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FRONTIER

Colorectal cancer screening and surveillance in patients with inflammatory bowel disease in 2021

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Abstract

The detection of dysplasia in patients with inflammatory bowel disease (IBD) continues to be important given the increased risk of colorectal cancer in this population. Therefore, in 2017, we performed a review and update of the recommendations for the management and follow-up of patients with IBD based on the clinical practice guidelines of various scientific societies. The present manuscript focuses on new aspects of the detection, follow-up, and management of dysplasia according to the latest studies and recommendations. While chromoendoscopy with targeted biopsy continues to be the technique of choice for the screening and detection of dysplasia in IBD, the associated difficulties mean that it is now being compared with other techniques (virtual chromoendoscopy), which yield similar results with less technical difficulties. Furthermore, the emergence of new endoscopy techniques that are still being researched but seem promising (*e.g.*, confocal laser endomicroscopy and full-spectrum endoscopy), together with the development of devices that improve endoscopic visualization (*e.g.*, Endocuff Vision), lead us to believe that these approaches can revolutionize the screening and follow-up of dysplasia in patients with IBD. Nevertheless, further studies are warranted to define the optimal follow-up strategy in this patient population.

Key Words: Colitis surveillance; Colitis screening; Chromoendoscopy; Colorectal cancer; Inflammatory bowel disease; Ulcerative colitis

Core Tip: Patients with inflammatory bowel disease (IBD) are at greater risk of

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colorectal cancer over time. Therefore, a series of recommendations has been made for the follow-up of this population. We carried out a review in which we set out the main new developments in the detection, follow-up, and management of dysplasia in patients with IBD in recent years.

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INTRODUCTION

The worldwide consensus among scientific societies is that screening for colorectal cancer (CRC) in patients with inflammatory bowel disease (IBD)[1-5] should be carried out by means of colonoscopy, preferably during the remission phase and with appropriate bowel preparation[6-10]. The objective is to detect potentially resectable premalignant lesions (dysplasia) and CRC in the early stages, thus improving prognosis[11-15]. While CRC-related mortality has decreased since the introduction of endoscopic screening techniques[16-19], the risk of CRC remains unchanged[20].

The differences in the recommendations of scientific societies on screening and surveillance in affected patients led us to review and update the recommendations of the various scientific societies and research groups in 2017[21]. Nevertheless, novel aspects and updates that have appeared since then are worthy of review, even though research in some areas remains incomplete.

SUMMARY OF OUR RECOMMENDATIONS

Below is a summary of our previously published recommendations

To whom should CRC screening be offered? Screening for CRC should be offered to the following patients affected by IBD: patients with ulcerative colitis (UC), regardless of its extent; patients with Crohn's disease (CD) affecting at least one-third of the colon or accompanied by complex perianal disease; patients with an ileoanal pouch; and patients with indeterminate or unclassified colitis. Endoscopy should be performed preferably in periods of clinical-biological remission and should enable an estimation of the individual risk of CRC, as well as the extent of the disease[21].

When should the first colonoscopy screening be performed? The first colonoscopy screening should be offered eight years after a diagnosis of CD or UC. In patients with primary sclerosing cholangitis (PSC), colonoscopy should be performed as soon as possible. In patients with an ileoanal pouch, colonoscopy should be performed one year after the surgical intervention. Patients with first-degree relatives who were diagnosed with CRC before age 50 should be offered the first endoscopy ten years before the age the family member was when affected by CRC or eight years after a diagnosis of IBD (whichever occurs earlier).

Who should be offered endoscopic surveillance? After screening, all patients should undergo endoscopy-based follow-up, except for those with ulcerative proctitis, CD with the involvement of less than one-third of the colon, and those in whom the risks outweigh the possible benefits.

Should the same endoscopic surveillance intervals be followed for all patients? No. We recommend that patients be stratified according to their individual risk.

Are there individual risk factors that allow us to stratify endoscopic surveillance? Yes. Among patients with left-sided UC or pancolitis and CD affecting at least one-third of the colon, high-risk patients are defined as those having any of the following: PSC, extensive involvement, moderate-severe active inflammation sustained over time (endoscopic or histological), a first-degree relative with CRC before age 50, or stenosis

or dysplasia detected during the previous five years. Intermediate-risk patients are defined as those having any of the following: extensive colitis with mild or moderate sustained active inflammation (endoscopic or histological), inflammatory polyps, or a first-degree relative with CRC after age 50. A diagnosis of IBD at a young age should be taken into account as a relative risk factor (due to the long duration of the disease). Factors other than high- and intermediate-risk factors should be considered low-risk factors. High-risk factors in patients with an ileoanal pouch are as follows: dysplasia or previous CRC, PSC, and type C mucosa in the pouch (persistent atrophy and severe inflammation) (Table 1).

How long should the endoscopic follow-up intervals be? Endoscopic follow-up intervals are recommended as follows: for patients with IBD, according to the presence of risk factors for each patient; for patients with high-risk factors, annual colonoscopy; for patients with intermediate-risk factors, colonoscopy every three years; for patients with low-risk factors or no other risk factors, colonoscopy every five years; for patients with an ileoanal pouch, according to the presence of risk factors; for patients with risk factors, annual colonoscopy; and for patients with no risk factors, colonoscopy every five years (Table 1).

What is the recommended endoscopic technique for screening and surveillance? Chromoendoscopy with endoscopic resection or biopsies aimed at visible lesions is the technique of choice. If this is not possible, high-definition video colonoscopy should be used, and four biopsies should be taken every ten cm of the colon.

How is chromoendoscopy performed? Methylene blue (0.04%-0.1%) or indigo carmine (0.1%-0.03%) is used. Caecal intubation is performed, and a dye is applied to the mucosa of the colon as the endoscope is removed, if possible, using a catheter spray. One segment should be examined before applying a dye to the next segment.

Does the occurrence of dysplasia require confirmation? The occurrence of dysplasia must be confirmed by a second pathologist.

What terminology should be used to describe lesions detected with endoscopy? The terms "dysplasia-associated lesion or mass (DALM)" and "flat lesions" should be discontinued. The modified Paris Classification, in which lesions are divided into visible dysplasia and invisible dysplasia depending on whether the biopsy has been taken from a lesion visualized in the colonoscopy or not, should be used. Visible dysplasia is divided into polypoid and non-polypoid depending on whether the lesion protrudes from the lumen ≥ 2.5 mm. The descriptions of visible lesions should also include a mention of whether they are ulcerated and whether the borders are easily distinguished from the surrounding mucosa (Table 2).

How should a visible lesion be managed? Visible lesions that are well delimited, with no evidence of dysplasia in the mucosa adjacent to the lesion and no synchronous dysplasia, should be resected endoscopically regardless of the degree of dysplasia.

How should invisible dysplasia be initially managed? Dysplasia that is not endoscopically visible but found in serial biopsies of the colon must be confirmed by an independent pathologist after chromoendoscopy is performed by an expert endoscopist. If dysplasia is confirmed, management will depend on the degree.

How should invisible dysplasia be managed in relation to the degree of dysplasia? Endoscopically invisible high-grade dysplasia is an indication for colectomy. The management of low-grade, invisible dysplasia should be agreed upon in a multidisciplinary committee and with the patient: the two possible options are colectomy and endoscopic follow-up.

How should endoscopically resected lesions be followed? The follow-up for resected lesions in healthy mucosa not affected by colitis should be the same as that for sporadic adenomas. Lesions that are endoscopically resected in areas affected by colitis should be examined endoscopically at three months and annually thereafter.

NOVEL ASPECTS IN SCREENING AND FOLLOW-UP

The most recent updates to clinical practice guidelines[22-24] do not contain substantial modifications with respect to candidates for screening, the periodicity of

Table 1 Risk factors for the development of colorectal cancer in patients with inflammatory bowel disease and recommended surveillance

	High risk	Intermediate risk	Low risk
Risk factors	(1) PSC; (2) Extensive involvement; (3) Moderate-severe active inflammation sustained over time (endoscopic or histological); (4) First-degree relative with CRC before age 50; (5) Stenosis or dysplasia detected during the previous five years; (6) Appearance of IBD at a young age; (7) If ileo-anal pouch: (a) Dysplasia; (b) Previous CRC; (c) PSC; and (d) Type C mucosa in the pouch	(1) Extensive colitis with mild or moderate sustained inflammatory activity (endoscopic or histological); (2) Inflammatory polyps; and (3) First-degree relative with CRC after age 50	(1) Factors other than high and intermediate risk; and (2) If ileo-anal pouch: Without risk factors
Surveillance	Annual	Every three years	Every five years

CRC: Colorectal cancer; IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis.

Table 2 SCENIC international consensus

Term	Definition
Visible dysplasia	Dysplasia identified on targeted biopsies from a lesion visualized in colonoscopy
Polypoid	Lesion protruding from the mucosa into the lumen ≥ 2.5 mm
Pedunculated	Lesion attached to the mucosa by a stalk
Sessile	Lesion not attached to the mucosa by a stalk: entire base is contiguous with the mucosa
Nonpolypoid	Lesion with little (< 2.5 mm) or no protrusion above the mucosa
Superficially elevated	Lesion with protrusion but < 2.5 mm above the lumen (less than the height of the closed cup of a biopsy forceps)
Flat	Lesion without protrusion above the mucosa
Depressed	Lesion with at least a portion depressed below the level of the mucosa
General descriptors	
Ulcerated	Ulceration (fibrinous base with depth) within the lesion
Border	
Distinct border	Border of the lesion is discrete and can be distinguished from surrounding mucosa
Indistinct border	Border of the lesion is not discrete and cannot be distinguished from surrounding mucosa
Invisible dysplasia	Dysplasia identified on random (non-targeted) biopsies of colon mucosa without a visible lesion

screening, or risk factors for CRC in these patients. Similarly, no novel aspects in this regard have been published in recent reviews[25]. While neither new research nor changes in current knowledge are expected in this field, it is worth taking into account the observation made by Burke *et al*[26], who concluded that patients with IBD and high-risk factors should be closely followed up at short intervals. These authors performed a retrospective review of interval CRC associated with IBD and not associated with IBD diagnosed between January 2007 and December 2014 in a large-scale American health system. When they compared cases of interval CRC associated and not associated with IBD, they found that associated cases were more common in younger patients (54.5 *vs* 70.4 years; $P < 0.001$); these had appeared within a shorter interval after the index colonoscopy (20.7 *vs* 35.1 mo; $P = 0.09$). An evaluation of adherence to American Society for Gastrointestinal Endoscopy guidelines revealed that 53% (8/15) of cases of interval CRC in patients with IBD were detected in line with surveillance guidelines. All of the patients with interval CRC detected after the surveillance period recommended in the guidelines had high-risk factors during the index colonoscopy, namely, active inflammation, multiple pseudopolyps, previous low-grade or indefinite dysplasia, or a family history (first-degree) of CRC[26].

A recent study analysed 9398 pouchoscopy procedures performed in 3672 patients and concluded that the low incidence rate for neoplasm recorded suggests that pouchoscopy was not routinely necessary in asymptomatic patients, in patients with a history of primary sclerosing cholangitis, or in patients with chronic pouchitis, although it is recommended when there is a personal or family history of CRC. More evidence is necessary before guidelines can be changed[27].

NOVEL ASPECTS OF THE RECOMMENDED ENDOSCOPIC TECHNIQUE

Up to the time of our review[21], there was a general consensus on using chromoendoscopy with methylene blue or indigo carmine as the technique of choice, mainly since the publication of the SCENIC CONSENSUS[28]. In situations where chromoendoscopy was not possible, the technique of choice was high-definition video colonoscopy and serial biopsies every ten cm of the colon. New subsequent guidelines maintained this recommendation[29], and real-life studies supported the decision[30].

However, the recommendation soon began to be called into question, and the new ACG guidelines stated the following: *When using high-definition colonoscopes in patients with UC undergoing surveillance, we suggest white-light endoscopy with narrow-band imaging or dye-spray chromoendoscopy with methylene blue or indigo carmine to identify dysplasia (conditional recommendation, low quality of evidence)*[23]. Furthermore, the difficulties arising with chromoendoscopy were reviewed by our group and included a long examination time, the need for optimal bowel preparation, specialist training, and higher costs[31]. These difficulties could have led to chromoendoscopy being performed less frequently than desired, despite recommendations from scientific societies on the application of the technique. Such were the findings of a recent online survey administered to academic gastroenterologists belonging to the Canadian Association of Gastroenterology, which revealed low uptake of chromoendoscopy as a surveillance tool for dysplasia in patients with IBD. The main barriers reported were the long duration of the procedure, cost, and the lack of experience or training in the technique[32].

The situation was recently evaluated by Bisschops *et al*[33], who performed a multicentre prospective randomized controlled trial of 131 patients with UC and found that chromoendoscopy and virtual chromoendoscopy (VCE) with narrow-band imaging (NBI) presented no significant differences for the detection of dysplasia or neoplasms associated with UC. Furthermore, the authors concluded that, given the reduced withdrawal time and greater ease of use, VCE with NBI should replace traditional chromoendoscopy. Clarke *et al* performed a retrospective analysis of cases and controls in which they concluded that there were no statistically significant differences in the detection of dysplasia using dye-spray chromoendoscopy compared with high-definition white-light colonoscopy, although the chromoendoscopy procedure took longer[34]. Gulati *et al*[35] performed a multifaceted randomized crossover trial to evaluate study feasibility and obtain preliminary comparative procedural and patient experience data. The authors found that the diagnostic accuracy of VCE was higher, although the differences were not statistically significant [93.7% (85.5%-98.2%) *vs* 76.9% (66.9%-85.1%) for chromoendoscopy]. In addition, the authors found that biopsy based on VCE was less frequent, with fewer dysplastic lesions overlooked than with chromoendoscopy and a failure rate of 9.1%, compared with 18.2% for chromoendoscopy. Patients generally reported a significant preference for VCE; most had a high risk of cancer requiring more intense and frequent surveillance. While the reasons for this preference were not explored, the authors suggest that it could be because the technique is less time-consuming than chromoendoscopy and there is no need for contrast or abdominal distension.

A study of 270 patients compared three different techniques for surveillance colonoscopy to detect colonic neoplastic lesions in IBD patients: high-definition, chromoendoscopy, and VCE using i-SCAN image–enhanced colonoscopy[36]. A randomized noninferiority trial was conducted to determine the detection rates of neoplastic lesions in IBD patients with longstanding colitis. The authors concluded that neither VCE nor high-definition colonoscopy was inferior to dye-spray colonoscopy for the detection of colonic neoplastic lesions during surveillance. Data from a study performed in Valencia, Spain, revealed no differences between VCE with standard i-Scan chromoendoscopy and VCE with indigo carmine 0.4% in the detection of dysplasia in the colons of patients with longstanding IBD[37]. However, VCE with i-Scan was a less time-consuming alternative. A meta-analysis from 2020 that included 17 randomized clinical trials and almost 2500 patients showed the superiority of dye-spray chromoendoscopy compared with standard-definition white-light endoscopy. No differences were detected when dye-spray chromoendoscopy was compared with high-definition white-light endoscopy or narrow-band imaging[38].

Therefore, it is very likely that we can use video chromoendoscopy as the technique of choice for the screening and follow-up of affected patients (Table 3).

Table 3 Summary of endoscopic detection techniques

Technique	Recommendation	Future
Standard-definition colonoscopy	None	No longer used
High-definition white-light video colonoscopy and serial biopsies every 10 cm of the colon	Avoid	No longer used
High-definition white-light video colonoscopy with dye-spray chromoendoscopy (methylene blue or indigo carmine)	High	Second choice
High-definition white-light video colonoscopy with narrow-band imaging	High	First choice
Full-spectrum endoscopy	Await further evidence	Under investigation
Autofluorescence imaging	None	No longer used
Confocal laser endomicroscopy	Await further evidence	Under investigation
Endocytoscopy	Investigate	Investigate

METHOD FOR TAKING BIOPSY SPECIMENS

The methods for taking targeted and serial biopsy specimens during chromoendoscopy might also be subject to change. This aspect was evaluated in a French randomized controlled trial[39], in which the authors evaluated whether taking serial biopsy specimens increased the detection rate for neoplasms in patients who had undergone screening for CRC and UC. They observed that while the yield was low, random biopsies for the detection of dysplasia should be combined with chromoendoscopy in patients with IBD and concomitant PSC, a personal history of neoplasm or the lead pipe sign. Furthermore, the rate of detection of additional neoplasms was 15% in this subgroup of patients. Along the same lines, Hu *et al*[40] recently published the results of a retrospective study of 300 patients with IBD in which they analysed the detection of dysplasia with targeted and random biopsy in 422 colonoscopies. The authors found that in up to 18% of the colonoscopies, dysplasia was identified using random biopsy. Risk factors such as concomitant PSC, a longer duration of disease, and endoscopically active disease increased the likelihood of a dysplastic lesion being detected in random biopsy, suggesting that specific high-risk patients could benefit from this strategy for the detection of dysplasia.

Previous reviews have suggested that additional biopsy specimens should be taken from the area of flat mucosa surrounding dysplastic polypoid lesions resected in IBD to confirm the absence of residual neoplasm *in situ*[28]. Nevertheless, recent studies consider these biopsies to be of low and even insignificant diagnostic yield and not predictive of findings in subsequent follow-up colonoscopy[41]. The findings reported cast doubt on the usefulness and yield of this procedure. New, high-definition techniques and chromoendoscopy may obviate the need for the biopsy of the adjacent mucosa to detect invisible dysplasia in many patients[42,43]. A recent retrospective cohort study compared the long-term effectiveness of targeted biopsy of suspected lesions with that of random biopsy and found robust evidence for targeted biopsy in the prevention of death from colon cancer. However, since panchromoendoscopy was used in only 4.6% of patients, the findings cannot be extrapolated[44].

Today, doubt remains as to which surveillance technique is best for patients with IBD and the involvement of the colon but no other risk factors for CRC: high-definition colonoscopy with random biopsy, VCE and targeted biopsy, or high-definition colonoscopy with targeted biopsy.

ENDOSCOPIC-HISTOLOGIC EVALUATION

The Paddington International Virtual ChromoendoScopy ScOre (PICaSSO) is a recently reported VCE scoring system in UC to redefine endoscopic findings of mucosal and vascular healing developed by international experts in optical diagnosis. Interobserver agreement on the pretest and the post-test evaluation was very good for the Mayo endoscopic score, Ulcerative Colitis Endoscopic Index of Severity, Robarts Histological Index, and a full spectrum of histological changes[45] (Table 4). Furthermore, a recent real-life study revealed that the PICaSSO score correlated

Table 4 The Paddington International Virtual ChromoendoScopy ScOre in ulcerative colitis

PICaSSO Mucosal Architecture	PICaSSO Vascular Architecture
0 - No mucosal defect	**0 - Vessels without dilatation**
A: Continuous/regular crypts	A: Roundish following crypt architecture
B: Crypts not visible (scar)	B: Vessels not visible (scar)
C: Discontinuous and or dilated/elongated crypts	C: Sparse (deep) vessels without dilatation
I - Micro erosion or cryptal abscess	**I - Vessels with dilatation**
1: Discrete	A: Roundish with dilatation
2: Patchy	B: Crowded or tortuous superficial vessels with dilatation
3: Diffuse	
II – Erosions, size < 5 mm	**II - Intramucosal bleeding**
1: Discrete	A: Roundish with dilatation
2: Patchy	B: Crowded or tortuous superficial vessels with dilatation
3: Diffuse	
III – Ulcerations, size > 5 mm	**III - Luminal bleeding**
1: Discrete	A: Roundish with dilatation
2: Patchy	B: Crowded or tortuous superficial vessels with dilatation
3: Diffuse	

PICaSSO: Paddington International Virtual ChromoendoScopy ScOre.

strongly with multiple histological indices and that, similar to histology, it predicted specified clinical outcomes at 6 and 12 mo. The authors concluded that PICaSSO can be a useful endoscopic tool in the therapeutic management of UC[46].

SERRATED EPITHELIAL CHANGE

In recent years, several studies have investigated the involvement of serrated epithelial changes in patients with IBD. This histopathological finding, which appears in patients with a long history of colitis in areas of nodular mucosa, is characterized by crypt distortion with diffuse striae and an epithelium rich in goblet cells. Parian *et al* recently performed a case-control study and a systematic review with a meta-analysis of 196 patients with UC in which they found higher rates of synchronous or metachronous tumours in patients with IBD and serrated epithelial change, thus leading us to believe that these patients require closer monitoring, possibly with yearly endoscopy[47].

PATIENTS WITH A LARGE NUMBER OF POLYPS IN SURVEILLANCE ENDOSCOPY

When pseudopolyps are found along an extensive area of the colon and it is impossible to remove them all appropriately to evaluate dysplasia, possible options should be discussed with the patient. Given the risk of nonidentified neoplasms, prophylactic proctocolectomy should be considered. In specific cases (patients with advanced age, high surgical risk, and refusal to undergo proctocolectomy), closer follow-up should be considered as an alternative[1,23]. With respect to this clinical situation, there have been no changes in the recommendations. This aspect should be evaluated in future studies, mainly with emerging endoscopic techniques.

INTEROBSERVER AGREEMENT IN THE HISTOLOGY ANALYSIS

The difficulties involved in interpreting histology specimens are well known with respect to the presence/absence and grade of dysplasia[48].

A recent study analysing interobserver agreement on histology findings in inflammatory bowel disease found that, with respect to the presence of dysplasia, interobserver agreement was moderate, with greater agreement for high-grade dysplasia. In addition, when endoscopic data and histological data were combined, dysplasia-associated lesions or masses were more common than adenoma-like masses. Therefore, the authors proposed using immunohistochemistry to increase the capacity for detecting these lesions, as well as a review of samples by a pathologist specializing in digestive diseases[49].

NEW ENDOSCOPIC TECHNIQUES

Full-spectrum endoscopy

Full-spectrum endoscopy (FUSE) comprises 2 Lateral cameras in addition to the traditional front camera, and this configuration makes it possible to see behind the folds and blind spots, thus providing a panoramic view.

Leong *et al*[50] performed a prospective, randomized, crossover tandem colonoscopy study of 52 patients to compare standard forward-viewing colonoscopy (FVC) with FUSE for the detection of dysplasia in patients with IBD. FUSE revealed significantly more dysplastic lesions than FVC, although the withdrawal time was significantly greater. Targeted biopsy revealed significantly more dysplastic lesions than random biopsy ($P < 0.0001$).

Autofluorescence imaging

Autofluorescence imaging (AFI) is based on the detection of natural tissue fluorescence emitted by endogenous molecules (fluorophores) such as collagen, flavins, and porphyrins. After excitation by a short-wavelength light source, these fluorophores emit light of longer wavelengths (fluorescence). The overall fluorescence emission differs between the various tissue types owing to the corresponding differences in fluorophore concentration, metabolic state, and/or spatial distribution. These colour differences in fluorescence emission can be captured in real time during endoscopy and used for the detection or characterization of lesions[51]. A multicentre international prospective randomized controlled trial compared images taken with AFI with chromoendoscopy for the detection of dysplasia in 210 patients with UC. The relative detection rate for dysplasia had to be greater than 0.67 (based on an 80% confidence interval) to justify a subsequent noninferiority trial. Dysplasia was detected in 20 patients (19%) using chromoendoscopy and in 13 (12%) using AFI. Therefore, AFI proved to be inferior to chromoendoscopy, and the criteria for performing a large-scale noninferiority trial were not fulfilled. The authors concluded that AFI should not be investigated as an alternative approach for monitoring the presence of dysplasia[52].

Confocal laser endomicroscopy

Confocal laser endomicroscopy (CLE) is a new method used for obtaining *in vivo* images of abnormalities of the mucosa at the subcellular level. The technique uses intravenous fluorescent agents, contrast, and a specialized probe that can be passed through the working channel of an endoscope, thus enabling real-time 1000-fold magnification of the mucosa of the colon. CLE has been shown to help differentiate among neoplasms, solitary adenomas, and benign regenerative changes (which can be seen in much the same way as dysplasia), with 97.8% accuracy[53].

Pilot trials have been performed with variations of this technique. One study evaluated the potential role of CLE combined with a fluorescein-labelled peptide to stain and detect dysplasia associated with UC. A heptapeptide derived from phages with marked affinity for dysplastic tissue was synthetized and labelled with fluorescein (VRPMPLQ peptide). The study included 9 patients who underwent the resection of 11 Lesions. The different affinities of fluorescein for nondysplastic tissue, inflammatory polyps, and dysplastic tissue enabled better characterization. The authors concluded that VRPMPLQ is a promising approach for the detection of dysplasia in patients with IBD. More *in vivo* studies in larger populations are required to evaluate the effective contribution of this molecular probe in the management of lesions detected during the surveillance of patients with UC[54]. However, the results of these studies should be confirmed in larger series. CLE is currently restricted to

research.

Endocytoscopy

Endocytoscopy is a novel ultra-high magnification endoscopic technique designed to provide excellent *in vivo* assessment of lesions found in the gastrointestinal tract. When used with intraprocedural stains, endocytoscopy enables microscopic visualization of the gastrointestinal mucosal surface[55,56].

Nevertheless, the application of endocytoscopy in the diagnosis of colitis-associated CRC has received little attention. A recently published case report revealed that endocytoscopy can be used to obtain information on the nuclei and lumen of the glands in colitis-associated CRC. Furthermore, colitis-associated CRC is often difficult to diagnose owing to the effects of inflammation, and the use of endocytoscopy can reduce the frequency of unnecessary biopsies[57].

DEVICES FOR IMPROVING ENDOSCOPIC VISION

Endocuff Vision (ARC Medical Design Ltd) is a distal colonoscopic accessory with smooth projections in the form of fingers that aims to flatten mucosal folds, and it has been shown to improve the rate of the detection of adenomas in patients with IBD undergoing screening for CRC[58]. The device is not currently recommended in patients with severe colitis because of concerns over mucosal injury[59], although it has not been previously studied in clinically active patients with IBD undergoing surveillance colonoscopy for the detection of dysplasia. A study of 25 patients evaluated the safety and viability of Endocuff Vision–assisted high-definition chromoendoscopy in patients with clinically active UC undergoing surveillance for dysplasia. The authors concluded that the technique is feasible and safe in patients with UC undergoing surveillance colonoscopy to rule out dysplasia[60]. Other studies reported that Endocuff could facilitate polypectomy, especially in the flexible folds of the sigmoid colon, thus enabling greater stabilization of the endoscope when facing the polyp[61]. In the future, it will be necessary to determine whether these devices can increase the rate of detection of polyps and facilitate their extraction without increasing the rate of complications in patients with UC.

COLON CAPSULE ENDOSCOPY

A priori, capsule endoscopy is an attractive option for screening CRC in patients with IBD because it is noninvasive and has proven useful in certain situations, namely, after incomplete colonoscopy, when the patient refuses colonoscopy, or when sedatives are contraindicated.

Regarding its yield, second-generation capsule endoscopy (CE-2) is almost at the same level as colonoscopy, with 80%–95% sensitivity for the detection of polyps ≥ 6 mm. The limitations of CE-2 in CRC are that it cannot be used for biopsy, for obtaining samples of the intestinal mucosa, or for resecting any lesions detected[62].

This is a field for future research, which will undoubtedly provide huge surprises in the coming years. Prototypes with steerable and self-propelling technology have already been designed[63].

COMPUTER-AIDED DETECTION

Nonblinded trials have shown that colonoscopy with computer-aided detection (CADe) improves the detection of polyps and adenomas in the colon by providing visual alarms during the procedure[64]. The technique was developed in China in a randomized, double-blind clinical trial aimed at evaluating the efficacy of a CADe system. The study excluded patients with IBD. The rate of the detection of adenomas was significantly greater in the CADe group than in the control group[65]. In the future, it will be necessary to evaluate whether computational devices that facilitate detection are valid for patients with IBD.

COSTS ASSOCIATED WITH SCREENING

Few studies have evaluated the costs—and more especially the cost-effectiveness—of screening in patients with UC. One of the first studies to do so was published by Provenzale *et al*[66] in 1998, who analysed the cost-effectiveness of CRC screening programmes and found a favourable association with screening. However, endoscopic technology has changed considerably since then.

The study by Konijeti *et al*[67] in 2014 evaluated 3 strategies with different surveillance intervals—chromoendoscopy with targeted biopsy, white-light endoscopy with random biopsy, and no surveillance—taking into account the financial costs and the characteristics of the United States health system. However, compared with no surveillance, chromoendoscopy was only cost-effective at intervals of at least 7 years, with an incremental cost-effectiveness of $77176. Chromoendoscopy was the most cost-effective strategy, with a sensitivity > 0.23 for the detection of dysplasia and a cost < $2200, regardless of the sensitivity of WLE for the detection of dysplasia. The estimated population lifetime risk of developing CRC ranged from 2.5% (annual chromoendoscopy) to 5.9% (chromoendoscopy every 10 years).

This aspect should be evaluated in the different health systems, given the large potential differences in the cost of screening programmes between countries.

CHEMOPREVENTION

Cancer chemoprevention involves the chronic administration of a synthetic, natural, or biological agent to reduce or delay the occurrence of malignancy[68]. In addition, the agent administered must be effective, safe, acceptable to patients, and inexpensive.

Chronic inflammation of the mucosa in patients with UC is the main risk factor for the development of CRC[69].

No medical treatment has proven able to sufficiently prevent dysplasia or CRC and thus obviate the need for colonoscopic surveillance in UC[23].

Chemoprevention is considerably controversial owing to the diversity of studies and varying reports on adherence to treatment, and available evidence supports the role of 5-ASA as chemopreventive therapy[70]. Immunomodulators could play a role owing to their control of mucosal inflammation, at least in a subgroup of patients[71]. The control of inflammation achieved with anti-TNFa agents would probably reduce the risk of CRC in patients with UC. Even in more recent observational studies, the number of patients exposed to tumour necrosis factor-alpha inhibitor agents is too low to adequately evaluate the potential chemopreventive effects of these agents[71]. In UC and concomitant PSC, low-dose ursodeoxycholic acid could reduce the risk of CRC, although evidence for this hypothesis is weak[72,73].

While much remains to be done in the field of the chemoprevention of CRC in patients with UC, the main barrier in the coming years will be the difficulty in performing clinical trials that provide more robust evidence.

CONCLUSION

Despite the advances made in the field, the following areas warrant further study: (1) The tailoring of surveillance intervals to the individual patient; (2) Continuous updating of recommendations based on the best available evidence; (3) Greater adherence by physicians to the recommendations of scientific societies; (4) The development of noninvasive biomarkers that could support or act as a screening approach before endoscopy; and (5) Efforts to reduce barriers to surveillance, including safe extension of intervals in patients with a lower risk.

The challenge in the coming years will be to match the individual patient with the best option for endoscopic surveillance. We must bear in mind the large number of newly diagnosed patients, in addition to those currently under surveillance and the fact that they are ageing, together with the increasing price of new technology and surveillance modalities. The maintenance of an adequate and efficient physician-patient relationship in decision making will be the greatest challenge we have to face in the coming years.

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MINIREVIEWS

Viral hepatitis: Innovations and expectations

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Abstract

Viral hepatitis is a significant health problem worldwide, associated with morbidity and mortality. Hepatitis B, C, D, and occasionally E viruses (HBV, HCV, HDV, and HEV) can evolve in chronic infections, whereas hepatitis A virus (HAV) frequently produces acute self-limiting hepatitis. In the last years, different studies have been performed to introduce new antiviral therapies. The most important goal in the treatment of viral hepatitis is to avoid chronic liver disease and complications. This review analyzes currently available therapies, in particular for viruses associated with chronic liver disease. The focus is especially on HBV and HCV therapies, investigating new drugs already introduced in clinical practice and clinical trials. We also describe new entry inhibitors, developed for the treatment of chronic HDV and HBV and currently available treatments for HEV. The last drugs introduced have shown important efficacy in HCV, with achievable target HCV elimination by 2030. Concurrently, renewed interest in curative HBV therapies has been registered; current nucleotide/nucleoside analogs positively impact liver-related complications, ensuring high safety and tolerability. Novel approaches to HBV cure are based on new antivirals, targeting different steps of the HBV life cycle and immune modulators. The improved knowledge of the HDV life cycle has facilitated the development of some direct-acting agents, as bulevirtide, the first drug conditionally approved in Europe for HDV associated compensated liver disease. Further studies are required to identify a new therapeutic approach in hepatitis E, especially in immunosuppressed patients.

Key Words: Viral hepatitis; Chronic liver disease; Treatments; Antiviral; Immunotherapy; Vaccination

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Core Tip: Viral hepatitis is a known worldwide health problem, with a risk of evolution in chronic liver disease. Novel therapies have shown increased efficacy in curing the hepatitis C virus (HCV), with the goal of HCV elimination by 2030. New concurrent interest in hepatitis B virus (HBV) curative therapies has been recently registered: New antivirals targeting different steps of HBV life cycle and immune modulators. In hepatitis D, the improved knowledge of the life cycle has facilitated the development of some direct-acting agents, more effective than interferon-based therapies. New studies are required to improve the treatment of hepatitis E.

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INTRODUCTION

Viral hepatitis represents a health problem that affects millions of people worldwide and is associated with high mortality. Except for hepatitis A virus (HAV), all hepatotropic viruses, including hepatitis B, C, D, and E viruses (HBV, HCV, HDV, and HEV), can produce chronic infections, whereas HAV causes acute self-limiting hepatitis that normally resolves spontaneously. In this review, we provide a brief outline of currently available therapies for major hepatotropic viruses associated with chronic liver disease. We focus especially on therapies that halt HBV progression and achieve a definitive HCV cure, investigating new drug clinical trials. We also describe bulevirtide, an entry inhibitor, developed for the treatment of chronic HDV and HBV infections and approved in the European Union for the therapy of chronic HDV infection in patients affected by liver disease, without decompensation. We describe currently available treatments for HEV with their limitations, pointing out the emergent need for novel antivirals not only safe but above all effective particularly for patients not eligible for interferon (IFN)-based regimes.

HEPATITIS B

HBV infection is the main cause of chronic liver disease in the world, affecting 257 million people globally, and is responsible for about 54% of the cases of hepatocellular carcinoma (HCC)[1].

The current goal for HBV treatment is to suppress viral replication, halt disease progression, and prevent cirrhosis development, liver failure, and HCC. The approved agents for the treatment of chronic HBV infection are not curative and can be classified into two categories: Pegylated IFN (peg-IFN) and nucleotide or nucleoside analogs (NAs)[2].

In particular, there are eight approved drugs: IFN and peg-IFN, lamivudine, adefovir, telbivudine, entecavir, tenofovir disoproxilfumarate (TDF), and tenofovir alafenamide fumarate (TAF). Besifovir dipivoxil is only approved in Korea. Their action is limited to suppress HBV replication and decelerate progression to cirrhosis and HCC.

Current and future aims of treatment

The current aim of antiviral treatment is the induction of two different responses: Virologic and biochemical[3].

Virologic response: A virologic response during NA therapy is defined as undetectable HBV-DNA. In the course of IFN treatment, a virologic response is obtained if serum HBV-DNA level falls below 2000 IU/mL, 6 mo after the start of treatment and at the end of treatment.

Biochemical response: A biochemical response is obtained when alanine aminotransferase falls into the normal range.

Functional cure: Corresponding to a durable hepatitis B surface antigen (HBsAg) loss, with or without seroconversion, is achieved rarely in the natural history of chronic hepatitis B: In only 10%-20% of Caucasian patients and less than 5% Asian patients[4]. HBsAg seroclearance correlates with a reduced risk of HCC, therefore it represents an advisable goal of treatment. The functional cure aspires to sterilize cells neither from integrated HBV-DNA nor from covalently closed circular DNA (cccDNA), a persistent form of the HBV genome in infected hepatocytes.

Complete cure: Complete cure is a functional cure with the elimination of cccDNA, which acts as a potential reservoir for reactivation. Complete HBV elimination is difficult, due to the existence of intrahepatic cccDNA and its ability to self-replenish: cccDNA serves as a template for the transcription of pre-genomic RNA, allowing the production of viral DNA and proteins even without detectable HBV-DNA or HBsAg [5,6]. A complete HBV cure consists of a full HBV eradication through the removal of all viral elements from an infected patient. To achieve this goal, combination therapy is probably required[7]. Treatments currently available, peg-IFN and NAs, have no direct effect on cccDNA that persists in the hepatocyte nucleus.

An additional obstacle to HBV elimination is the viral DNA integration into the host genome, sufficiently intact to support the translation of viral protein[8]. Integrated HBV-DNA can be a source of circulating HBsAg; definitive clearance of HBV would require the removal of hepatocytes that harbor this DNA.

The persistence of HBV in the liver of patients who have recovered from acute HBV infection can explain the risk of HBV reactivation in the course of potent immunosuppressive therapy and the potential transmission of HBV infection when these livers are transplanted into seronegative recipients[9].

An additional limitation to HBV elimination is impaired innate and adaptive immune responses in HBV patients. Chronic HBV patients fail to unleash efficient immune responses to HBV if cytotoxic T cells are hypo-responsive to HBV itself. For a complete cure, the restoration of suppressed host immunity is required[10].

New biomarkers in diagnosis and management of HBV infection

To diagnose acute and chronic HBV infections, some tests are routinely used. The identification of HBV presence and activity is essential in the diagnostic and therapeutic algorithm of infected patients. In clinical practice, there are routinely used assays to detect and measure serum levels of HbsAg, HbeAg, anti-HBs, and HBV DNA. Some of them are strong risk predictors of the development of HCC[11].

New diagnostic tools are needed to assess the efficacy of therapy and monitor with precision the response. The most interesting includes tests to measure HBV RNA and hepatitis B core-related antigen (HBcrAg). Levels of serum RNA indicate the presence of cccDNA transcriptional activity and might be used to monitor response to treatment and identify patients eligible for the safe discontinuation of NA. Also, HBcrAg might be used as a marker of cccDNA and its quantification could help to monitor response to current therapies[12]. New biochemical tools are in development to improve the diagnostic approach to virus activity and reservoir.

Available treatments: Pros and cons

Peg-IFN has lower antiviral activity but a higher rate of HBeAg and HBsAg loss than NAs, thanks to its immunomodulatory effect. It is administered as subcutaneous injections, which is a clear drawback, once weekly. It amounts to multiple adverse effects. And its use is strictly prohibited in patients with decompensated cirrhosis and autoimmune and psychiatric illnesses. Caution is mandatory in patients with compensated cirrhosis because it may precipitate hepatic decompensation. A 1-year course of pegylated IFN results in a rate of HBeAg seroconversion and HBsAg loss of 30% and 3%, respectively, in patients who are HBeAg-positive. Undetectable HBV-DNA is achieved in 25% of cases. The response is durable and rates of HBeAg and HBsAg loss increase after the end of treatment (to about 8%). The finite duration of treatment (48-52 wk) is a strong point[13].

