Int J Clin Exp Pathol 2018;11(2):481-489
www.ijcep.com /ISSN:1936-2625/IJCEP0071113

Original Article

Characteristics of bile microbiota in liver transplant recipients with biliary injury

Ying Liu[1], Ji-Dong Jia[1], Li-Ying Sun[1,2], Zhi-Jun Zhu[1,2], Jian-Rui Zhang[1], Lin Wei[1], Wei Qu[1], Zhi-Gui Zeng[1]

[1]Beijing Friendship Hospital, Capital Medical University, Beijing, China; [2]Beijing Key Laboratory of Tolerance Induction and Organ Protection in Tansplantation, Beijing, China

Received December 18, 2017; Accepted January 12, 2018; Epub February 1, 2018; Published February 15, 2018

Abstract: Objective: To identify the species and abundance of microbes in the bile of patients with biliary injury after liver transplantation. To explore the potential microbial involvement, we evaluated the differences in biliary microbiota in patients with and without biliary injury after liver transplantation. Methods: Bile was collected by percutaneous transhepatic cholangiography drainage from 5 liver transplant recipients with biliary injury (case group) and from the T-tube in 4 liver transplant recipients without biliary injury (control group). 16S rRNA sequencing was performed on the bile samples. Results: The biliary bacterial phyla in patients after liver transplantation were *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Acidobacteria* in order of abundance from highest to lowest. There were differences in genus *Prevotella* ($P = 0.03$), Porphyromonas ($P = 0.02$), and Fusobacterium ($P = 0.02$) between the two groups, which were significantly abundant in the case group. There was no significant difference in the diversity analysis between the two groups ($P > 0.05$). The terpenoid backbone biosynthesis pathway and the histidine degradation pathway were significantly enriched in the bile samples in the case group. Compared with the control group, the pyruvate ferredoxin oxidoreductase pathway was depleted in the samples from the case group. Conclusion: This is the first bile microbiota report using high-throughput 16S rRNA gene sequencing of bile material in liver transplant recipients. There were significant differences in bacterial abundance between patients with and without biliary tract injury after liver transplantation but no significant differences in the diversity. KEGG analysis showed that there were differences in metabolic pathways between the two groups.

Keywords: Liver transplantation, biliary injury, microbiota

Introduction

Biliary injury is one of the major complications after liver transplantation with an incidence of 20-30% [1, 2]. Mortality rates secondary to biliary complications range from 8% to 15% [3]. However, the etiology of these complications is often unknown.

Liver transplant recipients with biliary injury are considered to be at high risk of biliary infection. However, as multiple bacteria coexist in the biliary tract, culture-dependent methods are insensitive and biased for bacterial identification and are inadequate to study the entire microbial community [4]. In the past decade, high-throughput sequencing technology has been used to investigate microbial diversity. Variation of the intestinal microbiome during the perioperative period in liver transplant patients has been studied [5]. However, the bile duct microbiota of liver transplant recipients has not been investigated.

In this study, metagenomic sequencing technology was used to explore the species and abundance of microbes in bile of patients with biliary injury after liver transplantation.

Patients and methods

Patient selection

The case group comprised of 5 patients after liver transplantation in our Liver Transplant Center who met the following criteria: (1) age > 18 years; (2) imaging examination (ultrasound, magnetic resonance cholangiopancreatography) showing biliary stricture and segmental expansion; (3) bile duct radiography showing multiple stenosis or segmental expansion with

filling defect, involving the left and right hepatic duct; (4) the T-tube or percutaneous transhepatic cholangiography drainage (PTCD) tube was kept in place; (5) laboratory examinations showing abnormal liver function (alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, alkaline phosphatase, and γ-glutamyltransferase); and (6) informed consent had been signed.

The exclusion criteria were as follows: (1) clinical and pathological diagnosis of acute and chronic rejection; (2) imaging examinations showing biliary anastomosis stenosis after liver transplantation; (3) clinical and pathological diagnosis of recurrence of primary diseases (virus hepatitis or tumor); and (4) invasive systemic infection.

The control group was comprised of 4 patients after liver transplantation in our center who met the following criteria: (1) age > 18 years; (2) laboratory examinations showing normal liver function and B ultrasound showing normal blood flow and bile duct of the graft; (3) the T-tube was reserved and bile duct radiography showed no stenosis or expansion; and (4) informed consent had been signed.

