

Association study of gut flora in Wilson's disease through high-throughput sequencing

Hao Geng, MD^{a,b}, Shan Shu, MD^b, Jianjian Dong, MD^b, Hai Li, MD^a, Chenchen Xu, MD^b, Yongsheng Han, MD^{a,b}, Jiyuan Hu, MD^{a,b}, Yongzhu Han, MD^{a,b}, Renmin Yang, MD^{a,b}, Nan Cheng, MD^{a,b,*}

Abstract

In this study, we analyzed the difference of intestinal flora polymorphisms between Wilson's disease (WD) patients and healthy people by high-throughput sequencing technology, and explored the correlation between WD and intestinal flora polymorphism.

A total of 22 cases of WD patients and 22 healthy persons as control were recruited. The total DNA was extracted from the fecal specimens of all the subjects, V4 high variable region of 16S rRNA gene was amplified and sequenced by high-throughput sequencing. The sequencing results were analyzed by α diversity and β diversity. The unweighted UniFrac distance matrices were calculated and trees were built by unweighted-pair group method with arithmetic mean (UPGMA).

A total of 2,548,262 sequences were obtained after the data are optimized, the average sequences in the WD group was $36,836 \pm 4104$ and it was $35,051 \pm 3075$ in the normal control group, there was no significant difference in the average sequence number between the 2 groups. OTU analysis showed that 2663 OTU were obtained in WD group, and 3271 OTU were obtained in the control group, of which 941 were common OTU. Colony diversity analysis showed that the intestinal flora of WD group and control group belonged to 5 phyla, they were Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, and Tenericutes, respectively. In WD group, the abundance of Bacteroidetes was significantly lower than that of the control group (67.19% vs 76.75%, $P < .001$), and the abundance of Firmicutes (26.18% vs 19.83%, $P < .001$), Proteobacteria (4.31% vs 3.09%, $P < .05$), Fusobacteria (1.88% vs 0.04%, $P < .001$) were significantly higher than that of control group. Compared with the control group at the level of the genus, the abundance of Bacteroides (4.85% vs 4.6%, $P < .05$), Faecalibacterium (2.92% vs 2.13%, $P < .05$), Megamonas (0.84% vs 0.22%, $P < .001$), Lachnospira (0.16% vs 0.09%, $P < .001$) significantly increased in WD group, while the abundance of Prevotella (1.63% vs 2.48%, $P < .001$), Roseburia (0.75% vs 1.39%, $P < .001$) and Phascolarctobacterium (1.72% vs 2.45%, $P < .001$) significantly decreased in WD group. PCoA and UPGMA tree analysis showed that there were significant differences of gut microbial compositions between the 2 groups.

The diversity and composition of intestinal flora in the WD patients were significantly lower than those in the healthy controls, and the diversity of intestinal flora may be associated with the presence of WD.

Abbreviations: ATP = adenosine triphosphate, OTUs = operational taxonomic units, PCoA = orincipal coordinate analysis, UPGMA = unweighted-pair group method with arithmetic mean, WD = Wilson's disease.

Keywords: flora diversity, high throughput sequencing, principal coordinate analysis (PCoA), Wilson's disease

1. Introduction

Wilson's disease (WD), also known as hepatolenticular degeneration, is an autosomal recessive inherited disease caused by

ATP7B gene defect.^[1] It has occurred all over the world and mainly in children and young people. The incidence of men is slightly higher than that of women, which maybe because of the difference between the level of estrogen and the metabolism of iron.^[2] The incidence of WD in the world is about 1:30000,^[3] its carrier rate is about 0.011 and the gene frequency is about 0.56.

The *ATP7B* gene contains 21 exons and 20 introns, its expression product is P type copper transporter ATP enzyme, which is located in the liver cell Golgi body and is responsible for the copper transport of hepatocytes. The function of ATP enzyme is lost because of the *ATP7B* gene defect, and the copper in the cell can not be transported normally and deposited in the liver, brain, kidney, cornea and other tissues and organs. The clinical manifestations of WD mainly are progressive injury of the liver and nervous system.^[4] In some patients, corneal Kayser–Fleischer ring (K-F ring), renal injury, bone and muscle damage, and hematopoietic and immune system damage can also be found. WD has no radical cure, once a case is diagnosed, he needs a life-long low copper diet, and the treatment of copper resistance.^[5]

Intestinal flora is an important part of the normal microbial community of the human body, and is also an important basis for the normal operation of various physiological functions of the human body. Many studies have shown that changes in intestinal flora are associated with a variety of diseases, such as obesity,

Editor: Anser Azim.

HG and SS equally contributed to this study

This work was supported by the National Natural Science Fund of China (Grants 81673948).

The authors have no conflicts of interest to disclose.

^aAnhui University of Chinese Medicine, ^bThe Affiliated Hospital of the Neurology Institute of Anhui University of Chinese Medicine, Hefei, Anhui, China.