NAs are administered orally, have irrelevant adverse effects, and can be safely administered in patients with decompensated cirrhosis or acute liver failure[14]. Their main drawback is the need for long-term treatment in the majority of the patients.

Among NAs, ETV, TDF, and TAF are normally preferred; they boast high antiviral activity and barriers to resistance. After 1 year of treatment of HBeAg positive patients, NAs allow to get HBeAg seroconversion and HBsAg loss, respectively, in 10%-21% and < 1%-3%. Rates of undetectable HBV-DNA (61%-76%) are higher than those achieved by IFNs. HBeAg negative patients treated for a year with pegylated

IFN, achieve a higher rate of HBsAg loss (4% *vs* 1%) than the same duration of ETV, TDF, or TAF, despite a lower rate of HBV-DNA clearance (63% *vs* 94%). TAF is a prodrug of TDF, a nucleotide analog that inhibits reverse transcription of the human immunodeficiency virus (HIV) and HBV[15]. It has similar antiviral activity as TDF but its superior plasma stability and more effective active metabolites, allow the use of lower doses with similar antiviral activity and less systemic exposure. It has, therefore, a smaller damaging impact on glomerular filtration rate and bone mineral density.

What's in store for the future? Antivirals

Following the mirage of a sterilizing cure able to eliminate both cccDNA and integrated HBV-DNA, new approaches are being developed for the treatment of HBV.

Here we describe new drugs for HBV, including drugs that target different steps of the HBV life cycle and immune modulators. Some of them are under phase 2 trials.

Entry inhibitors: Some novel drugs aim to block HBV entry into hepatocytes that may protect hepatocytes not yet infected or antagonize *de novo* infections. The sodium taurocholate co-transporting polypeptide (NTCP) is the entry receptor for HBV/HDV into hepatocytes. Bulevirtide (Myrcludex B) is a peptide that mimics the NTCP-binding domain of HBV, blocking HBV and HDV entry into naïve hepatocytes[16].

Bulevirtide is approved in the European Union for the treatment of chronic HDV infection in compensated liver disease.

A multicenter, open-label phase 2 clinical trial (MYR203) assessed the efficacy of myrcludex B in combination with peg-IFNα2a in HDV/HBV co-infected patients. Sixty co-infected patients were randomized to receive peg-IFN or peg-IFN plus bulevirtide, or bulevirtide for 48 wk. Among the 60 patients, 27% of them in the peg-IFN plus bulevirtide group achieved HBsAg loss at 48 wk of treatment[17].

NTCP is a transporter of conjugated bile salts from the plasma into hepatocytes. Consequently, adverse events such as elevated plasma bile acids were reported in a dose-dependent manner but without clinically relevant effects (such as cholestasis and consequent pruritus or steatorrhea)[18].

Most of these studies are focused on HBV/HDV co-infection, but we expect the result of a new trial in chronic hepatitis B mono-infection.

Capsid assembly modulators: Inhibiting nucleocapsid formation is a promising strategy for HBV therapy. Nucleocapsid contains the viral DNA needed for replication. Two core protein allosteric modulators (CpAM-capsid assembly modulators) exist: Class I CpAMs lead to the formation of misassembled capsids. Class II CpAMs accelerate capsid assembly and form morphologically normal capsids that are empty.

NVR 3–778, first-in-class CpAM, has been evaluated in different studies. In a phase 1 study of HBeAg-positive patients with chronic infection, NVR 3-778 was well tolerated[19]. The largest drop in HBV-DNA levels was achieved in the cohort treated with NVR 3-778 in combination with pegIFN.

GLS4 is an inhibitor of HBV capsid assembly that induces the formation of aberrant nucleocapsid structures. A randomized, open-label phase 1b study examined the efficacy of GLS4 (at different doses) in combination with ritonavir for treating chronic HBV infection compared with entecavir alone. GLS4 120 mg administrated for 28 d, resulted in a reduction in serum HBV-DNA and HBV pre-genomic RNA (pgRNA) levels without major side effects[20].

RO7049389 disrupts HBV nucleocapsid assembly and induces the depletion of core proteins, thereby effectively inhibiting HBV replication. A multicenter, ongoing phase 1 study is investigating the efficacy of single and multiple doses of RO7049389 in healthy volunteers and chronic HBV participants. RO7049389 administration to HBV patients at different dosages, for 28 d, achieved a drop of HBV-DNA and RNA, in both HBeAg positive and negative patients with HBV-DNA levels lower than the lower limit of quantification in 81.3% of them[21].

JNJ-6379 (JNJ-56136379) is a class II CpAM that interferes with capsid assembly and inhibits *de novo* formation of cccDNA. A phase II study is ongoing. JNJ-6379 administrated for 4 wk to treatment-naïve patients with CHB, showed potent antiviral activity: HBV-DNA and HBV-RNA decreased from baseline in all patients. On day 29, 32% of 41 patients had levels of HBV-DNA below the lower limit of quantification[22]. The most interesting drop of HBV-DNA and RNA was found in the Asian cohort treated at a dose of 75 mg/d. After discontinuation of treatment, the viral load returned to baseline levels[23]. JNJ-6379 did not interfere with HBsAg or HBeAg.

ABI-H0731 (vebicorvir) is a potent and selective class II CpAM. In the 101B study, ABI-H0731 was administrated to HBeAg-positive patients for 28 d and obtained a

dose-dependent HBV-DNA reduction (maximum decline of 4.0 $\log_{10}$ IU/mL) with a parallel HBV RNA decline. No changes in HBsAg, HBeAg, or HBV core-related antigen were reported[24].

JNJ-64530440 and QL-007**,** two assembly modulators, and ABI-H2158, a core protein binding, are further interesting objects of study.

We need to further studies to establish whether CpAMs can eliminate HBsAg, HBeAg, and/or cccDNA but the combination strategy seems to be the better one.

Post-transcriptional inhibitors: Synthetic small interfering RNAs (iRNA) or silencing RNAs, are a class of non-coding RNAs that recognize complementary viral mRNA and pgRNA, inducing mRNA degradation after transcription and preventing translation [25]. In the contest of HBV infection, small iRNAs bind HBV mRNA in hepatocytes and induce its degradation. Their target is all RNA transcripts, derived from cccDNA [26].

ARC-520 was the first iRNA therapeutic targeting HBV. A single-dose phase 2 study showed that HBsAg was reduced more in HBeAg positive patients than in HBeAg negative or treatment-experienced patients[8]. To explain these findings, it should be recalled not only that HBsAg production mostly depends on integrated HBV-DNA, which is the main source in HBeAg negative and NA-experienced patients but also that ARC-520 is not able to target mRNA produced by integrated HBV-DNA.

Two randomized multicenter studies evaluated the HBsAg decrease after multiple doses of ARC-520 compared to placebo, in NA–experienced HBeAg negative or positive patients[27]. A high dose of ARC-520 (2 mg/kg) reduced HBsAg. The absolute reduction was larger in the HBeAg positive cohort, confirming that a significant proportion of HBsAg, in HBeAg negative patients, could derive from integrated HBV sequences. Ideal iRNA should target all viral transcripts. A second-generation iRNA targeting all HBV transcripts, ARC-521, reduces HBsAg and viral load as demonstrated in a phase I trial (NCT02797522). Both ARC-520 and ARC-521 studies were stopped for the lethal toxicity of specific delivery vehicles in non-human primates. A modified iRNA, JNJ-3989 (formerly ARO-HBV), is under investigation (NCT03365947). The HBsAg decline is strong in treatment naïve and experienced patients regardless of HBeAg status due to its ability to silence mRNA from cccDNA and host integrated viral DNA. ARB-1467 was tested in a phase II study, showing a consistent decline of HBsAg in 63.6% of patients (NCT02631096). An ongoing study is evaluating the efficacy of VIR-2218 in infected volunteers[28]. Preliminary results are promising to show a decline in HBsAg, but we are waiting for official data.

Inhibitors of HBsAg release: HbsAg proteins are not only incorporated into the viral envelope but also stored in non-infectious subviral envelope particles (SVP) produced by HBV, independently of viral replication. These particles, without capsid or genome, account for an important source of HBsAg in the blood and induce immune exhaustion against HBsAg. To eliminate spherical SVP is a major goal of a functional cure. Nucleic acid polymers (NAPs), such as REP 2139, target the assembly/secretion of SVPs. An open-label, phase 2 study tested the effectiveness of REP 2139 or REP 2165 combined with TDF and peg-IFN in HBeAg-negative CHB patients. The addition of NAPs to TDF and peg-IFN, without reducing the tolerability, significantly increased rates of HBsAg loss and HBsAg seroconversion during therapy[29]. After 12 mo of treatment, HBsAg levels were very low in more than half of the patients and after a temporal interval of 1 year, virologic control persisted in about half of them.

Neutralization: Lenvervimab is a recombinant human monoclonal IgG1-type anti-S antibody that binds HBsAg, inhibiting viral penetration into hepatocytes and reducing HBsAg levels. The neutralization of virions or HBsAg is obtained by immune complexes. The HBV neutralizing activity of lenvervimab had previously been demonstrated in a chimpanzee animal model[30].

In a prospective, open-label phase I trial, a single injection or 4 weekly doses of lenvervimab were administered (doses from 80.000 to 240.000 IU) to HBeAg-positive patients, reducing levels of HBsAg to below undetectable levels for up to 1 mo[31].

Inhibitors of cccDNA: As mentioned above, viral transcription requires a template, consisting of cccDNA. Curative treatment for HBV infection would require a disablement of cccDNA and several mechanisms have been studied to eradicate or silence cccDNA expression. The possibility of using sequence-specific RNA-guided nucleases (RGNs) and proteins has been explored. Among RGNs, the best present results are linked to transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) systems[32-34].

Nucleases cleave sequences in the HBV genome, resulting in mutated cccDNA, which is, in turn, transcribed into mutated viral proteins, not adequate to viral replication. Preclinical experiments showed that the CRISPR/Cas9 system, the most efficient method for gene inactivation, elicits inactivation of cccDNA, through mutations and deletions, but the application of this technology in a clinical setting requires further studies. For the clinical application, several challenging issues must be solved, first of all, the risk of genome instability.

Table 1 summarizes the main molecules to treat hepatitis B with the synthesis of the mechanism of action.

What's in store for the future? Immunotherapy

The progression to chronic HBV infection seems to be favored by weak immune responses and labile innate immune responses. It has been demonstrated that, in "recovered" patients, the early HBsAg clearance is fostered by activation of different cellular receptors [Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)] that allow the production of antiviral cytokines (IFN-α primarily), and, activation of natural killer (NK) cells. Activated innate immunity also boosts the adaptive immune system, leading to the maturation of B- and T-cell clones that target infected hepatocytes.

The progression to chronic HBV infection is determined by dysfunctional NK cells and plasmacytoid dendritic cells (pDCs). High levels of HBsAg or HBeAg not only block maturation of antigen-presenting cells such as DCs, causing a tolerogenic environment, but also deregulate the production of IFN and other cytokines by NK cells, NK T cells, and Kupffer cells. Prolonged exposure to viral antigens could be associated with functionally impaired immune response against the virus. Furthermore, HBV-specific cytotoxic T cell response, essential for clearance of infected hepatocytes, is inadequate with a poor cytotoxic activity and impaired cytokine production. This phenomenon has been described as "T cell immune exhaustion" and is responsible for HBV persistence. Several studies have reported overexpression of programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) in CD4+ or CD8+ T cells during chronic HBV infection.

Among compounds highly active against innate immunity, some immune stimulators (such as inarigivir) and TLR agonists (such as TLR7 and TLR8) have been studied. Among immunologic agents with activity against adaptive immunity, we must remember therapeutic vaccines (*e.g.*, GS-4774) and anti-PD-1 antibodies (*e.g.*, nivolumab) that are objects of ongoing studies.

TLR agonists: TLRs constitute the first line sensors of microorganisms and activate the production of mediators including IFNs and cytokines. TLR agonists promote up-regulation of type I IFN and cytokines, stimulating innate immunity response directed against the virus. The activation of TLR mediated pathways leads to the suppression of HBV replication. As an agonist of TLR-7 involved in IFN production, GS-9620 was tested in a human hepatocyte cell line infected with HBV; it showed suppression of HBV replication but no reduction in cccDNA levels. In woodchucks and chimpanzees, the TLR-7 agonist showed similar results. When used in humans with HBV-DNA suppression in the course of NA therapy, TLR-7 agonist (vesatolimod GS-9620) did not obtain any effect on HBsAg, HBeAg, and HBV-RNA levels despite inducing an HBV specific immune response. Another agonist for TLR-8 (Selgantolimod GS-9688) was investigated in patients with CHD and chronic suppression of the viral load, showing no significant impact on HBsAg, HBV-DNA, or RNA. Now this study is in phase II [35]. Several other TLR-7 and TLR-8 and 9 agonists are now entering clinical trials.

Retinoic acid-inducible gene-1 agonists: Type III IFNs are produced in human hepatocytes against HBV, through RIG-I. Viral RNA activates this intracytoplasmic receptor, leading to the IFN response that is essential for antiviral immunity[36]. Inarigivir is an immune modulator with an antiviral effect. It acts as a RIG-I agonist, inducing type I and type III IFN production, thus boosting immune response. In the ACHIEVE study, 80 treatment naïve non-cirrhotic patients with chronic HBV infection were enrolled. They received, through a random sampling, different doses of inarigivir or placebo for 3 mo followed by tenofovir for 3 mo. Both HBeAg-positive and negative patients achieved HBV-DNA and RNA reductions in a dose-dependent manner, and an HBsAg decline of > 0.5 $\log_{10}$ at 12 or 24 wk was found in more than 20% of patients.

Immune checkpoint inhibitors: Apoptosis is a fundamental mechanism at the basis of HBV-specific CD8 T cell depletion. Interruption of this mechanism restores CD8 T-cell responses against HBV. In the setting of chronic HBV infection, inhibitory receptors, like PD-1 and programmed death-ligand 1 (PD-L1) are overexpressed on exhausted T

Table 1 New antiviral drugs for hepatitis B

	Molecule(s)	Mechanism of action	Ref.
Entry inhibitors	Bulevirtide (Myrcludex B)	Block HBV entry into hepatocytes to protect cells not yet infected or antagonize *de novo* infection	[16, 17]
Capsid assembly modulators	NVR 3-788; GLS4; RO7049389; JNJ-6379; JNJ-0440; ABI-H0731	Inhibition of nucleocapsid formation	[19-24]
Post-transcriptional inhibitors	ARC-520; ARC-521; ARB-1467	Induction mRNA degradation after transcription and translation prevention	[27, 28]
Inhibitors of HBsAg release	REP 2165; Lenvervimab	Removal of subviral envelope particle. Neutralization activity	[29-31]
Inhibitors of cccDNA	CRISP-Cas system	Disablement of cccDNA	[32-34]

HBsAg: Hepatitis B surface antigen; cccDNA: Covalently closed circular DNA; HBV: Hepatitis B virus.

cells, limiting the effector function[37]. The block of the PD-1/PD-L1 pathway seems to enhance their function. In a few words, checkpoint inhibitors may restore T cell dysfunction.

In a recent phase I pilot study, the efficacy of the PD-1 inhibitor nivolumab was investigated. Twenty-four NA-suppressed anti-HBe positive patients received nivolumab at two different dosages, combined or not with the therapeutic vaccine GS4774. Patients that received higher doses had a noticeable decline in HBsAg levels and one of the patients receiving both nivolumab and the therapeutic vaccine GS4774 lost HBsAg[38]. Exhausted CD8+ T cells present also co-inhibitory molecules such as CTLA-4 and CD244/2B4 whose blockade, *in vitro* studies, seems to achieve restoration of immune function.

Stimulator of interferon genes agonists: The use of synthetic agonists for activation of the stimulator of interferon genes (STING) seems to stimulate the production of cytokines, especially IFNs. In mouse models, intraperitoneal infusion of a STING agonist stimulated the expression of IFN genes and mitigated HBV replication in mouse hepatocytes[39]. Such agonists may be exploited for the development of novel anti-HBV strategies.

Problem of antiviral resistance in chronic hepatitis B

Nucleoside and nucleotide analogs inhibit the viral DNA polymerase, causing an early stop of HBV replication. Drug-resistant strains of HBV have mutations in the viral polymerase gene. Resistance mutations alter the interaction and the inhibition of the drug on the viral polymerase. These mutants persist even after the end of treatment and determine cross-resistance to the next drug[40]. To prevent resistance development, guidelines recommend, as the first approach, the use of NAs with a high barrier to resistance. Sequential monotherapies with a low barrier to resistance should be avoided. In all cases of treatment failure, it is essential to precociously identify possible resistance mutations without forgetting to always check compliance to NAs[2].

What's in store for the future? Therapeutic vaccination

The therapeutic vaccine has shown encouraging results in animal models; non-similar results were demonstrated in humans for both vaccines, with adjuvant or with DNA. They are developed to stimulate the host immune response to suppress HBV replication and foster HBsAg loss. Therapeutic vaccination, alone or associated with oral antiviral therapy, is not sufficient to restore HBV immunity in chronically infected patients. Combination strategy failure was demonstrated by some studies, as the randomized study of Vandepapelière *et al*[41]: The difference between the HBe seroconversion rate obtained by the co-administration of vaccine and lamivudine (18.8%) or by lamivudine alone (16.1%) was not significant[41].

Previous vaccines only targeted HBsAg, but the new ones, under development, are against multiple HBV proteins and novel adjuvants. GS-4774 and TG-1050 are precisely the results of approaches based on multiple HBV proteins.

GS-4774 is a vaccine that uses recombinant *Saccharomyces cerevisiae* yeast to express surface, core, and X proteins. Virally suppressed patients, on treatment with oral NAs, were randomized to continue antiviral therapy alone or associated with different yeast

units of GS-4774. GS-4774 did not determine significant reductions in HBsAg levels. Five HBeAg-positive patients treated with GS-4774 showed HBeAg loss while none in the control group showed seroconversion to anti-HBe. Increased production of IFN-α, TNF-α, and IL2 by CD8+ T cells was documented[42].

TG1050 is an adenovirus-based vaccine that encodes a fusion protein composed of HBV polymerase and domains of HBcAg and HBsAg. This is a T-cell-inducing vaccine and has demonstrated antiviral effects in mice. In CHB patients treated with NAs, TG1050 is safe and effective in inducing HBV-specific cellular immune response. It induced functional IFN-γ producing cells and despite that serum HBsAg did not decrease significantly following TG1050, a robust decrease of HBcAg was shown in some patients[43].

Despite unsatisfactory results, it is now clear that a therapeutic vaccine could effectively achieve a functional cure only if associated with NAs or immunomodulatory therapies.

HEPATITIS D

HDV is a highly pathogenic virus that causes acute, fulminant, and chronic hepatitis, causing cirrhosis in more than 70% of cases. HDV has been defined as a defective virus because it needs the helper function of HBV for its transmission. The two possible scenarios are simultaneous infection of HDV and HBV (coinfection) that leads to chronic hepatitis D in < 5%, or HDV superinfection in chronic hepatitis B patients (superinfection); in this case, chronic hepatitis occurs in up to 90% of patients[44,45].

Permanent HDV-RNA suppression is associated with improved clinical outcomes. The treatment for chronic HDV infection with IFN therapy achieves a virologic response rate of about 17%-47%[46,47]. This translates into a rare curative outcome. Moreover, late viral relapses frequently occur in patients with chronic HDV hepatitis who have achieved sustained virological response (SVR). The treatment of choice in patients with HDV and HBV infection with compensated liver disease is peg-IFNa for 48 wk. The serious side effects (influenza-like illness, alopecia, leucopenia, thrombocytopenia, and emotional lability) make IFN a drug with huge limits.

NA treatment is also recommended when HBV-DNA levels are constantly less than 2.000 IU/mL and in patients with advanced liver disease[2].

In the last years, many efforts have been made to identify new therapeutic targets for HDV treatment. As mentioned above, NTCP is an important receptor for HBV to enter hepatocytes. HDV virions also attack the viral receptor NTCP before membrane fusion and release of the ribonucleoprotein into the cytoplasm. The entry of HDV into new cells can be blocked by bulevirtide (Myrcludex B) according to its action on a specific receptor NTCP. Bulevirtide is a chemically synthesized peptide composed of amino acids derived from large HBV surface proteins. Bulevirtide was recently approved in the European Union for treatment of chronic hepatitis D in HDV-RNA positive adult patients when liver disease is compensated. Bulevirtide blocks the virus entry into the cells and limits the HDV replication capacity. As mentioned above, two main studies showed that bulevirtide is effective at clearing HDV-RNA from the blood. In the first study, the MYR202 study, 55 out of 90 patients who received bulevirtide and TDF lost HDV-RNA after 6 mo, compared with 1 out of 28 patients treated with TDF alone. The combination treatment was associated with a significant normalization in alanine aminotransferase and an interesting improvement of liver function, as compared to patients who received TDF alone[48].

Similar results were seen in the second study, the MYR203, where patients were randomized to bulevirtide plus peg-INF alpha-2a, bulevirtide alone, and PEG-IFN alpha-2a alone for 48 wk[17]. At 48 wk, HDV-RNA was undetectable in 80% of patients treated with bulevertide 2 mg plus peg-INF alpha-2a while lower rates were obtained in the other two groups. Final results at week 72 confirmed the effectiveness of the association strategy, showing undetectable HDV-RNA in 53.3% of cases *vs* 0% in the IFN group[49].

A fundamental step in the virus assembly process consists of the modification (prenylation) of a specific HDV protein. This protein is the large delta antigen (HDAg). Prevention of prenylation blocks the formation of viral particles or more precisely, the assembly of mature HDV. A new class of antiviral agents, capable of inhibiting prenylation, have been developed for use in humans[50]. Lonafarnib is an oral prenylation inhibitor. A proof-of-concept phase 2A double-blinded randomized placebo-controlled study showed a decline in serum HDV-RNA levels in patients treated with lonafarnib (100 mg or 200 mg twice daily) compared with placebo. The

decline in virus levels was proportional to serum drug concentration. Two-thirds of patients treated with the higher dose had a viral rebound and ALT flare after the end of treatment[51].

Triple combination therapy with lonafarnib, ritonavir, and peg-IFN for 6 mo showed antiviral efficacy in patients with chronic HDV infection, as demonstrated in a recent study (NCT03600714; n = 26)[52]. At the end of treatment (week 24), 77% of patients achieved an important goal, the decrease of HDV RNA (> 2 log).

Recent studies are investigating the capacity of NAPs to block HBsAg release from infected hepatocytes by some non-immunostimulatory mechanism. NAPs clear circulating HBsAg by hindering the release of HBV and HDV particles. The REP-2139 is the first NAP selected for human studies. Unpublished interim results from an ongoing proof of concept trial have confirmed the ability of REP 2139 to achieve clearance of HDV-RNA in 50% of treated patients up to 12 mo after the end of treatment. The adverse events (pyrexia, chills, conjunctival hyperemia, headache, and asthenia) could represent a serious limit.

Further studies are needed to assess the efficacy and tolerability of NAPs. Bulevirtide and lonafarnib are antiviral agents in an advanced phase of investigation with a proven anti-HDV activity.

HEPATITIS C

HCV infection is one of the most important causes of chronic liver disease in the world. The primary goal of HCV therapy is the infection cure, achieving an SVR consisting of undetectable HCV-RNA. SVR also means normalization of transaminase, reduction of liver necro-inflammation and fibrosis, improvement in liver function, and reduction of HCC risk[53].

The approval of direct-acting antivirals (DAAs) in 2014 revolutionized the treatment of HCV, allowing nearly all patients to be cured. DAAs are highly effective and well-tolerated and represent the gold standard for the treatment of patients with chronic infection, in all stages of liver disease[54]. Most of the progression in HCV treatment is due to the possibility to treat many populations, once excluded for the discouraging side effects of IFN-based therapies. At present, high SVR rates are achieved in cirrhotic patients, HIV/HCV co-infected patients with chronic renal failure, or transplant patients. The efficacy is not undermined by the short duration of DAA therapy.

Thanks to the IFN-free regimens developed, the World Health Organization (WHO)'s goal for HCV infection is a 90% reduction in the incidence of new infections and reduction of HCV-related deaths by approximately 65% in 2030[55,56].

There are many available EMEA- and FDA-approved DAAs for HCV treatment, divided into three major classes based on their HCV proteins targets: Protease NS3/4A inhibitors (glecaprevir, grazoprevir, paritaprevir, simeprevir, and voxilaprevir), NS5A serine protease inhibitors (PIs) (daclatasvir, elbasvir, ledipasvir, ombitasvir, pribrentasvir, and velpatasvir), NS5B RNA-dependent RNA nucleoside polymerase (sofosbuvir), and non-nucleoside polymerase (dasabuvir) inhibitors[57]. NS3/4A PIs inhibit HCV polyprotein processing; NS5A inhibitors inhibit viral replication and assembly; NS5B polymerase inhibitors block HCV-RNA replication.

The HCV drug combinations available in Europe are sofosbuvir and velpatasvir in a single tablet administered once daily; sofosbuvir, velpatasvir, and voxilaprevir available in a combination product, glecaprevir, and pibrentasvir or grazoprevir and elbasvir available in a two-drug fixed-dose combination. The non-pan-genotypic combination of grazoprevir and elbasvir can also be used in patients infected with HCV genotype 1b. Pan-genotypic HCV drugs, in particular sofosbuvir plus velpatasvir and glecaprevir plus pibrentasvir, can be used even if the virus genotype is not available.

Contraindications to current DAAs are few. The pharmacological interactions are worthy of attention: Cytochrome P450/P-gp-inducing agents are often not switchable. The use of grazoprevir, glecaprevir, or voxilaprevir is allowed only in a contest of compensated cirrhosis (Child-Pugh B or C) and in patients without previous episodes of decompensation (EASL guidelines)[54]. Patients with decompensated cirrhosis and patients with compensated cirrhosis with a medical history characterized by previous episodes of decompensation should be treated with sofosbuvir and velpatasvir and ribavirin for 3 mo. If there are contraindications to the use of ribavirin or poor tolerance, a fixed-dose combination of sofosbuvir and velpatasvir for 24 wk without ribavirin can be used.

Two innovative and promising strategies are required and expected to obtain a global HCV elimination: A deep enhancement of screening and linkage to care for so-called "hard-to-reach" populations. The strengthening of new strategies to improve access to care for vulnerable and marginalized populations with HCV infection is becoming urgent[58]. Moreover, it has been demonstrated that a drastic decrease in HCV incidence requires proper education on injection safety, appropriate screening blood transfusions, and delivery of sterile syringes to people who inject drugs[55].

Political will and financing issues, thus, are essential to achieve elimination or a significant reduction in HCV infection prevalence and incidence.

Apart from the implementation of measures to reduce the prevalence of HCV infection, a big innovation in the HCV field is linked to a paper, published in 2020, that suggests DAA therapy as the prevention of HCV transmission. A single-arm trial evaluated short-course prophylactic therapy in patients (n = 30) receiving solid organ transplants from donors with HCV infection[59].

Prophylaxis with glecaprevir-pibrentasvir combined with the HCV entry blocker ezetimibe (from 6-12 h to 7 d after transplantation) showed prevention of chronic HCV infection in all 30 transplant recipients. This innovative study could help to reduce ethical concerns regarding the transplantation of HCV-infected organs into recipients without HCV.

In conclusion, the treatment with new oral DAA combinations is efficient and safe even in special populations and in patients with advanced liver disease or severe comorbidities. Eradication of HCV by 2030 is not a utopian goal if all countries direct efforts towards the same objective: Preventing transmission measures, improving screening programs (especially in marginalized populations without contact with healthcare services or incarcerated individuals), and enforcing educational approach among people who inject drugs who represent the most vulnerable group for HCV transmission.

Problem of DAA resistance in the treatment of hepatitis C

A large part of the responsibility for DAA failure, which involves a minority of patients (4%-5%), is the inadequate adherence to therapy. In some cases, resistance-associated variants (RAVs) directly cause relapse or viral breakthrough. RAVs affect all DAA classes but mostly occur in the NS5A region. RAVs arising after treatment are more difficult to treat; however, patients who fail to achieve SVR after first-line DAA therapy, respond to sofosbuvir/velpatasvir/voxilaprevir and glecaprevir/pibrentasvir [60].

HEPATITIS E

HEV infection has recently been known to represent a major health problem in industrialized countries, not only in developing countries. This peculiar virus, transmitted as a zoonotic infection (uncooked meat consumption) or through infected blood or blood products, is endemic in most high-income countries (mainly genotypes 3 and 4) and is an important cause of acute and chronic viral hepatitis, the latter affecting immunosuppressed patients (such as solid transplant recipients, patients with hematological malignancies receiving chemotherapy, and HIV infected patients). The incidence of acute HEV infection is about 3 million human infections *per* year worldwide. In the endemic areas, HEV is transmitted predominantly by the fecal-oral route while in the non-endemic areas, HEV is a foodborne illness. Immunocompetent individuals develop self-limiting acute icteric hepatitis. It is a mild form providing long-term protection. HEV is the leading cause of 1%-4% of acute viral hepatitis cases in the general population and 30% in pregnant women. Few patients have severe illnesses leading to fulminant hepatic failure. In immunocompromised patients, acute infections progress in over 60% of cases to chronicity and in 10% to liver cirrhosis. The presence of HEV-RNA for more than 12 wk stands for chronic HEV infection. HEV chronicity is most common among liver transplant recipients. Interestingly, chronic HEV infection has been reported to be associated with a variety of extra-hepatic manifestations, for example, neurological and renal (neuralgic amyotrophy, Guillain-Barré syndrome, and membranoproliferative and membranous glomerulonephritis) [61]. HEV can also act as a potential trigger of acute-on-chronic liver failure (ACLF) that causes a worsening of the liver function with clinical complications such as ascites and encephalopathy. Acute HEV infection is responsible for 3.2% of the cases of decompensation in patients with chronic hepatic disease, as shown in a large prospective study including 343 patients, with elevated mortality[62].

In chronic HEV infection, the reference tests for the confirmatory diagnosis are the detection of HEV-RNA in serum or stool samples by reverse transcriptase-polymerase chain reaction or loop-mediated isothermal amplification for a minimum of 3–6 mo duration[63]. Antibody tests are not useful in the diagnosis of chronic HEV.

There are no approved treatments for this infection although reduction of immunosuppression can lead to HEV clearance in one-third of patients. The study of Kamar *et al*[64] showed that reduction in immunosuppressive therapy that specifically targets T cells could promote HEV eradication in transplant recipients, in the face of a high risk of graft rejection[64]. However, dose reduction of immunosuppressive medications, particularly those targeting T lymphocytes, is the first-line therapeutic approach for solid organ transplant recipients with HEV; mycophenolate mofetil seems able to suppress viral replication.

The currently recommended drug for the treatment of severe acute and chronic hepatitis E in solid transplant recipients is off-label oral ribavirin. The EASL published guidelines (2018) recommended the use of ribavirin as a standard of care; however, the optimal ribavirin treatment regimen is not known. Ribavirin inhibits HEV-RNA replication by depleting guanosine triphosphate pools.

In a large, retrospective, multicenter case series authors showed that a 3 mo course of ribavirin is a proper strategy to obtain SVR in chronic HEV infection[65]. Ribavirin can cause anemia, in up to 50% of patients. Peg-IFN-α has been reported as a possible treatment in the setting of liver transplant recipients: Peg-IFN-alpha 2a administered to three liver transplant patients with chronic HEV infection for 2 mo, resulted in viral RNA clearance. Peg-IFN-γ with or without ribavirin has also been shown to be effective in the treatment of HEV-HIV patients and non-transplant immunosuppressed patients with hematological disorders[66]. IFN can be considered in liver transplant recipients and patients undergoing hemodialysis; IFN is contraindicated in many solid-organ transplant patients (kidney, heart, and lung) because of the risk of rejection.

Based on available data, a decrease in immunosuppression (if feasible) and ribavirin at a dose of 600-800 mg *per* day for 3 mo are recommended in patients with chronic HEV hepatitis. In the case of ACLF provoked by HEV, the efficacy of ribavirin is not clear. Ribavirin is contraindicated in pregnancy due to teratogenic potential, and can be suggested for pregnant women only in the last trimester of pregnancy.

Sofosbuvir was also reported to reduce HEV viral load without achieving SVR in seven chronic HEV-infected patients. Sofosbuvir has therapeutic activity against HEV with an additive effect to ribavirin. Sofosbuvir with ribavirin, in chronic HEV infection, promotes a reduction in HEV levels and ALT during therapy, but a rise has been documented after stopping treatment, confirming its inability to provide SVR in chronic HEV infections.

Further studies are needed to assess the efficacy and safety of Hecolin, the first vaccine produced by China, not approved yet for commercialization. This vaccine (30 mcg of purified recombinant hepatitis E antigen *per* dose) given at 0, 1, and 6 mo showed 100% efficacy in phase III clinical trial[67]. The safety and efficacy of the vaccine in patients with chronic liver disease and other populations, for example, immunosuppressed patients, are needed before recommended for its widespread use.

There is a clinical need for new therapeutic options for HEV in immunosuppressed patients. Large scale studies are required to understand the impact of some promising compounds such as 2'-C-methylguanosine, which suppresses the growth of HEV in cell cultures, zinc that could act as an adjuvant therapy in ribavirin resistant/relapsed HEV infections, and silvestrol that diminished fecal HEV-RNA in mice but has not been tested on humans[68,69].

CONCLUSION

Novel DAAs have shown an unimaginable efficacy of curing HCV to the point that the WHO defined it as an achievable target elimination of HCV by 2030. To pursue this goal, marginalized and stigmatized patients should be better engaged in the healthcare system to diagnose infection and encourage treatment adherence.

Concurrently with the approval of DAAs for HCV, starting in 2014, a renewed interest in pursuing curative therapies for HBV infection has been registered. Current NAs positively impact liver-related complications, ensuring a high level of safety and tolerability. The need for long, or even lifelong, therapy and the oncogenic role of HBV, fostered by integration into the hepatocytes genome, has increased the effort to develop definitive and curative therapy. Novel approaches to HBV cure are based on

new antivirals, targeting different steps of the HBV life cycle and immune modulators. Molecules acting on the HBV cycle have obtained encouraging results. Immune-based approaches are also emerging but must be further examined. Ideal and utopistic therapy should pursue a sterilizing aim, consisting of elimination of cccDNA or silencing its activity. A combination of traditional and new anti-HBV agents may favor HbsAg clearance and eliminate the cccDNA reservoir from the patient's liver, reducing the oncogenic effect.

About HDV, the refined knowledge of its life cycle has facilitated the study of some direct-acting agents, bulevirtide, lonafarnib, and NAPs, which are more effective than IFN-based therapies. In particular, bulevirtide is the first drug approved for the treatment of HDV in adults with compensated liver disease in Europe.

Severe side effects and limited effectiveness of current HEV therapies have also made it essential to identify new therapeutic approaches for HEV, especially in immunosuppressed individuals. Further studies are required to assess the efficacy of adjuvant zinc therapy, 2'-C-methylguanosine, sofosbuvir, or the natural compound silvestrol.

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ORIGINAL ARTICLE

Basic Study

Effect of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* supernatants on serotonin transporter expression in cells and tissues

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Author contributions: Wang YM, Wang BM and Zhang J conceived and designed the study; Chen YM and Wang ZL conducted the experiments; Li Y and Wang X rote the manuscript; Su S, Hou JJ, Zhong WL and Xu X analyzed data; Chen YM and Li Y contributed equally to this work; all authors read and approved the manuscript.

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Abstract

BACKGROUND

Bacillus subtilis (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of irritable bowel syndrome (IBS). Whether the supernatants of these three probiotics can improve gastrointestinal sensation and movement by regulating the serotonin transporter (SERT) expression needs to be clarified.

AIM

To investigate whether *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants can upregulate SERT expression *in vitro* and *in vivo*.

METHODS

Caco-2 and HT-29 cells were stimulated with probiotic culture supernatants for 12 and 24 h, respectively. A male Sprague-Dawley rat model of post-infectious irritable bowel syndrome (PI-IBS) was established and the rats were treated with phosphate-buffered saline (group A) and three probiotics culture supernatants (groups B, C, and D) for 4 wk. The levels of SERT were detected by quantitative PCR and western blotting.

RESULTS

The levels of SERT at post-treatment 12 and 24 h were significantly elevated in Caco-2 cells treated with *B. subtilis* supernatant compared with those in the control group ($^aP < 0.05$). Those levels were markedly upregulated in Caco-2 cells stimulated with *E. faecium* and *E. faecalis* supernatants at 24 h ($^aP < 0.05$). In addition, SERT expression in groups B, C, and D was significantly higher than

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that in group A in the 2nd wk ($^aP < 0.05$). Increased SERT expression was only found in group D in the 3rd wk ($^aP < 0.05$). However, there was no significant difference in SERT expression between the groups in the last week ($P > 0.05$).

CONCLUSION

The supernatants of B. *subtilis*, *E. faecium*, and *E. faecalis* can upregulate SERT expression in intestinal epithelial cells and the intestinal tissues in the rat model of PI-IBS.

Key Words: *Bacillus subtilis* supernatant; *Enterococcus faecalis* supernatant; *Enterococcus faecium* supernatant; Serotonin transporter expression; Irritable bowel syndrome

Core Tip: Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that can cause chronic symptoms and changes in bowel habits. Serotonin and serotonin transporter (SERT) play significant roles in the development of IBS. *Bacillus subtilis* (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of IBS. Here, we explored the effects of *B. subtilis*, *E. faecium,* and *E. faecalis* supernatants on the mRNA and protein expression of SERT *in vitro* and *in vivo,* highlighting the significance of the regulation of SERT expression in the treatment of IBS patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that can cause chronic symptoms, such as abdominal pain and changes in bowel habits[1]. According to the Rome IV criteria, IBS is divided into four subtypes: IBS with predominant constipation (IBSC), IBS with predominant diarrhea (IBSD), IBS with mixed bowel habits (IBSM), and unclassified IBS (IBSU)[2]. With a prevalence varying between 5% and 10% worldwide, IBS can reduce the health-related quality of life and lower work productivity. Some symptoms of IBS such as an abnormal psychological state, anxiety, and depression may impose an economic burden on individuals, healthcare systems, and society[3,4]. The etiology of IBS is complex and still elusive. The widely recognized pathogenic mechanisms include gastrointestinal dysmotility, varied visceral sensitivity, brain-gut axis disorder, gut microenvironment, and psychosocial/psychosomatic behaviors[3]. For decades, the significant decrease in expression of the serotonin transporter (SERT) in the intestinal mucosa has been considered one of the most important pathophysiology in the development of IBS, leading to the gastrointestinal motility disorders[5-7]. SERT terminates serotonin or 5-hydroxytryptamine (5-HT) activity by combining with it from the interstitial space. Reduced SERT expression and function may result in excess 5-HT, along with motor, sensory, and secretary dysfunctions of the gut, which in turn leads to the development of diarrhea and abdominal pain associated with the pathogenesis of IBS[8,9]. In addition, alterations in 5-HT metabolism have been postulated to play a role in the pathogenesis of IBS[10,11]. Overall, 5-HT and SERT contribute significantly to the development of IBS[12].

