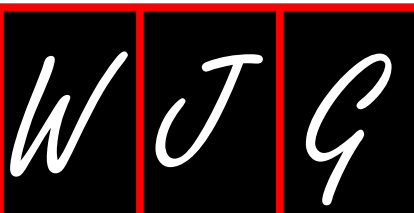


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Follow-up after curative resection for gastric cancer: Is it time to tailor it?

Paolo Aurello, Niccolò Petrucciani, Laura Antolino, Diego Giulitti, Francesco D'Angelo, Giovanni Ramacciato

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Abstract

There is still no consensus on the follow-up frequency and regimen after curative resection for gastric cancer. Moreover, controversy exists regarding the utility of follow-up in improving survival, and the recommendations of experts and societies vary considerably. The main reason to establish surveillance programs is to diagnose tumor recurrence or metachronous cancers early and to thereby provide prompt treatment and prolong survival. In the setting of gastric malignancies, other reasons have been put forth: (1) the detection of adverse effects of a previous surgery, such as malnutrition or digestive sequelae; (2) the collection of data; and (3) the identification of psychological and/or social problems and provision of appropriate support to the patients. No randomized controlled trials on the role of follow-up after curative resection of gastric carcinoma have been published. Herein, the primary retrospective series and systematic reviews on this subject are analyzed and discussed. Furthermore, the guidelines from international and national scientific societies are discussed. Follow-up is recommended by the majority of institutions; however, there is no real evidence that follow-up can improve long-term survival rates. Several studies have demonstrated that it is possible to stratify patients submitted to curative gastrectomy into different classes according to the risk of recurrence. Furthermore, promising studies have identified several molecular markers that are related to the risk of relapse and to prognosis. Based on these premises, a promising strategy will be to tailor follow-up in relation to the patient and tumor characteristics, molecular marker status, and individual risk of recurrence.

Key words: Gastric cancer; Follow-up; Surgery; Gastric carcinoma; Chemotherapy; Surveillance; Recurrence; Markers; Imaging

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Core tip: There is still no consensus on the utility, frequency and regimen of follow-up after curative resection for gastric cancer. Surveillance programs may allow the following: (1) the early diagnosis of recurrence; (2) the detection of adverse effects of a previous surgery; (3) the collection of data; and (4) the detection of psychological and social problems. This editorial discusses the main studies, systematic reviews and guidelines on this subject. Several studies have demonstrated that patients may be stratified according to the risk of recurrence. A promising strategy will be to tailor follow-up in relation to the patient and tumor characteristics, molecular marker status, and individual risk of recurrence.

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INTRODUCTION

The main goal of follow-up programs after curative surgery is the early detection of cancer recurrence, such that rapid and efficacious treatment may be administered and long-term survival may be improved^[1]. Other objectives of follow-up are to identify treatment-related complications and to collect data concerning cancer history and treatment outcomes^[2]. International guidelines recommend postoperative follow-up for the majority of cancers, even if the role of follow-up in improving overall survival has not been demonstrated for all types of tumors.

According to the GLOBOCAN data, gastric cancer represents the fifth most common malignancy in the world, with 952000 new cases estimated to have occurred in 2012. Stomach cancer is the third leading cause of cancer death in both sexes worldwide, with an estimated 723000 deaths in 2012^[3]. In the United States, there were an estimated 26370 cases in 2016, with 10730 deaths^[4].

Recurrence occurs within the first 3 years in the majority of cases, and fewer than 10% of recurrences occur after 5 years^[1,5,6]. Survival after recurrence is poor, and in the majority of recurrence cases, potentially curative treatments are not possible^[1,7].

There is still no consensus on the frequency and regimen of follow-up after curative resection. Moreover,

controversy exists regarding the utility of follow-up in improving survival, and the recommendations of experts and societies vary considerably. The problem of cost-effectiveness has also been raised^[8]. The objective of this editorial is to review the current literature concerning follow-up after curative resection for cancer. We discuss the rationale of follow-up, the methods of follow-up, including clinical examination, biochemical analyses and radiological tools to detect gastric cancer recurrence, the main articles evaluating the role of follow-up in improving overall survival and the current guidelines of the most important international societies. Furthermore, the role of molecular analysis in predicting gastric cancer recurrence is analyzed, and future directions of research are suggested.

RATIONALE OF FOLLOW-UP

The main reason to establish surveillance programs after curative gastrectomy is to diagnose tumor recurrence or metachronous cancers early to thereby provide prompt treatment and prolong patient survival. In the setting of gastric malignancies, other reasons have been advocated, including (1) the detection of adverse effects of a previous surgery, such as vitamin and iron depletion or malnutrition or digestive sequelae^[9]; (2) the collection of data to evaluate the efficacy and outcomes of treatments; and (3) the detection of psychological and/or social problems subsequent to the disease and the provision of appropriate support to the patients^[10].

Gastric cancer recurrence may be classified into five patterns^[11-14]. The first is the locoregional pattern, defined as tumor relapse at the resection margin (proximal, including the esophagus or the proximal stomach, and distal, including the duodenal stump) or in the adjacent tissue of the surgical bed. The second is the nodal pattern, which is relapse within the regional and distant lymph nodes, including the retropancreatic, retrocrural and para-aortic nodes. The third pattern is peritoneal recurrence, with intraperitoneal tumor spread. The fourth pattern is hematogenous relapse, defined as metastatic lesions in distant organs (*e.g.*, liver, lung, or bones). Finally, some tumors have a mixed pattern of recurrence, including different synchronous routes of relapse.

The majority of patients relapse within the first 3 years, with a median time to recurrence ranging from 14 to 29 mo in recent series^[13-17]. After laparoscopic gastrectomy, similar times to recurrence and patterns have been demonstrated^[16,17].

Among clinicopathological prognostic factors, the Italian Research Group for Gastric Cancer identified nodal status, nodal ratio, and stage, and proposed a prognostic score that was able to predict the likelihood of recurrence for high-risk patients better than the TNM stage^[14].

The majority of gastric cancer recurrences are

not surgically curable. The majority of patients with liver metastases are not candidates for resection, and treatment for peritoneal carcinosis is experimental^[18,19]. Chemotherapy is the primary treatment for recurrent gastric carcinoma and may prolong survival and improve the quality of life. The median survival with chemotherapy is poor, ranging from 6 to 13 mo^[13-21].

Follow-up also plays a role in evaluating and treating the long-term adverse effects of gastrectomy^[1]. Proper follow-up allows the detection of digestive problems, such as dyspepsia, nausea, vomiting, early satiety, reflux, and anorexia, which occur in approximately 30% of patients. Furthermore, postgastrectomy syndromes, such as dumping syndrome, bile reflux, Roux-en-Y stasis syndrome and afferent and efferent loop syndromes, can be identified and treated^[22]. Malabsorption may cause iron deficiency anemia (approximately 30% of patients), megaloblastic anemia due to vitamin B12 deficiency, and bone diseases (osteopenia, osteoporosis)^[23,24].

METHODS OF FOLLOW-UP

Follow-up is based on the case history, clinical examination, blood tests, including tumor marker assays, imaging and endoscopy.

Tumor markers

The level of tumor markers is commonly used during follow-up because the assay is simple and inexpensive to perform^[25,26]. However, the specificity and sensitivity are low. Markers are less useful in patients without an increase in the preoperative level of a tumor marker. As reported by Takahashi *et al.*^[27] 54.7% of patients had a first-time increase in carcinoembryonic antigen (CEA) levels at recurrence, and 40% had a first-time increase in carbohydrate antigen 19-9 (CA19-9) at recurrence. In that study, the sensitivity of the two markers for recurrence was 85.0%^[27]. In contrast, when the preoperative level of CEA was elevated, the CEA level increased again at the time of recurrence in more than 90% of patients. Additionally, the CA19-9 level increased again at recurrence in more than 90% of patients who had high preoperative levels^[27]. In a study by Kim *et al.*^[28] on 1117 patients, CEA and/or CA72-4 were found to be independent risk factors for recurrence. According to the findings of Choi *et al.*^[29], increased CEA was more frequent in cases of liver recurrence, whereas increased CA 19-9 was more frequent in cases of peritoneal recurrence. Furthermore, it has been reported that the elevation of tumor markers occurs earlier than imaging abnormalities (approximately 2-5 mo)^[27,30].

Endoscopic surveillance

Endoscopic surveillance aims to detect intraluminal recurrence, metachronous tumoral lesions, pre-cancerous gastric stump diseases and anastomotic

strictures. The role of endoscopic surveillance is fundamental after the endoscopic treatment of early gastric cancer. Hahn and colleagues reviewed a series of 1347 patients who underwent curative endoscopic submucosal dissection (ESD) for early gastric cancer^[31]. These authors found an annual incidence of recurrence of 0.84% at the previous endoscopic resection site and 2.48% at other sites in the stomach. Surveillance endoscopy at an interval of ≤ 12 mo permitted the detection of lesions at an earlier stage^[31]. For these reasons, the majority of authors recommend endoscopy exams with short time intervals after the endoscopic resection of early stage gastric cancer^[32]. The incidence of gastric stump recurrence or metachronous cancer ranges between 1% and 7% depending on the study and geographical area^[33]. The time to the development of tumoral lesions in the remnant stomach is variable, ranging from months to decades^[34]. Endoscopic surveillance may allow early diagnosis and potentially curative treatment, with survival advantages for resectable patients^[35].

Imaging modalities

Computed tomography (CT) represents the most-used imaging technique for the surveillance of patients with previous gastric cancer. However, only a few studies have evaluated the ability of CT to detect recurrence after gastrectomy. The accuracy is reported to be approximately 60%-70%, with a low predictive value in cases of peritoneal recurrence^[36]. Many reports have investigated the role of positron emission tomography (PET)/CT^[37-40]. One meta-analysis^[37] that included fourteen studies (828 patients) has shown the usefulness of PET/CT in this setting: the pooled sensitivity, specificity, positive likelihood ratio, negative likelihood ratio and diagnostic odds ratio of 18F-fluoro-2-deoxy-D-glucose PET (18F-FDG PET) or PET/CT were 0.85 (95%CI: 0.75-0.92), 0.78 (95%CI: 0.72-0.84), 3.9 (95%CI: 2.9-5.4), 0.19 (95%CI: 0.11-0.34), and 21 (95%CI: 9-47), respectively. On a per-lesion basis, the pooled sensitivity was 0.75 (95%CI: 0.61-0.86). The area under the SROC curve of PET/CT per patient was 0.86.

One study compared CT and PET/CT during the follow-up of 139 patients, 28 of whom had recurrence^[41]. The authors did not find statistically significant differences between the sensitivity, specificity and accuracy of PET/CT (53.6%, 84.7%, and 78.4%, respectively) and those of CT (64.3%, 86.5%, and 82.0%, respectively) for detecting tumor recurrence, except in the detection of peritoneal carcinomatosis. Among 36 recurrent lesions, 8 lesions (22.2%) were detected only with PET/CT, and 10 lesions (27.8%) were detected only with CT. PET/CT detected secondary malignancies in 8 patients. According to this study, additional PET/CT or CT scans may improve the detection rate of tumor recurrence and provide other

critical information, such as an unexpected secondary malignancy. Integrated PET/CT has an accuracy ranging from 75% to 97%. However, with both CT and PET, the accuracy is lower for detecting peritoneal disease. Inoue *et al.*^[42] have proposed the use of second-look laparoscopy and have shown that this approach is feasible in patients at a high risk of relapse.

WHAT IS THE EVIDENCE?

No randomized controlled trials have been published on the role of follow-up after the curative resection of gastric carcinoma, and there is no proven evidence that follow-up can provide a survival advantage due to the early identification and treatment of tumor recurrence.

Cardoso *et al.*^[43] published a systematic review on this topic in 2012. In this study, the authors searched the literature from 1999 to 2009 and selected five retrospective studies that reported data on follow-up after gastric resection and included a total of 810 patients^[44-48]. Follow-up was performed using several modalities, including the following: history and physical examination, abdominal ultrasonography, CT, endoscopy, endoscopic ultrasound (EUS), chest radiography, blood counts, chemistry profile, tumor marker assays, barium enema, and bone scintigraphy. Only one study reported that DFS was significantly shorter in the intensive follow-up group than in the standard follow-up group (11.5 mo vs 19.2 mo, $P = 0.02$)^[44]. In the included studies, survival post-recurrence was significantly longer in the asymptomatic than in the symptomatic patients. However, no significant differences were found concerning overall survival according to the two studies reporting the information. Therefore, the authors of the systematic review concluded that there is no evidence that follow-up may provide any survival benefit after gastric cancer resection and that further prospective studies are required to determine whether a subgroup of patients may benefit from more intensive follow-up.

Two studies included in the review reported data on overall survival according to follow-up intensity. The first, a retrospective study by Tan *et al.*^[44] was published in 2007 and included the data from 102 patients submitted to curative gastrectomy in Singapore from 1995 to 1998. Forty-nine patients were intensively followed-up, whereas 53 received the standard follow-up. Intensive follow-up was defined as employing regular physical examinations, serum tumor marker assays and performing CT scans more than once every 12 mo. The preoperative characteristics of the patients in the two groups were similar. Neoadjuvant therapy was not administered, and adjuvant therapy was administered to 36 patients, 30 in the intensive follow-up group and 6 in the standard group ($P < 0.01$). Recurrences were detected significantly earlier in the intensive follow-up group. However, no significant difference in overall survival was found between

the two groups (43% vs 34% at 5 years for intensive and standard follow-up, respectively, $P = 0.36$). The second study was published in 2003 by Kodera *et al.*^[48] from Nagoya. This retrospective study included 211 patients with relapsing gastric cancer, treated between 1985 and 1996. In this analysis, patients were divided into groups with and without cancer-related symptoms at the time the recurrent disease was diagnosed. Survival was analyzed in these two groups. The follow-up program consisted of an interim history, physical examinations, blood tests and tumor marker assays, repeated every 3 mo for the first postoperative year and every 6 mo thereafter for at least 5 years. Either abdominal ultrasonography or CT was performed every 6 mo, as was chest radiography. Endoscopy was performed annually to screen for cancer in the gastric remnant, beginning 1 to 1.5 years after surgery. In addition to this regular follow-up, patients consulted their doctors whenever the patients had clinical symptoms. Eighty-eight (45%) patients were asymptomatic at the time of diagnosis of a recurrence, whereas 109 had symptoms and consulted the physician or reported the symptoms during a scheduled follow-up. A greater proportion of the patients with asymptomatic recurrence was treated with chemotherapy and underwent resection of metastatic lesions (although these data were not statistically significant). Survival after the diagnosis of recurrent disease was better when the recurrence was detected at an asymptomatic stage ($P = 0.0001$). Longer survival in patients with asymptomatic recurrences was observed even in patients whose recurrences were not treated with chemotherapy. However, because symptomatic recurrences were diagnosed later after surgery than were asymptomatic recurrences, the overall survival after curative resection of the primary tumor was not significantly affected by the presence or absence of symptoms at the time of cancer recurrence. The authors conclude that the early detection of asymptomatic gastric cancer recurrence did not improve the overall survival of patients with recurrence after curative resection. Until the development of a more effective treatment for this disease, close follow-up may offer no survival benefit. Both of these studies and, consequently, the systematic review (the conclusions of which are based on these two articles) have several limitations: the retrospective nature of the studies, the period of treatment (prior to 2000), the protocol and administration of perioperative treatment, and the obsolescence of imaging modalities to detect recurrence.

The most recent studies report interesting results. Park *et al.*^[49] in 2016 reviewed the clinical data of 376 patients with intra-abdominal recurrence after curative gastrectomy. These patients were classified according to the surveillance interval. A total of 101 patients (26.9%) composed the 3 mo or less group, while 137 (36.4%) composed the 3- to 6-mo group, and 108 (28.7%) composed the 6- to 12-mo group. The

remaining 30 patients (8%) with a surveillance interval longer than 12 mo were excluded. The 3 mo or less group and the 3- to 6-mo group had higher proportions of stage 3 cancers and early recurrences within 24 mo after gastrectomy than did the 6- to 12-mo group [stage 3 cancer: 87.1% (3 mo) vs 81.0% (3-6 mo) vs 60.2% (6-12 mo), $P < 0.001$]. The recurrence rates within 24 mo after gastrectomy were 86.1% (3 mo) vs 78.8% (3-6 mo) vs 57.4% (6-12 mo) ($P < 0.001$). The proportion of patients with symptoms at the time of recurrence did not differ among the three groups ($P = 0.122$). The post-recurrence survival did not differ among the three groups ($P = 0.057$). According to the authors, although the detection of recurrence before symptoms enabled the prolongation of both post-recurrence survival and overall survival, shortening the surveillance interval to less than 6 mo was not useful in improving survival.

In 2016, Fujiya *et al*^[50] retrospectively analyzed 218 patients with recurrent gastric cancer after curative gastrectomy. The patients were divided into an asymptomatic group ($n = 117$) and a symptomatic group ($n = 101$). Peritoneal recurrence was less frequent in the asymptomatic group (22.2%) than in the symptomatic group (62.4%).

The median time to recurrence was shorter in the asymptomatic group than in the symptomatic group (12.7 mo vs 18.9 mo, $P < 0.001$), and the median survival time after recurrence was longer in the asymptomatic group than in the symptomatic group (18.7 mo vs 7.5 mo, respectively, $P < 0.001$). The median overall survival time after gastrectomy was not significantly different between the groups (30.1 mo for asymptomatic recurrence vs 30.0 mo for symptomatic recurrence, $P = 0.132$). In a multivariate analysis, the overall survival after gastrectomy was not significantly different between the groups (HR = 0.86, $P = 0.402$). Among the patients with a nonperitoneal recurrence, the time to recurrence was similar between the asymptomatic and symptomatic groups (12.2 mo vs 15.2 mo, $P = 0.062$), but the survival time after recurrence was significantly longer in the asymptomatic group than in the symptomatic group (20.8 mo vs 7.5 mo, $P < 0.001$). The overall survival time after gastrectomy was significantly greater in asymptomatic patients with nonperitoneal recurrence than in symptomatic patients (35.9 mo vs 24.0 mo, $P = 0.039$).

According to this study, the detection of nonperitoneal recurrence before the appearance of symptoms may provide a survival benefit, and regular follow-up is recommended.

Lee *et al*^[51] analyzed the data of 192 cancer patients with gastric cancer recurrence after curative resection. Of these patients, 126 (65.6%) had asymptomatic recurrences. The patients were divided into two groups: asymptomatic and symptomatic recurrence. The median recurrence-free survival did not differ between the two groups ($P = 0.507$), whereas the median post-

recurrence ($P < 0.001$) and overall survival times ($P = 0.022$) were longer in the asymptomatic group, suggesting the utility of follow-up programs.

In 2005, Marrelli *et al*^[52] proposed a scoring system to predict recurrence in patients with previous gastric cancer, with the aim of identifying the categories of patients at higher risk. These authors demonstrated that the risk of recurrence increased remarkably with the score values; the risk of recurrence was only 5% in patients with a score below 10 and rose to 95.4% in patients with a score of 91 to 100. Their model correctly predicted recurrence in 227 of 272 patients (sensitivity, 83.5%), whereas the absence of recurrence was correctly predicted in 214 of 264 patients (specificity, 81.1%); the overall accuracy was 82.2%. This scoring system was further validated with a group of 635 patients from 5 Italian Research Group for Gastric Cancer (GIRCG) centers^[53]. In the validation group, the observed recurrence rates ranged from 5% to 92% in the different scoring strata. The area under the receiver operating characteristic curve was 0.889 (95%CI: 0.864-0.914; $P < 0.001$), indicating a high discrimination value of the score for recurrence. A good calibration was observed by comparing the predicted risk with the actual risk of recurrence. With a score cut-off value of 50, the sensitivity, specificity, and overall accuracy were 74%, 86%, and 81%, respectively. An inverse correlation between the time to recurrence and score level was also estimated ($R^2 = 0.119$, $P < 0.001$). In addition, Barchi *et al*^[14] validated this scoring system on 185 patients with gastric cancer who underwent an operation with the intention of a cure, demonstrating that the GIRCG's prognostic score was more accurate than the TNM system in predicting recurrence mainly for high-risk patients, whereas the score did not have the same effectiveness for low-risk patients and overestimated the chance of recurrence even for disease-free patients.

A final study from Baiocchi *et al*^[54] included 814 patients with recurrent cancer. Ninety-four percent had recurrence within 2 years, and 98% had recurrence within 3 years. In this study, thoracoabdominal CT and 18F-FDG PET detected more than 90% of recurrences, whereas abdominal ultrasound detected 70%, and tumor marker assays detected 40%. Less than < 10% of tumor relapses were identified by physical examination, chest X-ray, and upper gastrointestinal (GI) endoscopy. Twenty-six percent of patients with recurrence were treated, but only 3.2% were treated with the intention of a cure. On the basis of these results, the authors affirmed that follow-up should be focused on the first 3 years and based mainly on thoracoabdominal CT and 18F-FDG PET.

GUIDELINES

Different protocols have been proposed by scientific societies and groups. The lack of strong evidence is

responsible for the heterogeneity of the guidelines.

NCCN

The NCCN recommends a systematic follow-up for all patients. A history and physical examination should be undertaken every 3 to 6 mo for 1 to 2 years, every 6 to 12 mo for 3 to 5 years, and annually thereafter^[55]. However, a CBC, a chemistry profile, radiologic imaging, or upper GI endoscopy should be performed if clinically indicated. Monitoring and treatment of vitamin B12 and iron deficiency is recommended in surgically resected patients.

ESMO

ESMO recommends regular follow-up for the investigation and treatment of symptoms related to previous treatments, for psychological support and for the early detection of recurrence^[56]. A follow-up tailored to the individual patient and the stage of the disease is recommended. Furthermore, ESMO recommends dietary support for patients on either a radical treatment or palliative pathway with reference to vitamin and mineral deficiencies. If relapse or disease progression is suspected, then a clinical history, a physical examination and directed blood tests should be conducted.

Association of upper gastrointestinal surgeons of Great Britain and Ireland, the British Society of Gastroenterology and the British Association of Surgical Oncology

A regular review of patients following the treatment of esophageal and gastric cancer is recommended for symptom management, supportive care and surveillance. These organizations assert that although regular review may identify an early recurrence, there is no evidence for specific investigations or that such an approach can affect OS^[57].

GIRCG

The GIRCG recommends routine follow-up for all patients for the following reasons: oncological (detection and management of cancer recurrence), gastroenterological (endoscopic surveillance and management of postgastrectomy symptoms), research (the collection of data on treatment toxicity, time to and site of recurrence, and survival and cost-benefit analyses), and pastoral (psychological and emotional support)^[58]. The nutritional sequelae of gastrectomy, including, but not limited to, adequate vitamin B12 and iron, and calcium replacement should be investigated. Follow-up should be offered by members of the multidisciplinary team who managed the initial diagnosis, staging, and treatment, and modalities should be tailored to the individual patient, to the stage of their disease, and to the treatment options available. Cross-sectional imaging is recommended to detect asymptomatic recurrence. Upper GI endoscopy may be used to detect

local recurrence or metachronous primary GC in patients who have undergone a subtotal gastrectomy. Routine screening for the asymptomatic recurrence of GC may be discontinued after 5 years, as recurrence beyond that interval is infrequent.

French guidelines: HAS

The French Haute Autorite de Sante recommends follow-up to detect recurrent or metachronous cancer, symptoms and adverse effects of treatment, to control the quality of life and to provide supportive care and social assistance^[59]. Follow-up is recommended for at least for 5 years by the multidisciplinary team that cared for the patient and, after this period, by the general practitioner. Clinical examinations should be performed every 3–6 mo during the first 1–3 years, every 6 mo for the first 5 years, and then, after 5 years, performed once a year. Abdominal ultrasound or thoracoabdominal CT should be performed every 6 mo for 3 years and then every year for 2 years. GI endoscopy should be performed in the case of remnant stomach and precancerous lesions or *Helicobacter pylori* infections on initial biopsies (frequency of endoscopy is not specified). Blood tests for tumor markers are not recommended.

The American Society of Clinical Oncology and the Japanese Gastric Cancer Association have not provided guidelines for the follow-up of gastric cancer.

MOLECULAR BIOMARKERS

Recent research on gastric cancer has focused on molecular biomarkers. Some markers have prognostic significance; other markers are potential targets for novel chemotherapeutics. In the setting of surveillance, some of the biomarkers may represent potential prognostic indicators, able to differentiate patients according to the risk of recurrence, and potential early markers of tumor relapse. Among the prognostic markers, the expression of vascular endothelial growth factor, a regulator of angiogenesis, has been associated with a poor prognosis by several authors^[60,61]. VEGF receptors are the targets of novel monoclonal antibodies that are currently being evaluated in clinical trials as potential treatments for advanced gastric cancer, such as ramucirumab and bevacizumab^[62,63].

Several microRNAs, such as miR-328^[64,65], have been found to be potential biomarkers for recurrence after curative resection, even though none are currently used in clinical practice.

The hypermethylation of various genes, such as cadherin 1, E-cadherin, hMHL1 and others, has been associated with the prognosis of gastric cancer^[66].

Among the cell cycle regulators, cyclin E is considered a significant regulatory factor and useful prognostic parameter in gastric cancers^[67]. Alterations in the *p53* gene are also associated with less favorable

prognoses in advanced gastric cancer^[68]. Apoptosis-related factors are associated with the prognosis of gastric cancer patients. Bcl-2 and Fas expression are related to the progression and prognosis of gastric carcinoma^[69,70]. The expression of markers such as VEGF or Bcl-2 is analyzed in the surgical specimen and may permit a better stratification of the risk of recurrence. Blood circulating biomarkers, on the other hand, may be quantified during follow-up with specific blood tests. We must point out that for novel biomarkers, the validation of a prognostic role to detect recurrence in a large cohort is still required.

Future studies will help to better characterize the role of each molecular factor and the ability of that marker to predict recurrence, allowing personalization of follow-up according to the individual risk of relapse.

CONCLUSION

Recommendations concerning the follow-up after curative resection of gastric cancer are heterogeneous, reflecting the absence of solid and high-grade evidence. The majority of international societies and authors recommend surveillance after gastrectomy on the basis of retrospective studies, although there is no consensus on how the follow-up should be conducted or how often the follow-ups should be scheduled. Surveillance permits the identification and treatment of postoperative digestive and nutritional problems, with a potential impact on quality of life. The ability of an early diagnosis to increase survival has not been demonstrated. Indeed, the outcomes after recurrence are poor according to the published series, even in cases of early diagnosis. The development of new agents based on new molecular targets may be a possible strategy to improve the survival of patients with recurrence. Several studies have demonstrated that it is possible to stratify patients submitted to curative gastrectomy into different classes according to the risk of recurrence. Prognostic scores may help clinicians to modulate the intensity and methods of surveillance.

In the future, better characterization of the molecular prognostic factors for gastric cancer will permit a better understanding of the biology of each resected gastric cancer and its risk of recurrence, enabling the establishment of a more individualized and tailored follow-up based on the tumor and patient characteristics. Tailoring the follow-up based on an accurate stratification of the recurrence risk may be a strategy to limit useless and expensive surveillance and to promptly identify recurrence patients who may benefit from treating the relapsing tumor, thereby improving survival outcomes.

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Cautiously using natural medicine to treat liver problems

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Abstract

Natural medicine is a system of therapy that administers natural agents and their derivatives to treat human diseases. This medicine has been used to treat many kinds of human diseases for thousands of years. The treatment protocols of natural medicine

are integrative in nature, and are required to utilize the most appropriate therapies to address the needs of the individual patient. Because of the relative convenience, safety and efficacy, natural medicine is now increasing worldwide. Naturopathic doctors are licensed in many areas of the world and regulated partly by law in these areas, which is quite different from various other forms of complementary and alternative medicine. Liver diseases, such as hepatitis, liver cirrhosis and liver carcinoma, are serious health problems worldwide. Nearly half of the natural agents used in treatment of liver diseases today are natural products and their derivatives. Although natural medicine is beneficial and safe, physicians should pay close attention to the potential side-effects of the naturopathic agents, which lead to liver injury, interstitial pneumonia and acute respiratory failure. Therefore, when administrating naturopathic protocols to patients for the treatment of liver diseases, we should try our best to prevent and avoid as much as possible the negative impact of these medicines. This article highlights the current practice and recommended improvement of natural medicines in the treatment of liver diseases and gives some specific examples to emphasize the prevention and management of adverse reactions of the natural agents and suggests that natural medicine should be cautiously used to treat liver problems.

Key words: Caution; Natural medicine; Herb; Natural nutraceutical; Liver disease; Adverse reactions

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Core tip: We discuss recent experiences in administering natural medicines to treat liver problems, and the adverse reactions of some natural medicines. Natural medicines provide benefits to patients with liver diseases, such as hepatitis, liver cirrhosis and liver cancer. Close attention should be paid to the prevention of side effects of the natural medicines, however, when liver diseases are treated to avoid as far as possible the negative impact of these medicines.

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INTRODUCTION

Natural medicine is a system of therapy in which the practice of medicine relies on natural agents and their derivatives to treat human diseases^[1,2]. It is also defined as a practice of diagnosis and prevention of human sickness^[3]. This medicine has been used for thousands of years in the treatment of many kinds of human diseases^[4-6]. Natural medicine contains many complementary and alternative methods in the prevention and treatment of diseases^[7]. Agents used in naturopathy must exist in nature, with no chemical additives, and have undergone no or very little processing, such as herbs, nutrients, diet supplements, etc. Both naive healthcare workers and the general public appreciate the use of natural medication^[8]. Unlike various other forms of complementary and alternative medicine, natural medicine is regulated in part by law. And, naturopathic doctors are now licensed in many regions of the North American continent. They have offered patient-centered care, more accessibly discounted care and more time for consulting, and more and more patients prefer to seek healthcare from them^[7,8]. Nutraceuticals are used as one of the naturopathic approaches to treat human diseases.

The liver is the largest internal digestive organ of our body, which is indispensable in many essential physiologic processes and vulnerable to be impaired by a wide variety of factors, such as toxins, micro-organisms, metabolic products, circulatory materials and neoformations^[9]. There are many types of liver diseases that result from different causes, such as viral hepatitis, alcohol abuse and non-alcoholic fatty liver disease^[10]. Recent research on functional foods such as nutraceuticals showed that many natural agents exert protective and therapeutic effects on the liver, and some of the other herbal and nutritional supplements also have mechanisms of action that make them beneficial to the liver^[11].

Naturopathy is now increasing worldwide and gives merit to the diseased liver in a natural manner, showing effective and curative action for several liver diseases^[2,3,7,12]. Knowledge about correct eating and lifestyle can be integrated into the everyday practice of managing liver problems^[11,13]. The aim of natural medicine is to treat the cause of a disease rather than just the symptoms, like allopathic medicine often does. Naturopathy also utilizes evidence-based medicine and modern scientific research to combine conventional and complementary and alternative medicine to treat

the diseases. Naturopathy follows some principles which underlie and are determined by its practice, such as relying on the healing power of nature, finding the root causes of diseases, treating the whole person, personalization, prevention, intent to do no harm, and the doctor serving as teacher for patient education^[3,14]. Most important of all, these medicines should be given only by physicians (*i.e.*, naturopathic doctors) who are licensed and certified, and who keep in mind good medical ethics and a sense of responsibility. If these rules are not followed, even those natural agents which are claimed to have hepatoprotective effects can also cause serious adverse drug reactions.

This article is essentially about natural medications, natural herbal medicines, food and natural nutraceuticals rather than prescribed medications of the liver, and highlights the current practice and recommended improvement of some natural medicines in the treatment of liver diseases; it also discusses the side effects of natural agents for liver disease and suggests that we should pay close attention to such and apply them cautiously.

HERBS FOR TREATMENT OF LIVER DISEASES

In both ancient western medicine and traditional oriental medicine, herbs have been used for centuries for the treatment of liver diseases^[4,6,9]. In the western world, Avicenna, who was one of the most famous physicians of the old era, authored *The Canon of Medicine*. In the Canon, Avicenna introduced many hepatoprotective plants and compound drugs, and some formulas that have the effect of treatment of liver diseases^[9].

Chinese herbal medicine is based on clinical experience and practice, and has been established over thousands of years^[4]. The formation of prescriptions with combination herbal formulas has experienced a long history. In a formula, the selection of individual herbs must be strictly guided by the theory of traditional Chinese medicine, and must highlight the overall concept of personalized treatment^[15]. Now, the efficacy and safety of a number of herbal products in the treatment of liver diseases have been demonstrated by correlated clinical studies^[9].

HERBAL FORMULAS FOR LIVER DISEASES

Sho-saiko-to, also known as Xiao-Chai-Hu-Tang and Minor Bupleurum Formula in Chinese, is commonly used to treat chronic hepatitis and is also effective for liver cirrhosis. This herbal medicine also has the effect of preventing development of hepatocellular carcinoma^[4,15]. It is the first herbal drug approved by the Food and Drug Administration (FDA)^[5]. Approximately

Table 1 Information about typical natural herbal formula "Sho-saiko-to" for treatment of liver diseases

Natural medicine	Ref.	Literature type	Effects and mechanisms/possible mechanisms	Adverse reactions
Sho-saiko-to	Shimizu ^[4] , 2000	Review	Preventive and therapeutic effects on experimental hepatic fibrosis; inhibition of oxidative stress in hepatocytes and hepatic stellate cells	Not mentioned
	Yamashiki <i>et al</i> ^[5] , 1992	Article	Efficacious against chronic liver diseases, malignant diseases and acute infectious diseases	Not mentioned
	Yamashiki <i>et al</i> ^[6] , 1999	Article	Suppressing liver cancer development; increasing interleukin-12 production and macrobiotic activity in liver cirrhosis patients	Not mentioned
	Lee <i>et al</i> ^[15] , 2011	Review	1 Protecting against development of hepatocellular carcinoma in cirrhotic patients 2 Reducing hepatocyte necrosis and enhancing liver function 3 Inhibiting hepatic fibrosis by reducing activation of stellate cells 4 Inhibiting hepatic lipid peroxidation, promoting matrix degradation, and suppressing extracellular matrix accumulation	Interstitial pneumonia and acute respiratory failure

**Figure 1** Leaves and blooming flower of silymarin.

1500 years ago, this herbal drug was introduced into Japan from China as an oriental classical medicine, and it used to be the most representative agent in Kampo medicine (traditional Japanese medicine). Here the word "Kampo" stands for "Han method", coming from a culture source in the Han era (from 206 before Christ to 220 Anno Domini) of China^[16].

Various clinical trials have shown that this herb drug can protect against the development of hepatocellular carcinoma in patients with cirrhosis, and some basic science studies have demonstrated that it also could enhance liver function and reduce hepatocyte necrosis. Although the therapeutic efficacy of Sho-saiko-to has been well studied and the formula is widely used in the treatment of liver diseases, the mechanism by which the formula protects hepatocytes against hepatic fibrosis and carcinoma remains unclear^[15]. In 1994, on the basis of large amount of studies, the Ministry of Health and Welfare of Japan approved the use of this Kampo in enhancing liver health and listed it in Japanese national formulary^[5,16]. Since then, Sho-saiko-to has become a widely used ethical drug in the treatment of hepatitis and liver cirrhosis in Japan^[6,16].

At one time, in this country, Sho-saiko-to had been widely prescribed to patients with all types of hepatitis

for long-term treatment^[15]. This led to the spectacular scenario that over 1.5 million hepatitis patients consumed this traditional Chinese herbal formula in Japan^[6,16]. Unfortunately, the long-term consumption of Sho-saiko-to, resulted in some severe adverse effects, such as interstitial pneumonia and acute respiratory failure. In March 1996, the media disclosed that in the past 2 years after this Kampo was approved in Japan, 88 hepatitis patients developed interstitial pneumonitis, including 10 deaths resulting from acute respiratory failure, due to taking of this drug^[15]. Information was urgently put out by the Japanese authorities, and the situation became known as the "Sho-saiko-to Event"^[17] (Table 1).

HERBAL MEDICINES FOR LIVER DISEASES

There are several herbal plants worth describing because they have significant effects of hepatoprotection as well as therapeutic activities (Table 2).

Silymarin

Silymarin (*Silybum marianum*), also called the milk thistle plant (Figure 1), is native to Asia and Southern Europe^[18,19]. The uses of *Silybum marianum* as a hepatoprotective agent to treat different types of liver and biliary disorders in European countries can be dated back to 2000 years ago^[11,18-20]. The oldest reported use of milk thistle was from the ancient Greeks and Romans, who used this plant as a treatment for liver ailments and snake bites. Then, during the Middle Ages, milk thistle was recommended to treat liver toxicity. Now, the German Commission E, the German governmental equivalent of the FDA, recommends it for treatment of toxin-induced liver problems and liver cirrhosis, and as a supportive treatment for chronic diseases of the liver^[19].

Although milk thistle has a considerably long history in the treatment of liver diseases, it was not

Table 2 Information of some natural herbal medicines for treatment of liver diseases

Natural medicines	Ref.	Literature type	Effects and mechanisms/possible mechanisms	Adverse reactions
Silymarin	Bahmani <i>et al</i> ^[18] , 2015	Review	Antioxidant; anti-inflammatory; cell permeability regulator and membrane stabilizer; stimulating liver regeneration and inhibiting deposition of collagen fibers	Mild gastrointestinal disorders
	Zhu <i>et al</i> ^[21] , 2016	Review	Anticancer by regulating cancer cells growth, proliferation, apoptosis, angiogenesis, such as hepatocellular carcinoma; antioxidant, immunomodulatory, anti-fibrotic, anti-proliferative, and antiviral activities	Not mentioned
	Csupor <i>et al</i> ^[22] , 2016	Review	Stimulating liver regeneration; antioxidant, anti-inflammatory and hepatoprotective; treatment of mushroom poisoning, hepatitis, cirrhosis and fibrosis of liver	Mild gastrointestinal and allergic reactions
Long pepper	Kumar <i>et al</i> ^[23] , 2011	Review	Hepatoprotective activity; treatment for chronic bronchitis, asthma, constipation, gonorrhea, paralysis of tongue, diarrhea, cholera, chronic malaria, viral hepatitis, stomachache, spleen diseases, cough, and tumors	Contraceptive activity; should be avoided during pregnancy and lactation
	Mansour <i>et al</i> ^[24] , 2009	Article	Increasing activity of transglutaminase; enhancing antioxidant activities in fibrotic liver; anti-tussive, anti-asthmatic, anti-allergic, antitubercular, antipyretic, hypotensive, hypoglycemic, antihelmentic and coronary vasodilatory	Not mentioned
Holy Basil	Jiang <i>et al</i> ^[25] , 2013	Review	Anti-hepatitis B virus activity <i>in vitro</i>	Not mentioned
	Lahon <i>et al</i> ^[26] , 2011	Article	Hepatoprotective activity and synergistic with silymarin; anti-inflammatory activity	Not mentioned
	Baliga <i>et al</i> ^[27] , 2013	Review	Anti-inflammatory, analgesic, antipyretic, antidiabetic, hepatoprotective, hypolipidemic, antistress, and immunomodulatory activities	Not mentioned but being considered non-toxic
	Singh <i>et al</i> ^[28] , 2007	Review	Anti-inflammatory, antipyretic, hypotensive, anticoagulant and immunomodulatory activities; chemopreventive and hypolipidemic activities	Not mentioned
	Prakash <i>et al</i> ^[30] , 2005	Review	Antifertility, anti-cancer, anti-diabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic, adaptogenic and diaphoretic actions	Not mentioned

**Figure 2** Fruits of long pepper.

until 1968 that silymarin (the medicinal ingredient of milk thistle) was found in milk thistle seed extract. Silymarin is a complex mixture of flavonolignans, including silybin, isosilybin, silychristin, silydianin, and taxifolin. It has been shown that its hepatoprotective effects are mainly contributed by its free radical scavenging property and antioxidant activity^[19]. Although its mechanisms of action are not yet fully understood, the hepatoprotective activity of silymarin has been shown to act in different ways, such as through antioxidant and anti-inflammatory activities, regulation of permeability of the cell wall, stabilization of cellular membranes, stimulation of liver regeneration

and anti-fibrotic, immunomodulatory, antiviral and anti-cancer activities^[18,21]. It is also used as a dietary supplement for food remedy, as the leaves of this plant have been used in salads and traditionally the fruit has been roasted as a coffee substitute^[18,19]. The preparations of milk thistle are safe, well tolerated, and few adverse side effects have been reported, except allergic reactions and mild gastrointestinal symptoms. Long-term use of this herb is considered to be safe and no incidence of significant abnormality has been reported^[18,19,22].

Long pepper

Long pepper (*Fructus Piperis Longi*; Figure 2) is another herb commonly used in Chinese medicine, and also has been used in the Ayurvedic system of medicine. This plant is low in cost, easy-to-obtain, and effective for various diseases, such as hepatotoxicity, inflammation, diabetes, obesity, depression and cancer^[23]. It has been demonstrated as capable of modulating liver function by enhancing antioxidant activities.

A study has shown that the extract of *Fructus Piperis Longi* treats liver diseases by reducing the activities of transglutaminase, such as serum aspartate amino transaminase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase, which are the main causes of the development of liver cirrhosis, and also by reducing bilirubin (total, direct



Figure 3 Holy basil as green vegetable.

and indirect) content, which leads to jaundice^[24]. The ethanol extract of long pepper was found to possess superior activity against hepatitis B virus *in vitro*^[25]. In rodents, this plant was assessed for its hepatoprotective effect against CCl₄-induced acute, chronic reversible and irreversible liver damage.

Furthermore, as a spice used in cooking, the plant is free from adverse effects, as no deaths have been reported with the use of high doses of the plant extracts. However, some studies reported that the plant had exerted contraceptive activity in experimental models; hence, use of this plant should be avoided during pregnancy and lactation^[23].

Holy basil

Ocimum sanctum L. (Labiatae; popularly known as "Tulsi" in Hindi and "Holy Basil" in English) is known as an herb which is an important hepatoprotective plant in various traditional and folk systems of medicine, including traditional Chinese medicine, Ayurveda, Greek, Roman, Siddha, and Unani. It is also commonly used as a green vegetable (Figure 3) in a delicious Thai cuisine, a stir-fry with rice, seafood or meat^[26-29]. This plant contains many important phytochemicals that present various activities of analgesia, anti-diabetes, anti-inflammation, antipyresis, antistress, hepatoprotection, hypolipidemia, immunomodulation and anti-neoplasia^[27,29,30]. For the treatment of liver diseases, some studies have shown that the combination use of holy basil and silymarin demonstrated synergistic hepatoprotective activity^[26]. No significant side effects of this plant have been reported, and it is considered non-toxic to humans and could be used as a chemopreventive and radioprotective agent^[27].

FOOD AND NUTRITIONAL SUPPLEMENTS FOR LIVER DISEASES

Several natural foods and drinks with nutritional supplements have been shown to be both safe and effective in the therapy of liver diseases^[10,11]. Natural foods, especially plant-based foods, have many advantages in

promoting liver health^[10,31]. So, traditionally, they have been usually used in the treatment and prevention of liver diseases throughout the world^[26]. The mechanisms of hepatoprotective effects in naturopathic medicine include anti-oxidative activity, inhibition of hepatic stellate cell activation, activation of physiological defense, and suppression of hepatocarcinogenesis by cell cycle arrest and induction of apoptosis^[4,32]. Natural foods in our everyday life also could be beneficial for patients with liver disease (Table 3).

Coffee

Coffee is the most popular beverage in the world and is a rich source of dietary antioxidants. Several epidemiological and case-controlled studies showed that coffee drinking is associated with better results of serum liver function tests and with a reduced risk of cirrhosis and hepatocellular carcinoma. The mechanisms involved are related to the anti-fibrosis, anti-carcinogenesis, and antioxidant effect of the drink. Coffee intake more than 2 cups per day protects against progression of almost all forms of liver diseases^[10,33]. However, there is no direct evidence that increasing the consumption of a coffee drinker will be beneficial or starting to drink coffee for an abstainer from coffee will improve their liver disease. And, caffeine is a psychoactive drug, increasing alertness and cognition; so, coffee drinkers have some common features of drug abuse, such as clinical dependency, withdrawal, tolerance or intoxication^[10].

Fruits

Popular plant foods, such as apple and citrus, also have been shown to have beneficial effects against a number of chronic diseases. Apple and mandarin are also rich in antioxidants. An animal study confirmed that a diet made of dried apple and mandarin orange is protective against liver impairment induced by tamoxifen in rats^[34]. Another study revealed the hepatoprotective efficacy of apple pomace aqueous extract, as it had inhibited CCl₄-induced hepatocyte apoptosis^[35]. However, fruit allergy which represents one of the most common allergenic responses, is hard to avoid. When some patients eat a particular fruit (apple, citrus, peach, etc.), oral allergy syndrome may be evoked^[36].

Plant phytoalexin

Natural resveratrol is a plant phytoalexin that is produced by plants in response to damage, and is found in many plants, such as mulberries, red grapes, peanuts, soy, knotweed, and sickle pod^[37,38]. Resveratrol has been shown to improve lipid metabolism, prevent expression of liver inflammatory markers and protect against nonalcoholic fatty liver disease. Furthermore, it activates sirtuins, which could extend lifespan in many organisms^[39].

Vegetables, especially green leafy vegetables, can be taken as measures against the lack of dependable hepatoprotective drugs in modern allopathic medicine to treat and prevent liver damage^[26,40]. Many other

Table 3 Information of some natural plants for treatment of liver problems

Natural plants	Ref.	Literature type	Effects and mechanisms/possible mechanisms	Adverse reactions
Coffee	Masterton <i>et al</i> ^[10] , 2010	Review	Potential to reduce risk of abnormal liver function tests, cirrhosis and hepatocellular carcinoma	Clinical dependency, withdrawal, tolerance or intoxication
	Wadhawan <i>et al</i> ^[33] , 2016	Review	Reducing incidence of fibrosis and cirrhosis, hepatocellular carcinoma rates, and decreasing mortality	Not mentioned
Apple	Codoñer-Franch <i>et al</i> ^[34] , 2013	Article	Protective action against oxidative stress induced by tamoxifen in rats	Not mentioned
	Sharma <i>et al</i> ^[35] , 2016	Article	Antioxidant activity; able to induce protein expression of nuclear factor (erythroid-derived 2)-like 2 to inhibit CCl ₄ -induced apoptosis	Not mentioned
Resveratrol	Burns <i>et al</i> ^[38] , 2002	Review	anti-oxidative, anti-carcinogenic, and antitumor properties	Not mentioned
	Andrade <i>et al</i> ^[39] , 2014	Article	Improving lipid metabolism, decreasing incidence of nonalcoholic fatty liver disease, pro-inflammatory profile in liver of mice fed with obesity-inducible diets	Not mentioned

natural foods and nutritional supplements contain natural compounds that are beneficial to liver health, such as bioflavonoids, carotenoids, crude plant extracts, polyphenols, terpenoids, sulphoraphane, and vitamins^[37]. A number of different natural agents protect against liver diseases, as well^[26]. There is a long list of these agents, including quercetin, genistein, catechins, curcumin, and rosemary essential oil^[41].

DISCUSSION

The natural formula has a diversity of therapeutical activities coming from multiple active elements^[1]. This diversity is the most suitable treatment for the heterogeneity of liver cancer cells, which causes in most circumstances the treatment failure in modern allopathic medicine^[42]. A naturopathic formula often contains several active elements that protect against liver diseases, such as antioxidant, anti-inflammatory, antiviral and anti-cancer elements^[15]. The mechanisms of anti-cancer properties are closely associated with the activity of pro-apoptosis, inhibition of proliferation, and cell cycle arrest that are presented by these active elements^[4,27].

Naturopathy is regulated in part by law, which is quite different from various other forms of complementary and alternative medicine^[7]. The scientific debate on natural medication and its integration with the conventional or mainstream medicine has continued for many years in western society^[43]. Safety of natural medication is concerned with treatment of liver problems^[44,45]. A formula composed of two or more drugs has been demonstrated as more beneficial for disease treatment, but the selection of individual herbs in the formula must be strictly guided by the principles underlying Chinese herbal medicine.

Had the fundamental essence of traditional Chinese medicine been followed, the "Sho-saiko-to Event" in Japan would have been avoided^[4,15]. The event was not an accident, but the inevitable result of western medicine physicians administering Chinese medicine without applying the underlying principles of traditional

Chinese medicine theory in Japan. Some experts suggest that under the theory and method of modern western medicine pharmacology, the mechanism and the curative effect of traditional Chinese medicine are often difficult to illustrate.

Based on traditional Chinese medicine theory, it is inappropriate to apply the Sho-saiko-to decoction to all patients with liver disease, and the drug also should not be administered to all the patients without change. This herbal medicine has been used to treat pyretic diseases in China for around 3000 years. However, there are no reports on large-scale pulmonary side effects as occurred in China^[4,5]. The essence underscores that diagnosis and treatment must be based on an overall analysis of the illness and the patient's personalized condition. Long-term high-dose administration of the decoction is fatal, and it is difficult to believe that one of the patients had been taking this formula continuously for 3 years with a total of 7.5 kg of the drug^[16].

Many factors may affect the efficacy of drugs and even enhance the side effects. For Sho-saiko-to, it is reported that higher risk of adverse reactions is related to elderly age, co-administration of interferon and most importantly the duration of medication^[15]. Even acute hepatitis was reported after long-term continual consumption of this decoction, which reminds us that even some herbs which are claimed to have hepatoprotective effects could probably lead to an adverse drug reaction^[46]. Although the therapeutic effect of herbs is beneficial, side effects must be treated with the greatest possible care.

Besides the time duration and amount of consumption, dynamic modification of this Kampo formula is necessary^[47]. Serious adverse effects can be prevented when the strong ginseng is replaced by the gentler dangsen (*Codonopsis pilosula*)^[48]. Banxia (*Rhizoma pinelliae*), another herb in the formula, is poisonous to the gastrointestinal tract and inhibitory for the respiratory center and peripheral nerves^[49]. In routine traditional Chinese medicine practice, when the patient has the symptom of cough, ginseng, jujube and fresh ginger must be removed from the formula

and the fruit of Chinese magnoliavine and dried ginger are used instead; moreover, pollen is employed instead of banxia if the patient has the symptom of thirst^[50,51]. Therefore, personalized modification of each herb in the Sho-saiko-to formula is of paramount importance^[52].

CONCLUSION

Natural medicine is a holistic approach to treat liver diseases. Naturopathy for liver disease is developing rapidly in clinical practice and theoretical research. However, the mechanisms of function and safety remain incomprehensive and even controversial. Natural agents have multiple therapeutic effects, based upon their antioxidant, anti-inflammatory, antiviral and antitoxic properties, etc. Because of the diverse therapeutical effects and relatively mild side effects, patients usually appreciate the use of natural agents to treat liver diseases. And for the sake of patients' safety, it is appropriate to try natural medication before allopathic medicine in the treatment of liver problems.

Although the therapeutic effect of natural medication is beneficial, side effects must be treated with the greatest possible care. The best way to prevent the complications is to ensure that these medicines be given only by physicians who are licensed and certified. And, good medical ethics and sense of responsibility are also absolutely necessary for the naturopathic doctors. Naturopathic doctors must follow the principles which underlie and are determined by the practice of natural medicine. For patients, it is necessary to follow the guidance of the doctor when natural drugs are used in the treatment of liver diseases.

There are safety problems that still require further study. On the one hand, natural medicines are often mistaken as agents with no side effects and the cheap prices make them easy to get and use. On the other hand, there exists a lack of regulators to oversee the use of natural medicine. These are the main issues that lead to long-term and large amount of consumption of natural drugs and bring about adverse reactions. Both the doctor and the patient should be aware of the potential risks of drugs and be cautious when using them. Each drug has certain indications, specific usage and dosage. An overdose more than the maximum dosage will certainly lead to some adverse reactions. Even the so-called natural medicines or the common foods cannot be abused to treat diseases.

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Renin angiotensin system in liver diseases: Friend or foe?

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Abstract

In the last three decades, the understanding of the renin angiotensin system (RAS) has been changed by the discoveries of functional local systems, novel biologically active peptides, additional specific receptors, alternative pathways of angiotensin (Ang) II generation, and new roles for enzymes and precursor components other than those in Ang II synthesis. In this regard, the discovery that Ang-(1-7) opposes the pressor, proliferative, pro-fibrotic, and pro-inflammatory effects mediated by Ang II has contributed to the realization that the RAS is composed of two axes. The first axis consists of the angiotensin-converting enzyme (ACE), with Ang II as the end product, and the angiotensin type 1 (AT₁) receptor as the main effector mediating the biological actions of Ang II. The second axis results from ACE2-mediated hydrolysis of Ang II, leading to the production of Ang-(1-7), with the Mas receptor as the main effector conveying the vasodilatory, anti-proliferative, anti-fibrotic, and anti-inflammatory effects of Ang-(1-7). Experimental and clinical studies have shown that both axes of the RAS may take part in the pathogenesis of liver diseases. In this manuscript, we summarize the current evidence regarding the role of RAS in hepatic cirrhosis and its complications, including hemodynamic changes and hepatorenal syndrome. The therapeutic potential of the modulation of RAS molecules in liver diseases is also discussed.

Key words: Renin angiotensin system; Angiotensin II; Angiotensin-(1-7); Hepatic cirrhosis; Liver fibrosis; Hepatorenal syndrome

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Core tip: This Editorial reports recent advances on the understanding of the renin angiotensin system in regard to the role of the two main and counter-regulatory mediators, Angiotensin II and Angiotensin-(1-7), in liver diseases and in their main complications. Experimental and clinical findings so far show that Angiotensin-(1-7) by binding to Mas receptor opposes Angiotensin II actions mediated by AT₁ receptors in liver tissue, by eliciting anti-inflammatory, anti-oxidative and anti-fibrotic effects. This knowledge may help in paving the way for the development of novel treatments for liver diseases and their complications.

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INTRODUCTION

The renin angiotensin system (RAS) is classically conceived as a hormonal system mainly responsible for blood pressure control and hydroelectrolyte balance^[1]. In this context, renin, the first enzyme of the classic RAS, is produced in the juxtaglomerular cells of the afferent renal arteriole in response to glomerular hypoperfusion, reduced sodium intake, and increased activity of the sympathetic nervous system^[2]. Next, circulating renin cleaves its substrate angiotensinogen to form the decapeptide angiotensin I (Ang I), which is converted by angiotensin-converting enzyme (ACE) to the major active component of the RAS, angiotensin II (Ang II). The major biological actions of Ang II are mediated by angiotensin type 1 (AT₁) receptors^[3]. The excessive activity of ACE-Ang II-AT₁ arm frequently contributes to several pathophysiological changes including excessive renal sodium reabsorption, abnormal vascular smooth muscle cell contraction, disproportionately high aldosterone secretion and inappropriate cardiovascular responses^[4]. Additionally, pro-inflammatory, pro-thrombotic and pro-fibrotic pathways are stimulated by AT₁ receptor activation^[5].

On the other hand, recent advances have changed our understanding of the RAS. These have included the discovery of functional local systems, novel biologically active peptides, additional specific receptors, alternative pathways of Angiotensin peptides generation, and new roles for enzymes and precursor components other than Ang II synthesis^[4,6]. Especially relevant for the reconceptualization of the RAS was the identification of the heptapeptide Ang-(1-7)^[7], the ACE homologue enzyme responsible for the conversion

of Ang II into Ang-(1-7), ACE2^[8,9], and the Mas receptor, a G-protein coupled receptor which mediates the main effects of Ang-(1-7)^[10]. Also important, a considerable amount of evidence supports a counter-regulatory role for Ang-(1-7). This peptide opposes several Ang II AT₁ receptor-mediated effects, including vasoconstriction, cell proliferation, inflammation and tissue fibrosis^[11-13]. Considering the opposite roles of the two main mediators of the RAS, Ang II and Ang-(1-7), independent research groups have proposed a new view of the RAS. In this model, the RAS can be envisioned as a dual function system in which the vasoconstrictor/proliferative or vasodilator/anti-proliferative actions are primarily driven by the balance between two axes of the RAS, the classical one formed by ACE-Ang II-AT₁ and the counter-regulatory comprising ACE2-Ang-(1-7)-Mas^[4,12-15]. In general, these axes present opposite effects in physiological and pathological states, including liver diseases^[16]. It is well-established that RAS blockers, ACE inhibitors and angiotensin receptor antagonists (ARAs), can inhibit the ACE-Ang II-AT₁ arm, but also stimulate the activity of ACE2-Ang-(1-7)-Mas axis^[15,17,18]. Both agents have been broadly used in the clinical practice for congestive heart failure, hypertension, chronic kidney disease^[19] and seem to exert beneficial effects on liver diseases^[20]. Table 1 displays the main opposite effects of both RAS axes.

In this editorial, we summarize recent evidence on the role of both RAS axes in liver diseases and their complications. Furthermore, the general idea that the final RAS effect represents a balance between the "friend", ACE2-Ang-(1-7)-Mas axis, and the "foe", ACE-Ang II-AT₁ axis, is discussed in the context of liver diseases and potential therapeutic strategies.

RAS IN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL CONDITIONS INVOLVING THE LIVER

The RAS was classically described as a circulating hormonal system that plays a pivotal role in the maintenance of blood pressure and fluid homeostasis^[21]. However, this view changed when the concept of local RAS was introduced. The concept of "local RAS" was first based on the discoveries of RAS components in unlikely places, such as renin, an enzyme originally described in the kidney, found in the brain. New hypotheses about local functions of the RAS were raised based on the tissue-based synthesis of Ang II with independent actions. Lately, contemporary concepts of local RAS have become function-oriented^[22].

The local angiotensin-generating system is important for regulating tissue/organ functions with clinical implications *via* autocrine, paracrine or intracrine actions^[21]. The local hepatic RAS is not well-defined, although studies about RAS involvement in hepatic

Table 1 Main and opposite actions of both renin angiotensin system axes

Organ/tissue	ACE-Ang II - AT ₁ axis	ACE2-Ang-(1-7)-Mas axis
Blood vessels	Vasoconstriction	Vasodilation
Heart	Hypertrophic, arrhythmogenic	Anti-hypertrophic, anti-arrhythmogenic
Kidney	Inflammation, fibrosis	Anti-inflammatory, anti-fibrogenic
Lung	Allergic, fibrosis	Anti-allergic, anti-fibrosis
Brain	Ischemia	Reduction of ischemia
Adipose tissue	Increase insulin resistance	Decrease insulin resistance

ACE: Angiotensin-converting enzyme; Ang II: Angiotensin II, AT₁: Angiotensin type 1 receptor; Ang-(1-7): Angiotensin-(1-7); Mas: Angiotensin-(1-7) receptor.

diseases have indicated a role for this system in the liver^[21]. Ang II is a pro-inflammatory, pro-oxidant, and pro-thrombotic agent that interferes in several steps of intracellular insulin signaling. In sharp contrast, Ang-(1-7) enhances glucose tolerance, insulin sensitivity, insulin-stimulated glucose uptake as well as decreases triglyceride and cholesterol levels and reduces abdominal fat mass. Furthermore, Ang-(1-7) has been demonstrated to decrease liver gluconeogenesis and the Mas receptor is an essential component of the insulin receptor signaling pathway^[23].

Liver diseases are major causes of morbidity and mortality worldwide. The most common liver diseases are hepatitis B and hepatitis C virus infections, alcoholic liver disease and nonalcoholic fatty liver disease (NAFLD). Without proper treatment, all types of chronic hepatitis will progress into end-stage liver diseases, including cirrhosis, liver failure and hepatocellular carcinoma, which ultimately lead to death^[20,24]. The pathological characteristics of chronic liver diseases include enhanced fibrosis, oxidative stress and inflammatory markers. These processes are associated with sinusoidal capillarization and increased hepatic vascular resistance, eventually resulting in portal hypertension. Edema, ascites, hyperdynamic circulation and hepatorenal syndrome can occur because of compensatory mechanisms attempting to restore hepatic function^[20]. The RAS is associated with all these processes. Furthermore, the local (*i.e.*, hepatic) RAS, in addition to the systemic RAS, is thought to contribute to the pathophysiology of liver diseases.

For instance, Ang II caused a rapid and pronounced rise in portal pressure in an experimental model of cirrhosis^[25]. In line with this result, the plasma concentration of Ang II was elevated in patients with cirrhosis, while losartan, an AT₁ receptor antagonist, was capable of reducing the hepatic venous pressure gradient in patients with moderate to severe portal hypertension^[26]. Altogether, these data point to the involvement of Ang II in cirrhosis-related portal hypertension. Ang II is thought to exert its vasoconstrictive effects on the postsinusoidal venules^[21].

Ang II effects in the liver go far beyond vasoconstriction. For instance, by activating AT₁ receptors, Ang II can induce hepatic stellate cell proliferation and up-regulate the expression of transforming

growth factor (TGF)- β 1 *in vitro*, indicating that Ang II plays an important role in the development of liver fibrosis^[27]. Hepatic fibrosis is a dynamic process commonly resulting from different causes of chronic hepatic injury. Fibrosis is a complex process that involves several cell types and mediators, including cytokines, chemokines and growth factors, leading to a disruption of homeostatic mechanisms in the liver. Ultimately, hepatic tissue remodeling depends on the balance between collagen degradation and synthesis. Several mediators are involved in this process, including the RAS components. Notably, Ang II acts as pro-inflammatory and pro-fibrotic mediator, while Ang-(1-7) appears to exert opposite effects in liver tissue^[28], comparable to heart and kidney effects^[15]. Corroborating these findings, both the AT₁ receptor antagonist Candesartan and the ACE inhibitor Perindopril significantly attenuated fibrosis and the associated pathological markers in an animal model of fibrosis^[27]. The fibrogenic effect of Ang II can also be mediated by Kupffer cells, specialized phagocytic cells located in the liver that are known to be actively involved in the fibrotic process. AT₁ receptors are expressed in Kupffer cells, as are renin and ACE^[21]. The presence of RAS components in Kupffer cells and in the liver itself (where AT₂ receptors and, obviously, angiotensinogen can also be found)^[21] corroborates the hypothesis of the existence of a local RAS in the liver. Accordingly, experimental hepatic fibrosis was associated with RAS activation, characterized by increased levels of plasma renin activity, Ang I, Ang II and Ang-(1-7). Additionally, treatment with the Mas receptor antagonist, A-779, worsened hepatic fibrosis, thus suggesting a protective role for endogenous Ang-(1-7)^[29].

NAFLD is the most common chronic liver disease worldwide and an important risk factor for non-alcoholic steatohepatitis, type 2 diabetes and cardiovascular diseases^[30,31]. Ang II actions are associated with a series of deleterious effects that together contribute to the spectrum of histological changes observed in NAFLD and its progressive form, non-alcoholic steatohepatitis. The deleterious effects of Ang II include the stimulation of insulin resistance, *de novo* lipogenesis, mitochondrial dysfunction, reactive oxygen species generation, and pro-inflammatory cytokine production as well as the activation of hepatic stellate cells to

trigger fibrogenesis^[31,32]. Accordingly, experimental studies and clinical trials have shown that either the inhibition of the classical arm (composed by ACE-Ang II-AT₁)^[33-37] or the activation of the counter-regulatory arm (ACE2-Ang-(1-7)-Mas)^[38-42] is beneficial in NAFLD and associated syndromes.

EXPERIMENTAL AND CLINICAL EVIDENCE ON THE ROLE OF RAS IN CIRRHOSIS

Cirrhosis is the end stage of progressive hepatic fibrosis, mainly characterized by liver architecture disruption due to fibrous scars and development of regenerating tissue. Fibrosis leads to significant changes in hepatic perfusion, enhanced portal blood flow resistance as well as liver dysfunction^[43,44]. The leading causes of liver fibrosis are chronic viral hepatitis B and C, alcohol use and steatohepatitis related to obesity. These disorders have accounted for a significant increase in the incidence of cirrhosis and the deaths of at least 800000 people worldwide annually^[45]. Although the pathophysiology of hepatic fibrosis remains not fully clear, current views have postulated that cirrhosis might be potentially reversible, particularly in a compensated stage, thus making the search for drug targets a scientific goal of highest priority^[46].

Emerging evidence has supported the existence of RAS not only in the circulation but also in several organs such as heart, kidney and liver^[47]. Locally, the classical RAS axis components, especially through AT₁ receptor signaling, have been implicated in the modulation of cell growth and proliferation, generation of reactive oxygen species, apoptosis, hormone secretion, inflammatory and pro-fibrogenic processes in response to physiological and pathophysiological stimuli^[48]. Accordingly, an up-regulation of RAS components including angiotensinogen, renin, ACE, Ang II and AT₁ receptors has been reported in experimental and clinical liver injury studies, pointing out a role for this system in hepatic fibrosis and cirrhosis^[28,47,49-51]. For instance, elevated serum levels of ACE were reported as a marker of fibrosis in patients diagnosed with chronic hepatitis B^[52], whereas increased expression of ACE and AT₁ receptors was found in human cirrhotic liver autopsies and biopsies^[50]. In line with these studies, the beneficial effects of ACE inhibitors, such as Captopril, were reported on the analysis of liver biopsies of subjects with hepatitis C virus-related fibrosis^[53]. *In vitro* approaches involving human culture-activated hepatic stellate cells, the main cells involved in the pro-fibrogenic process in the liver, showed that they increase their proliferation and acquire contractile properties in response to Ang II through AT₁ receptors^[54]. Culture-activated hepatic stellate cells from human cirrhotic livers also expressed high levels of active renin and ACE and

secreted Ang II, indicating that locally generated components of the classical RAS axis contribute to tissue fibrosis in human liver^[47]. There is evidence that Ang II effects on hepatic stellate cells are mediated, at least in part, by the activation of NADPH oxidase. Ang II, by means of phosphorylation of p47phox, a regulatory subunit of NADPH oxidase, induces reactive oxygen species formation and stimulates DNA synthesis, cell migration, pro-collagen alpha1 mRNA expression, and secretion of TGF-β1 and inflammatory cytokines, all of which contribute to hepatic fibrosis. Mice lacking p47phox exhibited attenuated liver injury and fibrosis associated with decreased hepatic concentration of TGF-β1 and inflammatory cytokines (TNF-α, IL-1β, IL-8 and MCP-1) following two weeks of bile duct ligation^[55]. Moreover, blockade of Ang II activity by Lisinopril (an ACE inhibitor), Losartan (AT₁ receptor antagonist) or N-acetylcysteine and diphenylene iodonium (NADPH oxidase inhibitors) prevented RAS pro-fibrogenic effects^[47,54,55].

A recent meta-analysis revised the effectiveness of RAS inhibitors in randomized controlled trials in patients with liver fibrosis and reported significant reduction in fibrosis score and area^[56]. A significant decrease in serum fibrosis markers, including TGF-β1, collagen I, IV, and matrix metalloproteinase-2 (MMP2), after treatment with RAS inhibitors was also reported in clinical studies with hepatic fibrosis or cirrhotic patients^[57].

Over the past decades, several studies investigated the role of the classical RAS arm as well as the mechanisms underlying its deleterious effects on liver function by employing different models of liver fibrosis, including bile duct ligation, carbon tetrachloride (CCl₄) treatment or continuous Ang II infusion^[27,49,58-61]. For instance, both ACE and AT₁ receptor genes were up-regulated in rats submitted to bile duct ligation. Additionally, Ang II induced an increase in mRNA expression of TGF-β1 in culture-activated hepatic stellate cells from rats. This finding supports the concept that RAS, especially the interaction of Ang II with AT₁ receptors, plays a pivotal role in liver fibrosis development, mainly through the activation of hepatic stellate cells^[27,49]. Accordingly, infusion of Ang II (25 ng/kg per hour) in bile duct-ligated rats *via* a subcutaneous pump significantly increased hepatic fibrosis by enhancing inflammation, TGF-β1 concentration, collagen deposition, lipid peroxidation product and phosphorylation of c-Jun and p42/44 mitogen-activated protein kinase (JAK2 and MAPK)^[59]. By employing *in vivo* and *in vitro* approaches, Granzow *et al.*^[61] showed that Ang II, acting at AT₁ receptors, promotes pro-fibrotic processes by phosphorylation of JAK2 and subsequent RhoA/Rho-kinase activation in rodents and human liver. Pharmacological inhibition of JAK2 prevented liver fibrosis in rats, indicating that inhibition of this pathway might be a promising therapy for this condition. Moreover, AT₁ receptor-deficient mice presented less liver fibrosis than wild type mice

following bile duct ligation or CCl₄ administration. The protective effect of the absence of the AT₁ receptor was associated with a significant decrease in inflammatory mediators, TGF-β1, lipid peroxidation products and phosphorylation of JAK2 and p42/44 MAPK, suggesting that the blockade of AT₁ receptor signaling might also be a promising approach to treat liver fibrosis^[58,60,62]. Indeed, blocking Ang II activity either by ACE inhibition or AT₁ receptor antagonism prevented liver injury and fibrosis in different experimental models^[16,28,48]. A more recent study revealed that the treatment with Captopril, an ACE inhibitor, accelerates liver regeneration in mice following partial hepatectomy^[63]. The beneficial effects of Captopril were potentially due to reduction of the inflammatory response, paving the way for the hypothesis that blocking the classical RAS arm might not only prevent but also reverse severe liver damage^[63].

More recently, it has been proposed that liver fibrosis and hepatic cirrhosis depend on the balance between the classical (ACE-Ang II-AT₁ receptor) and the counter-regulatory (ACE2-Ang-(1-7)-Mas receptor) RAS axes^[16,28,64-67]. In cirrhotic patients and rats with liver fibrosis, the balance between both RAS axes also accounts for hemodynamic changes, including splanchnic vasodilatation and portal hypertension^[62,66].

An elegant study showed widespread parenchymal expression of ACE2 in the liver of rats submitted to bile duct ligation as well as in cirrhotic patients, providing the first evidence of a potential role of the counter-regulatory RAS axis in chronic liver disease^[68]. Similar findings were found with the progression of liver fibrosis induced by CCl₄ administration in rats^[65]. Importantly, as liver fibrosis progresses, liver tissue expression of ACE2 and plasma levels of Ang-(1-7) increase^[16,29,64,66,67]. Both RAS axes, the counter-regulatory and the classical, seem to be simultaneously activated in patients with liver cirrhosis and experimental models of chronic liver disease^[16,29,64,66,67]. This finding supports the concept that the activation of the counter-regulatory RAS axis is a compensatory mechanism to counteract the deleterious effects of Ang II-AT₁ receptor-mediated actions^[16,29,64,66,67]. Accordingly, *in vivo* and *in vitro* inhibition of ACE under conditions of liver injury up-regulated the mRNA expression of ACE2 and Mas receptor, contributing to liver protection^[65]. It is worth mentioning that ACE2 activity seems to be important as an endogenous negative regulator of RAS in chronic, but not acute liver injury, primarily by promoting the conversion of Ang II into Ang (1-7). This statement is supported by the fact that ACE2 knockout mice only presented increased hepatic fibrosis 21 d after bile duct ligation or following chronic administration of CCl₄. On the other hand, no differences were found between ACE2 knockout mice and wild type littermates when animals were subjected to acute liver injury. Moreover, genetic ablation of ACE2 in one-year-old mice resulted in spontaneous inflammatory cell infiltration and mild

liver fibrosis^[69].

In this scenario, components of the counter-regulatory RAS axis may be regarded as promising targets for the development of novel anti-fibrotic therapies for chronic liver diseases. Our group has previously demonstrated that pharmacological blocking of Mas receptor with its antagonist A-779 aggravated bile duct ligation-induced liver fibrosis, which was associated with elevation in hepatic hydroxyproline and TGF-β1 concentrations^[29]. Conversely, infusion of Ang-(1-7) markedly attenuated hepatic fibrosis in bile duct ligated rats, decreased hydroxyproline content and down-regulated key genes involved in liver fibrosis and angiogenesis such as collagen 1A1, α-SMA (smooth muscle actin), VEGF (vascular endothelial growth factor) and CTGF (connective tissue growth factor)^[64]. In line with these findings, culture hepatic stellate cells treated with Ang-(1-7) or the Mas receptor agonist AVE 0991 expressed less α-SMA and hydroxyproline, while treatment with the Mas receptor antagonist A779 induced opposite effects^[70]. Cultured hepatic stellate cells express Mas receptor and binding of Ang-(1-7) with Mas inhibits Ang II-induced phosphorylation of extracellular signal-regulated kinase (ERK)1/2, a classical pathway of tissue fibrosis^[69].

Conversion of the pro-fibrotic peptide Ang II in the anti-fibrotic peptide Ang-(1-7) depends on ACE2 catalytic action, making this enzyme a very interesting therapeutic target for liver fibrosis^[65,69]. A recent study investigated the long-term therapeutic effect of recombinant ACE2 by employing a liver-specific adeno-associated viral genome 2 serotype 8 vector (rAAV2/8-ACE2) with a liver-specific promoter in chronic liver disease models, including bile duct ligation and CCl₄ administration^[71]. The rAAV2/8-ACE2 therapy promoted a rapid up-regulation of hepatic ACE2 and an attenuation of liver fibrosis. These findings were associated with reduction in hepatic Ang II levels concomitant with increased concentrations of Ang-(1-7) in liver tissue. Also revealed were reductions in NADPH oxidase activity, oxidative stress, ERK1/2 and p38 phosphorylation without unwanted systemic effects^[71].

RAS IN COMPLICATIONS OF LIVER DISEASES

Hemodynamic changes

Hemodynamic changes including portal hypertension and hyperdynamic circulation are the main cause of morbidity and mortality in patients with cirrhosis. Hemodynamic disorders can have widespread impact on the body according to the severity of the cirrhosis^[72]. Portal hypertension and hyperdynamic circulation are characterized by elevated cardiac output and low systemic vascular resistance. Arterial vasodilation in the splanchnic circulation and the resulting decrease in systemic vascular resistance are associated with portal hypertension in cirrhosis.

Compensatory mechanisms following the reduction of systemic vascular resistance lead to hyperdynamic circulation. The effective arterial blood volume and the circulating levels of RAS components and antidiuretic hormone remain normal at early stages of the disease, even with reduced systemic vascular resistance. Nevertheless, hyperdynamic circulation is insufficient to correct the effective arterial hypovolemia when the disease progresses and arterial vasodilation increases, resulting in arterial hypotension and consequent activation of the circulating RAS and the sympathetic nervous system and secretion of antidiuretic hormone. Maintenance of arterial pressure is a result of vasoconstrictive effects of Ang II in extra-splanchnic vascular areas, as the splanchnic circulation is resistant to Ang II, noradrenaline and vasopressin effects^[66].

Evaluating patients at different stages of cirrhosis, one study showed that the circulating RAS is not activated at early stages of the disease. In contrast, patients at the advanced stages of cirrhosis presented an activation of peripheral and splanchnic RAS, and a metabolic deviation toward the RAS vasodilator axis in the splanchnic circulation. Furthermore, these authors observed a positive correlation between the Ang-(1-7)/Ang II ratio and cardiac output as well as a negative correlation between Ang-(1-7)/Ang II ratio and systemic vascular resistance, concluding that the final effect of the RAS may reflect a balance between the two opposing axes^[66]. In this regard, the positive effects observed with blockade of the classical RAS arm are due, at least in part, to activation of the RAS counter-regulatory axis. Indeed, the hemodynamic effects of Ang-(1-7) has already been demonstrated in rats, in which Ang-(1-7) produced a significant increase in cardiac index (30%) and a decrease in total peripheral resistance^[73]. However, chronic treatment with propranolol (a β -adrenergic receptor antagonist) in cirrhotic patients resulted in marked changes in the precursors of the RAS cascade, *i.e.*, Renin and Ang I, with inhibition of both RAS arms at splanchnic and peripheral circulation. The chronic use of propranolol produced hemodynamic changes that were probably able to control the hyperdynamic circulation of cirrhotic patients. These effects were associated with overall RAS inhibition instead of changes in the balance between the two RAS arms^[74] (Figure 1).

Hepatorenal syndrome

Hepatorenal syndrome (HRS) has been defined as progressive renal failure that occurs in patients with chronic liver disease and advanced hepatic failure in the absence of any apparent clinical cause for renal insufficiency^[75-77]. HRS represents the final stage of a process that gradually reduces renal blood flow and the glomerular filtration rate (GFR) due to marked renal vasoconstriction^[75-77]. Despite the severity of renal failure, no significant histological abnormalities are found in the kidneys.

The pathophysiology of HRS is still poorly understood. The progressive reduction in systemic vascular resistance leads to effective arterial hypovolemia. To maintain arterial pressure within normal limits in this setting, there is activation of systemic vasoconstrictor systems, including the RAS, sympathetic nervous system and in late stages, nonosmotic hypersecretion of vasopressin. Although these systems help to maintain blood pressure, they have a negative influence on kidney function, leading to the retention of sodium and free water, and at late stages of the disease, producing an intense kidney vasoconstriction, which in turn decreases the glomerular filtration rate resulting in HRS. Indeed, hypoperfusion of the kidney due to the exaggerated action of renal vasoconstrictors has been considered the hallmark of HRS^[76,78] (Figure 2).

The activation of the classical RAS arm, ACE-Ang II-AT₁ receptor, is one of the main factors responsible for renal vasoconstriction in HRS^[16,20]. Plasma renin activity and Ang II levels are increased in HRS^[79]. Ang II infusion stimulates renal vasoconstriction and there is an inverse correlation between renal hypoperfusion and activation of the classical RAS in cirrhotic patients^[80,81]. It has been considered that at the early stages of hepatic injury, the renal effects of Ang II can represent a compensatory mechanism against the decrease in renal blood flow^[76,78,82]. However, the continuous, uncontrolled and exacerbated action of Ang II may progressively compromise renal function. Pereira and colleagues have previously shown that bile duct ligated rats exhibited increased circulating levels of Ang II and Ang-(1-7) even at early stages of hepatic damage^[29]. Bile duct ligation is an experimental that leads to hepatic fibrosis and HRS^[83]. However, it is difficult to know whether the changes in the components of the RAS preceded or were caused by the decline in renal function. The liver, or perhaps the kidney, can produce Ang peptides, which, in turn, act either as systemic hormones or as locally generated factors. Accordingly, Paizis and colleagues^[68] detected an up-regulation of ACE2, the main enzyme responsible for Ang-(1-7) synthesis^[84], in liver tissue from cirrhotic patients and bile duct ligated rats. Herath *et al.*^[64] showed increased expression of the Mas receptor in experimental biliary fibrosis, suggesting a role for ACE2-Ang-(1-7)-Mas arm in liver injury.

Although the renal effects of Ang II are well documented in patients with chronic liver disease, it is not yet clear whether Ang-(1-7) influences renal hemodynamics and renal tubular handling of sodium and free water. It has been previously shown that Ang-(1-7) exerts complex renal actions (see reference^[85], for review). *In vivo* and *in vitro* administration of Ang-(1-7) can increase water reabsorption by acting at the distal nephron and by interacting with vasopressin V2 receptor^[86-88]. In contrast, other studies reported that Ang-(1-7) has natriuretic and diuretic effects by inhibiting sodium reabsorption at proximal

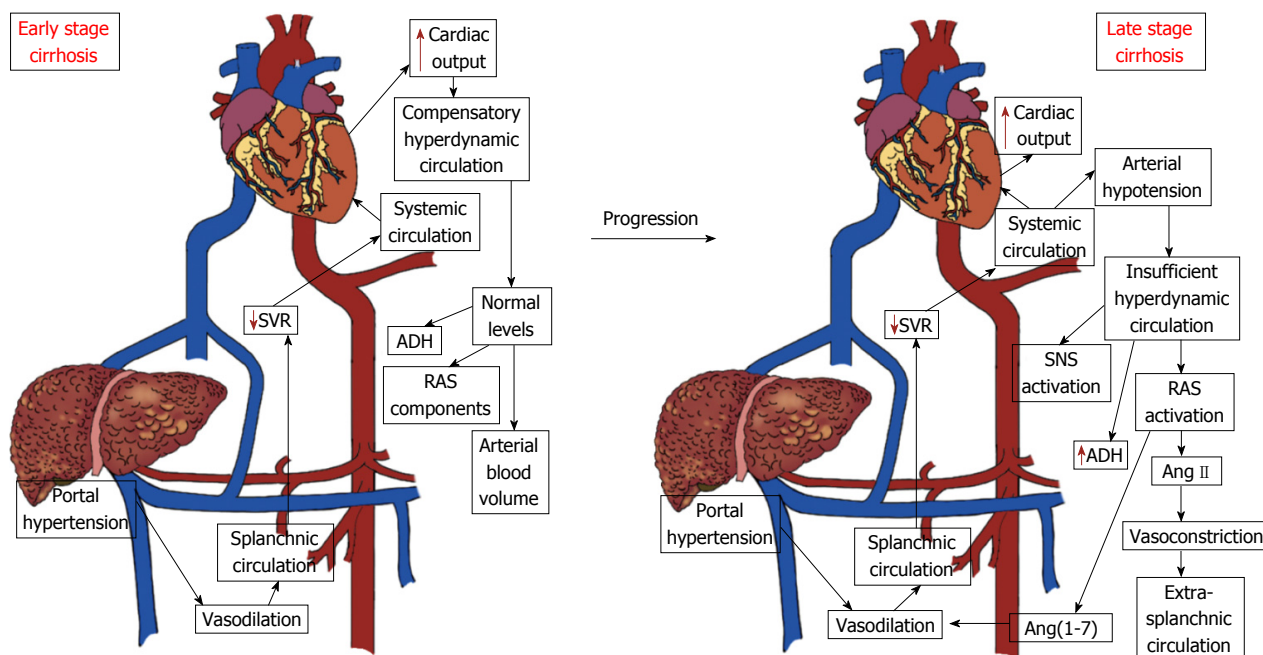


Figure 1 Hemodynamic changes in early and advanced stages of liver cirrhosis. The early phase of cirrhosis is characterized by elevated cardiac output and low systemic vascular resistance without changes in the circulating levels of renin angiotensin system (RAS) components and antidiuretic hormone (ADH). However, as the disease progresses, activation of the circulating RAS and of the sympathetic nervous system and secretion of the antidiuretic hormone occur in response to persistent arterial hypotension. Legend: Ang II: Angiotensin II; Ang(1-7): Angiotensin (1-7); SNS: Sympathetic nervous system; SVR: Systemic vascular resistance.

