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EDITORIAL

Advances in endoscopic balloon therapy for weight loss and its limitations

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Abstract

The field of medical and surgical weight loss is undergoing an explosion of new techniques and devices. A lot of these are geared towards endoscopic approaches rather than the conventional and more invasive laparoscopic or open approach. One such recent advance is the introduction of intrgastric balloons. In this article, we discuss the recently Food and Drug Administration approved following balloons for weight loss: the Orbera™ Intragastric Balloon System (Apollo Endosurgery Inc, Austin, TX, United States), the ReShape ® Integrated Dual Balloon System (ReShape Medical, Inc., San Clemente, CA, United States), and the Obalon (Obalon® Therapeutics, Inc.). The individual features of each of these balloons, the method of introduction and removal, and the expected weight loss and possible complications are discussed. This review of the various balloons highlights the innovation in the field of weight loss.

Key words: Weight loss; Gastric balloons; Endoscopic balloons; Orbera; Obalon; Reshape

Core tip: This review has been elucidated through a comparison of the strengths and weaknesses of recent balloon approaches, highlighting the indications and possible complications.

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INTRODUCTION

Throughout the last decade, the treatment of obesity has slowly undergone a paradigm shift. The advent of endoscopic balloon therapy has had a profound impact on long-term weight management. Three intra gastric balloons have been recently approved by the Food and Drug Administration (FDA) for the treatment of Class 1 and 2 Obesity. (Body Mass Index, BMI 30-40 kg/m^2).

Intragastric balloons have been used for the treatment of obesity since 1985. It was during this time that the FDA approved the Garren-Edwards Gastric Bubble, an orally inserted cylindrical device. The device was placed inside the stomach and filled with 220 cc of air. It was designed to be left in the stomach for 3 to 4 mo and then removed[1]. After the product's approval, randomized clinical trials showed that its use did not result in significant weight loss when compared to diet and behavioral modification only. It was furthermore associated with a large number of clinical complications including migrations, erosions, and bowel obstructions[2,3]. The device was thus taken off the market in 1992.

Intragastric balloons have been used outside the US for overweight individuals (BMI 25-29.9 kg/m^2), obese individuals (BMI 30-39.9 kg/m^2) and morbidly obese individuals (BMI 40 kg/m^2 and above) as a bridge therapy prior to definitive surgical procedures.

CURRENT APPROVED DEVICES

Currently, three intragastric balloon devices are FDA approved in the United States: Orbera™ Intragastric Balloon System (Apollo Endosurgery Inc, Austin, TX, United States), the ReShape® Integrated Dual Balloon System (ReShape Medical, Inc., San Clemente, CA, United States), and the Obalon (Obalon® Therapeutics, Inc.). These devices are indicated for patients with Class 1 and 2 obesity (BMI 30-40 kg/m^2).

Intragastric balloon systems operate on the principle of inducing an anatomical sensation of fullness secondary to the space they occupy in the stomach cavity. Consequently, post-procedure patients remain full for longer periods of time between meals. The Orbera Intragastric Balloon and Reshape Integrated Dual Balloon is placed into the gastric cavity through the mouth *via* a gastroscope. The Obalon balloon is swallowed by the patient through guided fluoroscopy and endoscopy is required to remove the balloon[4-6].

The balloons in the ReShape Integrated Dual Balloon System have a fill volume of 750-900 cc and are designed to conform to the natural shape of the stomach. This dual balloon design reduces the potential for migration of the device from the stomach to the intestines if a balloon deflation occurs, thus reducing the risk of intestinal obstruction[7]. Methylene blue due is injected into the saline solution present inside the balloon, serving as an indicator of balloon deflation by turning the patient's urine blue-green.

The Orbera system entails one balloon containing 400-700 cc of saline. Studies and trials have shown low rates of deflations in this system, leading to minimal migration and obstruction[8,9].

The Obalon balloon is a gas filled balloon system that functions using similar principles. It consists of up to 3 intragastric balloons placed over the first 3 mo. The patient swallows the catheter-balloon capsule, which also contains a radiopaque marker assisting in confirming its position under the gastroesophageal junction with fluoroscopy or X-ray. Once this is achieved, the catheter is used to inject gas (nitrogen-sulfur hexafluoride mixture) into the balloon. Each balloon has a volume of approximately 250 cc, totaling 750cc with 3 balloons[10].

The saline/air-filled End-Ball® and the Spatz Adjustable Balloon System (ABS) are two additional modalities that can be used and function in similar means to the approaches above. The SPATZ-ABS anchoring device is unique in preventing the migration of the balloon. This is especially advantageous when encountering acute angles where traditional metal anchoring modalities may not pass as easily.

LONG-TERM IMPLICATIONS

Typically, balloons are kept in place for no more than 6 mo and then removed endoscopically. Monthly follow up is suggested for the 6 mo in which the balloon system is in place and for a 6 month period after the balloon is replaced. Thus, 12 mo of medical supervision from an experienced bariatric multidisciplinary team is required. During the appointments, an integrated approach is used to support the patient in adhering to the weight loss program. Specifically, goal setting, weight management, and progress follow up is tracked during these appointments.

As shown in the Table 1, the intragastric balloons provided up to 25%-29% excess body weight loss at 12 mo using the various balloons.

Statistically significant and clinically pertinent comorbid improvements were observed in patients with diabetes, hypertension, and hyperlipidemia, and these improvements were sustained through 48 weeks of follow up in the REDUCE pivotal trial for the Reshape balloons[11].

Adverse events included post-implantation accommodative symptoms of nausea, (as high as 86.9%), vomiting, and abdominal pain. The most common adverse event was early removal of the device due to intolerance. The United States pivotal study showed a

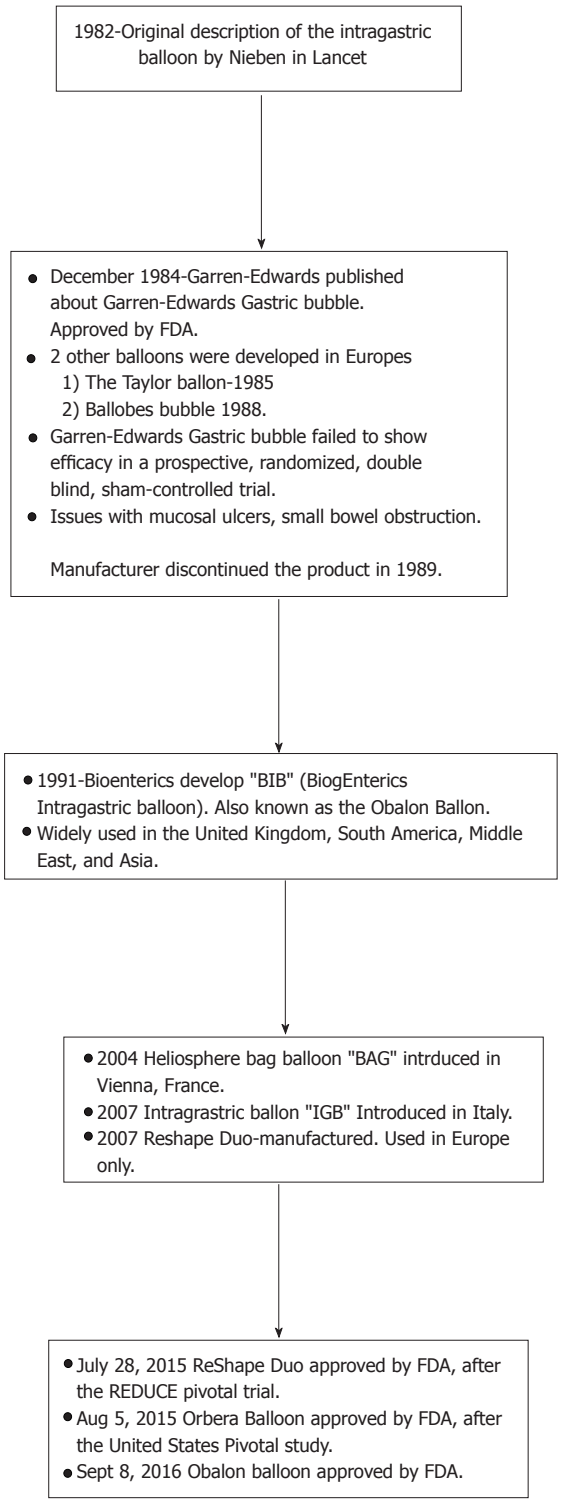

Figure 1 The history and development of Intragastric balloons: Depicting the timeline of developed interventions and devices involved for treatment of surgical and medical weight loss. Approvals by FDA and region specific utilizations are highlighted, illustrating the dynamic progression of techniques in the field. FDA: Food and Drug Administration.

4.25% rate of early removal of implanted devices[12].

Absolute contraindications for placement include previous gastric surgery, hiatal hernia > 5 cm, coagulation disorder, potential bleeding lesion of the foregut, pregnancy, alcoholism/drug addiction, and severe liver disease. Relative contraindications include esophagitis, Crohn's disease, NSAID use, and uncontrolled psychiatric illnesses.

Balloon deflation has become a rare event since manufactures have improved the design of the devices. However, it is still imperative that patients and providers remain aware of this possibility and the need for immediate removal to avoid balloon migration.

Table 1 Features of the Food and Drug Administration approved balloons

	Orbera™	Reshape®	Obalon®
Delivery/ insertion	Needs endoscopy	Needs endoscopy	Patient swallows, X-ray
Removal	Needs endoscopy	Needs endoscopy	Needs endoscopy
Capacity	400-700 cc (1 balloon)	750-900 cc (2 balloons)	750 cc (3 balloons)
Weight loss	29% EWL at 12 mo	25% EWL at 12 mo	25.2% EWL at 12 mo

EWL: Excess weight loss.

ReShape Integrated Dual Balloon System

The REDUCE trial showed deflation in up to 6% of patients and an absence of migrations. In order to detect the presence of deflation, the ReShape system monitors change in the color of urine from normal to blue-green[13].

Orbera Intragastric Balloon System

Studies using the Orbera Intragastric Balloon System, showed an absence of any spontaneous deflations. Deflation in this system can be detected through patient-stated loss of satiety or weight changes, however, common practice dictates a relatively easy means of detection through monitoring the change in urine output.

The Obalon System did not report any deflations in the 336 patients that were studied as a part of the SMART clinical trial.

Overall, the newly FDA approved intragastric balloons provide a viable option for weight loss in patients with BMIs between 30-40 kg/m^2. Studies have documented cases in which treatment with intragastric balloons have shown to incur better weight loss than diet and lifestyle modification alone. However, there is still much controversy on this topic, and the evidence is inconclusive for definitive guidelines, thus, further long-term monitoring and randomized control trials are needed to quantify benefits. The added benefit of patients being able to avoid surgical procedures such as gastric bypass or sleeve gastrectomy allows for this modality of treatment to appeal to certain patient groups. These may include patients that are not adequately fit or prepared to undergo a surgical procedure. It is imperative, however, to understand that the balloons work best when placed and cared

for by an experienced multidisciplinary bariatric team, well equipped with not only handling complications of balloons but providing dietary and emotional support to these patients. Ultimately, these new devices have the potential to serve as a novel instrument in the tool box of the bariatric surgeon.

APPROACH LIMITATIONS

One of the biggest concerns noted is that the balloons are unable to provide long term, substantial weight loss when compared with traditional bariatric procedures. The bypass and the sleeve provide up to 60%-75% EWL at 1 year, when compared to the 25%-30% EWL with the balloon. Patients with substantially higher BMIs looking for a durable procedure for sustained weight loss may not benefit from the balloons. In addition, the co-morbidity resolution profiles of the gastric bypass and sleeve gastrectomy are superior to that of the balloons. It may be premature to compare rigorously tested established surgical procedures to the newly approved less invasive gastric balloons. Overall, the balloons have the potential to serve as a powerful tool in select niche patient populations.

RECENT CONTROVERSIES

Recently, a few cases have been documented by the FDA entailing five deaths with liquid-filled intragastric balloon systems used to treat obesity since 2016[14]. Of these deaths, four involved the Orbera Intragastric Balloon System and one involved the ReShape Integrated Dual Balloon System. The FDA, however, has also stated that the "root cause" of these case fatalities is not known, as the evidence only depicts a one month or less temporal relationship between balloon placement and death. It was thus uncertain if the cause of death was gastric or esophageal perforation, intestinal obstruction, or through an alternate means. As further study into the controversy unfolds, it is important to note the possibility of significant confounding variables such as pre-existing morbidities, operator placement errors, and spontaneous overinflation, in determining the root cause of the recent case fatalities.

CONCLUSION

In order to better advance patient care and diagnostic as well as therapeutic approaches in gastroenterology, a meticulous analysis of endoscopic modalities is warranted. There is still much controversy regarding the post-intervention effects, however, modern advances have come a long way since the origin of the intragastric balloon, as highlighted in Figure 1. With new technologies and innovative devices such as the intragastric balloons, one has to be mindful about the legal aspects of introduction of the device and hospitals and clinics may need to institute a peer review process for credentialing and quality assurance purposes[15,16].

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ORIGINAL ARTICLE

Basic Study

Prediction of early-stage hepatocellular carcinoma using OncoScan chromosomal copy number aberration data

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Author contributions: Yu MC and Tsai CN designed the research; Lian JH, Liu YP and Wu CH performed the research; Lee YS, Lian JH and Tsai CL contributed to the analysis; Yu MC and Lee CW analyzed the clinical data; Yu MC and Tsai CN wrote the paper.

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Abstract

AIM

To identify chromosomal copy number aberrations (CNAs) in early-stage hepatocellular carcinoma (HCC) and analyze whether they are correlated with patient

prognosis.

METHODS

One hundred and twenty patients with early-stage HCC were enrolled in our study, with the collection of formalin fixed, paraffin-embedded (FFPE) specimens and clinicopathological data. Tumor areas were marked by certified pathologists on a hematoxylin and eosin-stained slide, and cancer and adjacent non-cancerous tissues underwent extraction of DNA, which was analyzed with the Affymetrix OncoScan platform to assess CNAs and loss of heterozygosity (LOH). Ten individuals with nonmalignant disease were used as the control group. Another cohort consisting of 40 patients with stage Ⅰ/Ⅱ HCC were enrolled to analyze gene expression and to correlate findings with the OncoScan data.

RESULTS

Copy number amplifications occurred at chromosomes 1q21.1-q44 and 8q12.3-24.3 and deletions were found at 4q13.1-q35.2, 8p 23.2-21.1, 16q23.3-24.3, and 17p13.3-12, while LOH commonly occurred at 1p32.3, 3p21.31, 8p23.2-21.1, 16q22.1-24.3, and 17p 13.3-11 in early-stage HCC. Using Cox regression analysis, we also found that a higher percentage of genome change ($\geqslant$ 60%) was an independent factor for worse prognosis in early-stage HCC (P = 0.031). Among the 875 genes in the OncoScan GeneChip, six were independent predictors of worse disease-free survival, of which three were amplified (*MYC*, *ELAC2*, and *SYK*) and three were deleted (*GAK*, *MECOM*, and *WRN*). Further, patients with HCC who exhibited $\geqslant$ 3 CNAs involving these six genes have worse outcomes compared to those who had < 3 CNAs (P < 0.001). Similarly, Asian patients with stage I HCC from The Cancer Genome Atlas harboring CNAs with these genes were also predicted to have poorer outcomes.

CONCLUSION

Patients with early-stage HCC and increased genome change or CNAs involving *MYC*, *ELAC2*, *SYK*, *GAK*, *MECOM*, or *WRN* are at risk for poorer outcome after resection.

Key words: Early-stage hepatocellular carcinoma; Copy number aberration; Prognosis; OncoScan; Molecular inversion probe

Core tip: In this paper, we report that patients with early-stage hepatocellular carcinoma presenting a higher percentage of genome change or copy number aberrations affecting *MYC*, *ELAC2*, *SYK*, *GAK*, *MECOM*, or *WRN* are predicted to have worse outcomes, and they should be intensively followed after resection.

Yu MC, Lee CW, Lee YS, Lian JH, Tsai CL, Liu YP, Wu CH, Tsai CN. Prediction of early-stage hepatocellular carcinoma using OncoScan chromosomal copy number aberration data. *World J Gastroenterol* 2017; 23(44): 7818-7829 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7818.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7818

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer death worldwide[1,2]. Partial hepatectomy, ablation therapy, and liver transplantation are considered curative treatments for HCC; however, the high probability of recurrence frequently results in unsatisfactory outcomes, which has led to the increased importance of combined or multimodal treatments in recent years[3-6]. Among patients with HCC, most patients with early-stage (stage Ⅰ/Ⅱ) cancer have a favorable outcome; nevertheless, a proportion of patients have poor prognosis after resection, which may arise from increased genomic instability[7]. Emergent efforts to resolve this dilemma include the integration of genomics, proteomics, metabolomics data, and clinical variants to predict outcomes for patients with early-stage HCC at both the research and clinical levels[8].

In particular, it appears that a differential gene expression profile in HCC arises from genetic instability or mutation[9,10]. Chromosome instability and copy number aberrations (CNAs) in HCC and other solid tumors could lead to the activation of oncogenes and the inactivation of tumor suppressor genes, which induce tumor invasiveness[11]. The common chromosome imbalances in HCC comprise of gains (amplification) at 1q, 8q, and 20q or losses (deletion) at 1p, 4q, 8p, 13q, 16q, and 17p across HCC specimens of different etiologies and cell lines using comparative genomic hybridization[12]. Some studies have also used formalin-fixed paraffin-embedded (FFPE) specimens for genome-wide copy number variation (CNV) analysis *via* high-density array, which disclosed common CNV regions, such as gains of 1q, 8q, 7q, 5p, 7p, Xq, 5q, and Xp and losses of 17p, 4q21.21-q26, 8p, 1p36.11-pter, and 9p[13,14]. In addition to these regions, chr12q13, 13q12, and 6p21-p24 may also contribute to the invasive phenotype of HCC[15,16]. However, little survival analysis has been performed in prior HCC FFPE studies owing to the limited number of cases or incomplete survival data[13,16].

Since FFPE specimens represent the most abundant bioresources in hospitals and the clinical outcome of some patients is already known, these factors allow scientists to integrate both complete clinical data and

genomic information to reveal potential biomarkers either for cancer diagnosis or prognosis, especially for rare tumors or early-stage cancers. Therefore, an increasing number of studies have analyzed FFPE specimens using a global analysis of chromosome imbalance *via* the Affymetrix OncoScan FFPE Express 2.0 system with molecular inversion probes (MIPs) in ovary, breast, colon, and brain tumors[17-20].

In the current study, global chromosomal CNAs in early-stage (stage Ⅰ/Ⅱ) HCC FFPE samples were analyzed using the Affymetrix OncoScan platform to: (1) disclose genomic alterations; (2) determine their correlation with patient characteristics; (3) predict long-term outcomes with CNA percent change; and (4) identify the most significant altered genes.

MATERIALS AND METHODS

Patients

This study was approved by the Institutional Review Board of the Chang Gung Memorial Hospital (CGMH) in Linkou, Taiwan (#104-3511C). The inclusion criterion for participants was defined as having a resectable single HCC lesion (stage Ⅰ or Ⅱ as defined in the American Joint Committee on Cancer/International Union Against Cancer TNM system) and the exclusion criteria were the presence of distant metastasis or abnormal liver function tests[6]. One hundred and twenty patients with early-stage HCC were enrolled in this study, with the collection of FFPE specimens and clinicopathological data.

DNA extraction, FFPE sample gene chip analysis, and analysis of MIP data

FFPE samples were sliced into 10-μm sections and the tumor area was marked by certified pathologists on a hematoxylin and eosin-stained slide. Cancer and adjacent non-cancerous tissues underwent DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen, Sussex, United Kingdom) according to the manufacturer's instructions. DNA concentration and purity were determined using the Qubit Fluorometer (Thermo Fisher Scientific UK Ltd., Paisley, United Kingdom). The extracted samples were further processed at the Genomic Medicine Core Laboratory at CGMH and analyzed with the Affymetrix OncoScan platform (Santa Clara, CA, United States) to assess CNAs and loss of heterozygosity (LOH)[21,22].

MIP data and the percentage of the genome that changed (percent genome change) were analyzed using the Nexus Copy Number software included in the Affymetrix OncoScan FFPE Express Service (Biodiscovery, El Segundo, CA, United States). The OncoScan GeneChip includes 875 gene targets representing tumor suppressor genes and oncogenes; each gene is represented by 20-40 probes depending on the length of the gene.

RNA extraction, microarray data processing, and Affymetrix GeneChip Human Genome U133 Plus 2.0 Array

Another cohort consisting of 40 patients with stage Ⅰ/Ⅱ HCC were enrolled to analyze gene expression and to correlate findings with the OncoScan data (IRB No.96-1371C, 99-1127B, 101-1186B, and 201600707B0). Total RNA was extracted with TRIzol as recommended by the manufacturer followed by RNA cleanup using the MinElute Kit (Thermo Fisher Scientific Inc.). RNA labeling, hybridization, washes, and processing were performed by the Genomic Medicine Core Laboratory of CGMH. To filter the lower variance genes, we used a standard deviation > 0.5 to filter 6522 probe sets from the original 22215. The differentially expressed genes between cancer and non-cancerous tissues were identified with paired *t*-tests, and *P*-values of gene expression were calculated as previously described[23].

TaqMan copy number assay

Validation of chromosome aberrations was performed with TaqMan copy number real-time polymerase chain reaction (PCR) using genomic DNA extracted from FFPE samples. Twenty nanograms of genomic DNA were mixed with 1 μL of target gene, RNASEP primer/probes (Thermo Fisher Scientific Inc.), and 1 × TaqPath ProAmp Master Mix (Thermo Fisher Scientific Inc.) to a final volume of 20 μL. Copy number PCR was performed in QuantStudio 3 (Thermo Fisher Scientific Inc.) and then further analyzed with CopyCaller v2.1 (Thermo Fisher Scientific Inc.) by normalization with RNASEP Ct values and calculating the log2 ratio of ΔCt between RNASEP and the target gene, which were further normalized to their normal counterpart tissues to show as $2^{-\Delta\Delta CT}$ (fold change).

Survival and long-term outcome analysis

Categorical data were analyzed using the chi-square test or Fisher's exact test. Continuous variables were analyzed using Student's *t* test. Survival rates in each group were determined by the Kaplan-Meier method and differences between groups were analyzed using log-rank test. Tumor recurrence was analyzed with area under the receiver operating characteristic curve (AUROC) comparisons using the percent genome change, α-fetoprotein (AFP), and tumor size for each patient. Long-term outcomes were determined using Cox regression analysis incorporating CNAs from the OncoScan data. All *P*-values calculated were two-tailed and significance was defined at the 95% level ($P < 0.05$). Statistical analyses were performed using SPSS

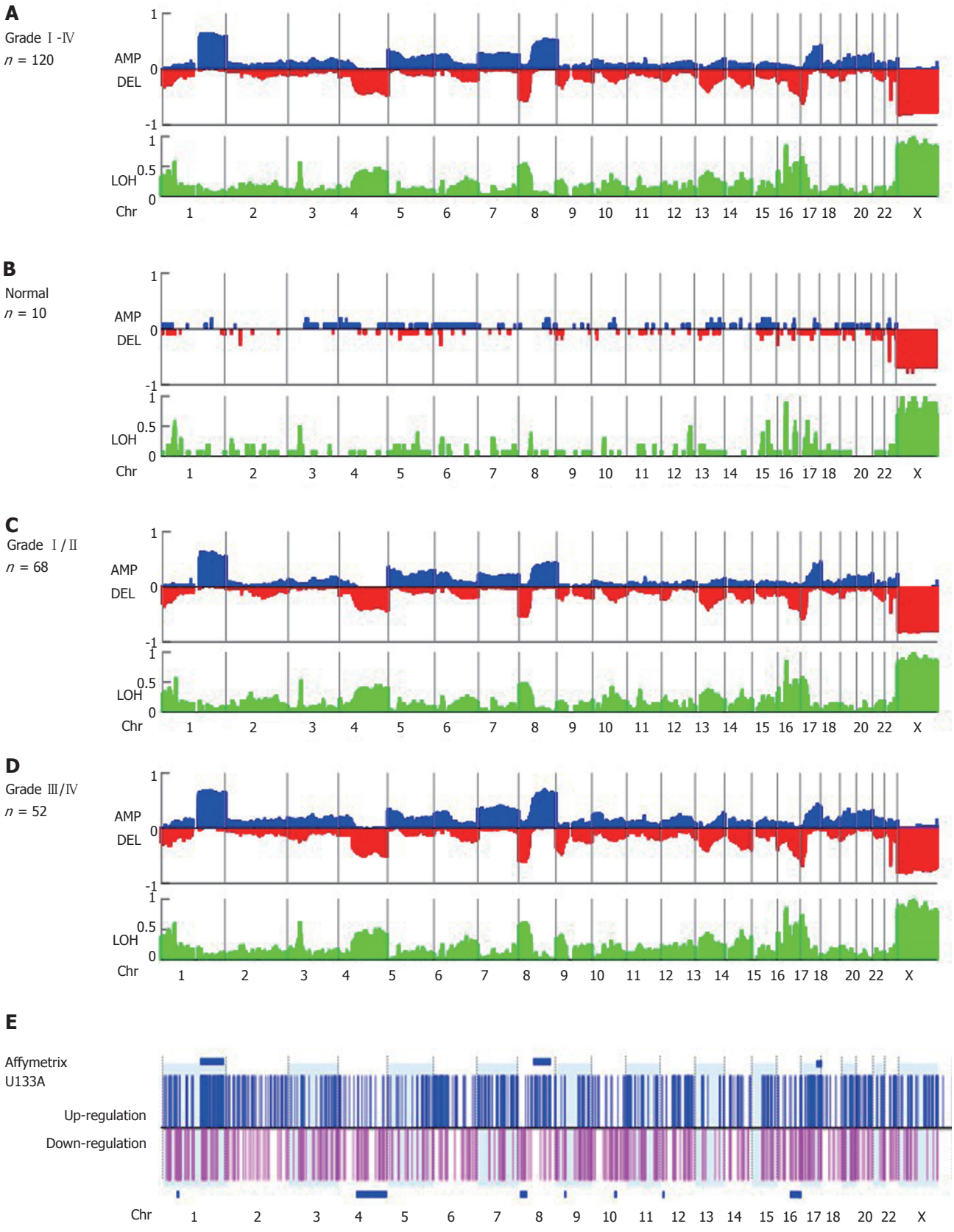

Figure 1 Virtual karyotyping analysis of amplification, deletion, and loss of heterozygosity in 120 formalin fixed, paraffin-embedded specimens of early-stage hepatocellular carcinoma. The frequency of copy number amplification (AMP), deletion (DEL), and loss of heterozygosity (LOH) in (A) specimens from all 120 patients with hepatocellular carcinoma (HCC), (B) patients with benign liver lesions (n = 10), (C) Edmonson grade I/II HCC specimens (n = 68), and (D) Edmonson grade Ⅲ/Ⅳ HCC specimens (n = 52) are shown on the Y-axis of each panel. Relative chromosomal position is shown on the X-axis. Blue and red plots represent the frequency of copy number amplification and deletion, respectively (upper panel), and LOH (shown in green, lower panel). Note that +1, 0, and -1 represent a frequency of 100% amplification, no alteration, and 100% deletion, respectively. (E) The gene expression profiles of another set of early-stage HCC tumors (n = 40). The log ratio of each probe set was normalized by the normal counterpart of each HCC tumor [log(T-N)] and fold changes > 2 or ⩽ 2 were considered up- (shown in blue) or down-regulated (shown in pink), respectively. The P-value for differential gene expression in tumor versus normal tissue was calculated and considered significant with $P < 0.05$. Clustered chromosome regions associated with genome change are shown as horizontal blue bars ($P < 0.05$).

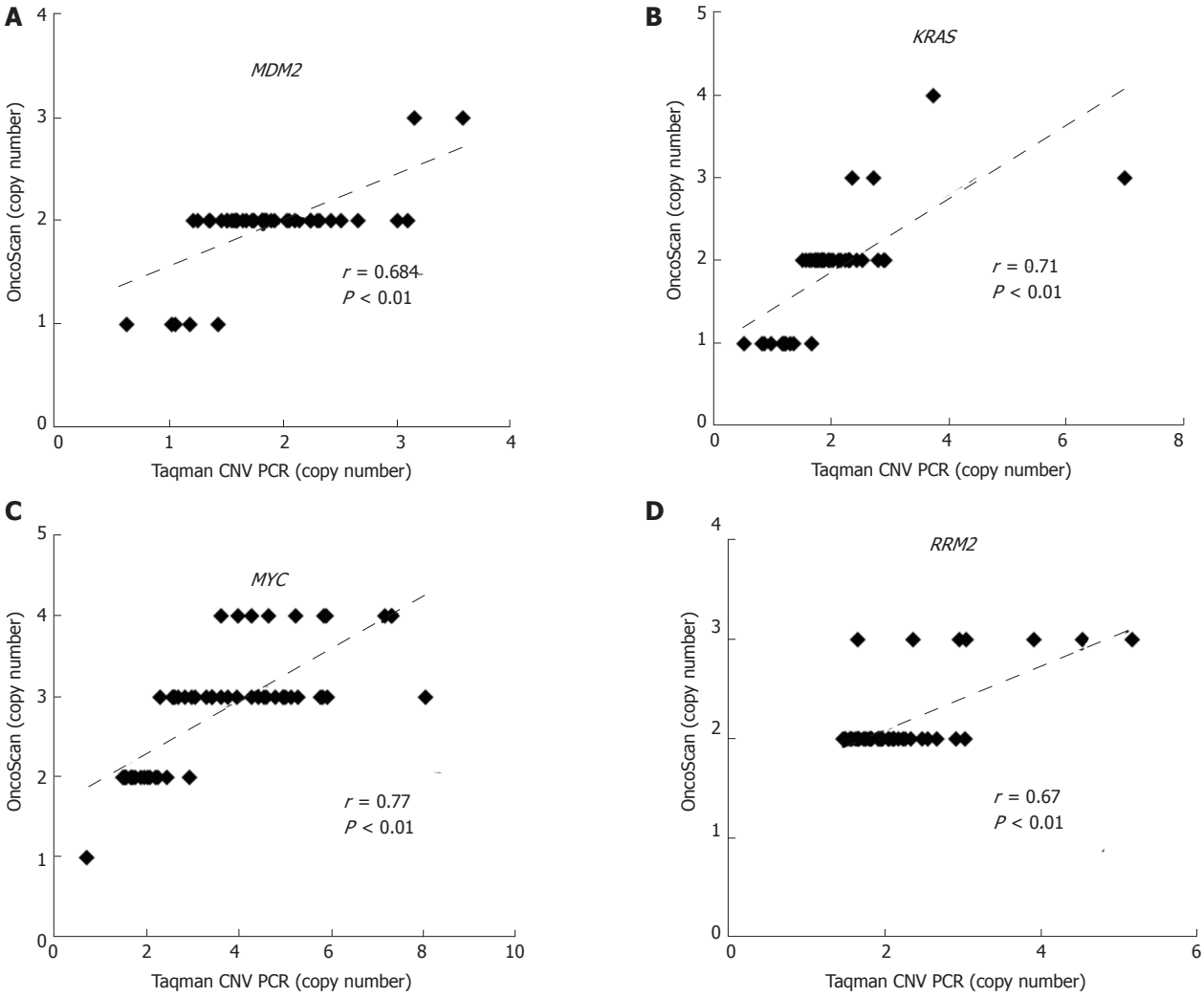

Figure 2 Validation of OncoScan copy number aberration data. The CNAs of (A) *MDM2*, (B) *KRAS*, (C) *MYC*, and (D) *RRM2* in early-stage HCC were validated by TaqMan copy number assay. Correlations between OncoScan CNAs and TaqMan PCR-determined CNAs were calculated *via* Pearson's correlation *r* and *P*-value. The X-axis shows the CNA results from TaqMan PCR and the Y-axis shows that of the OncoScan data. CNA: Copy number aberration.

statistical software version 17.0 (SPSS, Inc., Chicago, IL, United States).

RESULTS

Virtual karyotyping analysis and validation of OncoScan data via TaqMan copy number assay

Global genomic alterations in early-stage HCC FFPE were analyzed using the Affymetrix OncoScan platform. The most frequent CNAs identified by virtual karyotyping of chromosomes were amplifications of 1q21.1-q44 and 8q12.3-24.3 as well as deletions of 4q13.1-35.2, 8p23.2-21.1, 16q23.3-24.3, and 17p13.3-12. In addition, LOH was commonly identified at 1p32.3, 3p21.31, 8p23.2-21.1, 16q22.1-24.3, and 17p13.3-11 (Figure 1A). These CNAs were rarely found in patients with nonmalignant liver tumors or normal HCC counterpart tissue (Figure 1B). To confirm the OncoScan data, the same genomic DNA samples extracted from FFPE specimens were tested *via* TaqMan copy number assay using real-time PCR. Four target genes including MDM2 proto-oncogene (*MDM2* at 12q15), KRAS proto-oncogene, GTPase (*KRAS* at 12p12.1), *MYC* at 8q24.21, and ribonucleotide reductase regulatory subunit M2 (*RRM2* at 2q25.1) were chosen to validate the OncoScan CNV data. We found comparable results between the TaqMan copy number PCR results of these four genes and the corresponding OncoScan data, with robust Pearson correlation coefficients (*r*) of 0.684, 0.71, 0.67, and 0.77 for *MDM2*, *KRAS*, *RRM2*, and *MYC*, respectively (Figure 2).

Correlation of Affymetrix OncoScan results with gene expression microarray analysis

Next, gene expression of the global chromosome CNAs was investigated in the second cohort of 40 cases with stage Ⅰ/Ⅱ HCC, and six clustered regions were identified. Overexpressing genes in HCC tumors were found in 1q21.1-44, 8q12.3-24.3, and 17q22-25.3 with *P*-values of 7.72×10^{-25}, 0.002, and 0.042, respectively. These regions were also found

Table 1 Summary of chromosome imbalances in 120 hepatocellular carcinoma specimens and normal paired tissues

Chromosome region	Copy number	Cytoband	Grade Ⅰ/Ⅱ HCC	Grade Ⅲ/Ⅳ HCC	*P* value
1q	Gain	1q21.1-44	57.86%	60.63%	NS
4q	Loss	4q13.1- 35.2	39.56%	47.51%	NS
8p	Loss	8p 23.2-21.1	48.56%	56.28%	NS
8q	Gain	8q12.3-24.3	39.99%	62.01%	0.006[a]
16q	Loss	16q23.3-24.3	38.96%	47.32%	NS
17p	Loss	17p13.3-12	52.22%	53.73%	NS
17q	Gain	17q22-25.3	37.76%	36.37%	NS

[a]$P < 0.05$, statistically significant (Grade Ⅰ/Ⅱ *vs* Grade Ⅲ/Ⅳ). NS: Not significant; HCC: Hepatocellular carcinoma.

to be amplified based on OncoScan data (Figure 1E, blue bar). In addition, we found down-regulated genes in HCC tumors were clustered in 4q13.1-35.2, 8p23.2-21.1, and 16q23.3-24.3 with *P*-values of 0.002, 0.03, and 0.047, respectively, and these regions were deleted in tumors (Figure 1E, blue bar). These findings indicate that differential gene expression arises from CNVs in the HCC cancer genome.

Analysis of chromosome aberrations in early-stage HCC

Regarding the clinical characteristics of our patients with early-stage HCC, 60% were stage Ⅰ and 40% were stage Ⅱ; 16.7% had grade Ⅰ tumors, 40% had grade Ⅱ tumors, and 43.3% had grade Ⅲ/Ⅳ tumors. In our cohort, the mean tumor size was 4.2 cm, 7.5% presented with satellite lesions, 28.3% with vascular invasion, 0.8% with a microscopic margin, and all were Child-Pugh grade A (supplementary table 1). The mean disease-free survival (DFS) and overall survival were 43.8 ± 4.3 mo and 108.1 ± 10.5 mo, respectively. Tumor relapse occurred in 62.5% (75/120) of patients.

There were no correlations between CNAs and clinicopathological factors except for tumor grade. We found that amplification of chromosome 8q12.3-24.3 was associated with tumor differentiation and recurrence (%CNA = 39.99% and 62.01% in grade Ⅰ/Ⅱ *vs* Ⅲ/Ⅳ, respectively, $P = 0.006$, Table 1, Figure 1C and D, Supplementary figure 1). Furthermore, we found from univariate analysis that factors conferring worse prognosis for early-stage HCC included older age ($P = 0.004$), larger tumor size ($P = 0.001$), higher tumor grading ($P = 0.004$), elevated AFP > 100 ng/mL ($P = 0.006$), elevated alkaline phosphatase level > 120 U/L ($P = 0.016$), and higher percent genome change ≥ 60% ($P = 0.004$), whereas Cox regression analysis showed that tumor size > 4.5 cm ($P = 0.012$), the presence of satellite lesions ($P = 0.013$), elevated serum alkaline phosphatase > 120 U/L ($P = 0.042$), and percent genome change ($P = 0.031$) were independent predictors (Table 2). Taken together, our findings show that HCC genomic alteration patterns are consistent with those of previous reports using fresh or FFPE specimens, and a higher percentage of genome change itself is a prognostic factor for early-stage HCC.

Area under the receiver operating characteristic curve (AUROC) analysis for percent genome change, AFP, and tumor size in early-stage HCC

We next compared percent genome change with current risk factors, such as AFP and tumor size, by performing AUROC analysis. We found that the AUROC of percent genome change was higher than that found with AFP and tumor size with values of 0.657, 0.598, and 0.633, respectively (Figure 3A). The sensitivity and specificity of percent genome change were 0.467 and 0.8 for HCC recurrence at a cutoff of 30% (Supplementary table 2). Additionally, patients with a higher percentage of genome change (≥ 60%) were associated with extremely poor outcomes as determined by Kaplan-Meier curve analysis ($P = 0.004$, Figure 3B).

Predictive genes with copy number alteration for early-stage HCC

Of the 875 oncogenes and tumor suppressor genes evaluated, we found that 83 amplified genes, 14 deleted genes, and 35 LOH genes were associated with recurrence in DFS analysis (Supplementary table 3). Cox regression analysis for DFS identified six independent genes: three of which were amplified, *ELAC2* ($P = 0.023$), *MYC* ($P = 0.025$), and *SYK* ($P = 0.001$); and three deleted, *GAK* ($P < 0.001$), *MECOM* ($P = 0.001$), and *WRN* ($P = 0.009$) (Table 3). Patients with stage Ⅰ/Ⅱ HCC and any CNAs affecting these six genes have poor prognosis in Kaplan-Meier curve analysis ($P = 0.0036$, Figure 4A, dashed line). Furthermore, early-stage HCC with ≥ 3 CNAs affecting these six genes was associated with an extremely unfavorable outcome ($P < 0.001$, Figure 4B, dashed line). Subgroup analysis using tumor stage showed that CNAs of these six genes were associated with an unfavorable outcome in stage Ⅰ HCC ($n = 72$, $P = 0.0209$) but not in stage Ⅱ HCC ($n = 48$, $P = 0.15$) (Figure 4C and D, dashed line). Thus, genes within CNAs may predict poor outcome in early-stage HCC, especially patients with stage Ⅰ cancer, using OncoScan GeneChips.

Validation of CNAs as prognostic indicators for patients with early-stage HCC using The Cancer Genome Atlas Liver Hepatocellular Carcinoma database

CNAs involving these six putative prognostic genes for early-stage HCC were further analyzed using The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) database[24,25]. Of the 431 cases with HCC, we excluded those patients with liver dysfunction or lacking CNA or DFS follow-up data as well as patients with stage Ⅲ/Ⅳ HCC, and selected

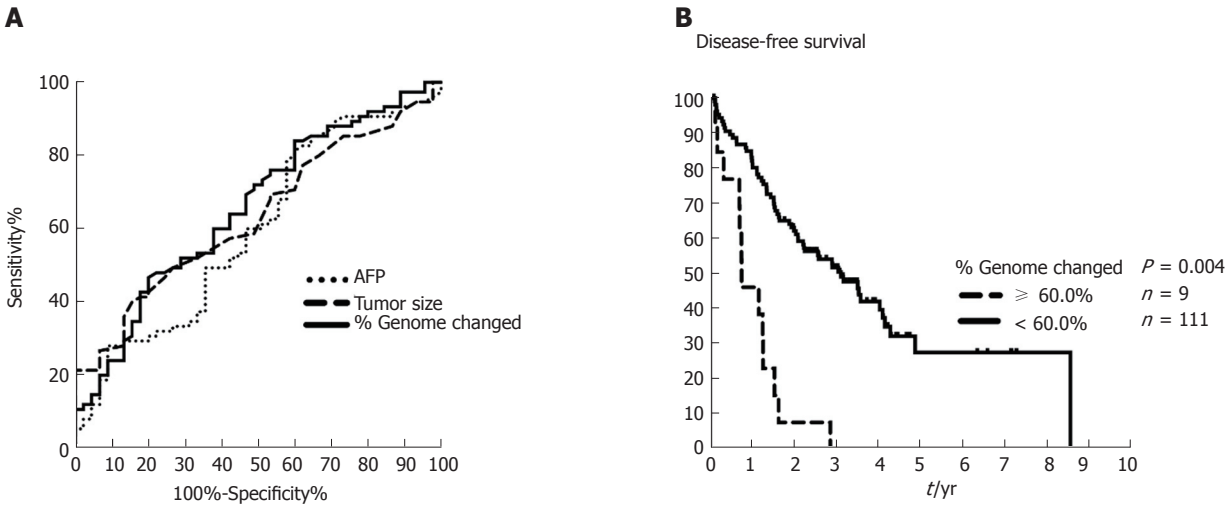

Figure 3 Comparisons of area under the receiver operating characteristic curve (AUROC) for percent genome change, α-fetoprotein, and tumor size in early-stage hepatocellular carcinoma, and Kaplan-Meier plot for 10-yr disease-free survival in patients with hepatocellular carcinoma with or without ⩾ 60% genome change. A: AUROC analysis of AFP (dotted line, 0.598), tumor size (dashed line, 0.633), and percent genome change (solid line, 0.657) shows predictive ability for early-stage HCC recurrence. Cutoffs for AFP, tumor size, and percent genome change are 5.2 ng/mL, 4.25 cm, and 30%, respectively (Supplementary table 3). B: The Kaplan-Meier plot for 10-yr disease-free survival in patients with HCC with ⩾ 60% (dashed line) or < 60% (solid line) genome change as determined from Affymetrix OncoScan CNA data. AFP: α-fetoprotein; HCC: Hepatocellular carcinoma.

Table 2 Disease-free survival analysis of clinicopathological data and percentage genome change of 120 patients with hepatocellular carcinoma

Clinicopathological factor	Log-rank test, *P* value	Cox regression analysis, *P* value	HR (95%CI)
Age (yr), ⩽ 49 (22.5%) *vs* > 49 (77.5%)	0.004[a]	0.169	0.674 (0.384-1.183)
Sex (M/F), M (81.7%) *vs* F (19.3%)	0.146		
Tumor size (cm), ⩽ 4.5 (69.2%) *vs* > 4.5 (30.8%)	0.001[a]	0.012[a]	1.959 (1.160-3.309)
Satellite lesions (%), Yes (7.5%) *vs* No (92.5%)	0.088	0.013[a]	2.900 (1255-6.702)
Vascular invasion (%), Yes (28.3%) *vs* No (71.7%)	0.240		
Grading Ⅰ, Ⅱ, Ⅲ, Ⅳ (%), Ⅲ & Ⅳ (43.3%) *vs* Ⅰ & Ⅱ (56.7%)	0.004[a]	0.063	1.626 (0.974-2.717)
Margin < 0.5 cm (%), ⩽ 0.5 cm (47.5%) *vs* > 0.5 cm (52.5%)	0.210		
Cirrhosis (%), Yes (54.2%) *vs* No (45.8%)	0.258		
AFP (100 ng/mL), £100 (77.5%) *vs* > 100 (22.5%)	0.006[a]	0.462	1.244 (0.695-2.227)
Alkaline phosphatase (120 U/L), ⩽ 120 (88.3%) *vs* > 120 (11.7%)	0.016[a]	0.042[a]	1.976 (1.025-3.808)
Staging Ⅰ/Ⅱ, Ⅱ (40.0%) *vs* Ⅰ (60.0%)	0.104		
Percentage genome changed (60%), ⩾ 60% (7.5%) *vs* < 60% (92.5%)	0.004[a]	0.031[a]	2.346 (1.080-5.097)

[a]*P* < 0.05, statistically significant. AFP: α-fetoprotein; HCC: Hepatocellular carcinoma; HR: Hazard ratio; CI: Confidence interval; M: Male; F: Female.

Table 3 Cox regression analysis for disease-free survival in early-stage hepatocellular carcinoma

Gene	Cytoband	CNA	HR	95%CI	*P* value
ELAC2	17p12	Amplification	2.784	1.153-6.724	0.023[a]
MYC	8q24.21	Amplification	1.772	1.074-2.924	0.025[a]
SYK	9q22.2	Amplification	4.204	1.813-9.748	0.001[a]
GAK	4p16.3	Deletion	2.916	1.635-5.199	< 0.001[a]
MECOM	3q26.2	Deletion	5.932	2.012-17.489	0.001[a]
WRN	8p12	Deletion	1.9	1.172-3.080	0.009[a]

[a]*P* < 0.05, statistically significant. HCC: Hepatocellular carcinoma; CNA: Copy number aberration; HR: Hazard ratio; CI: Confidence interval.

155 patients with early-stage HCC, of which 108 had stage Ⅰ and 47 had stage Ⅱ cancer. We found from this cohort those cases with a CNA that involved any of these six genes (*MYC*, *WRN*, *ELAC2*, *MECOM*, *GAK*, and *SYK*) have a trend towards worse prognosis (Figure 5C and D). Cases with stage Ⅰ HCC and these CNAs showed a borderline difference in DFS (*P* = 0.0583, Figure 5A), while Asian cases with stage Ⅰ (*n* = 73) and CNAs involving these genes have poorer outcome (*P* = 0.0203, Figure 5B). These findings from TCGA-LIHC patients were comparable to those found in our cohort.

DISCUSSION

Patients with early-stage HCC usually have a good survival outcome after curative treatment; however, the purpose of this study was to determine the underlying cause why some of those patients have a poor outcome[3,7]. In addition to driver gene mutations, and towards improvements in HCC treatment

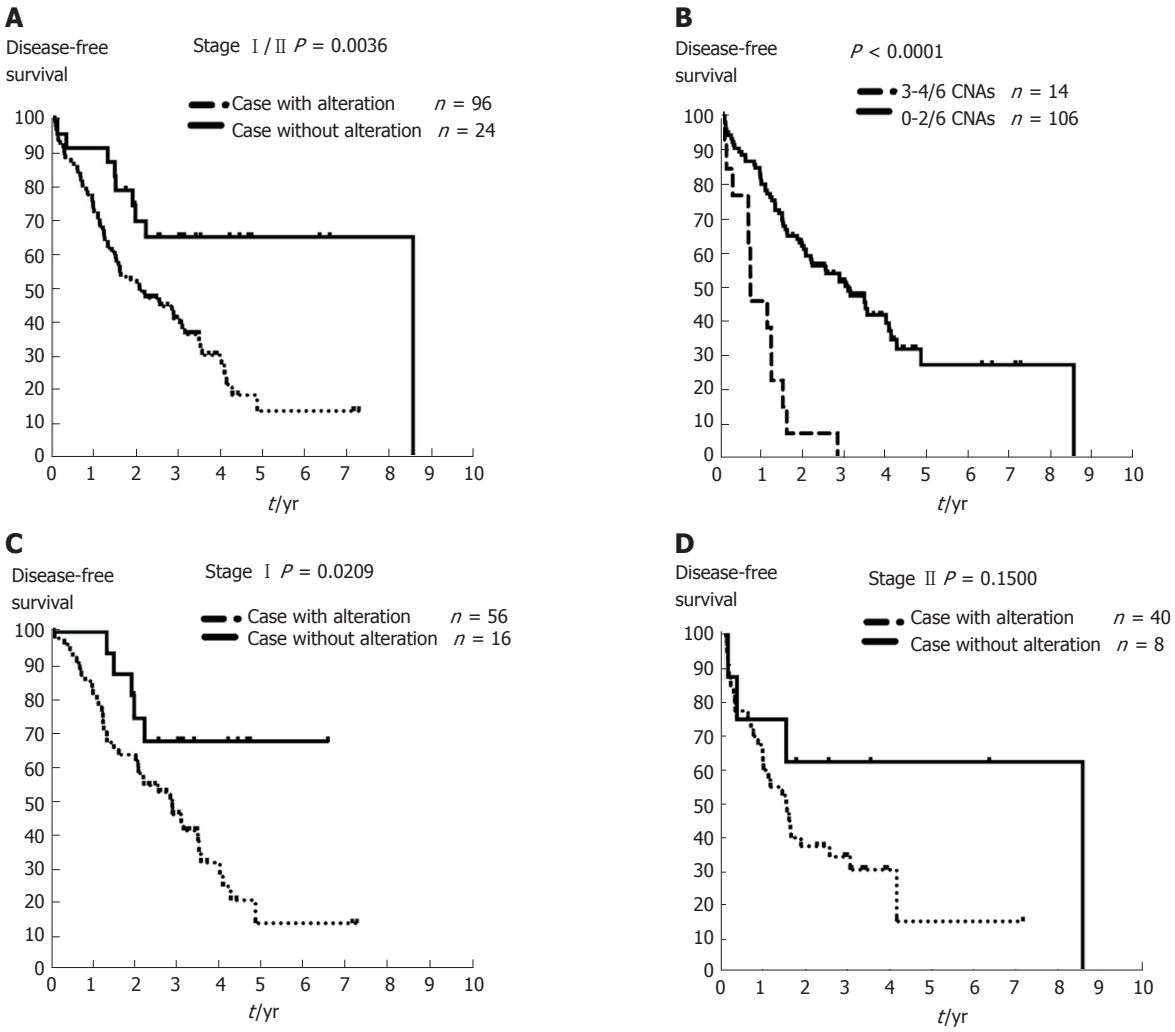

Figure 4 Disease-free survival analysis in patients with early-stage hepatocellular carcinoma with or without copy number aberrations involving six important genes identified using OncoScan data. A: Patients with stage Ⅰ/Ⅱ hepatocellular carcinoma (HCC) (n = 120) and copy number aberrations (CNAs) involving critical genes (dashed line) exhibited worse outcome compared to those without the predictive gene-containing CNAs (solid line) (P = 0.0036). B: Patients with early-stage HCC with three or more CNAs affecting these six identified genes had worse outcome (≥ 3 CNAs affecting these genes, dashed line, n = 14 *vs* < 3 CNAs affecting these genes, solid line, n = 106; P < 0.0001). C: Patients with stage Ⅰ HCC (n = 72) and CNAs affecting any of the six identified genes (*MYC*, *WRN*, *ELAC2*, *GAK*, *SYK*, or *MECOM*) were associated with worse outcome after resection (dashed line, P = 0.0209). D: Patients with stage Ⅱ HCC (n = 48) with/without any of the CNAs affecting these six genes (*MYC*, *WRN*, *ELAC2*, *GAK*, *SYK*, or *MECOM*) showed no difference in DFS (P = 0.15). DFS: Disease-free survival.

outcomes, we found that CNAs were also important in cancer genomics. In this study, we found that a higher percentage of genome change in CNAs identified *via* the Affymetrix OncoScan platform was of clinical significance, a finding which suggests that high-risk patients should be intensively followed after resection. The most important CNA region was gene amplification at chromosome 8q12.3-24.3, a region consistently reported in previous studies[13,26]. Within chromosome 8q12.3-24.3, *MYC* encodes a transcription factor with a basic helix-loop-helix leucine zipper domain that regulates various kinds of cellular processes. *MYC* has also been previously identified as a prognostic marker in HCC[27-30]. In our study, we found that *MYC* showed copy number amplification in 50.8% of patients with HCC and it was also an independent predictor of long-term survival for patients with early-stage HCC.

In contrast to *MYC*, the other five genes identified in our study have rarely been reported in either HCC carcinogenesis or prognosis[30]. *WRN* is associated with the autosomal recessive disorder Werner syndrome in which patients show premature aging and sarcomas because of chromosomal instability[31,32]. WRN's functions are related to its DNA helicase and exonuclease activities, and therefore, it interacts with p53 in DNA replication, repair, and recombination pathways to influence genomic instability[33]. The functions of MECOM in HCC carcinogenesis are linked with hepatitis B X protein carcinogenesis and antagonized growth inhibition induced by transforming growth factor-β (TGF-β) signaling[34]. Another CNA-linked gene, *ELAC2,* encodes a protein that contributes to endoribonuclease activity for tRNA 3′ processing[35]. In addition, ELAC2 is associated with Smad2 and its nuclear partner, forkhead box H1 (also known as FAST-1), which suggests that ELAC2 may be

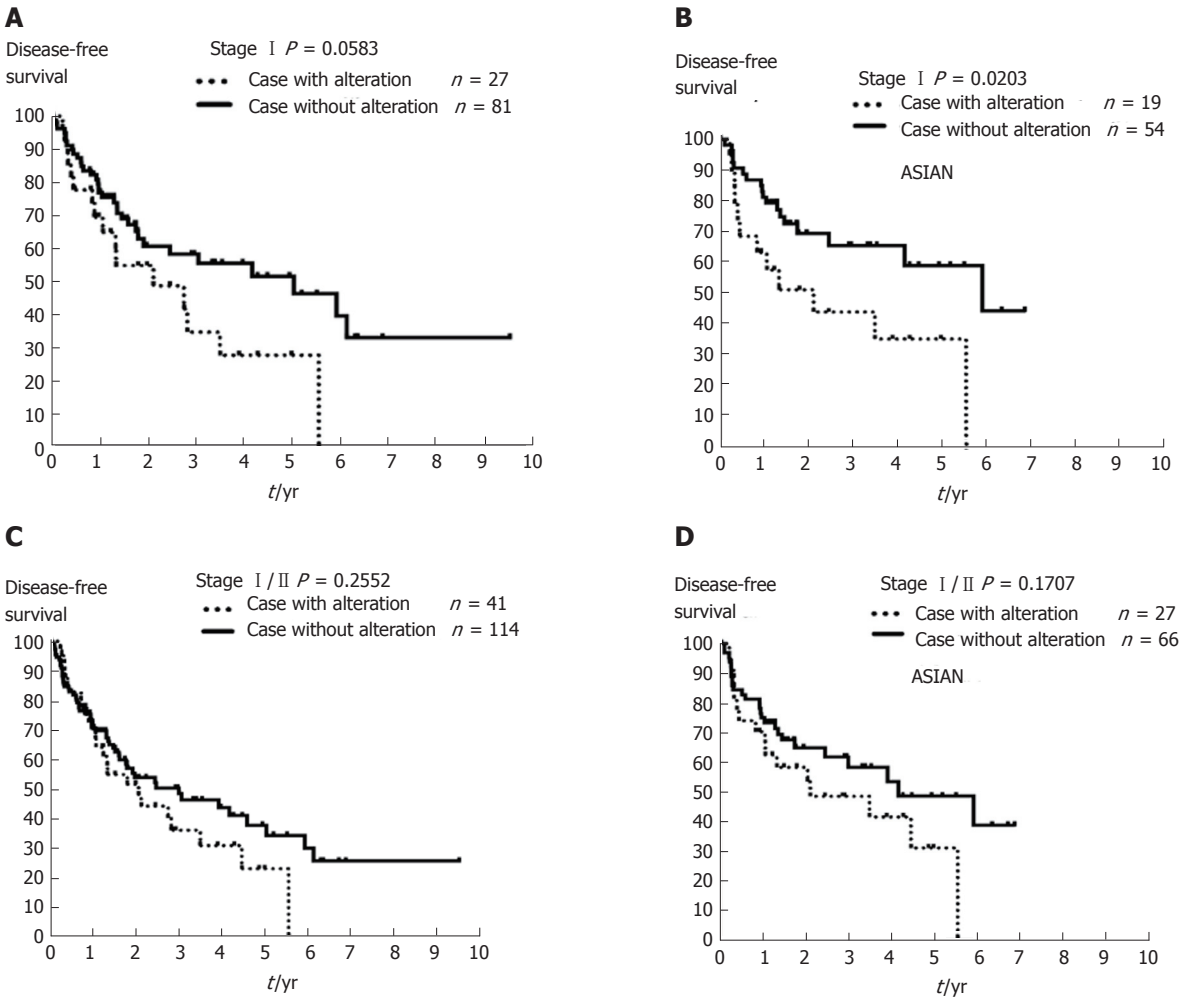

Figure 5 The Kaplan-Meier plot for 10-year disease-free survival in patients with stage Ⅰ/Ⅱ hepatocellular carcinoma with or without copy number aberrations involving *MYC*, *WRN*, *ELAC2*, *GAK*, *SYK*, or *MECOM* using The Cancer Genome Atlas Liver Hepatocellular Carcinoma data. A: Patients with stage Ⅰ hepatocellular carcinoma (HCC) (n = 108) with any copy number aberrations (CNAs) affecting one of the six genes identified in this study (*MYC*, *WRN*, *ELAC2*, *GAK*, *SYK*, or *MECOM*) had a trend for worse outcome after resection (dashed line, P = 0.0583). B: Asian patients with stage Ⅰ HCC (n = 73) and CNAs affecting these six genes (dashed line) exhibited poorer outcome compared to those cases without such CNAs (solid line, P = 0.0203). C: Patients with stage Ⅰ/Ⅱ HCC (n = 155), and D: Asian patients with stage Ⅰ/Ⅱ HCC (n = 93) with/without any of these CNAs affecting *MYC*, *WRN*, *ELAC2*, *GAK*, *SYK*, or *MECOM* showed no change in DFS (P = 0.2552 and P= 0.1707, respectively). DFS: Disease-free survival.

involved in TGF-β/Smad signaling in prostate cancer cells[36]. SYK, a non-receptor tyrosine kinase, is widely expressed in hematopoietic cells[37]. Down-regulation of *SYK* has also been reported in epithelial malignancies implicated in tumor formation and progression[38,39]. The protein of another gene, *GAK*, regulates clathrin-mediated membrane trafficking and functions as a transcriptional repressor of the androgen receptor[40,41]. It has been reported that knockdown of *GAK* activates the spindle-assembly checkpoint to induce misaligned or abnormally condensed chromosomes, a finding which indicates that GAK has a role in the maintenance of chromosome stability[42]. These findings suggest that the roles of these five genes in HCC carcinogenesis may involve genome stability, although their specific functions require further exploration. It would be worthwhile in the future to evaluate the genetic signature of *MYC*, *ELAC2*, *SYK*, *WRN*, *GAK*, and *MECOM* with respect to the survival of patients with early-stage HCC using another large cohort.

This study demonstrated the value of using FFPE samples to investigate CNAs in patients. In fact, some patients in our cohort had samples taken over 14 years ago and there were no issues regarding their quality and quantity for use in our study. FFPE samples may be a vital resource for cancer genomic studies enabling the prediction of long-term outcomes in patients who had surgery years ago. Furthermore, the TCGA database has been used to validate findings for various cancer genomic studies[24,25]. Our finding of a trend towards survival outcome using the TCGA dataset conferred further validation of our finding that Asians have a worse outcome, especially regarding cases with stage Ⅰ HCC. Although there was a different outcome based on the BRIDGE study, which may be because of ethical and/or regional differences in treatment choice, our use of the TCGA database consistently demonstrated that differences exist in the genomic

background of patients with HCC[43].

In conclusion, our identification of percent genome change and six independently predictive genes from CNA data obtained using the Affymetrix OncoScan platform illustrates that chromosomal alterations are crucial regarding the outcome of patients with early-stage HCC after resection. Adoption of this platform could be useful in precision medicine for the prediction of early-stage HCC prognosis using FFPE specimens.

ARTICLE HIGHLIGHTS

Research background

Most patients with early-stage (stage Ⅰ / Ⅱ) hepatocellular carcinoma (HCC) have a favorable outcome; nevertheless, increased genomic instability possibly leads to postoperative recurrence.

Research motivation

Previous studies using frozen HCC tumor tissue with array comparative genomic hybridization were of limited clinical value because of the absence of patient survival data. Since formalin fixed, paraffin-embedded (FFPE) samples are the largest bio-resource with long-term patient survival data found in every hospital worldwide, the aim of this research was to determine whether FFPE specimens of early-stage HCC with long-term survival data may be used with OncoScan GeneChips towards prognostic analysis of patients.

Research objectives

The study enrolled 120 patients with early-stage HCC and ten nonmalignant liver tumors or normal HCC counterpart tissues to explore genome instability and copy number aberrations (CNAs) in early-stage HCC.

Research methods

Extracted DNA was processed at the Genomic Medicine Core Laboratory and analyzed with the Affymetrix OncoScan platform to assess CNAs and loss of heterozygosity (LOH). We reliably obtained global genome amplification/deletion and overall percentage genome change from all FFPE samples in our cohort.

Research results

CNA amplifications were clustered at chromosomes 1q21.1-q44 and 8q12.3-24.3 and deletions at 4q13.1-q35.2, 8p 23.2-21.1, 16q23.3-24.3, and 17p13.3-12 in patients with early-stage HCC. We found that percentage of genome change ⩾ 60% was an independent factor for worse prognosis and *MYC*, *ELAC2*, and *SYK* (amplification) as well as *GAK*, *MECOM*, and *WRN* (deletion) were the most powerful predicting genes. Using Asian patients with stage I HCC from The Cancer Genome Atlas as an independent cohort, we found that patients harboring CNAs affecting these genes were also predicted to have poorer outcomes.

Research conclusions

The identification of percent genome change and six independently predictive genes from the Affymetrix OncoScan platform illustrates that chromosomal alterations are crucial for outcome of patients with early-stage HCC after resection, which may be further applied for clinical practice using OncoScan or a custom-designed chip covering these six genes regions.

Research perspectives

Genome instability was related to early-stage HCC clinical outcome and patients with CNAs affecting *MYC*, *ELAC2*, *SYK*, *GAK*, *MECOM*, or *WRN* are at risk for poorer outcome after resection. In the era of precision medicine, the identification of CNAs in these six genes could be further applied for clinical practice using a small custom-designed chip.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Basic Study

Composition and immuno-stimulatory properties of extracellular DNA from mouse gut flora

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Abstract

AIM

To demonstrate that specific bacteria might release bacterial extracellular DNA (eDNA) to exert immuno-modulatory functions in the mouse small intestine.

METHODS

Extracellular DNA was extracted using phosphate buffered saline with 0.5 mmol/L dithiothreitol combined with two phenol extractions. TOTO-1 iodide, a cell-impermeant and high-affinity nucleic acid stain, was used to confirm the existence of eDNA in the mucus layers of the small intestine

and colon in healthy Male C57BL/6 mice. Composition difference of eDNA and intracellular DNA (iDNA) of the small intestinal mucus was studied by Illumina sequencing and terminal restriction fragment length polymorphism (T-RFLP). Stimulation of cytokine production by eDNA was studied in RAW264.7 cells *in vitro*.

RESULTS

TOTO-1 iodide staining confirmed existence of eDNA in loose mucus layer of the mouse colon and thin surface mucus layer of the small intestine. Illumina sequencing analysis and T-RFLP revealed that the composition of the eDNA in the small intestinal mucus was significantly different from that of the iDNA of the small intestinal mucus bacteria. Illumina Miseq sequencing showed that the eDNA sequences came mainly from Gram-negative bacteria of Bacteroidales S24-7. By contrast, predominant bacteria of the small intestinal flora comprised Gram-positive bacteria. Both eDNA and iDNA were added to native or lipopolysaccharide-stimulated Raw267.4 macrophages, respectively. The eDNA induced significantly lower tumor necrosis factor-α/interleukin-10 (IL-10) and IL-6/IL-10 ratios than iDNA, suggesting the predominance for maintaining immune homeostasis of the gut.