All of the patients received tacrolimus (FK506)- or cyclosporine-based triple immunosuppressive therapy after liver transplantation and did not use antibiotics. There were no organs from executed prisoners used. The study was approved by the Ethics Committee in Beijing Friendship Hospital.

Sample collection

In the case group, bile was collected by PTCD. In the patients with a T-tube, bile was collected from the tube after sterilization. In the control group, bile was collected aseptically from the T-tube. The samples were stored at -80°C until being used for DNA extraction.

DNA extraction and quality control

The samples were treated using a mini-bead beater (Biospec Products, Bartlesville, OK, USA) and DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA extracts were stored at -80°C until use. The concentration of DNA was determined spectrophotometrically. Agarose gel electrophoresis was used to detect DNA integrity.

PCR amplification

The V4-V5 region of the bacterial 16S rRNA gene was amplified by PCR (95°C for 3 min, followed by 28 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 10 min) using primers 338F 5'-barcode- ACTCCTACGGGAGGCAGCAG)-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3'. PCR was performed in triplicate in a 20-μL mixture containing 4 μL 5 × FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL each primer (5 μM), 0.4 μL FastPfu Polymerase, and 10 ng template DNA.

Illumina MiSeq sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor-ST (Promega, Madison, WI, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited in the NCBI Sequence Read Archive (SRA) database.

Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered by Trimmomatic, and merged by FLASH with the following criteria: (1) The reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window. (2) Primers were exactly matched allowing 2 nucleotide mismatching and reads containing ambiguous bases were removed. (3) Sequences whose overlap longer than 10 bp were merged according to their overlap sequence.

Operational taxonomy units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1, http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU115) 16S rRNA database using a confidence threshold of 70% [6].

Statistical analysis

Species richness was evaluated by Chao, ACE, and OBS. Species diversity was evaluated by Shannon's, Simpson's, and Inv Simpson's index. The calculations of principal coordinates

Table 1. Demographics and characteristics of liver transplant patients with or without biliary injury

Case No.	Primary disease	Age (y)	Sex	Time since transplantation	Operation
S-1	Hepatocellular carcinoma	52	Male	11 m	LT
S-2	Primary biliary cirrhosis	55	Female	4 m	LT
S-3	Idiopathic portal hypertension	24	Male	12 m	LDLT
S-4	Hepatitis B cirrhosis	30	Male	5 y	LT
S-5	Hepatocellular carcinoma	18	Male	2 m	LT

S: case group; C: control group; LT: liver transplantation (Donor type was deceased donor); LDLT: living-donor liver transplantation.

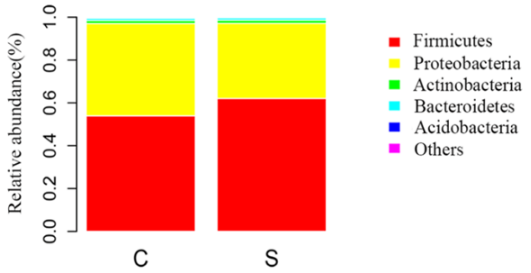

Figure 1. Top 5 bacterial phyla in the samples of the two groups (C, control group; S, case group), *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Acidobacteria.*

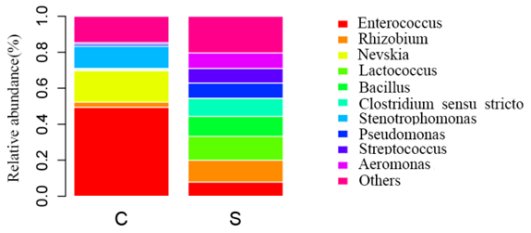

Figure 2. Top 10 bacterial genera in the samples of the two groups (C, control group; S, case group), *Enterococcus, Rhizobium, Nevskia, Lactococcus, Bacillus, Clostridium sensu stricto, Stenotrophomonas, Pseudomonas, Streptococcus, and Aeromona.*

analysis were performed by Hellinger distance, Jensen-Shannon Divergence (JSD) distance, Spearman's rank correlation distance, and (un) weighted UniFrac distance. LDA Effect Size (LEfse) analysis was performed to find species in which the relative abundance was significantly different among the various populations. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was evaluated by PICRUSt version 1.0.0 pipeline. Wilcoxon rank sum test was performed to evaluate the differences between the two groups in alpha diversity, principle coordinates, and KEGG module profiles.

