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REVIEW

Role of one-step nucleic acid amplification in colorectal cancer lymph node metastases detection

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Abstract

Current histopathological staging procedures in colorectal cancer (CRC) depend on midline division of the lymph nodes (LNs) with one section of hematoxylin and eosin staining. Cancer cells outside this transection line may be missed, which could lead to understaging of Union for International Cancer Control Stage II high-risk patients. The one-step nucleic acid amplification (OSNA) assay has emerged as a rapid molecular diagnostic tool for LN metastases detection. It is a molecular technique that can analyze the entire LN tissue using a reverse-transcriptase loop-mediated isothermal amplification reaction to detect tumor-specific cytokeratin 19 mRNA. Our findings suggest that the OSNA assay has a high diagnostic accuracy in detecting metastatic LNs in CRC and a high negative predictive value. OSNA is a standardized, observer-independent technique, which may lead to more accurate staging. It has been suggested that in stage II CRC, the upstaging can reach 25% and these patients can access postoperative adjuvant chemotherapy. Moreover, intraoperative OSNA sentinel node evaluation may allow early CRC to be treated with organ-preserving surgery, while in more advanced-stage disease, a tailored lymphadenectomy can be performed considering the presence of aberrant lymphatic drainage and skip metastases.

Key Words: Colorectal malignancies; One-step nucleic acid amplification; Diagnostic accuracy; Negative predictive value; Upstaging; Organ-sparing surgery; Tailored lymphadenectomy

Core Tip: Our findings suggest that the one-step nucleic acid amplification (OSNA) assay has high diagnostic accuracy and negative predictive value in detecting metastatic lymph nodes in colorectal cancer (CRC). The short turnaround time renders OSNA an attractive intra-operative method. OSNA results in upstaging in about 25% of stage II CRC cases. Moreover, organ-sparing surgery in early CRC and tailored lymphadenectomy, in more advanced cases, can be performed.

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INTRODUCTION

Of the gastrointestinal cancers, the colorectal cancer (CRC) is the most represented. Among the indication criteria for chemotherapy, lymph node (LN) positivity (stage III) is the most important[1]. The histopathological study of the LNs is performed on one or at most two sections of each LN with hematoxylin and eosin (HE). Therefore the conventional study presents the possibility of not detecting micro-metastases (MMs) or macro-metastases leading to an "understaging". The high relapse rates (20%–25%) in patients with negative LNs could be due to this "understaging"[2]. Multilevel LN sectioning combined with immunohistochemistry (IHC) can improve the detection rate of small nodal tumor infiltrates [*i.e.* isolated tumor cells (ITCs) and MMs], although it is a costly and protracted process [3-6].

Tsujimoto *et al*[7] were the first to describe the one-step nucleic acid amplification (OSNA) assay for detecting LN metastases (LNMs) in patients with breast cancer (BC). Numerous studies have followed which have confirmed the high sensitivity of OSNA in detecting LNMs of breast, gastric and CRCs[8-15]. Other studies[16-18] have underlined the usefulness of the OSNA assay as a complementary tool for diagnosing LNMs and upstaging in histologically node-negative stage II CRC.

The sentinel LN (SLN) is gaining more and more consensus because it allows to perform a more conservative surgery with considerable advantages, when applicable for the patient and for the operating times. Obviously in the early stages of CRCs this could play an important role, allowing to realize, in case of absence of lymph node metastases on SLNs, an organ preserving surgery.

In this review, we analyzed the use of OSNA in detecting LNMs in CRC.

LITERATURE SEARCH

Search strategy

After developing and piloting search terms, MEDLINE, SCOPUS, ClinicalTrials.gov, and Cochrane Database were used to conduct a comprehensive computerized literature search for articles pertaining to OSNA use in detecting LNMs in CRC. Medical subject headings terms and keywords were combined: colorectal malignant, cancer, colorectal tumor, colorectal neoplasm, carcinoma, lymph node metastasis, SLN, one-step nucleic acid amplification, OSNA, cytokeratin 19, CK-19, predictive value, upstaging, organ-sparing surgery, and tailored lymphadenectomy. The electronic search was supplemented by reviewing reference lists of included studies and previous systematic reviews. No time limitation was stipulated for the search, which was last updated December 20, 2021. In addition, we retrieved and cited high-quality references using the Reference Citation Analysis database (https://www.referencecitation-analysis.com/).

Study selection, data extraction, and quality assessment

The retrieval of articles was completed in three consecutive stages. Following reduplication of the sum of collected articles, their titles and abstracts underwent further screening and those deemed ineligible were removed. For duplicates, the most recent or complete publication was chosen. The remaining papers were evaluated in full text. Two reviewers (MB, SV) extracted data in duplicate using a standardized data extraction sheet.

LYMPHATIC DRAINAGE IN CRC

The American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) staging score divides the stages according to how many metastatic lymph nodes are present. Based on the location of the primary tumor, those with a course adjacent to the main vascular branches near the affected colon are considered regional lymph nodes. In particular, starting from the rectum up to the right colon, in addition to the peri-colic lymph nodes, regional lymph nodes are considered, those adjacent to the rectal arteries, the sigmoid arteries, the left colonic artery, the inferior mesenteric artery, the middle colic artery, the right colic artery, the ileocolic artery[19].

In AJCC/UICC tumor-node-metastasis (TNM) staging system[20-22], patients with no metastatic LNs are N0, cases with one to three metastatic LNs are N1, and cases with more than three positive LNs are N2. Moreover, the N1 category is subdivided into N1a (1 metastatic LN), N1b (2–3 metastatic LNs), and N1c (no regional LNs are positive but there are tumor deposits in the subserosa, mesentery or non-peritonealized pericolic or perirectal/mesorectal tissues), whereas the N2 category is subdivided into N2a (4–6 metastatic LNs) and N2b (7 or more metastatic LNs). The minimum number of examined LNs needed for adequate staging should not be less than 12 to minimize the possibility of stage migration[19, 23-27].

The Japanese Society for Cancer of the Colon and Rectum (JSCCR) staging score classifies the involved LNs based on location and number. This system divides the regional LNs into three groups: main, intermediate and peri-colic. Regional LNs depend on their adjacency to the blood vessels following the primary tumor site. LNs adjacent to the marginal arcade are pericolic nodes, the LNs along the course of the main vessels of the colon are intermediate nodes (sigmoid arteries, the left colonic artery, the inferior mesenteric artery, the right and left middle colic artery, the right colic artery, the ileocolic artery). Lymph nodes located proximal to the origin of the main colonic vascular branches of the inferior and superior mesenteric artery are the main nodes. LNMs are classified as N1 if up to 3 peri-colic or intermediate LNs are involved, N2 if they are ≥ 4, N3 when the main LNs are involved[28, 29].

ITCs and MMs

When single or few tumor cells smaller than 0.2 mm are found, these are called ITCs, if instead the deposits have a diameter between 0.2 and 2.0 mm these are called MMs. When ITCs or MMs with HE or IHC are found, they are classified as pN0 (i +), if instead the deposits are diagnosed only by reverse transcriptase polymerase chain reaction (RT-PCR), they are classified as pN0 (mol +)[19,20]. MMs, ITCs, and occult metastasis have been reported in 4.2%, 19.3%, and 5% of patients with stage I and II CRC, respectively, and attracted interest as prognostic factors[30-33].

Tumor deposits

In the literature, tumor deposits (TDs) are defined as foci of tumor separated from the main neoplasm and found in peri-rectal or peri-colonic adipose tissue or in mesocolon in the lymphatic drainage area, in the absence of identifiable LN tissue.

It is postulated that they are produced either by discontinuous dissemination of the tumor or by vascular/perineural dissemination. TDs can be found in 10.2%–22% of CRC cases and it has been suggested that TDs may represent a LN, a vascular structure, or a nerve completely replaced by carcinoma[34].

Several studies[35,36] have shown decreased disease-specific survival and overall survival (OS) in the presence of TDs. Moreover, the survival outcomes worsen when TDs occur concomitantly with LNMs. Other studies confirmed this evidence in CRC. It has been suggested that TDs have negative prognostic value but are not sufficiently categorized in the current TNM staging and the number and/or presence of the TD should be added to the number of LNMs to define the final N stage creating a specific category for TDs with LNM, which could be called category N2c or N3[37-41].

CLINICAL STAGE OF NODAL METASTASES

Diagnostic imaging

Diagnostic imaging assessment of lymphadenopathy in CRCs is challenging. Individual imaging modalities face specific intrinsic limitations, for example, transrectal ultrasound is operator-dependent, detrimentally affected by a small field of view, and cannot be employed in stenosing or rectal cancers. Computed tomography (CT) is hampered by its low soft tissue contrast resolution which, besides negatively impacting detection, precludes evaluation of fine LN details and therefore must rely only on LN size for assessing lymphadenopathy. Magnetic resonance imaging (MRI) requires long acquisition times and is prone to artifacts in the case of poor patient cooperation. Fluorodeoxyglucose (FDG)-positron emission tomography (PET)/CT and PET/MRI necessitate exposure to radiation, and its yields are influenced by the amount of metabolically avid cells in the affected LNs.

Size

Lymphadenopathy is also intrinsically challenging. Despite malignant LNs tending to be larger than their benign counterparts, there is wide size superimposition between malignant and benign LNs. In one study that evaluated only LNs ≥ 5 mm, short axis range was 6-12 mm for malignant LNs and 5-13 mm for benign LNs[42,43]. Moreover, the size of metastatic LNs is often at the lower limits of imaging spatial resolution. In a study, median LN short axis was 3.2 mm for malignant LNs and 2.8 mm for benign LNs[44]. Additionally, 30%-94% of metastatic LNs from rectal cancer are < 5 mm in short axis [45-48]. To make things even more challenging, some benign etiologies, especially inflammation, might increase LNs size.

MRI

Due its superior anatomic layout and high soft tissue contrast resolution, MRI can explore LN nature trough size and morphologic criteria. LN size criteria have yielded different results in different studies. However, across studies, the bigger the short axis threshold, the higher the specificity and lower the sensitivity; a 3 mm short axis has been associated with 92% sensitivity, 3% specificity, and 40% accuracy; a short axis threshold of 9 mm has been associated with the opposite trend, giving 8% sensitivity, 100% specificity, and 62% accuracy[42]. Short axis thresholds of 7.2-7.5 mm have reached 32%-87% sensitivity and 70%-94% specificity, with 68% of accuracy[42,43]. However, the most accepted short axis cut off for lymphadenopathy in rectal cancer is 5 mm, yielding sensitivities of 50%-72%, specificities of 46%-60%, and an accuracy of 57%[42,46-50].

Beside size, several other MRI criteria can be used, with diffusion weighted imaging (DWI) believed to be one of the most promising tools. However, DWI has proven inadequate for this purpose so far. High b-value DWI is a powerful tool for LN detection. However, DWI and even apparent diffusion coefficient values[43,51,52] are unable to discriminate benign from malignant LNs (accuracy 40%). Therefore, in our practice, we use DWI just to detect all LNs, relegating the differentiation of benign from malignant ones to morphologic, size and/or metabolic criteria.

Chemical shift effect (CSE) refers to a black or bright border outlining organ contours, including LNs. In the case of neoplastic growth in the subcapsular sinus, the resonance frequencies of hydrogen protons in the subcapsular sinus and in the adjacent fat are similar, resulting in the loss of CSE.

Four patterns of LN CSE have been described: continuous and smooth, continuous, and irregular, discontinuous, and irregular, or absent[44]. Once neoplastic cells have colonized the subcapsular sinus, they easily spread outside of the LN, likely more rapidly than toward the medulla. This results in irregular or obscure LN contours, reported in 60%-65% of malignant LNs and in 16%-20% of benign LNs, leading to sensitivity of 88%, specificity of 23%, and accuracy of 50% in one study. On the other hand, a smooth external contour has been described in 80%-84% of benign LNs and in 34%-40% of malignant LNs[42-44].

The internal structure of LNs may be heterogenous in the settings of metastases; this has been reported in 26%-52% of benign and 54%-91% of malignant LNs, with a sensitivity of 84%, specificity of 31%, and accuracy of 53%; on the other hand, homogeneous internal structure, has been observed in 48%-73% of benign and 8%-46% of malignant LNs[42-44]. The combination of inhomogeneous signal intensity and indistinct/irregular borders has been shown to yield sensitivity, specificity, and accuracy of 56%, 91%, and 77%, respectively[42].

Currently, LN size is the most used criterion to discriminate between malignant and benign LNs on MRI, but given the previously discussed inherent limitations, it is often integrated with the morphologic criteria described above. According to the European Society of Abdominal and Gastrointestinal Radiology (ESGAR) guidelines, a LN is considered metastatic in the case of[53]: Short axis diameter ≥ 9 mm, short axis 5-8 mm plus ≥ 2 morphologic criteria, short axis < 5 mm plus 3 morphologic criteria, or mucinous LN regardless of the size. Morphologic criteria chosen by ESGAR are round shape, irregular borders, and heterogenous signal.

However, despite all of the above efforts, even MRI, the most promising imaging modality for LN evaluation is still inadequate for the scope. A recent study that explored the staging performance of MRI in rectal cancer, using surgical pathology as a standard of reference, showed that MRI LN status was correctly assigned in 68% of cases, overstaged in 28%, and understaged in 4%. Moreover, only 40% of MRI-positive LN cases were pathologically confirmed[54]. These results are in line with a FDG-PET/MRI study where N status was overstaged by MRI in 22.6% of patients and by PET/MRI in 8% of cases; correct N status was assigned by MRI in 58% of patients and by PET/MRI in 79% of patients[55].

PET

FDG-PET, in addition to structural information, includes the advantage of assessing the metabolic activity of colorectal patient LNs. However, even with metabolic information, sensitivity is limited. A recent meta-analysis including 13 studies published between 2007 and 2019 evaluated the pretreatment ability of 18F-FDG PET/CT as a staging modality to detect metastatic LNs in CRC[15]. The pooled sensitivity, specificity, positive and negative likelihood ratios were 65%, 75%, 4.57, and 0.37, respectively. Prospective studies have demonstrated higher sensitivity and specificity compared to retrospective studies, and studies with sample sizes greater than 100 and that used a cut off value of

maximum standardized uptake value (SUV) ≤ 2.5 revealed better accuracy. An older meta-analysis of CRC patients found an even lower pooled sensitivity of 42.9% for detecting LN metastasis, but a higher specificity of 87.9%[56,57]. Differences in meta-analysis outcomes are thought to be due to the heterogeneity of baseline patient characteristics and included article methodologies. Regardless of the variances between the two meta-analyses, FDG-PET has limitations in sensitivity[58-61], likely due to a partial volume effect when assessing the SUV of small LNs (< 10 mm), as well as limitations in spatial resolution when differentiating between extension of primary tumor and adjacent positive LNs[62-64]. Specificity on the other hand is limited by false positives seen most often in reactive LNs.

Innovations

Advancements in the imaging evaluation of LNs in CRC are going to happen in the very near future due to innovative scanning technologies such as PET/MRI, which can investigate tumor biology, phenotypes, improve diagnosis, and impact the management of several solid organ malignancies including CRC[55,65-72], innovative radiopharmaceuticals such as fibroblast activation protein inhibitor (FAPI), which is already outperforming FDG in several settings[73,74], and due to the endless possibilities opened by artificial intelligence. Regarding FAPI, a study[74] comparing 68Ga-FAPI and 18F-FDG uptake in 35 patients with gastric, duodenal, and CRCs, showed a significantly higher sensitivity with 68Ga-FAPI PET/CT compared to 18F-FDG PET/CT (79% *vs* 54%) but an equivalent specificity (82% *vs* 89%). Artificial intelligence is going to play a major role in diagnostic imaging evaluation of LNs. A recently published metanalysis, which focused on LN staging in CRC, showed that deep learning and radiomics outperform radiologists, with deep learning also being superior to radiomics. In rectal cancer, on a per patient basis, pooled area under receiver operator characteristic curve was 0.017 for deep learning, 0.808 for radiomics, and 0.727 for radiologists; and sensitivity and specificity were 89% and 94% for deep learning, 78% and 73% for radiomics, and 68% and 70% for radiologists respectively[75].

CONTROVERSIES IN LN DISSECTION IN CRC

Several studies have shown that in more than 80% of cases, the first metastatic LN in CRC is a paracolic LN located 5 cm or less from the tumor[76-81]. Besides this classic lymphatic drainage, aberrant drainage within the regional LNs can exist. Such drainage leads directly to main LN stations near the superior and inferior mesenteric vessels or to colic and paracolic LNs located a significant distance from the tumor. The prevalence of aberrant lymphatic drainage is reportedly up to 20%[82,83]. Drainage of this nature influences the scope of lymphadenectomy since "aberrant" LNs are potential locations for "skip metastases"[76,77,84-86].

In some individual studies, a higher rate of aberrant lymphatic drainage reaching up to 29% has been observed in patients undergoing lymphatic mapping[87]. There are some different points of view on the resection type between East and West. The Japanese concept is partial resection of the bowel according feeding artery (short bowel specimen, long lymph vascular pedicle), and the opposite European concept is wide resection of the bowel such as hemicolectomy or extended hemicolectomy.

European Society of Medical Oncology (ESMO) recommends that local excision could be considered in the early colon cancer (CC) Stage 0 (Tis) and in selected T1N0M0 (G1-2, N0). ESMO and National Comprehensive Cancer Network (NCCN) recommend wide surgical resection with a safe margin (ESMO suggests at least 5 cm from the tumor), and en bloc removal of LNs with the feeding arterial arcade (regional nodes). NCCN suggests removing only suspicious LNs that are not contained in the arcade[25,26,88,89].

The best results in terms of prognosis after the introduction of the TME concept in the treatment of rectal cancer led to the Hohenberger *et al*[90] hypothesis in which surgical dissection according to embryological planes could lead to a similar improvement also in surgery of the. On this hypothesis is based the complete mesocolic excision (CME) associated with the concept of central vascular ligation (CVL) in which the main blood vessels are tied at the origin after a dissection according to the embryological planes (*i.e.* removing the surgical trunk of Gillot in right-sided CC)[90].

JSCCR provides a detailed description of the extent of surgical lymphadenectomy, based on tumor stage. In brief, JSCCR advocates central (D3) lymphadenectomy in selected T2 and in all T3-T4 cancers, as well as in all N-positive patients.

In the study by West *et al*[91] the CME with CVL does not show significant differences with the Japanese D3 as regards the quality of the mesocolon surgical plan and the free margins. In the Japanese school, as indicated in the JSCCR guidelines, the longitudinal extension is less important; consequently the number of lymph nodes and the mesenteric surface were lower. Even if the operative pieces in western countries have a greater longitudinal extension than in the Asian ones, the TNM system does not include in its nomenclature the localization of regional lymph nodes. The indications to D3 Lymphadenectomy with CVL in the Western countries are still a subject of debate.

CONTROVERSIES IN LN DISSECTION IN RECTAL CANCER

Rectal cancer surgery is based on[92-94] TME or tumor-specific mesorectal excision (TSME). TME is a procedure that resects all of the mesorectum just above the anal canal[92]. TSME is a procedure for partially resecting the mesorectum according to the location of the tumor[94]. In Western countries, in the rectal cancer, lateral LNM is generally considered a metastatic disease and neoadjuvant chemoradiotherapy combined with TME is widely used. By contrast, the Japanese Classification of Colorectal Carcinoma[95-98] defines lateral LNs as regional LNs in the internal iliac, obturator and common iliac subregions. The JSCCR Guidelines for the treatment of CRC[28,29] consider mesorectal excision as D1 resection and recommend a D3 procedure (TME with lateral LN dissection) as standard treatment for T3 or more middle and lower located rectal cancer.

Some studies have shown that extensive lymphadenectomy is associated with improved prognosis in patients with more advanced stage CRC, even though numerous postoperative complications related to this extensive surgery are described.

Among colorectal surgeons, it is now accepted that "one size does not fit all", and there is increasing agreement regarding the need for a more targeted surgery and a tailored lymphadenectomy[98-100]. The real challenge is careful patient selection.

HYSTOPATHOLOGICAL DIAGNOSIS OF LNMs IN CRC

A relevant clinical finding is the fact that up to 30% of patients with CRC diagnosed as pN0 following surgery will die within 5 years due to regional recurrence or distant metastases[87,101-104].

A discussion to establish criteria for defining high-risk stage II patients who could benefit from adjuvant therapy was undertaken by the Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer and National Surgical Adjuvant Breast and Bowel Project studies. Presently, the high-risk group, according to ESMO and NCCN treatment standards, comprises patients with T4 tumors (especially T4b), a high grade of histological malignancy, infiltration of vessels and perineural tissue, tumor budding (TB), a small number of removed LNs (< 12), and emergency surgery[101,105,106]. Several studies have identified prognostic genes that may select high-risk patients for adjuvant treatment[107-112], but only a few are routinely used in clinical practice.

Mismatch repair (MMR) genes act in DNA repair pathways. MMR deficiency results from the loss of function of their products (MMR-D), leading to microsatellite instability (MSI). MSI increases CRC risk by increasing tumor mutational burden and the number of tumor-infiltrating lymphocytes (TILs). There are two categories of CRC with MSI: MSI-high (MSI-H) and MSI-low (MSI-L). Instability in more than 30% of the markers as detected by PCR is defined as MSI-H, and alteration in 10%–30% of the markers is considered MSI-L. The MSI-H is associated with a high mutational burden in DNA.

Frameshift mutations can create antigenic epitopes that make MSI-H/MMR-D tumors more immunogenic compared with microsatellite-stable tumors. MSI-induced frameshift mutations produce a significant number of neoantigens. Accordingly, MSI-H/MMR-D tumors manifest a great number of TILs, many of which can be directed against tumor-related neoantigens[107].

Despite this, the most important risk factor is the presence of unidentified LN MMs and macrometastases. Rahbari *et al*[113] concluded that the presence of LN MMs is associated with poor OS and shorter disease-free survival (DFS) in stage II CRC patients. Therefore, the problem is to identify diagnostic methods that can improve selection based on this criterion in terms of both cost and effectiveness[101,114,115]. The relevant literature shows that examination of only one LN slide using HE staining leaves up to 33% of metastases unidentified. A single slide with HE staining through the center of a node 1 cm in diameter provides information on < 1% of its volume[114-118].

Additional HE histopathologic analyses of serial sections allows for the identification of micrometastatic disease in up to 20% of LNs determined to be negative by standard HE methods[119]. However, performing HE histopathologic analyses of sections can be technically challenging and time consuming, as well as entailing significantly greater cost. Other histopathologic methods utilized for more accurate assessment of the status of the regional LNs, such as IHC using antibodies against human cytokeratin (CK) or RT-PCR, require even more time and incur an even higher cost.

IDENTIFICATION OF AN OPTIMAL mRNA MARKER FOR THE OSNA ASSAY IN CRC METASTATIC NODES

Yamamoto *et al*[13] reported the background for the identification of CK19 mRNA as an optimal marker for the OSNA assay in CRC. Yamamoto *et al*[13] examined 98 candidate mRNA genetic markers, which were from a genome-wide database, by comparing an expression frequency in CC. After four sequencing phases, CK19, carcinoembryonic antigen (CEA), and CK20 mRNAs were evaluated using

the OSNA assay. The expression of CK19 mRNA was observed in all pathologically positive LNs; however, CEA and CK20 mRNAs were not found in metastatic nodes.

DIAGNOSTIC PERFORMANCE OF THE OSNA ASSAY IN CRC

A novel technique for pathological examination, OSNA, uses the reverse transcription loop-mediated isothermal amplification method to amplify CK19 mRNA. In contrast to the current routine histopathological examination, it can examine whole LNs and detect metastases in a sufficiently short time (Table 1). A standard curve previously determined with three calibrators containing different CK19 mRNA copy numbers was used to calculate the amount of CK19 mRNA. Positive and negative control samples were used to ensure the quality of the assay.

A limit value of 250 copies/mL of CK19 mRNA copy had been choosed. A value less than 250 copies/mL was considered negative for metastasis, on the contrary, a value ≥ 250 copies/mL was considered positive. Previous studies defined this by the logarithmic midpoint between the maximum value of the CK19 mRNA copy number in non-metastatic patients and minus 2 or 3 standard deviations (SDs) from the average of CK19 mRNA copy number in node-positive patients. These studies also defined the MM threshold between 250 and 4999 CK19 mRNA copies/mL. LNs with 5000 or more mRNA copies/mL were considered macrometastases[7,120,121]. The utility of conventional OSNA as a molecular staging method has been demonstrated for various cancers[9,17,122-124].

Although most of the studies evaluated in this review were prospective in design, none was a randomized controlled trial (Table 1). The studies comparing the diagnostic performance between OSNA and pathological examination for the detection of LNMs in CRC are shown in Tables 2 and 3. Our review on OSNA and CRC shows high sensitivity, few false negatives results, and a concordance rate with pathological findings ranging from 61.8% to 98.7% (Table 2). Moreover, studies have shown that OSNA results in upstaging in about 25% of initially nodal-negative CRC patients after conventional HE analysis (Table 3). With the OSNA approach, the lymph node is homogenized without the need for other preparations and the results are ready in less than 40 min for 3 or 4 LNs, 20 min for a single LN. The stage of the tumor and the number of lymph nodes analyzed correlates with upstaging. Notably, the OSNA upstaging rate in Croner's investigation for stage UICC I and II patients was 16.2% and 30.3%, respectively. Therefore, it was suggested that stage UICC I and II patients, who suffer from recurrent disease, were understaged by conventional HE analyses[9,124-126].

In a study of Yamamoto *et al*[13] OSNA-positive patients (2.0% of stage I CRC and 17.6% of stage II CRC) had more advanced features of CRC, such as deeper invasion to the colonic wall and severe invasion to lymphatic invasion compared with OSNA-negative cases. They found a 95% concordance rate between OSNA and classical histological analyses with HE and IHC. Yamamoto *et al*[13] concluded that OSNA is comparable to a 2-mm interval histopathological examination in its ability to detect LNMs.

In our previous published study[127], OSNA was superior to HE in identifying LNMs, with a false negative rate of 0% *vs* 44.4% and accuracy of 100% *vs* 76.4%, respectively (Table 2). As represented in Tables 2 and 3, few studies evaluated HE and IHC, few performed multi-sliced tissue sections using HE and the remaining single slice HE tissue section *vs* OSNA[11,128-131]. While the detection of small metastatic foci in LNs is influenced by the skill and experience of the pathologists, the advantage of the OSNA assay is the possibility to perform standard evaluations without being influenced by operator skill or experience. This explains the reason why the use of OSNA has recently garnered interest for detecting MMs[9,16,17,128].

Methods of LN division and pooled OSNA

Previous reports have detailed three major methods to compare LN status between pathological examination and the OSNA assay (Table 3). The first method[14,125] involves dividing LNs in half and sending each 50% portion for pathology and OSNA (half-division method). The second method[11,12,15] involves dividing LNs into four equal sections and sending two of these sections (50%) for pathology and OSNA (four-section method). In the third method[14,124], only 1 mm from the center of LNs are sent for pathological examination and the rest are used for OSNA measurement (center-cut method). The latter two methods described above are thought to be technically difficult for evaluating small LNs. By contrast, dividing in half and sending each 50% portion for pathology and OSNA is the simplest method.

In previous studies using classic OSNA (cOSNA), 50% of each LN was submitted for pathologic examination, followed by evaluation of each remaining half by OSNA. The obstacles for clinical applications of cOSNA include a need to simplify the procedure for halving the dissected LNs and reducing the operating costs associated with the equipment used for OSNA analyses.

Rakislova *et al*[125] conducted a study comparing two methods of LN evaluation by OSNA in CRC: an individual analysis of each LN (cOSNA) and a new approach involving pooling several LNs, known as the "pooling method". The diagnostic performance of pooled OSNA (pOSNA) was comparable to that of cOSNA. In the pOSNA method, the LNs are pooled together in a test tube for OSNA analysis. The weight limitation for the LNs per tube was ≤ 600 mg, with those exceeding this limit placed in another

Table 1 Characteristics of one-step nucleic acid amplification studies in colorectal cancer patients

Ref.	Nation	Type of study	Patients number (sample number)	Tumor type	Purpose of the OSNA analysis
Croner *et al*[11], 2010	Germany	Prospective study	184 (184)	Colorectal	Diagnosis of LN metastasis
Yamamoto *et al*[16], 2011	Japan	Prospective multicenter study	85 (385)	Colorectal	Diagnosis of LN metastasis
Güller *et al*[12], 2012	Switzerland	Prospective study	22 (313)	Colon	Diagnosis of LN metastasis
Yamamoto *et al*[13], 2013	Japan	Not shown	30 (66)	Colorectal	Identification of CK19
Croner *et al*[14], 2014	Germany	Prospective multicenter study	103 (1594)	Colon	Pathologically nodenegative CC
Vogelaar *et al*[15], 2014	Switzerland	Prospective multicenter study	128 (325)	Colon	Diagnosis of SLN metastasis
Yamamoto *et al*[17], 2016	Japan	Prospective multicenter study	204 (1925)	Colorectal	Diagnosis of LN metastasis
Aldecoa *et al*[133], 2016	Spain	Prospective multicenter study	149 (1940)	Colorectal	Correlation between TTL and tumor's characteristics
Rakislova *et al*[125], 2017	Spain	Observational study	188 (3206)	Colon	Diagnosis of pooled LN metastasis
Miyake *et al*[193], 2017	Japan	Prospective study	25 (306)	Rectum	Indication of LPLN dissection
Marhic *et al*[184], 2017	France	Prospective study	17	Colon	Diagnosis of SLN metastasis
Colling *et al*[129], 2-17	United Kingdom	Prospective study	19 (82)	Colorectal	Diagnosis of LN metastasis
Aldecoa *et al*[124], 2017	Spain	Prospective study	71 (936)	Colon	OSNA with endoscopic tattooing
Yeung *et al*[130], 2018	United Kingdom	Prospective study	16 (78)	Colorectal	OSNA with ICG detection
Brito *et al*[126], 2018	Portugal	Prospective multicenter study	59 (753)	Colon	Pathologically node negative CRC
Esposito *et al*[127], 2019	Italy	Prospective study	34 (51)	Colorectal	Diagnosis of SLN metastasis
Diaz-Mercedes *et al* [109], 2019	Spain	Prospective study	17 (980)	Colorectal	Budget impact analysis
Itabashi *et al*[18], 2020	Japan	Prospective multicenter study	195	Colorectal	Prognostic value of the OSNA assay for pStage IICRC patients
Archilla *et al*[145], 2021	Spain	Retrospective multicenter study	342 (5931)	Colorectal	Correlation between the TTL with patient outcome
Weixler *et al*[147], 2021	Netherlands, Germany, Switzerland	Retrospective multicenter study	87	Colon	Prognostic value of OSNA
Tani *et al*[33], 2021	Japan	Prospective multicenter study	92	Colon	Diagnosis of pooled LN metastasis
Numata *et al*[188], 2021	Japan	Prospective study	34	Rectum	Indication of LPLN dissection

CRC: Colorectal cancer; ICG: Indocyanine green; LPLN: Lateral pelvic lymph node; OSNA: One-step nucleic acid amplification; SLN: Sentinel lymph node; TTL: Total tumor load.

tube for measurement. In the study by Tani *et al*[33], pOSNA and the half-division method were combined and used in the diagnosis of pericolic LNMs and applied in clinical practice[33]. These results revealed that the pOSNA with the half-division method might be useful as a clinical molecular staging method.

In this study, the upstaging rate for early-stage CC patients was 9.1% (6/66). The upstaging rates of the study by Tani *et al*[33] were slightly lower than those previously reported (Table 3).

Table 2 A comparison of the diagnostic accuracy of the one-step nucleic acid amplification assay in colorectal cancer patients

Ref.	Pathological evaluation	IHC	LN number inspected by OSNA, mean	Sensitivity, %	Specificity, %	Concordance, %	PPV, %	NPV, %
Croner *et al*[11]	Multi-slice	CK19	1.0	92.5	96.5	93.6	88.1	97.9
Yamamoto *et al*[16]	Multi-slice	CK19	4.5	95.2	97.7	97.1	91.9	98.7
Güller *et al*[12]	Multi-slice	CK19	14.2	94.5	97.6	97.1	89.7	98.8
Yamamoto *et al*[17]	Single-slice	None	9.4	86.2	96.5	95.7	66.5	98.8
Colling *et al*[129]	Single-slice	None	4.3	92.9	97.1	96.3	86.7	98.5
Yeung *et al*[130]	Single-slice	None	4.9	100	98.4	98.7	94.1	100
Esposito *et al*[127]	Multi-slice	None	1.5	69.2	100	88.2	100	84.0
Rakislova *et al*[125]	Not shown	None	20.5 (pOSNA)	88.9	79.2	80.2	33.3	98.4
Vogelaar *et al*[15]	Multi-slice	Anti pan-cytokeratin	15.3	51.6	84.1	67.7	76.7	63.1
Miyake *et al*[193]	Single-slice	CEA	11	100	86	88	57	100
Marhic *et al*[184]	Not shown	None	Not shown	50	100	70.6	100	58
Numata *et al*[188] (predictive value for pathological LatLNM testing OSNA-MesLNM)	Not shown	None	17	100	55	61.8	28	100
Tani *et al*[33]	Not shown	CK19	6.9 (pOSNA)	84.6	90.9	89.1%	78.6	93.7

CEA: Carcinoembryonic antigen; CK19: Cytokeratin 19; IHC: Immunohistochemistry; LatLNM: Lateral lymph node metastasis; MesLNM: Mesorectal lymph node metastasis; NPV: Negative predictive value; OSNA: One-step nucleic acid amplification; pOSNA: Pooled one-step nucleic acid amplification; PPV: Positive predictive value.

COMPARISON OF THE NUMBER OF POSITIVE NODES AND QUANTITATIVE OSNA RESULTS

The OSNA assay of retrieved LNs does not allow the number of involved LNs typically used for TNM staging, and therefore cannot be used for conventional cancer staging. Nevertheless, the OSNA assay can potentially be used to infer the size of metastatic foci based on the detected copy numbers[113,132]. Patient's total tumor load (TTL) resulted from the sum of all CK19 mRNA tumor copies/μL of each positive LN from the colectomy specimen. Yamamoto *et al*[17] found that the sum of CK19 mRNA increased as the number of histologically positive LNs increased. Indeed, the median value of CK19 mRNA was significantly smaller in patients with < 3 regional LNMs than in those with ≥ 4 regional LNMs. The median TTL values of pN0, pN1 (1-3 positive LNs), and pN2 (4 or more positive LNs) were 1550 copies/mL (300-320000 copies/mL), 24050 copies/mL (250-890000 copies/mL), and 90600 copies/mL (7700-1635100 copies/mL), respectively. The TTL significantly increased as the node status increased.

In the study of Aldecoa *et al*[133] the TTL was related to pT stage ($P = 0.01$) and tumor size ($P < 0.01$) in low-grade tumors. In that study TTL, correlated with classical high-risk factors in stage I–II CC patients. These findings indicate that the sum of CK19 mRNA assessed by OSNA displays a trend compatible to the current pathological diagnosis system. These findings suggest the future possibility of novel molecular staging using OSNA, based on metastasis volume (amount of CK19 mRNA) rather than the number of LNMs. It has been suggested a correlation between CRC risk factors[11,12,16,18,122,126,134] such as pN, pT, tumor grade, male sex, tumor size, lymph vascular invasion (LVI), poor prognosis, worse DFS and TTL.

Correlation of node TTL with TB and poorly differentiated clusters

The physiological process that can lead epithelial cells to acquire mesenchymal properties and the potential for migration and stromal invasion, essential for the development of metastases, is the morphological manifestation of the epithelial-mesenchymal transition phenotype that can lead to the formation of TB and clusters poorly differentiated (PDCs). The presence of isolated tumor cells or cell clusters of ≤ 4 cells on the invasive front of the tumor is termed tuberculosis[134]. 5 or more neoplastic cells in the tumor stroma not organized into glandular structures constitute the PDCs. In stage II CC, both PDCs and TB are independent prognostic factors[134-136], associated with LNMs, distant

Table 3 Differences in lymph node processing methods and upstage rates of previous reports

Ref.	Subject (patients)	OSNA method	Harvested LN, *n*	Harvested LN, median	Dividing method of LN	Pathological staining	Measured LN by OSNA, *n*	Measured LN by OSNA, median	Upstage rate (pStage I and II)
Yamamoto *et al*[16]	Stage 0, I (85)	cOSNA	434	N/A[a]	Four[b]; 4 mm over diameter of LN	HE and IHC	385	4.5	16.5% (2/16)
Güller *et al* [12]	Stage I, II, III (22)	cOSNA	313	30 (16–60)	Four[b]; 3 mm over diameter of LN	HE and IHC	56	13 (6–24)	15.3% (2/13)
Croner *et al* [14]	Stage I, II (103)	cOSNA	N/A[a]	N/A[a]	Center[c]; 6 mm over half[d]; 4 mm to 6 mm diameter of LN	HE	1594	14 (1–46)	25.2% (26/103)
Vogelaar *et al*[15]	Stage I, II (128)	cOSNA	N/A[a]	N/A[a]	Four[b]; 10 mm over half[d]; 10 or less than 10 mm diameter of LN	HE and IHC	317	Mean 15.3 (4–40)	20.2% (20/90)
Yamamoto *et al*[17]	Stage I, II, III (204)	cOSNA	4324	19 (3–25)	Half[d]	HE	1925	8 (2–25)	17.6% (13/74)
Aldecoa *et al*[133]	Stage I, II (149)	cOSNA	2483	15	Center[c]	HE	1940	12	51% (76/149)
Rakislova *et al*[125]	Stage I, II, III (188)	cOSNA, pOSNA	cOSNA 1828, pOSNA 1992	cOSNA 17 (13-22), pOSNA 20.5 (17-27)	Center[c]	HE	cOSNA 1757, pOSNA 1449	cOSNA 13 (10-18), pOSNA 18 (13-25)	cOSNA 55.4% (51/92), pOSNA 20.7% (16/77)
Brito *et al* [126]	Stage I, II (59)	cOSNA	1046	13 (9-19)	Center[c]; 5 mm over half[d]; 4 or less diameter of LN	HE	753	12 (7-16)	28.8% (17/59)
Itabashi *et al*[18]	Stage I, II, III (195)	cOSNA	Not shown	19 (1-75)	Half[d]; 4 mm over diameter of LN	HE	Not shown	8 (2-25)	15.7% (11/70) in stage II patients
Tani *et al* [33]	Stage II, IIIA (92)	pOSNA	2236	24.3 (5–66)	Half[d]; 4 mm over diameter of LN	HE	636	6.9 (1–35)	9.1% (6/66)

[a]Not applicable.
[b]Four section method: dividing lymph nodes into four equal sections and sending two of these sections (50%) for pathology and one-step nucleic acid amplification (OSNA) measurement.
[c]Center-cut method: 1 mm from the center of lymph nodes are sent for pathological examination and the rest are used for OSNA.
[d]Half-division method divides lymph nodes in half and sends each 50% portion for pathology and OSNA.
HE: Hematoxylin and eosin; IHC: Immunohistochemistry; LN: Lymph node; OSNA: One-step nucleic acid amplification; pOSNA: Pooled one-step nucleic acid amplification.

metastases, extramural vascular invasion, LVI, perineural invasion (PNI), tumor grade and high pT stage[136-142].

International Consortium on TB Recommendations indicates how to count the number of TB at the invasive front of the tumor[135]. The classification for TB was as follows: Bd1/low (≤ 4 buds), Bd2/intermediate (5–9 buds), and Bd3/high (10 one more buds). The classification for PDCs evaluated [143] at the invasive front or in the center of the tumor was as follows: G1 (≤ 4 clusters), G2 (5–9 clusters), and G3 (10 or more clusters). Barresi *et al*[144] suggested not classifying tumor cells within mucin pools in mucinous carcinomas as TB, considering only tumor cells infiltrating the stroma with minimal extracellular mucin. While, the PDCs were evaluated within mucin lakes.

Recently Archilla *et al*[145] suggested the correlation of the TTL with patient outcome, TB, and PDC. The use of molecular methods to assess LN status, together with other pathological risk factors, could help improve risk stratification and management of patients with early-stage CRC.

Indeed, OSNA positivity was found in 38.3% of the cases (131/342) with a mean TTL of 36662 copies/mL among positive cases. The TTL present in the LNs evaluated by the OSNA test correlated positively, with both PDC (r = 0.266 by IHC; r = 0.257 by HE) and TB (r = 0.249 by IHC; r = 0.243 by HE) (P = 0.001). Low and intermediate TB had similar mean TTL (Bd1: 3292 copies/mL and Bd2: 18002 copies/mL), with no significant differences between both groups (*P* = 0.154). The mean TTL of high-Bd3

TB was 45331 copies/mL, and. it was significantly different from Bd1 and Bd2. Likewise, the mean TTL of PDC G1, with 4962 copies/mL and G2, with 13146 copies/mL did not show significant differences (P = 0.068), while PDC G3 had 61108 copies/mL, significantly different than low and intermediate grades. Thus, the authors grouped low and intermediate grades of TB and PDC into one category, obtaining two groups with significant differences for both TB and PDC (P < 0.001) as well. The authors also concluded that TTL can be used as an alternative method to to better stage patients compared to the classic HE because it is able to identify real stage II or III patients, thereby selecting those who are candidates for adjuvant therapy[145].

SURVIVAL ANALYSES

In the meta-analysis of Wild *et al*[146] it is emphasized that long-term outcomes and the use of adjuvant therapy in those upstaged by OSNA should be clarified before routine use of OSNA test.

Itabashi *et al*[18] showed that pStage II patients with OSNA positive LN had a lower 3-year DFS than negative patients (55% *vs* 86%; P = 0.005), with no significant differences in 3-year OS (P = 0.914). In this study, the upstaging occurred for patients with pStage II, of whom 11 of 70 patients (15.7%) were OSNA-positive. Most of the OSNA positive LNs were located in the peri-colic or peri-rectal area (10 out of 11 OSNA-positive stage II CRC patients).

Weixler *et al*[147] showed that the detection of positive LN by HE staining but not by OSNA as significant predictors of cancer-specific survival, cancer-specific and recurrence-free survival, and DFS. He concluded that in patients with CC, OSNA offers no prognostic advantage compared to conventional LN staging with HE contrasting findings in other cancers. It is important to highlight that the methodology of the histopathological evaluation for detection of the LNMs differed among studies. In Weixler's multicenter study[147] all harvested LNs > 3 mm in greatest dimension or a short axis ≥ 10 mm was cut into four slices: two were stored for later OSNA analysis and two were allocated to conventional standard HE staining, multilevel HE staining, and IHC for CK19. Multilevel sectioning with IHC leads to relevant upstaging of 15.4%-26% of otherwise negatively classified patients[148,149]. In addition, stage I-III patients were included in this study, whereas most of the OSNA studies focused on stage I-II patients. Therefore this HE + IHC *vs* OSNA study including stage I-III patients, although well conducted and of great value and interest, is not amenable to a comparison with studies in which HE *vs* OSNA in stage I-II patients are evaluated.

ORGAN-PRESERVING SURGERY

CC

Early CRC can be treated by endoscopic mucosal resection or endoscopic submucosal dissection (ESD). In determining the indication for endoscopic treatment and the treatment method, information on the depth of invasion and morphology of the tumor is essential. Colorectal ESD is an "endoscopic resection technique, which enables en bloc resection of a tumor, regardless of size" and avoids piecemeal resection. It is of great importance to differentiate Tis and T1a cancers from T1b cancers (T1 cancer with ≥ 1000 μm submucosal invasion depth), as the former can be treated by endoscopy while the latter requires surgical operation with nodal status assessment[29,150-154]. Moreover, innovative organ-preserving procedures such as endoscopic full-thickness resection or transanal minimally invasive surgery have been proposed to perform high-quality resections with decreased incidence of specimen fragmentation without resorting to demolitive interventions[155]. Nevertheless, it is estimated that overall 10%–20% of patients in stage T1 will have LNMs, and such patients subjected to a localized resection are undertreated[151].

Laparoscopic endoscopic cooperative surgery (LECS) can also lead to full thickness local resection by means of combined use of laparoscope and endoscope. The development of modified LECS procedures, such as non-exposed endoscopic wall-inversion surgery and closed LECS has almost resolved these drawbacks. This has led to a recent increase in the indication of modified LECS to include patients with gastric epithelial neoplasms. The LECS concept is also beginning to be applied in other organs such as the duodenum, colon and rectum. Further evolution of LECS procedures is expected in the future. SLN mapping could also be combined with LECS, resulting in a portion of early gastrointestinal cancers being treated by LECS with SLN mapping[156].

Rectal cancer

TEM, first described by Gerhard Buess[157-161], due to its ability to perform high-quality resections with decreased incidence of fragmentation, is superior to standard transanal excision for treating benign and malignant rectal lesions, most notably[162,163].

Transanal minimally invasive surgery (TAMIS) was initially born as a fusion between trans anal endoscopic microsurgery (TEM) and single-site laparoscopy. This technique was designed as a cost-

effective and easily reproducible alternative to TEM without specialized equipment.

The indications for TAMIS are similar to standard transanal resection for benign and malignant lesions determined by EUS or MRI[164,165]. TAMIS is also indicated for early malignant neoplasms confined to the submucosa[166]. T1 neoplasms of the rectum can still be divided into low-risk lesions [167] and at high risk for poor histopathological features (TB, poor differentiation, LVI or perineural invasion). Studies[155,168-170] identified a higher risk of LN metastasis in T1 sessile tumors with deeper submucosal invasion (sm^2 or sm^3).

THE OSNA ASSAY FOR THE DETECTION OF CRC METASTASIS IN SLN

LN status plays a crucial role in oncologic therapeutic strategies, and despite the use of increasingly sophisticated imaging techniques, pre-operative metastatic LN identification in patients with CRC is unsatisfactory[171,172].

The study of the SLN is gaining more and more popularity because it can avoid extensive lymphadenectomies, reduce operating times and morbidity. This can change the surgical strategy in patients with an apparent early stage of CRC, as patients with intraoperative positive SLN are submitted, during the same surgical procedure, to an adequate lymphadenectomy, whereas those with negative SLN can be treated with an organ-preserving surgery, avoiding unnecessary lymphadenectomy. The extemporaneous intraoperative examination performed on frozen specimen has a lower sensitivity than the classic postoperative analysis. The problem is mainly due to the low detection rate of MMs and ITC[173,174]. The disease detection rate increased with the technique of multi-step formalin-fixed tissue sections (FFTS) stained by HE with or without IHC[6,175-179]. Nonetheless, a significant number of MMs can still be overlooked, as during histological workup usually only small parts of LNs are screened.

Therefore, there are two methods for SLN mapping: staining and radioguided[180]. Three tracers have been used to detect SLNs: Dye, radioisotope, and indocyanine green (ICG). Each tracer has its respective disadvantages. The use of the ICG fluorescence method has officially been approved in Japan for LNs of BC and malignant melanoma; thus, it appears that ICG can be an acceptable tracer for the detection of LNs in gastric and CC.

ICG tattooing method is very useful for the marking of early gastric and CCs, especially when using a laparoscopic approach[181]. It has been suggested that SLN mapping with fluorescent dye can play an important role in the treatment of CC, particularly those at early stages, and can lead to ultraconservative surgery[182]. Because the results of OSNA are available in a relatively short time compared to the conventional technique, intraoperative OSNA analysis of SLNs may be employed easily in clinical practice.

In the study by Vogelaar *et al*[15] OSNA proved to be a promising method for the detection of SLN metastases in CC patients after *ex vivo* SLN mapping. OSNA appeared to outperform routine pathological examination with HE-stained slides with an upstaging rate of 20.2%. In the study by Yeung *et al*[130] OSNA was used intraoperatively, together with the technique of retrieving colorectal LNs by fluorescence imaging, to analyze the status of these specific LNs. In this study, OSNA is highly concordant with standard histology.

The results of the meta-analysis by Tranoulis *et al*[183] indicate that the the use of OSNA can allow to identify the status of the LNs even when applied intraoperatively. Marhic *et al*[184] proposed that the OSNA technique may be a new method to reduce time to adjuvant chemotherapy after surgery for CC. In this study, SLN status was determined intraoperatively with the OSNA assay; when positive, a port-a-cath was placed during the procedure for upcoming adjuvant chemotherapy. In this study, there was no difference between the groups regarding cancer staging, duration of hospitalization, and major morbidities but the time interval between surgery and adjuvant chemotherapy was significantly shorter in the OSNA group at 35 ± 8 d *vs* 67 ± 36 d ($P = 0.021$).

In our previous study[127], we showed that SLN analysis with OSNA in combination with ICG-near infrared (NIR) lymphangiography is feasible and may allow intraoperative prediction of LN status in patients with CRC (Table 4). Patients with SLN positive by the OSNA method were considered pN-positive and subjected to adjuvant chemotherapy. The time to start chemotherapy was lower in OSNA (+) patients [39.1 ± 1.9 d *vs* 50.2 ± 4.1 d in the OSNA (−) group; $P = 0.01$].

Both *ex vivo* and *in vivo* ICG fluorescence imaging are feasible for the detection of SLNs in CRC. The submucosal injection technique and subserosal were both used. A NIR 30° laparoscope (Olympus, Tokyo, Japan) was used to inspect the mesocolon. The LNs found using fluorescence were considered SLNs and analyzed intraoperatively. More work needs to be done to define protocols, indications for its use, a standard number of LNs that need to be removed and to test its efficacy in larger patient populations.

Implementation SLN analysis with OSNA in combination with ICG-NIR lymphangiography could allow more precise staging, reducing the delay between surgery and the onset of adjuvant chemotherapy. SLN evaluation by intraoperative OSNA analysis combined with a LECS approach may allow, in case of OSNA-negative early CRC, to apply an organ-preserving surgery avoiding the complic-

Table 4 Studies analyzing colorectal cancer metastasis in sentinel lymph nodes with one-step nucleic acid amplification

Author	Patients (samples), *n*	Injected dye	Intraoperative OSNA assay	Examined SLNs, *n*
Vogelaar *et al*[15]	128 (325)	Patent blue dye V or indocyanine green	No	3.0 (median)
Marhic *et al*[184]	17	Blue dye	Yes	Not shown
Yeung *et al*[130]	16 (78)	Indocyanine green	No	4.9 (mean)
Esposito *et al*[127]	34 (51)	Indocyanine green	Yes	1.0 (median)

OSNA: One-step nucleic acid amplification; SLN: Sentinel lymph node.

ations related to an unnecessary lymphadenectomy.

By contrast, in intraoperative OSNA-positive early CRC, a colorectal resection associated with selective lymphadenectomy can be performed, also taking into consideration the presence of aberrant lymphatic drainage and skip metastases. Despite the attractiveness of the previously exposed concept, studies in this field are lacking or very few (Table 4). However, several studies underline the role of OSNA SLN evaluation in more advanced stages and in case of positivity, the consequent upstaging and access to adjuvant treatment (Table 4).

LATERAL PELVIC LNs AND OSNA

There is disagreement in the international literature regarding the use of prophylactic lymphadenectomy in comparison with preoperative radiochemotherapy to improve prognosis in patients with locally advanced rectal cancer, due in part to the complex anatomy of the pelvic floor which makes diagnosis of lateral pelvic LNs (LPLNs) metastasis difficult. In Japan, the evolution in the surgical oncology approach has been toward LN clearance and, as a result, LPLNs have been considered local-regional disease from the outset[185].

Nevertheless, the rate of pathological lateral (Lat) LNM (p-LatLNM) in patients without clinical LatLNM (c-LatLNM) remained low at 7%, and lateral LN dissection (LatLND) is generally considered technically demanding and can prolong the operative time[186-188]. As Sammour *et al*[189] proposed, patients who are candidates for curative-intent treatment should be stratified depending on their risk to have LPLN metastasis in high, moderate, and low risk in order to select the best option to manage the pelvic compartment. Nevertheless, with preoperative images or traditional criteria, it is difficult to predict LatLNM. Obtaining a preoperative or intraoperative diagnosis is essential to select patients with LatLNM.

If SLN navigation surgery could be applied in cases of middle and lower rectal cancer, unnecessary LatLND procedures could be avoided. SLN anlysis may be useful in deciding both the indication of LatLND and which side of the lateral pelvic wall should be dissected[190]. In the study of Noura *et al*[191] the existence of a lateral pelvic region SLN in 53 lower rectal cancer patients was investigated. The lateral pelvic region was observed using a NIR camera system (photodynamic eye) after the ICG has been injected into the submucosa along the dentate line. If SLNs were positive for metastasis a Bilateral LatLND was performed, if instead SLNs were negative for metastasis mesorectal excision only was performed. In 49 (92.5%) of the 53 patients the lateral SLNs were successfully identified, 4 of these patients (8.2%) had lymph node metastases; the mean number of lateral SLNs per patient was 2.0 (range, 1–4).

The results of Yasui *et al*[192] suggested the potential use of SLN with ICG strategy to identify cases with non-metastatic LPLN, and to omit LatLND in such cases, and thereby avoid both LatLND-related surgical complications and radiation-induced adverse events. Moreover, the author suggested that further studies are needed to shorten the required time and improve the accuracy of SLN biopsy by the intraoperative rapid diagnosis with different methods such as molecular biological diagnosis.

Miyake *et al*[193] attempted to perform an intraoperative OSNA assay to detect perirectal LNMs to predict LPLN metastasis in rectal cancer patients undergoing surgical resection plus LatLND. In their study, LPLN metastases were present in 16% of patients (4/25), and all of these patients were positive on an OSNA for perirectal LNMs. The sensitivity of OSNA was 100%, specificity 86%, positive predictive value 57%, and negative predictive value 100% for predicting LPLN metastasis, and the authors concluded that the OSNA of perirectal LNs might be useful for selecting candidates for omission of LatLND in rectal cancer surgery. OSNA can be associated with SLN to intraoperatively identify foci of metastasis in LPLNs.

With respect to risk factors for p-LatLNM, three previous studies reported that pathological mesorectal LN (MesLN) metastasis (p-MesLNM) is a consistent risk factor for p-LatLNM[194-196]. Furthermore, previous studies have shown that p-LatLNM rarely occurs without p-MesLNM[16,197].

Negative OSNA diagnosis for mesorectal LNM (MesLNM) (OSNA-MesLNM) is highly correlated with negative p-LatLNM; hence, negative confirmation of OSNA-MesLNM may be useful in selecting patients in whom LatLND can be omitted[189].

In conclusion, the role of LatLND is still under discussion. Nevertheless, it has been suggested a selective and mono- or bilateral LatLND in advanced low and middle rectal cancer, based on OSNA positive mesorectal nodes or OSNA-positive ICG-stained sentinel LPLNs.

COSTS

A disadvantage of cOSNA is that it is more costly than pathologic examination. Depending on the number of samples analyzed in each lot, there is a variation in the cost of consumables and additional reagents. For example, a 12 LN analysis would have an indicative cost of single use products is £ 550–£ 590 per patient [excluding value added tax (VAT)]. If consumables are maximized and samples from more than 1 patient are tested in one batch, the cost could be as high as £ 33.50 per LN. The annual maintenance contract for the system (which would apply from the 2nd year after installation) is priced at £ 6628.48 (excluding VAT) with a 12 mo warranty.

The lifespan of the OSNA system declared by the manufacturer is at least 6 years. The duration of the analysis is approximately 90 min for 12 LNs, therefore it is possible to analyze samples of approximately 5 patients per day (7.5 h), and 1200 in 1 year (considering 240 annual working days). Average cost per patient (including capital, maintenance and disposable costs) ranges from £ 568 to £ 608, using the standard annuity method with a 3.5% discount rate[198]. Nonetheless, the OSNA use may reduce the reinterventions and allow earlier commencement of adjuvant treatment. The financial implications of OSNA have been previously investigated in BC, with an estimated saving between 400 and 700 £ per patient[199,200].

With pOSNA, only one measurement is required, at a cost of $ 225 (¥ 24000). In contrast, cOSNA requires three or more measurements, at a cost of $ 670 (¥ 72000) or more. In pOSNA, multiple LNs can be measured in one tube, taking into consideration the upper limit of the LN weight that can be assayed in one tube by the OSNA method is 600 mg. However, in cOSNA, only one LN can be measured in a single tube (max 50 mg). Using cOSNA to measure 12 or more halved LNs would require at least 12 tubes and three or more measurements per tube, whereas if 12 pericolic LNs are measured, pOSNA would require only one or two tubes for each case. The OSNA measuring device can measure up to four tubes at once; however, because pOSNA requires only one or two measurements per case, this allows the device to take measurements for two cases simultaneously depending on the number and weight of the removed LNs.

Diaz-Mercedes *et al*[109] analyzed the budget impact of introducing an OSNA assay in early-stage CRC patients and suggested that OSNA might have not only an economic benefit but also a clinical benefit in CRC patients, since it enabled more accurate staging, thereby avoiding unnecessary treatment. The results of Diaz-Mercedes *et al*[109] indicate that the Spanish National Health System would have saved over € 19 million from 2017 to 2019 if OSNA had been introduced in clinical practice for surgically treated CRC patients. In this study, HE-positive patients and OSNA-positive patients, both underwent adjuvant therapy. Savings are explained by the fact that OSNA ensures a more accurate diagnosis in CRC patients, allowing a reduction in treatment costs after initial surgery, as well as costs of adjuvant treatments and surgery after recurrence, compared with HE techniques. Although patients' LN staging is more expensive with OSNA than with HE, savings regarding treatment costs after surgery and treatment costs due to recurrence are high enough to justify the investment in OSNA analysis.

Although the costs of OSNA are high, the speed, simplicity, and reproducibility could allow a reduction in the hours of work of individual pathologists. Furthermore, two cases can be studied during a single procedure using the pOSNA method. Adding, as demonstrated by Diaz-Mercedes *et al*[109], the reduction in treatment costs after surgery and the reduction in costs relating to the treatment of recurrences, this method could be also attractive for developing countries.

ADVANTAGES AND DISADVANTAGES

Advantages

The innovative aspects of OSNA are that unlike standard histopathology, OSNA can analyze the whole LN as well as partial LNs. This may improve cancer staging accuracy. It can detect metastatic foci regardless of their size or location. It is seemingly superior to conventional FFTS in detecting MMs and ITC, as it can identify metastatic foci as small as 0.35 mm[201]. Yamamoto *et al*[17] found that the sum of CK19 mRNA increased as the number of histologically positive LNs increased. Indeed, the median value of CK19 mRNA was significantly smaller in patients with < 3 regional LNMs than in those with ≥ 4 regional LNMs. In the study of Aldecoa *et al*[133], the TTL was related to pT stage (P = 0.01) and tumor size (P < 0.01) in low-grade tumors. In this study, TTL correlates with classical high-risk factors in stage

I–II CC patients. These findings indicate that the sum of CK19 mRNA assessed by OSNA displays a trend compatible to the current pathological diagnosis system. These findings suggest the future possibility of novel molecular staging using OSNA, based on metastasis volume (amount of CK19 mRNA) rather than the number of LNMs.

Archilla *et al*[145] have suggested the correlation of TTL with TB and PDCs. TTL could be used as a new prognostic factor in CRC as it is related to the outcome and. The combination of the TTL as a new prognostic factor, TB and PDC, could help to better stratify and manage patients with early-stage CRC at risk of recurrence.

The application of OSNA in addition to the current standard pathology would likely provide an additional risk factor for disease recurrence regarding patients with stage II CRC. LNM is a reliable prognostic marker of CRC; it is used as the ''gold standard'' for post adjuvant chemotherapy after curative surgery. One may question whether OSNA-positive CRC patients should receive post-adjuvant chemotherapy after curative surgery. Further clinical trials are needed to determine if adjuvant therapy is beneficial in this upstaged group. On the other hand, the short turnaround time renders OSNA an attractive intra-operative method. Based upon the BC accumulated experience, the turnaround time is less than 40 min for one LN, whereas it ranges from 50 to 62 min for assessing four LNs[7]. The OSNA rapid turnover time may potentially be useful in circumventing the major issues associated with SLN biopsy. Moreover, OSNA is automated and the results are quantifiable; hence, easily reproducible, less operator-dependent with short learning curve[201-203]. Therefore, implementation of OSNA in routine clinical practice may ease the burden on pathologists.

Finally, it is laborious and rather expensive to perform molecular tests. Nonetheless, the OSNA use may reduce the re-interventions and could allow earlier commencement of adjuvant treatment. The financial implications of OSNA have been previously investigated in BC[199,200]. The results showed that pOSNA can simplify the process of cutting harvested LNs in half while reducing the equipment-related costs associated with OSNA assays used in clinical practice. Additionally, pOSNA demonstrated an upstaging rate for pNNCC equivalent to that reported in previous studies, suggesting its feasibility for molecular staging in clinical practice. With pOSNA, the possibility of fewer measurements per patient and of studying more cases simultaneously with the same panel further reduces costs[33,125]. In an era of stringent economics, health systems should undergo cost-effectiveness analyses upon which a progressive integration of OSNA in their daily clinical practice could be based on[109,200].

Limitations

The few OSNA limitations should be acknowledged. OSNA can be used to analyze LNs more than 50 mg; if the LN diameter is inferior to 3-4 mm, it cannot be divided and analyzed with OSNA and conventional histology (Table 3). The examination of the whole LN by OSNA inevitably precludes FFTS examination. One limitation of all OSNA studies performed in CRC, is that the analysis has been performed using only a part of the LNs, while using the rest of the LN tissue for conventional histological analysis and pN staging[145].

The technique of nodal division must be taken into account when evaluating the results because it can vary widely between different studies. Discordant results between OSNA and FFTS were reported. The three main reasons for these discrepancies are: tissue allocation bias, low CK19 expression, and tissue contamination. Moreover, discordances may also arise from the presence of metastases from primary tumors that do not express CK19[147]. The latter is considered an important limitation of OSNA. The accuracy of OSNA is seemingly higher amongst CK19 positive primary tumors by IHC compared to those with CK19-negative primary tumors[183]. As such, the positive CK19 IHC in primary tumors has been proposed as a prerequisite for the OSNA use by some authors[183]. An additional challenge is the fact that 36% and 49% of CK19-negative primary tumors have CK19-positive LNs[183]. Peigné *et al*[203] also reported that CK19 mRNA can also be detected by OSNA even in cases with CK19-negative primary lesion.

The tendency toward a loss of CK19 expression in poorly differentiated cancers may represent a challenge for assays using CK19 IHC or PCR for detecting MMs. It is of note that upregulation of CK19 in tumors derived from cells that are CK19-negative can also be linked to unfavorable tumor features. CK19 is highly expressed in positive LNs from BC patients even when its expression is not observed in primary tumors. Targeted studies on the upregulation of CK19 mRNA in LNM of CRC are needed[13].

In light of the potential for false-negative results, the incorporation of additional markers would be a possible direction in improving the diagnostic performance of OSNA. The cut-off point of 250 copies/mL is established in BC and seemingly sensitive in CRC while the optimal diagnostic cut-off point is a matter of debate[184]. The aforementioned pitfalls remain to date a field of contention necessitating a shift in the focus of future research into incorporation of novel biomarkers and evaluation of the optimal diagnostic cut-off point.

At this time there is no exact definition of true LN positives or negatives, cancer-related relapse and death were used as real positive LN indicators, disease-free survival as negative real LN[147]. The false-negative rate of pOSNA is a point to be considered when applying OSNA in clinical practice (Table 2). However, Aldecoa *et al*[133] observed that high-grade (G3) tumors or tumors with vascular invasion presented lower levels of TTL making it not a reliable prognostic tool for these specific pathologic features.

Finally, in this review, we found few reports dealing with CRC and SLN evaluation and there is obviously the need for future research in this field.

CONCLUSION

OSNA analysis is potentially more accurate than conventional pathologic methods for identifying metastasis because it solubilizes the entire LN and analyzes CK19 mRNA levels in the resulting sample. The advantages of OSNA include a short analysis time of approximately 30–40 min from start to completion, and the ability to automate the OSNA assay eliminates interlaboratory differences based on pathologist skill and experience. The short turnaround time renders OSNA an attractive intra-operative method. Patients with pN0 OSNA-positive CRC might also need chemotherapy after curative surgery. To achieve this goal, it needs several studies to compare the recurrence rate between the groups of no treatment or adjuvant chemotherapy after surgery both in OSNA-positive pStage II CRC patients. The result would clarify whether adjuvant chemotherapy is beneficial to patients with OSNA-positive pStage II CRC. Anyway, it can be suggested that OSNA may be considered as the route to tailormade surgery.

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FOOTNOTES

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MINIREVIEWS

Current perspectives on the role of liver transplantation for Langerhans cell histiocytosis: A narrative review

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Abstract

Langerhans cell histiocytosis (LCH) is a malignant disease of the histiocytes involving various organ systems. The spectrum of liver involvement in LCH ranges from mild transaminitis to end-stage liver disease. The hallmark of hepatic LCH is secondary sclerosing cholangitis, which manifests due to a progressive destruction of the biliary tree by malignant histiocytes. Chemotherapy remains the mainstay of treatment for active LCH. Early recognition, diagnosis and a systematic approach to the management of LCH can ameliorate the disease process. Nonetheless, the liver involvement in these patients may progress despite the LCH being in remission. Liver transplantation (LT) remains central in the management of such patients. Various facets of the management of LCH, especially those with liver involvement remain unclear. Furthermore, aspects of LT in LCH with regards to the indication, timing and post-LT management, including immunosuppression and adjuvant therapy, remain undefined. This review summarises the current evidence and discusses the practical aspects of the role of LT in the management of LCH.

Key Words: Langerhans cell histiocytosis; Liver transplantation; Outcomes; Management; Chemotherapy; Ethics

Core Tip: Involvement of the liver in Langerhans cell histiocytosis (LCH) is considered a high-risk disease and the management algorithm needs to factor in the malignancy and the severity of liver disease. Liver transplantation (LT) is usually offered to LCH patients in remission with decompensated liver disease. However, given the paucity of currently available literature, the role of LT in LCH remains undefined. There is hence a need for large collaborative, multicentre studies to provide recommendations on the management algorithm for LCH.

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INTRODUCTION

Langerhans cell histiocytosis (LCH) is a malignant disease of the histiocytes involving various organ systems including the liver, spleen, skin, bone, lymph nodes, lung, and the gastrointestinal tract[1]. Involvement of the liver in LCH occurs in many ways. The spectrum ranges from mild transaminitis to end-stage liver disease (ESLD). The hallmark of hepatic LCH is secondary sclerosing cholangitis (SSC), which manifests due to a progressive destruction of the biliary tree by malignant histiocytes[2]. Chemotherapy remains the mainstay of treatment for active LCH[3]. However, conventionally used vinblastine-based chemotherapy may not be tolerated by patients with concomitant liver involvement and modified regimens which balance the risk of liver decompensation with the oncological efficacy need to be instituted[3,4].

By the time the bile duct injury presents clinically, the primary disease is usually burnt out and the characteristic histiocytes are absent. ESLD and portal hypertension develop as a sequalae of SSC[5]. These patients are offered liver transplantation (LT) with an intent to cure the liver damage[6,7]. Various facets of the management of LCH, especially those with a liver involvement remain unclear. Furthermore, aspects of LT in LCH with regards to the indication, timing and post-LT management, including immunosuppression and adjuvant therapy, remain undefined. This review summaries the current evidence and discuss the practical aspects of the role of LT in the management of LCH.

LIVER INVOLVEMENT IN LCH

Liver is involved in 20%-60% of patients with LCH and is more common when there is multiorgan involvement[2]. LCH-related liver disease is conventionally divided into two stages: Early infiltrative and late sclerosis[8]. The early stage is characterised by reactions elicited by the malignant histiocytes and other inflammatory cells, manifesting as periportal infiltration and hepatic nodular lesions. In the late fibrous stage, progressive destruction of biliary tree occurs resulting in cirrhosis and portal hypertension[8,9].

Hepatic involvement as defined by the European Consortium for Histiocytosis includes a palpable liver 3 cm below the coastal margin confirmed by ultrasound and liver dysfunction as defined by hyperbilirubinemia (at least 3 times the upper limit of normal) hypoalbuminemia (< 30 g/dL) , alanine transaminase (ALT) and/or aspartate transaminase (AST) (more than 3 times the upper limit of normal), -glutamyl transpeptidase (GGT) over twice the upper limit of normal, ascites, oedema, or intrahepatic nodular mass[3]. Sclerosing cholangitis in LCH is defined as involvement of extrahepatic/intrahepatic biliary tree with strictures, dilatation, pruning detected on imaging (computed tomography or magnetic resonance imaging) and/or on liver biopsy with or without elevated GGT[10,11]. The characteristic features of bile duct injury can be detected by magnetic resonance cholangiopancreatography. It is noteworthy that despite resolution of active malignancy, the biliary injury continues to progress. The major site of LCH involvement is the large bile duct, and hence sites of active disease may be missed on liver biopsy[8].

With a mortality risk of over three times that of patients without liver involvement, hepatic LCH is considered a high-risk disease. In a study of patients with LCH, liver involvement drastically reduced survival from 97% to 52%[12]. SSC may be observed in up to 20%, which invariably progress to ESLD requiring LT[13]. It is noteworthy that an acute hepatic involvement by active LCH may be reversible with timely instituted chemotherapy[12]. Later stages and/or burnt out disease on the other hand may continue to have worsening liver injury. Furthermore, this progression of SSC is more rapid than what is observed in primary sclerosing cholangitis[14]. In a series of adults with LCH, nearly 30% died due to complications of SSC and ESLD[14].

LIVER PATHOLOGY IN LCH

LCH exhibit diverse morphological features in the liver, which vary depending on the phase, stage and activity of the disease. Aggregates of LCs having lobulated, coffee-bean-shaped or contorted nuclei with a fine chromatin pattern and abundant eosinophilic cytoplasm admixed with eosinophils, polymorphs, lymphocytes, plasma cells, non-Langerhans histiocytes and rare multinucleated giant cells forming masses of variable sizes have been described (Figure 1A). Most cases with hepatic infiltration show marked tropism of the bile ducts. Active infiltrations of the bile ducts cause mucosal injury and fibrosis that lead to a progressive pattern of SSC, ultimately resulting in biliary cirrhosis[11,15,16]. There is destruction of the walls of bile ducts which may lead to cystic dilatation with intraluminal biliary sludge and rupture (Figure 1B). LCs can be identified within the basement membrane of the bile ducts, displacing the epithelial cells off the wall. Many cases demonstrate concentric periductal fibrosis. Cholangiopathy causes periportal bile ductular proliferation (Figure 1C), duct loss (Figure 1D), and features of chronic cholestasis with periportal cholestasis and stainable granules of copper and copper-binding protein. It is important to note that, despite evidence of biliary damage, the characteristic LCs may not be detected on liver biopsy. The diagnosis then rests on the presence of a concomitant infiltrate in other sites. Reassuringly, examination of liver explants in patients with LCH-induced biliary cirrhosis usually shows no evidence of active disease[17].

CHEMOTHERAPEUTIC AGENTS IN LCH

Liver, bone marrow and spleen are defined as the high-risk organs and their involvement makes the prognosis worse in patients with LCH[3]. The standard chemotherapy regimen for high-risk LCH as per the LCH IV protocol includes an induction phase of 6 wk with vinblastine (6 mg/m^2) once weekly for 6 wk and prednisolone (40 mg/m^2/d) daily for 4 wk, tapered over the next 2 wk[3,18]. LCH is invariably avid on positron emission tomography (PET), and presence of active disease can easily be ascertained. At the end of 6 wk, a PET scan is recommended, and the maintenance phase of therapy is commenced only after confirmed remission of LCH. Nonresponders conventionally receive another 6-wk cycle of the same regimen. If after the second cycle, remission is not achieved, second-line salvage chemotherapeutic agents are used[3,18].

Maintenance-phase therapy consists of vinblastine (6 mg/m^2) given once in 3 wk along with prednisolone (40 mg/m^2/d) for 5 d in the same week for 1 year. A few other drugs have been tried with doubtful benefit. These include methotrexate, fludarabine, 6-mercaptopurine, cyclophosphamide and etoposide[17,19]. Clinical trials have shown that newer targeted agents like BRAF inhibitors (see below) have shown promise in achieving remission in patients with refractory disease[20].

LT FOR LCH

In contrast to other hepatic malignancies like hepatoblastomas or haematological cancers, hepatic involvement in LCH is unique. While the latter result in cirrhosis progressing to ESLD, the former do not. Hence management of LCH with liver involvement needs a meticulous balance of two major decision domains, *i.e.*, malignancy and ESLD.

In patients who have normal liver function, standard chemotherapeutic regimens (vinca alkaloids and steroids) can be safely used. However, in those with features of decompensated liver disease (DCLD) and/or synthetic failure, these drugs can result in serious adverse effects. Vinblastine is inherently hepatotoxic and can worsen liver dysfunction[16,21,22]. Also, since vinblastine is metabolised in the liver, a failing liver could lead to severe drug-related toxicity (peripheral neuropathy, myelosuppression, *etc.*)[16,21,22]. Nonetheless, a delay in initiating chemotherapy for the fear of compromising liver function potentially worsens the malignant process. Deferring LT may result in morbidity and morbidity from cirrhosis and decompensation[13]. However, premature LT in the presence of active malignancy is not a standard recommended practice, and risks recurrent LCH in the graft.

The oncologist and the transplant clinician are faced with four clinical scenarios in patients who have LCH with liver involvement. These include patients with CLD and LCH in remission, patients with DCLD and LCH in remission, those with CLD and active LCH, and lastly, those with DCLD and active LCH.

Compensated liver disease with LCH in remission

These are a subset of patients who have completed chemotherapy and have the disease in remission. As mentioned above, they could still have progressive liver disease as sequalae of LCH. It could be many years before they present with symptoms of liver disease, which may be in the form of cirrhosis and/portal hypertension[23]. LT is offered to these patients independent of their previous history of LCH. Apart from decompensation, other indications for LT in these patients include, portal

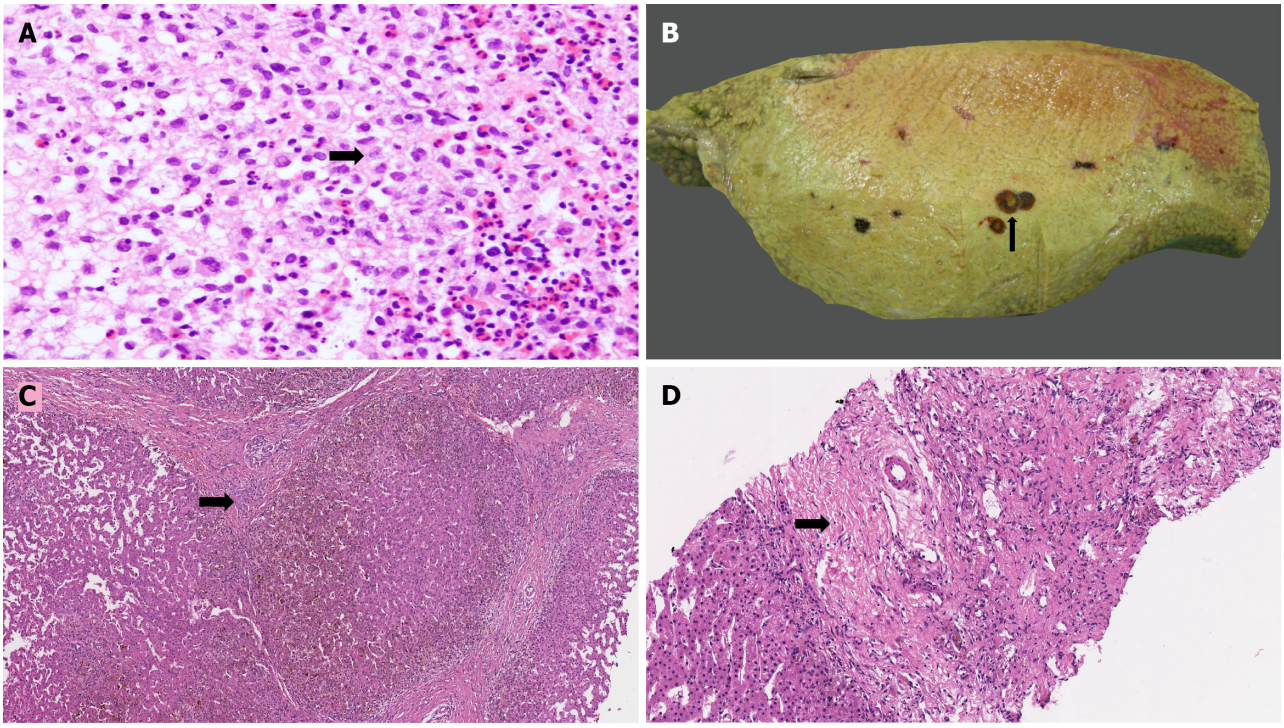

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Figure 1 Langerhans cell histiocytosis exhibit diverse morphological features in the liver. A: Aggregates of Langerhans cells admixed with inflammatory cells (arrow, H&E, 40 ×); B: Explant liver with dilated bile ducts and sludge (arrow); C: Liver wedge biopsy with evolving biliary cirrhosis and mild peripheral bile ductular proliferation (arrow, H&E, 20 ×); D: Liver biopsy with ductopenia (arrow, H&E, 40 ×).

hypertension, intractable pruritus and growth retardation. Nonetheless, irrespective of the lag-time between LCH remission and LT, prior to LT, a PET scan is performed to confirm the absence of active LCH.

Decompensated liver disease with LCH in remission

When indicated, LT may be performed in these patients. Evidence shows that the post-LT outcomes including disease-free survival in these patients is similar to those who have undergone LT for other indications[7].

Compensated liver disease with active LCH

Standard chemotherapeutic regimens (vinca alkaloid based) directed at LCH treatment should be initiated after appropriate staging workup for the disease. If the liver does not show signs of decompensation, patients should complete the full course of chemotherapy (induction and maintenance)[3]. They should however remain on close follow-up for LCH remission, signs of worsening of the liver disease and consequently the need for LT. The total duration of 12 mo chemotherapy is recommended in patients with LCH and an LT is offered after a minimum interval of 3 wk, when indicated[3]. The need for adjuvant chemotherapy in these patients is debatable, and a decision is usually made on a case-by-case basis. There may be a situation wherein the liver decompensates after initiating chemotherapy. In these patients, the severity of liver decompensation and the state of LCH remission guides further course of management.

In patients where the liver decompensation can be managed supportively, chemotherapy is continued with an aim to achieve remission. In this case however, the chemotherapy needs to be switched to relatively nonhepatoxic agents like cytarabine[24]. Once remission is confirmed following induction chemotherapy, these patients may be offered LT. It is nonetheless imperative to complete the full chemotherapeutic course. A chemotherapy-free interval of at least 3 wk is recommended prior to LT. This helps reduce the risk of chemotherapy-related damage to the liver allograft. Evidence is divisive with regards to the timing, dosing and regimen of post-LT chemotherapy. It is nonetheless unequivocally clear that the maintenance phase of chemotherapy needs to be completed. The agents are reintroduced usually 3 wk following LT. This allows for the transplanted liver to cope with the toxic agents better. Furthermore, it is advisable to reintroduce vinca alkaloids when the bilirubin is near normal (< 3 mg/dL)[22].

Decompensated liver disease with active LCH

Striking the balance between achieving remission with chemotherapy, abating worsening liver dysfunction and postponing LT remains a challenge in the management of these patients. The prudent approach would be to attempt achieving remission with relatively nonhepatotoxic, low-dose chemotherapy. Should this be achieved, as mentioned above, LT may be offered following a negative PET scan. A rarer and unfortunate situation is when a patient with active LCH requires LT. While LT may not be justified as per protocol, these are exceptional situations requiring a multi-disciplinary team decision.

ETHICS OF LT IN LCH

LCH is a malignancy; therefore, before offering these patients LT, the ethical aspects need to be debated. Immunosuppressants mute the native immune system's antitumour action, potentially resulting in a higher risk of recurrence. Furthermore, the ideal latency period between disease remission and LT remains undefined. There are also data to suggest that in patients with DCLD, delaying LT may result in worse outcomes[9]. Hence, for selected patients with DCLD, potential benefit and risk must be evaluated on an individual basis[25]. It does however, seem reasonable to indicate LT only when the oncological probability of survival disregarding immunosuppression is at least 50% at 5 years. The caveat in these cases is that the LCH should be amenable to sustained remission prior to LT and there should always remain the option of post-LT adjuvant therapy. Living donor LT (LDLT), especially in regions where deceased donor LT (DDLT) is uncommonly performed, permits optimal timing of the LT and avoidance of delays between the end of chemotherapy and LT.

LCH is a relatively rare oncological condition, and despite advances in medical oncology, there are few approved chemotherapeutic agents and even fewer clinical trials in this regard. An ethical dilemma also arises when despite chemotherapy, disease remission cannot be achieved in patients with DCLD. This subgroup of patients along with those who have active LCH and urgently require LT are outliers who need to have their management tailored according to the disease state; ideally in a clinical trial setting.

Nonetheless, prior to offering LT, it is imperative to satisfy the time-tested tenets of justice, utility and normal feasance for DDLT, and that of balancing the donor risk *versus* recipient benefit (principle of double equipoise) in living donor LT.

OUTCOMES OF LT IN LCH

Paediatric transplant oncology is a niche area that aims at optimising post-LT oncological outcomes. Given that LCH is a rare oncological condition, the literature remains sparse. Earlier series of LCH noted a recurrence of 30%-55% with a patient survival of 60%–67%[26,27]. With advances in immunosuppression, chemotherapeutic agents, and a better understanding of the oncopathology, more recent series including ours, have shown remarkably improved outcomes which are on par with LT for other indications[7,28] (Table 1). In a recent meta-analysis of 60 LT recipients with LCH, the 1-year, 3-year and 5-year patient survival rates were 86.6%, 82.4%, and 82.4%, respectively[25]. Furthermore, a cumulative recurrence of LCH in the grafts was 8%[25].

OUR UNIT POLICY AND OUTCOMES

We offer LT in patients with acute decompensation, portal hypertension with sclerosing cholangitis or growth retardation or intractable pruritus. If any patient decompensates in between chemotherapy, a LT is offered, following which the chemotherapy cycles are completed. A 18-fluorodeoxyglucose (FDG)-PET scan is mandatorily done before considering a LT in these patients. Patients who do not tolerate the full course of chemotherapy are also considered for LT during mid-cycle if the FDG-PET shows disease in remission. An interval of 2-3 wk is considered between the chemotherapy cycle and LT. This is followed by restarting of chemotherapy (if the cycles are not completed prior to surgery) after 3 wk.

Our unit's protocol includes the use of standard chemotherapy regimen in patients who are likely to withstand the full course. We use a modified chemotherapy in patients with liver dysfunction. This regimen includes low-dose cytarabine (100 mg/m^2) every 3 wk along with prednisolone (40 mg/m^2/d) daily for 4 wk tapered over the next 2 wk (each cycle is of 6 wk duration). Maintenance chemotherapy includes the same dose of cytarabine and prednisolone as the standard regimen, administered for 1 year. In our experience of six patients who underwent a LDLT, all are alive after mean follow up 36 mo (18-80 mo).