CONCLUSION

Our results indicated that degraded bacterial genomic DNA was mainly released by Gram-negative bacteria, especially Bacteroidales-S24-7 and Stenotrophomonas genus in gut mucus of mice. They decreased pro-inflammatory activity compared to total gut flora genomic DNA.

Key words: Bacterial extracellular DNA; Flora; Immune-stimulatory property; Gut microbiota; Mouse; Small intestine

Core tip: Our results revealed that degraded bacterial genomic DNA was mainly released by Gram-negative bacteria, especially Bacteroidales-S24-7 and Stenotrophomonas genus in gut mucus of mice. They decreased pro-inflammatory activity compared to genomic DNA of total gut flora. Our study highlights that bacteria derived DNA plays an important role in modulating local immune response in mouse gut.

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INTRODUCTION

The intestinal mucosal immune system of mammals evolved to coexist with densely populated microorganisms that reside in the intestinal mucus layer and lumen. The central physiological process for homeostatic immune response in the gut is induced by specific bacterial products. Unmethylated cytosine-guanine (CpG)-rich DNA is typical microbial products that are recognized by the vertebrate innate immune system[1]. Exposition to the TLR9 ligand CpG induces strong protective effects in different models of intestinal inflammation[2,3]. TLR9 activation by bacterial DNA has also been demonstrated to induce degranulation of Paneth cells and to induce increased resistance to *Salmonella typhimurium* infection[4]. However, the specific effect of the physiologic microbiota DNA on TLR9 pathway status within the intestine so far remains elusive. Because in the mucosal environment, dendritic cells (DCs) and enterocytes permanently monitor the bacterial burden and structure in the gut[5], it is conceivable that this physiologic interaction significantly contributes to gut homeostasis. It has been demonstrated that extracted DNA of gut lumen flora limited potently regulatory T cell (Treg) induction by DCs of the lamina propria, thus controlling the balance between Treg and effector T cell frequency and function[6].

Because of the large number of bacteria present in the gut, the amount of cell-free bacterial DNA is likely to be more significant. Small intestinal mucosa-associated bacteria might find it easier to release extracellular DNA (eDNA) because of the action of antimicrobial peptides[5], which would contact epithelial cells after penetration of the thin mucus layer. However, evidence is still lacking to support the existence of bacterial eDNA within the mucus layer.

It is worth to note that intestinal epithelial cells do not respond equally to bacterial DNA, and are capable of distinguishing between DNA from probiotic strains and DNA from pathogenic strains[7]. A bioinformatic analysis revealed that small intestine specific bacteria *Enterococcus faecalis*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus*, whose strains have been marketed as probiotics, had high counts of GTCGTT motifs, the optimal motif stimulating human TLR9[8]. The quantity and resource of CpG DNA can also be viewed as detrimental, depending on the host's physiological status. Estimation of the load of bacterial released DNA by mucosa-associated bacteria could shed new light on host-microbe interactions across a range of diseases.

The present study aimed to demonstrate the existence of mucosa-associated bacteria released eDNA in the mucus layer in the mouse intestine. Furthermore, the major bacterial genera responsible for the release of eDNA in the small intestinal mucus layer were identified.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (four weeks old) were purchased from the Model Animal Research Center of Nanjing

University (Nanjing, China). The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to normal chow and water) for one week prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection after being fasted overnight.

Staining of gut mucus and bacterial eDNA

The distal colon and small intestine were dissected longitudinally and placed in Carnoy's solution (ethanol: chloroform: acetic acid = 6:3:1) for 3 h. The firm mucus layer of the colon was fixed using the same method except for scraping the surface slightly. The fixed tissues were then washed twice in absolute ethanol for 20 min each, followed by two washes in xylene for 15 min each, before paraffin embedding and sectioning as 4-μm-thin sections. The sections were placed on glass slides after floating on an air bath according to standard procedures[9].

An alcian blue-periodic acid Schiff (AB-PAS) staining kit was used to visualize the gut mucus. Visualization of eDNA in the intestinal biofilm was performed using the fluorescent dye TOTO-1 (Molecular Probes, Eugene, OR, United States).

Isolation of mucus bacterial eDNA

Ileums were opened longitudinally and food debris was removed carefully. The mucus was harvested with PBS containing 0.5 mmol/L dithiothreitol (PBS-DTT) and incubated for 3 min with gentle shaking. It was centrifuged for 10 min at 10000 rpm to harvest released DNA. This step was repeated twice and the supernatant was pooled. Then, 10% CTAB in 0.7 mol/L NaCl was added, and ethanol precipitation was used to concentrate DNA. Bead beating and the QIAamp DNA Stool Mini Kit (QIAGEN) were used to extract genomic DNA of mucoid bacteria.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

Primers 334F/939R or 338F/806R[10] were labeled with 5′6-carboxyfluorescein (6-FAM) (forward) or 5′ 6-hexachlorofluorescein (HEX) (reverse). The 25-μL PCR reaction contained 1 × PCR buffer, 200 μmol/L of each deoxynucleoside triphosphate, 1.5 mmol/L $MgCl_2$, 0.1 μmol/L of each primer, 100 ng of DNA template, and 0.5 U of Takara Taq DNA polymerase[11]. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

After purification, the amplification products were digested with DdeI or AluI. The restriction enzyme digestion reaction mixture (20 μL), containing 2 U of DdeI or AluI, 2 μL of 1 × NEB buffer, and 500 ng of PCR product, was incubated at 37 ℃ overnight. Finally, the fluorescently labeled terminal fragments of sizes between 50 bp to 500 bp were analyzed by electrophoresis on an ABI PRISM 310 Genetic Analyzer in the GeneScan mode.

Bacterial 16S rRNA gene amplification and illumina MiSeq sequencing

The V4-V5 domains of 16S rRNA genes were amplified using primers 515F and 907R (see supplementary methods). The resulting amplicons were submitted to the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for Illumina paired-end library preparation, cluster generation, and 300-bp paired-end sequencing on a MiSeq instrument in two separate runs. Details of the PCR amplification and sequencing are described in supplementary information. The raw reads were deposited in the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP072153).

Stimulation of RAW264.7 cells for cytokine production

RAW264.7 cells (4×10^6 cells/mL) were treated with medium, lipopolysaccharide (LPS) (1 μg/mL), eDNA (1 ng/mL), iDNA (1 ng/mL), eDNA (1 ng/mL) + LPS (1 μg/mL), or iDNA (1 ng/mL) + LPS (1 μg/mL) for 12 h. Culture supernatants were analyzed by enzyme linked immunosorbent assays (ELISAs) for TNF-α, IL-6, IL-10, or IL-12p40. All recombinant murine cytokines and antibodies specific for murine cytokines were purchased from Xiamen Huijia Biotechnology Co., Ltd (Xiamen, China). Purified DNA was tested for contaminating endotoxins by using a Limulus amebocyte lysate kit (Xiamen Agent Company (Fujian, PR China). Only preparations with endotoxin levels not exceeding 0.05 endotoxin units/mL were used.

Statistical analysis

The statistical significance of the comparisons between multiple groups was carried out by ANOVA, followed by Tukey's test. A 95% confidence interval was considered significant and was defined as $P < 0.05$. All values are expressed as the mean ± standard error of the mean (SEM). Each value is the mean of at least three separate experiments. Principal component analysis (PCA) and cluster analysis were used to analyze the terminal restriction fragment (TRF) profiles generated from the T-RFLP experiment, and were combined with the diversity index to study the bacterial communities. PCA plots were generated using the multivariate statistics software Canoco (version 4.5, Microcomputer Power, Ithaca, NY, United States). The biodiversity was measured using the Shannon-Wiener index ($H = -\Sigma pi \bullet \ln pi$); the Simpson Index ($D = 1-\Sigma pi^2$); and the Evenness Index ($E = H/\ln S$) according to the relative height of each TRF (pi) and sum of the number of TRFs (S) in a sample.

RESULTS

Staining of gut mucus and eDNA

As shown in Figure 1, AB-PAS staining showed apparent differences in mucus thickness between

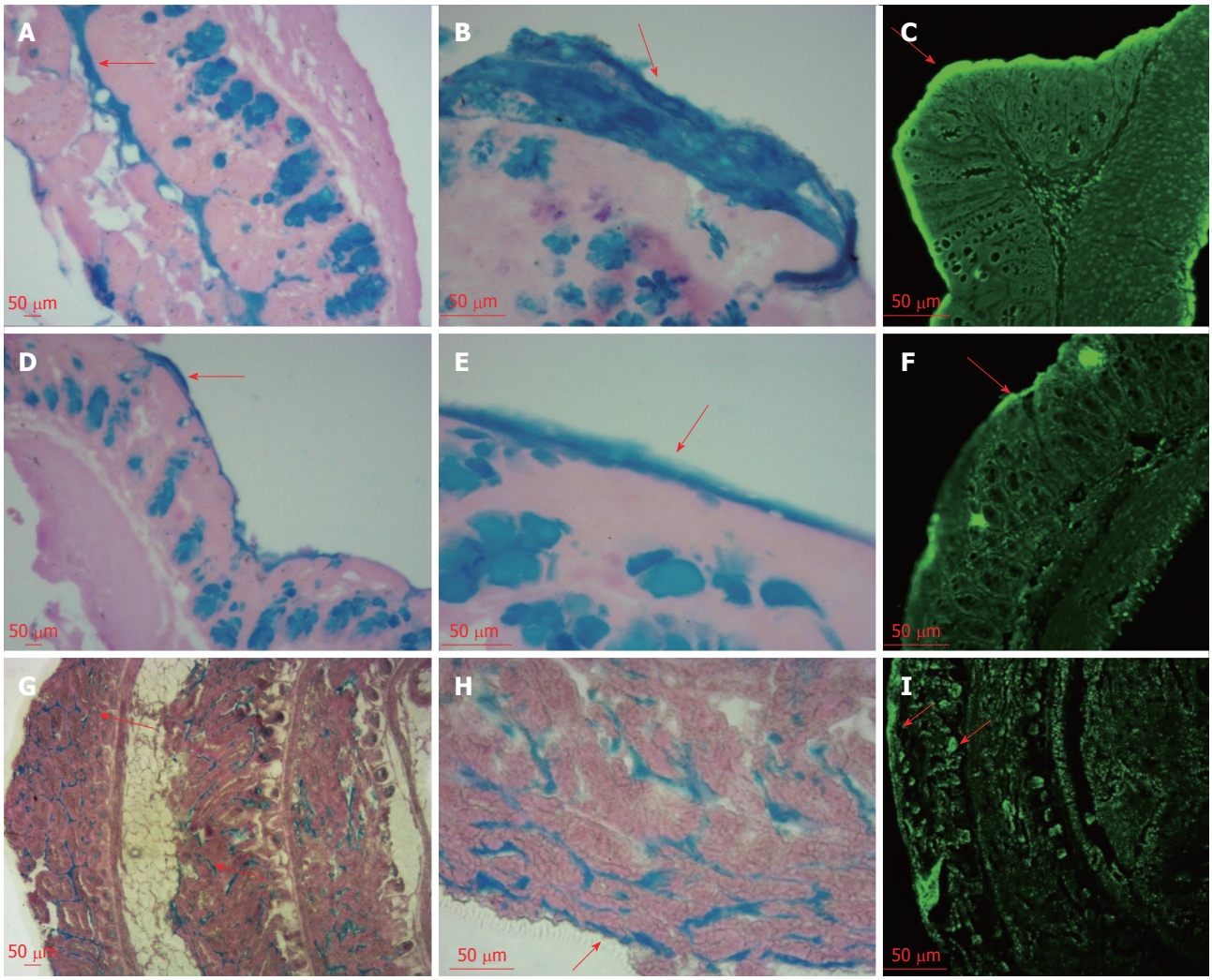

Figure 1 Mucus layer and eDNA in the mouse small intestine and colon. The first two columns are images obtained by optical microscopy with AB-PAS staining, and the third one are images obtained by laser scanning confocal microscopy with TOTO-1 staining. Pictures from the first two rows are the colon mucus layer, while those of the third row are the small intestinal mucus layer. A-F: Colon; G-I: Small intestine; A, D, G: AB-PAS 10×; B, E, H: AB-PAS 40×; C, F, I: TOTO-1 10 ×.

the intact mucosa and post-suction mucosa. The loose layer and inner layer of the colon mucus were separated by gentle suction and scraping, which were stained blue after PAS staining. The impermeant nucleic acid dye TOTO-1 was used to visualize the eDNA. This fluorescent stain can bind DNA molecules *via* its positive charge and emits green fluorescence when excited at 514 nm. The mucus is reported to be composed of two layers with different characteristics and completely different distribution patterns of bacteria[12]. The loose layer contains bacteria, whereas the firm layer is reported to be free of bacteria[12]. We could see a clear deepening of the green fluorescent band on the colon surface, which proved the existence of specific microbial communities, which we attributed to the release of eDNA in the gut mucus loose layer. By contrast, the fluorescence intensity of the inner mucus was much weaker, which suggested no eDNA release because of the absence of bacteria in this firm layer. In the small intestine, the thin surface mucus layer was positive for TOTO-1 staining. Note that the bottom of the crypt lumen was positive and stained green under a confocal microscope, which might be explained by the accumulation of eDNA around such cells.

Results of T-RFLP

The T-RFLP data representing the gut microbial community profiles were analyzed using multivariate statistics for the intestinal mucus separately. First, the T-RFLP data from each individual were normalized and entered into a data matrix that comprised the TRFs as variables and individuals as objects. A consensus T-RFLP profile from each biological replicate was constructed by averaging the technical duplicates[13].

The PCA in Figure 2 clearly demonstrates remarkably different TRF profiles between eDNA and iDNA, using either AluI (Figure 2A) or DdeI (Figure 2B). Samples from eDNA or iDNA were found to gather in a concentrated area and were separate from each other. The results indicated that the components of eDNA varied greatly from those of iDNA, indicating that they were derived from two different bacterial

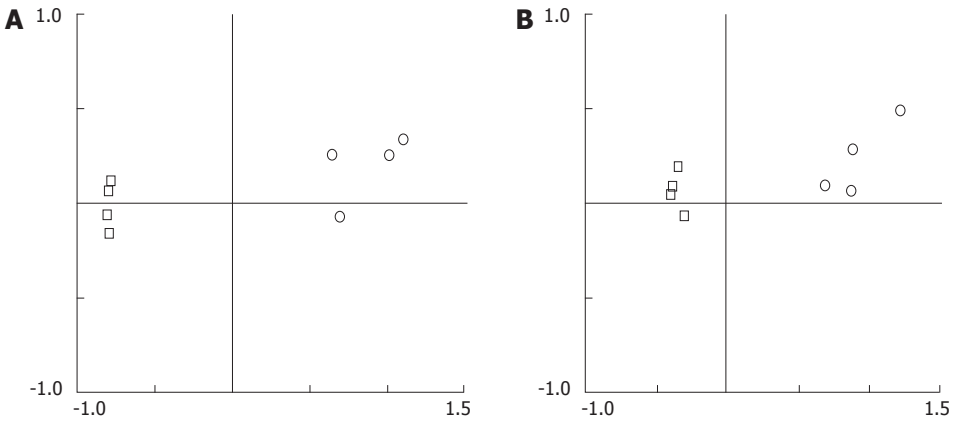

Figure 2 Principal component analysis plots for terminal restriction fragment length polymorphism profiles (including TRF size and relative abundance data). A: with AluI. B: with DdeI; Empty circle, eDNA; Empty square, iDNA.

Table 1 The analysis of diversity index

	Shannon-wiener index	Equitability index	Simpson's diversity index
iDNA	2.09 ± 0.04	0.87 ± 0.01	0.84 ± 0.00
eDNA	1.64 ± 0.09[a]	0.77 ± 00.04[a]	0.73 ± 0.04[a]

Values are expressed as mean ± SEM (n = 6). [a]P < 0.05, significant differences between iDNA and eDNA.

communities.

The cluster analysis in supplementary figure 1 showed that eDNA was clustered separately from iDNA, which agreed with the conclusion of PCA. The unique TRFs were extracted that belonged to the iDNA or eDNA. Some unique TRFs from the same set could be filtered for further confirmation. The TRFs in supplementary figure 1A and C were specific for the iDNA using AluI and DdeI, respectively, while the TRFs in supplementary figure 1B and D belonged to eDNA using the two endonucleases.

By analyzing the diversity index (Table 1), the values of Shannon-wiener index, equitability index, and Simpson's diversity index from the eDNA were observed to be smaller than those from the iDNA (P < 0.05). The lower index values indicate poorer abundance and stability of the eDNA. The special properties of eDNA microbiota could be the factor that distinguishes them from the iDNA resource. However, further studies are needed to provide more evidence to authenticate the particularity of eDNA.

Results of illumina MiSeq sequencing

We failed to amplify the 16S rRNA gene from the eDNA using primers 338F/806R, which should have generated a 468 bp amplicon (data not shown). Using primers 515F/907R, we produced a 392-bp product successfully. Consistent with our T-RLFP analysis, the sequencing results of this amplicon indicated a significant difference in proportions of major phyla between the eDNA and the iDNA (Figure 2). *Firmicutes* was the most abundant group in the iDNA (68%-77%), while 11% of *Bacteroidetes* occupied the second place. Whereas in the eDNA, we found that *Bacteroidetes* and *Proteobacteria* were more dominant at 54 % and 29%, respectively. Analysis at the genus level (Figure 3 and supplementary table 1) provided more detailed information. The results revealed that genera of two Gram-negative bacteria, *Bacteroidales S24-7* and *Stenotrophomonas,* were the dominant genera in the eDNA resource, reaching a proportion of 77%-91%. While two Gram-positive genera, *Staphylococcus* and *Allobaculum,* were main components in iDNA. The iDNA also contained a small quantity of *Bacteroidales S24-7.* Gram-negative genera represented 83.4% of the genera in the eDNA and Gram-positive ones represented 86.1 % of the genera in the iDNA

The results of fluorescence *in situ* hybridization with probes for *Bacteroidales* and *Staphylococcus* demonstrated different degrees of positive reaction in the crypt lumen (supplementary figure 2). These results indicated that eDNA of Gram-negative genera often migrated to the crypt.

Supplementary figure 3 showed that other detectable bacteria, such as *Lactobacillus, Facklamia,* and *Ralstonia*, were significantly different between eDNA and iDNA. All these results suggested that the different constituents and proportions of micro-community in the eDNA and iDNA might explain their specific functions.

Furthermore, the 16S rRNA marker gene sequences were used to establish the predictive functional profiles of the microbial communities using PICRUST (supplementary figure 4). Interestingly, the operational taxonomic unit (OTU) abundances representing certain specific functions in eDNA were generally higher than those of the iDNA, for example, cell motility (N), vesicular transport (U), and cell membrane biogenesis (M), which have been confirmed to be closely associated with the function of the eDNA.

Cytokine production from Raw264.7 cells is stimulated differently by eDNA and iDNA

As shown in Figure 4, the production of inflammatory cytokines TNF-α and IL-6 in Raw264.7 macrophages

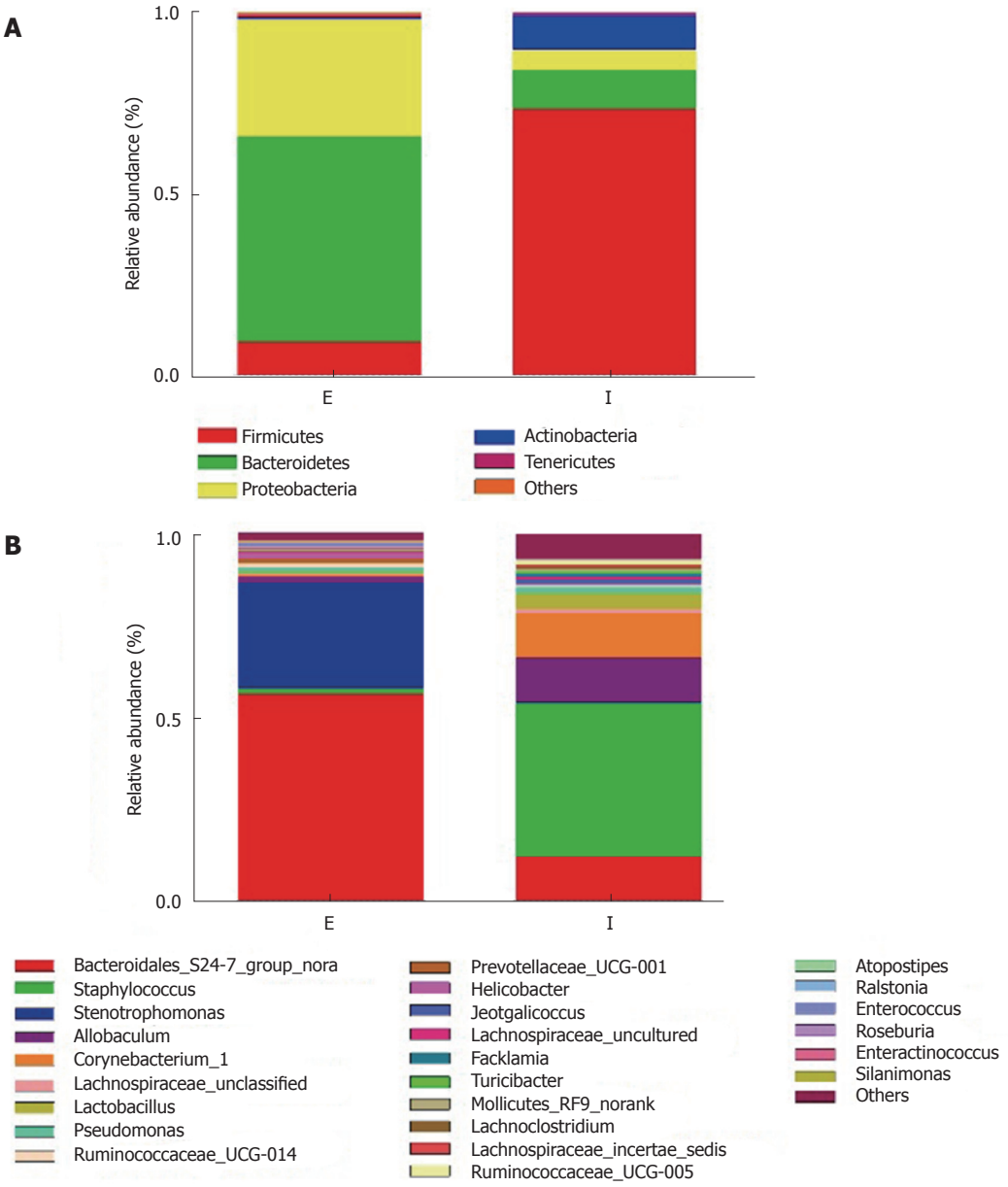

Figure 3 Community structure component diagram at the phylum (A) and genus (B) levels. Samples were from eDNA (E) and iDNA (I) in triplicate, respectively. eDNA: Extracellular bacterial DNA; iDNA: Intracellular bacterial DNA.

were stimulated significantly by both eDNA and iDNA ($P < 0.05$). Only eDNA promoted a very low, but significantly higher level of IL-12 compared with the iDNA ($P < 0.05$). However, iDNA suppressed the production of the anti- inflammatory cytokine IL-10 significantly ($P < 0.05$). LPS stimulated the production of all cytokines significantly ($P < 0.01$), which were greatly suppressed when cells were exposed to iDNA simultaneously ($P < 0.05$). Only the iDNA showed an inhibitory effect on LPS stimulated IL-12 production ($P < 0.05$). Considering the ratio of proinflammatory cytokine to IL-10, the iDNA showed a stronger proinflammatory effect than the eDNA and LPS, which was reflected by the significantly higher TNF-a/IL-10 and IL-6/IL-10 levels ($P < 0.01$).

DISCUSSION

In the present study, we extracted eDNA from the mouse small intestine and colon and established a suitable PCR-TRFLP protocol to distinguish the microorganism diversity, which consisted of eDNA and iDNA. Analysis of the Illumina MiSeq sequencing data demonstrated the significantly different constitutions and functions between the eDNA and iDNA. Our results provided a sound basis for research into the structure and function of eDNA from the aspect of microorganism diversity.

AB-PAS and TOTO-1 staining revealed that eDNA was enriched in the mucus layer of the colon and small intestine. Some of the eDNA would be derived from

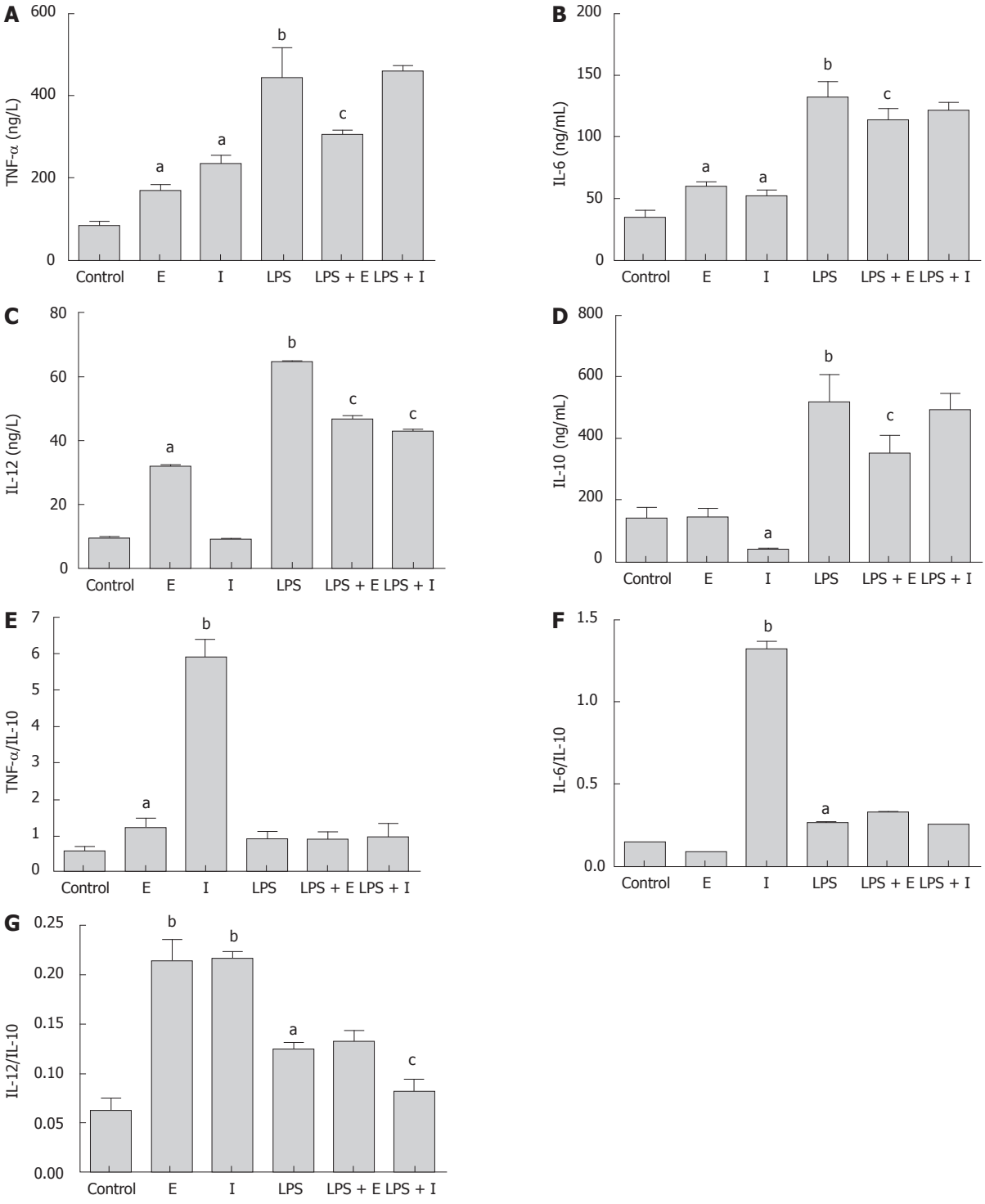

Figure 4 Effect of iDNA and eDNA on cytokine production by Raw267.4 cells, including TNF-α (A), IL6 (B), IL-12 (C), IL-10 (D), and ratio of TNF-α (E), IL6 (F) and IL-12 (G) to IL-10. Cells were treated for 12 h with medium, lipopolysaccharide (LPS) (1 μg/mL), eDNA (1 ng/mL), iDNA (1 ng/mL), eDNA(1 ng/mL) + LPS (1 μg/mL), or iDNA (1 ng/mL) + LPS (1 μg/mL) for 12 h. Values are expressed as mean ± SEM (n = 6). [a]P < 0.05, Significant differences between control and treatment; [b]P < 0.01, significant differences between control and treatment; [c]P < 0.05, significant differences between LPS treatment and treatment. E: Extracellular bacterial DNA; I: Intracellular bacterial DNA; LPS: Lipopolysaccharide.

shed epithelial cells, and approximately 1400 cells are shed from each villus every 24 h[14]. Our study confirmed that some mucus DNA originated from intestinal bacteria. In contrast to pig small intestinal crypts[15], eDNA was observed frequently at the bottom of the crypts of Lieberkühn in mice. This might be the result of the accumulation of eDNA migrating from the mucus layer, which cannot prevent the diffusion of linear DNA[16]. The eDNA might also be released by bacteria killed by antimicrobial peptides secreted by Paneth cells residing at the base of the small intestinal crypts[17]. The sentinel role of Paneth cells requires the interaction between TLR9 and DNA containing CpG sequences[4,18]; therefore, crypt eDNA might play a

significant role in maintaining small intestinal immune homeostasis.

We used DTT in eDNA extraction. It is a strong reducing agent that can break protein disulfide bonds to aid its dissolution. Using DTT allowed the efficient extraction of eDNA, demonstrating disruption of the interaction between eDNA and mucin. This is in agreement with the finding of Macierzanka *et al* that both large and small DNA particulates appeared to form a network or were held in place by the mucin network[15].

In consideration of lower integrity of eDNA compared with iDNA, primer selection was carried out before T-RFLP analysis, to recover a high percentage of bacterial species. Primers 27F/1492R and 530F/1492R, which have been used widely in previous studies, were found to be unsuitable for the amplification of 16S rRNA fragments from mucus eDNA (data not shown). The primers 334F/939R (V3-V5) produced a relatively small product that was highly specific for the spectrum of bacterial species in mucus eDNA, suggesting that eDNA was degraded genomic DNA. This is in agreement with the finding in pigs that degraded nuclei embedded in mucus was often fragmented[15]. This was confirmed by Illumina MiSeq sequencing analysis, where primers 515F/907R were more successful in PCR compared with primers 338F/806R.

PCA of T-RFLP and Illumina MiSeq sequencing data revealed significant differences between iDNA and eDNA, suggesting that the eDNA release characteristics varied among strains in the small intestinal microbiota. The eDNA was mainly released by Gram-negative bacteria of the *Bacteroidales S24-7* and *Stenotrophomonas* genera. Gram negative-bacteria contain eDNA specifically associated with the outer membrane vesicle (OMV)[19,20]. The presence of OMV-associated DNA within Gram-negative bacteria biofilms has also been confirmed[21]. In the present study, the result of PICRUST analysis of eDNA and iDNA sequences showed that the functions of vesicular transport and cell membrane biogenesis were related to the bacterial source of the eDNA. This result might suggest the presence of OMV-associated DNA in the eDNA. In particular, the genus *Stenotrophomonas* released eDNA into the mucus, as demonstrated by a significant difference in their proportions in the eDNA and iDNA. Members of this genus are strict aerobic bacteria that belong to the γ-β subclass of *Proteobacteria*[22]. *Stenotrophomonas* is reported as a dominant member of the plant-associated bacterial community[23]. It has also been reported in the colonic mucosa-associated microbiota of healthy humans[24] and the mouse colonic crypt[25]. Notable features of this genus are their weak invasiveness, variety of colonization mechanisms, and strong ability to form biofilms, making them a successful colonizer of various hosts[26]. Further studies should be carried out to clarify the mechanism involved in the release of *Stenotrophomonas* eDNA into the mucus layer and the physiological significance. Our findings are also in agreement with those of Ou *et al*[27], who reported a predominance of *Streptococci* in the small intestinal mucus. Itzek *et al*[28] reported that certain oral *Streptococci* produce H_2O_2, which causes the release of eDNA to promote biofilm formation. However, in the present study, this genus released trace eDNA, indicating that habitat is a key factor in their eDNA releasing property. *Streptococci* have been reported to attach to *Bacteroides*-produced OMV[29], which might help their incorporation into the mucus biofilm without actively releasing eDNA.

Bacterial DNA stimulates not only potent pro-inflammatory activities, but also the interferon regulatory factor pathway that induces anti-inflammatory activities[30]. In the present study, iDNA and eDNA showed distinct cytokine stimulation patterns. The eDNA showed a lower pro-inflammatory effect, according to the low TNF-α/IL-10 and IL-6/IL-10 levels, and induced very low levels of IL-12. TLR-9 activation by bacterial DNA is dependent on the individual CpG content and intracellular delivery rate[31]. Thus, it is necessary to further study the difference in the CpG contents of eDNA and iDNA.

In conclusion, our results indicated that eDNA is located in the intestinal mucus layer and at the bottom of the crypt lumen in the small intestine. DTT promoted the release of bacterial eDNA from the small intestinal mucus layer. The eDNA was degraded bacterial genomic DNA mainly released by Gram-negative bacteria, especially by the *Bacteroidales-S24-7* and *Stenotrophomonas* genera. The eDNA showed decreased pro-inflammatory activity compared with total gut flora genomic DNA. Further studies are needed to clarify the actual source of eDNA, and its relationship with the gut immune response, especially the production of AMPs in Paneth cells of the small intestinal crypt.

ARTICLE HIGHLIGHTS

Research background

Many studies strongly suggest that signals, including bacterial DNA, from colonizing microbes greatly alter host local immune system in the gut. Bacterial cells do not contact with enterocytes in normal physiological status. They might release DNA into the mucus layer to influence host innate immune cell through specific receptors, like Toll-like receptor 9. Evidence supporting this hypothesis is needed.

Research motivation

This research investigated the existence of extracellular bacterial DNA (eDNA) in the mouse gut mucus layer, their resource, and immune modulatory function. There were differences in DNA's immuno-stimulatory properties among different bacteria as reported by other researchers. Therefore, host immune response would be modulated by targeted change of DNA releasing bacteria in the mucus through specific medicine or food components.

Research objectives

This study aimed to confirm the existence of bacterial eDNA in the mouse gut mucus layer, and to identify bacterial genera that release them. Immuno-stimulatory properties of eDNA were also studied *in vitro*. This provided basic knowledge about bacteria and host interaction through bacterial DNA and related signal pathways. This will also promote nutritional strategy development to modulate local immune response through changing DNA releasing microbiota.

Research methods

Bacterial eDNA in the mucus layer and crypts was visualized by TOTO-1 staining. Small intestinal mucosal microbiota and eDNA were analyzed using T-RFLP and Illumina MiSeq amplicon sequencing. Immuno-stimulatory effects of microbiota and eDNA were determined after incubation with mouse RAW264.7 macrophages.

Research results

TOTO-1 iodide staining confirmed existence of eDNA in the mucus layer. The composition of the eDNA was significantly different from that of the intracellular DNA (iDNA). The eDNA sequences came mainly from Gram-negative bacteria of *Bacteroidales* S24-7. The eDNA induced significantly lower TNF-α/IL-10 and IL-6/IL-10 ratios in LPS stimulated RAW264.7 cells than iDNA. This is the first report related to bacteria genus responsible for DNA release in the gut mucus layer.

Research conclusions

Our results indicated that eDNA was located in the intestinal mucus layer. The eDNA was degraded bacterial genomic DNA mainly released by Gram-negative bacteria especially *Bacteroidales* S24-7. They showed decreased pro-inflammatory activity compared with total gut flora genomic DNA.

Research perspectives

Further studies are needed to clarify the specific bacterial species/strains that release eDNA, and its relationship with the gut immune response, especially the production of antimicrobial peptides in Paneth cells of the small intestinal crypt.

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ORIGINAL ARTICLE

Case Control Study

Transmitted cardiovascular pulsations on high resolution esophageal impedance manometry, and their significance in dysphagia

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Author contributions: Chaudhry NA was responsible for study planning and design, collecting and compiling, and also did preliminary statistical analysis. She also complied the manuscript, tables and images; Zahid K assisted in data collection, and manuscript compilation; Keihanian S provided feedback during manuscript compilation and also was part of the final approval of the manuscript; Dai Y was responsible for the final data analysis and review; Zhang Q was responsible for study planning and design, review of analytical process, feedback during manuscript compilation, and approval of the final manuscript.

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Informed consent statement: The informed consent was waived by the IRB due to the retrospective nature of the study.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: Statistical code and anonymized data set available from the corresponding author at naueen.chaudhry@medicine.ufl.edu. The stipulation for informed consent was waived by IRB due to the retrospective design of the study. No other data besides the one included in the manuscript are available.

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Abstract

AIM

To investigate the behavior of pulsatile pressure zones (PPZ's) as noted on high resolution esophageal impedance manometry (HREIM), and determine their association with dysphagia.

METHODS

Retrospective, single center case control design scr-

eening HREIM studies for cases (dysphagia) and controls (no dysphagia). Thoracic radiology studies were reviewed further in cases for (thoracic cardiovascular) thoracic cardiovascular (TCV) structures in esophageal proximity to compare with HREIM findings. Manometric data was collected for number, location, axial length, PPZ pressure and esophageal clearance function (impedance).

RESULTS

Among 317 screened patients, 56% cases and 64% controls had PPZ's. Fifty cases had an available thoracic radiology comparison. The distribution of PPZ's in these 50 cases and 59 controls was similar (average 1.4 PPZ/patient). Controls (mean 31.2 ± SD 12 years) were a significantly younger population than cases (mean 67.3 ± SD 14.9 years) with $P < 0.0001$. The upright posture PPZ pressure was higher in controls (15.7 ± 10.0 mmHg) than cases (10.8 ± 9.7 mmHg). Although statistically significant ($P = 0.005$), it was a weak predictor using logistic regression and ROC model (AUC = 0.65). Three dysphagia patients had partial compression from external TCV on radiology (1 aberrant subclavian artery, 2 dilated left atrium). The posture (supine vs upright) with more prominent PPZ's impaired bolus clearance in 9 additional cases by > 20%.

CONCLUSION

Transmitted TCV pulsations observed in HREIM bear no significant impact on swallowing. However, in older adults with dysphagia, evidence of impaired bolus clearance on impedance should be evaluated for external TCV compression. These associations have never been explored previously in literature, and are novel.

Key words: High resolution esophageal manometry; Dysphagia; Dysphagia lusoria; Dysphagia cardia; Esophageal motility; Thoracic cardiovascular structures; Esophageal disorders

Core tip: Transmitted pulsations from thoracic cardiovascular structures are frequently observed on HREIM, and usually bear no significant impact on swallowing. However, in older adults with dysphagia, evidence of impaired bolus clearance on impedance should be further reviewed using clinical data and functional esophageal swallow studies in order to assess the possibility of external compression from cardiovascular structures.

Chaudhry NA, Zahid K, Keihanian S, Dai Y, Zhang Q. Transmitted cardiovascular pulsations on high resolution esophageal impedance manometry, and their significance in dysphagia. *World J Gastroenterol* 2017; 23(44): 7840-7848 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7840.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7840

INTRODUCTION

The esophagus has a close correlation with several thoracic cardiovascular (TCV) landmarks along its course. Prominent arterial structures include ascending aorta, arch of the aorta, left atrium and descending aorta. Cases of TCV compression on the esophagus have been classically associated with congenital anomalies, *e.g.,* dysphagia lusoria (aberrant subclavian artery causing esophageal compression)[1]. It has also been proposed that the esophagus can be potentially compressed between the left atrium and descending aorta[2] if it takes a left course on transition from thorax to abdomen. There are case reports of previously asymptomatic patients diagnosed with external cardiovascular compression on esophagus when they presented with pill induced esophagitis[3] or pill related dysphagia[4]. Initial clinical suspicion of TCV compression on esophagus is never paramount in dysphagia. However in older patients with an established history of atherosclerosis, cardiomegaly, or left atrial dilatation[5], there can be a suspicion due to secondary causes[6], *e.g.,* atrial fibrillation, mitral valve regurgitation or stenosis. There are reports of Ortner's syndrome (Cardiovocal syndrome) which is hoarseness caused by cardiopulmonary disease compressing the left recurrent laryngeal nerve[7]. This interplay of the physiologic dynamics resulting from the anatomical proximity of these structures needs to be studied further. High Resolution Esophageal Impedance Manometry (HREIM) can provide an interesting insight on the significance and diagnosis of these cases. Effect of TCV pulsations, or pulsatile pressure zones (PPZ's), on the esophagus can be noted on HREIM[8]. They present as a steady pulsation that remains unaffected with the stage of swallowing, and have been reported in previous literature as a high pressure barrier[9]. Their characteristics and role in patients with or without dysphagia remain uninvestigated. Our aim with this study is to investigate the characteristics and significance of transmitted TCV pulsations on the esophagus as noted on HREIM, especially in terms of dysphagia.

MATERIALS AND METHODS

This is a retrospective single center case control study using HREIM studies of 317 patients conducted at a tertiary care center with institutional IRB approval.

Selection of cases

Studies of 250 patients undergoing HREIM for dysph-

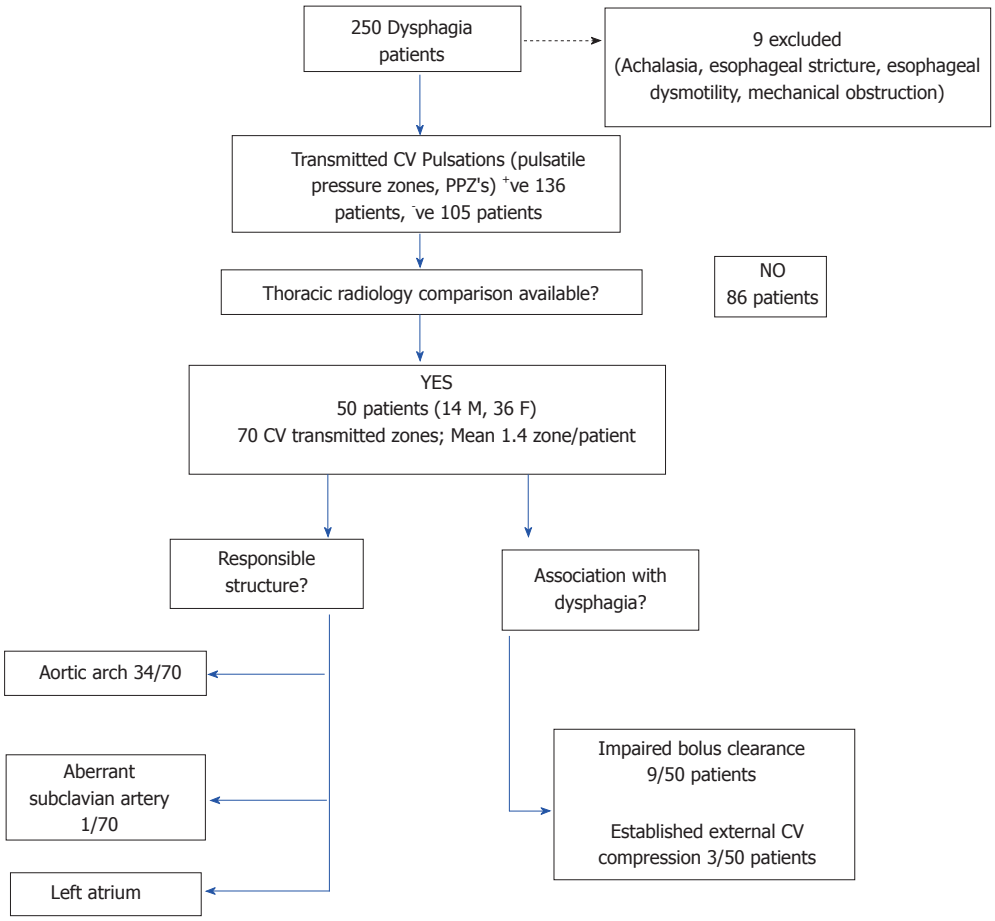

Figure 1 Selection of cases (dysphagia patients) with transmitted cardiovascular pulsations as noted on high resolution esophageal impedance manometry. CV: Cardiovascular.

agia from July 2012 to January 2014 were screened. Nine patients were eliminated on basis of achalasia, stricture, poor esophageal motility or mechanical obstruction. Cases were selected based on findings of PPZ's noted on HREIM, which were independent from the swallows, and hence representative of transmitted TCV pulsations on the esophagus. In these patients further electronic medical record was searched for available thoracic imaging, showing TCV structures likely responsible due to their anatomical proximity to esophagus. Fifty dysphagia patients with available radiology (CT/MR chest, esophagogram) were identified (Figure 1) and their data was used for statistical analysis.

Selection of controls

HREIM studies of 67 patients without dysphagia (per chart review) were screened as controls. Eight were eliminated due to presence of artifacts on study and catheter malfunction. The remaining 59 were reviewed for presence or absence of PPZ. In subjects with PPZ' s data were collected regarding number and location of PPZ, axial length, PPZ pressure in upright posture, postural effect and impedance. Comparative thoracic radiology was not available for the controls hence only their HREIM data was included for analysis.

Procedure and analysis

As per the institutional HREIM protocol (modified from Chicago protocol[10]), patients received 10 swallows each in the supine and then in the upright position using 5 mL Gatorade™. Some patients had also received additional 10 mL swallows but these were excluded from the results analysis. A 4.2 mm outer diameter solid-state combined manometry and impedance recording catheter assembly incorporating 36 circumferential pressure sensors spaced at 1-cm intervals and 18 impedance segments spaced at 2-cm intervals were used (ManoScan™ Eso, Given Imaging Yogneam, Israel). The catheter was calibrated prior to each procedure and was inserted transnasally and positioned to record from the hypopharynx to the stomach with a goal of three intragastric sensor recording loci. Data were analyzed using ManoView ESO 3.0 analysis software (Given Imaging). Failed peristalsis was defined as no peristaltic contraction following a swallow. PPZ was included if pressure across it was > 5 mmHg. Pressure was determined

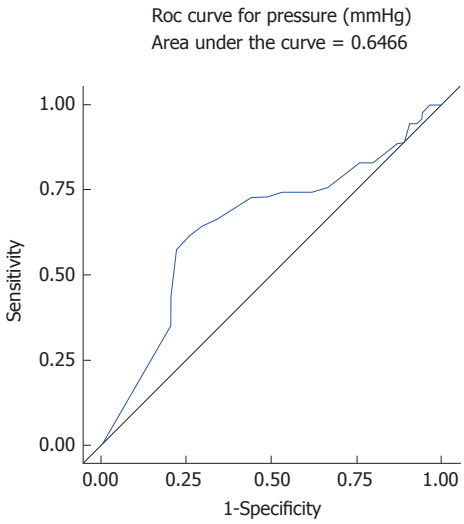

Figure 2 The receiver operator curve for pressure across pulsatile pressure zone with dysphagia shows area under the curve 0.65 demonstrating weak significance. Hence even though pressure was a statistically significant variable on Wilcoxon Rank Sum analysis, it is a poor predictor of dysphagia caused by transmitted cardiovascular pulsations on esophagus by this model.

using the isobar contour and measured in mmHg.

Data were collected from HREIM studies of selected patients regarding number of PPZ's present, axial length, PPZ pressures in upright posture, postural effect, location along esophagus (upper, middle or lower third) and impedance. The esophagus was divided into upper, middle and lower thirds. For our study, we only recorded pressures in the upright posture since prior research has demonstrated decreased integrated relaxation pressure, distal contractile integers, and elimination of vascular artifacts in a sitting posture[11]. Since we were interested in measuring the impact of transmitted pulsations on dysphagia, we considered the physiological posture (upright) of swallowing more appropriate for pressure measurement. Even though some PPZ's were observed with the patient both in upright and supine posture, there were instances when it appeared more prominent in one posture as compared to the other. This was considered as a *dominant posture* for the PPZ. For PPZ's present only in one posture, this was documented as its dominant posture. Available radiology images were reviewed by the investigators for correlating TCV structures, and where possible it was also assessed if the patients had a noticeable left turn of esophagus on transition from thorax to abdomen.

The data was tabulated in descriptive format and statistical analysis was done using SAS 9.4 (Copyright © 2013, SAS Institute Inc., Cary, NC, United States). Characteristics of age, sex, anatomical location of PPZ (upper, middle or lower esophagus), PPZ pressure and axial length were analyzed using Wilcoxon rank sum analysis and logistic regression was performed for marginally significant variables. A *P*-value of < 0.05 was considered statistically significant for the purposes of our study. We performed logistic regression analysis using the disease status (dysphagia vs. no dysphagia) as the response variable and gender, location, axial length, pressure and posture as explanatory variables. A backward selection procedure at the 0.2 significance level identified that pressure was the only explanatory variable significantly associated with dysphagia. We decided to assess the strength of the association by using pressure as a predictor for dysphagia.

RESULTS

In 241 patients with dysphagia (cases), 136 (56.4%) had PPZ's in either supine or upright posture on HREIM, in comparison to 38 (64.4%) in non-dysphagia group (controls). Since some patients had more than one independently behaving pulsatile zone, 70 PPZ's were noted in these 50 patients (Average 1.4 PPZ per patient). This was similar in prevalence to the 38 controls with PPZ's, 54 PPZ's were observed with an average 1.4 per patient. Demographic and clinical characteristics are presented in Table 1. The prevalence of PPZ's, was similar in both the cases and controls. The cases were markedly older (67.3 ± 14.9, median 70 years) than controls (31.2 ± 12.2, median 27 years) with $P < 0.0001$.

Average pressure of PPZ's (upright postures) in cases was 10.8 ± 9.7 mmHg, which was significantly lower than controls 15.7 ± 10.0 mmHg ($P < 0.01$). However, since rising external pressure does not correlate with resolution of dysphagia physiologically, we evaluated its strength as a predictor (see methods). The prediction model had an associated receiver operating characteristic (ROC) curve (Figure 2) with the area under the ROC curve (AUC) of 0.65 (weak significance). Hence, even though low PPZ pressure was significantly associated with dysphagia, the quality of predicting dysphagia by pressure was low; AUC, sensitivity and specificity were quite low.

Some patients had impairment in bolus clearance with regards to dominant posture, but there was no clear association between PPZ pressure and Impedance. (Impairment was considered to be > 20% change in bolus clearance on Impedance.) Impaired bolus clearance was observed in 12/50 (24%) dysphagia patients and was associated with the PPZ becoming more prominent. Bolus clearance in these patients decreased by > 20% if PPZ was dominant regardless of upright or supine posture, hence showing no relation to posture itself. Only 3 of these 12 patients had clear evidence of partial compression from external TCV structures on radiology (1 from aberrant subclavian artery with dysphagia lusoria, 2 from dilated left atrium). In the patient with dysphagia lusoria, bolus clearance was 10% in supine swallows

Table 1 Demographic and morphologic characteristics of transmitted cardiovascular pulsations, or pulsatile pressure zones, on High Resolution Esophageal Impedance Manometry *n* (%)

Variable		Dysphagia (*n* = 50)	No dysphagia (*n* = 38)	*P* value
Age, yr	mean ± SD	67.3 ± 14.9	31.2 ± 12.2	< 0.0001
	Median (min, max)	70 (24, 89)	27 (19, 61)	
Gender	Male	14 (28)	18 (47)	0.097
	Female	36 (72)	20 (53)	
Number of pulsatile zones	Total (% prevalence overall)	70 (56.4)	54 (64.4)	-
	Average per patient	1.4	1.4	-
Location of pulsatile zone in esophagus	Upper third	12 (17)	7 (13)	0.769
	Middle third	24 (34)	21 (39)	
	Lower third	34 (49)	26 (48)	
Axial length of pulsatile zone (cm)	mean ± SD	2.2 ± 1.4	2.4 ± 1.2	0.258
	Median (min, max)	2 (0.5, 6)	2 (0.5, 5)	
	Avg upper third (min, max)	1 (0.5-2)	1 (0.5-2)	
	Avg middle third (min, max)	0.7 (0.5-2.5)	2.2 (0.5-4)	
	Avg lower third (min, max)	3.3 (0.5-6)	2.3 (1.5-5)	
PPZ	mean ± SD	10.8 ± 9.7	15.7 ± 10.0	< 0.01
Pressure (mmHg)	Median (min, max)	10 (0, 35)	17 (0, 45)	
	Avg upper third	9.8	16.7	
	Avg middle third	5.9	13.3	
	Avg lower third	14.3	16.9	
Dominant posture for pulsatile zone	Supine	21 (30)	10 (18.5)	0.282
	Upright	10 (14.3)	6 (11.1)	
	Both postures	39 (55.7)	38 (70.4)	

This table shows a comparison between patients with dysphagia (cases) and without dysphagia (controls).

and 90% upright swallows (Figure 3). In one of the patients with enlarged left atrium, bolus clearance was 80% in supine swallows and 10 % upright (Figure 4). No impedance data was available for the third patient. PPZ pressure (upright) in these 3 patients ranged from 25 to 35 mmHg. There was no radiological evidence of obstruction caused by TCV structures in the remaining 47/50 patients. Figure 5 shows PPZ's observed in non-dysphagia patients.

The behavior of PPZ's was observed with regards to change in posture. In cases, majority (39/70, 56%) of the PPZ's were observed in both upright and supine postures, 10 in upright, and 21 in supine posture only. Similarly in controls, the majority (38/54, 70%) of them were noted in both upright and supine postures. Hence, this presentation was more common in controls. The average axial length of the PPZ in cases was 2.2 ± 1.4 cm, which was comparable to controls 2.4 ± 1.2 cm.

The aortic arch was related to 34 PPZ's, 11 of which were in upper and 23 in the middle third. One remaining PPZ in the upper third was due to an aberrant subclavian artery (dysphagia lusoria). The left atrium was related to 35 PPZ's including all 34 PPZ's in the lower third and one in the middle third. Hence the characteristics described for the lower PPZ's in Table 1 can be attributed to the left atrium.

Among the 50 patients with dysphagia, 20 had dysphagia to solids, 4 to both solids and liquids whereas 26 were unspecified per chart review. Esophageal orientation on lower thoracic cavity could be ascertained in 27 patients, 24 of them had a left turn of esophagus causing it to be situated between the descending thoracic aorta and left atrium. This finding was present in all patients with PPZ's causing bolus impairment on impedance in lower third of esophagus.

DISCUSSION

Per our results, transmitted cardiovascular pulsations were seen with a comparable frequency in patients with and without dysphagia. However the older age of the dysphagia population indicates that advancing age and/or development of atherosclerosis and valvular heart disease could be a risk factor. The most common causes for a pulsatile pressure zone were the left atrium and the aortic arch likely because they are most closely related CV structures to the esophagus during its thoracic course. Prior studies have reported dysphagia aortica seen in elderly patients especially women with a history of hypertension and kyphosis[12,13]. Even though a majority of the PPZ's were present in both upright and supine posture, it was apparent that most of these had a more prominent appearance in one posture than the other. We called this the "dominant posture" for the PPZ, and noted that this had some relation with impaired bolus clearance in the dysphagia group. Pressure, likely by itself is not a key determinant of bolus impairment unless there are other factors at play. We opted to measure pressure only in the upright posture (rather

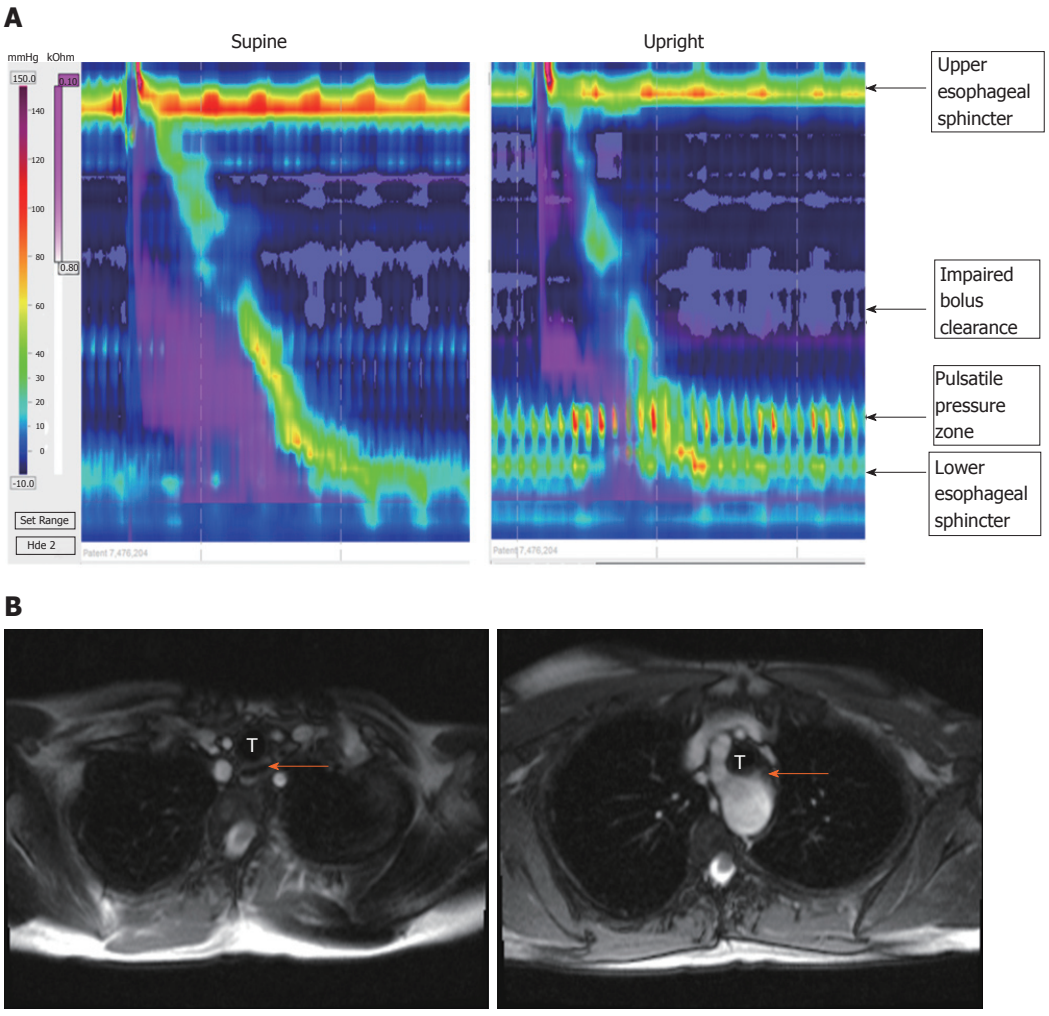

Figure 3 In the patient with dysphagia lusoria, bolus clearance was 10% in supine swallows and 90% upright swallows. A: Pulsatile pressure zones (PPZ) on HREIM due to transmitted pulsations of aberrant subclavian artery and aortic arch in a patient with dysphagia lusoria, with impaired bolus clearance; B: Magnetic Resonance Angiogram of patient with dysphagia lusoria; *LEFT* patent esophagus (arrow) *RIGHT* esophageal compression secondary to aberrant subclavian artery (dysphagia lusoria); T: Trachea.

than the dominant posture) since it is the physiological posture of swallowing. Hence, pressure from a vascular source of dysphagia would be more relevant if the PPZ was observed in the upright posture. Similar PPZ pressures in non-dysphagia patients had no impact on impedance, whereas they did in some dysphagia patients. More research needs to be done regarding dysphagia in the elderly in correlation with the history of atherosclerosis and cardiomegaly.

HREIM has evolved into quite an important field over the past couple of decades, and has established itself in the research and clinical arena. It provides us with a wealth of information in the realm of esophageal functional disorders and their diagnostics[14]. Transmitted cardiovascular pulsations are commonly encountered by the observing gastroenterologist, however there is still a lack of knowledge regarding their impact in patients with dysphagia. Our results demonstrate that even though there were a few cases of dysphagia secondary to TCV structures, these can only be diagnosed with confidence with the combined evidence of impedance and fluoroscopic studies such as barium swallow[15]. HREIM also does not fully account for patients with dysphagia to solids, which was the case in most of our patients when type of dysphagia was specified.

In elderly patients with dysphagia, the differential diagnosis usually focuses on swallowing difficulties secondary to advancing age or dementia, or intra-esophageal pathology, *e.g.,* strictures, rings. External compression by cardiovascular landmarks can be another possible, yet uncommon, cause of dysphagia in this population. It can cause considerable frustration for both the physician and the patient when an extensive work up is unsuccessful in yielding answers as to the cause of the symptoms. As per literature review an esophagus lying to the left of the vertebral column in the lower thorax has greater chances of being compressed between the left atrium and descending thoracic aorta[2] (two dominant vascular structures). We also identified this trend in our data, however it's difficult to establish a clear correlation

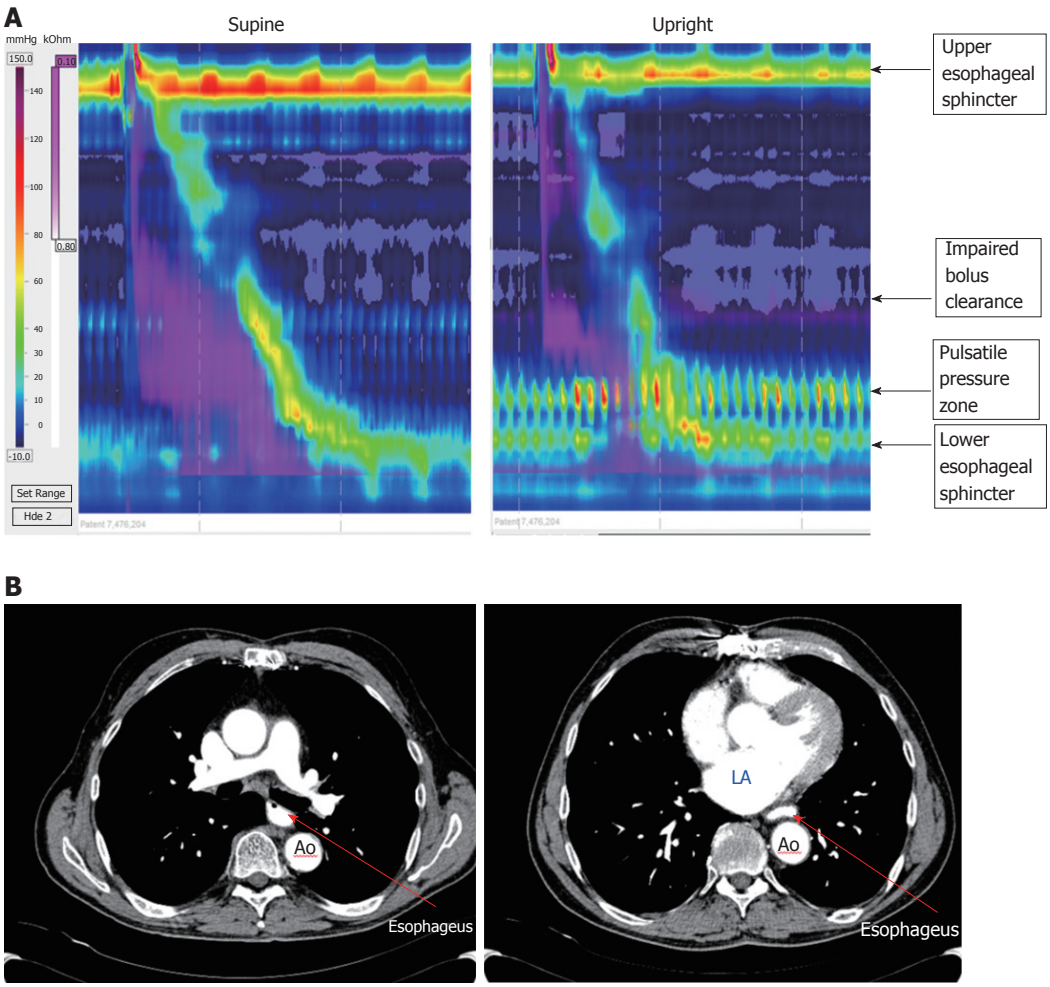

Figure 4 In one of the patients with enlarged left atrium, bolus clearance was 80% in supine swallows and 10 % upright. A: Pulsatile pressure zone with impaired bolus clearance easily visible in a dysphagia patient in upright posture due to left atrial enlargement; B: Corresponding CT Scan Images of the same patient (Left) Esophagus patent in upper thorax (Right) Esophagus compressed between dilated left atrium (LA) and descending aorta (Ao).

