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World Journal of Gastroenterology

Contents

Weekly Volume 23 Number 45 December 7, 2017

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

Case Control Study

Retrospective Cohort Study

Retrospective Study

AIMS AND SCOPE

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MINIREVIEWS

Pancreatic acinar cell carcinoma: A review on molecular profiling of patient tumors

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Abstract

Pancreatic carcinomas with acinar differentiation are rare, accounting for 1%-2% of adult pancreatic tumors; they include pancreatic acinar cell carcinoma (PACC), pancreatoblastoma, and carcinomas of mixed differentiation. Patients with PACC have a prognosis better than pancreatic ductal adenocarcinomas but worse than pancreatic neuroendocrine tumors. Reports of overall survival range from 18 to 47 mo. A literature review on PACCs included comprehensive genomic profiling and whole exome sequencing on a series of more than 70 patients as well as other diagnostic studies including immunohistochemistry. Surgical resection of PACC is the preferred treatment for localized and resectable tumors. The efficacy of adjuvant treatment is unclear. Metastatic PACCs are generally not curable and treated with systemic chemotherapy. They are moderately responsive to chemotherapy with different regimens showing various degrees of response in case reports/series. Most of these regimens were developed to treat patients with pancreatic ductal adenocarcinomas or colorectal adenocarcinomas. Review of PACC's molecular profiling showed a number of gene alterations such as: *SMAD4*, *BRAF*, *BRCA2*, *TP53*, *RB1*, *MEN1*, *JAK-1*, *BRCA-1*, *BRCA-2*, and DNA mismatch repair abnormalities. PACCs had multiple somatic mutations with some targetable with available drugs. Therefore, molecular profiling of PACC should be an option for patients with refractory PACC.

Key words: Pancreatic acinar cell carcinoma; Molecular profiling; Targeted therapy

Core tip: This is a review article on pancreatic acinar cell carcinoma, which is a rare type of pancreatic cancer, with a series of molecularly profiled cases and an insight on how to potentially target specific mutations and genetic abnormalities with different systemic treatments including tyrosine kinase inhibitors, immunotherapy and cytotoxic chemotherapy agents.

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INTRODUCTION

Pancreatic carcinomas with acinar differentiation are a very rare pancreatic neoplasm, comprising about 1%-2% of all pancreatic tumors in adults[1-3]. These tumors also occur in children, accounting for 6% of all childhood tumors and more importantly, 15%, of all pediatric pancreatic tumors[1,2,4-6]. These pancreatic neoplasms include pancreatic acinar cell carcinoma (PACC), pancreatoblastoma, and carcinomas of mixed differentiation. They typically occur in late adulthood, with a mean age of 62 at diagnosis. They are more common in males with a male to female incidence ratio of 2:1[1,7]. Prognosis of patients with PACC is worse than that of pancreatic neuroendocrine tumors but better than that for patients with pancreatic ductal carcinomas, which has the worst prognosis of the three tumors[8-11]. Overall survival for PACC ranges from 18-47 mo[8,9,11,12]. Although acinar cells are the most common cell types found in the pancreas, malignant transformation of these cells is very rare, as they involve only 1% of exocrine tumors of the pancreas[13]. In contrast, pancreatic ductal adenocarcinomas (PDACs), which arise from the epithelial lining of the pancreatic ducts, comprise the majority of pancreatic malignancies[1,9].

Morphologically, PACCs have different histological patterns, as they can appear to be normal pancreatic acini or they can be solid tumors with sheets of neoplastic cells that are poorly differentiated (Figure 1). This wide variation in histology can make it difficult to distinguish PACCs from other types of pancreatic tumors. One method to distinguish PACCs from other pancreatic tumors is the use of immunohistochemistry to show the proteins that the tumor produces. In the case of PACC, they tend to make acinar-specific enzymes including proteases, lipases, and amylases.

LITERATURE RESEARCH

The main search tool for identifying articles from MEDLINE was the PubMed system. The keywords used were "pancreatic cancer," "acinar cell carcinoma," "molecular biology," and "sequencing." Relevant articles that were quoted in publications from the original search were also used. Google Scholar was another search strategy and the search phrase was "pancreatic acinar cell carcinoma".

CLINICAL PRESENTATION

Patients who present with PACCs usually have non-specific symptoms. Their complaints consist of abdominal pain or discomfort, nausea, vomiting, weight loss, and diarrhea. Jaundice is less frequently seen at presentation. In about 50% of patients serum lipase is elevated, and up to 10% of these patients with lipase hypersecretion develop subcutaneous nodules erythematous and edematous subcutaneous nodules that occur in conjunction with eosinophilia, and polyarthralgia[6,12,14]. This condition, known as Schmid's triad, is associated with a poor prognosis[15,16].

DIAGNOSIS

Diagnosing PACCs, like other pancreatic tumors, includes use of various imaging modalities including CT and/or MRI scans. PACCs are well-circumscribed tumors and can be large in diameter, although findings vary[7,17]. Imaging is followed by fine-needle aspiration guided by endoscopic ultrasound (EUS) or other techniques dependent upon tumor location to obtain a tissue diagnosis. Resectability is the most important prognostic factor for this disease, and the staging system used in PACCs is the same as the TNM staging used for PDACs.

As mentioned above, PACCs have distinctive features, but there are some variations in appearance that make it difficult to distinguish PACCs from other pancreatic tumors. PACCs are usually highly cellular without a prominent stroma normally observed in PDACs[6]. The individual cells are uniform in size and shape with large nucleoli located in the center of the nucleus[6]. Other variants of PACC include acinar cell cystadenoma, which are grossly cystic neoplasms lined with simple cuboidal or columnar acinar cells. When a PACC has 25% to 30% of neoplastic endocrine cells, it is called a mixed acinar-endocrine carcinoma. In mixed acinar-endocrine carcinoma, there is evidence of ductal and endocrine differentiation, but only immuno-histochemical staining with markers for acinar cells (trypsin, chymotrypsin, lipase, *etc.*) and endocrine cells (synaptophysin or chromogranin A) can identify the different lines of differentiation. Even though this is a very rare entity, two forms of mixed acinar-endocrine carcinoma have been observed. In one form this cancer has expression of acinar and endocrine markers in histologically distinct cell types, while in the other form both markers are co-expressed in the same cells that show both acinar and endocrine histologies[18,19]. However, the majority of mixed acinar-endocrine

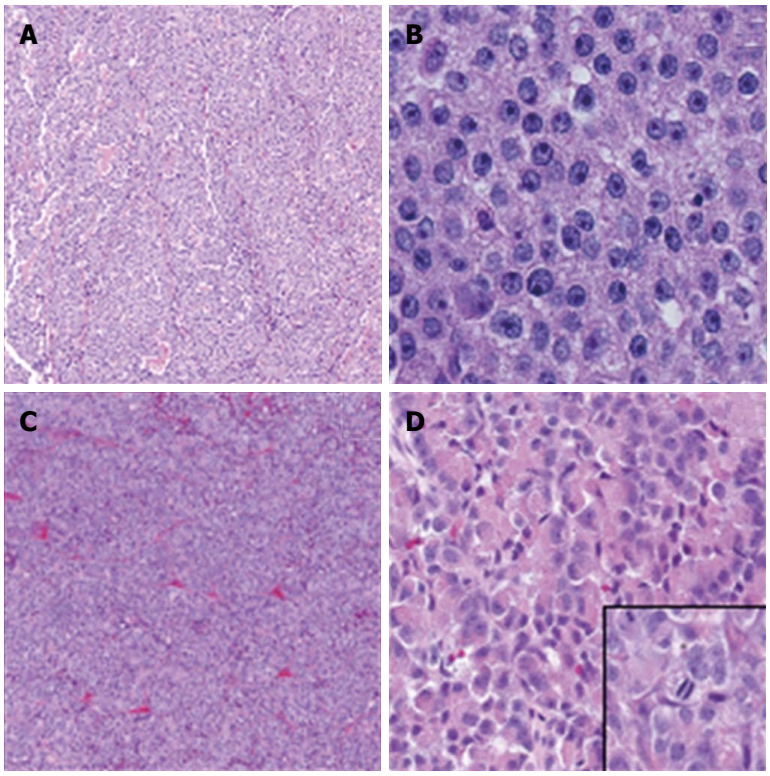

Figure 1 Different histological forms of pancreatic acinar cell carcinomas. A: A case of PACC displaying nested to glandular growth patterns (HE 40 ×); B: Higher magnification of the same tumor in Panel A showing monotonous cells with eosinophilic/granular cytoplasm with well-defined cell borders and uniform nuclei with minimal atypia and prominent nucleoli (HE 200 ×); C: Tumor from a different patient showing a predominantly sheet-like growth with no distinct pattern (HE 40 ×). D: Higher magnification of the same tumor in C showing uniform cells with eosinophilic granular cytoplasm (prominent zymogen granules) with minimal pleomorphism (HE 200 ×). Inset: mitotic figures were identified throughout the tumor (HE 400 ×).

carcinomas have a predominance of acinar cells[20].

MOLECULAR BIOLOGY

Understanding the role of signal transduction pathways responsible for cell growth and survival is important for development of cancer therapies. Secretion of pancreatic enzymes is a central role for acinar cells and this is evidenced by the fact they have the highest levels of protein synthesis of any adult cell[21]. Secretions from the acinar cells are correlated with the stage of digestion, hence pancreas secretion is regulated by hormones secreted by the digestive tract, principally cholecystokinin (CCK) and acetylcholine *via* the vagal nerve, as well as secretin. Increases in intracellular calcium levels play a key role in the control of digestive enzyme secretion, but extracellular stores are required too[22]. Protein synthesis, primarily digestive enzymes, are regulated by a number of pathways in the acinar cell including the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/A kinase binding (AKT)/mammalian target of rapamycin (mTOR) pathway[23]. In terms of PACC, up to 25% of the PACCs were reported to have inactivating mutations in genes of the adenomatous polyposis coli (*APC*)-β-catenin pathway[5]. Additionally, these authors reported finding activating mutations in the *CTNNB1* gene in PACC samples.

Table 1 summarizes the molecular abnormalities noted in PACC. Recently, one study reported finding DNA mismatch repair (MMR) abnormalities present in some acinar cell carcinomas. They examined the expression of DNA MMR genes by immunohistochemistry (IHC) in 36 PACC cases and detected the loss of MMR proteins in 5 of the 36 cases (14%) examined. At least two of these five patients with a loss of MMR protein expression had a known history of Lynch syndrome[24]. In another case series, microsatellite instability (MSI) was present in 2 out of 42 specimens (5%) examined[25].

Comprehensive genomic profiling has been performed on a number of tumor samples taken directly from patients with PACCs, including those with a mixed acinar carcinoma phenotype and pancreatoblastomas. Genomic profiling using next-generation sequencing (NGS) - based platforms including whole exome sequencing was performed on these tumor samples. Unlike PDACs, where > 90% of cases contain various types of *KRAS* gene mutations, mutation in *KRAS* was observed only in one (a mixed acinar/neuroendocrine tumor) out of the total 78 PACC cases sequenced in the three studies[12,26,27]. In addition, genomic alterations in *SMAD4*, *CDKN2A*, and *TP53* genes were observed in PACCs but less frequently than in PDACs. Other mutations noted in PACCs were in the *BRCA1*, *BRCA2*, *RB1* genes, along with mutations in the WNT-β-catenin

Table 1 Somatic genetic alterations observed in pancreatic acinar cell carcinoma specimens *n* (%)

Gene	Ref.	Number of patients	Number of patients with mutation (frequency)
TP53	Jiao *et al*[12]	23	3 (13)
	Chmielecki *et al*[26]	44	10 (23)
BRAF/RAF1[1]	Jiao *et al*[12]	23	3 (13)
	Chmielecki *et al*[26]	44	11 (25)
	Bergmann *et al*[25]	42	0 (0)
SMAD4	Jiao *et al*[12]	23	6 (26)
	Chmielecki *et al*[26]	44	6 (26)
BRCA2	Jiao *et al*[12]	23	1 (4)
	Chmielecki *et al*[26]	44	9 (20)
	Furukawa *et al*[27]	7	3 (43)
CDK2NA	Jiao *et al*[12]	23	4 (17)
	Chmielecki *et al*[26]	44	6 (14)
MMR/MSI	Liu *et al*[24]	36	5 (14)
	Bergmann *et al*[25]	42	2 (5)
RB1	Jiao *et al*[12]	23	3 (13)
	Chmielecki *et al*[26]	44	5 (11)
APC and CTNNB1	Jiao *et al*[12]	23	2 (9)
	Abraham *et al*[5]	17	4 (24)
	Chmielecki *et al*[26]	44	4 (9)
BRCA1	Jiao *et al*[12]	23	0 (0%)
	Chmielecki *et al*[26]	44	4 (9)
JAK1	Jiao *et al*[12]	23	4 (17)
MEN1	Jiao *et al*[12]	23	1 (4)
	Chmielecki *et al*[26]	44	3 (7)
GNAS	Jiao *et al*[12]	23	2 (9)
	Chmielecki *et al*[26]	44	2 (5%)
FAT	Furukawa *et al*[27]	7	4 (57)
Allelic Loss on Chromosome 11p	Abraham *et al*[5]	12	6 (50)

[1]Including *BRAF* gene fusion and point mutations.

pathway (*APC* and *CTNN1* genes). Interestingly, only one patient was found to have mutations in both the *BRCA2* and *CTNNB1* genes[26]. Additionally, changes were found in the *BRAF/RAF1*, *ATM* and *GNAS* genes[5,12,26,27]. Refer to Table 1 for more details.

TREATMENT

The treatment of choice for PACC is surgical resection if the tumor is localized and resectable. Surgical resection significantly improved survival; the 5-year survival was 72% in patients with resected PACC compared to 16% in their counterparts with PDAC[8]. The efficacy of adjuvant chemotherapy and/or radiotherapy is not clear for patients with PACC; 50% of cases present with metastatic disease, most commonly to regional lymph nodes and the liver. In addition, 25% of patients develop metastatic disease after resection of the primary tumor[1].

PACCs are moderately responsive to chemotherapy. Due to the few cases that present each year, there are no prospective data to guide treatment decisions and most therapeutic regimens used are the same as those utilized for PDACs or colorectal carcinomas. Responses have been seen in PACC patients treated with gemcitabine- or 5-fluorouracil-based combination therapies with irinotecan, a platinum analog, docetaxel, or erlotinib[1,6,28-30]. About 50%-60% of patients with metastatic PACC achieve stable disease or a better response with first-line combination chemotherapy regimens[6]. Table 2 describes systemic regimens that have shown activity in patients with PACCs.

For patients with limited metastatic disease, resection of the sites of metastasis may result in greater long-term survival than patients without resection. One case series included six patients with metastatic PACC who underwent resection of metastatic disease (liver metastases or omental metastases); they had a two-year overall survival rate of 67% (OS: 6-47+ mo). Four of these patients received adjuvant gemcitabine treatment in addition to resection of metastases[31].

CONCLUSION

Various genetic alterations were detected in PACCs; some of these mutations are potentially targetable by Food and Drug Administration (FDA)-approved medications or agents being studied for use in treating other malignancies. For example, BRAF inhibitors like vemurafenib are approved for treatment of melanoma with the *BRAF* V600E mutation[32], but these inhibitors have clinical activity against melanomas with the less common *BRAF* V600R mutation as well[33]. Tumors with DNA repair abnormalities, including *BRCA* gene mutations, show increased sensitivity to platinum agents. Poly ADP ribose polymerase inhibitors are efficacious in some *BRCA* mutated tumors as well[34,35]. One PACC patient with a loss of function mutation

Table 2 Chemotherapeutic regimens that showed activity in patients with pancreatic acinar cell carcinomas (9, 16, 18, 19, 22)

Regimen	Ref.	Total number of patients	Best responses
Gemcitabine	Lowery *et al*[6]	3	SD at 1 yr
Gemcitabine + erlotinib	Lowery *et al*[6]	4	PR at 5 mo, SD at 10 mo
Gemcitabine + irinotecan	Lowery *et al*[6]	2	SD at 25 mo
Cisplatin + irinotecan	Lowery *et al*[6]	1	PR at 12 mo, POD at 25 mo
Gemcitabine + cisplatin	Lowery *et al*[6]	2	PR at 4 mo
FOLFIRI	Lowery *et al*[6]	4	PR at 1.5 mo, POD at 11 mo
Gemcitabine + capecitabine	Lowery *et al*[6]	1	SD then POD at 9 mo
Gemcitabine + docetaxel + capecitabine	Lowery *et al*[6]	2	SD at 11 mo
Gemcitabine + oxalipatin	Lowery *et al*[6]	5	PR at 6 mo, POD at 15 mo
Capecitabine + erlotinib	Lowery *et al*[6]	1	SD at 15 mo, stopped because of toxicity
Folfirinox	Schempf *et al*[30]	1	PR with regression of primary disease and liver mets
Cisplatin + S1[1]	Furukawa *et al*[27]	1	CR with resolution of Liver mets. NED after 5 yr
Panitumumab[2]	Morales *et al*[40]	2	Clinically stable at 4 mo
Liposomal doxorubicin[3]	Armstrong *et al*[29]	1	PR for ⩾ 1 yr. Treatment discontinued due to cardiac toxicity risk
Docetaxel + irinotecan + cetuximab	Cananzi *et al*[41]	1	PR for 7 mo

[1]*BRCA-2* mutated; [2]*KRAS* wild-type gene; [3]The patient was treated with liposomal doxorubicin. DNA microarray and IHC analyses of a biopsy of liver metastases showed elevated topoisomerase Ⅱ expression and growth inhibition by doxorubicin (a topoisomerase Ⅱ inhibitor) *in vitro* in a cell line derived from the patient's tumor. FOLFIRI: 5-FU/Leucovorin/Irinotecan; FOLFIRINOX: 5-FU/Leucovorin/Oxaliplatin/Irinotecan; PR: Partial Response; POD: Progression of disease; SD: Stable disease; CR: Complete Response; NED: No evidence of disease.

in the *BRCA2* gene developed liver metastasis after receiving gemcitabine and S-1 as first line treatment; subsequently, the patient achieved a complete remission using cisplatin and S-1 therapy and the patient is alive with no evidence of disease after 5 years[27]. This was the only patient out of 11 in this study cohort treated with cisplatin. The results of this therapeutic intervention suggest that PACCs with *BRCA2* mutations might respond well to cisplatin-containing chemotherapy regimens.

As mentioned earlier, 14% of PACCs tested were revealed to have a MMR deficiency status[24]. Mismatch repair status in different malignancies (including colorectal, endometrial, gastric, pancreas, and small bowel carcinomas) predicted clinical benefit for use of immune checkpoint blockade by treatment with the PD-1 receptor blocker, pembrolizumab. Patients with MMR-deficient tumors showed a significantly higher objective response rate and progression-free survival compared to MMR proficient patients[36]. These findings suggest PD-1 and PD-L1 inhibitors might have antitumor activity in patients with MMR-deficient PACCs. Pembrolizumab has been recently approved by the FDA for the treatment unresectable or metastatic mismatch repair deficient solid tumors that have progressed on prior treatment and those who have no satisfactory alternative treatment options.

Comprehensive genomic analysis revealed that 23% of PACCs had fusions in *BRAF* and *RAF1* genes. One in particular, the staphylococcal nuclease and tudor domain containing 1 (*SND1*) -*BRAF* fusion which results from an amplified, chromosomal rearrangement between chromosome 7q32 and 7q34. This rearrangement results in an overexpression of the SND1 -BRAF fusion protein which is constitutively active, and confers resistance to c-Met receptor tyrosine kinase inhibition[37]. Several drugs were studied *in vitro* for use against the *SND1-BRAF* fusion mutation, including trametenib (a MEK inhibitor), sorafenib (a multikinase inhibitor), and TAK-632 (a pan-Raf inhibitor). Trametinib was shown to be superior to the other agents[26]. JAK-1 somatic mutations were seen in some cases of PACC, so JAK inhibitors such as ruxolitinib (a JAK-1 and 2 inhibitors), which is FDA approved for myelofibrosis[38], would be an option to consider for PACCs containing mutations in *JAK1/JAK2*. Additionally, there are investigational drugs that target the Wnt pathway and could be potentially studied in patients with PACC tumors with *APC* or *CTNNB1* gene mutations[39].

Unlike PDACs, the vast majority of PACCs are *KRAS* wild-type[12,26]. Two patients with PACC with liver metastases (they received two and three lines of prior chemotherapy, respectively) were treated with single agent panitumumab (an EGFR inhibitor). The *KRAS* gene was wild type in both patients. One patient was clinically stable for 4 mo with panitumumab therapy, while the other patient deteriorated clinically after 3 doses of the drug and then succumbed to the disease[40]. Panitumumab could be considered for refractory PACC patients with *KRAS* wild type tumors for which no other actionable targets are identified.

In summary, with this review of the literature, some potentially interesting targets were identified for treatment of patients with PACCs. Whether targeting tumors with mutations in the *BRAF* or *BRCA2* genes or a lack of MMR expression will be helpful to patients remains an issue to be settled in future clinical trials.

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MINIREVIEWS

Probiotics for gastrointestinal disorders: Proposed recommendations for children of the Asia-Pacific region

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Abstract

Recommendations for probiotics are available in

several regions. This paper proposes recommendations for probiotics in pediatric gastrointestinal diseases in the Asia-Pacific region. Epidemiology and clinical patterns of intestinal diseases in Asia-Pacific countries were discussed. Evidence-based recommendations and randomized controlled trials in the region were revised. Cultural aspects, health management issues and economic factors were also considered. Final recommendations were approved by applying the Likert scale and rated using the GRADE system. *Saccharomyces boulardii CNCM I-745* (Sb) and *Lactobacillus rhamnosus GG* (*LGG*) were strongly recommended as adjunct treatment to oral rehydration therapy for gastroenteritis. *Lactobacillus reuteri* could also be considered. Probiotics may be considered for prevention of (with the indicated strains): antibiotic-associated diarrhea (LGG or Sb); *Clostridium difficile*-induced diarrhea (Sb); nosocomial diarrhea (LGG); infantile colic (*L reuteri*) and as adjunct treatment of *Helicobacter pylori* (Sb and others). Specific probiotics with a history of safe use in preterm and term infants may be considered in infants for prevention of necrotizing enterocolitis. There is insufficient evidence for recommendations in other conditions. Despite a diversity of epidemiological, socioeconomical and health system conditions, similar recommendations apply well to Asia pacific countries. These need to be validated with local randomized-controlled trials.

Key words: *Lactobacillus rhamnosus*; Gastroenteritis; Guidelines; Probiotics; Children; Recommendations; Asia-Pacific; *Saccharomyces boulardii*

Core tip: This paper includes recommendations based on guidelines and local data for use of probiotics strains in the prevention or treatment of intestinal diseases in children of Asia-Pacific region. Notwithstanding major differences in health management and local conditions between countries, recommendations for children living in various countries of Asia-Pacific region are similar. *Saccharomyces boulardii* and *Lactobacillus rhamnosus GG* may be used in gastroenteritis, nosocomial infections, antibiotic-associated diarrhea. Selected strains may have a place in infantile colic, *Helicobacter pylori* treatment, and necrotizing enterocolitis. This document provides a helpful guidance to use probiotics in children based on local data and considerations.

Cameron D, Hock QS, Kadim M, Mohan N, Ryoo E, Sandhu B, Yamashiro Y, Jie C, Hoekstra H, Guarino A. Probiotics for gastrointestinal disorders: Proposed recommendations for children of the Asia-Pacific region. *World J Gastroenterol* 2017; 23(45): 7952-7964 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/7952.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.7952

INTRODUCTION

Gastrointestinal illnesses are a major cause of morbidity and mortality in children in developing countries[1], with diarrheal diseases being one of the top five causes of death in children younger than five years[2]. Worldwide, the most important enteric pathogen is rotavirus[2,3]; other major pathogens include *Escherichia coli*, *Vibrio cholera*, and *Shigella, Campylobacter, Salmonella*, and *Cryptosporidium* species[4,5].

In the Asia-Pacific region, the distribution of enteric pathogens shows marked regional variations, with differences largely corresponding to the predominant socioeconomic status of each region[2]. The Asia-Pacific region, comprising more than half of the world' s total population, is an area that is particularly diverse politically, economically, and culturally, including some of the world's least and some of the most developed nations[6]. While many in the Asia-Pacific region struggle to meet the most basic survival needs, a substantial proportion of the population in this region is moving towards Western diets and unhealthy lifestyles[6].

The human gut microbiota is emerging as a major determinant of intestinal and non-intestinal diseases. The human gut is home to a diverse collection of microbes, collectively termed gut microbiota, the disruption of which is associated with gastrointestinal diseases[7]. Moreover, it is becoming clear that geographic variations in the composition of the gut microbiota are likely to affect the risk of developing specific diseases[8]. These geographic variations may be due to a number of factors, including diet, culture, and genetics[9]. The relationship between disruption of gut microbiota and the risk of disease development and symptom severity suggests that the use of certain probiotics strains may prevent or reduce the progression of damage caused by some gastrointestinal illnesses.

Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit[10]. While there exists a substantial body of literature regarding the use of probiotics in numerous human diseases, the quality of evidence for their use is variable, with evidence lacking for many indications. A number of organizations and institutions have reviewed the currently available evidence and developed recommendations for probiotics use at national or international level[10-14]. In order to propose recommendations for the use of probiotics in children of the Asia-Pacific region, a group of experts met to review and discuss existing recommendations, as well as the relevant available data regarding the proposed recommendations for Asia-Pacific.

METHODS

With the objective of formulating recommendations for

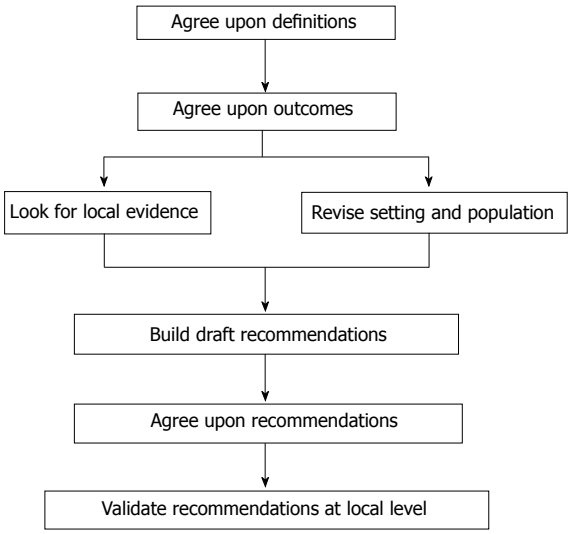

Figure 1 Steps for designing the recommendations for the use of probiotics in pediatric population in the Asia-Pacific region.

use of probiotics in pediatric gastrointestinal disorders in the Asia-Pacific region, a working group of international experts in adult and pediatric gastroenterology from Asia-Pacific countries (Australia, China, India, Indonesia, Japan, South Korea, and Singapore), as well as from several countries outside the region (United States, Uruguay, United Kingdom, The Netherlands, and Italy) met to discuss the indication for use of probiotics based on local epidemiological conditions. Workshops on the gastrointestinal epidemiology of the represented countries and the use of probiotics in both adults and children were held. This article describes the proposed recommendations for use of probiotics in intestinal diseases in children in the Asia-Pacific region. The main considerations in the development of these consensus recommendations were the epidemiology and etiology of gastrointestinal diseases in the Asia-Pacific region, and the evidence from the region and internationally to support the use of probiotics for different pediatric gastrointestinal conditions.

The method used to develop the recommendations consisted of multiple steps (Figure 1). Initially, target diseases and their impact were defined, and the definitions and outcomes for each disease were agreed upon. Published data and available evidence-based recommendations and guidelines from the Asia-Pacific region and from other organizations, such as the World Gastroenterology Organization (WGO), European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN), Latin American Society for Pediatric Gastroenterology and Nutrition (LASPGAN), were studied and discussed. Subsequently, the local epidemiological and clinical features of the main intestinal diseases commonly seen in the Asia-Pacific region were discussed to determine their impact on the recommendations. Randomized-controlled trials (RCTs) on the effect of probiotics on gastrointestinal diseases, which were conducted in the Asia-Pacific region, written in English and published in peer review journals after 2005 were also evaluated. The discussion included epidemiological data, local practices and traditions, availability of probiotics and other drugs , standards of care, perception of specific disease risk and the need for prevention, economic considerations, and other health management issues (*e.g.*, private *vs* public medicine).

The final recommendations were based on these steps using the GRADE system[15] and the strength of each individual recommendation was rated as weak or strong.

An overview of the steps used for designing the recommendations for acute gastroenteritis is shown in Figure 2.

Once the recommendations were designed, the participants from the Asia-Pacific region were asked to provide a consensus on the proposed recommendations using the Likert scale[16]. The recommendations were further revised based on the feedback received after circulating the proposed recommendations to countries in the Asia-Pacific region until final consensus was achieved as judged by agreement by all participants with grade 4 or 5 in the Likert scale (corresponding to "agree" and "strongly agree", respectively). The final steps will be the validation of recommendations through field trials to evaluate their applicability and efficacy using previously tested methodology[17].

THE HUMAN GUT MICROBIOME

The human gut microbiota includes a vastly diverse community of microorganisms that, together with the collection of all of the genomic elements of the specific microbiota, comprises the human gut microbiome[18]. With the development of increasingly advanced molecular methods in recent years, such as high-throughput sequencing, our interest in and understanding of the human gut microbiome have significantly increased.

It has become apparent that the microbiome is influenced by numerous factors, including age, geography, diet, lifestyle, disease, and antibiotic exposure[9,19,20]. Notably, while it has long been known that a course of antibiotics impacts the gut microbiome in the short term, there is also evidence to suggest that exposure to antibiotics may have a long-term impact on the human intestinal microbiota that persists for as long as 2 years after treatment[19].

While much of the function of the human gut microbiome remains to be fully elucidated, we do know that the microbiota plays an important role in maintaining health[20]. The role of microbiota includes the prevention of colonization by pathogens and development and activation of the immune system, as well as an important role in stability of metabolism[20]. When the gut microbiota is functioning as expected in a healthy

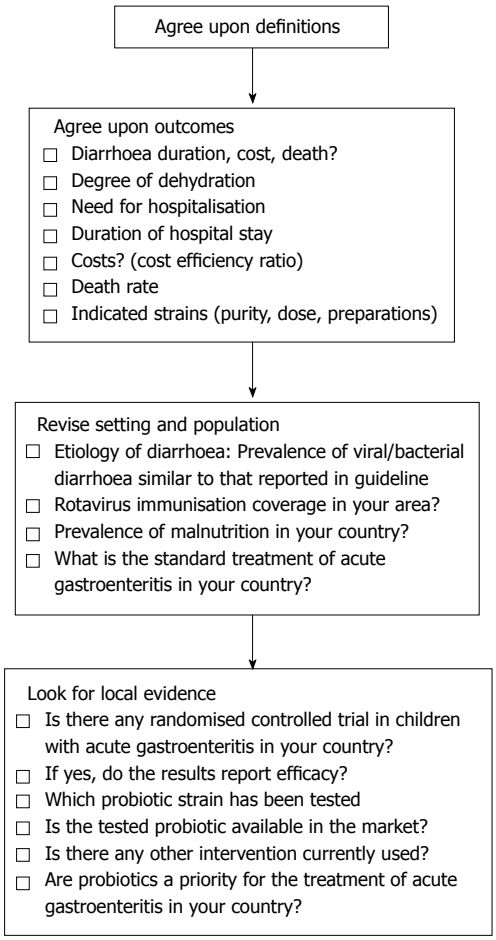

Figure 2 Steps for designing the recommendations for acute gastroenteritis.

person, it is in balance or eubiosis. In contrast, in a number of diseases the gut microbiota exists in a state of dysbiosis[20].

The composition of microbiota is altered in certain disease states, including enteric infections, *Helicobacter pylori* (*H. pylori*) infection and antibiotic-associated diarrhea (AAD)[20]. Furthermore, aberrations in microbial configuration are often associated with specific chronic diseases, producing a "microbial signature" of the specific disease[21,22], although the cause-effect relationship between microbial change and disease is not clear. There appears to be a link between dysbiosis and disease, including inflammatory bowel diseases (IBD), celiac disease, diabetes, cancer, obesity, and cystic fibrosis[22-24].

EPIDEMIOLOGY OF GASTROINTESTINAL DISEASES IN THE ASIA-PACIFIC REGION

While patterns of diseases worldwide are constantly changing, diarrhea remains a leading cause of mortality among children younger than five years[25]. The burden of this disease is not shared equally, but is disproportionately borne by children in low income countries, largely due to water and sanitation issues, as well as nutritional factors[26]. Moreover, effective interventions are not provided equitably across all communities[26].

Infectious diarrhea is a major concern in the Asia-Pacific region. Worldwide, the most frequent causative pathogen for infectious diarrhea is rotavirus, responsible for approximately 40% of all hospital admissions for diarrhea of infants and young children[5]. A specific feature of certain Asian countries is the circulation of *V. cholerae* that may be multidrug-resistant and cause severe diarrhea[27]. Other major pathogens include *E. coli,* Shigella, Campylobacter, and Salmonella species, as well as the protozoan pathogen Cryptosporidium that is an important enteric pathogen in children of some Asian Countries[5]. The pathogens responsible for diarrheal deaths vary between regions[2]. In a recent study, 5304 stool samples from Africa and Asia (Bangladesh, India and Pakistan) were analyzed and results show that approximately 80% of gastroenteritis in children are caused by six pathogens (Shigella, Rotavirus, Adenovirus 40/41, Heat-stable enterotoxin-producing *E coli* or ST-ETEC, Cryptosporidium and Campylobacter)[28]. Interestingly, *Clostridium difficile* (*C. difficile*) was an important pathogen in high-income countries, while Shigella and Aeromonas infections occurred at a higher frequency in areas with poor sanitation[2].

In addition to infectious diarrhea, there are several other diseases of importance in the Asia-Pacific region, such as AAD (including *C. difficile*-associated diarrhea), *H. pylori* infection, irritable bowel syndrome (IBS), and IBD. The incidence of the latter condition is sharply increasing in some locations; however, the relative prevalence of individual diseases differs from region to region, as well as across countries within the Asia-Pacific region[29-31]. For example, *H. pylori* is found in half of the world's population, although its prevalence and clinical manifestations vary markedly in relation to numerous factors, including geography, age and socioeconomic factors, with prevalence much higher in developing countries[32].

In addition to epidemiological factors, the organizational and economic features that differ throughout Asia-Pacific countries play a major role in the implementation of treatment guidelines[33].

PROBIOTICS

Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit[10]. A substantial body of evidence suggests that the clinical effects of probiotics in humans include prevention and treatment of diarrhea, immunomodulation, and modulation of intestinal flora[14]. The short-term effects of probiotics, such as those seen in patients with acute diarrhea, are due to the direct effect of specific probiotic strains on pathogens present in the human gut, whereas many of their long-term effects are the

result of an interaction between selected strains and the host immune response or the reconstitution of the disrupted intestinal barrier[34].

In general, the effects of probiotics are strain-, disease- and age-specific[14]. Administration of multiple strains is not necessarily better or worse than a single strain, rather use of specific probiotic strains or preparations should rely on clinical data from at least one well-designed trial of a well-characterized probiotic preparation[34]. Several meta-analyses combining data from studies of different probiotic strains in different patient populations produced results that were largely inconclusive[35-39]. Furthermore, the definitions and outcome parameters used in different studies are often heterogeneous, which may limit the interpretation of data available. Thus, although meta-analyses can be used to establish the effects of probiotics, they may underestimate the benefits of selected strains or amplify the efficacy of less effective strains if not performed appropriately. In addition, there is a high level of heterogeneity among microbial preparations, as well as a lack of quality control for commercially-available products. On the other hand, for selected strains and pathologies, there are a high number of well-conducted RCTs with compelling proof of efficacy[40,41]. Finally, it is recognized that the dose of probiotics used in children may be important, particularly when evaluating their efficacy in growing children, although currently data are limited.

Safety and regulatory issues are also important considerations and may act as barrier against the use of probiotics. Overall, the available evidence suggests that probiotics are generally safe[34], with long-term studies demonstrating the safety of *L. rhamnosus GG* in very low birth weight preterm infants[42] and probiotic strains (*L. rhamnosus GG, Bifidobacterium lactis Bb-12, Lactobacillus paracasei ST11,* and *B. longum BL999)* administered during the perinatal period[43]. However, it should be noted that there are a number of potential risks associated with the use of probiotics, and there is limited data specifically addressing the safety of these agents[14,44]. To date, known risks associated with use of probiotics are largely confined to specific patient populations, including premature infants, immunocompromised individuals and critically ill patients[14,44]. A study on probiotics from dietary supplements reported the presence of antibiotic resistance in selected strains, although further studies exploring the mechanism by which this resistance is conferred are warranted[45].

The current regulatory scenario of probiotics provides a scattered picture, with probiotics classified as food additives or as drugs in different countries. For instance, in Indonesia, probiotics are classified as supplements, and some strains (including *Saccharomyces boulardii*) are not available due to these regulations. A recent paper provides an overview of how probiotics are categorized in the world[46].

Furthermore, *L. rhamnosus GG* is not commonly used in Japan, where *L. casei* Shirota-fermented milk is widely available. The implementation of the recommendations for the use of probiotics is likely to be influenced by those factors.

Based on these findings, and keeping in mind that the main basis for prescribing probiotics is the data on clinical efficacy, the experts produced the following recommendations for intestinal diseases of children in the Asia Pacific region. The proposed recommendations could be modified according to the country-specific level of evidence.

PROPOSED RECOMMENDATIONS FOR PROBIOTICS

Acute gastroenteritis

Infectious diarrhea is the best established indication for probiotics administration in childhood. A position paper by ESPGHAN for the use of probiotics in the management of acute gastroenteritis in infants and children proposed that since the effects of probiotic are strain-specific, the recommendations for the use of an individual probiotic should be made based on the efficacy and safety of the probiotic from well-established RCTs and that the efficacy and safety of a particular strain should not be extrapolated to another strain[47]. Furthermore, a probiotic preparation should be selected from a manufacturer who has adequate quality control, and the lack of current evidence on the use of a particular probiotic does not mean that it will prove to be ineffective in future studies[47]. According to the ESPGHAN/European Society of Pediatric Infectious Diseases guidelines for the management of acute gastroenteritis in children in Europe, probiotics provide an "active therapy" to reduce the duration and severity of symptoms. The guidelines strongly recommend the use of probiotics as an adjunct to rehydration therapy. Based on large number of high quality RCTs, *L. rhamnosus GG* and *S. boulardii* were recommended (Table 1)[40]. *L. reuteri DSM 17938* was also included in the list of effective strains, albeit with a weak recommendation. Few RCTs are available for other strains or combinations of strains in children with acute gastroenteritis. Some RCTs were conducted in Asian countries with variable results[48]. A double-blind randomized trial found probiotics to be ineffective in acute diarrhea in Indonesian children[49]. However, because data are limited or have methodological issues, many strains were not considered for inclusion in the present recommendations.

A position similar to ESPGHAN was taken in the United States, where the indications for probiotics were discussed in the 4th Triennial Yale/Harvard Workshop on Probiotic Recommendations[12]. Similar recommendations were published in South America[50], and the Latin-American consensus guidelines on the use of probiotics in pediatric gastroenterology suggest

Table 1 Recommendations for use of probiotics in childhood intestinal diseases by geographic region

Diseases		Europe[14,40]	United States[36]	Latin America[50]	World[51]
Acute gastroenteritis	T	*L. rhamnosus GG, S. boulardii, L reuteri*	*L. rhamnosus GG, S. boulardii*	*L. rhamnosus GG, S. boulardii, L. reuteri*	*S. boulardii, L. rhamnosus GG,* Indian Dahi
AAD	P	*L. rhamnosus GG, S. boulardii*	*L. rhamnosus GG, S. boulardii*	*L. rhamnosus GG, S. boulardii*	*S. boulardii; L. rhamnosus GG, , B. lactis* Bb12 + *S. thermophilus, L. rhamnosus* strains E/N, Oxy and Pen
CDAD	P	*S. boulardii*			
Nosocomial diarrhea	P	*L. rhamnosus GG*	*L. rhamnosus GG*	*L. rhamnosus GG, B. lactis Bb12, S. thermophilus, B. bifidum S. boulardii*	*L. rhamnosus GG, B. lactis Bb12 + S. Thermophilus*
Traveler's diarrhea	P				
Functional intestinal disorders (IBS)	T	Insufficient evidence		*L. rhamnosus GG,* VSL#3	*L. rhamnosus GG, L. reuteri DSM 17938*
Infant colic	T	*L. reuteri DSM 17938*		*L. reuteri DSM 17938*	*L. reuteri DSM 17938*
IBD (CD, UC, pouchitis)	T	*E. coli Nissle 1917,* VSL#3[1]		VSL#3[1]	VSL#3[2]
Helicobacter pylori infection	T			Not recommended	*L. casei DN-114 001*

[1]Available evidence supports use in UC but not CD or pouchitis; [2]For mildly active UC. T: Treatment; P: Prevention; AAD: Antibiotic-associated diarrhea; CDAD: *Clostridium difficile*-associated diarrhea; CD: Crohn's disease; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; UC: Ulcerative colitis; VSL#3: Proprietary mixture of eight probiotic strains.

that *L. rhamnosus GG*, *S. boulardii,* and (with a weak recommendation) *L. reuteri* are effective against diarrhea and should be introduced at the onset of gastroenteritis[50]. The WGO also published a position paper on the indications for probiotics in adults and children[51]. The recommendations made for the use of probiotics in acute gastroenteritis, according to main geographic region and indication, are summarized in Table 1.

Other national and international guidelines provide recommendations for the management of gastroenteritis. A group of experts examined 15 guidelines from all over the world for management of acute gastroenteritis in children, of which nine guidelines recommended the use of probiotic strains for treatment of gastroenteritis[52]. In most guidelines, the recommended strains included *L. rhamnosus GG* and *S. boulardii*. Notably, selected countries in the Asia-Pacific region have adopted guidelines recommending effective use of probiotics for management of gastroenteritis.

A study conducted in India explored deviations from the WHO guidelines in the management of diarrhea in children[53]. The most common deviation from the WHO guidelines was in relation to the addition of probiotics. This study also found that the use of probiotics reduced the duration of symptoms[53].

The panel of experts also discussed the etiological patterns, features, and the risk factors for acute gastroenteritis. It was observed that the epidemiology of enteric pathogens, the features of disease, the incidence of risk factors (mainly childhood malnutrition) and the organization of health care provide a broad and variable picture in the Asia-Pacific region. While the efficacy of probiotics is not strictly related to the etiology, it is likely that the highest efficacy is exerted in viral rather than bacterial gastroenteritis. Overall, most guidelines recommend the same probiotic strains for active therapy of gastroenteritis, based on consistent data showing similar efficacy of recommended strains in various settings and conditions. However, the progressive introduction of immunization against rotavirus may alter the etiologic and epidemiologic pattern of gastroenteritis, as already reported in many countries including countries in the Asia-Pacific region[54]. Norovirus is an emerging etiologic agent, and data regarding the efficacy of *L. rhamnosus GG*, *S. boulardii*, and other probiotics against this virus are limited. It was also noted that there are no clear data about the efficacy of probiotics in patients with cholera, although there is weak evidence that probiotics may have some preventive effect[55]. Furthermore, there is little evidence regarding the efficacy and safety of probiotics in malnourished children. Since the incidence of malnutrition is high in several countries with poor living conditions in the Asia-Pacific region, additional studies are needed to specifically address the use of probiotics in gastroenteritis in undernourished pediatric populations living in these regions.

After discussing the current evidence and issues regarding the use of probiotics, the expert panel agreed upon recommending *L. rhamnosus GG* and *S. boulardii* CNCM I-745 for management of gastroenteritis in the Asia-Pacific region as an adjunct to rehydration. Inclusion of *L. reuteri* DSM 17938 may also be used although the quality of evidence for the latter strain is weak[39].

Antibiotic-associated diarrhea including C. difficile-induced diarrhea

AAD is a major global issue, with children being at a higher risk than adults. AAD has become an important problem due to the general overuse of antibiotics in children in most Asia-Pacific countries[29,56]. *C. difficile* is a major causative agent for AAD, both in terms of

frequency and severity[57]. Available data suggest that probiotics, including *L. rhamnosus GG* and *S. boulardii CNCM I-745,* are effective in the prevention of AAD[58] and *C. difficile* infections in pediatric populations[57,58].

The ESPGHAN working group recommendations on the use of probiotics for prevention of AAD in children suggest that physicians should evaluate the risk factors for the occurrence of AAD or *C. difficile*-associated diarrhea, such as the class of antibiotics, duration of antibiotic treatment, need for hospitalization, age, comorbidities, and previous episodes of AAD or *C. difficile*-associated diarrhea, before making decisions on the use of probiotics for the prevention of AAD in children[41]. The working group provides strong recommendations for the use of *L. rhamnosus GG* or *S. boulardii* CNCM I-745 for AAD prevention and a weak recommendation for the use of *S. boulardii* CNCM I-745 for the prevention of *C. difficile*-associated AAD[41].

Direct clinical data are available from the Asian region. A study conducted in China evaluated the efficacy of *S. boulardii* for the prevention and treatment of diarrhea in children receiving intravenous antibiotics for lower respiratory infections[59]. Administration of *S. boulardii* was associated with a significantly reduced risk of developing AAD (RR = 0.22, 95%CI: 0.1-0.5) and a reduction in the duration and severity of diarrhea[59]. But, in a recent Korean study, the use of probiotics was not associated with any significant differences with regard to prevention of *C. difficile* infection[60].

The expert group supports probiotic administration for the prevention of AAD. Either *L. rhamnosus GG* or *S. boulardii* CNCM I-745 should be administered as treatment, whereas based on available clinical evidence, the best option for prevention of *C. difficile*-associated diarrhea in children is administration of *S. boulardii* CNCM I-745.

Nosocomial diarrhea

Nosocomial diarrhea is an important issue in the Asia-Pacific region, mainly due to the high frequency of rotavirus infection[61]. Any coexisting respiratory infection can amplify the impact of nosocomial diarrhea, resulting in exacerbation of the clinical condition. Although evidence regarding the efficacy of probiotics in the prevention of nosocomial infections remains unclear[62], available data show that *L. rhamnosus* GG may be effective in the treatment of intestinal nosocomial infections in children[63,64].

Based on available data, the expert group recommends that administration of *L. rhamnosus GG* can be considered in hospitalized children for the prevention of intestinal infections.

Traveler's diarrhea

Traveler's diarrhea was also discussed during the meeting and it was observed that there is no strong evidence of an increased risk of diarrhea among children who live within the Asia-Pacific region and travel elsewhere. In addition, data on the efficacy of probiotics in preventing traveler's diarrhea are insufficient to provide recommendations[65]. Nevertheless, the Latin-American consensus guidelines recommend *S. boulardii* for the prevention of traveler's diarrhea in children based on RCT evidence[50]. *L. rhamnosus GG* and *L. acidophilus* appear to be ineffective, while there is some limited evidence that *S. boulardii* administration is associated with a significant reduction in diarrhea incidence[50].

The expert group does not recommend the use of probiotics for the prevention of traveler's diarrhea in children in the Asia-Pacific region.

Functional intestinal disorders

Early studies on the epidemiology of functional intestinal disorders in the Asia-Pacific region suggest that these conditions are common[66]. According to the WGO global guidelines, several studies have demonstrated significant therapeutic gains with probiotics over placebo in the symptomatic treatment of IBS, although most trials were performed in adults[34]. A reduction in abdominal bloating and flatulence as a result of probiotic treatment is a consistent finding in published studies, and some strains (including *B. infantis* 35624) may also reduce abdominal pain and provide systemic relief. However, there is only limited evidence on the efficacy of probiotics in the treatment of functional intestinal disorders in children[37,67,68].

Based on available data, there is insufficient evidence to recommend probiotics in the treatment of functional intestinal disorders.

Infant colic

Colic is a disturbing condition in infants that frequently requires medical consultation and therapy. Dysbiosis may affect gut motor functions and gas production, resulting in colicky abdominal pain in infants. Data from RCTs indicate that probiotics may be effective for the treatment of infant colic, with *L. reuteri* being identified as the most effective strain[69,70].

If infant colic is perceived as a problem in need of active intervention, administration of effective probiotics, particularly *L. reuteri* DSM 17938, may be considered.

Inflammatory bowel diseases

As shown in Table 1, the WGO global guidelines address the role of probiotics in IBD, particularly ulcerative colitis and Crohn's disease (including pouchitis)[34]. Regarding pouchitis, there is good evidence for the efficacy of VSL#3 (a mixture of eight probiotic strains including Lactobacilli, Bifidobacteria, and Streptococcal species) in preventing the initial episode and further relapses of pouchitis after induction of remission

with antibiotics. Overall, the guidelines conclude that probiotics are recommended for patients with pouchitis of mild activity or as maintenance therapy for those in remission. For ulcerative colitis, the guidelines suggest that *E. coli Nissle* may be as effective as mesalazine in maintaining remission of ulcerative colitis, and that VSL#3 has efficacy in the induction and maintenance of remission of mild to moderate ulcerative colitis. For Crohn's disease, the guidelines note that probiotics are less effective, as reported in a Cochrane systematic review, which concluded that there is no evidence to indicate a beneficial effect of probiotics in the maintenance of remission in Crohn's disease[34,71]. Other guidelines are less open to consider probiotics as a general intervention for intestinal inflammatory conditions (Table 1).

The expert panel concluded that overall, there appears to be limited evidence to support the use of probiotics for the treatment of IBD in children. In pouchitis, VSL#3 may be considered based on evaluation of individual cases.

H. pylori infection

H. pylori infection is a major concern in most Asian countries[72-74]. In addition the rate of *H. pylori* antibiotic resistance is high in Asian population[75]. A review on the use of probiotics in the treatment of *H. pylori* infections in children indicates that probiotics may be beneficial in improving the eradication rate of *H. pylori* infections and a meta-analysis of RCTs provided further support. However, these effects are strain-specific and additional long-term studies are needed to establish the efficacy of different strains and doses of probiotics in the management of these infections[76]. A systematic review and meta-analysis of 11 RCTs, including a total of 2200 participants (among whom 330 were children), found an increase in *H. pylori* eradication rate with the addition of *S. boulardii* to antibiotic treatment[38]. Eighty percent of patients in the *S. boulardii* group achieved eradication of *H. pylori* compared with 71% of patients in the control group (RR = 1.11, 95%CI: 1.06-1.17). Moreover, compared with controls, *S. boulardii* reduced the overall risk of *H. pylori* therapy-related diarrhea. However, since the number of children included in the studies was limited, additional studies in children are warranted.

WGO global guidelines report that several Lactobacilli strains appear to reduce the side effects of antibiotic therapies for *H. pylori* and improve patient compliance[34]. However, while there is evidence to suggest that supplementation of *H. pylori* antibiotic treatment regimens with certain probiotics may help eradication, the guidelines state that "there is currently insufficient evidence to support the concept that a probiotic alone, without concomitant antibiotic therapy, would be effective"[34]. An international consensus document, developed following the 5th Maastricht/Florence Consensus Conference in 2016, concluded that certain probiotics (such as Lactobacilli and *S. boulardii*) show favorable results as adjuvant treatment in *H. pylori* infection for reducing side effects, with a positive effect on patient compliance with the extensive antibiotic regime[77]. In a recent meta-analysis the efficacy of probiotics administration in improving eradication rates and in reducing side effects of therapy was confirmed. In subgroup analysis there was no difference between Asia and Europe, but probiotics were more effective in children than in adults. However several strains, including *S. boulardii,* were effective and may be considered in adjunct eradication therapy[78].

Based on available data, the Asia-Pacific expert panel suggests that probiotic strains with evidence of efficacy, such as *S. boulardii* CNCM I-745, may be considered for the prevention of side effects of antibiotic therapy for *H. pylori* and for improving eradication rates in children.

Necrotizing enterocolitis

There is a strong debate within the scientific community about the use of probiotics for the prevention of necrotizing enterocolitis (NEC). Several studies have reported that probiotics reduce the incidence of NEC and NEC-associated mortality in preterm infants and several meta-analysis have confirmed these results[79,80]. However, a rigorous phase 3 RCT recently showed no effect of *B. breve BBG-001* on NEC[81]. Several guidelines and position papers reported that although a substantial body of evidence supports the use of probiotics, there is insufficient evidence for a strong recommendation nor is there consensus on specific strain[36].

The expert panel agreed that probiotics may be considered for the prevention of NEC as there is evidence that the risk of NEC and the associated mortality may be reduced in high-risk populations. However, since there is no conclusive agreement on whether to administer probiotics, the decisions should be taken in agreement with the parents, in light of current evidence and the benefits expected.

A summary of all recommendations for the use of probiotics in children is shown in Table 2.

DISCUSSION

Clinical practice guidelines are important tools for translating the evidence available into clinical best practice[82]. The development of guidelines for probiotics use is often hampered by several factors, including lack of characterization of probiotics, inadequate quality control of preparations and a paucity of high quality studies. Nevertheless, well defined clinical indications for the use of probiotics have been established, and more are emerging.

Table 2 Proposed recommendations for the Asia-Pacific region with grade and strength of recommendations

Disease		Recommendation	Strains	Grade	Weak
Acute gastroenteritis	T	Should be considered[1]	*S. boulardii*,	Moderate quality	Strong
			L. rhamnosus GG		Strong
			L reuteri		Weak
AAD	P	May be considered	*L. rhamnosus GG*	Moderate quality	Strong
			S. boulardii		Strong
CDAD	P	May be considered	*S. boulardii*	Low quality	Weak
Nosocomial diarrhea	P	Can be considered	*L. rhamnosus GG*	Moderate quality	Weak
Traveler's diarrhea	P	Not recommended		Very low quality	Weak
Functional intestinal disorders	T	Not recommended		Very low quality	Weak
Infant colics	T	May be considered	*L. reuteri*	Moderate quality	Weak
IBD (Crohn's disease, ulcerative colitis)	T	Not recommended		Low quality	Weak
Helicobacter pylori infection	T	May be considered	*S. boulardii*,	Very low quality	Weak[2]
			others		Weak
Necrotizing enterocolitis		Decision to be discussed with parents	Various (Bifidobacterium, Lactobacillus species)		

[1]In adjunct to oral rehydration therapy and with the exclusion of malnourished children; [2]For prevention of antibiotic-induced side effects and possible increase in eradication rates. T: Treatment; P: Prevention; AAD: Antibiotic-associated diarrhea; CDAD: *Clostridium difficile*-associated diarrhea; IBD: Inflammatory bowel disease.

Guidelines that have been developed in one country may not be applicable in other countries, depending on the prevalence and etiology of the clinical condition, as well as the availability or cost of recommended interventions and the organization of health care. Therefore, guidelines should generally be developed for individual countries or regions.

As outlined in the previous sections, the burden of intestinal diseases differs across geographic regions, particularly between countries in the Asia-Pacific region. These differences are due to numerous factors that must be taken into account when developing guidelines for the prevention and treatment of intestinal illnesses in children in the Asia-Pacific region. However, despite the largely socioeconomic disparities that exist between countries, the proposed recommendations were agreed upon by all participants to the panel coming from different countries in the Asia Pacific region, as judged by the Likert scale voting and provide guidance for physicians on the use of probiotics in specific intestinal diseases, thereby filling a gap that exists, as similar documents are available for other major regions of the world.

Factors that must be taken into consideration in terms of the applicability of the Asia-Pacific guidelines include the availability of specific probiotic strains in local markets, costs, patient and physician access to probiotic products, and personal/cultural beliefs. Moreover, current regulations regarding probiotics are country-specific in the Asia-Pacific region, as well as in other countries in the world. Standardization of a regulatory framework for probiotics would be of substantial benefit in terms of furthering research into and the use of these agents in human medicine.

Given this complex scenario and the need for supporting evidence, the recommendations are provided as "proposed" and their potential benefits need to be confirmed at the local level with well-conducted RCTs.

SUMMARY OF RECOMMENDATIONS FOR THE ASIA-PACIFIC REGION

While it is acknowledged that more data specific to the Asia-Pacific region are needed for many of the indications, the working group proposes the following recommendations for pediatric intestinal diseases based on available data:

Acute gastroenteritis: Administration of probiotic strains for which there is good quality evidence of efficacy should be considered in adjunct to oral rehydration therapy in children with acute gastroenteritis. This recommendation does not include children with severe malnutrition. At present, *S. boulardii* CNCM I-745 and *L. rhamnosus* GG are the two strains for which there is compelling evidence of efficacy. *L.reuteri* DSM 17938 may be considered albeit proof of efficacy is less consistent.

Antibiotic-associated diarrhea: Upon evaluation of local conditions and risk factors, probiotics administration may be considered on a case by case basis for the prevention of AAD. *S. boulardii* CNCM I-745 and *L. rhamnosus GG* are two strains for which the quality of evidence is good.

***C. difficile* infection:** For the prevention of *C. difficile*-associated diarrhea, probiotics may be considered based on evaluation of individual cases. At present, the recommended strain is *S. boulardii* CNCM I-745 for which the quality of evidence is low.

Prevention of nosocomial diarrhea: Upon evaluation of local conditions and risk factors, probiotics

may be considered to prevent hospital-acquired intestinal infections and diarrhea on a case by case basis in children admitted to hospital. Although the evidence remains weak, *L. rhamnosus GG* is the strain recommended for this indication.

Traveler's diarrhea: Data on the efficacy of probiotics in preventing traveler's diarrhea are insufficient to provide recommendations for the use of probiotics.

Functional intestinal disorders: Based on available data, there is insufficient evidence to recommend probiotics in the treatment of functional intestinal disorders.

Infant colic: Probiotic administration may be considered for the treatment of infantile colic. At present, the recommended strain is *L. reuteri* DSM 17938, for which the quality of evidence is weak.

Inflammatory bowel disease: There is no strong evidence supporting the treatment of IBD with probiotics. In pouchitis probiotic therapy may be considered based on evaluation of individual cases. At present, the recommended probiotic preparation is VSL#3 for which the quality of evidence is weak.

***H. pylori* treatment:** Probiotics administration may be considered for the prevention of side effects and improving eradication rates in children undergoing therapy for *H. pylori*. The recommended strains include *S. boulardii* CNCM I-745 and others for which the quality of evidence is weak.

Necrotizing enterocolitis: Probiotics may be considered for prevention of NEC in high-risk populations as there is evidence that the risk of NEC and the associated mortality may be reduced. However since there is no agreement on strains, indications and scheme, the decision should be taken in agreement with the parents, in the light of current evidence.

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ORIGINAL ARTICLE

Basic Study

Down-regulation of miR-30a-3p/5p promotes esophageal squamous cell carcinoma cell proliferation by activating the Wnt signaling pathway

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Author contributions: Qi B and Zhao S contributed to the conception and design of the study; Chen ZJ, Li XN, Qi Y, Yang Y and Cui GH provided the animals and collected and managed the patients; Wang Y, Guo HZ, and Li WH performed statistical analysis and computational analysis; Qi B, Wang Y, and Zhao S wrote, reviewed, and made critical revision of the manuscript.

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Abstract

AIM

To investigate the potential role of microRNA-30a (miR-30a) in esophageal squamous cell carcinoma (ESCC).

METHODS

Expression of miR-30a-3p/5p was analyzed using microarray data and fresh ESCC tissue samples. Both *in vitro* and *in vivo* assays were used to investigate the effects of miR-30a-3p/5p on ESCC cell proliferation. Furthermore, Kyoto Encyclopedia of Genes and Genomes analysis was performed to explore underlying mechanisms involved in ESCC, and then, assays were carried out to verify the potential molecular mechanism of miR-30a in ESCC.

RESULTS

Low expression of miR-30a-3p/5p was closely associated with advanced ESCC progression and poor prognosis of patients with ESCC. Knock-down of miR-30a-3p/5p promoted ESCC cell proliferation. Increased miR-30a-3p/5p expression inhibited the Wnt signaling pathway by targeting Wnt2 and Fzd2.

CONCLUSION

Down-regulation of miR-30a-3p/5p promotes ESCC cell proliferation by activating the Wnt signaling pathway through inhibition of Wnt2 and Fzd2.

Key words: miR-30a-3p/5p; Proliferation; Esophageal squamous cell carcinoma; Wnt signaling pathway; Wnt2; Fzd2

Core tip: In this work, we found that low expression of miR-30a-3p/5p was closely associated with advanced esophageal squamous cell carcinoma (ESCC) progression and poor prognosis of patients with ESCC. Down-regulation of miR-30a-3p/5p suppressed ESCC cell proliferation both *in vitro* and *in vivo*. Furthermore, miR-30a-3p and miR-30a-5p could inhibit the activity of the Wnt signaling pathway by targeting the 3' untranslated regions of Wnt2 and Fzd2, respectively. This study provided further evidence suggesting that miR-30a-3p/5p are diagnostic and prognostic biomarkers for ESCC, as miR-30a-3p/5p participate in the activation of the Wnt signaling pathway and subsequently, the regulation of ESCC cell proliferation.

Qi B, Wang Y, Chen ZJ, Li XN, Qi Y, Yang Y, Cui GH, Guo HZ, Li WH, Zhao S. Down-regulation of miR-30a-3p/5p promotes esophageal squamous cell carcinoma cell proliferation by activating the Wnt signaling pathway. *World J Gastroenterol* 2017; 23(45): 7965-7977 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/7965.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.7965

INTRODUCTION

Esophageal cancer is one of the most common human malignancies, ranking sixth among cancer-related deaths worldwide[1]. Esophageal squamous cell carcinoma (ESCC) is the major histological type and the leading cause of death from all esophageal cancer types in Asian countries, especially in China[2,3]. Because of the lack of early detection, the majority of patients with ESCC are diagnosed at advanced stages with high risk of metastasis and recurrence[4]. The 5-year overall survival rate of ESCC is less than 20%[1]; therefore, further investigation of the molecular mechanisms involved in ESCC is urgent and essential for developing early diagnostic and therapeutic strategies.

The development and progression of ESCC involve synergic effects of various pathogenic factors, including particular dietary factors (chemical and physical), human papillomavirus infection, and genetic susceptibility[5]. To further investigate genetic susceptibility and develop personalized targeted therapy for ESCC, high-throughput techniques have been used. Genetic landscapes of ESCC obtained by whole genome and exome sequencing have illustrated that genomic alterations in ESCC include single nucleotide variants, copy number alterations, and alterations in multiple signaling pathways, such as cell cycle regulation, DNA damage control, RTK-Ras-MAPK-PI3K-Akt, Notch, and Wnt[6,7]. Many researchers have recently reported the significance of both canonical and non-canonical Wnt signaling pathways in ESCC, thereby indicating the potential of the Wnt signaling pathway markers as prognostic and therapeutic targets[8-12]. However, the regulation of the Wnt signaling pathway in ESCC remains largely unknown.

MicroRNAs are a class of small (21-23 nt), single-stranded non-coding RNAs that regulate gene expression post-transcriptionally by binding to the 3'-untranslated region (UTR) of target mRNAs. This typically causes mRNA degradation or translation repression[13]. Highly conserved across species, microRNAs not only participate in biological processes[14], but also in the pathogenesis of human cancers[15].

MicroRNA-30 (miR-30) family is evolutionarily conserved and consist of five members, microRNA-30a (miR-30a) through miR-30e. miR-30 family members play different roles, as oncogenes or tumor suppressor genes, in different kinds of cancer. For instance, miR-30 family members inhibit non-small-cell lung cancer[16,17], breast cancer[18,19], and colorectal cancer[20,21], but promote glioma[22], gastric cancer[23], and pancreatic cancer[24]. The miR-30 family is involved in the regulation of cancer cell apoptosis, proliferation, invasiveness, and metastasis, as well as in epithelial-mesenchymal transition. In particular, miR-30 targets oncogenes and tumor suppressor genes under different circumstances, the detailed/complete mechanism of which remains to be explored.

Emerging evidence has indicated that the two strands of miR-30a (miR-30a-3p and miR-30a-5p) are involved in various kinds of cancer. Recent studies have

revealed that miR-30a-3p/5p are closely associated with the Wnt signaling pathway in breast cancer, multiple myeloma, and glioma[19,25,26]; however, little has been reported about the expression and roles of miR-30a-3p/5p in ESCC progression. Analysis of public microarray data along with our previous experiment results has shown that miR-30a-3p/5p are down-regulated in ESCC in comparison with matched adjacent normal tissues. Additionally, bioinformatics analyses have indicated that the target genes of miR-30a-3p/5p were significantly enriched in the Wnt signaling pathway. Based on these findings, we sought to investigate the relationship between miR-30a-3p/5p expression and ESCC prognosis and the underlying mechanisms of the Wnt signaling pathway in ESCC.

MATERIALS AND METHODS

Patients and specimens

This study was conducted on 99 pairs of fresh ESCC tissue biopsies and matched adjacent normal tissues from the operating room of our hospital. Medical records of corresponding patients provided information on gender, age, differentiation, TNM stage, survival time. The fresh biopsies were stored in liquid nitrogen before usage. Prior patient consent and approval from the Institutional Research Ethics Committee were obtained.

Cell culture

The human ESCC cell lines KYSE30 and KYSE150 and normal esophageal epithelial cell line Het-1A were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, United States), supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, United States) at 37 °C with 5% CO_2.

RNA isolation, reverse transcription, and real-time quantitative PCR

Total RNA from cultured cells and ESCC tissues was isolated using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, United States) according to the manufacturer's instruction. Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, United States) was then used to synthesize cDNA from total RNA. Using the iQ™ SYBR Green Supermix (BioRad Laboratories, Hercules, CA, United States) and the Applied Biosystems 7500 Sequence Detection System, quantitative polymerase chain reaction (qPCR) was performed. The positive control (genomic DNA) and negative controls (PBS and samples processed without the RT step) were included. Data were normalized to the geometric mean of the housekeeping gene GAPDH or U6 values (internal control of small nuclear RNA expression) and analyzed using the $2^{-\Delta\Delta CT}$ method. Sequences of the primers for qPCR are summarized in Table S1.

Western blot

Proteins were isolated, subjected to SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes, and incubated with anti-Cyclin D1 (Abcam, Cambridge, MA, United States), anti-p27 (Abcam, Cambridge, MA, United States), anti-p21 (Abcam, Cambridge, MA, United States), anti-WNT2 (Bioworld Technology Inc. St. Louis Park, MN, United States), and anti-FZD2 (Bioworld Technology Inc. St. Louis Park, MN, United States) antibodies. α-tubulin (Sigma-Aldrich; Merck Millipore) was used as a loading control. Immunoreactive proteins were then detected by chemiluminescence. All the above operations were performed according to standard methods[27].

Plasmids and transfection

To construct the plasmids expressing miR-30a-3p or miR-30-5p, the fragments of pri-miR-30a were amplified using PCR, and then, respectively, cloned into the lentiviral vector pLVTHM (Addgene Inc., Cambridge, MA, United States). The mimics, negative controls, and inhibitors of miR-30a-3p or miR-30a-5p were purchased from Genecopoeia (Guangzhou, Guangdong, China), and transfected into cells with Lipofectamine 2000 reagent (Invitrogene) according to the manufacturer's instructions. To perform luciferase assay, small regions containing the target sequences of miR-30a-3p or miR-30a-5p in 3'-UTR were generated by PCR amplification, and cloned into psi-CHECK luciferase reporter plasmid (Promega). Two concentrations of miR-30a-3p or miR-30a-5p-mimics (10 nmol/L and 20 nmol/L) plus wild-type or mutant 3'-UTR of the target genes were applied. The primers used are listed in Table S2.

MTT assays

Cells (1×10^3) were seeded on 96-well plates and cultured for 24 h. Twenty microliters of 5 g/L of 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO, United States) was added to each well and incubated for 4 h. After MTT was removed, 150 μL of dimethyl sulphoxide DMSO (sigma, St, Louis, MO, United States) was added to the wells. Absorbance was measured at 490 nm with a Microplate Autoreader (Bio-Rad, Hercules, CA, United States). The experiment was repeated three times. Data are presented as the mean ± SD.

Colony formation assay

Cells were trypsinized, plated on 6-well plates (200 cells/well), and cultured for 2 wk. The colonies were stained with 1% crystal violet for 30 s after fixation with 4% paraformaldehyde for 5 min. The number of colonies, defined as > 50 cells/colony, were counted. Data are presented as the mean ± SD for three

dependent experiments.

Soft agar assay

Five hundred cells were suspended in 2 mL of complete medium containing 0.3% agar (Sigma, St Louis, MO). Then, the agar-cell mixture was plated on top of a bottom layer with 1% complete medium-agar mixture. Ten days later, the colonies were measured with an ocular micrometer. Colonies larger than 0.1 mm in diameter were counted. The experiment was repeated independently three times for each cell line. Data are presented as the mean ± SD.

Luciferase assay

Cells (4×10^4) were seeded in 24-well plates and settled for 24 h. Then, 1.5 μg of the TOPFlash (b-catenin/TCF reporter) and its mutant control FOPFlash luciferase reporter plasmid, plus 0.15 μg of pRL-TK Renilla plasmid (Promega), were, respectively, transfected into cells using the Lipofectamine 2000 reagent according to the manufacturer's recommendation. Luciferase and Renilla signal was measured 24 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega corp., Madison, WI, United States) according to the protocol provided by the manufacturer. All the experiments were performed in triplicate.

Tumorigenesis assay

Xenograft tumours were generated by subcutaneous injection of ESCC cells (2×10^6) into the hindlimbs of 4-6 week-old Balb/C athymic nude mice (nu/nu; Animal Center of XinXiang Medical University, HeNan China. $n = 6$ for each group). All mice were housed and maintained under specific pathogen-free conditions, and all experiments were approved by the Animal Care and Use Committee and performed in accordance with institutional guidelines. Tumour size was measured using a slide calliper and tumour volume was determined by the formula $0.44 \times A \times B^2$, where A represents the diameter of the base of the tumour and B represents the corresponding perpendicular value. After euthanasia, the tumours were excised, fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 μm sections, and stained with haematoxylin.

Accession number of public dataset

The public microarray data for analysis of miR-30a-3p/5p expression in this study was downloaded from the GEO database (GSE43732).

Statistical analysis

All statistical analyses were performed using SPSS version 20.0 for Windows (IBM, Armonk, NY, United States). Statistical tests included log-rank test, χ^2 test, and the Student's t-test. The two-tailed Student's *t*-test was used to compare intergroup differences. Survival data were analyzed by the Kaplan-Meier method and were compared using the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of miR-30a-3p/5p is down-regulated in ESCC tissues

To investigate the role of miR-30a-3p/5p in ESCC, we initially analyzed the expression of miR-30a-3p/5p in ESCC tissues and normal tissues, as well as using public microarray data (GSE43732, $n = 338$) from GEO database (https://www.ncbi.nlm.nih.gov/geo/). Results showed that both miR-30a-3p and miR-30a-5p were down-regulated in ESCC tissues when compared with adjacent normal tissues (Figures 1A and B). Additionally, we detected the expression of miR-30a-3p/5p in 99 cases of fresh ESCC biopsies and their paired adjacent normal tissues by qPCR. Consistent with the public microarray data, miR-30a-3p/5p were down-regulated in 81.82% (81/99) of ESCC tissues compared to their expression in the matched adjacent normal tissues (Figure 1C).

Down-regulation of miR-30a-3p/5p correlates with ESCC progression and clinical prognosis

Statistical analyses further revealed no difference in the miR-30a-3p/5p expression between different differentiation statuses of ESCC (Figure 2A), but miR-30a-3p/5p expression was inversely correlated with classifications of primary tumor and lymphatic metastasis (Figures 2B and C). Most importantly, survival analysis indicated that the group of lower miR-30a-3p/5p expression had shorter 5-year overall survival, and was associated with poorer prognosis of patients with ESCC (Figure 2D, log-rank, $P < 0.05$).