Table 1 Liver transplantation for Langerhan cell histiocytosis

Author (year)	No. of patients	Age at diagnosis (mo)	Age at transplant (mo)	Immunosuppression (No. of patients)	Surgical complications (No. of patients)	Medical complications (No. of patients)	Survival (follow-up in mo)
Stieber (1990)	3 (1adult/2 paediatric)	-	-	Cyclosporine, steroid	-	Recurrent rejection (3); retransplant (2)	66% (1 adult died of long bone fracture)
Whitington (1992)	2 (paediatric)	36 & 19	60 & 30	Cyclosporine, steroid	Roux-en Y anastomotic leak	CMV hepatitis (2); rejection (2); GI bleed (1)	100% (30-34)
Zandi (1995)	5 (paediatric)	23 ± 13	151 ± 43	Cyclosporine, steroid, azathioprine (3); cyclosporine, steroid (1); OKT3, steroid (1)	-	Rejection (4); CMV infection (2); GI bleed (1); Kidney injury (1)	60% (0.25-88.00)
Newell (1997)	6 (paediatric)	15 (12-30)	36	Cyclosporine, steroid, azathioprine (6)	Nil	PTLD (4); rejection (6); retransplantation (4); recurrence (2)	67% (24-74)
Hazdic (2000)	2 (paediatric)	16 & 17	34 & 14	Cyclosporine, steroid, MMF (1); tacrolimus, steroid (1)	Bowel perforation due to PTLD (1); PVT (1)	Recurrence (2); PTLD (1); rejection (2)	100% (5 & 60)
Braier (2002)	5 (paediatric)	-	-	Cyclosporine, steroid, azathioprine (5)	HAT & Retransplant (1)	CMV (1); rejection (1)	60% (14-37)
Chen (2020)	5 (paediatric)	15 (13-28)	53 (24-81)	Tacrolimus, steroid, MMF (5)	HAT (1)	EBV (4), CMV (1); LCH recurrence (1); DILI (1)	100%: 32 (2-67)
Our experience	6 (paediatric)	25 (9-48)	52.5 (33-204)	Tacrolimus, steroid (6)	Nil	Nil	100%: 36 (18-80)

CMV: Cytomegalovirus; GI: Gastrointestinal; MMF: Mycophenolate mofetil; EBV: Epstein–Barr virus; HAT: Hepatic artery thrombosis.

POST-LT COMPLICATIONS

Patients transplanted for LCH are known to have a higher incidence of rejections and post-transplant lymphoproliferative disorder (PTLD) (0%-67%)[7,8,27]. A purported theory is that of increased release of proinflammatory cytokines from the neoplastic cells predisposing to rejection. There remain concerns that immunosuppression could potentially increase risk an early recurrence of the index cancer, making LT futile. Hence the relative hesitancy at increasing immunosuppression in the early experiences of LT in LCH. There is no evidence till for the role of the mechanistic target of rapamycin inhibitors like sirolimus for preventing the occurrence of PTLD in these patients.

FUTURE DIRECTIONS

There is some encouraging evidence on the use of targeted therapy in LCH refractory to standard chemotherapy. *BRAF* gene mutations are observed in up to 67% of patients with LCH[29]. Vemurafenib and dabrafenib are two such agents that have shown potential in the clinical trial settings. Vemurafenib showed 83% response rate in refractory LCH[30]. In another trial of children with refractory LCH, 65% showed a remission with dabrafenib[31]. While vemurafenib may be hepatotoxic, dabrafenib is safe in patients with liver disease[32]. The authors concluded, that these agents may be used as the second- or third-line therapy for patients with refractory LCH and DCLD.

Another target for such novel therapy is the MEK1 mutation. It is observed in 19% of patients with LCH. Cobimetinib and trametinib are MEK1 inhibitors, and have shown some promise in clinical trials [33]. There however, remains a need for further research especially in those with concomitant liver disease to assess the true efficacy of these novel agents.

There have been studies evaluating the role of PD-1 (programmed cell death-1)/PD-L1 (programmed cell death ligand-1) check points in pathogenesis of LCH, especially when it involves the musculoskeletal system[34]. Hence anti PD-1 immunotherapy (pembrolizumab/nivolumab) may have a role in treating patients of LCH if there is an active musculoskeletal disease[35,36]. The role of anti-PD-1-based immunotherapy in the post-LT population remains a matter of intense debate. Given that these drugs act primarily by potentiating the native immune response, there have been studies demonstrating a high likelihood of rejections and allograft loss[37]. Therefore, the indications of anti PDL-1 therapy in the LCH patients who are likely to need LT remains undefined.

CONCLUSION

Early recognition, diagnosis and a systematic approach to the management of LCH can ameliorate the disease process. Nonetheless, the liver involvement in these patients may progress despite the LCH in remission. LT remains the mainstay in the management of such patients. Over the last decade significant advances in immunosuppression protocols, and availability of effective chemotherapeutic agents, have translated into better long-term allograft and recurrence-free patient survival. The post-LT outcomes are now comparable with those for other indications. However, given the rarity of the disease and the paucity of currently available literature, there is a need for large collaborative international, multicentre, society-based studies to provide recommendations on evidence-based algorithm for LCH, especially in patients with liver involvement.

FOOTNOTES

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MINIREVIEWS

Gut microbiota, inflammatory bowel disease and colorectal cancer

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Abstract

The gut microbiota is a complex community of microorganisms that inhabit the digestive tracts of humans, living in symbiosis with the host. Dysbiosis, characterized by an imbalance between the beneficial and opportunistic gut microbiota, is associated with several gastrointestinal disorders, such as irritable bowel syndrome (IBS); inflammatory bowel disease (IBD), represented by ulcerative colitis and Crohn's disease; and colorectal cancer (CRC). Dysbiosis can disrupt the mucosal barrier, resulting in perpetuation of inflammation and carcinogenesis. The increase in some specific groups of harmful bacteria, such as *Escherichia coli* (*E. coli*) and enterotoxigenic *Bacteroides fragilis* (ETBF), has been associated with chronic tissue inflammation and the release of pro-inflammatory and carcinogenic mediators, increasing the chance of developing CRC, following the inflammation-dysplasia-cancer sequence in IBD patients. Therefore, the aim of the present review was to analyze the correlation between changes in the gut microbiota and the development and maintenance of IBD, CRC, and IBD-associated CRC. Patients with IBD and CRC have shown reduced bacterial diversity and abundance compared to healthy individuals, with enrichment of *Firmicute* sand *Bacteroidetes*. Specific bacteria are also associated with the onset and progression of CRC, such as *Fusobacterium nucleatum*, *E. coli*, *Enterococcus faecalis*, *Streptococcus gallolyticus*, and ETBF. Future research can evaluate the advantages of modulating the gut microbiota as preventive measures in CRC high-risk patients, directly affecting the prognosis of the disease and the quality of life of patients.

Key Words: Gut microbiota; Dysbiosis; Ulcerative colitis; Crohn's disease; Inflammatory bowel disease; Colorectal cancer

Core Tip: Dysbiosis is present in patients with inflammatory bowel disease (IBD) and colorectal cancer (CRC). Dysbiosis can lead to a disruption of the mucosal barrier of the digestive tract lining, resulting in the perpetuation of inflammation and carcinogenesis, thus increasing the risk of developing CRC in IBD patients. Therefore, the aim of this review was to analyze the correlation between changes in gut microbiota and the development of IBD, CRC, and IBD-associated CRC. Further studies should be carried out to identify bacterial species that cause imbalances in the gut microbiota, enabling the development of prevention strategies or treatment of IBD-associated CRC.

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INTRODUCTION

The human microbiome is composed of several types of microbes that colonize different niches of the human body, such as the skin, lungs, vagina, and gastrointestinal tract[1]. Of these, the gut microbiota is the most studied, due to the presence of greater diversity and number of microbial species compared to other parts of the body[1,2]. The gut microbiota is a complex community of approximately 100 trillion microorganisms, such as fungi, viruses, and protists, that inhabit the digestive tracts of humans, living in symbiosis with the host[3,4].

Alterations in the gut microbiota are associated with several diseases, including inflammatory bowel disease (IBD) and colorectal cancer (CRC)[2]. Thus, studies aimed at elucidating the role of the microbiota in each phase of each disease are necessary. Based on changes in the intestinal microbiota, new diagnostic tools and possible treatments can be developed. Further, studies aiming to correlate alterations in the microbiota or in specific bacterial populations with the diagnosis, prognosis, and/or treatment of diseases are necessary. Therefore, this mini-review was conducted to determine the correlation between changes in the gut microbiota and IBD, CRC, and IBD-associated CRC.

At birth, the intestinal tract is sterile[2], with bacterial colonization beginning as early as during the passage of the baby through the birth canal[5]. Colonization continues through feeding and other environmental contacts. Several factors are known to influence colonization, including gestational age, mode of delivery (vaginal birth *vs* cesarean delivery), diet (breast milk *vs* formula) and exposure to antibiotics, and sanitation[2].

During the first year of life, the microbial composition of the mammalian intestine is relatively simple and characterized by low diversity with relative dominance of species from the phyla *Proteobacteria* and *Actinobacteria*[2,5]. Over time, the microbiota becomes more diverse, with the *Firmicutes* and *Bacteroidetes* phyla later emerging as the predominant microbes characterizing the adult microbiota[2,6-8].

In an adult individual, over 90% of intestinal bacteria belong to *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, or *Actinobacteria* phyla[4,7]. The gut microbiota can be differentiated into beneficial bacteria and opportunistic bacteria, the latter of which can cause infection[8]. *Lactobacillus*, *Bifidobacterium*, *Enterococci*, and *Propionobacteria* are some of the beneficial microbes, while the opportunistic groups include *Bacteriodes*, *Bacilli*, *Clostridia*, *Enterobacteria*, *Actinobacteria*, *Peptococci*, *Staphylococci*, and *Streptococci*[8].

The benefits of the gut microbiota to the host's physiology are vast, including nutrition, immune development, and host defense[4,7]. In the field of nutrition, some bacterial species, such as *Bifidobacterium*, participate in the biosynthesis of several components as vitamin K and vitamin B[7]. In addition, some can provide short-chain fatty acids (SCFAs) by fermenting dietary fibers[7,9]. SCFAs are a group of small fatty acids (2 to 5 carbons) produced by anaerobic microorganisms that, after absorption, have systemic immunomodulatory and anti-inflammatory properties[9].

The most abundant SCFAs in the colon are acetate, propionate, and butyrate[7,9,10], which promote the proliferation of beneficial bacteria and stimulate regulatory T cells to reduce inflammatory mediators, associated with an increase in colonic oxygen consumption by epithelial cells, and enhancing immune regulation, and gut barrier function[9-11].

Imbalance between the beneficial and opportunistic groups causes dysbiosis, which is associated with gastrointestinal disorders such as IBS, IBD, and CRC[4,7,8].

IBD AND GUT MICROBIOTA

IBD, a heterogeneous chronic and relapsing inflammatory illnesses of the digestive system, is traditionally classified into ulcerative colitis (UC) and Crohn's disease (CD)[4,12,13]. Both UC and CD can cause several symptoms like diarrhea, rectal bleeding, and abdominal pain. However, inflammation in CD is transmural and can involve any part of the gastrointestinal tract. In contrast, inflammation in UC is more superficial and limited to the colon[14].

Although the cause of IBD is not fully known, genetic history is likely involved in the pathophysiology of IBD, thus, several studies have identified a number of susceptible genes[12]. However, environmental factors, such as stress, sleeping patterns, antibiotic use, hygiene, diet, and smoking, are also associated with the development of IBD[4].

While IBD is a disease with the highest rates in industrialized countries, its incidence varies considerably worldwide, and newly industrialized areas such as India and South America presented a rapid increase in case numbers[7,13,14]. This enhancement can be related to dietary habits such as increased consumption of processed foods, sugars, and fats; overutilization of antibiotics; and an overall improvement in sanitary conditions and hygiene[13].

Nevertheless, the exact factors that trigger the first episode of inflammation and subsequent relapse remain unknown, it is now know that inflammatory episodes can result, in genetically predisposed people, from an immune response against intestinal microbial antigens under several environmental conditions[14]. Several IBD-associated susceptible genes are correlated with host responses to gut bacteria, suggesting that the gut microbiota also participate in the pathogenesis of IBD[7]. Dysbiosis is a reduction in microbial diversity, or a combination of the loss of beneficial bacteria and an increase in pathogenic bacteria[15], that may influence the pathogenesis of IBD.

The most prominent changes in the microbiota of IBD patients are the decreased diversity in bacteria species associated with decreased abundance of *Bacteroidetes* and *Firmicutes*[4,7] alongside an increase in the abundance of *Proteobacteria*[4].

In patients with IBD, the mucus layer is compromised, allowing luminal bacteria to penetrate and invade the submucosal layers, leading to the proliferative and inflammatory processes[4,13]. Then, mucosal destruction due to inflammatory injury further exposes the submucosa to more bacteria, leading to a vicious, positive feedback cycle of antigenic exposure and mucosal damage[13].

Faecalibacterium prausnitzii is one of the most abundant human gut bacteria and can be used as synonym of the gut health because of its anti-inflammatory and immunoregulatory properties[14]. One of the its products is butyrate, which is associated with an anti-inflammatory effect[7]. Several authors have correlated decreases in *Faecalibacterium prausnitzii* (*F. prausnitzii*) abundance with CD development [7,14,16] and in UC patients, a decrease was also observed during remission, being the recovery of the *F. prausnitzii* population associated with the maintenance of clinical remission[17].

Roseburia spp, including *Roseburia faecis*, *Roseburia intestinalis* (*R. intestinalis*), *and Roseburia hominis* (*R. hominis*), another genus of butyrate-producing bacteria, were significantly lower in healthy individuals with a high genetic risk for IBD compared to healthy individuals with low genetic risk[7]. *R. hominis*, specifically, was significantly reduced in UC patients, and an inverse correlation was observed between *R hominis* and disease severity[18]. In contrast, *R. intestinalis* supernatant suppressed the expression of interleukin (IL)-6 as well as the signal transducer and activator of transcription 3 (STAT3) *via* macrophage regulation in an *in vitro* experiment. Additionally, in dextran sodium sulfate- and 2,4,6-trinitrobenzene sulfonic acid-induced intestinal inflammation models, *R. intestinalis* supernatant reduced macrophages and Th17 cells in the colon, which was associated with the downregulation of IL-6 and STAT3[19].

In the field of pathogenic bacteria, several authors have described a relative increase in *Proteobacteria*, mainly *Escherichia coli* (*E. coli*), in IBD patients[7,20-22]. The exact mechanisms that lead to an increase in *Proteobacteria* during inflammation are not completely understood; however, Rizzatti *et al*[20] proposed two mechanisms: The oxygen hypothesis and the presence of nitrate. In a normal colon, epithelial cells deplete oxygen in the lumen through beta oxidation, creating an anaerobic environment[23]. In contrast, during an inflammatory episode, the beta oxidation capacity of colonic cells is decreased, thus increasing oxygen availability, which promotes dysbiosis and *Proteobacteria* growth[24]. Nitrate generated as a by-product during the inflammatory process conferred a growth advantage to the commensal bacteria *E. coli* in the large intestine, which then became predominant[25].

The increase in pathogenic bacteria in the digestive tract that can adhere to the colonic mucosa could alters the gut permeability, modifying the diversity and composition of intestinal microbiota, and ultimately leading to intestinal inflammation by regulating inflammatory genes expression[7].

Dysbiosis can further alter bacterial metabolites; decreased concentration of SCFAs has been reported as a result of a diminish in butyrate-producing bacteria like *F. prausnitzii*, in patients with IBD and in animal models of intestinal inflammation[7,9,10]. Decreased levels of SCFAs affects the differentiation and expansion of Treg cells and the growth of epithelial cells[26], leading to the loss of intestinal homeostasis.

CRC AND GUT MICROBIOTA

CRC is the third most common cancer, and the second most frequent cause of cancer deaths worldwide being more common in developed than in developing countries[27,28]. Notwithstanding cases in all countries are on the rise, most CRC cases occur in Western countries, where there is an increase in incidence[28]. CRC rates in older adults in the United States have declined in recent years probably due to increased screening, but rates in younger adults have been rising, which may be correlated with higher incidence of obesity and other diet and lifestyle trends in the western hemisphere[29].

The exact mechanism for CRC onset and progression has not been fully elucidated, but it is generally believed to be the result of extensive and complex interactions between genetic and environmental factors[30]. According to Olovo *et al*[28], the progression of adenoma to carcinoma could be linked to the gut microbiota and conversely, a healthy microbiota is correlated with a minor risk of advanced adenoma[28].

Several factors that affect the gut microbiota are thought to be related to colon carcinogenesis, such as obesity, a high-fat diet, smoking, and frequent consumption of alcohol[30].

Patients with CRC have shown reduced bacterial diversity and abundance compared to healthy individuals, with enrichment of *Firmicutes* and *Bacteroidetes*[31]. Specific bacteria are also associated with the onset and progression of CRC, such as *Fusobacterium nucleatum*, *E. coli*, *Enterococcus faecalis*, *Streptococcus gallolyticus*, and enterotoxigenic *Bacteroides fragilis* (ETBF)[32].

The abundance of *Fusobacterium nucleatum*, an opportunistic pathogen found normally in the oral cavity, is correlated with age and tumor diameter in CRC patients[31,33,34]. Additionally, an overabundance of *F. nucleatum* is associated with poor prognosis in metastatic CRC[32]; thus, *F. nucleatum* could be considered a potential biomarker for predicting the prognosis in patients with proximal colon cancer[34].

There is plenty of evidence of the tumor-promoting effects of *Streptococcus gallolyticus* in colon cells [35-37]. Colonic cells incubated with *Streptococcus gallolyticus* (*S. gallolyticus*) presented increased levels of β-catenin, c-Myc, and proliferating cell nuclear antigen (PCNA), all of which are transcription factors associated with cancer development[35]. In addition, in mice, administration of *S. gallolyticus* leads to more tumors, higher tumor burden, and dysplasia grade, and increased cell proliferation and β-catenin staining in colonic crypts compared to mice incubated with control bacteria[35]. Furthermore, CRC patients present high levels of this bacterium compared to healthy individuals[37]. However, CRC-specific conditions, such as the increased concentration of bile acids, could also promote *S. gallolyticus* colonization, creating a maintenance cycle for high levels of this bacterium in the gut[37].

ETBF is the most frequent anaerobe isolated from cases of diarrhea, peritonitis, intra-abdominal abscesses, and sepsis, and there is a positive correlation between the presence of ETBF and active IBD and CRC[38]. According to Appunni *et al*[39], co-colonization of toxigenic *E. coli* and ETBF in mice resulted in increased production of pro-inflammatory IL-17 and subsequent DNA damage, which could accelerate the development of CRC[39]. In addition, the toxin in ETBF could induce c-myc expression and IL-8 secretion causing oxidative DNA and epithelial barrier damage, and STAT3/Th17 immune responses activation, which are further correlated with an increased risk of CRC[38].

IBD-ASSOCIATED CRC AND GUT MICROBIOTA

Intestinal inflammation is one of the most common risk factors for developing CRC. Besides being two to six times more likely to develop CRC than healthy people, IBD patients with cancer are affected younger than sporadic CRC patients[40]. Disease duration, extension of the lesions, inflammation, sclerosing cholangitis, age at onset and onset in childhood are factors linking IBD with an increased incidence of CRC[41,42].

Chronic inflammation initiates and drives tumorigenesis[42], leading to an "inflammation-dysplasia-carcinoma" sequence, not the "adenoma-sequence" classically described in sporadic CRC[40].

Popov *et al*[43]proposed the three main theories about bacterial involvement in the development of IBD-associated CRC: The alpha-bug hypothesis, driver-passenger hypothesis, and common ground hypothesis. In the alpha-bug hypothesis, a single bacterium (normally ETBF) is thought to cause all modifications and damage that lead to carcinogenesis. The driver-passenger hypothesis is similar, but after the attack by the first bacteria, other opportunistic bacteria start to grow and contribute to cancer development. Finally, in the common ground hypothesis, endogenous and exogenous factors form a "leaky gut," allowing the passage of bacteria into the submucosal tissue, leading to chronic inflammation and the consequent emergence of cancer[43].

It is known that bacteria, through pathogen-associated molecular patterns (PAMPs), are capable of communicating with the tool-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs), and nucleotide binding oligomerization domain-receptors (NLRs), and trigger an immune response [42]. Nuclear factor kB (NF-kB) can be activated by the TLR and tumor necrosis factor-α (TNF-α), inducing the transcription of several tumorigenesis genes such as COX-2, which then leads to the apoptosis of intestinal epithelial cells *via* tumor suppressor p53 pathways[40] and consequent

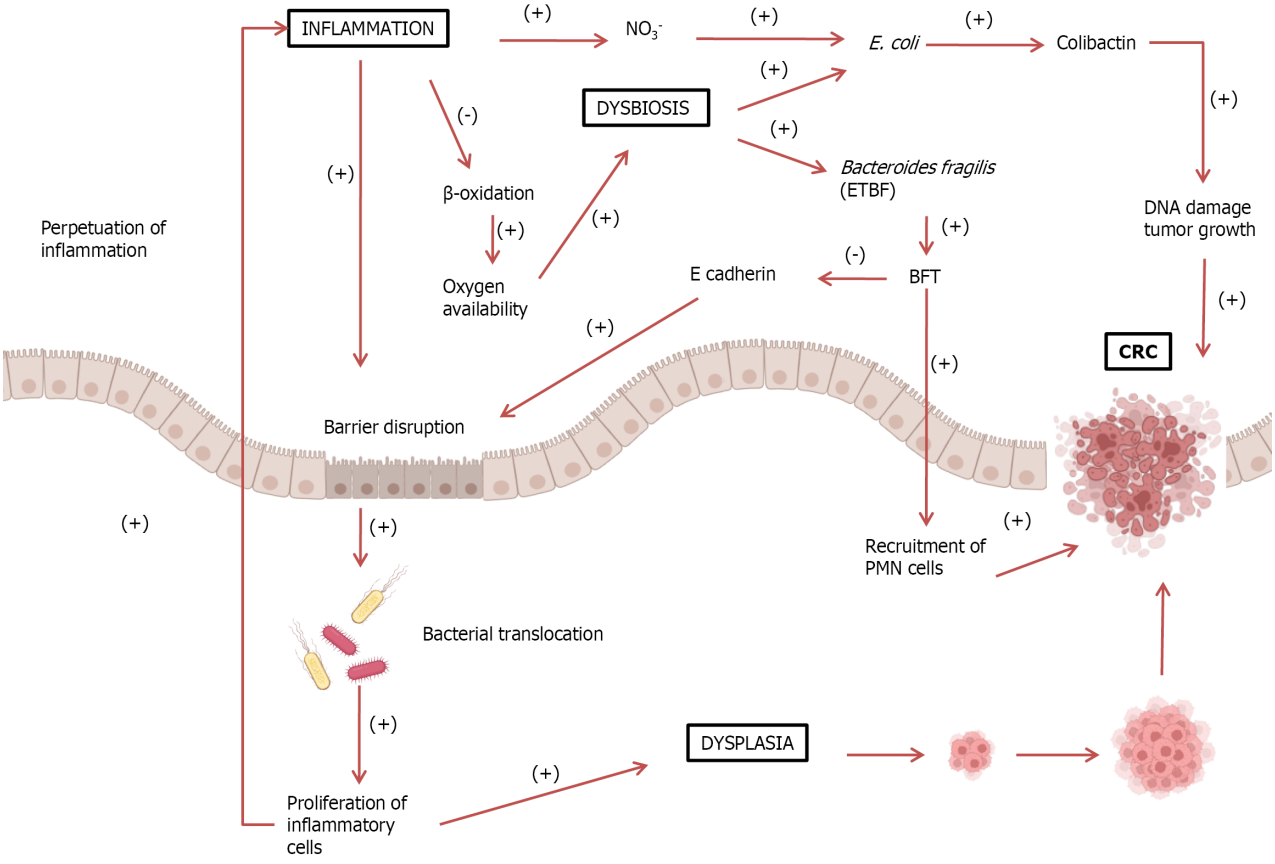

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Figure 1 Increased inflammatory response can lead to barrier disruption, allowing bacterial translocation into the intestinal lumen. These pathogens trigger an immune and inflammatory response, perpetuating the inflammatory process. Inflammatory cells can also cause dysplasia, leading to the development of colorectal cancer (CRC). Under normal conditions, epithelial cells, during β-oxidation, decrease oxygen availability, thus creating an anaerobic environment. During chronic inflammation, β-oxidation decreases, enhancing oxygen availability. This also leads to an increase in nitrate (NO3-) formation, leading to dysbiosis and growth of proteobacteria such as *Escherichia coli* (*E. coli*). *E. coli* produces colibactin, which could damage DNA and stimulate tumor growth. The growth of Bacteroidetes fragilis may also occur, which produces Bacteroides fragilis toxin (BFT). This toxin cleaves E-cadherin, a major constituent of the zonula adherens, which is responsible for cell adhesion, leading to further barrier disruption. Additionally, BFT also stimulates epithelial cells to recruit polymorphonuclear leukocytes (PMN) cells, promoting the development of CRC. BFT: Bacteroides fragilis toxin; ETBF: Enterotoxigenic *Bacteroides fragilis*; CRC: Colorectal cancer; PMN: Polymorphonuclear leukocytes.

breakdown of the intestinal barrier, allowing microbial translocation.

Increased quantities of *Proteobacteria* are associated with decreased levels of SCFAs and increased colonic inflammation[43]. In addition, intestinal Dysbiosis and barrier defects, associated with alterations in mucin secretion may happen even in the absence of active inflammation[43], perpetuating a favorable environment for the emergence of CRC.

Several studies have demonstrated the participation of bacteria such as *E. coli*, ETBF, and *Fusobacterium nucleatum* in chronic inflammation and cancer development in IBD patients[40,42-45].

A review by Yu *et al*[44] concluded that commensal bacteria, *E. coli* and *E. faecalis*, isolated from healthy subjects are capable of initiating intestinal inflammation in genetically deficient mice, suggesting that opportunistic commensal bacteria may become pathogenic only in genetically predisposed hosts[44]. Nevertheless, some types of *E. coli* can produce colibactin, a genotoxic compound capable of promoting tumor growth, alkylating DNA, and inducing tumorigenic double-stranded breaks[45]. According to Yang *et al*[45], inflammation may upregulate bacterial virulence genes, such as colibactin, and facilitate colonization of the mucosa, leading to increased colibactin-induced DNA damage in colonic epithelial cells, allowing this bacterial strain to exert its carcinogenic activity[42].

The enterotoxin produced by ETBF known as bacteroides fragilis toxin, acted as a metalloprotease cleaving E-cadherin, a major constituent of the zonula adherens, which is responsible for cell adhesion [46]. This toxin can trigger an inflammatory, pro-tumoral signaling caspase in colonic epithelial cells that cause the recruitment of polymorphonuclear immature myeloid cells to promote colon cancers[44].

CONCLUSION

There is a consensus that dysbiosis is present in both IBD and CRC, and that dysbiosis could lead to a disruption of the mucosal barrier with perpetuation of inflammation and carcinogenesis. Dysbiosis with a consequent increase in bacteria, such as *E. coli* and ETBF, is believed to lead to a breakdown of the intestinal mucosal barrier, allowing the translocation of more bacteria from the lumen to the interior of the tissue. This condition leads to chronic tissue inflammation, with the release of inflammatory and pro-carcinogenic mediators increasing the risk of developing CRC. This positive feedback loop of dysbiosis could be the basis for the inflammation-dysplasia-cancer sequence (Figure 1).

Further studies should be carried out to identify which bacteria or which set of bacteria may be responsible for this feedback cycle. Understanding the mechanism behind dysbiosis cycles may be the basis for preventing or treating IBD-associated CRC and CRC. Future research should evaluate the advantages of modulating the intestinal microbiota as a protective factor for the development of IBD-associated CRC; thus, the use of prebiotics, probiotics, or diet-based treatment can be used as preventive measures in CRC high-risk patients, directly affecting the prognosis of the disease and the quality of life of patients living with IBD.

FOOTNOTES

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MINIREVIEWS

Thrombocytopenia in chronic liver disease: Physiopathology and new therapeutic strategies before invasive procedures

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Abstract

Chronic liver disease is characterized by several hematological derangements resulting in a complex and barely rebalanced haemostatic environment. Thrombocytopenia is the most common abnormality observed in these patients and recent advances have led to researchers focus the attention on the multifactorial origin of thrombocytopenia and on the key role of thrombopoietin (TPO) in its physiopathology. Severe thrombocytopenia (platelet count < 50000/μL) complicates the management of patients with chronic liver disease by increasing the potential risk of bleeding for invasive procedures, which may be therefore delayed or canceled even if lifesaving. In the very last years, the development of new drugs which exceed the limits of the current standard of care (platelet transfusions, either immediately before or during the procedure) paves the way to a new scenario in the management of this population of patients. Novel agents, such as the TPO-receptor agonists avatrombopag and lusutrombopag, have been developed in order to increase platelet production as an alternative to platelet transfusions. These agents have demonstrated a good profile in terms of efficacy and safety and will hopefully allow reducing limitations and risks associated with platelet transfusion, without any delay in scheduled interventions. Altogether, it is expected that patients with chronic liver disease will be able to face invasive procedures with one more string in their bow.

Key Words: Thrombocytopenia; Chronic liver disease; Thrombopoietin agonists; Platelet transfusions; Avatrombopag; Lusutrombopag

Core Tip: Recent advances have shed light on the pathophysiology of thrombocytopenia in chronic liver disease and on the key role of thrombopoietin (TPO). Severe thrombocytopenia complicates the management of patients with liver disease by increasing the potential risk of bleeding for invasive procedures, possibly delaying lifesaving interventions. In the very last years, novel agents such as the TPO-receptor agonists avatrombopag and lusutrombopag have been developed in order to increase platelet production as an alternative to platelet transfusions, with positive efficacy and safety outcomes.

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INTRODUCTION

Thrombocytopenia, usually defined as any decrease in platelet count below the lower normal limit of 150000/μL, is the most common haematological abnormality in patients with chronic liver disease[1]. Current data report a prevalence ranging from 6% to 78%, which progressively increases from patients with compensated to those with decompensated cirrhosis[2]. The clinical significance of mild (100000/μL-150000/μL) and moderate (50000/μL-100000/μL) thrombocytopenia is minimal and does not interfere with the regular clinical practice. Otherwise, severe thrombocytopenia (< 50000/μL) can be associated with many sequelae and could have a negative impact on the management of patients with advanced chronic liver disease.

PHYSIOPATHOLOGY OF THROMBOCYTOPENIA IN CHRONIC LIVER DISEASE

In chronic liver disease, thrombocytopenia has been classically attributed to hypersplenism[1,3]. Over the last decades, however, advances in the understanding of thrombopoiesis have led to a wider and better understanding of its physiopathology. As a result, thrombocytopenia is considered a more complex and multifactorial process involving multiple different mechanisms. These are generically divided into those leading to decreased production or increased destruction of thrombocytes and splenic sequestration[1] (Figure 1).

Decreased platelet production

Decreased platelet production is a consequence of decreased production of thrombopoietin (TPO) and direct bone marrow suppression. Currently, the production of TPO is believed to play a pivotal role in thrombopoiesis. TPO is primarily produced in the liver and, after being secreted into the circulation, it binds to the surface of platelets and megakaryocytes through the c-MPL receptor[4]. TPO-receptor ligation activates a number of intracellular signalling pathways *via* Janus kinase type 2 and tyrosine kinase 2[5], which ultimately lead to the differentiation of bone marrow stem cells into mature megakaryocytes and to the production of platelets which are released into the peripheral circulation[6]. Of note, after binding to its receptor, TPO is internalized and destroyed in order to reduce further platelet and megakaryocyte exposure[4]. Platelet production is therefore mainly regulated by platelet levels in the blood through a negative feedback circuit[1]. The role of a decreased hepatic production of TPO in the development of thrombocytopenia in chronic liver disease is supported by the immediate increase in TPO levels and platelet production after liver transplantation[7]. Animal models and human clinical studies have confirmed decreased expression of *TPO* mRNA in liver tissue with the progression of cirrhosis[8], which is probably associated with specific regulatory mechanisms for the expression of *TPO* gene and is not regulated by bone marrow[9]. Moreover, a correlation between reduced c-MPL expression and the progression of liver cirrhosis has been demonstrated and may play an additional role in the development of thrombocytopenia[10]. Some chronic liver diseases may also cause decreased platelet production through direct bone marrow suppression or toxicity, as observed during viral infection (particularly hepatitis C virus (HCV) infection[11], alcohol abuse[12], iron overload[12], and drug consumption[1,13]).

Increased platelet destruction

Increased platelet destruction is a multifactorial process that may involve decreased levels of A disintegrin-like and metalloprotease with thrombospondin type 1 motif 13 (ADAMTS13), immunologically mediated platelet destruction, and bacterial activity. ADAMTS13 is a metalloproteinase

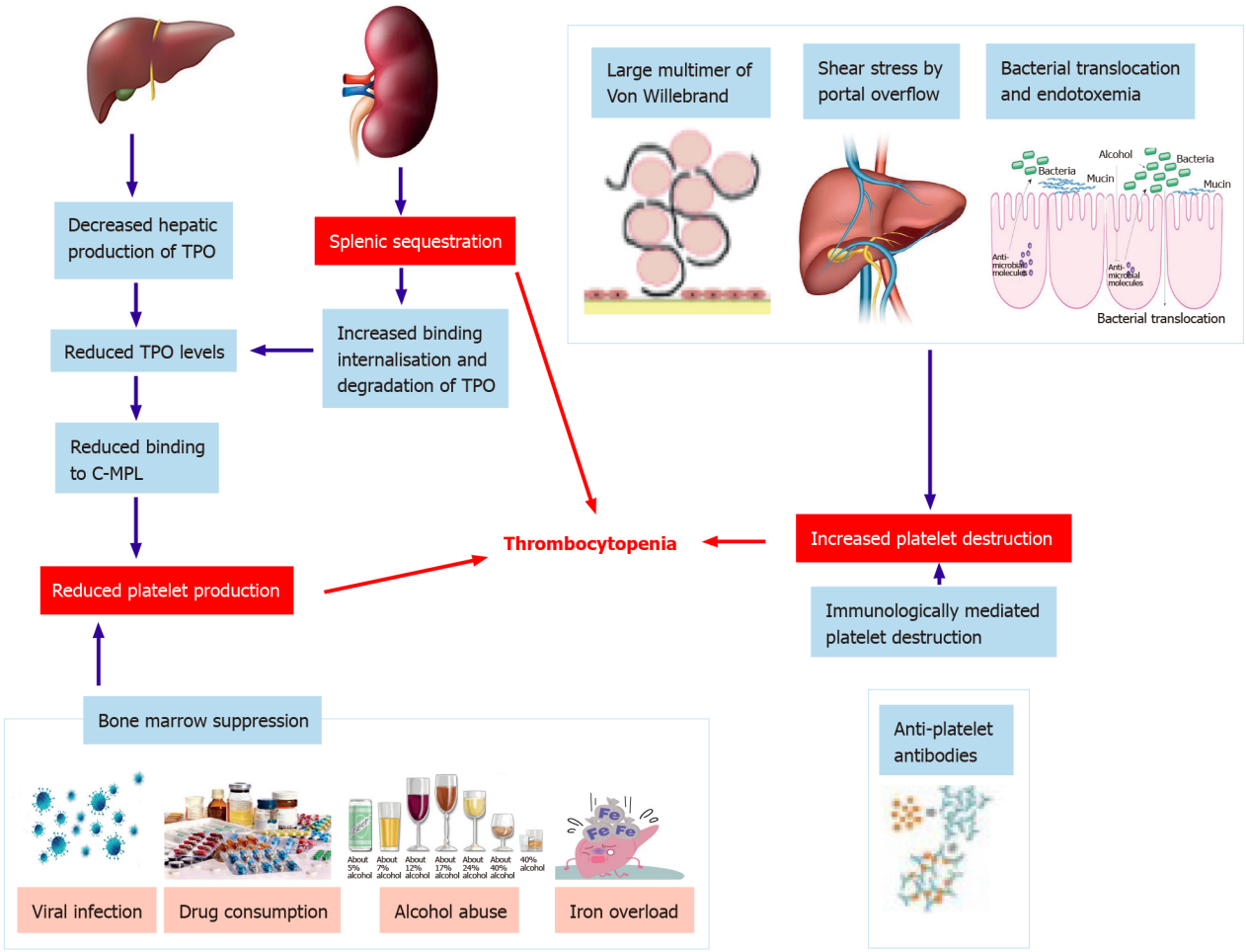

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Figure 1 Pathophysiology of thrombocytopenia. TPO: Thrombopoietin.

produced by hepatic stellate cells, whose physiological role is to cleave large von Willebrand factor (vWF) multimers[14]. In cirrhosis, decreased levels and activity of ADAMTS13 drive the accumulation of vWF multimers, which mediates an enhancement of shear-stress induced platelet aggregation[14]. Additionally, anti-platelet antibodies are a frequent finding in patients with liver cirrhosis, being detectable in up to 64% of cases[15]. The inverse relationship between platelet-associated immunoglobulin G (IgG) levels and platelet count evidences that immunologic destruction contributes to the genesis of thrombocytopenia at least in some chronic liver diseases[16]. Immune-mediated thrombocytopenia is most likely to occur in the course of autoimmune liver diseases (particularly primary biliary cholangitis) and HCV infection[1,17].

HCV can cause immune-mediated thrombocytopenia through multiple mechanisms[1]. First, it can be associated with idiopathic thrombocytopenic purpura (ITP), as supported by a prevalence of anti-HCV antibodies of approximately 10% in patients with ITP[18]. The virus can also directly bind to platelets interacting with multiple surface receptors, leading to the attachment of anti-HCV antibodies to platelets. This will ultimately determine either platelet phagocytosis by the reticuloendothelial system or alterations in the platelet membrane epitopes that induce the production of anti-platelet antibodies[19]. Finally, HCV infection can be associated with the production of cryoglobulins, which can accelerate platelet clearance by the reticuloendothelial system[20]. Thrombocytopenia can be found in about 48% of patients with bacterial infection and sepsis[21], confirming that the inflammatory cascade plays a role in the development of thrombocytopenia. This is confirmed in the hospitalized cirrhotic population[22]. In sepsis, thrombocytopenia is mainly dependent on the increased activation of the coagulative system, resulting in clot formation and platelet consumption[23].

Splenic sequestration

Hypersplenism has been classically considered the main determinant of thrombocytopenia during chronic liver disease[1,3], even after that many other physiopathologic mechanisms have been progressively identified. During chronic liver disease, the inception of portal hypertension causes a redistribution of splanchnic venous blood flow, ultimately responsible for congestion of the spleen and consequent enlargement of the organ, leading to a significant increase of the splenic pool of platelets

[24]. Actually, hypersplenism is the clinical syndrome in which splenomegaly is associated with splenic hyperactivity, *i.e.*, a reduction in one or more peripheral blood cell types, in patients with an appropriate proliferative bone marrow response. This syndrome can be reverted with splenectomy[1,24].

COAGULOPATHY AND HAEMORRHAGIC RISK ASSESSMENT IN CHRONIC LIVER DISEASE

Chronic liver disease is characterized by alterations of the entire hemostatic system[25]. Thrombocytopenia is just one face of a wider coagulation disorder whose relevance is mirrored by the inclusion of coagulation indices in all functional and prognostic scores of liver disease[26]. Traditionally, coagulopathy in cirrhosis was considered as a bleeding diathesis disorder[27], alongside the well-known thrombocytopenia, and the impaired coagulation tests were perceived as indicators of hemorrhagic risk [28]. In the last decades, several studies led to significant changes in knowledge, with a renewed vision concerning the coagulopathy of liver cirrhosis. A new paradigm of a balanced, *albeit* precarious, hemostatic state has emerged and the net effect is a rebalanced equilibrium[26,27], which can be easily disturbed by many different clinical events, alternatively leading to hemorrhagic as well as to thrombotic manifestations, with the latter being even more frequent indeed[27] (Figure 2).

It has been shown that the reduction in liver-derived pro-coagulant factors is counteracted by the concomitant decrease of the liver-derived anti-coagulant ones, especially protein C[28,29]. Thrombocytopenia and platelet abnormal function are offset by increased vWF and decreased ADAMTS13 levels. In cirrhosis, even if diminished in number, platelets are able to support normal thrombin generation at least until they are in the range 50-60000/mL, therefore assuring a normal primary hemostasis. This is possible thanks to the compensatory action of vWF and to the upregulation of intracellular activating signalling pathways[27], leading to an enhanced thrombocyte response. Furthermore, decreased clearance of tissue plasminogen activator and plasminogen activator inhibitor, and decreased synthesis of alpha 2-antiplasmin and thrombin activable fibrinolysis inhibitor are all factors contributing to hyperfibrinolysis. The latter is observed in up to 30% of patients with advanced liver disease[1], confirming a re-arrangement of the whole hemostatic system.

In cirrhotic patients, even though reduced in number, platelets are still able to ensure an adequate haemostatic function. Consequently, the sole platelet count is not able to predict bleeding risk in liver cirrhosis. Actually, for patients with cirrhosis undergoing invasive diagnostic or therapeutic procedures, the risk of procedure-related bleeding remains a clinical issue[30], and risk stratification is a great challenge. This is mainly due to the inaccuracy, in the context of cirrhosis, of the laboratory tests that are routinely used for the assessment of the hemocoagulative system[25,27,30]. Indeed, it is now well established that the standard clotting tests do not reflect the actual bleeding risk[30-32], and current evidence does not support prothrombin time (PT)/international normalized ratio (INR) as clinical targets[25,33]. Conversely, assessing platelet count and fibrinogen levels before high-risk procedures is recommended, as well as it is the pre-procedural correction of these parameters, having these laboratory parameters been proposed as more reliable indicators of the bleeding risk in patients with cirrhosis[34].

Moreover, many studies have shown that cirrhotic portal hypertension and kidney injury are more essential in determining the risk of bleeding[25,35]. As a matter of fact, renal failure can lead to platelet impairment resulting from reduced adhesive and aggregative capacities *via* alterations of serotonin concentration, of calcium flow and of thromboxane metabolism[25]. Patient with cirrhosis can also develop accelerated intravascular coagulation and fibrinolysis, described as a bleeding entity similar to disseminated intravascular coagulation, but different for the imbalance between pro- and anti-fibrinolytic factors, resulting in hyper-fibrinolysis with an increased bleeding risk[35]. Despite all such evidence, most current guidelines still recommend correcting elevated PT/INR values through plasma transfusions, while tests capable of better capturing the hemostatic function of cirrhotic patients (thrombin generation tests, thromboelastography, *etc.*) are not readily available in everyday clinical practice[25].

NON-PHARMACOLOGICAL TREATMENT OPTIONS FOR MANAGEMENT OF THROMBOCYTOPENIA

The management of thrombocytopenia in chronic liver disease has been a primary and challenging endpoint for decades. In the 1960s, surgical splenorenal shunts were performed with this purpose, but they were soon abandoned due to high mortality rates and the risk of liver decompensation[36]. Total and partial splenectomy were therefore developed. They gained popularity in the 1990s thanks to limited complication rates, mainly after the introduction of the laparoscopic technique. Later, less invasive techniques, namely, splenic artery embolization or spleen radiofrequency ablation, opened new scenarios[36].

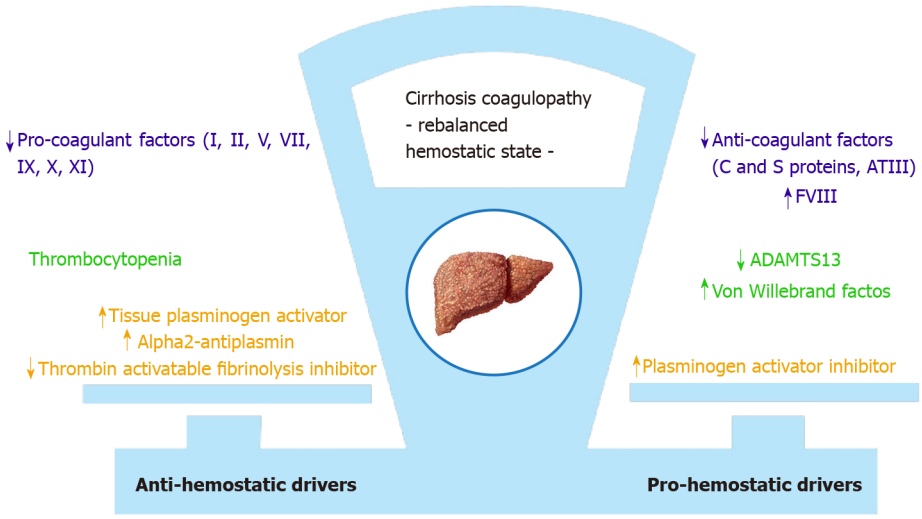

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Figure 2 Pathophysiology of coagulopathy. ATIII: Antithrombin III; FVIII: Coagulation factor VIII; ADAMTS13: A disintegrin-like and metalloprotease with thrombospondin type 1 motif 13.

Splenectomy

In the past, open splenectomy has been considered among the strategies for treating thrombocytopenia. Actually, this procedure was associated with a high risk of bleeding and consequent hepatic decompensation, particularly following the open technique. For these reasons, subsequent studies supported the laparoscopic technique over open surgical splenectomy or the shunt techniques over splenectomy[37,38]. Overall, also with less invasive procedures, the rate of complications range from 2.5% to 17%, and the risk of portal and splenic vein thrombosis is elevated (about 10%)[39]. Altogether, due to their invasive nature and to the high risk of complications, these strategies are restricted to rare and specific cases, while they have been virtually abandoned in the ordinary clinical practice.

Splenic artery embolization

Splenic artery embolization has been introduced since the 1970s as an alternative to splenectomy in surgically unfit patients. For the increased risk of developing splenic abscesses after total embolization, partial splenic embolization has become the preferred option for candidate patients[40], with a lower risk of complications, sepsis and mortality compared to total splenectomy[36,41]. Unfortunately, the extent of the beneficial effect on platelet count depends on the amount of the splenic mass embolized, which is proportional to the complications observed.

Radiofrequency ablation of the spleen

Radiofrequency ablation of the spleen is a minimally invasive procedure with promising results in patients with cirrhosis and severe thrombocytopenia[36]. The main benefits of this minimally invasive procedure are cost-effectiveness and lower complication rates over other invasive procedures. The major side effects are hemorrhagic shock and intra-abdominal bleeding, while complications such as splenic abscess or rupture are not a concern with this procedure[42]. Besides, to date, more clinical trials with longer follow-up would be needed to estimate the effectiveness of this strategy.

Shunt procedures

Shunt procedures [portocaval shunt, splenorenal shunt, and transjugular intrahepatic portosystemic shunt (TIPS)] are other possible techniques which have been experimented in chronic liver disease. They are aimed at decreasing splenic congestion and, therefore, platelet sequestration[36]. To date, however, these approaches are not supported by available studies; indeed, there is an absence of clear benefits on the platelet number and well-known complications. Actually, the use of these procedures, in particular TIPS, is currently restricted to selected cases where the aim is not to increase platelet levels, but to control bleeding from oesophageal varices or to manage refractory ascites[43].

Platelet transfusion

Platelet transfusion has become the mainstay of treatment in patients with chronic liver disease and severe thrombocytopenia who require an invasive procedure[44]. The choice to transfuse platelets is variable and controversial, depending upon patient comorbidities and the risk of bleeding. Most data suggest that invasive procedures may be performed without a significantly increased risk of bleeding in patients with more than 50000/μL platelets, while there is less consensus about the risk in patients with

a lower count, as reflected also in the latest guidelines and a recent retrospective study[45,46]. In a sub-analysis of 2740 liver biopsies from the Hepatitis C Antiviral Long-term Treatment against Cirrhosis trial, there were only 16 bleeding events (0.6%), and the highest bleeding risk (5.3%) was recognized for platelet counts less than 60000/μL[47]. Conversely, in another study evaluating the safety of liver biopsies (177 patients), the frequency of bleeding in patients with a platelet count lower than 50000/μL was not significantly different from that in those with a normal platelet count, and the only independent risk factor for bleeding was an underlying malignancy[32].

Guidelines vary as to the threshold below which periprocedural bleeding risk justifies intervening to treat thrombocytopenia; however, transfusions are more frequently indicated with a platelet threshold < 50000/μL[48]. Although platelet transfusion has been the standard of care for correcting thrombocytopenia for a long time, relevant side effects limit its use. The most common issues include febrile or allergic reactions, risk of infection (even if very low), hemolysis, transfusion-related graft-*versus*-host disease, and alloimmunization[41,49]. Even the procedure of transfusion itself may be critical. Potential risks include errors in patient identification, in blood typing, and in cross-matching[50]. Concerning efficacy, platelet transfusions determine an overall modest increase in platelet count, without a significant impact on the risk of bleeding[51], and treatment effect has a limited duration. Moreover, up to 70% of patients receiving repeated platelet transfusions become refractory to subsequent ones. Finally, platelet transfusions are limited by donor supply, and the reduced availability may have a great impact on the management and clinical outcomes of these patients.

NEW THERAPEUTIC OPTIONS FOR THRMBOCYTOPENIA IN CHRONIC LIVER DISEASE

Recent advances in understanding the physiopathology of thrombocytopenia in chronic liver disease, with the discovery of the central role of TPO in thrombocytopoiesis, has led to the development of many drugs with TPO activity.

Recombinant TPO and human cytokines

Two recombinant TPOs that stimulate platelet production in humans and showed potential promise were recombinant human TPO and pegylated recombinant megakaryocyte growth and development factor[52,53]. They have shown clinical benefits in clinical trials of haematological patients without safety concerns, but have been withdrawn from clinical development for the induction of neutralizing antibodies[53]. Similarly, the use of recombinant human cytokines was limited because of side effects and the occurrence of toxicities as observed after the subcutaneous injection of the recombinant human interleukin-1, approved by the Food and Drug Administration (FDA) for the treatment of the thrombocytopenia induced by chemotherapy for solid tumors, which can cause cardiovascular side effects and flu-like symptoms[54].

TPO agonists

Activation of thrombopoiesis through TPO receptor agonists is an alternative method to stimulate platelet production with the use of drugs, which are not homologous to endogenous TPO but activate the same receptor working on a different site. These drugs have been primarily investigated in patients with chronic ITP and subsequently in chronic liver disease for treating thrombocytopenia before invasive procedures. The effect of TPO agonists is mediated by the interaction with TPO receptors on megakaryocytes, specifically c-MPL ligand-mediated activation of Janus kinases and signal transducer and activator of transcription proteins and mitogen-activated protein kinase pathways[55].

Romiplostin: Romiplostin is a polypeptide linked to an IgG heavy-chain Fc molecule and has no amino acid sequence homology to endogenous TPO. It acts by competing with the same site of the TPO-receptor and activating intracellular transcriptional pathways aimed to increase platelet production[55]. Due to a different molecular structure, its use has not been associated with antibodies reacting against endogenous TPO. This drug is administered subcutaneously once a week, but it is currently approved only for treatment of ITP when refractory to other drugs[54,56].

Indeed, most of the experience with romiplostin was derived from clinical studies in patients with refractory ITP[57], while only anecdotal case reports and small series involved patients with chronic liver disease[58-61]. A single-centre study in Egypt involved 35 patients with HCV-related cirrhosis and severe thrombocytopenia who required elective surgery[60]. Patients received romiplostin once weekly for a maximum of 4 wk. The primary endpoint - achieving a threshold of platelet count of 70000/μL - was reached in 94% of patients, and 20% of them had maintained a count > 50000/μL 3 mo after the last injection. Headache was reported as the only adverse event. In another single centre, prospective, randomized, double-blind study, 65 subjects with chronic liver disease and thrombocytopenia (less than 60000/μL) undergoing percutaneous liver biopsy received a TPO agonist or platelet transfusion[61]. Romiplostin determined significantly higher pre-biopsy and post-biopsy platelet counts compared to eltrombopag and platelet transfusion, and it was cost-effective and safe.

Eltrombopag: Eltrombopag is an orally available, small non-peptide TPO mimetic molecule more largely studied for use in liver disease. Its binding to a specific human transmembrane domain of TPO-receptor induces proliferation and differentiation of megakaryocytes and precursor cells[62]. It is taken orally once daily and is approved for thrombocytopenia: (1) In chronic ITP refractory to other treatments; (2) In HCV chronic hepatitis candidates for treatment with interferon-based regimens; and (3) In patients with severe aplastic anaemia[63].

Eltrombopag safely increased platelet number in patients with cirrhosis and HCV infection[64]. In a phase II multicentre randomized trial, eltrombopag was effective in increasing platelet count to more than 100000/μL at week 4 in 75%-95% of patients, compared to 0% of patients in the placebo group. Consequently, these patients were significantly more likely to initiate and complete 12 wk of antiviral treatment with respect to those on placebo (36%-65% *vs* 6%). In a phase II trial in Japan on 38 patients with chronic liver disease, eltrombopag increased platelet count in a dose-dependent manner[65].

The most recently published data in chronic liver disease are derived from the Eltrombopag Evaluated for Its Ability to Overcome Thrombocytopenia and Enable Procedures study, a phase 3 double-blind, placebo controlled trial that assessed the utility of this drug to increase platelet count and reduce the need for transfusion in patients undergoing elective procedures[66]. In this trial, 86% of patients recruited had liver cirrhosis with a platelet count less than 50000/μL. Patients were randomized to receive 75 mg eltrombopag daily or placebo in the 2 wk preceding the invasive procedure performed within 5 d from the last dose. Primary endpoint was the number of subjects who did not require a platelet transfusion before, during, and up to 7 d after the procedure. This was achieved in 72% (104/145) of subjects who received eltrombopag, compared to 19% (28/147) in the placebo group (P < 0.001). However, this study was prematurely terminated since six patients in the treatment group developed thrombotic events (2 patients in placebo, odds ratio for eltrombopag 3.04, 95% confidence interval: 0.62-14.82). *Post-hoc* analysis suggested an association of platelet count > 200000/μL with the occurrence of portal vein thrombosis[66].

The other two international phase III trials included patients with chronic hepatitis C and platelet counts less than 75000/μL[67]. Eltrombopag to Initiate and Maintain Interferon Antiviral Treatment to Benefit Subjects with Hepatitic C-Related Liver Disease (ENABLE)-1 and ENABLE-2 assessed the ability of eltrombopag to increase platelet count and, so, allow subjects to initiate and maintain antiviral treatment with pegylated interferon and ribavirin. In both trials, significantly more patients on eltrombopag achieved a sustained virological response at 24 wk of antiviral therapy, with similar adverse events. However, the absolute benefit over placebo was less than 10% and the use of this drug was associated with an increased risk of hepatic decompensation (10% *vs* 5% placebo) and thromboembolic events (3% *vs* 1% placebo). The most frequent adverse events reported were anaemia, pyrexia, and neutropenia.

Avatrombopag: Avatrombopag is an orally available drug that has a similar mode of action to eltrombopag, and it does not compete with endogenous TPO for its receptor site-binding. It is taken once daily with food for 5 d a week, with the dose adjusted according to baseline platelet count. Differently from eltrombopag, it exhibits significant drug-drug interactions based on the cytochrome P450 2C9 (CYP2C9) and CYP3A cytochrome systems[68]. In a phase II study, 130 patients with chronic liver disease and platelet count less than 60000/μL received two different formulations of the drug 1 wk prior to an elective invasive procedure, and both groups of avatrombopag-treated patients achieved the primary endpoint of an platelet increase of > 20000/μL from baseline, and to > 50000/μL, at least once during the treatment days 4-8.

After that, avatrombopag was approved by the FDA in 2018 and the European Medicines Agency (EMA) in 2019 for the treatment of severe thrombocytopenia in patients with chronic liver disease undergoing invasive procedures, with the recommendation to take it 10-13 d before the procedure scheduled within 5-8 d from the last dose administration[69]. The safety and efficacy were evaluated in two pivotal randomized phase 3 studies[69]. ADAPT-1 and ADAPT-2 (Table 1) randomized 430 patients with cirrhosis and severe thrombocytopenia undergoing scheduled procedures to receive avatrombopag at different doses (according to platelet baseline count) or placebo for 5 d. The primary endpoint was the need for platelet transfusion or rescue procedures for bleeding in the 7 d after the procedures[70]. Significantly more patients met this endpoint in avatrombopag groups: In ADAPT-1, 65.6% and 88.1% compared to 22.9% and 38.2% of patients receiving placebo; in ADAPT-2, 68.6% and 87.9% compared to 34.9% and 33.3% (P < 0.001 for both) (Figure 3). Overall, the safety profile was similar to placebo and the most frequent adverse events were nausea, fatigue, abdominal pain, pyrexia, and headache. Serious adverse events occurred in 16%-19% of patients with avatrombopag and 6%-14% of those with placebo, including one patient who developed portal vein thrombosis during post-treatment follow-up. Finally, the safety and efficacy of avatrombopag were recently confirmed in a real-world setting, where cirrhotic patients mainly undergoing esophageal varices band ligation received the drug without requiring platelet transfusion, and with a good profile of adverse events[71,72].

Lusutrombopag: Lusutrombopag is an orally administered synthetic small molecule that acts as an agonist of human TPO, activating the signal transduction pathways to upregulate platelet production. Earlier studies have demonstrated that lusutrombopag raises platelet count and is a manageable drug

Table 1 ADAPT 1-2 key characteristics

Key characteristic	ADAPT 1-2
Key inclusion criteria	Chronic liver disease (MELD score ≤ 24) and thrombocytopenia (platelet counts < 50000/μL). Age ≥ 18 yr
Key exclusion criteria	ADAPT-1/2: History of thrombosis or hematologic disorders. Significant cardiovascular disease. Portal or splenic mesenteric system thrombosis at screening; portal vein blood flow < 10 cm/s at screening. Platelet transfusion within 7 d of screening. Use of heparin, warfarin, FANS, aspirin, verapamil, or antiplatelet therapy with ticlopidine or glycoprotein IIb/IIIa antagonists, or erythropoietin-stimulating agents within 7 d of screening. Advanced hepatocellular carcinoma (BCLC C or D)
Dosing	Low baseline platelet count cohort (≤ 40000/μL): 60 mg avatrombopag or placebo once daily with a meal on days 1-5. High baseline platelet count cohort (40000-50000/μL): 40 mg avatrombopag or placebo once daily with a meal on days 1-5
Type of study	ADAPT-1/2: Global, multicenter, randomized, double-blind, placebo- controlled, phase 3 studies
Patients number	ADAPT-1: 231; ADAPT-2: 204
Endpoints	Efficacy assessment: (1) Primary: Proportion of patients not requiring a platelet transfusion or rescue procedure for bleeding bleeding in the 7 d after the procedures; and (2) Secondary: Proportion of patients achieving the target platelet count of 50000/μL on procedure day; the change in platelet count from baseline to procedure day. Safety assessment: The incidence of adverse events adverse drug reactions, treatment-emergent adverse events

MELD: Model for end-stage liver disease.

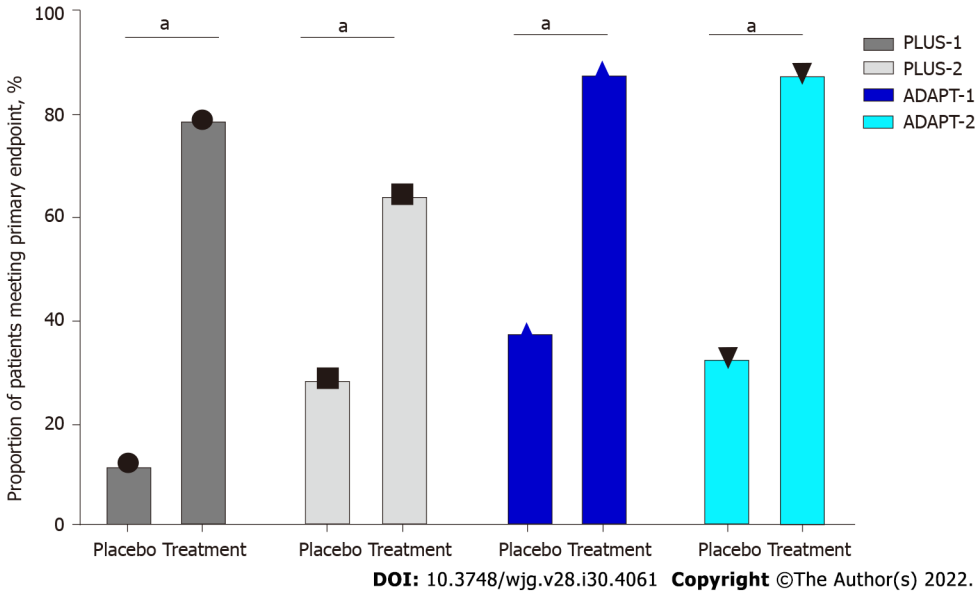

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Figure 3 Overview of primary endpoints of phase 3 studies (ADAPT-1, ADAPT-2, L-PLUS 1, and L-PLUS 2). [a]*P* ≤ 0.0001.

that does not require food restrictions, and has no clinically significant drug-drug interactions[73,74]. Furthermore, it has demonstrated a dose-proportional pharmacokinetic with no clinically significant differences in the pharmacokinetic grounded on age, liver function (Child-Pugh classes A and B), and renal function (creatinine clearance greater than 30 mL/min)[75]. It is primarily metabolized by CPY4 enzymes, including CYP4A11, and it is mainly excreted by the faecal route (83% of dose). Moreover, it has a low potential to inhibit or induce transporter systems and CYP enzymes[73]. The recommended dose of the drug is 3 mg once daily for 7 d, beginning 8-14 d prior to the scheduled procedure.

Lusutrombopag was approved in Japan in 2015 for use in patients with thrombocytopenia and chronic liver disease who needed invasive procedures and received a positive opinion from the EMA Committee for Medicinal Products for Human Use in 2018[76]. The approval of this drug was based on the results of Lusutrombopag for the Treatment of Thrombocytopenia in Patients with Chronic Liver Disease Undergoing Invasive Procedures trial (L-PLUS-1), a phase 3 double-blind study, carried out in Japan with 96 patients with chronic liver disease and severe thrombocytopenia (platelet count < 50000/μL) undergoing invasive procedures[77] (Table 2). In this study, the proportion of patients that did not require pre-operative platelet transfusions was significantly greater in the lusutrombopag group *vs* placebo (79.2 % *vs* 12.5%, respectively, $P < 0.0001$) (Figure 3). The median platelet count reached more than 50000/μL after 5 d in the drug group, with the greatest value observed after a mean of 13.4 d.

Table 2 Lusutrombopag for the Treatment of Thrombocytopenia in Patients with Chronic Liver Disease Undergoing Invasive Procedures trial 1-2 key characteristics

Key characteristic	L-PLUS 1-2
Key inclusion criteria	Chronic liver disease (Child Pugh A or B) and thrombocytopenia (platelet counts < 50000/μL). Age ≥ 18 yr (≥ 20 yr in L-PLUS 2)
Key exclusion criteria	L-PLUS 1: (1) Hematopoietic tumors; aplastic anemia, myelodysplastic syndrome, myelofibrosis, congenital, immune, or drug-induced thrombocytopenia; (2) Splenectomy, any other causes of thrombocytopenia; (3) History of portal vein thrombosis; (4) Active malignant tumor other than primary hepatic cancer; (5) Therapies that could influence platelet count (L-PLUS 1); (6) Chronic liver disease with Child-Pugh C; (7) Portal vein tumor embolism; and (8) Past or present thrombosis or prothrombotic condition. L-PLUS 2: (1) Hematopoietic tumors; aplastic anemia, myelodysplastic syndrome, myelofibrosis, congenital, immune, or drug-induced thrombocytopenia; (2) Portal vein thrombosis within 28 d prior to randomization or a history of portal vein thrombosis; absence of hepatopetal blood flow in the main trunk of the portal vein as demonstrated by doppler ultrasonography within 28 d prior to randomization; (3) Chronic liver disease with Child-Pugh C; (4) Portal vein tumor embolism; and (5) Past or present thrombosis or prothrombotic condition, history of splenectomy
Dosing	3 mg lusutrombopag or placebo once daily for up to 7 d
Type of study	L-PLUS 1: Double-blind, parallel-group, phase 3 study; L-PLUS 2: Global, phase 3, randomized, double-blind, placebo-controlled study
Patients number	L-PLUS 1: 96; L-PLUS 2: 215
Endpoints	Efficacy assessment: (1) Primary: Proportion of patients who required no platelet transfusions before the primary invasive procedure and no rescue therapy for bleeding; and (2) Secondary: Rate of response (defined as proportion of patients who achieved platelet count of more than 50000/μL with an increase of ≥ 20000/μL from baseline at any time during the study); the duration of sustained platelet count increase; time courses of changes in platelet count. Safety assessment: The incidence of adverse effects, adverse drug reactions, treatment-emergent adverse effects, bleeding-related adverse effects, and thrombotic events

L-PLUS: Lusutrombopag for the Treatment of Thrombocytopenia in Patients with Chronic Liver Disease Undergoing Invasive Procedures trial.

Moreover, no significant concerns were raised in this study, and no significant adverse drug reactions were observed.

The safety and efficacy of this drug were confirmed in a larger study, the L-PLUS-2[78]: 215 patients with chronic liver disease and a platelet count < 50000/μL were randomly assigned to once-daily lusutrombopag at a dosage of 3 mg or placebo for ≤ 7 d before an invasive procedure. The procedure was performed within 7 d after the last dose. In the intention to treat analysis, significantly more patients in the lusutrombopag group (64.8%) met this endpoint compared with placebo (29%, $P < 0001$). This percentage was greater in the per-protocol analysis, with the endpoint reached in 72.5% of patients in the active drug group *vs* 20% in the placebo group ($P < 0.0001$). The median duration of the achievement of a target of platelet count > 50000/μL was 19.2 d in the lusutrombopag group *vs* 0 d for the patients who received placebo. Moreover, the median maximum change in platelets from baseline was over 4 times higher for patients treated with the active drug, who did not receive platelet transfusions, compared with patients who did receive transfusions (45000 *vs* 11000/μL)[78]. Finally, 47.7% of patients in the lusutrombopag group and 48.6% in the placebo group had at least one adverse event. These side effects were mainly mild or moderate in severity and the most common were headache, abdominal pain, fatigue, peripheral edema, and nausea. There were only three mild bleeding events in three patients in the lusutrombopag group (2.8%) *vs* seven bleeding events (4 moderate and 1 severe; 5.6%) with placebo. Only three thromboembolic events were recorded (1 in the lusutrombopag group), which were not related to platelet count.

The first real-life study in Japan enrolled 25 patients with cirrhosis, who were treated with lusutrombopag prior to invasive treatments (radiofrequency ablation, transarterial chemoembolization, and endoscopic variceal ligation)[79]. In this group, platelet count significantly increased compared with baseline (82000 ± 26000 *vs* 41000 ± 11000 /μL). The proportion of patients who needed platelet transfusions before procedures was very low (only 4, 16%) compared to those not treated with lusutrombopag (69 patients, 54%). Moreover, platelet counts after treatment and before invasive procedures were lower in patients with a count less than 30000/μL, and this cut-off, together with a spleen index > 40 cm^2, was predictive of a lower response rate to the drug[80]. This was probably due to a larger number of platelets sequestered in the spleen in this subgroup of patients. No haemorrhagic complications were observed, and only a single case of recurrent portal vein thrombosis was observed and successfully treated.

Another real-life setting retrospective study was carried out in patients with chronic liver disease and severe thrombocytopenia. In this study[81], 74.2% of patients who received treatment did not require platelet transfusion before invasive procedures. This percentage increased to 82.1% of treatments if patients who repeated lusutrombopag use more times were included, thus demonstrating the efficacy of repeated use of the drug. Furthermore, only one serious adverse event was observed during/after treatment, *i.e.*, one case of portal thrombosis disappearing after anticoagulation. Notably, this study

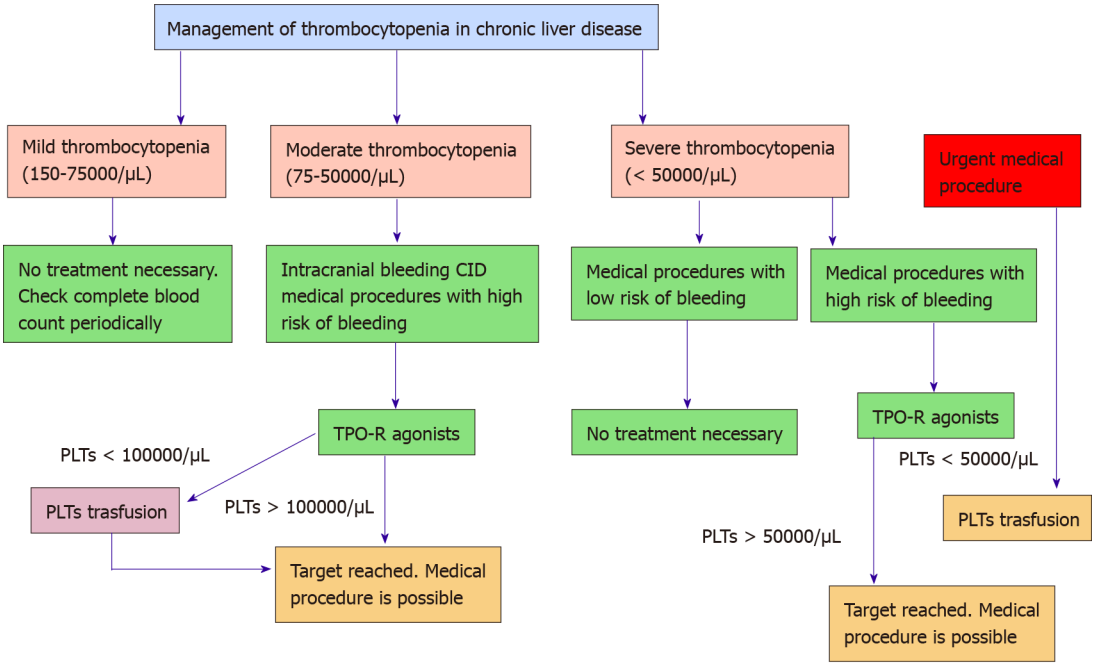

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Figure 4 Operative flow chart for management of thrombocytopenia in chronic liver disease. TPO-R: Thrombopoietin receptor; PLTs: Platelets; CID: Disseminated intravascular coagulation.

confirmed that a lower platelet count at baseline was a predictive factor for failure to reach the target of > 50000/μL platelets. Indeed, median basal platelet count was higher in responders *vs* non-responders (38000/μL *vs* 12000/μL).

Moreover, the safety and efficacy of repeated use of lusutrombopag have been confirmed also in 66 patients who underwent radiofrequency ablation for recurrence of hepatocellular carcinoma[82]. Later, others reports have confirmed the efficacy and safety of lusutrombopag in real life in patients with thrombocytopenia due to chronic liver disease. In a case report, Kaneko *et al*[83] showed lusutrombopag to be a successful substitute for platelet transfusion in a patient with chronic liver disease undergoing endoscopic spinal surgery. Kawata *et al*[84] reported three patients treated with lusutrombopag before tooth extraction: Platelet count increased, preventing the need for transfusion in two of three cases. There were no adverse events. In addition, the efficacy and safety of the drug have been confirmed in a retrospective Japanese study based on hospital administrative databases. Here the incidence of bleeding events was lower in the lusutrombopag group than in the platelet transfusion group (3.7% *vs* 8.2%, $P < 0.001$), with a consequently lower average medical cost[85]. Finally, real-world data for adverse events (spontaneously reported by healthcare professionals and consumers in a database including about 4000 patients exposed to lusutrombopag from December 2015 to April 2018) confirm the efficacy and the safety of the drug (93% of patients did not require pre-procedural platelet transfusion; 1.2% of serious adverse events, with 0.4% cases of portal vein thrombosis)[86].

CONCLUSION

Thrombocytopenia represents one of the main coagulation disorders in patients with chronic liver disease. Recent awareness in its physiopathology shed light on the central role of TPO. The development of TPO receptor agonists has opened a new scenario in the management of patients with liver disease who need invasive procedures (Figure 4). Avatrombopag and lusutrombopag have demonstrated their efficacy and safety in increasing platelet count without an increased risk of thrombosis. Moreover, they reduce the overall clinical risk associated with platelet transfusion without any delay in the management of these patients. With careful logistical planning and coordination between drug availability and medical procedures, patients with chronic liver disease and severe thrombocytopenia should now be able to undergo more easily invasive procedures.

FOOTNOTES

Author contributions: Vespasiani-Gentilucci U and Gallo P conceived the study; Gallo P, Terracciani F, Di Pasquale

G, and Esposito M wrote the manuscript; Picardi A and Vespasiani-Gentilucci U helped in drafting and revising the manuscript.

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ORIGINAL ARTICLE

Basic Study

P2X7 receptor blockade decreases inflammation, apoptosis, and enteric neuron loss during *Clostridioides difficile* toxin A-induced ileitis in mice

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Abstract

BACKGROUND

Clostridioides difficile (*C. difficile*) is the most common pathogen causing health care-associated infections. *C. difficile* TcdA and TcdB have been shown to activate enteric neurons; however, what population of these cells is more profoundly influenced and the mechanism underlying these effects remain unknown.

AIM

To characterize a specific population of TcdA-affected myenteric neurons and investigate the role of the P2X7 receptor in TcdA-induced ileal inflammation, cell death, and the changes in the enteric nervous system in mice.

METHODS
Swiss mice were used to model TcdA-induced ileitis in ileal loops exposed to TcdA (50 μg/Loop) for 4 h. To investigate the role of the P2X7 receptor, Brilliant Blue G (50 mg/kg, i.p.), which is a nonspecific P2X7 receptor antagonist, or A438079 (0.7 μg/mouse, i.p.), which is a competitive P2X7 receptor antagonist, were injected one hour prior to TcdA challenge. Ileal samples were collected to analyze the expression of the P2X7 receptor (by quantitative real-time polymerase chain reaction and immunohistochemistry), the population of myenteric enteric neurons (immunofluorescence), histological damage, intestinal inflammation, cell death (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling), neuronal loss, and S100B synthesis (immunohistochemistry).

RESULTS
TcdA upregulated ($P < 0.05$) the expression of the P2X7 receptor gene in the ileal tissues, increasing the level of this receptor in myenteric neurons compared to that in control mice. Comparison with the control mice indicated that TcdA promoted ($P < 0.05$) the loss of myenteric calretinin+ (Calr) and choline acetyltransferase+ neurons and increased the number of nitrergic+ and Calr+ neurons expressing the P2X7 receptor. Blockade of the P2X7 receptor decreased TcdA-induced intestinal damage, cytokine release [interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α], cell death, enteric neuron loss, and S100B synthesis in the mouse ileum.

CONCLUSION
Our findings demonstrated that TcdA induced the upregulation of the P2X7 receptor, which promoted enteric neuron loss, S100B synthesis, tissue damage, inflammation, and cell death in the mouse ileum. These findings contribute to the future directions in understanding the mechanism involved in intestinal dysfunction reported in patients after *C. difficile* infection.

Key Words: *Clostridioides difficile*; *Clostridioides difficile* toxin A; P2X7 receptor; Enteric nervous system; Enteric neuron; Enteric glia

Core Tip: There is a knowledge gap regarding the population of enteric neurons affected by TcdA and the role of the P2X7 receptor, which is a low-sensitivity adenosine triphosphate-gated cation channel, in TcdA-induced alterations in enteric neurons and enteric glial cell (EGC)-derived mediators, particularly S100B. The findings of the present study demonstrated the mechanism of P2X7 receptor-driven enteric neuronal loss induced by TcdA in the mouse ileum. TcdA promoted the upregulation of the P2X7 receptor, which promoted cell death in enteric neurons and induced the release of proinflammatory mediators, which in turn promoted S100B synthesis in EGCs. However, the blockade of the P2X7 receptor abrogated ileal damage induced by TcdA.

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INTRODUCTION

Clostridioides difficile (*C. difficile*) continues to be the leading cause of nosocomial diarrhea worldwide[1]. TcdA, TcdB, and *C. difficile* binary toxin are the main virulence factors of *C. difficile* infection-related intestinal damage. These toxins have been shown to play an important role in secretory diarrhea and inflammation during the infection[2,3]. The clinical disease ranges from mild diarrhea to toxic megacolon, colonic perforation, and death.

Intestinal dysfunction has been identified in patients after the acute phase of *C. difficile* infection[4-7]. Growing evidence suggests that the enteric nervous system (ENS) plays an important role in the regulation of intestinal inflammation. Alterations in the ENS components, including enteric neurons and glia, contribute to the amplification of inflammatory immune response and intestinal dysfunction under inflammatory conditions.

The P2X7 receptor is a low-sensitivity adenosine triphosphate (ATP)-gated cation channel expressed by several cell types, such as macrophages[8], EGCs[9], and enteric neurons[10]. Once activated, the P2X7 receptor increases the intracellular Ca^{2+} concentrations, which in turn promote the release of proinflammatory cytokines and neuromodulators[11,12]. Additionally, high levels of the P2X7 receptor have been reported in enteric neurons during colitis induced by dinitrobenzene sulfonic acid[13] and intestinal ischemia[10].

TcdA and TcdB have been shown to excite enteric neurons, stimulating the release of substance P and vasoactive intestinal peptide *via* the inhibition of noradrenergic transmission and the interleukin (IL)-1β pathway, respectively, resulting in neutrophil recruitment and secretory diarrhea[14-16]. However, there is a knowledge gap regarding the population of enteric neurons affected by TcdA and the role of the P2X7 receptor in TcdA-induced alterations in enteric neurons and enteric glial cell (EGC)-derived mediators, particularly S100B.

In the present study, we characterized the population of myenteric neurons affected by TcdA during ileitis in mice. In addition, we investigated the role of the P2X7 receptor in ileal damage, inflammation, and enteric glial and neuronal changes in TcdA-induced ileitis in mice. Our hypothesis was that TcdA affects specific types of neurons and induces reactive gliosis and that activation of P2RX7 is involved not only in ileal damage and inflammation but also in the activation of enteric glia and neuronal loss induced by this toxin.

MATERIALS AND METHODS

Animals

Swiss mice (8-week-old) were provided by the central vivarium of the Federal University of Ceara. All mice were maintained under standard conditions at 24 °C at a 12-h light-dark cycle, and all groups were provided water and food *ad libitum*. All mouse procedures were conducted according to current regulations regarding animal experiments approved by the local Animals Care and Use Committee (protocol no. 7028200418).

Mouse ileal loop model

A mouse model of TcdA-induced ileitis was established as described previously with[17] some modifications. Swiss mice (n = 5 *per* group) were fasted for 4 h with free access to water and deeply anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After a midline laparotomy, a single 4-cm ileal loop was ligated and injected with 50 μg of TcdA in 100 μL of phosphate-buffered saline (PBS). The control loops were injected with 100 μL of PBS alone. After 4 h, the mice were euthanized, and the ileal loops were removed for subsequent analysis. Alternatively, some mice were injected with Brilliant Blue G (BBG, Sigma–Aldrich, 50 mg/kg, i.p.)[10], a nonspecific P2X7 receptor antagonist, or with A438079 (Abcam, 10 μM/200 μL, i.p.), a competitive P2X7 receptor antagonist[18], one hour prior to PBS or TcdA (50 μg) injection in the ileal loops. The experimental groups were as follows: Control (loops were injected with 100 μL of PBS alone), TcdA (loops were injected with 50 μg of TcdA in 100 μL of PBS), BBG (injected with BBG one hour prior to the injection of 100 μL of PBS in the loop), A438079 (injected with A438079 one hour prior to the injection of 100 μL of PBS in the loop), TcdA + BBG (injected with BBG one hour prior to the injection of 50 μg of TcdA in 100 μL of PBS), and A438079 (injected with A438079 one hour prior to the injection of 50 μg of TcdA in 100 μL of PBS).

TcdA was provided by Prof. Carlos Quesada from the University of Costa Rica. BBG was kindly provided by Dr. Patricia Castelucci from the University of São Paulo. A43807 was kindly provided by Dr. Henning Ulrich from the University of São Paulo.

Analysis of histological damage in the ileum

The ileal samples were fixed in 10% formalin solution for 20 h and processed by the NEMPI-UFC Research Histology Core. The severity of ileal damage was measured by a blinded expert in histopathology based on a scoring system ranging from 0 to 3 as described previously with some modifications as follows: (0) Absence of alterations; (1) Mild loss of the integrity of the villi, mild edema, and neutrophil infiltration; (2) Partial loss of the villi, moderate edema, and neutrophil infiltration; and (3) Complete loss of the villi, extensive edema, and intense neutrophil infiltration[19].

Analysis of enteric neuron population

Fresh ileal samples were flushed with PBS, dissected, and opened along the mesenteric border. Then, the samples were fixed in 4% paraformaldehyde (in 0.2 M sodium phosphate buffer, pH 7.4) overnight at 4 °C. Then, the samples were washed three times with 100% dimethyl sulfoxide for 10 min, followed by three washes with PBS for 10 min each. All samples were stored at 4 °C in PBS containing 0.1% sodium azide. The fixed tissues were dissected to remove the mucosa, submucosa, and circular layers, yielding longitudinal muscle-myenteric plexus whole mounts as described previously[10].

Table 1 Primary and secondary antibodies used

Antigen	Host	Dilution	Manufacturer
nNOS	Sheep	1:2000	Millipore (AB1529/Lot 2488802)
ChAT	Goat	1:50	Millipore (AB144P/Lot 1978747)
P2X7 receptor	Rabbit	1:100	Millipore (AB5246/Lot 2361386)
Calretinin	Goat	1:100	Molecular
Donkey anti-rabbit IgG Alexa 594	Donkey	1:200	Probes (A21206/Lot 1182675)
Donkey anti-sheep IgG Alexa 488	Donkey	1:400	Molecular
			Probes (A11016/Lot93D1-1)

nNOS: Neuronal nitric oxide synthase; ChAT: Choline acetyltransferase; IgG: Immunoglobulin G.

Whole-mount preparations of the ileal myenteric samples were preincubated in 10% horse serum in PBS containing 1.5% Triton X-100 for 45 min at room temperature to reduce nonspecific binding and permeabilize the tissue. The antibodies used in the present study are described in Table 1. Double labeling was achieved using the combinations of primary antibodies (Table 1) overnight at 4 °C. Then, the samples were washed (with PBS three times for 10 min each) and incubated with secondary antibodies (Table 1). After washing with PBS, the samples were mounted in glycerol buffer (in 0.5 M sodium carbonate, pH 8.6). The immunostaining images were acquired using confocal microscopy by a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. The images were acquired at a resolution of 512 × 512 pixels, and the thickness of each optical section was 0.5 μm. The Z-stacks of immunoreactive cells were captured as a series of optical sections with a center spacing of 0.2 μm. Confocal images were collected using Zeiss LSM 5 image processing software and further processed using Corel Photo Paint and Corel Draw software[10].

