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Contents

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EDITORIAL

Evolving role of the endoscopist in management of gastrointestinal neuroendocrine tumors

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Abstract

Neuroendocrine tumors (NETs) are uncommon gastrointestinal neoplasms but have been increasingly recognized over the past few decades. Luminal NETs originate from the submucosa of the gastrointestinal tract and careful endoscopic exam is a key for accurate diagnosis. Despite their reputation as indolent tumors with a good prognosis, some NETs may have aggressive features with associated poor long-term survival. Management of NETs requires full understanding of tumor size, depth of invasion, local lymphadenopathy status, and location within the gastrointestinal tract. Staging with endoscopic ultrasound or cross-sectional imaging is important for determining whether endoscopic treatment is feasible. In general, small superficial NETs can be managed by endoscopic mucosal resection and endoscopic submucosal dissection (ESD). In contrast, NETs larger than 2 cm are almost universally treated with surgical resection with lymphadenectomy. For those tumors between 11-20 mm in size, careful evaluation can identify which NETs may be managed with endoscopic resection. The increasing adoption of ESD may improve the results of endoscopic resection for luminal NETs. However, enthusiasm for endoscopic resection must be tempered with respect for the more definitive curative results afforded by surgical treatment with more advanced lesions.

Key words: Carcinoid; Gastrointestinal; Endoscopy; Endoscopic submucosal dissection; Neuroendocrine tumor

Core tip: Neuroendocrine tumors (NETs) are uncommon but increasingly recognized gastrointestinal neoplasms. Management of NETs requires full understanding of tumor size, depth of invasion, lymphadenopathy, and location within the gastrointestinal tract. Small NETs can be removed by endoscopic techniques, while NETs > 2 centimeters typically require surgery. For tumors 11-20 mm in size, careful evaluation can

identify which NETs may be managed with endoscopic resection. Endoscopic submucosal dissection has been increasingly used for treatment of luminal NETs. However, enthusiasm for endoscopic resection must be tempered with respect for the more definitive curative results afforded by surgical treatment with more advanced lesions.

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INTRODUCTION

Neuroendocrine tumors (NETs) are an uncommon finding during endoscopic procedures, though the management of these neoplasms requires full understanding of tumor stage and prognosis, often with use of a multidisciplinary approach. Luminal NETs arise within the submucosa of the gastrointestinal (GI) tract and can be underappreciated without a careful examination. Increased recognition of NETs in recent years has been attributed to multiple factors, including improved detection (due to advanced imaging, laboratory and endoscopic techniques), a true rise in tumor incidence and greater awareness of NETs among physicians[1]. This rising incidence along with higher than previously thought mortality rates creates a challenge for gastroenterologists. These tumors have traditionally been characterized by indolent growth and a generally good prognosis, though more recent data illustrates subtypes of NETs with aggressive behavior and poor long-term survival. Treatment of NETs has traditionally been limited to endoscopic removal of small lesions (< 20 mm) and surgical excision of larger lesions, though advances in endoscopic techniques and the increasing use of endoscopic mucosal dissection (ESD) are allowing endoscopic therapy for an increasing proportion of these neoplasms.

EPIDEMIOLOGY

The incidence of NETs has increased over the past several decades in the United States and a similar rise has also been noted in Europe[2-4]. Data obtained from National Cancer Institute (NCI) registries in the United States identified 13715 NETs over 5 decades and the incidence was highest in the GI tract (67.5%)[2]. In addition, a study utilizing data on 35618 subjects with NETs from the Surveillance, Epidemiology, and End Results (SEER) Program registry reported a significant increase in age-adjusted incidence of NETs from 1.09 per 100000 person in 1973 to 5.25 per 100000 person in 2004[5]. Despite the reputation of NETs as relatively benign neoplasms, these large studies revealed an overall 5-year survival rate of only 50%-67.2%[2-3]. A recent SEER based review of gastorenteropancreatic NETs revealed similar overall 5-year survival rate of 68.1%[6]. Survival was lowest in pancreatic NETs (37.6%) and highest in rectal NETs (88.5%) with other sites being in between (colonic 54.6%, gastric 64.1%, small intestinal 68.1%, and appendiceal 81.3%). This marked variability in prognosis according to location has important implications for when surgical or endoscopic treatment should be chosen.

EMBRYOLOGY AND DISTRIBUTION

NETs of the GI tract are heterogeneous tumors and arise from the endocrine system mainly in the gastric submucosa, the small and large intestine and the rectum, as well as in the pancreas. The embryologic origin and vascular supply of NETs play a role in their classification, as some prefer to distinguish them based on origin by embryologic segments such as foregut (lung, stomach, liver, biliary tract, pancreas, the first portion of the duodenum, and the ovaries), midgut (the distal duodenum, small intestine, appendix, right colon, and the proximal transverse colon), and hindgut (the distal transverse colon, left colon, and the rectum)[7]. NETs can be either functional with secretion of hormones into the bloodstream (gastrinoma, glucagonoma, insulinoma, somatostatinoma and VIPoma) or non-functional[8]. Functional NETs may initially be diagnosed based on the patient's symptoms and serologic assays for the secreted hormone (such as the measurement of elevated insulin levels for an insulinoma); endoscopy may then follow as part of the attempt to localize the underlying NET. Nonfunctional NETs are typically discovered incidentally on endoscopy or cross-sectional imaging.

These tumors are not uniformly distributed within the GI tract. In the SEER 17 registry[6], gastorentero-pancreatic NETs made approximately 61% of NETs. In GI tract, the following sites were identified as common locations for NETs: rectum (17.7%), small intestine (17.3%), colon (10.1%), pancreas (7.0%), gastric (6.0%) and appendix (3.1%). This updated analysis showed a continued increase in the incidence of NETs, particularly in locations such as the rectum, stomach and small intestine, areas in which flexible and video capsule endoscopy have been utilized more often by gastroenterologists over the past few decades[6].

ENDOSCOPIC MANAGEMENT

GI NETs may be encountered during endoscopy under several circumstances. The first scenario is during endoscopic localization for an NET diagnosed by serologic or biochemical means (for instance, a suspected gastrinoma based on markedly elevated gastrin level and diarrhea). Secondly, hormonally inactive NETs may be discovered during evaluation of other symptoms such as GI bleeding or abdominal pain

caused by the tumors themselves. Finally, NETs may be incidentally discovered during endoscopy for upper GI symptoms or during screening colonoscopy. Once the diagnosis of a GI NET has been made by biopsy and histologic evaluation, staging must be performed to determine the appropriate treatment. If small and localized, these lesions can be effectively treated with endoscopic therapy. However, failure to recognize the size, depth, local invasion, or lymphatic spread may lead to incomplete treatment with endoscopic means. It is essential to recognize when surgical excision is the superior modality, and multidisciplinary evaluation of GI NETs is recommended prior to treatment.

ESOPHAGUS

Esophageal NETs comprise only 0.2% of GI NETs[6], and thus their endoscopic and histological features are not well characterized. A 2009 review identified only 25 reported cases in the previous 4 decades[9]. There are no established guidelines for treatment, which is thus dictated by provider experience and patient preference. Case reports describe a favorable prognosis in most subjects. Esophageal NETs may present incidentally as discrete polypoid lesions, or in association with adenocarcinoma in the setting of Barrett's esophagus[10,11]. Low-grade carcinoid lesions have been described, and these have a good prognosis following resection. However, atypical esophageal NETs (classified as large cell esophageal carcinoma or small cell esophageal carcinoma) may present at late stages with large fungating masses. These lesions have high mortality within a year despite surgical resection and subsequent chemotherapy[12-14].

Historically surgical resection has been the preferred treatment for esophageal NETs[15], though endoscopic resection is now considered safe and effective for small or superficial lesions. Esophageal NETs limited to the submucosal layer (without involvement of the muscularis propria) can be removed easily and safely[16]. In fact, endoscopic removal has been utilized frequently for esophageal NETs localized to submucosal layer and $\leqslant$ 10 mm in diameter without ulceration or erosion as these lesions had low probability for lymph node metastasis[9]. The threshold of 10 mm as the maximum size recommended for endoscopic resection of esophageal NET is based not on a large body of evidence for this location, but rather on extrapolation of data from gastric and rectal NETs, which have shown higher rates of lymph node metastases when lesions exceed 10 mm in size.

Endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) can each be considered for removal of low-grade esophageal NETs. EMR can allow an en bloc resection of a small lesion, though some authors have posited that ESD is preferable as EMR can lead to mechanical damage and limited pathological evaluation of the resected specimen[9]. ESD can enable complete removal of the tumor while maintaining an adequate horizontal margin for histologic review to ensure complete removal. Endoscopic ultrasound is recommended prior to removal to ensure the lesion does not extend to the muscularis propria, though there are no high-quality studies to show the efficacy of endoscopic ultrasonography (EUS) in delineating esophageal NET margins prior to resection.

STOMACH

Gastric carcinoids (GCs) can be asymptomatic and found incidentally. However, in certain subjects they are found during endoscopic evaluation of dyspepsia, abdominal pain or early satiety[17]. They are categorized into three groups in the following order in terms of frequency: Type 1 GCs (75%) and Type 2 GCs (5%-10%), which are well differentiated, and Type 3 GCs (15%-25%) which demonstrate aggressive behavior[17]. Type 1 GCs are typically small and multiple, seen in the setting of chronic atrophic gastritis with resulting stimulation of enterochromaffin cells by elevated gastrin levels. Type 2 GCs are similarly expressed due to excess gastrin levels in the setting of gastrinoma or multiple endocrine neoplasia type 1 (MEN-1). Type 3 GCs are sporadic, typically solitary and often larger when compared to types 1 and 2, and occur in the setting of normal gastrin levels.

GCs have been removed safely with endoscopy both in adults[18] and in children[19]. Various techniques can be used for resection of these lesions. ESD and EMR with utilization of cap aspiration, a ligation device, or grasping forceps are the most commonly used approaches, and all have been successful. However, initial studies comparing EMR and ESD have shown higher en bloc resection of lesions with ESD when compared to EMR[20,21]. In a recent study comparing the vertical and horizontal margins of 12 subjects who underwent either EMR or ESD, horizontal margins were negative in all subjects regardless of technique[22]. However, 66.7% of subjects in the EMR group had positive vertical margins compared to 0% of subjects in the ESD group. This small study suggests the superiority of ESD in complete removal of small GCs. Additional studies will be needed to confirm these findings and determine their clinical importance.

Metastatic progression of type I GCs is exceedingly rare, but has been described, so it is important not to overlook this possibility when considering endoscopic removal. A study examining prognostic factors in 20 patients with Type 1 GCs identified several factors associated with metastasis: tumor size of $\geqslant$ 1 cm, elevated Ki-67 index of tumor proliferation, and high serum gastrin levels (mean value 2138.4 mI/L)[17]. Careful examination to determine tumor size and depth of invasion can help in identifying those rare Type 1 or 2 GCs which should be managed with surgery and lymph node sampling.

Type 3 sporadic GCs are generally managed surgically due to their size and stage at the time of

diagnosis. Endoscopic management is rare but has been described. One center has described a series of 50 cases in which endoscopic resection of NETs confined to the submucosa and < 2 cm in size was attempted (41 EMR, 9 ESD)[23]. Complete removal was achieved in 80% of cases, and in 13-60 mo of follow-up there were no recorded instances of tumor recurrence, regardless of the completeness on initial resection. Another investigation utilized SEER data and identified 984 subjects with localized GCs who had cancer-directed surgery between 1983 and 2005. Results revealed that tumor size and depth predict lymph node metastasis and endoscopic therapy can be an option for intraepithelial GCs < 2 cm and GCs < 1 cm that invades into the submucosa or lamina propria[24]. Societal guidelines such as the National Comprehensive Cancer Network (NCCN) recommend staging of type 3 GC with EUS, multiphasic computerized tomography (CT) or magnetic resonance imaging (MRI), or somatostatin receptor scintigraphy to determine the appropriate stage and treatment modality. If EUS shows no evidence of lymphadenopathy, then surgical wedge resection or endoscopic resections are appropriate; otherwise, radical resection with lymphadenectomy is preferred[25]. The American Society for Gastrointestinal Endoscopy recommends that all type 3 GCs should be considered for surgical removal based on a high incidence of lymph node invasion, and only very small (< 1 cm), well-differentiated lesions should be considered for endoscopic removal[26]. As in other areas of the GI tract, proper assessment and staging of the lesion are critical for determining the threshold for endoscopic versus surgical removal of gastric NETs.

SMALL INTESTINE

The small intestine is one of the most common sites for NETs (17.3%)[6], though a large proportion of these lesions may not be accessible by standard bidirectional endoscopy. Duodenal NETs make only a small percentage of small bowel NETs[27] but can be candidates for endoscopic resection if the lesion is < 1 cm and confined to the mucosa and submucosa. Lesions of the ampulla or the medial wall of the duodenal C-sweep may be easily missed with use of standard forward-viewing endoscopes, and any survey of the duodenum for localization should ideally include use of a side-viewing duodenoscope. Duodenal bulb NETs are particularly likely to be found incidentally and with small size, with a small likelihood of metastatic disease. Although duodenal NETs < 2 cm have been shown to have limited metastatic potential and can be managed with local excision, tumor size alone does not predict risk of metastatic disease or lymphatic spread[28,29]. Cases of duodenal NETs as small as 5 mm with metastatic lymph node lesions have been reported[27,30]. Duodenal carcinoid tumors that are less than 1cm and limited to the submucosa with no evidence of lymphatic or metastatic disease are candidates for EMR or ESD[31,32]. Novel techniques for endoscopic resection include full-thickness resection with the use of an over-the-scope clip[33]. Careful follow-up examination for local recurrence is needed if decision is to remove these lesions with endoscopic resection[31]. When feasible, endoscopic resection is supported by the 2016 NCCN guidelines[25]. Surgical resection has been recommended for duodenal NETs larger than 1 cm, especially when there is imaging evidence of lymph node involvement or higher mitotic index[34]. Once again, careful examination of the lesion by endoscopic ultrasound is important to determine size and depth of invasion, as well as lymph node metastases.

NETs of the jejunum and ileum are classified as midgut tumors. They may be associated with carcinoid syndrome along with other midgut NETs such as appendiceal and cecal NETs[35]. Jejunal or ileal carcinoids may also present with anemia or overt bleeding, in which case they may be identified during video capsule endoscopy, deep enteroscopy, or colonoscopy with intubation of the terminal ileum[36,37]. Larger NETs may present with obstructive symptoms, including retention of video capsule endoscopy requiring retrieval of the capsule[38]. The majority of NETs of the small intestine are located in the distal ileum. Population based studies revealed that only 29% of NETs located in jejunum and ileum are localized and 71% have either regional or distant metastases[35]. Given the multifocal nature and potential technical difficulty of endoscopic resection of midgut small bowel carcinoids, surgical excision is preferred. The role for endoscopy in these NETs is limited to treatment of bleeding, or histologic confirmation by biopsy and localization by tattoo placement adjacent to the lesion[39]. Even with surgery, the 5-year survival rates for NETs located in these regions are 65% if localized and 71% if there is regional involvement[35]. While partial small bowel resection can be considered for proximal tumors, in such cases the remaining small intestine needs to be examined during resection to exclude multifocal disease[40].

COLONIC

Colorectal NETs comprise the majority of GI NETs (27.8%) and rectal NETs have been recognized more frequently over the past decade due to the increased utilization of screening colonoscopy[6]. Colonic NETs are often locally advanced or metastatic at the time of diagnosis, with a poorer prognosis than NETs located in other parts of GI tract. The 5-year survival rate is only 40% to 70% depending on the location and stage[41]. The larger size, invasive features, and (sometimes) anatomically challenging positions are contraindications to endoscopic management of many colonic NETs, similar to lesions in the jejunum and ileum. Endoscopic therapy with ESD has been reported, but only in small case series and with a higher risk of postprocedural

complications and incomplete resection[42]. Thus, surgical resection with lymphadenectomy is the approach recommended by NCCN guidelines and utilized frequently for these NETs.

RECTAL

Surgical resection with removal of associated lymphatic tissue remains the treatment for rectal NETs greater than 20 mm, due to the high risk of lymphatic invasion and metastasis. However, endoscopic resection is used for rectal NETs of < 20 mm without signs of deep invasion or lymphadenopathy. There is extensive experience with EMR of rectal NETs, mainly due to its ease and low complication rates. Conventional freehand EMR, cap-assisted EMR, or band ligation-assisted EMR have all been used with success and with minimal adverse events in NETs of < 1 cm in size[43-45]. However, with rectal NETs of 11-20 mm in size, complete resection of an en bloc specimen may prove more difficult using EMR[46-48]. This has spurred interest in the use of either ESD or modified EMR techniques to improve the rate of R0 resection while maintaining safety. A hybrid technique employing a "circumferential incision to EMR" (CIEMR) has been adapted to treat rectal NETs without regional lymph node enlargement[49]. When compared to conventional EMR in a randomized prospective trial of rectal NETs < 15 mm, procedure time was longer in CIEMR but R0 resection was superior (96.7% in CIEMR group compared to 82.14% in EMR group (P = 0.043)[50]. Other modifications include combining a circumferential mucosal incision with rubber band ligation (ESD-L)[51]. These techniques provide the advantage of a circumferential incision to ensure a clear lateral margin during resection, but allow the endoscopist to skip the time-consuming submucosal dissection in favor of snare-based resection.

ESD was initially pioneered for treatment of superficial gastric neoplasms and provides additional advantages in regards to en bloc removal and complete histological resection[20,52]. A comparison of ESD and EMR in subjects with rectal NETs < 16 mm without lymphadenopathy revealed similar en bloc resection rates in both groups, but a significantly higher histologic R0 resection rate in ESD group (90.3%) compared to EMR group (71%)[53]. Complication rates were similar for both groups. A retrospective analysis of 239 patients with colorectal NETs < 20 mm showed further evidence of the safety and efficacy of ESD; all but 6 of these lesions were located in the rectum. En bloc resection was achieved in all cases, and in all cases no local recurrence was noted over a median follow up period of 52 mo. Of note, distant metastases were noted in 6 patients (2.51%) during follow-up, underscoring the need for accurate assessment of deep invasion and lymphadenopathy prior to endoscopic removal[42]. ESD appears to increase the probability of complete histological resection when compared to EMR, and may provide an advantage in those NETs 11-20 mm where EMR techniques may not reliably provide a complete resection. A recent meta-analysis looked into 14 studies that included 782 subjects to compare the efficacy and safety of EMR or modified EMR (m-EMR) versus ESD for the treatment of rectal NETs[54]. Results revealed significantly higher rates of pathological complete resection among subjects treated with ESD or m-EMR compared to those treated with conventional EMR (OR = 0.42 and OR = 0.10, respectively) but no significant differences between m-EMR versus ESD groups. In summary, current data supports that m-EMR or ESD can be utilized safely in experienced hands for removal of colorectal NETs less than 2 cm without high-risk features.

The feasibility of endoscopic resection of rectal NETs by EMR or ESD is supported by treatment guidelines, as long as accurate staging is performed. The European Neuroendocrine Tumor Society (ENETS) consensus guidelines from 2012[41] note the importance of high risk features and recommended that rectal or colonic NETs larger than 2 cm or with high-risk features (advanced stage, high mitotic index, muscularis propria invasion or nodal disease) be removed surgically. Other NETs were considered to be candidates for endoscopic resection. These recommendations are mirrored by the NCCN, in which transanal surgical resection or endoscopic techniques are both recommended (following examination by MRI or EUS) for rectal NETs < 2 cm in size.

PANCREAS

Pancreatic NETs (PanNETs) make approximately 7% of GI NETs[6]. They have slightly higher predominance in males and Caucasians[55,56] and peak during the sixth and seventh decades of life[3]. They can be categorized into two groups as functioning versus non-functioning depending on the presence or absence of clinical syndromes related to hormone production. Functioning PanNETs have been reported in the following frequencies: Insulinomas (45%), gastrinomas (20%), glucagonomas (13%), VIPomas (10%) and somatostainomas (less than 5%)[54]. Cumulative 5-year survival has been reported to range between 30% to 97% in PanNETs[57]. The wide variability likely reflects heterogeneity of presentation, with hormonally active tumors being diagnosed at earlier stages during investigation of symptoms.

CT and MRI have been utilized frequently as imaging modalities during diagnosis of PanNETs. The sensitivity and specificity of these imaging modalities have been reported to differ in CT (60%-83% and 83%-100%, respectively) depending on lesion size and also in MRI (85%-100% and 75%-100%, respectively)[57]. Endoscopists play a crucial role in identification and evaluation of PanNETs by EUS. EUS provides not only key information about morphological features of these lesions, but also enables tissue

sampling for histopathological evaluation. Due to its high sensitivity for small lesions, EUS can also identify PanNETs undetected by cross-sectional imaging studies. A review of 81 subjects referred for EUS-guided fine-needle aspiration (EUS-FNA) for a suspected PanNET revealed diagnostic yield of EUS-FNA to be 90.1%[58]. Another large, single-center prospective series studied the utility of EUS early in the diagnostic evaluation of subjects with PanNETs who subsequently had surgical confirmation of tumor localization[59]. In this investigation overall sensitivity and accuracy of EUS was 93% and investigators pointed out the role of EUS as a primary diagnostic modality during evaluation and management of PanNETs. Another study of 72 subjects with PanNETs demonstrated EUS to be not only highly accurate in localizing PanNETs but also cost effective when utilized early in the preoperative course by decreasing the need for further invasive tests[60]. Surgical resection is the recommended treatment modality in PanNETs, and type of resection in PanNETs is mainly determined by size and location of the lesion. EUS - in combination with cross-sectional imaging - can provide crucial information regarding the location and stage of PanNETs to optimize treatment planning.

Besides accurately determining the size and characteristics of lesions, EUS also provides key histological information when combined with FNA. In a retrospective study, 75 out of the 81 patients underwent EUS-FNA and the yield of EUS-FNA reached up to 97.3%[58]. In a recent study, the utilization of FNA along with EUS characterized the nature of the pancreatic NETs in all cases[61] by providing accurate pathological information showing the critical role of EUS-FNA in preoperative management of these patients. In addition, the sampling rate for histological diagnosis by EUS-FNA was shown to be 100% and the concordance rate was 87.5% when it was compared with surgical specimens[62]. EUS thus has a crucial role in planning treatment strategies for PanNETs.

Given the importance of staging for luminal NETs prior to treatment, EUS is also a critical tool for evaluating tumor size, depth of invasion, and presence of lymphadenopathy in these lesions. Multiple studies have demonstrated the utility of EUS for accurately determining resectability of small NETs in the stomach, duodenum, and rectum[63]. EUS has been used to confirm a lack of muscular invasion in a case series of ESD for rectal carcinoids as well[64]. The information gleaned by accurate staging allows the endoscopist to choose the correct strategy (endoscopic or surgical) to afford a definitive cure in a minimally invasive fashion.

NOVEL TECHNIQUES

While EMR and ESD remain the most common endoscopic techniques for treatment of luminal NETs, newer techniques have been employed in an attempt to completely excise these submucosal tumors. For example, submucosal tunneling with endoscopic resection can be utilized to resect esophagogastric NETs arising from the deep submucosal layer. Following its initial use[65], investigators have reported several case series which revealed success rates up to 100% without major complications[66]. Another new technique that is limited to pioneering centers with defined protocols is peroral endoscopic tumor resection (POET). As a technique adapted from the successful management of achalasia with per oral endoscopic myotomy (POEM), POET also utilizes submucosal tunneling approach and provides an opportunity for en-bloc removal of the tumor followed by mucosal closure. POET can provide definitive en-bloc resection, excellent long-term results, and can be applied in cases where surgical resection is not an option due to comorbidities, though its use is limited to tumors of the esophagus, GE junction, and gastric cardia[67]. POET requires experience with POEM and ESD, and has only been utilized in specialized centers.

Endoscopic full-thickness resection (EFTR) has been employed for treatment of some gastric submucosal tumors. Case series have described successful resection in all subjects without laparoscopic assistance and success rate for complete resection was 100%[68]. In a different investigation, mean operative times, length of stays and complete resection rates were found to be similar among subjects who had EFTR (n = 32) *vs* laparoscopic surgery (n = 30) for treatment of gastric stromal tumors[69]. Another study that included 48 subjects with mean tumor size of 1.59 cm (largest lesion 4.8 cm) reported successful removal in all cases and there was no early recurrence during the follow-up period[70]. However, these techniques are not widely available and should be applied only by experts in dedicated centers. In addition, EFTR is ideal for tumors arising from the muscularis propria (such as GISTs) and may not provide superior outcomes when compared to ESD, as most GI NETs remain confined to the submucosa. Future studies will define the roles of these techniques in the management of GI NETs.

CONCLUSION

GI NETs are uncommon neoplasms which may represent a therapeutic challenge for the endoscopist. The choice of proper treatment depends on the location of the NET as well as proper evaluation of size, depth of invasion, and local lymphadenopathy. Endoscopic resection techniques continue to evolve, with the growth of endoscopic mucosal dissection showing promising results in achieving complete and safe en bloc resection of lesions as large as 2 centimeters. Despite the improvements in technique, the enthusiasm for endoscopic resection of larger lesions must be balanced against the superior ability of surgical resection to detect and treat lymphatic spread. Future directions for research should focus not only on optimizing the techniques for endoscopic treatment, but improving the recognition of factors that should

prompt surgical referral.

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EDITORIAL

Current research and treatment for gastrointestinal stromal tumors

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and have gained considerable research and treatment interest, especially in the last two decades. GISTs are driven by mutations commonly found in the *KIT* gene and less commonly in the platelet-derived growth factor receptor alpha gene, *BRAF* gene and succinate dehydrogenase gene. GISTs behave in a spectrum of malignant potential, and both the tumor size and mitotic index are the most commonly used prognostic criteria. Whilst surgical resection can offer the best cure, targeted therapy in the form of tyrosine kinase inhibitors (TKIs) has revolutionized the management options. As the first-line TKI, imatinib offers treatment for advanced and metastatic GISTs, adjuvant therapy in high-risk GISTs and as a neoadjuvant agent to downsize large tumors prior to resection. The emergence of drug resistance has altered some treatment options, including prolonging the first-line TKI from 1 to 3 years, increasing the dose of TKI or switching to second-line TKI. Other newer TKIs, such as sunitinib and regorafenib, may offer some treatment options for imatinib-resistant GISTs. New molecular targeted therapies are being evaluated, such as inhibitors of BRAF, heat shock protein 90, glutamine and mitogen-activated protein kinase signaling, as well as inhibitors of apoptosis proteins antagonist and even immunotherapy. This editorial review summarizes the recent research trials and potential treatment targets that may influence our future patient-specific management of GISTs. The current guidelines in GIST management from Europe, North America and Asia are highlighted.

Key words: Gastrointestinal stromal tumors; *KIT* gene; Platelet-derived growth factor receptor alpha gene; *BRAF* gene; Succinate dehydrogenase gene; CD117; Tyrosine kinase inhibitor; Molecular targeted therapy

Core tip: Research in the histogenesis of gastrointestinal stromal tumors (GISTs) identified gene mutations in *KIT*, platelet-derived growth factor receptor alpha and

BRAF. The discovery of tyrosine kinase inhibitors (TKIs) has allowed targeted therapy in metastatic and high-risk resected GISTs. However, the emergence of TKI-resistant GISTs has raised some important treatment issues. Newer TKIs and alternative targeted therapy within the domain of BRAF and the mitogen-activated protein kinase signaling pathway, heat shock protein 90 and succinate dehydrogenase inhibition are being investigated and appear promising. Many clinical trials have been undertaken and are still ongoing to define the best molecular targeted therapy for GISTs. The European, American and Asian guidelines on GISTs provide useful resources for specialists dealing with these interesting tumors.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) account for less than 1% of all gastrointestinal tumors, and the prevalence of histological type comes after adenocarcinoma and lymphoma. GISTs are, however, the most common mesenchymal tumors of the gastrointestinal tract[1]. Historically, GISTs were classified as leiomyomas or leiomyosarcomas due to smooth muscle features observed under light microscopy.

GISTs were first termed in 1983 by Mazur and Clark[2], who discovered that the majority of gastric wall tumors were not derived from smooth muscle and nerve sheath origin using immunohistochemistry. GISTs are believed to arise from the interstitial cells of Cajal or their precursors and are heterogeneous histologically, showing spindle cells (70%), epitheloid cells (20%) and mixed cells (10%)[3]. The histogenesis of GISTs has since gained considerable research and treatment interest.

A systematic review of population-based cohort studies on GISTs by Søreide *et al*[4] showed that incidence ranges from low 0.43 per 100000 per year in Shanxi Province, China to high 1.6-2.2 per 100000 per year in South Korea. The cohort of 13550 patients from 19 countries gave the reported age ranging from 10-100 years, with median age in the 60 s; both male and female populations had about equal distribution. The anatomical locations of GISTs are frequently the stomach (55.6%) and small bowel (31.8%), and are less commonly found in the colon and rectum (6%), other various locations (5.5%) and esophagus (0.7%)[4].

Primary GISTs are commonly symptomatic (in about 80% cases), presenting with gastrointestinal bleeding or obstructive symptoms and abdominal pain. Incidental asymptomatic GISTs are discovered in less than 20% of cases during other gastrointestinal endoscopy or imaging investigations.

The diagnostic tests for GISTs may include gastrointestinal endoscopy (Figure 1), computed tomography (CT) scan (Figure 2), magnetic resonance imaging (MRI) scan and ^{18}fluoro-deoxyglucose-positron emission tomography (^{18}FDG-PET) scan (Figure 3). Endoscopic ultrasound scan (Figure 4) with fine needle aspiration biopsy (Figure 5) may be useful in confirming GISTs histologically.

Open or laparoscopic complete surgical R_0 resection of GISTs (Figure 6) represent the only potentially curative treatment, but certain high risk features of the resected GISTs give rise to recurrence of the disease. DeMatteo *et al*[5] reviewed 200 patients with GISTs treated and followed-up at a single institution and found that 46% had primary disease, 47% had metastasis and 7% had isolated local recurrence. Eighty patients with primary disease who underwent complete resection had 5-year survival rate of 54%. Survival was predicted by tumor size, but not by microscopic resection margin. However, tumor recurrence was noted to occur at the original primary tumor site, peritoneum and liver. These data predated the use of tyrosine kinase inhibitors (TKIs). In later years, the treatment options for residual or progressive liver metastases of GISTs included hepatic artery embolization, radiofrequency ablation or liver resection[6-8].

Historical assessment of the malignant potential in GISTs were based on the criteria of tumor size, mitotic count, proliferating cell nuclear antigen and proliferation index, which allowed classification into low- and high-risk subgroups[9]. Subsequently, different risk stratification systems for GISTs were proposed, such as the National Institutes of Health (NIH) consensus criteria (Fletcher's criteria based on size and mitotic count) and the Armed Forces Institute of Pathology criteria (Mittinen's criteria based on size, mitotic count and tumor site) and the 8th edition of the International Union Against Cancer utilizing TNM classification in addition to a grade category based on mitotic count[10-12].

According to the NIH criteria for primary GISTs, the distribution of risk is categorized as very low risk (15%), low risk (30%), intermediate risk (22%) and high risk (33%)[10]. Table 1 shows the commonly used criteria for assessing malignant risk of GISTs. Other factors associated with a higher malignant risk of GISTs are the presence of necrosis, high cellularity, invasion to serosa or adjacent structure and rich vascularity. In addition, factors associated with a higher risk of recurrence of GISTs are now recognized to be incomplete R_1 or R_2 resection margin, tumor rupture and spillage during surgery.

GENETIC MUTATIONS IN GISTs

The landmark article by Hirota *et al*[13] discovered that GISTs express the proto-oncogene KIT and that

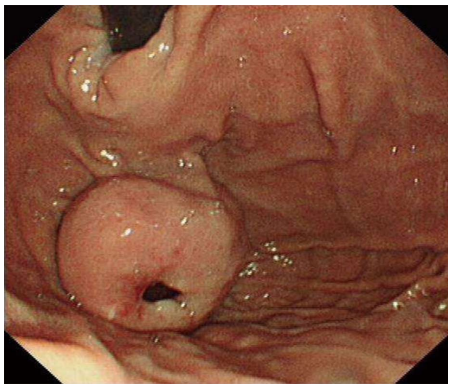

Figure 1 Gastroscopy view of a gastric gastrointestinal stromal tumors. A 4 cm × 4 cm in diameter gastric fundal submucosal tumor with a central ulceration associated with a recent bleed is shown.

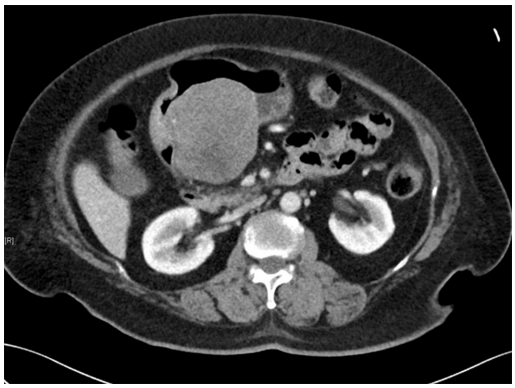

Figure 2 Computed tomography scan image of a gastric gastrointestinal stromal tumors. A submucosal tumor measuring 7.7 cm × 7.6 cm × 7.2 cm in dimension was located on the posterior wall of gastric antrum. An ill-defined hypodensity within the mass could represent an area of necrosis.

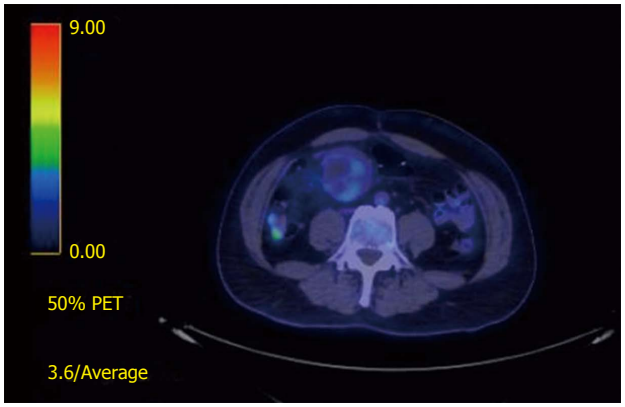

Figure 3 ^{18}Fluoro-deoxyglucose-positron emission tomography scan views of a small bowel gastrointestinal stromal tumors. There is a mildly fluoro-deoxyglucose avid mass adjacent to the jejunal anastomosis with SUVmax 3.4 suggestive of a local recurrence.

this *KIT* gene mutation provides growth stimulation of GISTs. c-KIT, also known as CD117, is a protein and a type of a receptor tyrosine kinase found on the surface of a variety of cell types; it is also a type of tumor marker. The binding of stem cell factor to

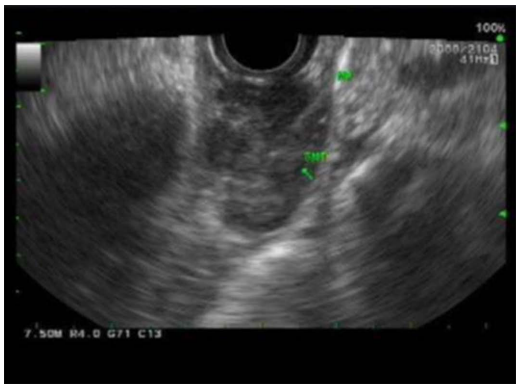

Figure 4 Endoscopic ultrasonography images of esophageal gastrointestinal stromal tumors. A distal esophageal submucosal lesion measuring 2.6 cm × 1.3 cm in diameter was noted to be well circumscribed, heterogeneous with hypoechoic echotexture without disruption of wall architecture and with no perilesional lymph node.

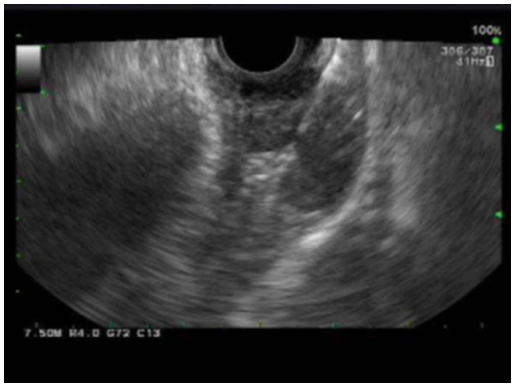

Figure 5 Endoscopic ultrasound scan and fine needle aspiration image of an esophageal gastrointestinal stromal tumors. The procedure was performed using a 22F procore needle with on-site cytotech.

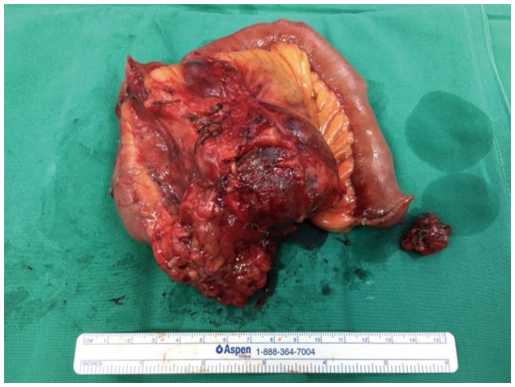

Figure 6 Macroscopic image of a recurrent small bowel gastrointestinal stromal tumors. A picture of a large enbloc resection specimen of a small bowel mesentery and jejunum was taken along a separate smaller metastatic mesenteric nodule.

the extracellular domain of c-KIT induces receptor dimerization and activation of downstream signaling pathways responsible for pro-growth signals within the cells.

Table 1 National Institutes of Health *vs* Armed Forces Institute of Pathology criteria for assessing malignant risk of gastrointestinal stromal tumors

Degree of risk	NIH criteria	AFIP criteria
Unknown	-	< 2 cm and ⩽ 5 mitotic index
Very low	< 2 cm and < 5 mitotic index	⩽ 5 cm and ⩽ 5 mitotic index
Low	2-5 cm and < 5 mitotic index	Gastric: > 5 cm and ⩽ 5 mitotic index Others: 2-5 cm and ⩽ 5 mitotic index
Intermediate or moderate	5-10 cm and < 5 mitotic index > 5 cm and 6-10 mitotic index	Gastric: > 10 cm and ⩽ 5 mitotic index or > 2-5 cm and > 5 mitotic index Others: 5-10 cm and ⩽ 5 mitotic index
High	> 5 cm and > 5 mitotic index > 10 cm and any mitotic index Any size and > 10 mitotic index	Gastric: > 5 cm and > 5 mitotic index Others: > 10 cm and > 5 mitotic index

Mitotic index = number of mitoses per 50 high-power field. AFIP: Armed Forces Institute of Pathology; NIH: National Institutes of Health.

Table 2 Frequency of genetic mutations in gastrointestinal stromal tumors

KIT mutation (about 85%)	*PDGFRA* mutation (about 5%)	*BRAF* mutation (< 1%)	*SDH* mutation (12%-15% adult, 90% pediatric GIST)
Exon 11 (about 70%)	Exon 18 (about 5%)	Exon 15 V600E	Subunit B, C and D
Exon 9 (10%-15%)	Exon 12 (1%)		
Exon 13 (1%-3%)	Exon 14 (< 0.5%)		
Exon 17 (1%)	Exon 18 D842V (about 0%)		

PDGFRA: Platelet-derived growth factor receptor alpha; SDH: Succinate dehydrogenase.

Another landmark article by Heinrich *et al*[14] later discovered GISTs lacking KIT expression have mutations related to platelet-derived growth factor receptor alpha (PDGFRA). Overall, *KIT* or *PDGFRA* mutations are found in 85% and 5% of GISTs respectively.

Agaram *et al*[15] later discovered *BRAF* mutation in imatinib-naïve and imatinib-resistant GISTs. This *BRAF* mutation in GISTs is quite rare, accounting < 1% of cases[16]. It is noted that these *KIT*, *PDGFRA* and *BRAF* gene mutations are mutually exclusive.

"Wild-type" GISTs were previously referred to GISTs lacking any mutation in *KIT* and *PDGFRA*. This "wild-type" terminology should be avoided now that new mutations have been discovered in *BRAF* genes and in genes encoding the protein succinate dehydrogenase (SDH). About 12%-15% of adult GISTs and 90% of pediatric GISTs lacking *KIT*, *PDGFRA* or *BRAF* mutations are classified into SDH-deficient and non-SDH-deficient groups. The SDH-deficient group includes Carney triad (GISTs, pulmonary chondroma and extra-adrenal paraganglioma) and Carney-Stratakis syndrome (GISTs and paraganglioma)[17].

The vast majority of *KIT* mutations are localized in exon 11 (juxtamembrane domain; about 70%), exon 9 (extracellular dimerization motif; 10%-15%), exon 13 (tyrosine kinase 1 domain; 1%-3%), and exon 17 (tyrosine kinase 2 domain and activation loop; 1%-3%)[18]. Secondary *KIT* mutations in exons 13, 14, 17 and 18 are commonly identified in post-imatinib biopsy specimens, after the patients have developed the acquired resistance. The mutations of *PDGFRA* are noted to be localized in exon 12, 14 and 18, and more specifically as 18 D842V. The mutation of *BRAF* is identified and localized to exon 15 V600E[15]. Mutations of the *SDH* gene are found to be localized to subunit B, C and D[17]. Table 2 summarizes the frequency of different genetic mutations in GISTs.

TKIs AND BIOLOGICAL THERAPY IN GISTs

Whilst complete surgical resection of GISTs can offer the best cure, targeted therapy in the form of TKIs has altered our management options. A landmark case report by Joensuu *et al*[18] described the effect of a TKI called STI571 in a patient with a metastatic GIST, for which the evaluation of MRI and ^{18}FDG-PET scans showed a very dramatic reduction of the GIST.

STI571 was the first TKI, also called imatinib, approved by the United States Food and Drug Administration (FDA) in 2002 for the treatment of unresected or metastatic GISTs. In 2008, imatinib was approved for adjuvant use in high-risk resected GISTs patients to prevent recurrence[19]. In 2012, the FDA granted the extension of standard 1 year imatinib therapy to 3 years, due to increase in overall patient survival[20,21]. An important study demonstrated that imatinib when used as a neoadjuvant therapy decreased the tumor volume and was associated with improved complete surgical resection in the locally advanced primary GISTs[22].

In a trial examining the relationship between kinase genotype and treatment outcome for 428 patients treated with either 400 mg or 800 mg daily doses of imatinib confirmed the favorable impact of *KIT* exon 11 genotype, when compared with KIT exon 9 and wild-

Table 3 Implication of gastrointestinal stromal tumors mutations and response to targeted therapy

	Imatinib[23]	Sunitinib[25]	Regorafenib[28]
KIT mutation			
Exon 11	OR 63%	CB 34%	Increased sensitivity
Exon 9	OR 37%. Intermediate sensitivity. Higher dose 800 mg more effective in metastatic disease than 400 mg daily	CB 34%	Unknown
Exon 13	OR 40%. Sensitivity as primary mutation. Resistance as secondary mutation	CB 100%	Unknown
Exon 14	Resistance as secondary mutation	Unknown	Unknown
Exon 17	OR 25%. Primary mutation sensitive *in vitro*. Resistance as secondary mutation	CB 0%	Unknown
PDGFRA mutation			
Exon 18	OR 50%	CB 0%	Unknown
Exon 12	Increased sensitivity	CB 0%	Unknown
Exon 14	Increased sensitivity *in vitro*	Unknown	Unknown
Exon 18 D842V	Decreased sensitivity	Decreased sensitivity	Unknown
BRAF mutation	Resistance	Resistance	Unknown
SDH mutation	Decreased sensitivity	Unknown	Increased sensitivity
No *KIT*, *PDGFRA* or *BRAF* mutation	OR 28%	CB 56%	Some activity

Objective response (OR) is defined as a complete or partial response by Response Evaluation Criteria for Solid Tumors (RECIST) criteria, excludes non-evaluable patients. Clinical benefit (CB) is defined as response or stable disease for 6 mo or more according to RECIST. PDGFRA: Platelet-derived growth factor receptor alpha; SDH: Succinate dehydrogenase.

type genotype for patients with advanced GISTs[23].

The American College of Surgeons Oncology Group led a trial studying the long-term outcome of patients categorized as high risk of recurrence who underwent complete gross GISTs resection followed by adjuvant imatinib at 400 mg/d for 1 year. After a median follow-up of 7.7 years, the 1-, 3- and 5-year overall survival rates were 99%, 97% and 83% respectively, which compared favorably with a historical 5-year overall survival rate of 35%. The 1-, 3- and 5-year recurrence-free survival rates were 96%, 60% and 40% respectively. On univariate analysis, age and mitotic rate were associated with overall survival. On multivariate analysis, the recurrence-free survival rate was lower with increasing tumor size, small bowel site, *KIT* exon 9 mutation, high mitotic rate, and older age[24].

TKIs other than imatinib are considered as second-generation TKIs, and include sunitinib, regorafenib, sorafenib, nilotinib, dasatinib and pazopanib. Table 3 summarizes the implication of different mutations in GISTs and their response to TKI therapy.

Sunitinib was approved by the FDA for the treatment of imatinib-resistant GISTs in 2006 and is considered as second-line TKI[25]. Heinrich *et al*[26] discovered clinical activity of sunitinib after imatinib failure is significantly influenced by both primary and secondary mutations in the predominant pathogenic kinases that implicate the optimum treatment of patients with GISTs.

Regorafenib was approved by the FDA in 2013 to treat advanced GISTs that cannot be surgically removed and are resistant to other TKIs, and it is considered as third-line TKI[27]. The long-term follow-up results of the multicenter phase Ⅱ trial of regorafenib in patients with metastatic or unresectable GISTs after failure of imatinib and sunitinib showed benefit in patients with primary *KIT* exon 11 mutations and SDH-deficient GISTs[28].

The use of other TKIs, apart from imatinib, sunitinib and regorafenib, is still being evaluated and remains debated. In a Korean clinical trial in 2012, sorafenib was shown to maintain disease control in one-third of the patients with metastatic GISTs who had otherwise failed with two or more TKIs[29].

In a phase Ⅰ study of single-agent nilotinib or in combination with imatinib in patients with imatinib-resistant GISTs showed some partial clinical response but required phase Ⅱ doses for further evaluation[30].

In a phase Ⅱ study of imatinib-resistant GISTs treated with dasatinib, there was a significant activity by objective response rate but it did not meet the predefined 6 mo progression-free survival rate of 30%[31]. There is an American phase Ⅱ clinical trial of dasatinib in advanced sarcoma including GISTs patients and a European phase Ⅱ trial of dasatinib as first-line therapy in GISTs patients[32,33]. Both trials have stopped recruiting participants and the conclusion of the study is expected in the future.

In a phase Ⅱ French trial, Mir *et al*[34] showed that pazopanib plus best supportive care improves progression-free survival compared with best supportive care alone in patients with advanced GISTs resistant to imatinib and sunitinib. This trial provides reference outcome data for future studies of targeted inhibitors in the third-line setting for this group of patients.

Other TKIs identified in clinical trials include masitinib (AB1010), crenolanib (CP-868,596), AZD2171, vatalanib (PTK787), OSI-930, TKI258 and DCC-2618 (Table 4). A biologics inhibitor of KIT and PDGFRA called olaratumab (IMC-3G3) was trialed (NCT01316263) but the development was put on hold and the stage 2 of this study was not completed.

Table 4 Potential treatment targets for gastrointestinal stromal tumors

Category	Name	ClinicalTrials.gov Identifier
TKI of KIT and PDGFRA	Masitinib (AB1010)	NCT00998751 (U)[57]
	Crenolanib (CP-868,596)	NCT02847429 (R), NCT01243346 (C)[58]
	AZD2171	NCT00385203 (C)[59]
	Vatalanib (PTK787)	NCT00117299 (C), NCT00655655 (A)
	OSI-930	NCT00513851 (C)
	TKI258	NCT01478373 (C), NCT01440959 (C)
	DCC-2618	NCT02571036 (R)
Biologic inhibitors of KIT and PDGFRA	Olaratumab (IMC-3G3)	NCT01316263 (C)
HSP90 inhibitors	Retaspimycin (IPI-5040)	NCT00276302 (C), NCT00688766 (T)
	BIIB021 (CNF2024)	NCT00618319 (C)
	Ganetespib (STA-9090)	NCT01039519 (C)
	AUY922	NCT01389583 (R), NCT01404650 (C)
	AT13387	NCT01294202 (C)
Inhibitors of pathways downstream of KIT and PDGFRA	RAS/RAF/MEK/ERK/MAPK inhibitors: MEK162	NCT01991379
	AKT inhibitors: perifosine	NCT00455559 (C)[60]
	mTOR inhibitors: everolimus (RAD001)	NCT01275222 (C), NCT00510354 (C), NCT02071862 (R)
	mTOR inhibitors: temsirolimus (Torisel)	NCT00700258 (R)
Cell cycle inhibitors	Alvocidib (Flavopiridol)	NCT00098579 (C)
Insulin-like growth factor pathway inhibitors	OSI-906	NCT01560260 (C)[61]

R: Recruiting; T: Terminated; C: Completed; A: Active, not recruiting; U: Unknown. PDGFRA: Platelet-derived growth factor receptor alpha.

CURRENT RESEARCH IN GISTs

The emergence of TKI-resistant GISTs has led to further research in understanding of this treatment failure and the alternative signaling mechanism conferring GIST survival. The research to find new drugs, particularly targeted therapy, is being evaluated.

Agaram *et al*[15] found that *BRAF* mutations appear to be associated with a higher malignant risk and resistance to TKI compared to *KIT* and *PDGFRA* mutations. Kinase inhibitors targeting BRAF may be considered as an effective therapeutic option in this GISTs subset. Falchook *et al*[35] published the first report on BRAF inhibitor, dabrafenib (GSK2118436), which showed prolonged anti-tumor activity in V600E *BRAF*-mutated GIST patients. There is presently no trial in GISTs looking at BRAF inhibitors.

In a phase Ⅱ trial study of heat shock protein (HSP)90 inhibitor, BIIB021, given to patients with GISTs refractory to imatinib and sunitinib, promising response was shown[36]. This result encourages future development of HSP90 inhibitors in TKI-resistant GISTs. A next phase study evaluating BIIB021 in GISTs is therefore warranted.

Testing for germline mutations in *SDH* is presently recommended for patients with GISTs lacking mutations in *KIT*, *PDGFRA* and *BRAF*[37]. There is an ongoing phase Ⅱ trial of vandetanib in children and adults with "wild-type" GISTs but it is currently not recruiting participants and the estimated study conclusion will be available in 2023[38]. Another study currently recruiting participants is the glutamine inhibitor CB-839 trial in solid tumors including SDH-deficient GISTs[39].

Ran *et al*[40] recently reported the combined inhibition of mitogen activated kinase (MAPK) and KIT signaling synergistically destabilizes the transcription factor called ETV1 and suppresses GIST growth. The combination of MAPK inhibitors and TKIs to target ETV1 may provide an effective therapeutic strategy in GISTs clinical management. There is currently a trial recruiting participants to study MEK162 in combination with imatinib in patients with untreated advanced GISTs[41].

In another emerging target category, Falkenhorst *et al*[42] discovered inhibitor of apoptosis proteins (IAPs) such as XIAP and survivin are commonly dysregulated in GISTs. Future study to assess the combination of imatinib with an IAP antagonist such as YM155 to enhance the pro-apoptotic activity in GISTs is therefore needed.

There was a clinical trial study looking at the role of immunotherapy by combining pegylated-interferon α-2b with imatinib for treatment of stage Ⅲ/Ⅳ GISTs that yielded highly promising clinical outcomes. The trial was terminated early in 2012 in preparation for a larger future trial[43]. Table 4 summarizes the potential treatment targets in GISTs under clinical trials. The trials' information was obtained from https://clinicaltrials.gov/ct2/home online.

CURRENT TREATMENT GUIDELINES FOR GISTs

Most countries have their own clinical practice guidelines for GISTs, such as the American National Comprehensive Cancer Network (NCCN) (2010 update), the European Society of Medical Oncology (ESMO) (2012), the French National Federation of Cancer Centers consensus guidelines (in French) (2005),

the Japan Society of Clinical Oncology (2008), the Korean GISTs guidelines (2012 Update), the Canadian Advisory Committee on GISTs statement (2006) and the Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland (AUGIS) (2009)[44-50]. Most recently, the Asian Consensus Guidelines (2016) for the Diagnosis and Management of GISTs was published to promote optimal care for Asian populations[51].

The NCCN task force report update on GISTs management is quite comprehensive and detailed, and covers over 41 pages. It described the epidemiology from the Surveillance, Epidemiology and End Results data from the National Cancer Institute, the clinical presentation, the pathology and differential diagnosis using immunohistochemistry and gene expression profiling, the recommendations for diagnosing GISTs, the significance of *KIT* and *PDGFRA* mutation status, the recommendations for mutational analysis, the management of adult *vs* pediatric patients with GISTs, the principles of surgery for GISTs, the need for multidisciplinary management for primary, recurrent or metastatic GISTs and the imaging of GISTs[44].

The ESMO clinical practice guidelines on GISTs describes the incidence of GISTs in Europe, the strategy to diagnose GISTs, the stage classification and risk assessment (does not recommend TNM classification), the staging procedure using CT, MRI and FDG-PET scan, the treatment planning involving multidisciplinary team for localized and metastatic disease, the response evaluation and optimal follow-up for different risk categories[45].

In the Asian consensus guidelines for diagnosis and management of GISTs, some points were highlighted. Firstly, it recommends the minimal 3-year treatment with imatinib before and after surgery for high-risk GISTs. Secondly, it recommends early evaluation of tumor response after 1 mo of neoadjuvant imatinib treatment, when genotyping is not feasible for primary gastric GISTs. Thirdly, it suggested a prospective study on the feasibility and efficacy of high-dose imatinib therapy in Asian patients. Lastly, it recommends imatinib rechallenge instead of discontinuing TKI treatment if third-line regorafenib is not available or failed[51].

In summary of these published treatment guidelines, the general consensus is complete surgical resection of GISTs as the first step when possible. Surgery is potentially curative for primary GISTs that have not metastasized and the probability of recurrence will depend on the malignant potential risk stratification criteria.

GIST cases that are initially inoperable may be given neoadjuvant therapy with the first-line TKI imatinib to improve resectability. Following complete removal of primary GISTs, patients with a higher risk of tumor recurrence may consider adjuvant therapy with first-line TKI. Patients with metastatic GIST disease, even if removed, will benefit from TKI to maintain disease control.

For patients with imatinib-resistant GISTs, sunitinib is a second-line drug treatment whilst regorafenib is the third-line drug for imatinib- or sunitinib-resistant GISTs. Some drugs approved for other conditions may be prescribed off-label for GISTs at a physician's discretion but with a caveat, and clinicians are advised to follow the local guidelines. New molecular targeted drugs are being tested in many clinical trials and some are still under development. An algorithm for the management of GISTs based on the summary of current guidelines is included (Figure 7).

PROGNOSIS

Data from pooled analysis of 2560 patients diagnosed with operable GISTs who were not given adjuvant therapy gave the estimated 15-year recurrence-free survival after surgery at 59.9%[52]. Whilst in a trial previously alluded to with resected GISTs deemed high risk were subsequently treated with imatinib showed the 5-year overall survival rate of 83%[24]. In a different follow-up study to assess the long-term survival of 695 patients with metastatic GISTs who were treated with imatinib, the estimated 10-year overall survival is 23%[53].

There was an interesting Dutch study which highlighted that severe fatigue occurred in 30% of patients with GISTs and in 33% of patients with GISTs who took TKIs. The disabling fatigue was associated with psychological distress and physical function[54]. In another survey study, the long term functional outcomes of laparoscopic resection of gastric GISTs were investigated by utilizing the Gastrointestinal Quality of Life Index (GIQLI). Most patients reported no change in symptoms and the GIQLI scores were within the normal range, with minimal effect on long-term quality of life[55].

FUTURE GIST TREATMENT TRENDS

About 5 years ago, Dematteo[56] proposed a concept of personalized therapy for GISTs. With accumulating research data in biology, such as genetic mutations and adjuvant or neoadjuvant therapy with systemic drugs, it is considered true that personalized assessment and therapy may appear to be the future trend for GIST management.

Complete surgical resection of GISTs is the gold standard of primary treatment when possible, with or without the adjunct of molecular targeted drug therapy. Through the understanding of the mutations of GISTs and addressing treatment resistance with TKIs, new treatment ideas such as combination trials of TKI plus other drugs, TKI plus surgery in specified sequences, newer line TKIs, inhibitors of BRAF, HSP inhibitors, inhibitors of downstream pathways such as MAPK, IAP inhibitors and immunotherapy may play

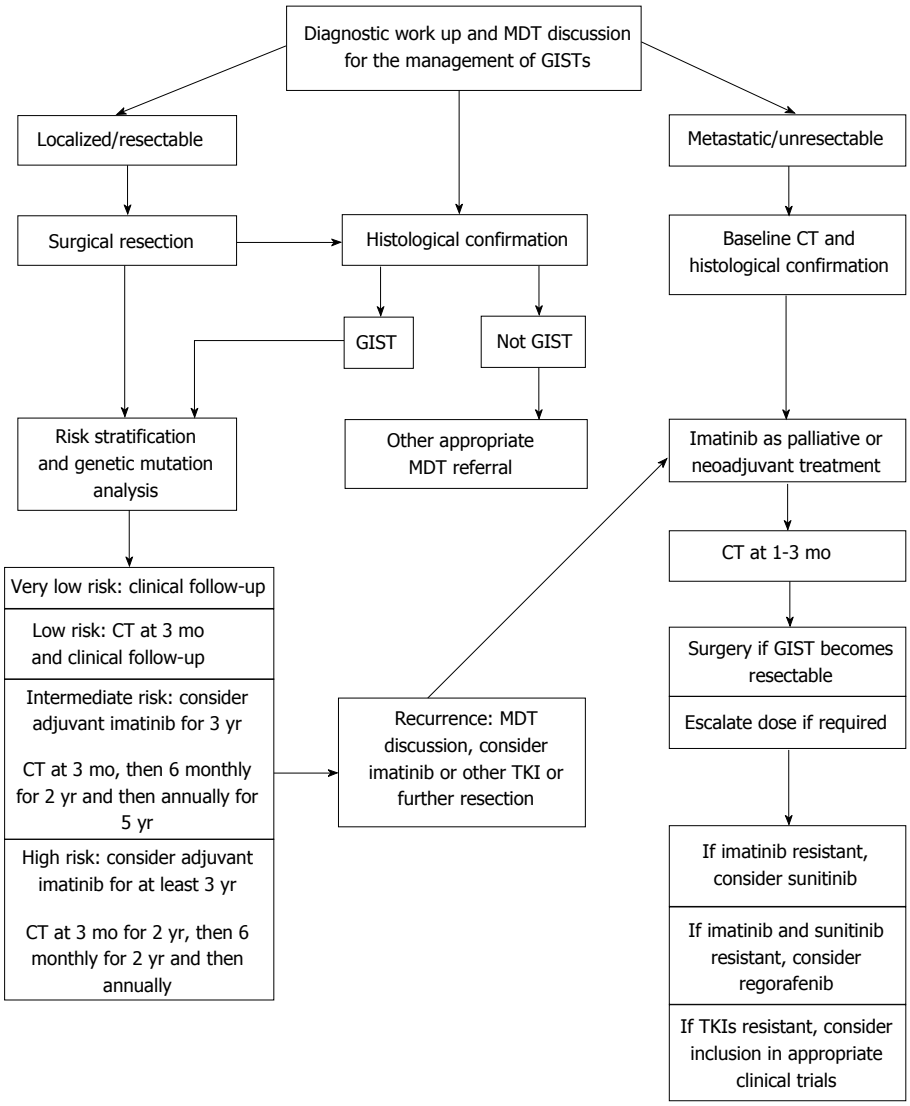

Figure 7 Algorithm for the management of gastrointestinal stromal tumors. GIST: Gastrointestinal stromal tumor; CT: Computed tomography; TKI: Tyrosine kinase inhibitor.

important roles as molecular targeted therapy in the future.

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REVIEW

Significance of dormant forms of *Helicobacter pylori* in ulcerogenesis

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Abstract

Nearly half of the global population are carriers of *Helicobacter pylori* (*H. pylori*), a Gram-negative bacterium that persists in the healthy human stomach. *H. pylori* can be a pathogen and causes development of peptic ulcer disease in a certain state of the macro-organism. It is well established that *H. pylori* infection is the main cause of chronic gastritis and peptic ulcer disease (PUD). Decontamination of the gastric mucosa with various antibiotics leads to *H. pylori* elimination and longer remission in this disease. However, the reasons for repeated detection of *H. pylori* in recurrent PUD after its successful eradication remain unclear. The reason for the redetection of *H. pylori* in recurrent PUD can be either reinfection or ineffective anti-Helicobacter therapy. The administration of antibacterial drugs can lead not only to the emergence of resistant strains of microorganisms, but also contribute to the conversion of *H. pylori* into the resting (dormant) state. The dormant forms of *H. pylori* have been shown to play a potential role in the development of relapses of PUD. The paper discusses morphological *H. pylori* forms, such as S-shaped, C-shaped, U-shaped, and coccoid ones. The authors proposes the classification of *H. pylori* according to its morphological forms and viability.

Key words: *Helicobacter pylori*; Forms of *H. pylori*; Dormant forms of *H. pylori*; Viable forms of *H. pylori*; Non-viable forms of *H. pylori*; Physiological states of *H. pylori*; Culturable forms of *H. pylori*; Unculturable forms of *H. pylor*; Resuscitation of dormant *H. pylori*; Ulcerogenesis

Core tip: The administration of antisecretory and antibacterial drugs can lead to the conversion of *Helicobacter pylori* (*H. pylori*) into the resting (dormant) state. C-shaped and U-shaped forms of *H. pylori*, most likely, are dormant forms of the bacteria. C-shaped

and U-shaped forms of *H. pylori* are capable of reverse transition into the vegetative replicative state and of causing development of recurrence of peptic ulcer disease (PUD). The induction of process reversion occurs under the influence of specific molecules. The identification and study of these compounds will allow development of new drugs aimed at preventing recurrent PUD associated with dormant forms of *H. pylori*.

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INTRODUCTION

Peptic ulcer disease (PUD) is a problem that is traditionally the center of attention of gastroenterologists[1]. Many aspects of this pathology have been well studied. The disease develops as a result of the influence of a set of various exogenous and endogenous etiological factors. There are many theories of PUD development, including vascular, inflammatory-gastritis, allergic, hormonal, motor-primacy, corticovisceral, neurogenic, psychosomatic, acido-peptic and infectious ones. Each of them deserves attention, as it reflects one of the facets of this complex problem. The diversity of the causes that lead to the pathological process allows PUD to be considered as a polyetiologic and polypathogenic disease.

PUD with a frequently recurrent or long-term healing ulcer of the stomach or duodenum generally occurs in the presence of chronic active gastritis or chronic active duodenitis, both of which are associated with *Helicobacter pylori* (*H. pylori*) infection. Decontamination of the gastric mucosa (GM) with various antibiotics results in *H. pylori* eradication and longer remission in PUD[2-6]. However, the reasons for repeated detection of *H. pylori* in relapsed PUD after its supposedly successful eradication remain unclear. This may be due to either reinfection or ineffective anti-Helicobacter therapy. In most cases, the administration of antibacterial drugs leads to complete *H. pylori* eradication, but can give rise to resistant bacterial strains and facilitate the conversion of *H. pylori* into the resting (dormant) forms[7]. It is therefore relevant to study dormant *H. pylori* forms, as well as their values in ulcerogenesis[8].

H. PYLORI IS ONE OF THE ETIOLOGICAL FACTORS OF PUD

Landmarks in the history of H. pylori studies

The accumulated scientific data can confirm that *H. pylori* infection is important in the mechanism of PUD development[9]. *H. pylori* was first reported in 1875 when Bottcher and Letulle observed it on the margins of peptic ulcers[10]. The bacterium did not grow in the artificial nutrient media that were known at that time, and this accidental discovery was long forgotten. In the 1980s, Australian pathologist Robin Warren together with Barry Marshall isolated *H. pylori* from human gastric mucosal biopsy specimens and cultured it in artificial nutrient media. Warren and Marshall suggested that most gastric ulcers and gastritis in humans might be associated with *H. pylori* infection[11,12]. Marshall demonstrated the role of *H. pylori* infection in the development of gastrointestinal diseases in 1983. He drank a culture of the bacterium to prove the etiological role of *H. pylori* in the development of antral gastritis. Thereafter, he developed *H. pylori*-associated antral gastritis. After that, many researchers concentrated on the study of *H. pylori*[13].

There has been gradually increasing evidence that duodenal ulcers and duodenitis are also associated with *H. pylori* infection[14-16]. In 2005, Warren and Marshall received the Nobel Prize in Physiology or Medicine for the discovery of *H. pylori* pathogenicity[12] and rekindled interest in the study of this microorganism. Since then, the association of *H. pylori* with digestive system diseases has been the subject of much research attention[10,16].

Risk of digestive system diseases caused by H. pylori

H. pylori is one of the most common infections worldwide. *H. pylori* infection is highly prevalent throughout the world, especially in developing countries[10]. Nearly half of the global population are carriers of *H. pylori*, a Gram-negative bacterium that persists in the human stomach and duodenum[12,17-21]. *H. pylori* gastric colonization is acquired early in life (almost always before the age of 10 years), and, in the absence of antibiotic therapy, generally persists for life[12,22,23]. The prevalence of *H. pylori* ranges from 35% to 90% in different populations[21,24-28]. It presents in 70%-90% of the population in developing countries and 35%-40% in developed ones[21,29].

Moscow falls into a group of cities with extremely high *H. pylori* infection prevalence, with the predominance of virulent bacterial strains[30]. It is reported that 88% of the Moscow working population is infected with *H. pylori*. Its prevalence is 78% in people younger than 30 years and about 97% in individuals older than 60 years[30]. The prevalence of *H. pylori* infection is high in developing countries, especially among children. In India, the prevalence of this infection is 22%, 56% and 87% in the 0-4, 5-9 and 10-19 year age groups, respectively[21,31]. *H. pylori* is usually the numerically dominant gastric microorganism[13]. However, PUD occurs only in a small percentage of *H. pylori* carriers[32].

H. pylori does not typically cause any adverse

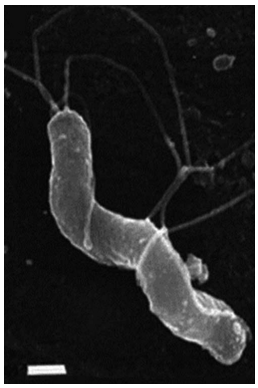

Figure 1 Morphology of *Helicobacter pylori*. S-shaped *H. pylori* with five to seven sheathed polar flagella. Field emission SEM, bar = 0.5 μm. (Field emission SEMs courtesy of L. Thompson and negative stains courtesy of S. Danon, School of Microbiology and Immunology, University of New South Wales). From: *Helicobacter pylori*: Physiology and Genetics. Mobley HLT, Mendz GL, Hazell SL, editors. Washington (DC): ASM Press; 2001. Chapter 6, Morphology and Ultrastructure[54].

effects[13]. Many people infected with *H. pylori* are shown to remain asymptomatic carriers[33]. It has turned out that *H. pylori* may behave as a commensal or symbiont, depending upon the circumstances[34-37]. The idea that *H. pylori* might actually confer benefits to humans has engendered considerable controversy among investigators. The data of the potential importance of health benefits that might be afforded by *H. pylori* are considered and debated in the review by Cover and Blaser[13]. It has been presumed that the conserved microbiota have specific adaptations that permit persistence at particular locales[13].

In the 1980-1990s, researchers studied the role of *H. pylori* as an important factor in the etiopathogenesis of PUD. Human gastric colonization by the bacterium *H. pylori* is a predisposing factor for gastrointestinal diseases, such as gastritis and PUD[13,38]. Strong links exist between PUD and *H. pylori* infection[39]. So, *H. pylori* detection rates in PUD vary from 60% to 100%. There is also a strong relationship between *H. pylori* and duodenal ulcer[21]. *H. pylori* has been shown to be one of the important local factors involved in the development of ulcerative defect[40,41]. The manifestations associated with chronic *H. pylori* infection vary considerably among distinct geographic regions and these differences have been attributed at least in part to polymorphisms of *H. pylori* genes, particularly those encoding virulence factors[15]. *H. pylori* is an important gastrointestinal pathogen associated with gastritis, PUD, and an increased risk of gastric carcinoma[22]. The presence of *H. pylori* in the gastroduodenal mucosa and its involvement in the development of chronic gastritis, PUD, carcinoma and other diseases are well documented[9,15,23,42].

Blaser considers that *H. pylori* shows its pathogenicity, by regulating the expression of different genes to the extent that is dictated by the response of a macroorganism[13,43]. The microorganism and macroorganism create a finely tuned balance system, the resulting impairment of which develops a specific disease with certain clinical signs and prognosis[44]. In the vast majority of cases, long-lasting *H. pylori* infection induces chronic gastritis, while only some patients develop PUD and gastric cancer. For this reason, the bacterium is considered to be a risk factor for the development and recurrence of PUD[45,46]. Therefore, *H. pylori* is assigned to the group of pathogenic bacteria. Antibiotic treatment of PUD results in bacterial disappearance and ulcer healing[12]. Marshall and Warren reported that eradication of the bacteria significantly reduced the duodenal ulcer relapse rate[12].

Characteristics of H. pylori

The genus *Helicobacter* (helix and bacteria) is heterogeneous[47]. The *Helicobacter* genus now includes at least 26 formally named species, and more that are still being studied[48,49]. Some of them were previously known by other names. Humans have been found to have only 11 *Helicobacter* species: *H. pylori*, *H. heilmannii*, and *H. felis* in the GM, *H. cinaedi* (*H. westmeadii*, *H. canadensis*), *H. fennelliae*, *H. canis*, *H. pullorum*, and *H. rappini* in the small intestinal mucosa. Some *Helicobacter* species have been isolated from the human hepatobiliary system: *H. pylori* from the liver, *H. bilis*, *H. pullorum*, and *H. rappini* from the bile ducts. *H. pylori* is the best known bacterium. *H. pylori* includes several strains[46]. *H. pylori* strains isolated from unrelated humans exhibit a high level of genetic diversity (reviewed in Blaser MJ and Berg DE[50]). The genetic structure of the pathogenic genes of *H. pylori* varies largely, which contributes to the differences in virulence among various strains and in clinical symptoms[15]. *H. pylori* strains differ in resistance to drugs, adhesive specificity and production of cytotoxins.

H. pylori (Figure 1) is a small, Gram-negative, asporogenous, S-shaped or slightly spirally curved, microaerophilic bacterium[51-53].

Thirty-seven degrees Celsius and pH 4.0-6.0 are the most favorable conditions for the life, growth, and reproduction of *H. pylori*; although, the species also survives at pH 2.5. *H. pylori in vivo* and under optimum *in vitro* conditions exists as an S-shaped bacterium with 1 to 3 turns, 0.5 μm × 5 μm in length, and a tuft of 5 to 7 polar sheathed flagella[54-56]. The bacterial cell is covered with a smooth sheath. The flagellum of *H. pylori* is 30 nm in diameter, consisting of an internal filament approximately 12 nm in diameter surrounded by a sheath, the outer membrane of which is continuous with the outer membrane of the cell[54-56].

Unipolar flagella are essential for the unique motility of *H. pylori*[57]. Qin *et al*[57] employed cryo-electron tomography to visualize intact *H. pylori* cells, with a particular focus on the flagella. Remarkably, the unipolar flagella of *H. pylori* are driven by one of the largest flagellar motors found in bacteria. The flagellar motor provides

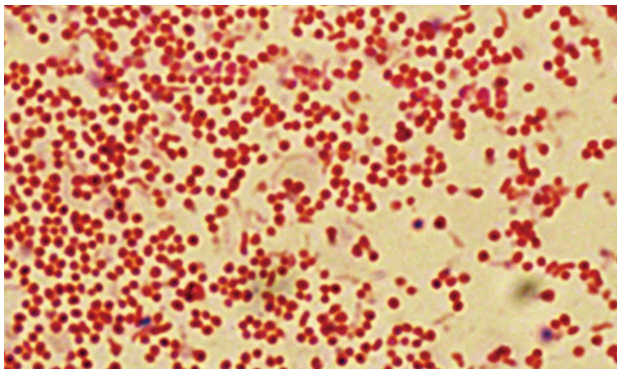

Figure 2 Microscopic images of coccoid forms of *Helicobacter pylori* after 6-d exposure to antibiotics. From: Faghri *et al*[65].

higher torque needed by the bacterium to navigate the viscous environment of the human stomach. Thin sections of *H. pylori* reveal the typical cell wall detail of a Gram-negative bacterium that consists of outer and inner, or plasma, membranes separated by the periplasm of approximately 30 nm thickness[54]. *H. pylori* has inherent corkscrew motility. The presence of flagella, a smooth cell sheath, a spiral shape, and corkscrew motility allows this microorganism to move in the mucus thickness along the pH gradient and serves as its virulence factor. In addition, the flagella contribute to the aggregation of *H. pylori* to colonize the latter on the epithelial surface of GM[58].

The stomach is the major habitat of *H. pylori*[13], but it may also survive in other environments[13,18,59]. The habitat of *H. pylori* may be the proximal duodenum or distal esophagus. This is usually accompanied by gastric metaplasia at these sites[13]. A gene that is pathognomonic for duodenal ulcer and called *dupA* (duodenal ulcer promoting gene), which encompasses the two *H. pylori* genes of *jhp0917* and *jhp0918*, has been discovered[60]. This gene increases the survival of the microorganism at low pH values. The presence of the *dupA* gene is associated with a high risk for duodenal ulcer and with a low risk for gastric atrophy and cancer[61].

COCCOID AND DORMANT FORMS OF *H. PYLORI*

Morphological forms of H. pylori

All living organisms are equipped with mechanisms that allow extended survival in adverse environments. For a number of them, this response involves, besides metabolic adaptations, changes in cell morphology[62]. *H. pylori* is mainly present as a spiral-shaped form in human gastric biopsy specimens. On aging, the bacterial cells lose their typical spiral-shaped form and convert to coccoid ones (Figure 2)[54]. When influenced by adverse factors (temperature or pH changes, prolonged fasting when cultivated, or use of antibacterial drugs), non-spore-forming microorganisms can be transformed into a latent coccoid form[63].

The ability of *H. pylori* to transform from the spiral-shaped form to the coccoid form is one of its most important, but not exclusive properties. Through the course of evolution, *H. pylori* has evolved special adaptive mechanisms and acquired vital physiological properties allowing it to survive extreme situations in the human organism, when cultivated, and to survive in the external environment[64].

The bacterium transforms from spiral to coccoid under mild circumstances, whereas under extreme ones it is unable to undergo shape modification. This strongly supports the view that transformation into the coccoid form is an active, biologically led process, switched on by the bacterium as a protection mechanism[62,65]. This study demonstrates that the coccoid shape is in fact a manifestation of cell adaptation to less than optimum environments.

Saito *et al*[66] identified three types of coccoid forms of *H. pylori*. The authors claim to represent different *H. pylori* transformation processes and consist of bacteria that are dead, living and cultivated, and viable but non-culturable[62,66]. Controversy exists as to whether these cells are viable, dormant or just dead[54].

The initial stage of *H. pylori* transformation in the coccoid form is accompanied by the condensation of the protoplasmic matrix and an increase in the periplasm on one side of the microorganism (usually at the pole opposite to the flagellar basal complex). An increase in the volume of the periplasmic space leads to stretching of the cell wall, displacement of the protoplasmic matrix to the periphery, and accumulation of dense material, that results in the formation of C-shaped and/or U-shaped cells (Figure 3)[54].

These C-shaped and/or U-shaped forms then convert to the coccoid cells, with an increase in the protoplasmic cylinder and maintenance of the double membrane system (Figures 3 and 4)[54]. In their work, Mouery *et al*[67] have shown transmission electron micrographs of typical stages of the helical-to-coccoid transformation. C-shaped and/or U-shaped forms of *H. pylori* are an intermediate state of the bacteria[67-69].

The C-shaped and/or U-shaped forms of *H. pylori* are an intermediate bacterial type transforming into an inactive phase (dormancy). Dormancy is understood to be a reversible state of bacterial cells with a low metabolic activity, in which they can be for a long time without replication[63,70,71]. In microbiology, this condition has, until recently, been associated with the presence of forms, such as spores and cysts. In the late 20th century, the literature began to discuss the possibility of formation of dormant (resting) forms by non-spore-forming bacteria[63,72] that encompass most Gram-negative pathogenic bacteria, including *H. pylori.* Dormancy is characterized by the increased resistance of bacterial cells to extreme stresses (deficiency of nutrients, effects of antisecretory drugs, antibiotics, *etc.*) and favors their survival[73]. The ability

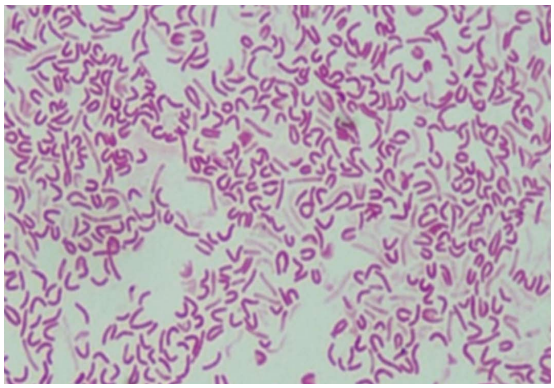

Figure 3 Microscopic images of morphological forms of *Helicobacter pylori* after exposure to antibiotics: S-shaped, U-shaped, C-shaped and coccoid-shaped. From: Faghri *et al*[65].

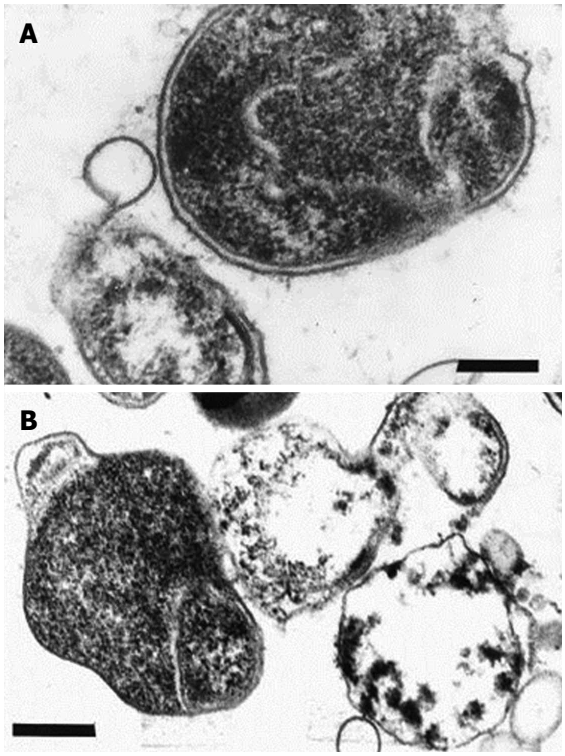

Figure 4 Electronograms of coccoid forms of *Helicobacter pylori*. A: Initial ingrowth in the periplasmic space resulting in the formation of U-shaped cells; B: Conversion to the coccoid form. Ultrathin section, bar = 0.5 μm. From: *Helicobacter pylori*: Physiology and Genetics. Mobley HLT, Mendz GL, Hazell SL, editors. Washington (DC): ASM Press; 2001. Chapter 6, Morphology and Ultrastructure[54].

of *H. pylori* to go into a dormant state may be an important factor in the epidemiology and spread of helicobacteriosis. The C-shaped and U-shaped forms of *H. pylori* can be considered truly a dormant form capable of reinfection[53]. The role of these forms in the pathogenesis and transmission of infection needs to be clarified.

The C-shaped and/or U-shaped forms of *H. pylori* keep the polar membrane associated with the flagellar basal complex[56,65]. Only a small number of intermediate forms of *H. pylori* possesses a complete set of flagella and retains metabolic activity to ensure mobility comparable to that of spiral-shaped forms[68].

The fully-formed coccoid forms maintain the basic pattern of a bacterial cell structure (Figure 4). They have a cell wall, cytoplasmic membrane and cytoplasm[56,62,67,69,74]. The coccoid cells differ in details of the cell wall structure, which leads to impairment of recognition of the bacteria by the host immune system (bacterial mimicry)[68].

The accumulated scientific data suggest that there are three morphological forms of *H. pylori*: (1) S-shaped forms; (2) U-shaped and C-shaped (intermediate or transitional) forms; and (3) Coccoid forms.

The intermediate and coccoid forms can coexist in the mucosa or in the culture in various ratios[75]. Their ratio depends on the time of *H. pylori* being present under adverse conditions and on the level of exposure to adverse factors. Spiral-shaped forms were predominant in a 3-d culture of *H. pylori*; about half of the bacteria are coccoid forms at 6 d[76-78]. Mouery *et al*[67] show the graphical distribution of the ratio of morphological forms of *H. pylori* after 12, 24 and 48 h of cultivation. The distributions of morphologies of more than 100 cells of each genotype for each time point are shown[67]. The number of coccoid forms increases with the longer time of cultivation. This happens due to the transition of C-shaped and U-shaped forms of *H. pylori* to coccoid ones.

The C-shaped, U-shaped and coccoid forms of *H. pylori* lose enzyme activity and show a lower metabolism[79]. The urease activities of resting (dormant) and coccoid cells were found to be lower than those of the spiral-shaped form of *H. pylori*[80,81]. A significant transformation of *H. pylori* into coccoid forms may result in loss of urease activity. However, urease-encoding genes continue to be identified in *H. pylori* by polymerase chain reaction (PCR)[69,81,82]. The minimization of enzyme activity and energy metabolism in the transformable *H. pylori* forms is adaptive and aimed at preserving the viability of microorganisms[65,83]. Bacterial viability has been confirmed by the fact that the transformed forms of *H. pylori* can be detected by acridine orange staining[69,84]. The C-shaped, U-shaped and coccoid forms of *H. pylori* tolerate a wider pH range to a greater extent than the spiral-shaped forms, are resistant to unfavorable factors and antibiotics, and cannot lose virulence. All this creates favorable conditions for the preservation of bacteria in the human body or in the external environment.

The triggers of *H. pylori* transformation from spiral-shaped to coccoid forms in the environment may be physical factors: higher insolation, low humidity, and lack of food substrates[77,85]. During bacteriological cultivation, transformation into the coccoid forms occurs due to the depletion of adequate components of the nutrient medium[85,86]. *H. pylori* transforms in the human body due to changes in the habitat conditions upon exposure to antisecretory and antibacterial

Table 1 Detection of *Helicobacter pylori* in gastric mucosa biopsy specimens from in-patients with duodenal ulcer by quantitative urease test and polymerase chain reaction before and one month after anti-Helicobacter therapy

Detection of *Helicobacter pylori*	Quantitative urease test	PCR	Difference between methods
Before treatment, %	93.4	98.7	5.3
After treatment, %	11.1	24.1	13.0

PCR: Polymerase chain reaction.

drugs. Khomeriki *et al*[69] have studied the time course of changes in the transformation of *H. pylori* in the GM. They have indicated that the spiral-shaped forms transform into the coccoid ones a few hours after adhesion to the cell surface of GM cells[69].

The conversion of the spiral forms of *H. pylori* to its C-shaped, U-shaped and coccoid forms in the GM and in the nutrient medium is due to the accumulation of toxic metabolic products of *H. pylori* vital functions. Reactive oxygen species generated by phagocytes or by *H. pylori* itself in the presence of specific pyridine nucleotides may trigger the formation of transitional and coccoid forms in the GM of untreated patients[69]. When an unfavorable situation occurs, there is accumulation of factors that induce the conversion of cells in the bacterial populations to a dormant state.

Loginov *et al*[8] carried out a comparative analysis in which *H. pylori* in GM biopsy specimens from patients with duodenal ulcer was detected by a quantitative urease test and PCR before and a month after anti-Helicobacter therapy. Prior to anti-Helicobacter therapy, the detection rate of *H. pylori* in the GM biopsy specimens from patients with PUD was 93.4% and 98.7%, as shown by the quantitative urease test and PCR, respectively (Table 1). The difference of 5.3% in the detection rate of *H. pylori* may be due to the different sensitivities of these methods.

One month after eradication therapy, these patients had *H. pylori* detected by the quantitative urease test in 11.1% of cases and by PCR in 24.1%. The difference between the methods was 13%, *i.e.*, almost twice that as before the treatment. These findings suggest that *H. pylori* were not completely eliminated in some patients at 1 mo after the anti-Helicobacter therapy, and the *H. pylori* diagnosed by PCR were at least partially in a dormant (resting) state and partly in coccoid forms. The low urease activity (or lack thereof) of coccoid *H. pylori* forms precludes identifying them by the quantitative urease test. The data presented allow us to indirectly suggest that after anti-Helicobacter therapy, the dormant forms of *H. pylori* are present in patients as a result of their incomplete elimination[7,87].

Viability of dormant and coccoid forms of H. pylori

The pleiomorphic nature of *H. pylori* has been the subject of intensive debate for many years, with part of the scientific community arguing that the coccoid shape represents a degraded, nonviable form of the cell[62,85,86,88-91]. Evidence supporting the concept that the coccoid forms are degenerate and not capable of growth comes from a number of studies showing that as the cells age, the levels of DNA and RNA and mRNA expression decrease with degradation of the nucleic acids, nonrandom fragmentation of the ribosomal RNA, and no evidence of a membrane potential necessary for processes such as oxidative phosphorylation[54]. There is evidence that the coccoid forms lose their reproductive ability, are unculturable in artificial nutrient media, have no characteristic features under a light microscope, and do not produce urease or produce it in low amounts[65,79,81,86,92,93]. The infectivity of coccoid *H. pylori* forms is still controversial[94].

There are opposing data regarding the viability of the C-shaped, U-shaped and coccoid form of *H. pylori.* A study by Khomeriki and Morozov[69] indicated that the structural transformation of spiral-shaped forms of *H. pylori* into the coccoid forms is not always a sign of functional disintegration of the microorganism. There is evidence confirming the viability of the transformed forms of *H. pylori*[81]. They maintain cell structure, exhibit respiratory activity, support protein metabolism and expression, and, in some cases, are capable of reverse transition into the vegetative spiral-shaped bacteria (on their passage through animals)[92]. Cell respiration is detected in up to 40% after 45 d *in vitro* cultivation of *H. pylori*[54,95-97]. The findings of Poursina *et al*[94] suggest that the induced coccoid form of *H. pylori* is not a passive entity but can actively infect a human by expression of the virulence genes after a long stay in the stomach and may contribute to the development of chronic and severe disease. Flow cytometry analyses show that the majority of the induced coccoids (90%-99.9%) are viable[94].