Over-expression of miR-30a-3p/5p represses ESCC cell proliferation in vitro and in vivo

As shown in Figure 3A, expression of miR-30a-3p/5p was significantly lower in ESCC cell lines KYSE30 and KYSE150 than in normal esophageal epithelial cell line Het-1A. To evaluate the potential roles of miR-30a-3p/5p in ESCC pathogenesis, we over-expressed miR-30a-3p/5p in KYSE30 cells by transfecting miR-30a-3p/5p-mimic oligonucleotides (Figure 3B). MTT and colony formation assays indicated that over-expression of miR-30a-3p/5p significantly repressed the proliferation of HYSE30 cells in comparison with the control group. It should be noted that the anchorage-independent growth ability of KYSE30 cells was also attenuated by over-expression of miR-30a-3p/5p in the soft-agar assays. To confirm this effect *in vivo*, KYSE30 cells were engineered to stably over-express miR-30a-3p/5p, and then, subcutaneously injected into the nude mice to perform tumorigenesis assays. Results showed that the miR-30a-3p/5p over-

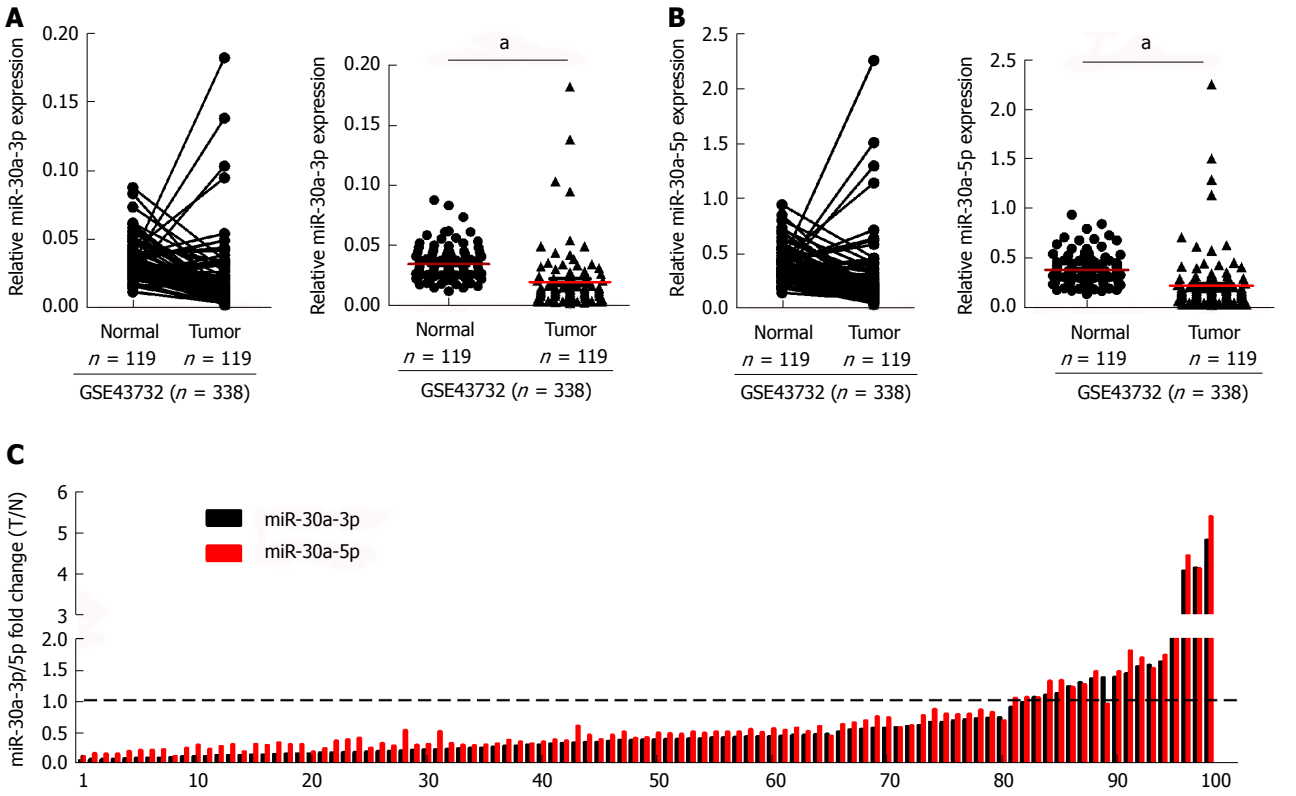

Figure 1 Expression of miR-30a-3p and miR-30a-5p in esophageal squamous cell carcinoma tissues and matched adjacent normal tissues. A and B: Analyses of miR-30a-3p and miR-30a-5p expression in ESCC tissues (n = 119) and matched adjacent normal tissues (n = 119). Accession number of the public microarray data from GEO database is GSE43732; C: Validation through qPCR of miR-30a-3p and miR-30a-5p expression patterns in 99 cases of fresh ESCC biopsies and their paired adjacent normal tissues. The expression levels of mRNA were normalized with U6. Fold changes of miR-30a-3p/5p were calculated by the ratio of miR-30a-3p/5p expression in ESCC tissues (T) and matched to adjacent normal tissues (N); $^{a}P < 0.05$.

expression group exhibited remarkably smaller tumor volume and slower tumor growth rate in comparison with the control group (Figures 3F, G, and H).

Inhibition of miR-30a-3p/5p promotes ESCC cell proliferation in vitro and in vivo

To further confirm the role of miR-30a-3p/5p in ESCC cell proliferation, we suppressed the expression of miR-30a-3p/5p in KYSE150 cells by expressing miR-30a inhibitor (Figure 4A, top). As indicated by the MTT and colony formation assays (Figure 4A bottom and B), inhibition of miR-30a-3p/5p significantly increased the proliferation of KYSE150 cells in comparison with the control groups. In addition, the anchorage-independent growth ability of KYSE150 cells was enhanced by inhibition of miR-30a-3p/5p in the soft-agar assays (Figure 4C). Tumorigenesis assays performed in the nude mice showed that inhibition of miR-30a-3p/5p increased the tumor volume and tumor growth rate in comparison with the control group (Figure 4D, E, and F).

Down-regulation of miR-30a-3p/5p enhances the activity of the Wnt signaling pathway

Both *in vitro* and *in vivo* experiments indicated that miR-30a-3p/5p served as tumor suppressors in ESCC. To figure out the underlying mechanisms, target genes of miR-30a-3p and miR-30a-5p were predicted using public algorithm miRWalk2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html), and KEGG pathway enrichment analyses were performed. Results suggested that target genes of miR-30a-3p or miR-30a-5p were involved in the Wnt signaling pathway (Figure 5A). The luciferase reporter assay showed that over-expression of miR-30a significantly attenuated the activity of the Wnt signaling pathway, while inhibition of miR-30a remarkably enhanced it (Figure 5B). Analyses of downstream genes of the Wnt signaling pathway further indicated that over-expression of miR-30a decreased the expression of Cyclin D1 and increased the expression of p27 and p21. In contrast, inhibition of miR-30a increased the expression of Cyclin D1 and decreased the expression of p27 and p21, both at protein and mRNA levels, as indicated by the results of Western blot and qPCR analyses, respectively (Figures 5C, D, E, and F).

miR-30a-3p/5p directly target the 3'-UTRs of WNT2 and FZD2 and inhibit their expression, respectively

We next performed qPCR analyses to screen the potential target genes of miR-30a-3p/5p that are

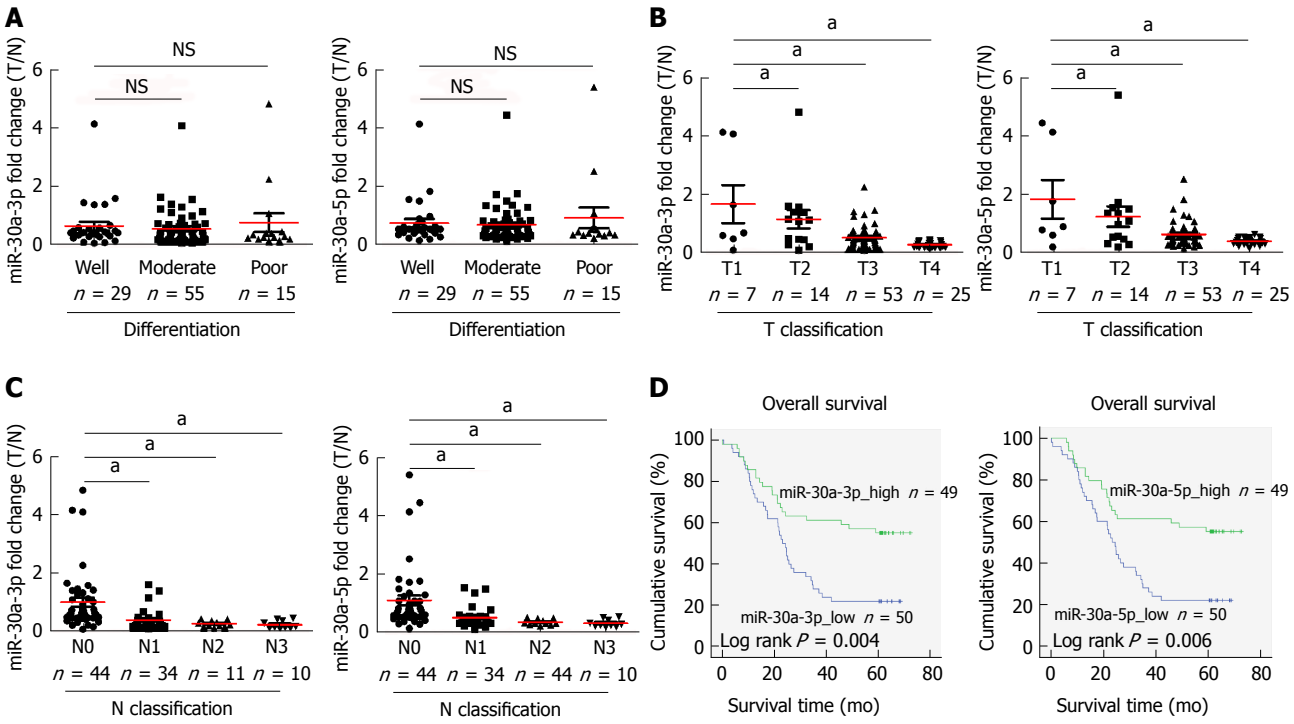

Figure 2 Down-regulation of miR-30a-3p/5p correlates with esophageal squamous cell carcinoma progression and clinical prognosis. Analyses of miR-30a-3p and miR-30a-5p expression were performed in 99 cases of esophageal squamous cell carcinoma tissues, which was normalized by U6 expression. Red lines within figures indicate the mean values of miR-30a-3p and miR-30a-5p expression fold changes. A: Expression fold changes of miR-30a-3p (left) and miR-30a-5p (right) in different statuses of differentiation of ESCC, with no significant difference found between different groups; B: Expression fold changes of miR-30a-3p (left) and miR-30a-5p (right) in different T classifications of ESCC, $^{a}P < 0.05$; C: Expression fold changes of miR-30a-3p (left) and miR-30a-5p (right) in different N classifications of ESCC, $^{a}P < 0.05$; D: Overall survival time was analyzed by the Kaplan-Meier method in ESCC patients with relatively high ($n = 49$) or low ($n = 50$) expression of miR-30a-3p (left, $P = 0.004$) and miR-30a-5p (right, $P = 0.006$), respectively.

related to Wnt signaling. Results showed that mRNA expression of Wnt2 was repressed by transfecting miR-30a-3p-mimics, and Fzd2 was inhibited by miR-30a-5p-mimics (Figure 6A). Analyses using public algorithms miRWalk2.0 indicated that Wnt2 and Fzd2 might be the respective targets of miR-30a-3p and miR-30a-5p (Figure 6B). Then, we respectively validated the effects of miR-30a-3p and miR-3a-5p on Wnt2 and Fzd2 expression. As shown in Figure 6C, over-expression of miR-30a-3p decreased the expression of Wnt2, while inhibition of miR-30a-3p increased it, at both protein and mRNA levels. The miR-30a-5p showed the same effects on Fzd2 expression. Luciferase reporter assays also demonstrated that over-expression of miR-30a-3p significantly reduced luciferase activity of the wild-type Wnt2-3'-UTR in a dose-dependent pattern, while it had no effects on the mutant type. Likewise, miR-30a-5p had the same effects on the wild-type FZD2-3'-UTR and the mutant one (Figure 6D).

DISCUSSION

MicroRNAs regulate protein translation at the post-transcriptional level by binding to mRNAs of target genes. Emerging evidence has shown that the dysregulation of microRNAs plays an important role in multiple tumor-related processes, such as cell differentiation, proliferation, apoptosis, autophagy, angiogenesis, invasion, and metastasis. In this study, we found that, according to analyses of public microarray data and validation in ESCC biopsies, miR-30a-3p/5p were down-regulated in ESCC tissues in comparison with matched adjacent normal tissues. It was interesting that aberrant expression of miR-30a-3p/5p was found in many kinds of cancer and showed opposite tendencies. miR-30a-3p/5p were down-regulated in bladder cancer[28], breast cancer[29,30], lung cancer[31,32], hepatocellular carcinoma[33], cutaneous squamous cell carcinoma[34], and ESCC[35], but were up-regulated in glioma[36], nasopharyngeal carcinoma[37], papillary thyroid carcinoma[38], ovarian serous adenocarcinoma[39], and head and neck squamous cell carcinoma[40]. These opposite tendencies of miR-30a-3p/5p in different cancers may lie in the different cell types and different genetic background.

It has been found that because of the particular expression patterns of microRNAs in cancers, some signatures consisting of microRNAs are linked with cancer progression and prognosis. For instance, the 5-microRNA classifier, including miR-210, miR-182, miR-486-5p, miR-30a, and miR-140-3p, could distinguish lung squamous cell carcinoma from normal lung tissues[41]. Another miRNA signature, including miR-451, miR-221, miR-30a,

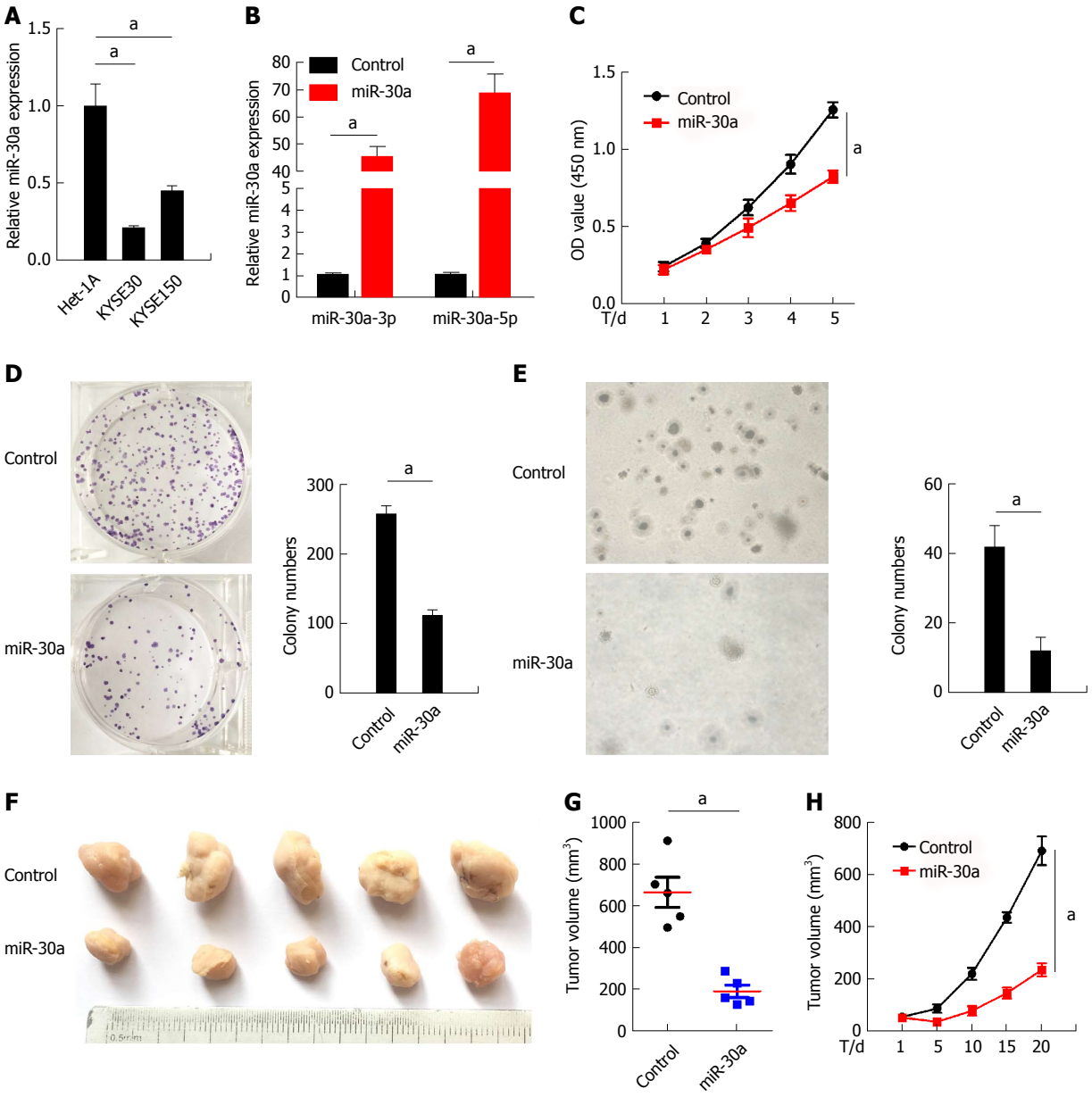

Figure 3 Over-expression of miR-30a-3p/5p represses esophageal squamous cell carcinoma cell proliferation. A: miR-30a expression in human esophageal epithelial cell line Het-1A and ESCC cell lines KYSE30 and KYSE150; B: miR-30a-3p (left) and miR-30a-5p (right) were, respectively, over-expressed in KYSE30 cells; C: Over-expression of miR-30a suppressed KYSE30 cells proliferation, as indicated by the MTT assay; D: Over-expression of miR-30a suppressed KYSE30 cell proliferation, as indicated by the colony formation assay. Colonies containing more than 50 cells were counted. E: Over-expression of miR-30a suppressed anchorage-independent growth ability of KYSE30 cells, as indicated by the soft-agar assay. Colonies containing more than 50 cells were counted. F: Subcutaneous tumorigenesis assay performed in nude mice (n = 6/group) indicated that over-expression of miR-30a inhibited ESCC growth *in vivo*. Tumor volumes were measured on the 1st, 5th, 10th, 15th, and 20th d; G and H: Statistical analyses of tumor volumes in the negative control and miR-30a over-expression groups. Error bars represent the mean ± SD of three independent experiments; $^aP < 0.05$.

miR-10b, and miR-29a, has been identified to distinguish between metastatic and non-metastatic clear cell renal cell carcinoma (ccRCC)[42]. In our study, down-regulation of miR-30a-3p/5p expression was found in ESCC tissues, and it was significantly correlated with advanced status of primary tumor and lymph node metastasis, as well as poor prognosis of patients with ESCC. The abnormal expression pattern of miR-30a-3p/5p in ESCC might also serve as potential diagnostic and prognostic markers in ESCC.

Apart from the expression pattern, it has been observed that miR-30a-3p/5p exhibited multiple roles in the regulation of tumor progression. miR-30a could suppress breast cancer cell migration and invasion[19]. In non-small cell lung cancer (NSCLC), miR-30a has been found to be inversely associated with invasive ability, and it suppressed epithelial to mesenchymal transition (EMT) of NSCLC cells[16]. In a similar fashion, down-regulation of miR-30a expression in hepatocellular carcinoma accelerated tumor cell migration, invasion,

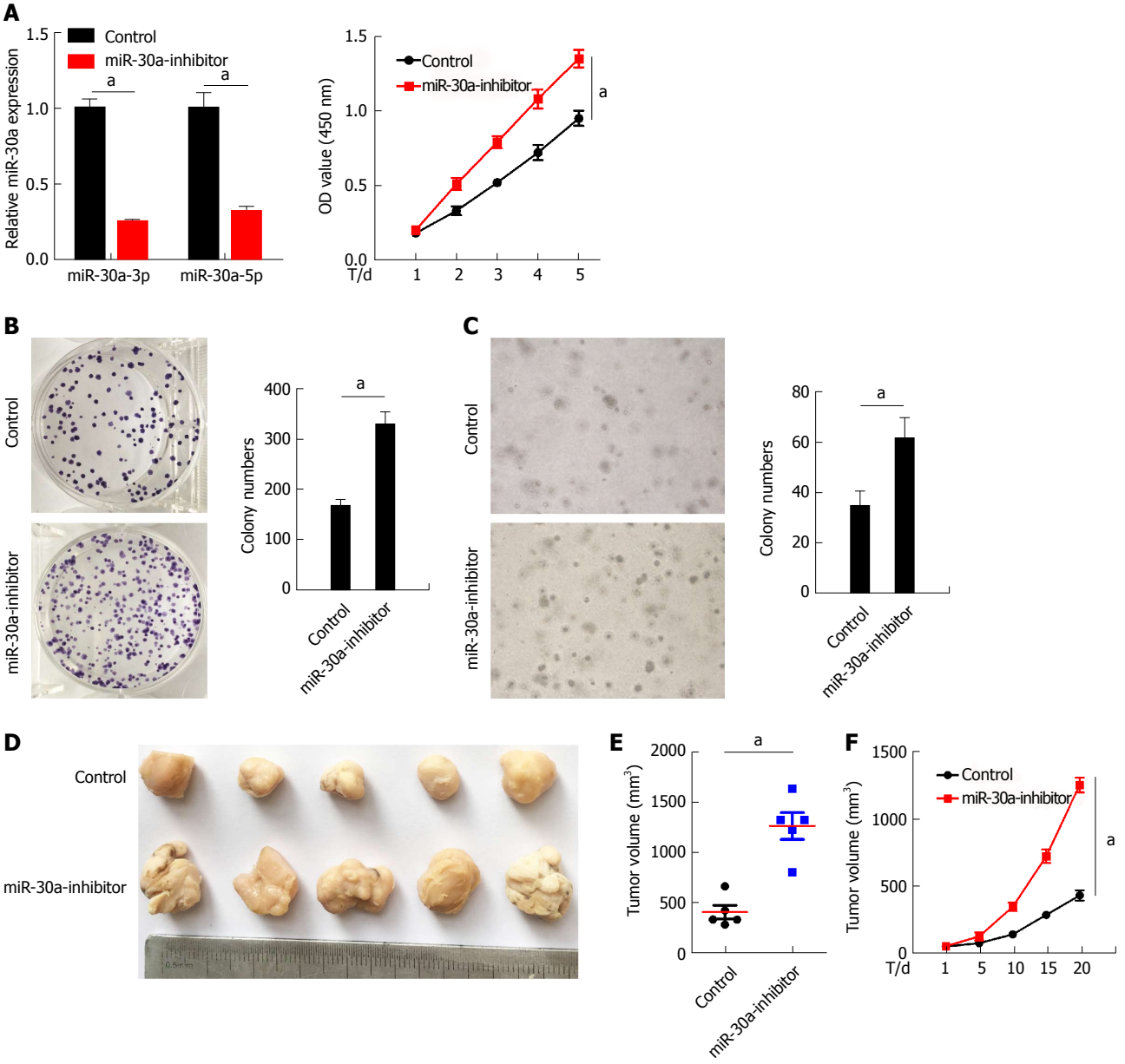

Figure 4 Inhibition of miR-30a-3p/5p expression promotes esophageal squamous cell carcinoma cell proliferation. A: miR-30a-3p and miR-30a-5p were knocked down using miR-30a inhibitor in KYSE150 cells (top), and inhibition of miR-30a expression promoted KYSE150 cell proliferation, as indicated by the MTT assay (bottom); B: Inhibition of miR-30a expression promoted KYSE150 cells proliferation, as indicated by the colony formation assay. Colonies containing more than 50 cells were counted; C: Inhibition of miR-30a expression promoted anchorage-independent growth ability of KYSE150 cells, as indicated by the soft-agar assay. Colonies containing more than 50 cells were counted; D: Subcutaneous tumorigenesis assay performed in nude mice (n = 6/group) indicated that inhibition of miR-30a expression promoted ESCC growth *in vivo*. Tumor volumes were measured on the 1st, 5th, 10th, 15th, and 20th d; E and F: Statistical analyses of tumor volumes in the negative control and miR-30a over-expression groups. Error bars represent the mean ± SD of three independent experiments; $^aP < 0.05$.

and EMT[43]. In contrast, miR-30a-5p has been identified as an oncogenic factor in glioma, and knock-down of miR-30a-5p inhibited glioma cell growth and cell invasion, while over-expression of miR-30a-5p had had opposite effects[44]. In this study, we revealed that over-expression of miR-30a-3p/5p suppressed the proliferation of ESCC cells, while down-regulation of miR-30a-3p/5p expression had the opposite effect both in *in vitro* and *in vivo* assays.

Our functional approach showed that miR-30a-3p/5p function as tumor suppressors in ESCC. The published microarray analysis, displaying that the down-regulation of miR-30a-3p/5p expression was correlated with the activation of Wnt signaling in ESCC, supplemented our findings. There are still gaps in the literature, as the molecular mechanisms by which miR-30a-3p/5p regulate the activation of Wnt signaling are unclear. Wnt signaling plays an essential role in various diseases and is considered a hallmark of colorectal tumorigenesis. Colorectal tumorigenesis is initiated by mutations in the Wnt signaling pathway (*e.g.*, APC or beta-catenin) that constitutively activate the pathway, and also

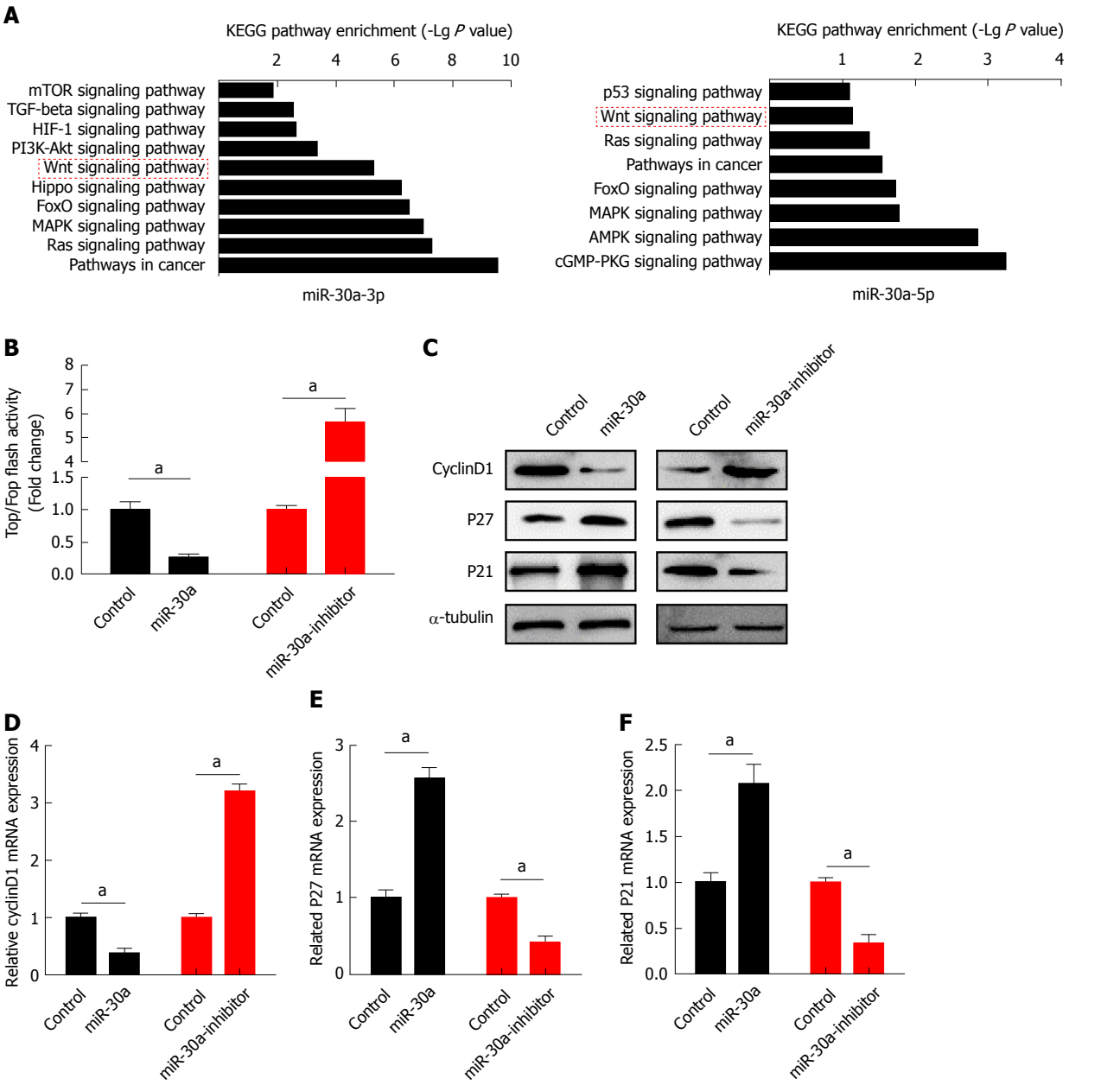

Figure 5 Down-regulation of miR-30a-3p/5p expression enhances the activity of Wnt signaling pathway. A: Respective KEGG pathway enrichment analyses of miR-30a-3p (top) and miR-30a-5p (bottom) target genes; B: Luciferase reporter assay suggested that down-regulation of miR-30a expression enhanced the activity of Wnt signaling pathway, while over-expression of miR-30a attenuated it; C: Western blot assay indicated that miR-30a negatively regulated the protein expression of downstream genes of the Wnt signaling pathway, including Cyclin D1, p27, and p21. α-tubulin was used as a loading control; D, E and F: qPCR assay revealed that miR-30a negatively regulated the mRNA expression of Cyclin D1, p27, and p21. Error bars represent the mean ± SD of three independent experiments; $^{a}P < 0.05$.

by binding a Wnt-protein ligand to a Frizzled family receptor, passing the biological signal to the dishevelled protein inside the cell, and leading to the regulation of gene transcription[45]. Accumulating literature has recently indicated that miRNAs regulate components of Wnt signaling in various cancers[46]. For example, miR-200a directly targets beta-catenin (beta-carotene) to inhibit cell proliferation in meningiomas[47], while miR-34 mediates suppression of Axin-2 to increase nuclear GSK3-beta and decrease Snai1 in colorectal cancer progression[48]. In addition, miR-30a-5p has been found to directly suppress PRDM1, resulting in activation of Wnt/beta-catenin (carotene) signaling in glioma[49].

We demonstrated that miR-30a-3p and miR-30a-5p directly target the 3′-UTRs of Wnt2 and Fzd2 and inhibit their expression, respectively. This leads to the inhibition of the Wnt signaling pathway, which might be the dominant component in miR-30a-3p/5p in regulating ESCC cell proliferation.

In conclusion, low expression of miR-30a-3p/5p was closely associated with advanced ESCC progression and poor prognosis of patients with ESCC. Down-

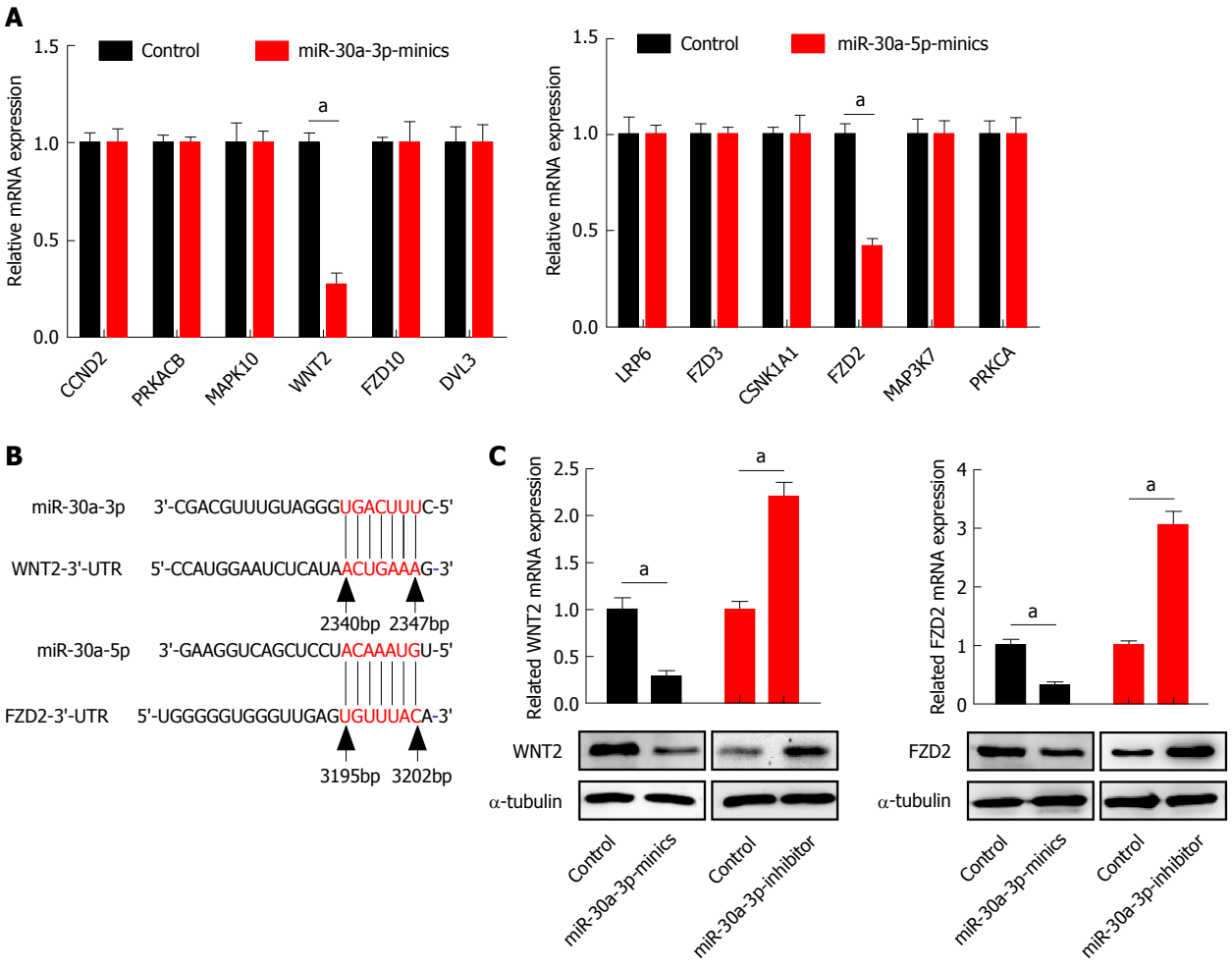

Figure 6 miR-30a-3p/5p directly target the 3'-UTRs of Wnt2 and Fzd2, respectively. A: Target gene screening of miR-30a-3p (left) and miR-30a-5p (right) by qPCR suggested that miR-30a-3p targeted Wnt2 and miR-30a-5p targeted Fzd2 in the Wnt signaling pathway; B: Predicted target sequences of miR-30a-3p (top) and miR-30a-5p (bottom) in the 3'-UTRs of WNT2 and FZD2, respectively; C: qPCR (top) and Western blot (bottom), respectively, confirmed the inhibitory effects of miR-30a-3p and miR-30a-5p on Wnt2 and Fzd2 at both mRNA and protein levels. GAPDH and α-tubulin were, respectively, used as loading controls for qPCR and Western blot; D: Luciferase reporter assay indicated that miR-30-3p and miR-30a-5p directly targeted the wild-type 3'-UTRs of Wnt2 and Fzd2, respectively, but had no effect on the mutant ones. Two concentrations of miR-30a-3p- and miR-30a-5p-mimics (10 nmol/L and 20 nmol/L) plus wild-type or mutant 3'-UTRs of target genes were applied. Error bars represent the mean ± SD of three independent experiments; $^{a}P < 0.05$.

regulation of miR-30a-3p/5p suppressed ESCC cell proliferation both *in vitro* and *in vivo*. Furthermore, miR-30a-3p and miR-30a-5p could inhibit the activity of the Wnt signaling pathway by targeting the 3'UTRs of WNT2 and FZD2, respectively. This study provided further evidence suggesting that miR-30a-3p/5p are diagnostic and prognostic biomarkers for ESCC, as miR-30a-3p/5p participate in the activation of the Wnt signaling pathway and subsequently, the regulation of ESCC cell proliferation.

ARTICLE HIGHLIGHTS

Research background

MicroRNA-30a (miR-30a) serves as a post-transcriptional regulator by directly targeting mRNAs in many biological processes, and it shows multiple roles in different kinds of cancer. Wnt signaling pathway is well known in the development and progression of various cancers. MiR-30a was recently found to be closely associated with Wnt signaling pathway in cancers; however, the potential role and underlying mechanism of miR-30a in esophageal squamous cell carcinoma (ESCC) have not been illustrated.

Research motivation

To investigate the potential role of microRNA-30a (miR-30a) in ESCC.

Research objectives

The study investigated the potential role of microRNA-30a (miR-30a) in ESCC, which is urgent and essential for developing early diagnostic and therapeutic strategies.

Research methods

Expression of miR-30a-3p/5p was analyzed using microarray data and fresh ESCC tissue samples. Both *in vitro* and *in vivo* assays were used to investigate the effects of miR-30a-3p/5p on ESCC cell proliferation. Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to explore underlying mechanisms involved in ESCC, and then, assays were carried out to verify the potential molecular mechanism of microRNA-30a (miR-30a) in ESCC.

Research results

Low expression of miR-30a-3p/5p was closely associated with advanced ESCC progression and poor prognosis of patients with ESCC. Knock-down of miR-30a-3p/5p promoted ESCC cell proliferation. We further demonstrated that increased miR-30a-3p/5p expression inhibited the Wnt signaling pathway by targeting Wnt2 and Fzd2.

Research conclusions

Down-regulation of miR-30a-3p/5p promotes ESCC cell proliferation by activating the Wnt signaling pathway through inhibition of Wnt2 and Fzd2.

Research perspectives

This study will provide an example for investigating the relationship between the expression of microRNAs and ESCC prognosis and the underlying mechanisms of the Wnt signaling pathway in ESCC. The direction of the future research is to provide more evidence for developing novel strategies by targeting microRNA-30a in ESCC. In our future research, the long-acting microRNA-30a will be used to treat the ESCC cells or animal models, and to observe the inhibitory effect of microRNA-30a on ESCC cells.

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ORIGINAL ARTICLE

Basic Study

Mesenchymal stem cells rescue acute hepatic failure by polarizing M2 macrophages

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Abstract

AIM

To investigate whether M1 or M2 polarization contributes to the therapeutic effects of mesenchymal stem cells (MSCs) in acute hepatic failure (AHF).

METHODS

MSCs were transfused into rats with AHF induced by D-galactosamine (DGalN). The therapeutic effects of MSCs were evaluated based on survival rate and hepatocyte proliferation and apoptosis. Hepatocyte regeneration capacity was evaluated by the expression

of the hepatic progenitor surface marker epithelial cell adhesion molecule (EpCAM). Macrophage polarization was analyzed by M1 markers [CD68, tumor necrosis factor alpha (TNF-α), interferon-γ (IFN-γ), inducible nitric oxide synthase (INOS)] and M2 markers [CD163, interleukin (IL)-4, IL-10, arginase-1 (Arg-1)] in the survival and death groups after MSC transplantation.

RESULTS

The survival rate in the MSC-treated group was increased compared with the DPBS-treated control group (37.5% *vs* 10%). MSC treatment protected rats with AHF by reducing apoptotic hepatocytes and promoting hepatocyte regeneration. Immunohistochemical analysis showed that MSC treatment significantly increased the expression of EpCAM compared with the control groups ($P < 0.001$). Expression of EpCAM in the survival group was significantly up-regulated compared with the death group after MSC transplantation ($P = 0.003$). Transplantation of MSCs significantly improved the expression of CD163 and increased the gene expression of IL-10 and Arg-1 in the survival group. IL-4 concentrations were significantly increased compared to the death group after MSC transplantation (88.51 ± 24.51 pg/mL *vs* 34.61 ± 6.6 pg/mL, $P < 0.001$). In contrast, macrophages showed strong expression of CD68, TNF-α, and INOS in the death group. The concentration of IFN-γ was significantly increased compared to the survival group after MSC transplantation (542.11 ± 51.59 pg/mL *vs* 104.07 ± 42.80 pg/mL, $P < 0.001$).

CONCLUSION

M2 polarization contributes to the therapeutic effects of MSCs in AHF by altering levels of anti-inflammatory and pro-inflammatory factors.

Key words: Acute hepatic failure; Mesenchymal stem cells; Macrophages; Polarization; Inflammation

Core tip: M1 or M2 polarization governs the therapeutic effect of acute hepatic failure (AHF). Mesenchymal stem cells (MSCs) were transfused into rats with AHF induced by galactosamine. It was found that MSCs alleviated the survival rate and biochemical indicators by promoting hepatocyte regeneration. Immunohistochemistry, flow cytometry, and RT-PCR showed that M2 polarization contributes to the rescue of AHF by MSCs in the survival group after MSC transplantation. In addition, in the death group after MSC transplantation, the number of M1 macrophages increased significantly. Our findings suggest that M2 polarization contributes to the rescue of AHF by MSCs, which result in altered levels of anti-inflammatory and pro-inflammatory factors.

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INTRODUCTION

Acute hepatic failure (AHF) is a lethal condition characterized by widespread hepatocyte necrosis, acute deterioration of liver function, and subsequent multiorgan failure. Although many animal and preliminary human studies have shown that stem cell transplantation has substantial potential in treating AHF[1-4], transplantation has rarely produced satisfying therapeutic effects. The exact mechanism through which stem cells assist in organ repair remains elusive. Recent studies have also indicated a substantial role for paracrine effects in delivering overall benefits, although specific cells and signaling molecules have not been identified to mediate these paracrine effects[5]. However, macrophages in the liver play an indispensable role in paracrine mechanisms. There has been a major paradigm shift in the field of macrophage biology with the recognition that macrophages play an important role in homeostasis[6]. Several studies employing selective Kupffer cell depletion in rodents have explored the role of this cell type in hepatocyte proliferation and liver regeneration following partial hepatectomy[7,8]. However, there is little information available regarding the role of macrophages in hepatocyte proliferation. This may be due to the multi-phenotype and multi-functional roles of macrophages in liver regeneration, as they are a major source of both pro-proliferative and anti-proliferative mediators in the liver. Classically activated macrophages (M1 macrophages) mediate host defenses from a variety of bacteria, protozoa, and viruses and have roles in anti-tumor immunity. M2 macrophages have an anti-inflammatory function and regulate wound healing[9,10]. Different phenotypes play various roles in tissue damage and maintenance[11-17]. For example, M1 macrophages are induced by exposure to CD68[18] and are associated with the phagosomes of macrophages, which is consistent with enhanced phagocytosis. These macrophages are characterized by the expression of high levels of inducible nitric oxide synthase (INOS) induced by interferon-γ (IFN-γ) that liberates pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6, which are increased in inflammatory reactions and tissue injury. In contrast, M2 macrophages are characterized by exposure to CD163[19], which is expressed by liver Kupffer cells. These macrophages are activated by IL-4, express high levels of arginase-1 (Arg-1), and release immune-modulatory mediators (such as IL-10) to modulate the inflammatory response and to promote

tissue remodeling.

MSC transplantation may be useful in treating AHF conditions. In the present study, we evaluated the contribution of M1 and M2 macrophages in survival and death groups after MSC transplantation to investigate whether macrophage polarization contributes to the rescue of AHF by MSCs.

MATERIALS AND METHODS

AHF animal model

Male Wistar rats weighing 190 ± 20 g were obtained from the Huafukang Experimental Animal Center (Beijing, China). The study was reviewed and approved by the Ethics Committee of Shengjing Hospital of China Medical University. The animal study protocol, in compliance with the Guidelines of China for Animal Care, conformed to internationally accepted principles in the care and use of experimental animals. Animals were housed at room temperature (22 ± 2 ℃) with light cycles between 08:00 and 22:00 and free access to food and water. A total of 52 rats were randomly divided into four groups: an experimental group (group A, n = 16), a control group (group B, n = 10), an MSC–treated group (group C, n = 16), and a DPBS (Dulbecco phosphate-buffered saline)–treated group (group D, n = 10). Rats in group A were injected intraperitoneally (i.p.) with D-galactosamine (DGalN) (1.2 g/kg; Sigma-Aldrich, St. Louis, MO, United States). Rats in group B were injected i.p. with 2 mL of 0.9% phosphate buffered saline (PBS). At 12 h after DGalN treatment, rats in group C underwent intravenous tail vein transplantation of 5.5 × 10^5 MSCs dissolved in 1.0 mL of DPBS, and rats in group D were given 1.0 mL of DPBS. All rats were selected for survival analysis at 72 h after treatment. The survival rate of rats remained unchanged at 48 h after treatment. The rats in the survival group were still in good physical condition at 48 h after MSC treatment. The rats in the death group were in poor physical condition or in the state of death before they died at 48 h after MSC treatment. Serum and liver tissues were collected at 48 h after $MSCs^{GFP}$ transplantation for biochemical analyses, inflammatory factor detection, and further evaluation.

$MSCs^{GFP}$ culture and $MSCs^{GFP}$ transplantation

Wistar bone marrow MSCs were obtained from a cell bank (Shanghai, China) and cultured in α-MEM medium with GlutaMAX-I (Gibco, United States), supplemented with 10% fetal bovine serum (Gibco, United States), 100 IU/mL penicillin and 100 μg/mL streptomycin (Thermo, United States). When cells reached 80%-90% confluence, they were trypsinized with 0.05 g/L trypsin-EDTA (Gibco, United States) and replated at a density of 1 × 10^4/cm^2 for further expansion. After cells were passaged to the fourth generation, they were infected with an adenovirus encoding the gene encoding green fluorescent protein (GFP), and the multiplicity of infection was determined by fluorescence inverted phase-contrast microscopy.

Biochemical assay and histological evaluation

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), and total bilirubin (TBIL) were monitored with an automatic analyzer (Roche, United States) and liver biochemical indicators were estimated. The liver was fixed in 4% paraformaldehyde for hematoxylin and eosin (HE) or immunohistochemical staining. Paraffin-embedded liver tissue was cut into 3-μm thick sections for histopathological evaluation, deparaffinized in xylene, and rehydrated through a series of decreasing concentrations of ethanol. Sections were stained with HE and analyzed under a light microscope.

Immunohistochemistry and immunofluorescence staining

Immunohistochemistry was performed with primary rabbit or mouse anti-rat antibodies (Abcam, Cambridge, MA, United States) for EpCAM, CD68, and CD163. Liver sections were deparaffinized in xylene and rehydrated through a series of decreasing concentrations of ethanol. Heat-mediated antigen retrieval was performed using citrate buffer (MVS-0100, MXB Biotechnologies, Fujian, China). Blocking solution and secondary antibodies (KIT-9710, MXB Biotechnologies, Fujian, China) were applied according to standard protocols. Sections were incubated overnight with a primary antibody at 4 ℃ and visualized with DAB (ZLI-9017, ZSGB-BIO, Beijing, China).

Indirect immunofluorescence was used to detect the phenotype of M1/M2 following overnight incubation at 4 ℃ with primary antibodies. Secondary antibodies (Abcam, Cambridge, MA, United States) were used at room temperature for 4 h with goat anti-mouse IgG-H&L (Abcam, Cambridge, MA, United States) and goat anti-rabbit IgG-H&L (Abcam, Cambridge, MA, United States). Nuclear staining was performed using DAPI (ZSGB-BIO, Beijing, China). A standard *in situ* TUNEL (Roche, Indianapolis, IN, United States) method was used for detection of DNA fragmentation in apoptotic cells according to the manufacturer's instructions. Cell proliferation was determined using anti-Ki67 (Novus, NB500-170).

To determine engraftment of MSCs after GFP transfection, the livers from rats in the survival and death groups were dissected out, fixed in 4% formaldehyde and optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura, Japan), and preserved at -20 ℃. GFP expression by transplanted MSCs was detected by fluorescence inverted phase-contrast microscopy.

Measurement of cytokine production

Cytokine production was measured in serum centrifuged at 1500 r/min for 15 minutes. IL-4 and IFN-γ were tested using Multi-Analyte Flow Assay Kit

Table 1 Primers used in mRNA expression analysis

Gene name	Sequence
GAPDH	(Forward) 5′-GGCACAGTCAAGGCTGAGAATG-3′
	(Reverse) 5′-ATGGTGGTGAAGACGCCAGTA-3′
CD68	(Forward) 5′-TCGGGCCATGCTTCTCTT-3′
	(Reverse) 5′-AGGGGCTGGTAGGTTGATTGT-3′
CD163	(Forward) 5′-CTGGGATGTCCAACTGCCAT-3′
	(Reverse) 5′-AATGCTTCCCCCATTCCTGG-3′
Arg-1	(Forward) 5′-GCTGTGGTAGCAGAGACCCAGA-3′
	(Reverse) 5′-CATCCACCCAAATGACGCATAG-3′
IL-10	(Forward) 5′-CAGACCCACATGCTCCGAGA-3′
	(Reverse) 5′-CAAGGCTTGGCAACCCAAGTA-3′
Nos2	(Forward) 5′-TCCTCAGGCTTGGGTCTTGTTAG-3′
	(Reverse) 5′-TTCAGGTCACCTTGGTAGGATTTG-3′
TNF-α	(Forward) 5′-CCGATTTGCCACTTCATACCA-3′
	(Reverse) 5′-TAGGGCAAGGGCTCTTGATG-3′

(BioLegend, CA, United States) and analyzed by flow cytometry. Each analysis was performed in duplicate. In this quantitative assay system, specific antibodies directed against each cytokine are conjugated to the surface of fluorescence-coded microbeads, with each fluorescence-coded microbead type being conjugated to one specific capture antibody.

Quantitative real-time PCR

Total RNA was extracted from liver tissue (~100 mg) using TRIzol reagent (Invitrogen, United States) according to the manufacturer's instructions, and the amount of isolated RNA was estimated by ribogreen fluorescence. Purity was assessed by the absorbance ratio at 260 and 280 nm. A total of 3 μg was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Promega, United States). Real-time quantitative PCR was performed using SYBR Green I Master and the appropriate primers on a LightCycler 480 instrument. In parallel, mRNA levels of human housekeeping GAPDH were analyzed as an internal normalization control. Primers used are shown in Table 1. Data were calculated using the ΔCt method and were normalized to GAPDH.

Statistical analysis

Survival statistics were assessed using Log-rank (Mantel-Cox) test. Data are expressed as the mean ± SD. Differences between groups were analyzed by independent sample *t*-test. Serum concentration and gene expression of cytokine assays were performed in duplicate or triplicate for each specific sample. All data points are the mean of duplicate or triplicate measurements. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Survival rate is increased and biochemical indicators are altered by MSC transplantation

Implanted MSCs were observed in the liver of treated rats (Figure 1). At 24 h after treatment, 50% (8/16) and 30% (3/10) of the animals had survived in the MSC- and DPBS-treated groups, respectively. At 48 h after transplantation, survival in the MSC- and DPBS-treated groups decreased to 37.5% (6/16) and 10% (1/10), respectively (Figure 1C). Although there was no statistical significance, survival rate was increased by MSC transplantation. At 24 h and 48 h after MSC transfusion, biochemical indicators (ALT/AST/ALB/TBIL) had significantly changed compared with rats in the DPBS-treated group (Figure 1). To investigate the liver histology of rats with AHF after MSC transplantation, HE staining was conducted (Figure 1). At 48 h after MSC transfusion, no obvious histopathological changes were observed in rats infused with MSCs, and most of the tissue showed generalized necrotic areas. Five days after transplantation, most of the tissue had returned to normal with only a few necrotic areas, indicating liver tissue repair after liver function repair.

MSC transfusion promotes hepatocyte regeneration

To determine whether MSC treatment promotes hepatocyte regeneration compared to rats in the DPBS-treated group, Ki67-positive hepatocytes were detected. Ki67-positive hepatocytes significantly increased at 48 h after transplantation ($P < 0.001$) (Figure 2). In DPBS-treated rats with AHF, many TUNEL-positive hepatocytes were observed, yet only a few hepatocytes were observed after MSC treatment ($P < 0.001$).

EpCAM has been shown to be expressed in a population of rat oval cells, which are composed of liver progenitors[16,17]. In the present study, significant up-regulation of EpCAM was observed in the MSC-treated group compared with DPBS-treated group ($P < 0.001$) (Figure 3). Compared with the death group, EpCAM expression was increased in the survival group after MSC transplantation ($P = 0.003$), suggesting the vital roles of progenitor cells in the regeneration process after MSC transplantation.

Enhanced M2 polarization in the survival group after MSC transplantation

To investigate the role of macrophage subsets in AHF, liver sections were stained for the recently described M1/M2 specific markers CD68 and CD163, which preferentially detect invading macrophages. The number of CD68+ macrophages was obviously up-regulated in the DGalN-treated group (Figure 4). However, compared to the death group after MSC transplantation, a significantly greater number of CD163+ macrophages was observed in the survival group, while the number of CD68+ macrophages was decreased (Figure 5). Serum protein levels of IL-4 were significantly higher in the survival group than in the death group (88.51 ± 24.51 pg/mL *vs* 34.61 ± 6.6 pg/mL, $P < 0.001$) (Figure 6). The mRNA expression of IL-10 and Arg-1 was significantly up-regulated in the survival group ($P < 0.001$) (Figure 7).

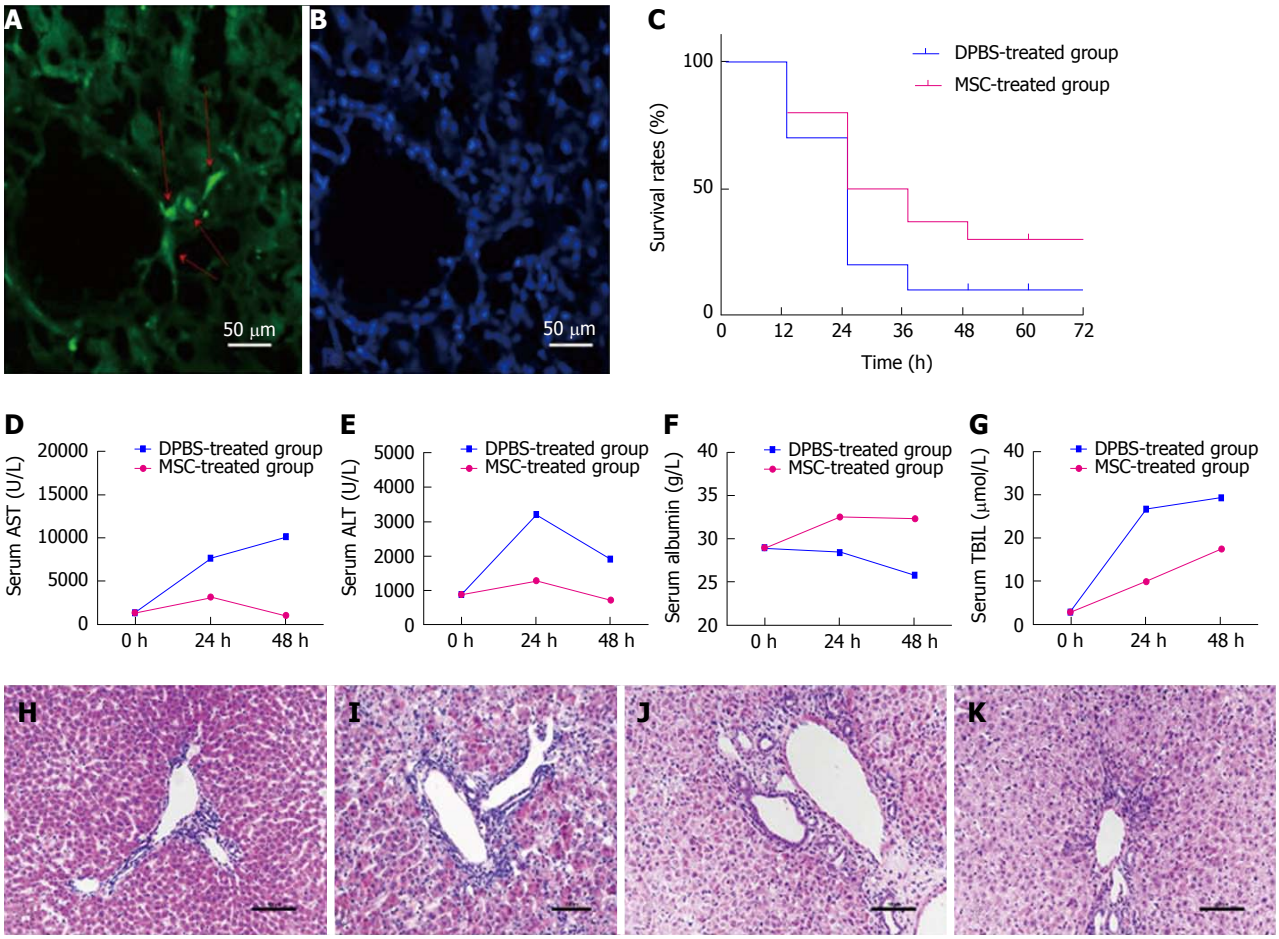

Figure 1 Survival rate and biochemical indicators in rats are improved after mesenchymal stem cell treatment. Colonization of mesenchymal stem cells (MSCs) was observed in the liver (A and B). The red arrow on the left shows engraftment of MSCs and nuclear staining in the same slice. Survival rates were compared between the MSC-treated group and the DPBS-treated group at each time point (C) (P = 0.36). Serum samples collected at various times (0 h, 24 h, 48 h) after MSC treatment were analyzed for levels of ALT, AST, ALB, and TBIL and compared with the DPBS-treated group (D-G). HE staining of liver sections was performed in each group. Compared with the PBS-treated group (H), we observed necrosis of centrilobular hepatocytes, characterized by cell shrinkage and lost nuclei, interstitial hemorrhage, and inflammatory cell infiltration in the DPBS-treated group (I). Liver histomorphology at 48 h after MSC treatment (J) did not change significantly compared with the DPBS-treated group, but the number of hepatocytes with edema, shrinkage, and lost nuclei decreased significantly, with massive inflammatory cell infiltration and increased number of cells observed. The liver histomorphology was gradually repaired after 5 d (K).

Enhanced M1 polarization in the death group after MSC transplantation

In the death group after MSC transplantation, the number of CD68+ macrophages was significantly increased and the number of CD163+ macrophages was markedly reduced. We investigated serum IFN-γ protein levels and showed that the concentration of IFN-γ was significantly up-regulated in the death group (542.11 ± 51.59 pg/mL *vs* 104.07 ± 42.80 pg/mL, P < 0.001) (Figure 6). TNF-α and INOS gene expression was dramatically increased (P < 0.001) (Figure 7).

DISCUSSION

As a heterogeneous population of cells, MSCs have the potential for multilineage differentiation. MSCs can differentiate into a variety of liver cells under appropriate culture conditions[20-23]. Many clinical studies have indicated that MSCs are safe and effective in clinical studies and are useful to treat hepatic failure[24-26]. In the present study, MSC infusion was beneficial in improving the survival rate and liver histopathology after altering the concentration of biochemical indicators. To study the reasons for increased survival in the MSC-treated group, we analyzed the expression of EpCAM, which is a marker used to assess liver regeneration[27,28]. The additional study showed that in the death group, implanted MSCs did not fully translate into functional liver cells after treatment and that subsequent liver cell hepatocyte proliferation was unsatisfactory, which cannot completely improve hepatocyte inflammatory necrosis.

There is growing evidence that MSCs increase angiogenesis and improve local cell function through paracrine effects, which are involved in releasing growth factors and signaling molecules[5,29-33]. The pivotal role of paracrine effects in stem cell therapies has been recognized to contribute to many biological processes, such as preventing inflammation, inhibiting apoptosis, improving metabolism, and promoting regeneration.

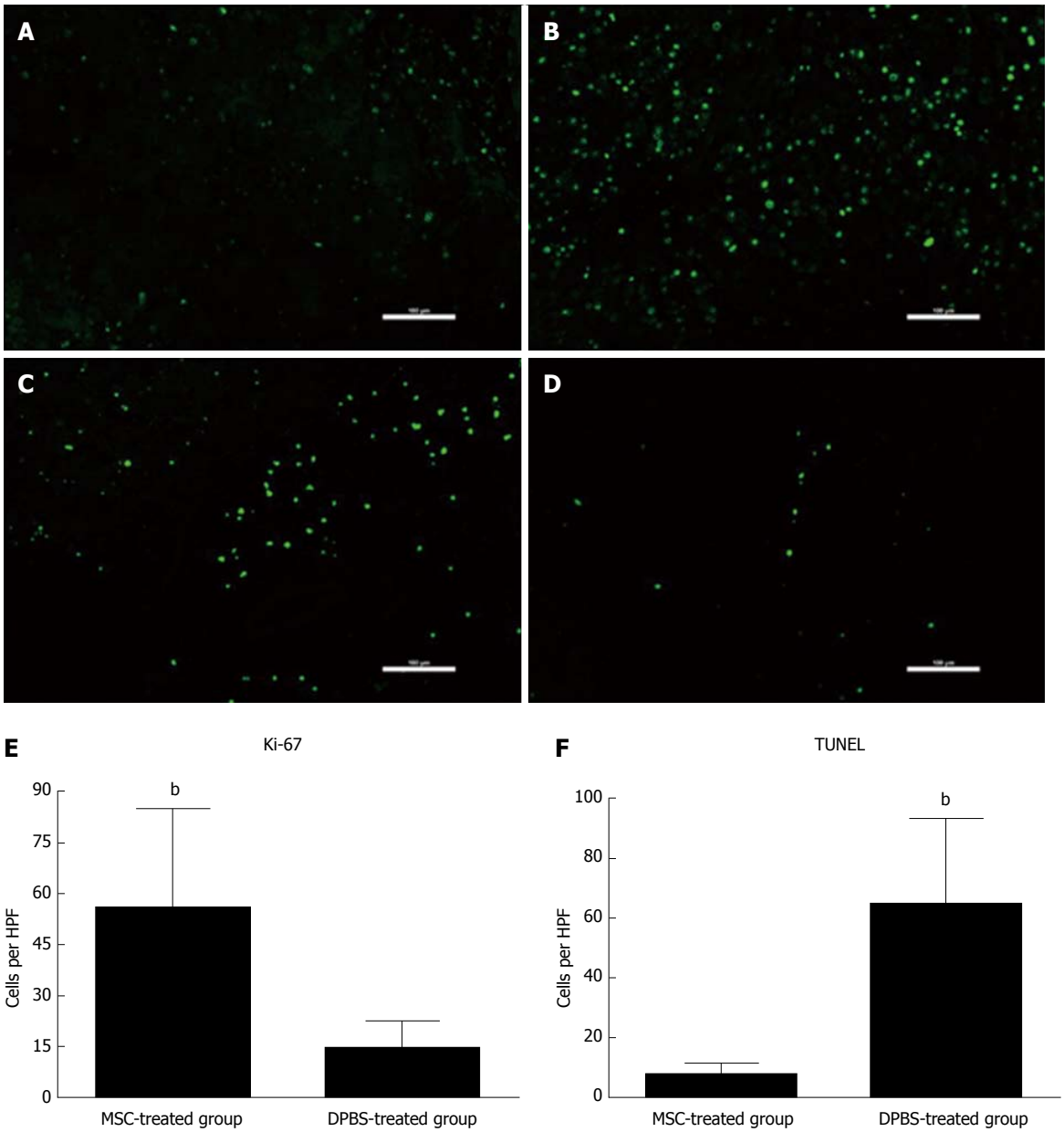

Figure 2 Assessment of hepatocyte apoptosis and proliferation after mesenchymal stem cell transplantation. Immunofluorescence for Ki-67 (A and B) and terminal deoxyribonucleotide transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) (C and D) staining in MSC-treated and DPBS-treated livers. A and C: MSC-treated group; B and D: DPBS-treated group. The numbers of Ki-67-positive and TUNEL-positive hepatocytes were observed in the DPBS- and MSC-treated groups (E and F). Bar represents the mean ± SD. (n = 5, ^{b}P < 0.001). MSC: Mesenchymal stem cell.

Macrophages are the major cells involved in paracrine effects. We found that M2 macrophages and their associated cytokines can contribute to AHF rescuing by MSCs. The number of CD163+ macrophages and levels of IL-10 and Arg-1 were significantly up-regulated in the survival group. In contrast, CD68+ macrophages and levels of TNF-α and INOS were significantly up-regulated in the death group. During type 2 helper T (TH2)-mediated immune responses, IL-4 can induce macrophages undergoing M2 activation[34], leading to expansion beyond a continuum in multiple activation states. In response to IFN-γ, macrophages undergo M1 activation during type 1 (TH1)-mediated immune responses and represent another extreme in terms of activation states. Our study demonstrates that high IL-4 levels drive M2 polarization, which occurred in the survival group after MSC transplantation. High expression of IFN-γ in the death group stimulated macrophages to undergo M1 activation.

In this study, we investigated the role of macrophage polarization in AHF rescuing by MSCs and found that polarized macrophages from the M2 anti-inflammatory phenotype promote MSC activity. Macrophages to M2 polarization also increase infused MSC activity during myocardial and spinal cord injuries[35,36]. Tremendous research efforts have corroborated the concept that hepatic macrophages are central in the pathogenesis of acute hepatic injury. Elsegood *et al*[37] showed that

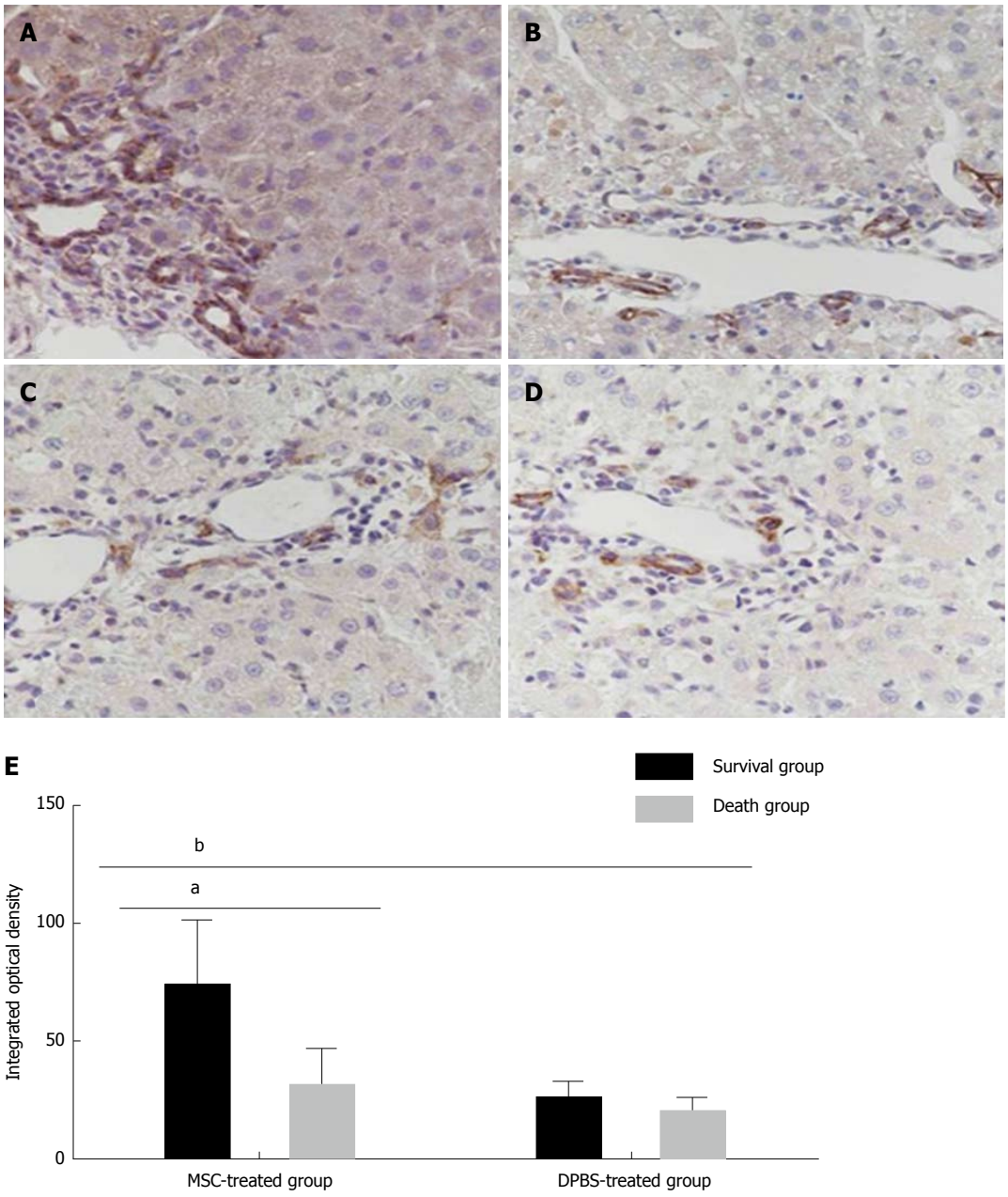

Figure 3 Immunohistochemical staining for epithelial cell adhesion molecule expression in each group. A: Survival group after mesenchymal stem cell (MSC) treatment; B: Death group after MSC treatment; C: Survival group after DPBS treatment; D: Death group after DPBS treatment; E: Integrated optical density of immunohistochemical staining for EPCAM+ hepatocytes. Bar represents the mean ± SD (n = 5, $^{a}P < 0.05$, $^{b}P < 0.001$).

the number of macrophages increases in the liver to induce liver progenitor cell proliferation in chronic liver injury models. Our data suggest that the number of macrophages was increased in the pathogenesis of acute hepatic injury. Importantly, the number of M1 macrophages was increased significantly compared to M2 macrophages. Lanthier *et al*[11] reported that higher liver macrophage expansion could increase proliferative hepatocytes and is associated with a favorable outcome. Here, we determined that TNF-α expression depressed hepatocyte regeneration in AHF. These results differ from those of Lanthier *et al*[11] and Bihari *et al*[38], who reported that TNF-α levels contribute to liver cell proliferation in chronic hepatic injury. This disparity could reflect differences in the mechanisms of hepatocyte repair in acute and chronic liver injury. Our results show an increase in IL-10 gene expression in the survival group. Interestingly, these results are in agreement with the suggestion that IL-10 released by MSCs has the potential for therapeutic recovery of liver fibrosis[39,40].