Quantitative analysis of myenteric neuron immunostaining

The antigen colocalization was determined in fluorescently labeled preparations. Initially, the neurons were identified by immunofluorescence. Then, the filter was switched, and the labeling of the second antigen was evaluated. The proportion of the neurons labeled with the antigen pairs was thus determined. The cohort size was 100 neurons, and the data were collected from the preparations obtained from five mice *per* experimental group. The percentage of double immunoreactive neurons was calculated and is expressed as the mean ± standard error of the mean (SEM). The density of the neurons immunoreactive (neurons/cm^2) to the P2X7 receptor, neuronal nitric oxide synthase (nNOS), calretinin (Calr), and choline acetyltransferase (ChaT) and neuronal morphological profiles were assessed in the whole-mount preparations at 100 × magnification. The number of the cell bodies of immunoreactive neurons in the myenteric ganglia in each visual microscopic field (0.04909 mm^2) was estimated. To quantify two whole-mount preparations (1.0 cm^2 each), the counts were estimated in 40 microscopic fields selected at random for each antigen in each animal. The perikaryon profile areas (μm^2) of 50 randomly selected neurons from each animal were obtained using a semiautomatic morphometry device and measured using the Image-Pro Plus software package.

Immunohistochemistry

Immunostaining of S100B (an enteric glia-derived mediator), HuC/D (a neuronal marker), and the P2X7 receptor was performed in paraffin-embedded ileal formalin-fixed sections (4-μm thick) using the streptavidin-biotin-peroxidase method; the sections were mounted on poly(L)-lysine-coated microscope slides as described previously[20]. Briefly, the samples were deparaffinized and rehydrated by incubation with xylene and graded alcohol solutions, respectively. Then, the samples were immersed in antigen retrieval solution (*EnVision™ FLEX target retrieval solution, pH = 6.0; Dako, Denmark A/S*) for 20 min on a PT Link system (Dako), incubated in 3% hydrogen peroxide (*EnVision™ FLEX peroxidase-blocking reagent; Dako)* to block endogenous peroxidase for 15 min at room temperature, and washed with PBS. Then, the samples were incubated with primary antibodies (rabbit anti-P2X7 receptor (Invitrogen), mouse anti-HuC/D (Invitrogen), or goat anti-S100B (Santa Cruz Biotechnology, 1:100) in antibody diluent solution (*EnVisionTM FLEX antibody diluent; Dako*) overnight at 4 °C. Then, the samples were incubated with *EnVision™ FLEX/HRP (Dako)* as recommended by the manufacturer. P2X7 receptor, HuC/D, and S100B were detected using the chromogen 3,3'-diaminobenzidine (DAB, *EnVision™ FLEX DAB+ chromogen; Dako*). The negative control sections were processed simultaneously as described above; however, the primary antibody was replaced with antibody diluent solution (*EnVision™ FLEX antibody diluent; Dako*). The slides were counterstained with Mayer's hematoxylin. The images were acquired by a Leica DM100 microscope and analyzed using Adobe Photoshop 8.0 software. The

percentages of P2X7 receptor-, S100B- and HuC/D-stained tissue sections were measured by using Adobe Photoshop as described previously[20].

Total RNA extraction, reverse transcription, and real-time polymerase chain reaction

Total RNA was isolated from the ileum using an *Aurum™ total RNA fatty and fibrous tissue* kit (Bio-Rad, CA, United States), and 1 μg of the RNA was reverse transcribed using iScript™ (Bio-Rad) according to the manufacturer's instructions. Real-time polymerase chain reaction (qPCR) was performed on a 7900HT fast real-time PCR system (Applied Biosystems) using the following specific primers (IDT, Coralville, IA): P2X7 receptor (forward: GCACGAATTATGGCACCGTC and reverse: CCCCACCCTCT-GTGACATTCT) and GAPDH (forward: TGCACCACCAACTGCTTAG and reverse: GGATGCAGG-GATGATGTTC)[21]. The reaction mixture was prepared in a final volume of 20 μL as follows: 10 μL of master mix iQTM SYBR® Green (Applied Biosystems), 2 μL of each primer (200 nM), 1 μL of cDNA, and 5 μL of nuclease-free water. The gene amplification included the following steps: 10 min at 95 °C (initial denaturation), 15 s at 95 °C and 60 s at 60 °C for 40 cycles; thus, a melting curve was obtained. Relative gene expression was determined using the 2-Δ Δ Ct method with GAPDH as a housekeeping gene.

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assay

Ileal samples were processed for terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) using an *ApopTagR S 7100* kit (Merck Millipore, Germany) to quantify apoptotic and necrotic cells. Briefly, paraffin-embedded sections were hydrated and incubated with proteinase K (Sigma, United States, 20 mg/mL) for 15 min at room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 5 min at room temperature. After a washing step, the sections were incubated with TdT buffer containing TdT enzyme and reaction buffer in a humidified chamber at 37 °C for 1 h. The specimens were incubated for 10 min at room temperature with stop/wash buffer and then incubated with an anti-digoxigenin–peroxidase conjugate at room temperature in a humidified chamber for 30 min. After washing with PBS, the slides were covered with peroxidase substrate (DAB) to develop the color and were counterstained with methyl green.

Cytokine quantification by enzyme-linked immunosorbent assay

To measure inflammatory markers, the ileal samples were processed to evaluate the levels of IL-1β, IL-6, keratinocyte chemoattractant (KC, a human IL-8 analog), and tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay (ELISA) using a mouse cytokine kit (R&D Systems) according to the manufacturer's instructions. The absorbance of the samples was detected at 450 nm using an ELISA plate reader (Biotech Epoch, United States). The data are expressed as pg *per* mg of tissue.

Statistical analysis

The results are expressed as the mean ± SEM calculated by GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA). The differences between more than two experimental groups were evaluated using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Student's t test was performed to analyze the differences between two groups. The histopathological score data were compared by using Kruskal–Wallis nonparametric test followed by Dunn's test. Statistical significance was defined as $P < 0.05$.

RESULTS

TcdA upregulates the P2X7 receptor transcripts in the ileum of mice and increases the population of myenteric enteric neurons expressing the P2X7 receptor

Initially, we investigated whether TcdA alters the expression of the *P2RX7* gene in the ileum of mice by using qPCR. We demonstrated that TcdA upregulated the P2X7 receptor in the ileum of mice compared with that in control mice ($P < 0.05$, Figure 1A). The data of the assay of the P2X7 receptor protein by immunofluorescence showed an increase in the percentage of positive P2X7 receptor immunostaining in the ileal samples of TcdA-challenged mice compared with that in the control samples ($P < 0.05$, Figure 1B). An increase in the expression of the P2X7 receptor was observed in epithelial cells, the lamina propria, and the myenteric plexus (Figure 1C).

Enteric neurons are an important component of the myenteric plexus, which is a part of the ENS; hence, we investigated whether the level of the P2X7 receptor is increased in these cells in the myenteric plexus by using immunofluorescence analysis. Comparison with the control group indicated an increase in the density of enteric neurons expressing the P2X7 receptor in the ileum myenteric plexus in mice challenged with TcdA ($P = 0.01$, Figure 1D and E).

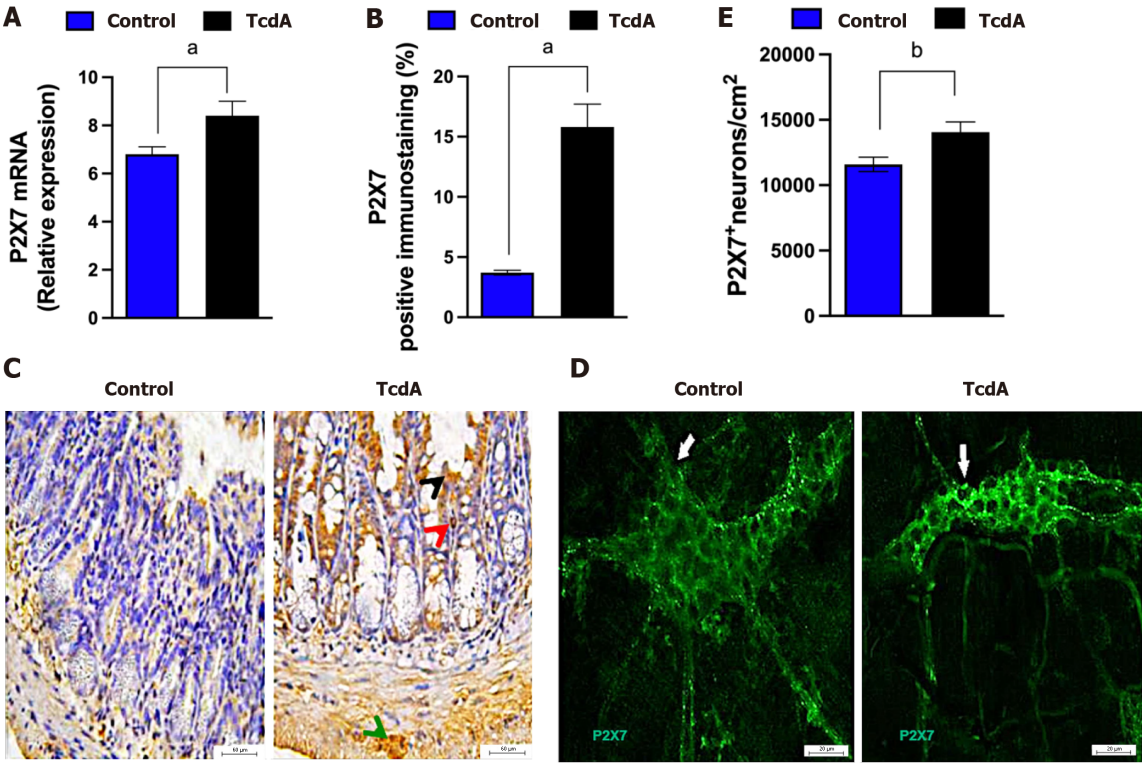

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Figure 1 *Clostridioides difficile* toxin A increases the expression of the P2X7 receptor in the ileum myenteric plexus of mice. A: The expression of the *P2RX7* gene [mean ± standard error of the mean (SEM)] assayed by quantitative real-time polymerase chain reaction in the ileal samples of mice injected with TcdA [TcdA, 50 μg in phosphate-buffered saline (PBS)] or PBS alone in the ileal loops (Control) (n = 4); B: Quantification of the percentage (mean ± SEM) of the P2X7 receptor-immunopositive area in the ileum from control and *TcdA*-challenged mice in 5-6 microscope fields *per* sample (n = 4 animals *per* group); C: Representative immunohistochemical images of the expression of the P2X7 receptor in the ileum of control and *TcdA*-challenged mice. Increased expression of the P2X7 receptor (arrowhead) was detected in the intestinal epithelial layer (black arrowhead), lamina propria (red arrowhead), and myenteric plexus (green arrowhead). Scale bars, 50 μm; D: Representative photomicrographs ofimmunostaining of the P2X7 receptor (arrow indicates the region stained green) in the ileum myenteric plexus from control and *TcdA*-challenged mice; E: Quantification of the number of P2RX7$^+$ neurons/cm^2 (mean ± SEM) in the ileum of control and *TcdA*-challenged mice (n = 4 animals *per* group); A, B and E: Unpaired two-tailed Student's t test ($^aP < 0.05$; $^bP = 0.01$).

TcdA decreases the density of enteric Calr+ and ChaT+ neurons in the ileum myenteric plexus of mice

Subsequently, to better understand how TcdA affects the myenteric enteric neuron population, we immunostained the ileum myenteric plexus to detect nNOS, Calr, and ChaT, which were stained in the main population of enteric neurons. As shown in Figure 2A, the density of Calr+ ($P < 0.03$) and ChaT+ neurons ($P < 0.002$) in the ileum myenteric plexus of mice challenged with TcdA was decreased compared to that in control mice. In addition, all these subtypes of the neurons expressed the P2X7 receptor (Figure 2B).

Comparison with the control group of mice indicated that the enteric neuron population expressing the P2X7 receptor in the ileum myenteric plexus had a higher number of nNOS+P2RX7+ and Calr+P2RX7+ neurons, but not of ChaT+P2RX7+ neurons ($P < 0.05$, Figure 2C-E).

Overall, these findings indicated that TcdA decreased the enteric neuron population, specifically Calr+ and ChaT+ cells and upregulated the P2X7 receptor in a specific population (nNOS and Calr) of the neurons in the ileum myenteric plexus in mice.

Blockade of the P2X7 receptor decreases ileal damage induced by TcdA in mice

Then, we blocked the P2X7 receptor by pretreating mice using a pharmacological approach, including administration of BBG and A438079 one hour prior to the challenge with TcdA to determine whether P2X7 receptor activity is required for ileal damage induced by TcdA. Hematoxylin and eosin-stained slides of ileum samples were analyzed for evidence of epithelial damage, edema, and neutrophil infiltration, with a maximal severity score of 3 (Figure 3). TcdA induced complete epithelial disruption, extensive edema, and intense neutrophil infiltration in the ileum of mice, resulting in a high damage score (score = 3) compared with those in the undamaged ileum in control mice ($P < 0.007$, Figure 3). However, both P2X7 receptor antagonists (BBG and 438079) induced a substantial decrease in the ileal damage promoted by TcdA, resulting in a reduction in the damage score (score = 1) compared to that in untreated TcdA-challenged mice ($P < 0.04$, Figure 3).

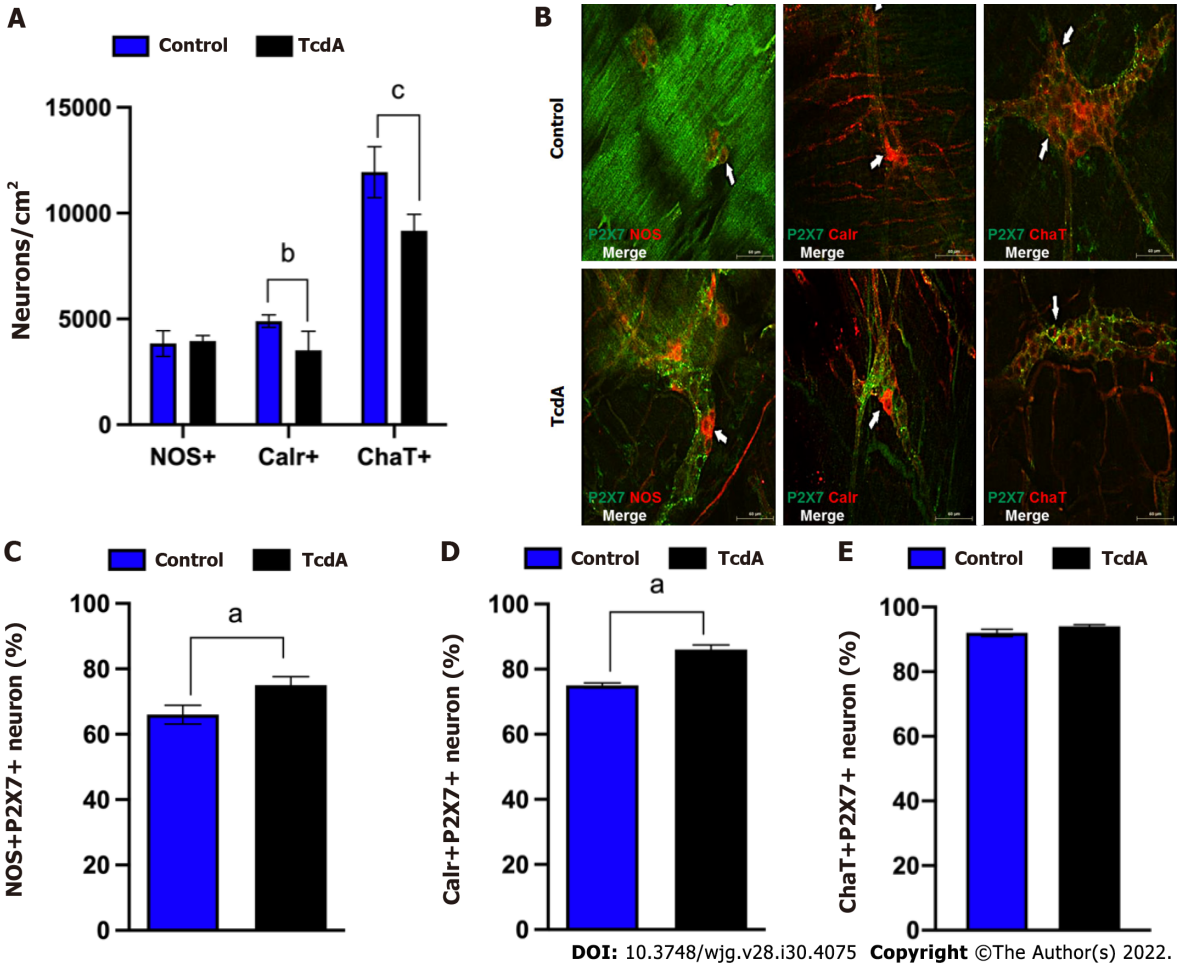

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Figure 2 *Clostridioides difficile* toxin A induces alterations in enteric neuronal coding in the myenteric plexus in the ileum of mice. A: Quantification of the number of neuronal nitric oxide synthase+ (nNOS+), calretinin+ (Calr+), and choline acetyltransferase+ (ChaT+) neurons/cm^2 [mean ± standard error of the mean (SEM)] in the ileum myenteric plexus in control and *TcdA*-challenged mice; B: Representative photomicrographs of the P2X7 receptor (green), nNOS (left panels, red), Calr (center panels, red), and ChaT (right panel, red) immunostaining and DAPI (blue) nuclear staining in control and *TcdA*-challenged mice. White arrows indicate positive immunostaining. Scale bars, 50 μm; C-E: Percentage of NOS$^+$P2X7$^+$ neurons (C), Calr$^+$P2RX7$^+$ neurons (D), and ChaT$^+$P2RX7$^+$ neurons (E) (mean ± SEM) in the ileum myenteric plexus of control and *TcdA*-challenged mice (n = 4); A, C, D and E: Unpaired two-tailed Student's t test ($^aP < 0.05$; $^bP < 0.03$; $^cP < 0.002$). NOS: Nitric oxide synthase; Calr: Calretinin; ChaT: Choline acetyltransferase.

Blockade of the P2X7 receptor decreases ileal inflammation and cell death induced by TcdA in mice

Subsequently, we evaluated whether P2X7 receptor activity is involved in ileal inflammation and cell death induced by TcdA in mice. We demonstrated that both P2X7 receptor blockers (BBG and A438079) reversed a TcdA-induced increase in IL-1β (P = 0.008 and P = 0.03, Figure 4A), TNF-α (P = 0.0002 and P = 0.0001, Figure 4B), and IL-6 (P = 0.03 and P < 0.0001, Figure 4C) in the ileal samples of mice. However, comparison with TcdA-challenged mice, which were not pretreated with the blockers, indicated that blockade of the P2X7 receptor by A438079, but not by BBG, decreased the levels of KC (P = 0.01, Figure 4D) and the number of TUNEL+ cells (P = 0.01, Figure 4E) in the ileum of mice challenged with TcdA.

Overall, these data indicated that the P2X7 receptor was involved in intestinal damage, inflammation, and cell death induced by TcdA in mice.

Blockade of the P2X7 receptor decreases enteric neuron loss and S100B synthesis induced by TcdA in mice

Since we demonstrated that TcdA promoted a decrease in ileum enteric neurons in mice, we assessed whether the P2X7 receptor accounts for this alteration. Comparison with TcdA-challenged mice, which were not pretreated with the blockers, indicated that a P2X7 receptor blocker (A438079) increased the percentage of positive immunostaining of HuC/D, which is a pan-marker of enteric neurons, in the ileum of mice challenged with TcdA (Figure 5).

Furthermore, we evaluated whether the activation of the P2X7 receptor is required to induce S100B expression in the ileum of TcdA-challenged mice; high levels of S100B are released by EGCs under inflammatory conditions. As shown in Figure 5, the P2X7 receptor antagonist A438079 induced a

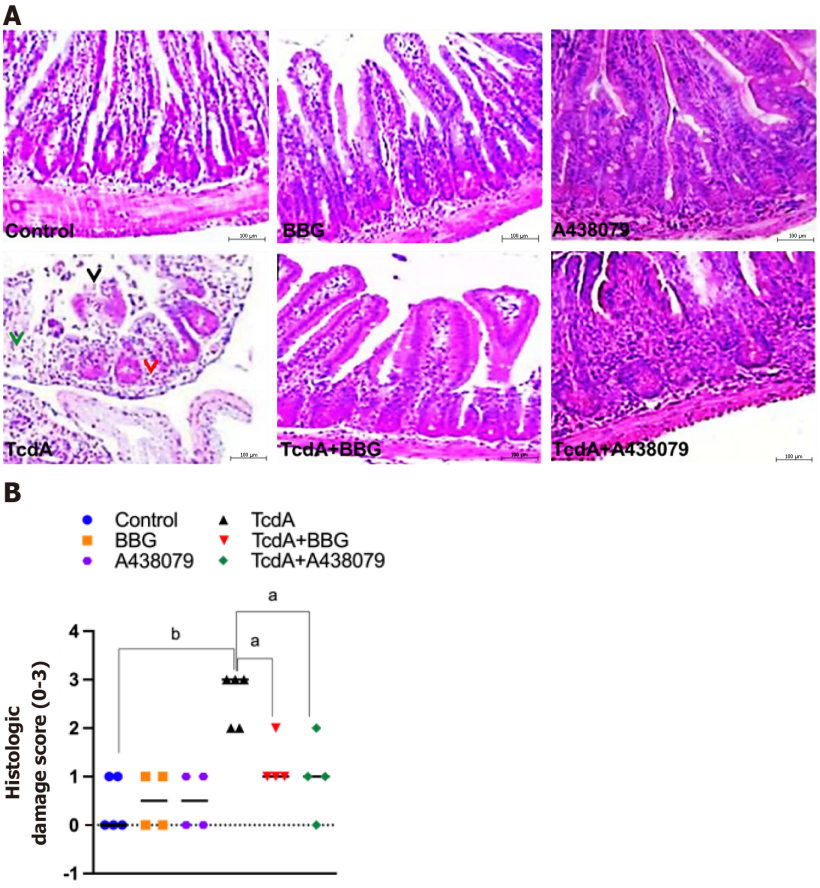

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Figure 3 Inhibition of the P2X7 receptor decreases *Clostridioides difficile* toxin A-induced ileal damage in mice. Mouse ileal loops were injected with TcdA [TcdA, 50 μg in phosphate-buffered saline (PBS)] or PBS alone (control) (n = 5), and the animals were pretreated with a P2X7 receptor antagonist [Brilliant Blue G (BBG) or A438079] one hour prior to TcdA challenge. A: Representative histological images (magnification of 200 ×) of TcdA-unchallenged (control, BBG, and A438079) and challenged (TcdA, TcdA + BBG, and TcdA + A438079) mice. TcdA induced epithelial disruption (black arrowhead), edema (green arrowhead), and neutrophil infiltration (red arrowhead); B: Histopathological score (median; 0 corresponds to-no damage and 3 corresponds to intense damage) performed by a blinded histopathological expert and based on epithelial damage, submucosal edema, and infiltration of inflammatory cells. Kruskal–Wallis nonparametric test followed by Dunn's test. $^{a}P < 0.04$; $^{b}P < 0.007$. BBG: Brilliant Blue G.

considerable decrease in the percentage of S100B-positive immunostaining in the ileum of mice challenged with TcdA compared with that in the ileum TcdA-challenged mice, which were not pretreated with the blockers (P = 0.009).

Overall, these data indicated that the P2X7 receptor was involved in enteric neuronal loss and S100B synthesis induced by TcdA in mice.

DISCUSSION

The data of the present study indicated that TcdA upregulated the P2X7 receptor in the ileum of mice. An increase in the expression of P2RX7 has been reported in colonic biopsies from Crohn's disease patients[21] and in preclinical models of intestinal inflammation, such as colitis induced by trinitrobenzene sulfonic (TNBS) acid[22] and sepsis[23]. Thus, the upregulation of the P2X7 receptor is a common phenomenon under inflammatory conditions[24].

In the present study, we also demonstrated that the level of the P2X7 receptor was increased in the epithelial layer, lamina propria, and myenteric plexus. In the myenteric plexus, we detected an increase in the density of neurons expressing the P2X7 receptor, including the nNOS+ and Calr+ subtypes. In addition to enteric neurons, other cell types can express the P2X7 receptors, such as mast cells, T cells, and dendritic cells[21]. However, we focused on enteric neurons in the myenteric plexus because this component of the ENS is a major functional unit of the system that moves the luminal contents along the intestine by coordinating muscle contraction and relaxation[25]. In addition, *C. difficile* infection is characterized by intense diarrhea in the acute phase of the disease, and the mechanism of these events is poorly understood.

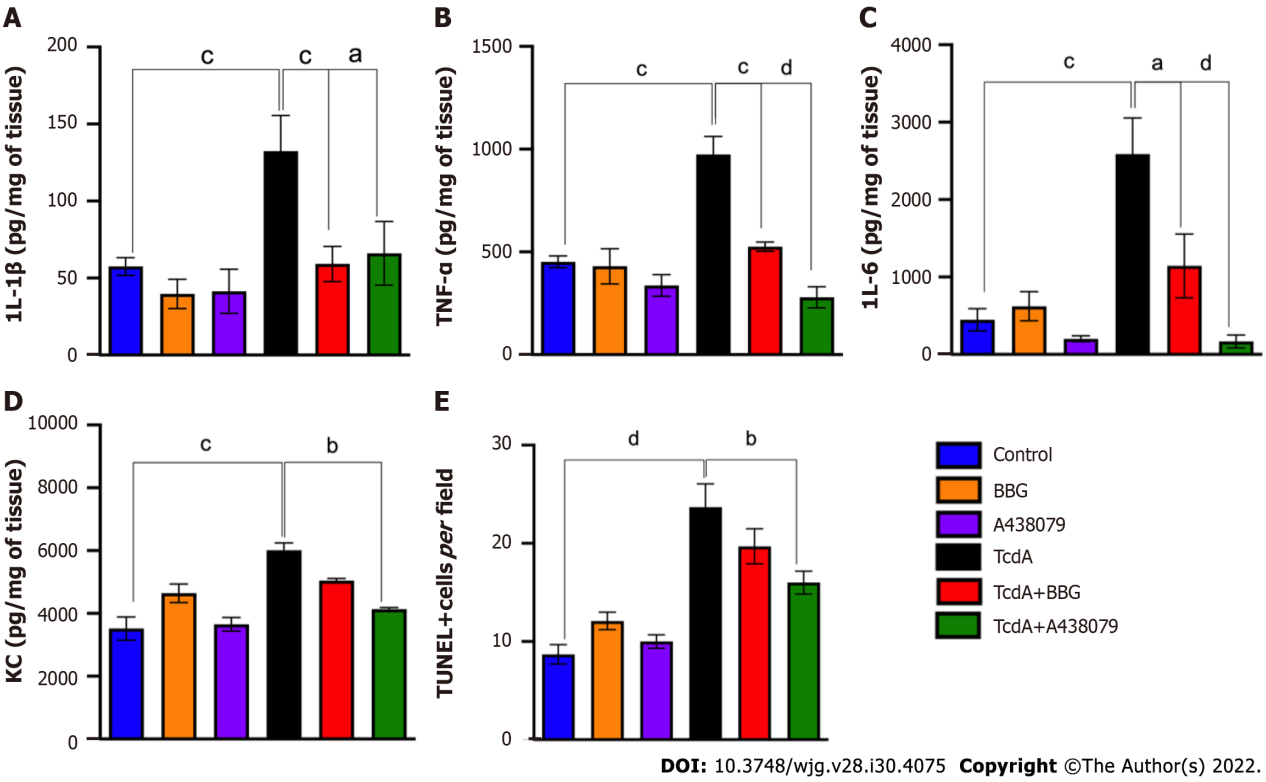

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Figure 4 P2X7 receptor inhibition abrogates *Clostridioides difficile* toxin A-induced inflammation and cell death in mice. A-D: The levels of interleukin (IL)-1β (A), tumor necrosis factor-α (B), IL-6 (C), and keratinocyte chemoattractant (D) in the ileal samples of TcdA-unchallenged [control, Brilliant Blue G (BBG), and A438079] and challenged (TcdA, TcdA + BBG, and TcdA + A438079) mice measured by ELISA (n = 5); E: The number of TUNEL+ cells *per* field in the ileal samples of TcdA-unchallenged (control, BBG, and A438079) and challenged (TcdA, TcdA + BBG, and TcdA + A438079) mice. The data are expressed as the mean ± standard error of the mean. ANOVA followed by Tukey's test was used (^{a}P = 0.03; ^{b}P = 0.01; ^{c}P < 0.008; ^{d}P < 0.0001). IL: Interleukin; TNF: Tumor necrosis factor; BBG: Brilliant Blue G; KC: Keratinocyte chemoattractant; TUNEL: Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling.

We demonstrated that TcdA promoted neuron loss specifically by reducing the density of the Calr+ and ChaT+ neuronal populations. Acetylcholine, which is synthesized in a reaction of choline with acetyl-CoA catalyzed by ChaT, is the primary transmitter in excitatory motor neurons, intrinsic afferent neurons, and interneurons, and Calr is the primary transmitter in excitatory cholinergic neurons[26]. Excitatory motor neurons are involved in coordinated muscle contraction[25]; thus, a reduction in the density of Calr+ and ChaT+ neuronal populations induced by TcdA may be involved in the functional disorders manifested after *C. difficile* infection. Accordingly, a study performed in the United States military personnel reported functional gastrointestinal disorders (including gastroesophageal reflux disease, dyspepsia, irritable bowel syndrome, or constipation) after *C. difficile* infection recovery[6]. In the present study, alterations in the myenteric enteric neuron population induced by TcdA could have been related to these post-*C. difficile* infection-related intestinal dysfunctions. However, more studies are needed to better understand how these alterations specifically contribute to intestinal dysfunction induced by *C. difficile* infection.

In the present study, we also showed that the activation of the P2X7 receptor was involved in the TcdA-induced loss of enteric neurons because inhibition of the receptor by known P2RX7 antagonists (BBG and A438079) resulted in a substantial decrease in the loss of these ENS cells during ileitis induced by TcdA. In agreement with these data of the present study, another study demonstrated that the activation of P2RX7 promotes cell death in mucosal regulatory T cells in colitis induced by TNBS[27]. The P2X7 receptor regulates cell death pathways, such as apoptosis, pyroptosis, necrosis, and autophagy[28].

ATP released from dead cells can increase the activation of the P2X7 receptor and promote the secretion of proinflammatory cytokines, such as IL-1β, which in turn can induce the secretion of other cytokines[29,30]. In the present study, the blockade of the P2X7 receptor markedly decreased IL-1β, IL-6, KC, and TNF-α synthesis in the TcdA-challenged mouse ileum, suggesting that this receptor plays an important role in inflammation induced by *C. difficile* toxin. Similarly, in a model of TNBS-induced colitis, P2X7 receptor blockade reduces the severity of inflammation by decreasing the infiltration of macrophages in the lamina propria[31]. In contrast, deletion of P2RX7 increases the susceptibility to toxoplasmic ileitis[32], suggesting that the activation of this receptor plays a role in the response against intracellular pathogens. In contrast, *C. difficile* releases toxins, which in turn enter the cells to inhibit Rho GTPases, and the P2RX7 antagonists have positive effects in this case.

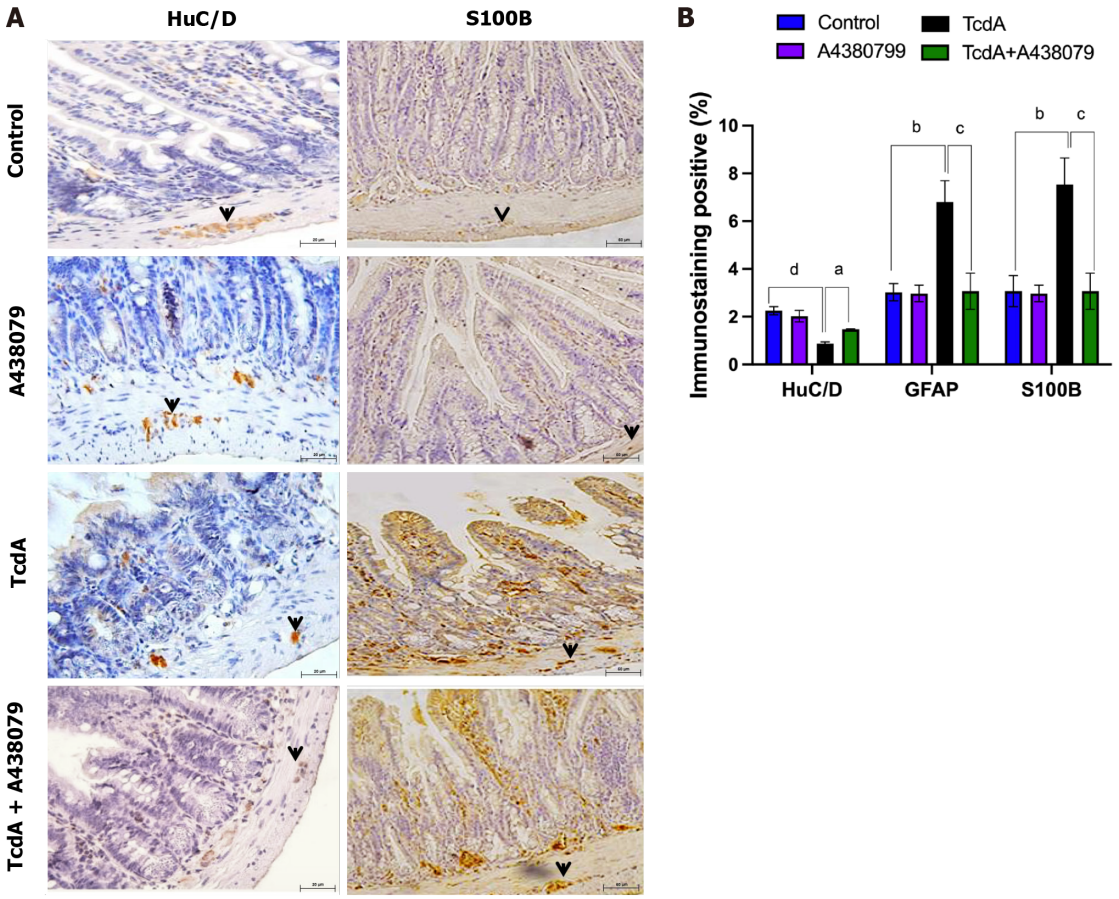

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Figure 5 P2X7 receptor inhibition attenuates *Clostridioides difficile* toxin A-induced enteric neuron loss and S100B synthesis in mice. A: Representative immunohistochemical images of the expression of HuC/D (neuronal marker) and S100B (enteric glia marker) in the ileum of TcdA-unchallenged (control and A438079) and challenged (TcdA and TcdA + A438079) mice. Black arrows indicate positive immunostaining. Scale bars, 50 μm; B: Quantification of the percentage (mean ± standard error of the mean) of HuC/D- and S100B-immunopositive areas in the ileum of TcdA-unchallenged (control and A438079) and challenged (TcdA and TcdA + A438079) mice (n = 4 animals *per* group). ANOVA followed by Tukey's test was used (^aP = 0.04; ^bP = 0.01; ^cP < 0.009; ^dP < 0.0001). GFAP: Glial fibrillary acidic protein.

In addition, we demonstrated that blockade of the P2X7 receptor decreased S100B synthesis in the ileum of mice challenged with TcdA. S100B functions as a proinflammatory mediator when released at higher levels by activating the nuclear activation factor-κB[20] and is an important mediator during *C. difficile* infection[33]. In the myenteric plexus, EGCs express S100B[34] and are involved in the control of motility and epithelial barrier[35]. In a rat glioblastoma cell line, IL-6 promotes S100B synthesis[36]. In the present study, a reduction in proinflammatory cytokines related to P2X7 receptor blockade could have contributed to a decrease in S100B synthesis induced by TcdA, which in turn reduced intestinal inflammation and neuronal death.

Additional studies are needed, for example, using a *C. difficile* infection model, to explore how P2RX7 blockage can influence the *C. difficile* infection outcome and to better understand physiological benefits of this blockade for relief of intestinal permeability and dysmotility during the infection. However, it is important to emphasize that investigations of the role of this receptor in the damaging effects induced by one of the main virulence factors released by *C. difficile* will help to understand the pathogenesis of these effects and to develop alternative cotreatments to control the deleterious and exacerbated host response to the *C. difficile* toxins.

CONCLUSION

In conclusion, the results of the present study revealed the mechanism of P2X7 receptor-driven loss of enteric neurons induced by TcdA in the mouse ileum. TcdA promoted the upregulation of the P2X7 receptor, which promoted cell death in enteric neurons and induced the release of proinflammatory mediators (IL-1β, IL-6, KC, and TNF-α) in epithelial/immune cells, which in turn promoted S100B synthesis in EGCs. However, blockade of the P2X7 receptor abrogated ileal damage induced by TcdA (Figure 6). Overall, the findings of the present study open new avenues to better understand how *C.*

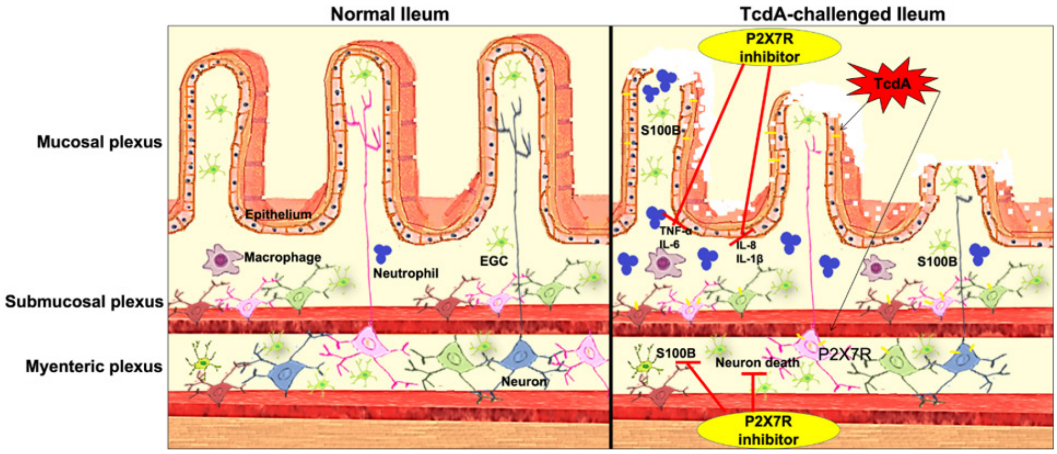

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Figure 6 Scheme of the hypothetical role of the P2X7 receptor during *Clostridioides difficile* toxin A-induced ileitis. *Clostridioides difficile* TcdA promotes epithelial damage and the upregulation of the P2X7 receptor in enteric neurons, intestinal epithelial cells, and immune cells. Once activated, the P2X7 receptor promotes the death of enteric neurons and the release of proinflammatory mediators by epithelial/immune cells [interleukin (IL)-1β, IL-6, keratinocyte chemoattractant, and tumor necrosis factor-α]. These proinflammatory mediators induce S100B synthesis by enteric glial cells. Blockade of the P2X7 receptor using a pharmacological approach attenuates these deleterious effects of TcdA. IL: Interleukin; TNF: Tumor necrosis factor; EGC: Enteric glial cell.

difficile toxins promote the changes in the ENS components that can be related to intestinal dysfunction after *C. difficile* infection.

ARTICLE HIGHLIGHTS

Research background

The P2X7 receptor, a low-sensitivity adenosine triphosphate-gated cation channel, is expressed in several cell types, including enteric neurons. Once activated, the P2X7 receptor promotes the release of proinflammatory cytokines and neuromodulators. High levels of P2X7 receptor have been reported in enteric neurons during experimental colitis.

Research motivation

There is a knowledge gap regarding the population of enteric neurons affected by TcdA and the role of the P2X7 receptor in TcdA-induced alterations in enteric neurons and enteric glial cell-derived mediators, particularly S100B.

Research objectives

We characterized the population of myenteric neurons affected by TcdA during ileitis in mice. In addition, we investigated the role of the P2X7 receptor in ileal damage, inflammation, and the changes in enteric glia and neurons in TcdA-induced ileitis in mice.

Research methods

Swiss mice were used to model TcdA-induced ileitis in ileal loops exposed to TcdA (50 μg/Loop) for 4 h. To investigate the role of the P2X7 receptor Brilliant Blue G (50 mg/kg, i.p.), which is a nonspecific P2X7 receptor antagonist or A438079 (0.7 μg/mouse, i.p.), which is a competitive P2X7 receptor antagonist, were injected one hour prior to TcdA challenge. Ileum samples were collected to analyze the expression of the P2X7 receptor (quantitative real-time polymerase chain reaction and immunohistochemistry), the population of myenteric enteric neurons (immunofluorescence), histological damage, intestinal inflammation, cell death (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling), neuronal loss, and S100B synthesis (immunohistochemistry).

Research results

TcdA upregulated ($P < 0.05$) the expression of the P2RX7 gene in the ileal tissues, increasing the level of this receptor in myenteric enteric neurons compared with that in control mice. Comparison with control mice indicated that TcdA promoted ($P < 0.05$) the loss of myenteric calretinin+ (Calr) and choline acetyltransferase+ neurons and increased the number of nitrergic+ (nitric oxide synthase+) and Calr+ neurons expressing the P2X7 receptor. Blockade of the P2X7 receptor decreased TcdA-induced intestinal damage, cytokine release (interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α), cell death, enteric

neuron loss, and S100B synthesis in the mouse ileum.

Research conclusions

The findings of the present study demonstrated that TcdA induced the upregulation of the P2X7 receptor, which promoted enteric neuron loss, S100B synthesis, tissue damage, inflammation, and cell death in the ileum of mice.

Research perspectives

These findings contribute to future directions in understanding the mechanism involved in intestinal dysfunction reported in patients after *Clostridioides difficile* infection.

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FOOTNOTES

Author contributions: Santos AAQA and Costa DVS contributions equally; Santos AAQA participated in the design and performed the experiments, analyzed the data and wrote the manuscript; Costa DVS analyzed the data and wrote the manuscript; Foschetti DA, Duarte ASG, Martins CS, and Soares PMG helped in the acquisition of the data and review of the manuscript; Castelucci P participated in the initial experimental design and helped to revise the manuscript; Brito GAC, the main investigator of the laboratory where the experiments were performed, conceptualized the main ideas, supervised the study and reviewed the manuscript.

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ORIGINAL ARTICLE

Case Control Study

Serological profiling of Crohn's disease and ulcerative colitis patients reveals anti-microbial antibody signatures

Mahasish Shome, Lusheng Song, Stacy Williams, Yunro Chung, Vel Murugan, Jin G Park, William Faubion, Shabana F Pasha, Jonathan A Leighton, Joshua LaBaer, Ji Qiu

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Abstract

BACKGROUND

The healthcare burden of inflammatory bowel disease (IBD) is rising globally and there are limited non-invasive biomarkers for accurate and early diagnosis.

AIM

To understand the important role that intestinal microbiota play in IBD pathogenesis and identify anti-microbial antibody signatures that benefit clinical management of IBD patients.

METHODS

We performed serological profiling of 100 Crohn's disease (CD) patients, 100 ulcerative colitis (UC) patients and 100 healthy controls against 1173 bacterial and 397 viral proteins from 50 bacteria and 33 viruses on protein microarrays. The study subjects were randomly divided into discovery (n = 150) and validation (n = 150) sets. Statistical analysis was performed using R packages.

RESULTS

Anti-bacterial antibody responses showed greater differential prevalence among the three subject groups than anti-viral antibody responses. We identified novel

antibodies against the antigens of *Bacteroidetes vulgatus* (BVU_0562) and *Streptococcus pneumoniae* (SP_1992) showing higher prevalence in CD patients relative to healthy controls. We also identified antibodies against the antigen of *Streptococcus pyogenes* (SPy_2009) showing higher prevalence in healthy controls relative to UC patients. Using these novel antibodies, we built biomarker panels with area under the curve (AUC) of 0.81, 0.87, and 0.82 distinguishing CD *vs* control, UC *vs* control, and CD *vs* UC, respectively. Subgroup analysis revealed that penetrating CD behavior, colonic CD location, CD patients with a history of surgery, and extensive UC exhibited highest antibody prevalence among all patients. We demonstrated that autoantibodies and anti-microbial antibodies in CD patients had minimal correlation.

CONCLUSION

We have identified antibody signatures for CD and UC using a comprehensive analysis of anti-microbial antibody response in IBD. These antibodies and the source microorganisms of their target antigens improve our understanding of the role of specific microorganisms in IBD pathogenesis and, after future validation, should aid early and accurate diagnosis of IBD.

Key Words: Inflammatory bowel disease; Anti-microbial antibody; Protein microarray; Crohn's disease; Ulcerative colitis; Gut microbiome

Core Tip: We performed the largest serological profiling of anti-microbial antibodies to date in using 100 Crohn's disease (CD) and 100 ulcerative colitis (UC) patients. We identified novel anti-microbial antibodies with differential prevalence in inflammatory bowel disease (IBD) patients compared with healthy controls. There was minimal correlation between anti-microbial antibodies and our previously reported autoantibodies in CD patients. We combined novel anti-microbial antibodies to build biomarker panels distinguishing CD *vs* control, UC *vs* control and CD *vs* UC with an area under the curve of 0.81, 0.87, and 0.82, respectively. Subgroup analysis revealed that IBD patients with severe disease had the highest antibody prevalence.

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INTRODUCTION

Inflammatory bowel disease (IBD) represents a group of intestinal disorders that causes chronic inflammation in the digestive tract. The two main clinical phenotypes are ulcerative colitis (UC) and Crohn's disease (CD). The public health burden of IBD is rising globally[1]. Early and accurate diagnosis is key to reducing this burden. Gastroenterologists often use a combination of relatively invasive procedures, like ileocolonoscopy with biopsy for diagnosis, and to determine the disease extent and activity. There is a need for serological biomarkers that can reveal the disease state non-invasively. Herein, our objectives were to discover anti-microbial antibody signatures in IBD patients and understand the association of microbial infection with IBD pathogenesis.

IBD is caused by a combination of genetic predisposition, faulty immune responses, and environmental factors[2]. The interaction of microbes with the gut mucosa in a genetically susceptible individual and the corresponding immune response play a pivotal role in the initiation and progression of IBD[3]. After birth, a limited diversity microbial community develops into a complex community due to the influence of diet and environmental factors[4]. During the second or third decade of life, a dysbiosis is observed in IBD patients which leads to an imbalance between commensal and potentially pathogenic microorganisms[5]. The healthy gut microbiota predominately comprises Firmicutes and Bacteroidetes, and to a lesser extent, Actinobacteria and Proteobacteria[6,7]. In IBD, dysbiosis is observed with reduced abundance of Firmicutes and either higher or similar abundance of Proteobacteria. Besides compositional changes, genetic alterations also contribute to gut dysbiosis that leads to disease initiation and progression. For example, NOD2 variants were found in 20%-40% of European and American CD patients[8,9]. NOD2 encodes an intracellular receptor for the bacterial peptidoglycan muramyl dipeptide, which helps maintain the balance of commensal bacterial flora[10].

Immune response to microbes results in the production of antibodies to microbial antigens[11]. Anti-*Saccharomyces cerevisiae* antibodies (ASCA) are associated with CD patients, with sensitivities and specificities ranging between 55% to 65% and 80% to 95%, respectively[12]. Perinuclear antineutrophil cytoplasmic antibodies (pANCA) are associated with UC patients, with sensitivities and specificities ranging between 50% to 71% and 75% to 98%, respectively[13]. Outer membrane protein of *Escherichia coli* (OmpC) and flagellin (CBir1) antibodies are prevalent in CD patients, with prevalence ranging between 24%-55% and 50%-56%[13]. The number and response magnitude of anti-microbial antibodies have previously been shown to indicate the presence of IBD, its severity and its clinical course; however, the clinical utility of available antibodies in diagnosis and clinical management of IBD patients has been limited. The techniques used to discover the known anti-microbial antibodies associated with IBD are of low throughput, and have only been applied to test on small number of candidate microorganisms or microbial antigens[14]. We have performed a large-scale comparative profiling of anti-microbial antibodies in CD and UC patients and healthy controls using an innovative protein microarray technology, namely Nucleic Acid Programmable Protein Array. We selected 1570 microbial proteins from our microbial protein collection (DNASU.org) from 50 bacteria and 33 viruses based on preliminary studies in our laboratory and a review of the literature, displayed them on microarrays and probed them against 100 CD, 100 UC and 100 healthy control serum samples.

MATERIALS AND METHODS

Patients and samples

All the serum samples were acquired from Serum Biobank at Mayo clinic with approval from institutional review board. CD patients were randomly selected, followed by age and gender matched healthy controls and UC patients. The samples (100 CD, 100 UC and 100 controls) were divided evenly into two non-overlapping discovery and validation sets randomly (Table 1). Disease status for study participants was assessed by clinicians at Mayo clinic.

Microbial protein array fabrication

We analyzed 1570 microbial proteins, of which 1173 proteins were from 50 different species of bacteria, 397 proteins were from 33 different species of viruses and the remaining proteins were autoantigens. These proteins were selected from our large collection of microbial antigens (DNASU.org) with reference to our anti-microbial antibody studies on other diseases (unpublished data). Microbial protein arrays were fabricated as described earlier[15,16]. Briefly, plasmids with genes of interest cloned in the pANT7_cGST expression vector were obtained from the DNASU plasmid repository, prepared, and printed into silicon nanowells using a piezoelectric dispensing system to produce microbial protein arrays. On the day of experiment, proteins were freshly expressed from printed plasmids using an *in-vitro* transcription and translation protein expression kit (Fisher Scientific) and captured by anti-GST antibody co-printed in each nanowell. After expression, microarrays were incubated with 1:100 diluted serum samples. We randomized the case and control serum samples while profiling on microarray to reduce bias. IgG and IgA anti-microbial protein antibodies were detected by Alexa-647 goat anti-human IgG (H+L) and Cy3 goat anti-human IgA (Jackson ImmunoResearch). After washing and drying, the microarrays were scanned in a Tecan PowerScanner and the raw fluorescence intensity data were extracted using the ArrayPro Analyzer Software. Raw fluorescence intensity of each protein on the microarray was divided by the median intensity of all the proteins on the microarray for normalization. The normalized value was termed as Median Normalized Intensity (MNI) and used for all analysis. Seropositivity of antibody for a particular antigen was defined as MNI ≥ 2 as we have done for our other studies[17,18].

Statistical analysis

Pairwise comparisons of numbers of IgG or IgA antibodies for each bacterial species among the 3 subject groups were performed using Chi-squared tests to assess statistical significance (Supplementary Figure 1). For each pairwise comparison, the Chi-squared *P* values were adjusted using the FDR (false discovery rate) method to reduce the likelihood of false positives. In addition to the multiple comparison adjustment at the antibody level, we performed adjustment at the species level.

For univariate analysis between two comparison groups, we calculated sensitivity for one group at the 96th percentile of the other group or the MNI of 2, whichever was larger. Antibodies with ≥ 14% sensitivity in the discovery set were selected as candidates for further validation. If an antibody had ≥ 14% sensitivity at 96% specificity in both discovery and validation sets, then it was considered as a "validated marker". Venn diagram for the overlap of microbial antigen targets were plotted using Venny[19].

We used a three-stage approach to build our multi-antibody panels. In the first stage, we selected all candidate biomarkers that passed the criteria above, *i.e.*, sensitivity was greater or equal than 14% at 96% specificity. Next, we applied the minimum redundancy maximum relevance algorithm to further select biomarkers that were possibly the most important and least correlated[20]. In the third stage, we

Table 1 Clinical information of the samples

	Discovery set			Validation set		
	CD	UC	HC	CD	UC	HC
N	50	50	50	50	50	50
Gender (female, male)	29, 21	29, 21	29, 21	28, 22	28, 22	28, 22
Age (median ± SD)	41 ± 17.66	44 ± 17.25	42 ± 18.47	39.5 ± 17.49	44.5 ± 17.23	39.5 ± 16.02
Disease behavior (B1/B2/B3)	9/10/6			16/8/2		
Disease location (L1/L2/L3/L4)	12/6/7/0			12/7/7/0		
Disease extent (E1/E2/E3)		0/32/18			0/34/16	
Surgery (Yes, No)	24, 25	8, 42		22, 27	7, 42	

Fischer's exact test *P* value is equal to 1 for the gender difference among CD, UC and HC in both discovery and validation set. Kruskal-Wallis test *P* value for the age difference among CD, UC and HC in discovery and validation set were 0.3159 and 0.1737 respectively. CD: Crohn's disease; UC: Ulcerative colitis; HC: Healthy control.

fit a logistic regression model using the selected biomarkers from the first two stages and generated its receiver operating characteristic curve and AUC value to evaluate the model's discriminatory performance between CD, UC, and healthy controls.

Pair-wise subgroup comparisons based on the Montreal classification were performed for the odds ratio (OR) of each antibody using the seropositivity threshold defined as the maximum of MNI 2 and the 75th percentile of all samples. Chi-squared tests were used to test global significance between all groups with a slight modification by adding 0.5 to each cell of the table to avoid zero cell counts. *P* values from the Chi-squared method were adjusted for each pair of comparisons and for all candidate biomarkers. The number of antibodies with significant difference in prevalence among classifications were counted based on OR > 1 and OR < 1 for each pair of classification of CD behavior, CD location, UC extent and the surgery history of CD patients. The difference in total number of antibodies with significant difference between classification groups were computed using two sample proportion test. We did not perform a subgroup analysis for the UC patients based on the surgery history because most (84 out of 100) had no surgeries (Table 1).

Spearman's rank correlation analysis was performed to assess the correlation between autoantibody and anti-microbial antibody reactivity for CD patients and healthy controls. The R "pheatmap" package was used to generate the heatmap for correlation coefficients.

Bioinformatics analysis

The NCBI Taxonomy browser was used to find the taxonomical details of all the bacteria and viruses used in our study. The taxa were downloaded as phylip tree file and was used as an input in interactive tree of life software. Two phylogenetic trees were created for bacteria and viruses with different colors distinguishing the phylum.

For sequence homology analysis, a pair-wise BLAST analysis was carried out on the antigen protein sequences of validated antibodies for CD *vs* healthy control analysis. E-values were used to generate a heatmap using Python Seaborn package.

RESULTS

Anti-microbial antibody profiling in IBD on microbial protein arrays

We studied IgG and IgA anti-microbial antibody profiles of 100 CD and 100 UC patients and 100 age-gender matched healthy controls (Table 1) against 1570 microbial antigens including 1173 antigens from 50 different bacteria and 397 antigens from 33 different viruses using our protein microarray platform (Figure 1, Supplementary Table 1). This study provided a representative overview of the anti-microbial antibody response in IBD patients (Supplementary Figure 1). The numbers of IgG antibodies against bacterial proteins from *Bacteroidetes vulgatus* (*B. vulgatus*) and *Citrobacter koseri* (*C. koseri*) were significantly higher in CD patients compared with those in healthy controls (Chi-square test, $P < 0.01$) (Supplementary Figure 1). On the contrary, the numbers of IgG antibodies against proteins from several bacteria, such as *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenza* (*H. influenzae*), *Staphylococcus aureus* (*S. aureus*), *Helicobacter pylori* (*H. pylori*) and *Parvimonas micra* (*P. micra*) were significantly lower in CD and UC patients compared with those in healthy controls (Chi-square test, $P < 0.05$)

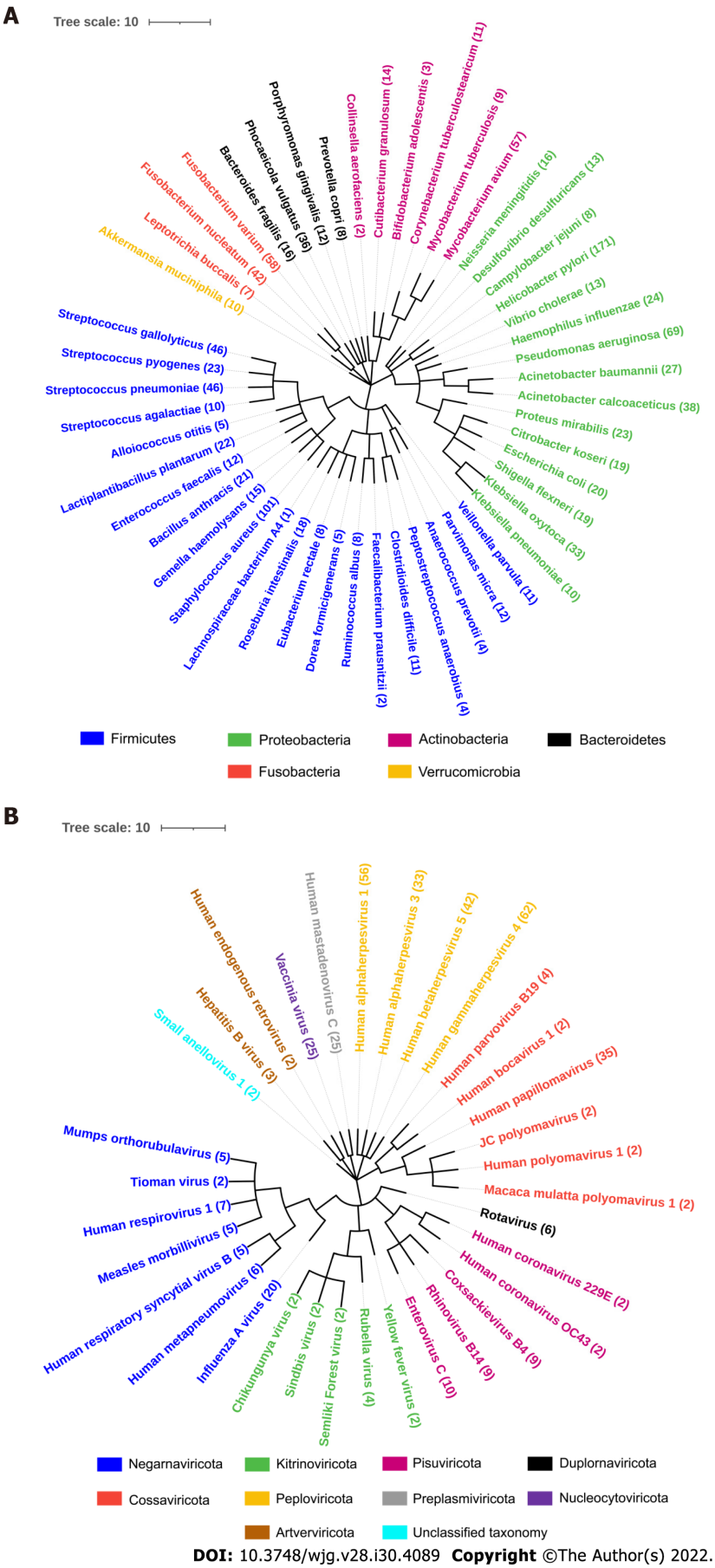

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Figure 1 Phylogenetic tree of microbes studied with their corresponding number of proteins analyzed. A: Fifty species of bacteria with 1173 proteins were segregated into 6 phyla; B: Thirty-three species of viruses with 397 proteins were segregated into 10 phyla.

(Supplementary Figure 1). Overall, fewer IgA anti-microbial antibodies were found than IgG antibodies. The numbers of IgA antibodies against *S. pneumoniae*, *H. influenzae*, *S. aureus*, and *H. pylori* were significantly lower in UC patients compared with those in healthy controls (Chi-square test, $P < 0.01$). On the other hand, anti-viral IgG and IgA antibodies showed heterogenous prevalence with no clear trend of differences among CD, UC, healthy controls (data not shown). Therefore, we focused our analysis on anti-bacterial antibodies.

Antibodies distinguishing CD from healthy controls

We compared prevalence for individual anti-microbial antibodies between CD patients and healthy controls. We randomly split samples evenly into discovery and the validation sets (Table 1). For antibodies with elevated prevalence in CD patients, 13 IgG antibodies passed the criteria (sensitivity ≥ 14% at 96% specificity) in both discovery and validation sets (Table 2). Anti-A4-Fla2_IgG, a well-studied anti-bacterial flagellin antibody in CD, had the best performance with 47% sensitivity at 96% specificity in the full sample set (Table 2). Beside the flagellins, we found antibodies to four novel target antigens from *B. vulgatus* (BVU_0562), *P. mirabilis* (PMI_RS06815), *S. flexneri* (SF_Lpp) and *S. pneumoniae* (SP_1992) (Table 2) with no significant sequence homology to flagellins (Figure 2A).

To our surprise, 12 validated IgG antibodies showed elevated prevalence in healthy controls relative to CD patients (Supplementary Table 2). Among these 12 antibodies, anti-bacterial antibodies performed better in differentiating CD patients from healthy controls than anti-viral antibodies (Supplementary Table 2). Antibody against SPy_2009, an anchoring protein located in the cell wall of *Streptococcus pyogenes* (*S. pyogenes*), had the highest sensitivity of 24% at 96% specificity in healthy controls relative to CD patients. Seven validated IgA antibodies showed higher prevalence in healthy controls relative to CD patients (Supplementary Table 4).

Antibodies distinguishing UC from healthy controls

For anti-microbial antibodies with elevated prevalence in UC patients relative to healthy controls, 4 IgG antibodies passed the criteria in both discovery and validation sets (Table 2). Antibodies to A4-Fla2_IgG and a flagellin from *C. koseri* had a sensitivity of 18% and 15% respectively. For IgG antibodies with higher prevalence in healthy controls relative to UC patients, 32 antibodies got validated (Supplementary Table 3). Source microorganisms for the target antigens of these 32 antibodies were enriched for *S. pneumoniae, S. aureus,* and *H. influenzae* (2-sample proportion test, $P < 0.05$). 2.7% of the proteins on the microbial protein microarray were from *S. pneumoniae* while 18.7% of antigens for validated antibodies were from *S. pneumoniae*, 6.1% of the proteins on the microarrays were from *S. aureus* while 18.7% of antigens for validated antibodies were from *S. aureus,* and 1.4% of the proteins on the microarrays were from *H. influenzae* while 12.5% of antigens for validated antibodies were from *H. influenzae*. Nine validated IgA antibodies showed higher prevalence in healthy controls relative to UC patients (Supplementary Table 4).

Fewer anti-viral antibodies than anti-bacterial antibodies were validated comparing CD or UC patients with healthy controls (Table 2, Supplementary Tables 2 and 3). Anti-viral antibodies to Rhinovirus B14, Enterovirus C, Influenza A virus, Human metapneumovirus had higher prevalence in healthy controls compared with CD and UC patients (Supplementary Tables 2 and 3).

Comparison of anti-microbial antibody response between CD and UC

We found 46 IgG and 22 IgA validated anti-microbial antibodies with higher prevalence in CD patients compared to UC patients while 28 IgG and 9 IgA validated anti-microbial antibodies with higher prevalence in UC patients compared to CD patients. There was minimal overlap of the target antigens of these validated IgG and IgA antibodies (Figure 2B).

Multivariate analysis to distinguish CD, UC, and healthy controls

We built multi-antibody panels that could distinguish CD *vs* control, UC *vs* control, and CD *vs* UC with an area under the curve (AUC) of 0.81, 0.87, and 0.82 respectively. For CD *vs* control, antibodies against novel flagellins (HP_0115, CK_LafA, CK_LafA.1, VC_flaD, VC_flaB, VC_flaE, VC_flaA) had an AUC of 0.73, antibodies against non-flagellins (BVU_0562, SP_1992, PMI_RS06815, and SF_Lpp) had an AUC of 0.75 and the combined AUC of antibodies against novel flagellins and non-flagellins was 0.81 (Figure 3A). For UC *vs* control, a combination of seven antibodies, four against *S. pneumoniae* and one each against *S. aureus*, *H. influenzae* and *B. vulgatus* had an AUC of 0.87 (Figure 3B). For CD *vs* UC, combination of seven antibodies, two against *H. pylori* and one each against *E. coli*, *S. pneumoniae*, *S. pyogenes*, *C. jejuni* and *L. bacterium A4* had an AUC of 0.82 (Figure 3C).

Subgroup analysis

We investigated the association of CD behavior (B1, B2, B3), CD location (L1, L2, L3), and UC extent (E1, E2, E3) based on the Montreal classification with the anti-microbial antibody prevalence. We calculated the 4th quartile odds ratio for each antibody between the two classification groups and compared the number of antibodies with significant odds ratio (P value < 0.05) in each group. We found B3 (penetrating) had the highest prevalence of antibodies followed by B2 (stricturing) and B1 (non-

Table 2 Sensitivities of validated IgG antibodies comparing Crohn's disease and ulcerative colitis with healthy controls in the discovery, validation, and the entire set at 96% specificity

		Antigen	Protein name	Organism	Discovery	Validation	Entire
Crohn's disease	Bacteria	HP_0115	Flagellin B	*H. pylori*	28	48	38
		BVU_0562	Uncharacterized protein	*B. vulgatus*	26	22	25
		CK_LafA	Lateral flagellin	*C. koseri*	20	22	21
		CK_LafA.1	Lateral flagellin	*C. koseri*	16	26	24
		A4-Fla2	Flagellin	*L. bacterium A4*	40	54	47
		PMI_RS06815	Hypothetical protein	*P. mirabilis*	14	16	15
		VC_flaD	Flagellin	*V. cholerae*	24	18	19
		VC_flaB	Flagellin	*V. cholerae*	28	22	24
		VC_flaE	Flagellin	*V. cholerae*	26	28	23
		VC_flaA	Flagellin	*V. cholerae*	20	22	21
		SF_Lpp	Outer membrane lipoprotein	*S. flexneri*	14	18	14
		SP_1992	Cell wall surface anchor	*S. pneumoniae*	20	16	18
	Virus	BILF2	Glycoprotein BILF2	Human herpesvirus 4	18	18	18
Ulcerative colitis	Bacteria	CK_flgG	Flagellar basal-body rod protein	*C. koseri*	14	16	15
		A4-Fla2	Flagellin	*L. bacterium A4*	22	16	18
	Virus	BVRF2	Capsid scaffolding protein	Human herpesvirus 4	14	16	14
		UL139	Membrane glycoprotein UL139	Human herpesvirus 5	14	20	17

stricturing, non-penetrating) (Table 3). For CD location, we found L2 had the highest prevalence of antibodies followed by L3 (ileocolonic) and L1 (Table 3). For UC extent, E3 (extensive UC) had higher prevalence of antibodies compared to E2 (left sided UC). In addition to the Montreal classification, we also performed subgroup analysis based on the surgery history of CD patients. We found that patients who had surgery possessed higher prevalence of antibodies compared to those without surgery (Table 3).

Correlation of anti-microbial antibodies and autoantibodies in CD patients

We previously reported novel autoantibodies in CD patients using the same set of CD patients and healthy controls[21]. We profiled both IgG and IgA autoantibodies and anti-microbial antibodies in all 100 CD and 100 healthy controls. It is interesting to note the antibodies showing differences for autoantibodies were mostly IgA, but the anti-microbial antibodies were mostly IgG[21]. Anti-SNRPB_IgA had the highest sensitivity of 20% at 96% specificity among all autoantibodies compared with 47% sensitivity at 96% specificity for the best performing anti-microbial antibody, anti-A4-Fla2_IgG.

We compared the novel autoantibodies and validated anti-microbial antibody profiles to determine if correlation existed between their reactivity. Overall, we did not observe high correlation between autoantibodies and anti-microbial antibodies in CD patients (Figure 4). Anti-microbial antibodies formed two clusters, one with anti-flagellin antibodies, and the other with SF_Lpp_IgG and PMI_RS06815_IgG. Five autoantibodies, PRPH_IgA, SNAI1_IgA, PPP1R13L_IgA, SNRPB_IgA and PTTG1_IgA, formed a cluster. The remaining antibodies had relatively unique reactivity patterns.

DISCUSSION

We have performed a microbiomics study to understand the connection between host anti-microbial responses and IBD and to identify antibody signatures that can aid in the accurate diagnosis of IBD. We found antibody responses to novel non-flagellin antigens with elevated prevalence in CD patients compared with healthy controls. On the contrary, we observed many anti-microbial antibodies with

Table 3 Subgroup analysis of inflammatory bowel disease patients

Classification	Comparison	Number of antibodies with		Two sample proportion test
		OR > 1	OR < 1	
Disease behavior: B1: non-stricturing, non-penetrating; B2: stricturing; B3: penetrating	B1 *vs* B2 ($P < 0.05$)	0	32	$P < 0.001$
	B2 *vs* B3 ($P < 0.05$)	0	19	$P < 0.001$
	B1 *vs* B3 ($P < 0.05$)	2	41	$P < 0.001$
Disease location: L1: ileal; L2: colonic; L3: ileocolonic	L1 *vs* L2 ($P < 0.05$)	0	38	$P < 0.001$
	L2 *vs* L3 ($P < 0.05$)	9	5	$P = 0.131$
	L1 *vs* L3 ($P < 0.05$)	5	16	$P < 0.001$
Disease extent: E2: left sided UC; E3: extensive UC	E2 *vs* E3 ($P < 0.05$)	11	39	$P < 0.001$
Surgery in CD patients	No *vs* Yes ($P < 0.05$)	6	25	$P < 0.001$

For each comparison, the number of antibodies with significant difference in prevalence between two classifications were counted based on odds ratio (OR) > 1 and OR < 1. The difference in total number of antibodies for each comparison were computed using two sample proportion test. CD: Crohn's disease; UC: Ulcerative colitis; OR: Odds ratio.

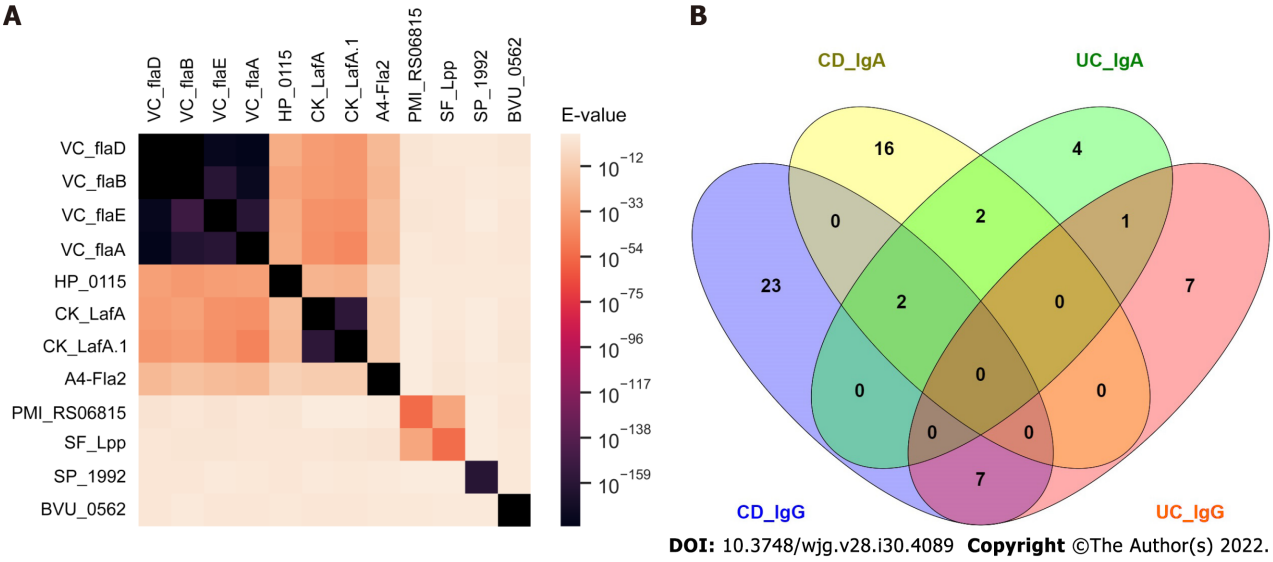

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Figure 2 Sequence homology of target antigens of validated antibodies and overlap of antibodies among Crohn's disease and ulcerative colitis. A: Heatmap showing sequence homology among target antigens for antibodies with validated performance of ≥ 14% sensitivity at 96% specificity comparing Crohn's disease (CD) patients with healthy controls; B: CD_IgG, ulcerative colitis (UC)_IgG, CD_IgA and UC_IgG represent the overlap of anti-microbial antibodies of IgG and IgA isotypes in CD and UC patients with ≥ 14% sensitivity at 96% specificity against healthy controls in the discovery set. CD: Crohn's disease; UC: Ulcerative colitis.

lower prevalence in UC patients relative to healthy controls. We built antibody panels that could distinguish CD *vs* control, UC *vs* control and CD *vs* UC with AUCs of 0.81, 0.87, and 0.82, respectively. Lichtenstein *et al*[22] reported an integrated serological (ASCA-IgA, ASCA-IgG, anti-OmpC, anti-CBir1, anti-I2, pANCA) and genetic (SNP8, SNP12, SNP13) marker panel with an AUC of 0.80 to distinguish CD *vs* control. A panel of serological markers (ASCA-IgA, ASCA-IgG, ANCA, pANCA, OmpC, and CBir1) built by Plevy *et al*[23] yields an AUC of 0.78 to distinguish CD *vs* UC. Our novel antibody marker panels had comparable or better performance in IBD diagnosis or distinguishing CD from UC subtypes. We observed a stronger anti-microbial antibody response with more aggressive disease in both CD and UC patients. Finally, we demonstrated that anti-microbial antibodies and autoantibodies had different reactivity patterns in CD patients.

Our comprehensive study of anti-microbial antibodies in IBD patients provided interesting insight into its pathogenesis. Antibody responses to proteins from *B. vulgatus*, *P. mirabilis*, *S. flexneri* and *S. pneumoniae* were elevated in CD patients. *B. vulgatus* has been reported to induce colitis in IBD-susceptible mice[24,25]. *P. mirabilis* in gut can induce inflammation in cells and a colitis mouse model and has been associated with CD pathogenesis[26]. Our results suggest that *B. vulgatus* and *P. mirabilis*

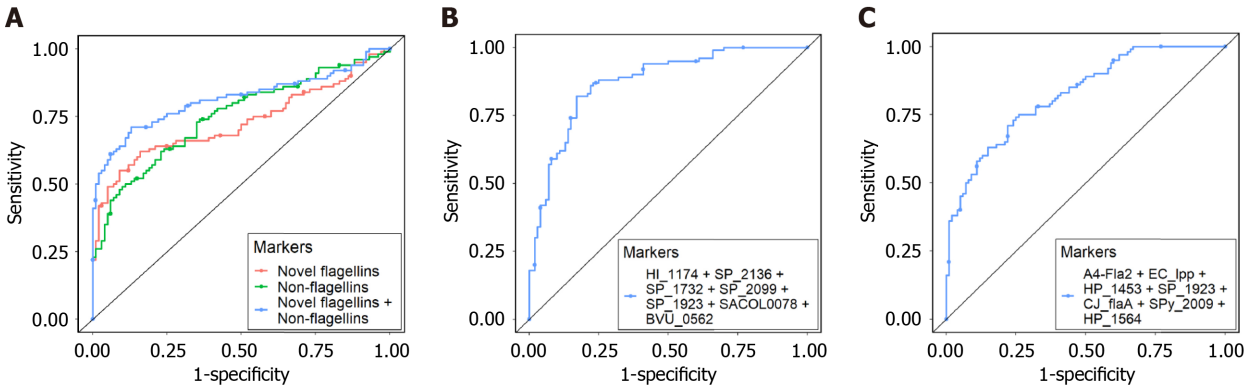

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Figure 3 Receiver operating characteristic curves to discriminate Crohn's disease, ulcerative colitis, and healthy controls. A: Receiver operating characteristic (ROC) curve for Crohn's disease (CD) *vs* healthy controls. Area under the curve (AUC) values of novel anti-flagellin antibodies (HP_0115, CK_LafA, CK_LafA.1, VC_flaD, VC_flaB, VC_flaE, VC_flaA) and anti-non-flagellin antibodies (BVU_0562, SP_1992, PMI_RS06815, SF_Lpp) was 0.73 and 0.75, respectively. The AUC value obtained with a combination of novel anti-flagellin and anti-non-flagellin antibodies was 0.81; B: ROC curve for ulcerative colitis (UC) *vs* healthy controls. The AUC value obtained with a combination of 7 markers was 0.87; C: ROC curve for CD *vs* UC. The AUC value obtained with a combination of 7 markers was 0.82.

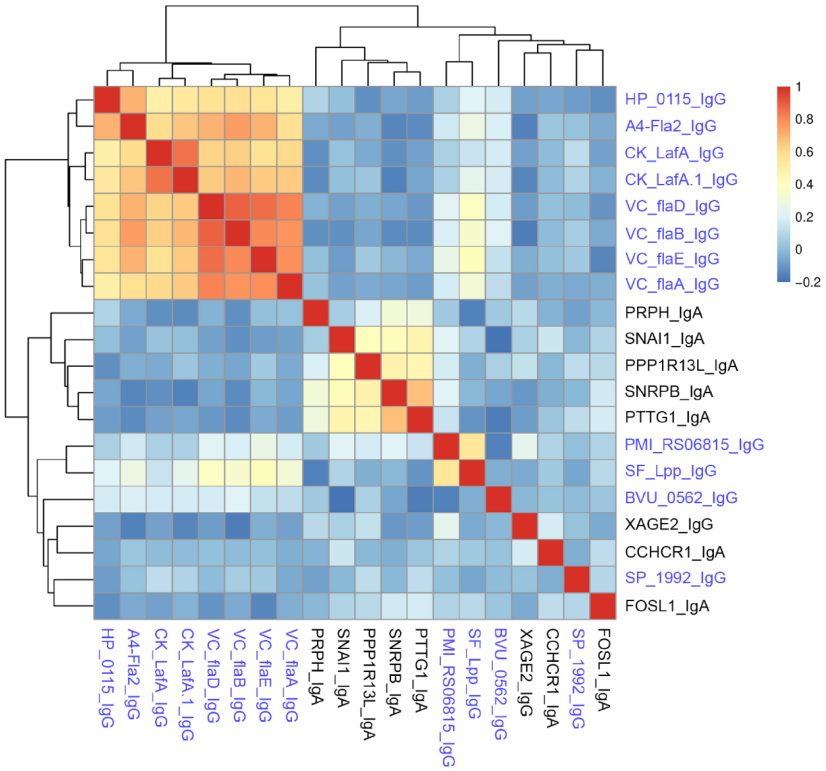

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Figure 4 Spearman's rank correlation coefficient heatmap of anti-microbial antibodies and autoantibodies in Crohn's disease patients. The names of anti-microbial antibodies are colored in blue while autoantibodies are colored in black.

may also play a role in human CD development. We observed reduced antibody responses in UC patients to several genera of the Firmicutes phylum including *P. micra*, *S. pyogenes*, *S. aureus*, which were often reduced in abundance in UC patients' gut microbiota[27]. For several genera belonging to Proteobacteria phylum, such as *H. influenzae*, *H. pylori*, *K. oxytoca*, we observed overall reduced antibody responses; however, their abundance in the gut microbiota of UC patients has been reported to be either increased or remained the same compared with healthy controls[6,28].

Beyond exposure alone, antibody response requires functional immunological interaction between a microorganism and the host; however, anti-microbial antibodies by themselves do not prove causality. As such, source microorganisms whose antibodies show significant changes between IBD patients and healthy controls warrant future confirmation and functional assessment in causing IBD. A4-Fla2

flagellin included in our study showed IBD-specific prevalence with performance similar to that reported in the literature[14,29]. We also identified several antibodies to flagellins with higher prevalence in CD patients relative to healthy controls.

Previous studies mostly focused on antibodies with higher prevalence in IBD patients[30]. Our unbiased data-driven approach revealed the existence of many anti-microbial antibodies with higher prevalence in healthy controls relative to CD and especially UC patients (Supplementary Tables 3 and 4). The reduction observed in CD and UC patients may be attributed to the dysbiosis and reduced diversity of gut microbiota in CD and UC patients[28,31]. It is also possible that the reduction in anti-microbial antibodies in some CD and UC patients was in part because of immunosuppressive therapies they received. The greater number of antibodies having high prevalence in CD patients compared with UC patients indicates stronger anti-microbial humoral immunity in CD than in UC, which is consistent with reports in the literature that most known anti-microbial antibodies, such as ASCA, anti-OmpC, anti-Cbir1, and anti-I2, had higher prevalence in CD patients than in UC patients[30]. This agreement, together with comparable performance of anti-flagellin antibodies in our study and that reported in the literature, suggests that our results reflect the microbial association of IBD etiopathology. However, the use of samples from patients with established disease and the lack of information on immunosuppressive therapies of these patients limited the interpretation of the results.

We studied the association between anti-microbial antibody prevalence and various disease classifications based on Montreal classification and surgery history. We found more antibodies with significantly higher prevalence in patients with more aggressive disease behaviors relative to those with milder disease behavior. We also found more antibodies with significantly higher prevalence in colonic CD patients relative to those in ileal CD patients. Our results were consistent with previous reports that increasing diversity and magnitude of anti-microbial immune response was correlated with increased frequency of penetrating and/or stricturing disease behavior[13,32]. It is known that the colon has a microbial density of 10^{11}-10^{12} anaerobic bacteria/gram while the ileum is colonized by 10^{7}-10^{8} anaerobic bacteria/gram[7]. Kleessen *et al*[33] found higher percentage of bacterial invasion of mucosa in colon compared to ileum. CD patients requiring surgery usually had more severe disease compared with those who did not need surgery. Stronger anti-microbial immune response in patients with severe CD or UC suggests a higher abundance of the source microorganisms for the target antigens of the differential antibodies and/or a stronger more conducive immune microenvironment at the disease site in severe disease.

Both autoantibodies and anti-microbial antibodies associated with IBD have been reported[30]. One popular hypothesis for the autoantibody elicitation is molecular mimicry, where anti-microbial antibodies cross react with human proteins. However, we found minimal correlation between the anti-microbial antibodies and the autoantibody profiles in the same set of CD samples. The lack of correlation suggests that IBD-specific autoantibodies and anti-microbial antibodies are elicited independently through different underlying mechanisms, and cross-reactivity may play less of a role in eliciting CD-associated autoantibodies. The breakdown of immune tolerance to human proteins might have occurred due to the damaged gut epithelial cells and the faulty immunological microenvironment partly caused by microbial infections. In addition, the elicitation of autoantibodies may be associated with the infections of multiple microorganisms, and the correlation with individual anti-microbial antibodies may not be great.

Strengths of our study include the broadest analysis to date of IgG and IgA antibodies against individual antigens from many different microorganisms in both CD and UC patients and the use of a two-stage approach with discovery and independent validation of antibody markers. There are some limitations to our study. Except for a few microbes, the number of proteins studied for each species is small, which might limit our interpretation of antibody response in IBD at the species level. Furthermore, many samples used in studies were collected from patients with established disease. Future studies with access to more clinical information, such as information about the use of immunosuppressive or antibiotics treatment, could aid in the interpretation of our results. In addition, samples collected from newly diagnosed patients will strengthen our ability to identify diagnostic markers and microbial connections for IBD development.