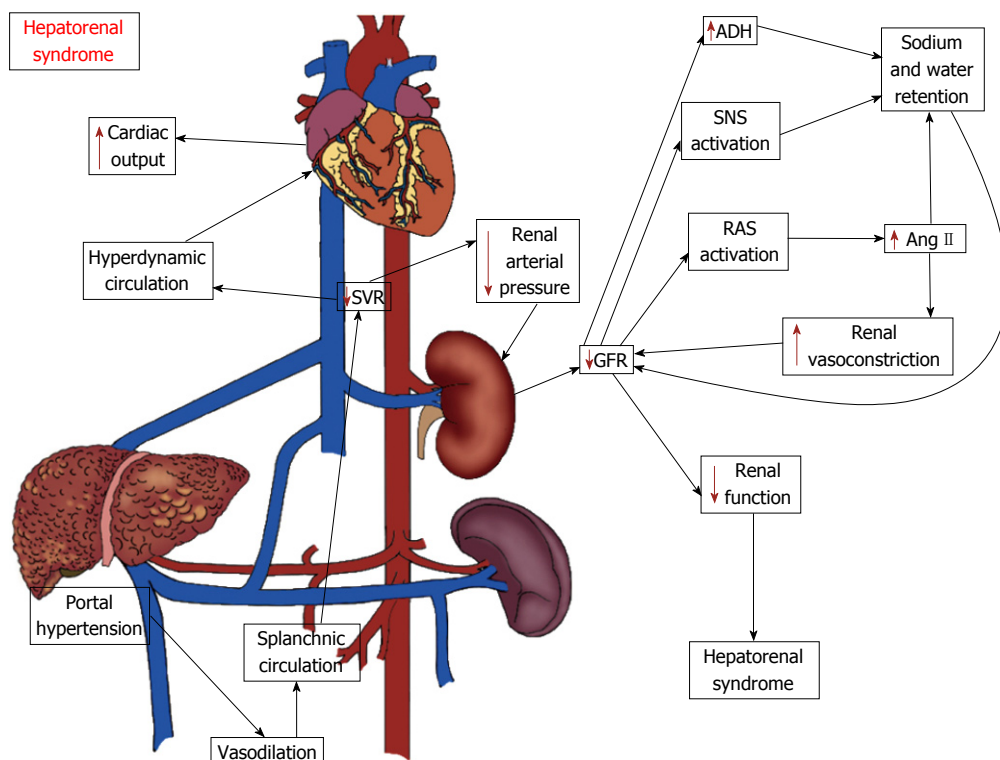


Figure 2 Potential mechanisms of hepatorenal syndrome. The hemodynamic changes associated with advanced stages of cirrhosis may lead to reductions in renal blood flow and in the glomerular filtration rate (GFR) as a compensatory mechanism to the low systemic vascular resistance and arterial hypotension. The kidney hypoperfusion is the hallmark of hepatorenal syndrome (HRS) and occurs as a consequence of the activation of systemic vasoconstrictor factors, including the classical axis of the renin angiotensin system (RAS), sympathetic nervous system and antidiuretic hormone (ADH). The increase in kidney vasoconstriction negatively influences its function, ultimately leading to HRS. Legend: Ang II: Angiotensin II; SNS: Sympathetic nervous system; SVR: Systemic vascular resistance.

tubule^[89-91]. Additionally, Ang-(1-7) has a vasodilator effect on pre-constricted rabbit afferent arterioles *in vitro* via Mas receptor and the release of nitric oxide, and attenuates pressor response to Ang II in rat renal vasculature^[73,92,93]. However, the role of Ang-(1-7) in modifying renal vascular responses in HRS has not been investigated yet.

The treatment of HRS remains a significant challenge. HRS progresses rapidly. Therefore, liver transplantation evaluation should begin promptly to achieve ideal clinical conditions for successful transplantation^[77]. Pharmacological and surgical interventions have not shown survival benefits but serve as temporary modalities to be used as a bridge to liver transplantation^[77]. Therapy with systemic vasoconstrictors has been established as first line for HRS. The combination of terlipressin and albumin remains the standard of care for treating HRS, based on the available evidence^[94]. Therefore, novel and alternative therapeutic approaches are needed to improve survival rate of HRS and to maintain patients in satisfactory clinical conditions for liver transplantation^[77,94]. In this regard, evaluation of the role of molecules that modulate both RAS axes may be investigated in HRS^[20].

CONCLUSION

RAS plays multiple roles in the pathophysiology of liver diseases. The classical RAS axis with its major mediator Ang II exerts pro-oxidant, fibrogenic, and pro-inflammatory actions in the liver. Conversely, the counter-regulatory RAS axis with its main effector Ang-(1-7) produces opposite actions in liver tissue, including anti-inflammatory, anti-oxidative and anti-fibrotic effects. Therefore, the balance between both RAS axes most likely affects the clinical and histopathological expression of liver diseases.

Pharmacological agents that inhibit Ang II formation (e.g., ACE inhibitors) or its binding to AT₁ receptors (e.g., ARAs) have exhibited beneficial effects in chronic liver diseases. However, further studies are needed to incorporate them into clinical practice. Another relevant aspect to be better investigated is the elevation of circulating levels of Ang-(1-7) during chronic RAS inhibition. In particular, an altered balance between Ang II and Ang-(1-7) might be involved in the mechanisms of action of ACE inhibitors and AT₁ receptor antagonists^[95-97]. Therefore, these agents may not only blunt the effects of the classical RAS axis ("the foe") but may also activate the counter-regulatory RAS axis ("the friend"). Most studies showing the therapeutic potential of ACE2-Ang-(1-7)-Mas axis are still pre-clinical. To date, Ang-(1-7) has only been administered in phase I / II studies as a putative anti-proliferative and anti-angiogenic agent to patients with advanced cancer refractory to standard treatment and as a hematopoietic agent to patients with multilineage cytopenias following chemotherapy^[98,99]. These

studies were very limited in scope but no dose-limiting toxicities have been reported. Therefore, further research on the contribution of the ACE2-Ang-(1-7)-Mas axis to the pathophysiology of liver diseases might lead to the development of pharmacological approaches. These new approaches may, in turn, result in the design of molecular or genetic methods to increase the expression of ACE2 and increased tissue levels of Ang-(1-7) and/or activation of the Mas receptor.

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Relationship between adipose tissue dysfunction, vitamin D deficiency and the pathogenesis of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. Its pathogenesis is complex and not yet fully understood. Over the years many studies have proposed various pathophysiological hypotheses, among which the currently most widely accepted is the "multiple parallel hits" theory. According to this model, lipid accumulation in the hepatocytes and insulin resistance increase the vulnerability of the liver to many factors that act in a coordinated and cooperative manner to promote hepatic injury, inflammation and fibrosis. Among these factors, adipose tissue dysfunction and subsequent chronic low grade inflammation play a crucial role. Recent studies have shown that vitamin D exerts an immune-regulating action on adipose tissue, and the growing wealth of epidemiological data is demonstrating that hypovitaminosis D is associated with both obesity and NAFLD. Furthermore, given the strong association between these conditions, current findings suggest that vitamin D may be involved in the relationship between adipose tissue dysfunction and NAFLD. The purpose of this review is to provide an overview of recent advances in the pathogenesis of NAFLD in relation to adipose tissue dysfunction, and in the pathophysiology linking vitamin D deficiency with NAFLD and adiposity, together with an overview of the evidence available on the clinical utility of vitamin D supplementation in cases of NAFLD.

Key words: Adipose tissue dysfunction; Vitamin D; Non-alcoholic fatty liver disease; Steatosis; Non-alcoholic steatohepatitis; Obesity; Adipokines

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Core tip: Obesity-associated chronic low-grade inflammation plays a pivotal role in the development of non-alcoholic fatty liver disease (NAFLD). Vitamin D deficiency is associated with both obesity and NAFLD, and its anti-inflammatory and immune-modulatory properties provided plausible mechanisms by which hypovitaminosis D may link adipose tissue dysfunction and NAFLD. Animal studies showed beneficial effect of vitamin D supplementation on systemic inflammation and NAFLD, but these data are not confirmed by the results of clinical trials so far conducted in humans.

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INTRODUCTION

Definition and epidemiology of non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a condition characterized by the accumulation of excessive fat in the liver in individuals with no history of alcohol abuse (< 30 g/d in men and < 20 g/d in women) and no competing etiologies for hepatic steatosis. NAFLD represents a spectrum of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which may evolve into hepatic fibrosis, cirrhosis, and eventually hepatic carcinoma^[1-5].

NAFLD is a global public health problem^[6]: it is currently the most common chronic liver disease worldwide, affecting approximately 20%-35% of adults in the general population^[7]. Furthermore, the number of patients affected is growing rapidly, and the disease has now reached epidemic proportions. The reported prevalence of NAFLD is 20%-30% in western countries and approximately 15% in Asian countries. In individuals who are of normal weight and who have no metabolic risk factors, the prevalence of NAFLD is about 16%. Though, it rises dramatically in high-risk individuals such as patients with diabetes (60%), hyperlipidemia (90%) and obese patients (91%)^[8-12]. In addition to diabetes and obesity, other independent risk factors identified for the disease to progress toward NASH and to develop fibrosis and cirrhosis are

age, female sex, Hispanic ethnicity, smoking^[13,14].

Notably, 55% of patients with NAFLD have normal aminotransferase levels^[15], suggesting that studies using liver enzymes as a surrogate for NAFLD significantly underestimate the prevalence of this condition. NAFLD diagnosis is usually made by ultrasonography, which enables moderate and severe steatosis to be detected with acceptable sensitivity but only once the level of fat accumulated in the liver exceeds 33%. More sensitive techniques, including nuclear magnetic resonance imaging and spectroscopy, are hindered by the high costs involved and the lack of feasibility in large populations. The American Association for the Study of Liver Diseases sets the limit for the diagnosis of NAFLD at a biopsy-proven hepatic fat content greater than 5%^[7]. Liver biopsy is therefore still considered to be the gold standard, although its widespread use is restricted by a number of factors, including the cost and lack of feasibility in population-based studies on both ethical and practical grounds.

The clinical implications of the alarming prevalence of NAFLD, our limited knowledge of its underlying pathophysiologic mechanisms, and the difficulties in both its diagnosis and treatment explain why NAFLD is currently a field of such intensive research.

Pathogenesis of NAFLD

A histological grading and staging system for non-alcoholic steatohepatitis was proposed by Brunt *et al.*^[16] in 1999. The amount of fat, fibrosis and necro-inflammation were the parameters included in the Brunt's criteria for grading and staging NASH. The presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) defined the diagnosis of NASH, according to recently recommended guideline of the American Association for the Study of Liver Disease^[7].

In patients with NAFLD, studies have shown that the vast majority of hepatic fat (59%) originates from adipose tissue lipolysis, 26% comes from *de novo* lipogenesis and 15% originates from the diet^[17]. Hepatic steatosis results when the balance between delivery and synthesis of free fatty acids exceeds the liver capacity to oxidize or export them. Accumulation of lipids can exert toxic effects on the liver by inefficient oxidation or by activation of inflammatory pathways. Further, increased lipid metabolites such as diacylglycerol and ceramides may themselves cause cell injury and insulin resistance (IR) by interfering with the ability of insulin to phosphorylate insulin receptor substrate-2 through activation of protein kinase C-epsilon^[18-21].

In 1998, Day *et al.*^[22] presented the "two hits" hypothesis to describe the pathogenesis of NAFLD. They proposed that the "first hit" was represented by lipid accumulation in the hepatocytes and consequent IR, and that the "second hit" was represented by increased oxidative stress that resulted in hepatic

inflammation, fibrosis and necrosis. This model is now considered obsolete, because it is inadequate to explain the several molecular and metabolic changes which lead to the development of NAFLD.

According to the current "multiple-hits" theory, the "first hit" sensitizes the liver to further insults, which are represented by a variable combination of different hits, such as oxidative stress and subsequent lipid peroxidation, mitochondrial dysfunction, gut microbiota, adipose tissue dysfunction, and adipokine secretion, all of which are ultimately capable of inducing hepatic injury^[23-26].

In this context, novel data has unraveled the role of adipose tissue dysfunction as a central player in the ectopic fat distribution associated with obesity and dysmetabolic conditions. In this review, we are therefore focusing on recent findings that provide an insight into the role of adipose tissue dysfunction in the pathogenesis of NAFLD.

ADIPOSE TISSUE DYSFUNCTION AND NAFLD

Obesity is a major risk factor for the development of NAFLD, but not all patients with obesity go on to develop NAFLD. In the National Health and Nutrition Examination Survey III, 7.4% of lean adults and 27.8% of overweight/obese adults had hepatic steatosis which could be detected by ultrasound^[27].

One reason for this incomplete overlap between obesity and NAFLD is related to the use of Body Mass Index (BMI) to define obesity, meaning that, although BMI cut-off points have good specificity for detecting excess adiposity, they lack sensitivity^[28] and also fail to provide information about the distribution, type and quality of body fat.

Traditionally, adipose tissue was regarded as an inert organ for the storage of energy, but in the last few years this conventional view has been radically altered. Currently, adipose tissue is considered to be the major, and possibly the largest, endocrine organ, having the ability to synthesize and release a variety of hormones, cytokines, both complement and growth factors, extracellular matrix proteins and vasoactive agents, collectively known as adipokines. Therefore, it has been shown that adipose tissue biology is much more complex than previously considered^[29] and visceral adipose tissue (VAT) dysfunction has been proposed as a major contributor to NAFLD^[30,31].

VAT consists of a loose connective tissue that is predominately populated with tightly packed adipocytes that are vascularized by a dense network of capillaries. A second component of VAT is represented by the stromal vascular fraction and includes pre-adipocytes, multi-potent stem cells, fibroblasts, vascular endothelial cells, and immune cells surrounded by the extracellular matrix (ECM). The ECM contains a variety of structural proteins and

collagen networks that anchor adipocytes to maintain the structural and functional integrity of the tissue^[32].

In obese subjects, excessive nutrient intake and the consequent accumulation of triglycerides result in an expansion of VAT that causes adipocytes hypertrophy and alters the stromal vascular compartment^[33,34]. Progressive adipocytes hypertrophy is associated with increased adipokine and pro-inflammatory cytokine production^[35] and leads to hypoxia and adipocyte cell death^[36,37]. Dysfunctional VAT also undergoes excessive fibrosis by enhancing the expression of different ECM components such as collagen VI^[32,38,39]; progressive fibrosis may also limit the amount of fat stored in the adipocytes, thus promoting the deposition of ectopic fat in liver and muscle^[40].

One hallmark of adipose tissue dysfunction is the accumulation of inflammatory cells in the context of VAT; in particular, active macrophages infiltrate VAT^[41,42] and surround dead adipocytes in typical "crown-like structures"^[43] (Figure 1).

Two major subtypes of macrophage are found in adipose tissue: "alternatively activated" M2 macrophages and "classically activated" M1 macrophages, and the proportions of these cell populations in VAT are dependent on the tissue microenvironment. M2 macrophages maintain VAT homeostasis in lean individuals through the secretion of anti-inflammatory cytokines, such as IL-10, whereas in obese individuals, pro-M1 polarized macrophages secrete pro-inflammatory cytokines, including TNF α , IL-1 β and IL-6, which can promote the proliferation of other inflammatory immune cells, chronic local and systemic inflammation, and can directly alter insulin receptor signaling in adipocytes, leading to IR^[44,45]. The mechanisms leading to increased infiltration of macrophages into VAT are not entirely clear; however, it is known that in obese individuals adipocytes increase their expression of monocyte chemoattractant protein 1 (MCP-1) in order to recruit macrophages^[46].

Moreover, adipose tissue secretes a large number of adipokines. These are delivered directly to the liver *via* its portal vein, and then exert local and peripheral effects. It is increasingly recognized that an impaired pattern of adipokines secretion could play a pivotal role in the development of NAFLD^[47].

Adipokines and NAFLD

Several investigators have attempted to demonstrate a role for adipokines in the pathogenesis of NAFLD and in the progression to NASH, although the data on many of the adipokines apparently involved are sometimes controversial. Adipokines are characterized by complex interactions and the role they may exert in the pathogenesis of NAFLD is often difficult to interpret^[48]. Those adipokines whose effects on the liver are defined and supported by solid data are adiponectin, leptin, TNF- α and IL-6.

TNF- α is the most commonly investigated and

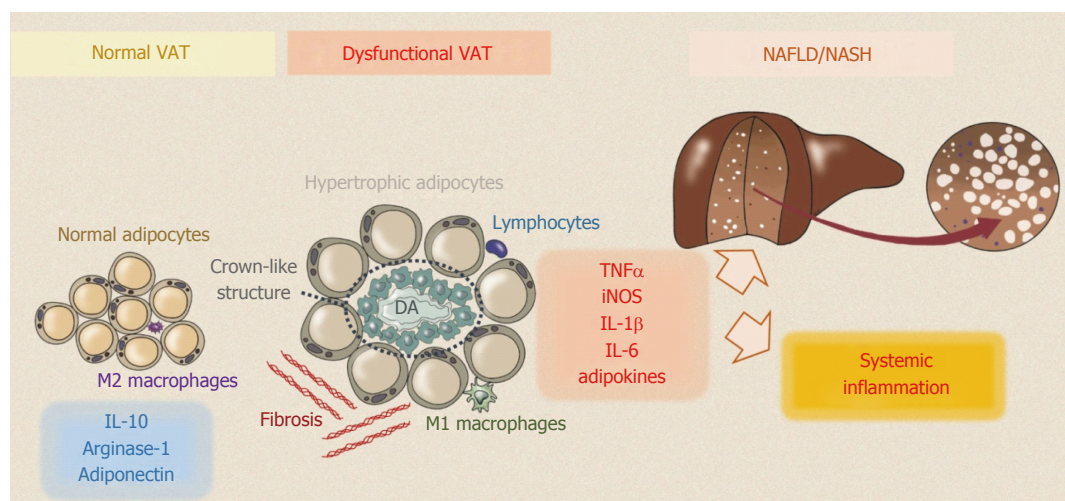


Figure 1 Normal visceral adipose tissue consists of a loose connective tissue that is populated with tightly packed adipocytes. In lean individuals VAT homeostasis is maintained by adiponectin released by adipocytes and by M2 macrophages through the secretion of anti-inflammatory cytokines, such as interleukin (IL)-10 and arginase-1. During obesity, dysfunctional visceral adipose tissue (VAT) undergoes excessive fibrosis and accumulation of inflammatory cells. Active macrophages surround dying adipocytes (DA) in typical "crown-like structures". Pro-M1 polarized macrophages secrete pro-inflammatory cytokines including TNF α , IL-1 β and IL-6, which can promote chronic local and systemic inflammation. VAT secretes a large number of adipokines which could play a pivotal role in development of NAFLD. NAFLD: Non-alcoholic fatty liver disease.

characterized. It is secreted by AT-associated macrophages as a response to chronic inflammatory activity. Human and experimental studies have suggested that TNF- α plays a role in all of the phases of fatty liver disease, from simple steatosis to steatohepatitis and cirrhosis; TNF- α also enhances IR^[49-51] and promotes the development of IR complications *in vitro*^[52,53]. Human studies have revealed that a high TNF- α level increases the risk of developing NAFLD in healthy individuals^[49] and predicts the progression and severity of NASH^[49,54-60].

In the pathogenesis and progression of IR and NAFLD, IL-6 exerts a role extensively investigated in both experimental models of steatosis and liver injury, and in humans. *In vitro* studies have shown that IL-6 promotes IR *via* several mechanisms^[61-63], and in animal models this effect was evident in the liver^[64-66]. Serum IL-6 levels were increased in subjects with biopsy-proven NAFLD compared to controls^[67], and these levels correlated with the degree of inflammation, the stage of the fibrosis and with IR^[68].

Leptin plays a role in both the development and progression of NAFLD, contributing to IR, steatosis and, through a pro-inflammatory role in the regulation of hepatic stellate cells, to hepatic fibrosis^[69]. In NASH patients leptin levels were found to be increased and to be related to the grade of hepatic steatosis^[70-72].

Adiponectin is produced specifically by differentiated adipocytes^[73] and is an anti-inflammatory and insulin-sensitizing hormone^[74]. It has been largely demonstrated that adiponectin prevents hepatocytes lipid accumulation by enhancing β -oxydation and by reducing synthesis of free fat acids^[75-77], that it mediates anti-inflammatory activity by lowering NF κ B action^[78], and antagonizes leptin-induced STAT3

phosphorylation in activated hepatic stellate cells^[79].

Certain *in vivo* studies have shown that low serum levels of adiponectin are associated with NAFLD^[75,76,80-85] and that low adiponectin was an independent risk factor for NAFLD^[86]; furthermore, that adiponectin is a good predictor of necro-inflammation and fibrosis in animal and *in vitro* models of NAFLD^[78,87-89]. Moreover, studies in humans have shown reduction of adiponectin in the serum, as well as reduction of the expression of its receptor in the liver of patients with NASH when compared to BMI-matched patients with steatosis^[54,83,90], providing robust evidence that decreased adipocyte production of adiponectin plays an important role in the progression of NAFLD.

The identification of serum adipokines associated with liver histology and, more specifically, with the severity of steatosis, fibrosis and inflammation might provide useful information about NAFLD pathogenesis and form the basis for new diagnostic and therapeutic approaches.

VITAMIN D, ADIPOSITY AND NAFLD

There is growing evidence to indicate that in addition to maintaining calcium and phosphorus homeostasis and bone health^[91], vitamin D also displays pleiotropic actions on several tissues, organs and metabolic processes^[92-96]. Vitamin D deficiency is currently a global health issue and may contribute to the pathogenesis of many disorders, such as obesity, metabolic syndrome and type 2 diabetes^[97-99].

Despite several epidemiological studies showing the existence of a close relationship between obesity and hypovitaminosis D^[100-105], the mechanisms underlying

this association are largely unknown. Interestingly, many studies have suggested that adipose tissue could be a direct target of vitamin D, and that this molecule might have a role in modulating adipose tissue pathophysiology^[106-116].

Notwithstanding the inverse association between BMI and fat mass^[114], higher plasma 25OHD has been associated with lower amounts of VAT and with reduced omental adipocyte size^[115-119], suggesting a link between vitamin D status and fat distribution. This is further substantiated by reports of the regulatory effects of vitamin D on adipose tissue and lipid storage and by the fact that vitamin D receptor (VDR) is expressed in adipocytes both in animals models and in humans. In particular, the expression of VDR gene has been reported in cultured adipocytes^[110], in human pre-adipocytes^[111] and human subcutaneous and visceral adipose tissue^[112,113].

Interestingly, it has been suggested that vitamin D may provide a protective effect in obese individuals who have healthy metabolic profiles as characterized by the absence of IR-related conditions and low systemic inflammation despite an increased body fat mass^[120-122].

Thus, these data suggest an involvement of vitamin D in the regulation of adipose tissue inflammation. The transduction of inflammatory pathways in adipose tissue involves the activation of nuclear factor κ -B (NF- κ B) that regulates the transcription of a wide range of inflammatory mediators. Several *in vitro* studies showed that vitamin D exerts an anti-inflammatory action on both mouse and human adipocytes by decreasing chemokines and cytokines expression *via* the involvement of p38 MAP kinase and the NF- κ B classical inflammatory pathway^[123-125]. Very recently, Karkeni *et al.*^[126] demonstrated that vitamin D modulates the expression of miRNAs in adipocytes *in vitro* and in adipose tissue *in vivo* through the NF- κ B signaling pathway, representing, thus, a new mechanism of regulation of adipose tissue inflammation by vitamin D.

In line with these data, vitamin D supplementation has been recently demonstrate to decrease circulating pro-inflammatory adipokines, in particular IL-6 and TNF- α , in diet-induced obese mice^[127,128]. Moreover, in a large cohort of human patients, serum 25OHD concentration correlated with low leptin^[129] and high adiponectin levels, irrespective of their BMI^[130].

The role of vitamin D in the pathogenesis of NAFLD is an active area of research^[131]. The existence of an independent association between hypovitaminosis D and NAFLD has been largely demonstrated in studies conducted using liver imaging^[132-136] and biopsy^[137,138]. In particular, low vitamin D levels were associated with the histological severity of NAFLD/NASH^[137-143] and with the prevalence of NAFLD among individuals with normal liver enzymes^[131]. Overall, in the only meta-analysis available in the literature, a 26% additional risk of vitamin D deficiency has been reported in

subjects with NAFLD compared to controls subjects^[139].

Experimental studies have shown that vitamin D also directly exerts anti-inflammatory, anti-proliferative and anti-fibrotic activities in the liver^[144,145] by linking VDR, widely expressed throughout the liver, in hepatocytes, cholangiocytes, and lymphocytes^[146-148]. There is extensive evidence to show that the VDR function in the liver regulates not only the hepatic lipid metabolism but also hepatic necro-inflammation and fibrosis; notably, in chronic hepatic diseases VDR expression negatively correlates with the inflammatory damage^[149].

In vitro and *in vivo* preclinical studies have found that vitamin D decreased hepatic stellate cell activation, suggesting it may have a potential role to protect against hepatic fibrosis^[150,151].

Data from animal studies further support the notion that vitamin D plays an immunomodulatory role in NAFLD. Roth *et al.*^[152] showed that a lack of vitamin D intake in obese rats led to the progression of NAFLD with increased lobular inflammation and a higher NAFLD activity score as evaluated by liver histology; at the same time, mRNA levels of resistin, IL-6 and TNF- α , were increased in the liver. All the above markers are involved in oxidative stress and hepatic inflammation. Therefore, in another study on NASH rat, phototherapy, by increasing the serum active form of vitamin D, reduced hepatocyte inflammation and fibrosis, improved insulin resistance, and increased serum adiponectin, while at the same time reducing the hepatic expression of inflammatory genes TNF- α and TFG- β ^[153].

It has also been demonstrated that a vitamin D-deficient high-fat diet hampers the enterohepatic circulation of bile acids, leading to NASH^[154]. Furthermore, a recent study evidenced that long-term dietary vitamin D depletion could generate spontaneous liver fibrosis in a mice model^[155]. Han *et al.*^[156] demonstrated that vitamin D supplementation in mice with NASH reduced the hepatic levels of cytokeratin 18 apoptotic fragment M30, a widely validated marker of hepatic damage^[157].

These observations regarding the link between vitamin D serum levels and the development and progression of NAFLD suggest that vitamin D supplementation might represent a new therapeutic option in the management of NAFLD. Nevertheless, controversies exist due to the limited number of studies and the conflicting results of prospective randomized clinical trials in humans designed to examine the role of vitamin D supplementation in NAFLD.

Our group has recently published the results of a randomized, double-blind, placebo-controlled trial involving 55 patients with type 2 diabetes and MRI-diagnosed NAFLD. In our study, the participants underwent a 24-wk course of high-dose oral vitamin D supplementation and no effect was shown on either the hepatic fat content or on markers of hepatic injury,

i.e., serum transaminases, CK-18 and PIIINP^[158].

Lorvand Amiri *et al.*^[159] conducted a randomized placebo-controlled double-blind clinical trial to evaluate the potential beneficial effects of oral calcium plus calcitriol supplementation versus calcitriol alone on liver enzymes and ultrasound-measured fat liver content in 120 patients with NAFLD, showing decreased improved serum ALT in the calcium plus calcitriol treated group.

Previously, a prospective small pilot study evaluated the impact of a 24-wk course of high-dose oral vitamin D supplementation on the liver histology of 12 non-cirrhotic NASH patients. The study found no beneficial effects of this treatment on hepatic damage or insulin sensitivity^[160].

Sharifi *et al.*^[161] also investigated the effect of oral vitamin D supplementation in patients with NAFLD in a placebo-controlled trial and their results showed no effect on serum levels of hepatic enzymes, HOMA-IR, or on the degree of hepatic steatosis. However, their study did demonstrate the beneficial effects of vitamin D on serum malondialdehyde, a marker of lipid peroxidation, and on CRP levels.

Studies with a longer intervention period are warranted in order to explore the effects of long term exposure to vitamin D on NAFLD and on the associated systemic inflammation it causes, as well as on the prevention of NAFLD.

CONCLUSION

There is a well-established inverse relationship between vitamin D status and obesity, and hypovitaminosis D is associated with an unfavorable metabolic and inflammatory profile. Obesity-related systemic low grade inflammation characterized by alterations in levels of circulating adipokines is suggested to be involved in the pathogenesis of NAFLD and in its progression to NASH. Vitamin D deficiency is also associated with NAFLD and has even been correlated with the severity of the disease. Recent data has suggested that vitamin D's anti-inflammatory and immune-modulatory properties provide plausible mechanisms by which hypovitaminosis D may link adipose tissue dysfunction to the various steps in the progression of NAFLD. Several animal studies have added further weight to this hypothesis by showing the beneficial effect of vitamin D supplementation on systemic inflammation and on NAFLD in a murine model, although these data are not yet confirmed by the results of the clinical trials conducted to date in humans. Further specifically-designed long-term randomized placebo-controlled trials are needed to clarify the therapeutic impact of vitamin D supplementation in NAFLD.

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Recent advances in the management of pruritus in chronic liver diseases

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Abstract

Pruritus is a symptom found in patients with chronic liver diseases, especially cholestatic liver diseases such as primary biliary cholangitis. This symptom impairs

patient quality of life by disturbing sleep and may lead to consideration of liver transplantation. Mechanisms implicated in pruritus have been associated with the peripheral and central nervous systems, leading to the development of various therapeutic options. Little evidence for the efficacy of most of these treatments is currently available, indicating a need for further investigations.

Key words: Pruritus; Cholestasis; Autotaxin; Opioid receptor antagonist

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Core tip: Pruritus is a symptom influencing the quality of life in patients with chronic liver diseases especially with cholestatic liver diseases. Complex underlying mechanisms have been identified and various therapeutic options developed. More evidence is needed for these treatments, as well as improvements in their tolerability.

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INTRODUCTION

Pruritus is one of the symptoms encountered in patients with chronic liver diseases, especially in those with cholestatic liver diseases such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC)^[1-3]. Pruritus in patients with cholestasis is characterized by a circadian rhythm, with the highest intensity during the evening and early at night^[4]. Chronic

pruritus generally tends to increase with warmth and at night^[4,5]. Women with cholestasis frequently show worsening of pruritus during the progesterone phase of the menstrual cycle, in late pregnancy, and during hormone replacement treatment^[4-6]. Although pruritus may not be directly associated with the prognosis or outcome of liver diseases, a recent systematic review showed that pruritus has an impact on health-related quality of life in patients with cholestatic liver diseases^[7]. Pruritus may be an indication for liver transplantation even in the absence of liver failure^[8,9]. Recently, several mechanisms underlying pruritus, as well as treatment advances have been identified. This review describes recent advances in the management of pruritus in chronic liver diseases.

MECHANISMS OF PRURITUS IN LIVER DISEASES

Several lines of evidence have suggested mechanisms by which pruritus is induced in cholestatic conditions. First, accumulated bile salts are thought to act as pruritogens^[10]. Bile salts have been reported to induce degranulation of mast cells *in vitro*, which may contribute to pruritus in cholestatic patients. However the relationship between bile salts and pruritus has not been clarified^[11], although some metabolites of bile salts may contribute to pruritus^[12]. Second, endogenous opioid levels have been reported increased in cholestatic patients^[12,13]. Activation of μ -opioid receptors may cause pruritus by reducing pain signaling, with μ -opioid receptor antagonists showing antipruritic effects in patients with chronic cholestasis^[14,15]. However the correlation between those increased opioid levels and pruritus remains unclear^[12,13]. Thus, these mechanisms could not fully explain the pathogenesis of pruritus. Furthermore, conditions that may cause skin itching are often found in cirrhotic patients. These include hyperhemodynamic conditions and skin dryness caused by administration of diuretics for hepatic edema^[16], complicating the mechanism by which pruritus is induced in cirrhotic patients.

MECHANISM OF PRURITUS IN THE PERIPHERY

Research in recent decades has clarified mechanisms of pruritus induction in the periphery (Figure 1). The first involves an itch-selective pathway, consisting of slow-conducting C-fibers insensitive to mechanical stimuli, which convey itch signals that are distinct from pain transmission^[17,18]. Mechanical stimuli such as pain and touch are transmitted through myelinated fast-conducting fibers with larger diameter and competitively inhibit itch-transmission^[19-21], thus explaining reason why itching is diminished by scratching^[22]. Scratching damages the skin barrier, inducing the release of

substance P or calcitonin gene-related peptide, thereby increasing pruritus^[23].

Several pruritogens and their receptors specific to itch signaling have been identified. Histamine and the histamine H1 and H4 receptors are considered the main contributors to itch signaling^[24]. The H1 receptor interacts with phospholipase C β 3 (PLC β 3)^[25] and transient receptor potential (TRP) vanilloid receptor subfamily V1 (TRPV1), which constitute a nociceptive ion channel^[26]. Protease-activated receptors (PAR) 2 and 4 are thought to be involved in itch signaling^[27], and PAR2 activation may sensitize TRPV1, thereby contributing to itch^[28,29]. Serotonin is another pruritogen, which, together with PLC β 3, acts on G-protein-coupled receptors (GPCR)^[26], such as NK-1, a GPCR shown to play a critical role in serotonin-mediated itch^[30]. The Mrg subtype A3 (MrgA3), a type of GPCR, is also involved in itch signaling^[31,32] by interacting with TRPV1^[32]. Thus, the mechanisms underlying itch signaling is complicated. Other pruritogens may indirectly trigger itch signaling. For example, mast cells are associated with itch not only by releasing histamine but other pruritogens^[33]. Cytokines produced by immune cells are also involved in itch^[22]. Keratinocytes (KCs) may also be associated with itching. These cells express the potential itch-associated molecules TRPV3 and 4^[34], with TRPV3 expression associated with allergic dermatitis^[35]. Skin inflammation suggests the involvement of immune cells^[22].

Gastrin-releasing peptide (GRP) may act as an itch transmitter^[36]. GRP is an itch-selective neurotransmitter of dorsal root ganglia (DRG) neurons that activates the GRP receptor (GRPR) on spinal neurons specific to itch not but to pain^[37]. The vesicular glutamate transporter (VGLUT) 2 was also shown to be involved in itch-selective neurotransmission^[38,39].

Lysophosphatidic acid (LPA) has been regarded as a specific target pruritogen/neurotransmitter in patients with cholestasis^[11,12]. LPA is generated from lysophosphatidylcholine (LPC) by autotaxin (ATX)^[40,41]. LPA and ATX were shown to be increased in cholestatic patients, suggesting they may be potential therapeutic targets^[11,12,42]. Serum ATX level was also shown to be increased by the administration of oral contraceptives to healthy females, and was a potential good indicator of intrahepatic cholestasis of pregnancy (ICP)^[43]. The metabolism of sex hormones is impaired in cirrhotic livers, accompanied by overt feminization^[44], thereby partly explaining the mechanism of ATX-induced pruritus in cirrhotic patients. In addition, the G-protein-coupled bile acid receptor 1, TGR5, encoded by *GPBAR1* and expressed on sensory nerves, was recently shown to be involved in pruritus by stimulating the release of neuropeptides in the spinal cord^[45]. TGR5 was also found to activate the transient receptor potential ankyrin 1 (TRPA1) and to induce pruritus^[46]. Thus many pruritogens, especially those specific to cholestatic conditions, have been identified.

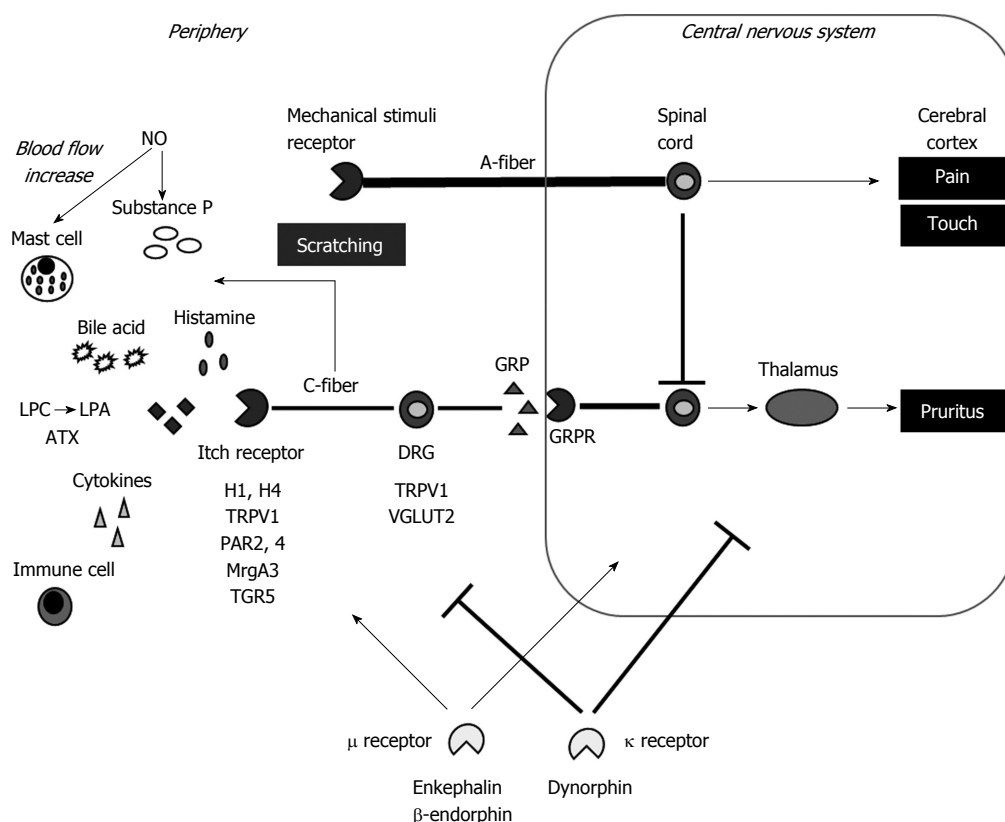


Figure 1 Mechanism of itch transmission in cholestatic conditions. Pruritogens such as histamine, bile acids, bile acid metabolites, LPA and cytokines act on itch receptors. Itch signals are transmitted through C-fibers to the cerebral cortex. The μ and κ opioid receptors are expressed in both the central nervous system and the periphery nerve and act to promote and suppress pruritus, respectively. Signals generated by mechanical stimuli compete with itch signals. Scratching induces C-fibers to secrete substance P, and acts as a pruritogen. NO: Nitric oxide; LPC: Lysophosphatidylcholine; ATX: Autotaxin; LPA: Lysophosphatidic acid; GRP: Gastrin-releasing peptide; GRPR: Gastrin-releasing peptide receptor; TRPV1: Transient receptor potential vanilloid receptor subfamily V1; PAR: Protease-activated receptors; MrgA3: Mrg subtype A3; TGR5: G-protein-coupled bile acid receptor 1; VGLUT2: Vesicular glutamate transporter 2.

MECHANISM OF PRURITUS IN THE CENTRAL NERVOUS SYSTEM

Pruritus also involves the central nervous system (CNS). For example, the most common adverse event of the μ -opioid receptor agonist morphine is pruritus^[47,48]. The μ -opioid receptor antagonist naloxone, however, inhibits morphine-induced pruritus^[49], and suppresses pruritus in patients with chronic cholestasis^[50]. Plasma concentrations of the μ -opioid receptor agonists methionine enkephalin and β -endorphin were shown to be increased in patients with cirrhosis, as ascites increased due to decreased hepatic elimination^[51,52]. The liver plays a major role in the elimination of blood-derived opioid peptides^[53]. These findings suggest that the μ -opioid receptor system is involved in pruritus sensations in patients with liver diseases^[54]. In contrast, the κ -opioid receptor was shown to suppress pruritus. The κ -receptor agonist, nalfurafine (TRK-820) [(E)-N-[17-(cyclopropylmethyl)-4,5 α -epoxy-3,14-dihydroymorphinan-6 β -yl]-3-(furan-3-yl)-N-methylpiperidine-2-enamide monohydrochloride], was shown to suppress anti-histamine-resistant pruritus in a mouse model^[54], whereas pruritus was not neutralized by the peripheral administration of the κ -opioid receptor

antagonist nor-binaltorphimine^[54]. Nalfurafine was also found to suppress pruritus induced by the intracisternal administration of morphine^[55]. Similar results were observed in a rat model of cholestasis induced by treatment with ethynylestradiol (EE), in which the levels of expression of the κ -receptor agonist dynorphin and nitric oxide (NO) were decreased^[56]. Nalfurafine showed anti-pruritic activity in this model, an activity partly mediated by NO systems^[56]. These model indicates that NO is involved in mediating the antipruritic effect of κ -receptor action^[56].

NO expression is enhanced in patients with cirrhosis and primary biliary cholangitis, with NO being a main contributor to hyperdynamic circulation in patients with cirrhosis^[16,57]. NO was shown to enhance substance P-induced scratching in the periphery, whereas a NO synthase inhibitor suppressed this scratching in a dose dependent manner^[58]. NO induces vasodilatation^[59], suggesting it increases peripheral blood flow. Thus the contribution of NO to pruritus remains still controversial and requires further investigation. Furthermore β and κ receptors have been shown to be distributed also in peripheral nerves and contribute to the development of pruritus^[60,61]. Thus their mechanisms of action are complicated between the periphery and CNS,

especially in patients with chronic liver diseases, making understanding of the pathogenesis of pruritus in these patients difficult and making them refractory to treatment.

PREVALENCE AND BURDEN OF PRURITUS IN LIVER DISEASES

Chronic cholestatic diseases

Primary biliary cholangitis (PBC), formerly called primary biliary cirrhosis, is a representative chronic cholestatic liver disease manifesting pruritus. Pruritus is found in about 70% of patients with PBC^[2,62] and precedes the diagnosis of PBC in about 75%^[62]. Pruritus has been shown to impair quality of life, such as sleep, in patients with PBC^[62,63].

Primary sclerosing cholangitis (PSC) is also associated with pruritus during the course of disease progression. In contrast to PBC, most patients with PSC are asymptomatic at the time of diagnosis; therefore the exact prevalence of pruritus in patients with PSC remains unclear^[64].

Pruritus in patients with PBC and PSC manifests frequently in the limbs, particularly in the palms and soles^[1,65]. Multivariate analysis showed that serum alkaline phosphatase activity and Mayo risk score were independent predictors of pruritus in patients with PBC^[66]. Severe pruritus limits daily life activities and causes fatigue, depression and even suicidal tendencies, becoming an indication for liver transplantation in some patients^[8,67,68].

Chronic hepatitis and cirrhosis

Pruritus was observed in four of 49 (8%) patients with chronic hepatitis B and 42 of 210 (20%) with chronic hepatitis C^[69]. Studies of large cohorts found that the proportion of HCV-infected patients with pruritus ranged from 2.5% of 1060 patients^[70] to 15% of 1614 patients^[71]. Pruritus in these patients was not caused by cholestasis^[70], whereas liver fibrosis progression was a risk factor contributing to pruritus^[71].

Other cholestatic conditions

Pruritus is a defining symptom of intrahepatic cholestasis of pregnancy (ICP), a condition characterized by increases in serum bile acid concentrations and increased rates of adverse fetal outcomes^[72]. Pruritus in ICP is usually localized to the palms and soles^[73]. The incidence of ICP was reported to be 1.5%, with increased fetal complications occurring at serum bile acid concentrations > 40^[74].

Familial intrahepatic cholestasis, such as benign recurrent intrahepatic cholestasis (BRIC), is an autosomal recessive disorder associated with canalicular transport defects resulting from mutations in *ATP8B1*, *ABCB11* and *ABCB4*. The phenotype is ranging from BRIC to progressive familial intrahepatic cholestasis according to the severity of disease^[75].

BRIC is characterized by intermittent jaundice and pruritus, and the clinical symptoms may be severe, last from several weeks to months and usually resolve spontaneously^[75,76].

Benign obstructive jaundice has been associated with a lower rate of pruritus than malignant obstruction. For example, pruritus was observed in 16% of patients with benign biliary obstruction such as choledocholithiasis, but in up to 45% of patients with malignant obstruction such as carcinoma of the pancreatic head^[77].

TREATMENT OF PRURITUS

The guidelines of the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) include criteria for the management of cholestatic pruritus in patients with PBC and PSC^[78,79]. Recommendations to all patients should include the use of moisturizing and cooling ointments and shortening of the fingernails to avoid secondary skin damage. The principal goals of treatment include^[5]: (1) removal of pruritogens such as cholestyramine or biliary drainage in the absence of cholestasis; (2) alteration of the metabolism of presumed pruritogens such as rifampicin; (3) modulation of itch signaling such as opioid receptor acting agents; and (4) removal of potential pruritogens by anion absorption, plasmapheresis or extracorporeal albumin dialysis (Figure 2).

Ursodeoxycholic acid (UDCA) is an established drug for the management of PBC and PSC^[80,81]. Although associated with biological and histological improvements and improving overall survival^[80,81], UDCA was ineffective in relieving cholestatic pruritus in both PBC and PSC^[66,82]. In patients with ICP, however, UDCA not only improved biological parameters such as aspartate aminotransferase and alkaline phosphatase concentrations, but ameliorated pruritus^[74,83]. UDCA is used for BRIC to stimulate hepatobiliary secretion of bile salts. Antiapoptotic effects of UDCA are also expected to protect hepatocytes in the treatment for BRIC^[75].

Cholestyramine, a bile acid resin, has been recommended as the treatment of choice for patients with cholestatic pruritus, as it was shown effective in randomized studies with small numbers of patients (eight and 10, respectively)^[84,85]. Although generally well-tolerated, cholestyramine has several side effects, including unpleasant taste, fat malabsorption, constipation, anorexia and gastrointestinal discomfort^[86].

A meta-analysis of five prospective randomized control trials showed that rifampicin, a pregnane X receptor (PXR) agonist commonly used to treat mycobacterial infection, was effective in treating chronic pruritus^[87]. Rifampicin was shown to reduce ATX expression *in vitro* by a PXR-dependent mechanism^[42]. Although safe as short-term treatment of chronic pruritus, rifampicin was associated with hepatotoxicity in up to 13% of patients after treatment

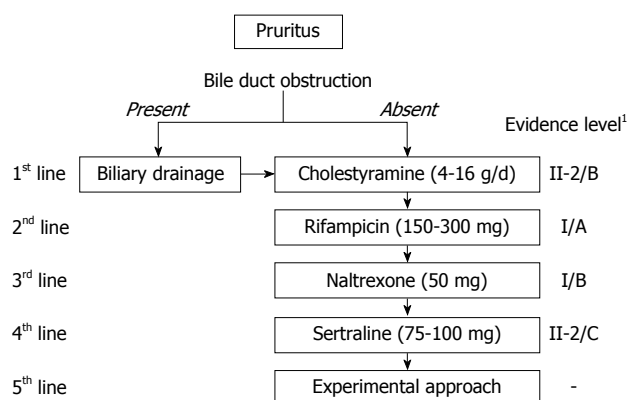


Figure 2 Therapeutic recommendations for the management of cholestatic pruritus modified from American Association for the Study of Liver Diseases guidelines. ¹Evidence level involves categories of evidence and evidence grading. Categories of evidence include: I, randomized controlled trials; II-1, controlled trials without randomization; II-2, cohort and case-control analytic studies; II-3, multiple time series, dramatic uncontrolled experiments; and III, opinions of respected authorities, descriptive epidemiology. Evidence grading includes A: High quality, indicating that further research is unlikely to change confidence in the estimate of effect; B: Moderate quality, indicating that further research may have an important impact on confidence in the estimate of effect and may change that estimate; and C: Low quality, indicating that further research is very likely to have an important impact on confidence in the estimation of effect and is likely to change that estimate. Any change of estimate is uncertain.

for several weeks or months^[88]. Adverse effects that may lead to discontinuation of therapy include nausea, loss of appetite, hemolytic anemia, renal failure and thrombocytopenia^[87,89]. Careful monitoring of blood count and liver function tests is required during administration of rifampicin for cholestatic pruritus, and administration for more than 2 wk is not recommended^[90].

Prospective placebo-controlled showed that the μ -opioid receptor antagonist naltrexone was effective in treating cholestatic pruritus^[91-94]. Common side effects of opioid antagonists include opiate withdrawal reactions, particularly during the first days of therapy. Contraindications to naltrexone include acute liver injury and severe liver insufficiency. Opioid antagonists should be avoided in patients with drug addictions and those taking opioid containing medications^[95]. In Japan, nalfurafine, a κ -opioid receptor agonist, is available for the treatment of pruritus in chronic liver diseases (2.5-5 μ g/d). Nalfurafine is metabolized predominantly by cytochrome P450^[96], but its main metabolite has no pharmacological activity, suggesting its availability and effectiveness for treatment of patients with advanced liver diseases^[97]. Recently a randomized controlled trial showed the effectiveness of nalfurafine by small dose (2.5 or 5 μ g/d) for refractory pruritus with chronic liver diseases^[98].

Sertraline, a selective serotonin re-uptake inhibitor (SSRI), is a fourth-line therapeutic option for patients with cholestatic pruritus. Sertraline (75-100 mg/d) was well-tolerated and showed moderate anti-pruritic effects in a randomized trial with a small number of

patients^[99]. Because sertraline is largely metabolized in the liver, careful administration (e.g., lower or less frequent dosing) should be considered in patients with advanced liver diseases. Sertraline should not be administered to patients who treated with monoamine oxidase inhibitors for the previous 14 d or those concurrently taking pimozide, and oral sertraline concentrate should not be administered together with disulfiram^[90].

The AASLD and EASL guidelines both recommend that patients who show no improvement on these standard therapies be treated by experimental approaches. Case studies have described methods such as plasmapheresis^[100,101], albumin dialysis using a molecular absorbent recirculating system (MARS)^[102-104], plasma separation and anion absorption^[105], ultraviolet B phototherapy^[106], nasobiliary drainage^[107] and surgical intervention such as partial biliary diversion^[75]. Little evidence is available for the effectiveness of these approaches, suggesting a need for validation prior to standard use. Furthermore therapeutic options recommended by guidelines lack strong evidence, except for rifampicin as second line-treatment. However, rifampicin cannot be administered for longer than 2 wk. Because pruritus is found in patients with chronic liver diseases, especially cirrhosis, therapeutic modalities tolerable for longer times are needed. A large-scale ($n = 337$), placebo-controlled study showed that nalfurafine was effective and safe in hemodialysis patients with uremic pruritus resistant to conventional treatments^[108] and that this treatment was tolerable for 52 wk^[109]. Its effectiveness in treating pruritus in patients with chronic liver diseases was also shown by a randomized controlled study ($n = 318$)^[98]. Its tolerability is under investigation. The effectiveness and tolerability in non-Japanese or non-Asian people are desired.

Recent studies have assessed the association of nuclear receptors with the homeostasis of bile acids, with farnesoid X receptor (FXR) shown to regulate bile acid synthesis^[110,111]. Obeticholic acid, a FXR agonist, showed significant improvements in biochemical parameters, but increased pruritus rates^[112]. The incidence and severity of pruritus were reported to be independent of PBC disease stage^[113,114], and the mechanism of FXR-induced pruritus remains unknown. The effect of FXR agonist on ATX level should be evaluated.

CONCLUSION

The mechanism of cholestatic pruritus in chronic liver diseases is complex. The various mechanisms at the periphery and in the central nervous system result complicate determinations of its pathogenesis and treatment strategies. Pruritus occurs frequently in patients with chronic liver diseases, especially those with chronic cholestatic diseases, and impairs patient

quality of life. Current treatments for cholestatic pruritus are inadequate, and additional, more effective therapeutic options are required.

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Basic Study

Oxidative stress-induced mitochondrial dysfunction in a normal colon epithelial cell line

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Abstract

AIM

To determine how a normal human colon cell line reacts to microbial challenge as a way to study oxidative stress-induced responses associated with inflammatory bowel disease.

METHODS

Normal human colon epithelial cells (ATCC® CRL.1790™) were stimulated with either heat killed *E. coli* or heat killed murine cecal contents (HKC) and examined for several relevant biomarkers associated with inflammation and oxidative stress including cytokine production, mitochondrial autophagy and oxidant status. TNF α , IL-1 β and IL-8 protein concentrations were measured within the supernatants. Fluorescent microscopy was performed to quantify the production of reactive oxygen species (ROS) using an oxidation responsive fluorogenic probe. Mitochondrial morphology and mitochondrial membrane potential was assessed by dual staining using COXIV antibody and a dye concentrating in active mitochondria. Mitochondrial ROS scavenger was used to determine the source of ROS in stimulated cells. Autophagy was detected by staining for the presence of autophagic vesicles. Positive controls for autophagy and ROS/RNS experiments were treated with rapamycin and chloroquine. Mitochondrial morphology, ROS production and autophagy microscopy experiments were analyzed using a custom acquisition and analysis microscopy software (ImageJ).

RESULTS

Exposing CRL1790 cells to microbial challenge stimulated cells to produce several relevant biomarkers associated with inflammation and oxidative stress. Heat killed cecal contents treatment induced a 10-12 fold increase in IL-8 production by CRL1790 cells compared to unstimulated controls at 6 and 12 h ($P < 0.001$). Heat killed *E. coli* stimulation resulted in a 4-5 fold increase in IL-8 compared to the unstimulated control cells at each time point ($P < 0.001$). Both heat killed *E. coli* and HKC stimulated robust ROS production at 6 ($P < 0.001$), and 12 h ($P < 0.01$). Mitochondrial morphologic abnormalities were detected at 6 and 12 h based on reduced mitochondrial circularity and decreased mitochondrial membrane potential, $P < 0.01$. Microbial stimulation also induced significant autophagy at 6 and 12 h, $P < 0.01$. Lastly, blocking mitochondrial ROS generation using mitochondrial specific ROS scavenger reversed microbial challenge induced mitochondrial morphologic abnormalities and autophagy.

CONCLUSION

The findings from this study suggest that CRL1790 cells may be a useful alternative to other colon cancer cell lines in studying the mechanisms of oxidative stress events associated with intestinal inflammatory disorders.

Key words: Colon cancer cell line; CRL1790 cells; Inflammation; Mitochondria; Microbial stimulation; Interleukin-8; Autophagy

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Core tip: The normal human colon cell line, CRL1790, can recapitulate oxidative stress-induced responses associated with inflammatory bowel disease following microbial challenge including enhanced production of reactive oxygen species (ROS), inflammatory cytokines, and enhanced mitochondrial autophagic responses. Scavenging mitochondrial ROS inhibited mitochondrial morphologic changes and autophagy suggesting that CRL1790 cells can be used to study oxidative events associated with intestinal inflammatory disorders.

Packiriswamy N, Coulson KF, Holcombe SJ, Sordillo LM. Oxidative stress-induced mitochondrial dysfunction in a normal colon epithelial cell line. *World J Gastroenterol* 2017; 23(19): 3427-3439 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3427.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3427>

INTRODUCTION

Oxidative stress-induced damage to intestinal epithelial cells is a key event in the initiation and progression

of pathologies associated with multiple intestinal inflammatory disorders including ulcerative colitis, colon cancer and enteritis^[1-3]. The intestinal epithelial layer is uniquely tasked with maintaining tolerance to commensal bacteria while recognizing and initiating immune responses to pathogenic infectious agents. Once tolerance is breached, immune and epithelial cells respond to commensal and pathogenic bacteria in an exaggerated manner and produce inflammatory mediators and reactive oxygen species (ROS) that can not only damage DNA, proteins and lipids^[4,5], but also eventually lead to activation of apoptotic pathway that destroys the epithelial cell layer. Mitochondria are a primary target for ROS-induced damage to epithelial cells and are also the primary source of intracellular ROS produced by oxidative phosphorylation. Exposure of organelles to modest amounts of ROS will activate important cytoprotective processes such as the autophagy pathway, which is designed to maintain cellular homeostasis during times of stress by clearing or recycling damaged organelles. However, excessive ROS generation overwhelms the protective function of the autophagy pathway leading to activation of apoptotic cell death and eventually causing loss of mucosal barrier^[6,7].

In vitro models studying oxidative stress response in intestinal epithelial cells are needed to understand the pathophysiology of oxidative stress in causing cellular damage. Currently, there are many colon cancer cell lines including HCT116, SW620, and Caco-2 that are used to assess the oxidative damage induced dysfunction of epithelial cells in conditions like microbial gastro-enteritis, ulcerative colitis, and Crohn's disease^[8,9]. Many of these cell lines tend to underestimate or overestimate the cellular oxidative responses because of their inherent resistance to oxidative stress, changes in endogenous antioxidant levels, altered expression or activation of detoxifying systems, and altered susceptibility of mitochondria and genetic components to ROS attack^[10,11]. Additionally, these cancer cell lines likely respond differently to microbial stimuli compared to normal human intestinal epithelium. For example, intestinal neoplastic cells have abnormal chromosome numbers (chromosome number: Caco-2 -96, HCT116-45, sw620-50)^[12-14] and react differently to various stimuli and stress factors compared to primary cells^[15,16]. Proteomic studies comparing cancer cell lines with primary cells lines showed distinct alterations in metabolic pathways suggesting that neoplastic cell lines may not be the best choice for disease models^[17]. Primary colon epithelial cells obtained from patient biopsy samples can be used to model oxidative stress during gastrointestinal disorders. However, limited cell recovery, a lack of reproducibility of experimental data, and procedural costs make the use of primary cell model impractical^[18]. The CRL1790 cells are an intestinal epithelial cell line isolated from normal human

neonatal intestine and are successfully maintained under laboratory conditions^[19,20]. The CRL1790 cells have a normal diploid chromosome number, are easy to propagate at laboratory conditions and are cost effective. The current study proposes an *in vitro* cell culture model using the CRL1790 normal human colon epithelial cells as an alternative to using other cancer cell lines to study oxidative stress responses to microbial exposure. Murine heat killed cecal contents (HKC) and heat killed *E. coli* were used to induce inflammation and associated oxidative stress. Inflammatory cytokine production, ROS generation, mitochondrial and autophagic responses were measured. Our results suggest that CRL1790 cells may be used to model *in vitro* characteristics of epithelial cell mitochondrial dysfunction during inflammation-induced oxidative stress.

MATERIALS AND METHODS

Cell culture

CCD 841 CoN (ATCC® CRL1790™; Manassas, VA, United States) normal human colon epithelial cells were obtained from ATCC and maintained at 37 °C, 5% CO₂ in MEM supplemented with 3% FBS, 2 mmol/L L-glutamine, penicillin-G (100 U/mL), and streptomycin (100 µg/mL). Colon cells ≤ 9 passages were grown as monolayers until confluent, harvested with trypsin-treatment at 37 °C for 5 min and plated for experiments. Media was replaced 24 h after plating and the cells were allowed to adhere for 48 h prior to experimental treatments.

Heat killed *Escherichia coli* and heat-killed cecal contents

Escherichia coli (ATCC® 25922™) was obtained from ATCC. *E. coli* was heat killed and used for experiments. Briefly, *E. coli* were grown in trypticase soy broth with gentle shaking to 37 °C to stationary phase. The bacteria were washed with PBS before cultures were adjusted to 1.0×10^5 cells per 1 µL. Bacterial cultures were then heat-killed at 80 °C for 30 min and penicillin-G (100 U/mL) and streptomycin (100 µg/mL) added prior to freezing and storage at -80 °C. To ensure complete killing of *E. coli*, aliquots were plated on trypticase soy agar and checked for growth. Murine HKC contents were prepared according to previously published methods^[21]. Briefly, 25 mg of cecal contents were mixed with 1 mL sterile HBSS and filtered twice through nylon mesh to remove large particles. Filtered supernatants were heat-killed at 80 °C for 30 min then centrifuged at $150 \times g$, for 5 min to remove remaining large particulate matter. Penicillin-G (100 U/mL) and streptomycin (100 µg/mL) were added to supernatants and aliquots frozen and stored at -80 °C.

Epithelial cell treatments

Epithelial cell monolayers were treated with heat killed *E. coli* (ATCC® 25922™) at multiplicity of infection

(MOI) = 1 or with 200 µg of HKC contents per 2.0×10^5 cells^[21] which served as a positive control. To study mitochondrial dysfunction, a positive control was created by adding carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, St. Louis, MO, United States) to a final concentration of 10 µmol/L to control wells for 90 min at 37 °C, 5% CO₂ to induce mitochondrial fission and abrogate outer membrane potential^[22,23]. Positive controls for autophagy-induction were treated with 500 nmol/L rapamycin and 10 µmol/L chloroquine for 16 h at 37 °C, 5% CO₂^[24].

ROS generation measurement

ROS generation from CRL1790 cells was measured by loading Carboxy-H₂DCFDA dye before exposure to microbial ligands as described earlier^[25]. Carboxy-H₂DCFDA is non-fluorescent but in the presence of ROS, this reagent is oxidized, and becomes green fluorescent, which is then detected using fluorescence plate reader. Briefly, CRL1790 cells were cultured in 96 well tissue culture treated plate at a concentration of 5×10^5 cells/well for 18 h. Supernatants were removed and incubated with HBSS containing 5 µmol/L of the Carboxy-H₂DCFDA dye for 30 min. Cells were then washed to remove excess dye and fresh media containing serum was added. Additionally, the cells were treated with heat-killed *E. coli*, HKC or H₂O₂ (100 µmol/L for 1 h) for specified time points. ROS generation was then detected by measuring fluorescence at 490/520 (Ex/Em) wavelengths using Tecan Infinite M200 Plate reader. Background fluorescence from the cells was subtracted from the fluorescence values obtained after loading the cells with carboxy-H₂DCFDA dye. Data are represented as fluorescence intensity.

Additionally, ROS generation was measured microscopically using cell permeable CellROX® Deep Red dye (Life Technologies Corp., Grand Island, NY, United States). Briefly, CRL1790 cells were grown as monolayers to confluence, harvested, and seeded onto sterile cover slips within 6-well dishes at 4×10^5 cells per well. Cells were allowed to adhere for 48 h at 37 °C before performing treatments. Cells were then treated with microbial ligands for specific time points. 5 µmol/L of CellROX® Deep Red dye was added to each well and cells were incubated at 37 °C, 5% CO₂ for 30 min. Media containing CellROX stain was removed and monolayers were washed with 1 × PBS and fixed with 3.7% paraformaldehyde for 15 min at 37 °C. Coverslips were processed for microscopy as described below.

Immunofluorescence microscopy

For microscopy experiments, CRL1790 cells were grown as monolayers to confluence, harvested, and seeded onto sterile cover slips within 6-well dishes at 4×10^5 cells per well. Cells were allowed to adhere for 48 h at 37 °C before performing treatments as described above. For mitochondria experiments,

500 nmol/L MitoTracker® (Life Technologies Corp., Grand Island, NY, United States) was added to each well and incubated for 30 min at 37 °C. MitoTracker stain is preferentially absorbed by mitochondria with intact outer membrane potential and reflects viable mitochondria. Diminished staining by mitochondria reflects disrupted membrane potential of non-viable mitochondria. Excess MitoTracker stain was removed after the 30 min incubation, chased with 1 × PBS for 15 min at 37 °C and fixed with 3.7% paraformaldehyde at 37 °C for 15 min. Following fixation, cells were permeabilized for 10 min with 0.1% Triton X-100 and blocked for 1 h with PBS containing 3% bovine serum albumin (BSA). Mouse monoclonal anti-COXIV (cytochrome c oxidase IV) (1:1000) antibody (Abcam, Cambridge, MA, United States), rabbit polyclonal anti-DRP1(1:100) (Santa Cruz Biotechnology, TX, United States), anti-MFN2 (1:100) (Santa Cruz Biotechnology, TX, United States) were used to stain mitochondria and incubated at room temperature for 1 h. Anti-mouse Alexa Fluor® 555 and anti-rabbit Alexa Fluor® 488 (1:500 dilution) were used as secondary antibodies to detect COXIV, DRP1 and MFN2 (Life Technologies Corp. Grand Island, NY, United States).

For experiments using MitoTempo as mitochondrial ROS scavenger, a final concentration of 25 nmol/L MitoTempo (Sigma Aldrich, St. Louis, MO, United States) was added to the cell culture 12 h prior to stimulation with HKC or *E. coli*. All cell nuclei were stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) for 15 min at room temperature. Autophagy experiments utilized the Cyto-ID® Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, United States) to detect autophagic vesicles and cells were processed according to manufacturer specifications. Briefly, cells were washed with assay buffer and incubated with a dual detection reagent containing Cyto-ID Green Detection Reagent and Hoechst stain for 30 min at 37 °C. After incubation, cells were washed with PBS and fixed with 4% paraformaldehyde. Cells were imaged immediately. Coverslips with cells for all experiments were mounted with Prolong® Gold anti fade mounting agent (Life Technologies Corp, Grand Island, NY, United States). Fluorescent images were taken at 60x (oil) magnification with a Zeiss Axiovert 200M and black and white AxioCam MRm (Zeiss) camera. All treatments were performed in duplicate and experiments were repeated a minimum of 3 times.

Cytokine and chemokine measurements

Cytokines including TNF α , IL-1 β and IL-8 were measured in the supernatants from CRL1790 cells treated with HKC- and heat-killed *E. coli*. Briefly, CRL1790 cells were grown as monolayer to confluence, harvested, and seeded in 6-well dishes at 4 × 10⁵ cells per well. Cells were treated with HKC contents or heat-killed *E. coli* for 6 or 12 h and supernatants were collected. TNF α , IL-1 β and IL-8 kits were obtained from eBiosciences (San

Diego, CA, United States) and samples were analyzed per manufacturer's instructions. All values were represented as pg/mL of media. All treatments were performed in duplicate and experiments were repeated a minimum of three times.

Microscopy quantification and statistical analysis

For each coverslip, 10 fields were captured and analyzed resulting in 20 fields per treatment for each experiment. CellROX and Mitotracker and autophagy microscopy experiments were analyzed using ImageJ 1.46/Java 8 software (National Institute of Health, Bethesda, MD, United States) as described previously^[26]. Briefly, individual cells in each image were selected and analyzed using the measurement command. Area, integrated density and mean gray value were collected. Additional measurements were made of areas without fluorescence adjacent to cells as background. Corrected total cell fluorescence (CTCF) was calculated using the equation: CTCF = integrated density - (area of selected cell × mean fluorescence of background). Mitochondrial morphology was analyzed from MitoTracker images using ImageJ, Mito-Morphology Plugin as described previously^[27]. Measurements of mitochondrial area, perimeter, circularity, minor and major axis as well as total mitochondrial counts were collected for each imaged field. Mitochondrial morphology was characterized by average circularity, area/perimeter ratio as a measure of interconnectivity and inverse circularity reported as a measure of elongation^[27-29]. Statistical significance of cytokine measurements, ROS, and morphology measurements was examined by ANOVA and Tukey's HSD *post hoc* comparison ($P = 0.05$) using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, United States). All results were expressed as mean ± SE.

RESULTS

CRL1790 cells respond to microbial stimulation

Excessive microbial stimulation of colonic epithelial cells is a key event in the progression of many intestinal disorders. Heat killed cecal contents obtained from wild type mice to mimic the population of intraluminal antigens and heat killed *E. coli* (ATCC 25922) were chosen to induce inflammatory responses in CRL1790 cells. The cells were treated with HKC contents or heat killed *E. coli* and supernatants were analyzed for the production of inflammatory cytokines TNF α , IL-1 β and IL-8 using ELISA. Of the 3 cytokines measured, only IL-8 production was significantly increased in HKC and *E. coli* treatment groups compared to untreated controls. Treatment with HKC contents induced 10-12 fold increase in IL-8 production by CRL1790 cells compared to unstimulated controls at 6 and 12 h. Heat killed *E. coli* stimulation resulted in a 4-5 fold increase in IL-8 compared to the unstimulated control cells at

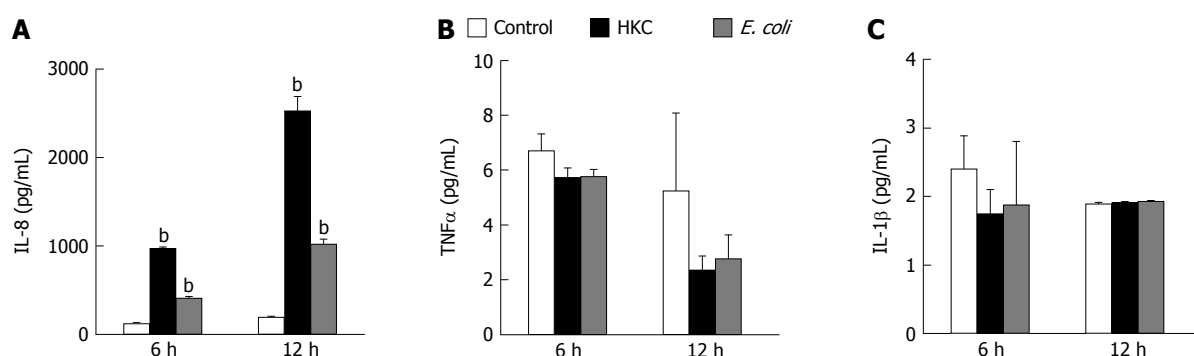


Figure 1 CRL1790 cells produce IL-8, but not TNF α and IL-1 β in response to microbial stimulation. CRL1790 cells were stimulated with heat-killed cecal contents (HKC) or heat-killed *E. coli* for 6 and 12 h. Cellular supernatants were collected and assayed for the production IL-8 (A), TNF α (B) and IL-1 β (C) using ELISA ($n = 3$). ^b $P < 0.01$ vs Control. Data are expressed as mean \pm SE.

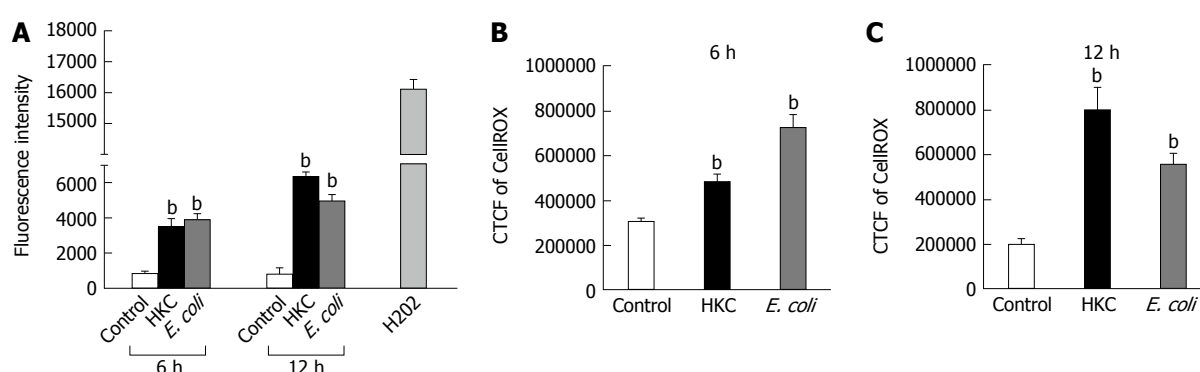


Figure 2 CRL1790 cells generate robust reactive oxygen species upon microbial stimulation. CRL1790 cells were stimulated with heat killed cecal contents (HKC) or heat killed *E. coli* for 6 h and 12 h. A: Fluorescence intensity of the CRL1790 cells preloaded with carboxy-H2DCFDA dye; B: CellROX staining intensity (CTCF- corrected total cell fluorescence) was measured to determine ROS generation in CRL1790 cells ($n = 3$). ^b $P < 0.01$ vs Control. Data are expressed as mean \pm SE. ROS: Reactive oxygen species; CTCF: Corrected total cell fluorescence.

each time point. (Figure 1A). No significant effects of either HKC or killed *E. coli* on TNF α or IL-1 β production were detected at 6 or 12 h (Figure 1B and C).

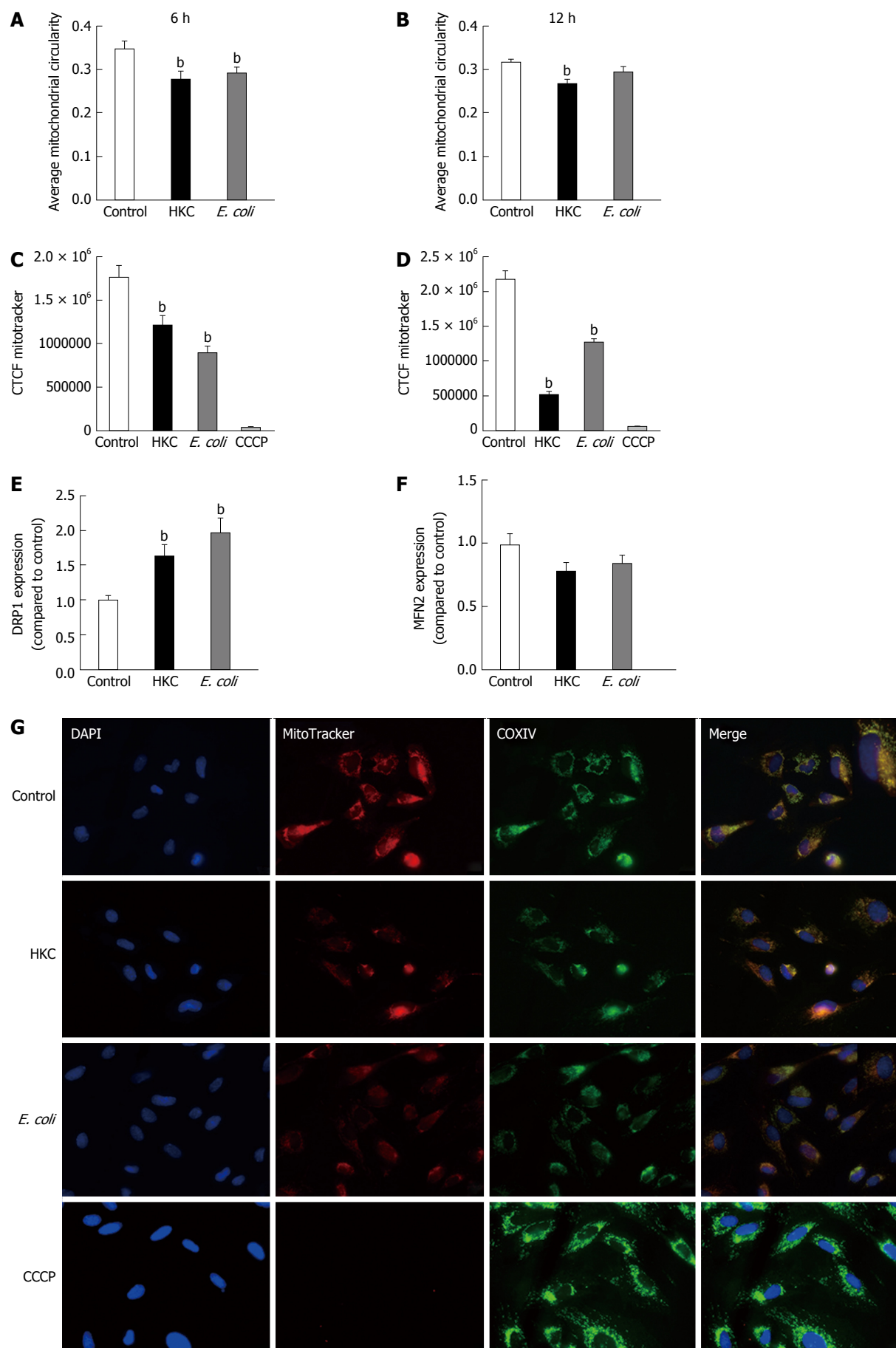
CRL1790 cells generate ROS upon microbial stimulation

ROS are generated during mitochondrial oxidative metabolism as well as in response to bacterial invasion, xenobiotic metabolism and cytokine stimulation. ROS generation was detected microscopically using cell permeable CellROX deep red dye and also by measuring fluorescence emission using carboxy-H2DCFDA dye. CRL1790 cells were grown on coverslips or 96 well plate and treated with HKC contents and heat-killed *E. coli* for 6 and 12 h. ROS generation was quantified microscopically by calculating corrected total cell fluorescence (CTCF) or by measuring fluorescence intensity using a fluorescence plate reader as described in the materials and methods. Both treatments induced a significantly higher production of ROS compared to untreated controls. At 6 h post-treatment, HKC contents and *E. coli* treatment induced 1.6 fold and 2 fold more ROS than untreated controls, respectively (Figure 2). By 12 h post-treatment, HKC and killed-*E. coli* produced 4 and 2.7 fold increases in ROS production, respectively, compared to untreated controls (Figure 2).

Mitochondrial dysfunction is evident during microbial stimulation in CRL1790 cells

To test the impact of ROS generated in response to microbial stimulation, CRL1790 cells treated with HKC contents and heat-killed *E. coli* were assessed microscopically for mitochondrial outer membrane potential and fission fusion dynamics, which are reflective of mitochondrial integrity.

Mitochondrial fission-fusion dynamics can be measured by calculating and comparing average circularity of mitochondria^[27]. Increased average circularity measurements denote more balanced fusion/fission dynamics and increased mitochondrial health. A decrease in average circularity measurements indicate accelerated fission suggestive of mitochondrial dysfunction and stress. Our results showed that average circularity was diminished in the cells stimulated with either HKC contents or *E. coli* (Figure 3A and B). At 6 h post stimulation a small but significant reduction in circularity was measured for both HKC contents and *E. coli*-treated cells. However, by 12 h, HKC stimulation alone showed a significant decrease in mitochondrial circularity. These data clearly demonstrate that CRL1790 cells undergo alteration in mitochondrial fission/fusion dynamics during microbial



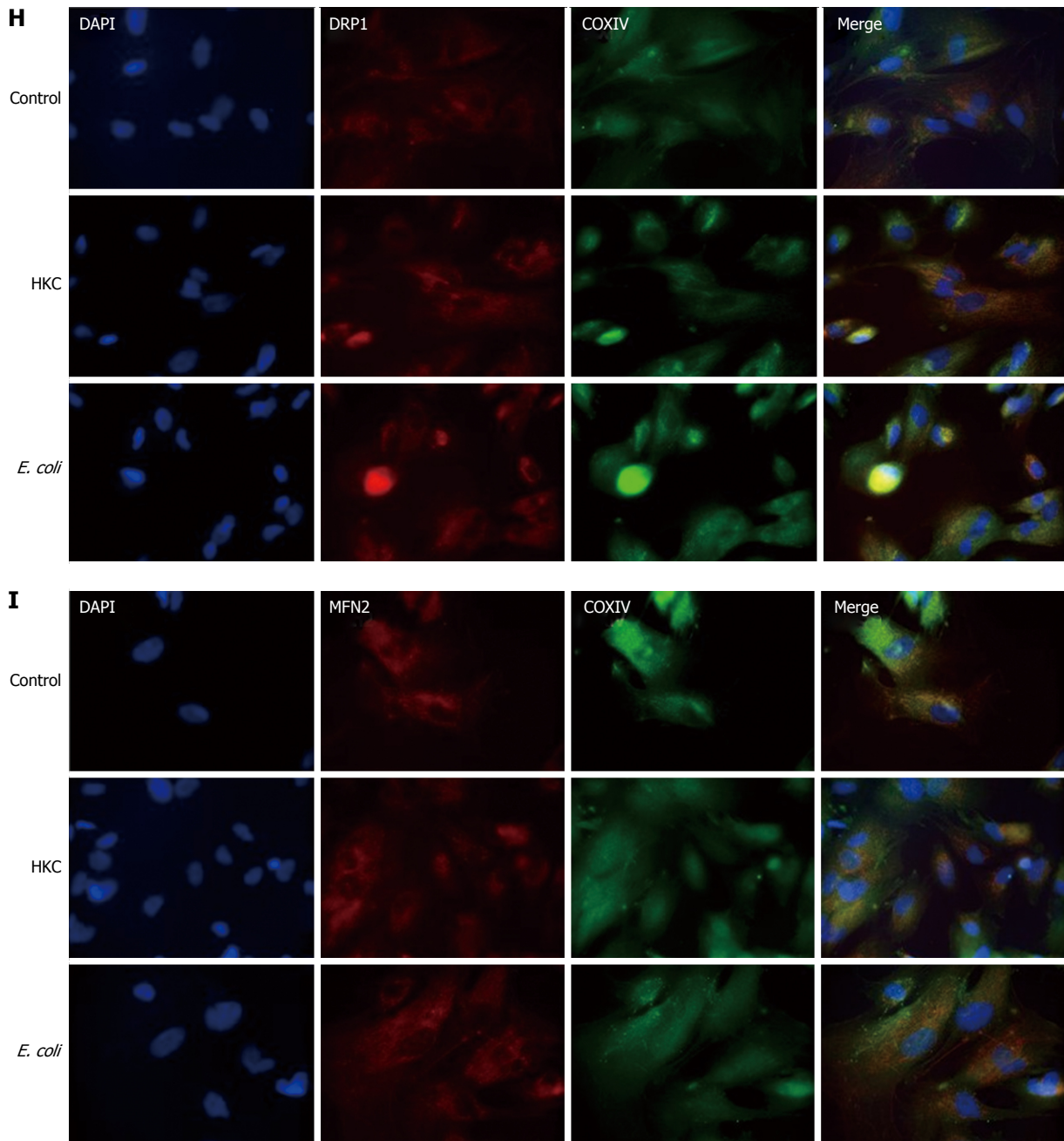


Figure 3 Mitochondrial dysfunction in CRL1790 cells is evident during microbial stimulation. CRL1790 cells stimulated with heat killed cecal contents (HKC) or heat killed *E. coli* for 6 h and 12 h were assessed for mitochondrial function using mitotracker staining. A and B: Denotes average circularity of mitochondria assessed 6 h and 12 h post stimulation respectively; C and D: Denotes intensity of mitotracker staining in cells 6 h and 12 h post stimulation respectively; E and F: Expression of DRP1 and MFN2 in CRL1790 cells treated with HKC or *E. coli* for 6 h and 12 h post stimulation respectively; G: Representative images showing mitotracker staining ($n = 3$) (magnification $\times 60$); H and I: Representative images showing expression of Drp1 and MFN2 (magnification $\times 60$). $^bP < 0.01$ vs Control. Data are expressed as mean \pm SE. CTCF: Corrected total cell fluorescence; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone.

stress potentially contributing to mitochondrial damage. Additionally, expression of Dynamin related protein 1 (DRP-1) (Marker for mitochondrial fission) and Mitofusin (MFN2) (Marker for mitochondrial fusion) were also measured after treating the cells with microbial ligands for 6 h. DRP1 expression was significantly increased in the HKC and *E. coli* treated groups suggesting increased mitochondrial fission. MFN2 expression was decreased in the HKC and *E. coli* treated groups, however the changes weren't significant.

