammatory damage caused by *H. pylori* to gastric mucosa would be reduced. The effects of these three aspects could preliminarily explain why BXXXT has good effects *in vivo*. However, clarifying the action mechanism of BXXXT is very complicated and difficult, for there are many components of BXXXT, among which many are anti-*H. pylori*, the



DOI: 10.3748/wjg.v29.i18.2818

Figure 1 Therapeutic effects of BanXiaXieXin decoction aqueous extract on mice with *Helicobacter pylori*-resistant acute gastritis. A: The amount of *Helicobacter pylori* colonization in model mice infected with drug-resistant strains; B: The expression of inflammatory factor IL-1β in model mice; C: The expression of inflammatory factor IL-6 in model mice; D: The expression of inflammatory factor tumor necrosis factor-alpha in model mice; E: The gastric mucosa injury and the expressions of apoptotic genes Bcl-2 and Bax, × 200. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; OPZ: Omeprazole; AC: Amoxicillin clarithromycin; PBS: Phosphate-buffered saline.

transcriptome and proteome analyses in this study also suggested that many membrane transporter genes were involved, such as ABC transporter, *etc*. Besides, we also found that berberine and other components of BXXXT could inhibit *HefA* gene to reverse drug resistance[25] and *CFAs* in this study might also be related to drug resistance[36,37]. Therefore, the action mechanism of BXXXT will be further studied.

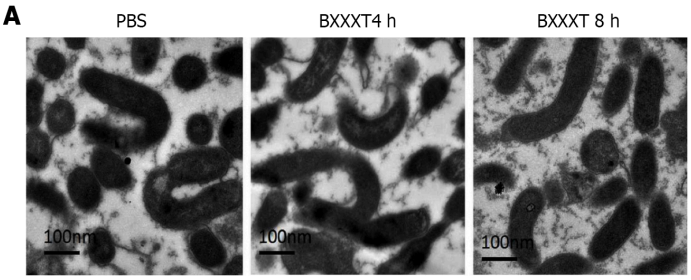


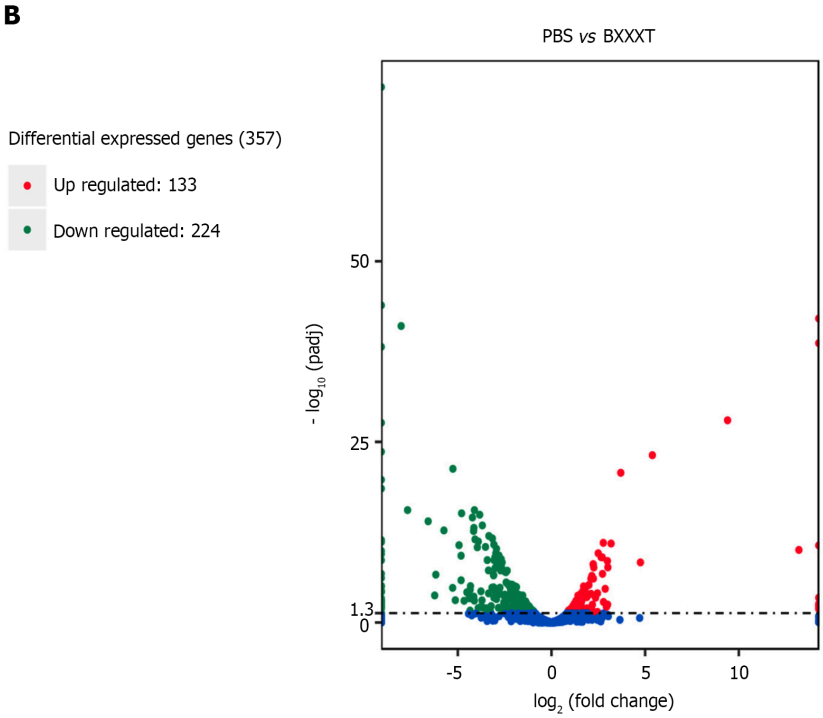
DOI: 10.3748/wjg.v29.i18.2818

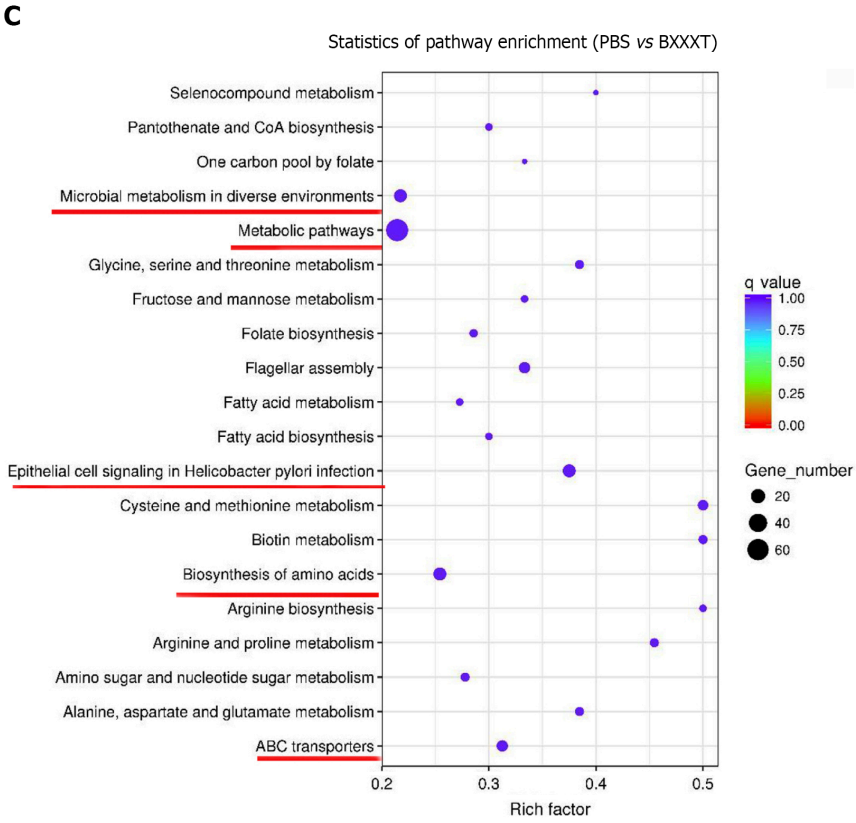
Figure 2 Immunobactericidal effects of BanXiaXieXin decoction aqueous extract on mice. A: Lymphocyte expression; B: CD3T cell expression; C: CD4T and CD8T cell expression; D: TH1 and TH2 expression; E: CD3T to lymphocyte ratio; F: CD4T to lymphocyte ratio; G: CD8T to lymphocyte ratio; H: CD4T /CD8T ratio. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

CONCLUSION

BXXXT aqueous extract could demonstrate good therapeutic effects on drug-resistance *H. pylori in vitro*


A
PBS
BXXXT4 h
BXXXT 8 h
100nm
100nm
100nm


B
PBS *vs* BXXXT
Differential expressed genes (357)
Up regulated: 133
Down regulated: 224
50
25
1.3
0
- log10 (padj)
-5
0
5
10
log2 (fold change)


C
Statistics of pathway enrichment (PBS *vs* BXXXT)
Selenocompound metabolism
Pantothenate and CoA biosynthesis
One carbon pool by folate
Microbial metabolism in diverse environments
Metabolic pathways
Glycine, serine and threonine metabolism
Fructose and mannose metabolism
Folate biosynthesis
Flagellar assembly
Fatty acid metabolism
Fatty acid biosynthesis
Epithelial cell signaling in Helicobacter pylori infection
Cysteine and methionine metabolism
Biotin metabolism
Biosynthesis of amino acids
Arginine biosynthesis
Arginine and proline metabolism
Amino sugar and nucleotide sugar metabolism
Alanine, aspartate and glutamate metabolism
ABC transporters
0.2
0.3
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60

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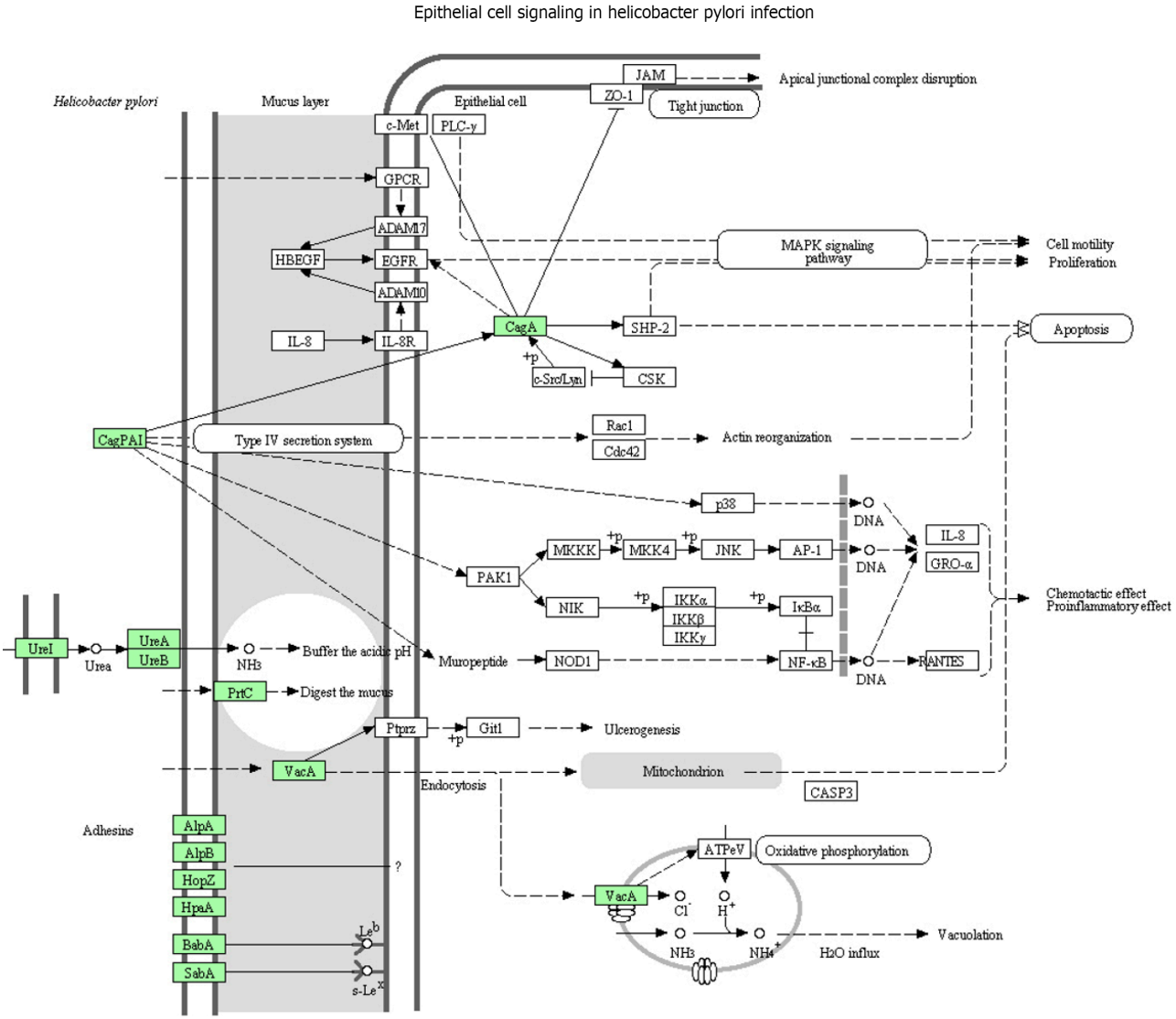
Epithelial cell signaling in helicobacter pylori infection
Helicobacter pylori
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Epithelial cell
JAM
ZO-1
Tight junction
Apical junctional complex disruption
c-Met
PLC-γ
GPCR
ADAM17
HBEGF
EGFR
ADAM10
MAPK signaling pathway
Cell motility
Proliferation
IL-8
IL-8R
CagA
SHP-2
Apoptosis
+p
c-Src/Lyn
CSK
CagPAI
Type IV secretion system
Rac1
Cdc42
Actin reorganization
p38
DNA
MKKK
MKK4
JNK
AP-1
IL-8
GRO-α
PAK1
NIK
IKKα
IKKβ
IKKγ
IκBα
Chemotactic effect
Proinflammatory effect
UreI
Urea
UreA
UreB
NH3
Buffer the acidic pH
Muropeptide
NOD1
NF-κB
RANTES
PrtC
Digest the mucus
Ptprz
Git1
Ulcerogenesis
VacA
Endocytosis
Mitochondrion
CASP3
Adhesins
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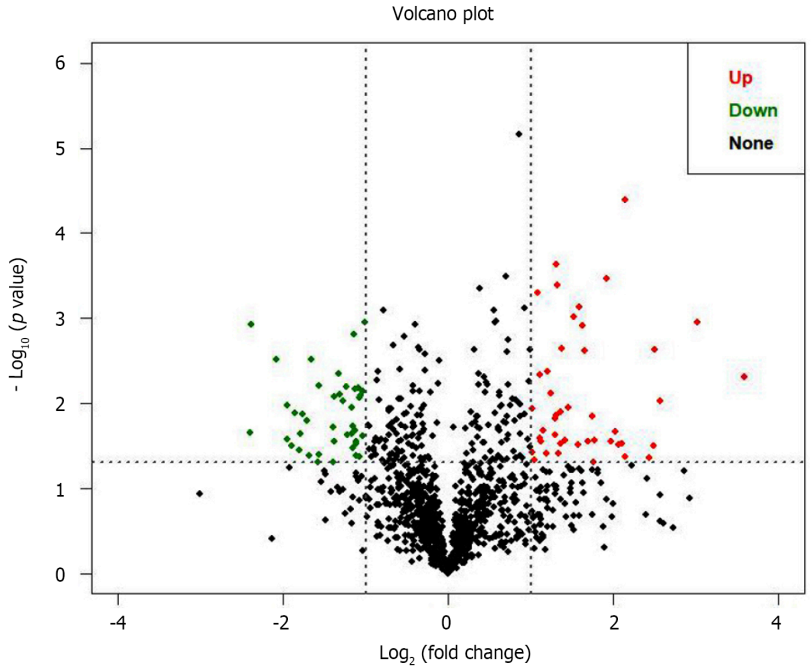
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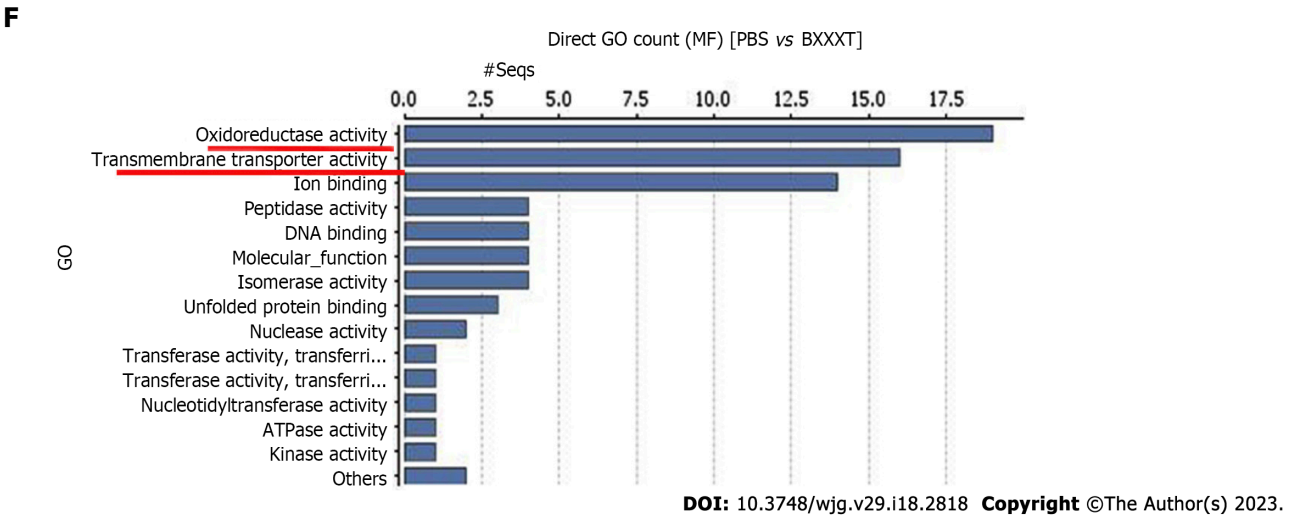
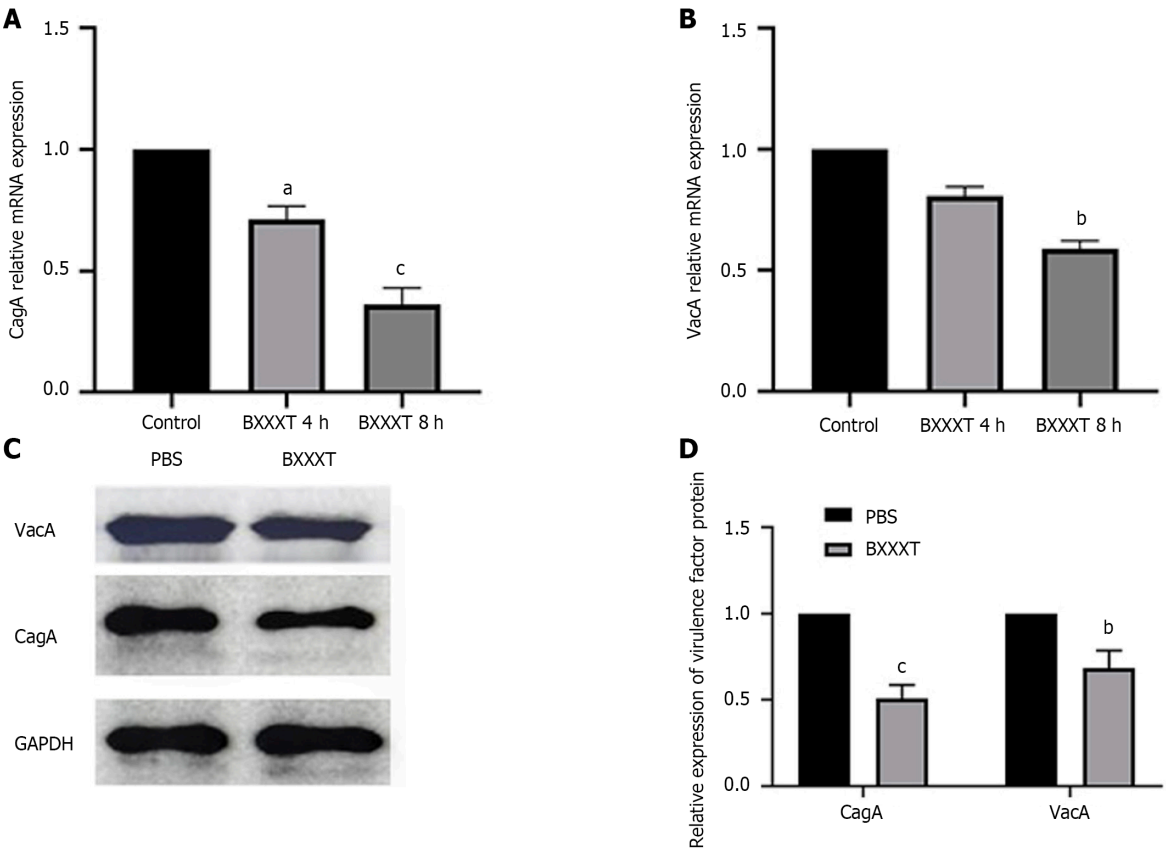
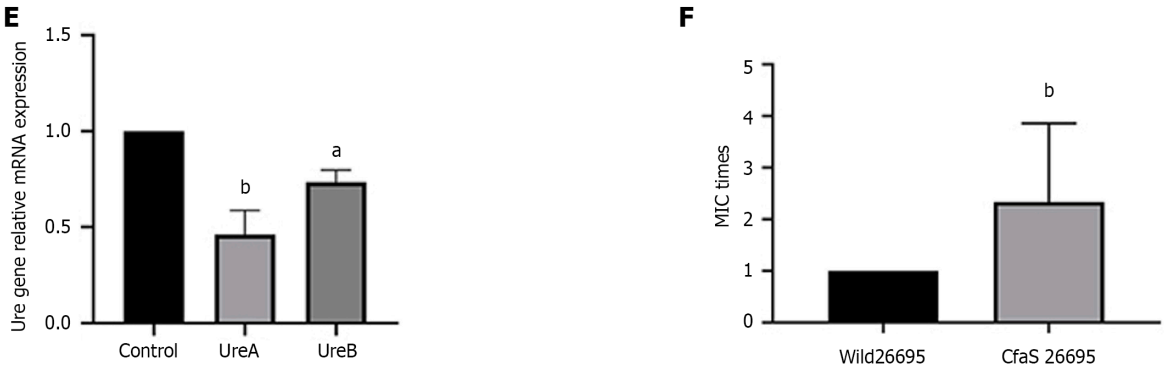


DOI: 10.3748/wjg.v29.i18.2818

Figure 3 Detection of changes of *Helicobacter pylori* after 8 h treatment with BanXiaXieXin decoction using an electron microscopy, transcriptome, and proteome analyses. A: Observation of changes of *Helicobacter pylori* (*H. pylori*) using an electron microscope; B: The number of significantly differential genes in the transcriptome; C: Significantly differential genes concentrating in the GO enrichment pathway in the transcriptome; D: The epithelial cell signaling in *H. pylori* infection; E: The number of significantly differential genes in the proteome; F: Significantly differential genes concentrating in the GO enrichment pathway in the proteome. BXXXT: BanXiaXieXin decoction; PBS: Phosphate-buffered saline.

and *in vivo* and its mechanism comes down to the synergistic or additional antibacterial effects of berberine, emodin and luteolin, the main components of the extract; the extract could activate the immune function and enhance bactericidal effects; BXXXT aqueous extract, with main targets of BXXXT aqueous extract related to urease, virulence factors, *etc*., could reduce the urease and virulence of *H. pylori*, weaken its colonization, and reduce its inflammatory damage to the gastric mucosa.





DOI: 10.3748/wjg.v29.i18.2818

Figure 4 Main targets of BanXiaXieXin decoction aqueous extract action. A: mRNA expression of *cagA*; B: mRNA expression of *VacA*; C: Protein expression of *CagA* and *VacA*; D: Quantitative expressions of *CagA* and *VacA* proteins; E: Mutant strain *CFAs* with a low urease expression; F: Changes of minimum inhibitory concentration of BXXXT against mutant strains with a low expression of urease. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; NS: Not significant. BXXXT: BanXiaXieXin decoction; PBS: Phosphate-buffered saline.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*)' s drug resistance brings challenges to the current clinical work. In China, traditional Chinese medicine could be used to treat a variety of refractory diseases, reducing drug-resistance, and improving the eradication rate of *H. pylori*. The study is to explore the therapeutic effects of Banxia Xiexin Decoction (BXXXT) on drug-resistant *H. pylori*.

Research motivation

H. pylori' s drug resistance brings challenges to the current clinical work. The study is to explore the therapeutic effects of BXXXT on drug-resistant *H. pylori*, avoid, overcome *H. pylori*' s drug resistance.

Research objectives

To confirm that BXXXT demonstrates therapeutical effects *in vivo* and *in vitro* on gastritis mice with drug-resistant *H. pylori* and explain its mechanism.

Research methods

The aqueous extract of BXXXT was gained by water decocting method. The inhibitory effect of the aqueous extract on *H. pylori* was detected by dilution in vitro. In terms of mechanism exploration, the main medicinal compositions of BXXXT aqueous extract and the synergistic bacteriostatic effects they had demonstrated were analyzed using mass spectrometry; the immune function of peripheral blood cells such as $CD3^+$ T and $CD4^+$ T of mice were detected using a flow cytometry; the *H. pylori* transcriptome and proteome were detected. Differently expressed genes were screened and verification was performed thereon with knockout expression.

Research results

BXXXT aqueous extract against *H. pylori* was better therapeutical effects *in vivo* and *in vitro*; BXXXT aqueous extract was found to stimulate the expressions of $CD3^+$ T and $CD4^+$ T and increase the number of $CD4^+$ T/$CD8^+$ T in gastritis mice; the detection of transcriptome and proteome, quantitative polymerase chain reaction, Western blot and knockout verification revealed that the main targets of BXXXT aqueous extract are *CFAs* related to urea enzymes, and *CagA*, *VacA*, *etc*.