There is evidence of the viability of the transformed forms of *H. pylori* obtained using biochemical methods. The cultures consisting of intermediate and coccoid forms have been found to retain oxidative metabolism at the same level as spiral-shaped forms for several months[98]. They maintain a high level of alkaline and acid phosphatases and a stable ATP level that increases if a number of fresh nutrient medium is added to the old culture[80-82,99]. Incorporation of a bromodeoxyuridine label into the transformed forms is suggestive of their continuing synthesis of new DNA[65,69]. So far, it is unconfirmed whether these data indicate the viability of *H. pylori* or the persistence of cells as "bags of enzymes"[98].

The contradictory data on the viability of the transformable forms of *H. pylori* are likely due to the fact that *H. pylori* coexists in coccoid and transitional (intermediate C-shaped and U-shaped) forms under unfavorable conditions in the human body or culture media. It is impossible to isolate data on the viability of intermediate and coccoid forms co-existing in the same culture. Apparently, one part of transformed cells in the population of *H. pylori* truly is degenerative,

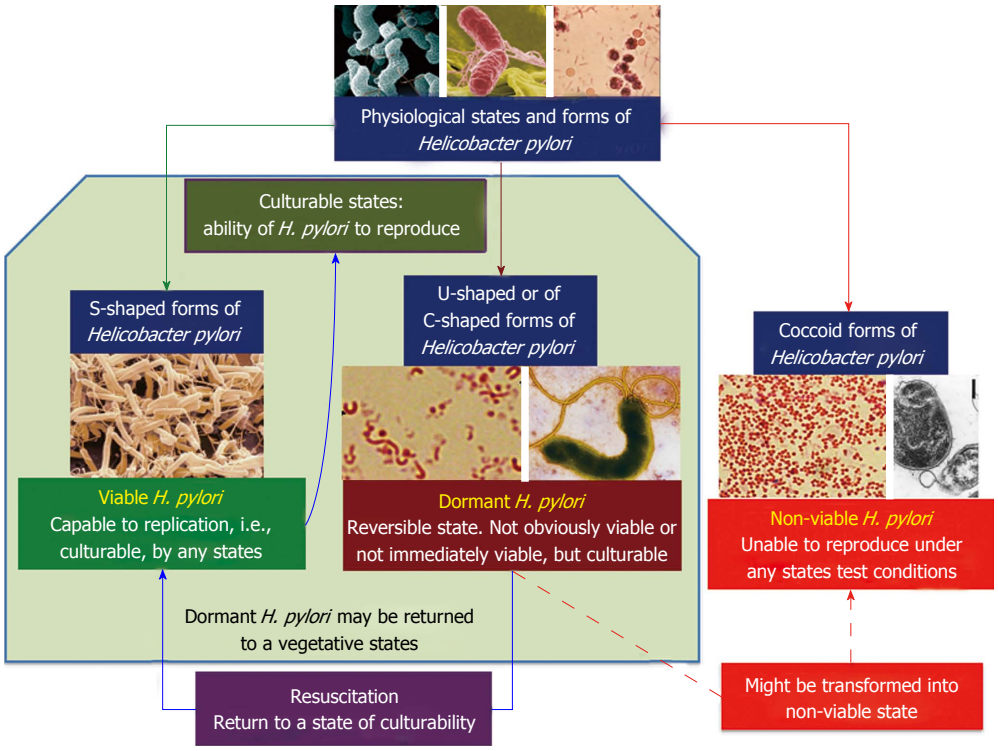

Figure 5 Major physiological states and forms of *Helicobacter pylori*[101]. *H. pylori*: *Helicobacter pylori*.

dead cells[100] (most likely it is the coccoid forms), and the other is dormant cells, reversible forms (most likely it is the C-shaped and U-shaped forms). There is evidence confirming the concept of viability of *H. pylori* dormant forms that indicate saving of cellular integrity and DNA synthesis in 3-mo-old cultures[54].

Available literature data may suggest the existence of the following conditions for various forms of *H. pylori* (Figure 5): (1) Viable and culturable (spiral-shaped forms of *H. pylori*) states; (2) Dormant (resting) and culturable (most likely it is the C-shaped and U-shaped forms of *H. pylori*) states; and (3) Non-viable and unculturable (most likely it is the coccoid forms of *H. pylori*) states.

However, there are not enough solid data to associate the particular morphological type (C-shaped, U-shaped and coccoid forms) of *H. pylori* with the functional characteristics of viability and culturability[65]. As a rule, the literature presents data on the viability of either spiral or coccoid forms. When describing the latter, the presence of dormant (transitional, intermediate, resting) forms is not generally taken into account. The C-shaped and U-shaped forms of *H. pylori* are most likely in a dormant (resting) state and can be a viable and culturable (Figure 5)[101].

And if so, once under favorable conditions, the dormant (resting) forms of *H. pylori* can revert to a vegetative spiral-shaped form. By using electron microscopy, Konstantinova *et al*[102] have shown that there are various defects in the cell wall of the transformed forms of *H. pylori*. The authors point out that before the reversion of the dormant forms of *H. pylori* to vegetative forms, there is a need for certain conditions for the repair of cellular damages.

"Reanimated" *H. pylori* can play an important role in the development of recurrent PUD after anti-*Helicobacter* treatment[81,96]. The "revived" forms of *H. pylori* are able to colonize the GM to subsequently develop peptic ulcer relapse[78,95]. Continuation of investigations in this area may reveal new important aspects of the pathogenesis of *H. pylori* infection and to find new ways to treat diseases associated with this microorganism[69,89,96].

Figure 5 suggests a classification of major physiological states and forms of *H. pylori* that is a hypothetical scheme and requires further evidence. Existing conflicting data on viability and culturability of various forms of *H. pylori* fit well with this scheme and pass into the category of comparable data.

DORMANT FORMS OF *H. PYLORI* IN THE DEVELOPMENT OF RECURRENT PUD

The main challenge is to prove the reversion of transformable forms of *H. pylori* into a normal replicative state. There is still no clear separation between the true "revivals" of transformed forms of *H. pylori* that are usually present in the population and secondary infection with the microorganism.

Genetic typing of the same strain of the microorganism has become possible with advances in molecular diagnosis. By using the PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis,

H. pylori strains in patients with duodenal ulcer were genotyped before and 1 mo after anti-Helicobacter therapy with incomplete elimination[7,8,87,103,104]. The *flaA* gene (1.5 kb in size) encoding the flagellar protein is one of the polymorphic ones in *H. pylori*. This gene is used in genetic typing of *H. pylori* strains. Identical *H. pylori* strains were detected in the same patient before and after the anti-Helicobacter therapy. At that, heterogeneous *H. pylori* strains were found in different patients. The given data suggested that there was neither superinfection nor reinfection with a new strain at 1 mo after anti-Helicobacter therapy.

Warren and Marshall reported that eradication of the bacteria markedly reduced the relapse rate of duodenal ulcer[12]. *H. pylori* eradication decreases the recurrence rate of PUD from 50% to 0%-10% of cases per year[105]. Current treatment modalities allow eradication of the *H. pylori* bacterium in up to 90% of cases (less if there is clarithromycin resistance)[106]. During the first years after effective anti-Helicobacter therapy, the rate of *H. pylori* reinfection in adults is 0%-35%[107]. The rate of *H. pylori* reinfection varies according to geographical area[106]. Reinfection in developed countries is less common, in 0%-7% of cases[108]. In regions with higher socioeconomic status and lower prevalence of *H. pylori*, it is only 1.68% of cases[106]. The *H. pylori* reinfection rate in Lithuania is relatively high (the annual rate being 3.36%), probably because of the high prevalence of *H. pylori*[105]. This could indirectly reflect differences in the socioeconomic status between Western and Eastern European countries[106]. In contrast, in developing countries, the reinfection rate could be much higher and has been reported to reach 9.63%[106,109,110].

In some cases, recrudescence or reinfection of *H. pylori* may occur[106]. According to Loginov *et al*[7], 18.4% of *H. pylori*-positive patients were identified among those with *H. pylori*-associated duodenal ulcer a year after successful treatment. The recurrence rate of PUD in these patients was 14.3%, which comprised 2.6% of the total number of patients included in the study patients. Reinfection of *H. pylori* is observed rarely and occurs during later periods. Reinfection is considered when *H. pylori* is found after confirmed *H. pylori* eradication. *H. pylori* strains genetically different from the original ones are generally identified in reinfection.

PCR-RFLP was used to detect *H. pylori* strains in patients with duodenal ulcer before and 1 year after anti-Helicobacter therapy[7,103,104]. *H. pylori* strains were genotyped by the *flaA* gene. Genetic typing of *H. pylori* strains revealed both cases of the same strain of the bacterium in a patient before and 1 year after anti-Helicobacter therapy, as well as cases of its different strains. Gastroduodenoscopy (EGD) at 1 year after treatment revealed that all *H. pylori*-positive patients had a pattern of exacerbation of chronic antral gastritis. At that, 1 mo after anti-*Helicobacter* therapy, these patients were found to have no signs of any gastric and duodenal changes during EGD.

Identification of the pattern of chronic antral gastritis and different strains of the bacterium a year after anti-Helicobacter therapy performed in patients with duodenal ulcer could reveal a case of reinfection with a new *H. pylori* strain in the patient successfully treated against *H. pylori*. Identification of the pattern of chronic antral gastritis and the same strain of *H. pylori* a year after anti-Helicobacter therapy in patients with duodenal PUD most likely suggests that the bacterium is transformed from a dormant (resting) state into the vegetative form. Hence, for successful therapy, it is essential not only to eradicate the spiral forms of *H. pylori*, but to eliminate its viable dormant forms.

Factors contributing to the transformation of dormant (resting) forms of bacteria into the vegetative ones

Mukamolova *et al*[111] have identified specific bacterial cytokines from *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Micrococcus luteus*, *etc.,* and showed their important role in the activation and reproduction of the dormant forms of the bacterium[111-114]. Cultivation of *M. luteus* in the presence of a small number of colony-forming cells in the starving population has been ascertained to greatly facilitate the resuscitation of dormant cells[115]. The authors have suggested that the viable cells are able to secrete certain substances promoting the transition of dormant forms into an active reproduction state[111,112,116]. A 16-17 kDa protein, named resuscitation-promoting factor (Rpf), has been isolated[112-115]. The protein promoted the "revival" of the starving cells and reduced the lag phase of an active culture in both the depleted and enriched nutrient media. Using *M. luteus* as an example, Rpf has been shown to stimulate the "reanimation" of dormant cells. Rpf is a pheromone and belongs to the bacterial cytokines[111].

Structural changes in the reversion of coccoid forms of *H. pylori* in the vegetative state have not been studied and their triggers are unknown. There is no evidence that there are cytokine factors for the activation of *H. pylori* reversion and growth. The slightly acidic environment (pH of 5 to 3.5) is known to be a factor that activates the process of protein synthesis in *H. pylori*. Interestingly, in acting on coccoid and spiral-shaped forms, the same acidic pH values induce the synthesis of various proteins in them[78,117]. Some of the *H. pylori* proteins, heat shock protein (Hsp) in particular, have a trophic effect on the bacteria themselves and can cause rearrangement of the cell cytoskeleton, which may be a trigger for the reverse transformation of dormant forms into vegetative ones. Hsp synthesis is enhanced under the influence of a number of environmental factors.

These subcellular proteins belong to the chaperones essential for viability of the entire cellular profile of

proteins involved in the processes of assembling for a variety of high-molecular-weight proteins[118]. *H. pylori* possesses two of the most studied chaperones: HspA (smaller) and HspB (larger), which are associated with urease assembling. HspA differs in its properties from analogous proteins of other bacteria. Being strong antigens, *H. pylori* chaperones take a certain part in the activation of lymphocytes, the regulation of cytokine and chemokine expression, the induction of apoptosis, *etc*. However, heat shock proteins play a much more important role in the induction of an autoimmune response due to the fact that they are highly antigenically similar to the orthologic structures of the GM[119]. It is possible that cytokine factors of the macroorganism can play an important role in the activation of bacterium dormant forms.

CONCLUSION

It is necessary to continue studies aimed at identifying specific cytokines or other metabolites of *H. pylori*, which are able to activate the transition of dormant forms of the microorganism into the vegetative spiral state. This will be able to design new anti-Helicobacter drugs to prevent the activation of dormant *H. pylori* forms, as well as recurrent duodenal ulcer.

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MINIREVIEWS

Prognostic significance of red blood cell distribution width in gastrointestinal disorders

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Abstract

The red blood cell distribution width (RDW) is a routinely measured and automatically reported blood parameter, which reflects the degree of anisocytosis. Recently, the baseline RDW was found to have clinical significance for assessing clinical outcome and severity of various pathological conditions including cardiovascular diseases, sepsis, cancers, leukemia, renal dysfunction and respiratory diseases. A myriad of factors, most of which ill-defined, have an impact on the red cell population dynamics (*i.e.*, production, maturation and turnover). A delay in the red blood cell clearance in pathological conditions represents one of the leading determinants of increased anisocytosis. Further study of RDW may reveal new insight into inflammation mechanisms. In this review, we specifically discuss the current literature about the association of RDW in various disease conditions involving the gastrointestinal and hepatobiliary systems. We also present some of the related measurements for their value in predicting clinical outcomes in such conditions. According to our data, RDW was found to be a valuable prognostic index in gastrointestinal disorders along with additional inflammatory biomarkers (*i.e.*, C reactive protein, erythrocyte sedimentation rate, and platelet count) and current disease severity indices used in clinical practice.

Key words: Red blood cell distribution width; Hepatitis; Pancreatitis; Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Colon cancer; Hepatocellular carcinoma; Acute mesenteric ischemia; Gastrointestinal diseases

Core tip: Mounting evidences show that red blood cell distribution width can be used as a prognostic marker in gastrointestinal disorders. A number of retrospective studies have been published about the use of this index of anisocytosis in prognostication of gastrointestinal disorders, especially inflammatory bowel disease and viral hepatitis among others. However, only a few have included confounding factors which could affect red blood cell distribution width. Our objective is to consolidate the current literature to better understand the use and further investigate the significance of red blood cell distribution width in gastrointestinal disorders.

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INTRODUCTION

The red blood cell distribution width (RDW) is a measure of size variability and heterogeneity of erythrocytes in the peripheral blood (*i.e.*, anisocytosis)[1]. RDW is a part of complete blood count and is a routinely measured and automatically generated blood parameter that has lately gained considerable interest due to its ability to help assessing the prognosis of various diseases. The value of RDW in assessing the severity of disease and clinical outcome has been proven in various conditions including, but not limited to, sepsis, renal dysfunction, cardiovascular and pulmonary diseases and malignancies. RDW was also proven useful in assessing mortality rates and survival of hospitalized patients, including those admitted to the intensive care unit (ICU)[2].

In a healthy adult, nearly 2 million red blood cells (RBCs) per second enter the peripheral circulation. The lifespan of a typical RBC is 100-120 d. During this period, a kaleidoscope of factors, many of which still unknown, may impact the RBC population dynamics (production, maturation, turnover and clearance). It is also known that RBCs, while in circulation, undergo a process of reduction (fast and slow phases) of volume (approximately 30%) and hemoglobin content (approximately 20%), leading to a slight increase in the relative hemoglobin concentration of RBCs (mean corpuscular hemoglobin; MCH) toward the end of their lifespan[3]. RDW increases with aging and this is clearly attributable to the gradual increased prevalence of comorbidities, which may actually contribute to derange erythrocyte biology and lead to release of a population of RBCs with heterogeneous size[4]. Hemoglobin concentration of RBCs is tightly controlled, because it is essential for adequate oxygen delivery to the tissues. In addition, recent studies showed that RBC clearance is another process under tight regulation, and that a delay in RBC clearance appears to be modulated in pathological conditions. RDW may increase in many diseases due to the impaired turnover of RBCs, which may both lead to increased permanence of aged cells in the circulation or release of immature and larger cells from the bone marrow due to increased turnover. Using a semi-mechanistic mathematical model of *in vivo* RBC population dynamics, Patel *et al*[3] showed that a delay in RBC clearance leads to a longer persistence of smaller volume RBCs in peripheral circulation, thereby increasing anisocytosis and consequently, RDW.

Most of the conditions affecting the gastrointestinal (GI) tract necessitate evaluation using minimally invasive and invasive procedures, such as endoscopy with or without biopsy. These procedures require specialized, expensive equipment and trained technical personnel. These may not be easily available in certain regions of the world, and may not be affordable to all the patients. Moreover, even with these evaluations, it is sometimes challenging to predict the clinical outcomes of GI conditions. The identification of a reliable and reproducible parameter that may provide important information on the evaluation of GI diseases is therefore of paramount importance.

In this manuscript, we review the most recent literature about the prognostic value of RDW for assessment of disease severity and clinical outcome in both benign and malignant conditions affecting the GI and pancreaticohepatobiliary systems.

ROLE OF RDW IN GUT DISORDERS

RDW and esophageal disorders

The prognostic value of RDW in esophageal disorders has only been studied in esophageal cancer (EC). One of the first studies was conducted by Chen *et al*[5] who carried out a retrospective analysis of 277 esophageal squamous cell carcinoma (ESCC) patients undergoing radical esophagectomy without preoperative neoadjuvant therapy. Patients were followed every 3-6 mo for two years, and annually thereafter, with a median follow-up of 42.5 mo. The mean initial RDW was 14.5% ± 2.3%. The patients were divided in to two groups (*i.e.*, RDW $\geqslant$ 14.5% and RDW < 14.5%). Patients with RDW < 14.5% had significantly better 5-year cancer-specific-survival than those with RDW $\geqslant$ 14.5%. Increased RDW was then found to be an independent prognostic factor for cancer specific survival, with mortality being nearly twice higher in patients with RDW $\geqslant$ 14.5% that in those with lower values[5]. Wan *et al*[6] studied 179 patients with EC (133 patients with squamous and 46 with adenocarcinomatous pathology) with median

follow-up of 21 mo, and found that patients with high RDW > 15% exhibited a shorter disease-free survival (P = 0.043, HR = 1.907, 95%CI: 1.020-3.565) and unfavorable overall survival (P = 0.042, HR = 1.895, 95%CI: 1.023-3.508) independently from other cancer-related factors.

Hirahara *et al*[7] also investigated 144 patients undergoing esophagectomy for ESCC, observing that a high RDW value was independently associated with cancer-specific survival poor prognosis in patients aged 70 years or younger. Sun *et al*[8] proposed to calculate the ratio of hemoglobin (Hb) to RDW (*i.e.*, Hb/RDW) as a novel prognostic factor in patients with ESCC. They observed that patients with a Hb/RDW ratio of < 0.989 had a 1.4-time higher risk of death during follow up compared to patients with Hb/RDW ratio > 0.989 (95%CI: 1.024-1.958, P = 0.035)[8]. Recently, Hu *et al*[9] studied 2396 patients (1822 men and 574 women) from a Fujian prospective cohort, who underwent three-field lymphadenectomy for ESCC and with median follow-up of 38.2 mo. Interestingly, RDW was found to be an independent predictor of ESCC mortality in the male gender only (adjusted HR = 1.5, 95%CI: 1.08-1.22, P < 0.001). In females, only lymphocyte count was marginally significant for predicting survival[9].

All of the above studies have consistently shown the existence of a relationship between increased RDW and poor disease-specific and overall survival in patients with EC, especially ESCC. However, these studies were mainly conducted on Asian population in China and Japan, where ESCC is more common than adenocarcinomatous EC in Western countries. This likely explains why all these studies were conducted mainly in patients with ESCC. At this time, it remains hence unclear whether or not the prognostic role of RDW can be extended to ESCC affecting western population.

RDW and gastric disorders

The role of RDW in gastric disorders has not been extensively studied. Since one of the most common features of gastric disorders is anemia, which can also significantly impact RDW values, challenges remain to carry out studies for evaluating the relationship between RDW and gastric disorders. Tüzün *et al*[10] performed a retrospective study in 122 patients with autoimmune gastritis (AIG) who were compared with 101 patients with functional dyspepsia. RDW was found to be significantly increased in patients with AIG (16.11% ± 3.04% *vs* 13.41% ± 0.95%, P < 0.001). Receiver operating characteristics (ROC) curve analysis suggested 13.95% as the optimum cut-off point (AUC: 0.860)[10]. They further analyzed the same patients to define the potential role of RDW in AIG and gastric carcinoid tumor type Ⅰ (94 AIG without gastric carcinoid and 28 AIG with gastric carcinoid), but failed to find a significant association[10]. However, the hemoglobin values were not included in a multivariate regression analysis to establish whether or not RDW was independent predictor of AIG compared to functional dyspepsia. This is a substantial drawback since the patients in the AIG cohort had significantly lower values of both vitamin B12 and ferritin.

In a retrospective study, Pietrzyk *et al*[11] studied RDW in gastric cancer and healthy individuals, concluding that gastric cancer patients had higher mean RDW values (14.9 ± 3.9) than healthy individuals (12.2 ± 0.7). It was hence suggested that elevated RDW, when combined with symptoms, can be used as an alert for upper endoscopy to early detect gastric cancer[11]. In another recent study in gastric cancer patients undergoing curative surgery, high preoperative RDW values were found to be significant predictors of 60-d mortality (17.9 ± 4.3 *vs* 16.0 ± 3.2; P = 0.015). The incidence of advanced gastric cancer was higher in patients with RDW ⩾ 16% than in those with lower values (75% *vs* 51%, P = 0.002), whereas disease free and overall survival was found to be reduced (P = 0.04)[12].

Isik *et al*[13] conducted a retrospective study on 147 patients with upper gastrointestinal hemorrhage, and found that these patients had significantly higher median RDW value compared to the standard reference value (15.3% *vs* 14.5%). This was not surprising since anemia and anisocytosis are tightly linked. The eight patients who died during the study period had significantly higher median RDW value (18.9%) compared to those who did not (P = 0.02), so concluding that RDW could be used as a predictor of mortality in upper gastrointestinal hemorrhage[13]. In an interesting investigation, Fatemi *et al*[14] studied 6689 patients with myocardial infarction who underwent percutaneous coronary intervention (PCI). Higher RDW values were significantly associated with post-procedural in-hospital GI bleeding (P < 0.001), a risk increasing in parallel with RDW values[14]. Despite these results, the authors did not exclude confounding factors known to be associated with anisocytosis. The results of this large study suggest that physicians may use RDW for identifying patients at higher risk of GI bleeding post PCI.

RDW and celiac disease

Due to increasing incidence and prevalence, the need of simple laboratorial markers to diagnosing and monitoring adherence to gluten-free diet and the treatment of celiac disease have recently emerged. Brusco *et al*[15] studied 126 untreated histologically diagnosed celiac disease patients, and found that increased RDW was the most frequent hematological abnormality in these patients (73 of 126; 58%); followed by anemia (31%) and iron deficiency (29%). Forty three out of 73 patients with increased RDW were restudied after a median follow-up of 12 mo and RDW was found to be decreased to normal value

except in five patients ($P < 0.001$) when on gluten-free diet[15]. Balaban *et al*[16] compared patients with newly diagnosed celiac disease, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS), and found that almost two-thirds of patients with celiac disease had elevated RDW as compared to only 9% of patients with IBS. However, the possible mechanisms of increased RDW in IBS were not explained. Importantly, they also found that a spleen diameter to RDW ratio < 6 had 0.73 sensitivity and 0.89 specificity for detecting celiac disease. The AUC for predicting celiac disease was 0.737 (95%CI: 0.597-0.877)[16]. Sategna Guidetti *et al*[17] also showed that increased RDW values may be reliable predictors of celiac disease even in the presence of normal hemoglobin value. They also observed that gluten-free diet was effective to significantly reduce RDW values within one year. Many other studies showed that RDW values significantly improved with gluten-free diet in patients with celiac disease[18-20]. Therefore, normalization of RDW in selected celiac disease patients can seemingly be used as a simple but indirect index for assessing patients' response to gluten-free diet. Notably, no study in the current scientific literature has explored the role of RDW for predicting development of intestinal lymphoma or refractory sprue in patients with celiac disease.

Harmanci *et al*[19] retrospectively studied 49 newly diagnosed celiac disease patients and compared their RDW values according to the presence of intestinal atrophy. A RDW value of > 17.25% was found to be the most significant predictor of atrophy in these patients ($P = 0.003$). The authors also concluded that RDW values > 17.7% predict intestinal atrophy with 0.76 sensitivity, 1.00 specificity, 0.79 PPV and 1.00 NPV when combined with tissue transglutaminase antibody titers of > 200 U/L in patients with celiac disease[19].

RDW in the IBD

IBD is characterized by chronic relapsing and remitting inflammatory changes of bowel, which are increasing in incidence and prevalence in the general population. Multiple blood tests such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), interleukin (IL)-6, white blood cell count (WBC) (among others) have been evaluated as surrogate markers of disease activity in IBD, but showed variable diagnostic performance. Therefore, there is still wiggle room for searching an ideal, noninvasive, simple, inexpensive and highly specific laboratory test for monitoring disease activity. In a retrospective study, Clarke *et al*[21] found that mean RDW at diagnosis was significantly higher in Crohn's disease (CD) compared to ulcerative colitis (UC) patients (14.9% *vs* 14.3%, $P = 0.027$). A higher prevalence of malabsorption-related anemia due to distribution of lesions in CD relative to UC has been brought to explain this finding, and it was finally suggested that RDW may be used as a marker for differentiating between the two distinct forms of IBD (CD and UC)[21]. However, the role of additional confounding factors such as hemoglobin, other causes of anemia (*e.g*., blood loss) and severity of disease at diagnosis was not explored.

Molnar *et al*[22] performed a retrospective study to investigate whether RDW value may be helpful in differentiating CD and UC in both active state and remission. It was hence observed that RDW value was increased in 53.2% of inactive CD *vs* 36.85% of inactive UC (mean RDW 14.3% *vs* 13.8%, $P = 0.05$), while no significant difference was found in the active state of both diseases (14.7% *vs* 14.4%, $P = 0.393$). Cakal *et al*[23] also showed that a cutoff 14% for RDW had 0.88 sensitivity and 0.71 specificity, so making this index the most sensitive and specific serologic inflammatory parameter (even better than fibrinogen, CRP, ESR and platelet count) for detecting active UC in the patient cohort. However, different results were obtained for active CD, since a value of 0.54 mg/dL for CRP had 0.92 sensitivity and 0.63 specificity, whereas a threshold of 14.1% for RDW was characterized by 0.78 sensitivity and 0.63 specificity. Authors concluded that this difference may be due to the variability in presentation of active UC and CD. More active UC patients have rectal bleeding and iron deficiency, whereas malabsorption of other micronutrients and vitamins in terminal ileitis (CD) may affect RDW value[23]. A study by Yeşil *et al*[24] yielded different results. In their study of patients with active CD, a RDW cut off of 14% had 0.79 sensitivity and 0.93 specificity (AUC = 0.935; $P < 0.001$), so making it the best overall parameter for identifying active CD. Interestingly, an increased RDW value was identified earlier than changes in hemoglobin and mean corpuscular volume (MCV), so supporting the notion that RDW may have a role as early indicator of active disease in both CD and UC[24]. Similarly, Ipek *et al*[25] showed that RDW may be a marker of disease activity in UC, with 0.41 sensitivity and 0.91 specificity (AUC = 0.65; $P < 0.001$). Song *et al*[26] investigated the relationship between RDW and disease activity in IBD patients with and without anemia, reporting that RDW was the best independent predictor of disease activity in both UC and CD irrespective of the presence of anemia. In another study by Oliveira *et al*[27] RDW was found to be associated with disease activity in CD (defined by CDAI $\geqslant$ 150); a RDW cutoff value of 16% had 0.88 specificity and 0.86 negative predictive value for active CD. These results support the role of RDW as an important inflammatory marker, as the positive correlation between anisocytosis and increase in systemic inflammation has been consistent throughout many studies (Table 1).

In an interesting study, Oustamanolakis *et al*[28] evaluated the value RBC indices for differentiating iron deficiency anemia (IDA) and anemia of chronic disease

Table 1 Published studies on role of red blood cell distribution width in inflammatory bowel disease

Ref.	No. of subjects	Study period	Activity index	Outcome measures	RDW Value	Statistics		Other laboratory studies	Main findings
						Sensitivity (%)	Specificity (%)		
Clarke *et al*[21], 2008	156 CD 128 UC	January 1st 2004 to December 31st 2005		Differential diagnosis CD *vs* UC	CD 14.9 UC 14.3	*P* = 0.027			RDW value was significantly higher in CD relative to ulcerative colitis patients
Cakal *et al*[23], 2009	74 UC, 22 CD, and 20 age/sex-matched controls		CDAI > 150 = active	Active UC or CD	UC 14	88	71	CRP, ESR, Fibrinogen, PLT, WBC, Hb	RDW and CRP were the most significant indicators of active UC and active CD, respectively
			Truelove-Witts scale for UC moderate and severe = active		CD 14.1	78	63		
Oustamanolakis *et al*[28], 2011	51 CD		CDAI	Anemia (IDA/ACD)	14 (cut off)	93	81	Ferritin, Tsat, sTfR	High RDW and low RSF values were the best markers for the diagnosis of IDA
	49 UC		> 150 active	IDA				RDWR-C, RSF, IRF,	
	102 age matched controls		< 150 inactive Simple Clinical Colitis Activity Index for UC Active ⩾ 3					Hb, ESR CRP	
Yeşil *et al*[24], 2011	56 CD 61 UC 44 age/sex matched controls		CDAI > 150 = active Truelove-Witts scale for UC	Active UC/CD	14 (cut off)	79 (CD) 17 (UC)	93 (CD) 84 (UC)	CRP, ESR, PLT, Hb	RDW was elevated in IBD and markedly increased in active disease. RDW may be a marker of active CD, whereas ESR is for active UC
Song *et al*[26], 2012	101 CD 120 UC	January 2003 and December 2010	CDAI remission < 150, mild 150-220, moderate to severe ⩾ 220; Mayo score for UC remission < 3, mild 3-6, moderate to severe ⩾ 6	Active UC/CD	14.1 CD without anemia (cut off) 13.8 UC without anemia (cut off)	82 (CD) 76 (UC)	83 (CD) 86 (UC)	CRP, ESR, PLT, WBC, Hb,	RDW was associated with active CD/UC in patients with or without anemia
Ipek *et al*[25], 2015	206 active UC 104 remission UC	January 2009 to December 2011	Endoscopic Rachmilewitz activity index > 4 = Active UC	Active UC *vs* remission	Active UC 16.8 Remission UC 15.5	*P* < 0.001		CRP, ESR, PLT, WBC, Hb	RDW can be used as a marker for disease activity in ulcerative colitis, but not in the non-anemic group
Oliveira *et al*[27], 2016	20 Active CD 99 remission CD	January 1st and September 30th 2013	CDAI ⩾ 150 = active CD	RDW association with Active CD	16 (Cut off)	30	88	CRP, ESR, PLT, WBC, Hb, MCV	RDW was associated with the severity of CD. The RDW cutoff 16% showed possible clinical applicability

CD: Crohn's disease; UC: Ulcerative colitis; CDAI: Crohn's disease activity index; CRP: C-reactive protein; ESR: Erythrocyte Sedimentation Rate; PLT: Platelets; WBC: White blood cells; Tsat: Transferrin saturation; sTfR: Soluble transferrin receptor; RSF: Red blood cell size factor; IRF: Immature reticulocyte fraction; RDWR-CV: Reticulocyte distribution width-coefficient of variation; IDA: Iron deficiency anemia; ACD: Anemia of chronic disease.

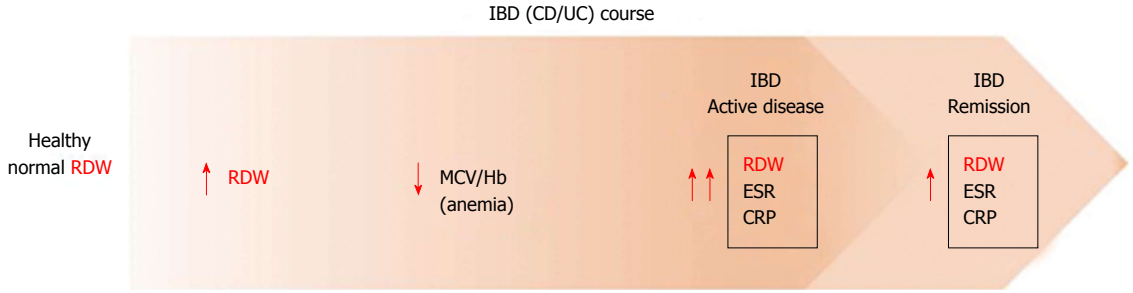

Figure 1 Changes in red blood cell distribution width levels during clinical course of inflammatory bowel disease (Crohn's disease/ulcerative colitis). RDW: Red blood cell distribution width; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; Hb: Hemoglobin; MCV: Mean corpuscular volume; ESR: Erythrocyte sedimentation rate.

in IBD. They found that higher values of RDW in IBD performed better than ferritin values as markers of IDA, displaying 0.93 sensitivity and 0.81 specificity[28].

Therefore, RDW was shown to have good potential as indicator of disease severity and as a differentiating marker in IBD. This is particularly important considering that the use of this readily available hematological parameter would permit physicians to better evaluating and managing IBD patients (Figure 1).

RDW and colon cancer

Several lines of evidence attest that RDW may be useful for diagnosing and assessing the survival of patients with solid and hematological cancers. Riedl *et al*[29] investigated various RBC parameters in a prospective cohort of 1840 cancer patients, confirming that an increased RDW value was associated with enhanced risk of mortality (HR = 1.72, 95%CI: 1.39-2.12, $P < 0.001$). The association was virtually unaltered after adjustment for age, sex, hemoglobin, leukocyte and platelet count (HR = 1.34, 95%CI: 1.06-1.70, $P = 0.016$)[29]. In a retrospective case-control study, Spell *et al*[30] showed that the RDW had high sensitivity (0.84) and specificity (0.88) for identifying right-sided colon cancer, so concluding that this parameter may be seen as a cost-effective screening tool for colon cancer. In a more recent investigation, Ay *et al*[31] found that RDW values were significantly higher in patients with colon cancer compared to those with colonic polyps ($P = 0.01$). Patients with anemia and other hematological disorders were already excluded from the analysis, so enhancing the value of this parameter for differentiating neoplastic lesions of the colon[31].

ROLE OF RDW IN LIVER DISORDERS

An impressive amount of literature exists about the clinical significance of RDW in liver disorders. Many scientists have investigated the role of RDW for predicting severity, fibrosis, inflammation and monitoring therapy in liver disorders. The clinical usefulness of RDW has then been established in several studies in patients with liver disorders such as non-alcoholic fatty liver disease (NAFLD), viral hepatitis, hepatocellular carcinoma and primary biliary cirrhosis (PBC).

Cengiz *et al*[32] showed that patients with non-alcoholic steatohepatitis had higher RDW values compared to those with simple steatosis and healthy individuals. They also observed that RDW values were independently associated with severity of fibrosis, wherein more severe fibrosis was accompanied by higher RDW values[32]. Gao *et al*[33] showed that an increase in the viral load was accompanied by enhanced RDW in patients with hepatitis B, thereby suggesting that RDW may serve as an indicator of disease stage and treatment response. Xu *et al*[34] also compared RDW values in hepatitis B patients with moderate to severe liver fibrosis and those with absent or mild fibrosis, and found that RDW values were useful for predicting both liver fibrosis and necrotic inflammatory changes. Similarly, Lou *et al*[35] showed that RDW values could be used to assess the disease states in patients with hepatitis B virus infection. In patients with chronic hepatitis B (CHB) and liver cirrhosis related to CHB, Huang *et al*[36] observed that RDW values were high and correlated with the severity of cirrhosis in terms of Child-Pugh scores and model for end-stage liver disease (MELD) scores. Wang *et al*[37] confirmed these findings by demonstrating that RDW may be useful for predicting liver fibrosis in patients with chronic autoimmune hepatitis and PBC, while globulin value may help assessing liver inflammation (Table 2)[38,39]. Several investigators studied the RDW to platelet ratio (RPR) in liver disorders, and analyzed its efficiency for predicting severity of liver fibrosis and cirrhosis. Cengiz *et al*[40] reported that the RPR index has a good predictive value for significant and advanced liver fibrosis in NAFLD. Taefi *et al*[41] found that the RPR ratio was a stronger predictor of severity of fibrosis and cirrhosis in patients with chronic hepatitis having native liver compared to RDW and MELD score. No significant correlations of these variables were found in transplanted livers. Karagoz *et al*[42] showed that RPR and mean platelet volume (MPV) are associated with severity of fibrosis in patients with chronic hepatitis C, so allowing prognostic evaluation

Table 2 Published studies on role of red blood cell distribution width in liver disorders

Ref.	No. of subjects	Study period	Liver pathology	Outcome measures	RDW value (%)	Statistics	Other laboratory studies	Main findings
Lou *et al*[35], 2012	16 AHB 61 CHB 46 CHB-severe 48 healthy controls	August 1st, 2010 August 1st, 2011	AHB, CHB, CHB-severe MELD score	RDW association with HBV related liver disease states and mortality	14.38 ± 1.72 (AHB) 16.37 ± 2.43 (CHB) 18.3 ± 3.11 (CHB-severe) 13.03 ± 1.33 healthy controls	$P < 0.05$ $P < 0.001$ $P < 0.001$	ALT, total bilirubin, total protein, albumin, WBC, Hb, MCV, INR, Creatinine, BUN, HBsAg HBeAg, HBcAb IgM, HBV DNA	RDW is significantly increased in HBV infected patients compared to controls, and RDW is an independent predicting factor for the 3 mo mortality rate in HBV infected patients.
			NASH (Brunt's criteria)			$P < 0.01$	Liver biopsy,	Patients with NASH had higher RDW relative to simple steatosis and healthy control groups.
Cengiz *et al*[32], 2013	62 NASH 32 simple steatosis 30 healthy controls	Jan-10 May-13	Advanced fibrosis (2-4 points) Mild fibrosis (0-1)	RDW association with NASH and fibrosis	NASH 14.28 ± 0.25 Simple steatosis 13.37 ± 0.12 Healthy controls 12.96 ± 0.14 Advanced fibrosis 15.86 ± 0.4 Mild fibrosis 13.63 ± 0.67	$P < 0.01$	Hb, platelets, MPV, WBC, lymphocytes, ALT, AST, GGT Albumin, BUN, Creatinine, alkaline phosphatase	RDW was higher in patients with advanced fibrosis compared to mild
Yang *et al*[38], 2013	1637 normal control 619 NALFD	Individuals were initially enrolled during 2010	NAFLD criteria presence of definite hepatic steatosis on US scan (grade 3), and exclusion of secondary hepatic steatosis.	RDW in NAFLD patients	12.96 ± 1.08 (control) 13.23 ± 1.01 (NAFLD)	$P = 0.000$	Total cholesterol, TG, Fasting glucose, Hb	RDW was increased in NAFLD patients
Kim *et al*[55], 2013	24547 NAFLD patients	Individuals were enrolled in 2010 (January 1st to December 30th)	NAFLD diagnosis by US and questionnaires about alcohol consumption. Degree of liver fibrosis by BARD and FIB-4 scores	RDW and the level of fibrosis in NAFLD	12.59±0.62 BARD score (0,1) 12.99 ± 0.85 (BARD score 2-4) 12.61±0.77 (FIB-4 score < 1.3) 12.89 ± 0.71(FIB-4 score ⩾ 1.3)	$P < 0.001$ $P < 0.001$	Hb, MCV, LDL, TG, HDL, HbA1C, high sensitivity CRP, ferritin, Platelet	Increased RDW is independently associated with advanced fibrosis in NAFLD
Karagoz *et al*[42], 2014	229 biopsy proven naïve chronic hepatitis B (CHB) patients	January 2010 and November 2013	Fibrosis in CHB (Ishak score)	Relationship of RDW and MPV with the severity of fibrosis in CHB patients	12.6 (cut off)	91.50% Sensitivity 42.50% Specificity	Liver biopsy, WBC, Hb, Ht, platelets, MPV, PDW, AST, ALT, total bilirubin, albumin,	RDW and MPV are significantly higher in HBV infected patients with severe fibrosis
Huang *et al*[36], 2014	69 CHB 61 HBV liver cirrhosis 41 controls	January 2011 and October 2013	HBV related liver cirrhosis Child-Pugh and MELD scores	Correlation of RDW with HBV cirrhosis, CHB; Child-Pugh and MELD scores	16.07 ± 2.41 (HBV cirrhosis) 13.29 ± 1.09 (CHB) 12.75 ± 0.7 (controls)	$P < 0.01$	AST, ALT, total bilirubin, albumin, WBC, Hb, platelets, INR, Creatinine, BUN. HBeAg, HBV DNA	RDW was elevated in HBV related cirrhosis and CHB relative to control, and was positively correlated with severity of HBV related cirrhosis
Dogan *et al*[39], 2015	54 NASH 39 controls	Dec-10 Mar-12	NASH (NAFLD activity score) Fibrosis, 0 not significant (F0-F1); 1 significant (F2-F4) Steatosis, 0 mild (grade 1); 1 moderate to severe (grade 2-3) 0 lobular inflammation (0-1); 1 moderate-severe (2-3)	Inflammation in NASH	13.3 (cut off)	79.50% Sensitivity 73.30% Specificity	Liver biopsy, Ht, MCV platelets, ALT, AST, GGT LDL, HDL, TG, Fasting glucose, insulin, Alkaline phosphatase	RDW is a specific and sensitive method to assess inflammation in NASH patients

Xu *et al*[34], 2015	446 HBV infected patients who underwent liver biopsy	January 2010 and December 2011	Liver fibrosis (no significant S0-S2, fibrosis *vs* advanced, S3-S4) Inflammation (no significant (G0-G2) *vs* significant (G3-G4)	RDW in liver fibrosis and inflammation	13.3 (S0-S2) 13.6 (S3-S4) 13.2 (G0-G2) 13.7 (G3-G4)	$P = 0.01$ $P < 0.001$	Liver biopsy, AST, ALT, total bilirubin, albumin, WBC, Hb, platelets, MCV, MPV, HBeAg, HBV DNA	RDW, together with other serum markers, could be useful in predicting liver fibrosis and necroinflammation in HBV infected patients
Wang *et al*[37], 2016	116 CHB 65 PBC 37 AIH	January 2010 to January 2015	Liver fibrosis and inflammation: absent-mild (S0-S1, G0-G1) *vs* moderate-severe (S2-S4, G2-G4)	RDW association with liver fibrosis and inflammation in chronic hepatitis	13.4 (S0-S1) 14.5 (S2-S4) 13.0 (G0-G1) 14.2 (G2-G4)	$P < 0.001$	AST, ALT, alkaline phosphatase, GGT, globulin, total bilirubin, total bilirubin acid, total protein, albumin, WBC, RBC, Hb, MCV, platelets	RDW and globulin could be useful predictors of liver fibrosis and inflammation in chronic hepatitis patients, respectively.

RDW: Red blood cell distribution width; Hb: Hemoglobin; HbA1C: Hemoglobin A1C; Ht: Hematocrit; CRP: C reactive protein, AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cell; RBC: Red blood cell; MCV: Mean corpuscular volume; PDW: Platelet distribution width; MPV: Mean platelet volume; GGT: Gamma-glutamyl transpeptidase; HBV: Hepatitis B virus; HbeAg: Hepatitis B e antigen; HbsAg: Hepatitis B s antigen; HbcAb: Hepatitis B core antibody; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; TG: Triglyceride; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; BUN: Blood urea nitrogen; INR: International normalized ratio; AHB: Acute hepatitis B; HBVDNA: Hepatitis B virus DNA; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis.

and minimizing the need for liver biopsy. These authors also confirmed the significance of MPV and RDW in prognostic evaluation of CHB[42]. Chen *et al*[43] also reported that RPR can predict significant fibrosis and cirrhosis in CHB patients. Lee *et al*[44] found that the RPR may be useful for assessing liver fibrosis in patients with CHB, so reducing the need for liver biopsy. On the other hand, a study by Thandassery *et al*[45] compared noninvasive scores with liver biopsy fibrosis stages in patients with chronic hepatitis C, and showed that MPV and RPR had low predictive accuracy for fibrosis stages. The role of RDW in liver disorders is double. First, it may represent a significant prognostic indicator of liver disease severity, fibrosis and inflammation. Then, its use in clinical practice could reduce the need for liver biopsy.

RPR was also shown to be related to histologic severity in treatment-naïve PBC. Seventy three patients were divided in two groups: Early stage (stage Ⅰ) and advanced stage (Stage Ⅱ, Ⅲ and Ⅳ) of liver fibrosis as per Ludwig and Scheuer criteria. RPR was found to have 0.47 sensitivity and 0.96 specificity, performing better than Fibrosis-4score for predicting the severity of liver fibrosis[46]. In another study, RPR was not associated with histologic severity in PBC. However, in this investigation patients were divided in two groups, early (Ⅰ and Ⅱ) and late (Ⅲ and Ⅳ) stage, as per histologic criteria[47] and did not consider the impact of confounding factors on results. In another study, 194 patients with biliary obstruction were studied. A RDW value of 14.8% was found to predict malignant obstruction with 0.72 sensitivity and 0.69 specificity. Both the hemoglobin value and MCV were not significantly different in either group[48].

The significance of RDW has also been evaluated in HCC. Smirne *et al*[49] performed a retrospective study in a training cohort (n = 208) and in an independently prospectively collected validated cohort (n = 106) of patients with HCC. In both cohorts, median survival time was significantly lower in patients with RDW ⩾ 14.6% at the time of diagnosis, and RDW remained independently associated with survival in multivariate analysis[49]. In another study, Wei *et al*[50] retrospectively evaluated 110 treatment-naïve HCC patients, reporting that the RDW admission value was significantly higher in HCC patients than in healthy controls. RDW was also found to be correlated with liver function tests but not with tumor staging at the time of diagnosis[50]. Zhao *et al*[51] investigated the significance of RDW in patients undergoing curative radical resection of HCC, and found that patients with high preoperative RDW value (> 14.5%) had significantly worse survival than those with lower values. RDW remained independently associated with overall survival in multivariate analysis[51].

In a recent retrospective study, Caire *et al*[52] investigated the utility of RDW for predicting mortality in post-liver transplant patients. They found that at-transplant RDW values was a prognostic factor of 1-year mortality in liver transplant patients. Notably, RDW outperformed all other liver pre-transplant prognostic laboratory value, including serum total bilirubin, prothrombin time, bicarbonate, WBC count and MELD score[52].

Despite these promising results, some authors raised concerns about the use of RDW alone in the prognostic evaluation of liver disorders. Gulcan Kurt *et al*[53] and Balta *et al*[54] argued about the specificity of RDW for predicting the extent of fibrosis in chronic liver diseases, pointing out that other confounding factors (*e.g.*, inflammatory biomarkers) should also be considered. Kim *et al*[55] showed that RDW was an independent predictor of nonalcoholic fatty liver disease. However, Kang

and Kim highlighted that this index should be used in combination with other inflammatory markers for reaching more efficient diagnostic efficiency[56].

RDW AND PANCREATIC DISORDERS

Acute pancreatitis (AP) is one of the most frequent GI causes of hospital admissions in the United States, with an annual incidence of 13 to 45/100000 persons[57]. Despite most of patients with AP have mild and self-limited disease, nearly 20% of them develop severe disease, which is in turn associated with a mortality rate of 7%-42%. Due to high mortality and high rate of complications, patients with severe pancreatitis need an early diagnosis. Multiple scoring systems, including Ranson's criteria, Acute Physiology and Chronic Health Evaluation, Glasgow scores, Blathazar score, BISAP score, Revised Atlanta criteria and classification, have been developed and evaluated for predicting severity of pancreatitis in early disease course, but these are not easily usable by physicians due to the cumber-some calculation, especially in emergency settings[58]. Recently, Blood Urea Nitrogen has emerged as a promising prognostic marker in AP, but its value has not been extensively evaluated in patients with chronic kidney disease. Therefore, a simple, reproducible, cost-effective and specific prognostic marker in AP is still lacking.

Uçar Karabulut *et al*[59] studied 104 patients with AP and found that the mean admission RDW value was significantly higher during the acute phase compared to after recovery ($P < 0.01$). Although patients with severe pancreatitis were excluded and the severity of disease was not analyzed in this study, an increased RDW was identified as a reliable marker of AP, so suggesting that this parameter can be used for early detection and prognostic evaluation[59]. Cetinkaya *et al*[60] performed both univariate and multivariate analyses in a retrospective cohort study of 102 patients, identifying both RDW and RPR on admission were independent and significant factors for predicting the risk of in-hospital mortality in APs ($P = 0.001$). The association between RDW and mortality in AP was also confirmed by Yao *et al*[61] in a cross-sectional study, showing that non-survivors had higher values than survivors. The RDW displayed 0.75 sensitivity and 0.90 specificity for predicting mortality in AP[61]. Şenol *et al*[62] found that RDW was the only admission variable predicting AP mortality in multivariate analysis. A RDW value of 14.8% was found to predict mortality in 77% cases[62]. Wang *et al*[63] also showed that AP patients with RDW > 13.4% had significantly higher mortality rate than those with lower RDW values. A RDW value of 14.3% was characterized by 0.88 sensitivity and 0.92 specificity for predicting mortality in AP[63]. In another study based on the Multiparameter Intelligent Monitoring in Intensive Care Ⅱ (MIMIC Ⅱ) database showed that higher RDW value predicts mortality in AP with an AUC of 0.66 (95%CI: 0.52-0.81)[64]. However, authors could not comment on confounding factors causing elevation in RDW[65]. Despite the differences in the cut-off values used in the various studies and relative heterogeneity of sensitivity and specificity values associated with these cut-offs, the available data supports the conclusions that RDW may be a sensitive predictor of mortality in patients with AP. Only one study failed to show an association between RDW and mortality in AP. However, patients with anemia, malignancy, kidney and hepatic diseases were also included in the final analysis, so potentially flawing the outcome[66]. Notably, Peng *et al*[67] also showed that a high RDW at admission was an independent risk factor for acute pancreatitis-associated lung injury (OR = 2.671, 95%CI: 1.145-6.138; $P = 0.026$).

The significance of RDW has been evaluated in only one study in pancreatic cancer. Yilmaz *et al*[68] studied 104 patients undergoing pancreatic cancer surgery, who were divided them in two groups with high (> 14%) and low (< 14%) RDW values. A positive correlation was hence observed between pancreatic cancer staging and RDW, but no association was noticed between RDW and postoperative complications or morbidity[68].

RDW AND OTHER DIGESTIVE DISORDERS

Huang *et al*[69] studied 35 patients with intestinal tuberculosis (ITB) who were compared with healthy controls ($n = 22$). Patients with ITB had higher RDW, which overall displayed a better diagnostic efficiency (AUC = 0.812) than CRP (AUC = 0.176) and ESR (AUC = 0.804) in diagnosing ITB[68]. In an interesting study, Yazıcı *et al*[70] showed that patients with acute cholecystitis undergoing surgery had a significant decrease in RDW compared to those subjected to conservative management.

Acute Mesenteric ischemia (AMI) is a relatively uncommon, but life-threatening, condition. Early recognition and management is imperative to prevent the related complications. Kisaoglu *et al*[71] carried a cross-sectional study by comparing AMI patients ($n = 49$) with patients with abdominal pain who did not undergo surgery ($n = 110$). A RDW value of 15.4% had 0.41 sensitivity and 0.81 specificity for identifying AMI patients after adjustment for anemia. However, no correlation was found between RDW and size of ischemia or mortality[71]. Bilgiç *et al*[72] investigated the preoperative RDW value in a retrospective cohort of 61 patients with AMI, and found that increased RDW predicted both the extent of necrosis and mortality. A cut-off values of 14.8% predicted mortality in nearly 70% of cases[72].

CONCLUSION

Several lines of evidences, summarized in this article,

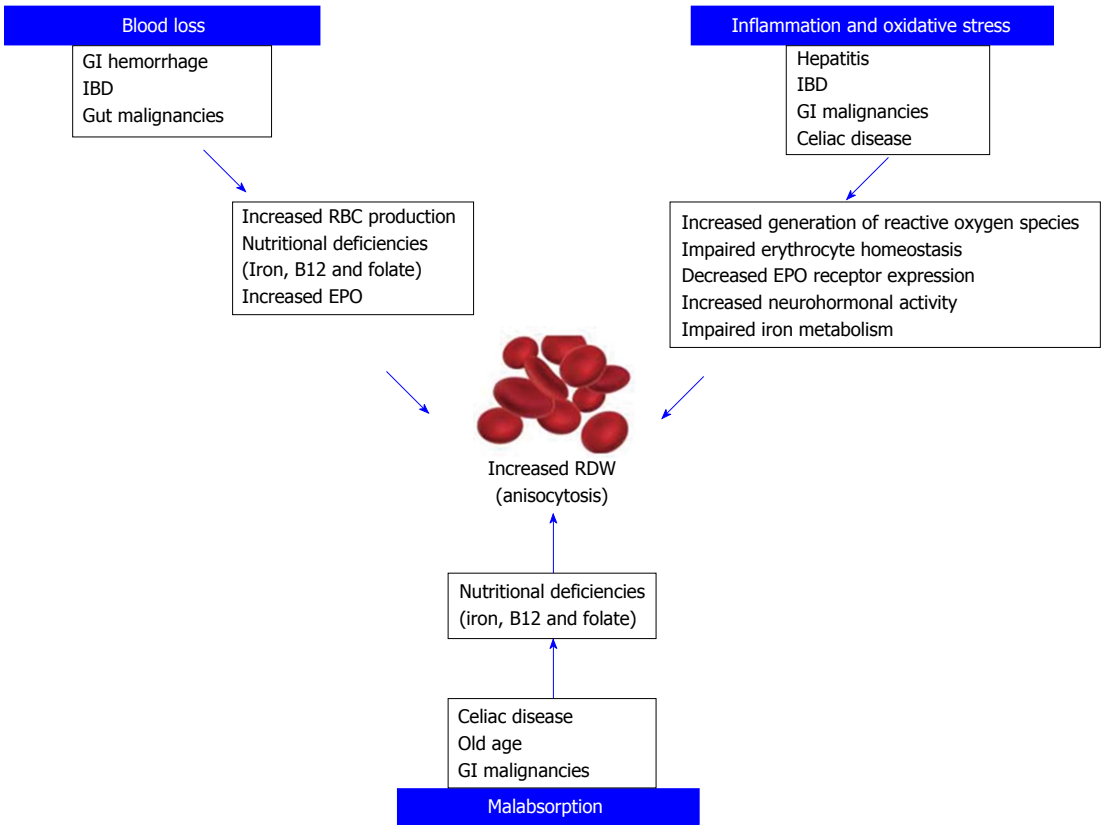

Figure 2 Possible mechanisms of increased red blood cell distribution width in gastrointestinal disorders. GI: Gastrointestinal; RBC: Red blood cell; EPO: Erythropoietin; RDW: Red blood cell distribution width; IBD: Inflammatory bowel disease.

attest that RDW may be a useful prognostic factor in a variety of GI conditions, including IBD, celiac disease, cancer of colon and esophagus, liver disorders including hepatitis and liver cancer[73], pancreatic disorders, especially AP (Figure 2).

Since RDW can be easily measured with routine blood tests, without additional technical requirements and at a rather affordable cost (*i.e.*, that of a complete blood cell count), this parameter may be considered a valuable perspective for prognostic assessment of patients with GI disorders. Along with RDW, other related parameters such as the RPR and the hemoglobin to RDW ratio were found have a prognostic value in various conditions. Due to its efficiency as an inflammatory biomarker, as a measure of disease activity and as a prognostic indictor in GI, RDW offers several advantages over other additional tests. RDW measurement is noninvasive, So that widespread use may help minimizing the use of invasive procedure such as endoscopy and biopsy to assess the prognosis in various clinical conditions. This is especially significant in certain regions where medical facilities may be limited or inaccessible. Nevertheless, despite a large number of studies showing utility and benefit from measuring and monitoring RDW values, some factors still make the use of results challenging. These encompass the lack of prospective studies, the heterogeneity of the diagnostic and prognostic cut-offs as well as the poor generalizability of outcomes due to unmet standardization of the available techniques for measuring RDW[74]. The cut-offs of RDW may vary according to the technique used for its measurement. This may explain why some subjects may display higher or lower values when RDW is measured with different analyzers. Moreover, some people in the general population may display values higher than the reference range due to the presence of undiagnosed conditions, which may ultimately lead to increase anisocytosis. However, a single cut-off for RDW cannot be identified so far, since the various analyzers use different techniques. Therefore, the application of a universal cut-off is unfeasible until a major degree of standardization can be reached[74]. In modern haematological analysers, the RDW is conventionally calculated from the histogram of erythrocyte volumes. It is, hence, predictable that further studies aimed to more deeply investigate the full distribution graph (*i.e.*, identifying extraordinarily large or small cells) may provide more meaningful information than the simple numerical value of RDW. Although, increased RDW seems to be a marker for severe GI disease, perhaps, but possibly not for functional GI disorders such as irritable bowel syndrome. This is probably due to the fact that functional disorders have a much lower impact on erythrocyte biology, so that RBC still display a normal turnover. Then, the role of the many confounding factors which may have an impact on RBC biology is still incompletely understood and evaluated.

Therefore, due to the mounting evidence regarding the usefulness and the considerable diagnostic potential of RDW,further prospective investigations are needed to validate its effectiveness and concrete steps should be undertaken to define standards that can serve as guidelines for effectively using RDW as a tool for diagnostic and prognostic assessment.

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MINIREVIEWS

Endoscopic ultrasound-guided radiofrequency ablation in gastroenterology: New horizons in search

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Abstract

Radiofrequency ablation (RFA) has been widely used for the treatment of various solid organ malignancies. Over the last decade, endosonographers have gradually shifted the application of RFA from porcine models to humans to treat a spectrum of diseases. RFA is performed in patients with pancreatic carcinoma who are not candidates for surgery. In this paper, we will discuss various indications for RFA, its procedural details and complications. At present, endoscopic ultrasound-guided RFA is gradually incorporated into the management of various diseases and opens a new avenue for disease treatment.

Key words: Pancreatic carcinoma; Radiofrequency ablation

Core tip: Endoscopic ultrasound-guided radiofrequency ablation (RFA) is a rapidly emerging modality, whose application has shifted from porcine models to humans over the last decade. In this review, we provide details on the indications, thermokinetic principles and complications related to RFA, which should be judiciously applied in the management of various diseases.

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INTRODUCTION

Over the last two decades, palliation techniques for pancreatic adenocarcinoma have changed significantly. New developments in endoscopic ultrasound-guided

therapies have also rapidly emerged[1]. Radiofrequency ablation (RFA) utilizes high frequency alternating current and can result in coagulative necrosis[2,3], and it can be applied percutaneously, intraoperatively or in combination with endoscopic ultrasound (EUS). This modality is gradually gaining popularity among endosonographers at tertiary centers. EUS-RFA is now an established anti-tumor therapy and an alternative to surgery[4].

Pancreatic adenocarcinoma is an aggressive tumor with a dismal survival rate due to delayed diagnosis. Only 10% of patients qualify for curative surgery[5]. The majority of patients have an unresectable locally advanced disease with encasement of vessels (superior mesenteric vessels, portal vein and/or hepatic artery)[6]. One-year survival rate in these patients is less than 5% after diagnosis[7].

EUS-guided RFA was first used in a porcine model by Goldberg *et al*[8] in 1999. EUS is used for various therapeutic procedures as it can be precisely applied in pancreatic lesions and helps delineate the area of interest for ablation[9-11].

EUS provides real-time imaging of deeply located anatomical structures such as the pancreas which is difficult to approach via the percutaneous route[12]. RFA has been widely utilized in the treatment of liver, lung and kidney tumors[13-15].

MECHANISM OF RFA

RFA is based on the principle that high frequency alternating current is converted into thermal energy which results in coagulative necrosis of surrounding tissue[16]. Thermal exposure above 45 ℃ results in denaturation of cell proteins and is utilized in the treatment of various tumors[17].

There are three important components in this procedure: the generator, the needle and the tissue.

The generator utilizes alternating current and converts it into thermal energy which is transferred through the exposed part of the needle[8,18].

RFA also causes thermal damage to the epithelium with a gradual rise in temperature, which results in destruction of cyst epithelium[19].

CLINICAL APPLICATIONS OF RFA

RFA is principally utilized in various benign and malignant conditions, including intraoperative applications. Studies have suggested that RFA leads to tumor necrosis and a reduction in tumor volume[20].

RFA can also be used in patients with malignant biliary obstruction for endobiliary ablation in the self-expandable metallic stent to improve stent patency[21].

A cryothermal probe (ERBE, Elektromedizin GmbH) has been used for palliation in locally advanced pancreatic carcinoma patients, with a technical success rate of 72.8% and median survival of 6 mo post ablation with manageable complications including jaundice, duodenal stricture and cystic fluid collection[20-22].

INDICATIONS

EUS-guided RFA is indicated in various diseases including: (1) pancreatic adenocarcinoma[23]; (2) patients after chemoradiotherapy; (3) patients with progressive tumor growth causing biliary or gastric outlet obstruction[24]; (4) liver metastasis[25]; (5) intraductal papillary mucinous neoplasms (IPMN)[26,27]; and (6) insulinoma[28,29].

PROCEDURE

A 19 G needle is usually used to puncture the pancreatic tissue under EUS guidance, the stylet is removed to introduce a thin wire which is connected to the generator, and then the tissue is ablated. This principle has been applied using a Habib EUS-RFA catheter (EMcision Ltd., London, United Kingdom) where a monopolar probe with a diameter of 1 Fr and length of 220 cm is utilized with a 2 cm active electrode tip to ablate the tissue[28,30,31]. It ablates for 2 min, which is considered one ablation with a break of 60 s for cooling. Up to 10 ablations can be applied to the tissue with interspersed cooling periods (Figures 1 and 2). In the case of a cyst, the lesion is aspirated prior to ablation. This technique should not be used in patients with cardiac pacemakers or other active implants.

Another novel 18 G RFA electrode (EUSRA RF Electrode; STARmed, Koyang, South Korea) with a total working length of 150 cm is also used. This electrode has the unique feature of two 0.8 mm diameter holes which are located 5 mm away from the tip, and can be used for aspiration and injection. The active electrode length is 7 mm while the tip exposure length is 10 mm. This RF electrode is attached to the RF generator (VIVA Combo system; STARmed) to ablate the tissue[32]. It results in the ablation of 1-3 cm of localized tissue from the needle tip[32-34].

A new flexible hybrid bipolar probe also known as the cryotherm probe (ERBE Elektromedizin, Tubingen, Germany) has recently been introduced, which combines cryotechnology with RFA[35]. This probe has an advantage over a monopolar probe in that it causes less collateral damage, but it is less efficient than a monopolar probe[36-38].

Cooling using a cryogenic gas increases the effect of RFA and interstitial devitalization[12]. It also proves that cooling does not affect the efficacy of ablation[39].

TIME AND TEMPERATURE UTILIZED IN RFA

RFA was successfully used in other organs such as the liver, intrahepatic tumors and muscle to achieve maximum coagulation within 6 min, prior to its application in the pancreas[40]. The Manchester group was

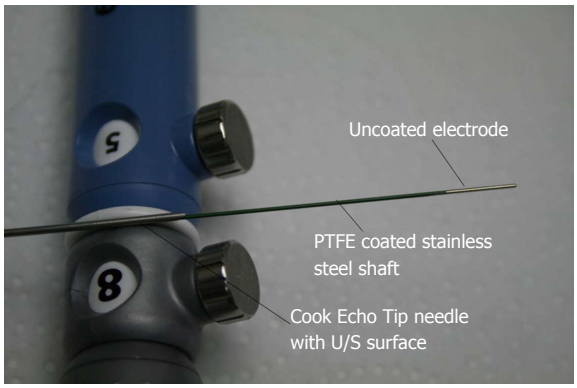

Figure 1 Habib RF needle with Cook Echo Tip needle. Courtesy of EMcision International Inc.

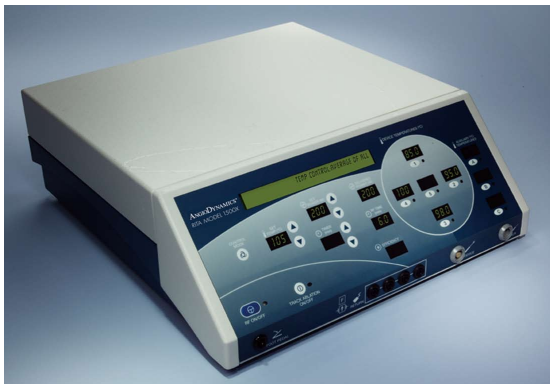

Figure 2 Radiofrequency generator (RITA 1500 X, ANGIODYNAMICS).

among the first to validate and define the thermo-kinetic principles in the pancreas[41]. As the distance from the electrode increases, the temperature tends to decrease[26]. The optimal temperature for thermal ablation was demonstrated in a porcine model by Date[42] in 2005. It was concluded that optimal thermo-kinetics was generated at a temperature of 90 ℃ when applied for 5 min. This leads to ablation of pancreatic tissue without injury to adjacent organs.

A few other studies have also established the relationship between temperature and the rate of complications[43,44].

It was again established in a study by Girelli *et al*[45] that a decrease in temperature from 105 ℃ to 90 ℃ leads to an overall reduction in the complication rate from 24% to 8%.

Wu *et al*[36] showed that when a temperature of 30 ℃ was applied, this led to a high rate of post-operative morbidity, where complications included pancreatic fistula, portal thrombosis, septic shock and massive bleeding.

EUS-RFA OF THE PANCREAS IS DIFFERENT TO THAT OF OTHER ORGANS

EUS-RFA is better than planned palliative R2 resections in pancreatic carcinoma patients as it results in decreased morbidity, mortality and reduced hospital stay. There are certain important and significant differences in ablation of the pancreas compared with other organs: (1) the RFA protocol for other organs cannot be applied to the pancreas as the physical properties of the pancreas are entirely different from those of other organs; (2) the pancreas is surrounded by other organs (the stomach and duodenum), vessels and bile ducts and thus has an increased risk of thermal-induced injury; and (3) pancreatic cancer usually has diffuse margins, whereas hepatic carcinoma or metastasis has discrete margins; therefore, it is difficult to completely ablate pancreatic carcinoma in a single session[16].

EVALUATION OF EFFICACY OF RFA TREATMENT

Lesion size can be evaluated by imaging at repeated intervals. Tumor progression can be estimated by an improvement in symptoms (abdominal pain, back pain) or biochemical indices (CA19-9 levels)[46,47].

RATIO OF PASSES TO THE SIZE OF THE LESION

The ratio of the number of passes to the size of the lesion is extremely variable in different studies with a median value of 0.5 (range, 0.36-19). This can be explained by the application of different devices[41,46,48].

COMPLICATIONS OF RFA

The fear of adverse events related to EUS-RFA also limits its application by clinicians in pancreatic carcinoma patients.

Most complications are related to thermal injury to pancreatic parenchyma (acute pancreatitis) and surrounding structures including thermal damage to superior mesenteric vessels, bile ducts, the portal vein, stomach and duodenum[12,49-51]. Mild abdominal pain was reported by 25%-33% of patients in various studies[33]. Frequent complications were gastro-intestinal hemorrhage, pancreatic fistula, bile leak, portal vein thrombosis, pseudocyst and sepsis. The overall postoperative morbidity rate was 28.3% and mortality was approximately 4%[52].

The pancreas is different to other organs such as the liver and kidney where RFA has been successfully utilized for the treatment of carcinomas. Optimal thermokinetic characteristics of the pancreas have not been completely determined, thus there is no standardized protocol for pancreatic RFA. Usually two or more sessions of RFA are required for pancreatic carcinoma ablation[12,32,33]. Retroperitoneal location, proximity to major vessels, distal bile duct crossing the head of the pancreas and closeness to the stomach

and duodenum are also major hurdles[44].

CONCLUSION

Normal pancreatic tissue is thermosensitive, thus RFA can lead to an inflammatory response with fibrosis and occasionally cystic collections. A clearer understanding of the principles of thermokinetics in humans is required to effectively ablate abnormal tissues. Better ablation devices with minimal side effects and complications may ensure improved results in the future. Further studies with a large number of subjects will provide a better understanding of this novel technique.

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ORIGINAL ARTICLE

Basic Study

Genetic association and epistatic interaction of the interleukin-10 signaling pathway in pediatric inflammatory bowel disease

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Abstract

AIM

To study the genetic association and epistatic interaction of the interleukin (IL)-10 and IL-10/STAT3 pathways in pediatric inflammatory bowel disease (IBD).

METHODS

A total of 159 pediatric inflammatory IBD patients (Crohn's disease, *n* = 136; ulcerative colitis, *n* = 23) and 129 matched controls were studied for genetic association of selected single nucleotide polymorphisms (SNPs) of the *IL-10* gene and the genes *IL10RA*, *IL10RB*, *STAT3*, and *HO1*, from the IL-10/STAT3 signaling pathway. As interactions between SNPs from different loci may significantly affect the associated risk for disease, additive (a) and dominant (d) modeling of SNP interactions was also performed to examine high-order epistasis between combinations of the individual SNPs.

RESULTS

The results showed that IL-10 rs304496 was associated with pediatric IBD (*P* = 0.022), but no association was found for two other IL-10 SNPs, rs1800872 and rs2034498, or for SNPs in genes *IL10RA*, *IL10RB*, *STAT3*, and *HO1*. However, analysis of epistatic interaction among these genes showed significant interactions: (1) between two IL-10 SNPs rs1800872 and rs3024496 (additive-additive *P* = 0.00015, Bonferroni *P* value (Bp) = 0.003); (2) between IL-10RB rs2834167 and HO1 rs2071746 (dominant-additive, *P* = 0.0018, Bp = 0.039); and (3) among IL-10 rs1800872, IL10RB rs2834167, and HO1 rs2071746 (additive-dominant-additive, *P* = 0.00015, Bp = 0.005), as well as weak interactions among IL-10 rs1800872, IL-10 rs3024496, and IL-10RA (additive-additive-additive, *P* = 0.003; Bp = 0.099), and among *IL10RA*, *IL10RB*, and *HO1* genes (additive-dominant-additive, *P* = 0.008, Bp = 0.287).

CONCLUSION

These results indicate that both the *IL-10* gene itself, and through epistatic interaction with genes within the IL-10/STAT3 signaling pathway, contribute to the risk of pediatric IBD.

Key words: Pediatric inflammatory bowel disease; Interleukin-10; HO1; Single nucleotide polymorphism; IL10-STAT3 pathway; Epistatic interaction

Core tip: Inflammatory bowel disease (IBD) affects not only adults, but also children and newborn infants. Of the 163 genes currently associated with risk for development of IBD, only a few have been studied in pediatric patients. In this study, we found that one interleukin (IL)-10 genetic variation, rs304496, is associated with risk for pediatric IBD. IL-10 restricts excessive immune responses during intestinal inflammation. We also demonstrated epistatic interactions between genetic variants within the IL-10/STAT3 signaling pathway that contribute to a higher associated risk for pediatric IBD. These findings emphasize the importance of the IL-10 pathway in a subgroup of IBD patients.

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INTRODUCTION

Pediatric inflammatory bowel disease (IBD) has a distinct clinical phenotype from adult IBD[1]. Few of the 163 genes identified to be associated with adult IBD have been identified and functionally studied in pediatric IBD. A genome-wide association study (GWAS) in the Polish population revealed that the genetic architecture is different between pediatric and adult-onset IBD[2]. Adult IBD-associated genes *NOD2* (Leu1007insC) and *IRGM* have been shown to be associated with increased risk of Crohn's disease (CD) and *ORMDL3* variant with susceptibility to ulcerative colitis (UC) in Lithuanian early-onset IBD patients[3]. The TRIM22-NOD2 network, signaling pathways and genetic factors are associated with very early-onset (VEO) and adult IBD. Functional studies showed that variants of the tripartite motif containing 22 gene (*TRIM22*) disrupted its ability to regulate NOD2-dependent activity of interferon-β signaling and nuclear factor-kappa B (NFκB)[4].

In addition, novel association of major histo-compatibility complex haplotype with pediatric-onset IBD has been reported[5]. The multi-drug resistance gene *MDR1* single nucleotide polymorphisms (SNPs) C1236T and G2577A/T have also been shown to be associated with CD in an Algerian pediatric CD population[6].

Mutations in IL-10 and IL-10 receptors IL10RA and IL10RB have been linked to VEO IBD[7-12]. Knockout mice lacking IL-10 develop IBD[13]. *IL-10* and *STAT3* have been identified as IBD-associated genes in children and adults[10,14-20]. The *IL-10* gene encodes an anti-inflammatory cytokine and the IL-10/STAT3 signaling pathway plays an important role in controlling inflammation and protecting the intestine tissue from

Table 1 Study samples

Sample	*n*	Sex, *n*	Race, *n*	Age at diagnosis
IBD	159	Male, 85; female, 74	White, 153; black, 6	13.1
CD	136	Male, 77; female, 59	White, 130; black, 6	13.1
CCFA	118	Male, 65; female, 53	White, 112; black, 6	13.0
Hershey	18	Male, 12; female, 6	White, 18	13.4
UC	23	Male, 9; female, 14	White, 23	13.3
CCFA	10	Male, 5; female, 5	White, 10	12.9
Hershey	13	Male, 4; female, 9	White, 13	13.6
Control	129	Male, 65; female, 64	White, 121; black, 7; unknown, 1	17.5

IBD: Inflammatory bowel disease; CD: Crohn's disease; CCFA: Crohn's and Colitis Foundation of America; UC: Ulcerative colitis.

damage[21,22]. During the IL-10 signaling transduction, IL-10 binds to receptors IL10RA and IL10RB, and activates Jak1 and Tyk2, leading to phosphorylation of STAT3. Then, the activated STAT3 translocates into the nucleus and regulates target gene transcription to promote an anti-inflammatory response[23,24].

Despite pronounced evidence of the role of the genes comprising IL-10 and genes within the IL-10/STAT3 signaling pathway, our knowledge about how they may interact with each other to determine IBD development is still very limited. Genetic interactions between different loci, *i.e.*, epistasis, have been thought to be of paramount importance in complex diseases[25,26]. Given the underlying complex pathways, it is reasonable to hypothesize that the genes detected affect IBD through a network of gene-gene interactions between genes, or SNP-SNP interactions within a gene. In this paper, we used a computational model[27] to analyze how epistatic interactions among polymorphic loci in the *IL-10* gene and IL-10/STAT3 pathways govern pediatric IBD in a case-control setting. The model cannot only estimate low-order epistasis between a pair of loci, but also detect high-order epistasis among three loci, thereby it is equipped with a capacity to unravel etiological complexities of pediatric IBD. Furthermore, by integrating classic quantitative theory, this model dissects overall epistatic interaction into its underlying components. With this, one may better understand the genetic machinery of this disease from a mechanistic aspect.

MATERIALS AND METHODS

Study samples

Genomic (g)DNA samples obtained from a total of 159 pediatric IBD patients (CD, *n* = 136; UC, *n* = 23) were studied. The age of diagnosis for all patients was < 17-years-old. The patients were Caucasian (*n* = 153) and African American (*n* = 6). gDNA samples were obtained from the Crohn's and Colitis Foundation of America (CCFA) DNA Databank (IBD, *n* = 128 including 118 with CD and 10 with UC) and the Pennsylvania State University IBD Biobank (IBD, *n* = 31 including 18 with CD and 13 with UC)[28]. Healthy gDNA control samples (*n* = 129) were obtained from CCFA (*n* = 70) and the Hershey Medical Center (*n* = 59). The race, age and sex of the controls were matched to the study cases; none of the controls were identified with gastrointestinal-related diseases (Table 1).

Informed consent was obtained for all patient samples retrieved from the Pennsylvania State University IBD Biobank and the Hershey Medical Center. All study protocols were approved by the Penn State University College of Medicine Institutional Review Board. CCFA gDNAs were collected from samples originating from the University of North Carolina at Chapel Hill, University of Chicago, Cedars-Sinai Hospital, Massachusetts General Hospital, University of Pittsburgh, and Mt. Sinai Hospital, with written informed parental or guardian consent.

DNA isolation

gDNA samples were obtained from CCFA as noted above. The gDNA from Hershey Medical Center was isolated from blood samples or Epstein Bar virus-immortalized B cell lines using Qiagen DNA Mini Kits (Qiagen Inc., Valencia, CA, United States). After DNA concentration was measured with a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States), the gDNA samples were stored at -80 ℃ until use.

Selection criteria and study of SNPs from IL-10, IL10RA, IL10RB, STAT3, and HO1

Seven SNPs from these five genes were studied. These are rs1800872 (C-592>A), rs3024498 and rs3024496 from *IL-10*[23]; rs3135932 from *IL10RA*[7]; rs2834167 from *IL10RB*[7,29]; rs744166 from *STAT3*; and rs2071746 from *HO1*[30,31]. The criteria for SNP selection were based (1) on the potential relevance of these SNPs in the function and the regulation of genes, which have been associated with IBD and other diseases, and/or play a role in inflammatory processes; (2) on the gene location, either within the coding region that changes the encoded amino acid, or at 5' upstream or 3'UTR potentially affecting RNA transcription, RNA stability or protein translation; and (3) being polymorphic in the study samples as tested in our preliminary study and having minor allele frequency information in existing databases. A summary of these SNPs is provided in Table 2, including genetic variation, chromosomal position, gene location, and disease implication.

Genotype analysis

The genotypes of all seven SNPs were determined with PCR-based RFLP/cRFLP as described previously[32]. The PCR primers and related information are given in Table 3. Briefly, 100 ng DNA were used for PCR in a 30 μL reaction volume. The PCR cycling profile

Table 2 Study single nucleotide polymorphisms for *IL-10*, *IL10RA*, *IL10RB*, *STAT3* and *HO1* genes

Gene	SNP ID	Chromosomal position	Variation	Gene location	Disease implication	Ref.
IL-10	rs1800872	206946407	C-592>A	5'-upstream	associated with IBD	[37]
	rs3024498	206941529	c.T>C	3'-untralated region	associated with colorectal cancer	[41]
	rs3024496	206941864	A>G	3'-untralated region	associated with IBD and colorectal cancer, with decreased IL-10, with increased IgE levels	[37-39,41]
IL10RA	rs3135932	117864063	c.A247>G, p.Ser159Gly	coding region	mutations (other than the studied SNP) associated with pediatric IBD	[7,10,12]
IL10RB	rs2834167	34640788	c.A>G, p.Lys(A)47Glu(G)	coding region	mutations (other than the studied SNP) associated with pediatric IBD	[7-11]
STAT3	rs744166	404514201	A>G	Intron 1 (closer to exon2)	associated with IBD	[20]
HO1	rs2071746	3577672	A413>T	5'-upstream	no association with IBD, associated with asthma and allergy, anti-inflammation, anti-oxidant	[30,31]

IBD: Inflammatory bowel disease; SNPs: Single nucleotide polymorphisms.

Table 3 PCR-RFLP method for genotyping *IL-10*, *IL10RA*, *IL10RB*, *STAT3* and *HO1* genes

Gene	SNP ID	Variant	PCR amplification		RFLP	
			Primers[1]	Product, bp	Restriction enzyme	Recognition site
IL-10	rs1800872	G>T	IL-2f: 5'-AACTTAGGCAGTCACCTTAGG-3' IL-2r: 5'-CATCCTGTGACCCCTCCAGT-3'	149	ScaI	T yes; G No
	rs3024498	T>C	IL-5f: 5'-GCTCCtTGGTTTCTCTTCCTA AG-3' IL-5r: 5'-AGAAGCTTCCATTCCAAGCC TGA-3'	137	HpyCH4V	C yes; T No
	rs3024496	A>G	IL-4f: 5'-GTATCAGAGGTAATAAATATTCcAT-3' IL-4r: 5'-TAGAAGCATACATGACAATGAAG-3'	178	NlaIII	G yes; A No
IL10RA	rs3135932	A>G	RA3f: 5'-CCCGCAAATGACACATATGgA-3' RA3r: 5'-AGTTCCCAATGGCACACAAGG-3'	172	MnI	G yes; A No
IL10RB	rs2834167	A>G	RB3f: 5'-GCCATAGAGGAGAACCAAGTG-3' RB3r: 5'-GCTGTGAAAGTCAGGTTCTCTT-3'	206	CarI	G yes; A No
STAT3	rs744166	A>G	ST2f: 5'-CAGGAGTGCCAACATTGAGAG-3' ST2r: 5'-G TAATGTCTTGAGGGAATCGAG-3'	106	AluI	A yes; G No
HO1	rs2071746	A>T	HO4f: 5'-TCAGCAGAGGATTCCAGCAGG-3' TG-3' HO4r: 5'-AGGCAGCGCTGCTCAGAGCAC-3'	110	BfaI	A yes; T No

[1]Lowercase letter indicates a mismatched nucleotide.

was as follows: 95 ℃ for 2 min, 5 cycles at 95 ℃ for 30 s, 50 ℃ for 1 min, and 72 ℃ for 1 min, then 30 cycles at 95 ℃ for 30 s, 58 ℃ for 1 min, and 72 ℃ for 1 min, followed by a final extension step at 72 ℃ for 4 min. PCR products (5 μL) were digested with an appropriate restriction enzyme (Table 3) according to manufacturer's instructions. The digested PCR products were separated by polyacrylamide gel electrophoresis (8%), and the genotypes were scored according to the gel pattern of the digested PCR products.

Statistical analysis

Single SNP analysis was statistically assessed by associating each single SNP with the disease. Specifically, we calculated the genotype-based OR and *P* value based on Fisher's exact test. We also calculated 95%CIs for each OR. The difference was considered as significant when $P < 0.05$.

Epistatic interaction analysis of IL-10 and IL-10 pathway genes

Epistatic analysis: Epistasis, due to the interaction between two different loci, may play an important role in disease progression. By using two different SNPs simultaneously, epistasis may detect information that cannot be detected by single SNP analysis. We have developed a model of epistatic detection[27] which allows high-order epistasis due to the interaction among more than two loci to be characterized. This model was used to test high-order epistasis between, *IL-10* and *IL-10* receptors, *IL-10* and *STAT3*, *IL-10* and *HO1*, and *STAT3* and *HO1*. This model not only allows the testing of additive (a) and dominant (d) effects at single SNPs, but is also able to detect the epistatic effects between two or three SNPs in a case-control study[27].

Four types of epistatic interactions for two SNPs, namely additive-additive (aa), additive-dominant (ad), dominant-additive (da), and dominant-dominant (dd) and eight types of epistatic interactions for three SNPs, namely aaa, aad, ada, add, daa, dad, dda and ddd, were estimated and are discussed in this paper.

We estimated the pair-wise linkage disequilibria (LD) between these epistatic loci, which were detected to be non-significant, showing that these loci are segregating randomly in the population.

Table 4 Genetic association of *IL-10*, *IL10RA*, *IL10RB*, *STAT3* and *HO1* genes with pediatric inflammatory bowel disease

Gene	SNP ID	Genotype	Disease, *n*	Control, *n*	OR	95%CI	*P* value
IL-10	rs1800872	CC	89	78	0.863	0.564-1.313	0.71
		CA	68	50			
		AA	2	1			
		C allele	246	206			
		A allele	72	52			
	rs3024498	CC	86	77	0.830	0.552-1.244	0.616
		CT	65	47			
		TT	8	5			
		C allele	237	201			
		T allele	81	57			
	rs3024496	AA	57	27	1.487	1.055-2.099	0.022
		AG	69	69			
		GG	33	33			
		A allele	183	123			
		G allele	185	135			
IL10RA	rs3135932	AA	108	85	0.925	0.595-1.433	0.160
		AG	39	40			
		GG	12	4			
		A allele	255	210			
		G allele	63	48			
IL10RB	rs2834167	AA	91	64	1.318	0.884-1.968	0.203
		AG	66	60			
		GG	2	5			
		A allele	248	188			
		G allele	70	70			
STAT3	rs744166	GG	64	49	0.943	0.662-1.340	0.352
		AG	66	63			
		AA	29	17			
		A allele	194	161			
		G allele	124	97			
HO1	rs2071746	AA	32	30	0.957	0.680-1.348	0.634
		AT	96	71			
		TT	31	28			
		A allele	158	131			
		T allele	160	127			

SNP: Single nucleotide polymorphism.

RESULTS

IL-10 rs304496 is associated with pediatric IBD

There is limited information in terms of genetic association studies for pediatric IBD. The present study of pediatric IBD builds upon and extends findings from our previous genetic association study on adult IBD. We initially wished to confirm previous findings[20] as to whether *IL-10* was involved in pediatric IBD. Since published studies of *IL-10* were done in adult IBD, we carried out a pilot study with adult IBD. We studied *IL-10* association with 122 adult IBD (74 with CD, 48 with UC) cases (mean age of 51 years) and 172 unrelated healthy controls from Hershey Medical Center using the SNPlex Genotyping System[33,34]. The results indicated that two *IL-10* SNPs are significantly associated with IBD: rs1800872 $P = 0.0056$, OR = 1.753, and 95%CI: 1.190-2.643; and rs304498 $P = 0.0008$ OR = 0.43, and 95%CI: 0.26-0.7. This pilot genetic association study as well as other association studies of adult IBD, guided our selection of genes and SNPs for the present study.

In the present study, we wished to know whether *IL-10*, shown previously to be associated with adult IBD is associated with pediatric IBD, and whether the IL-10/STAT3 pathway plays a role in pediatric IBD. The study samples were 159 IBD (136 with CD and 23 with UC) and the three SNPs genotyped were rs1800872, rs3024496, and rs3024498. The results indicated (Table 4) that neither of the two SNPs, rs1800872 and rs3024498, that have been previously observed to be associated with adult IBD were associated with pediatric IBD ($P = 0.71$ and $P = 0.616$, respectively). The rs3024496 was the only SNP found to significantly associate with pediatric IBD ($P = 0.022$).