MSCs improve liver function, although the specific mechanism of action is still unknown. Several studies have shown that MSCs have immunomodulatory properties, focusing on their paracrine effect. Studies of macrophage functions in hepatocyte repair have typically not distinguished between M1 and M2 after MSC transplantation. EpCAM+ hepatocytes are able to

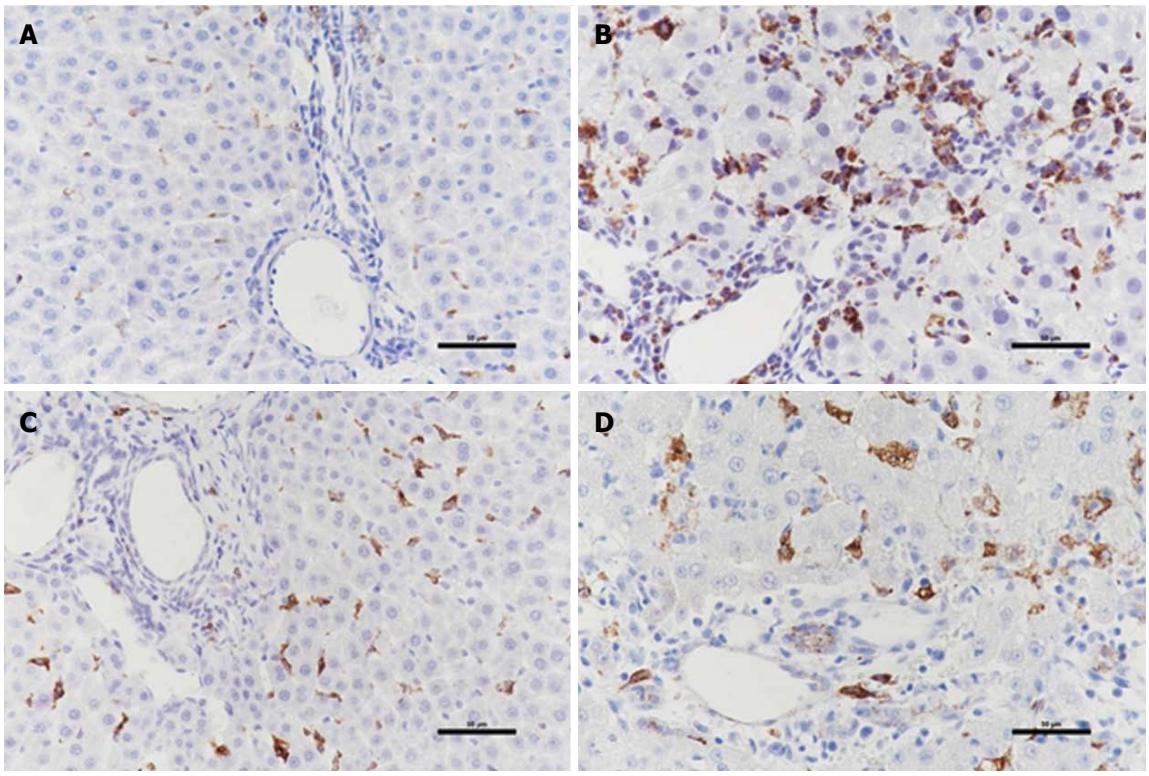

Figure 4 Immunohistochemistry for polarization of macrophages in liver tissue. A: Distribution of macrophages reacting to CD68 for M1 in PBS-treated group; B: Distribution of macrophages reacting to CD68 for M1 in DGalN-treated group; C: Distribution of macrophages reacting to CD163 for M2 in PBS-treated group; D: Distribution of macrophages reacting to CD163 for M2 in DGalN-treated group (n = 4).

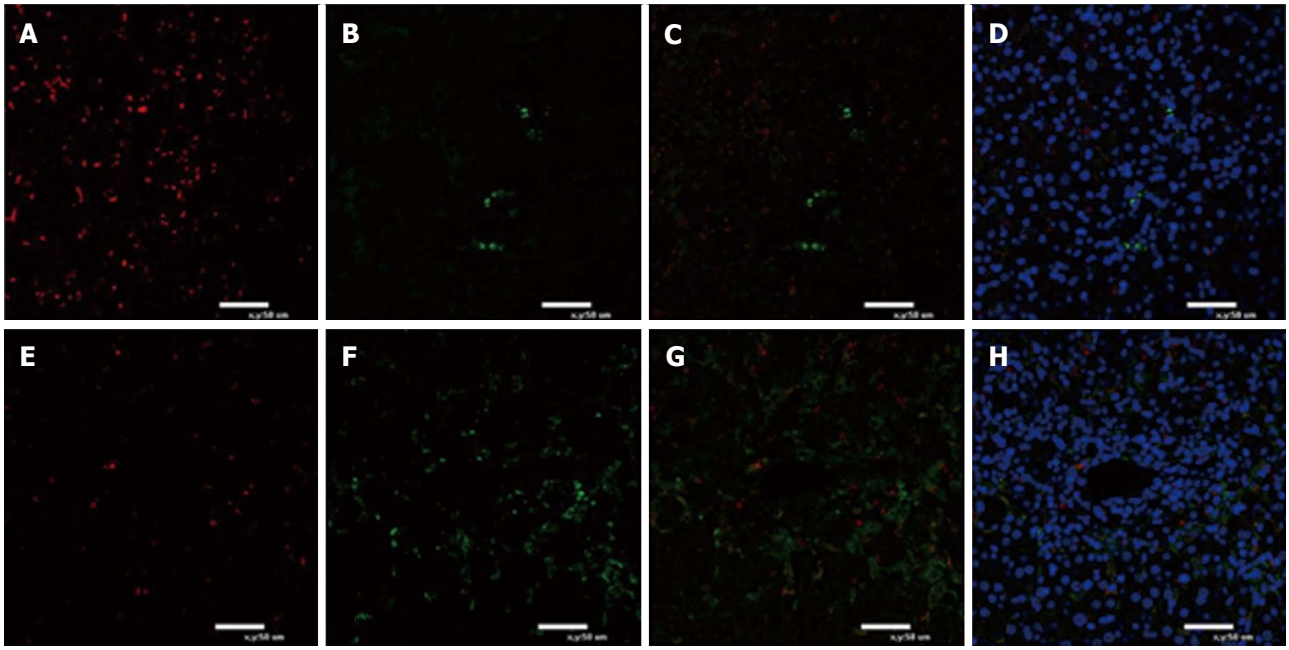

Figure 5 Immunofluorescence for polarization of macrophages in liver tissue. A-D: Death group after mesenchymal stem cell (MSC) treatment; E-H: Survival group after MSC treatment. Green fluorescence indicates CD163+ macrophages. Red fluorescence indicates CD68+ macrophages. Nuclei are stained blue with DAPI. Immunofluorescence for M1 macrophages reacting to CD68 and M2 macrophages reacting to CD163 is shown (n = 5).

differentiate into cholangiocytes or hepatocytes and are located in the portal area. CD68+ macrophages and CD163+ macrophages are mainly located in the portal zone. However, a specific signaling pathway between macrophage polarization, associated cytokines, and hepatocyte regeneration has not been examined to

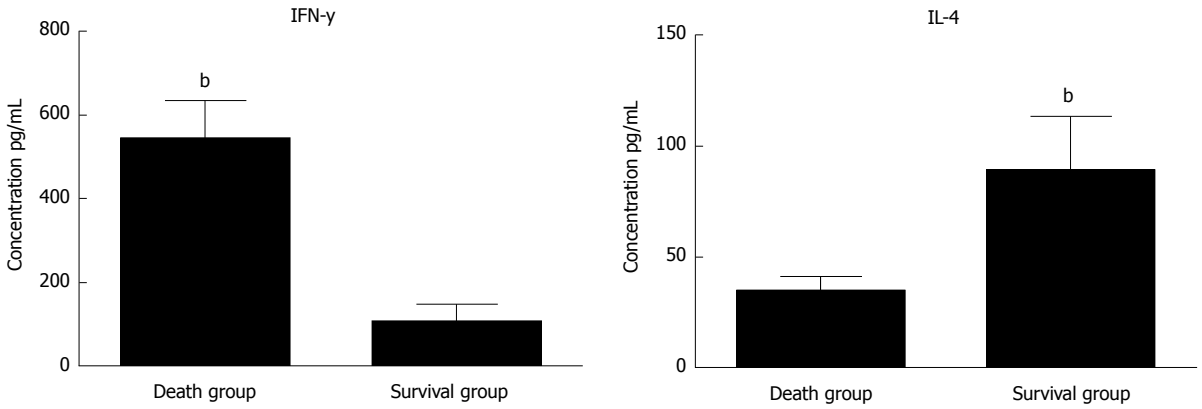

Figure 6 Flow cytometry analysis for serum levels of IFN-y and IL-4 in survival and death groups after mesenchymal stem cell treatment. Bar represents the mean ± SD (n = 6, ^{b}P < 0.001).

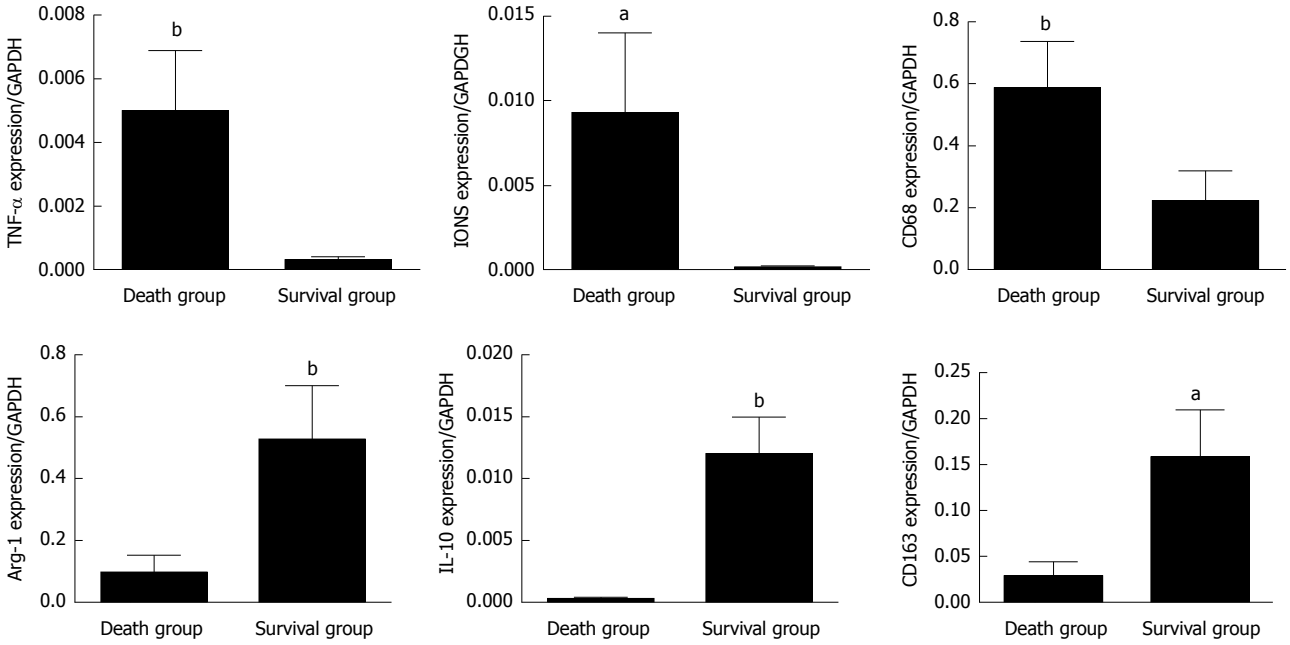

Figure 7 mRNA expression of M1- and M2-related factors in survival and death groups after mesenchymal stem cells treatment. The mRNA expression of M1-related factors (TNF-α, INOS, and CD68) and M2-related factors (Arg-1, IL-10, and CD163) was detected in the total liver tissues of death group and survival group after MSC treatment. Bar represents the mean ± SD (n = 6, ^{a}P < 0.05, ^{b}P < 0.001).

date.

In conclusion, MSCs transfused into rats were recruited and increased the survival rate by inhibiting apoptotic hepatocytes and promoting hepatocyte regeneration. This study demonstrates that expression of hepatic progenitor surface marker (EpCAM) is the key to improving the prognosis of AHF. Although this study lacks specific cell numbers of macrophage polarization in the liver, we detected macrophage polarization by cell markers and related cytokines. Importantly, M2 plays a crucial role in the prognosis of AHF, which results in altered levels of anti-inflammatory and pro-inflammatory factors. The mechanism by which M2 macrophages participate in the activation of infused MSCs remains unclear. In such a situation, the observed differential effects of M1 and M2 macrophages suggest that M2 polarization may provide a potential therapeutic application in AHF after MSC transplantation.

ARTICLE HIGHLIGHTS

Research background

Recent studies have demonstrated that macrophages promote stem cell activity *via* paracrine action. Macrophages can express multi-phenotype and multi-functional roles in the liver and are a major source of both pro-proliferative and anti-proliferative mediators in liver pathology. There is little information available on the role of macrophage polarization in rescuing acute hepatic failure by mesenchymal stem cells.

Research motivation

Different macrophage phenotypes play various roles in tissue damage and maintenance. It is not clear whether M1 or M2 polarization contributes to the

therapeutic effects of mesenchymal stem cells (MSCs). Macrophages to M1 or M2 polarization can increase infused MSCs activity during MSC transplantation, and improve the clinical efficacy of MSCs in the treatment of acute hepatic failure.

Research objectives

To investigate whether M1 or M2 polarization contributes to the therapeutic effects of MSCs.

Research methods

The rats were divided into a survival group and a death group at 48 h after MSC treatment. The rats in the survival group were still in good physical condition at 48 h after MSC treatment. The rats in the death group were in poor physical condition or in the state of death before they died at 48 h after MSC treatment. The polarization of M1 and M2 was compared between the two groups. Macrophage polarization was analyzed by M1 markers [CD68, tumor necrosis factor alpha (TNF-a), interferon-γ (IFN-γ), and inducible nitric oxide synthase (INOS)] and M2 markers [CD163, interleukin (IL)-4, IL-10, and arginase-1 (Arg-1)].

Research results

The number of CD163+ macrophages and levels of IL-4, IL-10, and Arg-1 were significantly up-regulated in the survival group. In contrast, CD68+ macrophages and levels of TFN-γ, TNF-α, and INOS were significantly up-regulated in the death group. However, a specific signaling pathway between macrophage polarization, associated cytokines, and hepatocyte regeneration has not been examined to date.

Research conclusions

This study demonstrates that expression of hepatic progenitor surface marker (EpCAM) is the key to improving the prognosis of AHF. We detected macrophage polarization by cell markers and related cytokines. M2 macrophages play a crucial role in the prognosis of AHF, which results in altered levels of anti-inflammatory and pro-inflammatory factors. The mechanism by which M2 macrophages participate in activation of infused MSCs remains unclear. The observed differential effects of M1 and M2 macrophages suggest that M2 polarization may provide a potential therapeutic application in AHF after MSC transplantation.

Research perspectives

M2 macrophages and their associated cytokines can contribute to AHF rescuing by MSCs. It is unclear whether M2 related cytokines originate from the liver or from the implanted MSCs. Further localization studies and relevant cell experiments are needed to confirm the results.

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ORIGINAL ARTICLE

Basic Study

Improved experimental model of hepatic cystic hydatid disease resembling natural infection route with stable growing dynamics and immune reaction

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Abstract

AIM

To investigate a safer way to set up the disease model of cystic echinococcosis without contamination risk and develop a novel experimental murine model of hepatic cystic echinococcosis.

METHODS

C57B/6 mice were injected with human protoscolices

of three different concentrations *via* the portal vein. The mice were followed for 10 mo by ultrasound, gross anatomy, and pathological and immunological examinations. The protoscolex migration in the portal vein, hydatid cyst growth, host immune reaction, and hepatic histopathology were examined periodically.

RESULTS

The infection rates in the mice in the high, medium, and low concentration groups were 90%, 100%, and 63.6%, respectively. The protoscolices migrated in the portal vein with blood flow, settled in the liver, and developed into orthotopic hepatic hydatid cysts, resembling the natural infection route and course.

CONCLUSION

We have established an improved experimental model of hepatic cystic echinococcosis with low biohazard risk but stable growing dynamics and immune reaction. It is especially useful for new anti-parasite medication trials against hydatid disease.

Key words: Echinococcosis; Echinococcosis granulosus; Protoscolex; Hydatid disease; Experimental model

Core tip: In this experimental study, we developed a novel murine model of cystic echinococcosis. This orthotopic model resembles primary infection route and natural infectious course with low biohazard risk and high efficiency.

Zhang RQ, Chen XH, Wen H. Improved experimental model of hepatic cystic hydatid disease resembling natural infection route with stable growing dynamics and immune reaction. *World J Gastroenterol* 2017; 23(45): 7989-7999 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/7989.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.7989

INTRODUCTION

Hydatid disease caused by *Echinococcus granulosus* is a worldwide zoonosis. It is highly prevalent in Xinjiang, China[1]. Humans are accidentally infected by *Echinococcus granulosus* egg-contaminated areas. Infection causes serious economic, medical, veterinary, and public health impact[2]. Animal model plays an important role in the study for novel drugs, surgical approaches, and vaccine development. An ideal experimental model should orthotopically induce hydatid disease in the most affected organ, *i.e.,* the liver. The model should resemble the natural infection route and course with a stable and predictable growth pattern. However, traditional animal models exhibit a biohazard risk when feeding animals with parasitic eggs and induce the parasite cyst in the abdomen cavity as the secondary infection[3]. In humans, hydatid disease demonstrates a chronic infectious course and it takes decades for the parasite to settle and grow in the liver[2]. The life cycle includes six stages: (1) adult *Echinococcus granulosus*, which is about 3-6 mm in length, resides in the bowel of its definite host; (2) gravid proglottids release eggs that are passed in the feces; (3) these eggs are then ingested by a suitable intermediate host, including sheep, goat, pigs, cattle, horses, and camels. The eggs then hatch in the bowel and release oncospheres that penetrate the intestinal wall. These oncospheres then migrate through the circulatory system to various organs of the host; (4) at the organ site, the oncosphere develops into a hydatid cyst. The cyst enlarges gradually, producing protoscolices and daughter cysts that fill the cyst interior; (5) the cyst-containing organs are then ingested by the definite host, causing infection. After ingestion, the protoscolices evaginate, producing protoscolexes; and (6) the scolexes of the organisms attach to the intestine of the definite host and develop into adults in 32-80 d. After invading into the gastrointestinal tract, its life cycle then continues in humans. The eggs then release oncospheres in the small intestine. In the liver, oncospheres migrate through the circulatory system and produce hydatid cysts.

To develop such an experimental animal model in order to mimic the natural life circle is expensive and time-consuming. In addition to the time and cost, the biohazard risk also cannot be ignored. Oral feeding with parasitic eggs can cause high infection risk for researchers and requires a high-level biohazard lab to perform the studies[4-7]. Thus, the development of a highly accurate animal model with low contamination risk to interpret short-term research results would be beneficial. In this study, we established a mouse model by injecting mice with protoscolices obtained from human hydatid cysts *via* the portal vein. Ultrasound studies detected cysts within 4 mo. The protoscolex migration, hydatid cyst formation, growing dynamics, pathological development, and immune reactions were followed until 10 mo.

This study proposes a way to circumvent many problems linked to an animal model for hydatid cyst closer to natural infection, *i.e.*, ingestion of oncospheres. Feeding animals Echinococcus eggs in the lab is risky because of biohazard for the lab personnel that can accidentally ingest or inhale the eggs. For this reason, most experimental work was done on the peritoneal injections with protoscolices, which does not reproduce the natural route of infection (ingestion of oncospheres) but the natural route of secondary echinococcosis (which is what happens when the contents of cysts are spilled into the peritoneal cavity). In addition, the disease model also has the following benefits: (1) small rodents were used so that the experiment can save labor and cost on big animals; (2) injection was performed *via* the portal vein instead of feeding from mouth so that biohazard

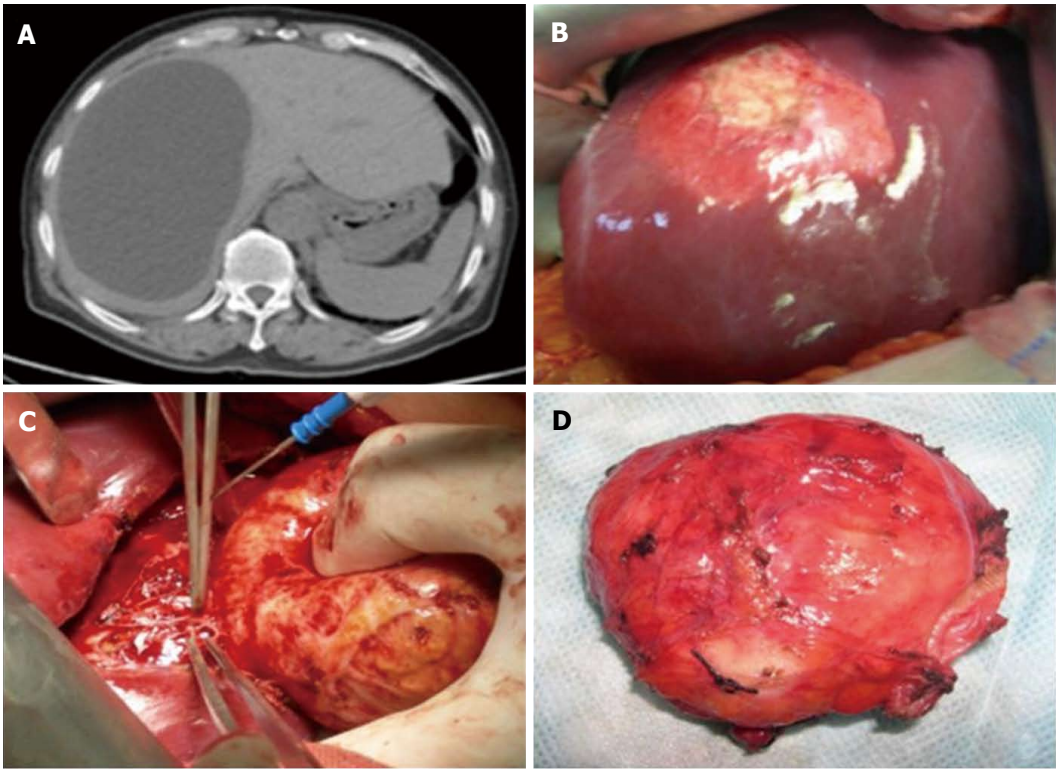


Figure 1 ***Echinococcus granulosus*** **protoscolex collection.** The protoscolices were collected from patients with hepatic hydatid cysts. A: Type I hydatid cyst (WHO classification) presents as a well-defined unilocular and fluid attenuation lesion in the liver; B: The single cyst appearance during an open surgery; C: The complete removal of the hydatid cyst from the liver; D: The hydatid cyst is full of protoscolices.

of collecting parasite eggs can be avoided; and (3) the model bypassed hatching in the small intestine so that time can be shortened and evacuation contamination be avoided. Using this model, we further proved that injected parasite can steadily grow up into hydatid cysts in the liver and stimulate host immune reaction.

MATERIALS AND METHODS

Echinococcus granulosus protoscolex collection

The protoscolices in this study were collected from hydatid cysts in naturally infected patients during an open surgery in the First Affiliated Hospital of Xinjiang Medical University (Figure 1). Written informed consent and an image release agreement were obtained in advance from all subjects. The number of protoscolices was adjusted in 0.9% NaCl solution with a 95% viability rate.

Three different concentrations were prepared for a parallel experiment design with long-term follow-up (Figure 2): group A (2000 protoscolices/100 μL), group B (200 protoscolices/100 μL), and group C (100 protoscolices/100 μL).

Viability test of Echinococcus granulosus protoscolices

Viability was confirmed using the 1% eosin exclusion test to determine the viability of the protoscolices. The viable protoscolices could exclude the eosin such that they were colorless and mobile, while dead protoscolices stained red. The viability was calculated by the number of viable cells divided by the total number of protoscolices. The protoscolices used for injection had more than 95% viability.

Mice

Eight-week-old female C57B/6 mice were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). The mouse weight varied from 20 to 24 g. They were maintained in an SPF level Experimental Animal Center of the First Affiliated Hospital, Xinjiang Medical University and acclimatized in the animal facility for one week before injection.

Portal vein injection

The animals were shaved, scrubbed, and then moved to a sterile surgical area. The animals were anesthetized with chloral hydrate (300 mg/kg) and remained anesthetized during the operation. A 1.5-cm incision was made from the bladder up to the level of the xiphoid. The skin and muscle layers were retraced by tissue retractors to hold them on the left and right sides. The intestines were carefully moved to one side with sterile gauze to expose the portal vein. The portal vein was located under the pancreas. The needle connected to the syringe filled with protoscolices was inserted into the portal vein and the protoscolex solution was released. After injection, the needle was

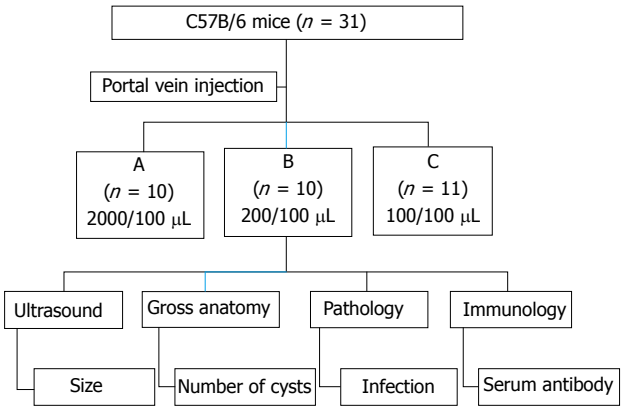


Figure 2 Experiment design and follow-up plan. Three different concentrations were prepared for a parallel experiment design with long-term follow-up: group A (2000 protoscolices/100 μL), group B (200 protoscolices/100 μL), and group C (100 protoscolices/100 μL). After injection, the hydatid cyst formation, location, distribution, and size, pathology, and immunology were followed regularly till the 10th month post injection.

slowly retracted and a piece of gauze was pressed on the puncture site to prevent backflow of blood for 5 min. The intestines were placed back into the abdomen and the abdominal wall was closed. Mice were maintained on the heating pad for recovery with frequent inspection, and no occurrence of bleeding or infection was found.

Animal grouping and long-term follow-up after injection

In total, 31 mice were randomly divided into three groups (Figure 2): group A: 2000 protoscolices in 100 μL saline, n = 10; group B: 200 protoscolices in 100 μL saline, n = 10; group C: 100 protoscolices in 100 μL saline, n = 11 mice.

After injection, the mice were observed regularly by non-invasive animal ultrasound to measure the cyst formation, location, distribution, and size. One mouse from each group was euthanized every month and examined for the presence of cysts. The liver tissue and hydatid cysts were examined microscopically to record the morphological and pathological changes. The liver and hydatid cyst wall were examined histologically by H&E staining to track the migration path of the protoscolices from the portal vein to the liver. Blood samples were collected to detect IgG production. The experiment grouping and follow-up design are illustrated using a flowchart in Figure 2.

Histological examination

Livers and hydatid cysts were fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stained with haematoxylin-eosin, and images were obtained using light microscopy to evaluate the tissue structure and pathological changes.

Detection of IgG

Blood samples were collected at different time points for detection of IgG antibodies using a nephelometric technique (Beckman Array 360; Beckman Coulter Instruments, Brea, United States)

Ethical committee approval

All experimental protocols were approved by the Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University (Approved project number: 20141217003). Informed consent was obtained from all subjects. All methods were performed according to the relevant guidelines and regulations of the Declaration of Helsinki and National Institutes of Health Guide for Care and Use of Laboratory Animals.

Statistical analysis

SPSS Software 17 for Windows (SPSS Inc., Chicago, United States) was used for statistical analyses. The differences between groups were determined using *t*-tests, and *P*-values less than 0.05 were considered significant. A standard score was used to evaluate the normal distribution of cyst formation efficiency among the three groups.

RESULTS

Hydatid cysts develop in mouse liver

In natural infection cycle, the adult parasite worms release eggs from feces and contaminate the environment. Eggs can survival for a year even in the drought and freezing environment and accidentally infect human residence *via* feces-oral route. In human digestive tract, the parasite eggs hatch and release oncospheres that penetrate the intestinal mucosa. They migrate passively through blood in the portal vein to reach the liver for final settlement. One oncosphere develops into a hydatid cyst. The hydatid cyst grows up with cyst fluid and infective protoscolices. In our experimental model, by injecting the protoscolices into the portal vein directly, we bypassed the contractable egg hatch stage in the intestine, and obtained the primary hydatid cyst in the liver. The final number of the developed cysts in fact depends on many factors, *e.g.,* space in the liver and the viability of the protoscolex.

After the mice were injected with protoscolices at three different concentrations (2000/100 μL in group A, 200/100 μL in group B, and 100/100 μL in group C), the hydatid disease infection rates in the mice in the three groups were 90% (9/10 in group A), 100% (10/10 in group B), and 63.6% (7/11 in group C), respectively (Table 1). There was no significant difference in the infection rates among the three groups (P < 0.05).

Hydatid cyst location in the liver

Four weeks after portal vein injection, visual lesions on the liver could be found. After 4 mo, the hydatid cysts presented significant growth. Table 2 presents the anatomical locations of the hydatid cysts in the mouse

Table 1 Hydatid disease infection rate in mice in the three groups injected with different concentrations of protoscolices

Group	Concentration of injected protoscolices	Number of infected mice	Number of non-infected mice	Total	Infection rate (%)[a]
Group A	2000/100 μL	9	1	10	90.0
Group B	200/100 μL	10	0	10	100.0
Group C	100/100 μL	7	4	11	63.6
Total		26	5	31	84.55

[a]$P = 0.096$, no significant difference in the infection rates among the three groups.

Table 2 Lobe position and quantity of hydatid cysts in the mouse liver

Mouse ID	Group A	Group B	Group C
1	Middle lobe: 2 Upper right lobe: 1 Lower right lobe: 1	Middle lobe: 2 Lower right lobe: 2 Lateral left lobe: 3	Right lobe: 3 Lateral left lobe: 1
2	Middle lobe: 6 Upper right lobe: 5	Middle lobe: 1 Lateral left lobe: 1	Lateral left lobe: 2
3	Multiple cysts all over the liver	Lower right lobe: 1	None
4	Middle lobe: 6 Upper right lobe: 2 Lower right lobe: 2 Lateral left lobe: 2	Upper right lobe: 2	Middle lobe: 3 Lateral left lobe: 3
5	Multiple cysts all over the liver	Middle lobe: 3 Upper right lobe: 1	Lower right lobe: 1 Lateral left lobe: 3
6	None	Lower right lobe: 1	None
7	Multiple cysts all over the liver	Upper right lobe: 1	Middle lobe: 1
8	Middle lobe: 1 Upper lobe: 1	Lateral left lobe: 4	Middle lobe: 2 Lateral left lobe: 3
9	Multiple cysts all over the liver	Lateral left lobe: 1	Middle lobe: 1
10	Multiple cysts all over the liver	Upper right lobe: 1 Lower right lobe: 2	None
11	-	-	None

liver (Table 2). Hydatid cysts occurred in any part of the liver and there was no significant preference in any of the liver lobes.

The fundamental structure of the four major liver lobes of rat and mouse livers and the segmentation of human liver according to Couinaud are similar and the fundamental structure is comparable. These findings allow the previous use of rodent models in experimental hepatobiliary surgery. The murine and human livers are comparable due to the similarity of the fundamental structures. These findings allow the use of mice to develop experimental models of hydatid disease.

Pathogenicity and efficiency

After 6 mo, when the hydatid cysts were fully developed in the mouse liver, the ratio of developed cysts/number of protoscolices was evaluated using two markers: pathogenicity (number of cysts per protoscolex) and number of hydatid cysts per mouse. The gross anatomy and column illustration are shown in Figure 3. Cyst abundance in each mouse reflects protoscolex immune reaction, which stimulates the host immune system to produce IgG against the parasite. Although group A had the highest parthenogenesis (2.395 ± 0.7424) and cyst abundance (47.90 ± 14.848), the condensed lesion made observation of the individual cyst impossible.

Optimization of injection concentration using a standard score

During the 10-mo long follow-up period, no mouse died due to portal vein bleeding, surgery related infection, or cachexia, unless a mouse was euthanized during the monthly routine examination. In terms of the hydatid disease model success rate, there was no significant difference among the three groups. However, the experimental model on hydatid requires a more reliable normal distribution. Thus, the standard score was used in this study to compare the reliability and efficiency of the animal models (Figure 4).

The standard score was used in this study to compare the reliability and efficiency of animal models. Standard score was calculated as (raw score - mean)/SD (Figure 4). This value indicates how well the model reflected the normal distribution compared to other models (the normal distribution of groups A, B, and C is shown in Figure 4, upper panel). This value allows comparisons to be made between the three models with different distribution characteristics, *i.e.*, mean and SD. Thus, a score of 1.39 in group B indicates that its performance was better compared with groups A

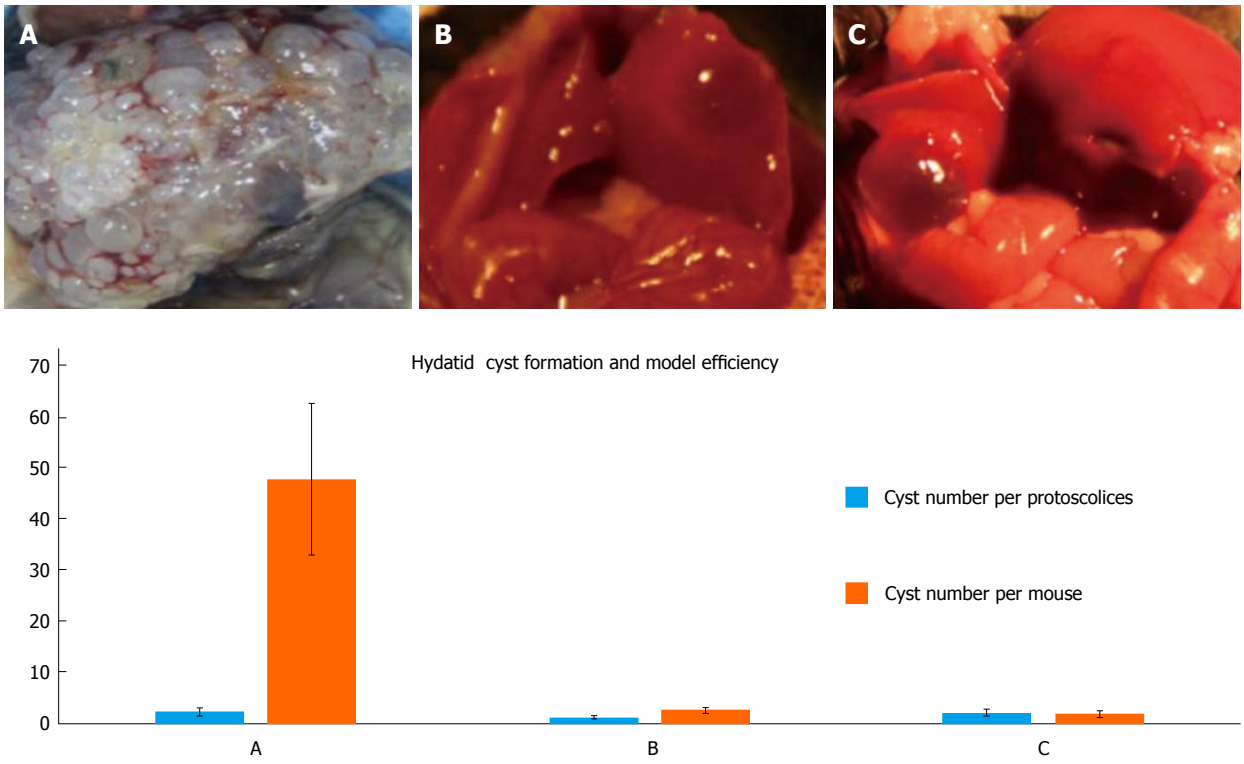
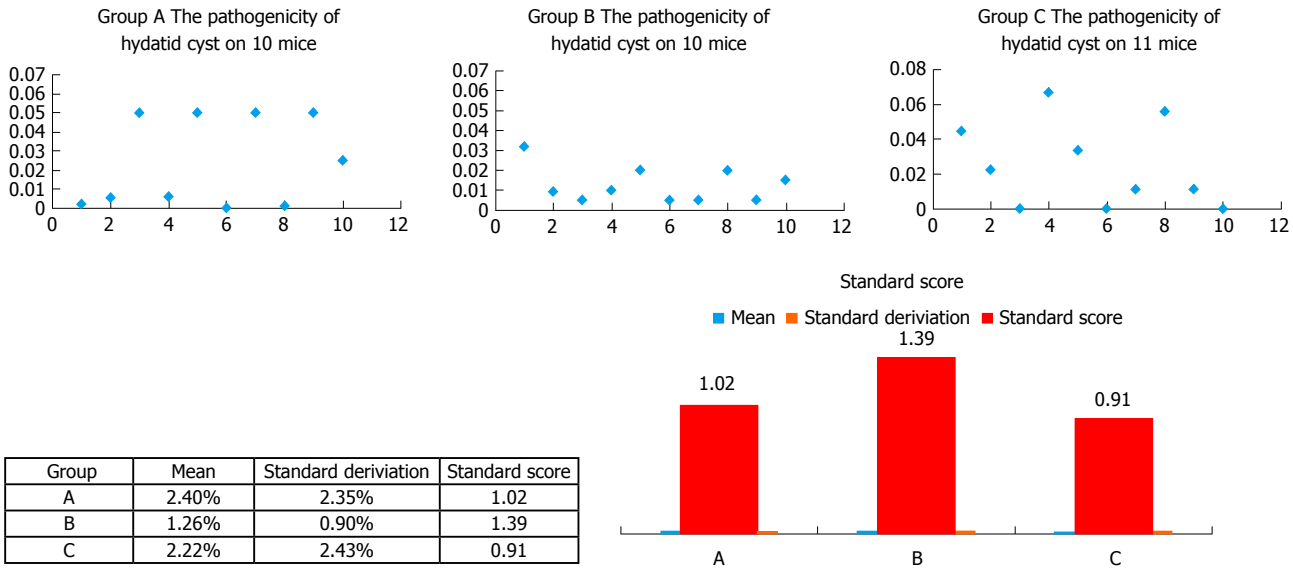


Figure 3 Pathogenicity and efficiency evaluated by cyst per protoscolex. After 6 mo when the hydatid cysts fully developed in the mouse liver, the injection efficiency was evaluated by two markers: The pathogenicity (cyst number per protoscolex) and hydatid cyst number per mouse.



Group	Mean	Standard deriviation	Standard score
A	2.40%	2.35%	1.02
B	1.26%	0.90%	1.39
C	2.22%	2.43%	0.91

Figure 4 Injection concertation optimization by standard score. The standard score was used in this study to compare the reliability and efficiency of animal models. The normal distribution of groups A, B, and C is shown in upper panel. A standard score of 1.39 of group B indicates that its performance was better compared with groups A (1.02) and C (0.91).

(1.02) and C (0.91).

Migration of protoscolices from the portal vein to liver lobe

The path and course of the protoscolex migration from the portal vein to the liver were tracked by open examination, pathology, and ultrasound. On the day of portal vein inoculation with human *Echinococcus granulosus* protoscolices, the branches of the portal vein diameter increased. With congestion of condensed protoscolices (Figure 5, middle panel), 1 d after inoculation, the inflammatory cell migration was incarcerated; 3 d after inoculation, a significant inflammatory reaction zone formed; 3 wk later

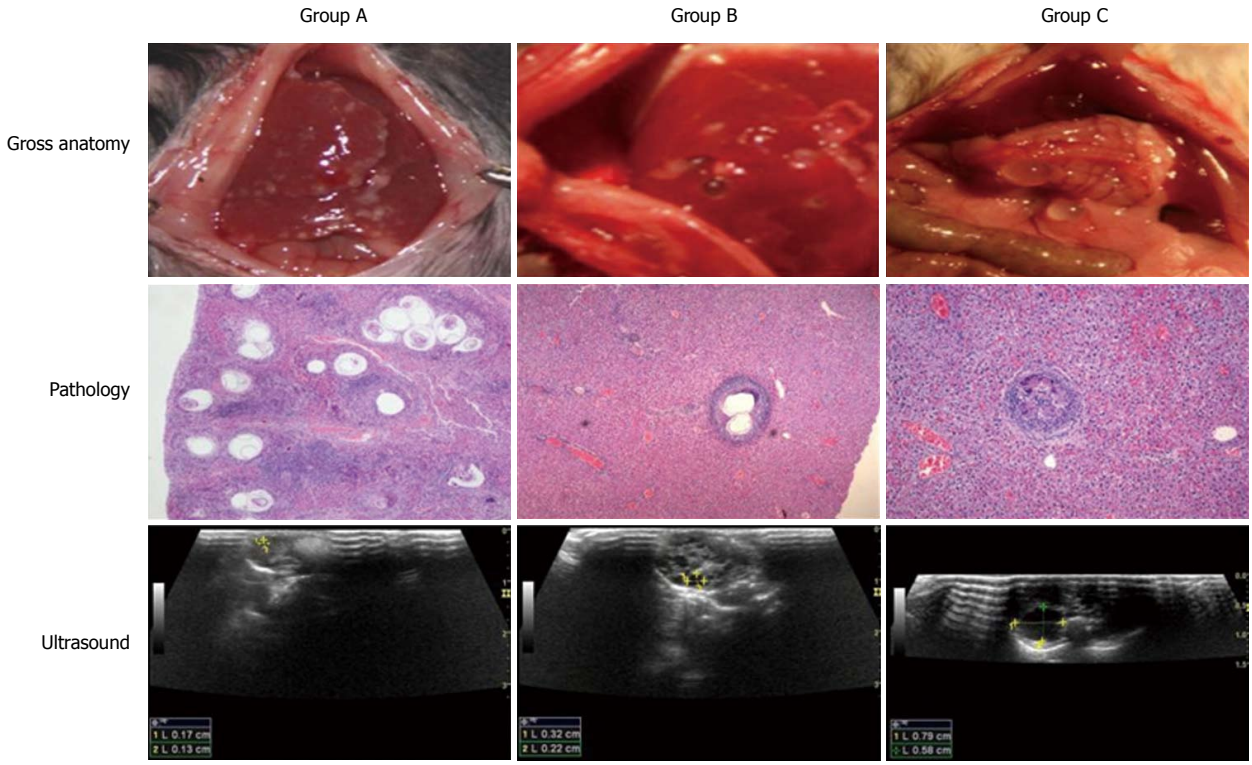


Figure 5 Migration of the protoscolices in the portal vein and liver. The path and course of the protoscolex migration in the portal vein and liver were tracked by open examination, pathology, and ultrasound. When the human *Echinococcus granulosus* protoscolices were injected, they travelled from small branches of the portal vein to the liver with the blood flow, causing inflammatory cell infiltration. When the hydatid cyst formed, the infected liver presented the infection zone around the parasite lesion. The open examination showed the distribution and cyst abundance in the livers of groups A, B, and C. After 4 mo, ultrasound detected spherical, fibrous rimmed cyst with surrounding host reaction. After 6 mo, the even larger parent cyst with satellite daughter cysts within or outside the parent cyst was found.

the protoscolex developed into vesicles (Figure 5, middle panel); and 6 wk after inoculation, none of the protoscolex could be found but visible vesicular structures of hydatid cyst were observed (Figure 5, middle panel). The open examination showed the distribution and cyst abundance in the livers of groups A, B, and C (Figure 5, upper panel). After 4 mo, ultrasound detected spherical, fibrous rimmed cysts with surrounding host reaction. After 6 mo, an even larger parent cyst with satellite daughter cysts within or outside the parent cyst was found (Figure 5, lower panel). The rodents have the four major liver lobes similar to human hepatic Couinaud segments. Murine and human livers are comparable due to the similarity of the fundamental structures. These findings allow the use of mice to develop the experimental hydatid disease model.

Hydatid cyst growing dynamics measured by ultrasound

After 6 mo, ultrasound could detect a stable increase in the number of hydatid cysts (Figure 6). The average cyst diameters in group B on the 24th, 28th, 29th, 32nd, and 36th weeks were 2.48 ± 0.91 mm, 3.29 ± 1.86 mm, 3.87 ± 2.26 mm, 5.00 ± 2.57 mm, and 7.98 ± 2.75 mm, respectively (Table 3), indicating a significant increase in diameter over time after 6 mo (P < 0.001).

Immunological changes evaluated by IgG

When the protoscolices migrated in the portal vein, the host had a low level of IgG (40 ng/mL at week 1). After approximately 6 mo, the hydatid cyst became fully developed, and the cyst began to release different antigens to modulate the host immune surveillance. IgG increased in parallel with the hydatid cyst volume (500-800 ng/mL during weeks 24-32). Parasitic antigens stimulated a series of complex host immune responses, which may benefit both the host and parasite for a symbiotic relationship (800-900 ng/mL at week 36) (Figure 6).

Histopathological changes in the hydatid cyst-infected liver

Microscopic examination of the mouse liver revealed parasitism related pathological changes. After injection, the protoscolices congested the portal vein. They caused dilatation of the vessel sinusoids. Dead protoscolices resulted in focal degeneration and necrosis, and the mouse liver reacted with an increased diameter of central veins. The mouse liver also showed protective immune reactions, such as lymph cell infiltration and fibrosis capsules (Figure 7).

Table 3 Diameter of the hepatic cystic echinococcosis lesion over time

	Week 24	Week 28	Week 29	Week 32	Week 36	*P*
Cyst diameter (mm)	2.48 ± 0.91	3.29 ± 1.86	3.87 ± 2.26	5.00 ± 2.57	7.98 ± 2.75	0.001

Data shown are mean ± SD.

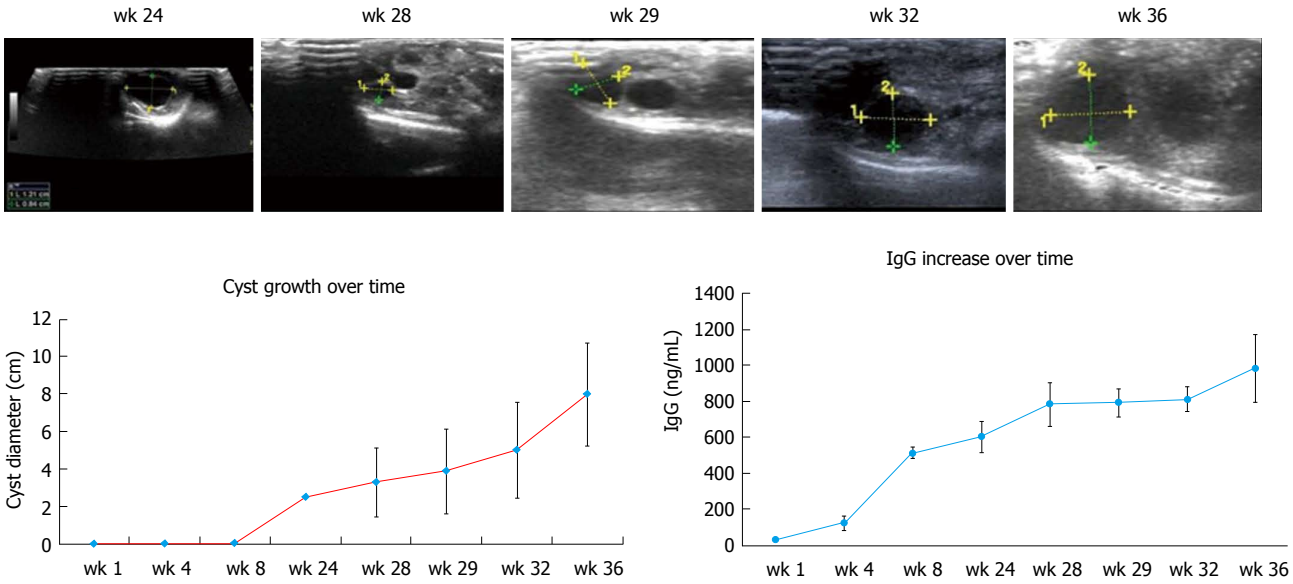


Figure 6 Hydatid cyst growing dynamics measured by ultrasound and accompanied IgG increase. Ultrasound measured the detectable hydatid cysts after 4 mo. After 6 mo, the hydatid cysts were fully developed and stimulated strong host immune reaction marked by IgG increase.

DISCUSSION

Hydatid disease is defined as a zoonotic disease or neglected tropical disease. It is a public health problem worldwide. As one of the most serious endemic diseases, it is extremely hazardous in Xinjiang, China due to poor health education and a lack of effective medication[8,9]. Thus, an animal model is needed as the basis for the development of new medication against hydatid disease[10].

To establish a mouse model of echinococcosis granulosus, different infection routes have been investigated: orally, intraperitoneally, or intravenously. Different parasitic stages have been employed, such as parenterally with eggs, hatched eggs, or activated oncospheres. In addition to the low infection rate, the generation of experimental animals orally with eggs might pose potential contamination risks to the laboratory personnel who are exposed to feces of the infected mouse and the liver with hydatid cysts[3,5-7].

In this study, an effective animal model was established to mimic the natural infection route and course of echinococcosis in humans. This animal model showed the following specific advantages.

Safe operation and low biohazard risk

Echinococcus granulosus poses the greatest risk because it is the most common and widely distributed species. Accidental ingestion of infective eggs is the primary laboratory hazard. A single infective egg from the feces of the definitive host could potentially result in serious infection. Handling parasites requires special care and a special lab facility[4].

Cystic echinococcosis is considered an occupational infection. Certain people, *e.g.,* shepherds, slaughters, stockbreeders, and farmers, are at higher risk of the disease because their career makes them to work closely with animals. When producing a disease model, the researchers are exposed to the risk due to the oral feeding of parasite eggs to animals.

In this study, an improved protocol without feeding high risk eggs orally into the gut, but instead, with injecting protoscolices *via* the portal vein, reduced the occurrence of laboratory-acquired infection in the laboratory and is safe for animal care personnel.

Most popular intermediate host

Hydatid disease can use many other wild herbivores as an animal model, such as sheep, goats, cattle, camels, buffalos, pigs, and kangaroos, but small rodents show high feasibility in the general animal center. A mouse model is easy for performing biochemical examinations with mouse-derived antibodies. In this study, the animal model was established using the most popular intermediate host mice. Good susceptibility to human protoscolices and a high yield of hydatid cysts were

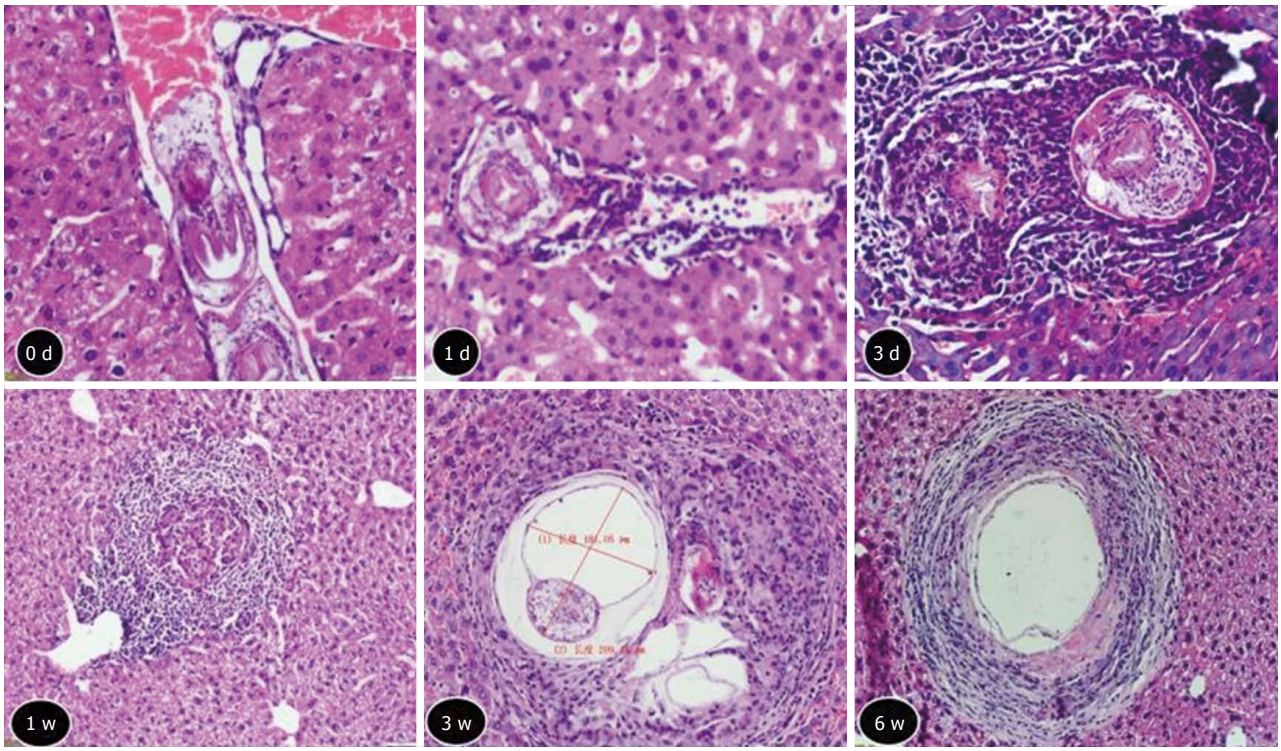


Figure 7 Pathological images of mouse liver injected with 200 protoscolices at different time points. 0 d: On the same day of portal vein injection, the protoscolices congested the portal vein. 1 d: The injected protoscolice caused the dilatation of vessel sinusoids. The central vein size increased. 3 d: Those dead protoscolices ended up as focal degeneration; mouse liver reacted with increased diameter of central veins, inflammatory cell infiltration (blue stained), and collagen deposition (red stained). 1 wk: Mouse liver had protective immune reactions such as lymphocyte infiltration. The vessel fibrosis was found in biliary ducts and portal vein area. 3 wk: The newly developed hydatid cyst was found full of the germinal layer, the laminated layer, and the beginning of the adventitious layer. 6 wk: The mouse localized the hydatid cyst with compressed and fibrotic host tissue (HE staining, × 10).

observed in the liver.

Natural infectious route and course of orthotopic and primary infection organ (liver)

The hydatid cysts formed by the metacestode (larval stage) migrate from the intestine to the liver *via* the portal vein and finally develop into hydatid cysts most often in the liver[1,2]. C57B/6 mice were injected *via* the portal vein with protoscolices from humans. This animal model mimics the natural infectious route. Protoscolices migrated from the portal vein into the liver lobe, forming hydatid cysts on the orthotopic and primary infection organ.

Stable hydatid cyst formation and growth

Small cystic larvae were observed macroscopically in the liver 3 wk post-injection. The laminated layer was found 6 wk post-injection. At four months post-injection, larger larval cysts were found in the orthotopic liver. A laminated layer with mature protoscolices was observed to be surrounded by lymphocytes.

Convenient anatomy location

Mouse and human liver anatomies are similar except that the human liver has a larger right lobe and a large right portal vein compared with the left side. When the human superior mesenteric vein and splenic vein are confluent in the portal vein, the majority of blood flow goes into the right portal vein, thus carrying more parasites in the flow into the right lobe. The majority of human hepatic hydatid cysts (60%-80%) were found in the right lobe. However, in the mouse model, the liver lobes showed no significant difference in this proportion. The middle lobe exhibited the highest volume percentage (approximately 30% of the total liver volume). Thus, the lesion location in the mouse liver showed no lobe preference and no lobe appeared in the liver. Mice injected with 2000 protoscolices produced more than 100 vesicles by the end of the study (9 mo). Mice injected with 200 protoscolices yielded 1-4 vesicles. Technically, the location of the hydatid lesions could be controlled by fixing the right lobe by selectively blocking the portal vein. To decrease the confounding factors, selective blockade of the portal vein was avoided in this study but it was technically possible in specific circumstances.

Active hydatid cysts

In human beings, the final fate of chronic hydatid cysts in the liver are quite different. Some cysts keep on expanding slowly in decades of years without obvious symptoms. Some cysts grow up to certain volume (*e.g.,* when the cyst diameter is larger than 5 cm) and become symptomatic. Some cysts rupture

spontaneously and the spillage of parasite tissue causes the secondary echinococcosis. Some cysts have necrotic processes leading to a solidification and/or calcification of the cysts. The cysts collapse and gradually disappear. According to ultrasonographic features of the hydatid cyst, the WHO classified cystic echinococcosis from CE1-CE5: CE1 and CE2 are active, CE3 is transitional, and CE4 and CE5 are inactive. In this study, the hydatid cysts can form in months (*vs* many years in humans) and the ultrasonography showed the cysts formed in murine livers are CE1, the most active type, making the model a reliable animal model for any further study.

Detectable host-parasite immune reaction and pathological changes, which are proportional to the expansion of the hydatid cyst

The mice produced the host immune protection following primary injection of protoscolices. Accordingly, the protoscolices developed cyst membranes and capsules that are highly effective in protecting the parasite from host immune destruction. IgG is a marker that reflects the host-parasite immune reaction. When a hydatid cyst develops in the liver, host IgG in serum is significantly elevated and can be used as an indirect marker for a coarse estimate of hydatid cyst volume and parasitic burden[11].

Optimal injection concentration of 200 protoscolices

Group B was optimally injected with a concentration of 200 protoscolices, and the cyst number (2.60 ± 0.618) left sufficient space for intervention and further follow-up observation. In group B, the number of cysts and protoscolices was proportional to the volume of the cysts. The radius of the individual cyst gradually increased accordingly over time (not linearly). In group B, 100% of the mice developed hydatid cysts with ultrasound detectable lesions. The hydatid cysts became distended and palpable in 4 mo. Group B was superior for research due to its low dose of infection and predictable cyst development as well as better normal distribution. It will benefit experimenters to observe the *in vivo* efficacy of new treatments against hydatid without the need for sacrificing the mouse[12-15].

In summary, we have developed a model of hydatid disease not on sheep, dogs, or humans, but on small rodents so that the experiment can save labor and cost, and avoid ethic issue on sheep or humans. Injection *via* the portal vein instead of feeding from mouth can avoid collecting parasite eggs with biohazard risk. This model can bypass the hatching stage in the intestine so that it saves time and avoid contamination. Using the animal model, we further showed the animal model can steadily grow up into hydatid cysts in the liver and steadily stimulate host's specific immune reaction. The proper cyst density and anatomical localization enable accurate monitoring. In this study, larval *Eechinococcus granulosus* infection was induced in mice, the most popular experimental intermediate host. Using this experimental model, the parasite cyst growth and immune reaction proportional to the cyst volume can be examined.

ARTICLE HIGHLIGHTS

Research background

Hydatid disease is caused by *Echinococcus granulosus*. It is a worldwide zoonosis. It is highly prevalent in Xinjiang, China. The animal disease model is of great significance for the drug development against parasite disease.

Research motivation

Echinococcosis is mostly caused by close contact with infected dogs or occupational exposures. The researchers producing hydatid disease model have the high risk of infection because they handle parasite eggs to feed animals through oral route.

Research objectives

To avoid contamination risk of handling parasite eggs, this study investigated a safer way for developing an experimental murine model of cystic echinococcosis in the liver.

Research methods

Bypassing the oral feeding of contaminant parasite eggs, human protoscolices were injected *via* the portal vein. Using this method, the tapeworm eggs that may contaminate lab and consequently enable transmission to human beings are avoided.

Research results

The pathological results confirmed that protoscolices kept alive and moved from the portal vein into different liver segments and lobes, and the three different protoscolice injection concentrations led to different infection rates of 90%, 100%, and 63.6%, respectively.

Research conclusions

By sterile injection with human protoscolices *via* the portal vein, a novel murine model was developed with echinococcosis vacuoles formed in the liver. Without contamination risk to researchers, this disease model is suitable for anti-hydatid treatment trials.

Research perspectives

The good experience that can be learnt from this study is the portal vein injection, which can bypass the oral feeding with parasite eggs. The protoscolices migrate in the portal vein with blood flow, settle in the liver, and develop into orthotopic hepatic hydatid cysts, resembling the natural infection route and course. The lesson that can be learnt from this study is protoscolex collection. Only the fresh protoscolex can result in success parasite growth.

With this model, the further anti-hydatid medicines and interventional treatment can be tried. With the quantitative immune results, the effects can be monitored by blood test.

Using standard score calculated as (raw score - mean)/SD, the best injection method has been screened. This value allows comparisons to be made between the three models with different distribution characteristics. The portal vein injection at 200 protoscolices in 100 μL saline is the best method for the future model.

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ORIGINAL ARTICLE

Case Control Study

Recurrence in node-negative advanced gastric cancer: Novel findings from an in-depth pathological analysis of prognostic factors from a multicentric series

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Abstract

AIM

To analyze the clinicopathological characteristics of

patients with both node-negative gastric carcinoma and diagnosis of recurrence during follow-up.

METHODS

We enrolled 41 patients treated with curative gastrectomy for pT2-4aN0 gastric carcinoma between 1992 and 2010, who developed recurrence (Group 1). We retrospectively selected this group from the prospectively collected database of 4 centers belonging to the Italian Research Group for Gastric Cancer, and compared them with 437 pT2-4aN0 patients without recurrence (Group 2). We analyzed lymphatic embolization, microvascular infiltration, perineural infiltration, and immunohistochemical determination of p53, Ki67, and HER2 in Group 1 and in a subgroup of Group 2 (Group 2bis) of 41 cases matched with Group 1 according to demographic and pathological characteristics.

RESULTS

T4a stage and diffuse histotype were associated with recurrence in the group of pN0 patients. In-depth pathological analysis of two homogenous groups of pN0 patients, with and without recurrence during long-term follow-up (groups 1 and 2bis), revealed two striking patterns: lymphatic embolization and perineural infiltration (two parameters that pathologists can easily report), and p53 and Ki67, represent significant factors for recurrence.

CONCLUSION

The reported pathological features should be considered predictive factors for recurrence and could be useful to stratify node-negative gastric cancer patients for adjuvant treatment and tailored follow-up.

Key words: N0 gastric cancer; Recurrence; Prognostic factors; Pathological analysis; Lymphatic embolization; Perineural infiltration; p53; Ki67

Core tip: We analyze the clinicopathological characteristics of patients with both node-negative gastric carcinoma and diagnosis of recurrence during follow-up in 4 Italian centers belonging to the Italian Research Group on Gastric Cancer between 1992 and 2010. Lymph node metastasis is the most important prognostic factor in patients undergoing radical surgery for gastric carcinoma. In-depth pathological analysis of two homogenous groups of pN0 patients, with and without recurrence during long-term follow-up, revealed two striking patterns: lymphatic embolization and perineural infiltration (two parameters that pathologists can easily report), and p53 and Ki67, represent significant factors for recurrence. The reported pathological features should be considered predictive factors for recurrence and could be useful to stratify node-negative gastric cancer patients for adjuvant treatment and tailored follow-up.

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INTRODUCTION

Nodal metastases are a well-known prognostic factor after radical treatment of gastric cancer. Although patients with adequately staged pN0 cancer have a good prognosis and usually do not undergo adjuvant chemotherapy, nevertheless a subset of them has a recurrence and, later, dies of this disease. Therefore, the identification of prognostic factors for cancer recurrence becomes extremely important for the identification of the small proportion of cases at risk for recurrence.

In some published studies focusing on the clinical-pathological features and prognostic factors of node-negative gastric cancer patients, surgical approaches were not homogenous, particularly in terms of lymph node dissection[1-10]. Other limitations were a too short follow-up, as well as the inclusion of T1 cases and of patients with less than 15 retrieved nodes. A previous multicentric series, which we published as members of the Italian Research Group for Gastric Cancer (GIRCG), studied 301 patients from 7 Italian centers[11]. These patients were treated during a 10-year period from 1992 to 2002, underwent almost 5 years of regular follow-up, and about 10% developed tumor recurrence. The much valuable feedback and insightful suggestions of peer-reviewers, who have refereed our earlier article, inspired our next project and current paper. Those peer-reviewers strongly encouraged us to carry out an in-depth pathological analysis of the subgroup of patients experiencing recurrence despite having negative nodes on e-e staining; this group clearly represents a very interesting sample for a biological study.

MATERIALS AND METHODS

The 4 Italian centers participating in this study were the University of Brescia, the Forlì Morgagni Pierantoni Hospital, the University of Siena, and the University of Verona. Inclusion criteria were patients with pN0 gastric carcinoma, treated during the period 1992-2010, who developed cancer recurrence during the 5-year follow-up. All patients had more than 15 retrieved nodes analyzed after surgery, all resulting negative

for metastases at routine hematoxylin-eosin staining. Exclusion criteria were (1) patients with gastric stump cancer; (2) patients with T1 or T4b tumors or peritoneal dissemination; (3) patients with fewer than 15 retrieved nodes; and (4) patients who underwent pre- or post-operative chemotherapy. As a result, out of 1725 patients who underwent radical gastrectomy for T2-T4a gastric cancer, 478 patients without lymph node metastases were studied, and among those 41 developed tumor recurrence (8.57%, Group 1) after a mean period of 17 months (range 9-89). Control group comprises the remaining 437 pN0 patients who did not develop cancer recurrence (Group 2).

We then performed a retrospective review of prospectively collected data, taking into account patient's age, sex, tumor markers CEA and CA 19.9 when available, tumor size, tumor location (upper/medium/lower third), depth of tumor invasion (T2/T3/T4a), histological grading (G1/G2/G3), Lauren histotype (intestinal/diffuse/mixed or other), the type of operation (subtotal, GST/total gastrectomy, GT), the number of retrieved nodes, associated resections, post-operative major morbidity, and post-operative mortality. Survival rate was reported as mean + SD. We adopted the 2012 American Joint Committee on Cancer (AJCC) TNM staging system[12], whereas histological evaluation was performed according to the Japanese General Rules for Gastric Cancer Study in Surgery and Pathology[13].

We discontinued the enrollment of patients on the 31st of December, 2010, whereas we conducted follow-up until the 31st of December, 2015 or until the patient's death. The median follow-up interval for 313 patients, who were alive at the cut-off date, was 113.4 months (range, 61-178 mo). Twenty-one patients (4.39%) had been lost to follow-up. There were 9 (1.99%) in-hospital deaths. Lost cases and operative mortality cases were censored for the analysis of survival.

For in-depth histological analysis, we selected a subgroup (Group 2bis) of 41 controls from Group 2 from one of the four centers participating to our study (the University of Brescia), matched with Group 1 according to age, sex, tumor size, tumor location, tumor invasion (T), histological grading (G), Lauren histotype, and the number of retrieved nodes. We selected cases from the database of the University of Brescia. The same pathologist (CB) retrieved and analyzed all the specimens of tumors from Group 1 and Group 2bis, and recorded lymphatic embolization, as well as microvascular and perineural infiltration, expressed as present/absent. Also, p53, Ki67, and HER2 were studied using immunohistochemical analysis, performed on formalin-fixed, paraffin-embedded sections of surgical specimens with commercially available primary antibodies Abcam anti-ErbB2 (CB11)ab8054, MIB-5 (DAKO) for ki67 and Clone BP-53-12 (Genemed Biotechnologies, United States, 1:150) for p53. For HER2 evaluation, the following classification was used: 0, 1+, 2+, 3+ for < 10%, > 10% partial staining, > 10% moderately complete staining, and > 10% intensely complete staining, respectively; 0 and 1+ were considered negative testing, 2+ and 3+ positive testing. The presence of p53 and ki67 was expressed by nuclear staining: the rate was calculated as the number of positive nuclear reactions over 100 cells, and for every case a final expression rate was estimated.

Statistical analysis

We classified variables as discrete (gender, tumor markers, location, T, histotype, grading, associated resections, post-operative morbidity and mortality, lymphatic embolization, vascular infiltration, perineural infiltration, HER2), or continuous (age, size, the number of retrieved nodes, p53, Ki67). We used χ^2 and the Student *t* test to evaluate the statistical significance of differences for discrete and continuous variables, respectively. We then computed survival rates using the Kaplan-Meier method. *P* values less than 0.05 were considered statistically significant. The statistical methods of this study were reviewed by Professor Giovanni Parrinello (Department of Biotechnologies, Section of Medical Statistics, University of Brescia, Italy).

RESULTS

Table 1 summarizes the clinical and pathological features of pN0 patients. They represent a typical Western series, with a significant rate of diffuse or mixed (34.0%) and proximal (35.8%) cancers. At the same time, N0 patients had most frequently T2 (60.2%) and G1-2 (72.2%) neoplasms, compared to the general gastric cancer population. The standard lymphadenectomy was D2, with a mean number of retrieved nodes equal to 26.2. However, this data should be interpreted taking into account the exclusion from the analysis of patients with less than 15 retrieved nodes. Less than 10% of the patients underwent associated splenectomy, whereas pancreas tail resection was done in only 7 cases. Major morbidity rate was 12.7% and included 9 cases of anastomotic and 3 cases of duodenal leak (3 of these complications required re-intervention), 8 subphrenic abscesses (all treated by percutaneous drainage), 4 pancreatic fistula, 2 splenic necroses requiring splenectomy, 3 postoperative hemorrhages, and 7 mechanical occlusions requiring re-intervention. Nine patients (1.88% of the sample) died before hospital discharge (8 because of surgical complications and 1 because of myocardial infarction).

Of the whole series of N0 patients, 41 developed cancer recurrence during the follow-up. Table 2

Table 1 Clinical, pathological, and surgical features of 478 N0 gastric cancer patients *n* (%)

	Values
Male	291 (60.9)
Female	187 (39.1)
mean age (range)	67.1 (31-90)
Tumor markers[1] (positive)	33/282
Tumor location	
Upper	67 (14.1)
Middle	104 (21.7)
Lower	261 (55.6)
Multiple	46 (9.6)
Tumor mean size (range), cm	4.311 (0.8-14)
Tumor invasion (T)	
T2	278 (58.2)
T3	181 (37.8)
T4a	19 (4.0)
Lauren histotype	
Intestinal	315 (65.9)
Diffuse	81 (16.9)
Mixed / Unavailable	82 (17.1)
Histological grading (G)	
G1	87 (18.2)
G2	287 (60.0)
G3	104 (21.8)
Type of surgery	
Subtotal gastrectomy	266 (55.7)
Total gastrectomy	212 (44.3)
Mean retrieved node number[2]	26.2
(range)	(15-85)
Associated resections	53 (11.1)
(splenectomies)	[41 (8.5)]
30-d mortality	9 (1.88)
Major morbidity	61 (12.7)

[1]CEA and/or CA19.9 above the normal values of 7 ng/mL and 36 UI/mL, respectively; [2]This value reflects the exclusion from our analysis of patients with less than 15 retrieved nodes.

shows the comparison between Group 1 (N0 patients with recurrence) and Group 2 (N0 patients with no recurrence). Significant prognostic factors for recurrence in N0 patients were T4a and diffuse histotype, while gender, age, tumor markers, location, size, grading, the number of retrieved nodes, associated resections, and major complications were not. Of the 41 patients with cancer recurrence, 11 developed their recurrence during the first year after surgery, 16 during the second year, 8 during the third, and 6 in the following years. The sites of recurrence were liver in 21 cases, peritoneum in 27 cases, loco-regional/lymphatic in 7 cases, and other sites in 5 cases. In only 2 cases, cancer recurrence was treated by surgery with potentially radical intent; other 23 patients underwent palliative chemotherapy.

Table 3 reports the comparison between Group 1 (41 N0 patients with recurrence) and Group 2bis (41 N0 patients without recurrence, matched to those of Group 1 according to demographic and pathological characteristics), for which we carried out in-depth histological analysis. Lymphatic embolization and perineural infiltration, which are histological parameters usually available with e-e staining, resulted highly significant in predicting recurrence; microvascular infiltration was not. Moreover, immunohistochemical study of p53, Ki67, and HER2 revealed that p53 and Ki67 were expressed in a significantly greater rate of cancer cells of patients who subsequently developed cancer recurrence, while this was not true for HER2.

At the time of the analysis, 313 (65.5%) patients were alive and free of disease, including 2 patients having a recurrence that was surgically treated with success (hepatic metastasis resection and total gastrectomy for gastric stump recurrence), 39 patients (8.15%) had died because of cancer recurrence, whereas 96 patients (20.0%) had died of other diseases. The remaining patients had died after surgery (9) or lost at follow-up (21). Due to the long period of follow-up after ending the recruitment of patients, no patient with recurrence is currently alive. Mean and median survival times were 93.4 and 81.1 months, respectively. The 3, 5, and 10-year overall survival and cancer-related survival rates of the whole group were 79.2%, 72.6%, 41.4% and 91.7%, 87.2%, 76.8%, respectively.

DISCUSSION

As mentioned earlier, the helpful feedback and suggestions of peer-reviewers, who have refereed an earlier article of ours[11] based on an Italian multicentric series of patients with pN0 gastric cancer, inspired our next project and current paper. Those peer-reviewers encouraged us to carry out an in-depth pathological analysis of the subgroup of patients experiencing recurrence despite having negative nodes on e-e staining; this group clearly represents a very interesting sample for a biological study. Meanwhile, the increased number of available pN0 cases also enabled us to perform a confirmation analysis of the significant clinico-pathological basic factors found in our earlier study.

In our current study we present a large, multicentric series of pN0 patients with gastric cancer, who have undergone radical surgery with reliable nodal harvesting (more than 15 analyzed nodes). Our main goal is to investigate which factors, easily available after standard pathological examination, are related to a higher likelihood of recurrence; this information is clearly very valuable to both the oncologists when deciding whether to use adjuvant chemotherapy, and the referring physicians when planning the targeted follow-up.