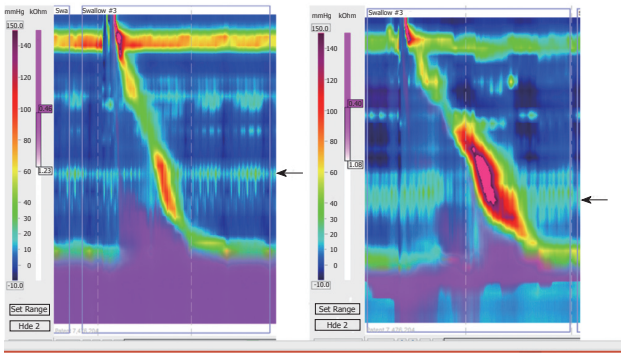

Figure 5 Pulsatile pressure zones observed in patients without dysphagia (see arrows).

given that this is the pre-dominant anatomical location for the esophagus, and no statistical evidence was determined to the effect.

It is interesting though, how a PPZ caused by the same cardiovascular structure, behaves differently in presentation across the subjects. It has previously been proposed that some patients have an underlying pre-disposition for dysphagia due to CVS causes which becomes manifest with super-imposed factors (pill-induced esophagitis[3], gastritis, atherosclerosis[16], aortic aneurysm[17,18]). No clear association was observed between any specific CV structure with properties of its esophageal transmitted pulsations, *e.g.,* appearance in upright vs supine posture, axial length, PPZ pressure, or presence or absence of impaired bolus clearance. The only obvious anatomical association was the presence of Aortic arch in the middle third of the esophagus on HREIM and the left atrium in the lower third.

When there is pre-existing history of mitral valve stenosis/regurgitation, atrial fibrillation or cardio-megaly[4,6] in a dysphagia patient, a dilated left atrium compressing the esophagus can be the culprit. Cardiac dysphagia was the cause in two of our 3 pts in which we could determine clear association between dysphagia and pressure caused by a cardiovascular structure. The third patient was of dysphagia lusoria due to an aberrant subclavian artery. Treatment of cardiovascular associated dysphagia is initially always

dietary modification[13], however some cases have described endovascular stenting[16] as well as surgical options[2] in advanced cases.

The limitations of our study include a smaller control group as compared to cases, which could not be matched on basis of age. Since our study was retrospective and most HREIM's are performed on patients with dysphagia, we were unable to compensate for this size difference and our control group was limited. Thoracic radiology was also not available for the control group. We were also unable to incorporate the pulse rate and blood pressure of the subjects in the analysis due to the retrospective design. The dysphagia status of the patient was also determined from chart review rather than a standardized patient reported measure. Future research can focus on older populations with underlying cardiovascular disease in the presence of noticeable transmitted CV pulsations on the esophagus and their association with dysphagia.

In conclusion transmitted pulsations from TCV structures are frequently observed on HREIM usually having no significant impact on swallowing. However, evidence of impaired bolus clearance on impedance can be correlated with clinical data and functional esophageal swallow studies in older adults with dysphagia to assess for external compression from cardiovascular structures.

COMMENTS

Background

Transmitted pulsations of thoracic cardiovascular (TCV) structures are often visible on high resolution esophageal impedance manometry (HREIM) as pulsatile pressure zones (PPZ's). However, their significance has never been established especially in light of dysphagia. This study investigated the characteristics of these common observations, to determine their impact on swallowing and dysphagia.

Research frontiers

The field of HREIM has shown rapid development in the past decade and its diagnostic importance, especially for swallowing disorders, is without question. There is a close anatomical relationship between thoracic cardiovascular structures and the esophagus, and the advent of HREIM enables us to study their physiological relationship in better detail.

Innovations and breakthroughs

This study investigates a commonly observed, but poorly understood, phenomenon on HREIM and seeks better comprehension of its relevance. This study is one such step in this direction, and seeks to answer questions about transmitted cardiovascular pulsations and dysphagia. This findings point towards an age-related association with dysphagia which is novel.

Applications

This study was retrospective in nature, and was carried out with liquid swallows. Future studies, with more control data and a prospective design, can be designed to study the impact of transmitted pulsations on a solid bolus, barium pill, or the impact of posture.

Terminology

TCV structures: describes the heart and the major vessels. In our study, reference is mostly to arterial structures due to the transmission of pulses across to the esophagus. HREIM and PPZ's are the representative areas of transmitted arterial pulsations to the esophagus on HREIM.

Peer-review

This is a well written article. It is an interesting topic. High resolution manometry is still evolving and there are various features in it that are not very clear.

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ORIGINAL ARTICLE

Case Control Study

Intestinal parameters of oxidative imbalance in celiac adults with extraintestinal manifestations

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Abstract

AIM

To evaluate selected intestinal parameters of oxidative stress, and antioxidant capacity in adult celiac disease patients with extraintestinal manifestations.

METHODS

The study involved 85 adult patients divided into the following subgroups: (1) patients with newly diagnosed celiac disease (CD) (n = 7); (2) celiac patients not adhering to a gluten-free diet (GFD) (n = 22); (3) patients with CD on the GFD (n = 31); and (4) patients with functional disorders of the gastrointestinal tract, serving as controls (n = 25). Celiac patients presented with non-classic symptoms or extraintestinal manifestations. Standard blood tests including serum antioxidant levels (uric acid, bilirubin, and vitamin D), celiac antibody levels, and histopathological status of duodenal biopsy specimens have been determined. The expression of mRNA for tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 10 (IL-10), superoxide dismutase (SOD), heat-shock protein 70 (HSP-70), hypoxia-inducible factor 1 (HIF-1α), and BAX in the duodenal mucosa of patients was analyzed by reverse transcriptase-polymerase chain reaction.

RESULTS

The mean plasma uric acid level in patients with active CD (newly diagnosed and nonadherent patients) and treated celiac patients was significantly higher than in controls (260.17 ± 53.65 *vs* 190.8 ± 22.98, P < 0.001, and 261.7 ± 51.79 *vs* 190.8 ± 22.98, P < 0.001, respectively). The mean bilirubin concentration in active and treated celiac patients was significantly lower than in controls (8.23 ± 5.04 *vs* 10.48 ± 4.08, P < 0.05 and 8.06 ± 3.31 *vs* 10.48 ± 4.08, P < 0.05, respectively). The mean plasma vitamin D level was significantly lower in active celiac patients than in treated celiac patients and controls (19.37 ± 9.03 *vs* 25.15 ± 11.2, P < 0.05 and 19.37 ± 9.03 *vs* 29.67 ± 5.12, P < 0.001, respectively). The expression of TNF-α, IL-10, and HSP-70 mRNAs was significantly elevated in the celiac groups regardless of the diet when compared with controls. Patients on the GFD presented a significantly lower mRNA expression of TNF-α and IL-10 than in newly diagnosed and nonadherent patients (P < 0.05). The expression of SOD mRNA was significantly elevated in celiac patients compared with controls (P < 0.05), with a significant difference between treated and untreated patients (P < 0.05). The expression of HIF-1α mRNA and BAX mRNA was significantly higher in patients with active CD compared with controls and patients on GFD, while no difference was observed between the latter two groups.

CONCLUSION

Increased intestinal expression of HSP-70 despite GFD indicates that GFD only partially reduced oxidative stress. CD patients exhibited an oxidative imbalance and inflammatory response despite GFD. Uric acid may act as an important antioxidant in CD.

Key words: Celiac disease; Oxidative stress; Superoxide dismutase; Heat-shock protein 70; Apoptosis; Hypoxia-inducible factor; Uric acid; Vitamin D; Tumor necrosis factor alpha

Core tip: Oxidative stress has been implicated in gliadin toxicity. Additional measures aimed at reducing oxidative imbalance may prove to be effective supplementary therapy. We demonstrated increased duodenal expression of hypoxia-inducible factor 1 (HIF-1α), heat-shock protein 70 (HSP-70), and superoxide dismutase in adult celiac patients with extraintestinal manifestations as a defensive reaction to oxidative stress. Hence, HSP-70 and HIF-1α might be potential novel biomarkers of celiac disease (CD). Increased HSP-70 expression, both in treated and untreated celiac patients, suggests that oxidative stress as well as histopathological alterations in duodenal mucosa persist despite gluten-free diet. Our data confirm the increased serum levels of uric acid in patients with CD compared with controls as a result of oxidative stress.

Piatek-Guziewicz A, Ptak-Belowska A, Przybylska-Felus M, Pasko P, Zagrodzki P, Brzozowski T, Mach T, Zwolinska-Wcislo M. Intestinal parameters of oxidative imbalance in celiac adults with extraintestinal manifestations. *World J Gastroenterol* 2017; 23(44): 7849-7862 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7849.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7849

INTRODUCTION

Celiac disease (CD) is an inflammatory disorder of the small intestine, which is caused by the gluten fraction of wheat or the homologous proteins from barley and rye in genetically predisposed individuals[1]. Histologically, these lesions include intraepithelial lymphocytosis, crypt hypertrophy, and villous atrophy, resulting in an inadequate absorption of micronutrients and macronutrients from the intestinal tract. The clinical presentation of CD is heterogeneous and varies with the age of patients, duration and intensity of the disease, and possible presence of extraintestinal disorders[1]. In adults, a variety of clinical manifestations have been described, including the non-classic or asymptomatic form of CD.

The pathogenesis of CD is complex and not fully understood. Besides genetic predisposition, the immunologic mechanism has been proposed because both the innate and adaptive immune responses contribute to the mucosal inflammation in patients with CD[2]. The disruption of the intestinal epithelial barrier makes it more permeable to gluten peptides, thus exacerbating the inflammatory process if gluten peptides are present in the intestinal lumen.

Recent studies have indicated a direct cytotoxic effect of gluten on enterocytes[3]. Moreover, it has been proposed that oxidative stress is one of the

mechanisms responsible for gliadin toxicity[4]. Recent data have also suggested the importance of hypoxia-inducible factor 1 (HIF-1) in maintaining the functions of the intestinal epithelial barrier[5]. Although activation of HIF-1 is mainly regulated by hypoxia[6], it is now established that HIF-1 signaling can also be triggered under inflammatory conditions[7-10]. Vannay *et al*[11] have shown the increased mucosal expression of HIF-1α in children with untreated CD, suggesting the involvement of this signaling factor in the pathomechanism of the disease. The regulation of HIF-1 is a complex process. Among these regulatory mechanisms, a direct effect of reactive oxygen species (ROS) on the HIF-1α subunit has received a great deal of attention, but there are contradictory literature data with respect to association between HIF-1α and ROS[12]. Some studies have indicated that heat shock proteins and the family of chaperones could play important roles in the pathology of CD[13-15]. The expression of HSPs can be markedly upregulated in epithelial cells under extreme conditions by the mechanism involving the expression and release of proinflammatory mediators, such as tumor necrosis factor alpha (TNF-α) or an activation of oxidative stress[16]. All these factors can trigger apoptosis[17,18], but the decision for a cell to undergo apoptosis depends on the balance between proapoptotic and antiapoptotic signals. For instance, HSPs may exert antiapoptotic effects and contribute to preservation of intestinal epithelial barrier integrity[19]. This process can be executed either by the extrinsic or intrinsic apoptotic pathways. While the role of the extrinsic apoptotic pathway activation in the mucosa of patients with CD has been proposed in the literature, studies on the intrinsic and common apoptotic pathways in patients with CD are sparse[18].

It is likely that the development of CD depends on the balance between proinflammatory and anti-inflammatory factors, proapoptotic and antiapoptotic signals, as well as prooxidant processes and antioxidant capacity of the cell. This imbalance is reflected in an impairment of the epithelial barrier and increased permeability, leading to activation of the immune response (native and adaptive) that contributes to cell damage and villous atrophy in patients with CD.

The aim of our study was to determine the involvement of oxidative imbalance in the mechanism of mucosal injury of the small intestine and to assess the effect of oxidative stress on the course of CD in adult patients with non-classic symptoms and extraintestinal manifestations. Apart from routine blood parameters, the serum concentrations of total vitamin D, uric acid, and bilirubin were measured. Moreover, in biopsy specimens collected during endoscopy of the proximal small intestine from these groups of patients, the expression of mRNA for proinflammatory cytokines TNF-α and interleukin 1β (IL-1β) as well as an anti-inflammatory cytokine interleukin 10 (IL-10) was determined by reverse transcription–polymerase chain reaction (RT-PCR) with specific primers. Oxidative stress may influence the expression of HSP-70, another marker examined in our study. Since ROS were shown to affect the stabilization of HIF-1α RNA and activate the intrinsic apoptotic pathway associated with overexpression of proapoptotic BAX, we also examined the gene expression of HIF-1α, antioxidant enzyme SOD, and proapoptotic factor BAX in the duodenal tissues of the enrolled patients.

MATERIALS AND METHODS

All individuals gave informed consent to participate in the study. The protocol of the study was approved by the Ethical Committee at Jagiellonian University Medical College in Cracow, Poland (No KBET/174/B/2013) and was run in accordance with the Declaration of Helsinki.

The study included 85 patients of the Outpatient Clinic and the Department of Gastroenterology and Hepatology of the University Hospital in Cracow (Table 1). Patients were divided into the following subgroups: (1) 7 patients with newly diagnosed CD (age range, 19-62 years; mean age, 34.7 ± 14.9 years); (2) 22 patients with CD who did not adhere to GFD (nontreated CD group; age range, 22-68 years; mean age, 38.2 ± 10.7 years); (3) 31 patients with CD who were on GFD for at least two years and who tested negative for celiac antibodies (treated CD group; age range, 28-65 years; mean age, 45.7 ± 16.1 years; mean duration, 10 ± 7.7 years); and (4) 25 patients with functional disorders of the gastrointestinal tract without abnormalities on upper gastrointestinal endoscopy and on serological and histological examinations (control group; age range 19-66 years; mean age, 38.5 ± 13.2 years). Groups 1 and 2 represented patients with active CD.

CD was diagnosed on the basis of clinical symptoms, positive test results for celiac antibodies [antitissue transglutaminase antibodies (TGAs) or antiendomysial antibodies (EmAs) or both], and the characteristic histological features of duodenal biopsies. Celiac patients presented with non-classic symptoms or extraintestinal manifestations such as iron deficiency, anemia, chronic abdominal pain without typical malabsorption syndrome, osteoporosis, osteopenia, as well as asymptomatic disease (Table 2). We excluded patients with diabetes, inflammatory bowel disease, current infectious disease, history of cancer, chronic hepatobiliary disease, chronic renal impairment, and alcohol abuse, or those who received therapy with nonsteroidal anti-inflammatory drugs, antioxidant supplements, oral contraceptives, immunosuppressants, and immunostimulants. All patients were nonsmokers.

All patients underwent upper gastrointestinal endoscopy, and at least four well-oriented duodenal

specimens were taken for histological examination and determination of the IL-1β, TNF-α, IL-10, HSP-70, HIF-1α, SOD, and BAX mRNA expression in the duodenum. The degree of intestinal mucosal damage was evaluated according to the Marsh classification[20]. The histological assessment was performed by an experienced pathologist in the Department of Pathology at Jagiellonian University Medical College.

We determined the serum levels of TGAs and EmAs, blood cell count, serum activity of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase, and total protein, as well as serum levels of antioxidants: uric acid, bilirubin, and vitamin D. The TGA concentration was assessed using a commercial ELISA kit (Aesku Diagnostics GmbH, Germany), and the results were expressed as unit (U)/mL of serum. A value higher than 15 U/mL was considered positive. EmAs were assessed with immunofluorescence. A value higher than 1:10 was considered positive. The other biochemical tests were performed in the Department of Diagnostics of the University Hospital in Cracow.

Expression of IL-1β, TNF-α, IL-10, HSP-70, HIF-1α, SOD, and BAX transcripts in the human intestinal samples determined by RT-PCR

The expression of IL-1β, TNF-α, IL-10, HSP-70, HIF-1α, SOD, and BAX transcripts in human samples was determined by RT-PCR. Each specimen was immediately frozen in liquid nitrogen and stored at -80 ℃ until analysis. Total RNA was then isolated according to the method by Chomczynski and Sacchi[21], using Trizol Reagent (Invitrogen, Carlsbad, United States) following the manufacturer's protocol. First-strand cDNA was synthesized from total cellular RNA (2 μg) using Reverse Transcription System (Promega, Madison, United States). The RT-PCR was carried out in an automatic DNA thermal cycler, using 1-μg cDNA and Promega PCR reagents. For amplification of IL-1β, TNF-α, IL-10, HSP-70, HIF-1α, SOD, and BAX cDNA, gene-specific primers were used (SIGMA-Aldrich St. Louis, United States) (Table 3). Amplification of control human β-actin was performed on the same samples to verify the RNA integrity. PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 μg/mL of ethidium bromide and then visualized under ultraviolet light. Location of the predicted PCR product was confirmed by using the O'Gene Ruler 50 bp DNA ladder (Fermentas, Life Sciences, San Francisco, United States) as standard marker.

Statistical analysis

A statistical analysis was performed by a biomedical statistician using a nonparametric Mann-Whitney test. For the comparison of normally distributed variables between the groups, the Student's *t*-test was used. The results were reported as mean ± SE or mean ± SD, and a significance level was defined as a *P* value of less than 0.05. The analysis was performed using Statistica 10 software (StatSoft® Inc., United States).

RESULTS

Blood test in controls and celiac patients

The results of biochemical tests are presented in Table 4. The mean leukocyte and platelet counts were similar between the celiac groups and controls. The mean red blood cell count was lower in the active CD and treated CD groups as compared with controls (4.49 ± 0.39 *vs* 4.7 ± 0.37, $P < 0.05$; 4.45 ± 0.42 *vs* 4.7 ± 0.37, $P < 0.05$, respectively). The mean hemoglobin and hematocrit levels were lower in patients with active CD than in controls (12.6 ± 1.8 *vs* 13.4 ± 1.4, $P < 0.05$; 37.6 ± 4.4 *vs* 41.6 ± 9.6, $P < 0.05$, respectively) and treated celiac patients (12.6 ± 1.8 *vs* 13.3 ± 1.1, $P < 0.05$; 37.6 ± 4.4 *vs* 39.5 ± 3.2, $P < 0.05$, respectively). Only 2 patients (3.3%) with CD were anemic (hemoglobin < 11 g/dL), but reduced mean corpuscular volume was observed in 11 patients (37.9%) with active CD (range, 59.9-81.4 fL), in 3 patients (3.2%) with treated CD (range, 80.9-81.1 fL), and in two controls (8%; range, 80.1-80.4 fL).

The mean serum levels of total protein, alanine aminotransferase, alkaline phosphatase, and γ-glutamyltransferase were similar to those observed in controls. The mean serum levels of aspartate aminotransferase were significantly higher in the active CD and treated CD groups compared with controls (28.0 ± 19.3 *vs* 18.0 ± 8.5, $P < 0.05$; 23.2 ± 6.5 *vs* 18.0 ± 8.5, $P < 0.05$, respectively), without significant differences between the two CD groups. Hypertransaminasemia was reported in 7 patients (24.1%) with active CD, in 5 patients (16.1%) with treated CD, and in none of the control patients.

Serum uric acid concentrations were elevated only in celiac patients, namely, in 3 patients (10.3%) with active CD and in 2 patients (6.5%) on GFD. Uric acid levels were significantly higher in the celiac groups than in controls ($P < 0.001$), while bilirubin levels were significantly lower in patients with CD than in controls ($P < 0.05$).

Reduced vitamin D levels were reported in 26 patients (89.6%) with active CD, in 21 patients (67.7%) with treated CD, and in 14 controls (56%). Moderate vitamin D deficiency (10-19 ng/mL) was reported in 11 patients (37.9%) with active CD and 12 patients (< 10 ng/mL) was reported in 3 patients (10.3%) with active CD and only in 1 patient (3.2%) with treated CD. Moderate to severe vitamin D deficiency was not observed in the control group. The mean vitamin D

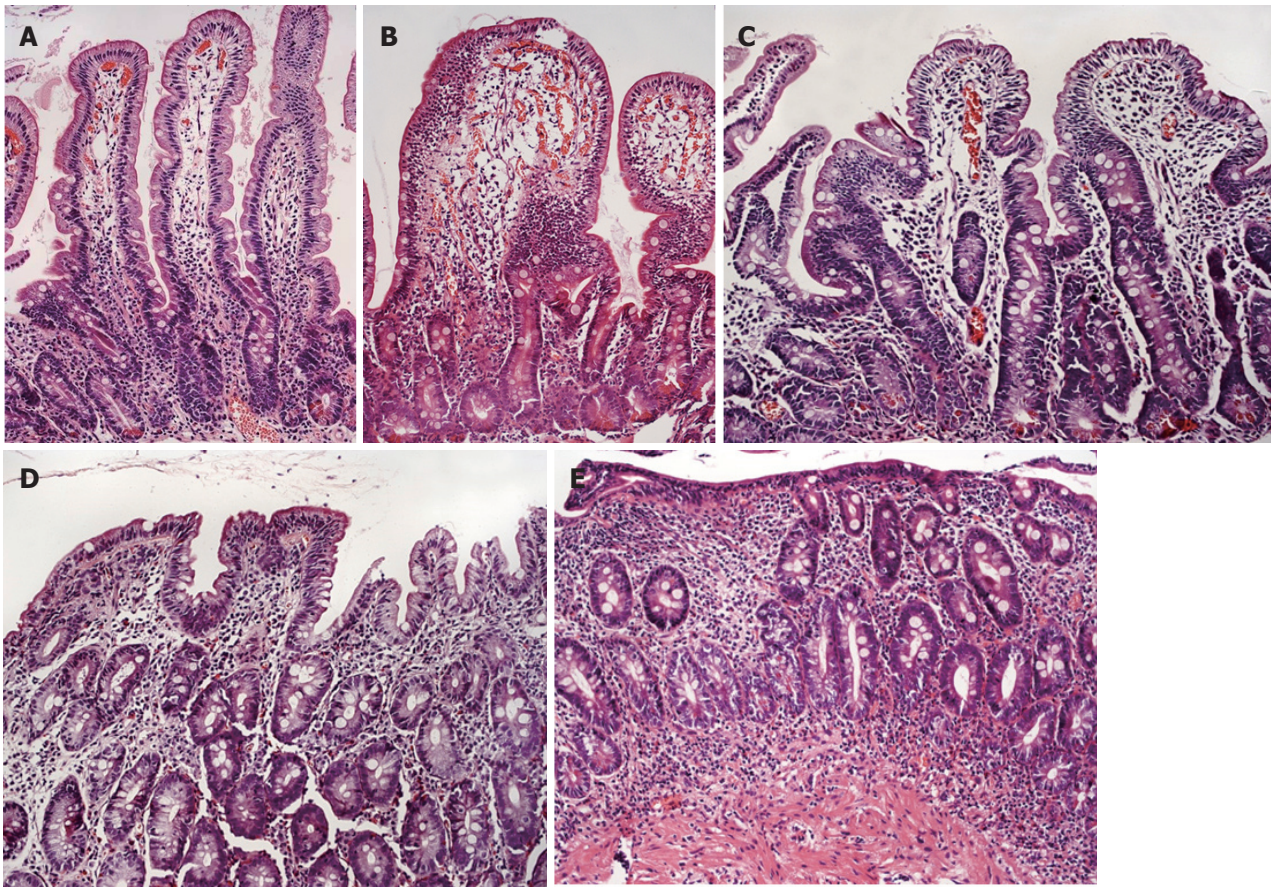

Figure 1 The spectrum of small intestinal damage in the study groups. Hematoxylin and eosin stained biopsy specimens obtained by gastroscopy. A: Normal duodenal mucosa: normal villus-to-crypt ratio; intraepithelial lymphocytes (IEL) within the normal range; B: Marsh 1: lymphocytic enteritis (an increase in IEL count); C: Marsh 3a: partial villous atrophy with hypertrophic crypts and an increase in IEL count; D: Marsh 3b: subtotal villous atrophy with hypertrophic crypts and an increase in IEL count; E: Marsh 3c: total villous atrophy with hypertrophic crypts and an increase in IEL count.

Table 1 Characteristics of the study groups

Groups of patients	Age (yr, mean ± SD)	*n* (%)
Total	70.74 ± 14.22	85
Female		71 (83.5)
Male		14 (16.5)
Active CD		
Newly diagnosed CD	34.7 ± 14.9	7
Female		5 (71.4)
Male		2 (28.6)
Nontreated CD	38.2 ± 10.7	22
Female		18 (81.8)
Male		4 (18.2)
Treated CD	45.7 ± 16.1	31
Female		28 (90.3)
Male		3 (9.7)
Control	38.5 ± 13.2	25
Female		20 (80)
Male		5 (20)

Active CD: Celiac patients with active disease; Newly diagnosed CD: Celiac patients at diagnosis of CD; Nontreated CD: Celiac patients not adhering to a gluten-free diet; Treated CD: Celiac patients on a gluten-free diet.

level was significantly lower in patients with active CD than in controls or treated celiac patients ($P < 0.001$ and $P < 0.05$, respectively), and was lower in treated celiac patients than in controls ($P < 0.05$).

Serum levels of celiac antibodies and the degree of intestinal mucosal damage in the study population

The serum levels of celiac antibodies were negative in the control group (Table 5). As expected, the significantly higher levels of celiac antibodies were observed in patients with active CD. In treated CD patients, the levels of antibodies were significantly lower compared with untreated CD patients ($P < 0.001$). The degree of intestinal mucosal damage evaluated according to the Marsh classification as shown in Figure 1 and Table 5 was the most severe in newly diagnosed CD patients, followed by nontreated CD patients, treated CD patients, and controls. The differences between the groups were significant. Data are presented in Table 5.

Expression of transcripts in human intestinal samples determined by reverse-transcriptase polymerase chain reaction

Expression of IL-1β, TNF-α, and IL-10: Figure 2 shows the mRNA expression of proinflammatory cytokines IL-1β and TNF-α and the alterations in RT-PCR mRNA expression of anti-inflammatory cytokine

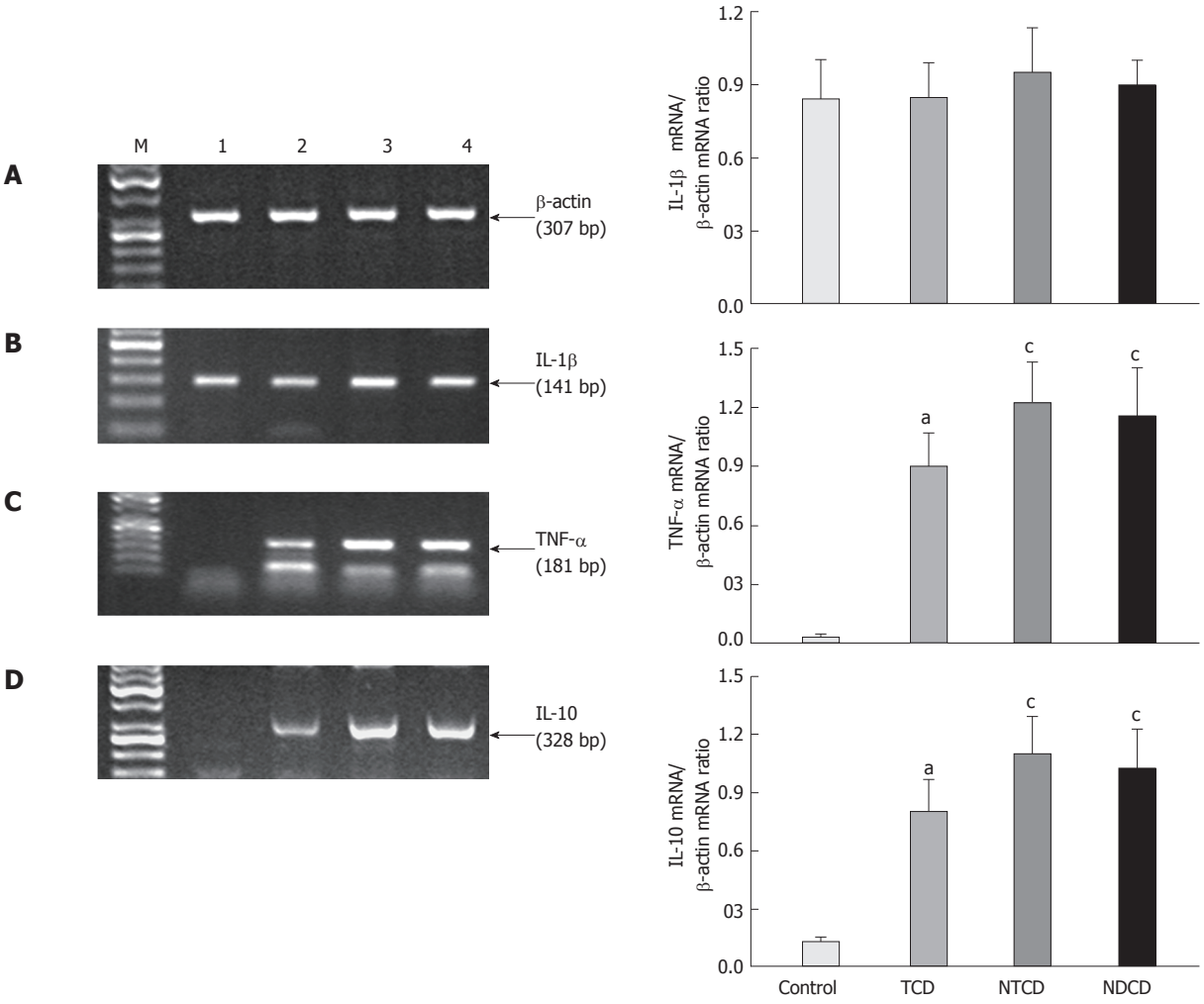

Figure 2 The RT-PCR expression of mRNA for β-actin (A), interleukin 1β (B), tumor necrosis factor α (C), and interleukin 10 (D) in duodenal tissue of celiac patients and controls. The bands densities [as a ratio of interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and IL-10 to the β-actin levels, respectively] are expressed as the mean ± SE of 4 determinations in selected patients. TCD: Treated celiac group; NTCD: Nontreated celiac group; NDCD: Newly diagnosed celiac group. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* control and treated CD groups.

Table 2 Clinical characteristic of study groups *n* (%)

Groups of patients	Iron deficiency/anemia	Chronic abdominal pain	Osteopenia/osteoporosis	Menstrual disorders	Abnormal liver tests	Others
Active CD ($n = 29$)	11 (37.9)	7 (24.1)	4 (13.8)	1 (3.4)	5 (17.2)	1 (3.4)
Treated CD ($n = 31$)	15 (48.4)	4 (12.9)	5 (16.1)	-	5 (16.1)	2 (6.5)

Active CD: Celiac patients with active disease; Treated CD: Celiac patients on a gluten-free diet.

IL-10 in the biopsies of duodenal mucosa. The IL-1β mRNA expression was not significantly different between the study groups, although it was slightly higher in active CD as compared with controls and patients on GFD. The expression of mRNA for TNF-α was significantly increased in all celiac groups when compared with controls ($P < 0.05$). The TNF-α RNA expression was similar in both groups of active CD (high degree of mucosal damage), but was significantly higher than in treated patients (low grade of mucosal damage).

The expression of IL-10 mRNA in the study groups was similar to the trend observed for the expression of TNF-α mRNA. In intact intestinal mucosa, the signal for IL-10 mRNA expression was faint. However, we observed a significant increase in the IL-10 mRNA expression in the celiac groups when compared with controls ($P < 0.05$). Celiac patients on GFD had a lower expression of IL-10 mRNA in the mucosa than patients with active disease, and this difference based on the semi-quantitative assessment of the ratio of IL-10 mRNA expression to β-actin mRNA expression was significant (Figure 2).

Expression of HSP-70 and SOD: As shown in Figure 3, the signal for the expression of HSP-70 mRNA was

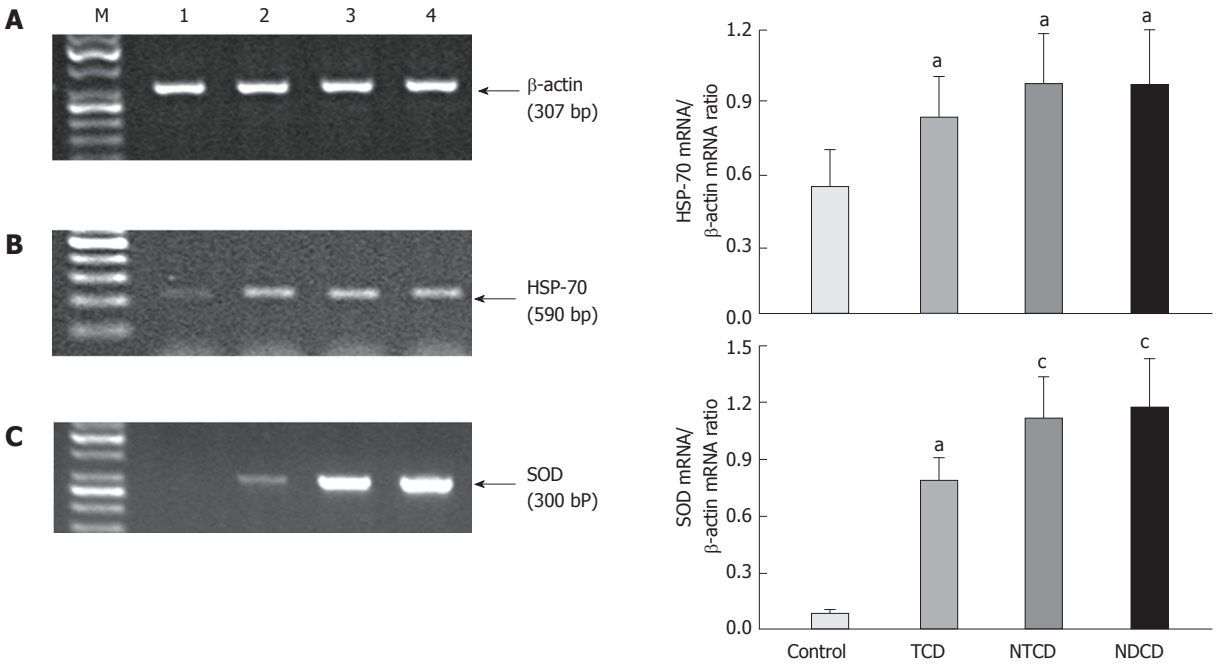

Figure 3 The RT-PCR expression of mRNA for β-actin (A), heat-shock protein 70 (B) and superoxide dismutase (C) in duodenal tissue of celiac patients and controls. The band densities [as a ratio of heat-shock protein 70 (HSP-70) and superoxide dismutase (SOD) to the β-actin levels, respectively] are expressed as the mean ± SE of 4 determinations in selected patients. TCD: Treated celiac group; NTCD: Nontreated celiac group; NDCD: Newly diagnosed celiac group. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* control and treated CD groups.

Table 3 Human oligonucleotide primers for detection of mRNA by RT-PCR

Gene	Primer sequence	*t*	PCR product
IL-1β	Forward 5′-ACA TCA GCA CCT CTC AAG -3′, Reverse 5′-AGT CCA CAT TCA GCA CAG -3′	60 ℃	141 bp
TNF-α	Forward 5′-GCC CAG GCA GTC AGA TCA TCT TC -3′, Reverse 5-TGA GGT ACA GGC CCT CTG ATG G-3′	58 ℃	181 bp
IL-10	Forward 5′-AGC TAT CCC AGA GCC CCA GAT CCG ATT TTG G-3′, Reverse 5′-AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG CG-3′	60 ℃	328 bp
HSP-70	Forward: 5′-GCC CCA ACA GAT TGT TGT CTT-3′, Reverse: 5′-CCA CCA AGC AGA CGC AGA T-3′	59.5 ℃	111 bp
HIF-1α	Forward 5′-GGT TCT CAC AGA TGA TGG TG-3′, Reverse 5′-TTC TTC CTC GGC TAG TTA GG-3′	60 ℃	239 bp
SOD	Forward: 5′-GAA GGT GGG AAG CAT TA-3′, Reverse: 5′-ACC TTT GCC CAA GTC ATC TG-3′	57 ℃	300 bp
BAX	Forward 5′-CGT CCA ACC CAC CCT GGT CT-3′, Reverse 5′-TGG CAG CTG ACA TGT TTT CTG AC-3′	55 ℃	195 bp
β-actin	Forward 5′-GGG TAC ATG GTG GTG CCG-3′, Reverse 5′-AGC GGG AAA TCG TGC GTG-3′	54 ℃	307 bp

t: Annealing temperature; IL-1β: Interleukin 1β; TNF-α: Tumor necrosis factor alfa; IL-10: Interleukin 10; HSP-70: Heat-shock protein 70; HIF-1α: Hypoxia-inducible factor 1α; SOD: Superoxide dismutase.

markedly increased in the celiac groups compared with controls, regardless of compliance with the diet ($P < 0.05$). The differences between the celiac groups were not significant.

The ratio of SOD mRNA expression to β-actin mRNA expression confirmed that the expression of this antioxidant enzyme was significantly elevated in celiac patients compared with controls ($P < 0.05$). The signal for SOD mRNA expression in treated CD patients was significantly lower than in untreated and newly diagnosed ones (Figure 3).

Expression of HIF-1α and BAX: The ratio of HIF-1α mRNA expression to β-actin mRNA expression confirmed that the expression of HIF-1α was significantly elevated in the mucosa of patients with active CD compared with controls and patients with treated CD ($P < 0.05$). HIF-1α mRNA expression was slightly increased in the duodenal mucosa of patients with treated CD compared with controls, but the difference was not significant (Figure 4). A significant increase in the expression of BAX mRNA as determined by the ratio of BAX mRNA expression to β-actin mRNA expression in the mucosa of patients with active CD was observed compared with controls and patients

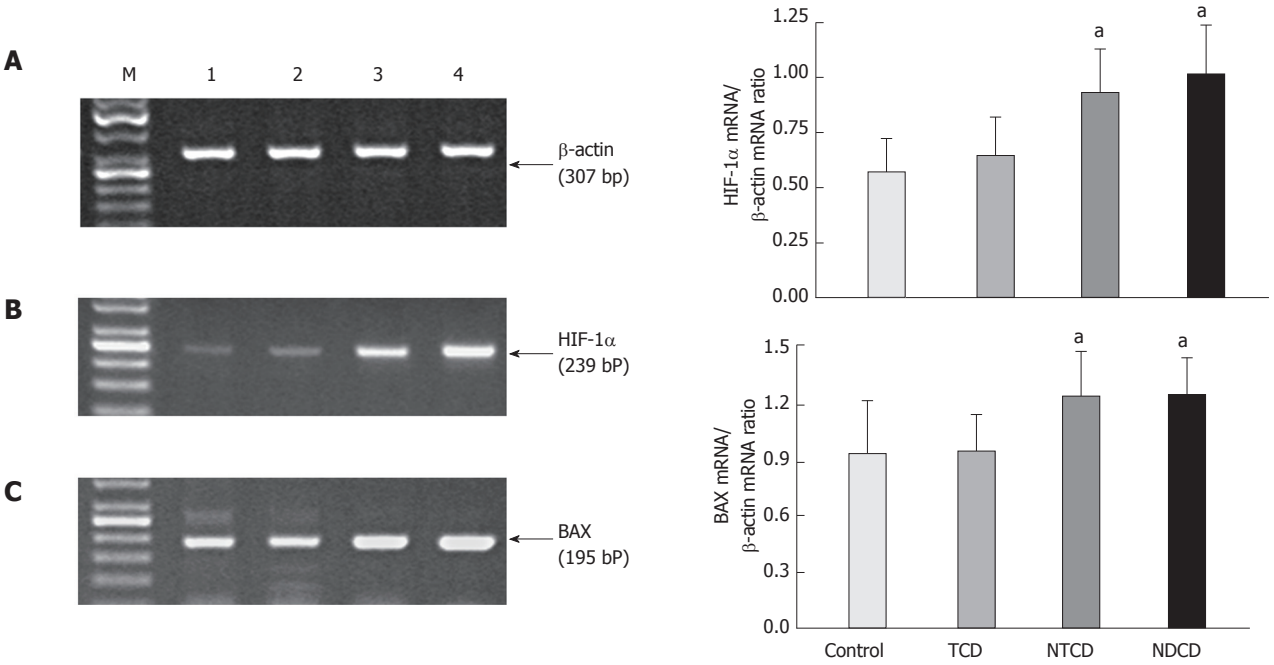

Figure 4 The RT-PCR expression of mRNA for β-actin (A), hypoxia-inducible factor 1α (B) and BAX (C) in duodenal tissue of celiac patients and controls. The band densities [as a ratio of hypoxia-inducible factor 1α (HIF-1α) and BAX to the β-actin levels, respectively] are expressed as the mean ± SE of 4 determinations in selected patients. TCD: Treated celiac group; NTCD: Nontreated celiac group; NDCD: Newly diagnosed celiac group. [a]*P* < 0.05 *vs* control and treated CD groups.

Table 4 Blood test results in the study groups

	Controls (*n* = 25)	Active CD (*n* = 29)	Treated CD (*n* = 31)
WBC (10^3/μL)	5.69 ± 1.55	5.81 ± 1.58	5.36 ± 1.36
RBC (10^6 cells/μL)	4.7 ± 0.37	4.49 ± 0.39[a]	4.45 ± 0.42[a]
Hemoglobin (g/dL)	13.4 ± 1.4	12.6 ± 1.8[a,c]	13.3 ± 1.1
Hematocrit (%)	41.6 ± 9.6	37.6 ± 4.4[a,c]	39.5 ± 3.2
PLT (10^3/μL)	248.52 ± 47.63	267.18 ± 96.8	252.52 ± 64.38
Vitamin D (ng/mL)	29.7 ± 5.1	19.4 ± 9.0[b,c]	25.2 ± 11.2[a]
Total protein (g/L)	73.2 ± 6.1	71.2 ± 7.9	71.6 ± 3.3
AST (U/L)	18.0 ± 8.5	28.0 ± 19.3[a]	23.2 ± 6.5[a]
ALT (U/L)	22.0 ± 4.3	26.7 ± 20.1	24.2 ± 12.5
AP (U/L)	59.6 ± 19.7	62.2 ± 29.1	59.2 ± 27.3
GGTP (U/L)	24.4 ± 14.6	21.3 ± 13.6	21.3 ± 22.62
bilirubin (μmol/L)	10.5 ± 4.1	8.2 ± 5.0[a]	8.1 ± 3.3[a]
Uric acid (μmol/L)	190.8 ± 23.0	260.2 ± 53.7[b]	261.7 ± 51.8[b]

Data are given as mean ± SD. [a]*P* < 0.05, [b]*P* < 0.001 *vs* controls; [c]*P* < 0.05 *vs* Treated CD. Active CD: Patients with active CD; Treated CD: Celiac patients on a gluten-free diet; WBC: White blood cell count; RBC: Red blood cell count; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AP: Alkaline phosphatase; GGTP: γ-glutamyltransferase.

with treated CD (*P* < 0.05). We failed to observe any significant difference in the expression of BAX mRNA in the duodenal mucosa of patients with treated CD compared with controls (Figure 4).

DISCUSSION

Most studies concerning the pathomechanism of CD and intestinal changes focused on children with classic clinical symptoms of malabsorption syndrome[4,22-24]. However, malabsorption alone does not explain the pathophysiology and clinical course of numerous extraintestinal manifestations as well as non-classic symptoms that predominate in adult patients with CD. Other mechanisms have been proposed including gluten toxicity with oxidative imbalance and autoimmunity[23,24].

In this study, we have examined less extensively studied, factors implicated in CD, such as HSP-70, HIF-1α, and the proapoptotic factor BAX. We found these 3 factors to be overexpressed in active CD, with varying degrees of activity in patients on GFD. This overexpression could be triggered by oxidative imbalance linked with an increase in ROS generation. Each of these factors was shown to influence the intestinal barrier integrity. For instance, HSP-70 and HIF-1α can contribute to preservation of intestinal barrier integrity[5,19], while apoptosis manifested by the rise in the BAX expression may lead to disruption of the intestinal barrier[25]. The impaired barrier function may be involved in several immune-mediated diseases, including CD and its extraintestinal manifestations or coexisting disorders[26,27].

HSP, a known chaperone, has potential epithelial barrier protecting, antiapoptotic, and immunologic properties[19], but its role in the pathogenesis of CD remains unexplored. Our results presented in this work revealed that HSP-70, which is expressed under normal conditions, can also play a particularly important role in extreme conditions such as gluten cytotoxicity. It is noteworthy that the expression of HSP-70 was significantly increased in each celiac group in our study

Table 5 Clinical characteristics of the study groups: serum levels of celiac antibodies and the degree of intestinal mucosal damage *n* (%)

	Control (*n* = 25)	Treated CD (*n* = 31)	Nontreated CD (*n* = 22)	Newly diagnosed CD (*n* = 7)
Antibody titer				
0	25	30 (96.7)	0	0
1	0	1 (3.3)	9 (40.9)	1 (14.3)
2	0	0	3 (13.6)	2 (28.6)
3	0	0	10 (45.5)	4 (57.1)
Antibody titer	0	0.03 ± 0.2	2.0 ± 0.9[b,d]	2.4 ± 0.8[b,d]
Degree of intestinal mucosal damage[1]				
Normal mucosa 0	24 (96)	13 (41.3)	2 (9)	1 (14.3)
Marsh 1 1	1 (4)	5 (16.1)	7 (31.8)	0
Marsh 2 2	0	0	0	0
Marsh 3a 3	0	5 (16.1)	6 (27.3)	1 (14.3)
Marsh 3b 4	0	7 (22.6)	5 (22.7)	3 (42.9)
Marsh 3c 5	0	1 (3.2)	2 (9)	2 (28.6)
Intestinal mucosal damage[1]	0.04 ± 0.2	1.7 ± 1.8[b]	2.5 ± 1.6[b,c]	3.7 ± 1.4[b,c]

[1]Intestinal mucosal damage was classified according to Marsh parameters, and each stage was given a score from 0 (normal mucosa) to 5 (total villous atrophy). [b]$P < 0.001$ *vs* controls; [c]$P < 0.05$, [d]$P < 0.001$ *vs* treated CD. Data are frequency counts (percentage of total) or the mean ± SD. Treated CD: Celiac patients on a gluten-free diet; Nontreated CD: Celiac patients not adhering to a gluten-free diet; Newly diagnosed CD: Celiac patients at diagnosis of CD; antibody titer 0: Negative; 1: Low [TGA <3x the upper limit of normal (ULN); EmA (+)]; 2: High [3xULN<TGA<10x ULN ; EmA (++)], 3: Very high [TGA>10x ULN; EmA (+++)].

regardless of the degree of compliance with the diet. A few previous studies evaluated the role of HSP in intestinal pathology of patients with CD[19,28-30]. Iltanen *et al*[31] reported enhanced expression of epithelial cell mitochondrial HSP-65 in 80% of study children with CD and in only 7% of control subjects. Sziksz *et al*[19] reported an increased HSP-72 mRNA expression in the duodenal mucosa of children with untreated CD as well as children with treated CD compared with that in controls. These observations are consistent with the results of our study on HSP-70 expression in adult patients. Our results indicate that HSP-70 in adult CD patients, similarly as HSP-72 in children, was overexpressed due to oxidative stress. The increased HSP-70 expression may constitute a protective mechanism against gliadin-induced cytotoxicity associated with antiapoptotic effects, thus contributing to preservation of intestinal epithelial barrier integrity.

It should be noted, however, that significant percentage of patients with CD on GFD, in our study, showed the persistence of duodenal damage despite clinical improvement and evident decline in celiac antibodies. The main criteria for inclusion in this group involved a specialist assessment by gastroenterologist and dietitian of patients proper dietary adherence, clinical recovery and above all, the negativity of serologic markers. Interestingly, a gap has emerged between the clinical and mucosal recovery, mainly in the adult population, since when re-biopsing treated CD patients only half of them had healed mucosa, despite the negativity of celiac antibodies[32,33]. Following the GFD, the clinical symptoms and mucosal architecture usually improve very quickly in children[34], while in a mixed population including adults, the recovery of duodenal mucosa assessed by histology requires longer time to heal[35]. These previous observations seem consistent with the results of our present study because the morphological alterations persisted in some of our CD patients despite the clear disappearance of specific antibodies. The increased expression of HSP-70 in treated and untreated celiac patients indicates that oxidative stress in patients with CD may still persist despite GFD and serological and clinical remission, and may be responsible for histopathological alterations observed in our study. Finally, the enhanced expression of HSP-70 suggest incomplete elimination of all sources of gluten in modern diet. Perhaps the expression of HSP-70 could be considered as a more sensitive marker than celiac antibodies in the detection of the trace amounts of gluten in diet. Thus, HSP-70 could be considered a potential novel biomarker of this disease.

It is known that ROS and HIF-1 signaling are involved in numerous diseases including cancer, inflammatory diseases, and ischemic disorders[12]. Furthermore, the increase of ROS levels is one of the main factors stabilizing HIF-1α. It has been shown that exogenous ROS, in the form of H_2O_2, can enhance the synthesis of inflammatory mediators such as TNF-α and IL-1β[36], which in turn can influence the protein transcription and activity of HIF-1α under normoxia[37]. We provided evidence for the increased mucosal HIF-1α expression in untreated adult patients with active CD compared with controls and treated CD patients, while we did not observe any significant difference between treated celiac patients and controls. This observation is consistent with that of Vannay *et al*[11], who suggested the role of HIF-1α in the pathomechanism of CD. In addition, involvement of HIF-1 in inflammatory bowel disease has been reported[10]. The increased expression of HIF-1α in our study can be explained by the initial intestinal damage or the direct effect of gluten in diet. These data suggest that increased HIF-1α expression may

be a consequence rather than a primary cause of CD. Moreover, the decreased mucosal expression of HIF-1α in treated CD may confirm the efficacy of GFD.

In general, data on the status of apoptosis in patients with CD are conflicting, but increased apoptotic cell death of intestinal epithelial cells was reported in untreated CD, as detected by DNA fragmentation assay using terminal uridine deoxynucleotidyl nick end labelling in small intestinal biopsies[38]. In that study, apoptosis was well correlated with proliferation and returned to normal in patients treated with GFD[38]. It is likely that increased apoptosis may be responsible for villous atrophy in CD. Therefore, our study included RT-PCR analysis of the proapoptotic member of the Bcl-2 family, that is, BAX, which in normal mucosa showed constitutive expression. This remains in keeping with the observation that the mucosa of healthy individuals undergoes a high rate of constitutive epithelial proliferation[17]. In our study, the expression of BAX showed a similar trend to that observed for the expression of HIF-1α. We revealed a significantly elevated BAX mRNA expression in the duodenal mucosa of patients with active CD compared with controls and patients with treated CD. Interestingly, the expression of BAX mRNA in the duodenal mucosa was not significantly different between treated CD patients and controls. Our results suggest that the increased expression of BAX results from the severity of intestinal inflammation and gluten-induced oxidative stress and leads to duodenal villous atrophy. Moreover, the decreased mucosal expression of BAX in treated CD patients may indicate the relief of inflammation and thus the efficacy of treatment. In contrast to our study, van der Woude *et al*[38] failed to demonstrate any changes in the expression of BAX, Bcl-2, and Bcl-xl between their study groups, which were similar to those in our study. However, Cherñav*s*ky *et al*[39] found that only Bak mRNA was significantly overexpressed in the mucosa of CD patients, whereas BAX and Bcl-2 transcription levels were unchanged with respect to control mucosa.

Oxidative imbalance seems to be involved in the molecular mechanisms of CD. In normal conditions, the harmful effects of ROS are opposed by the antioxidant defense system consisting of antioxidant enzymes (glutathione peroxidase, glutathione reductase, SOD, and catalase), non-enzymatic antioxidants (such as glutathione, albumin, bilirubin, ceruloplasmin, and uric acid) as well as nutritional antioxidants (carotenoids and vitamins A, C, and E)[40]. The reduced antioxidant defense may make the inflamed mucosa more sensitive to oxidative tissue damage and may disrupt its recovery and integrity.

Using thiobarbituric acid reactive substances as a marker of oxidative stress, Odetti *et al*[41] showed that redox equilibrium is impaired in patients with CD. They also observed decreased serum α-tocopherol levels in patients with silent CD in comparison with controls. Earlier studies also showed that the activity of SOD is markedly increased in pediatric patients with CD, while the activity of glutathione peroxidase is significantly decreased[24].

SOD, which reduces the most abundant free radical $^{\bullet}O_2$, is considered as the major intracellular antioxidant enzyme[40]. In agreement with previous data, our results demonstrated overexpression of SOD mRNA in the mucosa of celiac patients compared with controls. We observed an increased expression of SOD mRNA in active disease, and this increase was attenuated in the treated celiac group. These results suggest that the increased expression of SOD, reflecting the severity of oxidative stress in duodenal mucosa, could be a consequence of either intestinal impairment or of oxidative imbalance. Our observations may indicate that some markers of oxidative stress persist even in treated CD patients, but GFD partially counteracts the impairment of intestinal mucosa observed in active CD patients.

There is increasing experimental and clinical evidence showing that uric acid acts as an important antioxidant *in vivo*[42]. Interestingly, an increase in serum uric acid concentrations occurs as a physiological response to enhanced oxidative stress[43]. Despite being a major antioxidant in the human plasma, uric acid correlates with and may predict the development of conditions associated with oxidative stress such as obesity, hypertension, and cardiovascular disease[44]. Our results indicate that higher serum levels of uric acid in patients with CD compared with controls may be a consequence of oxidative stress and that uric acid may function as an antioxidant. Additional well-designed clinical studies are needed to clarify the potential use of uric acid (or uric acid precursors) in CD and to examine its role as a marker of oxidative stress and a potential therapeutic antioxidant.

In contrast to transaminases, the levels of bilirubin in patients with CD were significantly lower than in the control group. Bilirubin is an antioxidant that blocks vascular cell adhesion molecule 1 signals through ROS *in vitro*[45]. An Australian study[46] reported that bilirubin levels were significantly lower in severe asthma, suggesting altered regulation of inflammation in asthmatics by antioxidant vitamins and bilirubin. This observation is consistent with our results, indicating the relationship between the altered concentration of bilirubin and oxidative imbalance. However, the role of bilirubin in oxidative imbalance in CD requires further research.

A significant number of CD patients with intestinal malabsorption syndrome present vitamin D deficiency or insufficiency. In our study, vitamin D deficiency was noted in celiac patients despite the absence of clinical syndrome of malabsorption, possibly because inflammation may also lead to vitamin D deficiency.

It is likely that inflammatory cytokines, such as TNF-α, cause CYP27B1-mediated conversion of 25(OH)D to 1,25(OH)$_2$D in the intestines, thereby reducing serum 25(OH) D levels[47]. In turn, the active form of 1,25(OH)$_2$D inhibits the proliferation and secretion of inflammatory cytokines by type 1 helper T cells, thereby reducing inflammation[48]. This inverse relationship between the activity of CD and serum vitamin D levels was observed in our study. A similar observation concerns the degree of TNF-α expression and the degree of vitamin D deficiency, which is consistent with the results obtained in previous studies in healthy individuals[49,50]. The antioxidant property of vitamin D is rather less well recognized. Cholecalciferol (vitamin D$_3$) is likely to act as a membrane antioxidant by stabilizing the membrane against lipid peroxidation[51]. The antioxidant activity of vitamin D may involve an interaction with SOD[52]. We showed that a decrease in serum vitamin D levels in patients with CD was accompanied by an increase in the intestinal mucosal expression of TNF-α, suggesting that overexpressed TNF-α may lead to a reduction in the serum level of vitamin D. In turn, an increase in SOD expression may result from enhancement of TNF-α expression and a prominent fall in serum vitamin D levels which activate the antioxidative defense. This indicates that early diagnosis of vitamin D deficiency is particularly important in patients with CD, especially in those who do not comply with GFD. Therefore, the supplementation of vitamin D is recommended not only for bone metabolism but also for effective treatment of intestinal damage in patients with CD by reducing the oxidative stress.

A drawback of this study is a relatively small number of patients in each celiac subgroups, and definitely a further research with higher number of enrolled subjects is required to support our observations. It is noteworthy that the morphology of duodenal mucosa failed to show a full recovery despite the proper adherence to GFD, clinical improvement and the status of seroconversion, *i.e.* the decline in the value of antibodies in this group of patients to a negative result. Hence, further research with only subjects presenting full mucosal healing would add more to our understanding of pathomechanism of CD and intestinal recovery associated with GFD.

In conclusion, by its association with intestinal damage, the course of the disease, and perhaps extraintestinal disorders, oxidative imbalance appears to be one of the major factors implicated in the pathogenesis of CD. Our results support the hypothesis that HSP-70 may be a potential novel biomarker in CD. The increased intestinal expression of HSP-70 in patients with active CD and in treated celiac patients indicates that oxidative stress persists despite the exclusion of gluten from diet which deepens our knowledge on multifaceted mechanisms of this disease. This persistent oxidative imbalance may be responsible for sustained intestinal damage in CD despite GFD. In fact, the significant overexpression of HSP-70 despite dietary compliance may suggest refractory nature of CD. Furthermore, particularly noteworthy are non-enzymatic antioxidants, such as uric acid and bilirubin, whose concentration may be easily assessed in patients with CD.

Considering that several nutrients exert antioxidant effects and influence gene expression, they represent a useful approach for nutritional intervention in CD subjects, as confirmed by recent studies *in vitro*. These studies have revealed phytonutrients and docosahexaenoic acid efficacy in protection against the cytotoxic effect of gliadin[53-55].

To become aware of the usefulness of nutritional genomics as a tool for targeted medical nutrition therapy, further basic research, epidemiological studies and controlled intervention trials are needed to investigate whether some nutrients such as antioxidant vitamins modulate *in vivo* predisposition of chronic inflammatory conditions and thus, have a role in the therapy of celiac disease, in addition to the rigorous GFD.

ARTICLE HIGHLIGHTS

Celiac disease (CD) is a common condition. The only effective treatment available is a strict life-long gluten-free diet (GFD). Untreated CD can have serious complications, such as osteoporosis or malignancy. Some patients do not report symptomatic improvement after starting treatment, and some will still have persisting symptoms after 6 to 12 mo. The literature suggests that complete normalization of duodenal lesions is exceptionally rare in adult celiac patients despite adherence to GFD.

Research motivation

There is an increasing body of evidence suggesting a relationship between oxidative stress and CD. It has been proposed that oxidative stress is one of the mechanisms responsible for gliadin toxicity and persistent oxidative imbalance may be responsible for sustained intestinal damage in CD despite GFD.

Research objectives

The assessment of the severity of oxidative stress, including the evaluation of antioxidant capacity, in patients with CD may have therapeutic implications. The indication of a proper new biomarkers useful in assessing the individual susceptibility to oxidative stress, which may help elucidate the pathogenesis of the disease and implement an appropriate treatment.

Research methods

To determine the involvement of oxidative stress in the mechanism of mucosal injury of the small intestine and to assess the effect of oxidative stress on the course of CD in adult patients with non-classic symptoms and extraintestinal manifestations, we determined the expression of IL-1β, TNF-α, IL-10, HSP-70, HIF-1α, SOD and BAX transcripts in human duodenal samples by reverse transcriptase–polymerase chain reaction.

Research results

The authors found HSP-70, HIF-1α, and BAX to be overexpressed in active CD, with varying degrees of activity in patients on GFD. This overexpression could be triggered by oxidative imbalance linked with an increase in ROS generation. We observed an increased expression of SOD mRNA in active disease, and this increase was attenuated in the treated celiac group. These results suggest that the increased expression of SOD, reflecting the severity of oxidative stress

in duodenal mucosa, could be a consequence of either intestinal impairment or of oxidative imbalance.

Our results indicate that oxidative stress persists even in CD patients treated with GFD. Moreover, the results suggest that HSP-70 and HIF-1α may be potential novel biomarkers of this disease. The overexpression of HSP-70 despite dietary compliance may suggest refractory nature of CD. The increased levels of uric acid in patients with CD compared with controls resulting from oxidative stress indicates that uric acid may function as an antioxidant compound.

Further research with a greater number of participants is needed to confirm our results. Further clinical studies are needed to clarify the potential therapeutic role of uric acid as an antioxidant in CD.

Research conclusions

This study deepens the current knowledge on the role of oxidation products on the CD. By its association with intestinal damage, the course of the disease, and perhaps extraintestinal disorders, oxidative imbalance appears to be one of the major factors implicated in the pathogenesis of CD. Our observations may indicate that some markers of oxidative stress persist even in treated CD patients, but GFD partially counteracts the impairment of intestinal mucosa observed in active CD patients. Persistent oxidative imbalance may be responsible for sustained intestinal damage in adult celiac patients despite GFD. Perhaps the expression of HSP-70 could be considered as a more sensitive marker than celiac antibodies in the detection of the trace amounts of gluten in diet. Thus, HSP-70 could be considered a potential novel biomarker of this disease. Additional well-designed clinical studies are needed to clarify the potential use of uric acid (or uric acid precursors) in the diagnosis and prognosis of CD and to examine its role as a marker of oxidative stress and a potential therapeutic utility as an antioxidant. Considering that oxidative stress is involved in the molecular mechanisms of CD, additional measures aimed at reducing oxidative imbalance, such as administration of antioxidants, deserve attention as potential supplementary therapy in the treatment of CD, in addition to the rigorous GFD.

Research perspectives

Studies comparing the different assays for antioxidant capacity measurement in patients with CD are needed to select the method of choice that would best reflect susceptibility to oxidative stress in these patients. These assays might be particularly useful in clinical practice as a tool for therapy monitoring in patients with CD. It should be hypothesized that oral antioxidant supplementation may reduce the toxic effects of peptides contained in gluten on enterocytes and help alleviate histological lesions, thus exerting beneficial effects on the course of the disease. To become aware of the usefulness of nutritional genomics as a tool for targeted medical nutrition therapy, further basic research, epidemiological studies and controlled intervention trials are needed to investigate whether some nutrients such as antioxidant vitamins modulate *in vivo* predisposition of chronic inflammatory conditions and thus have a role in the therapy of celiac disease, in addition to the rigorous GFD.

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ORIGINAL ARTICLE

Retrospective Study

Prediction of hepatocellular carcinoma development by aminotransferase to platelet ratio index in primary biliary cholangitis

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Author contributions: Cheung KS designed and performed the study, were involved in statistical analysis and interpretation of the data, and wrote the manuscript; Seto WK and Mak LY performed the study, statistical analysis and interpretation of the data; Fung J, Lai CL and Yuen MF revised and edited the manuscript.

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Abstract

AIM

To investigate the usefulness of aspartate aminotransferase to platelet ratio index (APRI) in predicting hepatocellular carcinoma (HCC) risk in primary biliary cholangitis (PBC).

METHODS

We identified PBC patients between 2000 and 2015 by searching the electronic medical database of a tertiary center. The hazard ratio (HR) of HCC with different risk factors was determined by Cox proportional hazards model.