To assess mitochondrial integrity, the CRL1790 cells were stained with MitoTracker after treatments and images were collected and analyzed using ImageJ software as described in the methods. MitoTracker stain is only absorbed by mitochondria with intact outer membrane potential (OMP). Mitochondria were double-labeled with anti-COXIV antibodies as a non-OMP dependent marker of mitochondria, confirming the presence of those organelles lacking fluorescent stain. A subset of cells were treated with CCCP,

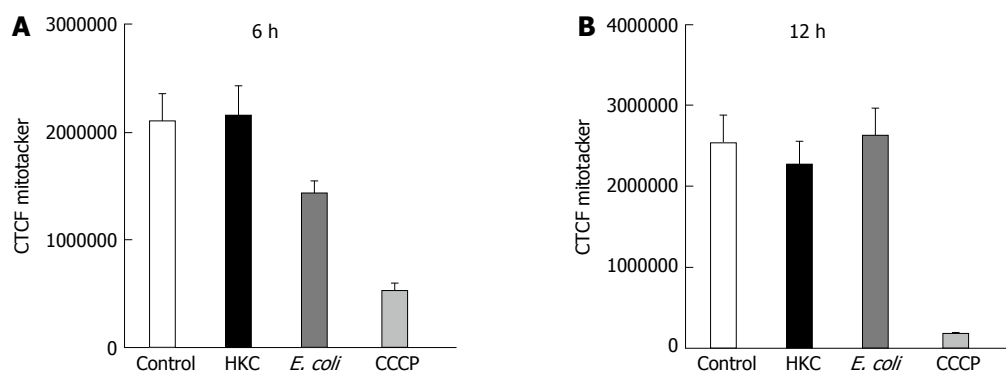


Figure 4 Mitochondrial reactive oxygen species scavenging ameliorates mitochondrial damage in CRL1790. CRL1790 cells pretreated with mitoTempo (Mitochondria specific reactive oxygen species scavenger), stimulated with heat killed cecal contents (HKC) or heat killed *E. coli* for 6 h and 12 h were assessed for mitochondrial function using mitotracker staining. A and B: Denotes intensity of mitotracker staining in cells 6 h and 12 h post stimulation respectively ($n = 3$). Data are expressed as mean \pm SE. CTCF: Corrected total cell fluorescence; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone.

a potent mitochondrial oxidative phosphorylation uncoupler to inhibit mitochondrial activity and serve as negative control. Corrected total cell fluorescence (CTCF) was measured to assess OMP, which indicates intact mitochondrial integrity. Higher fluorescence intensity indicates functioning mitochondria and lower fluorescence reflects destabilization of the mitochondrial membranes. Our results show that, mitochondrial integrity was significantly decreased following both HKC and heat-killed *E. coli* treatments at 6 and 12 h (Figure 3C-E). These data demonstrate that CRL1790 cells respond to microbial stress and undergo alteration mitochondrial function and integrity.

Mitochondrial ROS scavenging ameliorates mitochondrial damage in CRL1790

To test whether ROS produced during the microbial stress was responsible for the decrease in mitochondrial OMP, a mitochondrial-targeted oxidant scavenging molecule (MitoTempo) was used to inhibit ROS-induced mitochondrial damage. Briefly, MitoTempo was added to the cells for 12 h prior to treatments. Cells were stained with MitoTracker to assess the antioxidant impact on the OMP to evaluate mitochondrial integrity. Corrected total cell fluorescence was measured using ImageJ software as described in the methods. There was no significant difference in MitoTracker CTCF between HKC and untreated cells or heat-killed *E. coli* and untreated cells at 6 or 12 h of microbial stimulation. These data demonstrate that scavenging mitochondrial ROS prevented mitochondrial damage and dysfunction (Figure 4), suggesting that most of the ROS produced in the CRL1790 cells following HKC and heat-killed *E. coli* treatment was of mitochondrial origin.

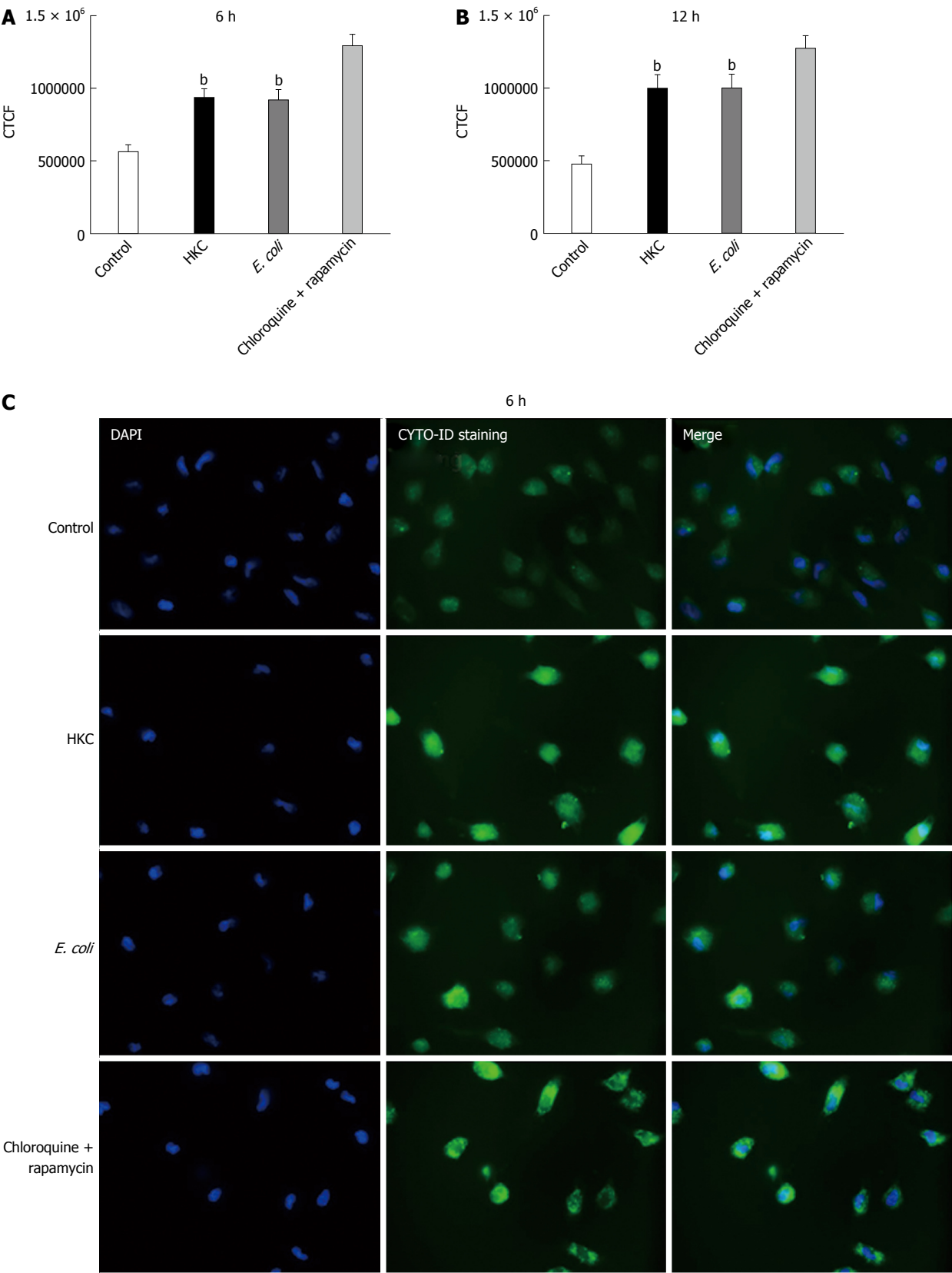
Autophagy is activated upon microbial stimulation in CRL1790 cells

Excessive ROS production, damages organelles mainly targeting lipid and DNA molecules. Under normal conditions, the autophagy pathway promotes cell

repair and survival. However, excessive production of ROS and the resultant oxidative stress can overwhelm the autophagy system and promote cell death. Thus, we evaluated autophagy activation in *E. coli* and HKC treated CRL1790 cells. Staining of autophagic vacuoles using Cyto-ID kit was determined to measure autophagy activation through labeling of autophagic vacuoles. At 6 and 12 h post-stimulation both HKC and *E. coli* treatment induced a significant increase in autophagic vacuoles compared to untreated controls (Figure 5). These data suggest that CRL1790 cells undergo sufficient damage to induce autophagy activation during microbial stress.

DISCUSSION

Studies have shown that cancer cell lines might be ineffective in recapitulating the oxidative stress response and mitochondrial dysfunction evident in inflammatory based intestinal diseases^[10]. Studies comparing oxidative stress responses in normal and cancerous cell lines showed normal cell lines behaved differently when compared to cancerous cell lines like caco-2 cells, likely owing to their altered metabolic and genetic profile. Studies like these highlight the importance of using a cell line that is closely related to normal cells, but at a same time, are practical and efficient for *in vitro* studies. In this study, we tested the usability of CRL1790 normal epithelial cells as a replacement for cancerous colon epithelial cell lines to study oxidative stress response and mitochondrial dysfunction induced by microbial ligands. The results of this study suggest that human colon epithelial CRL1790 cells may be a good alternative for cancer cell lines used in studying cellular oxidative stress response and specifically mitochondrial dysfunction during microbial exposure. The CRL1790 cells successfully recapitulated inflammatory response, induction of oxidative stress, mitochondrial dysfunction and autophagic responses which are key events leading to loss of epithelial cell barrier functions.



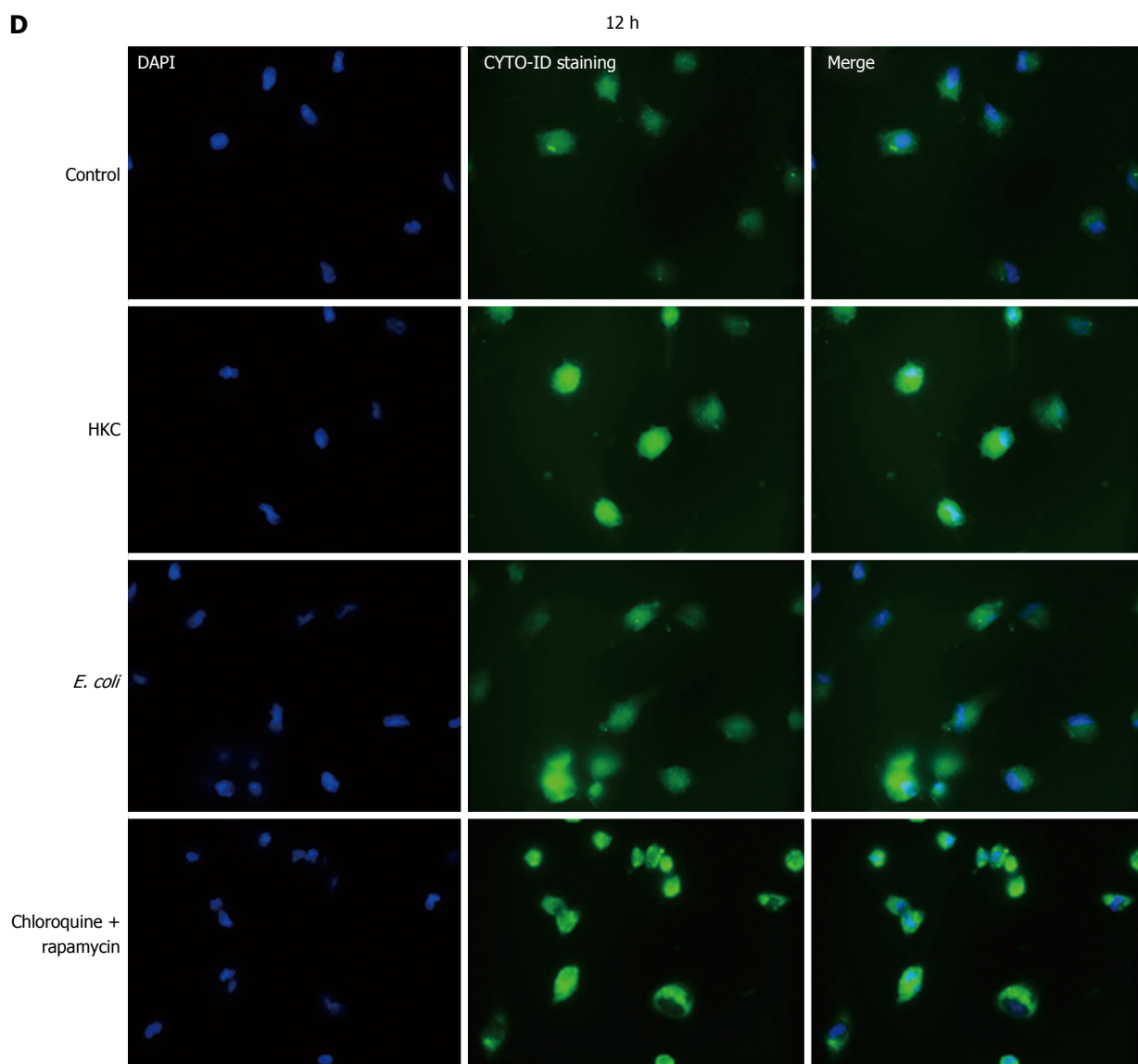


Figure 5 Increased autophagy observed in CRL1790 cells with microbial stimulation. A and B: CRL1790 cells stimulated with heat killed cecal contents (HKC) or heat killed *E. coli* for 6 h and 12 h were assessed for presence of autophagic vesicles using ENZO cytoID ($n = 3$). Chloroquine and rapamycin treated cells were used as positive control; C and D: Representative images showing staining of autophagic vesicles (magnification $\times 40$). $^bP < 0.01$ vs Control. Data are expressed as mean \pm SE. CTCF: Corrected total cell fluorescence.

The CRL1790 epithelial cells demonstrated pro-inflammatory responses following microbial challenge as evidenced by production of IL-8, which is a neutrophil attractant. Other cytokines specifically $\text{TNF}\alpha$ and $\text{IL1}\beta$ were not significantly increased compared to unstimulated controls. This finding is in concurrence with other studies demonstrating that colon epithelial cells respond to $\text{TNF}\alpha$ ^[30,31] and $\text{IL1}\beta$ ^[32] produced by immune cells but do not produce large quantities of these cytokines^[33]. Similarly, in human patients suffering from Crohn's disease and ulcerative colitis, only serum concentration of IL-8 is increased compared to control patients^[34] suggesting IL-8 production can be served as a good biomarker to test colon epithelial cell responses.

During inflammatory responses, increased mitochondrial oxidative phosphorylation is required to

meet increasing cellular demand and this may result in oxidative stress^[29,35]. Excessive accumulation of ROS that leads to oxidative stress can elicit cellular damage through the oxidation of various macromolecules and thus alter their biological functions and potentiate cell death. For example, previous studies^[36-38] highlighted the contribution of mitochondrial dysfunction to loss of epithelial cell integrity, leading to increased epithelial permeability promoting microbial translocation. One mechanism that promotes mitochondrial damage and dysfunction is excessive production of ROS^[37,38]. Mitochondrial ROS generation is considered to be a continuous physiological process under aerobic conditions. During times of microbial stress, however, mitochondrial oxidative phosphorylation is increased leading to generation of additional ROS. Increased ROS production must be neutralized by anti-oxidant

systems to prevent oxidative damage to mitochondria and other cellular organelles. CRL1790 cells exhibited significant mitochondrial morphology changes including reduced circularity and diminished mitochondrial membrane integrity in response to microbial treatments. Previous studies^[39] associated increased mitochondrial fission with decreased oxidative capacity, increased ROS generation and increased autophagy. In our current study, the damage to mitochondria also induced significant activation of autophagic responses demonstrating the ability of these cells to respond to oxidative stress and also potentially initiate recovery processes. We did not test activation of apoptotic pathway as a direct consequence of microbial stimulation in this study. However, mitochondrial stress and alterations in mitochondrial functions are observed in multitude of colon associated disease condition like ulcerative colitis, Crohn's disease and colon cancer. Our results concur with other studies showing mitochondrial dysfunction may be an early event leading to epithelial cell dysfunction observed in and other intestinal infections^[40] and the CRL1790 cells could provide a model system for studying this potential initiating phase of the disease.

Finally, to determine whether scavenging mitochondrial ROS can reverse mitochondrial dysfunction, MitoTempo, a mitochondrial specific ROS scavenger was added prior to treatments and mitochondrial damage was studied. Our results clearly show, by pre-treating CRL1790 cells with MitoTempo decreased the mitochondrial damage and dysfunction, confirming the mitochondria was one of the major sources of ROS generation. Additionally our study also revealed, HKC contents are more potent in inducing inflammation (IL-8 production and ROS generation) compared to heat killed *E. coli*. The increased activity of HKC contents is likely due to multiple microbial sources (bacterial, protozoal, fungal and viral antigens) and possibly due to presence of other luminal antigens, advantageous for researchers trying to model colitis. The different potencies of the microbial challenges may also account for the differential timing of ROS responses following exposure to either *E. coli* or HKC.

In conclusion, our findings indicated that the normal cell line, CRL1790, could be used in a convenient and reliable way to recapitulate both physiological and pathological mitochondrial function associated with intestinal inflammatory disorders. CRL1790 respond to microbial stimulation by increasing ROS and induced autophagic responses that serve as a good model to study oxidative stress responses.

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COMMENTS

Background

Cellular oxidative stress is implicated in the multifactorial etiology of inflammatory bowel diseases and is a key initiating event propagating cellular damage. Cancerous colon cell lines are used to model inflammatory and oxidative stress responses in inflammatory bowel disease (IBD), but their inherent alterations in genome and metabolism may lead to confounding results when investigating underlying mechanisms of disease. There is a need to determine if normal human colon cell lines can react to microbial challenge in a way that can recapitulate oxidative stress-induced responses that are associated with intestinal inflammatory disorders.

Research frontiers

Previous studies have already proven that oxidative stress and mitochondrial dysfunction are key events leading to intestinal inflammatory disorders in humans and animal models.

Innovations and breakthroughs

This is the first study to evaluate the use of normal colonic epithelial cell line as a model to recapitulate oxidative stress-induced mitochondrial dysfunction that is known to be critical in the development of inflammatory bowel diseases.

Applications

The normal cell line, CRL1790, can respond to microbial stimulation by increasing reactive oxygen species (ROS) and inducing autophagic responses that serve as a good model to study oxidative stress responses associated with intestinal inflammatory disorders. The results from this study confirm that CRL1790 can be used in a convenient and reliable way to investigate both physiological and pathological mitochondrial function in response to microbial challenge as a way to investigate the underlying mechanisms associated with intestinal inflammatory disorders.

Terminology

Oxidative stress is an imbalance between the production of ROS and other free radicals that can damage tissues and anti-oxidant defenses of the body that are needed to counteract pro-oxidant damage.

Peer-review

The authors investigated how normal human colon cells can react to microbial challenge as a way to investigate the role of oxidative stress on mitochondrial dysfunction. They were able to show that microbial challenge of CRL1790 could induce oxidative stress-induced responses associated with IBD and that scavenging ROS within the mitochondria during microbial challenge could overcome mitochondrial dysfunction.

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Basic Study

Role of AXL in invasion and drug resistance of colon and breast cancer cells and its association with p53 alterations

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Abstract

AIM

To characterize AXL receptor tyrosine kinase (AXL) expression in relationship to tumor protein P53 (*TP53* gene, p53 protein) and its role in tumor invasion and response to therapy.

METHODS

We used 14 cell lines, including 3 isogenic pairs carrying mutant/knockout p53, to gain insight into the relationship between AXL and *TP53*. These included HCT116, HCT116.p53 mutant, RKO, and RKO.p53^{-/-} lines (all from colon cancers) as well as breast cancer cell lines MCF7 and 1001 (MCF7-p53 mutant clone). HeLa cell line was used as a positive control for epithelial to mesenchymal transition (EMT). AXL expression was determined by Western blotting using rabbit monoclonal antibody clone C89E7. AXL siRNA silencing was

performed and followed by collagen invasion assay. Cell viability analysis using the sulforhodamine B assay and the invasion assay were performed after exposure to chemotherapeutic agents (doxorubicin for breast cancer cells; 5FU or irinotecan for colon cancer cells).

RESULTS

We showed that the introduction of p53 mutations or knockout increased expression levels of AXL in isogenic cells compared to the matching p53 wild-type parental cells. Overall, we found a trend for correlation between the potential EMT candidate AXL, p53 alterations, and EMT markers in colorectal and breast cancers. The expression of AXL in RKO cells, a rare colon cancer cell line with inactive Wnt signaling, suggests that the AXL oncogene might provide an alternative genetic pathway for colorectal carcinogenesis in the absence of Wnt signaling activation and *TP53* mutation. AXL silencing in the *TP53* mutant isogenic cell lines 1001, HCT116. p53 mutant and RKO.P53^{-/-} was > 95% efficient and the silenced cells were less invasive compared to the parental *TP53* wild-type cells. AXL silencing showed a subtle trend to restore colon cancer cell sensitivity to 5FU or irinotecan. Importantly, AXL expressing cells developed more invasive potential after exposure to chemotherapy compared to the AXL-silenced cells.

CONCLUSION

AXL is influenced by p53 status and could cause the emergence of aggressive clones after exposure to chemotherapy. These findings could have applications in cancer management.

Key words: AXL; Breast cancer; Chemotherapy; Colon cancer; Invasion

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Core tip: AXL receptor tyrosine kinase (AXL) is emerging as an attractive molecular target in cancer therapy. We showed that it is regulated by *TP53* in colon and breast cancer cells, and it contributes to epithelial to mesenchymal transition and response to therapy in these tumors. We also showed that it could be linked to other carcinogenic pathways, such as the Wnt/ β -catenin signaling pathway in colorectal cancer. These interactions should be considered carefully when designing AXL based therapy, because AXL could trigger the emergence of aggressive clones after inappropriate therapy.

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INTRODUCTION

Colorectal cancer is the third most common cancer in men (746000 cases, 10.0% of the total) and the second most common in women (614000 cases, 9.2% of the total) worldwide. Additionally, the latest estimates indicate that colorectal cancer is the third leading cause of cancer-related deaths in the United States^[1]. Mortality is higher (52%) in the less developed regions of the world^[1]. Colorectal cancers in regions/ethnicities other than the West, such as the Middle East, are often associated with intriguing clinical characteristics. Examples include a younger age at onset, advanced stage at presentation, and poor prognosis^[2]. Studies have identified contrasting molecular features in specific subsets of colorectal cancers including those that occur in the Middle East^[3]. These data highlight the need for novel molecular therapeutic targets to enable the design of personalized therapy for treatment of colorectal cancer. The receptor tyrosine kinases are attractive targets in this regard^[4,5].

AXL receptor tyrosine kinase (AXL) is a receptor tyrosine kinase in the TAM family (Tyrp3, AXL and Mer). It transduces signals from the extracellular matrix into the cytoplasm by binding to its main ligand: the growth factor "growth arrest-specific 6 (Gas6)"^[6]. Ligand binding induces dimerization and autophosphorylation of AXL, which then binds and induces tyrosine phosphorylation of PI3-kinase, GRB2, PLCG1, LCK and PTPN11. Other downstream substrate candidates for AXL are CBL, NCK2, SOCS1 and TNS2. Recruitment of GRB2 and PI3-kinase regulatory subunits by AXL leads to the downstream activation of the AKT kinase. Thus, AXL signaling plays a role in a wide array of processes, including cell survival, cell proliferation, migration, invasion and epithelial to mesenchymal transition (EMT)^[7]. AXL is considered a proto-oncogene that is overexpressed in lung, breast, ovarian, gastric, pancreatic and prostate cancers. It was first cloned from chronic myelogenous and lymphoblastic leukemia cells^[8]. AXL is regulated by tumor suppressor microRNAs, such as miR-34a^[9]. Furthermore, AXL is associated with increased metastasis potential^[10,11], poor prognosis^[6,12,13] and resistance to therapy^[14-16] in many cancer types. More recently, TAM receptors were shown to foster immune escape through regulation of PD-L1 in breast cancer cells^[17].

Many oncogenic mechanisms mediated by AXL, such as EMT, are known to be controlled by tumor protein P53 (*TP53* gene, p53 protein), which often induces apoptosis in invading cancer cells in a hostile micro-environment. *TP53* is one of the most commonly mutated/altered genes involved in colorectal carcinogenesis^[18,19]. Previous work suggested a relationship between AXL and the p53/miR-34a axis in B-cell chronic lymphocytic leukemia^[20]. Therefore, we hypothesized that AXL could be controlled by *TP53* in

Table 1 Cell lines sources and maintenance

Cell line	Source	Media	FBS %	Additives
MCF7	France ¹	DMEM	10%	1% P/S
1001	France	DMEM	10%	1% P/S
ZR-75-1	Helsinki ²	RPMI 1640	10%	1% P/S + Sodium pyruvate (1 mmol/L)
RKO	Horizon ³	RPMI 1640	10%	1% P/S + Sodium pyruvate (1 mmol/L)
RKO.p53 ^{-/-}	Horizon	RPMI 1640	10%	1% P/S + Sodium pyruvate (1 mmol/L)
HCT116	Horizon	RPMI 1640	10%	1% P/S + Sodium pyruvate (1 mmol/L)
HCT116.p53	Horizon	DMEM	10%	1% P/S + 50% F12
HeLa	France	DMEM	10%	1% P/S
MDA-MB-361	France	DMEM	10%	1% P/S
MDA-MB-231	France/ATCC	DMEM	10%	1% P/S + 50% F12 + insulin (10 mg/mL in 25 mmol/L HEPES) + hydrocortisone (0.5 µg/mL)
CAL-51	Helsinki	DMEM	20%	1% P/S
T47D	France	DMEM	10%	1% P/S + 50% F12 + EGF (20 ng/mL)
BT-549	Helsinki	RPMI 1640	10%	1% P/S + Sodium pyruvate (1 mmol/L) + insulin (10 mg/mL in 25 mmol/L HEPES)
SW480	Helsinki	DMEM	10%	1% P/S + 50% F12 + insulin (10 mg/mL in 25 mmol/L HEPES) + hydrocortisone (0.5 µg/mL) + EGF (20 ng/µL)

¹France: Kind Gift from Professor Salem Chouaib, Institute Gustave Roussy, France; ²Helsinki: Kind gift from Professor Paivi peltomaki; Department of Medical Genetics, Helsinki University, Finland; ³Horizon Discovery, Cambridge, United Kingdom.

colorectal cancers where a substantial fraction of tumors has mutant/alterd *TP53*. Additionally, many of the downstream mechanisms of AXL remain unclear. Based on our recent data^[21], we hypothesized that AXL might activate Wnt signaling and play an important role in development of some rare subsets of colon cancer when Wnt signaling activity is lacking^[21].

Therefore, we sought to determine how AXL expression levels are affected by *TP53* in colorectal and breast cancers and to address its role in response to chemotherapy.

MATERIALS AND METHODS

Cell lines and cell culture

A total of 14 cell lines were used. The breast cancer cell lines were MCF7, 1001 (a Tumor Necrosis Factor (TNF)-resistant/*TP53* mutant clone from MCF7 parental cell line), CAL-51, MDA-MB-231, MDA-MB-361, ZR-75-1, T47D and BT-549. Colon cancer cell lines were HCT116, HCT116.p53 mutant, RKO, RKO.P53^{-/-} and SW480. A HeLa cell line was used as a positive control for EMT. The abovementioned cell lines were cultured in their corresponding growth media (Table 1), maintained at 37 °C in a humidified 5% CO₂ incubator. The cells were split when their confluency reached 80%-90% according to supplier instructions. Sources and maintenance conditions of the cell lines are detailed in Table 1. The origin and p53 status of the cells were detailed on the commercial supplier data sheet, the ATCC data sheets/website, the Cancer Cell Line Encyclopedia (CCLE), and/or the supplied references in Table 2.

Total protein extraction and Western blot analysis

Cell lysates were made using RIPA buffer (150 mmol/L NaCl; 1% NP-40; 0.5% deoxycholic acid;

0.1% SDS; 50 mmol/L Tris-HCl pH 7.6 and 1 × protease/phosphatase inhibitors (Cell Signaling Technology). Protein concentration was quantified using the BCA protein assay (Thermo Fisher Scientific). Approximately 30 µg of total protein was subjected to SDS-PAGE (7.5% resolving gel, 4% stacking gel). The gel, with the separated proteins, was transferred to a PVDF membrane (Bio-Rad) using a Trans-Blot[®] Turbo[™] Blotting system (Bio-Rad) according to the standard transfer protocol. The membrane was blocked for 1 h at room temperature using 5% non-fat dry milk (Sigma-Aldrich) in 1 × TBST (TBS + 1% tween), followed by incubation with the primary antibody overnight at 4 °C on a shaker. The primary antibodies were against AXL (clone C89E7, rabbit monoclonal antibody, Cell Signaling), beta-actin (clone 13E5, rabbit, rabbit monoclonal antibody, Cell Signaling) and E-Cadherin (clone 24E10, rabbit monoclonal antibody, Cell Signaling). All primary antibodies were used at a 1:1000 dilution. Next, the membrane was washed with 1 × TBST 3 times for 10 min each. Then, it was incubated with horseradish peroxidase labeled secondary antibody for 1 h at room temperature (anti-rabbit IgG HRP-linked antibody at dilution of 1:2000, Cell Signaling). After washing with 1 × TBST for 10 min 3 times, the membranes were incubated with HRP labeled substrate (Pierce[™] ECL Western Blotting Substrate) for 1 min, exposed to film (Kodak Cl-XPosure TM Film; catalog No: 34090; Company: Thermo Scientific), fixed and developed. Pictures were then evaluated and scanned.

Gene silencing using siRNA

We used siRNA for AXL protein (siRNA ID: s1847, Life Technologies, United States). We transfected the 1001 cell line, according to the Life-Technologies protocol, using reverse transfection of the MCF7 cell line. The

Table 2 Cell lines origin and p53 status

Cell lines	Cell line description ¹	p53 status ¹
Breast cancer cell lines		
MCF7	Adenocarcinoma of the mammary gland, derived from 69-year-old female, cells were obtained from metastatic site; pleural effusion	Wild-type ^[34]
1001	1001 is derived from its parental MCF7 (MCF7/R-A1; which are cells exposed to increasing dose of recombinant TNF, transfected by p55 TNF receptor cDNA, Mutation in R280K) ^[35]	TNF resistant associated with loss of p53 function ^[35]
CAL51	Adenocarcinoma isolated from a malignant pleural effusion of a 44-year-old female with metastatic breast cancer, normal karyotype with genetic stability ^[36]	Wild-type ^[37]
ZR-75-1	Ductal carcinoma of the mammary gland, derived from 63-year-old female, cells were obtained from metastatic site: ascites	Wild-type
MDA-MB-361	Adenocarcinoma of the mammary gland, derived from 40-year-old female, cells were obtained from metastatic site; Brain	Wild-type
T47D	ductal carcinoma of the mammary gland, derived from 54-year-old female, cells were obtained from metastatic site; pleural effusion	Heterozygous mutant.
MDA-MB-231	Adenocarcinoma of the mammary gland, derived from 51-year-old female, cells were obtained from metastatic site; pleural effusion.	Homozygous mutant
BT549	Ductal carcinoma of the mammary gland, derived from 72-year-old female, cells were obtained from mammary gland.	Homozygous mutant
Colon Cancer Cell lines		
RKO	poorly differentiated colon carcinoma cell line developed by Michael Brattain	Wild-type.
RKO-p53	RKO parental cell line double negative mutant for p53	Homozygous knock out ²
HCT 116	Colorectal carcinoma of Adult Male derived from primary tumor site	Wild-type
HCT 116.p53	HCT116 parental cell line with hemizygous p53 mutation and knock out of the homologous p53	R284w/- ²
SW480	Dukes' type B, colorectal adenocarcinoma, derived from 50-year-old male, cells obtained from primary tumor site	Homozygous mutant
Positive control for EMT		
HeLa	Cervical adenocarcinoma of 31-year-old female derived from primary tumor site	Wild-Type but HPV inactivated ^[34]

¹The cell line origin and the p53 status were from ATCC data sheets/website, the Cancer Cell Line Encyclopedia or the indicated references; ²As per the source/supplier: Horizon Discovery, Cambridge, United Kingdom.

protocol is available at <https://www.lifetechnologies.com/content/dam/LifeTech/migration/en/filelibrary/pdf/protocols.par.23973.file.dat/human-breast-cancer.pdf>.

Invasion assay

For the invasion assay, we used QCM™ High Sensitivity Non-cross-linked Collagen Invasion Assay kits, available from Millipore, and followed the supplied manufacturer protocol. Briefly, the assay was performed using a modified chamber with filter inserts (pore size 8 µm) coated with matrigel in 24-well dishes. Approximately 0.5 million cells were prepared in serum-free media (RPMI1640). Two hundred and fifty µL of the cell suspension was added into the inserts (top chamber) and 500 µL of 15% FBS-containing media was added to the bottom chamber. After a 48-h incubation, cells remaining in the top chamber were removed, and 400 µL of cell stain was applied to the invasion chamber insert for 15 min. After several washes with water, the inserts were dried, viewed under the microscope and photographed. Inserts were then transferred into 200 µL of extraction buffer and allowed to incubate for 15 min at room temperature. The dye mixture was then assessed by a plate reader at a wavelength of 630 nm.

Doubling times and sulforhodamine assay

Population doubling times were calculated according

to standard protocols from the American Type Culture Collection (ATCC) available at <https://www.atcc.org/en/Guides/Guides.aspx>. The IC50 concentration of drugs used in the experiments were prepared by serial dilution in DMEM medium prior to performing the experiment. The IC50 concentrations were determined from published literature^[22-24]. In all of the experiments, the highest dimethyl sulfoxide (DMSO) concentration did not exceed 1% for drugs dissolved in DMSO. The cytotoxicity of the drugs was determined using the *sulforhodamine B* (SRB) method^[25]. Cells were seeded in 96-well plates at a concentration of 10⁴ cells/well. After 24 h, cells were incubated with drug-free medium, medium containing DMSO (1%), or medium containing the drug at varying concentrations (0.1-100 µmol/mL) in a final volume of 200 µL/well. Triplicate wells were prepared for each concentration. Cells were incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO₂. Cells were fixed for 1 h at 4 °C by adding 50 µL of 50% trichloroacetic acid (TCA) to the culture medium in each well. Cells were then rinsed with water several times, dried and stained with 0.4% SRB for 30 min. Next, cells were washed several times with 1% acetic acid to remove unbound stain. After drying, the dye was solubilized with 10 mmol/L Tris base. The optical density was measured at 564 nm with an ELISA microplate reader. The absorbance of cells incubated with only DMSO was subtracted from

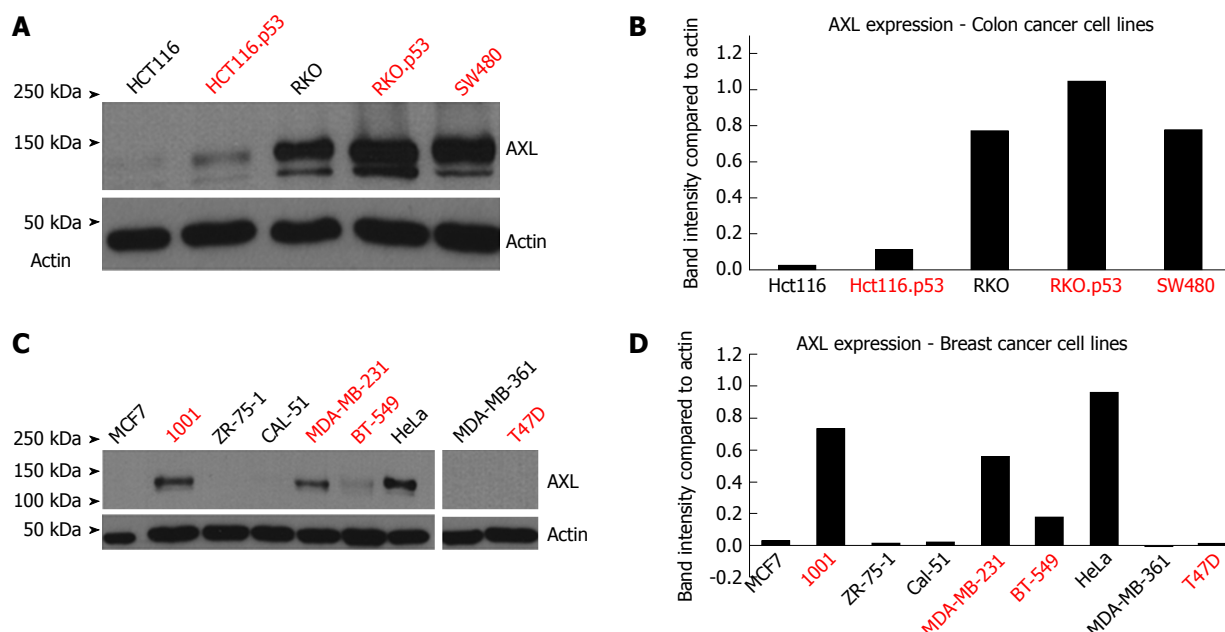


Figure 1 Western blot analysis of AXL protein levels in cancer cell lines. A: Colon cancer cell lines HCT116, HCT116.p53, RKO, RKO.p53^{-/-} and SW480. A band of 140 kDa was observed in AXL positive samples. Actin was used as a loading control; B: This graph shows the quantification of band intensity in comparison to Actin using the "ImageJ" program; value analysis was done using MS Excel. Red font indicates p53 mutation; C: Breast cancer cell line MCF7, MCF7-TP53 mutant clone 1001, ZR-75-1, CAL-51, MDA-MB-231, BT-549, MDA-MB-361, and T47D. HeLa was used as positive control for EMT. Actin was used as a loading control; D: This graph shows the quantification of band intensity in comparison to Actin using the "ImageJ" program and value analysis was done using MS Excel. Red font indicates p53 mutation.

the absorbance reading of each well (to account for the DMSO effect). Fractions of viable cells were calculated by dividing the reading at the respective concentration by the control (untreated cells). Survival curves were constructed by plotting the fraction of viable cells against the concentrations used.

Statistical analysis

When appropriate, Fisher's exact probability test, χ^2 test, or the student *t*-test was used to evaluate differences between groups. Person correlation analysis was performed to test relationships between variables. Analyses were performed using MS Excel and/or VassarStats Web-based statistical program, found at <http://faculty.vassar.edu/lowry/VassarStats.html>. All reported *P*-values were two-tailed and *P* values < 0.05 were considered significant. The "ImageJ" program (<http://imagej.nih.gov/ij/>) and MS Excel were used to quantify the Western blot band intensity.

RESULTS

Upregulation of AXL results from introducing TP53 mutation/knockout

Introduction of *TP53* mutations upregulated AXL expression in the HCT116.p53 mutant cells compared to its parental HCT116 *TP53* wild-type cell line. The same was true for RKO.p53^{-/-} compared to its parental RKO p53 wild-type cell line (Figure 1A and B). It should be noted that the parental RKO cell line already expressed AXL protein, but knocking out p53 further increased the level of expression, as detected by band

intensity quantification. In breast cancer, the 1001 cell line showed high AXL expression in comparison to its parental MCF7 cell line, which has wild-type *TP53* functionality (Figure 1C and D). The 1001 cell line originated from a *TP53*-mutant clone developed from the MCF7 cell line.

AXL upregulation is correlated to p53 alterations in colorectal and breast cancer cells

As shown in Table 3, there was association between the p53 mutation/knockdown and increased AXL expression in both colon cancer (with the exception of RKO) and breast cancer cell lines (with the exception of T47D).

AXL is negatively associated with E-Cadherin in both colorectal and breast cancer

Table 3 shows there was also a negative association between increased AXL expression in both colon cancer (with the exception of SW480) and breast cancer cell lines and E-cadherin.

Microarray analysis of AXL, p53, and Wnt signaling

Colorectal cancer cell lines: We extended our analysis of the relationship between AXL and *TP53* to available data sets from our own laboratory (Table 4)^[21]. The data, acquired from assessing 8 colon cancer cell lines, contradicted the hypothesis that mutations occurring naturally in *TP53* (compared to those introduced artificially by manipulating cell lines) can directly cause AXL upregulation. Two p53 mutant cells (HCA7, KM12) were AXL negative and

Table 3 Summary results of proteins investigated

Cell lines	p53 status	AXL	E-Cadherin
Positive control			
HeLa	HPV inactivated	Highest	Negative
Colon			
HCT116	Wild-Type	Negative	High
RKO	Wild-Type	High	Negative
HCT116.p53	Mutant	High	Lowest
RKO.p53 ^{-/-}	Mutant	Highest	Negative
SW480	Mutant	Highest	High
Breast			
MCF7	Wild-Type	Negative	Low
MDA-MB-361	Wild-Type	Negative	Highest
ZR-75-1	Wild-Type	Negative	Highest
CAL-51	Wild-Type	Negative	Low
1001	Mutant	Highest	Negative
MDA-MB-231	Mutant	High	Negative
T47D	Mutant	Negative	Highest
BT-549	Mutant	Lowest	Negative

the RKO (p53 wild-type cell) was strongly positive (Table 4) as confirmed by Western blotting above. This suggested the relationship between *TP53* and AXL is not direct and that other molecules or signaling pathways are involved. Since RKO is a unique colon cancer cell line that has an inactive Wnt signaling pathway, we hypothesized that AXL is potentially the oncogenic target involved in colorectal carcinogenesis in the absence of active Wnt signaling. We searched the Cancer Cell Line Encyclopedia (CCLE) collection of 62 colon cancer cell lines to further elucidate this hypothesis. We considered AXL to be positive in the CCLE colon cancer cells when it had an expression value of +0.4 or higher, as guided by our Western blot analysis. There was no significant relation between AXL expression and *TP53* mutation. We found that AXL was upregulated in 12/62 (20%) colon cancer cell lines. Of these, only 6/12 (50%) were *TP53* mutants. However, 3 (RKO, HS698T, HS255T) of these 6 had no mutations in either the APC or CTNNB1 (β -catenin) gene, suggesting inactive Wnt signaling.

Breast cancer cell lines: When a threshold of 0.4 or greater was set for identifying AXL positive expression in breast cancer cell lines, we found AXL positive expression in 22/60 (37%), but no significant relationship to *TP53* mutation was identified in the CCLE collection.

AXL silencing reduces invasiveness of both colon and breast cancer cells

AXL silencing decreased the invasion of breast cancer cell line 1001, and colorectal cell lines RKO.p53^{-/-} and HCT116.p53 mutant.

AXL predisposes to emergence of invasive clones after chemotherapy

AXL silencing did not show a slight trend to restore breast cancer cells sensitivity to Doxorubicin and colon

cancer cell sensitivity to 5FU or irinotecan (Figure 2A and B). Interestingly, analysis of cell invasion after therapy showed that AXL expressing cells developed more invasive potential after exposure to chemotherapy compared to the AXL-silenced cells (Figure 2C and D)

DISCUSSION

The current work sheds light on the relationship between AXL and *TP53* in cancer cells. Our data from isogenic cell lines showed that the introduction of *TP53* mutation and/or knockout upregulated AXL expression. As such, this finding indicates AXL is closely regulated by p53 protein. Recent data suggest few alternative mechanisms to explain this effect. Boysen *et al.*^[20] showed that the 3'UTR of the AXL gene includes a binding site for miR-34a. The expression of miR-34a is regulated by p53 in B-cell chronic lymphocytic leukemia, ovarian cancer^[26] and some other solid tumors^[27]. Conversely, miR-34 family members did not correlate with AXL mRNA or protein levels in other tumors, such as renal cell carcinoma^[28]. The p53-miR-34a-AXL link in colorectal and breast cancers still needs to be clarified.

Alternatively, mutant *TP53* was shown to up-regulate AXL in lung cancer cells at both the RNA and protein level^[29]. This effect was noted for mutant p53-R175H, R273H, and D281G but was refractory to mutations at positions 22 and 23 in the p53 transactivation domain. Mutant p53 was shown to directly nucleate and induce histone acetylation on the AXL promotor region and knockdown of mutant *TP53* was shown to reduce histone acetylation on the AXL promotor. In the presence of mutant *TP53*, the transcription factors binding capacity to the AXL promotor increased by up to 8-fold. This action was independent of p53-mediated transactivation because the p53/p63 binding site in the AXL promotor was not needed. This work also concluded that AXL partially mediated a change in functional activity of *TP53* mutants^[29]. It would be interesting to prove this relationship in other tumor lineages, such as colorectal and breast cancers. However, we could not find a consistent association between AXL expression and *TP53* mutation status in our microarray data (Table 4) and, more generally, in the CLLE cell lines. This suggests that the relationship between AXL and *TP53* could depend on tumor lineage, or more likely, on the carcinogenic mechanisms and molecular subtypes of the individual tumor.

Thus, we were intrigued by the exceptionally high levels of AXL expression in the *TP53* wild-type cell line RKO. Because RKO cells have inactive Wnt signaling, a rare phenomenon in colon cancer types, AXL expression might be an alternative oncogenic pathway for colorectal carcinogenesis in the absence of β -catenin/Wnt signaling activation and *TP53* mutation^[21]. After searching the literature for potential links between AXL and Wnt signaling, we found that AXL was shown to modulate

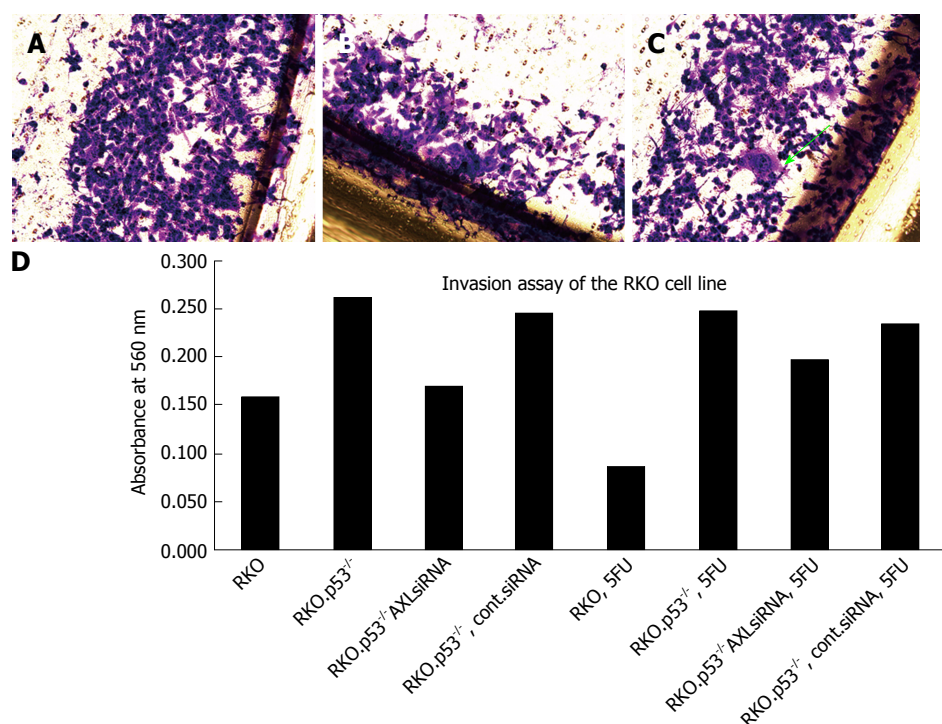


Figure 2 Invasion assays of different RKO clones. A: The base line invasion of RKO.p53⁺, which was not silenced or exposed to treatment, was high, as indicated; B: The same cell line after AXL siRNA silencing shows some decreased invasion; C: RKO.p53⁺ treated with siRNA mock control has increased invasiveness and showed some aggressive cells with increased nuclear size, clumped chromatin, increased cytoplasmic extensions (arrow), after exposure to 5FU treatment; D: Quantitative evaluation of different cells after dye elution and spectrophotometric reading at 560 nm.

Table 4 AXL mRNA expression from our microarray of colon cancer cell lines^[21]

	RKO	HCA7	KM12	LoVo	DLD1	HCT116	SW48	LIM 1215
P53 mutation	WT	mut	mut	WT	WT	WT	WT	WT
AXL expression ¹	+	-	-	-	-	-	-	-

¹Based on Affymetrix probe 202685_s_at considering a cut off value of 70 to differentiate negative from positive.

miR-374a and miR548b. These miRNAs play essential roles in gefitinib-induced apoptosis, EMT, tumorigenesis and migration of gefitinib-resistant lung cancer cells by targeting Wnt5a and CCNB1, respectively^[15]. MiR-374a was already known to induce canonical Wnt signaling activation in breast cancer cells^[30]. More recently, Wang *et al.*^[16] showed that AXL stimulation by Gas6 significantly increased β -catenin levels and induced its nuclear translocation, while AXL knockdown caused a decrease in nuclear β -catenin in MCF7 breast cancer cells. Collectively, these data support our argument that AXL potentially acts as an alternative carcinogenic mechanism to exert β -catenin activation in colorectal development, which is one of the very common early changes, in the absence of APC/ β -catenin mutations.

Our study also concluded that silencing AXL expression in breast and colorectal cell lines decreases their invasiveness. Clearly, this related to the well-established causative role of AXL in induction of EMT^[4,6,10,14,16,31]. Because the association between AXL overexpression and EMT markers is currently well-established, we only used E-cadherin expression

to confirm this relationship. Overall, we observed a correlation between *TP53*, AXL and E-cadherin expression (Table 3), but a few exceptions were noted, which may explain the differences in the prognosis observed below. After using siRNA transfection to silence AXL gene expression, we observed a clear reduction in invasiveness of colorectal and breast cancer cell lines, which is consistent with the published literature^[10,14,16]. It is important to note that AXL and myeloid zinc finger (MZF-1) were overexpressed in resected colonic cancer compared to normal tissue. MZF-1 increased migration and invasiveness of cancer cells, in part by binding to the promotor region of AXL and enhancing the protein expression. Knockdown of AXL using shRNA decreased migration and invasiveness induced by MZF-1 in RKO cell lines^[31]. This finding may explain alternative mechanisms of AXL upregulation and induction of EMT independent of *TP53*.

Finally, we showed that after exposure to chemotherapy, the AXL expressing cells became more invasive as compared to the AXL-silenced cells. This

finding could explain why AXL expression is sometimes associated with poor clinical outcome, often in early stages of colorectal cancer^[14]. A recent study showed that AXL overexpression (among other proto-oncogenes) was associated with short overall survival in patients with colorectal cancer and mutations in the tumor suppressor p53^[32]. However, after analyzing public datasets we found an inconsistent relationship between AXL and prognostic parameters, such as response to therapy and overall survival in patients with colorectal and breast cancer. Some recent publications^[14,33] also hinted at this result; therefore this observation should be considered when designing personalized AXL-based therapy. Our data and analysis suggest that these differences could be explained by the complex AXL signaling pathways activated in different cells, including *TP53* and Wnt signaling. These potential factors need to be elucidated for successful use of AXL in tumor therapy.

In conclusion, our data support the role of AXL in EMT and resistance to treatment in colorectal and breast cancer. We also showed that AXL could be regulated by *TP53*, and AXL could play an oncogenic role in activating the Wnt/ β -catenin pathway in colon cancer. The complexity of the interaction of AXL with various signaling pathways should be taken into consideration when designing therapeutic approaches targeting AXL.

COMMENTS

Background

Colorectal and breast cancers remain deadly diseases; hence, there is continuous effort to develop novel molecular therapeutic targets to improve the outcome of these cancers. AXL Receptor Tyrosine Kinase (AXL), a receptor tyrosine kinase, is an attractive candidate because it was shown to play a role in epithelial to mesenchymal transition; thus, it is also involved in cancer invasion and metastasis. The molecular regulations and interactions of AXL must be well understood before it can be used as a target for new therapeutics.

Research frontiers

AXL is an attractive molecule because it was shown to be associated with poor prognosis, resistance to therapy and immune escape in many cancer types. Recent research is focused on its usefulness as a potential therapeutic target.

Innovations and breakthroughs

The authors supply evidence that AXL is regulated by *TP53* in cancers of the colon and breast and it contributes to epithelial to mesenchymal transition, cellular invasion and response to therapy in these tumors. AXL could also be a marker for predisposition for emergence of aggressive clones after chemotherapy. This relationship could not be generalized, and we suggest that this could be linked to other carcinogenic pathways, such as Wnt/ β -catenin signaling in colorectal cancer. The interaction of AXL with such pathways should be considered carefully when designing AXL based therapy.

Applications

These data are directly applicable to aid in development and design of treatment options for colon and breast cancers based on the AXL gene.

Terminology

Receptor tyrosine kinases (RTKs) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. RTKs have been

shown not only to be key regulators of normal cellular processes but to also play a critical role in the development and progression of many types of cancer. The epithelial to mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties. EMT is essential for numerous developmental processes. It has been shown to occur in wound healing, and in the initiation of cancer invasion and metastasis.

Peer-review

The proposal has evaluated AXL expression in relationship to p53 status and its role in tumor invasion and response to therapy. The results have shown a directly application in designing therapeutic approaches targeting AXL.

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Basic Study

Effects of heme oxygenase-1-modified bone marrow mesenchymal stem cells on microcirculation and energy metabolism following liver transplantation

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Abstract

AIM

To investigate the effects of heme oxygenase-1 (HO-1)-modified bone marrow mesenchymal stem cells (BMMSCs) on the microcirculation and energy metabolism of hepatic sinusoids following reduced-size

liver transplantation (RLT) in a rat model.

METHODS

BMSCs were isolated and cultured *in vitro* using an adherent method, and then transduced with HO-1-bearing recombinant adenovirus to construct HO-1/BMSCs. A rat acute rejection model following 50% RLT was established using a two-cuff technique. Recipients were divided into three groups based on the treatment received: normal saline (NS), BMSCs and HO-1/BMSCs. Liver function was examined at six time points. The levels of endothelin-1 (ET-1), endothelial nitric-oxide synthase (eNOS), inducible nitric-oxide synthase (iNOS), nitric oxide (NO), and hyaluronic acid (HA) were detected using an enzyme-linked immunosorbent assay. The portal vein pressure (PVP) was detected by Power Lab ML880. The expressions of ET-1, iNOS, eNOS, and von Willebrand factor (vWF) protein in the transplanted liver were detected using immunohistochemistry and Western blotting. ATPase in the transplanted liver was detected by chemical colorimetry, and the ultrastructural changes were observed under a transmission electron microscope.

RESULTS

HO-1/BMSCs could alleviate the pathological changes and rejection activity index of the transplanted liver, and improve the liver function of rats following 50% RLT, with statistically significant differences compared with those of the NS group and BMSCs group ($P < 0.05$). In term of the microcirculation of hepatic sinusoids: The PVP on POD7 decreased significantly in the HO-1/BMSCs and BMSCs groups compared with that of the NS group ($P < 0.01$); HO-1/BMSCs could inhibit the expressions of ET-1 and iNOS, increase the expressions of eNOS and inhibit amounts of NO production, and maintain the equilibrium of ET-1/NO ($P < 0.05$); and HO-1/BMSCs increased the expression of vWF in hepatic sinusoidal endothelial cells (SECs), and promoted the degradation of HA, compared with those of the NS group and BMSCs group ($P < 0.05$). In term of the energy metabolism of the transplanted liver, HO-1/BMSCs repaired the damaged mitochondria, and improved the activity of mitochondrial aspartate aminotransferase (ASTm) and ATPase, compared with the other two groups ($P < 0.05$).

CONCLUSION

HO-1/BMSCs can improve the microcirculation of hepatic sinusoids significantly, and recover the energy metabolism of damaged hepatocytes in rats following RLT, thus protecting the transplanted liver.

Key words: Reduced-size liver transplantation; Bone marrow mesenchymal stem cells; Microcirculation; Heme oxygenase-1; Energy metabolism

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Core tip: Hepatic sinus is important in the liver

microcirculation, which is the basis for transplanted liver regeneration. Transplanted liver grafts with disturbed microcirculation of the hepatic sinus may affect liver energy metabolism. We investigated the protective effects of heme oxygenase-1-modified bone marrow mesenchymal stem cells (HO-1/BMSCs) on rat reduced-size liver transplantation in terms of the microcirculation and hepatic energy metabolism. HO-1/BMSCs promoted the equilibrium of ET-1/NO, repaired damaged hepatic sinusoidal endothelial cells, and lowered the portal vein pressure in rats following reduced-size liver transplantation, which improved the microcirculation of hepatic sinusoids and ATPase activity, and recover the energy metabolism of damaged hepatocytes.

Yang L, Shen ZY, Wang RR, Yin ML, Zheng WP, Wu B, Liu T, Song HL. Effects of heme oxygenase-1-modified bone marrow mesenchymal stem cells on microcirculation and energy metabolism following liver transplantation. *World J Gastroenterol* 2017; 23(19): 3449-3467 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3449.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3449>

INTRODUCTION

Liver transplantation is the only effective treatment for end-stage liver diseases; however, a shortage of donor graft remains the major impediment to the development of liver transplantation. Although reduced-size liver transplantation (RLT), split-liver transplantation, and living donor liver transplantation can make up the shortage of donor grafts to some extent^[1,2], transplantation-induced hepatic injury can seriously affect liver function, even leading to small-for-size syndrome (SFSS). SFSS is a clinical syndrome involving multiple factors, such as the volume and quality of the donor graft, recipient characteristics, and surgical techniques. The main pathophysiological characteristic is disturbance in the microcirculation of hepatic sinusoids^[3,4]. Hepatic sinusoids play an important role in the liver microcirculation. The integrity of sinusoidal structures and the stability of sinusoidal microcirculation are essential not only for normal liver function, but also for good physiological function and regeneration of a transplanted liver. Preservation of the donor graft, ischemia-reperfusion, and rejection may result in damage to the transplanted liver during transplantation. Ischemia-reperfusion promotes swelling, necrosis, and apoptosis of the sinusoidal endothelial cells (SECs), leading to sinusoidal obstruction. Kupffer cell activation and SECs injury after liver transplantation can lead to disturbance in the microcirculation of hepatic sinusoids, adhesion of leukocytes and platelets, and a series of inflammatory reactions, which cause dysfunction of the sinusoidal microcirculation^[5,6]. Furthermore, all these pathological

processes would lead to organ ischemia, and due to a lack of oxygen, a large amount of lipid peroxide in mitochondria was produced and ATP was gradually reduced, which caused tissue damage^[7]. A low baseline level of hepatic ATP leads to liver necrosis and apoptosis, although ischemic preconditioning and ATP pretreatment can increase the intrahepatic ATP level significantly, providing protective effects on liver function^[8,9]. Thus, disturbance in the microcirculation of hepatic sinusoids and disordered energy metabolism are important factors affecting the functions of transplanted livers, which still remain unsolved.

Bone marrow mesenchymal stem cells (BMMSCs), a group of non-hematopoietic stem cells derived from stromal cells, have multi-directional differentiation potential and can be differentiated into endothelia. BMMSCs promote angiogenesis, tissue repair, and paracrine signaling^[10-15], which can relieve ischemic reperfusion injury (IRI) of the liver, reduce hepatocyte injury and accelerate liver regeneration, and are involved in anti-inflammation and immunoregulation^[16-21]. BMMSCs have been investigated in the field of liver, kidney, small intestine, and islet transplantation^[22-27]; however, the proportion of BMMSCs surviving in the recipient's body for more than a week is less than 1%, which has affected its use in experimental studies^[22,24,28]. Thus, improving the survival time of BMMSCs is also a research hotspot.

Heme oxygenase-1 (HO-1) is a multifunctional microsomal oxidase related to heme metabolism, with anti-inflammatory, anti-oxidative stress, anti-apoptosis, anti-ischemia reperfusion injury, and microcirculation regulation effects that protect cells^[29-31]. HO-1 has been shown to alleviate rejection, prolong graft survival time, and induce immune tolerance in organ transplantation^[32-34]. HO-1 can regulate BMMSCs by reducing the apoptosis of BMMSCs under hypoxia and oxidative stress *in vitro*^[35], and prolonging the protective effects of BMMSCs on transplanted grafts^[22,24].

A previous study showed that transplantation of HO-1/BMMSCs could inhibit the apoptosis of hepatocytes and reduce IRI^[22]. How HO-1/BMMSCs exert their protective effects, and whether HO-1/BMMSCs can affect the microcirculation of hepatic sinusoids, portal vein pressure (PVP) and energy metabolism of hepatocytes following RLT have not been studied extensively. Therefore, the aim of this study was to determine whether HO-1/BMMSCs could protect the microcirculation of hepatic sinusoids and energy metabolism of the transplanted liver after RLT in order to provide a reliable experimental basis for solving the shortage of donors.

MATERIALS AND METHODS

Experimental animals

Specific-pathogen-free (SPF) adult inbred Brown-Norway (BN) rats and Lewis rats were purchased from the Academy of Military Medical Sciences, Beijing, China. Male BN rats (4-5 wk old; 100-120 g)

were inbred for the extraction and characterization of BMMSCs. Inbred male Lewis rats (6-8 wk old; 200-220 g) were the liver transplantation donors, and the inbred male BN rats were the recipients. The experimental animals were kept at 23 °C, with 50% humidity, and a 12 h light and dark cycle for 2 wk, with free access to water and food, and regular replacement of cage and clean bedding before the experiments. All experimental procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, 8th ed. 2011). All protocols were approved by the Animal Care and Research Committee of Tianjin First Central Hospital. All the rats were anesthetized with chloral hydrate to minimize their pain.

Instruments and reagents

The following instruments and reagents were used: Dulbecco's modified Eagle medium (DMEM)/F12, penicillin-streptomycin solution, and trypsin/EDTA solution (Gibco, Carlsbad, CA, United States); fetal bovine serum (FBS; Biowest, Nuaillé, France); dexamethasone phosphate sodium (5 mg/mL), sodium glycerophosphate (216 mg/mL), insulin (40 U/mL), 1-methyl-isobutyl-xanthine, vitamin C and indomethacin (Sigma Aldrich, St. Louis, MO, United States); Oil Red O powder (Dingguo Changsheng Biotechnology, Beijing, China); von Kossa cell staining kit (Genmed, Shanghai, China); recombinant adenovirus expressing rat HO-1 (Genechem Co., Ltd., Shanghai, China); phosphate buffer solution (PBS), highly sensitive radio immunoprecipitation assay (RIPA), bicinchoninic acid (BCA) protein assay kit (Solarbio, Beijing, China); Western blotting-associated reagents (Boster, Wuhan, China); normal goat serum (Minhai Biotechnology, Lanzhou, China); SuperPicture™ Polymer Detection Kit (Thermo, Waltham, MA, United States); diaminobenzidine (Dako, Glostrup, Denmark); flow cytometry-related antibodies (anti-rat CD34-fluorescein isothiocyanate (FITC), CD29-phycoerythrin (PE), CD45-PE, CD90-FITC, RT1A-PE, and RT1B-FITC; Biolegend, San Diego, CA, United States); rabbit antibodies for inducible nitric oxide synthetase (iNOS), endothelial nitric oxide synthetase (eNOS), von Willebrand factor (vWF), and mouse antibodies for endothelin (Abcam, Cambridge, United Kingdom); rabbit antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (SAB, College Park, MD, United States); goat anti-rabbit IgG labeled with horseradish peroxidase (HRP) and goat anti-mouse IgG labeled with HRP (Invitrogen, Carlsbad, CA, United States); ELISA kits (Biovalue, Shanghai, China); ATP assay kit (Jiancheng Biotechnology, Nanjing, China); inverted fluorescent microscope (Olympus, Japan); and FACSCalibur flow cytometric analysis (BD FACSAria III, Franklin Lakes, NJ, United States).

Isolation, culture, and characterization of BMMSCs

BMMSCs were isolated aseptically from the femur and

tibia of BN rats after sacrifice by cervical dislocation. After cutting off both ends of the epiphyseal plate, the marrow cavity was rinsed by DMEM/F12 containing 10% FBS. Red blood cells (RBCs) were lysed using 0.1 mol/L NH₄Cl, and the remaining cells were washed, resuspended as a single cell suspension, and cultured in T75 culture flask at 37 °C with 5 mL/L CO₂ in an incubator. Well-grown third-passage cells were resuspended and then labeled fluorescently with antibodies (anti-CD29-PE, anti-CD34-FITC, anti-CD45-PE, anti-CD90-FITC, anti-RT1A-PE and anti-RT1B-FITC) for 30 min for flow cytometric analysis.

Identification and induced differentiation of BMMSCs in vitro

Adipogenic differentiation: Well-grown third-passage BMMSCs were inoculated into 6-well plates at 2×10^5 cells/well. After complete adherence, BMMSCs were cultured in adipogenic differentiation medium (DMEM/F12 containing 10% FBS, 1 μmol/L dexamethasone, 10 μg/mL insulin, 0.5 mmol/L 1-methyl-3-isobutyl xanthine, and 0.1 mmol/L indomethacin). The medium was changed every 72 h. After induction for 8-10 d, BMMSCs were fixed by 4% paraformaldehyde, and stained with Oil Red O for 30 min. The BMMSCs were then rinsed with PBS and positive cells showed orange lipid droplets.

Osteogenic differentiation: Well-grown third-passage BMMSCs were also inoculated into 6-well plates at 2×10^5 cells/well. After complete adherence, BMMSCs were cultured in osteogenic differentiation medium (DMEM/F12 containing 10% FBS, 0.1 μmol/L dexamethasone, 10 mmol/L sodium glycerophosphate, and 50 μg/mL vitamin C). The medium was changed every 72 h. After induction for 13-15 d, BMMSCs were stained using a von Kossa staining kit, and positive cells showed black calcium deposits.

Preparation and identification of HO-1/BMMSCs

HO-1-bearing recombinant adenovirus (Adv/HO-1) was diluted to 10 pfu/cell with complete culture medium, which was used to replace the original medium of well-grown third-passage BMMSCs. After 6-8 h, the Adv/HO-1 culture medium was replaced with complete culture medium for continued cultivation of the BMMSCs. After 48 h, the proportion of cells containing green fluorescence was observed under a fluorescence microscope.

Establishment of a rat rejection model in 50% reduced-size liver transplantation

A 50% RLT rejection model was established with Lewis donor rats and BN recipient rats, using the two-cuff technique by a single operator. The donor rats were anesthetized with 5% chloral hydrate, and incised by abdominal median incision. After dividing the perihepatic ligaments, the portal vein (PV) and infrahepatic vena cava (IHVC) were isolated. The

anterior wall of the common bile duct was cut off and a stent was implanted, the hepatic artery was ligated and cut off, the PV was punctured and infused with 4 °C lactated Ringer's solution, and the IHVC and suprahepatic vena cava (SHVC) were both cut off as the outflow tract. When the liver turned yellowish and the outflow of perfusion fluid became clear, the PV was dissected and the donor liver was removed. The SHVC was trimmed, the PV and IHVC were prepared as vascular cuffs, and then the harvested graft was preserved at 4 °C. The perihepatic ligaments were divided in the recipient rats, the PV and IHVC were blocked, and the PV was punctured and infused with 1 mL normal saline (NS). The PV, SHVC, and IHVC were then dissected, and the liver was removed. The donor liver was placed orthotopically in the abdominal cavity of the recipient. The SHVC was anastomosed using an 8-0 nylon suture. Cuff anastomosis of the PV and IHVC was then performed. The graft was reperused by opening the PV, SHVC, and IHVC in turn. The bile duct was connected by a stent suture. After checking that all the cuff tubes were not distorted and no leak occurred in the SHVC and IHVC, the abdomen was washed and closed. The detailed surgical procedure was described previously^[36].

Treatment of experimental animals

Experimental rats were divided into three groups: the control group (receiving 1 mL NS), the BMMSCs group (receiving 5×10^6 BMMSCs resuspended in 1 mL), and the HO-1/BMMSCs group (receiving 5×10^6 HO-1/BMMSCs resuspended in 1 mL). All injections in the three groups were administered *via* the superficial dorsal veins immediately after RSL. Five rats in each of the three groups were euthanized on postoperative day (POD) 0, 1, 5, 7, or 14, respectively, and their peripheral venous blood and transplanted liver tissues (cooled in liquid nitrogen and stored at -80 °C) were collected for further analysis.

Biochemical analysis of liver function

The rat serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and mitochondrial AST (ASTm) were measured using an automatic biochemical analyzer (Hitachi, Japan) according to the manufacturer's instructions.

ELISA test

The rat serum levels of ET-1, eNOS, iNOS, nitric oxide (NO), and hyaluronic acid (HA) were detected using the ELISA kits according to the manufacturer's instructions.

Detection of PVP after RLT

The pathological injuries were most obvious on POD 7; therefore, POD 7 was chosen as the time point to measure the PVP. The rats were anesthetized by intraperitoneal injection of phenobarbital, and fixed in the supine position, the abdominal cavity was opened,

and the PV was exposed. An ileum mesenteric vein branch in the right side of anterior mesenteric vein near the PV was isolated, its distal end was ligated, a suitable nick to insert a catheter was made, and then the catheter was inserted along the anterior mesenteric vein upstream. After fixing the catheter and connecting it to the pressure transducer, the PVP was measured using a Power Lab ML880 (AD Instrument, Australia).

Detection of the protein levels of ET-1, eNOS, iNOS, and vWF by Western blotting

Liver tissues collected from different POD were treated with RIPA lysis buffer to extract total proteins, and the concentrations of the total proteins were detected using a BCA protein assay kit. The proteins were separated electrophoretically and then transferred to nitrocellulose membranes. After blocking with 5% skimmed milk for 2 h, ET-1 (1:250), eNOS (1:200), iNOS (1:250), vWF (1:250) and internal reference protein GAPDH (1:3000) antibodies were added and incubated at 4 °C overnight. The membranes were then rinsed with Tris Buffered Saline with Tween-20 (TBST), incubated with secondary antibodies (1:5000) for 2 h at room temperature, rinsed with TBST again, the chemiluminescence HRP substrate was added, and the membranes were exposed in a gel imaging analysis system (Alpha Innotech FluorChem FC2, CA, United States). The images were analyzed using the AlphaView SA 3.4.0 software (San Jose, CA, United States) to determine the grey scale. The relative abundance of a target protein was calculated as target protein band brightness value - background brightness value/internal reference protein GAPDH band brightness value - background brightness value. The resulting ratio was the relative abundance of the target protein. The samples were replicated three times in different batches at each time point.

Histopathological and immunohistochemical analysis of the transplanted liver

The transplanted livers on POD 0, 1, 5, 7, and 14 were sectioned, fixed, paraffin embedded, sliced, and stained with hematoxylin and eosin (HE). The histopathological changes in the liver tissues were observed in five randomly selected fields under a light microscope. Acute rejection was graded according to the Banff criteria^[37].

The transplanted liver was sliced, dewaxed by xylene, dehydrated by gradient ethanol, subjected to antigen retrieval, and blocked by normal goat serum at 37 °C for 1 h. After incubation with primary antibodies (1:500) at 37 °C for 1 h and at 4 °C overnight, the slides were incubated with secondary antibodies at 37 °C for 40 min, developed with DAB and stained with hematoxylin, differentiated using 1% hydrochloric acid ethanol, dehydrated using gradient ethanol, and clarified and encapsulated using xylene. The tissue

slices were observed to determine the presence of ET-1, eNOS, iNOS and vWF in the transplanted liver. The immunohistochemical results were analyzed using the Image-Pro Plus 6.0.0.260 software (IPP, Media Cybernetics, Rockville, MD, United States).

ATPase activity test

The activity of Na⁺-K⁺-ATPase in the liver tissues was detected using the chemical colorimetry method according to the instruction manual of the ATPase test kit.

Observation by transmission electron microscopy

The transplanted liver was double-fixed by glutaraldehyde and osmic acid, dehydrated by gradient acetone, immersed in embedding medium, ultrathin sectioned using an automatic microtome (LeicaRM2235, Leica, Germany), and stained with 1% uranyl acetate. The tissue slices were observed and filmed under a transmission electron microscope (Hitachi, Japan) to observe the status of mitochondria in the hepatocytes.

Statistical analysis

SPSS 17.0 (SPSS GmbH, Munich, Germany) was used for the statistical analysis. All data were presented as means ± SD. Different groups of data were compared by analysis of variance (ANOVA). Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Morphology, phenotype, induced differentiation, and identification of BMMSCs in vitro

Morphology and phenotype identification of BMMSCs: MSCs are spindle-shaped in morphology like fibroblasts in which the cytoplasm can stretch peripherally and maintain the ability to differentiate into a variety of other cell lines when cultured *ex vivo*. BMMSCs grew adherently when observed under microscope, and the cells were long spindle-shaped and partially vortexed or chrysanthemum-like, with typical morphological characteristics of MSCs (Figure 1A). Flow cytometry analysis showed that the positive rates of CD29, CD90 and RT1A in the third generation BN rat BMMSCs were 99.5%, 97.4%, and 96.9%, respectively; and the negative rates of CD34, CD45, and RT1B were all above 95% (Figure 1E-G).

In vitro differentiation and identification of BMMSCs

After induction by adipogenic differentiation medium for 8-10 d, BMMSCs showed multiple orange lipid droplets in their cytoplasm, which was consistent with the characteristics of adipocytes (Figure 1C). Similarly, BMMSCs showed black granular or lumpy calcium deposits in their cytoplasm after induction by osteogenic differentiation medium for 13-15 d, which was consistent with the characteristics of osteoblasts (Figure 1D). These results showed that the extracted BMMSCs had the potential to differentiate into

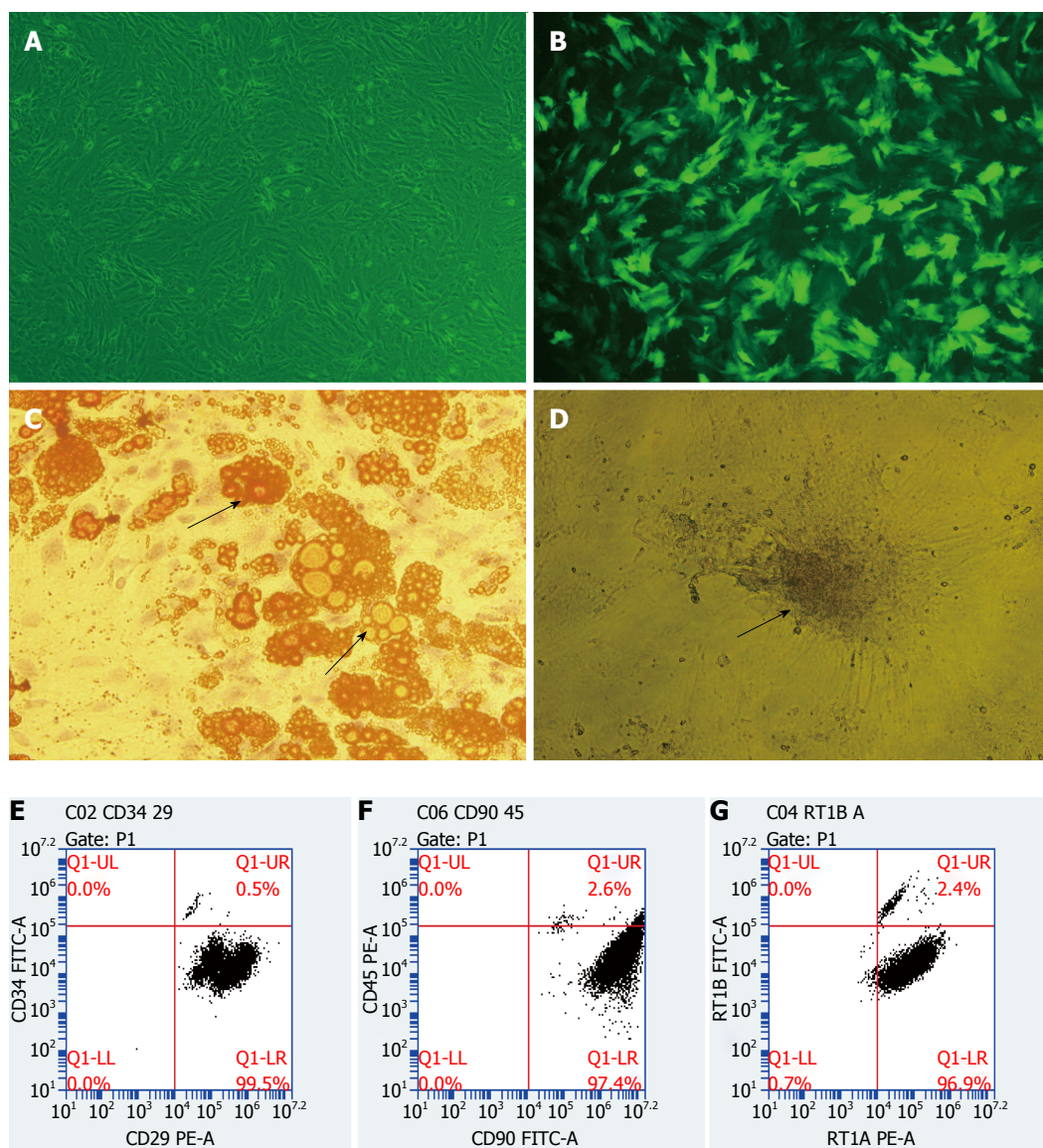


Figure 1 Morphology, transformation, and flow cytometric analysis of bone marrow-derived mesenchymal stem cells. A: The third generation of bone marrow-derived mesenchymal stem cells (BMMSCs) (normal field, $\times 100$): BMMSCs grew to complete adherence, and the cells were spindle-shaped, some were in a vortex or daisy-like arrangement; B: HO-1/BMMSCs (fluorescence field, $\times 100$): more than 80% of the BMMSCs expressed green fluorescence after transduction with HO-1; C: Adipogenic induction of BMMSCs, with orange lipid droplets stained by Oil red O in cytoplasm ($\times 200$, shown by an arrow); D: Osteogenic induction of BMMSCs, with black calcium deposits in cytoplasm stained by von Kossa's reagent ($\times 200$, shown by an arrow); E: The percentage of CD29⁺CD34⁻ cells was 99.5%; F: The percentage of CD90⁺CD45⁻ cells was 97.4%; G: The percentage of RT1A⁺RT1B⁻ cells was 96.9%.

adipocytes and osteoblasts *in vitro*.

Morphology and identification of HO-1/BMMSCs

BMMSCs were infected with recombinant adenovirus expressing HO-1 gene for 48 h, and the cells were observed under a fluorescence microscope. The rate of positive cells expressing green fluorescent protein was more than 80% (Figure 1B).

Effects of HO-1/BMMSCs on pathological changes and acute rejection in the transplanted liver

Histopathological manifestations of the transplanted liver in the NS group included obvious congestion in the hepatic sinus, red blood cell deposition in the sinus, peri-central vein and portal area, hepatocyte

swelling, eosinophilic degeneration, and punctate necrosis. The infiltration of a large volume of mixed lymphocytes in the central vein and the portal area was accompanied by necrosis of hepatocytes on POD5. Infiltration of inflammatory cells and necrosis of hepatocytes increased on POD7. The mixed lymphocyte infiltration became significant on POD14, with severe hepatic sinus congestion, hepatocyte destruction, disappearance of lobular structure, and significant liver fibrosis. The transplanted livers in the BMMSCs group showed no obvious hepatic sinus congestion, and the endothelial swelling and hepatocyte necrosis was less severe than those in the NS group. The histological changes showed disordered hepatic lobules on POD5, with slightly infiltrated inflammatory cells and mild

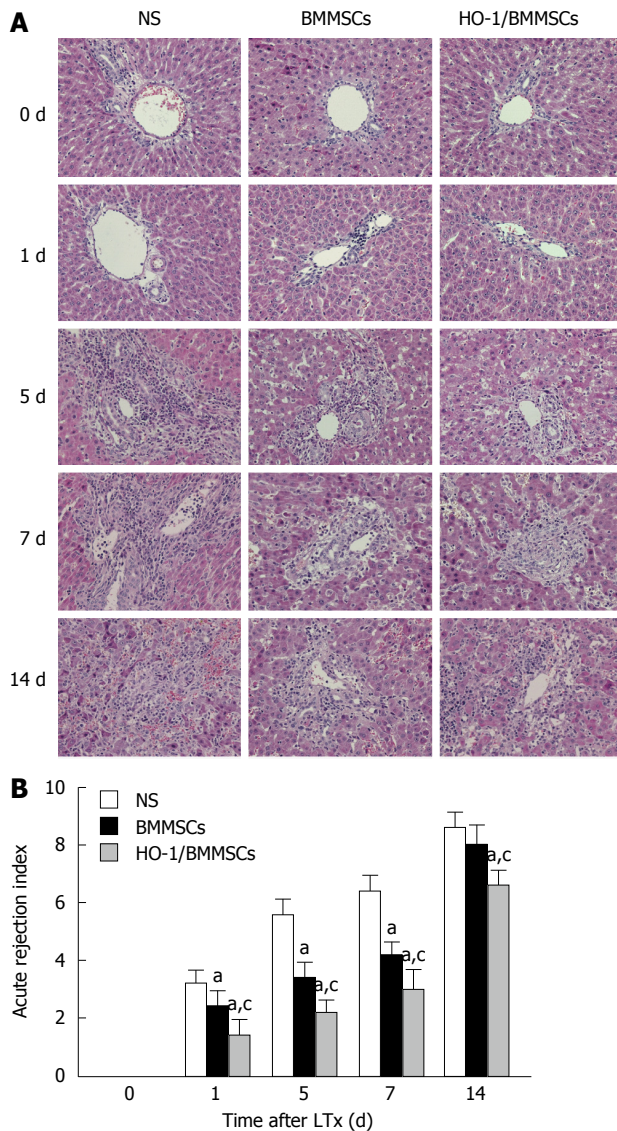


Figure 2 Histological changes of the rat liver and grading of acute cellular rejection after reduced size liver transplantation. A: Hematoxylin and eosin staining of the transplanted liver ($\times 200$): Rejection injuries were aggravated with increased post-operative time in all three groups. Histopathologically, the transplanted liver in the NS group showed obvious congestion in the hepatic sinus, hepatocyte swelling, mainly eosinophilic degeneration, and punctate necrosis. The infiltration of a large volume of mixed lymphocytes in the central vein and the portal area was accompanied by disordered hepatic lobules and necrosis of hepatocytes on postoperative day (POD) 5. Infiltration of inflammatory cells and necrosis of hepatocytes deteriorated on POD7. The mixed lymphocyte infiltration became significant on POD14, with severe hepatic sinus congestion, hepatocyte destruction, and disappearance of the lobular structure. Inflammatory cells infiltration, hepatocyte destruction, and lobular destruction of the transplanted liver in BMMSCs group were less severe than those in the NS group. HO-1/BMMSCs group showed less rejection, and fewer injuries of liver allografts compared with the BMMSCs and NS groups at each time point, without obvious hepatic sinus congestion, large volume of inflammatory cells infiltration, or disorder of the lobular structure; B: Grading of acute cellular rejection (ACR): The rejection injuries tended to deteriorate with increasing post-operative time in all groups. Rejection in the HO-1/BMMSCs group was significantly less severe than that in the BMMSCs and NS groups at all time points ($P < 0.05$). POD1: HO-1/BMMSCs group vs BMMSCs group vs NS group: 1.40 ± 0.55 vs 2.40 ± 0.55 vs 3.20 ± 0.45 ($P < 0.05$). POD5: HO-1/BMMSCs group vs BMMSCs group vs NS group: 2.20 ± 0.45 vs 3.40 ± 0.55 vs 5.60 ± 0.55 ($P < 0.01$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 3.00 ± 0.71 vs 4.20 ± 0.45 vs 6.40 ± 0.55 ($P < 0.01$). POD14: HO-1/BMMSCs group vs NS group: 6.60 ± 0.55 vs 8.60 ± 0.55 ($P < 0.01$); HO-1/BMMSCs group vs BMMSCs group: 6.60 ± 0.55 vs 8.00 ± 0.71 ($P < 0.01$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMMSCs group.

necrosis of hepatocytes. Inflammatory cell infiltration and hepatocyte necrosis progressed on POD7. On POD14, lymphocyte infiltration became obvious, liver sinus congestion increased, accompanied by liver fibrosis and incomplete hepatic lobule structures. The HO-1/BMMSCs group showed less rejection, and fewer injuries of the liver allografts compared with the BMMSCs and NS groups at all time points, without obvious hepatic sinus congestion, large volume of inflammatory cell infiltration, or disordered lobular structures; only mild hepatic fibrosis was observed (Figure 2A).