The secondary but potentially equally relevant purpose of our study is to discover if an in-depth pathological analysis, including three ancillary ematoxilin-eosin data (lymphatic embolization, microvascular infiltration, and perineural infiltration) and three immunohistochemical examinations (p53, Ki67, and HER2) may add valuable information for such clinical

Table 2 Prognostic factors for recurrence in N0 gastric cancer patients

	Group 1 (with recurrence) (n = 41)	Group 2 (no recurrence) (n = 437)	P value[3]
Gender (male)	28	263	0.308
Age (> 65)	19	201	0.966
Tumor markers[1]	6/25	27/207	0.138
Tumor location (upper)	7	60	0.555
Tumor size (> 5 cm)	16	132	0.242
Tumor invasion (T)			
T2	19	259	0.108
T3	13	168	0.395
T4a	9	10	0.001
Lauren histotype			
Intestinal	24	291	0.768
Diffuse	12	69	0.012
Mixed / Unavailable	5	77	0.509
Histological grading (G)			
G1	7	80	0.844
G2	22	265	0.382
G3	12	92	0.222
Retrieved nodes[2] (mean) > 25	26	273	0.905
Associated resections - yes	8	45	0.772
Major morbidity - yes	8	53	0.175

[1]Almost 1 tumor marker positive, including CEA, CA 19.9, or other tumor marker. Only 282 cases were available for analysis; [2]This value reflects the exclusion from our analysis of patients with less than 15 retrieved nodes; [3]P values of χ^2 test for gender, tumor markers, tumor location, tumor invasion (T), Lauren histotype, histological grading (G), associated resections, post-operative morbidity. P values of Student t-test for age, tumor size, and the number of retrieved nodes.

Table 3 Histological assessment of N0 patients with and without cancer recurrence, matched according to demographic and pathological characteristics

	Group 1 (41 N0 patients with recurrence)	Group 2bis (41 N0 patients without recurrence)	P value[3]
Lymphatic embolization	34	13	0.001
Microvascular infiltration	11	7	0.285
Perineural infiltration	19	6	0.001
p53[1]	49.1% (0-100)	33.8% (0-100)	0.041
Ki67[1]	52.7% (30-80)	33.1% (15-70)	0.038
HER2[2]	7	8	0.785

[1]Our immunohistochemical analysis of p53 and Ki67 differ from some of those found in other studies because we expressed them as the number of cancer cells showing immunohistochemical staining over 100 cells, thus as a rate and not as a binomial variable (yes/no). Statistical analysis was retrospectively performed by Student t-test; [2]Immunohistochemical detection, both 2+ and 3+ were considered positive; [3]P values of χ^2 test for lymphatic embolization, microvascular infiltration, perineural infiltration, and HER2. P values of Student t-test for p53 and Ki67.

decisions. Pathologists do not typically analyze those 6 ancillary data; however, these additional data, which are very easy and relatively inexpensive to analyze, may provide extremely helpful information in addition to the one from the basic pathological items.

We defined the study group taking into account the shortcomings of previous Western studies about node-negative gastric cancer patients, and, hence, excluding T1, T4b, and Nx patients. T1N0 patients have a very good outcome, thus making adjuvant chemotherapy unlikely to be valuable. In contrast, T4b patients usually undergo a kind of adjuvant therapy (chemo- or radiotherapy) irrespective of other pathological parameters. Patients with less than 15 retrieved nodes are not adequately staged, so we cannot define them N0 patients.

Factors associated with recurrence in N0 patients were only T4a and diffuse histotype. Nine out of 19 patients with a true, pathologically proven serosal and extragastric fat infiltration experienced recurrence (47.3%), mainly peritoneal (6/9 cases) and nodal (5/9 cases); this subgroup is thus suitable for adjuvant therapy. Therefore, T3 diffuse cases should also be considered for adjuvant therapy and intensive follow-up, considering that about a quarter of them will experience recurrence.

The most striking result of our study is that lymphatic embolization and perineural infiltration are significant parameters associated with recurrence in pN0 gastric cancer.[14] Out of 41 patients experiencing recurrence, 34 and 21 had lymphatic embolization and perineural infiltration, respectively. In Group 1 + Group2bis (82 cases), 15 patients had both parameters, and 10 of them subsequently developed

cancer recurrence (66.6%). In our earlier article including 301 patients from the same 4 Italian centers[11], data on these parameters were available for only 144 patients (confirming that basic pathological analysis does not typically provide information on these parameters); however, the 3 parameters were considered all together, with a rate of positive cases (26.3%) similar to the previously reported rate of about 20% of cases for microvascular infiltration and about 30% for perineural infiltration; they were not significantly related to the recurrence rate both at univariate and multivariate analysis.

According to the Japanese classification of gastric carcinoma[15], further research to classify lymphatic invasion and venous invasion into three more specific grades would help clarify the association between these anatomo-pathological characteristics of tumor and the rate of recurrence. Also, a further study in which also a subgroup among non-T4a and intestinal histotype are added, would provide a more in-depth analysis of prognostic factors of recurrence.

From an immunohistochemical point of view, the p53 protein, encoded by a tumor suppressor gene located on the short arm of chromosome 17, is involved in different cell functions, including apoptosis and cell cycle regulation[16]. This protein is usually poorly expressed in normal cells, but overexpressed and/or mutated in a number of human malignancies[17]; overexpression of p53 generally reflects an underlying mutation(s) in the *p53* gene[16]. A correlation among high protein levels, older age, advanced stage, and poor prognosis has been shown for colorectal, breast, and lung carcinoma[18]. Studies that have evaluated p53 expression and/or mutation in gastric cancer, have confirmed enhanced expression in cancer cells in comparison with normal cells (33.8% *vs* 4% in the series published by Zhou and Coll), but the benchmark value is different, ranging from 19%-29%[19] to 34%-65%[20,21]; however, correlation of p53 expression and worse prognosis was shown in some[16,22], but not in other series[23].

Antigen KI-67 (Ki67) is a nuclear proliferation associated antigen, involved in cycle regulation and cell proliferation. It is considered a reliable marker of tumor biology, having a clear prognostic value in several types of cancer, such as GIST[24]; in two recent retrospective analyses of patients with gastric cancer, Ki67 expression was associated with distant metastasis and survival[25], T stage and 3-year disease free survival[26].

Human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor, a 185 kDa glycoprotein encoded by a gene located in the long arm of chromosome 17. HER2 is involved in various cancerogenesis steps, including de-differentiation, angiogenesis, and metastasis[27]. As noticeably shown in the case of breast cancer, expression of HER2 is a marker of more aggressive behavior and may be related to a worse prognosis compared with HER2 negative tumors. In gastric cancer, an overexpression of HER2 has been reported in approximately 10%-30% of cases; however, a clear relationship with histopathological parameters or survival has not been established[28,29]. A comprehensive literature review published in 2011[30] confirmed the lack of association between HER2 overexpression and patient-related, as well as cancer-related, main parameters. Other small series, however, suggested an association with older age, larger tumors, and advanced stage[31,32]. Our results clearly confirm that HER2 overexpression, determined only by immunohistochemical analysis and not by FISH method, is not related to a greater risk of cancer recurrence in pN0 cases. The overall expression of HER2 in the whole series is similar to the one previously reported, with a marginal proportion of cases having a clear 3+ overexpression; none of the 41 patients with recurrence had 3+ HER2 expression, and the sum of 3+ and 2+ was also greater in group 2bis (*i.e.,* patients without recurrence).

In conclusion, despite a good overall prognosis, about 10% of pN0, R0 gastric cancer patients actually have cancer recurrence and die of this disease. The prognostic factors analyzed and reported in our study may help identify node-negative patients who would significantly benefit from existing or future adjuvant strategies. Moreover, for these patients an appropriate surveillance protocol in terms of follow-up should be proposed.

Further research with extra pathological examinations could be useful to define an algorithm for the management of pN0 gastric cancer patients.

COMMENTS

Background

Lymph node metastasis is the most important prognostic factor in patients undergoing radical surgery for gastric carcinoma. Even if node-negative patients have better outcomes, a subgroup of them develops recurrence. Hence, this is a key group to study for adjuvant treatment and follow-up.

Research frontiers

Clinicopathological characteristics of patients with both node-negative gastric carcinoma and diagnosis of recurrence during follow-up are key factors in the management of gastric cancer patients.

Innovations and breakthroughs

In-depth pathological analysis of two homogenous groups of pN0 patients, with and without recurrence during long-term follow-up, revealed two striking patterns: lymphatic embolization and perineural infiltration (two parameters that pathologists can easily report), and p53 and Ki67, represent significant factors for recurrence.

Applications

The reported pathological features should be considered predictive factors for recurrence and could be useful to stratify node-negative gastric cancer patients for adjuvant treatment and tailored follow-up.

Peer-review

It is very important in-depth pathological analysis added in this study.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Albumin as a prognostic marker for ulcerative colitis

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Author contributions: Khan N, Patel D, Shah Y and Yang YX have contributed to the study concept and design, analysis and interpretation of data as well as drafting of the manuscript; Khan N, Shah Y and Trivedi C have contributed to the acquisition of data; Yang YX has performed the statistical analysis; Trivedi C has contributed in the analysis and interpretation of data as well as drafting of the manuscript; Khan N and Yang YX have contributed to the critical revision of the manuscript for important intellectual content; Khan N has supervised the study.

Institutional review board statement: This study was reviewed and approved by Philadelphia VA Medical Center Institutional Review Board Committee.

Informed consent statement: This was a retrospective study and informed consent was not needed as no intervention was undertaken.

Conflict-of-interest statement: There were no conflicts of interest for this study.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at nabeel.khan@va.gov. Also, this was a retrospective cohort study and consent was not obtained but the presented data are anonymized and risk of identification is low.

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Abstract

AIM

To evaluate the role of albumin at the time of ulcerative colitis (UC) diagnosis in predicting the clinical course of disease.

METHODS

Nationwide cohort of patients with newly diagnosed UC in the Veterans Affairs health care system was identified and divided into two categories: hypoalbuminemia (*i.e.*, ⩽ 3.5 gm/dL) or normal albumin levels (*i.e.*, > 3.5 gm/dL) at the time of UC diagnosis. The exposure of interest was presence of hypoalbuminemia defined as

albumin level ⩽ 3.5 g/dL at the time of UC diagnosis. Patients were then followed over time to identify the use of ⩾ 2 courses of corticosteroids (CS), thiopurines, anti-TNF medications and requirement of colectomy for UC management.

RESULTS

The eligible study cohort included 802 patients, but 92 (11.4%) patients did not have their albumin levels checked at the time of UC diagnosis, and they were excluded. A total of 710 patients, who had albumin levels checked at time of UC diagnosis, were included in our study. Amongst them, 536 patients had a normal albumin level and 174 patients had hypoalbuminemia. Patients with hypoalbuminemia at diagnosis had a higher likelihood of ⩾ 2 courses of CS use (adjusted HR = 1.7, 95%CI: 1.3-2.3), higher likelihood of thiopurine or anti- TNF use (adjusted HR = 1.72, 95%CI: 1.23-2.40) than patients with normal albumin level at diagnosis. There was a trend of higher likelihood of colectomy in hypoalbuminemic patients, but it was not statistically significant (Adjusted HR = 1.7, 95%CI: 0.90-3.25).

CONCLUSION

Hypoalbuminemia at disease diagnosis can serve as a prognostic marker to predict the clinical course of UC at the time of diagnosis.

Key words: Prognostic marker; Albumin; Ulcerative colitis; Disease course; Colectomy

Core tip: The current literature states that pancolitis, anemia and steroid use at ulcerative colitis (UC) diagnosis are considered poor prognostic features in UC. Ours is a first community based nationwide multicenter study on this subject evaluating serum albumin at the time of UC diagnosis as a prognostic marker. We identified a new easily measurable prognostic marker for the clinical course of UC, which in conjunction with other prognostic markers would help us identify a subset of UC patients who will eventually develop severe disease. This subset of patients may benefit from closer follow up and early escalation of therapy.

Khan N, Patel D, Shah Y, Trivedi C, Yang YX. Albumin as a prognostic marker for ulcerative colitis. *World J Gastroenterol* 2017; 23(45): 8008-8016 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8008.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8008

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory condition of unknown etiology characterized by relapsing and remitting episodes of inflammation limited to the mucosal layer of the colon. The age and gender-adjusted prevalence is 214 per 100000 persons in the United States[1]. The clinical presentation and course of UC is variable and in severe cases it can lead to colectomy[2,3]. Unlike Crohn's disease (CD), in which several clinical and epidemiological factors at diagnosis are associated with a poor prognosis, there are limited prognostic indicators available at the time of UC diagnosis to predict the course of disease[4,5].

It would be beneficial for physicians to have predictive markers available at the time of UC diagnosis that can help them identify a subset of patients likely to develop a more severe disease course in the future. These patients may be a potential target for early effective management strategies that can induce steroid-free remission and reduce the need of colectomy. Albumin is an example of a negative acute phase reactant and decreased levels may be found during inflammatory conditions. A chronic inflammatory condition like UC can affect albumin concentration in the body in several ways. Chronic inflammation is associated with a greater fractional catabolic rate (FCR) of albumin and it also increases the transfer of albumin out of the vascular compartment[6]. Also, other conditions associated with UC such as malnutrition and malabsorption can cause low albumin level[7].

We hypothesize lower albumin level is associated with higher inflammatory activities in UC and thus, may be associated with poor clinical outcomes related to higher inflammatory activity, such as higher chances of ⩾ 2 courses of CS, ⩾ 1 courses of thiopurines/anti-TNF and colectomy. Previous studies have also shown that lower serum albumin level "at the time of UC exacerbation" predicted treatment failure and colectomy[8-11]. However, none of the studies to our knowledge have reported the association of albumin level in newly diagnosed UC patients and disease severity over time. Also, most of the previous studies that have looked at prognostic markers at time of UC diagnosis were done prior to the widespread use of immunomodulators and biologics and primarily looked at colectomy as an outcome, while we looked at escalation of therapy and colectomy both.

Our aim in this study was to evaluate albumin level at the time of UC diagnosis as a predictive marker for long-term outcomes in a population-based nationwide cohort of incident UC cases. To achieve this goal, we evaluated patients from the Department of Veterans Affairs (VA) which is the largest integrated health care system in the United States.

MATERIALS AND METHODS

Data source and study design

We conducted a retrospective cohort study in a previously validated nationwide inception cohort of UC patients[12,13] from the VA Database. The VA is the

largest integrated health care system in the United States serving approximately 8.3 million Veterans per year[14]. National VA-wide data were obtained from the VA Pharmacy Benefits Management and Corporate Data Warehouse databases. This data source allows automated data extractions to collect information regarding patients' demographics, pharmacy records, medical diagnoses, laboratory findings and procedures. Individual patient records were then evaluated by two reviewers to extract the data. Our validated VA Database represents 48 states plus Puerto Rico and 118 sites across the country.

Study population

We used a previously validated cohort of newly diagnosed UC patients to conduct this study[12,13]. Veterans who were seen and followed in the VA health care system from October 1, 2001, to October 1, 2011, were identified using International Classification of Diseases, Ninth Revision (ICD-9) codes for UC (556.xx). We have used a definition that has been validated in previous studies by Khan *et al*[12,13] to identify incident cases of UC in the VA system to form an inception cohort.

Inclusion criteria

(1) patients have ⩾ 4 UC ICD-9 codes in the VA database and ⩾ 3 5-ASA prescriptions; (2) patients have at least 20 encounters within the VA prior to the date of the first evidence of UC; (3) UC must be diagnosed after October 2001; (4) patients have their index scope in the VA system within 6 months of the first evidence UC; (5) did not have colectomy during the first attack of UC; (6) did not develop features of CD or inflammatory bowel disease unclassified (IBDU) over the course of the follow up; and (7) did not receive thiopurines or anti-TNF agents within 90 days of initial UC diagnosis. The records of the patients identified in this cohort were then individually reviewed by two investigators to collect the relevant data. (Figure 1)

The purpose behind requirement of > 20 encounters within VA system was to allow enough exposure to the health care provider after which any history of UC should have been recorded; furthermore, this helped ensure good adherence of the patients in seeking health care through the VA system and, subsequently, increased the validity of our outcome detection. The requirement of index colonoscopy within 6 mo of UC diagnosis helped to ensure that the reported UC diagnosis was based on endoscopic findings.

Exposure and follow-up

The exposure of interest was presence of hypoalbuminemia defined as albumin level ⩽ 3.5 g/dL at the time of UC diagnosis. All study patients were dichotomously categorized as having hypoalbuminemia (*i.e.*, ⩽ 3.5 g/dL) or normal albumin levels (*i.e.*, > 3.5 g/dL) based on their laboratory tests results for albumin level at the time of UC diagnosis. For all patients, the follow-up began on the day of UC diagnosis and ended at the first of outcome occurrence, colectomy for UC-related reason or for colorectal cancer, death, date of loss to follow-up (*i.e.*, last documented contact in VA electronic medical records), or the last date of follow-up (*i.e.*, October 2015).

Outcome

The outcomes were (1) the use of 2 or more courses of oral corticosteroids, separated by a three month time interval between the courses, during the follow-up subsequent to the UC diagnosis; (2) use of thiopurines or anti-TNFs during the follow up course; and (3) colectomy for UC-related reasons during follow-up. Information regarding relevant medication prescriptions, occurrence of colectomy and the indication for colectomy was collected based on manual chart review. Medication data elements that were collected included: dispense dates and stop dates, refill dates. Medications dispensed for reasons other than UC and non-UC related colectomies were not included in the outcome.

Covariates

Covariate data collected by manual chart review included patients' demographic at the time of UC diagnosis, age, gender, race (*i.e.*, Caucasian, African American, Hispanics, others, unknown), and extent of UC at index endoscopy based on Montreal classification (E0 = no involvement, E1 = proctitis, E2 = left sided disease till splenic flexure, E3 = right sided disease or pancolitis)[15].

Statistical analysis

We report crude incidence rates (IRs) for colectomy for both exposure groups. The Kaplan-Meier analysis was conducted to generate the survival curves for each group. Multivariable Cox proportional hazards models including all covariates were used to estimate the adjusted hazard ratios (HR) along with 95%CIs for the risk of the respective outcome associated with baseline hypoalbuminemia. The PH-assumption was tested using Schoenfeld's residuals for the treatment variable[16] and graphically. All analyses were conducted in STATA, version 14 (STATA Corp., College Station, TX, United States).

Biostatistics statement

The statistical methods of this study were reviewed by Dr. Yu-Xiao Yang who is affiliated with The University of Pennsylvania, Perelman School of Medicine as well as Corporal Michael J. Crescenz VA Medical Center in Philadelphia as an associate professor for medicine and epidemiology and also a senior scholar in the

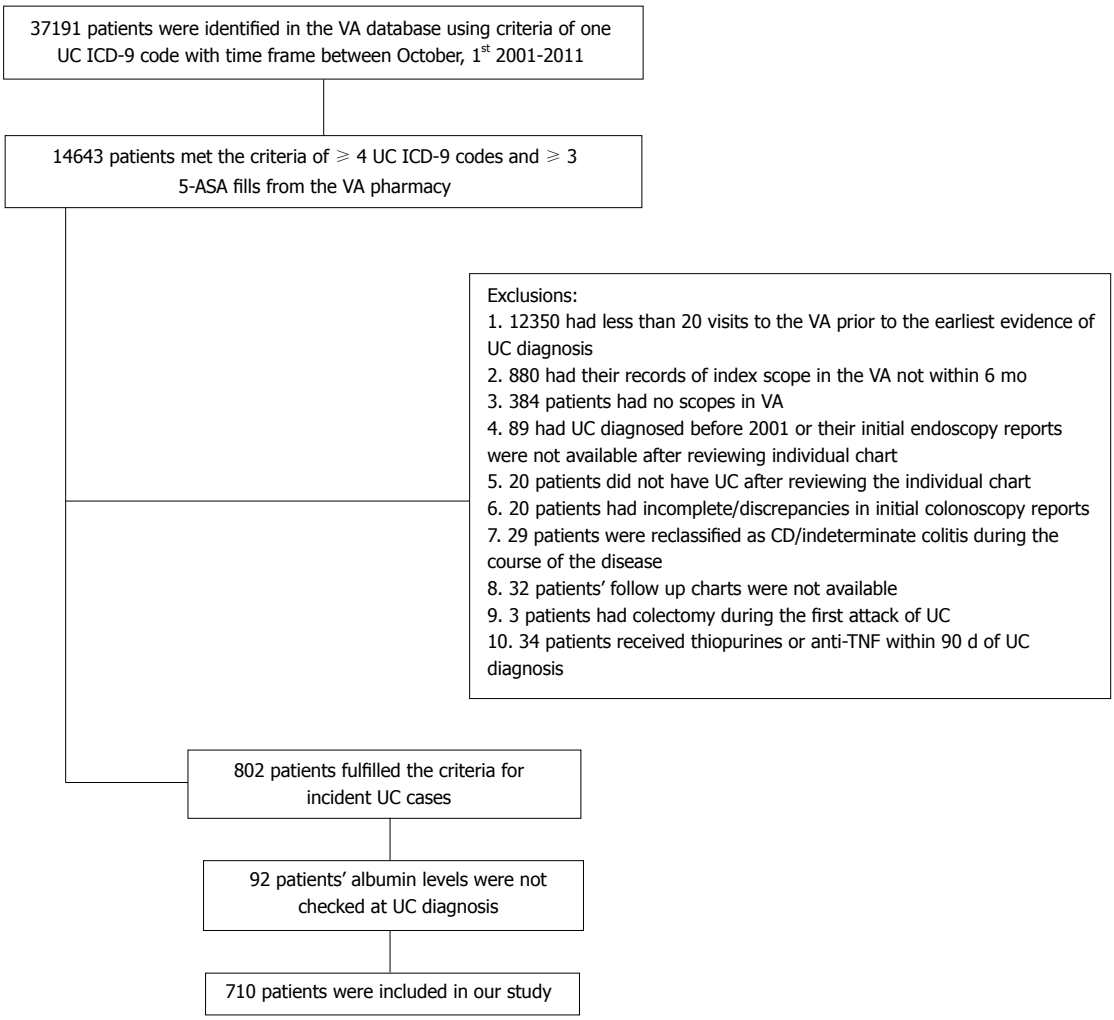

Figure 1 Flow chart for assembly of the study cohort. CD: Crohn's disease; UC: Ulcerative colitis.

department of epidemiology and biostatistics.

RESULTS

The eligible study cohort included 802 patients. Among them, 209 patients had received ⩾ 2 courses of corticosteroid therapy and 178 patients had received at least one course of thiopurine or anti-TNF therapy during follow up duration. In addition, 48 underwent colectomy for UC-related reasons during the follow-up. Baseline albumin level was not checked in 92 (11.4%) patients, leaving 710 patients available for analysis (Table 1). Of 710 patients, 174 patients (24.5%) had hypoalbuminemia (albumin level ⩽ 3.5 gm/dL) and 536 patients (75.5%) had normal albumin level (> 3.5 gm/dL) at new diagnosis of UC.

⩾ *1 course of thiopurine or anti-TNF therapy*

The incidence rate of receiving ⩾ 1 course of thiopurine or anti-TNF therapy was 3 per 100 person-year and 6 per 100 person-year among those without and with hypoalbuminemia at UC diagnosis, respectively. In the multiple variable Cox regression analysis, presence of hypoalbuminemia at UC diagnosis was associated with a significantly higher risk of receiving ⩾ 1 course of thiopurine or anti-TNF therapy during follow-up (adjusted HR = 1.72, 95%CI: 1.23-2.40) (Figure 2A).

⩾ *2 courses of corticosteroid therapy*

The incidence rate of receiving ⩾ 2 courses of corticosteroid therapy was 3 per 100 person-year and 7 per 100 person-year among those without and with hypoalbuminemia at UC diagnosis, respectively. In the multiple variable Cox regression analysis, presence of hypoalbuminemia at UC diagnosis was associated with a significantly higher risk of receiving ⩾ 2 courses of corticosteroid therapy during follow-up (adjusted HR = 1.7, 95%CI: 1.3-2.3) (Figure 2B).

The median time between the use of first and second course of CS in patients with hypoalbuminemia at UC diagnosis was 11 mo compared to 14 mo in patients with normal albumin level at UC diagnosis (P = 0.06).

UC-related colectomy

The incidence rate of UC-related colectomy was 0.7

Table 1 Baseline characteristics of the study cohort by absence or presence of hypoalbuminemia status at ulcerative colitis diagnosis *n* (%)

	Normal albumin at UC diagnosis (*n* = 536)	Hypoalbuminemia at UC diagnosis (*n* = 174)	*P* value
Age at anemia diagnosis (mean, SD, yr)	55 (13)	59 (12)	0.001
Sex			0.65
Male	500 (93.3)	164 (94.3)	
Female	36 (6.7)	10 (5.7)	
Race			0.433
Caucasian	397 (74.1)	130 (74.7)	
AA	82 (15.3)	24 (13.8)	
Hispanic	7 (1.3)	2 (1.2)	
Others	8 (1.5)	0 (0)	
Unknown	42 (7.8)	18 (10.3)	
Disease extent at baseline			< 0.001
E1	118 (22.0)	14 (8.1)	
E2	263 (49.1)	84 (48.3)	
E3	155 (28.9)	76 (43.7)	

UC: Ulcerative colitis; SD: Standard deviation; AA: African American.

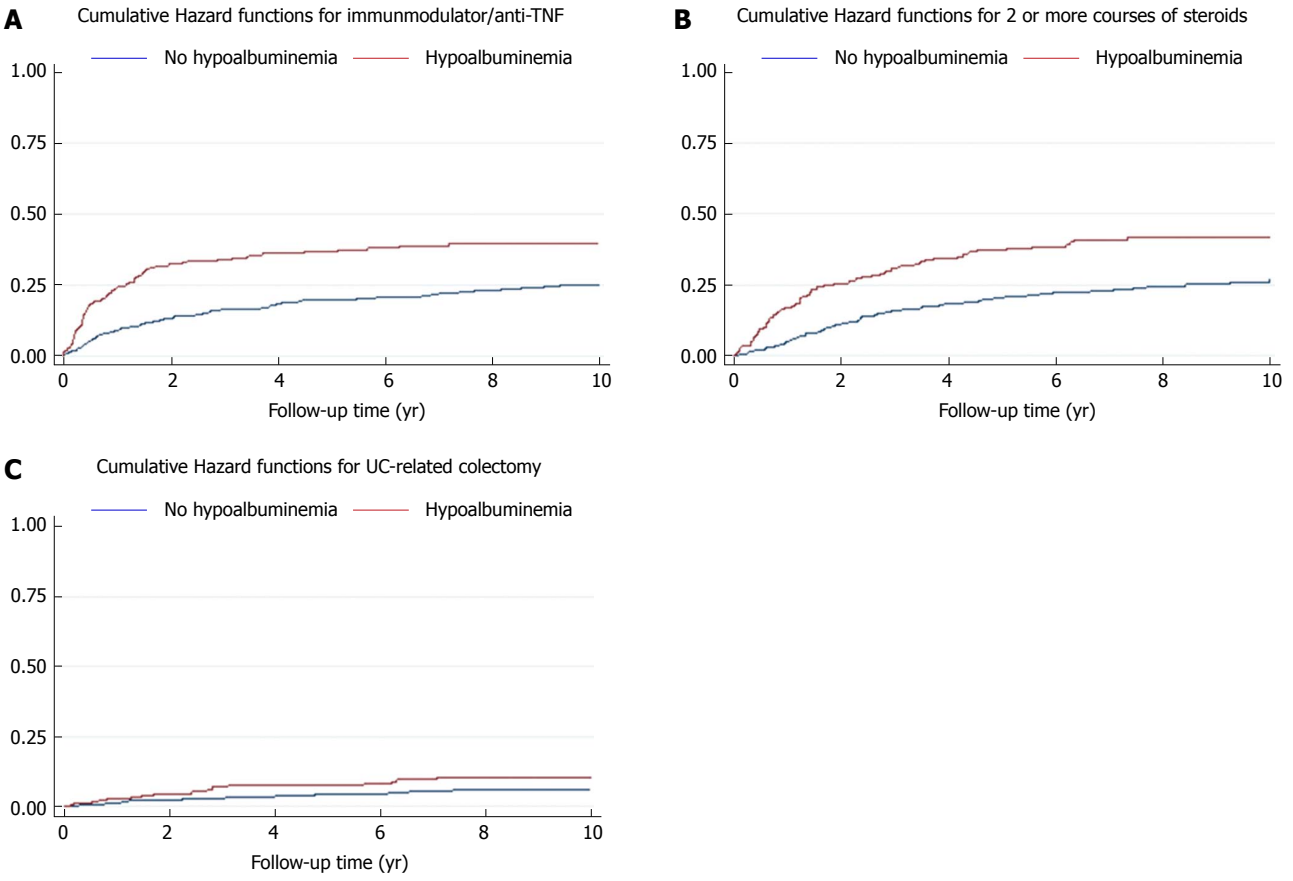

Figure 2 Cumulative hazard functions for immunomodulator/anti-TNF (A), 2 or more courses of steroids (B), and ulcerative colitis-related colectomy (C).

per 100 person-year and 1.2 per 100 person-year among those without and with hypoalbuminemia at UC diagnosis, respectively. In the multiple variable Cox regression analysis, presence of hypoalbuminemia at UC diagnosis was not statistically associated with a higher risk of UC-related colectomy during follow-up, but showed a higher trend of colectomy in the future (Adjusted HR = 1.7, 95%CI: 0.90-3.25) (Figure 2C).

Use of 5-ASA and their relation with low albumin level

Of 174 patients who had low albumin level (≤ 3.5 gm/dL) at UC diagnosis, 72 patients (41.4%) used oral formulation of 5-ASA only, 4 patients (2.3%) used rectal formulation of 5-ASA only, and 98 patients (56.3%) used both oral and rectal formulation of 5-ASA during the follow up course.

DISCUSSION

Utilizing a large nationwide population based cohort of newly diagnosed UC patients, we found that low albumin level (≤ 3.5 gm/dL) in newly diagnosed UC

patients is a poor prognostic indicator. It is associated with the higher likelihood of requiring ⩾ 2 courses of CS and ⩾ 1 course of thiopurines or anti-TNF medications over the course of disease. Also, our result showed a trend of higher chances of colectomy in the future in patients with lower albumin level at disease diagnosis compared to patients with normal albumin level.

Different clinical and epidemiological factors in newly diagnosed UC patients have been studied as prognostic indicators in UC at diagnosis (younger age[17,18], extensive colitis at diagnosis[17], non-smoker[19,20], higher ESR[17], and early use of CS[12]). Our goal was not to replace these established factors but to find an additional factor which is routinely available, easily measurable and a part of initial laboratory work up in newly diagnosed UC patients. Our study is the first study, to our knowledge, to evaluate the prognostic role of albumin level in newly diagnosed UC patients in predicting disease outcome. Additionally, in contrast to most previous studies we have not just looked at colectomy as an endpoint but have also evaluated the use of immunomodulators and biologics which are being increasingly used. In conjunction with other above mentioned factors albumin levels at the time of UC diagnosis could be used to develop a comprehensive prediction model to prognosticate the disease course.

Goals of UC management are induction and maintenance of steroid free remission as well as preventing the development of colorectal cancer or colectomy in the future[21]. Multiple population-based studies focusing on natural history of UC showed that rates of CS use range from 34% to 63% over a variable period of follow up ranging from 1 year to 7 years[12,22-26]. Among patients who require one course of CS therapy, almost half never required further CS therapy and remained in prolonged remission[22,23]. Thus, almost ¾th of UC patients have less aggressive phenotype of disease. It is the remaining group of patients which requires ⩾ 2 courses of CS therapy that have a poor prognosis. It is for this reason that we used ⩾ 2 courses of CS as one of our outcomes to predict poor prognosis. These patients would frequently require thiopurines as well as biologics and may even require colectomy to control their disease[12,22-24]. It is thus appropriate for physicians to recognize a subset of patients at diagnosis who will have a high propensity for aggressive disease in the near future.

Albumin has never been evaluated as a prognostic marker for long term disease outcomes, but has been evaluated as a marker for response to therapy. Hypoalbuminemia in acute severe UC predicts treatment failure and colectomy[8-11] within the same hospitalization. Furthermore, low serum albumin level during disease course is associated with thiopurine failure[27] and non-responding to anti-TNF therapy in UC patients[28]. Our study is the first study to evaluate the role of low albumin to severe long term disease outcome. Our study showed that hypoalbuminemia defined as ⩽ 3.5 gm/dL, at the time of disease diagnosis is a poor prognostic indicator. Multivariate analysis showed patients with hypoalbuminemia at diagnosis are associated with almost two-fold increase in ⩾ 2 courses of CS use and almost two-fold increase in thiopurine or anti- TNF use than patients with normal albumin level at diagnosis. Furthermore, low albumin level at diagnosis shows a trend of higher chances of colectomy in the future disease course.

Identifying prognostic factors at the time of diagnosis can assist in disease management. Patients with low albumin levels at UC diagnosis could potentially benefit from higher dose of 5-ASA at disease onset. However, no trials have specifically looked at 5-ASA dosing based upon risk- stratification at the time of UC diagnosis to induce remission and prevent relapses in the future. This is an area of further research. However, in the absence of such data, a meta-analysis from Ford *et al*[29] on the use of 5-ASA in UC patients showed that higher dose of 5-ASA in quiescent UC appears to reduce the risk of disease relapse than lower dose of 5-ASA. [Relative risk (RR) of relapse = 0.79, 95%CI: 0.64-0.97, P = 0.02].

Additionally, it has been shown that combination therapy with oral and rectal 5-ASA products is more effective than oral alone. A study by d'albasio *et al*[30] on efficacy of a combination of oral and rectal 5-ASA for the maintenance treatment of ulcerative colitis showed that patients in combined group had lower relapse rate than patients in oral treatment group alone. (39% *vs* 69%, P = 0.036). Other studies[31,32] showed that combined therapy with oral and rectal formulation of 5-ASA is superior to oral formulation alone for induction of remission and improving endoscopic and histologic severity in extensive mild-to-moderate active UC. In our study, only 56% patients with low albumin level were treated with a combination of oral and rectal 5-ASA therapy. We suggest that the finding of low albumin level should prompt all physicians to prescribe combination therapy with an aim to preventing recurrent steroid use and immunosuppressive agents.

Adherence with medications has been identified as the key factor in maintaining disease remission in UC. A study by Kane *et al*[33] showed that non-adherence to a prescribed regimen of 5-ASA dramatically increased the risk of symptomatic relapse (61% *vs* 11%, P = 0.001). 30%-45% of UC patients showed non-adherence to oral medication. A study by Hawthorne *et al*[34] showed that a combination of educational and behavioral strategies tailored to individual patient optimized 5-ASA adherence in UC patients. We suggest that patients with a low albumin level at disease diagnosis will help guide patient-physician discussion of the disease course, which in turn will lead to improve medication compliance and decrease flare rates in the future. Also, patients with a low albumin level at disease diagnosis

may benefit from more frequent monitoring through clinical follow up and evaluation of different biomarkers.

There are certain limitations in our study. In utilizing the VA data base, we had to conduct a retrospective study which has its own inherent limitations. Certain demographic characteristics such as smoking status which may impact disease course are not uniformly recorded. The VA population is predominately composed of males, which could potentially limit the generalizability of the findings to females. However, none of the previous studies has shown that gender plays a role in the prognosis of UC. Because we used the VA pharmacy database to estimate corticosteroid/thiopurine/biologics exposure rates, prescriptions filled outside the VA were not captured by our analysis. We anticipate the magnitude of such misclassification to be negligible as previous reports have shown that veterans have very good adherence in using the VA pharmacy[35-37]. Furthermore, all veterans included in this study had to have more than 20 visits prior to UC to ensure and maximizing the chance that they receive all their medical care within the VA system. Our cohort is comprised of only 802 patients with newly diagnosed UC in nationwide VA database (2.2% of total populations and 5.7% of the population meeting first inclusion criteria). There are various reasons behind this. Approximately half the veterans in VA systems are from Vietnam and Korean War. Thus, they most likely developed their UC prior to 2001. Also, veterans from the first Gulf war who form a large percentage of the remaining block also probably developed UC prior to 2001. A large proportion of veterans are diagnosed with UC while they are still in service and once they retire and join the VA, they already have a UC diagnosis. There is also a group of patients who do not get their clinical care at the VA but only use it to obtain medicines. We required all our patients to have an endoscopy in the VA. We also required all our patients to have > 20 visits in VA prior to UC diagnoses plus $\geqslant$ 4 UC ICD-9 codes and $\geqslant$ 3 5-ASA prescription, in order to increase the validity of our cohort and to ensure good adherence of the patients in seeking health care through the VA system.

The strength of this study was based on population-representative nation-wide VA data rather than data from tertiary centers, thus minimizing the likelihood of referral bias. We had patients from 118 sites spanning 48 states including Puerto Rico. We had complete records on all the patients including endoscopic and pathological records. To our knowledge this is the largest cohort of UC patients and the first multicenter nationwide study conducted in the US. Patients were followed for the median observation period of 8 years to identify CS use, thiopurine use, anti-TNF use and colectomy after their UC diagnosis.

In conclusion, we observed that low albumin at disease diagnosis was associated with a more severe subsequent disease course of UC. Assessing albumin levels at UC diagnosis could help physicians identify patients who may require escalation of therapy and closer follow up.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) has a variable clinical course ranging from disease remission to a severe exacerbation leading to colectomy. It is very difficult to predict the disease course at the time of UC diagnosis. There are several clinical and epidemiological factors available for Crohn's disease to predict the disease course, but there are limited prognostic markers available at the time of UC diagnosis. We believe that identifying these predictive markers will help physicians identify a subset of patients likely to develop a more severe disease course in the future and subsequently manage them more effectively at disease onset. Inflammatory activity of UC can lower albumin level by various mechanisms, such as malnutrition, malabsorption, greater fractional catabolic rate of albumin and increase transfer of albumin out of the vascular system. In view of this, we hypothesized that hypoalbuminemia at the time of UC diagnosis can be predictive of a more severe disease course of UC. Previous studies have shown that lower serum albumin level "at the time of UC exacerbation" predicted treatment failure and colectomy but none of these studies have looked at the level of albumin at the time of UC diagnosis and its relationship with the clinical course of the disease.

Research motivation

It is very hard to predict the disease course at the time of UC diagnosis. There are limited numbers of prognostic factors available to determine the course of UC at the time of diagnosis. None of the studies have considered the role of albumin in predicting UC course which is easy to measure on routine blood work by general practitioner as well as gastroenterologist. We thought that by identifying this new prognostic factor that can predict the course of UC at the time of UC diagnosis, it would be helpful for the physicians to identify a subset of patients at high risk of developing severe course and manage them more effectively early on in the disease course. In future studies, the impact of more aggressive therapy at the time of UC diagnosis among patients with poor prognostic factors could be evaluated.

Research objective

Our main objective was to identify a new prognostic marker which is cheap, reliable and readily available at the time of UC diagnosis that predicts the long-term clinical course of the disease. We hypothesized that hypoalbuminemia at the time of UC diagnosis is associated with a more severe disease course of UC. We were able to show that that low albumin at disease diagnosis was associated with a more severe subsequent disease course of UC. This in conjunction with other prognostic markers would help us identify a subset of UC patients who will eventually develop severe disease. This subset may benefit from closer follow up and early escalation therapy. The impact of early escalation of therapy can be evaluated in future studies.

Research method

We conducted a nationwide retrospective cohort study of newly diagnosed UC patients followed in the VA healthcare system. These patients were divided into 2 groups based on their albumin levels at the time of UC diagnosis, *i.e.,* hypoalbuminemic ($\leqslant$ 3.5 gm/dL) or normal albumin levels (> 3.5 gm/dL). Our outcomes of interest were 1) the use of 2 or more courses of oral corticosteroid, separated by a three month time interval between the courses; (2) use of thiopurines or anti-TNFs; and (3) colectomy for UC-related reasons during the clinical course of their UC. We also included other covariate data like patient demographics as well as the extent of UC at index colonoscopy based on the Montreal classification. We then subjected this data including all the covariates to statistical analysis and evaluation using the multivariate cox proportional hazard models to estimate the adjusted Hazard ratio (HR) along with 95%CI. The Kaplan Meier analysis was also conducted. The pH assumption was tested using Schoenfeld's residuals. All this analysis was done using the STATA software, version 14 (STATA Corp., College Station, TX, United States).

Research results

Our cohort included 710 newly diagnosed UC patients who had their albumin levels checked at the time of diagnosis and amongst them, 174 patients, *i.e.,* 24.5% had hypoalbuminemia. It was observed that there was a higher predilection to ⩾ 2 courses of CS use as well as thiopurine or anti-TNF use during the clinical course of disease among hypoalbuminemic patients. The chances of requiring subsequent colectomy were also higher in this group, although it was not statistically significant. Thus, we identified a new factor that is readily available and measurable at the time of UC diagnosis which can prognosticate the disease course. The problem that needs to be resolved is the impact of more aggressive therapy at the time of UC diagnosis among patients with poor prognostic factors on the clinical course of the disease.

Research conclusions

Low albumin at disease diagnosis was associated with a more severe subsequent disease course of UC. People with poor prognostic factors may benefit from aggressive therapy at onset of disease. We outlined the clinical and epidemiological factors in newly diagnosed UC patients which have been studied as prognostic indicators in UC at the time of diagnosis. This study identified a new prognostic factor which is readily available to predict the disease course of UC at the time of diagnosis. We hypothesized and confirmed that albumin level at the time of disease diagnosis can predict disease course of UC. Assessing albumin levels at UC diagnosis could help physicians identify patients who may require escalation of therapy and closer follow up.

Research perspectives

By using a large nationwide cohort of patients, we were able to identify a new prognostic factor to determine the clinical course of UC. This was made possible by having complete medical records and long duration of follow up. Using other large nationwide cohorts future studies may be able to identify other predictive factors. The future research should address the impact of more aggressive therapy at the time of UC diagnosis among patients with poor prognostic factors on the clinical course of the disease. The methodology that was employed by us can be utilized in future research.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Pretransplantation fetal-maternal microchimerism in pediatric liver transplantation from mother

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Abstract

AIM

To investigate the rates of pretransplantation fetal-maternal microchimerism (MC) and its effect on rejection in children receiving maternal liver grafts.

METHODS

DNA or blood samples before liver transplantation (LT) were available in 45 pediatric patients and their mothers. The presence of pretransplantation MC to non-inherited maternal antigens (NIMAs) (NIMA-MC) in the peripheral blood was tested using nested PCR-single-strand conformation polymorphism analysis for the human leukocyte antigen (HLA)-DRB1 alleles. NIMA-MC was successfully evaluated in 26 of the 45 children. Among these 45 pediatric LT recipients, 23 children (51.1%) received transplants from maternal donors and the other 22 from non-maternal donors.

RESULTS

Among these 26 children, pretransplantation NIMA-MC was detected in 23.1% (n = 6), 6.1 (range, 0.8-14) years after birth. Among the children with a maternal donor, the rate of biopsy-proven cellular rejection (BPCR) was 0% in patients with NIMA-MC positivity (0/3) and those with HLA-DR identity with the mother (0/4), but it was 50% in those with NIMA-MC negativity (5/10). Patients with NIMA-MC positivity or HLA-DR identity with the mother showed significantly lower BPCR rate compared with NIMA-MC-negative patients (0% *vs* 50%, P = 0.04). NIMA-MC-positive patients tended to show lower BPCR rate compared with NIMA-MC-negative patients (P = 0.23).

CONCLUSION

The presence of pretransplantation NIMA-MC or HLA-DR identity with the mother could be associated with BPCR-free survival in pediatric recipients of LT from maternal donors.

Key words: Liver transplantation; Microchimerism; Maternal graft; Graft survival; Non-inherited maternal antigen; Biopsy-proven cellular rejection

Core tip: We firstly investigated the rates of pretransplantation microchimerism (MC) to non-inherited maternal antigens (NIMAs) (NIMA-MC) in pediatric liver transplantation (LT) patients and its effect on biopsy-proven cellular rejection (BPCR) in those receiving maternal grafts. NIMA-MC for HLA-DRB1 alleles was successfully evaluated in 26 of the 45 children, and pretransplantation NIMA-MC was detected in 23.1% of the patients. Our study demonstrated that none of the patients with NIMA-MC positivity or HLA-DR identity with the mother developed BPCR. The presence of pretransplantation NIMA-MC or HLA-DR identity with the mother could be associated with BPCR-free survival in pediatric LT recipients from maternal donors.

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INTRODUCTION

Liver transplantation (LT) represents the gold standard of treatment for liver failure. Over 70% of pediatric recipients of LT exhibit 10-year patient and graft survival, which is higher than that observed in adults[1,2]. However, there are several challenges specifically associated with pediatric LTs: the implantation of relatively small grafts in children undergoing growth and development, the possibility of rare disease entities and accompanied congenital anomalies, and the requirement for life-long immunosuppression. In particular, a critical consideration in pediatric LT is the management of risks associated with life-long immunosuppression. Tolerance induction is utilized in order to enable discontinuation of immunosuppressant use, or the use of low-dose immunosuppressant in pediatric LT recipients.

Donor-specific microchimerism (MC), which develops following solid organ transplantations, especially LT, is considered to underlie tolerance induction and graft acceptance[3-5]. MC, which is defined as the presence of less than 1% of foreign cells in peripheral blood or tissues, may develop following transfusion, solid organ transplantation, and pregnancy[6]. Bidirectional cellular trafficking between mother and fetus during pregnancy results in postpartum fetal-maternal MC, which may persist in mothers and children for decades, and potentially over the course of a lifetime[7]. Exposure of the fetus to maternal cells results in MC to non-inherited maternal antigens (NIMAs) (NIMA-MC). Conversely, exposure of the mother to fetal cells results in MC to inherited paternal antigens (IPAs) (IPA-MC). The presence of maternal cells in the fetus generates fetal regulatory T cells that suppress the fetal response to NIMA and the presence of fetal cells in the mother suppresses the maternal response to IPAs; however, the underlying immune mechanism in the latter is far more complex.

Recent clinical studies investigating human leukocyte antigen (HLA)-haploidentical hematopoietic stem cell

transplantation have suggested the association of NIMA-MC with acquired immunologic hyporesponsiveness to NIMAs; this has been termed the "NIMA effect"[8-10]. The NIMA effect on allograft survival in renal transplant recipients is controversial[11-14]. Reports of this effect in LT are scarce; the NIMA effect has been observed only in pediatric patients with biliary atresia (BA), with lower rates of graft failure reported in children receiving maternal grafts compared with those receiving paternal or deceased donor grafts[15]. Most studies on the NIMA effect in organ transplant recipients have focused on the donor-recipient relationship in the family or the presence or absence of NIMAs in the donor. Investigation of donor-specific MC in pretransplantation blood samples has rarely been performed[14].

In the present study, we investigated the rate of NIMA-MC in pretransplantation blood samples from pediatric recipients of LT, and examined the effect of NIMA-MC on rejection-free graft survival in patients with a maternal donor.

MATERIALS AND METHODS

Population

A total of 73 children below the age of 18 years, who underwent pediatric LT at the Seoul National University Hospital between 1999 and 2012, were retrospectively reviewed. None underwent ABO-incompatible LT. Informed consent was obtained from guardians of the 65 pediatric patients before commencing the investigation, and the study protocol was reviewed and approved by the Institutional Review Board of the Seoul National University Hospital (H-1210-134-438 and H-1208-030-121). Pretransplantation DNA was preserved according to protocol. A total of 45 patients for whom pretransplantation DNA or blood samples were available for investigation were included in this study (Figure 1). Among these 45 pediatric LT recipients, 23 (51.1%) received transplants from maternal donors and the other 22 from non-maternal donors; the latter from either deceased donors (n = 10) or other family members (n = 12).

Only one of the 45 recipients (2.2%) showed preoperative positive results for complement-dependent cytotoxicity crossmatch of T cells; a titer of 1: 8 was obtained *via* the antihuman globulin-augmented method. As the titer for the crossmatch test was not sufficiently high for preoperative desensitization[16], desensitization was not performed in this patient. Induction therapy was not used in this study population. A routine dual immunosuppressant protocol in pediatric LT comprising tacrolimus and steroid was employed; steroid was tapered off over 6 mo, according to the protocol. The median follow-up period for the 45 patients after LT was 73 (range, 30-176) mo. At the time of examination, immunosuppressive monotherapy (tacrolimus) was administered to 44 patients (97.8%), except one, who was treated with tacrolimus and low-dose steroid, owing to side effects of tacrolimus. The median level of tacrolimus was 2.0 (2.0-5.0) ng/mL.

HLA-DRB1 MC analysis

Out of the 73 pediatric patients who underwent LT between 1999 and 2012, HLA-DRB1 MC analysis was attempted in 45 patients and their mothers, for whom DNA or blood samples were available for the study. Bidirectional HLA-DRB1 MC between the child and mother was analyzed, *i.e.,* NIMA-MC in the child and IPA-MC in the mother (Figure 1). Genomic DNA was extracted from peripheral blood samples using the LaboPass Blood kit (Cosmo Genetech, Seoul, South Korea).

HLA-DRB1 typing: Pretransplantation HLA-DRB1 typing data were available for all 45 patients and 27 maternal donors, and blood sampling and HLA-DRB1 typing were performed later, at the time of the study, in 18 maternal donors. Low-to-intermediate resolution HLA-DRB1 typing was performed *via* the PCR-sequence specific oligonucleotide (SSO) typing method, using either the Dynal RELI™ SSO HLA typing kit (Dynal Biotech Ltd., Wirral, United Kingdom) or the Luminex-based, WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan). For HLA-DRB1 MC analysis, high-resolution HLA-DRB1 allelic data are required for mismatched HLA-DRB1 alleles between the mother and child, *i.e.,* NIMA for the child and IPA for the mother. Allele-level HLA-DRB1 typing was performed *via* the PCR-single-strand conformation polymorphism (SSCP) method, as previously described[17].

HLA-DRB1 MC analysis*:* HLA-DRB1 MC was analyzed *via* the nested PCR-SSCP method, as described previously[14,18]. For nested PCR amplification of the *HLA-DRB1* gene, we used primers to amplify exon 2 of the *HLA-DRB1/B3/B4/B5* genes[19] for the first-round PCR, and *HLA-DR* group-specific primers (for eight different groups) to amplify exon 2 of *HLA-DRB1*[17] for the second-round PCR. The first-round PCR products were diluted to 1:200 in water and used as templates for the second-round PCR. The nested PCR product was analyzed *via* SSCP analysis, and single-strand DNA fragments separated in the gel were visualized with silver staining. For the determination of the presence or absence of NIMA-MC, the band pattern of DNA from pediatric LT recipients was compared with those of maternal DNA and reference DNA with known DRB1 allele types (obtained from the University of California at Los Angeles DNA Exchange Program); these were simultaneously analyzed by gel electrophoresis (Figure 2).

Evaluation of NIMA-MC was possible in only 26 of the 45 children. NIMA-MC could not be tested in the following 19 cases: DR identical (n = 8), DR 0

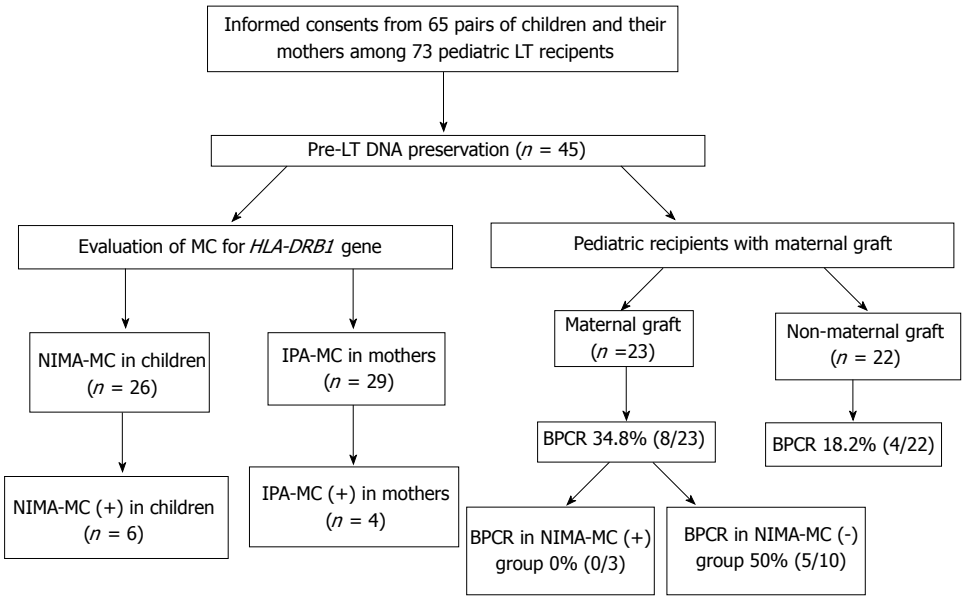

Figure 1 Evaluation of pretransplantation fetal-maternal microchimerism in pediatric recipients of liver transplants and their mothers and the rates of biopsy-proven cellular rejection in various recipient groups. LT: Liver transplantation; MC: Microchimerism; NIMA: Non-inherited maternal antigen; IPA: Inherited paternal antigen; BPCR: Biopsy-proven cellular rejection.

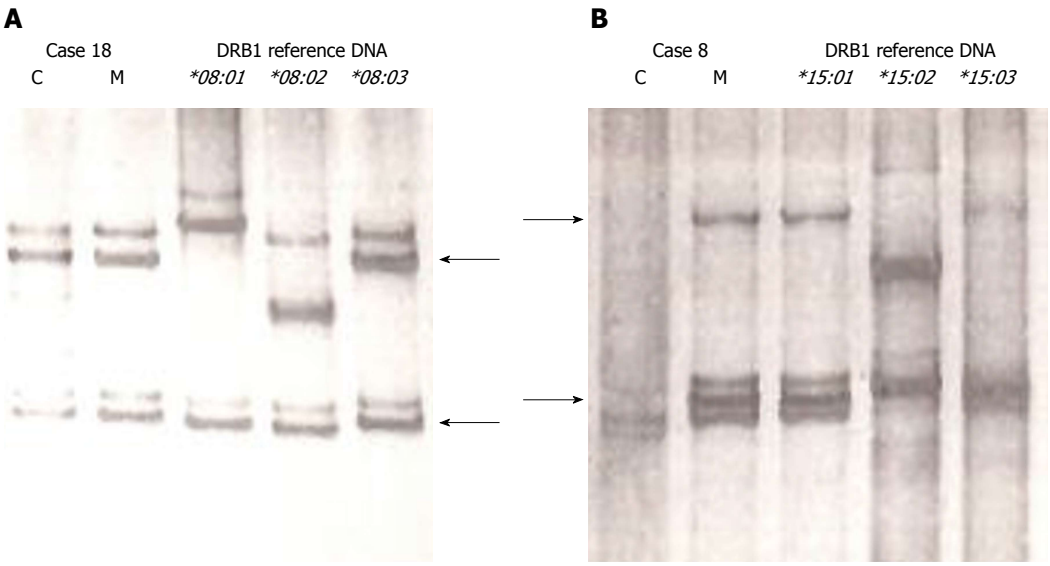

Figure 2 Detection of pretransplantation HLA-DRB1 microchimerism to non-inherited maternal antigen in pediatric recipients of liver transplants, using nested PCR-SSCP method. A: MC to HLA-DRB1 NIMA (*DRB1*08:03*) is positive in the child; B: MC to HLA-DRB1 NIMA (*DRB1*15:01*) is negative in the child. SSCP analyses were performed using nested PCR products in the recipients (children), non-nested PCR products in their mothers, and reference DNA. The SSCP band pattern of DNA of the recipients is compared with that of their mothers (arrows) and corresponding reference DNA (arrows), run in the same gel. C: Child; HLA: Human leukocyte antigen; M: Mother; SSCP: Single-strand conformation polymorphism.

mismatch (n = 8), and different DR alleles in the child and mother amplified by the same group-specific PCR (n = 3). Evaluation of IPA-MC was possible in only 29 of the 45 mothers; MC could not be tested in 16 cases: DR identical (n = 8), DR 0 mismatch (n = 6), and different DR alleles in the child and mother amplified by the same group-specific PCR (n = 2) (Figure 1).

Statistical analysis

The rate of pretransplantation NIMA-MC in the pediatric recipients and the rate of IPA-MC in the paired mothers were calculated. The factors associated with the presence or absence of NIMA-MC were evaluated in 26 recipients for whom NIMA-MC could be tested. The rate of biopsy-proven cellular rejection (BPCR) and associated factors were reviewed in order to investigate the impact of pretransplantation NIMA-MC and HLA-DR match status in 23 recipients with a maternal donor.

Continuous data are presented as median values with range, and categorical data as numbers with percentage. The categorical variables were compared

Table 1 Characteristics of the pediatric patients receiving liver transplantation (*n* = 26) according to the presence or absence of HLA-DR NIMA-MC *n* (%)

Factors[1]	NIMA-MC (+) (*n* = 6)	NIMA-MC (-) (*n* = 20)	*P* value
Age at the time of transplantation in years (range)	6.1 (1-14)	6.7 (0-16)	0.57
Age at the time of transplantation (less than 12 mo)	3 (50.0)	7 (35.0)	0.64
Sex, male: female	4 (66.7): 2 (33.3)	6 (30.0): 14 (70.0)	0.16
Recipient weight, kg (range)	28.9 (10.8-54.5)	27.8 (8.1-56.3)	0.92
Original liver disease			0.65
Biliary atresia	4 (66.7)	9 (45.0)	
Others	2 (33.3)	11 (55.0)	
ABO identical to that of mother	3 (50.0)	13 (65.0)	> 0.99

[1]Continuous data are presented as median values with range. NIMA-MC: Microchimerism (MC) to non-inherited maternal antigen (NIMA).

using the Fisher's exact test, and the continuous variables using the Mann-Whitney test. The level of significance was set at $P < 0.05$. Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc. and Microsoft Corp., Chicago, IL, United States).

RESULTS

Rate of pretransplantation NIMA-MC in the pediatric recipients

NIMA-MC was detected in 6 (23.1%) of 26 pre-transplantation cases (Figure 1). The characteristics of these 26 patients, with respect to the presence or absence of NIMA-MC, are compared in Table 1. There was no definite factor associated with the presence of NIMA-MC: age at the time of transplantation, gender, recipient weight, original liver disease (BA *vs* non-BA), ABO compatibility with mother (identical *vs* non-identical) (each, $P > 0.05$).

Rate of IPA-MC among paired mothers

IPA-MC was detected in 4 (13.8%) of 29 blood samples from paired mothers (Figure 1). Bidirectional MC was not observed in any of the child-mother pairs. There was no definite factor associated with the presence of IPA-MC: age of mothers at the time of study, gender mismatch with paired child, history of multi-parity, and ABO compatibility (data not shown).

Rate of BPCR in patients receiving maternal grafts

BPCR rates in patients receiving maternal grafts (34.8%, 8 of 23) and those receiving non-maternal grafts (18.2%, 4 of 22) were not significantly different ($P = 0.31$) (Figure 1). BPCR rates in the 23 patients that received maternal grafts, according to the presence or absence of NIMA-MC and HLA-DR match status, are shown in Table 2. BPCR rates in NIMA-MC-negative patients were as high as 50% (5 of 10). In comparison, none of the NIMA-MC-positive patients ($n = 3$) or those whose HLA-DR status was identical to that of their mother ($n = 4$) developed BPCR. Compared with NIMA-MC-negative patients, NIMA-MC-positive patients tended to show lower BPCR rates ($P = 0.23$).

In addition, patients with NIMA-MC positivity or HLA-DR identity with mother exhibited significantly lower BPCR rates ($P = 0.04$). All BPCRs occurred within 7 mo after transplantation (Figure 3). Rejection activity indexes varied from 3 to 5; rejection activity index of one of the recipients was 5, while that of the other 4 recipients was 3.

In order to investigate other factors associated with lower BPCR rates, various factors were compared in these two groups of patients (Table 3). No significant differences were found in the two groups of patients when compared by sex, sex mismatch, age and weight at the time of transplantation, liver disease, crossmatch positivity, ABO compatibility, donor age, PELD score, and graft versus recipient weight ratio (each, $P > 0.05$).

In order to examine the long-term effect of NIMA-MC positivity on graft function, we compared the levels of aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transferase at 1, 2, 3 and 5 years after LT. However, none of these markers were significantly different between the NIMA-MC-positive and -negative groups of patients who received a maternal graft (data not shown).

DISCUSSION

In this study, we investigated the incidence of NIMA-MC (maternal MC) in pretransplantation peripheral blood samples of pediatric LT recipients, and its effect on BPCR rates in recipients of maternal grafts. To our knowledge, this is the first study demonstrating the possible beneficial effect of pretransplantation fetal-maternal MC on LT outcomes, with verification of the presence or absence of MC in recipients.

The positive rate of pretransplantation NIMA-MC was 23.1% in the present LT recipients, which is similar to that in mother-to-child kidney transplant recipients (22.7%) as reported in our previous study[14]. We

Table 2 Biopsy-proven cellular rejection rate in pediatric patients receiving liver transplantation with maternal graft (*n* = 23) according to the presence or absence of pretransplantation HLA-DR NIMA-MC and HLA-DR match status *n* (%)

NIMA-MC and HLA-DR match status	Biopsy-proven cellular rejection	*P* value[a]
NIMA-MC (-) (*n* = 10)	5 (50.0)	
NIMA-MC (+) (*n* = 3)	0 (0)	0.23
NIMA-MC (+) or HLA-DR 0 mismatch with mother (*n* = 11)	2 (18.2)	0.18
NIMA-MC (+) or HLA-DR identity with mother (*n* = 7)	0 (0)	0.04
Total transplantation with maternal graft (*n* = 23)	8 (34.8)	

[a]*P* value for comparison with NIMA-MC (-) group, by two-tailed Fisher's exact test. NIMA-MC: Microchimerism (MC) to non-inherited maternal antigen (NIMA).

Table 3 Characteristics of pediatric patients receiving liver transplantation with maternal graft (*n* = 17) according to the presence or absence of pretransplantation HLA-DR NIMA-MC and HLA-DR match status *n* (%)

Factors[1]	NIMA-MC (+) or HLA-DR identity with mother (*n* = 7)	NIMA-MC (-) (*n* = 10)	*P* value
Age at the time of transplantation in years, median (range)	5.6 (0.8-10)	7.3 (0.8-16)	0.42
Age at the time of transplantation (less than 12 mo)	3 (42.9)	4 (40.0)	> 0.99
Sex, male: female	3 (42.9): 4 (57.1)	4 (40.0): 6 (60.0)	> 0.99
Recipient weight, kg, median (range)	27.4 (6.2-54.5)	26.0 (9.6-56.3)	> 0.99
Original liver disease			0.64
Biliary atresia	4 (57.1)	4 (40.0)	
Others	3 (42.9)	6 (60.0)	
Crossmatch positivity	0 (0)	1 (10.0)	> 0.99
ABO identical to that of mother	7 (100)	7 (70.0)	0.23
PELD score, median (range)	11.2 (-7.7-39)	2.3 (-10.0-14.1)	0.27
The year of LT (before 2007)	2 (28.6)	3 (30.0)	> 0.99
Donor age in years, median (range)	34 (26-39)	38 (33-43)	0.16
Sex match, yes: no	4:3	6:4	0.65
Operation time, median, min, median (range)	443 (300-595)	492 (285-710)	0.46
Graft *vs* recipient weight ratio, %, median (range)	2.68 (1.62-3.23)	2.15 (1.23-2.96)	0.40
Follow-up duration, mo, median (range)	84 (63-176)	66 (30-114)	0.32
Graft failure	0 (0)	0 (0)	> 0.99
Biopsy-proven cellular rejection	0 (0)	5 (50)	0.04

[1]Continuous data are presented as median values with range. NIMA-MC: Microchimerism (MC) to non-inherited maternal antigen (NIMA); LT: Liver transplantation.

were unable to identify specific factors, such as age, sex, liver disease, and mother-child ABO blood group identity, which were significantly associated with the presence of NIMA-MC in the LT recipients (Table 1).

As to the liver disease, patients with BA have been reported to have increased maternal MC in their livers[20-22], suggesting increased maternal MC in their peripheral blood as well; however, this has rarely been studied. In the present LT recipients, the positive rate of NIMA-MC in the peripheral blood was higher in BA patients (4/13, 30.8%) than in non-BA patients (2/13, 15.4%). However, this difference was not statistically significant as the number of patients in the present study was small. Further investigation in a larger number of patients is therefore needed.

We evaluated the clinical effect of NIMA-MC in 23 LT recipients with maternal grafts in terms of BPCR rate (Table 2). BPCR rate in recipients with NIMA-MC negativity was as high as 50%, whereas none of the recipients with NIMA-MC positivity or mother-child HLA-DR identity showed BPCR (P = 0.04). Nijagal *et al*[15] reported beneficial effects of maternal graft in patients with BA, in terms of decreased risk of hepatic graft failure. Furthermore, it was suggested that these patients with BA exhibit allograft tolerance to maternal grafts[15,23,24]. Sanada *et al*[24] described the importance of sex matching of parental grafts; in particular, the authors showed that maternal grafts were associated with lower incidence of acute cellular rejection than paternal grafts in daughters. This finding represents important clinical evidence of the beneficial impact of preexisting NIMA-MC on post-transplantation outcomes in patients with BA.

As patients with BA were found to exhibit increased number of maternal cells and inflammation of the biliary tree in their livers, the disease was viewed as an inflammatory disease of fetal-maternal circulation; further, graft-versus-host reaction was proposed to represent the underlying pathophysiology of BA[20-22]. Accordingly, beneficial effects of maternal liver grafts

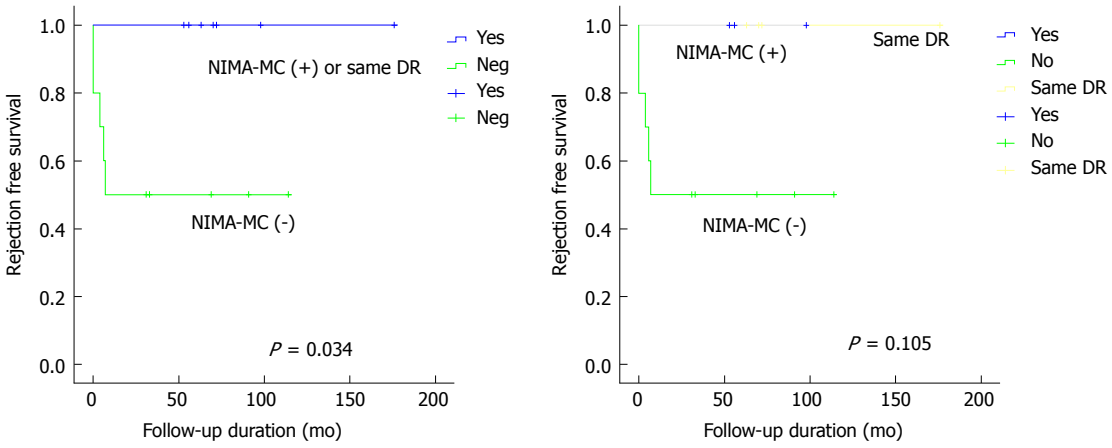

Figure 3 Biopsy-proven cellular rejection-free survival curve according to microchimerism to non-inherited maternal antigen positivity or same HLA DR status to the maternal graft. BPCR: Biopsy-proven cellular rejection; NIMA-MC: microchimerism (MC) to non-inherited maternal antigen (NIMA).

have been demonstrated in patients with BA, but not in non-BA LT recipients[15,23,24]. However, in these studies, donors were analyzed by parental relationship (maternal *vs* paternal) to the recipient, and the presence or absence of maternal MC was not tested. As only a proportion of children exhibit maternal MC (23.1% in this study), beneficial effects of maternal graft recipients that do not have BA may not be detectable by simple analysis of the donor-recipient relationship. *Via* determination of the presence or absence of fetal-maternal MC in renal transplantation from family donors, we reported beneficial effects on rejection-free graft survival in pretransplantation MC-positive patients in a previous study[14]. Further investigation of clinical outcomes, with determination of maternal MC, in patients with conditions other than BA is needed. Future studies should aim to determine whether such patients experience beneficial effects from maternal grafts when positive for the presence of pretransplantation maternal MC. Findings should be of significance for counseling in cases of pediatric LT, particularly with regard to donor selection when both parental donors are available. On the other hands, we can consider less immunosuppressant during life-long follow-up, if the patient has NIMA positivity or HLA-DR identity with mother and can get a maternal graft.

Apart from studies of pretransplantation MC, most reports on MC in solid organ transplantations (*i.e.,* kidney, liver, heart, and lung) investigated the development of MC following transplantation[5,25-30]. The overall positive rate of donor-specific MC detected at various intervals after transplantation has been reported to be high (> 50%)[20-23,25]; this is much higher than that of the pretransplantation fetal-maternal MC detected in the present LT recipients. However, posttransplantation MC exhibits fluctuation and variable patterns over time[20-23,25,26], and is generally detected at higher frequencies during the early period and at lower frequencies in the late period[20-23,25,30]. Posttransplantation MC has been considered to be associated with tolerance induction, as this phenomenon was initially detected in long-term hepatic and renal allograft recipients receiving minimal immunosuppressive therapy[3]. However, this association was not confirmed in a meta-analysis of the association of posttransplantation MC with graft survival in solid organ transplantations[31]. Posttransplantation MC was found to be generally associated with a higher incidence of acute rejection for heart, lung, and kidney transplants and a lower incidence for liver transplants. Privileged tolerogenicity of the liver compared with other organs may be explained by the large population of migratory cells in, and their migration from, hepatic grafts, resulting in balanced lymphodendritic cell chimerism[4].

This study has some limitations. First, MC detection *via* the amplification of *HLA-DRB1* gene is not a robust method applicable to all transplant cases, especially between one haplotype-matched mother and child pairs: in the present work, *HLA-DRB1* NIMA-MC could be tested only in 26 (58%) of the 45 children. Second, this was a retrospective study and posttransplantation follow-up of the persistence of NIMA-MC or the development of de novo donor-specific MC was not performed. NIMA-MC detected before transplantation is expected to persist for a long time following transplantation as fetal-maternal MC, when present, has been shown to persist for decades[7]. However, this has to be verified in a prospective study. Third, the number of cases examined in this study was small, and further studies on a larger number of patients are warranted.

In conclusion, we investigated the effects of pre-transplantation NIMA-MC on allograft outcomes in pediatric LT recipients of maternal grafts, but failed to show that pretransplantation NIMA-MC was associated with a lower risk of cellular rejection. Only the presence of pretransplantation NIMA-MC or HLA-DR identity with the mother could be associated with BPCR-free survival

in pediatric recipients of LT from maternal donors. This study was limited in terms of the small number of patients examined; future studies on a larger number of patients are warranted. The confirmation of the present beneficial effects of pretransplantation NIMA-MC on allograft outcomes *via* larger scale multi-center studies should have implications for donor selection and the development of protocols, including reduced immunosuppression, for managing pediatric LT recipients.

ARTICLE HIGHLIGHTS

Research background

A critical consideration in pediatric liver transplantation (LT) is the management of risks associated with life-long immunosuppression. Tolerance induction is utilized in order to enable discontinuation of immunosuppressant use in pediatric LT recipients. Donor-specific microchimerism (MC) is considered to underlie tolerance induction and graft acceptance. During pregnancy, exposure of the fetus to maternal cells results in natural MC to non-inherited maternal antigens (NIMAs) (NIMA-MC). Recent clinical studies have suggested the association of NIMA-MC with acquired immunologic hyporesponsiveness to NIMAs; this has been termed the "NIMA effect". However, reports of this effect in LT are scarce.

Research motivation

To investigate the pretransplantation status of NIMA-MC and its effect on cellular rejection in children receiving maternal liver grafts, HLA-DRB1 MC between the child and mother was analyzed. This blood DNA test was already reported by this research team, and we found that the presence of pretransplantation MC was associated with beneficial effects on rejection-free survival in family donor renal transplantation.

Research objectives

In the present study, we aimed to investigate the rate of NIMA-MC in pretransplantation blood samples from pediatric recipients of LT, and examine the effect of NIMA-MC on rejection-free graft survival in patients with a maternal donor.