Research conclusions

BXXXT aqueous extract could demonstrate good therapeutic effects on drug-resistance *H. pylori in vitro* and *in vivo* and its mechanism is related to reduce the urease and virulence of *H. pylori*, weaken its colonization, and reduce its inflammatory damage to the gastric mucosa, *etc*.

Research perspectives

BXXXT aqueous extract has good therapeutic effects on drug-resistance *H. pylori*, can overcome *H. pylori* 's drug resistance.

ACKNOWLEDGEMENTS

Thank Professor Bi Hongkai of Nanjing Medical University for the gift of *Helicobacter pylori* strain.

FOOTNOTES

Author contributions: Li XH and Xu JY consulted literature, performed experiments, collected and analyzed data and wrote the first draft, making equal contributions to this work; Wang X and Liao LJ corrected it; Huang L, Huang YQ, and Zhang ZF designed, checked, modified, and completed the manuscript, making equal contributions to this work as co-corresponding authors, Huang L (youyihuangl@163.com) is the first corresponding author; all authors approved the final version of the article.

Supported by the National Natural Science Foundation of China, No. 81760739; Special Fund Projects for Guide Local Science and Technology Development by the China government, No. GUIKEZY20198004; and 2022 Guangxi Graduate Education Innovation Plan Project, No. YCBZ2022071.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Youjiang Medical University for Nationalities Institutional Review Board (approval No. 20220110).

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: No additional data are available.

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

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: A
P-Editor: Zhang XD

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World Journal of Gastroenterology

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World J Gastroenterol 2023 May 14; 29(18): 2836-2849

DOI: 10.3748/wjg.v29.i18.2836

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Endoscopic and pathological characteristics of *de novo* colorectal cancer: Retrospective cohort study

Shi-Yang Li, Mei-Qi Yang, Yi-Ming Liu, Ming-Jun Sun, Hui-Jing Zhang

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Schijven MP, Netherlands; Tie J, Australia

Received: March 28, 2023
Peer-review started: March 28, 2023
First decision: April 10, 2023
Revised: April 14, 2023
Accepted: April 19, 2023
Article in press: April 19, 2023
Published online: May 14, 2023

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Abstract

BACKGROUND

Endoscopy has rapidly developed in recent years and has enabled further investigation into the origin and features of intestinal tumors. The small size and concealed position of these tumors make it difficult to distinguish them from nonneoplastic polyps and carcinoma in adenoma (CIA). The invasive depth and metastatic potential determine the operation regimen, which in turn affects the overall survival and distant prognosis. The previous studies have confirmed the malignant features and clinicopathological features of *de novo* colorectal cancer (CRC).

AIM

To provide assistance for diagnosis and treatment, but the lack of a summary of endoscopic features and assessment of risk factors that differ from the CIA prompted us to conduct this retrospective study.

METHODS

In total, 167 patients with small-sized CRCs diagnosed by endoscopy were reviewed. The patients diagnosed as advanced CRCs and other malignant cancers or chronic diseases that could affect distant outcomes were excluded. After screening, 63 cases were excluded, including 33 *de novo* and 30 CIA cases. Patient information, including their follow-up information, was obtained from an electronic His-system. The characteristics between two group and risk factors for invasion depth were analyzed with SPSS 25.0 software.

RESULTS

Nearly half of the *de novo* CRCs were smaller than 1 cm (n = 16, 48.5%) and the majority were located in the distal colon (n = 26, 78.8%). The IIc type was the most common macroscopic type of *de novo* CRC. In a Pearson analysis, the differential degree, Sano, JNET, and Kudo types, surrounding mucosa, and chicken skin

mucosa (CSM) were correlated with the invasion depth ($P < 0.001$). CSM was a significant risk factor for deep invasion and disturbed judgment of endoscopic ultrasound. A high degree of tumor budding and tumor-infiltrating lymphocytes are accompanied by malignancy. Finally, *de novo* CRCs have worse outcomes than CIA CRCs.

CONCLUSION

This is the first comprehensive study to analyze the features of *de novo* CRCs to distinguish them from nonneoplastic polyps. It is also the first study paying attention to CSM invasive depth measurement. This study emphasizes the high metastatic potential of *de novo* CRCs and highlights the need for more research on this tumor type.

Key Words: *De novo* colorectal cancer; Carcinoma in adenoma; Endoscopic features; Clinical characteristics; Pathological features

Core Tip: *De novo* colorectal cancer (CRC) is a specific tumor with a small lesion. Many different features of *de novo* CRCs exist to distinguish them from non-neoplastic polyps. Moreover, the study highlights that *de novo* CRCs have special endoscopic and pathological features that distinguish them from traditional adenocarcinomas. Different pit pattern types indicate various tumor types; for example, the III-type pit pattern often occurs in tubular adenomas. For CRCs, invasion depth evaluation is a vital issue. Computed tomography imaging and endoscopic ultrasound are used for judging invasive depth. Besides, chicken skin mucosa may also be a risk factor.

Citation: Li SY, Yang MQ, Liu YM, Sun MJ, Zhang HJ. Endoscopic and pathological characteristics of *de novo* colorectal cancer: Retrospective cohort study. *World J Gastroenterol* 2023; 29(18): 2836-2849
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2836.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2836

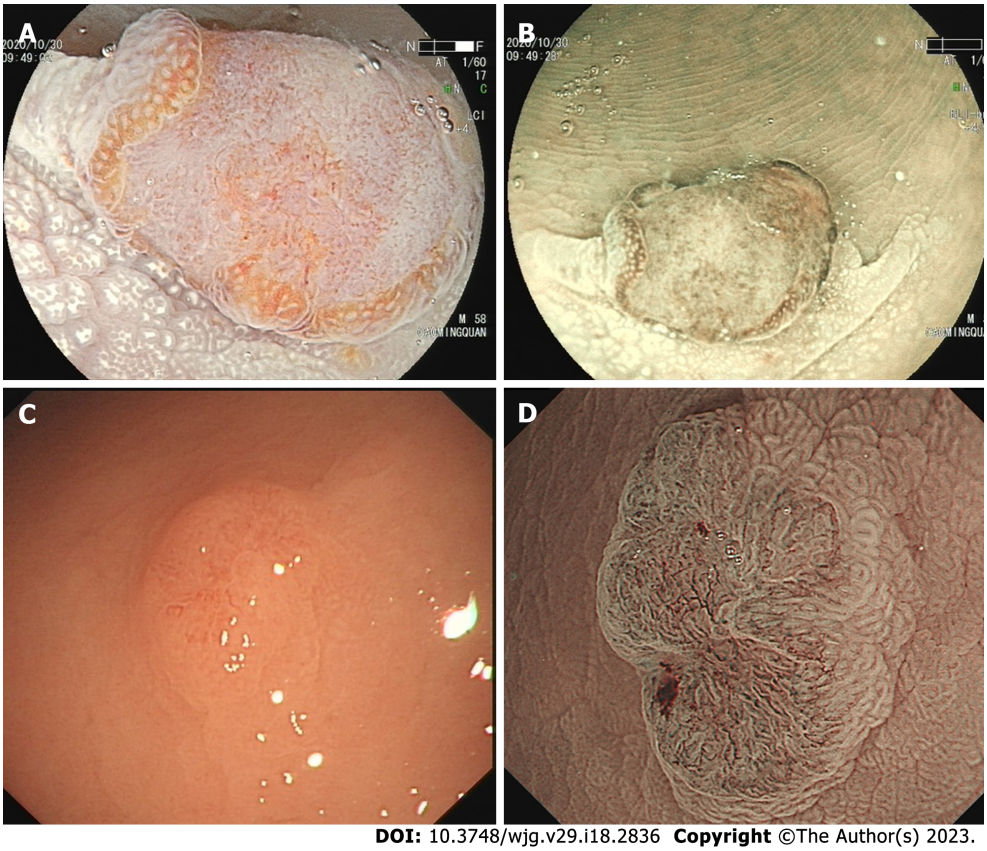
INTRODUCTION

A *de novo* colorectal cancer (CRC) is a unique CRC that represents a significant public health challenge with high morbidity and mortality globally[1,2]. At present, CRCs have four recognized pathways of development: The carcinoma in adenoma (CIA) pathway (aka chromosomal instability pathway), the *de novo* carcinoma pathway, the serrated lesion-carcinoma pathway, and the inflammatory-carcinoma pathway. In the 1950s, *de novo*-originated CRCs emerged increasingly after the definition "de novo" was proposed. In recent decades, the Japanese proposed a *de novo* carcinoma pathway[3]. Unlike other pathways, a *de novo* CRC is a tumor derived from cancer cells without progression of adenomatous changes, as shown in Figure 1, and its true pathway remains unknown. Normal I type around tumor and lack of III or IV type pits is a hallmark of *de novo* CRC under endoscopy[4-6].

For endoscopists, the endoscopic featrures occupied a key position in diagnosis and treatment. Magnified chromoendoscopy and narrow-band imaging (NBI) technology can be used to observe pit pattern and extimate depth of invasion[7-11]. Apart of pit pattern, there existed several indicators could forecast depth of invasion, such as chicken skin mucosa (CSM), mucosa pulling and converging. The CSM is an area of 0.5 mm of pale-yellow speckles adjacent to colonic neoplasms resulting from fat accumulation in the lamina propria[12]. CSM was more common in neoplastic and advanced polyps [13]. In this study, we concentrate on relationship between CSM and depth of invasion. For the whole CRCs, we should get to know three things: Get to know who they are firstly, estimate depth of invasion secondly, and predict the risk of metastasis at last.

Epithelial-mesenchymal transition (EMT) refers to a cellular reprogramming process in which epithelial cells acquire a mesenchymal phenotype[14]. EMT has an important role in development, wound healing, and malignant progression[15]. Mueller *et al*[16] have found that E-cadherin express lower in *de novo* than ex adenoma carcinomas through immunohistochemistry (IHC)[16]. We further want to know the relationship between E-cadherin proportion and distant metastsis.

Until now, there was no a complete study about *de novo* CRC, so we make this study to construct a comprehensive system for diagnosis and treatment of *de novo* CRC.



DOI: 10.3748/wjg.v29.i18.2836

Figure 1 The *de novo* colorectal cancer under endoscopy. A: A *de novo* colorectal cancer (CRC) under linked color imaging; B and D: The surface structure under the narrow bind imaging pattern; C: A *de novo* CRC under white light.

MATERIALS AND METHODS

We retrospectively reviewed patients diagnosed with CRCs between 2010 to 2020 year. We selected all *de novo* CRC and CIA-type patients on the His Electric System. All lesions were diagnosed by a senior pathologist and confirmed by two pathologists. The results from two pathologists had more than 90% similarity, and discrepant results were determined by a cheif pathologist. These patients did not have other comorbidities that could significantly impact the distant outcomes. Essential clinical information was collected, including endoscopic features and pathological results (size, endoscopic features, macroscopic type, pit pattern, clinical diagnosis, pathologic diagnosis, infiltration (IFN), immunohistology, invasive depth, tumor budding (TB), grading, lymphocyte IFN, perineural IFN, growth pattern, and lymph node metastasis). Pearson and χ^2 tests were performed using SPSS (25.0) software. The difference between each group was statistically significant ($P < 0.05$). Oral informed consent was obtained from all participants *via* telephone calls.

The degree of staining was scored according to the staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong) and the proportion of positive cells (0: 0%; 1: < 25%; 2: < 50%; 3: < 75%; 4: ≥ 75%). The score of each slice was determined by the staining index (staining intensity × proportion of positive cells). A staining index > 4 was classified as high-grade expression, while an index ≤ 4 was defined as low-grade expression.

The pathological film used in the study was IHC, with the patient's consent, and the patient's information was kept confidential; therefore, ethical approval was not required.

RESULTS

Clinical features of de novo CRCs

Nearly half (n = 16, 48.5%) of the *de novo* CRCs were less than 1 cm in size and located in the distal colon (n = 26, 78.8%). In contrast, CIA CRCs were mostly 2 cm (n = 21, 70.0%) and located at the proximal colon (n = 21, 70.0%). There were no significant differences in the other risk factors between the *de novo* and CIA groups ($P > 0.05$). In addition, we focused on the choice of treatment. All submucosa *de novo* CRCs were treated surgically, and seven CIA submucosa CRCs were treated endoscopically. The results are presented in Table 1.

Table 1 The clinical information of *de novo* and carcinoma in adenoma colorectal cancers

		De novo (*n* = 33)	CIA (*n* = 30)	χ^2	*P* value
Clinical information					
Age (%)	> 55	30 (90.9)	25 (83.3)	0.814	> 0.05
	< 55	3 (9.1)	5 (16.7)		
Gender (%)	Male	16 (48.5)	16 (53.3)	0.148	> 0.05
	Female	17 (51.5)	14 (46.7)		
Smoking history (%)	Yes	27 (81.8)	19 (63.3)	2.725	> 0.05
	No	6 (18.2)	11 (36.7)		
Location (%)	Proximal	7 (21.2)	21 (70.0)	15.149	< 0.05
	Distal	26 (78.8)	9 (30.0)		
Depth of invasion	M	8 (24.2)	9 (30.0)	1.504	> 0.05
	SMp	9 (27.3)	11 (36.7)		
	SMd	16 (48.5)	10 (33.3)		
Treatment (%)	Surgery	21 (63.6)	16 (53.3)	0.688	> 0.05
	Endoscopy	12 (36.4)	14 (46.7)		
Supplement chemotherapy (%)	Yes	21 (63.6)	12 (40.0)	3.520	> 0.05
	No	12 (36.4)	18 (60.0)		
Distal metastasis (%)	Yes	5 (15.2)	2 (6.7)	1.145	> 0.05
	No	28 (84.8)	28 (93.3)		
Overall survival (%)	1 yr	33 (100.0)	30 (100.0)		
	3 yr	30 (90.9)	28 (93.3)		
	5 yr	23 (69.7)	28 (93.3)		
DSS	Yes	26 (78.8)	28 (93.3)	2.715	> 0.05
	No	7 (21.2)	2 (6.7)		
Endoscopic gross characteristics					
Size%	1 cm < S ≤ 2 cm	14 (42.4)	9 (30.0)	30.514	< 0.05
	≤ 1 cm	16 (48.5)	0 (0.0)		
	> 2 cm	3 (9.1)	21 (70.0)		
Macroscopic type%	Is	5 (15.2)	6 (20.0)	35.187	< 0.05
	IIa + IIc	11 (33.3)	1 (3.3)		
	IIc	17 (51.5)	4 (13.3)		
	Is + IIc	0 (0.0)	19 (63.3)		
Surface change	Erosion	24 (72.7)	13 (43.3)	9.457	< 0.05
	Ulceration	2 (6.1)	11 (36.7)		
	None	7 (21.2)	6 (20.0)		
Color change	Reddened mucosa	26 (78.8)	2 (6.7)	33.104	< 0.05
	None	7 (21.2)	28 (93.3)		
NICE	Type 1	0 (0.0)	0 (0.0)		> 0.05
	Type 2	0 (0.0)	0 (0.0)		
	Type 3	33 (100.0)	30 (100.0)		
Sano	I	0 (0.0)	0 (0.0)	5.292	< 0.05
	II	0 (0.0)	0 (0.0)		

	IIIA	17 (51.5)	7 (23.3)		
	IIIB	16 (48.5)	23 (76.7)		
JNET	1	0 (0.0)	0 (0.0)	3.094	> 0.05
	2A	0 (0.0)	0 (0.0)		
	2B	12 (36.4)	5 (16.7)		
	3	21 (63.6)	25 (83.3)		
Chemical staining (crystal violet)					
Pit pattern	Vi	1 (3.0)	6 (20.0)	15.774	< 0.05
	Vn	13 (39.4)	15 (50.0)		
	Vi + Vn	19 (57.6)	5 (16.7)		
	IV + Vi	0 (0.0)	4 (13.3)		
Pit pattern at the edge of the tumors	I	33 (100.0)	0 (0.0)	63.000	< 0.05
	III + VI	0 (0.0)	30 (100.0)		
Surrounding mucosa					
Pulling	Yes	9 (27.3)	21 (70.0)	11.501	< 0.05
	No	24 (72.7)	9 (30.0)		
Converging folds	Yes	27 (81.8)	15 (50.0)	7.159	< 0.05
	No	6 (18.2)	15 (50.0)		
CSM	CSM1	11 (33.3)	4 (13.3)	3.553	> 0.05
	CSM2	17 (51.5)	21 (70.0)		
	CSM3	5 (15.2)	5 (16.7)		

CRC: Colorectal cancer; DSS: Disease specific survival; CSM: Chicken skin mucosa; CIA: Carcinoma in adenoma; NICE: Narrow-band imaging International Colorectal Endoscopic.

Endoscopic characteristics of de novo CRCs

Based on endoscopic appearance, small CRCs were classified as type I (polypoid), type IIa (slightly raised), type IIb (flat), or type IIc (depressed)[12]. Type IIa + IIc is the most common type of *de novo* CRC. Notably, 15 (45.5%) *de novo* CRCs were Is type. We showed different macroscopic types of *de novo* CRCs, as shown in Figure 2. For CIA CRCs, the Is + IIc type (polypoid with depression) was the most common presentation (n = 19, 63.3%). Another significant finding was surface changes: We found that erosion and reddened mucosa are hallmark features of *de novo* CRCs. In the CIA group, erosion was the same as ulceration. Furthermore, reddened mucosa was seldom observed in the CIA group (n = 28, 93.3%). The endoscopic results are shown in Table 2.

There was a significant difference in the Sano and pit pattern types (P < 0.05) but no significant difference in the NBI International Colorectal Endoscopic (NICE) classification and JNET types between the two groups (P > 0.05). About half of the *de novo* CRCs were IIIA type (n = 17, 51.5%) with a Vi + Vn type pit pattern (n = 19, 57.6%). The most striking difference was in the pit pattern around the edges of the lesions: I type pit patterns (small and round pits) were observed in all *de novo* CRCs (n = 33, 100%), and III + VI type pit patterns were observed in all CIA CRCs (n = 30, 100%).

We also analyzed the risk factors associated with the invasive depth (Table 3). A third (33.3%) of the *de novo* group had a CSM1 × *vs* 13.3% of the CIA group. CSM was associated with the depth of invasion [r (correlation coefficient) = -0.796, P < 0.001]. CSM1 has a high invasive potential for CRC. In addition to CSM, pulling and converging folds also appear in the mucosa surrounding CRCs. Mucosal pulling appeared more frequently in the CIA group (n = 21, 70.0%) than in the *de novo* group (n = 9, 27.3%). In contrast, the converging fold was more frequent in the *de novo* group (n = 27, 81.8%) than that in the CIA group (n = 15, 50%). After analyzing the relationship between these parameters and invasive depth, we found that mucosal pulling (r = 0.567, P < 0.001) and converging folds (r = 0.620, P < 0.001) were also significantly associated with invasive depth.

Pathological characteristics of de novo CRCs

Pathological results revealed that TB was significantly associated with invasive depth (r = 0.669, P < 0.001) (Table 4). Although there was no significant difference between the two groups, budding grade 3 (BD3) accounted for 33.3% of the *de novo* group, which is far more than that of the CIA group (n = 6,

Table 2 The endoscopic characteristics in *de novo* and carcinoma in adenoma groups

		De novo (*n* = 22)	CIA (*n* = 20)	χ^2	*P* value
Budding	BD1	8 (24.2)	4 (13.3)	6.161	> 0.05
	BD2	8 (24.2)	6 (20.0)		
	BD3	11 (33.3)	6 (20.0)		
	None	5 (18.2)	14 (46.7)		
Grading	G1	6 (18.2)	13 (43.3)	8.878	> 0.05
	G2	11 (33.3)	9 (30.0)		
	G1-2	10 (30.3)	8 (26.7)		
	G3-2	6 (18.2)	0 (0.0)		
TIL	Yes	27 (81.8)	19 (63.3)	2.725	> 0.05
	No	6 (18.2)	11 (36.7)		
Perineural infiltration	Yes	16 (48.5)	3 (10.0)	11.050	< 0.05
	No	17 (51.5)	27 (90.0)		
Lymphovascular invasion	Yes	16 (48.5)	7 (23.3)	4.289	< 0.05
	No	17 (51.5)	23 (76.7)		
Growth pattern	INFa	0 (0.0)	27 (90.0)	63.000	< 0.05
	INFb	0 (0.0)	3 (10.0)		
	INFc	33 (100.0)	0 (0.0)		
Lymph node metastasis	Yes	10 (30.3)	1 (3.3)	7.931	> 0.05
	No	23 (69.7)	29 (96.7)		
Adjacent lesions	Inflammation	33 (100.0)	0 (0.0)	63.000	< 0.05
	Adenoma	0 (0.0)	17 (56.7)		
	Hyperplasia	0 (0.0)	13 (43.3)		
IHC-E-cadherin	No staining	0 (0.0)	0 (0.0)	22.609	< 0.05
	Weak	17 (51.5)	2 (6.7)		
	Moderate	15 (48.5)	19 (63.3)		
	Strong	0 (0.0)	9 (30.0)		
IHC-Vimentin	No staining	0 (0.0)	0 (0.0)	23.182	< 0.05
	Weak	13 (39.4)	18 (60.0)		
	Moderate	1 (3.0)	10 (33.3)		
	Strong	19 (57.6)	2 (6.7)		

CIA: Carcinoma in adenoma; TIL: Tumor Infiltrating Lymphocyte; IHC: Immunohistochemistry; BD: Budding grade.

Table 3 The risk factors are related to the depth of invasion

Depth of invasion	M	SM-S	SM-d and deeper	*r*	*P* value
Size, %				-0.002	> 0.05
1 cm < S ≤ 2 cm	1 (12.5)	0 (0.0)	13 (81.3)		
≤ 1 cm	7 (87.5)	9 (100.0)	0 (0.0)		
> 2 cm	0 (0.0)	0 (0.0)	3 (18.8)		
Growth pattern					> 0.05

IFNa	0 (0.0)	0 (0.0)	0 (0.0)		
IFNb	0 (0.0)	0 (0.0)	0 (0.0)		
IFNc	8 (100.0)	9 (100.0)	16 (100.0)		
Differential degree				0.904	< 0.001
G1	5 (62.5)	1 (11.1)	0 (0.0)		
G2	3 (37.5)	8 (88.9)	0 (0.0)		
G1-2	0 (0.0)	0 (0.0)	10 (62.5)		
G3-2	0 (0.0)	0 (0.0)	6 (37.5)		
Macroscopic type				-0.093	> 0.05
Is	2 (25.0)	0 (0.0)	13 (81.3)		
IIa + IIc	6 (75.0)	9 (100.0)	0 (0.0)		
IIc	0 (0.0)	0 (0.0)	3 (18.8)		
Is + IIc	0 (0.0)	0 (0.0)	0 (0.0)		
NICE					> 0.05
Type 1	0 (0.0)	0 (0.0)	0 (0.0)		
Type 2	0 (0.0)	0 (0.0)	0 (0.0)		
Type 3	8 (100.0)	9 (100.0)	16 (100.0)		
Sano				0.938	< 0.001
I	0 (0.0)	0 (0.0)	0 (0.0)		
II	0 (0.0)	0 (0.0)	0 (0.0)		
IIIA	8 (100.0)	9 (100.0)	0 (0.0)		
IIIB	0 (0.0)	0 (0.0)	16 (100.0)		
JNET				0.649	< 0.001
1	0 (0.0)	0 (0.0)	0 (0.0)		
2A	0 (0.0)	0 (0.0)	0 (0.0)		
2B	5 (62.5)	7 (77.8)	0 (0.0)		
3	3 (37.5)	2 (22.2)	16 (100.0)		
Pit pattern				-0.491	< 0.001
Vi	1 (12.5)	0 (0.0)	0 (0.0)		
Vn	0 (0.0)	0 (0.0)	13 (81.3)		
Vi + Vn	7 (87.5)	9 (100.0)	3 (18.8)		
IV + Vi	0 (0.0)	0 (0.0)	0 (0.0)		
Pit pattern at the edge of the tumors					> 0.05
I	8 (100.0)	9 (100.0)	16 (100.0)		
III + VI	0 (0.0)	0 (0.0)	0 (0.0)		
Surrounding mucosa					
Pulling				-0.567	< 0.001
Yes	0 (0.0)	0 (0.0)	9 (56.3)		
No	8 (100.0)	9 (100.0)	7 (43.8)		
Converging folds				-0.62	< 0.001
Yes	3 (37.5)	8 (88.9)	16 (100.0)		
No	5 (62.5)	1 (11.1)	0 (0.0)		
CSM				-0.796	< 0.001

CSM1	0 (0.0)	0 (0.0)	11 (68.8)
CSM2	3 (37.5)	9 (100.0)	5 (31.3)
CSM3	5 (62.5)	0 (0.0)	0 (0.0)

IFN: Infiltration; CSM: Chicken skin mucosa; NICE: Narrow-band imaging International Colorectal Endoscopic.