The rejection injuries tended to increase in all the groups with time after operation. Rejection in the HO-1/BMMSCs group was the least severe at all time points, and was significantly less severe than that in the BMMSCs and NS groups ($P < 0.05$). Rejection in the BMMSCs group was significantly less severe than that in the NS group on POD1, 5, and 7 ($P < 0.05$). There was no significant difference between the BMMSCs group and the NS group on POD14 (Figure 2B).

Improvement of liver function by HO-1/BMMSCs after RLT

The serum ALT and AST in the three groups increased initially and then decreased gradually after operation, while TBIL tended to increase with time. The serum levels of liver enzymes and TBIL in the HO-1/BMMSCs group were significantly lower than in the BMMSCs and NS groups on POD1, 5, 7, and 14 ($P < 0.05$). The serum liver enzymes and TBIL in the BMMSCs group were significantly lower than in the NS group ($P < 0.05$; Figure 3).

Reduction of PVP by HO-1/BMMSCs after RLT

The PVP correlates with the blood flow and resistance of the PV, and plays an important role in maintaining the blood flow in the liver. The PVP was measured on POD7 after RLT. The PVP in the HO-1/BMMSCs and BMMSCs groups was significantly lower than that in the NS group ($P < 0.05$; Figure 4).

Improvement of liver sinus microcirculation by HO-1/BMMSCs after RLT

Effects of HO-1/BMMSCs on ET-1 expression in the transplanted liver: ET-1 positive cells were expressed in the hepatic sinusoids of transplanted livers surrounding the Glisson system. The proportions of ET-1 positive cells in the BMMSCs and HO-1/BMMSCs groups were significantly lower than that in the NS group, and HO-1/BMMSCs group showed fewer positive cells than the BMMSCs group. There were relatively more ET-1-positive cells in the NS group, which increased with time after operation (Figure 5A and B).

The levels of ET-1 in the transplanted liver decreased initially and then increased with time after operation. The level of ET-1 was the lowest in the HO-1/BMMSCs group, while the NS group showed

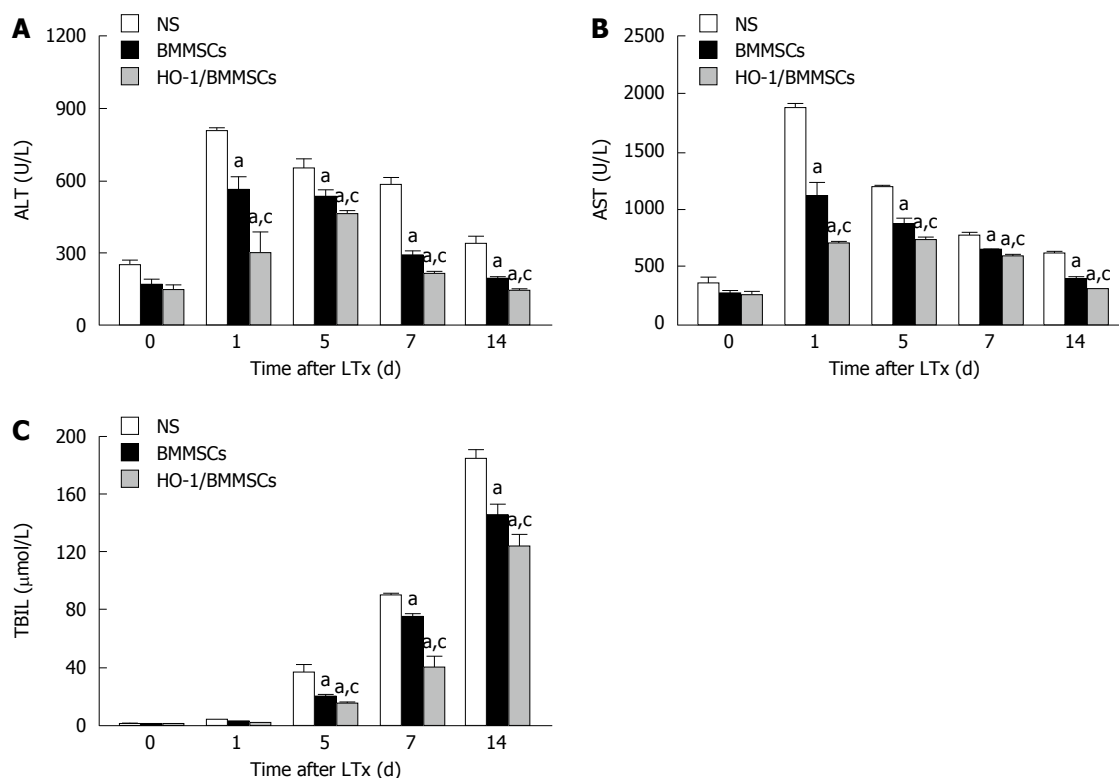


Figure 3 The level of liver function indexes after liver transplantation. A: Alanine aminotransferase (ALT); B: Aspartate aminotransferase (AST); C: Total bilirubin (TBIL). The serum ALT and AST in the three groups increased initially and then decreased with increasing post-operative time. The serum liver enzymes in the HO-1/BMSCs group were the lowest, and NS group were the highest on postoperative day (POD) 1, 5, 7, and 14 ($P < 0.05$). TBIL showed a trend to increase with time. TBIL of the HO-1/BMSCs group were the lowest, and were highest in the NS group on POD5, 7 and 14 ($P < 0.05$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMSCs group.

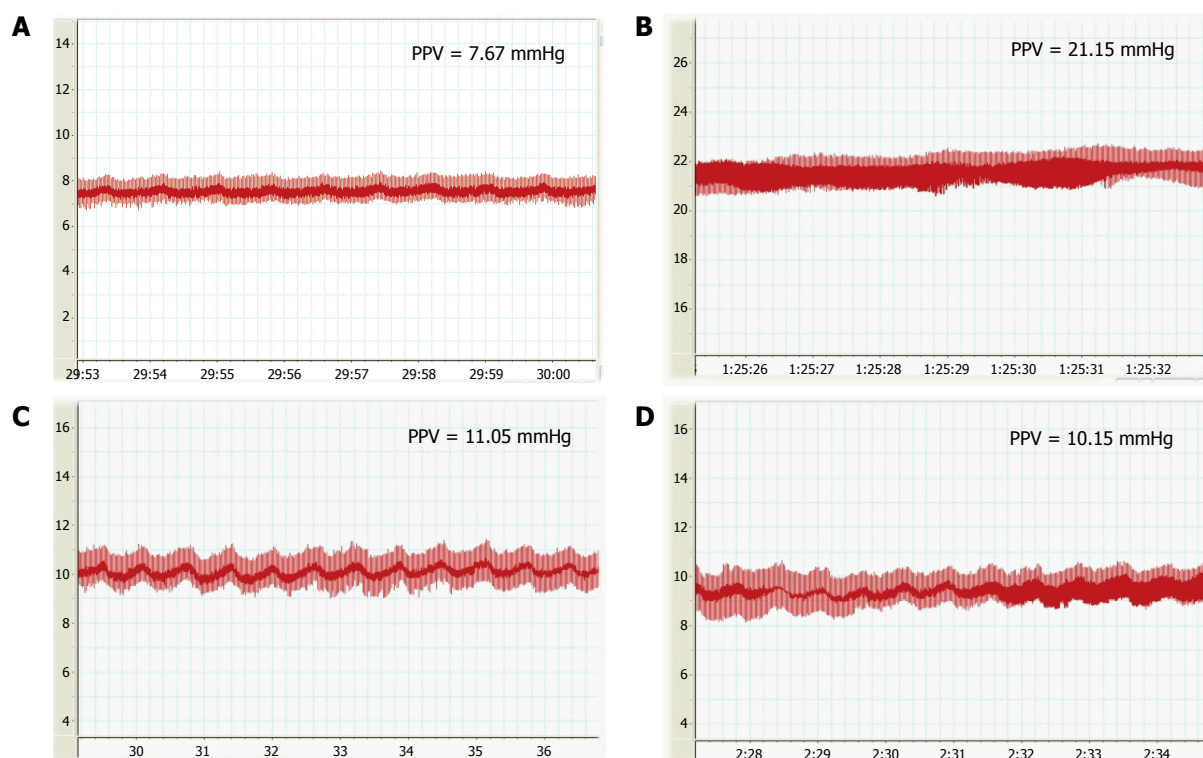


Figure 4 Portal vein pressure on the 7th d after liver transplantation. A: Portal vein pressure (PVP) of normal BN rats; B: PVP of normal saline (NS) group on postoperative day (POD) 7; C: PVP of bone marrow-derived mesenchymal stem cells (BMSCs) group on POD7; D: PVP of HO-1/BMSCs group on POD7. PVP of both the HO-1/BMSCs group and the BMSCs group was significantly lower than that of NS group on POD7 ($P < 0.05$). HO-1/BMSCs group vs NS group: 10.67 ± 0.35 vs 21.26 ± 0.20 ($P < 0.01$); BMSCs group vs NS group: 10.95 ± 0.22 vs 21.26 ± 0.20 ($P < 0.01$).

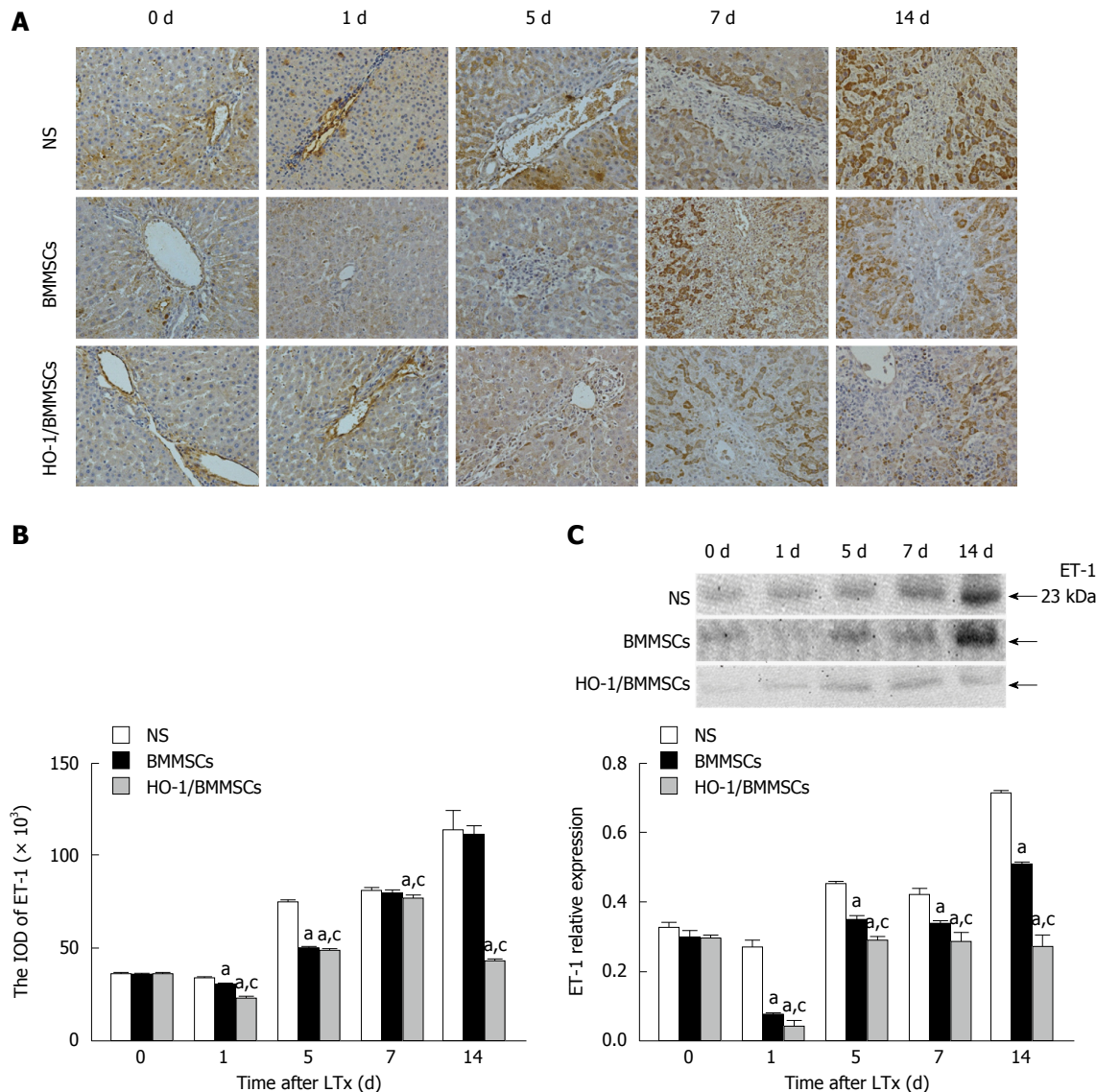


Figure 5 Endothelin-1 expression in the liver after liver transplantation. A: Immunohistochemistry (IHC) of endothelin-1 (ET-1); B: IHC integrated optical density (IOD) of ET-1; C: ET-1 protein levels. ET-1 protein levels: postoperative day (POD) 1: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.04 ± 0.02 vs 0.07 ± 0.01 vs 0.27 ± 0.02 ($P < 0.05$). POD5: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.29 ± 0.01 vs 0.35 ± 0.01 vs 0.45 ± 0.01 ($P < 0.01$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.28 ± 0.03 vs 0.34 ± 0.01 vs 0.42 ± 0.02 ($P < 0.05$). POD14: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.27 ± 0.04 vs 0.51 ± 0.01 vs 0.71 ± 0.01 ($P < 0.01$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMMSCs group.

the highest levels on POD1, 5, 7, and 14 ($P < 0.05$). The results indicated that BMMSCs could inhibit the secretion of ET-1 by SECs, thereby reducing sinusoidal contraction, decreasing sinusoidal vascular resistance, and improving hepatic sinus perfusion. The sinusoidal vasoconstriction alleviating effects of HO-1/BMMSCs were superior to those of BMMSCs (Figure 5C).

The serum ET-1 levels after RLT decreased initially and then increased gradually after operation. The level of ET-1 in the HO-1/BMMSCs group was lower than that in the NS group on POD1 ($P < 0.01$). The level of ET-1 in the HO-1/BMMSCs group was the lowest among the three groups, while the NS group showed the highest levels on POD5 and POD7 ($P < 0.01$). The level of ET-1 in the HO-1/BMMSCs group was significantly lower than that in the BMMSCs and NS groups on POD14 ($P < 0.01$). The results indicated

that BMMSCs could inhibit the secretion of ET-1 in rats after liver transplantation, and the inhibitory effects of HO-1/BMMSCs were better than those of BMMSCs (Figure 6A).

Effects of HO-1/BMMSCs on eNOS expression in the transplanted liver: The immunohistochemical results showed that eNOS positive cells were present in the hepatic sinusoids around the Glisson system. The ratios of eNOS positive cells in the BMMSCs and HO-1/BMMSCs groups were significantly higher than in the NS group. HO-1/BMMSCs group showed more eNOS-positive cells than the BMMSCs group, and the number of eNOS-positive cells was low in the NS group (Figure 7A and B).

The level of eNOS in the transplanted liver tended to increase with time after operation. The level of

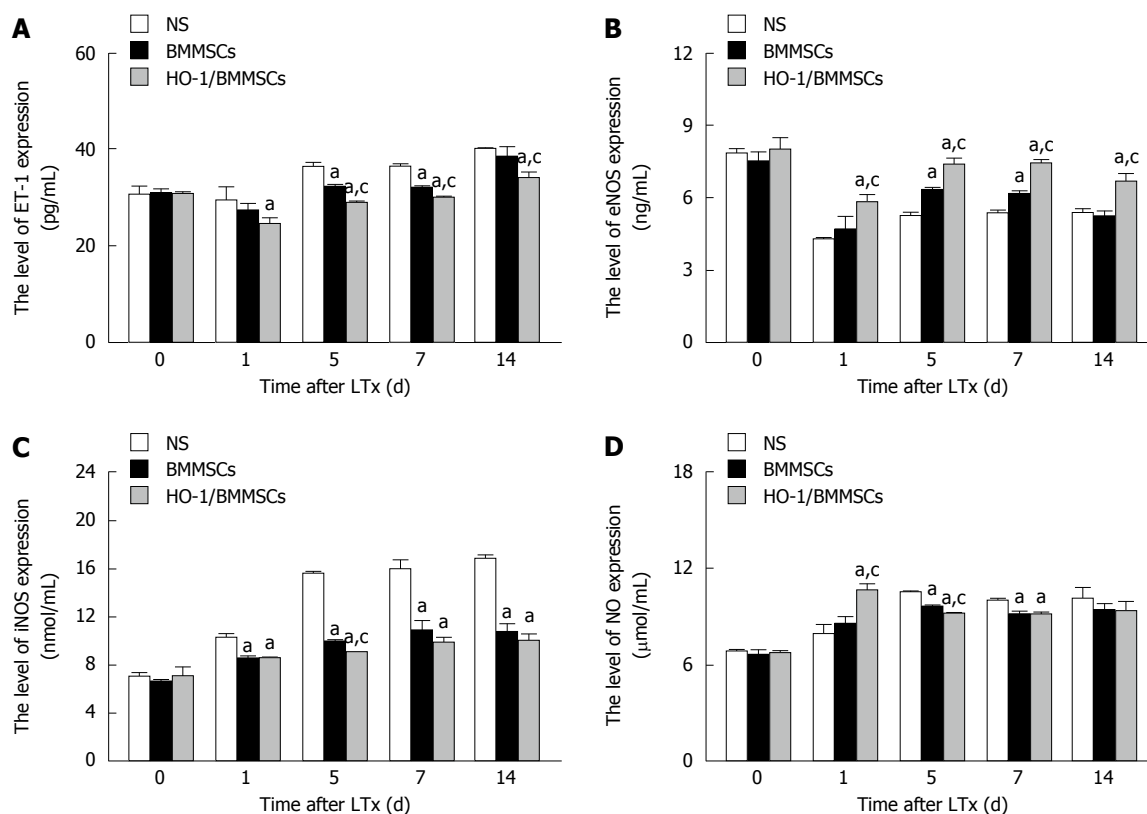


Figure 6 Endothelin-1/nitrous oxide related factors expression in serum after liver transplantation. A: Expression of endothelin-1 (ET-1); B: Expression of endothelial nitric-oxide synthase (eNOS); C: Expression of inducible nitric-oxide synthase (iNOS); D: Expression of NO. The serum ET-1 after RLT decreased initially and then increased with the extension of post-operative time. Postoperative day (POD) 1: HO-1/BMMSCs group vs NS group: 24.53 ± 1.22 vs 29.29 ± 2.97 ($P < 0.01$). POD5: HO-1/BMMSCs vs BMMSCs vs NS group: 28.84 ± 0.17 vs 32.18 ± 0.30 vs 36.27 ± 0.92 ($P < 0.01$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 29.92 ± 0.28 vs 31.87 ± 0.50 vs 36.25 ± 0.54 ($P < 0.01$). POD14: HO-1/BMMSCs group vs NS group: 33.82 ± 1.44 vs 40.03 ± 0.17 ($P < 0.01$); HO-1/BMMSCs group vs BMMSCs group: 33.82 ± 1.44 vs 38.35 ± 2.09 ($P < 0.01$). The serum levels of eNOS increased with the extension of post-operative time. POD1: HO-1/BMMSCs group vs NS group: 5.86 ± 0.30 vs 4.30 ± 0.07 ($P < 0.01$); HO-1/BMMSCs group vs BMMSCs group: 5.86 ± 0.30 vs 4.74 ± 0.54 ($P < 0.01$). POD5: HO-1/BMMSCs vs BMMSCs vs NS group: 7.39 ± 0.29 vs 6.36 ± 0.07 vs 5.27 ± 0.16 ($P < 0.01$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 7.43 ± 0.19 vs 6.17 ± 0.12 vs 5.37 ± 0.12 ($P < 0.01$). POD14: HO-1/BMMSCs group vs NS group: 6.72 ± 0.30 vs 5.40 ± 0.15 ($P < 0.01$); HO-1/BMMSCs group vs BMMSCs group: 6.72 ± 0.30 vs 5.26 ± 0.20 ($P < 0.01$). The serum levels of iNOS increased with the extension of post-operative time, and was most significant in NS group. POD1: HO-1/BMMSCs group vs NS group: 8.57 ± 0.07 vs 10.24 ± 0.42 ($P < 0.01$); BMMSCs group vs NS group: 8.56 ± 0.20 vs 10.24 ± 0.42 ($P < 0.01$). POD5: HO-1/BMMSCs vs BMMSCs vs NS group: 9.06 ± 0.05 vs 9.97 ± 0.08 vs 15.66 ± 0.13 ($P < 0.01$). POD7: HO-1/BMMSCs group vs NS group: 9.87 ± 0.42 vs 15.96 ± 0.74 ($P < 0.01$); BMMSCs group vs NS group: 10.85 ± 0.90 vs 15.96 ± 0.74 ($P < 0.01$). POD14: HO-1/BMMSCs group vs NS group: 10.04 ± 0.61 vs 16.85 ± 0.31 ($P < 0.05$); BMMSCs group vs NS group: 10.73 ± 0.64 vs 16.85 ± 0.31 ($P < 0.01$). The serum levels of NO tended to increase initially and then decrease with the extension of post-operative time. POD1: HO-1/BMMSCs group vs NS group: 10.64 ± 0.36 vs 7.92 ± 0.59 ($P < 0.01$); HO-1/BMMSCs group vs BMMSCs group: 10.64 ± 0.36 vs 8.56 ± 0.44 ($P < 0.01$). POD5: HO-1/BMMSCs vs BMMSCs vs NS group: 9.17 ± 0.08 vs 9.65 ± 0.09 vs 10.50 ± 0.07 ($P < 0.01$). POD7: HO-1/BMMSCs group vs NS group: 9.19 ± 0.12 vs 10.00 ± 0.10 ($P < 0.01$); BMMSCs group vs NS group: 9.41 ± 0.39 vs 10.00 ± 0.10 ($P < 0.01$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMMSCs group.

eNOS was the highest in HO-1/BMMSCs group, and the lowest in the NS group on POD1, 7, and 14 ($P < 0.05$). The level of eNOS in the HO-1/BMMSCs group was higher than that in the BMMSCs and NS groups on POD5 ($P < 0.05$). These results suggested that BMMSCs could promote the synthesis of eNOS in the transplanted liver, and more significantly in the HO-1/BMMSCs group (Figure 7C).

The serum levels of eNOS increased from POD1 to POD14 after RLT, but were lower than that on POD0. The levels of eNOS in the HO-1/BMMSCs group were significantly higher than those in the BMMSCs and NS groups on POD1 and POD14 ($P < 0.01$). The level of eNOS was highest in HO-1/BMMSCs group and lowest in the NS group on POD5 and POD7 ($P < 0.01$). This suggested that BMMSCs could promote the synthesis

of eNOS in rats after RLT, and the promotive effects of HO-1/BMMSCs were more significant than those of BMMSCs (Figure 6B).

Effects of HO-1/BMMSCs on iNOS expression in the transplanted liver:

The immunohistochemical results showed that iNOS positive cells were present in hepatic sinusoids around the Glisson system, and that iNOS was expressed in the cytoplasm of hepatocytes. The ratio and staining intensity of iNOS positive cells in the BMMSCs and HO-1/BMMSCs groups were significantly lower than those in the NS group, and those in the HO-1/BMMSCs group were lower than in the BMMSCs group. The number and staining intensity of iNOS positive cells in the NS group were relatively high (Figure 8A and B).

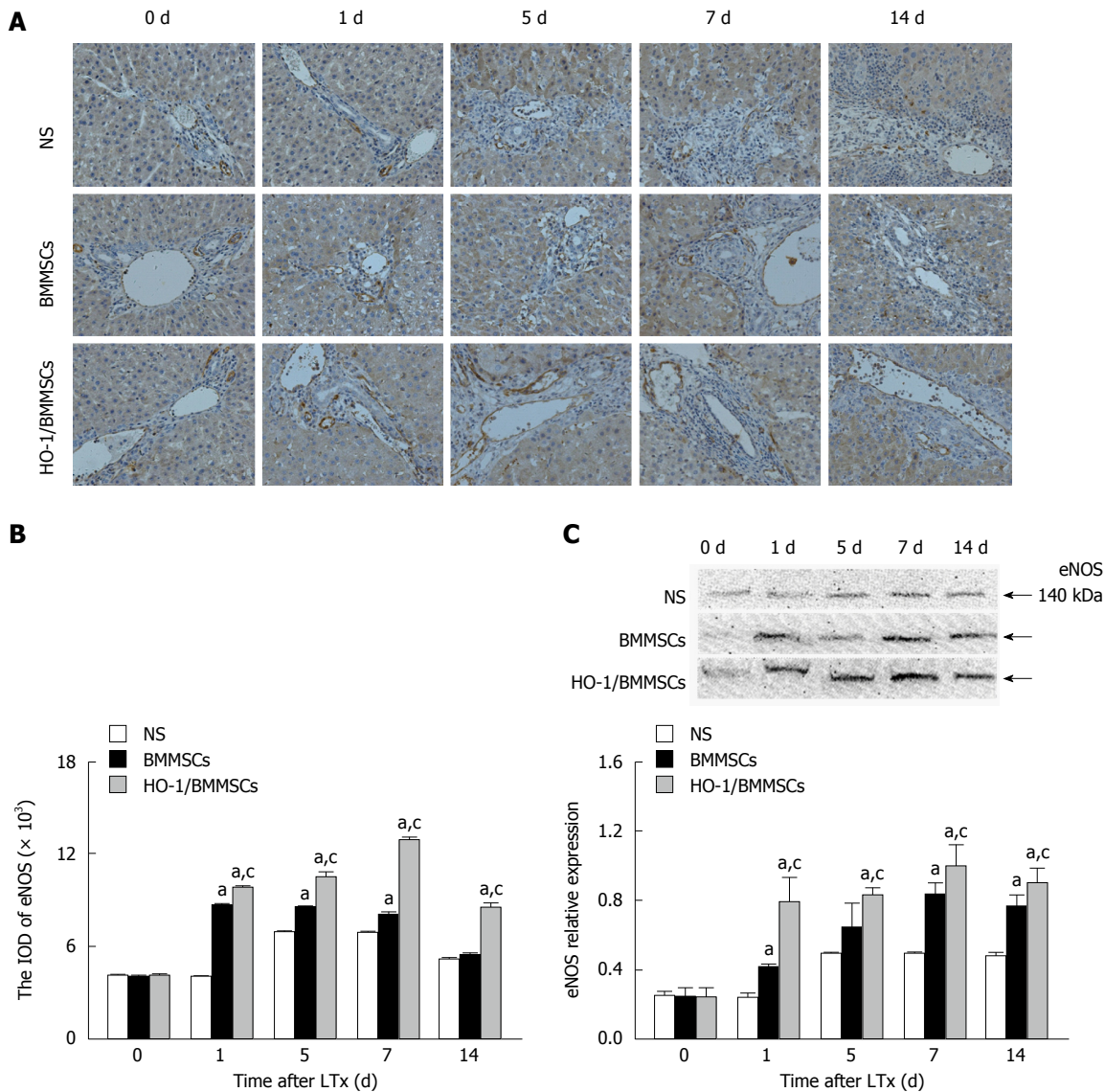


Figure 7 Endothelial nitric-oxide synthase expression in liver after liver transplantation. A: Immunohistochemistry (IHC) of endothelial nitric-oxide synthase (eNOS); B: IHC integrated optical density (IOD) of eNOS; C: Western blotting and eNOS protein levels. eNOS protein levels: postoperative day (POD) 1: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.79 ± 0.14 vs 0.42 ± 0.02 vs 0.25 ± 0.02 ($P < 0.05$). POD5: HO-1/BMMSCs vs NS group: 0.84 ± 0.04 vs 0.50 ± 0.01 ($P < 0.05$); HO-1/BMMSCs group vs BMMSCs group vs NS group: 1.00 ± 0.12 vs 0.84 ± 0.06 vs 0.50 ± 0.01 ($P < 0.05$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.90 ± 0.09 vs 0.77 ± 0.07 vs 0.48 ± 0.02 ($P < 0.01$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMMSCs group.

The level of iNOS in the transplanted liver tended to increase with the extension of post-operative time. The level of iNOS in the HO-1/BMMSCs and BMMSCs groups was lower than that in the NS group at all time points. The level of iNOS in the HO-1/BMMSCs group was significantly higher than that in the NS group on POD1 ($P < 0.05$), and was significantly higher than that in BMMSCs and NS groups on POD5 ($P < 0.05$). The expression of iNOS was highest in the HO-1/BMMSCs group and lowest in the NS group on POD7 and POD14 ($P < 0.05$). This suggested that BMMSCs could inhibit the synthesis of iNOS in the transplanted liver, and that the inhibitory effects of HO-1/BMMSCs were more significant than those of BMMSCs (Figure 8C).

The serum expression of iNOS increased with the

extension of post-operative time. The expression of iNOS in HO-1/BMMSCs group and BMMSCs group was significantly lower than that in NS group on POD1, 7, and 14 ($P < 0.01$). The expression of iNOS was the lowest in HO-1/BMMSCs group and highest in NS group on POD5 ($P < 0.01$). This suggested that BMMSCs could inhibit the synthesis of iNOS in rats after RLT, and the inhibitory effects of HO-1/BMMSCs were more significant than that of BMMSCs (Figure 6C).

Effects of HO-1/BMMSCs on NO production in rats after RLT:

The serum expression of NO tended to increase initially and then decrease with time after operation; however, the decreasing tendency was not significant in all groups. The serum NO in the

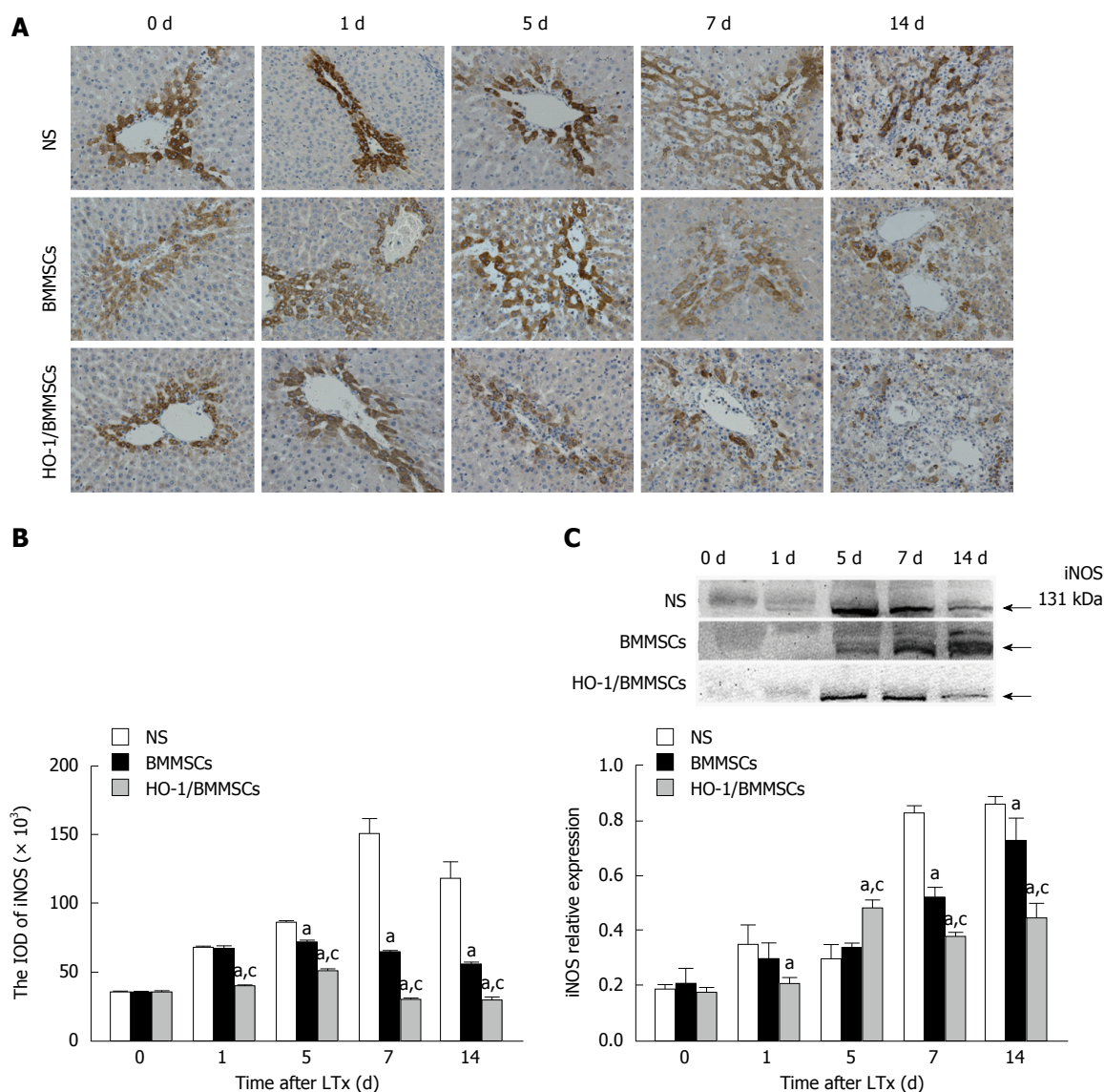


Figure 8 Inducible nitric-oxide synthase expression in the liver after liver transplantation. A: Immunohistochemistry (IHC) of inducible nitric-oxide synthase (iNOS); B: IHC integrated optical density (IOD) of iNOS; C: iNOS protein levels. iNOS protein levels: postoperative day (POD) 1: HO-1/BMMSCs group vs NS group: 0.21 ± 0.02 vs 0.35 ± 0.07 ($P < 0.05$). POD5: HO-1/BMMSCs vs NS group: 0.48 ± 0.03 vs 0.30 ± 0.05 ($P < 0.01$); HO-1/BMMSCs group vs BMMSCs group: 0.48 ± 0.03 vs 0.34 ± 0.02 ($P < 0.01$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.38 ± 0.01 vs 0.52 ± 0.04 vs 0.83 ± 0.03 ($P < 0.01$). POD14: HO-1/BMMSCs group vs BMMSCs group vs BMMSCs group vs NS group: 0.45 ± 0.06 vs 0.73 ± 0.08 vs 0.86 ± 0.03 ($P < 0.05$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMMSCs group.

HO-1/BMMSCs group was higher than that in the BMMSCs and NS groups on POD1 ($P < 0.01$). The serum NO on POD5 was lowest in the HO-1/BMMSCs group and highest in the NS group ($P < 0.01$). The serum NO in the HO-1/BMMSCs and BMMSCs groups was significantly lower than that in the NS group on POD7 ($P < 0.01$). This suggested that BMMSCs could inhibit the synthesis of NO in the transplanted liver, and the inhibitory effects of HO-1/BMMSCs were more significant than those of simple BMMSCs (Figure 6D).

Effects of HO-1/BMMSCs on vWF expression in hepatic sinusoids: vWF was expressed in sinusoidal endothelial cells and SECs of transplanted liver. Immunohistochemical staining for liver vWF showed that the ratios of vWF positive cells in the BMMSCs

and HO-1/BMMSCs groups were significantly higher than that in the NS group, and the level in the HO-1/BMMSCs group was higher than that in the BMMSCs group. The number of vWF positive cells in the NS group was relatively low and the cells were scattered. Whereas the vWF positive cells in the BMMSCs and HO-1/BMMSCs groups were arranged regularly and were consistent with hepatic sinusoids. These results showed that HO-1/BMMSCs could promote the proliferation of SECs and the remodeling of hepatic sinusoids more significantly than BMMSCs. The results suggested that HO-1 could enhance the ability of BMMSCs to promote the regeneration of hepatocytes (Figure 9A and B).

Intrahepatic expression of vWF tended to increase initially and then decrease after POD7; however, the

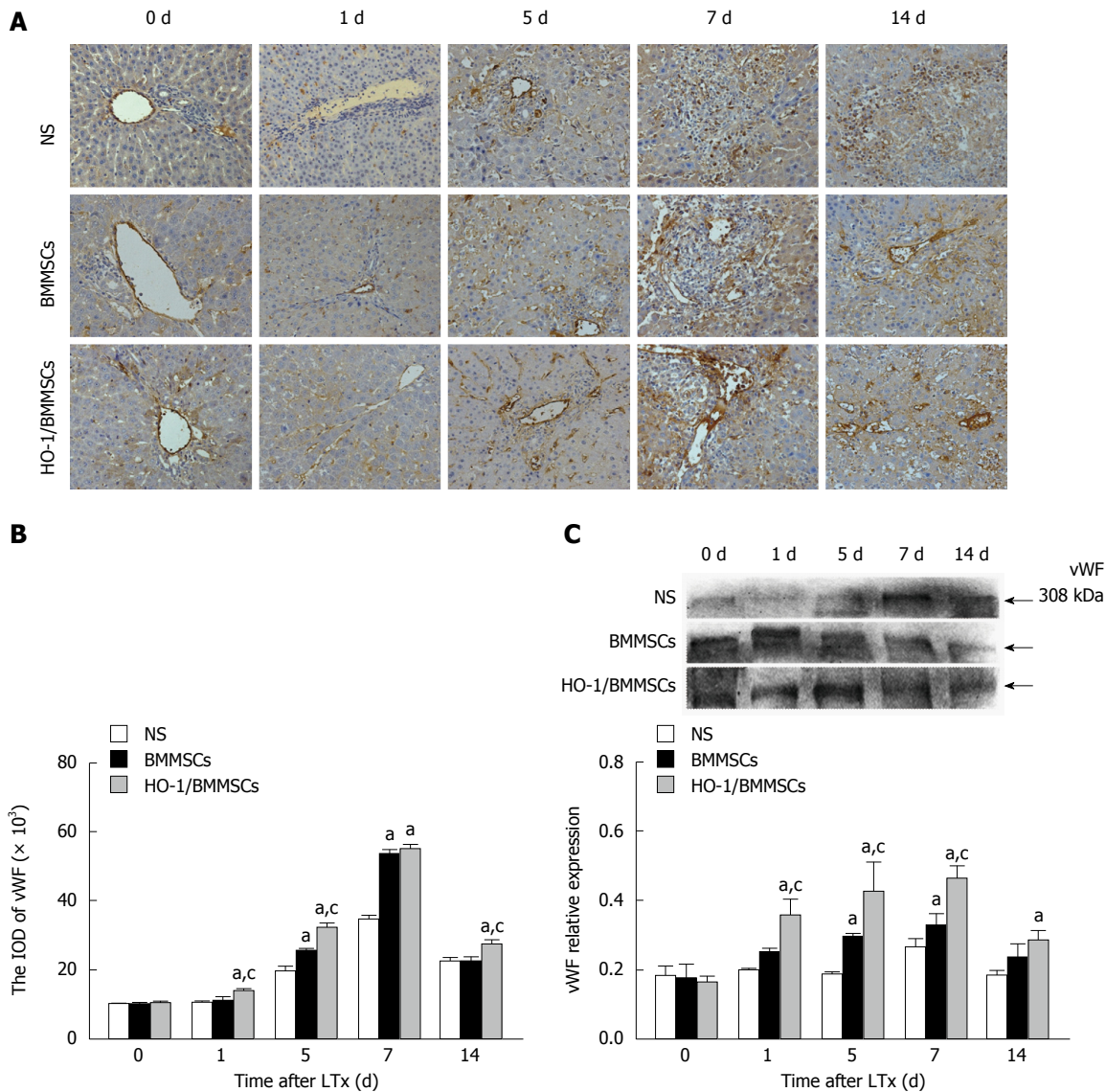


Figure 9 von Willebrand factor expression in the liver after liver transplantation. A: Immunohistochemistry (IHC) of von willebrand factor (vWF); B: IHC integrated optical density (IOD) of vWF; C: Western blotting and vWF protein levels. vWF protein levels: Postoperative day (POD) 1: HO-1/BMMSCs group vs NS group: 0.36 ± 0.05 vs 0.20 ± 0.00 ($P < 0.01$); HO-1/BMMSCs group vs BMMSCs group: 0.36 ± 0.05 vs 0.25 ± 0.01 ($P < 0.01$). POD5: HO-1/BMMSCs vs BMMSCs group vs NS group: 0.43 ± 0.08 vs 0.30 ± 0.01 vs 0.19 ± 0.01 ($P < 0.05$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 0.46 ± 0.04 vs 0.33 ± 0.03 vs 0.26 ± 0.03 ($P < 0.05$). POD14: HO-1/BMMSCs group vs NS group: 0.28 ± 0.03 vs 0.18 ± 0.01 ($P < 0.01$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMMSCs group.

NS group showed no significant increasing trend. The expression of vWF in the HO-1/BMMSCs group was significantly higher than in the BMMSCs and NS groups on POD1 ($P < 0.05$). The level of vWF was highest in the HO-1/BMMSCs group and lowest in the NS group on POD5 and POD7 ($P < 0.05$). The level of vWF in the HO-1/BMMSCs group was higher than that in NS group on POD14 ($P < 0.05$). These results suggested that BMMSCs could promote the synthesis of vWF in the transplanted liver graft, and that the effect of HO-1/BMMSCs was more significant than that of BMMSCs. However, the effects of BMMSCs decreased with time after operation (Figure 9C).

Effects of HO-1/BMMSCs on the degradation of HA by SECs: The serum HA in all groups tended to

increase initially, then decrease, and later increase again with increasing post-operative time. Serum HA was lowest in the HO-1/BMMSCs group and highest in the NS group on POD5 and POD7 ($P < 0.01$). Serum HA in the HO-1/BMMSCs group was lower than that in the NS group on POD14 ($P < 0.01$). This suggested that BMMSCs could promote the degradation of HA in rats after RLT, and the effect of HO-1/BMMSCs was more significant than that of simple BMMSCs. However, the effects of BMMSCs decreased with increasing post-operative time (Figure 10).

Hepatic mitochondrial function ameliorated by HO-1/BMMSCs

Effects on mitochondrial morphology under electron microscope: There were various degrees of

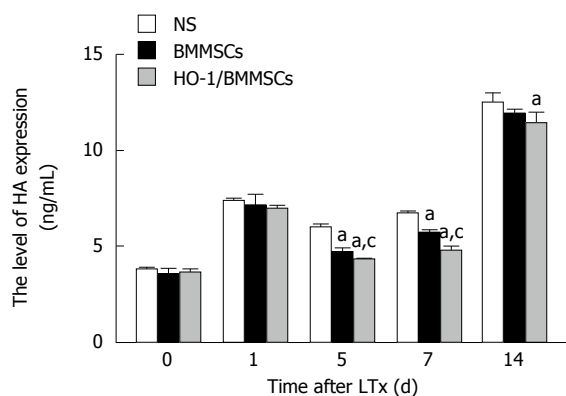


Figure 10 Hyaluronic acid expression in serum after liver transplantation. The serum hyaluronic acid (HA) level tended to increase initially, then decrease, and later increase again with the extension of post-operative time. Serum HA in the HO-1/BMMSCs group was lower than that of the BMMSCs and NS groups on postoperative day (POD) 5: HO-1/BMMSCs vs BMMSCs group vs NS group: 4.31 ± 0.07 vs 4.66 ± 0.24 vs 5.99 ± 0.15 ($P < 0.01$). POD7: HO-1/BMMSCs group vs BMMSCs group vs NS group: 4.78 ± 0.23 vs 5.73 ± 0.11 vs 6.72 ± 0.14 ($P < 0.01$). POD14: HO-1/BMMSCs group vs NS group: 11.43 ± 0.52 vs 12.52 ± 0.51 ($P < 0.01$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMMSCs group.

damage to mitochondria in hepatocytes after RLT. The damage was more severe on POD7 in the NS group, with obvious mitochondrial swelling, vacuolization, and disturbed structures of mitochondrial cristae (some even disappeared). Mitochondria in the HO-1/BMMSCs and BMMSCs groups showed mild swelling, no vacuolization, and integrated structures of the mitochondrial ridge. These results suggested that HO-1/BMMSCs could ameliorate the damage to mitochondria (Figure 11).

Alterations in ATPase activity

ATPase can degrade ATP to produce ADP and inorganic phosphorus, thus the amount of inorganic phosphorus reflects the activity of ATPase. The activities of ATPase in the transplanted liver tended to increase initially and then decrease after POD 7. The activities of ATPase in the HO-1/BMMSCs group were higher than those of the BMMSCs and NS groups on POD1, 7, and 14 ($P < 0.05$). The activities of ATPase in the HO-1/BMMSCs and BMMSCs groups were both higher than that in NS group on POD5 ($P < 0.05$). The results suggested that HO-1/BMMSCs could improve the activity of hepatic Na^+/K^+ -ATPase and promote hepatic energy metabolism (Figure 12A).

Effects on ASTm

ASTm exists in the mitochondria of hepatocytes, and is released into the blood when hepatocytes are severely damaged. Thus, the serum ASTm level can indicate whether the liver function is normal or not, and is also a sign of mitochondrial damage. In the present study, ASTm showed a tendency to increase initially and then decrease with increasing post-operative time. The expression of ASTm was lowest in the HO-1/BMMSCs group and highest in the NS group on POD0, 1, 5, 7 and 14 ($P < 0.05$; Figure 12B).

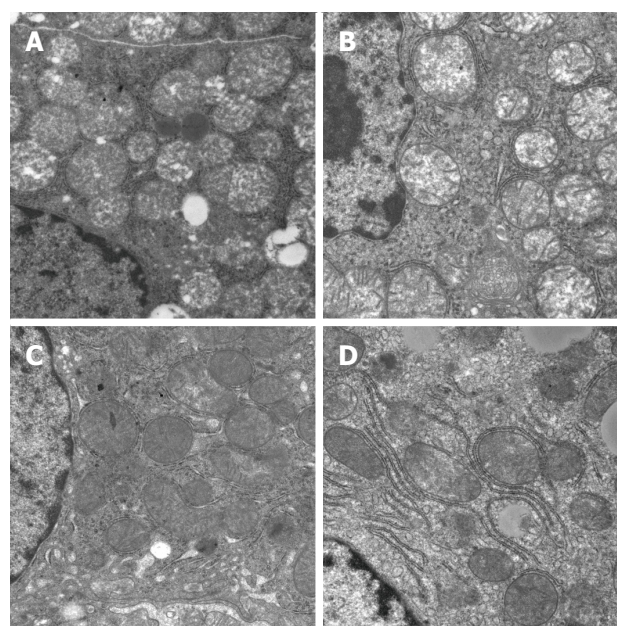


Figure 11 Mitochondrial ultrastructure changes on the 7th d after liver transplantation. A: Postoperative day (POD) 0; B: Normal saline (NS) group on POD7; C: Bone marrow-derived mesenchymal stem cells (BMMSCs) group on POD7; D: HO-1/BMMSCs group on POD7 ($\times 25000$). The mitochondrial damage to hepatocytes was relatively severe in the NS group, with obvious swelling and vacuolized mitochondria and disturbed structures of mitochondrial cristae (some even disappeared). Mitochondria in the HO-1/BMMSCs and BMMSCs groups showed mild swelling, no vacuolization, and the integrated structures of the mitochondrial ridge were present.

DISCUSSION

Ischemia-reperfusion, reduction of liver volume, and rejection of the transplanted liver can lead to disturbance of hepatic microcirculation: the main changes are stasis in the hepatic sinusoids and collapse of Disse's space^[38]. Transient or sustained hypertension of the PV will cause mechanical damage to the transplanted liver, and induce the activation and release of a variety of cytokines, resulting in a vicious circle. Shear stress caused by excessive blood flow of the PV will break the balance between vasoconstrictor factors and vasodilator factors, leading to microcirculation disturbance in hepatic sinusoids^[39,40]. Reduction of the PVP and improvement of hepatic venous perfusion are important to improve liver function after RLT^[39]. BMMSCs have tissue repair, regulation of inflammation, and immune response functions, and have protective effects on transplanted livers^[22,27,41,42]. To solve the problem that BMMSCs have low activity in damaged tissue^[43], and have a short survival time^[22,24,44,45], adenovirus-transduced BMMSCs expressing HO-1 were used to study their protective effects on transplanted livers, sinusoidal microcirculation, and energy metabolism after RLT.

Hepatic microcirculation comprises the circulation of the terminal PV and hepatic artery through sinusoids to central veins. The central structure is the sinusoid. The wall of a hepatic sinusoid comprises

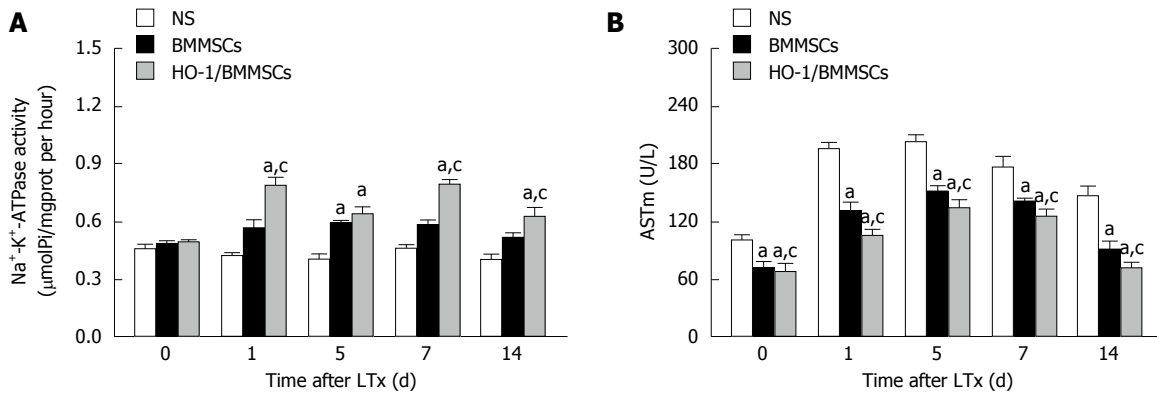


Figure 12 Liver Na⁺-K⁺-ATPase activity and mitochondrial aspartate aminotransferase level after liver transplantation. A: Na⁺-K⁺-ATPase activity; B: Serum mitochondrial aspartate aminotransferase (ASTm). Na⁺-K⁺-ATPase activity (μmolPi/mgprot per hour): The degradation of ATP per mg of protein per hour produces 1 μmol of inorganic phosphorus was defined as one unit of ATPase activity. The activities of ATPase in the HO-1/BMSCs group were higher than those of the BMSCs and NS groups. Postoperative day (POD) 1: HO-1/BMSCs group vs NS group: 0.79 ± 0.04 vs 0.42 ± 0.02 ($P < 0.01$); HO-1/BMSCs group vs BMSCs group: 0.79 ± 0.04 vs 0.57 ± 0.04 ($P < 0.01$). POD5: BMSCs vs NS group: 0.59 ± 0.01 vs 0.40 ± 0.03 ($P < 0.05$); HO-1/BMSCs vs NS group: 0.64 ± 0.04 vs 0.40 ± 0.03 ($P < 0.01$). POD7: HO-1/BMSCs group vs NS group: 0.79 ± 0.03 vs 0.46 ± 0.02 ($P < 0.01$); HO-1/BMSCs group vs BMSCs group: 0.79 ± 0.03 vs 0.58 ± 0.03 ($P < 0.01$). POD14: HO-1/BMSCs group vs NS group: 0.63 ± 0.05 vs 0.40 ± 0.03 ($P < 0.01$); HO-1/BMSCs group vs BMSCs group: 0.63 ± 0.05 vs 0.52 ± 0.03 ($P < 0.05$). ASTm showed a tendency to increase initially and then decrease with the extension of post-operative time. The expression of ASTm was the lowest in the HO-1/BMSCs group and highest in the NS group on POD0, 1, 5, 7 and 14 ($P < 0.05$). ^a $P < 0.05$ vs NS group, ^c $P < 0.05$ vs HO-1/BMSCs group.

SECs, Kupffer cells, hepatic stellate cells, and crypt cells. Among these cells, SECs account for 70%, protecting the hepatic parenchymal cells and maintaining the structural and functional integrity of hepatic lobules. SECs also show secretory functions of vascular endothelia, secreting ET-1 and NO to regulate the vascular tone and hepatic stellate cells, which plays an important role in maintaining hepatic sinusoidal microcirculation and intrahepatic homeostasis^[46,47]. Impaired SECs, and an imbalance in vasodilator substances, can lead to dysfunction of hepatic microcirculation, such as sinusoidal stasis, ET-1/NO imbalance, and increased reactive oxygen species (ROS)^[48]. ET-1 is produced mainly by vascular endothelial cells, and is the strongest endogenous vasoconstrictor discovered to date. Hypoxia is an important stimulating factor of ET-1 upregulation^[49]. When the liver is damaged, the expression of ET-1 in SECs increases, the production of NO is reduced, and the balance between vasoconstriction and vasodilatation is broken. The vasoconstrictive effects of ET-1 are dominant, leading to hepatic sinusoid stasis, vasoconstriction, increased intrahepatic vascular resistance, upregulation of leukocyte-endothelia interaction, and portal hypertension^[50,51]. IRI induces hepatic ischemia and hypoxia; the ET-1 secreted by all kinds of cells in the liver can lead to extensive sinusoidal vasoconstriction, decreased sinusoidal diameters, and dysfunction of hepatic sinusoidal microcirculation^[52]. The results of this study showed that the expression of ET-1 increased initially and then decreased gradually as the degree of liver injury and rejection increased. The levels of ET-1 in the BMSCs and HO-1/BMSCs groups were significantly lower than in the NS group. The level of ET-1 increased gradually with the disappearance of BMSCs, and the

level of ET-1 was the lowest and the duration of the effect was the longest in the HO-1/BMSCs group ($P < 0.05$). Taken together, these results suggested that HO-1/BMSCs could downregulate the expression of ET-1, and alleviate the damage to hepatic sinusoids induced by ET-1, indicating indirectly that HO-1 could prolong the effects of BMSCs.

NO has a protective effect against ET-1 by inhibiting the synthesis of ET, relaxing the smooth muscle to dilate blood vessels, improving microcirculation, and inhibiting platelet, leukocyte adhesion and antioxidation^[53,54]. Endogenous NO is produced by NOS-catalyzed oxidation of ammonia in the guanidine terminus of L-arginine. There are three isoenzymes of NOS (eNOS, iNOS, and nNOS). eNOS is only expressed continuously in the vascular endothelia of the liver, where it relaxes blood vessels, inhibits inflammation, scavenges free radicals, and inhibits platelet activation, adhesion and aggregation, and plays a protective role on the transplanted liver by improving the sinusoidal microcirculation and inhibiting hepatocytes apoptosis^[55]. iNOS is expressed in various kinds of intrahepatic cells, and can produce large amounts of NO under pathological conditions^[56]. Selective iNOS inhibitors were found to improve liver blood flow and reduce liver IRI, but aggravate liver injury under ischemia-reperfusion and sepsis conditions; and eNOS-derived NO could alleviate IRI, while iNOS-derived NO promoted IRI^[57,58]. We found that HO-1/BMSCs and BMSCs promoted the synthesis of eNOS, and inhibited the synthesis of iNOS, while HO-1/BMSCs had more significant effects than BMSCs. As mentioned above, BMSCs were potent in anti-inflammation activity, and could differentiate into endothelial cells, and secrete vascular endothelial growth factor (VEGF)^[11,12]. We hypothesized that

BMMSCs could promote endothelial proliferation and angiogenesis to improve the ET-1/NO and vasodilation balance, thereby improving sinusoidal microcirculation.

ET-1 plays a key role in the regulation of liver microcirculation, and dysfunction of SECs leads to the upregulation of ET-1 to promote hepatic stellate cell contraction and portal hypertension^[59]. Plasma ET-1 levels in the early stage after reperfusion of a transplanted liver correlated with the PVP^[60]. We monitored the PVP on POD7 in this study, and the results suggested that both BMMSCs and HO-1/BMMSCs could reduce the PVP after liver transplantation, which was associated with an improved ET-1/NO balance, leading to downregulation of ET-1 expression and upregulation of eNOS expression. This dual regulation of vasodilatory effects could decrease the PVP and improve hepatic sinusoidal perfusion.

As a glycoprotein present in plasma and on the surface of endothelial cells, vWF is a marker of endothelial cell activation^[61,62]. In this study, rejection and liver damage increased with the extension of post-operative time in the RLT model. In addition, SECs injury gradually increased and vWF expression decreased. BMMSCs showed a potent ability to differentiate into endothelial cells, and promote angiogenesis, tissue repair and secretion of VEGF^[10-15]; therefore, injection of BMMSCs after RLT could reduce apoptosis of hepatocytes and SECs, and promote the proliferation of hepatocytes and SECs, mainly through the effects of VEGF secreted by BMMSCs^[13]. VEGF regulates the recruitment of hepatic sinusoidal endothelial progenitor cells and promotes the proliferation of SECs, which play an important role in the postoperative sinusoidal regeneration^[63,64]. Therefore, BMMSCs might relieve sinusoidal injury by recruiting hepatic sinusoidal endothelial progenitor cells and secreting VEGF to promote SECs proliferation. The expression of the vWF protein in HO-1/BMMSCs group was significantly higher than that in BMMSCs group ($P < 0.05$), which suggested that HO-1/BMMSCs could improve the proliferation of SECs and promote the angiogenesis of hepatic sinusoids, leading to improved blood circulation in hepatic sinusoids and delayed sinusoidal injury.

To evaluate the degree of SECs injury accurately, we examined the level of HA. As a macromolecular mucopolysaccharide synthesized by liver interstitial cells, HA is mainly metabolized by SECs. SECs bind HA through the HA receptor, ingest HA by pinocytosis, and catabolize it using hyaluronidase in lysosomes. About 85%-95% of HA in the blood is absorbed and metabolized by SECs, thus serum HA levels can reflect the severity of SECs injury accurately^[65,66]. Our results showed that the SECs damage was more severe and the degradation of HA was reduced with increasing post-operative time. The concentration of HA in the HO-1/BMMSCs group was significantly lower than that

in the BMMSCs group, suggesting less injury to SECs. Thus, we speculated that BMMSCs have a protective effect on SECs in the short-term after allogeneic RLT, leading to improved hepatic sinusoidal microcirculation, and HO-1 might prolong the effects of BMMSCs.

Disturbance of hepatic sinusoidal microcirculation can lead directly to intrahepatic ischemia and hypoxia, thereby affecting liver energy metabolism. Mitochondrial dysfunction results in excessive production of ROS, which can affect the activity of mitochondrial oxidation-respiratory chain complexes, thus disturbing mitochondrial oxidative phosphorylation and reducing intracellular ATP synthesis^[67]. The concentration of ATP in the liver is critical for the maintenance of transplanted liver function. Necrosis and apoptosis of hepatocytes are associated with low baseline levels of intrahepatic ATP to some extent, ischemic preconditioning and liposomally-entrapped ATP pretreatment could significantly improve the hepatic ATP level after ischemia-reperfusion, thus increasing the operation success rate and graft survival rate^[8,9,68]. The serum level of ASTm is a sign of mitochondrial damage, and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity can assess the energy metabolism of the transplanted liver. In our study, the ultrastructure of the transplanted liver tissue was observed, and the mitochondria was damaged significantly in the NS group on POD7 after RLT, while they were recovered in BMMSCs group and HO-1/BMMSCs group at the same time point. ASTm and $\text{Na}^+\text{-K}^+\text{-ATPase}$ were also measured, and the results showed that the ATPase activities in the BMMSCs and HO-1/BMMSCs groups were higher than those in the NS group, which may be related to the significant decrease of ATP during rejection and disordered energy metabolism^[69]. The level of ASTm was lowest in HO-1/BMMSCs group, and was relatively high in the NS group. We found that the activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ after treatment with BMMSCs was higher than that in the NS group, which was consistent with the results for ASTm in the HO-1/BMMSCs group and BMMSCs group, which were lower than those in the NS group. These results indicated that mitochondrial injury was lowest in HO-1/BMMSCs group, which was beneficial to the metabolism of the transplanted liver. The results suggested that BMMSCs and HO-1/BMMSCs not only ameliorated the effects on ATPase activity and mitochondrial damage, but also had protective effects on the energy metabolism of the transplanted liver, and the effects of HO-1/BMMSCs were more significant.

In conclusions, in the context of liver donor graft shortage, RLT is a research hotspot in clinical liver transplantation. HO-1/BMMSCs could improve the hepatic sinusoidal microcirculation in rats after RLT, and promote liver energy metabolism to protect the transplanted liver. These results provide a basis to improve the quality of transplanted livers by gene therapy

combined with stem cells transplantation, and offer a reliable method to expand the source of donor liver.

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COMMENTS

Background

Graft injury after reduced-size liver transplantation (RLT) can affect the quality of donor liver seriously. Disturbance of the hepatic microcirculation and disorder of the hepatic energy metabolism are important factors affecting liver function after LT. Bone marrow mesenchymal stem cells (BMMSCs) can alleviate hepatic ischemia-reperfusion injury, accelerate liver regeneration, and have anti-inflammatory and immunoregulatory effects; however, their survival rate is low and survival time is short. Heme oxygenase-1 (HO-1) can regulate BMMSCs by enhancing the regulatory effects of BMMSCs under the state of hypoxia and oxidative stress, and can prolong the survival time of BMMSCs. The aim of this study was to explore the effects of HO-1/BMMSCs on hepatic microcirculation and energy metabolism of the transplanted liver in a rat model of rejection following RLT.

Research frontiers

BMMSCs have the potential for multipotent differentiation, regeneration promotion, and anti-inflammatory and immunoregulatory effects, and have been used widely in a variety of cell therapy research. However, the survival rate and survival time of BMMSCs in the diseased tissue are low. Therefore, how to improve the survival time of BMMSCs *in vivo*, and improve the microcirculation and energy metabolism of the transplanted liver at the same time, is a research hotspot.

Innovations and breakthroughs

HO-1/BMMSCs could improve the hepatic microcirculation after RLT significantly, and they decreased SECs injury and restored the energy metabolism of the damaged hepatocytes, showing a good protective effect on the transplanted liver.

Applications

In this study, BMMSCs modified by HO-1 could prolong the survival time and improve the activity of BMMSCs, and further demonstrated the protective effect of HO-1/BMMSCs on the transplanted liver after RLT. These results provided the basis for improving the quality of reduced-size transplanted livers by gene therapy combined with stem cells transplantation, and provide a reliable method to expand the source of donor livers.

Terminology

Sinusoids are central structures of the hepatic microcirculation, and the sinusoidal wall consists of SECs, Kupffer cells, hepatic stellate cells, and crypt cells, which play important roles in maintaining sinusoidal microcirculation and intrahepatic homeostasis. BMMSCs are non-hematopoietic stem cells derived from bone marrow, with multi-directional differentiation potential, and function in tissue repair, paracrine signaling, anti-inflammation, and immunoregulation. HO-1 is a microsomal oxidase in mammals, with protective effects, including anti-inflammation, anti-oxidative stress, anti-apoptosis, anti-ischemia reperfusion injury, and microcirculatory modulation.

Peer-review

This article demonstrates the protective effects of HO-1/BMMSCs on the transplanted liver in a rat model of rejection following RLT, which was studied from hepatic microcirculation and energy metabolism, hepatic microcirculation is very important and not well studied in liver transplantation. This study will be

of interest and the paper is clearly written.

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Basic Study

Diabetes recurrence after metabolic surgeries correlates with re-impaired insulin sensitivity rather than beta-cell function

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Abstract

AIM

To investigate factors causing diabetes recurrence after sleeve gastrectomy (SG) and duodenal-jejunal bypass (DJB).

METHODS

SG and DJB were performed on rats with diabetes induced by high-fat diet (HFD) and streptozotocin (STZ). HFD was used to induce diabetes recurrence

at 4 wk postoperatively. Body weight, oral glucose tolerance test, homeostatic model assessment of insulin resistance (HOMA-IR), insulin signaling [IR, insulin receptor substrate (IRS)1, IRS2, phosphatidylinositol 3-kinase and AKT in liver and skeletal muscle], oral glucose stimulated insulin secretion, beta-cell morphology (mass, apoptosis and insulin secretion), glucagon-like peptide (GLP)-1, PYY and ghrelin were compared among SG rats with common low-fat diet (SG-LFD), SG with HFD (SG-HFD), DJB rats with LFD (DJB-LFD), DJB with HFD (DJB-HFD) and sham-operation with LFD (Sham) at targeted postoperative times.

RESULTS

SG and DJB resulted in significant improvement in glucose tolerance, lower HOMA-IR, up-regulated hepatic and muscular insulin signaling, higher levels of oral glucose-stimulated insulin secretion, bigger beta-cell mass, higher immunofluorescence intensity of insulin, fewer transferase-mediated dUTP-biotin 3' nick end-labeling (TUNEL)-positive beta cells and higher postprandial GLP-1 and PYY levels than in the Sham group. The improvement in glucose tolerance was reversed at 12 wk postoperatively. Compared with the SG-LFD and DJB-LFD groups, the SG-HFD and DJB-HFD groups showed higher HOMA-IR, down-regulated hepatic and muscular insulin signaling, and more TUNEL-positive beta cells. No significant difference was detected between HFD and LFD groups for body weight, glucose-stimulated insulin secretion, beta-cell mass, immunofluorescence intensity of insulin, and postprandial GLP-1 and PYY levels. Fasting serum ghrelin decreased in SG groups, and there was no difference between HFD-SG and LFD-SG groups.

CONCLUSION

HFD reverses the improvement in glucose homeostasis after SG and DJB. Diabetes recurrence may correlate with re-impaired insulin sensitivity, but not with alterations of beta-cell function and body weight.

Key words: Apoptosis; Diabetes recurrence; Duodenal-jejunal bypass; Pancreatic beta cell; Sleeve gastrectomy

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Core tip: To investigate factors causing diabetes recurrence after sleeve gastrectomy (SG) and duodenal-jejunal bypass (DJB), we performed SG and DJB on diabetic rats and high-fat diet was used to induce diabetes recurrence at 4 wk postoperatively. The result showed that diabetes recurrence may correlate with re-impaired insulin sensitivity, but not with alterations of beta-cell function and body weight.

impaired insulin sensitivity rather than beta-cell function. *World J Gastroenterol* 2017; 23(19): 3468-3479 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3468.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3468>

INTRODUCTION

The accelerating twin pandemics of obesity and type 2 diabetes mellitus (T2DM) are recognized as two of the greatest global public health threats of our time. Randomized clinical trials have indicated that bariatric surgery achieves rapid and better glycemic control than medical therapy alone in severely obese patients with T2DM^[1-4]. Accordingly, bariatric surgery is recommended in the treatment algorithm of T2DM and endorsed by 45 worldwide medical and scientific associations^[5]. Although the evidence from clinical and basic research is sufficient to support Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG) as anti-diabetes interventions for obese patients, most studies have been restricted to short- and mid-term follow-up, and studies reporting long-term (≥ 5 years) diabetes remission rates have been limited, although they are now emerging^[6,7].

Recurrence of diabetes after initial remission has been observed and needs further investigation. In an earlier meta-analysis, Buchwald *et al.*^[8] found that the percentage of patients free of diabetes after gastric bypass decreased from 81.6% in the first 2 years to 70.9% after > 2 years. This suggests the potential for relapse of T2DM in some patients whose diabetes resolves after surgery. Several sporadic but convincing studies have documented a 12.1%-53% rate of recurrence of diabetes, with reference to RYGB, SG and biliopancreatic diversion (BPD) as selected procedures^[9-13]. Associated risk factors reported include longer duration of T2DM, preoperative use of insulin, old age, poor compliance with doctor's orders and postoperative high caloric intake^[9,14]. Weight regain, lower preoperative body mass index and less excess weight loss (EWL) are also regarded as risk factors, although this is controversial^[9,11].

Currently, the physiological and molecular mechanisms underlying diabetes control after bariatric surgery remain incompletely understood, even with regard to diabetes relapse. Against this background, the main goal of our study was to create an animal model of diabetes recurrence after metabolic surgery, and to evaluate alterations of insulin sensitivity, beta-cell function and related indexes in the process of diabetes remission and recurrence. As a secondary aim, we evaluated the association between weight changes, diabetes recurrence and high-fat diet (HFD), which was applied postoperatively to induce diabetes recurrence. In the present study, both SG and duodenal-jejunal bypass (DJB) were performed on rats with diabetes induced by HFD and streptozotocin

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(STZ). The antidiabetic effects of the two procedures were compared as a third aim. Our study could lead to a deeper understanding of diabetes remission after metabolic surgery and promote better strategies to enhance durable remission.

MATERIALS AND METHODS

Animals

All experiments were approved by the Animal Care and Utilization Committee of Shandong University, Jinan, China. Animals were housed under conventional conditions and had free access to tap water and food at the Laboratory Animal Center of Shandong University. Male Wistar rats (age, 8 wk; weight, 160–180 g) were fed an HFD (40% of calories as fat) rodent chow for a period of 8 wk, and then injected with STZ intraperitoneally (35 mg/kg). One week later, the rats were fasted overnight and received a 3-h oral glucose tolerance test (OGTT). Rats with a peak blood glucose of ≥ 11.1 mmol/L and ≤ 16.0 mmol/L were considered diabetic and selected for further studies.

Experimental protocol

Fifty-two diabetic rats were randomly assigned to SG-LFD ($n = 11$), SG-HFD ($n = 10$), SG-LFD ($n = 11$), DJB-HFD ($n = 10$) and Sham-operated ($n = 10$) groups. After surgery, all rats in the five groups were given a common low-fat diet (LFD, 15% of calories as fat) rodent chow for 4 wk. An HFD was then provided for the HFD groups for 8 wk. All Sham-operated rats were given LFD after surgery until the end of the study at 12 wk postoperatively. Body weight was monitored weekly during the study.

Surgical techniques

Before operations, all rats were fed with 10% Ensure (Abbott Laboratories, United States) for 2 d, then fasted overnight, and anesthetized with 10% chloral hydrate (3 mL/kg, Qilu Hospital, China) for surgery. Access to water was allowed at 2 h after surgery. Subsequently, the rats were fed with 10% Ensure for 3 d, followed by LFD or HFD rodent chow according to the protocol until end of the study.

SG: SG involved (1) a 4-cm midline epigastric incision; (2) ligation of all vessels around the greater curvature using 7-0 silk suture (Ningbo Medical Needle, Ningbo, China); (3) resection of the fundus and most of the stomach; and (4) closure of the remnant stomach using 5-0 silk suture (Ningbo Medical Needle).

DJB: DJB involved (1) a 4-cm midline abdominal incision; (2) transection of the duodenum at 0.5 cm from the pylorus and closure of the distal limb using a 7-0 silk suture; (3) transection of the jejunum at 10 cm from the ligament of Treitz; (4) end-to-end

anastomosis of the distal jejunal limb to the duodenal stump; and (5) end-to-side anastomosis of the proximal jejunal limb to the small intestine 10 cm distally.

Sham operations: For rats in the Sham group, laparotomy was performed to expose the stomach, esophagus, and small intestine. The operating time was prolonged to generate a comparable degree of anesthetic stress as in the SG and DJB groups. No other procedures were carried out.

OGTT

OGTT was performed at baseline and 2, 4 and 12 wk postoperatively, and areas under the curves for OGTT (AUC_{OGTT}) were calculated to evaluate the effect of diabetes control in each group. For OGTT, rats were fasted overnight and administered 1 g/kg glucose by oral gavage. Blood samples were obtained from the tail vein at baseline and 10, 30, 60, 120 and 180 min after administration, and glucose was measured using a glucometer (Roche One Touch[®] Ultra; Lifescan, Johnson & Johnson, Milpitas, CA, United States).

Homeostasis model assessment of insulin resistance

Homeostasis model assessment of insulin resistance (HOMA-IR) was adopted as a surrogate of insulin sensitivity and calculated at baseline, and 4 and 12 wk postoperatively, which was calculated according to the formula: $HOMA-IR = \text{fasting insulin (mIU/L)} \times \text{fasting glucose (mmol/L)} / 22.5$.

Insulin signaling pathway (liver and muscle)

All rats were sacrificed at 12 wk postoperatively. Liver and skeletal muscle were sampled and immediately frozen in liquid nitrogen and stored at -80°C until analysis. Alterations of the insulin signaling pathway were determined by Western blotting, as indicated by protein expression of insulin receptor, insulin receptor substrate (IRS)1, IRS2, phosphatidylinositol 3-kinase (PI3K) and Akt. For Western blotting, samples were mechanically dissociated and lysed in radioimmunoprecipitation assay 37 (RIPA) buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na₂-EDTA, 1% NP-40, 0.25% Na-deoxycholate) containing protease and phosphatase inhibitor cocktails (Roche, United States). After brief sonication and heating, the supernatants were subjected to SDS-PAGE and transferred to PVDF membranes. Blots were incubated overnight at 4°C with primary antibodies (anti-insulin receptor antibody, anti-IRS1 antibody, anti-IRS2 antibody, anti-PI3K P85 α antibody, anti-pan-AKT antibody, anti- β -actin antibody; all Abcam Cambridge, MA, United States) and were then incubated with secondary antibodies (Abcam). Blots were visualized with an enhanced chemiluminescence reagent (Millipore, Billerica, MA, United States) and quantified with Image Lab (Bio-Rad, Hercules, CA, United States).

Table 1 Body weight after operations *n* (%)

Group	<i>n</i>	Weeks after operations		
		0	4	12
SG-LFD	8	348.8 (20.1)	343.0 (15.3) ^a	418.1 (19.4) ^c
SG-HFD	7	353.0 (22.9)	345.6 (20.0) ^a	422.7 (21.9) ^c
DJB-LFD	9	349.4 (18.9)	346.2 (17.2) ^a	419.1 (18.4) ^c
DJB-HFD	8	352.3 (21.4)	349.6 (18.5) ^a	420.9 (21.2) ^c
Sham	10	352.4 (20.2)	368.4 (13.2)	450.7 (15.8)

Body weight of rats at baseline, 4 and 12 wk after operations. No difference in body weight was detected among rats fed with the same diet. Body weight in the four surgery groups was lower than the Sham group. ^a*P* < 0.05 *vs* Sham group at 4 wk postoperatively, ^c*P* < 0.05 *vs* Sham group at 12 wk postoperatively. SG: Sleeve gastrectomy; SG-LFD: SG rats with common low-fat diet; SG-HFD: SG with high-fat diet; DJB: Duodenal-jejunal bypass; DJB-LFD: DJB rats with LFD; DJB-HFD: DJB rats with HFD.

Oral glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion was measured in the serum samples as a surrogate index of beta cell function of insulin secretion at baseline, and postoperative weeks 4 and 12. Rats were deprived of food overnight and then administered 1 g/kg glucose by oral gavage. Blood was collected from the retrobulbar venous plexus at baseline and 15, 30, 60 and 120 min after gavage into tubes containing EDTA and dipeptidyl peptidase IV inhibitor. After centrifugation at 3000 rpm at 4 °C for 15 min, the separated serum was immediately removed to Eppendorf tubes and stored at -80 °C until analyzed. Insulin was measured with rat ELISA kits (Millipore).

Morphology of the pancreas

Beta-cell mass, insulin secretion and terminal deoxynucleotidyl transferase-mediated dUTP-biotin 3' nick end-labeling (TUNEL) assay were performed to evaluate changes in the morphology of beta cells. The rats were killed and pancreases were harvested, flattened and immersed in 4% paraformaldehyde. Tissues were embedded in paraffin after 24 h. Sections of 5 μm were rehydrated, and antigen retrieval was performed in citrate buffer (pH 6) in a pressure cooker. Beta cells were stained with guinea pig anti-insulin antibody (Abcam; 1/200). Apoptosis was assessed using the TUNEL assay (Roche). Nuclei were stained with DAPI (1 μg/mL). Affinity-purified secondary antibodies were from Abcam. Immunofluorescence images were captured on an Olympus FluoView FV1000 confocal microscope at 400 × magnification. Images of sections were analyzed using ImageJ software. The percentage of TUNEL-positive beta cells and the insulin fluorescence intensity of each islet were calculated.

GLP-1, PYY and ghrelin

Serum total GLP-1 and PYY levels after glucose gavage and fasting serum ghrelin were measured with the serum collected at 4 and 12 wk postoperatively. GLP-1 was measured with multi-species GLP-1 total ELISA kits (Millipore). PYY was measured with Rat Leptin

ELISA (Millipore). Ghrelin was measured with Rat/Mouse Ghrelin (total) ELISA (Millipore).

Statistical analysis

Data are expressed as mean ± SD. Data that were not normally distributed or did not satisfy homogeneity of variance were logarithmically transformed before analysis. AUC_{OGTT} was calculated by trapezoidal integration. All statistical analyses were performed with SPSS version 19.0. Body weight, AUC_{OGTT}, HOMA-IR and ghrelin data at each time point, band intensity of Western blotting, percentage of TUNEL-positive beta cells, and insulin fluorescence intensity of each islet were compared by one-way analysis of variance (ANOVA). Postprandial insulin, GLP-1 and PYY data were compared by two-factor repeated measures (RM) ANOVA. *Post hoc* comparisons adjusted by Bonferroni's correction, were performed when necessary. Differences were considered significant at *P* < 0.05.

RESULTS

General effects of treatments and weight changes

There was no significant difference among the groups in preoperative body weight and oral glucose tolerance. Table 1 shows the number of rats surviving in each group and their body weight. The body weight increased over time in all rats. Body weight in the four surgery groups was lower than the Sham group (4 wk postoperatively: 368.4 g ± 13.2 g; 12 wk postoperatively: 450.7 g ± 15.8 g) at 4 and 12 wk postoperatively (4 wk postoperatively: SG-LFD 343.0 g ± 15.3 g, *P* < 0.05 *vs* Sham; SG-HFD 345.6 g ± 20.0 g, *P* < 0.05 *vs* Sham; DJB-LFD 346.2 g ± 17.2 g, *P* < 0.05 *vs* Sham; DJB-HFD 349.6 g ± 18.5 g, *P* < 0.05 *vs* Sham; 12 wk postoperatively: SG-LFD 418.1 g ± 19.4 g, *P* < 0.05 *vs* Sham; SG-HFD 422.7 g ± 21.9 g, *P* < 0.05 *vs* Sham; DJB-LFD 419.1 g ± 18.4 g, *P* < 0.05 *vs* Sham; DJB-HFD 420.9 g ± 21.2 g, *P* < 0.05 *vs* Sham; Table 1). No difference in body weight was detected among rats fed with the same diet (all *P* > 0.05). Although body weight in the HFD groups was higher than in the LFD groups, no difference was detected between the HFD and LFD groups at postoperative weeks 4 and 12 (all *P* > 0.05).

HFD reversed improvement in glucose tolerance induced by DJB and SG

All SG and DJB groups exhibited a significant improvement in glucose tolerance after surgery, as shown by lower AUC_{OGTT} values than in the Sham group (4 wk postoperatively: 1766.4 ± 139.49; 12 wk postoperatively: 2304.62 ± 143.85) at 4 and 12 wk postoperatively (4 wk postoperatively: DJB-LFD 991.94 ± 198.9, *P* < 0.05 *vs* Sham; DJB-HFD 1052.88 ± 170.74, *P* < 0.05 *vs* Sham; SG-LFD 1031.16 ± 223.73, *P* < 0.05 *vs* Sham; SG-HFD 992.64 ± 105.68, *P* < 0.05 *vs* Sham; 12 wk postoperatively: DJB-LFD 1049.17

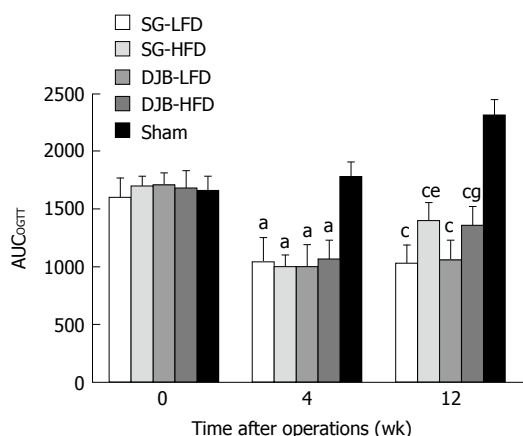


Figure 1 Areas under the curves for oral glucose tolerance test; of rats at baseline, 4 and 12 wk after operations. ^a $P < 0.05$ vs Sham group at 4 wk postoperatively; ^c $P < 0.05$ vs Sham group at 12 wk postoperatively; ^e $P < 0.05$ vs SG-HFD group at 4 wk postoperatively; ^g $P < 0.05$ vs DJB-HFD group at 4 wk postoperatively. AUC_{OGTT}: Areas under the curves for oral glucose tolerance test; SG: Sleeve gastrectomy; SG-LFD: SG rats with common low-fat diet; SG-HFD: SG with high-fat diet; DJB: Duodenal-jejunal bypass; DJB-LFD: DJB rats with LFD; DJB-HFD: DJB rats with HFD.

± 181.03 , $P < 0.05$ vs Sham; DJB-HFD 1344.82 ± 178.73 , $P < 0.05$ vs Sham; SG-LFD 1016.5 ± 170.1 , $P < 0.05$ vs Sham; SG-HFD 1387.53 ± 171.73 , $P < 0.05$ vs Sham; Figure 1). As expected, the improvement in glucose tolerance was reversed after HFD was provided for 8 wk. AUC_{OGTT} in the HFD groups at 12 wk postoperatively was significantly higher than at 4 wk postoperatively (DJB-HFD at 12 wk postoperatively, $P < 0.05$ vs DJB-HFD at 4 wk postoperatively; SG-HFD at 12 wk postoperatively, $P < 0.05$ vs SG-HFD at 4 wk postoperatively). Although the improved glucose tolerance was reversed, the HFD groups still had better glucose tolerance than the Sham group had, as shown by lower AUC_{OGTT} at postoperative week 12. SG and DJB groups fed with the same diet showed similar AUC_{OGTT} and no difference was detected at 4 and 12 wk postoperatively (4 wk postoperatively: DJB-LFD, $P > 0.05$ vs SG-LFD; DJB-HFD, $P > 0.05$ vs SG-HFD; 12 wk postoperatively: DJB-LFD, $P > 0.05$ vs SG-LFD; DJB-HFD, $P > 0.05$ vs SG-HFD).