RESULTS

One hundred and forty-four PBC patients were recru-

ited. Patients were diagnosed at a median age of 57.8 years [interquartile range (IQR): 48.7-71.5 years), and 41 (28.5%) patients had cirrhosis at baseline. The median follow-up duration was 6.9 years (range: 1.0-26.3 years). Twelve patients developed HCC, with an incidence rate of 10.6 cases per 1000 patient-years. The overall 5-, 10- and 15-year cumulative incidences of HCC were 2.3% 95%CI: 0%-4.8%), 8.4% (95%CI: 1.8%-14.5%) and 21.6% (6.8%-34.1%), respectively. Older age (HR = 1.07), cirrhosis (HR = 4.38) and APRI at 1 year after treatment (APRI-r1) > 0.54 (HR = 3.94) were independent factors for HCC development. APRI-r1, when combined with treatment response, further stratified HCC risk (log rank $P < 0.05$). The area under receiver operating curve of APRI-r1 in predicting HCC was 0.77 (95%CI: 0.64-0.88).

CONCLUSION

APRI-r1 can be used to predict the development of HCC in PBC patients. Combination of APRI-r1 with treatment response can further stratify the HCC risk.

Key words: Aspartate aminotransferase; Platelet ratio index; Hepatocellular carcinoma; Primary biliary cholangitis; Ursodeoxycholic acid; Cirrhosis

Core tip: Currently, no reliable predictive models exist for hepatocellular carcinoma (HCC) in primary biliary cholangitis (PBC). Our study showed that a higher aspartate aminotransferase to platelet ratio index (APRI) at 1 year after treatment (APRI-r1) was associated with a higher HCC risk. The performance of APRI-r1 in predicting HCC was satisfactory (area under the receiver operating curve: 0.77). Combination of APRI-r1 with treatment response further stratified HCC risk. Owing to its simplicity, non-invasiveness and cost-effectiveness, APRI can be used as a marker to streamline the HCC surveillance protocol in PBC patients.

Cheung KS, Seto WK, Fung J, Mak LY, Lai CL, Yuen MF. Prediction of hepatocellular carcinoma development by aminotransferase to platelet ratio index in primary biliary cholangitis. *World J Gastroenterol* 2017; 23(44): 7863-7874 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7863.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7863

INTRODUCTION

Primary biliary cholangitis (PBC) is an immune-mediated, chronic cholestatic liver disease due to the destruction of small-sized biliary ducts with progressive liver fibrosis[1]. The disease prevalence ranges from 19 to 402 per million[2] and 48 to 56 per million[3,4] in the western and Asian populations, respectively. If left untreated, patients will develop portal hypertension, cirrhosis and hepatocellular carcinoma (HCC) with resulting mortality[5].

Ursodeoxycholic acid (UDCA) is recommended in all PBC patients to delay histologic progression, reduce cirrhotic complications, and improve the long-term survival[6-9]. Recently, suboptimal treatment response to UDCA is recognized to be a risk factor for HCC development[10]. Various biochemical response criteria have been developed and validated, which include the Rotterdam criteria[11], Paris I criteria[12], Paris Ⅱ criteria[13], Barcelona criteria[7], and Toronto criteria[14].

In addition, the HCC risk was significantly increased in patients with significant fibrosis and cirrhosis. Traditionally, liver biopsy is regarded as the gold standard diagnostic method, but it may not be desirable in daily clinical practice due to its associated invasiveness which may lead to various complications[15]. Aspartate aminotransferase (AST) to platelet ratio index (APRI) is a serum marker shown to be able to assess liver fibrosis and cirrhosis across a wide array of chronic hepatic diseases[16-22]. It has the advantage of being easily calculated from routine laboratory results. Studies suggested that it could predict HCC risk in chronic hepatitis B (CHB)[23] and C (CHC) infection[24] and had prognostic value in CHB patients who underwent surgery for early stage HCC[25].

Recently, APRI is also found to be a prognostic marker in PBC patients independent of UDCA response[26]. This is attributed to its ability to not only capture fibrosis/cirrhosis, but also to reflect other biologically significant pathways like hepatic necroinflammation or non-cirrhotic portal hypertension[26-28]. Both APRI at baseline and APRI at 1 year after treatment (APRI-r1) have been shown to predict adverse outcomes (liver transplantation and/or death) in PBC patients[26]. In addition, when combined with the treatment response criteria, APRI-r1 can further stratify the risk of adverse outcomes and improve the predictive performances[26,29].

However, whether APRI can predict HCC risk in PBC patients remains uncertain. We aimed to demonstrate the role of APRI, alone and in combination with treatment response, in predicting HCC development in PBC patients receiving UDCA.

MATERIALS AND METHODS

Study subjects

PBC patients who followed up at the Clinic of Hepatology Unit of Queen Mary Hospital (QMH), a tertiary referral center, between January 2000 and October 2015 were recruited.

As all patients with PBC were prescribed with UDCA in QMH, we first identified patients receiving UDCA between 2000 and 2015 by searching the electronic

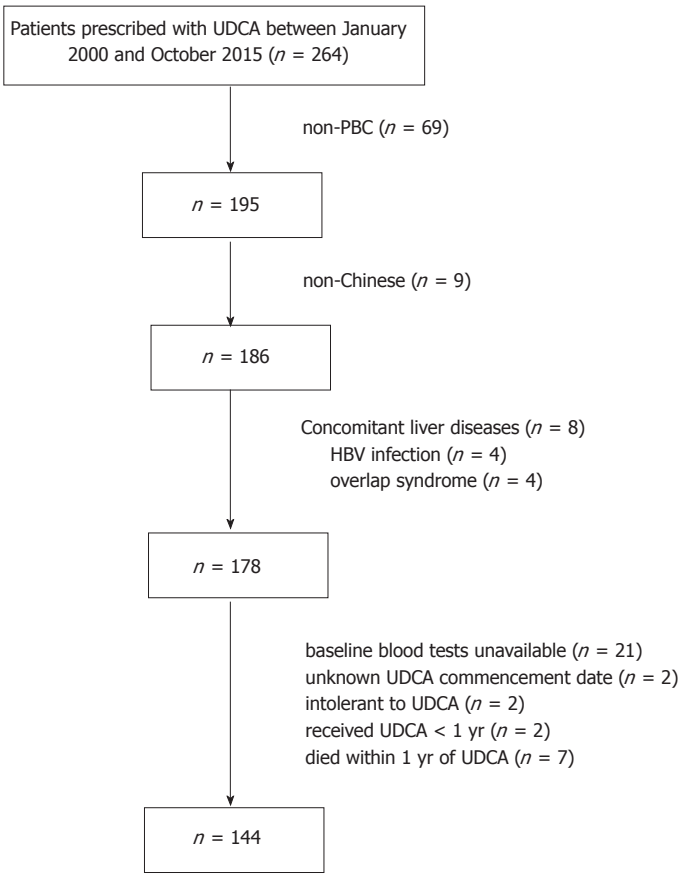

Figure 1 Flowchart illustrating case search and identification process. HBV: Hepatitis B virus; PBC: Primary biliary cholangitis; UDCA: Ursodeoxycholic acid.

medical database of QMH. Subsequently, we excluded non-PBC cases by reviewing the patient records, based on the criteria described in the subsequent section. Other exclusion criteria included non-Chinese ethnicity, cases with UDCA prescription for less than 1 year, overlap syndrome[30] and other coexisting hepatic diseases including CHB and CHC infection, steatohepatitis, alcoholic liver disease, and Wilson's disease. Figure 1 illustrates the patient recruitment process.

Ethics approval was issued by the Institutional Review Board, The University of Hong Kong and West Cluster of Hospital Authority, Hong Kong.

Diagnosis of PBC

A diagnosis of PBC was made if two out of the following three criteria were fulfilled: (1) cholestatic liver function pattern with raised alkaline phosphatase (ALP) ⩾ 1.5 times the upper limit of normal (ULN); (2) presence of anti-mitochondrial antibody (AMA); and (3) liver biopsy showing the histology of "nonsuppurative destructive cholangitis with destruction of interlobular biliary ducts"[5]. For liver biopsy cases, histologic staging was reported in accordance with Ludwig *et al*[31].

The Paris Ⅱ criteria were proposed by Corpechot *et al*[13] for predicting adverse events in PBC patients with early-stage disease. Early PBC can be defined either histologically (Ludwig's stages Ⅰ and Ⅱ) or biochemically (normal levels of albumin and bilirubin).

Diagnosis of adverse events

Patients had regular follow-up every 3 to 6 mo to monitor the platelet count, liver biochemistry, prothrombin time (PT) and alpha-fetoprotein level. Patients were recommended for ultrasonography (USG) of the liver every 6 mo for HCC[32].

A diagnosis of HCC was made by histology and/or imaging features [*i.e.* arterial enhancement and venous wash-out on triphasic computed tomography (CT) scan or magnetic resonance imaging (MRI)].

A diagnosis of cirrhosis was made by any one of the following: (1) imaging (USG, CT or MRI) showing small liver with surface nodularity, or signs of portal hypertension (including splenomegaly, ascites and varices); (2) fibrosis score > 16.9 kPa on transient elastography[33]; and (3) clinical features including thrombocytopenia, prolonged prothrombin time, ascites, varices, hepatic encephalopathy and hepatorenal syndrome.

APRI was calculated by the following formula proposed by Wai *et al*[16]: [(AST value/ULN)/platelet count (10^9/L)] × 100.

Suboptimal treatment response to UDCA

For the initial analysis, we used the Rotterdam criteria (abnormal levels of bilirubin or albumin)

to define suboptimal treatment response. This is because the Rotterdam criteria were shown to have better predictive performances than other treatment response criteria in predicting requirement for liver transplantation and death in Chinese PBC patients[34,35]. Analyses by using other treatment response criteria were also performed. Table 1 illustrates the description of other prognostic models[26]. Combination of APRI-r1 with treatment response could further stratify PBC patients into low-risk (APRI-r1 $\leqslant$ 0.54 with treatment response), intermediate-risk (APRI-r1 $\leqslant$ 0.54 with suboptimal treatment response, or APRI-r1 > 0.54 with treatment response) and high-risk (APRI-r1 > 0.54 with suboptimal treatment response) groups of developing adverse events in terms of liver transplantation or death.

Table 1 Descriptions of prognostic risk models for primary biliary cholangitis

	Time of evaluation	Definition of suboptimal treatment response
Rotterdam	1 yr	Abnormal bilirubin and/or albumin
Paris Ⅰ	1 yr	ALP $\geqslant$ 3 × ULN or AST $\geqslant$ 2 × ULN or bilirubin > 1 mg/dL
Paris Ⅱ	1 yr	ALP > 1.5 × ULN or AST > 1.5 × ULN or bilirubin > 1 mg/dL
Barcelona	1 yr	ALP > 1 × ULN and decrease in ALP < 40%
Toronto	2 yr	ALP > 1.67 × ULN
APRI	Baseline	AST/ULN of AST/platelet ($\times 10^9$) × 100
APRI-r1	1 yr	AST/ULN of AST/platelet ($\times 10^9$) × 100

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: AST to platelet ratio index; APRI-r1: APRI at 1-year; AST: Aspartate aminotransferase; PBC: Primary biliary cholangitis; ULN: Upper limit of normal.

Statistical analysis

Statistical analyses were performed using R version 3.2.3 (R Foundation for Statistical Computing) statistical software. We expressed continuous variables in terms of median and interquartile range (IQR). The correlation between continuous variables was assessed by Spearman's bivariate correlation. We used Mann-Whitney *U*-test to assess the difference in continuous variables of two groups. We used χ^2 test or Fisher's exact test for the comparison of categorical variables. The hazard ratio (HR) of HCC with different variables was derived from the Cox proportional hazards model. Patients not meeting the clinical endpoint (HCC) were censored at latest follow-up or death. The follow-up duration was calculated from the date of diagnosis to the censored date. Missing data in the Cox model were handled by multiple imputation, wherein 50 complete datasets were constructed by imputing the missing values[36]. The development of HCC was analyzed by the Kaplan-Meier method, and statistical significance was determined by the log-rank test. By plotting "sensitivity" against "1-specificity", the receiver operating curve was generated. The performances of different models were expressed by area under the receiver operating curve (AUROC), with the 95% CI being deduced from bootstrapping by sampling with replacement from the original dataset and repeating the process by 1000 times. A two-sided *P*-value of < 0.05 was used to define statistical significance.

RESULTS

Characteristics of study patients

One hundred and forty-four PBC patients were recruited, and 127 were female (88.2%). Table 2 shows the patient characteristics, laboratory and histology results. Patients were diagnosed at a median age of 57.8 years (IQR: 48.7 to 71.5 years). The median follow-up duration was 6.9 years (range: 1.0 to 26.3 years), making a total of 1136 patient-years. Twelve patients developed HCC, with an incidence rate of 10.6 cases per 1000 patient-years. Ten patients underwent liver transplantations, and there were 40 deaths (21 were liver-related and 19 were non-liver-related).

Cirrhosis was noted in 41 patients (28.5%) before treatment commencement, while the median APRI and APRI-r1 levels of the cohort were 1.00 (0.60 to 1.84) and 0.22 (0.13 to 0.43), respectively. A significantly higher proportion of patients who developed HCC had baseline cirrhosis compared with the non-HCC group (66.7% *vs* 25.0%, *P* = 0.005). The HCC group also had a higher median APRI-r1 level (0.54 *vs* 0.20, *P* = 0.002), with a larger proportion having APRI-r1 > 0.54 (50.0% *vs* 16.7%, *P* = 0.013). The difference in median APRI levels between the two groups was of borderline significance (2.02 *vs* 0.97, *P* = 0.050), while no significant difference existed for the proportions of patients with APRI > 0.54 (90.0% *vs* 77.5%, *P* = 0.689). For other prognostic scores, the HCC group had a higher median Mayo risk score (5.1 *vs* 4.6, *P* = 0.022), while no significant differences existed for the model for end-stage liver disease (MELD) or Child-Pugh (CP) scores between the two groups.

Patients were prescribed with UDCA at a median dose of 750 mg. The number of patients who had suboptimal treatment response was as follows: 61 (42.4%; Rotterdam criteria), 52 (36.1%; Paris Ⅰ criteria), 48 (33.3%; Barcelona criteria) and 50 (38.8%; Toronto criteria). None of our patients received fibric acid derivatives.

Liver biopsies were performed in 62 patients. Out of the 52 patients with histology reports available, 21 were regarded as having early-stage PBC. If only the biochemical criteria were considered, 52 patients had early-stage disease. None of these patients developed HCC, and therefore analysis could not be performed using the Paris Ⅱ criteria.

Correlation between baseline APRI and other variables

APRI had positive correlations with levels of AST (*r* = 0.86, *P* < 0.001), ALT (*r* = 0.68, *P* < 0.001), bilirubin

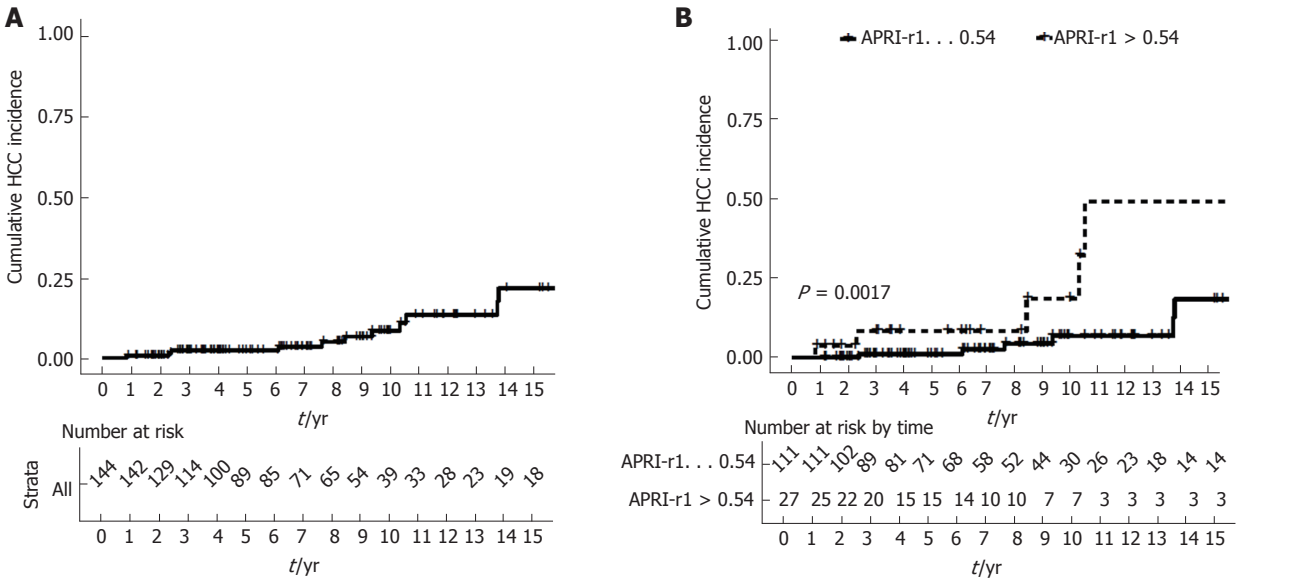

Figure 2 Cumulative hepatocellular carcinoma incidence. A: Whole cohort; B: Stratified by APRI-r1. APRI-r1: AST/platelet ratio index at 1-year.

Table 2 Baseline characteristics of the study cohort

Variable	Whole cohort, *n* = 144	Patients with HCC, *n* = 12	Patients without HCC, *n* = 132	*P* value
Age, yr	57.8 (48.7-71.5)	68.1 (56.2-74.6)	57.0 (48.2-70.7)	0.278
Female sex	127 (88.2)	9 (75.0)	118 (89.4)	0.153
Duration of follow-up, yr	6.9 (3.5-10.4)	8.9 (5.2-11.4)	6.8 (3.5-10.1)	0.499
Ursodeoxycholic acid, mg	750 (750-750)	750 (750-750)	750 (750-750)	0.576
Suboptimal treatment response, Rotterdam criteria	61 (42.4)	9 (75.0)	52 (39.4)	0.017[3]
Diabetes	29 (20.1)	6 (50.0)	23 (17.4)	0.016[3]
Smoking[1]	13 (9.5)	4 (33.3)	9 (6.8)	0.011[3]
Alcohol[1]	17 (13.7)	2 (16.7)	15 (11.4)	0.623
Cirrhosis	41 (28.5)	8 (66.7)	33 (25.0)	0.005[3]
Histological stage 3-4[2]	23 (44.2)	3 (50.0)	20 (43.5)	1.00
Platelet, x 10^9/L[1]	216 (152-262)	133 (95-150)	229 (175-266)	< 0.001[3]
Creatinine, μmol/L[1]	69 (60-82)	73 (60-79)	68 (60 - 82)	0.047[3]
Albumin, g/L[1]	40 (36-42)	24 (14-30)	40 (36-42)	0.087
Bilirubin, μmol/L[1]	14 (10-26)	30 (19-55)	14 (10-26)	< 0.001[3]
ALP (U/L)	284 (196-484)	343 (227-362)	273 (196-496)	0.991
ALT, U/L[1]	74 (54-130)	85 (64-109)	74 (53-133)	0.565
AST, U/L[1]	68 (51-115)	76 (56-109)	68 (51-115)	0.741
GGT, U/L[1]	517 (256-771)	626 (353-843)	490 (224-760)	0.285
PT, s	11.3 (10.5-11.7)	11.8 (11.7-12.5)	11.2 (10.5-11.7)	0.007[3]
AMA positivity	119 (82.6)	8 (66.7)	111 (84.1)	0.223
Globulin, mg/dL[1]	41 (37-46)	40 (37-44)	41 (37-46)	0.337
IgM, mg/dL[1]	363 (250-502)	446 (282-579)	359 (250-478)	0.563
Mayo risk score[1]	4.7 (3.8-5.5)	5.1 (4.8-6.6)	4.6 (3.8-5.4)	0.022[3]
MELD score	6 (6-8)	8 (6-9)	6 (6-7)	0.097
CP score[1]	5 (5-6)	6 (5-6)	5 (5-6)	0.125
CP class B/C[1]	29 (20.1)	2 (16.7)	25 (19.2)	1.00
APRI	1.00 (0.60-1.84)	2.02 (1.05-3.34)	0.97 (0.59-1.72)	0.05[3]
APRI > 0.54[1]	102 (78.5)	9 (90.0)	93 (77.5)	0.689
APRI-r1[1]	0.22 (0.13-0.43)	0.54 (0.31-0.70)	0.20 (0.13-0.38)	0.002[3]
APRI-r1> 0.54[1]	27 (19.6)	6 (50.0)	21 (16.7)	0.013[3]

Data are presented as *n* (%), and all continuous variables are expressed as median (interquartile range). [1]Missing data: smoking (7), alcohol (3), platelet (10), creatinine (1), albumin (2), bilirubin (2), ALT (2), AST (6), GGT (2), globulin (6), IgM (20), Mayo risk score (2), CP score (2), APRI (14), APRI-r1 (6); [2]Sixty-two patients had liver biopsies done, with reports available for review for 52; [3]*P* values < 0.05. All continuous variables expressed in median (interquartile range). ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AMA: Anti-mitochondrial antibody; APRI: AST/platelet ratio index; APRI-r1: APRI at 1 year after treatment; AST: Aspartate aminotransferase; CP: Child-Pugh; GGT: Gamma-glutamyl transferase; IgM: Immunoglobulin M; MELD: Model for end-stage liver disease; PT: Prothrombin time.

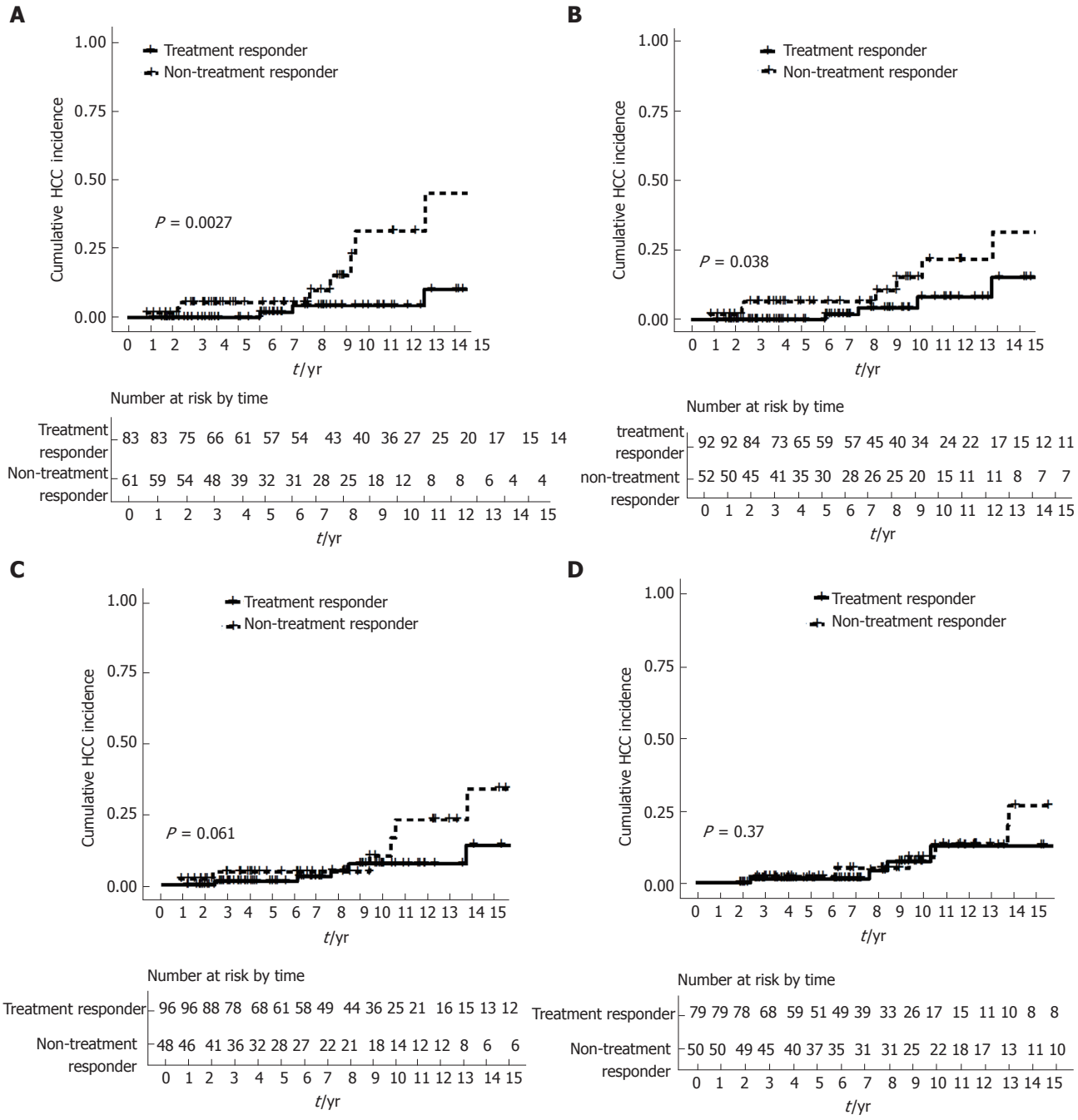

Figure 3 Cumulative hepatocellular carcinoma incidence stratified by treatment response. A: Rotterdam criteria; B: Paris I criteria; C: Barcelona criteria; D: Toronto criteria.

(r = 0.43, P < 0.001), ALP (r = 0.31, P < 0.001) and gamma-glutamyl transferase (GGT) (r = 0.31, P < 0.001). It had negative correlations with platelet counts (r = -0.43, P < 0.001) and albumin levels (r = -0.27, P = 0.002). The correlation between APRI and PT was of borderline significance (r = 0.17, P = 0.052). For the correlations with other prognostic models, there were positive correlations between APRI and Mayo risk score (r = 0.32, P < 0.001) and CP score (r = 0.43, P < 0.001), but not for the MELD score (r = 0.12, P = 0.664).

HCC risk factors

Table 3 shows the association between HCC development and various factors. On univariate analysis, significant factors for HCC development included older age, male sex, presence of cirrhosis, hypoalbuminemia and suboptimal treatment response (defined by the Rotterdam criteria). On multivariate analysis, only older age (HR = 1.07; 95%CI: 1.02-1.12), cirrhosis (HR = 4.38; 95%CI: 1.06-18.14) and APRI-r1 > 0.54 (HR = 3.94; 95%CI: 1.04-14.94) were independent risk factors (Table 4). Suboptimal treatment response was

Table 3 HRs and 95%CIs for the association between hepatocellular carcinoma development and different variables

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, yr	1.06	1.01-1.11	0.016[2]	1.07	1.02-1.12	0.004[2]
Male sex	5.22	1.27-21.44	0.022[2]	3.67	0.69-19.56	0.128
Diabetes mellitus	3.01	0.96-9.44	0.058			
Cirrhosis	8.02	2.35-27.29	< 0.001[2]	4.38	1.06-18.14	0.041[2]
APRI > 0.54	3.43	0.43-27.19	0.243			
APRI-r1 > 0.54	5.10	1.64-15.86	0.005[2]	3.94	1.04-14.94	0.043[2]
Creatinine, μmol/L	1.02	0.99-1.05	0.222			
Albumin, g/L	0.85	0.75-0.96	0.007[2]			
Bilirubin, μmol/L	1.01	0.98-1.03	0.514			
ALP, U/L	0.997	0.994-1.00	0.104			
ALT, U/L	0.996	0.987-1.00	0.331			
AST, U/L	0.996	0.975-1.01	0.467			
GGT, U/L	1.00	0.999-1.001	0.975			
PT, s	1.40	0.99-1.98	0.060[2]			
AMA positivity	0.52	0.16-1.75	0.292			
Globulin, mg/dL	0.99	0.90-1.08	0.804			
IgM, mg/dL	1.00	0.997-1.002	0.830			
Suboptimal treatment response, Rotterdam criteria[1]	5.95	1.59-22.26	0.008[2]	2.18	0.45-10.58	0.334

[1]In the multivariate analyses, albumin was not included as this variable was already included in the Rotterdam criteria. [2]*P* values < 0.05. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AMA: Anti-mitochondrial antibody; APRI: AST to platelet ratio index; APRI-r1: APRI at 1 year after treatment; AST: Aspartate aminotransferase; CI: Confidence interval; GGT: Gamma-glutamyl transferase; HR: Hazard ratio; IgM: Immunoglobulin M; PT: Prothrombin time.

Table 4 Adjusted HRs and 95%CIs for the association between hepatocellular carcinoma development and different variables

Criteria	HR	95%CI	*P* value
Rotterdam			
Age	1.07	1.02-1.12	0.004[1]
Male sex	3.67	0.69-19.56	0.128
Cirrhosis	4.38	1.06-18.14	0.041[1]
APRI-r1 > 0.54	3.94	1.04-14.94	0.043[1]
Suboptimal treatment response	2.18	0.45-10.58	0.334
Paris Ⅰ			
Age	1.07	1.02-1.12	0.003[1]
Male sex	3.04	0.54-17.12	0.207
Albumin	0.94	0.80-1.09	0.386
Cirrhosis	4.37	1.07-17.75	0.039[1]
APRI-r1 > 0.54	3.92	1.06-14.54	0.041[1]
Suboptimal treatment response	1.7	0.41-7.03	0.466
Barcelona			
Age	1.07	1.02-1.12	0.005[1]
Male sex	3.26	0.56-18.96	0.188
Albumin	0.93	0.80-1.07	0.307
Cirrhosis	4.44	1.06-18.56	0.041[1]
APRI-r1 > 0.54	4.47	1.26-15.93	0.021[1]
Suboptimal treatment response	1.22	0.33-4.49	0.768
Toronto			
Age	1.07	1.02-1.13	0.003[1]
Male sex	3.22	0.56-18.50	0.19
Albumin	0.94	0.80-1.09	0.425
Cirrhosis	4.56	1.09-19.17	0.038[1]
APRI-r1 > 0.54	4.16	1.10-15.69	0.036[1]
Suboptimal treatment response	1.46	0.31-6.89	0.631

[1]*P*-values < 0.05. The adjusted HR for suboptimal response was derived by multivariate analysis with other significant variables in Table 3 (age, male sex, cirrhosis and albumin) included. Separate multivariate analysis was performed for each criteria in defining suboptimal response. In the multivariate analyses, albumin was not included for the Rotterdam criteria. APRI-r1: AST/platelet ratio index at 1-year; HR: Hazard ratio.

not a significant independent risk factor irrespective of which treatment response criteria being used.

Patients were further stratified into low-, intermediate- and high-risk groups. High-risk patients (APRI-r1 > 0.54 with suboptimal biochemical response) had the highest risk of developing HCC, with consistent results for the Rotterdam, Paris Ⅰ and Barcelona criteria, while the difference was of borderline significance using the Toronto criteria (Table 5).

HCC cumulative incidence

The 5-, 10- and 15-year cumulative incidences of HCC were 2.3% (95%CI: 0%-4.8%), 8.4% (95%CI: 1.8%-14.5%) and 21.6% (6.8%-34.1%), respectively (Figure 2A).

Cumulative incidence of HCC was significantly higher for patients with APRI-r1 > 0.54 (log rank *P* = 0.002; Figure 2B). Among patients with APRI-r1 ⩽ 0.54, the 5-, 10- and 15-year cumulative incidences of HCC were 2.5%(95%CI: 0%-3.0%), 6.7%(95%CI: 0%-13.1%) and 18.3% (95%CI: 0.4%-33.0%), respectively. Among patients with APRI > 0.54, the 5-, 10- and 15-year cumulative incidences of HCC development were 8.3% (95%CI: 0%-18.7%), 18.5% (95%CI: 0%-37.2%) and 49.0% (95%CI: 0%-75.2%).

Cumulative incidence of HCC was significantly higher for patients who had suboptimal biochemical response by using the Rotterdam (log rank *P* = 0.003) and Paris I criteria (log rank *P* = 0.038). The difference was of borderline significance by using the Barcelona criteria (log rank *P* = 0.061), while there was no significant difference by using the Toronto criteria (log

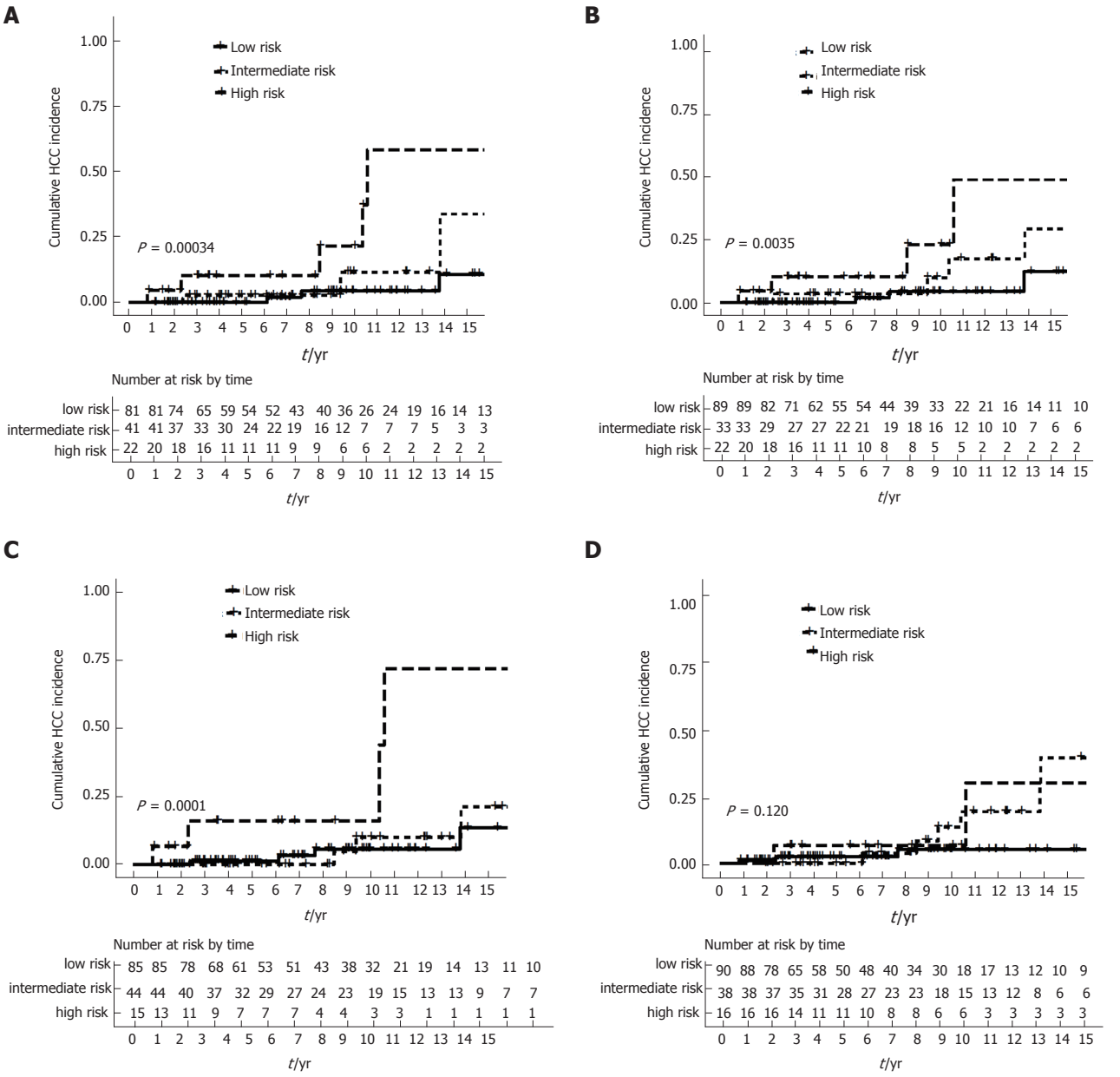

Figure 4 Kaplan-Meier survival plot stratified by APRI-r1 and treatment response. A: Rotterdam criteria; B: Paris I criteria; C: Barcelona criteria; D: Toronto criteria. APRI-r1: AST/platelet ratio index at 1-year; low-risk (biochemical response with APRI-r1 ≤ 0.54), intermediate risk (suboptimal biochemical response with APRI-r1 ≤ 0.54, or biochemical response with APRI-r1 > 0.54) and high risk (suboptimal biochemical response with APRI-r1 > 0.54).

rank $P = 0.370$) (Figure 3A-D).

Using the combination of APRI-r1 and biochemical response to define low-, intermediate- and high-risk groups further stratified HCC risk (all log rank $P < 0.05$), with the exception for the Toronto criteria (log rank $P = 0.120$) (Figure 4A-D). When APRI-r1 was combined with treatment response as defined by the Rotterdam criteria, the 5-, 10- and 15-year cumulative incidences of HCC were 0%, 4.3% (95%CI: 0%-10.0%) and 10.3% (95%CI: 0%-22.0%), respectively among the low-risk group. For the intermediate-risk group, the 5-year, 10- and 15-year cumulative incidences of HCC were 2.7% (95%CI: 0%-7.8%), 11.5% (95%CI: 0%-27.2%) and 33.7% (95%CI: 0%-63.5%), respectively. The high-risk group was at the highest risk, with the 5-, 10- and 15-year cumulative incidences of HCC being 10.2% (95%CI: 0%-22.7%), 21.4% (95%CI: 0%-41.9%) and 58.1% (95%CI: 0%-84.0%), respectively.

Predictive performances of various prognostic models

Table 6 shows the predictive performances of various prognostic models. APRI-r1 had the best performance in predicting HCC development (AUROC = 0.77, 95%CI: 0.64-0.88), although the 95%CI overlapped with that of Mayo risk score (AUROC = 0.70, 95%CI: 0.54-0.84), cirrhosis (AUROC = 0.71, 95%CI: 0.56-0.86) and thrombocytopenia (< 150 × 10^9/L) (AUROC = 0.75, 95%CI: 0.58-0.90). APRI, MELD CP scores and hyperbilirubinemia (> 17 μmol/L) did not

Table 5 Prediction of hepatocellular carcinoma development by APRI-r1 in combination with suboptimal treatment response

Criteria		Univariate analysis				Multivariate analysis[1]			
		HR	95%CI	*P* value	*P* trend	HR	95%CI	*P* value	*P* trend
Rotterdam									
Low-risk	Ref		-	-		Ref	-	-	
Intermediate-risk	2.81		0.56-14.01	0.208	< 0.001[2]	1.54	0.25-9.63	0.644	0.006[2]
High-risk	10.29		2.55-41.48	0.001[2]		7.95	1.56-40.45	0.012[2]	
Paris I									
Low-risk	Ref		-	-		Ref	-	-	
Intermediate-risk	2.81		0.63-12.60	0.177	0.003[2]	2.34	0.40-13.60	0.345	0.013[2]
High-risk	8.38		1.99-35.21	0.004		7.28	1.45-36.71	0.016[2]	
Barcelona									
Low-risk	Ref		-	-		Ref	-	-	
Intermediate-risk	1.28		0.29-5.72	0.75	0.002[2]	0.53	0.08-3.34	0.496	0.038[2]
High-risk	10.66		2.85-39.89	< 0.001[2]		5.54	1.29-23.71	0.021[2]	
Toronto									
Low-risk	Ref		-	-		Ref	-	-	
Intermediate-risk	3.25		0.81-13.06	0.097	0.052	4.4	0.97-19.90	0.055	0.061
High-risk	4.22		0.85-20.97	0.079		4.77	0.78-29.24	0.091	

[1]The adjusted HR for suboptimal response was derived by multivariate analysis with other significant variables in Table 3 (age, male sex, cirrhosis and albumin) included; [2]*P*-values < 0.05. Separate multivariate analysis was performed for each criteria in defining suboptimal response. In the multivariate analyses, albumin was not included for the Rotterdam criteria, as this variable was already included in the criteria. APRI-r1: AST/platelet ratio index at 1-year; CI: Confidence interval; HR: Hazard ratio.

Table 6 Predictive performances of prognostic models for hepatocellular carcinoma development

Categorical variable	Rotterdam	Paris I	Barcelona	Toronto	Cirrhosis	Thrombocytopenia, < 150 × 10^9/L	Hyperbilirubinemia, > 17 mmol/L
AUROC	0.68	0.67	0.64	0.64	0.71	0.75	0.64
(95%CI)	(0.52-0.80)	(0.52-0.81)	(0.48-0.78)	(0.47-0.78)	(0.56-0.86)	(0.58-0.90)	(0.49-0.77)
Sensitivity	75.00%	66.60%	58.30%	63.60%	66.70%	70.00%	66.70%
Specificity	60.60%	66.60%	68.90%	63.60%	75.00%	79.00%	60.80%
PPV	14.80%	15.40%	14.60%	14.00%	19.50%	21.20%	13.60%
NPV	96.40%	95.70%	94.80%	94.90%	96.10%	97.00%	84.90%
Continuous variable	APRI	APRI-r1	Mayo risk score	MELD score	CP score		
AUROC	0.68	0.77	0.7	0.63	0.62		
(95%CI)	(0.49-0.87)	(0.64-0.88)	(0.54-0.84)	(0.43-0.79)	(0.47-0.76)		
Sensitivity	-	-	-	-	-		
Specificity	-	-	-	-	-		
PPV	-	-	-	-	-		
NPV	-	-	-	-	-		

APRI: AST to platelet ratio index at baseline; APRI-r1: APRI at 1-year; AUROC: Area under receiver operating curve; CP: Child-Pugh; MELD: Model for end-stage liver disease; NPV: Negative predictive value; PPV: Positive predictive value.

have satisfactory performances, as the AUROCs were less than 0.70, with the 95%CI crossing 0.50.

The performances of various treatment response criteria were also unsatisfactory (all AUROCs less than 0.70), with the Rotterdam and Paris I criteria showing comparable AUROCs (around 0.68), while the 95%CI of the AUROCs of the Barcelona and Toronto criteria crossed 0.50. The Rotterdam criteria had the highest sensitivity and negative predictive value.

DISCUSSION

In the current study, a total of 144 Chinese PBC patients with UDCA use were recruited. A study with the same cohort of patients assessed by different prognostic models for prediction of long-term transplant-free survival was recently published[34]. Our patients were diagnosed at a slightly older age than that reported in the West (median: 57.8 *vs* 54.5 years)[37]. The female preponderance (88% of our cohort) and the treatment response (33%-42%) were consistent with those reported in the West[5,12].

APRI is widely used in the assessment of fibrosis/cirrhosis in various kinds of hepatic diseases (CHB and CHC infection, alcoholic liver disease and non-alcoholic fatty liver disease)[16-22]. A recent study proposed that APRI could be used to predict adverse outcomes in PBC patients[26]. However, its potential role in HCC prediction in PBC patients is still not clear. A meta-analysis reports an 18.8-fold increase of HCC risk in PBC patients compared with the general population[38], but it suggests that HCC may not be as common in PBC patients compared with other liver diseases[39]. Therefore, a non-invasive test that is simple and cost-

effective will be of significant clinical importance in the management of PBC patients.

Our study showed that APRI correlated with adverse liver function (in terms of both laboratory parameters and also traditional PBC prognostic models - Mayo risk score and CP score). In addition, a higher APRI-r1 level was associated with a higher HCC risk. Although being regarded as a fibrosis/cirrhosis marker, APRI-r1 retained the association with HCC development despite the inclusion of cirrhosis into the multivariate analysis (adjusted HR = 3.94). This is likely because APRI has an additional role of reflecting other pathological pathways including liver inflammation and non-cirrhotic portal hypertension[26-28].

Suboptimal treatment response was not an independent risk factor, in contrary to that reported by Trivedi *et al*[10]. There are a few possible reasons to account for this. First, as disease stage is known to affect the treatment response[11,12], the effect of treatment response on HCC risk would be attenuated by including APRI and cirrhosis into the multivariate analysis. Second, our study might not be adequately powered to detect this effect given the limited number of events. However, by combining both factors (APRI-r1 and treatment response), the HCC risk of individuals could be further stratified into low-risk, intermediate-risk and high-risk.

Hyperbilirubinemia was recognized to be a risk factor for liver transplantation and death in PBC patients in previous studies[37,40,41], but was not a significant independent risk factor for HCC development in the current study. This is likely related to the fact that hyperbilirubinemia in patients with PBC can also be due to cholestasis, while APRI is more specifically related to fibrosis/cirrhosis.

Our study also determined the predictive performances of APRI and APRI-r1 in addition to the traditional prognostic models and treatment response criteria. We showed that APRI-r1 had a satisfactory performance, with an AUROC of 0.77. It appears that APRI-r1 outperformed other prognostic models, although the result should be interpreted with caution as there was overlapping of the 95%CIs of some models (*e.g.,* Mayo risk score) due to the relatively small sample size of our cohort.

Cases were identified by searching the electronic database system of the hospital with subsequent review of the clinical records. This ensures the accurate and complete capture of all PBC cases. Another noticeable strength of the study is the long follow-up duration (up to 26 years), since a long lag time is usually required from PBC diagnosis to HCC development. Moreover, the inclusion of a homogenous group of Chinese patients and exclusion of concomitant liver diseases removed the confounding factors of ethnicity and hepatitis due to other liver diseases.

A few limitations are present in the current study. First, a relatively small sample size may render the study underpowered to confirm a significant association of some factors with HCC development (*e.g.,* smoking and alcohol), although previous study also failed to show an association with these factors[42]. Second, since this study was carried out in a tertiary center, selection bias may account for the higher HCC incidence rate in our cohort (10.6 cases per 1000 person-years). On the contrary, the study by Trivedi *et al*[10], which was a multi-center study involving 4565 patients, reported an incidence rate of only 3.4 cases per 1000 person-years. Third, our study recruited only Asian subjects, and therefore the applicability of this finding to other ethnicities remains to be investigated. Fourth, as this is only a single-center study with a relatively small sample size, validation studies from other centers are necessary. Lastly, while APRI was shown to be of both predictive and prognostic values in chronic viral hepatitis patients[23-25], studies on the usefulness of this marker in other chronic liver diseases are still lacking. Consistent findings are expected as fibrosis/cirrhosis is the major risk factor for HCC development, although further studies on other chronic liver diseases are warranted.

In conclusion, our study confirmed the usefulness of APRI-r1 in predicting HCC development in PBC patients receiving UDCA. The combination of APRI-r1 with treatment response allowed further stratification of HCC risk. Owing to its non-invasiveness and cost-effectiveness, APRI can be used as a marker to streamline the HCC surveillance protocol in PBC patients.

ARTICLE HIGHLIGHTS

Research background

No reliable predictive models exist for hepatocellular carcinoma (HCC) in primary biliary cholangitis (PBC). Aspartate aminotransferase (AST) to platelet ratio index (APRI) not only captures fibrosis/cirrhosis, but also reflects other biologically significant pathways like hepatic necroinflammation or non-cirrhotic portal hypertension. The usefulness of APRI in predicting HCC in PBC remains unknown.

Research motivation

A predictive marker for HCC development in PBC patients will help disease prognostication and streamline the follow-up and HCC surveillance strategy.

Research objectives

To investigate the usefulness of APRI in predicting HCC in PBC.

Research methods

The authors recruited all PBC patients who had follow-up in the Hepatology Clinic of Queen Mary Hospital (QMH) between January 2000 and October 2015. Patients were followed up every 3 to 6 mo with regular monitoring of platelet count, liver biochemistry, prothrombin time and alpha-fetoprotein level. In the analysis of the risk factors for adverse events, suboptimal response to UDCA was identified by using various treatment response criteria. APRI-r1 in combination with treatment response criteria enables further stratification of PBC patients into low-risk (biochemical response with APRI-r1 $\leqslant$ 0.54), intermediate-risk (suboptimal biochemical response with APRI-r1 $\leqslant$ 0.54, or biochemical response with APRI-r1 > 0.54) and high-risk (suboptimal biochemical response with APRI-r1 > 0.54) groups of developing adverse

outcomes in terms of liver transplantation or death. The Cox proportional hazards model was used to determine the hazard ratio (HR) of HCC with different variables. The Kaplan-Meier method was used to analyze the development of HCC. The performances of various prognostic models were expressed in terms of area under the receiver operating curve (AUROC).

Research results

A total of 144 patients were identified. The median age at diagnosis was 57.8 years (interquartile range: 48.7-71.5 years), and 41 (28.5%) had baseline cirrhosis. The median follow-up duration was 6.9 years (range: 1.0-26.3 years), with a total of 1136 patient-years. Twelve patients developed HCC, with an incidence rate of 10.6 cases per 1000 patient-years. The overall 5-, 10- and 15-year probabilities of HCC development were 2.3% [95% confidence interval (CI): 0%-4.8%], 8.4% (95%CI: 1.8%-14.5%) and 21.6% (6.8%-34.1%), respectively. Independent risk factors for HCC development were older age (HR = 1.07), cirrhosis (HR = 4.38) and APRI at 1 year after treatment (APRI-r1) > 0.54 (HR = 3.94). APRI-r1 in combination with treatment response further stratified the risk of HCC development (log rank $P < 0.05$). The AUROC of APRI-r1 in predicting HCC was 0.77 (95%CI: 0.64-0.88).

Research conclusions

APRI-r1 can be used as a predictive marker for HCC development in PBC patients. Combination of APRI-r1 with treatment response can further stratify the HCC risk.

Research perspectives

Our study confirmed the usefulness of APRI-r1 in predicting HCC development in PBC patients receiving UDCA. The combination of APRI-r1 with treatment response allowed further stratification of HCC risk. Owing to its non-invasiveness and cost-effectiveness, APRI can be used as a marker to streamline the HCC surveillance protocol in PBC patients. Future multi-center studies with larger sample size are warranted to confirm our findings.

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ORIGINAL ARTICLE

Clinical Trials Study

Role of combined propofol and sufentanil anesthesia in endoscopic injection sclerotherapy for esophageal varices

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Author contributions: Yu Y and Zhang Y designed research; Yu Y and Qi SL performed research and analyzed data; Yu Y wrote paper.

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Abstract

AIM

To investigate the efficacy and safety of a combination of sufentanil and propofol injection in patients undergoing endoscopic injection sclerotherapy (EIS) for esophageal varices (EVs).

METHODS

Patients with severe EVs who underwent EIS with sufentanil and propofol anesthesia between April 2016 and July 2016 at our hospital were reviewed. Although EIS and sequential therapy were performed under endotracheal intubation, we only evaluated the efficacy and safety of anesthesia for the first EIS procedure. Patients were intravenously treated with 0.5-1 μg/kg sufentanil. Anesthesia was induced with 1-2 mg/kg propofol and maintained using 2-5 mg/kg per hour of propofol. Information, regarding age, sex, weight, American Association of Anesthesiologists (ASA) physical status, Child-Turcotte-Pugh (CTP) classification, indications, preanesthetic problems, endoscopic procedure, successful completion of the procedure, anesthesia time, recovery time, and anesthetic agents, was recorded. Adverse events, including hypotension, hypertension, bradycardia, and hypoxia, were also noted.

RESULTS

Propofol and sufentanil anesthesia was provided in 182 procedures involving 140 men and 42 women aged 56.1

± 11.7 years (range, 25-83 years). The patients weighed 71.4 ± 10.7 kg (range, 45-95 kg) and had ASA physical status classifications of Ⅱ (79 patients) or Ⅲ (103 patients). Ninety-five patients had a CTP classification of A and 87 had a CTP classification of B. Intravenous anesthesia was successful in all cases. The mean anesthesia time was 33.1 ± 5.8 min. The mean recovery time was 12.3 ± 3.7 min. Hypotension occurred in two patients (1.1%, 2/182). No patient showed hypertension during the endoscopic therapy procedure. Bradycardia occurred in one patient (0.5%, 1/182), and hypoxia occurred in one patient (0.5%, 1/182). All complications were easily treated with no adverse sequelae. All endoscopic procedures were completed successfully.

CONCLUSION

The combined use of propofol and sufentanil injection in endotracheal intubation-assisted EIS for EVs is effective and safe.

Key words: Endoscopic injection; Esophageal varices; Propofol; Sclerotherapy; Sufentanil

Core tip: Propofol is widely used during painless endoscopy because of its rapid onset and rapid recovery properties. Intravenous injection of propofol during endoscopic esophageal varices therapy can reduce the complications associated with poor patient cooperation. Because of complications related to bleeding during endoscopic variceal ligation and endoscopic injection sclerotherapy (EIS), endotracheal intubation is essential for these procedures. However, due to its weak analgesic effect, intraoperative pain stimulation is greater, leading to overt physical movement, thus affecting the operation. Since analgesics are often required to ensure a successful operation, in this study, we used a combination of sufentanil and propofol injection for the endoscopic treatment of esophageal varices. In conclusion, sufentanil and propofol injection, with endotracheal intubation-assisted EIS is effective and safe.

Yu Y, Qi SL, Zhang Y. Role of combined propofol and sufentanil anesthesia in endoscopic injection sclerotherapy for esophageal varices. *World J Gastroenterol* 2017; 23(44): 7875-7880 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7875.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7875

INTRODUCTION

Esophageal varices (EVs), a form of port-systemic collateral vessels, occur as a result of portal hypertension. Rupture of EVs can cause variceal hemorrhage, which is among the most common lethal complications of liver cirrhosis[1]. Since the red color sign (RCS) is considered to be a sign of bleeding of EVs[2], identification of the RCS is thought to be necessary to prevent potentially fatal massive bleeding from EVs.

Although endoscopic findings are typically evaluated with the naked eye, this approach cannot be used to assess deep collateral vessels to identify the RCS. Therefore, endoscopic ultrasonography, which was developed to evaluate diseases of the mediastinum, is used to visualize the collateral channels that surround the distal esophagus and upper stomach[3-10], and may enhance variceal detection and improve therapeutic targeting[11-20]. Endoscopic variceal ligation (EVL) and endoscopic injection sclerotherapy (EIS) are the two major techniques used in endoscopic EV therapy[21].

Propofol is widely used during painless endoscopy because of its rapid onset and rapid recovery properties[22,23]. Intravenous injection of propofol during endoscopic EV therapy can reduce the complications associated with poor patient cooperation. Because of complications related to bleeding during EVL or EIS, endotracheal intubation is essential for these procedures. However, due to its weak analgesic effect, intraoperative pain stimulation is greater, leading to overt physical movements, thus affecting the operation. Since analgesics are often required to ensure a successful operation[24], in this study, we used a combination of sufentanil and propofol injection for the endoscopic treatment of EVs, and evaluated the safety and efficacy of this combination.

MATERIALS AND METHODS

Patients

Patients who underwent EIS for EVs at the Sixth People's Hospital of Dalian from April 2016 to July 2016 were enrolled in this study. The diagnosis of liver cirrhosis was based on histological or clinical factors. The patients had American Association of Anesthesiologists (ASA) classifications of Ⅱ (79 patients) or Ⅲ (103 patients) and Child-Turcotte-Pugh (CTP) classifications of A (95 patients) or B (87 patients). The inclusion criteria were EV grade ⩾ F2, as a prerequisite, and moderate to severe RCS, as indicated in the general rules for recording endoscopic findings for EV. The following cases were excluded: (1) emergency cases in which the EVs had ruptured; (2) cases in which anesthesia was not possible; (3) cases with portal venous obstruction; (4) cases with thrombocytopenia ($< 4 \times 10^4/\mu L$); and (5) cases with high CTP grades (C).

This study was approved by the Institutional Review Board and Ethics Committee of The Sixth People's Hospital of Dalian, Dalian, China. All patients voluntarily chose their own therapeutic course and provided written informed consent for their participation in this study.

Devices

Standard monitoring was performed in the endoscopy center and included electrocardiography, noninvasive arterial blood pressure monitoring, and pulse oximetry. Bispectral index (BIS) values (A2000 BIS XP monitor,

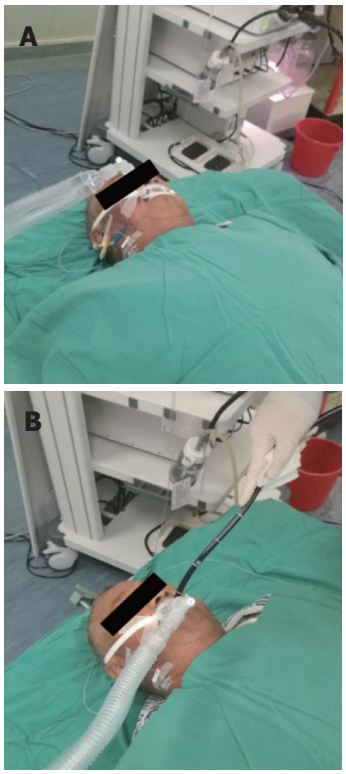

Figure 1 The patients were placed in the supine position. Electrocardiography, noninvasive arterial blood pressure monitoring, and pulse oximetry were performed as a part of the standard monitoring protocol. Sufentanil (0.5-1 μg/kg) was injected to induce mild sedation and restrain the stress response to intubation. Anesthesia was induced with 1-2 mg/kg of propofol and maintained with 2-5 mg/kg per hour of propofol. Scoline and cisatracurium were injected for the endotracheal intubation.

version 3.2; Aspect Medical System, Inc.; Newton, MA, United States) were obtained and recorded. The BIS smoothing period was set to 15 s.

EIS was performed using a standard endoscope (SV-290; Olympus Corporation; Tokyo, Japan). The endoscopic puncture needle used for EIS was a 23-gauge Varixer needle (Single Use Injector NM-400L-0423; Olympus Corporation; Tokyo, Japan). We performed 3-5 punctures per EV. We used lauromacrogol injection (10 mL/100 mg, Tianyu Pharmaceutical Co., Ltd., Xi'an, China) as the sclerosant in a low-cost and efficient EIS procedure that was technically easy to perform. All patients were treated by a single operator who had more than 10 years of experience as an endoscopist. The operator also had more than 5 years of experience as the main EIS operator.

Procedure

The patients were placed in the supine position. Electrocardiography, noninvasive arterial blood pressure monitoring, and pulse oximetry were performed. We injected 0.5-1 μg/kg sufentanil to induce mild sedation and suppressed the stress response during intubation. Anesthesia was induced using 1-2 mg/kg propofol and maintained using 2-5 mg/kg propofol per hour. Scoline and cisatracurium were injected as a part of the endotracheal intubation procedure. EIS was performed after endotracheal intubation (Figure 1).

Intravariceal injection sequential therapy was also performed (Figure 2). Patients with EVs of grades ≥ F2 and a moderate to severe RCS were included in the study. The sequential therapy was carried out over two sessions. During the first session, two EIS procedures were performed at a 7-d interval. The puncture was performed 3-5 times per esophageal varix, from the cardia to the esophagus. Two weeks after the first session, gastroscopy was performed to evaluate the EVs. If the EVs were still present, the patient was treated a second time. In order to exclude interclass bias, in this study, we evaluated data related to the first endoscopic procedure only.

The following data were obtained: Age, sex, weight, ASA physical status, CTP classification, indications, preanesthetic problems, endoscopic procedure, successful completion of the procedure, anesthesia time, and anesthesia agents. Adverse events were also recorded, including hypotension (defined as a blood pressure reduction of 20% from baseline and below normal for the patient's age), hypertension (defined as a blood pressure elevation of 20% from baseline and above normal for the patient's age), bradycardia (defined as a heart rate reduction of 30% from baseline and below normal for the patient's age), and hypoxia (defined as oxygen desaturation with SpO_2 < 90%). All anesthesia procedures were conducted by an experienced anesthetist.

Patient follow-up

After the endoscopic treatment, patients were followed up for at least 6 mo through bedside appointment or telephone contact to assess re-bleeding, complications, and mortality.

RESULTS

The patients received propofol and sufentanil injections during endotracheal intubation. Propofol anesthesia was provided during 182 procedures involving patients (140 men and 42 women) aged 56.1 ± 11.7 years (range, 25-83 years) and weighing 71.4 ± 10.7 kg (range, 45 - 95 kg). The mean anesthesia time was 33.1 ± 5.8 min. The mean recovery time was 12.3 ± 3.7 min. Hypotension occurred in two patients (1.1%, 2/182). In 1 patient, the blood pressure decreased from 135/80 mmHg to 100/60 mmHg during the endoscopic procedure. After rapid rehydration, the blood pressure returned to 130/75 mmHg. For the other patient, the blood pressure decreased from 145/90 mmHg to 108/65 mmHg during the endoscopic procedure. After rapid rehydration, blood pressure returned to 135/75 mmHg. No patient developed hypertension during endoscopy. Bradycardia and hypoxia occurred in one patient each (1/182; 0.5%). In this patient, the heart rate decreased from 82 beats per minute (bpm) to 56 bpm. After intravenous injection of 0.5 mg atropine, the heart rate returned to 77 bpm. All complications were

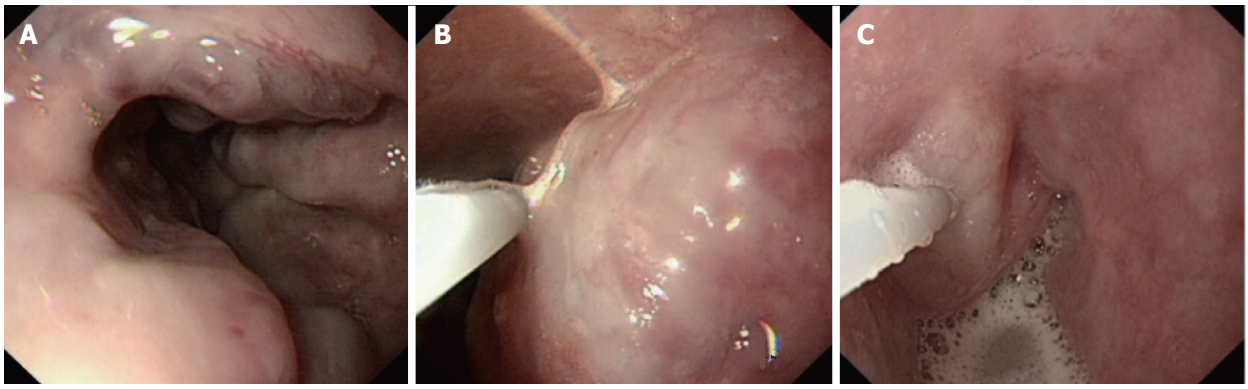

Figure 2 Endoscopic injection sclerotherapy for esophageal varices. A: The patient was EV grade ≥ F2 and had a severe red color sign, according to endoscopic findings; B: Endoscopic injection sclerotherapy (EIS) was performed with a standard endoscope (SV-290; Olympus Corporation; Tokyo; Japan). The endoscopic puncture needle used in EIS was a 23-gauge Varixer needle; C: The endoscopic finding after EIS.

easily treated, with no adverse sequelae. Intravenous anesthesia was successful. In addition, all endoscopic procedures were completed successfully.

In the study, the observed complications were minor and did not require intervention. After two therapy sessions, the recurrence rate of EVs was lower than 5% at the 1-year follow-up. Esophageal stenosis occurred in four patients at about 2 wk after the last EIS. The symptoms of esophageal stenosis were relieved after endoscopic balloon dilation.

DISCUSSION

In the present study, propofol was administered in combination with sufentanil to patients who underwent EIS for esophageal varices. The combination was found to facilitate safe and successful completion of the EIS procedure.

Portal hypertension increases blood flow and results in engorgement of the collateral vessels surrounding the lower esophagus and proximal stomach, leading to a build-up of gastroesophageal varices in approximately 50% of patients with cirrhosis[25]. Once varices have been diagnosed, variceal bleeding has been reported to occur at a yearly rate of 10% - 15%[26], and is associated with high morbidity and mortality in patients with liver cirrhosis. Treatment for prevention of EV bleeding is therefore required when large varices are present.

EIS or EVL is the initial endoscopic treatment selected for EVs. The 4th International Baveno Consensus[27] on Portal Hypertension recommends band ligation as the first-choice therapy, with sclerotherapy as a second-choice procedure. Ligation leads to lower complication rates and higher survival rates[28,29]. Additionally, EVL is popular worldwide because of the convenience of the procedure. However, the 5-year cumulative recurrence rate of EVs after EIS using intravariceal injection is 32%[30], which is markedly lower than that after EVL (80%)[31-33]. According to Triantos and colleagues[34], when band ligation is employed, it is necessary to withdraw the endoscope for system assembly, potentially increasing the duration of the procedure and the risk of complications. Hence, EIS was selected for the present study.