Bacillus subtilis (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of IBS[13-16]. These probiotics can alleviate IBS symptoms such as decreasing the intensity of abdominal pain and discomfort and reducing stool frequency. Our previous study revealed that *Lactobacillus rhamnosus GG* supernatants can upregulate the expression

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level of SERT in epithelial cells and colon tissues in rats with post-infectious IBS (PI-IBS)[7,17]. We also demonstrated that *Lactobacillus acidophilus* and *Bifidobacterium longum* supernatants have similar effects on SERT expression in intestinal epithelial cells[18]. However, whether the supernatants of *B. subtilis*, *E. faecium,* and *E. faecalis* can improve gastrointestinal sensation and movement by regulating SERT expression still needs to be clarified.

The current research explored the effects of *B. subtilis*, *E. faecium,* and *E. faecalis* supernatants on the mRNA and protein expression of SERT *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial culture

B. subtilis (CGMCC 1.3358), *E. faecium* (CGMCC 1.2136), and *E. faecalis* (CGMCC 1.2135) were obtained from China General Microbiological Culture Collection Center (Beijing, China). These bacteria were respectively inoculated into the corresponding liquid medium and cultured at 37 °C for 24 h (*B. subtilis*: nutrient broth medium, HB0105, Hope Biotechnology Co., Ltd., Qingdao, China; *E. faecium*: TSB, HB4114, Hope Biotechnology Co., Ltd., Qingdao, China; *E. faecalis*: TSB, HB4114, Hope Biotechnology Co., Ltd., Qingdao, China). Then they were diluted in the corresponding media, and continued to be cultured to reach the logarithmic stage with an optical density (OD) of 0.5 detected at a wavelength of 600 nm. The supernatants were collected by centrifugation (5000 g, 10 min) and filtered twice through a 0.22 μm filter.

Campylobacter jejuni 81-176 (BAA-2151; ATCC, Manassas, VA, United States) was grown on Skirrow's selective medium (Columbia Agar Base, Oxoid CM0331) at 42 °C for 24 h. Bacterial colonies were obtained with an inoculating loop and diluted with culture medium until reaching a concentration of 10^{10} CFU/mL. Determination of bacterial concentration was carried out by the conventional plate-counting method.

Cell culture

Caco-2, a human epithelial colorectal adenocarcinoma cell line, was grown in Eagle's minimal essential medium (MEM; Gibco, New York, NY, United States) supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% nonessential amino acids at 37 °C. The human colonic epithelial carcinoma cell line, HT-29, was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, NY, United States) supplemented with 10% FBS and 1% nonessential amino acids at 37 °C. The cells were incubated with the culture supernatants of *B. subtilis, E. faecium*, and *E. faecalis* diluted in MEM/DMEM (dilution ratios: 1:100, 1:50, and 1:20, respectively) for 12 and 24 h and classified as the 1:100 group, 1:50 group, and 1:20 group, respectively.

Animal studies

Sixty-six male Sprague-Dawley rats (270-310 g) were maintained in a room at 22 ± 1 °C under a 12-h light:12-h dark cycle in the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (Tianjin, China). Rats in all groups were given the same housing conditions and diet. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. The animal protocol was designed to minimize pain or discomfort to the animals. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (IACUC Protocol No. IRM-DWLL-2021142).

Intervention and evaluation of a rat model of PI-IBS

Rats were randomly divided into a PI-IBS group (n = 52) and control group (n = 14). Rats in the PI-IBS group were given 10^{10} CFU/mL *C. jejuni* for 7 days, while those in the control group were given phosphate-buffered saline (PBS) for 7 days. The rats in both groups were fed separately. *C. jejuni* stool culture and biochemical tests were carried out to evaluate the infection phase of rats in the PI-IBS group. Four rats were randomly chosen from the PI-IBS and control group to perform the intestinal motility test with carbon solution gavage. The specific experimental process was previously described by Wang *et al*[19]. After evaluation of the rat model of PI-IBS, the remaining rats were randomly assigned to four different experimental groups (n = 12 for each group): Group A, PBS (i.g); Group B, *B. subtilis* supernatant (i.g); Group C, *E. faecalis* supernatant (i.g); Group D, mixed supernatant *of B. subtilis* and *E. faecalis* with a dilution of 1:1 (i.g). These rats were treated for 4 wk, and changes in general and fecal

states were observed and recorded. Besides, three rats from each group were sacrificed, and protein expression levels of SERT in colonic tissues were detected weekly. The rats were fed 10% activated carbon suspension after fasting for 24 h and were killed 1 h later. The intestinal section from pyloric to terminal rectum was removed immediately. The total length of intestinal tract and the propelling distance of activated carbon were measured. Intestinal transit rate = propelling distance of activated carbon/total length of intestinal tract.

quantitative PCR

Total RNA was extracted from Caco-2 and HT-29 cells with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, United States). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The raw materials for PCR synthesis included cDNA, 2x iQSYBR Green Supermix (Thermo Fisher Scientific), primers, and double-distilled water. The primers used for quantitative PCR (qPCR) are listed in Table 1. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Proteins were extracted from HT-29 cells, Caco-2 cells, and colonic tissues of rats using radio-immunoprecipitation assay lysis buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Solarbio Science & Technology Co., Ltd., Beijing, China). A total of 40 g protein samples were isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isolated protein was transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% skimmed milk powder at room temperature for 1 h, and incubated with primary rabbit polyclonal antibodies (SERT, Cat. No. ab102048, Abcam, Cambridge, UK; β-actin, Cat No. KM9001, Tianjin Sungene Biotech Co., Ltd., Tianjin, China) at 4 °C overnight. The PVDF membranes were incubated with diluted secondary antibody (LK2001, Tianjin Sungene Biotech Co.) at room temperature for 1 h. Then the membranes were washed with Tris-buffered saline with 0.1% Tween® 20 Detergent and detected using an enhanced chemiluminescence kit (BB-3501; Amersham, Buckinghamshire, UK). The Bio-Rad Image Analysis System (Bio-Rad Laboratories) was utilized to visualize the protein bands. The protein quantitative analysis was undertaken using ImageJ software. The relative expression was calculated using the ratio of the gray value of protein band to that of glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

The Student's *t*-test was used to assess differences between two groups. All statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Effects of B. subtilis supernatant on the mRNA and protein expression levels of SERT in Caco-2 and HT-29 cells

Caco-2 and HT-29 cells were treated with supernatants of *B. subtilis* from 1:100, 1:50, and 1:20 groups. SERT mRNA and protein expression levels in Caco-2 and HT-29 cells were detected by qPCR and western blotting, respectively. SERT mRNA levels at both 12 and 24 h after treatment were significantly increased in the 1:50 and 1:20 groups in Caco-2 cells (Figure 1 Caco-2 A&B, all $^{a}P < 0.05$) compared with those in the control group. Increased SERT protein expression was found in the 1:20 group at 12 h after treatment (Figure 1 Caco-2C, $^{a}P < 0.05$). Both SERT mRNA and protein levels increased at 24 h (Figure 1 Caco-2B&D, $^{a}P < 0.05$). The mRNA and protein expression levels of SERT in the 1:20 group were significantly higher than those in the 1:100 group at 12 and 24 h ($^{a}P < 0.05$), which indicated that the effect of *B. Subtilis* supernatant on the mRNA and protein levels of SERT was concentration-dependent.

SERT protein expression in HT-29 cells was markedly elevated in the 1:50 and 1:20 groups at 12 h compared with that in the control group, and the level of SERT proteins in the 1:20 group was higher than that of the 1:100 and 1:50 group (Figure 1 HT-29C, $^{a}P < 0.05$). However, there was no significant difference in SERT mRNA level at 12 h among the treatment and control groups (Figure 1 HT-29A, $P > 0.05$). By contrast,

Table 1 Primer sequences for RT-PCR

Gene	Sequence (5'-3')
rGAPDH	Forward: 5'-CCATCAACGACCCCTTCATT-3'; Reverse: 5'-GACCAGCTTCCCATTCTCAG-3'
rSERT	Forward: 5'-ACTGTTACCAAGATGCCCTG-3'; Reverse: 5'-ATCTTCATTCCTCATCTCCGC-3'
hGAPDH	Forward: 5'-ACA GCA ACT CCC ATT CTT-3'; Reverse: 5'-TCC AGG GTT TCT TAC TCC-3'
hSERT	Reverse: 5'-AAT GGG TAC TCA GCA GTT CC-3'; Reverse: 5'-CCA CAG CAT AGC CAA TCA C-3'

hGAPDH: Human glyceraldehyde-3-phosphate dehydrogenase; hSERT: Human serotonin transporter; rGAPDH: Rat glyceraldehyde-3-phosphate dehydrogenase; rSERT: Rat serotonin transporter.

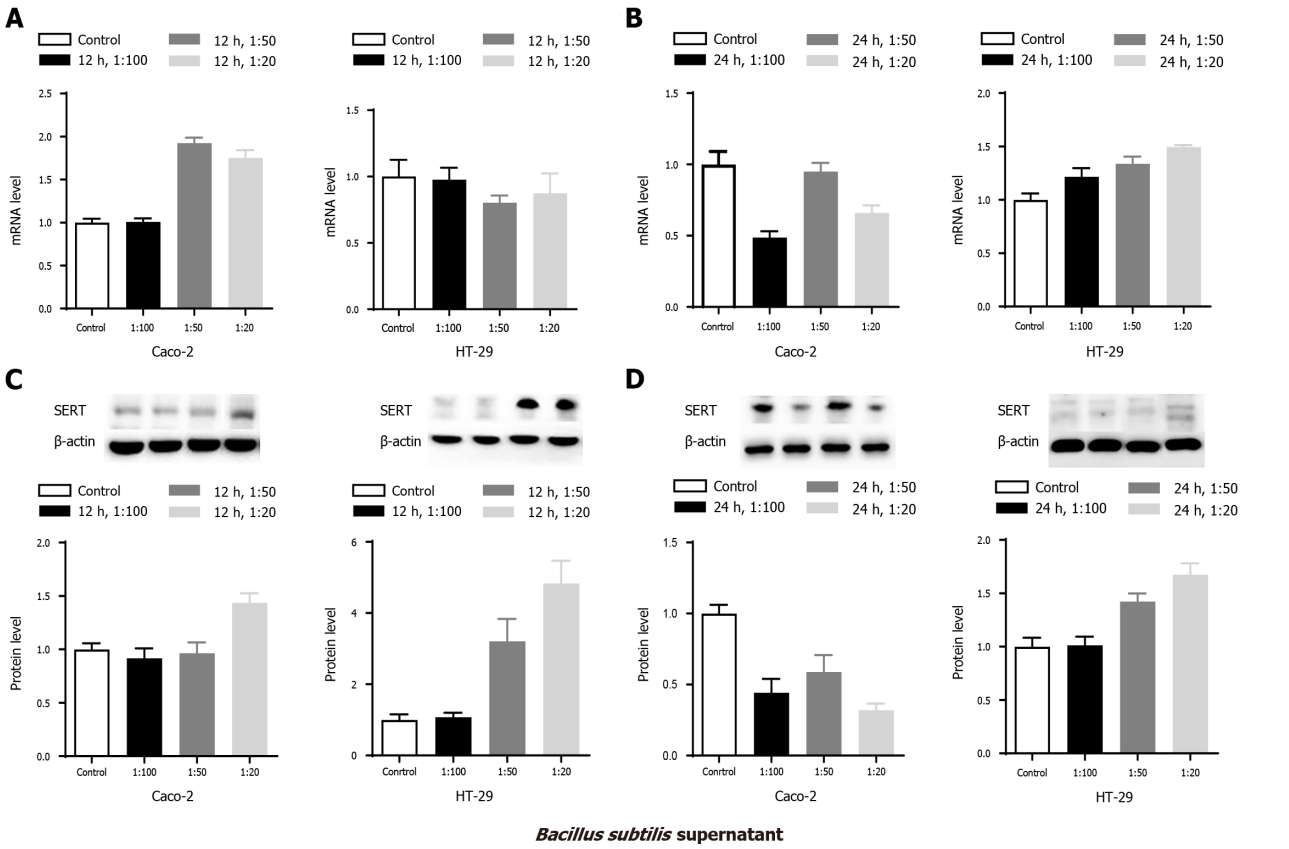

Figure 1 Effects of *Bacillus subtilis* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

significant reductions in the mRNA and protein levels of SERT were observed among the treatment groups at 24 h (Figure 1 HT-29-B&D, all $^{a}P < 0.05$).

Effects of E. faecium supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To explore the effects of *E. faecium* supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells, similar experiments were performed. The experimental results showed that there were no significant differences in the mRNA and protein levels of SERT at 12 h among the mentioned groups (Figure 2 Caco-2-A&-C, $^{a}P > 0.05$). SERT mRNA and protein levels were significantly upregulated in the 1:50 and 1:20 groups compared with the control group at 24 h (Figure 2 Caco-2-B&-D, $^{a}P < 0.05$), indicating that the effects of *E. faecium* supernatant on SERT mRNA and protein expression were time-dependent. Besides, SERT protein level in the 1:20 group was higher than that in the 1:50 and 1:100 group at 24 h (Figure 2 Caco-2D, $^{a}P < 0.05$), demonstrating that the effect of *E. faecium* supernatant was concentration-dependent.

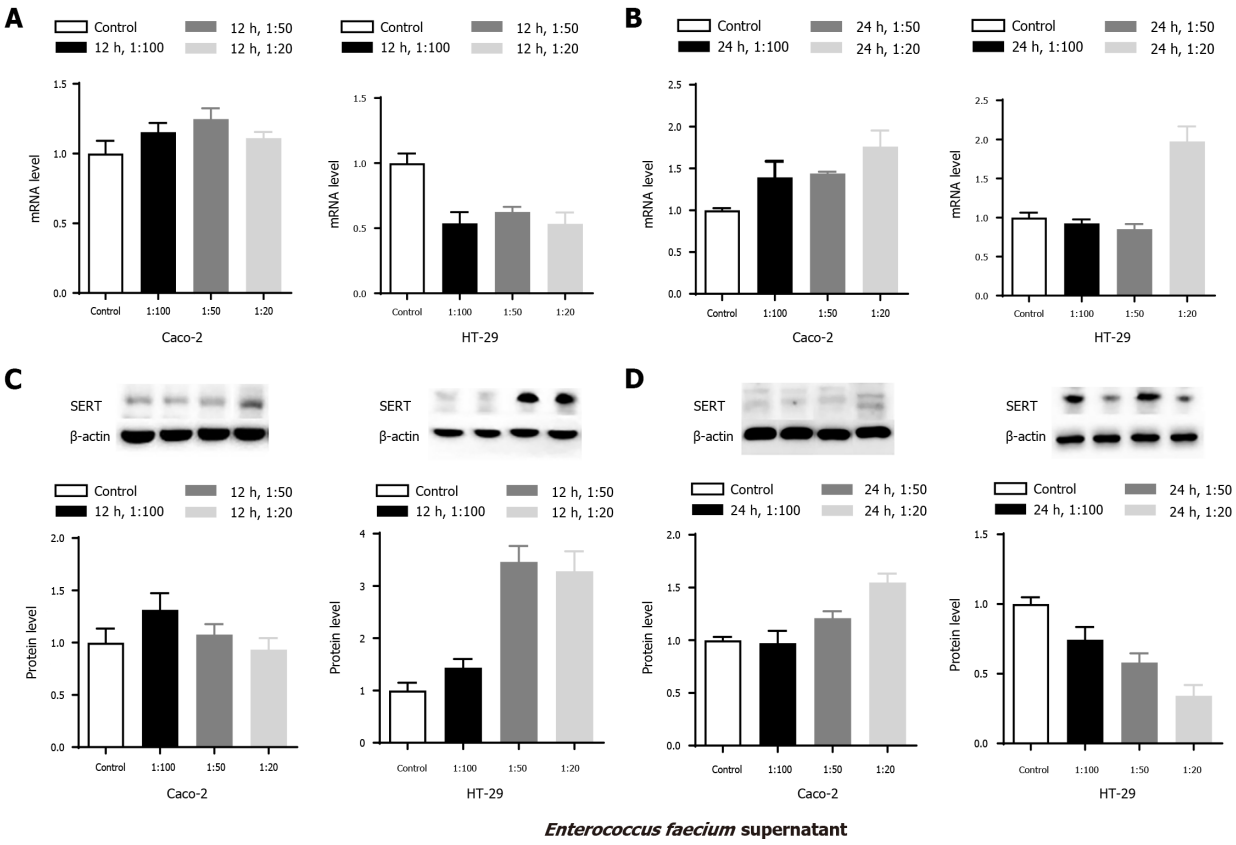

Figure 2 Effects of *Enterococcus faecium* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

SERT protein levels in the 1:50 and 1:20 groups were markedly higher than those in the control group in HT-29 cells at 12 h (Figure 2 HT-29C, $^{a}P < 0.05$). However, SERT mRNA levels in the three treatment groups were dramatically reduced at 12 h (Figure 2 HT-29-A, $^{a}P < 0.05$). Furthermore, compared to the control group, SERT mRNA level was notably elevated in the 1:20 group at 24 h (Figure 2 HT-29B, $^{a}P < 0.05$), while SERT protein level was significantly reduced in the 1:50 and 1:20 groups at 24 h (Figure 2 HT-29-D, $^{a}P < 0.05$).

Effects of mixed supernatant of B. subtilis and E. faecium on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To further investigate the abovementioned results, Caco-2 and HT-29 cells were stimulated with the mixed supernatants of *B. subtilis* and *E. faecium* in the 1:1 group. Compared with the control group, SERT mRNA level was upregulated in the 1:20 group in Caco-2 cells at 12 h (Figure 3 Caco-2 A, $^{a}P < 0.05$), and SERT protein levels were significantly elevated in the three groups at 12 h (Figure 3 Caco-2 C, $^{a}P < 0.05$). However, there were no significant differences in the mRNA and protein levels of SERT among these groups at 24 h (Figure 3 Caco-2 B&D, $P > 0.05$).

The expression of SERT mRNA in HT-29 cells was upregulated in the 1:100 and 1:20 groups at 12 h (Figure 3 HT-29A, $^{a}P < 0.05$) compared to the control group. An increased level of SERT protein was found in the 1:20 group at 12 h, whereas a significantly decreased level was observed in the 1:50 group compared with the control group (Figure 3 HT-29C, $^{a}P < 0.05$). Furthermore, compared with the control group, SERT mRNA levels were attenuated in the 1:100 and 1:20 groups at 24 h, whereas SERT protein levels were decreased in all the three treatment groups (Figure 3 HT-29 B&D, $^{a}P < 0.05$).

Effects of E. faecalis supernatant on mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To explore the effects of *E. faecalis* supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells, similar experiments were conducted. SERT mRNA

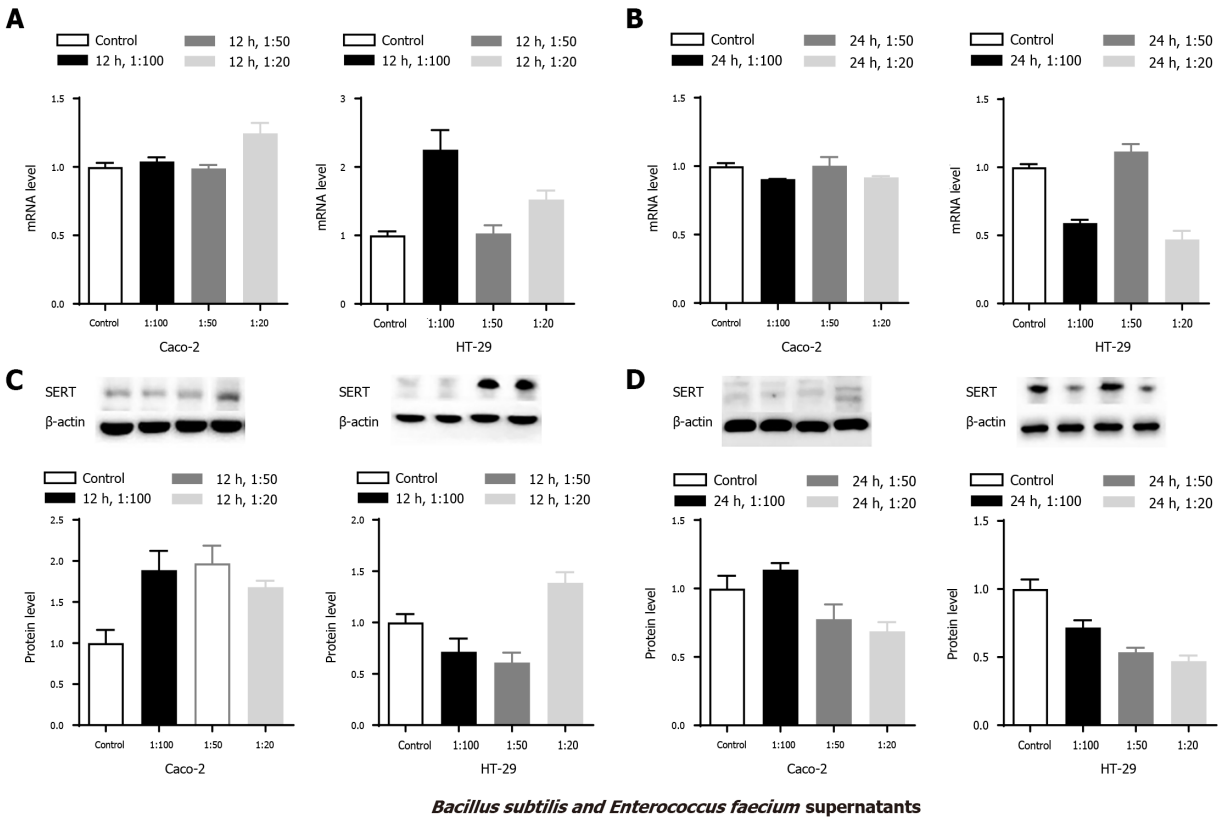

Figure 3 Effects of *Bacillus subtilis* and *Enterococcus faecium* supernatants on serotonin transporter mRNA and protein expression in Caco2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

levels in Caco-2 cells in the three treatment groups were significantly increased at 12 h (Figure 4 Caco-2 A, $^{a}P < 0.05$). Western blot analysis showed that there were no significant differences in SERT protein levels among the three treatment groups at 24 h, whereas a decreased level was observed in the 1:20 group at 12 h compared to the other two groups (Figure 4 Caco-2 C, $^{a}P < 0.05$). In addition, increased SERT mRNA level was only found in the 1:50 group at 24 h (Figure 4 Caco-2-B, $^{a}P < 0.05$). Similarly, the expression level of SERT protein was markedly upregulated in the 1:50 and 1:20 groups at 24 h (Figure 4 Caco-2 D, $^{a}P < 0.05$). SERT protein level in 1:20 group was significantly higher than that in the 1:50 and 1:100 groups at 24 h (Figure 4 Caco-2 D, $^{a}P < 0.05$). Besides, SERT protein level in the 1:50 group was also higher than that in the 1:100 group at 24 h (Figure 4 Caco-2 D, $^{a}P < 0.05$), indicating that the effect of *E. faecalis* supernatant on the mRNA and protein levels of SERT was concentration-dependent.

SERT mRNA level was in steady-state in HT-29 cells at 12 h among the treatment groups (Figure 4 HT-29A, $P > 0.05$), while western blot analysis showed that the SERT mRNA level was significantly elevated in all three groups (Figure 4 HT-29-C, $^{a}P < 0.05$) compared to the control group. Additionally, the expression level of SERT protein in the 1:50 group was markedly higher than that in the 1:20 group at 12 h (Figure 4 HT-29C, $^{a}P < 0.05$). SERT mRNA level was markedly increased in the 1:20 group at 24 h compared to the 1:100 group (Figure 4 HT-29B, $^{a}P < 0.05$). Similarly, the levels of SERT protein were significantly elevated in the 1:20 and 1:50 groups at 24 h. Meanwhile, the increase in SERT mRNA expression in the 1:20 and 1:50 groups was noticeably higher than that in the 1:100 group at 24 h (Figure 4 HT-29-D, $^{a}P < 0.05$), confirming that the effect of *E. faecalis* supernatant on the mRNA and protein levels of SERT was concentration-dependent in HT-29 cells.

The construction and evaluation of a rat model of PI-IBS

To further verify the effect of culture supernatant of probiotics in treating IBS, we constructed a rat model of PI-IBS. Before intervention, all rats were negative for *C. jejuni* stool culture and biochemical tests. Fifty-two of the rats were used to establish a rat model of PI-IBS as the PI-IBS group. The results of fecal bacteriological culture and

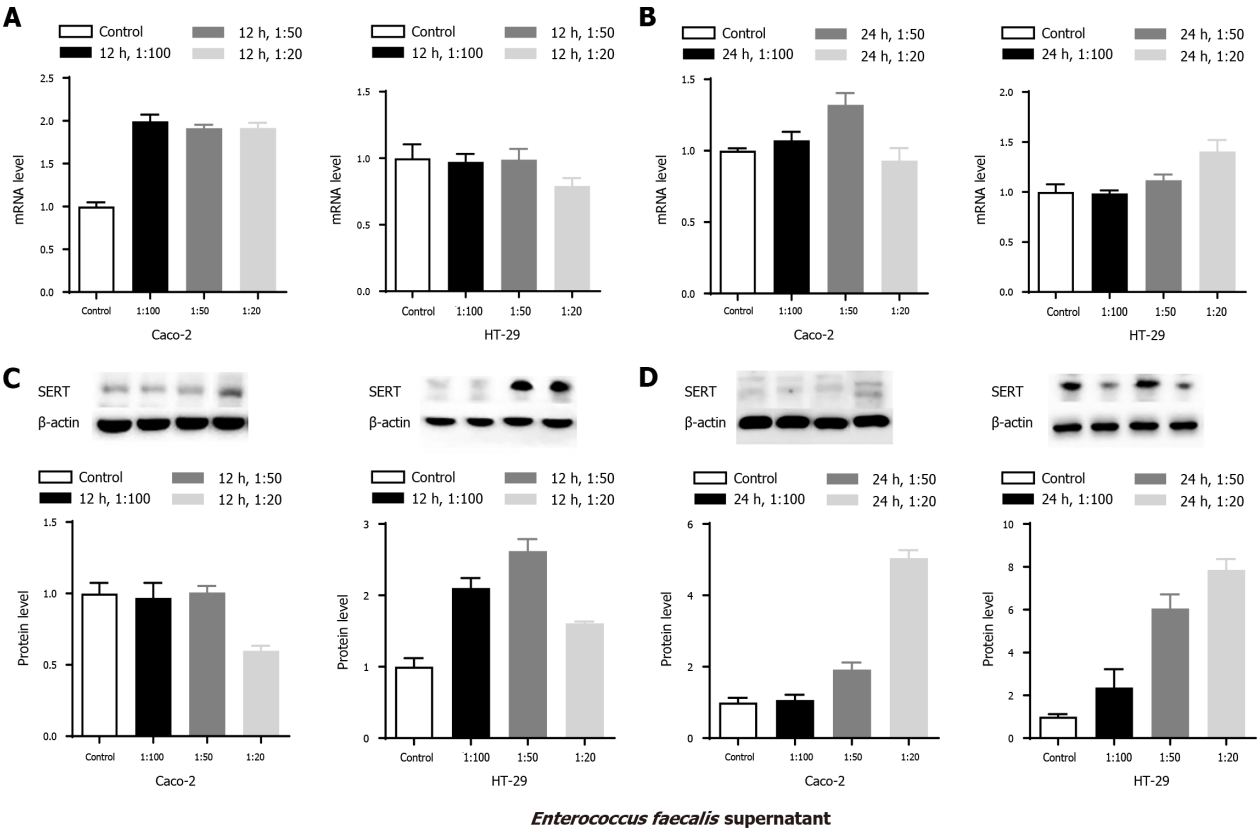

Figure 4 Effects of *Enterococcus faecalis* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells.
A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

biochemical tests were positive on the 7th d after gavage in the PI-IBS group. Twenty-four rats in the control group had lively spirits, quick reactions, bright hair, normal diet, and normal urine and feces. The activities of rats in the PI-IBS group were reduced, lack of luster was observed in the hair of rats, and their appearance was thinner compared with the control group (Figure 5A). However, there were no significant differences between the PI-IBS and control group from 5th wk to 7th wk after gavage. The negative rates of *C. jejuni* fecal culture and biochemical tests (Figure 5B) in the PI-IBS group were higher than 95% at the 7th wk and 9th wk. After 8 wk of *C. jejuni* gavage, four rats from each group were randomly selected for model's evaluation, and the rat model of PI-IBS was confirmed to be successfully constructed. The intestinal transport speed in the PI-IBS group were higher than those in the control group and the expression level of SERT protein in the PI-IBS group were lower than those in the control group (Figure 5C and 5D, $^{a}P < 0.05$).

The efficacy of probiotic therapy on the rat model of PI-IBS

After administration of several probiotic supernatants (group B: *B. subtilis* supernatant; group C: *E. faecalis* supernatant; group D: mixed supernatants of *B. subtilis* and *E. faecalis* with dilution ratio of 1:1), the expression level of SERT protein was further detected to evaluate the intestinal motility of rats.

SERT protein expression in groups C and D were elevated compared with that in the model group (group A) during the 1st wk, and the efficacy of group C was superior than that of group B (Figure 6 w_1, $^{a}P < 0.05$), demonstrating that the efficacy of *E. faecalis* supernatant was superior than *B. Subtilis* supernatant in the 1st wk. The expression levels of SERT protein in groups B, C, and D were markedly higher than that in group A in the 2nd wk (Figure 6 w_2, $^{a}P < 0.05$), and the increase was only observed in the group D in the 3rd wk (Figure 6 w_3, $^{a}P < 0.05$). Besides, the expression level of SERT protein in group D was greater than that in groups B and C in the 2nd wk and 3rd wk (Figure 6 w_2, w_3, $^{a}P < 0.05$), reflecting that the efficacy of mixed supernatant was more significant than single supernatant in the 2nd wk and 3rd wk. Meanwhile, the ratio of expression level of SERT protein in group B to that of group A was elevated from the 1st wk to the 2nd wk, indicating that the efficacy of *B. Subtilis*

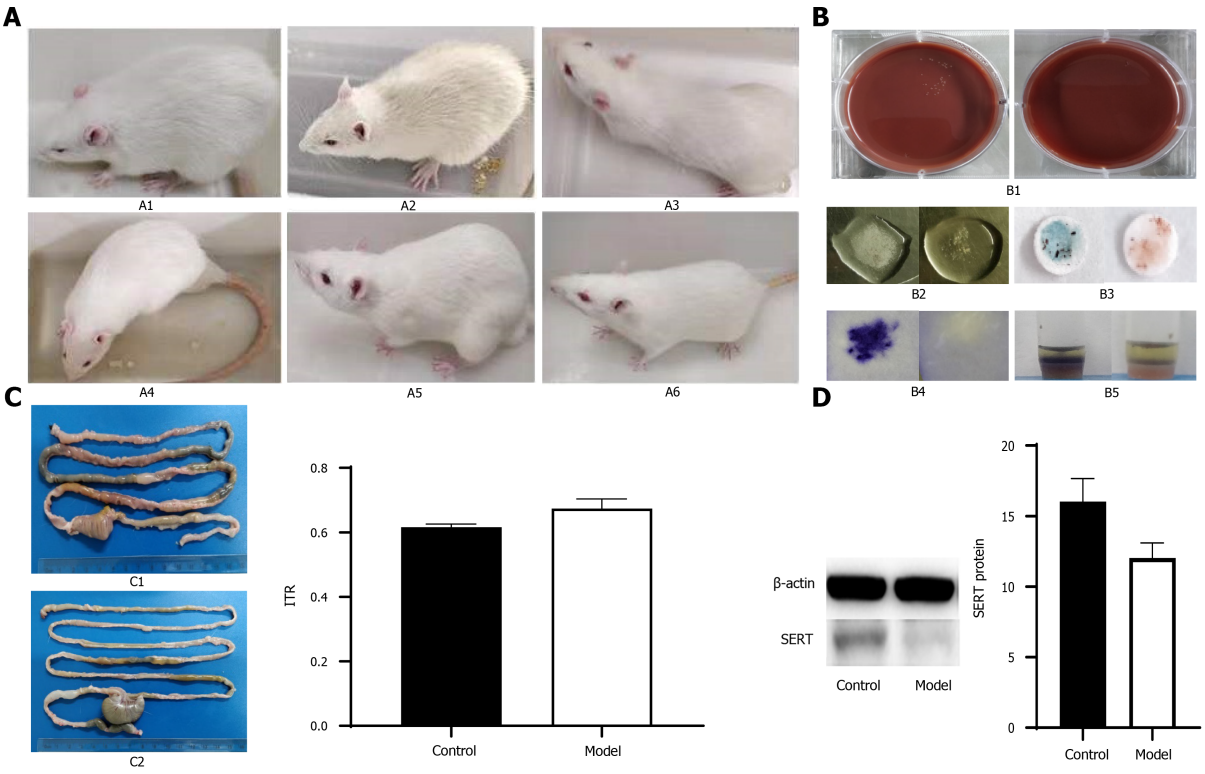

Figure 5 Rat model of post-infectious irritable bowel syndrome. A: Appearance changes of experimental rats; A1: model rats on the 7th day after gavage; A2: the 14th day after gavage; A3: The 28th d after gavage; A4: the 42nd d after gavage; A5: the 56th day after gavage; A6: the normal control group; B: Fecal culture and biochemical detection of *Campylobacter jejuni* B1: *Campylobacter jejuni* culture; B2: catalase test; B3: indole acetate test; B4: oxidase test; e: sodium hippurate hydrolysis test (a-e pictures show that the left picture is positive, the right picture is negative); C: Intestinal transport (ITR) experiment C1: Pylorus to rectal segment in model group; C2: Pyloric to rectal segment in control group; C3: Comparison of ITR histogram ($^aP < 0.05$); D: Expression of serotonin transporter (SERT) protein in the colon during the evaluation period after model evaluation and measurement of ITR experiment. Colon tissue was taken for western blotting to measure SERT level, and quantitative analysis of protein band gray level was used ($^aP < 0.05$).

supernatant was time-dependent. Besides, the ratio of expression level of SERT protein in group C to that of group A was reduced from the 2nd wk to the 3rd wk, representing that the efficacy of *E. faecalis* supernatant was not significant for long-term. There was no significant increase in the expression levels of SERT protein among the treatment groups at the end of the last week (Figure 6 w_4), demonstrating that the long-term efficacy of probiotic therapy was insignificant.

DISCUSSION

IBS is characterized by recurrent abdominal pain, and is associated with a change in stool frequency[3]. At present, there is no effective medical treatment for relieving the symptoms of IBS. Probiotic therapy is extensively applied in supporting the treatment of a broad range of gastrointestinal diseases, and has shown a modest effect on ameliorating the symptoms of IBS[20-23]. *L. rhamnosus GG*, *L. acidophilus*, *Bifidobacterium animalis subspecies lactis*, and *B. longum* are commonly used probiotics that can alleviate the symptoms of IBS[24-36]. A review of four clinical studies, which enrolled 214 IBS patients to evaluate the efficacy of a combination of *B. subtilis* and *E. faecium*, concluded that the combined therapy had a more significant effect on relieving abdominal pain without adverse events[16].

Previous animal studies have demonstrated that the combination of *E. faecium* and *B. subtilis* enhanced intestinal barrier function, increased the expression levels of zonula occludens-1 protein and occludin, and improved gut microbiota with a higher amount of *Lactobacilli*[37]. The expression level of ileal mucin 2 gene and bacteria population were increased in chickens intervened with *B. subtilis* in the 1st wk[38]. Besides, heat-killed *E. faecalis* EF-2001 has shown protective effects on a mouse model of colitis[39]. However, the underlying mechanism of therapeutic effects on IBS remains elusive and requires additional research.

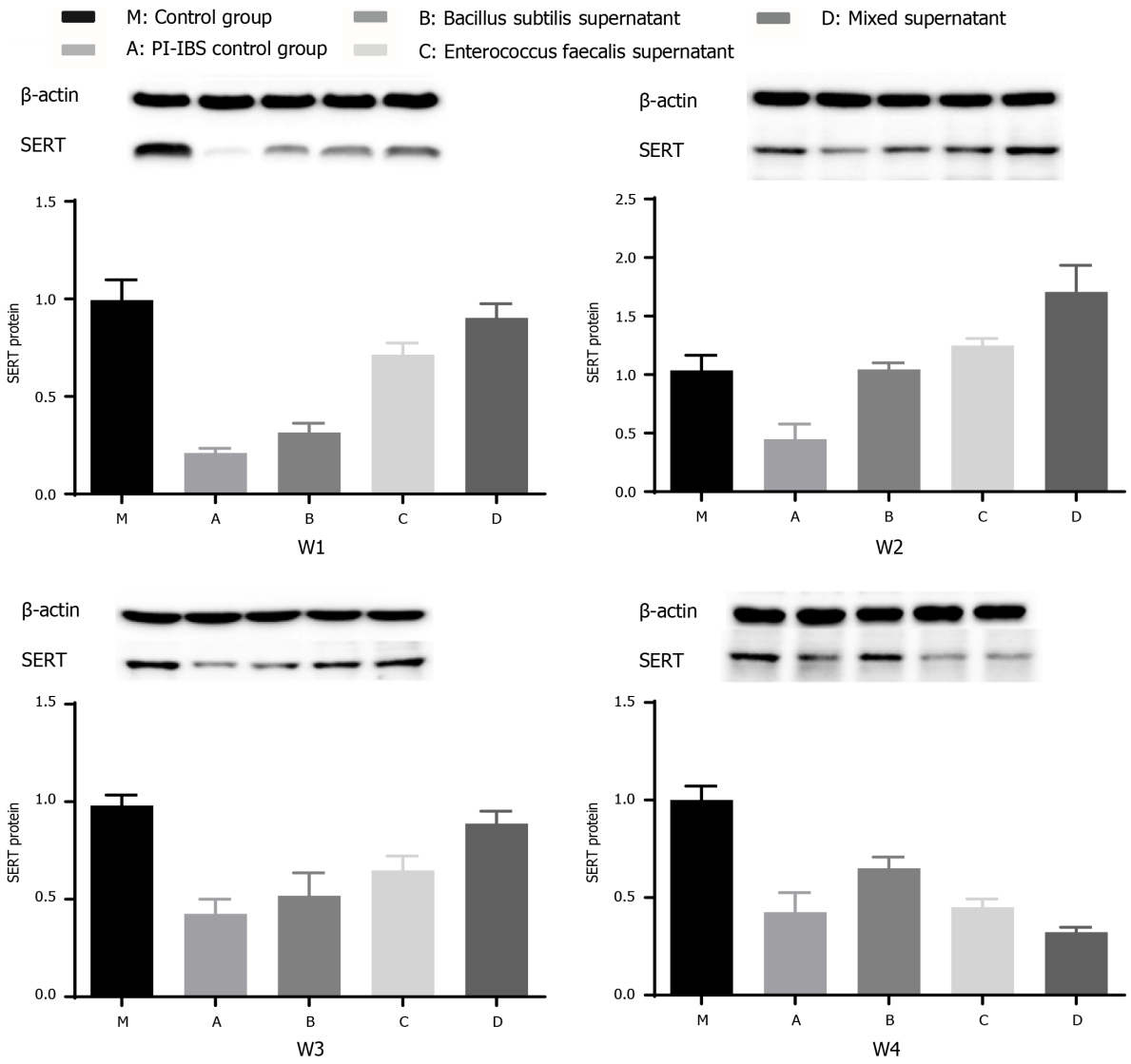

Figure 6 Effects of different supernatants of probiotics on serotonin transporter protein expression in rat intestinal tissues. Quantitative analysis of serotonin transporter (SERT) protein levels in the 1st wk (W1), 2nd wk (W2), 3rd wk (W3), and 4th wk (W4) analyzed by western blotting.