Table 4 The risk factors are related to tumor budding

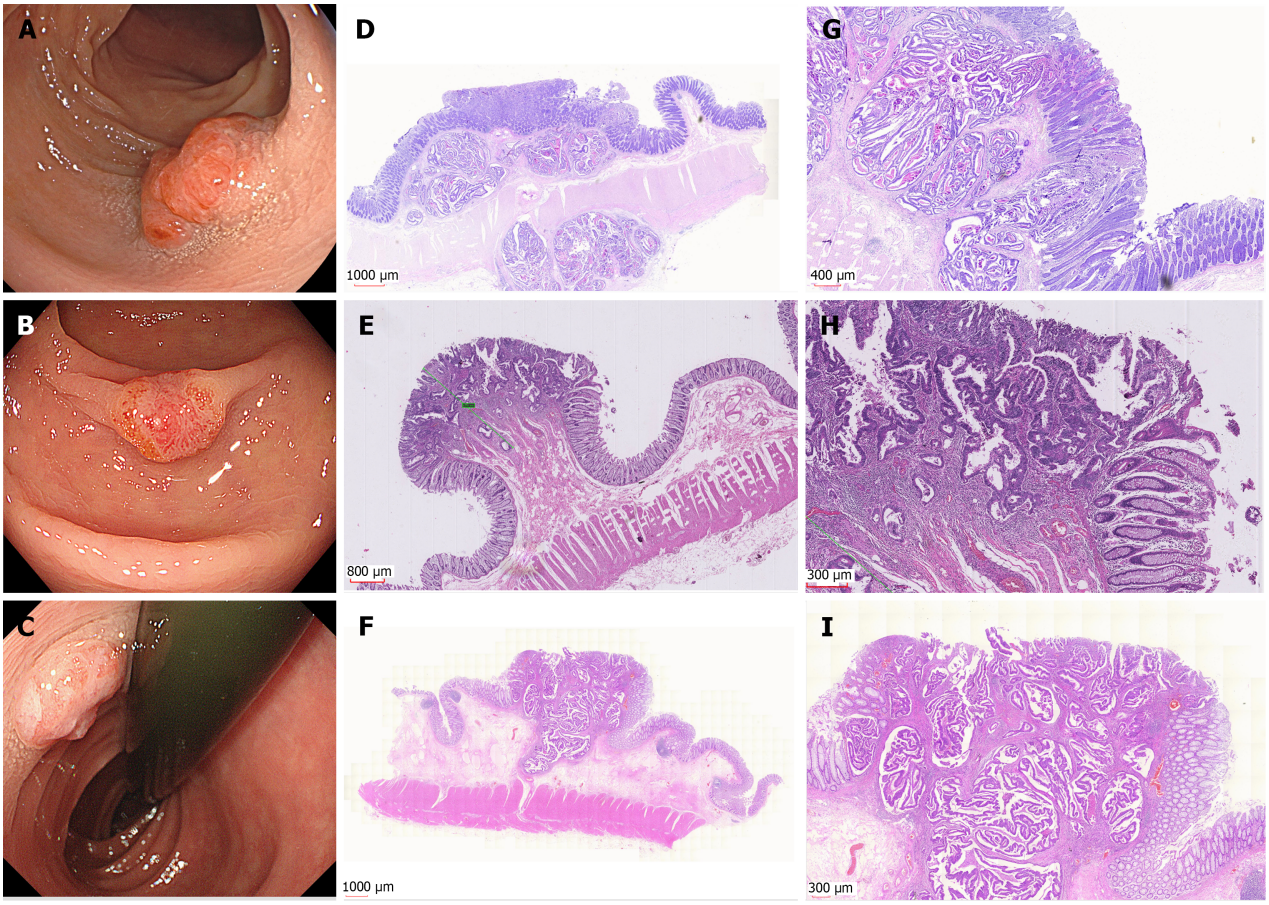
	BD1	BD2	BD3	No budding	*r*	*P* value
Infiltrative depth, %					0.669	< 0.001
M	0 (0.0)	3 (21.4)	0 (0.0)	14 (70.0)		
SMp	10 (83.3)	1 (7.1)	3 (17.6)			
SMd	2 (16.7)	10 (71.4)	14 (82.4)			
Lymphovascular invasion					0.663	< 0.001
Yes	2 (28.6)	4 (21.1)	16 (94.1)	1 (5.0)		
No	5 (71.4)	15 (78.9)	1 (5.9)	19 (95.0)		
Lymph node metastasis					0.489	< -0.001
Yes	1 (14.3)	1 (5.3)	9 (52.9)	0 (0.0)		
No	6 (85.7)	18 (94.7)	8 (41.7)	20 (100.0)		
Perineural infiltration					0.601	< 0.001
Yes	2 (28.6)	4 (21.1)	13 (76.5)	0 (0.0)		
No	5 (71.4)	15 (78.9)	4 (23.5)	20 (100.0)		
Tumor infiltrating lymphocytes					0.476	< 0.001
Yes	7 (100.0)	19 (100.0)	14 (82.4)	6 (30.0)		
No	0 (0.0)	0 (0.0)	3 (17.6)	14 (70.0)		
Differential degree					0.706	< 0.001
G1	0 (0.0)	0 (0.0)	0 (0.0)	19 (95.0)		
G1-2	2 (28.6)	9 (47.4)	7 (41.2)	0 (0.0)		
G2	5 (71.4)	10 (52.6)	4 (23.5)	1 (5.0)		
G3-2	0 (0.0)	0 (0.0)	6 (35.3)	0 (0.0)		

BD: Budding grade.

20%). Regarding other pathological characteristics, perineural IFN, lymph node metastasis, and tumor-infiltrating lymphocytes (TIL) have also emerged in CRC tissues. There was a significant difference in perineural IFN and lymph node metastasis between the two groups ($P < 0.05$).

Another critical issue that deserves attention is IHC. In both groups, approximately 70% of the cases were MLH1 (+), MSH2 (+), MSH6 (+), PMS2 (+), CDX2 (+), Ki67 (+), and P53 (+). We extracted several sections that were stained for E-cadherin and vimentin (VIM). In the *de novo* group, the E-cadherin expression was low in the epithelial regions and VIM was high in the mesenchyma. The CIA group showed opposite results to the *de novo* group, as shown in Figure 3. We analyzed risk factors for distant metastasis in *de novo* group, the results are shown in Supplementary Table 1. Differential degree, Sano classification, expression of E-cad and VIM were correlated with distant metastasis. High expression of E-cad was negatively related with distant metastasis and VIM had an opposite result.

In addition, we explored whether the *de novo* type was correlated with relapse and survival. In the *de novo* group, 11 subjects developed neoplastic polyps again within 5 years, and 10 relapsed within 5 years. The 5-year survival rate was 93.3% for CIA and 69.7% for *de novo* CRCs. Log-rank analysis revealed no significant difference in relapse between the two groups ($\chi^2 = 0.49$, $P = 0.515$); however, there was a significant difference in the survival probability between the two groups ($\chi^2 = 7.08$, $P = 0.020$). Survival curves are shown in Figure 4.



DOI: 10.3748/wjg.v29.i18.2836

Figure 2 The examples of I-type *de novo* colorectal cancer. A-C: Endoscopic images of different types *de novo* colorectal cancers (CRCs); D-I: Pathological images of *de novo* CRCs.

DISCUSSION

In this study, we present our novel findings pertaining to the characteristics of *de novo* CRC. The key findings included the following: (1) The incidence rate of *de novo* CRCs is considered lower than CIA CRCs, but our study showed that it is related to the classification of CRC. Although some *de novo* CRCs are limited to intramucosal cancer, they are not the same as high-grade dysplasia or cancer with high-grade intraepithelial neoplasia, which could be called advancing adenoma. An advancing adenoma is a precancerous lesion, whereas a *de novo* CRC is a type of early CRC. Some endoscopic physicians misdiagnose advanced adenomas as early CRCs; this will improve the diagnostic rate of CRC but reduce the incidence rate of *de novo* cancer. Therefore, we believe that the diagnostic bias of endoscopic physicians affects the incidence rate. One of the aims of this study was to bolster the standardized diagnostic requirements of endoscopic physicians and to understand *de novo* cancer from the molecular, pathological, and endoscopic perspectives. According to the Vienna classification, category 4 includes advanced adenoma/dysplasia and noninvasive carcinoma. The reason for the lower incidence of *de novo* CRCs in the previous study may be eliminated by comprehensively evaluating the characteristics comprehensively[17-20]; (2) In our study, 48.5% of *de novo* CRCs were < 1 cm in size. Some investigators excluded tumors > 1 cm because they thought that the adenomatous component might be obliterated by the expanding tumor mass[12]. In our study, 42.4% of the *de novo* CRC-derived tumors diagnosed by endoscopists and pathologists were ≥ 1 cm and ≤ 2 cm. In addition, there were three lesions larger than 2 cm. Therefore, warning regarding small lesions without adenomatous components should be escalated; and (3) In addition to platform lift, converging mucosa, pulling, and CSM are significant indicators of invasive depth. Fibroplasia causes surface changes during cancer cell invasion. Converging mucosa was more likely to occur in *de novo* CRCs than that in CIA group, and nearly half of the lesions (n = 16, 48.5%) had deeper invasion. Mucosal pulling may be caused by shrinkage of the mucosa or muscularis mucosa, indicating superficial layer invasion.

The cause of CSM may be fat accumulation in macrophages, which may result from the breakdown of lipids within colonocytes or adjacent tumors. These findings suggest that CSM is a valuable marker for differentiating between neoplastic and advanced polyps using conventional white colonoscopy[21]. Lee *et al*[13] divided CSM into three types based on their characteristics. Type 1 CSM was confirmed before

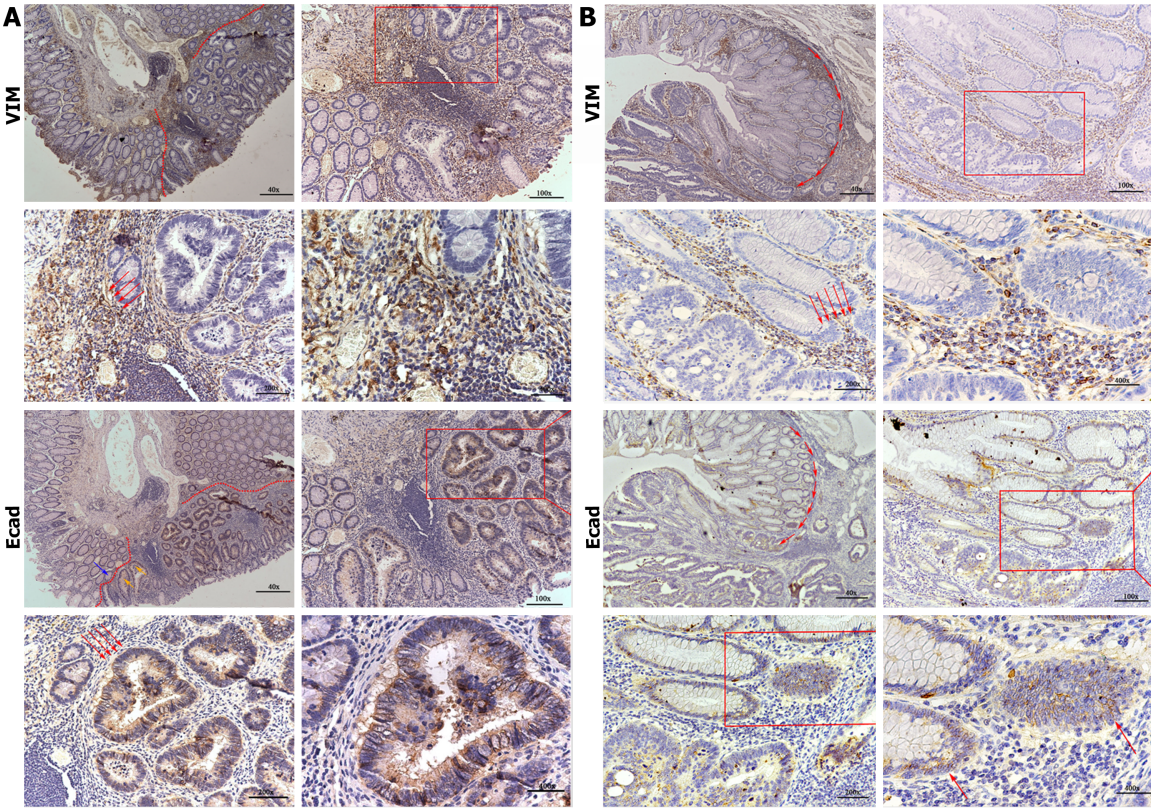


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Figure 3 The immunohistory results. A: A *de novo* colorectal cancer (CRC) pathological section stained with E-cad and vimentin (VIM); B: A carcinoma in adenoma CRC pathological section stained with E-cad and VIM. VIM: Vimentin.

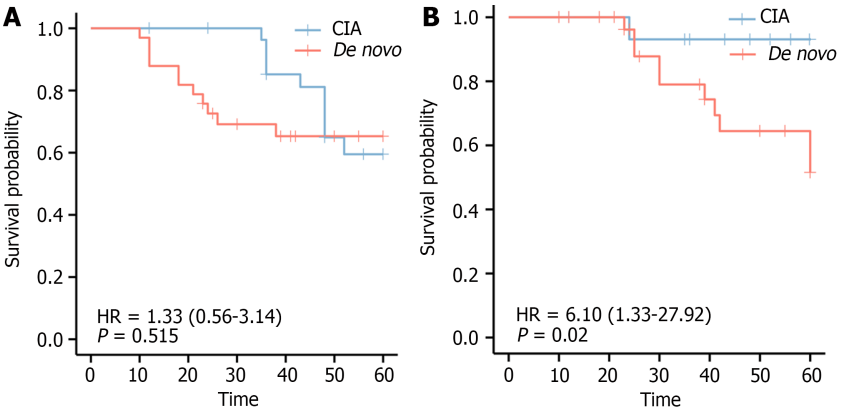

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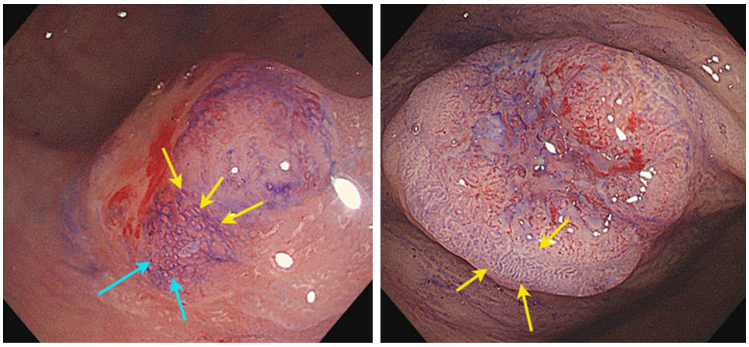
Figure 4 The association of *de novo* colorectal cancer and over survival. A: Relationship between relapse rate and colorectal cancer (CRC) types; B: Relationship between rate of death and CRC types. CIA: Carcinoma in adenoma.

injection; type 2 CSM was observed after injection; and type 3 CSM was not observed. Types 1 and 2 were considered positive CSM findings. CSM is accompanied by an increased IFN of foam macrophages in the lamina propria, representing a large-scale inflammatory reaction. The appearance of CSM is also related to the increased expression of Ki-67 and COX2, which suggests that the early appearance of CSM symbolizes a high malignancy. However, whether the appearance of CSM and the IFN of macrophages in the lamina propria affect the determination of the depth of IFN by endoscopic ultrasound (EUS) is unknown. After diagnosing white light endoscopy, magnifying endoscopy, and endoscopic ultrasonography, we concluded that the lesion was a *de novo* cancer with deep invasion, which was no longer suitable for endoscopic treatment. However, after surgical resection, pathology showed that the depth of invasion only reached the M2 layer. After further analysis of the pathology, we found that many inflammatory cells infiltrated the lesion, leading to errors in determining the IFN depth.



DOI: 10.3748/wjg.v29.i18.2836

Figure 5 The surrounding pit of *de novo* colorectal cancer and carcinoma in adenoma group. The surrounding pit around *de novo* colorectal cancer (CRC) is elongated I-type and IIIL-type around carcinoma in adenoma CRC.

Under NBI and magnifying endoscopy patterns, the surface and vascular microstructures can be clearly evaluated. The NICE classification is a method for assessing the characteristics of lesions under the NBI model, which is based on the color, vessel, and general surface pattern[22]. The NICE classification can help reduce the risk of failing to detect diminutive and small lesions, which are easily regarded as polys[23]. Nevertheless, the NICE classification had no differential ability for *de novo* CRCs and CIA CRCs. The pit pattern of CRC characteristics was categorized according to the Kudo and Tsuruta classification system. Type IIIL and IV patterns are often observed in adenomas and CIA CRCs. Type V is subclassified into Vi and Vn, and occurs in cancers. The surrounding I type pit is a landmark in the diagnosis of *de novo* CRCs. However, the normal I type often deforms and confounds with an abnormal pit due to the extrusion or invasion of cancer. The IIIs type pit was round and smaller than the I type pit, and IIIL was more irregular than the elongated I type pit. An example is shown in Figure 5.

De novo CRCs have greater metastatic potential, and all pathological indicators indicate that *de novo* carcinoma has a higher metastatic potential and worse prognosis. All *de novo* cancers were classified as microsatellite stability (MSS). In the *de novo* group, the degree of TB and lymphocyte IFN was higher than that in the CIA group and was positively correlated with the depth of invasion and poor prognosis. The IHC results also showed that the expression of E-cadherin in the *de novo* group was lower than that in the CIA group. At the same time, the interstitial-related index VIM was higher than that in the CIA group, which means that the metastatic potential of the tumor in the *de novo* group was higher than that in the CIA group. TB is defined as "a single cancer cell or a cell cluster of 5 cancer cells or less". Larger tumors are called poorly differentiated tumor cell nests. TB is closely correlated with disease recurrence and poor prognosis in SM-invasive CRCs[24-27].

In addition, the TB degree had a relationship with other pathological features. In general, tumor tissues with BD2/3 are considered for further surgery[28]. The lymphocytic reaction is another crucial part of pathological changes in CRCs, which includes Crohn's-like reaction, peritumoral reaction, intratumoral periglandular reaction, and TIL[29]. TIL are defined as lymphocytes on the top of cancer cells, which can be used to predict immunotherapy response and survival outcomes. One study reported that tumors with microsatellite instability-high and sufficient CD8+ TIL had better outcomes than those with MSS/microsatellite instability-low and lower CD8+ TIL[30]. Compared to typical pathological indicators, TIL are a better factor for overall survival[29,31,32]. TIL are related to the expression of exhaustion and senescence markers in the tumor microenvironment[33,34].

This study have several promising implications during clinical practice. Due to the highly invasive and metastatic ability of *de novo* CRC, endoscopic mucosal resection or endoscopic submucosal dissection could not be performed without exact observation when we find small protruded or depressed lesions. Observing the lesions with magnified endoscopy and chromoendoscopy is necessary to confirm the diagnosis. If we suspect the lesions may be *de novo* CRCs, it is crucial evaluating invasion depth by magnified endoscopy and chromoendoscopy, or by computed tomography imaging and EUS when necessary. Then, the treatment regimens should be chosen cautiously. In the future, we will further study the molecular biological difference between *de novo* CRC and CIA CRC to find out the molecular mechanism of invasion and metastasis of *de novo* CRCs.

However, there exists some limitations in the study. The sample size appear to be small for deficience of acknowledge of *de novo* CRCs. Moreover, this appears as a single center study, and the pathogenesis/incidence of *de novo* CRC might be different in other countries.

CONCLUSION

This review summarizes the characteristics of *de novo* CRCs and confirms the conclusions of our study.

A *de novo* CRC is a small, but malignant tumor that requires more attention during colonoscopy examination. *De novo* CRCs have special endoscopic and pathological features that distinguish them from the traditional adenocarcinomas. However, more studies are needed to determine the molecular characteristics to explain the genesis mechanism.

ARTICLE HIGHLIGHTS

Research background

The small colorectal small tumors usually be ignored during colonoscopy. However, many depressed or flat lesions have substantial invasion and metastasis. *De novo* colorectal cancer (CRC) is one type of small tumor related to poor prognosis. And some endoscopists could not distinguish *de novo* CRC during the examination.

Research motivation

Some small lesions were cut off directly in the examination without computed tomography imaging and endoscopic ultrasound. This may lead to mistreatment because endoscopists often ignore the judgment of invasion depth. The *de novo* CRC may have a deep invasion layer.

Research objectives

This study aimed to comprehensively review *de novo* CRCs and provide a reference atlas for future studies and analyze the features of *de novo* CRCs to distinguish them from non-neoplastic polyps.

Research methods

This study collected clinical and pathological information on *de novo* patients and stained E-cadherin and vimentin by immunohistochemistry. Based on this information, we analyzed the characteristic of *de novo* CRC and the relative correlation between different indicators.

Research results

This study highlights that *de novo* CRCs have special endoscopic and pathological features that distinguish them from traditional adenocarcinomas. It is also the first study paying attention to chicken skin mucosa invasive depth measurement. More importantly, this study summarized several factors relevant to invasion depth and provide tremendous help in clinical practice to increase diagnostic ability.

Research conclusions

This first study pointed out the relationship between *de novo* CRC and epithelial-mesenchymal transition related genes. And it is the first study put forward that chicken skin mucosa indicates the depth of invasion.

Research perspectives

We will further study the molecular biological difference between *de novo* CRC and carcinoma in adenoma CRC to discover the mechanism of invasion and metastasis of *de novo* CRCs.

FOOTNOTES

Author contributions: Zhang HJ had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; Zhang HJ, Li SY, and Yang MQ were responsible for protocol/project development; Li SY, Yang MQ, and Zhang HJ performed data analysis; Li SY, Yang MQ, Liu YM, and Sun MJ performed data collection or management; Yang MQ and Zhang HJ were responsible for manuscript writing/editing; Li SY and Yang MQ, these two authors, contributed equally to this work.

Supported by Natural Science Foundation of Liaoning Province, China, No. 2022-YGJC-71.

Institutional review board statement: The study was reviewed and approved by the Research Ethics Committee of the First Affiliated Hospital of China Medical University Institutional Review Board, No. [2021]2021-68-2.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Participants gave informed consent for data sharing.

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

Country/Territory of origin: China

ORCID number: Hui-Jing Zhang 0000-0002-6322-2335.

S-Editor: Fan JR
L-Editor: A
P-Editor: Cai YX

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World Journal of Gastroenterology

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World J Gastroenterol 2023 May 14; 29(18): 2850-2863

DOI: 10.3748/wjg.v29.i18.2850

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Prolonged hyperthermic intraperitoneal chemotherapy duration with 90 minutes cisplatin might increase overall survival in gastric cancer patients with peritoneal metastases

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Karaca CA, Turkey; Li K, China; Li L, New Zealand; Luo W, China; Segura-Sampedro JJ, Spain

Received: November 18, 2022
Peer-review started: November 18, 2022
First decision: February 15, 2023
Revised: February 27, 2023
Accepted: April 10, 2023
Article in press: April 10, 2023
Published online: May 14, 2023

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Abstract

BACKGROUND

Advanced gastric cancer with synchronous peritoneal metastases (GC-PM) is associated with a poor prognosis. Although cytoreductive surgery with hyperthermic intraperitoneal chemotherapy (CRS-HIPEC) is a promising approach, only a limited number of Western studies exist.

AIM

To investigate the clinicopathological outcomes of patients who underwent CRS-HIPEC for GC-PM.

METHODS

A retrospective analysis of patients with GC-PM was conducted. All patients were seen at the Department of General and Visceral Surgery, Hospital Barmherzige Brüder, Regensburg, Germany between January 2011 and July 2021 and underwent CRS-HIPEC. Preoperative laboratory results, the use of neoadjuvant

trastuzumab, and the details of CRS-HIPEC, including peritoneal carcinomatosis index, completeness of cytoreduction, and surgical procedures were recorded. Disease-specific (DSS), and overall survival (OS) of patients were calculated.