Results

Patients

From September 2015 to June 2016, four liver transplant recipients without biliary injury (control group) and five recipients with biliary injury (case group) were included in the study. They all underwent liver transplantation by our group. The primary diseases included hepatocellular carcinoma, primary biliary cirrhosis (PBC), idiopathic portal hypertension, hepatitis B cirrhosis, hepatitis C cirrhosis, and alcoholic cirrhosis. The demographics and characteristics of these patients are shown in **Table 1**.

Sequencing results

Nine samples from 2 groups were pooled into 3 libraries and 212,557 qualified reads from 1,271,996 raw reads were filtered for downstream analysis. We randomly chose 10,000 reads from each sample. Thus, 90,000 reads were chosen from all of the samples. Samples with > 5,000 reads were retained.

Eighty-nine qualified OTUs were clustered for downstream analysis. One hundred and seventy-three OTUs were discarded because of their low distribution among all samples (no more than 1 sample contained a specific OTU or no more than 2 reads were binned into this OTU). Among all of the qualified reads, 98.61% (not randomly chosen) could be clustered into qualified OTUs generated with randomly chosen qualified reads.

Distribution and abundance of bile microbiota

In the control group, there were 2 main bacterial phyla, *Firmicutes and Proteobacteria*. The two phyla covered 91-99% of organisms in the bile. The other bacterial phyla with abundance > 0.1% in each sample were *Actinobacteria, Bacteroidetes, Acidobacteria,* and unclassified bacteria.

In the case group, *Firmicutes and Proteobacteria* were also the main phyla. The two phyla cov-

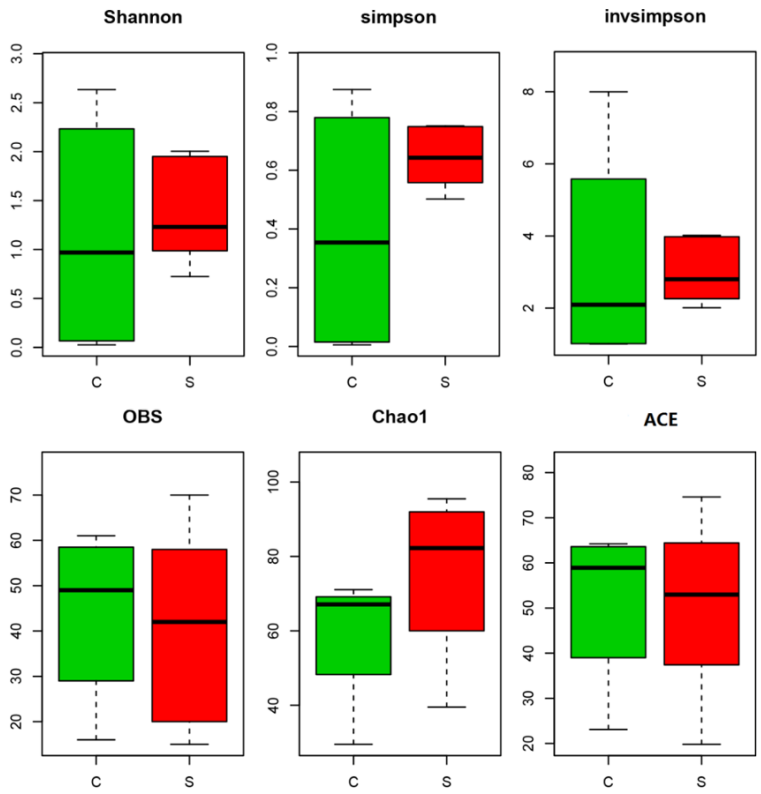

Figure 3. Alpha diversity and richness analysis. Shannon's, Simpson's, and inv Simpson's are common indexes to measure the species diversity including species richness and evenness. OBS, Chao1, and ACE are indexes to access the species richness. The greater the value means the higher the diversity and richness of the microbial community. No significant difference was found among all cohorts ($P > 0.05$).

ered 92-99% organisms in the bile. The other bacterial phyla with abundance > 0.1% in each sample were *Bacteroidetes, Actinobacteria, and Fusobacteria.*

In all of the samples, the top 5 phyla were *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Acidobacteria.* The top 10 genera were *Enterococcus, Rhizobium, Nevskia, Lactococcus, Bacillus, Clostridium sensu stricto, Stenotrophomonas, Pseudomonas, Streptococcus,* and *Aeromona* (**Figures 1** and **2**).