No association with pediatric IBD was found for the IL-10 pathway genes, IL10RA, IL10RB, STAT3, and HO1

The IL10-STAT3 signaling pathway plays an important role in controlling inflammation in intestine. The IL10RA, IL10RB and STAT3 are critical players in this pathway. *IL-10* and *STAT3* have previously been identified to be associated with IBD in adult, while mutations in both *IL-10 receptors A* and *B* have been demonstrated to be associated with early-onset IBD. The activated STAT3 pathway regulates expression of several critical anti-inflammatory genes, including HO1, a potent anti-inflammation and anti-oxidant

Table 5 Epistatic interaction between two single nucleotide polymorphisms in three *IL-10* single nucleotide polymorphisms studied

	Epistatic model	rs3024496, *P* = 0.022, Bp = 0.0226	rs3024498, *P* = 0.616; Bp = 1
rs1800872, *P* = 0.71; Bp = 1	aa	*P* = 0.00015; Bp = 0.003	*P* = 0.638; Bp = 1
	ad	*P* = 0.057; Bp = 1	*P* = 0.605; Bp = 1
	da	*P* = 0.010; Bp = 0.216	*P* = 0.977; Bp = 1
	dd	*P* = 0.239; Bp = 1	*P* = 0.049; Bp = 1
rs3024496, *P* = 0.022, Bp = 0.0226	aa		*P* = 0.371; Bp = 1
	ad		*P* = 0.222; Bp = 1
	da		*P* = 0.167; Bp = 1
	dd		*P* = 0.584; Bp = 1

The *P* and Bp values for each SNP are shown next to each SNP; the *P* and Bp values for each of two SNP interactions are shown in the 3rd and 4th column. In the 2nd column (epistatic model), a: Additive; d: Dominant. For a two SNP interaction, four different types of interactions may occur: additive-additive (aa), additive-dominant (ad), dominant-additive (da), and dominant-dominant (dd). Bp: Bonferroni *P* value; SNP: Single nucleotide polymorphism.

Table 6 Gene-gene interaction between *IL-10* with *IL10RA*, *IL10RB*, *STAT3*, or *HO1* in pediatric inflammatory bowel disease

Gene	SNP	Epistatic model	*IL10RA* rs3135932, *P* = 0.160; Bp = 1	*IL10RB* rs2834167, *P* = 0.203; Bp = 1	*STAT3* rs744166, *P* = 0.352; Bp = 1	*HO1* rs2071746, *P* = 0.634; Bp = 1
IL-10	rs1800872	aa	*P* = 0.046; Bp = 1	*P* = 0.029; Bp = 1	*P* = 0.248; Bp = 1	*P* = 0.910; Bp = 1
		ad	*P* = 0.739; Bp = 1	*P* = 0.107; Bp = 1	*P* = 0.954; Bp = 1	*P* = 0.617; Bp = 1
		da	*P* = 0.056; Bp = 1	*P* = 0.376; Bp = 1	*P* = 0.117; Bp = 1	*P* = 0.569; Bp = 1
		dd	*P* = 0.126; Bp = 1	*P* = 0.168; Bp = 1	*P* = 0.036; Bp = 1	*P* = 0.671; Bp = 1
	rs3024498	aa	*P* = 0.330; Bp = 1	*P* = 0.062; Bp = 1	*P* = 0.143; Bp = 1	*P* = 0.898; Bp = 1
		ad	*P* = 0.068; Bp = 1	*P* = 0.629; Bp = 1	*P* = 0.032; Bp = 1	*P* = 0.840; Bp = 1
		da	*P* = 0.884; Bp = 1	*P* = 0.307; Bp = 1	*P* = 0.316; Bp = 1	*P* = 0.607; Bp = 1
		dd	*P* = 0.265; Bp = 1	*P* = 0.644; Bp = 1	*P* = 0.029; Bp = 1	*P* = 0.790; Bp = 1
	rs3024496	aa	*P* = 0.021; Bp = 0.433	*P* = 0.425; Bp = 1	*P* = 0.538; Bp = 1	*P* = 0.346; Bp = 1
		ad	*P* = 0.020; Bp = 0.426	*P* = 0.495; Bp = 1	*P* = 0.306; Bp = 1	*P* = 0.741; Bp = 1
		da	*P* = 0.081; Bp = 1	*P* = 0.189; Bp = 1	*P* = 0.234; Bp = 1	*P* = 0.297; Bp = 1
		dd	*P* = 0.967; Bp = 1	*P* = 0.570; Bp = 1	*P* = 0.402; Bp = 1	*P* = 0.457; Bp = 1
IL10RB	rs2834167	aa	*P* = 0.403; Bp = 1		*P* = 0.251; Bp = 1	*P* = 0.128; Bp = 1
		ad	*P* = 0.384; Bp = 1		*P* = 0.956; Bp = 1	*P* = 0.369; Bp = 1
		da	*P* = 0.518; Bp = 1		*P* = 0.776; Bp = 1	*P* = 0.0018; Bp = 0.039
		dd	*P* = 0.176; Bp = 1		*P* = 0.072; Bp = 1	*P* = 0.289; Bp = 1

Bp: Bonferroni *P* value; SNP: Single nucleotide polymorphism.

enzyme. Our genetic association study results indicate that none of these genes is significantly associated with pediatric IBD (Table 4).

Epistatic interaction of SNP-SNP (rs3024496 and rs1800872) within the IL-10 gene in pediatric IBD

Based on previous genetic studies of the studied genes and their role in the IL-10 pathway[23,24], we speculated that some of the SNPs contribute to disease by interacting with other genes. To test this hypothesis we used our recently developed model[27] that has been demonstrated to be genetically meaningful in our previous studies on IBD susceptibility genes[30,35,36].

First, we studied SNP-SNP interaction among three SNPs (rs1800872 and rs3024496, rs1800872 and rs3024498, and rs3024496 and rs3024498) within the *IL-10* gene of all possible combinations of a and d models.

A significant epistatic interaction was only observed for rs1800872 and rs3024496 (*P* = 0.00015; Bp = 0.003) (Table 5). A graphical depiction of this aa model is shown in Figure 1. Although the *IL-10* rs1800872 was shown by itself to associate with adult IBD but not associate with pediatric IBD (*P* = 0.71) (Table 4), the present data indicate that it may still contribute to pediatric IBD *via* interaction with another IL-10 SNP, namely rs3024496.

Epistatic interaction of the IL-10 gene with the IL-10 signaling pathway genes, IL10RB and HO1, in pediatric IBD

We further analyzed gene-gene interactions between *IL-10* and the other four genes, *IL10RA*, *IL10RB*, *STAT3*, and *HO1*, involved in the IL-10 signaling pathway. The results showed that none of the three *IL-10* SNPs significantly interacted with the SNPs of the other four genes (Table 6). Although a low *P*-value was observed for the *IL-10* rs1800872 with either the *IL10RA* (aa, *P* = 0.046), *IL10RB* (aa, *P* = 0.029), or *STAT3* (dd, *P* = 0.036), and for the *IL-10* rs3024498 with the *STAT3* (ad, *P* = 0.032; dd *P* = 0.029), none of these stood as significant after Bonferroni correction was applied. Only the interaction of the *IL-10* rs3024496 with the *IL10RA* rs3135932 showed a low Bp (aa, *P* = 0.021, Bp = 0.433; ad, *P* = 0.020, Bp = 0.426) (Table 6). A graphic depiction of the

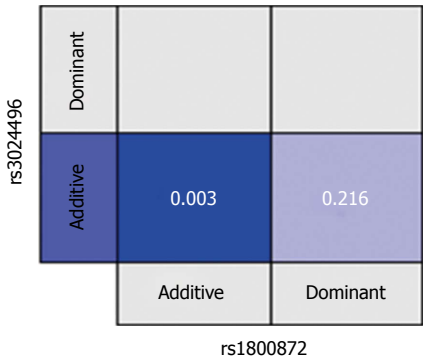

Figure 1 Epistatic interaction between *IL-10* single nucleotide polymorphisms rs3024496 and rs1800872. Graphic depiction of epistatic interaction between the *IL-10* SNPs, rs3024496 and rs1800872. Four interaction models for rs3024496 and rs1800872 are shown. The additive-additive model is significant (Bp = 0.003), the additive-dominant model is weak (Bp = 0. 216), and the other two models are not observed (Bp = 1, no color). Bp: Bonferroni *P* value; SNPs: Single nucleotide polymorphisms.

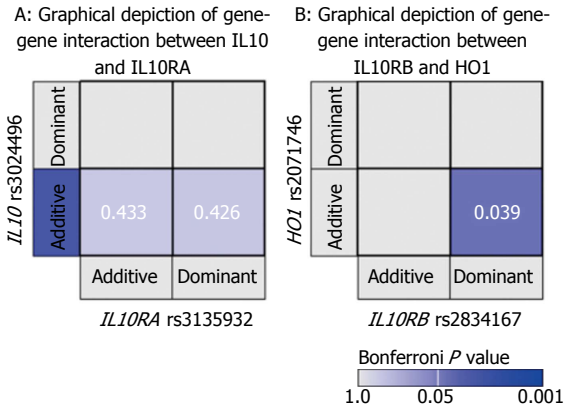

Figure 2 Gene-gene interaction between *IL-10* and *IL10RA*, *IL10RB* and *HO1* in pediatric inflammatory bowel disease. A: Graphic depiction of epistatic interaction between the *IL-10* SNP rs3024496 and the *IL10RA* rs3135932. Four interaction models for rs3024496 and rs3135932 are shown. The additive-additive and additive-dominant models are both weak (Bp = 0.433 and 0.426 respectively), and the other two models are not observed (Bp = 1, no color); B: Graphical depiction of epistatic interaction between the *HO1* rs2071746 and the *IL10RB* rs2834167. Four interaction models for rs3024496 and rs3135932 are shown. Only the additive-dominant model is significant (Bp = 0.039), and the other three models are not observed (Bp = 1, no color). The levels of Bp values are shown in the bar as shades of blue color from none (Bp = 1) to high (Bp = 0.001). Bp: Bonferroni *P* value; SNPs: Single nucleotide polymorphisms.

interaction model of *IL-10* with *IL10RA* is shown in Figure 2A. From a single association study as described above, none of the SNPs of the four genes in the IL-10 signaling pathway was associated with pediatric IBD. However, we found that SNPs of the *IL-10RB* and *HO1* genes contribute to pediatric IBD (da, $P = 0.0018$, Bp = 0.039) (Table 6) *via* gene-gene interaction. Graphic depictions for the model interactions between *IL10RB* and *HO1* are shown in Figure 2B.

Epistatic interaction of the IL-10 rs3024496 and rs1800872 with the IL10R, rs3135932 in pediatric IBD

We next investigated whether the interaction of two *IL-10* SNPs, rs1800872 and rs3024496, affects further

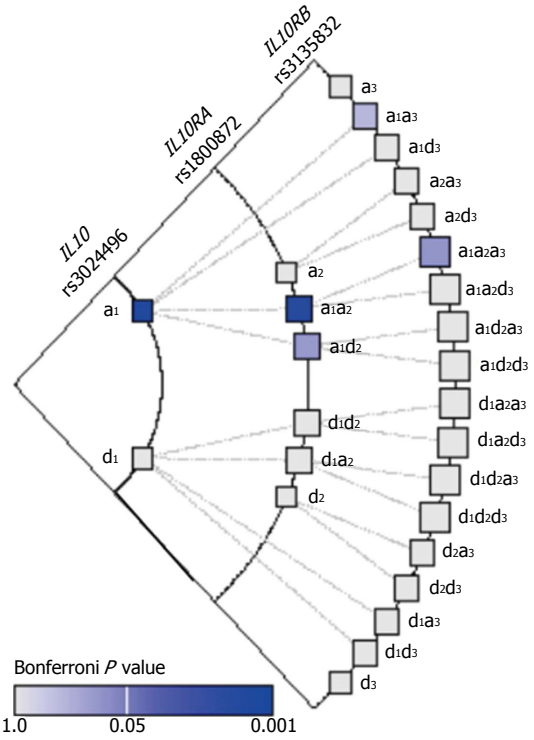

Figure 3 Epistatic interaction of two *IL-10* single nucleotide polymorphisms, rs1800872 and rs3024496, with *IL10RA*, *IL10RB*, *STAT3* and *HO1* in pediatric inflammatory bowel disease. Graphic depiction of epistatic interaction of the *IL-10* SNPs rs3024496 and rs1800872 with the *IL10RA* rs3135932. All the interaction models analyzed for the *IL-10* SNPs rs3024496 and rs1800872 with the *IL10RA* rs3135932 are shown. However, only one significant interaction model additive-additive-additive (a1a2a3) was observed ($P = 0.003$, Bp = 0.099). Levels of Bp values are shown in the bar as shades of blue color from none (Bp = 1) to high (Bp = 0.001). a1, d1, a2, d2, a3, d3: Letters, a and d, are for interaction model additive and dominant respectively; Numbers 1, 2, and 3 depict the *IL-10* rs3024496, *IL-10* rs1800872, and *IL10RA* rs3135932 respectively. The contribution of a single SNP in an interaction model could be either a or d (a1 and d1 for SNP #1, a2 and d2 for SNP #2, and a3 and d3 for SNP #3). The interaction models for two and three SNPs are the combination of the two and three SNPs; such as, a1a2 for SNPs 1 and 2, and a1a2a3 for SNPs 1, 2, and 3. Bp: Bonferroni *P* value; SNPs: Single nucleotide polymorphisms.

interaction of IL-10 with other genes. As shown in Table 7, in the presence of the two *IL-10* SNPs, the association of *IL10RA* rs3135932 is increased remarkably from $P = 0.16$ (when analyzed by itself), to $P = 0.046$ (with rs1800872), $P = 0.021$ (with rs3024496) (Table 5) to $P = 0.003$ (with both rs1800872 and rs3024496) (Table 7). This epistatic interaction is an aaa model (Figure 3). However, the interaction of the two *IL-10* SNPs did not exhibit any further observed effect on IL-10 interaction with the other genes, *IL10RB*, *STAT3*, and *HO1* (Table 7).

Epistatic interaction of the IL-10 pathway genes IL10RA, IL10RB, STAT3, and HO1 in pediatric IBD

Based on the epistatic interaction of *IL10RB* with *HO1* (Table 6, Figure 2B), we further analyzed the effect of the *IL10RB* and *HO1* interaction (in each of the four models aa, ad, da and dd) on the contribution to IBD in conjunction with *IL-10*, *IL10RA* and *STAT3*. Eight types of epistatic interactions in each set of three SNPs were studied. As shown in Table 8, a significant effect

Table 7 Epistatic interaction among the two *IL-10* single nucleotide polymorphisms, rs1800872 and rs3024496, and *IL10RA*, *IL10RB*, *STAT3*, or *HO1*

	Epistatic model	*IL10RA* rs3135932, *P* = 0.16; Bp = 1	*IL10RB* rs2834167, *P* = 0.203; Bp = 1	*STAT3* rs744166, *P* = 0.352; Bp = 1	*HO1* rs2071746, *P* = 0.634; Bp = 1
Two IL-10 SNPs: rs1800872 and rs3024496 aa: *P* = 0.0002; Bp = 0.003	aaa	*P* = 0.003; Bp = 0.099	*P* = 0.080; Bp = 1	*P* = 0.175; Bp = 1	*P* = 0.216; Bp = 1
	aad	*P* = 0.130; Bp = 1	*P* = 0.340; Bp = 1	*P* = 0.920; Bp = 1	*P* = 0.140; Bp = 1

The additive-additive (aa) interaction model for the two *IL-10* SNPs (rs1800872 and rs3024496) from Figure 1 was chosen for further analysis, in order to study the interaction of these two *IL-10* SNPs with SNPs of four other genes. The two interaction models, aaa and aad, were for the two *IL-10* SNPs and SNPs from each of the four genes. A significant *P* value (*P* = 0.003) was observed only with the *IL10RA* SNP (rs3135932). Bp: Bonferroni *P* value; SNPs: Single nucleotide polymorphisms.

Table 8 Epistatic interaction of *IL10RB* and HO1 with *IL-10* (three single nucleotide polymorphisms), *IL10RA*, or *STAT3*

	Epistatic model	*IL-10* rs1800872, *P* = 0.71; Bp = 1	*IL-10* rs3024498, *P* = 0.616; Bp = 21	*IL-10* rs3024496, *P* = 0.022; Bp = 0.0226	*IL10RA* rs3135932, *P* = 0.16; Bp = 1	*STAT3* rs744166, *P* = 0.352; Bp = 1
aa *IL10RB* rs2834167 and *HO1* rs2071746	aaa	*P* = 0.029; Bp = 1	*P* = 0.545; Bp = 1	*P* = 0.255; Bp = 1	*P* = 0.023; Bp = 0.820	*P* = 0.049; Bp = 1
	aad	*P* = 0.127; Bp = 1	*P* = 0.240; Bp = 1	*P* = 0.928; Bp = 1	*P* = 0.193; Bp = 1	*P* = 0.554; Bp = 1
ad *IL10RB* rs2834167 and *HO1* rs2071746	ada	*P* = 0.394; Bp = 1	*P* = 0.824; Bp = 1	*P* = 0.242; Bp = 1	*P* = 0.444; Bp = 1	*P* = 0.436; Bp = 1
	add	*P* = 0.976; Bp = 1	*P* = 0.406; Bp = 1	*P* = 0.327; Bp = 1	*P* = 0.644; Bp = 1	*P* = 0.649; Bp = 1
da *IL10RB* rs2834167 and *HO1* rs2071746	daa	*P* = 0.00015; Bp = 0.005	*P* = 0.489; Bp = 1	*P* = 0.047; Bp = 1	*P* = 0.008; Bp = 0.287	*P* = 0.014; Bp = 0.491
	dad	*P* = 0.072; Bp = 1	*P* = 0.140; Bp = 1	*P* = 0.388; Bp = 1	*P* = 0.205; Bp = 1	*P* = 0.977; Bp = 1
dd *IL10RB* rs2834167 and *HO1* rs2071746	dda	*P* = 0.199; Bp = 1	*P* = 0.808; Bp = 1	*P* = 0.568; Bp = 1	*P* = 0.766; Bp = 1	*P* = 0.4731; Bp = 1
	ddd	*P* = 0.694; Bp = 1	*P* = 0.958; Bp = 1	*P* = 0.007; Bp = 0.242	*P* = 0.181; Bp = 1	*P* = 0.057; Bp = 1

was observed on the interaction of these two genes, *IL10RB* and *HO1*, with the *IL-10* gene rs1800872 (*P* = 0.00015, Bp = 0.005). The model of the three gene interaction $d_{IL10RB}a_{HO1}a_{IL\text{-}10}$ is depicted in Figure 4. Moreover, weak interactions were also observed on the interaction of these two genes with *IL10RA* rs3135932 (aaa *P* = 0.023, Bp = 0.820; daa *P* = 0.008, Bp = 0.287) and with *STAT3* rs744166 (daa *P* = 0.014, Bp = 0.491). As shown in Figure 2B, the interaction model $d_{IL10RB}a_{HO1}$ for *IL-10* and *HO1* is significant (Bp = 0.039). The three SNPs interaction analysis indicated that this model is responsible for the interaction of *IL10RB* and *HO1* with the other genes, *IL-10*, *IL10RA*, and *STAT3*, in the IL-10 pathway, as $d_{IL10RB}a_{HO1}a_{IL\text{-}10}$, $d_{IL10RB}a_{HO1}a_{IL10RA}$, and $d_{IL10RB}a_{HO1}a_{STAT3}$, respectively.

The findings indicate that the interaction between *IL10RB* and *HO1* may enhance their action with *IL-10* and *IL10RA* and elevate the pathway activity in pediatric IBD. Considering that HO1 is an important mediator of the anti-inflammatory effect of IL-10 and several mutations in *IL10RB* are associated with pediatric IBD, these gene-gene interactions may affect pathway function by regulating anti-inflammatory activity in pediatric IBD.

DISCUSSION

In the present study, we identified a genetic association of the *IL-10* gene and the IL-10 signaling pathway with pediatric IBD and demonstrated that both SNP-SNP and gene-gene epistatic interactions contribute to pediatric IBD. The specific findings include the following: (1) *IL-10* rs3024496 is identified to be associated with pediatric IBD; (2) an aa interaction was found between *IL-10* SNPs rs3024496 and rs1800872; (3) the SNP-SNP interaction in the *IL-10* gene affects its action with the IL-10 receptor *IL10RA*; (4) the IL-10 signaling pathway genes *IL10RB* and *HO1* together are significantly associated with pediatric IBD *via* SNP-SNP interaction; and (5) a significant association of the three genes, *IL-10*, *IL10RB* and *HO1*, with pediatric IBD was identified from epistatic interaction analysis among three SNPs.

The *IL-10* gene has been shown to be associated

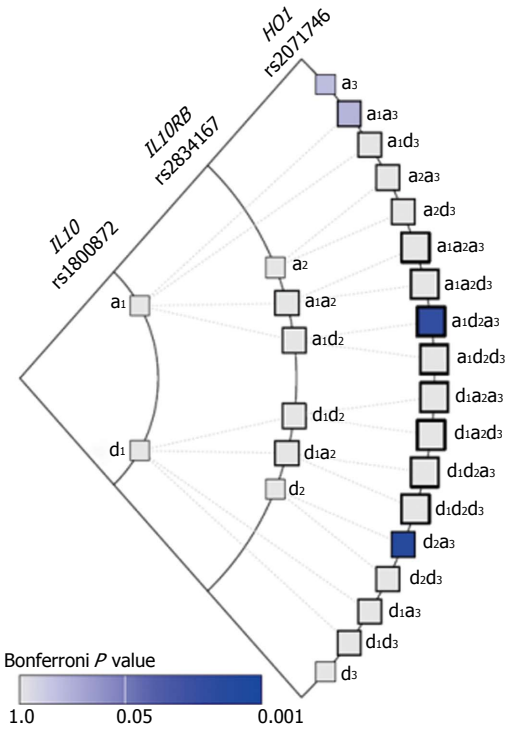

Figure 4 Epistatic interaction of *IL10RB* and *HO1* with *IL-10* (three single nucleotide polymorphisms), *IL10RA* and *STAT3* in pediatric inflammatory bowel disease. Graphic depiction of epistatic interaction of the *IL-10* rs1800872, *IL10RB* rs2834167 and *HO1* rs2071746. Of all the interaction models analyzed, a three SNP interaction (a1d2a3) for *IL-10* rs1800872, *IL10RB* rs2834167 and *HO1* rs2071746 is significant (Bp = 0.005). The levels of Bp values are shown in the bar as shades of blue color from none (Bp = 1) to high (Bp = 0.001). a1, d1, a2, d2, a3, d3: Letters, a and d, denote interaction models, additive and dominant respectively; Numbers 1, 2, and 3 are for *IL-10* rs1800872, *IL10RB* rs2834167, and *HO1* rs2071746, respectively. The contribution of a single SNP in an interaction model could be either a or d: a1 and d1 for SNP #1, a2 and d2 for SNP #2, and a3 and d3 for SNP #3. The interaction models for two and three SNPs are the combination of two and three SNPs (*e.g.*, a1a2 for SNPs 1 and 2, and a1a2a3 for SNPs 1, 2, and 3). Bp: Bonferroni *P* value; SNP: Single nucleotide polymorphism.

with adult IBD by GWASs. The most studied *IL-10* SNP, -1082A>G (rs1800896), is thought of as having potential for gene transcription regulation[7,14-16,37]. The *IL-10* SNP rs3024496 is shown to be related to inflammatory response with increased levels of IgE to dust mite[38], or decreased production of IL-10 by peripheral blood leukocytes[39], and with prostate[40] and colorectal[41] cancer, but has not been shown to be associated with IBD. The *IL-10* rs1800872 is associated with IBD[37] and also with increased serum IL-10 levels in CD[42,43], as well as with irritable bowel syndrome[44] and cancer susceptibility[42,43,45,46]. In this study, we found that *IL-10* rs3024496 is associated with pediatric IBD, and rs1800872, although by itself is not associated with pediatric IBD, appears to contribute to pediatric IBD *via* epistatic interaction with rs3024496.

Although currently more than 163 genes have been identified to be associated with IBD[47-50], only few of them have been studied in pediatric IBD. The estimate that a genetic contribution of the identified genes collectively represents only < 20% of the overall disease risk[47,51-55] indicates that other genetic/genomic and environmental factors may play a role in IBD pathogenesis. In the present study, we studied *IL-10* gene contribution in pediatric IBD by analyzing its association with disease as well as its epistatic interaction with IL-10 pathway genes. Our results indicate, in addition to disease association of *IL-10* itself, that SNP-SNP and gene-gene interactions contribute significantly to pediatric IBD.

Our results support that epistasis plays an important role in the formation and progression of human diseases[56,57]. Understanding gene-gene interaction is crucial to our understanding of the regulation of physiological function. When epistasis occurs, the presence of two or more particular loci may increase or reduce the risk of a disease more than would be expected from their independent effects[58]. A host of statistical models have been developed to analyze epistatic effects in different genetic designs[59,60].

Our recently developed model for multilocus epistatic interactions in case-control studies has proven to be genetically meaningful through the incorporation of traditional quantitative genetic principles into statistical models[27]. Using this model we have previously studied epistatic interaction between SNPs within the *DLG5* gene and between IBD genes *DLG5*, *OCTN1*, *IL23R* and *NOD2*[28,35,36], and found that epistatic interaction is an important component in IBD pathogenesis. In this study, we used the same method to study gene-gene interaction in IL-10 signaling transduction pathway. IL-10 signaling transduction occurs through binding of IL-10 to its receptors IL10RA and IL10RB to form a complex, with downstream molecules, Jak1 and Tyk2, activating STAT3[24,25]. IL10RA is specific to IL-10, but IL10RB also interacts with several other cytokines. When either *IL10RA* or *IL10RB* is mutated, the signals from IL-10 cannot be received and the resulting inflammation causes tissue damage in the gastrointestinal system[23,24]. A significant epistatic interaction was observed between two *IL-10* SNPs, resulting in a significant effect of *IL-10* interaction with *IL10RA*, but not with *IL10RB* (Table 7). This indicates that IL-10 may interact with receptor IL10RA, which plays a role in the initiation of the signaling pathway.

We also observed a significant epistatic interaction between *IL-10*, *IL10RB*, and the downstream pathway target *HO1* gene (Table 8). The key factor for interaction of these three genes is *IL10RB* that interacts strongly with *HO1*. This finding indicates that the IL-10 receptors IL10RA and IL10RB are likely to function differently in the IL-10 pathway in pediatric IBD. Although HO1 was not found to be associated with IBD[61], it has been shown to be associated with other diseases such as asthma and allergy[44,61]. HO1 is a potent enzyme of anti-inflammation, and has a very important function of the IL-10 pathway in controlling inflammation[14].

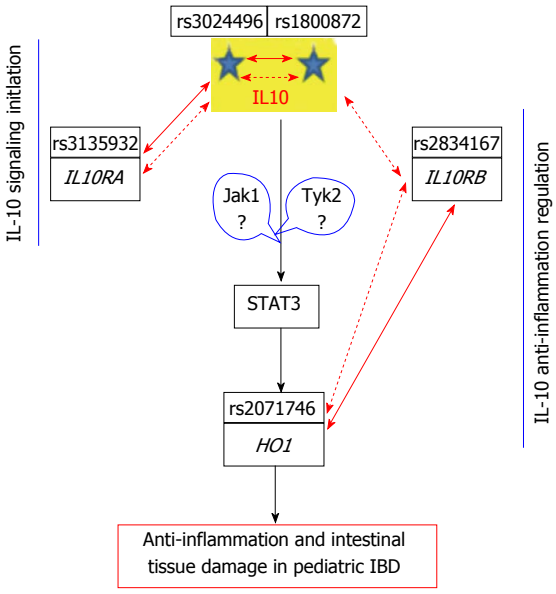

Figure 5 Summary of gene function and epistatic interaction of the IL-10/STAT3 signaling pathway in pediatric inflammatory bowel disease. In the IL-10 pathway genes, genetic variations in *IL-10* and *STAT3* are associated with adult IBD, and genetic mutations identified from *IL10RA* and *IL10RB* are associated with pediatric/very early onset inflammatory bowel disease (published data). In this study, we found SNP-SNP epistatic interaction within the *IL-10* gene, between *IL10* and *IL10RA* genes, and between *IL10RB* and *HO1* genes. We also found epistatic interaction among three SNPs between the two genes *IL-10* (two SNPs) and *IL10RA*, and among the three genes *IL-10*, *IL10RB* and *HO1*. Bp: Bonferroni *P* value.

Although STAT3 is a critical component of the IL-10/STAT3 pathway[23,24], no significant interaction was observed between *IL-10* and/or *IL-10 receptors* with *STAT3*, indicating that other factor(s) may play a role between these two genes in the pathway. We know that in the IL-10 pathway, upon binding of IL-10 to cell receptors IL10RA and IL10RB, the IL-10 receptor complex members JAK1 and Tyk2 are activated and catalyze phosphorylation of themselves and then of IL10RA[3,4], thereby forming a docking site for STAT3. STAT3 is phosphorylated by JAK1 and Tyk2, and this phosphorylation causes STAT3 dimerization and translocation to the nucleus where it can induce expression of its target genes including *HO-1*. Therefore, we speculate that JAK1 and Tyk2 play a role in pediatric IBD by their activity in the phosphorylation and activation of STAT3 in IL-10 signaling pathway.

Recently, pediatric/VEO IBD has been suggested to be a distinct form of IBD[11,62], and SNPs in *IL-10* and *IL-10 receptors* have been associated with VEO IBD[7-12]. In the present study, we identified *IL-10* SNP of rs3024496 to be associated with pediatric IBD; this has not been shown to be associated with adult IBD. However, our results did not show a genetic association of the *IL-10* rs1800872 and *STAT3* rs744166 with pediatric IBD, which have been shown to be associated with adult IBD. Our present study also showed that epistatic interactions of *IL-10* with genes *IL10RA*, *IL10RB*, *STAT3*, and *HO1* contribute to pediatric IBD. Their physiological function in the regulation of the anti-inflammatory pathway in response to pro-inflammatory stimulation, and protection of diseased tissues from damage is currently not well studied. IBD is a major gastrointestinal disease affecting 1.4 million people in the United States. About 15%-25% of IBD patients are diagnosed in childhood[16,23]. Specific investigation targeting the IL-10 signaling pathway in pediatric IBD pathogenesis will help to understand the pathogenesis of pediatric IBD, and may provide target molecules and pathways to potentially develop anti-inflammatory agents for clinical treatment of pediatric IBD.

Other cytokines are also shown to be involved in the inflammatory process of IBD. Studies on correlation between NO and IL17A, IL-23 and IL-6 levels in plasma of IBD patients indicated that the IL-23/IL17A axis and NO synthase pathway are involved in inflammation regulation in IBD[63].

In summary, *IL-10* is associated with pediatric IBD, and the IL-10 signaling pathway that plays an important role in anti-inflammation. We propose, as depicted in Figure 5, that in pediatric IBD pathogenesis, (1) *IL-10 via* its interaction with receptor *IL10RA*, and then together with receptor *IL10RB* are critical for the initial step of the signaling transduction; (2) *IL-10 via* its interaction with receptor *IL10RB* plays a key role in regulating gene transcription of anti-inflammation enzymes, such as *HO1*, that may lead to an anti-inflammatory response; and (3) no significant interaction was found between *IL-10* and *IL-10 receptors* with *STAT3*, a key molecule in the IL-10 pathway. However, further investigation may provide insight as to whether JAK1 and Tyk2 are involved in pediatric IBD, *via* potential interactions with *IL10RA* and *IL10RB* where together their gene products could phosphorylate and activate STAT3.

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COMMENTS

Background

Knockout mice lacking *IL-10* develop inflammatory bowel disease (IBD), and mutations in *IL-10* and IL-10 receptor genes *IL10RA* and *IL10RB* have been linked to very early-onset (VEO) IBD. Both *IL-10* and *STAT3* have been identified as IBD-associated genes in adults, but these are not well studied in pediatric IBD.

Research frontiers

Mutations of *IL-10* and genes encoding its receptors have been identified recently in VEO IBD. The authors studied genetic association of *IL-10* and genes in the IL-10/STAT3 pathway with pediatric IBD. Genetic interactions between different loci, *i.e.*, epistasis, have been thought to be of paramount importance in complex diseases. In this paper, we used a computational model

to analyze how epistatic interactions among polymorphic loci in the *IL-10* gene and IL-10/STAT3 pathway govern pediatric IBD in a case-control setting.

Innovations and breakthroughs

Despite pronounced evidence of the role of the genes comprising IL-10 and genes within the IL-10/STAT3 signaling pathway, our knowledge about how they interact with each other to determine IBD development is still very limited. In this paper, they have identified *IL-10* variation associated with pediatric IBD, and found a number of epistatic interactions of *IL-10* with genes in the IL-10/STAT3 pathway contributing to pediatric IBD. The contribution of interactions of the IL-10/STAT3 pathway and anti-inflammatory *HO1* gene to IBD indicated that *IL-10* plays a role in the control of inflammation in IBD.

Applications

The findings may help to understand the function of IL-10 and the IL-10 pathway in the control of inflammation in IBD, and identify target molecules for clinical investigation and drug discovery for controlling inflammation in IBD.

Peer-review

The authors report novel data. The obtained results indicate that both the *IL-10* gene and its epistatic interaction with genes within its signaling pathway are related to pediatric IBD.

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ORIGINAL ARTICLE

Basic Study

Generation of glyceraldehyde-derived advanced glycation end-products in pancreatic cancer cells and the potential of tumor promotion

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Author contributions: Takata T and Takeuchi M designed the research; Takata T performed the research; Takeuchi M contributed the reagents that were indispensable for this investigation; Takata T, Ueda T and Sakasai-Sakai A analyzed the data; Takata T and Takeuchi M wrote the paper.

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Abstract

AIM

To determine the possibility that diabetes mellitus promotes pancreatic ductal adenocarcinoma *via* glyceraldehyde (GA)-derived advanced glycation-end products (GA-AGEs).

METHODS

PANC-1, a human pancreatic cancer cell line, was treated with 1-4 mmol/L GA for 24 h. The cell viability and intracellular GA-AGEs were measured by WST-8 assay and slot blotting. Moreover, immunostaining of PANC-1 cells with an anti-GA-AGE antibody was performed. Western blotting (WB) was used to analyze the molecular weight of GA-AGEs. Heat shock proteins 90α, 90β, 70, 27 and cleaved caspase-3 were analyzed by WB. In addition, PANC-1 cells were treated with GA-AGEs-bovine serum albumin (GA-AGEs-BSA), as a model of extracellular GA-AGEs, and proliferation of PANC-1 cells was measured.

RESULTS

In PANC-1 cells, GA induced the production of GA-AGEs and cell death in a dose-dependent manner. PANC-1 cell viability was approximately 40% with a 2 mmol/L GA treatment and decreased to almost 0% with a 4 mmol/L GA treatment (each significant difference was $P < 0.01$). Cells treated with 2 and 4 mmol/L GA produced 6.4 and 21.2 μg/mg protein of GA-AGEs, respectively ($P <$

0.05 and $P < 0.01$). The dose-dependent production of some high-molecular-weight (HMW) complexes of HSP90β, HSP70, and HSP27 was observed following administration of GA. We considered HMW complexes to be dimers and trimers with GA-AGEs-mediated aggregation. Cleaved caspase-3 could not be detected with WB. Furthermore, 10 and 20 μg/mL GA-AGEs-BSA was 27% and 34% greater than that of control cells, respectively ($P < 0.05$ and $P < 0.01$).

CONCLUSION

Although intracellular GA-AGEs induce pancreatic cancer cell death, their secretion and release may promote the proliferation of other pancreatic cancer cells.

Key words: Tumor promotion; Glyceraldehyde-derived advanced glycation-end products; Pancreatic ductal adenocarcinoma

Core tip: The mechanisms promoting pancreatic ductal adenocarcinoma (PDAC) in the pancreas of Type 2 diabetes mellitus patients have not yet been elucidated. We hypothesized that glyceraldehyde (GA)-derived advanced glycation-end products (GA-AGEs) promote PDAC. PANC-1 cells were treated with GA, which induced the production of intracellular GA-AGEs and cell death. The high-molecular-weight complexes of heat shock proteins were produced after GA treatment in a dose-dependent manner. GA-AGEs-bovine serum albumin promoted the proliferation of PANC-1 cells. Although intracellular GA-AGEs induce pancreatic cancer cell death, their secretion and release may promote the proliferation of other pancreatic cancer cells.

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INTRODUCTION

Pancreatic cancer is a highly lethal disease with a 5-year survival rate of approximately 5%[1,2]. Pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 90% of malignancies in the pancreas[3]. The incidence of diabetes mellitus (DM) is increasing worldwide each year, and this condition may result in life-changing complications. A total of 415 million adults are already estimated to have DM (this will increase to 652 million adults by 2040)[4]. Type 2 DM (T2DM), the most common type of DM, has been shown to increase the risk of pancreatic cancer by more than 50%; furthermore, T2DM patients with pancreatic cancer have worse prognosis and shorter survival time than those without the disease[5,6].

However, the mechanisms promoting PDAC in the pancreas of T2DM patients have not yet been elucidated. Therefore, we focused on hyperglycemia, a characteristic of T2DM. Glucose and fructose have been shown to induce the production of advanced glycation-end products (AGEs)[7-12], which have, in turn, been implicated in the pathogeneses of a number of lifestyle-related diseases. Toxic and non-toxic AGEs exist among the various types of AGE structures generated *in vivo*. We previously identified AGEs derived from glyceraldehyde (GA), a glucose/fructose metabolism intermediate, also known as GA-AGEs[7-12]. We designated GA-AGEs as toxic AGEs (TAGE) because of their cytotoxicity and involvement in insulin resistance, hypertension, diabetic complications, cardiovascular diseases, dementia, non-alcoholic steatohepatitis, Alzheimer's disease, and cancer[7-12].

We hypothesized that the production of GA-AGEs in pancreatic ductal cancer cells in the pancreas of T2DM patients promotes PDAC. In the present study, we incubated PANC-1, a human pancreatic ductal cancer cell line, with GA to generate intracellular GA-AGEs. We analyzed cell viability, intracellular GA-AGEs, cell death-associated proteins, and the high-molecular-weight (HMW) complexes that appeared and increased in a GA dose-dependent manner. Then, we investigated whether secreted GA-AGEs from GA-AGEs-producing pancreatic cancer cells promoted tumors. In this study, we also examined the effects of GA-AGEs-bovine serum albumin (GA-AGEs-BSA) on the proliferation of PANC-1 cells.

MATERIALS AND METHODS

Materials

The human pancreatic cancer cell line PANC-1 (ATCC-CRL-1469) was purchased from ATCC (VA, United States). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin solution, and L-glutamine solution were obtained from Sigma-Aldrich (MO, United States). 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from Dojindo Laboratories (Kumamoto, Japan). GA was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The Western re-probe kit was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). The protein assay kit for the BCA method was purchased from Bio-Rad (CA, United States). The protein assay kit for the Bradford method was obtained from Takara Bio, Inc. (Otsu, Japan). All other reagents and kits were purchased from Wako Pure Chemical Industries, Ltd. (Osaka Japan). The non-glycated control bovine serum albumin (BSA) and GA-AGEs-BSA were prepared as described

previously[13].

Cell culture, cell seeds, and GA treatment

PANC-1 cells were grown in high glucose DMEM supplemented with 100 mL/L fetal bovine serum (FBS; Bovogen-Biologicals, VIC, Australia), 2.0 mmol/L) glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin under standard cell culture conditions (humidified atmosphere, 50 mL/L CO_2, 37 ℃). Cells were seeded (1.9×10^4 cells/cm^2) on various plates, culture dishes (Becton-Dickinson, NJ, United States), and glass chambers (Thermo Fisher Scientific Inc., MA, United States) and incubated for 24 h before GA treatment. GA was diluted in phosphate buffered saline (PBS) and filtered before being added to PANC-1 cells. The volume of PBS (including GA) was 2.0 μL/100 μL of the total medium volume. All experiments were performed 24 h after GA treatment.

Cell viability and proliferation

Cell viability and proliferation were assessed by the WST-8 assay. Three hours after medium change and treatment with the WST-8 reagent solution, absorbance was measured at 450 nm and 650 nm using a microplate reader (Labsystems Multiskan ascent, Model No. 354; Thermo Fisher Scientific Inc., Kanagawa, Japan). A blank value was obtained (OD 450 nm-OD 650 nm) from a well containing medium only. Cell viability and proliferation (%) = OD of GA-treated cells/OD of control cells.

Slot blotting analysis for GA-AGEs

Lysis buffer for slot blotting (SB): A solution containing 2 mol/L thiourea, 7 mol/L urea, 40 g/L CHAPS, and 30 mmol/L Tris (hydroxymethyl) aminomethane (Tris) was diluted in ultra-pure water. Then, this solution and 30 g/L of ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany) solution was mixed (9:1) to prepare the thiourea/urea lysis buffer.

Preparation of rabbit anti-GA-AGE antibody: An immunoaffinity-purified rabbit anti-GA-AGE antibody was prepared as described previously[13]. The immunoaffinity-purified anti-GA-AGE antibody did not recognize well-characterized AGE structures such as N^{ε}-(carboxymethyl) lysine, N^{ε}-(carboxyethyl) lysine, pyrraline, pentosidine, argpyrimidine, imidazolone, glyoxal-lysine dimers, methylglyoxal-lysine dimers, or GA-derived pyridinium. Additionally, it did not recognize other AGEs including glucose- and fructose-derived AGEs, the structures of which are currently unknown. This anti-GA-AGE antibody specifically recognized unique and unknown GA-AGE structures.

Neutralization of the anti-GA-AGE antibody for SB analysis: Tween 20 at a final concentration of 0.5 mL/L (GE Healthcare, Tokyo, Japan) was dissolved in PBS (PBS-T). Non-fat skimmed milk (SM; Nacalai Tesuque) at a final concentration of 5.0 g/L was dissolved in PBS-T (5.0 g/L SM-PBS-T). The anti-GA-AGE antibody (1:500) was incubated with a GA-AGEs-BSA solution at a final concentration of 250 mg/L in 5.0 g/L SM-PBS-T at room temperature for 1 h.

Preparation of cell lysates and SB analysis: Cells were washed with PBS and harvested with thiourea/urea lysis buffer. Protein concentrations were measured by the Bradford protein assay kit, using BSA as a standard. Cell lysates containing 2.0 μg of protein were maintained. Thiourea/urea lysis buffer was added to all samples to ensure equal final volimes.

The standard GA-AGEs-BSA solution and a horseradish peroxidase (HRP)-conjugated molecular marker (HRP-MM; Bionexus, CA, United States) were dissolved in thiourea/urea lysis buffer. Then, a final volume of 200 μL was obtained by adding PBS.

The polyvinylidene difluoride (PVDF) membrane (Millipore, MA, United States), which was set on the SB instrument (BIO-DOT SF, Bio-Rad), was washed with PBS. Each sample, standard GA-AGEs-BSA solution, and HRP-MM solution was then added to the membrane under vacuum conditions. PBS was added to wash the membrane. The membrane was then washed with ultra-pure water for 1 min, and cut to prepare two membranes: (1) the membrane for the anti-GA-AGE antibody; and (2) the membrane for the neutralized anti-GA-AGE antibody. Both membranes were blocked at room temperature for 1 h using 50 g/L SM-PBS-T. After being washed, each membrane was incubated with the anti-GA-AGE antibody (1:500) or the neutralized anti-GA-AGE antibody in 5.0 g/L SM-PBS-T at room temperature for 4 h. After being washed with 5.0 g/L SM-PBS-T, both membranes were incubated with an HRP-conjugated goat anti-rabbit IgG antibody (Dako, Glostrup, Denmark; 1:2000) in 5.0 g/L SM-PBS-T at room temperature for 1 h. Then, membranes were washed with PBS-T. Immunoreactive complexes were visualized using the ImmunoStar LD kit. The band densities on the membranes were measured using the LAS-4000 fluorescence imager (GE Healthcare, Tokyo, Japan) and expressed in arbitrary units (AU). The densities of HRP-MM bands were used to correct for differences in densities between membranes. The amount of GA-AGEs in samples was calculated based on a standard curve for GA-AGEs-BSA.

Immunostaining

PANC-1 cells were cultured in a glass chamber for 24 h. After removing medium and washing with PBS, fresh medium was added to the dish. Cells were then treated with GA and incubated at 37 ℃ for 24 h. Triton X-100 and BSA were dissolved in PBS (TritonX-100-PBS and BSA-PBS). Cells were fixed in 16 g/L paraformaldehyde at room temperature for 20 min, rinsed with PBS, permeabilized with 1.0 mL/L Triton X-100-PBS for 10

min and then rinsed with PBS, followed by 1.0 g/L BSA-PBS. Finally, cells were incubated with 30 g/L BSA-PBS (blocking step) for 1 h. After being washed with 1.0 g/L BSA-PBS, cultured cells were incubated with the anti-GA-AGE antibody dissolved in 10 g/L BSA-PBS (1:100) for 1 h. Cells were then washed three times with 1.0 g/L BSA-PBS and incubated with the HRP-conjugated goat anti-rabbit IgG antibody (1:1000) for 1 h. After being washed with 1.0 g/L BSA-PBS and PBS, cells were incubated for 5 min with 0.2 g/L 3,3'-diaminoenzidine tetrahydrochloride (Dojindo) and 5.0 mL/L H_2O_2 in PBS. Cells were briefly counterstained with hematoxylin. Optical microscopic examination was performed using a microscope system (OLYMPUS Co., Ltd. Tokyo, Japan).

Western blotting analysis

Cells were harvested with a radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific Inc.) solution with 3.0 g/L protease inhibitor cocktail (Roche Applied Science) solution. Protein concentrations were assessed by the BCA assay kit, using BSA as a standard. Lysates (15 μg protein/lane) were mixed with sodium dodecyl sulfate (SDS) sample buffer (Bio-Rad) and 2-mercaptoethanol (Sigma-Aldrich) and heated at 95 ℃ for 5 min. They were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 40-150 g/L gradient polyacrylamide gels (Bio-Rad). Proteins were transferred onto PVDF membranes using the semidry electron transfer system (ATTO Co., Ltd., Tokyo, Japan). The membranes were incubated in 50 g/L SM-PBS-T at room temperature for 30 min (blocking step). Proteins on PVDF membranes were probed with the following primary antibodies at 4 ℃ overnight: anti-GA-AGE antibody (1:1000), neutralized anti-GA-AGE antibody (1:1000), rabbit monoclonal [ERP3953] anti-HSP90α antibody (Abcam, Cambridge, United Kingdom; 1:2000; ab109248), rabbit polyclonal anti-HSP70 antibody (Abcam; 1:8000; ab94368), rabbit polyclonal anti-HSP27 antibody (Abcam 1:1000; ab1428), rabbit polyclonal anti-cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology Japan K.K.; 1:1000; #9661), and mouse monoclonal [6C5] anti-GA-3 phosphate dehydrogenase (anti-GAPDH) antibody (Abcam; 1:10000; ab8245). PVDF membranes were washed four times with 5.0 g/L SM-PBS-T and incubated with secondary antibody at room temperature for 1 h. The secondary antibodies were the following: HRP-conjugated goat anti-rabbit IgG antibody (Dako; 1:2000; REF0448), which was used to analyze GA-AGEs, HRP-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific Inc.; 1:5000; Product Number 31458), and HRP-conjugated donkey anti-rabbit IgG antibody (Thermo Fisher Scientific Inc.; 1:2000; Product Number 31432). Band densities were measured as well as SB. When the detection of HSP90β was performed, proteins on PVDF membranes were probed with a mouse monoclonal [5G4] anti-HSP90β antibody (Abcam; 1:20000; ab119833) and incubated at room temperature for 2 h. Then, proteins on PVDF were incubated in the secondary antibody at room temperature for 1 h. The anti-GAPDH antibody was used after the target antibody was removed by the Western re-probe kit.

Estimation of molecular weight of proteins

The molecular weight (MW) of proteins detected by (WB) was calculated based on a single logarithmic chart used by HRP-MM.

Statistical analysis

Stat Flex (ver. 6) software (Artech Co., Ltd.) was used for statistical analyses. Data are expressed as the mean ± SD. When statistical analyses were performed on the data, except those in the experiment of GA-AGEs-BSA, significant differences in the mean of each group were assessed by a One-Way Analysis of Variance. We then used a Dunnett's test for an analysis of variance. A statistical analysis of the data shown in the experiment of GA-AGEs-BSA was performed using the Student's *t*-test. In each statistical analysis, P values < 0.05 were considered to be significant.

RESULTS

Effects of GA treatment on cell viability and the production of GA-AGEs in PANC-1 cells

We employed the WST-8 assay to examine the viability of PANC-1 cells treated with GA for 24 h. The viability of PANC-1 cells decreased in a GA dose-dependent manner. PANC-1 cell viability was approximately 40% with a 2 mmol/L GA treatment and decreased to almost 0% with a 4 mmol/L GA treatment (Figure 1A). We then measured intracellular GA-AGEs using an SB analysis and detected these products after 24 h. The production of GA-AGEs in PANC-1 cells increased in a GA dose-dependent manner (Figure 1B). Cells treated with 2 and 4 mmol/L GA produced 6.4 and 21.2 μg/mg protein of GA-AGEs, respectively. A large amount of GA-AGEs was produced in cells treated with 4 mmol/L GA. The results of immunostaining using an anti-GA-AGE antibody are consistent with the SB results; namely, the production of GA-AGEs in PANC-1 cells increased in a GA dose-dependent manner (Figure 1C). Moreover, we observed areas lacking cells in 2 and 4 mmol/L GA treatment samples. The area without cells was larger in the samples treated with 4 mmol/L GA than in those treated with 2 mmol/L GA (Figure 1C).

Investigation of GA-AGEs

We performed a WB analysis on GA-AGEs. We compared the bands on PVDF membranes incubated with an anti-GA-AGE antibody and those on PDVF membranes incubated with a neutralized anti-GA-AGE antibody. The bands of GA-AGEs were confirmed and their MWs were analyzed. Bands were clearly observed

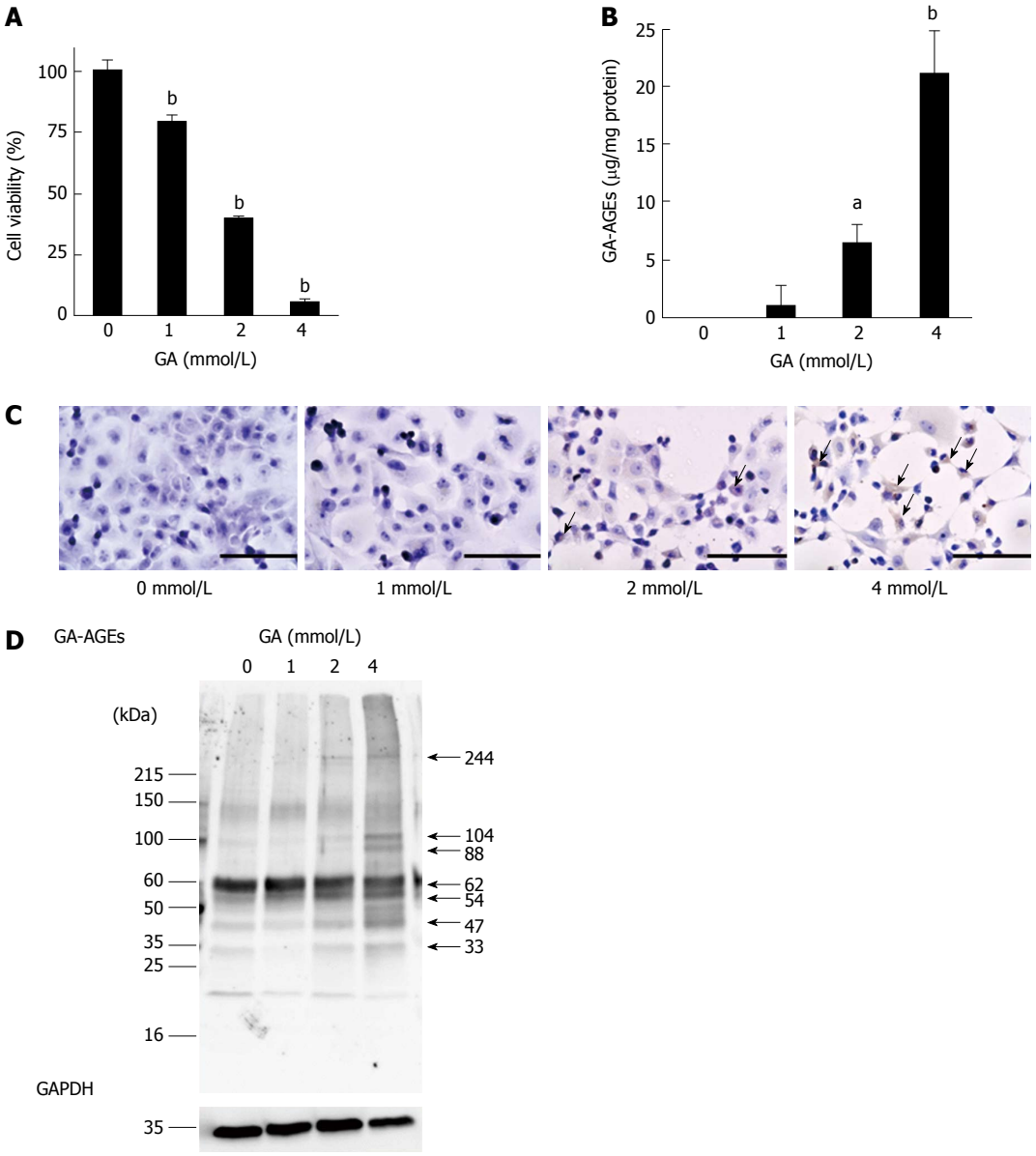

Figure 1 Analysis of cell viability, quantity of glyceraldehyde-derived advanced glycation-end products, immunostaining of glyceraldehyde-derived advanced glycation-end products, and molecular weight of glyceraldehyde-derived advanced glycation-end products in PANC-1 cells treated with glyceraldehyde for 24 h. A: Cell viability was assessed by the WST-8 assay. This assay was performed for three independent experiments. One assay was performed for $n = 7$. Data are shown as mean ± SD ($n = 7$); B: Slot blotting analysis of intracellular glyceraldehyde (GA)-derived advanced glycation-end products (GA-AGEs). Cell lysates (2.0 μg of protein/lane) were blotted onto polyvinylidene difluoride (PVDF) membranes. The amount of GA-AGEs was calculated based on a standard curve for GA-AGEs-BSA. Slot blotting was performed for three independent experiments. Data are shown as mean ± SD ($n = 3$); C: Immunostaining of GA-AGEs in PANC-1 cells. Cells were treated with 0, 1, 2 and 4 mmol/L GA. The arrow indicates the area stained by the anti-GA-AGE antibody. The scale bar represents 200 μm; D: Western blotting analysis of intracellular GA-AGEs in PANC-1 cells. Cell lysates (15 μg of proteins/lane) were loaded on a 40-150 g/L polyacrylamide gradient gel. Proteins on the PVDF membrane were probed with anti-GA-AGE and anti-GA-3-phosphate dehydrogenase (GAPDH) antibodies. The molecular weight of GA-AGEs was calculated based on a single logarithmic chart used by the molecular marker. GAPDH was used as the loading control. WB was performed for two independent experiments. A and B: *P* values were based on Dunnett's test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

at 33, 47, 54, 62, 88, 104, and 244 kDa (Figure 1D and Figure S1). The results of the WB indicated the production of GA-AGEs, and this was supported by the results of SB and immunostaining using an anti-GA-AGE antibody. The density of the GA-AGEs bands appeared to increase in a GA dose-dependent manner.

Effects of GA treatment on HSP90α and HSP90β

Expression levels of HSP90α and HSP90β, which are cell death-associated proteins that suppress the production of cleaved caspase-3 from pro-caspase-3, were analyzed by WB. Expression levels of the monomer HSP90α decreased in a GA dose-dependent manner (Figure 2A, B, and Figure S2), whereas that of the monomer HSP90β did not (Figure 2C, D and Figure S3). We only detected the 173 kDa band of HSP90β in PANC-1 cells treated with GA and the 173 kDa HSP90β/total HSP90β ratio increased in a GA dose-

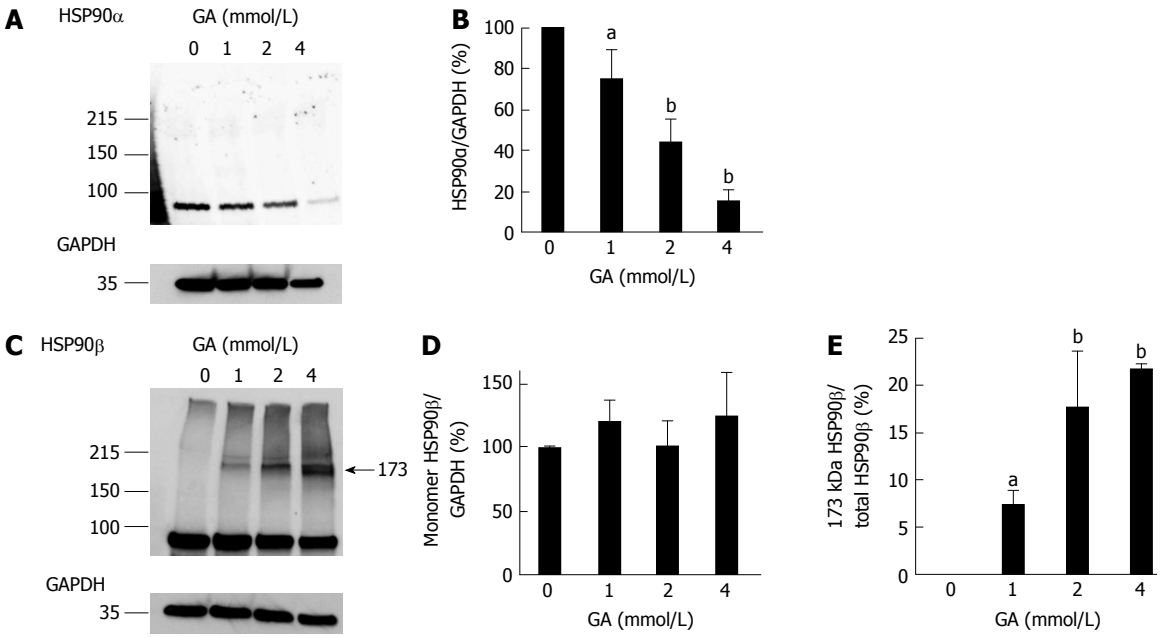

Figure 2 Western blotting analysis of HSP90α and HSP90β. PANC-1 cell lysates (15 μg of proteins/lane) were loaded on a 40-150 g/L polyacrylamide gradient gel. A: Proteins on the polyvinylidene difluoride (PVDF) membrane were probed with anti-HSP90α and anti-GAPDH antibodies; B: Expression levels of HSP90α were normalized with GAPDH; C: Proteins on the PVDF membrane were probed with anti-HSP90β and anti-GAPDH antibodies. A band of 173 kDa HSP90β only appeared in PANC-1 cells treated with GA; D: Expression levels of the monomer HSP90β were normalized with GAPDH; E: The 173 kDa HSP90β/total HSP90β ratio. A and C: Western blotting was performed for three independent experiments. GAPDH was used as the loading control; B, D and E: Data are shown as mean ± SD (n = 3). P values were based on Dunnett's test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. GA: Glyceraldehyde.

dependent manner (Figure 2C and E).

Effects of GA treatment on HSP70 and HSP27

WB analysis of HSP70 and HSP27, which suppress the production of cleaved caspase-3, HSP90α, and HSP90β, was performed. Expression of the monomer HSP70 was not affected by GA treatment, similar to HSP90β expression (Figure 3A, B and Figure S4). Furthermore, 138, 155, 184, and 244 kDa HSP70s were detected in PANC-1 cells treated with GA (Figure 3A, C-F). The ratio of these HSP70 bands to total protein (HSP70/total HSP70) increased in a GA dose-dependent manner (Figure 3A, C-F). However, expression of the monomer HSP27 was only affected by the 4 mmol/L GA treatment, in which it decreased to 20% (Figure 3G, H and Figure S5). The band of 54 kDa HSP27 was only detected in cells treated with GA and the 54 kDa HSP27/total HSP27 ratio increased in a GA dose-dependent manner (Figure 3G and I). The quantity of total HSP27 in cells treated with 4 mmol/L GA was 52% when compared to control cells (Figure 3G).

Effects of GA treatment on cleaved capsase-3

The production of cleaved caspase-3 was analyzed with WB. Two types of Jurkat cell lysates were used to confirm that the anti-cleaved caspase-3 antibody was functioning correctly: one lysate was treated with cytochrome c, whereas the other was not. Cleaved caspase-3 was not detected in any of the lanes (0-4 mmol/L) of PANC-1 cell lysates (Figure 4 and Figure S6).

Proliferation of PANC-1 cells treated with GA-AGEs-BSA

PANC-1 cells were treated with 10 and 20 μg/mL of the non-glycated control BSA and GA-AGEs-BSA and then incubated for 24 h. Cell proliferation was analyzed using the WST-8 assay. The proliferation of PANC-1 cells treated with 10 and 20 μg/mL GA-AGEs-BSA was 27% and 34% greater than that of control cells, respectively (Figure 5).

DISCUSSION

PDAC accounts for approximately 90% of pancreatic malignancies[3]. Another characteristic of pancreatic cancer is its association with T2DM, which increases the risk of pancreatic cancer by more than 50%[5,6]. However, the mechanisms through which T2DM promotes pancreatic cancer currently remain unknown. We consider PDAC to be one of the lifestyle-related diseases associated with T2DM. Therefore, we hypothesized that hyperglycemia, a characteristic of T2DM, contributes to the promotion of PDAC. Because hyperglycemia induces the production of AGEs, we herein investigated if AGEs promote PDAC. However, it is important to note that not all AGEs promote PDAC. GA-AGEs generated from GA (glucose and fructose metabolites) have been implicated in the pathogeneses of various diseases such as insulin resistance, hypertension, diabetic complications, dementia, cardiovascular diseases, non-alcoholic steatohepatitis, Alzheimer's disease, and cancer[7-12]. Therefore, we

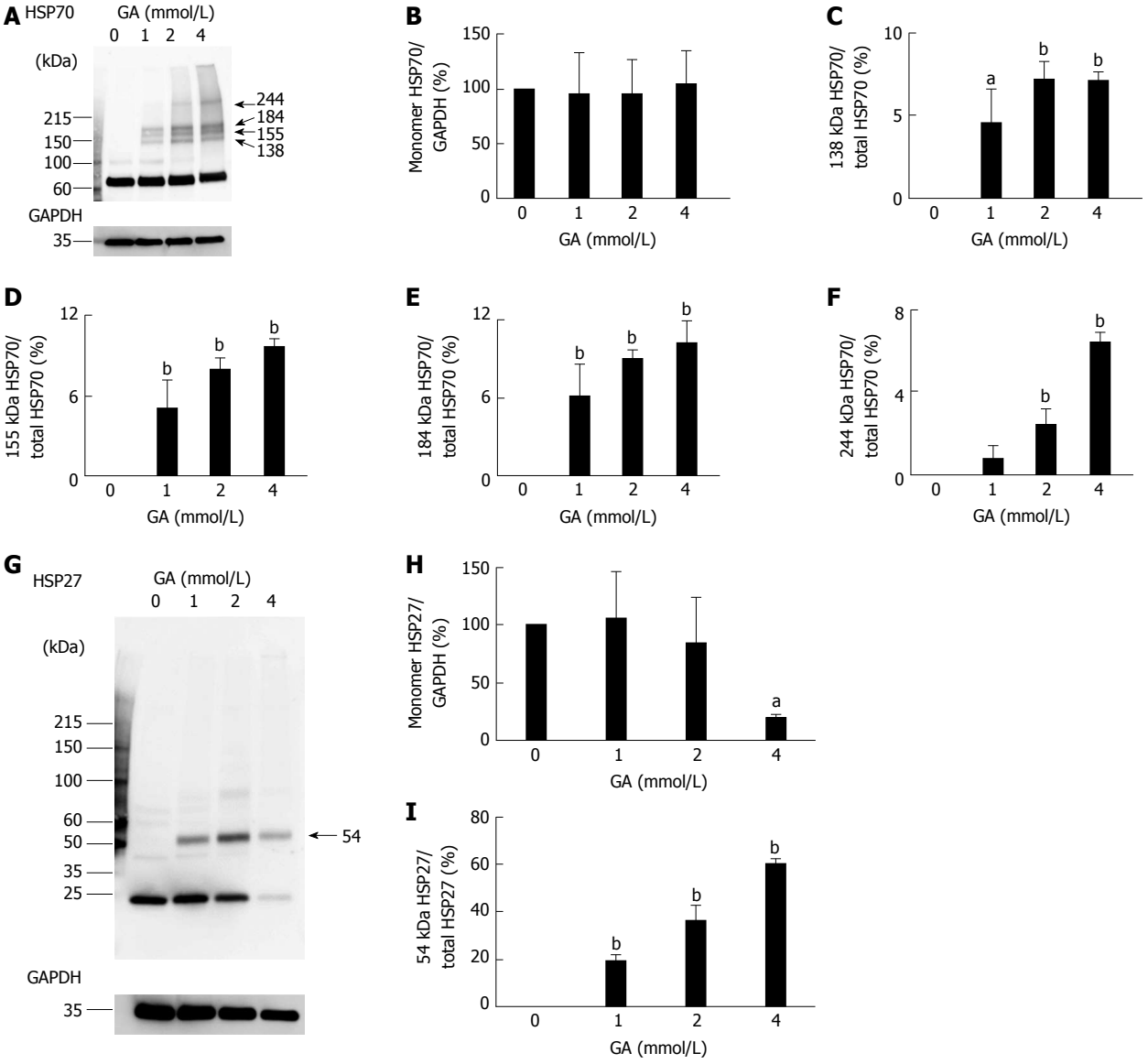

Figure 3 Western blotting analysis of HSP70 and HSP27. PANC-1 cell lysates (15 μg of proteins/lane) were loaded on a 40-150 g/L polyacrylamide gradient gel. A: Proteins on the polyvinylidene difluoride (PVDF) membrane were probed with anti-HSP70 and anti-GA-3 phosphate dehydrogenase (anti-GAPDH) antibodies. We found four high-molecular-weight (HMW) complexes of HSP70s only in PANC-1 cells treated with GA. Their MWs were 138, 155, 184 and 244 kDa; B: Expression levels of the monomer HSP70 were normalized with GAPDH; C-F: The 138 kDa HSP70/total HSP70, 155 kDa HSP70/total HSP70, 184 kDa HSP70/total HSP70, and 244 kDa HSP70/total HSP70 ratios; G: Proteins on the PVDF membrane were probed with anti-HSP27 and anti-GAPDH antibodies. A HMW complex with a MW of 54 kDa appeared only in PANC-1 cells treated with GA; H: Expression levels of the monomer HSP27 were normalized with GAPDH; I: The 54 kDa HSP27/total HSP27 ratio; A and G: WB was performed for three independent experiments. GAPDH was used as a loading control. B-F, H, I: Data are shown as mean ± SD (n = 3). P values were based on Dunnett's test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. GA: Glyceraldehyde.

designated these GA-AGEs as TAGE[7-12]. In the present study, we hypothesized that GA-AGEs contribute to the development of PDAC.

To prove this hypothesis, PANC-1 cells were incubated in high glucose medium, a model of T2DM blood, and treated with GA. We speculated that GA promotes the proliferation of PANC-1 cells. But our results indicated cell viability decreases in a GA dose-dependent manner (Figure 1A). In contrast, SB analysis revealed that the production of GA-AGEs in cells increased in a GA dose-dependent manner (Figure 1B), results that were supported by those obtained using immunostaining (Figure 1C). Our results indicated that cell death is induced by the production of GA-AGEs. Using WB, which was performed using an anti-GA-AGE antibody, we detected 7 clear bands (Figure 1D). Because the production of GA-AGEs induced cell death, we investigated cell death-associated proteins in PANC-1 cells. These proteins were selected from those with MWs of 33, 47, 54, 62, 88, 104, and 244 kDa, which represent bands that were detected by WB analysis with an anti-GA-AGE antibody (Figure 1D). As a result, HSP90 was selected as a candidate cell death-associated protein. This protein has been identified as an apoptosis-associated HSP in PANC-1 cells[14,15]. A previous study reported

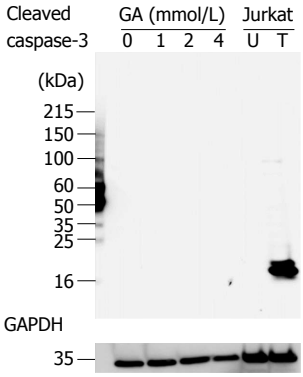

Figure 4 Western blotting analysis of cleaved caspase-3. PANC-1 cell lysates (15 μg of protein/lane) and Jurkat cell lysates (10 μg of protein/lane) were loaded on a 40-150 g/L polyacrylamide gradient gel. Proteins on the PVDF membrane were probed with anti-cleaved caspase-3 and anti-GA-3 phosphate dehydrogenase (anti-GAPDH) antibodies. U: The lysate of Jurkat cells not treated with cytochrome c. T: The lysate of Jurkat cells treated with cytochrome c. Western blotting was performed for three independent experiments. GAPDH was used as a loading control. GA: Glyceraldehyde.

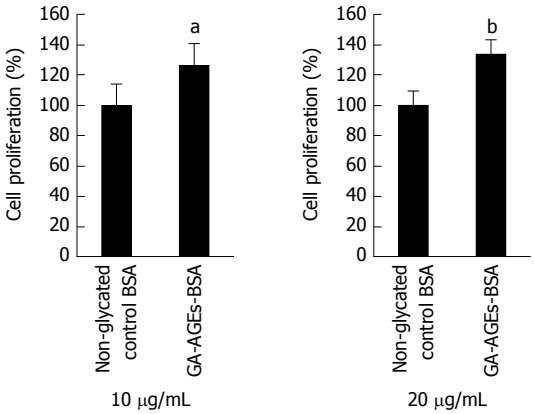

Figure 5 Analysis of the proliferation of PANC-1 cells treated with glyceraldehyde-derived advanced glycation-end products-bovine serum albumin. PANC-1 cells were treated with 10 and 20 μg/mL of non-glycated control BSA and glyceraldehyde-derived advanced glycation-end products-bovine serum albumin (GA-AGEs-BSA) to then be incubated for 24 h. This assay was performed for two independent experiments. One assay was performed for $n = 7$. Cell proliferation was assessed by the WST-8 assay. Data are shown as mean ± SD ($n = 7$). P values were based on the Student's t-test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

that GA-AGEs were generated from heat shock cognate 70 (Hsc70), which belongs to the HSP family, following treatment with GA in the human hepatic cancer cell line, Hep3B[16]. Apoptosis was induced in Hep3B cells because GA-AGEs induced the formation of Hcs70 aggregates, and therefore failed to exert its normal functions.

Furthermore, AGEs were shown to be produced from HSP27 by methylglyoxal, one of the by-products of glycolysis[17-19]. HSP90, HSP70, and HSP27 are important proteins in cell death because they suppress the production of cleaved caspase-3 from pro-caspase-3 in PANC-1 cells[14,15,20,21]. Because bands at 70 and 27 kDa were not clearly detected by WB with an anti-GA-AGE antibody (Figure 1D), we only targeted HSP90.

WB results on HSP90α revealed that band density decreased in a GA dose-dependent manner (Figure 2A and B). This result generally suggests a decrease in the expression or increase in the degradation of HSP90α; however, another possibility is that the GA derivative of HSP90α was generated and the epitope for the anti-HSP90α antibody lost its function.

However, the expression of the monomer HSP90β, an isomer of HSP90α[22], was not affected; furthermore, the 173 kDa HSP90β was detected and increased in a GA dose-dependent manner (Figure 2C-E). We were unable to confirm GA-induced modifications in the monomer HSP90β, but predicted that the 173 kDa HSP90β is a GA-AGEs-mediated homodimer. If two HSP90βs combine with GA-derived modifications, their MW is expected to be approximately 176 kDa, which is close to 173 kDa. Because we confirmed the presence of a 173 kDa HSP90β, we hypothesized that the HMW complexes with GA-derived modifications in HSP70 and HSP27 (*e.g.*, GA-AGEs-mediated dimers, trimers, and tetramers) may be generated and contain GA-AGEs detectable by WB (Figure 1D). These HMW complexes were generated in PANC-1 cells treated with GA (Figure 3A, C-G, I). We considered 138, 155, 184, and 244 kDa HSP70s to be the dimers and trimers containing the homo and hetero types with GA-AGEs-mediated aggregation. The possibility of 244 kDa HSP70 being GA-AGEs is greater than that of another HMW complexes of HSP70s because a clear band for 244 kDa GA-AGEs was detected in the WB analysis (Figure 1D).

Because a 54 kDa band was clearly detected by WB analysis using an anti-GA-AGE antibody, the 54 kDa HSP27 was likely to be a GA-AGE (Figure 1D). Moreover, we predict that 54 kDa HSP27 is a homodimer with GA-AGEs-mediated aggregation because its MW was twice that of a monomer. Monomers and total HSP27 only decreased with the 4 mmol/L GA treatment (Figures 3G-I). The production of GA-AGEs in PANC-1 cells treated with 4 mmol/L GA were greater than that seen with any other GA dose, and most cells died. A decrease in monomers and total HSP27 may have been induced due to the abnormal cellular conditions caused by the excessive amount of GA-AGEs. Previous studies identified a number of different routes for cell death in PANC-1 cells including apoptosis, necrosis, and autophagy[23-25]. HSP90, HSP70, and HSP27 regulate apoptosis by suppressing the production of cleaved caspase-3[14,15,20,21]. We were interested in the decrease in the normal monomer HSP90α and increase in the HMW complex of HSP90β, HSP70, and HSP27 after GA treatment. Although we speculated an increase in the production of cleaved caspase-3, this was not observed in PANC-1 cells, which generated intracellular GA-AGEs (Figure 4).

PANC-1 cells that produced GA-AGEs may have undergone a non-apoptotic form of cell death. Cell death may induce the release of intracellular GA-AGEs into conditioned medium. Live cells may also secrete

GA-AGEs. After analyzing intracellular GA-AGEs and proteins associated with cell death, we hypothesized that extracellular GA-AGEs promote the growth of pancreatic cancer cells. The proliferation of PANC-1 cells was promoted by both 10 and 20 μg/mL of GA-AGEs-BSA, a model of secreted or released GA-AGEs (Figure 5). This phenomenon may also be associated with the receptor for AGEs (RAGE) and induced *via* the GA-AGEs-RAGE system. Several reports have already been published on the proliferation of Hep3B, HepG2, IL90, and HuH7 cells, which are derived from human liver cancer cell lines, treated with GA-AGEs[26-28]. Although GA-AGEs-BSA did not promote the proliferation of Hep3B and HepG2 cells, it may promote the proliferation of IL90 and HuH7 cells. RAGE was previously shown to be weakly expressed in Hep3B and HepG2 cells[26]; therefore, the induction of proliferation did not appear to be induced *via* the GA-AGEs-RAGE system. On the other hand, high expression levels of RAGE in IL90 cells have already been demonstrated by WB[27]. RAGE was found to be weakly expressed in HuH7 and HepG2 cells; however, RAGE expression levels on the membrane of HuH7 cells were approximately 4-fold those of HepG2 cells[28]. RAGE expression levels on HuH7 cell membranes may be sufficient to promote cell proliferation through the GA-AGEs-RAGE system.

RAGE has been detected in human pancreatic cancer cell lines[29,30]. WB revealed high expression levels of RAGE in PANC-1 and MIA-PaCa-2 cells, and low levels in BxPC-3 cells[30]. GA-AGEs-BSA may promote the proliferation of PANC-1 as well as IL90 and HuH7 cells through the GA-AGEs-RAGE system.

In conclusion, intracellular and extracellular GA-AGEs induced PANC-1 cell death and proliferation, respectively. This suggests that, although intracellular GA-AGEs induce pancreatic cancer cell death, their secretion and release may induce the proliferation of other pancreatic cancer cells.

In this investigation, we did not examine GA-AGEs; however, we consider the monomer HSP90α, monomer HSP90β, and HMW complexes of HSPs to be GA-AGEs. However, two recent studies identified GA-AGEs generated by Hsc70 and heterogeneous nuclear ribonucleoprotein M in Hep3B cells treated with GA or fructose[16,31].

If the monomers and aggregates of HSPs are identified as GA-AGEs in the future, this indicates that GA-AGEs were first identified in human pancreatic ductal carcinoma cells. Identifying intracellular GA-AGEs will reveal the mechanism of cell death. In addition, GA-AGEs secreted or released into the conditioned medium of cultured PANC-1 cells, which generate intracellular GA-AGEs, may demonstrate that extracellular GA-AGEs promote cancer. If the mechanism through which T2DM promotes PDAC relies on GA-AGEs as key factors, the current research on drug therapy for PDAC may change[1,32].

COMMENTS

Background

Pancreatic cancer is a highly lethal disease and pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 90% of malignancies in the pancreas. On the other hand, the incidence of diabetes mellitus (DM) is increasing worldwide each year, and this condition may result in lifestyle-changing complications. Type 2 DM (T2DM) has been shown to increase the risk of pancreatic cancer by more than 50%. However, the mechanism promoting PDAC in the pancreas of T2DM patients has not yet been elucidated.

Research frontiers

The authors focused on hyperglycemia and how it induces the production of advanced glycation-end (AGEs). AGEs are formed by a Maillard reaction, a nonenzymatic reaction that occurs between the ketones or aldehyde of sugars and the amino group of proteins. The authors have previously identified AGEs derived from glyceraldehyde (GA-AGEs); therefore, they designated GA-AGEs as toxic AGEs (TAGE) because of their cytotoxicity and involvement lifestyle-related diseases. In this study, the authors examined the production of intracellular GA-AGEs in the human pancreatic cancer cell lines, PANC-1, and the proliferation of PANC-1 cells treated with GA-AGEs-bovine serum albumin (GA-AGEs-BSA).

Innovations and breakthroughs

This study reported the production of GA-AGEs in PANC-1 cells treated with GA, which resulted in cell death. HSP90β, HSP70, and HSP27, which are cell-death associated proteins, generated HMW complexes that could predict GA-AGEs-mediated aggregation. Moreover, GA-AGEs-BSA, a model of extracellular GA-AGEs, promoted proliferation of PACN-1 cells.

Applications

The experimental data can be used to analyze the relationship between T2DM and PDAC *via* the production of GA-AGEs. Studying the production and effects of intracellular and extracellular GA-AGEs in pancreatic cancer cells can be used in further investigation to develop PDAC therapies.

Terminology

AGEs derived from GA, a glucose/fructose metabolism intermediate, also known as GA-AGEs. They designated GA-AGEs as TAGE because of their cytotoxicity and involvement in insulin resistance, hypertension, diabetic complications, cardiovascular diseases, dementia, non-alcoholic steatohepatitis, Alzheimer's disease, and cancer.

Peer-review

The manuscript is well written, the study was conducted properly, and the conclusion is supported by the data presented.

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ORIGINAL ARTICLE

Basic Study

Anti-oxidant and anti-inflammatory effects of hydrogen-rich water alleviate ethanol-induced fatty liver in mice

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Abstract

AIM

To investigate the effects of hydrogen-rich water (HRW) treatment on prevention of ethanol (EtOH)-induced early fatty liver in mice.

METHODS

In vitro reduction of hydrogen peroxide by HRW was determined with a chemiluminescence system. Female mice were randomly divided into five groups: control, EtOH, EtOH + silymarin, EtOH + HRW and EtOH + silymarin + HRW. Each group was fed a Lieber-DeCarli liquid diet containing EtOH or isocaloric maltose dextrin (control diet). Silymarin was used as a positive control to compare HRW efficacy against chronic EtOH-induced hepatotoxicity. HRW was freshly prepared and given at a dosage of 1.2 mL/mouse trice daily. Blood and liver tissue were collected after chronic-binge liquid-diet

feeding for 12 wk.

RESULTS

The *in vitro* study showed that HRW directly scavenged hydrogen peroxide. The *in vivo* study showed that HRW increased expression of acyl ghrelin, which was correlated with food intake. HRW treatment significantly reduced EtOH-induced increases in serum alanine aminotransferase, aspartate aminotransferase, triglycerol and total cholesterol levels, hepatic lipid accumulation and inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6. HRW attenuated malondialdehyde level, restored glutathione depletion and increased superoxide dismutase, glutathione peroxidase and catalase activities in the liver. Moreover, HRW reduced TNF-α and IL-6 levels but increased IL-10 and IL-22 levels.

CONCLUSION

HRW protects against chronic EtOH-induced liver injury, possibly by inducing acyl ghrelin to suppress the pro-inflammatory cytokines TNF-α and IL-6 and induce IL-10 and IL-22, thus activating antioxidant enzymes against oxidative stress.

Key words: Hydrogen; Chronic plus binge EtOH feeding; Antioxidant; Protective cytokine; Acyl ghrelin; Female mice

Core tip: Hydrogen-rich water (HRW), a safe and effective antioxidant with minimal side effects, is used in preventive and clinical applications. Few studies have investigated the effects of hydrogen on early alcoholic liver disease. The present study evaluated the potential protective effects of HRW against chronic ethanol (EtOH)-induced early liver injury and the underlying mechanisms in female mice after chronic-plus-binge EtOH feeding. HRW pretreatment protected against mild EtOH-induced liver injury, possibly by inducing acyl ghrelin to suppress tumor necrosis factor-alpha and interleukin (IL)-6 and induce IL-10 and IL-22, thereby activating antioxidant enzymes against oxidative stress. These results suggest that HRW helps prevent and treat EtOH-induced early liver injury.

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INTRODUCTION

Sustained excessive alcohol consumption results in a spectrum of liver injury, from hepatic steatosis to hepatitis, fibrosis and cirrhosis, which can ultimately lead to hepatocellular carcinoma[1-4]. Among heavy drinkers, the incidence of hepatic steatosis is about 95%. Risk factors potentially associated with alcoholic liver disease (ALD) include sex, obesity, dietary factors, smoking and non-sex-linked genetic factors. Among humans and rodents, females are more susceptible to ALD, even if they consume less alcohol than males. This may be attributable to lower gastric alcohol dehydrogenase activity, lower distributed volume of alcohol and estrogen, which has a substantial effect on alcohol-induced hepatotoxicity[5-7].

ALD pathogenesis is mediated by increased steatosis, inflammatory factors, oxidative stress and immune responses. Ethanol (EtOH) impairs antioxidant defenses and mitochondrial functions and may trigger a burst of reactive oxygen species (ROS), thus resulting in hepatotoxicity, steatosis, inflammation and fibrosis[1,2]. ROS can also induce hepatocellular responses strongly associated with Kupffer cell activation, which increases inflammatory response and leads to liver injury. Moreover, activated Kupffer cells release ROS and cytokines that are crucial in activating hepatic stellate cells (HSCs) and inducing the pro-fibrogenic pathway[1,2].

Previous studies reported that oxidative stress and sensitization to endotoxins contributing to EtOH-induced liver injury are associated with release of pro-inflammatory mediators, promotion of lipid peroxidation and impaired hepatic antioxidant defense[1,2,8]. Activation of pro-inflammatory cytokines, particularly nitric oxide synthase, cyclooxygenase 2, transcription factor nuclear factor-κB, tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 is crucial in ALD progression, which leads to hepatocellular injury and death[1-3,6,9].

IL-22, a novel hepatoprotective factor, is a member of the IL-10 family of cytokines and appears to be an important effector molecule of activated T cells and natural killer cells. The main biological roles of IL-22 are to promote innate immunity, improve regeneration and protect against damage[10]. Evidence from several studies suggests that IL-22 - through antioxidant and anti-apoptotic pathways - exerts protective effects against hepatic injury induced by concanavalin A[11], carbon tetrachloride (CCl_4)[12] and EtOH[3]. Ghrelin, a 28-amino acid peptide produced in gastric mucosa, acts in the hypothalamus to promote appetite and inhibit sympathetic activity, thus increasing food intake while lowering metabolic rate[13,14]. Recent studies suggest that ghrelin has various biological functions, including anti-oxidation, anti-inflammation, anti-autoimmunity and promotion of vascular health[13-16].

Because of its effective scavenging of ROS, molecular hydrogen (H_2) has potent systemic antioxidant activity[17,18]. Approaches to administering H_2 include inhalation, injection, oral administration and immersion. Oral administration of H_2-rich water (HRW)

was easier, safer and more economical as a means to protect against oxidative stress-induced injury in multiple animal models of human diseases. H_2 was successfully used in a number of *in vivo* studies of hepatic injury, which examined conditions such as ischemia reperfusion injury, obstructive jaundice, acute hepatic failure and nonalcoholic steatohepatitis[19-25].

Our previous research indicated that electrolyzed reduced water and silica hydride, which contains H_2, ameliorated CCl_4-induced hepatotoxicity in mice by enhancing antioxidant enzyme activity and reducing lipid oxidation[26,27]. Additionally, consumption of more than 20 mL/kg per day of HRW had no observable adverse effects, which suggests a 60-kg human could safely drink at least 1.2 L/d of HRW[28]. Thus, HRW could be used in preventive and clinical applications as a safe and effective antioxidant with minimal side effects[17-21,23,28-31]. Recent clinical studies found that HRW reduced oxidative stress in persons with chronic hepatitis B[32] and metabolic syndrome[31].

The effects of H_2 on chronic EtOH-induced liver injury are not well understood. The Lieber-DeCarli liquid diet has been extensively used as the typical approach to establish a chronic-plus-binge EtOH feeding model that mimics some of the molecular and histological features of mild, early-stage human ALD[3,33,34]. This model induces mild steatosis and elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in C57BL/6 mice. The elevations are much more severe than those seen in mice on chronic EtOH or single EtOH gavage alone diets. This study investigated the potential protective effects of HRW against chronic EtOH-induced early-stage liver injury and the underlying mechanisms of such effects in female mice after chronic-plus-binge EtOH feeding.

MATERIALS AND METHODS

Production of H_2-rich water

HRW was prepared by inducing a chemical reaction between metallic magnesium and water [$Mg + 2H_2O/Mg(OH)_2 + H_2$][35]. The Daily Inner T505 Hydrogenerator apparatus (Unitiva Applied Materials Corp., Taipei, Taiwan) was used to generate HRW. Briefly, to produce HRW, a metallic magnesium stick (T505, 175 g Mg Chips) containing 99.99% pure metallic magnesium in a polypropylene and ceramic container was placed in distilled water with a flow rate set at 400 mL/min. The resulting H_2 content was 500-600 parts per billion (ppb). HRW was freshly prepared and immediately diluted to pre-specified concentrations for use *in vitro* and *in vivo*.