Research methods

The presence of pretransplantation NIMA-MC in the peripheral blood was tested using nested PCR-single-strand conformation polymorphism (SSCP) analysis for the HLA-DRB1 alleles. At first, low-to-intermediate resolution HLA-DRB1 typing was performed *via* the PCR-sequence specific oligonucleotide (SSO) typing method, using either the Dynal RELI™ SSO HLA typing kit (Dynal Biotech Ltd., Wirral, UK) or the Luminex-based, WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan). For HLA-DRB1 MC analysis, high-resolution HLA-DRB1 allelic data are required for mismatched HLA-DRB1 alleles between the mother and child. Allele-level HLA-DRB1 typing was performed *via* the PCR- SSCP method. For nested PCR amplification of the *HLA-DRB1* gene, we used primers to amplify exon 2 of the *HLA-DRB1/B3/B4/B5* genes for the first-round PCR, and *HLA-DR* group-specific primers (for eight different groups) to amplify exon 2 of *HLA-DRB1* for the second-round PCR. The first-round PCR products were diluted to 1:200 in water and used as templates for the second-round PCR. The nested PCR product was analyzed *via* SSCP analysis, and single-strand DNA fragments separated in the gel were visualized with silver staining. For the determination of the presence or absence of NIMA-MC, the band pattern of DNA from pediatric LT recipients was compared with those of maternal DNA and reference DNA with known DRB1 allele types; these were simultaneously analyzed by gel electrophoresis. The rate of pretransplantation NIMA-MC in the pediatric recipients was calculated. To evaluate the effect of NIMA-MC on transplantation outcome, the rate of biopsy-proven cellular rejection (BPCR) among different groups of NIMA-MC and/or HLA-DR match status was compared in patients with a maternal donor using the Fisher's exact test.

Research results

In this small cohort study, pretransplantation NIMA-MC tended to be associated with a lower risk of cellular rejection in pediatric LT recipients with maternal donors. BPCR rate in the patients with pretransplantation NIMA-MC or HLA-DR identity with the mother was significantly lower than those with NIMA-MC negativity (0% *vs* 50%). This is a novel finding, and further study for large cohort validation is required.

Research conclusions

The presence of pretransplantation NIMA-MC or HLA-DR identity with the mother could be associated with BPCR-free survival in pediatric recipients of LT from maternal donors. NIMA effect has been validated in HLA-haploidentical hematopoietic stem cell transplantation. Pretransplantation NIMA-MC could also exert a beneficial effect on rejection-free graft survival in pediatric LT with a maternal graft. Most studies on the NIMA effect in organ transplant recipients have focused on the donor-recipient relationship in the family or the presence or absence of NIMAs in the donor. By actual testing of the presence or absence of NIMA-MC in peripheral blood of the patients, the NIMA effect on outcomes of solid organ transplantations, including LT can be better investigated. Investigation of donor-specific MC in pretransplantation blood samples has rarely been performed. Presence of pretransplantation NIMA-MC could have a beneficial effect on the rejection-free survival in pediatric LT recipients with maternal grafts. Laboratory testing of pretransplantation NIMA-MC in the peripheral blood using nested PCR-single-strand conformation polymorphism analysis for the HLA-DRB1 alleles was previously reported by this research team in renal transplantation (Transplantation 2013; 95(11): 1375-1382). Pretransplantation NIMA-MC for HLA-DRB1 alleles was detected in 23.1% of the pediatric LT recipients; the presence of pretransplantation NIMA-MC or HLA-DR identity with the mother could be associated with BPCR-free survival in pediatric recipients of LT from maternal donors. The presence of pretransplantation NIMA-MC could be associated with BPCR-free survival in pediatric recipients of LT from maternal donors. If the beneficial effect of pretransplantation NIMA-MC on allograft outcomes can be confirmed *via* larger scale multi-center studies, it would have implications for donor selection when both parental donors are available and the development of protocols, including reduced immunosuppression, for managing pediatric LT recipients.

Research perspectives

Presence of microchimerism (MC) to non-inherited maternal antigen (NIMA) is known to be associated with acquired immunologic hyporesponsiveness to NIMA, which is called "NIMA effect". NIMA effect has been suggested in pediatric liver transplant patients with biliary atresia receiving maternal grafts. However, actual presence or absence of NIMA-MC has never been verified. This is the first study showing possible beneficial effect of NIMA-MC on rejection-free survival in pediatric liver transplant patients receiving maternal grafts with actual laboratory determination of the presence or absence of NIMA-MC. This study is based on a small number of patients and could not get a strong evidence of beneficial effect of NIMA-MC in pediatric liver transplant patients receiving maternal grafts. Further studies using a larger number of patients, and preferentially multicenter studies are warranted. If beneficial effects of NIMA-MC in pediatric liver transplant patients is verified, it will have useful clinical implications of selecting family donors and modifying life-long immunosuppression. MC detection *via* the amplification of HLA-DRB1 gene was used in this study, which is not a robust method applicable to all transplant cases, especially between one haplotype-matched mother and child pairs. MC detection methods using robust genetic markers other than HLA-DRB1 gene with good enough sensitivities had better be developed and used in the future research. In the present study, only the presence or absence of pretransplant NIMA-MC has been investigated and future studies are required to investigate the effect of posttransplant persistence of NIMA-MC on clinical outcome.

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ORIGINAL ARTICLE

Retrospective Study

Impact of homogeneous pathologic response to preoperative chemotherapy in patients with multiple colorectal liver metastases

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Abstract

AIM

To analyze the homogeneity of pathologic response to preoperative chemotherapy (PRPC) after chemotherapy in patients with multiple liver metastases (LM).

METHODS

From September 2011 to August 2014, patients with at

least two LM undergoing preoperative chemotherapy prior to resection were included in this retrospective, single-center study. The endpoints were PRPC homogeneity (according to both the Rubbia-Brandt and MD Anderson classifications), the impact of PRPC on the MDT decision, factors associated with homogeneous PRPC and overall survival of patients with *vs*. without homogeneous PRPC.

RESULTS

seventy-three patients with a total of 88 liver resections (including 15 two-stage procedures) were included in the study. The homogeneous PRPC rate was 55% according to the Rubbia-Brandt classification and 53% according to the MD Anderson classification. The MDT decision was modified by the PRPC in only 2.7% of patients (n = 2).

CONCLUSION

The PRPC was homogeneous in only one half of patients and had very little influence on the MDT decision.

Key words: Liver metastases; Pathological response; Homogeneity; Preoperative chemotherapy; Colorectal cancer

Core tip: Pathologic response to preoperative chemotherapy (PRPC) is correlated with survival after resection of liver metastases. This study analyzed the homogeneity of PRPC after chemotherapy in patients with multiple liver metastases. The study underlines that homogeneous PRPC rate was low (55% according to the Rubbia-Brandt classification and 53% according to the MD Anderson classification) and has little impact on the multidisciplinary team meeting decision (modified by the PRPC in only 2.7% of patients).

Sabbagh C, Chatelain D, Attencourt C, Joly JP, Chauffert B, Cosse C, Regimbeau JM. Impact of homogeneous pathologic response to preoperative chemotherapy in patients with multiple colorectal liver metastases. *World J Gastroenterol* 2017; 23(45): 8027-8034 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8027.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8027

INTRODUCTION

One half of patients with colorectal cancer develop liver metastases (LM) with a 5-year overall survival rate of 50%[1,2]. The curative management of LM includes surgical resection and chemotherapy (combined with targeted therapies, in some cases)[3-5].

Three main classifications of pathologic response to preoperative chemotherapy (PRPC) have been described (Rubbia-Brandt classification[6], MD Anderson classification[7] and the Sebagh classification)[8]. Two of these classifications, the Rubbia-Brandt classification and the MD Anderson classification, are used in routine clinical practice in our institution. The Rubbia-Brandt classification[6] is based on whether or not chemotherapy induces fibrosis in the metastasis, whereas the MD Anderson classification reflects the proportion of metastatic tumor cells that remain viable after chemotherapy[7]. A complete PRPC is defined as the absence of tumor cells at the liver site in both classifications[7]. However, the two classifications differ markedly in patients with multiple LM. The Rubbia-Brandt classification is based on the worst nodule, whereas the MD Anderson classification is based on the mean PRPC of all the nodules. Furthermore, the best category (in terms of survival) in the Rubbia-Brandt classification includes both complete tumor regression (tumor regression grade 1, TRG1) and a major response (TRG2), whereas the best category in the MD Anderson classification consists solely of a complete response[9-11].

PRPC scores appear to be correlated with survival after LM resection[6] but the really use of PRPC is a daily question. PRPC may be useful in three situations in which adjuvant chemotherapy may be required in patients with multiple LM, provided a homogeneous response is observed for all LM: (1) after neoadjuvant chemotherapy in a perioperative management setting; (2) between surgical stages in patients scheduled for two-stage hepatectomy for bilobar LM; and (3) in the case of recurrence. In these situations, PRPC could help to guide modification of the chemotherapy regimen when necessary.

For example, in the two-stage hepatectomy setting, Mentha et al. addressed this question by studying the difference in TRG grade between the two operative specimens from a given individual patient as a measure of chemotherapy resistance due to interruption of treatment or as a result of the immunosuppression that follows a surgical procedure[12]. However, Sebagh *et al*[8] did not assess the homogeneity of the PRPC in individual patients or whether a homogeneous PRPC had an impact on prognosis. Recently, Sebagh *et al* reported for the first time a 19.7% rate of PRPC heterogeneity.

The objective of the present study was therefore to analyze the homogeneity of PRPC after chemotherapy and to assess the impact of PRPC on the multidisciplinary team meeting (MDT) decision, on survival and on the management of two-stage procedures.

MATERIALS AND METHODS

Population

From September 2011 to August 2014, patients undergoing resection of at least two colorectal cancer LMs, who had received preoperative chemotherapy and for whom both PRPC classifications were available

Table 1 Details of the Rubbia-Brandt and MD Anderson classifications

The Rubbia-Brandt classification	The MD Anderson classification
TRG1: Absence of residual cancer and large amount of fibrosis	Complete response: No residual cancer cells
TRG2: Rare residual cancer cells scattered throughout the fibrosis	Major response: 1%-49% of residual cancer cells
TRG3: More residual tumor cells but fibrosis predominates	Minor response: More than 50% of residual cancer cells
TRG4: Residual cancer cells predominate over fibrosis	
TRG5: No signs of regression.	

TRG: Tumor regression grade.

were included in the present study.

Study design

This was a retrospective, single-center study. Data were extracted from a single-center database. The study was initiated following systematic use of PRPC by our institution (according to the MD Anderson and Rubbia-Brandt classifications) in pathology reports and MDTs.

Criteria studied

Patient-related: Age, gender, body mass index (BMI), and comorbidities. Tumor-related: Number of metastases, size of the metastases (after chemotherapy on pathological exam), site in the liver (central or peripheral), primary tumor stage (according to the TNM classification) and site (colon or rectum), and tumor markers (CEA, CA 19.9). Treatment-related: Type of chemotherapy, number of cycles, and association with targeted therapy. Related to surgery: The type of liver resection (minor or major). Related to pathologic response: A homogeneous PRPC (defined as the same classification for all metastases resected in a given patient, for example all metastases were classified as having a major regression when Rubbia-Brandt classification is considered and for example all metastases had a minor response to PRPC when the MD Anderson classification is considered). A heterogenous PRPC was determined if in a single specimen, one metastasis had a major regression and others have partial regression or no regression in the Rubbia-Brandt classification or if in a single specimen, one metastasis had a major PRPC and others have complete or minor PRPC in the MD Anderson classification. The homogeneity of PRPC was assessed separately for each classification (Rubbia-Brandt and MD Anderson), but the two classifications were not compared in terms of homogeneity of PRPC.

Endpoints

The primary endpoints were homogeneity of the PRPC according to the Rubbia-Brandt classification and the MD Anderson classification, and the impact of PRPC on the MDT decision.

The secondary endpoints were factors associated with homogeneous PRPC and the PRPC between the two surgical procedures for patients who underwent two-stage hepatectomy with preoperative chemotherapy.

Pathologic examination

Only operative specimens and no biopsies were examined. All specimens were examined independently by two pathologists (DC + AC). Tumors less than 2 cm in diameter were fully embedded, while an average of 5 slides were taken from tumors measuring more than 2 cm. All data were reported on a standardized pathology report form and included the Rubbia-Brandt and MD Anderson classifications for all LM[6,7]. The pathologists were blinded from each other for the analysis but in the event of disagreement between the two pathologists, a consensus was reached.

Rubbia-Brandt classification: The Rubbia-Brandt classification scores patients from TRG 1 to TRG 5 (Table 1)[6]. Patients were then categorized into three groups, defined as major regression (TRG1 or TRG2), partial regression (TRG3) or no regression (TRG4 or TRG5). Patients with multiple liver metastases and heterogeneous PRPC scores were categorized according to the poorest response.

MD Anderson classification: The MD Anderson classification scores patients as having complete, major or minor PRPC (Table 1)[7]. Patients with multiple liver metastases and heterogeneous PRPC scores were categorized according to the mean response.

Multidisciplinary team meeting

All operated patients were discussed at the MDT meeting before and after liver surgery. In the situation of liver metastases, at our institution, all patients had preoperative chemotherapy except for patients with small LM that could disappear with preoperative chemotherapy or patients with a limited number of metastases who had the resection of the primary tumour during the same procedure than liver resection. The decision to use a target agent was considered on a case-by-case basis. The MDT records were standardized and accessed with in-house software. The MDT decision (withdrawal from postoperative chemotherapy or modification of the chemotherapy regimen) was noted by the team leader (JPJ) in a register. The MDT records and decisions were analyzed retrospectively. The reason for modifying chemotherapy

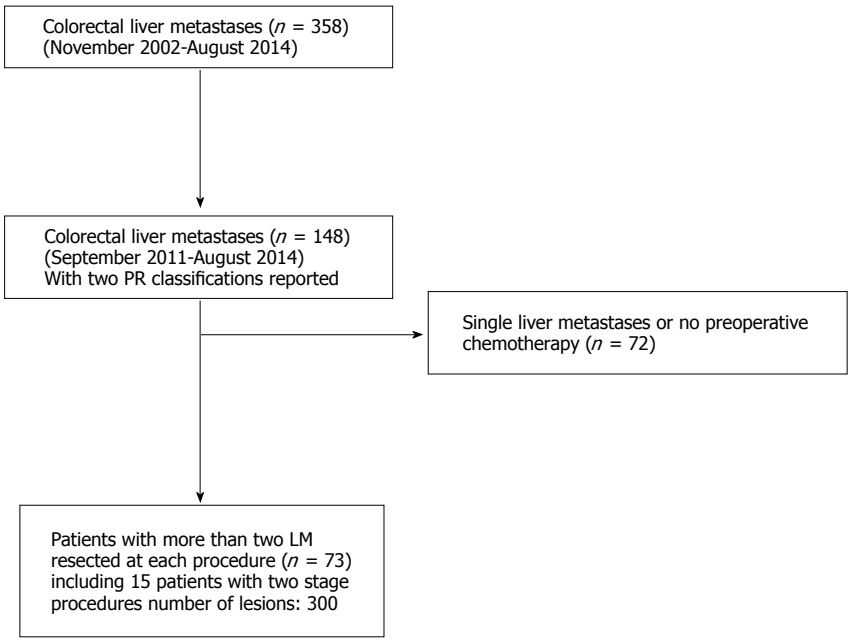

Figure 1 Study flowchart.

was always reported (disease progression, treatment response, or toxicity), thereby identifying all cases in which, the MDT decision was modified by either a complete PRPC or no PRPC.

Statistical analysis

Data are expressed as mean ± SD, median (range) or number (percentage). Logistic regression analysis was then performed to identify risk factors for homogeneous PRPC, with homogeneous PRPC as dependent variable. Only variables with a *P*-value < 0.2 in univariate analysis were included as independent variables in a multivariate model. Variables with a *P* value $\leqslant$ 0.1 in the multivariable model were considered to be risk factors. Statistical analyses were performed by a datamanager with SAS 9.2 statistical analysis software (SAS Institute Inc., Cary, NC, United States).

Ethical authorizations

The present study was reviewed and approved by the Commission Nationale de l'information et des libertés (CNIL) with the number DRCI T135.

RESULTS

Population

Seventy-three patients (with a total of 300 LM, including 15 two-stage procedures) met the inclusion criteria and were included in the study (Figure 1)**.** The study population had a median age of 62.5 years (range: 40-80) and included 45 men (61%). The primary tumor was located in the colon in 66% of patients (*n* = 48). It was on the ascending colon in 14% (*n* = 14), on the transverse colon in 7% (*n* = 5), on the descending colon in 45% (*n* = 45) and in the rectum in 34% (*n* = 25). The primary tumour was resected in 98% (*n* = 72) of the cases with a mean delay between the primary tumour resection and the first liver resection of 15.2 mo (range: 2-60). The median number of LM was 3 (range: 1-14), and metastases were synchronous with the primary tumor in 75% of patients (*n* = 55). The rate of patients with BRAF mutation was 5% (*n* = 4). The rate of patients with KRAS mutation was 9.5% (*n* = 7). The sites of LM are detailed in Table 2. The chemotherapy regimen included targeted therapy in 45% of cases (*n* = 40). The median number of preoperative cycles was 12 (range: 4-38) and the median number of overall cycles was 17 (range: 4-42) (Table 2). Median follow-up was 17 mo (ext: 2-78).

According to the Rubbia-Brandt classification, 15% of patients (*n* = 13) displayed major response, 14% (*n* = 12) displayed partial response and 71% (*n* = 63) had no response. The rate of concordance between the two pathologists for the Rubbia-Brandt classification was 98% (*n* = 86).

According to the MD Anderson classification, 9% of patients (*n* = 8) displayed a complete response, 30% (*n* = 26) displayed a major response and 61% (*n* = 54) displayed a minor response. The rate of concordance between the two pathologists for the MD Anderson classification was 96% (*n* = 85). A concordance was observed between the two classifications in 69% of cases (*n* = 61).

Primary endpoint

Homogeneity of PRPC: According to the Rubbia-Brandt classification, 55% of patients (*n* = 48) obtained a homogeneous PRPC. According to the MD Anderson classification, 53% of patients (*n* = 47) obtained a homogeneous PRPC.

Table 2 Characteristics of the study population *n* (%)

Variable	Study population
Demographic data	
Male gender	45 (61)
Age, median (range), yr	62.5 (40-80)
body mass index, mean ± SD, kg/m^2	25.36 ± 4.42
Tumor markers	
CEA level, mean ± SD (mg/L)	17 ± 3.5
Ca 19.9 level, mean ± SD (UI/L)	23 ± 5.2
Primary tumor site	
Ascending colon	10 (14)
Transverse colon	5 (7)
Descending colon	33 (45)
Rectum	25 (34)
Liver metastases	
Median (range) number of preoperative LM	3 (1-14)
Synchronous LM	55 (75)
Surgical procedure	
Right hepatectomy	15 (16)
Left lobectomy	4 (4)
Right lobectomy	3 (3)
Posterior segmentectomy	8 (11)
Wedge	58 (66)
Two-stage hepatectomy	15 (17)
Site of the 300 metastases (%)	
Ⅰ	2.5
Ⅱ	10
Ⅲ	17.5
Ⅳ	11
Ⅴ	16
Ⅵ	20
Ⅶ	13
Ⅷ	10
Preoperative chemotherapy	
Regimen	
Folfox	28 (32)
Folfiri/Folfox and bevacizumab	28 (32)
Folfiri with or without cetuximab	8 (9)
Campto or folfiri with or without cetuximab	20 (23)
Folfirinox	4 (4)
Median (range) number of preoperative cycles	12 (4-38)
Pathology	
T stage	
2	7 (9)
3	56 (77)
4	10 (14)
N stage	
0	18 (24)
1	37 (51)
2	11 (15)
X	7 (10)
Median (range) size of metastases, cm	3.1 (0.2-5)
PRPC	
Rubbia-Brandt classification	
Major response	13 (15)
Partial response	12 (14)
Absence of response	63 (71)
MD Anderson classification	
Complete response	8 (9)
Major response	26 (30)
Minor response	54 (61)

Impact of PRPC on the MDT decision: The PRPC changed the MDT decision in only 2 cases (2.7%; withdrawal of chemotherapy in both cases). For both patients, the PRPC was classified as major in the Rubbia-Brandt classification or complete in the MD Anderson classification. The PRPC was homogeneous in both patients and according to both classifications. Both patients had severe (grade Ⅲ) oxaliplatin-induced peripheral neuropathy. The absence of PRPC or the presence of heterogeneous PRPC did not change the MDT decision in any of the other cases.

Secondary endpoints

Factors associated with a homogeneous PRPC: For the Rubbia-Brandt classification, only the use of bevacizumab [OR (95%CI): 3.5 (1.2- 10.5); $P = 0.02$] was associated with a homogeneous PRPC (Table 3).

For the MD Anderson classification, no factor was associated with a homogeneous PRPC (Table 3).

PRPC in two-stage procedures: After the first stage of hepatectomy, a homogeneous PRPC was observed in 100% of cases ($n = 15$) with the Rubbia-Brandt classification and 73% of cases ($n = 11$) with the MD Anderson classification. After the second stage of hepatectomy, a homogeneous PRPC was observed in 53% of cases ($n = 8$) with the Rubbia-Brandt classification and 53% of cases ($n = 8$) with the MD Anderson classification.

DISCUSSION

A homogeneous PRPC was obtained in only 55% of cases according to the Rubbia-Brandt classification and in only 53% of cases according to the MD Anderson classification and PRPC had little impact on the MDT decision and patient survival. This study is the second to report these findings and to have identified factors associated with a homogeneous PRPC.

Recently, Sebagh *et al*[8] reported a heterogeneous PRPC in 19.7% of cases. In their study, the authors considered PRPC to be heterogeneous when at least 50% of metastases did not present the same PRPC. They also demonstrated the lack of impact of homogeneous PRPC on survival. In another study by the same group, the authors emphasized the limited impact of PRPC on survival according to the definition of heterogeneous PRPC. Thus, according to the MD Anderson classification, PRPC was not a prognostic factor when based on the mean value but tended towards significance when based on the median PRPC[8].

The high proportion of major or complete PRPC (*i.e.* similar to the rates reported in the literature) and the high quality of examination of our specimens support the robustness of the present study. Our findings therefore question the real value of PRPC in everyday practice. In our series, the PRPC influenced the MDT decision in only 2 cases. In everyday practice, the decision to prescribe adjuvant chemotherapy is based on laboratory data (decreased tumor marker levels), morphological data (RECIST score) and clinical data (postoperative performance status and tolerability of chemotherapy) and randomized controlled clinical

Table 3 Univariate and multivariate analyses of factors associated with a homogeneous pathologic response to preoperative chemotherapy

Variable	Homogeneity (Rubbia-Brandt)				Homogeneity (MD - Anderson)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	OR [95%CI]	*P* value	OR [95%CI]	*P* value	OR [95%CI]	*P* value	OR [95%CI]	*P* value
Age	2.33 [0.89-6.07]	0.82	/	/	1.5 [0.61-3.67]	0.37	/	/
Gender	1 [0.48-2.09]	0.99	/	/	1 [0.48-2.09]	0.99	/	/
Hypertension	1.13 [0.43-2.92]	0.81	/	/	1.13 [0.43-2.92]	0.81	/	/
Body mass index	0.99 [0.98-1.02]	0.95	/	/	1.01 [0.98-1.03]	0.86	/	/
Rectal cancer	1.14 [0.56-2.34]	0.72	/	/	1.73 [0.82-3.63]	0.15	/	/
Number of peroperative LM	0.96 [0.86-1.07]	0.45	/	/	0.99 [0.89-1.10]	0.87	/	/
Time interval between chemotherapy and surgery	3 [0.31-28.84]	0.34	/	/	1.5 [0.53-4.21]	0.44	/	/
Folfiri-based chemotherapy	0.007 [0.09-0.6]	0.90			0.8 [0.3-2.0]	0.60		
Metachronous liver metastases	2.11 [0.96-4.67]	0.14	2.8 [0.92-8.5]	0.06	1.33 [0.63-2.82]	0.45	/	/
T stage	1.26 [0.73-2.18]	0.41	/	/	1.17 [0.68-2.01]	0.58	/	/
N0 stage	0.8 [0.22-2.98]	0.74	/	/	0.8 [0.22-2.98]	0.74	/	/
ASA score	1.05 [0.88-1.25]	0.62	/	/	1.05 [0.88-1.26]	0.56	/	/
MSI	1.9 [0.2-18.3]	0.90			1.5 [0.2-9.8]	0.60		
RAS status	1.05 [0.0-99]	0.90			4.5 [0.8-23.9]	0.30		
Braf mutation	1.6 [0.0-120]	0.90			3.3 [0.32-34.6]	0.30		
Use of bevacizumab	3.20 [1.17-8.74]	0.02	3.5 [1.2-10.5]	0.02	1.33 [0.56-3.16]	0.51	/	/
Metastases in the left lobe of the liver	0.67 [0.24-1.87]	0.44	/	/	0.67 [0.24-1.87]	0.44	/	/
Number chemotherapy cycles	1.79 [0.93- 3.44]	0.12	1.06 [0.97-1.1]	0.10	1.44 [0.76- 2.72]	0.27	/	/

LM: Liver metastases.

trials and cohort studies have such a major impact on the decision to prescribe perioperative and adjuvant chemotherapy[5,13], that the potential impact of the PRPC in the MDT decision is negligible.

However, the proportion of patients with a homogeneous PRPC in our series was much lower than that published in the initial report by Rubbia-Brandt *et al*[6] (90%). Firstly, this disparity might be due to differences in chemotherapy regimens. In the study by Rubbia-Brandt *et al*[6] none of the patients received targeted therapies *vs* 61% of the patients in the present study. Secondly, the studies differed in terms of the number of slides prepared per metastasis. In the study by Rubbia-Brandt *et al*, specimens were prepared as 0.5-cm-thick slices. In the present study, a mean of 10 slides per metastasis were prepared, and metastases measuring less than two centimeters were fully embedded. One can argue that the retrospective design of the study is a limitation since no special analysis or additional slide for each metastasis was performed. Furthermore, no information on the distribution of the residual tumour cells in a single metastasis is available since there is no classification for that particular point.

Our findings concerning the proportion of patients with a homogeneous PRPC also question the conclusions reached by Mentha *et al*[12] on interval treatment in patients undergoing two-stage hepatectomy. Mentha *et al*[12] found that, when comparing the PRPC after the first and second stages, 10 out of 22 patients (45%) had a poorer PRPC at the second stage (compared with 23% in the present study). These authors suggested that this difference in PRPC might be due to interruption of treatment for 5-15 wk of chemotherapy[12]. Our results suggest another possible explanation for the difference in PRPC between the two stages of hepatectomy, as a heterogeneous PRPC was observed in one-half of our patients. The difference in PRPC classification therefore cannot be solely attributed to putative chemoresistance between the two stages of hepatectomy. Data on homogeneity also reflect the biological heterogeneity of liver metastases, derived from independent colonies with their own biological profile[14] and information on PRPC homogeneity is crucial regardless of the impact of PRPC on survival as it shows that the treatment strategy does not need to be adapted to the PRPC, which is variable from one metastasis to another.

Bevacizumab is known to be associated with an increased likelihood of complete PRPC. The present study is the first to report the association between bevacizumab and an increased likelihood of homogeneous PRPC[15]. One possible explanation is related to the mechanism of action of bevacizumab (necrosis and modification of vasculogenesis)[16]. The outcomes of this analysis should nevertheless interpret with caution, since there was a lot of tested variable of a limited number of patients and events[17]. An interesting extension of this work would be to perform the same analysis on patients who have received intra-arterial chemotherapy (which is known to influence the PRPC).

Although it has been clearly established that a complete PRPC is a major prognostic factor[6,7], it is a static variable (like age or the presence of metachronous *vs* synchronous metastases) in contrast

with the dynamic nature of tumor markers and the RECIST score, and therefore constitutes another limitation to the practical value of PRPC.

The present study nevertheless presents a number of limitations, due to the heterogeneous characteristics of liver resections, preoperative chemotherapy, inclusion of patients receiving targeted therapy and the number of preoperative cycles. Moreover, as previously demonstrated in the series published by Rubbia-Brandt *et al*[6] and Kishi *et al*[7], complete PRPC (but not homogeneous PRPC) is a prognostic factor but the presence of two classifications is disturbing[9] and contributes to the poor understanding and correct use of PRPC. All these points could have a direct impact on the homogeneity of PRPC.

In conclusion, the PRPC was homogeneous in only half of patients with multiple LM and had little impact on the MDT decision. Routine use of PRPC to guide treatment may be questionable (due to differences between classifications and the heterogeneity of the PRPC for multiple LMs in the same patient). Further investigations are therefore necessary in order to improve the value of the PRPC.

COMMENTS

Background

Pathologic response to preoperative chemotherapy scores appears to be correlated with survival after liver metastases (LM) resection. Pathologic response to preoperative chemotherapy may be useful in situations in which adjuvant chemotherapy may be required in patients with multiple LM. However a little is know about the homogeneity rate of pathologic response to preoperative chemotherapy and on its use in daily practice.

Research frontiers

Fifty percent of patients with colorectal cancer will develop LM with a 5-yr overall survival rate of 50%. The curative management of LM includes surgical resection and chemotherapy (combined with targeted therapies, in some cases.

Innovations and breakthrough

The study underlines that homogeneous PRPC rate was low and has little impact on the multidisciplinary team meeting decision.

Applications

Doing a liver biopsy to know the pathological response to preoperative chemotherapy is useless. Pathological response to preoperative chemotherapy is not a crutial point in MDT discussions.

Peer-review

The authors review a cohort of 73 patients undergoing liver resection for colorectal LM after systemic chemotherapy in order to assess the impact of homogeneity of pathological response to chemotherapy on survival and routine management of patients. They conclude that pathological response to chemotherapy is not a powerful prognostic factor and do not influence treatment or management in patients with advanced resectable LM. Overall this is a concise and well written manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Two-step method for creating a gastric tube during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy

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Abstract

AIM

To introduce a two-step method for creating a gastric tube during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy and assess its clinical application.

METHODS

One hundred and twenty-two patients with middle or lower esophageal cancer who underwent laparoscopic-thoracoscopic Ivor-Lewis esophagectomy at Liaoning Cancer Hospital and Institute from March 2014 to March 2016 were included in this study, and divided into two groups based on the procedure used for creating a gastric tube. One group used a two-step method for creating a gastric tube, and the other group used the conventional method. The two groups were compared regarding the operating time, surgical complications, and number of stapler cartridges used.

RESULTS

The mean operating time was significantly shorter in the two-step method group than in the conventional method group [238 (179-293) min *vs* 272 (189-347) min, $P < 0.01$]. No postoperative death occurred in either group. There was no significant difference in the rate of complications [14 (21.9%) *vs* 13 (22.4%), *P*

= 0.55] or mean number of stapler cartridges used [5 (4-6) *vs* 5.2 (5-6), *P* = 0.007] between the two groups.

CONCLUSION

The two-step method for creating a gastric tube during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy has the advantages of simple operation, minimal damage to the tubular stomach, and reduced use of stapler cartridges.

Key words: Minimally invasive srugery; Gastric tube; Ivor-Lewis esophagectomy

Core tip: The two-step method accomplishes totally laparoscopic-thoracoscopic Ivor-Lewis esophagectomy, by avoiding an additional abdominal incision and conducting operations *via* the operating port to simplify the complicated operation steps, thus greatly reducing the difficulty in creating the gastric tube after anastomosis, and shortening the operating time.

Liu Y, Li JJ, Zu P, Liu HX, Yu ZW, Ren Y. Two-step method for creating a gastric tube during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy. *World J Gastroenterol* 2017; 23(45): 8035-8043 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8035.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8035

INTRODUCTION

Esophageal cancer (EC) is a common malignancy of the upper digestive tract in China. Statistics show that EC ranks fifth in incidence among all malignancies and is the fourth leading cause of cancer death in China[1]. Surgery is the first-choice treatment for EC. Even for patients with locally advanced EC, surgery remains a very important component of multimodality therapy. Currently, many procedures for esophageal resection and reconstruction are available, and Ivor-Lewis esophagectomy combined with two-field lymphadenectomy has become the standard procedure for middle and lower EC[2-6]. Compared with open Ivor-Lewis esophagectomy, minimally invasive laparoscopic-thoracoscopic Ivor-Lewis esophagectomy has an obvious advantage of reducing the incidence of perioperative complications while having similar therapeutic outcomes[7-16].

After esophagectomy, the stomach is the most commonly used substitute for the esophagus. Because of its sufficient length, the gastric tube has the advantages of decreasing anastomotic tension, reducing the pressure on the lungs and heart by thoracic stomach expansion, reducing gastric retention, and not reducing the blood supply to the anastomosis site and surgical site. Therefore, it has been advocated by many surgeons in both China and other countries, and it has been applied clinically[17-22]. However, the creation of a gastric tube is time consuming, requires multiple gastric incisions, and increases patients' economic burden as many medical devices are required. To overcome these shortcomings, we proposed a two-step method for creating a gastric tube and assessed its feasibility during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy.

MATERIALS AND METHODS

Patients

One hundred and twenty-two patients with middle or lower EC treated at the Department of Thoracic Surgery, Cancer Hospital of China Medical University/Liaoning Cancer Hospital and Institute from March 2014 to March 2016 were included in this study. All patients underwent upper gastrointestinal radiography, fiber gastroscopic examination (including Lugol's iodine staining), and pathological biopsy to establish a definitive diagnosis. The inclusion criteria were as follows: (1) patients who had accurate preoperative staging following endoscopic ultrasonography (some patients did not undergo it due to severe obstruction), contrast-enhanced computed tomography (CT) scans of the chest and upper abdomen, color Doppler ultrasound of the neck, contrast-enhanced magnetic resonance imaging (MRI) of the head, and bone emission CT scans to exclude metastases to the head, lungs, bone, liver, gallbladder, spleen, kidneys, and adrenal glands as well as physical examination to exclude enlargement of the neck, supraclavicular and retroperitoneal lymph nodes; (2) Patients who had undergone heart, lung, liver and renal function examinations to exclude potential surgical contraindications; (3) patients who had no prior history of malignancy or gastrointestinal surgery; (4) patients who consented to the study protocol and signed the informed consent form; and (5) patients who had complete follow-up data. The exclusion criteria were: (1) Patients who had incomplete clinical data; and (2) patients who had poor compliance and withdrew from the study.

After admission, patients were divided into two groups based on the procedure used for creating the gastric tube. In one group of 64 patients (50 males and 14 females) with a mean age of 62 years, a two-step method for creating a gastric tube was adopted (two-step method group); and in the other group of 58 patients (46 males and 12 females) with a mean age of 61 years, the conventional method for creating a gastric tube was used (conventional method group). Both groups underwent laparoscopic-thoracoscopic Ivor-Lewis esophagectomy. The clinical data about the patients of the two groups are shown in Table 1.

Surgical techniques

The patients were placed in the supine position. A

Table 1 Clinical data in the two groups

Variable	Two-step method	Conventional method	t/χ^2-value	*P* value
Age (yr)	62.4 ± 7.6	61.4 ± 7.1	0.717	0.48
Gender (male/female)	50/14	46/12	0.873	0.53
Tumor location (middle/lower)	35/29	32/26	0.957	0.55
TNM stage (Ⅰ/Ⅱ/Ⅲ)	9/41/14	10/32/16	1.003	0.60

Veress needle was introduced below the umbilicus for CO_2 insufflation to maintain the pressure at 12-13 mm Hg. A 10-mm trocar was inserted below the umbilicus to create a camera port. A trocar was inserted 2 cm below the right anterior axillary line to create a main operating port. Two tool ports were made in the right and left midclavicular lines 2 cm above the umbilicus, respectively. An additional tool port was created below the xiphoid process to expose the liver. The stomach was mobilized with an ultrasonic scalpel along the greater curvature of the stomach with the right gastroepiploic vascular arch protected and short gastric artery branch severed. After mobilizing the lesser omentum, 16-19 lymph nodes were dissected. The left gastric artery and gastric coronary vein were severed with an ultrasonic scalpel after clipping three times with Hem-o-lok clips. For the conventional method, gastric tube creation was performed outside the abdominal cavity, while for the two-step method, gastric tube creation was performed inside the abdominal cavity. At this time, the abdominal phase was completed.

In the thoracic phase, the patient was placed in the left lateral decubitus position with elevation of the upper right arms. An artificial pneumothorax was then established by CO_2 insufflation (CO_2 pressure = 8 mmHg). A 10-mm trocar was inserted in the 7th intercostal space in the mid-axillary line to create a camera port. Two additional 5-mm ports were made in the 6th intercostal space outside the inferior angle of the scapula and 8th intercostal space in the posterior axillary line. The 3-cm main operating port was placed at the 4th intercostal space in the anterior axillary line, respectively. After the mediastinal pleura was longitudinally cut open along the esophagus, an ultrasonic scalpel was used to mobilize the middle and lower segments of the esophagus by separating and removing all the fat tissue around the lower segment of the esophagus. An electric hook and ultrasonic scalpel were alternately used to separate the tissues. After the arch of the azygous vein was fully exposed and severed, the upper segment of the esophagus was mobilized to the top of the right pleura, which was followed by the dissection of stations 4R and 2R lymph nodes and bilateral para-recurrent laryngeal lymph nodes. The esophagus was then severed below the top of the right pleura, and end-to-side anastomosis was performed between the esophagus and the gastric tube. The detailed procedure of anastomosis is described below.

Two-step method: In the abdominal phase, after the stomach was mobilized and lymphadenectomy was performed, a laparoscopic cutting stapler was used to create a 3-4 cm wide gastric tube along the direction from the severed site of the right gastric artery branch on the upper lateral side of the lesser curvature of the stomach to the gastric cardia. The joint area between the gastric cardia and stomach preserved, creating a gastric pouch, as shown in Figures 1 and 2[23]. At this time, the first step was completed. In the thoracic phase, after the large portions of the esophagus and lymph nodes were dissected, the esophagus was resected and the resection edge away from the upper border of tumor was more than 5 cm, in the region from the superior border of the azygos vein to about 3 cm below the thoracic inlet. Subsequently, the esophageal stump was ligated and sterilized, and the proximal esophageal stump was applied with a purse-string suture and placed in the anvil of an anastomat. After the purse-string suture was tightened and tied to fix the anvil (Figure 3), the distal esophagus and gastric pouch were pulled to lift the gastric tube out of the thoracic cavity *via* the main operating port. A cutting stapler was used to resect a portion of the fundus of the stomach in a retrograde manner (Figure 4A and B), with a connection region, whose width permitted an anastomat pass, between the gastric pouch and gastric tube preserved. A 2-cm incision was then made in the anterior wall of the gastric pouch 3-4 cm away from the connection region to place an anastomat, which was subsequently allowed to pass the connection region and travel up through the highest point of the fundus of the gastric tube (Figure 5A and B). Afterwards, the distal esophagus and gastric pouch were returned to the thoracic cavity to perform the anastomosis. The anastomat was then removed, and 8-character-pattern suturing was performed to close the incision in the anterior wall of the gastric pouch. A cutting stapler was used to resect the connection region, and the resected esophageal and cardiac tissues and the gastric pouch were removed (Figures 6 and 7). Thus, the second step was completed.

Conventional method: In the abdominal phase, after stomach mobilization and lymph node dissection, a laparoscopic cutting stapler was used to transect

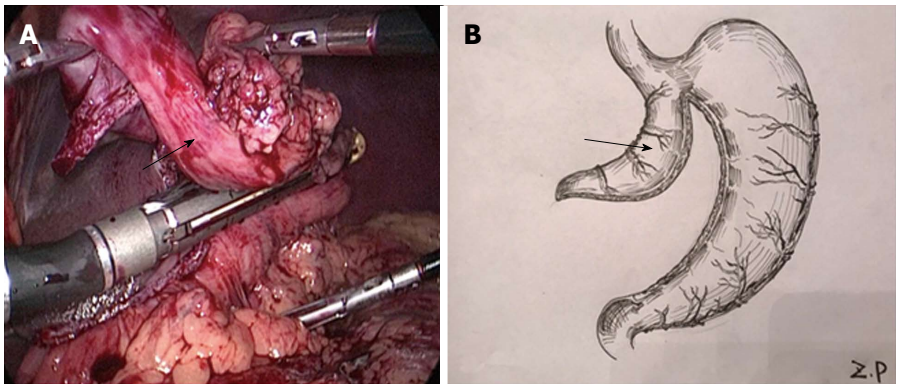

Figure 1 Intraoperative view (A) and schematic diagram (B) of the creation of a gastric pouch (arrows).

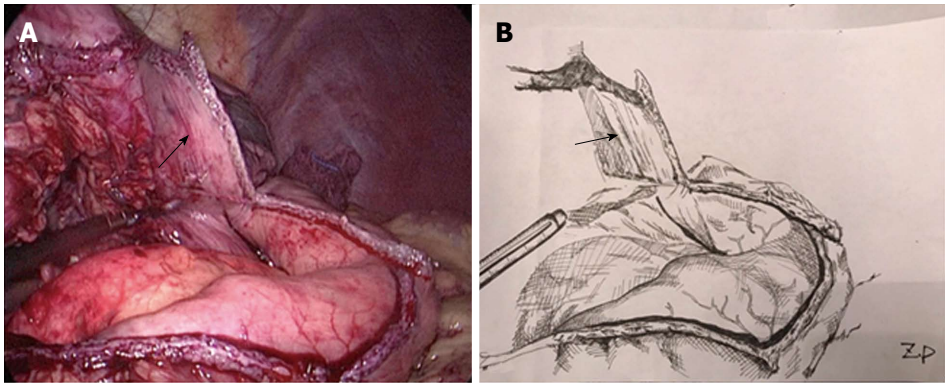

Figure 2 Intraoperative view (A) and schematic diagram (B) of the creation of a gastric tube (arrows indicate the gastric pouch).

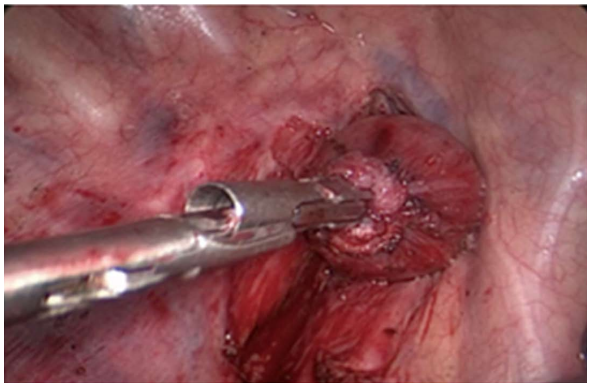

Figure 3 The purse-string suture was tightened and tied to fix the anvil.

the stomach and gastric cardia in a tumor-free cardia region. A 4-5 cm incision was made in the midline below the xiphoid process from which a suture was introduced to ligate the lower segment of the esophagus. The stomach was then lifted out of the abdominal cavity, and a gastric tube was created from the fundus along the greater curvature. The lesser curvature was transected at the first or second branch of the right gastric artery, which was followed by suturing with a stapler. The fundus was sutured with 3-0 silk suture as a fixation suture to tightly tie with the suture, ligating the lower segment of the esophagus. The created gastric tube was then returned to the abdominal cavity, and the abdominal incision was closed. In the thoracic phase, after mobilization of the esophagus and resection of lymph nodes, the esophageal resection and anvil fixation were the same as those described in the two-step method. After tissue retrieval, an incision was made in the anterior wall of the gastric tube to place an anastomat. The procedure of anastomosis was the same as that for the two-step method. Finally, a laparoscopic cutting stapler was used to resect the fundus tissue containing the incision in the gastric wall.

Outcome measures

The outcome measures included operating time, intraoperative material consumption, intraoperative blood loss, and postoperative complications.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 statistical software. Numerical data are expressed as the mean ± SD. Differences between two groups were compared using *t* tests. $P < 0.05$ was considered statistically significant.

RESULTS

Minimally invasive Ivor-Lewis esophagectomy was successfully performed in all patients in the two groups,

Table 2 Surgical complications in the two groups

	Two-step method	Conventional method	*P* value
[1]Pneumonia	[1]8	[1]6	0.78
Arrhythmia	4	5	0.74
Anastomotic leakage	1	1	1.00
Para-recurrent laryngeal nerve injury	3	1	0.62

[1]One case in the two-step method group developed concurrent pneumonia, anastomotic leakage, and para-recurrent laryngeal nerve injury, and one case in the conventional method group developed concurrent pneumonia and anastomotic leakage.

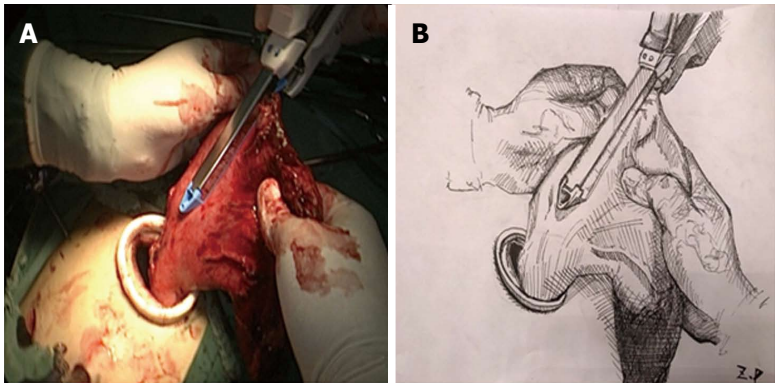

Figure 4 Intraoperative view (A) and schematic diagram (B) of the creation of a gastric tube *via* the main operating port.

Figure 5 Intraoperative view (A) and schematic diagram (B) of the placement of an anastomat *via* the gastric pouch and its course through the fundus of the gastric tube (arrows indicate the gastric pouch).

and there was no conversion to open thoracotomy. The mean operating time was 238 min (range, 179-293 min) for the two-step method, and 272 min (range, 189-347 min) for the conventional method. There was a significant difference between the two groups ($P < 0.01$).

In the two-step method group, 14 (21.9%) cases had surgical complications, including anastomotic leakage ($n = 1$), para-recurrent laryngeal nerve injury ($n = 3$), pneumonia ($n = 8$, including one case with concurrent para-recurrent laryngeal nerve injury and intrapulmonary infection secondary to anastomotic leakage), and arrhythmia ($n = 4$). In the conventional method group, 13 (22.4%) cases had surgical complications, including anastomotic leakage ($n = 1$), para-recurrent laryngeal nerve injury ($n = 2$), pneumonia ($n = 6$, including one case with concurrent anastomotic leakage), and arrhythmia ($n = 5$). No perioperative death occurred. Table 2 shows the surgical complications that occurred in the two groups.

Regarding intraoperative material consumption, the mean number of stapler cartridges used in the procedure was 4.1 (range, 4-5) for the two-step method group and 4.7 (range, 4-6) for the conventional method group, and there was a significant difference between the two groups ($P < 0.01$) (Table 3).

DISCUSSION

Ivor-Lewis esophagectomy can better expose the esophagus, which is beneficial to the dissection of abdominal and thoracic lymph nodes, especially

Table 3 Perioperative parameters for the two groups

Parameter	Two-step method	Conventional method	*P* value
Operative time (min)	238 (179-293)	272 (189-347)	< 0.001
Rate of complications (%)	21.9	22.4	0.560
Number of stapler cartridges used	5.0 (4-6)	5.2 (5-6)	0.007

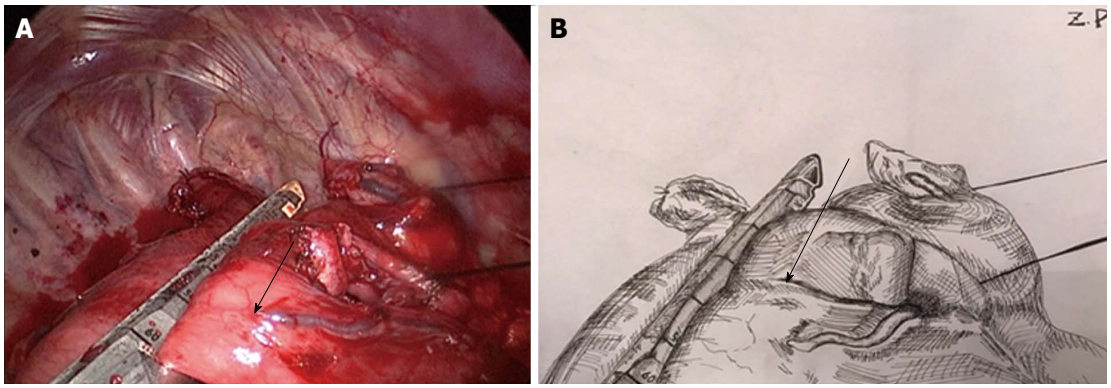

Figure 6 Intraoperative view (A) and schematic diagram (B) of the resection of the connection region (arrows indicate the gastric pouch).

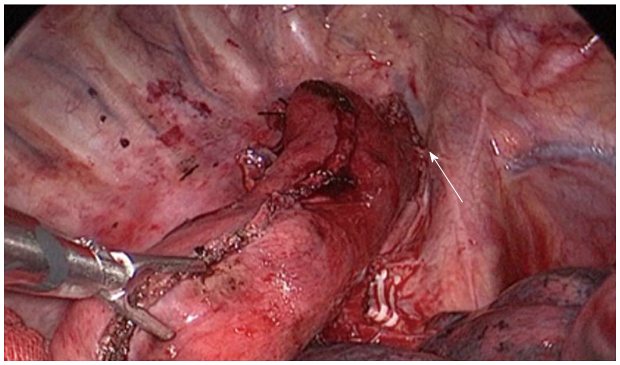

Figure 7 Anastomotic site and gastric tube after anastomosis. The arrow indicates the anastomotic site.

bilateral para-recurrent laryngeal lymph nodes. A prospective randomized study comparing Ivor-Lewis and Sweet esophagectomy for middle and lower EC demonstrated that the former is superior to the latter with respect to the extent of lymph node dissection and incidence of postoperative complications[6]. Ivor-Lewis esophagectomy has currently become the standard surgical procedure for middle and lower EC. With constant improvement of the minimally invasive technique, laparoscopic-thoracoscopic Ivor-Lewis esophagectomy has exhibited more advantages over open or hybrid Ivor-Lewis esophagectomy in terms of the perioperative complications, postoperative pain, length of hospital stay, and survival benefit[24-29]. However, gastric tube creation and esophageal-gastric anastomosis during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy still have significant technical difficulties. The two-step method for creating gastric tubes that we present here combine gastric tube creation and esophageal-gastric anastomosis, simplifying the surgical procedure, reducing the operating time, and achieving totally laparoscopic-thoracoscopic Ivor-Lewis esophagectomy.

During conventional laparoscopic-thoracoscopic Ivor-Lewis esophagectomy, an additional abdominal incision is required, and the gastric tube is created outside the abdominal cavity. Since the surgical assistant needs to lift the area on the greater curvature side and the area to be anastomosed in the fundus of the stomach to flatten the gastric tissue, this inevitably damages the upper part of the gastric tube, destroying the microvascular blood supply to the region and increasing the risk of poor blood supply to the anastomotic site. During the operation using the two-step method, the gastric tube is created in the thoracic cavity. Due to space limitations, the stomach cannot be flattened, increasing the operative difficulty. The stapling angle and length of the "first firing" are particularly important. According to our experience, the stapling should be performed in the gastric angle with an upward angle of approximately 40 ° with the lessor curvature of the stomach, and the stapling length should be approximately 40 cm (Figure 8). After the first firing, a gastric pouch is formed. By pulling the gastric pouch to adjust the cutting angle and range, a distal gastric tube is created. Since this procedure does not pull the gastric tissue in the anastomotic area to destroy the microvascular network, it is theoretically associated with a lower risk of poor blood supply to the anastomotic site. The creation of a gastric tube in the abdominal cavity requires fewer operative steps and can thus reduce the operating time once surgeons are skilled in the procedure.

During conventional laparoscopic-thoracoscopic Ivor-Lewis esophagectomy, the stomach and esophagus are

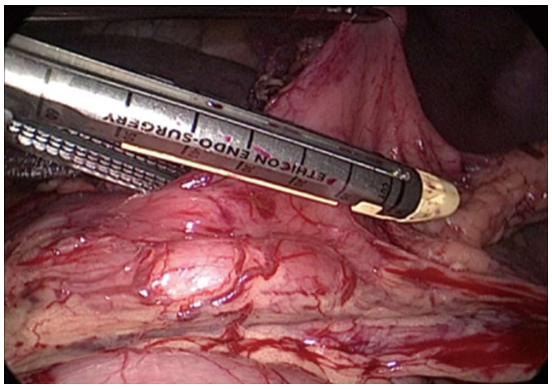

Figure 8 Stapling angle and length of the first firing.

transected and then connected by suturing. When the slender gastric tube is lifted behind the thoracic cavity, it may be twisted[30]. In contrast, the stomach and esophagus are not transected after the creation of a gastric tube by the two-step method, which makes the tension produced during the lifting process distribute uniformly, avoiding the angulation and twisting of the gastric tube. Moreover, the direction for lifting the gastric tube to the thoracic cavity is easily fixed, reducing the operative difficulty.

For anastomosis in the thoracic phase, the conventional method requires an additional incision in the wall of the gastric tube. After placing the anastomat, the fundus of the stomach is pulled anteroinferiorly. An anastomotic region is then selected in the posterior wall of the stomach from which the link rod of the anastomat goes through and enters the stapler cartridge. The fundus tissue that contains the incision in the gastric wall is excised. This anastomotic procedure will inevitably result in mechanical damage to the upper portion of the gastric tube. In addition, to reduce the risk of poor anastomotic healing, the mechanically damaged gastric fundus region is also resected, increasing the length of the resected gastric tube and the anastomotic tension. When anastomosis is performed in the posterior wall of the stomach, anastomotic tension in the posterior wall is greater than that in the anterior wall, leading to non-uniform tension. In the two-step method, these operations are performed *via* the operating port, and the gastric pouch and proximal end of the gastric wall can be lifted out of the thoracic cavity, reducing the operative difficulty of the remaining steps in the thoracic cavity, such as clipping of the gastric tube and creation of an incision in the gastric pouch to place the anastomat. The head-end gastric tube is created in a retrograde manner *in vitro*, and only the connection region is resected and sutured in the thoracic cavity, avoiding significant difficulty in clipping the remaining gastric tube due to the space limitation of the pleural top after high-level anastomosis as well as the anastomotic tension caused by pulling the gastric pouch. Since the majority of operations *via* the operating port by the two-step method are operated outside the abdominal cavity by the conventional method, this greatly simplifies the operating steps, reducing the operating time and saving the energy consumption of the surgeon and surgical assistants.

Compared to the conventional method, because of the lack of damage to the fundus of the stomach, the two-step method can better protect the microvascular network within the gastric wall, providing a better arterial blood supply and venous return. Moreover, the two-step method can, to the greatest extent, reduce anastomotic tension and make it uniformly distributed. In addition, the two-step method can decrease the number of stapler cartridges used (about 1), reducing the economic burden for patients.

In conclusion, the two-step method realize stotal laparoscopic-thoracoscopic Ivor-Lewis esophagectomy by avoiding an additional abdominal incision and conducting operations *via* the operating port to simplify the complicated operation steps, which greatly reduces the difficulty in creating the gastric tube after anastomosis and shortens the operating time. This newer approach is easy for surgeons to learn and can reduce the economic burden for patients and the energy consumption of the surgeons and surgical assistants. The temporary reservation of the gastric pouch that will be excised later makes all damage confined in the region to be excised, ensuring no damage to the gastric tube in the whole surgical process and reducing the anastomotic tension and making it more uniform. However, because this method only involves embedding suture of the cutting border and poorly stapled areas and fails to conduct full-length embedding suture of the gastric tube, the safety of the surgical border for the gastric tube remains to be observed.

COMMENTS

Background

Esophageal cancer is a common malignancy of the upper digestive tract in China. Ivor-Lewis esophagectomy combined with two-field lymphadenectomy has become the standard procedure for middle and lower esophageal cancer. The stomach is the most commonly used substitute for the esophagus, with advantages in creating gastric tube. However, the process is time consuming, requires multiple gastric incisions, and increases patients' economic burden as many medical devices are required. To overcome these shortcomings, this study proposed a two-step method for creating a gastric tube and assessed its feasibility during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy.

Research frontiers

Currently, many procedures for esophageal resection and reconstruction are available, and Ivor-Lewis esophagectomy combined with two-field lymphadenectomy has become the standard procedure for middle and lower esophageal cancer Compared with open Ivor-Lewis esophagectomy, minimally invasive laparoscopic-thoracoscopic Ivor-Lewis esophagectomy has an obvious advantage in reducing the incidence of perioperative complications while having similar therapeutic outcomes.

Innovations and breakthroughs

The two-step method realizes totally laparoscopic-thoracoscopic Ivor-Lewis

esophagectomy by avoiding an additional abdominal incision and conducting operations *via* the operating port to simplify the complicated operation steps, thus greatly reducing the difficulty in creating the gastric tube after anastomosis and shortening the operating time. It is easy for surgeons to learn and can reduce the economic burden of patients and the energy consumption of the surgeon and surgical assistants.

Applications

The two-step method will benefit the patients for minimally invasive Ivor-Lewis esophagectomy.

Peer-review

In this study, the authors introduced a two-step method for creating a gastric tube during laparoscopic-thoracoscopic Ivor-Lewis esophagectomy and assess its clinical application. The results are interesting.

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ORIGINAL ARTICLE

Observational Study

Clinical value of liver and spleen shear wave velocity in predicting the prognosis of patients with portal hypertension

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Abstract

AIM

To explore the relationship of liver and spleen shear wave velocity in patients with liver cirrhosis combined with portal hypertension, and assess the value of liver and spleen shear wave velocity in predicting the prognosis of patients with portal hypertension.

METHODS

All 67 patients with liver cirrhosis diagnosed as portal hypertension by hepatic venous pressure gradient in our hospital from June 2014 to December 2014 were enrolled into this study. The baseline information of these patients was recorded. Furthermore, 67 patients were followed-up at 20 mo after treatment, and liver and spleen shear wave velocity were measured by acoustic radiation force impulse at the 1st week, 3rd month and 9th month after treatment. Patients with favorable prognosis were assigned into the favorable prognosis group, while patients with unfavorable prognosis were assigned into the unfavorable prognosis

group. The variation and difference in liver and spleen shear wave velocity in these two groups were analyzed by repeated measurement analysis of variance. Meanwhile, in order to evaluate the effect of liver and spleen shear wave velocity on the prognosis of patients with portal hypertension, Cox's proportional hazard regression model analysis was applied. The ability of those factors in predicting the prognosis of patients with portal hypertension was calculated through receiver operating characteristic (ROC) curves.

RESULTS

The liver and spleen shear wave velocity in the favorable prognosis group revealed a clear decline, while those in the unfavorable prognosis group revealed an increasing tendency at different time points. Furthermore, liver and spleen shear wave velocity was higher in the unfavorable prognosis group, compared with the favorable prognosis group; the differences were statistically significant (P < 0.05). The prognosis of patients with portal hypertension was significantly affected by spleen hardness at the 3rd month after treatment [relative risk (RR) = 3.481]. At the 9th month after treatment, the prognosis was affected by liver hardness (RR = 5.241) and spleen hardness (RR = 7.829). The differences between these two groups were statistically significant (P < 0.05). The ROC analysis revealed that the area under the curve (AUC) of spleen hardness at the 3rd month after treatment was 0.644, while the AUCs of liver and spleen hardness at the 9th month were 0.579 and 0.776, respectively. These might predict the prognosis of patients with portal hypertension.

CONCLUSION

Spleen hardness at the 3rd month and liver and spleen shear wave velocity at the 9th month may be used to assess the prognosis of patients with portal hypertension. This is hoped to be used as an indicator of predicting the prognosis of patients with portal hypertension.

Key words: Liver cirrhosis; Portal hypertension; Liver and spleen shear wave velocity; Acoustic radiation force impulse

Core tip: Sixty-seven patients with liver cirrhosis with portal hypertension were assessed by acoustic radiation force impulse imaging at different time points after treatment. We found that the portal hypertension was significantly affected by spleen hardness at the 3rd month after treatment [relative risk (RR) = 3.481]. At the 9th month after treatment, the prognosis was affected by liver hardness (RR = 5.241) and spleen hardness (RR = 7.829). ROC analysis revealed that the area under the curve of liver and spleen hardness might be used to predict the prognosis of patients with portal hypertension.

Zhang Y, Mao DF, Zhang MW, Fan XX. Clinical value of liver and spleen shear wave velocity in predicting the prognosis of patients with portal hypertension. *World J Gastroenterol* 2017; 23(45): 8044-8052 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8044.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8044

INTRODUCTION

Portal hypertension is a common cause of cirrhosis and presents a series of symptoms[1,2]. In recent years, with the increase in incidence of liver cirrhosis, the number of patients with portal hypertension has rapidly increased[3]. The main clinical manifestations of portal hypertension are hepatosplenomegaly and ascites, which bring great negative impact on patients[4]. Due to its hard texture and obvious symptoms, splenomegaly associated with portal hypertension is significantly different from others, and is regarded as one of the main features of portal hypertension[5-7]. In clinical practice, severe complications of portal hypertension, including gastric fundus, esophageal varices, hepatic encephalopathy and gastrointestinal bleeding, have increased the risk of exacerbation, and even death[8-11]. In order to avoid complications and reduce the mortality of patients, early and effective evaluation indicators should be developed for predicting the prognosis.

As a new technology of ultrasonic elastography, acoustic radiation force impulse (ARFI) imaging can quantitatively reflect the advantages of tissue hardness by detecting the degree of deformation of the organ after compression, in order to assess the elasticity and hardness of tissues[12-15]. These detected results are displayed through imaging[16,17]. Although the clinical value of ARFI in predicting liver fibrosis, tumors and other diseases has been confirmed[18,19], researches on ARFI for detecting portal hypertension caused by cirrhosis have not been studied in detail. Hence, it remains to be determined whether ARFI has the ability to detect and evaluate portal hypertension prognosis.

Therefore, in this study, liver and spleen ARFI shear wave velocity values were determined to evaluate the clinical significance of ARFI for detecting portal hypertension caused by liver cirrhosis in patients, aiming to provide predictive indications for the prognosis of patients and avoid complication and death.

MATERIALS AND METHODS

Study objective

A total of 67 patients with liver cirrhosis, who were diagnosed with portal hypertension by hepatic venous pressure gradient (HVPG) in our hospital from June 2014 to December 2014, were included in this study. Among these patients, 42 were male and 25 were female; age of these patients ranged within 20-70 years old, with an average age of 52.68 ± 7.43 years-old. This research was approved by the Ethics Committee of our hospital, and all patients provided a signed informed

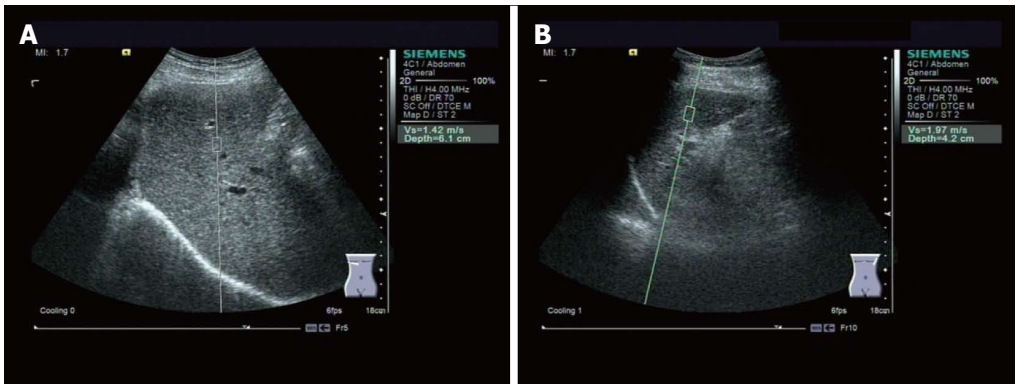

Figure 1 Measurement of liver and spleen hardness in patients by acoustic radiation force impulse. A: Liver hardness, SWV = 1.42 m/s; B: Spleen hardness, SWV = 1.97 m/s. SWV: Shear wave velocity.

consent.

Inclusion and exclusion criteria

Inclusion criteria: (1) patients diagnosed with liver cirrhosis through clinical symptoms combined with laboratory or image examinations; (2) patients with HVPG $\leqslant$ 12 mmHg; and (3) patients classified as grade A or B in the Child-Pugh grading criteria. Exclusion criteria: (1) patients whose shear wave velocity values could not be acquired by ARFI; (2) patients with liver cancer or other complications; (3) patients with acute heart failure, cardiogenic shock, or other vital organs diseases; (4) patients who underwent splenic surgery; (5) patients whose hepatosplenomegaly was caused by acute infection and other reasons; and (6) patients who were receiving propranolol hydrochloride or other drugs that can affect portal pressure.

Instruments and methods

All 67 patients underwent routine clinical examinations at the day of hospitalization, and the serological indicators of liver function and clinical symptoms of these patients were recorded. After treatment, liver and spleen ARFI shear wave velocity was measured continuously for all patients for 1 wk, and the results were recorded. Patients were instructed to fast at least 8 h before the measurement. A Siemens Acuson S2000 ultrasound system was used with a 4C1 convex array probe. During the liver shear wave velocity measurement, patients were asked to completely hold their breath and lie on the right side (Figure 1A). The probe was kept vertically fixed on the intercostal space to avoid larger blood vessels. This was repeated three times, and measurement results averaged. During the spleen shear wave velocity measurement, patients were instructed to hold their breath and lie on their left side (Figure 1B). These steps were repeated and the mean shear wave velocity values were recorded.

Follow-up

All 67 patients were followed-up for 20 mo after treatment. The first follow up was at the 1st month, and subsequent follow ups were performed by telephone every 3 mo. At the 3rd and 9th month, the liver and spleen ARFI shear wave velocity values of patients were measured, and the results were recorded. The endpoint of this study was the unfavorable prognosis of patients during the follow-up period, which include complications or death after treatment. After the follow ups, initial results upon admission and results of the last follow up were collected. In addition, the number of patients with unfavorable prognosis, serological indicators, the liver and spleen shear wave velocity values at the 1st week and at the 3rd and 9th month after treatment, and the diagnosis of the physician were recorded. Patients who were lost, refused visit, quit or died from other causes unrelated to the study were defined as censored.

Data processing after follow-up

During the 20-mo follow-up period, patients with favorable prognosis were assigned into the favorable prognosis group, while patients with unfavorable prognosis were as assigned into the unfavorable prognosis group. At the end of the follow-up period, baseline information, prognosis results, serological indicators of liver function, and liver and spleen ARFI shear wave velocity measurements were analyzed. The baseline information of patients included age and sex. The prognosis results were classified as either favorable prognosis or unfavorable prognosis. The serological indicators of liver function included albumin (ALB), alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Statistical analysis

SPSS 19.0 was used for statistical analysis. Shear wave velocity values, and the values of serological indicators ALB, AST and ALT and other measurement data were presented as mean ± SD. The unfavorable prognosis rate of patients and follow-up results were expressed *via* survival curve and pie chart, respectively, to analyze the prognosis of patients with portal hypertension. The variation and difference of shear wave velocity values in these two groups at the 1st week and the 3rd

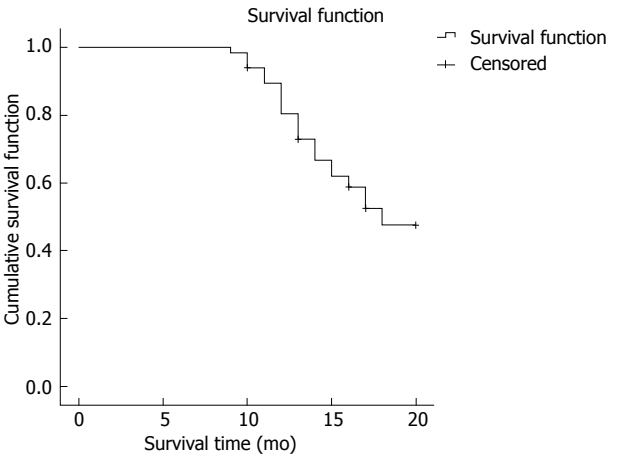

Figure 2 Analysis of the survival curve for the unfavorable prognosis rate of patients with portal hypertension.

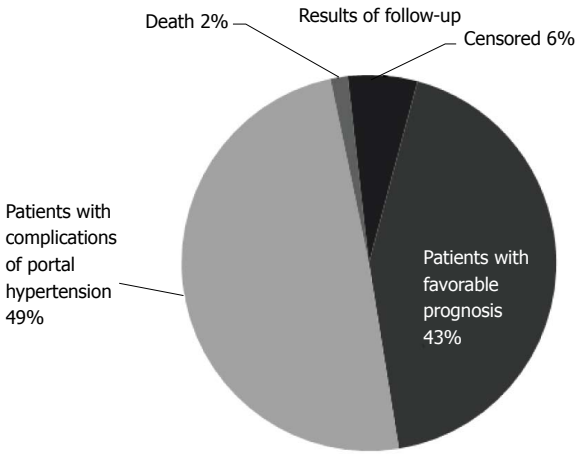

Figure 3 Results of follow up.

and 9th month after treatment were compared using repeated measures analysis of variance to explore the relationship between shear wave velocity values at different time points and portal hypertension. Age, sex, prognosis results, ALT, ALB and AST, shear wave velocity values and other possible influences were included in the Cox's proportional hazard regression model analysis. and indicators that affected prognosis were selected. On this basis, the receiver operating characteristic (ROC) curve was used to further compare with the area of all indicators that have statistically significant differences the area under the curve (AUC), and to investigate the ability of indicators that could predict the prognosis of patients with portal hypertension. $P < 0.05$ was considered statistically significant.

RESULTS

Analysis of follow-up results and unfavorable prognosis rate

The follow-up results revealed 29 patients in the favorable prognosis group and 34 patients in the unfavorable group. Among these patients, 11 patients died, 60 patients had adverse complications, and 4 patients were lost to follow-up. The Kaplan-Meier survival curve was used to analyze the incidence of portal hypertension complications after treatment. Results revealed that the unfavorable prognosis rate was 58.97%, as shown in Figures 2 and 3.

Comparison of liver shear wave velocity values between the favorable prognosis group and unfavorable prognosis group

The analysis results of variations in liver shear wave velocity values revealed that the liver shear wave velocity value in the unfavorable prognosis group exhibited an increasing trend, while a clear decline was observed in the favorable prognosis group ($F_{\text{within group}} = 2.106$, $P_{\text{within group}} = 0.039$). The liver shear wave velocity value was highest at the 1st week after treatment in the favorable prognosis group, which gradually decreased thereafter. This value was lowest at to 9th month after treatment. However, in the unfavorable prognosis group, the liver shear wave velocity value was highest at the 9th month and lowest at the 1st week after treatment.

The liver shear wave velocity values of these two groups were compared among different time points. Although, these values in the favorable prognosis group were slightly higher than those in the unfavorable prognosis group and the difference was not statistically significant ($P > 0.05$). Furthermore, the difference in liver shear wave velocity values at the 3rd and 9th month was statistically significant ($P < 0.05$), and these values in the favorable prognosis group were lower. Overall, the liver shear wave velocity values in the unfavorable prognosis group were higher than those in the favorable prognosis group, and the difference was statistically significant ($F_{\text{between groups}} = 2.193$, $P_{\text{between groups}} = 0.032$). Furthermore, these values correlated between the different groups or among different times points ($F_{\text{interaction}} = 2.457$, $P_{\text{interaction}} = 0.017$) (Table 1).

Comparison of spleen shear wave velocity values between the favorable prognosis group and unfavorable prognosis group

In the favorable prognosis group, the spleen shear wave velocity values declined from the 1st week to the 9th month after treatment. The value at the 1st week was highest and the value at the 9th month was lowest. However, spleen shear wave velocity values in the unfavorable prognosis group exhibited an increasing trend. The minimum and maximum values of the liver shear wave velocity were reached at the 1st week and 9th month after treatment, respectively. Values at the different time points of these two groups are presented in Table 2.