HFD re-impaired insulin sensitivity improved by DJB and SG

SG and DJB groups exhibited significantly lower HOMA-IR values than the Sham group (4 wk postoperatively: 3.60 ± 0.60 ; 12 wk postoperatively: 4.40 ± 1.27) at postoperative weeks 4 and 12 (4 wk postoperatively: DJB-LFD 2.56 ± 0.53 , $P < 0.05$ vs Sham; DJB-HFD 2.47 ± 0.42 , $P < 0.05$ vs Sham; SG-LFD 2.51 ± 0.41 , $P < 0.05$ vs Sham; SG-HFD 2.64 ± 0.36 , $P < 0.05$ vs Sham; 12 wk postoperatively: DJB-LFD 1.81 ± 0.28 , $P < 0.05$ vs Sham; DJB-HFD 3.0 ± 0.69 , $P < 0.05$ vs Sham; SG-LFD 1.70 ± 0.34 , $P < 0.05$ vs Sham; SG-HFD 3.19 ± 0.82 , $P < 0.05$ vs Sham; Figure 2). LFD groups resulted in better insulin

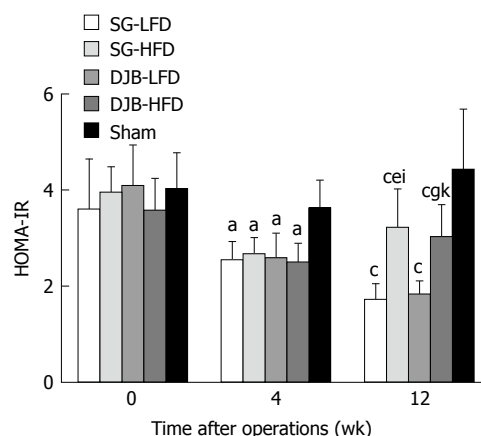


Figure 2 Homeostatic model assessment of insulin resistance of rats at baseline, 4 and 12 wk after operations. ^a $P < 0.05$ vs Sham group at 4 wk postoperatively; ^c $P < 0.05$ vs Sham group at 12 wk postoperatively; ^e $P < 0.05$ vs SG-HFD group at 4 wk postoperatively; ^g $P < 0.05$ vs DJB-HFD group at 4 wk postoperatively; ⁱ $P < 0.05$ vs SG-LFD at 12 wk postoperatively; ^k $P < 0.05$ vs DJB-LFD at 12 wk postoperatively. HOMA-IR: Homeostatic model assessment of insulin resistance; SG: Sleeve gastrectomy; SG-LFD: SG rats with common low-fat diet; SG-HFD: SG with high-fat diet; DJB: Duodenal-jejunal bypass; DJB-LFD: DJB rats with LFD; DJB-HFD: DJB rats with HFD.

sensitivity at postoperative week 12 than at week 4, as demonstrated by lower HOMA-IR at postoperative week 12 (DJB-LFD at 12 wk postoperatively, $P < 0.05$ vs DJB-HFD at 4 wk postoperatively; SG-LFD at 12 wk postoperatively, $P < 0.05$ vs SG-HFD at 4 wk postoperatively). However, the HFD groups resulted in re-impaired insulin sensitivity at postoperative week 12, as shown by higher HOMA-IR values than at postoperative week 4, but the difference did not reach significance (4 wk postoperatively: DJB-LFD, $P > 0.05$ vs SG-LFD; DJB-HFD, $P > 0.05$ vs SG-HFD; 12 wk postoperatively: DJB-LFD, $P > 0.05$ vs SG-LFD; DJB-HFD, $P > 0.05$ vs SG-HFD). HOMA-IR in the HFD groups at postoperative week 12 was higher than in the LFD groups (DJB-HFD at 12 wk postoperatively, $P < 0.05$ vs DJB-LFD at 12 wk postoperatively; SG-HFD at 12 wk postoperatively, $P < 0.05$ vs SG-LFD at 12 wk postoperatively).

HFD reversed up-regulation of hepatic and muscular insulin signaling induced by DJB and SG

Expression of IRS-1, IRS-2, PI3K and AKT increased in all DJB and SG groups (Figure 3), indicating that the insulin signaling pathway was up-regulated in the liver and skeletal muscle. However, expression of insulin receptor showed no difference among the groups. The HFD groups showed lower expression of IRS-1, IRS-2, PI3K and AKT than the LFD groups, indicating the re-impairment of hepatic and muscular insulin signaling.

DJB and SG protected, but failed to improve, beta-cell function of glucose-stimulated insulin secretion

At postoperative week 4, no significant difference in glucose-stimulated insulin secretion was observed

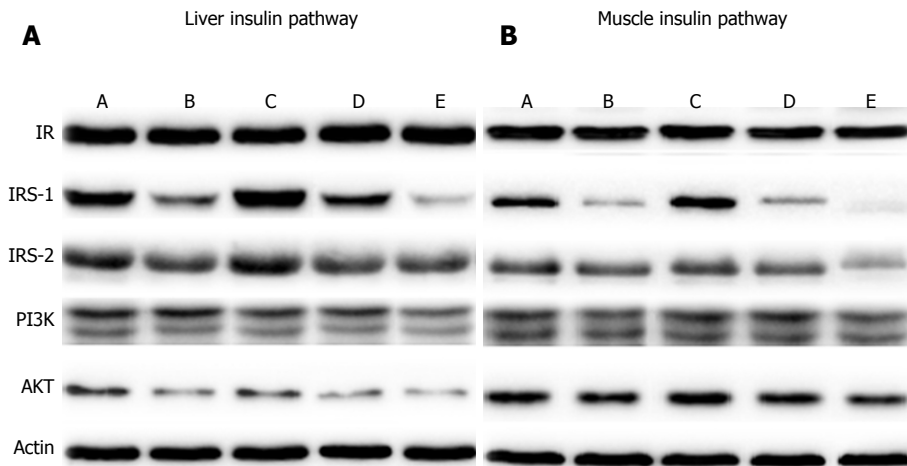


Figure 3 Western blot analysis of the insulin signaling pathway in liver and muscle. Expression of IR, IRS-1, IRS-2, PI3K, AKT in (A) liver and (B) muscle. A: SG-LFD group; B: SG-HFD group; C: DJB-LFD group; D: DJB-HFD group; E: Sham group. SG: Sleeve gastrectomy; SG-LFD: SG rats with common low-fat diet; SG-HFD: SG with high-fat diet; DJB: Duodenal-jejunal bypass; DJB-LFD: DJB rats with LFD; DJB-HFD: DJB rats with HFD.

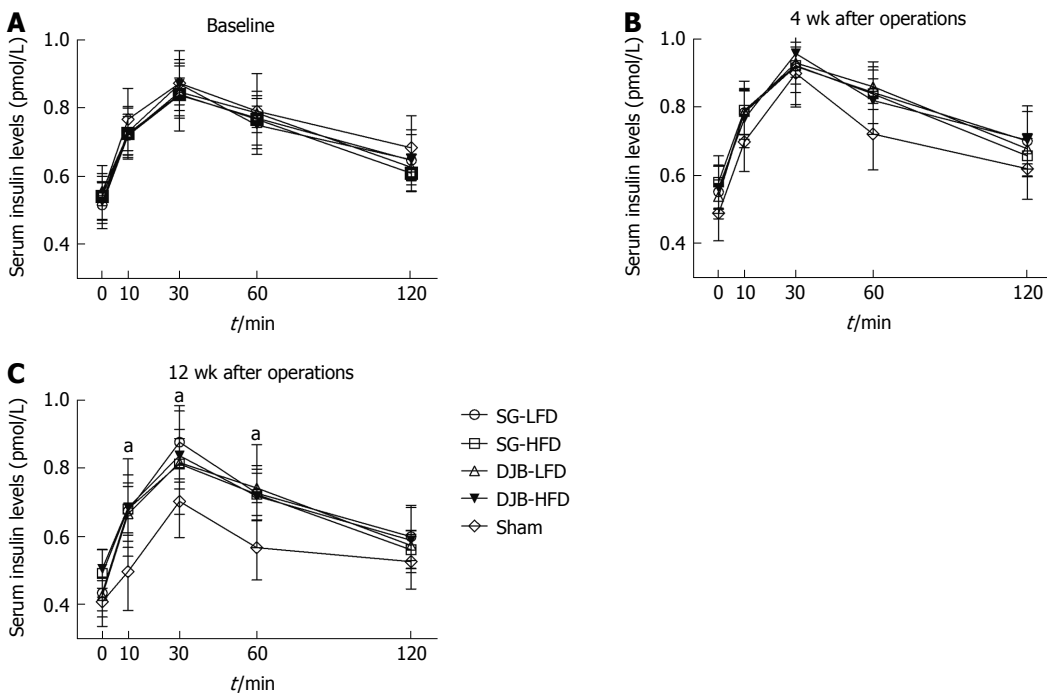


Figure 4 Serum insulin after oral glucose administration. Serum insulin levels after oral glucose gavage (1 g/kg) at baseline (A), 4 (B) and 12 (C) wk postoperatively. No significant differences were observed in SG and DJB groups at 4 or 12 wk postoperatively. Insulin levels in response to oral glucose gavage increased in HFD and LFD rats at 12 wk postoperatively. ^a $P < 0.05$ vs Sham group at 12 wk postoperatively. SG: Sleeve gastrectomy; SG-LFD: SG rats with common low-fat diet; SG-HFD: SG with high-fat diet; DJB: Duodenal-jejunal bypass; DJB-LFD: DJB rats with LFD; DJB-HFD: DJB rats with HFD.

among DJB, SG and Sham groups ($P > 0.05$, two-factor RM ANOVA; Figure 4B), suggesting that the beta-cell function of insulin secretion was not enhanced within a short time after surgery. At postoperative week 12, insulin levels in response to oral glucose gavage increased in HFD and LFD rats, demonstrated by higher insulin curves and peak insulin levels than in the Sham group ($P < 0.05$ vs Sham group, Figure 4C). However, the peak insulin levels showed no difference between baseline and postoperative week 12 in SG and DJB groups (DJB-LFD at 12 wk postoperatively $0.82 \text{ pmol/L} \pm 0.15 \text{ pmol/L}$, $P > 0.05$ vs DJB-LFD at

baseline $0.85 \text{ pmol/L} \pm 0.08 \text{ pmol/L}$; DJB-HFD at 12 wk postoperatively $0.84 \text{ pmol/L} \pm 0.08 \text{ pmol/L}$, $P > 0.05$ vs DJB-HFD at baseline $0.87 \text{ pmol/L} \pm 0.06 \text{ pmol/L}$; SG-LFD at 12 wk postoperatively $0.88 \text{ pmol/L} \pm 0.11 \text{ pmol/L}$, $P > 0.05$ vs SG-LFD at baseline $0.84 \text{ pmol/L} \pm 0.10 \text{ pmol/L}$; SG-HFD at 12 wk postoperatively $0.81 \text{ pmol/L} \pm 0.07 \text{ pmol/L}$, $P > 0.05$ vs SG-HFD at baseline $0.84 \text{ pmol/L} \pm 0.05 \text{ pmol/L}$; Figure 4), indicating no increase in the beta-cell function of insulin secretion.

DJB and SG protected beta cells from apoptosis

At postoperative week 12, all DJB and SG groups

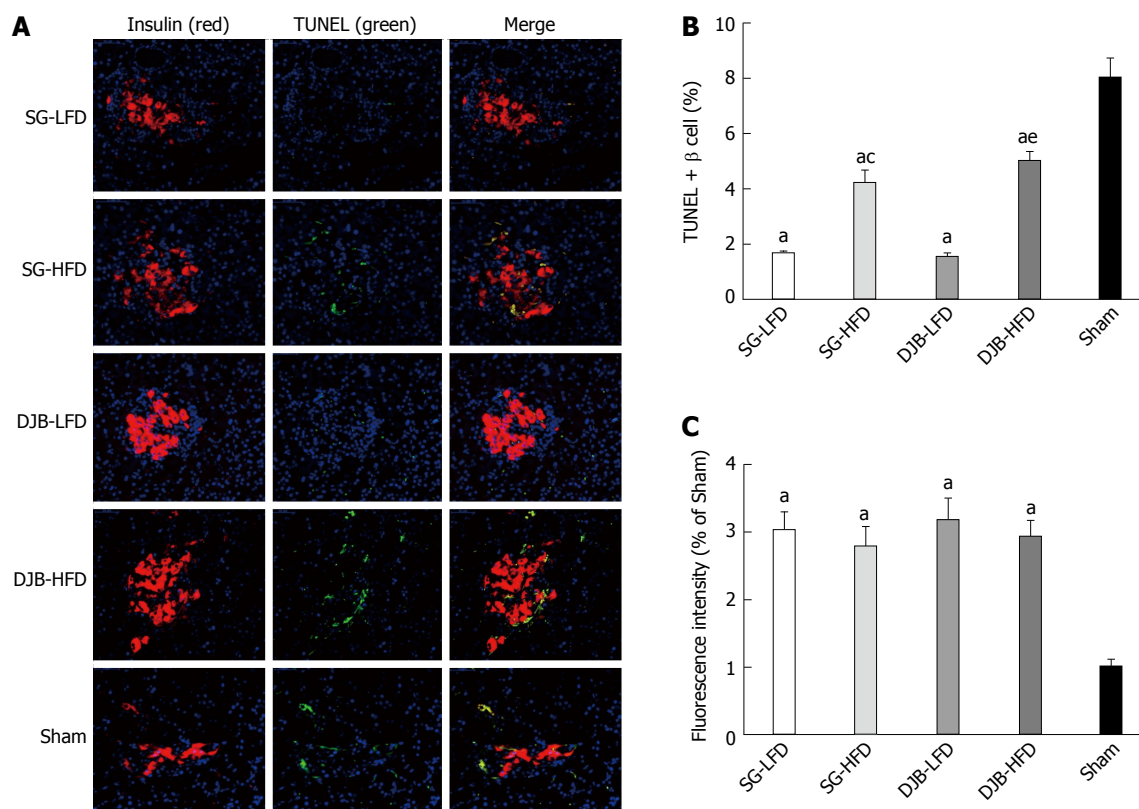


Figure 5 Pancreatic beta cell analysis. A: TUNEL-positive beta cell shown by immunofluorescence; B: and the percentage of TUNEL-positive beta cells was calculated. The two surgery group had fewer TUNEL-positive beta cells than the Sham group, and HFD group made the percentage higher than in the LFD group; C: The fluorescence intensity did not differ significantly between the four surgery groups ($P > 0.05$), but was higher than in the Sham group. ^a $P < 0.05$ vs Sham group at 12 wk postoperatively; ^c $P < 0.05$ vs SG-LFD group at 12 wk postoperatively; ^e $P < 0.05$ vs DJB-LFD group at 12 wk postoperatively. TUNEL: Transferase-mediated dUTP-biotin 3' nick end-labeling; SG: Sleeve gastrectomy; SG-LFD: SG rats with common low-fat diet; SG-HFD: SG with high-fat diet; DJB: Duodenal-jejunal bypass; DJB-LFD: DJB rats with LFD; DJB-HFD: DJB rats with HFD.

exhibited larger and more regulatory beta cell mass (Figure 5A) and fewer TUNEL-positive beta cells (Figure 5B) than in the Sham group. The immunofluorescence intensity of insulin in the DJB and SG groups was about threefold higher than in the Sham group (Figure 5C). These results indicated that DJB and SG resulted in less apoptosis of beta cells and better function of insulin secretion. The HFD groups exhibited more TUNEL-positive beta cells in than LFD groups did (Figure 5A and B), however, no significant difference in the immunofluorescence intensity of insulin was detected between the HFD and LFD groups (both $P < 0.05$).

Changes of GLP-1, PYY and ghrelin

Oral glucose gavage resulted in higher GLP-1 levels in all DJB and SG groups than in the Sham group at postoperative weeks 4 and 12 ($P < 0.05$ vs Sham group, two-factor RM ANOVA) and there was no significant difference between the HFD and LFD groups, and between the SG and DJB groups (Figure 6). Compared with the Sham group (4 wk postoperatively: 66.99 ± 4.27 ; 12 wk postoperatively: 57.13 ± 5.58), PYY levels in DJB-LFD (4 wk postoperatively: 59.09 ± 3.27 , $P < 0.05$ vs Sham group; 12 wk postoperatively: 106.78 ± 6.08 , $P < 0.05$ vs Sham group), DJB-HFD (4

wk postoperatively: 112.09 ± 5.77 , $P < 0.05$ vs Sham group; 12 wk postoperatively: 91.27 ± 5.58 , $P < 0.05$ vs Sham group), SG-LFD (4 wk postoperatively: 105.10 ± 4.67 , $P < 0.05$ vs Sham group; 12 wk postoperatively: 120.80 ± 5.50 , $P < 0.05$ vs Sham group), SG-HFD (4 wk postoperatively: 108.98 ± 6.31 , $P < 0.05$ vs Sham group; 12 wk postoperatively: 95.11 ± 6.01 , $P < 0.05$ vs Sham group) groups were much higher. SG groups showed lower fasting serum ghrelin values than the DJB and Sham groups.

DISCUSSION

RYGB and SG are currently the most frequently performed bariatric procedures worldwide and are included in diabetes treatment algorithms^[5,15]. Although growing evidence indicates that RYGB and SG improve T2DM with a BMI ≥ 30 kg/m², even with a lower BMI of 25-27.5 kg/m² in Asian patients^[16,17], diabetes recurrence appears after several years of remission. Few studies have specifically considered diabetes recurrence, compared with the large number that has reported rapid and dramatic remission after bariatric surgery. Identification of the predictors and causes of diabetes recurrence is beneficial to the postoperative

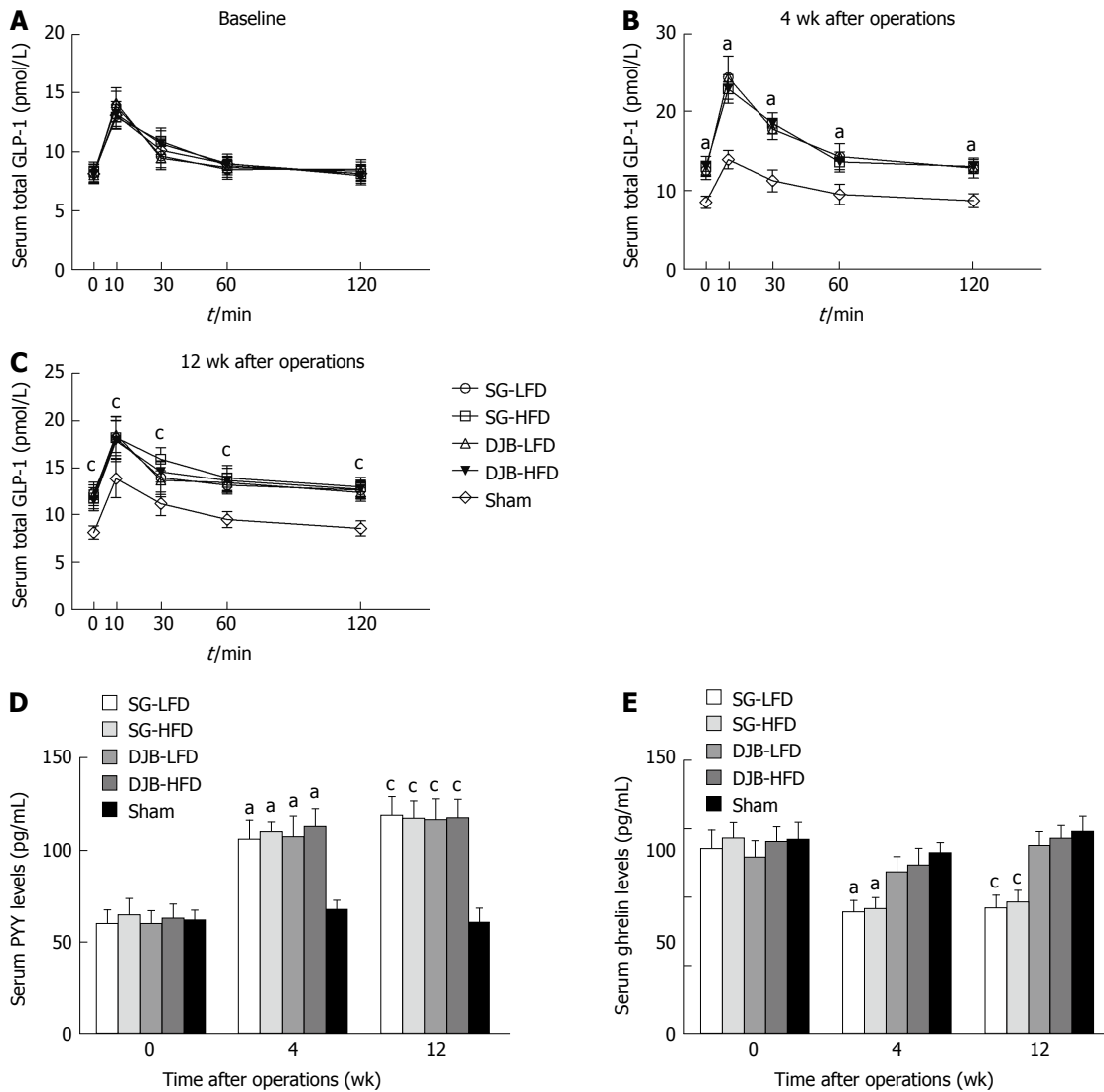


Figure 6 Glucagon-like peptide-1, PYY, ghrelin levels. GLP-1 levels after oral glucose gavage (1 g/kg) at baseline (A), 4 (B) and 12 (C) weeks after surgery. PYY levels 1h after the oral glucose gavage (D). And the fasting ghrelin levels (E). ^a $P < 0.05$ vs Sham group at 4 wk postoperatively; ^c $P < 0.05$ vs Sham group at 12 wk postoperatively. SG: Sleeve gastrectomy; SG-LFD: SG rats with common low-fat diet; SG-HFD: SG with high-fat diet; DJB: Duodenal-jejunal bypass; DJB-LFD: DJB rats with LFD; DJB-HFD: DJB rats with HFD.

glycemic control and reduction of revisional bariatric surgery. In one of our previous studies, we initially reported an animal model of diabetes recurrence after DJB in Goto-Kakizaki (GK) rats and HFD/STZ-induced diabetes in rats^[18]. In our study, SG and DJB were both performed on rats with HFD/STZ-induced diabetes. HFD was supplied after initial diabetes remission at 4 wk postoperatively and diabetes recurred at 12 wk postoperatively, which confirms that HFD can induce diabetes recurrence after gastrointestinal metabolic surgery. This remission-recurrence model helps us to understand the mechanisms of diabetes recurrence.

Moreover, it is more beneficial for studying surgery-induced diabetes remission, compared with animal models with long-term diabetes remission. IR is one of the two remarkable pathological characteristics of T2DM, which is usually caused by HFD. Previous studies have discovered that improvement in insulin

sensitivity contributes the most to diabetes remission after bariatric surgery, especially in the early stage^[19-21]. The present study confirmed this viewpoint with lower HOMA-IR, at 4 and 12 wk postoperatively, and up-regulation of hepatic and muscular insulin signaling provided further evidence for improvement in tissue insulin sensitivity. More importantly, with this remission-recurrence bariatric animal model, we found that the rapidly improved insulin sensitivity was re-impaired after 8 wk HFD gavage, which was shown by higher HOMA-IR in the HFD groups at 12 wk postoperatively compared with before surgery and in the LFD controls. Down-regulated expression of insulin signaling of liver and skeletal muscle in the HFD groups confirmed the re-impairment of tissue insulin sensitivity. HFD was obviously detrimental to durable improvement in our study. Accompanying alterations of gut microbiota, especially adverse higher abundance

of Bacteroidetes and *Escherichia coli*, are considered responsible for diabetes recurrence, possibly by influencing serum lipopolysaccharide and associated low-grade chronic inflammation^[22]. A low-calorie diet is included in postoperative instructions from bariatric surgeons, and non-adherence is an important factor associated with durable remission^[9-11].

Beta-cell dysfunction, including deficiency of insulin secretion and morphological disorders, is another important pathological characteristic of T2DM. Reports on alterations of beta-cell function after RYGB are controversial in patients or rodents with diabetes^[23-28]. In the present study, glucose-stimulated insulin secretion was higher in DJB and SG rats than in Sham rats at 12 wk postoperatively, and no difference was observed between postoperative and preoperative data. These results were identical to studies with non-obese diabetic GK rats^[19,23,24], which indicated that DJB and SG procedures preserved but did not increase insulin secretion, at least during 12 wk observation. Meirelles *et al.*^[25] reported decreased insulin secretion after RYGB in obese diabetic Zucker rats, and Cummings *et al.*^[26] reported a threefold increase in serum insulin after ileal transposition in UCD-T2DM rats. We ascribed these completely distinct alterations of insulin secretion after surgery to different diabetic animal models.

Similar to studies in animals, alterations of insulin secretion after bariatric surgery in patients were also inconsistent^[21]. Generally speaking, oral glucose-stimulated insulin secretion, which combines intrinsic and extrinsic regulation of insulin secretion, altered rapidly and significantly after RYGB, with an earlier and exaggerated postprandial rise in insulin concentration that reached a higher peak level than that achieved preoperatively^[29-33]. However, intravenous glucose-stimulated insulin secretion, which addressed only the intrinsic regulation, showed that beta-cell function improved minimally and remained significantly impaired after RYGB^[34].

We performed pathological examination of the pancreas, which supported the protective effect of DJB and SG on insulin secretion, with larger beta-cell mass, stronger insulin staining, and less apoptosis. Few studies have reported beta-cell morphology, and in all of these, including the present study, only larger beta-cell mass and/or stronger insulin staining were detected in RYGB, SG, DJB or IT animals compared with their diabetic control. It is regrettable that no comparison was performed between pre- and postoperative rats^[35-37]. Therefore, there has been no evidence supporting islets hyperplasia or beta-cell turnover after bariatric surgeries until now. DJB and SG preserved insulin secretion and protected beta cells from apoptosis.

Furthermore, we showed that glucose-stimulated insulin secretion was not decreased in DJB and SG rats with recurrent diabetes, and the beta-cell mass and insulin staining showed no difference between

the persistent remission and recurrence groups. Consequently, HFD-induced diabetes recurrence after DJB and SG was independent of alterations of beta-cell function during the short observation time. As reported, preoperative old age, higher BMI, lower C-peptide level and long duration of diabetes, which presented poor beta-cell function, predicted the failure of glycemic control^[14,38]. Given that more apoptotic beta cells were detected in the groups with recurrent diabetes, re-impaired beta-cell function appeared over the time.

It is controversial whether weight loss is essential to diabetes control after bariatric surgery. In the current study, all non-Sham-operated rats experienced a significant increase in body weight when killed, which was consistent with studies using HFD-STZ and GK rats. This indicated that remission of diabetes was independent of weight changes after surgery. In obese patients accepting bariatric surgery, weight loss undoubtedly benefited diabetes improvement, because the improvement in peripheral insulin sensitivity was significantly related to weight loss^[39,40]. EWL is reported to be the only predictor of diabetes remission that is influenced by bariatric procedures^[41]. Contradictory voices claim that diabetes resolves independently of weight loss because remission of diabetes occurs before significant weight loss appears, and baseline BMI is unrelated to diabetes remission^[42]. Based on the findings that body weight increased when the rats were killed, and no difference in body weight was detected between rats with persistent remission and recurrence of diabetes, we conclude that diabetes recurrence is independent of weight regain. On the contrary, DiGiorgi *et al.*^[9] reported that patients with recurrent or worsening diabetes regained a greater percentage of their lost weight, and weight regain was a significant predictor of T2DM recurrence^[11]. A possible explanation for the contrary results is that weight regain is a weak predictor of T2DM recurrence^[11], and recurrence is more likely to result from a postoperative unhealthy high-calorie diet, which more easily re-impairs insulin sensitivity.

SG and DJB were performed in the present study, and the effect of the two procedures on diabetes showed no significant difference. SG is currently the most performed bariatric procedure and DJB is also performed on diabetic patients, achieving satisfactory disease control^[43]. SG and DJB are combined and performed successfully on morbidly obese patients^[44]. Better glycemic control in T2DM patients is achieved than with SG alone^[45]. As expected and observed in other studies, secretion of GLP-1 and PYY increases after SG and DJB, and ghrelin decreases after SG. However, no alterations of these hormones were observed when diabetes recurred. These results demonstrate that GLP-1, PYY and ghrelin are not correlated with diabetes recurrence.

This study had some limitations. The observation

time was only 12 wk. Longer observation and HFD gavage might yield more information about beta-cell apoptosis. Furthermore, lipid profiles (triglycerides, cholesterol and free fatty acids) and inflammatory factors were not examined, and therefore, we cannot further discuss the effect of HFD on the recurrence of diabetes.

In conclusion, this study demonstrated that HFD induced diabetes recurrence after initial remission with SG and DJB surgery. The re-impairment of hepatic and muscular insulin sensitivity was likely responsible for the recurrence, and alterations of beta-cell function, body weight, and gastrointestinal hormones (GLP-1, PYY and ghrelin) seemed not to correlate with recurrence.

COMMENTS

Background

Gastrointestinal metabolic surgeries promote dramatic and durable improvement of type 2 diabetes. However, diabetes recurrence happened in part of patients with initial remission after surgeries.

Research frontiers

Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG) are currently the most frequently performed bariatric procedures worldwide and included in diabetes treatment algorithms. Diabetes recurrence appears after years of remission and frustrated both patients and surgeons, but few studies specifically consider diabetes recurrence. This "remission-recurrence" model helped to understand mechanisms of diabetes recurrence.

Innovations and breakthroughs

In this study, the authors created a rat model of diabetes recurrence after SG and duodenal-jejunal bypass (DJB) by feeding the rats a high-fat diet postoperatively. The rats with diabetes recurrence showed re-impairment of hepatic and muscular insulin sensitivity, but no alterations of beta-cell function, body weight, and gastrointestinal hormones (GLP-1, PYY and ghrelin).

Applications

The authors established a model using high-fat diet to induce diabetes recurrence after bariatric surgery and to find the mechanism of bariatric surgery to improve glucose metabolism. And they had found in this study that it should focus on the tissue insulin sensitivity but not beta cell apoptosis, and it seem not to depend on the change of gastrointestinal hormones like GLP-1, PYY and ghrelin.

Terminology

DJB is an experimental procedure that was initially designed to investigate the weight-independent anti-diabetic effects of Roux-en-Y gastric bypass surgery, which is the gold standard in patients with diabetes. SG is a popular bariatric procedures performed worldwide, which has a similar effect to RYGB, and less complications than RYGB.

Peer-review

The study is very interesting. In this study, the authors investigated factors causing diabetes recurrence after SG and DJB. HFD could reverse the improvement in glucose homeostasis induced by SG and DJB surgeries.

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Case Control Study

Polymorphisms of microRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* in gastric cancer

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Abstract

AIM

To evaluate associations between miRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* polymorphisms and gastric cancer (GC) in European population.

METHODS

Gene polymorphisms were analyzed in 508 controls and 474 GC patients from 3 tertiary centers in Germany, Lithuania and Latvia. Controls were patients from the out-patient departments, who were referred for upper endoscopy because of dyspeptic symptoms and had no history of previous malignancy. Gastric cancer (GC) patients had histopathological verification of gastric adenocarcinoma. Genomic DNA was extracted using salting out method from peripheral blood mononuclear cells. *IL12B* T>G (rs1368439), *INSR* T>C (rs1051690), *CCND1* A>C (rs7177) and *IL10* T>C (rs3024498) SNPs were genotyped by the real-time polymerase chain reaction. Associations between gene polymorphism and GC were evaluated using multiple logistic regression analysis with adjustment for sex, age and country of birth.

RESULTS

We observed similar distribution of genotypes and allelic frequencies of all polymorphisms between GC patients and controls except of *INSR* rs1051690. The frequency of the T allele of *INSR* gene was significantly higher in GC patients than in controls (23.26% and 19.19% respectively, $P = 0.028$). CT genotype was also more prevalent in patients compared to control group (38.48% and 30.12% respectively, $P < 0.021$). Logistic regression analysis revealed that only one polymorphism (rs1051690 in *INSR* gene) was associated with increased risk of GC. Carriers of CT genotype had higher odds of GC when compared to CC genotype (OR = 1.45, 95%PI: 1.08-1.95, $P = 0.01$). Similar association was observed in a dominant model for *INSR* gene, where comparison of TT+CT vs CC genotypes showed an increased risk of GC (OR = 1.44, 95%PI: 1.08-1.90, $P = 0.01$). Other analyzed SNPs were not associated with the presence of GC.

CONCLUSION

INSR rs1051690 SNP is associated with increased risk of GC, while polymorphisms in *IL12B*, *CCND1* and *IL10* genes are not linked with the presence of GC.

Key words: Gastric cancer; miRNA; Target genes; Single-nucleotide polymorphisms

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Core tip: Several studies have evaluated an association between single-nucleotide polymorphisms (SNPs) and gastric cancer (GC) risk. Here we used novel approach. Using bioinformatical analysis tools, several SNPs were identified as potential target sites of microRNAs that

previously have been linked with gastric carcinogenesis. This study evaluated an association between SNPs in the *INSR* (rs1051690), *IL12B* (rs1368439), *CCND1* (rs7177), and *IL10* (rs3024498) genes and risk of GC in subjects of European descent. The study found that *INSR* rs1051690 SNP was associated with increased risk of GC, while polymorphisms in *IL12B*, *CCND1* and *IL10* genes showed no association with GC.

Petkevicius V, Salteniene V, Juzenas S, Wex T, Link A, Leja M, Steponaitiene R, Skieceviciene J, Kupcinskas L, Jonaitis L, Kiudelis G, Malfertheiner P, Kupcinskas J. Polymorphisms of microRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* in gastric cancer. *World J Gastroenterol* 2017; 23(19): 3480-3487 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3480.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3480>

INTRODUCTION

Gastric cancer (GC) is one of the most prevalent cancers across the globe. Despite decline in the incidence over the last century, GC remains the third leading cause of cancer-related mortality worldwide^[1,2]. Furthermore, an upward trend of GC incidence was observed in young patients in recent years^[3]. The incidence and mortality of GC vary widely across different countries. Based on the GLOBOCAN 2012 estimates, the highest incidence is in East Asia. High rates are also observed in Central and Eastern Europe, where age-standardized GC mortality rates per 100000 are 16.8 in men and 7.1 in women^[1] and prevalence of *H. pylori* infection remains burdensome^[4].

Both environmental and genetic factors play a role in etiology of GC; however, as in most cancers, pathogenetic mechanisms in GC are still not fully understood. Demographic and environmental risk factors for GC include older age, male sex, family history, tobacco smoking, *H. pylori* infection and obesity^[5]. In recent years, different studies, including genome-wide association studies, examined genetic risk factors for GC. A number of gene polymorphisms have been shown to be related to gastric carcinogenesis, but this field mandates further research^[6,7].

The discovery of microRNAs (miRNAs) has opened new opportunities for understanding of pathophysiology and molecular biology of GC^[8]. Small non-coding miRNAs molecules (approximately 18-25 nucleotides) regulate gene expression through sequence-specific pairing with the target mRNA and inhibition of its translation^[9]. Previous studies have revealed that certain single-nucleotide polymorphisms (SNPs) of miRNA encoding genes may alter miRNA expression and influence cancer development^[10,11]. Moreover, genetic variations within miRNA binding sites affect the miRNA-mRNA interaction. SNPs within a miRNA target can

reinforce, weaken or disrupt the binding with miRNAs and change the expression of mRNA targets^[12-14].

Target gene identification may help to reveal specific functions of individual miRNAs. This process is challenging because miRNAs may bind to multiple target mRNAs. In order to identify potential miRNA targets computational modeling and experimental approaches are applied^[15]. In this study, selection of SNPs was carried out using freely available online database for miRNA target gene prediction^[16]. Using this bioinformatical approach we selected four SNPs: *IL12B* (rs1368439), *INSR* (rs1051690), *CCND1* (rs7177) and *IL10* (rs3024498) as putative miRNA-binding sites. Selected SNPs within the above mentioned genes are potential target sites of miR-27, miR-146a, miR-223 and miR-107, that have been linked with gastric carcinogenesis in different studies^[8,17].

The aim of this study was to evaluate potential associations between gene polymorphisms of predicted miRNA target genes *IL12B* (rs1368439), *INSR* (rs1051690), *CCND1* (rs7177) and *IL10* (rs3024498) and the presence of GC in European population. To date, these genetic variations have not been evaluated in case-control studies of GC.

MATERIALS AND METHODS

Study subjects

Patients and controls were recruited during the years 2005-2013 at three gastroenterology centers in Germany (Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg), Lithuania (Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas) and Latvia (Riga East University Hospital and Digestive Diseases Centre GASTRO, Riga). Controls were patients from the out-patient departments, who were referred for upper endoscopy because of dyspeptic symptoms and had no history of previous malignancy. GC patients had histopathological verification of gastric adenocarcinoma and were recruited from out-patient and stationary departments. The data from the most of the patients were in focus of our previous studies to genetic predisposition of GC^[18-20].

In total, 982 individuals were included in this study (508 controls and 474 GC). There were 206 subjects from Germany (104 controls and 102 GC), 285 subjects from Latvia (146 controls and 139 GC) and 491 subjects from Lithuania (258 controls and 233 GC). All patients were of European descent.

DNA extraction and genotyping

Genomic DNA from samples was extracted using salting out method from peripheral blood mononuclear cells and stored at -20 °C until analysis. *IL12B* T>G (rs1368439), *INSR* T>C (rs1051690), *CCND1* A>C (rs7177) and *IL10* T>C (rs3024498) SNPs were

genotyped by real time PCR (RT-PCR), using TaqMan® assays with a 7500 TM real-time cycler, in accordance with the manufacturer's instructions (Life Technologies, CA, United States). Dubious samples had repetitive genotyping analysis. Duplicate genotyping was performed in 5% of all samples with one hundred percent concordance rates.

Selection of putative miRNA target gene SNPs

In order to select the candidate SNPs falling within 3'-UTR of genes which are putative targets of frequently deregulated miRNAs in GC, the mirsnpscore database was used (<http://www.bigr.medisin.ntnu.no/mirsnpscore>). The database contains *in silico* predictions of SNP effects on miRNA-target gene regulation, which are measured by ΔS score. The higher the ΔS score, the higher the possibility that the miRNA-mRNA interaction is disrupted^[16]. The candidate SNPs had to meet the following criteria: a minor allele frequency (MAF) > 0.2, the ΔS value > 0.25 and the target gene had to be previously reported as associated with GC. The MAFs and positions of SNPs for Central European population (CEU) were retrieved from 1000 Genomes Browser [phase 3, dbSNP build 149 (Homo sapiens Annotation Release 105)]^[21]. The list of selected miRNA target gene polymorphisms is presented in Table 1.

Statistical analysis

Age is shown as means \pm SD. Mean values of age was compared using Student's *t*-test. Categorical data are presented as frequencies and comparisons were performed using the χ^2 test. Each polymorphism was tested to ensure the fitting with Hardy-Weinberg equilibrium with alpha threshold of 0.05. Associations between GC and gene polymorphisms were calculated using multiple logistic regression analysis and expressed as OR with 95%CI. The ORs were adjusted for sex, age and country of birth. The ORs and 95%CI were calculated for each genotype compared with the wild-type allele homozygous group. Recessive (variant homozygous genotypes vs heterozygotes for the variant and homozygotes for the wild-type allele) and dominant (homozygotes variant + heterozygotes versus homozygotes for the wild-type allele) models were also evaluated. The Bonferroni-corrected alpha level was set at 0.013 (0.05/4 SNPs).

The analysis was performed using freely available statistical program PLINK v.1.9 available at pngu.mgh.harvard.edu/~purcell/plink.

RESULTS

Characteristics of the study group

The characteristics of control ($n = 508$) and GC ($n = 474$) groups are presented in Table 2. Control subjects were significantly younger than GC patients ($P < 0.001$). Proportion of men was considerably higher in

Table 1 Selected target genes and their corresponding single-nucleotide polymorphisms

Chromosome	Target gene	miRNA	Delta S	SNP ID	Position	MAF
5	<i>IL12B</i>	miR-27	0.3249	rs1368439	15874204	0.202
19	<i>INSR</i>	miR-146a	0.2591	rs1051690	7116963	0.242
11	<i>CCND1</i>	miR-223	0.4614	rs7177	69466115	0.419
1	<i>IL10</i>	miR-107	0.7057	rs3024498	206941529	0.267

SNP ID: Single-nucleotide polymorphisms number; MAF: Minor allele frequency.

Table 2 Characteristics of gastric cancer patients and control subjects *n* (%)

	Controls (<i>n</i> = 508)	Gastric cancer patients (<i>n</i> = 474)	<i>P</i> value
Age (mean ± SD)	58.1 ± 17.4	62.5 ± 18.4	< 0.001 ¹
Gender			
Male	139 (27.4)	288 (60.8)	< 0.001 ²
Female	366 (72.0)	178 (37.5)	
Unknown	3 (0.6)	8 (1.7)	
Country of birth			
Latvia	146 (28.7)	139 (29.3)	0.866 ²
Lithuania	258 (50.8)	233 (49.2)	
Germany	104 (20.5)	102 (21.5)	

¹Student *t*-test; ²χ² test.

GC group than in control group, 60.8% and 27.4% respectively ($P < 0.001$). Individuals in both groups did not differ significantly by country of birth. In order to avoid the potential influence of gender, age and country of birth, these variables were included in further logistic regression analysis.

Hardy-Weinberg equilibrium

The distributions of all analyzed genotypes in the control group did not differ from those predicted by a Hardy-Weinberg equilibrium: $P = 0.013$ for *IL12B* (rs1368439), $P = 0.819$ for *INSR* (rs1051690), $P = 0.856$ for *CCND1* (rs7177) and $P = 0.412$ for *IL10* (rs3024498).

Association analysis of rs1368439, rs1051690, rs7177 and rs3024498 SNPs with gastric cancer

Genotype and allele distributions for analyzed gene polymorphisms are shown in Table 3. No significant differences in the frequencies of the *IL12B*, *CCND1* and *IL10* genotypes or alleles between control and GC groups were found. The rare G allele of *IL12B* gene had the lowest frequency (14.47% in controls and 15.30% in patients). C allele of *CCND1* gene was found in 44.18% of controls and 43.13% of GC patients, while C allele of *IL10* gene - in 28.04% and 25.53% respectively. Distribution of *INSR* genotypes and alleles differed between control and GC patients groups. The frequency of T allele was 19.19% in controls and 23.26% in GC patients ($P = 0.028$). Distribution of TT genotypes was similar in both groups, while CT genotype was more prevalent in patients than in controls (38.48% and 30.12% respectively, $P =$

0.021). Logistic regression analysis revealed that only one polymorphism (rs1051690 in *INSR* gene) was associated with increased risk of GC. Carriers of CT genotype had higher odds of GC when compared to CC genotype (OR = 1.45, 95%PI: 1.08-1.95, $P = 0.01$). A similar association was observed in a dominant model for *INSR* (rs1051690), where comparison of TT + CT vs CC genotypes showed an increased risk of GC ($P = 0.01$). A tendency for T allele vs C allele to be associated with higher risk of GC was observed; however, the difference did not reach the adjusted significance threshold (OR = 1.32, 95%PI: 1.04-1.67, $P = 0.02$). No associations with GC risk was found for other analyzed SNPs (Table 3).

DISCUSSION

This study evaluated the association between SNPs in the *INSR* (rs1051690), *IL12B* (rs1368439), *CCND1* (rs7177), and *IL10* (rs3024498) genes and risk of GC in subjects of European descent. These SNPs were selected as candidate miRNA-related genetic alterations that may change the expression of miRNAs linked to GC and potentially mediate carcinogenesis. The study found that *INSR* rs1051690 SNP was associated with increased risk of GC, while no link has been found for the polymorphisms in *IL12B*, *CCND1* and *IL10* genes and GC risks. To our best knowledge this is the first study which evaluated the effect of these SNPs for the development of GC.

The biological actions of insulin are mediated by *INSR* gene. de-Freitas-Junior *et al.*^[22] demonstrated that changes in the *INSR* gene can affect the insulin signaling pathway by modulating E-cadherin glycosylation and destabilization of cellular membranes that may have detrimental effects in gastric carcinogenesis. A recent study also identified *INSR* as new candidate gene for diffuse gastric cancer susceptibility^[23]. Landi *et al.*^[13] showed that alleles regulate differentially the amount of a reporter gene (luciferase) in an *in vitro* assay and may have a functional role in regulating the expression of *INSR* proteins. Several studies have described the role of miRNAs in the regulation of *INSR* gene in different cancers^[24,25]. In our study we selected rs1051690 of *INSR* gene which is a potential binding site for miR-146a^[16]. Previous case-control studies carried out in Czech Republic, Spain and Israel revealed an association between rs1051690 and colorectal cancer^[13,26,27]. The findings of our study are partly in line with the latter

Table 3 Genotype and allele frequencies in control and gastric cancer patients and odds ratio of gastric cancer by genotypes *n* (%)

Genotype	Controls (<i>n</i> = 508)	Gastric cancer patients (<i>n</i> = 474)	OR	95%CI	<i>P</i> value
<i>IL12B</i> (rs1368439)					
TT	366 (72.05)	338 (71.31)	1.00		
TG	137 (26.97)	127 (26.79)	0.87	0.63-1.18	0.39
GG	5 (0.98)	9 (1.90)	1.27	0.39-4.14	0.68
GG <i>vs</i> TG + TT			1.33	0.41-4.30	0.63
GG + TG <i>vs</i> TT			0.88	0.65-1.20	0.42
Allele T	869 (85.53)	803 (84.70)	1.00	0.83-1.45	0.63
Allele G	147 (14.47)	145 (15.30)	1.10		
<i>INSR</i> (1051690)					
CC	334 (65.75)	272 (57.51)	1.00		
CT	153 (30.12)	182 (38.48)	1.45	1.08-1.95	0.01
TT	21 (4.13)	19 (4.02)	1.30	0.66-2.60	0.44
TT <i>vs</i> CT + CC			1.15	0.58-2.30	0.70
TT + CT <i>vs</i> CC			1.44	1.08-1.90	0.01
Allele C	821 (80.81)	726 (76.74)	1.00		
Allele T	195 (19.19)	220 (23.26)	1.32	1.04-1.67	0.02
<i>CCND1</i> (rs7177)					
AA	160 (31.56)	159 (33.62)	1.00		
AC	245 (48.32)	220 (46.51)	0.92	0.68-1.26	0.63
CC	102 (20.12)	94 (19.87)	1.07	0.73-1.58	0.70
CC <i>vs</i> AC + AA			1.12	0.80-1.60	0.50
CC + AC <i>vs</i> AA			0.97	0.72-1.30	0.83
Allele A	565 (55.72)	538 (56.87)	1.00		
Allele C	449 (44.28)	408 (43.13)	1.02	0.84-1.24	0.81
<i>IL10</i> (rs3024498)					
TT	252 (50.30)	259 (54.87)	1.00		
TC	217 (43.31)	185 (39.19)	0.92	0.69-1.22	0.57
CC	32 (6.39)	28 (5.93)	1.05	0.58-1.87	0.88
CC <i>vs</i> TC + TT			1.08	0.29-1.61	0.78
CC + TC <i>vs</i> TT			0.94	0.71-1.23	0.64
Allele T	721 (71.96)	703 (74.47)	1.00		
Allele C	281 (28.04)	241 (25.53)	1.03	0.83-1.30	0.78

studies, suggesting that this SNP might mediate not only colorectal but also GC risks, pointing to a potential joint mechanism of gastrointestinal cancers. A study by Xiao *et al*^[28] showed that miR-146a was upregulated in 20 gastric cancer tissues compared with matched non-tumor adjacent tissues. Due to the design of the study we were not able to evaluate whether rs1051690 could mediate the expression of miR-146a and this remains to be evaluated in further studies.

Chronic inflammation plays a crucial role in GC development, thus multiple genes in inflammatory pathways may be associated with GC risk^[29]. To date, different gene polymorphism related to inflammatory pathways have been evaluated, with *IL-1B* and *IL-1RN* being the most widely studied ones^[18,30-34]. Computational analysis tools that we used in our study suggested two genes polymorphisms - *IL12B* (rs1368439) and *IL10* (rs3024498) - situated in inflammatory pathways, that might be the involved in miRNA-target gene interaction^[16]. The other studies evaluated some gene polymorphisms located in *IL12* and *IL10*; however, they were different from the ones selected for our study. *IL12B* encodes a subunit p40 of interleukin (IL) 12. Proinflammatory cytokine IL12 is expressed by activated macrophages and favors the differentiation of T helper 1 (Th1) cells^[35]. Th1 lymphocytes prevail over Th2 in *H. pylori* associated

chronic gastritis^[36]. *IL10* down-regulates the expression of Th1 cytokines and enhances B cell survival, proliferation, and antibody production^[37]. Our study did not find significant association between polymorphisms in *IL12B* or *IL10* genes with GC risk. Our results support the previous data to other populations, which analyzed associations between SNPs in genes regulating the inflammatory response and GC^[30-32,34].

CCND1 is an important regulator of the cell cycle. It plays essential role in the activation of G1/S transition, which increases cell proliferation and growth. Mutations, amplification and overexpression of this gene are observed frequently in a variety of tumors and may contribute to tumorigenesis^[38]. The study by Ma *et al*^[39] confirmed that high *CCND1* expression was related with poor prognosis in patients with resected gastric adenocarcinoma. A meta-analysis of associations between the most extensively studied *CCND1* polymorphism rs9344 and GC demonstrated negative results^[40]. In our study we did not find an association between *CCND1* (rs7177) SNP and the risk of GC. One study found no association between rs7177 and risk of head and neck cancer in a case control study^[41], but no data is available until now for GC.

Target site polymorphisms in gene may strengthen or weaken the miRNA-mRNA interaction and change expression of gene^[42]. This field still remains poorly

explored in different cancers including GC. The importance of miRNA related SNPs in gene regulation and the mechanism by which these SNPs can induce alteration in molecular pathways is largely unknown. Wang *et al.*^[43] suggested that rs4901706 SNP of *C14orf101* gene in the microRNA binding site might be used as a valuable biomarker when predicting GC risk. One other study showed that polymorphisms of the microRNA-binding sites in the 3' UTR region of integrin are associated with GC susceptibility (rs2675), tumor stage (rs2675, rs17664, and rs3809865), and lymphatic metastasis (rs17664) in Chinese Han population^[44]. In our previous studies we could not determine the link between *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* gene polymorphisms and the risk of gastric^[45] or colorectal cancers^[46].

Our study carries certain limitations that have to be taken into account. First of all, the selection of putative miRNA target genes and corresponding gene polymorphism is based on bioinformatical databases that may over- or underestimate real interaction effects. Future studies are needed to validate our findings in other cohorts and to investigate whether the gene variant affecting the insulin receptor (*INSR* gene) leads to changes in the expression level of the receptor. Since this is the first study on these SNPs in GC, direct comparison with the results of other studies is not possible yet. Nevertheless, overall our data provide important novel aspects on genetic susceptibility for GC.

The study showed that *INSR* rs1051690 SNP is associated with increased risk of GC. We did not find the association between polymorphisms in *IL12B*, *CCND1* and *IL10* genes and GC risks.

COMMENTS

Background

The discovery of microRNAs (miRNAs) has opened new opportunities for understanding of pathophysiology and molecular biology of gastric cancer (GC). MiRNAs regulate gene expression through sequence-specific pairing with the target messenger RNA (mRNA) and inhibition of its translation. Genetic variations within miRNA binding sites can affect the miRNA-mRNA interaction and change expression of gene. This study evaluated an association between single-nucleotide polymorphisms (SNPs) in the *INSR* (rs1051690), *IL12B* (rs1368439), *CCND1* (rs7177), and *IL10* (rs3024498) genes and risk of GC in subjects of European descent.

Research frontiers

Target site polymorphisms in gene may strengthen or weaken the miRNA-mRNA interaction. This field still remains poorly explored in different cancers including GC. The importance of miRNA related SNPs in gene regulation and the mechanism by which these SNPs can induce alteration in molecular pathways is largely unknown. Studied SNPs were selected as candidate miRNA-related genetic alterations that may change the expression of miRNAs linked to GC and potentially mediate carcinogenesis.

Innovations and breakthroughs

In this study, novel approach was applied. Using bioinformatical analysis tools, several SNPs were identified as potential target sites of microRNAs that previously have been linked with gastric carcinogenesis. The study found that *INSR* rs1051690 SNP was associated with increased risk of GC, while

polymorphisms in *IL12B*, *CCND1* and *IL10* genes showed no association to GC. Our data provide important novel aspects on SNPs of miRNA and their target gene interaction sites in GC.

Applications

Polymorphisms in microRNA binding site might be used as a valuable biomarker when predicting GC risk.

Peer-review

The authors investigated the association of selected polymorphisms with the risk of developing gastric cancer in European population. Their analyses were performed on relatively large population of patients. Overall, the manuscript presents the hypothesis and results well.

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Case Control Study

Insulin-like growth factor-1, IGF binding protein-3, and the risk of esophageal cancer in a nested case-control study

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Abstract

AIM

To assess the relationship between serum levels of insulin-like growth factor-1 (IGF1)/IGF-binding protein-3 (IGFBP3) and the risk of esophageal carcinoma.

METHODS

We assessed the relationship between the serum levels of these molecules and the risk of esophageal

cancer in a prospective, nested case-control study of participants from the Japan Collaborative Cohort Study. A baseline survey was conducted from 1988 to 1990. Of the 110585 enrolled participants, 35% donated blood samples. Those who had been diagnosed with esophageal cancer were considered cases for nested case-control studies. A conditional logistic model was used to estimate odds ratios for the incidence of esophageal cancer associated with serum IGF1 and IGFBP3 levels.

RESULTS

Thirty-one cases and 86 controls were eligible for the present assessment. The molar ratio of IGF1/IGFBP3, which represents the free and active form of IGF1, was not correlated with the risk of esophageal carcinoma. A higher molar difference between IGFBP3 and IGF1, which estimates the free form of IGFBP3, was associated with a decreased risk of esophageal carcinoma ($P = 0.0146$), and people in the highest tertile had the lowest risk (OR = 0.107, 95%CI: 0.017-0.669). After adjustment for body mass index, tobacco use, and alcohol intake, the molar difference of IGFBP3-IGF1 was inversely correlated with the risk of esophageal carcinoma ($P = 0.0150$).

CONCLUSION

The free form of IGFBP3, which is estimated by this molar difference, may be inversely associated with esophageal cancer incidence.

Key words: Esophageal cancer; Insulin-like growth factor; Insulin-like growth factor binding protein; Nested case-control study; Odds ratio

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Core tip: Insulin-like growth factor-1 (IGF1) is a potent mitogen, whereas IGF-binding protein-3 (IGFBP3) binds and inhibits IGF1. High circulating IGF1 and low IGFBP3 are associated with increased risk of several cancers. Here we assessed the relationship between these molecules and the risk of esophageal carcinoma in a prospective, nested case-control study from the Japan Collaborative Cohort Study. Free IGF1, represented by the molar ratio of IGF1/IGFBP3, was not correlated with the risk of esophageal carcinoma. The free form of IGFBP3, which is estimated by the molar difference of IGFBP3-IGF1, may be inversely associated with esophageal cancer incidence.

Adachi Y, Nojima M, Mori M, Yamashita K, Yamano H, Nakase H, Endo T, Wakai K, Sakata K, Tamakoshi A. Insulin-like growth factor-1, IGF binding protein-3, and the risk of esophageal cancer in a nested case-control study. *World J Gastroenterol* 2017; 23(19): 3488-3495 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3488.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3488>

INTRODUCTION

Esophageal carcinoma is one of the worst prognostic neoplasms internationally^[1,2]. The two main types of esophageal neoplasms are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). When diagnoses were made, most patients have either metastatic or unresectable cancers. Even if the patient received curative-intent surgery, the rest of time is still limited, and therapies for inoperable esophageal tumors are not so effective. Therefore, we must search for new risk factors for esophageal neoplasms to identify people who may develop early stages of the disease whose cancer is thus more readily treatable.

Several growth factor signals are needed for tumorigenesis and cancer progression^[3,4]. The insulin-like growth factor (IGF) system, which includes both the IGF1 and IGF2 ligands and the type 1 insulin-like growth factor receptor (IGF1R), may be an important molecular target for cancer therapy^[4-6]. After ligands bind to IGF1R, the receptor is autophosphorylated, and then several downstream signal pathways are activated^[7]. IGFs are secreted by hepatocytes and by several extra-hepatic components, such as cancer and stromal cells^[8]. In the homeostatic condition, the IGF axis is tightly regulated by multiple ways^[9]. The production of IGFs and IGF-binding proteins (IGFBPs) 1-6 is regulated by growth hormone, which is produced in the pituitary gland. IGF1R activation is controlled by the quantity of the free IGFs, which is modulated by those binding proteins and the nonstimulatory type 2 IGF receptor^[7,10]. In serum, almost all of IGF1 is inactive and bound with IGFBPs, which make a complex with IGF in a 1:1 molar ratio. IGFBP3 is the most plentiful binding protein and occupies around 80% of all IGF complexes. The IGF-IGFBP complex balance is controlled by proteases, including matrix metalloproteinase^[11]. Furthermore, IGFBPs have IGF-independent activities, however, these actions are not understood well^[10,12].

Normal esophageal epithelium express IGF1R, and IGFs could induce both cellular proliferation and DNA synthesis^[13-15]. Salivary IGF1 is in the free form (not bound to IGFBP, unlike the serum pool) and continuously bathes the esophageal epithelial cells^[16]. These data might suggest that the IGF system plays important parts not only in homeostasis but also premalignancy^[15]. Both IGF1R and its ligands are overexpressed in esophageal tumor components compared to normal ones^[17-19], and both serum levels of IGF1 and IGFBP3 are significantly elevated in patients with esophageal cancer compared with healthy subjects^[20,21]. Previously we revealed that expressions of IGF2 and IGF1R were detected in 50% and 60% of ESCC, respectively, and both molecules were related with advanced tumor stage, invasion depth, metastasis, and recurrence^[22]. Multivariate analysis

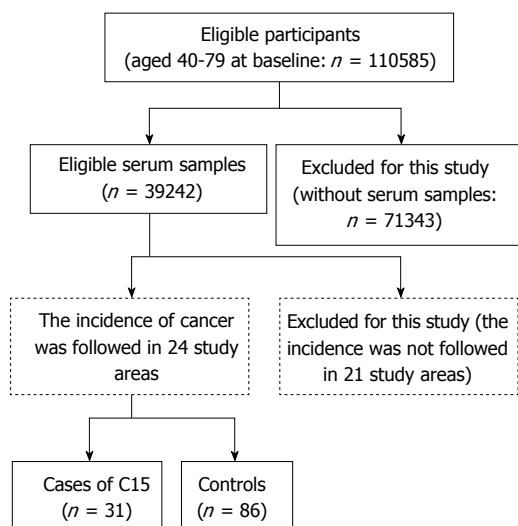


Figure 1 Flow chart for selection of cases and control.

revealed that patients with ESCC expressing both IGF2 and IGF1R show a significantly shorter survival than those expressing only one molecule or neither. A meta-analysis revealed that the expression of IGF1R is correlated with unfavorable prognosis in patients with EAC^[23]. IGF1R blockers, including antibodies, tyrosine kinase inhibitors, IGF1R dominant negative, and IGFBP3, suppress proliferation and up-regulate apoptosis^[22,24-26].

Elevated serum concentration of total-IGF1 or free-IGF1 levels, which could be calculated by the molar ratio of IGF1/IGFBP3, increase the future risk of several malignancies, including breast, colon, and prostate^[27-29]. Additionally, low serum levels of total-IGFBP3 or free-IGFBP3, which can be approximated by the molar difference (IGFBP3-IGF1), upregulate the future risk of neoplasms^[29,30]. However, the association between the risk of esophageal carcinoma and serum levels of IGF related compounds has not been reported. Although several correlations between the risk of cancer death and IGF axial proteins from the Japan Collaborative Cohort (JACC) Study were published^[30-32], the incidence of esophageal carcinoma has not been reported. Therefore, we assessed relationships between these parameters and esophageal tumor risk in a nested, case-control study in a prospective cohort study of participants in the JACC study.

MATERIALS AND METHODS

Study population and samples

We assessed data of the JACC Study, in which cancer risk associated with lifestyle factors in a Japanese population was evaluated. The study was described in detail elsewhere^[33-35]. Briefly, a baseline survey was conducted between 1988 and 1990. A total of 110,585 participants aged 40 to 79 years from 45 areas throughout Japan were enrolled in the study and

completed a self-administered questionnaire.

Informed consent was obtained from all participants. The ethical board of the Nagoya University School of Medicine approved this study.

Approximately 35% of the cohort participants (39242 subjects) provided blood samples, which were stored at -80 °C until analyzed.

Follow-up, identification of esophageal cancer, and selection of control

The incidence of cancer was followed in subjects living in 24 study areas beginning at the time of the baseline survey (Figure 1). Individuals who moved away from the study district were treated as dropouts, because deaths after such moves could not be detected in our system for follow-up. Participants with a history of malignant tumor at baseline were excluded. The malignancy was confirmed in population-based cancer registries or by reviewing the records of local major hospitals. We defined esophageal cancer as C15 (malignant neoplasm of esophagus) according to the International Statistical Classification of Diseases and Related Health Problems, 10th Revision (<http://www.who.int/classifications/icd/en/>). Subjects diagnosed with esophageal carcinoma by 1997 were regarded as cases for this nested case-control study. For each case, we randomly selected three or two controls that were matched for residential area, gender, and age (as near as possible). A total of 31 cases and 86 control participants were eligible for this study.

Biochemical assays

The trained staff who blinded to case-control status, measured all samples at a single laboratory (SRL, Tokyo, Japan) in 1999 and 2000. Serum levels of IGF1 and IGFBP3 were analyzed with an immunoradiometric assay using commercially available kits (Daiichi Radioisotope Lab., Tokyo, Japan). Details of the assay were reported previously^[36].

Statistical analysis

Proportions and mean values of baseline characteristics between cases and controls were assessed by a *t*-test or Fisher's exact test. The cross-sectional relationship between serum IGF1 and IGFBP3 was examined using the Spearman correlation coefficient. Serum values were divided into tertiles based on the distribution of serum values in all control subjects, with the first tertile used as a reference. IGF1 tertile values for tertiles 1, 2, and 3 were < 120; 120-150; and > 150.0 ng/mL, respectively. IGFBP3 tertile values for tertiles 1, 2, and 3 were < 2.88; 2.88-3.55; and > 3.55 ng/mL, respectively.

The odds ratios (ORs) for the incidence of esophageal carcinoma associated with serum IGF-related protein levels were estimated using conditional logistic regression. ORs were adjusted for alcohol intake, body mass index (BMI, computed as weight in kilograms

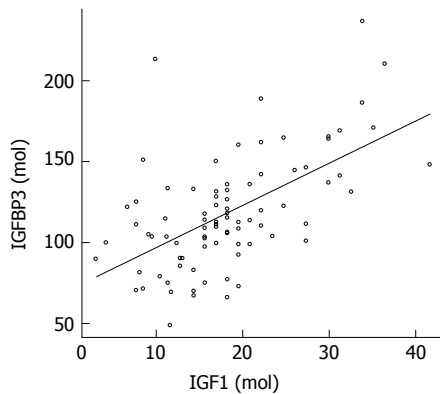


Figure 2 A scatter plot of insulin-like growth factor-1/insulin-like growth factor-binding protein-3. Spearman correlation coefficients between IGFBP3 and IGF1 among controls is 0.541 ($P < 0.0001$).

divided by the square of the height in meters), and tobacco smoking habit. The statistical significance of trends across exposure tertiles was assessed by including ordinal terms for each serum level tertile and entering the variable as a continuous term in the model. All P values and 95%CI presented in the tables were based on two-sided tests.

Because the molar ratio of IGF1 to IGFBP3 is believed to stand for free form IGF1, we also assessed the molar ratio IGF1/IGFBP3 (for conversion, 1 ng/mL is 0.130 nmol/L for IGF1 and 0.036 nmol/L for IGFBP3)^[29]. In addition, we assessed the molar difference between IGFBP3 and IGF1, which is considered to represent free IGFBP3^[30].

RESULTS

Baseline characteristics are shown in Table 1. No differences were found in height, weight, BMI, smoking habits, or alcohol consumption between case and control groups. The mean concentration of IGF1 tended to be higher in the control group, but the difference was not significant. The mean concentration of IGFBP3 was significantly higher in the control group than in the cases. Spearman correlation coefficient between IGFBP3 and IGF1 among controls is 0.541 ($P < 0.0001$; Figure 2).

Both concentrations of total IGF1 and total IGFBP3 were inversely associated the risk of esophageal carcinoma ($P = 0.036$ and 0.024 , respectively, Table 2). After adjusted for BMI, cigarette smoking, and alcohol intake, the latter still showed inverted relation with the risk of esophageal tumor but the former did not ($P = 0.042$ and 0.052 , respectively). After adjusted for the concentration of IGFBP3, total IGF1 was not associated with the risk of esophageal carcinoma. After adjusted for the concentration of IGF1, total IGFBP3 was not associated with the risk of esophageal carcinoma. After adjustment for alcohol consumption, BMI, and smoking status, and each other, both serum levels of IGF1 and IGFBP3 were not also related with the risk of esophageal cancer.

Table 1 Selected baseline characteristics of case and control group n (%)

	Cases	Controls	P value
Number of subjects	31	86	
Age	61.5 \pm 7.3	61.3 \pm 7.2	0.811
Male	25 (80.6)	75 (87.2)	0.383 ¹
Height	162.5 \pm 10.5	161.5 \pm 5.3	0.495
Weight	57.0 \pm 12.9	59.0 \pm 9.1	0.352
BMI (kg/m ²)	21.5 \pm 4.2	22.7 \pm 3.1	0.112
Cigarette smoking	30	77	0.384 ¹
Never	6 (20.0)	25 (32.5)	
Past	5 (16.7)	14 (18.2)	
Current	19 (63.3)	38 (49.3)	
Alcohol intake	29	84	0.410 ¹
Never	6 (20.7)	21 (25.0)	
Past	3 (10.3)	3 (3.6)	
Current	20 (69.0)	60 (71.4)	
IGF1	122.4 \pm 59.9	140.8 \pm 59.8	0.145
IGFBP3	2.82 \pm 0.95	3.30 \pm 0.97	0.020

¹Fisher's exact test. IGF1: Insulin-like growth factor-1; IGFBP3: IGF-binding protein-3.

A lower molar ratio of IGF1/IGFBP3, which means free IGF1, tended to be correlated with a decreased risk of esophageal cancer but the difference was not statistically significant ($P = 0.273$; Table 3). After adjustment for BMI, smoking status, and alcohol intake, the association was not observed ($P = 0.408$).

A lower molar difference of (IGFBP3-IGF1), which means free-form IGFBP3, was associated with an increased risk of esophageal carcinoma ($P = 0.015$; Table 3). After adjustment for BMI, smoking habit, and drinking status, the association was also observed ($P = 0.015$). The highest tertile of this molar difference showed the lowest risk of esophageal neoplasm (OR = 0.100, 95%CI: 0.015-0.674). Thus, the molecular difference of (IGFBP3-IGF1) might most precisely stand for the future risk of esophageal carcinoma in the current study.

In order to assess the interaction with age and gender, ORs were analyzed in subgroups. Higher free IGFBP3 was also related to a decreased risk of esophageal cancer in the male population ($P = 0.011$; Table 4). After adjustment for BMI, tobacco use, and alcohol intake, this association was confirmed ($P = 0.004$). However, we found no association between free IGFBP3 and the risk of esophageal cancer in the female population ($P = 0.517$), as the number of cases was too low. In non-elderly participants (population ≤ 65 years old), a higher molar difference (IGFBP3-IGF1) was related to a reduced risk of esophageal carcinoma ($P = 0.007$). After adjustment for BMI, tobacco use, and alcohol intake, this relationship was confirmed ($P = 0.007$). However, this correlation was not observed in elderly individuals (population > 65 years old, $P = 0.696$).

DISCUSSION

High serum concentrations of IGF1 and low IBFBP3

Table 2 Odds ratios and 95%CI for esophageal cancer with reference to serum concentrations of insulin-like growth factor-1 and insulin-like growth factor-binding protein-3

	Tertile			P value
	1 (referent)	2	3	
IGF1				
ng/mL (range)	< 120	120-150	> 150	
No. of case/control	17/27	8/33	6/26	
OR (95%CI)	1	0.300 (0.098-0.913)	0.263 (0.068-1.011)	0.036
OR adjusted 1 (95%CI)	1	0.292 (0.087-0.982)	0.265 (0.064-1.092)	0.052
OR adjusted 2 (95%CI)	1	0.281 (0.077-1.023)	0.387 (0.071-2.112)	0.247
OR adjusted 3 (95%CI)	1	0.298 (0.080-1.109)	0.417 (0.074-2.348)	0.249
IGFBP3				
ng/mL (range)	< 2.88	2.88-3.55	> 3.55	
No. of case/control	18/30	9/27	4/29	
OR (95%CI)	1	0.518 (0.171-1.564)	0.133 (0.023-0.786)	0.024
OR adjusted 1 (95%CI)	1	0.527 (0.155-1.792)	0.137 (0.021-0.911)	0.042
OR adjusted 4 (95%CI)	1	0.914 (0.241-3.468)	0.182 (0.025-1.329)	0.140
OR adjusted 5 (95%CI)	1	0.853 (0.210-3.468)	0.192 (0.023-1.585)	0.196

Adjusted 1: Adjusted for cigarette smoking, BMI, and alcohol intake; Adjusted 2: Adjusted for IGFBP3; Adjusted 3: Adjusted for cigarette smoking, BMI, alcohol intake, and IGFBP3; Adjusted 4: Adjusted for IGF1; Adjusted 5: Adjusted for cigarette smoking, BMI, alcohol intake, and IGF1.

Table 3 Odds ratios and 95%CI for esophageal cancer according to molar ratio and difference of insulin-like growth factor-1 and insulin-like growth factor-binding protein-3

	Tertile			P value
	1 (referent)	2	3	
IGF1/IGFBP3				
Molar ratio	< 0.137	0.137-0.177	> 0.177	
No. of case/control	9/29	11/28	11/29	
OR (95%CI)	1	1.733 (0.517-5.851)	2.127 (0.554-8.164)	0.273
OR adjusted (95%CI)	1	1.486 (0.416-5.309)	1.810 (0.453-7.226)	0.408
IGFBP3-IGF1				
Molar difference	< 87.77	87.77-108.14	> 108.14	
No. of case/control	18/29	9/28	4/29	
OR (95%CI)	1	0.432 (0.137-1.262)	0.107 (0.017-0.669)	0.015
OR adjusted (95%CI)	1	0.380 (0.115-1.250)	0.100 (0.015-0.674)	0.015

Adjusted: Adjusted for cigarette smoking, BMI, and alcohol intake.

Table 4 Odds ratios and 95%CI for esophageal cancer according to molar difference of insulin-like growth factor-1 and insulin-like growth factor-binding protein-3 (subgroup)

	Tertile			P value
	1 (referent)	2	3	
Molar difference	< 87.77	87.77-108.14	> 108.14	
Male				
No. of case/control	15 / 28	7/22	3/25	
OR (95%CI)	1	0.339 (0.085-1.349)	0.044 (0.004-0.527)	0.011
OR adjusted (95%CI)	1	0.186 (0.034-1.015)	0.022 (0.001-0.319)	0.004
≤ 65 years old				
No. of case/control	11/14	7/21	3/27	
OR (95%CI)	1	0.260 (0.052-1.300)	0.031 (0.025-0.400)	0.007
OR adjusted (95%CI)	1	0.226 (0.042-1.224)	0.028 (0.002-0.389)	0.007

Adjusted: Adjusted for cigarette smoking, BMI, and alcohol intake.

levels are risk factors for several malignancies^[27-29]. Furthermore, IGFs take several parts in tumorigenesis and cancer development of esophageal carcinoma, and IGFBPs could inhibit those effects of IGF ligands^[4,5,22,26]. In the present analysis, neither serum levels of IGF1 nor IGFBP3 were related to the OR for esophageal

cancer, after adjustment for each other. Moreover, serum free IGF1 did not show any association with esophageal tumor risk.

Salivary IGF1 continuously bathes the esophageal lumen and exists in a free and active form^[16]. Thus, the local concentration of IGF ligands around the

esophageal mucosal cells may be high, even though the serum concentrations are low. Salivary IGF1 could have higher binding ability to IGF1R on esophageal mucosal cells. This may be a reason why serum concentrations of IGF1 did not show significant association with the risk of esophageal carcinoma.

In serum, IGF and the binding protein make a complex in a 1:1 molar ratio, and the molar level of IGFBP3 is more than that of IGF1. Hence, the molar difference of (IGFBP3-IGF1) could represent the level of the free type of IGFBP3^[30]. The molar difference of IGFBP3-IGF1 was inversely and significantly correlated with the future risk of esophageal cancer. After adjustment for alcohol intake, BMI, and cigarette smoking, this molar difference was still significantly correlated with an inverse risk of esophageal carcinoma. Analyses of non-elderly or male subgroups might confirm that a high molar difference of (IGFBP3-IGF1) showed a low risk of esophageal tumors. Therefore, this parameter may be a candidate predictive marker of esophageal cancer. Moreover, we have reported that free IGFBP3 showed a reversed risk for hepatic cancer^[30]. Thus, the molar difference of IGFBP3-IGF1 may be an important parameter.

As serum IGF1 levels were higher in viscerally obese patients with esophageal cancer than non-obese patients^[21,37], visceral obesity may influence the IGF axis. Although this observation is seen in EAC in particular, more people have ESCC than EAC in Japan, as in Eastern Asia. Although our current study did not collect data about visceral obesity, free IGFBP3 was inversely associated with the risk of esophageal cancer after adjustment including BMI.

In a population-based case-control study, three polymorphisms in IGF and related genes such as IGF1 (CA)₁₇ 185-bp allele and two single-nucleotide polymorphisms, IGF1 rs6214 and growth hormone receptor rs6898743, were associated with EAC or its precursors, including reflux esophagitis and Barrett esophagus^[38]. These results also suggest that the IGF pathway may be involved in EAC development.

The advantage of the current analysis is that the subjects were from a large-scale JACC study of 110792 participants. One limitation is, however, that some data about alcohol intake, BMI, and smoking habit were lost due to the self-administered survey^[33]. Another is that both the numbers of serum samples and esophageal tumor cases were not so large (39242 subjects and 31 cases, respectively).

Our result might suggest that low free IGFBP3, estimated as the molar difference of IGFBP3-IGF1, represents an important predictive marker of a high incidence of esophageal carcinoma.

COMMENTS

Background

Insulin-like growth factor-1 (IGF1) is a potent mitogen, whereas IGF-binding protein-3 (IGFBP3) binds and inhibits IGF1. High circulating IGF1 and low

IGFBP3 are associated with increased risk of several cancers. However there are limited information about the relationship between the serum levels of these molecules and the risk of esophageal carcinoma.

Research frontiers

The authors assessed the relationship between the serum levels of IGF1 and IGFBP3 and the risk of esophageal carcinoma in a prospective, nested case-control study. They introduced a new concept of the free IGFBP3, which is estimated by molar difference IGFBP3 from IGF1.

Innovations and breakthroughs

The free form of IGFBP3, which is estimated by the molar difference of IGFBP3-IGF1, may be inversely correlated with the incidence of esophageal cancer. However, free IGF1, which is represented by the molar ratio of IGF1/IGFBP3, was not associated with esophageal carcinoma risk.

Applications

People with low free form of IGFBP3 might have higher future risk of esophageal carcinoma. However, the number of case was not so much, thus larger studies are needed. Moreover, some intervention studies for people with low free form of IGFBP3 are needed.

Terminology

The free form of IGFBP3 is estimated by the molar difference of IGFBP3-IGF1. The free form of IGF1 is represented by the molar ratio of IGF1/IGFBP3.

Peer-review

This is an interesting and valuable article in exploring the association between esophageal carcinoma, IGF1 and IGFBP3. This is the merit and value of the paper that can be referred and cited by other studies in future when the results are found. However, there are several concerns that should be clarified. It is the concern on the matter for the interested readers who can be involved in this research and can repeatedly practice it in future. I here illustrate some that are unclear, non-understandable, and non-readable letting shortcomings clearly limit the contribution of the paper.

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Retrospective Cohort Study

Tumor-associated autoantibodies are useful biomarkers in immunodiagnosis of α -fetoprotein-negative hepatocellular carcinoma

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Abstract

AIM

To determine the prevalence and diagnostic value of autoantibodies in α -fetoprotein (AFP)-negative hepatocellular carcinoma (HCC).

METHODS

Fifty-six serum samples from AFP-negative HCC cases, 86 from AFP-positive HCC cases, 168 from chronic liver disease cases, and 59 from normal human controls were included in this study. Autoantibodies to nucleophosmin (NPM)1, 14-3-3zeta and mouse double minute 2 homolog (MDM2) proteins in AFP-negative HCC serum were evaluated by enzyme-linked immunosorbent assay. Partially positive sera were further evaluated by western blotting. Immunohistochemistry was used to detect the expression of three tumor-associated antigens (TAAs) in AFP-negative HCC and normal control tissues.

RESULTS

The frequency of autoantibodies to the three TAAs in AFP-negative HCC sera was 21.4%, 19.6% and 19.6%, which was significantly higher than in the chronic liver disease cases and normal human controls ($P < 0.01$) as well as AFP-positive HCC cases. The sensitivity of the three autoantibodies for diagnosis of AFP-negative HCC ranged from 19.6% to 21.4%, and the specificity was approximately 95%. When the three autoantibodies were combined, the sensitivity reached 30.4% and the specificity reached 91.6%.

CONCLUSION

Autoantibodies to NPM1, 14-3-3zeta and MDM2 may be useful biomarkers for immunodiagnosis of AFP-negative HCC.

Key words: α -fetoprotein; Nucleophosmin 1; 14-3-3zeta; Mouse double minute 2 homolog; Immunodiagnosis; Autoantibody; Hepatocellular carcinoma

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Core tip: We firstly and specifically investigated the diagnostic value of autoantibodies in α -fetoprotein (AFP)-negative hepatocellular carcinoma (HCC). We retrospectively evaluated the prevalence and diagnostic value of autoantibodies to nucleophosmin (NPM)1, 14-3-3zeta and mouse double minute 2 homolog (MDM2) proteins and their different combinations in 56 AFP-negative HCC patients by enzyme-linked immunosorbent assay and western blotting. Immunohistochemistry was used to detect the expression of three tumor-associated antigens (TAAs) in AFP-negative HCC. Our study demonstrated that autoantibodies to NPM1, 14-3-3zeta and MDM2 may be useful biomarkers for immunodiagnosis of AFP-negative HCC.

Wang T, Liu M, Zheng SJ, Bian DD, Zhang JY, Yao J, Zheng QF, Shi AM, Li WH, Li L, Chen Y, Wang JH, Duan ZP, Dong L. Tumor-associated autoantibodies are useful biomarkers in immunodiagnosis of α -fetoprotein-negative hepatocellular carcinoma. *World J Gastroenterol* 2017; 23(19): 3496-3504 Available from: URL: <http://www.wjgnet.com/1007-9327/full/>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer-related death worldwide. Nearly 50% of the new cases of liver cancer and related deaths occur in China^[1]. Due to the lack of reliable methods for early diagnosis, most patients die within 1 year after diagnosis of HCC. Although ultrasound is used as an assistive tool for early detection of HCC, it is not sufficiently sensitive and is operator dependent. Computed tomography (CT) and magnetic resonance imaging (MRI) are not recommended as common screening tools for HCC because of the attendant radiation exposure and high cost^[2,3]. So far, α -fetoprotein (AFP) is still the only widely used clinical serum biomarker; however, some studies have shown that the sensitivity and predictive value of AFP for the diagnosis of HCC is only 41%-65% and 12%, respectively, especially for early HCC and AFP-negative HCC. There are still approximately 40% of cases of HCC with normal AFP levels that cannot be detected early^[4,5].

HCC can be diagnosed by significantly increased serum AFP levels and definitive imaging results. However, AFP-negative HCC cannot be diagnosed easily and depends largely on imaging results, which often leads to misdiagnosis^[6]. Thus, many HCC patients cannot obtain timely diagnosis and treatment. In recent years, numerous studies have been performed to identify a diagnostic biomarker for HCC^[7], however, all of the potential candidates have shown poor specificity and sensitivity, and there are few studies on AFP-negative HCC^[8,9].

Recently, many studies have shown that the serum of cancer patients contains autoantibodies that react with a unique group of autologous cellular antigens known as tumor-associated antigens (TAAs)^[10,11]. Unlike autoantibodies appearing in autoimmune diseases, TAA autoantibodies have been detected in a variety of tumors^[12]. Some autoantibodies are present several months to years before the clinical diagnosis of tumor^[13-15]. Furthermore, TAA autoantibodies may have greater advantages as immunodiagnostic markers, because their magnified signals can be easier to detect than TAAs themselves^[12,16]. One drawback of this method is the lower sensitivity when a single or individual TAA is used in diagnosis of HCC. However, this drawback can be overcome by using a panel of carefully selected TAAs to improve the sensitivity and specificity^[17,18]. Therefore, TAA autoantibodies seem to have great potential in early diagnosis of cancer.

Although many studies have been performed to determine the roles of autoantibodies to TAAs in

immunodiagnosis of HCC, no previous study has specifically evaluated the diagnostic value of TAA autoantibodies in AFP-negative HCC. Our previous studies have shown that the level of autoantibodies to nucleophosmin (NPM)1, 14-3-3zeta autoantibody and mouse double minute 2 homolog (MDM2) are all significantly higher in the serum of patients with HCC than other chronic liver diseases (CLDs) and normal human controls (NHCs). They were detected 6-9 mo before clinical diagnosis, which suggested that they may be potential biomarkers for early stage HCC screening and diagnosis^[8,14,19].

In the present study, we evaluated the diagnostic value of autoantibodies to NPM1, 14-3-3zeta and MDM2 and their different combinations in immunodiagnosis of AFP-negative HCC.

MATERIALS AND METHODS

Patient and serum samples

Sera from 56 patients with AFP-negative HCC, 86 with AFP-positive HCC and 168 with CLD, and from 59 NHC samples were obtained from outpatients or inpatients between January 2015 and January 2016 at Beijing You'an Hospital, Capital Medical University. The AFP levels of all patients were measured with a commercially available electrochemiluminescence immunoassay kit (reagents from Roche Ltd, Indianapolis, IN, United States). Samples with AFP < 20 ng/mL were defined as AFP-negative. The diagnosis of AFP-negative and AFP-positive HCC patients was based on ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) characteristics and biochemistry (AFP serology and liver function enzymes), according to the Primary Liver Cancer Treatment Protocols (2011 edition). No HCC patients received any surgical treatment, such as resection, ablation or transarterial chemoembolization, chemotherapy, radiotherapy or multikinase inhibitor (sorafenib). Patients with CLD were followed up for at least 12 mo to exclude individuals with autoimmune liver diseases. This study was approved by the Institutional Review Board of Capital Medical University, Beijing, China. All enrolled patients gave written consent.