Previous studies have demonstrated that the supernatants of *L. rhamnosus GG*, *L. acidophilus*, and *B. longum* can upregulate the expression of SERT protein in epithelial cells and PI-IBS rats[7,17,18]. SERT, a regulator of the level of 5-HT, plays a significant role in the pathogenesis of IBS and can serve as a novel therapeutic target for IBS. In the present study, we found that the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* upregulated the mRNA and protein levels of SERT in intestinal epithelial cells, and the trend of mRNA and protein expression changes were similar. The expression level of SERT protein was upregulated in the intestinal tissues in a rat model of PI-IBS receiving probiotic therapy. Meanwhile, the efficacy of combined supernatants of *B. subtilis* and *E. faecalis* was superior to a single supernatant.

The mRNA and protein levels of SERT at 12 and 24 h were significantly increased in Caco-2 cells treated with supernatant of *B. subtilis*. *E. faecium* supernatant upregulated the SERT mRNA level in Caco2 cells at 24 h, and the trend of SERT protein level alteration was similar to that of SERT mRNA level. The results were similar in HT-29 cells stimulated with *B. subtilis* supernatant or *E. faecium* supernatant. SERT protein level was elevated at 12 h and reduced at 24 h. Thus, we speculated that upregulation of SERT expression in HT-29 cells intervened with *B. subtilis* or *E. faecium* supernatant was significant in the short-term. Moreover, we explored the efficacy of combination of *B. subtilis* and *E. faecium* supernatants on SERT expression. We found that the mRNA and protein levels of SERT were elevated at 12 h in Caco-2 and HT-29 cells treated with mixed supernatants of *B. Subtilis* and *E. faecium*, and the trend of SERT protein expression was similar to that of mRNA expression. Furthermore, our results confirmed the efficacy of mixed supernatants in relieving the symptoms of IBS, which

was consistent with the findings of a previous study[14].

Besides, increased mRNA and protein levels of SERT were observed in Caco-2 and HT-29 cells treated with *E. faecalis* supernatant for 12 and 24 h. The upregulation of SERT expression treated with *E. faecalis* supernatant could decrease intestinal motility, confirming that ProSymbioflor containing *E. faecalis* and *E. coli* was effective on relieving the symptoms of IBS patients[15].

We also assessed the effects of mixed supernatants of *B. subtilis* and *E. faecalis* with dilution of 1:1 on the expression level of SERT in intestinal tissues in the rat model of PI-IBS. Western blot analysis revealed that SERT protein expression was upregulated in the three therapeutic groups except for the last week. The efficacy of mixed supernatants was superior than that of single supernatant in the first 3 wk. The abovementioned results indicated that the *in vivo* regulatory mechanisms might be more complicated. Due to the acidic environment in the stomach, the function of active ingredients in the supernatants might be attenuated, which might justify the weak efficacy of the supernatants in the last week. Thus, establishing a rat model of PI-IBS motivated us to explore a new approach for protecting important organs from possible damages.

The change in trend of mRNA and protein expression was similar, and the difference in SERT expression level between Caco-2 cells and HT-29 cells might be attributed to different types of cells. Occasionally, SERT protein level alterations in the stimulated groups were not identical to that of the mRNA level in the following conditions. First, the difference between reductions or no changes in the SERT mRNA level and significantly increased SERT protein expression could be explained by the fact that the process of mRNA transcription occurred earlier than protein translation, mRNA was easier degraded than protein, and the status of mRNA was less stable than protein. Second, the inconsistent results regarding an increased SERT mRNA level and a reduced SERT protein could be interpreted by unknown factors of supernatants that might be involved in the process of translation from mRNA to protein. Third, the non-change trend of SERT protein expression was in contrast with the increased SERT mRNA level, which was illustrated by the fact that the expression process of SERT protein was slower and more complicated.

The efficacy of the supernatants of *B. subtilis*, *E. faecium,* and *E. faecalis* was found time-dependent, which might help guide physicians to provide continuous treatment for patients with IBS in clinical practice. Moreover, the SERT expression level, which was regulated by the supernatants in a concentration-dependent manner, could be assessed by possible substances, such as bacterial components or small-molecule proteins regulating the SERT expression level. Such a soluble protein, p40, derived from LGG supernatant, could transactivate epidermal growth factor receptor (EGFR) signaling to reduce cytokine-induced apoptosis and epithelial barrier dysfunction *in vivo* and *in vitro*[40]. Benmansour *et al*[41] showed that EGF could upregulate the expression level of SERT in intestinal epithelial cells by activating the activator protien-1 (AP-1), and further interfering with the uptake of 5-HT. Cui *et al*[42] found that EGF could upregulate the expression level of SERT *via* EGFR signaling pathway. Other factors influencing the expression level of SERT include SERT gene polymorphisms, immune cells, inflammatory cytokines, microRNA 16 (MiR-16), and MiR-545[12]. *B. subtilis* has a highly adaptable metabolism and efficient protein expression and secretion system[43], including alkaline phosphatase and thermostable β-galactosidase[43]. A number of studies have demonstrated that *B. subtilis* and its conditioned media induced cytoprotective heat shock proteins in Caco-2 cells to protect colonic epithelial cells from damages[44-46]. Thus, it deserves to explore which specific factors in the supernatants of *B. subtilis*, *E. faecalis,* and *E. faecium* could regulate the expression level of SERT. The expression level of SERT was upregulated after stimulation with *B. subtilis* or *E. faecalis* supernatants for 12 and 24 h. However, the increase on the expression level of SERT was only observed in the cells stimulated with *E. faecium* supernatant at 24 h, and further research indicated that different potential active ingredients from probiotic supernatants might be effective at different time points or *via* different signaling pathways.

CONCLUSION

In conclusion, we found that the supernatants of *B. subtilis*, *E. faecium,* and *E. faecalis* could upregulate the expression level of SERT in intestinal cells, and the effect of combined supernatants of *B. subtilis* and *E. faecalis* was superior than that of single supernatant on the expression level of SERT in colonic tissues in the rat model of PI-

IBS. Additionally, 5-HT and SERT showed to play important roles in the development of IBS, thus, these proteins were significant to treat IBS patients with lower expression level of SERT or with symptoms of diarrhea. In the next research, we will attempt to explore which effective ingredients in the probiotic supernatants can regulate the SERT expression, and will also find out signaling pathways modulating the SERT expression.

ARTICLE HIGHLIGHTS

Research background

It is worthwhile to explore which effective ingredients in the supernatants of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* and which signaling pathways are regulating serotonin transporter (SERT) expression further.

Research motivation

The supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can upregulate SERT expression in intestinal epithelial cells and the intestinal tissues in the rat model of PI-IBS. And combined supernatants of *B. subtilis* and *E. faecalis* was more efficacious than single supernatant.

Research objectives

The levels of SERT (at post-treatment 12 and 24 h) were significantly elevated in Caco-2 cells treated with *B. subtilis* supernatant compared with those in the control group ($^aP < 0.05$). Those levels were markedly upregulated in Caco-2 cells stimulated with *E. faecium* and *E. faecalis* supernatants at 24 h ($^aP < 0.05$). In addition, the SERT expression in groups B, C and D was significantly higher than that in group A in the 2nd wk ($^aP < 0.05$). Increased SERT expression was found only in group D in the 3rd wk ($^aP < 0.05$). However, there was no significant difference in the SERT expression between the groups in the last week ($P > 0.05$).

Research methods

Caco-2 and HT-29 cells were stimulated with probiotic culture supernatants for 12 and 24 h, respectively. A rat (male Sprague-Dawley rat) model of post-infectious irritable bowel syndrome (PI-IBS) was constructed and the rats were treated with PBS (group A) and three probiotics culture supernatants (groups B, C, and D) for 4 wk. The levels of SERT were detected by quantitative PCR and western blotting.

Research results

The present study aimed to explore whether *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants could upregulate SERT expression in *vitro and in vivo.*

Research conclusions

5-HT and SERT contribute significantly to the development of IBS. Whether the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can improve gastrointestinal sensation and movement by regulating SERT expression needs to be clarified. The research is significant to the treatment of IBS patients with lower expression level of SERT or with symptoms of diarrhea.

Research perspectives

IBS is a functional gastrointestinal disorder, of which the onset and development are associated with serotonin and SERT. Recent studies have shown that *B. subtilis*, *E. faecium*, and *E. faecalis* play important roles in the clinical treatment of IBS. However, the underlying mechanism of therapeutic effects on IBS remains elusive and requires additional research.

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ORIGINAL ARTICLE

Basic Study

Connective tissue growth factor expression hints at aggressive nature of colorectal cancer

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Abstract

BACKGROUND

Connective tissue growth factor (CTGF) is a mediator of transforming growth factor-beta signaling and plays a key role in connective tissue remodeling, inflammatory processes and fibrosis in various illnesses including cancer.

AIM

To investigate the role of CTGF in colorectal cancer (CRC) progression and to compare the CTGF expression with different clinicopathological parameters.

METHODS

Real-time polymerase chain reaction, immunohistochemistry and Western blotting was performed to evaluate the CTGF expression and the results were statistically analyzed against the clinicopathological variables of patient data using STATA software version 16.

RESULTS

and proofreading.

Institutional review board statement: The study was reviewed and approved by the Institutional Ethics committee (IEC), Sher-I-Kashmir Institute of Medical Sciences, Srinagar, Jammu and Kashmir, India.

Conflict-of-interest statement: The authors declare no conflicts of interest to state regarding this study.

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CTGF expression levels in tumor specimens were significantly higher than their paired normal specimens. The higher protein expression levels showed a significant association with smoking, staging, tumor grade, invasion depth, necrosis of tumor tissue, and both lymphovascular and perineural invasion. As *per* the cox regression model and classification tree analysis, tumor-node-metastasis stage and perineural invasion were important predictors for CTGF expression and prognosis of CRC patients. Survival analysis indicated that CTGF overexpression was associated with poorer overall and disease-free survival.

CONCLUSION
Expression of CTGF was increased in CRC and was linked with poor overall and disease-free survival of CRC patients. These findings support prior observations and thus CTGF may be a possible prognostic marker in CRC.

Key Words: Connective tissue growth factor; Quantitative real time polymerase chain reaction; Immunohistochemistry; Western blotting; Colorectal cancer

Core Tip: This study demonstrates that connective tissue growth factor is overexpressed in colorectal carcinoma and is predominantly localized in the cytoplasm of the cell. The expression pattern of this gene showed a substantial association with aggressive phenotypes of colorectal cancer like late-stage tumor and lymph node metastasis. It also showed a significant correlation with the overall and disease-free survival of colorectal cancer patients, hence could act as a predictive biomarker for diagnostics and prognostics of colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) ranks 3rd worldwide in terms of prevalence and is the second most common type of cancer in terms of mortality, with bowel cancer being the fourth most common cancer and rectal cancer being the eighth most common cancer in world. In 2020, more than 1.9 million new CRC cases including the anus and 935000 deaths were expected, accounting for about one in every ten cases and deaths[1]. Transitioned countries have a four-fold higher incidence rate than transitional countries, but there is less difference in death rates due to the higher fatality rate in transitioning countries[2]. However, most of the cases and deaths are attributed to modifiable risk factors, such as smoking, certain types of diets, high alcohol consumption, sedentary lifestyle, and being over-weight or obese, and thus potentially evadable. Other clinicopathological characteristics like age, family history of CRC or personal history of inflammatory bowel disease or colon polyps and many other inherent risk factors that cannot be modified have progressively been recognized as contributing to a more individual prognosis prediction in CRC[3,4]. CRC is a disease of modernity and heterogeneity: It has the highest rates of incidence and almost 60% of cases are encountered in developed countries. In India, the age-standardized average for CRC is poor, at 7.2 *per* 100000 for men and 5.1 *per* 100000 for women[3].

Connective tissue growth factor (CTGF) or CCN-family protein 2 (CCN2), is a member of the CCN family. The acronym CCN was assigned to this family after the initials of the first three proteins which include cysteine rich-61 (Cyr61/CCN1), connective tissue growth factor (CTGF/CCN2) and nephroblastoma overexpressed (Nov/CCN3)[5]. WNT1 inducible signaling pathway proteins WISP1/CCN4, WISP2/CCN5 and WISP3/CCN6 are other members of this family[6]. These proteins manifest roles in cellular mechanisms like cell proliferation, apoptosis, cell-cell

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adhesion, ion-transport, extracellular matrix production, growth arrest, migration in numerous cell types, chemotaxis and motility[7]. CTGF is also related to growth of bone, cartilage, angiogenesis and is associated with a number of biological response modifiers[8,9]. CTGF expression has been linked to a variety of disorders, including diabetic nephropathy, arthritis, cardiovascular disease, and retinopathy as well as many types of malignancies[10-14]. CTGF shows an aberrant expression in variety of cancers like pancreatic cancer cells[12], breast[15], esophageal cancers[11], in gliomas [10] and invasive melanomas[16]. Expression studies of CTGF in CRC have been reported by some groups[17,18], but no such study has been conducted in our population to date. The Kashmir valley, located in India's extreme north, is very unique in its climate, diet and geology. CRC is the fourth most prevalent kind of cancer in the Kashmir valley, after stomach, esophageal, and lung with a significant increase in a number of cases[19]. To further explore the potential role of CTGF in CRC, we looked at its expression and localization and compared the findings to critical clinicopathological parameters like tumor grade, tumor-node-metastasis (TNM) stage, Duke stage, lymph node metastasis, lymphovascular invasion (LVI), perineural invasion (PNI) and so on. We also performed a survival study analysis on the CRC patients to see whether CTGF has a prognostic role in the disease. In patients with CRC, TNM staging remains the most important prognostic predictor. The systemic spread of tumor cells *via* metastasis is the major factor that leads to cancer recurrence and prognosis. As a result, including LVI and PNI into the present TNM staging method may provide a more accurate indication of patient prognosis in any stage of CRC, allowing for more effective adjuvant therapy planning and patient follow-up.

MATERIALS AND METHODS

Patient specimens

Seventy-one (n = 71) histopathological confirmed human CRC tissues along with adjacent normal tissues (study controls) were taken for the study, which included 38 males and 33 females. The specimens were taken from the subjects who underwent primary surgical resection for CRC at the Department of General and Minimal invasive Surgery, Sher-i-Kashmir Institute of Medical Science (SKIMS) Srinagar. Patients who received neoadjuvant or chemoradiotherapy were excluded from our study. Specimens were preserved in RNA Later (Sigma-Aldrich Burlington, MA) for RNA extraction and held in -80 ℃ till further processing. For immunohistochemistry (IHC), formalin fixed and paraffin embedded blocks of the same specimens and their adjacent normal blocks were collected from the Department of Pathology, SKIMS. Both the samples and blocks were taken in accordance with SKIMS Research Ethics Committee's approved protocol and with the patient's written consent.

RNA isolation and cDNA synthesis and real-time polymerase chain reaction

Total RNA was extracted by the Trizol method (Invitrogen Waltham, MA, United States)[20]. The absorbance at A260/280 of 1.8-2 was considered "pure" for RNA. After DNase-I (Qiagen) treatment, as *per* the manufacturer's instructions, complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (#K1622; Thermo Scientific Ltd, Waltham, MA, United States). In a final volume of 20 µL, 1g RNA was reverse transcribed using AMV Reverse Transcriptase and random hexamers. The cyclic conditions were 5 min at 25 ℃, then 60 min at 42 ℃ and finally 5 min at 70 ℃. Quantitative real time polymerase chain reaction (qRT-PCR) was done using SYBR Green Master Mix (Thermo Fisher Scientific Ltd) in Piko-Real PCR machine (Thermo). Experiments were carried out in triplicates with effects of each being standardized against the housekeeping gene GAPDH. The following primers were used; CTGF-fp: 5'CTCCTGCAGGCTAGAGAAGC3'; rp: 5'GATGCACTTTTTGCCCTTCTT3'. GAPDH-fp: 5'CACCACCAACTGCTTAG3'; rp: 5'CTTCACCACCTTCTTGATG'. The Cycle Threshold (Ct) was used to describe the expression of CTGF messenger RNA (mRNA). The relative expression levels were determined using Livak and Schmittgen's $2^{-\Delta\Delta ct}$ method[21].

The mean Ct values for the gene of interest (*CTGF*) and the control gene (*GAPDH*) were estimated. ΔCt was calculated by subtracting the average of triplicate CTGF Ct values from the average of triplicate GAPDH Ct values. The ΔΔCt stood for the difference between the paired tissues samples, which was determined using the formula ΔΔCt=ΔCt of tumor-ΔCt of normal. The fold-change in tumor levels relative to surrounding normals was estimated as $2^{-\Delta\Delta Ct}$. Denaturation at 95 ℃ for 7 min, 40 cycles of denaturation; 95 ℃ for 5 min, annealing; 59 ℃ for 50s and extension; 60 ℃ for 30s

were used in the qRT-PCR reaction. There was no non-specific product formation according to the melt curve review.

Protein extraction and Western blot assay

About 50-100 mg tissue specimens were washed thrice with icy-cold phosphate-saline buffer in a centrifuge at 3567g for 5-min. The Tissue sections were then homogenized in RIPA Lysis buffer (Cat#20-188; Lot#3283787), to which 1 mmol/L sodium fluoride, 1 mmol/L PMSF and protease inhibitor cocktail 10 µL *per* 1 mL of lysis buffer were added immediately prior to use. After lysis samples were vortexed for 15 s and then incubated on ice for 40 min. Centrifugation at 73 g for 20 min was done and the supernatant containing extracted protein was collected, the protein concentration was measured spectrophotometrically at 595 nm by using Bradford's assay. Protein extract was run on a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel after being preheated at 95 °C for 5 min in reducing SDS sample buffer containing 50 mmol/L TrisHCl (pH6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue and 100 mmol/L mercaptoethanol. The proteins isolated were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, United States) by semidry transfer method following the manufacturer's instructions (Hoefer) after gel electrophoresis. The PVDF membrane was processed according to a standardized protocol for immune detection. Rabbit polyclonal antibody anti-CTGF was used at 1:1000 dilution (ab5097; Abcam, Cambridge, United Kingdom). Rabbit monoclonal antibody against beta-actin was employed at 1:1000 dilution (Cell Signaling Technology; Danvers, MA, United States). The HRP-tagged secondary antibody (#70749; Cell Signaling Technology) was used for the detection of protein bands. Beta-actin served as a loading control. The blot was detected by chemiluminescence with Super Signal West Femto Maximum Sensitivity Substrate. (Cat No. 34095). Image J software version 15.3a was used to assess the densiometry of the blots to measure the quantity of proteins present.

Immunohistochemical analysis

For immunohistochemical analysis of CRC tissues, the formalin-fixed and paraffin embedded representative blocks were used. A manual microtome (Leica Biosystems, Mumbai, Maharashtra, India) was used to slice 5-µm thick tissue sections from paraffin blocks and mount on charged poly-L-lysin coated glass slides (LOT #180310 Bio-Optica Milano S.p.a *via* San Faustino, 58 20134 Milan-Italy). The sections were deparaffinized using xylene and ethanol, and then they were rehydrated in a graded series of ethanol (100%, 95%, 90%, 80% and 70%), followed by microwave/pressure cooker antigen retrieval. Rehydration of sections was done using distilled water. Hydrogen peroxide blocking solution (Biocare Medical, Condord, CA, United States) was added after rehydration to cover the whole section which acts as an inhibitor of endogenous peroxidase activity. After that was done the slides were incubated for 15 min while keeping the slides hydrated and letting them dry. Slides were washed two to three times with a phosphate buffer saline (PBS) (pH = 7.4). Tumor sections were then treated with 10 mmol/L citrate buffer (pH 6.0) at 95 °C to retrieve antigens. After which the slides were washed three times with PBS. To block non-specific background staining, the sections were covered with protein blocking solution (#BS966G Biocare Medical) which was followed by a 15 minute incubation. Washings were done with PBST (1 × PBS with Tween-20). Slide edges were dried and sections were outlined with boundary by PAP pen (Abcam). Primary antibody anti-CTGF (1:200; Abcam) was put on the whole sections and kept in overnight incubation. Next day the slides were washed thrice using PBS. Secondary antibody coated to goat anti-rabbit HRP-conjugated secondary antibody (#M2U522G; MACH 2 Universal HRP-Polymer Detection Kit) was put on the sections which were then kept in incubation for 15 min. PBST washings were done four times and later stained with HRP/DAB detection IHC kit (#BDB2004H; Biocare Medical). To prepare DAB solution, 30 µL of DAB chromogen (#BDB900C; Biocare Medical) was mixed with 1.5 mL of DAB substrate (#DS900H; Biocare Medical), which was then applied to the sections. The slides were again washed four times with PBST. The slides were immersed in distilled water, counterstained with hematoxylin and thereafter, dehydrated in xylene, mounted with DPX and covered by cover slips. Visualization was done by a light microscope (1 × 81; Olympus, 1 × 81; Tokyo, Japan). Sections from liver carcinoma were used as positive controls. For negative controls, PBS was used instead of primary antibody to tissue sections.

Statistical analysis

For statistical analysis, independent t test and logistic regression was used to calculate

the odds ratio (OR) and 95% confidence interval (95%CI). P value < 0.05 was considered as statistically significant. Analysis of the survival data was done by the Kaplan–Meier method. Kaplan–Meier curves were compared by a log-rank test. Regression analysis was utilized using an extended Cox regression model. All the statistical analysis was done using STATA software, version 16 (STATA 16 Corp., College Station, TX, United States).

Ethical clearance

A written consent was taken from all the patients prior to surgery and they were also apprised about the ongoing study which was approved by Ethical Clearance Committee of SKIMS (SIMS 1131/IEC-SKIMS/2018-330).

Follow-up

Patients were contacted by phone for follow-up. The deadline for contacting the patients was May 2021. The survival intervals were calculated from both the date of diagnosis as well as from the date of surgery to the last follow-up.

RESULTS

Patient characteristics

Seventy-one human histopathological confirmed CRC tissues and paired normal adjacent tissue specimens from patients who went through primary surgical resection for CRC with no chemo/radiotherapy received were used to assess the CTGF expression. Demographic and clinicopathological variables for this study are outlined in Table 1. The cases included 38 (53.52%) males and 33(46.47%) females. In all, 50 of 71 (70.42%) subjects were greater or equal to 50 years and 21 of 71 (29.71%) were less than 50 years having a mean age of 56.04 ± 13.48.

CTGF mRNA expression in CRC

We used qRT-PCR to analyze the expression of CTGF at mRNA level in CRC tissues and the adjacent normal tissues (n = 71). Overexpression of CTGF was detected in 80.28% (57/71) tumor tissues as compared to adjacent normal tissues. The average fold change of CTGF expression in CRC tissues in comparison to normal adjacent tissues was 2.49 ± 1.59. Figure 1 depicts the dot blots of CTGF mRNA indicating ΔCt values. To ensure accuracy, melt curve analysis was performed on every experiment that yielded zero nonspecific results.

CTGF protein expression and localization in CRC

CTGF protein localization and expression pattern was analyzed by IHC in 71 histopathological confirmed CRC specimens and their adjacent normal tissues. In CRC tissues, CTGF immunoreactivity showed a varying intensity of staining with most remarkable staining in cytoplasm of the tumor cells (Figure 2). A scoring system was set for evaluation of CTGF immunoreactivity as 0 (negative staining); +1(less than 5% of the tumor cells stained/Weak staining); +2 (less than 50% tumor cells stained/Moderate staining); and +3 (more than 50% tumor cells stained/Strong staining). Overexpression was said to be intense when cytoplasm stained more than three times in tumor compared to normal adjacent tissues. IHC Profiler plugin ImageJ software (ImageJ version 15.3a) was used for quantification of staining intensity. Out of 71 CRC cases, a total of 44/71 (61.97%) cases showed high expression compared to their normal adjacent tissues. Among all, 26 of 71 (36.61%) displayed a strong (+3) cytoplasmic staining intensity, 18 of 71 (25.35%) showed moderate (+2), 25 of 71 (35.21%) showed weak (+1) intensity and 2 of 71 (2.82%) CRC cases showed negative scoring intensity. The prevalence of high CTGF expression among the 4 TNM stages was 28%, 72%, 88.89% and 100% for Stage I, II, III, and IV respectively among all 71 CRC cases. Among the 44 cases which showed overexpression of CTGF, the stage wise contribution was 15.91% (7/44) in stage I, 40.91% (18/44) in stage II, 36.36% (16) in stage III and 6.82% (3/44) in stage IV disease (P < 0.001; Chi^2 = 20.7).

In order to confirm the results of immunohistochemical staining, western blot analysis was performed to evaluate the CTGF protein levels in the same patient specimens and their adjacent normal tissues. Densitometric image analysis was done by ImageJ version 1.53a software to assess the amount of protein present in the sample. CTGF protein expression was considered high in CRC tumor samples when the level was more than one-fold than that in adjacent normal tissues. Among the 71

Table 1 Clinicoepidemological and clinicopathological parameters of study population (*n* = 71)

Characteristics	*n* (%)
Age in years	
< 50	21 (29.58)
≥ 50	50 (70.42)
Gender	
Male	38 (53.52)
Female	33 (46.48)
Dwelling	
Rural	47 (66.20)
Urban	24 (33.80)
Social class	22 (30.99)
Low	
Middle and high	49 (69.01)
Family history	
Yes	20 (28.17)
No	51 (71.83)
Smoking status	
Yes	40 (56.34)
No	31(43.66)
Lifestyle	
Active	31 (43.66)
Sedentary	40 (56.34)
Salt tea intake	
Yes	65 (91.55)
No	06 (8.45)
Red meat consumption	
Yes	59 (83.10)
No	12 (16.90)
Sundried vegetables	
Yes	48 (67.61)
No	23 (32.39)
Source of drinking water	
Tap water (R)	46 (64.79)
Tap water (L)	07 (9.86)
Others[1]	18 (25.35)
Pickles	
Yes	41 (57.75)
No	30 (42.25)
Pesticide exposure	
Yes	33 (46.48)
No	38 (53.52)
Junk food consumption	

Yes	05 (7.04)
No	66 (92.96)
Site of tumor	
Colon	36 (50.70)
Rectum	20 (28.17)
Rectosigmoid	15 (21.13)
Tumor differentiation	
Well	18 (25.35)
Moderate	46 (64.79)
Poor	07 (9.86)
Invasion depth	
T1	08 (11.27)
T2	22 (30.99)
T3	31 (43.66)
T4	10 (14.08)
T1 + T2	30 (42.25)
T3 + T4	41 (57.75)
TNM staging	
I	25 (35.21)
II	25 (35.21)
III	18 (25.35)
IV	03 (4.22)
I + II	50 (70.42)
III + IV	21 (29.58)
Tumor grade	
1	18 (25.35)
2	46 (64.79)
3	07 (9.86)
DUKE stage	
A	05 (7.04)
B	44 (61.97)
C	22 (30.99)
Node status	
0	51 (71.83)
1 and 2	20 (28.17)
LVI	
Present	54 (76.06)
Absent	17 (23.94)
PNI	
Present	15 (21.12)
Absent	56 (78.87)
TALNR	
Present	62 (87.32)

Absent	09 (12.68)
Necrosis seen	
Yes	18 (25.35)
No	53 (74.65)
Recurrence	
Yes	12 (16.90)
No	59 (83.10)
Vital status	
Alive	66 (92.96)
Dead	05 (7.04)

[1]Others: Spring, Well, Pond.

N: Total Number of samples; R: River water through Tap; L: Lake water through Tap; TNM: Tumor-node-metastasis; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion.

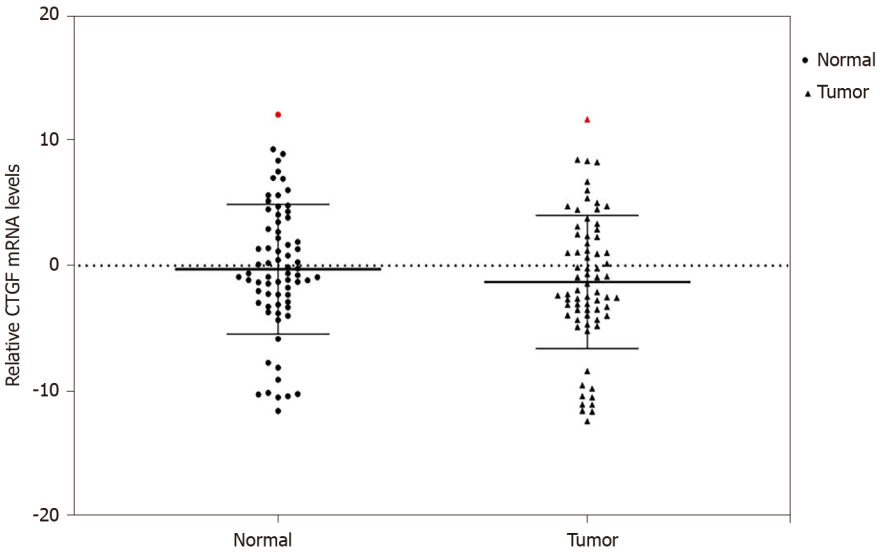

Figure 1 Quantitative real-time polymerase chain reaction analysis of connective tissue growth factor mRNA across the colorectal cancer tissues and their adjacent normals (*n* = 71). mRNA: Messenger RNA; CTGF: Connective tissue growth factor.

CRC cases, 60.56% (43/71) exhibited increased CTGF protein expression in tumor tissues as compared to adjacent normals. Figure 3A shows the representative blot of CTGF protein expression upregulated in CRC tumor tissues and Figure 3B depicts the corresponding bar chart of densiometric values of CTGF protein expression. The CRC samples which showed protein overexpression had also increased mRNA expression. Figure 4 depicts the average fold change in CTGF overexpression in all 71 CRC tumors relative to adjacent normal.

The correlation between real time PCR, western blotting and immunohistochemical analysis was high and significant (P = 0.0001).

Correlation of CTGF with various clinicopathological factors

To better understand the clinical significance of CTGF expression in CRC, expression of CTGF was correlated with a series of clinicopathological parameters. The overexpression of CTGF was associated with smoking, tumor differentiation, invasion depth, TNM stage, tumor grade, Duke staging both at mRNA level as well as at protein level. Node status, lymphovascular, PNI and necrosis of tumor tissues was also significantly associated with the overexpression of CTGF (P < 0.001). Gender was associated with mRNA expression of CTGF (P = 0.037), but not with its protein expression. No significant association with other parameters such as age, sex, dwelling, social class, family history, lifestyle, salt tea intake, sundried vegetables, junk food consumption,

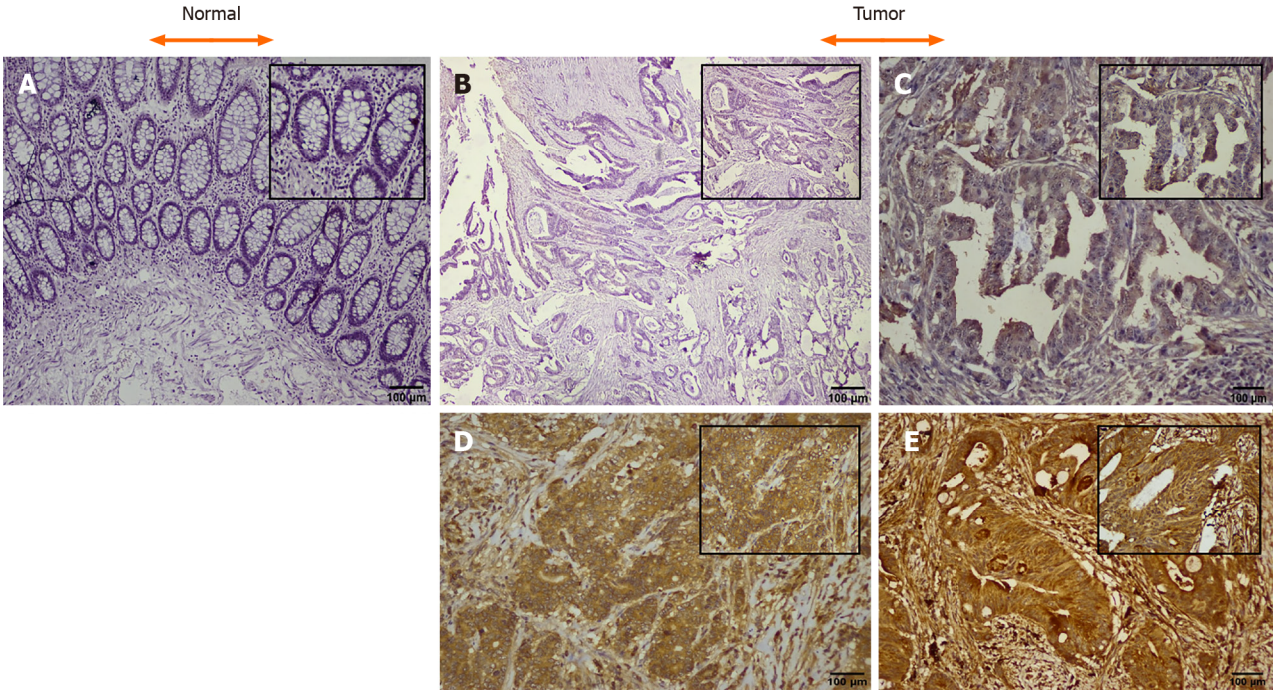

Figure 2 Representative images of immunohistochemical expression of the connective tissue growth factor protein in colorectal cancer and adjacent normal (H & E and DAB Chromogen × 100 insect × 400). A: Negative control; B: Tumor negative staining; C: Tumor + 1, weak staining; D: Tumor + 2, moderate staining; E: Tumor + 3, strong staining. Scale bars: 100 µM.

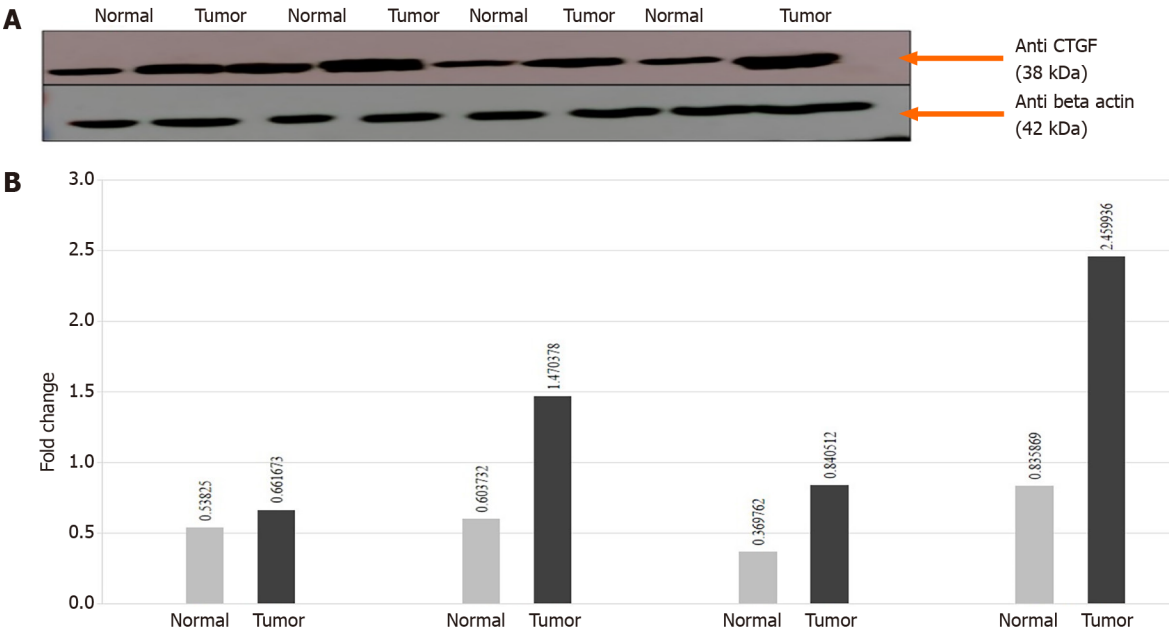

Figure 3 Connective tissue growth factor. A: The connective tissue growth factor (CTGF) is upregulated in human colorectal cancer (CRC) samples. Representative Immunoblot showing expression of tumors and their adjacent normals in CRC. Lane N: Normal; Lane T: Tumor. β-actin is used as a loading control. About 40 µg protein loaded on 12% sodium dodecyl sulphate-Gel transferred to polyvinylidene difluoride membrane, anti-β actin primary Ab (1:1000), anti-CTGF primary Ab (1:500); B: Normalized densitometric values. The experiments were performed in triplicates; mean and standard error was calculated. CTGF: Connective tissue growth factor.

pesticide exposure, use of pickles, source of drinking water and site of tumor was observed at mRNA or protein level. CTGF high expression was also significantly correlated with recurrence of the patients after surgery, but no association was seen with vital status of the CRC patients (Tables 2-4).