These results revealed that spleen shear wave velocity values at the 3rd and 9th month in the unfavorable prognosis group were higher, and the difference was statistically significant ($P < 0.05$). Furthermore, values at the 1st week in these two groups

Table 1 Comparison of the liver shear wave velocity values of the two groups at the 1st week and at the 3rd and 9th month after treatment

Group	1st wk after treatment, m/s	3rd mo after treatment, m/s	9th mo after treatment, m/s
Favorable prognosis group	1.88 ± 0.39	1.70 ± 0.41	1.67 ± 0.38
Unfavorable prognosis group	1.84 ± 0.43	1.92 ± 0.43	2.08 ± 0.35
t	0.384	2.068	4.455
P value	0.702	0.043	0.000

Data are presented as mean ± SD. (1) $F_{\text{within group}}$ = 2.106, $P_{\text{within group}}$ = 0.039; (2) $F_{\text{between groups}}$ = 2.193, $P_{\text{between groups}}$ = 0.032; (3) $F_{\text{interaction}}$ = 2.457, $P_{\text{interaction}}$ = 0.017.

Table 2 Comparison of spleen shear wave velocity values between the two groups at the 1st week, 3rd month and 9th month after treatment

Group	1st wk after treatment, m/s	3rd mo after treatment, m/s	9th mo after treatment, m/s
Favorable prognosis group	3.82 ± 0.44	3.71 ± 0.42	3.55 ± 0.34
Unfavorable prognosis group	3.83 ± 0.46	4.06 ± 0.44	4.29 ± 0.30
t	0.088	3.213	9.178
P value	0.930	0.002	0.000

Data are presented as mean ± SD. (1) $F_{\text{within group}}$ = 2.544, $P_{\text{within group}}$ = 0.013; (2) $F_{\text{between groups}}$ = 8.431, $P_{\text{between groups}}$ = 0.000; (3) $F_{\text{interaction}}$ = 3.422, $P_{\text{interaction}}$ = 0.001.

were similar, and the difference was not statistically significant ($P > 0.05$). The spleen shear wave velocity values were compared between these two groups. These results revealed that values in the unfavorable prognosis group were higher than in the favorable prognosis group, and the difference was statistically significant ($F_{\text{between groups}}$ = 8.431, $P_{\text{between groups}}$ = 0.000). The values in different groups or at different time points were correlated ($F_{\text{interaction}}$ = 3.422, $P_{\text{interaction}}$ = 0.001).

Evaluation of the effects of suspicious indicators using the Cox's proportional hazard regression model

A Cox's proportional hazard regression model was constructed to analyze the effects of all suspicious indicators on portal hypertension prognosis. These results revealed that the age and sex of patients had no effect on prognosis ($P > 0.05$), and serological indicators including ALB, AST and ALT did not influence the prognosis. At the same time, all liver and spleen shear wave velocity values at different time points were evaluated, and results revealed that liver shear wave velocity values at the 9th month and spleen shear wave velocity values at the 3rd and 9th month could affect the prognosis of patients ($P < 0.05$); other values had no significant effects ($P > 0.05$). All indicators that had statistically significant differences were compared. The spleen shear wave velocity value at the 9th month after treatment had the strongest effect on the prognosis of patients (relative risk (RR) = 8.829). The liver hardness value at the 9th month ranked second (RR = 6.271), followed by the spleen hardness value at the 3rd month (RR = 3.481), as shown in Table 3.

Comparison of the ability of liver shear wave velocity values at the 9th month and spleen shear wave velocity values at the 3rd and 9th month for predicting prognosis

In order to analyze the predictive ability of these three indicators for adverse prognosis, the ROC curve was established. This revealed that the AUC of spleen shear wave velocity values at the 3rd month and the AUC of the liver and spleen shear wave velocity values at the 9th month were 0.644, 0.579 and 0.776, respectively. It was found that the AUC of the spleen shear wave velocity value at the 9th month was highest. The sensitivity was 55.9%, specificity was 89.7% and the best diagnostic value was 0.455. Furthermore, the AUC of the spleen shear wave velocity value at the 3rd month was slightly lower, and the sensitivity, specificity and best diagnostic value was 70.6%, 58.6% and 0.292, respectively. The lowest AUC was the liver shear wave velocity value at the 9th month, and the sensitivity, specificity and best diagnosis value was 73.5%, 48.3% and 0.218, respectively (Figure 4).

DISCUSSION

Portal hypertension is a clinical syndrome caused by portal venous drainage obstruction. This occurs in middle-age men and develops slowly, and most of these cases are closely associated with cirrhosis[2,20-22]. In China, the number of new patients with cirrhosis increase year after year[23]. At the same time, the incidence of portal hypertension has also rapidly increased[24]. The majority of patients often present with liver dysfunction, bleeding, gastrointestinal vascular disease and other serious diseases, except common clinical symptoms, including hepatosplenomegaly and ascites[25].

At present, the main approaches for the clinical treatment of portal hypertension are surgery and symptomatic treatment[26]. Although these treatment approaches are diverse and effective, the mortality rate of patients who have this disease remains high due to delitescent pathogenetic condition, long disease duration

Table 3 Cox's proportional hazard regression model analysis of the prognosis of patients with portal hypertension

	B	SE	Wald	df	*P* value	RR	95%CI	
							Upper limit	Lower limit
ALB	-0.030	0.083	0.131	1	0.718	0.970	0.824	1.143
ALT	-0.014	0.021	0.415	1	0.520	0.986	0.946	1.028
AST	-0.007	0.028	0.068	1	0.794	0.993	0.939	1.049
Sex	-0.909	0.615	2.181	1	0.140	0.403	0.121	1.346
Age	0.016	0.036	0.192	1	0.661	1.016	0.947	1.090
Liver hardness in the 1st week after treatment	0.175	0.698	0.063	1	0.802	1.191	0.304	4.676
Liver hardness in the 3rd mo after treatment	1.155	0.769	2.258	1	0.133	3.175	0.704	14.329
Liver hardness in the 9th mo after treatment	1.657	1.123	3.930	1	0.047	5.241	1.026	83.802
Spleen hardness in the 1st week after treatment	0.034	0.024	2.089	1	0.148	1.035	0.988	1.084
Spleen hardness in the 3rd mo after treatment	1.247	0.583	4.576	1	0.032	3.481	1.110	10.914
Spleen hardness in the 9th mo after treatment	2.058	0.883	6.079	1	0.014	7.829	1.563	49.870

ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; df: Degrees of freedom; RR: Relative risk.

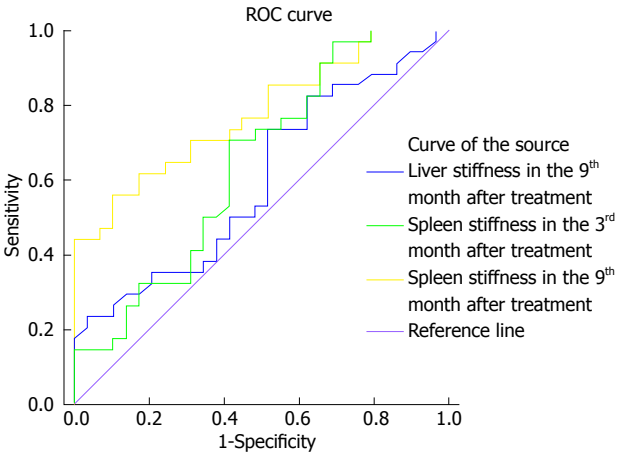

Figure 4 ROC curves of liver and spleen hardness for predicting prognosis of patients with portal hypertension.

and proneness to complications[27]. Therefore, it is important to establish a simple and effective system for the prognosis of portal hypertension, in order to monitor the disease, adjust the treatment approaches, and improve the survival rate of patients in real time. The varying degrees of hardness of the lesions are usually related to the severity of the disease[28]. Furthermore, the swelling and hardness of the hepatosplenomegaly of portal hypertension are more visible than those of other diseases[29,30]. This shows that there may be relationships between liver and spleen hardness and portal hypertension[31-33].

In the present study, it was shown that studies have investigated the relationship between liver and spleen hardness and liver fibrosis, chronic liver, or other liver diseases[34-36]. However, there is lack of further research on the relationship between liver and spleen hardness and portal hypertension, and the clinical value of liver and spleen hardness in evaluating the prognosis of portal hypertension could not be determined. In recent years, as a mature method of examination, ARFI imaging promotes the implementation of detecting tissue hardness to predict the development of diseases[37,38]. Due to its simple, convenient and good repeatability advantages, ARFI imaging has gained the attention of clinicians. Hence, we detected the liver and spleen ARFI shear wave velocity values of these patients and analyzed their prognosis, in order to evaluate the clinical application value of ARFI in predicting the prognosis of portal hypertension.

Analysis of unfavorable prognosis rates and the comparison of liver and spleen shear wave velocity values in these two groups

The common adverse complications of portal hypertension include esophageal and gastric variceal bleeding, hepatic encephalopathy, hepatorenal syndrome and others; these are the main indications of surgery for treating portal hypertension[39]. Among these complications, esophageal and gastric variceal bleeding were the most dangerous[40,41]. When this occurs, patients will be at risk due to acute upper gastrointestinal bleeding[42-44]. Therefore, it has been considered that the establishment of a prognostic detection system for portal hypertension has clinical value[45-47].

In this study, patients were enrolled according to the Child-Pugh criteria and HVPG results. Patients classified as grade C and having an HVPG $\geqslant$ 12 mmHg were excluded due to higher risk of surgery, lower survival rate and poor recovery. The Kaplan-Meier survival curve revealed that the unfavorable prognosis rate in all 67 patients was 58.97% at the end of follow-up, which reflects that it is unsatisfactory of the prognosis of patients with portal hypertension. In order to investigate the variation trend of liver and spleen shear wave velocity values, values at three different time points were collected and analyzed by repeated measures analysis of variance.

Results revealed that the liver and spleen shear wave velocity values in the favorable prognosis group exhibited a decreasing trend, and there were significant differences in these values at three different time points. Values are lowest in the favorable

prognosis group at the 9th month, but the variations in these values in the unfavorable prognosis group were the opposite. This suggests that there may be a link between the variation in liver and spleen shear wave velocity values and the development of portal hypertension. When comparing the overall values of liver and spleen shear wave velocity in these two groups, values in the unfavorable prognosis group was significantly higher than in the favorable prognosis group. This demonstrates that there is a potential relationship between liver and spleen shear wave velocity and the prognosis of patients with portal hypertension[9,48,49].

Comparative analysis of indicators that affect the prognosis of patients

In order to further investigate indicators that affect the prognosis of portal hypertension, Cox's proportional hazard regression model was performed on liver function serum markers, clinical data and liver and spleen shear wave velocity values at three different time points. As common clinical detection indicators, liver function serum markers were detected to reflect the degree of liver damage. In this study, ALB, ALT and AST were included in the Cox's regression model to evaluate their effect on the prognosis of patients with portal hypertension.

Results revealed that liver function serum markers ALB, ALT and AST have no significant effect on the prognosis of this disease. The reasons may be that the variation in ALB, ALT, and AST values are also associated with many diseases such as hepatitis, myocarditis and Japanese B encephalitis, except for cirrhosis. Hence, liver function markers have low sensitivity for the diagnosis and prediction of diseases. Therefore, it is unsatisfactory to take serum markers of liver function as a prognostic indicator of portal hypertension.

On the contrary, in analyzing liver and spleen shear wave velocity values at three different time points, it can be found that the spleen shear wave velocity value at the 3rd month and liver and spleen shear wave velocity value at the 9th month can significantly affect the prognosis of portal hypertension. Among these indicators, the effect of the spleen shear wave velocity value is the most significant, while the value at the 3rd month was the lowest. However, liver and spleen hardness at the 1st week after treatment had no significant effect. This may be due to the improvement of the liver and spleen in the short period after treatment. In addition, the liver shear wave velocity value also has no effect on the prognosis of this disease. The possible reasons are that that liver hardness is not a feature of portal hypertension, this value can be affected by many factors, and the variation degree is similar.

The results of this study show that spleen hardness at the 3rd month and liver and spleen hardness at the 9th month have the potential to assess the prognosis of portal hypertension.

Comparison of the ability of liver and spleen shear wave velocity values at different time points in predicting the prognosis of portal hypertension

Based on the above data, the ROC curve was constructed to further investigate the predictive ability of liver hardness at the 9th month and spleen hardness at the 3rd and 9th month. As a result, the AUC of the spleen shear wave velocity value at the 9th month was highest, which was over 0.7. This revealed that this value had a better predictive ability on the prognosis of portal hypertension, while the AUC of liver hardness at the 9th month and spleen hardness at the 3rd month are lower, and their predictive ability are slightly insufficient. The comprehensive analysis shows that the liver shear wave velocity values at the 9th month and the spleen shear wave velocity values at the 3rd and 9th month are expected to be used as predictive indicators for the prognosis of patients with portal hypertension. Furthermore, this can be combined with other prognosis detection indictors in evaluating the risk of patients.

However, our study still has some deficiency, which includes the small sample data, the inadequate time points for ARFI detection, and the lack of coverage on other types of portal hypertensions. Therefore, future studies with larger samples and adequate detection time points should be conducted to evaluate the other types of portal hypertensions and verify our findings.

In summary, liver and spleen ARFI shear wave velocity values have the potential to monitor the prognosis of portal hypertension, and liver shear wave velocity values at the 9th month and spleen shear wave velocity values at the 3rd and 9th month can reflect the prognosis of patients. It is hoped that this approach could be applied in clinic to reduce complications and improve the survival rate of patients.

COMMENTS

Background

Portal hypertension is a common cause of cirrhosis and presents a series of serious symptoms. In recent years, the incidence rate of liver cirrhosis as well as the portal hypertension rate in China are increasing. The main clinical symptoms of portal hypertension are hepatosplenomegaly and ascites, which bring great negative impact on patients. Because of the hard texture and obvious symptoms, splenomegaly associated with portal hypertension is regarded as one of the main features of portal hypertension. In clinical practice, severe complications of portal hypertension, including gastric fundus, esophageal varices, hepatic encephalopathy and gastrointestinal bleeding, have increased the risk of exacerbation, and even death.

Research frontiers

Acoustic radiation force impulse (ARFI) imaging can quantitatively reflect the advantages of tissue hardness by detecting the degree of deformation of the organ after compression, in order to assess the elasticity and hardness of tissues. These detected results are displayed through imaging. Although the clinical value of ARFI in predicting liver fibrosis, tumors and other diseases has been confirmed, research on ARFI for detecting portal hypertension caused by cirrhosis has not been carried out in detail.

Innovations and breakthroughs

ARFI could be used to determine liver and spleen hardness by detecting the degree of deformation of the organ after compression. ARFI is convenient, non-invasive and simple. In this study, we used the technology to figure out the relation between portal hypertension and to try to evaluate the predictive value for portal hypertension.

Applications

The study illustrated the ability for ARFI to be applied to detecting portal hypertension in clinical practice. The prognosis of patients with portal hypertension was significantly affected by spleen and liver hardness. Spleen hardness at the 3rd month, and liver and spleen shear wave velocity at the 9th month may be used to assess the prognosis of patients with portal hypertension. It is expected to be used as an indicator of predicting the prognosis of patients with portal hypertension.

Peer-review

This study illustrated that ARFI imaging could be used in detecting portal hypertension by detecting liver and spleen harshness. Shear wave velocity is a quantitative indicator that is accurate and objective. The detecting process of ARFI is simple, non-invasive, fast and widely used for detection and prediction in clinical practice. Thus, ARFI imaging is a helpful tool that has a significant clinical value and is worthy of developing.

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ORIGINAL ARTICLE

Prospective Study

Gender differences in ghrelin, nociception genes, psychological factors and quality of life in functional dyspepsia

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Abstract

AIM

to evaluate gender differences in the aspect of ghrelin, nociception-related genes and psychological aspects and the quality of life (QoL) in Korean functional dyspepsia (FD) patients.

METHODS

Total of 191 persons were prospectively enrolled between March 2013 and May 2016 in Seoul National Bundang Hospital, and classified into control and FD group based

on ROME Ⅲ criteria. Questionnaire included assessment for dyspepsia symptoms, QoL and anxiety or depression. Preproghrelin and nociception genes in the gastric mucosa and plasma acyl/des-acyl ghrelin were measured.

RESULTS

Lower level of plasma acyl ghrelin in FD patients compared to control was significant only in male (15.9 fmol/mL *vs* 10.4 fmol/mL, $P = 0.017$). Significantly higher mRNA expressions of nerve growth factor and transient receptor potential vanilloid receptor 1 were observed in male ($P = 0.002$ and $P = 0.014$, respectively) than in female. In contrast, female FD patients had a higher anxiety and depression score than male FD ($P = 0.029$), and anxiety score was correlated with epigastric pain only in female FD patients (female: Spearman rho = 0.420, $P = 0.037$). The impairment of overall QoL was more prominent in female FD patients than male patients (5.4 ± 0.3 *vs* 6.5 ± 0.3, $P = 0.020$).

CONCLUSION

Gender differences of ghrelin and nociception-related genes in male and psychological factors in female underlie FD symptoms. More careful assessment of psychological or emotional status is required particularly for the female FD patients.

Key words: Functional dyspepsia; Gender differences; Quality of life

Core tip: Gender-specific medicine has become a recently rising medical field in which differences between males and females are recognized and actively utilized in the clinical study, diagnosis and treatment. The lower level of plasma acyl ghrelin and higher expressions of nociception-related genes are associated with pathogenesis of functional dyspepsia (FD) in males, while female FD patients had more serious anxious and depressive mood. Underlying mechanism in FD could be different according to gender, and meticulous attention for psychological predisposition is required particularly in the treatment of female FD patients.

Choi YJ, Park YS, Kim N, Kim YS, Lee SM, Lee DH, Jung HC. Gender differences in ghrelin, nociception genes, psychological factors and quality of life in functional dyspepsia. *World J Gastroenterol* 2017; 23(45): 8053-8061 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8053.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8053

INTRODUCTION

Functional dyspepsia (FD) is a heterogeneous disorder characterized by recurrent upper abdominal discomfort or pain. According to the main symptom, FD is further classified into postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS). FD is not life-threatening but imposes a socio-economic burden due to its high prevalence. Several factors could underlie this disorder including abnormal motor function[1], visceral hypersensitivity[2], genetic predisposition[3] or psychosomatic feature[4]. Interestingly, most functional gastrointestinal (GI) disorder (FGID), including FD, shows female predominance[5].

Gender-specific medicine has become a recently rising medical field in which differences between males and females are recognized and actively utilized in the diagnosis and treatment. Gender is assumed to be a crucial factor in the pathogenesis, disease progression and even prognosis of certain diseases[6-8]. However, there have been only a few reported gender differences in FGIDs, and attention has focused mostly on irritable bowel syndrome (IBS). Moreover, the topic of most studies were restricted to the prevalence of FGID[9], specified GI symptoms[10] or quality of life (QoL)[11].

Ghrelin controls appetite[12] and modulates gastric motility. A reduced acyl ghrelin level has been correlated with impaired gastric emptying[13], leading to postprandial fullness or vomiting[14]. Transient receptor potential vanilloid-1 (TRPV1) is believed to be an important integrator of the transmission and modulation of pain with nerve growth factor (NGF) or glial cell line-derived neurotrophic factor (GDNF). Previously, we demonstrated that the genes encoding these nociception-related proteins are involved in the pathogenesis of FD, particularly in the EPS type[15]. Regarding the PDS type, we found an association of increased plasma acyl ghrelin levels with abatement of dyspepsia following *Helicobacter pylori* eradication[16]. However, we have not evaluated the differences of expression of ghrelin or nociception-related genes regarding gender specific manner. Female predominance of FGIDs maybe related with extraintestinal conditions such as hysterectomy, which was reported to be 3-fold higher in women with IBS[17]. However, its relationship with female FD has not been evaluated so far.

Against this background, we hypothesized that there might be a difference in the underlying mechanisms of FD which could cause a difference in QoL between males and females. To verify this hypothesis, we analyzed the possible etiological factors including ghrelin, nociception-related genes, psychological aspects and history of abdominal operation as well as basal characteristics, dyspepsia symptoms and QoL between male and female FD patients.

MATERIALS AND METHODS

Subjects

The subjects were enrolled prospectively at the Department of Gastroenterology of Seoul National University Bundang Hospital(SNUBH), between March 2013 and May 2016. All subjects were of Korean and received upper gastrointestinal endoscopies

and completed questionnaires about gastrointestinal symptoms including dyspepsia, emotional state and QoL under the supervision of a well-trained interviewer. History of abdominal operations including gynecological surgeries (*i.e.* hysterectomy, salpingooophorectomy or gynecologic surgery) was evaluated. Subjects were excluded if there was a history of gastrointestinal GI surgery, current duodenal/gastric ulcer and any history of malignancy. Users of non-steroid anti-inflammatory drugs/anticoagulants, patients with systemic diseases requiring chronic medication (except for hypertension and diabetes mellitus) were also excluded.

The subjects were classified into the FD or control group. FD was defined to the according to the Rome Ⅲ criteria[15,16]. FD patients were categorized into PDS, EPS and mixed subgroups on the basis of the Rome Ⅲ criteria[18]. Individuals without GI symptoms and any endoscopic lesion were assigned to the controls. The Institutional Review Board of SNUBH approved this study (B-1101/119-010), and written informed consent was obtained from all participants.

Dyspepsia symptom, emotional status and QoL assessment

The severities of epigastric pain/burning, postprandial fullness, early satiation and overall abdominal pain (not restricted to epigastric area) were scored using a five-point scale (0, none; 1, mild; 2, moderate; 3, severe; 4, very severe) using validated Korean version of Talley's bowel disease questionnaire[19]. Stool consistency based on Bristol Stool Form Scale[20] and the number of bowel habits was evaluated. In order to assess the anxiety and depression of participants, the hospital anxiety and depression scale (HADS) was used[21]. It is subdivided into anxiety and depression subscales, both containing seven items. Each response is ranked on a 4 point (0-3) scale. Higher HADS score indicates that the subject is more depressive or anxious, with a score > 7 of each subscale indicating potential anxiety disorder or depression[22]. History of abdominal operations including hysterectomy and gynecologic surgery were collected. World Health Organization quality of life scale field trial version (WHOQOL-BREF) was used to evaluate the QoL of each subject[23]. It consists of questions about overall QoL and general health with four domains of physical health, psychological health, social relationship and environmental domains. Results are expressed as an overall score (range 0-100) and domain score (range 0-20). Higher scores denote higher QoL. These three questionnaire s have been validated in Korea[19,24,25].

Upper endoscopy and biopsy

During endoscopy, biopsy specimens were obtained from the antrum, body and fundus for histological studies. Specimens taken from the fundus were used to measure the mRNA of preproghrelin, TRPV1, GDNF and NGF[16]. The baseline *H. pylori* infection status and histology using the updated Sydney scoring system[26] was evaluated (Supplementary document).

Measurement of preproghrelin and nociception-related gene expression

Preproghrelin- and nociception-related gene expression was measured based on previous studies[15,16]. Detailed method was described in Supplementary document.

Measurement of plasma ghrelin level

Acyl/desacyl ghrelin was measured according to previous study (Supplementary document)[13].

Statistical analysis

Categorical variables were analyzed by χ^2 test or Fisher's exact test. Continuous variables presented as mean or median were analyzed by Student *t*-test or Mann-Whitney test, respectively. Spearman correlation test was used to evaluate potential correlations between HADS score and dyspepsia symptoms. SPSS Statistics version 20.0 (IBM, Armonk, NY, United States) was used. All statistical tests were 2-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

General characteristics

A total of 191 subjects were included in this study. Among them, 87 subjects and 104 patients were classified into the control and FD group, respectively. Demographic characteristics of study population are summarized in Table 1. The control group was older than the FD group. The proportions of males were not significantly different between the control and FD groups (43.7% *vs* 37.5%. $P = 0.386$). The mean body mass index (BMI), *H. pylori* infection positivity, glandular atrophy and intestinal metaplasia were not significantly different between FD and control groups. However, there were more smokers in the FD group than the control group. The proportion of alcohol consumers was not significantly different between the groups.

Female FD patients had a lower BMI than male FD patients. A higher proportion of men smoked and consumed alcohol than women in the both FD and control groups (all $P < 0.001$). There were no significant gender differences in *H. pylori* infection positivity, glandular atrophy, intestinal metaplasia and the proportion of FD subtypes. Regarding history of gynecological surgeries, 87.8% of female subjects in the control and 73.8% of female subjects in the FD group responded. FD patients were more likely to have undergone gynecological surgeries, but this was insignificant (52.1% *vs* 30.2%, $P = 0.171$).

Comparison of expression of ghrelin and nociception-related gene

We analyzed whether gender and dyspepsia symptoms were associated with levels of plasma acyl-/desacyl ghrelin and expression of preproghrelin, NGF, GDNF

Table 1 Characteristics of the subjects *n* (%)

Variables	Control (*n* = 87)			FD (*n* = 104)			*P* value		
	Total (*n* = 87)	Male (*n* = 38)	Female (*n* = 49)	Total (*n* = 104)	Male (*n* = 39)	Female (*n* = 65)	Control *vs* FD	Control[1]	FD[1]
Age (mean ± SD, yr)	54.9 ± 12.1	57.4 ± 12.1	52.9 ± 11.8	50.5 ± 11.3	49.6 ± 11.6	51.3 ± 11.1	0.010	0.084	0.536
Male	38 (43.7)	38 (100)	0	39 (37.5)	39 (100)	0	0.386	-	-
BMI (mean ± SD, kg/m^2)	23.1 ± 2.9	22.4 ± 2.9	22.9 ± 3.30	22.4 ± 3.0	23.3 ± 2.7	21.9 ± 3.4	0.140	0.482	0.039
H. pylori	58 (66.7)	23 (60.5)	35 (71.4)	60 (57.7)	21 (53.8)	39 (60.0)	0.204	0.360	0.547
AG antrum	33 (37.9)	15 (39.5)	18 (36.7)	28 (26.9)	9 (23.1)	19 (29.2)	0.104	0.794	0.493
AG body	13 (14.9)	5 (13.2)	8 (16.3)	11 (10.6)	4 (10.3)	7 (10.8)	0.365	0.681	0.999
IM antrum/body	22 (25.3)	12 (31.6)	10 (20.4)	18 (17.3)	6 (15.4)	12 (18.5)	0.177	0.234	0.688
Smoking	13 (14.9)	10 (26.3)	1 (2.0)	24 (23.1)	17 (43.6)	2 (3.1)	< 0.001	< 0.001	< 0.001
Alcohol	20 (23.0)	16 (42.1)	4 (8.2)	30 (28.8)	21 (53.8)	9 (13.8)	0.342	< 0.001	< 0.001
Gynecologic surgery[2]	-	-	13/43 (30.2)	-	-	25/48 (52.1)	0.171	-	-
Subtype in FD	-	-	-	-	-	-	-	-	-
PDS	-	-	-	-	9 (23.1)	15 (23.1)	-	-	0.408
EPS	-	-	-	-	6 (15.4)	17 (26.2)	-	-	-
Mixed	-	-	-	-	24 (61.5)	33 (50.8)	-	-	-

[1]Between males and females; [2]Hysterectomy, salpingooophorectomy or cesarean section were included. Only some patients responded to this question. AG: Atrophic gastritis; BMI: Body mass index; EPS: Epigastric pain syndrome; mixed, postprandial distress syndrome and epigastric pain syndrome; FD: Functional dyspepsia; IM: Intestinal metaplasia; PDS: Postprandial distress syndrome; SD: Standard deviation; -: not available.

Table 2 Expression of plasma ghrelin, gastric peproghrelin and nociception-related genes in the control and functional dyspepsia groups

Variables	Control (*n* = 87)			FD (*n* = 104)			*P* value[1]		
	Total (*n* = 87)	Male (*n* = 38)	Female (*n* = 49)	Total (*n* = 104)	Male (*n* = 39)	Female (*n* = 65)	Control *vs* FD	Control[2]	FD[2]
Plasma acyl ghrelin (fmol/mL, median [IQR])	14.1 (9.1-20.8)	15.9 (9.2-33.7)	12.2 (9.0-18.6)	11.2 (6.6-16.8)	10.4 (6.5-18.4)	11.4 (6.8-16.5)	0.018	0.204	0.388
Plasma des-acyl ghrelin(fmol/mL, median [IQR])	67.9 (37.5-162.5)	108 (31.8-212.9)	65.4 (38.2-111.0)	62.1 (32.2-110.6)	58.5 (28.8-134.3)	63.6 (32.2-108.0)	0.297	0.389	0.913
Ghrelin mRNA (median [IQR])	2.6 (0.7-4.9)	2.1 (0.5-4.2)	3.2 (1.0-6.1)	1.7 (0.4-5.4)	1.9 (0.4-9.0)	1.5 (0.3-4.7)	0.435	0.076	0.308
NGF mRNA (median [IQR])	1.1 (0.7-1.7)	1.2 (0.7-1.8)	0.9 (0.5-1.6)	1.6 (0.9-2.3)	1.8 (1.1-2.7)	1.4 (0.8-2.2)	0.006	0.056	0.129
GDNF mRNA (median [IQR])	1.0 (0.7-1.6)	1.2 (0.8-1.5)	0.9 (0.7-1.7)	1.8 (1.0-2.9)	1.6 (1.1-3.0)	1.9 (0.9-2.9)	< 0.001	0.404	0.992
TRPV1 mRNA (median [IQR])	1.0 (0.6-1.5)	1.1 (0.8-1.6)	0.9 (0.6-1.2)	1.4 (0.9-2.3)	1.4 (0.9-2.2)	1.4 (0.8-2.3)	0.006	0.102	0.584

[1]Mann-Whitney test was used; [2]Male *vs* female. FD: Functional dyspepsia; GDNF: Glial cell-line derived neurotrophic factor; IQR: Interquartile range; NGF: Nerve growth factor; PDS: Postprandial distress syndrome; TRPV1: Transient receptor potential vanilloid receptor 1.

and TRPV1 mRNA (Table 2). While the levels of plasma acyl ghrelin in the control group was higher than in the FD group, those of NGF, GDNF and TRPV1 mRNA expressions in the control group were lower than in the FD group (Table 2) (all $P < 0.05$). When the comparison was restricted to control subjects, female control subjects tended to show lower levels of plasma acyl/desacyl ghrelin and mRNA expression level of NGF, GDNF and TRPV1 than male subjects, with no statistical significances. Preproghrelin mRNA was higher in female control subjects than male individuals, but was not significantly different. Among the FD group, there were no significant gender differences in expressions of the aforementioned proteins or genes.

The lower level of plasma acyl ghrelin in FD patients compared to controls subjects was significant only in men (15.9 fmol/mL *vs* 10.4 fmol/mL, $P = 0.017$; 12.2 fmol/mL *vs* 11.4 fmol/mL, $P = 0.348$). Higher expressions of most nociception-related genes were more prominent in men than in women (Table 3).

Dyspepsia symptoms and bowel habit

Supplementary Table 1 shows the severity of dyspepsia symptom and bowel habit in FD patients according to gender. The score of epigastric burning of female patients was higher compared to male patients (3.5 ± 0.1 *vs* 2.7 ± 0.2, $P = 0.047$). On the other hand, overall abdominal pain, early satiation and postprandial fullness were slightly more severe in male patients compared to female patients, but there was no statistical significance (Table 4). In case of nausea, the score was higher in female without statistical significance. In addition, there were no significant differences in stool consistency and number of defecations.

Table 3 Expression of plasma ghrelin, gastric peproghrelin and nociception-related genes in different gender

Variables	Male			Female		
	Control (n = 38)	FD (n = 39)	P value[1]	Control (n = 49)	FD (n = 65)	P value[1]
Plasma acylghrelin (fmol/mL, median [IQR])	15.9 (9.2-33.7)	10.4 (6.5-18.4)	0.017	12.2 (9.0-18.6)	11.4 (6.8-16.5)	0.348
Plasma des-acylghrelin (fmol/mL, median [IQR])	108.0 (31.8-212.9)	58.5 (28.9-134.3)	0.302	65.4 (38.2-111.0)	63.6 (32.2-108.0)	0.844
Ghrelin mRNA (median [IQR])	2.1 (0.5-4.2)	1.9 (0.4-9.1)	0.428	3.2 (1.0-6.1)	1.5 (0.3-4.7)	0.092
NGF mRNA (median [IQR])	1.2 (0.7-1.8)	1.8 (1.1-2.7)	0.002	0.9 (0.5-1.6)	1.4 (0.8-2.2)	0.119
GDNF mRNA (median [IQR])	1.2 (0.7-1.5)	1.6 (1.1-3.0)	0.003	0.9 (0.7-1.7)	1.9 (0.9-2.9)	0.018
TRPV1 mRNA (median [IQR])	1.1 (0.8-1.6)	1.4 (0.9-2.2)	0.014	0.9 (0.6-1.2)	1.4 (0.8-2.3)	0.089

1 Mann-Whitney test was used. FD: Functional dyspepsia; GDNF: Glial cell-line derived neurotrophic factor; IQR: Interquartile range; NGF: Nerve growth factor; PDS: Postprandial distress syndrome; TRPV1: Transient receptor potential vanilloid receptor 1.

Table 4 Dyspepsia symptoms, stool consistency and bowel movement between males and females

Symptoms	Male FD (n = 39)	Female FD (n = 65)	P value[1]
Overall abdominal pain[2] (mean ± SE)	3.4 ± 0.1	3.1 ± 0.2	0.195
Early satiation (mean ± SE)	2.8 ± 0.5	2.5 ± 0.3	0.434
Postprandial fullness (mean ± SE)	3.2 ± 0.3	3.1 ± 0.2	0.221
Epigastric burning/pain (mean ± SE)	2.7 ± 0.2	3.5 ± 0.1	0.047
Bloating (mean ± SE)	2.6 ± 0.3	2.2 ± 0.2	0.339
Nausea (mean ± SE)	1.5 ± 0.3	1.8 ± 0.2	0.327
Vomiting (mean ± SE)	0.5 ± 0.2	0.6 ± 0.1	0.203
BSFS (mean ± SE)	4.8 ± 0.2	4.4 ± 0.2	0.103
Number (per week) (mean ± SE)	4.8 ± 0.3	4.5 ± 0.2	0.532

[1]*t*-test was used; [2]Pain not restricted to the epigastric area. BSFS: Bristol stool form score (from 1 = very hard to 7= watery); FD: Functional dyspepsia.

Female FD patients were further classified according to the presence of history of gynecologic surgery. There were no answers from 17 patients. Twelve patients had received hysterectomy or salpingooophorectomy, and 13 patients underwent more than one Cesarean section. When patients with/without history of gynecologic surgery were compared, the patients who underwent these operations showed more frequent and severer overall abdominal pain and nausea compared to their counterparts (Supplementary Table 1).

Anxiety, depression and QoL

In order to evaluate the impact of mood and QoL on FD HADS scores and WHOQOL-BREF scores were analyzed between control and FD and between males and females.

When the FD and control groups were compared, FD patients showed higher mean HADS total score and higher mean HADS scores of both anxiety and depression than control subjects (Total, 15.8 ± 1.2 *vs* 11.0 ± 0.8; anxiety, 7.4 ± 0.7 *vs* 5.1 ± 0.4 and depression, 8.4 ± 0.6 *vs* 5.9 ± 0.4, all $P < 0.05$) (Table 5). In terms of quality of life with WHOQOL-BREF questionnaires, the scores of total, social domain and environmental domain was lower in the FD group than in the control group (Table 5).

In terms of gender, there were no significant differences in HADS score and every score of WHOQOL-BREF system between male and female in the control group. In contrast, FD group showed very clear gender difference. That is, female patients showed higher mean HADS total, anxiety and depression scores compared to male patients (total, 18.6 ± 2.1 *vs* 13.2 ± 1.3; anxiety, 9.0 ± 1.3 *vs* 6.0 ± 0.7; depression, 9.7 ± 1.0 *vs* 7.2 ± 0.8, all $P < 0.05$) (Figure 1). Moreover, the severity of epigastric pain correlated with HADS anxiety score only in female FD patients (males: Spearman rho = 0.232, P = 0.128; females: Spearman rho = 0.420, P = 0.037) (Figure 2).

Similar to HADS score WHOQOL-BREF scoring system showed gender difference only in FD group. That is, female FD patients scored lower in every domain including the scores of total, overall QoL and general health, physical, psychological, social and environmental domains compared to male FD patients (Table 5). In particular, the scores of overall QoL and general health, and physical domain in females were significantly lower than males (P = 0.020 and 0.016, respectively) (Table 5).

DISCUSSION

We demonstrated that differences in plasma acyl ghrelin and the gastric expressions of most nociception-related gene between FD and control groups were significant only in men. In contrast, female FD patients had a more anxious and depressive mood, and showed a more apparent impaired QoL compared to male FD

Table 5 HADS and WHOQOL-BREF scores in the control and functional dyspepsia groups according to gender

Variables	Control			FD			*P* value[1]		
	Total	Male	Female	Total	Male	Female	Control *vs* FD	Control[2]	FD[2]
	(*n* = 87)	(*n* = 38)	(*n* = 49)	(*n* = 104)	(*n* = 39)	(*n* = 65)			
HADS score[3] (mean ± SE)									
Total	11.0 ± 0.8	10.8 ± 1.2	11.2 ± 1.1	15.8 ± 1.2	13.2 ± 1.3	18.6 ± 2.1	0.001	0.780	0.029
Anxiety	5.1 ± 0.4	4.9 ± 0.6	5.3 ± 0.6	7.4 ± 0.7	6.0 ± 0.7	9.0 ± 1.3	0.005	0.587	0.036
Depression	5.9 ± 0.4	5.9 ± 0.7	5.9 ± 0.6	8.4 ± 0.6	7.2 ± 0.8	9.7 ± 1.0	0.001	0.984	0.047
WHOQOL-BREF score[4] (mean ± SE)									
Total	59.5 ± 1.6	60.7 ± 2.7	58.0 ± 1.5	54.0 ± 1.9	57.5 ± 1.9	51.6 ± 2.1	0.027	0.417	0.074
Overall quality of life and general health	6.2 ± 0.2	6.6 ± 0.3	5.8 ± 0.3	5.9 ± 0.3	6.5 ± 0.3	5.4 ± 0.3	0.278	0.080	0.020
Physical domain	13.4 ± 0.4	13.6 ± 0.7	13.1 ± 0.4	12.6 ± 0.4	13.6 ± 0.5	11.6 ± 0.7	0.185	0.575	0.016
Psychological domain	12.8 ± 0.4	13.2 ± 0.7	12.4 ± 0.5	11.8 ± 0.4	12.4 ± 0.5	11.4 ± 0.7	0.092	0.410	0.278
Social domain	13.7 ± 0.4	14.0 ± 0.6	13.3 ± 0.4	12.1 ± 0.5	13.0 ± 0.5	11.5 ± 0.6	0.012	0.370	0.064
Environment domain	13.3 ± 0.5	13.3 ± 0.7	13.3 ± 0.6	11.6 ± 0.4	12.1 ± 0.5	11.7 ± 0.7	0.010	0.990	0.617

[1]*t*-test was used; [2]Between males and females; [3]Higher score denotes more severe symptom; [4]Higher score denotes better quality of life. FD: Functional dyspepsia; HADS: Hospital anxiety and depression scale; WHOQOL-BREF: World health organization quality of life abbreviated version.

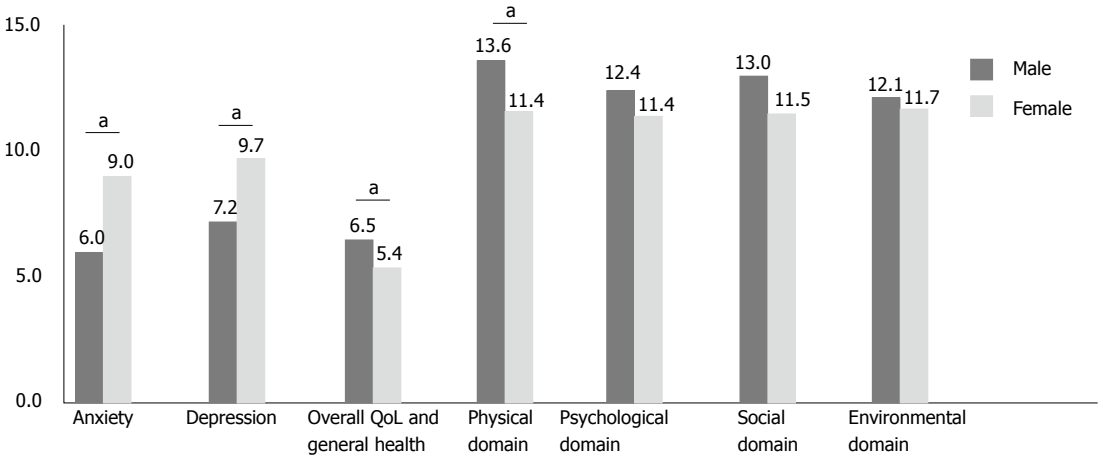

Figure 1 HADS and WHOQOL-BREF scores of patients with functional dyspepsia according to gender. HADS: Hospital anxiety and depression scale; WHOQOL-BREF: World health organization quality of life abbreviated version; QOL, quality of life. "a" denotes statistical significance.

patients. Epigastric burning or pain was correlated with anxiety score only in women. Women who underwent any gynecologic surgery showed more severe overall abdominal symptoms than women who did not. To our knowledge, this is the first study to evaluate the differences between males and females in terms of clinical characteristics of FD and the expression of ghrelin and nociception-related genes.

Women are more likely than men to meet the criteria for most FGIDs[5,9,27,28], although some studies reported no difference in the prevalence of FD between men and women[14,29,30]. One of the most essential factors characterizing an individual biologically male and female is the sex hormone. The representative female hormones estrogen and progesterone can interfere with gastric motility. Gastric emptying in premenopausal females is delayed compared to that in males[31-34] and gastric emptying during luteal phase when the levels of the sex hormones are high is prolonged in comparison with the follicular phase[33]. Generally accepted slower gastric emptying in females than in males[35] may be at least partially attributable to the female sex hormone, which hampers gastric motility by reducing gastric smooth muscle contractility[36].

However, sex hormones may not the single factor contributing to the delayed gastric emptying in females, because this has also been observed during the follicular phase of the menstrual cycle[32]. In terms with gastric motility, a reduced acyl ghrelin level has been reported to be correlated with an impairment of gastric emptying[13]. We previously published the data of decreased plasma acyl ghrelin levels in the PDS type of FD compared to the control group[16]. PDS symptom was thought to be associated with the delayed gastric emptying. Based on the literature, a decreased tendency of plasma acyl ghrelin in the female control group in the present study may reflect the delayed gastric emptying in women. Interestingly, the difference in plasma acyl ghrelin between FD patients and control was statistically significant in male but not

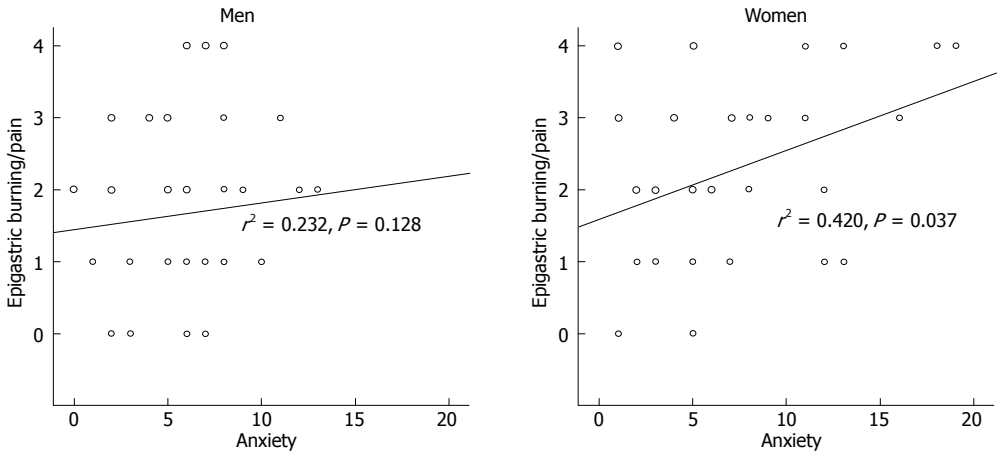

Figure 2 Correlation between epigastric burning/pain score and HADS anxiety score according to gender. Spearman correlation was used.

in female. Similarly, elevated level of NGF, GDNF and TRPV1 mRNA expression in the FD group was more prominent in men than in women. We also previously reported that FD patients showed an elevated level of NGF, GDNF and TRPV1 mRNA expression[15]. Indeed, visceral hypersensitivity is another important mechanism for the FD and the upregulation of TRPV1 has been proposed to be associated with visceral hypersensitivity of FD. The present results suggest that ghrelin and visceral hypersensitivity are important underlying mechanisms of male FD but female FD needs more other decisive mechanism.

There is evidence of a lack of the association between physiological mechanisms with dyspeptic symptoms[37]. Instead, recent epidemiological studies have provided increasing evidence for the positive association between anxiety or depression and functional gastrointestinal symptoms[4,38]. In the present study, female FD patients showed more anxious and depressive mood than male patients. Although it is unclear whether psychosocial factors mainly determine healthcare seeking or have a direct influence on symptom perception in FGID, anxiety has been recently reported to be negatively correlated with pain threshold[39]. It is interesting to note that the severity of epigastric pain correlated with HADS anxiety score only in female FD patients in the present study. This indicates that dyspepsia symptoms, particularly epigastric burning or pain in females, may be related more with psychological factors. Therefore, more attention is required in terms of psychological evaluation and management when treating female FD patients.

It is also conceivable that the gynecological condition of female may affect the varied clinical presentations in comparison with male, but most studies evaluated the association between GI symptoms and surgeries in IBS patients[17]. In the present study, female FD patients who received gynecologic surgery reported more severe general abdominal pain. However, because this abdominal pain was not restricted to the epigastric area, overlap with gynecologic conditions may contribute to dyspepsia in females. This needs further study.

Although women have been reported to be more likely than men to exhibit dysmotility-like symptoms and men are more likely to experience food regurgitation and heartburn[28,40], there were no significant differences in FD symptoms or FD symptoms subtypes between male and female FD in the present study. Only female FD patients suffered more epigastric pain than male FD patients. Although some studies in Europe and Japan reported that female predominance in the prevalence of FD[41-45], little gender analysis was conducted except for prevalence. The comparison of various aspects including prevalence, symptom subtype, dominant symptoms, natural course and QoL between males and females by different geographical areas or ethnicity could help to better understand the related pathophysiology of FD.

Concerning QoL, FD patients manifested more impaired QoL status than the control group, especially in the social and environmental domains, which indicates the need of more active intervention to ameliorate the FD symptoms. When comparing males and females, even though aspects of QoL did not reach statistical significance, every aspect of QoL was poorer in females than in males. Particularly, overall QoL and general health and physical domain scored significantly lower in females. Thus, modulating physical aspects would be effective for the alleviation of FD symptoms.

There are several limitations in the present study. This study is a single center study with a possible sample bias. Symptom scores were evaluated by questionnaire; this is not free from the risk of a recall bias. The sample size was relatively small. The effect of female sex hormones on the pathogenesis on FD was not evaluated. In spite of these limitations, our study clearly demonstrated gender differences of FD in terms of clinical characteristics of FD and the expression of ghrelin and nociception-related genes.

In conclusion, our study presents that the lower level of plasma acyl ghrelin and higher expressions of nociception-related genes are associated with pathogenesis of FD in males. On the other hand, female FD patients had more serious anxious and depressive mood, and anxiety score

was correlated with epigastric pain in female FD patients. This psychological predisposition might underlie the perception of symptom, especially in female FD patients. There was not a large difference in pattern or severity of FD symptoms, except for female predominance in epigastric pain. However, considering that the impairment of overall QoL and general health was more prominent in female FD patients than in male patients, more careful assessment of psychological or emotional status is needed for the better treatment of female FD patients.

ARTICLE HIGHLIGHTS

Research background

Although gender is assumed to be an important factor in the pathogenesis, progression and prognosis of certain diseases, there have been only a few reported gender differences in functional gastrointestinal disorders, and attention has focused mostly on irritable bowel syndrome.

Research motivation

Most functional gastrointestinal disorders, including functional dyspepsia, show female predominance.

Research objectives

We compared the possible etiological factors including ghrelin, nociception-related genes, psychological aspects and history of abdominal operation as well as basal characteristics, dyspepsia symptoms and quality of life between male and female functional dyspepsia patients.

Research methods

Total of 191 persons [87 subjects (male 38, female 49) and 104 patients (male 39, female 65)] were prospectively enrolled between March 2013 and May 2016 in Seoul National Bundang Hospital. They were classified into control and FD group (PDS, EPS and mixed subgroups) on the basis of ROME Ⅲ criteria. Questionnaire included assessment for dyspepsia symptoms, quality of life by WHOQOL-BREF scores and anxiety or depression by HADS scores were analyzed. Preproghrelin and nociception genes were analyzed by RT-PCR from the gastric mucosa. Plasma acyl/des-acyl ghrelin were measured by ELISA method.

Research results

Differences in plasma acyl ghrelin and the gastric expressions of most nociception-related gene between dyspepsia and control groups were significant only in men. In contrast, female functional dyspepsia patients had a more anxious and depressive mood, and showed a more apparent impaired quality of life compared to male dyspeptic patients. Epigastric burning or pain was correlated with anxiety score only in women. Women who underwent any gynecologic surgery showed more severe overall abdominal symptoms than women who did not.

Research conclusions

Different mechanisms might underlie the perception of dyspeptic symptom by gender and the negative impact of the functional dyspepsia on the quality of life can be more prominent in women than men.

Research perspectives

More careful assessment of psychological or emotional status is required particularly for the female FD patients.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Efficacy of combination therapy with natriuretic and aquaretic drugs in cirrhotic ascites patients: A randomized study

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Abstract

AIM

To assess the effects of a combination therapy with

natriuretic and aquaretic drugs in cirrhotic ascites patients.

METHODS

A two-center, randomized, open-label, prospective study was conducted. Japanese patients who met the criteria were randomized to trial group and the combination diuretic group (received 7.5 mg of tolvaptan) or the conventional diuretic group (received 40 mg of furosemide) for 7 d in addition to the natriuretic drug which was used prior to enrolment in this study. The primary endpoint was the change in body weight from the baseline. Vital signs, fluid intake, and laboratory and urinary data were assessed to determine the pharmacological effects after administration of aquaretic and natriuretic drugs.

RESULTS

A total of 56 patients were randomized to receive either tolvaptan (n = 28) or furosemide (n = 28). In the combination and conventional diuretic groups, the average decrease in body weight from the baseline was 3.21 ± 3.17 kg (P < 0.0001) and 1.75 ± 2.36 kg (P = 0.0006), respectively, when measured on the final dosing day. Following 1 wk of treatment, a significantly greater reduction in body weight was observed in the combination diuretic group compared to that in the conventional diuretic group (P = 0.0412).

CONCLUSION

Compared to a conventional diuretic therapy with only a natriuretic drug, a combination diuretic therapy with natriuretic and aquaretic drugs is more effective for patients with cirrhotic ascites.

Key words: Tolvaptan; Liver cirrhosis; Diuretic effect; Ascites; Hepatic edema

Core tip: Whether a combination therapy with natriuretic and aquaretic drugs is more effective than conventional therapy with natriuretic drugs only for liver cirrhosis patients with ascites remains unclear. To clarify this, we compared the pharmacological effects of combination therapy with conventional therapy in cirrhotic ascites patients. Compared to a conventional therapy with only natriuretic drugs, a combination therapy with natriuretic and aquaretic drugs is more effective for patients with cirrhotic ascites.

Uojima H, Hidaka H, Nakayama T, Sung JH, Ichita C, Tokoro S, Masuda S, Sasaki A, Koizumi K, Egashira H, Kako M. Efficacy of combination therapy with natriuretic and aquaretic drugs in cirrhotic ascites patients: A randomized study. *World J Gastroenterol* 2017; 23(45): 8062-8072 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8062.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8062

INTRODUCTION

Cirrhosis leads to portal hypertension and end-stage liver disease, with complications[1,2]. Pathophysiology of ascites, one of the most common complications in liver cirrhosis, involves a decrease in effective arterial blood volume due to splanchnic arterial vasodilatation leading to activation of potent vasoconstriction systems, such as the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system, and vasopressin release, which in turn results in retention of sodium and free water to restore blood homeostasis[3-6].

Progression of liver diseases is characterized by a large decrease in the excretion of urinary sodium, and accumulation of retained fluid within the abdominal cavity. For liver cirrhosis patients with ascites, current guidelines recommend the administration of a diuretic drug if the efficacy of sodium intake restriction is inadequate[7]. Conventional diuretics are natriuretic drugs that block sodium reabsorption in the nephrons, increasing renal sodium excretion to achieve a negative sodium balance[6,8]. Although ascites in the majority of patients can be controlled by restriction of sodium intake and administration of a natriuretic medication, 5%-10% of patients with ascites develop resistance to conventional therapy as refractory ascites. Refractory ascites is composed of diuretic-resistant ascites and diuretic-intractable ascites. Diuretic-resistant cannot be mobilized or the early recurrence because of a lack of response to dietary sodium restriction and conventional diuretics. For the treatment of diuretic-intractable ascites an effective diuretic dosage has not yet been determined because of the development of severe diuretic-related side effects[9]. The strategy of ascites refractory to diuretic therapy has still not been established.

Recently, several studies have evaluated the effects of aquaretic drugs such as tolvaptan for treating ascites resistant to conventional diuretics[10,11]. Tolvaptan, which blocks arginine vasopressin (AVP) from binding to V_2 receptors in the distal nephrons and thus restricts water reabsorption, is an ideal aquaretic drug for the treatment of delusional hyponatraemia in conditions associated with increased circulating levels of antidiuretic hormone such as decompensated liver cirrhosis[12]. However, whether a combination therapy with natriuretic and aquaretic is more effective than conventional therapy with natriuretic only for liver cirrhosis patients with ascites remains unclear. To clarify this, we compared the pharmacological effects of combination diuretics therapy with conventional diuretics therapy in cirrhotic ascites patients.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Review Board Ethics Committee of the Tokushukai Medical

Group (license number: TGE00406-024). Informed consent was obtained from patients or their families before study participation commenced. This study was registered at the UMIN Clinical Trials Registry as UMIN000015218. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Tokushukai Medical Group's human research committee.

Study design

A two-center, randomized, open-label, prospective study was conducted at Shonan Kamakura General Hospital and Shonan Atsugi Hospital in Kanagawa, Japan. This study consisted of a 2-d pretreatment observation period followed by a 7-d treatment period and 4-wk posttreatment observation period. Patients who met the criteria were randomized to receive aquaretic (7.5 mg of tolvaptan) or natriuretic (40 mg of furosemide) in a 1:1 ratio. Permuted block randomization was used for the creation of the randomization list prepared by an investigator with no clinical involvement in the trial, and a randomization code was pre-assigned to each trial drug and used during drug administration. Patients received oral tolvaptan or furosemide for 7 d in addition to the standard therapy identical to that administered prior to enrolment in this study, which included sodium intake restrictions (< 6 g/d), *ad libitum* fluid intake, and natriuretic therapy[8]. According to the standard diuretic regimen in Japan, high-dose diuretics such as furosemide at 160 mg/d or spironolactone at 400 mg/d is not recommended. All enrolled patients were admitted to the hospital, and the trial drug was administered orally between 07:00 and 08:00 after breakfast. The dosages of the natriuretic drug used prior to enrolment in this study were not changed, and therapeutic abdominal paracentesis procedures were not performed until the completion of the final assessment on the day following the final administration of the trial drug.

Eligibility criteria

Patients were required to meet the following inclusion criteria: Age > 20 years, liver cirrhosis with ascites even after undergoing a natriuretic with a loop diuretic and an anti-aldosterone agent for at least 7 d, and a daily dose of ⩾ 20 mg furosemide and ⩾ 25 mg spironolactone, a conventional diuretics regimen in Japan[13]. Additionally, the diuretic dosages should not have been changed for at least 7 d prior to initiating the trial, and an ineffective response was one in which body weight was not reduced in spite of administration of intensive diuretic therapy for the 7 d. A difference in dosage of diuretics was observed among patients because different doctors referred them for treatment. Diagnosis of liver cirrhosis was based on laboratory results and imaging tests (ultrasonography and computed tomography), revealing a hepatic cirrhotic appearance, splenomegaly, esophageal varices, and/or ascites[8]. Patients with hepatic encephalopathy (coma scale ⩾ Ⅱ), poorly controlled hepatocellular carcinoma, and patients receiving blood products including albumin for 7 d or less before initiating the trial drug treatment were excluded.

Clinical parameters

Baseline characteristics collected included demographic parameters, concomitant medication, ascites volume, mean 24-h urine volume, and baseline laboratory and urinary data obtained immediately preceding the start of the trial drug administration. All the patients exhibited ascites volumes of ⩾ 1000 mL as calculated by computed tomography[14]. Physical examination including measurement of body weight, supine blood pressure, and pulse rate was performed daily. Urine volume was measured over a 24-h period from 06:00. Mean differences in daily urine volume and body weight between the two groups were calculated. Cumulative 24-h urine samples were collected before drug administration each day from the day before initiating tolvaptan until the end of the posttreatment period. Laboratory and urinary data were obtained at 06:00 before drug administration and after drug administration on day 7.

Blood parameters measured included levels of hemoglobin, platelets, serum albumin, alanine aminotransferase (ALT), serum blood urea nitrogen (BUN), serum creatinine, serum total bilirubin, plasma brain natriuretic peptide, human atrial natriuretic peptide, serum sodium, serum potassium, serum osmolality (OSM), serum aldosterone, serum renin, ammonia, and plasma AVP. Urinary parameters measured included OSM, sodium, and potassium. The value for 24-h creatinine clearance (CCr) was calculated as urinary creatinine × urinary volume ÷ serum creatinine × 1440[15].

Evaluation

The primary endpoint of this trial was the change from baseline in body weight through the duration of the study. The day a patient completed or discontinued the treatment was defined as the final dosing day. We also studied the responder rate for the trial drug. According to a previous report, patients who lost ⩾ 2 kg and < 2 kg body weight after 1 wk of drug administration were defined as responders and non-responders, respectively[16]. Primary endpoint was the change in body weight measured 1 wk after administration of the trial drugs.

Secondary endpoints included changes in urine volume, ascites volume, and improvement of ascites-related symptoms. To determine the pharmacological effects of tolvaptan and furosemide, vital signs, fluid intake, and laboratory data on blood and urinary biomarkers were assessed.

Safety assessment

To evaluate drug safety during the treatment period, we assessed the adverse events following the trial drug administration. Data relating to all the adverse

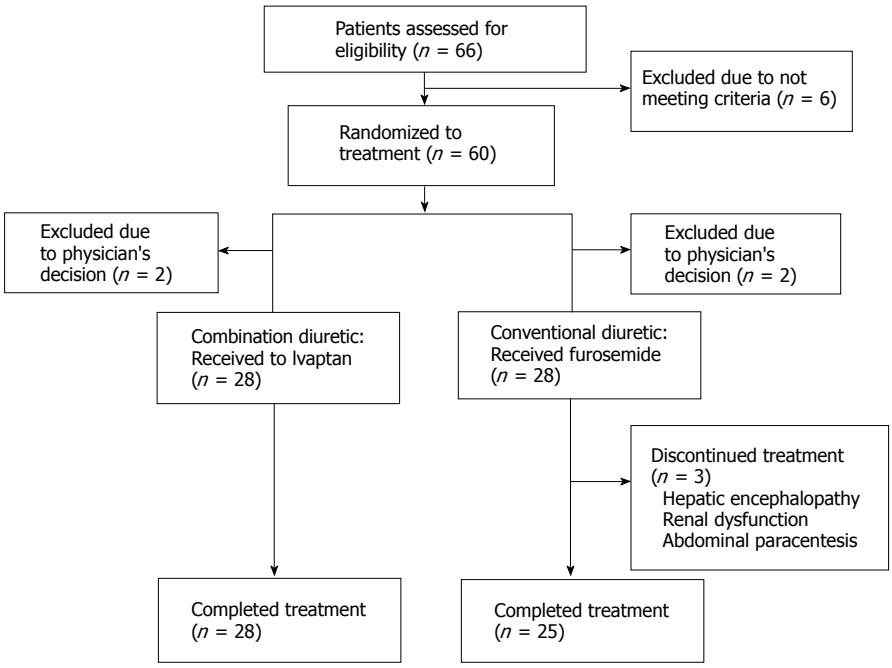

Figure 1 Study flow diagram.

events were collected from the start of the trial drug administration until the final dosing day. The adverse events were classified using the Medical Dictionary for Regulatory Activities, version 17.0, and their severity was graded using the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.

Statistical analysis

Our estimation of clinical effects using change in body weight after a combination diuretic therapy for a wk was based on a previous study[11]. The change of body weight after 1 wk was 1.95 kg in the combination diuretic group and 0.44 kg in the conventional diuretic group; and therefore, we suspect a difference of at least 1.5 kg/wk between the two groups (the standard deviation of the two groups was 1.8 kg). In order to detect a difference of this magnitude that is significant with a 95% confidence interval and a power of 80%, there was a minimum of 24 patients required in each group. Assuming that 20% of the patients would drop out of the study, the required sample size for this trial was, therefore, estimated to be approximately 60 patients (30 patients each in the furosemide and tolvaptan groups).

Analyses were based on the per-protocol analysis and the full analysis set. The full analysis set included all randomized patients who received the trial drugs at least once. Missing data at the final evaluation day were imputed by the last data obtained after the start of the study. Safety analyses were conducted on all patients who received at least one dose of either of the trial drugs. No interim analyses for this study's data were done.

Data were analyzed using the statistical software JMP 11.0.1 (SAS Institute) and expressed as mean ± SD. Continuous variables of the conventional diuretic groups and combination diuretic groups were compared using the Student's *t* test or the Mann-Whitney *U test*, whereas the paired *t* test or the Wilcoxon signed-rank test were used for paired data. Differences with a *P* value < 0.05 were considered significant. Statistical analyses were performed by the SATT Corporation, Tokyo, Japan.

RESULTS

Participating patients

A total of 66 patients were assessed in the present study from July 2014 through August 2016 (Figure 1). Six patients dropped out of the study during the run-in period for failing to meet the criteria for commencing treatment. The remaining 60 patients were randomized to receive either tolvaptan (*n* = 30) or furosemide (*n* = 30). However, four patients with unstable vital signs or acute renal failure were withdrawn from the study by their physicians before the drugs were administered (2 patients in each group). Three patients discontinued treatment, two owing to adverse events (hepatic encephalopathy and renal dysfunction, respectively), whereas the third required intervention following administration of standard diuretics therapy in the form of therapeutic abdominal paracentesis for uncontrollable ascites due to a bacterial infection.

Patient characteristics

Patient demographics and baseline characteristics are summarized in Tables 1 and 2. No significant differences

Table 1 Baseline patient characteristics *n* (%)

	Combination diuretic (*n* = 28)	Conventional diuretic (*n* = 28)	*P* value
Age, yr	69.3 ± 11.8	69.4 ± 12.6	0.9739
Gender: Male	17 (60.7)	15 (53.6)	0.7875
Weight, kg	61.7 ± 13.8	58.0 ± 13.5	0.3082
Body mass index, kg/m^2	24.2 ± 4.74	22.4 ± 3.48	0.1044
Etiology: HBV/HCV/alcoholic/other	2/12/9/5	2/10/9/7	0.9341
Child-Pugh score	10.4 ± 1.2	10.3 ± 1.4	0.7653
Child-Pugh stage: A/B/C	0/16/12	0/15/13	0.7881
Liver cancern	7 (25.0)	8 (28.6)	1.0000
Varicose veins	23 (82.1)	25 (89.3)	0.7049
Liver encephalopathy: Grade 0/I	24/4	24/4	1.0000
Concomitant medication:			
Spironolactone, mg/d	50.9 ± 29.3	50.0 ± 22.6	0.8987
Furosemide, mg/d	38.6 ± 18.4	37.7 ± 20.3	0.8639
BCAA	21 (75.0)	19 (67.8)	0.7674
Non-absorbable disaccharides	8 (28.5)	10 (35.7)	0.7748
ARB or ACE inhibitor	3 (10.7)	4 (14.3)	1.0000
Duration of diuretics therapy, d	25.9 ± 28.3	22.8 ± 20.1	0.6318
Pretreatment urine volume, mL/d	1356 ± 651	1517 ± 939	0.4213
Ascites volume, mL	2204 ± 1384	2104 ± 1084	0.6400
Grade of ascites: Mild/moderate/severe	5/15/8	6/13/9	0.8901
Amount of water drunk, mL/d	1032 ± 491.2	1071 ± 870.0	0.9710
Pulse rate, /min	78 ± 10.1	78 ± 11.2	0.8514
Systolic blood pressure, mmHg	121 ± 21.6	116 ± 12.9	0.3130
Diastolic blood pressure, mmHg	65 ± 13.5	72 ± 21.5	0.2115

Data are expressed as median, *n* (%) or mean ± SD. BCAA: Branched chain amino acids; ARB: Angiotensin receptor blocker; ACE: Angiotensin-converting enzyme.

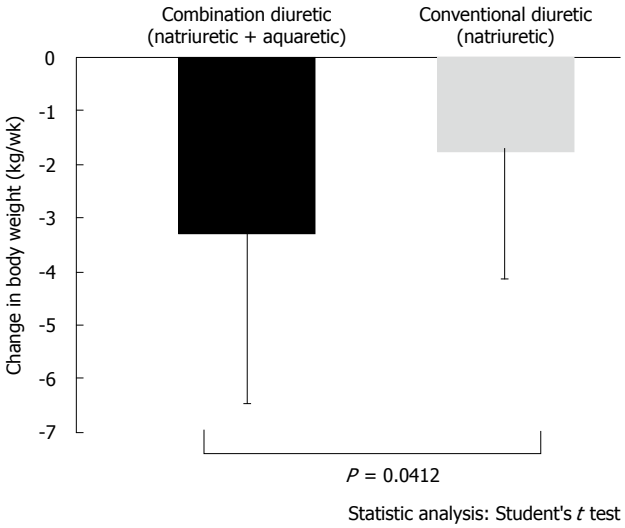

Figure 2 Change in mean body weight from baseline on the final dosing day. Comparisons between the combination and conventional diuretic groups were performed using Student's *t* test.

were found in sex, age, cause of liver cirrhosis, or the amount of diuretics used between the two groups. Hemoglobin levels were higher in the conventional diuretic group (P = 0.0481), and platelet counts were slightly elevated (P = 0.0598). No significant differences were found between the groups prior to trial initiation in terms of serum albumin, serum ALT, serum BUN, serum creatinine, serum total bilirubin, serum sodium, serum potassium, serum OSM, serum aldosterone, serum renin, plasma AVP, urinary OSM, urinary sodium, potassium, or 24-h CCr.

Effect of treatment on body weight

Change in body weight measured 1 wk after administration of the trial drugs is shown in Figures 2 and 3. In the combination diuretic group, the average decrease in body weight from baseline was -3.21 ± 3.17 kg (P < 0.0001) as measured on the final dosing day, with 18 (64.3%) responders. In the conventional diuretic therapy, the average decrease in body weight from baseline was -1.75 ± 2.36 kg (P = 0.0006) on the final dosing day, with 13 (46.4%) responders. Following 1 wk of treatment, a significantly greater reduction in body weight was observed in the combination diuretic group compared to that in the conventional diuretic group (P = 0.0412).

Effect on secondary endpoints

Change in urinary volume 1 wk after administration of the trial drugs is shown in Figure 4. Following the administration of tolvaptan, a significant increase in mean urine excretion volume from 1356 ± 651 mL pretreatment to 2439 ± 1179 mL posttreatment was observed (P < 0.0001). Following the administration of furosemide, a slight increase in mean urine excretion volume was observed, with a pretreatment value of 1517 ± 939 mL and a posttreatment value of 1759 ± 888 mL (P = 0.0253).

Following 1 wk of treatment, a significantly greater increase in urine volume was observed in the combination diuretic group compared to that in the conventional

Table 2 Baseline patient characteristics

	Combination diuretic (n = 28)	Conventional diuretic (n = 28)	P value
Hemoglobin, g/dL	9.43 ± 1.30	10.3 ± 1.62	0.0481
Platelets, × 10^3/μL	10.1 ± 7.96	12.3 ± 6.04	0.0598
Prothrombin time, s	12.5 ± 1.5	12.3 ± 1.1	0.4672
Serum albumin, g/dL	2.38 ± 0.49	2.36 ± 0.55	0.9934
BUN, g/dL	24.7 ± 14.6	27.1 ± 24.7	0.8058
Serum creatinine, mg/dL	1.14 ± 0.52	1.03 ± 0.42	0.4031
ALT, IU/L	31.3 ± 29.6	25.5 ± 12.2	0.7306
Total bilirubin, g/dL	3.20 ± 3.78	2.86 ± 2.96	0.7121
BNP, pg/mL	164 ± 310	97.1 ± 76.6	0.8957
HANP, pg/mL	79.5 ± 80.4	60.2 ± 33.6	0.8831
Ammonia, μg/dL	82.4 ± 48.5	75.6 ± 45.1	0.6314
Plasma-aldosterone, pg/mL	163 ± 178	185 ± 267	0.5221
Plasma-renin, pg/mL	9.04 ± 10.6	8.45 ± 14.3	0.8377
Plasma AVP, pg/mL	2.42 ± 1.52	2.35 ± 1.70	0.5007
Serum sodium, mEq/L	135 ± 5.4	135 ± 4.2	0.5869
Serum potassium, mEq/L	3.83 ± 0.52	3.99 ± 0.53	0.2301
Urinary OSM, mOSM/L	426 ± 163	413 ± 172	0.7742
Urinary sodium, mEq/d	88.8 ± 63.3	93.4 ± 65.3	0.5869
Urinary potassium, mEq/d	25.3 ± 13.8	26.8 ± 13.4	0.5497
24-h CCr, mL/min	57.3 ± 27.9	62.6 ± 38.4	0.9217

Data are expressed as median, n (%) or mean ± SD. BUN: Blood urea nitrogen; AVP: Arginine vasopressin; BNP: Brain natriuretic peptide; HANP: Human atrial natriuretic peptide; OSM: Osmolality; 24-h CCr: 24-hour creatinine clearance.

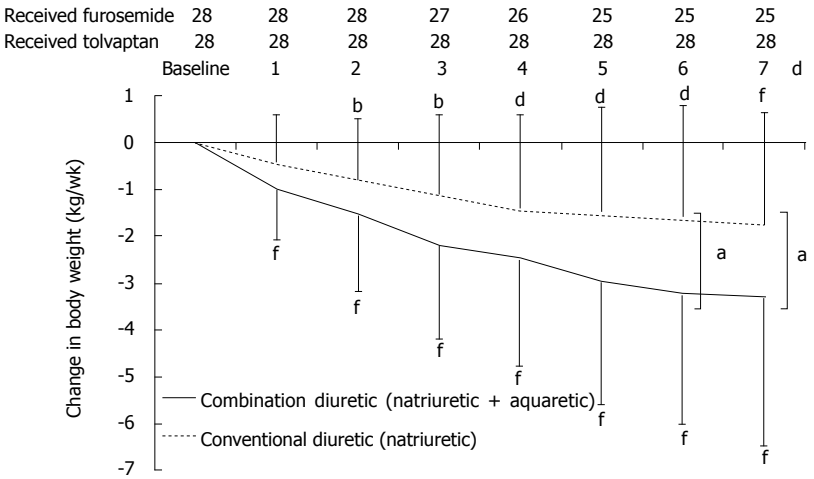

Figure 3 Time-course change in body weight from the baseline after administration of the trial drugs. Data are expressed as mean ± SD. Comparisons between final and baseline values for each group were performed using the paired t test. Paired t test: $^bP < 0.01$, $^dP < 0.001$, $^fP < 0.0001$; Student's t test $^aP < 0.05$.

diuretic group (P < 0.0001). In the combination diuretic group, body weight consistently decreased, whereas urine volume consistently increased. In the conventional diuretic group, body weight consistently decreased, whereas urine volume remained constant.