CONCLUSION

In summary, we have demonstrated the power of a microbiomics study of anti-microbial antibodies in IBD for the identification of anti-microbial antibody signatures to improve early accurate diagnosis and help understand IBD etiology. The elucidation of the source microorganisms of the target antigens of the antibody biomarkers could lead to novel strategies for the prevention of IBD.

ARTICLE HIGHLIGHTS

Research background

Early and accurate diagnosis of inflammatory bowel disease (IBD) can benefit the clinical management of IBD patients. The clinical utility of available non-invasive biomarkers is limited.

Research motivation

The gut microbiota plays an important role in the pathogenesis of IBD. Antibody responses to microbial antigens can be exploited to identify better diagnosis markers and improve our understanding of IBD pathology.

Research objectives

This study aimed to compare anti-microbial antibody profiles in Crohn's disease (CD) and ulcerative colitis (UC) patients and healthy controls.

Research methods

We employed an innovative protein microarray platform and profiled antibodies against 1570 microbial antigens in 100 CD, 100 UC, and 100 healthy controls.

Research results

Antibodies to bacterial proteins were better in distinguishing IBD patients from healthy controls compared with antibodies to viral proteins. We identified a set of novel anti-microbial antibodies against the antigens of *Bacteroidetes vulgatus* (BVU_0562) and *Streptococcus pneumoniae* (SP_1992) elevated in CD patients relative to healthy controls. In addition, anti-microbial antibodies against the antigen of *Streptococcus pyogenes* (SPy_2009) were found to be elevated in healthy controls relative to UC patients. We constructed antibody panels that could distinguish CD *vs* control, UC *vs* control, and CD *vs* UC with an AUC of 0.81, 0.87 and 0.82 respectively. Patients with severe disease had higher prevalence of anti-microbial antibodies. There was minimal correlation among the occurrence of autoantibodies and anti-microbial antibodies in CD patients.

Research conclusions

This study discovered novel anti-microbial antibodies with differential prevalence in CD and UC patients relative to healthy controls. In addition, this study revealed the potentially different roles of gut microbiota in CD and UC pathology.

Research perspectives

Our study demonstrated the power of a microbiomics approach to identify biomarkers that could aid in the early and accurate detection of IBD non-invasively, and shed light into the role of various microbes in IBD etiology.

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FOOTNOTES

Author contributions: Shome M and Song L designed and ran the experiment; Williams S and Chung Y performed the statistical analysis; Murugan V and Park JG helped in preparation of clones; Faubion W, Pasha SF and Leighton JA provided the serum samples and also provided intellectual guidance; LaBaer J and Qiu J supervised the project and secured funding; Shome M and Qiu J wrote the original manuscript which was then reviewed by remaining authors.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Trends in medication use and treatment patterns in Chinese patients with inflammatory bowel disease

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Abstract

BACKGROUND

Medications for inflammatory bowel disease (IBD) have changed dramatically over time. However, no study on long-term medication profiles has been conducted in the Chinese population.

AIM

To evaluate temporal changes in medication use and treatment patterns for Chinese patients with IBD.

METHODS

A multicenter retrospective cohort study was conducted among Chinese patients newly diagnosed with Crohn's disease (CD) and ulcerative colitis (UC) between January 1999 and December 2019. Baseline characteristics and drug prescriptions were collected. Trends in medication use and therapeutic patterns were analyzed.

RESULTS

In total, 3610 patients were analyzed. During follow-up, 5-aminosalicylates (5-ASA) and corticosteroids (CS) prescriptions gradually decreased, accompanied by a notable increase in immunosuppressants (IMS) and infliximab (IFX) prescriptions in patients with CD. Prescription rates of 5-ASA and CS were stable, whereas IMS and IFX slightly increased since 2007 in patients with UC. Subgroup (n = 957) analyses showed a switch from conventional medications to IFX in patients with CD, while 5-ASA and CS were still steadily prescribed in patients with UC. Logistic regression analyses revealed that surgical history, disease behavior, and disease location were associated with initial therapeutic strategies in patients with CD. However, medications before diagnosis, disease location, and diagnostic year might affect initial strategies in patients with UC.

CONCLUSION

Long-term treatment strategies analyses has provided unique insight into the switch from conventional drugs to IFX in Chinese patients with CD.

Key Words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Medication trends; Treatment pattern; Infliximab

Core Tip: Prescriptions of immunosuppressants and infliximab (IFX) increased in parallel with steady or decreasing prescriptions of 5-aminosalicylates and corticosteroids in Chinese patients with inflammatory bowel disease. Furthermore, a switching profile from conventional drugs to IFX was observed in patients with Crohn's disease. This is the first multicenter cohort study to depict temporal trends in long-term medication uses and periodic changes in treatment paradigms in the Chinese population, which may provide evidence for clinical practice in the foreseeable future.

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INTRODUCTION

Inflammatory bowel diseases (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are chronic debilitating disorders characterized by alternating periods of relapse and remission[1,2]. With a growing incidence worldwide, IBD cases in China are expected to exceed 1.5 million by 2025[3]. In light of the need for lifelong medical interventions, IBD poses a global public health challenge.

Owing to the rapid development of new drugs, IBD medications have launched in succession and changed in the past few decades, especially following the discovery of infliximab (IFX), the first anti-tumor necrosis factor agent, which has revolutionized the field[4,5]. Several attempts have been made by Western and other Asian countries to provide rough medication changes. Specifically, increasing proportions of immunomodulators and biological agents have been observed, accompanied by decreasing prescription rates of 5-aminosalicylates (5-ASA) and corticosteroids (CS)[6-8]. A few studies have been carried out on annual changes in IBD medications, including 5-ASA and CS[9,10]. However, no long-term analysis of medication tendency has been conducted in a large population of Chinese patients with IBD.

Drug diversification enables treatment combinations for better therapeutic effects[11]. However, combination treatment also correlates with increasing risks of side effects[12,13]. Therefore, patients might switch treatment strategies to acquire optimal effects from IBD medications under different circumstances. Currently, minimal attention has been paid to periodic changes in IBD treatment strategies. Additionally, initial treatment strategies affect follow-up therapeutic patterns to some extent; thus, investigating the possible predictors for initial treatment strategies may help to better understand periodic changes in therapeutic patterns in patients with IBD.

Therefore, this study aimed to provide fresh insights into temporal trends in medication prescriptions among the Chinese patients with IBD for over 20 years. The study also aimed to investigate long-term periodic changes in treatment paradigms and identify the possible factors that influence initial drug strategies.

MATERIALS AND METHODS

Study design

This multicenter retrospective cohort study included 12 IBD referral centers across all seven administrative regions in China with diverse socioeconomic backgrounds. The study protocol was reviewed and approved by Sir Run Run Shaw Hospital, College of Medicine Zhejiang University Institutional Review Board (Approval No. 20210714-31) with approval for all hospitals involved in the study. The requirement for patient informed consent was waived due to the retrospective nature of this study.

Study population

Incident adult patients with a definite diagnosis of CD or UC according to the Chinese consensus on IBD diagnosis, which was similar to that of the European Crohn's and Colitis Organization (ECCO) consensus, between January 1, 1999 and December 31, 2019 were included[14,15]. Patients were excluded from analyses for medication trends if the cessation date of medications was unclear. Additionally, patients were further excluded from analyses for treatment patterns if: (1) They were followed up for < 3 years since diagnosis; or (2) No prescription of either 5-ASA, CS, immunosuppressants (IMS), or IFX was administered throughout follow-up. Cases were followed from diagnosis until loss to follow-up, or December 31, 2020, whichever came first.

Data collection and definition

Patients were identified using International Classification of Diseases 8th and 10th revision codes for CD (563.00–563.09 and K50) and UC (563.19, 569.04 and K51), respectively. Data regarding baseline characteristics and prescriptions since diagnosis were collected by reviewing medical records and digitized into the Epidata 3.0. Data quality was ensured using standardized case report forms and careful manual scrutinization.

Demographic parameters including sex, date of first IBD-related symptoms, date of initial diagnosis, smoking status, region of urbanization, occupation, and clinical parameters including disease location, disease activity, extraintestinal manifestations (EIMs), IBD family history, gastrointestinal surgical history, and medication use within six months before diagnosis were collected from patients with IBD. Additionally, disease behavior, perianal involvement, and perianal surgical history were also collected from patients with CD. Other relevant variables were calculated, including median age of onset and diagnosis, median interval from onset of symptoms to diagnosis, median follow-up duration, and body mass index (BMI) at diagnosis.

Smoking status was defined by smoking habits at diagnosis. EIMs classification originated from the 2016 ECCO consensus[9]. Disease location for IBD and CD behavior were defined according to the Montreal classification[10]. Disease activity was acquired from medical records using the Crohn's Disease Activity Index (CDAI) and Mayo score for CD and UC, respectively, and was assessed and recorded by IBD nurses when patients were first diagnosed[16,17].

Statistical analysis

Continuous variables are presented as medians with interquartile ranges (IQRs), and categorical variables as numbers with percentages. A T-test or Mann-Whitney U test was used to compare

continuous variables, and Chi-square test or Fisher's exact test was used to compare categorical variables.

The initiation date of medications (including 5-ASA, CS, IMS, and IFX) since diagnosis was set as the first prescription date, and the last date was calculated as the first prescription date plus the number of prescription days. Annual percentage of each drug used among all available patients was plotted against every calendar year to demonstrate medication trends from 1999 to 2020. To conduct subgroup analysis for periodic changes in treatment paradigms, the study period was divided according to the following time points since diagnosis: 1, 3, 6, 12, 24, and 36 mo. The frequency and percentage of different drug combination strategies were plotted against different periods to illustrate periodic changes in drug patterns with a 3-year follow-up. To investigate possible factors for initial treatment strategies, we incorporated drugs into the following combination strategies. For CD, initial medications were classified as: (1) CS (CS monotherapy or combined with 5-ASA); (2) CS + IMS (CS combined with IMS); and (3) IFX [IFX monotherapy or combined with conventional drugs (5-ASA, CS, or IMS)]. For UC, initial medications were classified as: (1) 5-ASA (5-ASA monotherapy); (2) CS (CS monotherapy or combined with 5-ASA); and (3) IFX/IMS (IFX monotherapy, IMS monotherapy, or IFX combined with IMS). Predictors for initial drug strategies were identified using logistic regression analysis and presented as odds ratios and 95% confidence intervals.

P value < 0.05 were considered a statistically significant difference. Statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary NC, United States), and graphs were plotted using R software (version 4.1) with the "ggplot2" and "ggalluvial" packages[18,19].

RESULTS

Baseline characteristics

Altogether, 3610 patients were included in the analysis for temporal changes in medication use (2208 and 1402 patients with CD and UC, respectively). Furthermore, 957 patients were included in the analysis for periodic changes in treatment patterns (686 and 271 patients with CD and UC, respectively), after excluding 2452 patients who were followed up < 3 years since diagnosis, and 201 patients with no prescription of either 5-ASA, CS, IMS, or IFX throughout follow-up (Figure 1).

Patient baseline characteristics are summarized in Table 1. The median (IQR) follow-up duration was 1.6 (0.3-3.8) years, the median (IQR) age at diagnosis was 33.0 (25.0-48.0) years, and male accounted for 46.2% of patients. Similar patterns were found in the region of urbanization and IBD family history between CD and UC, except for male sex (P < 0.0001), median age of onset (P < 0.0001), median age at diagnosis (P < 0.0001), median interval from onset to diagnosis (P < 0.0001), median follow-up duration (P < 0.0001), smoking status (P < 0.0001), occupation (P < 0.0001), BMI category (P < 0.0001), EIM classification (P < 0.0001), gastrointestinal surgical history (P < 0.0001), and drug history (medication use within six months before diagnosis; P < 0.0001).

The differences in baseline characteristics between included and excluded patients in the analysis for periodic changes in treatment patterns are shown in Table 2. Briefly, patients who were included in further analysis were prone to be younger at the age of onset and diagnosis (P < 0.001), accompanied with EIMs (P = 0.0096), and underwent perianal surgeries before diagnosis (P < 0.0001). Moreover, patients with UC had a higher risk of proctitis and left-sided involvement (P = 0.0005), and patients with CD had higher proportion of moderate severity (P = 0.0002).

Temporal trends in medication use

Proportions of different prescriptions during the 20-year follow-up of patients with CD are depicted in Figure 2A and Supplementary Table 1. The prescription rate of 5-ASA showed a steady decline from 50.0% in 2002 to 5.5% in 2020, and a similar trend was observed for CS. Meanwhile, the prescription rate of IMS increased prominently from 2005 onwards, but decreased slightly between 2018 and 2020. The prescription rate of IFX has gradually increased since 2008, reaching 60.3% in 2020.

We also reported the proportions of different prescriptions among patients with UC (Figure 2B and Supplementary Table 2). 5-ASA and CS were steadily prescribed between 1999 and 2020. IMS was first prescribed in 2007, increasing slightly to 8.5% in 2020. Parallel to IMS, a gentle increase was observed in the proportion of IFX prescriptions between 2009 and 2020.

Periodic changes in treatment patterns

Therapeutic regimens were evaluated for three consecutive years after diagnosis. As IFX was first prescribed between 2008 and 2009 in China, we further divided the total cohort into Cohorts I (1999–2008) and II (2009-2020), to evaluate the potential impact of IFX availability on therapeutic strategies.

IFX (22.3%), IMS (7.1%), and IMS combined with IFX (12.0%) accounted for nearly half of initial (within the first month after diagnosis) treatment strategies for patients with CD (Figure 3A and Supplementary Table 3). Interestingly, 51.6% of patients ceased medical treatment within 1-3 mo, but 41.7% re-prescribed in the following period. Specifically, a prominent increase in monotherapy or

Table 1 Demographic and clinical characteristics of patients with inflammatory bowel disease in 1999-2020

	Total (*n* = 3610)	CD (*n* = 2208)	UC (*n* = 1402)	*P* value
Male sex, *n* (%)	1666 (46.2)	935 (42.3)	731 (52.1)	< 0.0001
Median age of onset (IQR), yr	31.0 (23.0-45.0)	27.0 (21.0-37.0)	42.0 (30.0-54.0)	< 0.0001
Median age at diagnosis (IQR), yr	33.0 (25.0-48.0)	29.0 (23.0-39.0)	45.0 (32.0-56.0)	< 0.0001
Median interval from onset to diagnosis (IQR), mo	12.2 (2.4-36.5)	12.4 (3.7-36.7)	6.17 (1.2-26.4)	< 0.0001
Median duration of follow-up (IQR), yr	1.6 (0.3-3.8)	2.0 (0.8-3.9)	0.7 (0.1-3.2)	< 0.0001
Smoking status at diagnosis, *n* (%)				< 0.0001
Non-smoker	2860 (79.2)	1828 (82.8)	1032 (73.6)	
Current smoker	356 (9.9)	198 (9.0)	158 (11.3)	
Former smoker	233 (6.4)	114 (5.2)	119 (8.5)	
Unknown	161 (4.5)	68 (3.0)	93 (6.6)	
Region of urbanization, *n* (%)				0.7918
Rural	1607 (44.5)	991 (44.9)	616 (43.9)	
Urban	1959 (54.3)	1189 (53.8)	770 (54.9)	
Unknown	44 (1.2)	28 (1.3)	16 (1.2)	
Occupation, *n* (%)				< 0.0001
Unemployed	495 (13.7)	316 (14.3)	179 (12.8)	
Employed	1851 (51.3)	1227 (55.6)	624 (44.5)	
Student	377 (10.5)	334 (15.1)	43 (3.1)	
Retire	258 (7.1)	95 (4.3)	163 (11.6)	
Unknown	629 (17.4)	236 (10.7)	393 (28.0)	
BMI category at diagnosis, *n* (%)				< 0.0001
Normal	1014 (28.1)	812 (36.8)	202 (14.4)	
Underweight	1524 (42.2)	912 (41.3)	612 (43.7)	
Overweight + Obese	379 (10.5)	183 (8.3)	196 (14.0)	
Unknown	691 (19.2)	300 (13.6)	391 (27.9)	
Disease location, *n* (%)				
L1—ileal location	-	626 (28.4)	-	
L2—colon location		224 (10.2)		
L3—ileocolon location		1142 (51.8)		
L4—including upper GI location		211 (9.6)		
E1—proctitis		-	201 (14.3)	
E2—left-sided			458 (32.7)	
E3—extensive colitis			660 (47.1)	
Unknown			83 (5.9)	
Disease activity (CDAI/Mayo), *n* (%)				
Remission	122 (3.4)	100 (4.5)	22 (1.6)	
Mild	719 (19.9)	417 (18.9)	302 (21.5)	
Moderate	1683 (46.6)	1156 (52.4)	527 (37.6)	
Severe	440 (12.2)	188 (8.5)	252 (18.0)	
Unknown	646 (17.9)	347 (15.7)	299 (21.3)	

Disease behavior, *n* (%)				
B1—inflammatory disease	-	1175 (53.3)	-	
B2—stricturing disease		690 (31.3)		
B3—penetrating disease		338 (15.4)		
Perianal involvement, *n* (%)				
Fistula	-	772 (35.0)	-	
Abscess		392 (17.8)		
Fissure		38 (1.7)		
Extraintestinal manifestations, *n* (%)	464 (12.9)	357 (16.2)	107 (7.6)	< 0.0001
Family history of IBD, *n* (%)	27 (0.7)	20 (0.9)	7 (0.5)	0.1671
History of gastrointestinal surgery, *n* (%)	594 (16.5)	524 (23.7)	70 (5.0)	< 0.0001
History of perianal surgery, *n* (%)	471 (13.0)	471 (21.3)	0 (0.0)	< 0.0001
Medication use before diagnosis (≤ 6 mo), *n* (%)	908 (25.2)	440 (19.9)	468 (33.4)	< 0.0001

The classification of body mass index: Underweight: < 18.5; Normal: 18.5-23.9; Overweight: 24-28; Obese: > 28. Disease behavior was not available in 5 patients. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IQR: Interquartile ranges; BMI: Body mass index; CDAI: Crohn's Disease Activity Index.

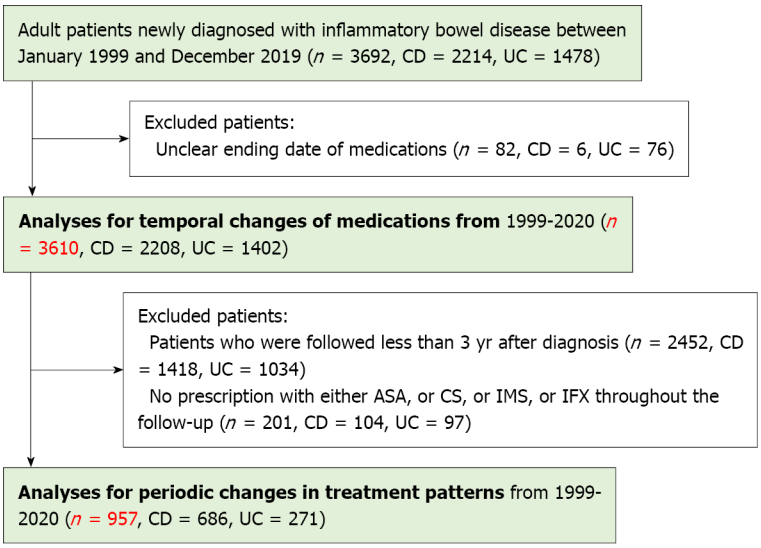

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Figure 1 Flowchart of inflammatory bowel disease selection and analysis. CD: Crohn's disease; UC: Ulcerative colitis; ASA: Aminosalicylates; CS: Corticosteroids; IMS: Immunosuppressants; IFX: Infliximab.

combination therapy containing IMS and IFX was observed during the subsequent 6-36 mo. Meanwhile, conventional therapies including 5-ASA, CS, and CS combined with IMS were steadily used. When comparing the two cohorts, we found that all initial prescriptions originated from conventional medications in Cohort I, while therapies comprising of IFX or IMS accounted for 45.0% of all initial prescriptions in Cohort II (Supplementary Figures 1 and 2).

Data on UC are presented in Figure 3B and Supplementary Table 4. 5-ASA (56.1%) was the most frequently prescribed medication in initial strategies, followed by CS (20.7%). Similar to CD, 60.1% of patients stopped medications within 1-3 mo, but 38.7% were put back on the same or other drugs for treatment progression, resulting in a steady increase of 5-ASA, CS, and IFX/IMS prescriptions in the following period. Compared with Cohort I in initial strategies, we still found 5-ASA and CS comprised a majority of prescriptions in Cohort II (Supplementary Figures 3 and 4).

Factors associated with initial drug strategies

Since initial therapeutic regimens may affect follow-up strategies to some extent, we further invest-

Table 2 Comparison of demographic and clinical characteristics of patients with inflammatory bowel disease included and excluded from analysis in 1999-2020

	Excluded (*n* = 2653)	Included (*n* = 957)	*P* value
Male sex, *n* (%)	1268 (47.8)	398 (41.6)	0.0009
Median age of onset (IQR), yr	32.0 (23.0-46.0)	30.0 (22.0-41.0)	< 0.001
Median age at diagnosis (IQR), yr	35.0 (25.0-49.0)	32.0 (24.0-44.0)	< 0.001
Median interval from onset to diagnosis (IQR), mo	12.2 (2.4-36.6)	12.2 (2.4-32.9)	0.3270
Smoking status at diagnosis, *n* (%)			0.0005
Non-smoker	2067 (77.9)	793 (82.8)	
Current smoker	273 (10.3)	83 (8.7)	
Former smoker	174 (6.6)	59 (6.2)	
Unknown	139 (5.2)	22 (2.3)	
Region of urbanization, *n* (%)			0.0411
City	1450 (54.7%)	509 (53.2)	
Rural	1164 (43.9)	443 (46.3)	
Unknown	39 (1.4)	5 (0.5)	
Occupation, *n* (%)			< 0.0001
Unemployed	371 (14.0)	124 (12.9)	
Employed	1226 (46.2)	625 (65.3)	
Student	272 (10.2)	105 (11.0)	
Retire	201 (7.6)	57 (6.0)	
Unknown	583 (22.0)	46 (4.8)	
BMI category at diagnosis, *n* (%)			< 0.0001
Normal	694 (26.1)	320 (33.4)	
Underweight	1100 (41.5)	424 (44.4)	
Overweight + Obese	278 (10.5)	101 (10.6)	
Unknown	580 (21.9)	111 (11.6)	
Disease location, *n* (%)			
L1—ileal location	441 (29.1)	185 (27.0)	0.3722
L2—colon location	158 (10.4)	66 (9.6)	
L3—ileocolon location	768 (50.6)	374 (54.6)	
L4—including upper GI location	151 (9.9)	60 (8.8)	
E1—proctitis	156 (13.8)	45 (16.6)	0.0005
E2—left-sided	360 (31.8)	98 (36.2)	
E3—extensive colitis	534 (47.2)	126 (46.5)	
Unknown	81 (7.2)	2 (0.7)	
Disease activity (CDAI), *n* (%)			0.0002
Remission	75 (4.9)	25 (3.6)	
Mild	314 (20.6)	103 (15.0)	
Moderate	748 (49.1)	408 (59.5)	
Severe	136 (9.0)	52 (7.6)	
Unknown	249 (16.4)	98 (14.3)	
Disease activity (Mayo), *n* (%)			< 0.0001

Remission	18 (1.6)	4 (1.5)	
Mild	231 (20.4)	71 (26.2)	
Moderate	406 (35.9)	121 (44.7)	
Severe	192 (17.0)	60 (22.1)	
Unknown	284 (25.1)	15 (5.5)	
Disease behavior (CD), *n* (%)			0.4273
B1—inflammatory disease	797 (52.5)	378 (55.2)	
B2—stricturing disease	488 (32.1)	202 (29.5)	
B3—penetrating disease	233 (15.4)	105 (15.3)	
Perianal involvement (CD), *n* (%)			0.0800
Fistula	524 (34.4)	248 (36.2)	
Abscess	262 (17.2)	130 (19.0)	
Fissure	24 (1.6)	14 (2.0)	
Extraintestinal manifestations, *n* (%)	318 (12.0)	146 (15.3)	0.0096
Family history, *n* (%)	19 (0.7)	8 (0.8)	0.7124
History of gastrointestinal surgery, *n* (%)	423 (15.9)	171 (17.9)	0.1687
History of perianal surgery, *n* (%)	263 (9.9)	208 (21.7)	< 0.0001
Medication use within 6 mo before diagnosis, *n* (%)	646 (24.3)	262 (27.4)	0.0643

The classification of body mass index: Underweight: < 18.5; Normal: 18.5-23.9; Overweight: 24-28; Obese > 28. Disease behavior was not available in 1407 patients. IQR: Interquartile ranges; BMI: Body mass index; CDAI: Crohn's disease Activity Index; CD: Crohn's Disease.

igated potential factors impacting initial drug strategies.

Table 3 presents factors for commencing different medications compared with a no prescription group among patients with CD. Specifically, variables including urban living, L3, B3, gastrointestinal surgical history, and diagnosis after 2009 yielded the most significant predictors of patients not commencing CS for initial treatment strategies. However, patients with a drug history had a higher probability of commencing CS. When compared with the no prescription group, patients of male sex, urban living, BMI ≥ 24.0 kg/m^2, B3, and gastrointestinal surgical history were less likely to be prescribed CS combined with IMS as initial treatment. In contrast, patients with L4, EIMs, drug history, and perianal surgical history were more likely to be prescribed CS combined with IMS strategy. In terms of comparison between the IFX and no prescription groups, patients who were smokers, had a BMI ≥ 24.0 kg/m^2, B2 or B3, and had a gastrointestinal surgical history had a fewer possibility to be prescribed IFX. Contrarily, patients who were students, L4, had moderate severity, and had a perianal surgical history were more likely to be prescribed IFX.

Table 4 presents the variables that determined initial therapeutic regimens in patients with UC. Particularly, patients with a drug history tended to maintain treatments after diagnosis, regardless of type (5-ASA, CS, IMS, or IFX). Variables including current smoker, severe disease activity, and diagnosis after 2009 were the most significant factors for not commencing 5-ASA, whereas variables including employed or retired and E3 were related to a higher probability of commencing 5-ASA. Likewise, patients with a BMI ≥ 24.0 kg/m^2 and diagnosed after 2009 were less likely to be prescribed CS, and those with E3 and EIMs involvement were likely to be prescribed CS. Moreover, patients with a drug history were more likely to be prescribed IFX/IMS for initial treatment.

DISCUSSION

To the best of our knowledge, no multicenter cohort study has been conducted to investigate temporal trends in long-term medication use and periodic changes in treatment paradigms in Chinese IBD patients. Our findings showed that prescriptions of IMS and IFX gradually increased in IBD over the past 20 years, paralleled by decreasing prescriptions of 5-ASA and CS for CD but not for UC. Additionally, periodic changes in treatment patterns revealed a switching profile from conventional medications to IFX in CD, while 5-ASA and CS still took irreplaceable positions in UC.

Table 3 Influencing factors of initial drug strategies in patients with Crohn's disease

	CS (*n* = 132)		CS + IMS (*n* = 203)		IFX (*n* = 728)	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Sex						
Female	Ref	–	Ref	–	Ref	–
Male	0.86 (0.57-1.29)	0.4577	0.49 (0.33-0.73)	0.0005	0.89 (0.70-1.13)	0.3295
Age of onset	1.02 (0.95-1.10)	0.5165	1.01 (0.93-1.09)	0.8581	0.99 (0.95-1.02)	0.4621
Age at diagnosis	1.00 (0.93-1.08)	0.9428	0.99 (0.92-1.07)	0.8167	0.98 (0.95-1.02)	0.3752
Smoking status at diagnosis						
Non-smoker	Ref	–	Ref	–	Ref	–
Current smoker	0.91 (0.49-1.71)	0.7754	0.86 (0.48-1.54)	0.6041	0.64 (0.42-0.99)	0.0452
Former smoker	0.65 (0.26-1.62)	0.3560	0.66 (0.30-1.43)	0.2900	0.47 (0.28-0.81)	0.0062
Unknown	1.30 (0.46-3.71)	0.6211	NS	NS	1.11 (0.62-1.99)	0.7192
Region of urbanization						
Rural	Ref	–	Ref	–	Ref	–
Urban	0.60 (0.40-0.89)	0.0117	0.49 (0.34-0.69)	< 0.0001	1.07 (0.85-1.35)	0.5761
Unknown	NS	NS	NS	NS	1.84 (0.76-4.49)	0.1794
Occupation						
Unemployed	Ref	–	Ref	–	Ref	–
Employed	1.27 (0.72-2.23)	0.4031	1.01 (0.62-1.63)	0.9826	1.11 (0.78-1.58)	0.5578
Student	1.27 (0.53-3.05)	0.5960	0.98 (0.49-1.95)	0.9539	1.61 (1.02-2.53)	0.0408
Retire	0.62 (0.21-1.84)	0.3916	0.56 (0.16-1.94)	0.3619	1.74 (0.85-3.54)	0.1267
Unknown	0.46 (0.19-1.11)	0.0830	NS	NS	0.86 (0.54-1.37)	0.5216
BMI category at diagnosis						
Normal	Ref	–	Ref	–	Ref	–
Underweight	1.30 (0.83-2.04)	0.2498	0.88 (0.60-1.30)	0.5295	0.92 (0.71-1.21)	0.5645
Overweight + Obese	0.56 (0.25-1.26)	0.1601	0.42 (0.20-0.88)	0.0204	0.47 (0.30-0.73)	0.0008
Unknown	0.46 (0.23-0.94)	0.0320	0.14 (0.04-0.46)	0.0012	0.81 (0.57-1.14)	0.2241
Disease location						
L1 – ileal location	Ref	–	Ref	–	Ref	–
L2 – colon location	0.81 (0.41-1.57)	0.5317	0.74 (0.35-1.55)	0.4201	0.94 (0.63-1.43)	0.7869
L3 – ileocolon location	0.58 (0.37-0.90)	0.0162	1.05 (0.69-1.61)	0.8105	1.12 (0.85-1.46)	0.4211
L4 – including upper GI location	0.69 (0.31-1.56)	0.3708	1.91 (1.02-3.57)	0.0438	1.88 (1.20-2.96)	0.0061
Disease activity (CDAI)						
Remission	Ref	–	Ref	–	Ref	–
Mild	0.84 (0.28-2.51)	0.7498	2.17 (0.84-5.61)	0.1091	1.44 (0.79-2.63)	0.2346
Moderate	1.56 (0.57-4.33)	0.3888	2.35 (0.94-5.86)	0.0664	2.08 (1.18-3.69)	0.0118
Severe	0.77 (0.19-3.04)	0.7050	0.46 (0.08-2.53)	0.3710	1.62 (0.83-3.16)	0.1576
Unknown	1.37 (0.45-4.15)	0.5791	0.88 (0.28-2.78)	0.8290	1.05 (0.56-1.98)	0.8746
Disease behavior						
B1 – inflammatory disease	Ref	–	Ref	–	Ref	–
B2 – stricturing disease	0.82 (0.53-1.27)	0.3734	0.99 (0.67-1.47)	0.9531	0.63 (0.48-0.83)	0.0008
B3 – penetrating disease	0.22 (0.10-0.48)	< 0.001	0.12 (0.05-0.30)	< 0.0001	0.39 (0.28-0.56)	< 0.0001

Extraintestinal manifestations						
No	Ref	–	Ref	–	Ref	–
Yes	1.10 (0.65-1.87)	0.7105	1.88 (1.23-2.87)	0.0037	0.93 (0.67-1.28)	0.6605
Family history of IBD						
No	Ref	–	Ref	–	Ref	–
Yes	1.46 (0.14-15.30)	0.7514	1.70 (0.22-13.10)	0.6104	1.67 (0.37-7.62)	0.5082
History of gastrointestinal surgery						
No	Ref	–	Ref	–	Ref	–
Yes	0.35 (0.20-0.60)	0.0002	0.35 (0.20-0.59)	< 0.0001	0.55 (0.41-0.73)	< 0.0001
History of perianal surgery						
No	Ref	–	Ref	–	Ref	–
Yes	1.36 (0.77-2.40)	0.2948	1.86 (1.20-2.87)	0.0052	2.68 (1.92-3.73)	< 0.0001
Medication use before diagnosis (≤ 6 mo)						
No	Ref	–	Ref	–	Ref	–
Yes	1.94 (1.16-3.23)	0.0111	3.06 (2.04-4.58)	< 0.0001	1.33 (0.95-1.85)	0.0934
Calendar year of diagnosis						
< 2009	Ref	–	Ref	–	Ref	–
≥ 2009	0.19 (0.09-0.40)	< 0.0001	0.77 (0.25-2.33)	0.6393	NS	NS

CS: Corticosteroids; IMS: Immunosuppressants; IFX: Infliximab; OR: Odd ratio; CI: Confidence interval; BMI: Body mass index; CDAI: Crohn's Disease Activity Index; IBD: Inflammatory bowel disease; NS: Non-significant.

In China, more than 1.5 million people are expected to suffer from IBD by 2025[3]. Therefore, the study population accounted for merely 0.2% of the total estimated Chinese patients with IBD. However, because there is lack of national registries covering all IBD patients, it is difficult to acquire complete data on the diagnosis, treatment, and prognosis of all Chinese patients with IBD. A well-organized national IBD registry in the near future will help facilitate larger clinical studies in China. Besides, IBD referral centers in our study are distributed across all seven administrative regions in China with diverse socioeconomic backgrounds. To some extent, the study population represents in-patients from Chinese referral centers. Moreover, we found similar patterns in demographic and clinical characteristics by further comparing IBD patients in our study with those in other large-sample Chinese studies, which also reflects the representativeness of our study to a certain extent[20,21].

In this study, the median (IQR) age of onset and diagnosis was 31.0 (23.0–45.0) years and 33.0 (25.0–48.0) years, respectively, similar to other Asian and Western populations[22,23]. The total median duration of follow-up was short. There are two possible explanations for this. On one hand, the study population mainly stems from IBD referral centers and therefore many patients will go back to grass-roots hospitals for following treatment after acquiring definite IBD diagnosis and initial treatment strategies, which may result in loss to follow-up. On the other hand, due to the observational design of this study, most information originated from medical records or databases. It would be a huge cost to follow such a large number of patients. Despite the short follow-up period, which may affect medication trend analysis, there were still more than 1000 patients who were followed for more than three years, and our analysis towards long-term changes in treatment patterns provided a credible result, which can assist in clinical drug management. We also compared baseline characteristics between CD and UC patients. Regarding demographic differences, CD patients seemed more likely to be younger at onset or diagnosis and have longer intervals from onset to diagnosis and follow-up duration. This is possibly due to more sophisticated processes in CD diagnosis and treatment. However, UC patients were more likely to be overweight compared to those with CD. UC patients who seldom implicate small intestine that would cause malabsorption of nutrients and following weight loss might explain BMI differences. In regard to clinical discrepancies, more CD patients had EIMs involvement, family history, and gastrointestinal and perianal surgical histories than UC. Patients with CD often undergo intestinal resection for perforation, ileocecal junction inflammation, or perianal surgeries before diagnosis. Contrarily, more medications were likely prescribed for UC before diagnosis, which was probably due to the fact that 5-ASA was also prescribed for patients with undetermined intestinal ulcers before definite diagnosis. Furthermore, L3 was the most frequent disease location for CD, while B1 and B2 predominated disease behaviors, sharing similar clinical patterns with other Asian cohorts[22,24].

Table 4 Influencing factors of initial drug strategy in patients with ulcerative colitis

	5-ASA (*n* = 566)		CS (*n* = 213)		IFX/IMS (*n* = 43)	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Sex						
Female	Ref	–	Ref	–	Ref	–
Male	1.02 (0.78-1.34)	0.8770	0.90 (0.63-1.27)	0.5397	0.98 (0.50-1.92)	0.9493
Age of onset	0.98 (0.95-1.02)	0.4057	1.03 (0.97-1.09)	0.3654	0.93 (0.87-0.99)	0.0233
Age at diagnosis	1.02 (0.98-1.05)	0.3876	0.96 (0.91-1.02)	0.1792	1.04 (0.98-1.10)	0.2434
Smoking status at diagnosis						
Non-smoker	Ref	–	Ref	–	Ref	–
Current smoker	0.64 (0.41-1.00)	0.0490	0.67 (0.38-1.18)	0.1651	0.94 (0.32-2.79)	0.9156
Former smoker	0.76 (0.46-1.25)	0.2812	0.87 (0.45-1.67)	0.6713	2.04 (0.72-5.78)	0.1777
Unknown	3.39 (1.83-6.27)	0.0001	1.15 (0.42-3.19)	0.7864	3.42 (0.62-18.90)	0.1583
Region of urbanization						
Rural	Ref	–	Ref	–	Ref	–
Urban	0.80 (0.60-1.07)	0.1311	0.82 (0.57-1.19)	0.2945	1.03 (0.51-2.08)	0.9245
Unknown	0.11 (0.02-0.64)	0.0138	0.23 (0.02-2.21)	0.2010	NS	NS
Occupation						
Unemployed	Ref	–	Ref	–	Ref	–
Employed	2.85 (1.79-4.55)	< 0.001	1.23 (0.74-2.06)	0.4241	0.93 (0.34-2.53)	0.8866
Student	1.06 (0.39-2.87)	0.9159	0.57 (0.19-1.71)	0.3178	0.78 (0.12-5.10)	0.7976
Retire	2.39 (1.31-4.35)	0.0043	1.14 (0.55-2.39)	0.7216	2.02 (0.52-7.91)	0.3135
Unknown	2.07 (1.15-3.70)	0.0145	0.73 (0.36-1.46)	0.3691	1.93 (0.60-6.19)	0.2697
BMI category at diagnosis						
Normal	Ref	–	Ref	–	Ref	–
Underweight	0.85 (0.55-1.31)	0.4669	1.22 (0.74-2.03)	0.4325	0.90 (0.36-2.22)	0.8136
Overweight + Obese	1.10 (0.73-1.64)	0.6532	0.52 (0.28-0.99)	0.0455	0.49 (0.14-1.77)	0.2757
Unknown	0.78 (0.55-1.12)	0.1742	0.78 (0.49-1.23)	0.2900	0.42 (0.16-1.11)	0.0785
Disease location						
E1 – proctitis	Ref	–	Ref	–	Ref	–
E2 – left-sided	1.18 (0.78-1.78)	0.4254	1.67 (0.82-3.38)	0.1585	2.57 (0.54-12.20)	0.2331
E3 – extensive colitis	1.58 (1.05-2.38)	0.0288	3.06 (1.54-6.08)	0.0014	2.93 (0.63-13.60)	0.1695
Unknown	4.41 (1.95-9.97)	0.0004	3.99 (1.23- 12.9)	0.0209	NS	NS
Disease activity (MAYO)						
Remission	Ref	–	Ref	–	Ref	–
Mild	0.65 (0.22-1.92)	0.4374	1.04 (0.11-9.97)	0.9697	0.35 (0.03-3.86)	0.3905
Moderate	0.76 (0.26-2.22)	0.6156	2.99 (0.33-27.10)	0.3292	0.57 (0.06-5.61)	0.6308
Severe	0.32 (0.10-0.97)	0.0442	6.51 (0.71-59.60)	0.0972	1.91 (0.19-19.00)	0.5798
Unknown	0.39 (0.13-1.22)	0.1061	1.57 (0.16-15.30)	0.6994	0.09 (0.01-1.24)	0.0716
Extraintestinal manifestations						
No	Ref	–	Ref	–	Ref	–
Yes	0.74 (0.41-1.31)	0.2993	1.94 (1.07-3.51)	0.0287	1.83 (0.72-4.62)	0.2025
Family history of IBD						

No	Ref	–	Ref	–	Ref	–
Yes	0.68 (0.09-4.94)	0.7041	0.87 (0.08-10.00)	0.9096	NS	NS
History of gastrointestinal surgery						
No	Ref	–	Ref	–	Ref	–
Yes	0.68 (0.36-1.26)	0.2163	0.57 (0.25-1.31)	0.1824	0.88 (0.18-4.23)	0.8683
Medication use before diagnosis (≤ 6 mo)						
No	Ref	–	Ref	–	Ref	–
Yes	10.2 (7.19-14.5)	< 0.0001	7.23 (4.71-11.10)	< 0.0001	9.88 (4.77-20.50)	< 0.0001
Calendar year of diagnosis						
< 2009	Ref	–	Ref	–	Ref	–
≥ 2009	0.31 (0.22-0.45)	< 0.0001	0.51 (0.30-0.85)	0.0097	3.12 (0.41-23.90)	0.2748

5-ASA: 5-aminosalicylates; CS: Corticosteroids; IMS: Immunosuppressants; IFX: Infliximab; OR: Odd ratio; CI: Confidence interval; BMI: Body mass index; NS: Non-significant; IBD: Inflammatory bowel disease.

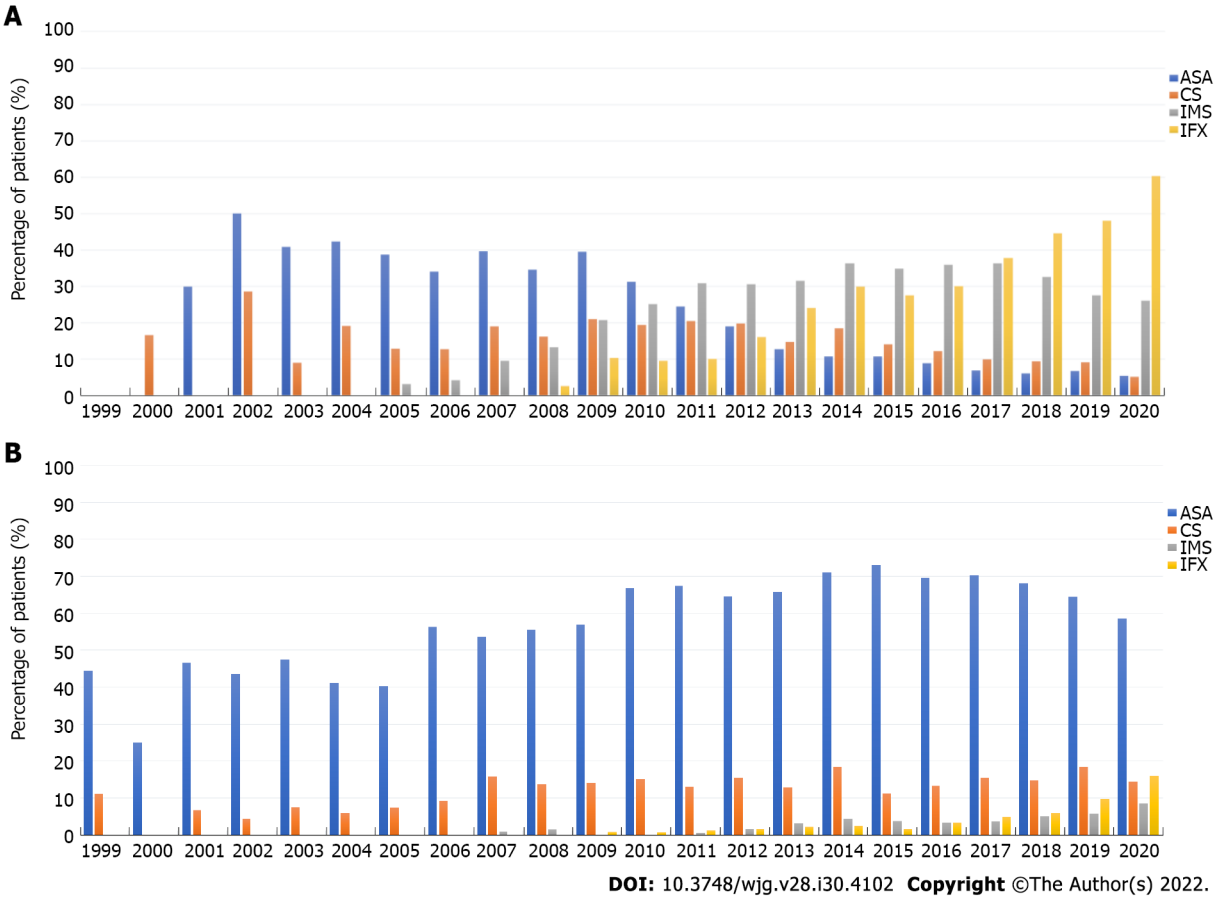

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Figure 2 Temporal trends in medication use in patients with inflammatory bowel disease. A: Trends in medication use in patients with Crohn's disease; B: Trends in medication use in patients with ulcerative colitis. ASA: Aminosalicylates; CS: Corticosteroids; IMS: Immunosuppressants; IFX: Infliximab.

Currently, IBD medications mainly include 5-ASA, CS, IMS, and biological agents. In our study, trend analysis for CD found a prominent decline in prescriptions of 5-ASA and CS in parallel to a significant increase in prescriptions of IMS and IFX from 1999 to 2020. A previous national study from the American database reported that 37.3% of patients with CD were prescribed 5-ASA between 2009 and 2014, despite the debatable effectiveness of 5-ASA[25]. Nevertheless, the latest ECCO guideline no longer recommends 5-ASA in patients with mild-to-moderate CD[26]. In our analysis, 5-ASA usage significantly decreased from 39.6% in 2009 to 5.5% in 2020, reflecting a positive trend in narrowing the

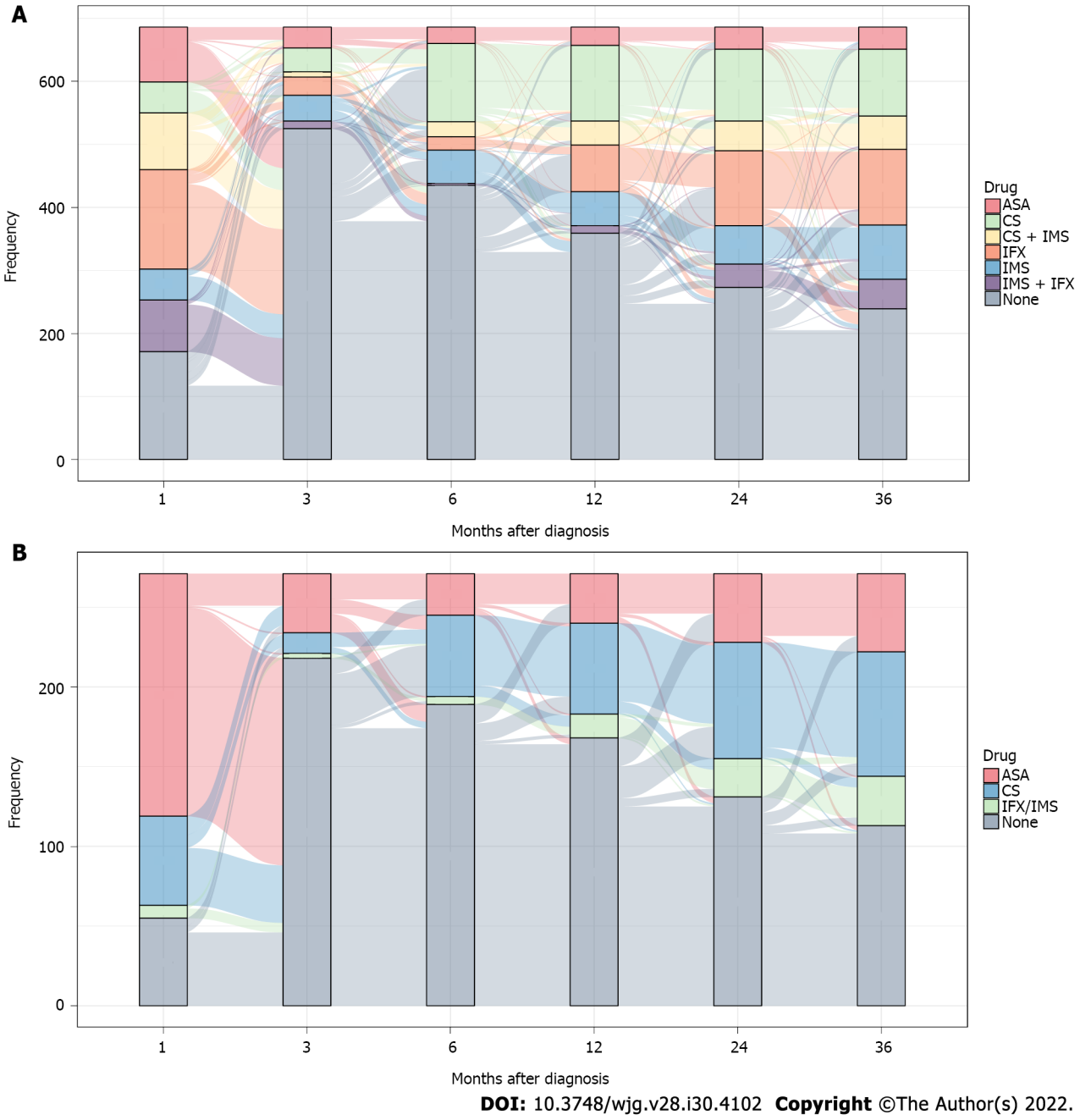

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Figure 3 Periodic changes in treatment patterns in patients with inflammatory bowel disease. A: Crohn's disease; B: Ulcerative colitis. ASA: Aminosalicylates; CS: Corticosteroids; IMS: Immunosuppressants; IFX: Infliximab.

knowledge gap between Chinese providers and IBD consensus. We also observed an apparent reduction in CS prescription onwards, which was probably due to improved awareness of steroid-sparing, earlier treatment after diagnosis, closer surveillance for disease relapse, or more attention on adverse effect of CS[27]. Moreover, IMS emerged in 2005, and prescription rates increased strikingly afterwards, except for a slight decrease between 2018 and 2020. The effect of maintenance treatment with IMS administered to patients with CD who are steroid-dependent has been testified[28]. IFX prominently increased between 2008 and 2020. These may reflect deeper market infiltration of IFX and better treatment adherence. It is worth noting that insurance coverage of IFX was not achieved until November 28, 2019, the day IFX entered the national medical insurance. This policy will influence the decision making of Chinese IBD patients on whether to choose IFX or other IMS and may explain treatment discrepancies between Chinese patients and those from other countries. Regarding trend analysis in UC patients, 5-ASA and CS were steadily prescribed, accompanied by a modest increase in IMS and IFX, demonstrating 5-ASA as a milestone during therapeutic armamentarium extension. The American Gastroenterological Association recommends treating patients with mild-to-moderate left sided UC with mesalamine or diazo-bonded 5-ASA compounds[29]. For patients with extensive mild-to-moderate UC, ECCO recommends the addition of rectal 5-ASA to oral therapy, as combined oral and

rectal therapy delivers a greater effective dose to the affected areas of colon and leads to higher rates of remission[30]. In our study, 51.6% of CD patients and 60.1% of UC patients ceased medical treatment within 1-3 mo. We realized the contradicting results from our study with the current strategy applied worldwide. Our treatment data are mainly derived from IBD referral centers where patients may cease treatment after returning home with only one-month prescriptions. This may be explained by the following reasons which reflect the specific situation of Chinese IBD management: (1) Lack of communication between referral centers and grass-roots hospitals during the early period of IBD treatment; (2) Poor medication adherence at the patient level[31]; and (3) Knowledge gap between doctors and guidelines worldwide. Overall, we are the first to depict temporal changes in medications among a large Chinese IBD population, which has not been well elucidated before.

To further investigate long-term changes in treatment patterns, we included patients who were followed for at least three consecutive years. Our analysis showed that monotherapy or combination therapy with IFX and IMS accounted for nearly half of initial treatment strategies for CD. In fact, 12.7% were prescribed 5-ASA as the initial drug, among which nearly two-thirds ceased treatment within 1-3 mo. However, most patients switched to up-level drugs afterwards, probably reflecting the limitations of 5-ASA for disease maintenance. According to a previous study, treatment strategies for patients with moderate-to-severe CD could be listed as: (1) Conventional step-care therapy (CS and IMS are prescribed sequentially); (2) Accelerated step-care therapy (a tapering process of CS together with IMS); and (3) Top-down therapy (early combination of IMS and IFX). In our study, 7.2% of cases were prescribed CS as the initial drug, most of whom received conventional step-care therapy three months after diagnosis[32]. Another 13.1% were prescribed CS combined with IMS as the initial strategy, most of whom followed an accelerated step-care regimen. Moreover, top-down therapy was the first choice for almost half of the population. Despite short-term interruption, most patients recovered with IMS, IFX, or a minority of CS or 5-ASA. We noted that conventional medications were the main initial strategies in Cohort I, comprising mostly of two step-care therapies. By contrast, the initial approach in Cohort II was predominated by top-down therapies. Regarding therapeutic patterns in UC patients, initial treatments mainly included 5-ASA and CS. As the principal therapy in initial strategies, 5-ASA was highly effective for UC[33]. Similar to CD patterns, patients with UC who stopped treatment within 1-3 mo were re-prescribed different levels of medications afterwards, reflecting better adherence and more standardized treatment. Additionally, 5-ASA and CS were only initial strategies in Cohort I, complying with clinical settings in the early period of IBD management. In general, our analysis provides a unique insight into long-term therapeutic paradigms in Chinese population.

Since initial treatments were crucial for long-term therapeutic paradigms, we next investigated potential predictors for initial drugs. In CD patients, L3 congruously reduced the possibility of commencing CS, whereas L4 increased the possibility of commencing CS combined with IMS or commencing IFX. This is likely due to the fact that patients with upper gastrointestinal involvement are prone to behave in a more sophisticated course, requiring IMS or IFX for maintenance of remission. Intriguingly, patients with penetrating behavior and gastrointestinal surgical history appeared to have a lower likelihood of being prescribed either drug, given the intricate and drug-refractory nature, calling for short-term surgical intervention after diagnosis. We also observed an increase in CS combined with IMS or IFX in patients who underwent perianal surgeries before diagnosis. A possible explanation is that poor healing after perianal surgeries provide useful evidence for making detailed diagnosis and more appropriate selection of therapeutic strategies. Regarding factors in UC, we found an appreciable correlation between all strategies and medication history. This finding should be carefully generalized to other UC populations. Thus, future investigation elucidating detailed association between treatments before diagnosis and initial therapeutic paradigms in larger population is warranted. Interestingly, patients with E3 reflected a higher risk of 5-ASA and CS medications, possibly explained by the fact that patients with extensive colitis naturally tended to adopt medications that might relieve symptoms. Moreover, our observations indicated that patients diagnosed after 2009 were less likely to be prescribed 5-ASA or CS as initial therapies, which is mainly due to IFX insurance coverage or improved awareness of steroid-sparing medications. Generally, these results suggest that baseline characteristics probably affect initial treatment strategies to a certain extent.

There are several strengths in this study. First, patients were from IBD referral centers, which covered all the administrative regions in China. The population represented a broad spectrum of Chinese patients with IBD for over 20 years, facilitating temporal trends evaluation in medication use and drug strategies. Second, detailed baseline characteristics and prescriptions through follow-up were retrieved from consecutive medical records, which may reflect actual drug intake to some extent. Lastly, no study has analyzed periodic changes in treatment paradigms for three consecutive years and associated factors with initial drug strategies in Chinese IBD patients.

The study also has limitations. First, extrapolation of medication trends to the entire country and other Asian countries remains unproven due to lack of the national registry in China and potential differences in racial phenotypes and treatment strategies worldwide. A wholesome IBD registry system and multicenter studies among Asian populations are needed in the near future. Second, potential information and confounding bias exist due to observational design. Therefore, we assessed baseline factors thoroughly and employed multivariate logistic regression analysis to minimize bias. Moreover, outcomes, including hospitalization, surgeries, or phenotype progression were not collected, limiting

further investigation in the correlation between different drug strategies and long-term outcomes.

CONCLUSION

In conclusion, this study provides insights into temporal trends in long-term medication use and periodic changes in treatment patterns for Chinese patients with IBD. The findings suggest that prescriptions of IMS and IFX increased in parallel with steady or decreasing prescriptions of 5-ASA and CS in real-world settings. The study also shed light on periodic changes in treatment patterns, reflecting a switching profile from conventional drugs to IFX in CD. Further research into the full breadth of trends in medication use and treatment patterns should be conducted for additional compliance with guidelines for IBD management.

ARTICLE HIGHLIGHTS

Research background

Medications for inflammatory bowel disease (IBD) have changed dramatically over time, especially following market availability of infliximab (IFX). Several attempts have been made by Western and other Asian countries to provide rough medication changes.

Research motivation

No study on long-term medication profiles has been conducted in Chinese population, and minimal attention has been paid to periodic changes in IBD treatment strategies. Additionally, investigating possible predictors for initial treatment strategies may help to better understand periodic changes in therapeutic patterns in patients with IBD.

Research objectives

This study was designed to leverage the real-world evidence in Chinese referral hospitals to provide fresh insights into temporal trends in medication prescriptions among the Chinese population with IBD for over 20 years, and to investigate long-term periodic changes in treatment paradigms and identify the possible factors that influence initial drug strategies.

Research methods

A multicenter retrospective cohort study was conducted to analyze trends in medication use and therapeutic patterns. Predictors for initial drug strategies were identified using logistic regression analysis.

Research results

Of 5-aminosalicylates (5-ASA) and corticosteroids (CS) prescriptions gradually decreased, accompanied by a notable increase in immunosuppressants (IMS) and IFX prescriptions in patients with Crohn's disease (CD). Prescription rates of 5-ASA and CS were stable, whereas IMS and IFX slightly increased since 2007 in patients with ulcerative colitis (UC). Subgroup analyses showed the switch from conventional medications to IFX in patients with CD, while 5-ASA and CS were still steadily prescribed in patients with UC. Logistic regression analyses revealed that surgical history, disease behavior, and disease location were associated with initial therapeutic strategies in patients with CD. However, medications before diagnosis, disease location, and diagnostic year might affect initial strategies in patients with UC.

Research conclusions

Parallel to increasing IMS and IFX use in IBD over the past two decades, a significant decrease in 5-ASA and CS use were observed for CD but not for UC. Long-term treatment strategies analyses provided a unique insight in switching from conventional drugs to IFX in Chinese patients with CD.

Research perspectives

The study was based on IBD referral centers, therefore the study population only accounted for limited proportion of total Chinese patients with IBD. A well-organized national registry system in the near future will help facilitate larger clinical studies in China. In addition, clinical outcomes, including hospitalization, surgeries, or phenotype progression were not collected. Further investigation in the correlation between different drug strategies and long-term outcomes are needed in future studies.

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FOOTNOTES

Author contributions: Cao Q formulated the research idea; Yao LY developed the study protocol; Yao LY, Tian F, Ye M, Li YQ, Wang XL, Wang L, Yang SQ, Lv XP, Jia Y, Wang XH, Zhang XQ and Wei YL conducted the patient identification and data collection; Shao BL and Yao LY conducted the data analysis; Yao LY, Shao BL and Cao Q developed the manuscript; and all authors have read and approve the final manuscript.

Institutional review board statement: The study protocol was reviewed and approved by Sir Run Run Shaw Hospital, College of Medicine Zhejiang University Institutional Review Board with approval for all hospitals involved in the study, No. 20210714-31.

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ORIGINAL ARTICLE

Retrospective Study

Salivary *Fusobacterium nucleatum* serves as a potential diagnostic biomarker for gastric cancer

Wen-Dan Chen, Xin Zhang, Meng-Jiao Zhang, Ya-Ping Zhang, Zi-Qi Shang, Yi-Wei Xin, Yi Zhang

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Abstract

BACKGROUND

As one of the most common tumors, gastric cancer (GC) has a high mortality rate, since current examination approaches cannot achieve early diagnosis. *Fusobacterium nucleatum* (Fn) primarily colonized in the oral cavity, has been reported to be involved in the development of gastrointestinal tumor. Until now, little is known about the relationship between salivary Fn and GC.

AIM

To determine whether salivary Fn could be a biomarker to diagnose GC and explore the influence of Fn on GC cells.

METHODS

The abundance of Fn in saliva was quantified by droplet digital polymerase chain reaction in 120 GC patients, 31 atrophic gastritis (AG) patients, 35 non-AG (NAG) patients, 26 gastric polyp (GP) patients, and 20 normal controls (NC) from Qilu Hospital of Shandong University from January 2019 to December 2020. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic value of Fn as well as traditional serum tumor markers, including carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, and CA72-4. Transwell assay and wound-healing assay were conducted to assess the influence of Fn infection on GC cells. The expression of epithelial-mesenchymal transition (EMT) markers was detected using western blot assay.

RESULTS

We found that the level of salivary Fn in GC patients was significantly increased compared with those in AG, NAG, and GP patients and NC ($P < 0.001$). ROC curve analysis showed a favorable capability of Fn (73.33% sensitivity; 82.14% specificity; area under the curve: 0.813) in GC diagnosis, which was superior to that of CEA, CA19-9, CA72-4, ferritin, and sialic acid. The Fn level in saliva of GC

patients was increased as the TNM stage increased. GC patients with lymph node metastasis had higher Fn levels than those without metastasis. Both transwell and wound-healing assays indicated that Fn infection promoted the migration and invasion of GC cells. Western blot analysis showed that Fn infection decreased the expression of E-cadherin and increased the expressions of N-cadherin, vimentin, and snail.

CONCLUSION

Fn abundance in saliva could be used as a promising biomarker to diagnose GC, and Fn infection could promote GC metastasis by accelerating the EMT process.

Key Words: Gastric cancer; *Fusobacterium nucleatum*; Saliva; Prognosis; Metastasis; Epithelial-mesenchymal transition

Core Tip: *Fusobacterium nucleatum* (Fn), known as a periodontal pathogen, is frequently detected in tissues of gastrointestinal tumor. Here, we found that the level of salivary Fn was increased in gastric cancer (GC) patients, which could be used as a potential biomarker for GC diagnosis, and its diagnostic capability was superior to that of traditional serum tumor markers. We also showed that salivary Fn level was positively associated with the GC TNM stage and lymph node metastasis. Further, experiments *in vitro* revealed that Fn could promote the migration and invasion of GC cells by promoting the epithelial-mesenchymal transition process. Our findings suggest that salivary Fn is a potential biomarker in diagnosis of GC.

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignant tumors, with morbidity and mortality ranking fifth and third in the world, respectively, posing a great threat to global human health[1]. Although the 5-year survival rate of GC is enhanced with the combination of chemotherapy and surgery, its mortality remains high due to the diagnosis at an advanced stage with a limited therapeutic scheme to choose from. Therefore, effective early diagnosis for GC is especially important.

The microorganisms in the human body are complex and they play important roles in each part of our body. Over 700 bacterial species have been detected in the human oral cavity, and they interact with each other as well as with the host[2]. The stomach was considered a sterile organ until the discovery of *Helicobacter pylori* (*H. pylori*) in 1984[3]. Subsequently, other microorganisms were found in the gastric mucosa[4]. Although the gut microbiota ecosystem is different from that of the oral cavity, there is also a partial similarity between them, since most of the bacteria in the oral cavity enter the stomach when swallowing food and saliva.

Human saliva is a unique biofluid resource with huge clinical diagnostic and risk assessment capacity. Moreover, it is easy to acquire in non-invasive and painless approaches. Most importantly, individual oral environment basically maintains a long-term relatively stable state except for little fluctuation caused by diet, infections, or other factors, and the dominant oral floras in different individuals are host specific[5]. *Fusobacterium nucleatum* (Fn) is a Gram-negative anaerobic bacterium, which is a normal composition of the oral microenvironment[6,7]. In recent years, due to its increased detection rate in oral infectious diseases, it has been identified as an opportunistic pathogen[8]. Chronic periodontitis is caused by the ecological imbalance of the subgingival plaque biofilm communities, leading to the growth of dominant species, which destroys the host immune response and leads to inflammation[9]. Fn has been proved to be one of the pathogens that grow abnormally before periodontal disease, leading to an imbalance in the composition of oral symbiotic bacteria, which leads to the occurrence of periodontitis[10]. It forms a bridge between the early symbiotic colonizers and the late pathogenic colonizers[11]. In recent years, Fn has also been found to be associated with extra-oral infections, including adverse pregnancy outcomes, rheumatoid arthritis, respiratory tract infections, and gastrointestinal neoplasm[12-14]. Accumulating evidence has shown that Fn is associated with the occurrence, development, and metastasis of colorectal cancer (CRC). However, the information for GC is

limited. In the present study, we aimed to detect the Fn abundance in saliva using digital droplet polymerase chain reaction (ddPCR), and establish a new simple and effective diagnostic approach to improve the early diagnosis of GC.

MATERIALS AND METHODS

Study population and sample collection

Subjects with GC (n = 120), atrophic gastritis (AG) (n = 31), non-AG (NAG) (n = 35), and gastric polyps (GP) (n = 26) and normal controls (NC) (n = 20) undergoing gastroscopy examination at Qilu Hospital of Shandong University from January 2019 to December 2020 were enrolled in this study. The selection of NC mainly depended on the normal results of gastroscopy examination, biochemical indexes, and tumor marker detection. GC, AG, NAG, and GP were further confirmed based on histopathological analyses. The pathological stage of GC was assessed by the 8th edition TNM staging system. The exclusion criteria were as follows: (1) Subjects who used any antibiotics within the last 4 wk; (2) Subjects with oral inflammation; and (3) Subjects without detailed general clinical data. Whole saliva specimens were collected with Salivette® tube before brushing teeth and eating breakfast in the morning. After collection, samples were immediately transferred to the laboratory with ice gauges and centrifuged at 3000 r/min for 10 min at 4 °C. All saliva samples were stored at -80 °C until further analysis. All study participants provided informed written consent prior to study enrollment. This study was approved by the Ethics Committee of the Qilu Hospital of Shandong University (Approval No. KYLL-2019-2-013).

DNA isolation

Total genomic DNA was extracted from 200 μL saliva using the QIAamp Blood Mini Kit (cat. 51106, Qiagen, Hilden, Germany), and purified DNA was finally dissolved in 80 μL AE buffer. The concentration of DNA was determined with a Qubit 3 Fluorometer using an Equalbit 1 × dsDNA HS Assay Kit (cat. EQ121-01, Thermo Fisher Scientific, United States) according to the manufacturer's instructions.

ddPCR

ddPCR was performed on the bio-digital PCR platform (Turtle Tech Ltd., Shanghai, China) in a 35 μL reaction system consisting of 5 μL genomic DNA, 3.5 μL 10 × dPCR buffer, 1 μL dPCR enzyme, 1 μM primers, and 0.5 μM probe of Fn (forward primer: 5'-TGGTGTCATTCTTCCAAAAATATCA-3'; reverse primer: 5'-AGATCAAGAAGGACAAGTTGCTGAA-3'; probe: 5'-ACTTTAACTCTACCATGTTCA-3'). After the automatic sample loading process was completed, the chips with samples added were transferred into a nucleic acid amplification instrument (Turtle Tech Ltd., Shanghai, China). Briefly, after an initial enzyme activation step at 50 °C for 10 min and then at 90 °C for 10 min, amplification (45 cycles) of Fn DNA was carried out. After amplification, the chips were transferred to the commercial digital PCR platform (BioDigital dPCR System, Turtle Tech. Ltd., Shanghai, China) to detect fluorescence amplitude signals.

Cell culture

The GC cell lines AGS and MKN-28 were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. (Shanghai, China) and maintained in appropriate medium (Hyclone, United States) supplanted with 10% fetal bovine serum (Hyclone, United States) under the standard conditions (50 mL/L CO_2, 37 °C).

Bacterial strains and growth conditions

Two strains of Fn (ATCC49256 and ATCC25586) used in our assays were purchased from American Type Culture Collection. The strains were first inoculated and cultured on Columbia Blood Agar under the anaerobic condition at 37 °C for 48 h. Then a single colony was transferred to a fluid thioglycolate medium, followed by incubation at 37 °C under anaerobic conditions (90 mL/L N_2, 50 mL/L CO_2, and 50 mL/L H_2) to reach an optical density (OD_{600}) of 0.2 for each bacterial strain.

Coculture

AGS and MKN-28 cells (2 × 10^5/per well) were seeded in six-well plates with 2 mL complete RPMI-1640 medium and cultured overnight. The plates were then incubated under an anaerobic condition mentioned above for 24 h. On the next day, the cultured Fn was centrifuged (4400 × g for 5 min at room temperature), and the bottom bacteria were sedimented and then re-suspended with fresh RPMI-1640 medium after the supernatant was discarded. After the OD_{600} was determined, the resuspended Fn was added to AGS and MKN-28 cells for co-culture. Cell treated with phosphate buffer solution served as a control group. The plates were centrifuged at 250 × g for 5 min and placed back in an anaerobic incubator for 24 h. For all the experiments in this study, Fn bacteria were added to the cells at an multiplicity of infection (MOI) of 100 based on the preliminary experiments, and this value was common in the published articles.

Transwell assay

After 24 h of infection, the cells were washed and seeded into the upper chamber of an 8-μm transwell insert (Costar, Corning, New York, United States). Transwell chambers used for invasion assay were pre-coated with Matrigel (Corning), while the chambers used for migration assay were not treated. The upper chamber of the transwell contained 2×10^4 cells in a volume of 200 μL serum-free medium, and 700 μL of medium containing 10% serum was added into the lower chamber. After being cultured for 24 h, the cells in the upper chamber were wiped with the cotton tip, and the adherent cells in the lower chamber were fixed with 40 g/L formaldehyde for 10 min and stained with 0.5% crystal violet for 30 min. The images were captured with an inverted microscope (Olympus, Tokyo, Japan).

Wound-healing assay

AGS and MKN-28 cells (5×10^5 cells/ per well) were seeded in six-well plates, and the later infection process was the same as the transwell assay above-mentioned. After 24 h of Fn infection, the cells were washed with RPMI-1640 medium (by this time, the cells were grown to 100% confluence, if not, cultured continually). A 200 μL pipette tip was used to make a straight scratch, simulating a wound. Subsequently, the medium and floating cells were removed and replaced with a serum-free culture medium, and the imagines were captured (0 h). After 48 h of cell culture, images were captured again. The cell migration rate was evaluated by comparing the healing degree between 0 h and 48 h.

Western blot analysis

The AGS and MKN-28 cells with or without Fn infection were lysed in radioimmune precipitation assay buffer for 30 min on ice. Then the cell lysates were centrifuged at 12000 r/min for 10 min at 4 °C, and the supernatant was collected. The bicinchoninic acid protein assay kit (Pierce, Rockford, United States) was used to quantify protein concentrations. Subsequently, equal amounts of proteins (30 μg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then electrophoretically transferred onto polyvinylidene fluoride membranes. The blots were blocked with 5% skim milk in Tris-buffered saline with tween (TBST) (BD Biosciences, San Jose, CA, United States) containing 0.1% tween-20 (Bio-Rad, Richmond, CA, United States) at room temperature for 1 h. The membranes were then incubated overnight at 4 °C with the primary antibodies against E-cadherin (1:1000, Cell Signaling, #3195S, Danvers, MA, United States), N-cadherin (1:1000, Cell Signaling, #13116T), vimentin (1:1000, Cell Signaling, #5741S, Danvers, MA, United States), Snail (1:1000, Cell Signaling, #3879S), and GAPDH (1:1000, Cell Signaling, #5174S). The membranes were washed three times with 1 × TBST buffer, followed by incubation with anti-rabbit HRP-conjugated secondary antibody (1:5000; Huaan, Hangzhou, China) at room temperature for 1.5 h. The immunoreactive bands were visualized using an Amersham Imager 680 (GE, Boston, United States). GAPDH served as a loading control.

Statistical analysis

Statistical analyses were conducted using SPSS software (SPSS Inc., Chicago, IL, United States) and graphs were prepared using GraphPad Prism 8.0 (La Jolla, CA). Statistical review was performed by a biomedical statistician. Fn abundance in different groups is expressed as the median and interquartile range. A χ^2 test was used for the comparison of categorical variables. A Kruskal-Wallis test was performed for the global comparison of multiple groups and post-hoc multiple comparisons were performed using the Mann-Whitney *U* test. Receiver operating characteristic (ROC) curve analysis was conducted *via* MedCalc15.2.2 to evaluate the diagnostic value of Fn as well as other serum tumor markers, and the best cut-off values were calculated by the Youden index. All statistical tests were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Abundance of Fn in the saliva of GC patients

Our ddPCR results showed a significant difference in salivary Fn among the patients with GC, AG, NAG, and GP, and NC. And the Fn level was significantly increased in GC patients compared with patients with AG, NAG, and GP and NC while there was no difference among AG, NAG, and GP patients and NC (Figure 1A). Moreover, the Fn level was increased as the TNM stage increased (Figure 1B). Further clinical characteristic analysis showed that GC patients with lymph node metastasis had higher Fn levels than those without (Table 1). However, the Fn level in GC was not significantly associated with age, gender, tumor size, pathological differentiation, or invasion depth.

Diagnostic performance of salivary Fn in GC patients

ROC analysis indicated some diagnostic power with a fitted area under the curve (AUC) of 0.813 (95% confidence interval: 0.756-0.861) (Figure 2A). When the Youden index was used to calculate the optimal cut-off value of the Fn copy number as 67.58 copies/μL, the sensitivity and specificity were 73.33% and 82.14%, respectively. To better evaluate the diagnostic value of Fn in saliva for GC, the routine clinical

Table 1 Association of *Fusobacterium nucleatum* levels in saliva with clinicopathologic features

Feature	Number of cases	*Fusobacterium nucleatum* level	*P* value
Age (yr)			0.780
< 61	56	153.00 (39.20-542.00)	
≥ 61	64	204.00 (57.30-566.00)	
Gender			0.961
Male	99	161.00 (53.52-620.60)	
Female	21	213.60 (46.63-335.90)	
Tumor size (cm)			0.450
< 5	76	152.90 (52.21-443.80)	
≥ 5	44	239.90 (47.31-846.80)	
Pathological differentiation			0.658
Well	77	147.50 (33.98-543.90)	
Moderate	33	155.80 (84.94-754.60)	
Poor	10	280.80 (78.46-518.70)	
Invasion depth			0.227
T1	29	124.60 (32.21-415.80)	
T2	60	298.30 (76.58-666.90)	
T3	21	102.10 (68.62-385.70)	
T4	10	223.00 (66.99-790.30)	
Lymph nodes metastasis			< 0.001
N0	43	80.14 (23.98-241.80)	
N1	25	324.50 (72.43-549.10)	
N2	22	239.60 (79.36-501.70)	
N3	30	478.00 (96.09-1054.00)	

test records of traditional serum tumor markers, including CEA, CA19-9, CA72-4, ferritin, and sialic acid were assessed. The upper cut-off values of these markers were determined according to the manufacturer's directions. Table 2 shows that the Fn level was significantly higher in GC patients compared with AG, NAG, and GP patients and NC, while CEA, CA19-9, CA72-4, and ferritin did not significantly different between GC patients and the other four groups. Although sialic acid was also significantly different among the five groups, its diagnostic value was still lower than that of Fn, according to the ROC curves.

The ROC curves presented that the AUC of CEA, CA19-9, CA72-4, ferritin, and sialic acid was 0.620, 0.570, 0.541, 0.648, and 0.693, respectively (Figures 2B-F). In addition, the sensitivity of Fn was higher compared with the other traditional serum markers. As for the specificity, the value of Fn was higher than those of CEA, CA72-4, ferritin, and sialic acid, but lower than that of CA19-9. Of note, although the specificity of CA19-9 was high, its sensitivity was relatively low (Table 3). These results indicated that the diagnostic capability of Fn was superior to that of CEA, CA19-9, CA72-4, ferritin, and sialic acid.

Role of Fn in GC metastasis

To evaluate the effect of Fn infection *in vitro*, we infected AGS and MKN-28 cells with Fn. The transwell assay indicated that Fn infection significantly enhanced the invasive and migratory capacities of AGS and MKN-28 cells (Figure 3). Consistent with these results, the wound-healing assay showed that Fn infection promoted the migration of these cells (Figure 4). Since epithelial-mesenchymal transition (EMT) is an important process of metastasis, we examined the effect of Fn infection on the expression of proteins involved in the EMT process. The western blot analysis revealed that Fn infection decreased the expression of epithelial markers, such as E-cadherin, but increased the expression of mesenchymal phenotype-associated molecules, such as N-cadherin, vimentin, and snail (Figure 5).

Table 2 Characteristics and levels of biomarkers among different subjects

	Normal control	Atrophic gastritis	Non-atrophic gastritis	Gastric polyps	Gastric cancer
Cases	20	31	35	26	120
Gender (male/female)	13/7	17/14	19/16	15/11	73/47
Age (yr)[1]	56.90 ± 16.02	62.13 ± 10.56	60.80 ± 14.54	58.17 ± 15.26	61.38 ± 10.31
CEA (mg/L)[2,3]	2.03 (1.22-2.56)	1.95 (1.49-53.37)	1.72 (1.44-2.60)	1.36 (1.10-1.85)	2.46 (1.38-4.63)
CA19-9 (pg/mL)[2,3]	6.38 (3.61-11.39)	10.67 (8.68-16.35)	10.30 (7.09-15.07)	9.20 (5.68-12.09)	11.46 (6.75-20.28)
CA72-4 (U/mL)[2,3]	2.86 (0.98-6.48)	0 (0-3.18)	2.41 (1.50-4.65)	1.71 (0.15-4.63)	2.58 (0-5.69)
Ferritin (ng/mL)[2,3]	143.15 (97.04-273.98)	96.98 (69.33-206.55)	126.70 (74.49-245.30)	182.95 (101.13-263.88)	86.20 (25.99-165.75)
Sialic acid (g/L)[2,4]	54.30 (51.45-56.75)	52.50 (46.50-55.30)	53.70 (48.25-60.55)	56.2 (50.53-59.65)	59.55 (53.28-65.33)
Fusobacterium nucleatum (copies/μL)[2,4]	16.70 (2.4-26.10)	28.611 (5.99-61.50)	12.18 (0.74-35.55)	16.32 (1.59-122.234)	177.09 (53.09-547.11)

[1]Data are presented as the mean ± SD.
[2]Data are presented as the median (interquartile range).
[3]Date are compared using Kruskal-Wallis test, $P < 0.05$.
[4]Date are compared using Kruskal-Wallis test, $P < 0.001$.
CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

Table 3 Diagnostic performance of *Fusobacterium nucleatum*, carcinoembryonic antigen, carbohydrate antigen 19-9, carbohydrate antigen 72-4, ferritin, and sialic acid for gastric cancer

Index	AUC	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Fn	0.813	67.58 copies/μL	73.33	82.14	81.48	74.19
CEA	0.620	5 ng/mL	55.00	69.64	66.00	59.09
CA19-9	0.570	69.2 pg/mL	26.67	86.61	68.09	52.43
CA72-4	0.541	6.9 U/mL	67.50	44.64	56.64	56.18
Ferritin	0.648	400 ng/mL	51.67	23.21	41.89	30.95
Sialic acid	0.693	75.4 mg/dL	60.83	70.54	68.87	62.70

AUC: Area under curve; PPV: Positive predictive value; NPV: Negative predictive value; Fn: *Fusobacterium nucleatum*; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

DISCUSSION

In the present study, we reported the relationship between the Fn level in saliva and GC development for the first time. First, a more sensitive ddPCR approach was used to determine the amount of Fn DNA in saliva, and the results showed that a higher number of Fn existed in the saliva of GC patients compared with NC and patients with benign disease. Importantly, salivary Fn had good diagnostic value for GC, which was superior to traditional serum tumor markers, such as CEA, CA19-9, CA72-4, ferritin, and sialic acid, providing a new direction for the diagnosis of GC. We also found that the Fn level was positively associated with TNM stage and lymph node metastasis. Furthermore, transwell and wound-healing assays verified the role of Fn in promoting the invasion and migration of GC cells. Subsequently, we indicated that Fn infection promoted GC metastasis by accelerating the EMT process.

It is essential to diagnose GC at an early stage. Currently, gastroscopy in combination with biopsy is considered the most accurate method to diagnose GC. However, early asymptomatic patients have poor compliance with gastroscopy due to its invasiveness, which directly reduces the rate of early diagnosis of GC. Serum tumor markers are another approach to screen GC. For example, CEA, CA19-9, and CA72-4 are widely used in clinical practice. However, serum tumor markers are not recommended for routine clinical screening due to the lack of specificity and sensitivity[15]. In a cohort consisting of 587 early GC patients, the positive rate of CA19-9, CA125, and CEA was 4.8%, 1.9%, and 4.3%, respectively[16]. For Fn in saliva, it is not only easy to obtain, but also shows a better diagnostic capacity for GC. Moreover, salivary Fn has a pleasurable tissue specificity as a diagnostic marker for GC.

H. pylori is a well-known risk factor for GC. However, other bacterial genera are also detected in the gastric mucosa, such as *Prevotella, Rothia, Fusobacterium,* and *Klebsiella*[17,18]. Fn, a resident colony of

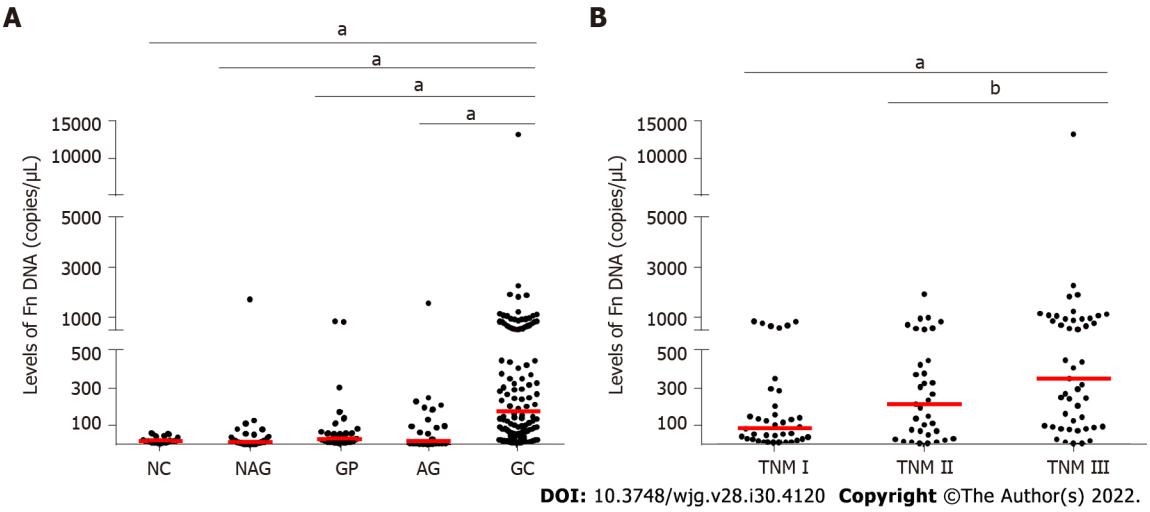

Figure 1 *Fusobacterium nucleatum* levels in different groups. A: *Fusobacterium nucleatum* (Fn) levels in normal control, atrophic gastritis (AG), non-AG, gastric polyps, and gastric cancer (GC) groups; B: Fn levels in different TNM stages of GC patients. Red lines represent the median. $^{a}P < 0.001$, $^{b}P < 0.05$ (Mann-Whitney *U* test). NC: Normal controls; NAG: Non-atrophic gastritis; GP: Gastric polyps; AG: Atrophic gastritis; GC: Gastric cancer; Fn: *Fusobacterium nucleatum*.