Characterization of H_2-rich water

HRW was analyzed by multiple devices. To analyze H_2 content and oxidation-reduction potential (ORP), HRW was freshly prepared in a capped vial and immediately measured with a dissolved H_2 portable meter (ENH-1000; Trustlex Co., Ltd, Tokyo, Japan) and ORP portable meter (MP220; Mettler Toledo, Zurich, Switzerland). Analysis of free radical scavenging activity was performed by modifying previously described methods[35,36]. In brief, a mixture of 0.1 mL of H_2O_2 solution (97 mmol/L in distilled water) and 0.4 mL of sample was loaded in the stainless steel container of a chemiluminescence analysis system (CLA-2100; Tohoku Electronic Co., Ltd, Sendai, Japan) for 60 s. Next, 0.1 mL of luminol solution (3 mmol/L in phosphate-buffered saline, pH 7.4) was immediately injected into the dark chamber of the chemiluminescence analyzer. Then, chemiluminescence intensity was continuously recorded for 120 s. Scavenging activity (%) was defined as [(Sum1 - Sum2)/Sum1] × 100%.

Animals

All procedures involving animals were reviewed by the Institutional Animal Care and Use Committee of Chung-Shan Medical University Experimental Animal Center (IACUC approval no. 1745). Female C57BL/6 mice (age 5 wk) were purchased from BioLasco Taiwan (Ilan, Taiwan) and acclimatized to the environment for 1 wk. All mice were handled under standard laboratory conditions (temperature 22 ± 2 ℃, humidity 55% ± 5% and 12-h light-dark cycle). Then, mice were allowed *ad libitum* access to a controlled Lieber-DeCarli diet for 1 wk, to acclimatize to the liquid diet before the experiment. The liquid diets provided 1 kcal/mL (prepared by Dyets, Inc., Bethlehem, PA, United States), in accordance with the Lieber-DeCarli formulation. This nutritional diet (containing 41.4 g/L casein, 0.5 g/L L-cystine, 0.3 g/L DL-methionine, 8.5 g/L corn oil, 28.4 g/L olive oil, 2.7 g/L safflower oil, 115.2 g/L maltose dextrin, 10 g/L cellulose, 8.75 g/L mineral mix, 2.5 g/L vitamin mix, 0.53 g/L choline bitartrate and 3 g/L xanthan gum) allowed for the prolonged exposure of EtOH in a rodent model and allowed for modification to calories provided by EtOH[2,3,5,33,34].

Mice in the present study were assigned to 5 groups (n = 8-10 each), as follows: (1) control group - mice receiving a controlled liquid diet and gavaged with distilled water; (2) EtOH group-mice receiving 5% EtOH (v/v) containing a liquid diet and gavaged with distilled water; (3) EtOH + silymarin group-mice receiving an EtOH diet and gavaged with silymarin (200 mg/kg); (4) EtOH + HRW group-mice receiving an EtOH diet and gavaged with HRW; and (5) EtOH + silymarin (200 mg/kg) + HRW group-mice receiving an EtOH diet and gavaged with silymarin and HRW.

Experimental design

Chronic and binge EtOH feeding was carried out by modifying a previously described protocol[33,34]. Both liquid diets were freshly prepared daily. HRW was orally administered (500 ppb, 1.2 mL/mouse) thrice daily for 13 continuous weeks. After 1-wk pretreatment with HRW, all mice (except for the control group)

underwent a 2-wk acclimation to their modified liquid diets; specifically, the EtOH content in the diet was graded from 7.2% to 36% of energy composition. All mice, including the control diet group, were regularly fed their assigned diets *ad libitum* for 10 wk. Subsequently, mice in the EtOH groups were gavaged with a single dose of EtOH (5 g/kg) and mice in the control group were gavaged with an isocaloric amount of dextrin maltose in the early morning. Nine hour after gavage, the mice were euthanized by CO_2 administration and blood was collected by caudal vena cava sampling. The whole liver was excised and washed immediately with ice-cold saline, to remove residual blood before weighing. The largest right lobe of each liver was fixed in 10% buffered formalin for histopathological assessment and the remaining tissues were immediately frozen at -80 ℃ for subsequent analysis.

Measurements of acyl ghrelin concentrations

After collection of whole blood, 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF) was immediately added to achieve a final concentration of 1 mg/mL for 30 min at room temperature, after which the sample was centrifuged (4 ℃, 2000 × *g*, 15 min). Next, serum was acidified with HCl to a final concentration of 0.1 N and samples were frozen at -80 ℃ for further analysis. The acyl form of ghrelin was measured in serum by the Active Ghrelin Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Millipore, MA, United States), according to the manufacturer's protocols.

Measurement of serum biochemistry parameters

To evaluate hepatic injury, levels of ALT, AST, triacylglycerol (TG) and total cholesterol (TC) were measured with commercial kits (Randox Laboratories Ltd, Antrim, United Kingdom), according to the assay protocol.

Hepatic histopathological assessment

Liver specimens were fixed in 10% neutral buffered formalin and embedded in paraffin, using standard microtechniques. Then, 4-μm-thick liver sections were stained with hematoxylin and eosin and observed with a microscope (IX71S8F-2; Olympus, Tokyo, Japan), to estimate fatty liver progression in hepatocytes. The semi-quantitative histological assessment of hepatic damage was graded 0-4, as follows: none (0), slight (1), mild (2), moderate (3) and severe (4).

Measurement of hepatic lipid accumulation

Extraction of hepatic lipids was performed by using the method of Folch *et al*[37], with some modifications. Then, 100 mg of liver tissue was homogenized in chloroform/methanol (v/v: 1/2). Next, chloroform and distilled water (v/v: 1/1) were loaded and mixed thoroughly. After centrifugation (1500 × *g*, 10 min), the organic layer was removed, placed in another glass tube and dried under nitrogen gas. The dried powder was dissolved in phosphate-buffered saline containing 1% Triton X-100. TG and TC content were measured with a commercial kit (Randox Laboratories Ltd).

Measurement of cytokine profiles in liver tissue and serum

For measurement of cytokine profiles, 100 mg of liver tissue was homogenized on ice with RIPA buffer (50 mmol/L Tris,150 mmol/L NaCl,1% Triton, 5 mmol/L EDTA, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor. After centrifugation (12000 × *g*, 10 min, 4 ℃), TNF-α and IL-6 content in serum supernatant was determined with ELISA kits (Enzo Life Science Inc., Farmingdale, NY, United States), according to the manufacturer's protocols. Tissue values were normalized to tissue wet weight. Serum IL-10 and IL-22 levels were determined with ELISA kits (Elabscience, Hubei, China), according to the manufacturer's protocols.

Measurement of hepatic antioxidant enzymes and lipid peroxidation

Livers were homogenized on ice with Tris-HCl (5 mmol/L, pH 7.4) containing 2 mmol/L EDTA. After centrifugation (10000 × *g*, 10 min, 4 ℃), the supernatant was immediately stored at -80 ℃ for additional antioxidant assays. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were determined by enzymatic assay kits (RANSOD and RANSEL, respectively; Randox Laboratories Ltd), according to the manufacturer's protocols. Catalase (CAT) activity was determined with the method proposed by Aebi[38]. The above antioxidant activities were normalized to hepatic total protein. GSH content was determined with a colorimetric assay kit (Bioxytech GSH-400; OXIS International Inc., Portland, OR, United States), according to the manufacturer's protocol.

Measurement of hepatic lipid peroxidation

Malondialdehyde (MDA) is a marker of lipid peroxidation. MDA reaction with thiobarbituric acid was determined by the method of Buege *et al*[39], with some modifications. In brief, deproteinized homogenates from liver were mixed thoroughly with 0.67% thiobarbituric acid in a 50% acetic acid solution and then placed in a boiling-water bath for 60 min. The supernatant was collected and measured at excitation/emission wave lengths of 515 nm and 555 nm in a microplate reader (Molecular Devices Flexstation 3; Molecular Devices, LLC, Sunnyvale, CA, United States).

Statistical analysis

Measurement data are expressed as mean ± SD and differences between groups were analyzed with the unpaired *t*-test. Associations between variables were assessed by the Spearman correlation test. All statistical analyses were performed using SPSS 12.0 software (SPSS Inc. IBM, Chicago, IL, United

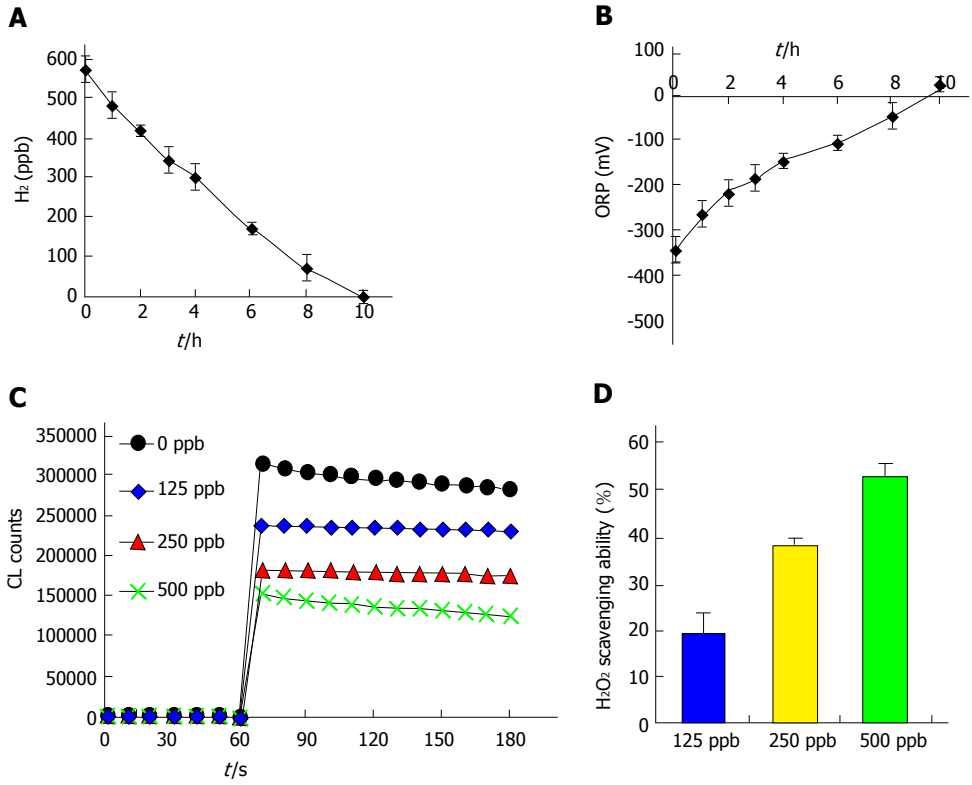

Figure 1 Scavenging of hydrogen peroxide by hydrogen-rich water *in vitro*. A: The hydrogen content in HRW displayed a time-dependent decline and reach zero at 10 h when exposed to air; B: Accordingly, the values of ORP also rapidly returned to baseline at 10 h; C: Scavenging ROS ability measured with chemiluminescence emission by luminol. Values for chemiluminescence intensity were showed as counts for every 10 s in 180 s; D: ROS scavenging ability was calculated as averages from 60 to 180 s using the area under the curve. Values are expressed as means ± SD of three independent experiments. HRW: Hydrogen-rich water; ORP: Oxidation-reduction potential; ROS: Reactive oxygen species.

States). When appropriate, $P < 0.05$ was considered statistically significant.

RESULTS

H₂-rich water directly scavenged H₂O₂ in vitro

To characterize H_2 solubility in water, a specific electrode was used to detect H_2 concentrations in freshly prepared HRW. Dissolved H_2 concentration decreased from the baseline (freshly prepared) concentration to undetectable in 10 h (Figure 1A). ORP is generally used as a measure of the antioxidant activity of a water sample. The present results show that HRW had a negative ORP and that ORP became positive at the same time that H_2 content became undetectable in water (Figure 1B).

H_2 markedly reduces ROS. Chemiluminescence emission *in vitro* was used to verify that HRW scavenged ROS in the present study. H_2O_2-generated free radicals were significantly and dose-dependently decreased by HRW treatment (125-500 ppb; approximately 7.5% to 30% saturation; Figure 1C). ROS scavenging ability was converted to the integral of the area under the curve. At concentrations of 125, 250 and 500 ppb, HRW treatment enhanced scavenging ability by 19.8%, 38.7% and 52.7%, respectively, as compared with the control group (Figure 1D). The presence of ROS scavenging ability *in vitro* suggests that HRW has hepatoprotective potential for *in vivo* EtOH-induced oxidative stress.

Effects of H₂-rich water on food intake, acyl ghrelin, body weight and liver weight in chronic-binge ethanol-fed C57BL/6J mice

To investigate the hepatoprotective potential of HRW *in vivo*, C57BL/6 mice were subjected to a chronic-plus-binge EtOH feeding model. Silymarin (200 mg/kg) was used as a positive control. The control and EtOH groups significantly differed in daily food intake ($P < 0.05$; Figure 2A). Silymarin, HRW and combination treatment significantly reversed the hypophagic effect induced by EtOH ($P < 0.01$), which indicates that HRW reversed EtOH-induced anorexia. After 12 wk of EtOH exposure, serum was collected for analysis of acyl ghrelin. The control and EtOH groups significantly differed in acyl ghrelin expression ($P < 0.001$; Figure 2B). Acyl ghrelin expression was significantly higher in the HRW and combination treatment groups than in the EtOH group ($P < 0.001$).

During the course of the experiment, body weight was lower in the EtOH group than in the control diet group (Supplementary Figure 1). Silymarin, HRW and combination treatment slightly restored body weight, especially from week 4 until week 10. The values for relative body weight gain among groups after 13 wk of feeding were 29%, 26.6% 28.5%, 25.4%

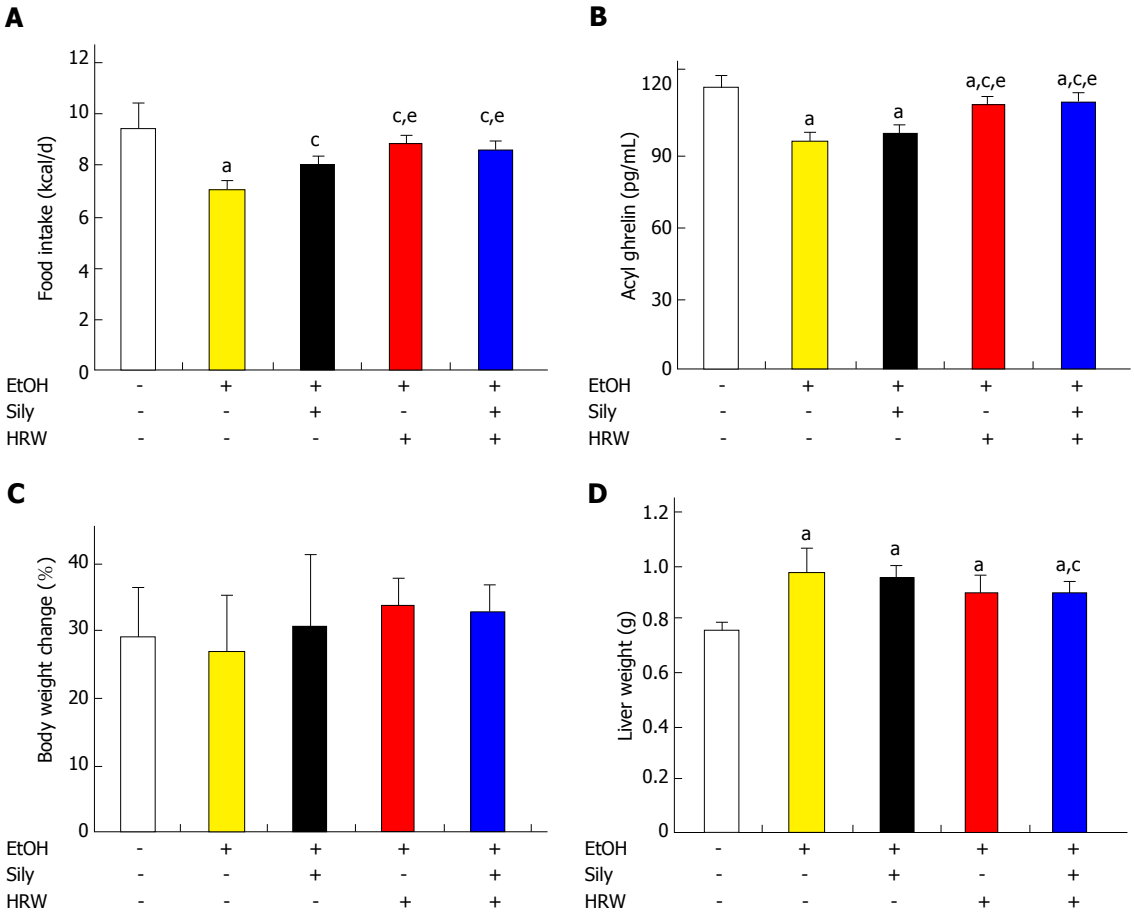

Figure 2 Effect of hydrogen-rich water on food intake, acyl ghrelin, body weight and liver weight in chronic-binge ethanol-fed C57BL/6J mice. The levels of acyl ghrelin in serum were measured. The values are the mean ± SD from 8-10 mice for each group and at least three independent measurements. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* EtOH group, and $^{e}P < 0.05$ *vs* silymarin group. EtOH: Ethanol; HRW: Hydrogen-rich water; Sily: Silymarin.

Table 1 Effects of hydrogen-rich water on the serum biochemical parameters and hepatic lipid contents in ethanol-fed C57BL/6J mice

Group	Control	EtOH	EtOH + Sily	EtOH + HRW	EtOH + Sily + HRW
ALT, U/L	43.4 ± 17.2	159 ± 16.9[a]	133.9 ± 15.3[a,c]	140.7 ± 15.5[a,c]	120.5 ± 19.7[a,c]
AST, U/L	98.4 ± 10.9	291 ± 43.7[a]	247.7 ± 20[a,c]	259.3 ± 10.9[a]	181.6 ± 22.3[a,c,e]
TG, mg/dL	153.1 ± 19.8	165 ± 26.7	136.9 ± 16.8[c]	142.1 ± 14.7[c]	122.2 ± 16.4[a,c]
TC, mg/dL	81 ± 15.8	106.3 ± 7.4[a]	94.9 ± 4.5[a,c]	95.4 ± 6.5[a,c]	87.1 ± 8.3[c,e]

Values are expressed as mean ± SD. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* EtOH group, and $^{e}P < 0.05$ *vs* silymarin group. Sily: Silymarin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; EtOH: Ethanol; HRW: Hydrogen-rich water; Sily: Silymarin; TC: Total cholesterol; TG: Triacylglycerol.

and 26.8%, compared to the baseline, respectively (Figure 2C). Body weight gain did not significantly differ among the groups. After the mice were sacrificed, liver tissues were excised and weighed. Liver weight significantly differed between the control and EtOH groups ($P < 0.001$), which suggests that EtOH administration resulted in liver enlargement (Figure 2D). Silymarin, HRW and combination treatment ($P < 0.05$) attenuated this EtOH-induced liver enlargement.

Effects of H_2-rich water on liver function in chronic-binge ethanol-fed C57BL/6J mice

ALT and AST activities are biomarkers of liver damage. As shown in Table 1, both significantly differed between controls and the EtOH group ($P < 0.001$), which indicates that EtOH administration caused hepatocellular injury. As compared with the EtOH group, ALT and AST levels were 15.8% and 14.8% lower, respectively, in the silymarin-treated group ($P < 0.01$ and $P < 0.05$) and 11.5% and 10.9% lower, respectively, in the HRW-treated group ($P < 0.05$ and $P > 0.05$). Combination treatment reduced ALT and AST by 24.2% and 26.7%, respectively ($P < 0.001$ for both). In addition, serum TG and TC levels were higher in the EtOH group than in the control diet group ($P > 0.05$ and $P < 0.001$, respectively). As compared with the EtOH group, TG and TC levels were 17% and 10.7% lower, respectively, after silymarin treatment ($P < 0.05$ and $P < 0.01$) and 13.9% and 10.3% lower, respectively, after HRW treatment ($P < 0.05$ and $P < 0.01$). Combination treatment yielded the greatest decreases; as compared with the EtOH group, TG and TC levels

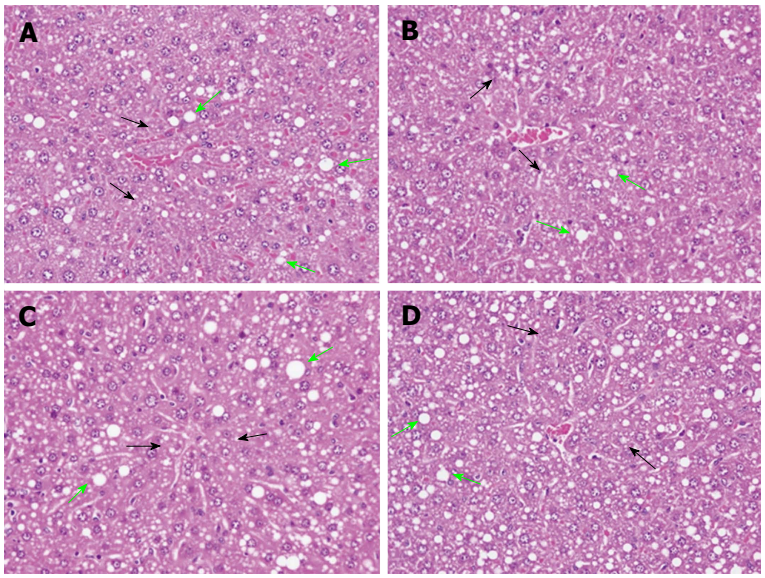

Figure 3 Histopathological alterations of livers treated with chronic-binge ethanol-fed C57BL/6J mice. Livers showed fatty change with micro- mixed macro-vesicles and were graded as mild in the EtOH (A), slight in the EtOH + silymarin (B), mild in the EtOH + HRW (C) and EtOH + silymarin + HRW (D) groups. H and E stain, 400 ×. Black arrow indicates the micro-vesicles and green arrow indicates the macro-vesicles. EtOH: Ethanol; HRW: Hydrogen-rich water.

Table 2 Effects of hydrogen-rich water on the hepatic lipid and cytokine contents in ethanol- fed C57BL/6J mice

Group	Control	EtOH	EtOH + Sily	EtOH + HRW	EtOH + Sily + HRW
Hepatic TG, mg/g tissue	82.4 ± 9.9	120.7 ± 6.8[a]	91.1 ± 8.8[c]	107.5 ± 6.6[a,c,e]	113.5 ± 7.3[a,c,e]
Hepatic TC, mg/g tissue	18.1 ± 2.4	41.4 ± 3.1[a]	34.4 ± 2.8[a,c]	38.0 ± 2.6[a,c,e]	36.5 ± 2.1[a,c]
Hepatic TNF-α, pg/mg tissue	29.0 ± 3.0	45.1 ± 3.4[a]	39.7 ± 2.5[a,c]	42.0 ± 2.4[a,c]	37.3 ± 3.8[a,c]
Hepatic IL-6, pg/mg tissue	10.7 ± 1.2	19.0 ± 1.2[a]	12.5 ± 2.3[a,c]	17.1 ± 2.5[a,e]	11.2 ± 2.7[c]

Values are expressed as mean ± SD. [a]$P < 0.05$ *vs* control group, [c]$P < 0.05$ *vs* EtOH group, and [e]$P < 0.05$ *vs* silymarin group. EtOH: Ethanol; HRW: Hydrogen-rich water; IL-6: Interleukin 6; Sily: Silymarin; TC: Total cholesterol; TG: triacylglycerol; TNF-α: Tumor necrosis factor-alpha.

were 25.9% and 18.1% lower, respectively ($P < 0.001$ for both).

Effects of H_2-rich water on hepatic lipid and inflammatory cytokines in chronic-binge ethanol-fed C57BL/6J mice

In normal liver, hepatic cells have well-preserved cytoplasm, a prominent nucleolus and portal vein. Hepatic steatosis is the most common EtOH-induced disorder and is characterized by accumulation of abnormal lipid droplets in hepatic cells. After 12 wk of EtOH exposure, hepatic TG and TC levels both significantly differed between the present control and EtOH groups ($P < 0.001$ for both; Table 2). These findings were consistent with the results of a histopathological examination of liver sections from the EtOH group, which revealed mild, diffuse and multifocal fatty change with microvesicular, macrovesicular and mixed steatosis (Figure 3A). As compared with the EtOH group, hepatic TG and TC levels were 24.5% and 16.9% lower, respectively, in the silymarin-treated group ($P < 0.001$ for both; Table 2).

Histopathological assessment yielded similar results. Silymarin treatment significantly attenuated EtOH-induced fatty change and macrovesicular steatosis (Figure 3B). HRW treatment resulted in significant reductions in hepatic TG and TC (10.9% and 8.9%; $P < 0.01$ and $P < 0.05$, respectively; Table 2), although there was no significant improvement in histopathological characteristics as compared with the EtOH group. Combination treatment resulted in 6% and 11.8% reductions ($P < 0.05$ and $P < 0.01$, respectively; Table 2).

We investigated the inflammatory profile of EtOH-induced liver injury. TNF-α and IL-6 levels were significantly higher for the EtOH group than for the control diet group ($P < 0.001$ for both; Table 2). As compared with the EtOH group, TNF-α and IL-6 levels were significantly lower in the silymarin-treated group (12% and 34.2% lower, respectively; $P < 0.01$ and $P < 0.001$). After HRW treatment, TNF-α and IL-6 levels were 6.4% and 10% lower than those of the EtOH group ($P < 0.05$ and $P > 0.05$, respectively). Combination treatment yielded the best results, with reductions of 17.3% and 41.1%, respectively ($P < 0.001$ for both). These results suggest that HRW inhibits EtOH-induced lipid accumulation and hepatic inflammation in the liver.

Anti-inflammatory effect of H_2-rich water on cytokine in chronic-binge ethanol-fed C57BL/6J mice

EtOH feeding significantly increased production of serum pro-inflammatory cytokines, including TNF-α

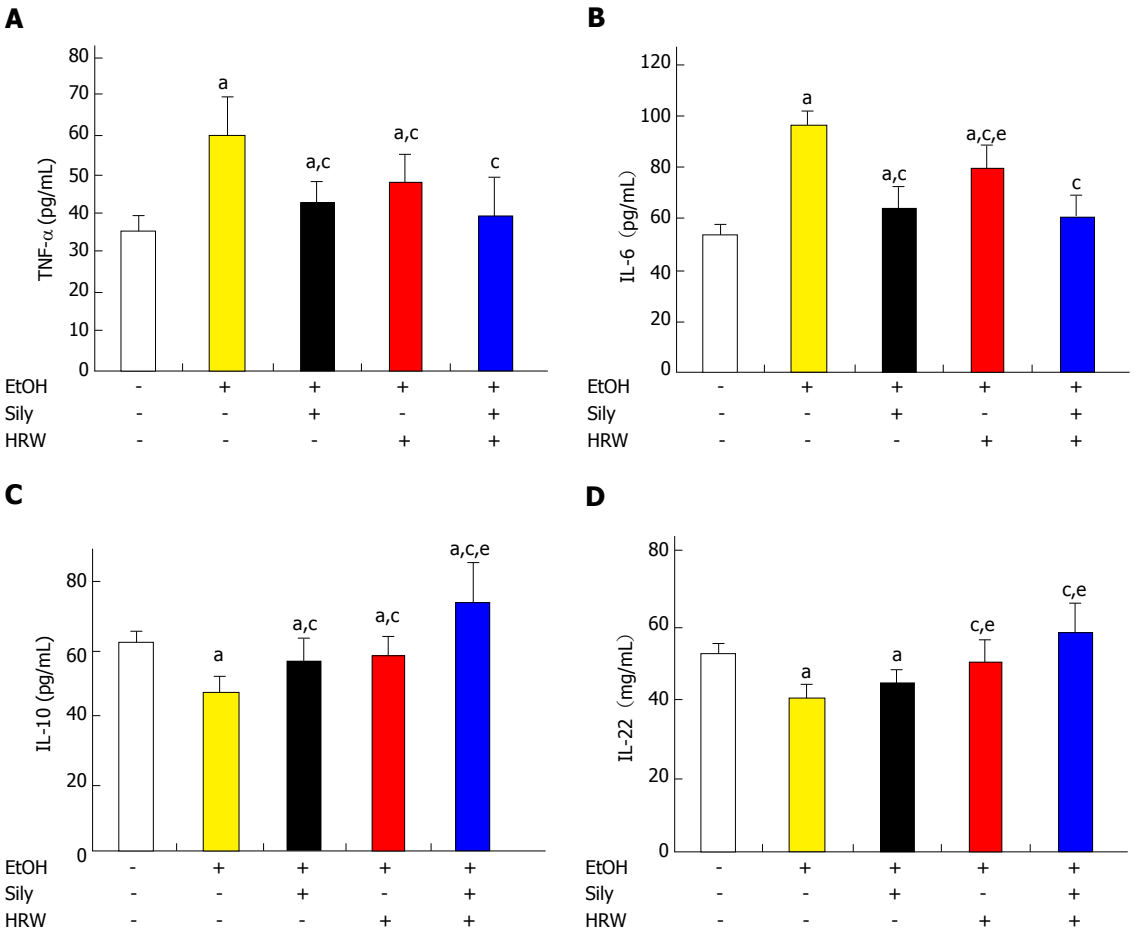

Figure 4 Anti-inflammatory effect of hydrogen-rich water on cytokines in chronic-binge ethanol-fed C57BL/6J mice. A and B: The levels of (A) TNF-α and (B) IL-6 in serum were measured as pro-inflammatory markers; C and D: In relative terms, (C) IL-10 and (D) IL-22 in serum were measured as anti-inflammatory markers. The values representare the mean ± SD from 8-10 mice for each group and at least three independent measurements. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* EtOH group, and $^{e}P < 0.05$ *vs* silymarin group. EtOH: Ethanol; HRW: Hydrogen-rich water; IL: Interleukin; Sily: Silymarin; TNF-α: Tumor necrosis factor-alpha.

and IL-6, as compared with the control diet group (Figure 3). TNF-α level was significantly reduced by silymarin, HRW and combination treatment ($P < 0.001$, $P < 0.05$ and $P < 0.001$, respectively; Figure 4A). In addition, IL-6 level was significantly reduced by silymarin, HRW and combination treatment ($P < 0.001$; Figure 4B). Moreover, levels of anti-inflammatory cytokines, including IL-10 and IL-22, were significantly lower in the EtOH group than in the control diet group. IL-10 level was significantly increased by silymarin, HRW and combination treatment ($P < 0.001$; Figure 4C). IL-22 level was also increased by silymarin, HRW and combination treatment ($P > 0.05$, $P < 0.01$ and $P < 0.001$, respectively; Figure 4D). In sum, these results suggest that HRW inhibits pro-inflammatory mediators and induces anti-inflammatory mediators in EtOH-induced liver injury.

Hepatic antioxidant effects of H_2-rich water on activities of related antioxidant enzymes and lipid oxidation in chronic-binge ethanol-fed C57BL/6J mice

EtOH-induced free radical oxidative stress is a hallmark of liver disease. Thus, we tested the hypothesis that HRW decreases oxidative neuronal stress by means of anti-ROS activity. The activities of hepatic antioxidant enzymes, including SOD, CAT, GSH-Px and GSH, in the EtOH group were significantly lower than in the control diet group (Figure 5). In addition, these activities were significantly promoted by silymarin and HRW. Moreover, significantly higher levels of hepatic antioxidant enzymes were observed in mice that received combined treatment with silymarin and HRW.

MDA concentration was used as a marker of oxidative stress. MDA level in hepatic tissue was significantly higher in the EtOH group than in the control diet group ($P < 0.001$). In addition, MDA was significantly lower in the silymarin group ($P < 0.01$) and HRW group ($P < 0.05$) than in the EtOH group. The strongest beneficial effect was seen in the combined treatment group. These findings suggest that HRW promotes antioxidant capacity and reduces lipid peroxidation, thus improving antioxidant defense.

Relationship of acyl ghrelin with inflammatory and oxidative markers

Spearman correlation analysis was used to evaluate associations of HRW-induced alterations in acyl ghrelin with anti-inflammatory and antioxidant markers. Acyl

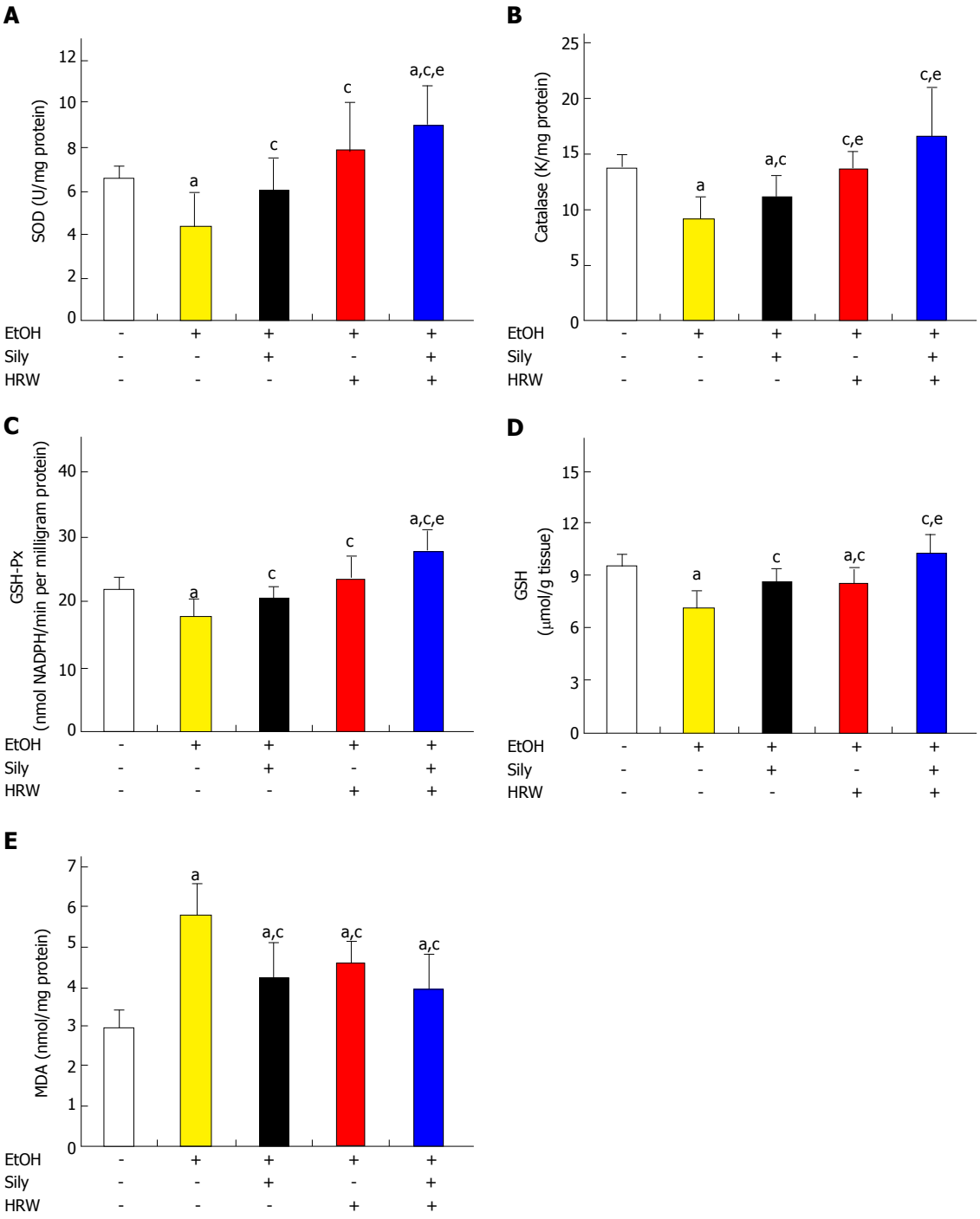

Figure 5 Antioxidative effect of hydrogen-rich water on activities of related antioxidant enzymes and lipid oxidation product in chronic-binge ethanol-fed C57BL/6J mice. A-D: Hepatic activities of antioxidant enzyme levels (A-C) and (D) GSH content were measured; E: Hepatic levels of MDA were shown as markers of oxidative stress. The values are the mean ± SD from 8-10 mice for each group and at least three independent measurements. [a]*P* < 0.05 *vs* control group, [c]*P* < 0.05 *vs* EtOH group, and [e]*P* < 0.05 *vs* silymarin group. EtOH: Ethanol; GSH: Glutathione; GSH-Px: Glutathione peroxidase; HRW: Hydrogen-rich water; MDA: Malondialdehyde; Sily: Silymarin; SOD: Superoxide dismutase.

ghrelin concentration was inversely correlated with the following pro-inflammatory and oxidative markers: serum TNF-α (ρ = -0.455, *P* > 0.05), serum IL-6 (ρ = -0.636, *P* < 0.01) and hepatic MDA (ρ = -0.542, *P* < 0.05). In contrast, acyl ghrelin was positively correlated with serum IL-10 (ρ = 0.811, *P* < 0.001; Figure 6A), serum IL-22 (ρ = 0.797, *P* < 0.001; Figure 6B), hepatic SOD (ρ = 0.539, *P* < 0.05), hepatic CAT (ρ = 0.814, *P* < 0.001; Figure 6C), hepatic GSH-Px (ρ = 0.679, *P* < 0.01; Figure 6D), hepatic MDA (ρ = 0.542, *P* < 0.05) and hepatic GSH (ρ = 0.478, *P* > 0.05). HRW-induced changes in IL-10 and IL-22 resulted in inverse correlations with TNF-α (ρ = -0.304, *P* > 0.05 and ρ = -0.508, *P* < 0.05, respectively) and serum IL-6 (ρ = -0.623, *P* < 0.01 and ρ = -0.703, *P* < 0.01) and positive correlations with hepatic SOD (ρ = 0.385, *P* > 0.05 and ρ = 0.630, *P* < 0.01, respectively), hepatic CAT (ρ = 0.659, *P* < 0.01 and ρ = 0.723, *P* <

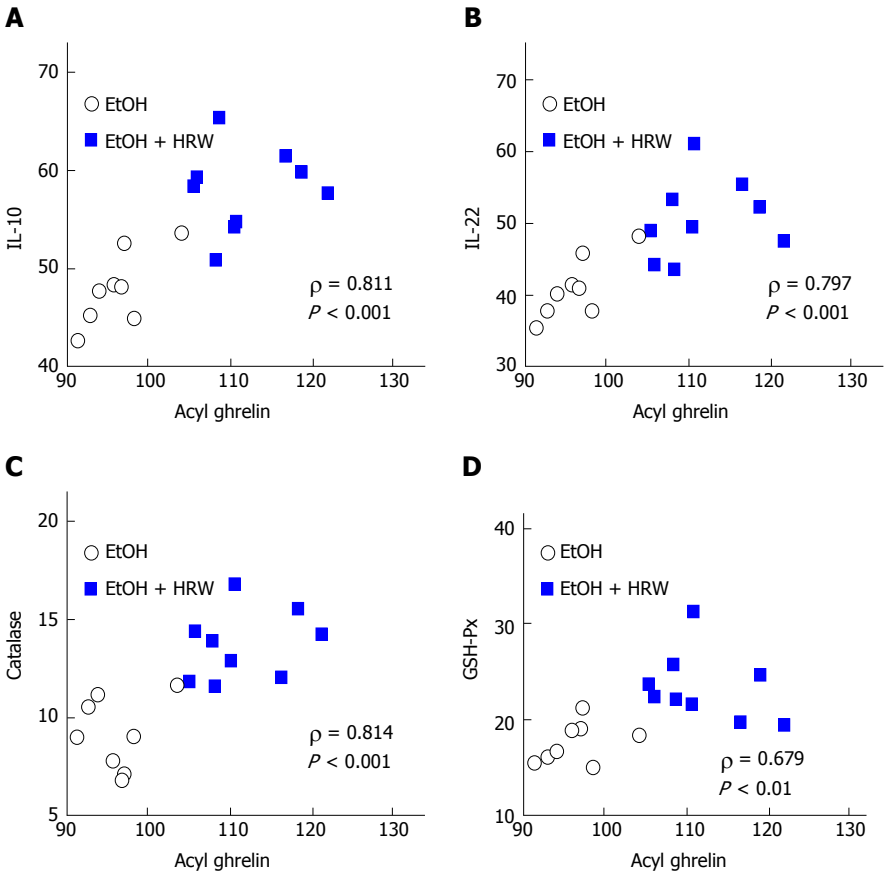

Figure 6 Relationship between acyl ghrelin with (A) interleukin-10, (B) interleukin-22, (C) Catalase, and (D) glutathione peroxidase. EtOH: Ethanol; GSH-Px: Glutathione peroxidase; HRW: Hydrogen-rich water; IL: Interleukin.

0.01, respectively) and hepatic GSH-Px (ρ = 0.711, P < 0.01 and ρ = 0.809, P < 0.001, respectively). These findings indicate that HRW-induced alterations in acyl ghrelin and the hepatoprotective cytokines IL-10 and IL-22 were associated with inflammatory and oxidative responses.

DISCUSSION

ALD not only causes lipopolysaccharide, which activates HSCs, but also affects synthesis and absorption of protein and vitamins, which leads to malnutrition, a secondary factor in hepatocyte damage. The mutual effects of these events eventually result in hepatic fat infiltration, inflammation, necrosis and cirrhosis[1,2,8]. If these conditions are not treated, the inevitable fibrosis and cirrhosis of the liver can result in numerous complications and death. Hence, there is a need for safe and effective agents that prevent or treat ALD. Future research should therefore investigate suppression and blockage of any of the steps that culminate in hepatic injury.

This study investigated whether HRW, alone or combined with silymarin, was beneficial for early-stage EtOH-induced liver injury in female mice. We found that HRW directly scavenged H_2O_2 *in vitro*. Our *in vivo* study showed that HRW pretreatment significantly attenuated increases in serum ALT, AST, TG and TC and hepatic lipid accumulation, which were induced by EtOH feeding. Ghrelin expression was higher after HRW treatment and was correlated with restoration of food intake and inflammatory cytokines, including TNF-α and IL-6, which were induced by EtOH feeding. HRW also attenuated MDA level, restored GSH depletion and increased SOD, GSH-Px and CAT activities in liver. Moreover, HRW reduced TNF-α and IL-6 levels and increased IL-10 and IL-22 levels. These results support the hypothesis that HRW has important antioxidant and anti-inflammatory effects in alcohol-related disease in mice. Previous studies reported that HRW treatment for 6 wk or 10 wk significantly attenuated oxidative stress and had the potential to improve liver function in patients with chronic hepatitis B[32] and metabolic syndrome[31], respectively. Therefore, HRW might be effective for prevention and clinical treatment of ALD such as steatosis, steatohepatitis and cirrhosis.

In the present study, a Lieber-DeCarli EtOH liquid diet was used to induce early ALD in female mice. This model closely reproduces the drinking behaviors of humans and the pathogenetic features of ALD[3,5,33,34]. The present mice fed an EtOH diet for 12 wk exhibited mild hepatic damage, as indicated by significant elevations in serum ALT and AST, hepatic TG and TC, which agreed with the findings

of previous studies[3,33]. These effects were abolished by HRW pretreatment, particularly in combination with silymarin treatment. HRW prevented progression of nonalcoholic steatohepatitis[19,40] and metabolic syndrome in previous studies which suggests that anti-fatty liver benefits are regulated by fatty acid and steroid metabolism through the peroxisome proliferator-activated receptor α (PPARα) signaling pathway[30]. Our results also indicate that prevention of ALD by HRW is partially mediated by lipid metabolism.

Acyl ghrelin acts within the hypothalamus to promote appetite and inhibit sympathetic activity, which increases food intake while lowering metabolic rate[13,14]. Our findings indicate that, as compared with a control liquid diet, an EtOH-containing liquid diet significantly decreases dietary intake and acyl ghrelin level, which suggests that EtOH affects appetite. This finding is consistent with the loss of appetite seen in long-term heavy drinkers. Silymarin, HRW and combination treatment significantly reversed the hypophagic effect of EtOH, indicating that HRW reversal of EtOH-induced anorexia may be mediated by restoration of acyl ghrelin levels. EtOH administration also altered liver weight but not body weight. This suggests that EtOH impairs body composition by means of liver enlargement and sarcopenia[41], which can be improved by HRW pretreatment. HRW might therefore reverse EtOH-induced effects on acyl ghrelin, which stimulate energy expenditure, thus resulting in loss of muscle mass and hypophagia. A previous study reported that ghrelin has a hepatoprotective role in nonalcoholic fatty liver[9]. In addition, after HRW treatment, acyl ghrelin had a neuroprotective effect in Parkinson's disease[42].

Acyl ghrelin and des-acyl ghrelin are both active signaling molecules; however, a limitation of the present study is that we did not measure des-acyl ghrelin. Measurement of the total ghrelin is not a surrogate for analysis of acyl ghrelin[13,43]. A recent study found that des-acyl ghrelin specifically binds to and acts on a subset of arcuate nucleus cells in a ghrelin receptor-independent manner and antagonizes the orexigenic effects of peripherally administered acyl ghrelin[44]. Furthermore, in a lethal rat model of burn trauma, survival was significantly better after resuscitation with saline containing des-acyl ghrelin than after resuscitation with saline alone[45].

ALD pathogenetic mechanisms are involved in increased steatosis, oxidative stress, inflammatory factors and immune response. EtOH-induced liver steatosis due to ROS accumulation and bacterial endotoxin leakage from damaged intestine triggers an inflammatory response[1-3,5,8,33]. TNF-α and IL-6 are widely considered to be the most important pro-inflammatory cytokines in ALD. In addition, pro-inflammatory cytokines and adipokines inhibit muscle-mass formation and promote fat-mass accumulation, a state that is associated with sarcopenia and obesity[41]. In the present study, HRW pretreatment reduced hepatic and systemic production of inflammatory mediators induced by EtOH feeding. Thus, HRW had a protective effect against early ALD.

The anti-inflammatory cytokine IL-10 protects against hepatic damage caused by viruses, alcohol and dietary autoimmunity[46]. IL-10 inhibited activation of HSCs and had antifibrogenic effects in rodents[47]. IL-22 is a survival factor for hepatocytes and prevents and repairs liver injury by enhancing pro-growth pathways *via* STAT3 activation. A previous study revealed that treatment with IL-22 protein contributed to liver regeneration in mice with concanavalin A-induced hepatitis after hepatectomy, which suggests that IL-22 acts as a protective cytokine that attenuates liver injury[11]. In the present study, levels of both anti-inflammatory cytokines were higher after HRW pretreatment than in the EtOH feeding group, which suggests that HRW pretreatment protects against chronic EtOH-induced liver injury and sarcopenia by suppressing the pro-inflammatory cytokines TNF-α and IL-6 and inducing the anti-inflammatory cytokines IL-10 and IL-22.

Oxidative stress is induced by overproduction of ROS, including superoxide anion, hydroxyl radical and H_2O_2 and has a key role in ALD pathogenesis. EtOH consumption leads to excessive ROS, which results in lipid peroxidation and membrane damage, as well as depletion of mitochondrial reduced GSH and its final precursor in liver[1,2]. Antioxidant enzymes such as SOD, CAT and GSH-Px protect against oxidative damage: SOD converts superoxide anion into H_2O_2 and GSH-Px and CAT metabolize H_2O_2 to H_2O. The balance between ROS and antioxidant enzymes is an important mechanism in preventing EtOH-induced oxidative damage. Therefore, antioxidant therapy is a potential strategy to improve outcomes in ALD.

In the present study, we found that EtOH decreased activities of hepatic SOD, CAT, GSH-Px and GSH and increased hepatic lipid oxidation, which is consistent with the findings of previous studies that used the chronic-plus-binge model[3]. These changes may be attributable to oxidative inactivation of enzymes by ROS accumulation. Our results suggest that HRW pretreatment abolished ROS induced by EtOH, resulting in enhanced antioxidant effects. This hypothesis is supported by the present *in vitro* studies (Figure 1C and D) and evidence from previous studies, which indicates that H_2 directly reduces ROS[17,35]. Similar antioxidant phenomena were observed in many oxidative stress-related diseases, especially CCl_4^-, endotoxin-, acetaminophen- and *ischemia/reperfusion*-induced hepatic injuries in rodents[21-27,32,40]. A number of studies reported that H_2 reduces oxidative stress not only directly but also indirectly, by regulating anti-oxidative signal transduction, including nuclear factor erythroid 2-related factor 2 (Nrf-2) and sirtuin 1 (Sirt1)[14,18,20,35]. Antioxidants regulated by Nrf-2 *via* an antioxidant response element-driven mechanism include SOD, GSH-Px, CAT, heme oxygenase-1 (HO-1),

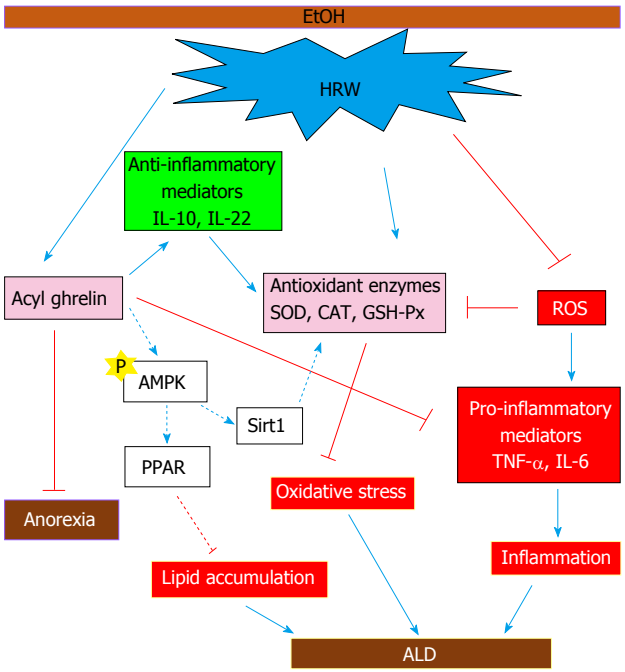

Figure 7 Schematic hypothesis of the mechanisms involved in the protective effects against ethanol-induced fatty liver of hydrogen-rich water. ALD: Alcoholic liver disease; AMPK: AMP-activated protein kinase; CAT: Catalase; EtOH: Ethanol; GSH-Px: Glutathione peroxidase; HRW: Hydrogen-rich water; IL: Interleukin; PPAR: Peroxisome proliferator-activated receptor; ROS: Reactive oxygen species; Sirt 1: Sirtuin 1; SOD: Superoxide dismutase; TNF-α: Tumor necrosis factor-alpha.

peroxiredoxins, *etc*[48]. Interestingly, our results indicate that the antioxidant effects of HRW are similar and/or stronger than those of silymarin, indicating that HRW pretreatment protects against EtOH-induced oxidative stress in EtOH-fed mice.

Acyl ghrelin has anti-inflammatory effects and may help mediate autoimmunity. Notably, acyl ghrelin acts on monocytes and T lymphocytes to suppress their production of TNF-α, IL-1β and IL-6 pro-inflammatory cytokines, which can induce anorexia during both infection and cancer progression[13,14,43]. Ghrelin administration had protective effects against high-fat diet-induced liver injury, oxidative stress, inflammation and apoptosis in rats, in part through the action of the LKB1/AMPK and PI3K/Akt pathways[9]. In the present study, HRW-induced alterations in acyl ghrelin were strongly associated with inflammatory and oxidative responses. This suggests that the hepatoprotective effect of HRW in ALD is mediated by acyl ghrelin production, which results in anti-inflammatory and antioxidant effects. However, future studies should investigate if acyl ghrelin directly regulates expression of IL-10 and IL-22.

HRW-induced alterations in IL-10 and IL-22 were strongly associated with inflammatory mediators and anti-oxidative enzymes. A previous study found that IL-10 was an anti-inflammatory cytokine that inhibits both secretion of pro-inflammatory cytokines by monocytes and/or macrophages and release of free oxygen radicals. Recombinant IL-10 blocks release of ROS[49]. IL-22 prevents oxidative and endoplasmic reticulum stress in mouse and human cells, where stress is induced by lipids, inflammatory cytokines or environmental ROS *via* STAT1- and STAT3-mediated up-regulation of antioxidant genes and suppression of oxidative stress-inducing genes[50].

H_2 inhibits secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, reduces the severity of intestinal inflammation and improves repair of intestinal cells[51,52]. EtOH-induced gastrointestinal dysfunction is caused by abnormalities in Kupffer cells - which result in reduced ability to detoxify endotoxins, disruption of intestinal barrier function and increased permeability to endotoxins and bacteria - and bacterial overgrowth in the gut, which leads to excessive generation of endotoxins[8]. Unfortunately, the limited sensitivity of the ELISA kit prevented us from analyzing endotoxins in the present study. However, previous studies reported that H_2 alleviated endotoxin-induced liver injury in rodents by reducing inflammation and cell apoptosis[20,22,23]. Taken together, the evidence indicates that HRW administration helps maintain permeability, mucosal structure and barrier function in intestine and improves the gastrointestinal microenvironment for bacteria that protect against EtOH-induced liver injury.

The response to EtOH ingestion in the present study appeared to depend on underlying hypertriglyceridemia and hypercholesterolemia and increased accumulation of hepatic TG and TC. AMPK has been implicated as a major regulator of energy metabolism at the cellular and systemic level, which suggests that it has a role in physiological regulation of lipid and glucose metabolism. Existing evidence indicates that EtOH consumption impairs AMPK-mediated regulation of fatty acid metabolism and results in facilitation of TG and TC accumulation in rodents[4]. By inhibiting AMPK and activating the p38-MAPK pathway, acyl ghrelin inhibits ROS-induced autophagy and cell death[15]. Our previous study revealed that the neuroprotective mechanisms of HRW were mediated *via* up-regulation of FoxO3a, which stimulated AMPK in a Sirt1-dependent manner[35]. Past and present evidence suggests that HRW inhibits hepatic lipid accumulation through induction of acyl ghrelin, which activates AMPK signaling after suppressing lipogenesis, inhibiting pro-inflammatory mediators and inducing hepatoprotective cytokines to activate anti-oxidative enzymes. In addition, HRW improved EtOH-induced anorexia *via* acyl ghrelin secretion (Figure 7).

Most importantly, use of HRW in combination with silymarin in the present study resulted in stronger hepatoprotective effects in EtOH-fed mice. These findings are consistent with those of our previous studies in which electronically produced H_2 co-administered with GSH increased the apoptosis-inducing effect in leukemia cells[36]. This finding suggests that HRW combination therapy has a beneficial effect. Alcoholic patients often develop protein calorie malnutrition, which can promote bacterial infection. Nutritional

support is recommended for patients with ALD and was found to improve liver function in histological analyses and to increase survival in short-term follow-up studies[2]. HRW administration might help improve appetite and treat malnutrition and can thus be regarded as an alternative nutritional strategy for treatment of patients with ALD.

In conclusion, this study is the first to show that, in female mice, HRW protects against early-stage chronic EtOH-induced liver injury, possibly by inducing acyl ghrelin to suppress the pro-inflammatory cytokines TNF-α and IL-6 and activate IL-10 and IL-22, which enhance antioxidant enzymes against oxidative stress. These findings indicate that long-term consumption of HRW is a potential strategy for prevention and clinical complementary treatment of ALD.

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COMMENTS

Background

Sustained excessive alcohol consumption results in a spectrum of liver injury, from hepatic steatosis to hepatitis, fibrosis and cirrhosis, which can ultimately lead to hepatocellular carcinoma. Females have an increased susceptibility to alcoholic liver diseases compared with males. This study investigated the potential protective effects of hydrogen-rich water (HRW) against chronic ethanol (EtOH)-induced early-stage liver injury and the underlying mechanisms of such effects in female mice after chronic-plus-binge EtOH feeding.

Research frontiers

Molecular hydrogen scavenges free radicals, thereby exerting a hepatoprotective effect. Approaches to administering hydrogen include inhalation, injection, oral administration and immersion. Oral administration of HRW was easier, safer and more economical as a means to protect against EtOH-induced early liver injury.

Innovations and breakthroughs

The authors investigated the effects of HRW on EtOH-induced early liver injury in female mice. The present study concluded that HRW protects against early-stage chronic EtOH-induced liver injury, possibly by inducing acyl ghrelin to suppress the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 and activate IL-10 and IL-22, which enhance antioxidant enzymes against oxidative stress.

Applications

Long-term consumption of HRW is a potential strategy for prevention and clinical complementary treatment of alcoholic liver disease.

Terminology

Acyl ghrelin has anti-inflammatory effects and may help mediate autoimmunity. Notably, acyl ghrelin acts on monocytes and T lymphocytes to suppress their production of TNF-α, IL-1β and IL-6 pro-inflammatory cytokines, which can induce anorexia during both infection and cancer progression. Acyl ghrelin administration had protective effects against high-fat diet-induced liver injury, oxidative stress, inflammation and apoptosis in rodents.

Peer-review

The manuscript is well documented and interesting. The performance of methodology and the statistical analysis of their results are very well established. They analyzed many biomarkers in the serum as well as in the liver tissue, so as to support their basic hypothesis that HRW can prevent progression of steatosis, liver damage and fibrosis. Their research will provide useful guidance for the treatment of alcoholic fatty liver disease in humans.

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ORIGINAL ARTICLE

Basic Study

Human liver chimeric mouse model based on diphtheria toxin-induced liver injury

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Author contributions: Ren XN and Zhou XH designed the experiments; Ren XN, Ren RR and Yang H performed the majority of experiments; Ren XN and Zhou XH analyzed the data; Qin BY, Peng XH, Chen LX, Yuan MJ and Wang C contributed to genotyping; Ren XN and Zhou XH wrote the paper; Li S revised the paper.

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Abstract

AIM

To establish an inducible liver injury mouse model and transplant human hepatocytes to obtain liver-humanized mice.

METHODS

We crossed three mouse strains, including albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient (SCID)-beige mice, to create Alb-cre/DTR/SCID-beige (ADSB) mice, which coincidentally harbor Alb-cre and DTR transgenes and are immunodeficient. As the Cre expression is driven by the liver-specific promoter Alb (encoding ALB), the DTR stop signal flanked by two loxP sites can be deleted in the ADSB mice, resulting in DTR expression in the liver. ADSB mice aged 8-10 wk were injected intraperitoneally (i.p.) with diphtheria toxin (DT) and liver damage was assessed by serum alanine aminotransferase (ALT) level. Two days later, mouse livers were sampled for histological analysis, and human hepatocytes were transplanted into the livers on the same day. A human ALB enzyme-linked immunosorbent assay was

performed 7, 14, 21 and 28 d after transplantation. Human CD68 immunohistochemistry was performed 30 and 90 d after transplantation.

RESULTS

We crossed Alb-cre with DTR and SCID-beige mice to obtain ADSB mice. These mice were found to have liver damage 4 d after i.p. injection of 2.5 ng/g bodyweight DT. Bodyweight began to decrease on day 2, increased on day 7, and was lowest on day 4 (range, 10.5%-13.4%). Serum ALT activity began to increase on day 2 and reached a peak value of 289.7 ± 16.2 IU/mL on day 4, then returned to background values on day 7. After transplantation of human liver cells, peripheral blood human ALB level was 1580 ± 454.8 ng/mL (range, 750.2-3064.9 ng/mL) after 28 d and Kupffer cells were present in the liver at 30 d in ADSB mice.

CONCLUSION

Human hepatocytes were successfully repopulated in the livers of ADSB mice. The inducible mouse model of humanized liver in ADSB mice may have functional applications, such as hepatocyte transplantation, hepatic regeneration and drug metabolism.

Key words: Liver disease; Liver injury; Diphtheria toxin; Liver chimeric mouse model

Core tip: We established a novel liver chimeric mouse model following liver damage caused by intraperitoneal injection of diphtheria toxin (DT), and transplanted human hepatocytes to obtain liver-humanized mice. After 28 d, human albumin was detected in these mice. Human hepatocytes were successfully repopulated in the livers of triple-crossed albumin-cre transgenic mice, inducible DT receptor transgenic mice and severe combined immune deficient-beige mice [*i.e.*, Alb-cre/DTR/SCID-beige (ADSB) mice]. Our inducible mouse model of humanized liver in ADSB mice may have functional applications, such as studies on hepatocyte transplantation, hepatic regeneration and drug metabolism.

Ren XN, Ren RR, Yang H, Qin BY, Peng XH, Chen LX, Li S, Yuan MJ, Wang C, Zhou XH. Human liver chimeric mouse model based on diphtheria toxin-induced liver injury. *World J Gastroenterol* 2017; 23(27): 4935-4941 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/4935.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.4935

INTRODUCTION

Liver diseases are a serious global health issue, particularly viral hepatitis infection and related diseases. The hepatitis B virus (HBV) and hepatitis C virus (HCV) are representative hepatotropic viruses. HBV is the prototype of the hepadnaviridae family of hepatotropic, partially double-stranded DNA viruses[1], while HCV is a single-stranded RNA virus[2]. Although different at the molecular level, they share many similarities as pathogens, and both infections can be acute or chronic[3]. Persistent HBV and HCV infections can lead to cirrhosis and/or hepatocellular carcinoma[4,5]. Despite the availability of vaccines and drugs, a huge number of patients suffer from the liver diseases related to these viral infections.

Animal models play a critical role in immunological or therapeutic drug development. The narrow spectrum of species that accommodate HBV and HCV infections restricts preclinical studies. Although chimpanzees have played an important role in studying HBV and HCV infections, there are few studies on chimpanzees due to high costs, ethics and their limited availability[6-8]. Other hepadnaviruses that infect woodchucks[9], ducks[10] and ground squirrels[11] harbor limitations due to genetic heterogeneity.

Fundamental questions regarding hepatotropic pathogen biology *in vivo* need to be addressed. However, this requires a suitable small animal model to guide the challenging and expensive studies. The transgenic 1.2 or 1.3 copy of the HBV genome in mice shows immunological tolerance to HBV antigens. Adenovirus-associated virus-based transduction or hydrodynamic transfection of mouse liver by the 1.2 or 1.3 copy of the HBV genome has also been used to study HBV immunobiology, but does not support viral replication for re-infection in the cycle. Human liver chimeric mouse models are useful in human liver disease research.

In this study, severe combined immune deficient (SCID)-beige mice were crossed with transgenic albumin (Alb)-cre mice which expressed cre enzyme[12] under the control of a liver cell-specific Alb promoter, and diphtheria toxin receptor (DTR)[13,14] transgenic mice, in which the DTR transgene is located in the ubiquitous *gt(ROSA26)Sor*(R26) locus after a loxp-flanked transcriptional stop sequence.

The resulting Alb-cre/DTR/SCID-beige (ADSB) mice specifically expressed DTR in the liver. Following administration of diphtheria toxin (DT), these mice developed liver injury. We further generated humanized liver in ADSB mice by the transplantation of human hepatocytes. The human hepatocytes were repopulated in the mouse liver, which were functional and secreted human albumin. Human Kupffer cells were also found to chimerize in the mouse liver.

Thus, we developed a novel animal model to investigate hepatocyte proliferation[15-17] and hepatotropic viruses.

MATERIALS AND METHODS

Generation of ADSB transgenic mice

To generate the ADSB mice, we crossed Alb-cre

mice (a gift from Dr. Qiang Deng, Institute Pasteur of Shanghai, Chinese of Academy Sciences, Shanghai, China) with DTR mice (a gift from Dr. Yue-Lei Shen, Beijing Biocytogen Co., Ltd, Beijing, China) to obtain Alb-cre/DTR mice. Transgenic mice were selected from the offspring by genomic PCR of tail DNA, and then Alb-cre/DTR transgenic mice were crossed with SCID-beige mice (purchased from the Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China), and ADSB mice were selected by genomic PCR of tail DNA.

Transaminase activity in the blood

DT (Sigma-Aldrich, St. Louis, MO, United States) was intraperitoneally administered (2.5 ng/g) to 8-10-wk-old ADSB mice, and blood was collected from these mice at different time points after DT administration. Samples were centrifuged at 600 × *g* for 15 min to separate the serum. Serum alanine aminotransferase (ALT) activity was measured with a commercially-available kit according to the manufacturer's instructions (Roche, Basel, Switzerland). Serum ALT activity levels in mice used for hepatocyte transplantation were measured 3 d after DT injection.

Histological assessments

The livers were fixed with 4% formaldehyde for 24 h and stored in 75% ethanol. They were then embedded in paraffin and serial sections were cut and stained with hematoxylin and eosin (H and E).

Humanization protocol

We found that a single DT dose of 2.5 ng/g body-weight was the maximum dose tolerated with a 100% survival. Using this dose, serum ALT activity levels were determined prior to cell transplantation. Human cryopreserved hepatocytes (Bioreclamation IVT, Baltimore, MD, United States) were thawed and the cryopreservation solution was removed by centrifugation at 100 × *g* for 5 min at 4 ℃ followed by resuspension in Dulbecco's modified Eagle's medium (DMEM). The resuspended hepatocytes were diluted 1:1 in trypan blue and then centrifuged again at 100 × *g* for 5 min at 4 ℃ and reconstituted in hepatocyte culture medium at 1 × 10^7 cells/mL, and 1 × 10^6 viable hepatocytes suspended in 100 μL DMEM were injected into the inferior splenic pole.

Human ALB ELISA

Starting 1 wk after transplantation, human ALB levels were monitored. Blood samples (10 μL) were collected and centrifuged at 600 × *g* for 15 min. Serum samples were assayed using the Quantitative Human Albumin ELISA Quantitation Kit (Bethyl Laboratory, Montgomery, TX, United States) according to the manufacturer's protocol.

Immunohistochemistry

At the time of harvest, the liver was fixed in 4% formaldehyde for 24 h and stored in 75% ethanol. Sections were then prepared and incubated with primary human CD68 antibody (1:200 dilution; Servicebio, Shanghai, China) and were used to detect specific Kupffer cells in the chimeric mice, and then incubated with horseradish peroxidase-goat anti-rabbit secondary antibody (1:200; Servicebio).

Statistical analysis

Statistical analyses were performed using Prism 5.0 software (GraphPad Software, San Diego, CA, United States). A *P* value of < 0.05 was considered significant.

RESULTS

Experimental design and PCR analysis of Alb-cre/DTR transgenic mice

The experimental design is outlined in Figure 1. In this study, we crossed Alb-cre with DTR and SCID-beige mice to obtain ADSB mice. In PCR used to identify the Alb-cre gene, Tg1, Tg2 and Tg4 mice were cre-positive (Figure 1B) and in PCR for the *DTR* gene, Tg1, Tg2 and Tg5 were found in homozygous DTR mice, and Tg3 and Tg4 in heterozygous DTR mice (Figure 1C). Genotyping of SCID-beige mice was performed as previously described[18]. The mice were then injected intraperitoneally with DT to induce liver injury, and adult human hepatocytes were transplanted to obtain chimeric mice.

Specific inducible liver injury in ADSB mice by DT

To examine the liver damage caused by DT, ADSB mice and non-transgenic mice (C57BL/6) were injected intraperitoneally with 2.5 ng/g bodyweight of DT in 200 μL phosphate-buffered saline. Both groups of mice were sacrificed 4 d later. The livers of non-transgenic mice appeared normal and dark red (Figure 2A), whereas the livers from ADSB mice were pale and almost white (Figure 2B). Liver sections from both types of mice were stained with H and E. Microscopically, the liver sections from non-transgenic mice were of normal histological appearance, the structure of the hepatic lobule was complete, the hepatic cord and hepatic sinusoid were appropriately arranged, and degeneration or necrosis of hepatocytes was not observed (Figure 2C). Hepatocyte nucleus fragmentation disappeared in ADSB mice, suggesting that ADSB mice had characteristic histological hepatocellular injury (Figure 2D).

Kinetic study of bodyweight and liver injury

ADSB mice and non-transgenic mice were injected intraperitoneally with 2.5 ng/g bodyweight of DT. At different time points, bodyweight was recorded and blood samples were collected to determine ALT activity. In ADSB mice, after DT injection, bodyweight began to decrease on day 2, was regained on day 7, and was lowest on day 4 (range, 10.5%-13.4%). No weight reductions were found in non-transgenic mice (Figure 3A). Serum ALT activity in ADSB mice

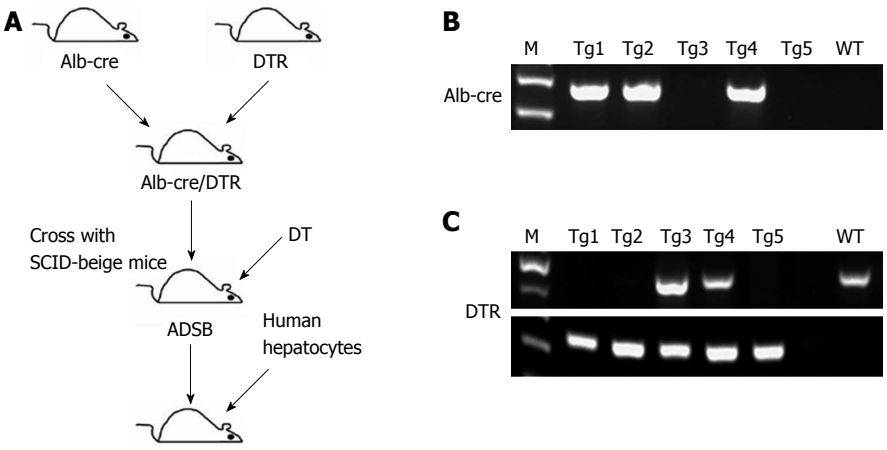

Figure 1 Experimental design and PCR analysis of Alb-cre/diphtheria toxin receptor transgenic mice. A: Experimental design used to characterize DT liver injury in ADSB mice, which were used for human hepatocyte transplantation; B: PCR analysis of the Alb-cre gene, Tg1, Tg2 and Tg4 mice are cre-positive; C: PCR analysis of the DTR gene, Tg1, Tg2 and Tg5 are homozygous DTR mice, and Tg3 and Tg4 are heterozygous DTR mice. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; DT: Diphtheria toxin.

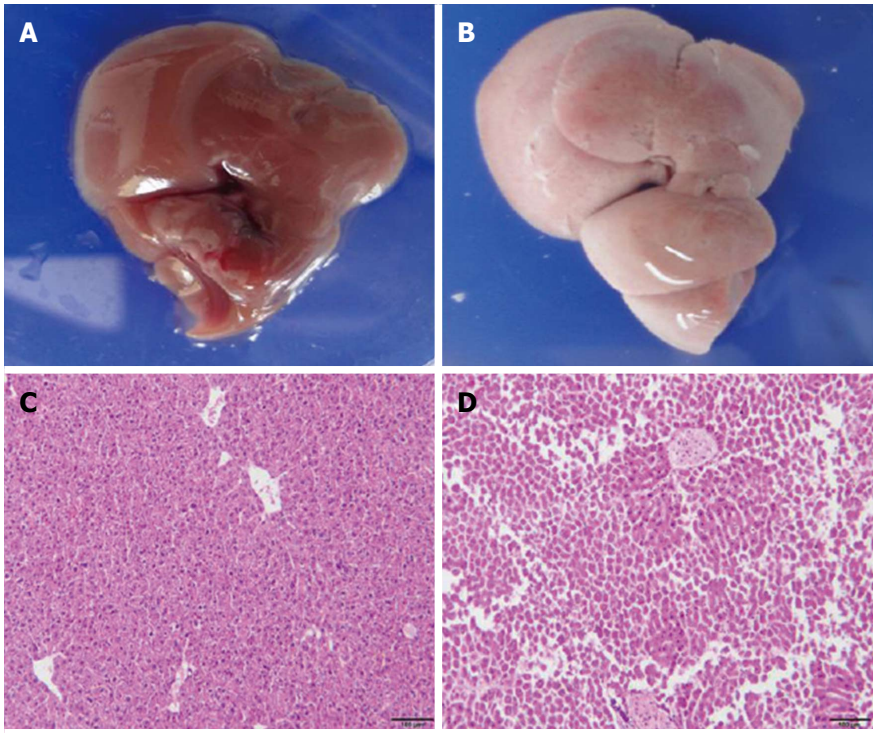

Figure 2 Histological analysis of liver injury. Two days after DT treatment, liver sections from non-transgenic mice and ADSB mice were stained with H and E. A: The liver from non-transgenic mice (C57BL/6); B: The liver from ADSB mouse; C: A representative liver section from non-transgenic mice showing normal histological appearance; D: A representative liver section from ADSB mice showing liver injury. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; DT: Diphtheria toxin; H and E: Hematoxylin and eosin.

began to increase on day 2, reached a peak value of 289.7 ± 16.2 IU/mL on day 4, and then returned to background values on day 7 (Figure 3B). In non-transgenic mice, ALT activity remained at basal levels (< 50 IU/mL). Therefore, from day 2 to day 7 after DT injection liver damage occurred, demonstrating that proliferation of transplanted hepatocytes took place in this mouse model.

Human hepatocyte reconstitution in ADSB mice

ADSB mice were transplanted 3 d after DT injection, and then peripheral blood ALB levels were determined on days 7, 14 and 21 after hepatocyte transplantation (Figure 4A). Serum levels of human ALB in ADSB mice are shown on days 7, 14, 21 and 28 after hepatocyte transplantation. Before 28 d, no human ALB was detectable either in ADSB mice or the non-transgenic mice. However, 28 d after transplantation we detected serum human ALB in ADSB mice at the level of 1580 ± 454.8 ng/mL (range, 750.2-3064.9 ng/mL), and no human ALB was detected in non-transgenic mice (Figure 4B). These results demonstrated that human

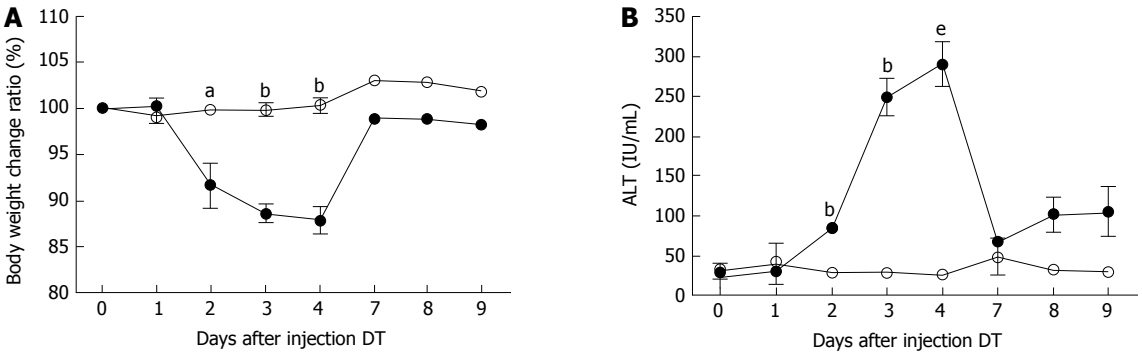

Figure 3 Body weight and alanine aminotransferase analysis after diphtheria toxin injection. ADSB mice (filled circle) and non-transgenic mice (open circle) were injected with 2.5 ng/g bodyweight DT. A: The bodyweight change ratio in the two groups of mice after injection of DT; B: Analysis of serum ALT activity in the mice. Data are shown as the mean of each group, and error bars represent SD (n = 3); $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; ALT: Alanine aminotransferase; DT: Diphtheria toxin.

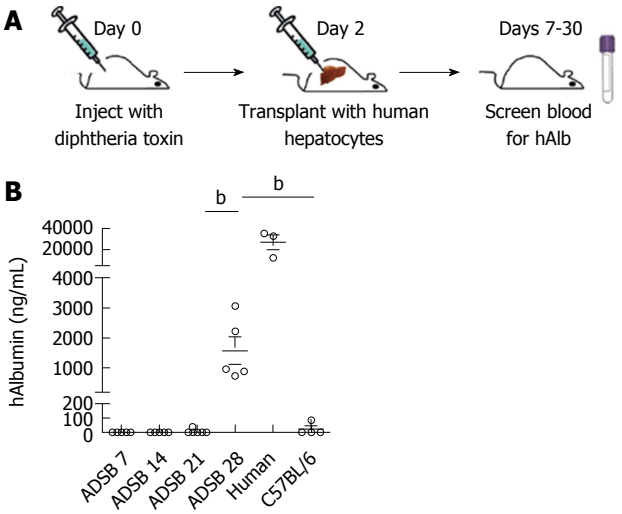

Figure 4 Human albumin plasma concentration in Alb-cre/DTR/SCID-beige mice after adult hepatocyte transplantation. A: Schematic of liver humanization. Two days after the intraperitoneal injection of DT, serum was collected for ALT assay. Human hepatocytes were transplanted into these mice on the same day; B: Serum levels of human albumin are shown for ADSB mice (n = 5) on day 7, 14, 21 and 28 after hepatocyte transplantation as detected by enzyme-linked immunosorbent assay. Results are mean ± SEM ($n \geqslant 3$); $^{b}P < 0.01$. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; ALT: Alanine aminotransferase; DT: Diphtheria toxin.

ALB was expressed at least 4 wk after hepatocyte transplantation.

Human Kupffer cells in the livers of ADSB mice

CD68 is considered a specific marker for activated Kupffer cells. Kupffer cells are essential for many hepatic functions and play a major role in inflammatory responses in this organ[19-21]. CD68 immunohistochemistry was used to measure CD68 expression in Kupffer cells. In ADSB mouse liver sections, $CD68^{+}$ cells were present 4 wk and 12 wk after transplantation, and more $CD68^{+}$ cells were found at 12 wk after transplantation than at 4 wk after transplantation (Figure 5).

DISCUSSION

Human liver chimeric mouse models are useful in human liver disease research. The urokinase-type plasminogen activator (uPA) transgenic mouse[22], was the first reported liver humanized mouse model; however, uPA mice have low breeding efficiency, are unhealthy and die due to hypofibrinogenemia; thus, the transplant time for uPA mice is limited. Two reports showed successful engraftment based on genetic knockout of the fumarylacetoacetate hydrolase (Fah) genes[23,24]. Fah is the last enzyme in the tyrosine breakdown pathway and its deficiency leads to lethal type Ⅰ hypertyrosinemia in humans and liver failure in mice. However, Fah mice also have mouse health problems, and 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione controls liver injury so that impacts its application in drug metabolism. More recently, two additional transgenic models have been developed, the TK-NOG[25] and the AFC8[26] models, which express active caspase 8 fused with the FK506 binding domain and has inducible suicidal activity in mouse liver under Alb promoter control, but its repopulation rate of human liver cells is only 30%. The FRG model was then developed. FRG[23] mice are immune-deficient, Fah knockout mice crossed with mice lacking the *Rag-2* gene and the common gamma chain of the interleukin receptor.

We report here a novel ADSB mouse model which can be efficiently repopulated with human hepatocytes. The transplanted human hepatocytes can reside in the mouse host's natural environment and maintain normal functions. Theoretically, these mouse models can be infected with HBV and HCV in a reproducible manner.

In this model, recipient mouse hepatocytes were destroyed by DT, and the transplanted human mature hepatocytes had a selective advantage in the mouse liver. We confirmed that these mice have the ability to engraft adult human hepatocytes, and the liver can harbor human Kupffer cells. Thus, this model provides

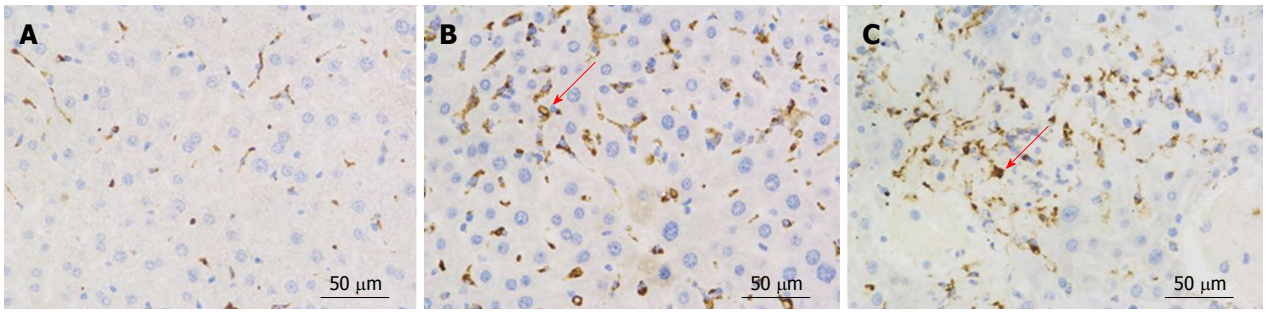

Figure 5 Human Kupffer cells in the liver of Alb-cre/DTR/SCID-beige mice. Human Kupffer cells stained with CD68, showing the high degree of liver chimerism. A: Non-transplanted C57BL/6 mice; B: ADSB mice 4 wk after transplantation; C: ADSB mice 12 wk after transplantation. Red arrows exhibit positive staining. Scale bar = 50 μm, × 400. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice.

a platform for basic biology in liver regeneration research and liver disease development.

Our mouse model has distinct advantages over the other chimeric models. First, ADSB mouse breeding is not as difficult as for uPA mice, making it possible to obtain sufficient ADSB mice for experiments. In addition, these mice are healthy and long-lived, and can be used for long-term transplantation studies. Second, the transplantation time points are flexible following DT injection to induce murine liver injury. Furthermore, we determined the appropriate dose of DT to be 2.5 ng/g bodyweight, which can sustain acute liver injuries with only one dose of DT, resulting in no death of mice, and can efficiently support the proliferation of transplanted hepatocytes.

In conclusion, this study introduced a new *in vivo* mouse model, which will serve as a promising tool for research into the interaction between host and virus *in vivo*, and in the development of new treatment approaches. This model is convenient for studies on hepatocyte transplantation, human drug metabolism research and drug-drug interactions[27,28]. Our model achieved the establishment of human liver without hemopoietic reconstitution. In a future study, we will attempt to establish human liver/immune dual chimeric mice in order to investigate HBV or HCV infections in these chimeric animals.

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COMMENTS

Background

Hepatitis B virus and hepatitis C virus are hepatotropic viruses that represent a serious global health issue. Humanized mouse models are useful in human liver disease research. However, mouse models have disadvantages and need to be improved.

Research frontiers

Recently, many humanized mouse models have been reported, such as the AFC8 mouse and Fah mouse models. However, these mouse models have disadvantages, such as low breeding efficiency, limited time window for transplantation and low repopulation rate.

Innovations and breakthroughs

In the present study, the authors developed a novel liver-chimeric mouse model. Liver failure was induced by diphtheria toxin (DT) and then human hepatocytes were transplanted and repopulated in the mice.

Applications

The results of this study suggest that the liver chimeric mouse model based on triple-crossed albumin-cre transgenic mice, inducible DT receptor (DTR) transgenic mice and severe combined immune deficient-beige mice (*i.e.*, ADSB mice) may provide a more stable platform for human drug metabolism research and viral hepatitis infections.

Terminology

A liver chimeric mouse is established using transgenic or knockout techniques to cause liver failure and human liver cells are transplanted to construct a chimeric mouse. In order to avoid host immune rejection following human hepatocyte transplantation, the mice used are usually immunodeficient.

Peer-review

The researchers provide a novel mouse model of human liver chimeric based on DTR transgenic mice, in which liver injury can be induced by DT injection. This model could serve as a promising tool for research on the interaction between host and hepatitis virus *in vivo*, and in the development of new treatment approaches against related liver diseases.

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ORIGINAL ARTICLE

Retrospective Study

Perinatal transmission in infants of mothers with chronic hepatitis B in California

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Abstract

AIM

To evaluate maternal hepatitis B virus (HBV) DNA as risk for perinatal HBV infection among infants of HBV-infected women in California.

METHODS

Retrospective analysis among infants born to hepatitis B surface antigen (HBsAg)-positive mothers who received post vaccination serologic testing (PVST) between 2005 and 2011 in California. Demographic information was collected from the California Department of Public Health Perinatal Hepatitis B Program databaseand matched to birth certificate records. HBV DNA level and hepatitis B e antigen (HBeAg) status were obtained from three large commercial laboratories in California and provider records if available and matched to mother infant pairs. Univariate analysis compared infected and uninfected infants. Multivariate analysis was restricted to infected infants and controls with complete maternal HBV DNA results using a predefined high HBV DNA level of $> 2 \times 10^7$ IU/mL, a 5:1 ratio of cases to controls and a two-sided confidence level of 95%.

RESULTS

A total of 17687 infants were born to HBsAg positive mothers in California between Jan 1 2005 and Dec 31, 2011. Among 11473 infants with PVST, only 125 (1.1%) were found to be HBV infected. Among these infected infants, lapses in Advisory Committee on Immunization Practices recommended post exposure prophylaxis (PEP) occurred in only 9 infants. However, PEP errors were not significantly different between infected and uninfected infants. Among the 347 uninfected and infected infants who had maternal HBeAg and HBV DNA level, case-control analysis found HBeAg positivity (70.4% *vs* 28.9%, OR = 46.76, 95%CI: 6.05-361.32, $P < 0.001$) and a maternal HBV DNA level $\geqslant 2 \times 10^7$ IU/mL (92.6% *vs* 18.5%, OR = 54.5, 95%CI: 12.22-247.55, $P < 0.001$) were associated with perinatal HBV infection. In multivariate logistic regression, maternal HBV DNA level $\geqslant 2 \times 10^7$ IU/mL was the only significant independent predictor of perinatal HBV infection.

CONCLUSION

In California, transmission is low and most infected infants receive appropriate PEP and vaccination. Maternal HBV DNA $\geqslant 2 \times 10^7$ IU/mL is associated with high risk of perinatal infection.

Key words: Perinatal transmission; Hepatitis B virus DNA; Pregnancy; Hepatitis B; Vaccination; Post-vaccine serology testing

Core tip: Most infants born to hepatitis B surface antigen positive woman in California received appropriate post exposure prophylaxis and vaccination but a low postvaccination serologic testing rate represents a missed opportunity to identify chronically infected infants needing lifelong medical care. Overall the perinatal transmission rate in California is low at 1.1% and only high maternal hepatitis B virus (HBV) DNA level predicts risk for perinatal transmission. Maternal HBV DNA is a vital prenatal screen if targeted antiviral therapy for high-risk mothers becomes a strategy to reduce transmission.

Burgis JC, Kong D, Salibay C, Zipprich J, Harriman K, So S. Perinatal transmission in infants of mothers with chronic hepatitis B in California. *World J Gastroenterol* 2017; 23(27): 4942-4949 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/4942.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.4942

INTRODUCTION

Perinatal hepatitis B virus (HBV) infection is associated with a 90% risk for chronic infection, which carries a 25% risk of death from liver failure and hepatocellular carcinoma[1]. The United States Advisory Committee on Immunization Practices (ACIP) recommends and California regulations require testing of all pregnant women for hepatitis B surface antigen (HBsAg)[2]. ACIP also recommends that infants born to HBsAg-positive mothers receive post-exposure prophylaxis (PEP) including hepatitis B immunoglobulin (HBIG) and the first dose of HBV vaccine within 12 h of birth; complete the HBV vaccine series; and receive postvaccination serologic testing (PVST), including testing for HBsAg (infection) and antibody to HBsAg (immunity) between 9-18 mo of age[3].