Alpha diversity and richness distribution among all samples

Alpha diversity and richness are two different measurements to assess species diversity within a sample. The greater the value means the higher the diversity and richness of the microbial community. No significant difference was found among all cohorts ($P > 0.05$) as measured by the common indexes including Shannon's, Simpson's, Chao1, and ACE (**Figure 3**).

Analysis of diversity differences between the two groups of samples: principal coordinates analysis (PCoA)

PCoA is based on the OTU classified taxon data, analyzing the microbial intrinsic composition structure between two groups. In our study, we used many methods to calculate the distance matrix including Hellinger, JSD, Spearman's coefficient, and (un) weighted UniFrac. There was no significant difference in the diversity between the two groups in this study ($P > 0.05$) (**Figures 4**-**6**).

Bacteria different in abundance between the two groups at the levels of phylum, class, order, family, and genus

At the level of the phylum, there were differences between the two groups in *Fusobacteria* ($P = 0.036$), which was abundant in the case group. At the level of the class, there were differences between the two groups in *Fusobacteria* ($P = 0.036$), which was enriched in the case group. At the level of the order, there were differences between the two groups in *Bacillales* ($P = 0.031$), *Fusobacteriales* ($P = 0.036$), and *Neisseriales* ($P = 0.030$), which were abundant in the case group. At the level of the family, there were differences between the two groups in *Leptotrichiaceae* ($P = 0.022$), *Prevotellaceae* ($P = 0.008$), and *Neisseriaceae* ($P = 0.030$), which were abundant in the case group. At the level of the genus, the bacteria difference between the two groups were *Prevotella* ($P = 0.03$), *Porphyromonas* ($P = 0.02$), and *Fusobacterium* ($P = 0.02$), which were enriched in the case group.

LDA Effect Size (LEfse) analysis

LEfse analysis was used to assess the significantly different microbiota in abundance

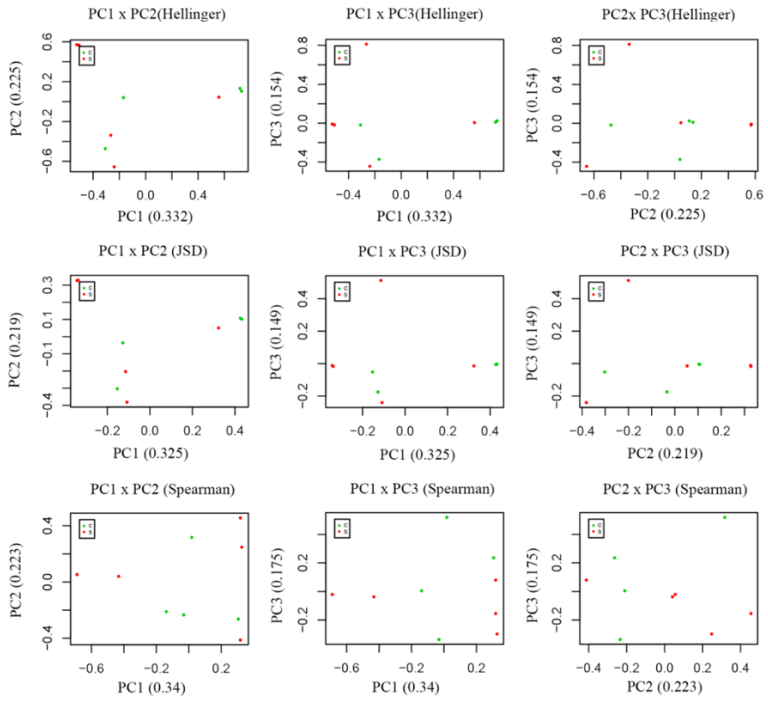

Figure 4. PCoA analysis based on OTU composition for all samples. The first line is the PCoA of Hellinger distance, the second line is of JSD, the third line is of the Spearman's coefficient distance, and different columns represent different PC combinations.