EIS is typically performed using one of the two methods. The first involves mucosal injection of sclerosant around the EVs, which results in a lower incidence of bleeding complications, while the other involves intravariceal injection of sclerosant, which is more effective. We performed intravariceal injection and sequential therapy in this study and achieved a success rate of 100%. Although EIS is an inexpensive, easily performed, and effective method, there are several complications associated with this technique. A previous study showed that minor complications such as low-grade fever, chest pain, and dysphagia can occur within the first 24-48 h after the procedure; however, they do not require treatment[1]. Local complications, such as esophageal ulcers, ulcer-related bleeding, and esophageal strictures, are also associated with EIS. Most of these complications occur due to incorrect injections or high sclerosant concentrations[1] and usually heal after omeprazole treatment. Esophageal stenosis occurs in 2%-10% of cases. In this study, the observed complications were minor and did not require intervention. After two treatment sessions, the recurrence rate of EVs was lower than 5% at the 1-year follow-up. Esophageal stenosis occurred in four patients about 2 wk after the last EIS. The symptoms of esophageal stenosis were relieved after endoscopic balloon dilation.

Esophagogastric varices are the most significant type of varices because their rupture results in variceal hemorrhage, which is among the most common lethal complications of cirrhosis. The presence of cirrhosis is independently associated with a 47% increase in the risk of postoperative complications and a greater than 2-fold increase in the risk of in-hospital mortality in patients undergoing elective surgery[35]. CTP scores have traditionally been used to assess the risk of mortality in patients with liver disease scheduled to undergo surgery[36-39]. Therefore, anesthesia for patients with liver cirrhosis is a significant challenge. The choice of anesthetic agent is based on variables such as protein binding, distribution, and drug metabolism. For

procedures requiring sedation, propofol is preferable to benzodiazepines, as it has a shorter time to sedation and a shorter recovery time in patients with cirrhosis[40]. Propofol is widely used because of its rapid onset and high recovery quality in painless endoscopy. Intravenous injection of propofol for endoscopic EV therapy can reduce the complications associated with poor cooperation of the patient and increase the comfort level of the patient during the endoscopic treatment. However, because of its weak analgesic effect, intraoperative pain stimulation is greater under propofol anesthesia and often appears in the form of marked physical movements, which may in turn affect the operation. Therefore, analgesics are often required to complete the operation. In the study by Zhang *et al*[22], pain levels in 439 patients were evaluated after injections of propofol and different combinations of fentanyl, sufentanil, or remifentanil at doses of 0.1 μg/kg or 0.05 μg/kg during gastrointestinal endoscopy. They observed that the incidence of pain in the group that was administered both propofol and half the dose sufentanil (0.05 μg/kg) was significantly lower (33%) than that in the other groups.

In this study, propofol was combined with sufentanil. Considering that this combination was effective at half the dose, we used this dose, as our study included patients with liver dysfunction. The dosage of anesthetic drug used did not affect anesthesia. The complication rate was very low, and the complications were easily treated with no adverse sequelae. The mean recovery time was less than 13 min.

In conclusion, propofol and sufentanil injection during endotracheal intubation-assisted EIS is effective and safe. Controlled clinical trials with larger sample sizes and longer follow-up periods are necessary to further evaluate the value and limitations of this technique.

ARTICLE HIGHLIGHTS

Research background

Propofol is widely used during painless endoscopy because of its rapid onset and rapid recovery properties. Intravenous injection of propofol during endoscopic esophageal varices therapy can reduce the complications associated with poor patient cooperation. Because of complications related to bleeding during endoscopic variceal ligation and endoscopic injection sclerotherapy, endotracheal intubation is essential for these procedures. However, due to its weak analgesic effect, intraoperative pain stimulation is greater, leading to overt physical movements, and thus affecting the operation. Since analgesics are often required to ensure a successful operation, in this study, authors used a combination of sufentanil and propofol injection for the endoscopic treatment of esophageal varices.

Research motivation

In the present study, propofol was administered in combination with sufentanil to patients who underwent EIS for esophageal varices. The combination was found to facilitate safe and successful completion of the EIS procedure.

Research objectives

To investigate the efficacy and safety of a combination of sufentanil and propofol injection in patients undergoing endoscopic injection sclerotherapy for esophageal varices (EVs).

Research methods

Patients with severe EVs who underwent EIS with sufentanil and propofol anesthesia between April 2016 and July 2016 were reviewed. Although at them hospital and sequential therapy were performed under endotracheal intubation, the authors only evaluated the efficacy and safety of anesthesia for the first EIS procedure. Patients were intravenously treated with 0.5-1 μg/kg sufentanil. Anesthesia was induced with 1-2 mg/kg propofol and maintained using 2-5 mg/kg propofol per hour. Information regarding age, sex, weight, American Association of Anesthesiologists (ASA) physical status, Child-Turcotte-Pugh (CTP) classification, indications, preanesthetic problems, endoscopic procedure, successful completion of the procedure, anesthesia time, recovery time, and anesthetic agents was recorded. Adverse events, including hypotension, hypertension, bradycardia, and hypoxia, were also noted.

Research results

Propofol and sufentanil anesthesia was provided in 182 procedures involving 140 men and 42 women aged 56.1 ± 11.7 years (range, 25-83 years). The patients weighed 71.4 ± 10.7 kg (range, 45-95 kg) and had ASA physical status classifications of Ⅱ (79 patients) or Ⅲ (103 patients). Ninety-five patients had a CTP classification of A and 87 had a CTP classification of B. Intravenous anesthesia was successful in all cases. The mean anesthesia time was 33.1 ± 5.8 min. The mean recovery time was 12.3 ± 3.7 min. Hypotension occurred in 2 patients (1.1%, 2/182). No patient showed hypertension during the endoscopic therapy procedure. Bradycardia occurred in 1 patient (0.5%, 1/182), and hypoxia occurred in 1 patient (0.5%, 1/182). All complications were easily treated with no adverse sequelae. In addition, all endoscopic procedures were completed successfully.

Research conclusions

The use of propofol and sufentanil injection in endotracheal intubation-assisted EIS for EVs is effective and safe.

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ORIGINAL ARTICLE

Observational Study

Health disparities are associated with gastric cancer mortality-to-incidence ratios in 57 countries

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Abstract

AIM

To evaluate the association between mortality-to-

incidence ratios (MIRs) and health disparities.

METHODS

In this study, we used the GLOBOCAN 2012 database to obtain the cancer incidence and mortality data for 57 countries, and combined this information with the World Health Organization (WHO) rankings and total expenditures on health/gross domestic product (e/GDP). The associations between variables and MIRs were analyzed by linear regression analyses and the 57 countries were selected according to their data quality.

RESULTS

The more developed regions showed high gastric cancer incidence and mortality crude rates, but lower MIR values than the less developed regions (0.64 *vs* 0.80, respectively). Among six continents, Oceania had the lowest (0.60) and Africa had the highest (0.91) MIR. A good WHO ranking and a high e/GDP were significantly associated with low MIRs (P = 0.001 and P = 0.001, respectively).

CONCLUSION

The MIR variation for gastric cancer would predict regional health disparities.

Key words: Gastric cancer; Mortality; Incidence; Mortality-to-incidence ratio; Gross domestic product; Expenditure; World Health Organization

Core tip: The mortality-to-incidence ratios (MIRs), defined as the ratio of the crude rate of mortality to the incidence, could reflect the clinical outcomes of disease. A total of 57 countries was included in this analysis to evaluate the association between MIR and health care disparities. The results showed the more developed regions had high gastric cancer incidence and mortality, but lower MIR than the less developed regions. Otherwise, good World Health Organization ranking and high total expenditures on health/gross domestic product were significantly associated with low MIRs. Therefore, the MIR variation for gastric cancer could predict regional health disparities.

Tsai MC, Wang CC, Lee HL, Peng CM, Yang TW, Chen HY, Sung WW, Lin CC. Health disparities are associated with gastric cancer mortality-to-incidence ratios in 57 countries. *World J Gastroenterol* 2017; 23(44): 7881-7887 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7881.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7881

INTRODUCTION

Gastric cancer was the leading cause of cancer mortality worldwide until the 1990s[1], but its incidence has since declined, especially in the developed world. Nevertheless, gastric cancer remains one of the most prevalent cancers in the world[2,3]. Gastric cancer is a multi-factorial disease, having a clear relationship with environmental risks, dietary habits, food storage, *Helicobacter pylori* infection, and geographic region[4-8]. Gastric cancer is more common in developing countries than in developed countries, and it occurs more frequently in men than in women[7,8].

Previous ethnic and migration studies have indicated that early exposure to environmental factors has a greater influence on the mortality and incidence rates of gastric cancer than is found for genetic factors[9,10]. Modern studies have also explored the mechanism of how *Helicobacter pylori* infection affects gastric cancer development in cancer stem cell lines and animal models[11,12]. Several recent large-scale database observational studies have documented the incidence of gastric cancer among patients with gastric precancerous lesions in western countries[13,14]. The early detection of precancerous lesions, such as atrophic gastritis, intestinal metaplasia and dysplasia, by esophagogastroduodenoscopy (EGD) and further endoscopic or surgical resection are helping to decrease the progression to malignancy[15] and therefore the morbidity and mortality associated with gastric cancer.

As already mentioned, the mortality rates due to gastric cancer have shown a steady decline globally, but regional differences are evident. Previous studies have demonstrated an annual percent decrease in gastric mortality rate of around 3% to 4% for European countries, the United States, the Republic of Korea, Japan and Australia between 1980 and 2005[16]. A 2% annual percent decrease was noted in gastric mortality rate among major Latin American countries and a less dramatic decline was observed in China[2]. This decline is at least in part due to the introduction of EGD, an important tool for early detection of gastric cancer, and even precancerous lesions. A recent Japanese study concluded that EGD screening was more powerful than either radiographic or photofluorography screening, and reduced the mortality rate from gastric cancer by 57%[17].

All these declines in gastric cancer prevalence suggest that the health care system may be able to improve precancerous lesion detection, early cancer detection, and treatment of gastric cancer to provide further declines in mortality. We therefore considered that the mortality-to-incidence (MIR) ratio for gastric cancer should be low in countries with good health care systems, in agreement with recent findings on prostate cancer[18-21]. The aim of the present study was therefore to clarify the association between human development, the World Health Organization (WHO) ranking, region, total expenditure on health/gross domestic product (GDP; e/GDP), life expectancy, and crude rates of incidence and mortality for gastric cancer in different countries. Our results provide a comprehensive overview of gastric cancer MIRs and health disparities in various countries across the globe.

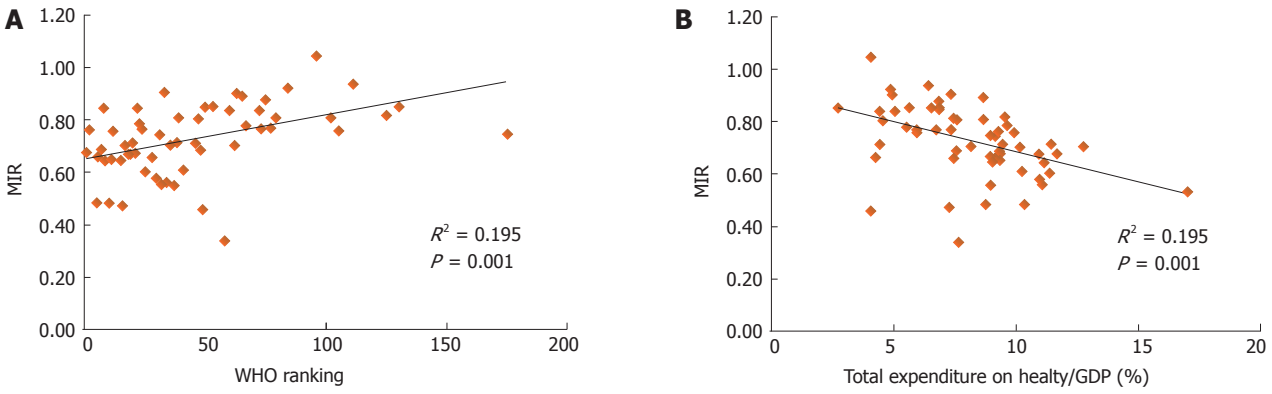

Figure 1 The (A) World Health Organization rankings and (B) total expenditures on health/gross domestic product are significantly associated with MIR in gastric cancer.

Table 1 Case number, rate and mortality-to-incidence ratio of the incidence and mortality of gastric cancer according to region

Region	Number		Crude rate		Age-standardized rate		Mortality-to-incidence ratio[1]
	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	
World	951594	723073	13.5	10.2	12.1	8.9	0.76
Development							
More developed regions	274509	174756	22.0	14.0	10.6	6.4	0.64
Less developed regions	677085	548317	11.7	9.4	12.7	10.2	0.80
WHO region categories							
WHO Africa region	19110	17589	2.2	2.0	4.0	3.7	0.91
WHO Americas region	85354	65130	8.9	6.8	6.9	5.1	0.76
WHO East Mediterranean region	23454	20789	3.8	3.3	5.5	4.9	0.87
WHO Europe region	161846	126315	17.9	14.0	10.0	7.4	0.78
WHO South-East Asia region	90558	83249	4.9	4.5	5.7	5.3	0.92
WHO Western Pacific region	571139	409897	31.0	22.2	22.8	15.7	0.72
Continent							
Africa	23806	21801	2.2	2.0	3.8	3.5	0.91
Latin America and Caribbean	60852	51435	10.1	8.5	9.7	8.1	0.84
Northern America	24502	13695	7.0	3.9	4.0	2.1	0.56
Asia	699954	527074	16.5	12.4	15.8	11.7	0.75
Europe	139667	107360	18.8	14.5	9.4	6.9	0.77
Oceania	2813	1708	7.5	4.5	5.1	3.0	0.60

[1]The percentage in the ratio of the crude rate of mortalities and the crude rate of incidences.

MATERIALS AND METHODS

The data acquisition was described previously[18]. In brief, the cancer epidemiological data were gathered from the GLOBOCAN 2012 database maintained by the International Agency for Research on Cancer (https://www.iarc.fr/). The WHO rankings of countries were obtained from the World's Health Systems of WHO. The e/GDP and life expectancies for 2012 were obtained from the World Health Statistics 2015.

Data for 184 countries were obtained from the GLOBOCAN 2012 database. Among these 184 countries, 22 were excluded from the study due to a lack of WHO ranking data. We excluded a further 105 countries due to the availability of mortality/incidence data mentioned in GLOBOCAN 2012 database (rankings E to G for incidence or rankings 4 to 6 for mortality were excluded). This resulted in 57 countries being further analyzed in this study. The MIR is defined as the ratio of the crude rate of mortality and the crude rate of incidence[21].

The methods used for statistical analyses were described previously[18]. Associations between the MIRs and variants among countries were estimated by linear regression. R-squared changes and analysis of variance (ANOVA) were determined using SPSS statistical software version 15.0 (SPSS, Inc., Chicago, IL, United States). P value < 0.05 of a two-sided test were considered statistically significant. Scatter plots were generated using Microsoft Excel 2010.

RESULTS

Incidence and mortality rates of gastric cancer according to regions

We examined the global trends in gastric cancer by analyzing the incidence and mortality numbers and rates according to different regions across the globe. The results are summarized in Table 1. The worldwide MIR is 0.76 and it is higher in less developed regions than in more developed regions (0.80 *vs* 0.64). The WHO values indicate that the Western Pacific region

Table 2 World Health Organization rankings, total expenditure on health/ gross domestic product, life expectancy, gastric cancer incidence, mortality and mortality-to-incidence ratio for gastric cancer in selected countries

Country	Ranking	Total expenditure on health/GDP, %	Life expectancy	Number		Crude rate		Age-standardized rate		Mortality-to-incidence ratio[1]
				Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	
France	1	11.6	82	6507	4412	10.3	7	4.7	2.9	0.68
Italy	2	9.2	83	13001	9917	21.3	16.3	8.2	5.6	0.77
Malta	5	8.7	81	68	33	16.2	7.9	8.0	3.3	0.49
Singapore	6	4.2	83	647	431	12.3	8.2	8.2	5.3	0.67
Spain	7	9.3	83	7810	5389	16.7	11.5	7.8	4.9	0.69
Oman	8	2.7	76	79	68	2.7	2.3	5.3	4.7	0.85
Austria	9	11.1	81	1314	853	15.6	10.1	6.8	4.0	0.65
Japan	10	10.3	84	107898	52326	85.3	41.4	29.9	12.4	0.49
Norway	11	9.3	82	475	311	9.6	6.3	4.6	2.8	0.66
Portugal	12	9.9	81	3018	2285	28.2	21.4	13.1	9.0	0.76
Iceland	15	9.0	82	28	18	8.5	5.5	5.0	2.9	0.65
Luxembourg	16	7.2	82	67	32	12.8	6.1	7.6	3.0	0.48
Netherlands	17	12.7	81	1953	1391	11.7	8.3	5.6	3.7	0.71
United Kingdom	18	9.3	81	6684	4534	10.6	7.2	4.7	2.9	0.68
Ireland	19	8.9	81	487	325	10.6	7.1	6.5	4.2	0.67
Switzerland	20	11.4	83	683	485	8.8	6.3	4.2	2.6	0.72
Belgium	21	10.9	80	1417	962	13.1	8.9	5.8	3.5	0.68
Colombia	22	6.8	78	5897	4981	12.4	10.5	13.4	11.2	0.85
Sweden	23	9.6	82	811	635	8.5	6.7	3.7	2.7	0.79
Cyprus	24	7.3	82	94	72	8.3	6.4	5.4	4.0	0.77
Germany	25	11.3	81	16015	9714	19.5	11.8	7.8	4.3	0.61
Israel	28	7.4	82	777	516	10.1	6.7	7.1	4.5	0.66
Canada	30	10.9	82	3342	1937	9.6	5.6	4.9	2.7	0.58
Finland	31	9.1	81	641	479	11.9	8.9	5.2	3.7	0.75
Australia	32	8.9	83	2049	1135	8.9	5.0	4.8	2.5	0.56
Chile	33	7.3	80	3712	3371	21.3	19.3	15.6	13.8	0.91
Denmark	34	11.0	80	625	351	11.2	6.3	5.6	2.9	0.56
Costa Rica	36	10.1	79	874	612	18.2	12.8	17.3	12	0.70
United States	37	17.0	79	21155	11758	6.7	3.7	3.9	2.0	0.55
Slovenia	38	9.4	80	468	335	22.9	16.4	10.4	6.8	0.72
Cuba	39	8.6	78	1126	916	10.0	8.1	5.9	4.6	0.81
New Zealand	41	10.2	82	393	240	8.8	5.4	5.2	2.9	0.61
Bahrain	46	4.4	77	29	21	2.1	1.5	3.9	3.5	0.71
Thailand	47	4.5	75	2841	2286	4.1	3.3	3.1	2.5	0.80
Czech Republic	48	7.5	78	1595	1099	15.1	10.4	7.4	4.9	0.69
Malaysia	49	4.0	74	1900	873	6.5	3.0	7.8	3.6	0.46
Poland	50	6.8	77	6105	5197	15.9	13.6	8.4	7.0	0.86
Jamaica	53	5.6	74	269	229	9.7	8.3	9.1	7.1	0.86
South Korea	58	7.6	82	31269	10746	64.4	22.1	41.8	13	0.34

Philippines	60	4.4	69	2415	2043	2.5	2.1	3.8	3.3	0.84
Slovakia	62	8.1	76	901	633	16.4	11.6	9.6	6.5	0.71
Egypt	63	4.9	71	1789	1584	2.1	1.9	2.5	2.3	0.90
Uruguay	65	8.6	77	577	514	17.0	15.2	10	8.4	0.89
Trinidad and Tobago	67	5.5	71	67	53	5.0	3.9	4.4	3.4	0.78
Belarus	72	5.0	72	2961	2495	31.1	26.2	18.8	15.3	0.84
Lithuania	73	6.7	74	867	668	26.3	20.3	13.8	10.2	0.77
Argentina	75	6.8	76	3738	3273	9.1	8.0	6.7	5.7	0.88
Estonia	77	5.9	77	370	286	27.6	21.3	13.8	9.7	0.77
Ukraine	79	7.5	71	11373	9216	25.3	20.5	14.3	11.6	0.81
Mauritius	84	4.8	74	121	112	9.2	8.5	8	7.4	0.92
Fiji	96	4.0	70	18	19	2.1	2.2	2.4	2.6	1.05
Bulgaria	102	7.4	75	1664	1354	22.5	18.3	10.3	8.3	0.81
Latvia	105	5.9	74	640	484	28.6	21.7	14.3	10.2	0.76
Ecuador	111	6.4	76	2401	2262	16.2	15.2	16.9	15.5	0.94
Brazil	125	9.5	75	19690	16077	9.9	8.1	9.2	7.4	0.82
Russian Federation	130	6.5	69	38417	32854	26.9	23	16	13.1	0.86
South Africa	175	8.9	60	2029	1529	4.0	3.0	5.1	3.9	0.75

[1]The percentage in the ratio of the crude rate of mortalities and the crude rate of incidences.

has the highest incidence and mortality for gastric cancer, regardless of whether this is based on the number, crude rate, or age-standardized rate (ASR) (Table 1). However, this region has the lowest MIR, at 0.72, among the six WHO regions. The highest MIR is reported for the WHO South-East Asia region (0.92). At a continent level, Africa has the lowest rate of incidence but has the highest MIR (0.91). The lowest MIR is found in North America.

WHO ranking and e/GDP were significantly associated with the MIRs for gastric cancer

We conducted a further comparison of the differences in epidemiology among countries by analyzing the 57 selected countries (Table 2). The e/GDP ranged from 2.7% (Oman) to 17.0% (United States of America) with mean ± standard deviation of 8.0% ± 2.6%. Japan had the longest life expectancy (84 years) and the Republic of South Africa had the shortest (60 years). Japan had the highest crude rates for gastric cancer, at 85.3 and 41.4 for incidence and mortality respectively. The MIR of Japan, at 0.49, was the fourth lowest value among the 57 countries. The lowest MIR was found for the Republic of Korea (0.34), which had the highest gastric cancer incidence in terms of ASR (41.8). Among these countries, five have MIR values greater than or equal to 0.90, including Fiji (1.05), Ecuador (0.94), Mauritius (0.92), Chile (0.91) and Egypt (0.90).

We also analyzed the association between the rates of incidence/mortality and the WHO ranking or e/GDP (SF1 and SF2). The results showed no significant association, except for the WHO ranking and the ASR of mortality (P = 0.005, SF2D). However, the use of the MIR for analyses revealed significant associations for both the WHO ranking and e/GDP and the MIR of the 57 countries (R^2 = 0.195, P = 0.001; R^2 = 0.195, P = 0.001, respectively, Figure 1).

DISCUSSION

In this study, we analyzed the correlation of the incidence, mortality and MIRs for gastric cancer with WHO rankings and e/GDP. The MIR, which was calculated as the ratio of the crude rate of mortality and the crude rate of incidence, is regarded as an important marker for cancer care disparities. The crude rates of incidence and mortality, which our results showed were higher in Japan and Korea, are similar to those reported previously[22]. The incidence of gastric cancer can be influenced by environmental hygiene, food storage, diet habits, ethnicity, geographic regions, and, most importantly, age[23].

Otherwise, in countries with high incidence of gastric cancer, more frequent survey or detection of cancer is performed. This might result in more cases detected in early stage and contribute to good clinical outcome. It is also observed in this database that countries with higher incidence of gastric cancer have lower MIR compared with those with lower incidence (crude rate *vs* MIR: R^2 = 0.104, P = 0.015; case number *vs* MIR: MIR: R^2 = 0.078, P = 0.035). Otherwise, a better WHO ranking and a higher e/GDP were correlated linearly with a longer life expectancy (R^2 = 0.0.689, P < 0.001; R^2 = 0.248, P < 0.001 respectively). This could explain the lack of a significant association between the WHO rankings, e/GDP, and incidence of gastric cancer in our analysis.

The mortality rates for gastric cancer can be reduced by screening programs, early endoscopic detection and management, surgical intervention availability[24,25], and the capability for chemotherapy or targeted therapy[26]. This may be why the ASR of mortality for gastric cancer was correlated with the WHO ranking, but had no significant correlation with total e/GDP. Previous data have shown that MIRs are lower in areas with better health care, and the present study shows that MIRs are also significantly lower in countries with better WHO rankings, with higher e/GDP, and in more developed regions. For the sex difference in the MIR and the health care disparities, previous study has shown that female patients with bladder cancer have higher MIR compared with male patients with bladder cancer[27]. However, unlike bladder cancer, there is no significant association in gastric cancer.

The limitations of this study include the fact that many countries are not participants in the WHO, and many of these countries are located in the least developed areas of the world. This limitation could influence the impact of e/GDP on gastric cancer incidence. Second, the use of the WHO rankings and e/GDP to represent the health care disparities of a country is not particularly specific. We were also unable to analyze ethnicity and national health insurance issues in our study. The reason why only the ASR mortality rate had a significant correlation with WHO rankings needs further investigation.

Our study indicates that gastric cancer has a higher incidence, mortality, and MIR value in less developed regions. Although the incidence, mortality, and MIR values are low in more developed regions, some differences were evident between the geographic regions; for example, the ASR incidence (9.4 *vs* 15.8 *vs* 3.8) and mortality (6.9 *vs* 11.7 *vs* 3.5) were higher in Europe and Asia than in Africa. The MIRs are generally lower in the more developed continents, as exemplified by North America, which showed the lowest MIR (0.56) and Africa, which showed the highest (0.91).

In conclusion, MIRs showed a significant correlation with WHO rankings and e/GDP in our analysis and we believe that this finding reflects the health care disparities of different countries. Our study provides a valuable documentation of the MIR and its relationship to the global geographic distribution of gastric cancer in 57 countries worldwide.

COMMENTS

Background

COMMENTS

MIRs of colorectal and prostate cancers are associated with health disparities, but similar associations between the MIRs for gastric cancer and health disparities among different countries have never been investigated.

Research frontiers

A total of 57 countries was included in this analysis. The more developed regions showed high gastric cancer incidence and mortality, but lower MIR than the less developed regions. Otherwise, good World Health Organization (WHO) ranking and high total expenditures on health/gross domestic product (e/GDP) were significantly associated with low MIRs.

Innovations and breakthroughs

The MIR variation for gastric cancer could predict regional health disparities.

Applications

The gastric cancer MIR could be used to evaluate the health disparities and ranking of countries.

Terminology

MIRs showed a significant correlation with WHO rankings and e/GDP in 57 countries, which reflects the health care disparities of different countries. Our study provides valuable documentation of the MIR and its relationship to the global geographic distribution of gastric cancer worldwide.

Peer-review

The novelty of this manuscript is good, and the result can help to explain the research purpose.

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ORIGINAL ARTICLE

Observational Study

Circulating miR-125a but not miR-125b is decreased in active disease status and negatively correlates with disease severity as well as inflammatory cytokines in patients with Crohn's disease

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Author contributions: Sun CM and Wu J contributed to study conception and design; Sun CM contributed to data acquisition, analysis, and interpretation and writing of the article; Wu J, Zhang H, Shi G, and Chen ZT contributed to editing and reviewing the article; all authors read and approved the manuscript.

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Abstract

AIM

To determine the association of circulating miR-125a/b expression with the risk and disease severity of Crohn's disease (CD), and with inflammatory cytokines.

METHODS

Plasma samples were collected from patients with active CD (A-CD), or CD in remission (R-CD) and from healthy controls (HCs). The levels of the inflammatory cytokines interleukin-17 (IL-17), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured by enzyme-linked immunosorbent assay. The expression of miR-125a/b was assessed by quantitative polymerase chain reaction (qPCR).

RESULTS

Twenty-nine A-CD patients, 37 R-CD patients, and 37 HCs were included in the study. Plasma miR-125a expression was decreased in A-CD patients compared

with that in R-CD patients (P < 0.001) and HCs (P < 0.001). miR-125a expression levels enabled the differentiation of A-CD from R-CD patients [area under curve (AUC) = 0.854] and from HCs (AUC = 0.780), whereas miR-125b expression did not. miR-125a was negatively correlated with C-reaction protein (CRP) (P = 0.017), erythrocyte sedimentation rate (ESR) (P = 0.026), Crohn's disease activity index (CDAI) (P = 0.003), IL-17 (P = 0.015), and TNF-α (P = 0.004) in A-CD patients. Furthermore, miR-125a was negatively associated with CRP (P = 0.038) and CDAI (P = 0.021) in R-CD patients. Regarding miR-125b, no association with CRP, CDAI, IL-17, TNF-α, or IFN-γ was found in A-CD or in R-CD patients. miR-125a levels gradually increased in A-CD patients who achieved clinical remission (P = 0.009) after 3-mo treatment, whereas they remained unchanged among patients who failed to achieve remission. No changes in miR-125b expression were detected in remission or non-remission patients after treatment.

CONCLUSION

Circulating miR-125a but not miR-125b is decreased in patients with active disease status and negatively correlates with disease severity and inflammatory cytokines in patients with CD.

Key words: Crohn's disease; miR-125; Disease risk; Disease severity; Inflammatory cytokines

Core tip: This study aimed to investigate the association of circulating miR-125a/b expression with the risk and severity of Crohn's disease (CD) and with inflammatory cytokines. Our results showed that miR-125a but not miR-125b is negatively correlated with the risk of active CD and disease severity and with inflammatory cytokines.

Sun CM, Wu J, Zhang H, Shi G, Chen ZT. Circulating miR-125a but not miR-125b is decreased in active disease status and negatively correlates with disease severity as well as inflammatory cytokines in patients with Crohn's disease. *World J Gastroenterol* 2017; 23(44): 7888-7898 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7888.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7888

INTRODUCTION

Crohn's disease (CD), an idiopathic chronic inflammatory disease, is a form of inflammatory bowel disease (IBD) and primarily affects the gastrointestinal tract. This disease causes the development of ulcers and complications, including abscesses and fistulas, affecting all layers of the intestinal wall[1,2]. It is reported that CD has an annual incidence of approximately 24 per 100000 in Europe and 19 per 100000 in North America. However, the incidence rate of CD is still increasing in developing countries, particularly in China due to its considerable increase in gross domestic product (GDP)[3,4] and improvement in quality of life[5].

MicroRNAs (miRNAs) are small endogenous RNAs that can degrade targeted mRNA or inhibit protein synthesis, and increasing evidence shows that miRNAs play a key role in regulating the intestinal immune system[6-9]. For example, miR-29b inhibits transforming growth factor-β (TGF-β)-induced intestinal fibrosis, and let-7 regulates the activity of nuclear factor-κB (NF-κB) and mediates interleukin (IL)-6 down-regulation in CD patients[10,11]. Furthermore, miR-192, miR-142-3p, and miR-21 are notably upregulated in paediatric IBD patients[12], whereas miR-495-5p and miR-19b are down-regulated in the inflamed bowel of CD patients[13,14].

miRNA-125 (miR-125) family, an miRNA family highly conserved throughout evolution, consists of miR-125a and miR-125b[15]. It has been shown that miR-125a inhibits innate macrophage responses by suppressing macrophage differentiation, and the expression level of miR-125a is down-regulated in systemic lupus erythematosus (SLE)[16,17]. miR-125b was correlated with rheumatoid arthritis (RA) disease activity and may serve as a potential biomarker for treatment response in early RA[18]. To date, few studies have investigated the impact of dysregulated miR-125a/b expression in CD patients. Therefore, the aim of this study was to determine the association of circulating miR-125a/b expression with the risk and disease severity of CD and with inflammatory cytokines.

MATERIALS AND METHODS

Participants

Twenty-nine clinically active CD (A-CD) patients and 37 patients with CD in clinical remission (R-CD) were enrolled in this study from May 2014 to June 2016 at the Department of Gastroenterology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology. The diagnosis of CD was determined according to the Lennard-Jones criteria[19], and active clinical disease was defined as Crohn's disease activity index (CDAI) above 150 points. Patients with the following conditions were excluded: arthritis with complications or other autoimmune diseases; history of malignant tumour or complications; severe renal and/or kidney dysfunction; and history of serious surgery or severe infection. In the meantime, 37 healthy controls (HCs) age- and gender-matched to the CD patients were enrolled.

The study was approved by the Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, and

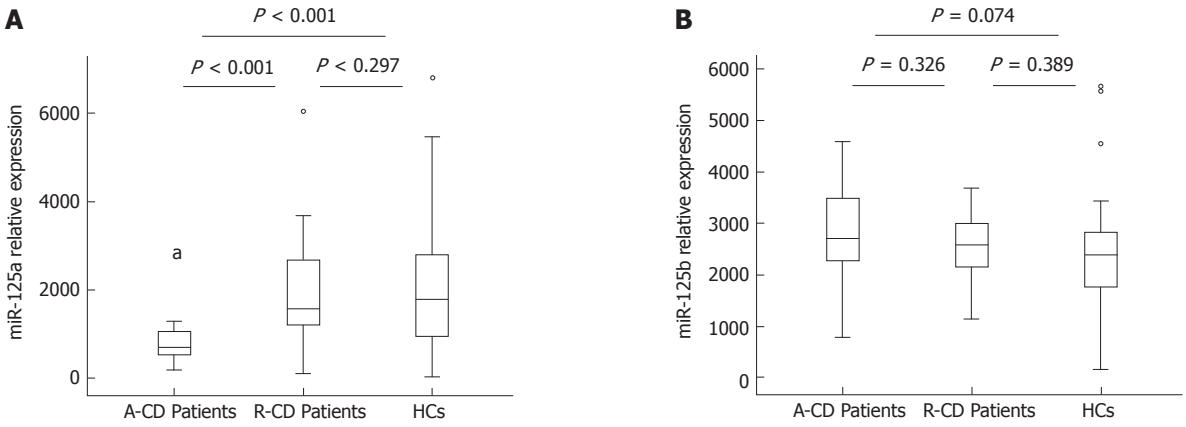

Figure 1 miR-125a/b expression in active Crohn's disease patients, patients with Crohn's disease in remission, and healthy controls. miR-125a was decreased in A-CD patients compared with R-CD patients and HCs (A), while no differences in miR-125b were detected between groups. Comparison between two groups was performed by the Wilcoxon signed-rank test. $P < 0.05$ was considered significant. A-CD: Active Crohn's disease; R-CD: Crohn's disease in remission; HCs: Healthy controls.

Table 1 Demographic and clinical characteristics of active Crohn's disease patients, patients with Crohn's disease in remission, and healthy controls

Parameter	A-CD patients (n = 29)	R-CD patients (n = 37)	HCs (n = 37)	P
Age (yr)	31.38 ± 9.51	31.97 ± 8.86	31.41 ± 5.69	0.941[a]
Gender (female/male)	17/12	20/17	18/19	0.719[a]
CRP (mg/L)	45.96 (32.27-67.26)	18.94 (10.78-26.71)	3.99 (3.33-6.06)	< 0.001[a]
ESR (mm/H)	43.93 (37.46-57.82)	16.73 (11.36-20.40)	13.02 (5.82-15.27)	< 0.001[a]
CDAI score	206.0 (170.5-253.0)	95.0 (75.5-112.0)	-	< 0.001[c]
IL-17 (pg/mL)	46.90 (38.46-60.06)	18.07 (10.21-20.17)	-	< 0.001[c]
TNF-α (pg/mL)	72.77 (53.46-83.39)	22.17 (19.49-30.43)	-	< 0.001[c]
INF-γ (pg/mL)	33.23 (24.59-39.54)	11.62 (9.16-15.07)	-	< 0.001[c]

Data are presented as mean ± SD, median (1/4-3/4 quarters), or counts. $P < 0.05$ was considered significant: [a]Comparison among three groups, determined by One-way analysis of variance (ANOVA), Kruskal-Wallis test by ranks, or χ^2 test; [c]Comparison between A-CD and R-CD groups, determined by Wilcoxon signed-rank test. A-CD: Active Crohn's disease; R-CD: Crohn's disease in remission; HCs: Healthy controls; CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; CDAI: Crohn's disease activity index.

all participants provided written informed consent.

Sample collection and total RNA extraction

Blood samples were collected from all participants into EDTA tubes when they were enrolled in this study and after a 3-mo treatment in the case of A-CD patients. After collection, the blood samples were centrifuged at 1000 *g* for 15 mins at 4℃; then, the supernatant (plasma) was removed and stored at -80℃ for further analysis. If red blood cell lysis was discovered, a repeat blood sample was collected from patients or HCs. Total RNA was extracted from the samples with TRIzol reagent (Invitrogen, CA, United States) according to the manufacturer's instructions.

miR-125a/b determination by qPCR

Total RNA was reverse transcribed using the PrimerScript Real-time Reagent kit (TAKARA BIO Inc. Shiga, Japan). Subsequently, miR-125 a/b expression levels were quantitated with SYBR Premix Ex TaqTM II (TAKARA BIO Inc., Shiga, Japan). The expression level of miR-125a/b was calculated using the $2^{-\Delta\Delta t}$ method and U6 was used as the internal reference.

IL-17, TNF-α, and IFN-γ measurement by ELISA

The measurement of IL-17, tumour necrosis factor (TNF-α), and interferon (IFN-γ) levels in plasma samples from A-CD and R-CD patients (but not HCs) was performed using commercial enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (all purchased from eBioscience, CA, United States).

Disease evaluation and treatment of A-CD patients

C-reaction protein (CRP), erythrocyte sedimentation rate (ESR), and Crohn's disease activity index (CDAI) were used to assess the severity of CD. In A-CD patients, effective treatments, including immunosuppressive agents and biologics, among others, were used, according to disease status and clinical experience. After 3-mo treatment, the CDAI and blood of patients were assessed again, and changes in miR-125a/b expression were analysed based on clinical remission achievement.

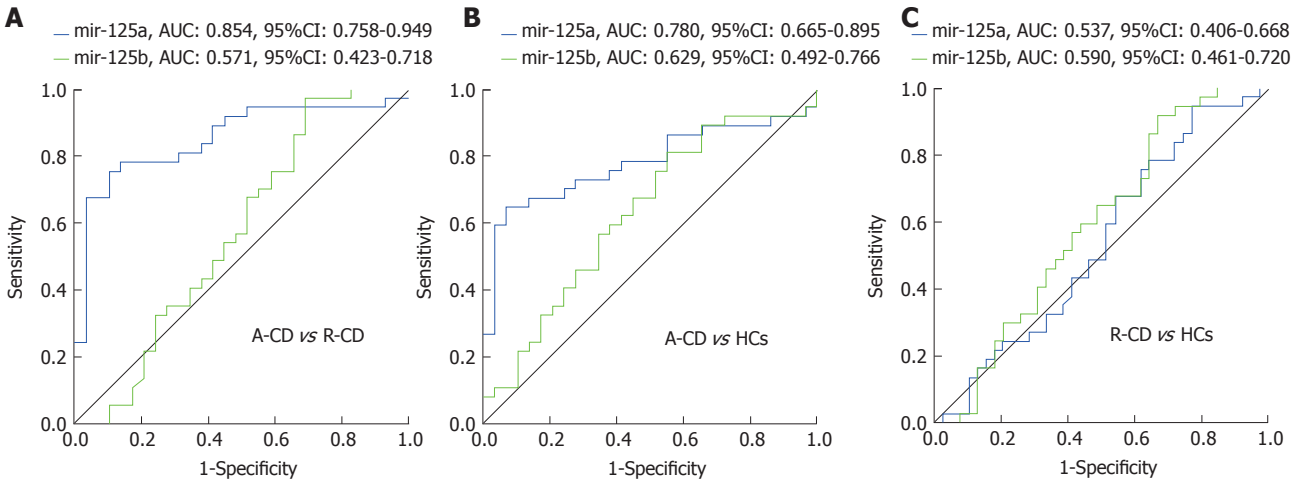

Figure 2 Receiver operating characteristic curve analysis of miR-125a/b for prediction of active Crohn's disease patients and patients with Crohn's disease in remission. Receiver operating characteristic curve was operated to assess miR-125a/b expression in differentiating A-CD patients from R-CD patients and from HCs. miR-125a was able to differentiate A-CD patients from R-CD (A) patients and from HCs (B), whereas miR-125b was not (A, B). Neither miR-125a nor miR-125b could differentiate R-CD from HCs (C). A-CD: Active Crohn's disease; R-CD: Crohn's disease in remission; HCs: Healthy controls.

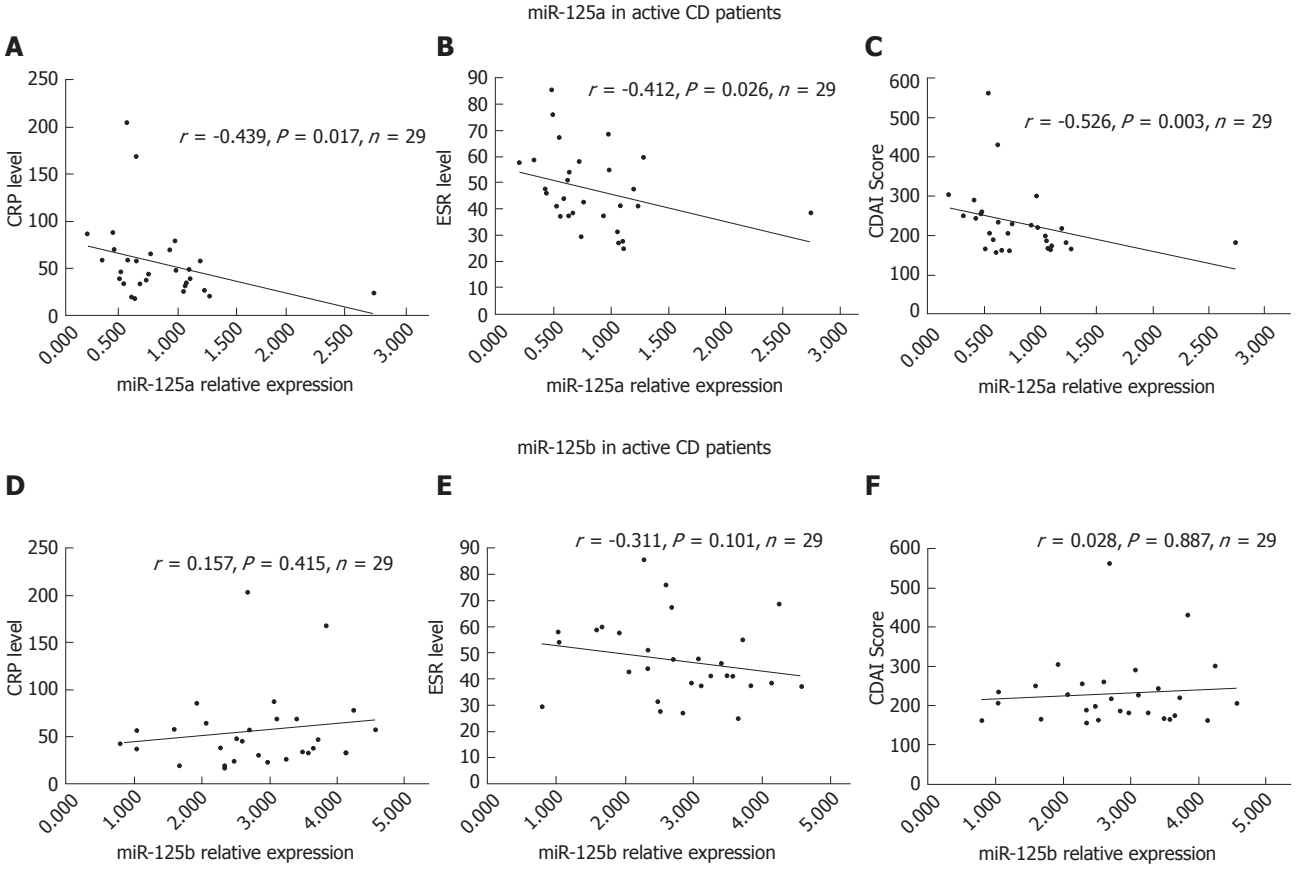

Figure 3 Correlations of miR-125a/b expression with disease severity in active Crohn's disease patients. A-C: Correlations of miR-125a expression with disease severity in A-CD patients; D-F: Correlations of miR-125b expression with disease severity in A-CD patients. Spearman's test was used to analyse the correlation of miR-125a/b expression with disease severity. $P < 0.05$ was considered significant. A-CD: Active Crohn's disease; R-CD: Crohn's disease in remission; HCs: Healthy controls.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software program (IBM, United States) and MS Office 2016 (Microsoft, United States). Data are mainly expressed as mean ± standard deviation, median (1/4-3/4 quarters), or counts. Comparisons among three groups were performed by one-way analysis of variance (ANOVA), Kruskal-Wallis test by ranks, or χ^2 test. Comparisons between groups or between each visit in the same group were performed using

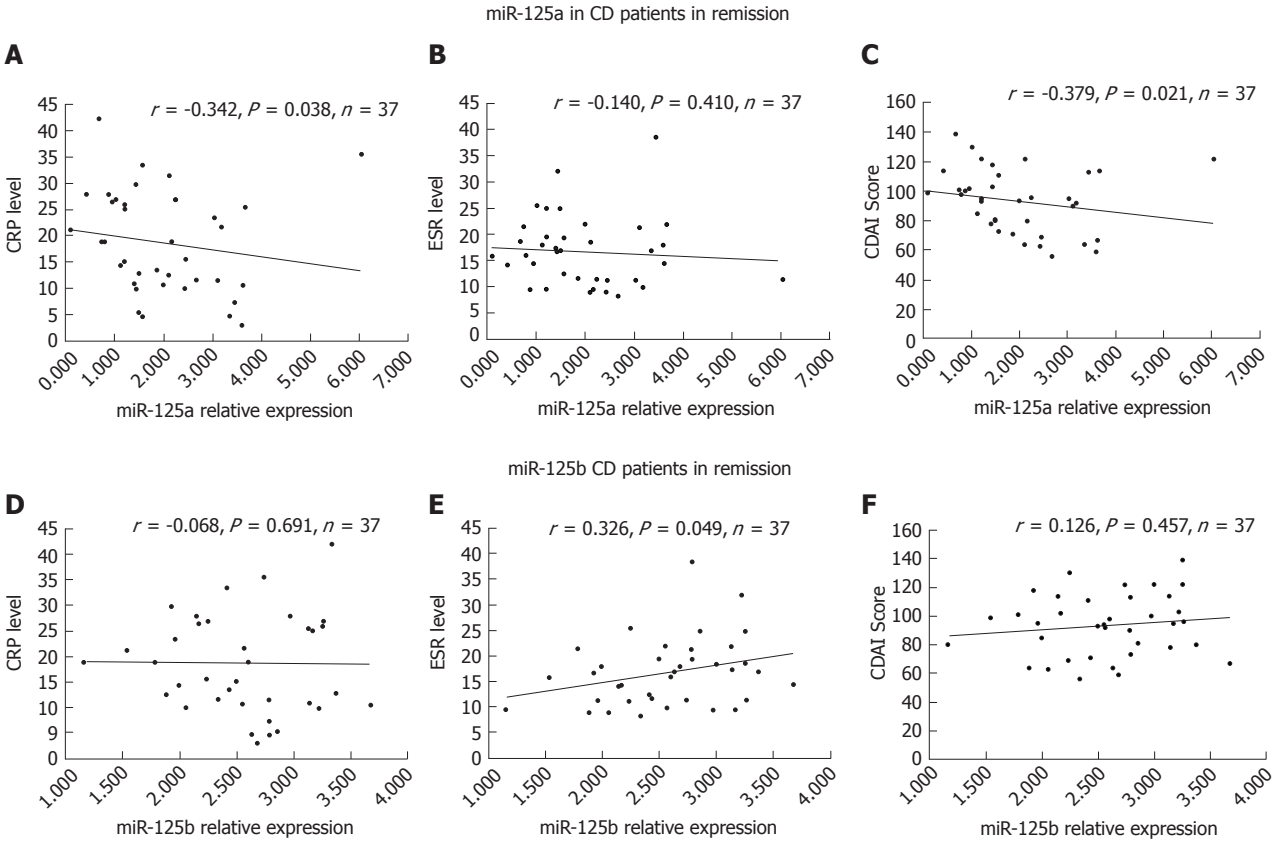

Figure 4 Correlations of miR-125a/b expression with disease severity in patients with Crohn's disease in remission. A-C: Correlations of miR-125a expression with disease severity in R-CD patients; D-F: Correlations of miR-125b expression with disease severity in R-CD patients. Spearman's test was used to analyse the correlation of miR-125a/b expression with disease severity. $P < 0.05$ was considered significant. A-CD: Active Crohn's disease; R-CD: Crohn's disease in remission; HCs: Healthy controls.

the Wilcoxon signed-rank test. Receiver operating characteristic (ROC) curve analysis was performed to assess the value of miR-125a/b expression in differentiating A-CD from R-CD patients and patients from HCs. Spearman's test was used to analyse the correlation of miR-125a/b expression with disease severity and inflammatory cytokines. $P < 0.05$ was considered significant.

RESULTS

Characteristics

As shown in Table 1, the 29 patients of the A-CD group had a mean age of 31.38 ± 9.51 years, whereas the 37 patients of the R-CD and the 37 HCs had a mean ages of 31.97 ± 8.86 and 31.41 ± 5.69 years, respectively. No difference in age or gender was found between groups ($P > 0.05$), although significant differences in CRP and ESR ($P < 0.001$) were observed between groups. Significant differences in inflammatory cytokines IL-17, TNF-α, and IFN and in CDAI were also found between A-CD and R-CD patients ($P < 0.001$).

miR-125a/b expression in A-CD patients, R-CD patients, and HCs

Plasma miR-125a expression was decreased in A-CD patients [0.719 (0.534-1.072)] compared with that of R-CD patients [1.564 (1.159-2.851), $P < 0.001$] and HCs [1.781 (0.874-2.873), $P < 0.001$]. However, no significant difference between R-CD patients and HCs ($P = 0.297$) was found (Figure 1A). No difference in miR-125b expression was observed between A-CD [2.707 (2.168-3.531)] and R-CD patients [2.600 (2.154-3.064)] and between patients and HCs [2.393 (1.705-2.852)], as shown in Figure 1B.

Interestingly, diagnostic value was discovered for miR-125a in differentiating A-CD from R-CD patients [area under ROC curve (AUC) = 0.854, 95%CI: 0.758-0.949) and patients from HCs (AUC = 0.780, 95%CI: 0.665-0.895] (Figure 2A and B). However, miR-125b failed as a predictive factor in differentiating A-CD from R-CD patients (AUC = 0.571, 95%CI: 0.423-0.718) and patients from HCs (AUC = 0.629, 95%CI: 0.492-0.766) (Figure 2A and B). Moreover, neither miR-125a nor miR-125b was able to differentiate R-CD patients from HCs (AUC = 0.537, 95%CI: 0.406-0.668; AUC = 0.590, 95%CI: 0.461-0.720, respectively) (Figure 2C).

Correlations of miR-125a/b expression with disease severity in CD patients

Negative correlations of miR-125a with CRP (r =

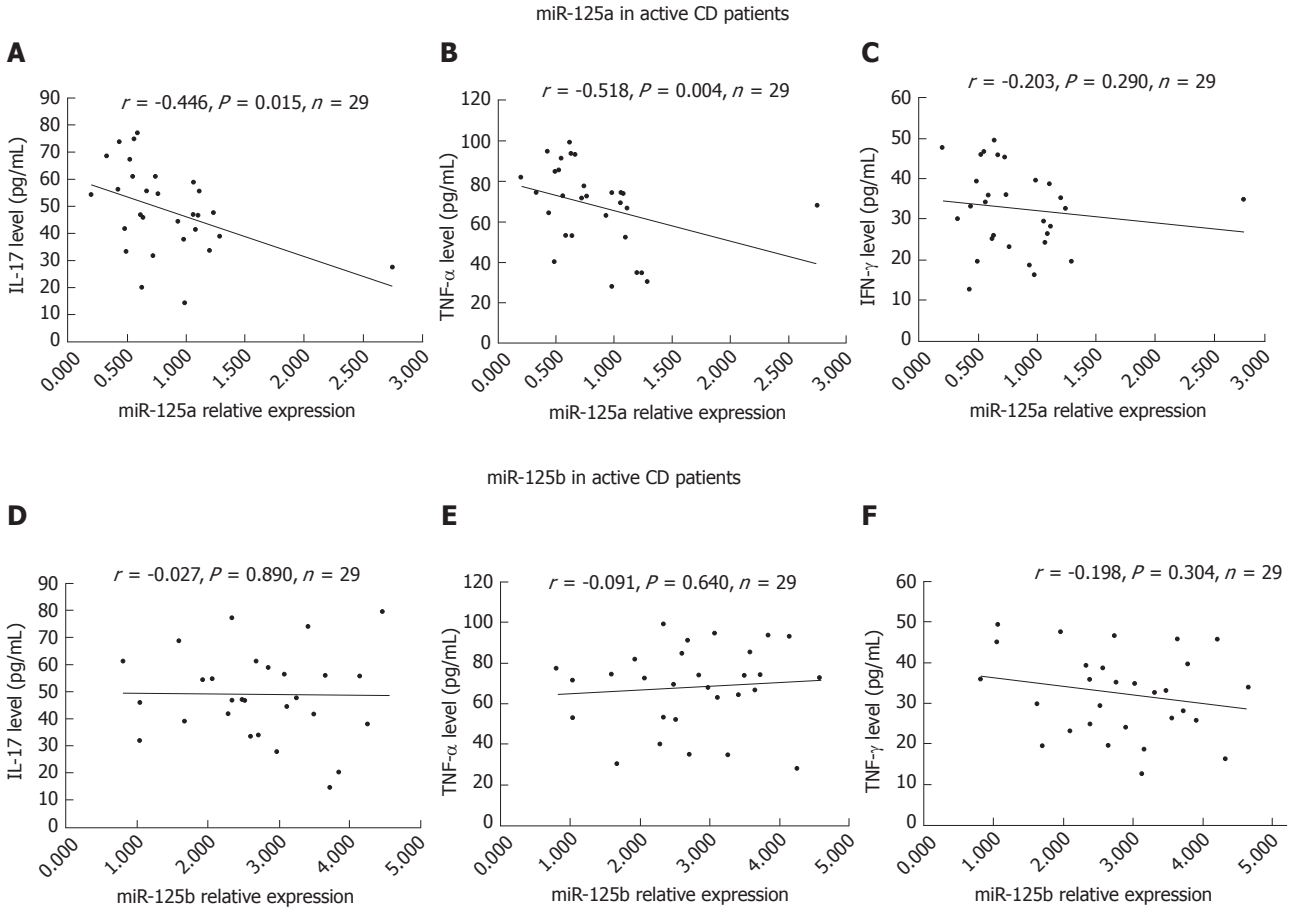

Figure 5 Correlations of miR-125a/b expression with inflammatory cytokines in active Crohn's disease patients. A-C: Correlations of miR-125a expression with inflammatory cytokines in A-CD patients; D-F: Correlations of miR-125b expression with inflammatory cytokines in A-CD patients. Spearman's test was used to analyse the correlation of miR-125a/b expression with inflammatory cytokines. $P < 0.05$ was considered significant. A-CD: Active Crohn's disease; R-CD: Crohn's disease in remission; HCs: Healthy controls.

-0.439, $P = 0.017$), ESR ($r = -0.412$, $P = 0.026$), and CDAI ($r = -0.526$, $P = 0.003$) in A-CD patients were observed, as shown in Figure 3A-C. However, no association was found between miR-125b and CRP, ESR, or CDAI in A-CD patients (Figure 3D-F).

Regarding R-CD patients, we found that the expression of miR-125a was negatively correlated with CRP ($r = -0.342$, $P = 0.038$) and CDAI ($r = -0.379$, $P = 0.021$), but not ESR ($r = -0.140$, $P = 0.410$) (Figure 4A-C). In R-CD patients, miR-125b was significantly associated with ESR ($r = 0.326$, $P = 0.049$), but not with CRP ($r = -0.068$, $P = 0.691$) or CDAI ($r = 0.126$, $P = 0.457$) (Figure 4D-F).

Correlations of miR-125a/b expression with inflammatory cytokines in CD patients

Negative correlations were detected for miR-125a with the inflammatory factors IL-17 ($r = -0.446$, $P = 0.015$) and TNF-α ($r = -0.518$, $P = 0.004$), but not with IFN-γ ($r = -0.203$, $P = 0.290$), in A-CD patients, as shown in Figure 5A-C. However, no association of miR-125b with the inflammatory factors IL-17 ($r = -0.027$, $P = 0.890$), TNF-α ($r = 0.091$, $P = 0.640$) and IFN-γ ($r = -0.198$, $P = 0.304$) was found in A-CD patients (Figure 5D-F). Regarding R-CD patients, we observed no correlation of miR-125a or miR-125b with the inflammatory factors IL-17, TNF-α or IFN-γ, as shown in Figure 6A-F.

miR-125a/b expression in A-CD patients after treatment

After 3-mo treatment, 19 of 29 A-CD patients achieved clinical remission (CDAI < 150), whereas 10 patients failed to achieve clinical remission. In patients with clinical remission, the miR-125a levels dramatically increased compared with the baseline ($P = 0.009$); conversely, in non-remission patients, miR-125a levels remained unchanged compared with the baseline. No changes in miR-125b expression were detected, either in remission or non-remission patients after treatment (Figure 7).

DISCUSSION

CD, a chronic debilitating syndrome, affects all layers of the gastrointestinal tract, causes severe diarrhea, abdominal pain, weight loss, metabolic disorder, and malabsorption, and is a major health concern, leading to huge financial losses of up to $2.2 billion dollars per year in the United States alone[20-23]. Although the pathogenesis of CD is still unclear, accumulating evidence shows that environmental factors, genetics,

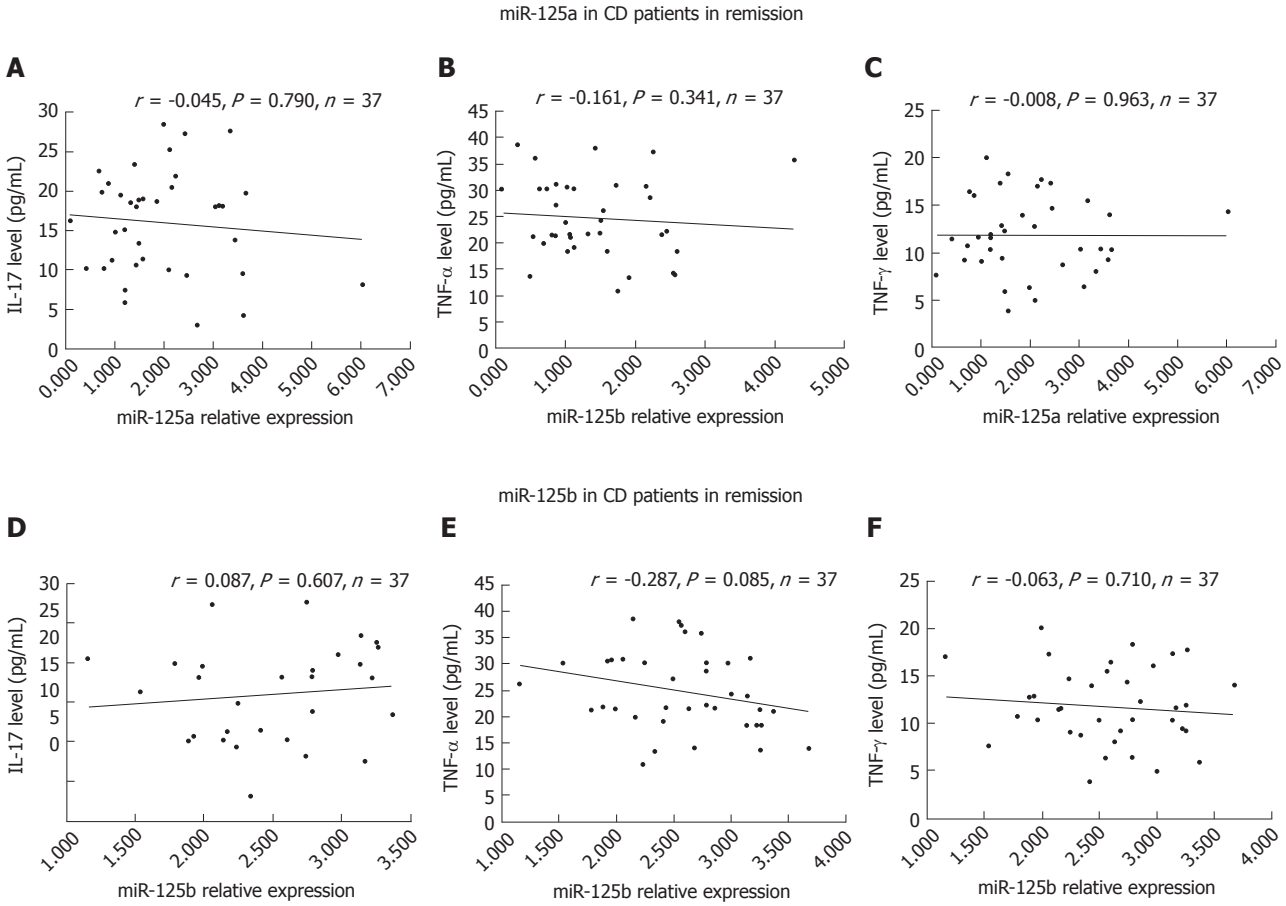

Figure 6 Correlations of miR-125a/b expression with inflammatory cytokines in patients with Crohn's disease in remission. A-C: Correlations of miR-125a expression with inflammatory cytokines in R-CD patients; D-F: Correlations of miR-125b expression with inflammatory cytokines in R-CD patients. Spearman's test was used to analyse the correlation of miR-125a/b expression with inflammatory cytokines. $P < 0.05$ was considered significant. A-CD: Active Crohn's disease; R-CD: Crohn's disease in remission; HCs: Healthy controls.

autoimmunity, and dietary habits may contribute to its development and progression[24,25]. As an important genetic factor, miRNA dysregulation is involved in the aetiological mechanism of several autoimmune diseases, including CD[26]; however, the role of miR-125a/b expression in CD risk and management is largely unstudied[27].

In this prospective study, we observed three notable findings. First, miR-125a expression was decreased in A-CD patients compared with R-CD patients and HCs. The results also showed the ability of miR-125a expression levels to distinguish A-CD from R-CD patients and from HCs, which was not possible using miR-125b expression levels. Second, miR-125a was negatively correlated with disease severity in A-CD patients, and negative correlations of miR-125a with inflammatory cytokines were also found in A-CD patients. Third, miR-125a levels were dramatically increased in A-CD patients who achieved clinical remission after 3-mo treatment, whereas miR-125a levels remained unchanged in non-remission patients. No changes in miR-125b expression were detected in remission or non-remission A-CD patients after treatment.