International studies have shown that maternal hepatitis B e antigen (HBeAg) positivity and high maternal HBV DNA levels ($\geqslant 2 \times 10^7$ IU/mL or 10^8 copies/mL) are the most significant risk factors for perinatal transmission[4-7]. Perinatal HBV infection has been shown to correlate linearly with increasing HBV DNA levels[6,7]. Studies have not shown a consistent relationship between perinatal transmission and other risk factors such as delivery mode, invasive procedures during pregnancy, or breastfeeding[8-10]. Antiviral therapy for pregnant women with high HBV DNA levels has been proposed to reduce perinatal transmission, but its use is not yet standard of care[11-14]. The exact viral load at which to initiate antivirals to prevent transmission has yet to be established.

To explore risk factors for perinatal transmission in California, we conducted a retrospective analysis of infants born between 2005 and 2011 to HBsAg-positive mothers. Our objective was to evaluate, the effect of maternal HBeAg status and HBV DNA levels on perinatal HBV infection.

MATERIALS AND METHODS

Each year, approximately 2500 pregnant women with chronic HBV infection are reported to the CDPH Perinatal Hepatitis B Prevention Program (PHPP). California local health jurisdictions monitor mother-infant pairs to ensure compliance with ACIP recommendations for the prevention of perinatal HBV infection and submit demographic data, laboratory results, times of HBIG and HBV vaccine administration at birth and completion of the HBV vaccine series, and PVST results to the CDPH PHPP. Data are entered and maintained at CDPH; internal logic checks and record reviews are used to verify data quality.

A retrospective study to determine risk factors for perinatal HBV infection was conducted. Eligible infants were those born to HBV-infected women between January 1, 2005 and December 31, 2011 whose HBV infection status was known *via* PVST. A case control analysis was conducted for mothers who had HBV DNA testing within one year of the infant's birth. Case infants were those with positive HBsAg results indicating perinatal infection; control infants were

those with negative HBsAg results. All control mothers also had HBeAg testing within one year of the infant's birth. Infants born during the study period who were younger siblings of another case or control infant were excluded.

Birth certificate data on maternal race and ethnicity, birthplace, education level and insurance status during pregnancy and details about the delivery were obtained for all infants. Information on infant sex, gestational age at birth, birth weight, the administration of HBIG and HBV vaccine at birth, and completion of a three or four dose HBV vaccine series for all infants was obtained from the CDPH PHPP, local health jurisdictions, clinic and birth hospital records, and birth certificates. Based on ACIP recommendations, infants without documentation of administration of HBIG and HBV vaccine within 12 h birth were considered to have a PEP error. Infants without documentation of at least three doses of HBV vaccine by 8 mo of age (245 d) or 4 doses of vaccine by 12 mo of age (366 d) were considered to have a vaccine series error. Infants weighing less than 2000 g at birth without documentation of three HBV vaccine doses after the birth dose were also considered to have a vaccine series error[3].

HBeAg and HBV DNA test results for women of childbearing age 14 to 45 years between January 1, 2005 and December 31, 2011 were requested from three large reference laboratories that serve most patients in California: Quest Diagnostics (Madison, NJ, United States), LabCorp (Burlington, NC, United States), and ARUP Laboratories (Salt Lake City, UT, United States). These results were matched to PHPP records by mother's name, date of birth, and proximity of mother's residence zip code to the ordering provider's zip code. Laboratory tests performed more than one year from the infant's date of birth were excluded. When multiple laboratory results matched to a mother, the result closest to the infant's date of birth was included. For all cases, maternal prenatal care providers were contacted to obtain any additional HBV DNA and HBeAg results that were available.

Given a limited case sample size of 27 infants, of whom 92% met the predefined exposure of HBV DNA level > 2×10^7 IU/mL, a 5:1 ratio of cases to controls, and a two-sided confidence level of 95%, our case control analysis had more than 95% power to detect an odds ratio of greater than 50. Group differences for continuous variables were examined using the Mann-Whitney test or Student's *t*-test ($\alpha = 0.05$, two-tail). OR and 95%CI were calculated for categorical variables using logistic regression. OR and CI for race were calculated using the Mantel Haenszel test. Variables with a *P* value < 0.10 in univariate analysis were included in a multivariate logistic regression analysis using the backward stepwise method with a removal standard of 0.05. Multivariate analysis was restricted to cases and controls with complete maternal HBV DNA results. All analyses were performed using SAS for Windows (version 9.3, SAS Institute, Cary, NC, United States). The statistical methods of this study were reviewed by Jennifer Zipprich from the Immunization Branch of the California Department of Public Health. Technical appendix, statistical code, and dataset are available upon request from Jennifer Zipprich (jennifer.zipprich@cdph.ca.gov). This study was reviewed by the California Health and Human Services Agency Committee for the Protection of Human Subjects and determined to be "not research" and was approved by the Stanford University Human Subjects Research and Institutional Review Board. A waiver of consent was granted for this study; the presented data are anonymized and the risk of identification is low. There are no conflicts of interest to disclose. No animal studies were conducted.

RESULTS

Of 17687 infants born and enrolled in PHPP during January 1, 2005 - December 31, 2011, 11473 (64.9%) had complete PVST results, and 347 (3%) of their mothers had HBV DNA testing within one year of the infant's birth. Of the infants with PVST results, 125 (1.1%) were HBsAg-positive.

Of the 125 infected infants, 115 (92%) infants received appropriate PEP at birth and the PEP status of one adopted infant was unknown. Of the 9 infected infants who did not receive appropriate PEP at birth, 3 did not receive HBIG and 6 received PEP greater than 12 h after birth. Additionally, three infected infants did not complete the vaccine series in the appropriate time period.

Of the 125 infected infants, 27 (21.6%) had maternal HBV DNA results and were eligible for case selection. Of the 11348 uninfected infants, 320 (2.8%) had maternal HBV DNA and HBeAg results and were eligible for control selection. Of eligible controls, 135 were randomly selected for a ratio of 5 controls for every case with maternal HBV DNA results for the case control analysis.

The case (infected) and control (uninfected) infants were similar with respect to sex, birthweight and gestational age at birth (Table 1). There was no significant difference in PEP errors between case and control infants. The most common error noted was late or incomplete HBV vaccine series. There was also no significant difference in infant age at administration of HBIG and HBV vaccine at birth or vaccine series completion between cases and controls (data not shown, *P* = 0.49, *P* = 0.79, *P* = 0.43, respectively).

Case and control mothers were similar with respect to age, foreign birth, education and insurance status during pregnancy (Table 2). There was no significant difference in maternal race with 96% of case mothers *vs* 81% of control mothers identified as API (OR = 4.790, 95%CI: 0.62-37.234, *P* = 0.129). Case mothers were more likely to be of Vietnamese or Hmong descent (OR = 19.6, 95%CI: 3.80-Undef, *P* <

Table 1 Univariate analysis of infant characteristics among those with maternal hepatitis B virus DNA results *n* (%)

Infant characteristics	Cases (*n* = 27)	Controls (*n* = 135)	OR	*P* value
Sex				
Male	17 (63.0)	73 (54.1)	Reference	
Female	10 (37.0)	62 (45.9)	0.693 (0.296-1.623)	0.398
Birthweight[1]				
> 2500 g	25 (92.6)	121 (89.6)	Reference	
< 2500 g	2 (7.4)	14 (10.4)	0.692 (0.148-3.235)	0.640
Gestational age[2]				
Full term	25 (92.6)	116 (85.9)	Reference	
Preterm	2 (7.4)	17 (12.6)	0.546 (0.118-2.515)	0.437
Unknown	0 (0)	2 (1.5)	-	-
Errors with HBIG or Birth Dose				
None	26 (96.3)	132 (97.8)	Reference	
Any	1 (3.7)	3 (2.2)	1.692 (0.169-16.912)	0.654
Late or incomplete series				
No	24 (88.9)	117 (86.7)	Reference	
Yes	3 (11.1)	18 (13.3)	0.813 (0.222-2.978)	0.754

[1]Birthweight defined by different Advisory Committee on Immunization Practices recommendations for vaccination at this weight threshold; [2]Full term defined as ⩾ 37 wk and preterm defined as < 37 wk gestation. HBIG: Hepatitis B immunoglobulin.

Table 2 Univariate analysis of maternal characteristics among infants with maternal hepatitis B virus DNA results *n* (%)

Maternal characteristics	Cases (*n* = 27)	Controls (*n* = 135)	OR	*P* value
Age (yr)				
< 25	5 (18.5)	17 (12.6)	Reference	
25-34	14 (51.9)	75 (55.6)	0.635 (0.201-2.002)	0.438
> 35	8 (29.6)	43 (31.9)	0.633 (0.181-2.209)	0.473
Race				
Not Asian/Pacific Islander	1 (3.7)	21 (15.6)	Reference	
Asian/Pacific Islander	26 (96.3)	114 (81.4)	4.790 (0.616-37.234)	0.129
Foreign born				
No	2 (7.4)	17 (12.6)	Reference	
Yes	25 (92.6)	118 (87.4)	1.801 (0.391-8.295)	0.450
Education				
Greater than High school	14 (51.9)	74 (54.8)	Reference	
High school or less	13 (48.2)	61 (45.2)	1.126 (0.492-2.577)	0.778
Insurance Prenatal Care				
Non-government	16 (59.3)	63 (46.7)	Reference	
Government	10 (37.0)	72 (53.3)	0.547 (0.232-1.292)	0.169
Unknown	1 (3.7)	0 (0)	-	-
Primigravid				
No	12 (44.4)	76 (56.3)	Reference	
Yes	15 (55.6)	59 (43.7)	1.610 (0.701-3.699)	0.262
Nulliparous				
No	11 (40.7)	65 (48.2)	Reference	
Yes	16 (59.3)	70 (51.9)	1.351 (0.584-3.124)	0.482
Delivery type				
Non cesarean	21 (77.8)	100 (74.1)	Reference	
Cesarean	5 (18.6)	35 (25.9)	0.680 (0.239-1.942)	0.472
Unknown	1 (3.7)	0 (0)	-	-

0.001; OR = 10.75, 95%CI: 1.69-Undef, $P = 0.031$, respectively). There were no significant differences in gravidity, parity, or delivery mode. In addition, no bleeding risks (including placenta previa, placental abruption or need for maternal blood transfusion) were identified in either case or control mothers, but one case mother had missing information. There were also no differences between case and control mothers with respect to premature rupture of membranes (> 12 h) and prolonged labor (> 20 h) (data not shown).

From provider reports to PHPP and electronically submitted data from three large references laboratories, HBV DNA results were only available for 27 (21.6%) mothers of the 125 infected infants, while 36 (28.8%) these mothers had HBeAg results. Among case mothers with HBV DNA results, all had detectable maternal HBV DNA levels compared to only 72.6% of control mothers ($P < 0.001$) (Figure 1). Case mothers were significantly more likely to have a HBV DNA level of ⩾ 2×10^7 IU/mL than control mothers (92.6% *vs*

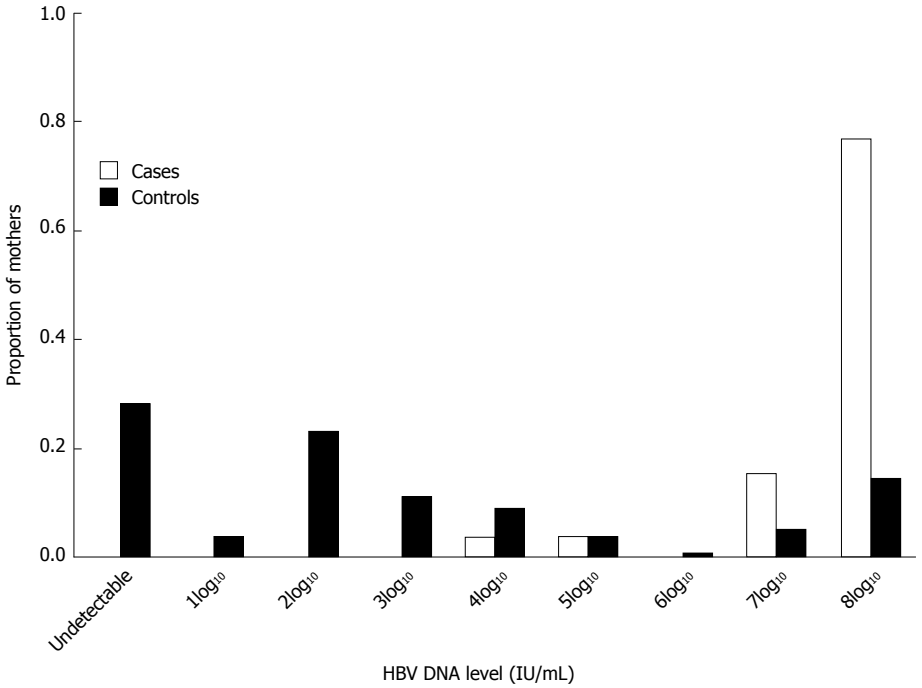

Figure 1 Distribution of hepatitis B virus DNA level between case and control mothers. HBV: Hepatitis B virus.

Table 3 Association between maternal laboratory results and infant hepatitis B virus infection *n* (%)

Maternal laboratory results	Cases (*n* = 27)	Controls (*n* = 135)	OR	*P* value
HBV DNA Levels				
< 2×10^7 IU/mL	2 (7.4)	110 (81.5)	Reference	
> 2×10^7 IU/mL	25 (92.6)	25 (18.5)	54.499 (12.219-247.550)	< 0.001
HBeAg Results				
Negative	1 (3.7)	96 (71.1)	Reference	
Positive	19 (70.4)	39 (28.9)	46.757 (6.051-361.317)	< 0.001
Unknown	7 (25.9)	0 (0.0)	-	

Some mothers had HBV DNA and HBeAg testing. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

18.5%, OR = 54.50, 95%CI: 12.219-247.550, P < 0.001) (Table 3). Significantly more control mothers than case mothers had HBV DNA results reported prior to delivery (57.8% *vs* 33.3%, P = 0.02). Among all mothers with known HBeAg results, significantly more case mothers than control mothers were HBeAg-positive (70.4% *vs* 29.9%, OR = 46.76, 95%CI: 6.051-361.617, P < 0.001).

Among both case and control mothers with detectable HBV DNA levels, mothers who were HBeAg-positive had higher median HBV DNA levels than those who were HBeAg-negative (1.1×10^8 IU/mL *vs* 178 IU/mL, P < 0.001). API mothers had significantly higher median HBV DNA levels (5745.50 IU/mL *vs* 162 IU/mL, P = 0.032) but there was no significant difference in the proportion of API mothers with HBV DNA levels above 2×10^7 IU/mL compared to other races (32.9% *vs* 18.2%, P = 0.217). API mothers were more likely to be HBeAg-positive than mothers of other races (38.6% *vs* 18.2%, P = 0.489) but this difference was not significant.

Variables with a P-value < 0.10 in the univariate analysis were included in the multivariate logistic regression. In the multivariate logistic regression analysis, only maternal HBV DNA level $\geqslant 2 \times 10^7$ IU/mL was a significant independent predictor of perinatal HBV infection.

Perinatal infection occurred in one female and one male infant with maternal HBV DNA levels < 2×10^7 IU/mL who received appropriate PEP at birth and completed the HBV vaccine series in the appropriate time period. Both infants were born at term *via* normal spontaneous vaginal deliveries. The mother of the female infant was HBeAg-negative and had an HBV DNA level of 1×10^4 IU/mL, although both laboratory results were only available 280 d after delivery. The mother of the male infant was HBeAg-positive, had an HBV DNA level of 1×10^5 IU/mL and laboratory results were available 24 d prior to delivery.

DISCUSSION

Our retrospective analysis and case-control study was designed to evaluate the role of maternal HBV DNA

level in perinatal HBV infection. From 2005-2011, 17687 at-risk infants were born to HBsAg-positive mothers in California which created an ideal sampling frame given the largest API population of any state and more than a quarter of the foreign-born population in the United States, both with high prevalence of chronic HBV as well as the extensive PHPP records at CDPH.

The overall HBV infection rate of 1.1% among at-risk infants with PVST is low and demonstrates that current immunoprophylaxis strategies continue to protect most at-risk infants from chronic infection. However, despite appropriate PEP and completion of the vaccine series some infants still become infected. Our study showed that approximately one-third of at-risk infants did not have PVST, a striking deficiency even in a state with the majority of counties with resources for local health departments to provide surveillance for perinatal hepatitis B cases. Similar limitations have been seen in the United States despite comprehensive surveillance programs[15,16]. Infected infants could have been missed. This would have effectively limited our sample size but should not change the nature of the observed association.

New guidelines are proposed to provide antiviral therapy in pregnancy to prevent perinatal transmission[17]. This requires both additional prenatal laboratory testing and a clear viral load threshold to offer therapy. Although retrospectively solicited, only 22% of case mothers of infected infants had HBV DNA results. During our study period, recommendations for routine prenatal screening did not specifically include HBV DNA PCR, but appropriate health care maintenance of chronic hepatitis B would have included this assessment. We relied upon matching laboratory results from three large reference laboratories that perform most of the hepatitis testing in California. Our matching algorithm has not been validated, but matches were individually verified by maternal name and date of birth. To optimize our yield, we included HBV DNA levels within one year of delivery. There is conflicting evidence regarding the variability of HBV DNA levels during pregnancy, as laboratory results obtained more distant from birth may not reflect the infant's true risk at the time of delivery[18,19]. Our study demonstrated that mothers of infected infants with HBV DNA results were more likely to be API, HBeAg-positive, and have higher HBV DNA levels than mothers of uninfected infants. Increased risk was clearly evident with maternal HBV DNA level of $\geqslant 2 \times 10^7$ IU/mL.

We identified two cases of perinatal HBV infection in children born to women with lower HBV DNA levels including one with HBeAg-negative status. This differs from other studies that have reported no transmission among infants born to HBeAg-negative mothers or mothers with low HBV DNA levels[8,20-23]. Due to the fluctuations inherent in HBV infection, HBV DNA levels and HBeAg results obtained distant from delivery may not reflect risk at the time of delivery. Infected infants whose mothers were HBeAg-negative have been described; infection in these infants is possibly due to pre-core mutations[24,25].

Our study had several limitations. Since all eligible mothers were selected based on documented laboratory results, selection bias was possible in our sampling. However, univariate analyses restricted to cases with HBV DNA results showed no significant differences when all infected infants were included. Finally, the sample size of our case control evaluation was small and designed to evaluate maternal HBeAg status and HBV DNA levels as risk factors, so the ability to assess other risk factors was limited. Our unique study evaluates perinatal HBV infection in California, with the largest single state sample size in the United States. The results are consistent with findings from prior smaller North American and international studies identifying a similar HBV DNA threshold for transmission risk, although some studies in Asia find higher perinatal transmission rates [26-29].

In California there is an overall low transmission rate of HBV with current PEP strategies, but PVST is not completed for all at-risk infants creating missed opportunity to screen for chronic hepatitis B infection. Infants of mothers with high viral load are at increased risk of chronic infection, clearly evident with a maternal HBV DNA level of $\geqslant 2 \times 10^7$ IU/mL[17].

Appropriate PEP, completion of the HBV vaccine series, and completion of timely PVST still remain critical and first line strategy to prevent perinatal transmission. The use of antiviral therapy in the third trimester for highly viremic mothers has been shown to decrease the incidence of chronic infection in at-risk infants who received appropriate PEP and the HBV vaccine series[14]. Safety profiles of these medications, risk of viral resistance and maternal disease flare should be considered and may delay efforts to make this prevention strategy standard of care[30-32]. Our study further corroborates existing evidence that a high maternal HBV DNA level is a significant risk factor for perinatal HBV infection and chronic HBV disease. More prospective studies are needed to investigate the specific HBV DNA threshold for treatment and the safety and comparative effectiveness of various antiviral therapies during pregnancy to further reduce the incidence of perinatal HBV infection in the infants of infected women.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection remains a significant public health burden in the United States and worldwide. Vaccination remains the most effective strategy to prevent transmission, especially in the high risk perinatal period when peak risk for chronic HBV infection occurs.

Research frontiers

Perinatal transmission despite post exposure prophylaxis (PEP) and vaccination has been shown to occur in hepatitis B surface antigen (HBsAg) positive mothers with hepatitis B e antigen positivity and/or high maternal HBV

DNA levels. Based on limited but ground-breaking international studies, practice guidelines have begun to suggest the use of antiviral therapy in the second or third trimester in woman with high HBV DNA level to reduce the risk of perinatal transmission. The certainty of evidence, the risk and benefit to both mother and infant, and the viral load threshold to recommend anti-viral therapy in the prenatal period remains under careful review.

Innovations and breakthroughs

This study is the largest single state sample size of perinatal transmission completed outside of Asia. In California, PEP and vaccination are widely used and highly effective to prevent perinatal HBV transmission with an overall perinatal transmission rate of only 1.1%. The incomplete post vaccination serologic testing (PVST) rate may underestimate the burden of pediatric chronic HBV infection in California and is a call to action for pediatricians caring for these at-risk infants. This study demonstrates that infants of mothers with high HBV DNA level of $\geqslant 2 \times 10^7$ IU/mL are clearly at an increased risk of perinatal transmission. Yet, the authors found very few pregnant women with maternal HBV DNA levels, limiting the ability for providers to risk stratify these women for possible anti-viral therapy to prevent perinatal transmission.

Applications

This study reveals a real-life assessment of the current HBV perinatal prevention strategies in California and identifies the opportunities for improvement. A comprehensive public health case managed program provides oversight to verify appropriate post-exposure prophylaxis and HBV vaccination for at-risk infants, and identify those with chronic HBV infection. A comprehensive prenatal screen including HBV DNA level has been recommended by ACOG for HBsAg positive pregnant woman. More research is crucial to determine the appropriate threshold for anti-viral therapy in mothers with high HBV DNA levels to safely advance the prevention strategies for chronic HBV infection.

Peer-review

The manuscript entitled "Perinatal transmission in Infants of mothers with chronic Hepatitis B in California" by Burgis *et al* is well written and well presented.

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ORIGINAL ARTICLE

Retrospective Study

Outcome of a session of extracorporeal shock wave lithotripsy before endoscopic retrograde cholangiopancreatography for problematic and large common bile duct stones

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Author contributions: Tao T designed the study and performed the majority of experiments; Zhang M, Zhu X and Zhang QJ provided analytical tools and were also involved in editing the manuscript; Li L, Li T, Li MD, Li GH and Sun SX co-ordinated and provided the collection of experimental data.

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Abstract

AIM

To compare the efficacy of a session of extracorporeal shock wave lithotripsy (ESWL) before endoscopic retrograde cholangiopancreatography (ERCP) *vs* ERCP only for problematic and large common bile duct (CBD) stones.

METHODS

Adult patients with CBD stones for whom initial ERCP was unsuccessful because of the large size of CBD

stones were identified. The patients were randomized into two groups, an "ESWL + ERCP group" and an "ERCP-only" group. For ESWL + ERCP cases, ESWL was performed prior to ERCP. Clearance of the CBD, complications related to the ESWL/ERCP procedure, frequency of mechanical lithotripsy use and duration of the ERCP procedure were evaluated in both groups.

RESULTS

There was no significant difference in baseline characteristics between the two groups. A session of ESWL before ERCP compared with ERCP only resulted in similar outcomes in terms of successful stone removal within the first treatment session (74.2% *vs* 71.0%, $P = 0.135$), but a higher clearance rate within the second treatment session (84.4% *vs* 51.6%, $P = 0.018$) and total stone clearance (96.0% *vs* 86.0%, $P = 0.029$). Moreover, ESWL prior to ERCP not only reduced ERCP procedure time (43 ± 21 min *vs* 59 ± 28 min, $P = 0.034$) and the rate of mechanical lithotripsy use (20% *vs* 30%, $P = 0.025$), but also raised the clearance rate of extremely large stones (80.0% *vs* 40.0%, $P = 0.016$). Post-ERCP complications were similar for the two groups.

CONCLUSION

Based on the higher rate of successful stone removal and minimal complications, ESWL prior to ERCP appears to be a safe and effective treatment for the endoscopic removal of problematic and large CBD stones.

Key words: Extracorporeal shock wave lithotripsy; Endoscopic retrograde cholangiopancreatography; Common bile duct stones

Core tip: Extracorporeal shock wave lithotripsy (ESWL) and endoscopic retrograde cholangiopancreatography (ERCP) are frequently used for patients with large common bile duct (CBD) stones. The effect of a session of ESWL prior to ERCP for problematic and large CBD stones has not previously been reported. The results of our research suggested that a session of ESWL can aid clearance of CBD in the following ERCP. Also mechanical lithotripsy usage was reduced and extremely large stone (≥ 30 mm) clearance rate can be raised.

Tao T, Zhang M, Zhang QJ, Li L, Li T, Zhu X, Li MD, Li GH, Sun SX. Outcome of a session of extracorporeal shock wave lithotripsy before endoscopic retrograde cholangiopancreatography for problematic and large common bile duct stones. *World J Gastroenterol* 2017; 23(27): 4950-4957 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/4950.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.4950

INTRODUCTION

Therapeutic endoscopic retrograde cholangiopancreatography (ERCP) was first introduced in the 1970s, and is the most frequently used endoscopic technique for the clearance of stones from the common bile duct (CBD)[1]. Conventional therapy for CBD stones involves sphincterotomy and stone extraction with either a Dormia basket or a Fogarty-type balloon[2]. About 80% to 90% of CBD stones can be extracted using conventional techniques[3]. Removal by ERCP is less invasive as compared with surgery but is more likely to fail when the stone is large[4,5]. For impacted or extremely large stones, or stones located intrahepatically or proximal to a bile duct stenosis, endoscopic removal may not be successful, and failure is generally due to the inability to grasp the large stones. In these patients, lithotripsy (electrohydraulic, electromagnetic or piezoelectric) has proved to be effective in terms of disintegrating stones into smaller fragments, facilitating the endoscopic clearance of CBD stones[6,7].

Extracorporeal shock wave lithotripsy (ESWL) is a novel technique, which uses shock waves to fragment stones. Clinical experience with ESWL for the fragmentation of kidney stones was first reported in 1980[8]. Its application was quickly extended to large biliary and pancreatic stones. Sauerbruch and his colleagues demonstrated the efficacy of ESWL in achieving CBD stone fragmentation in about 90% of patients with mild side effects[9]. The present prospective controlled trial was conducted to compare the therapeutic benefits and complications in patients having a session of ESWL before ERCP and patients having ERCP alone for the treatment of problematic and large CBD stones.

MATERIALS AND METHODS

This prospective study was conducted at the Department of Gastroenterology of Zibo Central Hospital, a tertiary referral hospital in Zibo, Shandong Province, China. The study was approved by the Ethics Committee of the Zibo Central Hospital. All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

From February 2013 to September 2016, 231 eligible patients were identified at the hospital. The inclusion criteria were adult patients with CBD stones who had undergone an unsuccessful initial ERCP. A nasobiliary tube (NBT) was placed in all subjects to irrigate the stones and visualize the calculi during ESWL. The number and diameter of the stones were assessed by pre-ESWL X-ray or computed tomography. If multiple stones were detected, the largest single stone diameter was tallied. Patients were treated with a session of ESWL (14-26 kV) before ERCP in the ESWL + ERCP group.

Patients who were admitted on Tuesday, Wednesday and Thursday were allocated to the ERCP-only group. These patients underwent conventional ERCP treatment for stone extraction using a side-view endoscope (JF-240; Olympus Optical Corporation, Tokyo, Japan),

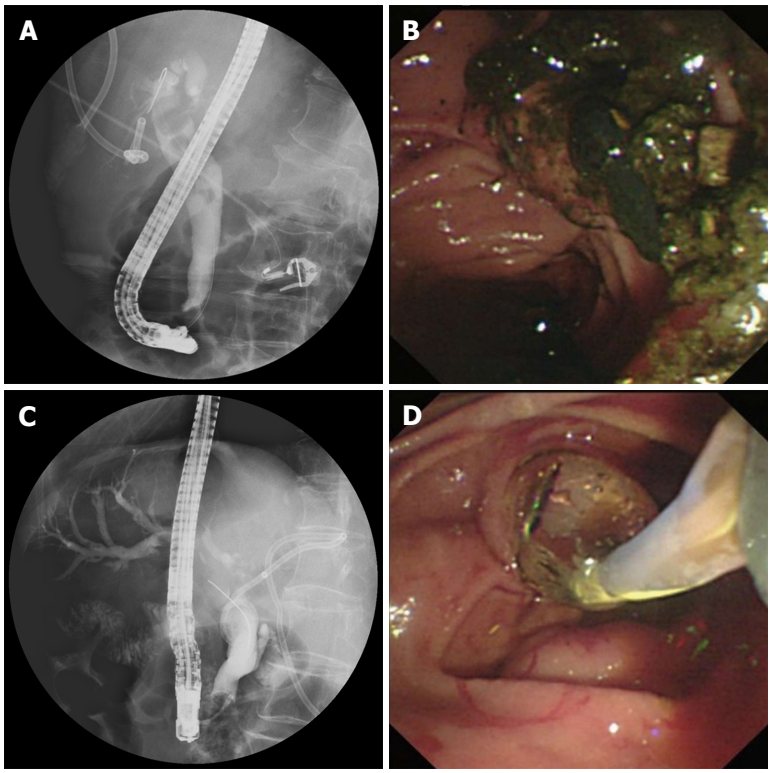

Figure 1 Large stones in the common bile duct were cracked by extracorporeal shock wave lithotripsy (A) and cleared by following endoscopic retrograde cholangiopancreatography (B and C), common bile duct strictures were dilated using a balloon, and passage dilating catheters were used to retrieve the stones (D).

performed by two senior endoscopists, each with the experience of more than 1000 ERCP procedures. Patients admitted on Friday, Saturday, Sunday and Monday were allocated to the ESWL + ERCP group. For these patients, a session of ESWL was performed 4 h before ERCP by experienced gastroenterologists using an electrohydraulic spark gap lithotripter (HealthTronics, Austin, TX, United States), even if no difficulty was anticipated in the following ERCP procedure in terms of removing the stones by means of basket extraction. Patients were treated in the prone position and under general anaesthesia with continuous monitoring. Stones were localized and targeted with an X-ray focusing system. ESWL was performed at a rate of 90 shocks/min for 10 min and at an intensity of 4 (on a scale of 1-6, corresponding to 11000-16000 kV). Patients were exposed to a maximum of 5000 shocks/session unless the stones were fragmented to less than 5 mm earlier. ERCP was performed 4 h after ESWL to clear the fragments using a retrieval basket or balloon catheter, unless the stones passed spontaneously. If present, CBD strictures were dilated using a balloon (4-15 mm), and passage dilating catheters were used to retrieve the stones (Figure 1). In cases where CBD stones could not be cleared successfully in two treatment sessions, the patient would be subjected to repeated biliary stenting or surgery.

CBD stone clearance was assessed after each ERCP session using procedure reports, plain films, ERCP films and/or abdominal MRCP (Figure 2). Separate records were made for each group that included information on post-ESWL complications, post-ERCP complications, number of mechanical lithotripsies used and the duration of each ERCP procedure. Post-ERCP complications were recorded in all patients and graded as mild, moderate or severe according to Cotton's criteria[10]. Successful clearance was defined as the clearance of more than 90% of the CBD stone fragments using a balloon or a basket.

Statistical analysis

The statistical methods of this study were reviewed by a biostatistician (Jia-Tong Liang) from Shandong University of Technology. Data are expressed as mean ± SD. Statistical analysis was performed using the χ^2 test or Fisher's exact test for non-continuous variables, and Student's *t*-test was used for continuous variables. Analyses were performed by using SPSS 12.0 (SPSS Inc, Chicago, IL, United States). A probability (*P*) value < 0.05 was considered statistically significant.

RESULTS

Two hundred and thirty-one patients were evaluated in this study (ESWL + ERCP group, *n* = 124; ERCP-only group, *n* = 107). Patient characteristics are shown in Table 1. There were no significant differences in clinical characteristics between the two groups including prothrombin time/international normalized ratio (1.14 ± 0.22 *vs* 1.19 ± 0.34, *P* = 0.382), periampullary

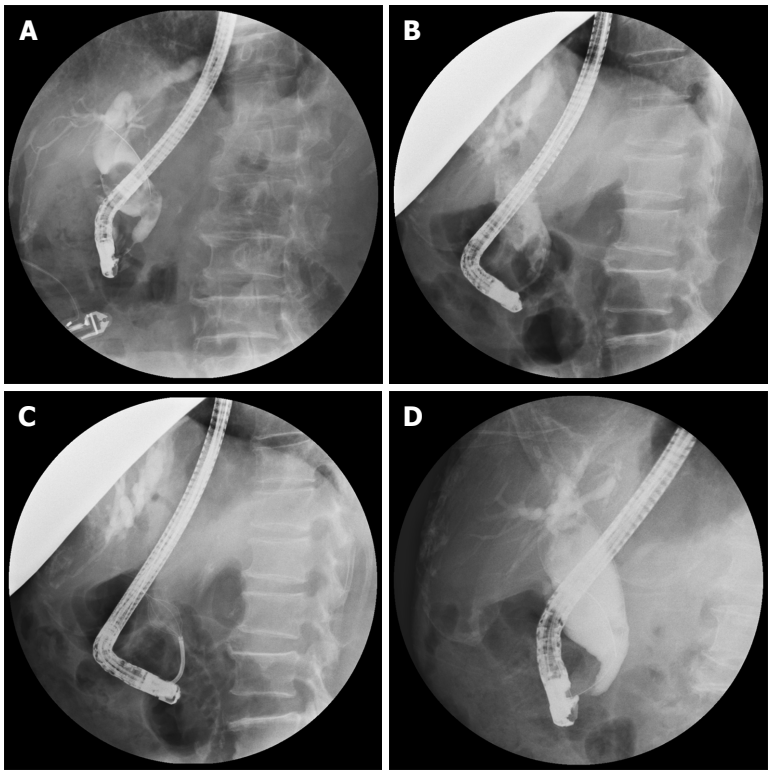

Figure 2 Common bile duct stone clearance was assessed after each endoscopic retrograde cholangiopancreatography session using procedure reports, plain films, endoscopic retrograde cholangiopancreatography films and/or abdominal magnetic resonance cholangiopancreatography. A: Pre-ESWL large common bile duct stones were very large; B: Post-ESWL reduction in diameter of CBD stones; C: Stones were tracked by a basket during the following ERCP; D: CBD was cleared successfully. ESWT: Extracorporeal shockwave lithotripsy; ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct.

Table 1 Baseline characteristics of patients in the extracorporeal shock wave lithotripsy + endoscopic retrograde cholangiopancreatography group and the endoscopic retrograde cholangiopancreatography-only group *n* (%)

Demographic characteristic	ESWL + ERCP (*n* = 124)	ERCP only (*n* = 107)	*P* value
Age (yr)	71.2 ± 4.6	68.4 ± 6.1	0.647
Male	63 (50.8)	56 (52.3)	0.318
Prothrombin time/INR	1.14 ± 0.22	1.19 ± 0.34	0.382
Periampullary diverticulum	54 (43.5)	48 (44.9)	0.263
Pre-cut sphincterotomy	65 (52.4)	60 (56.1)	0.187
Calculi characteristic			
Single	83	72	
Multiple	41 (33.1)	35 (32.7)	0.371
Stone size	18.3 ± 2.5	16.6 ± 3.8	0.084
1.5-3.0 cm	104	93	
> 3.0 cm	15 (12.1)	10 (9.3)	0.195

ESWL: Extracorporeal shock wave lithotripsy; ERCP: Endoscopic retrograde cholangiopancreatography; INR: International normalized ratio.

diverticulum (43.5% *vs* 44.9%, *P* = 0.263), pre-cut sphincterotomy (52.4% *vs* 56.1%, *P* = 0.187), stone size (18.3 ± 2.5 mm *vs* 16.6 ± 3.8 mm, *P* = 0.084), and the percentage of patients who have had multiple stones (33.1% *vs* 32.7%, *P* = 0.371) or extremely large (> 3.0 cm) stones (12.1% *vs* 9.3%, *P* = 0.195).

Stones passed spontaneously in six patients during ESWL treatment, in four patients during the first session and in two patients during the second session. Overall, there were 156 ESWLs and 150 ERCPs in the 124 patients in the ESWL + ERCP group, whereas there were 138 ERCPs in the 107 patients of the ERCP-only group. The outcome of the two groups is summarized in Table 2. In the first session, successful stone clearance did not differ significantly between the two groups (74.2% *vs* 71%, *P* = 0.135). However, in the second session, ESWL before ERCP produced a higher stone clearance than that observed in the ERCP-only group (84.4% *vs* 51.6%, *P* = 0.018), and the overall stone clearance also differed significantly (96.0% *vs* 86.0%, *P* = 0.029) between the two groups. Moreover, a session of ESWL before ERCP reduced the rate of mechanical lithotripsy (20.0% *vs* 30.0%, *P* = 0.025) and ERCP procedure time (43 ± 21 min *vs* 59 ± 28 min, *P* = 0.034). Successful stone removal by a conventional method (balloon or dormia basket) was similar between the two groups (97.0% *vs* 91.5%, *P* = 0.251); however, successful clearance rate of mechanical lithotripsy differed significantly (92.0% *vs* 75.0%, *P* = 0.041). Removal of extremely large-sized stones (≥ 3.0 cm) differed significantly between the groups [80.0% (12/15) in the ESWL + ERCP group *vs* 40.0% (4/10) in the ERCP-only group, *P* = 0.016] (Table 3).

In 20 patients, stones had not been extracted even after two sessions of treatment (5 patients in the

Table 2 Endoscopic stone removal observed after endoscopic retrograde cholangiopancreatography *n*(%)

Attempt	ESWL + ERCP (*n* = 124)	ERCP only (*n* = 107)	*P* value
First	92/124 (74.2)	76/107 (71.0)	0.135
Second	27/32 (84.4)	16/31 (51.6)	0.018[a]
Total	119/124 (96.0)	92/107 (86.0)	0.029[b]
Number (rate) of mechanical lithotripsy	30/150 (20)	41/138 (30)	0.025[c]
Mean duration of ERCP procedure (min)	43 ± 21	59 ± 28	0.034[d]

The CBD stone clearance rate observed after the second session of ESWL + ERCP procedure and the total clearance rate differed significantly ([a]$P < 0.05$, [b]$P < 0.05$). The use of mechanical lithotripsy and ERCP procedure time also differed significantly between the two groups ([c]$P < 0.05$, [d]$P < 0.05$). ESWL: Extracorporeal shock wave lithotripsy; ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct.

Table 3 Extraction methods and success rates after endoscopic retrograde cholangiopancreatography

Extraction method	Success rate		
	ESWL + ERCP (*n* = 124)	ERCP only (*n* = 107)	*P* value
Balloon or Dormia basket	96/99	65/71	0.251
Mechanical lithotripsy	23/25	27/36	0.041[a]
Stone size			
1.5-3.0 cm	102/104	90/93	0.473
⩾ 3.0 cm	12/15	4/10	0.016[b]
Total	119/124 (96.0%)	92/107 (86.0%)	0.029[c]

The use of mechanical lithotripsy differed significantly different between the two groups ([a]$P < 0.05$). The rate of successful clearance of stones sized 1.5-3.0 cm was similar in the two groups, whereas the clearance rate in patients with stones ⩾ 3.0 cm and total stone clearance were both greater in the ESWL + ERCP group ([b]$P < 0.05$, [c]$P < 0.05$). ESWL: Extracorporeal shock wave lithotripsy; ERCP: Endoscopic retrograde cholangiopancreatography.

ESWL + ERCP group and 15 patients in the ERCP-only group). Failure was due to either distal CBD strictures or patient intolerance, and thus these patients were subjected to repeated stenting or surgical treatment. No additional data are available.

Complications

Post-ERCP complication rates were similar in the two groups (6.7% *vs* 6.5%, $P = 0.673$). Complications included pancreatitis (3.3% *vs* 3.6%, $P = 0.357$), cholangitis (2.0% *vs* 2.2%, $P = 0.218$) and haemorrhage (1.9% *vs* 0.7%, $P = 0.074$; Table 4). Complications were mild with no serious consequences. Some patients experienced discomfort related to the presence of the NBT, but this was not recorded as a complication. Pancreatitis and haemorrhage required hospitalization for 1-3 d, while cholangitis was resolved with antibiotic therapy. Intensive care or surgery was not required for any of the patients. Complications related to ESWL (11 cases, 7.0%) included purpuric spots (5 cases, 3.2%) and skin ecchymosis (6 cases, 3.8%). These patients required no treatment and the symptoms generally disappeared within a week. Severe complications such as splenic rupture, ductal perforation and necrotizing pancreatitis did not occur. There was no procedure-related mortality among these patients.

Table 4 Complications in the extracorporeal shock wave lithotripsy + endoscopic retrograde cholangiopancreatography group and the endoscopic retrograde cholangiopancreatography-only group *n*(%)

Complication	ESWL + ERCP	ERCP only	*P* value
Post-ERCP	10/150 (6.7)	9/138 (6.5)	0.673
Pancreatitis (mild)	5/150 (3.3)	5/138 (3.6)	0.357
Cholangitis (mild)	3/150 (2.0)	3/138 (2.2)	0.218
Hemobilia (mild)	2/150 (1.9)	1/138 (0.7)	0.074
Bowel perforation	0	0	
Procedure-related mortality	0	0	
Post-ESWL	11/156 (7.0)		
Purpuric spots	5/156 (3.2)		
Skin ecchymosis	6/156 (3.8)		
Splenic rupture	0		
Lung trauma	0		
Necrotizing pancreatitis	0		
Procedure-related mortality	0		

ESWL: Extracorporeal shock wave lithotripsy; ERCP: Endoscopic retrograde cholangiopancreatography.

DISCUSSION

CBD stones may cause jaundice, cholangitis, pruritus and biliary pancreatitis. The prevalence of CBD stones increases with age and treatment is difficult. At present, surgical choledochotomy is no longer always the therapy of choice due to its invasive character and associated morbidity and mortality. Since the introduction of therapeutic ERCP in 1974, there has been much progress regarding this procedure for treating CBD stones[11]. However, in clinical practice it is not uncommon to see patients with large CBD stones, which cannot be removed by such conventional techniques. For difficult cases, various adjuvant treatments such as ESWL, electrohydraulic lithotripsy and lasers are recommended rather than just using a mechanical lithotripter[12].

Kidney lithotriptors can be used when performing ESWL of bile duct stones, and its efficacy in treating CBD stones has been reported in many studies[13,14]. But for some difficult cases, fragmentation alone may not be adequate because of size and other reasons[15]. Many researchers advised performing ERCP after ESWL to facilitate ductal clearance and decompression, clearing fragments and to address any ductal strictures by balloon dilation with or without stenting[16]. Tao *et al*[17]

reported that using cholecystokinin during ESWL can facilitate endoscopic clearance of large CBD stones. Therefore, ESWL overcomes the problem of the stone size by fragmenting the stones and reducing the stone burden, thus facilitating endoscopic clearance in the following ERCP procedure. According to the literature, complete clearance of CBD stones can be achieved in 40%-75% of the patients[18,19].

Tandan *et al*[20] reported improved stone clearance of large CBD stones using ESWL prior to ERCP. In the study, patients with large CBD stones were subjected to up to 7 ESWL sessions before CBD stones were decomposed to fragments less than 5 mm in diameter. The authors concluded that stones were best fragmented at a kV intensity of 4 (range, 1 to a maximum of 6, corresponding to 11000-16000 kV) and a frequency of 90 shocks/min. Patients were subjected to ESWL at a shock wave frequency of 90/min with continuous saline irrigation contributing to the complete fragmentation of CBD stones.

In our study, patients in the ESWL + ERCP group were subjected to an ESWL session 4 h prior to ERCP. The stones may or may not have been fragmented to a size less than 5 mm in diameter; however, in each case the following ERCP was performed as planned. The results were satisfactory. Although the rate of successful CBD clearance was similar in the first treatment session between the two groups (74.2% *vs* 71%, $P = 0.135$), a session of ESWL prior to ERCP showed a higher clearance rate in the second treatment session (84.4% *vs* 51.6%, $P = 0.018$) and in the overall outcome (96.0% *vs* 86.0%, $P = 0.029$). The rate of mechanical lithotripsy use also differed between the two groups (20% *vs* 30%, $P = 0.025$), potentially accounting for the statistical difference observed in the relative duration of ERCP (43 ± 21 min *vs* 59 ± 28 min, $P = 0.034$). Using the conventional methods (balloon or basket), stone clearance was high in both groups, while the removal of extremely large stones and successful clearance rates of mechanical lithotripsy differed significantly between the two groups (20% *vs* 30%, $P = 0.025$; and 92.0% *vs* 75.0%, $P = 0.041$, respectively). Although these differences might be due to various factors, such as the extent of ERCP, the size of the stone and the dilating balloon, the shape of the stone and the bile duct, we think that including a session of ESWL prior to ERCP is an important tool for reducing the rate of mechanical lithotripsy use and ERCP procedure time. In addition, it shows promise in terms of improving the clearance rate of extremely large stones.

In previous studies, stone fragmentation and clearance were influenced by the presence of a downstream stricture, stone size and location[21]. In our study, there was no significant difference in complete CBD stone clearance according to stone size or location, or accompanying downstream stricture. Strictures in the CBD were dilated using balloons in nearly all the cases. Such procedures did not have any negative impact on stone clearance in our study. Several authors reported that a stent should be placed for bile drainage if CBD stones could not be removed in the first session of ESWL[22-24]. Biliary stent placement has been established as a convenient and minimally invasive treatment for difficult stones, and we adopted it in our series.

Post-ERCP complications such as moderate pancreatitis, cholangitis and mild haemorrhage were similar in the two groups and are consistent with previous reports[25,26]. Post-ERCP pancreatitis is the most common complication of ERCP despite technological developments and improved endoscopist skill levels. The possible causes of the low rate of pancreatitis observed in the present study include proper patient selection for ERCP with appropriate indications and guidewire cannulation[27]. In our study, the presence of NBT helped us selectively cannulate CBD when performing ERCP, thus avoiding cannulating and excessive injection of the CBD. There was no increased incidence of pancreatitis after ERCP in the ESWL + ERCP group.

A number of rare and serious complications have been reported following ESWL[28-31]. These include perirenal hematoma, biliary obstruction, bowel perforation, splenic rupture, lung trauma and necrotizing pancreatitis. These severe complications did not occur in our study, probably because of accurate targeting achieved by the third-generation lithotripter and reduced patient movement. Pain at the site of shock wave delivery, skin ecchymosis, abdominal pain, occasional fever and hemobilia were observed in some of our patients. These complications were mild and minimal, all being managed conservatively without extension of hospital stay.

A recurrence rate of 14% for post-ESWL CBD calculi at 1-year follow-up has been reported[32]. Therefore, stones should be removed as completely as possible. In our study we found that saline irrigation is helpful and should be repeated several times until all the bile drainage is completely clean.

ESWL appears to be a valid, low-cost technique applicable to challenging bile duct stones and its utilization may be extended to urology. Hence, the same device can be used by different medical staff in hospitals, thus reducing management costs.

The main limitation of our study was that we evaluated short-term outcomes, such as successful clearance in the first and second session of ESWL + ERCP and complications of the procedure. However, long-term follow-up of the failed cases was not evaluated and included in this study. Second, the interpretation of the degree of CBD clearance could be subjective. Also, the treatment effect may be influenced by the skills of the treating physician. Other limitations include performing the study at only one centre and the relatively small number of patients. Further prospective randomized studies are therefore needed to prove efficacy and evaluate cost efficacy.

In conclusion, a session of ESWL prior to ERCP is an excellent therapeutic modality for problematic and large common bile duct stones, offering a high clearance rate, particularly in terms of removing extremely large CBD stones. This procedure moreover reduces the use of mechanical lithotripsy and ERCP procedure time. Therefore, we propose that a session of ESWL prior to ERCP is an effective and safe treatment for endoscopic removal of challenging and large CBD stones.

COMMENTS

Background

Extracorporeal shock wave lithotripsy (ESWL) uses electromagnetic waves to fragment problematic and large bile duct stones when endoscopic retrograde cholangiopancreatography (ERCP) fails. Fragmentation was considered satisfactory when the stones were broken down to < 5 mm diameter. For the patients in whom initial ERCP was unsuccessful, a session of ESWL before ERCP may aid the clearance of common bile duct stones.

Research frontiers

The results obtained with third generation electromagnetic lithotripters are optimal; however, technological improvements in lithotripters and other factors favoring stone fragmentation may further enhance our performance.

Innovations and breakthroughs

The authors' work emphasizes, in a wide patient population, that a session of ESWL before ERCP is a safe and effective method to the clearance of bile duct stones, and problematic and large common bile duct stone removal rate was higher in the final outcome. Also mechanical lithotripsy usage was reduced.

Applications

The study is of interest for physicians dealing with the common bile duct stones and particularly those managing problematic and large stone that failed in the first ERCP procedure. Based on these results, a session of ESWL before ERCP was confirmed to be helpful in the treatment of problematic and large common bile duct stones.

Terminology

Mechanical lithotripsy is an effective method of fragmenting stones in the bile duct. Retrieval basket, balloon catheter or passage dilating catheters are usual methods to retract stones and dilate bile duct to facilitate endoscopic clearance of stones.

Peer-review

Overall, the study helps to evaluate the outcome of a session of ESWL before ERCP in treatment of problematic and large common bile duct stones.

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ORIGINAL ARTICLE

Prospective Study

Genetic polymorphisms predict response to anti-tumor necrosis factor treatment in Crohn's disease

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Author contributions: Netz U, Eichenberger MR and Galandiuk S designed the research; Netz U, Carter JV, Eichenberger MR, Dryden GW and Galandiuk S collected the data; Netz U, Carter JV and Eichenberger MR performed the genetic labwork; Netz U, Carter JV, Eichenberger MR, Pan J, Rai SN and Galandiuk S analyzed and interpreted the data; all authors drafted the manuscript and approved the final version.

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Abstract

AIM

To investigate genetic factors that might help define which Crohn's disease (CD) patients are likely to benefit from anti-tumor necrosis factor (TNF) therapy.

METHODS

This was a prospective cohort study. Patients were

recruited from a university digestive disease practice database. We included CD patients who received anti-TNF therapy, had available medical records (with information on treatment duration and efficacy) and who consented to participation. Patients with allergic reactions were excluded. Patients were grouped as ever-responders or non-responders. Genomic DNA was extracted from peripheral blood, and 7 single nucleotide polymorphisms (SNPs) were assessed. The main outcome measure (following exposure to the drug) was response to therapy. The patient genotypes were assessed as the predictors of outcome. Possible confounders and effect modifiers included age, gender, race, and socioeconomic status disease, as well as disease characteristics (such as Montreal criteria).

RESULTS

121 patients were included. Twenty-one were non-responders, and 100 were ever-responders. Fas ligand SNP (rs763110) genotype frequencies, TNF gene -308 SNP (rs1800629) genotype frequencies, and their combination, were significantly different between groups on multivariable analysis controlling for Montreal disease behavior and perianal disease. The odds of a patient with a Fas ligand CC genotype being a non-responder were four-fold higher as compared to a TC or TT genotype (P = 0.009, OR = 4.30, 95%CI: 1.45-12.80). The presence of the A (minor) TNF gene -308 allele correlated with three-fold higher odds of being a non-responder (P = 0.049, OR = 2.88, 95%CI: 1.01-8.22). Patients with the combination of the Fas ligand CC genotype and the TNF -308 A allele had nearly five-fold higher odds of being a non-responder (P = 0.015, OR = 4.76, 95%CI: 1.35-16.77). No difference was seen for the remaining SNPs.

CONCLUSION

The Fas-ligand SNP and *TNF* gene -308 SNP are associated with anti-TNF treatment response in CD and may help select patients likely to benefit from therapy.

Key words: Anti-tumor necrosis factor; Fas ligand; Antibody; Response; Crohn's disease; Single nucleotide polymorphisms; Genotype; Tumor necrosis factor gene

Core tip: Predicting the subset of patients who do not respond to anti-tumor necrosis factor (TNF) treatment is important clinically and economically. Patients with Crohn's disease who received anti-TNF therapy were grouped as ever-responders or non-responders. Genomic DNA was extracted from peripheral blood, and 7 single nucleotide polymorphisms (SNPs) were assessed. 121 patients were included. Twenty-one were non-responders, and 100 were ever-responders. Fas ligand SNP (rs763110) genotype frequencies, *TNF* gene -308 SNP (rs1800629) genotype frequencies, and their combination, were significantly different between groups on multivariable analysis and may help select patients likely to benefit from anti-TNF therapy.

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INTRODUCTION

Crohn's disease (CD) is a transmural chronic inflammatory disease that can affect any part of the alimentary tract, but which often involves the distal ileum.

Anti-tumor necrosis factor-α (anti-TNF-α) monoclonal antibodies are generally used for inducing and maintaining remission and can be used alone or in combination with other drugs[1]. The most common drugs in this group, for CD, are infliximab (chimeric murine - human IgG1 monoclonal antibody targeting TNF-α), adalimumab (fully humanized IgG1 anti-TNF-α monoclonal antibody), and certolizumab pegol (a humanized monoclonal Fab' fragment with a high binding affinity for TNF-α)[2,3].

Although the majority of patients benefit from anti-TNF treatment, approximately one-third of patients treated with an induction dose of anti-TNF do not improve clinically, termed primary non-response[3]. An additional significant population who initially respond to treatment eventually lose responsiveness, termed a secondary non-response.

Identifying patients who will fail treatment with anti-TNF agents is of significant importance both from a clinical and economic perspective. Anti-TNF drugs have been associated with an increased risk of opportunistic infections, melanoma, and lymphoma[4-6]. Anti-TNF treatment is also very expensive, with 2013 annual per patient costs for adalimumab and infliximab at approximately $25000 and $24000 respectively[7,8].

Factors associated with the success of anti-TNF treatment include shorter disease duration, inflammatory (as opposed to fibrostenotic) disease phenotype, isolated colonic disease, young age, non-smoking status, as well as a serum high C-reactive protein that returns to normal after initiation of treatment[9-11]. Non-response can be due to multiple factors such as an alternative non-TNF mediated pathway of inflammation, due to a differential role of TNF in certain stages of disease and/or due to the presence or development of anti-drug antibodies. Additionally, individual differences in drug bioavailability and pharmacokinetics can be factors associated with non-response[9].

A possible conduit to predict response to anti-TNF therapy could be through genetic testing. Several genes have been implicated in the pathogenesis of

CD, including NOD2 and ATG16L1[12,13]. There is, however, limited data on the ability to predict anti-TNF treatment response in CD based upon genetic data. Some genes have been investigated without success[14]. Our aim was to investigate genetic factors that might help define which CD patients are likely to benefit from anti-TNF therapy and permit efficient and cost-effective treatment. We hypothesized that specific single nucleotide polymorphism (SNP) genotypes are associated with anti-TNF treatment response in patients with CD. We chose to examine a series of SNPs within genes that have been linked either with CD and/or with anti-TNF treatment response in order to determine whether these could aid in predicting response to anti-TNF treatment in CD patients.

MATERIALS AND METHODS

This study complies with the STROBE guidelines and the extension for genetic association studies[15].

Patient recruitment and data collection

This is a prospective cohort study approved by the University of Louisville Institutional Review Board. All patients signed a written informed consent. Consecutive patients with a diagnosis of CD were identified from a large prospectively maintained genetic database, from a large University digestive disease practice, encompassing the period 1/1998 to 4/2016. Inclusion criteria were CD patients who had received anti-TNF therapy, and whose medical records were available, with information about receipt of anti-TNF therapy, its duration, efficacy, and cessation where applicable. Included patients received appropriate drug doses and had a follow-up of at least 12 mo following treatment initiation[16,17]. Patients were excluded if anti-TNF treatment was stopped due to side-effects, local and/or systemic allergy, or if it was impossible to distinguish from the medical records whether the drug worked.

Additional data collected from the medical records included gender, race, socioeconomic status (patient's zip code of residence was used to obtain median household income based on United States census data from the American Community Survey 2014 - 5 year estimates)[18], surgical history, and clinical state of the disease according to the Montreal classification for CD, including age at diagnosis, location, disease behavior, and the presence or absence of perianal disease[19].

The main outcome measure (following exposure to the drug) was response to therapy. Participants were grouped as ever-responders if they had initial response to anti-TNF treatment (even if this was later lost due to antibody formation) or non-responders in accordance with the treating physician decision. The patient genotypes (see below) were assessed as the predictors of outcome. Possible confounders and effect modifiers included age, gender, race, and socioeconomic status disease, as well as disease characteristics (such as Montreal criteria).

DNA extraction

Peripheral blood was collected by venipuncture (after written informed consent) in EDTA-vacutainers (BD, Franklin Lakes, NJ, United States) and stored at 4 ℃ until further use.

Genomic DNA was extracted from blood samples using the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Pittsburgh, PA, United States) using the manufacturer's protocol[20]. Briefly, the blood was initially diluted with PBS buffer. Blood was then lysed: 1 μL of diluted blood was lysed with 1 μL of cell lysis solution (400 mmol/L KOH, 10 mmol/L EDTA, 100 mmol/L DTT), followed by the addition of 1 μL of neutralization buffer (400 mmol/L HCl, 600 mmol/L Tris-HCl, pH 7.5). Whole genome amplification was then performed: 17 μL of master mix [7 μL sample buffer, 9 μL reaction buffer, and 1 μL enzyme mix from the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Pittsburgh, PA, United States)] was added to each sample for a total reaction volume of 20 μL. Amplification was performed according to the following program: 30 ℃ for two hours, followed by 65 ℃ for 10 min, then cooled to 4 ℃. Following whole genome amplification, DNA concentration was determined using NanoDrop® 2000 spectrophotometry. The samples were diluted and stored at -20 ℃ until analysis.

SNP genotyping

SNPs selection: A PubMed literature search was conducted using the keywords "tumor necrosis factor-alpha", "anti-TNF", "infliximab", "adalimumab", "polymorphism", "Crohn's disease", "response", "biomarker" using Boolean operators (AND), (OR), (NOT). Results were narrowed down to original studies investigating SNPs including frequency of alleles and genotypes for different groups. We included SNPs that had demonstrated association with CD or anti-TNF treatment, those that had biological relevance, and those that had an expected minor allele frequency ⩾ 5%. Both new genetic associations and previously described efforts were investigated. SNPs were excluded if they had been extensively investigated and if there was no prognostic value for the combination of CD and anti-TNF treatment response. As a result of this search, the following seven SNPs within 5 genes were selected for study and assessed in each patient's DNA sample: ATG16L1 (rs10210302, T300A rs2241880), Fas ligand (-843 rs763110), IBD5 (rs2522057), FCGR 3A (rs396991), and TNF (-308 rs1800629, -238 rs361525).

SNP assessment was performed using TaqMan® predesigned genotyping assays (Life Technologies®, Carlsbad CA)[21]. The TaqMan® genotyping assays were diluted to a 20× working stock solution with 1× TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0, in DNase-free, sterile-filtered water) and stored at -20 ℃, as recommended by the manufacturer.

MicroAmpR Fast Optical 96-well reaction plates (Applied Biosystems, Foster City, CA, United States)

Table 1 Hardy weinberg equilibrium *n* (%)

SNP	Genotype distribution		Hardy Weinberg equilibrium	
	Genotype	Frequency	Expected frequency (*n*)	*P* value[1]
ATG16L1	CC	25 (21.0)	24	NS
rs10210302	TC	57 (47.9)	59	
	TT	37 (31.1)	36	
ATG16L1 rs2241880	AA	27 (22.5)	26	NS
	GA	57 (47.5)	60	
	GG	36 (30.0)	35	
Fas Ligand	CC	42 (36.5)	41	NS
rs763110	TC	54 (47.0)	55	
	TT	19 (16.5)	18	
IBD5 rs2522057	CC	35 (29.2)	29	0.045
	GC	49 (40.8)	60	
	GG	36 (30.0)	31	
FCGR 3A rs396991	AA	70 (58.3)	62	0.0005
	AC	33 (27.5)	48	
	CC	17 (14.2)	9	
TNF gene (-308)	AA	4 (3.4)	3	NS
rs1800629	GA	31 (26.1)	33	
	GG	84 (70.6)	83	
TNF gene (-238)	AA	2 (1.7)	1	NS
rs361525	GA	15 (12.5)	17	
	GG	103 (85.8)	102	

[1]Calculated using χ^2. TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

were used. Six microliters of master mix was used for each assay (5.5 μL of TaqMan® Universal Master Mix Ⅱ, no UNG [Applied Biosystems™] together with 0.5 μL of 20 × working assay [TaqMan® predesigned Genotyping Assays]). Five microliters of DNA (4.5 ng/μL) was added to the plate. PCR reactions were performed using a Step-One Plus® RT-PCR System (Life Technologies®, Carlsbad, CA, United States) and the following program: 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s, and then 60 ℃ for 1 min. Analysis was performed using Step-One Plus® software v2.1 (applied Biosystems, Foster City, CA, United States). Each genotype was independently assigned by two investigators. In cases of disagreement, assignment was reached by consensus. All laboratory work and genotyping was done at the Price Institute of Surgical Research, Louisville Kentucky, United States.

Statistical analysis

Descriptive and analytical statistics were performed using SAS version 9.4 statistical software[22]. Genotype frequencies, demographic, and disease characteristics were compared using a χ^2 test (or Fisher's exact test for 2 × 2 tables). Socioeconomic status was calculated according to the national percentile of the patient's median household income divided into quartiles (0-25, 26-50, 51-75 and 76-100) and compared using a χ^2 test. Comparison of continuous variables was performed using a two-sample *t*-test or ANOVA. In order to explore for the presence of bias in the cohort, a group of contemporary subjects who did not receive anti-TNF treatment were compared with patients included in this study. Following this, characteristics between ever-responders and non-responders in the study group were then compared[23].

Hardy-Weinberg equilibrium was determined for each SNP (Table 1). Univariable logistic regression was modeled for the probability of anti-TNF treatment failure for each covariate. Multivariable logistic regression models were used for separate SNPs and covariates exhibiting a trend towards a significant difference ($P < 0.15$)[24]. Final models included OR and 95%CI. A *P*-value of < 0.05 was considered statistically significant.

The statistical methods of this study were reviewed by Pan J and Rai SN.

RESULTS

Patient demographics

Table 1 shows a flow diagram of patient selection; 121 patients were selected for study. Of these, 21 (17.4%) patients were primary non-responders to anti-TNF treatment and 100 (82.6%) patients were ever-responders to anti-TNF treatment. A quarter of these initial ever-responders (25/100) lost response at a later time and were termed secondary non-responders. The patient population was predominantly Caucasian (92.6%), with a higher proportion of women (58.7%) (Table 2). With regards to clinical parameters, 90/121 (74.4%) patients were diagnosed between the ages of 17 and 40 years of age (Montreal A2). Most CD patients, 74/121 (61.2%), had combined ileocolonic disease (Montreal L3), whereas 15/121 (12.4%) had isolated ileal disease (Montreal L1), and 32/121 (26.4%) had only colonic disease (Montreal L2). Only 3/121 (2.5%) patients had upper GI (Montreal L4) involvement, all of whom were responders. Montreal L4 disease was analyzed separately from L1-3, due to the fact that, according to the Montreal classification, it is not mutually exclusive and can be added to any of the other locations when concomitant upper GI disease is present[19]. The population was fairly evenly distributed with respect to disease behavior with 36/121 (30%) patients having non-stricturing, non-penetrating disease (Montreal B1), 45/121 (37%) patients having stricturing disease (Montreal B2), and 40/121 (33%) patients having penetrating disease (Montreal B3). In addition, 37 of 121 (31%) patients had perianal disease (Montreal *P* designation). Table 2 shows the clinical and demographic data of the participants, as well as these data for the non-responder and ever-responder groups. None of the clinical or demographic characteristics were significantly different between these 2 groups.

Presence of bias

When comparing the characteristics of the patients who received anti-TNF treatment and included in the study ($n = 121$) with those who did not receive anti-TNF treatment ($n = 152$) in order to ascertain the

Table 2 Clinical and demographic patient characteristics *n* (%)

Variables	Total	Anti-TNF treatment		*P* value
		Non-responders	Ever responders	
Total	121 (100)	21 (17)	100 (83)	NA
Patient demographics				
Gender				
Female	71 (59)	15 (71)	56 (56)	NS
Male	50 (41)	6 (29)	44 (44)	
Race				
Caucasian	112 (93)	21 (100)	91 (91)	NS
African American	9 (7)	0 (0.0)	9 (9)	
Socioeconomic status[1]				
1st Quartile	24 (20)	4 (19)	20 (20)	NS
2nd Quartile	40 (33)	6 (29)	34 (34)	
3rd Quartile	29 (24)	3 (14)	26 (26)	
4th Quartile	28 (23)	8 (38)	20 (20)	
Montreal classification				
Age of onset (A)				
A1 - below 16 years old	14 (12)	1 (5)	13 (13)	NS
A2 - between 17 and 40 years old	90 (74)	17 (81)	73 (73)	
A3 - above 40 years old	17 (14)	3 (14)	14 (14)	
Location (L)				
L1 - ileal	15 (12)	1 (5)	14 (14)	NS
L2 - colonic	32 (26)	5 (24)	27 (27)	
L3 - ileocolonic	74 (61)	15 (71)	59 (59)	
Location (L4) upper				
No upper GI disease	118 (98)	21 (100)	97 (97)	NS
L4 - upper GI disease	3 (2)	0 (0)	3 (3)	
Behavior (B)				
B1 - non-stricturing, non-penetrating	36 (30)	4 (19)	32 (32)	0.1
B2 - stricturing	45 (37)	12 (57)	33 (33)	
B3 - penetrating	40 (33)	5 (24)	35 (35)	
Behavior (p) perianal disease				
No perianal disease	84 (69)	18 (86)	66 (66)	0.08
p - perianal disease present	37 (31)	3 (14)	34 (34)	
Anti-TNF treatment type				
Drugs received				
Infliximab	46 (38)	10 (48)	36 (36)	NS
Adalimumab	45 (38)	5 (24)	40 (40)	
Infliximab and Adalimumab[2]	29 (24)	6 (29)	23 (23)	
Certolizumab pegol	5	0	5	

[1]Calculated according to the national percentile of median household income; [2]Received sequentially. TNF: Tumor necrosis factor.

presence of bias, no difference was found in 3 of the 4 variables examined: gender (P = 0.27), race (P = 0.95), or socioeconomic status (P = 0.23). The patients included in the study who received anti-TNF treatment were, however, younger (41.6 years old, 95%CI: 39.2-44.0) than those that did not receive anti-TNF treatment (49.4 years old, 95%CI: 47.1-51.7) (P < 0.001).

We assessed 7 different SNPs associated with 5 different genes and observed less than 5% technical failure rate in all assays. Table 3 shows the SNPs tested as well as their genotype and allele distribution. Comparison of genotypes between ever responders and non-responders (Table 4) identified a significant difference in the Fas ligand SNP rs763110 genotypes (P = 0.042). Patients with a CC genotype (as compared to those with a TC or TT genotype) were more likely to be non-responders to anti-TNF treatment, (P = 0.016; OR = 0.31, 95%CI: 0.11-0.83). Genotypes of another SNP, such as -308 (rs1800629), within the *TNF* gene demonstrated a trend towards correlation with response to anti-TNF treatment (P = 0.088) when grouping genotypes AA and GA compared to genotype GG (P = 0.093, OR = 2.29, 95%CI: 0.85-6.17).

The vast majority of participants were Caucasian. Only 9 patients were African American, all of whom were ever-responders. Analyzing the Caucasians separately as a sensitivity analysis achieved similar results for the grouped Fas ligand SNP (P = 0.029) and for the grouped -308 *TNF* gene SNP (P = 0.049). No significant difference was observed for the remaining SNPs studied: ATG16L1 (rs10210302, T300A rs2241880), IBD5 (rs2522057), FCGR 3A (rs396991), and TNF (-238 rs361525).

Results of the univariable comparisons are shown in Table 5. In univariable analyses, the Fas ligand SNP (rs763110) demonstrated a difference between ever-responders and non-responders with borderline significance (P = 0.058) and significance when grouping TC and TT genotypes together (P = 0.020). The

Table 3 Single nucleotide polymorphisms tested with genotype and allele distribution for entire patients group *n* (%)

SNP	Location relative to gene (Chromosome number)	Nucleotide change	Assay successful	Genotype distribution		Allele distribution	
				Genotype	Frequency	Allele	Frequency
ATG16L1 rs10210302	2 kb upstream (2)	C/T	119 (98)	CC	25 (21.0)	Total	238
				TC	57 (47.9)	C	107 (45.0)
				TT	37 (31.1)	T	131 (55.0)
ATG16L1 rs2241880	Thr300Ala (2)	A/G	120 (99)	AA	27 (22.5)	Total	240
				GA	57 (47.5)	A	111 (46.3)
				GG	36 (30.0)	G	129 (53.7)
FAS ligand rs763110	-843 (1)	C/T	115 (95)	CC	42 (36.5)	Total	230
				TC	54 (47.0)	C	138 (60.0)
				TT	19 (16.5)	T	92 (40.0)
IBD5 rs2522057	Intergenic region (5)	C/G	120 (99)	CC	35 (29.2)	Total	240
				GC	49 (40.8)	C	119 (49.6)
				GG	36 (30.0)	G	121 (50.4)
FCGR 3A rs396991	Phe175Val (1)	C/G	120 (99)	AA	70 (58.3)	Total	240
				AC	33 (27.5)	A	173 (72.1)
				CC	17 (14.2)	C	67 (27.9)
TNF gene (-308) rs1800629	promotor region (6)	A/G	119 (98)	AA	4 (3.4)	Total	238
				GA	31 (26.1)	A	39 (19.6)
				GG	84 (70.6)	G	199 (80.4)
TNF gene (-238) rs361525	promotor region (6)	A/G	120 (99)	AA	2 (1.7)	Total	240
				GA	15 (12.5)	A	19 (7.9)
				GG	103 (85.8)	G	221 (92.1)

TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

Table 4 Single nucleotide polymorphism genotypes according to anti-tumor necrosis factor treatment response

SNP	Genotype	Anti-TNF treatment response		*P* value[1]
		Non-responders	Responders	
ATG16L1 rs10210302	CC	5 (25.0)	20 (20.2)	NS
	TC	9 (45.0)	48 (48.5)	
	TT	6 (30.0)	31 (31.3)	
ATG16L1 rs2241880	AA	5 (23.8)	22 (22.2)	NS
	GA	10 (47.6)	47 (47.5)	
	GG	6 (28.6)	30 (30.3)	
FAS Ligand rs763110	CC	12 (60.0)	30 (31.6)	0.042
	TC	5 (25.0)	49 (51.6)	
	TT	3 (15.0)	16 (16.8)	
FAS Ligand rs763110 (grouped)	CC	12 (60.0)	30 (31.6)	0.016 OR = 3.23, 95%CI: 1.20-8.78
	TC + TT	8 (40.0)	65 (68.4)	
IBD5 rs2522057	CC	6 (28.6)	29 (29.3)	NS
	GC	9 (42.9)	40 (40.4)	
	GG	6 (28.6)	30 (30.3)	
FCGR 3A rs396991	AA	11 (52.4)	59 (59.6)	NS
	AC	6 (28.6)	27 (27.3)	
	CC	4 (19.0)	13 (13.1)	
TNF gene (-308) rs1800629	AA	2 (10.0)	2 (2.0)	0.088
	GA	7 (35.0)	24 (24.2)	
	GG	11 (55.0)	73 (73.7)	
TNF gene (-308) rs1800629 (grouped)	AA + GA	9 (45.0)	26 (26.3)	0.093 OR = 2.29, 95%CI: 0.85-6.17
	GG	11 (55.0)	73 (73.7)	
TNF gene (-238) rs361525	AA	0 (0.0)	2 (2.0)	NS
	GA	2 (10.0)	13 (13.0)	
	GG	18 (90.0)	85 (85.0)	

[1]Calculated using χ^2. TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

comparison of -308 SNP (rs1800629) genotypes between ever-responders and non-responders (P = 0.130) became more different when grouping AA and GA genotypes together (P = 0.099). Univariate variables with P < 0.15 were included in the multivariable analysis for the comparison between anti-TNF treatment ever-responders and non-responders. Both Montreal disease behavior (P = 0.125) and perianal disease classification (P = 0.086) were included in the multivariable analysis.

Table 5 Univariable logistic regression data regarding factors associated with anti-tumor necrosis factor treatment failure

Covariate	*P* value
Demographic variables	
Gender	NS
Race	NS
Montreal Classification	
Age of onset (A) Montreal	NS
Location (L) Montreal	NS
Location (L4) upper GI Montreal	NS
Behavior (B) Montreal	0.13[1]
Behavior (p) perianal Montreal	0.09[1]
SNP variables	
ATG16L1 rs10210302	NS
ATG16L1 rs2241880	NS
FAS Ligand rs763110	0.057[1]
FAS Ligand rs763110 (TC + TT grouped)	0.02[1]
IBD5 rs2522057	NS
FCGR 3A rs396991	NS
TNF gene (-308) rs1800629	0.13[1]
TNF gene (-308) rs1800629 (AA + GA grouped)	0.099[1]
TNF gene(-238) rs361525	NS

[1]Covariates included in the subsequent multivariable analysis. TNF: Tumor necrosis factor; SNPs: Single nucleotide polymorphisms.

Logistic multivariable regression models were developed for the Fas ligand (rs763110) SNP, the *TNF* gene -308A/G (rs1800629) SNP, and their combination. The multivariable logistic regression models included genotype data for each of these two SNPs (with genotypes grouped as described above), the Montreal disease behavior classification, and the Montreal perianal disease classification (Table 6). The Fas ligand SNP (rs763110) CC genotype was predictive of non-response, as compared to the TC and TT genotypes (*P* = 0.009, OR = 4.30, 95%CI: 1.45-12.80). In the -308 *TNF* gene (rs1800629) SNP multivariable model, the AA and GA genotypes were significantly predictive of non-response as compared to the GG genotype (*P* = 0.049, OR = 2.88, 95%CI: 1.01-8.22). Patients with the combination of the Fas ligand (rs763110) CC genotype and presence of the TNF -308 A allele (genotypes AA or GA as opposed to GG) had nearly five-fold higher odds of being non-responders (*P* = 0.015, OR = 4.76, 95%CI: 1.35-16.77). This occurred in 16 (13%) of our patients. Montreal disease behavior and the presence of perianal disease were not found to be predictive in any of the multivariable models.

DISCUSSION

We identified two SNPs, Fas Ligand SNP (rs763110) and the *TNF* gene -308 (rs1800629), as being associated with CD patient response to anti-TNF treatment.

The Fas ligand SNP (rs763110) genotype frequencies were significantly different between non-responders and ever-responders (*P* = 0.042). This association became more significant when grouping the TC and TT genotypes as compared to the CC genotype (*P* = 0.016). According to our multivariable analysis, the odds of a patient with a Fas ligand CC genotype being a non-responder were four-fold higher as compared with a TC or TT genotype (*P* = 0.009, OR = 4.30, 95%CI: 1.45-12.80), when controlling for both Montreal disease behavior and perianal disease classification.

Abnormal regulation of apoptosis is one of the mechanisms of CD pathogenesis. Apoptosis (programmed cell death) can be induced through both extrinsic and intrinsic pathways[25]. The extrinsic pathway is controlled through plasma membrane receptors belonging to the TNF receptor superfamily that include, among others, the Fas/Fas ligand which has been implicated in inflammatory bowel disease (IBD)[26,27]. The SNP that we examined (rs763110) in the -843 position, which was located in a binding motif for the transcription factor CAAT/enhancer-binding protein β, has been implicated in carcinogenesis through the dysregulation of apoptosis[28]. Higher basal expression of Fas ligand has been significantly associated with the C allele compared with the T allele of this SNP[29]. The mechanism of action of anti-TNF drugs is complex; affecting many pathways, involving both soluble and membrane - bound TNF[30]. Currently, anti-TNF treatments have not been linked directly with Fas ligand; however, an interaction is possible either directly with the Fas ligand or indirectly by affecting the cells upon which the Fas ligand acts. Hlavaty *et al*[31] examined response to infliximab and the Fas ligand (rs763110) SNP and found the TT genotype to be correlated with non-response.

The *TNF* gene SNP was also found to be associated with response to anti-TNF treatment. Two polymorphisms: -308 (rs1800629) and -238 (rs361525), both in the promotor region have previously had conflicting data reported with respect to response to anti-TNF treatment in rheumatoid arthritis and in IBD[11,32-35]. The -308 (rs1800629) SNP has been shown previously to affect regulation of TNFα synthesis, with the minor allele (A) being a powerful transcriptional activator associated with increased TNFα production with the common allele (G)[36,37]. Our study demonstrated a possible correlation with anti-TNF treatment response with the -308 (rs1800629) SNP. Separately, -308 (rs1800629) when examined by itself demonstrated only a trend towards significance, but when combined with disease behavior and perianal disease, a significant correlation was demonstrated (*P* = 0.049, OR = 2.88, 95%CI: 1.01-8.22). The presence of the AA and GA genotypes (A being the minor allele) was correlated with non-response, implying a 2.88 higher odds of being a non-responder to anti-TNF treatment if the patient has an A allele. The fact that the A allele is observed fairly infrequently (19.6% in our study, and 9%-16% in others[38]) may explain the borderline statistical significance of this finding. Patients with the combination of the Fas ligand CC genotype and the TNF -308 A allele had nearly five-fold higher odds of being non-responders (*P* = 0.015, OR = 4.76, 95%CI: 1.35-16.77). This combination gives an

Table 6 Multivariable logistic regression models predicting anti-tumor necrosis factor treatment failure

Model	Variable	Class	Estimate	Global *P* value	OR (95%CI)
a	FAS ligand rs763110 (TC + TT grouped)	CC	1.460	0.009	4.30 (1.45-12.80)
		TC+TT	0		
	Montreal behavior (B)	B1	-0.083	NS	0.92 (0.21-3.96)
		B2	1.158		3.18 (0.89-11.34)
		B3	0		
	Montreal behavior (p) perianal	No	0.964	NS	2.62 (0.68-10.09)
		Yes	0		
b	*TNF* gene (-308) rs1800629 (AA+GA grouped)	AA+GA	1.056	0.049	2.88 (1.01-8.22)
		GG	0		
	Montreal Behavior (B)	B1	-0.153	NS	0.86 (0.20-3.59)
		B2	0.826		2.28 (0.68-7.61)
		B3	0		
	Montreal Behavior (p) perianal	No	1.213	NS	3.36 (0.87-12.93)
		Yes	0		
c	FAS ligand (CC genotype) and *TNF* gene -308 (AA or GA genotype) combined	CC and AA or GA	1.560	0.015	4.76 (1.35-16.77)
		Other	0		
	Montreal behavior (B)	B1	-0.022	NS	0.98 (0.23-4.22)
		B2	0.950		2.59 (0.73-9.17)
		B3	0		
	Montreal behavior (p) perianal	No	0.970	NS	2.64 (0.69-10.10)
		Yes	0		

additive effect compared to each SNP separately, most likely acting by different mechanisms. Considering that they are also found on different chromosomes (1 and 6), they are also in all probability inherited independently. The -238 TNF gene SNP (rs361525) did correlate with response to anti-TNF treatment. This combination was found in 16 patients in our study (13%).

The *ATG16L1* gene has been well described in CD and has an important role in autophagy[39]. A SNP in this gene (rs2241880) has been linked to diminished autophagy, predisposing to CD[40]. Our study did not show an association with anti-TNF treatment response for this SNP, nor for another SNP on this gene (rs10210302) that had shown promise as a predictor for response to adalimumab in Slovenian CD patients[41].

Another region, the *5q31* gene cluster (IBD5), has been described to confer CD risk in some populations[42-44]. A SNP in this cluster (rs2522057) may be associated with response to infliximab in CD[45]. We were, however, unable to demonstrate such a correlation.

Antibody-dependent cell-mediated cytotoxicity is important in the mechanism of action of anti-TNF drugs. It requires leukocyte receptors for the Fc portion of IgG. A polymorphism (rs396991) in the gene encoding FCGR 3A expressed on macrophages and natural killer cells was associated with the response to rituximab in follicular non-Hodgkin's lymphomas[46]. This polymorphism has also been associated with the response to infliximab in CD[47], but this was not able to be confirmed in our study.

Our study population included patients from a large university digestive practice. The patients included in the study and receiving anti-TNF drugs were younger than the excluded non-anti-TNF treated patients but were comparable with respect to race, gender, and socioeconomic status. Their younger age can be explained by the fact that younger patients tend to have a more severe disease[1] and would probably require anti-TNF drugs more frequently. Since patients were comparable in other aspects, we believe there was no selection bias. All CD patients were followed by gastroenterologists and surgeons, and as such, had a higher incidence of complicated CD. This may be a source of potential bias. This may also explain why we did not observe a difference with respect to likelihood of anti-TNF treatment response and disease behavior which has been observed in prior studies[9]. The clinical allocation into groups was performed by experienced clinicians (Galandiuk S, Dryden GW), who deal with this population on a daily basis. The assessment was made on the basis of the patient history, physical exam, patient follow-up, lab-work and endoscopy/pathology when clinically indicated. This study assessed non-response (no improvement whatsoever) *vs* ever-response. Since this was not a formal clinical trial and since only a quarter of patients had colonic disease, colonoscopy was not performed at defined intervals, but at the discretion of the treating physician. This could be a source of bias, but we believe that clinical assessments based on the above criteria are valid.

In summary, identification of patients whose anti-TNF treatment will fail is important, both from a clinical and from an economic perspective. We have identified two functional SNPs, Fas ligand (rs763110) and the *TNF* gene -308 (rs1800629), associated with non-response to anti-TNF treatment. Genotyping these SNPs from DNA obtained from peripheral blood may help define which CD patients are likely to benefit from anti-TNF therapy and permit efficient and cost-effective treatment by avoiding expensive therapy that is likely to fail and permitting selection of other treatments

more likely to succeed.

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COMMENTS

Background

Anti-tumor necrosis factor (TNF) agents will not be effective in a subset of patients with Crohn's disease (CD). Predicting the subset of patients who do not respond to anti-TNF treatment is important clinically and economically.

Research frontiers

A possible conduit to predict response to anti-TNF therapy could be through genetic testing. The authors chose to examine a series of single nucleotide polymorphisms (SNPs) within genes that have been linked either with CD and/or with anti-TNF treatment response in order to determine whether these could aid in predicting response to anti-TNF treatment in CD patients.

Innovations and breakthroughs

Two SNPs Fas ligand and *TNF* gene -308 were associated with response to anti-TNF treatment.

Applications

Genotyping these SNPs from DNA obtained from peripheral blood may help define which CD patients are likely to benefit from anti-TNF therapy and permit efficient and cost-effective treatment by avoiding expensive therapy that is likely to fail and permitting selection of other treatments more likely to succeed.

Terminology

Single nucleotide polymorphisms are a type of genetic variation in which a change is found in a single nucleotide at a specific position in the genome.

Peer-review

The authors investigate genetic factors that might help define which CD patients are likely to benefit from anti-TNF therapy. This is an interesting paper.

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ORIGINAL ARTICLE

Prospective Study

New formula for predicting standard liver volume in Chinese adults

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Author contributions: Feng LM, Wang PQ and Yu H contributed equally to this work; Feng LM collected all the clinical data and determined the morphological indices of computed tomography (CT), analyzed the data, participated in drafting the manuscript and prepared the revised materials; Wang PQ drafted the manuscript and assisted with data analysis; Yu H provided the CT instruments and measurements; Chen RT was involved with drawing the morphological indices of CT; Wang J participated in design of the study; Sheng X, Yuan ZL and Shi PM were involved with data collection; Xie WF helped in design and oversight of the study; Zeng X participated in design and oversight of the study and was involved with drafting of the manuscript.

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Abstract

AIM

To obtain a reference range of morphological indices and establish a formula to accurately predict standard liver volume (SLV) in Chinese adults.

METHODS

Computed tomography (CT)-estimated total liver volume (CTLV) was determined in 369 Chinese adults. Age, sex, body weight, body height, body mass index, and body surface area (BSA) were recorded using CT. Total splenic volume, portal venous diameter (PVD), splenic venous diameter (SVD), and portal venous cross-sectional area (PVCSA) were also measured by CT. Stepwise multiple linear regression analysis was performed to evaluate the impact of each parameter on

CTLV and to develop a new SLV formula. The accuracy of the new formula was compared with the existing formulas in a validation group.

RESULTS

The average CTLV was 1205.41 ± 257.53 cm^3 (range, 593.80-2250.10 cm^3). The average of PVD, SVD and PVCSA was 9.34 ± 1.51 mm, 7.40 ± 1.31 mm and 173.22 ± 48.11 mm^2, respectively. The CT-estimated splenic volume of healthy adults varied markedly (range, 46.60-2892.30 cm^3). Sex, age, body height, body weight, body mass index, and BSA were significantly correlated with CTLV. BSA showed the strongest correlation (r = 0.546, P < 0.001), and was used to establish a new model for calculating SLV: SLV (cm^3) = 758.259 × BSA (m^2)-124.272 (R^2 = 0.299, P < 0.001). This formula also predicted CTLV more accurately than the existing formulas, but overestimated CTLV in elderly subjects > 70 years of age, and underestimated liver volume when CTLV was > 1800 cm^3.

CONCLUSION

Our new BSA-based formula is more accurate than other formulas in estimating SLV in Chinese adults.

Key words: Standard liver volume; Reference range; Morphological indices; Chinese adults

Core tip: This was a prospective international phase Ⅱ trial with 16 patients seeking to evaluate the effect of selenomethionine on acute toxicity in the setting of concurrent chemoradiaiton for locally advanced, inoperable non-small cell lung cancer. Selenium proved to be well tolerated and led to significantly reduced rates of myelosuppression.

Feng LM, Wang PQ, Yu H, Chen RT, Wang J, Sheng X, Yuan ZL, Shi PM, Xie WF, Zeng X. New formula for predicting standard liver volume in Chinese adults. *World J Gastroenterol* 2017; 23(27): 4968-4977 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/4968.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.4968

INTRODUCTION

Liver volume, which can indirectly reflect liver function, is an important clinical indicator of liver disease. Changes in liver volume correlate with the prognosis and severity of liver cirrhosis, and a lower mean liver volume was observed in Child-Pugh C patients[1-3]. In liver transplantation, liver graft volume is one of the major factors determining outcome. A liver graft that is too large for the recipient will lead to poor perfusion, while a graft that is too small may cause postoperative small-for-size syndrome, primary non-function, and even severe liver failure[4-6]. Therefore, accurate estimation of total liver volume (TLV) is an important consideration for clinical condition assessment, pharmacological applications, and a variety of surgeries, especially liver transplantation.