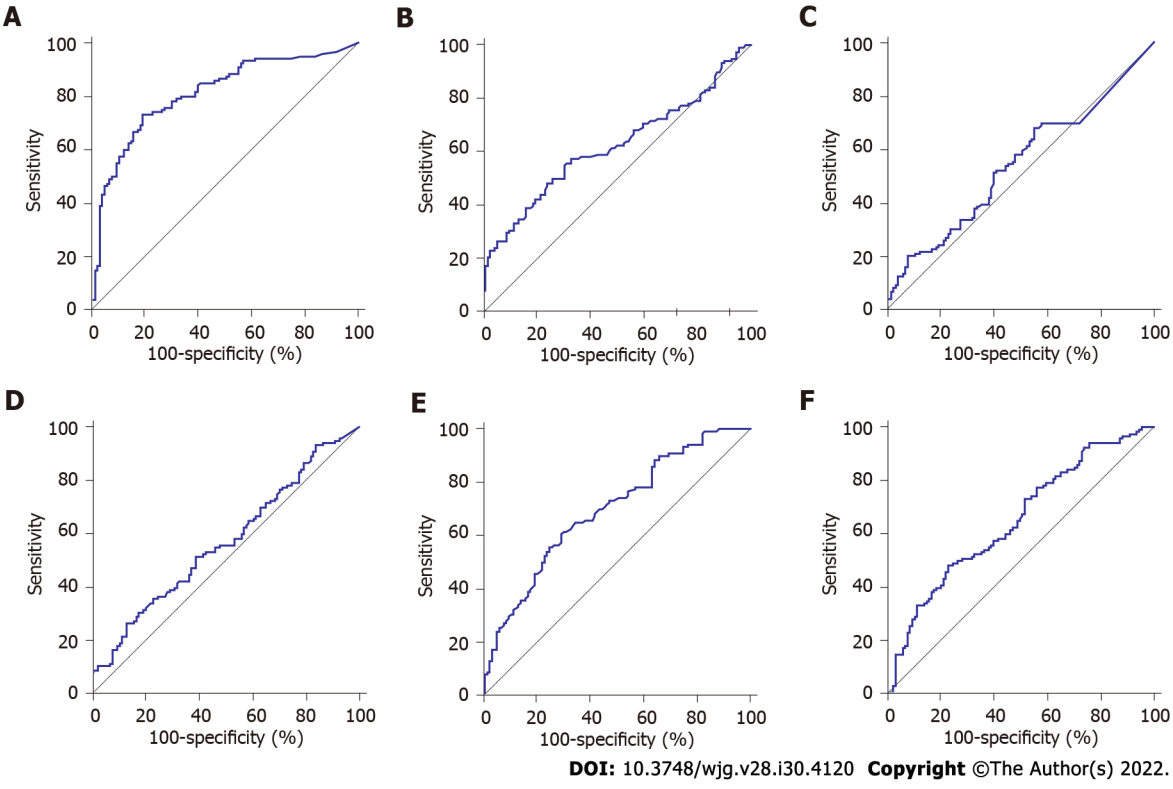

Figure 2 Diagnostic significance analysis of *Fusobacterium nucleatum* and traditional serum markers. Receiver operating characteristic curve analysis for the detection of gastric cancer. A: *Fusobacterium nucleatum*; B: Carcinoembryonic antigen; C: Carbohydrate antigen 72-4; D: Carbohydrate antigen 19-9; E: Ferritin; F: Sialic acid.

bacteria in the oral cavity, is detected significantly higher in the GC tissue compared with the para-cancerous tissue[19]. There is a microbial succession hypothesis to explain the decrease of *H. pylori* and elevation of the non-dominant flora in GC[18]. The hypothesis proposes that *H. pylori* can produce urease through the mucus layer, enhancing pH value to modify the microenvironment and triggering a strong inflammatory response. In such a process, gastric mucosal integrity is destroyed, and other secondary bacteria invade the mucus layer, causing more aggressive tumor occurrence. Therefore, Fn in the oral cavity can easily reach the stomach and colonize through the gastric mucosa barrier as the swallowing of saliva, forming an invasion focus. Thus, we think that Fn cooperates with *H. pylori* to promote GC development. Hsieh *et al*[20] have shown that Fn colonization leads to worse prognosis in GC patients with *H. pylori* positivity.

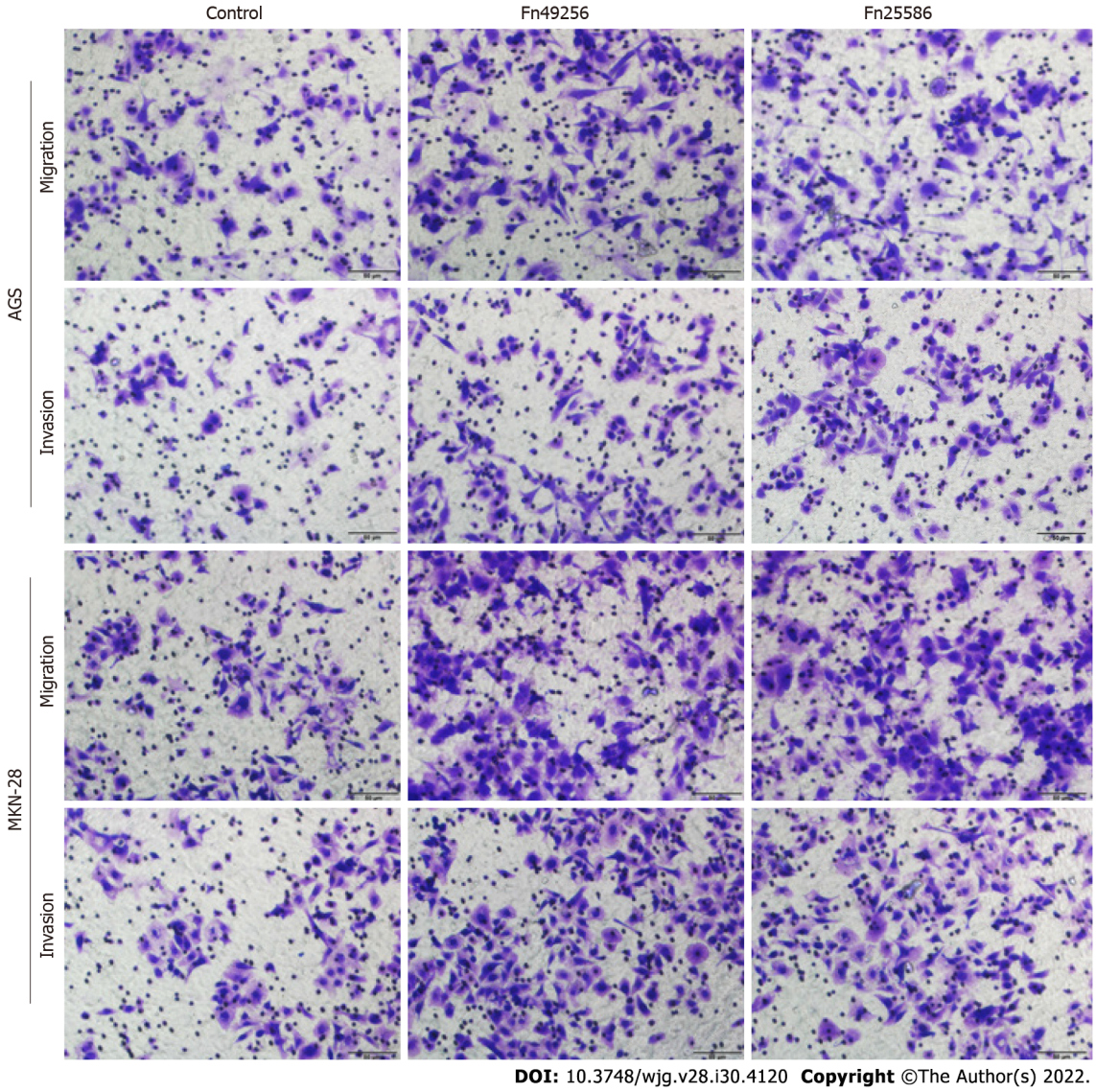

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Figure 3 *Fusobacterium nucleatum* promotes the invasion of gastric cancer cells. Transwell assay of AGS and MKN-28 cells was performed 48 h after coculture with *Fusobacterium nucleatum*. The cells in the control group were treated with phosphate buffer solution. Fn: *Fusobacterium nucleatum*.

As a high-frequency periodontal pathogen, Fn has been confirmed to promote the initiation, proliferation, invasion, and chemoresistance of CRC in recent years. There are several suggested mechanisms, such as enhancing adhesion and evasion ability, modulating host immune evasion, and improving autophagy to cause chemoresistance[21-23]. It is worth noting that Fn is considered to play a more important role in promoting the formation of tumor, rather than its development[24]. Consistent with these findings, Fn has been confirmed to have key pathogenic characteristics, such as virulence factor FadA and characteristic adhesion protein Fap2[25-27]. In addition to promoting the occurrence and development of CRC, accumulating evidence has shown that Fn also plays an important role in promoting CRC metastasis[28,29]. In our present study, a high level of Fn was found in GC patients with lymph node metastasis. Although its impact in promoting tumor metastasis has been recognized, the role of Fn in such a process and its potential mechanism remain largely unclear. Chen *et al*[30] have detected Fn colonization in metastatic lung lesions of nude mice, and they further verified that Fn infection up-regulates KRT7 (type II cytokeratin, which plays a role in maintaining the structural integrity of the cells as well as promoting motile activities) to promote CRC metastasis. Besides, Chen *et al*[31] have identified that Fn activates autophagy to promote CRC metastasis. It is worth mentioning that EMT is the most well-validated method by which Fn promotes CRC metastasis, and it has been discovered that the effect is enhanced when the MOI is increased[32-34]. EMT is a classical pathway promoting metastasis. It is a special program that enables settled epithelial cells to gain the ability to migrate as single cells, which can enhance mobility, invasion, and resistance to apoptosis, conferring metastatic properties of cancer cells[35]. The examinations conducted in CRC both *in vivo* and *in vitro* have verified that Fn can promote the EMT process[28]. We supplemented the evidence that Fn promoted the EMT process in GC cells. Moreover, Fn infection significantly decreased the expression of

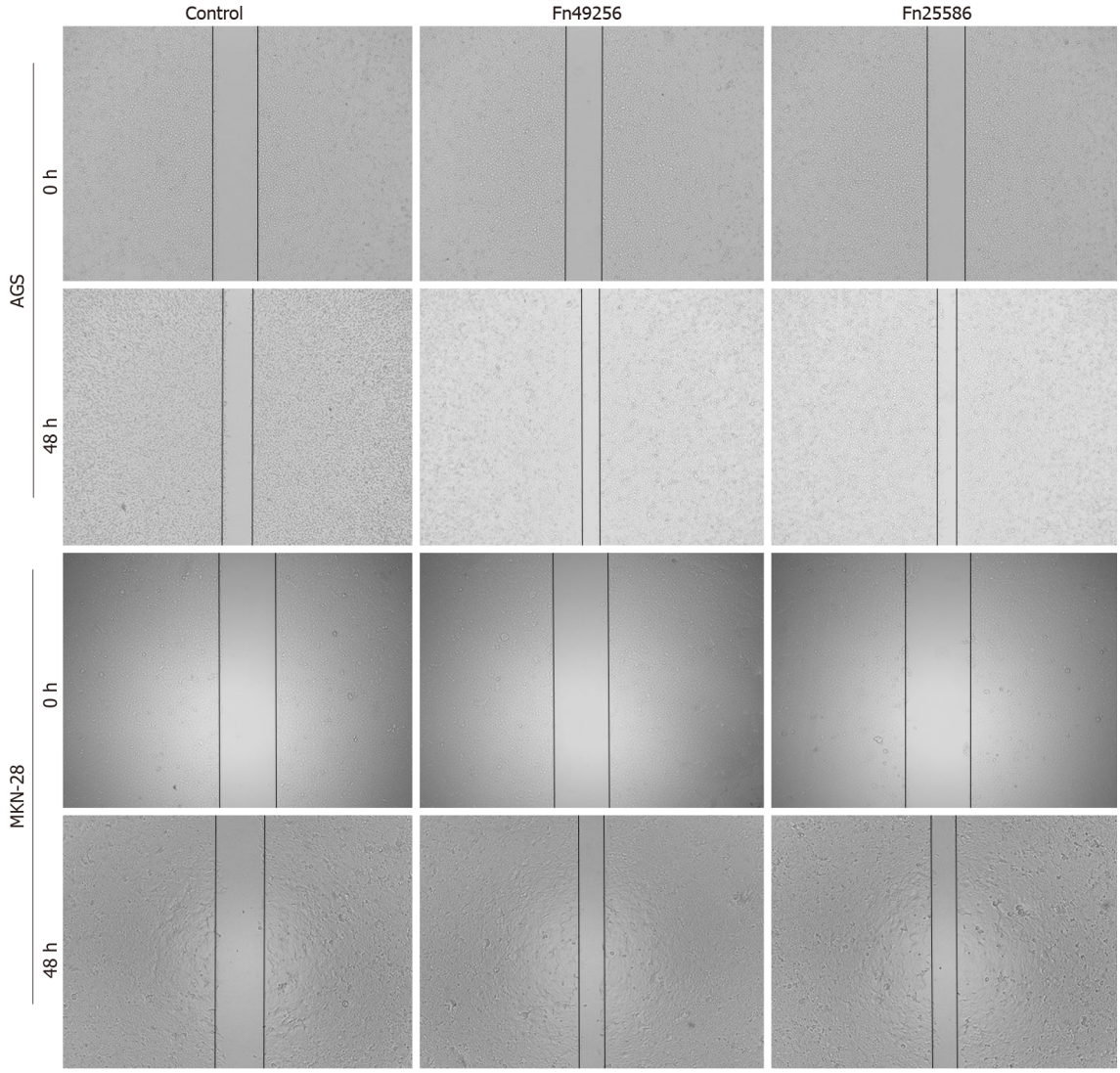

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Figure 4 *Fusobacterium nucleatum* promotes the migration of gastric cancer cells. Wound-healing assay of AGS and MKN-28 cells was performed 48 h after coculture with *Fusobacterium nucleatum*. The cells in the control group were treated with phosphate buffer solution. Fn: *Fusobacterium nucleatum*.

cell adhesion molecules and increased the expression of mesenchymal phenotype-associated molecules, eventually resulting in GC metastasis. Taken together, we identified that Fn promoted GC metastasis by facilitating the EMT process.

Targeting Fn may reduce the progression of related cancers to ascertain the origin of Fn in cancers, and determining the route by which it reaches the tumor is of therapeutic significance. It has been found for a long time that part of Fn detected in CRC tissue is originated from the oral cavity, while how Fn in the oral cavity is transported to other body sites is also a hot topic. Initially, Fn strains with similar sequences were found in human primary CRC tissues and paired liver metastases, suggesting that these bacteria can migrate to distant sites with CRC cells[29]. Therefore, some scholars believe that Fn enters tumor tissue *via* blood circulation. The existing data have shown that Fn is detected at higher loads in GC tissue[19], and our data suggested that Fn abundance was increased in the saliva of GC patients. Could this indicate that Fn could directly enter the gastrointestinal tract, and then colonize epithelial cells? For Fn can produce a variety of adhesion factors as well as invasive factors, it is easy to adhere to the gastrointestinal mucosa, and form an inflammatory microenvironment, which is conducive to the formation of tumor. However, some evidence has also shown that Fn in the blood is more successful in CRC colonization than being gavaged, indicating that Fn the in blood (from a bleeding wound in the gum or some other way) plays a more important role in forming colonization than the ones in the digestive tract, even if there is a disadvantage in quantity[36]. Which type of Fn transmission is dominant in tumor formation (or the two work together) remains to be further studied.

There are also some limitations in our study. First, the small sample size caused by high cost led to selection bias. In follow-up studies, larger, multicenter studies are required to consolidate the findings. Second, the flora in the oral cavity is diverse. Whether other floras have an impact on the occurrence and development of GC, or whether they have interacted with Fn needs to be further explored.

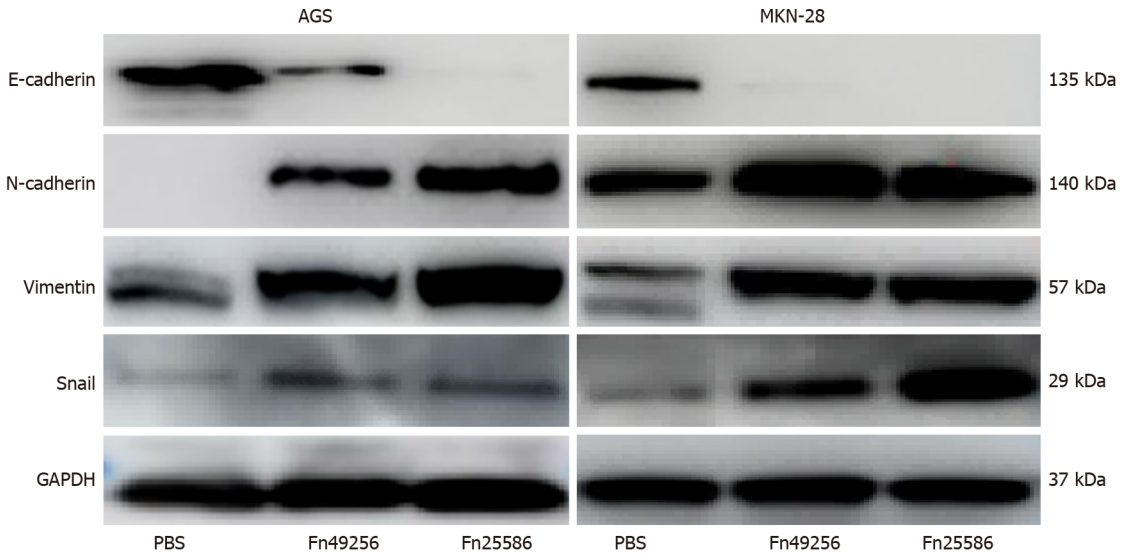

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Figure 5 *Fusobacterium nucleatum* enhances epithelial-mesenchymal transition of AGS and MKN-28 cells. Fn: *Fusobacterium nucleatum*; PBS: Phosphate buffer solution; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

CONCLUSION

Collectively, Fn in saliva exhibits a good predictive ability and represents a promising diagnostic marker for GC. Given the tumorigenicity and metastasis-promoting properties of Fn, we suggest that the elimination of Fn could reduce the incidence of GC and improve the treatment outcomes, which is similar to triple or quadruple anti-*H. pylori* therapy.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a common malignant tumor in the digestive tract. Although the 5-year survival rate of GC is enhanced with the combination of chemotherapy and surgery, its mortality remains high due to the diagnosis at an advanced stage. It is essential to develop a new method to diagnose GC at an early stage.

Research motivation

Fusobacterium nucleatum (Fn) primarily colonized in the oral cavity, has been detected in tissues of GC in recent years. We wondered whether there is a correlation between salivary Fn and GC.

Research objectives

The research purpose was to find a new simple and effective biomarker to diagnose GC.

Research methods

The abundance of Fn in saliva was quantified by droplet digital polymerase chain reaction in 120 GC patients, 31 atrophic gastritis (AG) patients, 35 non-AG (NAG) patients, 26 gastric polyp patients, and 20 normal controls (NC). The diagnostic value of Fn was evaluated and compared with traditional serum tumor markers, including carcinoembryonic antigen (CEA), carbohydrate antigen (CA)19-9, CA72-4, ferritin, and sialic acid. Transwell and wound-healing assays were conducted to assess the influence of Fn infection on GC cells. Western blot analysis was performed to detect the expression of epithelial-mesenchymal transition (EMT) markers.

Research results

Fn had favorable diagnostic value in classifying GC from NC and benign diseases, which was superior to those traditional serum tumor markers, such as CEA, CA19-9, CA72-4, ferritin, and sialic acid. Salivary Fn level was positively associated with the GC TNM stage and lymph node metastasis. Further, *in vitro* experiments revealed that Fn could promote the migration and invasion of GC cells. Western blot analysis indicated that Fn infection decreased the expression of epithelial marker such as E-cadherin, and increased the expression of mesenchymal markers such as N-cadherin, vimentin, and

snail.

Research conclusions

Fn in saliva could be used as a promising biomarker to diagnose GC, and Fn infection could promote GC metastasis by accelerating the EMT process.

Research perspectives

Human saliva is a unique bio-fluid resource with huge clinical diagnostic and risk assessment capacity. Salivary Fn has promising value for diagnosing GC.

FOOTNOTES

Author contributions: Chen WD and Zhang X contributed equally to this work; Chen WD performed the research; Zhang X contributed to conception and revised the manuscript; Zhang YP collected the samples; Chen WD, Zhang MJ, Zhang YP, Shang ZQ, and Xin YW performed the experiments together; Zhang Y managed and coordinated the research, and provided the financial support; and all authors approved the final version of the article.

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ORIGINAL ARTICLE

Retrospective Study

Development and validation of a nomogram for predicting overall survival in cirrhotic patients with acute kidney injury

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Abstract

BACKGROUND

Acute kidney injury (AKI) is a common and severe complication in patients with cirrhosis, and is associated with poor prognosis. Therefore, identifying cirrhotic patients with AKI who are at high risk of mortality is very important and may be helpful for providing timely medical interventions to improve the prognosis of these patients. However, studies focused on investigating the risk factors for the mortality of cirrhotic patients with AKI were scarce.

AIM

To identify risk factors for mortality and establish a nomogram for predicting the prognosis of these patients.

METHODS

Two hundred fifty consecutive patients with cirrhosis and AKI were recruited and randomly divided into training cohort (n = 173) and validation cohort (n = 77). In the training cohort, potential risk factors for death were identified by performing a Cox regression analysis, and a nomogram was established. The predictive performance of the nomogram was internally and externally validated by calculating the area under the receiver operating characteristic curve (AUROC), constructing a calibration curve and performing decision curve analysis.

RESULTS

The serum sodium level, international normalized ratio, peak serum creatinine level > 1.5 mg/dL, the presence of hepatic encephalopathy and diabetes were potential risk factors for mortality of cirrhotic patients with AKI in the training dataset. A prognostic nomogram incorporating these variables was established for

predicting the overall survival of these patients. Compared with Child-Turcotte-Pugh, the model for end-stage liver disease (MELD) and the MELD-Na scores, the nomogram in predicting 90- and 180-d mortality exhibited better discriminatory power with AUROCs of 0.792 and 0.801 for the training dataset and 0.817 and 0.862 for the validation dataset, respectively. With a nomogram score of 98, patients were divided into low- and high-risk groups, and high-risk patients had a higher mortality rate.

CONCLUSION

A prognostic nomogram displayed good performance for predicting the overall survival of cirrhotic patients with AKI, and will assist clinicians in evaluating the prognosis of these patients.

Key Words: Acute kidney injury; Cirrhosis; Nomogram; Prognosis

Core Tip: We investigated the potential risk factors for death in cirrhotic patients with acute kidney injury (AKI). A nomogram incorporating these risk factors was developed and evaluated by calculating the area under the receiver operating characteristic curve, constructing a calibration curve and performing decision curve analysis. Compared with Child-Turcotte-Pugh, the model for end-stage liver disease (MELD) and MELD-Na score, the nomogram has a better discriminative ability in predicting the overall survival of cirrhotic patients with AKI. Moreover, the nomogram was used to select patients with a high risk of death and assist clinicians in making clinical decisions.

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INTRODUCTION

Acute kidney injury (AKI) is a common and severe complication occurring in patients with cirrhosis, manifesting as an abrupt increase in serum creatinine (SCr) levels and an acute significant reduction in urine output (UO) in a short period[1,2]. Many diagnostic criteria and classifications of AKI have been developed to identify AKI and improve prognosis over the last two decades, such as the Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease (RIFLE) classification in 2004[3], the Acute Kidney Injury Network (AKIN) classification in 2007[4], and the Kidney Disease: Improving Global Outcomes (KDIGO) classification in 2012[5]. However, the concept of AKI in patients with cirrhosis was debated for many years until the International Club of Ascites (ICA) proposed a new classification of AKI in 2015[1]. These classifications are based on SCr levels and/or UO. One of the main differences between the ICA classification and other classifications is the abandonment of the UO criteria[1]. Although these criteria have been established, a single criterion that comprehensively evaluates and diagnoses AKI is still unavailable.

The incidence and mortality of AKI in patients with cirrhosis varies substantially among studies[2,6,7]. In some studies, the incidence of AKI ranges from 20%-50% in patients with cirrhosis[2,8]. Moreover, studies have reported that the mortality rate of AKI was also unacceptably high in patients with cirrhosis, reaching up to 80%[6,9]. Therefore, identifying cirrhotic patients with AKI who are at high risk of mortality is very important and may be helpful for providing timely medical interventions to improve the prognosis of these patients. Currently, many studies have investigated risk factors for mortality in patients with cirrhosis[10] and other complications, such as gastroesophageal variceal bleeding[11,12], ascites[13], bacterial infections[14] and metabolic acidosis[15]. Moreover, a number of studies have focused on identifying the risk factors for the development of AKI in patients with cirrhosis[16-19] and other conditions[20-22]. Furthermore, numerous studies have also evaluated the effect of AKI on the mortality of patients with cirrhosis[16,23-25]. A systematic review reported that the risk of death from AKI was increased more than 6-fold in patients with cirrhosis[24]. However, few studies have focused on analyzing the risk factors for mortality in patients with cirrhosis who are diagnosed with AKI. Additionally, based on clinician demand, a quantitative predictive model for determining the prognosis of cirrhotic patients with AKI also must be developed to predict the risk of mortality and distinguish patients with a high risk of mortality.

Therefore, the purpose of the present study was to investigate the risk factors for mortality of cirrhotic patients with AKI and establish a nomogram for predicting the overall survival of these patients to identify high-risk patients and guide timely treatments to improve the prognosis of these patients.

MATERIALS AND METHODS

Patients

We conducted a retrospective study at the Department of Gastroenterology and Hepatology and the Department of Infectious Diseases, The First Affiliated Hospital of Nanchang University, a tertiary care referral hospital in China, between January 2015 and December 2016. The inclusion criteria were as follows: (1) Patients who were diagnosed with cirrhosis; and (2) Patients who met the criteria for at least one of the RIFLE, AKIN, KDIGO and ICA classifications. These patients were hospitalized. The exclusion criteria were as follows: (1) Patients aged less than 18 and more than 80 years; (2) Patients with a previously history renal dysfunction or chronic kidney disease; (3) Patients who underwent liver or renal transplantation; (4) Patients who received renal replacement therapy (including hemodialysis and peritoneal dialysis) before admission; (5) Patients with malignancy or severe cardiopulmonary disease; (6) Patients who were discharged or died < 48 h after admission; (7) Pregnant patients; and (8) Patients without complete data.

According to the inclusion and exclusion criteria, 382 consecutive patients with cirrhosis who were diagnosed with AKI were screened, and 250 eligible patients were recruited and randomly divided into the training cohort (n = 173) and the validation cohort (n = 77) using the built-in random packet function of SPSS 23.0 software according at a ratio of 1:2. The flow chart for inclusion and exclusion is shown in Figure 1.

The study was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (No. AF-SG-04-2.0).

Data collection

Demographics and clinical variables, namely, demographic data (age, sex and body weight), mean arterial pressure (MAP), underlying liver disease, cirrhosis-related complications, diabetes, biochemical analysis (routine blood tests, serum biochemical tests, coagulation function tests, *etc*.), days of the hospital stay, and comorbidities were recorded and collected from every cirrhotic patient with AKI. UO was recorded for all patients during hospitalization. The Child-Turcotte-Pugh (CTP), model for end-stage liver disease (MELD) and MELD-Na scores at admission were calculated accordingly.

Definitions

Cirrhosis was diagnosed based on the medical history, physical signs and symptoms, endoscopic signs of portal hypertension, radiological evidence of liver nodularity, and liver biopsy[26].

According to the consensus of the Asian Pacific Association for the Study of the Liver[27], acute-on-chronic liver failure (ACLF) was defined as acute hepatic insult manifesting as jaundice and coagulopathy complicated within 4 wk by ascites and/or encephalopathy in patients with previously diagnosed or undiagnosed chronic liver disease associated with high mortality.

AKI was defined and classified using the RIFLE, AKIN, KDIGO or ICA classifications (Supplementary Table 1). The peak AKI stage based on the SCr level or UO criterion during hospitalization was used. For the RIFLE, KDIGO and ICA classifications, the last SCr level recorded within the previous 3 mo before admission was used as the baseline SCr level, but the SCr level at admission was used as the baseline SCr level for patients without a previous SCr measurement[1]. The SCr level at admission was also considered the baseline SCr level for the AKIN classification. The glomerular filtration rate (GFR) was used for the RIFLE classification, and the eGFR was calculated using the revised Schwartz method[28]. UO was used in the RIFLE, AKIN and KDIGO classifications.

Calculations of the CTP, MELD and MELD-Na scores

The CTP score was calculated as described in previous reports and classified as grade A (5-6 points), grade B (7-9 points), and grade C (10-15 points)[29]. The computed formula for the MELD score was 3.8 × ln (bilirubin, mg/dL) + 11.2 × ln (international normalized ratio) + 9.6 × ln (creatinine, mg/dL) + 6.4 (constant for liver disease etiology)[30,31]. The computed formula for the MELD-Na score was MELD + 1.59 × [135 – (Na, mmol/L)], and sodium ion concentrations ranged from 125 to 140 mmol/L[32].

Management of patients

A comprehensive medical intervention was administered to every patient, including supportive therapy, prevention and treatment of complications, and reduction or withdrawal of all unnecessary nephrotoxic medications. Patients also received albumin, vasoconstrictors (norepinephrine and terlipressin), intravenous antibiotics, diuretics, proton pump inhibitors or continuous renal replacement

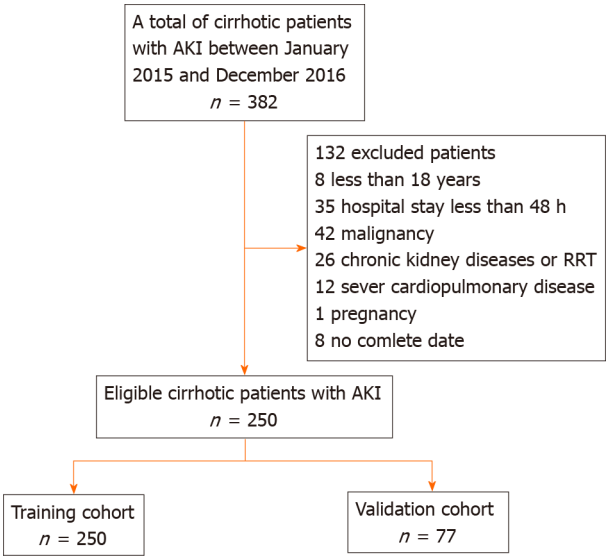

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Figure 1 Flow chart of patient inclusion and exclusion. AKI: Acute kidney injury; RRT: Renal replacement therapy.

therapy if required. Twenty-six patients (10.4%) received continuous replacement therapy.

Follow-up and outcomes

After the diagnosis of AKI, regular follow-up was performed for all cirrhotic patients with AKI by telephone and electronic medical records. Patients were followed for a maximum of 180 d or until death, liver or renal transplantation, or the end of the study period. Follow-up data on the prognosis were collected and evaluated. The primary endpoint of the study was the mortality rate.

Statistical analysis

All continuous variables were tested for a normal distribution using the Kolmogorov–Smirnov test. Variables that met the normal distribution are presented as the mean ± SD and were analyzed using Student's *t* test, while nonnormally distributed variables are summarized as medians and interquartile ranges and were analyzed using the Mann–Whitney *U* test. Categorical variables are presented as numbers and percentages and were analyzed using the χ^2 test or Fisher's exact test. Cumulative survival was evaluated using the Kaplan–Meier method and compared between groups using the log-rank test. The univariate analysis of risk factors of death in cirrhotic patients with AKI was performed using Cox regression analysis, and multivariate Cox analysis with the forward Wald method was subsequently performed for all variables with *P* values less than 0.2.

A nomogram was developed based on the weighted sum of each independent variable, with weights equal to the hazard ratios from the multivariate Cox model to predict the prognosis of cirrhotic patients with AKI. The area under the receiver operating characteristic curve (AUROC), calibration curve and decision curve analysis (DCA) were used to evaluate its predictive performance. All tests were two-tailed, and *P* values less than 0.05 were considered indicative of statistical significance. Data were analyzed using SPSS software, version 23.0 (SPSS Inc., Chicago, IL, Untied States) and R software, version 3.5.3.

RESULTS

Baseline characteristics of the study patients

The characteristics of the patients in the training and validation cohorts are summarized in Table 1. Among the 250 patients, the mean age was 53.6 ± 13.3 years, and most of the patients were male (190/250, 76.0%). Notably, 25.6% (64/250) of the patients presented AKI at the time of admission, and 74.4% (186/250) developed AKI during hospital stay. Only 7 (2.8%) of these patients had compensated cirrhosis, and 243 (97.2%) patients had decompensated cirrhosis. The main etiology of these patients in both datasets was hepatitis B, accounting for 69.6% (174/250) of the entire cohort. Most of the patients (239/250, 95.6%) had ascites, and 60.8% (152/250) of the patients had ACLF. The main causes of AKI were hypovolemia (136/250, 54.4%), followed by infections (54/250, 21.6%), nephrotoxicity (21/250, 8.4%) and other factors (39/250, 15.6%). In the comparison of the training cohort and validation cohort, although the MAP, albumin level and serum sodium level were significantly different (all $P < 0.05$), a significant difference in mortality was not observed between the training (75.1%) and validation (79.2%)

Table 1 Demographic and baseline clinical characteristics of cirrhotic patients with acute kidney injury

Variables	Total (*n* = 250)	Training cohort (*n* = 173)	Validation cohort (*n* = 77)	*P* value
Age (yr), mean ± SD	53.6 ± 13.3	53.3 ± 13.1	54.4 ± 13.7	0.554
Male, *n* (%)	190 (76.0)	127 (73.4)	63 (81.8)	0.199
Heart rates > 100 bpm, *n* (%)	58 (23.2)	36 (20.8)	22 (28.6)	0.120
MAP (mmHg), mean ± SD	87.8 ± 13.9	89.2 ± 13.7	84.5 ± 13.9	0.013
Duration of hospitalization (d), median (IQR)	16.0 (8.0-28.0)	16.0 (9.0-33.5)	16 (8.0-23.5)	0.180
Etiology of cirrhosis				> 0.05
Hepatitis B, *n* (%)	174 (69.6)	124 (71.7)	50 (64.9)	
Alcoholic liver disease, *n* (%)	16 (6.4)	9 (5.2)	7 (9.1)	
Hepatitis B and alcoholic, *n* (%)	20 (8.0)	14 (8.1)	6 (7.8)	
Other, *n* (%)	40 (16.0)	24 (13.9)	14 (18.2)	
Diabetes, *n* (%)	25 (10.0)	17 (9.8)	8 (10.4)	0.527
Ascites, *n* (%)	239 (95.6)	168 (97.1)	71 (92.2)	0.083
Infections, *n* (%)	62 (24.8)	42 (24.3)	20 (25.9)	0.445
HE, *n* (%)	93 (37.2)	68 (39.3)	25 (32.5)	0.187
ACLF, *n* (%)	152 (60.8)	111 (64.2)	41 (53.2)	0.068
Previous esophagogastric variceal bleeding, *n* (%)	57 (22.8)	40 (23.1)	17 (22.1)	0.497
UO (mL/kg/h), mean ± SD	0.61 ± 0.39	0.53 ± 0.38	0.61 ± 0.41	0.130
WBC count (10^9/L), mean ± SD	8.3 ± 6.2	8.0 ± 5.1	8.1 ± 6.8	0.853
Platelet count (10^9/L), median (IQR)	75.5 (48.0-123.7)	73.0 (47.5-124.0)	77.0 (48.0-124.5)	0.623
Albumin (g/L), mean ± SD	28.6 ± 5.4	29.1 ± 5.3	27.5 ± 5.4	0.029
Total bilirubin (mg/dL), mean ± SD	14.6 ± 10.8	15.4 ± 10.8	12.6 ± 10.7	0.060
AST (U/L), median (IQR)	127.5 (63.7-280.7)	130 (65.5-262.5)	118.0 (56.5-304.0)	0.842
ALT (U/L), median (IQR)	76.0 (32.7-203.5)	76.0 (37.0-206.0)	76.0 (30.0-204.0)	0.904
INR, mean ± SD	2.0 ± 0.8	2.0 ± 0.7	2.1 ± 0.9	0.361
Serum sodium (mmol/L), mean ± SD	132.9 ± 5.7	132.3 ± 6.2	133.9 ± 4.8	0.024
Admission SCr (mg/dL), (IQR)	1.17 (0.8-1.8)	1.17 (0.80-1.75)	1.26 (0.77-1.79)	0.897
Peak SCr > 1.5 mg/dL during hospitalization, *n* (%)	211 (84.4)	148 (85.5)	63 (81.8)	0.283
HDL (mmol/L), mean ± SD	0.34 ± 0.33	0.36 ± 0.33	0.37 ± 0.31	0.857
LDL (mmol/L), mean ± SD	1.2 ± 1.0	1.2 ± 0.9	1.1 ± 1.1	0.783
CTP score, mean ± SD	10.6 ± 1.8	10.7 ± 1.8	10.4 ± 1.9	0.396
MELD, mean ± SD	23.6 ± 8.8	24.0 ± 8.2	22.7 ± 9.9	0.304
MELD-Na, mean ± SD	29.0 ± 13.0	30.1 ± 12.7	26.5 ± 13.3	0.044
Mortality within 180 d, *n* (%)	191.0 (76.4)	130 (75.1)	61 (79.2)	0.298
Period of follow up (d), median (IQR)	35.0 (17.0-143.0)	38.0 (18.0-151.0)	32.0 (15.0-119.0)	0.512

MAP: Mean arterial pressure; IQR: Interquartile range; bpm: Beat per minute; SCr: Serum creatinine; HE: Hepatic encephalopathy; INR: International normalized ratio; ACLF: Acute on chronic liver failure; WBC: White blood cell; UO: Urine output; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CTP: Child-Turcotte-Pugh; MELD: The model for end-stage liver disease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

datasets (P = 0.298). Based on RIFLE, AKIN, KDIGO and ICA classifications, 234, 221, 241 and 211 of all 250 patients were diagnosed with AKI, respectively. No patients who underwent liver transplantation.

Mortality of cirrhotic patients with AKI

The median period of follow-up for all 250 patients after the diagnosis of AKI was 35.0 (17.0-143.0) d. The overall 30-d mortality, 90-d mortality and 180-d mortality rates were 46.4% (116/250), 68.8% (172/250) and 76.4% (191/250), respectively. Among these patients who died, the most common cause of mortality was hepatic failure (74/191, 38.7%), followed by infections (41/191, 21.5%), renal failure (34/191, 17.8%), variceal bleeding (24/191, 12.6%), and other causes (19/191, 9.9%). The median follow-up periods in the training and validation cohorts were 38.0 (18.0-151.0) and 32.0 (15.0-119.0) d, respectively. The mortality rates of the training cohort were 45.1%, 68.2% and 75.1% at 30 d, 90 d and 180 d, respectively. The mortality rates at 30 d, 90 d and 180 d were 49.4%, 70.1% and 79.2% in the validation cohort, respectively. No significant difference in mortality was observed during follow-up in either dataset (P = 0.511; Figure 2).

Potential risk factors for mortality in the training cohort

During the follow-up period, 130 cirrhotic patients with AKI in the training cohort died. We performed a Cox regression analysis to identify risk factors of death from cirrhotic patients with AKI (Table 2). The univariate Cox regression analysis showed that a heart rate > 100 bpm, UO, presence of diabetes, total bilirubin level, international normalized ratio (INR), serum sodium level, high-density lipoprotein level, admission SCr level, peak SCr level > 1.5 mg/dL, overt hepatic encephalopathy (HE) or ACLF, and CTP, MELD and MELD-Na scores were potential risk factors of mortality. Multicollinearity between CTP, MELD and MELD-Na scores and other predictors was avoided by excluding these predictive models from the multivariate analysis. Subsequently, the multivariate Cox regression analysis revealed that the presence of diabetes [Hazard ratio (HR) = 1.795; 95% confidence interval (CI): 1.048-3.076; P = 0.001], HE (HR = 1.986; 95%CI: 1.381-2.856; P < 0.001), INR (HR = 1.390; 95%CI: 1.081-1.787; P = 0.010), serum sodium level (HR = 0.964; 95%CI: 0.937-0.992; P = 0.011) and peak SCr level > 1.5 mg/dL (HR = 2.026; 95%CI: 1.109-3.702; P = 0.022) were potential risk factors for mortality in cirrhotic patients with AKI.

Establishment of a prediction nomogram in the training cohort

A nomogram incorporating these potential risk factors recorded after the diagnosis of AKI during hospitalization was constructed to predict the probability of death within 180 d for cirrhotic patients with AKI (Figure 3). Each predictor had a number with weights equal to the HR of the multivariate Cox regression model, and the estimated probability of death for cirrhotic patients with AKI was calculated by adding the scores of each predictor.

Validation of the prediction nomogram

In the training cohort, the AUROC of the nomogram in predicting mortality at 30, 90 and 180 d were 0.757 (95%CI: 0.685-0.830), 0.792 (95%CI: 0.726-0.859) and 0.801 (95%CI: 0.726-0.870), respectively, which exhibited good discrimination for the prognosis of cirrhotic patients with AKI (Figure 4A). The calibration plots of 30-, 90- and 180-d survival showed satisfactory agreement between the predicted prognosis and actual prognosis probability in the training cohort (Figure 4B-D). In the validation cohort, the nomogram also displayed good discrimination with AUROCs of 0.763 (95%CI: 0.656-0.869), 0.817 (95%CI: 0.716-0.917) and 0.862 (95%CI: 0.765-0.958) in predicting mortality at 30, 90 and 180 d, respectively (Figure 5A). The calibration plots also showed good agreement between the predicted and actual prognosis probabilities in the validation cohort (Figure 5B-D). Based on the results, the nomogram had good discrimination and calibration in predicting the prognosis of cirrhotic patients with AKI.

Performance of the nomogram in predicting mortality

We compared the performance of the nomogram with CTP, MELD and MELD-Na scores in predicting the overall survival of cirrhotic patients with AKI, and the results are shown in Table 3. There were no significant differences between the nomogram and the CTP, MELD and MELD-Na scores in predicting 30-d mortality for either the training or validation cohort (all P > 0.05; Figure 6A and B). However, the AUROC of the nomogram in predicting mortality at 90 d was significantly higher than that of the CTP, MELD and MELD-Na scores in both the training and validation cohort (all P < 0.05; Figure 6C and D). Moreover, the AUROC of the nomogram in predicting mortality at 180 d was also larger than that of the CTP, MELD and MELD-Na scores in both the training and validation cohort (all P < 0.05; Figure 6E and F). With regard to clinical usefulness, DCA of the nomogram was depicted and compared with the CTP, MELD and MELD-Na scores. Compared with the CTP, MELD and MELD-Na scores, medical intervention guided by the nomogram increased the net benefit for 90- and 180-d overall survival but nor 30-d overall survival in the training dataset (Figure 7A-C). In the validation dataset, the similar result was obtained: The nomogram showed the best net benefit for 90- and 180-d overall survival but not 30-d overall survival (Figure 7D-F). Taken together, these results showed that the nomogram better predicted the overall survival of cirrhotic patients with AKI than the CTP, MELD and MELD-Na scores.

Table 2 Cox regression analysis of predictors for death of cirrhotic patients with acute kidney injury in the training cohort

Variable	Univariate			Multivariate		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (≤ 60 *vs* > 60 yr)	1.003	0.989-1.017	0.670			
Male (male *vs* female)	0.779	0.522-1.161	0.220			
MAP (mmHg)	0.993	0.981-1.005	0.226			
Heart rates (55 ≤ HR ≤ 100 *vs* HR > 100 bpm)	1.317	0.867-2.001	0.197			
Etiology (viral hepatitis *vs* non-viral hepatitis)	0.897	0.521-1.248	0.335			
Diabetes (absent *vs* present)	1.486	0.878-2.514	0.140	1.795	1.048-3.076	0.033
Previous esophagogastric variceal bleeding (absent *vs* present)	0.834	0.549-1.268	0.397			
Ascites, (absent *vs* present)	1.594	0.507-5.012	0.425			
Infections (absent *vs* present)	1.127	0.756-1.680	0.559			
HE (absent *vs* present)	2.308	1.627-3.276	< 0.001	1.986	1.381-2.856	< 0.001
ACLF (absent *vs* present)	1.545	1.064-2.244	0.022			
UO (mL/kg/h)	0.679	0.420-1.098	0.114			
WBC count (10^9/L)	1.018	0.987-1.050	0.264			
Platelet count (10^9/L)	1.000	0.998-1.003	0.805			
Albumin (g/L)	1.001	0.971-1.032	0.963			
Total bilirubin (≤ 4.0 *vs* > 4.0 mg/dL)	2.225	1.350-3.666	0.002			
INR	1.625	1.282-2.061	< 0.001	1.390	1.081-1.787	0.010
Serum sodium (mmol/L)	0.957	0.933-0.982	0.001	0.964	0.937-0.992	0.011
Admission SCr (mg/dL)	1.121	0.975-1.289	0.109			
Peak SCr (≤ 1.5 *vs* > 1.5 mg/dL)	2.407	1.326-4.371	0.004	2.026	1.109-3.702	0.022
HDL (mmol/L)	0.477	0.258-0.883	0.018			
LDL (mmol/L)	0.871	0.696-1.090	0.227			
CTP score	1.328	1.192-1.478	< 0.001			
MELD score	1.057	1.034-1.082	< 0.001			
MELD-Na score	1.036	1.022-1.050	< 0.001			

HR: Hazard ratio; MAP: Mean arterial pressure; bpm: Beat per minute; SCr: Serum creatinine; HE: Hepatic encephalopathy; INR: International normalized ratio; ACLF: Acute on chronic liver failure; WBC: White blood cell; UO: Urine output; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CTP: Child-Turcotte-Pugh; MELD: The model for end-stage liver disease; CI: Confidence interval.

According to the nomogram, the score of each patient was calculated and utilized for a stratified analysis. Based on the Youden index, the optimal cutoff value of the nomogram score in predicting mortality within 180 d in the training cohort was 98, with a sensitivity of 56.1% and a specificity of 90.7%. Based on the threshold of 98, patients in the training cohort were divided into two subgroups, with high risk (> 98 points, n = 77) and low risk (≤ 98 points, n = 96) of mortality. The high-risk group had a significantly higher mortality rate within 180 d than the low-risk group (94.8% *vs* 59.4%, P < 0.001; Figure 8A). In the validation cohort, the same cutoff value was used, and the sensitivity and specificity of the nomogram were 55.6% and 92.9%, respectively. Patients from the validation cohort were also stratified into a high-risk group (n = 36) and a low-risk group (n = 41), and the mortality rate was significantly different between both risk groups (97.2% *vs* 68.3%, P < 0.001; Figure 8B).

Table 3 Comparison of models in predicting the death of cirrhotic patients with acute kidney injury

Datasets	Time of mortality	Predictive model	AUROC (95%CI)	*P* value
Training dataset	30-d	Nomogram	0.757 (0.685-0.830)	Ref.
		CTP	0.694 (0.616-0.772)	0.210
		MELD	0.669 (0.585-0.752)	0.114
		MELD-Na	0.702 (0.622-0.782)	0.332
Validation dataset	30-d	Nomogram	0.763 (0.656-0.869)	Ref.
		CTP	0.689 (0.569-0.809)	0.416
		MELD	0.745 (0.631-0.859)	0.979
		MELD-Na	0.763 (0.649-0.876)	> 0.05
Training dataset	90-d	Nomogram	0.792 (0.726-0.859)	Ref.
		CTP	0.696 (0.614 – 0.778)	0.041
		MELD	0.657 (0.574-0.740)	0.003
		MELD-Na	0.670 (0.586-0.755)	0.003
Validation dataset	90-d	Nomogram	0.817 (0.716-0.917)	Ref.
		CTP	0.667 (0.523-0.809)	0.031
		MELD	0.650 (0.515-0.785)	0.008
		MELD-Na	0.642 (0.502-0.783)	0.005
Training dataset	180-d	Nomogram	0.801 (0.726-0.870)	Ref.
		CTP	0.722 (0.630-0.814)	0.045
		MELD	0.663 (0.575-0.752)	0.008
		MELD-Na	0.680 (0.588-0.771)	0.009
Validation dataset	180-d	Nomogram	0.862 (0.765-0.958)	Ref.
		CTP	0.708 (0.540-0.878)	0.027
		MELD	0.659 (0.512-0.806)	0.002
		MELD-Na	0.652 (0.491-0.812)	0.002

CTP: Child-Turcotte-Pugh; MELD: The model for end-stage liver disease; AUROC: The area under the curve of the receiver operating characteristic curve; CI: Confidence interval.

DISCUSSION

In this study, we conducted a Cox regression analysis to identify the risk factors for mortality within 180 d in cirrhotic patients with AKI, and these predictors included the presence of diabetes, HE, INR, serum sodium level and peak SCr levels. A nomogram incorporating these predictors was developed and showed good predictive discrimination and calibration for the mortality of these patients. Moreover, compared with the CTP, MELD and MELD-Na scores, the nomogram showed better performance for predicting the overall survival of cirrhotic patients with AKI. To our knowledge, this is the first to establish a quantitative nomogram based on these risk factors for the overall survival of cirrhotic patients with AKI.

AKI is a very common and life-threatening complication in patients with cirrhosis and has an unacceptably high mortality rate[33]. Therefore, many AKI criteria and classifications have been developed over the past two decades to comprehensively evaluate and diagnose AKI as early as possible and improve the prognosis. Currently, four main classifications are used: The RIFLE[3], AKIN [4], KIDGO[5] and ICA[1] criteria. SCr level and UO, which are established markers of kidney function, were the basis for the development of these AKI classifications. The use of the SCr level alone to diagnose with AKI may not be appropriate. First, SCr levels are influenced by various factors, such as age, race, sex, body weight, tubular creatinine secretion and the effect of bilirubin on creatinine assays [34,35]. Second, in patients with advanced cirrhosis in particular, the diagnostic value of SCr levels was diminished because of the reduced hepatic production of creatinine from creatine and muscle wasting[7, 36]. Finally, a greater number of AKI cases could be detected earlier using UO in combination with the

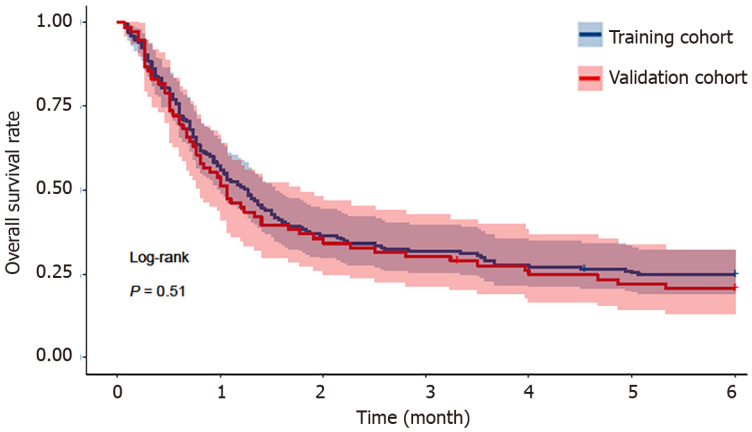

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Figure 2 Kaplan–Meier analysis of the overall survival of cirrhotic patients with acute kidney injury within 180 d in both the training and validation cohorts.

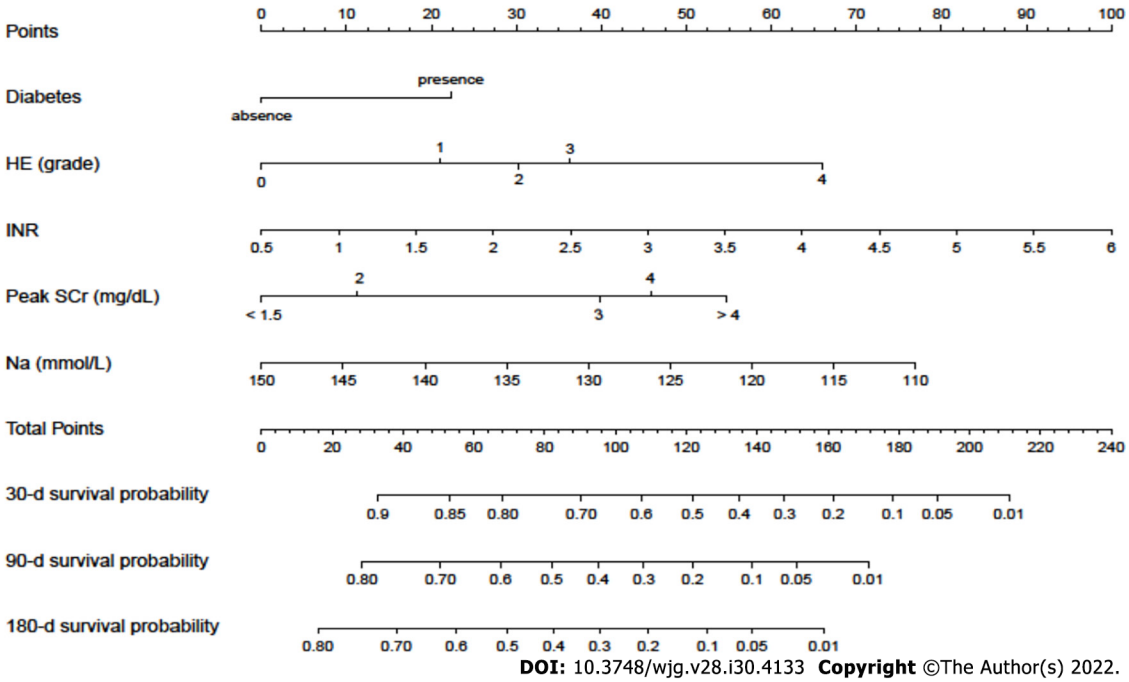

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Figure 3 A nomogram for predicting the 30-d, 90-d and 180-d overall survival of cirrhotic patients with acute kidney injury. HE: Hepatic encephalopathy; SCr: Serum creatinine; INR: International normalized ratio.

SCr level[37-39]. Notably, UO is also affected by many factors, such as the body fluid volume of the patients and the use of diuretics, which may decrease the diagnostic and predictive value of the UO criteria for AKI[37]. As a result, any single criterion for AKI may not be able to comprehensively identify and evaluate these patients. Therefore, in the present study, patients who met the criteria for at least one of the RIFLE, AKIN, KDIGO and ICA classifications were recruited as comprehensively as possible, to analyze the clinical characteristics of cirrhotic patients with AKI and identify risk factors for mortality.

The mortality rate of cirrhotic patients with AKI displays substantial heterogeneity among studies. Xiong *et al*[9] reported that the mortality rates were 76.1% and 86.7% in-hospital and at 6 mo, respectively, for patients with cirrhosis who were diagnosed with AKI upon admission to the intensive care unit (ICU), which was consistent with the results of previous studies[7,40]. The results were significantly higher than the values reported in our present study (52.4% in-hospital mortality and 76.4% 180-d mortality). Another study conducted by Kumar *et al*[41] showed that the mortality rate at 30 d was 46.7% for cirrhotic patients with AKI, which was consistent with our result of 46.4%. A meta-analysis conducted by Tariq *et al*[42] revealed that the overall in-hospital mortality, 30-d mortality, and 90-d mortality rates of patients with liver cirrhosis were 34.6%, 42.4%, and 47.1%, respectively, which was lower than the results from our study (52.4% in-hospital mortality, 46.4% 30-d mortality, and 68.8% 90-d mortality). The heterogeneity of the mortality rate may be mainly affected by the difference in the

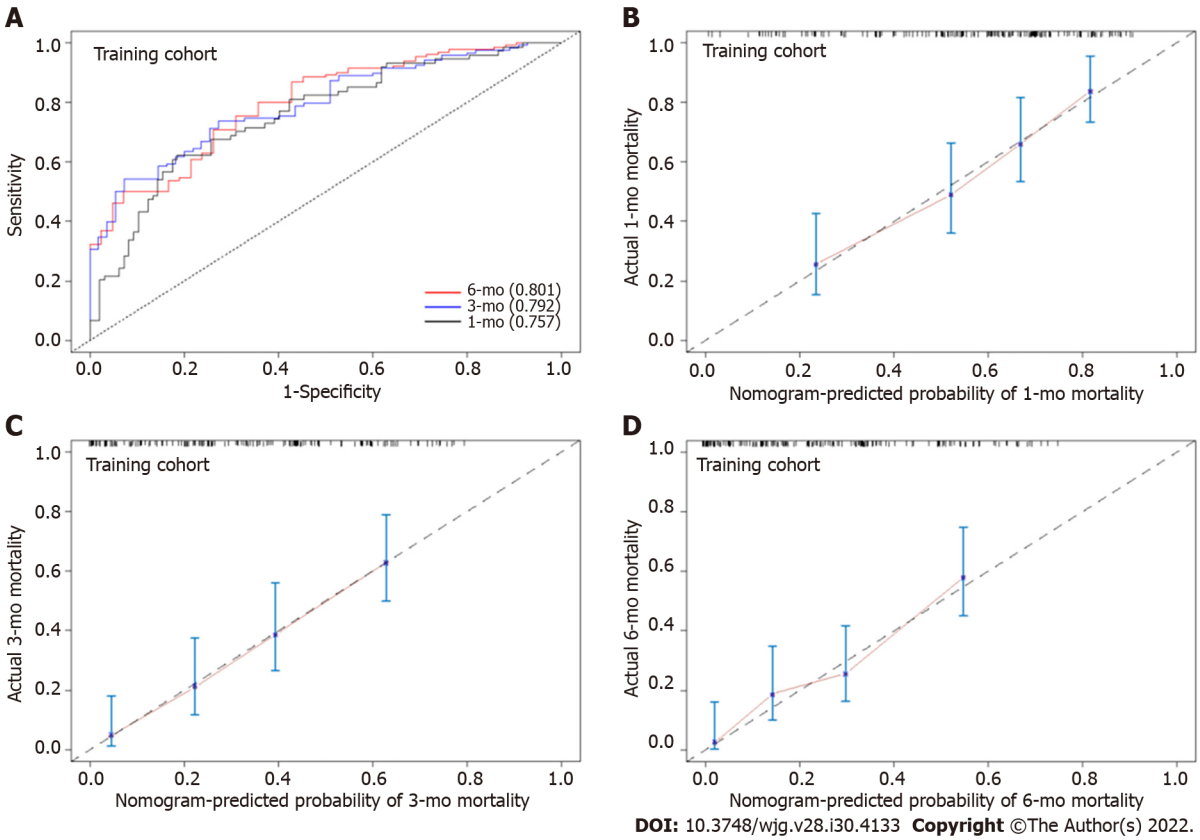

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Figure 4 The area under the receiver operating characteristic curve and calibration curve of the nomogram for predicting the overall survival of cirrhotic patients with acute kidney injury in the training cohort. A: The area under the receiver operating characteristic curve of the nomogram for mortality at 30-, 90- and 180-d in the training cohort; B: Calibration curve of the nomogram in predicting 30-d overall survival in the training cohort; C: Calibration curve of the nomogram in predicting 90-d overall survival in the training cohort; D: Calibration curve of the nomogram in predicting 180-d overall survival in the training cohort.

severity of the patient's condition. Moreover, some serious complications of patients with cirrhosis may also contribute to the high mortality rate. For instance, in the present investigation, 60.8% (152/250) of patients had cirrhosis combined with ACLF, resulting in an increase in the AKI mortality rate, which was supported by previous studies by Zang *et al*[43] and Shi *et al*[44]. Furthermore, the literature has indicated that the mortality of AKI in patients with cirrhosis who were admitted to the ICU was significantly higher than that of patients admitted to regular wards[7,42]. In addition, the use of different AKI criteria to identify patients among studies also resulted in different the mortality rate[6,7]. Notably, the types of AKI may also affect the prognosis of these patients. AKI is usually divided into three subtypes: Prerenal AKI, acute tubular necrosis (ATN) and hepatorenal syndrome (HRS)[9]. AKI is closely associated with the prognosis of patients with cirrhosis. A retrospective study showed that compared with non-AKI patients, patients with prerenal AKI had a 2.37-fold higher risk of in-hospital death, patients with ATN had a 6.878-fold higher risk, and patients with HRS had a 12.98-fold higher risk[9]. The result seems to indicate that cirrhotic patients with HRS have a higher risk of death than those with ATN and prerenal AKI. Moreover, patients with HRS-AKI have a worse prognosis than those with non-HRS-AKI[2]. Taken together, the varying mortality of AKI in patients with cirrhosis may be influenced by the severity of the liver disease, the diversity of AKI classifications, survey populations, complications of cirrhosis, and the subtypes of AKI, making the evaluation of AKI difficult and in many cases impossible.

Considering the high mortality rate of AKI in patients with cirrhosis, the identification of risk factors for mortality in these patients is meaningful. However, few studies conducted to date have focused on investigating the risk factors for death in cirrhotic patients with AKI. A study conducted by Kumar *et al*[41] showed that the presence of jaundice, HE, SCr level > 1.5 mg/dL at admission, CTP score, and MELD score were independent predictors of mortality for cirrhotic patients with AKI. Another study by Pan *et al*[40] found that MAP, total bilirubin levels, acute respiratory failure and sepsis were closely associated with the prognosis of cirrhotic patients with AKI who were admitted to the ICU. In the present study, the serum sodium level, INR, peak SCr level > 1.5 mg/dL, the presence of diabetes and HE were considered potential risk factors for death in cirrhotic patients with AKI. Notably, the CTP, MELD and MELD-Na scores were excluded from the multivariate Cox regression analysis because of the increased probability of multicollinearity. An increase in INR is one of the manifestations of

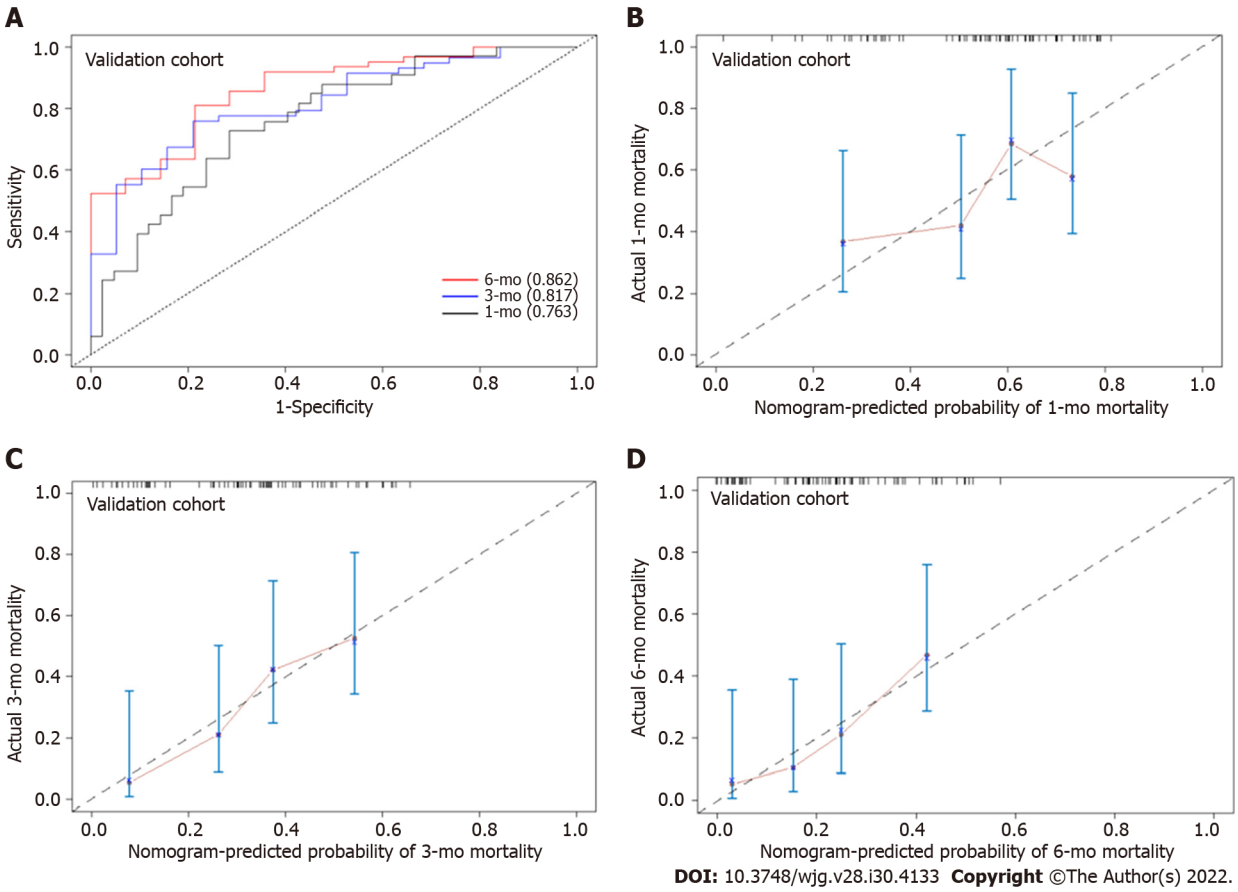

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Figure 5 The area under the receiver operating characteristic curve and calibration curve of the nomogram for predicting the overall survival of cirrhotic patients with acute kidney injury in the validation cohort. A: The area under the receiver operating characteristic curve of the nomogram for mortality at 30-, 90- and 180-d in the validation cohort; B: Calibration curve of the nomogram in predicting 30-d overall survival in the validation cohort; C: Calibration curve of the nomogram in predicting 90-d overall survival in the validation cohort; D: Calibration curve of the nomogram in predicting 180-d overall survival in the validation cohort.

synthesis dysfunction in the liver. Similarly, HE is also an important feature of liver failure. Once HE occurs in patients with chronic liver disease, the prognosis is very poor, with a 1-year survival rate of less than 50% and a 3-year survival rate of less than 25%[46]. Studies have shown that INR is associated with the death of patients with cirrhosis[46,47]. Moreover, a serum sodium imbalance is very common in cirrhotic patients with AKI and affect the prognosis of these patients[48-50]. Furthermore, the SCr level represents the status of renal function. Patients with a peak SCr > 1.5 mg/dL had a higher short-term mortality rate than those with a peak SCr ≤ 1.5 mg/dL[51,52]. A study showed that a SCr > 1.5 mg/dL was an independent predictor of mortality in cirrhotic patients with AKI[41]. In addition, diabetes was also considered an independent risk factor for mortality in cirrhotic patients with AKI in this study. Although the mechanisms underlying the relationship between diabetes and mortality in cirrhotic patients with AKI remain unclear, several studies have documented that the presence of diabetes and poorly controlled blood glucose levels are associated with the prognosis of patients with cirrhosis[53-55]. Diabetes is an independent risk factor for the development of AKI or acute kidney disease[56,57]. Consequently, we postulate that the presence of diabetes is associated with the prognosis of cirrhotic patients with AKI. Overall, the recovery of liver and kidney function, correction of electrolyte imbalances, and monitoring and control of blood glucose levels in patients with diabetes may improve the poor prognosis of cirrhotic patients with AKI.

Currently, many studies have investigated the risk factors for the development of AKI in patients with numerous acute and chronic diseases, or have established a model for the development of AKI[20,58-60]. However, studies focused on predicting the prognosis of cirrhotic patients with AKI are scarce. According to Kumar *et al*[41], the AUROCs of CTP and MELD scores were 0.82 and 0.84, respectively, for predicting 30-d mortality in cirrhotic patients with AKI who met the ICA classification. Fang *et al*[45] reported that CTP and MELD scores had AUROCs of 0.61 and 0.757, respectively, for predicting in-hospital mortality of cirrhotic patients with AKI. Moreover, another prospective study conducted by Pan *et al*[40] indicated that CTP and MELD scores had AUROCs of 0.622 and 0.776, respectively, for predicting the in-hospital mortality of cirrhotic patients with AKI who met the RIFLE classification. In the present study, the AUROCs of CTP and MELD scores were 0.694 and 0.669, respectively, for predicting 30-d mortality of cirrhotic patients with AKI in the training cohort, respectively. The discrim-

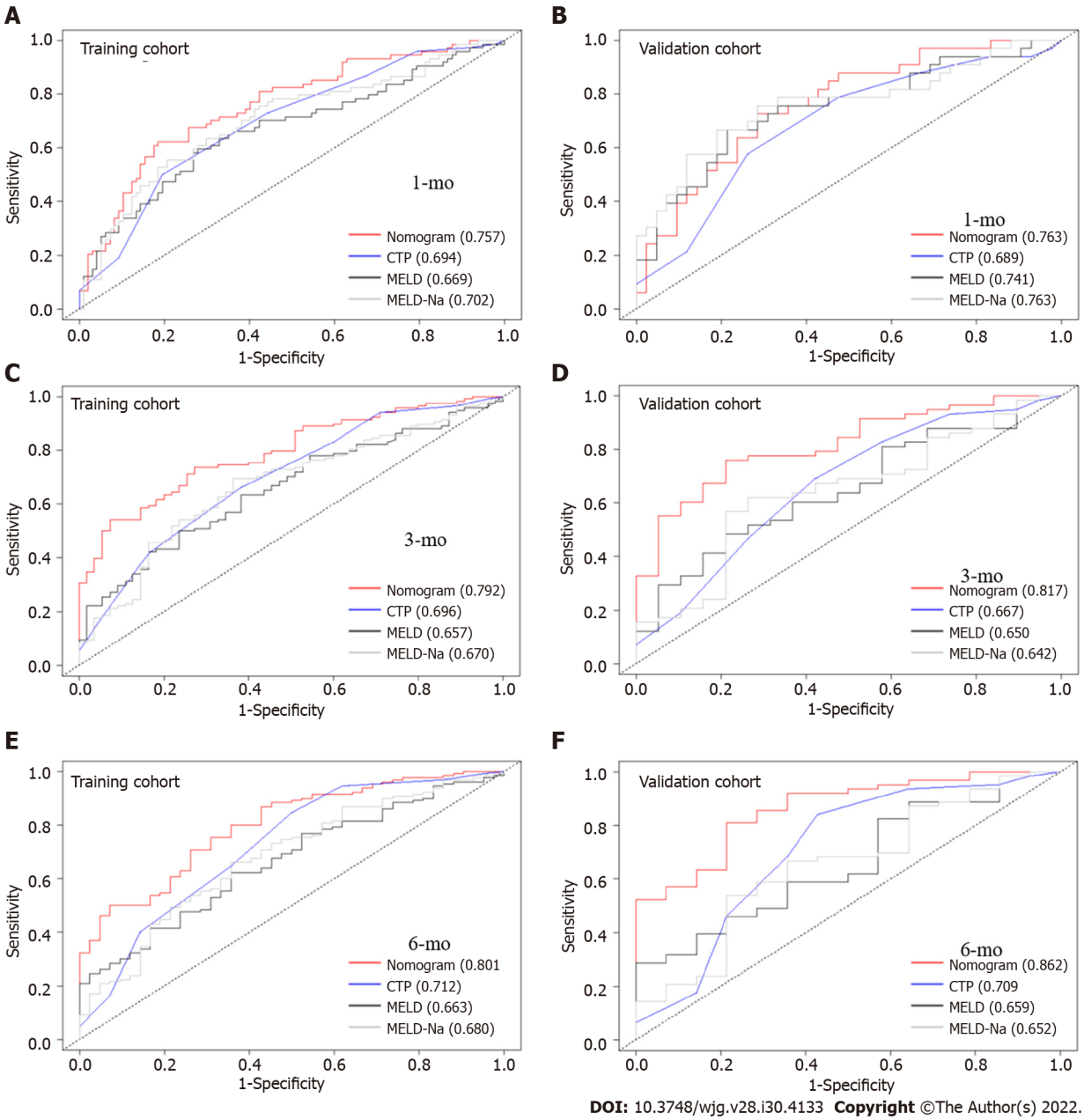

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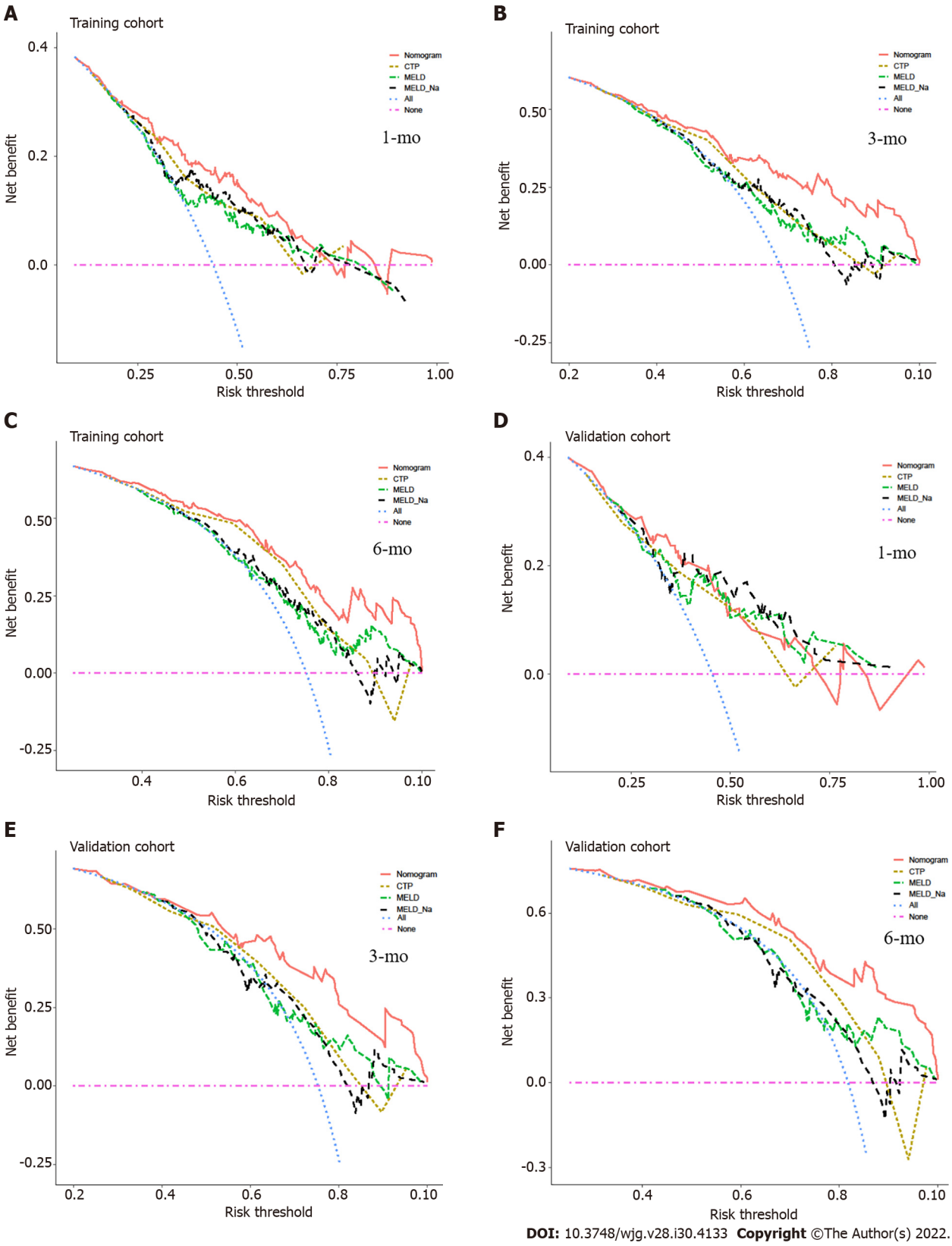
Figure 6 Comparison of the performance of the nomogram and the other scores in predicting mortality of cirrhotic patients with acute kidney injury. A: The area under the receiver operating characteristic curve (AUROC) of the nomogram and the Child-Turcotte-Pugh (CTP), model for end-stage liver disease (MELD) and MELD-Na scores in predicting 30-d mortality in the training cohort; B: The AUROCs of the nomogram and the CTP, MELD and MELD-Na scores in predicting 30-d mortality in the validation cohort; C: The AUROCs of the nomogram and the CTP, MELD and MELD-Na scores in predicting 90-d mortality in the training cohort; D: The AUROCs of the nomogram and the CTP, MELD and MELD-Na scores in predicting 90-d mortality in the validation cohort; E: The AUROCs of the nomogram and the CTP, MELD and MELD-Na scores in predicting 180-d mortality in the training cohort; F: The AUROCs of the nomogram and the CTP, MELD and MELD-Na scores in predicting 180-d mortality in the validation cohort. CTP: Child-Turcotte-Pugh; MELD: Model for end-stage liver disease.

inative ability of CTP and MELD scores differed among studies, which may be explained by the analysis of different patients. Patients with cirrhosis who were diagnosed with AKI in the study by Kumar *et al* [41] only met the ICA criteria, and those patients in the studies by Fang *et al*[45] and Pan *et al*[40] only met the RIFLE criteria, while the study population in our cohort met the criteria for at least one of the main four classifications. In addition, Fang *et al*[45] developed an MBRS (MAP + bilirubin + respiratory failure + sepsis) score (AUROC = 0.898) (including MAP, serum bilirubin level and presence of acute respiratory failure and sepsis) in 2008, a predictive model for cirrhotic patients with AKI, that had better discriminative ability than the CTP and MELD scores. Another prospective study by their team published in 2012 further validated the discrimination and calibration of the MBRS score[40]. The AUROCs and goodness-of-fit of the MBRS were 0.863 and 1.160, respectively, which were superior to the CTP and MELD scores in terms of discriminative ability[40]. Notably, the development of the MBRS score was based on patients admitted to the ICU who may suffer from multiple organ failures and comorbidities, but the authors did not clearly determine whether the MBRS score was applicable to



DOI: 10.3748/wjg.v28.i30.4133

Figure 7 Comparison of the decision curve analysis curve of medical interventions in patients based on the nomogram and the other scores. A: The decision curve analysis (DCA) curve of medical interventions in patients from the training cohort based on the nomogram, Child-Turcotte-Pugh (CTP), model for end-stage liver disease (MELD) and MELD-Na scores at 30 d; B: The DCA curve of medical interventions in patients from the training cohort based on the nomogram, CTP, MELD and MELD-Na scores at 90 d; C: The DCA curve of medical interventions in patients from the training cohort based on the nomogram, CTP, MELD and MELD-Na scores at 180 d; D: The DCA curve of medical interventions in patients from the validation cohort based on the nomogram, CTP, MELD and MELD-Na scores at 30 d; E: The DCA curve of medical interventions in patients from the validation cohort based on the nomogram, CTP, MELD and MELD-Na scores at 90 d; F: The DCA curve of medical intervention in patients from the validation cohort based on the nomogram, CTP, MELD and MELD-Na scores at 180 d. CTP: Child-Turcotte-Pugh; MELD: Model for end-stage liver disease.

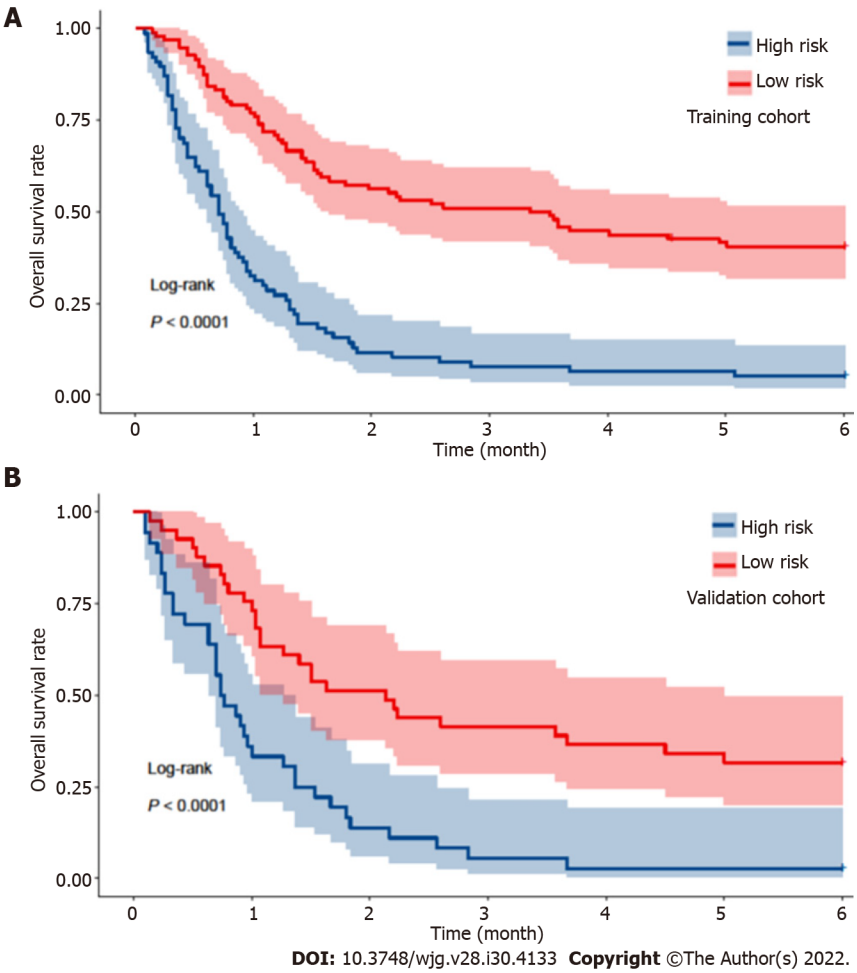


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Figure 8 Kaplan–Meier analysis of the cirrhotic patients with acute kidney injury stratified into high-risk and low-risk mortality groups according to the nomogram score, with cutoff of 98. A: Overall survival rates were compared between patients with a high and low risk of mortality in the training cohort; B: Overall survival rates were compared between patients with a high and low risk of mortality in the validation cohort.

patients admitted to regular wards, which requires further validation in the future. Moreover, some clinical data incorporated in the MBRS score, such as the fraction of inspired oxygen, are difficult to obtain in the regular ward and have a complex calculation, which may result in the score not being widely used in clinical practice.