The *miR-125a* gene is located on chromosome 19 and in a cluster with miR-99b and miR-7e[28]. In the healthy population, the largest contributor of circulating miR-125a may be germinal center (GC) and hematopoietic stem cells (HSCs). Shaham *et al* suggest that miR-125a is enriched in HSCs (up to 23-fold more than in total bone marrow), particularly in long-term HSCs (up to 6-fold). Moreover, miR-125a is not restricted to the stem cell population, and its cluster members, miR-99b and let-7e, are preferentially expressed by centroblasts in the GC[16]. Studies show that miR-125a is down-regulated in peripheral $CD3^+$ T cells and negatively correlated with RANTES (also known as CCL5 chemokine) expression by targeting the *KLF13* gene in SLE patients[17]. Interestingly, miR-125a expression is also decreased in oral lichen planus, which is a T-cell-mediated autoimmune disease of the oral mucosa[29]. Furthermore, miR-125a is identified as a key regulator of $CD4^+$ T-cell differentiation that prevents autoimmune pathogenesis by controlling the balance between tolerance and autoimmunity[30]. Recently, it was reported that miR-125a participates in immune thrombocytopenic purpura (ITP), by modulating Tregs and Th17[31], which play a key role in CD development and progression and are correlated

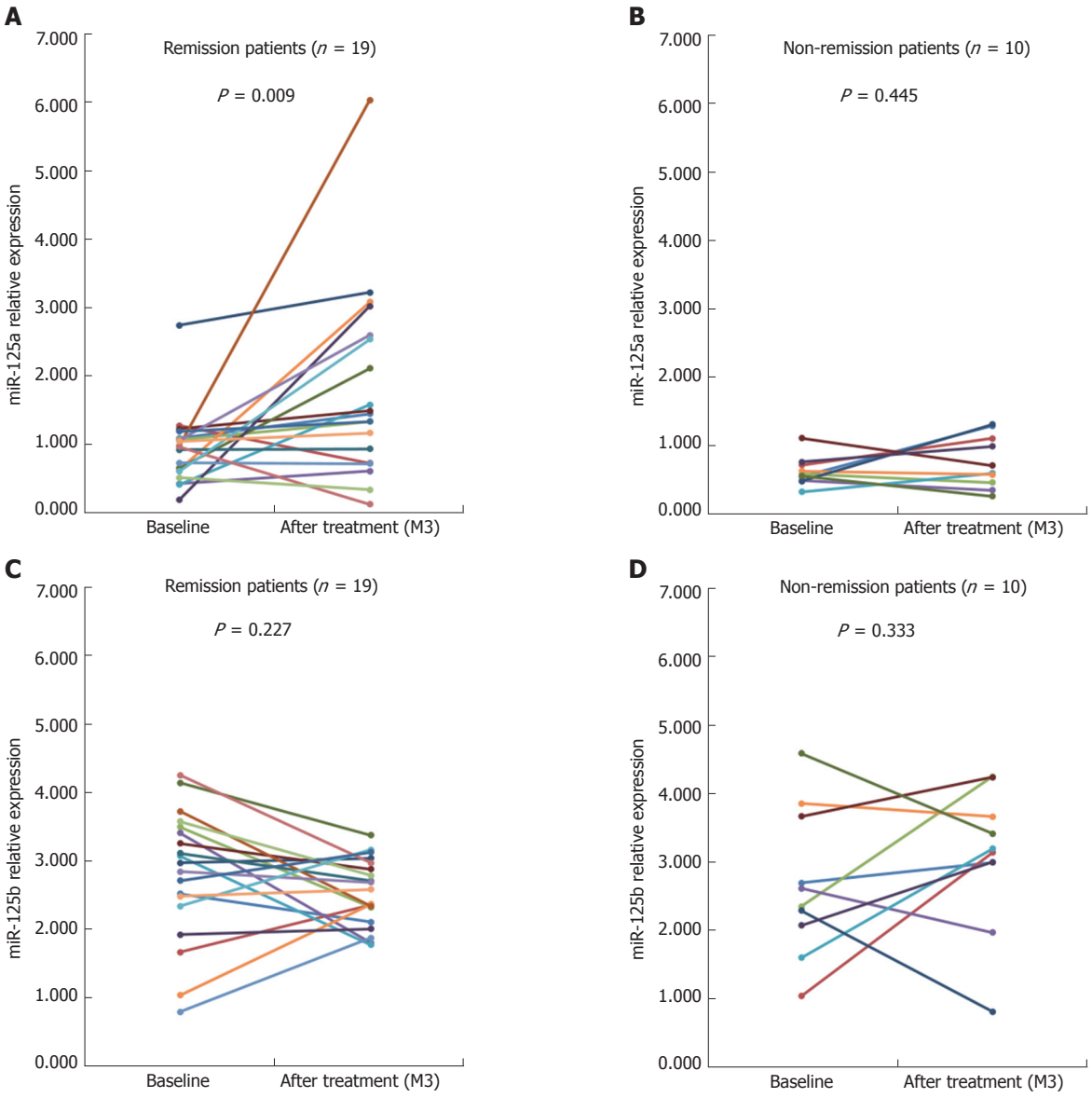

Figure 7 miR-125a/b expression in active Crohn's disease patients after treatment. After 3-mo treatment, miR-125a expression was increased in A-CD patients who achieved clinical remission but remained stable in patients who failed to achieve remission. Conversely, no changes in miR-125b expression were observed in either remission or non-remission patients after 3-mo treatment. Comparison between visits in the same group was performed by the Wilcoxon signed-rank test. $P < 0.05$ was considered significant. A-CD: Active Crohn's disease.

with CD disease activity[32-34]. These studies indicate that miR-125a is an anti-inflammatory gene that plays a key role in regulating autoimmune diseases, which is consistent with our results showing that miR-125a expression is decreased in A-CD patients and may be used to differentiate A-CD from R-CD patients and from HCs. This might be due to the anti-inflammatory effect of miR-125a, because the levels of inflammatory cytokines were markedly increased in A-CD patients compared with those of R-CD patients and HCs, whereas the extent of inflammation in R-CD patients was similar to that of HCs. The results showing a negative correlation between miR-125a and the inflammatory cytokines IL-17 and TNF-α further confirmed this point of view.

In addition, we found a negative association of miR-125a with disease severity in A-CD patients. Partly in line with our results, Murata *et al*[35] reported that miR-125a was negatively correlated with some indices of disease activity including CRP in RA, and reduced levels of miR-125a were associated with severe trauma through inflammatory cytokine IL-10 regulation, as shown in polytrauma patients[36], most likely, because of the powerful anti-inflammatory effect of miR-125a. Thus, miR-125a was able to predict the disease severity of CD. Furthermore, we found that the miR-125a levels were dramatically increased in A-CD patients who achieved clinical remission after 3-mo treatment, whereas they remained unchanged in patients who failed to achieve remission. The results proved once again that miR-125a is closely related to inflammation and might be a therapeutic target for CD in the future.

miR125-b has been shown to target the 3' untranslated region of the *TNF-α* gene to negatively regulate the inflammatory response[37]. However, another study has shown that miR-125b could promote macrophage mediated inflammation by increasing the expression of co-stimulatory factor[38]. These studies suggest that miR-125b has both anti- and pro-inflammatory effects. We found no correlation of miR-125b with inflammatory factors in A-CD patients, and miR-125b was not able to differentiate A-CD from R-CD patients and from HCs, most likely because miR-125b has both anti- and pro-inflammatory effects, which has been reported in previous studies. Thus, the role of miR-125b in regulating inflammation in CD remains unclear[16,39,40]. Then, we analysed the target genes of miR-125a and miR-125b by Validated Target *Module-MicroRNA-gene* analysis using the software miRWalk 2.0[41], which showed that miR-125a had 234 reported target genes, whereas miR-125b had 391 reported target genes, and that they shared 110 target genes. Conversely, most miR-125a and miR-125b target genes were different, which may explain the differences between miR-125a and miR-125b in CD.

To the best of our knowledge, this was the first study investigating the correlation between circulating miR-125a/b expression and the risk and disease severity of CD and with inflammatory cytokines. Notwithstanding, there were still some limitations in our study. First, we did not detect the expression of miR-125a/b in the intestinal tract, where miRNA dysregulation might be more frequent than in blood. However, it is difficult to obtain normal intestinal tract tissue from HCs and CD patients who remain remission. Second, the sample size of our study was relatively small, and a larger sample is needed for further studies. Third, IL-17, TNF-α, and IFN-γ expression was not detected in HCs in this study, which would help to further elucidate the role of miR-125a/b in inflammation, which we will investigate in future studies. Finally, the levels of Tregs and Th17 cells, which are essential for CD development and progression, were not determined in the present study. We will also assess these levels in future studies.

In summary, circulating miR-125a but not miR-125b is decreased in active disease status and negatively correlates with disease severity and inflammatory cytokines in patients with Crohn's disease. Therefore, this study sheds some light on the measurement of circulating miR-125a for the diagnosis and treatment of CD patients.

COMMENTS

Background

Crohn's disease (CD), an idiopathic chronic inflammatory disease, is a form of inflammatory bowel diseases (IBD) and primarily affects the gastrointestinal tract. Few studies have investigated the impact of dysregulated miR-125a/b expression in CD patients.

Research frontiers

Accumulating evidence shows that environmental factors, genetics, autoimmunity, and dietary habits may contribute to the development and progression of CD. As an important genetic factor, dysregulated miRNA has been involved in the aetiological mechanism of several autoimmune diseases, including CD; however, the role of miR-125a/b expression in CD risk and management is largely unstudied.

Innovations and breakthroughs

This was the first study that investigated the correlation between circulating miR-125a/b expression and the risk and disease severity of CD and inflammatory cytokines. The authors showed that miR-125a but not miR-125b is negatively associated with the risk for A-CD and disease severity and with inflammatory cytokines.

Applications

miR-125a is negatively associated with the risk for A-CD patients and disease severity and with inflammatory cytokines. In the near future, miR-125a may be an important marker for the diagnosis and treatment of CD patients.

Terminology

Crohn's disease activity index (CDAI) was used to differentiate A-CD from R-CD patients. Patients with a CDAI above 150 points were defined as A-CD patients, and those with a CDAI below 150 points were defined as R-CD patients.

Peer-review

This work focused on two miRNA molecules and their potential relevance to IBD. It is a well-written and interesting manuscript.

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ORIGINAL ARTICLE

Prospective Study

Dramatic response of hepatitis C patients chronically infected with hepatitis C virus genotype 3 to sofosbuvir-based therapies in Punjab, Pakistan: A prospective study

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Author contributions: Iqbal S designed the study, performed the research, analyzed the data and wrote the paper; Yousuf MH and Yousaf MI treated the patients, collected and analyzed the patient data.

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Abstract

AIM

To prospectively evaluate the efficacy of sofobuvir (SOF) in hepatitis C patients infected with hepatitis C virus (HCV) genotype 3 in Pakistan.

METHODS

The present study was performed with the coordination of gastroenterology and pathology departments of Shalamar Hospital Lahore from August 2014 to May 2016. The total number of patients included in this study was 1375 and all of them were infected with HCV genotype 3. On the basis of drug combinations, all the patients were separated into two groups. The first group of patients was treated for 24 wk with SOF (Sovaldi® by Gilead Sciences) plus ribavirin (RBV) [Ribazol® by Getz Pharma Pakistan (PVT) Ltd], while the patients of the second group were treated with SOF + RBV + pegylated-interferon (pegIFN) alfa-2a (Ropegra by Roach) for 12 wk. HCV genotyping and viral load measurement were performed on fully automated Abbott Real-Time PCR system (Abbott m24sp automated nucleic acid extraction system and Abbott m2000rt amplification system; abbott Molecular, Des Plaines, IL, United States). For the assessment of sustained virological response (SVR), all HCV RNA negative patients were followed for 12

weeks after the treatment completion. Any patient with less than 12 IU/mL viral load after 12 wk of treatment completion was considered as a sustained virological responder (SVR-12).

RESULTS

A total of 1375 patients chronically infected with HCV genotype 3 were treated with two drug combinations SOF + RBV and SOF + RBV + pegIFN alfa-2a. On the basis of these drug combinations, patients were divided into two groups (first and second). Overall SVR-12 was excellent in both groups (99.17% and 97.91%). Older patients (> 40 years) of second group showed lower SVR-12 (93.46%) compared to first group older patients (98.79%), while in the younger patients of both groups, the SVR-12 rate was almost the same (99.54% in first group and 99.05% in second group). No such difference regarding SVR-12 rate was seen in males and females of first group patients (99.68% and 98.88%, respectively), while in second group the males were found to be better responders compared to females (98.96% and 95%). The SVR-12 rate in previously treated patients of first group was better (99.34%) than second group (93.70%), while naïve patients of second group were marginally better responders (99.25%) than first group (97.80%). Rapid viral response at week-4 was found to be a very effective predictor for assessing the SVR rate at this stage of therapy in both groups. Headache, anemia and fatigue were common side effects in both groups either treated with SOF + RBV or SOF + RBV + pegIFN alfa-2a, while the overall percentage of the side effects was higher in second group.

CONCLUSION

The remarkable SVR response rate of HCV genotype 3 infected patients to SOF provided a new way to look forward to eliminate hepatitis C from our region.

Key words: Sofosbuvir; Sustained virological response; Pakistan

Core tip: Previously, hepatitis C was treated with interferon-based therapies. Intolerable side effects, prolonged treatment duration and unsatisfactory response rates were the major droughts of those therapies. The introduction of sofobuvir (SOF) was claimed as a highly responding oral drug for hepatitis C patients, with minimal side effects in different trials; thus, it was important to assess its efficacy in our population. We found an outstanding response rate of SOF in hepatitis C patients infected with genotype 3 of hepatitis C virus. These findings revealed that with SOF we may eliminate hepatitis C from our population.

Iqbal S, Yousuf MH, Yousaf MI. Dramatic response of hepatitis C patients chronically infected with hepatitis C virus genotype 3 to sofosbuvir-based therapies in Punjab, Pakistan: A prospective study. *World J Gastroenterol* 2017; 23(44): 7899-7905 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7899.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7899

INTRODUCTION

Approximately 2%-3% of the world's population (about 170 million) is chronically infected with hepatitis C virus (HCV)[1]. Prevalence of hepatitis C only in Europe and United States was estimated as 0.2%-2%[2,3]. In Pakistan, the situation regarding HCV infection rate was alarming. About 5.5% Pakistani population was infected with HCV; out of those 60%-80% had HCV genotype 3[4-6]. Hepatitis C patients always remain at risk for developing higher stages of disease like decompensated liver cirrhosis or hepatocellular carcinoma (HCC) that need liver transplantation[7-9].

Since the late 1980s, different categories of conventional interferon were known as a "key drug" to treat hepatitis C patient[10]. Although, the addition of RBV and improvement of conventional interferon with pegylation had enhanced the rate of sustained virological response (SVR)[11-13], yet most of the cases remained non-responders or relapsed after the termination of the treatment.

Because of prolonged treatment, adverse side effects and low SVR rates of interferon plus RBV based therapies, there was a need to improve the long-term viral clearance rate with more effective and less side effects containing drug for hepatitis C patients. In recent trials, newly approved drug "Sofosbuvir" drastically improved the SVR rate[14-16]. Sofosbuvir is thought the next milestone in the advancement of medication for hepatitis C[15]. Sofosbuvir is a nucleotide analogue that acts directly on virus and inhibits polymerase coding region NS5B of HCV and is thought to be more effective direct acting antiviral drug. Sofobuvir is also thought that it has rare side effects as compared to different categories of interferon those were associated with a long list of side effects[17-19].

Although some studies from Pakistan especially from province Punjab were reported regarding SOF based therapies response in HCV genotype 3 infected patients[20,21], yet the efficacy of SOF on such a large scale was never evaluated previously in this region. The main objective of the present study was to assess the response and side effects of SOF in hepatitis C patients infected with genotype 3. Additionally, we were also interested to see the influence of patient's age, gender and baseline viral load on treatment response and to evaluate the association of rapid viral response (RVR) at week-4 with SVR that may help to predict the viral response rate at the earliest stage of the treatment.

MATERIALS AND METHODS

Patients and study design

From August 2014 to May 2016, 1375 patients having chronic infection of HCV genotype 3 were registered at the Department of Gastroenterology in Shalamar Hospital Lahore. Out of these 1375 patients, 885

(64.36%) were either non-responders or relapsers against pegIFN alfa-2a plus RBV and 490 (35.64%) were naive. According to the drug combinations, all the patients were separated into two groups. Patients of the first group were treated with SOF (Sovaldi® by Gilead Sciences) and RBV (Ribazol® by Getz Pharma Pakistan (PVT) Ltd). For the patients of the second group; pegIFN alfa-2a (Ropegra by Roach) was added with SOF + RBV.

Before starting the treatment; baseline characteristics, clinical data and laboratory investigations of all the patients was collected. All the patients included in the present study adults with more than 18 years of age were infected with HCV genotype 3. Out of 1375 patients, 696 (50.62%) were males and 679 (49.38%) were females. HCV genotyping and viral load measurement was performed on fully automated Abbott Real-Time PCR system (Abbott m24sp automated nucleic acid extraction system and Abbott m2000rt amplification system, Abbott Molecular, Des Plaines, IL, United States). Viral load was measured at day-0, week-4 and week-12 of the treatment in the first group and at day-0, week-4 and week-24 in the second group. To evaluate the SVR rate, PCR for HCV RNA was done at week-12 after the termination of treatment. Limit of detection or limit of quantitation was 12 IU/mL on Abbott Real-Time PCR system.

Approved recommendations were followed to treat the patients[22]. The first group was treated for 24 wk with SOF + RBV. For the second group, pegIFN alfa-2a was included with SOF and RBV and treatment duration was reduced to 12 wk. SOF of 400 mg was given as a single pill per day and the dosage of RBV was adjusted according to the patient's body weight (1000-1200 mg/d). For the second group, additional 180 μg of pegIFN alfa-2a was subcutaneously injected once in a week.

Statistical analysis

Continuous data like age, hemoglobin was expressed as mean ± SD, whereas categorical data was expressed in the form of frequencies, proportion and percentages. A 95%CI was also calculated for various proportions. A $P < 0.05$ was considered statistically significant. Statistical analysis was performed using Epicalc 2000 software (version 1.2, Brixton Health, United States).

RESULTS

In the present study, 1375 patients, including 50.62% males and 49.38% females with chronic infection of HCV genotype 3 were enrolled. Out of 1375, 35.64% patients were fresh and 64.36% had previous treatment history of pegIFN alfa-2a + RBV (Table 1).

The response of first group patients was 100% (847/847) to SOF + RBV at the end of therapy (ETR). From 847 ETR responders 840 (99.17%) could sustain their response after 12 weeks of the therapy termination and were declared as SVR-12 responders. In the second group, 526 (99.62%) out of 528 patients showed response at the time of treatment completion and 515 (97.91%) out of 526 were able to sustained their response (SVR-12) (Tables 2 and 3).

Table 1 Baseline characteristics of the patients

Characteristics	Value
Total participants (*n*)	1375
mean age (yr) mean ± SD (range)	48 ± 13 (18-65)
Gender	
Male	696 (50.62%)
Female	679 (49.38%)
Treatment history	
Treatment naive	490 (35.64%)
Treatment experienced	885 (64.36%)
Laboratory investigations	
Hemoglobin(Hb) (g/dL), mean ± SD (range)	12.50 ± 3.50 (7.5-18.3)
Platelets (10^3/μL), mean ± SD (range)	160 ± 76 (50-450)
Creatinine (mg/dL)	0.65 ± 0.22 (0.38-1.50)
Mean viral load (10^6 IU/mL) mean ± SD (range)	3.54 ±2.56 (0.01-33.98)
HCV genotype 3	1375 (100%)

HCV: Hepatitis C virus.

The rate of relapse cases was higher (6.54%) in old age patients (> 40 years) of the second group as compared to first group's old age patients (1.21%). In less than 40 years of age from First and second group's patients the rate of relapse cases was on the lower side (0.46% and 0.95%) (Table 2 and 3). In previously treated patients of the First group, SVR-12 was higher (99.34%) than naïve patients (97.80%) while, in second group, the SVR-12 rate of previously treated patients was lower than naïve (93.70% and 99.25% respectively) (Tables 2 and 3).

Males of both groups were found better responders than females in the present study. In the first group the difference in the response (SVR-12) of males and females was marginal (99.68% and 98.88%, respectively) as compared to second group where the SVR-12 of males and females was examined 98.96% and 95% (Tables 2 and 3).

The SVR-12 rate of the patients from both groups was higher (99.68% and 99.05% respectively) who had less than or equal to 2 MIU/mL of HCV viral load at day-0 than the patients having more than 2 MIU/mL viral load at that stage (97.70% and 96.19% respectively) (Tables 4 and 5). The RVR of the patients (week-4 response) was found very effective predictor to assess the SVR rate at earlier stages of the study. All the patients with HCV RNA negative after four weeks of the treatment (week-4) were also able to sustain their response after 12 weeks of the treatment completion either they belong to first or second group. One patient from the first group (0.19%) and four from the second group (1.05%), who were not able to drop more than two logs of the viral load at the fourth week of the treatment but were negative at the end of treatment, relapsed within 12 wk after the treatment termination. The rate of SVR-12 from both groups was found lower (64.70% from first group and 76.67%

Table 2 Association of baseline characteristics of the patients with sustained virological response treated with sofosbuvir + ribavirin (First group) *n* (%)

Baseline characteristics (*n* = 847)	ETR (*n* = 847), overall ETR = 100%	SVR (*n* = 840), overall SVR = 99.17%	Relapse (*n* = 7), overall relapse rate = 0.83%
Age (yr)			
≤ 40 (*n* = 435)	435 (100)	433 (99.54)	2 (0.46)
> 40 (*n* = 412)	412 (100)	407 (98.79)	5 (1.21)
Previous antiviral treatment history			
Naïve (*n* = 91)	91(100)	89 (97.80)	2 (2.20)
Treated (*n* = 756)	756 (100)	751 (99.34)	5 (0.66)
Gender			
Male (*n* = 310)	310 (100)	309 (99.68)	1 (0.32)
Female (*n* = 537)	537 (100)	531 (98.88)	6 (1.12)

ETR: End of therapy response rate; SVR: Sustained virological response.

Table 3 Association of baseline characteristics of the patients with sustained virological response treated with sofosbuvir + ribavirin + peg-interferon alfa-2a (Second group) *n* (%)

Baseline characteristics (*n* = 528)	ETR (*n* = 526), overall ETR 0.9962	SVR (*n* = 515), overall SVR 0.9791	Relapse (*n* = 11), overall relapse rate = 2.09%
Age (yr)			
≤ 40 (*n* = 419)	419 (100)	415 (99.05)	4 (0.95)
> 40 (*n* = 109)	107 (98.17)	100 (93.46)	7 (6.54)
Previous antiviral treatment history			
Naïve (*n* = 399)	399 (100)	396 (99.25)	3 (0.75)
Treated (*n* = 129)	127 (98.45)	119 (93.70)	8 (6.30)
Gender			
Male (*n* = 386)	386 (100)	382 (98.96)	4 (1.04)
Female (*n* = 142)	140 (98.59)	133 (95)	7 (5)

ETR: End of therapy response rate; SVR: sustained virological response.

Table 4 Association of baseline characteristics of the patients with sustained virological response treated with sofosbuvir + ribavirin (First group) *n* (%)

Time point	Viral load (*n* = 847)	ETR (*n* = 847), overall ETR = 100%	SVR (*n* = 840), overall SVR = 99.17%	Relapse (*n* = 7), overall relapse rate = 0.83%
Baseline (d-0)	≤ 2 MIU/mL (*n* = 630)	630 (100)	628 (99.68)	2 (0.32)
	> 2 MIU/mL (*n* = 217)	217 (100)	212 (97.70)	5 (2.30)
RVR (weeks-4)	Negative (*n* = 290)	290 (100)	290 (100)	0 (0)
	≥ 2 log drop (*n* = 540)	540 (100)	539 (99.81)	1 (0.19)
	< 2 log drop (*n* = 17)	17 (100)	11 (64.70)	6 (35.30)

ETR: End of therapy response rate; SVR: Sustained virological response.

from second group) in those patients who were unable to drop more than two logs of viral load at week-4 (Tables 4 and 5).

The most common side effects in the patients of both group were headache, fatigue and anemia. Excluding hashimoto's thyroiditis, rash, acute psychosis, Bell's palsy and intracranial hemorrhage the percentage of other side effects was higher in the patients of second group in which pegIFN alfa-2a was included with SOF+RBV as compared to first group patients treated without pegIFN alfa-2a. Three cases of Bell's palsy from the first group and one from the second group were new findings of the present study. Intracranial hemorrhage (ICH) was also found in two patients of the first group only but not in second group's patients (Table 6).

DISCUSSION

The main purpose of antiviral therapy in hepatitis C is either to eradicate the infection from the patient's body or to slow down the chances of disease progression to advanced stages like cirrhosis and HCC[23]. Before the introduction of SOF, hepatitis C was treated with interferon and RBV. The response rate of those drugs was not satisfactory. The patient also had to face many side effects of those drugs for a long time due to prolonged therapy durations. The addition of SOF in the antiviral

Table 5 Association of baseline characteristics of the patients with sustained virological response treated with sofosbuvir + ribavirin + peg-interferon-alfa-2a (Second group) *n* (%)

Time point	Viral load (n = 528)	ETR (n = 526), overall ETR 0.9962	SVR (n = 515), overall SVR 0.9791	Relapse (n = 11), overall relapse rate = 2.09%
Baseline (d-0)	⩽ 2 MIU/mL (n = 316)	316 (100)	313 (99.05)	3 (0.95)
	> 2 MIU/mL (n = 212)	210 (99.06)	202 (96.19)	8 (3.81)
RVR (weeks-4)	Negative (n = 116)	116 (100)	116 (100)	0 (0)
	⩾ 2 log drop (n = 380)	380 (100)	376 (98.95)	4 (1.05)
	< 2 log drop (n = 32)	30 (93.75)	23 (76.67)	7 (23.33)

ETR: End of therapy response rate; SVR: Sustained virological response; RVR: Rapid virological response.

Table 6 Side effects (n = 1375) *n* (%)

Side effects	SOF + RBV (n = 847)	SOF + RBV + Peg (n = 528)	*P* value
Headache	248 (29.28)	198 (37.50)	0.083
Fatigue	147 (17.36)	168 (31.82)	0.001
Myalgia	38 (4.49)	43 (8.14)	0.830
Hashimoto's thyroiditis	2 (0.23)	1 (0.19)	0.001
Decreased appetite	71 (8.38)	89 (16.86)	0.179
Rash	7 (0.82)	3 (0.57)	0.001
Thrush	23 (2.71)	19 (3.60)	0.467
Hair loss	8 (0.94)	59 (11.17)	0.781
Aggressiveness	29 (3.42)	38 (7.20)	0.895
Pruritus	69 (8.14)	57 (10.79)	0.959
Insomnia	79 (9.32)	63 (11.93)	0.498
Depression	36 (4.25)	67 (12.69)	0.006
Acute psychosis	8 (0.94)	3 (0.57)	0.776
Hematologic abnormalities			
Anemia (< 10 g/dL)	238 (28.10)	215 (40.72)	0.001
Leukocytopenia (< $3 \times 10^3/\mu L$)	16 (1.89)	61 (11.55)	0.001
Thrombocytopenia (< $100 \times 10^3/\mu L$)	64 (7.56)	72 (13.64)	0.188
Bell's Palsy	3 (0.35)	1 (0.19)	0.628
Intracranial hemorrhage	2 (0.23)	0 (0)	

therapy regimen has not only dramatically improved the SVR rate, but has also minimized the side effects[23].

Sofosbuvir is a nucleotide analogue that acts as a NS5B polymerase inhibitor. It has become the key drug to treat the patients of hepatitis C[17-19,24,25]. No virological breakthrough (HCV RNA negative patients at early stages of therapy became again positive during the therapy) was examined so far in the previous studies in which SOF was used as part of drug combinations in hepatitis C patients[24, 26]. The same situation was observed in our findings where no virological breakthrough was seen. It confirms the efficacy of SOF based therapies.

The overall SVR-12 rate in first and second group was 99.17% and 97.91% respectively. These findings show that the addition of SOF in drug combinations for hepatitis C patients infected with genotype 3 is more effective as compared to the treatment regimen without SOF as was reported before[22-24]. This is encouraging to eliminate the hepatitis C disease from the Pakistani population where most of the patients are infected with HCV genotype 3[27-29].

Our findings also advocate that 12 wk regimen containing SOF + RBV + pegIFN alfa-2a is equally effective as the 24 wk regimen of SOF + sRBV in HCV genotype 3 patients. It indicates that the pegIFN is still effective if given with SOF based regimen. With the SOF + RBV + pegIFN alfa-2a combination, treatment duration and cost could also be cut down in HCV genotype 3 cases. But, more side effects due to pegIFN as shown in this study may be the major disadvantage.

The patients less than 40 years of age were better responders in both groups as compared to the patients with more than 40 years of age. The better response of younger group patients treated with pegIFN+RBV was also indicated in our[30] and many other previous studies reported from different areas of the world before the introduction of SOF[29, 30]. It revealed that younger group patients with hepatitis C are suitable candidates to treat. Intolerance of old age patients (More than 40 years) as compared to young patients (Less than 40 years) against pegIFNs may be a major cause of lower SVR-12 rate in older patients as was also indicated previously[30-32]. It shows that the use of SOF without pegIFN alfa-2a is more effective, especially in old age patients and it may help to manage the hepatitis C patient's treatment regimen in the future.

The SVR-12 rate of previously treated patients was higher in first group patients (99.34%) than the second group (93.46%). It indicates that six months therapy of SOF + RBV is more effective in previously non-

responders or relapsed patients to pegIFN alfa-2a + RBV and infected with HCV genotype 3 as compared to three months therapy of SOF + RBV + pegIFN alfa-2a. On the other hand, SVR-12 rate of naïve patients was on higher side in second group's patients compared to first group's patients. It revealed that in naïve patients, treatment could be reduced from six to three months with the addition of pegIFN alfa-2a.

Interestingly, the RVR rate at week-4 was found a good predictor of SVR in both groups. All HCV RNA negative patients at week-4 were also found SVR-12 responders either they belongs to first or second group. The SVR rate of both group patients who were able to drop more than two log viral load at week-4 of the therapy was more satisfactory as compared to those who were unable to drop more than two log viral load at that stage. The effect of baseline (Day-0) viral load was not so significant in the present study.

The overall percentage of side effects was higher in the patients treated with triple therapy regimen (SOF + RBV + pegIFN alfa-2a) as compared to those patients treated with double therapy regimen (SOF + RBV). Headache, fatigue and anemia were the common side effects in all the patients either treated with or without pegIFN alfa-2a. Myalgia, decreased appetite, hair loss, aggression, depression, leukocytopenia and thrombocytopenia were prominently on the higher side in the patients of the second group as compared to first group's patients. It was also reported previously in RBV and pegIFN alfa-2a treated patients[33].

Severe neuropsychiatric side effects were commonly reported previously in hepatitis C patients treated with pegIFN and RBV[33, 34]. Sometimes treatment has to discontinue because of the severity of such adverse side effects. But in newly introduced antiviral drug "SOF" the severe neuropsychiatric complications are not much reported so far.

Two cases of intracranial hemorrhage were found only in first group's patients. Intracranial hemorrhage was also reported before in untreated hepatitis C patients[35,36], but no evidence of ICH with pegIFN plus RBV was seen previously. In our findings ICH may also be due to HCV not because of SOF that needs further studies on a large scale.

In conclusion, SOF (SOVALDI®) based therapy was found safe, effective and well tolerated by the patients infected with HCV genotype 3 in Pakistan. Higher SVR rate of the present study indicates that this is the right time for full blooded attack on HCV to get rid of it permanently from our region. To achieve the target, there is a need of policy under the aegis of the Federal Government to provide drugs on discounted rates or free for non-affording patients.

COMMENTS

Background

In Pakistan about 5.5% population is infected with hepatitis C virus (HCV), out of those 60%-80% has HCV genotype 3. Due to lower response and intolerable side effects of peg-Interferon plus RBV therapy, it was difficult to treat hepatitis C patients in past. In recent trials, the newly introduced oral drug "Sofosbuvir" was claimed for its remarkable response in the chronically infected patients with HCV genotype 2 and 3. It was important to see its efficacy in Pakistani population where most of the patients were infected with HCV genotype 3.

Research frontiers

Outcomes of the present study revealed that the SOF is more effective drug to treat the hepatitis C patients especially infected with genotype 3 of HCV. Sofosbuvir has less side effects and short treatment duration that helps the patient to get relief from hepatitis C in short time. These outcomes are also encouraged to eliminate hepatitis C from Pakistan where most of the population is infected with genotype 3 of HCV.

Innovations and breakthroughs

The novel finding of this study was to find highly responding oral drug (SOF) with low side effects that may help to eradicate hepatitis C from Pakistan where most of the population is infected with HCV genotype 3. Furthermore, we were also able to declare that the use of SOF is equally effective with or without peg-Interferon that may also help to avoid the adverse side effects of peg-Interferon injections. Its better response in all age groups also make easy to the clinician to treat the hepatitis C patients of all age groups.

Applications

The use of this oral drug (SOF) that has a dramatic response rate in hepatitis C patients will help to eliminate hepatitis C from Pakistan. To achieve the target, there is a need for policy under the aegis of the Federal Government to provide free drugs or on discounted rates for non-affording.

Terminology

Scientific terms that have been used in this manuscript are familiar with most readers and have been described comprehensively in different sections of the manuscript.

Peer-review

The authors carried out a prospective study to assess the efficacy and safety of SOF based therapies for the patients with HCV genotype 3. This study is well designed and the results are relevant to clinical practice.

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META-ANALYSIS

Short-term clinical outcomes of laparoscopic *vs* open rectal excision for rectal cancer: A systematic review and meta-analysis

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Abstract

AIM

To review evidence on the short-term clinical outcomes of laparoscopic (LRR) *vs* open rectal resection (ORR) for rectal cancer.

METHODS

A systematic literature search was performed using Cochrane Central Register, MEDLINE, EMBASE, Scopus, OpenGrey and ClinicalTrials.gov register for randomized clinical trials (RCTs) comparing LRR *vs* ORR for rectal cancer and reporting short-term clinical outcomes. Articles published in English from January 1, 1995 to June, 30 2016 that met the selection criteria were retrieved and reviewed. The Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) statements checklist for reporting a systematic review was followed. Random-effect models were used to estimate mean differences and risk ratios. The robustness and heterogeneity of the results were explored by performing sensitivity analyses. The pooled

effect was considered significant when $P < 0.05$.

RESULTS

Overall, 14 RCTs were included. No differences were found in postoperative mortality ($P = 0.19$) and morbidity ($P = 0.75$) rates. The mean operative time was 36.67 min longer (95%CI: 27.22-46.11, $P < 0.00001$), the mean estimated blood loss was 88.80 ml lower (95%CI: -117.25 to -60.34, $P < 0.00001$), and the mean incision length was 11.17 cm smaller (95%CI: -13.88 to -8.47, $P < 0.00001$) for LRR than ORR. These results were confirmed by sensitivity analyses that focused on the four major RCTs. The mean length of hospital stay was 1.71 d shorter (95%CI: -2.84 to -0.58, $P < 0.003$) for LRR than ORR. Similarly, bowel recovery (*i.e.*, day of the first bowel movement) was 0.68 d shorter (95%CI: -1.00 to -0.36, $P < 0.00001$) for LRR. The sensitivity analysis did not confirm a significant difference between LRR and ORR for these latter two parameters. The overall quality of the evidence was rated as high.

CONCLUSION

LRR is associated with lesser blood loss, smaller incision length, and longer operative times compared to ORR. No differences are observed for postoperative morbidity and mortality.

Key words: Laparoscopic rectal resection; Open rectal resection; Laparoscopy; Rectal cancer; Postoperative morbidity; Short-term outcomes; Systematic review; Meta-analysis

Core tip: There is no consensus on which technique, between laparoscopic rectal resection (LRR) and open rectal resection (ORR), is more beneficial for the patient. A systematic review and meta-analysis exclusively based on randomized clinical trials comparing LRR *vs* ORR has been performed. The pooled analyses focused on the evaluation and comparison of short-term clinical outcomes and showed that postoperative morbidity and mortality are similar between the two surgical approaches. However, LRR is associated with lesser blood loss and smaller incision length, which may represent clinical advantages for the patient.

Martínez-Pérez A, Carra MC, Brunetti F, de'Angelis N. Short-term clinical outcomes of laparoscopic *vs* open rectal excision for rectal cancer: A systematic review and meta-analysis. *World J Gastroenterol* 2017; 23(44): 7906-7916 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7906.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7906

INTRODUCTION

The oncologic principles for the curative treatment of rectal cancer imply the complete removal of the tumor and the mesorectum[1]. In locally advanced rectal cancers, oncologic outcomes can be improved by tailored multi-disciplinary approaches that combine surgery with neoadjuvant chemoradiation therapy[2].

Laparoscopy is currently useful for the resection of rectal cancer. The results of multi-centric randomized clinical trials (RCTs) have shown that laparoscopic rectal resection (LRR) was associated with more favorable short-term outcomes compared to open rectal resection (ORR)[3,4]. Specifically, the COLOR II trial showed statistically significant differences in favor of LRR in terms of blood loss, bowel recovery, and the length of hospital stay, with no differences between the two approaches in postoperative morbidity and mortality[3]. The COREAN study achieved similar results, and showed less postoperative pain and better physical and intestinal recovery after LRR[4]. In the more recent ACOSOG Z6051 and ALaCaRT trials, LRR was associated with longer operative times, less blood loss, and faster post-surgery bowel movements [ACOSOG] or time to flatus compared to ORR [ALACART], despite no observed group differences in the length of hospital stay[5,6]. Two recent meta-analyses had compared the short-term clinical results of LRR *vs* ORR based on pooled actualized data from the relevant literature on rectal cancer. They shown, among others advantages, a significant lesser postoperative morbidity[7,8] and mortality[7] for patients undergoing LRR over those who received ORR[7,8]. However, they considered both RCTs and non-RCTs, a critical factor that dampens the strength of the results due to the quality of the selected studies and the risk of bias. Furthermore, the results of a recent RCTs-based meta-analysis focusing exclusively on the pathologic outcomes of LRR *vs* ORR reignited the debate regarding the oncological safety of laparoscopy for rectal cancer in terms of quality of mesorectal resection[9]. Thus, while waiting for the long-term data of the ongoing RCTs, we conducted a systematic review and meta-analysis on RCTs only to evaluate the best level of evidence available so far on the short-term clinical outcomes of laparoscopic *vs* open rectal resections in patients with rectal cancer.

MATERIALS AND METHODS

Literature search

A literature search was performed on the following online databases: Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE (through PubMed), EMBASE, and Scopus. To increase the

probability of identifying all relevant articles, a specific research equation was formulated for each database, using the following keywords and/or MESH terms: rectal/colorectal cancer/carcinoma, treatment, therapy, management, surgery, laparoscopy/laparoscopic surgery, open surgery/laparotomy, and randomized trial/trial. Moreover, the reference lists of the eligible studies and relevant review articles were crosschecked to identify additional pertinent studies. Grey literature was explored on the OpenGrey database and the ClinicalTrials.gov registry was also searched to look for any ongoing RCT whose results might be published in the near future. Articles published in English from January 1, 1995 to June, 30 2016 that met the selection criteria were retrieved and reviewed.

Study design

The methodological approach for this systematic review included the development of selection criteria, the definition of search strategies, the assessment of study quality, and an abstraction of relevant data. The Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) statements checklist for reporting a systematic review was followed[10].

The eligibility and selection criteria were defined before the data search was initiated to ensure the proper identification of all studies that were eligible to be included in the systematic review and meta-analysis. Only RCTs that compared LRR and ORR and reported at least one of the outcomes of interest were retrieved and analyzed. No trial duration limitation was applied. Non-randomized studies, retrospective studies, case reports, review articles, commentaries, and conference abstracts were not considered in the systematic review. Studies that reported the results of surgical teams during their learning curve in laparoscopic rectal resection were excluded.

By applying the PICO framework, the study selection criteria were as follows:

Participants: Adult patients with histologically confirmed rectal cancer that required a surgical resection.

Interventions: Laparoscopic (including laparoscopic-assisted) or open rectal resection. Studies were included independently of the surgical technique (*e.g.,* abdominoperineal resection or anterior resection) and the performance of a primary anastomosis.

Comparisons: LRR *vs* ORR.

Outcome measures: include short-term surgical and clinical outcomes that were divided into: (1) Intraoperative outcomes: mean operative time (min), intraoperative morbidity rate (%), mean estimated blood loss (ml), mean incision length (cm), ureter injury rate (%), gastrointestinal injury rate (%); and (2) postoperative outcomes: postoperative morbidity rate (%), postoperative mortality rate (%), mean length of hospital stay (days), anastomotic leak rate (%), reoperation rate (%), ileus rate (%), time to bowel recovery (day of first bowel movement in days), wound infection rate (%), chest infection rate (%), urinary infection rate (%).

Data extraction

Initially, titles and abstracts of the retrieved studies were independently and blindly screened for relevance by two reviewers (AM-P and NdeA) according to the 2010 CONSORT Statement for RCTs (Http://www.consort-statement.org). To enhance sensitivity, records were removed only if both reviewers excluded the record at the initial screening level. Subsequently, both reviewers performed a full-text analysis of the selected articles.

Risk of bias

Both reviewers independently assessed the risk of bias using the Cochrane "Risk of Bias" tool, as described in the Cochrane Handbook for Systematic Reviews of Interventions[11]. Additionally, the Grading of Recommendations Assessment Development and Evaluation (GRADE) system was used to grade the "body of evidence" that emerged from the review[12]. All disagreements between the two reviewers in the selection and evaluation processes were resolved by discussion with a third reviewer (FB).

Statistical analysis

Data from the included studies were processed using qualitative and quantitative analyses. For binary outcome data, the relative risk (RR) and 95% CI were estimated using the Mantel-Haenszel method. For continuous data, the mean differences (MD) and 95%CIs were estimated using inverse variance weighting. Outcome measures (mean and median values, standard deviations, interquartile ranges) were extracted for each surgical treatment. If necessary and possible, outcome variables were calculated based on the data available in the individual studies. If the SE was provided instead of a SD, the SD was calculated based on the sample size (SE = SD/variables were calculated based on the data available in the individual studies. Whether neither mean or SD values were reported, they were estimated from the median, ranges, interquartile ranges (IQR) or *P* values[13,14]. Heterogeneity was assessed by the I^2 statistic[11,15,16]. I^2 values of 25%, 50%, and 75% were considered as low, moderate, and high, respectively[11,16].

The pooled estimates of the mean differences were calculated using random effects models to consider potential inter-study heterogeneity and to adopt a more conservative approach. Then, the robustness of the results and the potential sources of heterogeneity were explored by performing sensitivity analyses (*e.g.*,

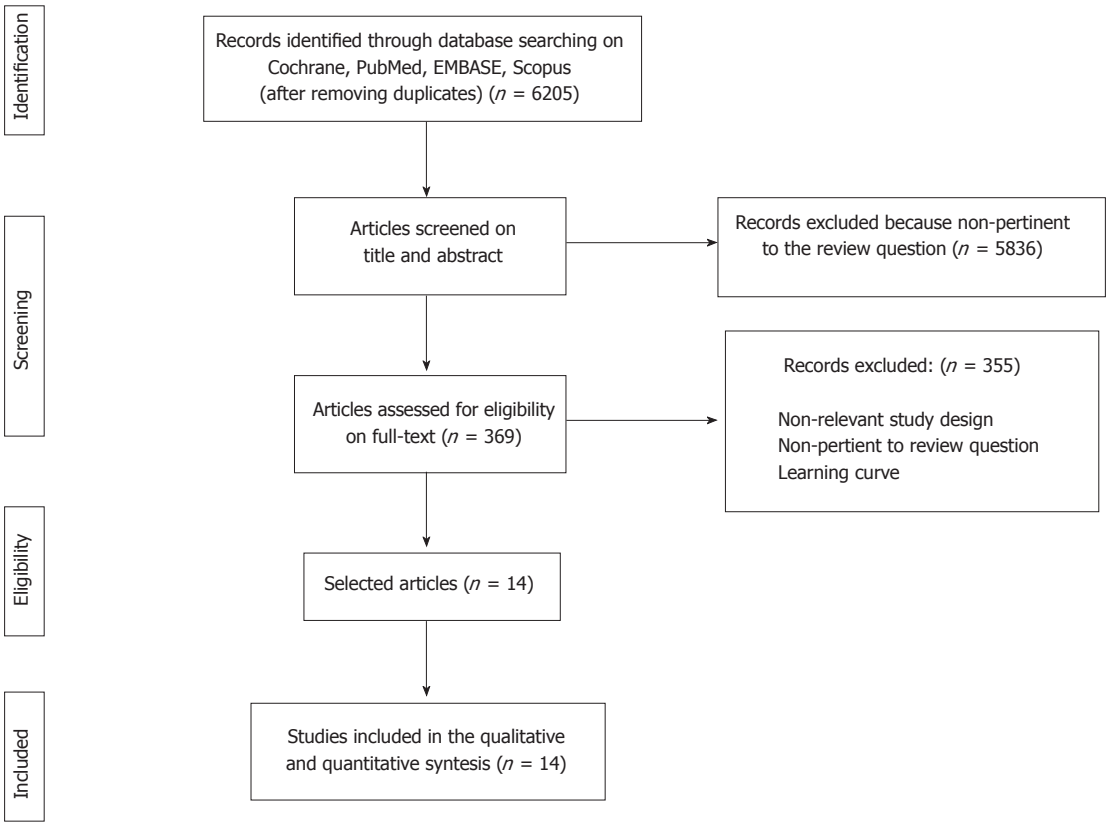


Figure 1 Flowchart of the literature search and study selection process according to the preferred reporting Items for systematic reviews and meta-analysis guidelines.

subgroup analyses; comparison using a fixed-effects model). The pooled effect was considered significant if $P < 0.05$. The meta-analysis was performed using Review Manager (RevMan, version 5.3, Cochrane Collaboration, Copenhagen, Denmark).

RESULTS

Study selection

Overall, the combined search identified 6205 articles, of which 5836 were rejected based upon the title and abstract evaluation. The remaining 369 articles underwent full-text evaluation; 355 were excluded because they were not RCTs, presented duplicate data of other RCTs included in the systematic review, did not report the outcomes of interest, or presented the results of laparoscopic rectal resections during the surgeon's learning curve. No additional study was identified through the manual search, reference lists crosschecks, grey literature or on ClinicalTrials.gov. Finally, 14 eligible articles were found and were included in the qualitative and quantitative analyses. The flowchart of the literature search and the study selection process is shown in Figure 1.

The 14 selected studies were published between 2003 and 2015. They included patients who had surgery between September 1993 and November 2014. Nine studies were performed in single centers[17-25], whereas 5 were multi-centric studies[3-6,26]. Overall, these studies analyzed a total of 4132 patients who underwent either open (n = 1819) or laparoscopic (n = 2313) rectal resections. In this latter group, 13.8% of patients (range: 0%-33.9%) required a conversion from laparoscopy to open surgery. Table 1 displays the baseline characteristics of patients who underwent LRR or ORR.

Intraoperative outcomes

Mean operative time was significantly longer, the estimated blood loss and the mean incision length significantly lower for LRR than ORR (Figure 2A-C). Conversely, no significant differences were observed for ureteric or gastrointestinal injury rates, or for overall intraoperative morbidity (Table 2).

Postoperative outcomes

The mean length of hospital stay was reported in 12 studies[3-6,17-23,26]. The overall MD was -1.71 d (95%CI: -2.84 to -0.58, $P < 0.003$) in favor of laparoscopy, with a high heterogeneity (I^2 = 90%). Bowel recovery, described as the day of the first bowel movement, was reported in 9 studies[3,5,6,17,20-24,26]. The overall MD was -0.68 d (95%CI: -1.00 to -0.36, $P < 0.00001$) in favor of laparoscopy, with a high heterogeneity (I^2 = 79%) (Figure 2D and E). Anastomotic leak[3-5,17,19-21,23,24], postoperative morbidity[3-5,17-23,25,26], and

Table 1 Summary of the included randomized clinical trials

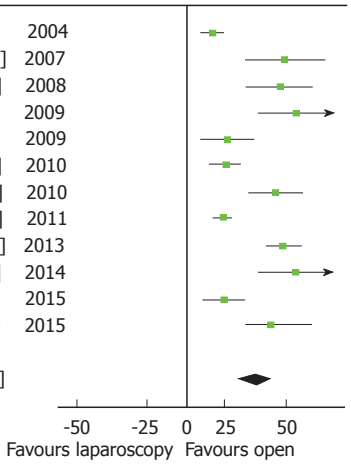
Ref.	Number of centers involved (country) in the RCT and in the study period	Inclusion criteria	Exclusion criteria	*n*	Surgical approach			Types of procedure		Preoperative treatment	
					Lap (*n*)	Conversion rate (%)	Open (*n*)	Lap (%)	Open (%)	Lap (%)	Open (%)
Fleshman *et al*[5], 2015	35 (United States-Canada) Oct 2008-Sep 2013	S. Ⅱ-Ⅲ rectal cancer ⩽ 12 cm from AV	1-11	462	240	11.25	222	LAR (74.6) APR (24.2) H (0.4) TPC (0.8)	AR (76.1) APR (21.2) TPC 6 (2.7)	CRT (95) RT (3.3) CT (1.7)	CRT (91.2) RT (5.5) CT (3.4)
Stevenson *et al*[6], 2015	24 (Australia-N. Zeal) Mar 2010-Nov 2014	T1-3 rectal cancer ⩽ 15 cm from AV	1, 2, 4, 7, 10, 12, 13	473	238	8.82	235	LAR (89) APR (11)	AR (90) APR (10)	RT (50)	RT (49)
Ng *et al*[20], 2014	1 (Hong Kong) Aug 2001-Aug2007	Rectal cancer low margin 5-12 cm AV	13, 16, 17, 23, 24, 25	80	40	7.5	40	LAR (100)	AR (100)	0	0
van der Pas *et al*[3], 2013	30 (Europe- Canada-South Korea) Jan 2004-May 2010	T1-3 rectal cancer ⩽ 15 cm from AV	1, 2, 9, 10, 13-22	1044	699	16.4	345	LAR (70) APR (29) U (1)	AR (77) APR (23)	RT (59) CT (32)	RT (58) CT (34)
Liang *et al*[24], 2011	1 (China) May 2004-April 2008	Rectal cancer	16, 25, 26, 31, 34, 35	343	169	0.59	174	LAR (50.9) APR (49.1)	AR (59.8) APR (40.2)	0	0
Kang *et al*[4], 2010	3 (South Korea) Apr 2006-Aug 2009	T1-3 rectal cancer ⩽ 9 cm from AV	1, 5, 10, 13, 16, 21, 23, 26	340	170	1.18	170	LAR (85.9) APR (14.1)	AR (88.8) APR (11.2)	CRT (100)	CRT (100)
Liu *et al*[18], 2010	1 (China) Feb 2005-Oct 2008	Rectal cancer	16, 17, 23	186	98	0	88	[1]LAR (69.4) H 14 (14.3) APR (12.2) O 4 (4.1)	AR (67) H (12.5) APR (15.9) O 4 (4.5)	0	0
Ng *et al*[21], 2009	1 (Hong Kong) Sep 1993-Oct 2002	Rectal cancer low margin 12-15 cm AV	1, 16, 23, 24, 27, 28	153	76	30.26	77	LAR (100)	AR (100)	0	0
Luján *et al*[19], 2009	1 (Spain) Jan 2002-Feb 2007	Mid or low rectal cancer	1, 13, 18, 29	204	101	7.92	103	LAR 77 (76.2) APR 24 (23.8)	AR (78.6) APR (21.4)	CRT (72.3)	CRT (74.8)
Ng *et al*[22], 2008	1 (Hong Kong) Sep1994-Feb 2005	Low rectal cancer	13, 16, 23, 24, 30	99	51	9.8	48	APR (100)	APR (100)	0	0
Braga *et al*[17], 2007	1 (Italy) Period n.a.	Rectal cancer	1, 2, 10, 13, 31	168	83	7.23	85	LAR (91.6) APR (8.4)	AR (87) APR (13)	CRT (16.9)	CRT (14.1)
Guillou *et al*[26], 2005	27 (United Kingdom) Jul 1996 - Jul 2002	Colorectal cancer (excl. transverse)	11, 16, 17, 21, 32, 33	381	253	33.88	128	AR 167 (66) APR (25) S (3) LH (2) O (3) U (1)	AR (62) RC 1 (1) S 7 (5) APR (27) O 4 (3) U (2)	NA	NA
Zhou *et al*[23], 2004	1 (China) Jun 2001 - Sep 2002	1.5 cm above AV to peritoneal reflection	1, 13, 29	171[24]	82	na	89	LAR (100)	AR (100)	0	0
Araujo *et al*[25], 2003	1 (Brazil) Sep 1997-Sep 2000	Low rectal cancer not responding RCT	1	28	13	0	15	APR (100)	APR (100)	CRT (100)	CRT (100)

[1]Hand-assisted procedures. Exclusion criteria: (1) Tumors other than histologically confirmed adenocarcinoma; (2) Age > 18 years; (3) body mass index (BMI) > 34, (4) Eastern Cooperative Oncology Group (ECOG) performance score ⩾ 3; (5) Not receiving neoadjuvant chemoradiotherapy/radiotherapy; (6) Operation not performed between 4-12 wk of the final radiation treatment; (7) History of invasive pelvic malignancy within 5 years; (8) psychiatric or addictive disorders that affected compliance with the protocol, (9) American Society of Anesthesiologists (ASA) classification IV or V, (10) Severe systemic disease, (11) Conditions that limited the success of the laparoscopic resection; (12) Life expectancy of at least 12 weeks; (13) T4 tumors/involved CRM pretreatment; (14) T1 tumor treated with local transanal excision; (15) History of other malignancy except basocellular carcinoma of the skin or in situ carcinoma of the cervix uteri, (16) Signs of acute intestinal obstruction, (17) Need for synchronous colorectal surgery; (18) Familial adenomatous polyposis coli/hereditary non-polyposis; (19) Colorectal cancer; (20) Active Crohn's disease/ulcerative colitis; (21) Pregnancy; (22) T3 rectal cancer within 2 mm from the endopelvic fascia; (23) Tumor perforation; (24) Tumor larger than 6 cm; (25) Neoadjuvant chemoradiotherapy; (26) Distant metastasis, (27) Distal tumor that needed an anastomosis within 5 cm of the dentate line; (28) Previous abdominal operations near the region of the colorectal operation; (29) Emergency surgery; (30) recurrent disease; (31) ongoing infection/plasma neutrophil level < 2 × 109/L; (32) Associated gastrointestinal disease that needed surgical intervention; (33) Malignant disease in the past 5 years; (34) BMI > 30 kg/m2 and (35) previous abdominal surgery. AV: Anal verge; Lap: Laparoscopy; LAR: Laparoscopic anterior resection; AR: Anterior resection; APR: Abdominoperineal amputation; TPC: Total Proctocolectomy; H: Hartmann; S: Sigmoidectomy; LH: Left Hemicolectomy; RC: Right colectomy; O: Other; U: Unknown; CRT: Chemoradiotherapy; RT: Radiotherapy; CT: Chemotherapy.

A

Study or subgroup	Laparoscopy Mean	SD	Total	Open Mean	SD	Total	Weight	Meta difference IV, random, 95%CI	Year
Zhou 2004	120	18.33	82	106	25	89	9.8%	14.00 [7.46, 20.54]	2004
Barga 2007	262	72	83	209	70	85	6.7%	53.00 [31.52, 74.48]	2007
Ng 2008	213.5	46.2	51	163.7	43.4	48	7.6%	49.80 [32.15, 67.45]	2008
Ng 2009	213.1	59.3	76	154	70.3	77	6.9%	59.10 [38.50, 79.70]	2009
Lujan 2009	193.7	45.1	101	172	59.4	103	8.3%	21.70 [7.24, 36.16]	2009
Liu 2010	161	35	98	140	20	88	9.6%	21.00 [12.91, 29.09]	2010
Kang 2010	244.9	75.4	170	197	62.9	170	8.2%	47.90 [33.14, 62.66]	2010
Liang 2011	138.08	23.79	169	118.53	21.99	174	10.0%	19.55 [14.70, 24.40]	2011
van der Pas 2013	240	85.92	699	188	66.66	345	9.3%	52.00 [42.51, 61.49]	2013
Ng 2014	211.6	53	40	153	41.1	40	6.9%	58.60 [37.82, 79.38]	2014
Stevenson 2015	210	66.67	238	190	59.25	235	9.0%	20.00 [8.64, 31.36]	2015
Fleshman 2015	266.2	101.9	240	220.6	92.4	222	7.6%	45.30 [27.88, 63.32]	2015
Total(95%CI)			2047			1676	100.0%	36.67 [27.22, 46.11]	

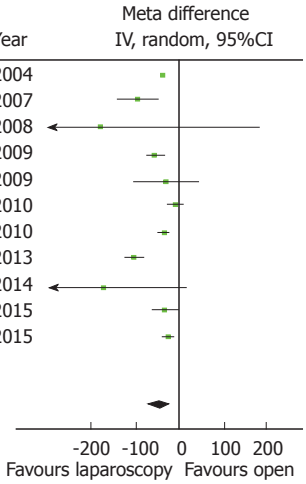
Heterogeneity. Tau^2 = 225.21; χ^2 = 96.98, df = 11 (P < 0.00001); I^2 = 89%
Test for overall effect: Z = 7.61 (P <0.00001)



B

Study or subgroup	Laparoscopy Mean	SD	Total	Open Mean	SD	Total	Weight	Meta difference IV, random, 95%CI	Year
Zhou 2004	20	19.1	82	92	25	89	15.7%	-72.00 [-78.64, -65.36]	2004
Barga 2007	213	236	83	396	367	85	5.9%	-183.00 [-276.09, 89.91]	2007
Ng 2008	321.7	750	51	555.6	1180	48	0.5%	-233.90 [-626.08, 158.28]	2008
Lujan 2009	127.8	113.3	101	234.2	174.3	103	12.0%	-106.40 [-146.67, -66.13]	2009
Ng 2009	280	500	76	337.3	423.67	77	3.0%	-57.30 [-204.24, 89.64]	2009
Kang 2010	200	148.15	170	217.5	185.18	170	12.7%	-17.50 [-53.15, 18.15]	2010
Liu 2010	310	96	98	380	85	88	14.0%	-70.00 [-96.01, -43.99]	2010
van der Pas 2013	200	192.59	699	400	370.35	345	11.8%	-200.00 [-241.61, -158.39]	2013
Ng 2014	141.8	500	40	361.1	623.75	40	1.2%	-219.30 [-467.04, 28.44]	2014
Fleshman 2015	256.1	305.8	240	318.4	331.7	222	9.5%	-62.30 [-120.62, -3.98]	2015
Stevenson 2015	100	111.11	238	150	181.48	235	13.8%	-50.00 [-77.16, -22.84]	2015
Total(95%CI)			1878			1502	100.0%	-88.80 [-117.25, -60.34]	

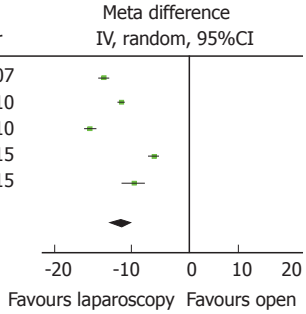
Heterogeneity. Tau^2 = 1335.06; χ^2 = 58.16, df = 10 (P < 0.00001); I^2 = 83%
Test for overall effect: Z = 6.12 (P <0.00001)



C

Study or subgroup	Laparoscopy Mean	SD	Total	Open Mean	SD	Total	Weight	Meta difference IV, random, 95%CI	Year
Barga 2007	5.8	0.8	83	19.1	3.1	85	20.1%	-13.30[-13.98, -12.62]	2007
Liu 2010	6	1	98	17	2	88	20.2%	-11.00 [-11.46, -10.54]	2010
Kang 2010	5	1.15	170	20	3.7	170	20.2%	-15.00 [-15.58, -14.42]	2010
Stevenson 2015	6	3.33	238	13	4.44	235	20.1%	-7.00 [-7.71, -6.29]	2015
Fleshman 2015	7	5.7	240	16.5	8.4	222	19.4%	-9.50 [-10.82, -8.18]	2015
Total(95%CI)			829			800	100.0%	-11.17 [-13.88, -8.47]	

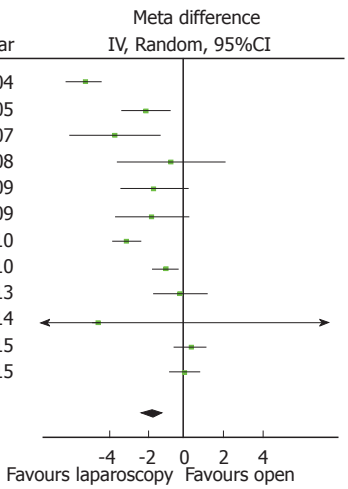
Heterogeneity. Tau^2 = 9.36; χ^2 = 333.13, df = 4 (P < 0.00001); I^2 = 99%
Test for overall effect: Z = 8.09 (P <0.00001)



D

Study or subgroup	Laparoscopy Mean	SD	Total	Open Mean	SD	Total	Weight	Meta difference IV, Random, 95%CI	Year
Zhou 2004	8.1	3.1	82	13.3	3.4	89	9.9%	-5.20 [-6.17, -4.23]	2004
Guillou 2005	11	4.44	253	13	6.66	128	9.4%	-2.00 [-3.28, -0.72]	2005
Barga 2007	10	4.9	83	13.6	10	85	7.2%	-3.60 [-5.97, -1.23]	2007
Ng 2008	10.8	5.5	51	11.5	8.25	48	6.5%	-0.70 [-3.48, 2.08]	2008
Ng 2009	8.4	5	76	10	6	77	8.5%	-1.60 [-3.35, 0.15]	2009
Lujan 2009	8.2	7.3	101	9.9	6.8	103	8.1%	-1.70 [-3.64, 0.24]	2009
Liu 2010	12	2	98	15	3	88	10.2%	-3.00 [-3.74, -2.26]	2010
Kang 2010	8	3.73	170	9	2.96	170	10.2%	-1.00 [-1.72, -0.28]	2010
van der Pas 2013	11.9	11.8	684	12.1	10.6	337	9.1%	-0.20 [-1.64, 1.24]	2013
Ng 2014	10.5	7.5	40	15	40.25	40	0.7%	-4.50 [-17.19, 8.19]	2014
Fleshman 2015	7.3	5.4	240	7	3.4	222	10.1%	0.30 [-0.52, 1.12]	2015
Stevenson 2015	8	4.44	238	8	4.44	235	10.1%	0.00 [-0.80, 0.80]	2015
Total(95%CI)			2116			1622	100.0%	-88.80 [-117.25, -60.34]	

Heterogeneity. Tau^2 = 3.10; χ^2 = 112.33, df = 11 (P < 0.00001); I^2 = 90%
Test for overall effect: Z = 2.97 (P = 0.003)



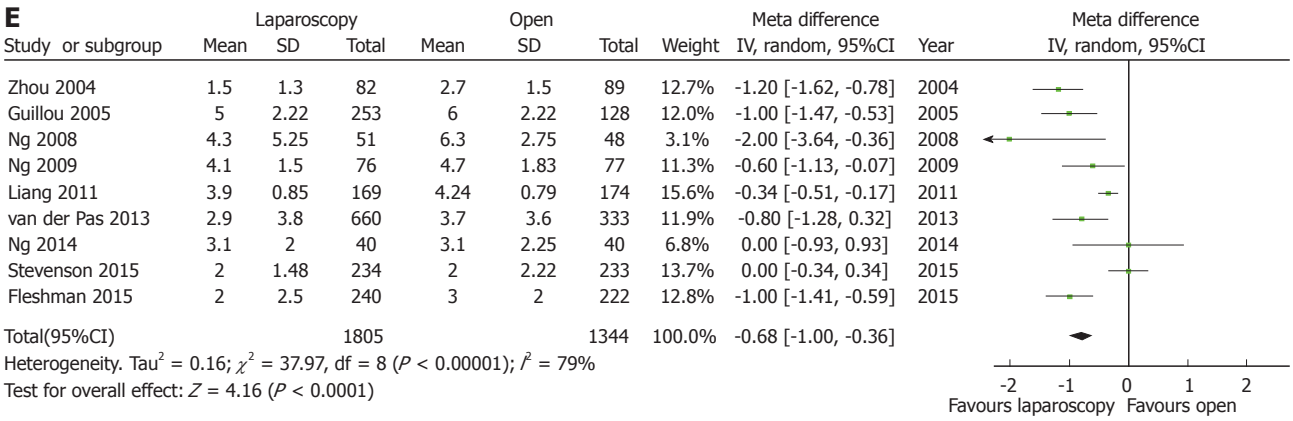


Figure 2 Forest plots of short-term outcomes showing significant differences between laparoscopic rectal resection and open rectal resection. A: Operative time; B: Estimated blood loss; C: Incision length; D: Length of hospital stay; E: Bowel recovery.

mortality[3-6,17-24] rates showed no significant differences between LRR and ORR (Table 2).