Although liver volume can be measured by the water overflow method or calculated indirectly by liver weight[7], these methods are limited to autopsy or intraoperative use. Numerous other methods have been developed for noninvasive measurement of liver volume based on imaging, including ultrasonography[8], computed tomography (CT), and magnetic resonance imaging (MRI)[1,9,10]. CT volumetric analysis is the most frequently used among these methods. The development of helical CT, refinements in imaging techniques, and the availability of sophisticated software for three-dimensional reconstruction has improved the estimation deviation of liver volume to within 5%[11,12]. Thus, CT liver volumetry is considered the gold standard for measuring TLV. Measurement of liver volumes based on CT analysis is also used in liver resection[6,13-15] and transplantation[16,17]. However, the associated post-procedural image processing of CT volumetry is costly, time-consuming, and has not yet been successfully automated, which are obstacles in some clinical settings, particularly in developing countries.

Due to the large variability in liver size based on race, sex, body shape and body size, the difference in actual liver volume and standard liver volume (SLV) is considered more sensitive than TLV alone[1,8,18,19]. Therefore, it is important to establish SLV criteria for healthy individuals. Since DeLand *et al*[20] first presented their formula for calculating liver size, at least 15 other formulas have been proposed for estimating SLV. However, as yet, no single formula is statistically comparable with the TLV for the absolute volume and the percentage error reported by Pomposelli *et al*[18]. Furthermore, the SLV formulas derived from various ethnic groups may not be comparable with each other. Indeed, discrepancies have been observed between formulas for various Asian populations[18,19]. Thus, at present there are no formulas for assessing SLV that are generally accepted in Chinese or Western centers[18,19]. Other morphological indices, including total splenic volume (TSV), portal venous diameter (PVD), splenic venous diameter (SVD) and portal venous cross-sectional area (PVCSA), have been used as indirect measures of portal vein (PV) pressure. However, as for TLV, differences in race, sex, body shape and size, and measurement method can also affect these indices. The aim of this study was to obtain a reference range of TLV and other morphological indices for healthy

Chinese adults, and to establish a new formula for assessing SLV in the Chinese population that is more accurate than current formulas.

MATERIALS AND METHODS

Data source

Patients who underwent CT examination between October 2014 and January 2016 in Shanghai Changzheng Hospital, Shanghai, China and who met the following criteria were included in the analysis. Eligibility criteria were as follows: (1) > 18 years of age; (2) normal liver function and no history of liver disease; and (3) either sex. Exclusion criteria were: (1) presence of hepatitis B or hepatitis C virus infection, alcoholic liver disease, non-alcoholic fatty liver disease, autoimmune liver disease, and hepatitis caused by other etiology; (2) liver cirrhosis or other liver diseases; (3) patients with confirmed or highly suspected diagnosis of primary or secondary liver cancer; (4) history of diseases affecting TLV or total spleen volume, such as cysts, hemangioma, and other benign lesions in the liver and spleen (1 cm in diameter, and 2 in number), hematologic diseases, connective tissue disorders, or history of severe infectious diseases; and (5) conditions with a hemodynamic effect on the PV, including PV thrombosis, embolism, or sponge appearance change.

A total of 244 subjects were included to establish a new formula. A further 125 subjects were included to validate the formula. For each subject, body weight (BW; kg) and body height (BH; m) were recorded at the time of CT examination. Body mass index (BMI) was calculated as: $BMI = BW/BH^2$. Body surface area (BSA) was calculated using Mosteller's formula[21] as follows: $BSA = \sqrt{BW\ (kg) \times BH\ (cm)/3600}$.

The study protocol was reviewed and approved by the Institutional Ethics Committee of Shanghai Changzheng Hospital, and all subjects provided signed informed consent.

Assessment of TLV using CT

Patients were examined using a multi-slice spiral CT scanner (Brilliance 256-slice spiral CT scanner; Philips Medical Systems, the Netherlands) with a collimating reconstruction thickness of 1 mm and an interval of 1 mm. Abdominal contrast-enhanced CT was completed by professional technicians in accordance with standard operating procedures. To improve the quality of examinations, patients were placed in the supine position and trained to hold their breath to reduce breathing and movement artifacts during scanning.

The morphological indices assessed included CT estimated TLV (CTLV), CT-based total splenic volume (CTSV), PVD, SVD, and PVCSA. These variables were measured on portal phase images by two medical students blinded to the clinical results under the supervision of an experienced radiologist using an image-analysis program (Phillips Intellispace Portal Workstation; Philips Medical Systems). CTLV and CTSV were obtained as a summation by manually tracing the boundaries on each transverse image, avoiding the large vessels, gallbladder, and fissures. PVD and PVCSA were measured at the midpoint between the portal bifurcations and the venous confluences, while SVD was detected at the point 1 cm proximal to the confluence of the PV and splenic vein.

Liver volume calculated using previous formulas

In the validation group, CTLV was measured as above, and SLV was calculated using our new formula and seven other previously reported formulas. The detailed information of each formula is shown in Supplementary Table 1.

Statistical analysis

All analytical tests were performed using SPSS 21 (IBM SPSS, Chicago, IL, United States). Data are expressed as mean ± SD. The correlations between CTLV and age, sex (men = 1, women = 0), BW, BH, BSA, and BMI were analyzed by Pearson correlation analysis. Stepwise multiple linear regression analysis was performed to evaluate the impact of each parameter and to develop a new equation that more accurately predicted CTLV. CTLV was compared with SLV calculated by formulas using a *t*-test, with absolute volume and percentage error as (SLV - CTLV)/CTLV × 100. We used ± 10% and ± 15% as acceptable ranges for differences between estimated SLV and CTLV[18,22], and the proportions of the estimates within these acceptable ranges were determined for each SLV formula. All statistical analyses were two-tailed, and *P* values less than 0.05 were considered to indicate statistical significance.

RESULTS

Characteristics and CTLV of Chinese adults

A total of 244 healthy adults (138 men and 106 women) were enrolled to establish the new SLV formula. The mean age was 48.81 ± 12.00 years, with no differences in age between the sexes (48.81 ± 12.00 years *vs* 48.00 ± 12.49 years, respectively; $P = 0.355$). Anthropometric data, including BH, BW, BMI and BSA, are shown in Table 1. The mean BH and BW of men were larger than those of women (BH: 172.10 ± 4.95 *vs* 161.04 ± 4.04, respectively; BW: 70.86 ± 9.35 *vs* 58.32 ± 8.47, respectively; $P < 0.001$ for both). BMI and BSA were also significantly different between the sexes (Table 1). The average CTLV in all subjects was 1194.31 ± 238.25 cm^3 (range, 593.80-2250.10 cm^3). Eleven of 138 men and 26 of 206 women had a liver volume smaller than 80% of the mean volume (955.4 cm^3), while 33 men and 7 women had a liver volume

Table 1 Physical characteristics and computed tomography estimated total liver volume in Chinese healthy adults

	Total, *n* = 244	Male, *n* = 138	Female, *n* = 106	*P* value
Age, yr	48.8 ± 12.00 (18-88)	49.4 ± 11.59 (18-81)	48.0 ± 12.49 (18-88)	0.355
Body height, cm	167.29 ± 7.14 (148-185)	172.10 ± 4.95 (156-185)	161.04 ± 4.04 (148-172)	< 0.001
Body weight, kg	65.41 ± 10.92 (38-96)	70.86 ± 9.35 (49-96)	58.32 ± 8.47 (38-80)	< 0.001
BMI, kg/m^2)	23.29 ± 3.06 (15.63-35.16)	23.91 ± 2.95 (17.16-35.16)	22.47 ± 3.02 (15.63-30.04)	< 0.001
BSA, m^2	1.74 ± 0.17 (1.25-2.21)	1.84 ± 0.13 (1.47-2.21)	1.61 ± 0.13 (1.25-1.94)	< 0.001
CTLV, cm^3	1194.31 ± 238.25 (593.80-2250.10)	1268.32 ± 228.09 (815.10-2250.10)	1097.96 ± 216.60 (593.80-2005.80)	< 0.001

BMI: Body mass index; BSA: Body surface area; CTLV: Computed tomography estimated total liver volume.

larger than 120% of the mean volume. As expected, there was a significant difference in CTLV between men and women (1268.32 ± 228.09 *vs* 1097.96 ± 216.60 cm^3, respectively; $P < 0.001$), which may be related to the difference in body frame between men and women.

CTSV, PVD, SVD and PVCSA in Chinese adults

We also measured CTSV, PVD, SVD and PVCSA in Chinese adults using CT (Table 2). The average CTSV in healthy Chinese adults was 210.48 ± 224.07 cm^3 (range, 46.60-2892.30 cm^3), with large individual variations observed (Table 2). The mean PVD, SVD and PVCSA were 9.34 ± 1.51 mm, 7.40 ± 1.31 mm and 173.22 ± 48.11 mm^2, respectively. Three of 213 subjects had a PV larger than 13 mm, the widely referenced upper limit. There were no differences in CTSV or SVD between men and women. Pearson correlation analysis showed that CTSV was not associated with anthropometric indices, including sex, BH, BW, BMI or BSA (Supplementary Table 2).

Factors related to TLV

Pearson correlation analysis showed a negative correlation between TLV and age, and a positive correlation between TLV and sex, BH, BW, BMI and BSA (Table 3; correlation coefficients = -0.117, 0.355, 0.421, 0.534, 0.416 and 0.546, respectively). Of these factors, BSA had the strongest correlation ($r = 0.546$, $P < 0.001$), while age had the weakest correlation ($r = -0.117$, $P = 0.067$). Conversely, stepwise multiple linear regression analysis showed that BSA and age were the only two definite independent correlation factors for CTLV. Because of the small partial regression coefficient for age (-2.246/year), we considered that its effect on TLV was negligible, and we thus removed this factor from the new SLV formula. The final formula used to calculate SLV based on BSA was: SLV (cm^3) = 758.259 × BSA (m^2) - 124.272; $R^2 = 0.299$, $P < 0.001$). The scatter plot is shown in Figure 1.

Comparison of the new formula with previously reported formulas

Based on the results of Pomposelli *et al*[18], John's formula[23] showed a calculated SLV closest to CTLV, while Poovathumkadavil's formula[19] showed the lowest percentage error. However, Urata's formula[11] is the most frequently used worldwide. Therefore, we selected these three formulas plus four others established in the Chinese population[24-27] and compared these with our new formula in the validation group ($n = 125$). Although the root of the mean predicted residual sum of squares and the intra-class correlation were similar between all formulas, only Urata's, Fu-Gui's, and our new formula had a mean percentage error of SLV *vs* CTLV lower than ± 10%. Our new formula and Urata's formula were the most accurate for assessing absolute volumes, which were very close to the CTLV. In contrast, the mean absolute values of difference calculated by the other six formulas were > 100 cm^3. Indeed, our formula underestimated the mean SLV by < 10 cm^3 compared with the mean CTLV, while the average deviation of Urata's formula was 37 cm^3 (Table 4). Nevertheless, there were no significant differences between the mean liver volume estimated by either of these formulas and the CTLV. Furthermore, the SLV derived from our formula showed almost perfect agreement with liver volume using Urata's formula ($R^2 = 0.993$, $P < 0.001$, Figure 2). Our new formula showed the highest proportion of estimated SLV with percentage errors compared with CTLV for both levels of agreement, which was greater than that for Urata's formula (60.8% *vs* 57.6%, and 44.0% *vs* 20.8%, respectively, Table 5).

Factors related to the difference between CTLV and SLV

Our formula was the most accurate for estimating both absolute volume and the percentage error. However, the proportions of estimated SLV with percentage errors compared with CTLV within ± 10% and ± 15% agreement using our new formula were just 44.0% and 60.8%, respectively. We suspect that factors related to differences between CTLV and SLV may have limited the application of our formula. A total of 369 subjects (244 for formula establishment, 125 for validation) were enrolled to analyze the latent factors (Table 6). The new formula overestimated the CTLV of elderly subjects (age > 70 years) ($P < 0.05$, Table 6), with a decreased correlation between SLV and CTLV in this population (Figure 3). Scatter plots and

Table 2 Computed tomography estimated total splenic volume, portal venous diameter, splenic venous diameter and portal venous cross-sectional area in Chinese healthy adults

	Total, n = 244	Male, n = 138	Female, n = 106	P value
CTSV, in cm^3	210.48 ± 224.07 (46.60-2892.30)	213.91 ± 172.76 (46.60-1490.30)	206.02 ± 277.87 (65.00-2892.30)	0.786
PVD, in mm	9.34 ± 1.5 (5.60-16.25)	9.84 ± 1.56 (6.10-16.25)	8.27 ± 1.23 (5.60-12.95)	< 0.001
SVD, in mm	7.40 ± 1.31 (3.20-12.50)	7.61 ± 2.95 (4.70-12.50)	7.17 ± 3.02 (3.20-10.50)	0.278
PVCSA, mm^2	173.22 ± 48.11 (92.22-451.00)	189.63 ± 50.88 (101.91-451.00)	153.74 ± 36.16 (92.22-253.34)	< 0.001

CTSV: Computed tomography estimated total splenic volume; PVCSA: Portal venous cross-sectional area; PVD: Portal venous diameter; SVD: Splenic venous diameter.

Table 3 Related factors to computed tomography estimated total liver volume

Factor	r value	P value
Age	-0.117	0.067
Sex	0.355	< 0.001
BH	0.421	< 0.001
BW	0.534	< 0.001
BMI	0.416	< 0.001
BSA	0.546	< 0.001

BH: Body height; BMI: Body mass index; BSA: Body surface area; BW: Body weight.

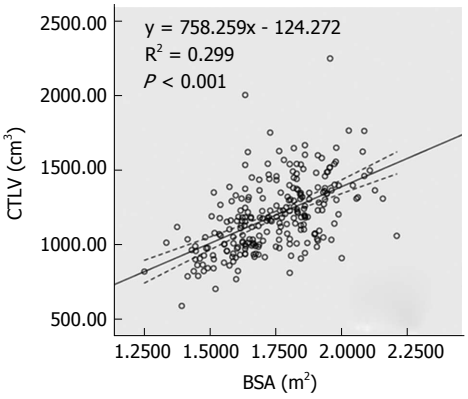

Figure 1 Correlation between computed tomography estimated total liver volume and body surface area of 244 healthy adults. The 95%CI is shown (Dotted line). BSA: Body surface area; CTLV: Computed tomography estimated total liver volume.

fitted lines (Figure 4) showed that SLV also tended to underestimate liver volume when the CTLV was > 1800 cm^3.

DISCUSSION

Precise measurement of liver volume and correct assessment of SLV have important implications for estimating liver function and making strategic medical decisions for liver transplantation. Due to the marked effects of race and body indices on SLV, it is important to develop a method for SLV calculation that can be applied to particular demographic groups. In the present study, we determined morphological variables including CTLV, CTSV, PVD, SVD and PVCSA using CT

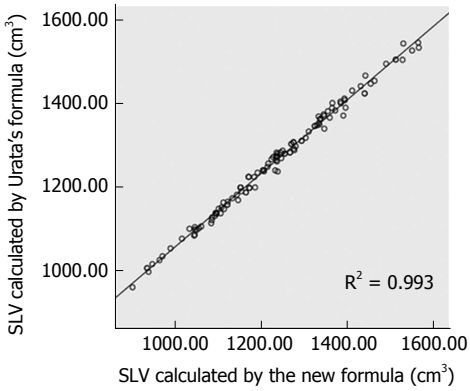

Figure 2 Correlation between standard liver volume calculated by the new formula and Urata's formula. SLV: Standard liver volume.

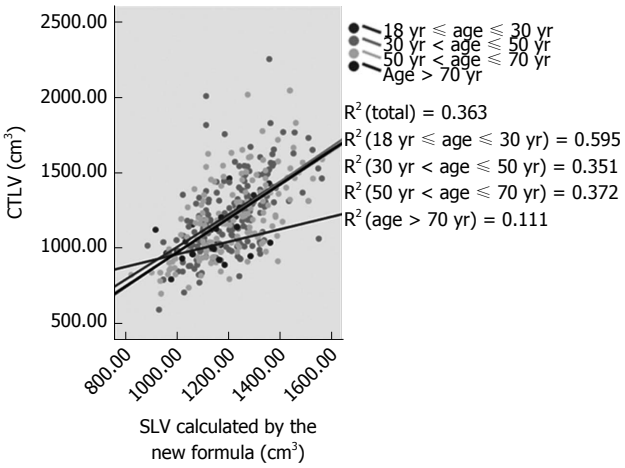

Figure 3 Correlation between standard liver volume calculated by the new formula and computed tomography estimated total liver volume stratified according to age. CTLV: Computed tomography estimated total liver volume; SLV: Standard liver volume.

in healthy adult Chinese subjects, and determined the medical reference ranges for Chinese men and women older than 18 years of age. We found a direct correlation between liver volume and various body indices, including sex, BH, BW, BMI and BSA. Based on Pearson's correlation and stepwise multiple linear regression analyses, BSA was selected to establish a new formula for estimating SLV. In addition, we confirmed the precision of our formula for calculating SLV in healthy Chinese adults by comparing it with

Table 4 Differences between total liver volume and standard liver volume approximated by various formulas

Study	Mean SLV-TLV, in cm^3	Mean error, as %	*P* value	Root of the mean PRESS	ICC
Urata *et al*[11], 1995	37.45	6.95	0.066	225.72	0.668
Johnson *et al*[23], 2005	232.94	22.92	< 0.001	216.79	0.668
Poovathumkadavil *et al*[19], 2010	169.96	10.82	< 0.001	216.67	0.686
Lin *et al*[24], 1998	271.54	25.74	< 0.001	225.40	0.652
Chan *et al*[25], 2006[1,2]	-165.31	-10.82	< 0.001	216.60	0.686
Yuan *et al*[26], 2008[3]	104.16	11.72	< 0.001	216.03	0.672
Fu-Gui *et al*[27], 2009	-103.28	-5.35	< 0.001	218.85	0.686
Our new SLV, 2016	8.10	4.17	0.680	219.43	0.679

[1]The formula can be directly converted into TLV from liver weight because liver density is 1 g/mL; [2]Sex factor: male = 1 and female = 2; [3]Age factor: < 40 yr = 1, 41-60 yr = 2, and > 60 yr = 3. SLV: Standard liver volume; TLV: Total liver volume.

Table 5 Proportions of estimated standard liver volume with percentage errors within acceptable agreement (10% and 15%) in comparison with total liver volume

Study	Proportion within acceptable agreement, as %	
	± 15%	± 10%
Urata *et al*[11], 1995	57.6	20.8
Johnson *et al*[23], 2005	30.4	23.2
Poovathumkadavil *et al*[19], 2010	44.8	29.6
Lin *et al*[24], 1998	29.6	17.6
Chan *et al*[25], 2006[1,2]	52.8	30.4
Yuan *et al*[26], 2008[3]	59.2	36.0
Fu-Gui *et al*[27], 2009	58.4	42.4
SLVn, 2016	60.8	44.0

Table 6 Factors related to the difference between computed tomography estimated total liver volume and standard liver volume calculated by the new formula

Factor	*n*	TLV, in cm^3	SLV-TLV, in cm^3
Total	369	1205.41 ± 257.53	
Sex			
Male	219	1285.64 ± 252.27	-1.82 ± 218.70
Female	150	1088.27 ± 217.76[a]	9.40 ± 187.04
Age, in yr			
18-30	14	1143.50 ± 192.33	-16.51 ± 122.80
30-50	184	1217.30 ± 259.75	-7.06 ± 210.37
50-70	158	1211.43 ± 263.84	4.52 ± 209.63
> 70	13	1030.55 ± 118.18[a]	140.69 ± 124.56[a]
BMI, in kg/m^2			
< 18.5	19	973.94 ± 163.74	4.65 ± 138.26
18.5-24.9	250	1162.63 ± 231.54[a]	12.70 ± 197.74
⩾ 25	100	1356.33 ± 262.51[a]	-22.50 ± 235.32

[a]*P* < 0.05. BMI: Body mass index; SLV: Standard liver volume; TLV: Total liver volume.

seven other reported formulas.

Previous studies have reported significant differences in TLV between Asian and Caucasian populations[5,23,28]. Kokudo *et al*[5] reported an average TLV of 1092 cm^3 in Japanese populations and 1622 cm^3 in Swiss populations, while TLV volumes in Chinese populations were reported to range from 1099.10-1220.1 cm^3[24-27]. Thus, there seem to be notable racial differences, rather than geographical differences, in TLV. The mean TLV

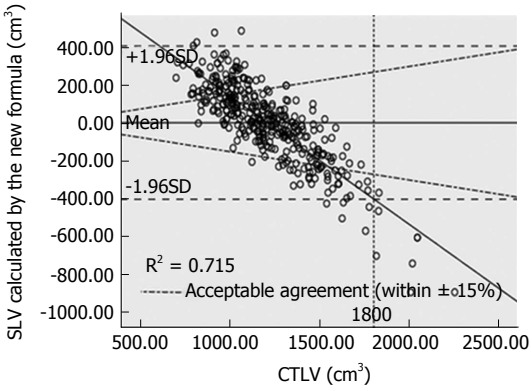

Figure 4 Modified Bland-Altman plots of the volume differences and computed tomography estimated total liver volume. CTLV: Computed tomography estimated total liver volume; SLV: Standard liver volume.

represented by CTLV in the present study was 1194.31 ± 238.25 cm^3, providing further support for racial differences in liver volume. There are 56 different ethnic groups in China, although Han is the major population. As there were few other ethnic groups included in the present study, differences in TLV between distinct ethnic groups were not analyzed, and the accuracy of our formula in ethnic minorities remains unclear.

A number of body indices have been reported to impact liver volume, including sex, age, BH, BW, BMI and BSA. Of these factors, BSA is the most widely accepted parameter for determining SLV. BSA is widely used to normalize measurement of biological function with respect to variations in body size. Several formulas have been developed to calculate BSA, including Du Bois's formula[29]: BSA (m^2) = BW (kg) 0.425 × BH (cm) 0.725 × 0.007184. However, due to its convenience and accuracy in the Chinese population, we selected Mosteller's formula[21] to estimate BSA. Since DeLand *et al*[20] first presented their formula for calculating liver size based on BSA, numerous studies have used BSA as an independent factor for liver size estimation[11,23,26]. As expected, BSA was also the best indicator for estimating liver volume in the present study, providing further support linking BSA with SLV.

BMI has also been used to adjust the influence of body size on biological function. Surprisingly, although many studies have reported that BMI can affect SLV, there are no SLV formulas based on BMI. This may relate to the even stronger association between BSA and TLV compared with that between BMI and TLV. Indeed, we found that BMI was positively correlated with CTLV in Pearson's correlation analysis (coefficient of correlation = 0.416). However, this index was excluded from our new SLV formula by stepwise multiple linear regression analysis. In addition, Hashimoto *et al*[30] reported that TLV may be underestimated in thin individuals with a BMI < 18.5. In contrast, in the present study, we did not find this association when comparing differences between TLV and SLV calculated using our formula.

Similar to the findings by DeLand *et al*[20], we found a correlation between TLV and both BH and BW (correlation coefficient = 0.421 and 0.534, respectively). With increasing BH or BW, there was a gradual increase in CTLV. Although BSA based on BW and BH showed an excellent correlation with TLV, liver size was not predicted exactly by BW and/or BH. Thus, both BW and BH were eliminated in the SLV formula by stepwise multiple linear regression analysis, and SLV estimated by formulas based on BW/BH[19,24,25,27] were significantly different from the actual TLV.

Sex is another important factor that can affect liver size, with the majority of studies showing that men have a larger TLV than women[7,31]. In the present study, there was a weak correlation between sex and TLV. However, sex is not widely used in the formula for calculating SLV. For example, sex was included as a factor in the SLV formula in two studies, as women (particularly those < 50 years old) showed significantly smaller liver volume compared with males with the same BMI[25,31]. In contrast, Pomposelli *et al*[18] reported that sex did not improve the accuracy of SLV calculation. Similarly, in the present study, sex was not an independent correlation factor of CTLV by stepwise multiple linear regression analysis. These contrasting findings may be explained by the fact that the effect of sex on TLV is contained in that of BSA. DeLand *et al*[20] and Urata *et al*[11] reported that men and women have similar LV/BSA or liver weight/BSA ratios. Similarly, there were no differences in the LV/BSA ratio between men and women in the present study (691.10 ± 117.27 *vs* 673.90 ± 116.53, respectively; $P = 0.166$), while there was a difference in the LV/BW ratio with sex ($P < 0.05$).

Age is also an important consideration when assessing SLV. Hashimoto *et al*[30] reported that SLV estimated using their equation overestimated the TLV of older donors (⩾ 50 years of age), while Pomposelli *et al*[18] found no benefit of adjusting for age in their SLV formula. In the present study, age was an independent predictor of SLV by multiple linear regression, with a similar coefficient to that of Kokudo *et al*[5]. To validate the precise impact of age on liver volumes, we used 8-year interval interpretation as a presentation for the liver volume measurement and established another new formula based on age stratification: SLVa = 857.088 × BSA - 21.228 × Age - 205.070 (aged between 18 and 26, value of Age was 1; aged between 27 and 34, value of Age was 2; and so on). Then we compared the difference of SLV, SLVa and TLV. Consistent with our expectation, the mean Error between SLVa and TLV was 3.63%, which was not significant different with the mean Error between SLV and TLV (4.17%, $P = 0.283$). Therefore, considering that the partial regression coefficient (-2.246/year) was very small, we considered that the effect of this variable in adults was negligible. Thus, age was excluded from our final formula, as reported by Vauthey *et al*[28]. However, it is still possible that age may have an effect on TLV in specific populations. In the present study, the correlation between TLV and SLV decreased in subjects > 70 years of age, and SLV was always overestimated in these individuals. Therefore, further studies are required to evaluate the validity of our new formula in a large elderly population.

At present, Urata's formula[11] is the most frequently used for calculating SLV worldwide. Pomposelli *et al*[18] compared 16 different SLV formulas and found that Johnson's formula[23] was the most accurate in terms of assessing absolute liver volume with a relatively low percentage error, while Poovathumkadavil's formula[19] provided the lowest percentage error *versus* actual TLV. However, these formulas have not been validated in the Chinese population. To the best of our knowledge, there are four formulas based on the Chinese population established by Lin *et al*[24], Chan *et al*[25], Yuan *et al*[26], and Fu-Gui *et al*[27]. Nevertheless, these formulas have not gained general acceptance in Chinese or Western centers. In the present study, our new formula based on SLV was more accurate for assessing absolute liver volume compared with the other formulas, with the exception of Urata's formula. Furthermore, our formula showed the strongest agreement with TLV. Thus, despite the limited adjusted R^2 value, our formula is easily calculated and should be considered when assessing SLV in the Chinese population.

There are few reports on spleen volume and SVD in the normal population, with evidence of marked differences in those studies[32,33]. For example, spleen size determined by CT was relatively larger than that measured at postmortem, which may be explained by differences in the measurement methods or by removal of blood from the spleen during surgery. In the present study, CTSV varied markedly in various subjects (average CTSV = 210.48 ± 224.07 cm^3), as reported in previous CT imaging studies[9,34]. Spleen volume was also reported to be related to age, BW,

and BSA, although this correlation was not confirmed by other investigators or in the present study[29].

PVD and PVCSA were significantly enlarged in the high portal venous pressure group. PVD is regarded as an essential indicator of portal hypertension[35]. However, there are limited studies assessing PVCSA, as it is difficult to measure. PVD values in healthy adults were reported to range from 9.6-12.5 mm, with 13 mm accepted as the upper limit of PVD in many major medical textbooks. Nevertheless, Stamm *et al*[36] reported that PVD measured by CT was larger than this upper limit, which may be related to the different methods used to measure PVD. Many earlier studies detected PVD using ultrasonography, while Stamm *et al*[36] used CT imaging. However, in contrast to the findings of Stamm *et al*[36], we found that PVD was 9.34 ± 1.51 mm (range, 5.6-16.25 mm), with only 3 of 213 subjects having a PVD larger than 13 mm. Thus, further studies are required to compare ultrasound and CT for the measurement of PVD and PVCSA.

There are some limitations in the present study. In addition to TLV, the liver segmentation proposed by Couinaud is used in everyday clinical practice, which divided the liver into 8 operatively relevant segments based on the anatomy of the PV and hepatic vein[37]. Some studies that have reported the change of volume in different locations of liver could hint at some liver diseases[38]. Our further study will continue to propose the range of normal value of the volume of the 8 liver segments and investigate the correlation between the volume of different liver segments and body indices. When TLV was large, especially > 1800 cm^3, the accuracy of our formula for SLV decreased. Liver steatosis may affect liver volume. Although we set very strict exclusion criteria, we did not obtain liver biopsy results, and our study may have included subjects with liver steatosis or mild fatty liver. Hwang *et al*[14] also reported that the calculated decrease in SLV was larger than the reduction in CTLV after body weight loss. Nevertheless, this formula has not been used in individuals with a short-term and rapid decrease in body weight. Our formula also used BSA as a major variable, which may be influenced considerably by the presence of ascites or edema. Hence, the application of our formula in patients with chronic liver disease and liver cirrhosis remains to be assessed.

In conclusion, by setting strict exclusion criteria, we obtained the TLV of healthy Chinese adults, and developed a new formula for estimating SLV based on BSA. This formula predicted the SLV in the Chinese population more accurately than other previously reported formulas.

COMMENTS

Background

Liver volume, which can indirectly reflect liver function, is an important clinical indicator of liver disease. Accurate estimation of total liver volume (TLV) is an important consideration for clinical condition assessment, pharmacological applications, and a variety of surgeries, especially liver transplantation. Computed tomography liver volumetry is considered the gold standard for measuring TLV. The standard liver volume (SLV) formulas derived from various ethnic groups may not be comparable with each other.

Research frontiers

The multiple comparison of liver volumes obtained by different methods were very similar without statistically significant differences, However, the three methods achieved an efficiency of 27.63 min, 1.26 min and 1.18 min on average, respectively, compared with the manual volumetry, which took 43.98 min (*Journal of Applied Clinical Medical Physics*, 2016).

Innovations and breakthroughs

By setting strict exclusion criteria, the authors obtained the TLV of healthy Chinese adults, and developed a new formula for estimating SLV based on BSA. This formula predicted the SLV in the Chinese population more accurately than other previously reported formulas.

Applications

The authors were able to use the SLV formula to obtain the SLV of patients with liver cirrhosis, resulting in its normal state of the liver volume prediction, compared with the actual liver volume, from which the reserved liver function can be evaluated. The optimal size of liver resection for hepatectomy in patients with hepatocellular carcinoma is of great significance for the evaluation of donor liver volume in donor and recipient liver transplantation.

Peer-review

The current manuscript would like to create a SLV and vascular structure in Chinese adult. It's an interesting and well-written paper.

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ORIGINAL ARTICLE

Prospective Study

Postoperative decrease of serum albumin predicts short-term complications in patients undergoing gastric cancer resection

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Author contributions: Liu ZJ and Ge XL contributed equally to this work; Ge XL, Liu ZJ and Guan WX contributed to study conception and design; Ge XL, Liu ZJ and Wang HK contributed to data acquisition; Chao AS, Sun F and Wang HK contributed to analysis and interpretation of data; Wang HK and Sun F contributed to drafting of the manuscript; Guan WX and Chen L contributed to critical revision of the manuscript.

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Institutional review board statement: This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, Nanjing, China. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and the guidelines of the Ethics Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, Nanjing, China.

Informed consent statement: All study participants provided written informed consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at guan-wx@163.com. No additional data are available.

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Abstract

AIM

To find an accurate and simple predictor for post-operative short-term complications after gastrectomy.

METHODS

Two hundred and twenty-three patients undergoing gastric cancer resection between October 1, 2015 and September 30, 2016 were enrolled in this study. Univariate and multivariate analyses were used to

identify risk factors for complications after gastrectomy. The cutoff values and diagnostic accuracy were examined by receiver operating characteristic curves.

RESULTS

Sixty-two (27.8%) patients had short-term complications after gastric cancer resection. The postoperative decrease in serum albumin (ΔALB) was an independent risk factor for complications (OR = 17.957, 95%CI: 6.073-53.095, $P < 0.001$). The cutoff value was 14.0% and the area under the curve was higher than that of C-reactive protein on postoperative day 3 (area under the curve: 0.806 *vs* 0.709). Patients with ΔALB ⩾ 14.0% were more likely to have short-term complications after gastrectomy (46.7% *vs* 5.0%, $P < 0.001$), prolonged hospital stay (17.2 ± 10.8 d *vs* 14.1 ± 4.2 d, $P = 0.007$) and higher comprehensive complication index ($P < 0.001$) than those with ΔALB < 14.0%.

CONCLUSION

Postoperative ΔALB with a cutoff of 14.0% can be used to recognize patients who have high risk of short-term complications following gastric cancer resection.

Key words: Gastric cancer; Postoperative complications; Gastrectomy; Serum albumin; Predictor

Core tip: In this work, we investigated whether postoperative decrease of serum albumin can predict short-term complications following gastric cancer resection. Results indicate that the decrease of serum albumin could be more accurate than postoperative C-reactive protein in predicting complications after gastrectomy. Surgeons are warned of potential postoperative complications in patients whose serum albumin levels reduce by more than 14.0%. This is the first evaluation of the relationship between decrease in albumin and postoperative complications in gastric cancer resection.

Liu ZJ, Ge XL, Ai SC, Wang HK, Sun F, Chen L, Guan WX. Postoperative decrease of serum albumin predicts short-term complications in patients undergoing gastric cancer resection. *World J Gastroenterol* 2017; 23(27): 4978-4985 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/4978.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.4978

INTRODUCTION

Gastric cancer is one of the most common malignancies and is the third leading cause of cancer-related mortality in China. Surgery provides the only possibility of cure in patients with gastric cancer. Despite improvements in perioperative care and surgical procedures, postoperative complications remain a major impediment and threat to survival after gastric cancer surgery[1-3]. Therefore, it is crucial to specify a reliable and simple risk assessment index to indicate the possibility of postoperative complications, and the likelihood of early and safe patient discharge.

Many preoperative or postoperative indexes, such as C-reactive protein (CRP; a proinflammatory cytokine increasing in parallel with postoperative surgical stress), white blood cell count and albumin (ALB), have been identified as risk factors for complications after gastrectomy[4-8]. There are many studies revealing the association between serum ALB and postoperative outcomes. For example, preoperative hypoalbuminemia can predict surgical site infections[9]. Ryan *et al*[10] found that postoperative hypoalbuminemia on postoperative day (POD) 1 was associated with complications following esophagectomy. Sang *et al*[11] also found that hypoalbuminemia on POD 2 was an independent risk factor for acute kidney injury in patients with living donor liver transplantation. However, these studies mainly focused on the impact of serum ALB on nutritional status of patients[12,13].

ALB is also a negative acute phase protein and decreases after surgery, because of trauma and increased capillary leakage[14]. Norberg *et al*[15] observed an immediate and sharp decrease of serum ALB level (ΔALB) by 33% after major abdominal surgery, which occurred even earlier than the change in CRP. Unfortunately, few studies have identified the change in serum ALB level as a marker in predicting the outcomes of gastrectomy.

This study aimed to clarify whether the reduction of ALB level after surgery could be a predictor for short-term complications following gastric cancer resection. Therefore, the ΔALB on POD 1 was examined and its diagnostic accuracy in gastric cancer was investigated.

MATERIALS AND METHODS

Patients

Written informed consent was obtained from all the patients enrolled in the investigation. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and the Ethics Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, Nanjing, China.

Data collection

The data of 317 consecutive patients who underwent surgery for gastric cancer between October 1, 2015 and September 30, 2016 at the Department of Gastrointestinal Surgery, Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School were prospectively collected and standard ethnic audit was conducted. Patients with ALB infusion preoperatively or within POD 1, reoperation for postoperative complications, non-resection of the stomach, severe organ dysfunction or comorbidity, multivisceral resection, or incomplete laboratory data

were excluded. Finally, a total of 223 patients were enrolled in the study.

Data were collected based on the following factors: age, sex, body mass index (BMI), American Society of Anesthesiologists (ASA) grade, initial clinical stage, comorbidity, surgical procedures (surgical approach, type of resection, degree of lymph node dissection), neutrophils, lymphocytes, hemoglobin, tumor makers such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9, preoperative CRP and ALB, CRP on POD 3[16], ALB on POD 1[11], operation time, estimated blood loss during surgery and intraoperative blood transfusion.

Definition of outcomes

Relative ΔALB was calculated as: (preoperative ALB level - ALB level on POD 1)/preoperative ALB level × 100%[17]. Receiver operating characteristic (ROC) curve was used to calculate the cutoff values of ΔALB. The postoperative outcomes were analyzed which included length of postoperative hospital stay and postoperative complications before discharge or < 30 d after surgery. Postoperative complications was identified by Clavien-Dindo classification, which showed that the Grades Ⅰ and Ⅱ were mild complications and Grades Ⅲ and Ⅳ were major complications in patients[18]. The comprehensive complication index (CCI) is a score developed to include all complications after surgery and is based on the Clavien-Dindo system[18,19]. The CCI of each patient was calculated using the Website http://www.assessurgery.com[20].

Statistical analysis

SPSS version 19.0 (SPSS Inc., Chicago, IL, United States) was used for the analysis. Categorical data were expressed by counts and percentages, while continuous data were expressed by mean ± SD or median (range). Fisher's exact test or Pearson's χ^2 test was used to analyze categorical variables, while Student's *t*-test was used to analyze continuous variables. $P < 0.05$ was considered statistically significant. ROC curve analysis was used to assess the predictive accuracy. Significant correlations ($P < 0.05$) on univariate analysis were used to verify independent predictors for postoperative complications by multivariate logistic regression analysis.

RESULTS

Study population and baseline characteristics

There were 159 men and 64 women, with a mean age of 62.3 ± 9.9 years. The clinical characteristics are summarized in Table 1. The preoperative mean BMI, and CRP and ALB level were 23.0 ± 3.4 kg/m^2, 6.6 ± 10.3 g/L and 38.3 ± 3.2 g/L, respectively. One hundred and seventeen patients received total gastrectomy, 82 distal gastrectomy and 24 proximal gastrectomy. One hundred and eighty patients underwent D2 or more lymphadenectomy. The operation time was 239.1 ± 66.9 min, with blood loss of 228.6 ± 146.2 mL. The length of postoperative hospital stay was 15.8 ± 8.6. Sixty-two patients (27.8%) had postoperative complications. According to Clavien-Dindo classification, 40 patients (17.9%) had mild complications (Grade Ⅰ or Ⅱ), and 22 (9.9%) had major complications (Grade Ⅲ or greater).

Predictive factors for postoperative complications

The results of univariate analyses of various clinical factors are shown in Table 2, including age, sex, BMI, ASA grade, clinical stage, tumor makers (CA19-9, CEA), lymphocyte count, comorbidity, preoperative hemoglobin, operation time, intraoperative blood loss, surgical procedures (mode of approach, type of resection, degree of lymph node dissection), preoperative CRP and serum ALB, CRP on POD 3, and ΔALB. Among these, diabetes mellitus (OR = 3.259, 95%CI: 1.128-9.414, P = 0.029), preoperative serum ALB (OR = 1.048, 95%CI: 1.018-1.109, P = 0.015), clinical stage (OR = 1.798, 95%CI: 1.255-2.686, P = 0.009), type of resection (OR = 1.291, 95%CI: 1.006-1.896, P = 0.013), CRP on POD 3 (OR = 4.653, 95%CI: 2.435-8.889, $P < 0.001$) and ΔALB (OR = 16.837, 95%CI: 6.403-44.275, $P < 0.001$) were significantly associated with postoperative complications. Therefore, a multivariate analysis model was used to identify the independent predictive factors for complications after gastrectomy. As shown in Table 3, ΔALB (OR = 17.957, 95%CI: 6.073-53.095, $P < 0.001$) remained as an independent risk factor in predicting complications after surgery. However, it was not strong enough for us to draw the conclusion that ΔALB can be an accurate predictor for complications after gastrectomy.

Predictive accuracy of ΔALB and CRP on POD 3 for complications after gastrectomy

CRP on POD 3 was identified as a practical predictor for complications after gastric cancer surgery in recent studies[7,16]. In this study, the predictive accuracy of ΔALB and CRP on POD 3 was analyzed by ROC curve. Figure 1 shows the ROC curve parameters. The area under the curve (AUC) of CRP on POD 3 was 0.709, sensitivity was 0.785, specificity was 0.661, Youden's index was 0.446, and the cutoff point was 131.9; comparatively, the AUC of ΔALB was 0.806, sensitivity was 0.808, specificity was 0.675, Youden's index was 0.483, and the cutoff point was 14.0%. Therefore, ΔALB was a better predictive index than CRP on POD 3 for postoperative complications in patients undergoing gastric cancer surgery.

Use of ΔALB to predict complications after gastrectomy

Based on the cutoff value of ΔALB, we divided patients into two groups. As shown in Table 4, patients with ΔALB ⩾ 14.0% had more complications after gastrectomy than those with ΔALB < 14.0% (46.7% *vs* 5.0%, P

Table 1 Patient characteristics

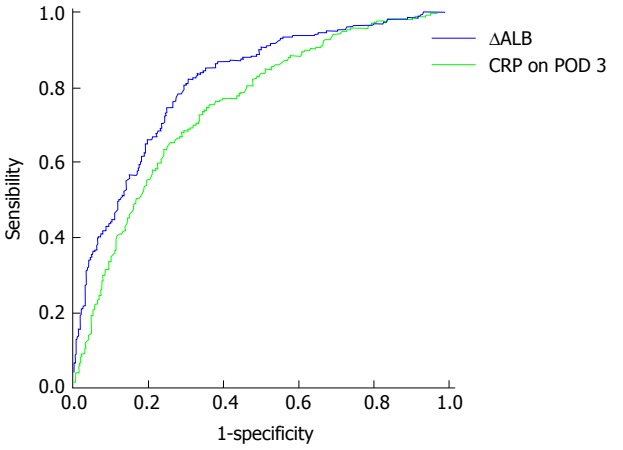
Characteristic	*n* = 223
Age in yr	62.3 ± 9.9
Sex, *n*	
Male	159
Female	64
BMI in kg/m^2	23.0 ± 3.4
Comorbidities, *n*	
Diabetes mellitus	15
Hypertension	72
Preoperative serum albumin in g/L	38.3 ± 3.2
Preoperative hemoglobin in g/L	123.4 ± 24.9
Preoperative CRP in g/L	6.6 ± 10.3
CA 19-9 in ng/mL	
≥ 37	43
< 37	180
CEA in ng/mL	
≥ 5	26
< 5	197
Lymphocyte count as × 10^9/L	
≥ 3	9
< 3	214
CRP on POD 3 in mg/L	99.7 ± 60.9
ALB on POD 1 in g/L	32.7 ± 3.6
ASA ≥ 3, *n*	122
Clinical stage Ⅰ / Ⅱ / Ⅲ / Ⅳ, *n*	67/44/103/9
Mode of surgical approach, *n*	
Laparoscopic	18
Open	205
Type of resection, *n*	
Distal gastrectomy	82
Proximal gastrectomy	24
Total gastrectomy	117
Degree of lymph node dissection ≥ 2, *n*	180
Operation time in min	239.1 ± 66.9
Blood loss in mL	228.6 ± 146.2
Postoperative complications as Clavien-Dindo grade, *n*	
Ⅰ and Ⅱ	40
≥ Ⅲ	22
Postoperative stay in d	15.8 ± 8.6

ALB: Albumin; ASA: American Society of Anesthesiologists; BMI: Body mass index; CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; CRP: C-reactive protein; POD: Postoperative day.

< 0.001). Patients with Δ < 0.001). Patients with Δ ALB ≥ 14.0% were found to have more mild or severe complications than those with ΔALB < 14.0% (30.3% *vs* 3.0%, *P* < 0.001 or 16.4% *vs* 2.0%, *P* < 0.001, respectively). In addition, patients who had Δ ALB ≥ 14.0% were suggested to have prolonged postoperative stay (17.2 ± 10.8 *vs* 14.1 ± 4.2, *P* = 0.007) and higher CCI (*P* < 0.001).

DISCUSSION

In this study, serum ALB was mainly considered as an acute phase protein. The ΔALB was an independent risk factor for prolonged hospital stay and complications after gastrectomy. Patients were distinguished as having low or high risk of complications after gastrectomy by the cutoff value of 14.0% in ΔALB, which was more accurate than CRP level on POD 3.



	ΔALB	CRP on POD 3
Cutoff point	0.140	131.9
AUC	0.806	0.709
Sensitivity	0.808	0.785
Specificity	0.675	0.661
Youden's index	0.483	0.446

Figure 1 Receiver operating characteristic curve showing decrease in serum albumin levels and C-reactive protein levels on postoperative day 3 predictive of postoperative overall complications. ROC: Receiver operating characteristic; CRP: C-reactive protein; ΔALB: (Albumin level before surgery-albumin on POD 1)/albumin level before surgery × 100%; POD: Postoperative day; AUC: Area under the curve.

Numerous studies have shown that preoperative and postoperative hypoalbuminemia are risk factors for complications after surgery[9-12], while few have specifically focused on the relationship between decreased serum ALB after surgery and clinical outcomes. Hübner *et al*[21] reported that stress response led to a reduction in postoperative albumin levels, which was consistent with the findings in our study. After major abdominal surgery, the albumin synthesis rate remained the same, whereas the fractional synthesis rate increased, leading to a decrease in plasma ALB[15]. However, sequestration into the interstitial space may contribute most to a postoperative drop in ALB level[14,21]. Capillary leakage is especially common in some malnourished patients with surgical trauma followed by an increased transcapillary escape rate of ≥ 100%[14,22,23]. To summarize, multiple factors affect the reduction in serum ALB after surgery and the stress response plays an important role in the change[23].

Nevertheless, increasing evidence shows that postoperative CRP level, as a risk factor for postoperative inflammation, can be used to predict complications after gastrectomy[7,24]. For instance, Kim *et al*[16] found that the CRP level on POD 4 was one of the most reliable predictors for complications following gastric cancer resection, when compared to many systematic inflammatory markers (white blood cells, neutrophils, platelet counts and CRP on POD 1). However, in previous studies, certain drawbacks were found by using postoperative CRP as a marker of

Table 2 Univariate analysis of risk factors associated with postoperative complications

Characteristic	OR	95%CI	*P* value
Age ⩾ 75 yr	1.330	0.476-3.715	0.586
Sex	0.642	0.342-1.202	0.166
BMI of < 18.5 kg/m^2	0.277	0.034-2.232	0.228
Comorbidities			
Diabetes mellitus	3.259	1.128-9.414	0.029
Hypertension	0.728	0.382-1.389	0.336
Preoperative serum albumin of < 35 g/L	1.048	1.018-1.109	0.015
Preoperative hemoglobin of < 120 g/L	1.487	0.817-2.705	0.194
Preoperative CRP of ⩾ 10 g/L	1.336	0.512-3.486	0.553
CA 19-9 of ⩾ 37 ng/mL	1.328	0.647-2.723	0.439
CEA of ⩾ 5 ng/mL	1.177	0.483-2.865	0.720
Lymphocyte count of ⩾ 3 × 10^9/L	0.314	0.038-2.560	0.279
CRP on POD 3 in mg/L	4.653	2.435-8.889	< 0.001
△ALB of ⩾ 14.0%	16.837	6.403-44.275	< 0.001
ASA of ⩾ 3	1.007	0.559-1.815	0.981
Clinical stage of ⩾ Ⅱ	1.798	1.255-2.686	0.009
Mode of surgical approach	1.206	0.588-2.475	0.610
Type of resection	1.291	1.006-1.896	0.013
Degree of lymph node dissection ⩾ 2	2.263	0.948-5.399	0.066
Operation time of ⩾ 250 min	1.621	0.896-2.931	0.110
Blood loss of ⩾ 200 mL	1.417	0.758-2.651	0.275

ΔALB: (Albumin level before surgery-albumin on POD 1)/albumin level before surgery × 100%; ASA: American Society of Anesthesiologists; BMI: Body mass index; CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; CRP: C-reactive protein; POD: Postoperative day.

Table 3 Multivariate analysis of risk factors associated with postoperative complications

Characteristic	OR	95%CI	*P* value
Age ⩾ 75 yr	1.053	0.248-4.470	0.945
Sex	0.572	0.237-1.380	0.214
BMI of < 18.5 kg/m^2	0.147	0.013-1.666	0.122
Comorbidities			
Diabetes mellitus	1.234	0.322-4.726	0.759
Hypertension	0.989	0.397-2.463	0.981
Preoperative serum albumin of < 35 g/L	0.914	0.897-1.067	0.093
Preoperative hemoglobin of < 120 g/L	1.804	0.748-4.353	0.189
Preoperative CRP of ⩾ 10 g/L	1.008	0.210-4.840	0.993
CA 19-9 of ⩾ 37 ng/mL	1.197	0.431-3.327	0.730
CEA of ⩾ 5 ng/mL	0.794	0.233-2.706	0.713
Lymphocyte count of ⩾ 3 × 10^9/L	0.192	0.018-2.048	0.172
CRP on POD 3 in mg/L	4.296	1.887-9.780	0.001
△ALB of ⩾ 14.0%	17.957	6.073-53.095	< 0.001
ASA of ⩾ 3	0.929	0.406-2.127	0.861
Clinical stage of ⩾ Ⅱ	1.198	0.355-2.286	0.109
Mode of surgical approach	1.737	0.576-5.235	0.327
Type of resection	0.791	0.506-1.896	0.063
Degree of lymph node dissection ⩾ 2	3.485	1.163-10.446	0.026
Operation time of ⩾ 250 min	1.448	0.624-3.355	0.389
Blood loss of ⩾ 200 mL	1.418	0.636-3.160	0.393

ΔALB: (Albumin level before surgery-albumin on POD 1)/albumin level before surgery × 100%; ASA: American Society of Anesthesiologists; BMI: Body mass index; CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; CRP: C-reactive protein; POD: Postoperative day.

complications after gastrectomy, which lacks accuracy in certain conditions[16,21,25]. Rettig *et al*[5] discovered that increased CRP levels after surgery were not early enough to identify patients at high risk for postoperative complications. In our study, ΔALB was an independent predictive marker for complications after gastrectomy in multivariate analysis. The AUC of ΔALB was larger than that of CRP on POD 3, suggesting ΔALB was a better positive predictive marker. According to these findings, ΔALB had a higher predictive value and tended to be more precise than CRP.

ΔALB could be more precise than CRP in predicting complications after gastrectomy because it is more accurate in reflecting the stress response after surgical trauma. As previously mentioned, the serum ALB level decreases earlier than CRP level after major abdominal surgery. The postoperative reduction in ALB level is associated with systemic inflammatory

Table 4 Univariate analysis of postoperative complications associated with median value of ΔALB

Characteristic	All, n = 223	ΔALB < 14.0%, n = 101	ΔALB ⩾ 14.0%, n = 122	P value
Overall[1,3]	62 (27.8)	5 (5.0)	57 (46.7)	< 0.001
Mild complications[1,3]	40 (17.9)	3 (3.0)	37 (30.3)	< 0.001
Major complications[1,3]	22 (9.9)	2 (2.0)	20 (16.4)	< 0.001
Postoperative stay in d[2]	15.8 ± 8.6	14.1 ± 4.2	17.2 ± 10.8	0.007
CCI all patients[4]	0 (0-20.9)	0 (0-0)	8.7 (0-26.2)	< 0.001

[1]Clavien-Dindo's classification of surgical complications; [2]Values are expressed as the median ± SD; [3]Values are expressed as n (%); [4]Values are expressed as median (interquartile range); ΔALB: (Albumin level before surgery-albumin on POD 1)/albumin level before surgery × 100%; CCI: Comprehensive complication index.

response syndrome, which leads to increased fractional synthesis and pathological capillary leakage of serum ALB[14]. Besides, serum ALB has a series of significant physiological functions, including free radical scavenging, maintenance of colloid osmotic pressure, change of capillary membrane permeability, and anticoagulant effects[26]. Hypoalbuminemia inhibits the innate immune response by promoting granuloma formation and reducing collagen synthesis. As a result, the systemic immune status is more sensitive to infection and other postoperative complications[27]. In contrast, CRP is involved in innate immunity as an early defense against infection, enhancing phagocytosis by macrophages and assisting complement binding to damaged cells or foreign matter[28]. From the above, the finding that ΔALB could be more precise than CRP in predicting complications after gastrectomy is understandable, and serum ALB is a better predictor of both systemic inflammation and nutritional status.

However, it remains to be established whether ALB supplementation benefits patients with a large ΔALB after gastrectomy. Golub *et al*[29] reported that routine ALB infusion was not beneficial to patients in the surgical intensive care unit. For patients with postoperative hypoalbuminemia, ALB infusion was deemed to be useless after major gastrointestinal surgery[30]. No studies have ever demonstrated the benefit for patients with preoperative hypoalbuminemia. Instead, exogenous ALB administration might increase risks of edema, extravasation of albumin, or other postoperative complications[31]. More intensive perioperative care might relieve an early ΔALB following gastric cancer resection and improve patient outcomes by reducing postoperative generalized inflammation[9].

There were several limitations to the current study. First, it was a retrospective observational analysis, so the possibility of residual confounding factors could not be entirely excluded. Second, it was a single-center study and the outcome might have been influenced by the small number of patients enrolled and perioperative management strategies in our hospital. To verify the conclusions, multicenter prospective studies involving a large volume of data are needed. Third, whether the findings in our study could be applied to other operations, such as esophagectomy or liver resection, is not known for sure.

In conclusion, this study confirmed that a postoperative ΔALB can predict short-term complications following gastric cancer resection. The ΔALB could be more accurate than postoperative CRP in predicting complications after gastrectomy. Surgeons are warned of potential postoperative complications in patients whose serum ALB levels are reduced by > 14.0%.

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COMMENTS

Background

To find an accurate and simple predictor for postoperative short-term complications after gastrectomy. A reduction of serum albumin (ALB) level is observed in many patients after gastrectomy, but it remains uncertain whether it could be used as a predictor for short-term outcomes following gastric cancer resection.

Research frontiers

Many preoperative or postoperative indexes, such as C-reactive protein (CRP; a proinflammatory cytokine increasing in parallel with surgical stress after operation), white blood cell count and ALB, have been identified as risk factors for complications after gastrectomy. There are many studies revealing the association between serum albumin and postoperative outcomes. However, these studies mainly focused on the impact of serum albumin on nutritional status of patients.

Innovations and breakthroughs

The authors investigated whether the postoperative decrease of serum ALB can predict short-term complications following gastric cancer resection. The decrease of serum ALB could be more accurate than postoperative CRP in predicting complications after gastrectomy. This study involves the first evaluation of the relationship between decrease of ALB and postoperative complications in gastric cancer resection.

Applications

Surgeons are warned of potential postoperative complications in patients whose serum ALB levels are reduced by more than 14.0%.

Peer-review

The authors investigated serum ALB changes after gastrectomy and found a

correlation between the ALB change with short-term complication rates. Study results are interesting and have novel findings. They know colorectal surgery correlated with ALB changes, but gastric cancer is a new finding and may add some contribution to the literature.

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SYSTEMATIC REVIEW

Management of inflammatory bowel disease with *Clostridium difficile* infection

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Abstract

AIM

To address the management of *Clostridium difficile* (*C. difficile*) infection (CDI) in the setting of suspected inflammatory bowel disease (IBD)-flare.

METHODS

A systematic search of the Ovid MEDLINE and EMBASE databases by independent reviewers identified 70 articles including a total of 932141 IBD patients or IBD-related hospitalizations.

RESULTS

In those with IBD, CDI is associated with increased morbidity, including subsequent escalation in IBD medical therapy, urgent colectomy and increased hospitalization, as well as excess mortality. Vancomycin-containing regimens are effective first-line therapies for CDI in IBD inpatients. No prospective data exists with regards to the safety or efficacy of initiating or maintaining corticosteroid, immunomodulator, or biologic therapy to treat IBD in the setting of CDI. Corticosteroid use is a risk factor for the development of CDI, while immunomodulators and biologics are not.

CONCLUSION

Strong recommendations regarding when to initiate IBD specific therapy in those with CDI are precluded by a lack of evidence. However, based on expert opinion and observational data, initiation or resumption of immunosuppressive therapy after 48-72 h of targeted antibiotic treatment for CDI may be considered.

Key words: Biologic therapy; *Clostridium difficile*;

Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Corticosteroids

Core tip: *Clostridium difficile* infection (CDI), common and increasing in inflammatory bowel disease (IBD), is associated with worse outcomes in IBD. Vancomycin-containing regimens are effective first-line therapies for CDI in IBD. Ambiguity exists on the treatment of IBD flare in patients with CDI; however, case reports suggest corticosteroid initiation after appropriate antibiotic therapy may be effective.

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INTRODUCTION

Inflammatory bowel disease (IBD), comprised of Crohn's disease (CD) and ulcerative colitis (UC), are chronic, idiopathic inflammatory gastrointestinal disorders. The pathogenesis of IBD, although incompletely understood, is thought to arise from interactions between environmental and host factors. CD and UC are characterized by recurrent episodes of relapsing inflammation of the gastrointestinal tract with variable clinical manifestations and potentially serious complications including bleeding, perforation and abscess formation[1,2].

Clostridium difficile (*C. difficile*), a gram-positive spore-forming anaerobe, is highly transmissible through the fecal-oral route and its exotoxins cause a spectrum of disease ranging from mild or moderate diarrhea to fulminant infectious colitis occasionally complicated by toxic megacolon, colonic perforation, sepsis, and death[3].

Several diagnostic assays exist to assess for *Clostridium difficile* infection (CDI). DNA-based tests or nucleic acid amplification tests *via* polymerase chain reaction (PCR) for *C. difficile* toxin genes (tcdA and tcdB) have been found to be more sensitive than toxin A and B enzyme immunoassays (EIA) and are currently recommended as the preferred diagnostic test for CDI[4]. Other, less commonly used diagnostic assays for CDI include EIA for glutamate dehydrogenase (GDH, a protein produced by both toxigenic and non-toxigenic strains) with confirmatory testing via EIA for toxin genes. This has fallen out of favor in view of the more sensitive and rapid PCR assay. The advent of DNA-based testing may partially contribute to the observed increased incidence of CDI. Toxigenic culture is considered to be the gold standard diagnostic assay, albeit the slowest, requiring several days to result and therefore possibly delaying initiation of therapy.

While *C. difficile* is often pathogenic and accounts for significant morbidity and mortality in the health-care and community setting, it has also been found to colonize the stool of healthy children and adults[5-7]. CDI is most commonly defined as the presence of *C. difficile* toxin in the context of characteristic clinical manifestations including diarrhea and abdominal pain[4]. CDI rates are increasing in the general population. Health care institutions have seen large outbreaks of CDI as well as the emergence of hypervirulent strains[8-10]. Surveillance of CDI in the United States has demonstrated a shift in the epidemiology to more community-acquired infections. A nationwide study of CDI in the United States using Emerging Infections Program data from the Centers for Disease Control estimated 453000 incident infections in 2011, of which only 24% were identified during hospitalization, as opposed to the outpatient setting[11].

Decreased intestinal microbial diversity along with an inadequate immune response is thought to play a causative role in the development CDI[12-14]. Antibiotic exposure, leading to alterations in the gut microbiota, has been identified as a traditional risk factor for CDI. IBD also predisposes to CDI and accounts for considerable excess morbidity and mortality along with increased systemic costs in IBD patients. Reductions in gut microbial diversity as well as an increase in pro-inflammatory species have been identified in IBD patients[15]. Although a causative role for this dysbiosis in the development of IBD has not been well established, it is plausible that dysbiosis may play a role in increasing CDI risk in IBD patients. Due to an overlap in symptomatology, CDI also gives rise to a series of diagnostic and therapeutic challenges in the IBD population.

This systematic review aims to summarize the management of patients with CDI and concurrent, suspected IBD flares. The epidemiology, risk factors, and methods of diagnosis for CDI in IBD patients are also summarized.

MATERIALS AND METHODS

Data sources and searches

We performed a systematic search of MEDLINE and Ovid EMBASE databases (Figure 1). Eligibility criteria for included studies were decided a priori. Two authors (D'Aoust J and Battat R) independently judged study eligibility. "Clostridium difficile", "pseudomembranous colitis", "inflammatory bowel disease", "Crohn's disease", and "ulcerative colitis" were used as search terms. MESH subheadings were combined using the Boolean operators "AND" and "OR" for full articles published in the English language between 1946 and the third week of January 2017. Additional publications

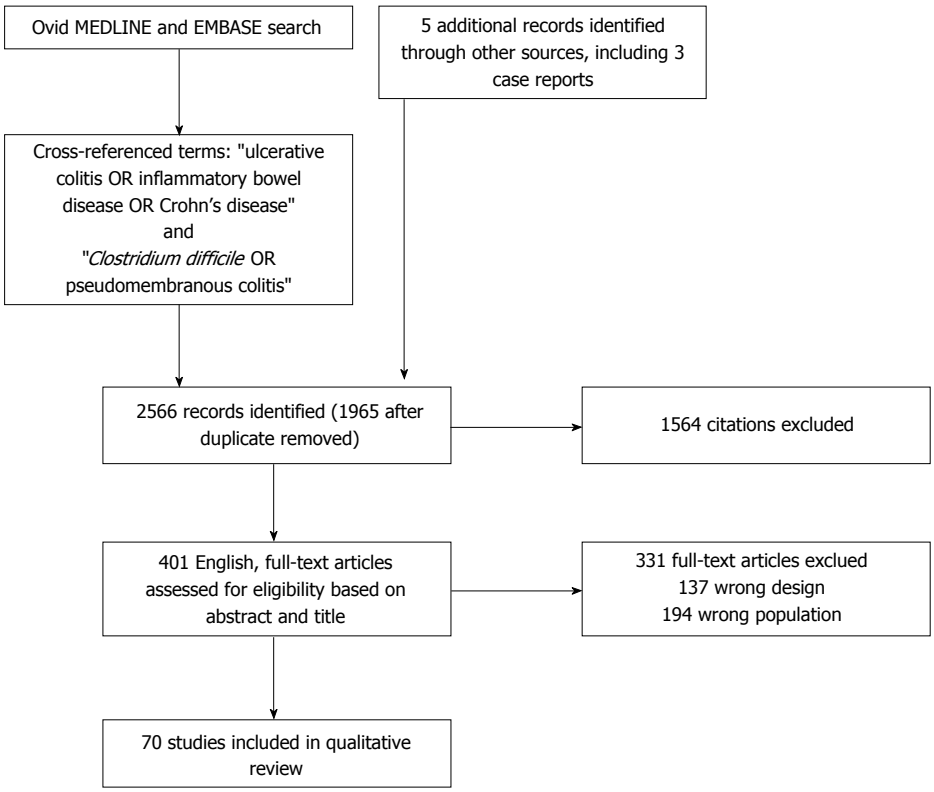

Figure 1 Search strategy for the selection of articles on *Clostridium difficile* infection in inflammatory bowel disease.

were retrieved from included studies and relevant review articles. Publications identified as duplicates were excluded. Cases of disagreement were resolved by discussion and joint analysis of articles by two reviewers (D'Aoust J and Battat R).

Study selection

Study titles and abstracts obtained from database searches were reviewed to identify those addressing CDI in IBD. Studies analyzing adult and pediatric patients were included. Case reports and case series were included if the management of IBD and CDI was discussed, due to limited data on this topic. Articles not pertaining to this topic in the title or abstract were excluded. Letters, editorials, and review articles were excluded. Data referring to the incidence, risk factors, diagnosis, management, and outcomes of *C. difficile* infection in patients with IBD were extracted from the articles. Data extraction was performed and agreed on by two authors (D'Aoust J and Battat R).

RESULTS

The search strategy, summarized in Figure 1, revealed 396 full-texts, English-language articles. Sixty-five articles were retained from the database search after applying the exclusion criteria. Two additional articles were retrieved from references. Two case reports discussing the management of CDI with corticosteroids were included. One additional case series on this topic was retrieved from relevant references for inclusion. Articles retained included a total of 932141 IBD patients or IBD-related hospitalizations (526765 UC; 312240 CD; 161 IC; 92 975 not-reported).

DISCUSSION

Epidemiology of CDI in IBD

Both an increasing burden of disease, as well as preponderance for community-acquired infection is reflected in the IBD population. Several studies have documented the changes in CDI epidemiology over time in IBD patients (Table 1). In adult inpatients with IBD, CDI incidence increased two to threefold in the early 2000s and more so in pediatric populations, with the largest rise in incidence among UC patients[16-19]. Several studies demonstrate a disproportionate rise in CDI in the IBD population as compared to the general population[16,17], while others do not[20].

The epidemiological studies of CDI in IBD are heterogeneous with regards to patient population, disease activity, sampling time frame, and diagnostic assay sensitivity. Reported incidences of CDI in pediatric and adult populations reflect this heterogeneity (Table 1). In mixed inpatient and outpatient adult IBD populations, the incidence of CDI ranges between 5.1%-16.7%[21-25].

Studies report the incidence of CDI in CD adult inpatients between 1.0 and 7.7%[26-30]. In adult UC inpatients, the incidence of CDI ranges from 2.8%

Table 1 Epidemiology of *Clostridium difficile* infection in inflammatory bowel disease

Ref.	Patient population	Sampling time frame	Diagnosis method	Disease activity	Conclusions
Keighley[92] (1983)	IBD adult inpatients	1978-1980	Stool culture on selective medium + cytotoxicity assay	Active	CDI incidence (%) IBD: 5.7; UC 4.7; CD 6.3
Gurian *et al*[93] (1983)	IBD adult inpatients and outpatients	1980-1981	Stool culture on selective medium + cytotoxicity assay	Active	CDI incidence (%) IBD: 0
Rolny *et al*[26] (1983)	IBD adult inpatients	1980-1981	Stool culture on selective medium + cytotoxicity assay	Active	CDI incidence (%) UC: 5; CD: 7.7
Greenfield *et al*[21] (1983)	IBD adult inpatients and outpatients	1980-1981	Stool culture on selective medium + cytotoxicity assay	Mixed	CDI incidence (%) UC: 13.7; CD: 13.2
Burke *et al*[94] (1987)	IBD adult outpatients	1984-1986	Stool culture on selective medium + cytotoxicity assay	Active	CDI incidence (%) IBD 3.2
Gryboski[95] (1991)	IBD pediatric inpatients and outpatients	1986-1990	Stool culture on selective medium + cytotoxicity assay	Active	CDI incidence (%) IBD 16; UC: 18; CD 14
Meyer *et al*[22] (2004)	IBD adult inpatients and outpatients	2000-2001	Immunoassay for Toxin A until 2001 then EIA for Toxin A/B	Active	CDI incidence (%) IBD: 16.7; UC: 12.5; CD: 23.8; IC: 11.1
Mylonaki *et al*[23] (2004)	IBD adult inpatients and outpatients	1997-2001	ELISA for Toxins A/B	Active	CDI incidence (%) IBD: 5.5; CD: 13.2
Issa *et al*[24] (2007)	IBD adult inpatients and outpatients	2005	ELISA for Toxins A/B	Active	CDI incidence (%) UC: 6.1; CD: 4.1 IBD patients accounted for 4% of the total CDI patient cohort in 2003, 7% in 2004, and 16% in 2005
Rodemann *et al*[16] (2007)	IBD pediatric and adult inpatients (United States)	1998-2004	Cell cytotoxic culture	Active	CDI incidence (%) UC: 3.9; CD: 1.6
			2002 onwards *C. difficile* Toxin A/B immunoassay		CDI incidence increase: UC > CD > non-IBD Non-IBD population: 8.5 to 15.9/1000 admissions CD: 9.5 to 22.3/1000 admissions UC: 18.4 to 57.6/1000 admissions
Shen *et al*[33] (2008)	UC adult outpatients with IPAA	2005-2006	ELISA for Toxin A/B	Mixed	CDI incidence (%) UC: 18.3
Bossuyt *et al*[20] (2009)	IBD and non-IBD CDI adult inpatients	2000-2008	EIA for Toxin A until 2005, then EIA for Toxins A/B	Active	All patients: 3.75-fold increase in CDI between 2000-2003 and 2004-2008
Balamurugan *et al*[96] (2008)	UC adult outpatients	2004-2005	PCR for *C. difficile* Toxin A/B ELISA	Mixed	CDI incidence (%) UC: 92
Ananthakrishnan *et al*[18] (2008)	IBD and non-IBD CDI adult inpatients	1998-2004	N/R	N/R	CDI incidence increase: UC: 24 to 39/1000 discharge ; CD: 8 to 12/1000 discharges
Nguyen *et al*[17] (2008)	IBD and non-IBD adult inpatients	1998-2004	N/R	N/R	CDI incidence increase: UC: 26.6 to 51.2/1000 discharges
Pascarella *et al*[35] (2009)	IBD pediatric inpatients	2005-2007	Enzyme immunoassay for toxins A/B	Mixed	CDI incidence (%) UC: 21.3; CD: 35
Ricciardi *et al*[27] (2009)	IBD adult inpatients	1993-2003	N/R	Active	CDI incidence (%) UC: 2.8; CD: 1.0 CDI incidence increase: IBD: 12.2 to 21/1000 discharges; CD + colonic involvement: 12.2 to 23.1/1000 discharges
Wultańska *et al*[36] (2010)	IBD pediatric outpatients	2005-2007	EIA for Toxins A/B or PCR	Mixed	CDI incidence (%) IBD: 60; UC: 61; CD: 59
Ananthakrishnan *et al*[58] (2011)	IBD adult inpatients	1998, 2004, 2007	N/R	N/R	CDI incidence increase: CD: 0.8 to 1.5% of hospitalizations; UC: 2.4 to 5.3% of hospitalizations Absolute mortality increase in CDI + IBD (5.9% to 7.2%)
Kaneko *et al*[46] (2011)	UC pediatric and adult inpatients and outpatients	2006-2009	ELISA for Toxin A	Active	CDI incidence (%) UC inpatient: 36.6; UC outpatient: 41.7
Mezoff *et al*[37] (2011)	IBD pediatric patients	2007-2008	EIA for Toxins A and B	Mixed	CDI incidence (%) UC: 5.8; CD: 7.8; IC: 11.1
Ott *et al*[28] (2011)	IBD adult inpatients	2001-2008	ELISA for Toxins A/B or characteristic histology	Active	CDI incidence (%) IBD: 4.0; CD: 13.2; UC: 4.7
Banaszkiewicz *et al*[38] (2012)	IBD pediatric inpatients	2007-2010	EIA for Toxins A and B	Mixed	CDI incidence (%) IBD: 47
Antonelli *et al*[29] (2012)	IBD adult inpatients	2007-2010	N/R	Active	CDI incidence (%) UC: 11.1; CD: 1.7
Murthy *et al*[31] (2012)	UC adult inpatients	2002-2008	N/R	Active	CDI incidence (%) UC: 9.0
Lamousé-Smith *et al*[97] (2013)	IBD pediatric inpatients and outpatients (United States)	2006-2012	PCR for Toxin B +/- ELISA for Toxin A/B	Mixed	CDI incidence (%) UC: 18.4; CD: 11.6

Masclee *et al*[47] (2013)	IBD adult outpatients	2009-2010	PCR for *C. difficile* and Toxin A/B	Active	CDI incidence (%) IBD: 4.9; UC: 3.4; CD: 5.9
Mir *et al*[39] (2013)	IBD pediatric patients	2010-2012	EIA or PCR for Toxin A/B	N/R	CDI incidence (%) IBD: 8.1; UC: 5.6; CD: 9.3 ; IBDU: 11.1 No significant variation in IBD incidence over 3 yr
Pant *et al*[98] (2013)	IBD pediatric inpatients	2000, 2003, 2006, 2009	N/R	N/R	CDI incidence increase: IBD: 21.7 to 28 cases/1000 IBD cases per year; UC: 28.1 to 42.2/1000 cases per year; CD: 18.3 to 20.3/1000 cases per year
Li *et al*[34] (2013)	IBD adult outpatients with IPAA	2010-2011	PCR for Toxin B gene	Active	CDI incidence (%) IBD: 10.7; UC: 10.4; CD: 0; IC: 25.0
Martinelli *et al*[40] (2014)	IBD pediatric inpatients and outpatients	2010-2011	EIA for Toxins A/B	Mixed	CDI incidence (%): IBD: 10.0; UC: 7.5; CD: 11.9
Regnault *et al*[30] (2014)	IBD adult inpatients	2008-2010	Stool culture on selective medium + cytotoxicity assay +/- toxigenic culture	Active	CDI incidence (% hospitalizations): IBD: 7.0; UC: 6.8; CD: 7.2
Negrón *et al*[32] (2014)	UC adult inpatients	2000-2009	EIA for Toxins A/B	Active	CDI incidence (%) UC: 6.1
Hourigan *et al*[99] (2014)	IBD and non-IBD pediatric and adult inpatients	1993-2012	N/R	N/R	CDI incidence increase: IBD: 19.9 to 67/1000 admissions Rate of increase in CDI not significantly different between patients with or without IBD
Krishnarao *et al*[25] (2015)	IBD adult inpatients and outpatients	2008-2011	EIA and PCR	Mixed	CDI incidence (%) IBD: 5.1
Sandberg *et al*[19] (2015)	IBD pediatric inpatients	1997-2011	N/R	N/R	Hospitalization rate increase: CDI + IBD: 2.8 to 14.4 per million population per year Rate of increase for UC + CDI = CD + CDI
Simian *et al*[100] (2016)	IBD adult and pediatric inpatients and outpatients	2014-2015	PCR	N/R	CDI incidence (%) UC: 5.0; CD: 5.0
Roy *et al*[101] (2016)	CD adult outpatients on chronic antibiotic therapy > 6 mo	1992-2015	N/R	N/R	CDI incidence (%) CD: 2.0

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's Disease; IC: Indeterminate colitis; IBDU: Inflammatory bowel disease unclassified; IPAA: Ileal anal-pouch anastomosis; EIA: Enzyme immunoassay; ELISA: Enzyme linked immunosorbent assay; PCR: Polymerase chain reaction ; N/R: Not reported.

to 11.1%[26-32]. In adult outpatients with ileal-anal pouch anastomosis (IPAA) for IBD, incidence of CDI is 10.7%-18.3%[33,34]. The incidence of CDI in IBD among pediatric patients is 7.8%-69%, similarly with a higher incidence among patients with UC as opposed to CD[35-40].

Risk factors for CDI in IBD

In patients with CDI and IBD, risk factors are categorized into environmental and host risk factors, including those specific to IBD. Several studies have demonstrated that IBD itself is an independent risk factor for CDI in both adult and pediatric populations[16,35,41].

In the general population, many host and environmental risk factors have been identified. These include antibiotic exposure, specifically broad-spectrum antibiotics, as well as recent hospitalization, immunosuppression, increased age, and comorbidities[42].

In IBD populations, risk factors for CDI appear to be partly distinct (Table 2). Evidence is contradictory regarding antibiotic use as a risk factor for CDI in IBD patients. Three retrospective studies identified recent antibiotic use as a risk factor for CDI and recurrent CDI in both CD and UC[23,43,44]. In one study, antibiotic exposure within 30 d prior to *C. difficile* testing was associated with a twelve-fold risk of CDI in UC patients (95%CI: 1.2-124.2)[43]. Several others contradict this[30,34,35,43,45-47]. Scarce evidence supports nonsteroidal anti-inflammatories (NSAIDs) and proton pump inhibitors (PPIs) as risk factors for CDI in IBD. One retrospective cohort study of 480 IBD patients hospitalized for a flare who also underwent *C. difficile* testing, describes NSAID use within two months prior to admission as a predisposing factor for CDI (OR = 3.8, 95%CI: 1.2-12.3, P = 0.02)[30]. No studies have identified gastric acid-suppressive therapy as a risk factor for CDI in the IBD population[30,34,43-46].

Most studies demonstrate ongoing steroid, biologic, or immunomodulator therapy does not increase the risk of CDI in IBD patients[30,34,35,43,45-47], however, some contradictory evidence exists. A retrospective cohort study of 999 IBD inpatients (737 CD and 262 UC) report a greater than two-fold increased risk of CDI with maintenance immunomodulator use, defined as azathioprine, 6-mercaptopurine, or methotrexate

Table 2 Risk factors for *Clostridium difficile* infection in inflammatory bowel disease

Ref.	Sampling time frame	Setting	Diagnosis method	Identified risk factors	
				HOST	ENVIRONMENT
Razik *et al*[44] (2016)	2010-2013	Inpatient	PCR	Non-ileal CD	Hospitalisation for CDI; recent antibiotic use; biologic therapy; 5-ASA; Steroids
McCurdy *et al*[54] (2016)	2005-2011	Inpatient and outpatient	PCR	CMV infection	N/A
Seril *et al*[45] (2014)	2010-2013	Inpatient and outpatient	PCR for Toxin B	Post-surgery mechanical intestinal complications; low serum immunoglobulin level	None identified
Regnault *et al*[30] (2014)	2008-2010	Inpatient	Stool culture on selective medium + cytotoxicity assay +/- toxigenic culture	None identified	NSAIDs
Connelly *et al*[52] (2014)	N/R	N/R	PCR for Toxin A gene	*IL-4* gene associated SNP rs2243250	Not studied
Ananthakrishnan *et al*[102] (2014)	1998-2010	Inpatient	N/R	Low vitamin D concentration	Not studied
Ananthakrishnan *et al*[56] (2013)	N/R	Inpatient and outpatient	ELISA for Toxin A/B	Female sex; pancolitis; IBD-related SNPs	Protective : Anti-TNF therapy
Monaghan *et al*[53] (2013)	2009-2012	N/R	Toxigenic culture	Impaired ability to generate: toxin-specific antibody, memory B-cell responses	Not studied
Li *et al*[34] (2013)	2010-2011	Outpatient	PCR for Toxin B	None identified	Recent hospitalization
Masclee *et al*[47] (2013)	2009-2010	Outpatient	PCR for *C. difficile* and Toxins A/B	None identified	None identified
Kaneko *et al*[46] (2011)	2006-2009	Inpatient and outpatient	ELISA for Toxin A	None identified	None identified
Kariv *et al*[43] (2011)	2000-2006	Inpatient and outpatient	EIA for Toxin A/B	Recent surgery	Recent antibiotic use; recent hospitalization
Ricciardi *et al*[27] (2009)	1993-2003	Inpatient	N/R	Colonic involvement	Not studied
Schneeweiss *et al*[49] (2009)	2001-2006	Inpatient and outpatient	N/R	Not studied	Corticosteroid initiation
Nguyen *et al*[17] (2008)	1998-2004	Inpatient	N/R	Colonic involvement Comorbidity	Not studied
Issa *et al*[24] (2007)	2005	Inpatient	ELISA for Toxin A/B	Colonic involvement	Maintenance immunomodulator use
Rodemann *et al*[16] (2007)	1998-2004	Inpatient	Cell cytotoxic culture 2002 onwards *C. difficile* Toxin A/B immunoassay	Age Comorbidity	Not studied
Mylonaki *et al*[23] (2004)	1997-2001	Inpatient	ELISA for Toxin A/B	None identified	Recent antibiotic use

CDI: Clostridium difficile infection; rCDI: Recurrent Clostridium difficile infection; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's Disease; IPAA: Ileal anal-pouch anastomosis; CMV: Cytomegalovirus; CF: Cystic fibrosis; EIA: Enzyme immunoassay; ELISA: Enzyme linked immunosorbent assay; NSAID: Non-steroidal anti-inflammatories PCR: Polymerase chain reaction; N/R: Not reported.

(OR = 2.56, 95%CI: 1.28-5.12, P = 0.008)[24]. In the general population, corticosteroid use increases the risk of CDI[48]. However, when analyzing CDI risk in IBD patients using corticosteroids, studies were observational and did not control for underlying disease activity. A large retrospective cohort study of 10662 IBD inpatients noted a greater than three times increased risk of CDI within 90 d of corticosteroid initiation (RR = 3.4; 95%CI: 1.9-6.1) but no increased risk with preceding biologic therapy. This risk remained constant after 90 d of corticosteroid therapy and was not dose-dependent[49]. Risk factors for recurrent CDI (rCDI), in addition to recent antibiotic use, included preceding steroid and biologic therapy. However, when further stratified, rCDI was associated with infliximab use but not adalimumab or immunomodulator therapy[44].

Although there appears to be more community-acquired CDI in the IBD population compared to the general population, recent hospitalization has also been identified as a risk factor for CDI and rCDI[43,44]. Patients who have undergone colectomy are still at risk of CDI. Ten point seven percent of symptomatic IBD patients with ileal anal-pouch anastomosis (IPAA) were found to be positive for *C. difficile* toxin in a prospective cohort of 196 patients[34]. A retrospective observational study of 284 UC patients who underwent IPAA found that 64 patients developed pouchitis. Three of the four patients in this cohort with antibiotic-refractory pouchitis were discovered to have CDI that responded to oral vancomycin[50].

Genetic and immunologic risk factors have been identified in IBD patients for the development of CDI[51]. In a retrospective cohort study of 172 IBD patients, an interleukin-4-associated single nucleotide polymorphism (rs2243250) is associated with CDI

in IBD[52]. Monaghan *et al*[53] studied the humoral response to *C. difficile* toxins A and B in patients with IBD, cystic fibrosis, and healthy controls, finding that an impaired ability to sustain or generate strong toxin-specific antibody and B-cell responses could play a role in CDI development in IBD patients. Furthermore, low serum immunoglobulins were reported as a risk factor for CDI in IBD patients with IPAA[34]. A retrospective case control study of 306 IBD inpatients and outpatients, found that those with CMV infection were at higher risk of being co-infected with *C. difficile*[54]. As in the general population, patient comorbidities increase the risk of CDI in the IBD population[16,17,55]. While adult IBD patients affected by CDI are younger than those in the general population, increasing age has also been reported as a risk factor for CDI[16].

IBD disease activity is difficult to differentiate from CDI. Therefore, it is not clear that disease activity is an independent risk factor for the development of CDI. Disease location may affect patient risk. CDI is more often identified in those with UC and CD patients with colonic involvement[16,24]. In a retrospective nested case-control analysis of a national hospital discharge database, the prevalence of CDI among IBD patients with only small bowel disease was significantly lower than UC patients or CD patients with ileocolonic disease and only slightly higher than non-IBD patients[17]. Extent of disease in UC patients may be a risk factor for CDI. A prospective cohort study of 319 UC patients found pancolitis to be a risk factor for CDI (OR = 2.52, 95%CI: 1.03-6.17)[56].

Impact of CDI in IBD

CDI negatively impacts short and long-term IBD-related outcomes, including rates of colectomy, escalation in IBD therapy, and mortality. It also results in longer hospitalizations, increased readmission rates, and increased in-hospital expenditures (Table 3).

Increased mortality among IBD patients with CDI has been reported in numerous adult inpatient studies compared to non-IBD patients with CDI[17] and IBD patients without CDI[31,55,57,58]. Furthermore, it appears that this excess mortality is not limited to the index hospitalization. A retrospective cohort study of 2016 adult UC inpatients described increased mortality among patients with CDI compared to those without CDI in the five years post-discharge (HR = 2.41, 95%CI: 1.37-4.22)[31].

Colectomy rates have been reported to be higher in IBD patients with CDI. A retrospective case control study of 99 adult UC inpatients reported CDI at index admission significantly predicted colectomy within one year[59]. Higher rates of colectomy among IBD patients with CDI have been similarly reported in other large adult inpatient studies compared to non-IBD patients with CDI (6.4% *vs* 0.3%)[44] and IBD patients without CDI (OR = 1.87-10.0) [32,57,59,60] during index admission and up to one year following the initial episode.

IPAA failure also is associated with a history of CDI. A retrospective chart-review study of 417 IBD patients undergoing IPAA found that a history of CDI prior to colectomy in IBD patients was independently associated with IPAA failure (HR = 3.02, 95%CI: 1.23-7.44)[61].

While CDI alone is associated with significant morbidity and mortality, it is thought that CDI may actually lead to a flare in IBD activity resulting in further morbidity. This is supported by a retrospective cohort study of 146 adult UC inpatients and outpatients reporting increased escalation in therapy among patients with CDI in the year after index admission compared to the year prior[60]. A retrospective nested case control study of 238 pediatric IBD inpatients with and without CDI similarly demonstrated significant escalation in therapy among those with CDI as compared to those without after the infection[62].

Diagnosis of CDI in IBD

The overlap in symptomatology between CDI and isolated IBD flare complicates the diagnosis of CDI in IBD patients. CDI and acute inflammatory colitis are clinically indistinguishable. Therefore, a diagnosis relies primarily on laboratory findings, and to a lesser degree endoscopic or histologic findings.

It is recommended to test all patients with acute flares presenting with diarrhea for CDI[63]. Despite its impact on outcome and management, many patients with newly diagnosed IBD or flaring IBD are not tested for CDI. A retrospective cohort study of adult IBD inpatients report that *C. difficile* testing within 48 h for patients hospitalized for an IBD flare was only performed on 59% of 813 consecutive hospitalizations. A diagnosis of UC or CD with colonic involvement was noted to be independent predictors of CDI testing[30]. In a retrospective cohort study of pediatric patients with newly diagnosed IBD, only 42% of 290 cases had testing for *C. difficile* around the time of diagnosis[39].

Compared to previously discussed diagnostic methods, pseudomembranes on colonoscopy are specific but not sensitive to diagnose CDI in IBD patients. In a multi-center retrospective study of 93 IBD patients hospitalized with CDI who underwent colonoscopy, only 13% were noted to have pseudomembranes. The presence of pseudomembranes was not found to significantly impact clinical outcomes[64]. A retrospective case-control study of CDI in IBD and non-IBD patients found that none of the IBD-CDI patients had pseudomembranes on endoscopy compared to nearly half of the non-IBD-CDI group[20]. A retrospective study of 37 flaring UC patients assessed histological changes on colonic biopsies with or without CDI. They reported that although those with CDI had significantly more microscopic pseudomembranes than the controls without CDI, less than half of the specimens of CDI patients had this finding[65].