After the administration of tolvaptan, a significant decrease in the ascites volume from 2204 ± 1384 mL pretreatment to 1501 ± 669 mL posttreatment was observed (P < 0.0001). Following the administration of furosemide, a significant decrease in the ascites volume from 2104 ± 1084 mL pretreatment to 1649 ± 401 mL posttreatment was observed (P < 0.0001). A significantly greater decrease in ascites volume was observed in the combination diuretic group compared to that in the conventional diuretic group (P = 0.0207) (Figure 5).

Improvement in ascites-related symptoms after administration of the tolvaptan or furosemide was 71.4% (20/28) and 57.1% (16/28), respectively. There were no significant differences between the groups.

Pharmacological effects

After the administration of tolvaptan, plasma AVP was higher on the final dosing day compared to that at baseline (2.42 ± 1.52 *vs* 3.87 ± 1.99, P < 0.0001), whereas urinary OSM was lower (426.2 ± 163.3 *vs* 318.4 ± 118.0, P = 0.0106) (Figure 6). Renal function

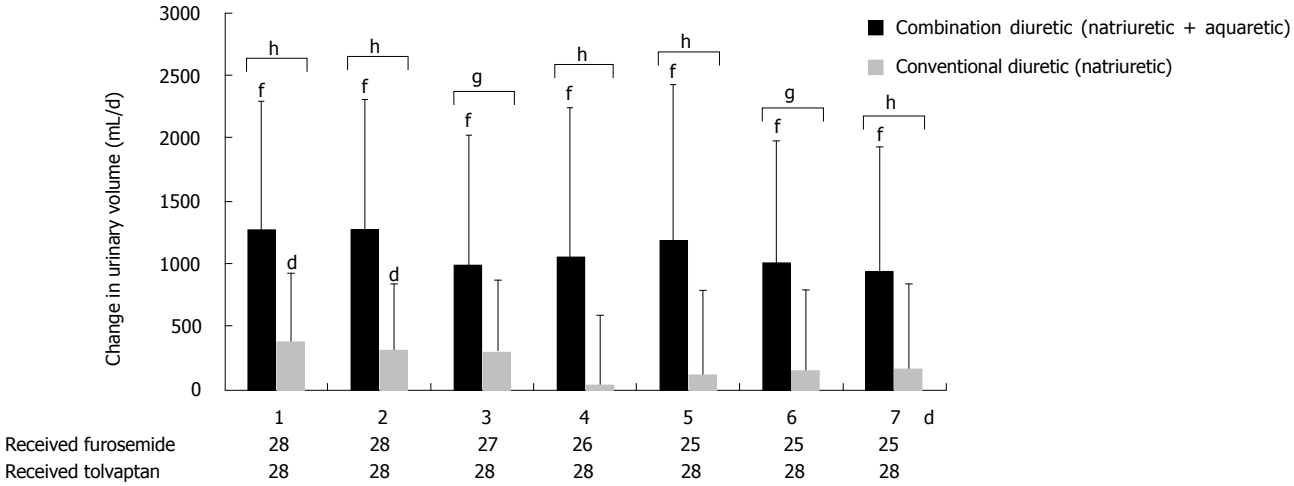

Figure 4 Time-course change in urinary volume from the baseline after administration of the trial drugs. Data are expressed as mean ± SD. Comparisons between final values and baseline values for each group were performed using the paired *t* test, $^{b}P < 0.01$, $^{d}P < 0.001$, $^{f}P < 0.0001$. Comparisons between the groups were performed using Student's *t* test, $^{g}P < 0.05$, $^{h}P < 0.001$.

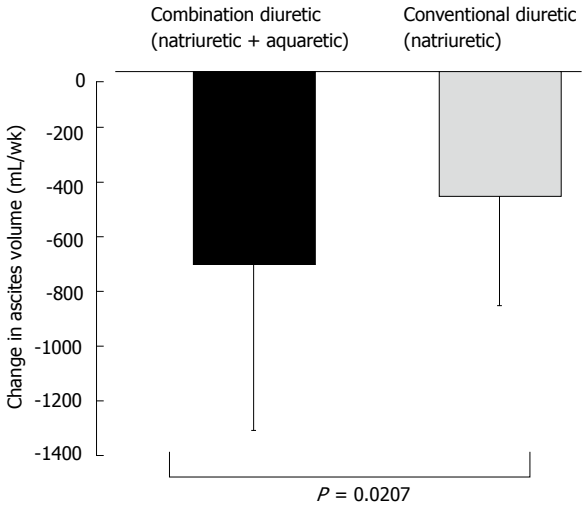

Figure 5 Change in the ascites volume from baseline on the final dosing day. Comparisons between the combination diuretic and conventional diuretic groups were performed using Student's *t* test.

represented by 24-h CCr was indistinguishable between that at the baseline and the final dosing day (57.2 ± 27.9 *vs* 55.3 ± 34.1, $P = 0.9980$), as were ammonia levels (82.4 ± 48.5 *vs* 73.5 ± 42.2, $P = 0.0854$) (Figure 7 and 8).

After the administration of furosemide, plasma renin was higher on the final dosing day compared to that at baseline (8.45 ± 14.3 *vs* 15.0 ± 20.2, $P = 0.0128$), whereas renal function represented by 24-h CCr was lower (62.6 ± 38.4 *vs* 49.9 ± 40.4, $P = 0.0231$). Ammonia levels were higher on the final dosing day compared to those at baseline (75.6 ± 45.1 *vs* 110.8 ± 66.0, $P = 0.0012$).

Posttreatment serum ammonia levels were higher in the conventional diuretic group compared to those in the combination diuretic group ($P = 0.0008$), whereas plasma AVP and urinary OSM were higher in the combination diuretic group ($P = 0.0003$; $P = 0.0296$, respectively). No significant differences were observed between the groups in platelet count, serum albumin, serum bilirubin, serum creatinine, serum sodium, plasma renin, plasma aldosterone, urinary sodium, and 24-h CCr after administration of the trial drugs for 1 wk.

Safety

No deaths occurred during the treatment period. Adverse events were observed in 13 (46.4%) of the patients receiving tolvaptan and 16 (57.1%) of the patients receiving furosemide (Table 3). Severe adverse events were reported in two patients in the conventional therapy (hepatic encephalopathy and a bacterial infection) during treatment and led to their withdrawal from the study. One patient with acute kidney injury was removed from the study at the request of the physician. Hepatic encephalopathy and acute kidney injury were more frequent in the conventional therapy. Dry mouth and frequent urination were more common in the combination diuretic group. There were no significant differences between the groups in heart rate and blood pressure. Hypokalemia was seen in three patients in the conventional diuretic group, and potassium supplements were given when serum potassium dropped to < 3.0 mEq/L during the study.

Clinical course after treatment for 1 week

After the treatment period, we conducted an observation for 1 mo. After the administration of tolvaptan, according to efficacy and tolerability, dose of tolvaptan was changed. Fifteen patients were kept on 7.5 mg, 8 patients were decreased to 3.75 mg; however, for

Table 3 Adverse events

	Combination diuretic: (n = 13) (%)		Conventional diuretic: (n = 16) (%)	
	Any grade	⩾ Grade 3	Any grade	⩾ Grade 3
Encephalopathy	1 (3.5)		3 (10.7)	1 (3.5)
Abdominal infection				1 (3.5)
Dry mouth	6 (21.4)		2 (7.1)	
Urinary frequency	4 (14.3)		2 (7.1)	
Hypopotassemia	0		3 (10.7)	
Acute kidney injury	2 (7.1)		4 (14.3)	

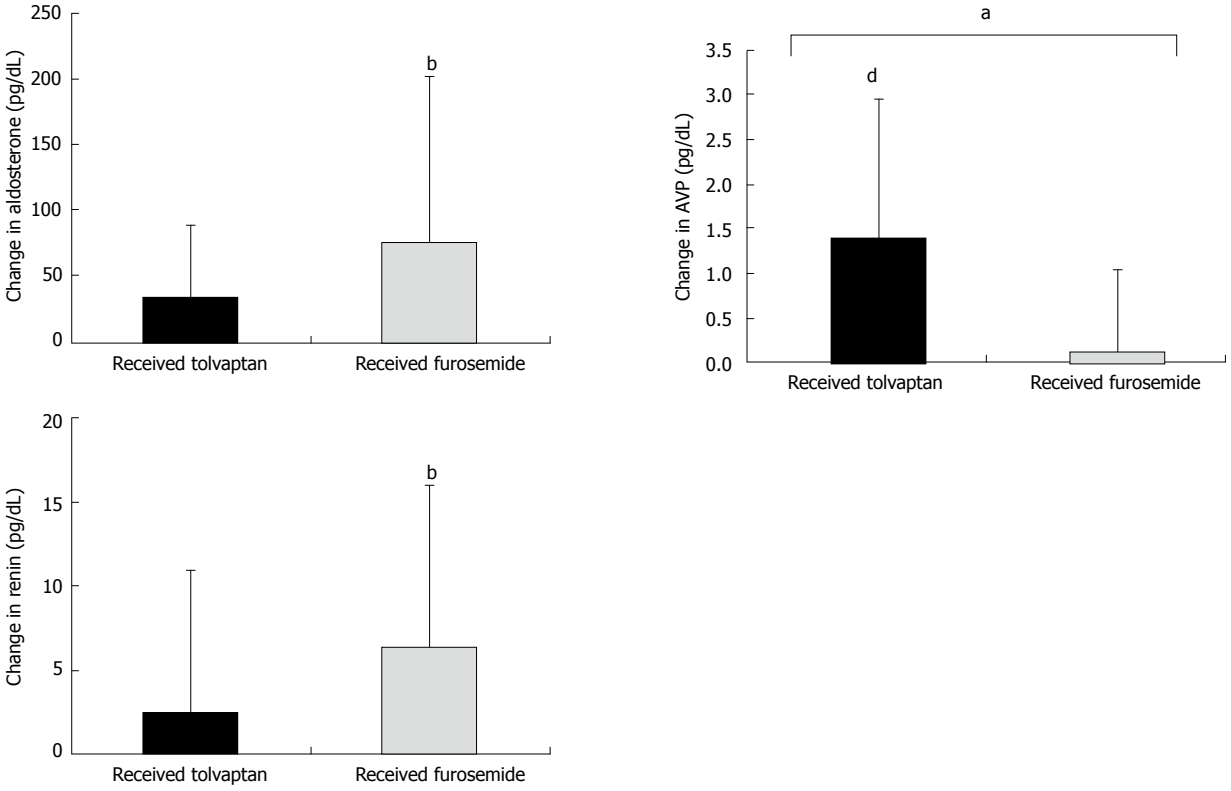

Figure 6 Changes in aldosterone, arginine vasopressin and renin from the baseline after administration of drugs. Comparisons between final values and baseline values for each group were performed using the Wilcoxon signed-rank test, $^{b}P < 0.01$; $^{d}P < 0.001$. Comparisons between the groups were performed using the Mann-Whitney *U* test, $^{a}P < 0.05$.

5 patients the therapy was discontinued because of inefficacy. The average decrease in body weight from baseline was -4.01 ± 3.97 and -4.31 ± 4.07 kg (2 w, 4 w; respectively).

After the administration of furosemide, tolvaptan was administered or the furosemide dose was changed. Twelve patients were treated with tolvaptan. The furosemide doses were decreased for 9 patients. No changes were made in the doses for 5 patients, and only 2 patients received an increased dose of furosemide. Except for the patients who received tolvaptan, the average decrease in body weight from baseline was -3.18 ± 3.15 and -3.61 ± 3.37 kg (2 w, 4 w; respectively).

DISCUSSION

A combination diuretic therapy including aquaretic resulted in a greater decrease in body weight compared to conventional diuretic with natriuretic only for cirrhotic ascites patients. Moreover, compared to conventional diuretic therapy, combination diuretic therapy reduced the occurrence of diuretic-related severe side effects.

For cirrhotic ascites patients, the developed resistance to the standard medical therapy with sodium intake restrictions and natriuretic therapy was one of the most challenging problems. Negative sodium balance is commonly pursued for managing liver cirrhosis with ascites[3]. Although natriuretic drugs are generally useful, only a minority of ascites patients respond to this clinical treatment[17]. Diuretic-resistant ascites is possibly because of the reduction in the glomerular filtration rate and the loss of nephrons with natriuretic potential[18,19]. Another common reason is the so-called "ceiling effect." Natriuretic drugs increase renal sodium excretion,

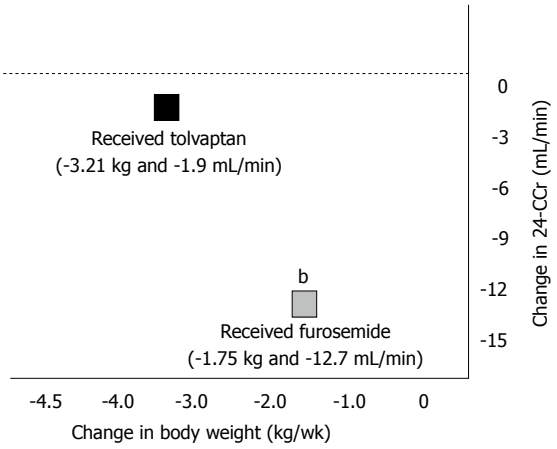

Figure 7 Changes in 24-creatinine clearance and body weight from the baseline after administration of drugs. Comparisons between final values and baseline values for each group were performed using the Wilcoxon signed-rank test, $^{b}P < 0.01$. Comparisons between the groups were performed using the Mann-Whitney *U* test.

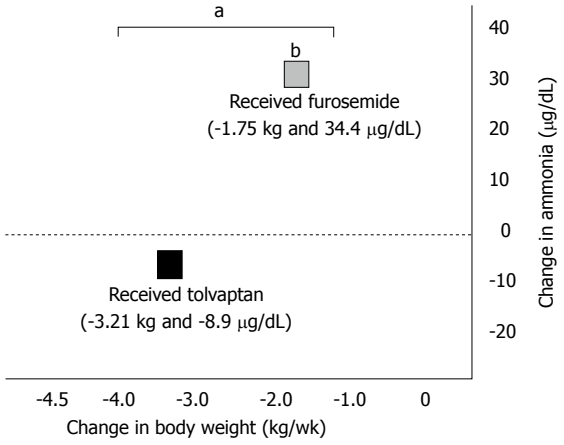

Figure 8 Changes in ammonia and body weight from baseline after administration of drugs. Comparisons between final values and baseline values for each group were performed using the Wilcoxon signed-rank test, $^{b}P < 0.01$. Comparisons between the groups were performed using the Mann-Whitney *U* test, $^{a}P < 0.05$.

and water excretion follows naturally[20-23], until a limit is reached. Ceiling doses of loop diuretics produce a maximal increase in fractional sodium excretion[24].

In the present study, the administration of furosemide in patients undergoing therapy with moderate doses of natriuretic was not change in urine excretion volume. Conventional diuretic therapy with a natriuretic drug only, which blocks sodium reabsorption, may be insufficient to restrict water reabsorption in cases of severe liver cirrhosis with increased circulating levels of AVP and RAAS components. In conditions associated with a lack of response to natriuretic drugs because of insufficient water excretion, concomitant use of aquaretic drugs may provide the ideal treatment. Consequently, combination therapy that involves the blocking of sodium and water reabsorption may be more effective in treating liver cirrhosis with ascites than conventional diuretic therapy, which only blocks sodium reabsorption[25].

The present study does not prove that an aquaretic drug alone is more effective than a natriuretic drug for patients with cirrhotic ascites. The basic therapy used for induction of a negative sodium balance in cases of liver cirrhosis with ascites consists of restricting salt intake and increasing renal sodium excretion[4]. Our previous report shows that administering tolvaptan is beneficial in cases with sufficient sodium excretion induced by a natriuretic drug, and that body weight decreased in proportion to levels of urine sodium excretion as a result of the combination therapy[16]. Therefore, we recommend measuring sodium excretion for a quick and easy determination of the diuretic regimen required.

It is noteworthy that this combination therapy was useful for diuretic-intractable ascites. Addition of tolvaptan had only a slight influence on ammonia levels, and that the incidence of hepatic encephalopathy was reduced in the tolvaptan group, compared to furosemide. Hepatic encephalopathy is related to impaired blood circulation, which decreases renal clearance of ammonia[26,27]. In contrast to furosemide, tolvaptan increases urine output without decreasing renal blood flow, leading to indistinguishable ammonia levels in the tolvaptan group between the baseline and the final dosing day.

This study has shown that addition of furosemide influenced 24-h CCr and plasma renin activity, with renal dysfunction more frequent in the furosemide group than that in the tolvaptan group. A diuretic agent preferably should not activate the sympathetic nervous system or the RAAS, because the pathophysiology of ascites formation in liver cirrhosis is associated with the activation of the RAAS, and occurs to help restore blood homeostasis[28-30]. Although concomitant medications such as branched-chain amino acids, nonabsorbable disaccharides, angiotensin II receptor blockers, or angiotensin converting enzyme inhibitors affect RAAS activation and ammonia levels, combination therapy including aquaretic and natriuretic drugs reduces the incidence of diuretic-related severe side effects. In contrast, increasing the dosage of furosemide above the ceiling dose increases the frequency of severe side effects.

Furthermore, hyponatremia and hypokalemia were not seen in the combination diuretic group. To avoid an electrolyte disturbance, combination diuretic therapy should be evaluated and may resolve hyponatremia with long-term treatment[7]. Hyponatremia, which is characterized by excessive renal retention of water increased release of AVP, is associated with increased mortality and numerous complications in cirrhotic patients. A combination therapy, including a vasopressin

V2-receptor antagonist, which increases the serum sodium concentration, has the potential to improve outcomes in liver cirrhosis patients with ascites[7].

Our final objective was to establish the strategy of ascites refractory to diuretic therapy. However, the present study was not suitable for the management of patients with refractory ascites in two points. First, refractory ascites is defined as immobilization of free fluid from the peritoneum despite 160 mg/d of furosemide and 400 mg/d of spironolactone. Therefore, doses of naturetic drugs were not matched, which is different from the standard Japanese guidelines[15]. Second, many patients with refractory ascites develop a chronic renal dysfunction with the progression of cirrhosis. Patients enrolled in this study had a relatively good renal function and good sodium excretion. If they had presented with mild or severe renal dysfunction, which would have affected the response to diuretics, the results would likely have been markedly different. A previous study reported that changes in body weight after administration of tolvaptan were dependent on the level of creatinine or eGFR (estimated glomerular filtration rate)[25].

The present study has a few limitations. First, the study was not a multicenter study. Hence, a relatively small number of patients were enrolled, resulting in patient selection bias. Second, current understanding of the influence of tolvaptan on circulating blood volume and renal clearance of ammonia is insufficient, warranting further study. Finally, it is not clear whether or not the combination therapy improved the long-term prognosis of these patients. Further detailed research of prognosis under combination therapy is warranted.

In summary, to our knowledge, this study is the first study that examines whether the addition of an aquaretic drug is a more effective therapy than only increasing the dose of a natriuretic drug for liver cirrhosis patients with ascites.

ARTICLE HIGHLIGHTS

Research background

Recently, several studies have evaluated the effects of aquaretic drugs such as tolvaptan for treating ascites resistant to conventional therapies.

Research motivation

Whether a combination therapy with natriuretic and aquaretic drugs is more effective than conventional therapy with natriuretic for liver cirrhosis patients with ascites remains unclear.

Research objectives

We assessed the effects of a combination therapy with natriuretic and aquaretic drugs in cirrhotic ascites patients.

Research methods

A two-center, randomized, open-label, prospective study was conducted. Japanese patients who met the criteria were randomized to trial groups and they received an aquaretic drug (7.5 mg of tolvaptan) or a natriuretic drug (40 mg of furosemide) for 7 d in addition to the natriuretic drug which was used prior to enrolment in this study. The primary endpoint was the change in body weight from the baseline. Vital signs, fluid intake, and laboratory and urinary data were assessed to determine the pharmacological effects of tolvaptan and furosemide.

Research results

A total of 56 patients were randomized to receive either tolvaptan (n = 28) or furosemide (n = 28). In the tolvaptan and furosemide groups, the average decrease in body weight from the baseline was 3.21 ± 3.17 kg (P < 0.0001) and 1.75 ± 2.36 kg (P = 0.0006), respectively, when measured on the final dosing day. Following 1 wk of treatment, a significantly greater reduction in body weight was observed in the tolvaptan group compared to that in the furosemide group (P = 0.0412).

Research conclusions

Compared to a conventional therapy with only natriuretic drugs, a combination therapy with natriuretic and aquaretic drugs is more effective for patients with cirrhotic ascites. Following 1 wk of treatment, a significantly greater reduction in body weight was observed in the combination therapy compared to that in the conventional therapy. Combination therapy that involves the blocking of sodium and water reabsorption may be more effective in treating liver cirrhosis with ascites than conventional therapy, which only blocks sodium reabsorption. Combination therapy including tolvaptan and conventional diuretics reduced the occurrence of diuretic-related severe side effects. A two-center, randomized, open-label, prospective study was conducted. The criteria were randomized to a trial group and the combination diuretic group (natriuretic and aquaretic drugs) or the conventional diuretic group (a natriuretic drug only). A combination diuretic therapy including an aquaretic drug resulted in a greater decrease in body weight compared to conventional diuretic with only a natriuretic drug for cirrhotic ascites patients. Moreover, compared to conventional diuretic therapy, combination diuretic therapy reduced the occurrence of diuretic-related severe side effects. Compared to a conventional diuretic therapy with only a natriuretic drug, a combination diuretic therapy with natriuretic and aquaretic drugs proved to be more effective for patients with cirrhotic ascites. Combination therapy may establish a strategy to treat ascites refractory to the standard diuretic therapy.

Research perspectives

The study compared to a conventional diuretic therapy with only a natriuretic drug, a combination diuretic therapy with natriuretic and aquaretic drugs is more effective for patients with cirrhotic ascites. It is not clear whether or not the combination therapy improved the long-term prognosis of these patients. Therefore, future long-term observational studies are warranted. The future research should assess the influence of renal function after the administration of tolvaptan and furosemide in liver cirrhosis patients with ascites for at least 24 wk.

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SYSTEMATIC REVIEW

Double-balloon enteroscopy-assisted dilatation avoids surgery for small bowel strictures: A systematic review

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Abstract

AIM

To evaluate the therapeutic role of double-balloon enteroscopy (DBE) in small bowel strictures and to propose a standard approach to small bowel strictures.

METHODS

Systematic review of studies involving DBE in patients with small bowel strictures. Only studies limited to small bowel strictures were included and those with ileo-colonic strictures were excluded.

RESULTS

In total 13 studies were included, in which 310 patients were dilated. The average follow-up time was 31.8 mo per patient. The complication rate was 4.8% per patient and 2.6% per dilatation. Surgery was avoided in 80% of patients. After the first dilatation, 46% were treated with re-dilatation and only 17% required surgery.

CONCLUSION

DBE-assisted dilatation avoids surgery in 80% of patients with small bowel strictures and is safe and effective. We propose a standardized approach to small bowel strictures.

Key words: Double-ballloon enteroscopy; Dilatation; Small bowel stricture; Enteroscopy; Crohn's disease; Systematic review

Core tip: At present there is a wealth of literature on the value of double-balloon enteroscopy (DBE) in the management of obscure gastrointestinal bleeding.

However, there is only few data regarding its role in small bowel strictures and these patients often face surgery. In our study we show that DBE-assisted endoscopic balloon dilation offers safe and effective treatment of small bowel strictures. Surgery can be avoided in 80% of cases. Moreover, we propose a flow-chart representing a standard approach to small bowel strictures.

Baars JE, Theyventhiran R, Aepli P, Saxena P, Kaffes AJ. Double-balloon enteroscopy-assisted dilatation avoids surgery for small bowel strictures: A systematic review. *World J Gastroenterol* 2017; 23(45): 8073-8081 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8073.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8073

INTRODUCTION

Small bowel strictures are associated with major comorbidity and adequate management is therefore required. The aetiology of small bowel strictures is varied and includes Crohn's disease (CD), post-surgical, ischemic, non-steroidal anti-inflammatory drugs (NSAIDs) induced, neoplastic and idiopathic[1,2]. Endoscopic access into the small bowel is not widely available. Hence, surgery has been the mainstay for treatment for small bowel strictures. However, surgery is associated with serious complications including bowel obstruction, intra-abdominal infections, wound infections, anastomotic leakages and fistulas. In patients with CD, the complication rate can be as high as 20%[3]. Besides the direct complications of surgery, there is also a > 70% chance of re-stricturing and it is known that multiple small intestinal resections can lead to short bowel syndrome and malnutrition[4-6]. Endoscopic balloon dilation (EBD) has been extensively described for fibrostenosing CD as it is minimally invasive and preserves intestinal length[7]. However, small bowel strictures are often more difficult to treat given their anatomical location.

In 2001 Yamamoto *et al*[8] first described double-balloon enteroscopy (DBE) as a new method to visualise the small bowel. Additionally, DBE provides the opportunity to perform therapeutics in the small bowel. Complications of DBE that have been described include bleeding, perforation and pancreatitis and however the rates are low ranging from 2%-18%[9,10]. There have been many publications regarding the diagnostic and therapeutic roles of DBE. However, most of these publications have been limited to obscure gastrointestinal bleeding and there is limited data on its role in the management of small bowel strictures[9,10]. Furthermore, previously published data are limited by small series and selection bias. Moreover, there is no clear data to recommend a safe and effective approach to endoscopic balloon dilatation (EBD) of small bowel strictures.

Our aim was to perform a systematic review of the published literature on DBE in small bowel strictures to evaluate the therapeutic role and safety of DBE in management of these strictures. Based on the reviewed data we aim to propose a standardized approach to EBD of small bowel strictures.

MATERIALS AND METHODS

A systematic search was performed in the Medline, PubMed and endbase databases including relevant references for English only articles using the following search terms: small bowel strictures, enteroscopy, balloon dilation/dilatation, double-balloon endoscopy. We only included small bowel strictures and excluded ileo-colonic anastomotic strictures. There were two papers from Hirai *et al*[11] that included the same patient population, so we excluded the first paper of Hirai *et al*[12] from 2010 as this data was updated in 2014. Moreover, Yamamoto's paper from 2004 included overlapping data with the Sunada paper from 2005[13,14]. As the Yamamoto paper had a longer inclusion period with more patients we excluded the paper from Sunada *et al*[14]. Pinho *et al*[15] published a multicenter survey on the use of device-assisted enteroscopy in Portugal, which mentioned 6 cases of DBE-assisted dilatation. However, as these data are soley based on a survey and data with regard to efficacy and follow-up is lacking we decided to exclude this paper. In total, 13 original articles were included[2,11,13,16-25].

We performed a descriptive analysis studying patient demographics, stricture and disease characteristics, dilatation techniques, long-term and short-term complication and success rates. Short-term success was defined as improvement of symptoms after the dilatation. The endoscopic dilatation was only considered successful when the patient was free of surgery at the end of the follow-up period. Major complications such as bleeding, perforation, pancreatitis or any event leading to hospitalization were considered in the safety assessment. Long-term success was defined as the number of patients who did not need surgery during the follow-up period. Re-dilatation was reported separately. In the studies that also included diagnostic enteroscopies or other double-balloon assisted endoscopic interventions, only the data with regard to the dilatation cohort were included.

RESULTS

Study characteristics

General characteristics: In total 13 studies met the inclusion criteria mentioned above. The baseline study characteristics are listed in Table 1. All studies had a retrospective study design and the number of patients enrolled ranged from 8-156 patients. Fifty percent of the 626 patients (n = 316) that were included were

Table 1 Descriptive analysis of the main variables in the studies

Author	Year of publication	# patients	# patients undergoing dilatations	Total # strictures dilated	Aetiology stricture	Length stricture (cm)	FU (months)
Yamamoto *et al*[13]	2004	23	6	6	Mixed	NR	NR
Pohl *et al*[16]	2006	19	9	13	CD	≤ 4	16 (4-26)
Ohmiya *et al*[17]	2009	66	22	47	Mixed	NR	16 (2-43)
Despott *et al*[18]	2011	11	9	18	CD	< 5	20.5 (2-41)
Hayashi *et al*[19]	2008	18	2	2	NSAID	NR	NR
Hirai *et al*[11]	2014	65	52	52	CD	≤ 5	41.8 ± 24.9
Gill *et al*[20]	2014	32	14	15	Mixed	NR	16 (3-60)
Irani *et al*[21]	2012	13	12	17	Mixed	≤ 2	46
Nishimura *et al*[22]	2011	8	7	11	Ischemic	≤ 3	16
Fukumoto *et al*[23]	2007	156	31	50	Mixed	NR	11.9 (1-40)
Sunada *et al*[24]	2016	99	85	291	CD	< 5	41.9
Kita *et al*[25]	2006	NR (at least 45)	45	45	Mixed	NR	NR
Kroner *et al*[2]	2015	71	16	16	Mixed	NR	NR
Total		626	310	583			

CD: Crohn's disease; NSAIDS: Non-steroidal anti-inflammatory drugs; NR: Not reported.

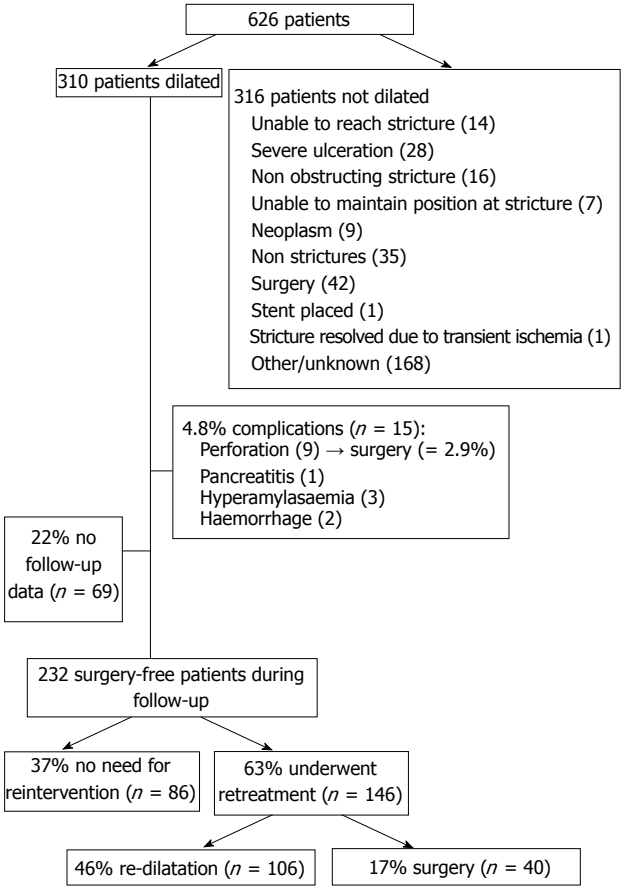

Figure 1 Study results. This flowchart summarizes the study results with the outcomes of the patients that were dilated.

not dilated. The reasons included technical difficulty reaching the stricture due to anatomical variations such as angulation or adhesions (n = 14) or once at the stricture, it was difficult to maintain a stable wire or scope position (n = 7). Other reasons were evidence of active inflammation at the stricture site which was then managed medically (n = 28), or the stricture was not obstructing and therefore not treated (n = 16) (Figure 1). In total, 581 dilatations were performed in the included 310 patients.

Study heterogeneity: There was a significant heterogeneity among the studies and they differed in terms of study aims, patient population and dilatation techniques. However, all studies described at least the short-term success and complication rates of the EBD. Moreover, the enrolled patients differed between the studies: seven studies included all causes of small bowel strictures, whereas in four series the study population was limited to patients with CD, one series only described NSAID-related strictures and one series was limited to ischemic strictures. The length of the stricture varied but none of the studies included strictures above 5cm in length. The inclusion and exclusion criteria of the studies are reported in Table 2.

Dilatation technique

Balloon characteristics: The details of the dilatation technique that was used are specified in Table 3. The controlled radial expansion (CRE) wire guided balloon dilator (Boston Scientific Corporation, Natick, Mass) was the most commonly used (at least 8/13 studies). Five papers did not specify the type of balloon that was used. Four studies reported a maximum dilatation diameter of 20 mm, whereas the other studies went up to 12 mm (one study), 13 mm (one study), 16.5 mm (one study) or 18 mm (two studies). In four studies the diameter was not reported. There was also some variability in the duration of each dilatation. Most studies dilated for 30-60 s, whereas in two studies the dilatation duration was up to 120 s per patient.

Fluoroscopy: Fluoroscopic guidance was used in five studies, whereas two studies performed the dilatation purely under direct vision. The other five papers did not make a remark regarding the use of fluoroscopy.

Table 2 Inclusion and exclusion criteria of the included studies

Author	Year of publication	Inclusion criteria	Exclusion criteria
Yamamoto *et al*[13]	2004	- Retrospective review of all DBEs - Dilatation criteria NR	- NR
Pohl *et al*[16]	2006	- Known or suspected CD and proven or suspicious small bowel strictures - Dilatation criteria NR	- Strictures > 5 cm or including significant angulation or severe active inflammation with ulcerations
Ohmiya *et al*[17]	2009	- Patients with SBO - The stricture was assumed to be restricted within narrow limits in the small bowel assessed by radiologic imaging - A second dilation session was only performed if obstructive symptoms recurred	- Acute obstruction with strangulation or suspected perforation - A stricture with a deep open ulcer
Despott *et al*[18]	2011	- CD patients with small bowel stricture - Dilatation criteria NR	- Strictures > 5 cm
Hayashi *et al*[19]	2008	- Retrospective case series of all patients who had undergone DBE - In the case of a diaphragm-like stricture, all the strictures were dilated	-NR
Hirai *et al*[11]	2014	CD patients with: - Small bowel strictures causing obstructive symptoms - Stricture length ⩽ 5 cm - No associated fistula or abscess - no deep ulcer - No severe curvature of the stricture	- Stricture of the ileocolonic anastomosis - Post-dilatation observation period < 6 mo - Patients who did not meet dilatation criteria
Gill *et al*[20]	2014	- Retrospective review: All patients with suspected strictures in the small bowel undergoing DBE - Dilatation criteria NR	- Patients with severely ulcerated or inflamed strictures - Patients in whom the scope could not traverse the stricture
Irani *et al*[21]	2012	- Clinical and radiological evidence (CT or small bowel follow through) of small bowel obstruction	- Malignant strictures and masses found either at video capsule endoscopy or DBE
Nishimura *et al*[22]	2011	- Patients with ischemic enteritis and a segment of intestine that could not be passed by the enteroscope - Dilation was indicated when there were symptoms of intestinal obstruction and evidence of caliber change by CT scan	- Deep ulcerations
Fukumoto *et al*[23]	2007	A stricture was defined by 1 or more of the following criteria: - DBE showed the internal diameter of the bowel lumen to be < 10 mm or the endoscope could not pass through the lesion - The patient complained of obstructive symptoms - Stricture was suggested or identified by other modalities.	-Asymptomatic patient (even when the endoscope did not pass through the stricture)
Sunada *et al*[24]	2016	- Retrospective review of all DBEs - Dilatation criteria NR	-NR
Kita *et al*[25]	2006	- Retrospective review of all DBEs - Dilatation criteria NR	-NR
Kroner *et al*[2]	2015	- Retrospective review of consecutive patients who were found to have small bowel stricture at the time of DBE - Benign appearance of the stricture	- Malignant (appearance of) strictures

CD: Crohn's disease; NR: Not reported; SBO: Small bowel obstruction; DBE: Double balloon enteroscopy.

Sedation: In four studies all dilatations were done under conscious sedation, and in one study all dilatations were done under conscious sedation or propofol. In another five studies the method of sedation was not mentioned. Despott *et al*[18] used general anesthesia in 9 procedures, with midazolam and pethidine in 2 procedures and anesthesist-delivered propofol sedation in 1 procedure. Irani *et al*[21] used general anesthesia in seven patients and conscious sedation in 6 others.

Study outcomes

Follow-up: The follow-up duration was reported in 9 out of 13 studies and varied from 11.9 - 46.0 mo. In 68 patients (22%) there was no follow-up data, which was either not reported in the study or the patient was lost to follow-up. The total follow-up time was 7750.5 mo for 241 patients leading to an average follow-up time of 31.8 mo per patient.

Clinical efficacy: Among those who underwent EBD, 80% achieved long-term success without the need for surgery during follow-up (*n* = 192/241 patients). In total, 20% required surgery during the study-period (*n* = 49/241 patients), either because of a complication

Table 3 Technical details of dilatations

Author	Year of publication	Balloon diameter (mm)	Duration of dilatation per stricture (s)	Type of balloon	Fluoroscopy	Sedation CS/ GA
Yamamoto *et al*[13]	2004	NR	NR	Boston Scientific, CRE	NR	CS
Pohl *et al*[16]	2006	Up to 20	120	Boston Scientific, CRE	Yes	NR
Ohmiya *et al*[17]	2009	8-20	60	NR	NR	NR
Despott *et al*[18]	2011	12-20	60	Boston Scientific, CRE	No	CS and GA
Hayashi *et al*[19]	2008	NR	NR	Boston Scientific, CRE	NR	NR
Hirai *et al*[12]	2014	12-18	30-120	Boston Scientific, CRE	NR	CS
Gill *et al*[20]	2014	10-16.5	NR	Boston Scientific, CRE	No	CS or propofol
Irani *et al*[21]	2012	10-18	30 or until waist effacement	NR	Yes	CS and GA
Nishimura *et al*[22]	2011	8-12	30 (and 30 s interval)	Boston Scientific, CRE	Yes	CS
Fukumoto *et al*[23]	2007	NR	NR	NR	Yes	NR
Sunada *et al*[24]	2016	8-20	30-60	Boston Scientific, CRE	Yes	CS
Kita *et al*[25]	2006	NR	NR	NR	NR	NR
Kroner *et al*[2]	2015	13	NR	NR	NR	GA

CS: Conscious sedation; GA: General anesthesia; CRE: Controlled radial expansion; NR: Not reported.

of the EBD (n = 9) or because of a relapse (n = 40).

Excluding those who needed surgery due to a complication of the EBD, 37% of patients did not need further treatment after the initial EBD. In total, 63% was retreated because of a relapse or underwent prophylactic re-dilatation. Unfortunately, one study with a high re-dilatation rate did not report the reason for re-dilatation and the relapse rate can therefore not be precisely reported[24]. Of the 146 patients who needed retreatment, 27% underwent surgery (n = 40) and 46% successfully underwent EBD (n = 106). The study results are summarized in Figure 1 below.

Complications: The complications are listed in Table 4. The total complication rate per patient was 4.8% (15/310 patients), whereas the total complication rate per dilatation was 2.6% (15/583 dilatations). This included 5 patients with a perforation, 3 patients with acute pancreatitis, 1 patient who suffered from hemorrhage that required blood transfusions and 3 patients with hyperamylasemia. The patient with pancreatitis recovered after a short hospital stay and the patients with perforations all underwent surgery.

DISCUSSION

In this systematic review we demonstrate that double-balloon assisted dilatation is a safe and effective treatment for small bowel strictures. Four out of five patients avoid surgery due to double-balloon assisted dilatation of their small bowel stricture in an average follow-up of 2.5 years per patient.

Small bowel strictures are associated with major comorbidity and usually require treatment. Surgery has been the main treatment option in the past. Moreover, the management of small bowel strictures, especially in CD, is often a clinical challenge because of the high recurrence rate and many patients need surgery more than once. Although surgery often results in symptomatic resolution, repeat surgery is often needed which can result in short bowel syndrome and malnutrition[26,27]. Our study showed that DBE-assisted balloon dilatation is effective in avoiding surgery as only 17% of patients required surgery due to a relapse.

In our study cohort, 47% of patients underwent re-dilatation during the follow-up period. It is known that strictures often recur, especially in CD[26,27]. Even after stricturoplasty, repeat surgery may be as high as 25% over a follow-up of 2.5 years[28]. In our study, surgery was avoided in 80% of cases.

Hirai *et al*[11] reported an unusually high repeat DBE rate (85.6%). This can be explained by the fact that their protocol included a routine re-examination and prophylactic re-intervention if a stricture was seen. In total 45 out of 52 successful EBD cases were re-examined to confirm the condition of the strictures after initial EBD. Of these 45 patients, 26 patients needed a secondary EBD of which 7 patients were asymptomatic. Sunada *et al*[24]. also reported a very high repeat DBE rate, but again they also performed repeat EBD in asymptomatic patients. They did not report how many patients actually needed retreatment because of a relapse. In the other studies, patients only underwent a repeat enteroscopy if they were symptomatic. There is currently no role for scheduled re-dilatation if patients are asymptomatic as there is no literature available to support scheduled re-dilatation. However, if patients are symptomatic re-dilatation would need to be considered.

In our study cohort a complication rate of 4.8% per patient and 2.6% per dilatation was demonstrated. This is comparable with previous studies. In 2007, Mensink *et al*[10] reported a complication rate of 4.3% in therapeutic double-balloon enteroscopic procedures. In the included study of Ohmiya *et al*[17] data of 668 DBE examinations were reported, of which there were 3 cases of complicating perforation that required surgery (0.45%). However, no complicating perforation occurred in patients during or after enteroscopic balloon dilation and these complications were therefore not included in our study cohort. In our cohort of 310

Table 4 Overview of endoscopic balloon dilation-associated complications per study

Author	Complications	Type of complication	Complication rate per patient	Complication rate per dilatation	Short-term success[2] (%)	Long-term success avoiding surgery[2] (%)	Surgery[2] (%)	Re-Dilatations[2,3] (%)
Yamamoto *et al*[13]	NO	NA	0%	0%	6/6 (100)	NR	NR	NR
Pohl *et al*[16]	NO	NA	0%	0%	9/9 (100)	6/9 (67)	3/9 (33)	2/9 (22)
Ohmiya *et al*[17]	NO[1]	NA	0%	0%	22/22 (100)	18/22 (82)	4/22 (18)	3/22 (14)
Despott *et al*[18]	YES	Perforation (n = 1)	11%	5.6%	8/9 (89)	8/9 (89)	1/9 (11)	2/9 (22)
Hayashi *et al*[19]	NO	NA	0%	0%	2/2 (100)	NR	NR	NR
Hirai *et al*[12]	YES	Haemorrhage (n = 1) Acute pancreatitis (n = 1) Perforation (n = 1) Hyperamylasemia (n = 3)	12%	12%	48/52 (92.3)	44/52 (85)	8/52 (15)	26/52 (50)
Gill *et al*[20]	YES	Perforation (n = 2)	13%	13%	11/14 (79)	11/14 (79)	3/14(21)	1/14 (7)
Irani *et al*[21]	YES	Perforation (n = 1)	8%	6%	10/12 (83)	10/12 (83)	2/12 (15)	2/12 (15)
Nishimura *et al*[22]	NO	NA	0%	0%	6/7 (86)	4/7 (60)	3/7 (43)	1/7 (14)
Fukumoto *et al*[23]	NO	NA	0%	0%	NR	27/31 (87)	4/31 (13)	5/31 (16)
Sunada *et al*[24]	YES	Perforations (n = 4) Bleeding (n = 1)	6%	2%	80/85 (94)	64/85 (75)	21/85 (25)	64/85 (75)[4]
Kita *et al*[25]	NO	NA	0%	0%	45/45 (100)	NR	NR	NR
Kroner *et al*[2]	NO	NA	0%	0%	16/16 (100)	NR	NR	NR
Total		Haemorrhage (n = 1) Acute pancreatitis (n = 1) Perforation (n = 9) Hyperamylasemia (n = 3) Bleeding (n = 1)	4.8%	3%	263/279 (94.3)	192/241 (80)	49/241 (20)	106/241(44)

[1]No complications mentioned in dilatation-cohort; [2]1 patient lost to follow-up; [3]Either because of a relapse or as prophylaxis; [4]No data reported on indication for repeat dilatation and relapse rate. NA: Not applicable; NR: Not reported.

patients, nine patients (2.9%) needed surgery directly after the dilatation due to a perforation. Our reported rate of intestinal perforation is within the range of 0% to 3.7% reported in previous studies[26,27,29,30].

Known risk factors for perforation include adhered or angulated strictures, active inflammation and dilating to more than 15 mm[18,20]. Sunada *et al*[24] demonstrated that the surgery-free interval in patients with a fistula was significantly shorter than in patients without a fistula. Moreover, most of the included studies did not perform dilatation when there was active ulceration.

In this study we assess the role of DBE-assisted dilatation of small bowel strictures. The data on other modalities of enteroscopy including single balloon enteroscopy and spiral enteroscopy is very limited with only small numbers and limited follow-up. We therefore decided to focus our systematic review on DBE-assisted dilatation only. There is a large multicenter survey from Portugal on the use of device-assisted enteroscopy, which mentioned 6 cases of DBE-assisted dilatation with just one reported complication. This confirms the safety we also show in our study[15].

Endoscopic and surgical management of small bowel strictures are not mutually exclusive options. On the contrary, these two modalities complement each other as even patients who have undergone surgical resections in the past may still be considered for endoscopic therapy. In one of the studies in our systematic review, 10 of the 11 patients had undergone previous surgery and yet the procedure was successful in 9 of them[18].

The current study is limited by its retrospective design. Due to heterogeneity of the study designs and lack of randomized controlled trials it was not possible to perform a meta-analysis. However, this large systematic review includes a large patient group with a long average follow-up time of over 2.5 years per patient.

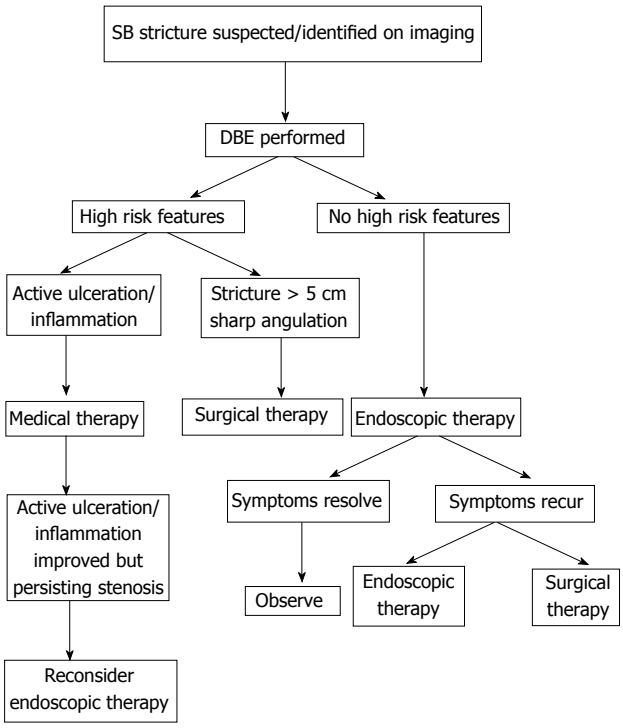

Figure 2 Suggested approach to small bowel. This algorithm proposes a standardized approach to small bowel strictures, taking into account the known risk factors previously demonstrated in literature.

Concomitant medical treatment was not evaluated, although we know this is important for preventing strictures and can influence the relapse-rate. Moreover, as the numbers are relatively small we could not analyze the outcomes stratified by disease. Finally, the published studies did not focus on multiple small bowel strictures. Therefore, based on this literature review we cannot analyze the outcomes of treatment of multiple small bowel strictures, nor can we comment on the optimal strategy to treat multiple small bowel strictures.

Recently, a clinical practice guideline to enteroscopy was published in Japan[31]. In this guideline the indications for enteroscopy-assisted balloon dilatation are discussed which include symptomatic strictures that are < 5 cm long and are not associated with active inflammation or fistula/abscesses. Although the indications to EBD are described in this guideline, the general, step-wise approach for the management of small bowel strictures is currently not available. A standardized approach is beneficial as it may reduce complication rates and therewith even further increase the beneficial effect of DBE-assisted balloon dilatation. Taking into account the known risk factors previously demonstrated in literature and discussed in the Japanese guideline we propose the algorithm demonstrated in Figure 2 as a standardized approach to small bowel strictures.

In this algorithm we propose that at first high risk features such as active inflammation/ulceration, large

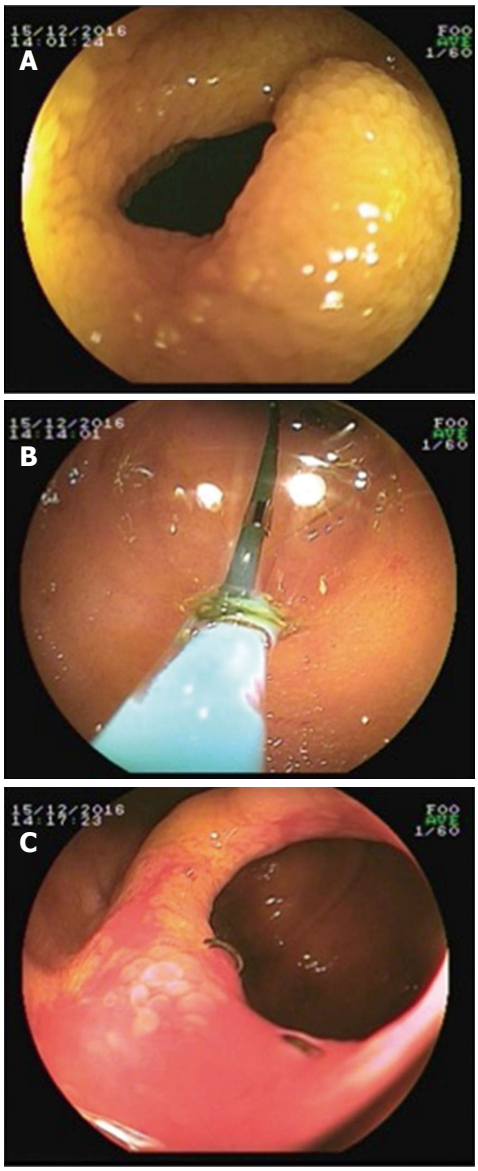

Figure 3 Double-balloon enteroscopy -assisted balloon dilatation. An example of a successful double-balloon enteroscopy (DBE)-assisted balloon dilatation is presented. A: shows the endoscopic image of a benign small bowel stricture in one of our patients. This patient was known with Crohn's disease and had had prior small bowel surgery. She presented with obstructive symptoms and a fibrotic stricture at the side of the anastomosis. B: The stricture was dilated with DBE-assisted balloon dilation; C: Shows the anastomotic stricture after successful dilatation. This picture reveals the surgical staples at the anastomosis and there were no signs of active Crohn's disease.

stricture and sharp angulation should be considered. In case there are no high risk features endoscopic therapy can be performed safely. An illustration of endoscopic balloon dilatation is shown in Figure 3A-C. If the symptoms have resolved after endoscopic therapy no further dilatation is required and the patient should be observed. However, if symptoms do recur, endoscopic re-dilatation or finally surgery has to be considered.

In conclusion, DBE is a safe and effective tool in the management of small bowel strictures and avoids unnecessary surgery in the majority of patients. DBE-assisted EBD should therefore be considered in all

patients with small bowel strictures.

ARTICLE HIGHLIGHTS

Research background

At present there is a wealth of literature on the value of double-balloon enteroscopy (DBE) in the management of obscure gastrointestinal bleeding. However, there is only few data regarding its role in small bowel strictures. The management of small bowel strictures is complicated and these patients often face surgery, which has a huge impact on their quality of life.

Research motivation

In this study we aimed to evaluate the therapeutic role of DBE in small bowel strictures. In addition, we aimed to propose a standard approach to the management of small bowel strictures.

Research objectives

The main objective of this manuscript was to assess the efficacy and safety of DBE-assisted balloon dilatation of small bowel strictures. This is important as many of these patients often face surgery. The authors aimed to assess the role of DBE-assisted dilatation as an alternative for surgery.

Research methods

This study is a systematic review of published papers on DBE-assisted dilatation of small bowel strictures. Only studies limited to small bowel strictures were included and those with ileo-colonic strictures were excluded.

Research results

In total 13 studies were included, in which 310 patients were dilated. The average follow-up time was 31.8 mo per patient. The complication rate was 4.8% per patient and 2.6% per dilatation. Surgery was avoided in 80% of patients. After the first dilatation, 46% were treated with re-dilatation and only 17% required surgery.

Research conclusions

In this systematic review we demonstrate that double-balloon assisted dilatation is a safe and effective treatment for small bowel strictures. Four out of five patients avoid surgery due to double-balloon assisted dilatation of their small bowel stricture in an average follow-up of 2.5 years per patient. Moreover, we propose a flow-chart representing a standard approach to small bowel strictures.

Research perspectives

This research shows that double-balloon assisted balloon-dilatation is a safe and effective treatment for small bowel strictures and should be considered as a first treatment options. Future research is needed to explore the options of balloon-assisted enteroscopy in other device-assisted enteroscopy modalities.

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META-ANALYSIS

Impact of inflammatory bowel disease activity and thiopurine therapy on birth weight: A meta-analysis

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Abstract

AIM

To investigate the effect of disease activity or thiopurine use on low birth weight and small for gestational age in women with inflammatory bowel disease (IBD).

METHODS

Selection criteria included all relevant articles on the effect of disease activity or thiopurine use on the risk of low birth weight (LBW) or small for gestational age (SGA) among pregnant women with IBD. Sixty-nine abstracts were identified, 35 papers were full text reviewed and, only 14 of them met inclusion criteria. Raw data were extracted to generate the relative risk of LBW or SGA. Quality was assessed using the Newcastle Ottawa Scale.

RESULTS

This meta-analysis is reported according to PRISMA guidelines. Fourteen studies met inclusion criteria, and nine reported raw data suitable for meta-analysis. We found an increased risk ratio of both SGA and LBW in women with active IBD, when compared with women in remission: 1.3 for SGA (4 studies, 95%CI: 1.0-1.6, P = 0.04) and 2.0 for LBW (4 studies, 95%CI: 1.5-2.7, P < 0.0001). Women on thiopurines during pregnancy had a higher risk of LBW (RR 1.4, 95%CI: 1.1-1.9, P = 0.007) compared with non-treated women, but when adjusted for disease activity there was no significant effect on LBW (RR 1.2, 95%CI: 0.6-2.2, P = 0.6). No differences were observed regarding SGA (2 studies; RR 0.9, 95%CI: 0.7-1.2, P = 0.5).

CONCLUSION

Women with active IBD during pregnancy have a higher risk of LBW and SGA in their neonates. This should be considered in treatment decisions during pregnancy.

Key words: Pregnancy; Inflammatory bowel disease; Thiopurines; Disease activity; Low birth weight; Small for gestational age

Core tip: There are conflicting data on the impact of disease activity and thiopurine use on birth weight in pregnant women with inflammatory bowel disease. The individual impact of these factors in low birth weight (LBW) and small gestational age (SGA) has not been systematically evaluated to date. For these reasons, we performed a meta-analysis to identify the effect of disease activity or thiopurine use on the rates of LBW and SGA in these patients. Since many women become non-adherent to medications during pregnancy, for fear of a negative effect on the fetus, further information would be useful in counseling women.

Gonzalez-Suarez B, Sengupta S, Moss AC. Impact of inflammatory bowel disease activity and thiopurine therapy on birth weight: A meta-analysis. *World J Gastroenterol* 2017; 23(45): 8082-8089 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8082.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8082

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease, often affecting young women of reproductive age. The potential impact of IBD on their pregnancy is one of the main concerns of these women. Several retrospective case-control studies have reported an increase of preterm delivery, low birth weight (LBW), small for gestational age (SGA) or congenital abnormalities (CAs) in patients with IBD, when compared to healthy controls[1-4] .These conclusions have been confounded by the individual roles that disease activity and maintenance medications may play in these birth outcomes. Population-based studies from Europe have concluded that disease activity did not significantly increase the risk of low birth weight in patients with Crohn's disease (CD), whereas other studies have reported an increase in small for gestational age (SGA) births[5,6]. A large prospective study including 332 pregnancy IBD women, reported that women with IBD had similar pregnancy outcomes women without IBD, but most of the IBD women were in remission or on maintenance therapy[7]. Since many women stop taking therapy during pregnancy, for fear of a negative effect on the fetus, further information on the impact of disease relapse while pregnant would be useful in counseling women[8] . A recent multicenter and prospective study[9], where IBD women were asked to complete a questionnaire about their concerns on pregnancy and medications, demonstrated that a lack of knowledge or inappropriate education and counseling may lead to inappropriate treatment decisions.

A key factor in preventing relapse is use of maintenance medication. Thiopurines (azathioprine/mercaptopurine) have been a mainstay of maintenance therapy in IBD globally since the 1980s. There have been conflicting data on the birth outcomes of women with IBD treated with thiopurines, with population-based studies showing no impact, but referral centers reporting an increased risk of SGA births or pre-terms births[10-12].

Since to women with IBD who are pregnant have a higher risk of adverse events, early consultation with specialists can help them to plan appropriate pregnancy and delivery[13]. Information on the role of disease activity and use of maintenance thiopurines can be critical in these discussions. The individual impact of these factors in LBW and SGA in women with IBD has not been systematically evaluated to date. For these reasons, we performed a systematic review and meta-analysis to identify the pooled effect of disease activity or thiopurine use on the rates of LBW and SGA in women with IBD.

MATERIALS AND METHODS

This systematic review and meta-analysis was conducted with guidance provided by the Cochrane Handbook for Systematic Reviews[14]. It is reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[15].

Study selection

We included observational studies in this meta-analysis that met the following inclusion criteria: (1) participants: Studies performed in pregnant women with IBD; (2) interventions: Described any disease activity or thiopurine use as defined by each study's primary definition of use. We then sub-classified based on duration of exposure; (3) comparators: Pregnant women with IBD not exposed to disease activity or thiopurines; (4) outcomes: Risk Ratio (RR) and 95%CI of development of either LBW (a birth weight < 2500 mg), or SGA (a weight below the 10th percentile for gestational age); and (5) studies: Reported with clear definitions of LBW and SGA. Provide sufficient data to allow estimation of effect size. Inclusion was not otherwise restricted by study size, language, or publication type. When there were multiple publications from the same cohort, data from the most recent comprehensive report were included.

Search strategy

The clinical investigators searched Medline, Embase, ClinicalTrials.gov, Web of Science and the Cochrane Library (up to October 2016), for all relevant articles on the effect of disease activity or thiopurine use on the risk of LBW or SGA among pregnant women with IBD. Medical subject heading (MeSH) or keywords used in the search included the following: "ulcerative colitis", "Crohn's disease", "Crohn's", "colitis", "inflammatory bowel disease", "IBD", "activity", "relapse", "thiopurine",

"azathioprine", "mercaptopurine", "low birth weight", "LBW", "small for gestational age", "SGA". We manually searched review articles and abstracts (2006-2016) from major gastroenterology conferences (American Gastroenterology Association, American College of Gastroenterology, British Society of Gastroenterology, United European Gastroenterology Week, European Crohn's and Colitis Organization). Only abstracts summarized in English were used for screening purposes.

Study selection

Two authors (BGS, SS) independently and without blinding identified and reviewed potentially relevant articles to determine if they fulfill the inclusion criteria. A third author (ACM) adjudicated on whether or not studies should be included, were consensus not reached.

Data collection process and items

An unblinded review author used specially designed assessment forms to extract the relevant data. The forms captured data, including: author, geographical region, year of publication, study design, nature of disease, outcomes (cancer/ dysplasia), and drug used, dose effect, duration of treatment, setting in which the study was performed, and adjusted and unadjusted OR. Study quality and risk of bias were assessed according to the Newcastle-Ottawa Scale (NOS) guidelines. Patient selection methods, comparability of the studies groups and outcome were evaluated (Supplementary Table 1)[16]. Studies are assigned points for different questions in each category and can have a maximum of nine points. We considered higher than seven points as high quality[16].

Analysis

Our analysis focused on the risk of the development of LBW or SGA among pregnant women with IBD, based on whether or not they had active disease or been treated with thiopurines. The random effects model as proposed by DerSimonian and Laird[17] was used to calculate pooled risk ratio (RR) and 95%CI. The effect estimate used was the RR, as a suitable estimate of relative risk. Potential publication bias was examined by funnel plots. Heterogeneity was examined by the Q statistic and the I2 statistic[18].

The Q and I^2 statistics were used to test statistical heterogeneity among studies. For the Q statistic, a *P* value of less than 0.1 is considered representative of statistically significant heterogeneity. An I^2 index of around 25% is considered to demonstrate low levels of heterogeneity, 50% medium, and 75% high. Sensitivity analyses were conducted, omitting each study in turn to screen for outliers with respect to results, study population, study design, or duration of thiopurines exposure. Analyses were performed using the Review Manager (RevMan, Version 5.3. Copenhagen: The Nordic Cochrane Centre, the Cochrane Collaboration, 2014).

RESULTS

Study selection and characteristics are shown in Figure 1; of the 69 abstracts identified, 35 papers were full text reviewed and, only 14 of them met inclusion criteria. Twenty-two papers were excluded for different reasons (supplementary Table 2) and 5 papers were not included in the meta-analysis because raw data was not available. Four studies reported data about disease activity in IBD patients and SGA; the same number of papers reported data about the prevalence of LBW. The prevalence of SGA in IBD patients on thiopurines treatment was described in 2 papers and, finally, LBW in newborns on thiopurines treatment and LBW were reported in 5 studies.

Nine studies reported raw data suitable for meta-analysis (Table 1)[5,6,19-25]. All but two of these studies had a retrospective design. Mean length of follow up was 18.7 years, ranging from 3 to 15 years.

The analysis on disease activity was carried out on a total of 1128 IBD pregnancies with active disease vs 1280 IBD pregnancies in remission. There was not a homogeneous definition of "active disease" in the included studies (Table 2). Pregnancies on or off thiopurines treatment (782 *vs* 3946 patients) were also analyzed. Outcomes evaluated were SGA and LBW. For the thiopurine-exposed groups, the proportions of patients exposed to other medications are shown in supplementary Table 3.

Effect of disease activity on SGA and LBW

Six studies reported data about disease activity and SGA, 2 prospective study and 4 with retrospective design[6,19-21]. Amongst the 1115 pregnancies in women with active disease, 12% (130/1115) resulted in SGA birth, compared to 9% in women with IBD in remission. The risk ratio for SGA with active disease was 1.3 (95%CI: 1.0-1.6, $P = 0.04$; Figure 2). There was no statistical heterogeneity amongst these studies ($I^2 = 0\%$).

Two studies did not report raw data, so was excluded from the meta-analysis[26,27]. Stephansson *et al*[27] reported a higher adjusted prevalence odds ratio of SGA in women with Ulcerative Colitis (UC) compared to controls (Adj POR 1.2) but this risk was higher in women with UC who had been hospitalized for UC (Adj POR 1.4) compared to outpatient women (Adj POR 0.95), suggesting that disease activity influenced this outcome.

Six studies reported data on LBW rates according to disease activity. Three had a prospective design and 3 were retrospective. Two studies provided no raw data[7,28], thus 4 were included in the meta-analysis[19-21,24]. Women with IBD with active disease

Table 1 Studies included in systematic review

Study ID	Yr	Study period	Type of study	Sample size	IBD patients with active disease	IBD patients with inactive disease	Outcomes
Disease activity and SGA							
Bröms *et al*[19]	2014	2006-2010	R	470110	988	972	SGA and LBW
Bush *et al*[20]	2004	1986-2001	R	56514	22	94	SGA and LBW
de Lima-Karagiannis *et al*[21]	2016	2008-2014	P	298	92	134	SGA and LBW
Moser *et al*[6]	2000	1993-1997	R	130	13	52	SGA
Stephansson *et al*[27]	2011	1994-2006	R	871579	No raw data provided		SGA
Mahadevan *et al*[26]	2012		P	797	No raw data provided		SGA
Disease activity and LBW							
Bortlik *et al*[24]	2013	2007-2012	R	41	10	31	LBW
Bröms *et al*[19]	2014	2006-2010	R	470110	988	972	SGA and LBW
Bush *et al*[20]	2004	1986-2001	R	56514	22	94	SGA and LBW
de Lima-Karagiannis *et al*[21]	2016	2008-2014	P	298	92	134	SGA and LBW
Molnár *et al*[28]	2010		R	167	No raw data provided		LBW
Bortoli *et al*[7]	2011	2003-2006	P	664	No raw data provided		LBW
Treatment and SGA					Thiopurine exposure	Non thiopurine exposure	
Bröms *et al*[19]	2014	2006-2010	R	470110	421	1539	SGA and LBW
Cleary BJ[23]	2009	1995-2007	R	1164030	315	676	SGA and LBW
Treatment and LBW							
Bröms *et al*[19] (AZA)	2014	2006-2010	R	470110	421	1539	SGA and LBW
Cleary *et al*[23]	2009	1995-2007	R	1164030	315	1676	SGA and LBW
Komoto *et al*[25] (AZA)	2016	2008-2014	R	72	7	29	LBW
Nørgård *et al*[5]	2007	1996-2004	R	900	20	628	LBW
Shim *et al*[22]	2011	1996-2006	R	93	19	74	LBW
Nørgård *et al*[10]	2003	1991-2000	R	19437	No raw data provided		LBW

R: Retrospective study; P: Prospective study; IBD: Inflammatory bowel disease; SGA: Small for gestational age; LBW: Low birth weight.

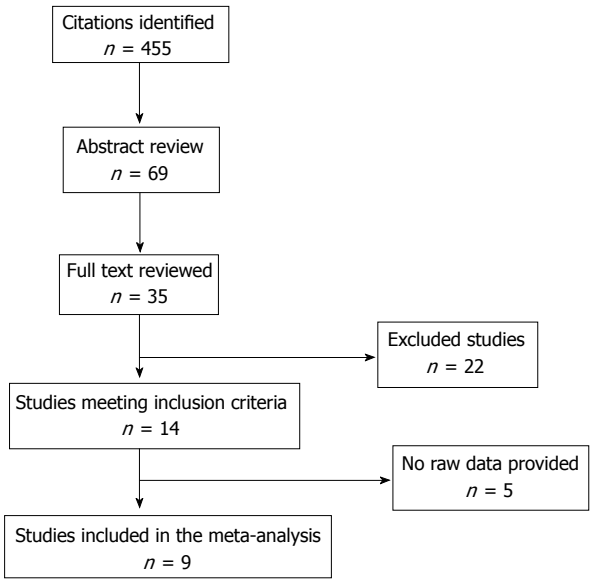

Figure 1 Flow of literature search.

had a higher risk ratio of LBW than women with inactive disease (RR = 2; 95%CI: 1.5-2.7, P < 0.0001; Figure 3). There was significant statistical heterogeneity amongst these studies (I^2 = 60%), based on the inclusion of one study[20] from a tertiary referral center, where the RR for LBW was 8.6. When this was excluded from the analysis the heterogeneity was 0%, but the RR for LBW remained significantly elevated (RR = 1.8, 95%CI: 1.5-2.7, 1.3-2.15, P = 0.0004).

One study was not included in the meta-analysis as it included no infants with birth weight less than 2500 g. This prospective and multi-centric European case-control study, called ECCO-EpiCom, only reported mean birth weight, which was lower in those with active disease[7]. In only one study[19], patients were grouped by timing of their flare-up (early pregnancy, late pregnancy or both). They reported a 3-4-fold risk increase of low birth weight if the flare event was in both early and late pregnancy.

Effect of thiopurine therapy on small for gestational age and low birth weight

Since thiopurines have been a mainstay maintenance medication in IBD in many studies, and there was conflicting data regarding its impact on birth weight, we next analyzed studies that examined this issue. For the outcome of SGA, only 2 studies reported the SGA rates in patients on thiopurines, compared to non-thiopurine patients[19,23]. No differences were observed between SGA risk in treated or non-treated women with IBD (RR = 0.9, 95%CI: 0.7-1.2, P = 0.50; Figure 4). There was significant statistical heterogeneity amongst these studies (I^2 = 70%). This is likely because the paper by Cleary did not control for disease activity[23].

Five studies reported raw data on LBW outcomes in women treated with thiopurines suitable for meta-analysis[3-7], including 782 patients on treatment and 3946 non -treated. There was a higher risk ratio of LBW in women on thiopurines (RR = 1.4, 95%CI:

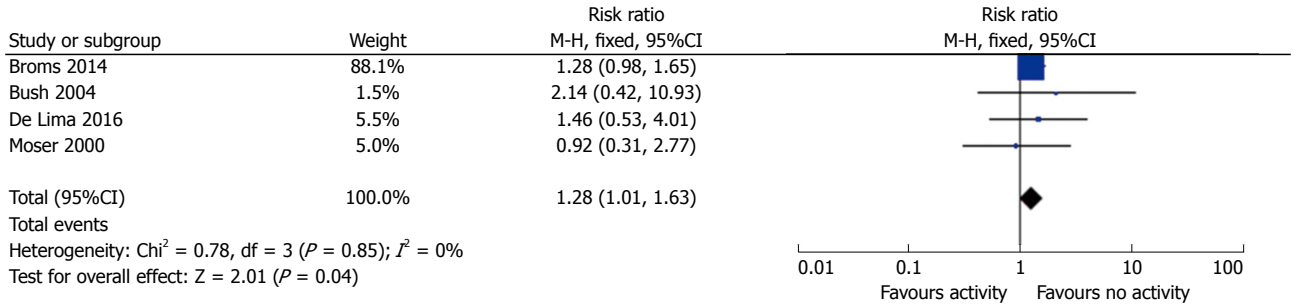

Figure 2 Risk ratio of small for gestational age based on disease activity. SGA: Small for gestational age.

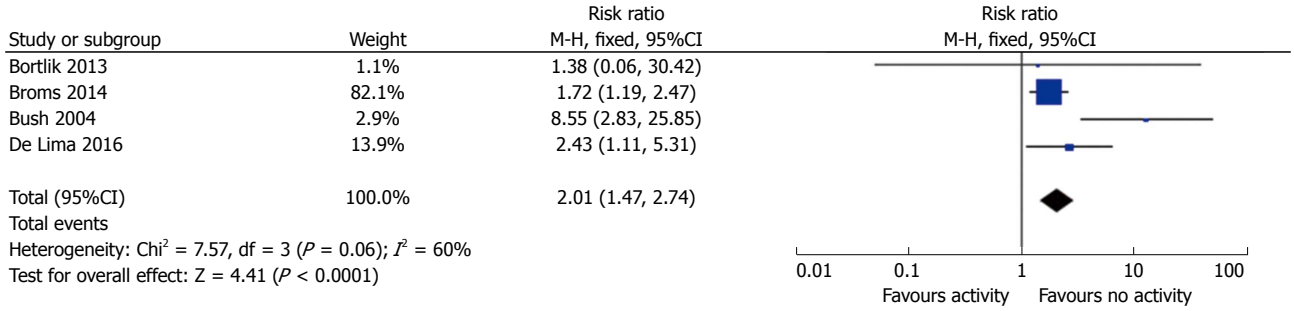

Figure 3 Risk ratio of low birth weight based on disease activity. LBW: Low birth weight.

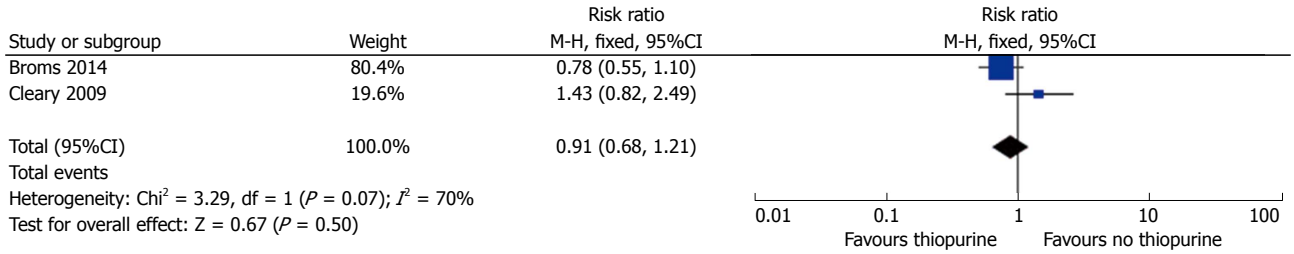

Figure 4 Risk ratio of small for gestational age based on thiopurine exposure.