RESULTS

A total of 73 patients were included in the study. Patients treated with neoadjuvant trastuzumab (n = 5) showed longer DSS (P = 0.0482). Higher white blood cell counts (DSS: P = 0.0433) and carcinoembryonic antigen levels (OS and DSS: P < 0.01), and lower hemoglobin (OS and DSS: P < 0.05) and serum total protein (OS: P = 0.0368) levels were associated with shorter survival. Longer HIPEC duration was associated with more advantageous median survival times [60-min (n = 59): 12.86 mo; 90-min (n = 14): 27.30 mo], but without statistical difference. To obtain additional data from this observation, further separation of the study population was performed. First, propensity score-matched patient pairs (n = 14 in each group) were created. Statistically different DSS was found between patient pairs (hazard ratio = 0.2843; 95% confidence interval: 0.1119-0.7222; P = 0.0082). Second, those patients who were treated with trastuzumab and/or had human epidermal growth factor receptor 2 positivity (median survival: 12.68 mo *vs* 24.02 mo), or had to undergo the procedure before 2016 (median survival: 12.68 mo *vs* 27.30 mo; P = 0.0493) were removed from the original study population.

CONCLUSION

Based on our experience, CRS-HIPEC is a safe and secure method to improve the survival of advanced GC-PM patients. Prolonged HIPEC duration may serve as a good therapy for these patients.

Key Words: Cytoreductive surgery; Hyperthermic intraperitoneal chemotherapy; Peritoneal metastasis; Stomach neoplasms; Gastric cancer

Core Tip: Advanced gastric cancer (GC) cases with peritoneal metastases are known for their poor survival rates. It has been previously reported that these patients benefit from cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) but available data on this treatment are scarce. In this study, we retrospectively analyzed the clinicopathological and laboratory data of 73 patients with advanced GC and synchronous peritoneal metastases. It was found that prolonged HIPEC duration after macroscopic complete CRS in the scope of multimodal treatment along with advanced perioperative chemotherapy and biologicals may serve as the best currently available therapy for these patients.

Citation: Steinhoff H, Acs M, Blaj S, Dank M, Herold M, Herold Z, Herzberg J, Sanchez-Velazquez P, Strate T, Szasz AM, Piso P. Prolonged hyperthermic intraperitoneal chemotherapy duration with 90 minutes cisplatin might increase overall survival in gastric cancer patients with peritoneal metastases. *World J Gastroenterol* 2023; 29(18): 2850-2863

URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2850.htm

DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2850

INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer, with a worldwide incidence of 1,089,103 new cases and 768,793 deaths based on the 2020 GLOBOCAN results[1,2]. The majority of the new cases are diagnosed in Asia, where occurrence is 6-fold higher than in Europe; a similar distribution is observed in GC mortality[1]. In Germany, 15322 new cases and 9196 deaths were reported in 2020[1]. GC is known for its morphological diversity[3], and the most commonly used classifications are those outlined by Nakamura *et al*[4], Lauren[5], and the World Health Organization (WHO)[6]. The treatment of gastric cancer is multidisciplinary and depends on the clinical staging of the tumor. While early-stage GC (stage T1a) can be endoscopically resected[7], stage T1 with positive lymph node(s) and T2-T4a tumors regardless of lymph node status are treated by surgical resection and peri- or postoperative chemotherapy[8]. Advanced resectable GCs are typically treated with neoadjuvant chemotherapy followed by gastrectomy and adjuvant chemotherapy[9]; if not amenable to resection, then the treatment of choice is chemotherapy[8].

A recent analysis of 18,000 United States patients showed that advanced GC with PM has a median survival of 8.6 mo if treated with chemotherapy only[10], while studies from the United States[11], China[12] and Germany[13] have shown that advanced GCs with peritoneal carcinomatosis benefit significantly from cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC)[11-15] when complete macroscopic resection of the tumor tissue can be achieved[16]. Nevertheless, the available data regarding the use of CRS and HIPEC in advanced GC with PM is scarce, and this multimodal therapy has infrequently been recommended in any national or international guidelines. To date, the Japanese[17] and the United States[18] guidelines do not include CRS and HIPEC as therapeutic options. In France, the guidelines for treatment of advanced GC with PM[19] are yet to be defined in future randomized phase III studies. The same is true in Germany, where an expert consensus-based recommendation calls for the implementation of CRS and HIPEC in clinical studies[8]. The European Society for Medical Oncology guidelines for the treatment of gastric cancer describe CRS and HIPEC as safe procedures, but with unclear oncological outcomes[20]. Accordingly, the aim of this retrospective study was to investigate the clinical outcome after administration of this multimodal therapy in a tertiary center to treat patients with primary advanced GC with PM.

MATERIALS AND METHODS

Patients and study design

The HIPEC database of a single tertiary care center was analyzed in a retrospective manner. A total of 73 patients seen at the Department of General and Visceral Surgery, Hospital Barmherzige Brüder, Regensburg, Germany between January 2011 and July 2021 with primary GC and synchronous PM were included (Figure 1). All patients gave written and verbal informed consent to be included in the national HIPEC registry, administered by the German Society for General and Visceral Surgery (DGAV), and for the use of their anonymized data for research purposes and quality assurance prior to any study-specific procedures. All 73 patients underwent CRS + HIPEC and were treated according to national or international multidisciplinary recommendations[8,20].

Details of CRS + HIPEC

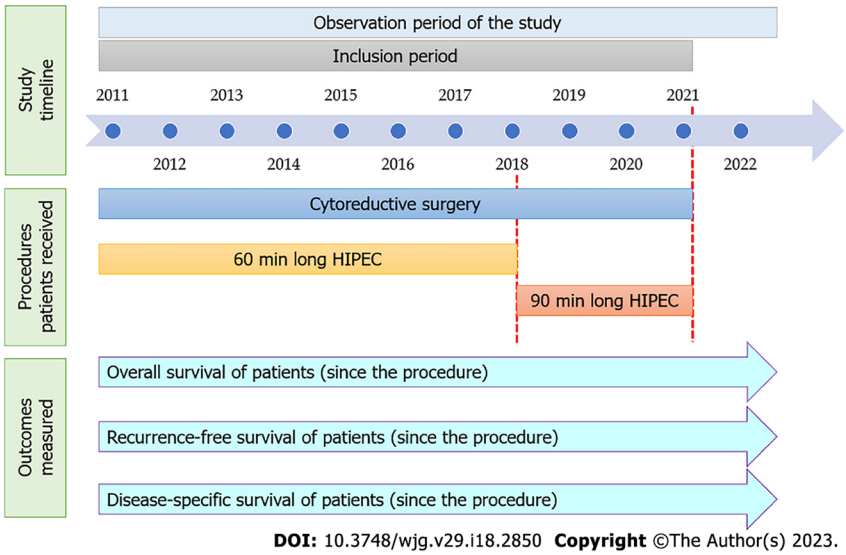
Each of the 73 cases was discussed by a multidisciplinary board of experts (oncologists, surgeons and anesthesiologists) before any treatment decision was made. Preoperatively, the extent of peritoneal dissemination was assessed using abdominal and chest computed tomography (CT) scans. The peritoneal carcinomatosis index (PCI)[21] was calculated based on diagnostic laparoscopy performed on tumors of T3 stage or higher or CT evidence of peritoneal carcinomatosis[22]. Prior to surgery, all patients were preconditioned as per the enhanced recovery after surgery (ERAS) protocol. During CRS, the completeness of cytoreduction (CC) was scored as proposed by Jacquet and Sugarbaker[21]: No residual disease, residual nodules measuring less than 2.5 mm, between 2.5 mm and 2.5 cm, or greater than 2.5 cm were defined as CC-0, CC-1, CC-2, and CC-3, respectively.

Closed HIPEC with a goal temperature of 42 °C with bidirectional HIPEC with cisplatin (75 mg/m^2) and doxorubicin (15 mg/m^2) was administered immediately after CRS for 60 min or 90 min duration (Figure 1). The duration of HIPEC was changed from 60 min to 90 min in 2018 based on the findings of van Driel's study[23]. The cytotoxic agents were added to a 3000 mL-4000 mL isotonic saline solution with a mean flow rate of 1400 mL/min-1800 mL/min. During the treatment, temperature probes for monitoring the 42 °C goal temperature were placed in the right subphrenic and pelvic areas.

Clinicopathological and laboratory data measurements

Clinicopathological and laboratory data were obtained from the DGAV HIPEC registry and the electronic medical system of Hospital Barmherzige Brüder, Regensburg, Germany. The staging of the tumors was unified using the 8th American Joint Committee on Cancer (AJCC) TNM system[24]. Histopathology types of the tumors were categorized as diffuse type adenocarcinoma (ACD), intestinal type adenocarcinoma (ACI), or signet-ring cell adenocarcinoma (SRC)[3]. Neoadjuvant chemotherapeutic treatment of patients was recorded as the latest lineage the patient received prior to CRS + HIPEC. Except for a single patient, all study participants were treated with at least docetaxel-based first-line chemotherapy (FLOT protocol: 5-fluorouracil, leucovorin, oxaliplatin, and docetaxel; or DCF protocol: docetaxel, cisplatin, and 5-fluorouracil). Chemotherapy was administered in accordance with the German guidelines on GC; when recommendation changed from EFC/ECX (Epirubicin, Cisplatin, Fluorouracil/Epirubicin, Cisplatin, Capecitabine) to FLOT after Al-Batran's FLOT-4 study in 2019[25], chemotherapy was accordingly changed. The additional use of trastuzumab (trade name: Herceptin) was also recorded.

Complete blood count, liver enzyme, lipase, creatinine, and tumor marker blood tests were performed at the Department of Laboratory Medicine, Microbiology, and Hospital Hygiene, Hospital Barmherzige Brüder, Regensburg, Germany. The Chronic Kidney Disease Epidemiology Collaboration equations were used to calculate estimated glomerular filtration rate[26]. The Clavien–Dindo Classification[27] was used to assess postoperative adverse events. Although some recent publications have



DOI: 10.3748/wjg.v29.i18.2850

Figure 1 Timeline of the study. HIPEC: Hyperthermic intraperitoneal chemotherapy.

suggested including all patient deaths within 90 d as post-procedure deaths[28,29], HIPEC-related post-procedure deaths were defined as follows: (1) Those occurring during the observation period at the intensive care unit or at the surgical inpatient unit prior to discharge; or (2) Between discharge and adjuvant chemotherapy. If a patient had started adjuvant chemotherapy, their death was defined as GC-related. Recurrence-free (RFS), disease-specific (DSS), and overall survival (OS) were calculated from the date of surgery (CRS + HIPEC) to the date of tumor recurrence, cancer-related death, or death from any cause, respectively. The follow-up of patients was terminated on 30 September 2022 and the patients alive at this time point were right censored (Figure 1).

Statistical analysis

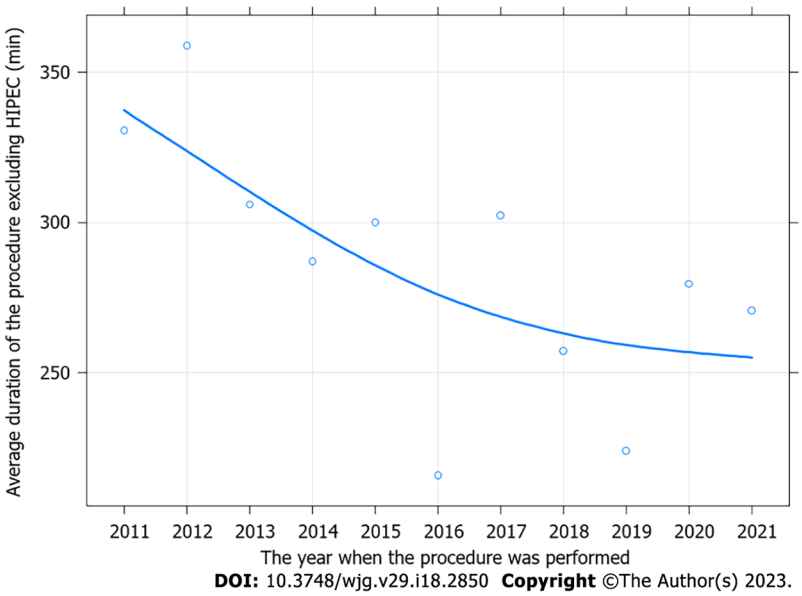
Statistical analyses were performed within the R for Windows version 4.2.1 environment (R Foundation for Statistical Computing, 2022, Vienna, Austria). Wilcoxon rank sum test and Fisher's exact test were used for group comparisons. Linear models were used to investigate whether there was an improvement in the duration of the procedure (learning curve). Matching of patient pairs was done *via* propensity score matching (R-package "Matching" version 4.10-8). DSS, OS, and RFS were determined using the cause-specific competing risk Cox survival model (R packages "survival" version 3.4-0 and "survminer" version 0.4.9). Parameter selection for multivariate survival models was not based on univariate P value, but on literature data and the medical/clinical importance of the given parameter. $P < 0.05$ was considered statistically significant, and P values were corrected with the Holm method[30] for the multiple-comparisons problem. Continuous, survival, and count data were expressed as the mean ± standard deviation (SD), the hazard ratio (HR) with a 95% confidence interval (95%CI), and the number of observations (percentage), respectively.

RESULTS

A total of 73 GC patients with PM were included in the study. Sixty-four cancer-related death events, 13 tumor recurrence events, and 1 death due to postoperative complications occurred. The complete list of pre-, peri- and postoperative clinicopathological characteristics of study participants are listed in Supplementary Table 1. In general, the average operating times (excluding the time for HIPEC) improved significantly over observation period ($P = 0.0097$; Figure 2).

First, it was investigated whether any of the CRS + HIPEC-related or clinicopathological features had a significant effect on patient survival. The need to remove any further organs, such as the removal of the bladder or the appendix during CRS ($n = 9$), was associated with a negative effect on DSS (HR: 2.0538; 95%CI: 1.2715-3.3179; $P = 0.0033$). Those patients who received additional trastuzumab treatment during neoadjuvant chemotherapy ($n = 5$) before the CRS + HIPEC procedure had better DSS (HR: 0.4446; 95%CI: 0.1989-0.9937; $P = 0.0482$). A trend towards longer RFS was found in patients who did not require pelvic peritonectomy (HR: 0.3382; 95%CI: 0.1099-1.0410; $P = 0.0588$). OS was significantly better in patients without pelvic peritonectomy (HR: 0.5459; 95%CI: 0.3152-0.9454; $P = 0.0307$).

Longer HIPEC duration (60 min *vs* 90 min) was associated with more advantageous median survival times: 12.86 mo (95%CI: 11.01-17.31) for the 60 min and 27.30 mo (95%CI: 16.20-NA) for the 90 min cohorts (Supplementary Table 1). However, despite the clinically different median survival times, the



DOI: 10.3748/wjg.v29.i18.2850

Figure 2 Improvement of the operating times of cytoreductive surgery (excluding hyperthermic intraperitoneal chemotherapy) in our surgical center during the study period. HIPEC: Hyperthermic intraperitoneal chemotherapy.

survival of the groups did not differ based on the results of the statistical models with respect to DSS (HR: 0.6239; 95%CI: 0.3413-1.1410; P = 0.1250; Figure 3), OS (HR: 0.6134; 95%CI: 0.3007-1.2510; P = 0.1790), or RFS (P = 0.9650). Furthermore, the type of histology (ACD *vs* ACI *vs* SRC) did not affect DSS (P = 0.4096; Supplementary Figure 1),OS (P = 0.2422), or RFS (P = 0.2799). However, the RFS survival curves of the different histology types seemed to be visually different (Figure 4).

The effect of pre-HIPEC laboratory results on patient survival was also investigated. Higher white blood cell counts (HR: 1.1319; 95%CI: 1.0037-1.2770; P = 0.0433) and carcinoembryonic antigen (CEA) levels (HR: 1.1490; 95%CI: 1.0422-1.2667; P = 0.0053) were associated with an increased risk for shorter DSS. In contrast, higher hemoglobin (HR: 0.7897; 95%CI: 0.6562-0.9505; P = 0.0125) and serum total protein (HR: 0.6795; 95%CI: 0.4330-1.0660; P = 0.0928) levels were associated with a significant and marginally decreased risk for shorter survival, respectively. The same results were found for OS (white blood cell count: P = 0.0945; CEA: P = 0.0052; hemoglobin: P = 0.0087; serum total protein: P = 0.0368), while shorter RFS times were observed in patients with higher RDW levels (HR: 1.2190; 95%CI: 1.0030-1.4810; P = 0.0466). Moreover, similar to that observed with respect to OS and DSS, marginally advantageous RFS was justified for higher serum total protein levels (P = 0.0875).

The effect of clinicopathological and laboratory data on survival was also further investigated in a multivariate setting (Table 1). DSS was marginally affected by the duration of HIPEC [60 min (ref.) *vs* 90 min: HR: 0.5252; 95%CI: 0.2565-1.0750; P = 0.0781] and by PCI (HR: 1.0630; 95%CI: 0.9982-1.1310; P = 0.0569), and significantly by preoperative serum CEA levels (HR: 1.2220; 95%CI: 1.0880-1.3720; P = 0.0007). Similar trends were observed for OS, while worse RFS was more likely associated with lower preoperative white blood cell count (HR: 0.4616; 95%CI: 0.2270-0.9385; P = 0.0327), lower T stage (HR: 13.1182; 95%CI: 1.0285-167.3080; P = 0.0475), and higher N stage (HR: 5.6893; 95%CI: 0.7616-42.4972; P = 0.0902).

Comparison of the 60 and 90-min-long HIPEC patient groups

Further comparison was performed by creating 2 groups according to the duration of HIPEC. Fifty-nine and 14 study participants were enrolled in the 60 min and 90 min groups, respectively. Except for the above-described median survival differences (12.86 mo *vs* 27.30 mo; Figure 3), no difference was found in any clinicopathological characteristic between the two groups after P value adjustment (Supplementary Table 1).

By investigating the results without P value adjustment, several observations were made. The length of CRS trended toward being shorter in the 90 min group (299 ± 76 min *vs* 264 ± 82 min; crude P = 0.0718). Peritonectomy of the omental bursa was more frequently performed in the 60-min group (30.5% *vs* 0%; crude P = 0.0157), while lesser omentectomy was more common in the 90-min group (33.9% *vs* 71.4%; crude P = 0.0153). Fresh frozen plasma (FFP) transfusion was needed only once in the 90 min group, while in the 60-min group, FFP was administered in 32 patients (7.1% *vs* 54.2%; crude P = 0.0009). On average, the length of hospital stay was shorter in the 90 min group (crude P = 0.0134); a more detailed examination of the data revealed that hospitalization longer than 20 d was more common in the 60 min group (39.0% *vs* 7.1%; crude P = 0.0276). Moreover, abnormal serum levels of gamma-glutamyl transferase (crude P = 0.0407, Figure 5A) and serum total protein (crude P = 0.0570, Figure 5B)

Table 1 *P* values of the multivariate survival model

Parameter	DSS	OS	RFS
HIPEC duration [60 min (ref.) *vs* 90 min]	0.0781	0.1541	0.5578
Age (yr)	0.1870	0.1331	0.3691
Sex [male *vs* female (ref.)]	0.3327	0.2943	0.8681
Stage T [1-2 (ref.) *vs* 3-4]	0.1205	0.1857	0.0475
Stage N [0 (ref.) *vs* 1-3]	0.5071	0.4511	0.0902
Histology			
ACD (ref.) *vs* ACI	0.3092	0.2335	0.2471
ACD (ref.) *vs* SRC	0.9456	0.8638	0.2227
Body-mass index (kg/m^2)	0.6394	0.8365	0.3049
Peritoneal carcinomatosis index	0.0569	0.2530	0.2752
White blood cell count (10^9/L)	0.1843	0.2387	0.0327
Hemoglobin (g/dL)	0.2783	0.2924	0.7656
Carcinoembryonic antigen (ng/mL)	0.0007	0.0005	0.1089

ACD: Diffuse type adenocarcinoma; ACI: Intestinal type adenocarcinoma; DSS: Disease-specific survival; ref.: Reference category; RFS: Recurrence-free survival; OS: Overall survival; SRC: Signet-ring cell adenocarcinoma.

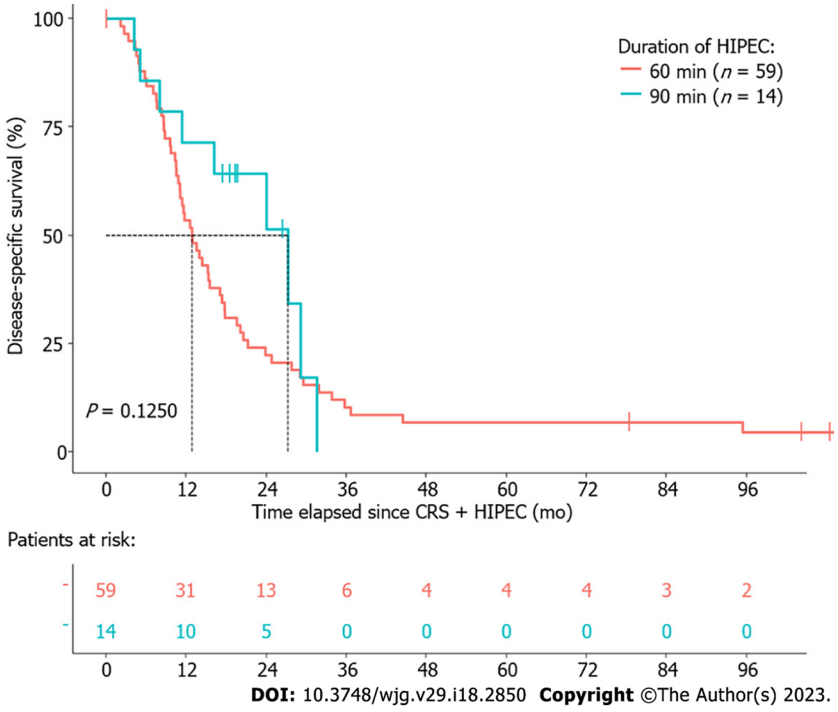


Figure 3 Differences in disease-specific survival between patients with gastric cancer who underwent cytoreductive surgery and 60 min or 90 min hyperthermic intraperitoneal chemotherapy. The dotted line represents median survival. CRS: Cytoreductive surgery; HIPEC: Hyperthermic intraperitoneal chemotherapy.

levels were observed more often in the 60-min group (Supplementary Table 1).

To further investigate the cause of the clinically significant difference in median survival, the following adjustments to the groups were performed with consideration of any possible confounding effects. First, propensity score-matched patient pairs (n = 14) were created in which patients were matched by age, sex, PCI score, CC score, time spent in the intensive care unit after CRS + HIPEC, duration of CRS, and the presence of lymph node metastasis (stage N = 0 *vs* stage $N \geq 1$). No differences in adjusted or in crude P values were found in any of the preoperative, perioperative, and postoperative

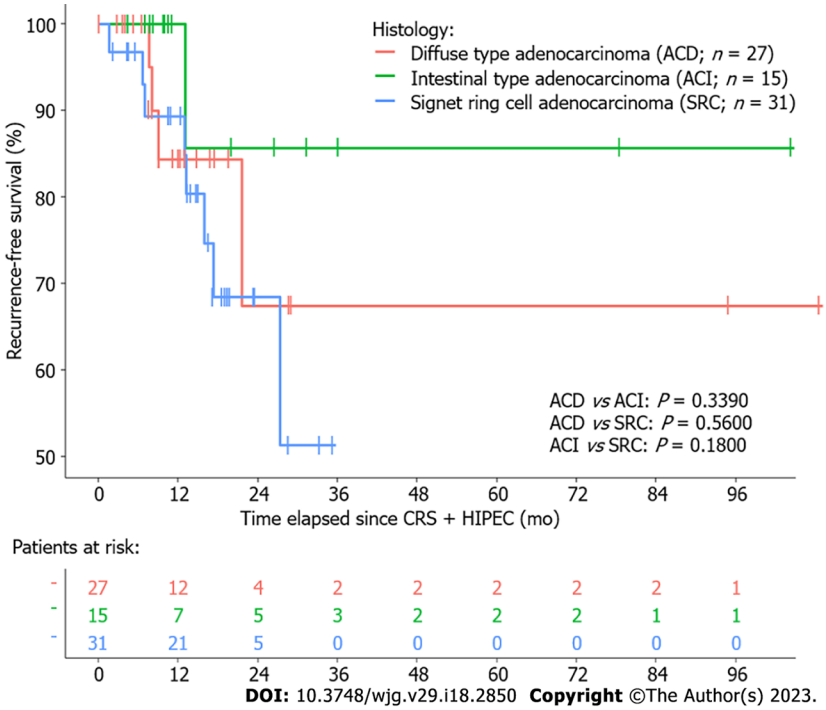


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Figure 4 Differences in recurrence-free survival between patients with gastric cancer of different histological types who underwent cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. ACD: Diffuse type adenocarcinoma; ACI: Intestinal type adenocarcinoma; CRS: Cytoreductive surgery; HIPEC: Hyperthermic intraperitoneal chemotherapy; SRC: Signet-ring cell adenocarcinoma.