Sensitivity analysis

Sensitivity analyses performed to test the impact of using fixed-effect models showed the same results for all variables that for random effect models. Subgroup analysis was also performed by including the four largest multi-centric trials only (namely, the ACOSOG Z6051, AlaCaRT, COLOR Ⅱ, and COREAN trials[3-6]). These 4 studies comprised 2319 patients (56.2% of the total). Although being a high-populated multi-centric RCT, the UK MRC-CLASICC trial[26] was not included in the sensitivity analysis because it was conducted in the early years of laparoscopic surgery and included both colon and rectal cancers. The estimated blood loss and the length of incision were significantly lower with an operative time that was significantly higher for LRR compared with ORR, but the heterogeneity remained high. For the postoperative variables, the length of hospital stay and bowel recovery did not reach statistical significance in favor of LRR. Heterogeneity decreased to moderate for length of hospital stay and remained high for bowel recovery (Table 2).

Study quality assessment

The assessment of study quality and the risk of bias, according to the Cochrane Collaboration tool for RCTs, are shown in supplemental table 1. Overall, 10 studies were classified as a low risk of bias[3-6,17,19-22,26], 1 at an unknown risk of bias[24] and 3 studies at a high risk of bias[18,23,25]. By applying the GRADE system, the overall quality of the evidence was rated as high.

DISCUSSION

This systematic review and meta-analysis focuses on the short-term clinical outcomes of laparoscopic *vs* open resection for the treatment of rectal cancer and shows that there are no differences in postoperative morbidity and mortality between the two approaches. However, LRR is associated with significantly longer operative time, lesser blood loss, and smaller incision than ORR. The length of hospital stay and the time to bowel recovery are shorter for LRR in the overall analysis but are not significantly different when considering the major RCTs only.

Previous meta-analyses have reported contrasting results about the benefits associated with the use of laparoscopy for rectal cancer instead of conventional open surgery. In 2013, Arezzo *et al*[27] analyzed 8 RCTs and 15 non-RCTs and showed a significantly lower postoperative mortality and morbidity in LRR than ORR. A more recent meta-analysis by Zhao *et al*[28] and the latest Cochrane review[29], both based on RCTs only, showed no differences in overall morbidity and mortality. However, they found better outcomes for laparoscopy in terms of blood loss, length of hospital stay, wound infection, and bowel recovery compared to open surgery. Noticeably, the above-mentioned meta-analyses were performed before the two largest and most recent RCTs being published, namely the ACOSOG Z6501 and ALaCaRT trials[5,6], which did not confirm the non-inferiority of laparoscopy and questioned the oncological safety of laparoscopy for rectal cancer. Indeed, the topic remains highly debated. Two recent meta-analyses published by Chen *et al*[8] and by Zheng *et al*[7] were performed to assess the outcomes of laparoscopy *vs* open surgery by including data from RCTs and non-RCTs. The study by Chen *et al*[8] demonstrated longer operative time, lesser blood loss, lesser overall complications, faster bowel recovery, shorter hospitalization, and major distal resection margin for laparoscopic surgery than open surgery[8]. However, there was found a considerable and arbitrary lack of data from the most populated RCTs[3-6] (which represented more than 50% of the patients included) for all the short-term variables analyzed, such as distance of distal resection margin[3,4,6], CRM involvement[5], lymph node harvest[3], operative time[3,4,6], hospital stay[4,6],

Table 2 Results of the meta-analyses comparing laparoscopic rectal resection vs open rectal resection

Outcome variables	Number of studies (Number of patients)	RR or MD	95%CI (Low; High)	*P* value	Heterogeneity, I2 (*P* value)	Sensitivity analysis by including the largest multi-centric RCTs				
						Number of studies (Number of patients)	RR or MD	95%CI (Low; High)	*P* value	Heterogeneity, I^2 (*P* value)
Intraoperative outcomes										
Operative time	12 (3723)	36.67	27.22; 46.11	<0.00001	89% (< 0.00001)	4 (2319)	41.18	24.88; 57.48	< 0.00001	85% (0.0002)
Intraoperative morbidity	4 (1909)	0.97	0.74; 1.27	0.82	8% (0.35)	2 (1500)	1.02	0.60; 1.72	0.94	61% (0.11)
Estimated blood loss	11 (3380)	-88.8	-117.25; -60.34	< 0.00001	83% (< 0.00001)	4 (2319)	-82.1	-158.87; -5.34	0.04	94% (< 0.00001)
Incision length	5 (1629)	-11.17	-13.88; -8.47	< 0.00001	99% (< 0.00001)	3 (1275)	-10.51	-16.16; -4.85	0.0003	99% (< 0.00001)
Ureter injury	5 (2256)	1.23	0.20; 7.72	0.82	51% (0.11)	2 (1500)	2.59	0.66; 10.11	0.17	0% (0.67)
Gastrointestinal injury	4 (2052)	1.14	0.25; 5.17	0.86	73% (0.02)	2 (1500)	1.05	0.13; 8.22	0.96	86% (0.007)
Postoperative outcomes										
Postoperative morbidity	12 (3313)	0.98	0.88; 1.09	0.75	16% (0.3)	3 (1844)	1.02	0.91; 1.15	0.69	0% (0.65)
Postoperative mortality	13 (3751)	0.65	0.34; 1.23	0.19	0% (1)	4 (2319)	0.60	0.27; 1.33	0.21	0% (0.89)
Length hospital stay	12 (3738)	-1.71	-2.84; -0.58	0.003	90% (< 0.00001)	4 (2296)	-0.25	-0.90; 0.39	0.44	52% (0.10)
Anastomotic leak	9 (2253)	0.97	0.69; 1.34	0.84	0% (0.6)	3 (1351)	1.19	0.79; 1.80	0.41	0% (0.53)
Reoperation rate	7 (2468)	0.93	0.64; 1.34	0.69	3% (0.4)	3 (1844)	1.19	0.77; 1.86	0.43	0% (0.42)
Ileus	10 (2930)	0.77	0.55; 1.06	0.11	0% (0.66)	3 (1875)	0.79	0.43; 1.44	0.44	62% (0.07)
Bowel recovery	9 (3149)	-0.68	-1.00; -0.36	< 0.0001	79% (< 0.00001)	3 (1922)	-0.59	-1.24; 0.07	0.08	87% (0.0005)
Wound infection	10 (2684)	0.81	0.61; 1.09	0.16	0% (0.5)	1 (1042)	0.82	0.43;1.47	0.5	NA
Chest infection	7 (1252)	1.55	0.82; 2.93	0.17	0% (0.61)	0 (0)	-	-	-	-
Urinary infection	7 (1075)	0.89	0.50; 1.57	0.68	15% (0.32)	0 (0)	-	-	-	-

RR: Risk ratios; MD: Mean difference; CI: Confidence interval; NA: Not applicable.

and estimated blood loss[3,4,6]. Similarly, the meta-analysis by Zheng *et al*[7] included 38 studies and 13,408 patients, but only less than a third of patients (3978, 29.7%) were treated in RCTs. Moreover, the 32.9% of the included patients (4405 patients) were coming from a unique multi-centric observational study involving 72 Spanish hospitals[30]. Despite the eager of pooling data to gain power and answer the hot question about the advantages of laparoscopic rectal resection, caution should be paid when interpreting meta-analytic results. Contrasting result may be generated by using different statistical models, or when pooling together RCTs with non-RCTs. In general, the choice of the effect model should be assessed prior to start the data analysis and based on the researcher's understanding of whether or not all the included studies share a common treatment effect. For surgical literature, a random-effect model seems to be more appropriate than a fixed-effect[31,32] due to the nature of the data retrieved from studies performed by researchers operating independently. Deciding between the models after performing the analysis or based upon the level of heterogeneity found (*e.g.*, whether the I^2 is higher than 40%[29] or 50%[7,8,27] or reaches significance[33]) is strongly discouraged[31,32]. Most importantly, the quality of a meta-analysis is strictly dependent on the quality of the original studies included and robustness of the findings should be tested by sensitivity analyses.

The present systematic review and meta-analysis aimed to analyze the best level of evidence available for LRR *vs* ORR, thus only high-quality RCTs were included. By applying a strict methodology, the present findings confer fewer advantages to LRR over ORR, especially when only the largest multi-centric RCTs were considered, when compared to the results of previous meta-analyses.

The main intra-operative benefit of LRR, confirmed by both the pooled data analysis and the sensitivity analysis, is a lower blood loss. This might justify the use of laparoscopy for rectal cancer resections despite longer operative times. Indeed, the amount of blood loss has been shown to be an independent predictor of adverse surgical outcomes, such as intra- and postoperative complications, cancer recurrence, and poorer survival[34,35]. Although the reasons why intraoperative blood loss would be associated with morbidity and poor survival remain unclear, some evidence supports that blood loss triggers stress and immune reactions, which may lead

to an increased susceptibility of infections and cancer recurrence. Thus, minimizing blood loss, and the consequent risk of blood transfusion by meticulous and gentle dissections in the anatomical planes, may contribute to better outcomes of oncological surgery. However, it remains to be assessed whether the difference observed between the two surgical approaches (*i.e*., 88.80 mL) is clinically relevant, and may potentially impact on the postoperative and long-term outcomes.

Other markers of surgical quality are the postoperative complication rates and the time to bowel recovery. Based on the pooled data analyses from the major RCTs[3-6], laparoscopy was not associated with a significantly different incidence of postoperative complications, time to bowel recovery or hospital stay compared to open surgery. Concerning bowel recovery, it can be measured with multiple clinical variables, such as the time to the first flatus, the time to a liquid or solid diet, or the time to the first bowel movement. Globally, bowel recovery was not significantly different between LRR and ORR but it must be noted that benefits in at least one of the recovery variables considered (*e.g*., time to flatus or time to regular diet) were found in all RCTs. Thus, caution should be paid before drawing definitive conclusions; differences among studies did not allow pooling data for all variables (*e.g*., the COREAN study[4] expressed bowel recovery in hours rather than days and could not be included in the meta-analysis), except for the time to first bowel movement. Despite the non-significant results in the sensitivity analysis, bowel recovery is probably faster after LRR than ORR, but further studies are needed to confirm this finding.

The evidence emerging from this systematic review and meta-analysis can be considered of high quality since it is based exclusively upon RCTs[36], most of which with low risk of bias. However, some limitations must be acknowledged. The pooled data analyses showed high degrees of heterogeneity; this may be linked to multiple factors, such as different sample size (*e.g*., some studies presented less than 100 patients per group[17,18,20-23,25]), different tumor characteristics (*e.g*., only upper[21] or lower rectal cancer[22,25]), and different study designs (*e.g*., non-inferiority study) or protocols. For instance, neoadjuvant treatments were not performed in all studies, and therapies were not standardized. It has been hypothesized that major pathologic responses might translate into greater postoperative morbidities because of the effects of neoadjuvant chemoradiation therapy on pelvic tissues[37]. To date, only a few studies have addressed the influence of the pathologic response to neoadjuvant chemoradiation therapy on intraoperative and short-term morbidity, with contrasting results[37-40], but its impacts could neither be confirmed nor ruled out in this meta-analysis. Finally, the results of this meta-analysis cannot be generalized to the application of LRR and ORR for all types of rectal cancer. Indeed, T4 rectal cancers were excluded from most of the studies[3,4,6,17,19,22,23]. Thus, the outcomes of laparoscopy for this specific subset of tumors cannot be assumed, although a recent propensity score-matched study showed that LRR also achieved similar outcomes to ORR in pT4 rectal cancer patients[41].

The short-term benefits of laparoscopy must be counterbalanced with its safety. Indeed, uncertainty persists concerning the oncological appropriateness of laparoscopy for rectal cancer. A recent meta-analysis[9] focused on the pathologic outcomes of LRR *vs* ORR and showed that LRR was associated with a significantly higher rate of non-complete mesorectal excision compared with ORR, which represents a critical issue on the choice of the surgical approach. Innovative techniques, such as transanal-TME and robotics, are receiving worldwide attention in the latest years and they may represent a valuable alternative to laparoscopy, especially if they are proved to be oncologically safe, clinically advantageous for the patient, and maybe less challenging for the surgeon[42-45]. Nevertheless, data on the long-term outcomes of the ongoing RCTs are pending and they may be crucial in the definitive assessment of the role of laparoscopy in rectal cancer resection.

In conclusion, LRR and ORR show similar rates of intra- and postoperative complications, as well as morbidity and mortality. However, LRR is associated with a significantly higher operative time, lesser blood loss, and smaller incision length than ORR.

ARTICLE HIGHLIGHTS

Research background

Laparoscopy is widely used for the resection of rectal cancer. The associated short-term benefits for the patient (*e.g.,* fewer postoperative morbidity) have been highlighted in several studies, but with contrasting results. We conducted a systematic review and meta-analysis by selecting only randomized clinical trials (RCTs) that evaluated the short-term clinical outcomes of laparoscopic rectal resection (LRR) *vs* open rectal resection, (ORR) in patients with rectal cancer.

Research motivation

The short-term advantages of laparoscopic rectal resection remain under debate due to controversial results, especially when analyzing the most recent RCTs. Pooled data analyses of the available literature represents the best way to summarize the current evidence and support the development and widespread of the most advantageous surgical approach.

Research objectives

The main objective of the present systematic review and meta-analysis was to analyze the current literature of RCTs on the surgical treatment for rectal cancer to compare the short-term outcomes of laparoscopy *vs* open surgery. The analysis of the literature has also highlighted the level of evidence and risk of bias inherent in the available studies, which should be used to design future research on the treatment of rectal cancer.

Research methods

This is a systematic literature review and meta-analysis that was conducted by following the guidelines of the Cochrane Collaboration as well as the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) statements checklist. Literature search was performed on different databases

for articles published in English from January 1, 1995 to June, 30 2016. Random-effect models were used to estimate mean differences and risk ratios between LRR and ORR. The robustness and heterogeneity of the results were explored by performing sensitivity analyses.

Research results

Overall, 14 RCTs were analyzed. The mean operative time was longer for LRR than ORR, whereas the mean estimated blood loss and the mean incision length were lower for LRR than ORR. No differences between the two surgical approaches were found in postoperative mortality, morbidity, length of hospital stay, and time to bowel recovery. Although the overall quality of evidence was judged as high, not all the studies evaluated the same parameters. Thus, future research should use standardized definitions of postoperative outcomes in order to increase comparability and decrease heterogeneity among studies.

Research conclusions

LRR is associated with lesser blood loss, smaller incision length, and longer operative times compared to ORR. No differences are observed for postoperative morbidity and mortality. The short-term advantages of laparoscopic rectal resection are mainly represented by a significantly lower intraoperative blood loss and better cosmetic results compared to open surgery. The overall level of evidence supporting these findings is high.

Research perspectives

Further studies should evaluate alternative minimally-invasive surgical techniques (*e.g.,* transanal TME or Robotics) and compare them with laparoscopic and open approaches.

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META-ANALYSIS

Anterior *vs* conventional approach right hepatic resection for large hepatocellular carcinoma: A systematic review and meta-analysis

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Author contributions: Tang JX and Li JJ acquired, analyzed and interpreted the data; Tang JX drafted the article; Weng RH revised the article; Liang ZM interpreted the data; Jiang N conceived and designed the study and critically revised the manuscript; all authors have read and approved the final manuscript.

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Abstract

AIM

To compare the clinical outcomes of right hepatectomy for large hepatocellular carcinoma *via* the anterior and conventional approach.

METHODS

We comprehensively performed an electronic search of PubMed, EMBASE, and the Cochrane Library for randomized controlled trials (RCTs) or controlled clinical trials (CCTs) published between January 2000 and May 2017 concerning the anterior approach (AA) and the conventional approach (CA) to right hepatectomy. Studies that met the inclusion criteria were included, and their outcome analyses were further assessed using a fixed or random effects model.

RESULTS

This analysis included 2297 patients enrolled in 16 studies (3 RCTs and 13 CTTs). Intraoperative blood loss [weighted mean difference = -255.21; 95% confidence interval (95%CI): -371.3 to -139.12; $P < 0.0001$], intraoperative blood transfusion [odds ratio (OR) = 0.42; 95%CI: 0.29-0.61; $P < 0.0001$], mortality (OR = 0.59; 95%CI: 0.38-0.92; $P = 0.02$), morbidity (OR = 0.77; 95%CI: 0.62-0.95; $P = 0.01$), and recurrence

rate (OR = 0.62; 95%CI: 0.47-0.83; *P* = 0.001) were significantly reduced in the AA group. Patients in the AA group had better overall survival (hazard ratio [HR] = 0.71; 95%CI: 0.50-1.00; *P* = 0.05) and disease-free survival (HR = 0.67; 95%CI: 0.58-0.79; *P* < 0.0001) than those in the CA group.

CONCLUSION
The AA is safe and effective for right hepatectomy for large hepatocellular carcinoma and could accelerate postoperative recovery and achieve better survival outcomes than the CA.

Key words Anterior approach; Conventional approach; Right hepatectomy; Hepatocellular carcinoma; Postoperative complication; Survival

Core tip: Anterior approach has been suggested as an alternative approach to conventional approach for right hepatectomy. However, comparative studies have shown conflicting results. To evaluate whether right hepatectomy using the anterior approach for large hepatocellular carcinoma results in better clinical outcomes when compared with the conventional approach, we investigated these two techniques in terms of estimated intraoperative blood loss, massive blood loss, intraoperative blood transfusion, operative time, mortality, morbidity, recurrence rate, hospital stay, overall survival and disease-free survival.

Tang JX, Li JJ, Weng RH, Liang ZM, Jiang N. Anterior *vs* conventional approach right hepatic resection for large hepatocellular carcinoma: A systematic review and meta-analysis. *World J Gastroenterol* 2017; 23(44): 7917-7929 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7917.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7917

INTRODUCTION

Conventional right hepatectomy (CRH), which is complete mobilization of the right liver with the right hepatic vein controlled outside the liver before parenchymal transection, is a standard treatment approach[1,2]. However, its use is often difficult and hazardous in cases of large hepatocellular carcinoma (HCC) involving the right liver with extrahepatic organ invasion in the right retrohepatic region. The conventional approach (CA) could result in excessive blood loss, hemodynamic instability, tumor metastasis, tumor rupture, and liver ischemia because of prolonged rotation of the liver remnant during the course of liver mobilization[3]. All of these drawbacks could be ameliorated using the anterior approach (AA) for right hepatectomy, which was first demonstrated by Lai and colleagues in 1996[4]. The AA involves initial vascular inflow control, completion of parenchymal transection, and complete venous outflow control before mobilization of the right liver. Lately, it has been recognized that the AA has some advantages over the CA, including less intraoperative blood loss, fewer requirements for transfusion, shortened operation time, lower hospital mortality, and better disease-free survival (DFS) or overall survival (OS) following right hepatectomy for HCC $\geqslant$ 5 cm[5]. However, using the AA, it is difficult to control the branches of the middle hepatic vein at the deeper parenchymal transection, thereby increasing the risk of major vessel injury, especially to the hepatic veins and inferior vena cava[6].

Our initial experience using the AA in a group of patients with large benign or malignant right-lobe liver tumors showed that it was a safe and effective option for selected patients undergoing right hepatectomy. Some prospective randomized controlled trials (RCTs) and retrospective controlled clinical trials (CCTs) documented the clinical outcomes of AA compared to CA for right hepatectomy; however, the clinical significance of the AA over the CA remains unclear.

A recent systematic review evaluated the feasibility, safety, and efficacy of CA *vs* AA right hepatectomy[7], but data regarding the operative and survival outcomes of patients undergoing surgery are insufficient. A comprehensive systematic review and meta-analysis of the AA over the CA to right hepatectomy in patients with right-lobe large HCC has not been published to date. Many questions on the AA remain unanswered, most notably its clinical and oncologic outcomes and long-term survival. Therefore, the current study aimed to perform a comprehensive systematic review of all available studies to evaluate the safety, feasibility, and effectiveness of AA *vs* CA right hepatectomy using a meta-analytical method.

MATERIALS AND METHODS

Here we acquire evidence through four steps: data sourcing and searches, application of inclusion and exclusion criteria, data extraction, and quality assessment and statistical analysis. We followed the systematic review methods of the Institute of Medicine's Standards for Systematic Reviews[8] with slight modifications. Our study results are reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses[9] and Meta-Analysis of Observational Studies in Epidemiology[10] standards.

Data sources and searches

An electronic search was performed of and relevant publications from January 2000 to May 2017 that were identified in Pubmed, Embase, and the Cochrane Library. The search was not restricted by language, region, or publication type. The search terms were ("anterior approach right hepatectomy" or "conventional approach right hepatectomy" or "anterior approach right hepatic resection" or "conventional approach right hepatic resection") and ("liver cancer"

or "liver tumor" or "hepatocellular carcinoma" or "liver neoplasms"). Related terms were used to broaden the search, and the computer search was supplemented with manual searches of the reference lists of all retrieved studies. When multiple similar studies describing the same population were identified, the most recent or complete study was included.

Inclusion and exclusion criteria

The included studies met the following criteria: (1) Comparing AA and CA right hepatectomy; (2) Patients underwent planned selective right-lobe hepatic resection of a large liver tumor; (3) Prospective RCT or retrospective CCT; (4) Adult patients (age ⩾ 18 years) who underwent right-lobe hepatic resection; (5) Primary or metastatic large liver tumors; and (6) Reporting at least one of the quantitative outcomes mentioned in these studies.

The excluded studies met the following criteria: (1) Non-comparative or irrelevant to the subject; (2) Lacking a comparison group of CA right hepatectomy; (3) Patients had distant metastases or malignancies in other organs; (4) Left-lobe large hepatocarcinoma resection or minor liver resection; (5) Non-adult patients (age ⩽ 18 years) who underwent right hepatectomy; (6) Duplicate publications, editorials, meeting abstracts, letters to the editor, review articles, case reports, and animal experimental studies; and (7) Studies that included no extractable data.

Data extraction

Studies that met all the inclusion criteria were retrieved as full-text articles. Data from the included studies were extracted and summarized independently by two authors (Tang JX and Weng RH). Any disagreement was resolved by the senior author (Jiang N).

The primary outcomes were intraoperative blood loss, massive blood loss, intraoperative blood transfusion, operative time, mortality, morbidity, overall survival, disease-free survival, and recurrence. Recurrence was subdivided into extrahepatic recurrence, intrahepatic recurrence, and extrahepatic plus intrahepatic recurrence. The secondary outcomes were hospital stay, R0 resection rate, bile leakage, and liver failure.

Quality assessment and statistical analysis

Two review authors (Tang JX and Li JJ) independently assessed the methodological quality of the studies. The methodological quality of the RCTs was assessed using the Jadad score[11], with a cumulative score ⩾ 3 indicating high quality. The methodological quality of the retrospective nonrandomized studies was assessed using the modified Newcastle-Ottawa scale[12], which consists of three elements: patient selection, comparability of the study groups, and outcome assessment. A score of 0-9 (allocated as stars) was allocated to all included studies (supplementary table 1). RCTs and nonrandomized studies achieving six or more stars were considered of high quality.

All included studies were rated at the level of evidence according to criteria provided by the Centre for Evidence-Based Medicine in Oxford, United Kingdom. The meta-analyses were performed using Review Manager 5.0 (Cochrane Collaboration, Oxford, United Kingdom) and Stata 12.0 (StataCorp, College Station, TX, United States). The weighted mean difference (WMD) was used to compare continuous variables, while odds ratio (OR) was used to compare dichotomous variables. We extracted hazard ratio (HR) with 95% CI from the publications as a relevant measure for the effects of overall survival and disease-free survival. We estimated the HR using log-rank χ^2 statistics, log-rank P values, the given numbers of events, or Kaplan-Meier curves as described by Parmar *et al* and Williamson *et al*[13,14]. We calculated the standard deviations of continuous data presented as means and ranges using the technique described by Hozo *et al*[15]. The results are reported with 95%CI. Statistical heterogeneity between the included studies was evaluated using the Q measure for statistical significance and the I^2 measure for quantifying heterogeneity, with values of $P < 0.1$ considered statistically significant and $I^2 > 50\%$ indicating substantial heterogeneity. The random-effects model was used in cases of interstudy heterogeneity; otherwise, the fixed-effects model was used[16]. A sensitivity analysis was performed of the high-quality studies. Funnel plots were used to screen for potential publication bias.

RESULTS

The initial search revealed 376 studies. After the title and abstract screening process, 36 studies were considered potentially useful for inclusion. We then retrieved and reviewed their full text; 20 of these 36 studies were ultimately excluded from the meta-analysis because they were meeting abstracts, not specifically about right hepatectomy, institution duplications, or had no extractable date. Finally, 16 studies fulfilled the predefined inclusion criteria and were included in the final analysis (Figure 1). The included studies were published from 2000 to 2017. All publications were full-text articles; we reviewed the reference list of each to identify additional possible studies for inclusion. Agreement between the two reviewers (Tang JX and Weng RH) was 94% for study selection and quality assessment.

Characteristics of the included studies

The characteristics of the included studies are shown in Table 1. Among them, there were three RCTs[5,6,17] and thirteen CCTs[3,5,17-29] including a total of 2297 patients (AA = 1076; CA = 1221). All of the patients in the studies underwent right hepatectomy or extended

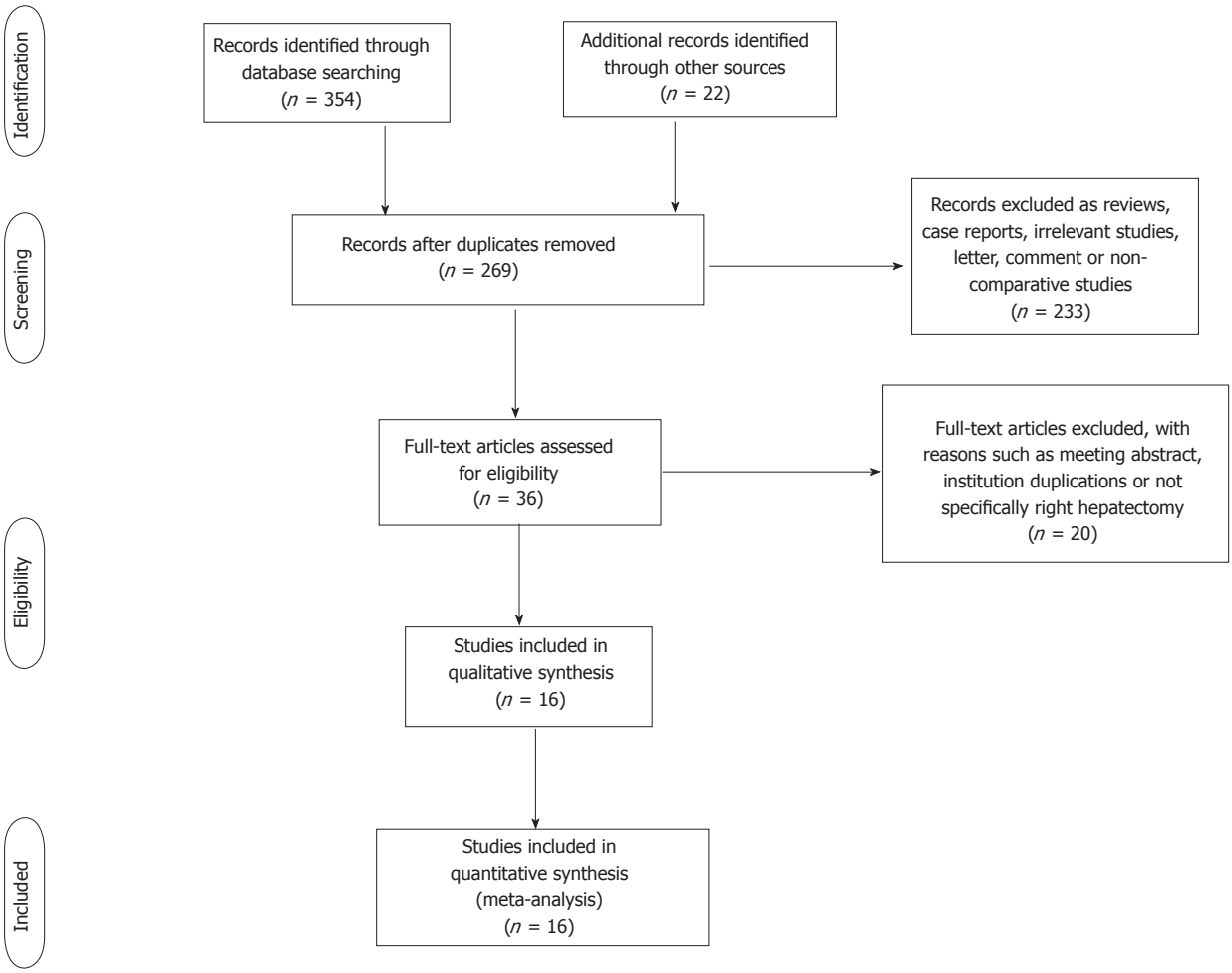


Figure 1 Flow diagram of study identification, inclusion, and exclusion.

right hepatectomy. Eight studies described simple right hepatectomy[5,6,20,23-26,28], while another eight studies detailed a mixture of right hepatectomy for large liver right-lobe tumors[3,17-19,21,22,27,29].

Methodological quality of the included studies

The objective quality of the included studies was generally high. True randomization was used in three RCTs[5,6,17]. Most of the retrospective CCTs adopted an appropriate protocol for treatment assignment, and allocation was usually at the physician's discretion. However, no studies provided information about allocation concealment or blinding method. Matching criteria between the groups were variable (Table 1). Methods for managing missing data and intention to treat right-lobe hepatectomy analyses were generally adequate among the majority of studies. Eleven studies[3,5,18,19,22,23,25-29] mentioned the length of follow-up, and most provided accurate data.

Primary outcomes

Intraoperative blood loss: Aggregation of the data from 14 studies[3,5,6,17-24,26-28] revealed that 2041 of the patients who underwent right hepatectomy (AA = 984; CA = 1057) experienced intraoperative blood loss. There was significant heterogeneity among the studies (χ^2 = 122.96; I^2 = 89%; P < 0.00001). The pooled data showed that intraoperative blood loss was significant lower in the AA group than in the CA group (WMD = -255.21; 95%CI: -371.30 to -139.12; P < 0.0001) (Figure 2A).

Massive blood loss: Seven studies[3,5,6,20,25,26,28] including 672 patients reported massive blood loss > 1 L (AA = 302; CA = 370). There was significant heterogeneity among the studies (χ^2 = 11.49; I^2 = 48%; P = 0.07) (Figure 2B). Meta-analysis using a random-effects model revealed that the OR of massive blood loss differed significantly between the two groups (OR = 0.42; 95%CI: 0.21-0.85; P = 0.02).

Intraoperative blood transfusion: Data concerning intraoperative blood transfusion were available in three RCTs[5,6,17] and nine CCTs[3,18,20,21,23,26-29] including 1400 patients who underwent large right-lobe hepatic cancer resection (AA = 659; CA = 741). Meta-analysis using a random-effects model revealed a significant decrease in blood transfusions in the AA group than in the CA group (OR = 0.42; 95%CI: 0.29-0.61; P < 0.00001) (Figure 2C).

Table 1 Characteristics of included studies

Study	Design	Level of evidence	Indication	Indications Patients (*n*)		Characteristic matching[1]	Follow-up, mean or median, ARH/CRH	Quality score
				ARH	CRH			
Beppu *et al*[18]	R	3b	MP	72	72	1, 2, 5, 6, 7, 8, 9, 10	27.2 ± 2.1/18.1 ± 2.8	*******
Capussotti *et al*[6]	RCT	2b	RH	33	32	1, 2, 3, 4, 5, 7, 9, 10, 11, 12	NA	RCT
Chan *et al*[19]	R	3b	MP	110	169	1, 2, 5, 6, 7, 8, 9, 12	60/60	******
Chen *et al*[20]	RP	3b	RH	11	13	1, 2, 5, 6, 8, 12	NA	*****
Cresswell *et al*[21]	RP	3b	MP	62	62	1, 2, 4, 7, 11	NA	*****
Habib *et al*[22]	RP	3b	MP	242	169	1, 2, 4, 7, 10, 11	30 ± 20.3	******
Hao *et al*[23]	P	3b	RH	107	111	1, 2, 4, 5, 6, 7, 8, 9, 10	49/38	*******
Higuchi *et al*[24]	R	4	RH	25	44	1, 2, 3, 5, 9, 12	NA	*****
Jabir *et al*[25]	R	3b	RH	40	98	1, 2, 5, 6, 7, 9, 11	36 ± 21.5	******
Li *et al*[26]	R	3b	RH	92	96	1, 2, 5, 6, 7, 8, 9	29 ± 7.8	*******
Liu *et al*[27]	R	3b	MP	54	106	1, 2, 5, 6, 7, 8, 9, 12	59.7/18.6	*******
Liu *et al*[27]	RCT	1b	RH	60	60	1, 2, 5, 6, 7, 8, 9, 10, 11, 12	21.6 ± 8.0/18.3 ± 5.4	RCT
Llado *et al*[28]	P	3b	RH	33	33	1, 2, 6, 7, 9, 10	24/24	******
Takács *et al*[29]	R	3b	MP	52	67	1, 2, 7, 9, 10, 11	32/32	*****
Wu *et al*[3]	R	3b	MP	33	38	1, 2, 5, 6, 7, 9, 11, 12	19 ± 12.7	*******
Zhou *et al*[17]	RCT	2b	MP	50	51	1, 2, 5, 6, 7, 8, 9, 10, 11	NA	RCT

[1]Matching: 1 = Age; 2 = Gender; 3 = Body mass index; 4 = American Society of Anesthesiologists score; 5 = Liver function test; 6 = Hepatitis status; 7 = Tumor number or maximum tumor size; 8 = Child-Pugh Classification; 9 = Vascular invasion; 10 = Distant metastasis; 11 = Tumor histology; 12 = Indocyanine green retention rate at 15 min. ARH: Anterior approach for right hepatectomy; CRH: Conventional right hepatectomy; NA: Data not available; R: Retrospective; P: Prospective; RP: Retrospective design, prospective data collection; RCT: Randomized controlled trail; RH: Right hepatectomy; ERH: Extended right hepatectomy; MP: Mixed procedures.

Operative time: Fourteen studies[3,5,6,17-20,22-24,26-29] including 2035 patients who underwent right hepatectomy (AA = 974; CA = 1061) reported operative time. There was significant heterogeneity among the studies (χ^2 = 289.73; I^2 = 96%; P < 0.00001). A meta-analysis indicated no significant difference in operative time between the two groups (WMD = -10.69; 95%CI: -37.22-15.87; P = 0.43) (Figure 2D).

Mortality: Twelve studies[3,5,6,19,21-24,26-29] including 1890 patients who underwent right hepatectomy for large liver tumors evaluated hospital mortality rate. The mortality rate was 3.54% (32/903 patients) in the AA group and 6.48% (64/987 patients) in the CA group. There was no significant heterogeneity among the studies (χ^2 = 10.83; I^2 = 0%; P = 0.46) (Figure 3A). Using a fixed-effects model, the pooled data showed that mortality rate in the AA group was significantly lower than that in the CA group (OR = 0.59; 95%CI: 0.38-0.92; P = 0.02).

Morbidity: Thirteen studies[3,5,6,17,18,21-26,28,29] including 1834 patients who underwent major right hepatectomy reported operative morbidity events. The overall morbidity rate was 29.30% (264/901 patients) in the AA group and 36.23% (338/933 patients) in the CA group. The operative morbidity rate of the AA group was significantly lower than that of the CA group (OR = 0.77; 95%CI, 0.62-0.95; P = 0.01) (Figure 3B).

Overall survival: One prospective RCT[5] and eight retrospective CCTs[3,18,19,23,25-28] reported overall survival events, including 1242 patients (AA = 562; CA = 680). There was significant heterogeneity among the studies (χ^2 = 47.12; I^2 = 83%; P < 0.00001). Meta-analysis using a random-effects model revealed that there was a significant increase in overall survival following the AA right hepatectomy in comparison with the CA right hepatectomy (HR = 0.71; 95%CI: 0.50-1.00; P = 0.05) (Figure 3C).

Disease-free survival: One prospective RCT[5] and eight retrospective CCTs[3,18,19,23,25-28] including 1227 patients (AA = 556; CA = 671) reported disease-free survival events. There was little significant heterogeneity among the studies (χ^2 = 15.05; I^2 = 47%; P = 0.06). Meta-analysis using a random-effects model showed that there was a significant increase in disease-free survival after AA right hepatectomy than after CA right hepatectomy (HR = 0.67; 95%CI: 0.58-0.79; P < 0.00001) (Figure 3D).

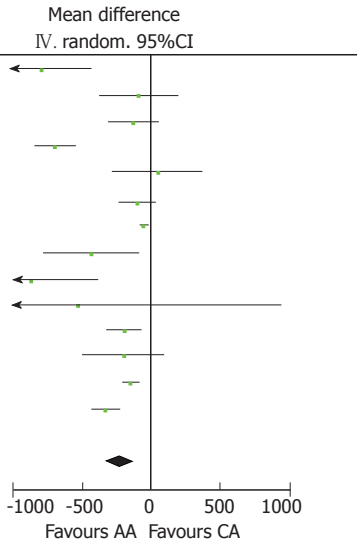
Tumor recurrence: Tumor recurrence rate was available for one prospective RCT[5] and eight retrospective CCTs[3,18,19,22,23,25,26,28] including 1682 patients who underwent right hepatectomy (AA = 788; CA = 840). The total recurrence rate was 47.21% (372/788 patients) in the AA group and 61.19% (514/840 patients) in the CA group. There was no significant heterogeneity among the studies (χ^2 = 13.82; I^2 = 42%; P = 0.09). Meta-analysis using a random-effects model showed that there was a significant decrease in tumor recurrence rate following the AA right hepatectomy in comparison with the CA right hepatectomy (OR = 0.62; 95%CI: 0.47-0.83; P = 0.001) (Supplementary figure 1). Then, patients were divided into three subgroups based on the recurrence location, including intrahepatic, extrahepatic, or both intrahepatic and extrahepatic tumor recurrence. There was no significant heterogeneity among the

A

Study or subgroup	AA Mean	AA SD	AA Total	CA Mean	CA SD	CA Total	Weight	Mean difference IV. random. 95%CI
Beppu, T 2017	480	895	72	1242	1264.167	72	5.3%	-762.00 [-1119.77, -404.23]
Capussotti, L 2012	437	664	33	500	532.3	32	6.4%	-63.00 [-355.13, 229.13]
Chan, K. M 2015	500	825	110	600	658.33	169	8.4%	-100.00 [-283.36, 83.36]
Chen, H. W 2016	227.27	91.44	11	892.31	275.26	13	8.9%	-665.04 [-824.13, -505.95]
Cressswell, A. B 2009	425	1317.25	62	355	277	62	5.6%	70.00 [-265.05, 405.06]
Habib, M 2012	348	228.333	242	420	878.167	169	9.3%	-72.00 [-207.49, 63.49]
Hao, S 2016	378	130	107	402	115	111	10.5%	-24.00 [-56.63, 8.63]
Higuchi, R 2016	1237	579	25	1646	953.75	44	5.2%	-409.00 [-770.84, -47.16]
Li, S. Q 2010	1291	1159	92	2129	2012	96	3.9%	-838.00 [1304.99, -371.01]
Liu, C. L 2000	2000	4850	54	2500	3300	106	0.6%	-500.00 [-1938.05, 938.05]
Liu, C. L 2006	800	225	60	1000	425	60	9.5%	-200.00 [-321.68, -78.32]
Llado, L 2016	941	348	33	1145	807	33	6.2%	-204.00 [-503.85, 95.85]
Wu, T. J 2010	500	106.25	33	660	178.25	38	10.3%	-160.00 [-227.28, -92.72]
Zhou, Y. M 2016	423	154	50	757	338	52	9.8%	-334.00 [-435.30, -232.70]
Total (95% CI)			984			1057	100.0%	-255.21 [-371.30, -139.12]

Heterogeneity: Tau^2 = 32986.50 χ^2= 122.96, df = 13 (P < 0.00001); I^2 = 89%

Test for overall effect: Z = 4.31 (P < 0.0001)

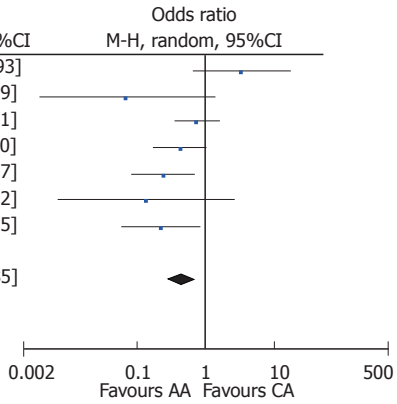


B

Study or subgroup	AA Events	AA Total	CA Events	CA Total	Weight	Odds ratio M-H, random, 95%CI
Capussotti, L 2012	6	33	2	32	11.4%	3.33 [0.62, 17.93]
Chen, H. W 2016	0	11	5	13	4.6%	0.07 [0.00, 1.39]
Jabir, M. A 2017	13	40	40	98	23.9%	0.70 [0.32, 1.51]
Li, S. Q 2010	8	92	18	96	21.9%	0.41 [0.17, 1.00]
Liu, C. L 2006	5	60	17	60	18.8%	0.23 [0.08, 0.67]
Llado, L 2016	0	33	3	33	4.7%	0.13 [0.01, 2.62]
Wu, T. J 2010	3	33	12	38	14.7%	0.22 [0.06, 0.85]
Total (95% CI)		302		370	100.0%	0.42 [0.21, 0.85]
Total events	35		97			

Heterogeneity: Tau^2 = 0.38 χ^2= 11.49, df = 6 (P = 0.07); I^2 = 48%

Test for overall effect: Z = 2.42 (P = 0.02)

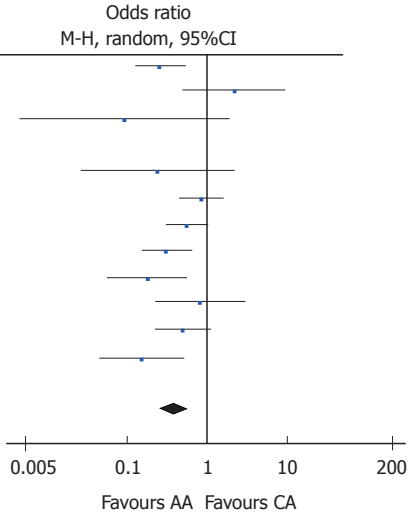


C

Study or subgroup	AA Events	AA Total	CA Events	CA Total	Weight	Odds ratio M-H, random, 95%CI
Beppu, T 2017	15	72	36	72	11.9%	0.26 [0.13, 0.55]
Capussotti, L 2012	6	33	3	32	5.0%	2.15 [0.49, 9.45]
Chen, H. W 2016	0	11	4	13	1.4%	0.09 [0.00, 1.93]
Cressswell, A. B 2009	1	62	4	62	2.6%	0.24 [0.03, 2.19]
Hao, S 2016	23	107	27	111	13.4%	0.85 [0.45, 1.60]
Li, S. Q 2010	38	92	54	96	14.3%	0.55 [0.31, 0.98]
Liu, C. L 2000	31	54	86	106	12.0%	0.31 [0.15, 0.65]
Liu, C. L 2006	4	60	17	60	7.1%	0.18 [0.06, 0.58]
Llado, L 2016	5	33	6	33	6.1%	0.80 [0.22, 2.95]
Taka cs, I 2006	30	52	49	67	11.4%	0.50 [0.23, 1.08]
Wu, T. J 2010	4	33	18	38	6.6%	0.15 [0.05, 0.52]
Zhou, Y. M 2016	6	50	15	51	8.2%	0.33 [0.12, 0.93]
Total (95% CI)		659		741	100.0%	0.42 [0.29, 0.61]
Total events	163		319			

Heterogeneity: Tau^2 = 0.17 χ^2= 19.54, df = 11 (P = 0.05); I^2 = 44%

Test for overall effect: Z = 4.49 (P < 0.0001)



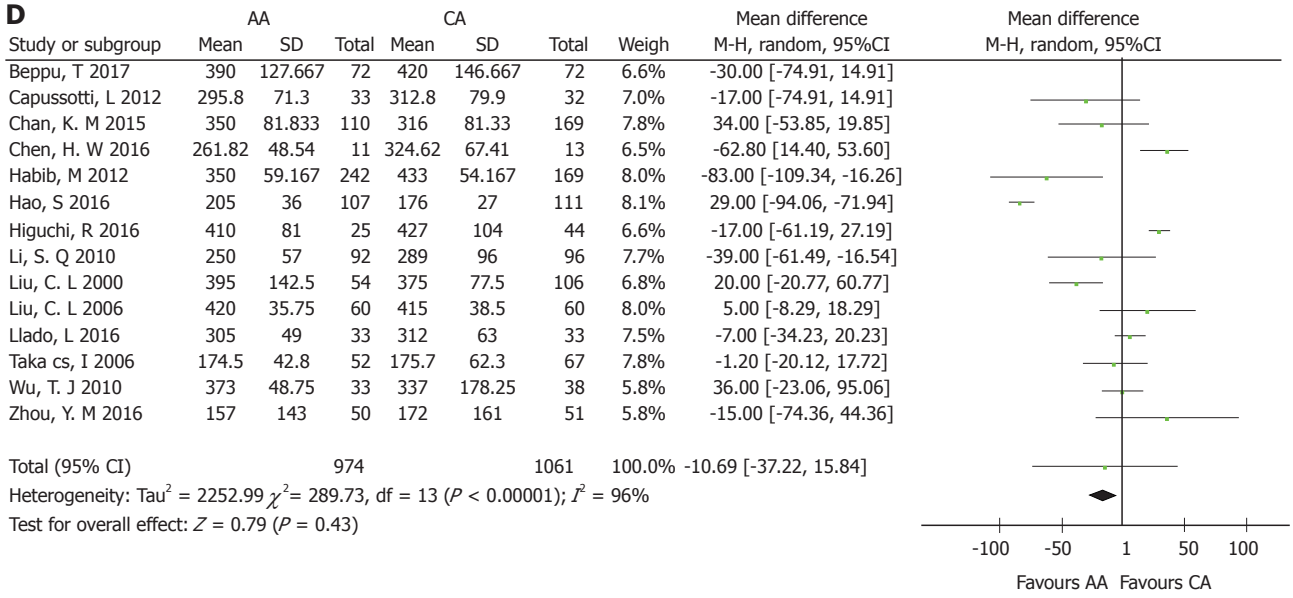


Figure 2 Forest plot and meta-analysis of clinical outcomes. A: Forest plot and meta-analysis of intraoperative blood loss; B: Forest plot and meta-analysis of massive blood loss; C: Forest plot and meta-analysis of intraoperative blood transfusion; D: Forest plot and meta-analysis of operative time. AA: Anterior approach; CA: Conventional approach.

Table 2 Results of meta-analysis comparison of anterior approach vs conventional approach right hepatectomy

Outcome of interest	Studies (*n*)	AA patients (*n*)	CA patients (*n*)	[1]WMD/OR/HR (95%CI)	[2]*P* value	Study heterogeneity			
						χ^2	df	I^2, %	[2]*P* value
Primary outcomes									
Intraoperative blood loss	14	984	1057	[1]-255.21 (-371.30, -139.12)	< 0.0001	122.96	13	89	< 0.00001
Massive blood loss	7	302	370	0.42 (0.21, 0.85)	0.02	11.49	6	48	0.07
Intraoperative blood transfusion	12	659	741	0.42 (0.29, 0.61)	< 0.00001	19.54	11	44	0.05
Operative time	14	974	1061	[1]-10.69 (-37.22, 15.87)	0.43	289.73	13	96	< 0.00001
Mortality	12	903	987	0.59 (0.38, 0.92)	0.02	10.83	11	0	0.46
Morbidity	13	901	933	0.77 (0.62, 0.95)	0.01	6.39	12	0	0.89
Overall survival	9	562	680	0.71 (0.50, 1.00)	0.05	47.12	8	83	< 0.00001
Disease-free survival	9	556	671	0.67 (0.58, 0.79)	< 0.00001	15.05	8	47	0.06
Recurrence	9	788	840	0.62 (0.47, 0.83)	0.001	13.82	8	42	0.09
Extrahepatic	7	436	502	0.67 (0.46, 0.97)	0.03	12.5	6	52	0.05
Intrahepatic	7	436	502	0.78 (0.59, 1.02)	0.07	6.32	6	5	0.39
Extrahepatic and intrahepatic	4	224	286	0.54 (0.29, 0.98)	0.04	2.1	3	0	0.55
Secondary outcomes									
Hospital stay	11	741	772	[1]-1.13 (-1.69, -0.58)	< 0.0001	10.69	10	6	0.38
R0 resection rate	6	527	681	1.10 (0.57, 2.14)	0.78	13.32	5	62	0.02
Bile leak	6	503	497	0.48 (0.19, 1.19)	0.11	2.87	5	0	0.72
Liver failure	4	239	294	0.50 (0.21, 1.20)	0.12	1.82	3	0	0.61

[1]Weighted mean difference; [2]Statistically significant results are shown in bold. AA: Anterior approach; CA: Conventional approach; WMD/OR/HR: Weighted mean difference/odds ratio/hazard ratio; CI: Confidence interval; df: Degrees of freedom.

three subgroups (Figure 4). Using a fixed-effects model, the data indicated that the AA group had significantly less extrahepatic or both intrahepatic and extrahepatic recurrence than the CA group (OR = 0.67; 95%CI: 0.46-0.97; *P* = 0.03; and OR = 0.54; 95%CI: 0.29-0.98; *P* = 0.04, respectively), but there was no significant intergroup difference in intrahepatic recurrence (OR = 0.78; 95%CI: 0.59-1.02; *P* = 0.07).

Secondary outcomes

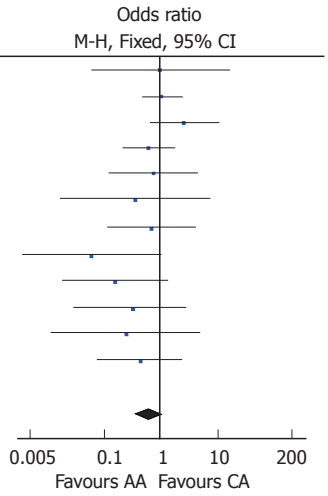
Hospital stay and R0 resection rate: Eleven studies[3,5,6,17,19,20,22,24,26,28,29] including 1513 patients who underwent right hepatectomy reported the length of hospital stay. The length of hospital stay was significantly shorter in the AA group than in the CA group (WMD = -1.13; 95%CI: -1.69 to -0.58; *P* < 0.0001). Six studies[18,21,22,24,25,27] including 1208 patients who underwent right hepatectomy reported the R0 resection rate. There was little significant heterogeneity among the studies (χ^2 = 13.32; I^2 = 62%; *P* = 0.02). Using a random-effects model, a meta-analysis indicated no significant difference in R0

A

Study or subgroup	AA Events	AA Total	CA Events	CA Total	Weight	Odds ratio M-H, Fixed, 95% CI
Capussotti, L 2012	1	33	1	32	1.8%	0.97 [0.06, 16.18]
Chan, K. M 2015	10	110	15	169	19.5%	1.03 [0.44, 2.38]
Cresswell, A. B 2009	7	62	3	62	4.8%	2.50 [0.62, 10.17]
Habib, M 2012	6	242	7	169	14.6%	0.59 [0.19, 1.78]
Hao, S 2016	2	107	3	111	5.2%	0.69 [0.11, 4.19]
Higuchi, R. 2016	0	25	2	44	3.3%	0.33 [0.02, 7.22]
Li, S. Q 2010	2	92	3	96	5.2%	0.69 [0.11, 4.22]
Liu, C. L 2000	0	54	14	106	17.7%	0.06 [0.00, 1.00]
Liu, C. L 2006	1	60	6	60	10.7%	0.15 [0.02, 1.31]
Llado, L 2016	1	33	3	33	5.3%	0.31 [0.03, 3.17]
Takacs, I 2006	0	52	2	67	3.9%	0.25 [0.01, 5.31]
Wu, T. J 2010	2	33	5	38	7.9%	0.43 [0.08, 2.36]
Total (95% CI)		903		987	100.0%	0.59 [0.38, 0.92]

Heterogeneity: χ^2= 10.83, df = 11 (P = 0.46); I^2 = 0%

Test for overall effect: Z = 2.35 (P = 0.02)

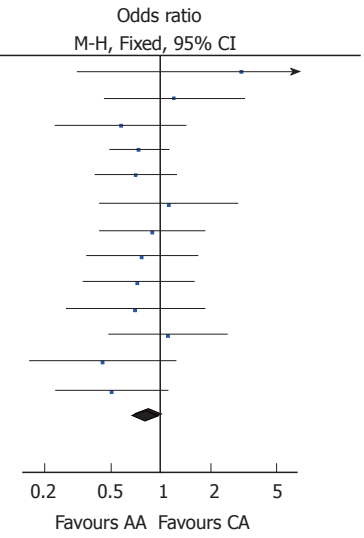


B

Study or subgroup	AA Events	AA Total	CA Events	CA Total	Weight	Odds ratio M-H, Fixed, 95% CI
Beppu, T 2017	3	72	1	72	0.5%	3.09 [0.31, 30.40]
Capussotti, L 2012	15	33	13	32	3.7%	1.22 [0.46, 3.26]
Cresswell, A. B 2009	9	62	14	62	6.1%	0.58 [0.23, 1.47]
Habib, M 2012	70	242	59	169	25.1%	0.76 [0.50, 1.16]
Hao, S 2016	29	107	38	111	13.8%	0.71 [0.40, 1.27]
Higuchi, R. 2016	12	25	20	44	3.8%	1.11 [0.41, 2.96]
Jabir, M. A 2017	18	40	47	98	7.6%	0.89 [0.42, 1.86]
Li, S. Q 2010	13	92	17	96	7.3%	0.76 [0.35, 1.68]
Liu, C. L 2006	16	60	20	60	7.4%	0.73 [0.33, 1.59]
Llado, L 2016	14	33	17	33	5.0%	0.69 [0.26, 1.83]
Takacs, I 2006	39	52	49	67	5.4%	1.10 [0.48, 2.52]
Wu, T. J 2010	8	33	16	38	5.7%	0.44 [0.16, 1.23]
Zhou, Y. M 2016	18	50	27	51	8.7%	0.50 [0.23, 1.11]
Total (95% CI)		901		933	100.0%	0.77 [0.62, 0.95]
Total events	264		338			

Heterogeneity: χ^2= 6.39, df = 12 (P = 0.89); I^2 = 0%

Test for overall effect: Z = 2.48 (P = 0.01)

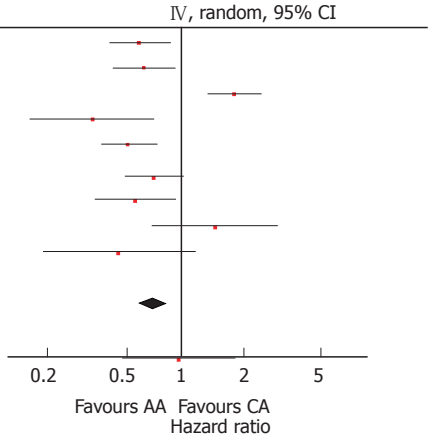


C

Study or subgroup	AA log [Hazard ratio]	AA SE	CA Total	Total	Weight	Hazard ratio IV, random, 95% CI
Beppu, T 2017	-0.5	0.18	71	71	12.6%	0.61 [0.43, 0.86]
Chan, K. M 2015	-0.45	0.19	78	78	12.4%	0.64 [0.44, 0.93]
Hao, S 2016	0.6	0.16	105	108	12.9%	1.82 [1.33, 2.49]
Jabir, M. A 2017	-1.0498	0.3684	40	98	8.9%	0.35 [0.17, 0.72]
Li, S. Q 2010	-0.63	0.16	90	93	12.9%	0.53 [0.39, 0.73]
Liu, C. L 2000	-0.33	0.17	54	106	12.8%	0.72 [0.52, 1.00]
Liu, C. L 2006	-0.56	0.24	60	60	11.4%	0.57 [0.36, 0.91]
Llado, L 2016	0.37	0.38	33	33	8.7%	1.45 [0.69, 3.05]
Wu, T. J 2010	-0.755	0.4621	31	33	7.3%	0.47 [0.19, 1.16]
Total events	64		338			
Total (95% CI)		562		680	100.0%	0.71 [0.50, 1.00]

Heterogeneity: Tau^2 = 0.22 χ^2= 47.12, df = 8 (P < 0.00001); I^2 = 83%

Test for overall effect: Z = 1.94 (P = 0.05)



D

Study or subgroup	AA log [Hazard ratio]	AA SE	CA Total	Total	Weight	Hazard ratio IV, random, 95% CI
Beppu, T 2017	-0.3	0.14	71	71	14.0%	0.74 [0.56, 0.97]
Chan, K. M 2015	-0.46	0.13	78	78	14.9%	0.63 [0.49, 0.81]
Hao, S 2016	-0.49	0.17	105	109	11.6%	0.61 [0.44, 0.85]
Jabir, M. A 2017	-0.821	0.2684	40	98	6.5%	0.44 [0.26, 0.74]
Li, S. Q 2010	-0.5	0.15	84	83	13.2%	0.61 [0.45, 0.81]
Liu, C. L 2000	-0.31	0.15	54	106	13.2%	0.73 [0.55, 0.98]
Liu, C. L 2006	-0.12	0.17	60	60	11.6%	0.89 [0.64, 1.24]
Llado, L 2016	-0.03	0.19	33	33	10.3%	0.97 [0.67, 1.41]
Wu, T. J 2010	-1.1087	0.3384	31	33	4.5%	033 [0.17, 0.64]
Total (95% CI)			556	671	100.0%	0.67 [0.58, 0.79]

Heterogeneity: Tau^2 = 0.03 χ^2= 15.05, df = 8 (P < 0.06); I^2 = 47%

Test for overall effect: Z = 4.97 (P < 0.0001)

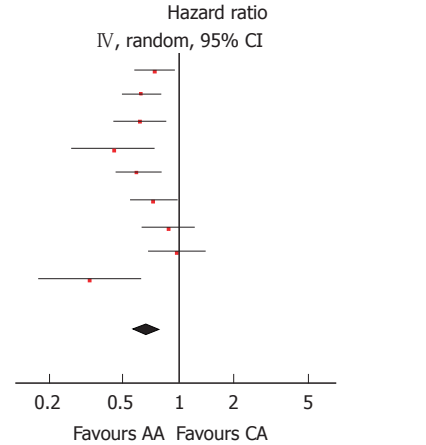


Figure 3 Forest plot and meta-analysis of postoperative outcomes. A: Forest plot and meta-analysis of mortality; B: Forest plot and meta-analysis of morbidity; C: Forest plot and meta-analysis of overall survival; D: Forest plot and meta-analysis of disease-free survival. AA: Anterior approach; CA: Conventional approach.

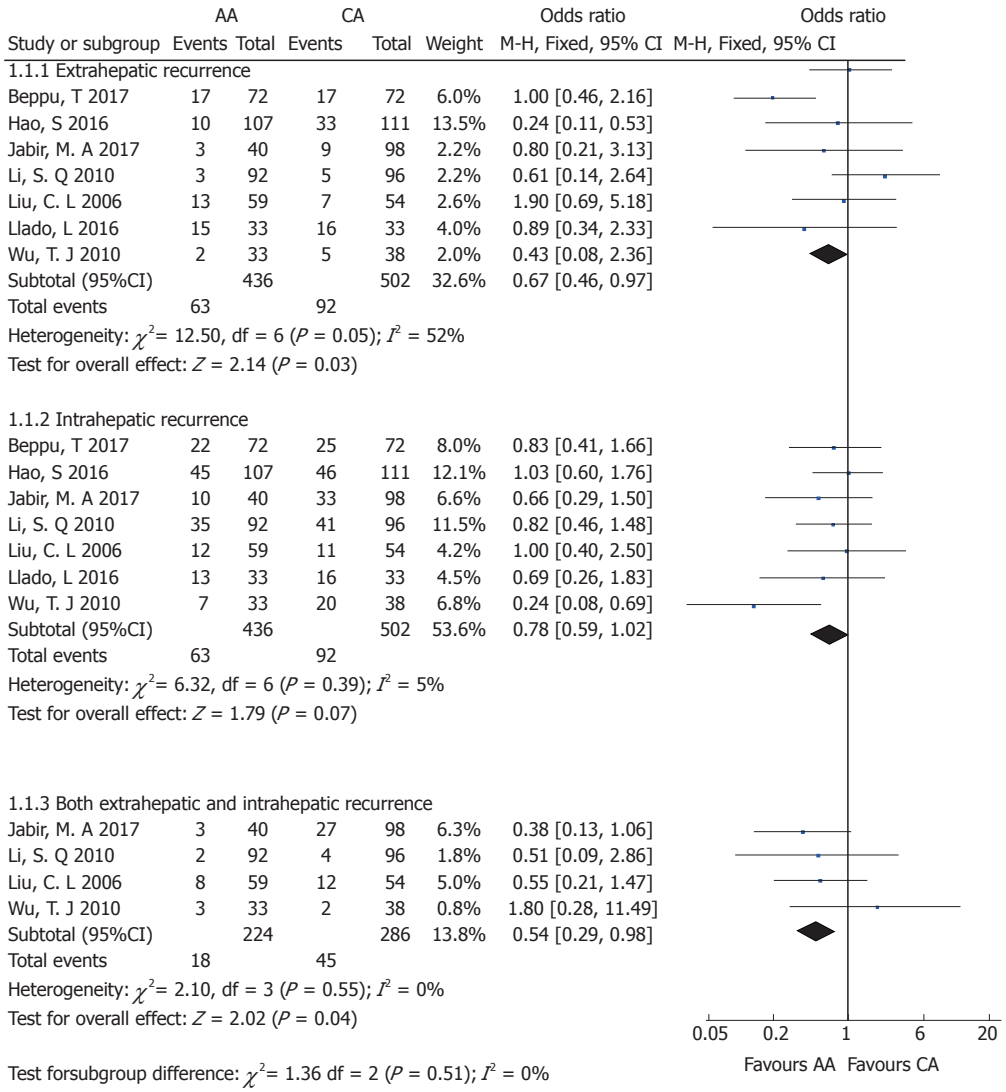


Figure 4 Forest plot and meta-analysis of tumor recurrence. AA: Anterior approach; CA: Conventional approach.

resection rate between the AA and CA groups (OR = 1.10; 95%CI: 0.57-2.14; *P* = 0.78) (Supplementary figure 2).

Bile leak and liver failure: Six studies[5,21,22,27-29] with a total of 1000 patients who underwent right hepatectomy for large liver rumor (AA = 503; CA = 497) reported a comparative incidence of bile leak. Using a fixed-effects model, a meta-analysis indicated no significant difference in bile leak after right hepatectomy surgery between the AA and CA groups (OR = 0.48; 95%CI: 0.19-1.19; *P* = 0.11) (Supplementary figure 2). Many studies did not provide the postoperative outcomes of liver failure. Therefore, we did a meta-analysis, including only four studies[5,6,26,27], to assess postoperative liver failure in 533 patients who underwent right hepatectomy due to large liver tumor. Analysis using a fixed-effects model revealed a lower rate of liver failure in the AA group than in the CA group, but this difference was not statistically significant (OR = 0.50; 95%CI: 0.21-1.20; *P* = 0.12).