In the present study, we developed a quantitative and visual nomogram for predicting the overall survival of cirrhotic patients with AKI based on these potential risk factors of mortality. The nomogram could be used to calculate the scores corresponding to each potential risk factor, and the predicted probability corresponding to the sum of the scores represented the risk of death for patients with cirrhosis and AKI. For example, patients with cirrhosis presenting AKI and diabetes had SCr level of 2 mg/dL, INR 2, grade 1 HE and blood sodium level of 130 mmol/L. The total score of the patient was approximately 118 points (SCr level of 2 mg/dL, approximately 11 points; INR 2, approximately 26 points; grade 1 HE, approximately 21 points; diabetes, approximately 22.5 points; and blood sodium level of 130 mmol/L, approximately 37.5 points.), and the probability of the overall survival at 30, 90 and 180 d was approximately 53%, 23% and 15%, respectively. Compared with the CTP, MELD and MELD-Na scores, the nomogram had better discriminative power for mortality at 90 and 180 d in both the training and validation datasets (all $P < 0.05$). Moreover, with regard to clinical decisions, the DCA curve indicated that the nomogram had more net benefits than the CTP, MELD and MELD-Na scores for predicting mortality at 90 and 180 d in both the training and validation datasets. However, the AUROC and DCA curves were not significantly different between the nomogram and the CTP, MELD and MELD-Na scores for predicting mortality at 30 d in both the training and validation datasets. Overall, the nomogram was also superior to the CTP, MELD and MELD-Na scores in predicting the prognosis of cirrhotic patients with AKI, especially 90- and 180-d mortality. Furthermore, a score for each patient was calculated based on the nomogram and utilized for stratifing patients into two risk groups for mortality. The high-risk group had a significantly higher mortality rate within 180 d than the low-risk group (all $P < 0.001$) in both the training and validation cohorts. The mortality rates of high-risk patients were as high as 94.8% in the training cohort and 97.2% in the validation cohort. As a result, the nomogram might select patients with a high-risk mortality to allow treatments to be provided as soon as

possible, which may improve the prognosis of cirrhotic patients with AKI. In addition, compared with the MBRS score, the five variables included in the nomogram were all convenient and easily accessible in clinical practice. Since the use of nomograms for evaluation in clinical practice can be time-consuming and complicated, our next step will be to develop software that can be embedded in electronic medical systems to guide clinicians in the timely treatment of these patients and reduce patient mortality without increasing the working time and burden of clinicians.

Several limitations of the present study should also be considered. First, this study analyzed a small sample and employed a retrospective design in which selection biases were unavoidable. Second, this investigation was performed at one academic tertiary-care medical center; the results may not be extrapolated to other centers. In addition, a prospective, large-scale and multicenter study will be needed to validate the reliability of the nomogram.

CONCLUSION

In conclusion, the presence of HE and diabetes, serum sodium level, INR and peak SCr level > 1.5 mg/dL were considered potential risk factors for mortality. A nomogram based on these risk factors was established, and compared with the CTP, MELD and MELD-Na scores, the nomogram exhibited better predictive performance for the overall survival of cirrhotic patients with AKI. According to the score obtained from the nomogram, patients with a high risk of mortality could be selected for suitable individualized treatments, which may improve the prognosis of cirrhotic patients with AKI.

ARTICLE HIGHLIGHTS

Research background

Acute kidney injury (AKI) is a life-threatening complication in cirrhotic patients and is closely associated with the prognosis of these patients. The mortality of AKI in patients with cirrhosis was as high as 80%. Therefore, the identification of patients with AKI at high risk of death is necessary to improve their prognosis.

Research motivation

The majority of studies have focused on investigating the risk factors for the development of AKI or establishing a risk score model for predicting the development of AKI. However, studies focused on identifying the potential risk factors for cirrhotic patients with AKI are scarce.

Research objectives

This study aimed to identify risk factors of mortality and establish a nomogram for predicting overall survival in cirrhotic patients with AKI.

Research methods

We included 250 eligible cirrhotic patients with AKI in this study. These patients were randomly divided into a training cohort and a validation cohort at a ratio of 2:1. Potential risk factors for death were investigated by performing a Cox regression analysis of the training cohort. A prognostic nomogram was developed and evaluated by calculating the area under the curve of the receiver operating characteristic curve, constructing a calibration curve and performing decision curve analysis.

Research results

The serum sodium level, international normalized ratio, peak serum creatinine level > 1.5 mg/dL, the presence of hepatic encephalopathy and diabetes were considered potential risk factors for death in cirrhotic patients with AKI. The nomogram based on these risk factors has a good performance in predicting the short-term prognosis of cirrhotic patients with AKI. The cutoff value of 98 for the nomogram score was used to stratify patients; patients were divided into low- and high-risk groups, and high-risk patients had a higher mortality rate.

Research conclusions

The nomogram was a practical tool to predict the short-term prognosis of patients with cirrhosis who were diagnosed with AKI, and assist clinicians in making clinical decisions.

Research perspectives

The reliability and practicability of nomogram must be validated by conducting prospective, large-scale and multicenter studies. Application programs or software that can be embedded in electronic medical systems will be developed to guide clinicians in the timely evaluation and treatment of these patients.

FOOTNOTES

Author contributions: Wan YP, Wang AJ and Zhang W equally contributed to this work; Wang AJ and Zhu X contributed to the concept; Wan YP, Wang AJ and Zhang W designed this study; Wan YP performed the manuscript writing; Zhang W performed data analysis; Zhang H and Peng GH contributed to samples collection and data collection and validation; Zhu X were clinical experts and performed the manuscript revision; all authors read and approved the final version of the manuscript.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (No. AF-SG-04-2.0).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of The First Affiliated Hospital of Nanchang University.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Retrospective Study

Cumulative incidence and risk factors for pouch adenomas associated with familial adenomatous polyposis following restorative proctocolectomy

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Abstract

BACKGROUND

The emergence of restorative total proctocolectomy has significantly reduced the lifetime colorectal cancer risk associated with familial adenomatous polyposis (FAP). However, adenomas may develop in the ileal pouch over time and may even progress to carcinoma. We evaluated the cumulative incidence, time to development, and risk factors associated with ileal pouch adenoma.

AIM

To evaluate the cumulative incidence, time to development, and risk factors associated with pouch adenoma.

METHODS

In this retrospective, observational study conducted at a tertiary center, 95 patients with FAP who underwent restorative proctocolectomy at our center between 1989 and 2018 were consecutively included. The mean follow-up period was 88 mo.

RESULTS

Pouch adenomas were found in 24 (25.3%) patients, with a median time of 52 mo to their first formation. Tubular adenomas were detected in most patients (95.9%). There were no high-grade dysplasia or malignancies. Of the 24 patients with pouch adenomas, 13 had all detected adenomas removed. Among the 13 patients who underwent complete adenoma removal, four (38.5%) developed recurrence. Among 11 (45.8%) patients with numerous polyps within the pouch, seven

(63.6%) exhibited progression of pouch adenoma. The cumulative risks of pouch adenoma development at 5, 10, and 15 years after pouch surgery were 15.2%, 29.6%, and 44.1%, respectively. Severe colorectal polyposis (with more than 1000 polyps) was a significant risk factor for pouch adenoma development (hazard ratio, 2.49; 95% confidence interval: 1.04-5.96; $P = 0.041$).

CONCLUSION

Pouch adenomas occur at a fairly high rate in association with FAP after restorative proctocolectomy, and a high colorectal polyp count is associated with pouch adenoma development.

Key Words: Adenomatous polyposis coli; Familial adenomatous polyposis; Adenoma; Intestinal polyps; Proctocolectomy, restorative; Ileal pouch anal anastomosis

Core Tip: This is a retrospective study that evaluated the cumulative incidence and risk factors for pouch adenoma in association with familial adenomatous polyposis following restorative proctocolectomy. The incidence of pouch adenoma was 25.3%, and the cumulative risk 15 years after pouch surgery was 44.1%. Severe colorectal polyposis was a significant risk factor for pouch adenoma development. In our series, 62% of adenomas did not recur after endoscopic removal, but 63% of patients under observation showed progression. There was no spontaneous adenoma diminution or disappearance. Close endoscopic pouch surveillance is essential, and new pouch adenoma management guidelines are needed.

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INTRODUCTION

Familial adenomatous polyposis (FAP) is a genetic syndrome caused by an adenomatous polyposis coli (*APC*) gene mutation that has an incidence of 1 in 10000 births[1]. This genetic variation is characterized by extensive polyposis in the colon and rectum, and patients with FAP have a lifetime colorectal cancer risk of 100% by the age of 35 to 40 years[2]. Hence, prophylactic surgical intervention is required for these individuals, and the currently favorable therapeutic option is a total proctocolectomy and ileal pouch-anal anastomosis (TPC/IPAA)[2]. TPC/IPAA increases life expectancy among patients with FAP by eliminating their metachronous colorectal cancer risk[3]. The postoperative disease course is determined by extracolonic manifestations, including duodenal adenoma and carcinoma, and desmoid tumors[4].

Cancers of the terminal ileum are extremely rare, and this region thus represents a far lower disease risk than the remaining rectal mucosa after ileorectal anastomosis. Notably, however, several studies have found a 35%-57% incidence of ileal pouch adenomas associated with FAP, which can progress to malignancy[5-11]. Given that most of these prior studies involved small sample sizes and were retrospective in nature, definitive results on these cancers are lacking, and the risk factors associated with pouch adenoma development remain to be established. Although these data are limited, the pouch adenoma incidence is reported to be quite high; therefore, long-term periodic surveillance is required. Most clinical guidelines recommend annual endoscopic surveillance of the anal transition zone (ATZ) mucosa after TPC/IPAA as a surgical management strategy for FAP patients. According to the National Comprehensive Cancer Network guidelines, this surveillance interval should be shortened to 6 mo if the adenoma is large or has advanced histology. However, there are currently no specific criteria directing more frequent surveillance. Moreover, with the currently implemented management approaches for pouch adenoma, uncertainty remains regarding the specific indications for endoscopic or surgical intervention[12-14]. More information is, therefore, needed regarding the incidence, natural course, and risk factors associated with pouch adenoma.

We investigated the cumulative incidence and time to development of pouch adenomas. In an FAP cohort after TPC/IPAA, we analyzed the clinical factors associated with pouch adenoma development.

MATERIALS AND METHODS

Study sample

Data on all patients with FAP who underwent pouch surgery at Asan Medical Center, Seoul, South Korea between November 1989 and December 2018 were identified from the hospital database. The FAP patients were identified by the presence of more than 100 colorectal adenomas. The indication for TPC/IPAA was FAP with or without malignancy. We excluded patients with attenuated FAP with fewer than 100 polyps (as per histopathology reports), as well as patients who did not receive TPC/IPAA. Demographic data, surgical details, original histopathology, and details of follow-up pouch endoscopic and pathologic findings were captured retrospectively. The definition of pouch adenoma included lesions that occurred in the ileum above the anastomosis site. Pouch adenoma progression was defined as an increase in the number or size of these lesions, as well as the development of dysplasia or malignancy, evident on histopathological examination. The severity of the duodenal polyposis in each case was assessed using the Spigelman classification[15] (Table 1). The study protocol was approved by the institutional review board of Asan Medical Center (registration No. 2021-0309) in accordance with the Declaration of Helsinki.

Surgical techniques of IPAA

An anal mucosectomy leaving a short rectal muscular cuff above the dentate line and transanal hand-sewn ileoanal anastomosis were performed for 72 of our enrolled FAP patients (75.8%). The remaining 23 patients (24.2%) underwent double-stapled anastomosis adjacent to the dentate line at the ATZ. The pouch construction in all patients was J-shaped using two ileal limbs of 15 cm in length.

Follow-up protocols

The regular follow-up protocols for our study patients included clinical examinations, pouch and upper gastrointestinal endoscopy, and abdominoperineal computed tomography. Pouch endoscopy was performed within 1 postoperative year. Subsequently, for patients who underwent a mucosectomy, endoscopic examinations were performed once every 2 years in the absence of any polyps. If polyps were observed during endoscopic follow-up, they were removed regardless of size. In patients with multiple polyps, those larger than 5 mm in diameter were removed, or a biopsy specimen was collected. More intensive follow-up was performed, at intervals of 6-12 mo, if warranted by the size, number, or pathologic characteristics of identified polyps. Patients with colorectal cancer in our cohort were followed at 6-mo intervals for 5 years and annually thereafter. Additionally, testing for carcinoembryonic antigens was conducted every 6 mo, and chest computed tomography was performed annually.

Statistical analysis

The quantitative variables in this study are expressed as medians with interquartile ranges (IQRs) or ranges, and categorical variables are summarized as frequencies and percentages. The clinicopathological variables for the patients with and without pouch adenomas were compared using the Kruskal-Wallis test and Fisher's exact test. The time to pouch adenoma formation was defined as the time from the date of surgery to the date of detection of the first histologically confirmed adenoma. Pouch adenoma–free survival was calculated using the Kaplan-Meier method. Multivariate Cox regression analysis based on backward elimination was used to assess the impact of variables on pouch adenoma–free survival with adjustment for variables reported to be associated with pouch adenoma–free survival in previous reports (colorectal polyp burden, time interval after IPAA, presence of desmoid tumors, and presence of gastric polyps and duodenal adenomas) in addition to basic patient characteristics, such as age and sex. Hazard ratios (HRs) with 95% confidence intervals (CIs) were also calculated. Statistical significance was established with a two-sided test at $P < 0.05$. All statistical analyses were performed using SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, United States).

RESULTS

A total of 175 patients with FAP who underwent pouch surgery were identified from the hospital database. Among these patients, we excluded 48 with attenuated FAP with fewer than 100 polyps according to histopathology reports. We also excluded six patients who underwent total colectomy procedures with ileorectal anastomosis, two who underwent end-ileostomy without ileal pouches, and one whose pouch was removed due to postoperative bleeding. We further excluded 23 patients who did not undergo postoperative follow-up sigmoidoscopy. The final study cohort, thus, comprised 95 patients with FAP who underwent TPC/IPAA (Figure 1).

The characteristics of the 95 FAP patients enrolled in this study are summarized in Table 2. The median follow-up period after pouch surgery was 88 mo (IQR, 61-141 mo). The median age at the time of IPAA was 32 years (IQR, 24-41 years). The median number of follow-up endoscopies was 5 (IQR, 3-7).

Table 1 Characteristics of familial adenomatous polyposis patients analyzed in this study

Variable	
Age at time of IPAA [yr, median (IQR)]	32 (24-41)
Sex, *n* (%)	
Male	52 (54.7)
Female	42 (45.3)
No. of colorectal polyps [*n*, median (IQR)]	350 (150-700)
Type of anastomosis, *n* (%)	
Hand-sewn with mucosectomy	72 (75.8)
Double stapling	23 (24.2)
No. of surveillances [*n*, median (IQR)]	
Upper GI endoscopy	5 (2-8)
Sigmoidoscopy	5 (3-7)
Pouch adenomas, *n* (%)	24 (25.3)
Time to pouch adenoma onset [mo, median (IQR)]	52 (28.3-114.3)
ATZ adenomas, *n* (%)	7 (7.4)
Gastric polyps, *n* (%)	70 (73.7)
Duodenal adenomas, *n* (%)	46 (48.5)
Spigelman stage, *n* (%)	
I	24 (25.3)
II	12 (12.6)
III	10 (10.5)
IV	0
Missing data	49 (51.6)
Desmoid tumors, *n* (%)	23 (24.2)
Colorectal cancer at time of IPAA, *n* (%)	43 (45.3)
Colon	24 (55.8)
Rectum	19 (44.2)

IPAA: Ileal pouch anal anastomosis; IQR: Interquartile range; ATZ: Anal transition zone; GI: Gastrointestinal.

We observed that 24 (25.3%) of our study patients had adenomas that had been histologically confirmed in the pouch mucosa above the anastomosis. The median time to first pouch adenoma detection was 52 mo (IQR, 28.3-114.3 mo). Adenomas arising from the ATZ below the anastomosis were detected in seven (7.4%) patients. Upper gastrointestinal endoscopy was also performed for all patients. Gastric polyps were present in 70 (73.3%) patients; these were mainly fundic gland polyps (75.7%) as per biopsy results. Duodenal adenomas were confirmed in 46 (48.5%) patients. Twenty-four, 12, and 10 patients were classified as having Spigelman stages I, II, and III lesions, respectively, and there were no stage IV cases. Twenty-three (24.2%) patients developed desmoid tumors. At the time of surgery, 43 (45.3%) patients had colorectal cancer.

Mutation analysis

Information on the underlying germline mutation was available for 40 (42.1%) FAP patients, among whom 28 (70.0%) harbored *APC* mutations, most commonly within exon 15 of the *APC* gene (64.3%). No significant difference was found in terms of the distribution of germline mutations between patients with and without pouch adenomas ($P = 0.21$).

Presence and distribution of pouch adenomas

The cumulative incidences of pouch adenomas at 5, 10, and 15 years after IPAA were 15.2%, 29.6%, and 44.1% (95%CI: 7.2%-22.4%, 15.8%-41.1%, and 25.3%-58.2%), respectively (Figure 2). Among the 24

Table 2 Clinical characteristics of study patients according to presence of pouch adenomas

Characteristic	Presence of pouch adenomas (*n* = 24)	Absence of pouch adenomas (*n* = 71)	*P* value
Age at time of IPAA [yr, median (IQR)]	29 (20-40)	32 (26-41)	0.10
Sex (male), *n* (%)	16 (66.7)	36 (50.7)	0.17
No. of colorectal polyps [*n*, median (IQR)]	700 (325-1000)	250 (110-500)	0.001
Colorectal polyps < 1000, *n* (%)	16 (66.7)	64 (90.1)	0.006
Colorectal polyps ≥ 1000, *n* (%)	8 (33.3)	7 (9.9)	
Time interval after IPAA [mo, median (IQR)]	142 (106-199)	116 (91-175)	0.01
Mucosectomy, *n* (%)	19 (79.2)	53 (74.6)	0.66
Gastric polyps, *n* (%)	19 (79.2)	51 (71.8)	0.49
Gastric polyp burden, *n* (%)			0.31
< 20	13 (54.2)	41 (57.7)	
20-49	0	11 (15.5)	
≥ 50	6 (25.0)	0	
Duodenal adenomas, *n* (%)	16 (66.7)	30 (42.3)	0.039
Spigelman stage, *n* (%)			0.30
I	6 (37.5)	18 (60.0)	
II	6 (37.5)	6 (20)	
III	4 (25.0)	6 (20)	
Desmoid tumor, *n* (%)	4 (16.7)	19 (26.8)	0.32
NSAIDs use, *n* (%)	1 (4.2)	14 (19.7)	0.07
Colorectal cancer, *n* (%)	9 (37.5)	34 (47.9)	0.38

IPAA: Ileal pouch anal anastomosis; NSAIDs: Nonsteroidal anti-inflammatory drugs; IQR: Interquartile range.

patients with pouch adenomas, 13 (54.2%) had fewer than 12 countable lesions, whereas others had numerous polyps within the pouch. All countable adenomas were removed endoscopically regardless of size. Only one patient had to undergo a transanal excision to remove a pouch adenoma 31 mm in diameter. The median value of the maximum diameter was 3 mm (range, 2.0-31.0 mm). Tubular adenomas were detected in 23 (95.9%) patients, and tubule-villous adenomas were detected in two (8.4%) patients. There were no cases of high-grade dysplasia or malignancy in our study sample. During follow-up for the 13 patients who underwent complete removal of all detected adenomas, eight (61.5%) patients had no recurrence, and four (38.5%) developed recurrent adenomas, which were endoscopically resected. The remaining patient in this group was lost to follow-up, and the disease course after adenoma removal was not documented. For the 11 (45.8%) patients with numerous polyps within the pouch, only those larger than 5 mm were removed, and surveillance biopsy specimens were collected. In four (36.4%) patients, the adenomas remained unchanged, while seven (63.6%) patients exhibited pouch adenoma progression (Figure 3).

Clinical characteristics of study patients with and without pouch adenomas

Comparisons of the clinical characteristics among the FAP patients according to the presence of pouch adenomas are presented in Table 2. Compared with patients without adenomas, the pouch adenoma group had a significantly higher mean number of colorectal polyps at the time of surgery (700 *vs* 250, $P = 0.001$). Severe polyposis involving more than 1000 colorectal polyps was also significantly more common in the pouch adenoma group ($P = 0.006$). Additionally, patients with pouch adenomas (relative to those without pouch adenomas) were more likely to have duodenal adenomas (66.7% *vs* 42.3%, $P = 0.039$). There was no significant intergroup difference in the Spigelman adenoma stage distributions ($P = 0.30$) and no differences in the gastric polyp status (absent or present) or gastric polyp burden. Nonsteroidal anti-inflammatory drug (NSAID) treatment for desmoid tumors-including with celecoxib and meloxicam—was more common among patients without pouch adenomas, but this difference was not statistically significant (4.2% *vs* 19.7%, $P = 0.07$). There were no differences between the clinical characteristics of the study patients when stratified by the presence of colorectal cancer at the time of surgery ($P = 0.38$).

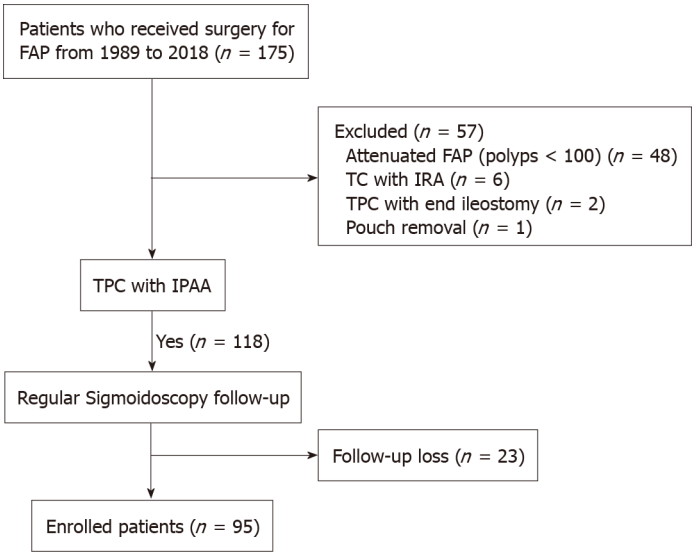

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Figure 1 Patient selection flow chart. FAP: Familial adenomatous polyposis; TPC: Total proctocolectomy; IPAA: Ileal pouch-anal anastomosis; IRA: Ileorectal anastomosis.

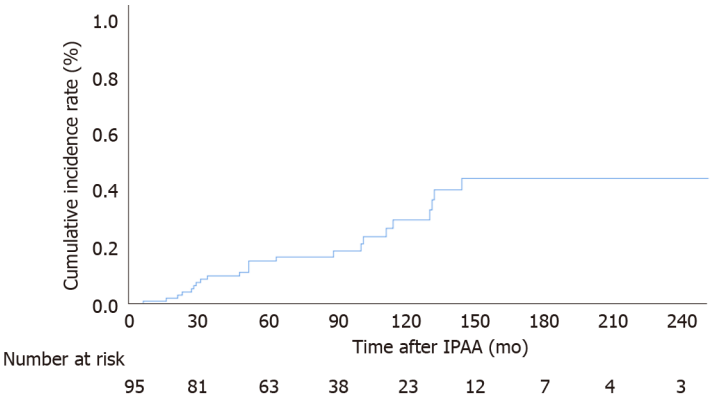

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Figure 2 Cumulative incidence of pouch adenomas. IPAA: Ileal pouch-anal anastomosis.

Associations between different clinical factors and pouch adenoma–free survival

Associations between different patient factors and pouch adenoma–free survival were evaluated (Table 3). Among the clinical factors analyzed, multivariate analysis revealed severe colorectal polyposis (with more than 1000 polyps) to be a significant risk factor for pouch adenoma development (HR, 2.49; 95%CI: 1.04-5.96; P = 0.041) (Figure 4). The presence of gastric polyps or a duodenal adenoma had no significant association with pouch adenoma development (P = 0.28 and 0.54, respectively).

DISCUSSION

TPC/IPAA is now being implemented as a standard treatment to eliminate the risk of colorectal cancer in patients with FAP. However, the postoperative development of adenomas in the ileal pouch in these individuals has raised new concerns about appropriate postoperative surveillance and management approaches. In this study cohort, the incidence of pouch adenoma was 25.3% at a median follow-up of 88 mo (IQR, 61-141 mo) after IPAA. The cumulative risk of pouch adenoma was 15.2%, 29.6%, and 44.1% at 5, 10, and 15 years after TPC/IPAA, respectively. There were no cases of high-grade dysplasia or carcinoma in our cohort. We found that a higher colorectal polyp burden at the time of surgery was positively associated with the occurrence of pouch adenoma formation (P = 0.001). These results are consistent with the findings reported in prior studies[9,10,16-18].

Several potential risk factors associated with pouch adenoma development have been investigated previously, but they remain controversial. Consistent with the findings of Tonelli *et al*[10], we found in

Table 3 Risk factors associated with pouch adenoma-free survival

Variable	Univariate analysis		Multivariable analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	0.97 (0.93-1.01)	0.12		
Sex (male)	1.46 (0.62-3.44)	0.39		
No. of colorectal polyps (≥ 1000)	2.80 (1.18-6.63)	0.019	2.49 (1.04-5.96)	0.041
Time interval after IPAA	0.996 (0.99-1.003)	0.29		
Mucosectomy	1.54 (0.56-4.24)	0.40		
Gastric polyps	1.73 (0.64-4.68)	0.28		
Duodenal adenoma	2.31 (0.99-5.41)	0.54	2.08 (0.88-4.93)	0.10
Spigelman stage		0.15		
I-II	2.27 (0.92-5.55)	0.07		
III-IV	2.46 (0.73-8.25)	0.14		
Desmoid tumor	0.82 (0.28-2.44)	0.72		
NSAID use	0.29 (0.04-2.13)	0.22		
Colorectal cancer	0.58 (0.25-1.37)	0.21		

IPAA: Ileal pouch anal anastomosis; NSAIDs: Nonsteroidal anti-inflammatory drugs; HR: Hazard ratio; CI: Confidence interval.

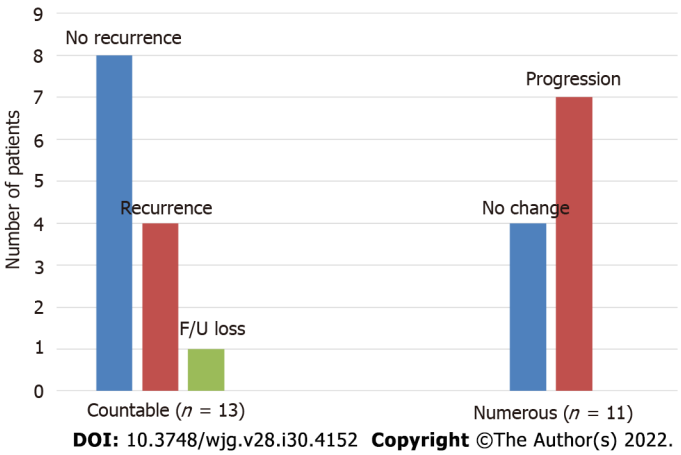

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Figure 3 Progression of pouch adenomas. Patients in the countable group had all adenomas removed, and numerous groups were only undergoing surveillance biopsy.

our present analyses that a high colorectal polyp count at the time of IPAA was positively associated with pouch adenoma development. This result is likely to be assumed to be more aggressive disease. However, other authors have not observed an association between the severity of colonic disease and development of pouch adenoma[5].

Mutations of the *APC* gene predispose the carrier to benign polyps and malignancies in the upper gastrointestinal tract. Therefore, the risk of developing small bowel adenomas remains in these individuals even after TPC/IPAA. In this context, gastric or duodenal adenomas may be relevant to development of pouch adenoma. Ganschow *et el*[9] analyzed these variables previously and found that the presence of a gastric adenoma was a significant risk factor for developing a pouch adenoma (P = 0.0019). Several investigator groups have also reported that the presence of a duodenal adenoma is associated with pouch adenoma formation[10,16]. Tonelli *et al*[10] observed that the severity of duodenal polyposis was also relevant in this regard. As with the findings of other studies, our experience has been that patients who develop pouch adenomas are more likely to have had duodenal adenomas, although this was not observed to be a significant risk factor in our present multivariate analysis.

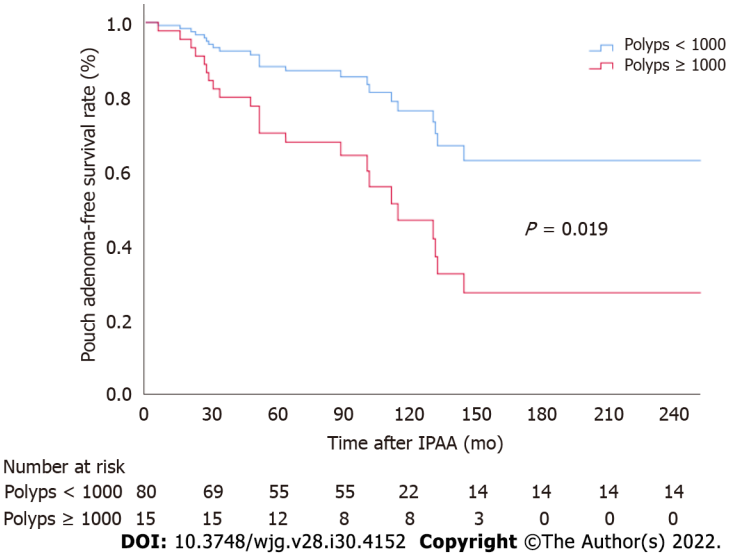

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Figure 4 Pouch adenoma-free survival according to colorectal polyp burden at the time of ileal pouch-anal anastomosis. IPAA: Ileal pouch-anal anastomosis.

There has been little research conducted on the association between the site of an *APC* mutation and the occurrence of pouch adenoma. No such association has been identified in earlier studies[9,10,16], nor was such an association identified in our present analysis. However, in previous reports and in our present series, *APC* mutation data were not available for all subjects. Moreover, the low number of patients who underwent this mutation analysis in our present series and in prior study cohorts could not yield the statistical power needed to determine whether the *APC* mutation site is associated with the tendency to develop pouch adenomas. Additionally, a recently published study of genotype-phenotype associations between *APC* mutations and pouch adenomas found that patients with either indel/deletion mutations or exon 15 mutations had a higher tendency for pouch adenoma formation (P = 0.002 and 0.019, respectively)[19]. Hence, further larger-scale research is required to investigate the association between underlying germline mutations and pouch adenoma development.

Several authors have implicated colonic metaplasia of the ileal mucosa—an adaptive response of the neorectum—as a predisposing condition to the onset of ileal adenoma development[20]. Advanced adenomas with high-grade dysplasia were observed in about 18% of previous study samples[5,18]. However, no case of an advanced adenoma was detected in our present study cohort, possibly because adenomas were aggressively removed from our patients, even if small. If ileal pouch adenomas progress to carcinoma, following the classic adenoma-carcinoma sequence, a higher number and larger size of polyps may be associated with the severity of the dysplasia. Tajika *et al*[18] reported that the postoperative time interval was significantly associated with the maximum ileal pouch adenoma size (P = 0.0214). Progression of a pouch adenoma to invasive carcinoma appears to be rare, with an incidence of less than 1% in patients that undergo IPAA[8,9,19]. Moreover, the majority of carcinomas observed after IPAA arise in the residual rectal mucosa or anal canal, although several cases of carcinoma inside the ileal pouch have been described in the past few years[18]. Tonelli *et al*[10] found that the development of malignancy in the terminal ileum can present with a rapid disease course and does not seem to follow the classic adenoma-carcinoma sequence. In some patients, ileal polyps are not detected by endoscopic follow-up until the development of a pouch carcinoma, and the mean interval between pouch construction and the diagnosis of carcinoma can be very short (range, 3-16.4 years).

Clinically, the treatment of pouch adenomas depends on the number, size, shape, and histological features—as for the gastric or duodenal polyps. According to the American Society for Gastrointestinal Endoscopy and British Society of Gastroenterology guidelines, all types of gastric or duodenal polyps detected *via* endoscopy need to be sampled using biopsy forceps for evaluation[21-23]. In the case of adenomatous polyps, these guidelines recommend complete endoscopic removal when it is safe to do so, given the higher probability of malignant transformation[22,23]. Endoscopic follow-up should be repeated at 6 mo for incompletely resected polyps or for those with high-grade dysplasia; it can be conducted again at 12 mo for all other polyps[22]. Although the data are currently limited, 62% of our cohort did not develop adenoma recurrence after adenoma removal, whereas 63% of our patients under observation showed progression. There was no spontaneous diminution or disappearance of any adenoma in our study cohort. Hence, it is important to be aware of the risk of pouch neoplasia when caring for FAP patients after IPAA, and—to enable evaluation and treatment at the same time—we recommend endoscopic resection when the adenoma is detected. If numerous polyps are present, random biopsy sampling is required, and polyps larger than 5 mm should be removed.

Interestingly, our FAP patients who used NSAIDs as a treatment for desmoid tumors rarely developed pouch adenomas. This finding is presumed to be related to the chemopreventive effect of these drugs. Chemoprevention with NSAIDs is a treatment option considered to facilitate the management of the remaining rectum or pouch in selected postoperative patients[13]. In a prior randomized controlled study, patients with FAP and attenuated FAP treated with celecoxib following prophylactic surgery showed reductions in polyp number and diameter[24]. NSAIDs may thus be considered if not all of the numerous polyps within the pouch can be removed, although there are currently no Food and Drug Administration–approved drugs for this indication.

There were some limitations to this study. First, this was a retrospective cohort study with a small sample size, and there may have been unknown confounders. However, as we were investigating a rare disease, there have been few studies to date that have analyzed a substantial number of affected patients. Therefore, in relative terms, our sample size was large, and the patients were followed for a considerable length of time. A second limitation was that whereas all of the patients had a clinical phenotype of 100 or more colorectal polyps and biopsy-confirmed FAP, *APC* genetic mutation analysis was only performed for about 50% of the patients. This limited the strength of our findings related to these mutations. Additionally, there was no consistent endoscopic treatment standard in our cohort. When a polyp was countable, it was removed regardless of its size. In patients with multiple polyps, however, the individual judgment of the endoscopist determined whether to perform endoscopic resection or observation. The indications used for polyp removal were, therefore, not clear.

Overall, our study findings provide further evidence for the need for standardized endoscopic surveillance of FAP patients following TPC/IPAA. Pouch endoscopy should be performed yearly for these patients, and standardized biopsy and removal protocols are needed if pouch adenomas are observed. Furthermore, surveillance should be tailored depending on the presence and characteristics of the pouch adenoma. In the future, validation of whether an annual endoscopic interval is appropriate is required, as is an evaluation of the feasibility and effectiveness of endoscopic resection of a pouch adenoma.

CONCLUSION

In conclusion, pouch adenomas occur at a fairly high rate over time in association with FAP after IPAA. A high colorectal polyp count increases the risk of developing a pouch adenoma in this patient population. In our experience, the progression of pouch adenomas to high-grade dysplasia or carcinoma is rare. However, this risk is not negligible, and the long-term risk of this cannot presently be well quantified. Close surveillance of the pouch should thus be mandatory, and new guidelines for the management of pouch adenomas are essential.

ARTICLE HIGHLIGHTS

Research background

Restorative total proctocolectomy is now being implemented as a standard treatment to eliminate the risk of colorectal cancer in patients with familial adenomatous polyposis (FAP). However, the postoperative development of adenomas in the ileal pouch in these individuals has raised new concerns about appropriate postoperative surveillance and management approaches.

Research motivation

More information is needed regarding the incidence, natural course, and risk factors associated with pouch adenoma.

Research objectives

To investigate the cumulative incidence and time to development of pouch adenomas and analyze the clinical factors associated with pouch adenoma development among patients with FAP after restorative proctocolectomy.

Research methods

A retrospective cohort study was carried out with 95 consecutive patients with FAP who underwent restorative proctocolectomy at Asan Medical Center (Seoul, South Korea) from November 1989 to December 2018.

Research results

The cumulative risks of pouch adenoma development at 5, 10, and 15 years after pouch surgery were 15.2%, 29.6%, and 44.1%, respectively. Severe colorectal polyposis (with more than 1000 polyps) was a

significant risk factor for pouch adenoma development (hazard ratio, 2.49; 95% confidence interval: 1.04-5.96; $P = 0.041$).

Research conclusions

We recommend endoscopic resection when the adenoma is detected. If numerous polyps are present, random biopsy sampling is required, and polyps larger than 5 mm should be removed. Close surveillance of the pouch should be mandatory, and new guidelines for the management of pouch adenomas are required.

Research perspectives

In the future, validation of whether an annual endoscopic interval is appropriate is required, as is an evaluation of the feasibility and effectiveness of endoscopic resection of a pouch adenoma.

FOOTNOTES

Author contributions: Ryu HS designed and performed the research and wrote the paper; Yu CS designed the research and supervised the report; Kim YI, Lee JL, Kim CW, Yoon YS, Park IJ, Lim SB, and Kim JC provided clinical advice and supervised the report.

Institutional review board statement: The protocol for this research project has been approved by a suitably constituted ethics committee of the institution (Committee of Asan Medical Center, Approval No. 2021-0309), and it conforms to the provisions of the Declaration of Helsinki.

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

Conflict-of-interest statement: This study did not receive a specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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ORIGINAL ARTICLE

Retrospective Study

Changes in the esophagogastric junction outflow obstruction manometric feature based on the Chicago Classification updates

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Abstract

BACKGROUND

The critical diagnostic criteria for esophagogastric junction outflow obstruction (EGJOO) were published in the latest Chicago Classification version 4.0 (CCv4.0). In addition to the previous criterion [elevated integrated relaxation pressure (IRP) in supine position], manometric diagnosis of EGJOO requires meeting the criteria of elevated median-IRP during upright wet swallows and elevated intrabolus pressure. However, with the diagnostic criteria modification, the change in manometric features of EGJOO remained unclear.

AIM

To evaluate the esophageal motility characteristics of patients with EGJOO and select valuable parameters for confirming the diagnosis of EGJOO.

METHODS

We performed a retrospective analysis of 370 patients who underwent high-resolution manometry with 5 mL water swallows × 10 in supine, × 5 in upright position and the rapid drink challenge (RDC) with 200 mL water from November 2016 to November 2021 at Peking University First Hospital. Fifty-one patients with elevated integrated supine IRP and evidence of peristalsis were enrolled, with 24 patients meeting the updated manometric EGJOO diagnosis (CCv4.0) as the EGJOO group and 27 patients not meeting the updated EGJOO criteria as the isolated supine IRP elevated group (either normal median IRP in upright position or less than 20% of supine swallows with elevated IBP). Forty-six patients with normal manometric features were collected as the normal high-resolution manometry (HRM) group. Upper esophageal sphincter (UES), esophageal body, and lower esophageal sphincter (LES) parameters were compared between groups.

RESULTS

Compared with the normal HRM group, patients with EGJOO (CCv4.0) had significantly lower proximal esophageal contractile integral (PECI) and proximal esophageal length (PEL), with elevated IRP on RDC ($P < 0.05$ for each comparison), while isolated supine IRP elevated patients had no such feature. Patients with EGJOO also had more significant abnormalities in the esophagogastric junction than isolated supine IRP elevated patients, including higher LES resting pressure (LESP), intrabolus pressure, median supine IRP, median upright IRP, and IRP on RDC ($P < 0.05$ for each comparison). Patients with dysphagia had significantly lower PECI and PEL than patients without dysphagia among the fifty-one with elevated supine IRP. Further multivariate analysis revealed that PEL, LESP, and IRP on RDC are factors associated with EGJOO. The receiver-operating characteristic analysis showed UES nadir pressure, PEL, PECI, LESP, and IRP on RDC are parameters supportive for confirming the diagnosis of EGJOO.

CONCLUSION

Based on CCv4.0, patients with EGJOO have more severe esophagogastric junction dysfunction and are implicated in the proximal esophagus. Additionally, several parameters are supportive for confirming the diagnosis of EGJOO.

Key Words: Esophagogastric junction outflow obstruction; High-resolution manometry; Esophageal motility disorders; Upper esophageal sphincter; Proximal esophagus

Core Tip: This is a retrospective study to evaluate the motility features of esophagogastric junction outflow obstruction (EGJOO). This is the first detailed study of EGJOO based on the latest Chicago Classification. Patients with EGJOO showed more substantial abnormalities at the esophagogastric junction than patients who met the previous criteria, and the motility disorder of EGJOO is implicated in the proximal esophagus. Additionally, the upper esophageal sphincter nadir pressure, proximal esophageal contractile integral, proximal esophageal length, lower esophageal sphincter resting pressure, and integrated relaxation pressure on rapid drink challenge contribute to confirming the diagnosis of EGJOO.

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INTRODUCTION

Esophagogastric junction outflow obstruction (EGJOO) is a common type of esophageal motility disorder in patients with dysphagia or chest pain. EGJOO includes a group of heterogeneous disorders with common manometric features for esophageal outflow obstruction. Progress has been made toward understanding the manometric features and symptoms of patients who meet the EGJOO criteria[1-5]. The Chicago Classification version 4.0 (CCv4.0) updated the critical diagnostic criteria for the manometric diagnosis of EGJOO, including increased median-integrated relaxation pressure (IRP) in supine and upright positions, ≥ 20% elevated intrabolus pressure (IBP) in the supine position, and evidence of peristalsis[1]. A clinically relevant conclusive diagnosis of EGJOO requires a manometric diagnosis of EGJOO as described above, and clinically relevant symptoms with at least one of the complementary tests, including timed barium esophagram and functional lumen imaging probe. Additional provocative tests including rapid drink challenge (RDC), solid test swallows, or pharmacologic provocation may also strengthen the confidence in an EGJOO diagnosis and helps to identify the causes of symptoms, particularly in borderline cases. Compared with the previous version (v3.0)[6], the new diagnostic criteria provided a more rigorous definition for EGJOO by adding criteria for median IRP in the secondary position, IBP, clinically relevant symptoms, and complementary tests, which aid in distinguishing pathological motility disorder and abnormal manometry caused by mechanical effect, opioid use, or other nonpathological motility disorders[7]. However, the change in manometric features of EGJOO with the diagnostic criteria modification remained unclear. This study aims to investigate the esophageal motility features of patients with the manometric diagnosis of EGJOO and to identify high-resolution manometry (HRM) parameters that are supportive for confirming the diagnosis of EGJOO.

MATERIALS AND METHODS

Patient and data selection

Patients who completed esophageal HRM and upper gastrointestinal endoscopy from November 2016 to November 2021 at Peking University First Hospital were retrospectively analyzed. Exclusion criteria included: (1) Patients under 18 years of age; (2) A history of upper gastrointestinal or mediastinal surgery; (3) Previous endoscopic treatment for esophageal motor disorders; (4) Diseases with abnormal intraabdominal pressure, such as intestinal obstruction or ascites; (5) Use of opiates; and (6) Secondary factors identified by upper gastrointestinal endoscopy or endoscopic ultrasonography, especially for hiatal hernia, infiltrative disease, mechanical obstruction, and extrinsic compression. The normal HRM group was obtained from patients who underwent HRM for mild symptoms such as dysphagia, retrosternal pain, regurgitation, or heartburn, with normal HRM results. The patients also fulfilled normal results in pH-monitoring and upper gastrointestinal endoscopy, in order to exclude the possibility of organic diseases. Demographic data, including age, gender, body mass index (BMI), previous medical and surgical history were collected, and symptoms were extracted from self-report questionnaires completed by patients before HRM. The Institutional Review Board (IRB) of the Peking University First Hospital approved the study protocol (2022-099). The IRB waived the requirement for informed consent because our retrospective analysis used completely anonymized data.

HRM protocol

HRM studies were conducted according to standard clinical protocol, using a 4.2-mm outer diameter, 36-sensor solid-state HRM catheter (ManoScan™, Medtronic, Los Angeles, CA, United States)[8]. Experienced nurses performed nasal canal anesthesia and transnasal placement of the solid-state manometry catheter with the patient sitting upright after an 8-h fast. Sensors were positioned to ensure a complete record of the hypopharynx, esophagus, and proximal stomach. The manometric protocol consisted of a landmark phase captured during a quiet rest in the supine position at the beginning, followed by ten 5-mL ambient temperature water swallows in the supine position, then five 5-mL water swallows in the upright position, with 30 s between each swallow, and finally a RDC of 200 mL water in the upright position.

HRM data analysis

HRM Clouse plots were analyzed using computerized HRM analysis software (Manoview, Medtronic). All pressure measurements were referenced to the gastric pressure.

Upper esophageal sphincter parameters[9,10]: Upper esophageal sphincter (UES) parameters were measured within the UES high-pressure zone defined by a 20-mmHg isobaric contour, consisting of UES length (UESL), UES resting pressure (UESP), nadir pressure (UESNP), and postdeglutitive UES contractile integral (PD-UESCI). PD-UESCI was measured by the smart mouse at the beginning of the deglutitive UES relaxation to the end of the proximal esophageal contraction or the beginning of the transition zone (Figure 1).

Proximal esophageal parameters[9,11]: Proximal esophageal parameters measured from the lower border of the UES to either a break between the proximal and distal segment or the area with the lowest pressure between the proximal and distal segment of the contraction in patients showed no break in the 20-mmHg isobaric contour. Measurements of the proximal esophageal segment included proximal esophageal segment length (PEL), proximal latency (PL, defined as the time interval between UES relaxation to the transition zone), and proximal esophageal contractile integral (PECI, = amplitude × duration × length, measured using a 20-mmHg pressure threshold).

Esophageal body parameters[12]: Esophageal shortening during RDC (ES-RDC) was defined as an upward lift of lower esophageal sphincter (LES) for more than 1 cm, as measured by the length variation between the baseline position of the LES before RDC and its maximal axial position during RDC or within 60 s after the start of RDC. The distal latency (DL) and distal contraction integral (DCI) were calculated automatically using the ManoView system.

Esophagogastric junction parameters[13]: The LES resting pressure (LESP), IBP were calculated automatically using the ManoView system. The median IRP in the supine and upright positions was selected from a list of IRP in each position. The IRP on RDC was assessed in the window beginning with dilatative UES relaxation to the end of esophagogastric junction (EGJ) relaxation for free drinking lasting less than 30 s, or during the first 30 s of the window for free drinking lasting longer.

Statistical analysis

Statistical analysis was performed using SPSS 25.0, and GraphPad Prism 8.0. The chi-square test was used for the comparison of categorical variables. The Student's *t*-test and analysis of variance were used to compare quantitative data with normal distribution between groups, and the results are expressed as mean ± SD. Multivariate analysis was performed with stepwise variable selection. The receiver-

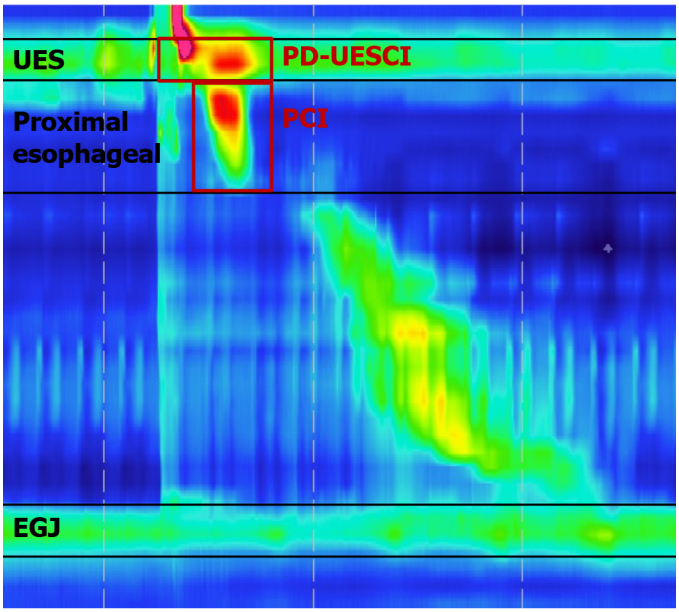

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Figure 1 Key metrics of Clouse plots used in our study. The postdeglutitive upper esophageal sphincter (UES) contractile integral was measured using a 20-mmHg pressure threshold from the beginning of the deglutitive UES relaxation to the end of the proximal esophageal contraction or the beginning of the transition zone. The proximal esophageal contractile integral was measured using a 20-mmHg pressure threshold from the lower border of the UES to either a break between the proximal and distal segment or the area with the lowest pressure between the proximal and distal segment of the contraction in patients showed no break in the 20-mmHg isobaric contour. UES: Upper esophageal sphincter; PD-UESCI: Postdeglutitive upper esophageal sphincter contractile integral; PCI: Proximal contractile integral; EGJ: Esophagogastric junction.

operating characteristics (ROC) curve was used to illustrate the diagnostic ability of the HRM parameters for EGJOO. A P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Fifty-one patients (33 female, 59.5 ± 1.7 years) fulfilling the criteria of EGJOO (CCv3.0) were identified, with supine IRP ≥ 15 mmHg (Medtronic) and evidence of peristalsis. Among them, 24 patients (14 female, 62.7 ± 2.7 years) met the manometric definitions of EGJOO (CCv4.0), while 27 patients (19 female, 56.6 ± 11.1 years) failed to meet the updated EGJOO criteria formed the isolated supine IRP elevated group, with either normal median IRP in upright position or less than 20% of supine swallows with elevated IBP. The normal HRM group comprised 46 patients (24 female, 50.2 ± 2.2 years) with normal HRM results (Figure 2). Patients in the EGJOO group were older than the normal HRM group. There was no difference in gender or BMI between these three groups. As for symptoms, among the 24 patients with manometric diagnosis of EGJOO (CCv4.0), there were seven with dysphagia and five with retrosternal pain that might be clinically relevant. Symptoms were also counted in isolated supine IRP elevated group and normal HRM group as shown in Table 1. The occurrence of dysphagia, retrosternal pain, and regurgitation did not differ between these three groups. Among the 24 patients with manometric diagnosis of EGJOO (CCv4.0), there were five patients with spastic features, five with hypercontractile features, two with ineffective motility, and twelve with no evidence of peristalsis disorders.

Esophageal HRM parameters

Table 2 details the differences in manometric parameters among EGJOO, isolated supine IRP elevated group, and the normal HRM group.

UES parameters: UESNP was significantly higher in the EGJOO group and the isolated supine IRP elevated group than in the normal HRM group. There was no significant difference in UESL, UESP, and PD-UESCI between the three groups.

Proximal esophageal parameters: Proximal esophageal contractile function was weaker in the EGJOO group than in the normal HRM group, specifically PEL and PECI. Consistently, PEL was lower in the EGJOO group than in the isolated supine IRP elevated group. There was no difference in PL in the EGJOO group, isolated supine IRP elevated group, and the normal HRM group.

Table 1 Patient characteristics

	EGJOO (*n* = 24)	Isolated supine IRP elevated (*n* = 27)	Normal HRM (*n* = 46)	*P* value
Demographics				
Age (yr)	62.7 ± 2.7[a]	56.6 ± 11.1	50.2 ± 2.2	0.001
Female, *n* (%)	14 (58.3)	19 (70.4)	24 (52.2)	0.312
BMI (kg/m^2)	23.29 ± 0.63	21.88 ± 2.50	23.17 ± 0.68	0.300
Dominant symptom				
Dysphagia, *n* (%)	7 (29.2)	6 (22.2)	7 (15.2)	0.380
Retrosternal pain, *n* (%)	5 (20.8)	3 (11.1)	8 (17.4)	0.630
Regurgitation, *n* (%)	8 (33.3)	9 (33.3)	15 (32.6)	0.997

[a]$P < 0.05$, compared with normal high-resolution manometry group.

BMI: Body mass index; EGJOO: Esophagogastric junction outflow obstruction; IRP: Integrated relaxation pressure; HRM: High-resolution manometry.

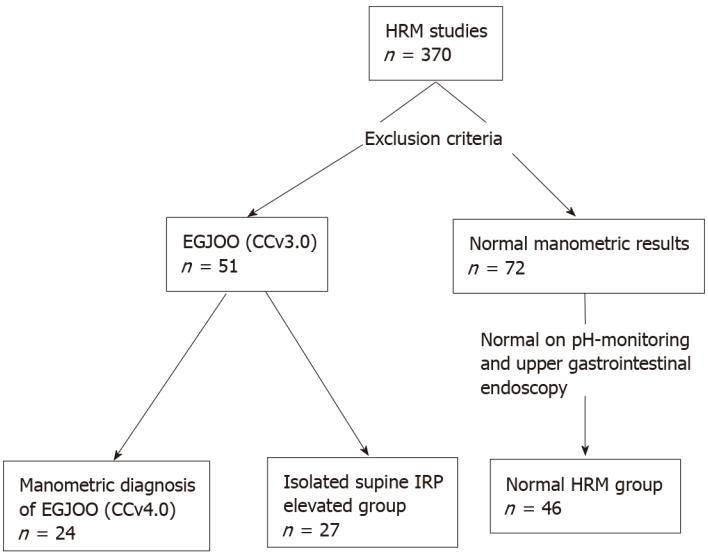

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Figure 2 Patient flow. HRM: High-resolution manometry; EGJOO: Esophagogastric junction outflow obstruction; CCv3.0: Chicago Classification version 3.0; CCv4.0: Chicago Classification version 4.0; IRP: Integrated relaxation pressure.

Esophageal body parameters: There was no significant difference in DL, DCI, and ES-RDC among the three groups.

EGJ parameters: Patients with EGJOO exhibited stronger contractile function in EGJ than in the isolated supine IRP elevated group and the normal HRM group, including LESP, IBP, median supine IRP, median upright IRP, and IRP on RDC.

The multivariate analysis revealed that PEL, LESP, and IRP on RDC are factors associated with EGJOO (Table 3).

Relationship between symptoms and parameters

We compared parameters based on symptoms for the 51 patients with elevated IRP. Patients with dysphagia showed significantly lower PD-UESCI (377.60 ± 36.67 *vs* 517.14 ± 30.47 mmHg·s·cm, $P = 0.017$), PECI (200.25 ± 39.18 *vs* 315.08 ± 30.24 mmHg·s·cm, $P = 0.048$) and PEL (4.43 ± 0.42 *vs* 5.75 ± 0.24 cm, $P = 0.008$) than patients without dysphagia. PEL was higher in patients with retrosternal pain, compared to patients without the symptom (6.43 ± 0.46 *vs* 5.23 ± 0.24 cm, $P = 0.046$) (Figure 3).

Predictors of EGJOO

The ROC analysis discovered HRM parameters that helped identify EGJOO (Table 4). The area under the curve (AUC) of LESP in predicting EGJOO is 0.85, with the optimal cutoff at 40.20 mmHg, yielding a

Table 2 High-resolution manometry parameters of the patients

HRM findings	EGJOO (*n* = 24)	Isolated supine IRP elevated (*n* = 27)	Normal HRM (*n* = 46)	*P* value
UES parameters				
UESL (cm)	3.13 ± 0.18	3.01 ± 0.67	3.34 ± 0.12	0.206
UESP (mmHg)	53.35 ± 5.28	68.12 ± 6.35	57.77 ± 3.35	0.123
UESNP (mmHg)	3.00 ± 1.43[a]	3.50 ± 6.57[a]	-4.08 ± 0.84	< 0.001
PD-UESCI (mmHg·s·cm)	430.01 ± 32.90	527.41 ± 37.48	534.13 ± 34.40	0.118
Proximal esophageal parameters				
PEL (cm)	4.89 ± 0.27[a]	5.89 ± 0.32[b]	6.08 ± 0.14	0.001
PL (s)	1.99 ± 0.08	2.05 ± 0.08	2.04 ± 0.07	0.872
PECI (mmHg·s·cm)	238.34 ± 35.18[a]	328.00 ± 35.15	367.99 ± 37.88	0.048
Esophageal body parameters				
DL (s)	6.14 ± 0.25	6.85 ± 0.32	6.25 ± 0.14	0.078
DCI (mmHg·s·cm)	1581.21 ± 276.20	1655.80 ± 170.33	1705.41 ± 144.27	0.897
ES-RDC (%)	37.5% (9)	18.5% (5)	13.0% (6)	0.053
EGJ parameters				
LESP (mmHg)	47.91 ± 4.05[a]	35.68 ± 1.80[a,b]	23.89 ± 1.34	< 0.001
IBP (mmHg)	12.29 ± 1.37[a]	7.41 ± 0.84[b]	5.45 ± 0.54	< 0.001
Median supine IRP (mmHg)	27.76 ± 2.39[a]	18.74 ± 0.52[a,b]	10.48 ± 0.60	< 0.001
Median upright IRP (mmHg)	23.69 ± 2.58[a]	7.32 ± 0.53[b]	5.41 ± 0.84	< 0.001
IRP on RDC (mmHg)	9.96 ± 1.78[a]	3.00 ± 0.98[b]	2.04 ± 0.58	< 0.001

[a]*P* < 0.05, compared with normal high-resolution manometry group.
[b]*P* < 0.05, compared with esophagogastric junction outflow obstruction group.
IRP: Integrated relaxation pressure; EGJOO: Esophagogastric junction outflow obstruction; UES: Upper esophageal sphincter; UESL: Upper esophageal sphincter length; UESP: Upper esophageal sphincter resting pressure; UESNP: Upper esophageal sphincter nadir pressure; PD-UESCI: Postdeglutitive upper esophageal sphincter contractile integral; PEL: Proximal esophageal length; PECI: Proximal contractile integral; PL: Proximal latency; DL: Distal latency; DCI: Distal contraction integral; ES: Esophageal shortening; RDC: Rapid drink challenge; LESP: Lower esophageal sphincter resting pressure; IBP: Intrabolus pressure; HRM: High-resolution manometry.

Table 3 Multivariate logistic regression analysis for factors associated with esophagogastric junction outflow obstruction

Effect variables	OR	95%CI	*P* value
PEL (cm)	0.543	0.30-0.99	0.044
LESP (mmHg)	1.106	1.05-1.17	0.001
IRP on RDC (mmHg)	1.197	1.02-1.41	0.028

PEL: Proximal esophageal length; LESP: Lower esophageal sphincter resting pressure; IRP: Integrated relaxation pressure; RDC: Rapid drink challenge.

sensitivity of 68.2% and specificity of 85.1%. IRP on RDC achieved an AUC value of 0.81, with the optimal cutoff at > 10.75 mmHg and sensitivity and specificity of 50.0% and 98.5%, respectively. UESNP, PEL, and PECI showed the best predictive value for EGJOO, with cutoff values of 1.15 mmHg (AUC 0.66), 4.76 cm (AUC 0.67), 312.35 mmHg·s·cm (AUC 0.67), respectively.

DISCUSSION

In this study, we mainly assessed the clinical and manometric characteristics of EGJOO based on the Chicago Classification version 4.0 (CCv4.0) to reveal potential changes in esophageal dynamics based on

Table 4 Receiver-operating characteristic analysis for esophagogastric junction outflow obstruction

	Cutoff	AUC	95%CI	Sensitivity (%)	Specificity (%)
UESNP (mmHg)	> 1.15	0.66	0.53-0.78	63.6	68.7
PEL (cm)	< 4.76	0.67	0.55-0.80	50.0	87.7
PECI (mmHg·s·cm)	< 312.35	0.67	0.55-0.80	83.3	46.6
LESP (mmHg)	> 40.20	0.85	0.75-0.94	68.2	85.1
IRP on RDC (mmHg)	> 10.75	0.81	0.70-0.91	50.0	98.5

UESNP: Upper esophageal sphincter nadir pressure; PEL: Proximal esophageal length; PECI: Proximal esophageal contractile integral; LESP: Lower esophageal sphincter resting pressure; IRP: Integrated relaxation pressure; RDC: Rapid drink challenge; AUC: Area under the curve.

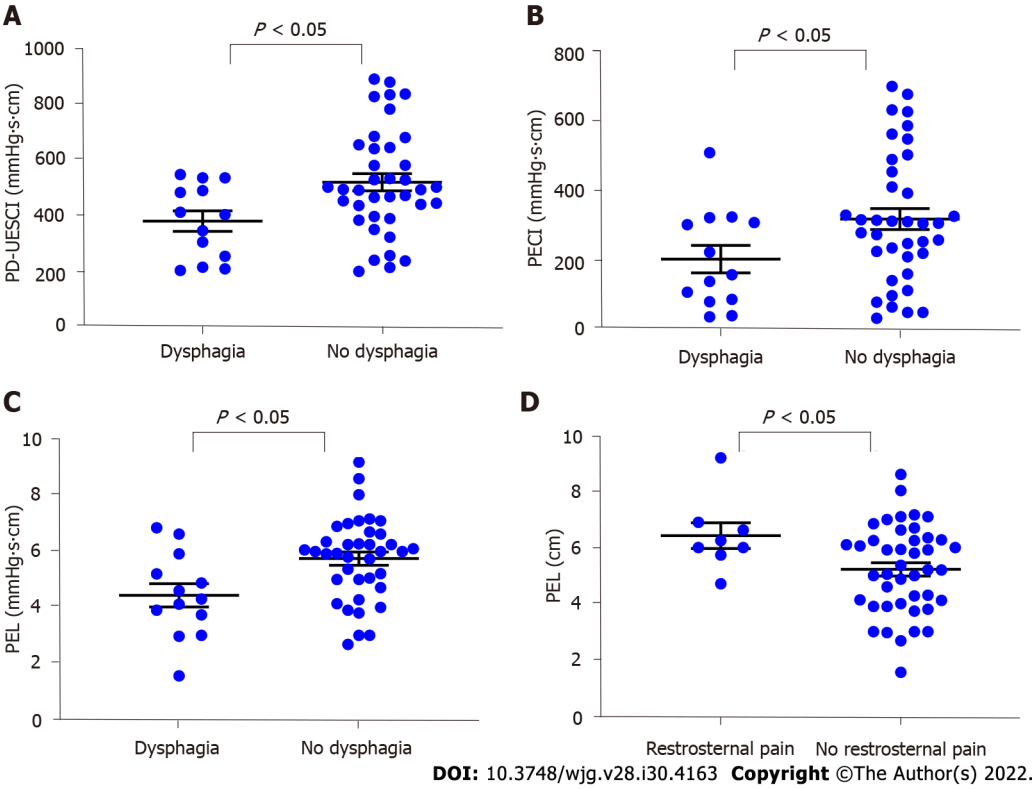

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Figure 3 Comparisons of parameters according to symptoms in 51 patients with elevated integrated relaxation pressure. A: Patients with dysphagia showed lower postdeglutitive upper esophageal sphincter contractile integral than patients without dysphagia; B: Patients with dysphagia showed lower proximal esophageal contractile integral than patients without dysphagia; C: Patients with dysphagia showed lower proximal esophageal length (PEL) than patients without dysphagia; D: Patients with retrosternal pain showed higher PEL than patients without retrosternal pain. PD-UESCI: Postdeglutitive upper esophageal sphincter contractile integral; PECI: Proximal esophageal contractile integral; PEL: Proximal esophageal length.

the new diagnostic criteria, and we identified parameters that help distinguish the EGJOO.

Based on our observations, older people had a higher likelihood of being diagnosed with EGJOO according to manometric results. The EGJOO group showed no significant difference in symptom distribution pattern compared with the isolated supine IRP elevated group and the normal HRM group. Therefore, more manometric features and additional examinations are required for a better understanding of EGJOO.

The nadir UES residual pressure (UESNP) was reported to be higher in EGJOO (CCv3.0) than in normal controls[2,14]. Within EGJOO, higher UESNP was observed in motor disorders compared to mechanical etiologies and was a potential predictor of symptom recurrence after myotomy, with a cutoff level of 2 mmHg[2]. Based on our initial findings, patients with EGJOO and isolated supine IRP had significantly higher UESNP than the normal HRM group. The findings support the hypothesis that the UES is hypertonic with impaired relaxation, which may serve as a protective mechanism to facilitate esophageal clearance and prevent aspiration pneumonitis under IRP elevation[15]. Further ROC analysis revealed that UESNP elevation might serve as a feature for confirming EGJOO.

Previous studies have linked proximal esophageal motility abnormalities to achalasia[9]. The PECI of type 1 achalasia patients was weaker than that of healthy volunteers, but there was no difference between EGJOO (CCv3.0) and health volunteers, and patients with aberrant PECI had more severe upper gastrointestinal symptoms than patients with normal PECI[9]. It is worth noting that EGJOO (CCv4.0) group had weaker PECI than the normal HRM group, while patients with isolated IRP elevation had no difference compared to the normal HRM group. PECI resulted in a limited value for confirming the diagnosis of EGJOO. Furthermore, our findings suggest that PEL is lower in patients with EGJOO than patients with isolated elevated supine IRP or normal HRM, which might also serve as a feature that strengthens the confidence in an EGJOO diagnosis. The above results indicate that based on the updated criteria, EGJOO dysfunction may involve the proximal esophagus, while patients with isolated supine IRP elevated had no such features. It is reasonable to speculate that patients with EGJOO have common changes in proximal esophageal dynamic features, although further studies are required to reveal the underlying pathophysiological mechanism.

With our current analysis of symptoms, postdeglutitive contraction of the UES and proximal esophagus were weaker, and PEL was significantly lower in patients with dysphagia compared to patients without the symptom, indicating that in patients with impaired EGJ relaxation, dysphagia may represent a potential dysfunction of the UES and proximal esophagus.

Esophageal shortening has been proposed as an outcome of longitudinal muscle contraction, and esophageal shortening during the rapid drink test was mainly associated with impaired EGJ relaxation or major peristalsis disorders, particularly achalasia[12]. In our study, the incidence of ES-RDC had a marginal difference between the three groups, which is comparable with the motor pattern observed in achalasia.

In this study, LESP increased progressively from normal HRM to the isolated supine IRP elevated and EGJOO groups, with significant differences between groups. LESP was critical in the evaluation of EGJ obstruction. Furthermore, ROC curve analysis revealed that LESP had the highest differential diagnostic efficacy of EGJOO, indicating that the value of LESP is helpful in the assessment of EGJOO.

CCv4.0 also highlights the role of ancillary manometric evaluations, such as RDC, to identify the causes of symptoms and elicit evidence of obstruction. Our study performed IRP on RDC, which was significantly higher in the EGJOO group than in other groups, while the isolated supine IRP elevated group showed no difference compared with the normal HRM group, indicating that Chicago Classification updates filtered out EGJ dysfunction with more severe obstruction. It is worth noting that IRP on RDC greater than 12 mmHg (Medtronic software) indicates achalasia and may correlate with symptom severity[1,13,16]. The ROC analysis revealed that a high IRP on RDC is useful for confirming the diagnosis of EGJOO with high specificity (98.5%), but low sensitivity (50.0%).

Based on CCv3.0, a significant proportion of EGJOO is associated with the effect of artifact, hiatal hernia, mechanical obstruction, opioid effect, or gastric volvulus, but not primary LES dysfunction. Hence, numerous studies focused on the identification of primary motility disorders and excluded motility patterns secondary to medication use, mechanical obstruction, previous surgery, or endoscopic interventions[3,4,17], which are critical in making appropriate therapeutic decisions. Since the morphology of LES is affected by position, the CCv4.0 defines IRP in the upright position and IBP; thus, the Chicago Classification update has reduced the number of clinically irrelevant diagnoses and improved the specificity for EGJOO diagnosis[7,17,18], enabling us to avoid irreversible treatment for these conditions. According to the results of this study, patients with EGJOO had multiple abnormalities in EGJ parameters compared with the isolated supine IRP elevated group, including LESP, IBP, median supine IRP, median upright IRP, and IRP on RDC, implying that the Chicago Classification update aids in the selection of EGJOO with more severe EGJ dysfunction.

Due to the limitations of the retrospective study, our study lacked data on treatment and outcomes of patients, larger cohorts are required to explore the prognostic value of the parameters mentioned above. Based on CCv4.0, additional provocative tests such as solid test swallows, or pharmacologic provocation were recommended, and complementary tests are required for a conclusive, actionable diagnosis of clinically relevant EGJOO, while our study did not include the tests mentioned above and mainly focused on the changes in the manometric diagnosis of EGJOO. Moreover, it is necessary to further investigate the pathophysiological mechanism of the changes in proximal esophageal motility of patients with EGJOO.

CONCLUSION

Conclusively, our current analysis revealed that patients with EGJOO had multiple changes in esophageal parameters based on Chicago Classification updates, especially more severe dysfunction at the esophagogastric junction than the previous diagnostic criteria, and showed multiple abnormalities at the proximal esophagus. The results illustrate that EGJOO is implicated in the proximal esophagus, and Chicago Classification updates improved the specificity for EGJOO diagnosis. Accordingly, we have expanded the valuable parameters for confirming the diagnosis of EGJOO based on CCv4.0, including UESNP, PEL, PECI, LESP, and IRP on RDC. With the advancement of EGJOO research, more

contributions will be provided to the diagnosis and treatment of this type of disorder.

ARTICLE HIGHLIGHTS

Research background

The critical diagnostic criteria for esophagogastric junction outflow obstruction (EGJOO) were published in the latest Chicago Classification version 4.0 (CCv4.0). However, as a result of the diagnostic criteria modifications, the changes in manometric features of EGJOO remained unclear.

Research motivation

To investigate the changes of EGJOO manometric features according to the Chicago Classification updates.

Research objectives

This study focused on evaluating the esophageal motility characteristics of patients with EGJOO, and selecting valuable parameters that are supportive for confirming the diagnosis of EGJOO.

Research methods

A total of 97 patients were enrolled, with 24 patients that met the updated manometric diagnosis of EGJOO (CCv4.0), 27 patients that only met the previous criteria, and 46 patients with normal manometric features served as the normal high-resolution manometry (HRM) group for this study. We collected clinical data, HRM parameters, and conducted comparisons among groups. Factors associated with EGJOO were illustrated by multivariate analysis. Furthermore, valuable parameters that strengthen the confidence in an EGJOO diagnosis were selected by the receiver-operating characteristic analysis.

Research results

EGJOO patients revealed significantly decreased proximal esophageal contractile integral (PECI) and proximal esophageal length (PEL) compared to the normal HRM group, and the features were related to dysphagia. EGJOO patients also had more severe dysfunction of the esophagogastric junction including lower esophageal sphincter resting pressure (LESP), intrabolus pressure, median supine integrated relaxation pressure (IRP), median upright IRP, and IRP on rapid drink challenge (RDC) than patients that only met the previous criteria. Further multivariate analysis revealed that the PEL, LESP, and IRP on RDC are factors associated with EGJOO. Additionally, the upper esophageal sphincter nadir pressure, PECI, PEL, LESP, and IRP on RDC contributes to confirming the diagnosis of EGJOO.

Research conclusions

The updates of Chicago Classification have improved the precision for identification of EGJ dysfunction that may reduce over-diagnosing for EGJOO. The motility disorder of EGJOO is implicated in the proximal esophagus, and the changes of proximal esophagus may relate to dysphagia. Additionally, there are valuable parameters that can be applied for confirming the diagnosis of EGJOO.

Research perspectives

Further investigations are required to reveal the pathophysiological mechanism of the abnormal proximal esophageal motility showed in EGJOO patients, and larger cohorts are required to explore the prognostic value of the parameters mentioned above.

FOOTNOTES

Author contributions: Li YY and Lu WT contributed equally to this work; Li YY, Lu WT, and Jiao HM conception and designed of research; Li YY and Lu WT analyzed data; Lu WT and Chen M performed the HRM and provided clinical information; Li YY, Liu JX, and Jiao HM interpreted of research; Li YY drafted manuscript; Jiao HM revised manuscript and approved the final version of manuscript; all authors approved the final version of the article.

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Ornella Sari Cassol, Gilmara Pandolfo Zabot, Rogerio Saad-Hossne, Alexandre Padoin

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Abstract

BACKGROUND

This is the first study on the epidemiology of inflammatory bowel diseases (IBDs) in Rio Grande do Sul (RS), the southernmost state of Brazil with the country's fifth largest population. Crohn's disease (CD) and ulcerative colitis (UC) are collectively termed IBDs. They have high incidence and prevalence rates in high-income countries, although in recent years there has been a change in the classic geographical distribution of IBDs, with growing rates in traditionally low-incidence regions.

AIM

To estimate the incidence and prevalence of IBDs in the RS state, Brazil, between 2014 and 2019.

METHODS

This is a cross-sectional descriptive observational study. Patients with IBD who had initiated treatment and met the inclusion criteria of the RS state free drug distribution program were included. Data were obtained from registration or renewal records of the RS state specialty pharmacy. The male, female, and total

populations were estimated according to mid-year data from the Brazilian Institute of Geography and Statistics, which served as a reference for calculating the incidence and prevalence rates of IBDs during the study period. Results were described using mean, standard deviation, and range.

RESULTS

We included 1082 patients with IBD, of whom 57.5% were female and 42.5% were male. Patients with CD accounted for 72.45% of the sample, and those with UC accounted for 27.54%. IBD prevalence during the study period was 9.51 per 100000 population, of which 6.89 corresponded to people with CD and 2.62, to people with UC. Incidence rates per 100000 population/year were 2.54 in 2014, 2.61 in 2015, 1.91 in 2016, 0.80 in 2017, 0.83 in 2018, and 0.96 in 2019. The mean IBD incidence rate per 100000 population was 1.61, of which 1.17 corresponded to CD and 0.44, to UC. The mean age was 41 years, and patients were mostly aged 30-40 years. Prevalence by region was higher in the state capital metropolitan area: 12.69 per 100000 population.

CONCLUSION

Our results demonstrated an IBD prevalence of 9.51% and incidence of 1.61 per 100000 population. The patients were predominantly female, and CD was more prevalent than UC.

Key Words: Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Epidemiology; Incidence; Prevalence

Core Tip: This is the first study in the state of Rio Grande do Sul to address the epidemiology of inflammatory bowel diseases (IBDs). We assessed the incidence and prevalence of IBDs between 2014 and 2019, including 1082 patients (57.5% female and 42.5% male). Crohn's disease corresponded to 72.45% of the analyzed cases and ulcerative colitis, to 27.54%. Prevalence was 9.51% and the mean incidence rate was 1.61 per 100000 population. The mean age was 41 years, and the capital metropolitan area had the highest prevalence. This study showed a similar prevalence to other studies and a decrease in annual incidence rates.

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are characterized by a chronic inflammatory process that compromises the digestive tract. The main presentations are Crohn's disease (CD) and ulcerative colitis (UC), which are chronic idiopathic diseases causing inflammation of the gastrointestinal tract, with individual clinical and pathophysiological characteristics[1-3]. Children and young adults are more commonly affected[4]. Although uncommon, the chronicity, severity, progression, and morbidity of IBDs can significantly affect the patient's quality of life and are associated with an increased risk of hospitalizations and surgery[3,4].

IBDs are contemporary health conditions of industrialized societies. The prevalence of IBDs continues to increase steadily in Western countries, and their incidence is growing in newly industrialized countries. The global spread of IBDs seems to be associated with the Westernization of human diets and environments, affecting the gut microbiota and increasing the risk of IBDs in genetically susceptible individuals. It is therefore important to deepen our understanding of these events to delay the progression of IBDs[5].

Despite the paucity of population-based studies from low- and middle-income countries, epidemiological studies of IBDs have shown increased incidence and prevalence of CD and UC in different parts of the world. The increasing epidemiology of IBDs in newly industrialized countries in Latin America resembles that of high-income countries. Although a stabilization of incidence rates has been observed in many Western countries, the global burden of IBDs is still high, with an estimated prevalence exceeding 0.3% of the population in North America and Europe and 0.7% in Canada in 2018[6].

In newly industrialized countries, obstacles to conducting epidemiological studies of IBDs include the lack of disease surveillance systems and reliable unified health care databases, which are available in high-income countries. In countries of continental dimensions with economic problems such as Brazil,

an additional challenge is the country's generally disorganized health care system, which can result in inadequate records and, consequently, few population-based studies[6]. In this respect, Rio Grande do Sul (RS), the southernmost state of Brazil with the country's fifth largest population, still does not have a specific study on the epidemiology of IBDs. Therefore, the purpose of this study was to estimate the incidence and prevalence of IBDs in the state of RS.

MATERIALS AND METHODS

Study design

This is a cross-sectional descriptive observational study. All patients who had initiated treatment for IBD and met the inclusion criteria for the free drug distribution program of the state of RS between 2014 and 2019 were included. Drugs for the treatment of CD and UC are dispensed by the specialty pharmacy [*Farmácia de Medicamentos Especializados* (FME), or FME for short, in Portuguese] according to clinical protocols and therapeutic guidelines published by the Brazilian Ministry of Health. In Brazil, access to care is provided through private health insurance or the Unified Health System, which is a universal health care system funded by federal taxes and operated by state or municipal governments that includes the public provision of core physician and hospital services without copayments or patient charges. Approximately 22% of the population in the state of RS have private health insurance and only occasionally use the public health system. Therefore, because the FME is a government program within the Brazilian Unified Health System, it is responsible for supplying specialty medications to approximately 78% of the state population, all of them users of the public health system. Data from private health insurance companies are not included in this study.

Demographic characteristics and incidence and prevalence data were obtained from registration or renewal records of the FME. We requested from the State Health Department and its affiliated School of Public Health the data of patients included in the specialty medication distribution programs of the FME, which were provided through administrative and judicial means[7].

As variables to be analyzed, we included CD or UC diagnosis using International Classification of Diseases codes K50 and K51, date of treatment initiation, sex, and age.

Data analysis

Quantitative variables, such as age, were described using mean, standard deviation, and range. Categorical variables were expressed as counts and percentages. Disease prevalence and incidence were estimated by dividing the annual case notifications by the total estimated population of that year and presented as cases per 100000 population. Binomial distribution was used for obtaining 95% confidence intervals. All comparisons between rates assumed the binomial distribution and were based on the chi-square test. Findings with $P \leq 0.05$ were deemed statistically significant. All estimates of incidence and prevalence were calculated considering the population of the state of RS between 2014 and 2019[8]. Data analysis was conducted using IBM-SPSS, version 25.0.

Ethical considerations

This study did not involve the collection of biological material from participants. Data obtained with the data collection instrument were coded to preserve the participants' privacy and anonymity. All investigators signed a data use agreement.

The study was conducted after approval by the Research Ethics Committees of Pontifícia Universidade Católica do Rio Grande do Sul, Certificate of Presentation for Ethical Appreciation No. 25551019 9 0000 5336, and of the State Health Department and its affiliated School of Public Health, Certificate of Presentation for Ethical Appreciation No. 25551019 9 3001 5312. The study followed the guidelines of Resolution No. 466/12 of the Brazilian National Health Council and the Brazilian General Data Protection Law No. 13709.

RESULTS

This study involved 1082 patients with IBD of a total population of 11377239 people. Of these, 784 (72.45%) had CD and 298 (27.54%) had UC.

Age

Patient age ranged from 1 to 91 years, with a mean of 41 years and standard deviation of 15 years. The highest IBD incidence was between 20 and 60 years of age, and patients were mostly aged 30-40 years (Figure 1A). The highest incidence occurred between the ages of 30 and 40 years for CD, and between the ages of 50 and 60 years for UC. UC affected a higher percentage of people aged 40 years or more ($P < 0.01$), whereas CD affected a higher percentage of people under 40 years of age ($P < 0.01$) (Figure 1B).

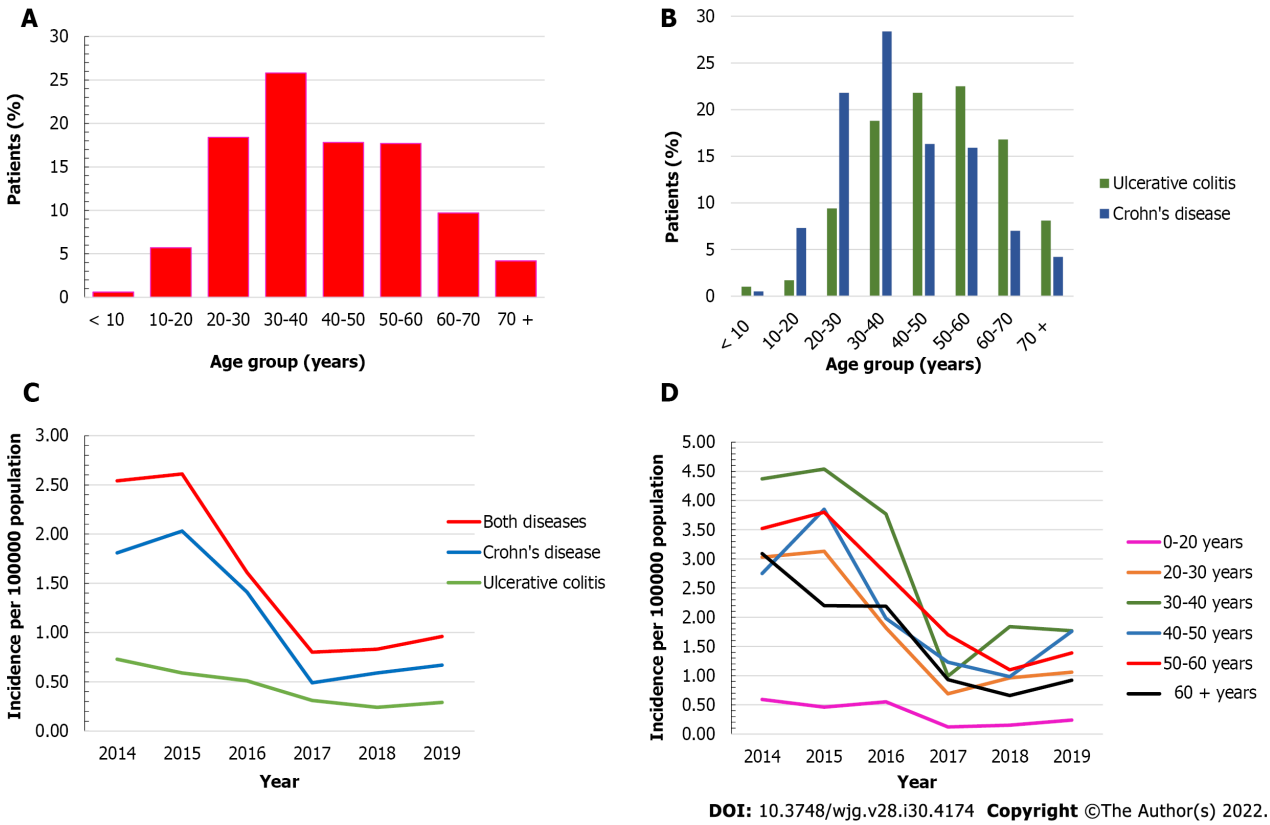

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Figure 1 Age distribution and incidence rates of patients with inflammatory bowel diseases. A: Age distribution of patients with inflammatory bowel diseases (IBD) (n = 1082), state of Rio Grande do Sul, Brazil, 2014-2019; B: Age distribution of patients with Crohn's disease (n = 784) and ulcerative colitis (n = 298), state of Rio Grande do Sul, Brazil, 2014-2019; C: Annual IBD incidence rates, new cases per 100000 population/year in the state of Rio Grande do Sul (Brazil), 2014-2019; D: Annual IBD incidence rates by age group, new cases per 100000 population/year in the state of Rio Grande do Sul (Brazil), 2014-2019.

Sex

Of all patients with IBD, 622 (57.5%) were female and 460 (42.5%) were male (female-to-male ratio of 1.35:1.00; $P < 0.001$).

Considering CD only, 432 (55.10%) patients were female and 352 (44.89%) were male (female-to-male ratio of 1.23:1.00; $P < 0.001$). For UC, 190 (63.75%) were female and 108 (36.25%) were male (female-to-male ratio of 1.76:1.00; $P < 0.001$).

Incidence and prevalence

Overall, IBD prevalence was 9.51 per 100000 population during the study period (2014-2019), of which 6.89 corresponded to CD and 2.62, to UC. Table 1 shows the sex distribution of prevalence for each disease.