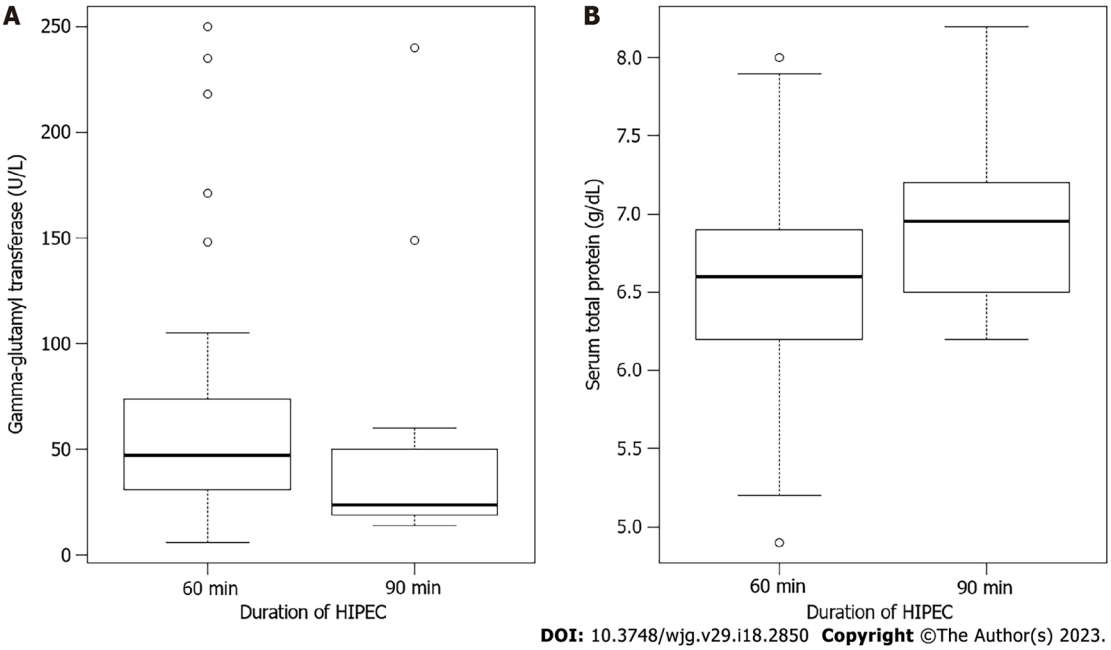

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Figure 5 Gamma-glutamyl transferase and total protein levels in 60 min and 90 min hyperthermic intraperitoneal chemotherapy groups. Clinically abnormal serum levels of gamma-glutamyl transferase (crude $P = 0.0407$) and total protein (crude $P = 0.0570$) were observed more often in those gastric cancer patients who received hyperthermic intraperitoneal chemotherapy (HIPEC) for 60 min after the cytoreductive surgery. Thick lines and hollow circles represent the median and outliers, respectively. A: Gamma-glutamyl transferase; B: Total protein.

parameters between propensity score-matched groups. However, the seemingly different survival between the 2 groups became statistically significant [60 min (ref.) *vs* 90 min: HR = 0.2843; 95%CI: 0.1119-0.7222; $P = 0.0082$; Figure 6] with 10.91 mo (95%CI: 9.56-17.77) and 27.30 mo (95%CI: 16.20-NA) median survivals for the 60 min and 90 min groups, respectively.

We also investigated whether results changed if patients who received trastuzumab and/or had immunohistochemically positive pathological results against human epidermal growth factor receptor 2

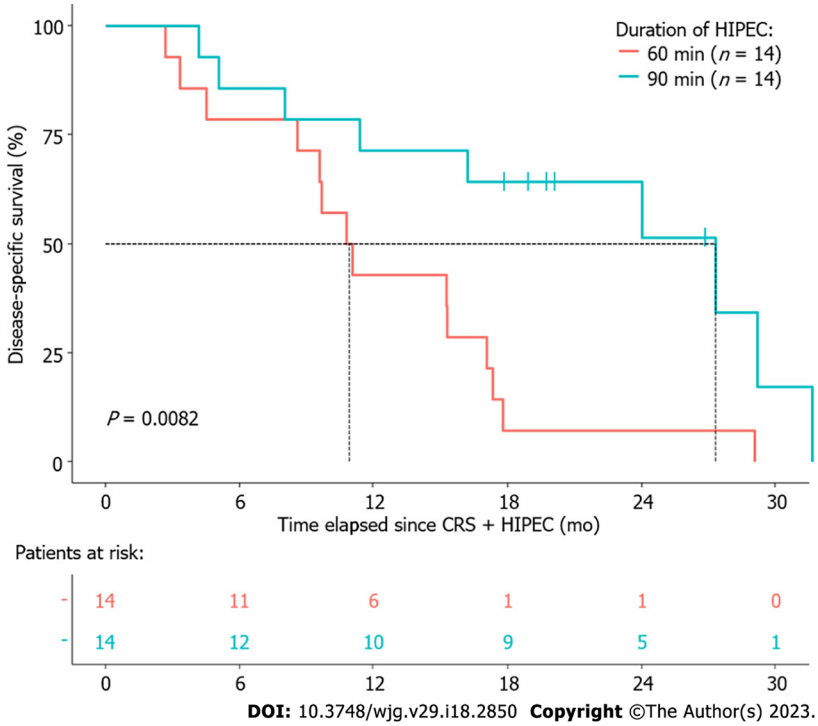

Figure 6 Differences in disease-specific survival between propensity score in matched gastric cancer patient-pairs. Patients were matched by age, sex, peritoneal carcinomatosis index score, Jacquet and Sugarbaker's completeness of cytoreduction score, time spent in the intensive care unit after cytoreductive surgery (CRS) + hyperthermic intraperitoneal chemotherapy (HIPEC), duration of CRS, and presence of lymph node metastasis (stage *N* = 0 *vs* stage *N* ≥ 1). The dotted line represents median survival.

(HER2; *n* = 7) or had the procedure before 2016 (*n* = 44) were removed from the original cohort. For the former, we obtained the same results as those for the full cohort. Median survivals of 12.68 mo and 24.02 mo were observed for the 60-min and 90-min groups, respectively, and no statistical difference was detected in the survival models (DSS: *P* = 0.1540; OS: *P* = 0.2040; Supplementary Figure 2A). However, the same difference was seen for the modified patient population that seen with propensity-matched pairs. Median survivals of 12.52 mo and 27.30 mo were found for the 60 min and 90 min groups, respectively (HR: 0.4225; 95%CI: 0.1789-0.9975; *P* = 0.0493; Supplementary Figure 2B).

DISCUSSION

There are only a few Western studies concerning the treatment of advanced GC with CRS and HIPEC. Although the positive effects of cytoreduction and HIPEC on survival have been described[11-13,31], the practical nonexistence of prospective clinical studies (except for two studies with small sample sizes [12,32]) on CRS and HIPEC highlights the need for additional primary research. Moreover, more randomized trials are required to substantiate the effect of CRS and HIPEC. For example, the results of the German phase III PREVENT study, in which the effect of HIPEC applied for prevention in lieu of FLOT-chemotherapy, is currently recruiting patients, and results are eagerly anticipated[33].

In the current retrospective study, we demonstrated prolonged survival with multimodal therapy in the treatment of primary GC patients with PM. The 27.3 mo median survival that we observed is in line with similar studies. For example, in the phase II trial by Badgwell *et al*[11], the median OS was 24.2 mo from the date of diagnosis and 16.1 mo from the date of CRS and HIPEC. Similarly, a recent Spanish multicenter study found a median survival of 21.2 mo[34], while in the German retrospective HIPEC-register study the median survival times ranged from 7.9 mo to 21.2 mo[35]. The same is true of median PCI-scores; median PCI was 2, 6, 6, and 8 in the studies of Badgwell *et al*[11], Bonnot *et al*[31], Manzanedo *et al*[34], and Rau *et al*[35], respectively; a median PCI of 3 was calculated in the current study. In addition, Rau *et al*[35] reported OS of 18 mo, 12 mo, and 5 mo for the 3 patient groups, with corresponding PCI scores of 0-6, 7-15, and 16-39, respectively; this finding suggests that significantly better outcomes are associated with higher CC. In our study, 93.2% of patients underwent complete macroscopic tumor reduction. An important conclusion of the above presented studies is that patients with small tumor burden (PCI < 6, but maximally 9) benefit the most from this multimodal therapy. Although in the current study we could not confirm the benefit of reduced PCI scores, our results were in line with the previously described observations (*i.e.* patients with higher PCI scores trended toward shorter survival). Furthermore, an interesting observation emerged during the analysis of our data over

time and with an increasing number of cases: The duration of surgery to reach complete cytoreduction became significantly shorter. These findings match with the results of a study outlining the technical aspects and learning curve of CRS/HIPEC by Vining *et al*[36], where the authors describe a steep learning curve and a correlation between CC and surgeon expertise. This observation underscores the idea that treatment of advanced GC with PM should be performed in specialized centers by expert surgeons. Recent studies have also found that sodium thiosulfate can prevent impairment of renal function following HIPEC[37,38]. In the patient population analyzed in the current study, sodium thiosulfate was not used; however, since January 2022, we have started to use it routinely in our center.

There is still no consensus regarding the ideal duration of HIPEC. In the current analysis, the median survival time was 27.30 mo in the 90 min group, which was significantly longer than that of the 60 min group (12.86 mo). Near the publication date of the van Driel *et al*[23] study for ovarian cancer and the PRODIGE-7 trial[39] for HIPEC in colorectal cancer, our institutional HIPEC protocol was changed in favor of the 90 min HIPEC perfusion. Our group has recently described the advantages of prolonged HIPEC duration have been recently described for primary peritoneal carcinoma, primary advanced epithelial carcinoma, and ovarian or fallopian tube carcinoma[40,41]. Longer HIPEC duration does not adversely affect perioperative morbidity and mortality, and a potential survival benefit could be realized by the application of prolonged HIPEC[40]. However, a recent study found that a secondary inflammatory reaction might occur after 90 min HIPEC with mitomycin C/doxorubicin or cisplatin, but not with shorter duration and oxaliplatin[41]. These and the current findings suggest that a prolonged peritoneal perfusion time may be more advantageous after complete cytoreduction; however, as the study of Roth *et al*[41] has shown, gathering additional data is essential.

Another possible reason for better survival in patients with longer HIPEC duration is enhanced cytotoxicity and anti-tumor effects of chemotherapeutic drugs in hyperthermia; the longer exposure may allow for more effective drug action[42]. The effect of cytoreductive surgery with macroscopic complete tumor reduction followed by HIPEC with effective neoadjuvant chemotherapy extends survival time of patients with advanced GC with PM, as recently shown in the CYTO-CHIP study[31]. Since 2016, the most frequently used neoadjuvant chemotherapy for advanced GC with PM is the FLOT-protocol; however, due to differences in cytochrome P450 family 2 subfamily A member 6[43], the S-1 regime (tegafur, gimeracil, and oteracil) is the standard adjuvant treatment in Asia[44,45]. The latest advancements in preoperative chemotherapy with[46] or without[25] biological agents can significantly extend the survival of GC patients. Recently, it has also been demonstrated that the 15%-20% of GC cases that overexpresses HER2 should be treated with monoclonal antibodies like trastuzumab in a neoadjuvant setting due to the positive influence of these drugs on patient survival and fewer side effects than traditional chemotherapies[47]. In the current study, the individual responses to pre- and/or postoperative chemotherapy were not known for most patients, which was one of the biasing factors affecting patient survival in our study.

SRC differentiation is described as a tumor with aggressive growth and a poorer prognosis than non-SRC carcinomas of the stomach[48]. In contrast, we found that the type of histology did not affect DSS, OS, or RFS. A similar finding was reported in an Asian study of 136 advanced GC patients, in which the authors described no difference in median survival between the histopathologic entities after R0-resection[49]. Moreover, we observed that if pelvic peritonectomy during CRS is not necessary, the OS of the patient improved. We hypothesize that the extent of the tumor may have a greater influence on patient survival than the histopathological differentiation. Improvement in patient survival may also be influenced by the experience of the surgical team; this factor may have also introduced additional bias in the current study.

We also investigated whether any preoperative laboratory result was predictive of patient survival. Strong correlations were found between patient survival and white blood cell count, hemoglobin, CEA, and serum total protein. These findings match with previously reported data of non-HIPEC-treated GC patients[50-55]. Furthermore, results of a recent German multi-center study[56] and the WHO urgent call[57] to implement blood management in surgical patients have shown that preoperative anemia is a serious threat to patient survival. Preoperative iron supplementation in preoperative anemia is also an important part of the recently published ERAS protocol for CRS and HIPEC[58]. As such, emphasis should be placed on iron supplementation and normalization of hemoglobin prior to surgery[58].

Limitations

The current study had a few limitations, including the small sample size, the retrospective nature of the study, the fact that data were available from a single center only, and the heterogeneity of the data. Also, during the study period, preoperative chemotherapy protocols changed and surgeon expertise grew. Furthermore, in this small cohort of patients with GC and PM, there were limited data regarding post-HIPEC treatment. Our follow-up data could only differentiate between alive and dead patients and tumor recurrence or no recurrence. Efforts were made to collect post-HIPEC patient data; however, we could not collect these in a timely manner, as routine oncological treatments were often performed in other hospitals. Moreover, the lack of chemotherapy-only control patients should also be mentioned as a limiting factor.

CONCLUSION

In summary, we conducted a single-center retrospective observational study to investigate what factors influence the survival of advanced GC patients with PM who underwent CRS and HIPEC. We confirmed that CRS followed by HIPEC applied over 90 min has a positive impact on DSS in comparison with CRS followed by 60 min of HIPEC. Of note, the learning curve of surgeons may confound the interpretation of this observation. Furthermore, the preparation of patients for surgery based on preoperative laboratory testing according to the current ERAS protocol might optimize the positive effect of CRS and HIPEC. To further expand upon our findings, multi-institutional and cooperative randomized group trials should be organized to further support and confirm survival and safety outcomes.

ARTICLE HIGHLIGHTS

Research background

Cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) is one of the last options in the treatment of advanced gastric cancer (GC) with peritoneal metastasis (PM); however, most national and international guidelines only recommend it to be performed within clinical trials. Despite this, CRS with HIPEC is a safe and effective method to treat advanced GC with PM, and recent studies have shown encouraging results with respect to increased patient survival.

Research motivation

CRS and HIPEC are safe and effective therapeutic options for the treatment of advanced GC with PM. To investigate the optimal length of HIPEC procedure, it is important to provide a basis for further research. Improving the composition of HIPEC medications could further improve the outcomes of this modern multimodal therapy. It is expected that ongoing research regarding antibody and checkpoint inhibitor therapies will strongly influence not only perioperative therapy but also the therapeutic agents used during HIPEC itself.

Research objectives

The aim of the study was to explore the effect of CRS and HIPEC in the treatment of advanced GC with PM and find parameters that could further improve patient survival.

Research methods

We conducted a retrospective observational study with the inclusion of 73 GC patients with synchronous PM. Details of CRS + HIPEC, preoperative laboratory results, and pre-, peri-, and postoperative surgical details of the patients were recorded. Overall survival (OS), disease-specific survival (DSS), and recurrence-free survival (RFS) were calculated.

Research results

In line with recently published data, we found that CRS + HIPEC had a measurable impact on the survival of advanced GC patients without significantly elevating the rate of postoperative complications. The effects of longer HIPEC duration, higher white blood cell count, lower hemoglobin and serum total protein, and higher carcinoembryonic antigen levels with respect to the survival of patients were found.

Research conclusions

In general agreement with previously published findings, we concluded that 90 min HIPEC treatment correlates with improvement in the OS and DSS of patients compared to that of 60 min HIPEC. Moreover, more complete cytoreduction also contributes to longer patient survival and better disease management.

Research perspectives

The improvement of CRS and HIPEC with respect to the duration and composition of HIPEC therapeutic agents is a controversial research topic. The current report provides evidence from a single center retrospective study that could be implemented in future randomized multicenter studies.

ACKNOWLEDGEMENTS

We are grateful to Madar-Dank V, research assistant of the Department of the Institute for Dispute Resolution of New Jersey City University, for English proofreading.

FOOTNOTES

Author contributions: Steinhoff H, Acs M, Szasz AM and Piso P built the study design; Steinhoff H, Acs M, Blaj S and Sanchez-Velazquez P were involved in the collection of patient data; Herold Z performed the statistical analysis of data; Steinhoff H, Acs M, Herold Z, Herold M, Herzberg J, Strate T and Szasz AM interpreted the data; Steinhoff H, Blaj S, Acs M, Sanchez-Velazquez P and Piso P were involved in patient selection; Steinhoff H, Acs M and Herold Z prepared the draft of the manuscript; All authors were involved in the manuscript editing and reviewing; Dank M and Piso P supervised the study; all authors have read and agreed to the published version of the manuscript.

Institutional review board statement: The study was conducted according to the guidelines of the Declaration of Helsinki. Ethical review and approval were waived for this study due to the retrospective nature of the study. All the patients had agreed to data recording for the national HIPEC registry and to the use of their anonymized data for quality assurance and research purposes by written and verbal informed consent prior to surgery. Therefore, no institutional or further approval of a review board was necessary.

Informed consent statement: Informed consent was obtained from all subjects before cytoreductive surgery with hyperthermic intraperitoneal chemotherapy (CRS + HIPEC). All patients agreed to their data being recorded for the national HIPEC registry, administered by the German Society for General and Visceral Surgery.

Conflict-of-interest statement: All the authors report having no relevant conflicts of interest for this article.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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S-Editor: Gao CC
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World Journal of Gastroenterology

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World J Gastroenterol 2023 May 14; 29(18): 2864-2874

DOI: 10.3748/wjg.v29.i18.2864

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Endoscopic ultrasound-guided fine-needle aspiration pancreatic adenocarcinoma samples yield adequate DNA for next-generation sequencing: A cohort analysis

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Li JJ, China; Martino A, Italy; Wang D, China

Received: December 31, 2022
Peer-review started: December 31, 2022
First decision: February 28, 2023
Revised: March 14, 2023
Accepted: April 18, 2023
Article in press: April 18, 2023
Published online: May 14, 2023

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Abstract

BACKGROUND

Genetic tests are increasingly performed for the management of unresectable pancreatic cancer. For genotyping aimed samples current guidelines recommend using core specimens, although based on moderate quality evidence. However, in clinical practice among the endoscopic ultrasound (EUS) guided tissue acquisition methods, fine needle aspiration (FNA) is the most widely performed.

AIM

To assess the adequacy for next generation sequencing (NGS) of the DNA yielded from EUS-FNA pancreatic adenocarcinoma (PDAC) samples.

METHODS

Between November 2018 and December 2021, 105 patients with PDAC confirmed by EUS-FNA were included in the study at our tertiary gastroenterology center. Either 22 gauge (G) or 19G FNA needles were used. One pass was dedicated to

DNA extraction. DNA concentration and purity (A260/280, A260/230) were assessed by spectrophotometry. We assessed the differences in DNA parameters according to needle size and tumor characteristics (size, location) and the adequacy of the extracted DNA for NGS (defined as A260/280 ≥ 1.7, and DNA yield: ≥ 10 ng for amplicon based NGS, ≥ 50 ng for whole exome sequencing [WES], ≥ 100 ng for whole genome sequencing [WGS]) by analysis of variance and *t*-test respectively. Moreover, we compared DNA purity parameters across the different DNA yield categories.

RESULTS

Our cohort included 49% male patients, aged 67.02 ± 8.38 years. The 22G needle was used in 71% of the cases. The DNA parameters across our samples varied as follows: DNA yield: 1289 ng (inter quartile range: 534.75-3101), A260/280 = 1.85 (1.79-1.86), A260/230 = 2.2 (1.72-2.36). DNA yield was > 10 ng in all samples and > 100 ng in 93% of them (one sample < 50 ng). There were no significant differences in the concentration and A260/280 between samples by needle size. Needle size was the only independent predictor of A260/230 which was higher in the 22G samples (P = 0.038). NGS adequacy rate was 90% for 19G samples regardless of NGS type, and for 22G samples it reached 89% for WGS adequacy and 91% for WES and amplicon based NGS. Samples with DNA yield > 100 ng had significantly higher A260/280 (1.89 ± 0.32 *vs* 1.34 ± 0.42, P = 0.013). Tumor characteristics were not corelated with the DNA parameters.

CONCLUSION

EUS-FNA PDAC samples yield DNA adequate for subsequent NGS. DNA amount was similar between 22G and 19G FNA needles. DNA purity parameters may vary indirectly with needle size.

Key Words: Pancreatic adenocarcinoma; Endoscopic ultrasound guided fine needle aspiration; Next generation sequencing; DNA yield; Needle size; Genetic testing

Core Tip: Genetic testing is increasingly undertaken in pancreatic adenocarcinoma (PDAC). The main diagnostic method is endoscopic ultrasound (EUS)-guided tissue acquisition and fine needle aspiration (FNA) is most frequently performed in clinical practice. However, current guidelines recommend using core specimens for genotyping aimed samples. In our cohort analysis, we show that EUS-FNA PDAC samples yield DNA of adequate amount and purity for subsequent next-generation sequencing (NGS). DNA amount was similar between 22G and 19G FNA needles. DNA purity parameters may vary indirectly with needle size. EUS FNA PDAC samples may be used for NGS.

Citation: Bunduc S, Varzaru B, Iacob RA, Sorop A, Manea I, Spiridon A, Chelaru R, Croitoru AE, Becheanu G, Dumbrava M, Dima S, Popescu I, Gheorghe C. Endoscopic ultrasound-guided fine-needle aspiration pancreatic adenocarcinoma samples yield adequate DNA for next-generation sequencing: A cohort analysis. *World J Gastroenterol* 2023; 29(18): 2864-2874

URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2864.htm

DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2864

INTRODUCTION

Pancreatic cancer (PC) is among the most the most aggressive malignancies with a mortality rate comparable to the incidence[1,2]. In more than 80% of cases it is detected in advanced stages that require systemic treatment[3]. Moreover, there is growing evidence about the benefits of neoadjuvant treatment over upfront surgery for the management of resectable pancreatic cancer[4]. Regardless of stage, anatomopathological confirmation of the disease is mandatory prior to chemo or radiotherapy initiation [5].

Endoscopic ultrasound (EUS) guided tissue acquisition (TA) is the main sampling method for the diagnosis of pancreatic solid lesions with high sensitivity (85%-89%) and specificity (96%-99%)[6]. Among the EUS-TA methods, fine needle aspiration (FNA) is the most widely performed[5].

Despite the high diagnostic accuracy, EUS-TA samples are still associated with an inconclusive diagnosis in 10% to 15% of cases[7]. Detection for KRAS mutations in inconclusive samples could decrease with up to 50% the rates of false negative results[6].

Precision medicine has changed the paradigm in oncologic treatment and although the targeted therapies are not yet routinely used in PC, it is known that up to 25% of the tumors harbor actionable alterations[8]. Molecular testing on tumor tissue for guiding personalized treatment is recommended in the current guidelines for the management of unresectable pancreatic adenocarcinoma (PDAC) amenable for anti-cancer therapy[9].

The roles of EUS-TA in PC have therefore broadened from tumor diagnosis to obtaining adequate samples for further molecular testing that enable the use of targeted therapies and may also increase diagnostic accuracy[6,9,10]. Although according to the current guidelines in pancreatic solid masses core tissue specimens should be used in downstream applications, there is increasing evidence on the feasibility of performing comprehensive genomic profiling based on FNA samples[5,11].