Sensitivity analysis and publication bias

Three RCTs[5,6,17] and nine CCTs[3,5,17-19,22,23,25-28] that scored six or more stars on the modified Newcastle-Ottawa scale were included in the sensitivity analysis (Supplementary table 2). There was no change in the significant differences of all outcomes compared with the original outcomes; however, the degree of study heterogeneity was decreased in terms of massive blood loss, mortality, hospital stay, and R0 resection rate.

Funnel plots of the studies included in this meta-analysis reported postoperative outcomes such as perioperative blood transfusion, mortality, disease-free

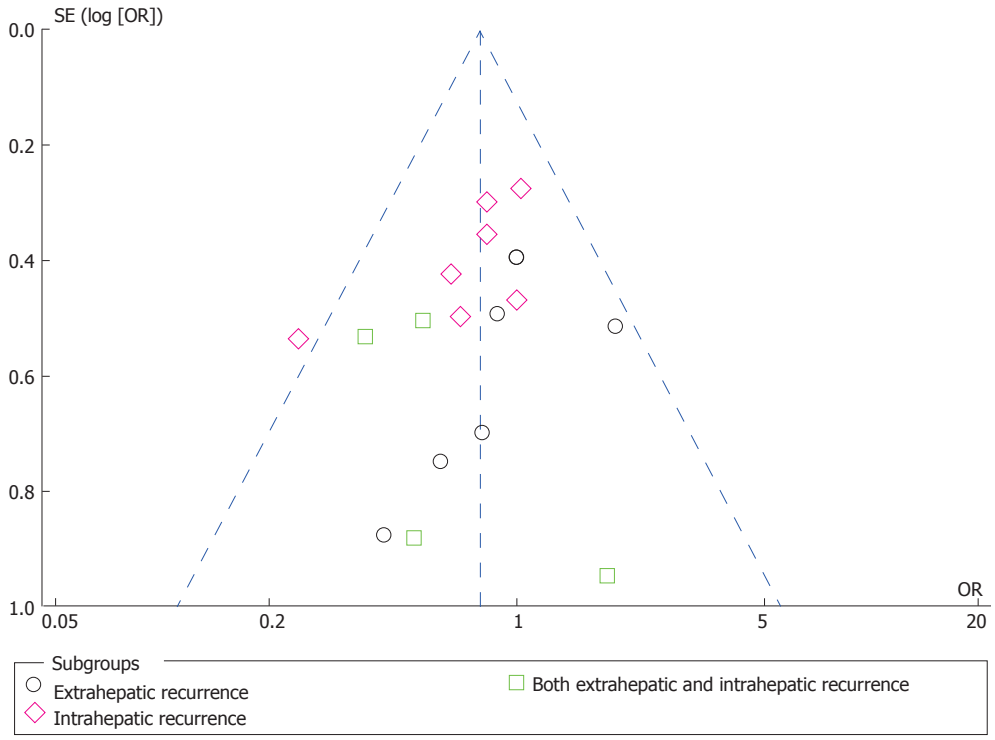


Figure 5 Funnel plots illustrating meta-analysis of tumor recurrence rates. SE: Standard error; OR: Odds ratio.

survival, and tumor recurrence (Figure 5). All studies were inside the 95%CI and evenly distributed around the vertical axis, indicating no obvious publication bias.

DISCUSSION

Hepatectomy for large right HCC is associated with significant operative morbidity and mortality and remains a major surgical challenge, especially when underlying liver cirrhosis is present[30,31]. With regard to the CA, operative complications may arise during the difficult mobilization of the right lobe of the liver, leading to unfavorable surgical outcomes including excessive intraoperative blood loss, tumor rupture, and the spillage of cancer cells into the systemic circulation[5]. The AA was first described by Ozawa *et al*[32] as a "nonconventional approach" to advanced liver cancer that attempts to avoid prolonged rotation and displacement of the hepatic lobes, which can lead to impairment of the afferent and efferent circulation.

In the current report, the AA, as a "no-touch" technique, was shown to result in favorable surgical and long-term survival outcomes compared to the CA in patients who underwent right hepatectomy for large HCC. The AA runs a risk of massive bleeding from the right or middle hepatic vein at the deeper plane of the parenchymal transection that is often uncontrollable and life-threatening. However, Belghiti *et al*[33] designed a liver hanging maneuver (LHM) using a tape inserted between the anterior surface of the vena cava and the liver and combined it with AA in 2001. The beneficial effects of this technique have been demonstrated and include better control of bleeding, protection of the inferior vena cava, good exposure during deeper parenchymal dissection, and guidelines for transection direction[34]. Besides, Chen *et al* reported a five-step stapling technique for right hepatectomy using the AA with the LHM for patients with HCC and liver cirrhosis that resulted in less intraoperative blood loss and significantly shorter parenchymal transection time. Nonetheless, retrograde bleeding is sometimes difficult to control during right hepatectomy for large HCC, especially in patients with liver cirrhosis and portal hypertension.

The long-term outcome after surgery remains unsatisfactory, although hepatectomy is an effective method for treating HCC. The 5-year recurrence rate of HCC after surgery reportedly exceeded 60%-80%[35], which represents a major factor for long-term outcomes. HCC recurrence is a complex process that involves many clinical and pathological factors. Moreover, the no-touch concept is an important principle in surgical oncology[36]. However, it is very difficult to follow it in conventional hepatectomy due to the special anatomical structures of the liver. Moreover, HCC exhibits strong vascular invasion[37]. Liver tumor cells are more easily spread through the portal vein or hepatic vein during right hepatectomy surgery. Hao *et al*[23] reported that macro- and microvascular invasion, blood transfusion, and the CA of hepatectomy were independent risk factors for HCC recurrence on multivariate analysis. Moreover, excessive blood loss and blood transfusion have been associated with increased morbidity and mortality

as well as poorer DFS and OS after right hepatic resection[38,39]. Perioperative transfusion has also been found to promote HCC recurrence after hepatic resection. Technical innovations have mainly focused on minimizing bleeding during hepatic parenchymal transection. Various devices have been developed to promote liver transection and reduce blood loss in right hepatectomy. However, none has proven superiority compared with previous techniques[40]. Therefore, we systematically summarized related studies using a meta-analysis to assess the safety and efficacy of AA and CA for right hepatectomy. In our analysis, the AA was associated with less intraoperative blood loss or massive blood loss, fewer transfusion requirements, lower mortality or morbidity, and less recurrence after right hepatectomy; otherwise, it was associated with longer OS and DFS. Besides, the incidences of extrahepatic or both extrahepatic and intrahepatic tumor recurrence were higher in cases using the CA than those using the AA, and this result seems to support the proposal that excessive blood loss and blood transfusions were associated with increased tumor recurrence as well as poorer DFS and OS after right hepatic resection. Our meta-analysis results indicated that the AA results in favorable surgical and long-term survival outcomes compared to the CA in patients who underwent right hepatectomy for large HCC. The better outcome achieved in the AA group might have been a result of using the no-touch technique, which fulfills the oncological principles of surgical resection.

From the surgical perspective, the AA can prevent complications related to mobilization of the right liver before parenchymal transection. In particular, mobilization of the right liver might be difficult in patients with a large HCC due to limited space, and the surgeon is likely to encounter excessive bleeding or iatrogenic tumor rupture, expansion of liver resection, and a risk of squeezing cancer cells into the blood circulation system[19]. Consequently, the rates of morbidity and operative complications, including bile leak, liver failure, and bleeding, were theoretically low in the AA group. However, data mentioning bile leak or liver failure were available in two RCTs and four CCTs. Our result indicated no significant difference in bile leak or liver failure after right hepatectomy surgery between the AA and CA groups. A few articles have reported on the incidence of intraoperative iatrogenic tumor rupture during mobilization of the right lobe of the liver. Only one clinical study by Liu *et al*[27] reported that the incidence of tumor rupture appeared to be higher in the CA group (seven patients, 6.6%) than in the AA group (one patient, 1.9%), although the difference was not significant (P = 0.268). However, one RCT and one clinical study[3,5] showed that the rates of tumor rupture were similar between the AA and CA groups. Therefore, additional RCTs with large samples are needed to resolve this conflict.

The AA has a potential advantage of liver function preservation since it does not require twisting the portal pedicle during right liver mobilization as in the CA. This advantage is consistent with the suggestion by Ozawa that the AA could contribute to better preservation of postoperative liver function by avoiding prolonged rotation during right hepatectomy[32]. The results of one CCT study by Capussotti *et al*[6] showed no difference in postoperative liver function tests such as serum transaminases, bilirubin and prothrombin time, which are considered the barometers of hepatocytic damage[41]. However, it is difficult to conduct an intensive credibility analysis since not all of the included studies reported detailed information about liver function. Whether there is a significant difference between the two approaches should be elucidated in the future.

Safety should be prioritized when selecting a surgical approach. Although the theoretical advantages of the AA over the CA are well established, right hepatectomy for large HCC using AA with or without the LHM remains a technically demanding method, making numerous surgeons reluctant to perform this approach. In addition, others see that the CA has the advantage of preventing critical bleeding during liver transection, while the AA can be an effective alternative when difficulty is encountered during liver mobilization. In our analysis, the AA technique used for right hepatectomy was associated with less intraoperative blood loss, fewer cases of massive blood loss, fewer transfusion requirements, and lower mortality or morbidity. Our meta-analysis results indicated that AA is a safe and effective technique for right hepatectomy for large HCC.

The limitations of this meta-analysis are as follows. The primary limitation is that most of the included studies were retrospective CCTs, evidence for which may be less feasible, except for three RCTs. Therefore, a meta-analysis of several RCTs would be perfect, but the limited number of RCTs prevented us from drawing a definitive conclusion. Besides, inter-study heterogeneity was significant for outcomes including intraoperative blood loss, massive blood loss, intraoperative blood transfusion, operative time, overall survival, disease-free survival, and tumor recurrence. Hence, we processed data using the random-effects model since it might reduce the effect of heterogeneity but does not abolish it. Finally, most studies lacked some available data about intraoperative and postoperative outcomes or insufficient data on factors such as tumor rupture and liver function. Therefore, studies with comprehensive and sufficient data and more RCTs are needed to resolve this limitation.

This meta-analysis was conducted at an appropriate time since sufficient data have accumulated for research, and right hepatectomy for large liver tumor using the AA or the CA is still a hot topic. In our analysis, patients who underwent right hepatectomy in the AA group had less intraoperative blood loss;

less frequent massive blood loss; reduced transfusion requirements and hospital stay; lower mortality, morbidity and recurrence, and better OS and DFS than those in the CA group. However, there are no advantages of the AA over the CA regarding operative time, intrahepatic tumor recurrence, R0 resection rate, bile leak, or liver failure. In summary, the AA is a safe, feasible, and effective technique for right hepatectomy for large liver tumor that could accelerate postoperative recovery and achieve better long-term survival outcomes than the CA.

ARTICLE HIGHLIGHTS

Research background

Conventional right hepatectomy (CRH), which is complete mobilization of the right liver with the right hepatic vein controlled outside the liver before parenchymal transection, has been used as the standard procedure. Anterior approach (AA) has been suggested as an alternative approach to conventional approach (CA) for right hepatectomy in recent years. However, comparative studies have shown conflicting results.

Research motivation

Some studies have compared AA and CA to evaluate their safety and efficacy in right hepatectomy for large hepatocellular carcinoma (HCC). Recently, no meta-analysis of the safety, clinical outcome and survival after AA right hepatectomy for HCC compared with the CA was published. Besides, in our article, several conclusions might be used to guide future clinical practice.

Research objectives

To evaluate whether right hepatectomy using the AA for large hepatocellular carcinoma results in better clinical outcomes when compared with the CA, and the safety, efficacy and clinical outcome of the two approaches.

Research methods

We comprehensively performed an electronic search of PubMed, EMBASE and the Cochrane Library that published between January 2000 and May 2017 for randomized controlled trials (RCTs) or clinical controlled trials (CCTs) concerning using AA and CA in right hepatectomy. Studies that met the inclusion criteria were included, and their outcomes analysis were further assessed using either a fixed or a random effects model.

Research results

The analysis included 2297 patients enrolled in 16 studies (3 RCTs and 13 CTTs). Intraoperative blood loss, intraoperative blood transfusion, mortality, morbidity, and recurrence rate were significantly reduced in AA group. Besides, patients in the AA group had better overall survival and disease-free survival than those in the CA group.

Research conclusions

The AA is a safe and effective technique for right hepatectomy for large HCC, and it could accelerate postoperative recovery and achieve more advantageous survival over the CA. AA can be an effective alternative when difficulty is encountered during liver mobilization and reduce the risk of bleeding.

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CASE REPORT

Metabolically based liver damage pathophysiology in patients with urea cycle disorders - A new hypothesis

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Abstract

The underlying pathophysiology of liver dysfunction in urea cycle disorders (UCDs) is still largely elusive. There is some evidence that the accumulation of urea cycle (UC) intermediates are toxic for hepatocyte mitochondria. It is possible that liver injury is directly caused by the toxicity of ammonia. The rarity of UCDs, the lack of checking of iron level in these patients, superficial knowledge of UC and an underestimation of the metabolic role of fumaric acid, are the main reasons that are responsible for the incomprehension of the mechanism of liver injury in patients suffering from UCDs. Owing to our routine clinical practice to screen for iron overload in severely ill neonates, with the focus on the newborns suffering from acute liver failure, we report a case of citrullinemia with neonatal liver failure and high blood parameters of iron overload. We hypothesize that the key is in the decreased-deficient fumaric acid production in the course of UC in UCDs that causes several sequentially intertwined metabolic disturbances with final result of liver iron overload. The presented hypothesis could be easily tested by examining the patients suffering from UCDs, for liver iron overload. This could be easily performed in countries with a high population and comprehensive national register for inborn errors of metabolism. Conclusion: Providing the hypothesis is correct, neonatal liver damage in patients having UCD can be prevented by the supplementation of pregnant women with fumaric or succinic acid, prepared in the form of iron supplementation pills. After birth, liver

damage in patients having UCDs can be prevented by supplementation of these patients with zinc fumarate or zinc succinylate, as well.

Key words: Urea cycle disorder; Citrullinemia; Neonatal liver iron overload; Fumaric acid; Succinic acid; Krebs' cycle; Transferrin; Zinc fumarate supplementation

Core tip: Underlying pathophysiology of liver dysfunction in urea cycle disorders (UCDs) is still largely elusive. We hypothesize that the key is deficient fumaric acid production in urea cycle in UCDs, which causes several sequentially intertwined metabolic disturbances with the final result of liver iron overload. Providing the hypothesis is correct, neonatal liver damage in patients having UCD can be prevented by the supplementation of pregnant women with fumaric or succinic acid, prepared in the form of iron supplementation pills. After birth, liver damage in patients having UCDs can be prevented by supplementation of these patients with zinc fumarate or zinc succinylate.

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INTRODUCTION

Urea cycle defects (UCDs) occur in approximately 1 of 30000 live births[1]. The clinical hepatic presentation of the UCDs may include acute liver failure (ALF), liver dysfunction, and hepatocellular injury. The UCDs are not considered as significant metabolic diseases that cause severe liver dysfunction and ALF[2,3]. However, according to the previous reports, people affected by hepatocellular injury and ALF are also diagnosed as having ornithine transcarbamylase (OTC) deficiency[4,5], the most common UCD. Other UCDs are also associated with hepatocellular injury and liver failure[6-14]. Although liver dysfunctions and histopathological liver changes in patients with the UCDs have been reported since the late 1970s[15], the underlying pathophysiology of hepatic dysfunction has remained unknown. There is some evidence that the accumulation of urea cycle intermediates is toxic for hepatocyte mitochondria[16]. Recently it was shown, *in vitro,* that liver injury could be caused by direct toxicity of ammonia[17]. The toxicity of carbamoyl phosphate has also been proposed, but this is conjectural[18,19]. A variety of mitochondrial changes have been demonstrated on the electron microscopy of liver samples of patients with UCDs: An enlarged mitochondria with a swollen cristae and a dense matrix and numerous electron dense bodies of different sizes in the mitochondrial matrix[20]. Notably, these mitochondrial changes in the reported patients, were present exclusively in liver cells.

Neonatal hemochromatosis is another disorder followed by severe hepatic injury and associated with extrahepatic siderosis. NH is one of the most commonly recognized causes of liver failure in a neonate[21]. The presence of the UCDs and NH among neonates have very similar clinical and laboratory presentations. While the mechanism of liver damage in the NH is known[22], the mechanism of liver damage in the UCDs has not still been discovered.

Herein we present a case of a newborn affected by severe liver and central nervous system (CNS) toxicity. Furthermore, we aimed to provide a brand new and metabolically based view on the pathophysiology of liver failure among patients diagnosed with the UCDs, with the prospect of its prevention.

CASE REPORT

The propositus was a 2-day-old male, born at 39 gestation weeks, weighing 3610 g. He was the sixth child of non-consanguineous parents. All previous pregnancies were normal, and all children are healthy. No previous abortions have occurred. The family history was negative for metabolic diseases. During the first hours of life, the health of the newborn male worsened due to vomiting, lethargy and focal seizures. He was immediately transported from a local hospital to University Children's Hospital for further examinations and treatment. Initial laboratory analyses showed an absence of hypoglycemia, total bilirubin level 42 μmol/L, conjugated fraction 28 μmol/L, alanine aminotransferase (ALT) level of 987 IU/L, aspartate aminotransferase (AST) level of 4165 IU/L and lactate dehydrogenase level 4760 IU/L. Ammonia level was 823 μmol/L. The coagulation test displayed extended partial thromboplastin time (PTT) of 59.5 s, extended prothrombin time (PT) of 22.2 s and international normalized ratio (INR) of 1.75. Fibrinogen level was normal (2.24 g/L). Having completed the routine screening of iron concentration (performed for neonatal iron overload), the results showed elevated iron serum concentration (30.7 μmol/L), very high ferritin (7145.6 ng/L), decreased serum transferrin concentration (1.35 g/L), TIBC 34.1 μmol/L and transferrin saturation 90% accordingly. A biopsy of submucosal oral salivary glands was done as well. Without knowing of histological findings of biopsy specimen, the patient was diagnosed as having NH. It was decided to treat the patient with blood exchange transfusion and intravenous immunoglobulin, but there was no improvement. Two days later, the pathologist reported that the biopsy specimen stained for iron deposits, was negative. The diagnosis of NH was immediately suspended and patient's blood and urine samples were urgently sent

to another medical institution for metabolic testing. The results of that testing showed: High citrulline (328 μmol/L, normal 10-21 μmol/L) and high alanine (847 μmol/L, normal range 274-384 μmol/L) levels, as well as high urine orotic acid excretion (0.9, normal < 0.14). This metabolic profile finding gave the final diagnosis of citrullinemia type Ⅰ, a defect in the urea cycle, caused by the deficiency of argininosuccinate synthetase. The treatment was continued according to the rules for the treatment of acute hypeammomiemia[23]. It included prompt removal of ammonia from the body and providing the organism with adequate calories and essential amino acids to halt further breakdown of endogenous proteins. The patient showed visible clinical and laboratory improvement. The ammonia level fell to 46 μmol/L, ALT level 70 IU/L, AST 86 IU/L and LDH level 538 IU/L. Unfortunately, on the 20th day of the recovery process in the intensive care unit, the patient contracted sepsis caused by the multi-resistant hospital species of *Enterobacter*. Despite vigorous antimicrobial and supportive therapy, the patient started to suffer from gastrointestinal, intracranial and pulmonary hemorrhage, accompanied by the failure of vital functions. Ten days later the patient died.

DISCUSSION

Clinical presentation, laboratory tests findings and course of the disease indicate that the patient had UCD (*i.e.,* citrullinemia). This is in accordance with diagnostic criteria used by Urea Cycle Disorders Consortium of the Rare Diseases Clinical Research Network[24], as well. In addition, we think that the patient had another very serious disease, a form of high level of iron concentration, which caused severe liver failure. Due to the absence of siderosis in the biopsy specimen of the oral salivary submucosal glands, neonatal hemochromatosis phenotype as a cause of the high level of iron concentration in the blood, hyperferritinemia and very high transferrin saturation was excluded. Besides in the above mentioned report, the following data did not match with the diagnosis of NH: (1) five previously born healthy children; (2) absence of late second and third trimester fetal loss[25]; (3) health of the patient immediately after birth; and (4) lack of improvement during the specific therapy applied in case of NH, *i.e.,* intravenous immunoglobulin and exchange transfusion, as well.

Owing to the clinic's routine practice of using screening for checking the high concentration of iron in neonates' bodies, with the focus on the newborn children who suffer from acute liver failure, the presented case is, to our best knowledge, the first report of the high concentration of iron in newborns with UCD. No other cases of UCDs with high concentration of iron have been reported in the literature to date.

CNS toxicity manifested by lethargy and seizures can be explained by ammonia toxicity. Namely, the ammonia in CNS is detoxified using its own possibility to be accepted by α-keto glutarate and form glutamate. This produces the lack of α-keto glutarate in Krebs cycle in CNS cells, which become energy deprived. Liver toxicity in all existing cases of UCDs was explained in a speculative way[15,16,18-20]. Acute liver failure, *i.e.,* liver toxicity in patients having UCD can be logically explained on a basis of an "*isolated hepatic iron overload*". According to our postulation, this specific iron overload, is an inevitable occurrence in lives of many patients suffering from UCD. It is, probably, the cause of the liver damage that is seen among many UCD patients in their post neonatal life, as well.

There are several reasons which are responsible for the incomprehension of the mechanism of the liver injury in patients suffering from the UCDs. Firstly, there is a rarity of the UCDs. Secondly, there is a lack of checking of iron levels in these patients. The third reason is the superficial knowledge of the urea cycle (UC) and the underestimation of the metabolic role of fumaric acid (FA) which is a *byproduct* in the UC and its physiologic importance. During the preparation of this work we even found one scientific article in which the metabolic map of the UC was depicted without FA[26]. It is worth mentioning that several of the components and reactions of citric acid cycle were analyzed the 1930s in the research of the Nobel laureate Albert Szent-Györgyi. He received the Nobel Prize in 1937 for his discoveries pertaining to fumaric acid, a key component of the cycle[27].

For better understanding of our theory, at this point we have to remind the readers that the liver is the predominant hematopoietic organ through the period of weeks 20th to 24th week of gestation[28]. According to our theory, there are compounds whose metabolism is the key for understanding and elucidation of the mechanism of hepatic injury in the UCDs. These are succinyl-CoA, *i.e.,* succinic acid (SA) and fumaric acid (FA), their intertwined pathways and their physiological roles.

Succinic acid

The sources of SA are exclusively located in mitochondria. SA is produced from α-ketoglutarate, methionine, isoleucine, valine, cholesterol and from β-oxidation of odd-chain fatty acids in the form of activated succinate, *i.e.,* succinyl-CoA. Succinyl-CoA, which is easily converted to succinic acid and vice versa (*via* the catalytic activity of succinyl-CoA synthetase, also known as succinate thiokinase or succinyl-CoA ligase) has three metabolic roles. The first one is the energy production, through the course of TCA, in the form of nucleoside triphosphates (ATP and GTP). The second role is also related to the energy production *via* the ketone body activation (KBA) and utilization. The third metabolic role of succinyl-CoA is heme formation. These three physiological functions of SA are basically enabled by the catalytic activity of succinate thiokinase (STK). Mammalian cells have two distinct STKs. One STK is specific for GDP/GTP (G-STK)

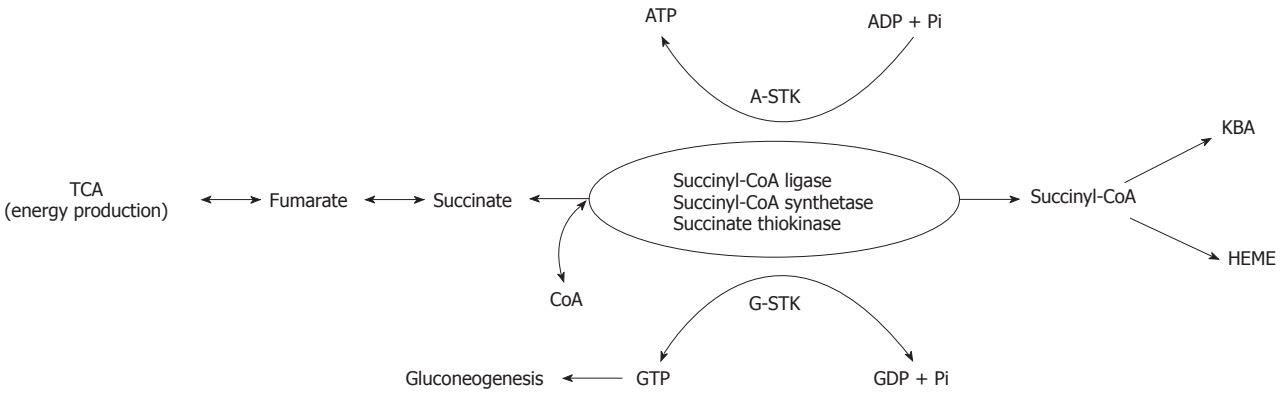

Figure 1 Metabolic catalytic activities of succinate thiokinase(s).

and the other STK is specific for ADP/ATP (A-STK). A-STK functions in TCA in the direction of succinyl-CoA breakdown for energy production in the form of ATP. G-STK has two metabolical roles. The first one is for the energy production in the form of GTP which is used for glucose production via gluconeogenesis. The second role presents reversing of SA into succinyl-CoA, at the expense of GTP, for ketone body activation and heme biosynthesis (Figure 1)[29-31].

Fumaric acid

While SA is produced exclusively in mitochondria, FA is produced both, in mitochondria and in the cytosol. In mitochondria FA is gained from SA upon the catalytic action of succinate dehydrogenase, an enzyme located in mitochondria. The second mitochondrial production of FA is through the catabolism of phenylalanine (Phe) and tyrosine (Tyr). FA produced in mitochondria is primarily used for the energy production by converting to malate, and then to oxaloacetate. However, in mitochondria FA can be easily converted to succinate (upon catalytic activity of succinate dehydrogenase) and then to succinyl-CoA (*via* the activity of G-STK, for ketone body utilization and heme production). The cytosolic production of FA comes from the UC through the division of argininosuccinate to arginine and fumarate, by the action of argininosuccinate liase (or argininosuccinase). After adding water to fumarate forms L-malate, and subsequent NAD^+-dependent oxidation converts L-malate to oxaloacetate. These two reactions are analogous to the reactions of the citric acid cycle, but are catalyzed by cytosolic fumarase and malate dehydrogenase. Oxaloacetate transforms by glutamate aminotransferase, then re-forms aspartate, which is used for the synthesis of citrulline[32]. Judging from the rate of gluconeogenesis[33], it could be concluded that the need for aspartate in the cytosol largely depends upon the supply from the mitochondria[34]. Therefore, it is reasonable to believe that most of the FA produced in the cytosol by the division of argininosuccinate is transferred into the mitochondria where it is metabolized by fumarase and malate dehydrogenase to oxaloacetate. In mitochondria, oxaloacetate is converted in aspartate which is, then, exported into the cytosol by citrin and is reused for urea synthesis (Figure 2).

In conclusion, in accordance with our theory, FA and SA form a basic, mitochondrial dynamic exchangeable pool. Depending on the metabolical needs of the cell (energy production, ketone body activation and heme production) the chemical equilibrium can be directed to the left or to the right (Figure 3).

Considering the law of chemical equilibrium reactions (the law of Guldberg-Waage, the law of reversible chemical reactions, A + B ↔ A′ + B′) in the case of the deficiency of a compound involved in the reaction, the equilibrium is directed toward the deficiency. In the presented case (citrullinemia), this means that because of complete deficiency of cytosolic FA production, the equilibrium is directed to the left, *i.e.,* toward FA, with the consequence of less SA available for ketone body activation and heme production. Compromised heme synthesis leads to compromised hemoglobin production. As a consequence, the decreased heme and hemoglobin production is "understood" (because evolution is still not perfect) as iron deficiency by the fetal hematopoietic hepatic tissue ("liver bone marrow"). An identical mechanism operates in the cases of thalassemias and other anemias with ineffective hematopoiesis, such as the congenital sideroblastic anemia and congenital dyserythropoietic anemia, where the defect is recognized as iron deficiency. In all these situations because of "iron deficiency" there is an increased iron influx because of decreased hepcidin production[35]. During fetal life placenta tightly controls the movement of iron from the mother to the fetus by mechanisms that are similar to those controlling the absorption of dietary iron from the intestine[36]. Therefore, in fetal deficient hematopoiesis, which is wrongly recognized as iron deficiency, expression of hepcidin is down regulated with consequential increase of placental iron influx. An excess of iron was transferred across the placenta through the hepcidin receptor ferroportin, bound to transferrin and most of this iron was transported directly into the liver, the main hematopoietic fetal organ. This could be a physiologic event, since humans possess

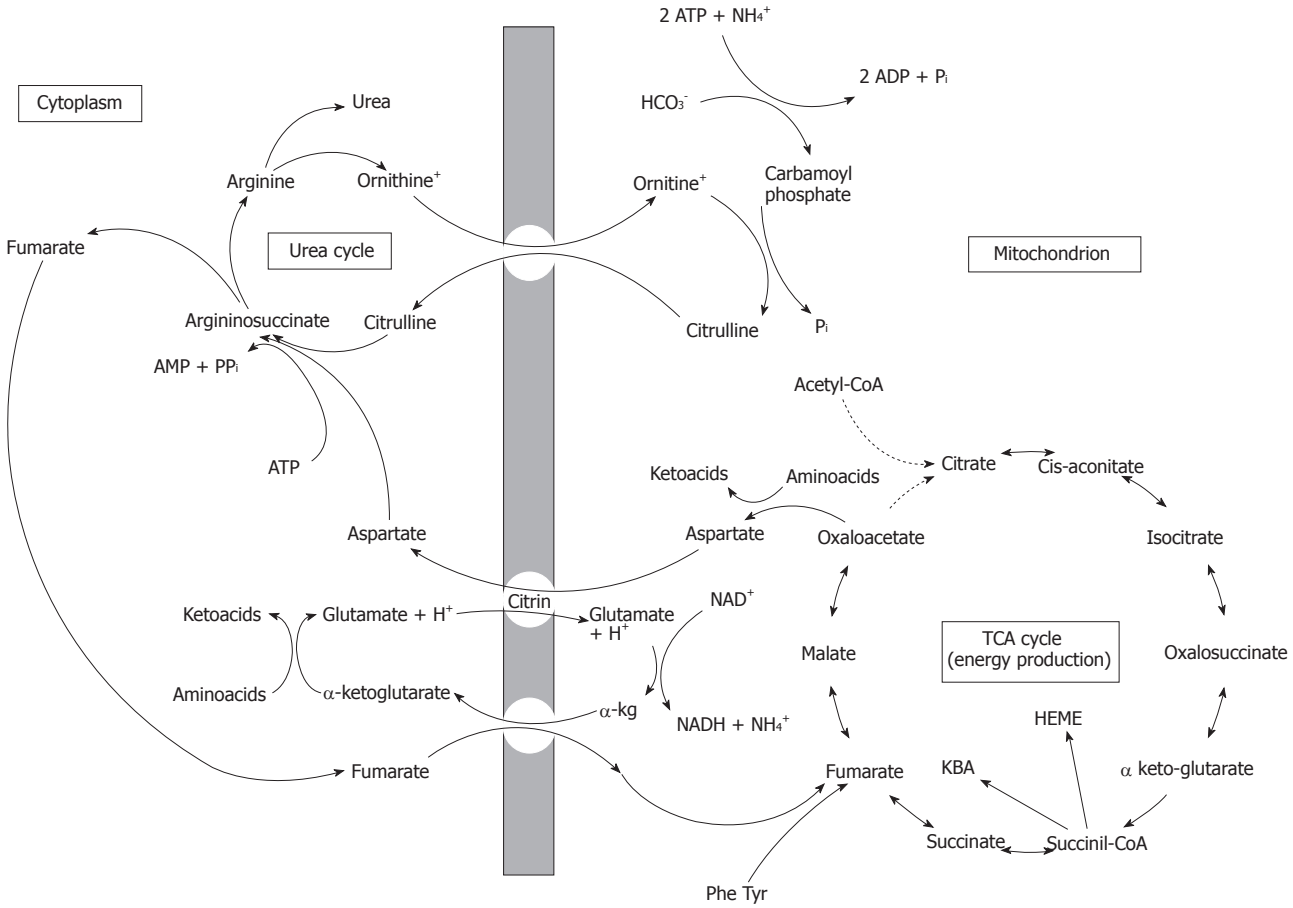

Figure 2 Metabolic interrelationships between urea cycle and TCA. Depicted biochemical pathways show the interrelationship of the urea cycle and TCA cycle. Note that all reactions of the urea cycle are unidirectional. In the TCA cycle most reactions are reversible, except for the initial reaction of the TCA, the condensation of acetyl-CoA with oxaloacetate to form citrate, and the reaction of oxidative decarboxylation of α-keto glutarate to form succinyl-CoA. Note that fumarate gained in the cytosol in the course of urea cycle (UC), to be reused in UC must be transported into the mitochondria to be converted to aspartate.

various transferrin isoforms, that differ in their degree of glycosylation[37,38]. It is possible that each transferrin isoform refers to a specific cell/tissue/organ tropism. We argue that probably one of low glycosylated transferrin isoforms, known as carbohydrate-deficient transferrin, was involved in the transportation of the placental iron exclusively into the hepatic hematopoietic tissue. This argumentation could explain the absence of extrahepatic siderosis and the existence of high concentration of isolated hepatic iron and hepatic injury in the presented case. Not being used by erythroblasts for heme/hemoglobin synthesis because of the lack of FA, *i.e.*, SA, the excess of the iron is deposited in hepatocytes and reticuloendothelial von Kupffer cells. In hepatocytes, the excess of iron is stored in the mitochondria (Fe^{++}) and ferritin (Fe^{+++}).

Iron toxicity in intrauterine and extrauterine earth life

After birth, human being lives in the oxygen-enriched atmosphere. Our bodies require oxygen for many metabolic processes. However, oxygen is highly reactive and its interaction with non-chelated, *i.e.*, free iron potentiates its toxicity. Normal cellular reactions, including respiration and "respiratory burst" generate reactive oxygen intermediates - superoxide free radical (O_2^-) and hydrogen peroxide (H_2O_2). Superoxide free radical is promptly dismutated into less toxic hydrogen peroxide, via the catalytic activity of superoxide dismutase. Superoxide and secondary reactive oxygen intermediates (H_2O_2), are potent antimicrobial agents. When the production of reactive oxygen species exceeds the processing capacity of the body, oxidative stress appears. Under these circumstances, reactive oxygen intermediates may be converted to much damaging radicals by the iron-catalyzed Fenton reaction[39], which is depicted bellow.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$

Hydroxyl radicals (HO•) and hydroperoxyl radicals (HOO•) promote the peroxidation of proteins, DNA and membrane lipids, problems that are exacerbated by the high concentration of iron. Certain organelles are particularly susceptible to the iron-dependent peroxidation. In the cells with the high concentration of iron, the injured mitochondria and lysosomes become leaky[40]. The mitochondrial damage and release of lysosomal proteases cause further cell injury and may ultimately lead to the cell death. This process causes

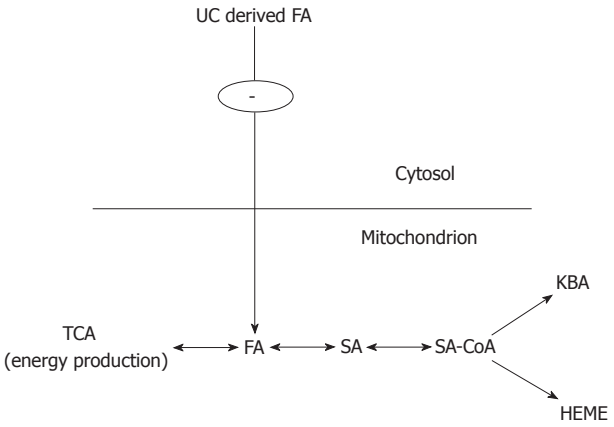

Figure 3 Metabolic exchangeable pool of fumaric acid and succinic acid. In urea cycle disorders (UCDs) there is decreased or completely absent FA supply from the cytosol. FA deficient supply is proportional to the enzyme activity deficiency.

severe tissue damage in the liver, heart, and endocrine organs of patients who have disorders due to the high concentration of iron. These deleterious properties of iron are threatening only when the element, *i.e.,* iron is in a "free" state or in an abnormal form within the cell. This happens in the cases of its high quantity and if this surplus is accompanied by life in oxygen-enriched atmosphere. During fetal life, a fetus lives in a sterile atmosphere of the uterine cavity and in relatively low oxygen pressure. It is well known that oxygen physiologically diffuses down decreasing rate of partial pressure from air in the lungs PO_2 (150 mmHg), pulmonary capillary vessels (105 mmHg), arterial blood (95 mmHg), placental vessels (30-40 mmHg), umbilical venous fetal blood (20-30 mmHg), equilibrate PO_2 with the fetal tissues at PO_2 of around 10-20 mmHg in a term fetus. Therefore, during the fetal life there is no peroxidation reaction and need for the "respiratory burst". As a consequence of low oxygen partial pressure, there is no generation of reactive oxygen intermediates, superoxide free radical and hydrogen peroxide. In that way, it is reasonable to speculate that iron accumulated in the fetal body does not exert its toxicity *via* the catalytic Fenton reaction properties. Problems start immediately after birth with the first breath and activation of the pulmonary function, reaching the arterial oxygen partial pressure between 75 mmHg and 100 mmHg, as well as the bacterial invasion of the newborn and start of the "respiratory burst".

Timing of organ-tissue injury in UCDs and NH

UCDs with neonatal presentation and NH have very similar clinical and laboratory characteristics. The main difference is the timing of the hepatic damage that conducts the mode of presentation at birth. Newborns with UCDs, immediately after birth, are healthy. The problems commence after the activation of the pulmonary function and bacterial invasion, with the activation of iron toxicity, too. On contrary, in case of the NH, the liver injury starts during the intrauterine life and is complement-mediated. At birth, the child having NH is already severely ill because of the liver injury with further deterioration of the hepatic function. It is complemented with the injury of extrahepatic organs which already contain high concentration of iron. After birth and development of the pulmonary function and bacterial colonization of the newborn, accumulated iron starts to exert its toxicity via the generation of injurious radicals (hydroxyl radicals (HO•) and hydroperoxyl radicals (HOO•) by iron-catalyzed Fenton reaction. The severity of liver damage in the UCDs is proportional to the amount of iron excess and the degree of FA deficiency, which is in a correlation with the degree of enzyme deficiency. Complete enzyme deficiency results in very severe, life threatening neonatal liver disease, urgent for an instant liver transplant.

Testing the hypothesis-theory

Presented hypothesis could be easily tested by the examining of patients suffering from UCDs, the newborns and the patients with post neonatal, late presentation, for liver iron overload by using specific magnetic resonance imagination (MRI) for pathologic iron deposition in their livers. Having in mind the rarity of UCDs, *i.e.,* its incidence of only 1: 30000 live births[1], follows that this could be easily performed in countries with a high population and comprehensive national register for inborn errors of metabolism. The countries of choice for this purpose are the United States, Republic of China, Japan, Italy, France, Great Britain. In the United States with its 300000000 inhabitants, and approximately 3000000 of births per year, and UCD incidence of 1:30000 live births, each year approximately a hundred children have UCD. These children should be screened by MRI for liver iron overload. Provided the presented theory is correct, these children should have positive MRI for liver iron overload and would have a prospect for physiologically based mode of prevention.

Mode of prevention of neonatal liver damage

The presented theory, provided correct, offers a prospect for the prevention of hepatic damage in UCDs, during the fetal life and after birth, throughout the whole life, as well. During the fetal life prevention could be achieved by the gestational fumarate supplementation of pregnant women, as it is a routine practice for pregnant women to be supplied with iron because of higher need for iron during the pregnancy. Proscribed iron according to the postulated theory, should be in the form of ferrous fumarate (or succinate). Taking into account Avogadro's law number (6.022×10^{23}), it could be easily calculated that one ferrous fumarate pill of 350 mg contains about 200 mg of FA, *i.e.,* 1.0344×10^{21} FA molecules. These molecules can be converted into the same number of SA and succinyl-CoA subsequently *via* the catalytic activity of G-STK, in

mitochondria. On the other hand, it is well known that one red blood cell contains about 280 million (28×10^7) hemoglobin molecules and that for the synthesis of one heme molecule there is a need of 8 SA molecules in the form of active succinate (succinyl-CoA)[41]. Since one hemoglobin molecule contains 4 globin chains and each globin chain binds one heme, it can be stated that for the synthesis of one hemoglobin molecule there is a need for 32 succinates, and for the synthesis of 280 million of hemoglobin molecules there is a need for $32 \times 28 \times 10^7$ succinate molecules (*i.e.,* 8.96×10^9 fumaric acid molecules). Thus, only one ferrous fumarate pill of 350 mg, of which 200 mg belong to FA, is sufficient for the production of 1.15×10^{11} red blood cells (1.0344×10^{21} FA molecules/8.96×10^9 FA molecules = 1.15×10^{11} red blood cells). When it comes to the fetus, at the 25th week of gestation, it has approximately 60 mL of blood volume[42] and a total of about 4.20×10^{11} fetal red blood cells (60 mL = 6×10^4 mm^3, multiplied by the fetal red blood cell number about $7 \times 10^6/mm^3$). In terms of the aforementioned points, the prevention of the fetal liver damage in fetuses having the UCDs, can be realized by the daily supplementation with 200 mg of fumaric acid in iron supplement formula during pregnancy.

Mode of prevention of the post-neonatal liver damage

Throughout their lives, many patients with the UCDs experience various degrees of the liver damage, without any explanation of the mechanism of the damage. In accordance with the presented theory, the pathophysiology is probably the same. It is caused by the high concentration of hepatic iron, because of the increased iron absorption and its transportation via the specific transferrin isoform into the liver, as a consequence of the compromised hematopoiesis (since the liver retains its genetically imprinted capability of hematopoiesis throughout the life). The postnatal " liver hematopoiesis" is compromised because of the fumarate deficiency, caused by the urea cycle enzymatic fumarate deficient production. After birth, the fumarate deficiency is further augmented by the protein restricted diet, which practically excludes protein catabolism and "expels" the UC from the hepatocytes. A physiologically required preventive dose of FA can be calculated upon the following consideration: Almost 1% of the body's red blood cells are generated each day and the balance between red blood cell production and the removal of aging red blood cells from the circulation is precisely maintained. The ceaseless hematopoietic process replenishes the senescent cells that leave the circulation and produces nearly 200000000000 (2×10^{11}) red blood cells per day[43]. As it was previously demonstrated, approximately 1.0344×10^{21} of FA molecules must be used for the heme synthesis of 200000000000 (2×10^{11}) red blood cells. This quantity of FA is concentrated in only one ferrous fumarate pill of 350 mg. In this way, adult patients suffering from the UCDs can prevent the high concentration of iron, by daily consumption of 200 mg of fumarate. An identical calculation can be used for the succinate supplement on the basis of ferric-succinate formulas. The prevention of the hepatic iron excess during childhood can be also achieved with the fumarate-succinate supplementation, *i.e.*, doses should be adjusted according to the age dependent erythropoietic kinetic properties. Bearing in mind that UCD patients are always subjected to an increasing risk of high concentrations of iron in their livers, we would suggest these patients to be supplemented with formulae on the basis of zinc-fumarate or zinc-succinate.

COMMENTS

Case characteristics

Urea cycle defects (UCDs) occur in approximately 1 of 30000 live births[1]. The clinical hepatic presentation of the UCDs may include acute liver failure (ALF), liver dysfunction, and hepatocellular injury.

Pathological diagnosis

This metabolic profile finding gave the final diagnosis of citrullinemia type Ⅰ, a defect in the urea cycle, caused by the deficiency of argininosuccinate synthetase.

Treatment

The treatment was continued according to the rules for the treatment of acute hypeammomiemia. It included prompt removal of ammonia from the body and providing the organism with adequate calories and essential amino acids to halt further breakdown of endogenous proteins. The patient showed visible clinical and laboratory improvement. The ammonia level fell to 46 μmol/L, alanine aminotransferase level 70 IU/L, aspartate aminotransferase 86 IU/L and LDH level 538 IU/L.

Experiences and lessons

The prevention of the hepatic iron excess during childhood can be also achieved with the fumarate-succinate supplementation, *i.e.,* doses should be adjusted according to the age dependent erythropoietic kinetic properties. Bearing in mind that UCD patients are always subjected to an increasing risk of high concentrations of iron in their livers, we would suggest these patients to be supplemented with formulae on the basis of zinc-fumarate or zinc-succinate.

Peer-review

The manuscript "Metabolically based liver damage pathophysiology in patients with urea cycle disorders-a new hypothesis" is and interested and very detail for the new hypothesis.

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CASE REPORT

First case of cross-auxiliary double domino donor liver transplantation

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Author contributions: Zhu ZJ planned and performed the operations; Wei L, Qu W and Zeng ZG participated in the operations; Wei L, Qu W, Sun LY and Liu Y performed the patient management after the operations; Qu W and Liu Y followed the patients after discharge; He EH monitored the blood flow by ultrasound; Zhang HM and Zhu ZJ wrote the case report; Jia JD and Zhang ZT contributed to the treatments and operations as expert consultants; all authors contributed to this article.

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Abstract

We report a case of double domino liver transplantation in a 32-year-old woman who was diagnosed with familial amyloid polyneuropathy (FAP) and liver dysfunction. A two-stage surgical plan was designed, and one domino graft was implanted during each stage. During the first

stage, an auxiliary domino liver transplantation was conducted using a domino graft from a 4-year-old female child with Wilson's disease. After removing the right lobe of the FAP patient's liver, the graft was rotated 90 degrees counterclockwise and placed along the right side of the inferior vena cava (IVC). The orifices of the left, middle, and right hepatic veins were reconstructed using an iliac vein patch and then anastomosed to the right side of the IVC. Thirty days later, a second domino liver graft was implanted. The second domino graft was from a 3-year-old female child with an ornithine carbamyl enzyme defect, and it replaced the residual native liver (left lobe). To balance the function and blood flow between the two grafts, a percutaneous transcatheter selective portal vein embolization was performed, and "the left portal vein" of the first graft was blocked 9 mo after the second transplantation. The liver function indices, blood ammonia, and 24-h urinary copper levels were normal at the end of a 3-year follow-up. These two domino donor grafts from donors with different metabolic disorders restored normal liver function. Our experience demonstrated a new approach for resolving metabolic disorders with domino grafts and utilizing explanted livers from children.

Key words: Domino liver transplantation; Familial amyloid polyneuropathy; Double graft; Wilson's disease; Ornithine transcarbamylase deficiency; Case report

Core tip: We implanted two domino graft livers into a familial amyloid polyneuropathy patient. One domino graft liver was from a child with Wilson's disease, and the other was from a child with ornithine carbamyl enzyme defect. The blood flows of the two grafts were balanced by a percutaneous transcatheter selective portal vein embolization. These two domino donor grafts from donors with different metabolic disorders restored normal liver function. Our experience demonstrated a new approach to resolving metabolic disorders with domino grafts and utilizing explanted livers from children.

Zhu ZJ, Wei L, Qu W, Sun LY, Liu Y, Zeng ZG, Zhang L, He EH, Zhang HM, Jia JD, Zhang ZT. First case of cross-auxiliary double domino donor liver transplantation. *World J Gastroenterol* 2017; 23(44): 7939-7944 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i44/7939.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i44.7939

INTRODUCTION

Liver transplantation has become a standard treatment for hereditary and metabolic liver diseases, such as familial amyloid polyneuropathy (FAP)[1,2]. Domino liver grafts from patients with some types of metabolic liver diseases, such as maple syrup urine disease[3] and methylmalonic acidemia[4], may function well in the recipient. Domino liver grafts are a good option for some recipients who might otherwise experience long wait times for liver transplantation, such as recipients with hepatocellular carcinoma (usually outside the Milan criteria)[5]. However, ethical concerns remain regarding the influence of the domino donor's genetic disease on the recipient[5,6]. Sometimes, a domino transplantation is used only as a bridging therapy for fulminant liver failure[7]. The indications for auxiliary liver transplantation are also limited to potentially reversible fulminant hepatic failure[8-10] and liver-based metabolic disorders[11,12]. Thus, limitations exist for both domino donors and recipients and restrict the application of this technique. Additionally, explanted livers from small children with certain metabolic diseases are more difficult to use as domino grafts in adult patients because of their small sizes and metabolic problems.

In this report, we present a case of a cross-auxiliary double domino donor liver transplantation. The implantation of the double domino grafts from the children increased the total volume, and the two grafts compensated for each other's metabolic defects and thus could be used for the complete replacement of the recipient's liver. Based on this work, we believe that simultaneous double domino graft transplantation may also be conducted in most adult liver transplantation candidates. The reconstruction of the outflow tract in this case has previously been reported as an operative technique[13].

CASE REPORT

A 32-year-old woman was admitted into Beijing Friendship Hospital on September 9, 2013, with diagnoses of FAP and digestive tract hemorrhage. The patient had abdominal distension and decreased sensation in the lower limbs for 5 years. She received Chinese medicine treatments for nearly 2 years. Then, hematemesis and hematochezia began to occur intermittently. FAP was diagnosed in February 2013 at Peking Union Medical College Hospital using Congo red staining of the intestinal mucosa.

The FAP patient was malnourished and exhibited symptoms of anemia. The laboratory test results were as follows: Blood group, A; HGB, 59 g/L; PT, 18.2 s; KPTT, 50.1 s; ALB, 28.4 g/L; and TBIL, 37.23 μmol/L. Transthyretin (TTR) protein was detected in the intestinal mucosa using immunohistochemical staining with an anti-TTR antibody. The Val30Met mutation in *TTR* was confirmed using *TTR* gene sequencing. The electrocardiogram results revealed a sinus rhythm and no microvoltage. Echocardiography revealed thickened cardiac walls (interventricular septum, 13 mm; posterior wall, 12 mm; left ventricle end-diastolic diameter, 42 mm; and left ventricular ejection fraction, 66%). Contrast-enhanced computed tomography (CT) of the liver revealed heterogeneous enhancement of the liver, an increase in liver volume, and multiple soft tissue nodules around the portal vein and in the retroperitoneal region. The patient was listed for liver transplantation, with diagnoses of FAP (with liver,

digestive tract, and myocardial involvement) and liver dysfunction.

Hematemesis and hematochezia continued after plasma transfusion and mucosal protector and acid suppression therapies. These symptoms occurred because of the digestive tract mucosal injury caused by amyloid deposition and coagulation disorders. The patient's condition deteriorated over time, with worsening intestinal dysfunction and anemia, which increased the patient's need for transplantation. However, no deceased donor liver was readily available. The FAP patient's mother, the only potential living donor, was not suitable for donation because she had severe hepatic steatosis, which was confirmed by ultrasound and pathological examinations. Two children were under evaluation for living donor liver transplantation at that time. One (donor 1) was a 4-year-old female child with type A blood group, who was diagnosed with Wilson's disease. The other (donor 2) was a 3-year-old female child with type O blood group, who was diagnosed with an ornithine transcarbamylase deficiency (OTCD). Neither of these children was an optimal domino donor because of their metabolic liver defects. Moreover, auxiliary liver transplantation would not be a good choice for FAP patients because the deposition of mutated *TTR* may persist and result in heart failure after auxiliary liver transplantation. Therefore, we decided to implant both domino grafts and remove the FAP patient' s native liver. Thus, the two grafts could compensate for each other's metabolic defects. However, it would have been difficult to perform all of the required operations at the same time (including two living donor liver transplantations and a double domino graft liver transplantation) because the number of liver transplant surgeons was insufficient. A two-stage surgical plan for the FAP patient was designed, and one domino graft was implanted after removing part of the liver in each stage. All of the procedures and potential risks were explained to the three patients or their parents, and written consents were obtained. These works were approved by the ethics committee of Beijing Friendship Hospital.

The donor of the first domino liver graft (donor 1) underwent liver transplantation because of central nervous system involvement and the failure of decoppering treatments due to a penicillamine allergy. Donor 1 received the left lateral liver lobe from her mother on September 16, 2013. The domino auxiliary liver transplantation of the FAP patient was performed at the same time.

The auxiliary liver transplantation was conducted by removing the right lobe of the FAP patient's liver (segments 5, 6, 7, 8 and the right part of segment 1) and implanting the whole donor liver (the domino liver from donor 1). The residual liver of the FAP patient included segments 2, 3, 4 and the left part of segment 1. The left and middle hepatic veins of the FAP patient were reserved. We retained the right branches of the portal vein, hepatic artery, and hepatic duct for as long as possible.

The domino donor liver from donor 1 was perfused with histidine-tryptophan-ketoglutarate (HTK) solution immediately after it was resected. Three separate orifices of the main branches of the hepatic vein were found in the domino graft, and they were reconstructed using a cold-preserved iliac venous patch graft from a deceased donor. For the convenience of vascular anastomosis, the caudate lobe was resected. The graft was rotated 90 degrees counterclockwise and placed along the right side of the inferior vena cava (IVC) in the FAP patient. The reconstructed hepatic vein of the graft was anastomosed to the open end of the FAP patient's right hepatic vein, which was extended with an incision at the IVC. Next, the main portal vein was anastomosed to the right branch of the FAP patient's portal vein. After graft reperfusion, the graft hepatic artery was anastomosed to the FAP patient's right hepatic artery under a microscope. The common hepatic duct of the graft was anastomosed to the FAP patient's right hepatic duct (Figure 1). The cold ischemia time of the domino graft from donor 1 was 796 min and the operative time of the first graft transplantation for the FAP patient was 610 min. The graft to recipient weight ratio (GRWR) calculated by the weight of first domino graft was 0.84%.

The immunosuppressive regimen consisted of tacrolimus, mycophenolate mofetil, and methylprednisolone. The FAP patient recovered well from the surgery. The coagulation indices, including PT, KPTT, and TT, returned to normal on day 3 after liver transplantation, and no hematemesis or hematochezia occurred thereafter. A ^{99}mTc-EHIDA SPECT examination conducted on day 30 revealed that the proportions of the functional volumes of the domino graft and the residual left liver were 70.9% and 29.1%, respectively. However, the 24-h urinary copper levels continued to increase beyond the normal range, and there was a significant reduction in the serum copper protein level on day 14 after transplantation.

The donor of the second domino liver graft (donor 2) underwent a living donor liver transplantation for OTCD on October 16, 2013-one month after the first liver transplantation in the FAP patient. The second domino graft transplantation in the FAP patient was performed on the same day. The residual left lobe of the FAP patient's liver was removed. The middle-left hepatic vein, left hepatic artery, left branch of the portal vein, and hepatic duct were reserved. During the back-table preparation, the second domino graft was perfused with HTK solution, and the orifices of the right hepatic vein and middle-left hepatic vein were reconstructed using an iliac venous patch in the same manner employed for the first graft. The caudate lobe of the second graft was also resected. The second graft was orthotopically positioned with its right lobe overlapping the first graft. The reconstructed hepatic vein of the graft was connected with the middle-left hepatic vein of the FAP patient. Then, the graft portal vein was connected to the left portal vein of the FAP patient. After reperfusion, the proper hepatic artery and the

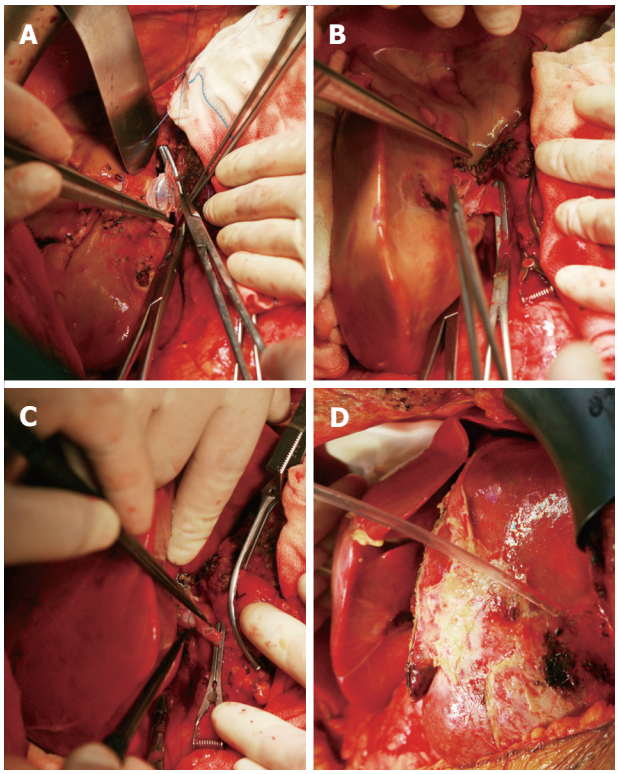

Figure 1 Implantation of the first graft. A: Reconstruction of the hepatic vein; B: Reconstruction of the portal vein; C: Reconstruction of the hepatic artery; D: Image of the first implanted graft.

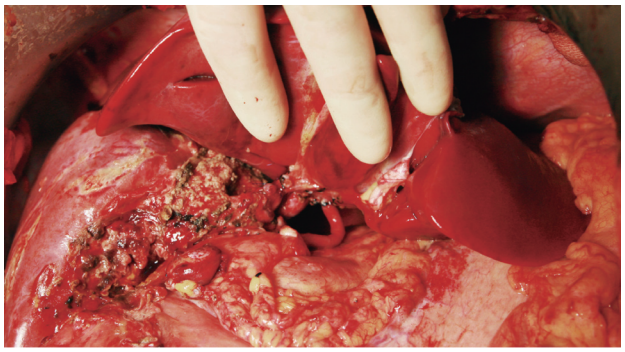

Figure 2 Image of the second implanted graft.

hepatic duct of the graft were anastomosed to the FAP patient's left hepatic artery and left hepatic duct, respectively (Figures 2 and 3). The cold ischemia time of the domino graft from donor 2 was 469 min and the operative time of the second graft transplantation for the FAP patient was 504 min. The GRWR calculated by the weight of second domino graft was 0.92%.

The immunosuppressive therapy regimen was the same as that for the first transplantation and included tacrolimus, mycophenolate mofetil, and methylprednisolone (tapered gradually). There were no episodes of rejection or surgical complications during recovery or follow-up. The liver function indices (ALT, AST, and TBIL) recovered smoothly. The test results for Wilson's disease and OTCD, including copper blue protein oxidase activity, blood copper, and blood ammonia, were negative. The hepatic arterial, portal

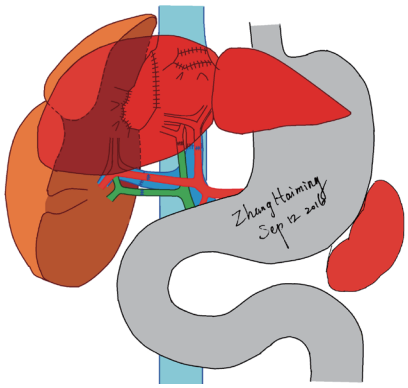

Figure 3 Illustration of the positions of the livers and anastomoses of the vessels and bile ducts.

venous, and hepatic venous blood flows were monitored using ultrasound and enhanced CT scans. Thirty days after the second domino transplantation, ^{99}mTc-EHIDA SPECT revealed that the proportions of the functional volumes of the first and second grafts were 75.6% and 24.6%, respectively.

Two hundred fifty-eight days after the second transplantation, the FAP patient's 24-h urinary copper excretion increased beyond the normal range. A contrast-enhanced CT scan revealed a markedly increased volume of the first graft, which received the greater part of the portal venous blood supply. To balance the function and blood flow between the two grafts, a percutaneous transcatheter selective portal vein embolization was performed, and the "left portal vein" of the first graft was blocked. Subsequently, the FAP patient's 24-h urinary copper excretion returned to normal. The FAP patient was followed by our hospital for over 4 years (Figure 4). The latest test results indicated that the ALT, AST, TBIL, serum ammonia, and 24-h urinary copper excretion were normal. Sensation in the lower limbs improved slightly. No symptoms of cardiac problems emerged, and there was no change in echocardiography. No surgical complications were found in the two domino donors, and their grafts functioned well at the end of a 4-year follow-up.

DISCUSSION

Auxiliary liver transplantation with a living related partial graft or a domino liver graft was initially introduced as a temporary or permanent support for patients, as it avoided small-for-size syndrome[14]. A complete domino liver from a child donor can be used without reducing the liver graft size, which may reduce surgical complications, such as liver graft cross-sectional bleeding, hemorrhage, and bile leakage. We rotated the graft 90 degrees counterclockwise, which facilitated anastomosis and reduced the potential complications caused by the limitation of space. However, the risks of outflow tract obstruction and portal vein angulation were increased. Extending the orifice of the right hepatic vein[13] contributed to the outflow tract patency. Additionally, the trends and lengths of the portal veins

Figure 4 Photos of patients and doctors involved. A: The second domino graft transplantation in the familial amyloid polyneuropathy (FAP) patient was performed on October 16, 2013; B: The FAP recipient (left), the first (right) and second (middle) domino donors one month after the second operation; C: Doctor Zhi-Jun Zhu (right) and the FAP patient (left) 4 years after transplantation.

of the graft and recipient should also be considered when the graft is positioned in the recipient. Resection of the caudate lobe may also be necessary to reduce the risk of portal vein angulation.

Domino liver grafts from small children can also be used in auxiliary liver transplantation. However, the metabolic deficiency of the domino liver graft limits the application of this approach. In this patient with FAP, we conducted a second domino liver transplantation instead of simply removing the remnant native liver. These two domino donor grafts, each from a donor with a different metabolic disorder, were used to restore full liver function. The metabolic disorder of a domino graft can be resolved in this manner.

Our experience with double domino transplantation will contribute to the improved utilization of explanted livers from children with metabolic disorders and expand the donor pool. Exchanging parts of livers between two patients with complementary metabolic liver diseases would also be practical when the body sizes and blood groups of the patients are suitable. "No donation liver transplantations" would represent a new mode of liver transplantation.

COMMENTS

Case characteristics

The main characteristics of familial amyloid polyneuropathy are pain, paresthesia, muscular weakness, autonomic dysfunction, and abnormalities caused by kidney and heart involvements.

Clinical diagnosis

Amyloid deposition can be found in many visceral organs.

Differential diagnosis

Familial Mediterranean fever, familial polyneuropathy, senile amyloidosis, amyloidosis of central nervous system, and localized amyloidosis.

Laboratory diagnosis

The mutation of the transthyretin gene can be found by genetic examinations.

Imaging diagnosis

Thickened cardiac walls can be found by echocardiography after heart involvement.

Pathological diagnosis

Depositions of amyloid can be found in the tissue sections after Congo red staining.

Treatment

Liver transplantation is the only curable treatment.

Related reports

Ando Y, Ueda M. Novel methods for detecting amyloidogenic proteins in transthyretin related amyloidosis. *Front Biosci* 2008;13: 5548-5558.

Term explanation

Domino donor liver transplantation: When a patient receives a liver transplantation, the explanted ill liver sometimes can be transplanted to another patient. The second transplantation is domino donor liver transplantation. Cold ischemia time: the time interval between liver graft explanting and implanting, during which liver graft is preserved in cold storage solution. Graft to recipient weight ratio (GRWR): graft weight/patient's body weight, which is used to

assess whether the graft is enough for a patient.

Experiences and lessons

Two domino donor grafts, each from a donor with a different metabolic disorder, can be used to restore full liver function in cross-auxiliary double domino donor liver transplantation.

Peer-review

This case report shows that cross-auxiliary double domino donor liver transplantation is practicable. However, details of this technique should be further discussed.

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