Table 2 Clinicopathological relevance of connective tissue growth factor mRNA expression in colorectal cancer patients as determined by quantitative real-time polymerase chain reaction

Characteristics	Normal expression (*n* = 14)	Overexpression (*n* = 57)	Odds ratio (95%CI)	*P* value	Chi²
Age					
< 50	2 (9.52)	19 (90.48)	Referent		
≥ 50	12 (24.00)	38 (76.00)	3.0 (0.6-29.9)	0.16	1.95
Gender					
Male	4 (10.53)	34 (89.47)	Referent		
Female	10 (30.30)	23 (69.70)	3.6 (0.9-17.8)	0.037[a]	4.36
Dwelling					
Rural	11 (23.40)	36 (76.60)	Referent		
Urban	3 (12.50)	21 (87.50)	0.5 (0.1-2.1)	0.27	1.19
Social class					
Low	7 (31.82)	15 (68.18)	Referent		
Middle and high	7 (14.29)	42 (85.71)	0.4 (0.09-1.4)	0.08	2.94
Family history					
Yes	3 (15.00)	17 (85.00)	Referent		
No	11 (21.57)	40 (78.43)	1.5 (0.3-9.7)	0.53	0.39
Smoking status					
Yes	4 (10.00)	36 (90.00)	Referent		
No	10 (32.26)	21 (67.74)	4.3 (1.04-20.68)	0.019[a]	5.46
Lifestyle					
Active	8 (25.81)	23 (74.19)	Referent		
Sedentary	6 (15.00)	34 (85.00)	0.5 (0.1-1.1)	0.25	1.28
Salt tea intake					
Yes	13 (20.00)	52 (80.00)	Referent		
No	1 (16.67)	5 (83.33)	0.8 (0.02-8.1)	0.844	0.03
Red meat consumption					
Yes	13 (22.03)	46 (77.97)	Referent		
No	1 (8.33)	11 (91.67)	0.3 (0.01-2.6)	0.37	1.22
Sundried vegetables					
Yes	9 (18.75)	39 (81.25)	Referent		
No	5 (21.74)	18 (78.26)	1.2 (0.2-4.7)	0.767	0.08
Source of drinking water					
Tap water (R)	12 (26.09)	34 (73.91)	Referent		
Tap water (L)	1 (14.29)	6 (85.71)	0.5 (0.01-5.3)		
Others[1]	1 (5.56)	17 (94.44)	0.4 (0.04-2.4)	0.54	1.21
Pickles					
Yes	8 (19.51)	33 (80.49)	Referent		
No	6 (20.00)	24 (80.00)	1.03 (0.3-3.9)	1.000	0.002
Pesticide exposure					
Yes	7 (21.21)	26 (78.79)	Referent		
No	7 (18.42)	31 (81.58)	0.84 (0.219-3.21)	0.8	0.07

Junk food consumption					
Yes	0 (0)	5 (100)	Referent		
No	14 (21.21)	52 (91.23)	0 (0-3.08)	0.25	1.32
Site of tumor					
Colon	7 (19.44)	29 (80.56)	Referent		
Rectum	6 (30.00)	14 (70.00)	1.8 (0.4-7.45)		
Rectosigmoid	1 (6.67)	14 (93.33)	0.3 (0.01-2.7)	0.23	2.91
Tumor differentiation					
Well	8 (44.44)	10 (55.56)			
Moderate	5 (10.87)	41 (89.13)	Referent		
Poor	1 (14.29)	6 (85.71)	0.15 (0.03-0.7)	0.009[a]	9.35
Invasion depth					
T1	5 (62.50)	3 (37.50)			
T2	7 (31.82)	15 (68.18)	Referent		
T3	2 (6.45)	29 (93.55)	0.3 (0.03-2.0)	0.001[a]	17.21
T4	0 (0)	10 (100)			
T1 + T2	12 (40.00)	18 (60.00)			
T3 + T4	2 (4.88)	39 (95.12)	0.08 (0.008-0.4)	0.000[a]	13.5
TNM staging					
I	11 (44.00)	14 (56.00)	Referent		
II	2 (8.00)	23 (92.00)	0.1 (0.01-0.6)	0.002[a]	14.5
III	1 (5.56)	17 (94.44)	0.07 (0.01-0.7)		
IV	0 (0)	3 (100.00)		0.040[a]	4.21
I + II	13 (26.00)	37 (74.00)			
III + IV	1 (4.76)	20 (95.24)	0.1 (0.01-1.1)		
Tumor grade					
1	8 (44.44)	10 (55.56)			
2	5 (10.87)	41 (89.13)	Referent		
3	1 (14.29)	6 (85.71)	0.1 (0.03-0.7)	0.009[a]	9.35
DUKE stage					
A	2 (2.82)	3 (4.23)			
B	11 (15.49)	33 (46.48)	Referent		
C	1 (1.41)	21 (29.58)	0.5 (0.05-6.8)	0.042[a]	5.20
Node status					
0	13 (25.49)	38 (74.51)	Referent		
1 and 2	1 (5.00)	19 (95.00)	0.1 (0.003-1.2)	0.041[a]	3.81
LVI					
Present	8 (14.81)	46 (85.19)	Referent		
Absent	6 (35.29)	11 (64.71)	3.1 (0.7-12.7)	0.064	3.42
PNI					
Present	0 (0.00)	15 (100)	Referent		
Absent	14 (25.00)	42 (75.00)	0 (0-1.08)	0.031[a]	4.67
TALNR					

Present	11 (17.74)	51 (82.26)	Referent		
Absent	3 (33.33)	6 (66.67)	2.3 (0.32-12.8)	0.272	1.20
Necrosis seen					
Yes	17 (94.44)	1 (5.56)	Referent		
No	40 (75.47)	13 (24.53)	2.7 (0.5-27.7)	0.080	3.10
Recurrence					
Yes	0 (0)	12 (100)	Referent		
No	14 (23.73)	45 (76.27)	0 (0-1.08)	0.060	3.55
Vital status					
Alive	13 (19.70)	53 (80.30)	Referent		
Death	1 (20.00)	4 (80.00)	1.4 (0.02-1.9)	0.85	0.07

[1]Others: Spring, Well, Pond.
[a]$P < 0.05$: Statistical significance.
R: River water through tap; L: Lake water through tap; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion; CI: Confidence interval.

CTGF and CRC prognosis

Kaplan-Meier survival curves from date of diagnosis of the disease and from date of surgery as shown in Figures 5 and 6 revealed that CRC patients with low CTGF protein expression had good overall survival (OS) and disease-free survival (DFS) than those with higher levels of CTGF. The patients had a median survival time of 37 mo from the time that CRC was diagnosed and 28 mo from when the surgery was done and the higher CTGF protein levels were significantly associated with the recurrence of disease (P=0.020). To determine the independent predictive value of CTGF expression, the influence of each clinicopathological parameter and the expression pattern of CTGF on patient OS and DFS was determined using extended Cox-regression model listed in Table 5. Although TNM stage, Duke Stage, Node status, Necrosis and CTGF Expression showed an accord with the DFS, but only TNM stage and PNI were significant independent predictors of OS in the multivariate analysis ($P < 0.05$). A classification tree analysis was also done to explore the relationship of CTGF overexpression with different variables of colorectal tumors (Figure 7). TNM Stage, Duke Stage, Node status and necrosis were important factors for recurrence of the disease and TNM stage with PNI positive tumors were important predictors for CTGF expression in CRC.

DISCUSSION

In the present study, the CTGF mRNA and protein expression in CRC tissues was evaluated and their histopathological confirmed adjacent normals using qRT-PCR, western blotting, and IHC. The results show that 80.28% of CRC tissues at mRNA level and 60.56% of CRC tissues at protein level had overexpression compared to their adjacent normals. CTGF was overexpressed at both the protein and mRNA levels in the majority of samples, showing that CTGF is upregulated at both the transcriptional and translational levels, although some samples showed overexpression at the mRNA level only. CTGF overexpression in CRC has previously been reported in some studies but they did not contain much clinicopathological data of patients. Higher expression levels of CTGF in CRC tissues compared to adjacent normal specimens have been found in cancers of the esophagus, pancreas and gliomas[11,22,23]. In this study, it was observed that CTGF expression was high in 61.97% of tumor sections as compared to their adjacent normals and was predominantly present in cytoplasm of CRC tumor cells. This is analogous to a variety of other tumor forms, such as esophageal and pancreatic cancers[11,22]. Previous CTGF immunohistochemical studies also revealed cytoplasmic or membranous staining[17,18]. Higher levels of CTGF are linked to more advanced disease in esophageal adenocarcinoma and breast cancer[11,24]. CTGF, a well-known transcriptional target of transforming growth factor-beta (TGF-β), was found to have higher mRNA and protein levels in more advanced Duke and TNM

Table 3 Clinicopathological relevance of connective tissue growth factor protein expression in colorectal cancer patients as determined by Western blot analysis

Characteristics	Normal expression (*n* = 28)	Overexpression (*n* = 43)	Odds ratio (95%CI)	*P* value	Chi²
Age					
< 50	6 (28.57)	15 (71.43)	Referent		
≥ 50	22 (44.00)	28 (56.00)	1.9 (0.6-7.1)	0.22	1.47
Gender					
Male	12 (31.58)	26 (68.42)	Referent		
Female	16 (48.48)	17 (51.52)	2.03 (0.7-6.0)	0.15	2.11
Dwelling					
Rural	20 (42.55)	27 (57.45)	Referent		
Urban	8 (33.33)	16 (66.67)	0.7 (0.21-2.1)	0.45	0.57
Social class					
Low	10 (45.45)	12 (54.55)	Referent		
Middle and high	18 (36.73)	31 (63.27)	1.4 (0.5-4.5)	0.57	0.48
Family history					
Yes	7 (35.00)	13 (65.00)	Referent		
No	21 (41.18)	30 (58.82)	0.7 (0.2-2.5)	0.63	0.23
Smoking status					
Yes	11 (27.50)	29 (72.50)	Referent		
No	17 (54.84)	14 (45.16)	3.2 (1.1-9.7)	0.019[a]	5.46
Lifestyle					
Active	12 (38.71)	19 (61.29)	Referent		
Sedentary	16 (40.00)	24 (60.00)	1.05 (0.4-0.1)	0.91	0.01
Salt tea intake					
Yes	25 (38.46)	40 (61.54)	Referent		
No	3 (50)	3 (50)	1.6 (0.2-12.8)	0.58	0.30
Red meat consumption					
Yes	24 (40.68)	35 (59.32)	Referent		
No	4 (33.33)	8 (66.67)	0.7 (0.1-3.1)	0.63	0.22
Sundried vegetables					
Yes	19 (39.58)	29 (60.42)	Referent		
No	9 (39.13)	14 (60.87)	1.0 (0.3-3.0)	0.97	0.01
Source of drinking water					
Tap water (R)	20 (43.48)	26 (56.52)	Referent		
Tap water (L)	1 (14.29)	6 (85.71)	0.21 (0.01-2.3)		
Others[1]	7 (38.89)	11 (61.11)	1.3 (0.3-4.6)	0.34	2.21
Pickles					
Yes	16 (39.02)	25 (60.98)	Referent		
No	12 (40)	18 (60)	1.04 (0.3-3.03)	0.93	0.01
Pesticide exposure					
Yes	15 (45.45)	18 (54.55)	Referent		
No	13 (34.21)	25 (65.79)	0.624 (0.2-1.8)	0.33	0.93

Junk food consumption					
Yes	0 (0)	5 (100)	Referent		
No	28 (42.42)	38 (57.58)	0 (0-1.1)	0.06	3.5
Site of tumor					
Colon	12 (33.33)	24 (66.67)	Referent		
Rectum	12 (60)	8 (40)	3 (0.84-10.89)		
Rectosigmoid	4 (26.67)	11 (73.33)	0.7 (0.13-3.1)	0.077	5.12
Tumor differentiation					
Well	14 (77.78)	4 (22.22)	Referent		
Moderate	13 (28.26)	33 (71.74)	0.1 (0.02-0.5)		
Poor	1 (14.29)	6 (85.71)	0.04 (0.01-0.64)	0.000[a]	15.33
Invasion depth					
T1	7 (87.50)	1 (12.50)			
T2	13 (59.09)	9 (40.91)	Referent	0.000[a]	18.61
T3	7 (22.58)	24 (77.42)	0.04 (0.01-0.44)		
T4	1 (10)	9 (90)			
T1 + T2	20 (66.67)	10 (33.33)			
T3 + T4	8 (19.51)	33 (80.49)	0.12 (0.03-0.4)	0.000[a]	16.12
TNM staging					
I	19 (76.00)	6 (24.00)	Referent		
II	7 (28.00)	18 (72.00)	0.12 (0.02-0.5)	0.000[a]	23.3
III	2 (11.11)	16 (88.89)	0.04 (0.003-0.3)		
IV	0 (0.00)	3 (100)	0 (0-0.5)		
I + II	26 (52.00)	24 (48.00)			
III + IV	2 (9.52)	19 (90.48)	0.1 (0.01-0.5)	0.001[a]	11.2
Tumor grade					
1	14 (77.78)	4 (22.22)	Referent		
2	13 (28.26)	33 (71.74)	0.1 (0.02-0.5)		
3	1 (14.29)	6 (85.71)	0.05 (0.01-0.6)	0.000[a]	15.33
DUKE stage					
A	4 (80.00)	1 (20.00)	Referent		
B	22 (50.00)	22 (50.00)	4 (0.3-205)		
C	2 (9.09)	20 (90.91)	0.02 (0.01-0.5)	0.001[a]	13.9
Node status					
0	26 (50.98)	25 (49.02)	Referent		
1 and 2	2 (7.14)	18 (41.86)	0.10 (0.01-0.53)	0.001[a]	10.10
LVI					
Present	17 (31.48)	37 (68.52)	Referent		
Absent	11 (64.71)	6 (35.29)	0.2 (0.06-0.90)	0.015[a]	5.97
PNI					
Present	1 (6.67)	14 (93.33)	Referent		
Absent	27 (48.21)	29 (51.79)	0.1 (0.01-0.58)	0.003[a]	8.55
TALNR					

Present	25 (40.32)	37 (59.68)	Referent		
Absent	3 (33.33)	6 (66.67)	0.7 (0.11-3.9)	0.688	0.16
Necrosis seen					
Yes	2 (11.11)	16 (88.89)	Referent		
No	26 (49.06)	27 (50.94)	0.2 (0.03-0.76)	0.004[a]	8.10
Recurrence					
Yes	1 (8.33)	11 (91.67)	Referent		
No	27 (45.76)	32 (54.24)	0.3 (0.05-1.34)	0.016[a]	5.85
Vital status					
Alive	27 (40.91)	39 (59.09)	Referent		
Death	1 (20.00)	4 (80.00)	0.4 (0.007-3.9)	0.36	0.85

[1]Others: Spring, Well, Pond.

[a]$P < 0.05$: Statistical significance.

R: River water through tap, L: Lake water through tap; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion; CI: Confidence interval.

stages of CRC[18,25].

In our study, we correlated the CTGF expression with a number of clinicopathological parameters of patients and we found that CTGF protein overexpression was significantly associated with smoking, tumor differentiation, invasion depth, TNM staging, Duke Staging, Tumor grade and lymph node metastasis ($P < 0.001$). This was also the first study which revealed that the presence of LVI ($P = 0.015$), PNI ($P = 0.016$) and necrosis ($P = 0.008$) in the tumor was significantly associated with the overexpression of CTGF. In consideration with a previous study, smoking is an important factor which might oxidatively activate the latent TGF-β members like CTGF[26]. In another study, rat models were exposed to cigarette smoke, and it was found that CTGF shRNA effectively suppressed the upregulation of CTGF and cyclin D1 expression in narrow, intrapulmonary arteries which indicated that CTGF plays a crucial role in smoke-induced pulmonary vascular remodeling while regulating cyclin D1 expression[27]. In a study, it was found that CTGF mRNA overexpression was significantly associated with Duke Stage which was particularly noticeable in Duke's A and B stage tumors, while as they found noticeable proportions of CTGF protein overexpression in Duke's stage C[18]. In addition to this, CTGF protein expression was higher in more advanced T-stage and lymph node positive CRC samples as *per* the previous studies[25]. The recurrent cases of colon cancer have been recorded in cases where the lesion was initially diagnosed as LVI negative but later revealed to be positive after re-evaluation with additional more deeply cut specimens when the recurrence was discovered. In the current study, LVI was observed in 54/71 (76.00%) CRCs, in which 86.09% had high expression of CTGF and was significantly associated with CTGF overexpression ($P = 0.043$), and the presence increased with tumor stage from 56% in stage I, 80% in stage II, 94.44% in stage III and in all the three cases of stage IV involved in this study. Since our study included only three cases of Stage IV CRC tumors as the samples taken are mostly from the early-stage primary CRC patients, so most of the samples were skipped due to exclusion criteria of chemo/radio therapy which included mostly the late stage or recurrent patients. Most of the CRC patients with stage I or II are treated with surgery, though some stage II patients can be benefited by adjuvant therapy. The preferable method to treat stage III CRC patients is surgery coupled with adjuvant chemotherapy[28,29]. Comparable to other investigations, our findings indicated that LVI was associated with aggressive tumor characteristics such as higher tumor grade and advanced tumor stage ($P = 0.05$). Presence of LVI was a significant prognostic indicator in patients with stage III CRC [30]. PNI was present in 15/71 (21.12%) and the presence increased with the tumor stage from 4% in Stage I, 20% in stage II and 33.33% in Stage III CRC patients and in all 14/15 (93.33%) PNI positive cases showed significant association with CTGF overexpression ($P = 0.020$). The connection of PNI with histological markers associated with tumor growth (lymphatic invasion and venous invasion) suggests that these components may all be involved in the metastatic processes that include vascular emboli, lymphatic invasion, and PNI. It has been shown that PNI status can be used to

Table 4 Clinicopathological relevance of connective tissue growth factor high and low expression status determined by immunohistochemistry in colorectal cancer patients

Characteristics	Low expression (*n* = 27)	High expression (*n* = 44)	Odds ratio (95%CI)	*P* value	Chi²
Age					
< 50	6 (28.57)	15 (71.43)	Referent		
≥ 50	21 (42.00)	29 (58.00)	1.8 (0.5-6.6)	0.28	1.13
Gender					
Male	11 (28.95)	27 (71.05)	Referent		
Female	16 (48.48)	17 (51.52)	2.3 (0.8-6.9)	0.09	2.86
Dwelling					
Rural	19 (40.43)	28 (59.57)	Referent		
Urban	8 (33.33)	16 (66.67)	0.7 (0.2-2.2)	0.56	0.33
Social class					
Low	10 (45.45)	12 (54.55)	Referent		
Middle and high	17 (34.69)	32 (65.31)	1.5 (0.5-5.0)	0.38	0.74
Family history					
Yes	7 (35.00)	13 (65.00)	Referent		
No	20 (39.22)	31 (60.78)	0.8 (0.2-2.7)	0.74	0.10
Smoking status					
Yes	10 (25.00)	30 (75.00)	Referent		
No	17 (54.84)	14 (45.16)	3.6 (1.2-11.3)	0.010[a]	6.61
Lifestyle					
Active	12 (38.71)	19 (61.29)	Referent		
Sedentary	15 (37.50)	25 (62.50)	0.9 (0.3-2.8)	0.92	0.01
Salt tea intake					
Yes	24 (36.92)	41 (63.08)	Referent		
No	3 (50.00)	3 (50.00)	1.7 (0.2-13.6)	0.53	0.41
Red meat consumption					
Yes	23 (38.98)	36 (61.02)	Referent		
No	4 (33.33)	8 (66.67)	0.8 (0.15-3.3)	0.71	0.13
Sundried vegetables					
Yes	19 (39.58)	29 (60.42)	Referent		
No	8 (34.78)	15 (65.22)	0.8 (0.2-2.5)	0.70	0.15
Source of drinking water					
Tap water (R)	20 (43.48)	26 (56.52)	Referent		
Tap water (L)	1 (14.29)	6 (85.71)	0.2 (0.004-2.06)		
Others[1]	6 (33.33)	12 (66.67)	0.6 (0.2-2.3)	0.29	2.41
Pickles					
Yes	16 (59.26)	25 (56.82)	Referent		
No	11 (40.74)	19 (43.18)	0.9 (0.3-2.6)	0.84	0.04
Pesticide exposure					
Yes	15 (55.56)	18 (40.91)	Referent		
No	12 (44.44)	26 (59.09)	0.5 (0.2-1.6)	0.23	1.4

Junk food consumption					
Yes	0 (0)	5 (100)	Referent		
No	27 (40.91)	39 (59.09)	0 (0-1.1)	0.07	3.30
Site of tumor					
Colon	11 (30.56)	25 (69.44)	Referent		
Rectum	12 (60.00)	8 (40.00)	3.4 (0.9-12.5)		
Rectosigmoid	4 (26.67)	11 (73.33)	0.8 (0.1-3.7)	0.06	5.81
Tumor differentiation					
Well	14 (77.78)	4 (22.22)	Referent		
Moderate	12 (26.09)	34 (73.91)	0.1 (0.02-0.4)		
Poor	1 (14.29)	6 (85.71)	0.05 (0.01-0.6)	0.000[a]	16.52
Invasion depth					
T1	7 (25.93)	1 (2.27)	Referent		
T2	12 (44.44)	10 (22.73)	0.2 (0.003-1.8)	0.001[a]	17.30
T3	7 (25.93)	24 (54.550)	0.04 (0.01-0.4)		
T4	1 (3.70)	9 (20.45)	0.01 (0.01-0.40)		
T1 + T2	19 (70.37)	11 (25.00)			
T3 + T4	8 (29.63)	33 (75.00)	0.14 (0.04-0.4)	0.000**a**	14.11
TNM staging					
I	18 (66.67)	7 (15.91)	Referent		
II	7 (25.93)	18 (40.91)	0.1 (0.03-0.6)	0.000[a]	20.7
III	2 (7.41)	16 (36.36)	0.05 (0.01-0.3)		
IV	0 (0)	3 (6.820)	0 (0-0.6)		
I + II	25 (92.59)	25 (56.82)			
III + IV	2 (7.41)	19 (43.18)	0.12 (0.01-0.5)	0.001[a]	10.31
Tumor grade					
1	14 (77.78)	4 (22.22)	Referent		
2	12 (26.09)	34 (73.91)	0.1 (0.02-0.4)		
3	1 (14.29)	6 (85.71)	0.05 (0.01-0.6)	0.000[a]	16.52
DUKE stage					
A	4 (80.00)	1 (20.00)	Referent		
B	21 (47.73)	23 (52.27)	0.2 (0.004-2.6)		
C	2 (9.09)	20 (90.91)	0.02 (0.014-0.5)	0.001[a]	13.3
Node status					
0	25 (49.02)	26 (50.98)	Referent		
1 and 2	2 (10.00)	18 (90.00)	0.1 (0.01-0.6)	0.002[a]	9.30
LVI					
Present	17 (31.48)	37 (68.52)	Referent		
Absent	10 (58.82)	7 (41.18)	0.3 (0.1-1.13)	0.043[a]	4.11
PNI					
Present	1 (6.67)	14 (93.33)	Referent		
Absent	26 (46.43)	30 (53.57)	0.1 (0.002-0.6)	0.020[a]	5.42
TALNR					

Present	24 (38.71)	38 (61.29)	Referent		
Absent	3 (33.33)	6 (66.67)	0.8 (0.1-4.1)	0.75	0.09
Necrosis seen					
Yes	24 (47.06)	27 (52.94)	Referent		
No	3 (15.00)	17 (85.00)	0.2 (0.03-0.82)	0.012[a]	6.21
Recurrence					
Yes	1 (8.33)	11 (91.67)	Referent		
No	26 (44.07)	33 (55.93)	0.11 (0.012-0.9)	0.020[a]	5.40
Vital status					
Alive	26 (39.39)	40 (60.61)	Referent		
Death	1 (20.00)	4 (80.00)	0.4 (0.07-4.2)	0.389	0.74

[1]Others: Spring, Well, Pond.
[a]$P < 0.05$: Statistical significance.
R: River water through tap; L: Lake water through tap; TALNR: Tumor associated lymph-node response; LVI: Lymphovascular invasion; PNI: Perineural invasion; CI: Confidence interval.

Table 5 Predictors for recurrence or mortality of colorectal cancer using extended cox-regression analysis model

Characteristics	Disease-free survival			Overall survival		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	1.66	0.4-6.4	0.45	1.03	0.9-1.1	0.32
Tumor differentiation	1.13	0.5-2.7	0.79	3.1	0.7-14.4	0.15
Tumor grade	1.12	0.4- 2.7	0.79	3.1	0.7-14.4	0.15
Depth invasion	1.6	0.8-3.2	0.18	1.91	0.6-5.7	0.30
TNM stage	2.8	1.38-5.97	0.005[a]	3.7	1.1-12.7	0.043[a]
Duke stage	9.6	2.3-40.5	0.002[a]	3.36	0.6-18.3	0.161
Node status	0.08	0.02-0.37	0.001[a]	0.25	0.04-1.5	0.139
Necrosis	0.26	0.1-0.82	0.022[a]	1.30	0.14-11.6	0.811
LVI	0.25	0.05-1.2	0.090	1.16	3.25e-17	0.08
PNI	0.47	0.14-1.6	0.230	0.16	0.02-0.98	0.041[a]
CTGF expression	0.13	0.01-1.06	0.013[a]	0.39	0.04-3.5	0.33

[a]$P < 0.05$: Statistical significance. HR: Hazard ratio; CI: Confidence interval; TNM: Tumor-node-metastasis; LVI: Lymphovascular invasion; PNI: Perineural invasion; CTGF: Connective tissue growth factor.

aid in identifying the stage II CRC patients for adjuvant treatment chemotherapy[31]. According to certain studies, PNI evaluation utilizing a grading system based on PNI location inside the gut could improve the value of cancer staging[32].

Furthermore, it was observed that a high CTGF expression was associated with high recurrence of the disease, but no significant association was seen with vital status of the patients. The difference in this study may be due to limited sample size and a shorter time period of 4-year survival analysis. Some other studies have looked into the connection between CTGF expression and CRC prognosis, with mixed results. Previously, studies were conducted to examine CTGF expression in primary CRC using IHC and it was observed that elevated CTGF protein levels were linked to a lower probability of peritoneal metastasis and a substantially increased OS and DFS [17,18]. A previous study has shown that CRC patients with low expression levels of CTGF were associated with shorter survival and shorter recurrence time and moreover overexpression of CTGF in human CRC cells showed a decrease in the invasiveness of tumor cells[17]. This seems to be at odds with our findings. The following could be a

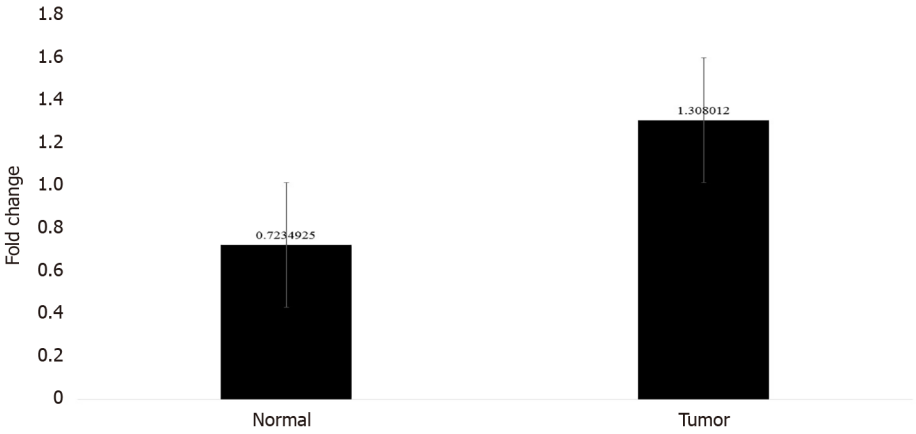

Figure 4 Bar graph showing the average fold change of connective tissue growth factor overexpression in colorectal cancer tumors and their adjacent normal tissue with error bars.

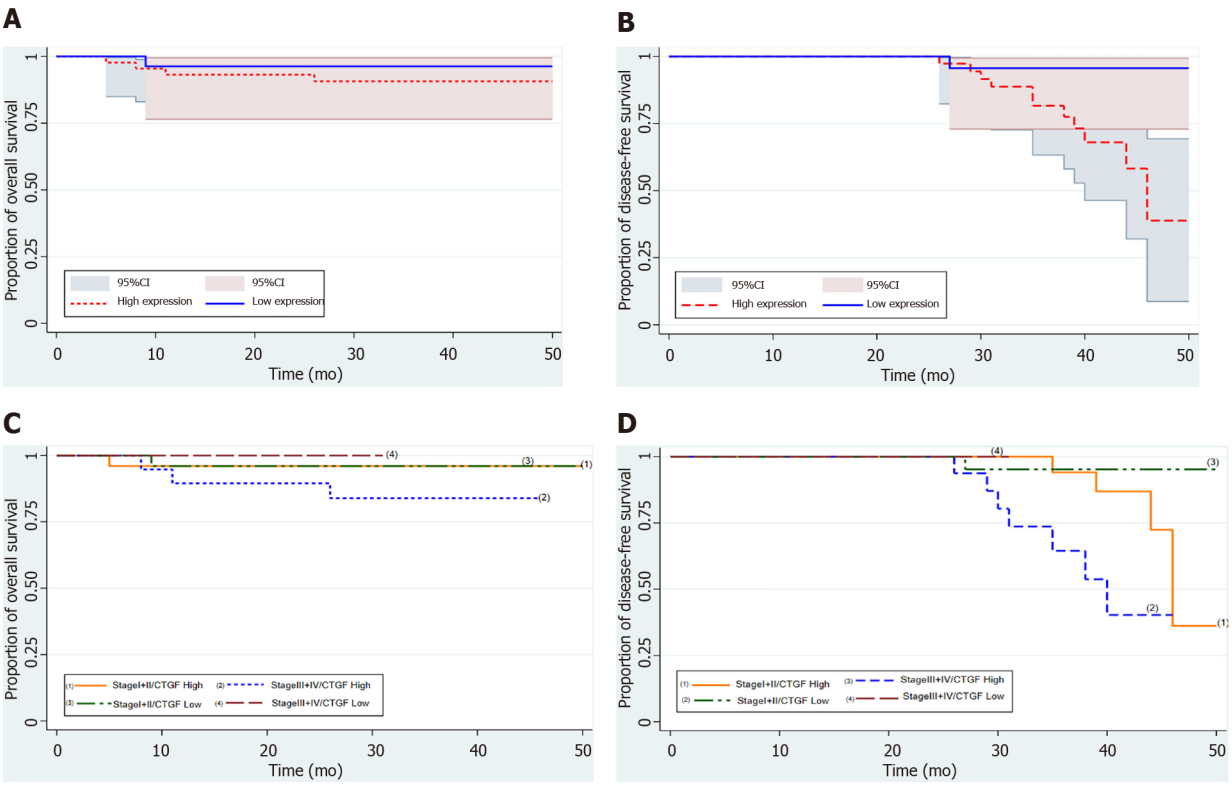

Figure 5 Kaplan-Meier survival analysis of 71 colorectal cancer patients from the time of diagnosis of the disease with connective tissue growth factor protein expression. A: Overall survival (OS) with connective tissue growth factor (CTGF) High and low expression; B: Disease-free survival (DFS) with CTGF High and Low expression; C and D: Depict OS and DFS of colorectal cancer patients respectively stratified as Stage I + II and III + IV.

possible explanation for the aforesaid inconsistency: They compared the CTGF expression with only stage II and III CRC disease, but we stratified the patients broadly into two merged categories of Stage I/II and Stage III/IV. The search for a more accurate mechanism is underway. Previous research has found that tumor stage is the biggest indicator of poor prognosis in people with colon cancer[33]. The results of the present study indicated that high expression levels of CTGF were associated with the TNM Stage which is the most important prognostic factor in CRC. After stratifying our population of 71 CRC patients as *per* TNM stage into I/II and III/IV groups, we found a significant correlation of TNM stage with OS of the patient (P = 0.001; Chi^2 = 17.05) as well as with recurrence of the disease (P = 0.034, Chi^2 = 8.64). Among all, 41.67% of the recurrent cases had Stage I/II CRC disease in which 55.93% had high expression of CTGF protein and about 58.33% recurrent cases had Stage III/IV CRC disease, in which 91.67% of recurrent cases had a high expression of CTGF

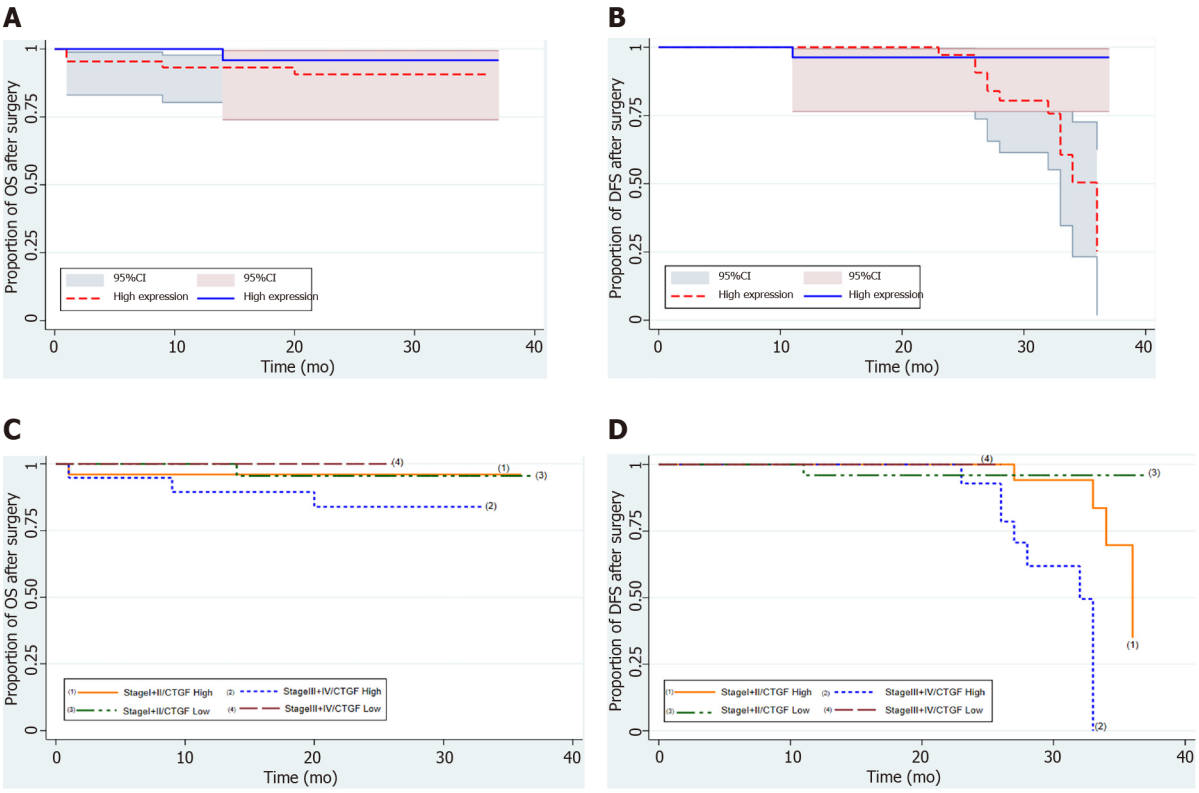

Figure 6 Kaplan-Meier survival analysis of 71 colorectal cancer patients after surgery with connective tissue growth factor protein expression. A and B: Depicts the overall and disease-free survival with connective tissue growth factor High and Low expression respectively; C and D: Shows the overall and disease-free survival of colorectal cancer patients respectively, stratified as Stage I + II and III + IV. CTGF: Connective tissue growth factor; OS: Overall survival; DFS: Disease-free survival.

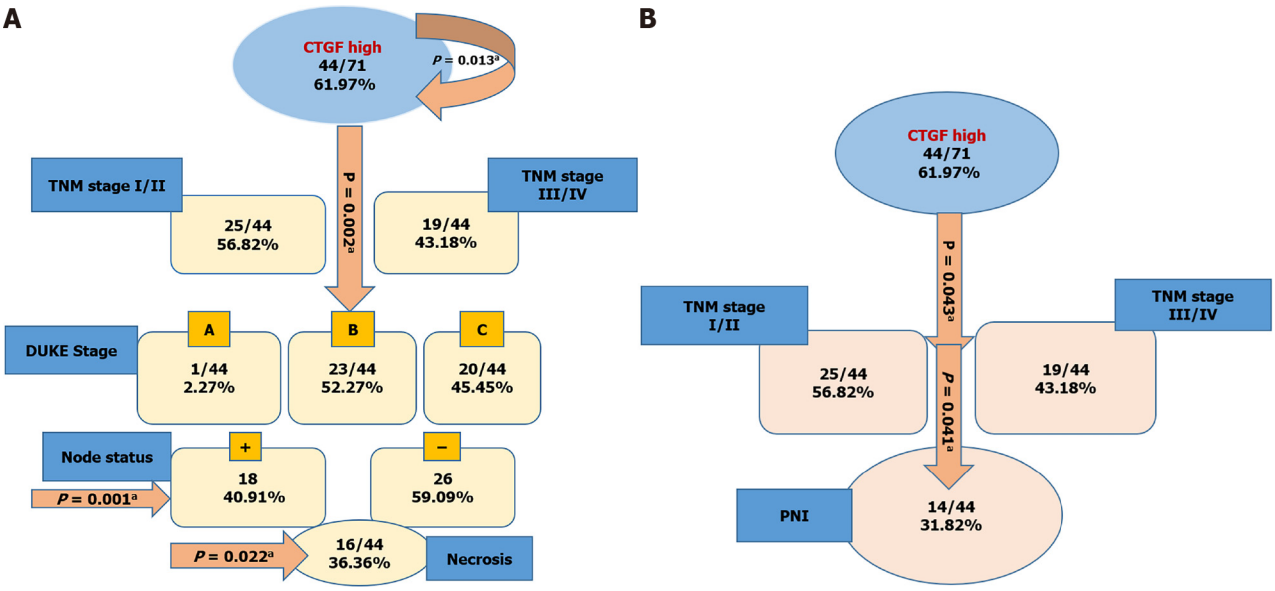

Figure 7 Connective tissue growth factor classification tree analysis for disease-free and overall survival of colorectal cancer patients; the numbers in the circles and boxes shows connective tissue growth factor high expression and percentage of the positive markers *per* total number of cases and arrows show the significant *P* values. A: Tree model of connective tissue growth factor high expression showing that tumor-node-metastasis (TNM) stage, duke stage, lymph node status and Necrosis of tumor tissue were independent predictors for disease-free survival; B: Depicts that TNM Stage and perineural invasion status were independent predictors for overall survival of colorectal cancer patients. $^{a}P < 0.05$. CRC: Colorectal cancer; CTGF: Connective tissue growth factor; PNI: Perineural invasion.