Figure 1C shows the annual incidence rates of IBD in the state of RS. The mean incidence rate was 1.61, of which 1.17 corresponded to CD and 0.44, to UC.

Table 2 shows annual incidence percentages. There was a 62% reduction in the accumulated incidence between 2014 and 2017 ($P < 0.015$). A statistically significant reduction in incidence was observed when comparing 2014 and 2019 ($P < 0.001$).

Figure 1D shows that, when listing annual incidence rates by age group, all groups showed reductions in 2017, with increases in the following years.

DISCUSSION

Historical data on the geographical distribution of IBDs worldwide have shown higher incidence and prevalence rates in high-income countries with predominantly White populations. More recently, IBDs have shown an increasing frequency in all continents, including low- and middle-income countries[2].

In Brazil, IBDs are not notifiable diseases. Therefore, data on the incidence and prevalence of CD and UC are scarce, and health systems lack adequate records. These data would be useful for better organization and political and economic planning of the public health system[9,6].

Table 1 Prevalence of inflammatory bowel diseases in the state of Rio Grande do Sul between 2014 and 2019 (per 100000 habitants)

Item	CD	UC	IBD
Female	7.40	3.26	10.66
Male	6.35	1.95	8.30
Total	6.89	2.62	9.51

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

Table 2 Annual percentages of incidence rates

Year	Percentage
2014-2015	↑ 3%
2014-2015	↓ 26%
2014-2015	↓ 58%
2014-2015	↑ 3%
2014-2015	↑ 15%

In North America, 6.30 to 23.82 new cases per 100000 population are estimated per year for CD, and 8.8 to 23.14 new cases per 100000 population for UC. Prevalence rates are far higher than those in Brazil, with 96.3 to 318.5 cases/100000 population for CD and 139.8 to 286.3 cases/100000 population for UC[1,10].

A cohort study published in the United Kingdom reported incidence and prevalence rates much higher than those in Brazil between 2000 and 2018. The study estimated incidence rates of 28.6, 10.2, and 15.7/100000 population for IBD, CD, and UC, respectively, and prevalence rates of 725, 276, and 397/100000 population[11].

In the state of RS, we observed a predominance of CD during the study period (CD 72.54% and UC 27.54%), which differs from previous studies published in Brazil reporting a predominance of UC[2,3,12,13]. This is consistent with a study conducted in the Southeast region of Brazil by Souza *et al*[14], who reported an increase in CD cases in comparison with UC. These differences between countries and regions may reflect differences in environmental risk factors and genetic predispositions, such as those observed in Europe, where the incidence of NOD2 mutations appears to be higher in the central part of the continent, corresponding to areas with a higher proportion of CD cases[15].

According to the present study, the state of RS had a mean annual IBD incidence rate of 1.61 new cases per 100000 population/year (CD = 1.17 and UC = 0.44 new cases per 100000 population/year) between 2014 and 2019, which is consistent with the results reported by Kaplan *et al*[5] in a systematic review considering 147 studies on IBD worldwide. This comprehensive review demonstrated a CD incidence of 0-3.5 new cases per 100000 population/year, and an UC incidence of 0.19-6.76 new cases per 100000 population/year in Brazil[1]. In comparison, the systematic review by Selvaratnam *et al*[16] on the epidemiology of IBD in South America showed higher incidence rates, ranging from 4.3 to 5.3 per 100000 population/year for UC and from 0.74 to 3.5 per 100000 population/year for CD between 1990 and 2018. Similarly, a Brazilian study conducted in the state of Sao Paulo reported higher incidence rates than those found in the state of RS, with rates of 6.14 for CD and 7.16 for UC (per 100000 population/year)[3].

Regarding IBD prevalence, the mean rate observed during the study period (2014-2019) was 9.51 cases per 100000 population, of which 6.89 corresponded to CD and 2.62, to UC. Prevalence in the state of RS was higher than that reported for Brazil in a systematic review by Ng *et al*[1], with rates ranging from 0.9 to 6.75 cases/100000 population for CD and from 2.42 to 21 cases/100000 population for UC. Prevalence was also higher in a systematic review on IBD epidemiology conducted in South America, reaching 15-24.1/100000 population for UC and 2.4-14.1/100000 population for CD[16]. Similarly, in the Brazilian study by Gasparini *et al*[3], overall prevalence rates were 24.3 for CD and 28.3 for UC per 100000 population.

If we were to analyze the state of RS according to the epidemiological transition theory proposed by Gilaad G Kaplan and Joseph W Windsor in 2021, where each region of the world is at an epidemiological stage (Emergence, Acceleration in Incidence, Compounding Prevalence, and Prevalence Equilibrium), RS would be in the Compounding Prevalence stage, where a steady increase in the population living with IBD is observed despite stabilization or even a decrease in incidence[17].

Regarding patient age, studies show a clear predominance of IBD in individuals aged 20 to 50 years. In our study, the mean age was 41 years. The highest IBD incidence was between 20 and 60 years of age, and patients were mostly aged 30-40 years. The highest incidence was at 30-40 years of age for CD, and at 50-60 years of age for UC. UC showed a trend toward an increased incidence among patients aged 50 to 60 years, and these results are consistent with those of national and international studies[3,11]. A limitation of this variable is that patients were selected through the FME, which provides the age that the patients begin to receive the free specialty medications rather than their age at diagnosis.

Regarding the sex distribution of patients with IBD, we observed a predominance of female patients for both CD and UC, which is consistent with the Brazilian studies conducted by Victoria *et al*[12] in the state of Sao Paulo and Lima Martins *et al*[13] in the state of Espírito Santo. However, our findings differ from those reported in the state of Piauí by Parente *et al*[2], who found a male-to-female ratio of 1.2:1.0 for patients with CD, but with no significant association when considering sex in statistical analyses. Patients with UC, on the other hand, were mostly female (male-to-female ratio of 1.8:1.0, statistically significant)[2].

CONCLUSION

Based on the methodology used in this study, our results demonstrated an IBD prevalence of 9.51% and incidence of 1.61 per 100000 population in the state of RS between 2014 and 2019. The patients were predominantly female, and CD was more prevalent than UC. Our prevalence rate was similar to that reported in previous Brazilian studies. Mean CD and UC incidence rates were, respectively, 1.17 and 0.44 new cases per 100000 population, decreasing until 2017 and increasing afterwards. Women were more affected than men by both CD and UC. IBD occurred more frequently in major urban centers, where the referral centers that care for patients with IBD are often located. The highest IBD incidence was observed in patients aged 20 to 60 years, predominantly between the ages of 30 and 40 years; this was slightly higher than the mean values reported in previous studies.

This is the first study to estimate IBD incidence and prevalence in the state of RS. Our findings suggest that detailed studies in this field are needed to properly understand in which epidemiological stage the state of RS currently is. We believe that the difficulty in keeping accurate epidemiological records and the consequent underestimation of disease burden result from a disorganized health care system, associated with economic problems, inadequate records, lack of population-based studies, and the inaccurate diagnosis of IBD as an infectious disease before the proper knowledge of CD and UC.

ARTICLE HIGHLIGHTS

Research background

First study on the epidemiology of inflammatory bowel diseases (IBDs) in Rio Grande do Sul (RS), the southernmost state of Brazil. IBDs have high incidence and prevalence rates in high-income countries, although in recent years there has been a change in the classic geographical distribution of IBDs, with growing rates in traditionally low-incidence regions.

Research motivation

The importance of this study lies in the lack of research on the incidence and prevalence of IBDs in the state of RS. A challenge for future studies is related to access to data of all patients with IBDs, as these are not notifiable diseases in Brazil.

Research objectives

To determine the incidence and prevalence of IBD in the state of RS. The relevance of the results lies in the lack of knowledge of such data until the present study.

Research methods

This is an observational study of IBDs from the state of RS between 2014 and 2019. Drugs for the treatment of Crohn's disease (CD) and ulcerative colitis (UC) are dispensed by the specialty pharmacy. The variables analyzed were incidence, prevalence, age, sex, and CD or UC.

Research results

These are the first data on the incidence and prevalence of IBDs in the state of RS. However, these are not comprehensive data, as only patients from the public health system were included. Studies also including patients from the private health insurance sector are needed.

Research conclusions

The most relevant of the following questions should be briefly answered: What are the new theories that this study proposes? This study showed a similar IBD prevalence to other Brazilian studies and a decreasing incidence. What are the new methods that this study proposed? We propose that studies covering patients from both public and private health sectors should be conducted.

Research perspectives

Future studies should include a larger sample to provide a more reliable understanding of the real epidemiology of IBDs in the state of RS.

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FOOTNOTES

Author contributions: Cassol OS contributed to data collection, investigation, writing-original draft, and statistical analysis; Zabot GP, Saad-Hossne R, and Padoin A contributed to writing-review and editing.

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Institutional review board statement: This study was performed after approval by the Research Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul, Certificate of Presentation for Ethical Appreciation No. 25551019 9 0000 5336; and the ESP/SES/RS Research Ethics Committee, Certificate of Presentation for Ethical Appreciation 25551019 9 3001 5312; this work respected the guidelines of Resolution No. 466/12 of the National Health Council and Law No. 13709 of the General Personal Data Protection Law.

Informed consent statement: This study did not involve the collection of biological material from participants. Data obtained with the data collection instrument were codified, aiming to preserve the participants' privacy and anonymity. A data use agreement form was signed.

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ORIGINAL ARTICLE

Observational Study

Hepatocellular carcinoma, decompensation, and mortality based on hepatitis C treatment: A prospective cohort study

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Abstract

BACKGROUND

Prospective studies of the long-term outcomes of patients with hepatitis C virus (HCV) infection after treatment with interferon-based therapy (IBT) or direct-acting antivirals (DAA) are limited in many Asian countries.

AIM

To elucidate the incidences of hepatocellular carcinoma (HCC) and

death/transplantation based on treatment with IBT or DAA, to compare the outcomes of the sustained virologic response (SVR) to IBT and DAA, and to investigate outcome-determining factors after SVR.

METHODS

This cohort included 2054 viremic patients (mean age, 57 years; 46.5% male; 27.4% with cirrhosis) prospectively enrolled at seven hospitals between 2007 and 2019. They were classified as the untreated group (n = 619), IBT group (n = 578), and DAA group (n = 857). Outcomes included the incidences of HCC and death/transplantation. The incidences of the outcomes for each group according to treatment were calculated using an exact method based on the Poisson distribution. A multivariate Cox regression analysis was performed to determine the factors associated with HCC or death/transplantation, followed by propensity score matching to confirm the results.

RESULTS

During a median of 4.1 years of follow-up, HCC and death/transplantation occurred in 113 and 206 patients, respectively, in the entire cohort. Compared with the untreated group, the incidences of HCC and death/transplantation were significantly lower in the IBT group [adjusted hazard ratio (aHR) 0.47, 95%CI: 0.28-0.80 and aHR 0.28, 95%CI: 0.18-0.43, respectively] and the DAA group (aHR 0.58, 95%CI: 0.35-0.96, and aHR 0.19, 95%CI: 0.20-0.68, respectively). Among 1268 patients who attained SVR with IBT (n = 451) or DAA (n = 816), the multivariable-adjusted analysis showed no differences in the risks of HCC (HR 2.03; 95%CI: 0.76-5.43) and death/transplantation (HR 1.38; 95%CI: 0.55-3.49) between the two groups. This was confirmed by a propensity score-matching analysis. Independent factors for HCC after SVR were age, genotype 1, and the presence of cirrhosis.

CONCLUSION

Treatment and achieving SVR with either IBT or DAA significantly reduced the incidences of HCC and mortality in the Asian patients with HCV infection. The risks of HCC and mortality were not significantly different regardless of whether SVR was induced by IBT or DAA.

Key Words: Hepatitis C virus; Direct-acting antiviral; Sustained virologic response; Hepatocellular carcinoma; Mortality

Core Tip: Treatment and sustained virologic response (SVR) with either interferon-based treatment (IBT) or direct-acting antiviral (DAA) significantly reduced the incidences of hepatocellular carcinoma and mortality in our Asian prospective cohort. The risks of HCC and all-cause of mortality were not significantly different regardless of whether SVR was induced by IBT or DAA. After achieving SVR, age, the presence of cirrhosis, and genotype 1 hepatitis C virus infection were indicators of worse clinical outcomes.

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver cirrhosis, hepatocellular carcinoma (HCC), and liver-related and overall mortality. In 2019, the World Health Organization estimated that 58 million people worldwide had chronic HCV infection[1]. Currently, this substantial public health burden can be reduced by active screening and treatment with highly tolerable direct-acting antivirals (DAA), which result in a cure rate of more than 95% in terms of the sustained virologic response (SVR)[2-4].

From the identification of HCV to the introduction of DAA therapy, interferon (IFN)-based therapy (IBT) was the only option for HCV treatment, with an approximate SVR rate of 50%[5]. Although IBT-induced SVR includes a wide variety of adverse effects and narrow indications, it significantly reduces the incidence of HCC and long-term mortality[6,7]. Furthermore, DAA-induced SVR has resulted in

reductions in hepatic fibrosis[8,9], portal hypertension[10], hepatic decompensation[11], HCC incidence [11,12], and liver-related and overall mortality[11,13]; however, the follow-up durations were relatively short.

To reach the HCV elimination goal of 2030, it is imperative to understand the regional epidemiology and outcomes of HCV. However, prospective studies of the long-term outcomes after treatment with IBT or DAA are limited in many Asian countries, where hepatitis B virus (HBV) is the major cause of liver-related complications. Additionally, comparative studies of the outcomes of SVR induced by IBT and DAA are scarce.

We established a prospective, nationwide, multicenter HCV cohort (the Korea HCV cohort study) funded by the Korean National Institute of Health in 2007. Using these data, we aimed to elucidate the clinical outcomes, including HCC, hepatic decompensation, and all-cause death, among Korean patients with chronic HCV infection. We compared the outcomes based on the antiviral treatments (untreated, IBT, and DAA) and analyzed patients after achieving SVR (IBT-SVR and DAA-SVR groups). Additionally, we investigated outcome-determining factors after SVR.

MATERIALS AND METHODS

Study subjects

The Korea HCV cohort is a prospective cohort of 2485 adult patients with HCV RNA positivity at seven tertiary centers nationwide enrolled from January 2007 to June 2019 in South Korea. Patients who met any of the following criteria were excluded: Positive serology for HBV surface antigen (n = 62) or HIV (n = 2); decompensated cirrhosis at enrollment (n = 79); previous antiviral treatment before cohort entry (n = 260); and less than 6 mo of follow-up (n = 28). Additionally, patients who had HCC before or at the time of cohort entry were excluded.

Therefore, 2054 viremic patients with or without compensated cirrhosis were analyzed as the entire cohort, which was further classified into three groups based on their treatment: untreated group (n = 619; 30.2%); IBT group (n = 578; 28.1%); and DAA group (n = 857; 41.7%). Patients in the untreated group did not receive IBT or DAA treatment during the entire follow-up period. Subject selection, classification, and overall outcomes are summarized in Figure 1.

In the entire cohort, 1267 patients achieved SVR (61.7%) with IBT (n = 451) or DAA (n = 816); these patients comprised the SVR cohort. Diagnostic criteria for liver cirrhosis were based on histology or at least one clinical sign of portal hypertension, such as cirrhotic features on radiological images, platelet count less than 100,000/mm^3, and documented gastroesophageal varices without hemorrhage. The study protocol was approved by the Institutional Review Board of seven hospitals, and each enrolled patient provided written informed consent.

Data collection at baseline and follow-up visits

At the time of enrollment, trained research coordinators at the seven hospitals interviewed the patients using a standardized questionnaire that included demographic and socioeconomic factors (age, sex, body mass index, education level, and occupation), health behaviors (smoking and alcohol consumption), comorbidities (extrahepatic cancers, thyroid disease, psychiatric disease, diabetes, kidney disease, cerebrovascular disease, and cardiovascular disease), and lifetime exposure to risk factors for HCV infection.

Laboratory data at baseline and follow-up visits were collected from the medical records, including the anti-HCV antibody, serum HCV RNA level, HCV genotype, HBV surface antigen, HBV core antibody immunoglobulin G (anti-HBc IgG), white blood cell count, hemoglobin, platelet count, aspartate aminotransferasel (AST), alanine aminotransferase, alkaline phosphatase, total bilirubin, albumin, creatinine, prothrombin time (PT) and alpha-fetoprotein (AFP). The following three non-invasive serum fibrosis assessment scores were calculated at the index date: The fibrosis-4 (FIB-4) index [14], the AST-to-platelet ratio index (APRI) score[15], and the albumin-bilirubin (ALBI) score[16].

The results of imaging studies, such as abdominal ultrasonography or computed tomography, liver pathology, and transient elastography (FibroScan®, Echosens, Paris, France), were collected when available. Detailed information about antiviral treatment, including therapeutic regimens, duration, and achievement of SVR, was collected from the patients' medical records. These data were entered into the established electronic case report form on the authorized website of the Korean Centers for Disease Control Korea HCV cohort study (http://is.cdc.go.kr/) by the research coordinators. All input data were quality-controlled by independent statistical researchers (Baik D, Choi HY, and Ki M) at least four times per year.

Follow-up evaluations

All patients underwent regular clinical assessments every 3 to 12 mo, and HCV treatment was recommended by their attending physicians according to the treatment guidelines for HCV infection unless there were contraindications or patient's refusal. If the patients were treated, then SVR was evaluated, and regular follow-up visits every 6 to 12 mo after SVR were encouraged. HCC surveillance

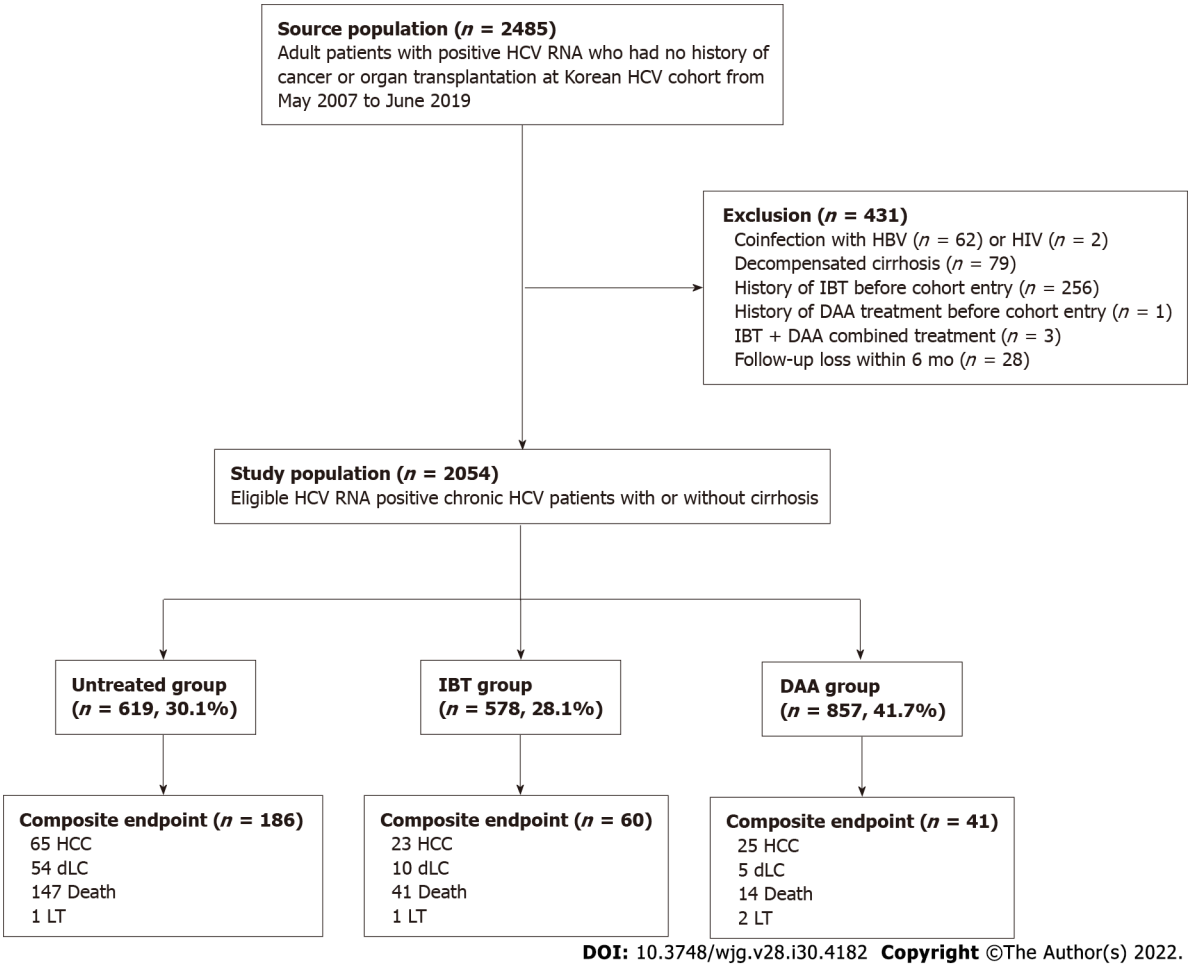

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Figure 1 Patient flow diagram. DAA: Direct-acting antivirals; dLC: Decompensated liver cirrhosis; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; IBT: Interferon-based treatment; HIV: Human immunodeficiency virus; LT: Liver transplantation.

using abdominal ultrasonography and serum AFP tests every 6 to 12 mo were recommended according to the pretreatment fibrosis stage. If the patients did not adhere to the regular follow-up schedule, then research coordinators called the patients or their families to encourage them to attend the clinic and to check their survival status, disease progression to hepatic decompensation, or development of HCC. The end of follow-up was defined as the date of death/liver transplantation (LT), or the last follow-up date (June 30, 2020).

Measurement of liver-related outcomes

Outcomes included the incidences of HCC, hepatic decompensation, and death/LT. HCC was diagnosed according to pathology or typical imaging criteria observed on dynamic computed tomography and/or magnetic resonance imaging in accordance with the Korean Liver Cancer Study Group guidelines (similar to major international guidelines)[17]. Decompensated liver cirrhosis was defined as the presence of ascites, jaundice, variceal bleeding, encephalopathy, or a combination of these [18]. All-cause mortality or death/LT was directly documented or indirectly indicated as disqualification from the National Health Insurance status provided in the electronic medical records. In Korea, enrollment in the National Health Insurance is compulsory for all individuals; therefore, disqualification from the National Health Insurance indicates death or emigration in most cases[19]. To verify the survival status, physician-confirmed death certificate data, including the date and cause of death, were obtained from the Statistics Korea mortality database, which was established in 1981.

To calculate the outcomes of the entire cohort, the index date of the entire cohort was defined as the date of cohort entry when HCV RNA positivity was confirmed by the referred clinics. To compare the outcomes of patients with SVR induced by IBT and patients with SVR induced by DAA, the index date for the SVR cohort was defined as the initiation day of the antiviral treatment. SVR was evaluated using an intention-to-treat analysis; therefore, patients who received at least one dose of IBT or DAA were included.

Statistical analysis

Baseline characteristics of the patients were compared using the chi-square test for categorical variables, and a one-way analysis of variance (ANOVA) or *t*-test was used for continuous variables. For multiple comparisons, a one-way ANOVA was used followed by a Bonferroni correction. The total follow-up (in person-years) of each group was calculated by multiplying the cohort population size by the average follow-up in years. Survival time was calculated as the time from cohort entry (the entire cohort) or the start of the first treatment (SVR cohort) until the date of death/LT or the last available follow-up date. A few patients who received a second course of DAA treatment because of failure of the first DAA treatment were censored at the time of retreatment.

The incidences and 95% confidence intervals (CI) of the outcomes for each group based on treatment were calculated using an exact method based on the Poisson distribution. Cumulative incidence curves for outcome development were estimated using the pseudo-Kaplan–Meier method with a clock reset procedure for patients treated with IBT or DAA during follow-up and compared using the log-rank test. Therefore, patients who had received IBT and subsequent DAA treatment were considered as the DAA group, but the period between IBT and DAA treatment was included in the IBT group with no event.

A time-varying Cox regression model was used to determine the factors associated with outcomes, and the adjusted hazard ratio was estimated for the entire cohort and the SVR cohort. In the models, baseline variables (sex, body mass index, alcohol, smoking, HCV genotype) were adjusted, and age, antiviral treatment, laboratory data, achievement of SVR, and presence of cirrhosis were considered time-dependent variables. A multivariate Cox regression analysis was performed to determine the factors associated with HCC or death/LT, and the adjusted hazard ratio was estimated for the SVR cohort at the time of IBT or DAA initiation. Covariates with $P < 0.05$ in the univariate Cox regression model were used as covariates for the multivariate Cox regression analyses. To confirm the multivariate analysis results for the SVR cohort, significant differences in characteristics at the time of initiation of each treatment were adjusted by propensity score (PS) matching for all possible variables, including the time from cohort entry to treatment. We used nearest-neighbor matching with a caliper size of 0.1 and matched the patients using a 1:1 ratio. The covariate balance was considered to be achieved if the absolute standardized difference between the two groups was ≤ 0.1.

All *P*-values were two-sided, and $P < 0.05$ was considered significant. SPSS (version 21; IBC Corp., Armonk, NY, United States) and R (version 4.0.4; http://cran.r-project.org/) software were used for statistical analyses. The R package of *MatchIt* was used for matching analyses.

RESULTS

Baseline characteristics of the entire cohort

The demographic, clinical, and laboratory characteristics of the entire cohort ($n = 2054$), untreated group ($n = 619$; 30.1%), IBT group ($n = 578$; 28.1%), and DAA group ($n = 857$; 41.7%) at the index date are provided in Table 1. The mean age of the patients was 57 years; 46.5% were men and 27.4% had compensated cirrhosis.

Compared with the untreated and DAA groups, the IBT group was significantly younger, had a higher proportion of genotype 2, lower rates of alcohol consumption, cirrhosis and high FIB-4 index values (≥ 3.25), and fewer comorbidities.

Among the DAA group, 14.0% had been treated with an IFN-based regimen after cohort enrollment, and 86.0% were treatment-naive. DAA treatments administered were sofosbuvir plus ribavirin (32.1%), daclatasvir plus asunaprevir (26.3%), elbasvir plus grazoprevir (13.7%), glecaprevir plus pibrentasvir (13.7%), ledipasvir plus sofosbuvir (9.1%), and ombitasvir plus paritaprevir plus ritonavir plus dasabuvir (3.9%).

Incidences of HCC, decompensation, and death/LT in the entire cohort according to treatment, SVR, or the presence of liver cirrhosis

The median time periods between cohort entry (index date) and the end of follow-up were 5.6 years [interquartile range (IQR), 3.4-8.2] for the untreated group, 7.9 years (IQR, 6.1-9.7) for the IBT group, and 3.5 years (IQR, 2.0-5.5) for the DAA group (Table 1). During this period, 113 patients developed HCC, 69 patients experienced hepatic decompensation, 202 patients died (119 Liver related and 83 non-liver related), and four patients underwent LT.

As shown in Table 2, the estimated HCC incidence rates per 100 person-years for the untreated group, IBT group, and DAA group were 1.98 (95%CI: 1.56-2.52), 0.59 (95%CI: 0.39-0.89), and 1.16 (95%CI: 0.78-1.71), respectively (Table 2). The incidence rates of hepatic decompensation per 100 person-years for the untreated group, IBT group, and DAA group were 1.62 (95%CI: 1.24-2.11), 0.25 (95%CI: 0.14-0.47), and 0.23 (95%CI: 0.10-0.55), respectively. The incidence rates of death/LT per 100 person-years for the untreated group, IBT group, and DAA group were 3.33 (95%CI: 2.85-3.91), 0.87 (95%CI: 0.64-1.18), and 0.65 (95%CI: 0.40-1.06), respectively. The incidence rates of all three outcomes were significantly lower in the IBT and DAA groups compared with the untreated group. However, the

Table 1 Baseline characteristics of the entire hepatitis C virus cohort according to the treatment

Variable, *n* (%)	Untreated group (*n* = 619)	IBT group (*n* = 578)	DAA group (*n* = 857)	*P* value (3 Gr.)	*P* value (U *vs* I)	*P* value (U *vs* D)	*P* value (I *vs* D)
Age (yr)	60.4 ± 13.4	51.4 ± 10.9	59.1±11.4	< 0.001	< 0.001	0.086	< 0.001
Male sex	280 (45.2)	277 (47.9)	412 (48.1)	0.511	0.351	0.281	0.955
HCV RNA, $\log_{10}$ IU/mL	5.89 (5.08-6.45)	5.83 (4.87-6.49)	6.04 (5.27-6.53)	< 0.001	0.979	0.017	< 0.001
HCV genotype				< 0.001	< 0.001	< 0.001	< 0.001
1	256 (41.4)	242 (41.9)	491 (57.3)				
2	299 (48.3)	323 (55.9)	355 (41.4)				
Others/missing	64 (10.3)	13 (2.2)	11 (1.3)				
Diagnosis status				< 0.001	< 0.001	0.002	0.058
Chronic hepatitis	417 (67.4)	456 (78.9)	639 (74.6)				
Compensated cirrhosis	202 (32.6)	122 (21.1)	218 (25.4)				
FIB-4 index				< 0.001	< 0.001	0.142	< 0.001
≤ 1.45	112 (18.9)	191 (33.3)	164 (19.9)				
1.45-3.25	218 (36.8)	200 (34.8)	337 (40.9)				
≥ 3.25	263 (44.3)	183 (31.9)	323 (39.2)				
APRI score				0.15	0.09	0.094	0.853
≤ 2.0	480 (80.3)	485 (84.1)	708 (83.7)				
> 2.0	118 (19.7)	92 (15.9)	138 (16.3)				
ALBI score				0.004	0.04	0.001	0.313
≤ -2.60 (Grade 1)	446 (73.0)	450 (78.1)	674 (80.3)				
> -2.60 (Grade 2 or 3)	165 (27.0)	126 (21.9)	165 (19.7)				
Ever smoker	264 (42.9)	283 (49.5)	380 (44.5)	0.058	0.022	0.519	0.068
Alcohol intake				< 0.001	< 0.001	< 0.001	< 0.001
None	326 (53.0)	226 (39.4)	419 (48.9)				
Social	227 (36.9)	308 (53.7)	229 (26.7)				
Significant[1]	61 (9.9)	40 (7.0)	209 (24.4)				
Fatty liver disease on imaging study, *n* = 1389	71 (19.3), *n* = 367	86 (21.1), *n* = 408	140 (22.8), *n* = 614	0.435	0.549	0.202	0.516
Anti-HBcIgG (+), *n* = 1442	47 (11.0), *n* = 429	33 (8.4), *n* = 391	64 (10.3), *n* = 622	0.46	0.225	0.73	0.33
BMI, kg/m^2	23.4 ± 3.2	23.8 ± 3.0	23.8 ± 3.1	0.02	0.143	0.02	1
Diabetes mellitus	120 (19.4)	77 (13.3)	128 (14.9)	0.01	0.005	0.024	0.391
Hypertension	145 (23.4)	107 (18.5)	243 (28.4)	< 0.001	0.037	0.034	< 0.001
Cardiovascular disease	16 (2.6)	3 (0.5)	25 (2.9)	0.006	0.004	0.701	0.001
Cerebrovascular disease	17 (2.7)	4 (0.7)	20 (2.3)	0.026	0.007	0.617	0.017
Laboratory study							
WBC, × 10^3/mm^3	5.09 (4.29-6.50)	5.05 (3.91-6.16)	5.40 (4.40-6.61)	< 0.001	0.145	0.164	< 0.001
Hemoglobin, g/dL	13.5 (12.3-14.5)	13.7 (12.7-14.8)	13.8 (12.7-14.8)	< 0.001	0.004	< 0.001	1
Platelet, × 10^3/mm^3	162 (118-212)	174 (130-220)	172 (129-215)	0.229	0.236	0.317	1
Albumin, g/dL	4.2 (3.9-4.4)	4.2 (4.0-4.5)	4.2 (4.0-4.4)	< 0.001	< 0.001	0.001	1
Total bilirubin, mg/dL	0.8 (0.5-1.0)	0.7 (0.6-1.0)	0.7 (0.6-1.0)	0.784	1	1	1
ALP, IU/L	87 (68-136)	79 (63-112)	121 (78-250)	< 0.001	0.001	< 0.001	< 0.001

AST, IU/L	50 (30-85)	50 (31-83)	46 (30-76)	0.026	1	0.139	0.038
ALT, IU/L	42 (23-80)	52 (29-100)	36 (22-70)	< 0.001	0.013	0.176	< 0.001
Creatinine, mg/dL	0.9 (0.7-1.0)	0.8 (0.7-1.0)	0.8 (0.7-1.0)	0.857	1	1	1
PT, INR	1.06 (1.01-1.12)	1.03 (0.98-1.08)	1.03 (0.97-1.09)	< 0.001	< 0.001	0.007	0.119
AFP, ng/dL	4.3 (2.5-9.7)	3.5 (2.3-7.1)	3.8 (2.4-6.6)	0.868	1	1	1
Cohort entry time				< 0.001	< 0.001	< 0.001	< 0.001
January 2007–June 2015	488 (78.8)	561 (97.1)	49 (5.7)				
July 2015–June 2019	131 (21.2)	17 (2.9)	808 (94.3)				
SVR rate	-	67.5 (451/668)	95.3 (817/857)	-	-	< 0.001	-
Follow-up duration (yr)	5.6 (3.4-8.2)	7.9 (6.1-9.7)	3.5 (2.0-5.5)	< 0.001	< 0.001	< 0.001	< 0.001

[1]Significant alcohol intake is defined as weekly ≥ 210 g for men and ≥ 140 g for women.

Data were presented as mean ± SD, median (interquartile range), or *n* (%). AFP: Alpha-feto protein; ALBI: Albumin-bilirubin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate aminotransferase to platelet ratio index; AST: Aspartate aminotransferase; BMI: Body mass index; DAA: Direct acting antivirals; FIB-4: Fibrosis-4; HCV: Hepatitis C virus; IBT: Interferon-based therapy; INR: International normalized ratio; PT: Prothrombin time; WBC: White blood cell.

incidence of HCC in the non-cirrhotic group was not significantly different between the untreated and DAA groups, while the incidences of decompensation and death/LT were significantly lower in the DAA group compared with the untreated group. The cumulative outcome incidences for the untreated, IBT, and DAA groups are shown in Figure 2, and those for the non-cirrhotic and cirrhotic subgroups are shown in Supplementary Figures 1 and 2, respectively.

Multivariable analyses of factors associated with HCC, decompensation, and death/LT in the entire cohort

A multivariable time-varying Cox regression analysis with the untreated group as a reference showed that IBT [hazard ratio (HR), 0.47; 95%CI: 0.28-0.80; $P = 0.005$] and DAA groups (HR, 0.58; 95%CI: 0.35-0.96; $P = 0.035$) were independently associated with a significantly lower risk of HCC (Table 3, model 1). Other independent HCC risk factors were older age (HR, 1.06; 95%CI: 1.03-1.08; $P < 0.001$), male sex (HR, 2.50; 95%CI: 1.37-4.55; $P = 0.003$), genotype 1 (HR, 2.25; 95%CI: 1.45-3.48; $P < 0.001$), the presence of cirrhosis (HR, 3.81; 95%CI: 2.38-6.10; $P < 0.001$), significant alcohol consumption (HR, 2.20; 95%CI: 1.14-4.24; $P = 0.027$), prolonged PT (HR, 2.66; 95%CI: 1.13-6.24; $P = 0.025$), and higher AFP level (HR, 2.12; 95%CI: 1.48-3.05; $P < 0.001$) (Table 3, model 1).

The death/LT incidence decreased independently after antiviral treatment with IBT (HR, 0.28; 95%CI: 0.19-0.43; $P < 0.001$) or DAA (HR, 0.19; 95%CI: 0.10-0.35; $P < 0.001$) (Table 3, model 1). In contrast, older age (HR, 1.05; 95%CI: 1.03-1.06; $P < 0.001$), male sex (HR, 1.70; 95%CI: 1.24-2.33; $P < 0.001$), the presence of cirrhosis (HR, 1.88; 95%CI: 1.34-2.64; $P < 0.001$), lower body mass index (HR, 0.94; 95%CI: 0.89-0.99; $P = 0.019$), lower albumin level (HR, 0.35; 95%CI: 0.25-0.51; $P < 0.001$), and prolonged PT (HR, 2.34; 95%CI: 1.06-5.16; $P = 0.034$) independently increased mortality/LT rates. Antiviral treatment, cirrhosis, albumin level, and PT were independently associated with the risk of decompensation (Table 3, model 1).

We established another multivariable model (model 2) by replacing the achievement of SVR instead of IBT or DAA treatment. A multivariate time-varying Cox regression analysis of the combined outcomes of the untreated and no SVR groups as a reference showed that SVR induced by either IBT or DAA significantly decreased the risk of HCC (HR, 0.41; 95%CI: 0.26-0.65; $P < 0.001$), decompensation (HR, 0.10; 95%CI: 0.04-0.29; $P < 0.001$), and death/LT (HR, 0.26; 95%CI: 0.17-0.39; $P < 0.001$) (Table 3, model 2).

Comparison of the clinical outcomes of the IBT-SVR group and DAA-SVR group in the SVR cohort: Unadjusted and PS-matched results

The SVR cohort comprised 1,268 chronic HCV patients who achieved SVR with IBT or DAA (IBT-SVR group, $n = 451$; DAA-SVR group, $n = 817$). The SVR rates of the IBT and DAA groups were 67.5% and 95.3%, respectively. The baseline characteristics at the time of initiation of treatment of the IBT and DAA groups according to SVR are shown in Supplementary Table 1. The incidence rates of HCC, decompensation, and death/LT were significantly lower in the IBT-SVR group than in the IBT-no SVR group (Supplementary Table 2). In contrast, the incidence rate of death/LT was significantly lower in the DAA-SVR group than in the DAA-no SVR group, whereas the incidence rates of HCC and decompensation did not reach statistical significance, probably because of the short follow-up or rare incidence of decompensation (Supplementary Table 2).

Table 2 Annual incidence rates of hepatocellular carcinoma, decompensation and death/transplantation according to treatment exposure and the presence of cirrhosis

	Group	PY	No. of events	No./100 PY (95%CI)	HR (95%CI)	*P* value
Entire cohort	Hepatocellular carcinoma					
	Untreated group	3285.4	65	1.98 (1.56-2.52)	Reference	-
	IBT group	3888.9	23	0.59 (0.39-0.89)	0.31 (0.19-0.49)	< 0.001
	DAA group	2158.0	25	1.16 (0.78-1.71)	0.59 (0.37-0.95)	0.029
	Decompensation					
	Untreated group	3333.6	54	1.62 (1.24-2.11)	Reference	-
	IBT group	3950.1	10	0.25 (0.14-0.47)	0.15 (0.07-0.29)	< 0.001
	DAA group	2178.7	5	0.23 (0.10-0.55)	0.16 (0.06-0.39)	< 0.001
	Death or transplantation					
	Untreated group	4438.3	148	3.33 (2.85-3.91)	Reference	-
	IBT group	4850.1	42	0.87 (0.64-1.18)	0.22 (0.16-0.31)	< 0.001
	DAA group	2467.2	16	0.65 (0.40-1.06)	0.30 (0.17-0.51)	< 0.001
No cirrhosis	Hepatocellular carcinoma					
	Untreated group	2364.6	15	0.63 (0.38-1.05)	Reference	-
	IBT group	2971.8	6	0.20 (0.09-0.45)	0.30 (0.11-0.77)	0.012
	DAA group	1569.4	8	0.51 (0.26-1.02)	0.90 (0.37-2.18)	0.812
	Decompensation					
	Untreated group	2369.9	6	0.25 (0.11-0.56)	Reference	-
	IBT group	2992.3	1	0.03 (0.00-0.24)	0.09 (0.01-0.74)	0.026
	DAA group	1573.4	0	0.00	0.00	N/A
	Death or transplantation					
	Untreated group	3176.3	63	1.98 (4.15-7.12)	Reference	-
	IBT group	3712.3	22	0.59 (0.39-0.90)	0.26 (0.16-0.42)	< 0.001
	DAA group	1784.6	7	0.39 (0.19-0.82)	0.33 (0.15-0.75)	0.008
Cirrhosis	Hepatocellular carcinoma					
	Untreated group	919.9	50	5.44 (4.15-7.12)	Reference	-
	IBT group	917.1	17	1.85 (1.16-2.97)	0.35 (0.20-0.61)	< 0.001
	DAA group	588.6	17	2.89 (1.81-4.61)	0.53 (0.30-0.92)	0.025
	Decompensation					
	Untreated group	962.8	48	4.99 (3.78-6.57)	Reference	-
	IBT group	957.7	9	0.84 (0.49-1.80)	0.18 (0.09-0.37)	< 0.001
	DAA group	606.3	5	0.82 (0.34-1.97)	0.17 (0.07-0.44)	< 0.001
	Death or transplantation					
	Untreated group	1262.1	85	6.73 (5.48-8.27)	Reference	-
	IBT group	1137.8	20	1.76 (1.14-2.71)	0.21 (0.13-0.35)	< 0.001
	DAA group	682.6	9	1.32 (0.69-2.52)	0.28 (0.14-0.56)	< 0.001

A Cox proportional hazards model was used to determine hazards ratios and *P* values. CI: Confidence interval; DAA: Direct acting antivirals; HR: Hazard ratio; IBT: Interferon-based therapy; N/A: Not applicable; PY: Person-year.

Table 3 Multivariate time-varying Cox regression analysis for hepatocellular carcinoma and death/transplantation in entire cohort

Variable	Hepatocellular carcinoma		Decompensation		Death/transplantation	
	aHR (95%CI)	*P* value	aHR (95%CI)	*P* value	aHR (95%CI)	*P* value
Model 1						
Untreated group	Reference	-	Reference	-	Reference	-
IBT group	0.47 (0.28-0.80)	0.005	0.16 (0.08-0.33)	< 0.001	0.28 (0.18-0.43)	< 0.001
DAA group	0.58 (0.35-0.96)	0.035	0.12 (0.03-0.33)	< 0.001	0.19 (0.10-0.35)	< 0.001
Age, yr	1.06 (1.03-1.08)	< 0.001	1.00 (0.98-1.03)	0.805	1.05 (1.03-1.06)	< 0.001
Male sex	2.50 (1.37-4.55)	0.003	-	-	1.70 (1.24-2.33)	< 0.001
HCV genotype			-	-	-	-
2	Reference	-				
1	2.25 (1.45-3.48)	< 0.001				
Others/unknown	1.72 (0.66-4.48)	0.266				
Cirrhosis	3.81 (2.38-6.10)	< 0.001	9.26 (4.03-21.03)	< 0.001	1.88 (1.34-2.64)	< 0.001
Ever smoker	1.47 (0.82-2.66)	0.192	-	-	-	-
Alcohol consumption			-	-	-	-
None	Reference	-				
Social	1.20 (0.73-1.98)	0.473				
Significant	2.20 (1.14-4.24)	0.027				
BMI, kg/m^2	-	-	-	-	0.94 (0.89-0.99)	0.019
Diabetes mellitus	0.96 (0.59-1.56)	0.872	-	-	1.12 (0.77-1.62)	0.563
Albumin, g/dL	0.71 (0.44-1.15)	0.162	0.35 (0.20-0.63)	< 0.001	0.36 (0.25-0.51)	< 0.001
Total bilirubin, mg/dL	0.95 (0.80-1.12)	0.568	1.03 (0.98-1.08)	0.264	-	-
PT, INR	2.66 (1.13-6.24)	0.025	3.32 (1.08-10.19)	0.036	2.34 (1.06-5.16)	0.034
AFP, $\log_{10}$ ng/dL	2.12 (1.48-3.05)	< 0.001	1.54 (0.95-2.51)	0.08	1.29 (0.94-1.77)	0.112
Model 2						
Untreated or no SVR	Reference	-	Reference	-	Reference	-
SVR	0.41 (0.26-0.65)	< 0.001	0.10 (0.04-0.29)	< 0.001	0.26 (0.17-0.39)	< 0.001
Age, yr	1.06 (1.04- 1.08)	< 0.001	1.02 (0.99-1.04)	0.152	1.06 (1.04-1.07)	< 0.001
Male sex	2.50 (1.38-4.54)	0.003	-	-	1.68 (1.23-2.30)	0.001
HCV genotype			-	-	-	-
2	Reference	-				
1	2.25 (1.45-3.48)	< 0.001				
Others/unknown	1.57 (0.60-4.09)	0.359				
Cirrhosis	3.75 (2.35-6.01)	< 0.001	7.54 (3.32-17.15)	< 0.001	1.72 (1.23-2.41)	0.002
Ever smoker	1.53 (0.85-2.75)	0.156	-	-	-	-
Alcohol consumption			-	-	-	-
None	Reference	-				
Social	1.24 (0.75-2.04)	0.397				
Significant	2.69 (1.40-5.16)	0.003				
BMI, kg/m^2	-	-	-	-	0.94 (0.89-0.99)	0.024
Diabetes mellitus	0.99 (0.61-1.61)	0.959	-	-	1.10 (0.76-1.60)	0.612
Albumin, g/dL	0.76 (0.47-1.20)	0.238	0.42 (0.24-0.71)	0.001	0.42 (0.30-0.60)	< 0.001

Total bilirubin, mg/dL	0.96 (0.81-1.13)	0.624	1.03 (0.99-1.08)	0.156	-	-
PT, INR	2.53 (1.02-6.28)	0.044	4.42 (1.48-13.20)	0.008	2.69 (1.22-5.92)	0.014
AFP, $\log_{10}$ ng/dL	2.10 (1.46-3.02)	< 0.001	1.48 (0.90-2.44)	0.12	1.17 (0.86-1.60)	0.32

Total number of patients, 2197; number of hepatocellular carcinomas, 113; number of deaths, 202; number of transplantations, 4; number of decompensations, 69. AFP: Alpha-feto protein; aHR: Adjusted hazard ratio; BMI: Body mass index; CI: Confidence interval; DAA: Direct acting antivirals; HCV: Hepatitis C virus; IBT: Interferon-based therapy; PT: Prothrombin time; SVR: Sustained virologic response.

In the SVR cohort (5880.4 patient-years), 30 patients developed HCC, 6 patients had decompensation, 35 patients died, and no patient underwent LT during follow-up. The cumulative incidence rates of HCC at 2 and 5 years were 0.6% and 1.6%, respectively for non-cirrhotic patients with SVR (n = 985), and 4.8% and 10.1%, respectively, for cirrhotic SVR patients (n = 283). The cumulative HCC risk according to the presence of cirrhosis, FIB-4 index, APRI score, and ALBI score were significantly different (Supplementary Figure 3). However, HBcIgG positivity (HR, 0.39; 95%CI: 0.05-2.89; P = 0.358) and the presence of fatty liver disease (HR, 0.16; 95%CI: 0.02-1.18; P = 0.072) were not significant risk factors for the development HCC.

Comparisons of the clinical characteristics and outcomes of the IBT-SVR and DAA-SVR groups before and after PS matching (Table 4) yielded 213 matched pairs of patients from the IBT-SVR and DAA-SVR groups with no significant between-group differences in all baseline variables. Before PS matching, the DAA-SVR group had a significantly higher risk of HCC than the IBT-SVR group (HR, 3.53; 95%CI: 1.47-8.49; P = 0.005) (Supplementary Figure 4A), whereas the risks of decompensation (HR, 2.45; 95%CI: 0.33-18.37; P = 0.382) and death/LT (HR, 1.44; 95%CI: 0.62-3.34; P = 0.400) did not reach statistical significance (Supplementary Figure 4B and C). However, after PS matching, the DAA-SVR group showed no significant differences in the risks of HCC (HR, 2.72; 95%CI: 0.63-11.81; P = 0.179), decompensation (HR, 9.74; 95%CI: 0.42-224.81; P = 0.155), and death/LT (HR, 2.18; 95%CI: 0.47-10.15; P = 0.318) compared with the IBT-SVR group (Figure 3).

Multivariable-adjusted analyses of factors associated with outcomes of the SVR cohort

According to the multivariable-adjusted analysis, the DAA-SVR group did not show any independent differences in the development of HCC (HR, 2.03; 95%CI: 0.76-5.43; P = 0.160) or death/LT (HR, 1.38; 95%CI: 0.55-3.49; P = 0.494) compared with the IBT-SVR group (Table 5). Covariates independently associated with a higher incidence of HCC were older age (HR, 1.05; 95%CI: 1.01-1.10; P = 0.025), genotype 1 (HR, 3.02; 95%CI: 1.18-7.68; P = 0.021), and the presence of cirrhosis (HR, 3.18; 95%CI: 1.33-7.68; P = 0.009) after adjustment for the antiviral treatment regimen, sex, alcohol consumption, diabetes mellitus, albumin level, PT, and AFP level (Table 5). Additionally, covariates independently associated with death/LT risk were the presence of cirrhosis (HR, 2.97; 95%CI: 1.42-6.20; P = 0.004) and PT (HR, 5.27; 95%CI: 1.01-27.53; P = 0.049).

DISCUSSION

We analyzed the incidence rates of HCC, decompensation, and all-cause death/LT in a large, prospective, Asian cohort including 2054 patients with chronic HCV infection. During a median follow-up period of 4.1 years, the risks of HCC, decompensation, and all-cause mortality were significantly lower after treatment with IBT or DAA compared with no treatment.

After statistical adjustment, including a time-varying Cox analysis and PS-matched analysis, the risks of HCC, decompensation, and all-cause mortality were not significantly different regardless of whether SVR was achieved after IBT or DAA treatment; however, the follow-up duration of the DAA group was shorter than that of the IBT group. After achieving SVR, independent factors associated with HCC risk were no treatment, age, male sex, HCV genotype 1, cirrhosis, alcohol consumption, PT, and pretreatment AFP level, whereas independent factors associated with all-cause mortality were cirrhosis and PT.

There are limited studies of the association between antiviral treatment (IBT or DAA) and all-cause mortality, especially those involving Asian cohorts. Many studies focused on liver-related mortality rather than all-cause mortality as the primary outcome after SVR achievement. However, extrahepatic mortality should be considered for patients with HCV because HCV is associated with increased cardiovascular disease events[20] and extrahepatic malignancies such as bile duct cancers and diffuse large B-cell lymphoma[21,22]. Moreover, Tada *et al*[23] reported that IBT could reduce all-cause mortality and HCC risk even in patients who did not achieve SVR. However, the effect of DAA treatment in the absence of SVR on clinical outcomes is unknown. Therefore, this study focused on the effect of treatment on the outcomes including all-cause mortality in the entire cohort, and the effect of SVR induced by IBT and DDA on the outcomes in the SVR cohort.

Table 4 Comparison of characteristics and outcomes between the interferon-based treatment-sustained virologic response and direct-acting antivirals-sustained virologic response groups: Unadjusted and propensity score matching groups

Variable	Before adjustment				PS matched			
	IBT-SVR group (*n* = 451)	DAA-SVR group (*n* = 817)	*P* value	SMD	IBT-SVR group (*n* = 213)	DAA-SVR group (*n* = 213)	*P* value	SMD
Age, yr	51.0 ± 10.6	59.1 ± 11.4	< 0.001	0.741	53.9 ± 9.8	53.7 ± 11.4	0.823	0.022
Male sex	209 (46.3)	394 (48.2)	0.52	0.036	102 (47.9)	97 (45.5)	0.627	0.047
HCV RNA, $xlog_{10}$ IU/mL	5.72 (4.77-6.37)	6.04 (5.26-6.52)	< 0.001	0.278	5.84 (4.91-6.53)	6.07 (5.21-6.50)	0.439	0.073
HCV genotype			< 0.001	0.406			0.731	0.058
1	167 (37.0)	464 (56.8)			95 (44.6)	101 (47.4)		
2	278 (61.6)	342 (41.9)			116 (54.5)	111 (52.1)		
Others	6 (1.3)	11 (1.3)			2 (0.9)	1 (0.5)		
Entry-to-treat, year	0 (0-0.3)	0.2 (0-2.1)	< 0.001	0.654	0.1 (0-0.4)	0.1 (0-0.4)	0.78	0.125
Diagnosis status			0.001	0.192			0.901	0.012
Chronic hepatitis	373 (82.7)	612 (74.9)			174 (81.7)	173 (81.2)		
Compensated cirrhosis	78 (17.3)	205 (25.1)			39 (18.3)	40 (18.8)		
FIB-4 index			< 0.001	0.294			0.363	0.071
≤ 1.45	156 (34.8)	156 (19.9)			69 (32.7)	59 (29.1)		
1.45–3.25	155 (34.6)	326 (41.6)			74 (35.1)	85 (41.9)		
≥ 3.25	137 (30.6)	302 (38.5)			68 (32.2)	59 (29.1)		
APRI score			0.562	0.096			0.108	0.048
< 2.0	383 (85.1)	676 (83.9)			186 (87.7)	170 (82.1)		
≥ 2.0	67 (14.9)	130 (16.1)			26 (12.3)	37 (17.9)		
ALBI score			0.958	0.044			0.339	0.054
≤ -2.60 (Grade 1)	360 (80.0)	639 (79.9)			172 (81.1)	172 (82.7)		
> -2.60 (Grade 2 or 3)	90 (20.0)	161 (20.1)			40 (18.9)	36 (16.3)		
Ever smoker	228 (50.8)	15 (37.5)	0.045	0.162	95 (44.6)	93 (43.7)	0.527	0.019
Alcohol intake			< 0.001	0.669			0.816	0.058
None	171 (38.0)	19 (47.5)			98 (46.0)	104 (48.8)		
Social	245 (54.4)	13 (32.5)			88 (41.3)	85 (39.9)		
Significant[1]	34 (7.6)	8 (20.0)			27 (12.7)	24 (11.3)		
Fatty liver disease on imaging study	77 (22.8), *n* = 337	131 (22.4), *n* = 586	0.863	0.039	34 (24.3)	35 (24.8)	0.917	0.013
Anti-HBc IgG positivity	25 (8.6), *n* = 290	62 (10.5), *n* = 592	0.386	0.062	12 (7.8)	15 (9.7)	0.546	0.062
BMI, kg/m^2	23.7 ± 3.0	23.9 ± 3.1	0.429	0.047	23.9 ± 2.9	23.9 ± 3.4	0.983	0.005
Diabetes mellitus	61 (13.6)	122 (14.9)	0.495	0.049	26 (12.2)	22 (10.3)	0.54	0.055
Hypertension	80 (17.7)	236 (28.9)	< 0.001	0.267	45 (21.1)	44 (20.7)	0.905	0.012
Cardiovascular disease	3 (0.7)	24 (2.9)	0.007	0.162	2 (0.9)	3 (1.4)	0.315	0.058
Cerebrovascular disease	2 (0.4)	19 (2.3)	0.012	0.172	1 (0.5)	3 (1.4)	0.315	0.141
Laboratory study								
WBC, $\times 10^3/mm^3$	4.99 (3.90-6.10)	5.40 (4.40-6.63)	< 0.0010.297	0.24	5.26 (4.25-6.35)	5.35 (4.50-6.52)	0.787	0.027
Hemoglobin, g/dL	13.6 (12.6-14.9)	13.8 (12.8-14.8)	0.218	0.061	13.9 (12.8-14.9)	13.9 (12.8-14.9)	0.916	0.01

PLT, × 10^3/mm^3	177 (136-220)	173 (130-216)	0.178	0.055	176 (136-222)	180 (139-219)	0.401	0.083
Albumin, g/dL	4.2 (4.0-4.5)	4.2 (4.0-4.4)	0.664	0.082	4.3 (4.0-4.5)	4.3 (4.0-4.5)	0.734	0.031
Total bilirubin, mg/dL	0.7 (0.6-1.0)	0.7 (0.6-1.0)	< 0.001	0.072	0.8 (0.6-1.0)	0.7 (0.6-1.0)	0.85	0.009
ALP, IU/L	80 (63-113)	122 (78-250)	0.012	0.612	90 (65-126)	96 (72-183)	0.524	0.072
AST, IU/L	50 (30-86)	46 (30-76)	< 0.001	0.156	50 (32-83)	42 (27-85)	0.899	0.012
ALT, IU/L	54 (29-105)	36 (22-70)	0.756	0.297	49 (29-85)	37 (21-91)	0.915	0.009
Creatinine, mg/dL	0.8 (0.7-1.0)	0.8 (0.7-1.0)	0.02	0.09	0.8 (0.7-1.0)	0.8 (0.7-0.9)	0.176	0.069
PT, INR	1.03 (0.98-1.08)	1.04 (0.99-1.10)	0.434	0.111	1.03 (0.98-1.08)	1.04 (0.87-1.09)	0.67	0.038
AFP, ng/dL	3.3 (2.3-6.3)	3.8 (2.3-6.5)		0.142	5.8 (4.9-6.5)	6.1 (5.2-6.5)	0.278	0.034

[1]Significant alcohol intake is defined as weekly alcohol consumption ≥ 210 g for men and ≥ 140 g for women.
Data were presented as mean ± SD, median (interquartile range), or *n* (%). AFP: Alpha-feto protein; ALBI: Albumin-bilirubin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: Aspartate aminotransferase to platelet ratio index; AST: Aspartate aminotransferase; BMI: Body mass index; DAA: Direct acting antivirals; FIB-4: Fibrosis-4; HCV: Hepatitis C virus; IBT: Interferon-based therapy; MELD: Model for end-stage liver disease; PLT: Platelet; PT: Prothrombin time; WBC: White blood cell; SVR: Sustained virologic response.

Table 5 Multivariate Cox regression analysis for hepatocellular carcinoma and death/transplantation among the sustained virologic response cohort

Variable	Hepatocellular carcinoma		Death/transplantation	
	aHR (95%CI)	*P* value	aHR (95%CI)	*P* value
IBT-SVR group	Reference	-	Reference	-
DAA-SVR group	2.03 (0.76-5.43)	0.16	1.38 (0.55-3.49)	0.494
Age, yr	1.05 (1.01-1.10)	0.025	-	-
Male sex	2.89 (0.97-8.61)	0.057	-	-
HCV genotype			-	–
2	Reference	-		
1	3.02 (1.18-7.68)	0.021		
Cirrhosis	3.18 (1.33-7.68)	0.009	2.97 (1.42-6.20)	0.004
Ever smoker	0.72 (0.26-1.99)	0.545	-	-
Alcohol consumption			-	-
None	Reference	-		
Social	2.62 (0.92-7.60)	0.072		
Significant	2.51 (0.71-8.82)	0.152		
BMI, kg/m^2	-	-	-	-
Diabetes mellitus	1.03 (0.42-2.52)	0.952	2.16 (0.98-4.78)	0.057
Albumin, g/dL	0.53 (0.19-1.49)	0.228	-	-
Total bilirubin, mg/dL	-	-	-	-
PT, INR	4.12 (0.89-19.17)	0.071	5.27 (1.01-27.53)	0.049
AFP, log_{10} ng/dL	1.07 (0.49-2.34)	0.858	-	-

Total number of patients, 1250; number of hepatocellular carcinomas, 30; number of deaths, 7; number of transplantations, 3. AFP: Alpha-feto protein; aHR: Adjusted hazard ratio; BMI: Body mass index; CI: Confidence interval; DAA: Direct acting antivirals; HCV: Hepatitis C virus; IBT: Interferon-based therapy; PT: Prothrombin time; SVR: Sustained virologic response.

Recent 5-year follow-up studies after DAA treatment showed that SVR is associated with a gradual but significant reduction in liver fibrosis in terms of FIB-4, METAVIR, transient elastography, and Child-Pugh score[24,25], whereas another study showed that induced SVR was associated with a reduced risk of clinical disease progression in patients with Child-Pugh A cirrhosis but not in patients

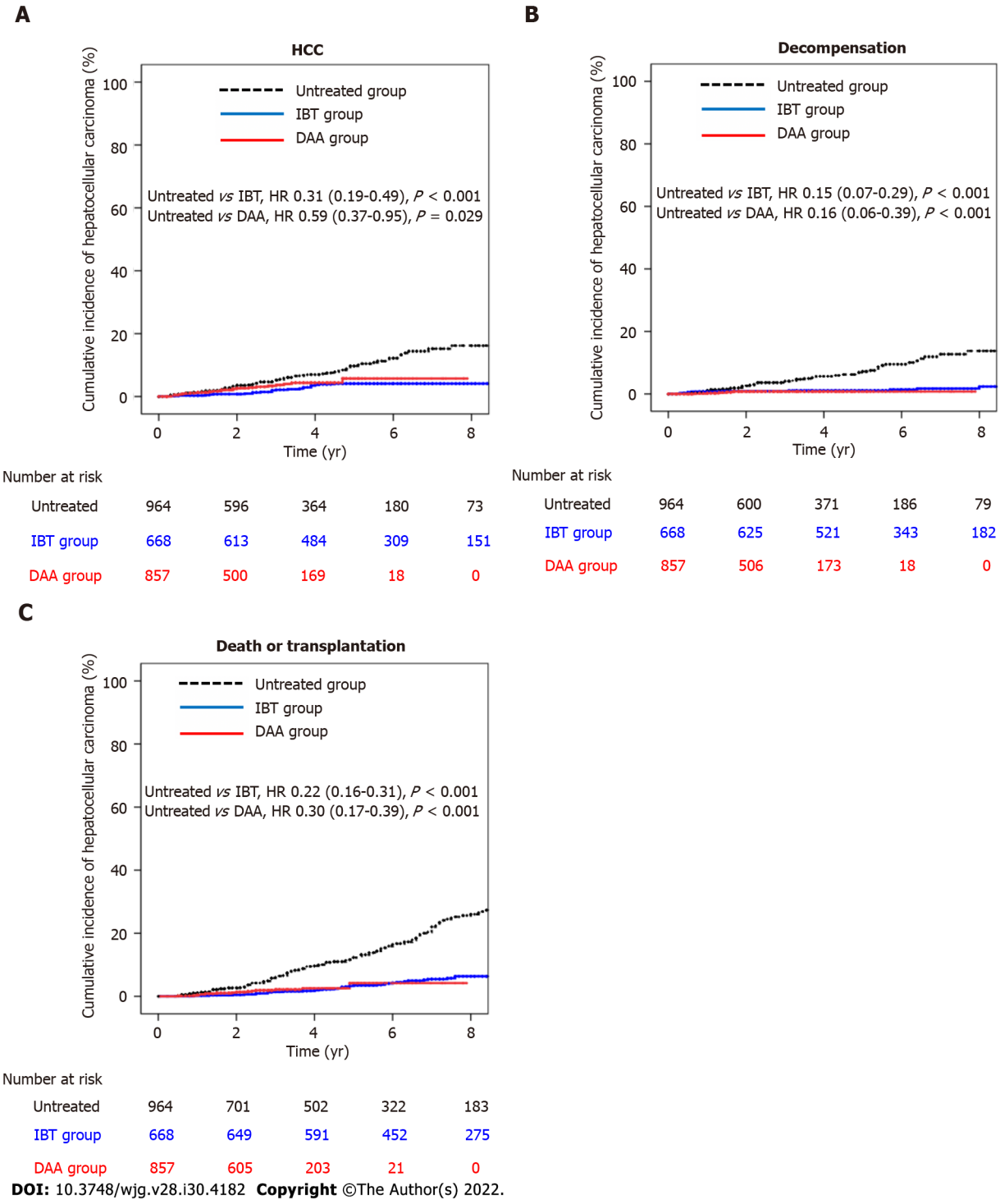

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Figure 2 Cumulative incidences of hepatocellular carcinoma, decompensation, and death/transplantation in entire cohort. A: Cumulative incidence of hepatocellular carcinoma in the untreated, interferon-based treatment (IBT), and direct-acting antivirals (DAA) groups; B: Cumulative incidence of decompensation in the untreated, IBT, and DAA groups; C: Cumulative incidence of death/transplantation in the untreated, IBT, and DAA groups. DAA: Direct-acting antivirals; HCC: Hepatocellular carcinoma; IBT: Interferon-based treatment.

with Child-Pugh B/C cirrhosis[26]. In this context, our study showed an approximately 85% reduction in the risk of decompensation after IBT or DAA treatment in Child-Pugh A patients, with decompensated cirrhotic patients being excluded from enrollment.

This study demonstrated that treatment with IBT and DAA resulted in risk reductions of 72% (median follow-up, 94.8 mo) and 81% (median follow-up, 42 mo) for all-cause mortality, respectively, compared with no treatment after multivariate adjustment. Similar to previous studies[27], our results showed that even IBT, with its probable adverse events and lower SVR rates, had long-term beneficial effects on mortality. Regarding the benefit of DAA treatment, Butt *et al*[28] (n = 6790), using the Electronically Retrieved Cohort of HCV Infected Veterans (ERCHIVES) data in the United States in

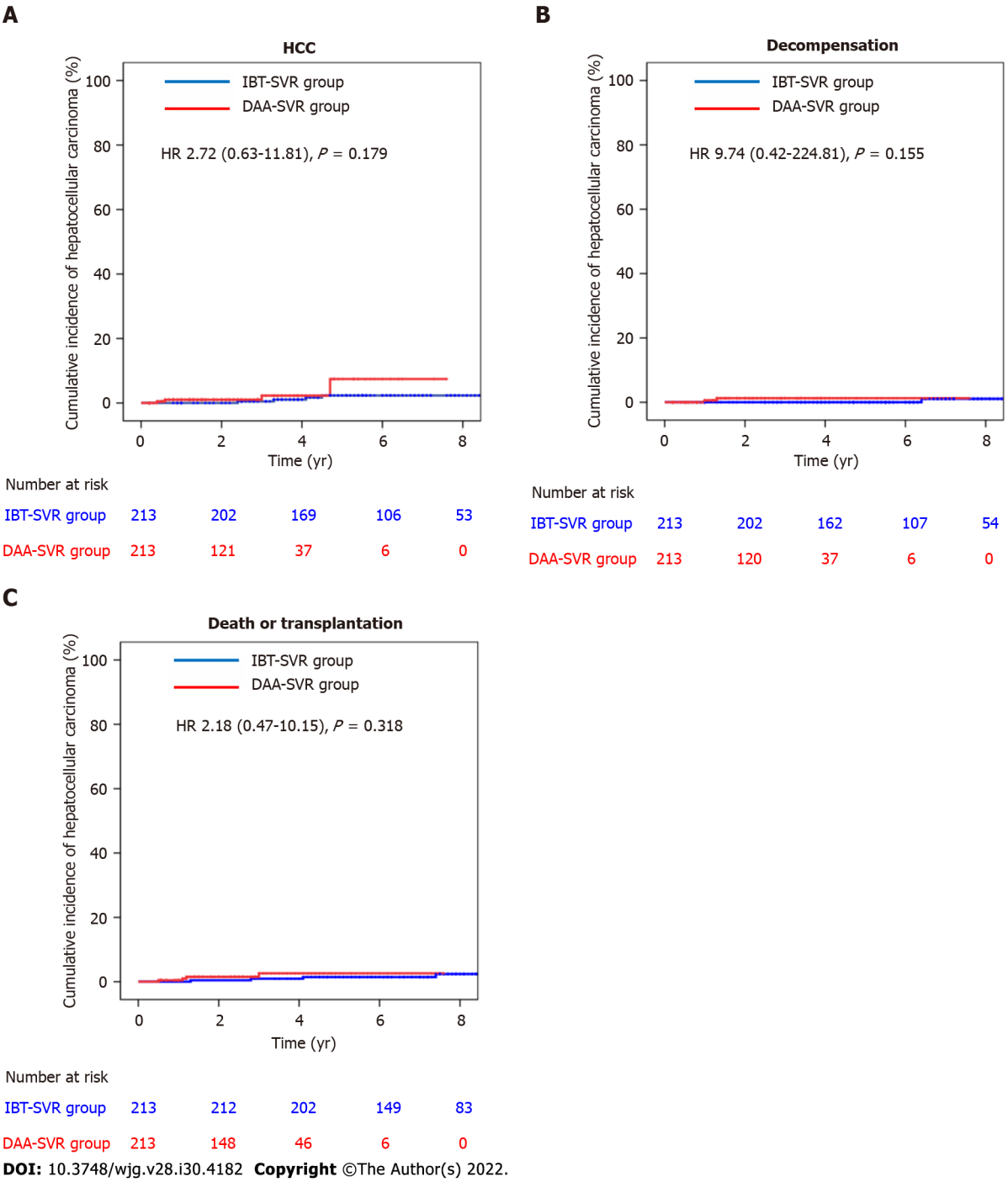

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Figure 3 Cumulative incidences of hepatocellular carcinoma, decompensation, and death/transplantation in the matched sustained virologic response cohort. A: Cumulative incidence of hepatocellular carcinoma in the interferon-based treatment-sustained virologic response (IBT-SVR) and direct-acting antivirals-sustained virologic response (DAA-SVR) groups; B: Cumulative incidence of decompensation in the IBT-SVR and DAA-SVR groups; C: Cumulative incidence of death/Liver transplantation in the IBT-SVR and DAA-SVR groups. DAA: Direct-acting antivirals; HCC: Hepatocellular carcinoma; IBT: Interferon-based treatment; SVR: Sustained virologic response.

2017, reported that DAA treatment and the achievement of SVR reduced all-cause mortality by 57% and 43%, respectively, within the first 18 mo of treatment. A prospective cohort study performed in France (n = 9895; follow-up, 33.4 mo) reported a 52% reduction in the risk of all-cause mortality in the DAA-treated group compared with the untreated group in 2019[11]. Compared with these studies, our results showed a higher reduction in the risk of all-cause mortality with DAA treatment. This may be related to the low proportion of patients with advanced fibrosis in the DAA group in our cohort. Additionally, the follow-up duration of this study was relatively longer than the durations used for those studies.

During this study, the achievement of SVR by IBT or by DAA resulted in a 59% reduction in the risk of HCC compared with no treatment or no SVR after multivariable adjustment, similar to previous studies[29,30]. Nonetheless, our results also showed that the HCC risk remained after SVR achievement.

Despite the achievement of SVR, the absolute risk of HCC remains high for patients with cirrhosis; therefore, according to international guidelines, they should be enrolled in an HCC surveillance program[2-4]. However, there is little evidence of the benefits of HCC surveillance for non-cirrhotic chronic HCV patients with SVR, because of the low residual HCC risk. During this study, the non-cirrhotic group had an HCC risk of 0.26 per 100 person-years (5-year cumulative incidence of 1.6%). In particular, the low FIB-4 (5-year cumulative incidence of 0.3%) group had a very low risk of HCC during this study. These results are similar to those of the retrospective REAL-C cohort study that targeted Eastern Asians (5-year cumulative incidence: 1.35 for the non-cirrhotic group and 0.13 for the low FIB-4 group)[30]. These results support the European guidelines, which do not recommend HCC surveillance for fibrosis 0 to 2[4]. However, more long-term follow-up data after achieving SVR are needed.

Identifying the risk factors associated with HCC after SVR is important to the development of a reasonable surveillance strategy. An East Asian retrospective study suggested that among the cirrhotic DAA-SVR group, age older than 60 years, ALBI scores of 2 or 3, and pretreatment AFP > 10 ng/mL were associated with HCC risk; however, among the non-cirrhotic group, only AFP > 10 ng/mL was significant[30]. Additionally, there are many factors associated with HCC risk after SVR, such as clinical factors (age, sex, presence of diabetes, HCV genotype 3, history of IBT), laboratory parameters (platelet count, AFP), and fibrosis stage (determined by histology or estimation by Fibroscan®, FIB-4, APRI, or the presence of esophageal varices) before DAA treatment and at follow-up (determined by Fibroscan®, FIB-4, APRI, alanine aminotransferase, AFP, albuminemia, or VITRO score)[31]. In our study, age, cirrhosis, and HCV genotype 1 (compared with genotype 2) were independent risk factors for HCC after SVR. Therefore, it is necessary to test the HCV genotype even in the therapeutic era of pangenotypic regimens to develop a follow-up strategy after SVR according to the regional distribution of HCV genotypes. These factors should be considered when establishing an optimal HCC prediction model.

Previous studies have suggested a similar risk of HCC development with IBT and DAA treatment [32]. Interestingly, a recent meta-analysis suggested that IBT is better than DAA for preventing the occurrence of HCC in chronic hepatitis C patients after achieving SVR[33]. Biologically, IFN family members, especially class I IFN (IFN-α, IFN-β), have important anti-HCC effects[32]. However, some investigators have suggested that the sudden decrease in viral load caused by DAA treatment causes immune distortion, thus deregulating the antitumor response and releasing precancerous foci from immune surveillance[34,35]. Although it can be explained theoretically, there is little clinical evidence regarding the difference in the HCC risk for patients with DAA-SVR and IBT-SVR. Our results showed significant clinical differences between the IBT-SVR and DAA-SVR groups. Both the multivariable analysis and PS matching analysis showed that the effect of IBT-SVR was not different from that of DAA-SVR on the HCC incidence and all-cause mortality. Recently, some studies reported different effects of IBT-SVR and DAA-SVR on the incidence of diabetes[36] and hematologic malignancies[37]. Therefore, long-term studies of the effects of DAA-SVR are warranted.

To meet the 2030 HCV elimination target of the WHO[1], active testing and enhanced linkage to treatment are important. In this study, 30.1% of patients remained untreated, and this percentage should be reduced. Indeed, the untreated group showed a higher mortality rate of 23.7% (88 Liver related deaths and 59 non-liver related deaths) during the median follow-up of 5.6 years. In the untreated group, 3 patients received IBT after HCC diagnosis, and 19 patients received DAA treatment after HCC diagnosis or a decompensation event. The majority of the untreated patients (78.8%, 457/619) were enrolled in the Korean HCV cohort before June 2015, when the first DAA was introduced, and 46.7% (289/619) died or were lost to follow-up before June 2015. Although DAA therapy is covered by the National Health Insurance System, 30% of the drug price is an out-of-pocket expense of the patients, equaling to approximately 3000 United States dollars. Even in the DAA era, the main reasons for non-treatment are the relatively high price of DAA, old age, and extrahepatic or advanced hepatic malignancy.

One of the strengths of our study was that it was a long-term study involving a prospective, Asian cohort that included both IBT and DAA groups. This study used a well-established protocol, guidelines and database, and the data, including death outcomes, were verified under government guidance. To date, few reports have evaluated and compared clinical outcomes (HCC, death, and decompensation) after IBT and DAA treatment, especially in Asia, where HCV is less prevalent than HBV.

This study had several limitations. First, because it was an observational study, the findings may show selection and confounding biases. Despite this limitation, it would be useful for comparing the effectiveness of IBT and DAA because of the relatively low incidence of clinical events. Second, we used clinical and radiological criteria to diagnose cirrhosis; therefore, some patients with advanced fibrosis may have been included in the non-cirrhotic group. However, we attempted to correct this point using a non-invasive liver fibrosis biomarker. Third, there was a disparity in the follow-up periods of the IBT and DAA groups because of the later introduction of DAA (since 2015 in Korea). Additional long-term follow-up studies evaluating the outcomes of patients with SVR are warranted. Forth, the presence of anti-HBc IgG in HCV patients has been implicated in HCC development, and a non-negligible risk of HBV inactivation during DAA treatment (0.91%)[38]. However, approximately 30% of our patients were not tested for anti-HBc IgG, and none of the anti-HBc IgG-positive patients were tested for HBV DNA during DAA treatment. Likewise, the role of fatty liver in the outcomes of HCV patients was not

completely evaluated owing to missing data. Finally, compared with the IBT era, diagnostic imaging modalities and treatment options for HCC have improved in the DAA era. Therefore, these points could have affected the clinical outcomes of the IBT and DAA groups.

CONCLUSION

This study showed that antiviral treatment significantly reduced the incidences of HCC, decompensation, and mortality in an Asian population, regardless of the use of IBT or DAA. After achieving SVR, age, the presence of cirrhosis, and HCV genotype 1 were indicators of worse clinical outcomes. Therefore, an adequate HCC surveillance strategy after SVR that considers age, the presence of cirrhosis, and genotype should be developed. Additional studies evaluating the long-term DAA outcomes of SVR patients are also warranted.

ARTICLE HIGHLIGHTS

Research background

Sustained virologic response (SVR) with either interferon-based therapy (IBT) or direct acting antivirals (DAA)-induced SVR significantly reduces the incidence of hepatocellular carcinoma (HCC) and long-term mortality in patients with hepatitis C virus (HCV) infection.

Research motivation

Prospective studies of the long-term outcomes for patients with HCV infection after treatment with IBT or DAA are limited in many Asian countries.

Research objectives

We aimed to elucidate the incidences of HCC and death/transplantation based on treatment with IBT or DAA. And, we aimed to compare the outcomes of the SVR to IBT and DAA. Finally, we aimed to investigate outcome-determining factors after SVR.

Research methods

The cohort included 2054 viremic patients (mean age, 57 years; 46.5% male; 27.4% with cirrhosis) prospectively enrolled at seven hospitals between 2007 and 2019. They were classified as the untreated group (n = 619), IBT group (n = 578), and DAA group (n = 857).

Research results

Compared to the untreated group, the incidences of HCC and death/transplantation were significantly lower in the IBT group and the DAA group. SVR induced by IBT or DAA did not show significant differences in the risk of HCC and all-cause mortality. After achieving SVR, age, presence of cirrhosis, and genotype 1 HCV infection were indicators of worse clinical outcomes.

Research conclusions

Treatment and SVR with either IBT or DAA significantly reduced the incidences of HCC and mortality in the Asian prospective cohort.

Research perspectives

This study was a long-term study involving a prospective, Asian cohort that included both IBT and DAA groups. This study used a well-established protocol, guidelines and database, and the data, including death outcomes, were verified under government guidance.

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FOOTNOTES

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META-ANALYSIS

Network meta-analysis of randomized controlled trials on esophagectomies in esophageal cancer: The superiority of minimally invasive surgery

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Abstract

BACKGROUND

Previous meta-analyses, with many limitations, have described the beneficial nature of minimal invasive procedures.

AIM

To compare all modalities of esophagectomies to each other from the results of randomized controlled trials (RCTs) in a network meta-analysis (NMA).

METHODS

We conducted a systematic search of the MEDLINE, EMBASE, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and CENTRAL databases to identify RCTs according to the following population, intervention, control, outcome (commonly known as PICO): P: Patients with resectable esophageal cancer; I/C: Transthoracic, transhiatal, minimally invasive (thoracolaparoscopic), hybrid, and robot-assisted esophagectomy; O: Survival, total adverse events, adverse events in subgroups, length of hospital stay, and blood loss. We used the Bayesian approach and the random effects model. We presented the geometry of the network, results with probabilistic statements, estimated intervention effects and their 95% confidence interval (CI), and the surface under the cumulative ranking curve to rank the interventions.

RESULTS

We included 11 studies in our analysis. We found a significant difference in postoperative pulmonary infection, which favored the minimally invasive intervention compared to transthoracic surgery (risk ratio 0.49; 95%CI: 0.23 to 0.99). The operation time was significantly shorter for the transhiatal approach compared to transthoracic surgery (mean difference -85 min; 95%CI: -150 to -29), hybrid intervention (mean difference -98 min; 95%CI: -190 to -9.4), minimally invasive technique (mean difference -130 min; 95%CI: -210 to -50), and robot-assisted esophagectomy (mean difference -150 min; 95%CI: -240 to -53). Other comparisons did not yield significant differences.

CONCLUSION

Based on our results, the implication of minimally invasive esophagectomy should be favored.

Key Words: Surgery; Esophageal cancer; Esophagectomy; Network meta-analysis; Minimally invasive; Laparoscopy

Core Tip: Minimally invasive laparoscopic techniques should be the preferred approach for the treatment of esophageal cancer, due to the lower incidence of postoperative pulmonary complications.

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INTRODUCTION

Esophageal cancer is the eighth most common type of cancer worldwide[1], with an incidence of 5.2 per 100000 for squamous cell cancer (SCC) and 0.7 per 100000 for adenocarcinoma (AC)[2]. While the prognosis varies between the two histological diagnoses, both AC and SCC are associated with poor clinical outcomes, with a 5-year survival rate of 20%[3].

Surgical therapy plays an essential role in the treatment of esophageal cancer. However, it cannot be routinely used due to the late diagnosis, as symptoms usually occur when the cancer is already unresectable[4]. Traditionally, open surgical interventions are performed, including transhiatal and transthoracic techniques. A meta-analysis comparing these two open surgical modalities did not find a significant difference in 5-year survival[5]. While both techniques are successful in terms of removing the neoplasm, open esophagectomies are associated with significant limitations, most importantly, postoperative morbidity[6,7].

A transition to non-open surgical techniques has been the trend in almost every field of surgery in recent years[8]. A wide variety of non-open techniques are available, including minimally invasive surgery (thoracolaparoscopic) surgery or even robot-assisted esophagectomy[9,10]. In the form of hybrid surgical intervention, a combination of open and non-open technique is available[11].

Previous meta-analyses have compared the different types of surgical techniques, with variable success and significant limitations[12-19]. To date, convincing evidence is missing regarding the optimal surgical approach of resectable esophageal cancer, as it is presented in a recent guideline[20].

Network meta-analysis (NMA) is a relatively novel methodology, which allows the direct and indirect comparison of multiple interventions, thus providing more information than traditional meta-analyses. Indirect comparisons can be made in the case of missing trials comparing two interventions if those are compared with a third intervention[21]. Several meta-analyses were carried out focusing on esophageal cancer surgery, but none of those addressed the problem of the wide variety of surgical techniques.

The purpose of our study was to provide objective evidence considering the surgical treatment of resectable esophageal cancer by comparing each treatment modality in the form of an NMA and possibly rank the different approaches.

MATERIALS AND METHODS

The NMA was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses-NMA guideline[22].

Protocol

The NMA protocol was registered in advance in PROSPERO under the number CRD42020160978. Analyses of the mortality and quality of life could not be carried out due to the low number of reporting articles. The risk of bias was assessed using an updated risk assessment tool.

Search strategy and inclusion criteria

We conducted a systematic search of the MEDLINE (*via* PubMed), EMBASE, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and Cochrane Central Register of Controlled Trials (CENTRAL) from initiation until 2019 November to identify studies, comparing at least two types of esophagectomies from transthoracic, transhiatal, hybrid, laparoscopic or robot-assisted approach treating esophageal cancer without the restriction of histological subtype and an NMA was performed. The following search key was used: (((esophagus OR oesophagus OR esophageal OR oesophageal) AND (tumor OR tumour OR malign* OR cancer OR adenocarcinoma OR carcinoma)) AND (esophagectomy OR oesophagectomy OR Ivor-Lewis OR „Ivor Lewis" OR hybrid OR laparoscop* OR „minimal invasive")) AND random*. We also reviewed the reference lists of eligible articles for further studies. Only randomized controlled trials (RCTs) were included.

Selection and data extraction

After the removal of duplications, two independent reviewers (Szakó L, Engh MA) executed the selection first by title, second by abstract, last by full text following pre-discussed aspects. Data extraction was done by the same two independent reviewers (Szakó L, Engh MA) onto a pre-established Excel worksheet (Office 365, Microsoft, Redmond, WA, United States). Extracted data consisted of the year of publication, name of the first author, study design, country, applied surgical modalities, mortality, overall survival rate (referred as survival), adverse events (AEs), blood loss, length of hospitalization, length of surgical procedure, and demographic data including age, male-female ratio, and SCC/AC ratio. Disagreements regarding both selection and data extraction were resolved by consensus. If consensus could not be reached, a third reviewer (Dömötör RZ) resolved the disagree-ment.