Recombinant proteins and antibodies

NPM1 construct GFP-NPM WT (plasmid ID: 17578), 14-3-3zeta construct GST-14-3-3 WT (plasmid ID: 1944) and MDM2 construct pGEX-4T MDM2 WT (plasmid ID: 16237) were purchased from Addgene (Cambridge, MA, United States), and the first two were subcloned into the pET28a vector. The recombinant protein NPM1 expressed in *Escherichia coli* BL21 (DE3) was purified using nickel column chromatography. The recombinant protein 14-3-3zeta expressed in ArcticExpress (DE3) RP was purified using nickel column chromatography. The recombinant

protein MDM2 expressed in ArcticExpress (DE3) RP was purified using SP-sepharose and DEAE Sephacel.

The recombinant proteins were examined in SDS-PAGE and the expected molecular size of expression products were determined using Coomassie blue staining. In addition, western blot analysis was used to confirm that the bands seen in SDS-PAGE were reactive with corresponding antibodies.

Enzyme-linked immunosorbent assay

Three recombinant proteins were respectively diluted in phosphate-buffered saline (PBS) to a final concentration of 0.5 µg/mL for coating a 96-well microtiter plate (No. 3590; Corning, Corning, NY, United States) overnight at 4 °C. The antigen-coated wells were blocked with 10% fetal bovine serum (FBS) at 37 °C for 1 h. Human serum diluted 1:200 was incubated in the antigen-coated wells for 60 min. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Zhongshan Golden Bridge Biological Technology Co Ltd, Beijing, China) as a secondary antibody was diluted 1:10000 for coating (1 h) followed by washing with PBS containing 0.1% Tween 20 (PBST). The 3,3',5,5'-Tetramethylbenzidine Liquid Substrate System (Solarbio Science & Technology Co Ltd, Beijing, China) was used as the detecting agent.

The optical density (OD) value of all wells was read at 450 nm, and the cut-off value for defining a positive reaction was designated as the mean OD value of the 59 normal sera plus three standard deviations (SDs). Each microtiter plate included 10 NHC samples representing a range of absorbance values above and below the mean of 59 NHC samples, and the average OD value of 10 NHC samples was used to normalize all OD values to the standard mean of the 59 NHC samples. Each sample was tested in triplicate.

Western blotting

The purified recombinant proteins of three TAAs were electrophoresed on 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane. After blocking in Tris-buffered saline with 5% nonfat milk and 0.1% Tween-20 for 1 h at ambient temperature, the membranes were cut into strips and incubated with patient sera diluted 1:200, polyclonal anti-NPM1 antibody diluted 1:1000, anti-14-3-3zeta antibody diluted 1:1000 or polyclonal anti-MDM2 antibody diluted 1:1000 separately, and finally incubated with HRP-conjugated goat anti-human IgG or HRP-conjugated goat anti-rabbit IgG diluted 1:10000 for 1 h. Positive signals were detected by the ECL kit (Thermo Scientific, Waltham, MA, United States).

Immunohistochemistry with tissue array slides

The liver cancer tissue array slides with normal tissue controls (9 AFP-negative HCC tissues/10 normal tissues, including pathological diagnosis and clinical

Table 1 Baseline characteristics of patients in α fetoprotein-negative hepatocellular carcinoma, α fetoprotein-positive hepatocellular carcinoma, chronic liver disease and normal human control groups

Variable	AFP(-)HCC (n = 56)	AFP(+)HCC (n = 86)	CLD (n = 168)	NHC (n = 59)
Age, yr	57 \pm 9	56 \pm 10	48 \pm 14	39 \pm 13
Sex, male/female	47/9	66/20	129/39	25/34
AFP, ng/mL	5.38 (3.4-8.2)	795.1(116.1-11244.0)	4.32 (2.5-10.4)	-
HBV/HCV/BC/NBNC	36/4/6/10	75/5/1/5	103/39/3/23	-
ALT, U/L	37.9 (26.2-53.6)	40.5 (26.8-79.6)	42.9 (24.5-135.1)	-
AST, U/L	41.6 (29.8-66)	63.9 (37.05-146.3)	47.1 (27.8-92.6)	-
TBIL, μ mol/L	22.4 (15.9-33.7)	28.4 (17.4-53.7)	23.8 (14.8-49.1)	-
DBIL, μ mol/L	5.9 (3.8-10.6)	7.3 (4.5-20.8)	6 (3.6-18.6)	-
ALB, g/L	34.2 \pm 5.9	34.8 \pm 5.7	38.1(32.4-43)	-
CR, μ mol/L	63 (55.3-73.2)	64 (52.2-74.3)	63.85 (55.1-73.3)	-
INR	1.09 (1-1.21)	1.13 (1.03-1.24)	-	-
PT, s	12.4 (11.4-13.8)	12.7 (11.5-14.1)	-	-
Child-Pugh score	6 (5-8)	7 (6-9)	-	-
Child-Pugh grade, A/B/C	6/1/49	62/19/5	-	-
Meld score	9 (8-11)	10 (8-13)	-	-
BCLC grade, A/B/C/D	24/16/13/3	21/17/42/6	-	-
Tumor size, > 5 cm/< 5 cm	19/37	42/44	-	-
Tumor no., single/double/multiple	4/18/34	31/7/48	-	-
Vascular invasion, yes/no	11/45	44/42	-	-
Metastasis, yes/no	5/51	11/75	-	-
Encephalopathy, non-/1-2/3-4	53/3/0	1/4/81	-	-
Ascites degree, non/low/medium/high	24/25/2/5	31/40/2/13	-	-

Continuous variables are expressed as the mean \pm SD or the medians (25th and 75th percentile). Count data are described as frequency. AFP: Alpha fetoprotein; CLD: Chronic liver disease; HCC: Hepatocellular carcinoma; NHC: Normal human control; NPM1: Nucleophosmin 1; MDM2: Mouse double minute 2 homolog.

information) were purchased (Outdo Biotech Co Ltd, Shanghai, China) and used to detect the expression of the three antigen proteins. Tissue array slides were baked for 1 h and deparaffinized with xylene, and dehydrated with ethanol. Antigen retrieval was performed by microwave heating method in citrate antigen retrieval solution for 20 min. After incubation with acid methanol for 15 min, goat serum blocking solution was used to prevent nonspecific binding of antibodies. The tissue microarrays were incubated with polyclonal NPM1 antibody, polyclonal 14-3-3zeta antibody or polyclonal MDM2 antibody (1:100 dilution) for overnight at 4 °C. The HRP Detection System (HRP streptavidin label and polyvalent biotinylated link) and DAB Substrate Kit (Zhongshan Golden Bridge Biotechnology Co Ltd) were used as detecting reagents. The sections were counterstained with hematoxylin, dehydrated, and mounted. The slides were observed by light microscopy (Model BX51; Olympus, Tokyo, Japan).

Statistical analysis

A χ^2 test with Yates' correction was used to determine whether the frequency of autoantibodies to three TAAs in each cohort of patient sera was significantly higher than that in sera from normal individuals. Two significant levels (0.05 and 0.01) were used. Methods for calculating the sensitivity, specificity and accuracy were based on the methodology provided in *Introduction to Epidemiology* (6th edition, by Ray M. Merrill, published by Jones & Bartlett Learning

Company, Burlington, 2012).

RESULTS

Baseline characteristics of patients in the AFP-negative HCC, AFP-positive HCC, CLD and NHC groups

The baseline characteristics of patients in the HCC, CLD and NHC groups are summarized in Table 1. Most of the patients with AFP-negative HCC at an early stage had good liver function, lower Child-Pugh score and Barcelona Clinic Liver Cancer (BCLC) grade, and were without ascites, hepatic encephalopathy, vascular invasion or metastasis.

Frequency of autoantibodies against NPM1, 14-3-3zeta and MDM2 in patients

Three recombinant proteins were used as coating antigens in enzyme-linked immunosorbent assay (ELISA) to screen for autoantibodies against NPM1, 14-3-3zeta and MDM2 in sera from patients with HCC and CLD as well as NHCs. The prevalence of autoantibodies against NPM1, 14-3-3zeta and MDM2 was 21.4% (12/56), 19.6% (11/56) and 19.6% (11/56) in AFP-negative HCC, which was significantly higher than in CLD and NHCs ($P < 0.01$) and higher than in AFP-positive HCC, although not significantly (Table 2). These results were confirmed by western blot analysis. Representative HCC sera with a positive reaction to NPM1, 14-3-3zeta and MDM2 in ELISA also had strong reactivity in western blotting compared to CLD and normal human sera (Figure 1).

Table 2 Frequency of autoantibodies against NPM1, 14-3-3zeta and MDM2 in human sera by enzyme-linked immunosorbent assay *n* (%)

Type of sera	No. tested	Frequency of autoantibody			
		NPM1	14-3-3zeta	MDM2	Combination of 3 proteins
AFP(-)HCC	56	12 (21.4) ^b	11 (19.6) ^b	11 (19.6) ^{a,b}	17 (30.4) ^b
AFP(+)HCC	86	9 (10.5)	8 (9.3)	14 (16.3)	18 (20.9)
CLD	168	9 (5.4)	11 (6.5)	15 (8.9)	18 (10.7)
NHC	59	1 (1.7)	0 (0)	1 (1.7)	1 (1.7)

Cut-off value, mean \pm 3 SD of NHCs. ^a*P* < 0.05 and ^b*P* < 0.01 (value relative to CLD and NHC). AFP: Alpha fetoprotein; CLD: Chronic liver disease; HCC: Hepatocellular carcinoma; NHC: Normal human control; NPM1: Nucleophosmin 1; MDM2: Mouse double minute 2 homolog.

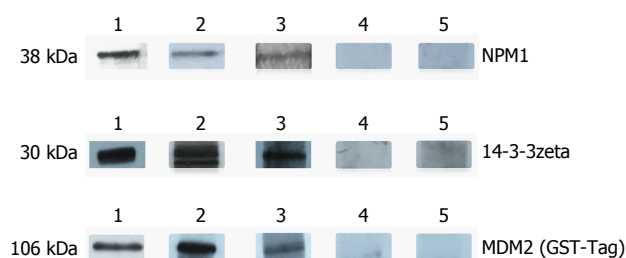


Figure 1 Western blot analysis of representative sera of three anti-tumor-associated antigens autoantibodies assessed by enzyme-linked immunosorbent assay. Lane 1: The polyclonal anti-NPM1 autoantibody and anti-14-3-3zeta autoantibody were used as positive control; Lanes 2 and 3: Two representative AFP-negative HCC serum samples which were positive in ELISA also had strong reactivity to 14-3-3zeta recombinant protein in western blot analysis; Lanes 4 and 5: Randomly selected chronic liver disease sera and normal human control, respectively, with negative reactivity to 14-3-3zeta recombinant protein. AFP: Alpha fetoprotein; ELISA: Enzyme-linked immunosorbent assay; HCC: Hepatocellular carcinoma.

Preferential reactivity of AFP-negative HCC sera with certain antigens

The presence or absence of co-expression of auto-antibodies to any combination of two of the three TAAs in AFP-negative HCC, AFP-positive HCC, CLD and NHCs is shown in Figure 2. The frequency of AFP-negative HCC sera co-expressing antibodies to NPM1 and MDM2 was highest among all the combinations and significantly higher than in AFP-positive HCC, CLD and NHCs. Most of the normal human sera showed a low level of co-expression of antibodies to any combination of two of the three TAAs. Similar results were observed in AFP-positive HCC and CLD.

Diagnostic value of three autoantibodies and different combinations in AFP-negative HCC

The sensitivity and specificity of diagnosis for AFP-negative HCC were 21.4% and 95.6% with NPM1 autoantibody, 19.6% and 95.2% with 14-3-3zeta autoantibody, and 19.6% and 93.0% with MDM2 autoantibody (Table 3). In a further analysis, with combined NPM1 with 14-3-3zeta autoantibodies, the sensitivity and specificity for immunodiagnosis of AFP-negative HCC reached 25% and 94.3%, respectively. When we combined 14-3-3zeta with MDM2 autoantibodies, the sensitivity and specificity reached 25% and 92.1%, respectively. When we

Table 3 Diagnostic value of autoantibodies and different combinations in α -fetoprotein-negative hepatocellular carcinoma

Type of sera	Sensitivity	Specificity	Accuracy
NPM1	21.4%	95.6%	80.9%
14-3-3zeta	19.6%	95.2%	80.2%
MDM2	19.6%	93.0%	78.4%
NPM1 + 14-3-3zeta	25.0%	94.3%	80.7%
NPM1 + MDM2	30.4%	93.0%	80.7%
14-3-3zeta + MDM2	25.0%	92.1%	78.8%
NPM1 + 14-3-3zeta + MDM2	30.4%	91.6%	79.5%

NPM1: Nucleophosmin 1; MDM2: Mouse double minute 2 homolog.

combined NPM1 with MDM2 autoantibodies, the sensitivity and specificity reached 30.4% and 93.0%, respectively. Finally, when we combined the three antigens, the sensitivity was still 30.4% and specificity was maintained at 91.6%. This suggested that the three TAA autoantibodies had higher consistency in the diagnosis of AFP-negative HCC. The accuracy for this TAA array was 79.5%.

Expression of three antigen proteins in AFP-negative HCC tissues and normal hepatic tissues detected by immunohistochemistry

The expression profiles of three proteins in AFP-negative HCC tissues and normal liver tissues was examined by immunohistochemistry of tissue array slides. Tissue array slides were commercially available for this study, and included 9 AFP-negative HCC tissues and 10 normal hepatic tissues. The polyclonal NPM1 antibody, 14-3-3zeta antibody and MDM2 antibody were used as primary antibodies to detect the expression of the three proteins in liver cancer and normal hepatic tissues. The characteristics of patients and protein expression in AFP-negative HCC are shown in Table 4.

All 9 HCC tissues and 2/10 normal hepatic tissues were positively stained in the NPM1 autoantibody group. Five of the 9 HCC tissues and 1/10 of the normal hepatic tissues were positively stained in the 14-3-3zeta autoantibody group, and 3/9 HCC tissues and 2/10 normal hepatic tissues were positively stained in the MDM2 autoantibody group. Due to the small sample size of tissues in this study, it was difficult

Table 4 Characteristics of patients and three proteins expression in α -fetoprotein-negative hepatocellular carcinoma tissues *n* (%)

Variable	Age, yr	Sex, male/female	Grade, I - II / III-IV	Frequency		
				NPM1	14-3-3zeta	MDM2
Liver cancer	52 \pm 9	8/1	6/3	9 (100)	5 (55.6)	3 (33.3)
Normal liver tissue	58 \pm 9	9/1	-	2 (20.0)	1 (10.0)	2 (20.0)

Continuous variables are expressed as mean \pm SD; Count data are described as frequency.

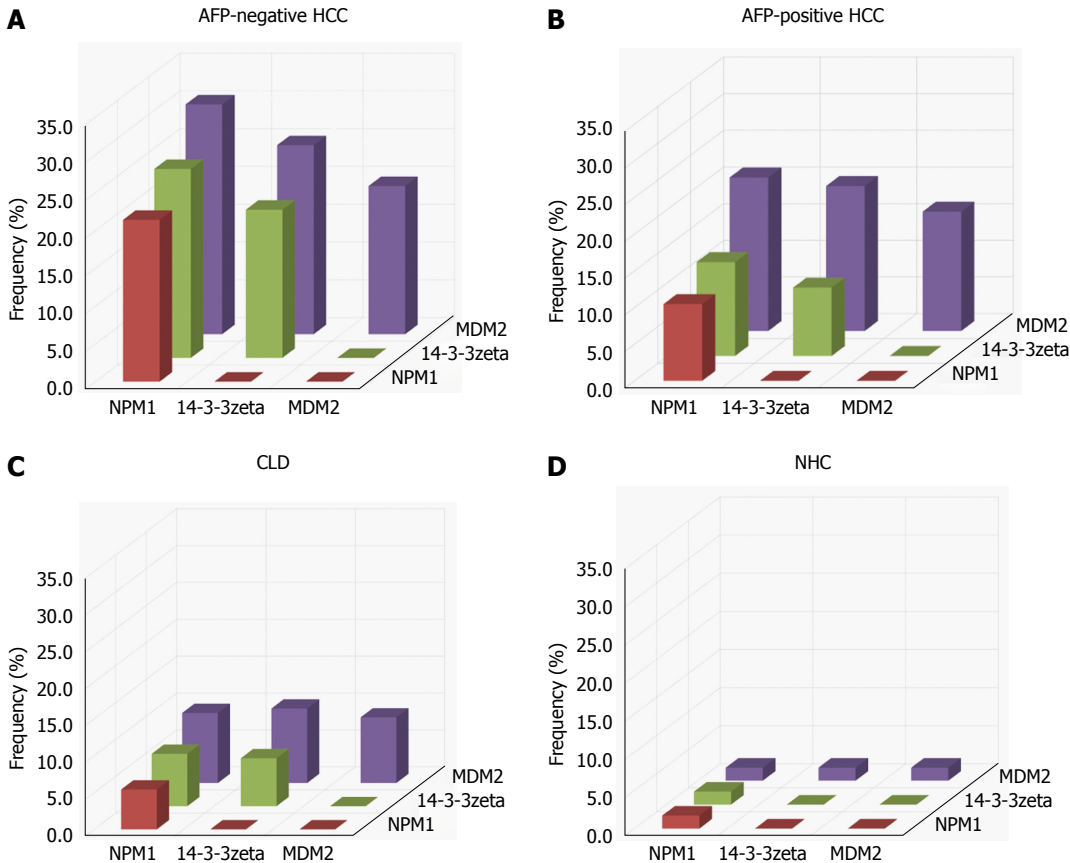


Figure 2 Analysis to determine the presence or absence of co-expression of antibodies to any combination of two of the three tumor-associated antigens in α fetoprotein-negative hepatocellular carcinoma, α fetoprotein-positive hepatocellular carcinoma, chronic liver disease and normal human control. The height of the bar represents the percentage of sera with co-expression of two antibodies, e.g., NPM1 antibody with 14-3-3zeta antibody, and NPM1 antibody with MDM2 antibody. AFP: Alpha fetoprotein; CLD: Chronic liver disease; HCC: Hepatocellular carcinoma; NHC: Normal human control; TAAs: Tumor-associated antigens.

to perform a statistical analysis. The expression of the three proteins in AFP-negative HCC tissues and normal hepatic tissues is shown in Figure 3.

DISCUSSION

In this study, we firstly and specifically evaluated the diagnostic value of three TAA autoantibodies and their different combinations in immunodiagnosis of AFP-negative HCC. The sensitivity of diagnosis for AFP-negative HCC was 19.6%-21.4% for the three TAA autoantibodies, and specificity was approximately 95%. When we combined two of the TAA autoantibodies, the diagnostic sensitivity for AFP-negative HCC was significantly increased. When we combined three of the autoantibodies, the sensitivity reached 30.4%, with a

higher level of specificity.

Furthermore, we examined the expression level of three TAA proteins in AFP-negative HCC tissues. The three proteins were all overexpressed in HCC tissues, and 30%-100% of AFP-negative HCC liver tissues were positively stained with three TAA autoantibodies. Due to the small sample size of AFP-negative HCC tissues in this study, it was difficult to perform a further statistical analysis.

With immunological proteomics technology, a variety of TAAs and TAA autoantibodies have been detected in HCC, such as Imp-1^[20], c-Myc^[21] and CIP2A/p90^[22]. NPM1 (also known as nucleolar phosphoprotein B23 or numatrin) is a member of the nucleoplasmin family, and has multiple functional roles, including in cell proliferation^[23], DNA repair^[24], tumorigenesis^[25] and

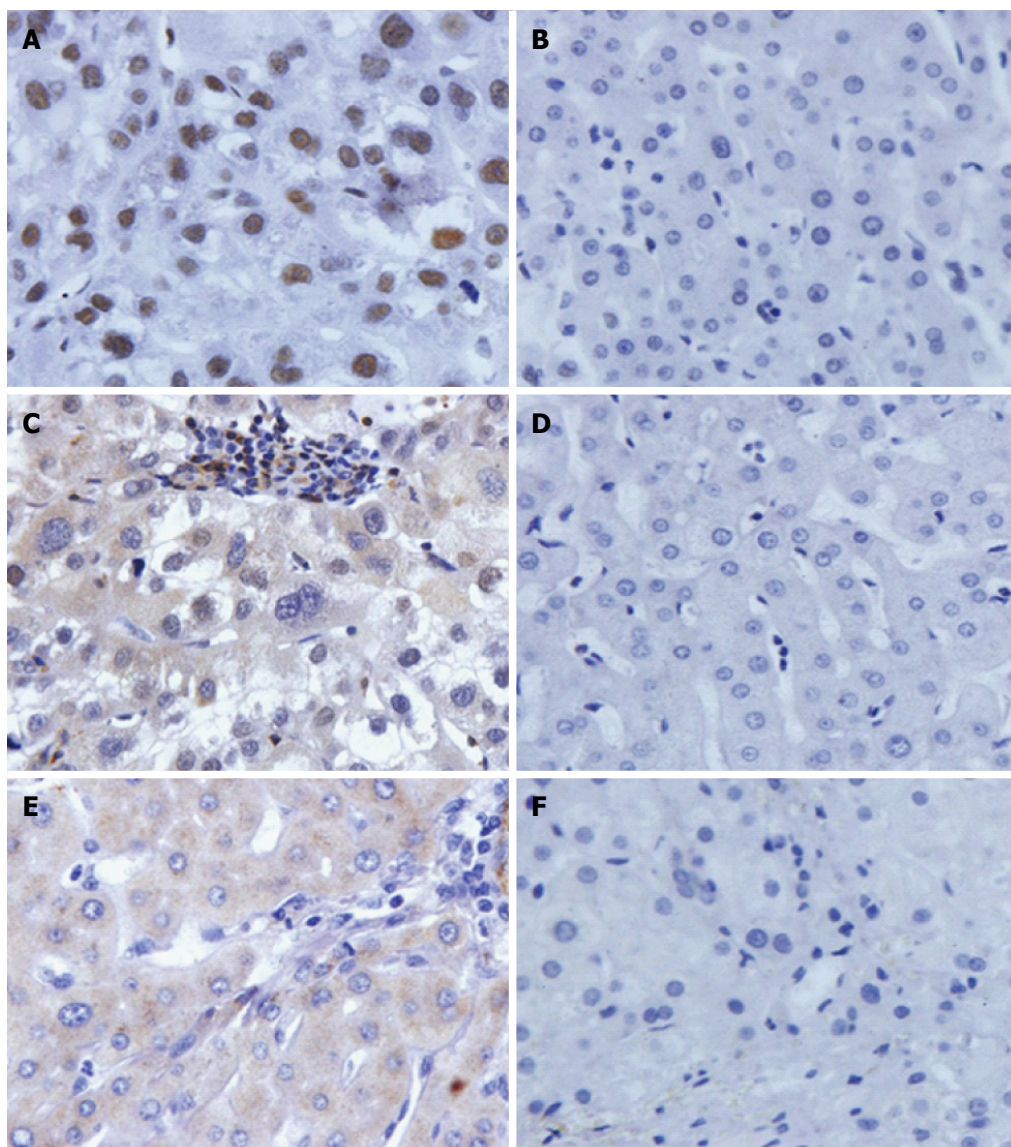


Figure 3 Expression of NPM1, 14-3-3zeta and MDM2 in α -fetoprotein-negative hepatocellular carcinoma tissues and normal hepatic tissues by immunohistochemistry. The three polyclonal anti-TAAs antibodies were used as a primary antibody to detect their expression in liver cancer and normal hepatic tissues. A and B: HCC tissue with positive staining and normal hepatic tissue with negative staining in anti-NPM1 antibody; C and D: HCC tissue with strong positive staining and normal hepatic tissue with negative staining in anti-14-3-3zeta antibody; E and F: HCC tissue with strong positive staining and normal hepatic tissue with negative staining in anti-MDM2 antibody. AFP: Alpha fetoprotein; HCC: Hepatocellular carcinoma; TAAs: Tumor-associated antigens.

apoptosis^[26]. A previous study has demonstrated that NPM1 is expressed highly in liver cancer cells and weakly in normal hepatocytes, which is closely related to tumor grade and poor prognosis; thus, it is possible that NPM1 can be a TAA biomarker for early HCC diagnosis^[27].

The 14-3-3zeta protein is one of the 14-3-3 protein family members, which is a group of highly conserved acidic proteins encoded by different genes and which includes the β , γ , ϵ , ζ (zeta), η , σ , and τ isoforms in mammals^[28]. Studies have shown that the 14-3-3zeta protein is overexpressed in a variety of tumor types, including HCC^[29,30].

The MDM2 oncogene, biochemically known as E3 ubiquitin protein ligase, is deregulated in many human cancers and exerts oncogenic activity predominantly

by binding to p53 and inhibiting p53 transactivation function as well as the p53 tumor suppressor, thus resulting in tumorigenesis^[31]. Our previous studies^[8,14,19] have shown that the levels of NPM1, anti-14-3-3zeta and anti-MDM2 autoantibodies were all significantly higher in the HCC patient sera, with a 16.7%-22.4% positive rate, which was confirmed in the present study. In addition, we specifically evaluated the diagnostic value of three TAA autoantibodies and their different combinations in immunodiagnosis of AFP-negative HCC.

Some researchers have tried to find serological biomarkers for diagnosis of AFP-negative HCC, but only a few have investigated the present antigenic proteins in serum or tissue of AFP-negative HCC patients. Zhang *et al.*^[32] found that the sensitivity of

AFP-L3 and GP73 for diagnosis of AFP-negative HCC was 50.0% and 66.0%, respectively, and combination of AFP-L3 and GP73 improved diagnostic accuracy and sensitivity. Li *et al.*^[33] tested liver tissue glypican (GPC)3 (GPC3L) expression to evaluate the diagnostic value of GPC3 in patients with AFP-negative hepatitis-B-related HCC and 80.0% of HCC samples were positive for GPC3L expression. However, antigen detection is often late and liver biopsy is an invasive procedure. Therefore, TAA autoantibodies have unique diagnostic value due to their early appearance and magnified signals.

However, there were some limitations to our study. First, our sample size was small. In addition, we only chose three autoantibodies to evaluate in AFP-negative HCC, which is not enough to measure sensitivity. Finally, we lacked serum samples with corresponding tissue samples to address the relationship between TAA expression in HCC and serum antibody positivity.

In conclusion, our study demonstrated that autoantibodies to NPM1, 14-3-3zeta and MDM2 may be useful biomarkers for immunodiagnosis of AFP-negative HCC. More potential TAA autoantibodies could be identified and added to the panel of TAAs identified previously, to create an optimized TAA array, which would be useful to increase the sensitivity for diagnosis of AFP-negative HCC. In addition, the mechanism underlying the production of TAA autoantibodies in AFP-negative HCC remains to be investigated in serial serum samples.

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COMMENTS

Background

Many autoantibodies to tumor-associated antigens (TAAs) have been reported in hepatocellular carcinoma (HCC), and have been suggested to be useful tools for immunodiagnosis of HCC. However, no previous study has specifically evaluated the diagnostic value of TAA autoantibodies in α -fetoprotein (AFP)-negative HCC.

Research frontiers

No previous study has specifically evaluated the diagnostic value of TAA autoantibodies in AFP-negative HCC.

Innovations and breakthroughs

This is believed to be the first study to evaluate specifically the diagnostic value of TAA autoantibodies in AFP-negative HCC.

Applications

This study demonstrated that autoantibodies to nucleophosmin 1, 14-3-3zeta and mouse double minute 2 homolog may be useful biomarkers for immunodiagnosis of AFP-negative HCC.

Peer-review

In this study, the authors determined the prevalence and diagnostic value of autoantibodies in AFP-negative HCC. Partially positive sera were further evaluated by western blotting. Immunohistochemistry was used to detect the expression of three TAAs in AFP-negative HCC and normal control tissues. The frequency of autoantibodies to three TAAs in AFP-negative HCC sera was significantly higher than in chronic liver diseases and normal human controls as well as AFP-positive HCC.

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Observational Study

Clinical course of ulcerative colitis patients who develop acute pancreatitis

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Abstract

AIM

To investigate the clinical course of ulcerative colitis (UC) patients who develop acute pancreatitis.

METHODS

We analyzed 3307 UC patients from the inflammatory bowel disease registry at Asan Medical Center from June 1989 to May 2015. The clinical course of UC patients who developed acute pancreatitis was compared with that of non-pancreatitis UC patients.

RESULTS

Among 51 patients who developed acute pancreatitis, 13 (0.40%) had autoimmune, 10 (0.30%) had aminosalicylate-induced, and 13 (1.73%) had thiopurine-induced pancreatitis. All 13 patients with autoimmune pancreatitis (AIP) had type 2 AIP. Two (15.4%) patients had pre-existing AIP, and three (23.1%) patients developed AIP and UC simultaneously. Compared to non-pancreatitis patients, AIP patients had UC diagnosed at a significantly younger age (median, 22.9 years *vs* 36.4 years; $P = 0.001$). AIP and aminosalicylate-induced pancreatitis patients had more extensive UC compared to non-pancreatitis patients. All patients with pancreatitis recovered uneventfully, and there were no recurrences. Biologics were used more frequently in aminosalicylate- and thiopurine-induced pancreatitis patients compared to non-pancreatitis patients [adjusted OR (95%CI), 5.16 (1.42-18.67) and 6.90 (1.83-25.98), respectively]. Biologic utilization rate was similar among AIP and non-pancreatitis patients [OR (95%CI), 0.84 (0.11-6.66)]. Colectomy rates for autoimmune, aminosalicylate-induced, and thiopurine-induced pancreatitis, and for non-pancreatitis patients were 15.4% (2/13), 20% (2/10), 15.4% (2/13), and 7.3% (239/3256), respectively; the rates were not significantly different after adjusting for baseline disease extent.

CONCLUSION

Pancreatitis patients show a non-significant increase in colectomy, after adjusting for baseline disease extent.

Key words: Ulcerative colitis; Pancreatitis; Autoimmune; Colectomy; Clinical course

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Core tip: Clinical course of ulcerative colitis (UC) patients who develop acute pancreatitis is not well known. In a large prospectively maintained inflammatory bowel disease cohort at Asan Medical Center, we found 51 cases of acute pancreatitis among 3,307 UC patients. Among these, there were 13 (0.4%) patients with autoimmune, 10 (0.3%) with aminosalicylate-induced, and 13 (1.73%) with thiopurine-induced pancreatitis, whose colectomy rates were 15.4% (2/13), 20% (2/10), and 15.4% (2/13), respectively. The colectomy rate for non-pancreatitis patients was 7.3% (239/3256), which was not significantly different from those of acute pancreatitis patients, after adjusting for baseline extent.

Kim JW, Hwang SW, Park SH, Song TJ, Kim MH, Lee HS, Ye BD, Yang DH, Kim KJ, Byeon JS, Myung SJ, Yang SK. Clinical course of ulcerative colitis patients who develop acute pancreatitis. *World J Gastroenterol* 2017; 23(19): 3505-3512 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3505.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3505>

INTRODUCTION

The incidence rates of ulcerative colitis (UC) and Crohn's disease (CD) are rising in parallel with rapid urbanization^[1]. Patients with inflammatory bowel disease (IBD) seem to be at risk for developing pancreatitis^[2]. Pancreatitis in IBD has several causes: it can be an extraintestinal manifestation (EIM)^[3-6], or it can be drug-induced^[7-10] or autoimmune-related^[11]. Gallstones and alcohol abuse are also long-established risk factors for acute pancreatitis^[12]. The relationship between IBD and autoimmune pancreatitis (AIP) is mainly confined to UC^[13,14]. Several studies have reported positive associations between risk for acute pancreatitis and IBD severity^[15-17]. To our knowledge, however, no study has evaluated the clinical course of UC patients who develop pancreatitis according to its etiology. Recently, we described the clinical course of UC in a cohort of 2802 Korean patients, and reported a cumulative colectomy rate of 14.2% during a follow-up period of 20 years^[18]. In this study, we aimed to describe the clinical course of UC patients who develop acute pancreatitis in a large prospective cohort, and to compare these patients with the rest of the UC cohort. In particular, AIP and drug-induced pancreatitis were analyzed, and the clinical outcomes were compared according to the etiology.

MATERIALS AND METHODS

Patients

This study enrolled patients with UC who were managed at Asan Medical Center, a tertiary university hospital in Seoul, South Korea, between June 1989 and May 2015. All patients were diagnosed with UC between 1977 and 2015, based on composite criteria of clinical, radiological, endoscopic, and histopathological findings^[19,20].

Diagnosis of acute pancreatitis

AIP was diagnosed using the International Consensus Diagnostic Criteria^[21]. Drug-induced pancreatitis was clinically diagnosed after excluding other potential causes of pancreatitis. In drug-induced pancreatitis cases where causal relationship was uncertain, patients were rechallenged. Severity of acute pancreatitis was classified using the revised Atlanta classification^[22].

Study design

The IBD registry of Asan Medical Center is a well-established prospectively maintained registry, and has been described previously^[18,23-25]. We used the clinical data from this prospectively maintained registry to retrospectively analyze the incidence of acute pancreatitis among patients with UC and the clinical course of UC patients who developed acute pancreatitis. The information obtained from the registry included sex, date of birth, date of symptom

Table 1 Baseline characteristics of ulcerative colitis patients who developed autoimmune pancreatitis and aminosalicylate-induced pancreatitis, each compared with non-pancreatitis ulcerative colitis patients *n* (%)

Variable	Autoimmune pancreatitis (<i>n</i> = 13)	<i>P</i> value vs no pancreatitis	Aminosalicylate- induced (<i>n</i> = 10)	<i>P</i> value vs no pancreatitis	No pancreatitis (<i>n</i> = 3256)
Male gender	9 (69.2)	0.223	8 (80.0)	0.125	1788 (54.9)
Age at diagnosis of UC, years, median (range)	22.9 (14.9-42.8)	0.001	31.7 (15.7-67.3)	0.444	36.4 (9.0-90.5)
Smoking status at diagnosis of UC		0.876		0.876	
Never smoked	8 (61.5)		6 (60)		1870 (57.5)
Ex-smoker	2 (15.4)		3 (30)		706 (21.7)
Current smoker	3 (23.1)		1 (10)		557 (17.1)
Not documented	0		0		121 (3.7)
Disease extent at diagnosis of UC, <i>n</i> (%)		0.012		< 0.001	
Proctitis	0		0		1381 (42.4)
Left-sided	6 (46.2)		0		862 (26.5)
Extensive	6 (46.2)		9 (90)		711 (21.8)
Not documented	1 (7.6)		1 (10)		302 (9.3)
Follow-up duration after UC diagnosis, mo, median (range)	48.3 (3.2-150.9)		97.2 (12.4-187.6)		87.2 (0.2-455.5)
Follow-up duration after pancreatitis diagnosis, mo, median (range)	27.8 (3.2-81.9)		91.6 (10.7-174.8)		

NA: Not applicable; UC: Ulcerative colitis.

onset, date of UC diagnosis, family history of IBD, smoking status, disease activity, disease extent at diagnosis and during the course, medication use, and colectomy. The extent of disease was determined on the basis of endoscopic findings. Proctitis was defined as disease < 15 cm from the anal verge, left-sided colitis as disease up to the splenic flexure, and extensive colitis as disease beyond the splenic flexure^[20]. To investigate the subsequent evolution of the disease, we evaluated the rates of proximal disease extension and of colectomy.

Treatment policy

Our treatment strategies for UC were detailed previously^[18,26] and are based on a step-up approach that is similar to that of Western countries. To briefly summarize, topical and/or oral 5-aminosalicylates were used to induce and maintain remission in mild to moderate UC; systemic corticosteroid therapy was used for moderately to severely active disease; and thiopurines (azathioprine or 6-mercaptopurine) and, in case of failure, anti-tumour necrosis factor (TNF) agents were used for steroid-dependent or steroid-refractory patients.

Pancreatitis was managed conservatively with fasting and antibiotics after discontinuation of the causative drug if present. Patients with AIP were treated with corticosteroids (prednisone 0.6 mg/kg to 1 mg/kg) for 2 to 4 wk, with a taper of 5 mg/d every week. After induction treatment with corticosteroids, immunomodulator agents were given, unless contraindicated.

Statistical analysis

Continuous variables are presented as either mean with SD or median with range. Fisher's exact test was used to compare proportions, and the Mann-Whitney *U* test was used to compare quantitative variables.

Logistic regression with a forward variable selection was used to calculate the adjusted OR and 95%CI for colectomy. In multivariable analysis, the variables with *P* < 0.05 on bivariate analysis were entered into the model. For drug-induced pancreatitis, clinical characteristics and disease course were compared with non-pancreatitis patients who had also been treated with the same drugs. Stata ver. 14.2 (StataCorp, College Station, TX, United States) was used for statistical analyses.

Ethical considerations

This study was approved by the Institutional Review Board of Asan Medical Center (IRB No. 2016-0688).

RESULTS

Patient population

A total of 3307 UC patients [1812 males (54.8%), Table 1] were included for analysis. The median age at diagnosis of UC was 36.3 (range, 9-90) years. At the time of diagnosis of UC, the disease extent was proctitis in 1387 (41.9%), left-sided colitis in 875 (26.5%), extensive colitis in 736 (22.3%), and unknown in 309 (9.3%). Overall, median follow-up time from diagnosis of UC to the last contact was 86.8 (range, 0.2-455.5) mo.

Incidence of acute pancreatitis

Among the 3307 study subjects, 51 (1.5%) developed acute pancreatitis. Among the acute pancreatitis patients, 23 (45.1%) had drug-induced (13 thiopurine-induced and 10 aminosalicylate-induced), 13 (25.5%) had autoimmune, 9 (17.6%) had idiopathic, and 6 (11.8%) had gallstone-induced pancreatitis.

AIP developed in 0.40% of 3307 UC patients. All cases of AIP were type 2 (four definitive, nine

Table 2 Diagnosis of autoimmune pancreatitis

Patient	Imaging	IDCP			ERP			Rt	Definitive/probable
		1H	2H	Negative	1D	2D	Negative		
1	Typical			+	+			+	Probable
2	Typical	+			+			+	Definitive
3	Indeterminate			+	+			+	Probable
4	Typical	+			+			+	Definitive
5	Typical			+	+			+	Probable
6	Typical			+		+		+	Probable
7	Typical	+				+		+	Definitive
8	Typical			+			+	+	Probable
9	Typical			+		+		+	Probable
10	Indeterminate			+	+			+	Probable
11	Typical	+			+			+	Definitive
12	Indeterminate		+			+		+	Probable
13	Indeterminate			+			+	+	Probable

D: Ductal imaging on ERP; ERP: Endoscopic retrograde pancreatography; H: Histology of the pancreas; IDCP: Idiopathic duct centric pancreatitis; Rt: Response to steroid, *i.e.*, rapid (< 2 wk) radiologically demonstrable resolution or marked improvement in manifestations.

Table 3 Comparison of baseline characteristics in ulcerative colitis patients who developed thiopurine-induced pancreatitis and non-pancreatitis ulcerative colitis patients *n* (%)

Variable	Thiopurine-induced pancreatitis (<i>n</i> = 13)	No pancreatitis (<i>n</i> = 704) ¹	<i>P</i> value
Male gender	4 (30.8)	434 (61.7)	0.040
Age at diagnosis of UC, yr, median (range)	37.9 (12.1-57.3)	34.8 (11.4-75.9)	0.667
Smoking status at diagnosis of UC			0.576
Never smoked	10 (76.9)	405 (57.6)	
Ex-smoker	2 (15.4)	168 (23.9)	
Current smoker	1 (7.7)	125 (17.8)	
Not documented	0	5 (0.7)	
Disease extent at diagnosis of UC			0.837
Proctitis	2 (15.4)	167 (23.7)	
Left-sided	3 (23.1)	167 (23.7)	
Extensive	6 (46.1)	232 (33.0)	
Not documented	2 (15.4)	138 (19.6)	
Follow-up duration after UC diagnosis, mo, median (range)	42.9 (10.7-169.2)	91.5 (0.3-356.3)	0.008
Follow-up duration after pancreatitis diagnosis, mo, median (range)	21.0 (0.3-89)		

¹Non-pancreatitis UC patients who had taken thiopurines. NA: Not applicable; UC: Ulcerative colitis.

probable, Table 2). Two (15.4%) patients had preexisting AIP, for 16 and 30 mo prior to the diagnosis of UC, respectively. Three (23.1%) patients developed both AIP and UC simultaneously. Among the eight AIP patients who had preexisting UC, median time to development of acute pancreatitis was 1046 (range, 294-2217) d after diagnosis of UC.

Aminosalicylate-induced pancreatitis developed in 0.30% of 3307 UC patients who were treated with aminosalicylates. The median interval from the start of aminosalicylate to the development of pancreatitis was 50 (range, 0-549) d. All cases of aminosalicylate-

induced pancreatitis occurred after taking oral forms.

Thiopurine-induced pancreatitis developed in 1.75% of 742 UC patients who were treated with thiopurines. The median interval from the commencement of thiopurines to the development of pancreatitis was 18 (range, 0-131) d.

There was one patient with AIP whose severity was classified as moderate; all other patients had mild acute pancreatitis.

Demographics and clinical characteristics of UC patients with pancreatitis

Baseline demographic and clinical characteristics of UC patients with and without pancreatitis are shown in Tables 1 and 3. Median age at diagnosis of UC was significantly younger among AIP patients compared to those without pancreatitis. Patients with autoimmune and aminosalicylate-induced pancreatitis had more extensive UC compared to those without pancreatitis.

Clinical course

All 13 patients with AIP showed a good response to corticosteroids, and there were no cases of recurrence during the median follow-up of 27.8 mo (range, 3.2-81.9) following diagnosis of AIP. All 23 patients with drug-induced pancreatitis recovered uneventfully after cessation of the causative agent and with conservative care. Six of ten patients with aminosalicylate-induced pancreatitis underwent aminosalicylate re-challenge, and all cases developed repeat episodes. After permanent cessation of aminosalicylate, all 10 patients showed no further recurrence of pancreatitis during the median follow-up of 91.6 (range, 10.7-174.8) mo. One of 13 patients with thiopurine-induced pancreatitis underwent thiopurine rechallenge and showed a positive response. There was no recurrence of acute pancreatitis during the median follow-up of 21.0 (range, 0.3-89) mo.

Among 13 patients with AIP, medical therapy for

Table 4 Odds ratios for anti-tumor necrosis factor use during follow-up according to cause of acute pancreatitis, adjusted for baseline disease extent of ulcerative colitis

Cause of pancreatitis	<i>n</i>	OR (95%CI)	<i>P</i> value
No pancreatitis (reference)	3256	1.00	
Autoimmune	11	0.84 (0.11-6.66)	0.873
No pancreatitis (reference) ¹	3256	1.00	
Aminosalicylate-induced	10	5.16 (1.42-18.67)	0.012
No pancreatitis (reference) ¹	704	1.00	
Thiopurine-induced	12	6.90 (1.83-25.98)	0.004

¹Non-pancreatitis patients (reference) who were treated with either aminosalicylates or thiopurines. Patients who had been on anti-TNF before diagnosis of pancreatitis were excluded (*n* = 3). UC: Ulcerative colitis.

UC included aminosalicylates in two, thiopurines in nine, and anti-TNF agents in three. Among 10 patients with aminosalicylate-induced pancreatitis, medical therapy for UC included thiopurines in eight, and anti-TNF agents in four. Among 13 patients with thiopurine-induced pancreatitis, medical therapy for UC included aminosalicylates in three and anti-TNF agents in ten.

Among anti-TNF agent-naïve patients, subsequent use of anti-TNF agents was observed in 9.1% (1/11) of autoimmune, 40% (4/10) of aminosalicylate-induced, and 75% (9/12) of thiopurine-induced pancreatitis cases. Among non-pancreatitis patients, anti-TNF agents were used in 8.26% (269/3256). The rate of anti-TNF agent use was significantly higher among aminosalicylate-induced and thiopurine-induced pancreatitis patients, after adjusting for baseline disease extent [adjusted OR (95%CI), 5.16 (1.42-18.67) and 6.90 (1.83-25.98), respectively] (Table 4).

Colectomy rates for autoimmune, aminosalicylate-induced, and thiopurine-induced pancreatitis patients, as well as for non-pancreatitis patients, were 15.4% (2/13), 20% (2/10), 15.4% (2/13), and 7.3% (239/3256), respectively. Compared to those without pancreatitis, patients with pancreatitis did not show a significant increase in colectomy rates during follow-up, after adjusting for baseline disease extent (Table 5).

DISCUSSION

In this study, we analyzed the frequency and clinical course of acute pancreatitis among UC patients in a large, well-established prospective cohort. To the best of our knowledge, this is the largest single study to date to describe the frequency and clinical course of AIP.

The most common causes of acute pancreatitis in IBD patients are reported to be gallstones and drugs^[27]. Thiopurines are the drugs most frequently implicated as a cause of acute pancreatitis in IBD patients, with a reported incidence of 3%-4%^[28,29]. In a prospective study among IBD patients, azathioprine-induced acute pancreatitis occurred in 37 of 510

Table 5 Odds ratios for colectomy according to cause of acute pancreatitis, adjusted for baseline disease extent of ulcerative colitis

Cause of pancreatitis	<i>n</i>	OR (95%CI)	<i>P</i> value
No pancreatitis (reference)	3256	1.00	
Autoimmune	13	1.65 (0.35-7.66)	0.525
No pancreatitis (reference) ¹	3256	1.00	
Aminosalicylate-induced	10	1.76 (0.67-8.41)	0.480
No pancreatitis (reference) ¹	704	1.00	
Thiopurine-induced	13	1.31 (0.32-6.60)	0.651

¹Non-pancreatitis patients (reference) who were treated with either aminosalicylates or thiopurines. Among 3307 UC patients, those with biliary pancreatitis (*n* = 6) and idiopathic pancreatitis (*n* = 9) were excluded from the analysis, and the results from the remaining 3292 are shown. UC: Ulcerative colitis.

patients (7.3%)^[30]. In our study, a higher rate of acute pancreatitis was observed in thiopurine-treated patients (1.75%), compared with the rates of autoimmune (0.40%) and aminosalicylate-induced cases (0.30%).

UC patients are reported to be at an increased risk of developing acute pancreatitis compared to the general population^[31]. According to a study performed in 2003, the annual incidence of acute pancreatitis in South Korea was 19.4 per 100000 persons^[32]. In our patients, the annual incidence of acute pancreatitis was 152.9 (95%CI: 113.4-206.1) per 100000 persons (data not shown). The incidence was higher among our patients, and further analysis using data from the general population is required to draw firm conclusions.

Ueki *et al.*^[11] reported that five (0.5%) of 961 Japanese patients with UC developed AIP during a mean follow-up period of 86 mo. This figure is comparable with the 0.4% in our study. Although AIP is uncommon among IBD patients, it is interesting to note that the reported prevalence of IBD in patients with AIP is 6% to 27%, predominantly UC^[27,33-35]. AIP is subclassified into two separate entities: type 1 AIP, or lymphoplasmacytic sclerosing pancreatitis (LPSP), and type 2 AIP, or idiopathic duct centric pancreatitis (IDCP)^[21,36]. Type 2 AIP is most commonly associated with IBD, with a reported frequency of 16% to 30%^[37,38]. All of our patients with AIP had type 2 AIP. It is interesting that two cases of AIP occurred before the diagnosis of UC. There have been several reports of AIP occurring before the diagnosis of CD^[31,32,39,40], but to the best of our knowledge, there have been no reports of AIP that preceded UC. Our results suggest that patients with repeated episodes of unexplained acute pancreatitis should be evaluated for inflammatory bowel disease.

Among the drug-induced pancreatitis patients, some cases were diagnosed only after a prolonged period since starting the drug. It is possible that objective diagnosis of pancreatitis was delayed, since symptoms of pancreatitis and UC, such as abdominal pain, can overlap. It is also possible that the patient

could have skipped the drug after experiencing the side effect, without notifying the attending physician. In previous studies, the median duration of azathioprine therapy before diagnosis of pancreatitis was 26 (range, 6-720) d^[41] and 25 (range, 5-30) d^[29], which was similar to that in our study [median, 18 d (range, 0-131)]. A case of pancreatitis after 18 mo of mesalamine treatment has also been reported^[42].

Patients with AIP were younger and had more extensive disease than those without acute pancreatitis. Most patients with aminosalicylate-induced pancreatitis had extensive disease, but the reason is not clear. There were significantly more females among thiopurine-induced pancreatitis patients compared to non-pancreatitis patients in our study (69.2% vs 38.3%; $P = 0.040$). In a prospective study of 37 patients with azathioprine-induced acute pancreatitis, 24 (64.9%) were female ($P = 0.06$)^[30]. A study on CD patients^[29] reported that females over 40 years of age had an increased risk for developing thiopurine-related adverse events, but the reasons for increased thiopurine-induced pancreatitis among females is not clear. The incidence of thiopurine-induced pancreatitis is reported to be 3%-4% among IBD patients^[27-29]. Among our patients, thiopurine-induced pancreatitis developed less frequently (1.75% of thiopurine users). The reason for this is not clear, but it might represent a distinct characteristic of our cohort.

In our study, there was no case of relapse of pancreatitis among AIP patients, once treated. All our AIP patients had type 2 AIP, and previous studies reported that relapse of pancreatitis is rare in type 2 AIP compared to type 1 AIP^[43,44]. In a large multicenter study involving 978 subjects with type 1 AIP and 86 with type 2 AIP, the relapse rate was 31% with type 1 and 9% with type 2 ($P < 0.001$)^[44].

Considering the usual step-up approach for treating UC, the higher rate of anti-TNF agent use among thiopurine-induced pancreatitis cases is expected. Aminosalicylate-induced pancreatitis patients also showed a higher rate of anti-TNF agent use, presumably because a high proportion had extensive disease (90%). Although the rate of anti-TNF use was high, the colectomy rates were not significantly different in acute pancreatitis patients compared to non-pancreatitis patients. The colectomy rate in our cohort was 7.3%, which is comparable to those of previous studies^[45-49].

AIP is a new diagnostic entity, only established in 2011^[21]. In addition, the tendency among Korean physicians to prescribe thiopurine only showed a rising trend in recent years^[18]. For these reasons, durations of follow-up for autoimmune and thiopurine-induced pancreatitis patients were shorter than those for non-pancreatitis patients. Therefore, directly comparing the colectomy rate with non-pancreatitis patients might have led to false-negative results. As physicians are becoming more aware of AIP, and as thiopurines are being used more frequently^[18], future studies using longer analysis times seem to be required.

This study has several limitations. First, since this study was conducted at a single tertiary referral center, the conclusions could have been biased. Second, we could not analyze the severity of pancreatitis in detail, due to limitations in clinically available data. Third, as mentioned above, the follow-up time of autoimmune and thiopurine-induced pancreatitis patients was relatively short, which could have led to false negative results regarding subsequent colectomy rates. Fourth, the follow-up interval was variable among patients, which could have led to the apparently low rate of thiopurine-induced pancreatitis.

In conclusion, we described the frequency and clinical course in UC patients who developed acute pancreatitis in a large, prospectively maintained cohort. Compared with non-pancreatitis UC cases, the baseline disease extent in patients with autoimmune and aminosalicylate-induced pancreatitis was greater, and age at diagnosis of UC with AIP was younger. Anti-TNF agents were used more frequently in UC patients who had developed aminosalicylate- or thiopurine-induced pancreatitis. Despite these differences, the clinical course of UC patients who developed acute pancreatitis was not significantly different. Further studies with longer follow-up are required.

COMMENTS

Background

Patients with ulcerative colitis (UC) seem to be at risk for developing acute pancreatitis, which can be an extraintestinal manifestation of the UC, drug-induced, or autoimmune-related. The clinical course of UC in patients who develop acute pancreatitis is not well known.

Research frontiers

Acute pancreatitis can cause a significant impact on the course of UC by requiring change in treatment or by acting as a prognostic factor itself. This study investigated the clinical course of UC in patients who developed acute pancreatitis in a large, well-established, prospectively maintained cohort. Particular focus was given to the clinical course of patients who developed autoimmune, aminosalicylate-induced and thiopurine-induced pancreatitis.

Innovations and breakthroughs

The results showed that UC patients who developed acute pancreatitis had a non-significantly higher colectomy rate compared with those without pancreatitis, after adjusting for disease extent of UC at baseline. The acute pancreatitis in most patients was mild. All patients with autoimmune pancreatitis (AIP) had type 2 and there was no recurrence of the pancreatitis.

Applications

The data in this article can be used to predict the clinical course of patients with UC who develop acute pancreatitis, which seems to be mild in most cases. There was no recurrence of AIP. Patients with aminosalicylate-induced and thiopurine-induced pancreatitis had higher rates of treatment with a biologic.

Terminology

AIP is a peculiar type of pancreatitis of presumed autoimmune etiology, subclassified as type 1, lymphoplasmacytic sclerosing pancreatitis, and type 2, idiopathic duct centric pancreatitis.

Peer-review

This is an interesting manuscript which describes the clinical course of patients

with UC who develop acute pancreatitis. The manuscript is well-structured, the methodology and the sample size seem appropriate and the topic is relevant for the field of inflammatory bowel disease epidemiology.

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Observational Study

Relationship between use of selective serotonin reuptake inhibitors and irritable bowel syndrome: A population-based cohort study

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Informed consent statement: The Institutional Review Board specifically waived the consent requirement, because the presented data are anonymized in Taiwan's National Health Insurance Research Database, with no risk of identification.

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Abstract

AIM

To investigate the relationship between selective serotonin reuptake inhibitor (SSRI) use and the subsequent development of irritable bowel syndrome (IBS).

METHODS

This retrospective, observational, population-based cohort study collected data from Taiwan's National Health Insurance Research Database. A total of 19653 patients newly using SSRIs and 78612 patients not using SSRIs, matched by age and sex at a ratio of 1:4,

were enrolled in the study from January 1, 2000 to December 31, 2010. The patients were followed until IBS diagnosis, withdrawal from the National Health Insurance system, or the end of 2011. We analyzed the effects of SSRIs on the risk of subsequent IBS using Cox proportional hazards regression models.

RESULTS

A total of 236 patients in the SSRI cohort (incidence, 2.17/1000 person-years) and 478 patients in the comparison cohort (incidence, 1.04/1000 person-years) received a new diagnosis of IBS. The mean follow-up period from SSRI exposure to IBS diagnosis was 2.05 years. The incidence of IBS increased with advancing age. Patients with anxiety disorders had a significantly increased adjusted hazard ratio (aHR) of IBS (aHR = 1.33, 95%CI: 1.11-1.59, $P = 0.002$). After adjusting for sex, age, urbanization, family income, area of residence, occupation, the use of anti-psychotics and other comorbidities, the overall aHR in the SSRI cohort compared with that in the comparison cohort was 1.74 (95%CI: 1.44-2.10; $P < 0.001$). The cumulative incidence of IBS was higher in the SSRI cohort than in the non-SSRI cohort (log-rank test, $P < 0.001$).

CONCLUSION

SSRI users show an increased risk of subsequent diagnosis of IBS in Taiwan.

Key words: Brain-gut axis; Irritable bowel syndrome; Selective serotonin reuptake inhibitor

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Core tip: Selective serotonin reuptake inhibitor (SSRI) users were associated with a risk of subsequently diagnosed irritable bowel syndrome. The brain-gut axis may play a key role in this relationship. In clinical practice, physicians should pay attention to the gastrointestinal symptoms of patients with psychiatric disorders and SSRI use.

Lin WZ, Liao YJ, Peng YC, Chang CH, Lin CH, Yeh HZ, Chang CS. Relationship between use of selective serotonin reuptake inhibitors and irritable bowel syndrome: A population-based cohort study. *World J Gastroenterol* 2017; 23(19): 3513-3521 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3513.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3513>

INTRODUCTION

Antidepressants are among the most widely used medications in clinical practice for the treatment of depressive disorder, panic disorder, generalized anxiety

disorder, and numerous other psychiatric diseases^[1,2]. Among the many classes of antidepressants, selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed because of their effectiveness in treating many psychiatric disorders. SSRIs are safer and have a more favorable side-effect profile than the previous generations of antidepressants^[3]. However, numerous studies have indicated that short-term use of SSRIs can cause many adverse side effects, including nausea, diarrhea and unstable mood swings. Additionally, suicide attempts, gastrointestinal bleeding, sexual dysfunction, and hyponatremia may occur during long-term use^[4-6].

Irritable bowel syndrome (IBS) is part of a large group of functional gastrointestinal disorders that are characterized by recurrent abdominal discomfort or pain and disturbed defecation in the absence of organic disease^[7,8]. IBS is one of the most commonly treated diseases by primary care physicians as well as gastroenterologists. The prevalence of IBS is estimated to be 7.5%-21% worldwide^[9,10]. IBS is a functional disorder and has no contribution towards mortality^[11]; however, it is chronic and significantly reduces patients' quality of life^[9,12,13]. The suggested treatments for IBS include antispasmodics, antidiarrheal agents, laxatives, prokinetics, probiotics, anxiolytics, SSRIs, tricyclic antidepressants (TCAs), 5-HT₃ antagonists, cGMP agonists, and antibiotics according to each patient's clinical symptoms^[14,15]. However, there is no universally accepted or recommended therapy that effectively cures this disease.

The pathophysiology of IBS is believed to be associated with abnormal gastrointestinal motility, visceral hypersensitivity, low-grade inflammation, stress and brain-gut interactions^[9,16]. Additionally, a high prevalence of psychiatric disorders in patients with IBS, in particular anxiety and depressive disorders, has been reported in previous studies^[16,17]. Antidepressants are often used to treat a variety of functional bowel disorders. Tricyclic antidepressants have been proven to offer statistically significant control of IBS symptoms in a previous meta-analysis^[18,19]. Additionally, several randomized controlled trials have evaluated the safety and efficacy of fluoxetine, citalopram and paroxetine for the treatment of IBS^[20-23]. However, the evidence regarding the effectiveness of SSRIs in providing symptom relief in IBS is inconsistent. The American Gastroenterological Association Institute guidelines advise against using SSRIs for patients with IBS, based on the lack of improvement in global relief of symptoms identified in pooled estimates of five randomized control trials^[24].

The present study aimed to explore the relationship of SSRIs with the subsequent development of IBS using the Taiwan National Health Insurance Research Database (NHIRD).

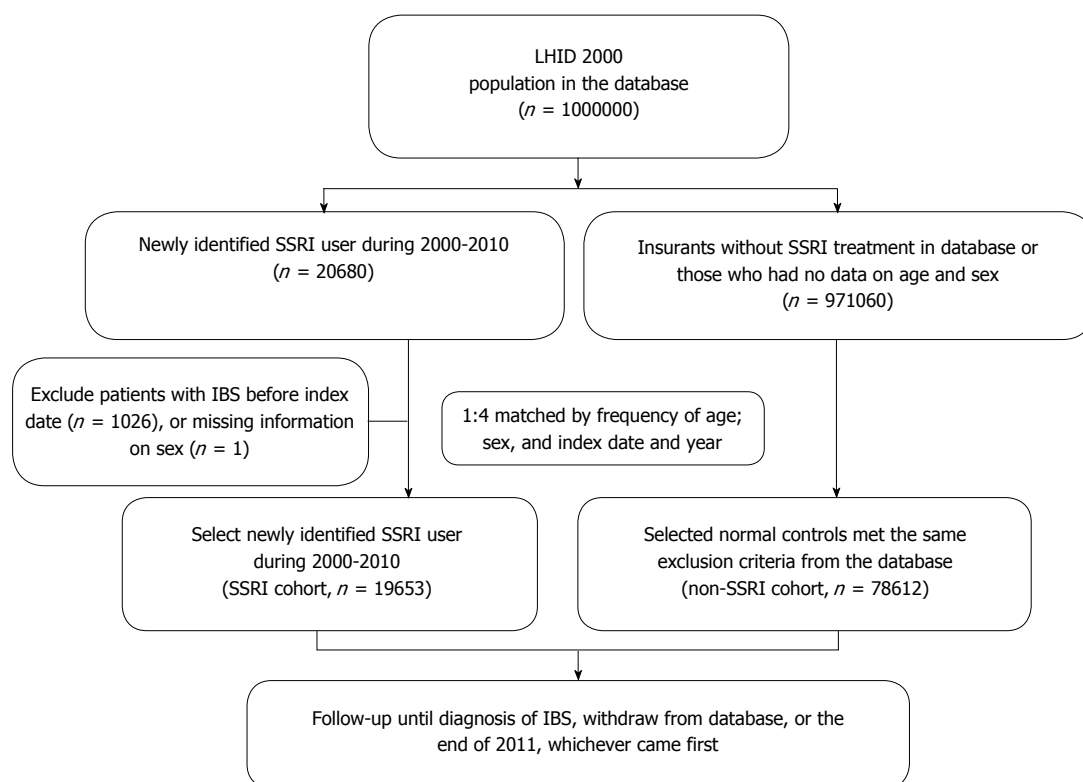


Figure 1 Flowchart of study design. IBS: Irritable bowel syndrome; LHID: Longitudinal Health Insurance Database; SSRI: Selective serotonin reuptake inhibitor.

MATERIALS AND METHODS

Data source

The database used in our study was the NHIRD of Taiwan. The National Health Insurance (NHI) program in Taiwan was initiated in 1995 and enrolled over 24 million people by the end of 2014, representing 99% of the population in Taiwan. In the present study, data from the Longitudinal Health Insurance Database (LHID) 2000 were analyzed. The LHID 2000 is a subset of the NHIRD that contains data from 1,000,000 randomly sampled patients, which is approximately 5% of the Taiwan's general population. We conducted a retrospective observational study on the correlation of SSRIs with and their possible influence on IBS. This study was approved by the Institutional Review Board (IRB) of Taichung Veterans General Hospital (IRB number: CE13152B-3), and because the data were obtained from the LHID 2000, informed consent from the participants was not obtained. The IRB specifically waived the requirement for consent.

Study population

We extracted data from the LHID 2000 for this retrospective study. Patients with a more than two-month medical prescription for an SSRI during one year between January 1, 2000 and December 31, 2010 were selected. The prescribed SSRIs included fluoxetine, citalopram, paroxetine, sertraline, fluvoxamine and escitalopram. We excluded any patients who were

diagnosed with IBS (ICD-9-CM code: 564.1) prior to medical treatment with SSRIs. The comparison cohort included patients without any medical history of SSRI use who were frequency-matched with the SSRI cohort by age, sex, index date and year at a ratio of 1:4 (Figure 1). To ensure the validity of the diagnosis, we included only patients who were diagnosed with IBS (ICD-9-CM code: 564.1) in more than three outpatient visits or more than one inpatient hospitalization. The included patients taking SSRIs were regularly followed at the database for at least three outpatient visits. Thus, we supposed that these patients needed these medication, and had compliance in medication.

In addition, patients with inflammatory bowel disease (ICD-9:555, 556) were excluded because some of these patients exhibit symptoms similar to those of IBS during the inactive or remission stage of inflammatory bowel syndrome^[25-29]. Additionally, patients with a diagnosis of infectious enterocolitis (ICD 9: 0078,0079, 0080-0088, 0090-0093, 558, 0030, 0062, 11285) within three months prior to the diagnosis of IBS were excluded due to the potentially increased risk of post-infectious IBS associated with bacterial, protozoan, helminth, or viral infections, all of which have been reported^[30-34]. The main outcome was the incidence of newly diagnosed IBS during the follow-up period, which was estimated as the duration from the index date until IBS, withdrawal from the insurance system, or the end of study in 2011.

Furthermore, common comorbidities diagnosed

Table 1 Baseline characteristics of the selective serotonin reuptake inhibitor cohort and the non-selective serotonin reuptake inhibitor cohort, 2000-2010 *n* (%)

Variable	SSRI use		<i>P</i> value
	No (<i>n</i> = 78612)	Yes (<i>n</i> = 19653)	
Sex			1.000
Female	45984 (58.5)	11496 (58.5)	
Male	32628 (41.5)	8157 (41.5)	
Age, yr			1.000
< 20	5548 (7.1)	1387 (7.1)	
20-29	11592 (14.8)	2898 (14.8)	
30-39	14312 (18.2)	3578 (18.2)	
40-49	15112 (19.2)	3778 (19.2)	
50-59	11720 (14.9)	2930 (14.9)	
≥ 60	20328 (25.9)	5082 (25.9)	
Urbanization ¹			< 0.001
1	24102 (31.3)	6275 (32.7)	
2	23134 (30.0)	5851 (30.5)	
3	12486 (16.2)	2827 (14.7)	
4	17325 (22.5)	4228 (22.0)	
Family income (NTD)			< 0.001
0	13932 (17.7)	3411 (17.4)	
1-15840	14871 (18.9)	4807 (24.5)	
15841-28800	33687 (42.9)	7915 (40.3)	
28801-45800	10243 (13.0)	2173 (11.1)	
≥ 45801	5874 (7.5)	1346 (6.9)	
Area			< 0.001
North	39708 (50.6)	10001 (51.0)	
Midland	14067 (17.9)	3265 (16.7)	
South	22823 (29.1)	5642 (28.8)	
East	1837 (2.3)	690 (3.5)	
Occupation			< 0.001
Public and military	5735 (8.2)	1531 (8.8)	
Industry	23168 (33.3)	5617 (32.3)	
Business	28266 (40.6)	5841 (33.6)	
Low income	559 (0.8)	385 (2.2)	
Other and retired	11898 (17.1)	3994 (23.0)	
Anti-psychotics			< 0.001
No	75615 (96.2)	16022 (81.5)	
Yes	2997 (3.8)	3631 (18.5)	
Comorbidity			
Diabetes mellitus	8126 (10.3)	3143 (16.0)	< 0.001
Hypertension	18319 (23.3)	6726 (34.2)	< 0.001
Hyperlipidemia	11823 (15.0)	4521 (23.0)	< 0.001
Colorectal cancer	290 (0.4)	96 (0.5)	0.017
Major depressive disorder	770 (0.98)	4274 (21.8)	< 0.001
Anxiety disorder	10007 (12.7)	9477 (48.2)	< 0.001
Bipolar disorder	130 (0.2)	273 (1.4)	< 0.001
Posttraumatic stress disorder	7 (0.0)	101 (0.5)	< 0.001
Eating disorder	26 (0.0)	141 (0.7)	< 0.001
Mean follow-up time (yr)	5.86 ± 3.00	5.53 ± 3.21	

¹Urbanization level: 1 indicates the highest level of urbanization and 4 the lowest. SSRI: Selective serotonin reuptake inhibitor; NTD: New Taiwan dollars.

before enrollment in this study, including hypertension, diabetes mellitus, dyslipidemia, colorectal cancer, major depressive disorder, anxiety disorder, bipolar disorder, and posttraumatic disorder, were compared between the SSRI and comparison cohorts.

Statistical analysis

All analyses were performed using SAS software, version 9.3 (SAS Institute, Cary, NC, United States).

The distributions of SSRI exposure based on subject's age, gender and clinical comorbidities were examined using χ^2 tests for categorical variables.

In the cohort study, multivariable Cox proportional hazard models were used to explore the relationship between exposure to SSRI and the diagnosis of IBS, after adjusting for age, gender and medical comorbidities. All statistical tests were two-sided, conducted at a significance level of 0.05, and reported using *P* values and/or 95% CIs.

RESULTS

Demographic characteristics of study subjects

The eligible study participants included 19653 patients in the SSRI cohort and 78612 persons in the comparison cohort, with a similar age and sex distribution (Figure 1 and Table 1). Males represented 41.5% and females 58.5% of the entire study population. The mean follow-up time in the present study was 5.9 ± 3.0 years in the non-SSRI cohort and 5.5 ± 3.2 years in the SSRI cohort. The mean follow-up period of SSRI exposure to IBS diagnosis was 2.05 years. The majority of psychiatric disorders leading to a prescription of SSRI included anxiety (48.2%) and major depressive disorders (21.8%). There was more concomitant anti-psychotic usage in the SSRI cohort than in the non-SSRI group. However, most participants in both groups did not use anti-psychotics. At the baseline, comorbid diabetes, hypertension, hyperlipidemia, colorectal cancer, major depressive disorder and anxiety disorder were more prevalent in the SSRI cohort than in the comparison cohort.

Risk of IBS in SSRI users

A total of 236 patients in the SSRI cohort (incidence, 2.17/1000 person-years) and 478 patients in the comparison cohort (incidence, 1.04/1000 person-years) had a new diagnosis of IBS during the follow-up period (Table 2). The incidence of IBS increased with advancing age. Comorbidities such as diabetes mellitus, hypertension, hyperlipidemia, colorectal cancer, and major depressive disorder did not influence the HR of IBS. However, patients with anxiety disorders had a significantly increased HR of IBS (HR = 1.33, 95%CI: 1.11-1.59, *P* = 0.002). The use of anti-psychotics did not affect the incidence of IBS, whereas the use of SSRIs was associated with an increased HR of IBS.

Incident rate and HR of IBS associated with SSRI use in Cox regression analyses

After adjusting for sex, age and other comorbidities including diabetes, hypertension, hyperlipidemia and colorectal cancer, the overall adjusted HR (aHR) in the SSRI cohort compared with the comparison cohort was 1.74 (95%CI: 1.44-2.10; *P* < 0.001) using Cox regression analysis. The subgroup analysis showed that the aHR was higher in SSRI users than in non-SSRI

Table 2 Comparisons of the incidence of irritable bowel syndrome by age, gender, comorbidity and drug use

Variable	Events	PT	Rate ¹	HR ²	(95%CI)	P value
Sex						
Female	414	335363	1.23	1.00	(reference)	
Male	300	233656	1.28	1.05	(0.91-1.23)	0.495
Age, yr						
< 20	13	43139	0.30	1.00	(reference)	
20-29	47	91408	0.51	1.62	(0.87-3.01)	0.128
30-39	93	110721	0.84	2.52	(1.40-4.55)	0.002
40-49	153	113198	1.35	3.94	(2.22-7.02)	< 0.001
50-59	133	80752	1.65	4.56	(2.54-8.17)	< 0.001
≥ 60	275	129801	2.12	5.57	(3.12-9.92)	< 0.001
Comorbidity (yes vs no)						
Diabetes mellitus	113	55858	2.02	0.93	(0.74-1.17)	0.549
Hypertension	269	129073	2.08	1.06	(0.87-1.28)	0.576
Hyperlipidemia	175	84352	2.07	1.07	(0.88-1.31)	0.482
Colorectal cancer	5	1527	3.27	1.48	(0.61-3.58)	0.386
Major depressive disorder	67	30292	2.21	1.19	(0.90-1.57)	0.217
Anxiety disorder	220	96735	2.27	1.33	(1.11-1.59)	0.002
Bipolar disorder	5	2435	2.05	1.03	(0.42-2.52)	0.820
Anti-psychotics						
No	630	535264	1.18	1.00	(reference)	
Yes	84	33755	2.49	1.18	(0.92-1.50)	0.193
SSRIs						
No	478	460307	1.04	1.00	(reference)	
Yes	236	108713	2.17	1.74	(1.44-2.10)	< 0.001

¹Per 1000 person-years; ²Adjusted by age, sex, urbanization, family income, area of residence, occupation, anti-psychotics and the comorbidities listed in Table 1. IBS: Irritable bowel syndrome; SSRIs: Selective serotonin reuptake inhibitors.

Table 3 Incidence rate and hazard ratio of irritable bowel syndrome associated with selective serotonin reuptake inhibitor use in Cox regression analysis

	SSRI use (2000-2010)						aHR ²	(95%CI)	P value
	No (n = 78612)			Yes (n = 19653)					
	Event	Person-years	Incidence rate of IBS ¹	Event	Person-years	Incidence rate of IBS			
Overall	478	460307	1.04	236	108713	2.17	1.74	(1.44-2.10)	< 0.001
Sex									
Female	282	270195	1.04	132	65169	2.03	1.65	(1.29-2.11)	< 0.001
Male	196	190112	1.03	104	43544	2.39	1.85	(1.38-2.48)	< 0.001
Age									
< 20	8	34575	0.23	5	8564	0.58	1.12	(0.23-5.41)	0.884
20-29	27	73594	0.37	20	17815	1.12	2.78	(1.36-5.70)	0.005
30-39	61	89305	0.68	32	21416	1.49	1.95	(1.13-3.34)	0.016
40-49	96	91299	1.05	57	21899	2.60	1.79	(1.19-2.69)	0.005
50-59	85	65244	1.30	48	15508	3.10	2.17	(1.40-3.36)	< 0.001
≥ 60	201	106290	1.89	74	23511	3.15	1.32	(0.97-1.79)	0.077

¹per 1000 person-years; ²aHR, adjusted by age, sex, urbanization, family income, area of residence, occupation, anti-psychotics and the comorbidities listed in Table 1. IBS: Irritable bowel syndrome; SSRI: Selective serotonin reuptake inhibitor.

users among females (aHR = 1.65; 95%CI: 1.29-2.11), males (aHR = 1.85; 95%CI: 1.38-2.48) and individuals aged between 20 and 60 years (Table 3).

The cumulative incidence of IBS was higher in the SSRI cohort than in the non-SSRI cohort (log-rank test, $P < 0.001$) (Figure 2).

HR of IBS associated with the duration of SSRI exposure

Table 4 shows the association between SSRI exposure days over one year and the HR of a subsequent diagnosis of IBS. The aHR was highest in individuals

with a one-year SSRI exposure of less than 90 d (aHR = 3.27, 95%CI: 2.61-4.08). The aHR remained significantly higher in patients with longer durations of SSRI exposure.

Hazard ratio of IBS among SSRI and antidepressant users

A subgroup analysis of SSRI and non-SSRI users showed a significantly increased aHR of IBS in SSRI only users (aHR = 1.82, 95%CI: 1.50-2.21, $P < 0.001$) but not in the users of other antidepressants only

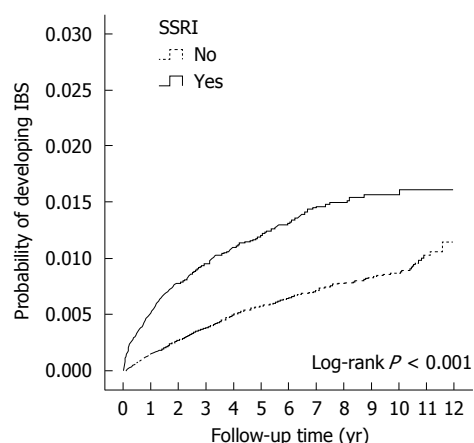


Figure 2 Comparison of the cumulative incidence of irritable bowel syndrome for selective serotonin reuptake inhibitor users and non-users. SSRI: Selective serotonin reuptake inhibitor; IBS: Irritable bowel syndrome.

(aHR = 1.33, 95%CI: 0.75-2.36, $P = 0.338$) or the combined SSRI and other antidepressant users (aHR = 1.30, 95%CI: 0.84-2.01, $P = 0.235$) (Table 5).

DISCUSSION

Based on our review of the literature, this is the first study using nationwide population database to investigate the relationship between SSRI prescriptions and IBS in Taiwan. Our results revealed that patients exposed to SSRIs had an increased risk of developing IBS (aHR = 1.74) after adjusting for sex, age, and major comorbidities.

In this study, we demonstrated that IBS in SSRI users tended to occur in older patients. The incidence of IBS became higher as age increased in both SSRI users and non-users, and this finding conflict with both the global data and a previous questionnaire survey conducted in Taiwan^[10,35]. A previous global meta-analysis and questionnaire study in Taiwan showed that the prevalence of IBS decreased with advancing age. However, the participants in the previous questionnaire study in Taiwan were healthy volunteers, and thus the prevalence of IBS in the Taiwan's general population may have been underestimated. Additionally, IBS symptoms may occur at early ages with relatively benign symptoms. Most patients may tolerate these symptoms and not seek medical advice; this tendency may have also led to an underestimation of the incidence of IBS in young individuals.

To evaluate the dose effect of SSRIs on the subsequent development of IBS, we analyzed the days of SSRI exposure within one year and determined the HR of subsequent IBS diagnosis. The aHR was highest in individuals with SSRI exposure for less than 90 d but remained significantly higher for patients with longer exposure times. Although SSRIs include widely used antidepressants that are more tolerable and have relatively benign adverse effects compared

Table 4 Hazard ratio of irritable bowel syndrome associated with duration of selective serotonin reuptake inhibitor exposure over one year

	aHR ¹	(95%CI)	P value
SSRI non-users	1.00	(reference)	
SSRI exposure < 90 d	3.27	(2.61-4.08)	< 0.001
SSRI exposure 90-180 d	1.38	(1.03-1.85)	0.029
SSRI exposure 181-270 d	1.49	(1.05-2.13)	0.027
SSRI exposure ≥ 270 d	1.52	(1.07-2.15)	0.020

¹aHR, adjusted by age, sex, urbanization, family income, area of residence, occupation, anti-psychotics and the comorbidities listed in Table 1. SSRI: Selective serotonin reuptake inhibitor.

Table 5 Hazard ratio of irritable bowel syndrome among selective serotonin reuptake inhibitor and antidepressant users

	n	aHR ¹	(95%CI)	P value
Overall				
Non-SSRIs	77327	1.00	(reference)	
Non-SSRIs + other antidepressants	1285	1.33	(0.75-2.36)	0.338
SSRIs + other antidepressants	2872	1.30	(0.84-2.01)	0.235
SSRIs only	16781	1.82	(1.50-2.21)	< 0.001

¹aHR, adjusted by age, sex, urbanization, family income, area of residence, occupation, anti-psychotics and the comorbidities listed in Table 1. SSRIs: Selective serotonin reuptake inhibitors.

to TCAs or monoamine oxidase inhibitors^[3], they continue to have some early onset adverse effects and problems associated with long-term treatment. The early onset adverse effects include gastrointestinal discomfort, nausea, dyspepsia and diarrhea and disappear within two to three weeks^[5]. The higher HR of IBS in individuals with lower SSRI exposure times may be due to these early onset gastrointestinal side effects that lead to misdiagnosis of IBS by clinical physicians. However, Table 4 shows that patients with SSRI exposure for more than 90 d had a significantly higher HR of IBS, and this finding cannot be explained by the early gastrointestinal adverse effects of SSRIs. In addition, the mean follow-up time from SSRI exposure to IBS diagnosis was 2.05 years, which is long enough to exclude transient adverse effects of SSRIs. Therefore, it can be hypothesized that the long duration of psychiatric disorders leads to an increased risk of subsequent IBS.

Many studies have identified a relationship between IBS and psychiatric disorders^[17,36]. In patients with IBS who seek treatment, the rates of comorbid psychiatric disorders range from 54% to 94%^[9,12]. Anxiety and depression disorders are associated with gastrointestinal symptoms in accordance with brain-gut interactions^[37-40]. Additionally, the communication between the central nervous system and enteric nervous system appears to be bidirectional^[36]. The biopsychological model of IBS suggests that deterioration of gastrointestinal symptoms could exacerbate

anxiety and depression (bottom-up model) and that psychological factors themselves similarly influenced physiological factors (top-down model)^[41]. Fond *et al.*^[17] demonstrated in a systematic review and meta-analysis that patients with IBS have significantly higher levels of anxiety and depression than healthy controls. To exclude the influence of other antidepressants, we performed a subgroup analysis of SSRI and non-SSRI users. Table 5 shows that the aHR was significantly higher in the users of SSRIs only compared with the users of non-SSRIs or users of combined SSRIs and other antidepressants. The use of other antidepressants only or in combination with SSRIs was not associated with an increased risk of IBS.

One possible reason for the higher incidence of IBS in the SSRI cohort is that the patients' underlying psychiatric disorders, particularly anxiety, may have deteriorated and thus their subclinical gastrointestinal symptoms became overt. Under these conditions, clinical physicians may provide new prescriptions for SSRIs, and patients may seek medical advice for IBS symptoms. This process is consistent with our study results, which showed that patients with anxiety disorders had a significantly higher HR of IBS. Poorly controlled anxiety disorders and unstable mood may exacerbate the symptoms of IBS. The increased HR of IBS in the SSRI cohort, compared with the comparison cohort in our study, may be due to the increased severity of anxiety disorders, poor compliance to SSRIs due to adverse medication effects or personal reasons.

There were some limitations to our study. First, there is only one code for IBS (ICD-9-CM code: 564.1) in the ICD-9 system, and further subgroup analyses were therefore not feasible. Second, data on lifestyle factors, such as smoking and alcohol use, were not available in the NHIRD. Third, information on drug compliance was not obtained from this health care database. Fourth, the severity of any psychiatric disorder upon enrollment in the study was also not available in the NHIRD. Fifth, there are always coding issues in database studies. There may be registration differences among the physicians and psychiatrists who did not code for IBS and the gastroenterologists who did not code for depression/anxiety. However, most physicians should adhere to the proper coding standards due to NHI payment rules.

In conclusion, SSRI use is associated with subsequently diagnosed IBS. In clinical practice, it is important to pay attention to the gastrointestinal symptoms of patients with psychiatric disorders who use SSRIs.

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COMMENTS

Background

Irritable bowel syndrome (IBS) is part of a large group of functional gastrointestinal disorders that significantly reduce patients' quality of life. Antidepressants, selective serotonin reuptake inhibitors (SSRIs) in particular, have been used to treat refractory IBS. The influence of SSRIs on the subsequent development of IBS remains unknown.

Research frontiers

The results regarding the clinical efficacy of SSRI in treating IBS are inconsistent. This study presents the first attempt to elucidate the relationship between SSRI use and subsequent diagnosis of IBS.

Innovations and breakthroughs

The overall adjusted hazard ratio was higher in the SSRI cohort than in the comparison cohort. The incidence of IBS in SSRI users increased with advancing age.

Applications

Physicians in clinical practice should pay attention to the gastrointestinal symptoms of patients with psychiatric disorders and SSRI use.

Terminology

IBS is characterized by recurrent abdominal discomfort or pain and disturbed defecation in the absence of an organic disease.

Peer-review

This study by Lin *et al* probes the relationship between SSRI use and the subsequent diagnosis of IBS over a 10-year span, using a national health insurance research database. The data indicate an adjusted increase in HR of 1.74 ($P = 0.002$) for the diagnosis of IBS in patients treated with SSRIs.

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Observational Study

Laparoscopic management of gastric gastrointestinal stromal tumors: A retrospective 10-year single-center experience

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Abstract

AIM

To determine the feasibility, safety, and oncological outcome of laparoscopic resection of gastric gastrointestinal stromal tumors (GISTs) based on favorable or unfavorable location.