In our study we evaluated the adequacy for NGS [targeted, amplicon based, whole exome sequencing (WES) and whole genome sequencing (WGS)] of the DNA extracted from fresh EUS FNA PDAC samples, in terms of quantity and purity as measured by spectrophotometry. Moreover, we assessed the influence of needle the size and tumor characteristics on the DNA parameters.

MATERIALS AND METHODS

Study design and settings

The research was conducted in Fundeni Clinical Institute-a tertiary gastroenterology referral center in Bucharest, Romania, between November 2018 and December 2021. This prospective observational study was approved by the Internal Review Board of Fundeni Clinical Institute. Patients provided informed consent for EUS-FNA and study enrolment before the procedure.

Study participants

For enrolment, we assessed the cases with pancreatic lesions referred to our department for EUS-TA. The eligibility criterion was-diagnosis of PDAC established by EUS-FNA. We only considered for inclusion in the study patients with a high suspicion of PDAC (as evaluated by clinical, biological, and imaging criteria). Patients with subsequent diagnoses other than PDAC were excluded from our analysis.

Highly experienced endosonographers performed all the procedures and all patients were under propofol sedation. A linear echoendoscope was used (EG-3870UTK, Pentax, equipped with a Hitachi Arietta v70 processor, Tokyo, Japan) and either 19 gauge (G) or 22G FNA needles (EchoTip Ultra Endoscopic Ultrasound Needle; Cook Medical, Bloomington, IN, United States). The endosonographer decided on the needle type. After obtaining the samples for the pathological diagnosis, one pass was dedicated to sample acquisition for DNA extraction. The needle stylet or air flushing were used to facilitate the specimen extrusion. The samples dedicated to diagnosis were smeared onto slides and fixed with ethanol and/or placed into 10% formalin for further paraffin fixation and cell block analysis, FNA yielded small tissue fragments (about 50% of cases). Dedicated pathologists with extensive experience in cytology techniques assessed the samples on site. The diagnosis of PDAC was confirmed if the pathology findings were positive or suspicious for malignancy[12]. The samples purposed for DNA extraction were placed directly in 1.5 mL of RNA later solution, in 2 mL Eppendorf tubes and kept at room temperature until DNA extraction that was performed within 4 h after sampling. For DNA extraction we used a commercially available silica membrane-based column DNA extraction kit (PureLink™ Genomic DNA, Invitrogen™, Waltham, MA, United States) according to the manufacturer's indications.

The purity and quantity of the extracted DNA were assessed immediately after extraction by spectrophotometry (Nanodrop, Thermo Fisher™, Waltham, MA, United States). The DNA concentration was measured in ng/μL and the purity was assessed by the absorbance ratios-A260/280 and A260/230 (the ratios between absorbances of the samples at 260 nm-which is characteristic for nucleic acids, and 280 nm and 230 nm respectively)[13]. The optimal DNA parameters for second generation high throughput sequencing were considered as follows: For DNA yield ≥ 100 ng will be sufficient for NGS of any type (whole genome-WGS, whole exome-WES or amplicon based targeted NGS), for WES at least ≥ 50 ng are required, whereas for amplicon based targeted NGS a minimum of 10 ng is necessary[14-16]. The main purity parameter assessed in terms of NGS adequacy is A260/280 and values ≥ 1.7 are considered optimal[17]. A260/230 is a secondary purity parameter with optimal values ranging between 2.0-2.2 [17]. Abnormal values of the purity parameters reflect mainly a contaminated sample with proteins, phenols, carbohydrates, salts among others[17]. To calculate the DNA yield we multiplied the measured concentration with the elution volume which was 25 μL in all cases. The DNA samples were stored at -80 °C until further analysis. We have successfully performed targeted amplicon based NGS in 20 of the collected samples, using the Illumina NextSeq500 platform.

Data sources

Two investigators, trainees in gastroenterology, collected the data using a customized form. Among the gathered information, we used in our study: Patients demographics (age, gender), tumor characteristics-

size (mm)-the maximum diameter measured during EUS) and location (coded as head/neck or body/tail respectively), if cross-sectional imaging was performed we also collected-TNM (tumor, node, metastasis) stage according to the American Joint Committee on 8th Cancer edition[18], and the vascular invasion status; information about the procedure: total number of passes, needle size (recorded either during the procedure or retrieved from the EUS registry of the department), the parameters for the extracted DNA-concentration and absorbance ratios. We included in our analysis only samples from patients with confirmed PDAC on the FNA specimens.

Outcomes

Our end-points were as follows: To evaluate the adequacy for NGS of the DNA extracted from EUS-FNA PDAC samples we generated several categorical variables based on the previously mentioned criteria: Three of them defining optimal DNA yield for the main types of NGS with the cut-offs: ≥ 100 ng for WGS, ≥ 50 ng for WES and ≥ 10 ng for amplicon based targeted NGS, and one for optimal DNA purity: A260/280 ≥ 1.7. NGS adequacy was defined as optimal parameters for both DNA yield and purity for each type of NGS (WGS, WES and amplicon based NGS adequacy respectively). The definitions of the categorical variables are detailed in Supplementary Table 1. The variables were coded as 1 if the corresponding criteria were accomplished and 0 if not. We also compared the purity parameters between the predefined categories of optimal DNA yield. Furthermore, we assessed the association between the FNA needle size and tumor characteristics (diameter and location) and the DNA parameters (DNA concentration [ng/μL], A260/280, A260/230).

Statistical analysis

Continuous variables are computed as either mean and standard deviation (mean ± SD) or median and inter quartile range. We analyzed the impact of needle size on DNA parameters by one way analysis of variance. We assessed the association between needle size and NGS adequacy using chi-square test. The impact of both needle size and tumor characteristics on the DNA parameters was evaluated by linear regression, while their impact on DNA NGS adequacy was evaluated by logistic regression respectively. The differences in purity parameters across the predefined DNA yield categories were assessed with independent sample *t*-test. Data was normalized using a two-step approach-transforming the variable into a percentile rank and subsequent inverse-normal transformation[19]. Two-tailed alpha of 0.05 defined statistical significance. Data analysis was performed with SPSS (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. IBM Corp, NY, Armonk, United States). The statistical review of the study was reviewed by Daniel Veres, MD, PhD who is a biomedical statistician at Center for Translational Medicine of Semmelweis University.

RESULTS

Patients characteristics

The characteristics of patients included in our analysis are summarized in Table 1. Out of the 128 patients who accepted to participate in our study, 105 were confirmed with PDAC subsequent to EUS-FNA (Figure 1). Sex distribution was almost equal and the mean age at diagnosis was 67.02 ± 8.38 years. Tumors were more frequently located in the head or neck of the pancreas than in the body or tail, and the tumor diameter was in average 43.66 ± 14.83 mm. More than half of the cases were metastatic at diagnosis. Most of the samples were acquired with 22G FNA needles-75 (71%).

Adequacy of the EUS-FNA PDAC samples for second generation high throughput analysis

In our cohort the DNA yield was in average 1289 ng (inter-quartile range: 534.75-2995). All samples resulted in more than 10 ng of DNA while 98 (93%) of them yielded more than 100 ng of DNA. All 7 samples from which less than 100 ng of DNA were extracted, were acquired with 22G needles, and only one yielded below 50 ng of DNA. In our analysis, needle size was not correlated with DNA NGS adequacy rate regardless of NGS type (Supplementary Table 2). For 19G needles, NGS adequacy rate was 90% for all types of NGS while for the 22 G needles the adequacy rate was 89% for WGS and 91% for both WES and amplicon based NGS. Tumor location and diameter did not significantly influence DNA NGS adequacy regardless of NGS type. When comparing the purity parameters across the predefined DNA yield categories (Table 2), the only significant difference we found was for the A260/280 parameter which was significantly higher in the samples with DNA yield ≥ 100 ng (1.89 ± 0.32 *vs* 1.34 ± 0.42, $P = 0.013$).

Association between FNA needle size and DNA parameters

The DNA parameters across our samples were as follows: median concentration 51.56 ng/μL (21.39-124.04), A260/280 = 1.85 (1.79-1.86), A260/230 = 2.2 (1.72-2.36). The association between FNA needle size and the extracted DNA parameters are summarized in Figure 2. Needle size did not influence the concentration of the extracted DNA nor the A260/280 ratio. The median A260/230 was significantly

Table 1 Characteristics of the enrolled patients

Variable	Amount
Number of PDAC cases	105
Age (yr)	67.02 ± 8.38
Male (number of cases)	51
Tumor diameter (mm)	43.66 ± 14.83
Tumor location (number of cases)	
Head/neck	60
Body/tail	44
Tumor stage (number of cases)[1]	
IA	1
IB	3
IIA	3
IIB	12
III	31
IV	55
Needle size (number of samples)	
22G	75
19G	30

[1]According to American Joint Committee on 8th Cancer edition.
PDAC: Pancreatic adenocarcinoma; FNA: Fine needle aspiration; G: Gauge; NGS: Next generation sequencing. Data is given as: mean ± standard deviation, or median (inter quartile range).

Table 2 Purity parameters across the different DNA yield categories

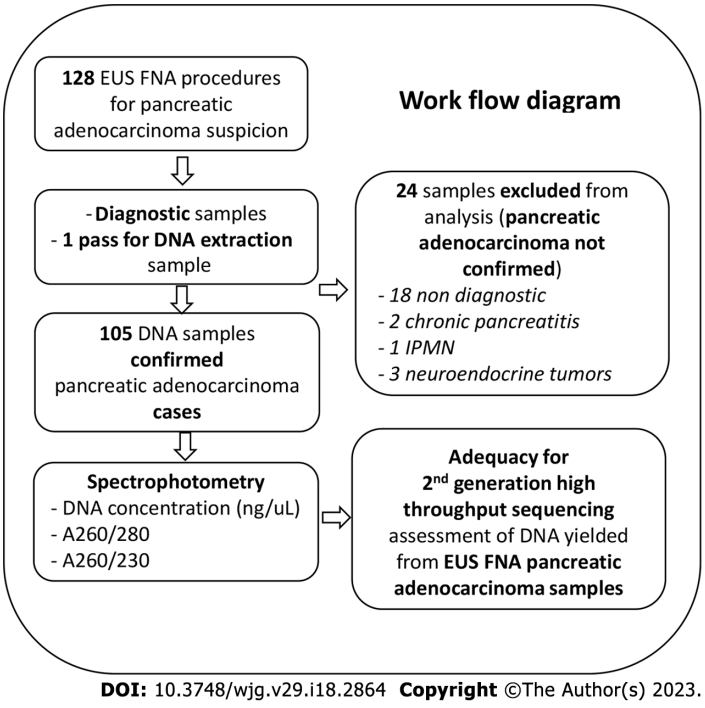
DNA yield	Purity parameters	
	A260/280	A260/230
≥ 100 ng	1.86 (1.80-1.89)	2.23 (1.79-2.35)
≥ 50 ng	1.85 (1.80-1.89)	2.21 (1.7-2.36)
≥ 10 ng	1.85 (1.79-1.86)	2.2 (1.72-2.36)

higher in the 22G samples than in 19G samples (P = 0.038). In multivariate analysis (on needle size, tumor location and tumor diameter) the only independent predictor of A260/230 was needle size (β = 0.36, $t(104)$ = 2.1, P = 0.038). None of the DNA parameters were significantly influenced by the tumor size or location.

DISCUSSION

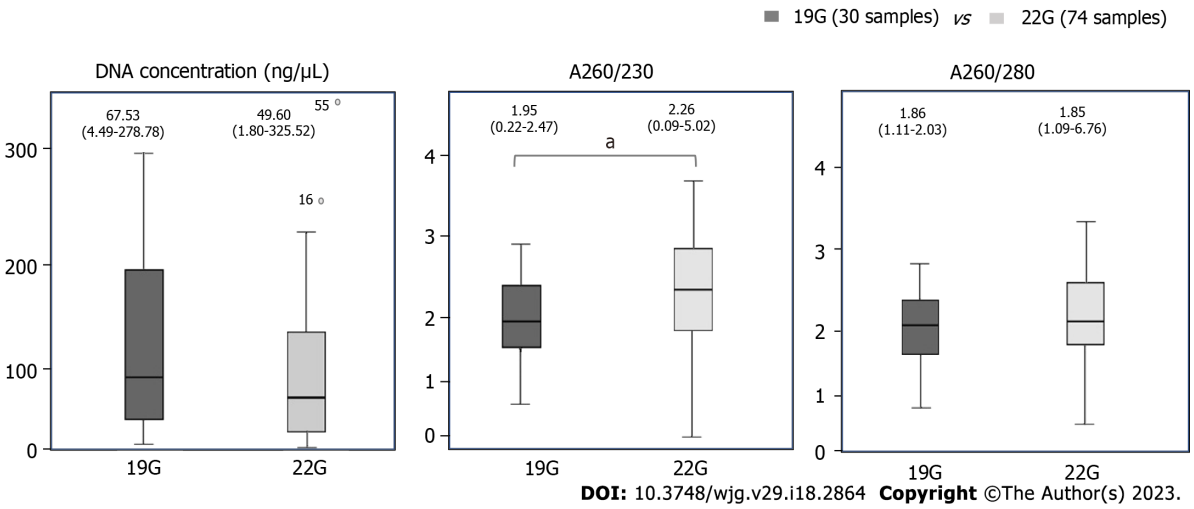
The quantity and quality of the genetic material extracted from tumor samples are essential for their adequacy for downstream genetic analyses performed to further guide the individualized cancer therapy[21]. The requirements for NGS dedicated specimens vary with extraction protocols and testing method, but also with acquisition method, sample manipulation, disease type[22]. Although prior research has demonstrated the suitability of several pancreatic EUS-TA sample types for a range of comprehensive genetic testing techniques, the best sampling protocol for this use is still up for debate [23]. The current guidelines recommend the use of core samples for molecular testing in pancreatic solid lesions based on a moderate level of evidence[6,11].

Our cohort analysis revealed that FNA needle size does not influence the concentration and the primary purity parameter (A260/280) of the DNA extracted from 19G or 22G EUS-FNA PDAC samples. However, needle size was the only independent predictor of A260/230. Tumor diameter and location



DOI: 10.3748/wjg.v29.i18.2864

Figure 1 Patients recruitment and tumor sampling. EUS FNA: Endoscopic ultrasound guided fine needle aspiration.



DOI: 10.3748/wjg.v29.i18.2864

Figure 2 The association between needle size and DNA parameters. G: Gauge. To complement the graphical information, the additional data is given as median (min-max). [a]*P* < 0.005.

did not influence the DNA purity and concentration parameters. Based on the purity and DNA yield thresholds there were no significant differences in NGS adequacy rates between the two FNA needle sizes. NGS adequacy rate was 90% for 19G needle samples regardless of NGS type, while for the 22G needle samples it was 89% for WGS and 91% for both WES and amplicon based NGS. Regarding purity parameters, A260/280 was significantly better in samples with DNA yield above 100 ng. We further successfully performed targeted amplicon based NGS on a subgroup of 20 samples.

Park *et al*[24] evaluated which of the EUS-TA related factors and tumor characteristics have a significant impact on the NGS success rate on a cohort of 190 PDAC confirmed cases. They used for DNA analysis the remaining material after acquiring cytologic or histologic specimens for PDAC diagnosis[24]. As needed, 19G, 22G or 25G FNA or Fine needle biopsy (FNB) needles were used[24]. While tumor location in the body or tail of the pancreas was associated with a higher NGS success rate in their cohort than head or uncinate process tumor location, in multivariate analysis the only independent predictor of NGS success was a greater needle size (19G and 22G *vs* 25G)[24]. Although we were able to evaluate only a surrogate marker of NGS success, respectively-the adequacy of the DNA parameters for high throughput analysis, did we did not find a significant impact of tumor character-

istics and neither of needle size on this outcome. Notably, we only used higher gauge needles (19G and 22G and not 25 G).

A group from Johns Hopkins evaluated the concordance of the mutational profiles assessed by targeted NGS between EUS-FNA snap frozen PDAC samples and the corresponding biopsy specimens from the primary tumors that were subsequently resected [25]. Although on a pool of 16 cases only, they detected a 100% concordance between the two sample types for KRAS mutations. They concluded that it is feasible to perform NGS on FNA samples and the results are reliable in complementing pathology results[25].

Besides the parameters of the extracted DNA, the total cellularity and the tumor cell content of the samples are also essential for the assessment of adequacy for NGS[22,26]. We did not evaluate these metrics in our cohort, since we used dedicated FNA samples, processed directly after collection, for DNA extraction. However, we must emphasize that the same needle types were used for the samples dedicated to diagnosis and we only report on confirmed PDAC cases. Moreover, more than 50% of specimens obtained by FNA yielded small tissue fragments and were subjected to cell block techniques to increase the diagnostic accuracy. The most widely used NGS platforms to date require a sample cellularity between 1000 and 5000 cells and a minimum 10% "lesional to-non-lesional cell ratio"[26-28]. A group from MD Anderson compared these parameters between concurrently acquired, percutaneous, image guided FNA and core needle biopsy samples in 24 various malignancy cases. They obtained higher cellularity and better tumor fraction in the cytologic specimens, arguing that by comparison with core biopsies, during FNA the proportion of acquired stromal cells is lower[27].

In their study Berry *et al*[21] performed molecular profiling of 66 snap frozen EUS-FNA pancreatic cancer samples. They revealed a high epithelial and tumor cell content in their specimens and a low contamination with inflammatory, gastric or duodenal cells[21]. Moreover, they compared the DNA yield between the FNA samples dedicated to molecular testing and formalin fixed paraffin embedded cell-block preparations used for PDAC diagnosis, obtaining 10 times higher amounts of DNA from the FNA samples which was in average 4.8 ± 3.7 μg[21]. This could be explained by the degrading effect of formaldehyde on nucleic acids and the cross-links formation between protein and DNA that it determines[22].

Cytologic specimens yield optimal DNA and have adequate cellularity for further NGS. The quality of nucleic acids is altered in samples previously fixed with formalin. More standardized procedures that are focused on specimen sparing and keeping costs and processing times within accessible ranges are needed for the optimization of the sample assessment in the preanalytical phase of NGS.

Implications for practice and research

Regarding the implications for practice our study has shown that both 19G and 22G fresh EUS-FNA PDAC samples yield DNA of adequate amount and purity for next generation high throughput sequencing, regardless of tumor size or location within the pancreas. Overall, the NGS adequacy rates corresponded to the diagnostic accuracy of EUS-FNA samples in PDAC in our cohort[6]. A randomized controlled trial could reliably evaluate the cause-effect relationship between EUS needle G and DNA output from pancreatic cancer samples. Since the technical requirements in various clinical scenarios may limit the feasibility of randomization, prospective cohort studies of greater sizes that allow propensity score matching could decrease the influence of confounders in analyzing this association. Besides targeted NGS, the EUS-FNA samples yielding below 100 ng of DNA could be also used in other downstream applications for genetic testing like droplet digital PCR or TaqMan assays, which, although addressing fewer targets, have the advantage of good sensitivity for samples with low DNA amount.

Strengths and limitations

Even though relatively similar analyses have been previously reported, our study included a high number of samples[21,27]. Besides measuring the yielded DNA concentration and purity ratios, we were able to successfully perform NGS on subgroup of samples, functionality in the downstream application being a reliable method of sample adequacy evaluation[26]. Nevertheless, several limitations should be pointed out: (1) Study design-one of the main limitations of our work-since lack of patients randomization precludes the evaluation of causality between needle size and samples' NGS adequacy; (2) our study was performed in a tertiary gastroenterology center and all involved personnel were experts in their fields (endosonographers, pathologists, biologists); (3) the analysis was not based on a prior sample size calculation therefore our results must be interpreted with caution especially since 71% of the procedures were performed with one EUS-FNA needle type; (4) we did not use 25G needles for our samples therefore our conclusions cannot be extrapolated to all FNA needle sizes; and (5) lack of a comparison group comprising samples obtained by EUS-FNB-another main limitation of our study; to this end however we cite the study of Razzano *et al*[29] that compared the performance for NGS between FNA, FNB and resection PDAC specimens. They obtained similar success rates for mutation and amplification analysis between FNA and FNB samples and proposed FNA material as a source for comprehensive molecular testing[29]. Moreover, the spectrophotometric methods may overestimate the quantity of amplifiable DNA by measuring not only the double stranded fragments, but also single stranded DNA, free and oligonucleotides[22].

CONCLUSION

Acquiring EUS-FNA specimens dedicated for genetic testing is associated with a high adequacy rate for NGS of the extracted DNA. Nineteen and 22G EUS-FNA PDAC samples generate similar amount of DNA. DNA purity may vary indirectly with needle size in EUS-FNA samples. Further standardization in sample handling and cellularity assessment in the pre-analytical phase of NGS will increase reliability of the results for EUS-FNA PDAC samples and hence their usability in current practice.

ARTICLE HIGHLIGHTS

Research background

Due to the opportunities for personalized treatment in pancreatic adenocarcinoma (PDAC), genetic testing is increasingly performed. Fine needle biopsy is the method recommended for endoscopic ultrasound (EUS) guided tissue acquisition to obtain samples dedicated to downstream comprehensive molecular analyses. In current practice however, fine needle aspiration (FNA) is more widely accessible.

Research motivation

We evaluated the EUS-FNA PDAC samples in terms of adequacy for next generation sequencing (NGS) of the yielded DNA to assess the possibility of using this type of samples for genetic testing.

Research objectives

To investigate the association between DNA parameters (amount and purity) measured by spectrophotometry and FNA needle size (19 gauge [G] or 22G), and also tumor characteristics.

Research methods

We performed an observational prospective study on PDAC cases diagnosed through EUS-FNA at a tertiary center of Gastroenterology in Romania. During EUS one pass acquired samples dedicated to genetic testing. NGS adequacy was a dichotomus variable defined based on DNA parameters (purity: A260/280 ≥ 1.7 and DNA amount: ≥ 100 ng for whole genome sequencing, ≥ 50 ng for whole exome sequencing or ≥ 10 ng for amplicon based targeted NGS).

Research results

Our cohort analysis comprised 105 confirmed PDAC cases. The majority of samples were acquired with 22G FNA needles-75 (71%). The DNA amount was in average 1289 ng (inter-quartile range: 534.75-2995). All samples yielded more than 10 ng of DNA while 98 (93%) of them yielded more than 100 ng of DNA. Needle size was not correlated with DNA NGS adequacy rate regardless of NGS type. Needle size did not influence the concentration or A260/280 ratio of the extracted DNA. The median A260/230 was significantly higher in the 22G samples than in 19G samples ($P = 0.038$). In multivariate analysis (on needle size, tumor location and tumor diameter) the only independent predictor of A260/230 was needle size ($\beta = 0.36$, $t(104) = 2.1$, $P = 0.038$).

Research conclusions

Both 22G an 19G EUS-FNA PDAC samples are adequate for downstream NGS. FNA needle size and tumor characteristics did not significantly influence sample NGS adequacy rate. Greater FNA needle size might be associated with decreased sample purity.

Research perspectives

PDAC FNA samples (22 and 19G) yield samples of adequate purity and amount for NGS and can be used both in current practice and for research purposes.

ACKNOWLEDGEMENTS

we would like to give special thanks to Daniel Veres, MD, PhD, for his guidance in performing the formal analysis for the project and Mihaela Topala, MD for her help with patients' enrolment.

FOOTNOTES

Author contributions: Bunduc S contributed to conceptualization, methodology, investigation, formal analysis, original draft, and data visualization; Varzaru B contributed to conceptualization, investigation, data curation, methodology, review and editing; Iacob RA contributed to conceptualization, supervision, review and editing,

methodology, funding acquisition, and resources; Sorop A contributed to methodology, investigation, data curation, review and editing; Manea I contributed to methodology, investigation, data curation, review and editing; Spiridon A contributed to investigation, data curation, review and editing; Chelaru R contributed to investigation, data curation, review and editing; Becheanu G contributed to conceptualization, methodology, investigation, review and editing; Dumbrava M contributed to conceptualization, methodology, investigation, review and editing; Croitoru A contributed to conceptualization, methodology, review and editing, Dima S contributed to conceptualization, supervision, funding acquisition, project administration, review and editing, resources; Popescu I contributed to conceptualization, supervision, funding acquisition, project administration, review and editing, Gheorghe C contributed to guarantor of the article, conceptualization, supervision, funding acquisition, project administration, review and editing; all authors certify that they have participated sufficiently in this work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. All authors have read the journal's authorship agreement, and reviewed and approved the final version of the manuscript.