Statistical analysis

The Bayesian method was used to perform pairwise meta-analyses and NMAs. All analyses were carried out using a random effects model. To ensure the interpretability of the NMA results (pooled of direct and indirect data), we presented the geometry of the network, the results with probabilistic statements, and estimates of intervention effects along with their corresponding 95% confidence intervals (CIs), as well as forest plots for ranking the interventions, we chose to use the surface under the cumulative ranking (SUCRA) curve, which provides a numerical summary of the rank distribution of each treatment.

Risk of bias assessment and quality of evidence

The risk of bias assessment was performed at the individual study level, according to the Revised Cochrane risk-of-bias tool for RCTs[23].

The Grading of Recommendations Assessment, Development, and Evaluation system was used to assess the certainty of evidence into four levels: high, moderate, low, and very low. The certainty of the evidence was classified into four levels: high, moderate, low, and very low. Two independent reviewers (Szakó L, Engh MA) decided the overall quality of the evidence[24]. Disagreements were resolved by consensus. If consensus could not be reached, a third reviewer (Dömötör RZ) resolved the

Table 1 Baseline characteristics of the included studies

Ref.	Year	Country	Design	Compared interventions	Number of patients	Male/female ratio	Age in yr, mean	Squamous cell cancer/adenocarcinoma ratio	Inclusion criteria
Straatman *et al*[25]	2012	Netherlands, Spain, Italy	Multicenter	MI-TT	59-56	43/16-46/17	62.3–61.8	24/35-19/36	cT1-3, N0-1, M0
van der Sluis *et al* [26]	2019	Netherlands	Single center	RA-TT	54-55	46/8-42/13	64-65	13/41-12/43	T1-4a, N0-3, M0
Mariette *et al*[27]	2019	France	Multi center	H-TT	103-104	88/15-175/32	59-61 (median)	46/57-84/123	T1-3, N0-1, M0
Guo *et al* [28]	2013	China	Single center	MI-TT	111-110	68/43-72/38	57.3-60.8	No information	T1-3, N0-1, M0
Ma *et al* [29]	2018	China	Single center	MI-TT	47-97	36/11-83/14	61-59.3	43/0-91/2	Resectable cancer
Jacobi *et al* [30]	1997	Germany	Single center	TH-TT	16-16	No information	54-55	13/3-13/3	Resectable cancer
Goldminc *et al*[31]	1993	Australia	Single center	TH-TT	32-35	31/1-33/2	57.4-57.4	32/0-35/0	Resectable squamous cell cancer
Chu *et al* [32]	1997	China	Single center	TH-TT	20-19	18/2-17/2	60.7-63.9	No information	Lower third resectable cancer
Hulscher *et al*[33]	2002	Netherlands	Multicenter	TH-TT	106-114	92/14-97/17	69-64	0/106-0/114	Resectable adenocarcinoma
Yang *et al* [35]	2016	China	Single center	MI-TT	120-120	82/38-87/33	62.5 -67.8	75/45-72/48	T1-3, N0-1, M0
Paireder *et al*[34]	2018	Austria	Single center	H-TT	14-12	10/4-10/2	64.5-62.5 (median)	4/10-1/11	Siewert I-II, resectable squamous cell cancer

Number of patients, male/female ratio, age, and ratio of squamous cell cancer and adenocarcinoma are presented according to the compared interventional arms. H: Hybrid esophagectomy; MIE: Minimally invasive esophagectomy; RA: Robot assisted esophagectomy; TH: Transhiatal esophagectomy; TT: Transthoracic esophagectomy.

disagreement.

RESULTS

Selection process

The database search yielded 3335 records, of which 2002 articles were left after removing duplicates. Twenty-one full-text articles were screened for eligibility. Finally, we included 11 RCTs (25-35), including 1525 patients, in the quantitative synthesis (Figure 1). Baseline characteristics of the enrolled studies are presented in Table 1[25-35].

Outcomes

A significant difference was found for pulmonary infection, which favored the minimally invasive intervention compared to transthoracic surgery (relative risk [RR]: 0.49, 95%CI: 0.23-0.99) (Figure 2). Operation time was significantly shorter for the transhiatal approach compared to transthoracic surgery (mean difference: -86 min, 95%CI: -150 to -29 min), hybrid intervention (mean difference -99 min, 95%CI: -190 to -9.4 min), minimally invasive technique (mean difference -130 min, 95%CI: -210 to -53 min), and robot-assisted esophagectomy (mean difference -150 min, 95%CI: -250 to -52 min) (Figure 3). We did not find significant differences regarding survival (Supplementary Figures 1-5), total AEs (Supplementary Figure 6), cardiac AEs (Supplementary Figure 7), anastomotic leakage (Supplementary Figure 8), atrial fibrillation (Supplementary Figure 9), wound infection (Supplementary Figure 10), total pulmonary AEs (Supplementary Figure 11), vocal chord paralysis (Supplementary Figure 12), length of hospital stay (Supplementary Figure 13), and blood loss (Supplementary Figure 14). The ranking and detailed results of the comparisons of the interventions are presented in the supplementary files (Supplementary Figures 1-14).

Table 2 The results of the risk of bias assessment by each domain

Ref.	Randomization process	Deviation from intended intervention	Missing outcome data	Measurement of the outcome	Selection of the reported results	Overall
Straatman *et al* [25]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
van der Sluis *et al* [26]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Mariette *et al*[27]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Guo *et al*[28]	Unclear risk	Low risk	Low risk	Low risk	Unclear risk	Unclear risk
Ma *et al*[29]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	High risk
Jacobi *et al*[30]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	High risk
Goldminc *et al* [31]	Unclear risk	Unclear risk	Low risk	Low risk	Low risk	Unclear risk
Chu *et al*[32]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	High risk
Hulscher *et al*[33]	Low risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk
Yang *et al*[35]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	High risk
Paireder *et al*[34]	Low risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk

Risk of bias is indicated according to each domain of the Revised Cochrane risk-of-bias tool for randomized trials[23]. By the assessment of overall risk of bias, low risk of bias was given in the case of low risk of bias by every domain; if one or two domains were assessed as unclear risk of bias, unclear overall risk of bias was given, and if at least three domains were accompanied with unclear risk of bias, the overall risk of bias was assessed as high risk of bias.

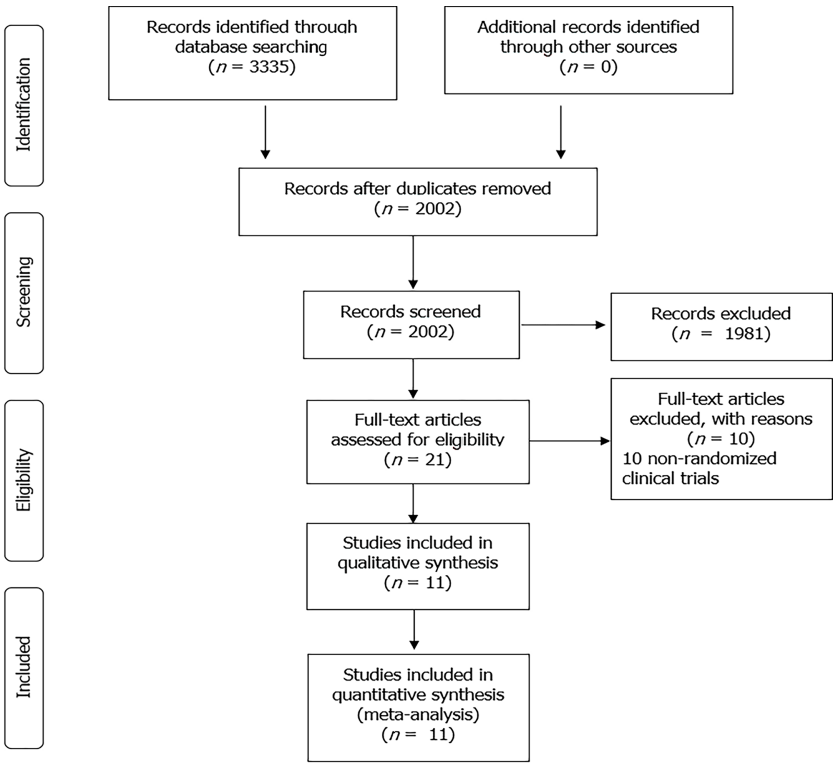

Figure 1 Results of the selection process according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Available from: https://prisma-statement.org//prismastatement/flowdiagram.aspx.

Risk of bias assessment and grade of evidence

Results of the risk of bias assessment for the outcome of survival were assessed following the Cochrane Risk of Bias Assessment Tool 2. Details are shown in Table 2.

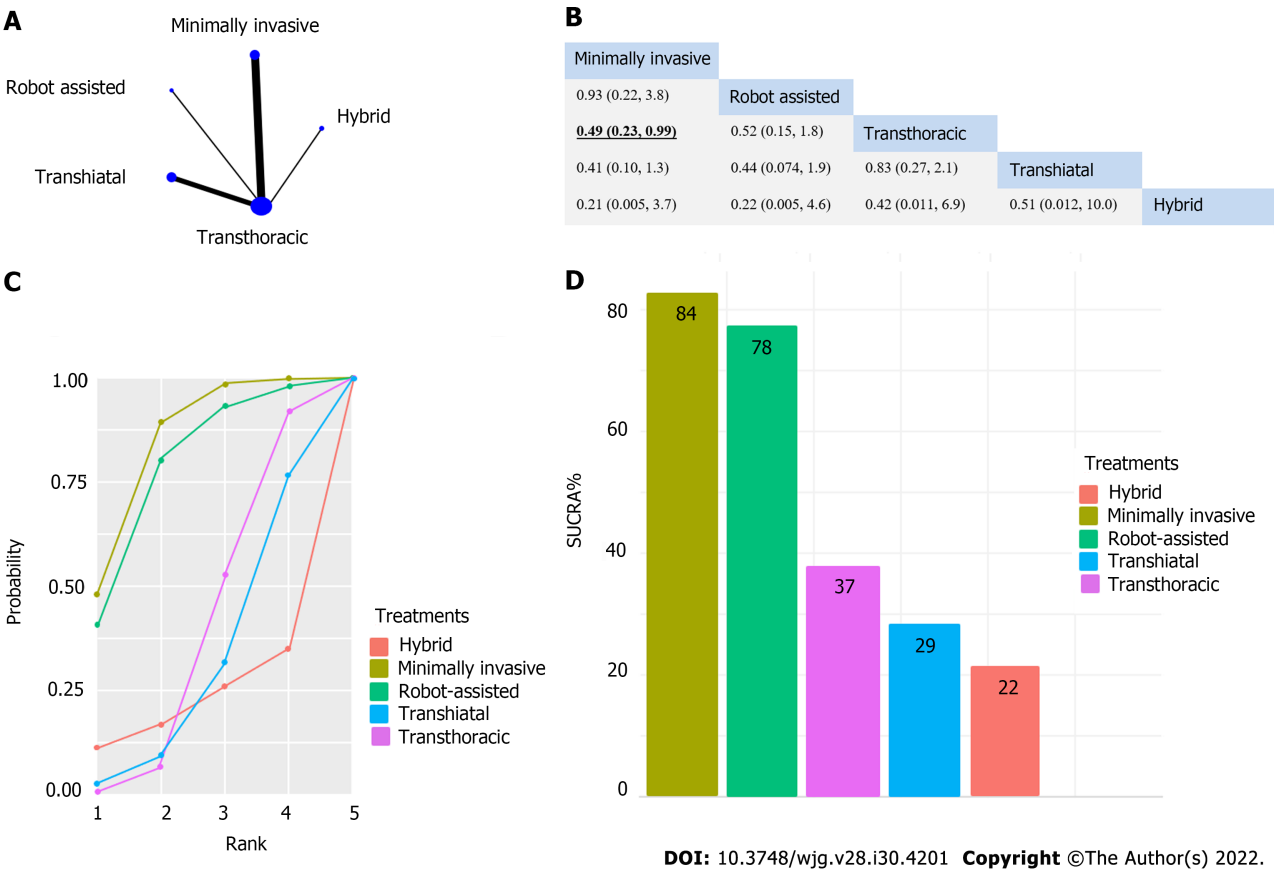

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Figure 2 A significant difference was found considering pulmonary infection, which favored the minimally invasive intervention compared to transthoracic surgery. A: The network of eligible studies for pulmonary infection (the width of the lines is proportional to the number of trials comparing every pair of treatments, and the size of every circle is proportional to the number of randomly assigned participants [sample size]); B: League table of the analysis for pulmonary infection. Comparisons should be read from left to right. The values are presented in risk ratios, with corresponding credible interval. Significant result is in TextTitle and underlined; C: Cumulative probability of treatment rank; D: Treatment rank in SUCRA% histogram.

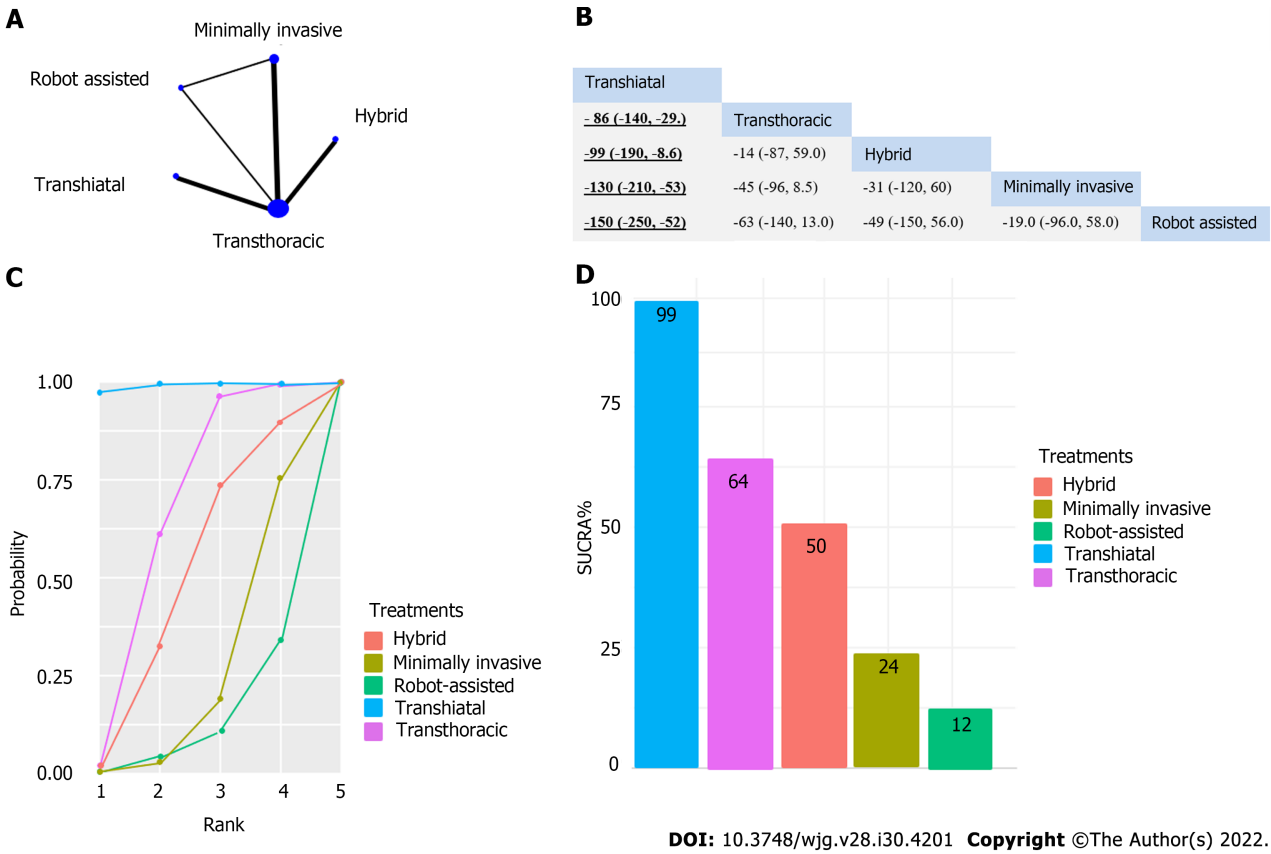
The results of the certainty of evidence are presented in Supplementary Table 1.

DISCUSSION

Our NMA confirmed the superiority of the minimally invasive esophagectomy over transthoracic open surgery regarding one of the main complications during these procedures, namely pulmonary infection. On the other hand, non-open surgical techniques require significantly more time to perform compared to open techniques. While statistically significant results were only achieved in the case of pulmonary infection, a clear tendency was demonstrated by the SUCRA curves, showing a preference for non-open techniques, which is also supported by the individual studies.

The results of previous meta-analyses and systematic reviews are not congruent regarding the comparison of minimally invasive and open surgical techniques. Kauppila *et al*[14] described the superiority of minimally invasive esophagectomy (MIE) regarding quality of life (QoL), which our work failed to analyze, as there were not enough RCTs reporting on QoL. Guo *et al*[13] also described the advantages of minimally invasive techniques regarding total complication rate, intraoperative blood loss, wound infection, and pulmonary infection, supporting our findings. MIE was also favorable in the analysis of Wang *et al*[19] considering blood loss. Besides blood loss and hospital stay, fewer respiratory complications were also shown by MIE in a meta-analysis conducted by Nagpal *et al*[15]. The work of Yibulayin *et al*[18] also supports the superiority of MIE in terms of in-hospital mortality and postoperative morbidity. By contrast, Dantoc *et al*[12] focused on oncological outcomes in their meta-analysis, where significant differences could not be proven. Sgourakis *et al*[17] showed that open surgery was more beneficial in terms of anastomotic stricture, while morbidity favored MIE. Oor *et al*[16] also described the benefit of open surgery in the case of hiatal hernia. The above comprehensive studies show that the inclusion of non-randomized studies carries a notable limitation.

Although the results of our analysis are only supportive in terms of pulmonary complication, the future perspectives are promising regarding minimally invasive esophagectomy, as the limelight shifts towards robot-assisted surgical techniques. The technique is time consuming, but with the development



DOI: 10.3748/wjg.v28.i30.4201

Figure 3 Operation time was significantly shorter for transhiatal approach compared to transthoracic surgery, hybrid intervention, minimally invasive technique, and robot-assisted esophagectomy. A: The network of eligible studies for operation time [the width of the lines is proportional to the number of trials comparing every pair of treatments, and the size of every circle is proportional to the number of randomly assigned participants (sample size)]; B: League table of the analysis for operation time. Comparisons should be read from left to right. The values are presented in weighted mean difference (minutes), with corresponding credible interval. Significant results are in TextTitle and underlined; C: Cumulative probability of interventions rank; D: Intervention ranking in surface under the cumulative ranking (SUCRA)% histogram.

of new robotic platforms, the benefit of less AEs and more precise procedure will overcome this limitation[36]. The steep learning curve will be possibly managed by allowing the intervention to be carried out only in larger centers, as it has been seen in northern countries[37]. Despite the missing cumulative evidence, minimal invasive techniques have become the gold standard interventions for esophageal cancer since the TIME study. The results of this RCT provide evidence for using minimally invasive surgery for patients with resectable esophageal cancer aimed toward improving postoperative outcomes (especially pulmonary complication) and QoL with comparable oncologic results[25].

Considering the strengths of our analysis, by the inclusion of only RCTs, we managed to achieve a higher quality of evidence than previous works. Furthermore, a thorough methodology was applied. With the application of NMA, we were also able to make indirect comparisons. To date, this work is the most comprehensive review of the available RCTs.

One of the limiting factors of our study was the low number of cases and limited number of direct comparisons. Other limitations were the different enrollment criteria of the individual studies considering the histological subtype and stage of esophageal cancer. Furthermore, our analysis included many indirect comparisons, with weak direct comparisons. Additionally, we only included studies published until 2019.

We emphasize the application of MIE over open surgical techniques. Further analyses should focus on the outcomes of robot-assisted esophagectomies, and direct comparisons should be carried out between robot-assisted esophagectomy and thoracolaparoscopic intervention. Following recent trends, the centralization of upper gastrointestinal surgery is suggested, thus achieving the possibility of the implementation of such techniques without the limitation originating from the low number of cases and the learning curve of minimally invasive techniques.

CONCLUSION

While practice is already shifting towards the application of minimally invasive techniques, it should be

noted that clear evidence is still needed to form guidelines. As we aimed to fill this void, we were only able to prove the beneficial nature of these techniques regarding pulmonary infection. To further assess any other potential differences between the techniques, RCTs and systematic analysis of these trials are needed.

ARTICLE HIGHLIGHTS

Research background

The differences considering esophagectomies as the most applied curative methodology in the case of esophageal cancer are not clearly described. Minimally invasive techniques have become more popular in the belief of their superiority, although objective evidence is missing.

Research motivation

Recent guidelines are not yet clear considering the usage of minimally invasive esophagectomies. The authors wanted to provide the most objective evidence available, considering the differences between every subtype of minimally invasive and open esophagectomies.

Research objectives

The authors aimed to find every randomized controlled trial (RCT) providing comparative information about at least two types of esophagectomies, and pool the results using NMA.

Research methods

After establishing our clinical question using the population, intervention, control, outcome (commonly known as (PICO) framework a systemic search was carried out using three different databases. The results of the search were pooled, duplications were removed, suitable studies were selected, from which the data extraction was carried out onto a data sheet. With the help of biostatisticians, a network meta-analysis was performed. The quality of the included studies was assessed, as well as the grade of evidence.

Research results

Eleven articles were included in our analysis, according to which the minimally invasive surgical technique was superior compared to the transthoracic open approach in terms of pulmonary infection, while transthoracic surgery took less time to perform than any other surgical technique.

Research conclusions

The authors conclude that minimally invasive surgical techniques should be performed, whenever possible, for resectable esophageal cancer.

Research perspectives

The conduction of additional RCTs evaluating the same problem would be welcomed, while we hope that our work will help clinicians in the decision-making of the selection of the right surgical technique.

FOOTNOTES

Author contributions: Szakó L conceptualized the work, contributed to establishment of the search key, selection strategy, data extraction, interpretation of the results, and writing of the manuscript; Németh D and Farkas N performed the bio-statistical analyses, and contributed to the interpretation of the results and writing of the manuscript; Kiss S helped was involved in the conceptualization, coordination of the work, and writing the manuscript; Dömötör RZ conceptualized, wrote, and critically appraised the manuscript; Engh MA was involved in the conceptualization, data extraction, risk of bias assessment, writing of the manuscript, and language revision of the manuscript; Hegyi P contributed to the conceptualization, interpretation of the results, critical appraisal, and writing of the manuscript; Erőss BM conceptualized the work, interpreted the results, critically appraised and wrote the manuscript; Papp A provided supervision, and was involved in the conceptualization, interpretation of the results, critical appraisal, and writing of the manuscript.

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CASE REPORT

Contrast-enhanced ultrasound of a traumatic neuroma of the extrahepatic bile duct: A case report and review of literature

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Abstract

BACKGROUND

Traumatic neuromas result from nerve injury after trauma or surgery but rarely occur in the bile duct. However, it is challenging to diagnose traumatic neuromas correctly preoperatively. Although some previous reports have described the imaging features of traumatic neuroma in the bile duct, no features of traumatic neuromas in the bile duct have been identified by using contrast-enhanced ultrasound (CEUS) imaging before.

CASE SUMMARY

A 55-year-old male patient presented to our hospital with a 3-mo history of abdominal distension and anorexia and history of cholecystectomy 4 years ago. Grayscale ultrasound demonstrated mild to moderate intrahepatic bile duct dilatation. Meanwhile, a hyperechoic nodule was found in the upper extrahepatic bile duct. The lesion approximately 0.8 cm × 0.6 cm with a regular shape and clear margins. The nodule of the bile duct showed slight hyperenhancement in the arterial phase and isoenhancement in the venous phase on CEUS. Laboratory tests showed that alanine aminotransferase and aspartate aminotransferase were increased significantly, while the tumor marker carbohydrate antigen 19-9 was increased slightly. Then, hilar bile duct resection and end-to-end bile ductal anastomosis were performed. The histological examination revealed traumatic neuroma of the extrahepatic bile duct. The patient had an uneventful recovery after surgery.

CONCLUSION

The current report will help enhance the current knowledge regarding identifying traumatic neuromas by CEUS imaging and review the related literature.

Key Words: Traumatic neuroma; Bile duct; Contrast-enhanced ultrasound; Enhancement; Cholangiocarcinoma; Case report

Core Tip: A traumatic neuroma results from nerve injury after trauma or surgery but rarely occurs in the bile duct. Herein, we present some of the sonographic features of ultrasound and contrast-enhanced ultrasound in a case of a traumatic neuroma. We report this unusual case and review the related literature to improve the diagnosis and differential diagnosis of a traumatic neuroma of the bile duct and related imaging findings.

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INTRODUCTION

A traumatic neuroma is a chronic reparative proliferative response of the nerve after trauma or surgery. It is composed of disorganized nerve fiber bundles with fibrous stroma, Schwann cells, perineural cells, axons, and endoneural fibroblasts[1]. The common sites of traumatic neuromas are the necks and extremities[2,3]. Although some studies have described traumatic neuromas in the bile duct, cases of sonographic features of contrast-enhanced ultrasound (CEUS) have not been published before. The clinical manifestation and imaging examination of a traumatic neuroma of the bile duct are not specific, which makes it challenging be accurately diagnosed preoperatively. Herein, we report a traumatic neuroma of the extrahepatic bile duct with detailed ultrasonographic imaging features. We also reviewed the literature on the imaging findings for traumatic neuromas.

CASE PRESENTATION

Chief complaints

A 55-year-old man was admitted to our hospital with unexplained abdominal distension and anorexia 3 mo ago.

History of present illness

The patient suffered from unexplained abdominal distension and anorexia for 3 mo. The patient developed darkened urine 2 mo ago. He experienced a weight loss of 5 kg over the course of the disease. He underwent contrast-enhanced computed tomography (CECT) examination at a local hospital, and a lesion was found in the extrahepatic bile duct, which was believed to be a tumor.

History of past illness

The patient underwent cholecystectomy for gallbladder stones with an uneventful postoperative recovery 4 years ago. He had a 10-year history of hypertension.

Personal and family history

There was no other personal or family history of acute or chronic disease.

Physical examination

The patient showed no tenderness, rebound tenderness or muscle tension on abdominal palpation.

Laboratory examinations

The liver function tests demonstrated increased levels of alanine aminotransferase (185 IU/L, normal range: < 50 IU/L), aspartate aminotransferase (148 IU/L, normal range: < 40 IU/L) and total bilirubin (37.0 μmol/L, normal range: 5 μmol/L to 28 μmol/L). Tumor markers included carbohydrate antigen 19-9 (98.6 U/mL, normal range: < 22 U/mL), carcinoembryonic antigen (0.97 ng/mL, normal range: < 5 ng/mL), and alpha-fetoprotein (4.67 ng/mL, normal range: < 7 ng/mL).

Imaging examinations

The patient underwent an abdominal ultrasound (US) examination by a Resona7 US system (Mindray Medical International, Shenzhen, Guangdong Province, China) equipped with an SC6-1U (1-6 MHz)

transducer. The US revealed mild to moderate dilatation of the intrahepatic bile duct, and the diameter of the upper extrahepatic bile duct was 1.2 cm (Figure 1A). A hyperechoic nodule sized 0.8 cm × 0.6 cm was found in the upper extrahepatic bile duct with an almost regular shape and slightly clear margins (Figure 1B). The patient underwent CEUS with the patient's consent for further diagnosis. A 2.4-mL US contrast agent SonoVue (Bracco, Milan, Italy) suspension was injected through the left cubital vein followed by a flush with 5 mL saline. In the arterial phase, the nodule showed slight heterogeneous hyperenhancement without rim-like enhancement (Figure 1C). The nodule appeared heterogeneous isoenhancement in the venous phase (Figure 1D). Additional CECT in our hospital showed a hypoenhancement nodule approximately 1.3 cm × 1.0 cm in size in the upper extrahepatic bile duct (Figure 2).

FINAL DIAGNOSIS

Based on the incidence of bile duct diseases, imaging findings and laboratory tests, the patient's clinical diagnosis was hilar cholangiocarcinoma. However, postoperative pathology of the common bile duct lesion showed a neoplastic proliferation of submucosal nerve tissue and fibrous tissue (Figure 3A), and an immunohistochemistry marker was positive for S-100 (Figure 3B). The above pathological findings indicated that the lesion in the bile duct was a traumatic neuroma.

TREATMENT

During the surgery, intraoperative frozen pathology showed no tumor cells within the bile duct lesion. Therefore, hilar bile duct resection and end-to-end bile ductal anastomosis (EE) were performed. The patient recovered uneventfully after surgery.

OUTCOME AND FOLLOW-UP

There was no obvious abnormality on CECT for half a year after the operation.

DISCUSSION

Extrahepatic bile duct masses are commonly malignant tumors, while benign tumors account for only 6%[4-7]. Consequently, the possibility that extrahepatic bile duct lesions are traumatic neuromas is easily overlooked. It has been reported that most traumatic neuromas of the biliary tract arise in the cystic duct stump after cholecystectomy[8]. If a nerve is transected and its continuity cannot be reestablished, a traumatic neuroma may develop[9].

We reviewed the literature from 2000 to 2021 and found 18 publications regarding the imaging features of traumatic neuromas in the bile ducts[2,10-26]. The clinical findings and imaging features of these 18 reported cases are summarized in Table 1. Finally, 22 patients were included in the literature review for further analysis. The age of patients ranged from 17 to 81 years of age, and there was a significant male predominance, with 15 males (68.2%), 2 females, and 5 patients of unreported sex. Most cases were secondary to cholecystectomy, but a few were secondary to liver transplantation, hepatectomy and hilar cholangiocarcinoma. The major symptoms found in these patients were jaundice, abdominal pain, and weight loss, while some patients had no apparent symptoms.

Unfortunately, no specific imaging features for traumatic neuromas of the bile duct have been found at present. Although some imaging modalities, such as US, computed tomography (CT), and nuclear magnetic resonance imaging (MRI), are valuable to some extent, it remains a challenge to diagnose traumatic bile duct neuromas preoperatively[17]. Imaging findings in these 22 patients varied from nodules or masses to localized bile duct stenosis with dilatation of the upper bile duct. It has been reported in the literature that the US imaging findings of extraabdominal nerve tumors and traumatic neuromas are generally hypoechoic masses, larger than the nerve trunk and continuous with the nerve [27]. However, the nerve injury related to cholecystectomy may be too small, so we could not find that the nerve is connected to traumatic neuroma of the bile duct. US was performed in 5 of the 22 patients, 2 of whom showed hypoechoic nodules, and the remaining 3 patients showed stenosis and dilatation of the bile ducts. However, our patient's US sonogram showed a hyperechoic nodule, indicating that the echogenicity of the nodule of traumatic neuroma was variable.

CECT was performed in 2 of the 18 cases, and an enhancing nodule was seen, which was consistent with the CECT findings of our patient. Traumatic neuromas also show enhancement on MRI when a contrast agent is used[28], which may be related to a damaged peripheral nerve blood barrier that occurred during a prior insult to the nerve[29-32]. One of these 18 cases described the enhancement

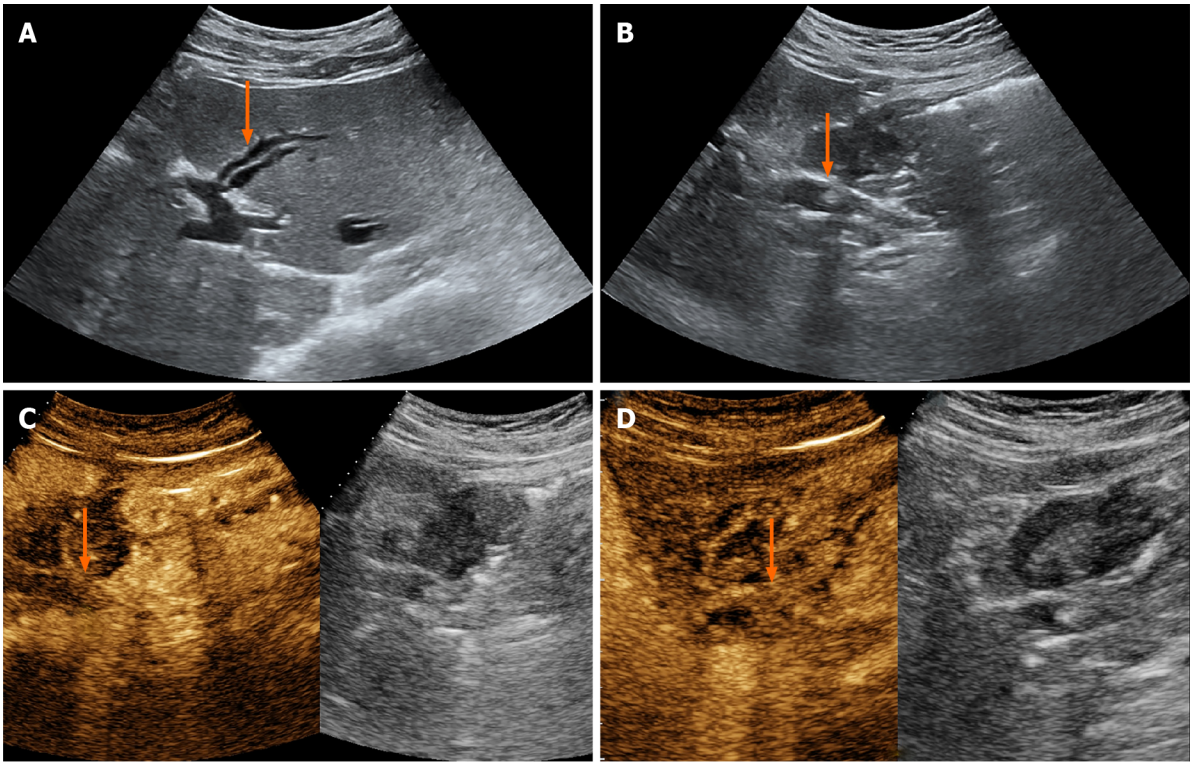

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Figure 1 Ultrasound images of the patient. A and B: The ultrasound (US) showed mild to moderate intrahepatic bile duct dilatation (orange arrow) and a hyperechoic nodule sized 0.8 cm × 0.6 cm (orange arrow) in the extrahepatic bile duct; C and D: In the arterial phase, contrast-enhanced US (CEUS) showed slight hyperenhancement (orange arrow); in the venous phase, CEUS showed isoenhancement (orange arrow).

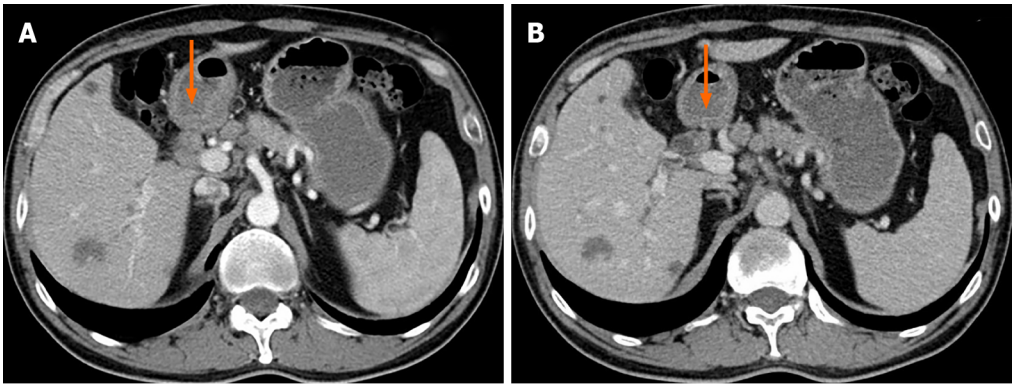

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Figure 2 Contrast-enhanced computed tomography images of the patient. Contrast-enhanced computed tomography showed a hypoenhancement nodule in the upper extrahepatic bile duct (orange arrow).

pattern of traumatic neuroma on MRI in detail, which showed a marked homogeneously enhanced nodule that was iso-intense to the aorta in the atrial phase and a homogeneously enhanced nodule that was iso-intense to the aorta in the portal phase. There have been a few reports of other imaging techniques for diagnosing traumatic neuromas, such as magnetic resonance cholangiopancreatography, endoscopic US, contrast-enhanced harmonic endoscopic ultrasonography, intraductal ultrasonography and percutaneous transhepatic cholangiography. None of these imaging methods revealed specific features for traumatic neuromas.

It is challenging to distinguish bile duct traumatic neuroma from other lesions before surgery, so it is often misdiagnosed. The diagnosis of bile duct traumatic neuroma was correctly diagnosed in 1 of the 18 cases examined and confirmed by biopsy. The remaining cases were not correctly diagnosed, and it was difficult to distinguish between benign and malignant lesions in most cases. Therefore, surgery would be performed on a large proportion of patients. Once the patient underwent surgery, an intraoperative frozen section examination helped to confirm that the lesion was benign and extensive surgical

Table 1 Traumatic neuroma of the bile duct reported in the literature between 2000 and 2020

Ref.	Age	Sex	Symptoms	Location	Imaging findings	Preoperative diagnosis	Treatment
Shimura *et al*[10]	70	F	Abdominal discomfort	Extrahepatic bile duct	US: Hypoechoic tumor, bile duct slightly dilated CT: Round, hyperdense, distinct margin tumor Angiography: No encasement of the surrounding major vessels Endoscopic retrograde cholangiography: A protuberant nodule Intraductal ultrasonography: A smooth hypoechoic tumor	Did not indicate bile duct carcinoma	Bile duct excision and a Roux-en-Y hepaticojejunostomy
Watanabe *et al*[11]	48	M	Jaundice	Extrahepatic bile duct	Cholangiogram *via* the percutaneous transhepatic biliary drainage tube: The extrahepatic bile duct severely stenotic	ND	Bile duct excision and a Roux-en-Y hepaticojejunostomy
Iannelli *et al* [12]	81	M	Jaundice	Common bile duct	US: Dilatation of the intrahepatic bile ducts MRCP: A focal stricture	ND	Bile duct excision and a Roux-en-Y hepaticojejunostomy
Ueno *et al*[2]	60	M	Jaundice	Mid-common bile duct	US: Dilatation of the bile ducts, a mildly echogenic mass CT: Dilatation of the bile ducts, a markedly enhanced nodule MRI: Dilatation of the bile ducts. Homogeneous enhanced nodule with an iso-intense to the aorta, both in the arterial and portal phase Percutaneous transhepatic cholangiography: Dilatation of the bile ducts and a smooth stricture	Could not confirm benign or malignant nature	Bile duct excision and a hepatojejunal anastomosis
Choi *et al* [13]	46	M	Increased liver enzymes	Right hepatic duct	CT: A mass approximately 2 cm MRI: A mass approximately 2 cm	A bile duct cancer could not be excluded	Right hemihepatectomy
Kim *et al*[14]	76	M	ND	Mid-bile duct	CT: A small enhancing nodule MRC: Eccentric wall thickening of the bile duct consistent with a neoplasm	ND	Segmental resection with a Roux-en-Y hepaticojejunostomy
Cheng *et al* [15]	33	F	Jaundice and weight loss	Remnant choledochal cyst	MRI: A mass	Cholangiocarcinoma	Excision of the remnant choledochal cyst and a new hepaticojejunostomy
Cheng *et al*	56	M	Jaundice, abdominal	Distal	US: Dilatation of bile	Ampullary or	Pancreaticoduodenectomy

[16]			pain and weight loss	extrahepatic bile duct	duct MRI: Dilatation of bile duct, a filling-defect in the distal bile duct and a thickened biliary wall around the ampulla of Vater	periampullary carcinoma	
Cheng *et al* [17]	68	M	Progressive jaundice and abdominal pain	Bifurcation of the left and right hepatic duct	MRI: A mass with enhancement, a stricture of the hilar bile duct, dilatation of bile ducts	Cholangiocarcinoma	Excision of the mass and a new Roux-en-Y hepaticojejunostomy
Navez *et al* [18]	ND	ND	Jaundice (3 patients) or liver function test alteration (1 patient), a retro-obstructive choleperitoneum on the downstream biliary stenosis (1 patient)	Anastomotic biliary stricture	CT: Anastomotic biliary stricture (4 patients) MRI: A markedly homogeneous high intensity nodule enhanced on portal-phase (1 patient), anastomotic biliary stricture (4 patients)	ND	Traumatic biliary neuromas resection combined with hepaticojejunostomy (1 patient); traumatic biliary neuromas resection and duct-to-duct biliary reconstruction protected by a T-tube (4 patients)
Terzi *et al* [19]	17	F	Persistent elevated transaminase and bilirubin levels	Anastomotic biliary	Percutaneous transhepatic cholangiography: A biliary stricture at the anastomosis	ND	Resection of the bile duct stricture and a Roux-en-Y hepaticojejunostomy
Toyonaga *et al*[20]	76	F	A bile duct nodule	Proximal common bile duct	CT: An 8 mm, smooth, and uniformly enhanced nodule Contrast enhanced endoscopic ultrasonography: A clear boundary and a low echoic nodule, uniformly enhanced at early Cholangioscopy: A smooth elevated lesion, covered with normal mucosa	Submucosal tumor	Biopsy, observation for 1 year, no changes to the nodule
Yang *et al* [21]	65	M	Jaundice	Right bile duct	MRI: A 1.0 cm × 1.5 cm mass	Cholangiocarcinoma	Resection of the mass and Roux-en-Y hepaticojejunostomy.
Hirohata *et al*[22]	60	F	No chief complaint	Junction of the cystic duct	US: A 6 mm round tumor, surrounding lymph nodes were not swollen MRI: A slightly high signal on T2 and the periphery remnant cystic duct of the tumor presented as a high-intensity lesion on T2 EUS: A residual cystic duct tumor with enhancement ERCP: Not invade the common bile duct	Cholangiocarcinoma	Surgery
Yasuda *et al* [23]	76	M	ND	Stump of the dilatated cystic duct	EUS: A hypoechoic oval mass with a hyperechoic rim on the surface, 14 mm in	Amputation neuroma	Biopsy, observation

					diameter, hypervascularity Cholangiogram: A hemispherical defect Cholangioscopy: A hemispherical mass covered with thin normal cystic duct epithelium		
Lalchandani *et al*[24]	41	M	Epigastric pain, weight loss, tea-colored urine	Common hepatic duct	US: Dilation of the bile ducts ERCP: A 3-4 cm stricture	Acute cholangitis	First: Biliary stent Finally: Bile duct resection and hepaticojejunostomy
Kim *et al*[25]	72	M	A duodenal subepithelial tumor during a medical checkup	Near the duodenal wall and the cystic duct stump	CT: A 1.4 cm mass EUS: An 18 mm hypoechoic mass	Duodenal subepithelial tumor	Resection of the mass and duodenal wall, en-block resection of the mass and cystic duct origin
Nechi *et al*[26]	76	M	Jaundice	The transition zone between the common hepatic duct and the main bile duct	US: Dilation of the bile ducts, a 5 mm hypoechoic nodule MRI: Dilation of the common hepatic duct	Could not confirm benign or malignant nature	Resection of the main bile duct with a choledocho-duodenal anastomosis

ND: Not described; US: Grayscale ultrasound; CT: Computed tomography; CECT: Contrast-enhanced computed tomography; MRI: Magnetic resonance imaging; MRC: Magnetic resonance cholangiogram; MRCP: Magnetic resonance cholangiopancreatography; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography.

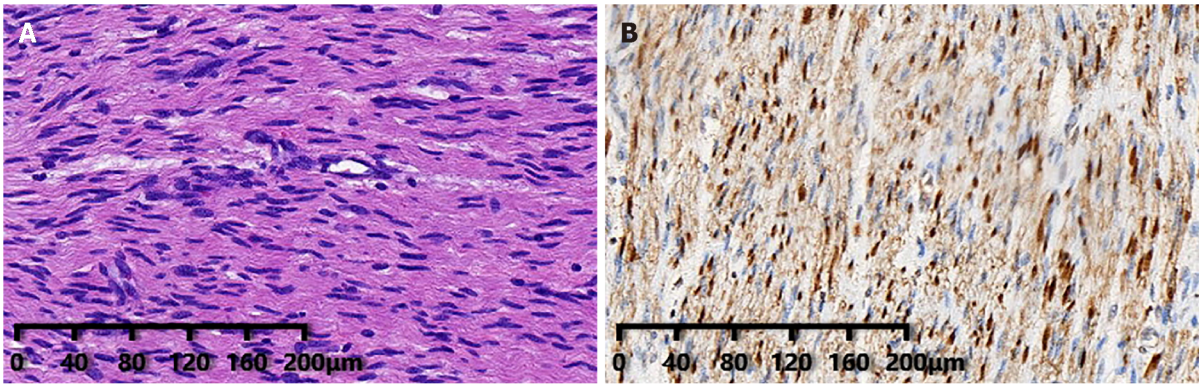

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Figure 3 Postoperative histopathological images of the patient. A: Hematoxylin and eosin staining showed proliferation of submucosal nerve tissue (magnification, × 100); B: Immunohistochemical staining displayed S100(+) (magnification, × 100).

resection of the traumatic neuroma was avoided[2,3]. The primary treatment reported in the literature consists of bile duct excision and hepaticojejunostomy (HJ). Although HJ is frequently recommended for reconstruction, the indications, surgical options and suture selection are also controversial. Some investigators also recommend EE because it is more physiological and can maintain physiological balance [33]. It is possible to achieve excellent long-term results and high quality of life using both HJ and EE when it is feasible for the proximal and distal ductal ends to permit EE[34]. Therefore, the choice of the optimum method is strictly correlated with the morphological nature of the lesion, which is different from one stage to the other, depending upon the moment of detection, and therefore have different surgical implications[35]. The surgeon found that the anastomosed edges blood supply was good and that there was no tension of the anastomosed edges in this patient. Therefore, according to the actual conditions of patients, as well as to maintain physiological balance, our hospital professor implemented EE for this patient.

In this patient, the symptoms of anorexia, weight loss and jaundice mimicked those often caused by malignant tumors of extrahepatic bile ducts. CEUS and CECT showed enhancement of the nodule. Based on the incidence of bile duct diseases and imaging findings, the surgeons misdiagnosed it as cholangiocarcinoma. Periductal infiltrative cholangiocarcinomas account for the majority of extrahepatic cholangiocarcinomas[36]. Extrahepatic cholangiocarcinomas may show hyperenhancement, isoenhancement, or hypoenhancement in the early phase of CEUS, and most of them show hypoenhancement in the late phase[37]. If we find a nodule in the bile duct, we should rule out the diagnosis of cholangiocarcinoma when the nodule does not show hypoenhancement in the late phase of CEUS. However, when traumatic neuroma presents as localized bile duct stenosis, it is relatively difficult to distinguish it from malignant lesions. When a patient has a history of biliary system surgery and the tumor markers are not significantly elevated, suspicion of traumatic neuroma increases. If conditions permit, patients can be protected from unnecessary surgeries by confirming the diagnosis with a biopsy. CEUS is beneficial for differentiating cholangiocarcinoma from traumatic neuromas, but more cases are needed to summarize the sonographic features of this disease. Recognizing of traumatic neuromas may aid in preoperative work up, planning, and patient counseling[24].

CONCLUSION

It is difficult to correctly diagnose traumatic neuroma of the bile duct before surgery. We should rule out malignant differential diagnoses, such as cholangiocarcinoma preoperatively, to avoid unnecessary surgery. The enhancement mode of CEUS may provide information to distinguish traumatic neuromas from malignant lesions. We need to combine the history of biliary tract surgery, clinical findings, imaging findings and laboratory tests to diagnose this disease.

FOOTNOTES

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LETTER TO THE EDITOR

Prognostic role of expression of angiogenesis markers in hepatocellular carcinoma: A bioinformatics analysis

Yan-Dong Miao, Xiao-Long Tang, Jiang-Tao Wang, Deng-Hai Mi

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Abstract

The expression of angiopoietin (ANGPT) 1, ANGPT2, vascular endothelial growth factor (VEGF) A, VEGFB, VEGFC, VEGFD, and placental growth factor (PGF) is significantly higher in tumor tissues than in normal tissues in both unpaired and paired hepatocellular carcinoma (HCC) samples. ANGPT2, VEGFB, VEGFC, and PGF are primarily involved in regulating the activation of the epithelial-mesenchymal transition pathway; ANGPT1 is primarily involved in regulating the activation of the RAS/mitogen-activated protein kinase and receptor tyrosine kinase (RTK) pathways; VEGFA is engaged in regulating the RTK activation pathway; and VEGFD is mainly involved in regulating the activation of the tuberous sclerosis protein/mammalian target of rapamycin pathway. There is a significant difference in overall survival between HCC patients with high and low expression of ANGPT2, PGF, VEGFA, and VEGFD. Disease free survival (DFS) is significantly shorter in HCC patients with high ANGPT2, PGF, and VEGFA expression than in those with low ANGPT2, PGF, and VEGFA expression.

Key Words: Hepatocellular carcinoma; Angiogenesis; Marker; Bioinformatics analysis; Pathway

Core Tip: We found that the expression of angiogenesis markers was significantly higher in tumor tissues than in normal tissues in both unpaired and paired hepatocellular carcinoma (HCC) samples. These angiogenesis markers are mainly involved in regulating the activation of the EMT pathway, the RAS/mitogen-activated protein kinase and receptor tyrosine kinase pathways, and the tuberous sclerosis protein/mammalian target of rapamycin pathway. In addition, there was a significant difference in overall survival between HCC patients with high and low expression of angiopoietin-2 (ANGPT2), placental growth factor (PGF), vascular endothelial growth factor A (VEGFA), and VEGFD. Disease free survival was significantly shorter in HCC patients with high ANGPT2, PGF, and VEGFA expression than in those with low ANGPT2, PGF, and VEGFA expression.

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TO THE EDITOR

We read with read interest the article by Choi *et al*[1], in which they initially evaluated plasma levels of angiogenesis biomarkers in hepatocellular carcinoma (HCC) patients, and then assessed their roles in forecasting overall survival (OS) and progression-free survival (PFS), indicating that the plasma level of angiopoietin (ANGPT) 2 was related to tumor stage, liver function, and cancer invasiveness, and that ANGPT2 performed better in predicting OS and PFS than alpha-fetoprotein (AFP), ANGPT1, and vascular endothelial growth factor (VEGF).

We appreciate the authors' unique perspective in exploring the prognostic role of plasma levels of ANGPT1, ANGPT2, and VEGF in HCC. However, there are some errors in the original text that may cause confusion for readers. For example, the survival curve in figure 3B in the original article should have represented the survival curve between the high and low ANGPT2 expression subgroups, which the authors incorrectly labeled as ANGPT1. Second, it is well known that the VEGF family includes VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, and placental growth factor (PGF)[2,3], so to which VEGF do the authors refer in the text? Usually, VEGF refers to VEGFA, but the authors should have clarified it in the text.

Moreover, it might make the results more significant if the authors could improve the outcome by demonstrating the differential expression of ANGPT1, ANGPT2, and VEGF in normal tissues and HCC tissues as a whole, for example, the analysis of HCC samples in the Cancer Genome Atlas database using bioinformatics. We found that the expression of ANGPT1, ANGPT2, VEGFA, VEGFB, VEGFC, VEGFD, and PGF was significantly higher in cancer samples than in corresponding normal samples in both unpaired and paired HCC samples (Figures 1A and B). Detailed statistical results are reported in Tables 1 and 2.

We also found that ANGPT2, VEGFB, VEGFC, and PGF are mainly involved in regulating the activation of the EMT pathway; ANGPT1 is prominently involved in regulating the activation of the RAS/mitogen-activated protein kinase and receptor tyrosine kinase (RTK) pathways; VEGFA is engaged in regulating the activation of the RTK pathway; and VEGFD is mainly involved in regulating the activation of the tuberous sclerosis protein/mammalian target of rapamycin pathway (Figure 1C). These results are consistent with those of previously reported studies[4-7]. Our findings could be a supplement to Choi *et al*'s study[1]. In the future, the roles of ANGPT1, ANGPT2, and VEGF in the development of HCC should be further explored.

Choi *et al*[1] found that OS was significantly shorter in the high ANGPT2 and high AFP subgroups than in the low ANGPT2 and AFP subgroups, respectively, though the differences in OS rates were not significant between the high and low ANGPT1 subgroups or between the high and low VEGF subgroups. Our study found that OS was significantly shorter in patients with high ANGPT2, PGF, VEGFA, or VEGFD expression than in those with low expression, respectively (Figures 2A-D; $P < 0.05$). However, there was no significant difference in survival time between patients with high and low expression of ANGPT1, VEGFB, VEGFC, and AFP (Figures 2E-H; $P > 0.05$). Prognostic data for HCC came from Liu *et al*[8].

In addition, we also analyzed the differences in disease free survival (DFS) between patients with high and low angiogenesis marker expression. We found that DFS was significantly shorter in the high ANGPT2, PGF, and VEGFA groups than in the low ANGPT2, PGF, and VEGFA groups, respectively (Figures 3A, B and C; $P < 0.05$). However, there was no significantly difference in DFS between groups with high and low expression of AFP, ANGPT1, VEGFB, VEGFC, and VEGFD (Figures 3D-H; $P > 0.05$). The above results confirm that the study performed by Choi *et al*[1] is of great value and that our discovery could be a supplement to their research.

Table 1 Detailed statistical results of differential expression analysis of angiogenesis markers in hepatocellular carcinoma

Gene	Group	Number	Minimum	Maximum	Median	IQR	Lower quartile	Upper quartile	Mean	SD	SE
ANGPT1	Normal	50	0.029	0.552	0.188	0.132	0.138	0.27	0.206	0.106	0.015
ANGPT1	Tumor	374	0	2.351	0.373	0.464	0.2	0.664	0.485	0.391	0.02
ANGPT2	Normal	50	0.043	1.351	0.278	0.33	0.195	0.525	0.394	0.289	0.041
ANGPT2	Tumor	374	0.116	3.339	0.848	0.769	0.513	1.282	0.963	0.581	0.03
VEGFA	Normal	50	1.616	3.901	2.687	0.473	2.439	2.911	2.717	0.445	0.063
VEGFA	Tumor	374	1.258	6.138	3.268	1.103	2.769	3.871	3.291	0.809	0.042
VEGFB	Normal	50	2.816	4.919	3.568	0.523	3.325	3.848	3.636	0.444	0.063
VEGFB	Tumor	374	0.978	8.003	4.532	2.234	3.223	5.458	4.292	1.521	0.079
VEGFC	Normal	50	0.408	1.901	1.019	0.453	0.787	1.239	1.057	0.355	0.05
VEGFC	Tumor	374	0.253	4.988	1.376	0.816	0.978	1.795	1.436	0.62	0.032
VEGFD	Normal	50	0.054	1.74	0.236	0.151	0.164	0.316	0.307	0.28	0.04
VEGFD	Tumor	374	0.014	6.756	0.422	0.622	0.241	0.863	0.838	1.14	0.059
PGF	Normal	50	0.182	0.992	0.471	0.204	0.37	0.575	0.501	0.188	0.027
PGF	Tumor	374	0.061	5.991	1.007	0.855	0.613	1.467	1.104	0.675	0.035
AFP	Normal	50	0.266	1.969	1.016	0.507	0.714	1.221	0.992	0.416	0.059
AFP	Tumor	374	0	13.118	1.644	2.855	0.844	3.699	2.965	3.15	0.163

ANGPT: Angiopoietin; VEGFA: Vascular endothelial growth factor; PGF: Placental growth factor; AFP: Alpha-fetoprotein; IQR: Interquartile range; SD: Standard deviation, SE: Standard error.

Table 2 Detailed statistical results of differential expression analysis of angiogenesis markers in paired samples of hepatocellular carcinoma

Gene	Group	Number	Minimum	Maximum	Median	IQR	Lower quartile	Upper quartile	Mean	SD	SE
ANGPT1	Normal	50	0.029	0.552	0.188	0.132	0.138	0.27	0.206	0.106	0.015
ANGPT1	Tumor	50	0.014	1.557	0.463	0.56	0.228	0.788	0.507	0.363	0.051
ANGPT2	Normal	50	0.043	1.351	0.278	0.33	0.195	0.525	0.394	0.289	0.041
ANGPT2	Tumor	50	0.193	2.324	1.056	0.77	0.747	1.517	1.111	0.517	0.073
VEGFA	Normal	50	1.616	3.901	2.687	0.473	2.439	2.911	2.717	0.445	0.063
VEGFA	Tumor	50	1.471	5.974	3.102	1.087	2.801	3.888	3.287	0.902	0.128
VEGFB	Normal	50	2.816	4.919	3.568	0.523	3.325	3.848	3.636	0.444	0.063
VEGFB	Tumor	50	1.164	7.789	4.833	1.993	3.323	5.317	4.328	1.575	0.223
VEGFC	Normal	50	0.408	1.901	1.019	0.453	0.787	1.239	1.057	0.355	0.05
VEGFC	Tumor	50	0.261	3.233	1.398	0.819	1.013	1.831	1.459	0.633	0.09
VEGFD	Normal	50	0.054	1.74	0.236	0.151	0.164	0.316	0.307	0.28	0.04
VEGFD	Tumor	50	0.014	5.746	0.367	0.562	0.231	0.793	0.832	1.207	0.171
PGF	Normal	50	0.182	0.992	0.471	0.204	0.37	0.575	0.501	0.188	0.027
PGF	Tumor	50	0.144	5.991	1.072	0.833	0.67	1.503	1.16	0.859	0.121
AFP	Normal	50	0.266	1.969	1.016	0.507	0.714	1.221	0.992	0.416	0.059
AFP	Tumor	50	0	5.824	1.033	1.31	0.725	2.036	1.62	1.383	0.196

ANGPT: Angiopoietin; VEGFA: Vascular endothelial growth factor; PGF: Placental growth factor; AFP: Alpha-fetoprotein; IQR: Interquartile range; SD: Standard deviation, SE: Standard error.

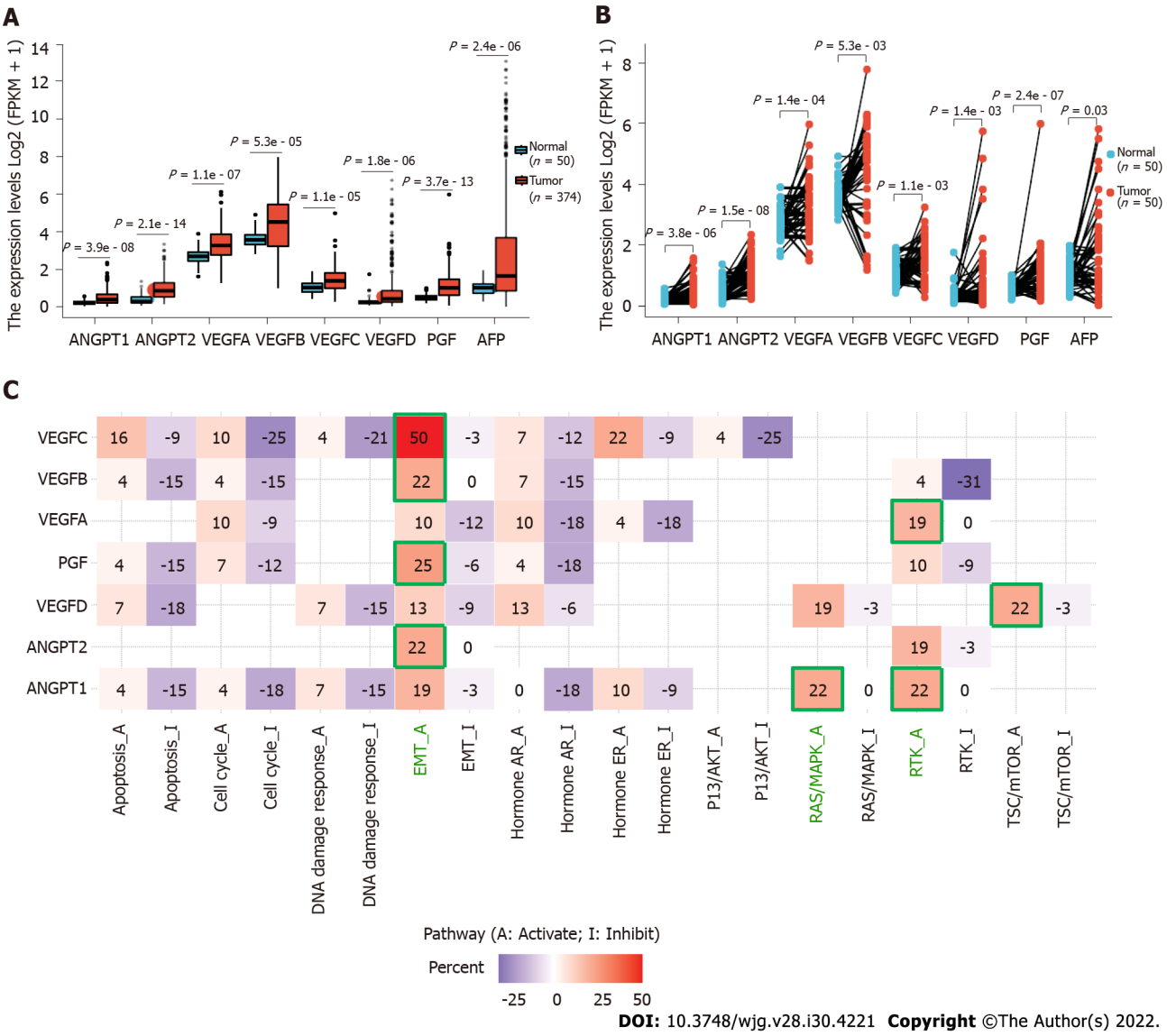

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Figure 1 Roles of angiopoietins 1 and 2, vascular endothelial growth factors A-D, and placental growth factor in development of hepatocellular carcinoma. Data source: UCSC XENA (https://xenabrowser.net/datapages/) mRNA-Seq data of TPM format for GTEx and TCGA processed uniformly *via* the Toil process[11]. Liver hepatocellular carcinoma tissue data from TCGA and corresponding normal tissue data from GTEx were used. A: Differential expression of angiopoietin (ANGPT) 1, ANGPT2, vascular endothelial growth factor (VEGF) A, VEGFB, VEGFC, VEGFD, and placental growth factor (PGF) in hepatocellular carcinoma (HCC) and normal tissue samples; B: Differential expression of ANGPT1, ANGPT2, VEGFA, VEGFB, VEGFC, VEGFD, and PGF in paired HCC and normal samples. The expression in cancer tissues is represented in orange, and that in normal tissues is displayed in blue; C: Pathway analysis for ANGPT1, ANGPT2, VEGFA, VEGFB, VEGFC, VEGFD, and PGF in HCC. ANGPT: Angiopoietin; PGF: Placental growth factor; VEGF: Vascular endothelial growth factor; AFP: Alpha-fetoprotein; EMT: Epithelial-mesenchymal transition; AR: Androgen receptor; ER: Estrogen receptor; P13K/AKT: Phosphatidylinositol 3 kinase/AKT; RAS/MAPK: RAS/mitogen-activated protein kinase; RTK: Receptor tyrosine kinase; TSC/mTOR: TSC/mammalian target of rapamycin.

Statistical analysis

We utilized R (version 4.0.3) to perform statistical analyses and display the results. The differential expression analysis of angiogenesis markers between HCC tissues and corresponding normal tissues was performed using the Wilcoxon rank-sum test, and the results are presented by using R-package "ggplot2"[9]. Survival analysis was completed through log-rank test and COX regression. Pathway analysis was performed based on the online database GSCALite (http://bioinfo.life.hust.edu.cn/web/GSCALite/)[10].

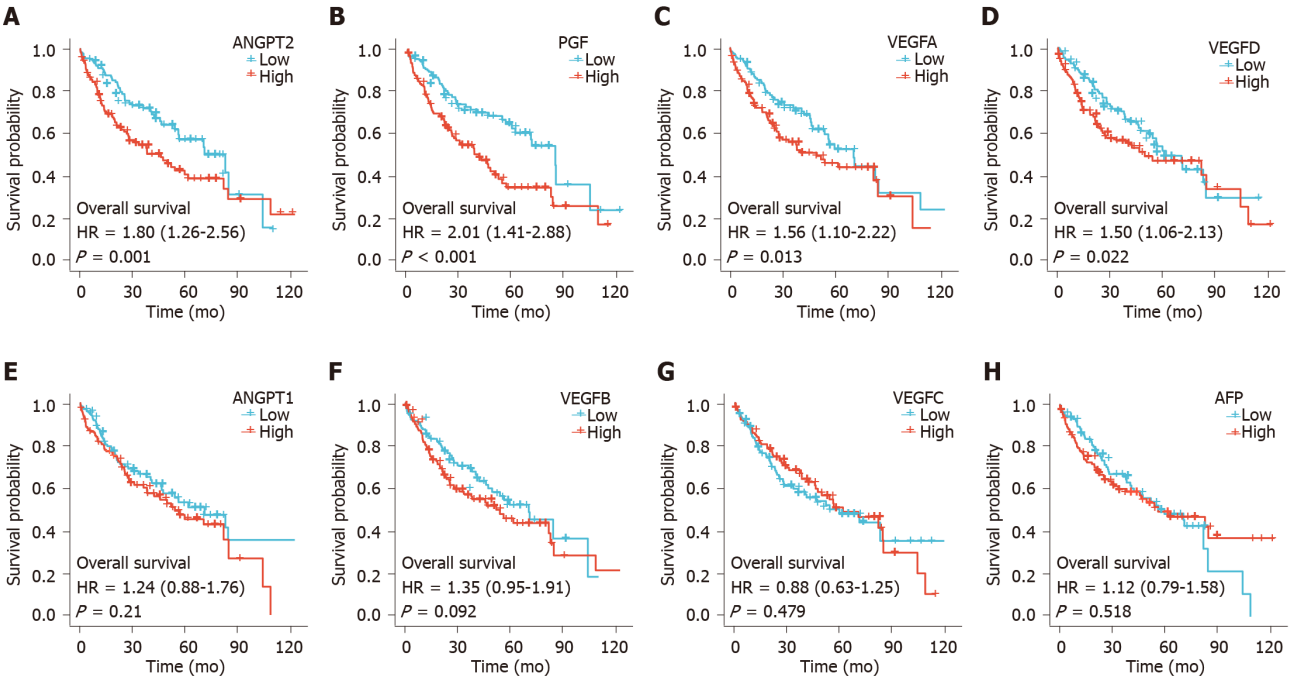

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Figure 2 Overall survival by angiogenesis marker expression in hepatocellular carcinoma. A: Angiopoietin (ANGPT) 2; B: Placental growth factor; C: Vascular endothelial growth factor (VEGF) A; D: VEGFD; E: ANGPT1; F: VEGFB; G: VEGFC; H: Alpha-fetoprotein. The red and blue lines indicate the survival curves of the high and low angiogenesis marker expression groups, respectively. ANGPT: Angiopoietin; PGF: Placental growth factor; VEGF: Vascular endothelial growth factor; AFP: Alpha-fetoprotein; HR: Hazard ratio.

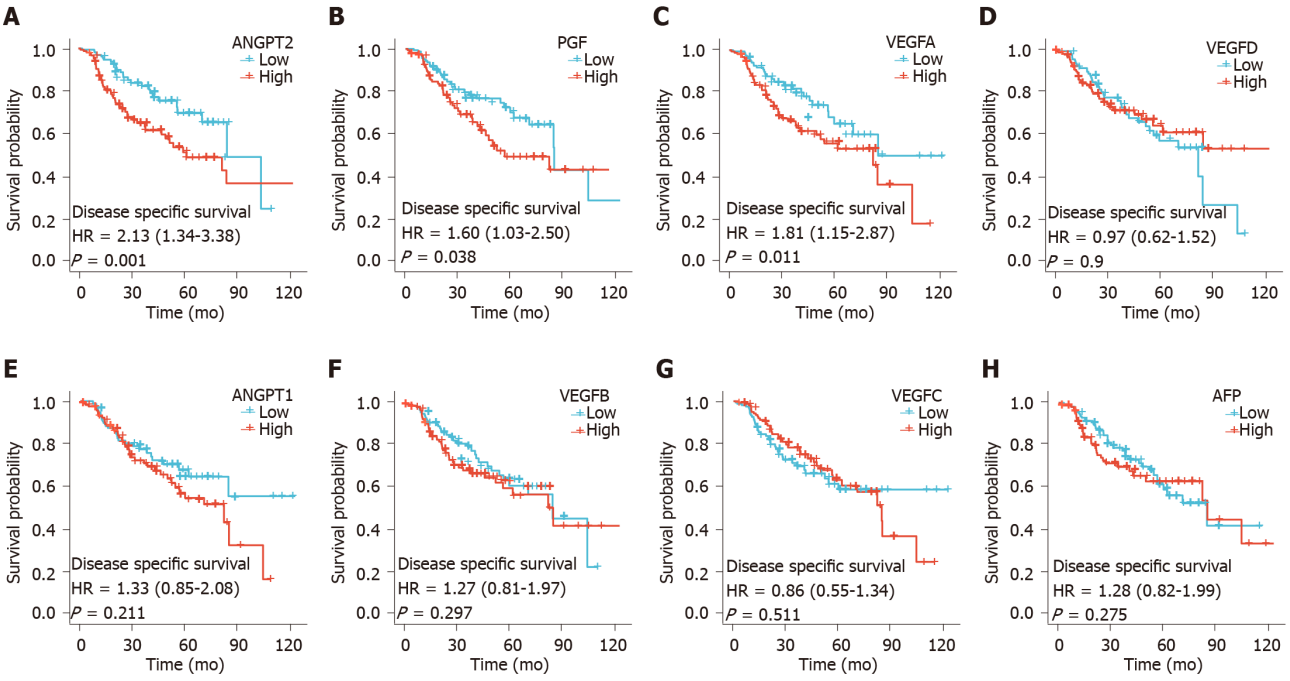

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Figure 3 Disease free survival by angiogenesis marker expression in hepatocellular carcinoma. A: Angiopoietin (ANGPT) 2; B: Placental growth factor; C: Vascular endothelial growth factor (VEGF) A; D: Alpha-fetoprotein; E: ANGPT1; F: VEGFB; G: VEGFC; H: VEGFD. The red and blue lines indicate the survival curves of the high and low angiogenesis marker expression groups, respectively. ANGPT: Angiopoietin; PGF: Placental growth factor; VEGF: Vascular endothelial growth factor; AFP: Alpha-fetoprotein; HR: Hazard ratio.

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FOOTNOTES

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LETTER TO THE EDITOR

Benefits of minimally invasive surgery in the treatment of gastric cancer

Simone Sibio, Francesca La Rovere, Sara Di Carlo

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Abstract

We read with great interest the article that retrospectively analyzed 814 patients with primary gastric cancer, who underwent minimally invasive R0 gastrectomy between 2009 and 2014 by grouping them in laparoscopic *vs* robotic procedures. The results of the study highlighted that age, American Society of Anesthesiologists status, gastrectomy type and pathological T and N status were the main prognostic factors of minimally invasive gastrectomy and showed how the robotic approach may improve long-term outcomes of advanced gastric cancer. According to most of the current literature, robotic surgery is associated with a statistically longer operating time when compared to open and laparoscopic surgery; however, looking at the adequacy of resection, defined by negative surgical margins and number of lymph nodes removed, it seems that robotic surgery gives better results in terms of the 5-year overall survival and recurrence-free survival. The robotic approach to gastric cancer surgery aims to overcome the difficulties and technical limitations of laparoscopy in major surgery. The three-dimensional vision, articulation of the instruments and good ergonomics for the surgeon allow for accurate and precise movements which facilitate the complex steps of surgery such as lymph node dissection, esophagus-jejunal anastomosis packaging and reproducing the technical accuracy of open surgery. If the literature, as well as the analyzed study, offers us countless data regarding the short-term oncological results of robotic surgery in the treatment of gastric cancer, satisfactory data on long-term follow-up are lacking, so future studies are necessary.

Key Words: Gastric cancer; Robotic gastrectomy; Laparoscopy; D2 lymphadenectomy; Long-term outcomes; Morbidity

Core Tip: Laparoscopic and robotic approaches are compared in the treatment of gastric cancer focusing on the prognostic factors as well as the oncological benefits brought about. While the long-term outcomes of laparoscopic surgery have been increasingly cited in recent years, only a few studies have analyzed the long-term results of the robotic approach, underlining the importance of future studies. A relevant aspect of robotic gastrectomy is the possibility to perform a more accurate lymph node dissection, which results in a longer survival with advanced gastric cancers.

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TO THE EDITOR

We read with interest the Nakauchi *et al*[1] study, which retrospectively examined 814 patients with primary gastric cancer undergoing a minimally invasive R0 gastrectomy, between 2009 and 2014 in Kanazawa (Japan), comparing the laparoscopic and robotic approach and looking at the 5-year overall survival (OS) and recurrence-free survival (RFS). We were pleased to see from the results of the study that the robotic approach could improve the long-term outcomes of advanced gastric cancer. The authors observed that the robotic approach led to significantly better RFS compared to the laparoscopic one in patients with p-Stage II/III tumors, although no significant difference in OS was detected, nor in OS and RFS in p-Stage patients treated with laparoscopy or robotics.

The study also revealed that age > 65 years, American Society of Anesthesiologists physical status 3, total or proximal gastrectomy, and disease status T4 and N positive, are all independent prognostic factors[1]. Since gastric cancer is the fifth most common malignancy in the world and the third cause of cancer death, it is worth it to identify the most appropriate technical approach for this disease being minimally invasive surgery the standard approach for several GI surgery procedures[2,3].

Surgical treatment remains the only therapeutic option with curative intent. Total or subtotal gastrectomy, associated with D2 lymphadenectomy, represents the therapeutic gold standard for gastric cancer. We must acknowledge that the traditional surgical approach, open surgery, remains the most widespread surgical technique. Although, laparoscopy has become almost constant in general surgery, the use of the laparoscopic technique for gastric surgery is yet scarce in the case of malignancies. As supported by various authors, laparoscopy has several technical drawbacks and limitations, including two-dimensional vision, stiffness of instruments, limited range of motion, amplification of hand tremors and uncomfortable surgical placement which makes some fundamental surgical steps, such as D2 lymphadenectomy, extremely complex[2,4].

According to the paper discussed, the pN factor is strongly associated with survival after gastric cancer treatment, confirming the thesis that laparoscopy in gastric cancer is more adequate in the earlier stages. In contrast, the safety and oncological adequacy of laparoscopic-assisted radical D2 gastrectomy for advanced gastric cancer are still under discussion[5]. From the meta-analysis, it emerges that the main variables associated with a statistically significant advantage of laparoscopic technique over open surgery are represented by: Reduced blood loss, lower complication rate, faster recovery and reduced pain at the expense of a longer surgical time and fewer lymph nodes removed, therefore a potential worse local control of the disease[6,7].

Alongside laparoscopy, robotic technology allows us to overcome the technical difficulties of laparoscopy, thanks to the three-dimensional vision, instruments' articulation and greater ergonomics for the surgeon, offering a better therapeutic approach to the minimally invasive treatment of stomach tumors. Thus, the short and medium term results of robotic gastric surgery can be almost compared with open and laparoscopic procedure when taken into account surgeon experience and technical implementation of the robotic system.

We fully agree with the authors, who have shown a significantly lower morbidity in the group of patients treated with robotics than in the laparoscopic group, as widely discussed in many studies. A recent meta-analysis, which compared laparoscopy with robotics in the treatment of gastric cancer, highlighted that the robotic approach appears to achieve better surgical results in the short term, also thanks to the ability to recover a greater number of lymph nodes, namely lymph nodes in station n. 7, 8a, 9 and 11p, which are avowedly more difficult to reach, ensuring a more appropriate staging and chemotherapy plan[8].

A study conducted in Japan reported, among the advantages of robotic surgery, a lower intraoperative blood loss, with a consequent reduction in the dissemination of cancer cells in the peritoneal cavity during surgery and, therefore, a better prognosis. Another aspect highlighted is a lower risk of dehiscence of the esophagus-jejunal anastomosis, along with a lower incidence of internal hernias[9-11]. From the short-term results it emerges that robotic gastrectomy is a safe technique that potentially allows to extend the number of patients treatable with a minimally invasive approach, overcoming the technical difficulties of laparoscopy, offering some benefits in terms of blood loss, conversion rate, overall number of lymph nodes removed and in suprapancreatic areas, procedure-specific postoperative morbidity and shorter length of hospital stay[12].

Robotic gastrectomy is a safe and effective surgical technique when performed by experienced surgeons, however, it is associated with a longer operative time and a higher economic value than laparoscopic and open approaches[13,14]. Indeed, one of the factors that slows down the spread of robotic surgery is the particular technical expertise required while handling the robotic devices, resulting in a steeper learning curve for the specialized operator. The cost and longer timeframe of robotics make future studies necessary[15], as well as the need for randomized controlled trials comparing the two techniques with a long-term follow-up, on which publications are still scarce given the relatively recent diffusion of the technique[16].

Given the greater cost of robotics, we want to underline one of the limitations of the study discussed here, represented by possible errors in the selection of patients. The availability of robotic devices is strongly dependent on the wealth of the country and of the individual; both patients who are aware of the advantages of the robotic approach and experienced surgeons who are able to perform this novel technique could lead to an overuse of the technique. In Western countries, robotic devices are associated with longer operative time, and higher costs but fewer post-operative complications resulting in lower hospitalization costs, and shorter hospital stays[17].

In conclusion, the study discussed here provides valid results on the correct therapeutic management of patients with gastric cancer, with the aim to bridge over some of the difficulties and technical limitations that laparoscopy encounters in major surgery. Essentially, laparoscopic D2 lymphadenectomy remains a challenging procedure: In particular, the dissection of the lymph nodes along the celiac, hepatic and splenic arteries makes this approach technically complicated and time-consuming even for well-trained surgeons. It is in this context that robotic surgery is worth looking at and it represents a useful tool that overcomes some limitations of conventional laparoscopic techniques, even if greater surgical and anesthetic times and the higher costs have to be considered when compared to open surgery.

Although, in accordance with the international literature that ascribes better results to robotic surgery in perioperative outcomes in terms of blood loss, and postoperative complications, future studies of higher quality are necessary due to the lack of data on long-term results, given the relatively recent diffusion of the technique. In a long-term perspective, considering the need for further studies on larger samples of patients from Western countries, we believe that robotic technology for gastric cancer surgery, taking into account the many advantages it offers, can become a gold standard[18].

FOOTNOTES

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LETTER TO THE EDITOR

Alcohol-related diseases and liver metastasis: Role of cell-free network communication

Manuel Muro, Aurelia Collados-Ros, Isabel Legaz

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Abstract

Alcohol intake is a risk factor for cancer development and metastatic disease progression. Extracellular vesicle (EV)-mediated interorgan communication is assumed to be significant in boosting tumorigenic pathways and disease progression. Recent research indicates that exosomes have a variety of roles in the development of cancer during pathophysiological conditions. The involvement of EV signaling during cancer progression in the alcohol environment is unknown. Therefore, understanding communication networks and the role of EVs as biomarkers can contribute significantly to developing strategies to address the serious public health problems associated with alcohol consumption and cancer.

Key Words: Exosomes; Liver metastasis; Alcohol-associated liver disease; Cancer

Core Tip: In this letter to the editor, we discussed the reality that alcohol consumption is a risk factor that acts by itself to favor the appearance of the carcinogenic process and its harmful evolution towards metastatic pathology. One of the hypotheses that have been suggested as important in metastasis and communication between cells and/or organs is the traffic of extracellular vesicles/exosomes that can play or promote tumorigenesis locally and even at a distance from the primary tumor. Unraveling these communication mechanisms and therapeutic possibilities may lead to new ways to combat cancer's worsening, as metastasis, in the future.

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TO THE EDITOR

We have read with great attention and particular interest the review by Kuracha *et al*[1] entitled: "Role of cell-free network communication in alcohol-associated disorders and liver metastasis". The authors highlight the many implications of extracellular vesicle (EV) (exosome) communications across organs in this review, focusing on the role of EVs in alcohol-related illnesses and cancer metastasis. It is crucial to consider the impact of EV cargo and release along a multi-organ axis on tumorigenic pathways and metastatic disease.

Alcohol consumption negatively impacts people's health and quality of life, contributing to more than 5% of the global disease burden and early death[2,3]. Alcohol intake has been linked to several neoplastic diseases, including colorectal, head and neck, esophageal, liver, breast, and pancreatic cancers[4,5]. On the other hand, recent research suggests that exosomes have different functions in disease progression during pathophysiological circumstances. Exosomes from tumors have been found to operate as regulatory factors in cancer development, promoting cell migration and proliferation and creating a pre-metastatic niche for cells resistant to treatment[6,7].

Hepatocytes and non-parenchymal cells produce and release EVs at higher rates in response to alcohol-mediated stress[8]. The EVs produced can alter gene expression and target cell function, prolonging liver damage[8]. Bidirectional exosomal communication between organs, including the liver, brain, intestine, and lungs, can also happen in addition to intra-organ transmission mediated by EVs. The gut-liver axis maintains bilateral interactions in an environment where alcohol is present, which results in gut dysbiosis and the progression of liver impairment[9,10].

In addition to persistent alcoholism, endotoxin transfer during sepsis and brain inflammation are caused by loss of intestinal barrier integrity. Alcohol dependency and its regulatory consequences, such as altered immunological function and neurological and endocrine signaling, are hypothesized to be influenced by alcohol-induced gut dysbiosis[11,12]. Acute respiratory distress syndrome, bacterial infection, and hepatopulmonary syndrome are also linked to persistent alcohol exposure on the liver-lung axis (ARDS)[13,14].

The significance of alcohol-induced EV communication in cancer initiation and progression is unknown until now because of the high prevalence of alcohol drinking and cancer-related risk. The therapeutic significance of the function of these exosomes has been highlighted by identifying EVs as critical mediators of communication networks within and across organ systems[7,15,16]. Clinical evaluation of EVs in body fluids provides another measure for understanding exosomes as valid and valuable diagnostic biomarkers and therapeutic targets.

Communication between malignant and non-cancerous cells, mediated by nanometric vesicles, is thought to be an essential part of tumor growth and its subsequent spread through the body. By promoting oncogene overexpression, stromal cell remodeling, immune system regulation, and angiogenesis, tumor-derived exosomes may control the course of cancer[17]. Cancer cells' ability to grow anchorage-independently is thought to be enhanced, and their morphological changes may be modulated by the transfer of tumor-causing material through EVs[18].

Additionally, miRNA-enriched EVs have also been demonstrated in cell-cell communications and the conversion of cells into populations with enhanced motility[19]. The involvement of EV signaling during cancer progression in the alcohol environment is unknown. Recent studies have shown that the exosomal content (proteins, miRNA, non-coding RNA) can help diagnose and treat cancer[20-22]. Therefore, comprehending EVs and communication networks as biomarkers can considerably aid in developing methods to deal with the serious public health issues brought on by alcohol intake and cancer.

FOOTNOTES

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