METHODS

Our hospital database included 207 patients who underwent laparoscopic removal of gastric GISTs from January 2004 to September 2015. Patient demographics, clinical presentation, surgery, histopathology, post-operative course, and oncological outcomes were reviewed and analyzed.

RESULTS

Gastric GIST in favorable locations was present in 81/207 (39.1%) cases, and in unfavorable locations in 126/207 (60.9%) cases. Overall mean tumor size was 3.28 ± 1.82 cm. No conversions occurred, and complete R0 resection was achieved in 207 (100%) cases. There were three incidences of iatrogenic tumor rupture. The feasibility and safety of laparoscopic surgery were comparable in both groups with no statistical difference between unfavorable and favorable

location groups, respectively: for operative time: 83.86 ± 44.41 vs 80.77 ± 36.46 min, $P = 0.627$; conversion rate: 0% vs 0%; estimated blood loss: 27.74 ± 45.2 vs 29.59 ± 41.18 mL, $P = 0.780$; tumor rupture during surgery: 0.90% vs 2.82%, $P = 0.322$; or postoperative complications: 3.74% vs 7.04%, $P = 0.325$. The follow-up period recurrence rate was 1.89% with no significant differences between the two groups (3.03% vs 0%, $P = 0.447$). Overall 5-year survival rate was 98.76% and survival rates were similar between the two groups: 98.99% vs 98.39%, $P = 0.623$ (unfavorable vs favorable, respectively).

CONCLUSION

The laparoscopic approach for gastric GISTs is safe and feasible with well-accepted oncological surgical outcomes. Strategies for laparoscopic resection should be selected according to the location and size of the tumor. Laparoscopic treatment of gastric GISTs in unfavorable locations should not be restricted in gastrointestinal centers.

Key words: Laparoscopic; Gastrointestinal stromal tumors; Gastrectomy; Minimally invasive surgery

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Core tip: In most guidelines laparoscopic surgery is suggested only for gastrointestinal stromal tumors (GISTs) in favorable locations, such as those in the greater curvature or anterior wall of the stomach. The feasibility, safety and oncological outcome of this technique for GISTs in unfavorable locations remain unclear. We aimed to determine the feasibility, safety, and oncological outcome of laparoscopic resection of gastric GISTs based on different location. To our knowledge, this retrospective study includes the largest series of patients with gastric GISTs treated with laparoscopic resection at a single center. We also used and describe three relatively new laparoscopic surgical techniques for GISTs.

Liao GQ, Chen T, Qi XL, Hu YF, Liu H, Yu J, Li GX. Laparoscopic management of gastric gastrointestinal stromal tumors: A retrospective 10-year single-center experience. *World J Gastroenterol* 2017; 23(19): 3522-3529 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3522.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3522>

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common type of subepithelial tumor, with more than half (50%-60%) located in the stomach^[1]. The standard treatment for localized GISTs is complete R0 surgical excision, avoiding tumor rupture, and without dissection of clinically-negative lymph nodes^[2,3].

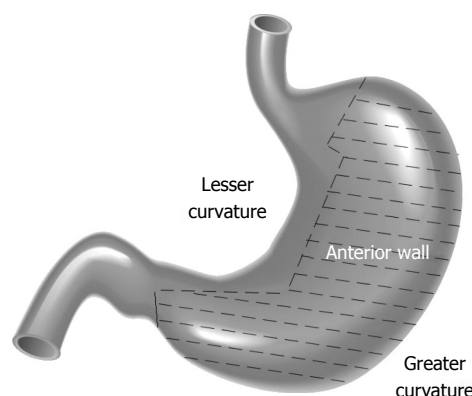


Figure 1 Illustration of favorable locations and unfavorable locations. The hatched area shows favorable locations (also known as the easy-to-access area), and which includes tumors located on the greater curvature and anterior wall of the gastric body, fundus, and antrum. The unhatched area shows unfavorable locations (also known as the difficult-to-access area), and which includes tumors located in the lesser curvature of the body, fundus, and antrum, the cardia, or prepyloric region.

Although the feasibility and safety of the laparoscopic approach for GIST resection has been demonstrated in many retrospective studies^[4], in the European Society for Medical Oncology, National Comprehensive Cancer Network (NCCN) and Asian GIST guidelines, laparoscopic surgery is suggested only for GISTs in favorable locations such as those in the greater curvature or anterior wall of the stomach^[2,3,5]. In unfavorable locations, due to the difficulty in exposing tumor position, there is a risk of stenosis of the lumen postoperatively, and guaranteed R0 resection is still difficult with laparoscopic procedures. The feasibility, safety and oncological outcome of this technique for GISTs in unfavorable locations remain unclear^[6,7].

We examined our most recent 10-year experience regarding the laparoscopic treatment of gastric GISTs based on different locations including favorable locations (tumors located in the greater curvature and anterior wall of the gastric body, fundus, and antrum) and unfavorable locations (tumors located in the lesser curvature or posterior wall of the gastric body, fundus, and antrum) (Figure 1) to determine the feasibility, safety, and oncological outcomes of laparoscopic resection of gastric GISTs. We also outline, herein, the technical details involved in the surgeries. To the best of our knowledge, this is the largest case series to date focusing on the laparoscopic management of GISTs.

MATERIALS AND METHODS

Patient data

All laparoscopically-resected gastric GISTs included in a maintained database at Nanfang Hospital, China, from January 2004 to September 2015, were retrospectively analyzed. All patients underwent preoperative endoscopy and abdominal computed tomographic imaging. The diagnosis of gastric GIST was established by positive immunohistochemical

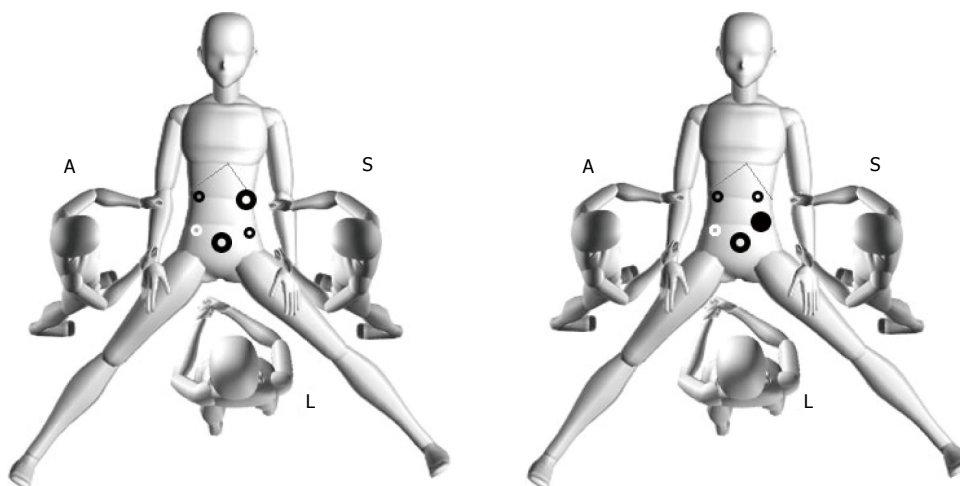


Figure 2 Operative positions and puncture trocars setting. The surgeon stood on the patient's left side with the assistant on the patient's right side. The laparoscopist stood between the patient's legs. A 12-mm trocar was inserted below the umbilicus, and used as the laparoscope port. One 10-mm trocar was placed 2 cm from the intersection of the right side of the outer rectus and under the costal margin was used as the dominant hand port. One 5-mm trocar was placed 2 cm from the intersection of the right side of the outer rectus and the umbilicus. Two additional 5-mm trocars were placed in the contralateral side, including a third (white circle) when necessary. When performing laparoscopic intragastric submucosal dissection, the laparoscope port position is shown in the figure on the right as a 12-mm trocar (black solid circle) that was inserted intragastrically with a laparoscopic monitor and changed to an intragastric monitor port.

staining of CD117 and/or CD34 in the surgical specimens, and all operations were performed at our hospital by experienced laparoscopic surgeons. Data on patient demographics, clinical presentation, surgery, histopathology, postoperative course, and oncological outcomes were reviewed and analyzed. Hand-assisted cases not expected preoperatively were classified as conversions. Tumor size was defined as the maximal tumor dimension of the resected specimen, and R0 resection was defined as removal of all gross disease at surgery without microscopic disease. This study was approved by the ethics committee of Nanfang Hospital.

Surgical approach

The surgical procedure following exploration was based on the location and size of the tumor. Laparoscopic procedures for managing GISTs were introduced in a previous study^[7]. The options for laparoscopic surgery included gastrectomy (total, subtotal, distal, gastric stump, or proximal), wedge resection, transgastric resection, and seromuscular dissection and laparoscopic intragastric submucosal dissection. After conventional endotracheal intubation anesthesia, the patient was placed in the supine position with legs apart. The position of the surgeons and the location of the puncture hole are shown in Figure 2. The technical details depended on tumor location, size, and morphology. The tumor specimen was extracted through a minilaparotomy in an endoscopic retrieval bag. The details of laparoscopic wedge resection, transgastric resection, seromuscular dissection and laparoscopic intragastric submucosal dissection are described below.

Laparoscopic wedge resection

Most lesions could be seen or palpated with laparoscopic

instruments on the anterior side of the stomach, and wedge resection was performed preferentially to anatomical resection whenever possible. Exophytic GISTs were treated with wedge resection using a linear endoscopic stapler at the base. Endophytic lesions of the anterior wall of the stomach were resected with a margin of normal stomach using an ultrasonic scalpel or EndoGIA (United States Surgical Corporation, Norwalk, CT, United States).

Laparoscopic seromuscular dissection (Video 1)

An ultrasonic scalpel was used to incise the seromuscular layer at the lower edge of the tumor, and the incised seromuscular layer was grasped by noninvasive forceps to lift the tumor. The tumor was then detached from the mucosa from proximal to distal direction using the ultrasonic scalpel. Once the tumor was removed, the mucosal integrity was evaluated, and if the mucosa was penetrated, it was repaired with absorbable sutures. The seromuscular layer was then sutured laparoscopically using continuous 3-0 absorbable sutures, and the incision was observed during gastric endoscopy for the presence of bleeding or stenosis.

Transgastric resection (Video 2)

When the endogenous tumor was located in the mesangial side or near the posterior wall of the cardia, it was necessary to fully free the surrounding gastric wall, reposition the tumor, and incise the full-thickness stomach wall containing the tumor at the lower 1-cm edge of the tumor along the longitudinal axis. After tumor resection, the stomach wall was sutured with 3-0 absorbable suture vertically to the stomach longitudinal axis, and the seromuscular layer was then embedded intermittently. A laparoscopic linear cutter stapler

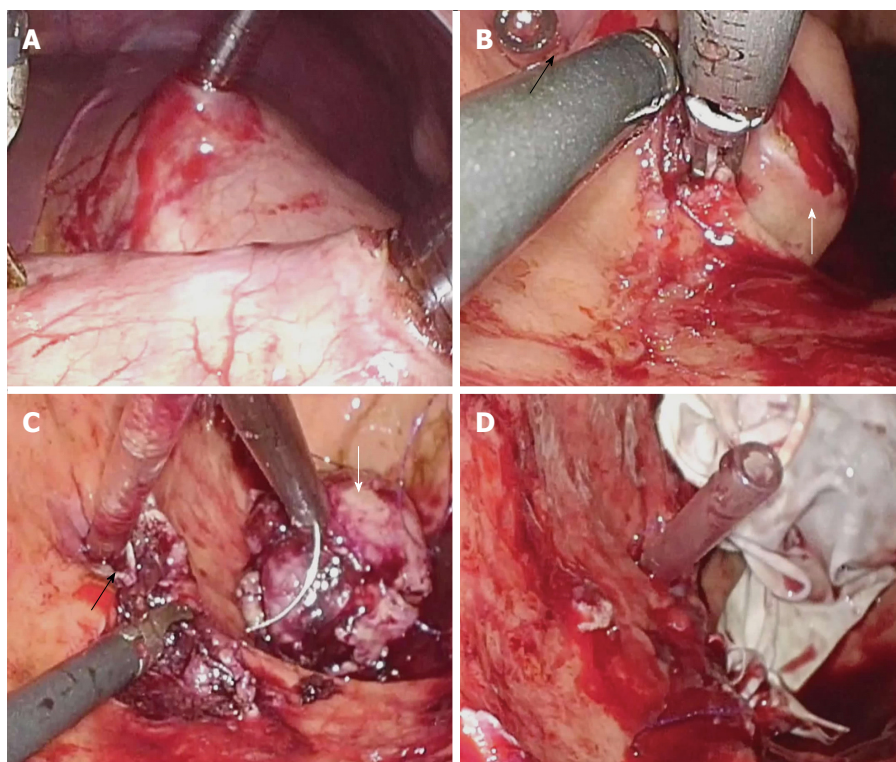


Figure 3 Laparoscopic intragastric submucosal dissection for gastrointestinal stromal tumor. A: Insertion of trocars into the stomach cavity under laparoscopic view; B and C: Stripping of the tumor (white arrow) from the gastric submucosal layer and suturing of the gastric wall incision in the stomach cavity (ostium pyloricum was indicated with black arrow); D: Removal of the tumor with the specimen bag through stomach wall and abdominal wall.

can also be used to close the gastric wall opening. Recently, a “dumpling making” method was used in our center, which involves cutting the stomach in a half circle, lifting the tumor, using the cutter stapler to remove the stomach wall and tumor together and closing the incision at the same time.

Laparoscopic intragastric submucosal dissection (Video 3)

A small incision was made in the anterior wall of the gastric body near the greater curvature using the ultrasonic scalpel. A 12-mm cannula in the left lower abdomen was inserted into the stomach cavity and air was delivered into the cavity. A laparoscopic lens was inserted through the cannula to determine the position of the tumor and the puncture site for another accessory port and assistant cannula in the stomach wall. After placement, the laparoscopic instrument was moved into the stomach cavity. The mucosa at the edge of the tumor was then incised, and the tumor was stripped from the submucosal layer using the ultrasonic scalpel. Dissection of the tumor was performed while the intracardial port was monitored (Figure 3).

Following removal of the tumor, the mucosal incision was sutured with 3-0 absorbable suture. The gastric cannula provided guidance and support to prevent cardia stenosis caused by the sutures. The tumor was placed in the specimen bag, the stomach wall was lifted to the abdominal wall through the appropriately expanded cannula incision, and the

specimen bag was then removed. The specimen bag could also be removed by intraoperative gastroscopy. The stomach wall incision was lifted through the cannula port outside the abdominal wall for suturing or was sutured directly under laparoscopy.

Follow-up

The majority of patients underwent close follow-up. However, 18 patients were lost to follow-up or had insufficient clinical records. Follow-up was conducted by telephone or outpatient visits, and follow-up data included adjunctive therapy, survival time, recurrence, and death.

Statistical analysis

Continuous variables are presented as mean \pm SD and were compared using Student's *t*-test. Categorical variables are expressed as valid percentages and were compared using the χ^2 test or Fisher's exact test, as appropriate. Recurrence and survival outcomes were calculated using the Kaplan-Meier method and were compared by the log-rank test. Univariate analysis of multiple clinicopathological variables was performed to determine the variables associated with poor outcomes. Factors deemed significant in univariate analyses were entered into multivariate analyses using logistic and Cox regression models. A *P* value < 0.05 was considered significant, and all statistical analyses were performed using SPSS 20.0 software (SPSS, Chicago, IL, United States).

Table 1 Clinical and pathological characteristics

Characteristics	
Age (yr)	54.09 ± 12.53
Male/female	92/106
Tumor location	
Favorable	81 (39.1)
Unfavorable	126 (60.9)
Tumor size (cm)	3.28 ± 1.82
Risk ¹	
Very low	38 (22.2)
Low	57 (33.5)
Intermediate	51 (30.2)
High	24 (14.1)
Application of endoscopy during surgery	27 (13.0)
Conversion	0
Tumor rupture during surgery	3 (1.58)
Tumor resection margin R0	100%
Operative time (min)	80.74 ± 38.96
Estimated blood loss (mL)	28.17 ± 44.99
Postoperative exhaust time	2.537 ± 0.88
Time to liquid diet (d)	2.91 ± 1.70
Length of postoperative stay (d)	6.10 ± 2.99
Classification of postoperative complications ²	
Grade 1	3 (0.17)
Grade 2	6 (0.34)

¹Fletcher's criteria (2002); ²Clavien-Dindo classification. Data are presented as *n* (%) or mean ± SD.

RESULTS

During the study period, a total of 207 patients underwent laparoscopic resection of gastric GISTs. Patient and tumor characteristics are presented in Table 1. The tumors were in favorable locations in 81/207 (39.1%) cases, and in unfavorable locations in 126/207 (60.9%) cases. Overall mean tumor size was 3.28 ± 1.82 cm. According to the 2002 Fletcher's criteria, 95/207 (55.7%) cases were in the low or very low risk group, 51/207 (30.2%) were in the intermediate risk group and 24/207 (14.1%) cases were in the high risk group. Twenty-seven (27/207, 13.0%) cases underwent endoscopy during surgery. No conversions occurred and complete R0 resection was achieved in all 207 (100%) cases. There were three incidences of iatrogenic tumor rupture and no major intraoperative complications. The overall postoperative complication rate was 0.51%. Nine cases had only mild complications, which resolved with observation and antibiotic treatment.

When comparing gastric GISTs in favorable locations with those in unfavorable locations according to the NCCN guidelines, we noted that the application of endoscopy in the unfavorable group was more frequent (20.72% vs 5.63%, respectively, *P* = 0.05). The feasibility and safety of laparoscopic surgery were comparable in both groups, with no statistical difference (favorable location group vs unfavorable location group, respectively) in operative time (83.86 ± 44.41 min vs 80.77 ± 36.46 min, *P* = 0.627), conversion rate, estimated blood loss (27.74 ± 45.2

Table 2 Comparison between favorable gastrointestinal stromal tumor location and unfavorable gastrointestinal stromal tumor location

	Unfavorable area	Favorable area	<i>P</i> value
Age (yr)	54.29 ± 12.29	55.59 ± 12.4	0.486
BMI	22.37 ± 3.28	23.17 ± 3.01	0.100
Tumor size (cm)	3.37 ± 1.85	4.07 ± 2.23	0.020
Operative time (min)	83.86 ± 44.41	80.77 ± 36.46	0.627
Estimated blood loss (mL)	27.74 ± 45.2	29.59 ± 41.18	0.780
Conversion	0	0	
Application of endoscopy during surgery	23 (20.72)	4 (5.63)	0.005
Tumor rupture during surgery	1 (0.90)	2 (2.82)	0.322
Postoperative complications	4 (3.74)	5 (7.04)	0.325
Postoperative exhaust time	2.64 ± 0.9	2.54 ± 0.86	0.452
Time to liquid diet (d)	3.05 ± 1.62	2.89 ± 1.79	0.516
Time to semiliquid diet (d)	4.17 ± 1.75	3.92 ± 1.83	0.345
Postoperative hospital stay (d)	7.17 ± 4.1	5.69 ± 2.46	0.007
Recurrence	3 (3.03)	0 (0.00)	0.447
5-yr overall survival rate	98 (98.99)	61 (98.39)	0.623

BMI: Body mass index. Data are presented as *n* (%) or mean ± SD.

mL vs 29.59 ± 41.18 mL, *P* = 0.780), tumor rupture during surgery (0.90% vs 2.82%, *P* = 0.322), or postoperative complications (3.74% vs 7.04%, *P* = 0.325) (Table 2).

During the follow-up period (7-107 mo), GISTs recurred in three patients in unfavorable location group, but there were no recurrences in the favorable location group. The total recurrence rate was 1.89%. Two patients with recurrences had the history of tumor rupture during the laparoscopic surgery. No laparoscopic technique was performed in patients with recurrent disease. An exploratory laparotomy was performed in one patient with local recurrence after 22 mo of the first operation. Although the recurrence rate was higher in the unfavorable location group compared with the favorable location group, there were no significant differences between the two groups (3.03% vs 0%, *P* = 0.447) (Figure 4A). One patient in the favorable location group suffered from recurrence at 12 mo and died at 28 mo after the surgery. One patient in the favorable location group presented with tumor rupture during surgery and died 70 mo after the surgery. The overall 5-year survival rate was 98.76%, and overall survival rates were similar (98.99% vs 98.39%, *P* = 0.623) (Figure 4B) when comparing the unfavorable location group versus favorable location group, respectively.

DISCUSSION

Many guidelines recommend laparoscopic surgery for GISTs dependent on tumor location. Laparoscopic surgery has been performed for tumors in unfavorable locations, especially the cardia and antrum, and has been reported to be safe and effective for GISTs^[8-13]; however, to our knowledge, no study has compared

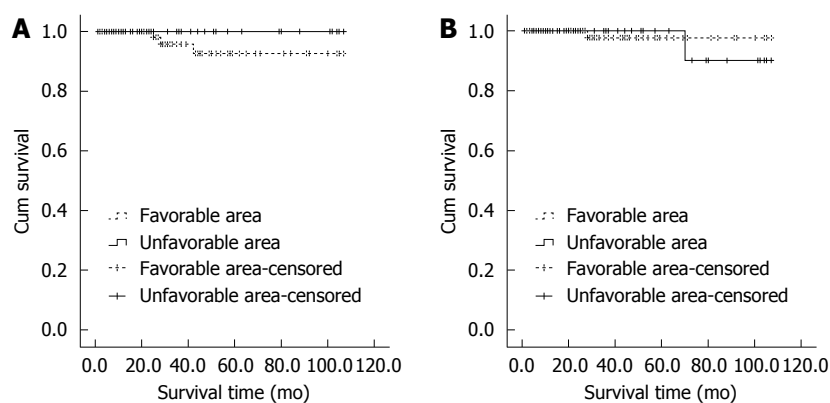


Figure 4 Disease-free survival curves (A) and survival curves (B) of the two groups.

the safety and efficacy of laparoscopic surgery in GISTs in unfavorable locations vs favorable locations. With similar operative morbidity, conversion risk, blood loss, postoperative recovery, and oncological results, our series demonstrates that compared with GISTs of the greater curvature or anterior wall, the resection of gastric GISTs in other locations is also feasible and can be safely accomplished laparoscopically.

To our knowledge, this retrospective study includes the largest series of patients with gastric GISTs treated with laparoscopic resection at a single center. Laparoscopic resection for proven GISTs even at challenging locations had 100% negative resection margins and excellent long-term oncological outcomes. Compared with open surgery, laparoscopic surgery for GISTs has similar outcomes and advantages that include less pain, less invasiveness, early recovery, and better cosmetic results. Our results indicate that a laparoscopic approach should be considered in all patients with gastric GISTs who have no contraindications to this approach. Three systematic review and meta-analyses comparing laparoscopic versus open resections for gastric GISTs showed that laparoscopic resection for gastric GISTs is a safe and feasible procedure with superior postoperative outcomes^[14]. Long-term survival was mainly associated with tumor stage and type, and laparoscopic surgery did not increase the risk of tumor relapse and metastasis^[15]. The low morbidity, no conversion to open surgery, and the long-term disease-free interval observed in our study indicate that laparoscopic resection is safe and effective for gastric GISTs irrespective of tumor locations.

Endoscopic cooperative surgery is being described increasingly as a technique to resect gastric submucosal lesions in unfavorable locations, such as the cardia or pyloric region^[16,17]. However, this approach requires facilities for endoscopic submucosal dissection and highly-advanced endoscopic skill, which limits the wide application of a combined laparoscopic-endoscopic approach^[17]. In our experience, most cases underwent laparoscopic surgery alone, and diagnostic gastroscopy was used when it was difficult to delineate the tumor's intraluminal extent and location. Different strategies

for laparoscopic resection should be selected according to the tumor location.

Wedge resection is the most prevalent procedure for laparoscopic resection of GIST. Although wedge resection can be performed successfully in the majority of cases, the technique should be chosen carefully, especially in patients with GISTs near the cardia or pylorus^[18]. The lesser curvature of the stomach and sites near the cardia or pylorus are considered unfavorable locations. When the tumor is located in these locations, wedge resection of the stomach wall can easily cause stenosis, and when a proximal or distal gastrectomy is performed, the scope of surgery for a stromal tumor increases.

In our center, we dissect the tumor from the gastric mucosa or incise and suture the gastric wall, which not only avoids gastric stenosis, but also maximizes retention of healthy stomach. Specifically, when tumors in unfavorable locations show exogenous growth, dissection of the tumor from the gastric mucosa should be chosen, and if the tumor shows endogenous growth, seromuscular dissection should be chosen when an intact mucosal layer surrounding the tumor is confirmed by preoperative gastroscopy.

For endogenous tumors treated with seromuscular dissection, mucosal layer identification is difficult, thus transgastric resection with suturing provides better visibility. When the tumor is located near the cardia or pylorus, if preoperative endoscopy or endoscopic ultrasonography suggests a clear tumor boundary, uniform texture, and endogenous growth, submucosal dissection of the tumor *via* the gastric cavity can be adopted. In these cases, surgery should be meticulous to prevent postoperative abdominal infection by placing gauze under the stomach wall incision and performing thorough aspiration of gastric contents. In patients with gastric retention, the stomach contents may flow into the abdominal cavity during gastric incision, thus preoperative gastrointestinal decompression and other relevant techniques should be performed.

Because this operation involves opening the gastric cavity, there may be potential risks of intraperitoneal infection; therefore, the safety of this treatment

remains controversial. Conrad *et al*^[6] operated on 11 cases using the following techniques: (1) a combined gastroscopic/laparoscopic approach when minimal manipulation of the lesion is needed; (2) multiport resection, which provides optimal triangulation and allows for resection of more complex lesions; (3) stapled removal of broad-based lesions; and (4) a single access technique with the device placed directly through the abdominal wall into the stomach. These techniques expand the surgeon's armamentarium to address more complex intragastric processes safely.

Because the reported number of surgical resections for endogenous tumors *via* the gastric cavity is small, it can not be confirmed if this method is better than other methods for stromal tumors in special locations. However, theoretically, preventing exposure of the gastrointestinal tract within the abdominal cavity could reduce the occurrence of abdominal infection. Tumor resection *via* the stomach cavity has been performed in only a few cases, thus its safety remains to be verified.

The 2010 NCCN report suggested that gastric GISTs larger than 5 cm may be resected using a laparoscopic or laparoscopic-assisted technique^[8]. Prior to 2015, a size limit in the NCCN guidelines for GISTs was not stated^[3]. Size is also not emphasized in the Asian GIST guidelines^[3]. On the other hand, the latest European Society for Medical Oncology practice guidelines clearly discourage a laparoscopic approach in patients with large tumors because of the risk of tumor rupture, which is associated with a very high risk of relapse^[5].

In our study, which included tumors ranging in size from 0.5-11 cm, tumor rupture or bleeding did not occur in relatively large tumors intraoperatively. When it is necessary to retract the tumor during surgery, the tissue around the tumor should be clamped to avoid or reduce direct contact with the tumor. Tumor size should not be considered a limitation for experienced laparoscopic surgeons using the no-touch technique. Recent retrospective studies showed that laparoscopic surgery did not increase the risks of tumor relapse and metastasis^[9,11]. Laparoscopic surgery should be considered as a standard approach in all cases irrespective of tumor size.

Despite recent advances in targeted oncological therapy for GISTs, for the majority of patients, complete surgical resection is sufficient to achieve long-term disease-free outcomes. Our experience echoes this fact. All curative-intent patients in this series underwent complete oncological resection with negative surgical margins on pathology. In this series, 146/170 (85.9%) of patients were classified as low or intermediate risk according to Fletcher's criteria, and only a small number of these cases (6/146) received adjuvant therapy. To date, none has recurred during a median follow-up period of 4.1 years (7-107 mo).

These data reinforce the importance of proper

oncological resection of GIST tumors, the primary merits of which are negative mucosal margins and avoidance of tumor rupture. Gastric GISTs recurred in three patients in the unfavorable location group, and there were no recurrences in the favorable location group. All of the patients who experienced recurrence belonged to the high risk group according to the Fletcher's criteria, and one died at 28 mo after surgery without receiving imatinib therapy. Survival analysis showed no significant difference in the disease-free survival time between the two groups; therefore, the long-term efficacy was similar between the two groups.

Our study has certain limitations. First, although the study included the largest known case series, it was a single center study. Second, early data were not fully recorded. Although most demographic and clinicopathological characteristics were comparable between the two groups with tumors in different locations, we acknowledge the potential imbalance in unknown factors that may have compromised the validity of the results. Third, the lack of long-term follow-up restricts the evaluation of survival benefits. Fourth, wedge resection was still the most prevalent procedure for laparoscopic resection of GIST in our study. In this study, only two patients underwent laparoscopic intragastric submucosal dissection. The present clinical data failed to conduct an analysis for the relationship between different localization with different techniques. Fifth, our study was a non-randomized controlled pilot study with a small sample size. The major purpose of the study was to determine the perioperative safety and efficacy of laparoscopic management of GISTs.

In conclusion, this study showed that a laparoscopic approach for gastric GISTs is a safe and feasible procedure with well-accepted oncological surgical results. Different strategies for laparoscopic resection should be selected according to tumor location.

COMMENTS

Background

Laparoscopic surgery is recommended for gastrointestinal stromal tumors (GISTs) located in the greater curvature or anterior wall of the stomach. We retrospectively examined our most recent 10-year experience regarding the laparoscopic treatment of gastric GISTs with a focus on unfavorable locations such as the lesser curvature or posterior wall of the gastric body, fundus and antrum. We aimed to determine the feasibility, safety, and oncological outcome of laparoscopic resection of gastric GISTs based on a favorable or unfavorable location. We also used and describe three new laparoscopic surgical techniques for GISTs.

Research frontiers

Although the feasibility and safety of the laparoscopic approach for GIST resection has been demonstrated in many retrospective studies, in the European Society for Medical Oncology, National Comprehensive Cancer Network (NCCN) and Asian GIST guidelines, laparoscopic surgery is suggested only for GISTs in favorable locations such as those in the greater curvature or anterior wall of the stomach. This study examined recent 10-year experience in a high value center regarding the laparoscopic treatment of gastric GISTs based

on different locations including favorable locations and unfavorable locations to determine the feasibility, safety, and oncological outcomes of laparoscopic resection of gastric GISTs.

Innovations and breakthroughs

The laparoscopic approach for gastric GISTs is safe and feasible with well-accepted oncological surgical outcomes. Strategies for laparoscopic resection should be selected according to the location and size of the tumor.

Applications

The data in this study suggested that laparoscopic treatment of GISTs in unfavorable locations should not be restricted in gastrointestinal centers.

Peer-review

This is a very interesting study concerning new techniques of GIST resection in stomach localization. To date it is widely accepted (NCCN guidelines) that laparoscopy is safe, with good outcome in disease-free survival time.

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Prospective Study

Short health scale: A valid measure of health-related quality of life in Korean-speaking patients with inflammatory bowel disease

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Institutional review board statement: This study was approved by the Institutional Review Board of Kangbuk Samsung Hospital, Korea.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment

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Abstract

AIM

To evaluate the short health scale (SHS), a new, simple, four-part visual analogue scale questionnaire that is designed to assess the impact of inflammatory bowel disease (IBD) on health-related quality of life (HRQOL), in Korean-speaking patients with IBD.

METHODS

The SHS was completed by 256 patients with Crohn's disease (CD) and ulcerative colitis (UC). Individual SHS items were correlated with inflammatory bowel disease questionnaire (IBDQ) dimensions and with disease activity to assess validity. Test-retest reliability, responsiveness and patient or disease characteristics with probable association with high SHS scores were analyzed.

RESULTS

Of 256 patients with IBD, 139 (54.3%) had UC and 117 (45.7%) had CD. The correlation coefficients between SHS questions about "symptom burden", "activities of daily living", and "disease-related worry" and their corresponding dimensions in the IBDQ ranged from 0.62 to 0.71, compared with correlation coefficients ranging from -0.45 to -0.61 for their non-corresponding dimensions. There was a stepwise increase in SHS scores, with increasing disease activity in both CD and UC (all *P* values < 0.001). Reliability was confirmed with test-retest correlations ranging from 0.68 to 0.90 (all *P* values < 0.001). Responsiveness was confirmed with the patients who remained in remission. Their SHS scores remained unchanged, except for the SHS dimension "disease-related worry". In the multivariate analysis, female sex was associated with worse "general well-being" (OR = 2.28, 95%CI: 1.02-5.08) along with worse disease activity.

CONCLUSION

The SHS is a valid and reliable measure of HRQOL in Korean-speaking patients with IBD.

Key words: Quality of life; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Disease activity

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Core tip: The short health scale (SHS) is a new, simple, four-part visual analog scale questionnaire that is designed to assess the impact of inflammatory bowel disease (IBD) on health-related quality of life (HRQOL). In Korean-speaking IBD patients, total SHS scores correlated with total IBDQ scores in both Crohn's disease (CD) and ulcerative colitis (UC). There was a stepwise increase in SHS scores with increasing disease activity in both CD and UC. Reliability was confirmed with test-retest correlations. Thus, SHS is a valid and reliable measure of HRQOL in Korean-speaking patients with IBD.

Park SK, Ko BM, Goong HJ, Seo JY, Lee SH, Baek HL, Lee MS, Park DI. Short health scale: A valid measure of health-related quality of life in Korean-speaking patients with inflammatory bowel disease. *World J Gastroenterol* 2017; 23(19): 3530-3537 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3530.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3530>

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the two most common variants of inflammatory bowel disease (IBD); both are chronic relapsing disorders that significantly affect daily life^[1,2]. The biological burden of IBD had been measured using disease activity scales

such as the Crohn's disease activity index (CDAI) in CD^[3]. However, these do not reflect the well-being of patients with chronic illness. In contrast, an important patient-reported outcome, the health-related quality of life (HRQOL), refers to the subjective perception of illness and disease impact on daily life and general well-being; the HRQOL is therefore an essential part of health assessment in patients with IBD in both clinical practice and clinical trials^[4,5]. Early HRQOL scales for IBD involved extensive questions relating to specific symptoms, social function, limitation of activities, and mental health, and were time-consuming to complete and evaluate^[6]. Since then, shorter scales have been developed, such as the short inflammatory bowel disease questionnaire (IBDQ)^[7], and short health scale (SHS)^[8]. The SHS consists of four simple 100-mm visual analog scales assessing factors traditionally associated with HRQOL (symptom burden, activities of daily living, disease related worry, and sense of general well-being). The questions were designed to be open-ended, so that patients could score any or all aspects of their life that they felt were important to them when completing the questionnaire. The SHS was validated in Swedish-speaking^[8,9], Norwegian-speaking^[10], and English-speaking^[11] patients with IBD.

Although the incidence of IBD in Asia has increased rapidly in recent years^[12-14], only a few studies have investigated HRQOL in Korean patients with IBD^[15]. Thus, we aimed to evaluate the SHS in Korean-speaking patients with IBD.

MATERIALS AND METHODS

Patients

A total of 272 patients with IBD, who visited the Kangbuk Samsung Hospital and Soonchunhyang University Hospital, Bucheon between March 2016 and August 2016 were invited to participate in this study. Of these, 265 patients agreed (97.4% response rate). After excluding 9 patients whose questionnaires did not have evaluable SHS data, 256 patients were finally enrolled. This study was approved by the Institutional Review Board of Kangbuk Samsung Hospital, South Korea.

Questionnaires

QOL was measured using the SHS. The questionnaire was designed to be self-administered and patients were asked to place a mark on the 10-cm visual analog scale that they thought was appropriate to their condition (Figure 1). Scores were presented for each of four dimensions including symptom burden, social function, disease-related worry, and sense of general well-being; the scores were then added for a total score. One gastroenterologist (SKP) translated the English SHS to Korean. QOL was also determined using the 32-item IBDQ that collects data on four dimensions, including bowel symptoms, social function,

How severe are the symptoms you suffer from your bowel disease?

No symptoms ○ ————— ○ Very severe symptoms

Do your bowel problems interfere with your activities in daily life?

Not at all ○ ————— ○ Interfere to a very high degree

How much worry does your bowel disease cause?

No worry ○ ————— ○ Constant worry

How is your general feeling of well being?

Very good ○ ————— ○ Dreadful

Figure 1 Short health scale questionnaire.

emotional function, and systemic symptoms. We used the Korean version of the IBDQ, which has been validated for IBD^[15].

Disease activity at the time of questionnaire completion was assessed by consulting physicians without knowledge of the questionnaire results. To assess disease activity, the CDAI was used for CD^[3], and the Mayo score for UC^[16]. CD in remission was defined as a CDAI < 150, mild disease as 150-220, moderate as 220-450, and severe as > 450. UC in remission was defined as a Mayo score 0-2, mild disease as 3-5, moderate as 6-10, and severe as 11-12.

Evaluation of SHS

Validity: We assessed validity by correlating both individual SHS items and total SHS score with IBDQ dimensions and total score. The score for each SHS question should be closely associated with other HRQOL measures reflecting the same dimension of health (convergent validity), whereas the association with variables that measure other health dimensions should be less (discriminant validity). The SHS item "symptom burden" was expected to be closely associated with the IBDQ dimension "bowel symptoms", the SHS item "social function" with the IBDQ dimension "social function", the SHS item "disease-related worry" with the IBDQ dimension "emotional function", and the SHS item "general well-being" with the IBDQ dimension "systemic symptoms" or "emotional function". We also compared SHS scores according to disease activity, as it can be assumed that disease activity would influence QOL (known-groups comparison/predictive validity).

Reliability: Test-retest reliability was determined from results 2 to 8 wk apart for SHS questions in patients in remission.

Responsiveness: Changes in SHS scores in patients who remained in remission and who experienced a change in disease activity, from remission to mild to moderate activity or vice versa, were measured for responsiveness. To evaluate responsiveness, the

patients were offered a second appointment 3-6 mo later, or earlier in the event of deterioration.

Influence of patient or disease characteristics on SHS: Patient or disease characteristics with probable association with high SHS scores were analyzed. High score was defined as ≥ 5 cm for each SHS question.

Statistical analysis

Continuous data were presented as medians and interquartile ranges and categorical variables were expressed as percentages. Correlations between continuous data were analyzed using Spearman's rank correlation coefficient (rs). For test-retest reliability, total score was analyzed using the Bland-Altman plot. Differences in unpaired and paired groups were assessed using the Kruskal-Wallis test and Wilcoxon signed-rank test, respectively. To investigate the influence of patient or disease characteristics on SHS, multivariate logistic regression analysis was performed. In the multivariate analysis, variables with probable association with high scores for each of the four SHS dimensions were included. These variables were age (< 40 years, ≥ 40 years), sex (female, male), education (beyond high school, junior school), smoking status (never, previous, or current smoker), family history (yes, no), disease type (CD, UC), disease duration (≥ 5 years, < 5 years), anti-tumor necrosis factor (TNF) or azathioprine use (yes or no), history of operation (yes or no), and disease activity based on CDAI for CD and Mayo score for UC (remission, mild, moderate, severe). For each variable, the odds ratio (OR) and 95%CI were provided. Two-sided *P* values < 0.05 were considered to be statistically significant. Statistical calculations were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patients

Demographic and disease characteristics are shown in Table 1. Among 256 patients who were finally enrolled, median age was 37 and 64% were male. Of these,

Table 1 Demographic and clinical details of patients *n* (%)

Variables	UC (<i>n</i> = 139)	CD (<i>n</i> = 117)	<i>P</i> value
Age, yr, median (range)	43 (16-70)	32 (17-60)	< 0.001
Sex, Male	83 (59.7)	81 (69.2)	0.11
Education ¹			0.04
Junior (education to age 16)	9 (8.5)	2 (1.9)	
Graduate (education to age 19)	35 (33.0)	28 (26.9)	
Third level education	62 (58.5)	74 (71.2)	
Family history ²	11 (10.3)	4 (3.9)	0.07
Smoking status ³			0.25
Non-smoker	64 (60.4)	64 (63.4)	
Ex-smoker	33 (31.1)	23 (22.8)	
Smoker	9 (8.5)	14 (13.9)	
Disease duration: years, median (range)	6.3 (0.1-26.2)	5.2 (0.1-14.9)	0.06
Disease location			
Proctitis (UC)/colon (CD)	39 (28.1)	27 (23.1)	
Left sided (UC)/small bowel (CD)	47 (33.8)	27 (23.1)	
Pancolitis (UC)/colon+Small bowel (CD)	53 (38.1)	63 (53.8)	
Disease activity			0.16
Remission	82 (59.0)	81/117 (69.2)	
Mild	42 (30.2)	21/117 (17.9)	
Moderate	14 (10.1)	14/117 (11.9)	
Severe	1 (0.7)	1/117 (0.8)	
Previous surgery ⁴	19 (18.6)	34 (33.3)	0.02
Medication			
Azathioprine/6-mercaptopurine	33 (23.7)	72 (61.5)	< 0.001
Anti-TNF	17 (12.2)	31 (26.7)	0.003

Data were available for 210¹, 209², 207³, and 204⁴ patients. CD: Crohn's disease; UC: Ulcerative colitis; TNF: Tumor necrosis factor.

Table 2 Spearman correlations (*r_s*) between short health scale items and inflammatory bowel disease questionnaire dimensions

	Symptoms <i>r_s</i>	Activities <i>r_s</i>	Worry <i>r_s</i>	Well-being <i>r_s</i>	Total score <i>r_s</i>
Crohn's disease					
IBDQ bowel symptoms	-0.66	-0.54	-0.45	-0.52	
IBDQ social function	-0.50	-0.71	-0.5	-0.46	
IBDQ emotional function	-0.49	-0.59	-0.64	-0.67	
IBDQ systemic symptoms	-0.60	-0.61	-0.51	-0.65	
Total score					-0.81
Ulcerative colitis					
IBDQ bowel symptoms	-0.68	-0.52	-0.49	-0.55	
IBDQ social function	-0.55	-0.71	-0.59	-0.56	
IBDQ emotional function	-0.47	-0.59	-0.62	-0.62	
IBDQ systemic symptoms	-0.45	-0.56	-0.50	-0.52	
Total score					-0.71

SHS: Short health scale; IBDQ: Inflammatory bowel disease questionnaire.

139 (54.3%) had UC and 117 (45.7%) had CD. The median duration of disease was 4.8 years.

Evaluation of SHS

Validity: Table 2 shows correlations between the four SHS dimensions and their corresponding IBDQ items. To establish convergent validity and discriminant validity, each SHS question score should be more closely associated with corresponding than with non-corresponding dimensions of the IBDQ questionnaires. In CD, the correlation coefficients between the SHS questions "symptom burden", "activities of daily living", and "disease-related worry" and their corresponding dimensions in the IBDQ ranged from 0.64 to 0.71, compared with correlation coefficients ranging from

-0.45 to -0.63 for their non-corresponding dimensions. In UC, correlation coefficients between the SHS questions "symptom burden", "activities of daily living", and "disease-related worry" and their corresponding dimensions in the IBDQ were also higher (0.61-0.71) than for their non-corresponding dimensions (0.45-0.59). The remaining SHS question score, "general well-being", showed closest correlation with the IBDQ score for emotional function, similar to that for the SHS question score for "worry", with a correlation coefficient of 0.67 for CD and 0.62 for UC.

Correlations between the four SHS dimensions and disease activity scores are shown in Figure 2. There was a stepwise increase in all SHS dimensions, with increasing disease activity. For both CD and UC

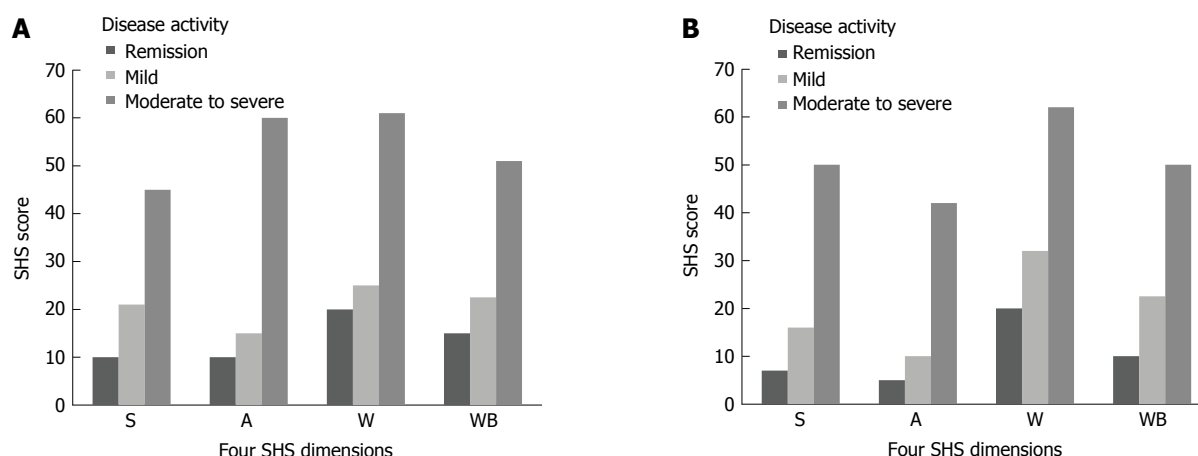


Figure 2 Correlation between the four short health scale dimensions and disease activity score in patients with Crohn's disease (A) and ulcerative colitis (B).

Table 3 Responsiveness to change in disease activity in the short health scale

SHS	Stable remission (<i>n</i> = 67)		Change in disease activity (<i>n</i> = 21)	
	Remission	Remission	Remission	Mild to moderate
Symptoms	4 (0-35)	7 (0-50)	8.5 (5-45)	15 (2-50)
Activities	3.5 (0-70)	8 (0-60)	10 (0-70)	8.5 (0-60)
Worry	17.5 (0-100)	7.5 (0-100) ^a	20 (7-70)	27 (7-60)
Well-being	8.5 (0-55)	6 (0-90)	17.5 (3-80)	30 (0-60)
Total score	40 (0-205)	40 (0-210)	59 (24-265)	87 (20-230)

^a*P* < 0.05 by Wilcoxon signed-rank test. SHS: Short health scale.

patients in remission, median "disease-related worry" scores were 20 (range 0-100), and were higher than for other dimensions.

Reliability: To assess reliability, test-retest results for 36 patients with IBD (20 with UC, 16 with CD) in remission were investigated. The Spearman rank correlation coefficients for test-retest scores for the SHS questions "symptom burden", "activities of daily living", "disease-related worry", and "general well-being" were 0.70 (*P* < 0.01), 0.63 (*P* < 0.01), 0.79 (*P* < 0.01), and 0.60 (*P* < 0.01), respectively. The correlation coefficient for the SHS total score was 0.74 (*P* < 0.01). In the Bland-Altman plot, mean difference was -2.81 (95%CI: -15.55-9.94) (Supplement Figure 1).

Responsiveness: Table 3 shows responsiveness in 88 patients with IBD. Seventy-five patients remained in stable remission and were reexamined after a median 4 mo. Their SHS scores remained unchanged, except for the SHS dimension "disease-related worry", which decreased during follow-up (17.5-7.5, *P* < 0.05). A change in disease activity occurred in 21 patients; 15 were in remission at baseline, but changed to mild to moderate disease activity during the follow-up period; 6 patients with mild to moderate activity at baseline repeated the questionnaires when in remission at the median 4-mo follow-up. There was no significant change in scores in the four SHS dimensions between

the two test occasions.

Influence of other patient and disease characteristics on SHS

In the multivariate analysis, disease activity was associated with high scores for each SHS question after adjusting for patient- or disease-related variables (Table 4). In addition, a 3.53-fold increase (95%CI: 1.18-10.56) in the adjusted odds for high score on "activities of daily living" was observed in young patients. In the SHS dimension "disease-related worry", the adjusted odds were 4.74 (95%CI: 1.06-21.2), 2.98 (95%CI: 1.43-6.20), and 2.56 (95%CI: 1.06-6.22) among patients with more than high school education, disease duration ≥ 5 years, and anti-TNF use, respectively. Female sex was associated with worse "general well-being" (OR = 2.28, 95%CI: 1.02-5.08) along with worse disease activity.

DISCUSSION

In this study, we verified appropriate psychometric properties for the SHS in Korean patients with IBD, as were previously shown in Western patients with IBD^[8-11]. We assessed the SHS for validity, reliability, and responsiveness, and all were confirmed except responsiveness in patients whose disease activity had changed. Associated patient and disease factors such as age, sex, and education were identified in our study.

Table 4 Influence of other demographic and disease factors on high short health scale score

	Symptoms	Activity	Worry	Well-being
Age < 40 (<i>vs</i> ≥ 40)	2.76 (0.79-9.56)	3.53 (1.18-10.56)	1.06 (0.47-3.28)	1.67 (0.70-3.94)
Sex, Female (<i>vs</i> male)	0.85 (0.25-2.90)	3.70 (0.98-7.45)	1.79 (0.83-3.85)	2.28 (1.02-5.08)
Education, beyond high school (<i>vs</i> junior school)	0.33 (0.02-5.51)	1.73 (0.25-11.95)	4.74 (1.06-21.2)	1.31 (0.22-7.75)
Smoking (<i>vs</i> non or ex-smoking)	3.79 (0.86-16.43)	3.43 (0.92-12.77)	1.02 (0.33-3.16)	2.78 (0.94-8.21)
Family history (<i>vs</i> no)	0.34 (0.06-1.92)	1.93 (0.20-18.62)	0.50 (0.14-1.80)	0.41 (0.10-1.59)
Disease, CD (<i>vs</i> UC)	0.54 (0.15-1.91)	0.52 (1.17-1.54)	0.81 (0.35-1.88)	1.41 (0.58-3.43)
Disease duration ≥ 5 yr (<i>vs</i> < 5 yr)	1.33 (0.45-3.89)	2.12 (0.81-5.52)	2.98 (1.43-6.20)	1.62 (0.77-3.45)
Anti-TNF use (<i>vs</i> no)	0.81 (0.18-3.59)	1.69 (0.53-5.44)	2.56 (1.06-6.22)	1.01 (0.48-2.68)
Azathioprine use (<i>vs</i> no)	0.55 (0.16-1.91)	1.39 (0.49-3.94)	1.39 (0.62-3.08)	1.69 (0.72-3.96)
Bowel operation history (<i>vs</i> no)	1.19 (0.28-4.94)	1.80 (0.50-6.47)	0.91 (0.40-2.06)	1.09 (0.44-2.68)
Disease activity, moderate to severe (<i>vs</i> mild or remission)	16.6 (4.19-66.18)	8.02 (2.43-26.43)	12.56 (3.66-43.09)	6.03 (1.98-18.40)

SHS: Short health scale; CD: Crohn's disease; UC: Ulcerative colitis; TNF: Tumor necrosis factor.

The SHS is a simple and standardized four-item questionnaire that is traditionally associated with HRQOL^[8,9]. It is quick and easy to administer, and our Korean patient population appeared to have little difficulty completing the survey. Validity was confirmed by correlation between the four SHS dimensions and their corresponding IBDQ items. In both CD and UC, the SHS questions for "symptom burden" and "activities of daily living" showed a close association with their corresponding dimensions in the IBDQ, and correlation coefficients were higher than for their non-corresponding dimensions, thus establishing convergent and discriminant validity. Previous studies examined either patient ratings of IBD concerns^[8,17] or IBDQ emotional function^[11], and both showed high correlations with the SHS "worry" dimension. In this study, we compared the "worry" dimension with IBDQ emotional function and confirmed convergent and discriminant validity. However, for "general well-being", previous studies examined different dimensions, *i.e.*, either IBDQ emotional function^[8] or IBDQ systemic symptoms^[11]. In this study, we found close associations between the SHS "well-being" dimension for both IBDQ emotional function and systemic symptoms in CD, and emotional function in UC. This is consistent with results in Swedish-speaking patients^[8], and different from those in English-speaking patients^[11]. This indicated that well-being is the most comprehensive among various dimensions associated with HRQOL, which is formed by the interaction of biological, psychological, social, and economic variables in patients with IBD. Validity was also supported by the significantly worse scores for all SHS questions in patients with mild to severe disease, compared with those in remission.

The SHS had good reliability, although it was assessed in a small group of patients in remission, using surveys 2 to 6 wk apart. The correlations between the two measurements were high in both CD and UC, and corresponded with the results of previous studies^[8-11].

Although patients in stable remission showed no significant change in score, the SHS was not responsive

to changes in disease activity. Patients who changed from remission to active disease or vice versa between the two follow-up visits had changes in symptom, worry, well-being, and total scores, but the results did not reach statistical significance, possibly due to the small number of patients. However, patients in stable remission showed no significant change in score, except in the worry dimension. "Disease-related worry" scores were relatively high in patients in remission, compared with scores for other dimensions, and improved after a median 4 mo. It is possible that worry will decrease when a patient is in remission for a certain period of time.

In this study, we also investigated patient and disease factors that might affect QOL. Only a study in Norwegian patients reported factors associated with SHS, and unemployment was adversely associated with SHS social function and general well-being in UC patients^[10]. Interestingly, we found that younger age, higher education, and female sex were adversely associated with SHS activity, worry, and well-being dimensions, respectively. In the worry dimension, longer disease duration and anti-TNF use were also adversely associated. As we expected, moderate to severe disease activity was associated with worse scores in all SHS questions, after adjusting for patient- or disease-related variables, especially in the "symptom burden" dimension.

This is the first study to test and validate the SHS in Asian patients. In addition, associated patient and disease factors in the SHS were identified in our study. The open-ended nature of the four SHS questions helps clinicians determine which component of health is affected, and enables decision-making for therapeutic intervention in bowel inflammation or the need for psychological support. The SHS is comprehensive and simple to complete, and can quickly provide information for use in both clinical practice and clinical trials.

Our study had several limitations. First, most subjects were outpatients in remission. Evaluation

of a population with a higher proportion of patients with moderate to severe disease is needed. Second, the number of patients who entered the study in remission and subsequently had a relapse during follow-up was small, and limited the confirmation of good responsiveness. Third, only Koreans were included, and further studies of other Asians should be performed to determine SHS reliability as an HRQOL instrument in Asia.

In conclusion, the SHS is a valid and reliable measure of HRQOL in Korean-speaking IBD patients. The SHS can be used in clinical trials and clinical practice to identify the main problems affecting QOL in IBD patients.

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COMMENTS

Background

The short health scale (sHS) was validated in Swedish-speaking, Norwegian-speaking, and English-speaking patients with inflammatory bowel disease (IBD). Although the incidence of IBD in Asia has increased rapidly in recent years, only a few studies have investigated health-related quality of life (HRQOL) in Korean patients with IBD. Thus, we evaluate the SHS in Korean-speaking patients with IBD.

Research frontiers

An important patient-reported outcome, the HRQOL, refers to the subjective perception of illness and disease impact on daily life and general well-being; the HRQOL is therefore an essential part of health assessment in patients with IBD in both clinical practice and clinical trials.

Innovations and breakthroughs

This is the first study to test and validate the SHS in Asian patients. We verified appropriate psychometric properties for the SHS in Korean patients with IBD, as were previously shown in Western patients with IBD. We assessed the SHS for validity, reliability, and responsiveness, and all were confirmed except responsiveness in patients whose disease activity had changed. Associated patient and disease factors such as age, sex, and education were identified in our study.

Applications

The open-ended nature of the four SHS questions helps clinicians determine which component of health is affected, and enables decision-making for therapeutic intervention in bowel inflammation or the need for psychological support. The SHS is comprehensive and simple to complete, and can quickly provide information for use in both clinical practice and clinical trials.

Peer-review

Both the total score for SHS and IBDQ in Crohn's disease and ulcerative colitis as well as reliability should be also presented as Bland-Altman plot.

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Prospective Study

Continuing episodes of pain in recurrent acute pancreatitis: Prospective follow up on a standardised protocol with drugs and pancreatic endotherapy

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Abstract

AIM

To assess the outcomes of drug therapy (DT) followed by pancreatic endotherapy for continuing painful episodes in recurrent acute pancreatitis.

METHODS

DT comprised of pancreatic enzymes and anti-oxidants failing which, endotherapy (ET; pancreatic sphincterotomy and stent placement) was done. The frequency of pain, its visual analogue score (VAS), quality of life (QoL), serum C peptide and faecal elastase were compared between baseline and after 1 year of follow up in all patients and in the two subgroups on DT and ET. Response was defined as at least 50% reduction in the severity of pain to below a score of 5.

RESULTS

Of the thirty nine patients analysed, 21 (53.9%) responded to DT and 18 (46.1%) underwent ET. The VAS for pain (7.0 ± 2.0 vs 1.3 ± 2.5 , $P < 0.001$) and the number of days with pain per month decreased [1.0 ($1.0, 2.0$) vs 1.0 ($0.0, 1.0$), $P < 0.001$], and the QoL scores [55.0 ($44.0, 66.0$) vs 38.0 ($32.00, 51.00$), $P < 0.01$] improved significantly during follow up. Similar

significant improvements were seen in patients in the subgroups of DT and ET except for QoL in ET. The serum C-peptide ($P = 0.001$) and FE ($P < 0.001$) levels improved significantly in the entire group and in the two subgroups of patients except for the C peptide levels in patients on DT.

CONCLUSION

A standardised protocol of DT, followed by ET decreased the intensity and frequency of pain in recurrent acute pancreatitis, enhanced QoL and improved pancreatic function.

Key words: Drug therapy; Endoscopy; Exocrine insufficiency; Pancreatic diabetes; Pancreatic duct stents; Quality of life; Recurrent acute pancreatitis; Surgery

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Core tip: This prospective case series provides evidence for the efficacy of a sequential approach to the treatment of patients with recurrent acute pancreatitis in whom painful episodes persisted after initial aetiological work up and appropriate interventions if any, with drugs and endoscopic therapy. Along with improvements in the intensity and average number of days with pain, the protocol also improved the quality of life, C-peptide levels and faecal elastase in these patients. The significance of our results needs to be explored in future studies on the effect of these interventions in preventing the progression of recurrent acute pancreatitis to chronic pancreatitis.

Pai CG, Kamath MG, Shetty MV, Kurien A. Continuing episodes of pain in recurrent acute pancreatitis: Prospective follow up on a standardised protocol with drugs and pancreatic endotherapy. *World J Gastroenterol* 2017; 23(19): 3538-3545 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3538.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3538>

INTRODUCTION

Recurrent acute pancreatitis (RAP) is an important cause of morbidity and mortality in gastroenterology practice^[1,2]. Many aetiological factors underlie RAP and a variable proportion of patients exhibit multiple causative factors. Up to a third of patients may have no cause evident and these have been variably designated as unexplained, idiopathic, or true idiopathic disease^[3-5]. Identifying the cause helps in unravelling the underlying patho-mechanisms and also directs therapy. Current recommendations on the treatment of RAP focus on the cause. However, the causative or therapeutic significance of some of these factors continue to be controversial. Biliary

sludge, crystals and microcalculi provide examples^[6,7]. Identifying some causative factors such as genetic mutations may not convert to effective therapy as of today. Similarly, while endoscopic sphincterotomy at the minor papilla appears to improve pain in patients with pancreas divisum presenting with RAP the very cause-effect relationship between these two conditions has been questioned^[8-10]. Patients may continue to smoke and drink despite advice to the contrary and even when they comply with such advice, painful episodes may continue to occur. Continuing attacks of pancreatitis even after an identified cause has been corrected suggest that other unrecognized or unknown factors may be operative in such patients. No therapy short of total pancreatectomy and islet cell transplantation is available for such patients who continue to have recurrent episodes of pancreatic pain^[7].

The natural history of acute pancreatitis (AP) and RAP progressing to chronic pancreatitis (CP) and the overlap in the causative factors of these three conditions suggest a continuum in their disease spectrum^[11]. The lack of definitive therapy in patients with idiopathic RAP and the continuing symptoms in some of those in whom the cause has been corrected means that these patients are potentially at risk of progression to CP with the consequent risks of developing pancreatic diabetes, steatorrhea and pancreatic cancer over time.

The mechanisms underlying inflammation and pain in RAP are poorly understood but are likely to overlap with those of CP^[12]. Supplementation of pancreatic enzymes and anti-oxidants, though controversial, are routinely recommended for the treatment of CP, but have not been tried in RAP^[13-15]. Endoscopic pancreatic sphincterotomy, an accepted therapy in CP has been used with variable success and attendant controversies, especially in the subgroups with pancreas divisum and sphincter of Oddi dysfunction^[5,8,16]. Most centers manage the pain of CP in a stepwise fashion once the underlying causative factors have been addressed - drug therapy with anti-oxidants and/or enzyme supplementation initially followed by endoscopic therapy and finally surgery for those who fail the former approaches^[17,18]. We hypothesized that patients with unexplained RAP and those in whom painful inflammatory episodes continue despite treatment of the identified causative factors may benefit from supplementation of pancreatic enzymes and anti-oxidants or endoscopic pancreatic sphincterotomy and temporary stent placement. This prospective case series was designed to assess the role of a standardized protocol of initial drug therapy (DT) followed by endoscopic therapy (ET) in those failing the former, in patients with continuing painful episodes of RAP even after initial work up for definite causative factors and treatment directed at any of these detected.

MATERIALS AND METHODS

Patients with RAP seen in the Department of Gastroenterology and Hepatology, Kasturba Hospital, Manipal University, Manipal, India between January 2013 and June 2014 were eligible for the study. An episode of pancreatitis was defined by any two of typical upper abdominal pain, elevation of serum amylase and lipase above three times the upper limit of normal and changes of pancreatitis on abdominal imaging. RAP was defined as 2 or more episodes of pancreatitis with complete resolution of symptoms in between in the absence of imaging changes of CP on at least two of the following imaging studies - abdominal ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography or endoscopic ultrasound (EUS). The patients underwent aetiological evaluation as per current standard of care^[3,4]. Sphincter of Oddi manometry and genetic testing were not routinely done. Patients with pancreatic or periampullary carcinoma were excluded. Those undergoing therapy specifically for pseudocysts, pancreatic ascites or pleural effusion were included only if they had recurrence of pancreatic pain after the fluid collections had been tackled. Patients with treatable causes such as bile duct stones, gall bladder microcalculi, hypercalcaemia, serum triglyceride levels more than 500 mg/dL were treated appropriately and included only if recurrent episodes of pancreatitis continued to occur^[19]. Those with alcoholic RAP underwent assessment and de-addiction therapy by a psychiatrist. Tobacco smokers were advised to discontinue smoking. Those with recurrence of an acute episode of pancreatitis after any treatable cause had been corrected and those in whom no treatable cause was evident were included.

Interventions

DT comprised of supplementation of pancreatic enzymes as 3 tablets (Digimax tablets, Shreya Life Sciences, Mumbai, containing protease activity of 93750 USP units per tablet) with meals per day and anti-oxidant capsules (Antoxid, Dr Reddy's Pharma, Hyderabad) three times a day. ET involved an initial pancreatogram for defining the ductal anatomy, a 3-4 mm long pancreatic sphincterotomy and the placement of a 5 cm long, 5 French pancreatic stent with multiple side holes and a single flange at the duodenal end. Biliary sphincterotomy was done only if the initial cannulation happened to be in the bile duct or if the pancreatic duct could not be accessed after repeated attempts^[20]. The stents were checked for spontaneous passage and removed if still seen *in situ* between 3 and 6 wk after placement. Response to therapy was defined as at least 50% reduction in the severity of pain [as defined by the visual analogue

score (VAS)] to a score below 5. Failure of DT, either as at entry to the study or after initiation into the study qualified the patient for ET. Those with no response to pain after initial ET were offered repeat endotherapy which involved sphincterotomy if stenosis of the pancreatic opening was encountered, and pancreatic stent placement since total pancreatectomy with islet cell transplantation is rarely performed in our country. Patients were explained the study protocol at entry, and written informed consent was obtained from all before enrollment. The protocol was approved by the Ethics Committee of Kasturba Hospital, Manipal.

Follow up

The patients were followed up at intervals of 6-12 wk or more frequently as clinically indicated. Follow up was continued for a minimum of 1 year on either of the two therapies. Abdominal pain was assessed at baseline and at each follow up visit for its severity using a VAS scale with a maximum score of 10^[21]. The number of days with pain since the last follow up was assessed at each visit and averaged to the number of days with pain per month for the entire period of follow up. Quality of life was assessed in all patients aged 18 years and above using the EORTC C 30 questionnaire at baseline, when there was a change of therapy and at the end of 1 year of follow on a given therapy. The EORTC C 30 questionnaire is a validated, self-administered questionnaire with 30 questions, available in the three languages spoken by our patients^[22].

Exocrine and endocrine function

Exocrine and endocrine functions were evaluated at baseline, when there was a change of therapy and at the end of 1 year of follow on a given therapy. Fasting plasma glucose and glycosylated haemoglobin (G-Hb) were used to diagnose diabetes mellitus as per the American Diabetes Association criteria^[23]. Serum C peptide levels were estimated in fasting morning samples by Enzyme Immunoassay (EIA) (Human C-Peptide EIA kit RayBio Norcross, United States) and values between 1.3-5.2 ng/mL were considered normal^[24]. Faecal elastase (FE) was estimated by enzyme-linked immunosorbent assay (ELISA) (Faecal Elastase 1 ELISA kit, ScheBo Biotech, Giessen, Germany) and a value less than 200 µg FE1/g was classified as exocrine insufficiency. All these tests were done at baseline, when there was a change of treatment and at the end of 1 year of follow up on a given treatment.

Statistical analysis

All study parameters were compared between two time points - at entry to either of the two therapies (DT or ET) and at the end of 1 year on the same therapy. The results are provided for all patients as a group and also separately in the two subgroups on DT and

Table 1 Characteristics of patients with recurrent acute pancreatitis completing the study *n* (%)

Number	39
Age in years, median (range)	26 (9-55)
Male:female	32 (82):7 (18)
Alcohol abuse	11 (28.2)
Smokers	10 (25.6)
Number of pain episodes (yr), median (range)	3.00 (1-30)
Duration of symptoms (mo), median (range)	12.00 (1-48)
Family history of pancreatitis	0
Drug therapy alone/endotherapy	21 (53.9)/18 (46.1)
Duration of follow up, median (range)	13 (12-24)

Table 2 Visual analogue score and average number of days with pain per month all patients with recurrent acute pancreatitis and in the subgroups

	Baseline	1 yr	<i>P</i> value
All patients (<i>n</i> = 39)			
VAS	7.7 (5.5, 8.3)	0 (0, 2)	< 0.001
Average number of days with pain per month	1.0 (1.0, 2.0)	1.0 (0, 1.0)	< 0.001
Patients on DT (<i>n</i> = 21)			
VAS	7.3 (5.1, 8.3)	0 (0, 2.4)	< 0.001
Average number of days with pain per month	2.0 (1.0, 2.0)	1.0 (0.0, 1.0)	< 0.01
Patients on ET (<i>n</i> = 18)			
VAS	7.1 (5.8, 8.4)	0 (0, 7.5)	< 0.01
Average number of days with pain per month	1.0 (1.0, 3.5)	1.0 (0.0, 1.0)	< 0.05

Data expressed as median (quartiles). VAS: Visual analogue scale.

ET. Continuous variables were expressed as median (quartiles) or as mean \pm SD and Wilcoxon Signed Rank Test or paired *t*-test were used as appropriate for comparison as appropriate. The package SPSS 16.0 was used for statistical analysis. The statistical review was performed by a biomedical statistician.

RESULTS

Patients

Forty five patients with RAP were enrolled of whom 39 (86.7%) who completed at least one year of follow up on either of the two therapies were analysed; the remaining 6 (13.3%) were lost to follow up. Eight (20.5%) of these were aged below 18 years. None had a family history of CP. The other characteristics of these patients are shown in Table 1.

Interventions

Twenty-one (53.9%) responded to drug therapy and did not undergo any further interventions. The other 18 (46.1%) underwent endoscopic therapy, 8 (20.5%) having already failed drug therapy at entry, and the rest failing drug therapy during the course of the study. The latter patients did not respond to DT over a median (quartiles) 3 (2.0, 5.0) mo, as evidenced by no improvement in the VAS 8.0 (5.9, 8.5) vs 6.6 (4.1, 8.0),

$P \geq 0.05$). All 18 in the endotherapy group underwent successful pancreatic sphincterotomy and stent placement. one (5.5%) patient had pancreas divisum and the sphincterotomy and stent placement were done at the minor papilla. Three (16.7%) patients on ET needed 1 additional endoscopic procedure and 1 (5.5%) needed 2 additional procedures during the one year follow up.

Pain

The VAS and the average number of days with pain per month decreased significantly in all patients with RAP at the end of follow up. Similar significant improvements were seen in the subgroups on DT and ET (Table 2).

Eleven (28.2%; 8 on DT and 3 on ET) patients had no recurrence of pain with appropriate therapy during the 1 year of follow up. Twenty one (53.9%, 13 on DT and 8 on ET) had partial relief of pain. None of these 32 patients needed re-admissions to the hospital for the control of pain. The remaining 7 (17.9%) failed both therapies. Four of these needed between 1 and 7 (median 2) re-admissions to the hospital for the control of acute episodes of pain.

Quality of life

The QoL scores improved significantly at the end of follow up in patients aged above 18 years (*n* = 31, 79.5%) and in the subgroup on DT (Table 3). However, the decrease seen in patients on ET alone did not reach statistical significance.

Pancreatic functions

No patient had diabetes mellitus or steatorrhea at baseline and none developed these sequelae during follow up. All patients had normal serum C peptide and FE levels at baseline. These parameters improved significantly in the entire group and in the two subgroups of patients except for the C peptide levels in patients on DT (Table 4).

Adverse events

Patients tolerated DT well and none discontinued drugs due to adverse events. Following ET, 3 (16.7%) patients developed acute exacerbation of pancreatitis, which subsided with conservative management. No other complications were encountered following ET.

DISCUSSION

By following up patients with RAP in whom painful episodes continued to occur after common, treatable causes had been ruled out or corrected, we have shown that more than three quarters of them improved on a standardized protocol of oral pancreatic enzyme replacement along with anti-oxidant supplementation followed by selective use of endoscopic pancreatic sphincterotomy and stent placement in non-responders

Table 3 Quality of life scores in patients with recurrent acute pancreatitis above the age of 18 years and in the subgroups on drug therapy and endoscopic therapy

	Baseline	1 yr	P value
All patients (n = 31)	55.0 (44.0, 66.0)	38.0 (32.00, 51.00)	< 0.01
Patients on DT (n = 22)	55.0 (47.0, 64.0)	40.00 (31.50, 54.00)	< 0.01
Patients on ET (n = 9)	59.5 (47.5, 67.5)	36.0 (32.50, 54.3)	0.084

Data expressed as median (quartiles). DT: Drug therapy; ET: Endoscopic therapy.

to the former therapy. The improvement in the pain was evidenced by a reduction in the VAS and the average number of days with pain per month, avoidance of hospitalisation for the control of pain in the responders and also an attendant improvement in the QOL. Such a stepwise approach to the management of pain has been previously described in patients with CP^[17,18,20]. However, this is the first time a similar approach has been shown to be effective in the treatment of the pain of RAP. This is also probably the first report on the response to the use of pancreatic enzymes and anti-oxidants in the treatment of RAP. Controversies surround the significance of the entity of sphincter of Oddi dysfunction (SOD) and the usefulness of endoscopic therapy for RAP with or without concomitant SOD^[16,25,26]. Given such controversies our results show that a difficult to manage subgroup of patients with RAP can be treated successfully using the protocol we used.

Pancreatic enzymes and anti-oxidants are often used for the treatment of pain in CP though their exact role remains controversial. A Cochrane review concluded that the former therapy is no better than placebo^[13]. The conflicting results of two recent, large trials on anti-oxidant therapy for CP appears to translate into only a small benefit in a meta-analysis^[14,15,27]. Nonetheless, our results indicate that randomised controlled studies with these drugs for the management of RAP are warranted in the future.

The role of endoscopic pancreatic sphincterotomy in the treatment of RAP is controversial. The response to pancreatic sphincterotomy or stent placement have been variably reported in 50%-100% of patients with idiopathic RAP irrespective of whether they had SOD or not in various case series^[4,5,28]. In a recent randomised trial, combined pancreatic and biliary sphincterotomy was no better than biliary sphincterotomy alone in patients with RAP and SOD, either treatment relieving pain in about half the patients^[16]. However, patients without SOD underwent only biliary but not pancreatic sphincterotomy in this study. Some of the differences in the outcomes of pancreatic endotherapy in RAP in different studies could be because of the differences in the patients enrolled. The patients who qualified for our study had few therapeutic options available

to them short of total pancreatectomy and islet cell transplantation.

RAP is a condition with diverse aetiologies and consequently one with variable natural history. The mechanisms underlying the pain in RAP are complex and not fully understood, but are likely to be similar to those in CP^[21,29,30]. Being those in whom painful episodes continued after an initial evaluation for causative factors and their treatment, the patients in the present study were uniform in one sense. No clear cut recommendations are available as to how to treat these patients short of total pancreatectomy and islet cell transplantation, a procedure available only in a few centres. On the other hand the age range of the patients was wide and the proved or presumed causative factors such as alcohol abuse, tobacco smoking or pancreas divisum were seen in varying proportions thereby suggesting that the group was diverse. The fact that more than 80% of the patients showed a complete or partial response during follow up suggests however that the treatment approach we followed is effective. The reason for this could be that the therapies we used targeted specific common pathways leading to recurrent episodes of pain in RAP irrespective of the etiology. For example the negative feedback induced by the enzyme supplementation and the reduction in the pancreatoduodenal pressure gradient brought about by the pancreatic sphincterotomy could both have acted by decreasing the pancreatic ductal pressure irrespective of whether SOD was present in our patients or not^[21,31].

Admittedly, the small numbers included in our study and the lack of a control group are its obvious limitations, especially because long, pain free intervals can occur spontaneously in RAP. Also, it is possible that some of the response seen could be attributed to the rigorous follow up and also the resultant close monitoring of compliance with abstinence from alcohol and tobacco use. Nonetheless, it cannot be forgotten that the type of patients studied have almost no treatment options left and in this sense form a particularly difficult-to-treat group. An example is the group of patients with alcohol or smoking as causative factors who continued to have painful episodes of pancreatitis despite initial interventions such as alcohol deaddiction therapy and advice on tobacco abuse. Nonetheless, the relative role of abstinence from alcohol or smoking, pancreatic enzyme supplementation and anti-oxidant therapy can only be teased out in larger, randomised controlled trials which, for obvious reasons, are not easy to conduct.

The significant improvement in serum C peptide and FE levels we have shown on follow up compared to baseline are probably being reported for the first time in RAP. Their significance can be questioned since none of the patients in the present study had pancreatic insufficiency to begin with, which is an expected line. But these results are also interesting because

Table 4 Comparison of serum C peptide and faecal elastase levels at baseline and end of follow up

	Baseline	1 yr	P value
All patients (<i>n</i> = 39)			
C Peptide (35)	3.2 (2.8, 4.3)	6.4 (2.6, 11.5)	0.001
F Elastase (38)	401.94 (215.5, 484.8)	559.6 (411.3, 597.4)	< 0.001
Patients on DT (<i>n</i> = 21)			
C Peptide (21)	4.13 (3.11, 4.35)	4.47 (2.55, 11.65)	0.079
F Elastase (24)	406.18 (220, 496.43)	559.55 (442.24, 597.30)	0.002
Patients on ET (<i>n</i> = 14)			
C Peptide (14)	2.85 (2.15, 3.53)	7.52 (2.33, 10.35)	0.004
F Elastase (14)	335.87 (207.3, 481.41)	562.70 (265.47, 597.35)	0.006

Data expressed as median (quartiles). DT: Drug therapy; ET: Endoscopic therapy.

it is reasonable to attribute such improvements to the reduction in the repeated episodes of pain and the associated inflammation within the pancreatic parenchyma. Mild, transient exocrine and endocrine dysfunction are known following acute episodes of pancreatitis and progression of RAP to CP has been attributed to recurrent episodes of inflammation^[18,32,33]. Also, progression of pancreatic insufficiency has been associated with recurrent painful episodes in patients with CP^[34]. Such data from these diverse studies taken together raise the possibility that interventions which decrease the painful episodes in RAP could possibly also prevent its progression to CP. Our results should provide the impetus for undertaking such long term studies to evaluate the effect of successful interventions that decrease pain and inflammation in RAP on its progression to CP.

In conclusion, a standardised protocol of DT with pancreatic enzymes and anti-oxidant supplementation followed by ET with pancreatic sphincterotomy and temporary stent placement in the non-responders to the former decreases the intensity and average number of days with pain per month, avoids repeated hospitalisations in those who respond, improves pancreatic exocrine and endocrine functions and enhances QoL. Our results pave the way for larger, randomised trials that can evaluate the effect of these therapeutic interventions on the progression of RAP to CP.

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COMMENTS

Background

Recurrent acute pancreatitis (RAP) is an important cause of morbidity and mortality in gastroenterology practice. Continuing attacks of pancreatitis even after an identified cause has been corrected suggest that other unrecognized or unknown factors may be operative in such patients. No therapy short of total pancreatectomy and islet cell transplantation is available for such patients who continue to have recurrent episodes of pancreatic pain.

Research frontiers

Many aetiological factors underlie RAP and a variable proportion of patients exhibit multiple causative factors. Up to a third of patients may have no cause evident and these have been variably designated as unexplained, idiopathic, or true idiopathic disease. Current recommendations on the treatment of RAP focus on the cause. However, the causative or therapeutic significance of some of these factors continues to be controversial. So it is important to understand the role of standardized therapy in patients suffering due to RAP.

Innovations and breakthroughs

This study focussed on the role of a standardized protocol of initial drug therapy (DT) followed by endoscopic therapy (ET) in those failing the former, in patients with continuing painful episodes of RAP even after initial work up and treatment of definite causative factors. In this study, they have shown that more than three quarters of them improved on a standardized protocol of oral pancreatic enzyme replacement along with anti-oxidant supplementation followed by selective use of endoscopic pancreatic sphincterotomy and stent placement in non-responders to the former therapy. The improvement in the pain was evidenced by a reduction in the pain scores and the average number of days with pain per month, avoidance of hospitalisation for the control of pain in the responders and also an attendant improvement in the quality of life (QoL). Such a stepwise approach to the management of pain has been previously described in patients with chronic pancreatitis (CP). However, this is the first time a similar approach has been shown to be effective in the treatment of the pain of RAP. This is also probably the first report on the response to the use of pancreatic enzymes and anti-oxidants in the treatment of RAP.

Applications

A standardised protocol of DT with pancreatic enzymes and anti-oxidant supplementation followed by ET with pancreatic sphincterotomy and temporary stent placement in the non-responders to the former decreases the intensity and average number of days with pain per month, avoids repeated hospitalisations for those in pain who respond, improves pancreatic exocrine and endocrine functions and enhances QoL. The results pave the way for larger, randomised trials that can evaluate the effect of these therapeutic interventions on the progression of RAP to CP.

Terminology

DT in this study comprised of supplementation of pancreatic enzymes as 3 Tablets (Digimax tablets, containing protease activity of 93750 USP units per tablet) with meals per day and anti-oxidant capsules (Antoxid) three times a day. ET involved an initial pancreatogram for defining the ductal anatomy, a 3 mm - 4 mm long pancreatic sphincterotomy and the placement of a 5 cm long 5 French pancreatic stent with multiple side holes and a single flange at the duodenal end. Biliary sphincterotomy was done only if the initial cannulation happened to be in the bile duct or the pancreatic duct could not be accessed after repeated attempts. The stents were checked for spontaneous passage and removed if still seen in situ between 3 and 6 wk after placement. Response to therapy was defined as at least 50% reduction in the severity of pain (as defined by visual analogue score) to a score below 5. Failure of DT, either as at entry to the study or after initiation into the study qualified the patient for ET.

Those with no response to pain after initial ET were offered repeat endotherapy which involved sphincterotomy if stenosis of the pancreatic opening was encountered and pancreatic stent placement since total pancreatectomy with islet cell transplantation is rarely performed in our country.

Peer-review

This article is unique since it emphasises on the use of a standardised protocol of DT followed by ET for those suffering in recurrent pain due to RAP. The results show that DT and/or ET helps to improve the pancreatic exocrine and endocrine functions and enhance the QoL of these patients.

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Prospective Study

Nissen fundoplication *vs* proton pump inhibitors for laryngopharyngeal reflux based on pH-monitoring and symptom-scale

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Abstract

AIM

To compare the outcomes between laparoscopic Nissen fundoplication (LNF) and proton pump inhibitors (PPIs) therapy in patients with laryngopharyngeal reflux (LPR) and type I hiatal hernia diagnosed by oropharyngeal pH-monitoring and symptom-scale assessment.

METHODS

From February 2014 to January 2015, 70 patients who were diagnosed with LPR and type I hiatal hernia and referred for symptomatic assessment, oropharyngeal pH-monitoring, manometry, and gastrointestinal endoscopy were enrolled in this study. All of the patients met the inclusion criteria. All of the patients

underwent LNF or PPIs administration, and completed a 2-year follow-up. Patients' baseline characteristics and primary outcome measures, including comprehensive and single symptoms of LPR, PPIs independence, and satisfaction, and postoperative complications were assessed. The outcomes of LNF and PPIs therapy were analyzed and compared.

RESULTS

There were 31 patients in the LNF group and 39 patients in the PPI group. Fifty-three patients (25 in the LNF group and 28 in the PPI group) completed reviews and follow-up. Oropharyngeal pH-monitoring parameters were all abnormal with high acid exposure, a large amount of reflux, and a high Ryan score, associated reflux symptom index (RSI) score. There was a significant improvement in the RSI and LPR symptom scores after the 2-year follow-up in both groups ($P < 0.05$), as well as typical symptoms of gastroesophageal reflux disease. Improvement in the RSI ($P < 0.005$) and symptom scores of cough ($P = 0.032$), mucus ($P = 0.011$), and throat clearing ($P = 0.022$) was significantly superior in the LNF group to that in the PPI group. After LNF and PPIs therapy, 13 and 53 patients achieved independence from PPIs therapy (LNF: 44.0% vs PPI: 7.14%, $P < 0.001$) during follow-up, respectively. Patients in the LNF group were more satisfied with their quality of life than those in the PPI group (LNF: 62.49 ± 28.68 vs PPI: 44.36 ± 32.77 , $P = 0.004$). Body mass index was significantly lower in the LNF group than in the PPI group (LNF: 22.2 ± 3.1 kg/m² vs PPI: 25.1 ± 2.9 kg/m², $P = 0.001$).

CONCLUSION

Diagnosis of LPR should be assessed with oropharyngeal pH-monitoring, manometry, and the symptom-scale. LNF achieves better improvement than PPIs for LPR with type I hiatal hernia.

Key words: Laryngopharyngeal reflux; Hiatal hernia; Laparoscopic Nissen fundoplication; Proton pump inhibitor; pH-monitoring; Gastroesophageal reflux disease

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Core tip: Laryngopharyngeal reflux disease is often associated with hiatal hernia and gastroesophageal reflux disease. Although the role of oropharyngeal pH-monitoring in the diagnosis of laryngopharyngeal reflux is clear, little is known regarding the anti-acid and anti-reflux therapeutic outcome by pH-monitoring and symptom-scale diagnosis. Laparoscopic Nissen fundoplication and proton pump inhibitors (PPIs) are effective in patients with laryngopharyngeal reflux and type I hiatal hernia. Nissen fundoplication shows better symptom relief than PPIs administration, and it also controls body mass index of patients. Our findings shed new insight into diagnosis and management for patients with laryngopharyngeal reflux disease.