Supported by The Executive Agency for Higher Education, Research, Development and Innovation Funding-research, No. PN-III-P1-1.2-PCCDI-2017-0797 (PANCNGS).

Institutional review board statement: This prospective observational study was approved by the Internal Review Board of Fundeni Clinical Institute, No. IRB:23878/14.06.2018.

Informed consent statement: Each participant gave informed consent for study enrolment.

Conflict-of-interest statement: All authors confirm they have no conflict of interest in relation to this manuscript.

Data sharing statement: The data underlying this article will be shared upon reasonable request to the corresponding author.

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

Country/Territory of origin: Romania

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S-Editor: Chang KL
L-Editor: A
P-Editor: Zhao S

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 May 14; 29(18): 2875-2887

DOI: 10.3748/wjg.v29.i18.2875

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Contributory roles of sarcopenia and myosteatosis in development of overt hepatic encephalopathy and mortality after transjugular intrahepatic portosystemic shunt

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Barone M, Italy; Srpcic M, Slovenia

Received: January 29, 2023
Peer-review started: January 29, 2023
First decision: February 22, 2023
Revised: March 8, 2023
Accepted: April 17, 2023
Article in press: April 17, 2023
Published online: May 14, 2023

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Abstract

BACKGROUND

Skeletal muscle abnormalities, such as muscle mass depletion (sarcopenia) and fatty infiltration of the muscle (myosteatosis), are frequent complications in cirrhotic patients scheduled for transjugular intrahepatic portosystemic shunt (TIPS).

AIM

To investigate the association and predictive value of sarcopenia and myosteatosis for overt hepatic encephalopathy (HE) and mortality after TIPS.

METHODS

The records of cirrhotic patients who underwent the TIPS procedure at our hospital between January 2020 and June 2021 were retrospectively retrieved. The transversal psoas muscle thickness (TPMT) and psoas muscle attenuation (PMA) measured from the unenhanced abdominal computed tomography (CT) at the level of the third lumbar vertebrae were used to analyze the sarcopenia and myosteatosis, respectively. The area under curve (AUC) was used to evaluate the discriminative power of TPMT, PMA, and relevant clinical parameters. Fur-

thermore, log-rank test was performed to compare the incidence of overt HE and survival between the different groups, and the association of risk factors with overt HE and mortality was analyzed using Cox proportional hazards regression models.

RESULTS

A total of 108 patients were collected. Among these patients, 45.4% of patients developed overt HE after TIPS treatment. Furthermore, 32.4% and 28.7% of these patients were identified to have myosteatosis and sarcopenia, respectively. Myosteatosis (51.0% *vs* 16.9%, $P < 0.001$) and sarcopenia (40.8 *vs* 18.6%, $P = 0.011$) were found to be more frequent in patients with overt HE, when compared to patients without overt HE. The receiver operating characteristics analysis indicated that the predictive power of TPMT and PMA in overt HE (AUC = 0.713 and 0.778, respectively) was higher when compared to the neutrophil lymphocyte ratio (AUC = 0.636). The cumulative incidence of overt HE was the highest in patients with concomitant sarcopenia and myosteatosis, followed by patients with myosteatosis or sarcopenia, while this was the lowest in patients without sarcopenia and myosteatosis. In addition, sarcopenia and myosteatosis were independently associated with overt HE and mortality after adjusting for confounding factors in post-TIPS patients.

CONCLUSION

CT-based estimations for sarcopenia and myosteatosis can be used as reliable predictors for the risk of developing overt HE and mortality in cirrhotic patients after TIPS.

Key Words: Sarcopenia; Myosteatosis; Hepatic encephalopathy; Transjugular intrahepatic portosystemic shunt; Transjugular intrahepatic portosystemic shunt

Core Tip: Few studies have investigated the relationship among sarcopenia, myosteatosis, and overt hepatic encephalopathy (HE) after transjugular intrahepatic portosystemic shunt (TIPS). The present study revealed that the cumulative incidence of overt HE was the highest in patients with concomitant sarcopenia and myosteatosis, followed by patients with myosteatosis or sarcopenia, and the lowest incidence was found in patients without myosteatosis and sarcopenia. Sarcopenia and myosteatosis were the independent risk factors for overt HE and mortality in patients following TIPS. Therefore, identifying strategies for improving muscle mass (sarcopenia) and muscle fatty infiltration (myosteatosis) may help to reduce the incidence of HE after TIPS.

Citation: Yin L, Chu SL, Lv WF, Zhou CZ, Liu KC, Zhu YJ, Zhang WY, Wang CX, Zhang YH, Lu D, Cheng DL. Contributory roles of sarcopenia and myosteatosis in development of overt hepatic encephalopathy and mortality after transjugular intrahepatic portosystemic shunt. *World J Gastroenterol* 2023; 29(18): 2875-2887
URL: https://www.wjgnet.com/1007-9327/full/v29/i18/2875.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i18.2875

INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS), which establishes an artificial shunt between the portal and hepatic vein, has been considered an effective method for treating portal hypertension, and can be used to manage refractory ascites and variceal hemorrhage in patients with liver cirrhosis[1]. However, the TIPS procedure leads to a significantly high incidence of overt hepatic encephalopathy (HE), which occurs in approximately 20%-50% of post-TIPS patients[2-4]. Previous studies have suggested that post-TIPS HE is associated with increased hospitalization rates, mortality, and poor quality of life[5]. The use of rifaximin and lactulose remains controversial in treating this pathological entity[6,7]. Thus, studies that can precisely predict post-procedure HE in cirrhotic patients would be beneficial in developing appropriate preventive measures.

More recent studies have revealed that abnormalities in the skeletal muscle, such as sarcopenia (muscle mass depletion) and myosteatosis (fatty infiltration of the muscle), are more prevalent[8,9], and that these are associated with the development of HE in cirrhotic patients[10]. Cirrhosis can lead to the excessive accumulation of ammonia in the skeletal muscle, in which a cascade of molecular alterations and metabolic disturbances may contribute to sarcopenia and myosteatosis. Consequently, both sarcopenia and myosteatosis may, in turn, further increase the circulating ammonia levels to induce

overt HE. These two skeletal muscle abnormalities can hypothetically be targeted for preventing overt HE.

In clinic, both sarcopenia and myosteatosis can be reliably and objectively diagnosed through the computed tomography (CT) image-based measurement of body composition. Compared to the skeletal muscle index (SMI) and total psoas muscle volume, the CT-based measurement of transversal psoas muscle thickness (TPMT) has become an easy-to-use method to diagnose sarcopenia through gauging the psoas diameter, and this has become an independent risk factor for mortality in cirrhotic patients [11]. Furthermore, myosteatosis can be quantitatively graded by CT measurements, which shows that lower muscle radiodensity for designating muscle attenuation can be considered as evidence of fat infiltration in the skeletal muscle[12]. Previous studies have demonstrated that psoas muscle attenuation (PMA) is associated with postoperative complications and mortality in elderly patients[13,14]. The present study employed these CT-based methods to evaluate the association and predictive value of sarcopenia and myosteatosis for overt HE after TIPS.

MATERIALS AND METHODS

The present study retrospectively analyzed the demographic, clinical, laboratory and radiological data of cirrhotic patients who underwent TIPS at our institution between January 2020 and June 2021. A total of 108 patients were included (Supplementary Figure 1). The inclusion criteria were, as follows: (1) Patients diagnosed with cirrhotic portal hypertension; and (2) patients with at least one episode of variceal hemorrhage or refractory ascites after treatment with vasoactive drugs, endoscopic treatment, or large-volume paracentesis. The exclusion criteria were, as follows: (1) Patients < 18 years old; (2) patients with a history of overt HE (grade ≥ 2, according to the West-Haven criteria[7]) within the past six months; (3) patients with hepatocellular carcinoma or other malignancies; (4) patients with no or poor quality preoperative abdominal CT scans; (5) patients with severe medical comorbidities, such as renal failure, pulmonary insufficiency, or extensive cardiovascular and cerebrovascular disease; and (6) patients who were lost to follow-up within three months.

Our institutional ethics review board approved the present retrospective study. According to the Declaration of Helsinki, all data of the study patients remained confidential throughout the study, and the informed consent for the study was waived due to the retrospective nature of the study.

The TIPS procedure

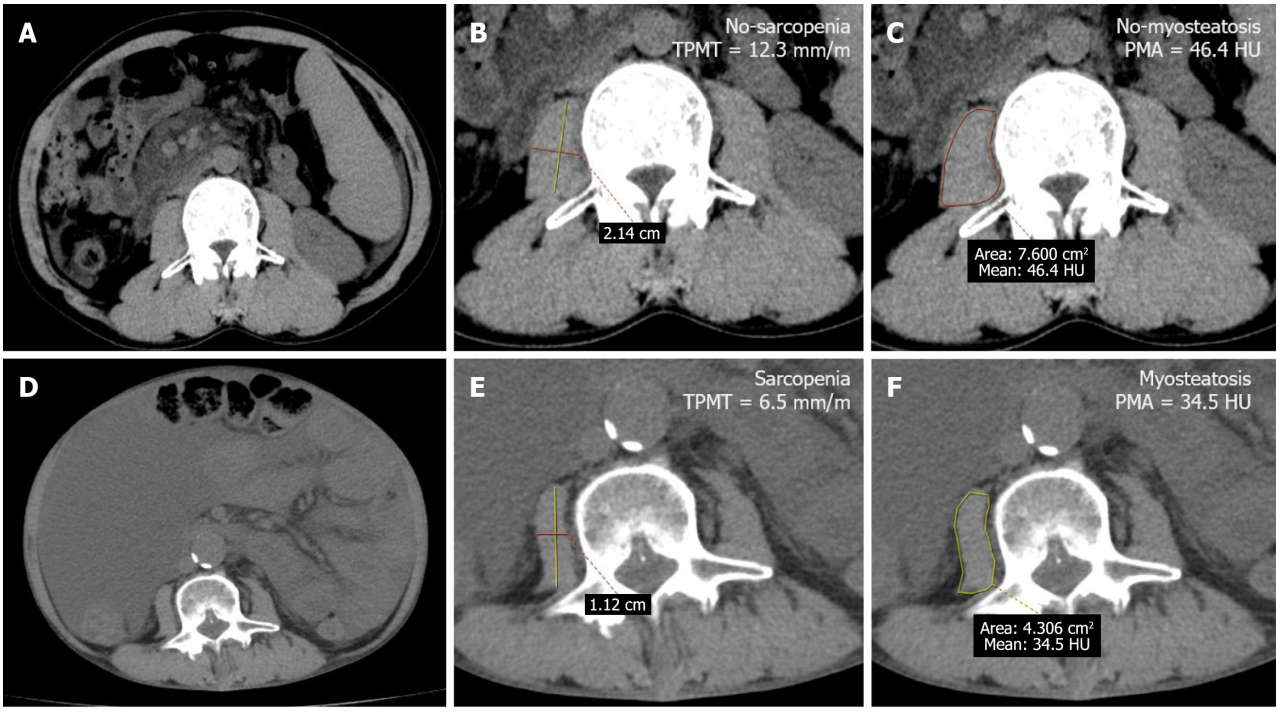
The TIPS procedure was performed based on the following steps: (1) After successfully puncturing the internal jugular vein, a transjugular liver access set (RUPS-100; Cook Incorporated, Bloomington, IN, United States) was introduced over the guidewire into the right hepatic vein; (2) the bifurcation of the left and right branches of the portal vein was punctured from the right hepatic vein; (3) the pre-shunt portosystemic pressure gradient (PPG) was measured; (4) a balloon catheter of 6-7 mm in diameter was passed over the guidewire to dilate the puncture channel; (5) an 8-mm polytetrafluoroethylene-covered stent (Viatorr stent, W. L. Gore & Associates, Flagstaff, AZ, United States) was implanted to establish the portosystemic shunt; (6) an 8-mm balloon catheter was used to further dilate the in-place stent and ensure that the expansion of the stent reaches 8 mm; (7) after the stent insertion, portography was performed to visualize the left and right branches of the portal vein; (8) if the patient was complicated with variceal rebleeding, the esophageal and gastric varicose vessels were embolized at the same time; and (9) finally, the post-shunting PPG was measured again to determine whether the target PPG was ≤ 12 mmHg or reduced by more than 50% from baseline.

CT image analysis

The abdominal CT scans of all studied patients were retrieved from our hospital's Picture Archiving and Communication System (Carestream, Canada). Then, the PMA and TPMT were measured at the level of the third lumbar vertebrae (L3) using the ADW4.4 workstation. In order to standardize the measurement, TPMT was obtained by gauging the transversal diameters of the right psoas muscle, further normalizing these to the body height, and presenting the values in mm/m. PMA was determined as the mean muscle attenuation in Hounsfield unit (HU) of the right psoas muscle at the L3 level (Figure 1). For the present study, sarcopenia was defined as TPMT < 10.7 mm/m in males and TPMT < 7.8 mm/m in females[11], while myosteatosis was defined as PMA < 41 HU for body mass index (BMI) < 25 kg/m^2 and PMA < 33 HU for BMI ≥ 25 kg/m^2[15].

Evaluation and follow-up of overt HE

All patients were followed-up at 1, 3 and 6 mo after TIPS, and every six months thereafter, during the inpatient rounds and outpatient visits. The follow-up evaluation included the following: General clinical manifestations, physical examination, laboratory tests, shunt patency, and HE. The different stages of HE were assessed and classified based on the detailed neurological examination, and according to the clinical criteria (West-Haven criteria). Overt HE was clinically diagnosed according to the grade 2 West-Haven criteria or higher, or based on the evidence of asterixis and disorientation[5].



DOI: 10.3748/wjg.v29.i18.2875

Figure 1 Abdominal computed tomography images at the level of the third lumbar vertebrae were used to measure the transversal psoas muscle thickness and psoas muscle attenuation in a 45-year-old man without sarcopenia and myosteatosis, and a 51-year-old man with sarcopenia and myosteatosis. A: The axial computed tomography (CT) scan image of a 45-year-old cirrhotic patient (height: 1.74 m, weight: 74 kg) who developed variceal rebleeding; B and C: The patient had normal transversal psoas muscle thickness (TPMT) and psoas muscle attenuation (PMA) levels: 12.3 mm/m (21.4 mm/1.74 m) and 46.4 HU, respectively; D-F: The axial CT scan image of a 51-year-old cirrhotic patient (height: 1.72 m, weight: 55 kg) who developed refractory ascites (D), and had low TPMT and PMA (6.5 mm/m [11.2 mm/1.72 m] and 34.5 HU) (E and F). TPMT: Transversal psoas muscle thickness.

Statistical analysis

The statistical analysis was performed using SPSS v26.0 (IBM, United States). The data were presented as mean ± SE, or in frequency and percentage. Before the analysis, tests were performed to verify the normal distribution of the variables. The comparison of two groups for quantitative variables was performed using Student's *t*-test or Mann-Whitney *U*-test, while the comparison of two groups with qualitative variables was analyzed using χ^2 test. Receiver operating characteristic (ROC) analysis was performed to determine the prognostic significance and precision of various relevant parameters in predicting the development of overt HE. The Kaplan-Meier survival estimates were graphed to plot the survival trends of the patients, and log-rank test was conducted to determine the cumulative rates for overt HE. Univariate and multivariate Cox proportional hazards regression models were used to assess the association of risk factors with HE and mortality, and forward regression analysis was subsequently performed to select the appropriate variables in the multivariate model. A two-sided P value of < 0.05 was considered statistically significant for all statistical tests.

RESULTS

Patient demographics

Among the 108 patients, 84 (77.8%) patients were male, with a mean age of 53.0 ± 10.8 years old. The detailed demographic data and clinical information of these 108 patients are presented in Table 1. The table shows that the most common etiology of cirrhosis was viral hepatitis (67.6%). Furthermore, 74 (68.5%) patients had ascites, and the mean Child-Pugh score, model for end-stage liver disease (MELD) score, and neutrophil-lymphocyte ratio (NLR) was 7.4 ± 1.5, 11.0 ± 4.4 and 4.4 ± 4.1, respectively. The mean levels for hepatic [total bilirubin (TBIL), 22.8 ± 12.9 umol/L; alanine aminotransferase, 27.5 ± 19.7 U/L; aspartate transaminase, 37.8 ± 28.6 U/L] and renal (creatinine, 67.8 ± 25.7 umol/L) function were within the normal range. The mean value for PMA and TPMT was 43.5 ± 6.9 HU and 11.6 ± 3.1 mm/m, respectively. In general, myosteatosis and sarcopenia were found in 35 (32.4%) and 31 (28.7%) patients, respectively, and overt HE was diagnosed in 49 (45.4%) post-TIPS patients.

Table 1 Demographic data, relevant clinical characteristics, and skeletal muscle abnormalities of patients in the study

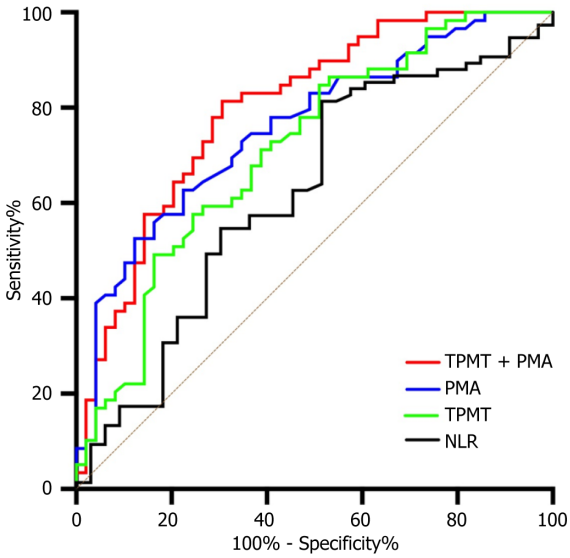
Variables	All patients (*n* = 108)	Non-HE (*n* = 59)	HE (*n* = 49)	*P* value
Age (years)	53.0 ± 10.8	50.3 ± 9.6	56.2 ± 11.5	0.005[a]
Gender (male), *n*, %	84 (77.8)	47 (79.7)	37 (75.5)	0.605
BMI (kg/m^2)	20.99 ± 2.59	21.00 ± 2.58	20.96 ± 2.63	0.856
Etiology (*n*, %)				
Viral hepatitis	74 (68.5)	42 (71.2)	32 (65.3)	0.512
Others	34 (31.5)	17 (28.8)	17 (34.7)	
Ascites (*n*, %)				
Yes	74 (68.5)	41 (69.5)	33 (67.3)	0.811
No	34 (31.5)	18 (30.5)	16 (32.7)	
Child-Pugh score	7.4 ± 1.5	6.9 ± 1.2	7.9 ± 1.8	0.002[a]
Child-Pugh class A/B/C (*n*)	38/59/11	25/30/4	13/29/7	0.155
MELD score	11.0 ± 4.4	9.3 ± 3.8	13.0 ± 4.3	< 0.001[a]
NLR	4.4 ± 4.1	3.9 ± 4.5	4.9 ± 3.5	0.015[a]
TBIL (umol/L)	22.8 ± 12.9	19.1 ± 9.4	27.3 ± 15.0	0.003[a]
ALT (U/L)	27.5 ± 19.7	27.1 ± 16.9	28.0 ± 22.8	0.610
AST (U/L)	37.8 ± 28.6	32.0 ± 16.5	44.9 ± 37.4	0.071
Albumin (g/L)	32.6 ± 5.0	33.2 ± 5.3	31.9 ± 4.7	0.161
Creatinine (umol/L)	67.8 ± 25.7	66.6 ± 23.4	69.1 ± 28.5	0.858
Reduction of PPG (%)	53.7 ± 9.1	51.5 ± 9.1	56.5 ± 8.5	0.004[a]
PMA (HU)	43.5 ± 6.9	46.3 ± 6.6	40.2 ± 5.7	< 0.001[a]
Myosteatosis	35 (32.4)	10 (16.9)	25 (51.0)	< 0.001[a]
No myosteatosis	73 (67.6)	49 (83.1)	24 (49.0)	
TPMT (mm/m)	11.6 ± 3.1	12.6 ± 2.9	10.4 ± 3.1	< 0.001[a]
Sarcopenia	31 (28.7)	11 (18.6)	20 (40.8)	0.011[a]
No sarcopenia	77 (71.3)	48 (81.4)	29 (59.2)	

[a]$P < 0.05$.

HE: Hepatic encephalopathy; BMI: Body mass index; MELD: Model for end-stage liver disease; NLR: Neutrophil-lymphocyte ratio; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate transaminase; PPG: Portosystemic pressure gradient; PMA: Psoas muscle attenuation; TPMT: Transversal psoas muscle thickness; HU: Hounsfield units.

Features associated with overt HE

The comparison between patients with and without overt HE is presented in Table 1. The table shows that patients with overt HE were older, had higher TBIL, MELD, Child-Pugh scores and reduction of PPG, and had lower NLR, PMA and TPMT values, when compared to patients without overt HE ($P < 0.05$). Furthermore, both myosteatosis (51.0% *vs* 16.9%, $P < 0.001$) and sarcopenia (40.8 *vs* 18.6%, $P = 0.011$) were more common in patients with overt HE, when compared to patients without overt HE. ROC curves were further plotted to determine the area under curve (AUC) values for PMA (AUC = 0.759, 95%CI: 0.669-0.849) and TPMT (AUC = 0.7143, 95%CI: 0.615-0.811), which were similar to the MELD score (AUC = 0.737, 95%CI: 0.642-0.833), but were greater than the NLR (AUC = 0.636, 95%CI: 0.530-0.743). The diagnostic sensitivity and specificity for the combined TPMT and PMA were 81.4% and 69.4%, respectively, and the AUC value was 0.799 (95%CI: 0.715-0.884) (Figure 2). The univariate Cox proportional hazards regression analysis revealed that the following were potentially associated with overt HE: age (HR = 1.044, 95%CI: 1.016-1.071, $P = 0.001$), Child-Pugh score (HR = 1.292, 95%CI: 1.091-1.531, $P = 0.003$), MELD score (HR = 1.137, 95%CI: 1.074-1.203, $P < 0.001$), TBIL (HR = 1.033, 95%CI: 1.014-1.053, $P = 0.001$), reduction of PPG (HR = 1.039, 95%CI: 1.007-1.071, $P = 0.017$), PMA (HR = 0.901, 95%CI: 0.863-0.940; $P < 0.001$), and TPMT (HR = 0.853, 95%CI: 0.774-0.940, $P < 0.001$). The further multivariate Cox proportional hazards regression analysis identified the following as independent predictive variables for overt HE after TIPS: MELD score (HR = 1.083, 95%CI: 1.020-1.449, $P = 0.009$),



DOI: 10.3748/wjg.v29.i18.2875

Figure 2 Receiver operating characteristic curves for the probability of transversal psoas muscle thickness, psoas muscle attenuation, and neutrophil-lymphocyte ratio in predicting overt hepatic encephalopathy. TPMT: Transversal psoas muscle thickness; PMA: Psoas muscle attenuation; NLR: Neutrophil-lymphocyte ratio.

PMA (HR = 0.930, 95%CI: 0.889-0.974, P = 0.002), and TPMT (HR = 0.895, 95%CI: 0.808-0.992, P = 0.035) (Table 2).

Incidence of overt HE

The 3-mo, 6-mo, 1-year, and 2-year cumulative incidence of overt HE after TIPS was 32.8%, 38.0%, 44.7% and 52.6%, respectively. The incidence of overt HE was significantly higher in patients with sarcopenia (log-rank P = 0.022) or myosteatosis (log-rank P < 0.001), when compared to those without either condition. Furthermore, the 108 studied patients were divided into the following groups: Concomitant sarcopenia and myosteatosis (13.9%, 15/108), myosteatosis alone (18.5%, 20/108), sarcopenia alone (13.0%, 14/108), and normal muscle (54.6%, 59/108) groups (Table 3). It was determined that the cumulative incidence of overt HE was the highest in the concomitant sarcopenia and myosteatosis group, followed by the myosteatosis alone and sarcopenia alone groups, while the incidence was the lowest in the non-myosteatosis and non-sarcopenia groups (Figure 3).