Testing *via* PCR should only be performed on

Table 3 Outcomes of inflammatory bowel disease patients with *Clostridium difficile* infection

Ref.	Patient population	Sampling time frame	Study design	*n*	Outcomes
Razik *et al*[44] (2016)	Adult CDI IBD + CDI Inpatient	2010-2013	Retrospective, single-center, cohort study	503	Incidence of rCDI IBD > non-IBD [2.04/100 person-months (95%CI: 1.55-2.64) *vs* 1.25 episodes per 100 person-months (95%CI: 1.05-1.48)] Colectomy IBD > non-IBD (6.4% *vs* 0.3%)
Skowron *et al*[61] (2016)	Adult IBD + IPAA Inpatient (United States)	2000-2010	Retrospective, observational, single-center cohort study	417	CDI pre-colectomy associated with post-reconstruction pouch failure (HR = 3.02 95%CI: 1.23-7.44)
McCurdy *et al*[54] (2016)	Adult IBD IBD + CMV IBD + CMV + CDI IBD + CDI Inpatient and outpatient (United States)	2005-2011	Retrospective, case-control, single-center, study	248	Colectomy-free survival at 1 yr IBD + CDI > IBD + CMV + CDI (71.5% *vs* 30%) IBD + CMV controls > IBD + CMV + CDI (57.1% *vs* 30%)
Negrón *et al*[32] (2014)	Adult UC Inpatient (Canada)	2000-2009	Retrospective, case-control, multi-center, database study	481	Emergent surgery CDI + UC > UC alone [OR = 3.39 (95%CI: 1.02-11.23)] Development of new infectious postoperative complication CDI + UC > UC alone (OR = 4.76, 95%CI: 1.10-20.63)
Horton *et al*[70] (2014)	Adult IBD Inpatient (United States)	2006-2010	Retrospective, observational, single-center study	114	Readmission: UC + CDI > CD + CDI (24% *vs* 10%, P = 0.04) IBD + steroids > no-steroids (29% *vs* 8%, P < 0.01) Colectomy: UC + CDI > CD + CDI, index admission (27.4% *vs* 0%, P < 0.01) IBD + steroids > no-steroids (32% *vs* 6%, P < 0.01)
Pant *et al*[98] (2013)	Pediatric IBD Inpatient (United States)	2000, 2003, 2006, 2009	Retrospective, nested case-control, nationwide database study	12610	LOS: CDI + IBD > IBD (8.0 *vs* 6.0, aRC = 2.1 d, 95%CI: 1.4-2.8) Hospitalization cost: CDI + IBD > IBD alone ($45126 *vs* $34703, aRC = $11506, 95%CI: 6192-16829) Parenteral nutrition: CDI + IBD > IBD alone (15.9% *vs* 12.1% aOR = 1.5, 95%CI: 1.1-2.0) Blood transfusions: CDI + IBD > IBD alone (17.7% *vs* 9.8%, aOR = 1.8, 95%CI: 1.4-2.4).
Li *et al*[34] (2013)	Adult IBD + IPAA Outpatient (United States)	2010-2011	Prospective, single-center, cohort study	196	42.9% cured by single course of Vancomycin 57.1% recurrent/refractory CDI
Chu *et al*[103] (2013)	Adult UC + CDI Inpatient (United States)	2002-2012	Retrospective, single-center, observational study	23	Morbidity and mortality after colectomy: UC + CDI + full antibiotic course pre-op = UC + CDI + incomplete antibiotic course pre-op
Ananthakrishnan *et al*[55] (2013)	Adult IBD Inpatient (United States)	2007	Retrospective, nested case-control, nationwide database study	67221 hospitalizations	Mortality: CDI + IBD *vs* IBD alone (OR = 3.23, 95%CI: 2.55-4.03).
Murthy *et al*[31] (2012)	Adult UC Inpatient (Canada)	2002-2008	Retrospective, database, cohort study	2016	Mortality: CDI + UC > UC alone, 5-yr risk (aHR = 2.40, 95%CI: 1.37-4.20) CDI + UC > UC alone, index hospitalization (aHR = 8.90, 95%CI: 2.80-28.3) CDI + UC > UC alone, 5 years post-discharge (aHR = 2.41, 95%CI: 1.37-4.22)

Navaneethan *et al*[60] (2012)	Adult UC Inpatient and outpatient (United States)	2002-2007	Retrospective, single-center, cohort study	146	UC-related ER visits: CDI + UC *vs* UC alone, 1 yr post index infection (37.8% *vs* 4%, $P < 0.001$) Colectomy: CDI + UC *vs* UC alone, 1 yr post index infection (35.6% *vs* 9.9%, $P < 0.001$) CDI associated with colectomy within 1 yr (OR = 10, 95%CI: 2.7-36.3) Escalation in therapy: CDI + UC year after CDI admission *vs* year prior (55.8% *vs* 12.9%, $P < 0.0001$)
Jen *et al*[57] (2011)	Adult IBD Inpatient (England)	2002-2008	Retrospective, nested case-control, nationwide database study	241478 hospitalizations	Mortality: IBD + CDI (defined as hospital-acquired > IBD alone (aOR = 6.32, 95%CI: 5.67-7.04) LOS: IBD + CDI > IBD alone (27.9 d longer) GI surgery: IBD + CDI > IBD alone (aOR = 1.87, 95%CI: 0.60-5.85)
Kariv *et al*[43] (2011)	Adult UC Inpatient and outpatient (United States)	2000-2006	Single-center	78	Colectomy within 3 mo not associated with CDI No UC or CDI associated mortality identified
Ananthakrishnan *et al*[58] (2011)	Adult IBD Inpatient (United States)	1998, 2004, 2007	Retrospective, nested case-control, nationwide database study	-	Mortality: IBD + CDI > IBD alone, from 1998 to 2007 (OR = 2.38, 95%CI: 1.52-3.72 to OR = 3.38, 95%CI: 2.66-4.29).
Kelsen *et al*[62] (2011)	Pediatric IBD Inpatient (United States)	1997-2007	Retrospective, nested case-control, single-center study	315	rCDI: CDI + IBD > CDI-alone (34% *vs* 7.5%, $P < 0.0001$) Escalation in therapy: IBD + CDI > IBD alone (67% *vs* 30%, $P < 0.001$)
Jodorkovsky *et al*[59] (2010)	Adult UC Inpatient (United States)	2004-2005	Retrospective, single-center, case-control study	99	UC-related hospitalizations: CDI + IBD > IBD alone, over 1 yr Colectomy: CDI at index admission predictor for colectomy within 1 yr (OR = 2.38, 95%CI: 1.01-5.6) CDI status not a significant predictor for requirement for emergent colectomy at index admission LOS: CDI + IBD = IBD alone
Ben-Horin *et al*[64] (2010)	Adult IBD + CDI Inpatient (Europe/Israel)	2000-2008	Retrospective, multi-center, cohort study	93	Morbidity and mortality: IBD + CDI patients + pseudomembranes on endoscopy = IBD + CDI without pseudomembranes
Nguyen *et al*[17] (2008)	IBD and non-IBD controls Inpatient (United States)	1998-2004	Retrospective, nested case-control, nationwide database study	116842 hospitalizations	Mortality: UC + CDI > CDI alone (OR = 3.79, 95%CI: 2.84-5.06) LOS: CD + CDI > CDI alone Hospitalization cost: UC + CDI > CDI alone

CDI: Clostridium difficile infection; rCDI: Recurrent clostridium difficile infection; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; IPAA: Ileal anal-pouch anastomosis; CMV: Cytomegalovirus; OR: Odds ratio; aOR Adjusted odds ratio; aRC: Adjusted regression coefficient; LOS: Length of stay.

unformed stools to limit false positives. Asymptomatic carriers of toxigenic *C. difficile* exist in both IBD patients and the general population. Asymptomatic carriage rates vary significantly with the patient population under study[66]. A rate of 8.2% has been reported in an adult outpatient IBD population with stable disease compared to 1.0% in healthy controls, with higher rates in UC patients compared to those with CD[67]. A prospective case-control study of 163 pediatric outpatients reports a significantly higher carriage rate in those with IBD than in healthy controls (17% *vs* 3%), which was not associated with recent hospitalization[68]. There are no studies evaluating treatment of the asymptomatic carriage of *C. difficile*. Evidence is lacking to suggest that treating asymptomatic *C. difficile* carriers has any future

impact on IBD disease activity or the development of symptomatic CDI. However, in the general population, carriage of *C. difficile* in the absence of symptoms carries a protective effect against future symptomatic CDI[7]. This protective effect has not been studied in the IBD population.

It has been demonstrated that the asymptomatic shedding of *C. difficile* spores can continue for weeks following the resolution of symptoms[69]. Therefore, test of cure is not recommended. However, in patients with IBD and CDI where symptom overlap creates both diagnostic and therapeutic challenges, repeat testing in patients with ongoing diarrhea may guide management, despite the risk of false-positive results.

Treatment of CDI in IBD patients

In patients with confirmed CDI, distinguishing between symptoms resulting from infection, as opposed to a flare of underlying IBD, creates a management dilemma. There are no randomized controlled trials (RCT) of therapy in IBD patients with CDI to help guide practice. Guidelines outlining the approach to eradication of *C. difficile via* antibiotic therapy or fecal microbiota transplant (FMT) in the setting of recurrent CDI also include recommendations for the IBD population[3]. IBD outpatients with non-severe CDI can be initially treated with metronidazole, however IBD inpatients regardless of disease severity should receive a vancomycin-containing regimen as first-line therapy (Table 4)[70]. In addition to medical therapy, specific infection control measures should also be put in place, including hand-washing to minimize fecal-oral transmission of *C. difficile* spores, as well as isolation of patients with CDI under contact-precautions.

Management of IBD flares in patients with CDI

While the treatment of isolated CDI is well studied, the initiation, maintenance or escalation of corticosteroid, immunomodulator or biologic therapy in IBD patient with CDI is not delineated and relies heavily on expert opinion.

Corticosteroids

In the setting of suspected IBD flare in a patient with known CDI, concurrent corticosteroid therapy is reasonable and supported by expert opinion[3,71]. Nevertheless, significant uncertainty exists among practitioners with regards to the initiation of corticosteroid therapy and its safety in the context of an ongoing CDI-mediated colitis. A survey of 169 North American gastroenterologists demonstrated divergence among clinicians with regards to initiating therapy in hospitalized UC patients with CDI; 54% opted for antibiotic monotherapy compared to 46% opting for a combination of antibiotics with either azathioprine or corticosteroids[71]. This concern originates from findings of several observational studies, detailed above, demonstrating increased risk of CDI, rCDI, and worse outcomes among IBD patients receiving corticosteroids[44,49,70]. However, these patients were receiving corticosteroids prior to CDI, and no analysis has been performed for initiation of corticosteroids in IBD patients with CDI on appropriate antimicrobial therapy.

Literature on initiating corticosteroids for IBD flares in patients with concomitant CDI is limited to case reports yielding promising results with patients experiencing remission of symptoms after starting corticosteroid therapy when appropriate antibiotics had failed to do so (Table 5). Similarly, data regarding the initiation of corticosteroids in patients with CDI in the general population is scarce. Corticosteroids have been successfully used as adjunctive therapy to antibiotics in infectious processes such as meningitis, pneumonia, and sepsis[72-74]. While the benefit of corticosteroids seen in these infections may not predict an effect in CDI, it does confer biologic plausibility.

Conversely, a European retrospective, non-randomized, multi-center study of 155 IBD patients hospitalized with CDI evaluated the effects of antibiotics and immunomodulators compared to antibiotics alone. Immunomodulators were defined as any of the following: corticosteroids at a dose equal to or above 20 mg of prednisone daily, thiopurines at any dose, methotrexate, cyclosporine, tacrolimus, or biologics of any kind. Furthermore, there was no indication of whether therapy was for induction or maintenance of IBD. Conclusions are thus limited by the heterogeneity in the definition of immunomodulator use and antibiotic regimens. Nonetheless, combination of antibiotic and immunomodulator therapy was associated with higher morbidity and mortality compared to antibiotic monotherapy[75]. Most recent AGA practice guidelines suggest postponing escalation of steroids in the setting of acute CDI until 72 to 96 h after the initiation of appropriate antibiotic therapy. However, they refrain from providing further guidance on when to withhold, continue, or escalate corticosteroid therapy given the current absence of prospective data[76].

Immunomodulators and biologic therapy

Recent CDI guidelines suggest, in IBD patients with CDI, maintaining, but not escalating, existing immunosuppressive therapy, including immunomodulators such as azathioprine and methotrexate, as well as biologic agents[3]. Guidelines for the management of opportunistic infections in IBD make no explicit recommendations regarding these therapies in this setting, citing the lack of data available[77]. As described above, conflicting evidence exists regarding immunomodulator and biologic therapies as risk factors for the development of CDI or rCDI. No published data exists regarding when initiation of immunomodulating therapy or biologic therapy is

Table 4 Treatment of clostridium difficile infection in inflammatory bowel disease[3,4]

Severity	Criteria	Treatment	Comments
First episode			
Stop all non-CDI related antibiotic therapy if possible			
Mild to moderate disease	Diarrhea and symptoms not meeting criteria for severe disease	Metronidazole 500 mg by mouth 3 times per day for 10 d to 14 d or Vancomycin 125 mg by mouth 4 times per day for 10 to 14 d	In hospitalized patients with UC and nonsevere CDI, treatment with a vancomycin-containing regimen *vs* metronidazole alone resulted in fewer readmissions and shorter LOS[70]
Severe disease	Serum albumin < 3 g/dL AND one of the following: WBC ⩾ 15000 cells/mm^3 Abdominal tenderness Creatinine ⩾ 133 μmol/L	Vancomycin 125 mg by mouth 4 times per day for 10 to 14 d	
Severe, complicated disease	Admission to intensive care unit Hypotension ± vasopressor requirement Fever ⩾ 38.5 ℃ Ileus Mental status changes WBC ⩾ 35000 cells/mm^3 or ⩽ 2000 cells/mm^3 Serum lactate ⩾ 2.2 mmol/L End organ failure	Vancomycin 500 mg by mouth or nasogastric tube 4 times per day and Metronidazole 500 mg IV every 8 h and, if ileus, Vancomycin 500 mg in 500 mL saline as enema 4 times per day	Consider early surgical consultation
Recurrent CDI			
First recurrence		Metronidazole 500 mg by mouth 3 times per day for 10 to 14 d or Vancomycin 125 mg by mouth 4 times per day for 10 to 14 d or Fidaxomicin 200 mg by mouth 2 times per day for 10 d	
Second recurrence		-Tapered and pulsed vancomycin or Fidaxomicin 200 mg by mouth 2 times per day for 10 d	
Subsequent recurrence		-Fecal microbiota transplant	

LOS: Length of stay; CDI: Clostridium difficile infection; UC: Ulcerative colitis.

safe in patients with both IBD and CDI. In a study of 14 pediatric patients with predominantly CD being treated with methotrexate and anti-TNF therapy, four patients developed CDI. They were treated with antibiotics with successful clearance of *C. difficile* but ultimately failed combination therapy[78]. It is difficult to draw conclusions regarding the safety of biologic and immunomodulator therapy from this due to the sample size. Figure 2 summarizes our approach to the patient with IBD who presents with an acute flare in symptoms for which a *C. difficile* assay is sent, based on existing literature.

Fecal microbiota transplant and recurrent CDI

Existing therapeutic options for rCDI in the general population include vancomycin pulsed and tapered regimens, fidaxomicin, as well as fecal microbiota transplant (FMT). FMT is appealing given the potential to treat both CDI and IBD simultaneously. The risk of rCDI increases with each episode and is higher in IBD patients, as demonstrated in a large retrospective cohort study (32% *vs* 24%, $P < 0.01$)[44]. FMT has been demonstrated to be a safe and effective therapy for rCDI in the general population on the basis of several large RCTs[79-81]. Several studies have analyzed treatment of rCDI in IBD patients. A retrospective study of immunosuppressed patients with CDI undergoing FMT included 36 IBD patients, of which 86% were cured of CDI after one transplant and 14% worsened in disease activity[82]. Another retrospective multicenter study of 67 IBD patients (35 CD; 31 UC; 1 IBDU), of which 64% were receiving immunosuppressive therapy at the time of FMT, found that 79% had either resolution of their diarrhea and/or negative CDI testing at week 12 and 46% had improved disease activity at 3 mo. Disease activity at 3 mo remained the same or worsened in 36%, and 18% of patients, respectively[83]. Adverse events occurred in 12% of patients at 3 mo. One patient received a colectomy and two had IBD related hospitalizations. In

Table 5 Case reports of corticosteroid initiation in *Clostridium difficile* infection

Reference (year of publication)	Patient data		Treatment regimen	Outcome
	Demographics	Clinical presentation		
Cavagnaro *et al*[104] (2003)	5M	Bloody diarrhea (> 10 loose stools/d), tenesmus, abdominal tenderness, fever	Oral vancomycin (40 mg/kg per day divided in 6-hourly doses) and IV metronidazole (20 mg/kg per day divided in 8-hourly doses) × 14 d	Resolution of diarrhea within 24 h of steroid initiation
		WBC 19000 cells/mm^3, albumin 21 g/L		Resolution of endoscopic changes at 6 wk
		Positive *C. difficile* toxin	IV methyldrnisolone (2 mg/kg per day in two divided doses) on day 14 × 3 d	
		Pseudomembranous colitis on flexible sigmoidoscopy on day 14	Prednisone 2 mg/kg per day tapered over one month	
Sykes *et al*[105] (2012)	54F	Moderate CDI that resolved with 10-d course antibiotics	Oral metronidazole × 10 d with resolution of symptoms (doses not specified)	Decreased stool frequency, normalization of vital signs, reduction in CRP to 132 within 48 h of steroid initiation
		Recurrent diarrhea and abdominal pain 10 d after completion of antibiotics with		Resolution of diarrhea, further reduction in CRP to 15 after 9 d of steroid therapy
		left colonic thickening on CT and positive *C. difficile* toxin	Oral vancomycin and metronidazole upon admission (doses not specified) × 4 d	Resolution of endosocopic changes at 1 mo
		Fever, tachycardia on day 4		Sustained clinical response at 5 mo
		with pseudomembranous colitis on flexible sigmoidoscopy	Oral vancomycin 125 mg every 6 h × 9 d	
		CRP increased from 149 on admission to 236 on day 4	IV hydrocortisone 100 mg every 6 h × 9 d	
			Prednisolone 30 mg daily with tapering regimen	
	73F	Moderate-severe CDI that resolved with 10-d course antibiotics	Metronidazole 400 mg every 8 h × 10 d with resolution of symptoms	Resolution of diarrhea, normalization of vital signs, reduction in CRP to 7 within 48 h of steroid initiation
		Recurrent moderate CDI 1 wk after completion of antibiotics that resolved with another 10-d course of antibiotics		Complete clinical response at 14 d with no further relapses
		Recurrent CDI 10 d after completion of antibiotics with fever, tachycardia, increased CRP 87	Oral vancomycin 125 mg every 6 h × 10 d with resolution of symptoms	
		Slow response to antibiotics with flexible sigmoidoscopy on day 8 with pseudomembranous colitis		
			Oral vancomycin 125 mg every 6 h × 8 d with tapering regimen over 14 d	
			Prednisolone 30 mg daily × 7 d followed by tapering regimen	
	91F	Moderate CDI with persistent diarrhea despite courses of metronidazole and vancomycin	Oral metronidazole 400 mg every 8 h × 10 d without resolution of symptoms	Resolution of diarrhea and normalization of CRP within 72 h of steroid initiation
		CRP 11		No further relapses
		Flexible sigmoidoscopy with pseudomembranous colitis	Oral vancomycin 125 mg every 6 h for prolonged course without resolution of symptoms	
			Prednisolone 30 mg daily × 14 d with continued vancomycin tapering regimen over 4 wk	

CDI: Clostridium difficile infection; CRP: C-reactive protein.

a prospective study of 35 IBD patients (13 CD; 22 UC) undergoing FMT for rCDI, 54% of patients required escalation of IBD therapy, despite disappearance of *C. difficile* toxin from the stool[84]. Another retrospective study of 272 IBD and non-IBD patients undergoing one FMT for rCDI demonstrated IBD patients had lower CDI clearance rates than non-IBD patients (74% *vs* 92% $P = 0.0018$), independent of immunosuppressive

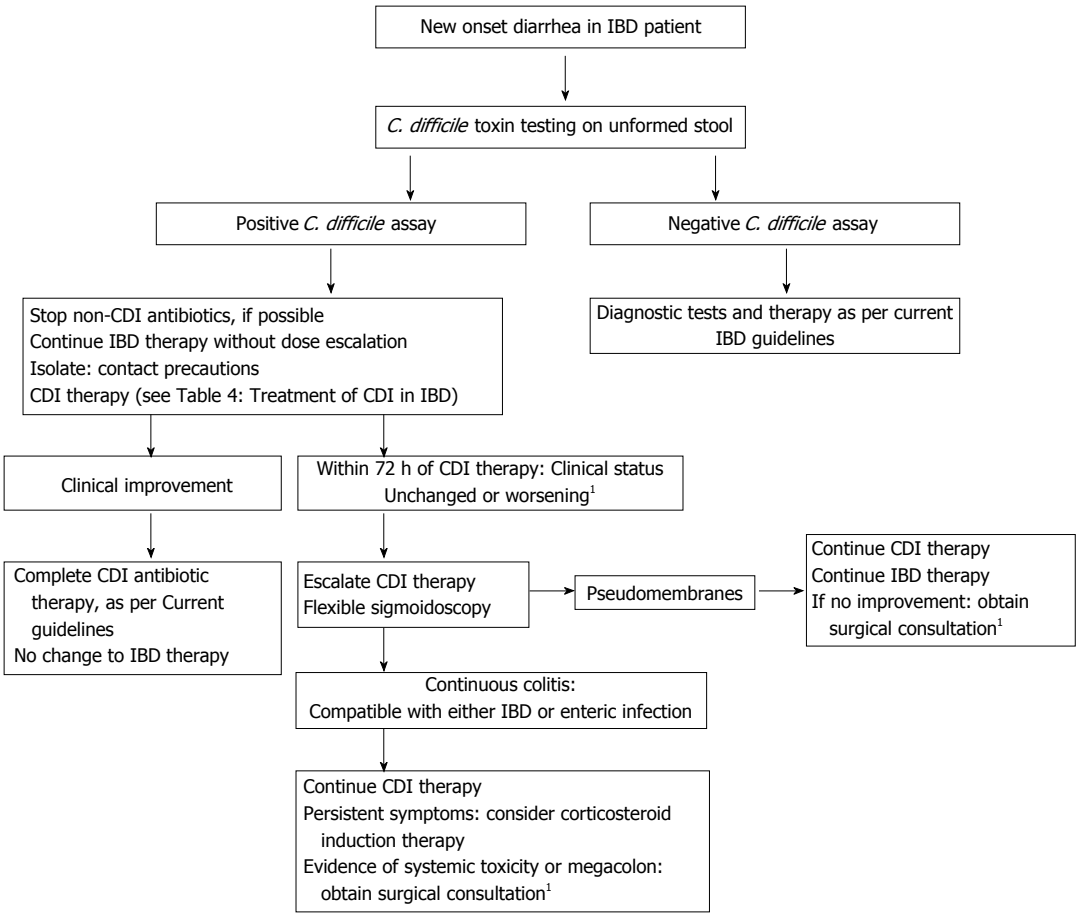

Figure 2 Approach to potential *Clostridium difficile* infection in inflammatory bowel disease patients. [1]Obtain surgical consultation earlier, as dictated by CDI guidelines, should there be evidence of toxic megacolon, or concern for rapid deterioration despite medical therapy. CDI: *Clostridium difficile* infection; IBD: Inflammatory bowel disease.

therapy[85]. In follow-up, despite *C. difficile* toxin clearance, 50% of UC patients worsened in disease activity requiring escalation of therapy[86].

FMT appears to effectively treat rCDI in IBD patients, albeit less-so than in the general population. However, subsequent worsening of disease activity is consistent throughout the literature. Furthermore, the effects of FMT on IBD activity are unclear. Outcomes are heterogeneous regarding FMT as treatment for IBD alone[87,88]. Although several meta-analysis exist[89,90] only 2 RCTs with conflicting results regarding UC patients are included. While one RCT of 70 patients showed FMT induced clinical remission compared to placebo[88], the other did not achieve a stringent composite primary end point of clinical remission and a > 1 point decrease in the endoscopic mayo score in 37 UC patients[87]. More recently, an RCT of intensive multidonor FMT (colonoscopic infusion followed by 5 enemas weekly for 8 wk) in 85 UC patients achieved a primary endpoint of steroid free clinical remission with endoscopic remission or response at week 8[91]. These results, combined with the efficacy of FMT in the treatment of rCDI in IBD patients, necessitates future RCTs analyzing intensive multidonor FMT for rCDI in IBD patients.

In conclusion, CDI commonly complicates the course of IBD but the lack of data precludes formal strong recommendations on the management of IBD in patients with CDI. Initiation of corticosteroids in IBD flares in the context of acute CDI is understudied but seems to be safe. Initiation or resumption of immunosuppressive therapy within 48 to 72 h of targeted antibiotic therapy may be appropriate. To better understand the treatment of IBD flares in the context of acute CDI, further studies are needed to determine the optimal timing and dosing of IBD-specific therapies.

COMMENTS

Background

Clostridium difficile (*C. difficile*) has been identified as an important nosocomial infection whose traditional risk factors include recent antibiotic use and exposure to a health care institution. Inflammatory bowel disease (IBD) is another important risk factor for *Clostridium difficile* infection (CDI), likely related to the decreased intestinal microbial diversity and disordered immune response seen in this population. Many observational studies have explored the epidemiology, risk factors, and outcomes of CDI in those with IBD and have reported its negative impact. CDI in IBD patients has been linked to excess morbidity, including longer hospitalization, higher risk of colectomy, and escalation in IBD therapy, as well increased mortality. IBD and CDI-related symptoms are often difficult to distinguish and beyond *C. difficile* eradication,

the appropriate IBD therapy is unclear. This review explored the existing evidence regarding the management of IBD in patients with CDI.

Research frontiers

Prospective studies evaluating the initiation and maintenance of IBD therapeutics in patients with CDI are lacking and are needed to help guide practice.

Innovations and breakthroughs

While the negative impact of developing CDI in those with IBD has been well established, the appropriate management of CDI in the IBD population is less well-defined. Risk factors for the development of CDI in IBD patients identified in this review include recent antibiotic exposure, hospitalization, and colonic involvement. Contradictory evidence exists as to whether maintenance immunosuppressive therapy is a risk factor for the development of CDI. On the basis of data presented in this study, vancomycin should be used as a first-line regimen for CDI. Case reports suggest that corticosteroid initiation, after appropriate antibiotic coverage, may be safe in those with CDI and IBD flare.

Applications

The symptoms of an IBD flare and CDI are often indistinguishable. As such, stool testing for *C. difficile* should be sent in every flaring IBD patient. Once CDI is diagnosed, a vancomycin-containing antibiotic regimen should be initiated. In the setting of ongoing symptoms, not warranting surgical intervention, it remains unclear when IBD-specific therapy can be initiated. However, case reports and expert opinion may allow for corticosteroid initiation after 3 d of appropriate CDI therapy.

Peer-review

It's a well done and well written extensive review on the epidemiology and therapy of CDI in IBD patients.

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META-ANALYSIS

Effect of silymarin on biochemical indicators in patients with liver disease: Systematic review with meta-analysis

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Abstract

AIM

To evaluate the effect of silymarin on the serum levels of alanine aminotransferase (ALT), aspartate amino-transferase (AST) and gamma glutamyl transpeptidase (γGT) in patients with liver diseases.

METHODS

A systematic review with meta-analysis of ramdomized and controlled clinical trials was performed, evaluating the effects of sylimarin in patients with hepatic diseases, published by January 31, 2016. Clinical trials were sought on the basis of The Cochrane Central Register of Controlled Trials in the Cochrane Library, PubMed/Medline, Scopus, Web of Science, Lilacs and Clinical Trials. The trials with adult and elderly patients of both sexes, with Liver Diseases who took oral silymarin supplementation, as extract or isolated, as well as Silymarin combined with other nutrients, were included. The trials should provide information about the intervention, such as dosages and detailing of the product used, besides the mean and standard deviation of serum levels of ALT, AST and γGT of the baseline and at the end of the intervention.

RESULTS

An amount of 10904 publications were identified. From those, only 17 were included in the systematic review and 6 in the meta-analysis, according to the used selection criteria. In this meta-analysis, the results indicated a reduction of 0.26 IU/mL (95%CI: -0.46-0.07, P = 0.007) at the level of ALT and 0.53 IU/mL

(95%CI: -0.74-0.32, P = 0.000) at the serum levels of AST after using the silymarin, both, statistically significant, but with no clinical relevance. There was no significant change in the γGT levels. Subgroup analyzes were also performed for the biochemical markers in relation to the type of intervention, whether silymarin isolated or associated with other nutrients and the time of intervention (whether ⩾ 6 mo or < 6 mo). Significant differences were not found. The evaluated studies presented a high degree of heterogeneity and low methodological quality in the carried out analysis.

CONCLUSION

Silymarin minimally reduced, but without clinical relevance, the serum levels of ALT and AST. It is necessary to carry out studies with more appropriate methodological designs.

Key words: Systematic review; Liver diseases; Milk thistle; Silymarin; Meta-analysis

Core tip: Silymarin is commonly prescribed in the practice of many professionals and ingested as self-medication for patients. Studies suggest benefits of its use in hepatic disorders, discussing its mechanisms of action and potential as a coadjutant in the treatment of those diseases. Favorable clinical outcomes as improvement of biochemical indicators and liver profile were observed in clinical trials. However, other studies are controversial or have not reported statistical significance in the improvement of these indicators. Facing the differences and methodological peculiarities of these studies, a systematic review with meta-analysis was performed to clarify the real benefits of silymarin in liver diseases.

de Avelar CR, Pereira EM, de Farias Costa PR, de Jesus RP, de Oliveira LPM. Effect of silymarin on biochemical indicators in patients with liver disease: Systematic review with meta-analysis. *World J Gastroenterol* 2017; 23(27): 5004-5017 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/5004.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.5004

INTRODUCTION

The most frequent liver diseases are of an inflammatory nature, which have different etiologies and characteristics. The most common causes of chronic inflammatory liver diseases are viral infections (hepatitis B and C viruses), autoimmune diseases, alcoholic liver disease and non-alcoholic fatty liver disease (NAFLD). Other diseases also occur with inflammation such as chronic biliary diseases, hereditary metabolic diseases and hepatic attacks by hepatotoxic substances[1]. Méndez-Sánches *et al*[2] have estimated approximately two million cases of chronic liver disease by the year 2050.

The nutritional treatment comprises a fundamental step in the clinical treatment of these patients, as well as in the minimization and/or postponement of the common symptomatology in these diseases[3] and, the prescription of herbal medicines can be a complementary tool to conventional dietary strategies[4].

Silymarin is part of the flavonoid group and is extracted from the plant *Silybum marianum*, an herbal remedy that has been extensively studied in various hepatic disorders. It is composed of approximately 50% silibinin, which is considered the biologically active component of silymarin[5,6]. *Silybum marianum* is one of the most commonly plants used in liver diseases treatments, because it is considered hepatoprotective and it has been widely used in patients with cirrhosis, chronic hepatitis and liver disease associated with alcohol consumption and exposure to environmental toxins[7-9]. Currently, it is one of the most studied medicinal herbs for the treatment of NAFLD and steatohepatitis (NASH) and its use has been shown to be safe, well tolerated, with limited adverse effects also for these patient groups[10-12].

Silymarin acts primarily as an antioxidant, reducing the production of reactive oxygen species and lipid peroxidation, increasing the endogenous concentrations of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase[13-16]. It exerts a significant anti-inflammatory effect, mainly by inhibition of nuclear transcription factor NFκB and consequently reduction of inflammatory cytokines in the hepatic parenchyma, in addition to interaction with protein kinases and downregulation of cyclooxygenase 2[17,18].

It also acts as an immunomodulator and anti-fibrotic agent, due to the reduction of the activation or stimulation of apoptosis of the hepatic stellate cells, or increasing the degradation of the collagen deposits in the hepatic parenchyma[19-21]. In addition, it's considered a hepatoprotective for the ability to stabilize the cell membranes of hepatocytes, preventing the entry of toxic chemicals into these cells. Silymarin binds to receptors present on these membranes, inhibiting the binding of toxins in these sites, reducing drug-induced hepatocellular damage[22,23]. It also stimulates the synthesis and activity of enzymes responsible for the hepatic biotransformation process, such as glutathione S-transferase[24,25].

Studies have shown that silymarin has an important effect on the reduction of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in liver diseases, being considered beneficial in the treatment of these patients[22,26-28].

However, it is important to point out that most of these studies present considerable methodological variations. In addition to having used different doses with different concentrations of silymarin and various formulations, which makes it difficult to perform a comparative analysis of the studies and a consensus

about the clinical use of this herbal medicine and its effects on biochemical indicators such as liver enzymes. Thus, the objective of this article is to perform a systematic review with meta-analysis on the effect of silymarin on the ALT, AST and gamma glutamyl transpeptidase (γGT) levels in patients with liver diseases. The present systematic review can be considered a useful publication to evaluate the real benefit of silymarin as commonly prescribed and used as a coadjutant in the treatment of liver diseases.

MATERIALS AND METHODS

Identification and selection of articles

This is a systematic review with a meta-analysis of randomized controlled trials evaluating the effect of silymarin in patients with liver disease, published by January 31, 2016. This review was carried out taking into account the provisions of PRISMA (Peferred Reposting Items for Systematic Reviews and Meta-Analyzes)[29]. We searched for randomized controlled trials in the Cochrane Central Register of Controlled Trails databases in the Cochrane Library, PubMed/Medline, Scopus, Web of Science, Lilacs and Clinical Trials. The research was conducted with no restrictions regarding the year of publication.

The terms "*silybum marianum*", "milk thistle", "silymarin", "silybin", "silibinin", "silydianin", "silychristin", "cardus marianus", "liver disease", "chronic liver disease", "end-stage liver disease", "drug-induced liver injury", "Non-alcoholic fatty liver disease", "fatty liver", "alcoholic fatty liver", "alcoholic liver disease", "fibrosis", "liver cirrhosis, "Hepatocellular carcinoma", "viral liver disease", "hepatitis B", "hepatitis C", "hemochromatosis", "liver steatosis", "alcoholic hepatitis" and "chronic hepatitis" were searched in English, Portuguese and Spanish. All these keywords were combined using the Boolean operators "OR" and "AND" in several databases. The construction of the search strategy took into account the research question structured by the acronym PICO. Only the terms for the components Population, Intervention and Control had been defined. The terms for outcome "O" were not defined to avoid assigning undesirable specificity at this stage of data collection[30].

Two reviewers independently carried out the active search of the scientific articles. The identified disagreements were evaluated and discussed by a third evaluator. The review team through the screening phase, reading the titles and abstracts, carried out a process of evaluation of the eligibility of the studies. Subsequently, reading the full text and identifying the duplicates in all databases described, the confirmation phase was performed. In this stage, the reason for the exclusion of each article was recorded in an article selection flow form. The third reviewer solved the disagreements between the former reviewers, regarding the eligibility of the articles.

Inclusion criteria

Randomized and controlled clinical trials with adult and elderly patients of both sexes with liver disease who took oral silymarin supplementation, as an extract or in its isolated form, as well as silymarin combined with other nutrients were included. We included studies in English, Portuguese and Spanish. The trials should provide information on the intervention such as doses and details of the product used, as well as mean and standard deviation of ALT, AST and γGT serum levels at baseline and at the end of the intervention.

Exclusion criteria

We excluded articles that reported the use of drugs associated with silymarin, did not provide descriptive data of the control or intervention group, used a crossover study design and also those who after contact, did not obtain answers from the authors to provide data not available in the articles. Studies using median and interquartile range with descriptive measures of outcome variables could not be included in the meta-analysis. Trials whose full accesses were not possible due to year of publication, or by online unavailability were also excluded.

Data extraction

Two reviewers independently reviewed eligible articles. For the data extraction process, the eligible articles were read in full and a standardized sheet was used for each article, with all the selection criteria established. The variables data in the baseline and at the end of the intervention were recorded in a spreadsheet in the Excel® program.

Evaluation of the methodological quality of articles

The methodological quality of the articles included in this review was evaluated according to the adapted Downs and Black[31] checklist. This checklist evaluates criteria such as description of the information in the studies, items to analyze external validity, items referring to participants, intervention and statistical tests, besides the internal validity, confounding factors and possible selection biases and the power of the studies.

The articles were evaluated based on the following criteria: (1) definition of the objectives/hypothesis; (2) description of results; (3) characterization of participants included in the study; (4) description of the exposure; (5) quality of the description of the main results; (6) reports of 95% confidence intervals and/or *P* value for the main outcomes; (7) representativeness of individuals invited to participate in the study; (8) representativeness of the individuals included in the research; (9) clarity if any of the studies were *post hoc*-based; (10) appropriate use of statistical tests to evaluate the main results; (11) validity and reliability of measures of the main outcomes; and (12) whether the statistical analysis includes adequate adjustment for the main confounding variables.

Clinical trials were also evaluated according to the following items: (1) information on the characteristics of the loss of follow-up; (2) analysis adjusted for

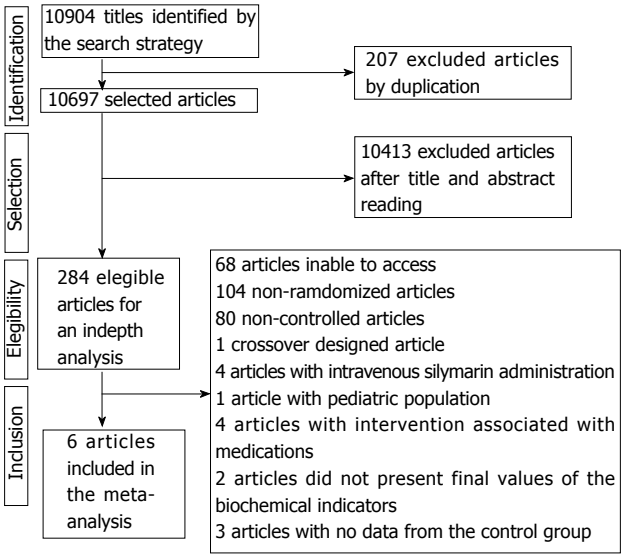

Figure 1 Flow of selection of articles included in the meta-analysis.

different follow-up times; (3) whether participants in the intervention and control groups were recruited from the same population; (4) whether participants in the intervention and control groups were recruited within the same time period; (5) reporting blinding of the intervention to participants and evaluators; and (6) whether follow-up losses were considered.

In order to evaluate the quality, a dichotomous response was defined as "yes", with a score of 1, or "no", with a score of 0, for each item in the checklist. At the end, a sum of the scores and the percentage for each publication was calculated. The percentage of ideal methodological quality was equal to or greater than 80%, according to Downs and Black[31].

The risk of bias in the studies was assessed according to the criteria of the Cochrane Collaboration for the development of systematic reviews of intervention[32]. It was not possible to assess publication bias by the Funnel plot and test its asymmetry by the Egger's test because of the small number of included studies.

Statistical analysis

For the data extraction process, the eligible articles were read in full and a standardized clinical record was used for each article, with all the selection criteria established. The data referring to the descriptive measures of the outcome variables at the baseline and at the end of the intervention were recorded in an Excel® worksheet.

The summary measure used in this meta-analysis was the difference of standardized means among the groups for each indicator evaluated (ALT, AST and γGT) and their respective confidence intervals, which were presented in Forest plot charts. The difference of global standard means was calculated using the random effects model, due to the high heterogeneity of the studies. The assumption of the homogeneity of the studies was tested by the extent of the heterogeneity interpreted by the total percentage of variation between the studies analyzed with the I^2 statistic (Higgins inconsistency test). This test of inconsistency greater than 50% was used as an indicator of moderate heterogeneity[33]. The statistical methods of this study were reviewed by Priscila Costa from the School of Nutrition of the Federal University of Bahia, Brazil.

Subgroup analyzes were also performed according to the type of intervention (isolated silymarin or silymarin associated with nutrients) and the intervention time (⩾ 6 mo or < 6 mo) to identify possible differences. The heterogeneity of the meta-analysis was evaluated by meta-regression and the influence of the variables: sample size, treatment time and type of intervention were tested. In all analyzes, a *P*-value less than 0.05 was considered significant.

Statistical analysis was performed using the STATA Program for MAC, version 12 (Stata Corp. College Sattion, TX, United States).

RESULTS

Selection of studies

The electronic search identified 10904 publications, excluding 207 duplicates and 10413 articles by reading the title and abstracts, totaling 284 eligible studies for in-depth analysis. A total of 267 articles were excluded due to issues such as: impossibility of access to the full article (n = 68), non-randomization (n = 104), uncontrolled clinical trials (n = 80), crossover design (n = 1), medication-associated intervention (n = 4), absence of biochemical markers (AST, ALT and γGT) after intervention (n = 2) and absence of Data from the control group (n = 3). Thus, the systematic review was performed with 17 publications and of these, only 6 were included in the meta-analysis (Figure 1), since 5 of them used the median as descriptive measure and 6 had no descriptive data necessary for the analysis.

Characteristics of the studies

Table 1 presents the main characteristics of the studies and patients included in the systematic review. Seven studies[8,19,26,34-37] were performed in Europe, six[27,28,38-41] in Asia, three[42-44] in Africa and one in America[45]. The year of publication varied from 1994 to 2016. The sample size varied from 30 to 370 individuals, totalizing 1558 adults and elderly, of both sexes. The studies evaluated drug-induced hepatic injury[8,40,41], with C virus[43,44,45], individuals with acute hepatitis[42], NAFLD and of these studies, three articles[28,38,39] included patients diagnosed with NASH and one[35] evaluated individuals with DHGNA and metabolic syndrome , being a pilot article[19].

The work of Loguercio *et al*[19] presented a subgroup with patients with hepatitis C virus, but it was decided not to include this subgroup in the analyzes, since it was considered impracticable to analyze this small

Table 1 Summary of clinical trial characteristics

Study	Year	Origin	Population	Silymarin dose	Intervention	Inclusion criteria	Follow-up	Outcomes
Loguercio *et al*[19]	2007	Italy	59 adult patients with NAFLD	4 × 94 mg silibin + 194 mg phosphatidylcholine + 90 mg vitamin E (Reasil®) daily	Silymarin Control untreated (diet + physical activity)	NAFLD with no chronic liver disease	6 mo and 12 mo	ALT, gGT, insulin and HOMA
Hashemi *et al*[38]	2009	Iran	100 adult patients with NAFLD (NASH)	2 × 140 mg silymarin (Livergol®) daily	Silymarin Control	USG evidencing steatosis, ALT elevation in more than 1.2 of the normal value, exclusion of conical diseases of the liver, histological evidence of NASH or presence of risk factor such as MD or obesity	6 mo	ALT, AST, gGT, FA, glycemia, triglycerides and cholesterol
Massodi *et al*[39]	2013	Iran	100 adult patients with NAFLD (NASH)	2 × 140 mg silymarin daily	Silymarin Control	NASH confirmada por USG e níveis elevados de AST e ALT	3 mo	AST and ALT
Solhi *et al*[28]	2014	Iran	64 adult patients with NAFLD (NASH)	3 × 70 mg silymarin (Livergol®) daily	Silymarin Control	NASH confirmada por USG abdominal e elevação persistente de AST e ALT mais de 1,2 acima do valor normal nos últimos 6 meses	8 wk	ALT and AST
Aller *et al*[34]	2015	Spain	36 adult patients with NAFLD	2 × Silybum marianum 540.3 mg + vitamin E - 36 mg (Eurosil 85®) daily	Silymarin Control untreated (diet + physical activity)	NAFLD confirmed by liver biopsy	3 mo	Glycemia, triglycerides, AST, ALT, gGT and HOMA IR
Sorrentino *et al*[35]	2015	Italy	78 adults with MS and NAFLD	2 × silymarin 210 mg+ 30 IU vitamin E (Eurosil 85®) daily	Silymarin Control untreated (diet)	MS and NAFD confirmed by USG	3 mo	Hepatic steatosis, lipid accumulation index, ALT, AST, gGT, triglycerides, cholesterol, LDL, HDL, glycated Hb and Glycemia
Abenavoli *et al*[36]	2015	Italy	30 overweight Caucasian adults with NAFLD	2 × Silibin 94 mg + phosphatidylcholine 194 mg + vitamin E 89.28 mg daily	Group A: Hypochloric diet Group B: Diet + silymarin Group C: control	Overweight and NAFLD confirmed by USG	6 mo	BMI, weight, waist circumference, blood pressure, AST, ALT, gGT, bilirubin, glycemia, HOMA-IR, insulin, triglycerides, total cholesterol, HDL, LDL, creatinine, azotemia, hepatic steatosis index
Luangchosiri *et al*[40]	2015	Thailand	55 adults and elderly with pulmonary tuberculosis	3 × silymarin 140 mg daily	Silymarin Control	Diagnosis of pulmonary tuberculosis, > 18 yr, treatment with anti-tuberculosis drugs	4 wk	ALT, AST, alkaline phosphatase, gGT, total proteins, albumin, bilirubin, SOD, glutathione, malonyldialdehyde, risk of hepatic injury by anti-tuberculosis drug, adverse events

El-Kamary *et al*[42]	2009	Egypt	105 adults with acute hepatitis of varied etiologies	3 × silymarin 140 mg daily (Legalon®)	Silymarin Control (multivitamin)	ALT > 100 IU/L with jaundice and 3 or more symptoms of acute hepatitis	8 wk	ALT, AST, bilirubin, acute hepatitis symptoms, adverse events
Fried *et al*[45]	2012	United States	154 adults with HCV	5 × silymarin 140 mg daily (Legalon®) - 700 mg 3 × silymarin 140mg daily (Legalon®) - 420 mg	Group 1: silymarin 420 mg Group 2: silymarin 700 mg Group 3: control	HCV and ALT > 65 U/L or unsuccessful patients on interferon therapy	24 wk	ALT, RNA HCV
Hajaghamohammadi *et al*[27]	2008	Iran	50 adults with NAFLD	1 × 140 mg silymarin (Livergol®) daily		NAFLD confirmed by USG and elevated levels of ALT and AST	2 mo	Weight, BMI, AST, ALT
Stiuso *et al*[37]	2014	Italy	30 adults with NASH	2 × 94 mg silibin + 194 mg phosphatidylcholine + 89.28 mg vitamin E (Reasil®) daily	Silymarin Control	NASH histologically confirmed	12 mo	Levels of substances that react with thiobarbituric acid, nitric oxide, SOD, catalase, BMI, glycemia, insulin, HOMA, AST, ALT, gGT, score for NAFLD
Velussi *et al*[26]	1997	Italy	60 diabetic adults and elderly with alcoholic cirrhosis	600 mg siymarin daily	Silymarin Control	Diabetics treated with insulin with alcoholic cirrhosis (biopsy), aged between 45 and 70 years old	12 mo	Glycemia, postprandial glycemia, glycated hemoglobin and malonildialdehyde
Yakoot *et al*[43]	2012	Egypt	66 adult and elderly patients with HCV genotype 4	3 × silymarin 140mg daily	Group 1: spirulina 500 mg Group 2: silymarin Group 3: control	HCV genotype 4, elevated liver enzymes, virgin antiviral therapy	6 mo	Virological response, ALT, quality of life score, adverse events
Zhang *et al*[41]	2015	China	370 adult patients with tuberculosis on antituberculosis therapy	2 × *S. marianum* 200 mg	Silymarin Control	> 12 yr with tuberculosis and in anti-tuberculosis therapy	8 wk	ALT, AST, bilirubin, gGT, alkaline phosphatase
Tanamly *et al*[44]	2004	Egypt	141 adults and elderly with HCV	3 × silymarin 140 mg daily (Legalon®)	Silynarin Control (multivitamin)	HCV	12 mo	RNA HCV, ALT, fibrosis, adverse events
Palasciano *et al*[8]	1994	Italy	60 adult women using psychotic drugs	2 × 400 mg sliymarin daily	Group 1A: drugs + silymarin Group 1B: drugs + control Group 2A: no drugs and with silymarin Group 2B: no drugs but control	Women between 40 and 60 yr of age, treated with phenothiazines and/or butyrenes for at least 5 yr, AST or ALT with values 2 × higher than the regular range	3 mo	AST, ALT, gGT, malonildialdehyde, bilirubin

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γGT: Gamma glutamyl transpeptidase; NAFLD: Non-alcoholic fatty liver disease; HCV: Hepatitis C virus.

number of patients. The duration of follow-up ranged from 4 wk to 12 mo, the dose of oral silymarin used was 210 mg to 700 mg and the frequency of ingestion was two to five times a day. Four studies[8,39,40,45] reported blinding, describing methodological design as double-blind. Twelve studies[8,26-28,38-45] used only dry extract of Silybum marianum, which contains silymarin or silymarin alone, two[34,35] used silymarin associated with vitamin E and three[19,36,37] studies used silybin with vitamin E and phosphatidylcholine. All articles evaluated ALT, however the study by Velussi *et al*[26] only evaluated liver enzymes in the baseline, four[16,43-45] did not evaluate AST and eight[27-29,36,39,42-44] did not evaluate γGT. Six studies[26-28,36,37,39] did not report data on adverse effects, six[8,19,34,35,41,45] did not identify any of these effects, four[40-43] performed specific evaluation and only one[39] described that serious adverse events were not observed and that side effects were similar in

frequency and uncommon in both groups.

Evaluation of the methodological quality of the studies included in the meta-analysis

Among the 6 studies included in this meta-analysis, only one[39] was double-blind and reported the randomization method used. No intention-to-treat analyzes were described in the studies evaluated. Only one study[39] presented methodological adequacy (92.5%), higher, therefore, to 80% according to the checklist score adapted from Downs and Black[31]. The main limitations observed in the studies were: (1) absence in the description of the characterization of participants with loss of follow-up[19,34,35]; (2) failure to report blinding for the intervention of the participants and evaluators[19,28,34,35,38]; (3) no adjusted analyzes were performed for different follow-up times[19,25,28,34,35,38]; (4) randomization was not concealed for patients and staff until complete recruitment[19,28,34,35,38]; and (5) absence of adequate adjustments for confounding factors in the analyzes of which the main findings were withdrawn[19,28,34,35,38,39].

Risk analysis of bias

Fungal plot analysis and the Egger test were not performed since these are recommended for meta-analyzes with at least 10 studies and are not indicated for this study[32].

A bias risk assessment of randomized controlled trials was performed according to the Cochrane Collaboration criteria[32] for the development of systematic reviews of intervention (Figure 1). There was a high risk of bias in relation to the blinding of the participants and the researcher, since only one study was double-blind[39] and reports on allocation, blinding of outcome evaluation and other potential biases were not well understood in the studies evaluated. There was a low risk of bias for selective information[28,38,39] and random sequence generation in half of the studies analyzed[28,34,39] (Figure 2). Only studies by Massodi *et al*[39] and Solhi *et al*[28] presented half of the items assessed as low risk for bias (Figure 2).

Meta-analysis results

The results of the meta-analysis are shown in Table 2, Figures 3-8. This meta-analysis included 437 individuals. All articles evaluated ALT levels. One study had no AST levels measurements and only three[19,34,35] had γGT dosages. The included studies evaluated only patients with NAFLD and publications evaluating other liver diseases were naturally excluded in the screening and confirmation stages of eligible articles. However, the work of Loguercio *et al*[19] presented a subgroup with patients with HCV, but it was decided not to include these patients in this meta-analysis. In the groups treated with silymarin, four studies[19,28,38,39] observed a significant reduction in serum ALT levels, three[28,38,39] showed a significant reduction in AST and only one[19] observed a significant decrease in γGT serum levels.

Thus, when the intervention groups were compared with the control groups of all studies included in the meta-analysis, a reduction of 0.26 IU/mL (95%CI: -0.46-0.07) was observed in the mean ALT serum values and a reduction of 0.53 IU/mL (95%CI: -0.74-0.32) in the mean AST serum values of the treated group, compared to the control group (Figure 4), both of which are statistically significant. No significant change in the Gamma γGT serum levels was identified (Figure 5).

A subgroup analyses was also performed to identify possible differences in relation to intervention characteristics. We considered as subgroups different studies that performed intervention with isolated silymarin[28,38,39] or silymarin associated with other nutrients[19,34,35], as well as the studies that presented different follow-up time (equal or superior to 6 mo and less than 6 mo) for both ALT and AST serum levels (Figures 6-8). It was not possible to consider these subgroups for the evaluation of γGT and for the AST levels regarding the intervention time due to the insufficient number of studies to enable these analyzes.

When comparing control and treatment groups, a reduction trend of 0.59 IU/mL (95%CI: -0.83-0.34) was found in the mean ALT serum values of subjects treated with isolated silymarin and 0.23 IU/mL in this same marker (95%CI: -0.08-0.53), in those treated with silymarin associated with other nutrients. However, there was no statistical significance (Figure 6). Therefore, no significant differences were observed in these forms of intervention (isolated or associated silymarin).

Analysis of the mean AST serum values showed a reduction of 0.86 IU/mL (95%CI: -1.12 to -0.61, $P = 0.003$) in subjects treated with isolated silymarin (Figure 7). These results also did not present significant differences between the types of intervention, similar to the analysis referring to ALT levels. Likewise, the assessment of intervention time subgroups and ALT levels did not show significant differences between them (Figure 8).

Heterogeneity and meta-regression

The present study observed that the studies evaluated presented a high degree of heterogeneity, with an inconsistency test (I^2) greater than 50%. Two meta-regressions were performed, one having ALT as the outcome and another for AST. It was not possible to perform meta-regression for γGT, considering the small number of studies. In the first meta-regression, the sample size ($P = 0.901$), treatment time ($P = 0.233$) and type of intervention (isolated silymarin and associated silymarin) ($P = 0.143$) did not explain the heterogeneity between the studies. Likewise, in the second meta-regression, the sample size ($P = 0.941$), treatment time ($P = 0.163$) and type of intervention ($P = 0.089$) also failed to explain the heterogeneity of the studies

Table 2 Results of selected studies for meta-analysis

Ref.	Used Indicators	Results
Loguercio *et al*[19], 2007	ALT, γGT	There were no adverse events in either group. The intervention group presented a significant reduction of hepatic steatosis in the ultrasonography score (change from 2-3 to 1-2) after 6 mo and 12 mo ($P < 0.01$). Significant reduction of ALT and γGT after 6 mo and 12 mo only in the intervention group ($P < 0.01$). Treatment affected the levels of ALT and γGT Range independent of changes in BMI of the participants. We did not evaluate data from the group with HCV patients
Hashemi *et al*[38], 2009	ALT, AST	There was a significant reduction in the average of ALT levels only in the intervention group (113.54 IU/mL *vs* 73.14 IU/mL) ($P < 0.001$). The percentage of patients with normalization (ALT < 40) was 32% after 3 mo and 52% after 6 mo in the intervention group and the difference in these percentage between control and intervention group was significant ($P = 0.001$). There was also a significant reduction in AST averages only in the intervention group (71.42 IU/mL *vs* 49.66 IU/mL) ($P = 0.006$). The percentage of patients with normalization (AST < 40) was 46% after 3 mo and 62% after 6 mo in the intervention group and the difference in these percentages between control group and intervention was also significant ($P = 0.0001$)
Massodi *et al*[39], 2013	ALT, AST	There were no serious adverse events and the side effects were similar in frequency and uncommon in both groups. There was a significant reduction in the average of ALT levels only in the intervention group (84.06 IU/mL *vs* 68.54 IU/mL) ($P < 0.001$) and in the average AST levels only in the intervention group (71.94 IU/mL *vs* 54.70 IU/mL) ($P < 0.001$)
Solhi *et al*[28], 2014	ALT, AST	There was a significant difference in the mean values of ALT levels only in the intervention group (91.3 IU/mL *vs* 38.4 IU/mL) ($P = 0.026$) and in the AST levels only in the intervention group (62.8 IU/mL *vs* 30.5 IU/mL) ($P = 0.038$).
Aller *et al*[34], 2015	ALT, AST, γGT	There were no adverse events in both groups. There was a significant improvement in the fibrosis score in both groups ($P < 0.05$). There was a significant difference in the reduction of the average γGT levels (81.5 IU/L *vs* 46.2 IU/L) ($P < 0.05$) in the intervention group and also in the control group (80.5 IU/L *vs* 50.3 IU/L) ($P < 0.05$). There was a significant reduction only in the average of ALT levels (70.8 IU/L *vs* 54.7 IU/L) ($P < 0.05$) and AST (41.6 IU/L *vs* 36 IU/L) ($P < 0.05$) in the control group.
Sorrentino *et al*[35], 2015	ALT, AST, γGT	No adverse events were reported in both groups. Mean levels of ALT, AST and γGT were within normal limits at the baseline. There was a significant reduction only in the average values of right lobe size of the liver by the USG (17.24 cm *vs* -0.96 cm) ($P = 0.044$)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γGT: Gamma glutamyl transpeptidase.

(data not shown in tables).

DISCUSSION

In this review, some intervention studies have observed an improvement in the biochemical and clinical indicators evaluated in patients with NAFLD, including hepatic steatosis and NASH, after the use of silymarin. Although the results of the meta-analysis indicate that the use of silymarin is associated with a reduction in serum levels of ALT and AST, the values found are not clinically relevant. The studies[19,34,35,39] also showed limited adverse effects and good tolerance to the use of silymarin as reported in other studies[10-12].

Some studies report that silymarin is capable to improving biochemical indicators in patients with liver diseases of different etiologies[40,46-50], in addition to the reduction of ALT and AST levels are commonly described in other studies[48,49,51,52]. The hypothesis described by the researchers is that the antioxidant properties of silymarin are capable of reducing reactive oxygen species, thus inhibiting cellular damage[53]. In addition to the improvement in the antioxidant system, observed in experimental studies, due to the increase of enzymes such as glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase, all with antioxidant function[13,16,54] and non-enzymatic antioxidants, through the modulation of associated transcription factors[55,56]. On the other hand, there are reports of similar studies that, despite showing differences in the values of these indicators, these were not statistically significant[45,57].

It is important to emphasize that there are a few trials with rigorous methodologies that consider important issues such as the use of well-characterized products, evaluation of specific liver diseases, adequate sample size, representativeness of the study population, adequate intervention time and appropriate statistical analysis. These factors are quite divergent among the studies, which may directly interfere both in the positive results and in the controversial findings, representing an important limitation for conclusions on this topic.

It was identified in this meta-analysis that a clinical trial[35] that found normal values of ALT, AST and γGT in the baseline, which is not surprising, since some patients may be carriers of NAFLD and do not present alterations in liver enzyme levels[58-60]. Thus, in the aforementioned study[35], there was no relevance in the results of these markers after intervention, since in the baseline; the patients no longer presented alteration in these markers. It was also found that another clinical trial[34] included in this analysis demonstrated a significant reduction of γGT in the control and intervention group, probably due to differences in the methodological design used. In this study, both groups had prescriptions for hypocaloric diet and physical activity, which probably influenced the clinical and

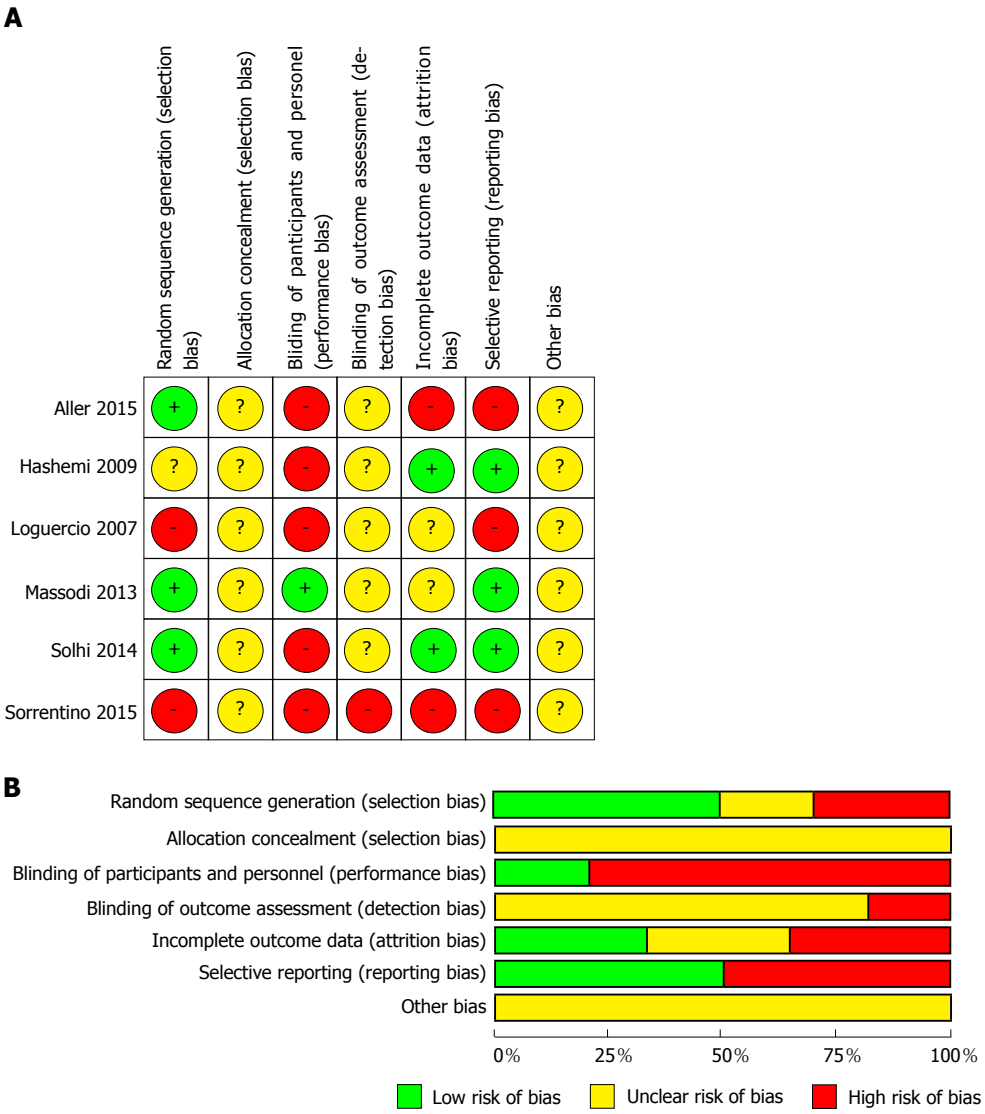

Figure 2 Risk of bias assessment. A: Risk of bias summary: review authors' judgments about each risk of bias item for each included study; B: Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies.

Study	Silymarin mean ± SD	Total	Control mean ± SD	Total	Weight	SMD (95%CI)	mean difference (95%CI)
Hashemi *et al*[38], 2009	73.1 ± 62.4	50	89.9 ± 41.8	50	23.79%	-0.32 (-0.71, 0.08)	
Aller *et al*[34], 2015	52.7 ± 26.0	18	54.7 ± 18.0	18	8.66%	-0.09 (-0.74, 0.56)	
Loguercio *et al*[19], 2007	59 ± 20.0	39	45.2 ± 34.0	20	12.32%	0.54 (-0.01, 1.09)	
Solhi *et al*[28], 2014	38.4 ± 11.8	33	52.3 ± 29.9	31	14.68%	-0.62 (-1.12, -0.12)	
Massodi *et al*[39], 2013	68.5 ± 5.5	50	73.3 ± 5.6	50	22.02%	-0.86 (-1.27, -0.45)	
Sorrentino *et al*[35], 2015	28.0 ± 22.3	43	24.8 ± 14.6	35	18.53%	0.17 (-0.28, 0.62)	
Total (95%CI)		233		204	100%	-0.26 (-0.46, -0.07)	
Heterogeneity: χ^2 = 22.26, *df* = 5 (*P* = 0.000) I^2 = 77.5%							
Teste of SMD = 0 *Z* = 2.70 *P* = 0.007							

Figure 3 Alanine aminotransferase levels.

biochemical parameters of patients with NAFLD.

Studies have associated high levels of ALT or the AST:ALT ratio > 1 in patients with NAFLD and with disease progression and the presence of hepatocellular fibrosis[61-63]. Several publications[64-66] have been considered to change lifestyle with dietary intervention

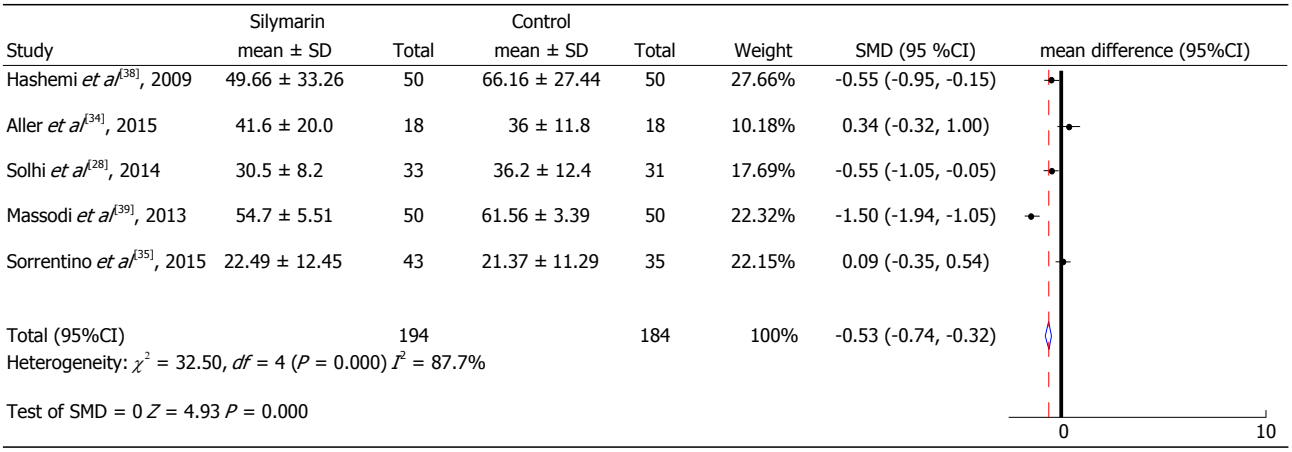

Figure 4 Aspartate aminotransferase levels.

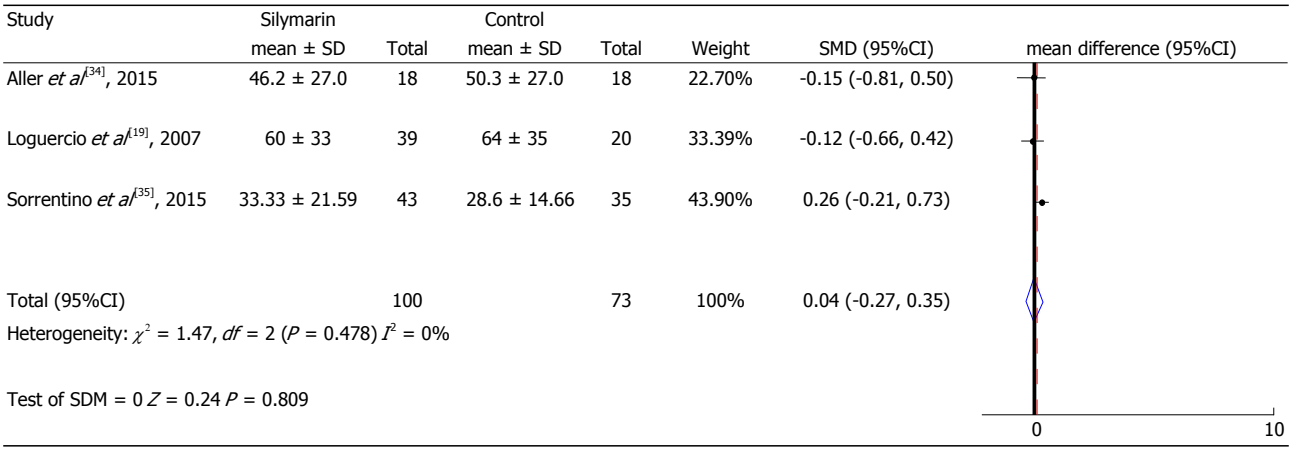

Figure 5 Gamma glutamyl transpeptidase levels.

and physical activity practice, as strategies with an impact on the improvement of markers and liver function for individuals with NAFLD. Despite this, it was observed that there are still more data available in the literature regarding the pattern of adherence of this profile of patients to lifestyle changes and nutritional guidelines provided by health professionals. It is also important to consider the growing increase in the prevalence of NAFLD in recent years and is even considered a global public health problem[1-3]. Considering this scenario, researchers have investigated adjuvant therapeutic strategies, such as phytotherapy and considered the use of silymarin as a possibility to improve biochemical indicators of these patients[28,38,39]. However, the available studies present low methodological quality and the positive results found are not of clinical relevance, as found in this meta-analysis. Therefore, there is still insufficient scientific evidence for the recommendation of silymarin as a possibility of adjunctive therapeutic alternatives for the reduction of biochemical indicators in patients with hepatic disease.

It is important to highlight that the inconsistency tests performed in this meta-analysis showed that the studies evaluated presented a high degree of heterogeneity, which is generally present in meta-analyzes involving clinical trials[30], especially when evaluating such specific topics and presenting few studies with well-designed methodological designs. The case of phytotherapy and specifically, the use of silymarin. In addition, details of intervention, blinding, selection and recruitment of the population and absence of adjustments in the statistical analyzes may be factors that interfere in the final results, as well as the high and medium risk of bias observed in the studies.

In this meta-analysis, trials with small samples and, therefore, little representativeness of the population were identified, which may have favored the high heterogeneity, since studies with larger samples provide greater precision in the association. Absence of intention-to-treat analyzes in the studies can also be considered factors that interfered in the final results and conclusions. Another relevant methodological factor refers to the blinding of the studies evaluated: only one is double-blind, representing another inconsistency of the studies evaluated. Although meta-regression did not identify interference with sample size, time of treatment and type of intervention in the results, it's considered that these results might have

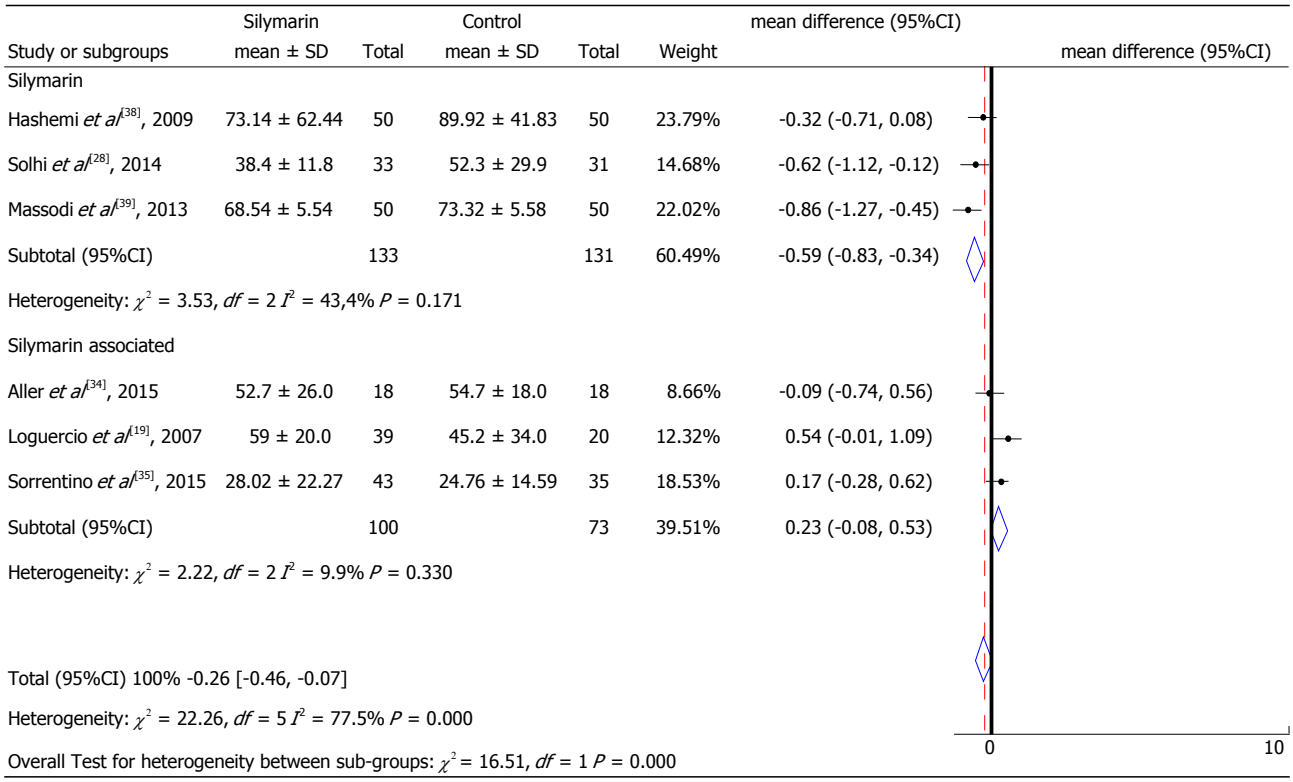

Figure 6 Alanine aminotransferase levels according to the type of product used.

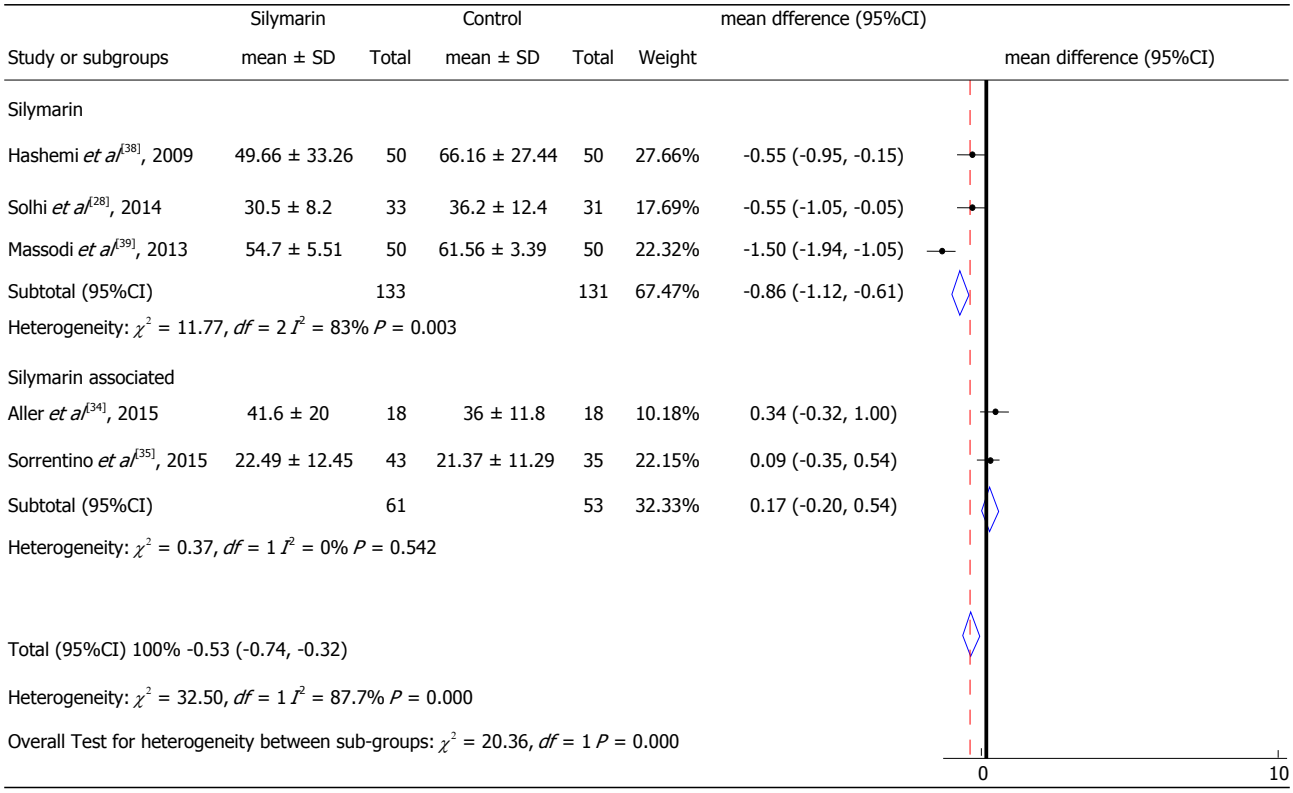

Figure 7 Aspartate aminotransferase levels according to the type of product used.

been strongly influenced by the low methodological quality, observed in all studies, in general, according to the used methods.

In conclusion, the results of this meta-analysis demonstrate that the use of silymarin minimally reduced, but without clinical relevance, the ALT and AST serum levels in patients with non-alcoholic fatty liver disease. Although the reductions observed do

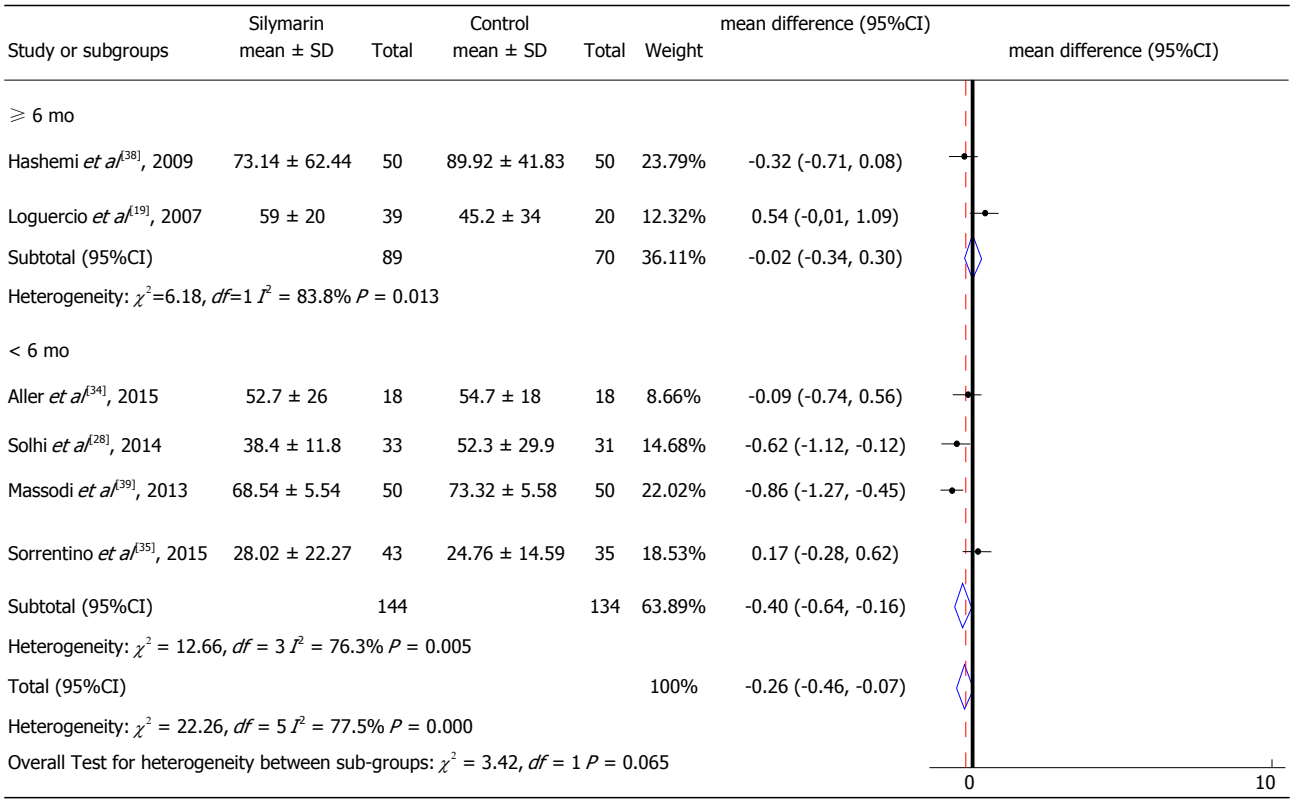

Figure 8 Alanine aminotransferase levels according to intervention time.

not translate into clinical relevance, they may signal to a possible additional therapeutic strategy in the control of NAFLD. When discussing the data found, it is important to consider the great variability and methodological fragility of these studies, a finding very common in publications that evaluate herbal medicines. Therefore, it is necessary to carry out new studies with more adequate methodological designs, with special attention in the accomplishment of the planning stages and execution of clinical trials. This will provide more consolidated scientific evidence and may contribute to a greater safety in the indication or not of doses of silymarin to be prescribed by qualified health professionals.

COMMENTS

Background

The use of phytotherapic medicines is very common and, for many individuals, represents a simple and easily accessible therapeutic option. Despite the use of silymarin in liver diseases be described as millenarian and prescribed by many professionals, there is still controversy in the literature about its real effects on biochemical indicators in patients with liver diseases.

Research frontiers

Chronic liver disease is one of the main causes of morbidity and mortality in the world. High levels of indicators, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (γGT), have been associated with progression of these diseases. Researchers have investigated supporting therapeutic strategies such as phytotherapy and discussed the use of silymarin as a possibility to improve the biochemical indicators of these patients.

Innovations and breakthroughs

In the present study, the authors investigated the effect of the use of silymarin on ALT, AST and γGT levels in patients with liver diseases. This is the first meta-analysis which evaluates the effect of oral use of silymarin on biochemical indicators of patients with liver disease and the methodological quality of the included studies.

Applications

This study allows us to understand the real effects of silymarin on ALT, AST and γGT levels, from patients with liver diseases, in addition to signaling to the need of new clinical trials with more appropriate methodological designs.

Peer-review

This manuscript describes the results of a meta-analysis evaluating effect of silymarin on the serum levels of ALT, AST and GGT in patients with liver diseases. Silymarin has been used in several studies of liver diseases for its hepatoprotective effects. Consequently, this systematic review with meta-analysis evaluating large majority of the literature is crucial to be understood of its actual effectiveness.

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META-ANALYSIS

High expression of anti-apoptotic protein Bcl-2 is a good prognostic factor in colorectal cancer: Result of a meta-analysis

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Author contributions: Li S, Cheng P and Deng M reviewed and screened the articles included in our meta-analysis; Huang Q and He X performed the analysis work needed in this work and draft the manuscript; Wang Z and Yang CH drew the essential diagrams; Zhao XY and Huang J designed the study and edited the manuscript.

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Abstract

AIM

To systematically evaluate the prognostic-predictive capability of Bcl-2 in colorectal cancer (CRC).

METHODS

A systematic literature search was conducted using PubMed, Web of Science and EMBASE databases. Any eligible study must meet the following criteria: (1) bcl-2 expression was evaluated in human CRC tissues by immunohistochemistry; (2) assessment of the relationships between bcl-2 expression and overall survival (OS), disease free survival (DFS), recurrent free survival (RFS) or clinic-pathological characteristics of CRC was included; (3) sufficient information was provided to estimate the hazard ratio (HR) or odds ratio and their 95% confidence intervals (CIs); and (4) the study was published in English. The impact of Bcl-2 expression on survival of CRC patients were evaluated through this meta-analysis.

RESULTS

A total of 40 eligible articles involving 7658 patients were enrolled in our final analysis. We drew the conclusion that Bcl-2 high expression was significantly correlated with favorable OS (pooled HR = 0.69, 95%CI: 0.55-0.87, P = 0.002) and better DFS/RFS (pooled HR = 0.65, 95%CI: 0.50-0.85, P = 0.001). Additionally, the subgroup analysis suggested that Bcl-2 overexpression was significantly associated with

prognosis (OS) especially in patients came from Europe and America but not Asian and patients who did not receive any adjuvant therapy before surgery. Finally, our present results indicated that expression of bcl-2 protein was associated with high differentiation grade and A/B Ducks' stage.

CONCLUSION

Bcl-2 high expression was significantly correlated with favorable OS and better DFS/RFS. Hence, we propose that Bcl-2 may be a valuable prognostic-predictive marker in CRC.

Key words: Bcl-2; Colorectal cancer; Meta-analysis; Prognostic; Apoptotic

Core tip: No consensus is available in the literature about the prognostic value of Bcl-2 expression in patients with colorectal cancer (CRC). This is the first systematic review and meta-analysis indicating that Bcl-2 is a good prognostic factor in CRC. We investigated the relation in terms of overall survival, disease free survival/recurrent free survival, number of patients, nations, therapy methods, pathological grade.

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INTRODUCTION

Bcl-2 family proteins are key regulators of apoptosis whose dysregulation can cause various pathological consequences including the development of cancer[1]. The anti-apoptotic protein Bcl-2 (B-cell lymphoma-2) is an important member of the Bcl-2 family which controls the release of proapoptotic factors responsible for the activation of caspases by stabilizing the mitochondrial outer membrane[2].

Colorectal cancer (CRC) is one of the most common malignancies worldwide[3,4]. Despite the great progress made in clinical treatment, the morbidity and mortality of CRC remains high. Aberrant expression of Bcl-2 has been implicated in several cancer types including CRC[5]. Nonetheless, data obtained by different researchers were often in disagreement[6-10].

An increasing body of evidence from many studies indicates that Bcl-2 expression may be associated with prognosis in malignancies including CRC[11]. Expression of Bcl-2 has been found to correlate with favorable clinicopathologic parameters and better prognosis by many investigators[7,8,12,13]. In contrast, some groups demonstrated that Bcl-2 was a poor prognostic for cancer patients[9,10,14]. And there are others who found no prognostic significance of Bcl-2 expression in CRC[6,15,16]. Thus, neither the function nor the prognostic value of Bcl-2 expression in patients with CRC is clear to us.

Herein, we carried out this meta-analysis to explore the reason for present contradictory observations and determine the prognostic value of Bcl-2 in patients with CRC.

MATERIALS AND METHODS

Literature search

We identified relevant articles by conducting searches in the PubMed, Web of Science and EMBASE databases using the following terms and all possible combinations: "Bcl-2", "colorectal carcinoma", "CRC", "colon cancer", "rectal cancer". More than this, we examined the references to identify additional eligible studies. The reviews and bibliographies were also retrieved to discern other relevant articles. The most recent search update was October 15th, 2016. After excluding non-related articles through browsing the titles and abstracts of the listed studies, full-text viewing of resting studies was performed. The largest population size study was chosen to avoid duplicate analysis when patients overlap partly or entirely.

Inclusion criteria

The eligible studies included in our meta-analysis must meet the following requirements: (1) bcl-2 expression was evaluated in human CRC tissues by immunohistochemistry (IHC); (2) assessment of the relationships between bcl-2 expression and overall survival (OS), disease free survival (DFS), recurrent free survival (RFS) or clinic-pathological characteristics of CRC was included; (3) sufficient information was provided to estimate the hazard ratio (HR) or odds ratio (OR) and their 95% confidence intervals (CIs); and (4) the study was published in English.