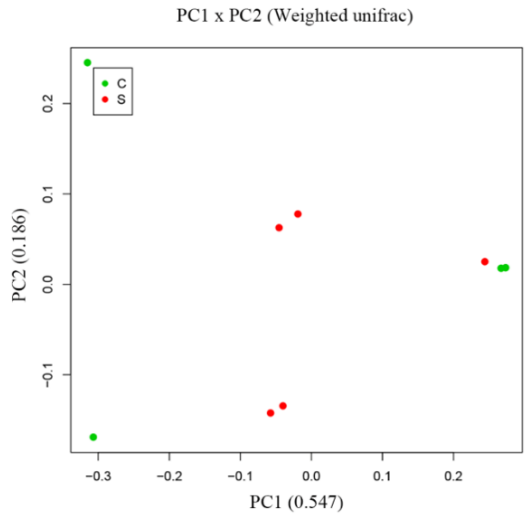

Figure 5. PCoA analysis of the weighted uniFrac distance. Each point represents a sample, the same color is from the same group, and the closer distance between the two points indicates that the difference in community composition is smaller. There was no significant difference in the diversity between the two groups in this study ($P > 0.05$).

between the two groups in all levels. The results showed that *Prevotellaceae, Prevotella,* and *Fusobacterium* were all more abundant in the case group (LDA score > 2) (**Figure 7**).

Differences in the level of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway between the two groups

There were differences between the two groups at the level of KEGG pathway. We identified 2 significantly enriched pathways and 1 depleted pathway in bile samples. Bile samples in the case group were significantly enriched in pathways related to C5 isoprenoid biosynthesis and histidine degradation. Compared to the control group, the case group was depleted in pyruvate ferredoxin oxidoreductase pathway (**Figure 8**).

Discussion

To the best of our knowledge, this study is the first to use high-throughput 16S rRNA gene sequencing of bile material from liver transplant recipients to explore the role of microbiota in the initiation, progression, and development of biliary injury in liver transplant recipients. In this study, the presence of bacteria in the bile of liver transplant recipients was evidenced by 16s rRNA high-throughput sequencing. There were differences in bacterial abundance between patients with and without biliary tract injury after liver transplantation.

In our study, we found that biliary microbiota having the highest abundance at the phylum level and in the population after liver transplan*tation were (from highest to lowest): Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Acidobacteria.* Those at the genus level were (from highest to lowest): *Enterococcus, Rhizobium, Nevskia, Lactococcus, Bacillus, Clostridium sensu stricto, Stenotrophomonas, Pseudomonas, Streptococcus,* and *Aeromona.* A study about bile and gall bladder microbiota of healthy pigs showed that the main phyla were *Proteobacteria, Firmicutes, and Bacteroidetes* [7]. Shen H et al. identified 13 novel biliary bacteria by metagenomic sequencing in gallstone patients. Bile samples had reduced

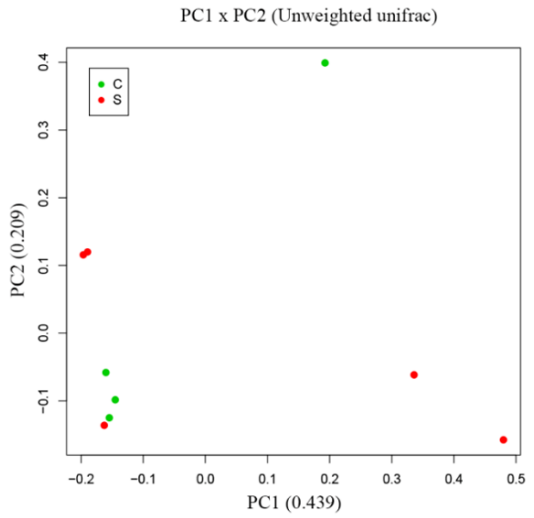

Figure 6. PCoA analysis of the unweighted uniFrac distance. There was no significant difference in the diversity between the two groups in this study ($P > 0.05$).