(P = 0.017). In this study, we found that TNM stage was a significant prognostic factor for both OS and DFS of CRC patients, as the stage progresses and CTGF expression increases, the chances of survival decrease in both pre- and postoperative conditions. Hence, proper staging considering LVI and PNI also is conducive to optimal care and has much to do with prognosis of CRC.

CONCLUSION

In conclusion, our results reported strong cytoplasmic localization and overexpression of CTGF in colorectal tumors compared to their adjacent normals. Moreover, the results of this study indicate that the overexpression of CTGF is associated with more intrusive clinicopathological parameters of CRC, suggesting that the expression of CTGF may be important in CRC progression. CTGF expression status could be an independent prognostic factor for CRC patients. LVI and PNI status could be used as complementary factors for TNM staging of CRC. However, because the current study included a limited sample size, more studies with a large number of samples is needed to fully comprehend the mechanism of CTGF induced CRC progression.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is one of the deadliest cancers, having a high death rate. Despite considerable advances in diagnostics and treatments, early detection remains difficult, resulting in a poor prognosis, which is exacerbated by deregulation of critical genes that contribute to disease development.

Research motivation

To investigate the role of connective tissue growth factor (CTGF) in CRC in order to improve the disease diagnosis and prognosis.

Research objectives

To evaluate the expression pattern and localization of CTGF in CRC and correlate it with various clinicopathological variables which might serve as effective preliminary predictive and therapeutic biomarker and aid in future CRC therapies in the Kashmir valley.

Research methods

A total of 71 histopathological confirmed CRC tissues and their adjacent normal specimens were included in this investigation. Real time polymerase chain reaction and western blot were employed to assess CTGF mRNA and protein levels, respectively. Immunohistochemistry was used for confirmation of the CTGF localization. CTGF expression was correlated with various clinicopathological characteristics, and survival analysis was performed on CRC patients to determine whether CTGF plays a predictive role in CRC.

Research results

CTGF expression in tumor samples were significantly higher than in adjacent normal samples. Higher levels of CTGF expression were associated with smoking, staging, tumor grade, invasion depth, necrosis of tumor tissue, lymphovascular and perineural invasion. Tumor-node-metastasis stage and PNI were major predictors of CTGF expression and prognosis in CRC patients, according to the cox regression model and classification tree analysis. CTGF overexpression was linked to poor overall and disease-free survival (DFS), according to a survival analysis.

Research conclusions

CTGF was shown to be overexpressed and cytoplasmic in CRC tumor tissues, according to the findings. Overexpression was related to an aggressive phenotype in CRC patients, as well as poor overall and disease-free survival.

Research perspectives

The study strongly indicates that higher CTGF levels in CRC patients can be employed

as predictive biomarker. Furthermore, CTGF overexpression may serve as a predictive biomarker for patients undergoing a distinct regimen of chemotherapeutic interventions. In order to validate these findings, future investigations with high sample size are needed to completely understand the mechanism of CTGF-induced CRC advancement.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Abnormal liver chemistries as a predictor of COVID-19 severity and clinical outcomes in hospitalized patients

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Abstract

BACKGROUND

Abnormal liver chemistries are common findings in patients with Coronavirus Disease 2019 (COVID-19). However, the association of these abnormalities with the severity of COVID-19 and clinical outcomes is poorly understood

AIM

We aimed to assess the prevalence of elevated liver chemistries in hospitalized patients with COVID-19 and compare the serum liver chemistries to predict the severity and in-hospital mortality.

METHODS

This retrospective, observational study included 3380 patients with COVID-19 who were hospitalized in the Johns Hopkins Health System (Baltimore, MD, United States). Demographic data, clinical characteristics, laboratory findings, treatment measures, and outcome data were collected. Cox regression modeling was used to explore variables associated with abnormal liver chemistries on admission with disease severity and prognosis

RESULTS

A total of 2698 (70.4%) had abnormal alanine aminotransferase (ALT) at the time of admission. Other more prevalent abnormal liver chemistries were aspartate

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aminotransferase (AST) (44.4%), alkaline phosphatase (ALP) (16.1%), and total bilirubin (T-Bil) (5.9%). Factors associated with liver injury were older age, Asian ethnicity, other race, being overweight, and obesity. Higher ALT, AST, T-Bil, and ALP levels were more commonly associated with disease severity. Multivariable adjusted Cox regression analysis revealed that abnormal AST and T-Bil were associated with the highest mortality risk than other liver injury indicators during hospitalization. Abnormal AST, T-Bil, and ALP were associated with a need for vasopressor drugs, whereas higher levels of AST, T-Bil, and a decreased albumin levels were associated with mechanical ventilation

CONCLUSION
Abnormal liver chemistries are common at the time of hospital admission in COVID-19 patients and can be closely related to the patient's severity and prognosis. Elevated liver chemistries, specifically ALT, AST, ALP, and T-Bil levels, can be used to stratify risk and predict the need for advanced therapies in these patients.

Key Words: Severe acute respiratory syndrome coronavirus 2; Liver injury; Liver tests; Aspartate aminotransferase; Alanine aminotransferase; bilirubin

Core Tip: Severe acute respiratory syndrome coronavirus-2 primarily infects the respiratory system. However, increasing evidence exists for the direct multiorgan effect. Liver injury in hospitalized patients is associated with a poor prognosis. We investigated whether abnormal liver chemistries in Coronavirus Disease 2019 (COVID-19) hospitalized patients can be of prognostic value. We show that abnormal liver chemistries were commonly observed on hospital admission and are associated with worse outcomes in COVID-19 patients, namely mortality, the need for vasopressor drugs, and mechanical ventilation. In hospitalized COVID-19 patients, elevated liver chemistries, specifically alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin levels, can be used to stratify risk and predict the need for advanced therapies. These results strongly suggest that abnormal liver chemistries at the time of hospital admission are associated with worse outcomes in COVID-19 patients and should be closely followed in admitted patients.

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INTRODUCTION

The Coronavirus Disease 2019 (COVID-19) infection is a global public health crisis that has spread rapidly throughout most of the world and has resulted in over 2 million deaths. Although it is primarily a respiratory disease, increasing evidence indicates that infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the virus that causes COVID-19, can affect multiple organ systems and cause long-term damage[1]. One possible explanation is the route of viral entry into cells *via* the angiotensin-converting enzyme 2 (ACE2) receptor, present on almost all human organs. The liver and the biliary system are no exception, and reports indicate that COVID-19 infection can induce varying degrees of liver injury, ranging from 19%-76% in reported cases[2-4]. The mechanism by which COVID-19 triggers liver injury remains poorly understood.

Direct infection of cholangiocytes *via* ACE2 is postulated as a potential mechanism for intrinsic liver injury from COVID-19[5]. The etiology of the abnormal liver chemistries frequently observed in patients with COVID-19 infection is multifactorial

and associated with major adverse clinical outcomes[6]. The incidence of abnormal liver enzymes significantly increases during the course of the disease, which may indicate the effect of SARS-CoV-2 on the liver or the side effects of medications used to treat the infection[7]. Patients with severe COVID-19 infections have been shown to have higher rates of abnormal liver chemistries[8]. While some studies revealed abnormal liver chemistries are associated with increased disease severity and mortality[9,10], other studies did not find an association with disease progression[11], intensive care unit (ICU) admission[12], or the length of hospital stay[13] and mortality [14]. Thus, study results are inconsistent, with a high degree of heterogeneity.

To address some of these inconsistencies, we examine whether abnormal liver chemistries in COVID-19 hospitalized patients can be of prognostic value. We determined the prevalence of elevated liver chemistries in a large cohort of hospitalized patients with COVID-19 infection and identified whether an independent association exists between abnormal liver chemistries and clinical severity or the risk of in-hospital mortality.

MATERIALS AND METHODS

Study design and participants

In this observational, retrospective cohort study, we analyzed consecutive adult patients (> 18 years of age) who were admitted at the Johns Hopkins Health System (Baltimore, MD, United States) between March 01, 2020, and January 21, 2021, who tested positive for SARS-CoV-2. The diagnosis of COVID-19 was made by at least one positive SARS-CoV-2 real-time PCR test performed on nasopharyngeal swab samples [8]. Only laboratory-confirmed patients were included in this study. This study was approved by the Institutional Review Board (IRB00249001) of the Johns Hopkins University School of Medicine, and the informed consent was waived for a retrospective review of patient charts.

Data collection

We obtained data from the COVID-19 Precision Medicine Analytics Platform Registry (JH-CROWN) database for this cohort study[15], which is a collection of data from the Johns Hopkins electronic health record (Epic) and available for analysis using an electronic database on the Precision Medicine Analystic Platform. Patients without any liver chemistry (n = 1874) results were excluded from the study. Demographic, clinical characteristics, laboratory tests, and treatment were retrieved from the medical records. Furthermore, the clinical outcomes were observed up to January 21, 2021, the final date of follow-up.

Definitions

Elevated liver enzyme levels were defined as patients having alanine aminotransferase (ALT) levels greater than 25 U/L for women and 35 U/L for men, according to the American Association for the Study of Liver Diseases definitions[16]. Other liver chemistry abnormalities were characterized as using the upper limit of the normal range (ULN) for serum levels of aspartate aminotransferase (AST), total bilirubin (T-Bil), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT). Additionally, liver injury was categorized based on the degree of liver enzyme elevation as mild (1-2 times of ULN), moderate (> 2-5 times of ULN), and severe (> 5 times of ULN).

Clinical classification

According to the World Health Organization interim guidance, patients in this study were classified based on COVID-19 disease severity[17]. Cases were defined as either: mild—if patients had mild symptoms of COVID-19 without abnormalities on chest imaging; moderate—if they had respiratory tract symptoms with no obvious hypoxemia and pneumonia manifestation by imaging; severe—if they had any of the following conditions: respiratory rate ≥ 30 breaths/minute; resting fingertip oxygen saturation <90%; a ratio of the arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) < 300mmHg or lung infiltrates critical—if they had respiratory failure requiring mechanical ventilation, or symptoms of shock, or respiratory failure combined with other organ dysfunction requiring intensive care. Identified COVID-19 patients were then stratified into two groups: non-severe (mild and moderate cases) and severe (severe and critical cases) disease, based on the above classification.

Study outcomes

We defined mortality as the primary clinical outcome. Death was assessed at the end of the study period. We also examined the need for vasopressor drugs and mechanical ventilation as the secondary outcomes.

Statistical analysis

Categorical variables were summarized as frequencies (percentages). Chi-squared tests were used to compare categorical variables, and the Mann-Whitney-Wilcoxon test was used for continuous variables. The results are presented as median with interquartile range (IQR). Interaction analyzes were performed as needed. Missing data were not imputed, and only complete cases were included. Patients were considered right-censored if they were discharged from the hospital alive or remained in the hospital at the end of follow-up. We measured time to event in days from the date of hospital admission to the date of in-hospital mortality or hospital discharge alive. Cox regression modeling was used to explore the relationship between abnormal liver biochemistries and mechanical ventilation and risk of death using hazard ratios (HRs) and 95% confidence intervals (CIs). Univariate analyses were used to identify independent risk factors associated with mechanical ventilation and risk of death, and these were ultimately included in a multivariate model with the grade of liver chemistry elevation. Age, gender, ethnicity, race, body mass index, and all the preexisting comorbidities were adjusted as confounders in the Multivariable Cox proportional hazards model. Cox proportional hazards regression models were also used to estimate HRs for the grade of liver chemistry elevation associated with mortality after controlling for the empirical prognostic elements. Kaplan-Meier (KM) method was used to assess differences in mortality by the degree of liver chemistry elevation. The event-free survival rate was estimated using the KM method, and significance was evaluated with the log-rank test. All tests were two-tailed, and statistical significance was determined at P values < 0.05. All statistical data analyzes were conducted with Stata software (version SE16; StataCorp, College Station, TX, United States).

RESULTS

Patients' demographic and clinical characteristics across disease severity groups

A total of 3830 hospitalized patients with confirmed SARS-CoV-2 infection were included in the analysis (Figure 1). Baseline clinical characteristics of the study cohort are summarized in Table 1. Among these patients, 2476 (64.6%) were non-severe cases, and 1354 (35.4%) were classified as severe cases during hospitalization. On hospital admission, abnormal liver chemistries were commonly seen (ALT 70.4%, AST 44.4%, T-Bil 5.9%, and ALP 16.1%) among all patients. The median age was 64.2 years (IQR 49.6-77.3), 51.1% were male, and 34.8% were African Americans. Obesity was present in 1494 (43.7%) of patients, and preexisting liver diseases in 392 (12.2%) patients. Severe disease was associated with older age and male sex. In patients with severe disease, the rate of coexisting diabetes mellitus without and with complications was significantly higher than in the non-severe group. In addition, these patients had higher cardiovascular disease, chronic respiratory disease, kidney disease, and neurological disease as comorbidities (P < 0.001), as well as significantly higher white blood cell and neutrophil counts and C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen, ferritin, prothrombin time (PT), international normalization ratio, D-dimer, lactate dehydrogenase (LDH), and cardiac troponin levels (P < 0.001). Levels of absolute lymphocyte, red blood cell, albumin, and total protein were lower (P < 0.001) in patients with severe disease

Prevalence and degree of abnormal liver chemistries according to COVID-19 disease severity

We compared the abnormal liver chemistries at different cut-off values as 1-2 ×, >2-5 ×, and > 5 × ULN, respectively, between the two groups (Table 1). On hospital admission, abnormal liver chemistries were commonly observed, and most patients had mild elevations within 1-2 × ULN. ALT, AST, T-Bil, and ALP levels were higher and more common in patients with severe disease. Overall, 2698 (70.4%) patients had an elevated ALT level, and the median ALT level was 28 U/L (IQR 18-47). A higher proportion of patients with severe disease had elevated ALT compared to the non-severe patients. The median ALT level was 27 U/L in non-severe disease compared to

Table 1 Baseline and clinical characteristics of patients with a positive test for severe acute respiratory syndrome coronavirus-2

Variables	All patients (*n* = 3830)	Non-severe[1] (*n* = 2476)	Severe[1] (*n* = 1354)	*P* value
Age in yr, median (IQR)	64.2 (49.6- 77.3)	62.1 (45.2-76)	67.4 (55.6-79.1)	< 0.001
Sex, *n* (%), *Male*	1959 (51.1)	1179 (47.6)	780 (57.6)	< 0.001
Ethnicity, *n* (%), *Hispanic*	817 (21.5)	565 (23)	252 (18.8)	0.003
Race, *n* (%)				0.12
White	1389 (36.6)	877 (35.7)	512 (38.2)	
Black	1323 (34.8)	848 (34.5)	475 (35.4)	
Asian	203 (5.3)	133 (5.4)	70 (5.2)	
Other	883 (23.2)	600 (24.4)	283 (21.1)	
BMI (kg/m^2), n (%)				0.11
≤ 18.5	794 (23.2)	519 (23.4)	275 (22.8)	
18.5-24.9	98 (2.9)	56 (2.5)	42 (3.5)	
25-29.9	1036 (30.3)	693 (31.3)	343 (28.4)	
≥ 30.0	1494 (43.7)	946 (42.7)	548 (45.4)	
Comorbidities, *n* (%)				
Chronic liver disease	465 (12.1)	295 (11.9)	170 (12.6)	0.56
Cardiovascular disease				
Congestive heart failure	869 (22.7)	395 (16)	474 (35)	< 0.001
HT without complications	2575 (67.2)	1766 (71.3)	717 (53)	< 0.001
HT with complications	1347 (35.2)	710 (28.7)	637 (47)	< 0.001
Diabetes				
Diabetes without complications	1459 (38.1)	856 (34.6)	603 (44.5)	0.017
Diabetes with complications	1270 (33.2)	679 (27.4)	591 (43.6)	< 0.001
Chronic respiratory disease	1065 (27.8)	618 (25)	447 (33)	< 0.001
Chronic neurological disease	1033 (27.0)	569 (23)	464 (34.3)	< 0.001
CKD of any stage	973 (25.4)	491 (19.8)	482 (35.6)	< 0.001
Anemia	1655 (43.2)	906 (36.6)	749 (55.3)	< 0.001
Hypothyroidism	557 (14.5)	330 (13.3)	227 (16.8)	0.004
Malignancies				
Primary cancer	458 (12)	276 (11.1)	182 (13.4)	0.036
Metastatic cancer	277 (7.2)	167 (6.7)	110 (8.1)	0.12
Laboratory findings, median (IQR)				
Liver biochemistries:				
ALT, median (IQR)	28 (18-47)	27 (18-45)	30 (19-49)	0.003
Normal, *n* (%)	1132 (29.6)	852 (34.4)	280 (20.7)	
Abnormal, *n* (%)	2698 (70.4)	1624 (65.6)	1074 (79.3)	< 0.001
1-2 ULN, *n* (%)	1225 (32)	829 (33.5)	396 (29.2)	
> 2-5 ULN, *n* (%)	1009 (26.3)	583 (23.5)	426 (31.5)	
> 5 ULN, *n* (%)	464 (12.1)	212 (8.6)	252 (18.6)	
AST, median (IQR)	36 (25-55)	34 (24-51.5)	42 (29-64)	< 0.001
Normal, *n* (%)	2046 (55.6)	1443 (60.5)	603 (46.4)	
Abnormal, *n* (%)	1637 (44.4)	941 (39.5)	696 (53.6)	< 0.001

1-2 ULN, *n* (%)	1187 (32.2)	704 (29.5)	483 (37.2)	
> 2-5 ULN, *n* (%)	361 (9.8)	194 (8.1)	167 (12.9)	
> 5 ULN, *n* (%)	89 (2.4)	43 (1.8)	46 (3.5)	
T.Bil, median (IQR)	0.5 (0.3-6.1)	0.4 (0.3-6.0)	0.5 (0.4-7.0)	< 0.001
Normal, *n* (%)	3496 (94.1)	2286 (95.3)	1210 (91.7)	
Abnormal, *n* (%)	221 (5.9)	112 (4.7)	109 (8.3)	< 0.001
1-2 ULN, *n* (%)	177 (4.8)	89 (3.7)	88 (6.7)	
> 2-5 ULN, *n* (%)	34 (0.9)	17 (0.7)	17 (1.3)	
> 5 ULN, *n* (%)	10 (0.3)	6 (0.3)	4 (0.3)	
ALP, median (IQR)	78 (61-103)	77 (61-100)	79 (61-108)	0.014
Normal, *n* (%)	3183 (83.9)	2101 (85.6)	1082 (80.7)	
Abnormal, *n* (%)	611 (16.1)	353 (14.4)	258 (19.3)	< 0.001
1-2 ULN, *n* (%)	525 (13.8)	311 (12.7)	214 (16)	
> 2-5 ULN, *n* (%)	78 (2.1)	38 (1.5)	40 (3)	
> 5 ULN, *n* (%)	8 (0.2)	4 (0.2)	4 (0.3)	
GGT, median (IQR)	119 (63-199)	116 (80-161)	144.5 (59-245)	0.54
Serum Albumin, g/dL median (IQR)	3.8 (3.4- 4.1)	3.9 (3.5-4.2)	3.6 (3.1-3.9)	< 0.001
Total protein (g/L), median (IQR)	6.5 (5.9- 7.1)	6.7 (6.1-7.2)	6.3 (5.7-6.9)	< 0.001
Coagulation test: median (IQR)				
PT (s)	11 (10.5-11.9)	10.9 (10.4-11.6)	11.4 (10.8-12.4)	< 0.001
INR	1.07 (1-1.14)	1.05 (1.0-1.1)	1.1 (1.02-1.20)	< 0.001
APTT (s)	26.2 (1.2-32.2)	25.9 (1.1-31)	25.7 (1.3-33.6)	< 0.001
D-Dimer	1.0 (0.57, 2.06)	0.83 (0.5-1.62)	0.4 (0.8-3.0)	< 0.001
Routine blood tests: median (IQR)				
Hemoglobin (g/dL)	11.9 (10.1, 13.3)	12.2 (10.7-13.6)	10.9 (9-12.7)	< 0.001
White blood cell (/mcL)	7.4 (5.1-10.5)	6.8 (4.7-9.3)	9 (6.4-13)	< 0.001
Red blood cells (/mcL)	4.06 (3.34-4.63)	4.19 (3.59-4.70)	3.81 (3.02-4.46)	< 0.001
Platelets (/mcL)	229 (168-309)	223 (166-298)	238 (172-327)	< 0.001
Neutrophils(/mcL)	36 (5.23-73)	36 (4.6-70.7)	38.9 (6.75-77.9)	< 0.001
Lymphocytes (/mcL)	15.4 (9.2-23.6)	17.8 (11.3-26.1)	11.5 (6.3-18.4)	< 0.001
Renal function tests: median (IQR)				
Creatinine (mg/dL)	0.9 (0.7-1.3)	0.85 (0.7-1.1)	1.03 (0.7-1.8)	< 0.001
Blood urea nitrogen, (mmol/L)	17 (11-28)	15 (10-22)	25 (16-42)	< 0.001
Sodium (mEq/L)	138 (136-141)	138 (136-140)	139 (136-143)	< 0.001
Potassium (mEq/L)	4.1 (3.8-4.5)	4.1 (3.8-4.4)	4.2 (3.8-4.6)	< 0.001
Inflammatory markers: median (IQR)				
Interleukin-6 (pg/mL)	37 (14.5-90.8)	24.7 (10.6-52.2)	81.83 (31.2-181)	< 0.001
Ferritin (ng/mL)	587 (265-1090)	482 (207-900)	810 (413.5-1462.5)	< 0.001
C reactive protein (mg/L)	8.4 (3.4-21.4)	6.4 (2.5-15.5)	13.6 (6.6-33.2)	< 0.001
Fibrinogen (mg/dL)	495 (387-622)	475 (374-579)	524 (393-653)	< 0.001
Lactate(mmol/L)	1.4 (1.1-2.0)	1.3 (1.0-1.7)	1.6 (1.2-2.3)	< 0.001
Cardiac markers: median (IQR)				
Cardiac troponin I (ng/L)	0.07 (0.04-0.18)	0.05 (0.03-0.1)	0.09 (0.05-0.27)	< 0.001

Lactate dehydrogenase (U/L)	327 (245-460)	303 (229-411)	385 (290-533)	< 0.001

[1]Based on the World Health Organization disease severity classification.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; IQR: Interquartile range; BMI: Body mass index; Fio2: Fraction of inspired oxygen; HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency síndrome; HT: Hypertension; CKD: Chronic kidney disease; ALT: Alanine aminotransferases; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; T-Bil: Total bilirubin; PT: Prothrombin time; INR: International normalized ratio; APTT: Activated partial thromboplastin time; ULN: Upper limit of normal.

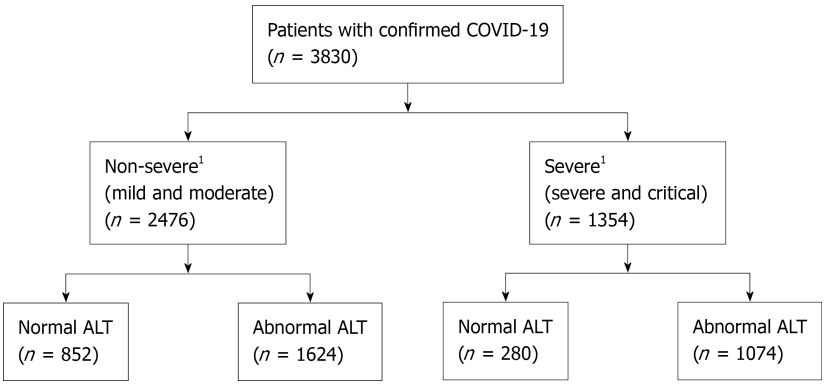

Figure 1 Flow chart of the study. [1]Severity of the diseases is based on the World Health Organization, Classification. COVID-19: Coronavirus Disease 2019 (COVID-19); ALT: Alanine aminotransferase. ALT cut of value was defined as patients having ALT levels greater than 25 U/L for women and 35 U/L for men, according to the AASLD definitions.

30 U/L in severe cases (P = 0.003). In addition, there was a significant difference in the degree of ALT elevation between the two groups (P < 0.001). An elevated ALT at > 2-5 × ULN and > 5 × ULN were significantly more common among patients with severe disease than non-severe. The median AST was 34 U/L (IQR, 24-51.5) in non-severe cases *vs* 42 U/L (IQR, 29-64) in severe cases (P < 0.001). The elevated AST level of 1-2 × ULN (37.2% *vs* 29.5%), > 2-5 × ULN (12.9% *vs* 8.1%) and > 5 × ULN (3.5% *vs* 1.8%) were significantly more common among severe patients compared to non-severe (P < 0.001). Patients with severe COVID-19 also had a higher median T-Bil compared with non-severe patients. However, there was no difference in T-Bil distribution at different levels between the two severities (P = 0.056). Again, in patients with severe disease, the median ALP elevation was higher in the non-severe patients (P = 0.014). However, only four patients in both groups at >5 × ULN had elevated ALP levels. Nevertheless, there was a significant difference in ALP distribution levels between the two severity groups (P < 0.001). Finally, there was no variation in GGT levels at the three thresholds between the two groups (P = 0.540)

Risk factors and predictors of liver injury: Univariate analyses showed that older age, Asian ethnicity, and being overweight were associated with liver injury, whereas other races and obesity were associated with severe liver injury (Figure 2).

In-hospital management and clinical outcomes

In-hospital management and clinical outcomes of patients with COVID-19 infection are shown in Table 2. Patients with severe diseases received more oxygen support and invasive ventilation (P < 0.001). Compared with the non-severe group, patients with the severe disease were more likely to receive antibacterial, antifungal, remdesivir, statins (P < 0.001), antiviral, and NSAID treatment (P = 0.005). The proportion of patients who received azithromycin and dexamethasone treatment was 24.7% and 40.7%, respectively. Moreover, 587 (40.7%) of the severe cases required vasopressor drugs for multiorgan failure (P < 0.001). Severe patients were more likely to develop kidney injury and need renal replacement therapy than non-severe patients. The median length of hospitalization prior to the death was 10.3 d (IQR, 4.5 to 18.8 d). The median length of stay was 6.3 d (IQR, 3.4 to 12.1 d), but patients with severe diseases had a significantly extended hospital stay compared with the non-severe group (median, 4.8 d *vs* 12.3 days, P < 0.001).

Major outcomes

Mortality: Overall, 461 (12.1%) patients died, and all the patients belonged to the

Table 2 In-hospital management and outcomes of patients with a positive test for severe acute respiratory syndrome coronavirus-2

Characteristics	All patients (*n* = 3830)	Non-severe[1] (*n* = 2476)	Severe[1] (*n* = 1354)	*P* value
Respiratory support, *n* (%)				
Non-rebreathing oxygen face mask	3147 (82.2)	1821 (73.5)	1326 (97.9)	< 0.001
High-flow nasal cannula oxygen therapy	843 (22)	18 (0.7)	825 (60.9)	< 0.001
Pharmacological treatment, *n* (%)				
NSAIDs	3303 (86.2)	2107 (85.1)	1196 (88.3)	0.005
Antiviral therapy	192 (5)	106 (4.3)	86 (6.4)	0.005
Antibacterial therapy	2649 (69.2)	1483 (59.9)	1166 (86.1)	< 0.001
Antifungal therapy	234 (6.1)	59 (2.4)	175 (12.9)	< 0.001
Azithromycin	947 (24.7)	530 (21.4)	417 (30.8)	< 0.001
Hydroxychloroquine	423 (11)	239 (9.7)	184 (13.6)	< 0.001
Oseltamivir	10 (0.3)	4 (0.2)	6 (0.4)	0.10
Remdesivir	1303 (34)	741 (29.9)	562 (41.5)	< 0.001
Vitamin D	391 (10.2)	227 (9.2)	164 (12.1)	0.004
Statins	1457 (38)	865 (34.9)	592 (43.7)	< 0.001
ACE inhibitors	378 (9.9)	215 (8.7)	163 (12)	< 0.001
ARB inhibitors	414 (10.8)	246 (9.9)	168 (12.4)	0.018
Immunomodulatory therapy, *n* (%)				
Dexamethasone	1560 (40.7)	881 (35.6)	679 (50.1)	< 0.001
Tocilizumab	95 (2.5)	3 (0.1%)	92 (6.8)	< 0.001
Advanced therapies, *n* (%)				
Vasopressors	587 (15.3)	2 (0.1)	585 (43.2)	< 0.001
Renal replacement therapy/dialysis	188 (4.9)	6 (0.2)	182 (13.4)	< 0.001
Clinical outcome, *n* (%)				
Discharged alive from hospital	3138 (87.2)	2372 (100)	766 (62.4)	< 0.001
Median length of hospital stay (IQR)	6.3 (3.4-12.1)	4.8 (2.8-7.7)	12.3 (7.1-22.3)	< 0.001

[1]Based on the World Health Organization disease severity classification.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus; IQR: Interquartile range; NSAIDs: Nonsteroidal anti-inflammatory drugs; ACE: Angiotensin-converting enzyme; ARB: Angiotensin II receptor blockers.

severe diseases group. 3138 (87.2%) were discharged at the time of data collection for this analysis. In addition, compared to survivors, non-survivors were older and had significantly higher rates of comorbidities (Table 3). In multivariable Cox proportional hazards analysis, increasing age, overweight, obesity, hypertension without complications, chronic neurological disease, and kidney disease were independently associated with an increased risk of in-hospital mortality after adjusting for confounders. Moreover, the results indicated that abnormal AST, T-Bil, ALP, and PT levels were significantly associated with all-cause mortality in all patients with COVID-19, but not the preexisting chronic liver disease, ALT, and albumin levels. Furthermore, a higher state of inflammation was also associated with mortality, with statistically significant increased levels of neutrophil count (P = 0.008), ferritin (P = 0.001), D-Dimer (P = 0.004), CRP, and IL-6 (P < 0.001).

Determining the association of changes in liver chemistries and mortality: The associated distribution of liver chemistries at different levels with in-hospital mortality in patients with COVID-19 was explored using the Cox proportional hazards model (Tables 4 and Figure 3). Unadjusted models showed that a stepwise increase in liver chemistries levels conferred an incremental risk of in-hospital death. Patients with abnormal AST, T-Bil, and ALP levels during hospitalization had a higher mortality

Table 3 Major outcome as the need for mechanical ventilation and mortality among patients with a positive test for severe acute respiratory syndrome coronavirus-2

Liver function test	No mechanical ventilation (*n* = 3205)	Mechanical ventilation (*n* = 625)	*P* value	Survivor (*n* = 3369)	Non-survivors (*n* = 461)	*P* value
Age in yr, median (IQR)	63.8 (48.4-77.7)	66.3 (54.9-75.2)	0.030	62.3 (47.7-74.7)	78.1 (66.9-87.6)	< 0.001
Comorbidities, *n* (%)						
Chronic liver disease	385 (12)	80 (12.8)	0.58	419 (12.4)	46 (10)	0.13
Cardiovascular disease						
Congestive heart failure	662 (20.7)	207 (33.1)	< 0.001	693 (20.6)	176 (38.2)	< 0.001
HT without complications	2112 (65.9)	463 (74.1)	< 0.001	2214 (65.7)	361 (78.3)	< 0.001
HT with complications	1059 (33)	288 (46.1)	< 0.001	1097 (32.6)	250 (54.2)	< 0.001
Diabetes						
Diabetes without complications	1161 (36.2)	298 (47.7)	< 0.001	1269 (37.7)	190 (41.2)	0.14
Diabetes with complications	979 (30.5)	291 (46.6)	< 0.001	1076 (31.9)	194 (42.1)	< 0.001
Chronic respiratory disease	865 (27)	200 (32)	0.011	910 (27)	155 (33.6)	0.03
Chronic neurological disease	810 (25.3)	223 (35.7)	< 0.001	835 (24.8)	198 (43)	< 0.001
CKD of any stage	764 (23.8)	209 (33.4)	< 0.001	787 (23.4)	186 (40.3)	< 0.001
Anemia	1281 (40)	374 (59.8)	< 0.001	1379 (40.9)	276 (59.9)	< 0.001
ALT, median (IQR)	27 (18-46)	33 (21-54)	< 0.001	28 (18-47)	27 (17-45)	0.28
Normal, *n* (%)	1055 (32.9)	77 (12.3)		1011 (30)	121 (26.2)	
1-2 ULN, *n* (%)	1065 (33.2)	160 (25.6)	< 0.001	1090 (32.4)	135 (29.3)	< 0.001
> 2-5 ULN, *n* (%)	786 (24.5)	223 (35.7)		902 (26.8)	107 (23.2)	
> 5 ULN, *n* (%)	299 (9.3)	165 (26.4)		366 (10.9)	98 (21.3)	
AST, median (IQR)	35 (24-54)	44.5 (31-70)	< 0.001	35 (25 -54)	45 (29 -71)	< 0.001
Normal, *n* (%)	1794 (58.3)	252 (41.7)		1856 (57.2)	190 (43.5)	
1-2 ULN, *n* (%)	946 (30.7)	241 (39.9)	< 0.001	1023 (31.5)	164 (37.5)	< 0.001
> 2-5 ULN, *n* (%)	283 (9.2)	78 (12.9)		300 (9.2%)	61 (14.0)	
> 5 ULN, < 0.001 (%)	56 (1.8)	33 (5.5)		67 (2.1%)	22 (5)	
Bilirubin, median (IQR)	0.5 (0.3-0.6)	0.5 (0.4-0.7)	< 0.001	0.5 (0.3-0.6)	0.5 (0.4-0.8)	< 0.001
Normal, *n* (%)	2943 (94.7)	553 (90.8)		3102 (94.9)	394 (88.1)	
1-2 ULN, *n* (%)	133 (4.3)	44 (7.2)	< 0.001	135 (4.1)	42 (9.4)	< 0.001
> 2-5 ULN, *n* (%)	23 (0.7)	11 (1.8)		26 (0.8)	8 (1.8)	
> 5 ULN, *n* (%)	9 (0.3)	1 (0.2)		7 (0.2)	3 (0.7)	
ALP, median (IQR))	77 (61-101)	80 (62-110)	0.062	77 (61-100)	87 (64-118)	< 0.001
Normal, *n* (%)	2686 (84.5)	497 (80.7)		2837 (85)	346 (75.7)	
1-2 ULN, *n* (%)	420 (13.2)	105 (17)	0.091	435 (13)	90 (19.7)	< 0.001
> 2-5 ULN, *n* (%)	65 (2)	13 (2.1)		58 (1.7)	20 (4.4)	
> 5 ULN, *n* (%)	7 (0.2)	1 (0.2)		7 (0.2)	1 (0.2)	
GGT, median (IQR)	116 (56-199)	144.5 (106-235.5)	0.483	117 (56-199)	180 (116.5-447.5)	0.28

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; IQR: Interquartile range; HT: Hypertension; CKD: Chronic kidney disease; ALT: Alanine

aminotransferases; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; T-Bil: Total bilirubin; ULN: Upper limit of normal.

Table 4 Multivariable Cox proportional hazards model for outcomes among hospitalized patients with a positive test for severe acute respiratory syndrome coronavirus-2

Clinical predictors	Mechanical ventilation[1]		Mortality[1]	
	Multivariable HR (95%CI)	*P* Value	Multivariable HR (95%CI)	*P* Value
Age	1.00 (0.99-1.01)	0.553	1.04 (1.03-1.05)	< 0.001
Male Gender	1.19 (0.99-1.43)	0.067	1.11 (0.90-1.37)	0.338
Overweight	0.93 (0.73-1.19)	0.577	0.75 (0.59-0.97)	0.030
Obesity	0.94 (0.74-1.19)	0.609	0.58 (0.44-0.77)	< 0.001
Liver diseases	0.84 (0.65-1.09)	0.198	0.78 (0.55-1.11)	0.164
Chronic respiratory disease	1.15 (0.95-1.38)	0.156	1.16 (0.94-1.45)	0.167
HT without complications	0.85 (0.68-1.06)	0.152	0.68 (0.53-0.89)	0.005
HT with complications	1.29 (1.01-1.66)	0.045	1.06 (0.78-1.430)	0.727
Congestive heart failure	0.78 (0.64-0.95)	0.014	1.00 (0.80-1.25)	0.987
Chronic neurological disease	0.78 (0.65-0.95)	0.012	1.44 (1.18-1.76)	< 0.001
Chronic kidney disease	0.94 (0.76-1.15)	0.535	1.55 (1.26-1.88)	< 0.001
ALT	1.00 (1.00-1.00)	0.420	1.00(1.00 - 1.00)	0.892
ALP	1.00 (1.00-1.00)	0.916	1.02 (1.02-1.03)	< 0.001
AST	1.00 (1.00-1.00)	0.003	1.00 (1.00-1.01)	< 0.001
T-Bil	1.06 (0.99-1.14)	0.008	1.21 (1.14-1.28)	< 0.001
Albumin	0.87 (0.76-1.01)	0.071	0.84 (0.71-1.01)	0.057
INR	0.91 (0.77-1.09)	0.312	1.12 (0.99-1.26)	0.071
PT	1.00 (0.98-1.02)	0.814	1.03 (1.02-1.05)	< 0.001
Neutrophil	1.00 (1.00-1.00)	0.858	1.00 (1.00-1.01)	0.008
BUN	1.01 (1.00-1.01)	<0.001	1.01 (1.01-1.02)	< 0.001
Creatinine	1.07 (1.02-1.13)	0.007	1.16 (1.11-1.22)	< 0.001
Interleukin-6	1.00 (1.00-1.00)	<0.001	1.00 (1.00-1.00)	< 0.001
CRP	1.02 (1.01-1.03)	0.001	1.04 (1.03-1.05)	< 0.001
Ferritin	1.00 (1.00-1.00)	0.002	1.00 (1.00-1.00)	0.001
D-Dimer	1.00 (0.98-1.02)	0.721	1.03 (1.01-1.05)	0.004
LDH	1.00 (1.00-1.00)	0.063	1.00 (1.00-1.01)	< 0.001

[1]Age, gender, ethnicity, race, body mass index, and all the preexisting comorbidities were adjusted as confounders in the Multivariable Cox proportional hazards model.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; HT: Hypertension; ALT: Alanine aminotransferases; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; AST: Aspartate aminotransferase; T-Bil: Total bilirubin; INR: International normalized ratio; BUN: Blood urea nitrogen; PT: Prothrombin time; CRP: C-reactive protein; LDH: Lactate Dehydrogenase; HR:Hazard ratio; CI: Confidence interval.

risk than patients with normal levels. Age, gender, ethnicity, race, BMI, and all the preexisting comorbidities were adjusted as confounders. Among these liver chemistries, elevated AST and T-Bil levels were associated with the highest risk of in-hospital mortality. Compared to patients with T-Bil in the normal level, all-cause mortality risk significantly increased 6-fold (95%CI, 2.90-12.41; $P < 0.001$) in patients with an elevated T-Bil level of >2-5 × ULN and increased 7.86-fold (95%CI, 1.88-32.96; $P = 0.005$) in patients with T-Bil > 5 × ULN (Table 5). A stepwise increase in the levels

Table 5 Association of abnormal liver chemistries and mortality in patients with a positive test for severe acute respiratory syndrome coronavirus-2

Parameters	Unadjusted, Cox regression		Adjusted[1], Cox regression		Log-rank test
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	*P* value
ALT, Abnormality type					
Normal,	Reference		Reference		
1-2 ULN	0.83 (0.65-1.07)	0.146	0.95 (0.72-1.25)	0.719	< 0.001
> 2-5 ULN	0.52 (0.40-0.68)	< 0.001	0.68 (0.53-0.92)	0.013	
> 5 ULN	0.84 (0.64-1.11)	0.219	1.31 (0.98-1.79)	0.092	
AST, Abnormality type					
Normal	Reference		Reference		
1-2 ULN	1.16 (0.94-1.43)	0.169	1.07 (0.84-1.35)	0.584	0.001
> 2-5 ULN	1.48 (1.11-1.98)	0.008	1.49 (1.06-2.10)	0.021	
> 5 ULN	2.13 (1.37-3.32)	0.001	2.19 (1.27-3.76)	0.005	
Bilirubin, Abnormality type					
Normal	Reference		Reference		
1-2 ULN	1.74 (1.27-2.40)	0.001	1.58 (1.04-2.22)	0.032	< 0.001
> 2-5 ULN	2.49 (1.24-5.02)	0.011	6.00 (2.90-12.41)	< 0.001	
> 5 ULN	2.78 (0.89-8.65)	0.078	7.86 (1.88-32.96)	0.005	
ALP, Abnormality type					
Normal	Reference		Reference		
1-2 ULN	1.52 (1.20-1.92)	< 0.001	1.42 (1.09-1.86)	0.009	0.001
> 2-5 ULN	2.13 (1.36-3.35)	0.001	1.81 (1.05-3.10)	0.032	
> 5 ULN	1.05 (0.15-7.45)	0.964	1.84 (0.25-13.38)	0.547	

[1]Age, gender, ethnicity, race, body mass index, and all the preexisting comorbidities were adjusted as confounders in the Multivariable Cox proportional hazards model.

SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ALT: Alanine aminotransferases; AST: Aspartate aminotransferase; T-Bil: Total bilirubin; ALP: Alkaline phosphatase; HR: Hazard ratio; CI: Confidence interval; ULN: Upper limit of normal.

of AST was associated with a significant increased risk of all-cause mortality (HR, 1.49; 95%CI, 1.06-2.10; $P < 0.001$ for > 2-5 × ULN; HR, 2.19; 95%CI, 1.27-3.76; $P = 0.005$ for AST > 5 × ULN). The degree of ALT ranging from > 2-5 × ULN (adjusted HR, 0.68; 95%CI, 0.53-0.92; $P = 0.013$) was associated with a decreased risk of all-cause mortality; however, 1-2 × and > 5 × ULN were not significantly associated with all-cause mortality. Lastly, compared to the patients with ALP in the normal range, all-cause mortality risk significantly increased 1.42-fold (95%CI, 1.09-1.86; $P = 0.009$) in patients with ALP > 1-2 × ULN and increased 1.81-fold (95%CI, 1.05-3.10; $P = 0.032$) in patients with ALP > 2-5 × ULN after adjusting for confounders. Interestingly, the ALP levels > 5 × ULN were not significantly associated with mortality.

Need for vasopressor support: Only two (0.1%) patients in the non-severe group required vasopressor drugs, whereas 585 (43.2%) patients with severe COVID-19 required vasopressors ($P < 0.001$). Multivariable adjusted Cox proportional hazard regression analysis revealed that patients with abnormal AST (HR, 1.00, 95%CI, 1.00-1.00; $P < 0.001$), T-Bil (HR, 1.09, 95%CI, 1.03-1.17; $P = 0.003$), ALP (HR, 1.02, 95%CI, 1.01-1.03; $P < 0.001$), blood urea nitrogen (BUN) (HR, 1.007, 95%CI, 1.00-1.01; $P < 0.001$), and PT (HR, 0.795, 95%CI, 0.72-0.88; $P < 0.001$) were associated with a need for vasopressor drugs. Similarly, a higher state of inflammation was also associated with this outcome, namely higher levels of ferritin (HR, 1.00, 95%CI, 1.00-1.00; $P = 0.035$), D-Dimer (HR, 1.02, 95%CI, 1.00-1.04; $P = 0.04$), and IL-6 (HR, 1.00, 95%CI, 1.00-1.00; $P < 0.001$). However, higher BMI and abnormal ALT were not independently associated

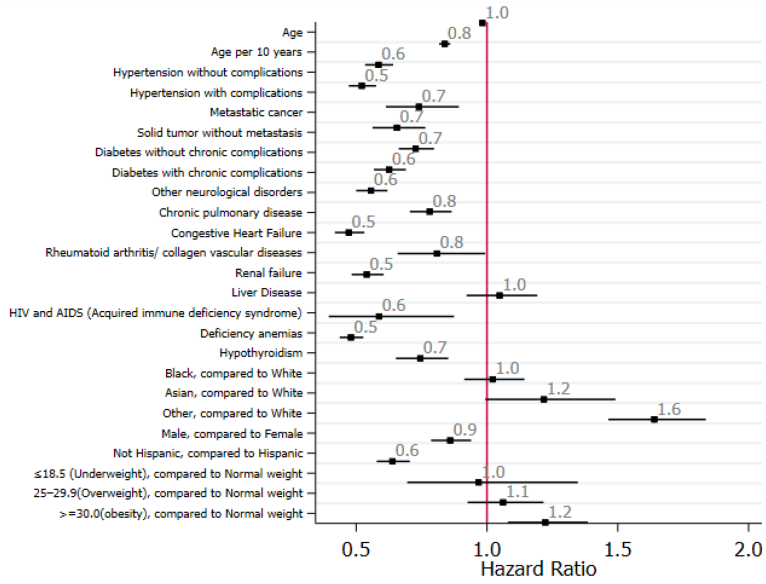

Figure 2 Standardized forest plot comparing selected clinical variables between elevated ALT and normal ALT among hospitalized. ALT: Alanine aminotransferase.

with the increased risk for this outcome. Of note, chronic neurological disease (HR, 0.77, 95%CI, 0.63-0.93; P = 0.004) and albumin (HR, 0.804, 95%CI, 0.69-0.93; P = 0.004) were associated with lower vasopressor support hazards in multivariate analyses.

Need for mechanical ventilation: Patients who received invasive mechanical ventilation were older, more likely to have preexisting comorbidities than were patients who did not receive invasive mechanical ventilation (Table 3). However, preexisting liver diseases did not differ between these groups. Median levels of ALT, AST, and T-Bil were significantly increased for patients who received mechanical ventilation (P < 0.0001) compared to those who did not receive it. However, no difference in ALP and GGT levels were seen between these two groups (ventilated P = 0.091 *vs* non-ventilated patients P = 0.483). Furthermore, patients who received invasive mechanical ventilation had varying degrees of abnormal liver chemistries (1-2 ×, > 2-5 ×, and > 5 × ULN), but in general, ALT, AST, and T-Bil values were significantly higher in these patients (P < 0.001). Multivariable Cox regression analysis adjusted for age, gender, ethnicity, race, BMI, and preexisting comorbidities revealed that older age, HT with complications, and abnormal levels of AST, T-Bil, BUN, and creatinine were associated with mechanical ventilation. In addition, a higher state of inflammation was also associated with this outcome, namely higher levels of neutrophil, ferritin, CRP, and IL-6.

DISCUSSION

This retrospective cohort study is one of the largest and most comprehensive to evaluate liver chemistries and clinical outcomes of hospitalized patients with COVID-19. Overall, the results show that liver injury, assessed by elevated liver enzyme levels, is commonly seen in hospitalized patients with COVID-19 and is associated with the risk of in-hospital mortality and other adverse clinical outcomes, such as the need for vasopressor drugs and mechanical ventilation. The key findings of the study are: (1) There is a high prevalence of liver injury (70.4%), defined by an elevation in ALT levels, in hospitalized patients with COVID-19; (2) Abnormal liver chemistries during hospitalization are strongly associated with mortality (ALT, T-Bil, and ALP); (3) Liver injury measured in patients with COVID-19 on admission is associated with the need for vasopressor drugs (AST, T-Bil, and ALP), and mechanical ventilation (AST, and T-Bil); (4) A strong and independent association of AST, T-Bil, and ALP correlates with the severity of COVID-19 infection; and (5) Elevated inflammatory markers (CRP, IL-6, ferritin, D-dimer, and LDH) are associated with increased risk of disease severity.

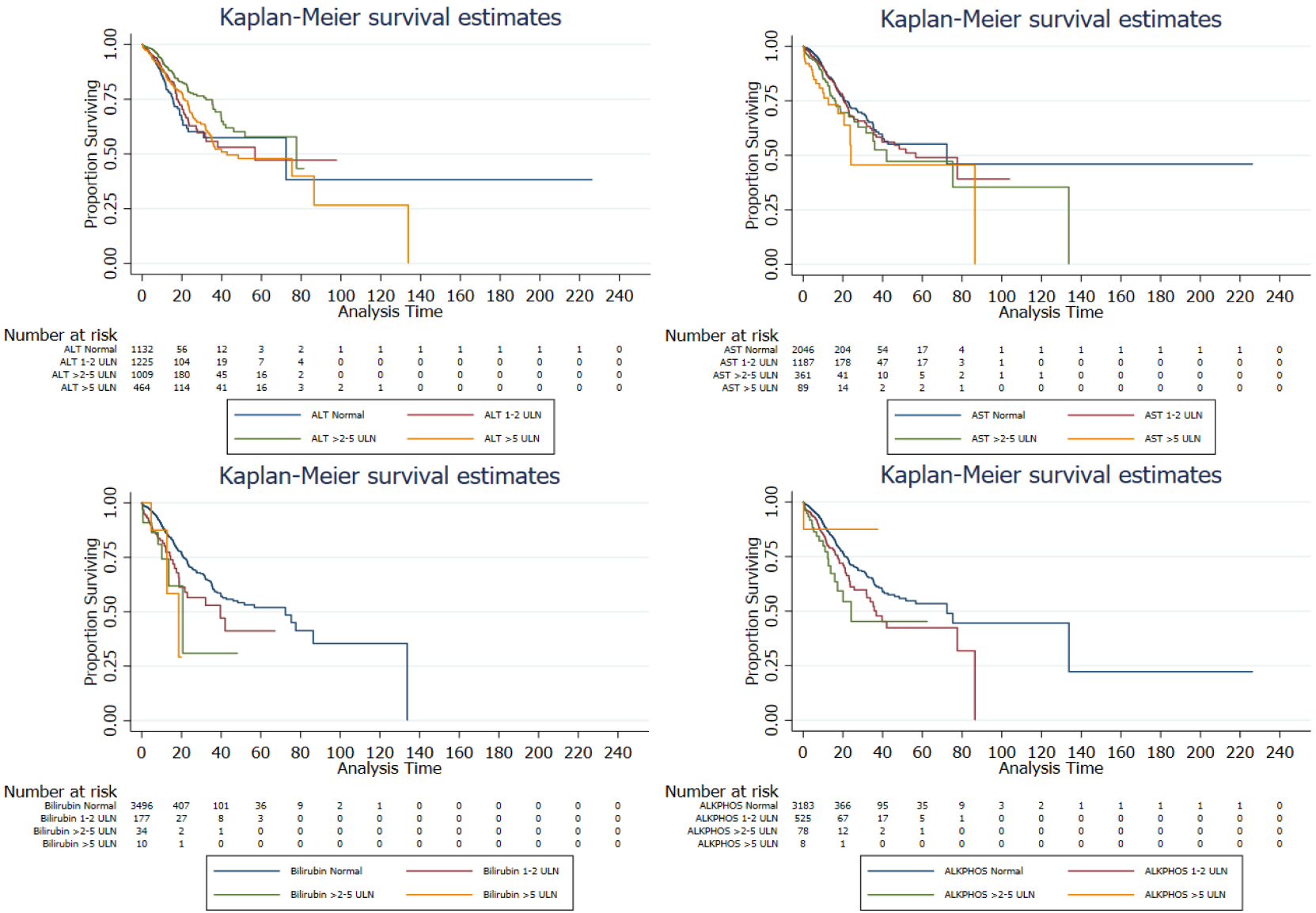

Figure 3 Kaplan-Meier analysis showed the association of abnormal liver chemistry results among patients with COVID-19. ALT: Alanine aminotransferase; AST: aspartate aminotransferase.

The pathophysiology of SARS-CoV-2 infection is similar to other coronavirus infections [SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV)] and shares a large genome sequence homology with other pathogenic human coronaviruses. SARS-CoV and MERS-CoV cause abnormal liver chemistries, and various degrees of hepatic injury[18,19]. SARS-CoV viral RNA is detected in autopsied human liver, suggesting a direct liver involvement[20]. Thus, similar liver injury in COVID-19 patients is not surprising. In particular, the overall ALT elevations were observed in 70.4% of patients with COVID-19 on hospital admission, and 79.3% of patients in the severe group had elevations much higher than previous reports. Based on a recent systematic review and meta-analysis (17 studies, 2711 patients), abnormal ALT is estimated to occur in ~15% of COVID-19 patients[14]. However, in Chinese cohorts, the prevalence of abnormal ALT among patients with COVID-19 ranged between 4% and 53.1%, and recent studies from the United States reveal even higher estimates, ranging between 39% and 64.9%[21-23].

In our cohort, only a small percentage of patients in our study had underlying liver disease (12.2%), suggesting that liver damage in these patients is directly caused by the COVID-19 infection and not an underlying condition or drug-induced liver injury from medications. Furthermore, our study showed varying degrees of liver enzyme levels, ranging from mild to severe. A previous study reported that 9.2% of ALT elevations were > ULN at the time of hospital admission[11], while another study reported higher rates of 36.5% >2 × ULN on admission[24]. Overall, our study showed that 38.8% of patients had abnormal ALT elevations > 2 × ULN (2-5 × ULN 26.7% and > 5 × ULN 12.1%).

Another potential cause of liver injury in our cohort is drug-induced liver injury. Self-medication before hospital admission was not reported in the data and is an unlikely cause. However, during hospitalization, treatment consists of a combination of antibiotics, antivirals, systemic corticosteroids, antipyretics, and analgesics to treat the COVID-19 infection, which may promote liver injury. In this study, these combined drug treatments positively correlated in patients with severe disease. Thus, drug-related liver injury during the treatment of COVID-19 infection needs to be considered. Future studies are needed to evaluate the possible effects of drugs on liver chemistries in patients with COVID-19.

Various studies have shown that older patients with COVID-19 have a higher case fatality rate[25], and having certain comorbidities may contribute to worse outcomes [26]. Similarly, our analysis revealed that risk factors for severe infection and death included older age, chronic heart disease, elevated inflammatory response, more prolonged PT, elevated liver enzymes, and bilirubin. In addition to ALT elevation, AST, T-Bil, and ALP were independently associated with the severity of COVID-19 infection, with over half of severe patients exhibiting AST elevation. The current study is consistent with prior studies that the pattern of liver injury is primarily hepatocellular instead of cholestatic, while our study indicates that elevations in T-Bil and ALP may be more common than previously reported[27]. Upon hospitalization, the percentage of the patients with elevated ALP and T-Bil in severe cases was 18.6% and 6.8%, respectively, which was significantly higher than 14.6 % and 4.6% in patients with the non-severe disease.

COVID-19 causes severe respiratory distress and pneumonia, with the latter being independently correlated with the need for ICU care, mechanical ventilation, and death[28]. Our study complements this knowledge and reveals that elevated liver chemistries can predict the risk of major in-hospital outcomes, such as the need for vasopressor drugs, mechanical ventilation, and death. Moreover, significant hypoalbuminemia was observed, particularly among patients with severe disease, and was also a predictor for the need for vasopressor drugs and mechanical ventilation. In the present study, the elevated levels of LDH, creatinine, BUN, IL-6, CRP, and ferritin are independently associated with mechanical ventilation risk.

COVID-19 induces a release of inflammatory cytokines, leading to organ dysfunction[19]. Inflammatory cytokine storm during COVID-19 infections is not uncommon and can result in sudden patient clinical deterioration and multiorgan failure. Direct hepatocyte injury caused by the SARS-CoV-2 may be closely related to systemic inflammatory response syndrome, and overproduction of cytokines is linked to the lung-liver axis[20]. An increase in systemic immune mediators that cause inflammation, oxidative stress, and underlying hypoxia facilitates and exacerbates liver function[29]. Our findings indicate an association between elevated inflammatory markers (CRP, IL-6, ferritin, D-dimer, and LDH) with increased risk of disease severity. IL-6 values are increased in patients with both severe COVID-19 and significantly increased in patients with elevated liver chemistries compared to patients with non-severe COVID-19. IL-6 is the primary driver of cytokine release syndrome,

and IL-6 inhibitors are effective in treating severe COVID-19 cases[30]. Moreover, the neutrophil levels and serum CRP are significantly increased in patients with liver injury from COVID-19. These data imply a potential association between liver injury and the inflammatory responses induced by SARS-CoV-2 infection. Clinical treatment against the cytokine storm might also reduce liver injury and liver injury-related mortality. We also found a reduction in red blood cells in severe patients, which coincides with the fact that SARS-CoV-2 destroys hemoglobin in red blood cells, dissociates deoxyhemoglobin and iron, and produces hypoxia and respiratory distress. Increased ferritin levels due to cytokine storm and secondary hemophagocytic lymphohistiocytosis have also been reported in severe COVID-19 patients[31]. A higher level of ferritin was observed in patients with severe disease on admission than patients with a non-severe disease in the present study. Hypoxia may also damage hepatocytes and induce liver injury; thus, elevated levels of serum ferritin and hypoxia are potential indicators of hepatocyte injury in patients with severe COVID-19 infection. In our study, abnormal levels of LDH were found in patients with severe COVID-19, which was also seen in patients with SARS and MERS and was an independent risk factor for severe disease[32]. LDH is an intracellular enzyme found in cells in almost all organ systems and can be released during tissue damage, and is involved in various pathophysiological processes. Abnormal levels of LDH seem to reflect that multiple organ injury and failure. Despite its lack of specificity, serum LDH can have great prognostic significance in patients with COVID-19.

Limitations: Despite analyzing a large cohort of patients, the study has some limitations. Data collection was a retrospective observational cohort study and used electronic health record extraction within a single health system. Our health system is a tertiary medical health system, potentially introducing referral bias. The analysis represents only patients who were hospitalized, *i.e.*, more likely to be in severe cases. Therefore, it cannot be entirely excluded that abnormal liver chemistries at admission might represent a more severe course in patients with COVID-19 with multiorgan involvement, including hepatobiliary manifestations. However, irrespective of the cause of liver injury at the time of hospitalization, we show a strong association between severity and liver chemistries at hospital admission rather than peak values, which may help guide clinical decisions early in the disease course. Furthermore, it was not feasible to describe all the potential causes of liver injury and all the causes of liver injury in the patients progressing to liver injury, such as the use of hepatotoxic medications and self-medication before hospitalization. Our study's data permit an initial evaluation of patients' clinical course and outcomes with COVID-19. The causes of death in COVID-19 patients may involve multiple organ injuries, and it is challenging to differentiate liver injury as the primary and direct cause of death. We were unable to obtain long-term outcomes due to a comparatively short observation period. Further studies with long-term periods are required to understand the long-term impact of COVID-19 on the liver and elucidate the pathogenic mechanisms.

CONCLUSION

This study found that abnormal liver chemistries (AST, ALT, T-Bil, and ALP) at the time of hospital admission are associated with worse outcomes in COVID-19 patients, namely mortality (ALT, T-Bil, and ALP), the need for vasopressor drugs (AST, T-Bil, and ALP), and mechanical ventilation (AST, and T-Bil). Consequently, in hospitalized COVID-19 patients, elevated liver chemistries, specifically ALT, AST, ALP, and T-Bil levels, can be used to stratify risk and predict the need for advanced therapies.

ARTICLE HIGHLIGHTS

Research background

Severe acute respiratory syndrome coronavirus 2 primarily infects the respiratory system. Abnormal liver chemistries are common findings in patients with Coronavirus Disease 2019 (COVID-19). In addition, increasing evidence exists for the direct multiorgan effect. However, the association of these abnormalities with the severity of COVID-19 and clinical outcomes is poorly understood.

Research motivation

To explore the impact of abnormal liver chemistries in hospitalized patients with COVID-19 and whether it is associated with worse outcomes, namely mortality, the need for vasopressor drugs, and mechanical ventilation.

Research objectives

We examine whether abnormal liver chemistries in COVID-19 hospitalized patients can be of prognostic value. We determined the prevalence of elevated liver chemistries in a large cohort of hospitalized patients with COVID-19 infection and identified whether an independent association exists between abnormal liver chemistries and clinical severity or the risk of in-hospital mortality.

Research methods

This retrospective, observational study included 3380 patients with COVID-19 who were hospitalized in the Johns Hopkins Health System. Demographic data, clinical characteristics, laboratory findings, treatment measures, and outcome data were collected. Cox regression modeling was used to explore variables associated with abnormal liver chemistries on admission with disease severity and prognosis.

Research results

A total of 2698 (70.4%) had abnormal ALT at the time of admission. Other more prevalent abnormal liver chemistries were AST (44.4%), ALP (16.1%), and T-Bil (5.9%). Factors associated with liver injury were older age, Asian ethnicity, other race, being overweight, and obesity. Higher ALT, AST, T-Bil, and ALP levels were more commonly associated with disease severity. Multivariable adjusted Cox regression analysis revealed that abnormal AST and T-Bil were associated with the highest mortality risk than other liver injury indicators during hospitalization. Abnormal AST, T-Bil and ALP were associated with a need for vasopressor drugs whereas, higher levels of AST, T-Bil, and a decreased albumin levels were associated with mechanical ventilation.

Research conclusions

This study found that abnormal liver chemistries (AST, ALT, T-Bil, ALP, and albumin) at the time of hospital admission are associated with worse outcomes in COVID-19 patients, namely mortality (ALT, T-Bil, and ALP), the need for vasopressor drugs (AST, T-Bil, and ALP), and mechanical ventilation (AST, and T-Bil). Consequently, in hospitalized COVID-19 patients, elevated liver chemistries, specifically ALT, AST, ALP, and T-Bil levels, can be used to stratify risk and predict the need for advanced therapies.

Research perspectives

Abnormal liver chemistries are common at the time of hospital admission are associated with worse outcomes in COVID-19 patients. In particular, abnormal levels of AST, T-Bil, ALP, and hypoalbuminemia correlate with the severity of COVID-19 infection, and abnormal liver chemistries (ALT, T-Bil, and ALP) during hospitalization are strongly associated with all-cause mortality in patients with COVID-19. Furthermore, liver injury measured in patients with COVID-19 on admission is associated with the need for vasopressor drugs (AST, T-Bil, and ALP) and mechanical ventilation (AST, and T-Bil).

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CASE REPORT

Simultaneous endoscopic and video-assisted retroperitoneal debridement in walled-off pancreatic necrosis using a laparoscopic access platform: Two case reports

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Author contributions: Lindgaard L, Novovic S, Hansen EF, and Schmidt PN designed the study; Lindgaard L and Schmidt PN drafted the manuscript; Karstensen JG edited the manuscript; all authors critically revised the manuscript and approved the final content.

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Abstract

BACKGROUND

Infected walled-off necrosis is a potentially life-threatening complication of necrotizing pancreatitis. While some patients can be treated by drainage alone, many patients also need evacuation of the infected debris. Central necroses in relation to the pancreatic bed are easily reached *via* an endoscopic transluminal approach, whereas necroses that involve the paracolic gutters and the pelvis are most efficiently treated *via* a percutaneous approach. Large and complex necroses may need a combination of the two methods.

CASE SUMMARY

Transluminal and percutaneous drainage followed by simultaneous endoscopic and modified video-assisted retroperitoneal debridement was carried out in two patients with very large (32-38 cm), infected walled-off necroses using a laparoscopic access platform. After 34 d and 86 d and a total of 9 and 14 procedures, respectively, complete regression of the walled-off necroses was achieved. The laparoscopic access platform improved both access to the cavities as well as the overview. Simultaneous transluminal and percutaneous necrosectomy are feasible with the laparoscopic access platform serving as a useful adjunctive.

CONCLUSION

This approach may be necessary to control infection and achieve regression in some patients with complex collections.

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Core Tip: Infected walled-off necrosis is a potentially life-threatening complication of necrotizing pancreatitis. In cases with large or complex walled-off necrosis, treatment combining endoscopic, transluminal and percutaneous video-assisted retroperitoneal debridement may be needed. We report on two patients who underwent a combination of endoscopic and percutaneous drainage and necrosectomy for their large, infected walled-off necroses. Furthermore, we introduce a laparoscopic access platform as a useful adjunctive to endoscopic necrosectomy and video-assisted retroperitoneal debridement.

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INTRODUCTION

Infected walled-off necrosis (WON) is a potentially life-threatening complication of necrotizing pancreatitis. Traditionally, such patients were treated *via* open surgery, but during the last decade an endoscopic or surgical step-up approach using minimally invasive techniques has become the treatment of choice[1-5].

Whereas some patients can be treated by drainage alone, many patients also need evacuation of the infected debris. In such cases, it is our experience that central necroses in relation to the pancreatic bed are most easily reached by endoscopic, transluminal drainage followed by endoscopic necrosectomy (EN), whereas necroses that involve the paracolic gutters and the pelvis are more effectively treated with percutaneous drainage and video-assisted retroperitoneal debridement (VARD).

We report on two patients who needed a combination of endoscopic and percutaneous drainage and necrosectomy for their large, infected WONs. Furthermore, we introduce a laparoscopic access platform (Figures 1 and 2) as a useful adjunctive to EN and VARD.

CASE PRESENTATION

Chief complaints

Case 1: A 36-year-old male with a history of proctocolectomy and J-pouch due to ulcerative colitis was admitted to a local hospital because of jaundice.

Case 2: An 18-year-old boy was admitted to another hospital with severe necrotizing pancreatitis of unknown etiology.

History of present illness

Case 1: Magnetic resonance imaging raised suspicion of a cholangiocarcinoma in the liver hilum, a diagnosis that was subsequently confirmed. An endoscopic retrograde cholangiopancreatography revealed multiple intra- and extrahepatic bile duct strictures consistent with primary sclerosing cholangitis but failed to relieve the obstruction. Subsequent percutaneous transhepatic biliary drainage was carried out. After the endoscopic retrograde cholangiopancreatography, the patient developed post-endoscopic retrograde cholangiopancreatography pancreatitis, severe sepsis, and multiple organ failure and was admitted to the intensive care unit (ICU).

Figure 1 GelPOINT path transanal access platform.

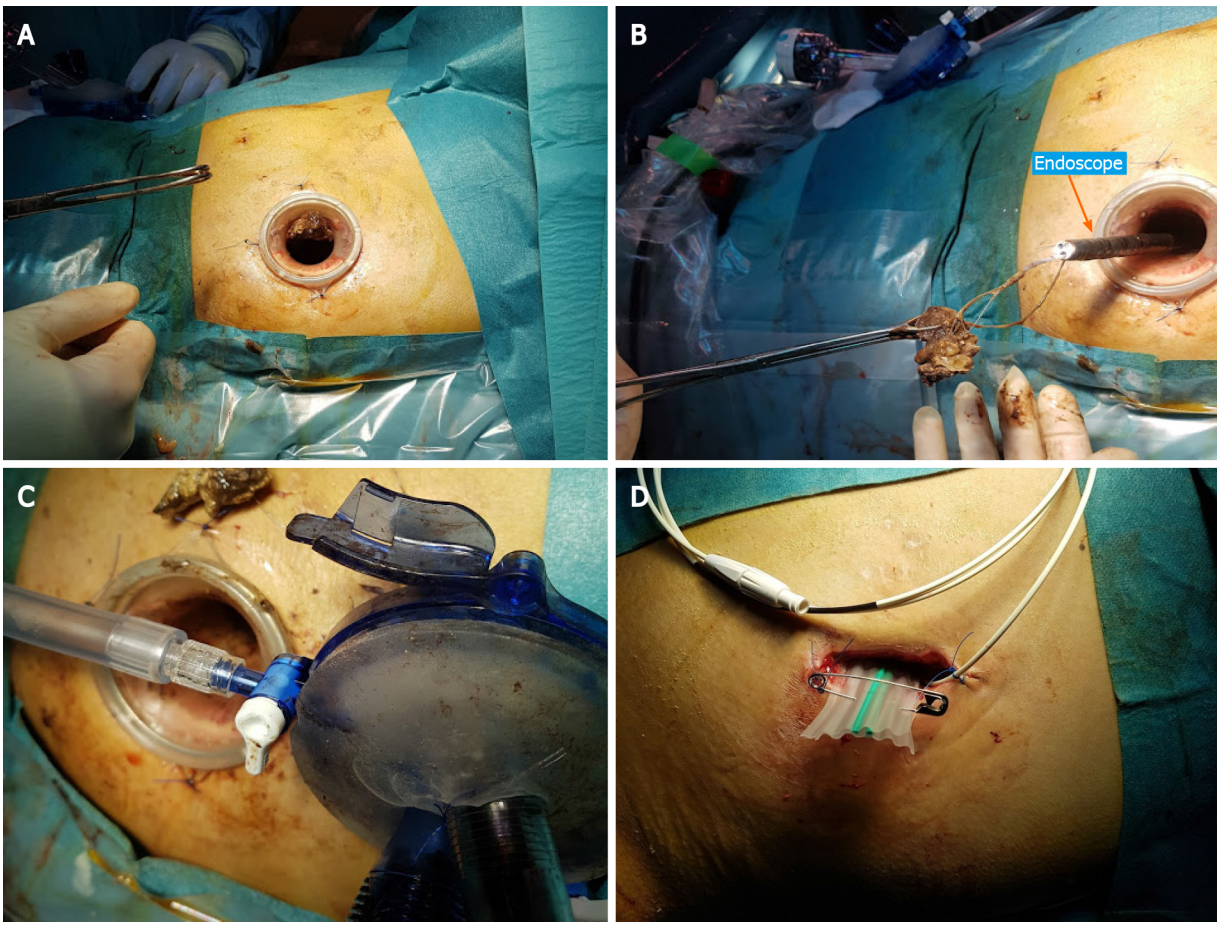

Figure 2 Endoscopic-laparoscopic retroperitoneal debridement. A: GelPOINT path transanal access platform with access channel placed in the video-assisted retroperitoneal debridement incision; B and C: Necrotic debris evacuated through the access channel during endoscopic, transluminal necrosectomy; D: Corrugated drainage sheet placed in the video-assisted retroperitoneal debridement incision at the end of the procedure.

Case 2: On day 2, he was referred to the ICU with respiratory and circulatory failure. On day 3, he developed intra-abdominal hypertension and renal failure with the need for dialysis.

FINAL DIAGNOSIS

Case 1: After 6 wk, which included severe episodes of gastrointestinal bleeding that needed coiling and laparotomy with creation of an ileostomy, he was referred to our hospital for endoscopic drainage of a large (12 cm × 14 cm × 38 cm), infected, left-sided WON extending from the spleen down through the left paracolic gutter into the left groin (Figure 3A).

Case 2: On day 43, he was referred to the ICU at our hospital with septicemia and a large (16 cm × 24 cm × 32 cm), infected, left-sided WON extending from the pancreatic bed into the pelvis (Figure 4A).

TREATMENT

Case 1: Between days 57 and 143, the patient underwent one endoscopic transluminal drainage, four EN, two VARD, and seven combined EN/VARD procedures.

Case 2: The same day, VARD was performed. Five days later, after only one VARD and one EN session, he was discharged from the ICU. The patient underwent a total of one endoscopic transluminal drainage, four VARD, two EN and two combined VARD/EN procedures, with the last procedure occurring on day 77.

OUTCOME AND FOLLOW-UP

Case 1: There was complete regression of the large WON (Figure 3B).

Case 2: There was complete regression of the large WON (Figure 4B).

DISCUSSION

In this paper, we describe 2 patients with necrotizing pancreatitis who needed treatment with a combination of EN and VARD. Both patients were referred to our hospital with signs of sepsis due to very large, infected WONs. We chose to combine two minimally invasive techniques to control the infection as quickly as possible in order to accelerate the treatment course.

EN is ideal for treating central WONs, whereas VARD is especially suitable in the case of lateral collections extending into the paracolic gutters and the pelvis. Even though flexible endoscopes can reach remote parts of the retroperitoneum, an isolated endoscopic approach can prove more time-consuming and delay evacuation of large collections as compared to a combined approach. Furthermore, large fluid collections may become compartmentalized over the course of treatment, creating isolated portions of the WON that can no longer be reached *via* the transluminal route. A successful combination of EN and VARD has been described elsewhere[6,7].

VARD has been thoroughly described in the literature[8,9]. However, use of a laparoscopic platform has not to our knowledge been documented. With the GelSeal cap closed and CO_2 insufflation through the platform, the large cavity could be expanded during the procedure, thus making debridement easier. During transgastric necrosectomy, debris was removed through the access channel of the platform by the surgeon or endoscopy assistants, reducing the procedure time. Finally, the platform facilitated debridement *via* the percutaneous route using the flexible endoscope. The success of the method needs to be investigated in prospective studies evaluating the risk of recurrence and fistula formation as well as matters relating to costs of the technique.

CONCLUSION

We conclude that simultaneous transluminal and percutaneous necrosectomy is practical and hypothesize that it may accelerate treatment in cases of complex WONs due to acute pancreatitis. Furthermore, a laparoscopic access platform is a useful adjunctive to the procedure.

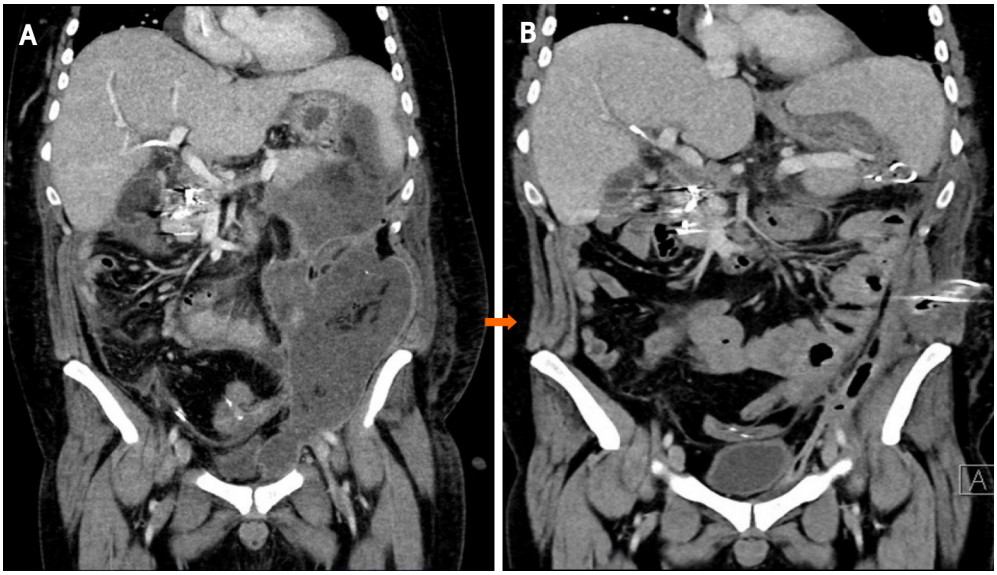

Figure 3 Case 1. Coronal computed tomography with walled-off necrosis. A: Before drainage; B: After 86 d and 14 procedures.

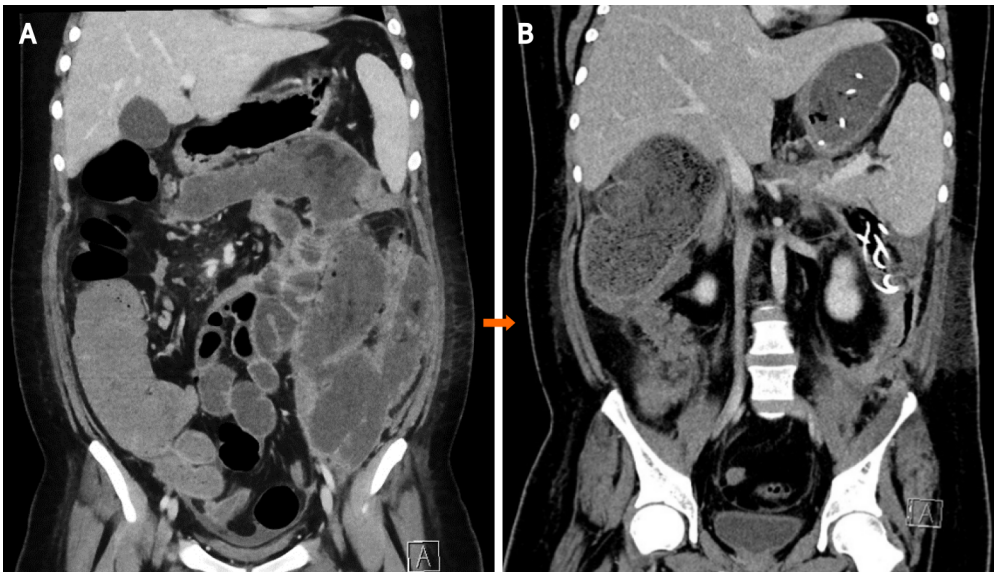

Figure 4 Case 2. Coronal computed tomography with walled-off necrosis. A: Before drainage; B: After 34 d and nine procedures.

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CASE REPORT

Curative resection with endoscopic submucosal dissection of early gastric cancer in *Helicobacter pylori*-negative Ménétrier's disease: A case report

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Author contributions: Fukushi K reviewed the literature and contributed to manuscript writing and data analysis; Goda K and Irisawa A contributed to draft conception and design; Fukushi K, Kino H, Kondo M, Kanazawa M, Kashima K, Kanamori A, Abe K, Suzuki T, Tominaga K, and Goda K were the clinicians involved in patient diagnostics, management, therapy, and surveillance; Yamagishi H performed pathological diagnosis; all authors have read and approved the final manuscript.

Informed consent statement: A written informed consent was obtained from the patient at the time of ESD.

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Abstract

BACKGROUND

Adult-onset Ménétrier's disease is strongly associated with *Helicobacter pylori* (*H. pylori*) infection and an elevated risk of carcinogenesis. Cases of early-stage gastric cancer developed in *H. pylori*-negative Ménétrier's disease are extremely rare. We report a case of early gastric cancer in *H. pylori*-negative Ménétrier's disease that was curatively resected with endoscopic submucosal dissection (ESD).