Exclusion criteria

The articles were excluded from our analysis if they have the following characteristics: (1) letters, reviews, case reports, and conference abstracts without original data; (2) lack of necessary data or survival curves to calculate HRs, ORs or the corresponding 95%CIs; and (3) overlapping studies.

Data extraction and assessment of study quality

Data extraction and quality assessment were conducted independently by two primary investigators (Qi H and Shu L) using a standardized form. Discrepancies were arbitrated by a third reviewer. The following characteristics were retrieved: first author's name,

year of publication, country of patients' origin, tumor location, number of patients, age of patients, tumor stage, treatment state before surgery, follow-up time, research technique used, antibody source and dilution, cut-off value, survival data and clinical-pathological data. The quality of each study was tested according to the Newcastle-Ottawa quality assessment scale (NOS).

Statistical analysis

All statistical analysis was performed using the STATA 12.0 software (Stata Corporation, Collage Station, TX, United States). We calculated the pooled HRs and the 95%CIs of all included articles. OS, DFS and RFS were all included in our outcome analysis. We used the raw data directly if HRs and their corresponding 95%CIs were described in the literature. Otherwise, they were extracted from Kaplan-Meier curves published in the article read by Engauge Digitizer version 4.1 (http://digitizer.sourceforge.net/) according to the methods described by Parmar *et al*[17]. At the same time, we also explored the correlation between Bcl-2 expression and clinical-pathological parameters of CRC such as tumor location, tumor grade, Ducks' stage and lymph node metastasis combining the ORs and their 95%CIs. A value of HR > 1 implies a worse prognosis of survival in patients who overexpressed Bcl-2, while a value of OR < 1 indicates an unfavorable parameters in those high Bcl-2 expression patients. The association between Bcl-2 and survival or clinical-pathological factors was considered statistically significant if the 95%CI did not span across 1. The heterogeneity among articles included in this meta-analysis was evaluated by χ^2-based Q statistical test according to Peto's method[18]. The inconsistency index (I^2) ranged from 0% to 100% was used to quantify the proportion of the total variation[19]. A *P*-value for the *Q*-test was presented to assess the heterogeneity among the studies. We chose the random-effects model (the DerSimonian and Laird method) when $P < 0.10$. Otherwise, the fixed-effects model (the Mantel-Haenszel method) was applied[19,20]. Begg's test was used to determine the potential publication bias when $P < 0.05$. Statistical significant was defined as $P < 0.05$.

RESULTS

Literature search and study description

We identified 2274 relevant articles upon screening the keywords from several databases and a total of 40 eligible studies were finally selected to explore the relationship between Bcl-2 expression and CRC patients' survival using the strategy depicted in Figure 1[6-10,12-16,21-50]. The detailed clinical features of each record were shown in Table 1, which enrolled an overall of 7658 CRC patients in this analysis published ranging from 1995 to 2016. Among the 40 studies, 6 studies were conducted in Italy, 5 in Greece, 4 each in China and United Kingdom, 2 each in Netherlands, Sweden, United States, Canada, Finland and Germany, 1 each in Romania, Switzerland, Brazil, South Korea, Ireland, Japan, Australia, India and Austria. As to the prognostic analysis, 34 studies evaluated the correlation between Bcl-2 expression and patients' OS while 13 articles reported the data of Bcl-2 related DFS or RFS.

In OS analysis, 22 of the included articles enrolled more than 100 patients and 12 manuscripts recruited less than 100 patients. Patients from 7 studies received treatment such as radiotherapy, chemotherapy or endocrine therapy before surgery while other 18 studies were not the case, another 9 articles did not provide therapy strategy before surgery.

Methodological quality of selected studies

Each of the 40 eligible studies included in our meta-analysis underwent quality evaluation according to the Newcastle-Ottawa Scale (NOS). NOS scores were judged on eight items of the methodology that categorized into three sections: selection, comparability, exposure and outcome. The quality score of enrolled studies ranged from 5 to 8 with a mean score of 6.5. Eighteen studies scored 7 or more in methodological assessment were defined as high quality (Table 1).

Correlation between Bcl-2 high expression and increased OS or DFS/RFS in CRC

34 studies were included in the analysis to evaluate the association between Bcl-2 high expression and OS. The pooled hazard ratio (HR) for OS was 0.69 (95%CI: 0.55-0.87, $Z = 3.14$, $P = 0.002$). A statistical heterogeneity ($I^2 = 80.0\%$, $P < 0.001$) was observed based on the random-effects model (Figure 2A). A meta-analysis on 13 studies was performed to analyze the correlation between Bcl-2 and DFS/RFS. The pooled HR for DFS/RFS was 0.65 (95%CI: 0.50-0.85, $Z = 3.19$, $P = 0.001$), accompanied with considerable heterogeneity ($I^2 = 59.0\%$, $P = 0.004$) (Figure 2B). These results indicate that high level expression of Bcl-2 is significantly associated with decreased mortality risk in CRC patients and Bcl-2 may be an independent prognostic factor in CRC.

Subgroup analysis and sensitivity analysis of the correlation between Bcl-2 high expression and OS in CRC

To address the heterogeneity in OS, we performed subgroup analysis on the number of patients involved in the study, the origin country of patients, the treatment situation before surgery and the NOS score (Table 2). We found that a significant relationship between high expression of Bcl-2 and OS was exhibited in subgroup with number of patients more than 100 (HR = 0.684, 95%CI: 0.54-0.866, $P = 0.002$) (Figure 3A) and subgroup with origin country of Europe and America (HR = 0.691, 95%CI: 0.553-0.864, $P = 0.001$) (Figure 3B). Additionally, Bcl-2 overexpression showed

Table 1 Main characteristics of the studies included in the meta-analysis

Ref.	Year	Country	Tumor location	Patient(P/N)	Age	Stage	Treatment before surgery	Follow-up time Median(range)	Detection method	Antibody source	Antibody dilution	Cut off value	HR(95%CI) estimation	Quality Score
Cai *et al*[21]	2016	China	Colon and rectum	117(34/83)	52.0 yr (24-87 yr)	Ⅰ-Ⅳ	No	NA	IHC	Thermo Scientific	1:50	> 10%	OS = 0.7 (0.34-1.45) Multivariate	7
Melincovici *et al*[6]	2016	Romania	Colon	31(12/19)	63 ± 11.71 yr	A-D (Ducks)	Yes	NA	TMA/IHC	Dako	1:100	> 5%	OS = 0.211 (0.026-1.718) Univariate	8
Huang *et al*[22]	2015	China	Colon and rectum	190(85/105)	NA	A-D (Ducks)	No	986 d (21-2572d)	IHC	Genetex	NA	Multiply the intensity score by the percentage of labeled cells > 150	OS = 2 (1.21-3.3) Multivariate RFS = 1.32 (0.82-2.13) Multivariate	8
Balzi *et al*[15]	2015	Italy	Colon and rectum	321 (153/168)	< 85 yr	Ⅰ-Ⅲ	No	NA	IHC	Dako	1:50	> 5%	OS = 0.87 (0.51-1.48) Univariate DFS = 0.971 (0.654-1.449) Multivariate	8
Belt *et al*[23]	2014	Netherlands	Colon	160 (81/76)	72.4 yr (34.5-94.0 yr)	T1-4, N1-2, M0	Yes	46.9 mo (3.0-148.6 mo)	TMA/IHC	Dako	1:300	Score ⩾ 1	DFS = 0.409 (0.256-0.653) Univariate	6
Fucini *et al*[9]	2012	Italy	Rectum	66 (27/39)	67 ± 9 yr	Ⅱ-Ⅲ	Yes	105.5 ± 39.6 mo	IHC	Dako	1:50	> 10%	OS = 2.526 (1.146-5.565) Univariate	5
Xu *et al*[10]	2009	China	Colon and rectum	119 (33/86)	57 yr (31-74 yr)	Ⅰ-Ⅳ	No	95 mo (1-203 mo)	IHC	Dako	1:50	> 10%	OS = 3.064 (1.217-7.718) Multivariate	5
Zlobec *et al*[24]	2008	Switzerland	Colon and rectum	1420 (NA)	NA	pT1-4, N0-N2	NA	NA	TMA/IHC	NA	NA	> 30%	OS = 1.15 (0.94-1.39) Multivariate	5
Torsello *et al*[7]	2008	Italy	Colon and rectum	1340 (650/690)	NA	A-D (Ducks)	Yes	5 yr	IHC	Dako	NA	> 30%	OS = 0.221 (0.105-0.464) Univariate	6
Cahlin *et al*[25]	2008	Sweden	Colon	22 (NA)	75 ± 9 yr	A-D (Ducks)	No	68 mo (3.6-127.4 mo)	IHC	Santa Cruz Biotechnology	0.25 μg/mL	NA	OS = 1.43 (1-2.06) Multivariate	5
Tsamandas *et al*[8]	2007	Greece	Rectum	28 (17/11)	64 yr (27-76 yr)	B2 and C (Ducks)	No	47.19 ± 6.2 mo	IHC	Dako	1:40	> 5%	OS = 0.032 (0.007-0.158) Univariate	5
Meleth *et al*[26]	2007	United Kingdom	Colon and rectum	491 (NA)	NA	Ⅰ-Ⅳ	No	5 yr	IHC	NA	NA	Score ⩾ 0.5	OS = 0.67 (0.493-0.92) Multivariate	6
Zavrides *et al*[12]	2006	Greece	Colon and rectum	100 (27/73)	NA	Ⅰ and Ⅲ	No	7 yr (5-9 yr)	IHC	Biogenex	1:10	> 5%	OS:0.273(0.139-0.534) Univariate	7
Georgiou *et al*[27]	2006	Greece	Colon and rectum	170 (64/106)	NA	B and C (Ducks)	NA	46 mo (3-93 mo)	IHC	Dako	1:80	> 10%	OS = 0.556 (0.326-1.031) Univariate	7
Chatla *et al*[28]	2005	United States	Colon and rectum	158 (89/69)	NA	Ⅱ and Ⅲ	No	7.31 yr (< 1-> 20 yr)	IHC	Cambridge Laboratories	1:80	Score ⩾ 0.5	RFS = 0.45 (0.083-2.441) Multivariate	6
Zhao *et al*[29]	2005	China	Colon and rectum	93 (53/40)	51 yr (median)	A-C (Ducks)	NA	60 mo (median)	IHC	NA	NA	Score ⩾ 2	OS = 0.505 (0.317-0.804) Univariate	6
Lustosa *et al*[16]	2005	Brazil	Colon and rectum	116 (58/58)	63.4 yr (30-87 yr)	Ⅰ-Ⅳ	No	28.5 mo (2-96 mo)	IHC	Dako	NA	> 10%	OS = 0.858 (0.433-1.698) Univariate	6
Krajewska *et al*[13]	2005	Sputh Korea	Colon and rectum	106 (NA)	NA	Ⅱ	No	66 mo (median)	TMA/IHC	NA	1:2000	NA	OS = 0.251 (0.111-0.567) Multivariate	6

Rosati *et al*[30]	2004	Italy	Colon and rectum	103 (41/62)	66 yr (29-79 yr)	B and C (Ducks)	Yes	5 yr	IHC	Dako	NA	> 10%	OS = 0.71 (0.37-1.35) Univariate DFS = 1 (0.51-1.96) Univariate	7
Garrity *et al*[31]	2004	Canada	Colon and rectum	366 (97/269)	NA	B2 and C (Ducks)	Yes	8.7 yr (median)	IHC	Dako	1:50	> 10%	OS = 0.99 (0.69-1.429) Multivariate DFS = 0.971 (0.654-1.449) Multivariate	5
Kouraklis *et al*[32]	2003	Greece	Colon	113 (55/58)	70.9 yr (42-94 yr)	B and C (Ducks)	No	NA	IHC	Dako	1:50	> 5%	OS = 0.523 (0.304-0.903) Univariate	8
Scopa *et al*[33]	2003	Greece	Colon and rectum	117 (76/41)	66 yr (25-82 yr)	A-D (Ducks)	No	97 mo (44-142 mo)	IHC	Dako	1:40	Cytoplasmic staining	OS = 1.55 (0.7-3.4) Multivariate	8
Sun *et al*[34]	2003	Sweden	Colon and rectum	138 (82/56)	71 yr (43-94 yr)	A-D (Ducks)	NA	NA	IHC	Nova Castra Laboratories Ltd	1:80	> 5%	OS = 0.504 (0.221-1.146) Univariate	7
Bendardaf *et al*[35]	2003	Finland	Colon and rectum	58 (45/13)	60.3 yr (24.3-78.2 yr)	T2-X,N0-X,M0-1	Yes	NA	IHC	Dako	1:50	Sum the intensity score and expression score ⩾ 1.10	OS = 1.02(0.7-11.5) Univariate	6
Elkablawy *et al*[36]	2001	United Kingdom	Colon and rectum	52 (18/34)	68.8 yr (33-93 yr)	pT2-4, N0-1,M0-1	NA	43.5 mo (2-111 mo)	IHC	Dako	4 μg/mL	Multiply the intensity score and expression score ⩾ 6	OS = 0.552 (0.231-1.319) Univariate	6
Meterissian *et al*[37]	2001	Canada	Colon	76 (62/14)	71.2 yr (40-89 yr)	B (Ducks)	No	59 mo (5-110 mo)	IHC	Dako	1:50	⩾ 30% or stain intensity scale ⩾ 1	OS = 0.35 (0.13-0.94) Univariate DFS = 0.49 (0.19-1.26) Univariate	7
Paradiso *et al*[38]	2001	Italy	Colon and rectum	80 (29/51)	NA	Advanced	No	NA	IHC	Dako	1:40	> 5%	OS = 1.287 (0.76-2.183) Univariate	6
Schwandner *et al*[39]	2000	Germeny	Rectum	160 (47/113)	66.7 yr (31-92 yr)	Ⅰ-Ⅲ	No	38 mo (12-72 mo)	IHC	Dako	1:20	> 10%	DFS = 0.181 (0.056-0.585) Univariate	7
Buglioni *et al*[40]	1999	Italy	Colon and rectum	171 (57/114)	64 yr (56-70 yr)	A-D (Ducks)	No	50 mo (median)	IHC	Dako	NA	A strong homogeneous cytoplasmic immunoreaction	OS = 0.192 (0.0439-0.84) Multivariate DFS = 0.178 (0.0508-0.625) Multivariate	7
Leahy *et al*[41]	1999	Ireland	Colon and rectum	102 (22/80)	69 yr (39.2-85.5 yr)	A-C (Ducks)	No	9.9 yr (9.0-11.2 yr)	IHC	Dako	1:50	> 5%	OS = 0.5 (0.2-1) Multivariate	7
Ishijima *et al*[42]	1999	Japan	Colon and rectum	33 (10/23)	61.6 yr (42-86 yr)	A-D (Ducks)	NA	NA	IHC	Santa Cruz Biotechnology	1:50	> 30%	DFS = 1.051 (0.202-5.464) Univariate	6
Sinicrope *et al*[43]	1999	United States	Colon	137 (71/66)	65.2 yr (26-89 yr)	T2-3, N0,M0	No	105.5 mo (2-281 mo)	IHC	Dako	1:20	> 20%	OS = 0.46 (0.21-1.05) Multivariate RFS = 0.45 (0.21-0.96) Multivariate	7
Hirvikoski *et al*[44]	1999	Finland	Rectum	92 (62/30)	72 yr (52-90 yr)	A-D (Ducks)	Yes	32 mo (0-306 mo)	IHC	Dako	1:200	> 20%	OS = 0.99 (0.55-1.79) Univariate	6
Biden *et al*[45]	1999	Australia	Colon and rectum	66 (49/17)	NA	A-D (Ducks)	NA	NA	IHC	Dako	1:40	> 5%	OS = 0.132 (0.057-0.306) Univariate	5

Ilyas *et al*[46]	1998	United Kingdom	Colon and rectum	66 (40/26)	NA	B (Ducks)	NA	NA	IHC	Dako	1:40	Stain	RFS = 0.77 (0.62-0.96) Univariate	5
Kaklamanis *et al*[47]	1998	United Kingdom	Colon and rectum	224 (73/151)	NA	A-C (Ducks)	NA	36 mo (1-72.5 mo)	IHC	Dako	NA	> 10%	OS = 0.605 (0.375-0.977) Univariate	7
Tollenaar *et al*[48]	1998	Netherlands	Colon and rectum	209 (99/110)	NA	A-C (Ducks)	No	NA	IHC	Boehringer Mannheim	1:200	Score ⩾ 2	OS = 0.978 (0.658-1.453) Univariate	8
Bhatavdekar *et al*[14]	1997	India	Colon and rectum	48 (29/19)	48 yr (25-74 yr)	B and C (Ducks)	NA	29.95 mo (2-36 mo)	IHC	Dako	NA	> 5%	OS = 7.813 (2.375-25.64) Univariate	7
Baretton *et al*[49]	1996	Germeny	Colon and rectum	95 (64/31)	63.8 ± 12.5 yr	pT2-3, N0,M0	NA	Up to 8 yr	IHC	Dianova	1:60	An unequivocally strong cytoplasmic immunoreaction	DFS = 0.504 (0.27-0.943) Univariate	6
Ofner *et al*[50]	1995	Austria	Colon and rectum	104 (47/57)	67.8 yr (35-90 yr)	pT1-4, N0-X,M0-1	NA	NA	IHC	Dako	1:300	Stain	OS = 0.443 (0.252-0.78) Univariate	7

P/N: Positive/Negative; NA: Not assessable.

a favorable OS when the patients adopted no therapy before surgery (HR = 0.696, 95%CI: 0.502-0.964, P = 0.029) (Figure 3C). Our results also indicated that the NOS quality score had no significant effect on the prognostic value of Bcl-2 expression (Figure 3D). Meanwhile, a sensitive analysis was conducted to assess the role of each study on the overall environment. To achieve this, studies were excluded one at a time while the rest were analyzed. HR of Bcl-2 high expression on OS ranged from 0.664 (95%CI: 0.532-0.830) to 0.730 (95%CI: 0.585-0.909) (Figure 4A), and pooled HR of Bcl-2 high expression on DFS/RFS ranged from 0.597 (95%CI: 0.461-0.775) to 0.687 (95%CI: 0.528-0.894) (Figure 4B).

Impact of Bcl-2 high expression on clinicopathological parameters

Twelve studies were selected to assess the association between Bcl-2 high expression and tumor differentiation grade. The pooled OR was 2.475 (95%CI: 1.307-4.685, P = 0.005) with statistical heterogeneity (I^2 = 68.4%, P = 0.000), which indicated that low expression of Bcl-2 was correlated with differentiation of CRC. Correlation between Bcl-2 overexpression and Ducks' stages were also evaluated in twelve studies. The pooled OR was 1.630 (95%CI: 1.009-2.632, P = 0.046) with significant heterogeneity (I^2 = 78.1%, P = 0.000), suggesting that downregulated Bcl-2 expression was associated with the progression of CRC. However, we did not find significant association between Bcl-2 expression and gender or the tumor location, the pooled OR being shown in Table 3.

Publication bias

Begg's test was used to assess the potential publication bias. The funnel plots for the OS (Figure 5A) and DFS/RFS (Figure 5B) indicated that there was no evidence of significant publication bias in our present meta-analysis.

DISCUSSION

It is well documented that defects in the mitochondrial apoptotic pathway are closely related with carcinogenesis. Bcl-2 is a key inhibitor of apoptosis, playing a major role in the maintenance of normal balance between apoptosis and cellular survival.

Currently, effective treatment of CRC remains a big challenge. The majority of patients will experience relapse or distant metastases within 5 years following surgical resection. Abnormal Bcl-2 activation has been implicated during the evolution of CRC. Up to this date, however, the exact role of Bcl-2 in CRC has not been established. The explanation of this inconsistency is not known, perhaps because of the variations with ethnicity and location in the patient population. By the same token, no consistent conclusion about the prognostic value of Bcl-2 expression in CRC patients has been made. So we speculate that the prognostic significance of

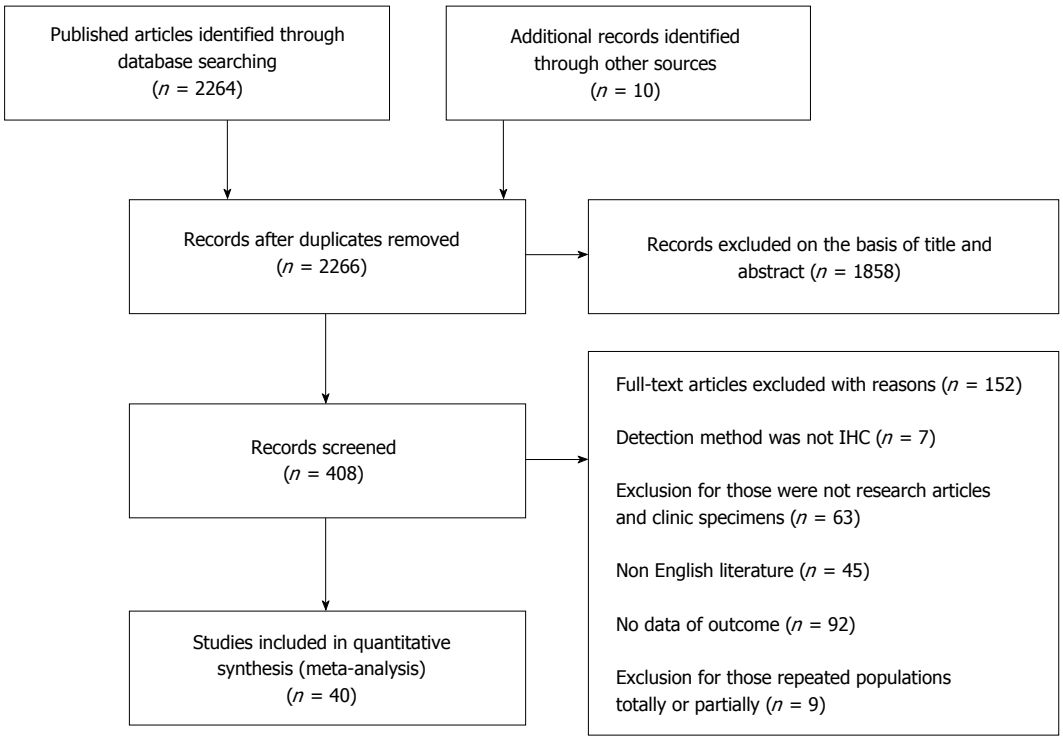


Figure 1 Flow diagram of the selection procedure for the studies.

Table 2 Subgroup analysis of pooled hazard ratios for colorectal cancer patients with overexpressed Bcl-2

Stratified analysis	No. of studies	No. of patients	Pooled HR (95%CI)	*P* value	Heterogeneity	
					I^2 (%)	*P* value
No. of patients						
⩾ 100	22	6274	0.684 (0.54-0.866)	0.002	75.9	0.000
< 100	12	712	0.693 (0.389-1.235)	0.214	85.8	0.000
Study location						
Asia	7	777	1.021 (0.488-2.136)	0.955	88.2	0.000
Europe and America	26	6143	0.691 (0.553-0.864)	0.001	73.9	0.000
Treatment before surgery						
Yes	7	2056	0.772 (0.55-0.947)	0.394	73.8	0.001
No	18	2615	0.696 (0.502-0.964)	0.029	79.9	0.000
Quality score						
⩾ 7	18	2471	0.678 (0.499-0.92)	0.013	71.8	0.000
< 7	16	4515	0.708 (0.503-0.996)	0.047	84.9	0.000

Table 3 Bcl-2 expression and clinicopathological features of colorectal cancer

Clinicopathologial features	No. of studies	No. of patients	Pooled OR (95%CI)	*P* value	Heterogeneity	
					I^2	*P* value
Gender (male *vs* female)	11	1671	1.125 (0.865-1.463)	0.381	30.2%	0.158
Tumor location (colon *vs* rectum)	8	1361	1.168 (0.922-1.480)	0.199	0%	0.628
tumor grade (1 + 2 *vs* 3)	12	1454	2.475 (1.307-4.685)	0.005	68.4%	0.000
Ducks' stage (A + B *vs* C + D)	12	1572	1.630 (1.009-2.632)	0.046	78.1%	0.000

Bcl-2 expression in CRC may be restricted to specific subgroups. To the best of our knowledge, this is the first meta-analysis pertinently investigating the prognostic value of Bcl-2 expression in CRC.

Our meta-analysis incorporated 40 eligible studies with the survival data of OS, DFS and RFS. From our analyses results we found that Bcl-2 high expression is of significant association with increased OS and DFS/RFS in patients with CRC. When the subgroup analyses were conducted, the pooled results demonstrated that high expression Bcl-2 was a favorable prognostic factor in subgroup with number of patients more than 100 and subgroup with origin country of Europe and America. Additionally, Bcl-2 overexpression showed an

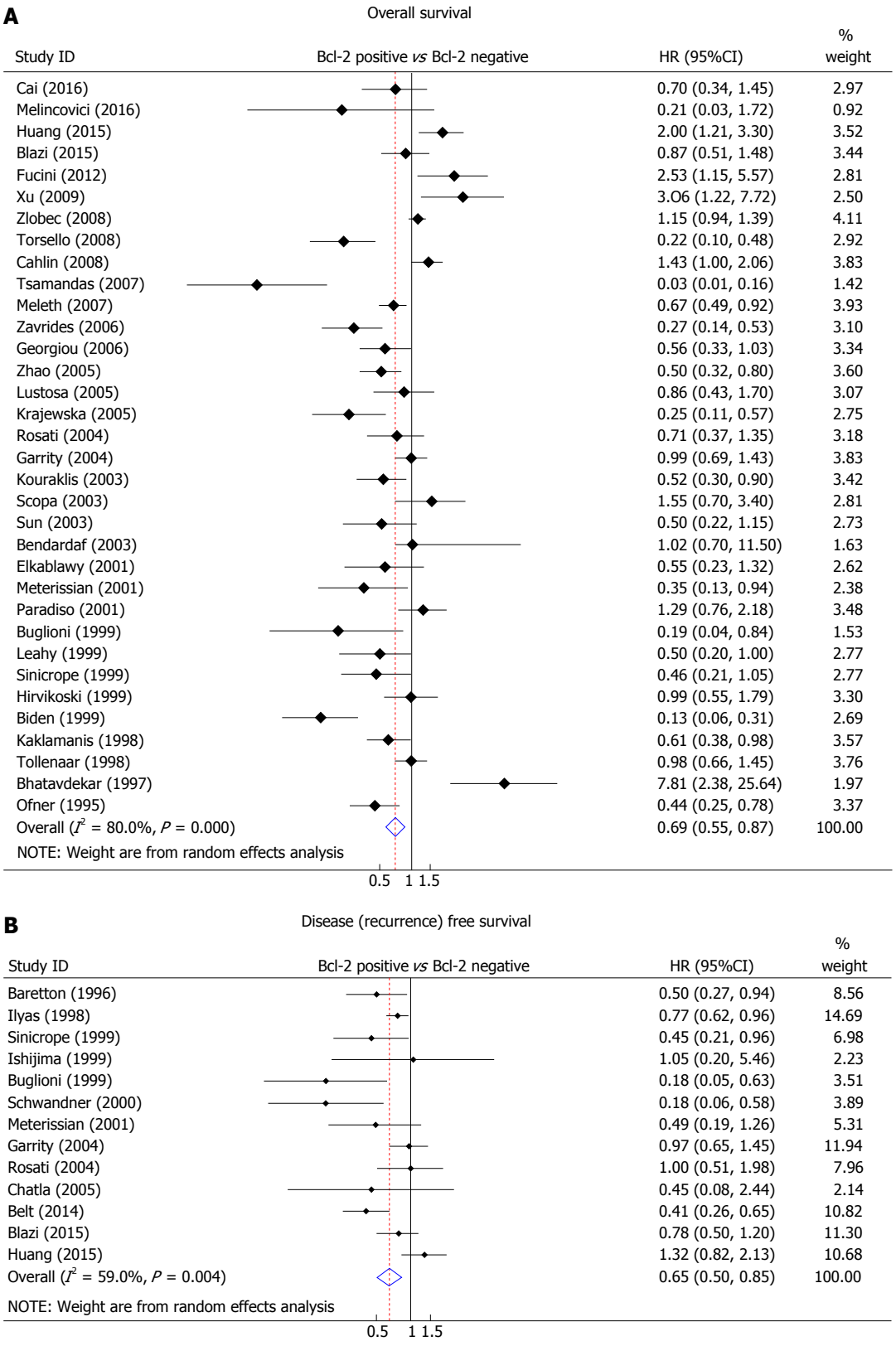
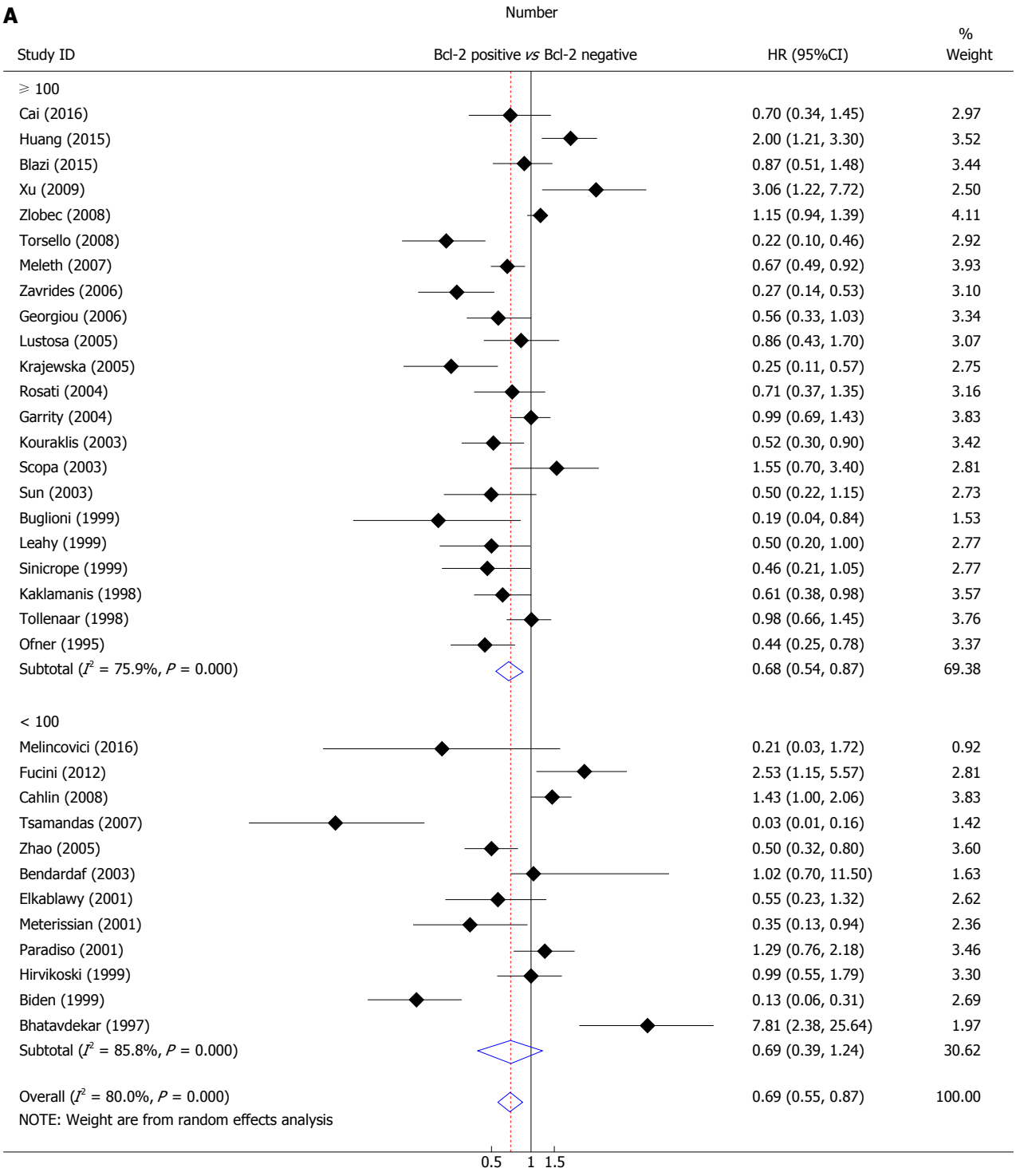
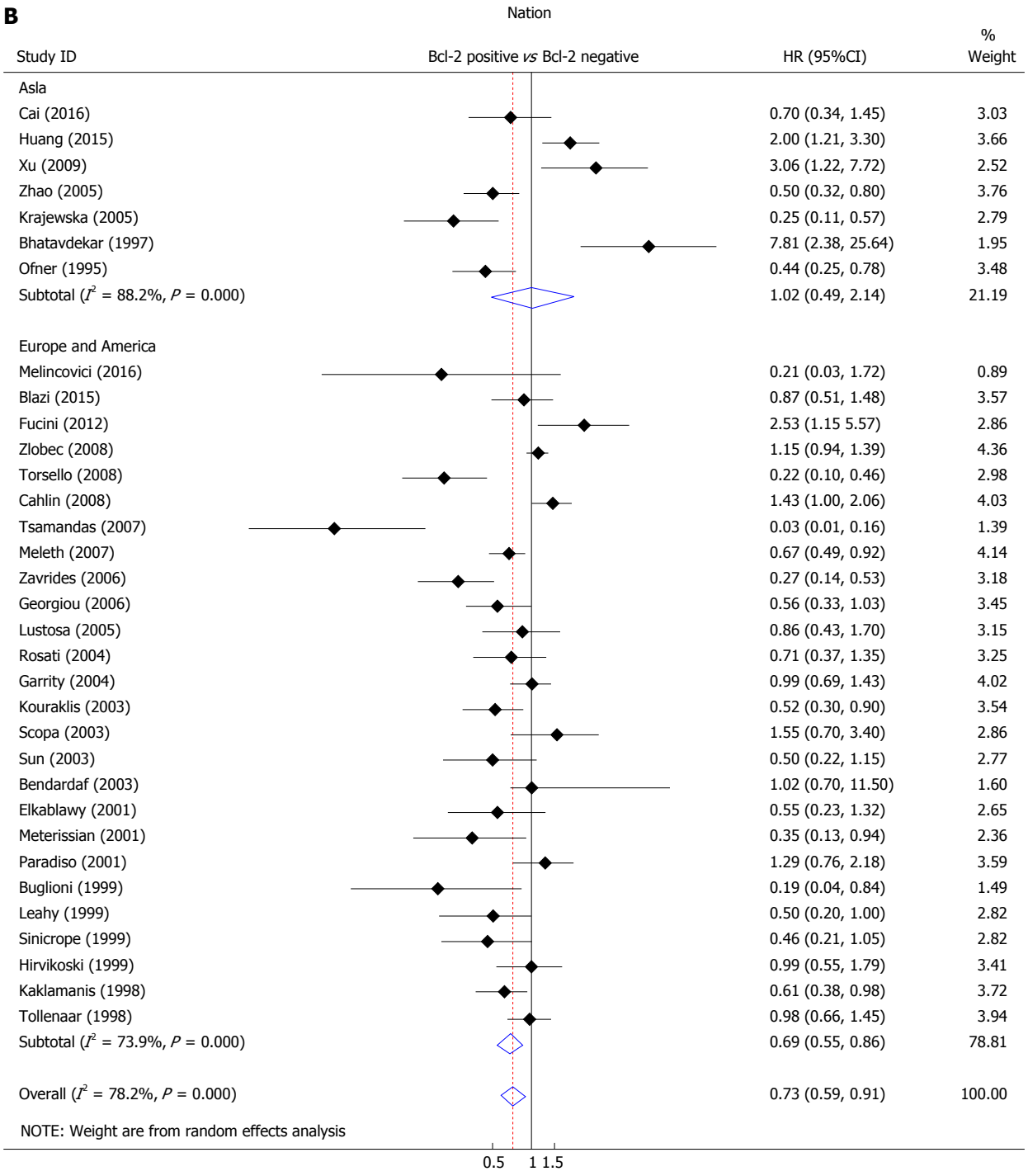


Figure 2 Hazard ratio of Bcl-2 expression associated with (A) overall survival and (B) disease free survival/recurrence free survival. DFS: Disease free survival; RFS: Recurrence frees survival.

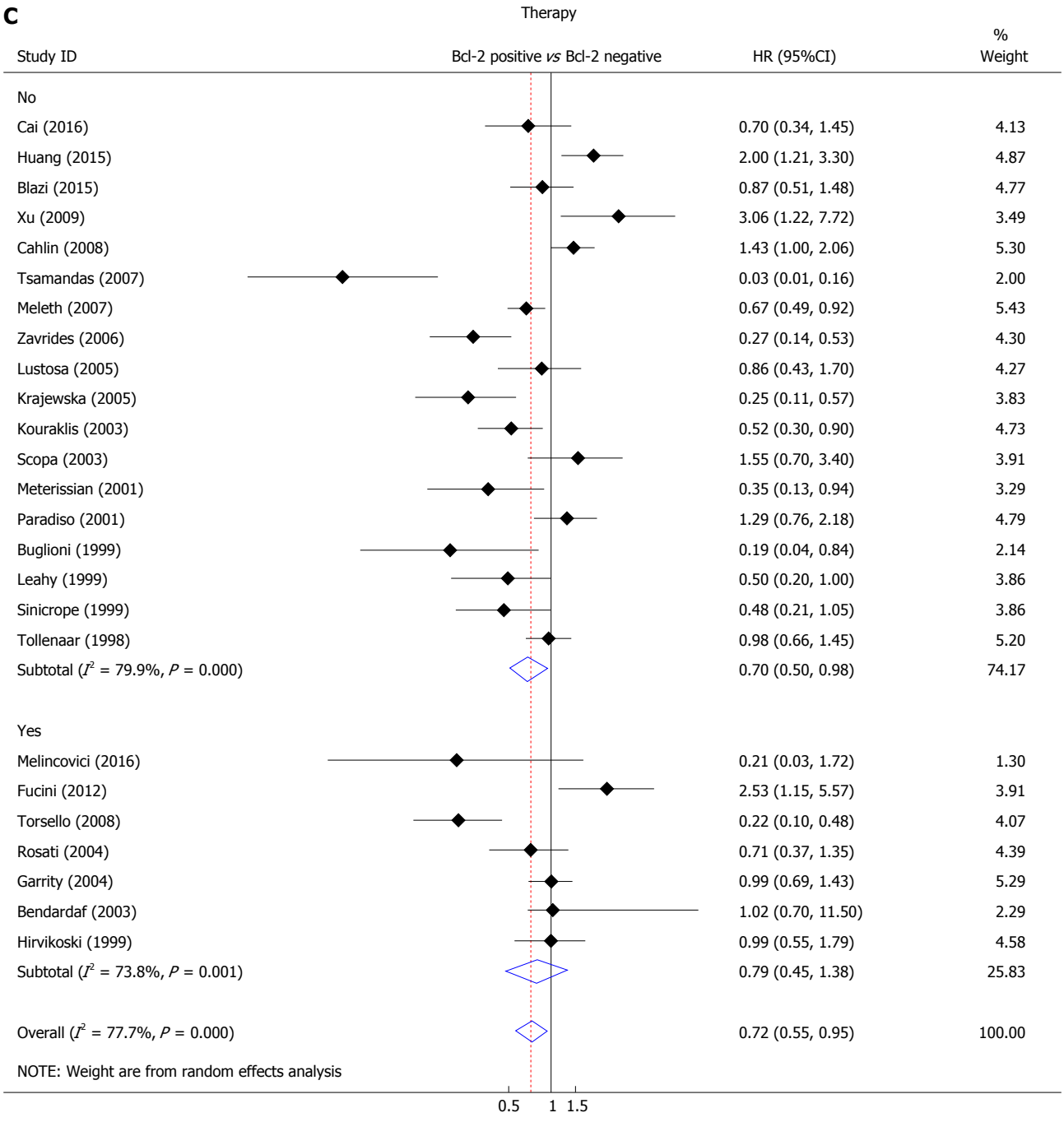
increased OS when the patients adopted no therapy before surgery. As to clinicopathological parameters analysis, Bcl-2 was found to express more frequently in tumors with high differentiation grade and A/B Ducks' stage. It should be noted that no publication bias was found in this meta-analysis.

Our study leads to several valuable conclusions. First, expression of Bcl-2 is a favorable factor for


A
Number
Study ID
Bcl-2 positive *vs* Bcl-2 negative
HR (95%CI)
% Weight
⩾ 100
Cai (2016) 0.70 (0.34, 1.45) 2.97
Huang (2015) 2.00 (1.21, 3.30) 3.52
Blazi (2015) 0.87 (0.51, 1.48) 3.44
Xu (2009) 3.06 (1.22, 7.72) 2.50
Zlobec (2008) 1.15 (0.94, 1.39) 4.11
Torsello (2008) 0.22 (0.10, 0.46) 2.92
Meleth (2007) 0.67 (0.49, 0.92) 3.93
Zavrides (2006) 0.27 (0.14, 0.53) 3.10
Georgiou (2006) 0.56 (0.33, 1.03) 3.34
Lustosa (2005) 0.86 (0.43, 1.70) 3.07
Krajewska (2005) 0.25 (0.11, 0.57) 2.75
Rosati (2004) 0.71 (0.37, 1.35) 3.16
Garrity (2004) 0.99 (0.69, 1.43) 3.83
Kouraklis (2003) 0.52 (0.30, 0.90) 3.42
Scopa (2003) 1.55 (0.70, 3.40) 2.81
Sun (2003) 0.50 (0.22, 1.15) 2.73
Buglioni (1999) 0.19 (0.04, 0.84) 1.53
Leahy (1999) 0.50 (0.20, 1.00) 2.77
Sinicrope (1999) 0.46 (0.21, 1.05) 2.77
Kaklamanis (1998) 0.61 (0.38, 0.98) 3.57
Tollenaar (1998) 0.98 (0.66, 1.45) 3.76
Ofner (1995) 0.44 (0.25, 0.78) 3.37
Subtotal (I^2 = 75.9%, P = 0.000) 0.68 (0.54, 0.87) 69.38
< 100
Melincovici (2016) 0.21 (0.03, 1.72) 0.92
Fucini (2012) 2.53 (1.15, 5.57) 2.81
Cahlin (2008) 1.43 (1.00, 2.06) 3.83
Tsamandas (2007) 0.03 (0.01, 0.16) 1.42
Zhao (2005) 0.50 (0.32, 0.80) 3.60
Bendardaf (2003) 1.02 (0.70, 11.50) 1.63
Elkablawy (2001) 0.55 (0.23, 1.32) 2.62
Meterissian (2001) 0.35 (0.13, 0.94) 2.36
Paradiso (2001) 1.29 (0.76, 2.18) 3.46
Hirvikoski (1999) 0.99 (0.55, 1.79) 3.30
Biden (1999) 0.13 (0.06, 0.31) 2.69
Bhatavdekar (1997) 7.81 (2.38, 25.64) 1.97
Subtotal (I^2 = 85.8%, P = 0.000) 0.69 (0.39, 1.24) 30.62
Overall (I^2 = 80.0%, P = 0.000) 0.69 (0.55, 0.87) 100.00
NOTE: Weight are from random effects analysis
0.5
1
1.5


B
Nation
Study ID
Bcl-2 positive vs Bcl-2 negative
HR (95%CI)
% Weight
Asla
Cai (2016) 0.70 (0.34, 1.45) 3.03
Huang (2015) 2.00 (1.21, 3.30) 3.66
Xu (2009) 3.06 (1.22, 7.72) 2.52
Zhao (2005) 0.50 (0.32, 0.80) 3.76
Krajewska (2005) 0.25 (0.11, 0.57) 2.79
Bhatavdekar (1997) 7.81 (2.38, 25.64) 1.95
Ofner (1995) 0.44 (0.25, 0.78) 3.48
Subtotal (I^2 = 88.2%, P = 0.000) 1.02 (0.49, 2.14) 21.19
Europe and America
Melincovici (2016) 0.21 (0.03, 1.72) 0.89
Blazi (2015) 0.87 (0.51, 1.48) 3.57
Fucini (2012) 2.53 (1.15 5.57) 2.86
Zlobec (2008) 1.15 (0.94, 1.39) 4.36
Torsello (2008) 0.22 (0.10, 0.46) 2.98
Cahlin (2008) 1.43 (1.00, 2.06) 4.03
Tsamandas (2007) 0.03 (0.01, 0.16) 1.39
Meleth (2007) 0.67 (0.49, 0.92) 4.14
Zavrides (2006) 0.27 (0.14, 0.53) 3.18
Georgiou (2006) 0.56 (0.33, 1.03) 3.45
Lustosa (2005) 0.86 (0.43, 1.70) 3.15
Rosati (2004) 0.71 (0.37, 1.35) 3.25
Garrity (2004) 0.99 (0.69, 1.43) 4.02
Kouraklis (2003) 0.52 (0.30, 0.90) 3.54
Scopa (2003) 1.55 (0.70, 3.40) 2.86
Sun (2003) 0.50 (0.22, 1.15) 2.77
Bendardaf (2003) 1.02 (0.70, 11.50) 1.60
Elkablawy (2001) 0.55 (0.23, 1.32) 2.65
Meterissian (2001) 0.35 (0.13, 0.94) 2.36
Paradiso (2001) 1.29 (0.76, 2.18) 3.59
Buglioni (1999) 0.19 (0.04, 0.84) 1.49
Leahy (1999) 0.50 (0.20, 1.00) 2.82
Sinicrope (1999) 0.46 (0.21, 1.05) 2.82
Hirvikoski (1999) 0.99 (0.55, 1.79) 3.41
Kaklamanis (1998) 0.61 (0.38, 0.98) 3.72
Tollenaar (1998) 0.98 (0.66, 1.45) 3.94
Subtotal (I^2 = 73.9%, P = 0.000) 0.69 (0.55, 0.86) 78.81
Overall (I^2 = 78.2%, P = 0.000) 0.73 (0.59, 0.91) 100.00
NOTE: Weight are from random effects analysis
0.5
1
1.5

C


Therapy
Study ID
Bcl-2 positive vs Bcl-2 negative
HR (95%CI)
% Weight
No
Cai (2016) 0.70 (0.34, 1.45) 4.13
Huang (2015) 2.00 (1.21, 3.30) 4.87
Blazi (2015) 0.87 (0.51, 1.48) 4.77
Xu (2009) 3.06 (1.22, 7.72) 3.49
Cahlin (2008) 1.43 (1.00, 2.06) 5.30
Tsamandas (2007) 0.03 (0.01, 0.16) 2.00
Meleth (2007) 0.67 (0.49, 0.92) 5.43
Zavrides (2006) 0.27 (0.14, 0.53) 4.30
Lustosa (2005) 0.86 (0.43, 1.70) 4.27
Krajewska (2005) 0.25 (0.11, 0.57) 3.83
Kouraklis (2003) 0.52 (0.30, 0.90) 4.73
Scopa (2003) 1.55 (0.70, 3.40) 3.91
Meterissian (2001) 0.35 (0.13, 0.94) 3.29
Paradiso (2001) 1.29 (0.76, 2.18) 4.79
Buglioni (1999) 0.19 (0.04, 0.84) 2.14
Leahy (1999) 0.50 (0.20, 1.00) 3.86
Sinicrope (1999) 0.48 (0.21, 1.05) 3.86
Tollenaar (1998) 0.98 (0.66, 1.45) 5.20
Subtotal (I^2 = 79.9%, P = 0.000) 0.70 (0.50, 0.98) 74.17
Yes
Melincovici (2016) 0.21 (0.03, 1.72) 1.30
Fucini (2012) 2.53 (1.15, 5.57) 3.91
Torsello (2008) 0.22 (0.10, 0.48) 4.07
Rosati (2004) 0.71 (0.37, 1.35) 4.39
Garrity (2004) 0.99 (0.69, 1.43) 5.29
Bendardaf (2003) 1.02 (0.70, 11.50) 2.29
Hirvikoski (1999) 0.99 (0.55, 1.79) 4.58
Subtotal (I^2 = 73.8%, P = 0.001) 0.79 (0.45, 1.38) 25.83
Overall (I^2 = 77.7%, P = 0.000) 0.72 (0.55, 0.95) 100.00
NOTE: Weight are from random effects analysis
0.5
1
1.5

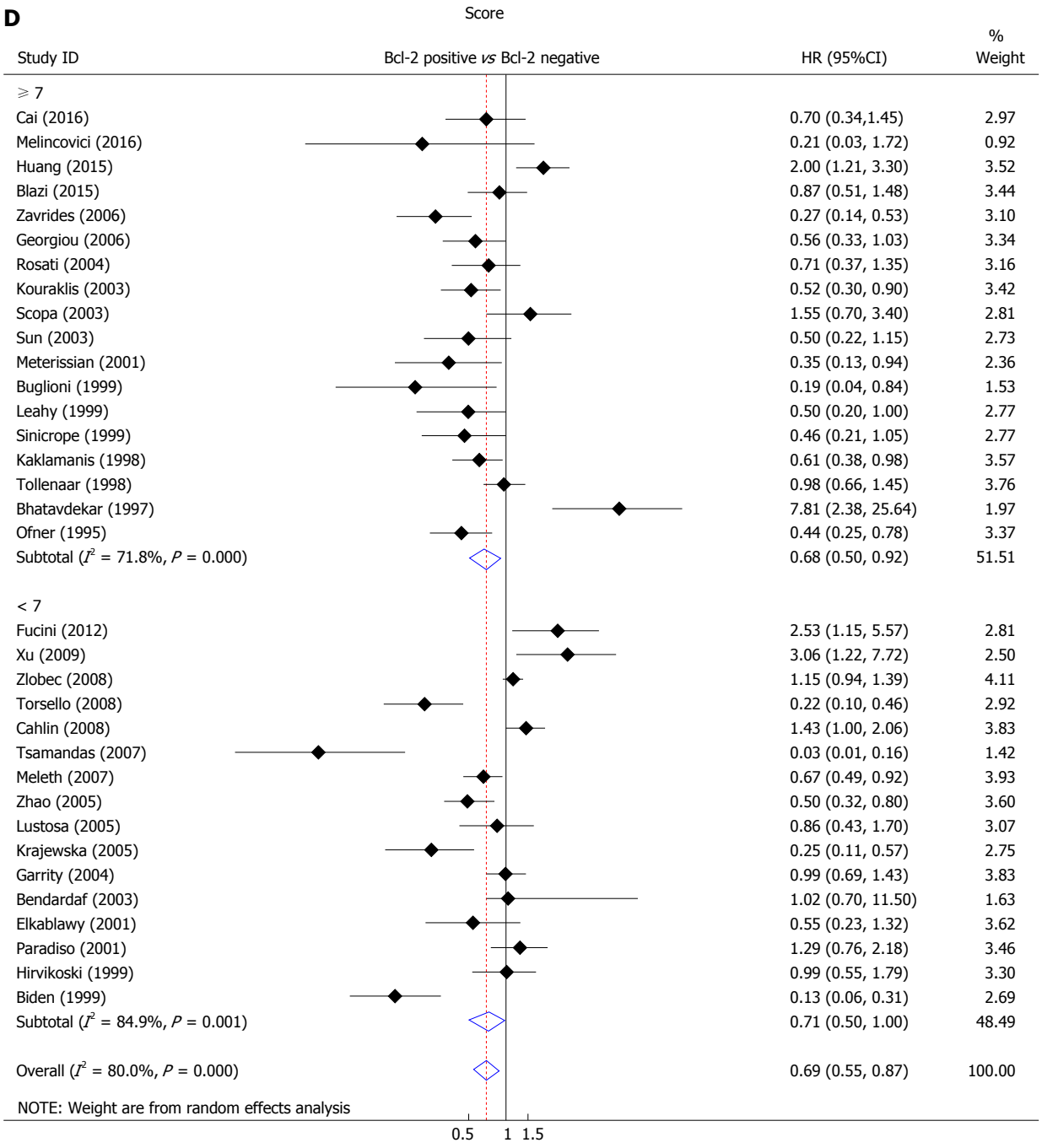


Figure 3 Hazard ratio of Bcl-2 expression associated with overall survival in the subgroup. A: Patients' number; B: Patients' country of origin; C: The condition of treatment before surgery; D: Quality score of study.

CRC. The relationship between Bcl-2 expression and transformation from normal epithelium to invasive cancer is not entirely clear. However, there is evidence to suggest that during the evolution of CRC, the role of Bcl-2 oncoprotein is believed to be in the early stages of carcinogens[51,52]. Moreover, lack of Bcl-2 expression has been proved to be correlated with invasion, metastasis and recurrence of CRC. Our meta-analysis revealed that the upregulation of Bcl-2 was related to favorable prognosis in both OS and DFS/RFS. This is contradictory to the anti-apoptotic function of Bcl-2, which may be due to the interactions of various proteins involved in apoptotic pathways such as p53, Fas and so on. Second, our present results indicated that expression of Bcl-2 protein was associated with pathological grade and clinical stage, consistent with what Zavrides *et al*[12] reported. The survival of CRC patients largely depends on disease stage at the time of diagnosis and differs greatly between stages. It was reported earlier that Bcl-2 expression correlated with improved survival, a significantly higher MFS for the subgroup of patients with Dukes' B[53]. It is logical to assume that the primary role of Bcl-2 during carcinogenesis and progression of CRC may depend

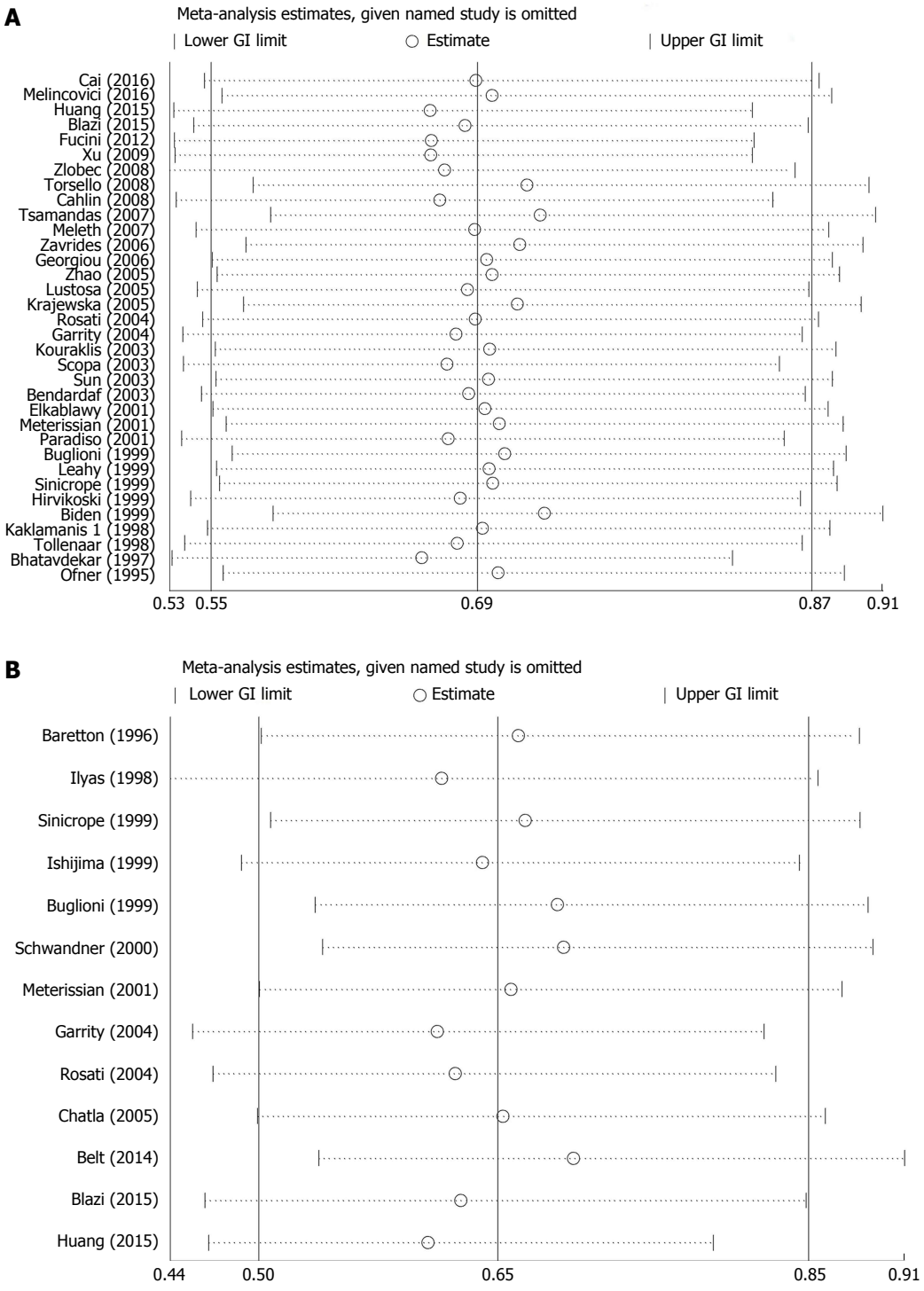


Figure 4 Sensitivity analysis for the meta-analysis of (A) OS and (B) disease free survival/recurrence free survival in all patients.

on disease stage. However, further study on large sample is needed to confirm this speculation. Third, the prognostic role of Bcl-2 expression on CRC patients is evident in Caucasian populations but not yet the case in Asians. Genomic polymorphisms among various ethnic groups may be the explanation. Thus, the clinical value of Bcl-2 should be studied separately based upon different population structures and aggregates in the future research. Additionally, the favorable effect of Bcl-2 expression on CRC patients' overall survival is insignificant in subgroup receiving preoperative treatment. Some studies have suggested that preoperative chemoradiation can influence cancer cell's apoptosis and treatment effect by changing Bcl-2 expression. Thus, it seems easy to explain such observation. However, Long-term prospective studies are needed to verify this.

Recently, targeting proteins involved in apoptotic pathways appeared as an attractive strategy to assist anticancer therapy. A particular concern has been

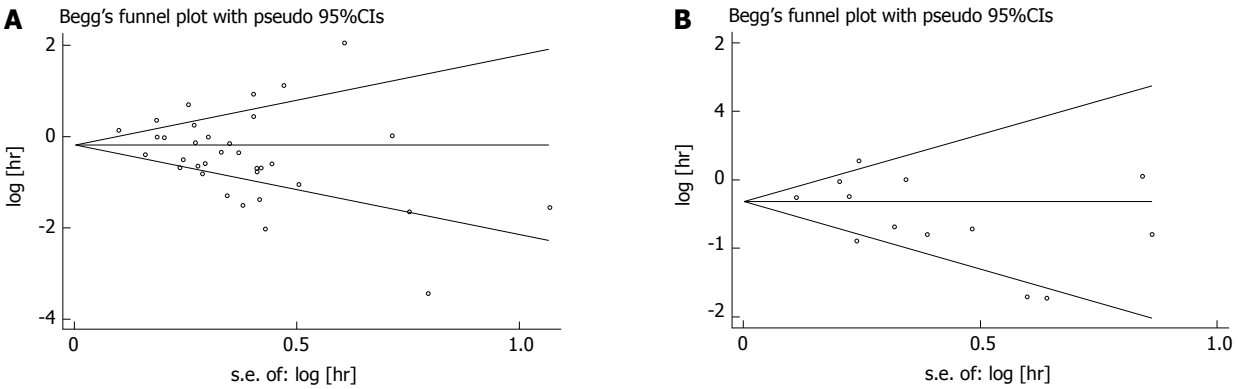


Figure 5 Begg's publication bias plot. It showed no publication bias for the studies regarding the association of Bcl-2 expression with (A) OS or (B) disease free survival/recurrence free survival in the meta-analysis. Each point represents a separate study for the indicated association.

focused on the development of agents capable of inhibiting the activity of Bcl-2 family members that are overexpressed in various malignancies[54,55]. In this regard, it seems that we need to reassess the of small-molecule drugs targeting Bcl-2. There is a serious need for more *in vivo* experiments to explicate the detailed mechanism. Finally, using Bcl-2 alone to evaluate prognostic information of CRC patients with different stages is probably limited. On the other hand, integration of multiple biomarkers may provid sufficient support for clinical application[56-59]. We suggest focusing on the combination of key markers within the prominent pathways that occupy an important role in clinical prognosis, which better reflects the overall molecular environment in CRC. A systematic study on the prognostic value of multi-marker proteins in CRC patients can also be performed in the future.

There exists some limitations that should be noted in our meta-analysis. We only recruited articles published in English, thus a language bias might exist. Some HRs and their corresponding 95%CIs were extracted from the survival curves. However, these data might be less reliable than those directly obtained from survival data. We use random effects model to deal with heterogeneity, however, the inter-study heterogeneity resulted from different populations, different antibody source and varying cutoff values was inevitable.

In summary, our meta-analysis suggests that expression of the Bcl-2 protein is associated with favorable prognosis in patients with CRC. Subgroup analysis showed that Bcl-2 overexpression may become a good prognosis factor in CRC where patients come from Europe and America but not Asian and patients not receive any adjuvant therapy before surgery. These significant associations were more remarkable in CRCs with high grade of differentiation and A/B Ducks' stage. Our analysis also found those significant associations only be find in populations more than 100. This told us that further prospective studies with larger sample sizes are required to validate the prognostic value of Bcl-2 expression in CRC.

COMMENTS

Background

An increasing body of evidence from many studies indicates that Bcl-2 expression may be associated with prognosis in malignancies including colorectal cancer (CRC). However, neither the function nor the prognostic value of Bcl-2 expression in patients with CRC is clear to us.

Research frontiers

Currently, effective treatment of CRC remains a big challenge. Abnormal Bcl-2 activation has been implicated during the evolution of CRC and speculated playing a major role in the prognosis of CRC. Up to this date, however, the exact role of Bcl-2 in CRC has not been established.

Innovations and breakthroughs

In the present study, the authors explored the reason for present contradictory observations and determine the prognostic value of Bcl-2 in patients with CRC. This is the first meta-analysis pertinently investigating the prognostic value of Bcl-2 expression in CRC.

Applications

The present study allows understanding the prognostic-predictive capability of Bcl-2 in CRC.

Peer-review

This systematic review and meta-analysis of retrospective studies adds useful information for practice and research, and probably for policy.

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CASE REPORT

Liver injury after aluminum potassium sulfate and tannic acid treatment of hemorrhoids

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Abstract

We are reporting a rare case of acute liver injury that developed after an internal hemorrhoid treatment with the aluminum potassium sulfate and tannic acid (ALTA) regimen. A 41-year-old man developed a fever and liver injury after undergoing internal hemorrhoid treatment with a submucosal injection of ALTA with lidocaine. The acute liver injury was classified clinically as hepatocellular and pathologically as cholestastic. We could not classify the mechanism of injury. High eosinophil and immunoglobulin E levels characterized the injury, and a drug lymphocyte stimulation test was negative on postoperative day 25. Fluid replacement for two weeks after hospitalization improved the liver injury. ALTA therapy involves injecting chemicals into the submucosa, from the rectum to the anus, and this is the first description of a case that developed a severe liver disorder after this treatment; hence, an analysis of future cases as they accumulate is desirable.

Key words: Aluminum potassium sulfate and tannic acid injection; Aluminum potassium sulfate and tannic acid therapy; Rectal submucosal injection; Drug-induced

lymphocyte stimulation test; Drug-induced liver injury

Core tip: The definition of drug-induced liver injury has diversified in recent years. This report describes the characteristics of a case of acute liver injury that developed after internal hemorrhoid treatment using the aluminum potassium sulfate and tannic acid regimen, and it is the first report of a case of drug-induced liver injury caused by a rectal submucosal injection.

Yoshikawa K, Kawashima R, Hirose Y, Shibata K, Akasu T, Hagiwara N, Yokota T, Imai N, Iwaku A, Kobayashi G, Kobayashi H, Kinoshita A, Fushiya N, Kijima H, Koike K, Saruta M. Liver injury after aluminum potassium sulfate and tannic acid treatment of hemorrhoids. *World J Gastroenterol* 2017; 23(27): 5034-5040 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/5034.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.5034

INTRODUCTION

Drug-induced liver injury (DILI) is a liver disorder caused by the administration of drugs, but in recent years the definition of this term has diversified to include supplements, health foods, and traditional Chinese medicines[1-3]. Drugs that cause hepatopathy are usually administered orally or intravenously, but there are few case reports that describe DILI cause by drugs administered by other routes, for example, subcutaneously or intravesically[4-6].

In this report, we describe a case of DILI that developed after internal hemorrhoid treatment with an aluminum potassium sulfate and tannic acid (ALTA) regimen with lidocaine. This is the first case report that describes the development and management of a severe liver disorder after ALTA therapy.

CASE REPORT

A 41-year-old Japanese man was admitted to our hospital with liver damage, itchy skin, and pyrexia. His medical history was unremarkable, but one year previously his gamma-glutamyl transpeptidase levels had risen to 271 IU/L (normal: < 30 IU/L). Prior to this, the patient had consumed 540 mL of sake three times a week, and he had been reducing the amount of alcohol he consumed for about one year. He did not smoke or take any recreational drugs. He was single, was not homosexual, and he had not had sexual intercourse for over one year.

The patient had undergone treatment of his internal hemorrhoids as an outpatient at a nearby hospital that comprised an aluminum potassium sulfate and tannic acid (ALTA) injection. He developed a fever of 38 ℃, dark colored urine, and itchy skin on postoperative day (POD) 1, and he took loxoprofen sodium, as required. He consulted another doctor on POD 7, because the symptoms had not improved, and his blood test results led to a diagnosis of liver dysfunction. His liver dysfunction had not improved by POD 14, and he was admitted to our hospital on POD 15.

The patient was 167 cm tall and weighed 52.5 kg. He presented with a blood pressure of 103/59 mmHg, a heart rate of 82 beats per minute, a temperature of 36.6 ℃, and 99% oxygen saturation in room air. No specific physical findings were evident during the clinical examination on the day of hospitalization, but the patient had a rash, edema, hepatosplenomegaly, lymphadenopathy, and jaundice. The subjective symptoms, comprising the fever, dark colored urine, and itchy skin, had disappeared by POD 10.

The laboratory test results showed that the patient's complete blood count was almost normal with a white blood cell count of 6900 cells/μL, a hemoglobin concentration of 14.7 g/dL, a hematocrit level of 43.1%, and a platelet count of 3.98 × 10^5 cells/μL, but a higher percentage of eosinophils was present (6.8%) (normal: 1.0%-5.0%), which peaked at 13.9% on POD 20. The patient's serum liver enzyme and bilirubin levels were elevated at the time of admission. The aspartate aminotransferase (AST) level was 432 IU/L (normal: 10-40 IU/L), the alanine aminotransferase (ALT) level was 911 IU/L (normal: 10-40 IU/L), the alkaline phosphatase (ALP) level was 473 IU/L (normal: 115-359 IU/L), and the total bilirubin level was 1.3 mg/dL (normal: 0.2-1.2 mg/dL). Thus, compared with the ALP level, the AST and ALT levels showed greater magnitudes of elevation. The patient's other blood test results were within the normal ranges, as follows: serum albumin: 4.0 g/dL (normal: 3.8-5.2 g/dL); serum creatinine: 0.86 mg/dL (normal: 0.65-1.09 mg/dL); C-reactive protein: 0.1 mg/dL (normal: 0-0.3 mg/dL); prothrombin time: 100% (normal: 80%-120 %); thyroid-stimulating hormone: 0.5 μIU/mL (normal: 0.34-4.04 μIU/mL); triiodothyronine: 2.98 pg/dL (normal: 2.36-5.00 pg/dL); and thyroxin: 1.18 ng/dL (normal: 0.88-1.67 ng/dL). The serological tests for autoantibodies generated weakly positive results (1:40) for anti-nuclear antibodies, and negative results for anti-smooth muscle and anti-mitochondrial antibodies. Negative results were obtained from the tests for the hepatitis B virus surface antigen, the hepatitis C virus antibody, rapid plasma reagin, the anti-Epstein-Barr virus immunoglobulin M (IgM) antibody, and the anti-cytomegalovirus IgM antibody, and from the *Treponema pallidum* hemagglutination test. The IgG level was within the normal range at 1415 mg/dL (normal: 800-1600 mg/dL) and the IgM level was within the normal range at 100 mg/dL (normal: 60-250 mg/dL). The IgE level was elevated at 1333 mg/dL (normal: < 250 mg/dL). Chest radiography did not reveal any abnormalities. No morphological changes were evident following abdominal ultrasonography, magnetic re-

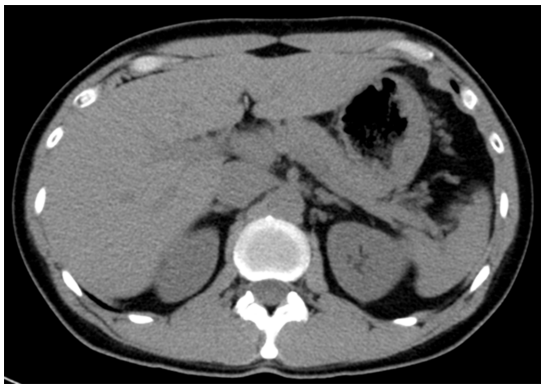

Figure 1 Abdominal computed tomography scans did not show any morphological changes on admission.

sonance cholangiopancreatography, and abdominal computed tomography (Figure 1).

When the patient was hospitalized, we considered viral, alcohol-induced, autoimmune, drug-induced, biliary tract, and thyroid function-based diseases as frequent causes of acute liver dysfunction for the differential diagnosis, and, based on the patient's medical history, and the serological and imaging results, we considered that alcohol and DILI were highly likely causes of the patient's acute liver dysfunction. However, the patient had stopped drinking during the past year, so we thought that there was a high likelihood of DILI. Therefore, we began conservative treatment with a small amount of extracellular fluid replacement.

Figure 2 presents the patient's symptoms, laboratory test results, and treatment over the entire course. The liver transaminase and bilirubin levels decreased rapidly after admission, and the maximum levels were as follows: ALT: 950 IU/L on POD 15; AST: 470 IU/L on POD 17; and total bilirubin: 2.1 mg/dL on POD 17. The ALP and gamma-glutamyl transpeptidase levels showed consistent downward trends from POD 7. The patient's symptoms disappeared on POD 10, and no new symptoms appeared after hospitalization. We performed a liver biopsy on POD 25, and a drug-induced lymphocyte stimulation test (DLST) of the components of the ALTA injection and lidocaine on POD 28. The patient was discharged on POD 29, and he was managed as an outpatient without any prescriptions. At the time of the patient's first outpatient appointment on POD 35, his liver transaminase and bilirubin levels had declined and were almost within the normal ranges.

Figure 3 presents hematoxylin and eosin (H and E)-stained sections of the liver biopsy performed on POD 25. The H and E staining showed that the basic structure of the liver had been maintained without any hepatocyte dropout or disruption, and that the bile duct had not been disrupted. At a higher magnification, the H and E staining showed that the parenchymal cells were partially dilated and that a mild inflammatory cell infiltration was present in the area of the central vein. Neither eosinophil nor plasma cell infiltrations were detected within the liver tissue. Bile plugs were found at multiple sites within the parenchyma and sinusoids. Masson staining did not show the presence of liver tissue fibrosis.

The lymphocyte proliferation activity levels, which were determined from the DLST performed on POD 28, were 676 counts per minute (cpm) for the control and 871 cpm for the ALTA with lidocaine. The stimulation index, which was calculated as the ALTA with lidocaine cpm divided by the control cpm, was 1.29 (normal in Japanese people: < 1.8).

DISCUSSION

This is the first case report that describes suspected severe acute liver injury caused by ALTA therapy for the treatment of internal hemorrhoids. Our discussion focusses on the cause of the acute liver injury, the components of the ALTA regimen used to treat the hemorrhoids in this case, the positions of the biopsy and the DLST in the diagnosis, and the properties of the administration route.

We primarily suspected DILI caused by the ALTA therapy in this case. The laboratory test results ruled out viral, autoimmune, and biliary tract diseases, and a thyroid gland malfunction as causes of the acute hepatic injury. Given that the IgG levels were normal and the autoantibody test results were negative or only weakly positive in this case, a comprehensive judgement based on a histopathological evaluation was necessary[7]. Accounting for the consumption of alcohol during the pathological assessments was necessary based on the patient's history. Interface hepatitis with lymphocytic/lymphoplasmacytic inflammatory activity, rosettes, and emperipolesis, which are characteristic of autoimmune hepatitis[8,9], and lobular neutrophilic infiltration, ballooning degeneration with apoptosis, and Mallory bodies, which are characteristic of alcoholic liver injury[10], were not observed in the pathological specimens from this case. Therefore, we diagnosed the patient as having DILI, which was a diagnosis of exclusion. This patient did not regularly use supplements or health foods, so the suspect drugs were loxoprofen and the ALTA with lidocaine injection. Loxoprofen is a non-steroidal anti-inflammatory agent, is one of the most frequently used drugs by patients with DILI[11], and it was used once by this patient. Given that it was used to treat the symptoms after their onset, we decided that loxoprofen did not cause this patient's liver injury. Therefore, we concluded that the ALTA with lidocaine injection caused the acute liver injury in this case.

ALTA is an improved formulation of Xiaozhiling®[12], and ALTA injections were approved by the Japanese Government in 2005 for the treatment of hemorrhoids. Traditionally, 5% phenol almond oil was injected into the submucosal layer to treat internal hemorrhoids, which reduced the blood flow to the thick hemorrhoidal vein by causing an inflammatory reaction in the surrounding area and promoting fibrosis, and the short-

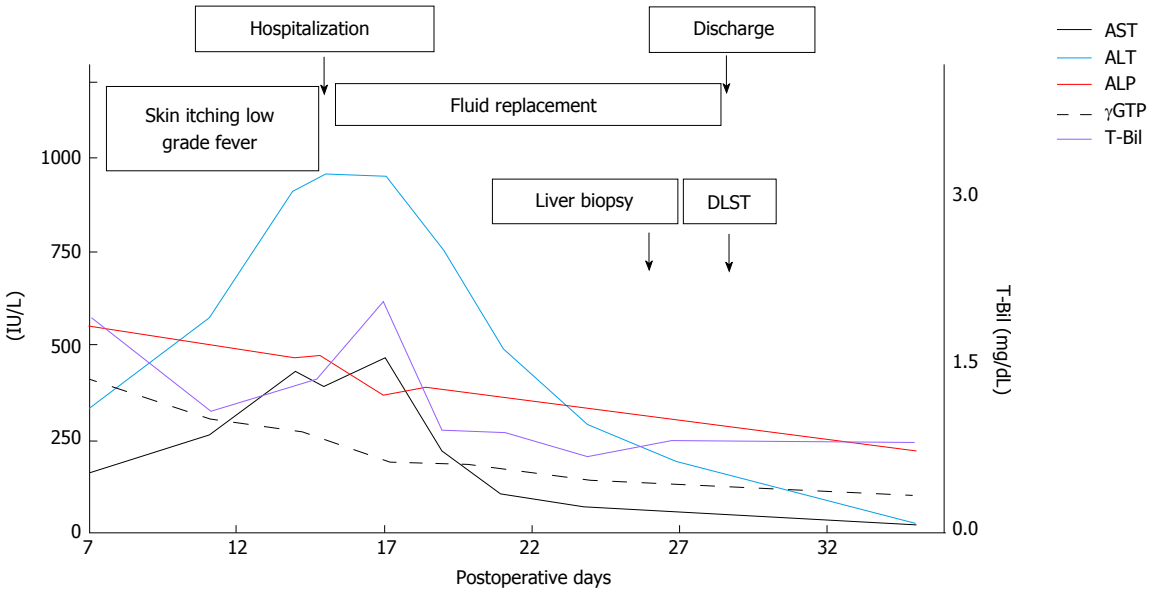

Figure 2 Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, and gamma-glutamyl transpeptidase levels in the patient at presentation and in response to treatment. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; γGTP: Gamma-glutamyl transpeptidase; T-Bil: Total bilirubin; DLST: Drug lymphocyte stimulation test.

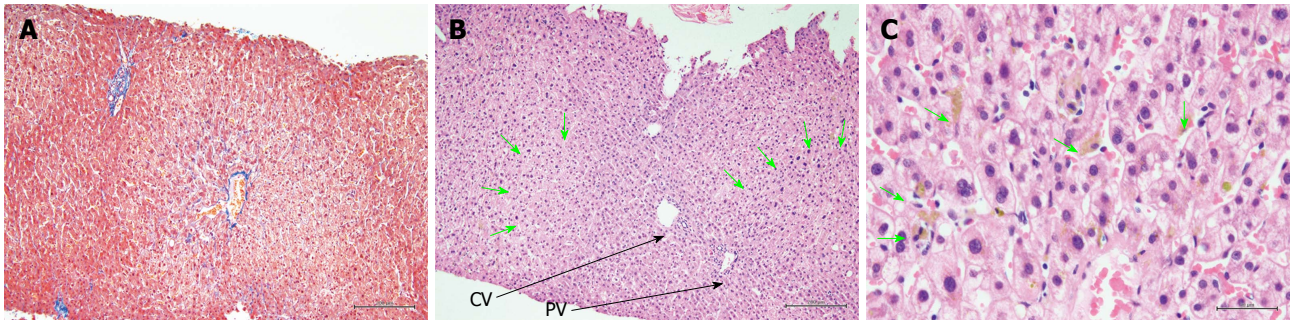

Figure 3 Micrographs of a liver biopsy specimen. A: Fibrosis around the central vein area and the fibrous expansion of the portal region were not detected (Masson staining × 40); B: Swelling of the parenchymal cells and a mild infiltration of inflammatory cells around the central vein area were evident (green arrows). Destruction of the bile duct had not occurred (hematoxylin-eosin staining × 40); C: Bile plugs were found in multiple sites within the hepatocytes and sinusoids (green arrows). (hematoxylin-eosin staining × 200). CV: Central vein; PV: Portal vein.

term one-year results were good[12]. However, the long-term outcomes from phenol almond oil injections were not positive in relation to curing hemorrhoids or regarding the treatment of internal Goligher grade Ⅲ and Ⅳ hemorrhoids. On the other hand, ALTA sclerotherapy is now widely used to treat internal hemorrhoids in Japan, because it performs well over one-to-five years, with cumulative successive rates at one, three, and five years of 95.9%, 89.3%, and 89.3%, respectively, for grade Ⅱ hemorrhoids, and 93.1%, 83.7%, and 78.2%, respectively, for grade Ⅲ hemorrhoids[13]. The treatment comprises a four-step process, and it is injected locally into the submucosa of the rectum and the anus *via* the lamina propria, and not into the vascular lumen[14]. Aluminum potassium sulfate causes inflammation inside the hemorrhoids followed by sclerosis, and tannic acid is an anti-inflammatory substance that prevents excessive inflammation. The complications associated with this treatment include bleeding (1.4%), pyrexia (1.4%), and rectal ulcers (1.4%)[15]; however, no case reports that describe acute liver injury following ALTA injections have been published.

The formulation used to treat the current case comprised aluminum potassium sulfate, tannic acid, and lidocaine. This patient had undergone local anesthesia involving the use of lidocaine in the past; hence, we considered that the likelihood of lidocaine involvement in the acute liver injury was low. Aluminum is less essential in living organisms, and it is generally considered to be less toxic. Clinically, aluminum encephalopathy can become a problem in dialysis patients, and the possibility of its accumulation in the central nervous system has been noted when renal excretion becomes insufficient[16]. Aluminum as aluminum chloride is used in an external preparation to manage hyperhidrosis, and sucralfate is a sucrose sulfate-aluminum complex that is used to treat peptic ulcers. Although a case

report has been published that describes liver injury following sucralfate treatment[17], it is very rare and the underlying mechanism is unknown. There are no data that describe the transferability of aluminum into the blood following its external application or oral administration. In a rat model, intraperitoneal administrations of aluminum increased the aluminum concentrations in the blood and caused morphological changes in the liver, but the mechanism that underlies these changes remains unclear[18,19]. Tannic acid is used to treat gastric bleeding, diarrhea, and ulcerative colitis[20,21]. Although a liver disorder has been described that involved an advanced degree of destruction in the region of the central vein following the administration of a barium enema containing tannic acid, the causative component was not identified. Lidocaine is used as an anesthetic or as an antiarrhythmic agent, and only two cases of hepatopathy caused by tocainide, which is a lidocaine analog that is used as an oral preparation for ventricular arrhythmias, have been published[22,23].

Some scoring systems have been used to diagnose DILI[24], and the Digestive Disease Week Japan 2004 scale and the Roussel-Uclaf Causality Assessment Method are considered particularly useful[25-27]. The current case scored six points using these scoring systems, which suggested that DILI was probable in this patient. DILI is classified in several ways based on its clinical presentation, histological findings, and the hepatotoxicity mechanism[28]. When the current case was admitted, the *R* value, defined as the ALT level/the upper limit of the normal (ULN) ALT level divided by the ALP level/the ULN ALP level, was 17.3, and given that it was $\geqslant$ 5, we diagnosed DILI with a hepatocellular injury pattern[27]. DILI mechanisms can be separated into the "intrinsic" type, which is dose-dependent and can be predicted, and the "idiosyncratic" type, which is dose-independent and unpredictable, and the idiosyncratic type can be further classified into the immune-mediated and metabolic types[29]. Immune-mediated DILI develops one-to-eight weeks after the administration of the drug, and T-cell-dependent liver injury is accompanied by a positive DLST, because the drug or its metabolite binds to protein to acquire antigenicity. Metabolic DILI develops against a background of the genetic variations in the enzymes that metabolize drugs, it develops after the long-term oral administration of drugs, and the DLST is often negative, because the mechanism is not mediated by T cells. A mechanism has not been described for any hepatic disorder caused by the drugs used to treat this case. The DLST was negative at one-to-two weeks after the administration of the drugs, and the elevated eosinophil and IgE levels suggested that the DILI was immune mediated. The mechanism underlying this patient's DILI cannot be specified, because there was a problem relating to the timing of the DLST that is described below; however, given that the DILI developed in association with specific agents, we think the mechanism was idiosyncratic hepatotoxicity in this case.

The histopathological patterns in DILI can be classified as the hepatocellular type, the cholestatic type, and the mixed type. A characteristic pathological feature of the hepatocellular type is cyclic necrosis in the central vein zone (zone 3), and biliary plugs in the bile capillaries are a characteristic pathological feature of the cholestatic type. The histological pattern is basically classified as cholestatic in the idiosyncratic clinical disease type[30], and the current case seemed to be consistent with the idiosyncratic type. A report that compares the clinical disease and the pathological types[31] explains that a pathological image can exhibit the cholestatic type, even if the clinical disease type is hepatocellular, which corresponds to the current case. Although the scoring systems can diagnose DILI with high levels of sensitivity and specificity, they may produce high scores for autoimmune hepatitis[31]. Drugs may cause autoimmune hepatitis, therefore, careful evaluations must be undertaken[32]. We confirmed the liver biopsy findings while taking this fact into account.

A DLST determines the proliferative response by lymphocytes to the addition of drugs. Based on the observed responses of sensitized T cells, positive DLST results tend to be generated by immune-mediated DILI[33]. Currently, DLST is positioned as one item that contributes to the scoring systems used to diagnose DILI, because it is only positive in association with some types of DILI[34], and because of the diversification of substances that have been determined to cause DILI in recent years. The number of reports describing DILI caused by supplements and health foods has increased in recent years[1-3], and the causal agents include garlic, egg yolk, turmeric, glucosamine, and noni juice[35]. Moreover, traditional Chinese medicine is thought to generate false-positive results from DLSTs[36], and their interpretation is often difficult. The DLST positive response rate for DILI overall is 45.7%[1].

On the other hand, Popple *et al*[37] and Pichler and Tilch[38] suggested that DLSTs should be undertaken following remission, that is, at about four-to-eight weeks, as the tests may generate false-negative results, because of the strong activation of regulatory T cells or the uneven distribution of memory T cells caused by ongoing hypersensitivity reactions[37,38]. Because the patient consent is not obtained, a second DLST cannot be conducted on samples from that patient. However, if the test appears to be positive, it may be possible to determine whether or not the mechanism is immune mediated.

DILI usually occurs following the oral or intravenous administration of drugs or when they are administered as suppositories. In relation to intravesical instillations, 18 cases (0.7%) of pneumonia or hepatitis occurred following Bacillus Calmette-Guérin therapy for bladder cancer[4]. Regarding the subcutaneous administration of drugs, insulin and interferon injections can cause hepatic injury[5,6]. This is the first report that describes a case of hepatic injury caused by the administration of

drugs to the rectal submucosa. The median rectal vein and the inferior rectal vein are present in the middle-to-lower regions of the rectum, and it is possible that the administered drugs entered the systemic circulation from the submucosal blood vessels and caused DILI in this patient.

We have described the first case of DILI following an ALTA injection to treat internal hemorrhoids. The accumulation and analysis of further cases are necessary to clarify the mechanism, frequency, and the clinical variations of this disease type.

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COMMENTS

Case characteristics

A Japanese male patient presented with fever, dark-colored urine, and itchy skin.

Clinical diagnosis

The authors diagnosed acute liver disorder after aluminum potassium sulfate and tannic acid (ALTA) therapy.

Differential diagnosis

The diseases to be considered are liver injury induced by autoimmune hepatitis, alcohol, and other drugs, which can be estimated by liver biopsy.

Laboratory diagnosis

The patient had elevated liver enzymes, with an aspartate aminotransferase level of 432 IU/L, an alanine aminotransferase level of 911 IU/L, an alkaline phosphatase level of 473 IU/L, and a total bilirubin level of 1.3 mg/dL.

Imaging diagnosis

Computed tomography scan showed no morphological changes.

Pathological diagnosis

Histological examination showed liver injury of the cholestatic type characterized by bile plugs within the parenchyma and sinusoids, with mild inflammation of hepatocytes around the area of the central vein.

Treatment

The patient received conservative treatment, with a small amount of extracellular fluid replacement.

Related reports

There is no other case report of acute liver injury associated with ALTA therapy. There are reports of a case of liver injury accompanying barium enema containing tannic acid and of two cases of liver injury accompanying lidocaine, but neither pathophysiology nor pathological features have been elucidated.

Term explanation

ALTA sclerotherapy is now widely used to treat internal hemorrhoids in Japan and consists of aluminum potassium sulfate, tannic acid and lidocaine.

Experiences and lessons

This is the first case of drug-induced liver injury following an ALTA injection to treat internal hemorrhoids and discusses the clinical and pathological features. The accumulation and analysis of further cases are necessary to clarify the mechanism, frequency and clinical variations of this disease type.

Peer-review

This case report is very informative and well written. It should be worth sharing to people in the field as well as to the general public.

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