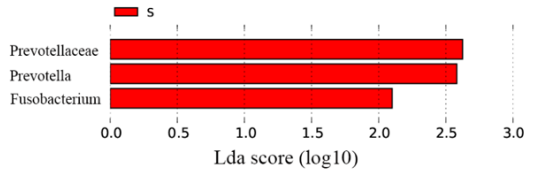

Figure 7. *LDA Effect Size (LEfse) analysis* between the two groups. LEfse analysis allows for comparisons between multiple groups to find species that are significantly different in abundance between groups. The logarithmic LDA score obtained by LefSe software analysis, which was significantly different, was set to 2. *Prevotellaceae, Prevotella,* and *Fusobacterium* were all enriched in the case group.

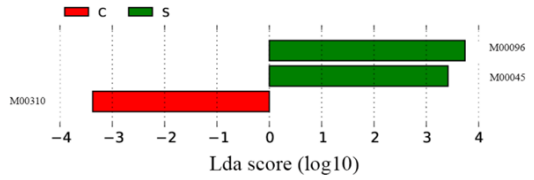

Figure 8. Differences in the level of KEGG pathway between the two groups. Bile samples in the case group were significantly enriched in terpenoid backbone biosynthesis pathway (M00096) and histidine metabolism pathway (M00045), whereas were depleted in pyruvate ferredoxin oxidoreductase pathway (M00310).

microbial diversity compared to healthy fecal samples [8]. The human gut includes approximately 10^{14} microorganisms, known as the intestinal microbiota, and contains four main phyla: *Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria.* Liver ischemia/reperfusion injury in liver transplant recipients can lead to severe intestinal barrier damage and intestinal microbiota changes. In addition, surgery and the application of postoperative antibiotics and immunosuppressive agents lead to intestinal microbial environment changes [9]. Intestinal microbiota of the population after liver transplantation has been reported. One study showed that *Eubacteria, Bifidobacterium* spp., *Faecalibacterium prausnitzii,* and *Lactobacillus* spp. were all significantly lower in liver transplantation recipients while *Enterobacteriaceae* and *Enterococcus* spp. were significantly higher. Except for *Enterococcus* spp., other bacteria have shown a tendency to return to normal levels with time after liver transplantation [10]. Bacteria normally present in the gut can invade the biliary tract by ascending from the duodenum or via the hematogenous route from the hepatic portal venous blood. Therefore, there could be a correlation between biliary and intestinal microbiota. In our previous study of intestinal microbiota in patients after liver transplantation, the major bacterial phyla included *Firmicutes* (75.88%), *Bacteroidetes* (12.95%), and *Proteobacteria* (4.24%) that was similar to bile microbiota in our study. Thus, in a future study, the fecal samples of the patients will be collected at the same time to further study the relationship between the two microbiota.

Biliary injury is one of the major complications after liver transplantation and is also the main cause of liver retransplantation, with an incidence of 20-30% [1, 2]. Biliary injury after liver transplantation can be caused by many factors and is accompanied by obstructive jaundice and secondary infection. The clinical manifestation is abnormal liver function, with imaging examination showing biliary stricture or obstruction. These patients must be treated with drugs, endoscopy, or PTCD. If the treatment is not effective, the patient can develop graft failure or even die. Biliary strictures can occur at the site of the anastomosis (anastomotic strictures) or in any other part of the biliary tract (nonanastomotic strictures). The former are associated with surgical technique and local ischemia. In our study, patients with biliary anastomotic strictures were excluded in the