CASE SUMMARY

A 60-year-old woman was referred to our hospital after her medical examination detected anemia. Contrast-enhanced upper gastrointestinal (UGI) radiography revealed translucency of the nodule-aggregating surface with giant rugae. Blood tests showed hypoproteinemia and were negative for serum *H. pylori* immunoglobulin G antibodies. The ^{99m}Tc-DTPA-human serum albumin scintigraphy showed protein loss from the stomach. UGI endoscopy showed a 40-mm protruding erythematous lesion on giant rugae of the greater curvature of lower gastric body, suggesting early-stage gastric cancer due to Ménétrier's disease. *En bloc* resection with ESD was performed for diagnosis and treatment. Histology of ESD showed well-differentiated tubular adenocarcinoma. The cancer was confined to the mucosa, and complete curative resection was achieved. Foveolar hyperplasia and atrophy of the gastric glands were observed in non-tumor areas, histologically corresponding to Ménétrier's disease. Three years after ESD, gastric cancer had not recurred, and Ménétrier's disease remained in remission with spontaneous regression of giant gastric rugae.

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CONCLUSION
Complete curative resection was achieved through ESD in a patient with early-stage gastric cancer and *H. pylori*-negative Ménétrier's disease.

Key Words: Ménétrier's disease; *Helicobacter pylori*; Gastric cancer; Endoscopic resection; Endoscopic submucosal dissection; Case report

Core Tip: We reported an extremely rare case of gastric cancer as a complication of *Helicobacter pylori*-negative Ménétrier's disease. The cancer was early-stage, and a minimally invasive curative resection was achieved with endoscopic submucosal dissection (ESD). There was no recurrence, and the patient's course was extremely good. This case also highlights that when endoscopic biopsy tissue from a cancer-suspected lesion showed atypical glands in Ménétrier's disease, total biopsy with ESD can be useful for diagnosis as well as treatment. ESD seems to be a beneficial therapy for early-stage gastric cancer in Ménétrier's disease because of its low invasiveness and high complete curative resection rate.

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INTRODUCTION

Ménétrier's disease (discovered in 1888) is a relatively rare gastric disease[1] characterized by giant rugae in the stomach accompanied by hypoproteinemia. Adult-onset Ménétrier's disease is strongly associated with *Helicobacter pylori* (*H. pylori*) infection and an elevated risk of carcinogenesis[2]. Our literature review suggests that *H. pylori*-negative cases of early-stage gastric cancer are extremely rare[3,4].

We report the first case of *H. pylori*-negative Ménétrier's disease with early gastric cancer in which complete curative resection was achieved through endoscopic submucosal dissection (ESD).

CASE PRESENTATION

Chief complaints

A 60-year-old woman was referred to our hospital for examination and treatment of anemia.

History of present illness

After observing transient melena, the patient's previous doctor conducted a medical examination and detected severe anemia (blood hemoglobin 6.1 g/dL).

History of past illness

The patient had undergone successful treatment of chronic hepatitis C with interferon therapy performed by a previous doctor.

Personal and family history

The patient had no history of smoking or drinking alcohol. There was no relevant family history.

Physical examination

On admission, the patient's temperature was 36.7 °C, heart rate was 81 bpm, blood pressure was 128/80 mmHg. There was conjunctival pallor and no spontaneous abdominal pain or no tenderness.

Laboratory examinations

The blood test revealed hypoproteinemia (serum albumin, 3.4 g/dL), and the serum *H. pylori* immunoglobulin G antibody level was negative (< 3).

Imaging examinations

Computed tomography (CT) of the abdomen revealed marked mucosal thickening of the body of the stomach, with no enlarged lymph nodes around the stomach (Figure 1A). Upper gastrointestinal (UGI) radiograph showed giant gastric rugae[5] and translucency of the nodule-aggregating surface in the greater curvature of the lower gastric body (Figure 1B). The ^{99m}Tc-DTPA-human serum albumin (HSA-D) scintigraphy showed protein loss from the stomach after 24 h (Figure 1C).

Endoscopic examination

UGI endoscopy revealed giant rugae in the gastric body, similar to contrast-enhanced radiographic findings (Figure 2A). A 40-mm broad-based, protruding erythematous lesion with lobular surface was observed on the giant rugae of the gastric body (Figure 2B). UGI endoscopy showed no evidence of atrophic gastritis and intestinal metaplasia.

Endoscopic ultrasonography (EUS) demonstrated the five layers of the gastric wall. In the lesion area, hypertrophy was observed in the first two layers (equivalent to the mucosa and the muscularis mucosae); however, no noticeable structural changes were evident in the third layer (equivalent to the submucosal layer) or deeper (Figure 2C). These findings were consistent with early tumor confined to the mucosal layer.

Low-magnification narrow-band imaging (NBI) showed granular surfaces with various sizes/forms and dilated vessels (Figure 2D). High-magnification NBI demonstrated irregular microstructures of various forms and tortuous microvessels with changes in caliber (Figure 2E).

Histopathological findings

Histological diagnosis based on endoscopic biopsy was Group 2 (non-tumorous changes suspected, though tumorous lesions cannot be ruled out)[6]. Considering the tumor size and high probability of a cancerous lesion based on the endoscopic images, *en bloc* resection with ESD was performed for definitive diagnosis and treatment (Figure 3A). Loupe image showed that the well-differentiated tubular adenocarcinoma was confined to the mucosal layer, with no lymphovascular invasion (ly0/v0) or ulceration (UL0), and both the lateral and vertical margins were negative (Figure 3B).

Histology of ESD specimens also showed proliferation of atypical cells with an irregular glandular structure (Figure 4A and B). Additionally, scattered p53 protein-positive cells were observed (Figure 4C). Based on these findings, the atypical glands were diagnosed with early gastric cancer of a well-differentiated tubular adenocarcinoma. Foveolar hyperplasia and atrophy of the proper gastric glands were observed in the non-tumor areas, a histological image corresponding to Ménétrier's disease (Figure 4D).

Immunohistochemical staining showed that the tumor cells were diffusely positive for MUC5AC (Figure 5A) and partially positive for MUC6 (Figure 5B), and negative for MUC2 (Figure 5C) and CD10 (Figure 5D). *H. pylori* were not observed in any biopsy or ESD specimens.

FINAL DIAGNOSIS

The final diagnosis of the presented case was early-stage gastric cancer and *H. pylori*-negative Ménétrier's disease.

TREATMENT

The patient underwent ESD.

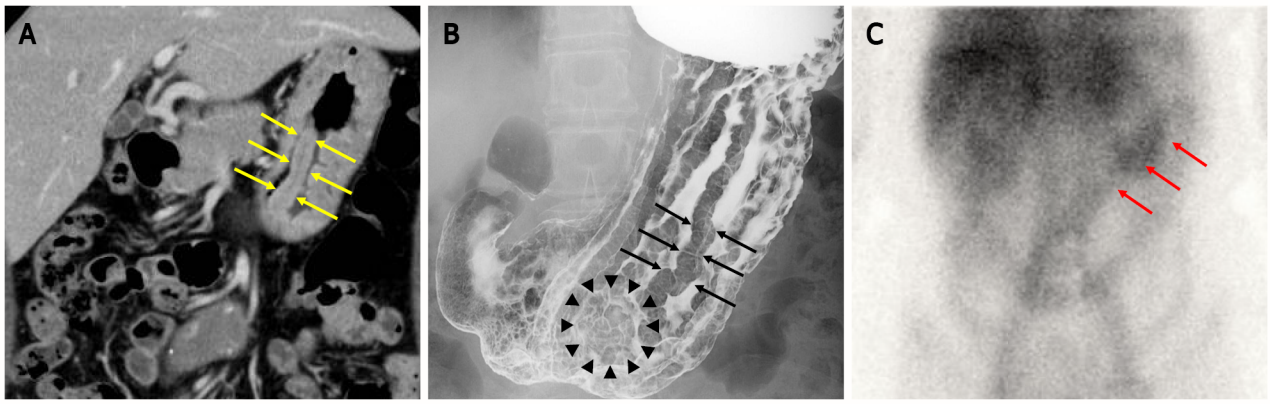

Figure 1 Images of computed tomography scan, upper-gastrointestinal radiograph and scintigraphy. A: Computed tomography scan revealed marked mucosal thickening of the body of the stomach (yellow arrow); B: The double-contrast technique shows remarkably enlarged fold width > 10 mm (black arrow) and translucency of nodule aggregating surface at the greater curvature in the lower gastric body (black arrowhead); C: ^{99m}Tc-DTPA-human serum albumin (HSA-D) scintigraphy: The arrows indicate accumulation in the stomach (red arrow).

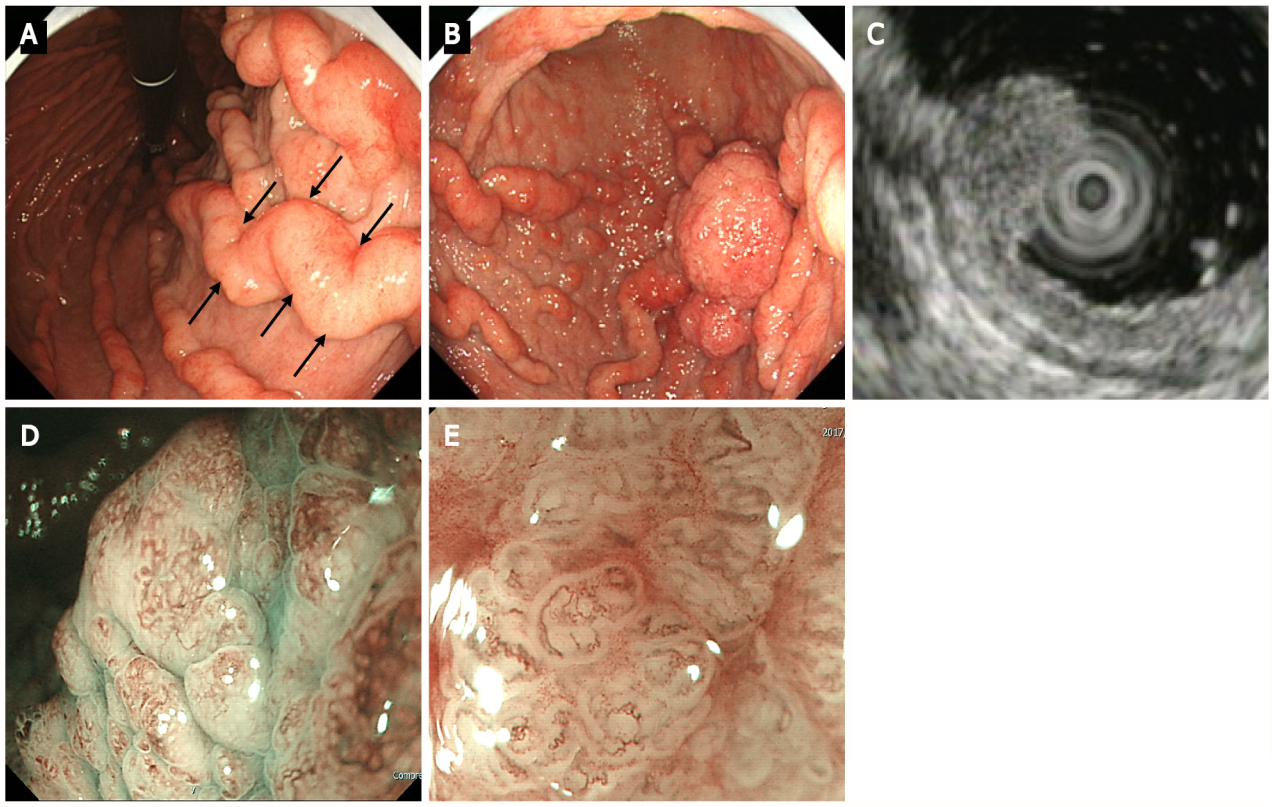

Figure 2 Upper gastrointestinal endoscopy. A: Upper gastrointestinal endoscopy shows marked fold enlargement from the ventricular angle to the fundus (black arrow); B: A 40-mm broad-based protruding lesion is observed near the posterior wall of the greater curvature of the lower gastric body; C: Endoscopic ultrasonography shows the five layers of the gastric wall. In the lesion area, hypertrophy was observed in the first two layers. No noticeable structural changes were evident in the third layer or deeper; D: Low-magnification narrow-band imaging (NBI) shows granular surfaces with various sizes/forms and dilated vessels; E: High-magnification NBI demonstrates irregular microstructures with various forms and tortuous microvessels with changes in caliber.

OUTCOME AND FOLLOW-UP

Histology of ESD specimens showed complete curative resection. Endoscopic follow-up was performed twice a year for 3 years after ESD, and no recurrence was detected (Figure 6). Even though no therapeutic agents were administered specifically for Ménétrier's disease, the giant rugae regressed spontaneously, hypoproteinemia and anemia improved gradually, and the remission was maintained until the last surveillance endoscopy.

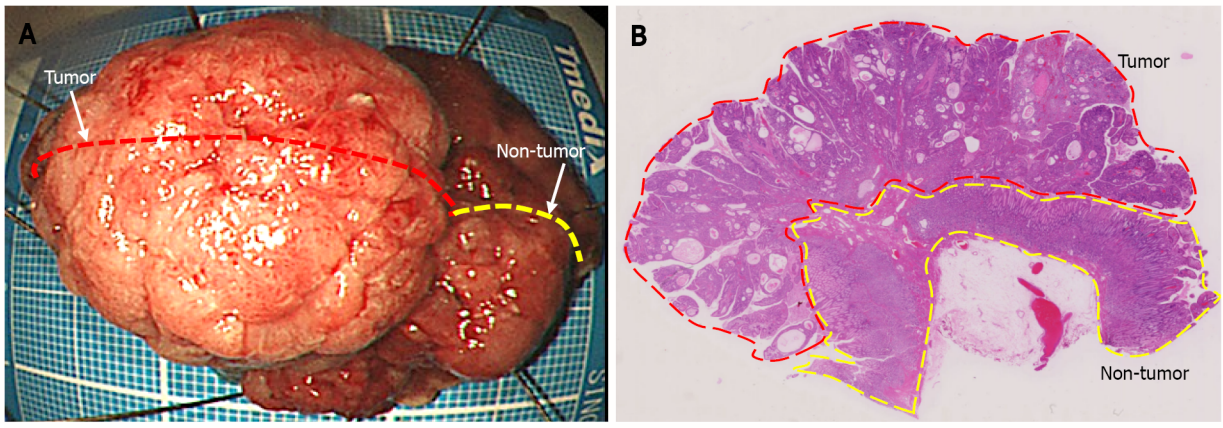

Figure 3 Endoscopic submucosal dissection specimen. A: 45 mm × 38 mm resection specimen comprising a broad-based protruding lesion with nodular surface and uninvolved (non-tumor) tissue with giant rugae; B: Loupe image shows that the well-differentiated tubular adenocarcinoma was confined to the mucosal layer, with no lymphovascular invasion (ly0/v0) or ulceration (UL0), and both the lateral and vertical margins were negative.

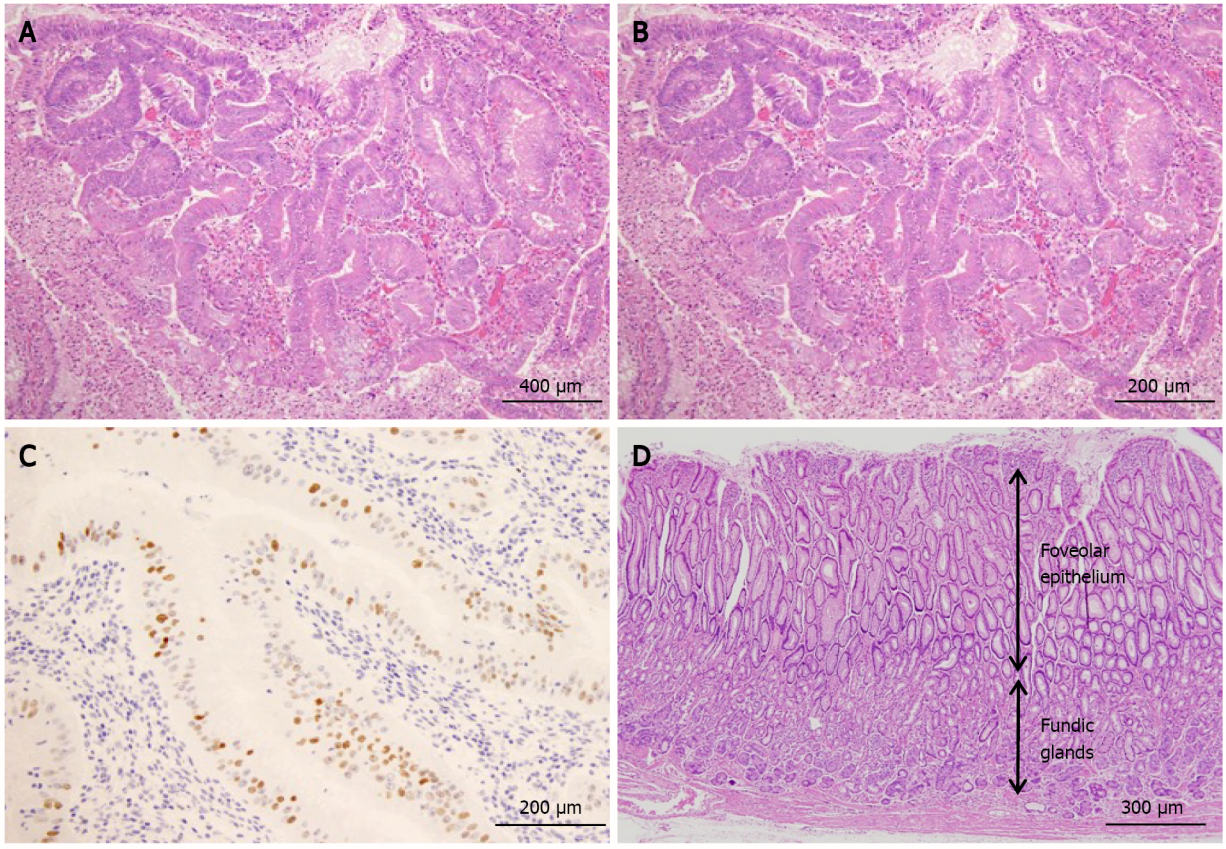

Figure 4 Histological findings. A: The protruding lesion exhibits proliferation of atypical glands with irregular tubular structures (HE; × 20); B: High magnification shows proliferation of atypical cells and enlarged nuclei (irregular or oval-shaped) in the irregular tubular structure (HE; × 20); C: Scattered positive image (in nuclei) in the region (p53; × 20); D: Foveolar hyperplasia and atrophy of the proper gastric glands in non-cancerous area, consistent with Ménétrier's disease (HE; × 20).

DISCUSSION

We reported an extremely rare case of early gastric cancer as a complication of *H. pylori*-negative Ménétrier's disease. We succeeded in curative resection of the cancerous lesion with ESD. Three years after the ESD, there was no recurrence of gastric cancer and Ménétrier's disease regressed spontaneously and had maintained regression for about 30 mo.

Although the present case was *H. pylori*-negative, there have been two reports of close associations between adult-onset Ménétrier's disease and *H. pylori* infection[7,8].

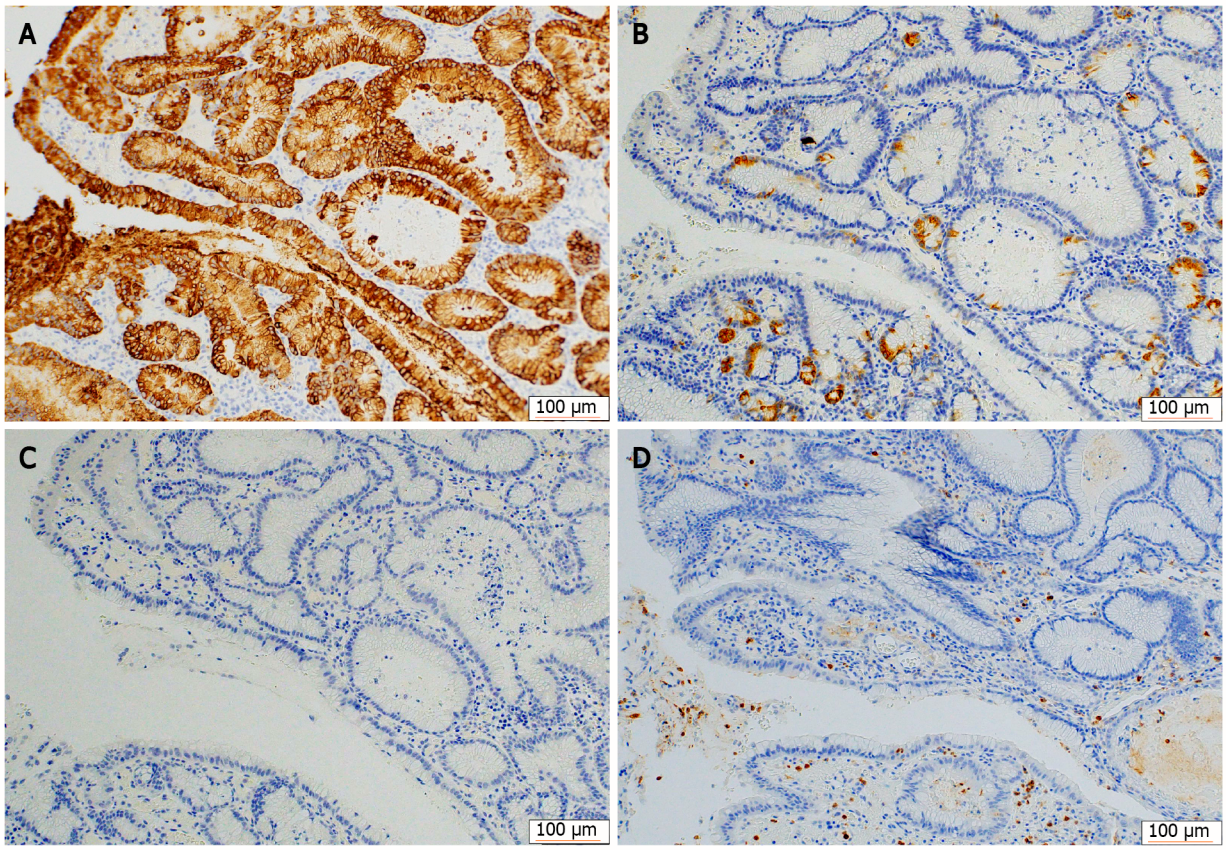

Figure 5 Immunohistochemical staining for mucin phenotypes. The tumor cells were diffusely positive for MUC5AC and partially positive for MUC6, and negative for MUC2 and CD10. A: MUC5AC; B: MUC6; C: MUC2; D: CD10.

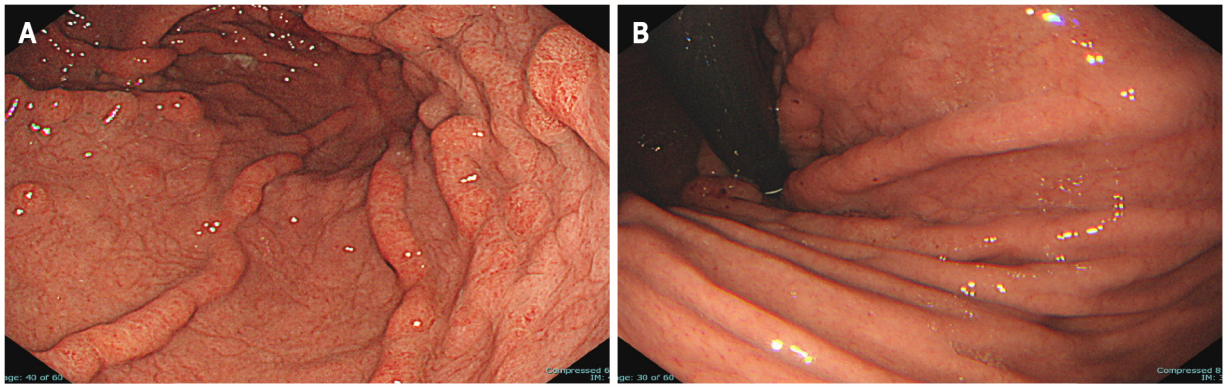

Figure 6 Uppeer-gastrointestinal endoscopy 3 yr after endoscopic submucosal dissection. A: Endoscopic submucosal dissection scar but no recurrence of tumor; B: The giant rugae with erythematous and edema has regressed spontaneously. Ménétrier's disease has remained in remission for the past 3 yr.

It has been reported that Ménétrier's disease with hypertrophic gastropathy increases the risk of gastric carcinogenesis[9]; however, the association between gastric carcinogenesis and *H. pylori* infection in Ménétrier's disease remains unclear.

There have been about 80 case reports on gastric cancer (including advanced cancer) in adult-onset Ménétrier's disease, though few have reported early-stage cancers. Our literature search found four case reports of Ménétrier's disease with early gastric cancer and recorded endoscopic findings and *H. pylori* infection status (Table 1); two of these cases were *H. pylori*-positive[10,11], and three cases, including the present one, were *H. pylori*-negative[3,4]. Our case was the second early gastric cancer to undergo endoscopic resection and the first resection with ESD instead of conventional endoscopic mucosal resection. Macroscopically, all the lesions of early gastric cancer were of protruding-type[12] and located on the oral side of the ventricular angle (corpus/fundus region)[13]. Hypoalbuminemia was observed in three cases and was

Table 1 Case reports on early-stage gastric cancer in patients with Ménétrier's disease

Ref.	Age	Gender	Hypoalbuminemia[1] (g/L)	*H. pylori*	Differentiation	Tumor invasion depth	Macroscopic types	Tumor location	Therapy	Outcome
Raderer *et al*[10]	79	F	Present (1.5)	Positive (Hp IgG-Ab)	Well	M	0-Is	Fundus	EMR	No recurrence for 3 yr
Johnson *et al*[11]	73	M	Present (2.4)	Positive (histology)	Well	SM	0-IIa + IIc	Corpus	TG	Unknown
Ozawa *et al*[3]	48	F	Present (1.5)	Negative (Hp IgG-Ab)	Poor	M	0-Is	Corpus to Antrum	TG	No recurrence for 2 yr
Charton-Bain *et al* [4]	62	F	Unknown	Negative (histology)	Well	M	0-Is	Corpus	TG	Death on the 7th day post-operation[2]
Present case	60	F	Present (3.4)	Negative (Hp IgG-Ab)	Well	M	0-Is	Corpus	ESD	No recurrence for 3 yr

[1]Hypoalbuminemia defined with less than 3.4 g/L.
[2]Death of pneumonia and liver failure.
EMR: Endoscopic mucosal resection; TG: Triglyceride; ESD: Endoscopic submucosal dissection; IgG: Immunoglobulin G.

unclear in one case.

These findings (protruding lesions located in the corpus or fundus region and hypoalbuminemia) may depict the clinicopathological features of Ménétrier's disease-associated early gastric cancer.

Ménétrier's disease generally presents with hypertrophic gastropathy, primarily in the fundic gland region, which may explain the tendency for Ménétrier's disease-associated early-stage gastric cancer to occur in this region[14]. Endoscopic gastric cancer screening of patients with Ménétrier's disease should focus on two characteristics: The location (corpus or fundus) and the macroscopic type.

In the present case, cancer could not be confirmed by preoperative biopsy; therefore, a definitive diagnosis of cancer was made using the specimen from the endoscopic resection specimen. Based on histopathological findings, Ménétrier's disease is considered a chronic inflammatory disease[15], making it difficult to differentiate between inflammatory atypia and neoplastic atypia *via* small tissue biopsy alone, as in the present case. When endoscopic biopsy tissue from a superficial tumor-like lesion exhibits atypical glands in patients with Ménétrier's disease, total biopsy with *en bloc* ESD can be useful for definitive diagnosis as well as treatment including complete curative resection, such as in this case.

Severe Ménétrier's disease cases with clinical symptoms such as abdominal pain and vomiting require total gastrectomy to control severe hypoalbuminemia[16,17]. Besides the present patient, the two other patients with *H. pylori*-negative early-stage cancer underwent total gastrectomy. There was one case of surgery-related death. Therefore, if Ménétrier's disease is not severe and the stomach can be preserved, ESD seems a beneficial therapy for early-stage gastric cancer, especially for mucosal cancer, because of its low invasiveness and high complete curative resection rate.

The mucin phenotype of the early gastric cancer in this case was gastric type with strongly positive for MUC5AC and low expression of MUC6. These suggest the gastric-type mucin of foveolar-dominant type. In the non-tumor area involved by Mén étrier's disease, although p53 staining was negative and Ki-67 index was low, significant hyperplasia of foveolar epithelium was shown in the fundic gland region. We deduced that early cancer in the present case could be developed from a polypoid lesion with foveolar hyperplasia along hyperplasia–dysplasia–carcinoma sequence [18], even though the mechanism of gastric cancer development in Ménétrier's disease is unknown.

CONCLUSION

To our knowledge, this is the first report of an extremely rare case of early-stage

gastric cancer detected in a patient with *H. pylori*-negative Ménétrier's disease in which complete curative resection was achieved with ESD.

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LETTER TO THE EDITOR

Artificial intelligence model validation before its application in clinical diagnosis assistance

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Abstract

The process of selecting an artificial intelligence (AI) model to assist clinical diagnosis of a particular pathology and its validation tests is relevant since the values of accuracy, sensitivity and specificity may not reflect the behavior of the method in a real environment. Here, we provide helpful considerations to increase the success of using an AI model in clinical practice.

Key Words: Artificial intelligence; Diagnostic assistance; Validation tests; Leave-one-out cross-validation; K-fold validation; Hold-out validation

Core tip: The validation tests and the process to adopt a particular artificial intelligence (AI) model are relevant. The percentages of accuracy, sensitivity and specificity obtained through validation techniques are strong indicators of whether the AI model is suitable for implementation in clinical practice or whether it will be necessary to

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continue acquiring samples.

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TO THE EDITOR

After studying the interesting article "Non-occlusive mesenteric ischemia: Diagnostic challenges and perspectives in the era of artificial intelligence" by Bourcier *et al*[1], who analyzed the current state of artificial intelligence (AI) in assisting clinical diagnosis and its possible application in diagnosing nonocclusive mesenteric ischemia, we are in full agreement with the AI techniques that the authors mention. However, a greater emphasis on the evaluation process for AI models could yield better results; when a rigorous testing stage is lacking, these models show poor performance upon transfer from the laboratory to real practice.

It is essential to mention that AI models using machine learning techniques, such as decision tree, support vector machine, artificial neural networks, naïve Bayesian classifier, Bayesian network K-nearest neighbor, and random forest, are predictive[2], are indispensable to performance of the three stages of training, validation and testing [3].

In this sense, the scarcity of validation tests provokes a reduction in the percentages of accuracy (Ac), sensitivity (Se) and specificity (Sp) of the AI models at the time of transferring them to a real environment[4].

These validation tests consist of segmenting the total of the samples available in different proportions to force the AI model to look for a robust solution (a representative pattern) due to the variance in the data. However, how to define the proportions in which the database will be segmented is a subject under development. Therefore, cross-validation strategies such as leave-one-out cross-validation (LOOCV) or k-fold cross-validation have been used more frequently than techniques such as hold-out validation because they obtain better Ac, Se and Sp in laboratory tests[4-6]; moreover, they consider a larger population in the training process compared to hold-out.

Following the LOOCV guidelines, a sample is left out of the database and the AI model is trained with the rest; once the training is finished, the separated spectrum is evaluated with the trained model. This process is repeated until all the collected spectra are evaluated, and the percentages of Ac, Se and Sp are calculated based on the number of correct and incorrect evaluations that the models have carried out. By involving most of the data in the training process, the result obtained by LOOCV usually reflects an overtrained model, making the generalization process of future samples complex by reducing their Ac, Se and Sp in a real scenario.

The k-fold model is similar to LOOCV, except that the database is divided into k groups with approximate numbers of samples instead of separating a single sample. Thus, one group is left out, and the rest is used for training; the process ends when all groups have been evaluated. The conflict with this strategy lies in defining the number of k groups created, since there is currently no formal methodology to calculate them. However, the most common values are k = 5 and 10; as such, the base data are segmented into five or 10 groups. In contrast, hold-out divides the populations that make up the database into percentages of 80–20 (one of the most used); that is, 80% of the samples from each population that make up the database are used for training. As this process is subjected to a more significant variance, the evaluation process usually shows lower percentages of Ac, Se and Sp compared to LOOCV and k-fold[4]. The above does not mean that the AI model is inadequate; instead, it indicates that the number of samples collected is insufficient to detect a sufficiently robust pattern. Thus, if the Ac, Se and Sp percentages are not reliable enough, acquiring more samples is a good option before using this AI methodology in clinical practice.

Although no studies have been carried out in this regard, an excellent strategy for evaluating whether an AI model is ready to be tested in a real environment is to

analyze several techniques, first using LOOCV and selecting the techniques with the best results to study their performance. Subsequently, k-fold evaluates the performance of the previously selected models thanks to the LOOCV strategy. As a result of the study of the models using the k-fold strategy, the model with the best performance should be selected. Finally, the best AI technique can be studied using the hold-out strategy; upon separating a considerable number of samples from each population according to the database (20%), the training/learning process of the AI models is subject to a more significant variance in the data of each population. In this way, they focus on particular features of the same group and not on characteristics of the samples that make up a particular database (overfitting), as could occur in the case of considering the LOOCV strategy only[4,5]. If the accuracy metrics of the model evaluated with hold-out are similar to those obtained when it was evaluated using LOOCV, it is possible to expect that the AI model will perform well in a real environment.

The use of AI methods in clinical diagnosis is new, and there are many subjects to investigate in this field; however, it is fascinating how the use of these technologies has reached medical science and how the new generations of researchers venture to use and combine the different sciences (physics, chemistry, mathematics, engineering, computer science, biology, among others) to generate new knowledge. We hope that the recommendations made here will help explore this AI field in the biological and medical sciences.

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LETTER TO THE EDITOR

Machine learning models and over-fitting considerations

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Abstract

Machine learning models may outperform traditional statistical regression algorithms for predicting clinical outcomes. Proper validation of building such models and tuning their underlying algorithms is necessary to avoid over-fitting and poor generalizability, which smaller datasets can be more prone to. In an effort to educate readers interested in artificial intelligence and model-building based on machine-learning algorithms, we outline important details on cross-validation techniques that can enhance the performance and generalizability of such models.

Key Words: Machine learning; Over-fitting; Cross-validation; Hyper-parameter tuning

Core Tip: Machine learning models are increasingly being used in clinical medicine to predict outcomes. Proper validation techniques of these models are essential to avoid over-fitting and poor generalization on new data.

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TO THE EDITOR

Con *et al*[1] explore artificial intelligence (AI) in a classification problem of predicting biochemical remission of Crohn's disease at 12 mo post-induction with infliximab or adalimumab. They illustrate that, after applying appropriate machine learning (ML) methodologies, ML methods outperform conventional multivariable logistic

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regression (a statistical learning algorithm). The area-under-the-curve (AUC) was the chosen performance metric for comparison and cross-validation was performed.

Their study elucidates a few important points regarding the utilization of ML. First, the use of repeated k-fold cross-validation, which is primarily utilized to prevent over-fitting of the models. This technique, while common in ML, it has not been traditionally used in conventional regression models in the literature so far. Especially in small datasets, such as in their study (n = 146), linear (and non-linear, in the case of neural networks) relationships risk being "learned" by chance, leading to poor generalization of the models when applied to previously "unseen" or future data points. It was evident from their analysis that the "naïve" AUCs (training the model on all the data), was significantly higher than the mean cross-validated AUCs, in all 3 models, suggestive of "over-fitting" when one does not cross-validate. Smaller datasets tend to be more susceptible to over-fitting as they are less likely to accurately represent the population in question.

Second, the authors utilized "hyper-parameter tuning" for their neural network models, where the otherwise arbitrarily selected "settings" (or hyper-parameters, such as the number of inner neuron layers and number of neurons per layer) of the neural network are chosen based on performance. Hyper-parameters cannot be "learned" or "optimized" by simply fitting the model (as it happens with predictor coefficients), and the only way to discover the best values is by fitting the model with various combinations and assessing its performance. The combinations can be evaluated stochastically (randomly or *via* a Bayes-based approach) or using a grid approach (*e.g.*, for 3 hyper-parameters that take 5 potential values, there are $5 \times 5 \times 5 = 5^3 = 125$ combinations to evaluate) over k times. One may ask, if one was to fit a model 125 × k times, on 146 observations, is not there a risk for over-fitting the "optimal" hyper-parameter values? To avoid such a problem, nested k-fold cross-validation must be performed: within each repeated k-fold training data subset, a sub-k-fold "inner" training/validation must be done to evaluate each hyper-parameter combination. In this way, we overcome potential bias to optimistic model performance, which can occur when we use the same cross-validation procedure and dataset to both tune the hyper-parameters and evaluate the model's performance metrics (*e.g.*, AUC)[2]. The authors did not elaborate on how the hyperparameter tuning was performed.

Another point to consider in k-fold cross-validation in small datasets is the number of k-folds used, specifically in classification problems (*i.e.*, yes/no binary outcomes). In this study[1], the outcome prevalence was 64% ($n \approx 93$). With a chosen k = 5, the training folds would comprise 80% of that data, leading to approximately 74 positive cases of biochemical remission. The number of positive outcomes in each training fold must be considered, especially in logistic regression, where the rule of thumb recommends at least ten positive events per independent predictor, to minimize over-fitting[3]. In this study[1], six predictors were eventually used in the multivariable model, making over-fitting less likely from a model-specification standpoint. Finally, k-folds are recommended to be stratified by the outcome, so the outcome prevalence is equal among the training and testing folds. This becomes crucial when the prevalence of outcome of interest is < 10%-20% (imbalanced classification problem). While imbalanced classification is not an issue in this study[1], the authors did not mention whether they used outcome-stratified k-folds.

Lastly, the endpoint utilized, CRP normalization, has poor specificity for endoscopic inflammation in Crohn's disease[4]. More robust endpoints would include endoscopic inflammation and/or deep remission using validated disease activity indices[5].

We congratulate the authors for their effort, which acts both as a proof-of-concept for using ML in improved prediction of outcomes in IBD, but also for the methodologies outlined to reduce over-fitting. In general, with the advent of AI and specifically ML-based models in IBD[6], it is important to recognize that while now we have the tools to construct more accurate models and enhance precision medicine, most ML-based models, such as artificial neural networks, lack in being intuitively interpretable (*i.e.*, "black-box"). Efforts in "explainable AI" are under way[7], hopefully eliminating the "black-box" concept in future clinical decision tools. Applying these to validated disease activity assessments will be essential for prediction models in future studies.

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