Survival analysis

The univariate Cox proportional hazards regression analysis revealed that age (HR = 1.042, 95%CI: 1.008-1.077, P = 0.016), Child-Pugh score (HR = 1.317, 95%CI: 1.064-1.630, P = 0.011), MELD score (HR = 1.089, 95%CI: 1.013-1.172, P = 0.021), albumin (HR = 0.904, 95%CI: 0.836-0.977, P = 0.011), PMA (HR = 0.887; 95%CI: 0.839-0.938, P < 0.001), and TPMT (HR = 0.851, 95%CI: 0.759-0.954, P = 0.006) were potentially associated with overall mortality. When these above parameters were reprocessed using the multivariate Cox proportional hazards regression model, merely albumin (HR = 0.903, 95%CI: 0.827-0.986, P = 0.023), PMA (HR = 0.901, 95%CI: 0.853-0.951, P < 0.001), and TPMT (HR = 0.867, 95%CI: 0.760-0.988, P = 0.032) were identified as independent predictors for overall mortality (Table 4). Based on the Kaplan-Meier survival analysis, the cumulative survival rate was significantly lower in patients with overt HE (log-rank P < 0.001), sarcopenia (log-rank P = 0.001), and myosteatosis (log-rank P = 0.002), when compared to patients without overt HE. Furthermore, the cumulative survival rate was the lowest in patients with concomitant sarcopenia and myosteatosis, followed by patients with sarcopenia alone and myosteatosis alone, while the cumulative survival rate was the highest in patients without myosteatosis and sarcopenia (Figure 4).

DISCUSSION

Skeletal muscle abnormalities are frequently observed in advanced cirrhosis and cancer, and are associated with unfavorable outcomes[16-18]. Few studies have investigated the relationship between sarcopenia and myosteatosis, and overt HE after TIPS. The present study revealed that the cumulative incidence of overt HE was the highest in patients with concomitant sarcopenia and myosteatosis, followed by patients with myosteatosis alone and sarcopenia alone, while the lowest incidence was identified in patients without myosteatosis and sarcopenia. Furthermore, both myosteatosis and

Table 2 Prognostic factors for overt hepatic encephalopathy in patients treated with transjugular intrahepatic portosystemic shunt

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (years)	1.044	(1.016-1.071)	0.001[a]	1.043	(1.017-1.069)	0.330
Gender (male)	1.207	(0.629-2.316)	0.572			
BMI (kg/m^2)	1.012	(0.902-1.134)	0.844			
Etiology (viral hepatitis/others)	1.277	(0.706-2.310)	0.418			
Ascites (yes/no)	0.845	(0.465-1.536)	0.580			
Child-Pugh score	1.292	(1.091-1.531)	0.003[a]	1.078	(0.832-1.397)	0.740
MELD score	1.137	(1.074-1.203)	< 0.001[a]	1.083	(1.020-1.449)	0.009[a]
NLR	1.041	(0.984-1.101)	0.161			
TBIL (umol/L)	1.033	(1.014-1.053)	0.001[a]	1.029	(1.000-1.058)	0.768
ALT (U/L)	1.003	(0.989-1.018)	0.681			
AST (U/L)	1.010	(0.998-1.023)	0.107			
Albumin (g/L)	0.956	(0.903-1.013)	0.128			
Creatinine (umol/L)	1.004	(0.993-1.015)	0.454			
Reduction of PPG (%)	1.039	(1.007-1.071)	0.017[a]	1.050	(1.014-1.087)	0.082
PMA (HU)	0.901	(0.863-0.940)	< 0.001[a]	0.930	(0.889-0.974)	0.002[a]
TPMT (mm/m)	0.853	(0.774-0.940)	0.001[a]	0.895	(0.808-0.992)	0.035[a]

[a]$P < 0.05$.

HE: Hepatic encephalopathy; BMI: Body mass index; MELD: Model for end-stage liver disease; NLR: Neutrophil-lymphocyte ratio; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate transaminase; PPG: Portosystemic pressure gradient; PMA: Psoas muscle attenuation; TPMT: Transversal psoas muscle thickness; HU: Hounsfield units.

Table 3 The different skeletal muscle abnormalities in patients

Skeletal muscle abnormalities (*n* = 108)		PMA	
		Low (*n* = 35); BMI < 25 kg/m^2: < 41 HU; BMI ≥ 25 kg/m^2: < 33 HU	Normal (*n* = 73); BMI < 25 kg/m^2: ≥ 41 HU; BMI ≥ 25 kg/m^2: ≥ 33 HU
TPMT	Low (*n* = 31); Male: < 10.7 mm/m; Female: < 7.8 mm/m	Sarcopenia; Myosteatosis; (*n* = 15)	Sarcopenia; No myosteatosis; (*n* = 14)
	Normal (*n* = 77); Male: ≥ 10.7 mm/m; Female: ≥ 7.8 mm/m	Myosteatosis; No sarcopenia; (*n* = 20)	No myosteatosis; No sarcopenia; (*n* = 59)

BMI: Body mass index; PMA: Psoas muscle attenuation; TPMT: Transversal psoas muscle thickness; HU: Hounsfield units.

sarcopenia were identified as independent risk factors for predicting the occurrence of overt HE and mortality in cirrhotic patients following TIPS.

Overt HE is a frequent complication of decompensated cirrhosis, which is eventually induced and/or aggravated by the TIPS procedure, and this can impact the quality of life and increase the mortality of patients[19]. The present study revealed that the overall incidence of overt HE after TIPS treatment was 45.4%, which is similar to the reports of previous studies[20,21]. However, merely the MELD score (HR = 1.083, 95%CI: 1.020-1.449, $P = 0.009$), myosteatosis (HR = 0.930, 95%CI: 0.889-0.974, $P = 0.002$) and sarcopenia (HR = 0.895, 95%CI: 0.808-0.992, $P = 0.035$) were identified as independent risk factors after adjusting for confounding variables in the present study. Two retrospective studies on 279 and 284 patients with TIPS reported that a unit of increase in the MELD score would lead to a 1.69 higher odds and a 1.06-fold increase in risk of post-TIPS HE development[22,23]. At the same time, a prospective study on 46 patients who underwent the TIPS procedure also reported that the MELD score and sarcopenia were independently associated with the development of HE after TIPS placement[24]. In addition, several studies reported that age, albumin, creatinine, Child-Pugh score, previous HE, PPG, proton pump inhibitors, and shunt size (> 8 mm *vs* 8 mm) are potential risks for developing post-TIPS

Table 4 The univariate and multivariable analysis results for predicting mortality in patients treated with transjugular intrahepatic portosystemic shunt

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (years)	1.042	(1.008-1.077)	0.016[a]	1.047	(1.012-1.084)	0.278
Gender (male)	1.187	(0.559-2.518)	0.656			
BMI (kg/m^2)	1.000	(0.871-1.148)	0.997			
Etiology (Viral hepatitis/other)	1.187	(0.559-2.518)	0.656			
Ascites (yes/no)	1.652	(0.714-3.823)	0.241			
Child-Pugh score	1.317	(1.064-1.630)	0.011[a]	1.194	(0.903-1.579)	0.740
MELD score	1.089	(1.013-1.172)	0.021[a]	1.050	(0.963-1.146)	0.845
NLR	1.060	(0.994-1.130)	0.075			
TBIL (umol/L)	1.022	(0.996-1.048)	0.096			
ALT (U/L)	1.003	(0.986-1.020)	0.745			
AST (U/L)	1.007	(0.998-1.016)	0.109			
Reduction in PPG (%)	1.030	(0.992-1.069)	0.120			
Albumin (g/L)	0.904	(0.836-0.977)	0.011[a]	0.903	(0.827-0.986)	0.023[a]
Creatinine (umol/L)	1.012	(1.000-1.024)	0.044[a]	1.005	(0.993-1.018)	0.342
PMA (HU)	0.887	(0.839-0.938)	< 0.001[a]	0.901	(0.853-0.951)	< 0.001[a]
TPMT (mm/m)	0.851	(0.759-0.954)	0.006[a]	0.867	(0.760-0.988)	0.032[a]

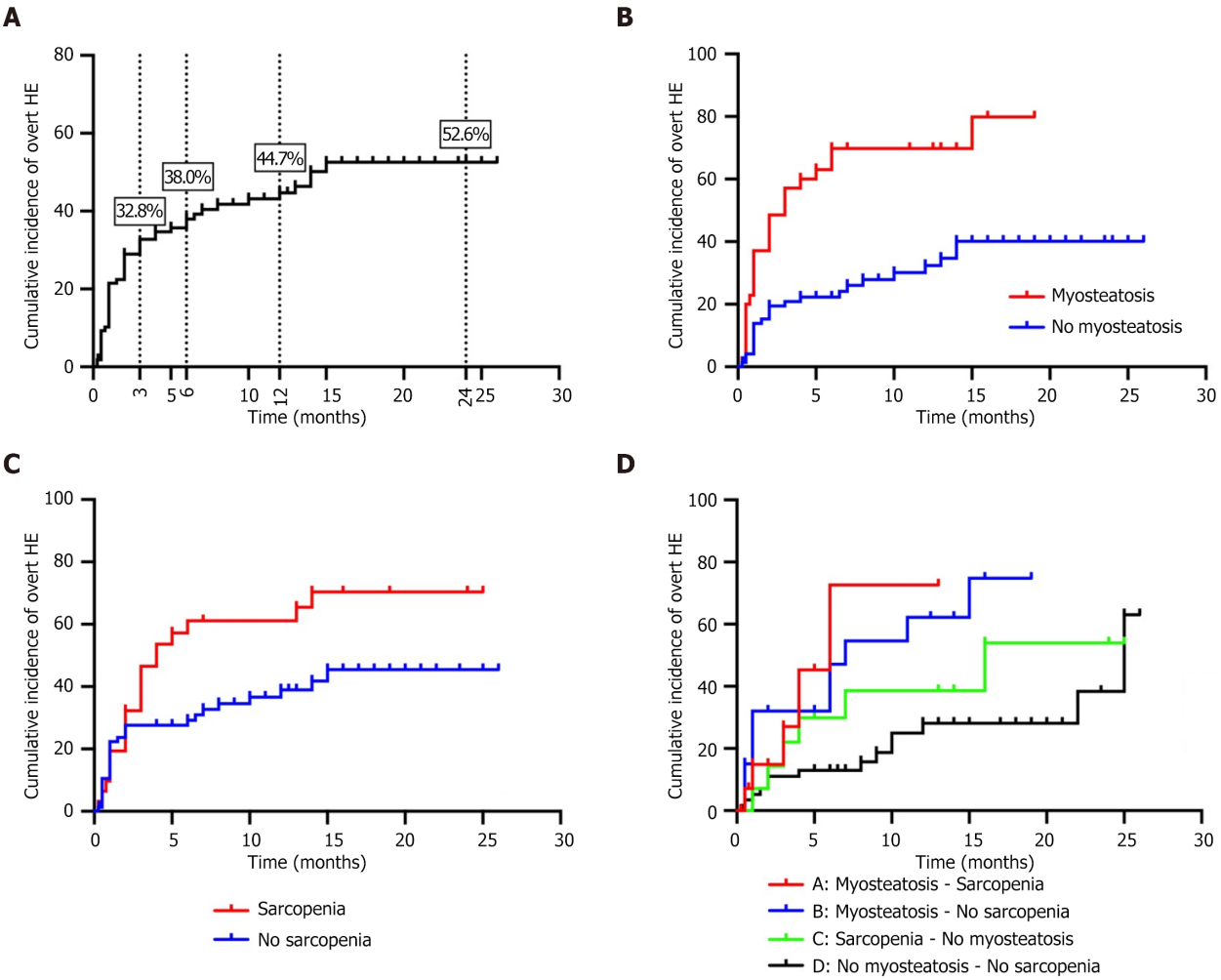
[a]$P < 0.05$.

BMI: Body mass index; MELD: Model for end-stage liver disease; NLR: Neutrophil-lymphocyte ratio; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate transaminase; PPG: Portosystemic pressure gradient; PMA: Psoas muscle attenuation; TPMT: Transversal psoas muscle thickness; HU: Hounsfield units.

HE[4,23,25-27]. Thus, correct patient selection and early intervention for TIPS based on these potential risk factors may contribute to preventing HE complications, and improving the life quality of patients.

The relationship among myosteatosis, sarcopenia and HE may have different pathophysiological mechanisms. Previous studies have suggested that sarcopenia is caused by impaired protein synthesis and reduced satellite cell function[28]. Furthermore, since the muscle has been considered as an alternative site for the detoxification of ammonia, the accumulation of ammonia may contribute to the development of sarcopenia in cirrhotic patients by interfering with the protein remodeling[29]. On the other hand, myosteatosis may reduce the detoxification of ammonia by inhibiting glutamine synthetase, and increasing inflammatory cytokines[30]. Previous systematic reviews and meta-analyses have revealed that sarcopenia and myosteatosis are highly associated with complications and poor overall survival in patients with various diseases[31,32]. In the present study, patients with sarcopenia (log-rank $P = 0.022$) or myosteatosis (log-rank $P < 0.001$) had a higher cumulative incidence of overt HE after TIPS. In addition, subgroup analysis revealed that the cumulative incidence of overt HE was the highest in patients with concomitant sarcopenia and myosteatosis, followed by patients with myosteatosis alone or sarcopenia alone, while the cumulative incidence was the lowest in patients with normal skeletal muscles. These findings are consistent with those reported by previous studies[10,33], showing that sarcopenia and myosteatosis are independent predictive factors for overt HE in patients with cirrhosis. Furthermore, the relationship between skeletal muscle abnormalities (sarcopenia and myosteatosis) and minimal HE in patients with cirrhosis was further suggested by the Firth's bias-reduced multivariate analysis[33].

Similar to the present study, Liu *et al*[34] observed that the incidence of overt HE after TIPS was higher in patients with sarcopenia, when compared to patients without sarcopenia (29% and 16%, $P = 0.04$). However, that study could only identify sarcopenia as the possible risk factor for post-TIPS HE in the univariate analysis (HR = 1.86, 95%CI: 0.99-3.46, $P = 0.004$), and its significance in the multivariate analysis could not be verified. Another study conducted by Gioia *et al*[35] revealed that the psychometric HE score, ammonia, and occurrence of minimal and overt HE significantly improved in post-TIPS patients, with an amelioration SMI of > 10%, suggesting that the reversal of sarcopenia after TIPS can reduce the risk of occurrence of minimal or overt HE.

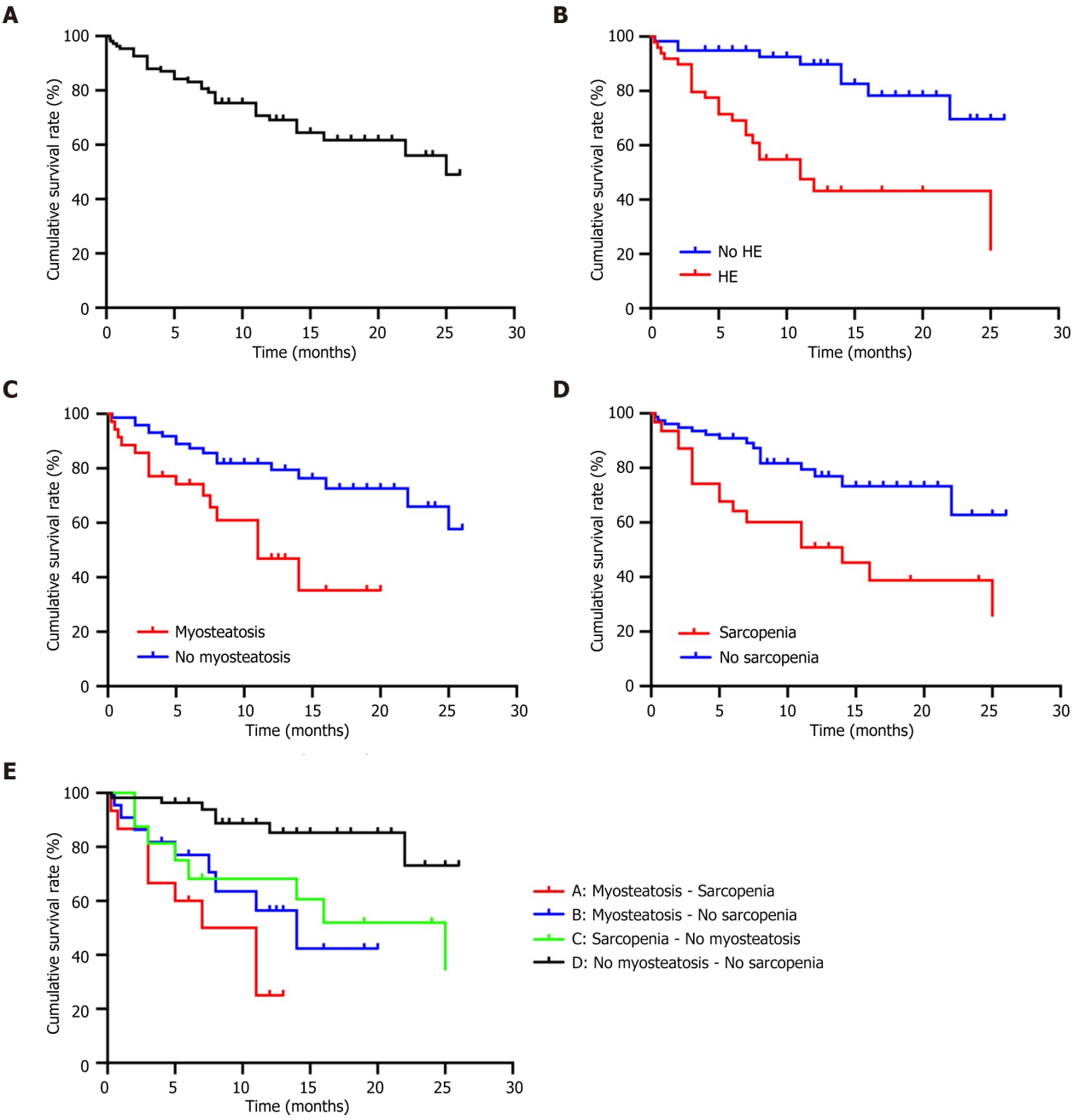


DOI: 10.3748/wjg.v29.i18.2875

Figure 3 Kaplan-Meier curves for the cumulative incidence of post-transjugular intrahepatic portosystemic shunt overt hepatic encephalopathy. A: For all patients; B: For patients with and without myosteatosis; C: For patients with and without sarcopenia; D: For patients with coexisting sarcopenia and myosteatosis. HE: Hepatic encephalopathy.

Furthermore, the present study revealed that patients with coexisting myosteatosis and sarcopenia had the lowest cumulative survival rate, and it was identified that hypoalbuminemia (HR = 0.903, 95%CI: 0.827-0.986, P = 0.023), sarcopenia (HR = 0.901, 95%CI: 0.853-0.951, P < 0.001) and myosteatosis (HR = 0.867, 95%CI: 0.760-0.988, P = 0.032) are independent predictors of post-TIPS morbidity. Although other relevant factors, including age, creatinine, Child-Pugh score and MELD score, were significant in the present univariate analysis, these lost its significance as independent predictors in the multivariate analysis. A possible explanation is the inherent complexity and heterogeneity of TIPS cohorts, in general, with a high number of factors potentially affecting the clinical outcomes. A retrospective cohort study on 855 patients with cirrhosis reported that myosteatosis and sarcopenia are associated with mortality after adjusting for factors, and it was even quantitatively extrapolated that one HU of increase in muscle radiodensity corresponds to a 2% decrease in mortality risk (HR = 0.98, 95%CI: 0.96-0.99, P < 0.001)[36]. Another recent retrospective study on 224 patients with TIPS also identified sarcopenia (determined by L3 SMI) as an independent risk factor for mortality after TIPS (HR = 3.0, 95%CI: 1.2-7.8), and that patients who converted from sarcopenic to non-sarcopenic had a higher cumulative survival rate, when compared to those who did not convert (96.4% *vs* 82.1%, log-rank P = 0.04)[34]. In the present study, the cumulative survival rates were significantly lower in patients with sarcopenia/myosteatosis, when compared to patients without sarcopenia/myosteatosis (log-rank P = 0.001 and log-rank P = 0.002, respectively), further confirming the predictive value of sarcopenia/myosteatosis in the unfavorable prognosis of cirrhotic patients with TIPS.

There were several limitations in the present study. First, the present study was a single-center retrospective cohort study with a limited number of patients, and the intrinsic limitation of the study design appeared to be inescapable. Second, the diagnostic values for sarcopenia and myosteatosis were heterogeneous across studies in the literature. Thus, more studies and external validation of data are needed to standardize the CT-derived diagnostic criteria for sarcopenia and myosteatosis. Third,



DOI: 10.3748/wjg.v29.i18.2875

Figure 4 Kaplan-Meier curves for the cumulative survival rate of post-transjugular intrahepatic portosystemic shunt. A: For all patients; B: For patients with and without hepatic encephalopathy; C: For patients with and without myosteatosis; D: For patients with and without sarcopenia; E: For patients with coexisting sarcopenia and myosteatosis. HE: Hepatic encephalopathy.

sarcopenia and myosteatosis were merely evaluated through CT measurements at admission, and these were not re-evaluated through CT imaging during the post-TPS follow-up period[35]. Thus, further well-designed studies are needed to determine whether the improvement in muscle abnormalities after TIPS can reduce the risk of overt HE and variceal rebleeding in post-TIPS patients[34].

CONCLUSION

The present study revealed that patients with coexisting myosteatosis and sarcopenia had the highest incidence of overt HE, and the lowest cumulative survival rate after TIPS placement, when compared to patients with myosteatosis alone or sarcopenia alone, or patients without muscle abnormalities. Sarcopenia and myosteatosis are independent variables for the development of overt HE and mortality in post-TIPS patients.

ARTICLE HIGHLIGHTS

Research background

Skeletal muscle abnormalities, such as muscle mass depletion (sarcopenia) and fatty infiltration of the muscle (myosteatosis), are frequent complications in cirrhotic patients scheduled for a transjugular intrahepatic portosystemic shunt (TIPS) procedure, leading to an incidence of approximately 20%-50% for overt hepatic encephalopathy (HE).

Research motivation

The motivation of the study was to provide computed tomography (CT) image-based methods for predicting overt HE and mortality after TIPS, based on the sarcopenia and myosteatosis.

Research objectives

The study aims to investigate the association and predictive volubility of sarcopenia and myosteatosis for overt HE, and mortality after TIPS.

Research methods

The records of cirrhotic patients, who underwent the TIPS procedure at our hospital, were retrospectively reviewed. Transversal psoas muscle thickness and psoas muscle attenuation, which were measured by unenhanced abdominal CT at the level of the third lumbar vertebrae, were used to diagnose the sarcopenia and myosteatosis, respectively. Then, the incidence of overt HE and mortality were compared based on the sarcopenia and myosteatosis status.

Research results

A total of 108 patients were collected. Myosteatosis (51.0% *vs* 16.9%, $P < 0.001$) and sarcopenia (40.8 *vs* 18.6%, $P = 0.011$) were identified to be more frequent in patients with overt HE, when compared to patients without overt HE. The cumulative incidence of overt HE was the highest in patients with concomitant sarcopenia and myosteatosis, followed by patients with myosteatosis or sarcopenia, while this was the lowest in patients without sarcopenia and myosteatosis. In addition, sarcopenia and myosteatosis were independently associated with overt HE and mortality after adjusting for confounding factors in post-TIPS patients.

Research conclusions

The CT-based diagnostic method of sarcopenia and myosteatosis can be used as a reliable predictor for the risk of developing overt HE and mortality in cirrhotic patients after TIPS.

Research perspectives

In the future, more well-designated trials are required to standardize the CT-derived diagnostic criteria for sarcopenia and myosteatosis. In addition, more validation studies are needed to confirm the predictivities of sarcopenia and myosteatosis in post-TIPS overt HE.

FOOTNOTES

Author contributions: Yin L and Chu SL designed the research, analyzed the data, and wrote the initial draft of the manuscript; Zhou CZ, Liu KC, Zhu YJ and Lu D collected the data, analyzed the data, and designed the study; Zhang WY, Wang CX and Zhang YH performed the research and collected the data; Lv WF and Cheng DL contributed to the central idea, designed the study, and wrote the manuscript.

Supported by Natural Science Foundation of Anhui Province, No. 2108085MH301; and The Key Project of University Research Found of Anhui Province Education Department, No. 2022AH040189.

Institutional review board statement: The retrospective study protocol was approved by the institutional review board of The First Affiliated Hospital of University of Science and Technology of China, No. 2022-RE-461.

Informed consent statement: Informed consent for the study was waived due to the retrospective nature of the study.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets generated and analyzed in the study are available from the corresponding author on reasonable request.

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

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S-Editor: Fan JR
L-Editor: A
P-Editor: Chen YX

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