case group. In a prospective study, Gotthardt et al. reported the results from 213 patients undergoing endoscopic retrograde cholangiography (ERC) after liver transplantation. Bile samples from first ERC showed Gram-positive bacterial isolates in 102/180 (57%) and Gram-negative isolates in 44/180 (24%). The main species were *Enterococcus* (40%; 72/180) and *Escherichia coli* (10%; 18/180). Both enteric bacteria (47%) and *Candida* spp. (18%) were associated with clinical signs of cholangitis [11]. However, culture-dependent methods are inadequate to study the entire microbial community. Therefore, improved understanding of biliary tract microbiota would be helpful for studying the pathogenesis of biliary injury after liver transplantation and antibiotic therapy. In our present study, compared with the group without biliary injury, there was enrichment of three types of bacterial genera in patients after liver transplantation with biliary injury: 1. *Prevotella* (P = 0.03); 2. *Porphyromonas* (P = 0.02); and 3. *Fusobacterium* (P = 0.02). These three microbiota are all anaerobic conditional pathogens. *Prevotella* is a Gram-negative, rod-shaped, and strictly anaerobic genus. *Prevotella* is more prevalent in populations with a plant-based diet, suggesting that it contributes to catabolism in the human gut. However, the presence of *Prevotella* in the human gut has also been linked to inflammation [12]. One study has suggested that *Prevotella copri* has a potential role in the pathogenesis of rheumatoid arthritis. It is abundant in the pro-inflammatory environment and it even enhances the advantage of inflammation [13]. *Porphyromonas* is also a Gram-negative anaerobic genus. The most frequently researched species is *Porphyromonas gingivalis*. It plays a crucial role during periodontal infection. Meanwhile, the contribution of this bacterium as well as its main pro-inflammatory component, lipopolysaccharide (LPS), in accelerating systemic diseases, including cardiovascular and central nervous system diseases, has been investigated in many long-term studies [14, 15]. LPS can even cause endotoxemia and result in inflammatory responses via several signaling pathways in immune cells [16, 17]. Fusobacterium is a genus in the phylum *Fusobacteria* of Gram-negative, anaerobic, rod-shaped bacteria. They are normal bacteria in the mouth, upper respiratory tract, intestinal tract, and urinary tract of humans and animals. No endospores are formed; some species are pathogenic and occur in various purulent or gangrenous diseases [18]. Chen Y et al. recruited 36 patients with liver cirrhosis and 24 healthy controls to study the changes of fecal microbiota. The proportion of phylum Fusobacteria was highly enriched in the cirrhosis group (P = 0.002) [19]. In patients with colorectal cancer the gut microbiota showed an increased population of Bacteroides-Prevotella and Fusobacterium [20].

Patients, after liver transplantation, require long-term use of immunosuppressive agents. In the case of immunodeficiency, the above-mentioned bacteria may cause opportunistic infections. Alteration of microbiota would alter many signals between the colonizing bacteria and epithelial or immune cells leading to changes in inflammation, epithelial cell cycle, proliferation, or mucus production which may lead to biliary injury. However, more studies are needed to determine whether they are associated with occurrence and progression of biliary injury.

Bile samples in the case group showed enrichment of pathways related to carbohydrate and lipid metabolism, terpenoid backbone biosynthesis, nucleotide and amino acid metabolism, and histidine metabolism. However, the samples in the control group showed enrichment of the pathway related to pyruvate ferredoxin oxidoreductase. Due to the limitation of 16s rRNA sequencing, the study of KEGG pathway deserves further research by whole-metagenomic shotgun (WMS) sequencing.

The sample size of this study was small. In the future, we will study a larger sample size combined with intestinal microbiota to further explore the mechanism of biliary injury after liver transplantation by genomics and metabolomics methods.

In conclusion, for the first time we used 16s rRNA sequencing to analyze the distribution and diversity of biliary microbiota in patients after liver transplantation. There were differences in bacterial abundance between patients with and without biliary tract injury after liver transplantation. KEGG analysis showed the differences in metabolic pathways between the two groups. Our results provide new insights

into biliary microbiota in liver transplant recipients which could be valuable for diagnosis and treatment of biliary injury after liver transplantation.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No.81570586) and Beijing Municipal Administration of Hospitals Ascent Plan (Code: DFL20150101).

Disclosure of conflict of interest

None.

Address correspondence to: Ji-Dong Jia and Li-Ying Sun, Beijing Friendship Hospital, Capital Medical University, 95 Yong-An Road, Beijing 100050, China. Tel: +86-10-63139246; Fax: +86-10-63139246; E-mail: jia_jd@ccmu.edu.cn (JDJ); Tel: +86-10-63138350; Fax: +86-10-63138350; E-mail: sunxlx@outlook.com (LYS)

References

[1] Dubbeld J, van Hoek B, Ringers J, Metselaar H, Kazemier G, van den Berg A, Porte RJ. Biliary Complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcome from a single center. Ann Surg 2015; 261: e64.