Table 2 Active disease definitions

Study	Active or inactive disease definition
Bröms *et al*[19]	Any change in the treatment
Bush *et al*[20]	Hospitalization
Moser *et al*[6]	Surgery due to a flare of the disease
Shim *et al*[22]	
de Lima-Karagiannis *et al*[21]	HBI > 5
Komoto *et al*[25]	SCCAI > 2; Partial Mayo Score
	Fecal calprotectin > 200 microgr/gr
Bortlik *et al*[24]	Consideration of treating physician

1.1-1.9, P = 0.007; Figure 5), however, when we only included the study that adjusted for disease activity, there was no significant effect of thiopurines on LBW (RR = 1.2, 95%CI: 0.6 - 2.2; P = 0.6)[19].

DISCUSSION

One of the most important questions for IBD physicians and patients is what effect disease activity and drug exposure will have on pregnancy outcomes. Previous studies reported an increased risk of LBW in women with IBD, but neither disease activity nor therapies were assessed separately[1-4]. On the other hand, some studies found increased odds of LBW and SGA among infants born to mothers with CD but not those with UC[2].

Active disease during conception or pregnancy was previously associated with fetal loss, LBW and preterm birth[20,29]. Based on this, the 2010 ECCO Consensus recommended that clinicians achieve clinical remission of CD patients before pregnancy[30]. In contrast, Mahadevan *et al*[31] did not identify any relationship between active disease and adverse birth outcomes in an insured cohort in California.

The present meta-analysis, that included 9 studies, suggests that women with active disease during pregnancy have an increased risk of SGA and LBW in their neonates. This effect estimate averages the risk across different patient populations, and provides an estimate to consider when encouraging women

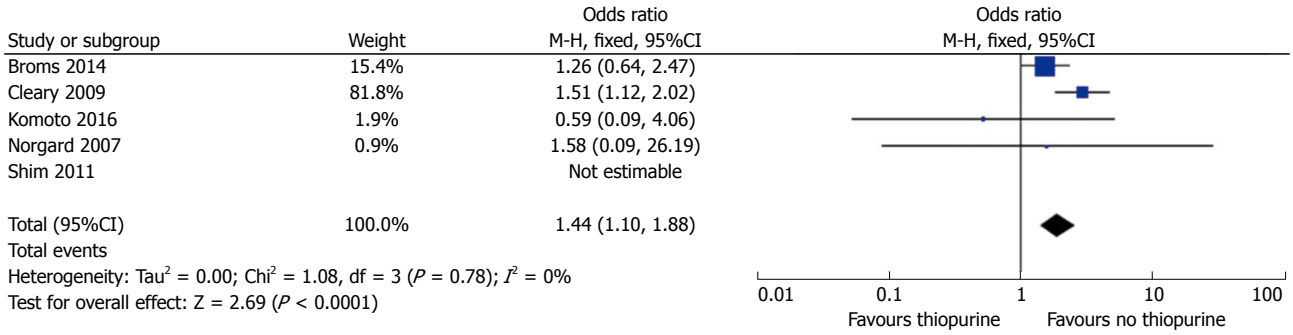

Figure 5 Risk ratio of low birth weight based on thiopurine exposure.

to continue maintenance medications for IBD. This should also factor-in the role of peri-conception disease activity on probability of remission during pregnancy, an issue upon which we have previously reported[32]. Due to lack of reporting of sub-group data, we were unable to undertake sensitivity analysis by disease (UC or CD) or definition of 'active' disease. The mechanisms for this risk are unknown, but possible mechanisms include the physiological disruption of inflammation, in parallel with dietary restrictions and steroid use, may increase the risk of LBW or SGA[33-35].

When the relationship between thiopurine exposure was analyzed, most studies did not discriminate between patients with active disease on thiopurines, and those in remission on thiopurines. The study by Bröms *et al*[19] was most informative on this matter; when risk of LBW was controlled for disease activity, no significant effect of thiopurines was noted. This conclusion is consistent with the population-based studies on this topic, with cohorts predominantly in clinical remission[10].

Limitations

There are several limitations to this meta-analysis. There is a great deal of heterogeneity regarding the definition of "active disease", and drug exposure. The criteria for clinical activity included medical record review, pharmacy data and patient contact, all of which have limitations. In addition, the influence of potentially clinically relevant confounding variables cannot be adequately evaluated. These include retrospective assessment of activity, endoscopic (not symptom) activity, duration of disease, anatomic extent, family history of LBW/SGA, nutritional status and smoking. We intended to performed sub-analyses for the case definition, (as reported in the Results) disease activity definitions, follow-up duration and protocol but there were too few similarly defined parameters within the included studies to further sub-group these for analyses. For some outcomes, less than 5 studies were suitable for meta-analysis, which may limit the application of the results to the pregnant IBD population as a whole.

Conclusion

The presence of active IBD during pregnancy is a risk factor for both SGA and LBW in women with IBD. Independent of disease activity, thiopurine use does not increase the risk of either SGA or LBW. These conclusions should be factored-into discussion with pregnant women about maintenance therapies during pregnancy. Future studies on these relationships should define disease activity according to standard criteria (*e.g.,* Mayo score), and control for disease activity when assessing the impact of medications on pregnancy outcomes.

COMMENTS

Background

The pregnancy outcomes in inflammatory bowel disease (IBD) patients are an important topic for physicians and patients. The drug exposure effect and the influence of active disease on low birth weight (LBW) and/or small gestational age (SGA) are not yet well-known. In fact, many women become non-adherent to medications during pregnancy, for fear of a negative effect on the fetus So further information would be useful in counseling IBD pregnancy women.

Research frontiers

Several retrospective case-control studies have reported an increase of preterm delivery, LBW, SGA or congenital abnormalities in patients with IBD, when compared to healthy controls. These conclusions have been confounded by the individual roles that disease activity and maintenance medications may play in these birth outcomes.

Innovations and breakthroughs

In the present meta-analysis, we have observed that the presence of active IBD during pregnancy is a risk factor for SGA and LBW in women with IBD. Independent of disease activity, thiopurine use does not increase the risk of either SGA or LBW. These conclusions should be factored-into discussion with pregnant women about maintenance therapies during pregnancy.

Applications

Since to women with IBD who are pregnant have a higher risk of adverse events, early consultation with specialists can help them to plan appropriate pregnancy and delivery. Future studies on these relationships should define disease activity according to standard criteria (*e.g.,* Mayo score), and control for disease activity when assessing the impact of medications on pregnancy outcomes.

Peer-review

This systematic review and meta-analysis adds useful information for practice

and research.

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CASE REPORT

Midgut neuroendocrine tumor presenting with acute intestinal ischemia

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Author contributions: Mantzoros I, Savvala NA, Ioannidis O and Tsalis K designed the report; Mantzoros I, Savvala NA, Ioannidis O, Parpoudi S, Loutzidou L and Tsalis K contributed to the clinical diagnosis, evaluation and management of the patient; Parpoudi S, Loutzidou L and Kyriakidou D collected the patient's clinical data; Cheva A performed the histopathologic analysis; Intzos V performed the radiological studies; Mantzoros I, Savvala NA, Ioannidis O and Tsalis K analyzed the data and drafted the paper; all authors discussed the results and commented and contributed to this manuscript.

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Abstract

Neuroendocrine tumors represent a heterogeneous group of neoplasms that arise from neuroendocrine cells and secrete various peptides and bioamines. While gastrointestinal neuroendocrine tumors, commonly called carcinoids, account for about 2/3 of all neuroendocrine tumors, they are relatively rare. Small intestine neuroendocrine tumors originate from intestinal enterochromaffin cells and represent about 1/4 of small intestine neoplasms. They can be asymptomatic or cause nonspecific symptoms, which usually leads to a delayed diagnosis. Imaging modalities can aid diagnosis and surgery remains the mainstay of treatment. We present a case of a jejunal neuroendocrine tumor that caused nonspecific symptoms for about 1 year before manifesting with acute mesenteric ischemia. Abdominal X-rays revealed pneumatosis intestinalis and an abdominal ultrasound and computed tomography confirmed the diagnosis. The patient was submitted

to segmental enterectomy. Histopathological study demonstrated a neuroendocrine tumor with perineural and arterial infiltration and lymph node metastasis. The postoperative course was uneventful and the patient denied any adjuvant treatment.

Key words: Small intestine; Carcinoid; Enterochromaffin cells; Jejunum; Enterectomy; Pneumatosis intestinalis

Core tip: The current case report underlines the indolent clinical course with nonspecific symptoms of a small intestine carcinoid that finally caused acute intestinal ischemia. This case also emphasizes the importance of simple imaging modalities, such as X-rays and the abdominal sonography, in the work-up of a patient with intestinal ischemia.

Mantzoros I, Savvala NA, Ioannidis O, Parpoudi S, Loutzidou L, Kyriakidou D, Cheva A, Intzos V, Tsalis K. Midgut neuroendocrine tumor presenting with acute intestinal ischemia. *World J Gastroenterol* 2017; 23(45): 8090-8096 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8090.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8090

INTRODUCTION

Gastrointestinal (GI) neuroendocrine tumors (NETs), commonly called carcinoids, are relatively quite rare, only representing approximately 0.5% of all human cancers. Their incidence is reported to be 1-2 cases per 100000 individuals per year[1]. The increased incidence of these tumors being reported in recent years can probably be explained by increased awareness and increased detection through new imaging techniques. While small intestine NETs are in general rare tumors, the ileum represents the commonest NET site of the human body[1]. Patients with NETs of the small intestine can manifest symptoms due to the local effect of the primary tumor, enlarging metastases or, indirectly, from secretion of hormones. These tumors tend to grow slowly, which explains their long and indolent course; this slow growth, in combination with their nonspecific symptoms, results in a high level of misdiagnosis[1,2]. When a GI NET is suspected, initial imaging is with computed tomography or magnetic resonance imaging and then somatostatin receptor scintigraphy with SPECT/CT are the standard approaches. In all other instances, the imaging modalities used would depend on the patient's symptomatology[3-7]. While the diagnosis of intestinal ischemia is usually established using contrast enhanced CT, and simple X-ray and abdominal ultrasound only sometimes aid the diagnosis, the present report underlines the importance of using simple imaging modalities, such as X-rays and abdominal sonography, in the work-up of a patient with intestinal ischemia originating from a midgut carcinoid. In localized disease, surgery is the mainstay of treatment[8-10]. In locally advanced and metastatic disease, new treatment modalities, such as octreotide and interferon, have led to longer survival rates[11].

CASE REPORT

A 70-year-old female presented to our emergency department with worsening abdominal pain, vomiting and symptoms of intestinal obstruction. The initial disease onset was one year prior to presentation in the emergency department, with episodes of crampy and paroxysmal abdominal pain, which led her to seek medical treatment. A previous abdominal computed tomography demonstrated distal small bowel thickening, but no further workup was performed due to clinical improvement.

Clinical examination revealed a diffuse abdominal tenderness, and laboratory exams showed severe leukocytosis with neutrophilia (WBC = 20000/mm^3) and increased CRP (4.7 mg/dL). Arterial blood gas revealed mild metabolic acidosis (pH: 7.32, PaO_2: 87 mmHg, $PaCO_2$: 37 mmHg, bicarbonate: 19 mmol/L, lactate: 2.3 mmol/L) and normal coagulation tests (PT: 13.1 s, INR: 1.07, APTT: 28.3 s). All other biochemical tests were within the normal limits.

Abdominal X-ray demonstrated a dilated small bowel loop with intestinal pneumatosis (Figure 1). This was also confirmed in an ultrasound exam, during which the affected loop was found to be slightly thickened, with no peristalsis and with intraluminal content and intestinal wall pneumatosis (Figure 2). The intramural gas pattern, along with the presence of fluid in the Douglas pouch, raised the suspicion of mesenteric ischemia. The abdominal CT angiography demonstrated a small bowel loop with wall thickening, pneumatosis, dilatation of the lumen and adjacent mesenteric fat inflammation (Figures 3 and 4).

Based on the radiologic and clinical findings, the patient was submitted to exploratory laparotomy due to the possibility of mesenteric ischemia. During surgery, the small bowel was thickened and dilated and gross areas of ischemic bowel were present with full thickness necrosis (Figure 5). A segmental enterectomy was performed. Ligation of the mesentery revealed several enlarged lymph nodes adjacent to the jejunal mesenteric arterial branches. Approximately 1m of the jejunum was resected and a side-to-side anastomosis was performed. There was no evidence of other metastatic disease.

Histopathological study of the specimen revealed areas of ischemia with destroyed mucosa and two neoplastic foci of well-differentiated low-grade neuroendocrine tumors that infiltrated the muscularis propria and the adjacent fat tissue (Figure 6). The surgical margins were free, but vascular and perineural

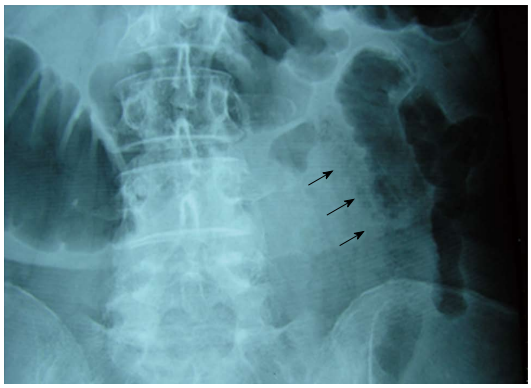

Figure 1 Plain abdominal X-ray depicts slightly dilated small bowel loop with pattern of intramural pearls of air (black arrows).

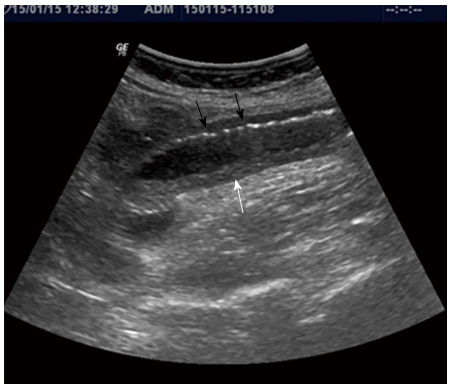

Figure 2 Sonographic features show dilated small bowel loop with absent peristalsis. Also depicted are increased intraluminal secretions within the ischemic small bowel segment (white arrow), slight mural thickening and intramural gas (black arrows).

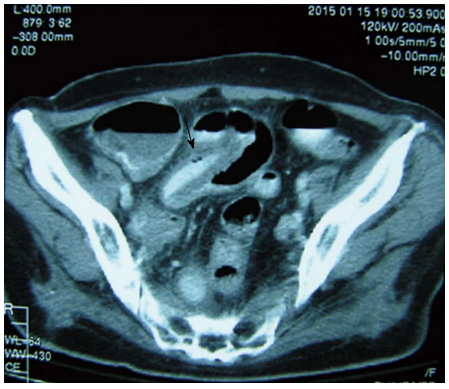

Figure 3 Axial contrast enhanced computed tomography demonstrates intestinal pneumatosis (long black arrow).

invasion was noted (Figure 7A). Interestingly, infiltration of the peripheral mesenteric artery was identified. Three out of twenty lymph nodes were infiltrated (Figure 6). Immunohistological stains for chromogranin A (Figure 7C), CD56 (figure 7E) and synaptophysin (Figure 7F) were positive and stains for ki 67 showed less than 1% proliferation (Figure 7D). The mitotic rate was < 2 per 10 high power fields (HPF).

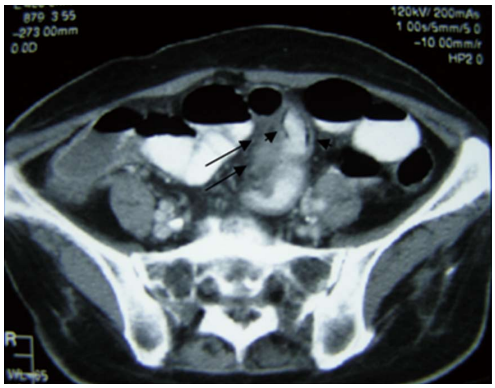

Figure 4 Axial contrast enhanced computed tomography demonstrates a homogeneous thickened soft tissue mass at the small bowel mesentery (long black arrows), as well as intestinal pneumatosis (small black arrows).

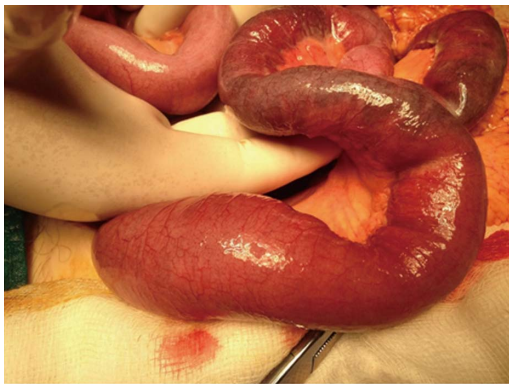

Figure 5 Intraoperative findings.

The patient had an uncomplicated postoperative course and was discharged on the 20th day. A whole-body pet octreotide scan and oncology consultation was recommended, but the patient denied any adjuvant treatments.

DISCUSSION

Nets consist of a heterogeneous tumor group which originate from neuroendocrine cells and secrete various bioamines and peptides[12]. The incidence of NETs has increased from 1.9 to 5.2 per 100000 people per year over the last three decades. Because of the slow growth pattern of NETs, along with their incidence, their prevalence is increased. The GI tract is the primary location of about 67% of NETs. These are also called GI NETs or carcinoids. Small intestine neuroendocrine tumors arise from intestinal enterochromaffin cells and can be recognized by typical tumor cell serotonin immunoreactivity. They account for 1/4 of all small intestine neoplasms and present at a mean age of 65 years with a slight male predominance[1,2,9,12]. Due to the absence of specific clinical manifestations except for the carcinoid syndrome, along with the absence of specific blood biomarkers, there is usually a notable delay in the establishment of the correct diagnosis[9,12].

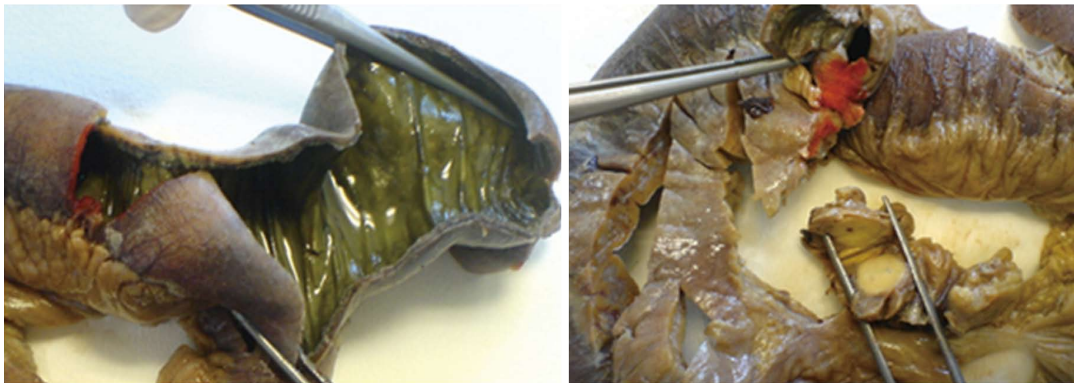

Figure 6 Gross pathology specimen of resected small bowel, showing ischemic bowel and enlarged mesenteric lymph nodes.

Similarly, in our case, the patient suffered from atypical episodes of abdominal pain and loss of weight a year before she irreversibly deteriorated and presented to the emergency department.

Mesenteric metastases occur in high frequency and mainly concern the regional mesenteric and paraaortic lymph nodes. In a recent large series of patients with small intestine NET, mesenteric metastases presented on 93% of operated patients at operation, whereas 61% also had liver metastases[9,12]. When the liver is involved, carcinoid syndrome may develop. In this case, a constellation of symptoms manifests, including flushing, diarrhea, bronchoconstriction and cardiac disease. These symptoms result from the non-inactivation, by monoamine oxidase in the liver, of serotonin and other vasoactive substances which are secreted by the tumor cells. Thus, these substances reach the systemic circulation by escaping the liver metabolization[13]. In our patient, liver disease was not present, which contributed to the elusive course of the disease.

The usual clinical manifestations of carcinoid tumors in the small intestine vary, but commonly include postprandial and colicky pain, gastrointestinal bleeding, obstruction and loss of appetite. However, a high percentage of cases may be asymptomatic[9]. Similarly, our patient presented weight loss and mild abdominal pain with no specific characteristics. The disease can also manifest catastrophically as a carcinoid abdominal crisis. In this case, the protruded symptoms are those of intestinal ischemia as a result of obstruction of the mesenteric artery due to infiltrated paraaortic lymph nodes or the development of elastic vascular sclerosis provoked by tumor-produced hormones[14,15]. However, in our case, the mesenteric lymph nodes adjacent to the arterial jejunal branches provoked intestinal ischemia with no other evidence of a carcinoid abdominal crisis. The elastic tissue infiltration probably led to chronic obstruction of the jejunal arteries and the deterioration of mesenteric circulation led to ischemia. Elastic vascular sclerosis and tumor vascular infiltration were identified in the pathologic examination of the specimen, thus proving the pathophysiology of ischemia. The pathogenesis of elastic sclerosis is related to a desmoplastic reaction associated with hormones produced by the carcinoid tumor and consists of the formation of an elastic tissue layer on the endothelium of the vasculature, diminishing its diameter[16-18]. In our case, the hormones produced by the carcinoid were not sufficient to develop a carcinoid clinical syndrome, but they were probably the cause of a severe desmoplastic reaction. However, because of the urgency of the case, we did not measure the hormones.

The establishment of a diagnosis is usually challenging. It demands a great level of suspicion, and usually the final diagnosis is only settled after a long course of expensive and futile laboratory and imaging examinations. Depending on the prominent symptoms, the work-up series differs. In the case of gastrointestinal bleeding, the used imaging modalities are endoscopy of the gastrointestinal tract, red blood scintigraphy and computed tomography[19]. In the case of intestinal obstruction, the primary used imaging modalities are computed tomography and magnetic resonance imaging. In the event of mesenteric ischemia, the standard approach is CT angiography[20]. In the current case, abdominal X-rays and ultrasound raised the suspicion of mesenteric ischemia, which was confirmed subsequently by CT angiography; however, the small intestine carcinoid was not depicted. Somatostatin receptor scintigraphy (octreoscan) can detect suspected neuroendocrine tumors and their metastases. It has 90% sensitivity and is very efficient for the identification of extra-abdominal tumor spread and can even demonstrate bone metastases better than bone isotope scan. It is also useful for staging neuroendocrine tumors, evaluating recurrence, determining somatostatin-receptor status and selecting patients with metastatic tumors for peptide receptor radionuclide therapy (PRRT)[21-23]. Finally, PET with the serotonin precursor 5-Hydroxytryptophan labelled with ^{11}C or ^{68}Ga with the highest sensitivity in identifying GI NETs and has been used increasingly to diagnose GI NETs, stage the disease and monitor the effects of therapy. FDG-PET imaging can be utilized to detect both intermediate-grade and high-grade poorly-

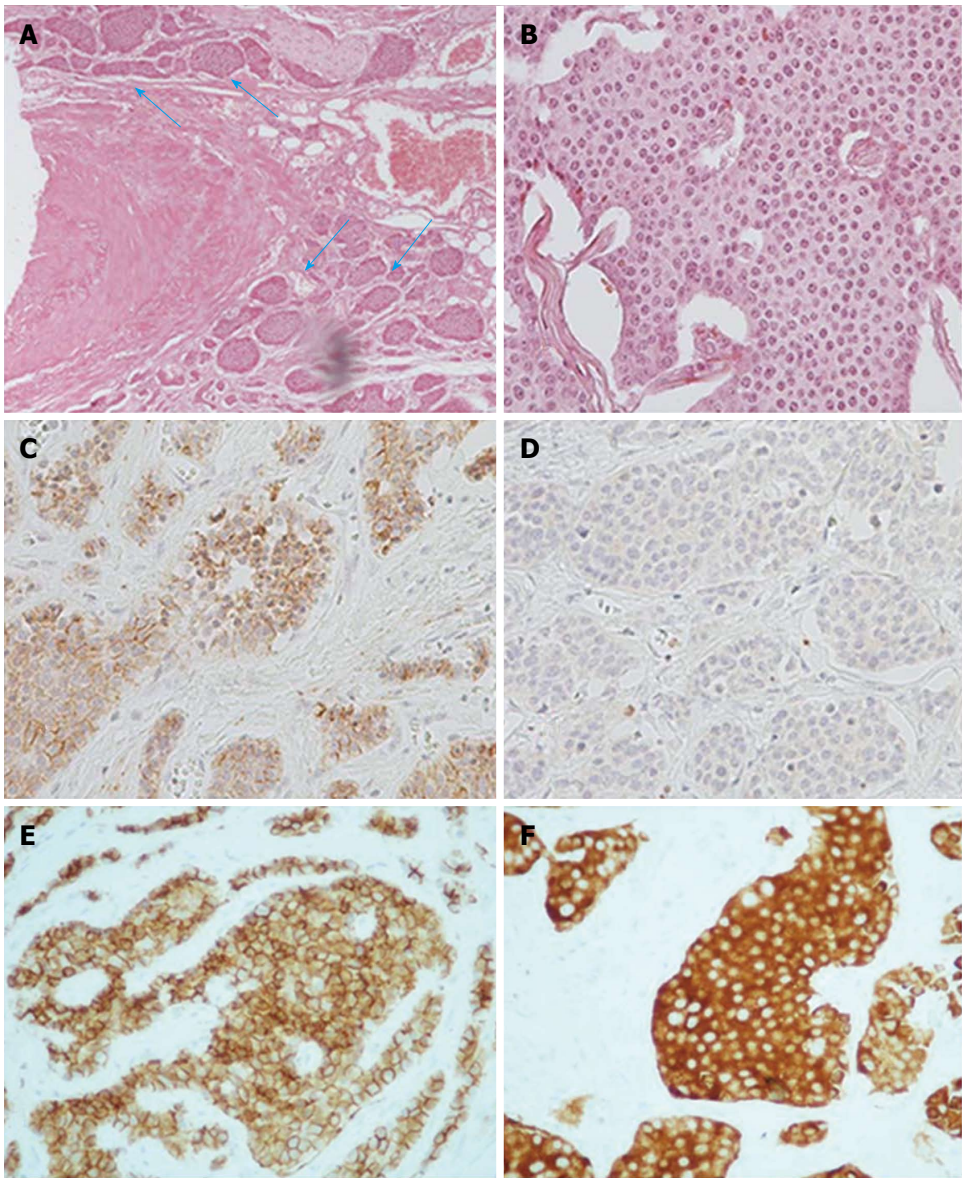

Figure 7 Histopathological findings. A: Vascular and perineural invasion of tumor cells (blue arrows), medium magnification (100 ×); B: Tumor cells with characteristic nuclear appearance, high magnification (400 ×); C: Immunohistochemical staining reveals strong positivity for chromogranin A marker, high magnification (400 ×); D: Less than 1% of tumor cells reveal positivity for proliferative marker Ki-67, high magnification (400 ×); E: Immunohistochemical staining reveals strong positivity for CD56 marker, high magnification (400 ×); F: Immunohistochemical staining reveals strong positivity for synaptophysin marker, high magnification (400 ×).

differentiated neuroendocrine carcinomas (ki > 15%), but it will not visualize low-grade well-differentiated lesions[4].

Surgery represents the mainstay of treatment for localized tumors and can be curative as it provides a 5-year survival rate from 80% to 100% in resectable tumors. So far, surgical treatment is the only curative option[8-10]. Apart from the indisputable value of surgical extirpation of the tumor, which is mandatory even in cases of metastatic disease, somatostatin analogs (lanreotide and octreotide) are the backbone of medical treatment for most functioning NETs, as their use may by stabilizing tumor growth and alleviating symptoms improve the quality of life. For the majority of patients with well-differentiated advanced midgut NETs, somatostatin analogs are appropriate for first-line systemic medical treatment and are very well tolerated[24]. IFNa has the potential also to control hormonal symptoms and inhibit tumor growth, but its usage is limited by its toxicity. Most guidelines suggest that IFNa should be given as second-line agent in patients with tumor progression or refractory carcinoid syndrome on somatostatin analogs[25]. Peptide receptor radionuclide therapy is actually an internal radiation therapy that depends on delivering therapeutic doses of radiation inside tumor cells. Radiolabeled somatostatin analogues, which have high affinity to somatostatin receptor subtype 2 (sstr2), are preferred in therapeutic use. Any tumor should be potentially treated if sstr2 overexpression is documented and confirmed on diagnostic somatostatin receptor imaging or histopathologically[22-24].

Recently, systemic chemotherapy agents and new molecular-targeted agents, such as everolimus, an mTOR inhibitor, and sunitinib, a tyrosine kinase inhibitor, have been approved for the treatment of well-differentiated pancreatic NETs, but their roles are not clear in GI NETs. These drugs are currently being evaluated in ongoing clinical trials[26,27]. The responses to these treatments should be evaluated by both imaging, including CT and MRI, and biochemical markers, mainly chromogranin A which is a stable marker that more importantly can be measured during long-term treatment of both non-functioning and functioning tumors[28,29].

During treatment with biological therapy or cytotoxic agents or PRRT, patients with malignant NETs should be followed every 3 mo in order to evaluate response to treatment. Patients who were submitted to curative surgery are usually followed at 3 to 6 mo intervals for > 5 years with biochemical testing performed every 3 mo and imaging every 6 mo[30,31].

The establishment of guidelines and the new therapeutic modalities concerning the management of NETs has contributed significantly to the improvement of the quality of life and survival of many patients with malignant NETs. Thus, patients with NETs today exhibit a median survival at centers of excellence of more than 16 years [31].

COMMENTS

Case characteristics

A 70-year-old female with acute abdominal pain and intestinal obstruction with a 1-year medical history of nonspecific paroxysmal abdominal pain.

Clinical diagnosis

Clinical examination revealed diffuse abdominal tenderness.

Differential diagnosis

Intestinal obstruction due to colonic tumor or acute mesenteric ischemia.

Laboratory diagnosis

Severe leukocytosis with increased CRP and mild metabolic acidosis.

Imaging diagnosis

Abdominal X-ray, abdominal ultrasound and abdominal computed tomography (CT) revealed intestinal pneumatosis, which was compatible with the diagnosis of mesenteric ischemia.

Pathological diagnosis

Small bowel ischemia and two neoplastic foci of well-differentiated low-grade neuroendocrine tumor.

Treatment

Segmental enterectomy and side-to-side anastomosis

Related reports

A high percentage of small bowel neuroendocrine tumors may be asymptomatic and the clinical symptoms vary and are nonspecific. They can manifest as a carcinoid abdominal crisis, where the protruded symptoms are those of intestinal ischemia as a result of obstruction of the mesenteric artery due to the development of elastic vascular sclerosis provoked by tumor-produced hormones or due to the infiltration of paraaortic lymph nodes.

Term explanation

Gastrointestinal neuroendocrine tumors were previously and are still commonly called carcinoids.

Experiences and lessons

Small bowel neuroendocrine tumors are highly misdiagnosed because they can be asymptomatic or present with nonspecific symptoms, but they can also manifest as acute abdomen. In most cases of intestinal ischemia, the diagnosis is established using contrast-enhanced CT; simple X-rays and abdominal ultrasound can establish the diagnosis in some cases. Therefore, simple imaging modalities should not go unnoticed, as they can aid diagnosis.

Peer-review

This paper reported a rare case of midgut neuroendocrine tumor presenting with acute intestinal ischemia. It provided sufficient data.

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CASE REPORT

Endoscopic submucosal dissection in a patient with esophageal adenoid cystic carcinoma

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Author contributions: All authors contributed equally to this work; Yoshikawa K and Kanayama H provided the treatment; Yoshikawa K analyzed the data and wrote the manuscript's text; Kinoshita A wrote the supplementary information; all of the authors discussed the results and commented on the manuscript at all stages.

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Abstract

We report the first use of endoscopic submucosal dissection (ESD) for the treatment of a patient with adenoid cystic carcinoma of the esophagus (EACC). An 82-year-old woman visited our hospital for evaluation of an esophageal submucosal tumor. Endoscopic examination showed a submucosal tumor in the middle third of the esophagus. The lesion partially stained with Lugol's solution, and narrow band imaging with magnification showed intrapapillary capillary loops with mild dilatation and a divergence of caliber in the center of the lesion. Endoscopic ultrasound imaging

revealed a solid 8 mm × 4.2 mm tumor, primarily involving the second and third layers of the esophagus. A preoperative biopsy was non-diagnostic. ESD was performed to resect the lesion, an 8 mm submucosal tumor. Immunohistologically, tumor cells differentiating into ductal epithelium and myoepithelium were observed, and the tissue type was adenoid cystic carcinoma. There was no evidence of esophageal wall, vertical stump or horizontal margin invasion with pT1b-SM2 staining (1800 μm from the muscularis mucosa). Further studies are needed to assess the use of ESD for the treatment of patients with EACC.

Key words: Adenoid cystic carcinoma of esophagus; Endoscope; Ultrasound; Esophageal; Tumor; Endoscopic submucosal dissection

Core tip: Adenoid cystic carcinoma of the esophagus (EACC) is a rare tumor that may be confused with squamous cell carcinoma and basaloid-squamous cell carcinoma. There is limited data regarding the frequency of metastasis, and the prognosis of patients with this tumor is poor. This is the first report of the use of endoscopic submucosal dissection (ESD) for the treatment of a patient with EACC. ESD may represent an additional treatment option for patients with this disease.

Yoshikawa K, Kinoshita A, Hirose Y, Shibata K, Akasu T, Hagiwara N, Yokota T, Imai N, Iwaku A, Kobayashi G, Kobayashi H, Fushiya N, Kijima H, Koike K, Kanayama H, Ikeda K, Saruta M. Endoscopic submucosal dissection in a patient with esophageal adenoid cystic carcinoma. *World J Gastroenterol* 2017; 23(45): 8097-8103 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8097.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8097

INTRODUCTION

Adenoid cystic carcinoma of the esophagus (EACC) is a rare tumor that may be confused with squamous cell carcinoma (SCC) and basaloid-squamous cell carcinoma (BSC). There is limited data regarding the frequency of metastasis and the prognosis in patients with EACC[1]. Many patients have been found to have metastases at the time of initial diagnosis, and the prognosis is thought to be poor[2]. Accurate preoperative diagnosis is difficult because the tumor primarily involves the submucosa and is not easily sampled with endoscopic biopsy[3]. Although treatment is usually surgical resection in principle, the degree of invasion and the frequency of lymph node or other distant metastases are unknown[4].

In this report, we describe the use of endoscopic submucosal dissection (ESD) for the treatment of a patient with EACC. In particular, we describe the image-enhanced endoscopy and endoscopic ultrasound (EUS) findings observed during endoscopy prior to the ESD. This is the first case report describing the use of ESD for the treatment of a patient with EACC; this approach may become a more commonly accepted therapeutic option in the future.

CASE REPORT

An 82-year-old Japanese woman visited our hospital for evaluation of an esophageal tumor. Her medications included clopidogrel for right internal carotid artery stenosis, and she had a history of a prior cerebral ischemic event. She was undergoing upper gastrointestinal endoscopy as part of her annual health examination. She denied any subjective symptoms, including dysphagia, and laboratory examination revealed no evidence of anemia or abnormalities of liver or renal function. She did not have any family history with esophageal disease, and the values of tumor markers for adenocarcinoma and SCC were within normal limits.

She was referred to our hospital for evaluation of what appeared to be a protruding submucosal lesion in the middle esophagus. The lesion was noted during an upper gastrointestinal endoscopic examination performed a month prior to consultation. Endoscopic examination with normal white light (GIF-H290Z and UM-3R-3-20 MHz; Olympus, Tokyo, Japan) in our hospital revealed a brownish submucosal tumor, located 25 cm from the incisor of the middle esophagus (Figure 1). The tumor surface showed mild reddening with a central planar depression, and was elastic, mobile and hard when compressed with the forceps. Image enhancement with narrow band imaging (NBI) magnification revealed a central brownish area with slightly dilated, non-uniform diameter intrapapillary capillary loops. The central planar depression stained slightly with the application of Lugol's solution.

EUS revealed a solid 8 mm × 4.2 mm mass, primarily involving the second and third layers of the esophagus; the tumor was hypoechoic and homogeneous with a thickened hyperechoic submucosa, and slight irregularity of the third layer was recognized (Figure 1C, white arrow). The biopsy showed esophagitis and no distinct tumor; enlarged lymph nodes or other lesions suspicious for metastases were not observed with contrast-enhanced computed tomography (CT). With the above endoscopic findings, SCC and gastrointestinal stromal tumor (GIST) were included in the differential diagnosis. In accordance with our treatment protocol, we planned on performing ESD if the lesion could be lifted with a local injection.

After completing an adequate clopidogrel washout period, the patient was admitted to the hospital for endoscopic treatment (Figure 2). After marking the lesion, saline was locally injected into the submucosal layer on the anal side of the lesion. Next, a mixed

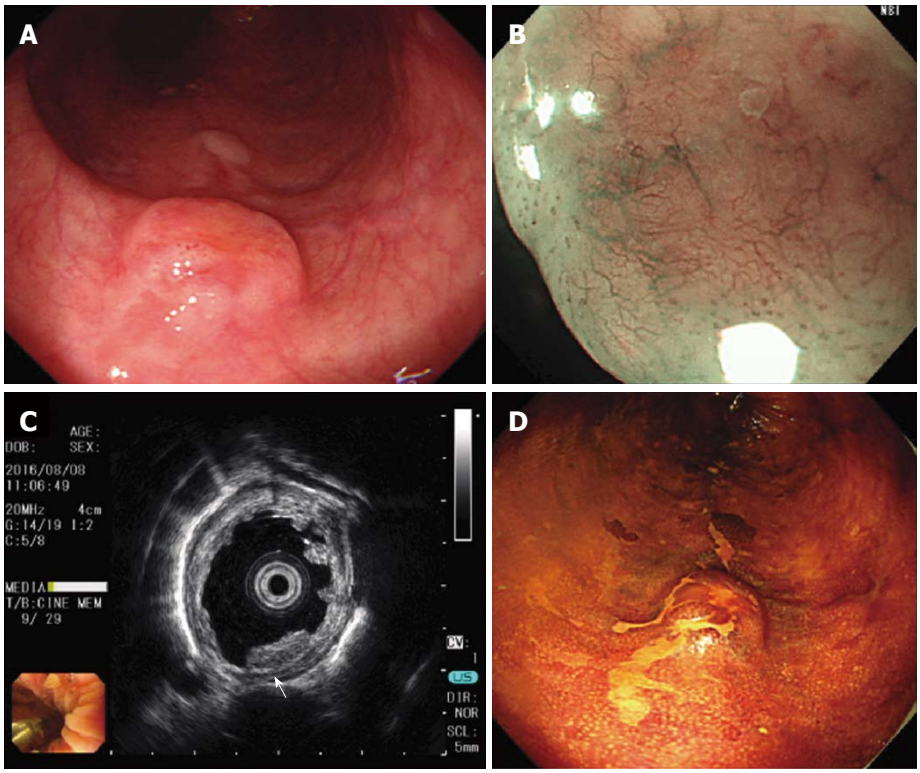

Figure 1 Preoperative endoscopy. A: Normal white light; B: Narrow band imaging with magnification; C: Endoscopic ultrasound, showing a tumor that was hypoechoic and homogeneous with a thickened hyperechoic submucosa slight irregularity of the third layer (white arrow); D: Lugol's solution application.

solution of glycerol and hyaluronic acid was locally injected, and a perimeter incision was made using a needle knife tip (DualKnifeTM; Olympus, Tokyo, Japan). The en block resection was performed after surrounding trimming and submucosal layer exfoliation were performed. During the procedure, cauterization for bleeding and exposed blood vessels on the resected surface was accomplished using bipolar hemostat forceps. The size of the excised specimen was 24 mm × 16 mm. The postoperative course was uneventful. At follow-up endoscopy 2 d after ESD, the wound was healed and scarred without stenosis (Figure 3). The patient was discharged on the 8th postoperative day and was symptom free at all outpatient visits at 6 mo postoperatively.

Hematoxylin and eosin staining findings are shown in Figure 4, and immunostaining findings are shown in Figure 5. With low power magnification, the submucosal layer showed proliferative heterotypic cells with cribriform nuclei distributed in an alveolar pattern and having numerous glandular cavities and small cyst-like structures in the alveoli. The upper border of the tumor partially extended to the luminal surface. With higher magnification, the nuclei of the heterotypic cells showed a dark chromatin core and a narrow eosinophilic border. Cells with an eosinophilic cytoplasm lined the wall of the glandular cystic cavity and formed a two-layer structure with small cells and having a high nuclear/cytoplasm ratio forming the outer layer. Immunostaining showed slight staining with cytokeratin CAM 5.2, which stains duct epithelium but not squamous epithelium. The glandular cavities and small cystic structures stained positively with epithelial membrane antigen, which stains glandular epithelium. Carcinoembryonic antigen staining, which stains ductal epithelium, was positive in the intraluminal epithelium and in those areas with differentiation into ductal components. p63 staining, which stains basal cells, was not observed in the intraluminal epithelium, and tumorization of the basal cells was not observed. Alpha-smooth muscle actin staining, which stains smooth muscle cells, was weakly positive in the cells of the outer layers of the cysts, suggesting smooth muscle differentiation. Staining with calponin, which stains smooth muscle cells, was slightly positive in the outer cyst wall cells.

In summary, the lesion was an 8 mm × 8 mm submucosal tumor with ductal epithelial and myoepithelial differentiation on immunohistochemical staining. The histological type was adenoid cystic carcinoma (ACC). The depth of penetration of the wall was pT1b-SM2, 1800 μm from the muscularis mucosa; both the horizontal margins and vertical stump were negative, and lymphovascular invasion was not observed with D2-40 staining. Also, no venous invasion was observed with elastic van Gieson staining.

DISCUSSION

EACC was first reported by Gregg and Stamler in 1954[5]. ACC is common in the salivary glands and

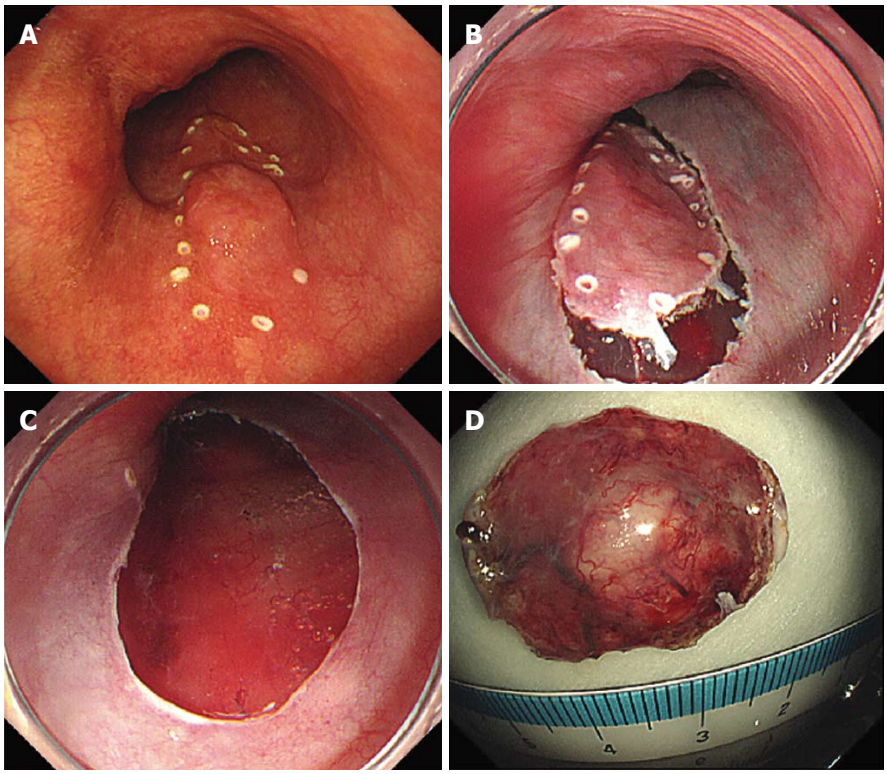

Figure 2 Intraoperative endoscopy. A: Marking of lesion; B: Incision of lesion perimeter; C: Resected surface after excision; D: Excised specimen.

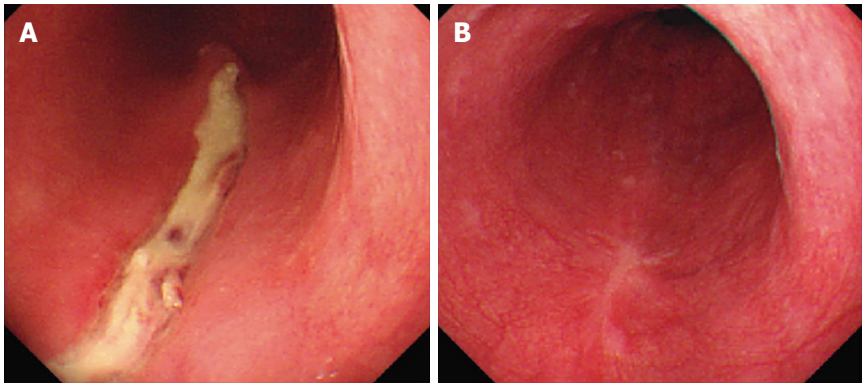

Figure 3 Postoperative endoscopy. A: 2 d postoperatively; B: 6 mo postoperatively.

respiratory system, but accounts for only 0.1% of esophageal malignancies[6]. EACC is a highly malignant tumor[1]. Based on previous reports[3,7,8], the average patient age is 60.4-66.4 years and the ratio of males to females is 2.75-5:1. Seven percent of EACCs are in the upper third of the esophagus, 63% are in the middle third, and 30% are in the lower third. Difficulty swallowing is the most common symptom, similar to what is seen in patients with SCC of the esophagus[3].

In our case, the lesion was located in the middle third of the esophagus, but the patient's sex, age and lack of symptoms were atypical. Although EACC is normally diagnosed by endoscopy, it is frequently misdiagnosed preoperatively[3]. Only 21.6% of EACC cases diagnosed before 1996 were successfully diagnosed by endoscopic biopsy preoperatively[9]. This is thought to occur because components of SCC and BSC are likely to be found in EACC[10]; and, because EACC predominately involves the submucosa, it is impossible to sample the structure of the entire EACC tumor with an endoscopic biopsy alone[11].

When observed endoscopically with white light, an infiltrative growth pattern was reportedly observed in 58.6% of tumors, and 24.1% of tumors were classified as an ulcerative growth[7,8]. With NBI magnification, one observes a mixture of glandular and squamous epithelial components. Tumors with an irregular vascular pattern with large vessels, tumors with an irregular mucosal pattern with mucous, and tumors with no clear pattern have been reported[12].

Chromoendoscopy is useful for discriminating between SCC and adenocarcinoma, but its utility in establishing

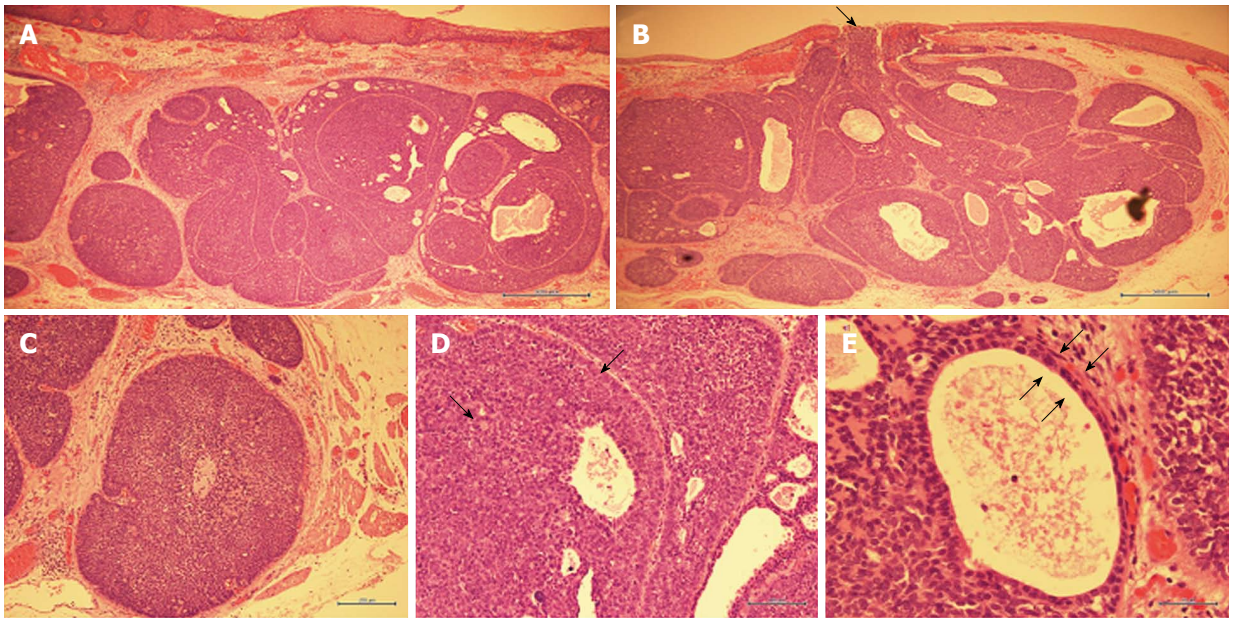

Figure 4 Hematoxylin and eosin staining of the resected specimen. A: Main locus of submucosal tumor (× 40); B: Tumor protrusion into esophageal lumen (black arrows, × 40); C: Cribriform structure of tumor cells (× 100); D: Heterotypic cells with eosinophilic cytoplasm (black arrows, × 200); E: Bi-layered structure of tumor duct cells (black arrows, × 400).

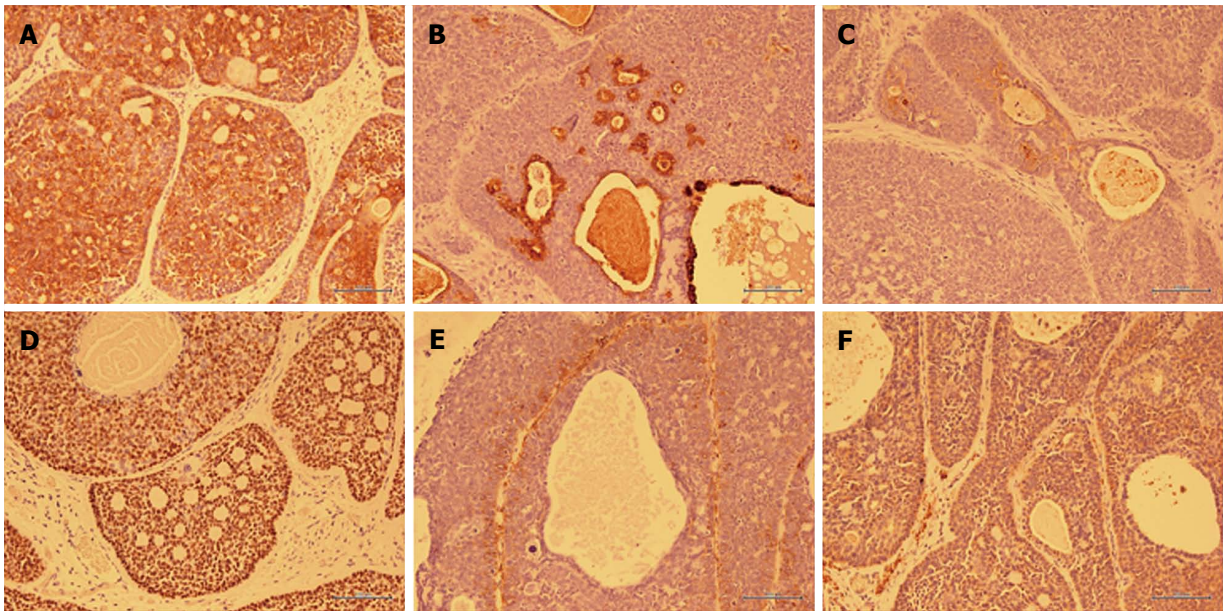

Figure 5 Immunostaining of resected specimen. A: Cytokeratin CAM 5.2 staining (× 200); B: Epithelial membrane antigen staining (× 200); C: Carcinoembryonic antigen staining (× 200); D: p63 staining (× 200); E: Alpha-smooth muscle actin staining (× 200); F: Calponin staining (× 200).

a diagnosis of ACC has not been determined. The utility of EUS in patients with EACC has only been discussed in a limited number of case reports. In one report, the EUS findings were described as a thickened submucosal layer and a relatively hypoechoic muscular layer[13]. In the present case, submucosal tumor-like lumen formation was observed, and with NBI enhancement some findings common to SCC were observed. Staining with Lugol's solution was poor in that portion of the tumor protruding into the lumen, and EUS findings were similar to those previously reported. Preoperative endoscopic biopsy did not result in an accurate diagnosis.

Histopathologically, EACC is characterized by tumor differentiation into two types of cells, duct-lining epithelial cells and myoepithelial cells, both of which are commonly seen in salivary gland tumors. Three different cellular patterns have been observed, a cribriform pattern, a lattice-like pattern, and a tubular pattern[14]. With Alcian blue staining, the tumor demonstrates pale blue mucus in the cystic alveolar cavities as well as outside the alveoli. Although it may be necessary to distinguish EACC from BSC because of its location, the

finding of myoepithelial cell differentiation with vimentin or S100 staining is considered useful in establishing a diagnosis of EACC[15].

In our case, tumor cell proliferation was primarily submucosal and myoepithelial differentiation was confirmed with immunostaining, and a diagnosis of EACC was made. Characteristics such as the formation of ductal epithelium, the biphasic nature of the tumor, and the presence of an eosinophilic substance in the cell cytoplasm were also typical of ACC.

Compared with ACC of the salivary gland, EACC has a poor prognosis[16], with a 5-year survival rate of 35% and an average life expectancy of 7 mo[2,8]. EACC with a solid growth pattern is also reported to have a poor prognosis[14]. Lymph node metastasis is more frequent than other organ metastasis, and patients with lymph node metastases have poor prognosis[17]. The presence of vascular invasion is associated with a worse prognosis in these patients. However, some cases previously diagnosed as EACC may have been confused with BSC and SCC, and additional larger studies are needed to clarify the data regarding metastasis, prognosis, and the presence of other tumor components in the tumors of patients with EACC.

The primary choice for the treatment of patients with EACC is radical surgery[4,18]; however, the surgical mortality rate was reported to be 15% in previous studies[8]. Adjuvant radiation therapy has been advocated if dysphagia is present or if surgical margins are positive for tumor involvement. A previous case report described the use of chemotherapy, including doxorubicin, mitomycin C and 5-fluorouracil, with local radiation[8], but chemotherapy is generally thought to be ineffective[4]. Overall, there is little data to support the use of chemotherapy, and the effects of adjuvant or primary chemotherapy are unknown[6,19].

One prior report described the endoscopic treatment of EACC with incisional endoscopic enucleation[20]. Ours is the first case to report the use of ESD for the treatment of a patient with EACC. It has been reported that lymph node metastases are rare in patients with esophageal SCC when the tumor is confined to the mucosal epithelial layer and the mucosal lamina propria. The incidence of metastasis is 9.3% when the tumor reaches the muscularis muscle plate and 19.3% when the tumor is within 200 μm of the submucosal layer.

In the present case, the stump of the resected specimen was negative, but invasion to within 1800 μm of the submucosal layer was observed. It is possible that tumor resection in our case will not be curative. The relationship between tumor depth of invasion and the frequency of lymph node metastasis in patients with EACC is not known, and additional studies are necessary. Our patient has been asymptomatic without evidence of recurrence or metastasis at 6 mo after ESD. We believe that continued rigorous monitoring for recurrence or metastasis with contrast-enhanced CT and upper gastrointestinal endoscopy is necessary.

In conclusion, We have described herein the first case of the use of ESD for the treatment of a patient with EACC. The accumulation and analysis of additional cases is needed to clarify the prognosis and most appropriate treatment for these patients.

ARTICLE HIGHLIGHTS

Case characteristics

A Japanese woman was asymptomatic, and the disease was diagnosed as a result of regular upper gastrointestinal endoscopy.

Clinical diagnosis

The authors diagnosed adenoid cystic carcinoma of the esophagus (EACC).

Differential diagnosis

The diseases to be considered are squamous cell carcinoma (SCC) and gastrointestinal stromal tumor (GIST), which can be estimated by total biopsy.

Laboratory diagnosis

The patient had nothing particular changeincluding hemoglobin and tumor marker.

Imaging diagnosis

Computed tomography scan showed no morphological changes.

Pathological diagnosis

Tumor cell proliferation was primarily submucosal and myoepithelial differentiation was confirmed with immunostaining

Treatment

The patient received endoscopic submucosal dissection (ESD).

Related reports

There is no other case report of ESD treatment for EACC. There are reports of cases with incisional enucleation, surgery, chemotherapy, and radiation therapy.

Term explanation

EACC is a rare tumor, that may be confused with SCC and basaloid-squamous cell carcinoma. There is limited data regarding the frequency of metastasis, and the prognosis of patients with this tumor is poor.

Experiences and lessons

This is the first report of the use of ESD for the treatment of a patient with EACC. ESD may represent an additional treatment option for patients with this disease.

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CASE REPORT

Simultaneous liver, pancreas-duodenum and kidney transplantation in a patient with hepatitis B cirrhosis, uremia and insulin dependent diabetes mellitus

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Author contributions: Guo QJ, Cai JZ, and Pan C performed transplant surgery and provided the intellectual content; Jiang WT summarized the clinical data; Li J performed the literature review and drafted the manuscript; Shen ZY contributed critical comments and revised the manuscript; all authors read and approved the final manuscript.

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Institutional review board statement: Approval from the ethics committee of the Tianjin First Central Hospital was obtained for this study.

Informed consent statement: The patient gave his written informed consent to this case report.

Conflict-of-interest statement: We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript.

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Abstract

Simultaneous liver, pancreas-duodenum, and kidney transplantation has been rarely reported in the literature. Here we present a new and more efficient *en bloc* technique that combines classic orthotopic liver and pancreas-duodenum transplantation and heterotopic kidney transplantation for a male patient aged 44 years who had hepatitis B related cirrhosis, renal failure, and insulin dependent diabetes mellitus (IDDM). A quadruple immunosuppressive regimen including induction with basiliximab and maintenance therapy with tacrolimus, mycophenolate mofetil, and steroids was used in the early stage post-transplant. Postoperative recovery was uneventful and the patient was discharged on the 15th postoperative day with normal liver and kidney function. The insulin treatment was completely withdrawn 3 wk after operation, and

the blood glucose level remained normal. The case findings support that abdominal organ cluster and kidney transplantation is an effective method for the treatment of end-stage liver disease combined with uremia and IDDM.

Key words: Insulin dependent diabetes mellitus; Cirrhosis; Chronic renal failure; Transplantation; *En bloc*; Liver-pancreas

Core tip: Combined orthotopic liver and heterotopic pancreas transplant has been usually reported in the literature. Here we present a new and more efficient *en bloc* technique for combined liver-pancreas transplant. Hepatectomy was performed in the standard fashion with caval cross-clamping. The liver, along with the *en bloc* duodenopancreatic graft, was then transplanted orthotopically without using veno-venous bypass. Graft kidney was implanted in right iliac fossa in accordance with the traditional classical manner. Postoperative recovery was uneventful and the patient was discharged on the 15th postoperative day with normal liver and kidney function. The insulin treatment was completely withdrawn 3 wk after operation, and the blood glucose level remained normal. The case findings support that abdominal organ cluster and kidney transplantation is an effective method for the treatment of end-stage liver disease combined with uremia and insulin dependent diabetes mellitus.

Li J, Guo QJ, Cai JZ, Pan C, Shen ZY, Jiang WT. Simultaneous liver, pancreas-duodenum and kidney transplantation in a patient with hepatitis B cirrhosis, uremia and insulin dependent diabetes mellitus. *World J Gastroenterol* 2017; 23(45): 8104-8108 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i45/8104.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i45.8104

INTRODUCTION

Combined liver-pancreas transplantation was formerly used as a salvage procedure for non-resectable upper abdominal malignancies, but the procedure was almost abandoned due to poor outcome[1]. Recently, the indication for combined liver-pancreas transplantation has been changed and transplant surgeons have started using this technique for patients with insulin dependent diabetes mellitus (IDDM) who are candidates for liver transplantation at the same time. There are several advantages to using simultaneous liver-pancreas transplant such as insulin independence after transplantation, improving patient management, and decreasing the risk of post-transplant cardiovascular diseases[2]. Diabetic patients who receive only liver graft are not only at increased risk of developing cardiovascular diseases but also remain diabetic after transplantation which may itself negatively affect the long-term graft survival[3-5]. Although there are so many advantages to combined liver pancreas transplantation, surprisingly only few cases have been reported in the literature[6]. We hereby report about our experience with *en bloc* liver-pancreas and kidney transplantation in one patient with end-stage liver disease, chronic renal failure, and IDDM, with some detail aspects about the surgical procedures and immunosuppressive regimens discussed.

CASE REPORT

A 44-year-old male complaining of abdominal distension and anorexia more than one year was referred to our center in April 2016. The patient was infected with hepatitis B virus for more than 30 years, and he developed abdominal distension and anorexia one year ago and suffered with melena two months ago. His past medical history was significant for IDDM for more than 10 years. Additionally, the patient suffered with chronic renal failure relying on regular dialysis (three times per week). He was on insulin therapy (60 units/d) and had a history of recurrent hypoglycemic episodes recently. At the time of admission, the laboratory parameters were as follows: total bilirubin 2.5 mg/dL, aspartate aminotransferase 106 U/L, alanine aminotransferase 50 U/L, alkaline phosphatase 683 U/L, albumin 33.1 g/L, blood urea nitrogen 22 mg/dL, serum creatinine 8.2 mg/dL, and hemoglobin 7.7 g/dL. Bleeding profile was within normal limits. Enhanced abdominal computed tomography indicated liver cirrhosis, splenomegaly, mild ascites, and esophageal varices. After much clinical testing and deliberation, the multi-disciplinary care management concluded that transplantation would offer the greatest survival benefit. The patient was listed for a combined liver, pancreas, and kidney transplant.

He received *en bloc* liver-pancreas and kidney graft from an ABO identical deceased donor aged 28 years. Liver biopsy of the graft showed no macro- or microsteatosis. Liver, pancreas, duodenum, and both kidneys were retrieved *en bloc* without disturbing the hepatoduodenal ligament. The superior mesenteric artery and celiac arteries were then anastomosed to a Y graft of the internal iliac artery and external iliac artery from the same donor on the back table so as to allow a single vascular anastomosis. Bile was washed out through the gallbladder as the bile duct was left intact. The superior mesenteric vein beyond the neck of the pancreas was closed (Figure 1).

Hepatectomy was performed in the standard fashion with caval cross-clamping. The liver, along with the *en bloc* duodenopancreatic graft, was then transplanted orthotopically without using veno-venous bypass. This was followed by the suprahepatic and

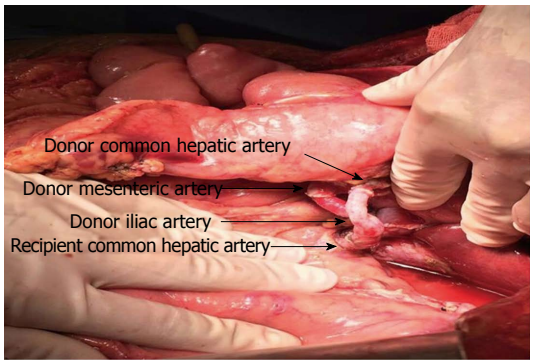

Figure 1 Arterial reconstruction for organ cluster.

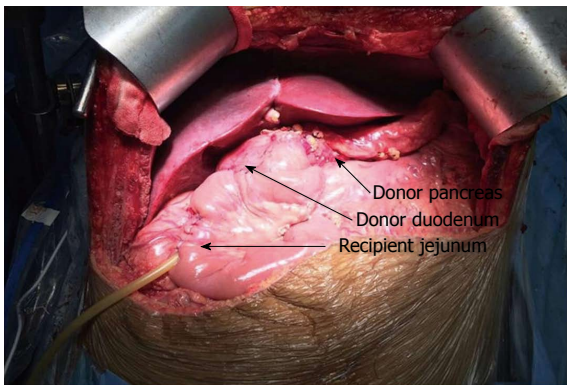

Figure 2 Final appearance of *en bloc* liver pancreas graft with enteric drainage of the graft duodenum to a Roux en Y limb.

infrahepatic inferior vena caval anastomoses. The recipient portal vein was then anastomosed to the back side of the donor portal vein between the superior border of pancreas and the liver. The innominate artery Y graft was anastomosed to the infrarenal aorta (Figure 2). After reperfusion, graft liver and pancreas were well perfused with normal color (Figure 1). Finally, a Roux-en-y enteroenteric anastomosis was performed between the recipient's jejunum and graft duodenum for exocrine pancreatic and biliary drainage. Graft kidney was implanted in right iliac fossa in accordance with the traditional classical manner. Total duration of the operation was 480 min and 10 units of packed cells were transfused. Total cold ischemic time was 1 h and 15 min with a warm ischemic time of 5 min.

Initial immunosuppression included induction with basiliximab (Simulect) and maintenance therapy with tacrolimus (target trough level 8-10 ng/mL) and mycophenolate mofetil (MMF) 1.5 g/d. Steroids were tapered down and completely withdrawn within 3 mo post transplantation. In addition, anti-infection, anticoagulation, inhibition of gastric acid and pancreatic enzyme secretion, and intravenous nutritional support treatment were provided.

Postoperative recovery was uneventful and the patient was discharged on the 15th postoperative day with normal liver and kidney function. The insulin treatment was completely withdrawn 3 wk after operation, and the blood glucose level remained normal.

DISCUSSION

Indications for abdominal organ cluster transplantation

The most accepted indication for combined liver-pancreas transplantation is chronic liver disease in patients who concomitantly suffer from IDDM. This approach not only corrects liver disease but also allows the patient to have insulin independence at the same time[7]. Multivisceral transplants have usually been in the form of kidney-pancreas, liver-kidney, and sometimes liver-pancreas transplant, with combined liver, pancreas, and kidney transplant being comparatively rare. Until now, there have been only three cases of combined liver, pancreas, and kidney transplant reported[8-10]. Certain liver diseases have strong association with diabetes mellitus; primary sclerosing cholangitis is one of the diseases which has been described in association with diabetes mellitus type 1[11,12]. Other diseases of the liver which have direct association with diabetes mellitus are NASH[13,14] and systic fibrosis[15]. In selected cases, these diseases justify combined liver-pancreas transplantation.

Choice of two surgical procedures

Technically, combined liver-pancreas transplantation can be performed in two ways. The first pattern is to perform a standard orthotopic liver transplantation followed by a standard heterotopic transplantation of the pancreas allograft onto the iliac vessels in the right lower quadrant. The separate transplantation of the liver and pancreas offers the advantage that if a complication occurs in one of the organ grafts, the other organ theoretically is less likely to become affected. The second technique described by Starzl *et al*[16] is to perform an orthotopic organ cluster of graft liver and pancreas with their entire arterial blood supply including the donor celiac axis and superior mesenteric artery. The recipient portal vein was anastomosed to graft portal vein in an end-to-side manner. A good metabolic control can be achieved for the *en bloc* technique, which provided the physiological position of the pancreas with a natural venous drainage on to graft liver[17]. Additionally, this technique greatly reduced the surgical difficulty since it involved only three or four large vascular anastomoses and one duodeno-jejunostomy without biliary anastomosis compared to the separate transplantation.

Comparison with combined liver and islet transplantation

Preexisting IDDM is a major risk factor for poor outcome after liver transplantation[4,18-20]. For patients with end-stage liver disease and type 1 diabetes

mellitus, there seems to be a strong rationale for replacing the pancreas in addition to the liver[6]. Adequate glycemic control without the need for endogenous insulin was achieved in our patient after combined liver and pancreas transplant, which indicated that combined liver and pancreas transplantation is an effective method for the treatment of end-stage liver disease and IDDM.

Liver transplant has also been combined with simultaneous islet cell transplant with some success dating back to the cluster operations[21] and again more recently[22]. Although islet transplantation is much easier to operate with fewer complications and can be repeated several times, long-term (> 5 years) islet survival is not ideal and patients usually return to insulin dependence. Compared with islet transplantation, separated pancreas transplantation is accompanied by more postoperative complications such as splenic vein thrombosis, anastomotic leakage, and pancreatic fistula. However, combined liver and pancreas transplantation greatly reduces the difficulty of surgery and risk of complications. Additionally, the pancreas transplantation can also solve the problem of insufficient islet.

Immunologic advantage for organ cluster transplantation

Combined liver-pancreas transplantation may have an immunologic advantage. Unlike the liver, the pancreas is considered a highly immunogenic organ[23] and when liver transplant is combined with other organ transplants such as pancreas or multiple organs, the liver can protect these organs from severe rejection episodes[24-26]. We applied the same immunosuppressive regimen (induction with basiliximab and maintenance therapy with tacrolimus, mycophenolate mofetil, and steroids) for liver transplantation in this case, and at the time of this writing, no liver, pancreatic, or kidney rejection was seen in our case. Despite the advantages of combined liver-pancreas transplantation, only few centers have reported this kind of transplantation. The establishment of more effective immunosuppressive regimen has to be based on the accumulation of more multi-organ transplantation cases.

COMMENTS

Case characteristics

Multivisceral transplants have usually been in the form of kidney-pancreas, liver- pancreas, or sometimes liver-kidney transplant, however, combined liver-pancreas and kidney transplant was rarely reported. More recently, the authors have successfully performed *en bloc* liver-pancreas and kidney transplant in one patient with end-stage liver disease, uremia, and insulin dependent diabetes mellitus (IDDM), although the follow-up period was shorter.

Clinical diagnosis

The patient was infected with hepatitis B virus for more than 30 years, and he developed abdominal distension and anorexia one year ago, and suffered with melena two months ago. Upon physical examination, he had a mild abdominal tenderness and palpable enlarged spleen. His past medical history was significant for IDDM for more than 10 years. Additionally, the patient suffered with chronic renal failure relying on regular dialysis (three times per week).

Differential diagnosis

Liver cirrhosis can be divided into viral hepatitis cirrhosis, alcoholic cirrhosis, metabolic cirrhosis, cholestatic cirrhosis, portal cirrhosis, autoimmune cirrhosis, toxic and drug-induced cirrhosis, malnutritional liver cirrhosis, and cryptogenic cirrhosis.

Laboratory diagnosis

At the time of admission, the laboratory parameters were as follows: total bilirubin 2.5 mg/dL, aspartate aminotransferase 106 U/L, alanine aminotransferase 50 U/L, alkaline phosphatase 683 U/L, albumin 33.1 g/L, blood urea nitrogen 22 mg/dL, serum creatinine 8.2 mg/dL, and hemoglobin 7.7 g/dL. Bleeding profile was within normal limits.

Imaging diagnosis

Enhanced abdominal computed tomography indicated liver cirrhosis, splenomegaly, mild ascites, and esophageal varices.

Pathological diagnosis

Histological examination showed diffuse nodular sclerosis which was in line with the performance of hepatitis B cirrhosis.

Treatment

A quadruple immunosuppressive regimen including induction with basiliximab and maintenance therapy with tacrolimus, mycophenolate mofetil, and steroids was used in the early stage post-transplant. In addition, anti-infection, anticoagulation, inhibition of gastric acid and pancreatic enzyme secretion, and intravenous nutritional support treatment were provided.

Related reports

Very few cases of simultaneous liver, pancreas-duodenum, and kidney transplantation have been reported in the literature. Additionally, combined liver- pancreas transplant was usually reported in the manner of separate transplantation, however, we performed orthotopic *en bloc* transplantation of the liver and pancreas grafts.

Experiences and lessons

This case report presents a new surgical type of the upper abdominal organ cluster transplant. Compared to the traditional separate transplantation, the main advantage of this technique is its rapidity and simplicity since it involves only three or four large vascular anastomoses and one duodeno-jejunostomy with no separate biliary anastomosis. Additional advantage of the *en bloc* technique is the physiological position of the pancreas with a natural venous drainage into the donor liver graft.

Peer-review

The author has described one case of simultaneous liver, pancreas-duodenum, and kidney transplantation. The article highlights surgical advantages of abdominal organ cluster transplant compared with traditional separate multivisceral transplants.

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