Zhang C, Hu ZW, Yan C, Wu Q, Wu JM, Du X, Liu DG, Luo T, Li F, Wang ZG. Nissen fundoplication vs proton pump inhibitors for laryngopharyngeal reflux based on pH-monitoring and symptom-scale. *World J Gastroenterol* 2017; 23(19): 3546-3555 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3546.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3546>

INTRODUCTION

Laryngopharyngeal reflux (LPR) is a common condition in patients with gastroesophageal reflux disease (GERD). The effect of GERD on the upper aero-digestive tract seriously affects the quality of life of patients, with symptoms such as hoarseness, rhinitis, pharyngalgia, foreign body sensation, throat clearing, chronic cough, and laryngospasm^[1,2]. All of these clinical presentations of LPR are considered as extraesophageal symptoms for distinguishing typical symptoms of GERD, such as heartburn and regurgitation. The incidence rate of reflux-induced laryngitis ranges from 18%-80%^[3,4]. The association between GERD and hiatal hernia has been well confirmed, including sliding hernia (type I), paraesophageal hernia (type II), and mixed hernia (types III and IV)^[5]. Recently, evidence has suggested that hiatal hernia is one of the major risk factors for the occurrence of LPR in patients with GERD^[4]. The management strategies for LPR, GERD, and type I hiatal hernia are similar, which involve controlling the occurrence of reflux and reducing reflux-induced symptoms. Common management methods include lifestyle modification, anti-acid therapy, and anti-reflux surgery. To the best of our knowledge, few studies have focused on the treatment outcome of patients with LPR and type I hiatal hernia, especially regarding comparison of anti-acid therapy and anti-reflux surgery with lifestyle modification.

Currently, diagnosis of LPR mainly includes empirical therapeutic trials of proton pump inhibitors (PPIs), the reflux symptom index (RSI) score, pH-monitoring and laryngoscopy^[6]. Among these diagnostic methods, PPIs do not have objective evidence for diagnosis of LPR. Additionally, the placebo effect of anxiety in patients without LPR cannot be excluded. Laryngoscopic findings, such as erythema and edema, are also nonspecific signs of LPR^[7]. The reflux finding score, a clinical severity rating scale based on laryngoscopic findings, has poor reliability in detecting LPR^[8,9]. Monitoring of pH can directly detect increased esophageal or laryngopharyngeal acid exposure by a pH probe. Therefore, it is regarded as the best evidence for diagnosis of LPR^[10]. Some studies have documented LPR using a new pH sensor^[9,11] and others have investigated the pH threshold for identifying patients with an abnormal pharyngeal pH environment^[12,13]. However, few studies have shown the therapeutic outcome in patients with LPR who were diagnosed by

oropharyngeal pH-monitoring. Moreover, abnormal laryngopharyngeal acid exposure can indicate the presence of pathological reflux, but it does not provide proof of causality for symptoms of LPR. Therefore, evidence of LPR should be combined with pH-monitoring and special symptom-scales, such as the RSI score^[14] and single symptom score^[15].

Therefore, in this study, we investigated two different therapeutic strategies for LPR with hiatal hernia: anti-reflux surgery and anti-acid therapy both with lifestyle modifications. We assessed the postoperative 6-mo and 2-year outcomes based on diagnosis by oropharyngeal pH-monitoring and the symptom-scales. In particular, we analyzed the integrated results of pH-monitoring, manometry, and endoscopy, which may demonstrate the characteristics of patients with LPR and hiatal hernia.

MATERIALS AND METHODS

Patients

Medical records of 70 patients with LPR and type I hiatal hernia who manifested laryngopharyngeal symptoms, who underwent laparoscopic Nissen fundoplication (LNF), or were administered PPIs therapy alone between February 2014 and January 2015, were obtained. The following criteria were met before enrolment: Patients complaint with laryngopharyngeal symptoms (hoarseness, globus, throat clearing/pain, mucus, and chronic cough) were suspected by the otolaryngologist, the LPR symptom occurred at least once a week, and lasted at least 6 mo; RSI score ≥ 13 ; type I hiatal hernia (increase of the squamo-columnar junction by > 2 cm, and without paraesophageal hernia); absence of significant esophagitis (Los Angeles grades A and B esophagitis); abnormal Ryan score during 24-h oropharyngeal pH-monitoring; and abnormal lower esophageal sphincter (LES) pressure as detected by esophageal manometry. Patients were symptomatically stable and generally medically fit for anti-acid or surgical anti-reflux treatments. Patients with broncho-pulmonary disease, central nervous system diseases, connective tissue diseases, previous pharyngolaryngeal, esophageal or gastric surgery, esophageal stricture, a shortened esophagus, impaired distal esophageal peristalsis, Barrett's esophagus, autoimmune diseases, collagen vascular disease, and/or coagulation disorders were excluded. This prospective, observational study was approved by the Institutional Review Board at the Second Artillery General Hospital of Chinese People's Liberation Army and Xuanwu Hospital. Informed consent was obtained from each participant according to the Helsinki Declaration.

Oropharyngeal pH-monitoring

The diagnosis of LPR was confirmed using the 24-h oropharyngeal Restech pH recorder system (Respiratory Technology Corp., San Diego, CA,

United States) and LPR symptom-scale (outcome assessment). Patients were instructed to stop taking any anti-acid medications at least 1 wk before insertion of the probe. The pH probe was inserted through the patient's nose and advanced slowly to its destination in the back of the oropharynx just 5 mm below the tip of the uvula. Placement of the probe in the oropharynx was verified by observing the flashing light at the tip of the probe. Patients were asked to keep a diary indicating the time of meals and the time spent in the supine and upright positions. Meal periods were excluded in the final analyses. The data recorder was downloaded to a dedicated software program (DataView Lite V3; Respiratory Technology Corp.) and correlated with the patient's diary. Tracings were all manually evaluated by a single operator. Thresholds for the detection of acidic reflux were 5.5 for the upright position and 5.0 for the supine position. The percentage of time spent below these thresholds was then calculated. The Ryan score was also calculated using the same pH thresholds for the upright and supine positions. This score was obtained by combining the following three different parameters: (1) the number of reflux episodes; (2) the duration of the longest reflux episode; and (3) the percentage of time spent below the defined threshold. A score greater than 9.41 in the upright position and/or 6.81 in the supine position was regarded as LPR^[12,16].

High-resolution manometry and gastrointestinal endoscopy

A solid-state manometric catheter assembly with 36 circumferential sensors spaced in 1-cm intervals was used (Sierra Scientific Instruments, Los Angeles, CA, United States). Before the recording, the transducers were calibrated, and a thermal compensation program was applied using external pressure. The catheter was passed *via* the nose and positioned to provide simultaneous recordings from the hypopharynx and the esophagus to the stomach. Ten 5-mL water swallows were provided to evaluate peristalsis. Upper esophageal sphincter (UES) and LES pressure, the size of hiatal hernia, and esophageal body contractions were recorded for data analysis. Moreover, hiatal hernia, reflux esophagitis, and esophageal metaplasia were determined by gastrointestinal (GI) endoscopy, which was independent of pH-monitoring and manometry. If esophagitis was present, it was graded according to the Los Angeles classification^[17].

Treatment

Patients were allocated to the PPI or LNF group according to their own preference and physical conditions after the following instructions: PPIs medication focused on the anti-acid, which need life-long medication but could not cause other damage or complication on upper gastrointestinal, whereas, LNF was an invasive operation, aiming to make a one-way flap by fundus for anti-reflux, with more possibility of injury and

complications, but a lower recurrence rate. Of the 70 patients, 39 were treated with esomeprazole 40 mg every day for 61–96 d (mean, 78 d). LNF was carried out in the remaining 31 patients. Briefly, LNF was performed with five ports under general anesthesia. After dissecting the gastrohepatic ligament with a harmonic scalpel, a widow was created behind the lower esophagus. The diaphragmatic crura were then carefully dissected and the distal esophagus was mobilized at approximately 5 cm. In all cases, the gastric fundus was dissected by dividing short gastric vessels. The diaphragmatic crura were sewn behind the esophagus with 1–2 non-absorbable sutures. A posterior 360° with a 2-cm-long fundoplication was constructed with 2–3 interrupted non-absorbable stitches. After operation, omeprazole 40mg i.v. was administered once for gastric mucosal protection.

We also suggested that lifestyle modifications (head elevation during bedtime, no fatty foods and eating close to bedtime, eating more frequently with smaller meals, and reduction of cigarettes, alcohol, or caffeine) should be adopted for all of the patients. Body mass index [BMI, body weight (kg) divided by the square of standing height (m)] was calculated before treatment and after treatment at a 2-year follow-up.

Assessment of outcome

Comprehensive symptom LPR was evaluated on the basis of symptom scoring using the RSI. The RSI accurately documents symptoms with LPR with a nine-item self-administered outcome instrument. An RSI score greater than 13 is considered to indicate LPR^[18,19]. The single symptom score was used to measure the frequency and severity of each symptom, including heartburn, regurgitation, cough, globus, mucus, hoarseness, throat pain and clearing. Data on these outcome measures were collected through a standardized questionnaire as previously described^[20,21]. More specifically, the total of the frequency score (5 points) and the severity score (5 points) for each of these measures was designed as the symptom score out of 10 points. The questionnaires were prepared in simplified Chinese and administered to the patients before and after treatment. Other outcome measures included PPI independence (PPIs was prescribed and administered continually over 3 d for recurrent GERD and LPR symptoms that were excluded from PPI independence in LNF or PPI group), satisfaction, and complications.

Statistical analysis

Data are expressed as mean \pm SD or number (%) unless specified otherwise. For statistical analyses, normality was assessed by the Kolmogorov-Smirnov test. Data were analyzed by the independent-/paired-sample Student's *t* test (Table 1) or nonparametric tests (Tables 2–4, Figures 1 and 2) based on the normality of data distribution. Independent-sample *t*-test and Mann-Whitney *U* test were performed for

independent samples in LNF and PPI groups (Figures 1 and 2, Tables 2–4), whereas paired-sample *t* test and the Wilcoxon test for within-group paired samples (Table 4). The statistical analysis software, SPSS-17.0 (SPSS Inc., Chicago, IL, United States), was used. Statistical review of the study was performed by a professional statistician. Differences were considered significant when $P < 0.05$.

RESULTS

Baseline measurements

Consecutive patients who were diagnosed with LPR with type I hiatal hernia and met our inclusion criteria were enrolled between February 2014 and January 2015 in this study. Patients were divided into two groups based on the patients' choice to undergo an LNF surgery or PPIs administration. A total of 39 patients were included in the PPI group and 31 patients were included in the LNF group. A total of 61 patients were still in the study at the 6-mo follow-up, and 53 patients (25 patients in the LNF group and 28 patients in the PPI group) completed the 2-year follow-up (follow-up time ranged from 1.7 to 2.5 years; average of 2 years). The demographic data for each group are listed in Table 1. Baseline demographic data were similar between the LNF and PPI groups, including the mean age, sex distribution, and pre-treatment values for the RSI and BMI. And 66.7%–74.2% patients also suffered from typical GERD symptoms (regurgitation and/or heartburn) in the LNF and PPI groups. The number of presenting complaints and the RSI were not significantly different between the two groups, except for globus (LNF group: 27/31 vs PPI group: 20/39, $P = 0.003$).

Characteristics of diagnostic examinations

The results of diagnostic examinations are summarized in Table 2, including 24-h oropharyngeal pH-monitoring, high-resolution manometry, and GI endoscopy. Almost all of the patients demonstrated more reflux events and a longer duration of acid exposure in the upright position, with a much higher Ryan value than the standard upright-threshold (Ryan score = 9.41), regardless of the groups. The mean supine Ryan score still exceeded 6.81, which was the upper limit of the normal value. Although the presenting complaints varied, there was no significant difference in pH-monitoring between the two groups. For manometric investigation, 13 of 70 patients presented with ineffective or abnormal esophageal motility, with a significant difference between the two groups (LNF group: 3/31 vs PPI group: 10/39, $P = 0.090$). LES pressure values ranged from 11.78 to 14.84 mmHg, which were lower than the normal value^[22,23]. UES pressure was lower in the LNF group than in the PPI group ($P = 0.045$). Hiatal hernia was assessed by high-resolution manometry and GI endoscopy. Manometry showed hiatal hernia in 57/70 patients (LNF

Table 1 Baseline demographics, clinical characteristics and proportions for laparoscopic Nissen fundoplication and proton pump inhibitor groups

Characteristic/parameter	LNF	PPI	P value
No.	31	39	
Age (yr)	47.2 ± 10.7	51.3 ± 12.5	0.218
Sex			0.670
Male	14 (45.2)	19 (48.7)	
Female	17 (54.8)	20 (51.3)	
BMI (kg/m ²)	23.9 ± 3.8	25.0 ± 3.1	0.285
RSI score	15.3 ± 3.5	14.2 ± 4.0	0.759
Presenting complaint			
Regurgitation ¹	21 (67.7)	26 (66.7)	0.926
Heartburn ¹	23 (74.2)	28 (71.8)	0.826
Cough	18 (58.1)	21 (53.8)	0.729
Mucus	14 (45.1)	12 (30.7)	0.222
Globus	27 (87.1)	20 (51.3)	0.003 ²
Hoarseness	12 (38.7)	10 (25.6)	0.248
Throat pain	12 (38.7)	10 (25.6)	0.248
Throat clearing	10 (32.2)	9 (23.1)	0.398

¹Represents gastroesophageal reflux disease typical symptoms; ²Represent statistical significant *P* values (*P* < 0.05). Values are given as mean ± SD or *n* (%). LNF: Laparoscopic Nissen fundoplication; PPI: Proton pump inhibitor; BMI: Body mass index; RSI: Reflux symptom index.

group: 24/31, PPI group: 33/39). However, endoscopy showed endoscopic hiatal hernia in 46/70 patients (LNF group: 16/31, PPI group: 30/39). Additionally, 13 patients in the LNF group and 12 in the PPI group had esophagitis as shown by endoscopy (Table 2).

Efficacy

When we completed the 2-year follow-up, all of the data were collected to assess the efficiency of controlling symptoms of LPR. To assess relief from symptoms, we evaluated each symptom *via* a questionnaire that was scaled by frequency and severity. There were no significant differences in the pre-treatment symptom scores between patients in the PPI and LNF groups. The LPR and GERD typical symptom scores for cough, mucus, globus, hoarseness, and throat pain and clearing improved in both groups at the 6-mo and 2-year follow-up. The overall mean value of the symptom score decreased from 7.71 to 1.12 after both treatments (Table 3). Evaluation at the 2-year follow-up showed significantly better improvement in cough, mucus, and throat clearing of the LNF group than in the PPI group (Figure 1), as well as typical symptoms of GERD, including regurgitation and heartburn (Figure 2). The symptom scores for globus, hoarseness and throat pain were not significantly different between the two groups. However, the post-treatment symptom score for globus was lower in the LNF group than in the PPI group (LNF group: 2.95 ± 2.75 vs PPI group: 5.43 ± 2.50, *P* = 0.013 at 6 mo and LNF group: 2.77 ± 2.87 vs PPI group: 5.28 ± 2.86, *P* = 0.017 at 2 years, Table 3). We also observed no improvement in the LPR and GERD symptom scores in a few patients in both groups. This finding indicated no effect of LNF or PPI in

Table 2 Characteristics on the oropharyngeal pH-monitoring, manometry and endoscopy between laparoscopic Nissen fundoplication and proton pump inhibitor groups

Characteristic/examination parameter	LNF	PPI	P value
Oropharyngeal pH-monitoring			
Acid exposure (upright, %)	11.77 ± 18.95	8.49 ± 15.66	0.416
Acid exposure (supine, %)	4.25 ± 12.79	3.28 ± 7.92	0.182
Number of reflux events (upright)	53.84 ± 97.48	34.93 ± 65.35	0.195
Number of reflux events (supine)	7.44 ± 18.42	5.14 ± 9.22	0.232
Ryan score (upright)	335.13 ± 491.08	274.57 ± 459.10	0.617
Ryan score (supine)	8.98 ± 16.18	7.23 ± 7.81	0.217
High-resolution Manometry			
LES pressure (mmHg)	11.78 ± 8.07	14.84 ± 9.73	0.236
UES pressure (mmHg)	43.8 ± 28.33	67.08 ± 42.51	0.045 ¹
Dysperistalsis	3 (9.7)	10 (25.6)	0.090 ¹
Hiatal hernia	24 (77.4)	33 (84.6)	0.449
GI endoscopy			
Esophagitis (grade A)	10 (32.2)	10 (25.6)	0.549
Esophagitis (grade B)	3 (9.7)	2 (5.1)	0.470
Hiatal hernia	16 (51.6)	30 (76.9)	0.027 ¹

¹Represent statistical significant *P* values (*P* < 0.05). Values are given as mean ± SD or *n* (%). LNF: Laparoscopic Nissen fundoplication; PPI: Proton pump inhibitor; LES: Lower esophageal sphincter; UES: Upper esophageal sphincter.

three patients (Figures 1 and 2).

Comprehensive assessment and satisfaction

The comprehensive LPR diagnostic scale of the RSI score was assessed in this study. All 70 patients reported one or more symptoms that were included in the RSI scale. Forty-three (81.1%) patients had an RSI score ≥ 13 during the 2-year follow-up. The RSI score decreased after treatment in the LNF and PPI groups. Importantly, the mean RSI score in patients who had LNF surgery was significantly lower (9.7 ± 4.1) than that in patients who had PPIs administration (12.8 ± 3.1) at the 2-year follow-up (*P* = 0.004). Similar results were observed in the rate of a positive RSI score between the two groups (*P* = 0.003). Interestingly, 11 (44.0%) patients in the LNF group achieved independence of PPIs at the 2-year follow-up. However, only two (7.1%) patients were completely weaned off of PPIs in the PPI group (*P* < 0.001). Moreover, we found that the mean BMI was significantly decreased after LNF compared with before LNF (24.9 kg/m² vs 22.2 kg/m², *P* < 0.001). However, the mean BMI of the PPI group did not significantly change before and after treatment (25.0-25.1 kg/m², *P* = 0.991). There was a significant difference in BMI between the LNF and PPI groups at the 2-year follow-up (LNF group: 22.2 ± 3.1 vs PPI group: 25.1 ± 2.9, *P* = 0.001), but not at pre-treatment (Table 4).

From baseline to the 2-year follow-up, the mean satisfaction score of patients improved by 62.49 ± 28.68 in the LNF group and 44.36 ± 32.77 in the PPI group. Patients were more satisfied with their quality of life after undergoing LNF than with PPI therapy (*P* = 0.004, Table 4). However, three (12%) patients

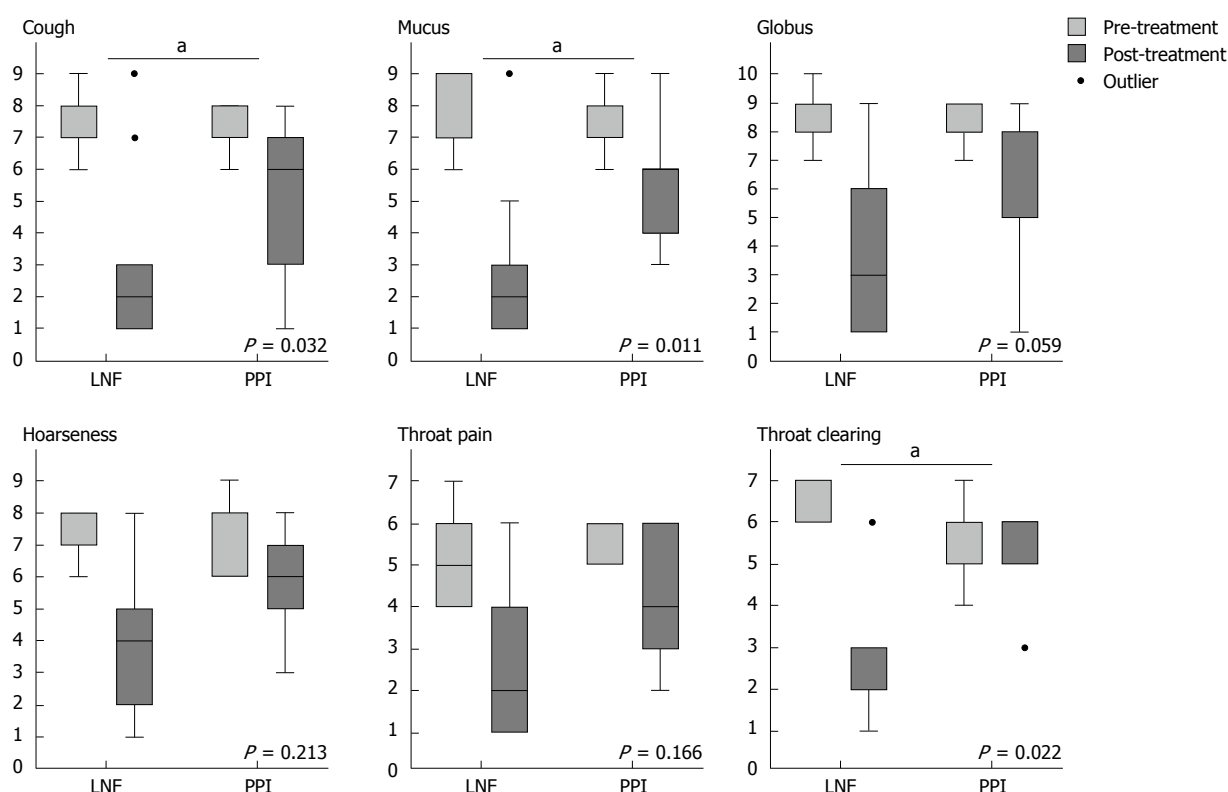


Figure 1 Comparison of the laryngopharyngeal reflux symptom score between the laparoscopic Nissen fundoplication and proton pump inhibitor groups before treatment and at the 2-year follow-up. Range, upper and lower quartiles, and median values are shown. Represents significant P values ($^aP < 0.05$) for a difference in improvement of symptoms between the LNF and PPI groups. P values are also shown in the right lower corner of each box. LNF: Laparoscopic Nissen fundoplication; PPI: Proton pump inhibitor.

Table 3 Comparison of the laryngopharyngeal and gastroesophageal reflux disease typical symptom score between laparoscopic Nissen fundoplication and proton pump inhibitor groups before treatment and at 6-mo and 2-year follow-up

Characteristic/symptom score (No. of LNF/PPI)	Baseline ($n = 70$)			6-mo follow-up ($n = 61$)			2-yr follow-up ($n = 53$)		
	LNF	PPI	P value	LNF	PPI	P value	LNF	PPI	P value
Regurgitation ² (17/20)	6.47 ± 0.62	6.24 ± 0.56	0.385	1.41 ± 1.69	2.53 ± 1.15	0.022 ¹	1.12 ± 1.47	2.53 ± 1.26	0.005 ¹
Heartburn ² (19/20)	6.33 ± 0.68	6.30 ± 0.59	0.100	1.33 ± 1.06	2.60 ± 1.69	0.008 ¹	0.94 ± 1.10	3.05 ± 2.20	0.001 ¹
Cough (15/16)	7.71 ± 0.82	7.67 ± 0.46	0.804	2.34 ± 2.37	4.40 ± 2.10	0.022 ¹	2.28 ± 2.12	5.00 ± 2.28	0.012 ¹
Mucus (11/9)	7.09 ± 0.83	7.22 ± 0.44	0.824	2.82 ± 2.24	4.77 ± 1.85	0.055 ¹	3.27 ± 2.18	5.29 ± 1.78	0.020 ¹
Globus (23/14)	6.10 ± 0.66	7.01 ± 0.88	0.268	2.95 ± 2.75	5.43 ± 2.50	0.013 ¹	2.77 ± 2.87	5.28 ± 2.86	0.017 ¹
Hoarseness (10/7)	7.30 ± 2.78	7.33 ± 2.55	0.954	3.80 ± 2.69	5.00 ± 2.65	0.409	3.50 ± 2.76	4.33 ± 2.53	0.546
Throat pain (11/4)	7.20 ± 1.03	7.50 ± 0.58	0.552	3.70 ± 2.98	5.50 ± 2.65	0.100	3.20 ± 3.46	4.25 ± 2.06	0.166
Throat clearing (6/7)	7.67 ± 0.52	7.14 ± 0.52	0.063	3.33 ± 2.06	6.00 ± 1.53	0.034 ¹	2.83 ± 2.40	6.28 ± 1.51	0.020 ¹

¹Represent statistical significant P values ($P < 0.05$); ²Represents gastroesophageal reflux disease typical symptoms. Values are given as mean ± SD. LNF: Laparoscopic Nissen fundoplication; PPI: Proton pump inhibitor.

suffered from severe dysphagia after LNF surgery, and this was relieved after bougie dilation treatment. No patients experienced perforation, infection, or death.

DISCUSSION

LPR remains a controversial issue with inconsistent data on epidemiology, etiology, diagnosis, and management, even though LPR and GERD are both caused by reflux of stomach contents. The association between GERD and hiatal hernia is well known; hiatal hernia is present in 83% of patients with GERD.

Additionally, the prevalence of GERD is 68% in patients with hiatal hernia^[24,25]. Recent studies have shown that LPR is found in 70% of patients with GERD, 53% patients with GERD and LPR have hiatal hernia, and approximately 50% of patients with GERD and hernia have common symptoms of LPR^[4,26]. In our study, we found that 67.7%-74.2% of patients with LPR and type I hiatal hernia had typical GERD symptoms, similar to a previous study^[26]. We also found that esophagitis was present in 35.7% of patients with LPR. Indeed, hiatal hernia appears to be an important risk factor for occurrence of LPR as GERD. A method of treating

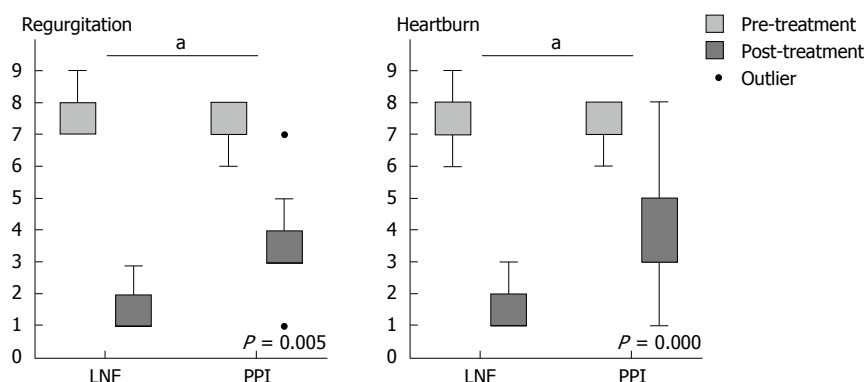


Figure 2 Comparison of the typical gastroesophageal reflux disease symptom score between the laparoscopic Nissen fundoplication and proton pump inhibitor groups before treatment and at the 2-year follow-up. Range, upper and lower quartiles, and median values are shown. Represents significant P values ($^2P < 0.05$) for a difference in improvement of symptoms between the LNF and PPI groups. P values are also shown in the right lower corner of each box. LNF: Laparoscopic Nissen fundoplication; PPI: Proton pump inhibitor.

Table 4 Comparison of outcome between laparoscopic Nissen fundoplication and proton pump inhibitor administration by 2-year follow-up

Characteristic/parameter	LNF (n = 25)	PPI (n = 28)	P value
BMI (kg/m ²)			
Pre-treatment	24.9 ± 3.8	25.0 ± 3.1	0.285
Post-treatment	22.2 ± 3.12	25.1 ± 2.9	0.001 ¹
RSI score (value)			
Pre-treatment	15.3 ± 3.5	14.2 ± 4.0	0.759
Post-treatment	9.7 ± 4.12	12.8 ± 3.12	0.004 ¹
RSI score (≥ 13), n (%)			
Pre-treatment	19 (76.0)	24 (85.7)	0.284
Post-treatment	7 (28.0) ²	19 (67.9) ²	0.003 ¹
PPI independence, n (%)	11(44.0)	2 (7.14)	0.000 ¹
Satisfaction, n (%)	62.49 ± 28.68	44.36 ± 32.77	0.004 ¹

¹Represent statistical significant P values between LNF and PPI group;

²Represent statistical significant P values between pre-treatment and post-treatment for 2-year follow-up ($P < 0.05$). Values are given as mean ± SD or n (%). LNF: Laparoscopic Nissen fundoplication; PPI: Proton pump inhibitor.

hernia or GERD might also improve symptoms of LPR. Therefore, this study was designed to focus on the comparison of diagnosis and treatment for LPR with hiatal hernia.

Currently, diagnosis of LPR mainly includes empirical therapeutic trials of PPIs, the RSI scale, laryngoscopy, and pH-monitoring^[6]. Among these diagnostic methods, PPI trials and the RSI scale are subjective methods, which cannot provide direct pathophysiological evidence of LPR. The reflux finding score, which is based on laryngoscopic findings, also has poor reliability in detecting LPR^[8,9]. Recent evidence has suggested no relationship between clinical findings of LPR, laryngoscopy, and the reflux finding score^[26]. Monitoring of pH can directly detect increased esophageal or laryngopharyngeal acid exposure by a pH probe, and is thus regarded as the best evidence for diagnosis of LPR. Advances in oropharyngeal pH-monitoring have been made, such as the oropharyngeal pH-monitoring system. This

system is a sensitive and minimally invasive device for determining acid reflux in oropharynx^[10,13]. Our study was designed to diagnose LPR by combining a comprehensive RSI scale/single symptom score and pH-monitoring to assess the consistent reflux events and the occurrence of symptoms. Monitoring of pH showed that all patients had positive pH-monitoring (Ryan score) with an RSI score ≥ 13. This finding suggested that oropharyngeal pH-monitoring and the RSI have the same diagnostic value for LPR. Moreover, oropharyngeal pH-monitoring showed more reflux events and a longer duration of acid exposure in the upright position than in the supine position. The potential reasons for these findings could be due to the following: (1) increased abdominal pressure induces high-level reflux and acid has direct laryngeal contact in the upright position; (2) feeding and acid secretion stimulate the vagal afferents to promote irregular contraction of the distal/proximal esophagus in the daytime; and (3) activity of the laryngopharynx in the daytime affects the nozzle structure^[27], as hypothesized by our group. A recent study proposed that, in the upright position, intragastric air rushes proximally with the assistance of increased intra-abdominal pressure, and the resultant gastric distension triggers relaxation of the intra-thoracic portion of the LES *via* stretch receptors in the stomach^[28]. Our result of a higher Ryan score in the upright position than in the supine position is consistent with the LPR characteristics of aerosol of acidic contents.

The primary determinants of severity of GERD are a dysfunctional anti-reflux barrier and impaired esophageal clearance^[29]. Disruption of the anti-reflux barrier can be related to a hypotensive LES (< 10 mmHg), dysperistalsis, and hiatal hernia^[29-31]. All of these factors may contribute to the occurrence of symptoms of LPR. Our study showed that LES pressure was reduced to approximately 10-15 mmHg. Additionally, a high incidence of dysperistalsis and hiatal hernia was shown by esophageal high-resolution manometry and GI endoscopy. High-resolution

manometry had a greater sensitivity than endoscopy for diagnosis of hiatal hernia in this study. Additionally, we analyzed the UES pressure of patients with LPR. Unfortunately, we obtained a different baseline of UES pressure before treatment, and no studies have focused on normal values of UES pressure or the relationship between UES pressure and LPR. However, our center has proposed a mechanism of LPR by using a special pharyngeal nozzle structure, and measured hypertensive UES pressure in a rat model^[32,33].

Hiatal hernia impairs LES function by reducing its length and pressure, and appears to be an important risk factor for the occurrence of LPR. LPR symptoms are likely to be cured by surgery for hernia repair and fundoplication. However, the guideline for management of GERD^[34] suggests that the strength of evidence is insufficient, with no consistent benefit attributed to surgery for LPR. PPIs therapy is the first choice for LPR in patients who also have typical symptoms of GERD or objective evidence of GERD by endoscopy or reflux monitoring.

The efficacy of empirical PPIs therapy for suspected LPR has been previously investigated. PPIs therapy reduced the incidence rate of LPR symptoms by 50.3% in one study (range: 38%-90%)^[35]. Another study specified that abnormal pH testing was an inclusion criterion, and the rate for responding to PPIs was 59.1%^[36]. In our study, comprehensive symptoms (RSI scale) markedly improved in 10 patients, and the mean RSI score significantly decreased after PPIs treatment. However, the rate of independence from PPIs (2 patients, 7.14%) was low. Additionally, some symptoms were not significantly relieved in some patients in the PPI group, such as globus, hoarseness, and throat pain and clearing. Some studies have suggested that symptoms persisted or recurred in the long-term follow-up, even though some patients used a double dose of PPI twice daily^[37,38].

LNF has become the surgical gold standard for GERD treatment. LNF can repair diaphragmatic crura to correct the anatomical problem of hiatus, and establish a wrap to provide an anti-reflux barrier. Nevertheless, the guideline^[34] suggests that controlling symptoms of LPR is not satisfied by Nissen fundoplication. In our study, we focused on patients with LPR with a clear diagnosis of hiatal hernia, and compared LNF with PPIs administration, which is the first recommended choice for LPR. We found that LNF was effective in reducing the RSI score, and the frequency and severity of LPR and typical GERD symptoms. Additionally, LNF was superior to empirical PPIs therapy in all aspects, especially in independence from PPIs, and patients' satisfaction with LNF tended to be better than that with PPIs therapy. These findings demonstrated that anti-reflux surgery could be as effective for LPR as for GERD. However, the accuracy of diagnosis of LPR could be a problem. With strict screening *via* multichannel intraluminal impedance-pH^[39,40] or oropharyngeal pH-

monitoring, symptoms of LPR are likely to demonstrate improvement following anti-reflux surgery.

Another important factor related to the occurrence of GERD is BMI, which affects the efficacy of anti-reflux surgery for LPR^[41]. In our study, BMI ranged from 21.1 to 28.1 kg/m², with no difference between the LNF and PPI groups. However, all of the patients were instructed to adopt lifestyle modifications. BMI was remarkably decreased only in the LNF group during the 2-year follow-up. Some studies have suggested that obesity is associated with GERD^[42] and an increased BMI is associated with increased esophageal acid exposure^[43]. Therefore, LNF could play a role in maintaining or reducing BMI, which affects the progress of GERD, as well as symptoms of LPR. However, this effect of LNF still needs to be observed in a long-term investigation.

A limitation of this study is that it was an uncontrolled, nonrandomized study, which made it impossible to control for baseline demographics. Although all of the patients underwent either anti-reflux or anti-acid therapy, the methods of therapy were not randomly chosen. Oropharyngeal pH-monitoring is a costly and time-consuming technique, which is still not widely available for use in patients for follow-up. Only improvement of symptoms was used to evaluate the effect of treatment, such as the RSI and specific symptoms score. Another limitation of the study is its small sample size and the loss of follow-up. Only 75.7% of patients finally completed the 2-year follow-up. A multicenter, randomized, controlled trial with more samples is required to reach a conclusion regarding the superiority of anti-reflux surgery for controlling LPR.

In conclusion, current knowledge on diagnosis of LPR needs to be expanded with multiple diagnostic strategies, including oropharyngeal pH-monitoring and high-resolution manometry. These strategies should be combined with classical techniques, such as GI endoscopy and symptom-scale assessment. Anti-reflux surgery and anti-acid therapy are effective in patients with LPR and type I hiatal hernia. LNF shows better improvement than PPIs administration, and it also controls BMI of patients in short time. Our findings shed new insights into the diagnosis and management for patients with LPR.

COMMENTS

Background

Laryngopharyngeal reflux (LPR) is considered as extraesophageal symptoms for distinguishing typical symptoms of gastroesophageal reflux disease (GERD). Evidence has suggested hiatal hernia is a risk factor for the occurrence of LPR as well as GERD. Common diagnosis and management for LPR depend on empirical proton pump inhibitors (PPIs) therapy; however, it cannot solve the hiatal hernia. Few studies have focused on the outcome of LPR patients following anti-reflux surgery, like laparoscopic Nissen fundoplication. The current trial was designed to evaluate anti-acid and anti-reflux therapy for LPR with type I hiatal hernia.

Research frontiers

LPR symptoms are much harder to be improved than GERD typical symptoms,

such as heartburn and regurgitation. GERD could be cured by anti-reflux surgery, thus increasing studies focused on the outcome of laparoscopic fundoplication to find the best therapeutic strategy for LPR. Meanwhile, oropharyngeal pH-monitoring is still in debate for diagnosing LPR, the integrated results of symptom-scale and oropharyngeal pH-monitoring could demonstrate the characteristics of LPR.

Innovations and breakthroughs

The literature suggests that the LPR patient should be assessed with oropharyngeal pH-monitoring and symptom-scale, combination of manometry and endoscopy, which could avoid the misdiagnosis of hiatal hernia. LNF achieves better improvement than PPIs for LPR with type I hiatal hernia, and could play a role in controlling BMI in short-term.

Applications

This study provides clinical evidence to support the effect of laparoscopic Nissen fundoplication on LPR patients diagnosed by oropharyngeal pH-monitoring and symptom-scale.

Terminology

Oropharyngeal pH-monitoring: A pH probe is inserted in laryngopharyngeal to detect acid exposure for continual 24 h. The pH-monitoring system includes a pH sensor with a teardrop shape to avoid becoming covered with food or mucus, a recorder to store the message and a new parameter calculation system (Ryan score) with different pH thresholds for upright and supine positions. Reflux symptom index (RSI): the scale accurately documents laryngopharyngeal symptoms with 9 item self administered outcome instrument, ranges from 0 to 45 (worst possible score). RSI more than 13 is considered as LPR diagnosis.

Peer-review

The authors have performed an interesting single-centre, comparative study. The outcomes of interest are well described and defined in the manuscript.

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Prospective Study

Diagnosis of eosinophilic gastroenteritis is easily missed

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Abstract

AIM

To analyze the clinical characteristics of eosinophilic gastroenteritis (EGE) and to investigate the situations of missed diagnosis of EGE.

METHODS

First, the clinical characteristics of 20 EGE patients who were treated at our hospital were retrospectively summarized. Second, 159 patients who underwent gastroscopy and 211 patients who underwent colonoscopy were enrolled. The pathological diagnosis showed only chronic inflammation in their medical records. The biopsy slides of these patients were reevaluated to determine the number of infiltrating eosinophils in order to assess the probability of a missed diagnosis of EGE. Finally, 122 patients who experienced refractory upper gastrointestinal symptoms for at least one month were recruited. At least 6 biopsy specimens were obtained by gastroscopy, and the number of eosinophils that had infiltrated was evaluated. Those who met the pathological diagnostic criteria of EGE underwent further examination to confirm the diagnosis of EGE. The probability of a missed diagnosis of EGE was prospectively investigated.

RESULTS

Among the 20 patients with EGE, mucosal EGE was found in 15 patients, muscular EGE was found in 3 patients and serosal EGE was found in 2 patients. Abdominal pain was the most common symptom. The number of peripheral blood eosinophils was elevated in all 20 patients, all of whom were sensitive to corticosteroids. Second, among the 159 patients who underwent gastroscopy, 7 (4.40%) patients met the criteria for pathological EGE (eosinophil count ≥ 25 /HPF). Among the 211 patients who underwent colonoscopy, 9 (4.27%) patients met the criteria for pathological EGE (eosinophil count ≥ 30 /HPF). No patients with eosinophil infiltration were diagnosed with EGE in clinical practice before or after endoscopy. Although these patients did not undergo further examination to exclude other diseases that can also lead to gastrointestinal eosinophil infiltration, these might be the cases where the diagnosis of EGE was missed. Finally, among the 122 patients with refractory upper gastrointestinal symptoms, eosinophil infiltration was seen in 7 patients (5.74%). The diagnosis of EGE was confirmed in all 7 patients after the exclusion of other diseases that can also lead to gastrointestinal eosinophil infiltration. A positive correlation was observed between the duration of the symptoms and the risk of EGE ($r = 0.18$, $P < 0.01$). The patients whose symptoms persisted longer than 6 mo more readily developed EGE. None of the patients were considered to have EGE by their physicians before endoscopy.

CONCLUSION

Although EGE is a rare inflammatory disorder, it is easily misdiagnosed. When a long history of abdominal symptoms fails to improve after conventional therapy, EGE should be considered.

Key words: Eosinophilic gastroenteritis; Missed diagnosis; Eosinophil; Gastroscopy; Colonoscopy

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Core tip: Eosinophilic gastroenteritis (EGE) is a rare but easily missed disorder. In our study, the biopsy slides from the patients who underwent gastroscopy or colonoscopy were reevaluated. We found that a diagnosis of EGE might have been missed in 4.40% (7/159) patients who underwent gastroscopy and in 4.27% (9/211) who underwent colonoscopy. Finally, a prospective study was performed and showed that in patients with refractory upper gastrointestinal symptoms, 5.74% (7/122) of patients represent a missed diagnosis of EGE. Therefore, physicians should increase their alertness and improve communication with pathologist to reduce the rate of missed diagnosis of EGE.

Abassa KK, Lin XY, Xuan JY, Zhou HX, Guo YW. Diagnosis of eosinophilic gastroenteritis is easily missed. *World J Gastroenterol* 2017; 23(19): 3556-3564 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3556.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3556>

INTRODUCTION

Eosinophilic gastroenteritis (EGE), which is a type of eosinophilic gastrointestinal disorder (EGID), is a rare chronic inflammatory disease characterized by patchy or diffuse infiltration of eosinophils into different layers of the gastrointestinal tract^[1-3]. Accurate epidemiologic data are lacking because most of the current studies are limited to small case series and single case reports. The incidence of EGE is estimated to be approximately 1-30/100000^[4-6]. Moreover, no effective consensus statement exists to guide clinical practice, and it is always a challenge for clinicians to diagnose EGE. Although recent studies and case reports have demonstrated that the incidence of EGE has been increasing, we believe that the incidence of EGE is underestimated.

Due to the non-specific nature of the symptoms of EGE, especially in those patients with mild symptoms, many clinicians seldom think of EGE unless these symptoms are refractory or elevated peripheral blood eosinophils are found. It is known that not all EGE patients present with an elevated level of peripheral blood eosinophils^[7-10], which might result in the missed diagnosis of some patients with normal counts of peripheral blood eosinophils. Furthermore, a definite diagnosis of EGE often relies on gastrointestinal endoscopy and histopathology, especially for the determination of the total number of infiltrating eosinophils per high power field^[11-15], but pathologists do not evaluate the exact number of infiltrating eosinophils unless the clinician has a special request to do so.

How many patients with EGE are there worldwide who are missed and how does this occur? Few studies have sought to answer this question. To improve clinicians' understanding of EGE and to increase its diagnosis rate, the clinical characteristics of patients with EGE who were treated at our hospital were retrospectively summarized. Then, patients who underwent gastroscopy and colonoscopy and whose pathological diagnosis showed only chronic inflammation in the medical records, were enrolled; the probability of a missed diagnosis of EGE was then retrospectively reviewed. Finally, patients with refractory upper gastrointestinal symptoms for at least one month were recruited, and the probability of a missed diagnosis of EGE was prospectively investigated.

MATERIALS AND METHODS

Patients and methods

Retrospective analysis of patients with EGE: All the patients diagnosed with EGE at our hospital from 2008 to 2015 with complete medical records were grouped together. The diagnosis and classification of EGE were performed according to Klein's criteria, as follows: the presence of gastrointestinal symptoms; pathological evidence of one or more areas infiltrated by eosinophils; other causes of eosinophilia were excluded^[16]. The age, gender distribution, symptoms, CRP, WBC, serum albumin, pathology report, Hp infection, treatment regimen, and response to treatment, among other parameters, were obtained and analyzed. The number of infiltrating eosinophils on the biopsy slide was recalculated.

The method of calculation of the number of infiltrating eosinophils was as follows: all biopsy samples were observed under the microscope with maximum magnification ($\times 400$) by specialists according to the "sweeping" technique, which consists of counting downward, then upward and finally from left to right. The mean number of eosinophils equaled the number of eosinophils counted in each field, divided by the number of fields present on the slide. Two specialists performed this analysis independently to calculate the mean value, which was the final number of infiltrating eosinophils. The pathological diagnostic criteria of EGID were as follows: esophagus, eosinophil count ≥ 15 /HPF; Stomach and duodenum, eosinophil count ≥ 25 /HPF; Colon and rectum, eosinophil count ≥ 30 /HPF^[17].

Retrospective study of cases of potential missed diagnosis: Patients who underwent gastroscopy from January 2014 to December 2014, and those who underwent colonoscopy from January 2010 to December 2014 at our hospital were enrolled.

Admission criteria: cases diagnosed as chronic mucosal inflammation after histopathologic study of a biopsy specimen obtained during gastroscopy or colonoscopy. Exclusion criteria: age < 18 years or ≥ 70 years; presence of ulcers, polyps, tumors, esophageal and gastric varices, portal hypertensive gastropathy, reflux esophagitis disease, or Barrett's esophagus observed during the endoscopy procedure; a history of gastrectomy or colectomy; previously diagnosed inflammatory bowel disease or autoimmune disease.

The biopsy slides of all the patients who met the criteria for this study were observed under a microscope, where the number of infiltrating eosinophils per high power field was obtained. Those who met the pathological diagnosis of EGE were selected and were analyzed statistically in terms of their age, sex, and results of a previous endoscopy report, among other characteristics.

Prospective study on eosinophilic gastroenteritis:

Patients who underwent gastroscopy in our Department of Endoscopy from August 2016 to December 2016 were enrolled. The admission criteria were as follows: patients with non-specific gastrointestinal symptoms such as abdominal pain, nausea, vomiting, dysphagia, dyspepsia, abdominal distension, unexplained weight loss, diarrhea for more than a month, and failure to respond to conventional treatments such as antacids, proton-pump inhibitors, and others for at least one week. The exclusion criteria were as follows: age < 18 years or ≥ 70 years; evidence or presence of tumors, esophageal varices, portal hypertension gastropathy, ulcers, reflux esophagitis disease, and polyps; a history of autoimmune disease, chronic liver diseases, severe diseases of the lung and cardiovascular system, diabetes mellitus, untreated coagulopathies, and chronic use of steroids, clopidogrel or aspirin; patients who refused or who were unable to give consent.

All the patients who fulfilled the criteria for the present study were classified into 3 groups according to the duration of their symptoms. Group one contained patients with symptoms that persisted for less than 3 mo, group two contained patients with symptoms that persisted between 3 mo and 6 mo, and group three contained patients whose symptoms persisted for more than 6 mo. All patients signed a written consent form before the procedure. A questionnaire that asked the name, age, sex, symptoms, and previous medical history was then completed and signed by the physician before gastroscopy was performed.

Among the patients who met the above criteria, biopsies were obtained from their antrum and duodenum (3 pieces from each site) for pathologic study, and the number of infiltrating eosinophils per high power field on each slide was calculated. Patients who met the requirements for the diagnosis of EGE by microscopy were classified as positive patients. To exclude other causes of eosinophilia of the gastrointestinal tract, the positive patients underwent examinations such as routine blood routine tests, routine stool tests, examination of the stool for ova and parasites, chest X-ray, abdominal ultrasound, anti-nuclear antibody (ANA), and detection of cancer markers, among other tests. A statistical analysis was then performed that included the age, sex, symptoms, laboratory examinations, treatment and follow-up protocol of the positive patients.

Statistical analysis

Statistical data were expressed as a mean \pm SD or as a percentage. A *t*-test was used to compare means of continuous variables between two groups. A χ^2 test was used to compare the constituent ratio of non-continuous variables between two groups. A Spearman correlation was used to study the correlation of non-continuous variables. All data analyses were performed

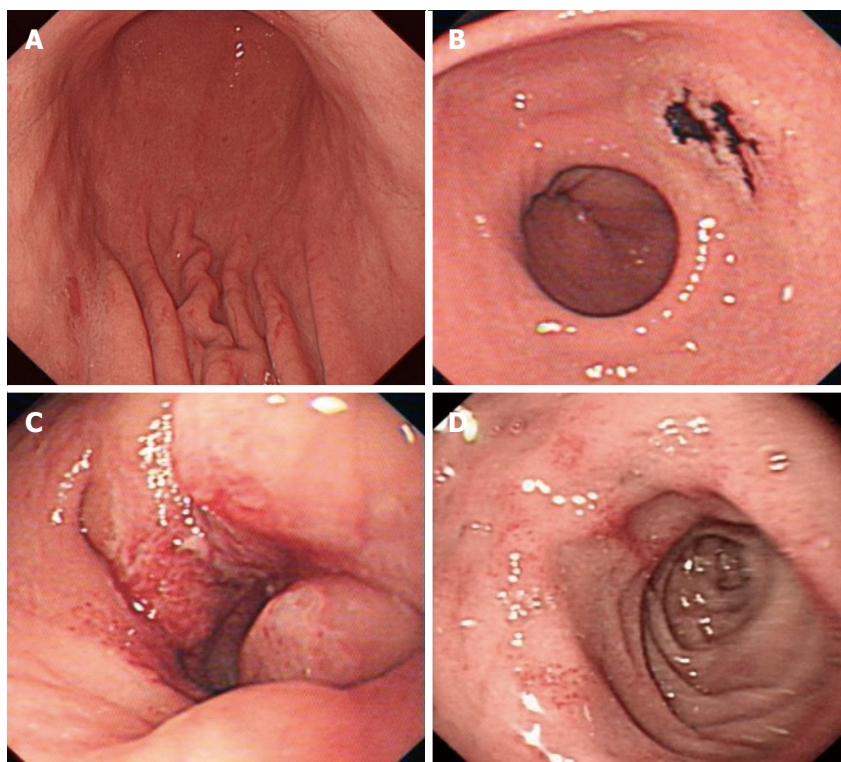


Figure 1 Endoscopic presentation of the eosinophilic gastroenteritis patients on gastroscopy. A: Mucosal edema and hyperemia of the greater curvature of stomach; B: Large sheet erosion in antrum; C: Pyloric stenosis with ulcers in duodenal bulb; D: Edema and hyperemia of the descending duodenum.

using SPSS 22.0. A statistical significance threshold of $P = 0.05$ was adopted.

RESULTS

Retrospective analysis of patients with EGE

From 2008 to 2015, 20 patients were diagnosed with EGE according to complete medical data obtained in our hospital; these patients included 8 males and 12 females, with a mean age of 46.1 ± 15.2 years. With respect to the affected layer, 15 patients were diagnosed with mucosal EGE, three patients were diagnosed with muscular EGE, and two patients were diagnosed with serosal EGE. The most common symptom was abdominal pain (70%), followed by abdominal distention (65%), nausea and vomiting (35%), and diarrhea (20%). The duration of symptoms of the 20 patients ranged from 2 wk to 6 years.

Elevated numbers of blood eosinophils were found in all 20 patients (100%), who had an average eosinophil count of $7.12 \pm 9.25 \times 10^9/L$, and 8 patients (40%) showed an elevated level of WBC with mean WBC count of $11.82 \pm 7.29 \times 10^9/L$. An elevated CRP level was detected in 7 patients (35%) and a low albumin level was detected in 5 patients (25%). All the patients underwent fecal testing for ova and parasites, ANA, X-ray, abdominal ultrasound or CT scan, to exclude all other causes of eosinophilia.

All patients underwent gastroscopy, but only ten underwent colonoscopy at the same time. The most common endoscopic presentation was mucosal edema

and hyperemia (100%), followed by mucosal erosion and hemorrhage; moreover, duodenal stenosis was found in two patients (Figure 1). Pathological infiltration of the esophagus by eosinophils was not observed in any of the 20 patients. Eosinophil infiltration was found in the antrum in 10 patients (50%) with a mean eosinophil count of $27.8 \pm 6.9/HPF$, and in the duodenum in 13 patients (65%) with a mean eosinophil count of $29.8 \pm 6.6/HPF$. Among the 10 patients who underwent colonoscopy, the most common endoscopic presentations were mucosal congestion, edema, and spotty or segmental mucosal erosion. Superficial ulcers were observed in one patient (Figure 2). Eosinophil infiltration was found in the distal ileum in 6 patients (60%) with a mean eosinophil count of $30.4 \pm 35.4/HPF$; eosinophil infiltration in the colon was observed in 2 patients (20%) with a mean eosinophil count of $35.2 \pm 12.4/HPF$. None of the patients exhibited eosinophil infiltration in the rectum. A large number of eosinophils were observed in ascites of two patients (Figure 3).

All patients received corticosteroid treatment, which consisted of oral prednisone at an initial dose of 30–45 mg/d or intravenous dexamethasone at an initial dose of 5–10 mg/d. Within 5 to 7 d, all 20 patients reported complete remission of symptoms. After 7–8 d of treatment, abdominal ultrasound confirmed the absence of ascites in two patients with serosal EGE. The duration of corticosteroid treatment was 21.3 ± 13.7 d. Other treatments included dietary restrictions, proton pump inhibitors, mucosal protective agents, antispasmodics, as well as antidiarrheal and

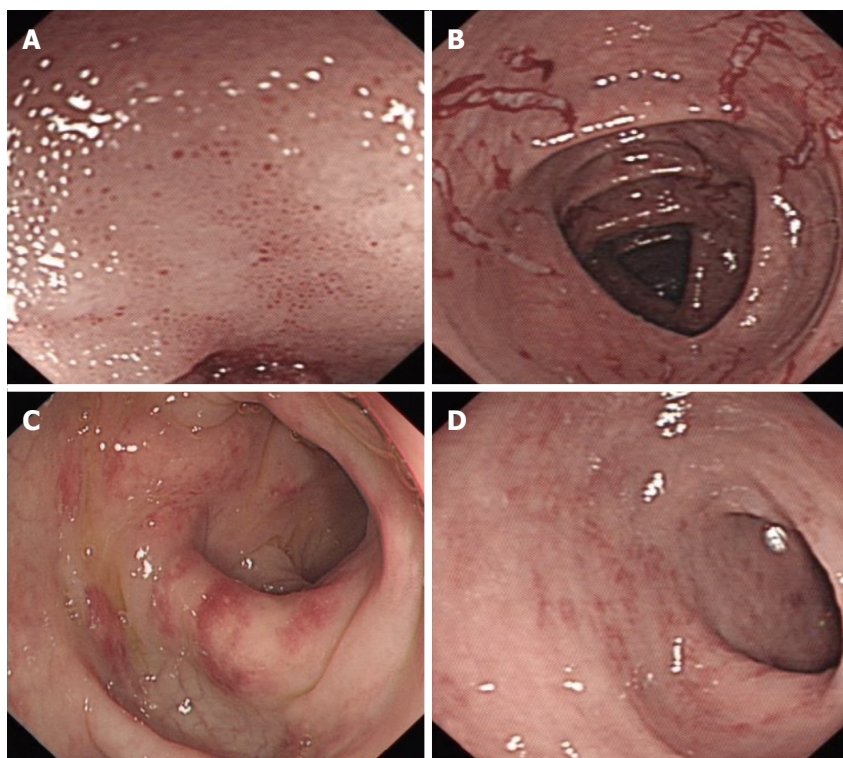


Figure 2 Endoscopic presentation of the eosinophilic gastroenteritis patients on colonoscopy. A: Mucosal edema and small hemorrhagic spot in the distal ileum; B: Mucosal edema and erosions in the transverse colon; C: Segmental erythematous edema and hyperemia in the descending colon; D: Erythematous edema and hyperemia in the sigmoid colon.

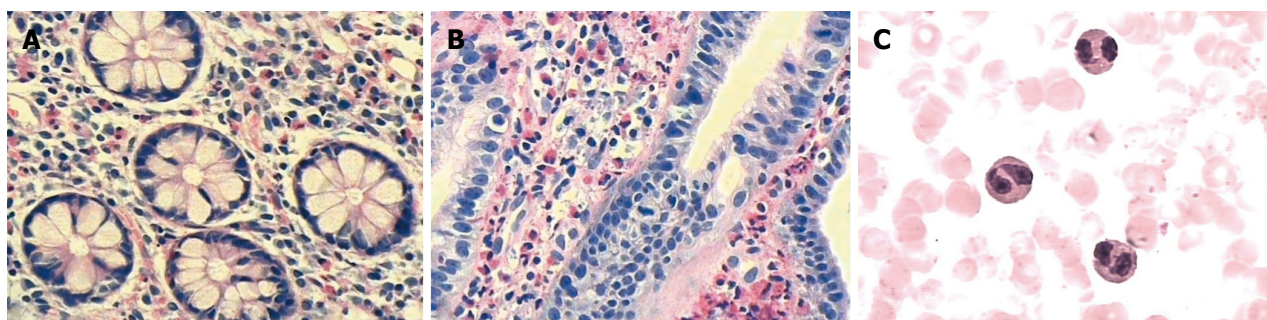


Figure 3 Pathology presentation of biopsy specimens from eosinophilic gastroenteritis patients (HE stain $\times 400$). A: Massive infiltration of eosinophils in the gastric mucosa of an eosinophilic gastroenteritis (EGE) patient; B: Massive infiltration of eosinophils in the colonic mucosa of an EGE patient; C: Massive infiltration of eosinophils in the ascites fluid of an EGE patient.

antianaphylaxis agents. After 2 years of follow-up, the symptoms of two patients (in the muscular group) recurred, and these patients were readmitted to the hospital for another steroid regimen, which was able to control the symptoms.

Retrospective study of cases of potential missed diagnosis

According to the admission and exclusion criteria, data from a total of 159 patients who underwent gastroscopy from January 2014 to December 2014 were collected. Among those patients, 7 patients (4.4%) met the criteria of the pathological requirement for the diagnosis of EGE by microscopy (eosinophil count ≥ 25 /HPF). Among these 7 patients, 5 were male and 2

were female, and the mean age was 40.0 ± 14.1 years. According to the admission and exclusion criteria, data from 211 patients who underwent colonoscopy from January 2010 to December 2014 were collected. Among those patients, 9 patients (4.26%) met the criteria for the pathological requirement for the diagnosis of EGE by microscopy (eosinophil count ≥ 30 /HPF). Among these 9 patients, 4 were male and 5 were female, and the mean age was 40.0 ± 14.1 years.

According to the medical records, all 7 patients who underwent gastroscopy and the 9 patients who underwent colonoscopy were not considered to have EGE in clinical practice before or after endoscopy. They were simply diagnosed with “chronic gastritis” or “non-specific enteritis”, and because of this, the

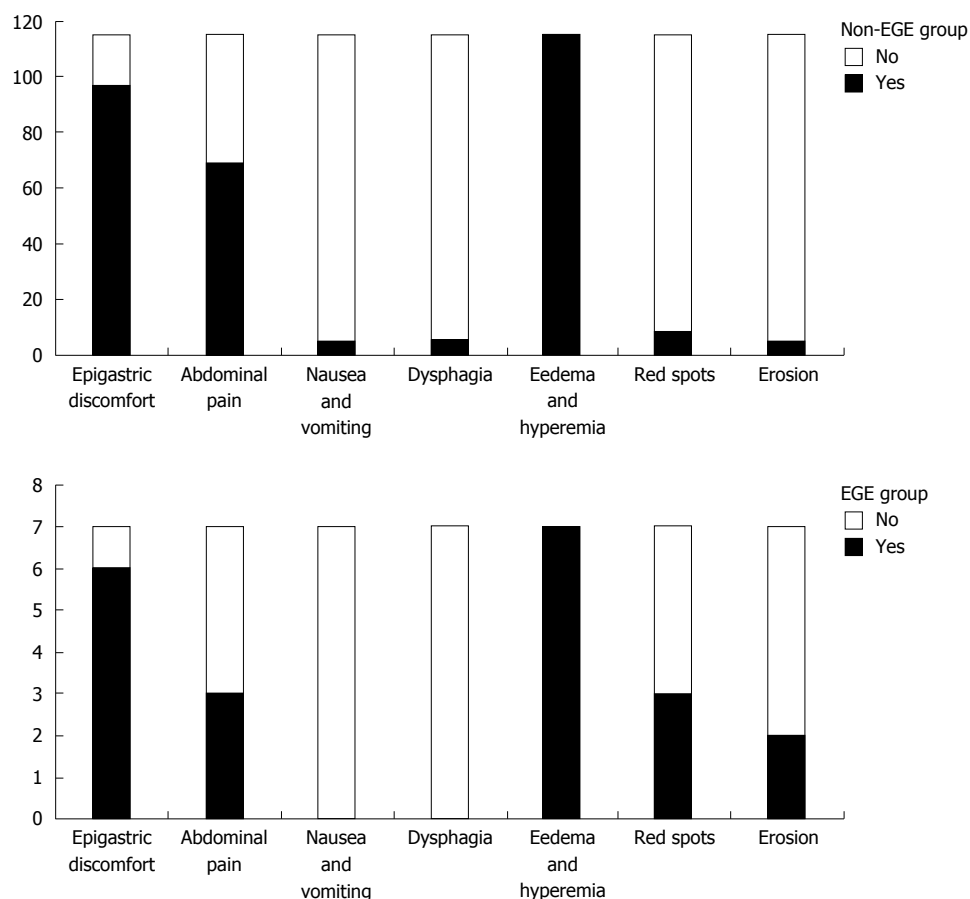


Figure 4 Clinical and endoscopic presentation of eosinophilic gastroenteritis and non- eosinophilic gastroenteritis patients. No statistical significance was shown between eosinophilic gastroenteritis (EGE) and non-EGE patients in terms of clinical and endoscopic presentation (all $P < 0.05$).

number of infiltrating eosinophils per high power field was not obtained. Although these patients did not undergo further examination to exclude other diseases that may also lead to gastrointestinal infiltration by eosinophils, they might represent the cases in which a diagnosis of EGE might have been missed.

Prospective study on eosinophilic gastroenteritis

Patients admitted to the study were all from an outpatient department (OPD) and were therefore in a relatively stable general condition. Their OPD files showed that their physicians did not consider the possibility of EGE in the differential diagnoses. Most of the diagnoses and treatments were directed toward gastritis and gastroesophageal reflux disease, among others.

A total of 122 patients, including 59 males (48.4%) and 63 females (51.6%) with a mean age of 38.3 ± 12.2 years, met the criteria of this study. Among the 122 patients, 7 (5.74%) met the pathological diagnostic criteria of EGE. The diagnosis of EGE was confirmed in all 7 patients through the exclusion of other diseases that can also lead to gastrointestinal eosinophil infiltration. Two out of the 122 (1.64%) patients had a history of allergy, one from the EGE group ($n = 7$) and the other from the non-EGE group

(those who didn't meet the diagnostic criteria of EGE, $n = 115$).

Among all recruited patients, the common clinical presentations included epigastric discomfort (84.4%), abdominal pain (59%), dysphagia (4.9%), nausea and vomiting (4.1%). No difference was observed between the EGE and non-EGE groups in terms of clinical presentation (all $P > 0.05$) (Figure 4). According to the duration of the symptoms, the 122 patients were divided into three groups. Among the 27 patients whose symptoms persisted for less than 3 mo, no EGE was observed. Among the 60 patients whose symptoms persisted between 3 and 6 mo, 2 EGE cases were observed. Among the 35 patients whose symptoms persisted longer than 6 mo, 5 EGE cases were observed. A Spearman correlation analysis revealed a positive correlation between the duration of symptoms of the patients and the probability of the development of EGE ($r = 0.209$, $P < 0.05$).

All 7 patients in the EGE group underwent further laboratory examination to exclude other causes of eosinophilia. The mean blood WBC of the 7 patients was $4.97 \pm 1.66 \times 10^9/L$ and only 2 patients (28.6%) showed elevated blood eosinophils with a mean count of $0.46 \pm 0.39 \times 10^9/L$. No abnormal result was noted in terms of stool parasites, ANA, cancer markers, X-ray,

abdominal ultrasound or CT scan.

Eosinophil infiltration was found in the duodenal biopsies of all 7 patients with EGE (mean value of $34.7 \pm 6.7/\text{HPF}$) and in the antral biopsies of 2 patients with EGE (mean value of $26.0 \pm 1.4/\text{HPF}$). Positive *Helicobacter pylori* infection was detected in 17 patients (14.8%) in the non-EGE group and in 2 patients (28.6%) in the EGE group, but these differences were not statistically significant ($\chi^2 = 0.954$, $P > 0.05$). The common endoscopic presentations were mucosal edema and hyperemia, red spots, and erosion in all 122 patients. No difference was observed between the EGE and non-EGE groups in terms of endoscopic presentation (all $P > 0.05$) (Figure 4).

All 7 patients with EGE received oral prednisone treatment at an initial dose of 30-40 mg/d, combined with dietary restrictions, proton-pump inhibitors or mucosal protective agents. After a week of treatment, all the patients noticed a remarkable improvement in their symptoms. The dosage of prednisone was gradually decreased to 5-10 mg/d. The complete treatment course varied from 4 and 12 wk. A follow-up of all 7 patients revealed that, to date, none of them complained of symptom relapse.

DISCUSSION

Eosinophilic gastrointestinal disorder (EGID) was first described by Kaijer in 1937^[18] and is characterized by infiltration of eosinophils into different layers of the gastrointestinal tract in the absence of secondary causes. EGID primarily involves eosinophilic esophagitis (EoE) and eosinophilic gastroenteritis (EGE). In Asian patients, EGE occurs more frequently than EoE compared with Caucasian patients^[19].

According to previous studies, the incidence of EGE is estimated to be approximately 1-30/100000^[4-6]. Recent studies and case reports have demonstrated that this incidence has been increasing. In their study, Reed *et al.*^[20] revealed that among all the biopsies obtained through upper endoscopy at their center, 0.67% of them met the criteria for EGE. This indicates that EGE is not as rare as previously thought. The percentage of missed diagnoses of EGE may be very high. Our retrospective study revealed the possibility that 4.26% of cases were missed diagnoses of EGE in patients whose gastroscopy and histopathology results showed only chronic inflammation. Our study also revealed the possibility that 4.40% of cases were missed diagnoses of EGE in patients whose colonoscopy and histopathology results showed only chronic inflammation. The prospective study revealed that 5.74% of patients with chronic refractory upper gastrointestinal symptoms might represent cases where the diagnosis of EGE was missed. Thus, we believe that the incidence of EGD is underestimated, not only because the incidence of EGD itself is on the rise but also because it is easy for a diagnosis of EGE to be missed in clinical practice.

In terms of the reasons for the missed diagnosis of EGE, we consider the non-specificity of EGE symptoms and endoscopic presentations, insufficient understanding of EGE, and poor communication between clinicians and pathologists, among other reasons. The clinical presentations of EGE greatly depend on the site and depth of infiltration of eosinophils. The most common clinical presentations are abdominal pain, nausea, vomiting, diarrhea, weight loss, abdominal distention, dysphagia, and in some cases, gastrointestinal bleeding^[2-4]. Most EGE patients do not exhibit any specific symptoms, and this number may be as high as 80% of all EGE patients^[21]. As shown in our study, these patients always present with abdominal pain, epigastric discomfort, abdominal distention, nausea and vomiting, and diarrhea, among other symptoms. With the exception of those who present with some "severe" symptoms or signs such as weight loss, gastrointestinal bleeding, anemia, pyloric stenosis, intestinal obstruction, or ascites, patients are always diagnosed with "gastritis" or "non-specific enteritis". Even in those patients assigned to undergo endoscopy, their endoscopic presentations also lack specificity. The endoscopic presentations primarily present as mucosal hyperemia, edema, hemorrhage, erosions, and ulcers^[9,22,23]. If a clinician does not consider EGE and if eosinophils are not detected in blood and biopsy samples, EGE is very easily missed.

An increase in the level of peripheral eosinophils is an important factor in the diagnosis of EGE^[24]. It is known that not all EGE patients present an elevated level of peripheral blood eosinophils^[25,26]. Present studies have revealed that approximately 70%-90% of EGE patients have elevated peripheral eosinophil counts^[7-10]. According to our retrospective study, all 20 EGE patients exhibited an elevated peripheral eosinophil count, whereas in our prospective study, only 2 out of 7 patients diagnosed with EGE showed an elevated peripheral eosinophil count. Thus, the high level of peripheral eosinophils is very important in the diagnosis of EGE, but it is not mandatory. Waiting to observe an increase in the peripheral eosinophil count before considering EGE is a huge mistake, which commonly leads to missed diagnoses and unnecessary medical tests. Although our prospective study contained a relatively small number of samples, we observed milder clinical and endoscopic presentations in those 7 patients compared with the 20 patients in the retrospective study. Therefore, we believe that a missed diagnosis of EGE occurs more easily in patients with mild presentations and those with a better overall condition.

Since a normal level of peripheral eosinophils is seen in some patients with EGE, evidence of one or more areas infiltrated by eosinophils is more reliable and necessary for a diagnosis. Unlike the esophagus, the healthy gastrointestinal tract normally contains a certain number of eosinophils. Therefore, various studies have established the number of infiltrating

eosinophils as an indication of pathological infiltration at deferent levels^[7,12-13,25]. The stomach and small intestine, especially the antrum and the duodenum, are the most affected sites in EGE, which was also confirmed in our retrospective analysis of EGE patients. For this reason, we selected the antrum and the duodenum as the sites for biopsy in the prospective study. At the same time, it is necessary to obtain at least 5-6 biopsy specimens in order to improve the positive detection of eosinophil infiltration^[11,14]. One study asserted that nearly 50% of EGE patients exhibited the abnormal presence of eosinophils in the colon and rectum^[8]. Therefore, for highly suspected patients with negative founding by gastroscopy, a colonoscopy is necessary to acquire evidence of EGE. Furthermore, in some cases a repeat endoscopy may be useful. More importantly, as pathologists do not routinely calculate the number of eosinophils on biopsy slides, a special reminder should be sent to pathologists once EGE is suspected.

Diet control and corticosteroids are the main treatments for patients with EGE^[27-30]. For patients in whom diet-induced EGE is suspected and in those with a prior history of allergy, EGE can be managed *via* the sequential elimination of possible food allergens. Corticosteroids are the primary treatment modality for patients with EGE. The starting dose is 15-40 mg/d of oral prednisone and 40 mg/d of methylprednisolone infusion in more severe cases. The dosage is then slowly decreased until complete cessation, but this is dependent on the different response of patients. In cases of relapse of the disease, which occur while the dosage of steroids is decreased, it is recommended that the dose be increased and that the treatment time be extended. It is not uncommon for the disease to relapse once the steroid treatment ceases. It is advisable to treat the patient again with the same regimen for a longer duration. However, to date, no standard length of the treatment duration has been established for EGE. In the present study, all patients with EGE were sensitive to prednisone.

Of course, the present study has some limitations that should be mentioned. In the retrospective analysis of patients with EGE, only the patients with complete data were included. Those with incomplete data who were diagnosed with EGE were not enrolled. This increased the gap between the actual number of EGE patients and those considered in this study. In the retrospective study on possible cases of missed diagnosis, because clinicians did not consider the probability of EGE as a diagnosis, these patients did not undergo a full workup to exclude other causes of eosinophil infiltration. Thus, this portion of the study can only give a possibility of diagnosis and not a confirmation of EGE. This study is a single-center study that was performed over a relatively short period of time with a relatively small number of samples, especially the prospective study. Therefore, the results obtained in this study require further investigation.

In conclusion, eosinophilic gastroenteritis is a relatively rare chronic inflammatory disease, but we have underestimated its incidence. Since the clinical presentation of EGE lacks specificity, patients with symptoms of chronic gastritis that fail to improve after repeated treatments should undergo examinations such as endoscopy and histopathology to exclude EGE. The longer these symptoms persist, the more likely the person has EGE. Peripheral blood hypereosinophilia is not mandatory for the diagnosis of EGE, and a multiple-site biopsy and an eosinophil count under a microscope are more important. Good communication among clinicians, endoscopists and pathologists can help decrease the rate of missed diagnosis of this disease.

COMMENTS

Background

Eosinophilic gastroenteritis (EGE) is a rare chronic inflammatory disease. Due to the non-specific nature of the symptoms of EGE and no effective consensus statement exists to guide clinical practice, EGE always a challenge for clinicians to diagnose EGE. This study was designed to explore the clinical characteristics of EGE and the situations of missed diagnosis of EGE.

Research frontiers

Because the diagnosis of EGE is easily missed, many current researchers mainly focus on how to increase diagnostic efficiency of EGE. It is rare for research to investigate how many cases miss the diagnosis and analyze the reason.

Innovations and breakthroughs

The rate of missed diagnosis of EGE is very high, especially in patients with refractory upper gastrointestinal symptoms. The longer these symptoms persist, the more likely the person has EGE. Peripheral blood hypereosinophilia is not mandatory for the diagnosis of EGE, and a multiple-site biopsy and an eosinophil count under a microscope are more important.

Applications

Clinicians should be aware of the possibility of EGE in the patients with refractory gastrointestinal symptoms. Good communication among clinicians, endoscopists and pathologists can help decrease the rate of missed diagnosis of this disease.

Terminology

PHG: portal hypertension gastropathy, is a gastric disorder associated with portal hypertension. The endoscopic appearance of gastric mucosa shows a characteristic mosaic-like pattern. The patient with PHG can present with abdominal pain, nausea, vomiting, dyspepsia, dominant or recessive upper gastrointestinal bleeding.

Peer-review

Very interesting work on a little discussed topic in the gastroenterology community. Given the rarity of the disease, it is not expected that both retrospective and prospective studies could have a large number of patients to allow definitive conclusions.

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Fecal microbiota transplantation cured epilepsy in a case with Crohn's disease: The first report

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Abstract

Fecal microbiota transplantation (FMT) is a promising strategy that involves reconstruction of gut microbiota. Recently, it has been considered as a treatment of Crohn's disease (CD) and certain neurological diseases. Here, to the best of our knowledge, we report the first case that used FMT to achieve remission of intestinal and neurological symptoms in a girl with CD and a 17-year history of epilepsy. During the 20 mo of follow-up, FMT has proved its efficacy in preventing relapse of seizures after withdrawing the antiepileptic drugs. Furthermore, this finding highlights the role of microbiota-gut-brain axis and inspires a novel treatment for epilepsy through remodeling gut microbiota.

Key words: Fecal microbiota transplantation; Epilepsy; Crohn's disease; Gut microbiota; Brain-gut axis

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Core tip: We report a case of 17-year history of epilepsy which fortunately showed improvement as a result of fecal microbiota transplantation (FMT) treatment for

Crohn's disease. This is the first report that FMT has been used in epilepsy treatment to our knowledge. This case might open a new window into disease mechanism focusing on the microbiota-gut-brain axis and inspire a novel treatment for epilepsy through remodeling of gut microbiota.

He Z, Cui BT, Zhang T, Li P, Long CY, Ji GZ, Zhang FM. Fecal microbiota transplantation cured epilepsy in a case with Crohn's disease: The first report. *World J Gastroenterol* 2017; 23(19): 3565-3568 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i19/3565.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i19.3565>

INTRODUCTION

Considerable evidence has shown the effects of microbiota on neuropsychiatric disorders^[1]. However, very few studies have reported the clinical use of microbiota in brain diseases. Fecal microbiota transplantation (FMT), the most effective strategy for reconstruction of the gut microbiota, has been considered as a treatment of *Clostridium difficile* infection^[2], inflammatory bowel disease^[3-5], constipation and other diseases^[6]. In this study, we report the first case that used FMT as a treatment of long-term epilepsy in a patient with Crohn's disease (CD). FMT showed positive response of more than 20 mo seizure free without using antiepileptic drugs.

CASE REPORT

A 22-year-old girl, with a 17-year history of epilepsy, was referred to the Second Affiliated Hospital of Nanjing Medical University in May 2015 because of unsuccessful CD treatment. The initial presentation was at the age of 6 years, with generalized seizures of loss of consciousness and unexplained chronic diarrhea. The patient had more than 120 seizures every year between the ages of 6 to 13. After that, she was diagnosed with epilepsy by typical electroencephalogram (EEG) and started to take sodium valproate. That treatment achieved extended stabilization in the seizures, but she still experienced 2-3 generalized seizures every year if she had forgotten to take the antiepileptic drug. Diagnosis of CD was made at the age of 17, and at that time she started treatment for the chronic diarrhea and achieved symptom improvement after oral mesalamine. She had growth retardation, mild malnutrition and started the first menarche at age 17, which was followed by menstrual cycle disorder.

After administration, abdominal/pelvic magnetic resonance imaging (MRI) showed severe strictures in sigmoid colon and anus with perianal fistula; brain MRI was normal. CD activity index (CDAI) was 361 points. The patient underwent endoscopic balloon dilation for

the intestinal strictures, and then was administered the first FMT through mid-gut by gastroscopically (Trial: NCT01793831) under anesthesia^[7]. The stool for FMT was obtained from a primary school girl and scanned after signing an informed consent from her parents. The laboratory protocol and clinical work flow were noted in our recent report^[8].

The 200 mL fresh fecal microbiota suspension was prepared under an automatic purification system (GenFMTer; FMT Medical, Nanjing, China) in our fecal microbiota bank system. After the FMT, the patient was given professional food instruction related to CD. In addition, she was given oral mesalamine at 3.0 g per day during the follow-up. She underwent the second endoscopic balloon dilation for colonic stricture before her third FMT. Based on our initial expectation on the role of FMT in epilepsy, we decided to stop sodium valproate after the first FMT and getting her informed consent. Since then, the patient never had recurrence of epilepsy during the entire 20 mo of follow-up and has remained in seizure-free without antiepileptic drugs up to the date of this submission. Importantly, a male infant was born by normal spontaneous vaginal delivery before this final approval for this article. Therefore, there was no need for EEG during the follow-up.

The clinical response of CD to the FMT was evidenced by decreasing CDAI to 104 points after 12 mo, and this remission maintained after the third FMT until the end of 20 mo follow-up. In addition, the patient showed sustained improvement of quality of life and started to work. More interestingly, her menstrual cycle after FMT tended to shorten and became regular every 6 wk, with normal menstruation quantity during each cycle. The key clinical parameters before and after the FMT were shown in Table 1.

DISCUSSION

Epilepsy entails a major burden in seizure-related disability, mortality, comorbidities, stigma and costs^[9]. Although the number of available antiepileptic drugs has increased substantially during the past 20 years, about a third of patients remain resistant to medical treatment^[10]. Despite the development of surgical procedures, epilepsy surgery is still done in a small subset of drug-resistant epilepsy cases. Here, we report a case of 17-year history of epilepsy which fortunately showed improvement as a result of FMT treatment for CD. Although the patient never took any antiepileptic drugs after the FMT, she had a more than 20-mo seizure free and this status is maintained to date.

Unfortunately, in this case report, there is no confirmed focal pathology, no potential pathogen identification, no microbiome analysis, and no gene mutation detection. There are very few reported cases in the literature on epilepsy comorbid CD^[11,12], which may be the key reason that the mechanism linking intestinal microbiota, intestinal inflammation

Table 1 Clinical parameter changes of the patient during follow-up

Parameter (normal range)	Before the 1 st FMT	After the 1 st FMT					
		1 mo	3 mo	6 mo	12 mo	15 mo	20 mo
CDAI score	361	174	158	87	104	112	131
Body weight in kg	42	42	43	47	49	50	52
Hemoglobin (110-160) in g/L	95	99	120	117	113	103	111
CRP (0-10) in mg/mL	8	3	7	10	1	3	10
ESR (0-20) in mm/h	59	51	27	30	36	21	61
Album in g/L	39.6	ND	47.7	39.7	45.5	48.4	41.5
Total cholesterol (≤ 5.2) in mmol/L	4.08	ND	4.98	ND	ND	5.71	ND
Triglycerides (≤ 2.3) in mmol/L	1.68	ND	1.03	ND	ND	0.32	ND
HDL-C (≥ 0.9) in mmol/L	0.7	ND	1.2	ND	ND	1.8	ND
IgA (0.70-4.00) in g/L	5.71	ND	5.47	4.39	ND	ND	5.4
Menstrual cycle length in d	60-75	ND	45	45	45	45	30

CDAI: Crohn's disease activity index (remission: < 150 ; moderate: 150-450; severe: > 450); CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; FMT: Fecal microbiota transplantation; HDL-C: High density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; ND: No detection.

and epilepsy remains unclear. CD with associated nutrient deficiencies could have symptoms like tetany and seizures, which may be related to the deficit of magnesium and/or calcium^[12]. However, the present patient in this report was diagnosed as epilepsy at the age of 13 and only had mild malnutrition.

Although it has been mentioned that FMT might be helpful for certain neurological diseases^[6] and CD^[7], as far as we know, this article is the first report of successful epilepsy treatment using FMT. We are conducting a randomized controlled clinical trial (Trial: NCT02889627) to investigate the efficacy of FMT for epilepsy. It is notable that the level of blood lipid of the patient returned to the almost normal level after FMT. In our previous studies^[7,13], we also found similar results, showing that the gut microbiota could affect host lipid metabolism. These evidence suggested that FMT may be one of the therapeutic options for metabolic diseases.

Although there has been an at least 1700-year old history of using FMT in human diseases^[14], to the best of our knowledge, no previous report on using FMT in epilepsy is present in the publicly available literature. This interesting finding might open a new window into disease mechanism focusing on the microbiota-gut-brain axis and inspire a novel treatment for epilepsy through remodeling of the gut microbiota.

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COMMENTS

Case characteristics

A Chinese girl with long-term epilepsy was referred to our hospital because of unsuccessful treatment for Crohn's disease (CD).

Clinical diagnosis

Clinical symptoms showed chronic diarrhea, growth retardation, mild malnutrition and menstrual cycle disorder.

Differential diagnosis

The differential diagnosis included intestinal tuberculosis and viral infection.

Laboratory diagnosis

Laboratory evaluation revealed low hemoglobin and elevated erythrocyte sedimentation rate.

Imaging diagnosis

Magnetic resonance imaging confirmed the severe strictures in sigmoid colon and anus with perianal fistula, and negative finding in brain.

Pathological diagnosis

The patient was diagnosed definitely with no pathological examination, although this was important.

Treatment

The patient underwent three fecal microbiota transplantations and two endoscopic balloon procedures during the 12 mo after her first visit.

Related reports

There is no report on fecal microbiota transplantation for epilepsy.

Term explanation

Fecal microbiota transplantation involves infusing healthy donor microbiota into the intestines of a patient to restore the intestinal microbiota.

Experiences and lessons

This case highlights the disease mechanism, focusing on the microbiota-gut-brain axis and possibly inspiring a novel treatment for epilepsy through remodeling of the gut microbiota.

Peer-review

The paper is well written. The nutrient deficiencies associated with CD is usually subclinical but, occasionally, can cause weight loss, growth retardation, anemia and, even, tetany and seizures.

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