[2] Nemes B, Gámán G, Doros A. Biliary complications after liver transplantation. Expert Rev Gastroenterol Hepatol 2014; 21: 1-20.

[3] Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation 2011; 92: 373-379.

[4] Theron J, Cloete TE. Molecular techniques for determining microbial diversity and community structure in natural environments. Crit Rev Microbiol 2000; 26: 37-57.

[5] Lu HF, He JQ, Wu ZW, Zhang H, Ye P, Yang J, Zhen S, Li L. Assessment of microbiome variation during the perioperative period in liver transplant patients: a retrospective analysis. Microb Ecol 2013; 65: 781-791.

[6] Amato KR, Yeoman CJ, Kent A, Righini N, Carbonero F, Estrada A, Gaskins HR, Stumpf RM, Yildirim S, Torralba M, Gillis M, Wilson BA, Nelson KE, White BA, Leigh SR. Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. ISME J 2013; 7: 1344-1353.

[7] Jiménez E, Sánchez B, Farina A, Margolles A, Rodríguez JM. Characterization of the bile and gall bladder microbiota of healthy pigs. Microbiologyopen 2014; 3: 937-949.

[8] Shen H, Ye F, Xie L, Yang J, Li Z, Xu P, Meng F, Li L, Chen Y, Bo X, Ni M, Zhang X. Metagenomic sequencing of bile from gallstone patients to identify different microbial community patterns and novel biliary bacteria. Sci Rep 2015; 5: 17450.

[9] Doycheva I, Leise MD, Watt KD. The intestinal microbiome and the liver transplant recipient: what we know and what we need to know. Transplantation 2016; 100: 61-8.

[10] Wu ZW, Ling ZX, Lu HF, Zuo J, Sheng JF, Zheng SS, Li LJ. Changes of gut bacteria and immune parameters in liver transplant recipient. Hepatobiliary Pancreat Dis Int 2012; 11: 40-50.

[11] Gotthardt DN, Weiss KH, Rupp C, Bode K, Eckerle I, Rudolph G, Bergemann J, Kloeters-Plachky P, Chahoud F, Büchler MW, Schemmer P, Stremmel W, Sauer P. Bacteriobilia and fungibilia are associated with outcome in patients with endoscopic treatment of biliary complications after liver transplantation. Endoscopy 2013; 45: 890-896.

[12] Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, Rostron T, Cerundolo V, Pamer EG, Abramson SB, Huttenhower C, Littman DR. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife 2013; 2: e01202.

[13] Hofer U. Microbiome: pro-inflammatory Prevotella? Nat Rev Microbiol 2014; 12: 5.

[14] Belstrøm D, Holmstrup P, Damgaard C, Borch TS, Skjødt MO, Bendtzen K, Nielsen CH. The atherogenic bacterium Porphyromonas gingivalis evades circulating phagocytes by adhering to erythrocytes. Infect Immun 2011; 79: 1559-1565.

[15] Poole S, Singhrao SK, Chukkapalli S, Rivera M, Velsko I, Kesavalu L, Crean S. Active invasion of Porphyromonas gingivalis and infection-induced complement activation in apoe-/- mice brains. J Alzheimers Dis 2015; 43: 67-80.

[16] Ding PH, Wang CY, Darveau RP, Jin LJ. Nuclear factor-kappaB and p38 mitogen-activated protein kinase signaling pathways are critically involved in Porphyromonas gingivalis lipopolysaccharide induction of lipopolysaccharide binding protein expression in human oral keratinocytes. Mol Oral Microbiol 2013; 28: 129-141.

[17] Jitprasertwong P, Charadram N, KumphuneS, Pongcharoen S, Sirisinha S. Female sex hormones modulate Porphyromonas gingivalis lipopolysaccharide induced toll-like receptor signaling in primary human monocytes. J Periodontal Res 2016; 51: 395-406.

[18] Lee Y, Eun CS, Han DS. Clinical Fusobacterium mortiferum isolates cluster with undifferentiated Clostridium rectum species based on 16S

rRNA gene phylogenetic analysis. Ann Clin Lab Sci 2016; 46: 279-281.

[19] Chen Y, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L. Characterization of fecal microbial communities in patients with liver cirrhosis. Hepatology 2011; 54: 562-572.

[20] Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res 2012; 22: 299-306.