* Correspondence: Nan Cheng, Anhui University of Chinese Medicine, No.103 Mesishan Road, The Affiliated Hospital of the Neurology Institute of Anhui University of Chinese Medicine, No. 357 Changjiang Road, Hefei, Anhui, China (e-mail: azychengnan@163.com).

Copyright © 2018 the Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial License 4.0 (CCBY-NC), where it is permissible to download, share, remix, transform, and buildup the work provided it is properly cited. The work cannot be used commercially without permission from the journal.

Medicine (2018) 97:31(e11743)

Received: 28 February 2018 / Accepted: 6 July 2018

<http://dx.doi.org/10.1097/MD.00000000000011743>

asthma, type 2 diabetes, arthritis and cardiovascular disease.^[6–11] The development of some mental diseases is also associated with the imbalance of intestinal flora.^[12] It was shown that extrapyramidal symptoms were associated with intestinal flora in Parkinson's disease.^[13] The absorption and utilization of trace elements in the intestinal tract regulates the balance of intestinal flora.^[14] Iron absorption barrier caused by the deficiency of iron regulation protein gene IRP-2 can change the composition of intestinal flora.^[15] Therefore, we speculated that intestinal flora diversity may be associated with WD.

In this study, we analyzed the difference of intestinal flora polymorphisms between Wilson's disease (WD) patients and healthy people by high-throughput sequencing technology, and explored the correlation between WD and intestinal flora diversity.

2. Materials and methods

2.1. Subjects

A total of 22 WD patients with first diagnosis and taking any medications for <7 days were recruited in the Affiliated Hospital of the neurology Institute of Anhui University of Chinese medicine between May 2015 and May 2016. The diagnosis based on clinical manifestations, medical and neurological examination, family history, low serum ceruloplasmin levels, 24 hours high urinary copper excretion, liver function test, liver ultrasonography, brain MRI and/or CT. All cases were confirmed by gene diagnosis. The healthy people with matching gender and age from the Anhui University of Chinese medicine who have no disease or drugs affecting the autonomic nervous system are recruited as control. The exclusion criteria are as follows: patients who have used antibiotics, probiotics, and microecologic agents within 30 days; patients with organic diseases of the digestive system; patients with gastrointestinal surgery; patients with alcohol addiction, diabetes and other affecting intestinal flora diseases; patients who have fever, cough, congestion, and other respiratory tract infections and other inflammatory diseases within 30 days.

2.2. Ethical review

All subjects signed informed consent forms. This study was approved by the Ethics Committee of the Anhui University of Chinese medicine.

2.3. Specimen collection and DNA extraction

The subjects in WD group had low copper diet, others had a normal diet. The first fresh stool (2–5 g) in the morning was collected. DNA was extracted using Tiangen stool mini kit (Tiangen, Beijing, China) in 5 hours after collection according to the instruction manual.

2.4. DNA library construction and high throughput sequencing

The extracted DNA was quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), they were detected by electrophoresis with 0.8% agarose. The DNA samples were amplified using MetaVx_ Library Preparation Kit (Genewiz, New Zealand) by PCR according to the instruction manual. The primers used in this study are as follows: F: 5'-GTGCCAGCMGCCGCGG-3'; R: 5'-GGACTACHVGGGTWCTAAT-3'. The AxyPrepDNA gel Recovery Kit (XYGEN company) was used to purify the PCR

products according to the instruction manual. Library validation was performed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Sequencing was conducted using a 2 × 250 paired-end (PE) configuration, and image analysis and base determination were performed using the PE250 Control Software on the PE250. Preliminary classification and analysis were performed with Illumina BaseSpace platform.

2.5. Data analysis

The data were analyzed with CASAVA software (V1.8.2), and Pass Filter Data were obtained after analysis. Pandaseq software (V2.7) was used to compare the sequencing results of the WD patients and the control group. Trimmomatic software (V0.30) was used to eliminate primers and joint sequences. Then the Usearch software (V8.0) was used to compare the splicing sequence and the database, the final effective sequence was obtained after the chimerism sequence was removed. A taxonomic analysis was carried out in the representative sequences of 97% similar level Operational taxonomic units (OTUs) by using RDP classifier Bayes algorithm. The community composition of each sample was counted at different classification levels: domain, Kingdom, phylum, class, order, family, genus (genus), and species. The comparison databases are as follows: Silva_111 16S rRNA database (<https://www.arbsilva.de>); RDP (Release 11.1, <http://rdp.cme.msu.edu/>); Greengene (Release 13.5, <http://greengenes.secondgenome.com/>); Unite (Release 6.0, <http://unite.ut.ee/index.php>).

According to the results of OTUs analysis, α diversity analysis including Simpson, Shannon, Chao 1, ACE and Good's Coverage were carried out by Mothur (version v.1.30.1, http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity) software. β diversity analysis was carried out using PCoA to calculate the diversity distance matrix by the QiimeV1.7 software (http://qiime.org/tutorials/otu_picking.html). The trees were built by unweighted-pair group method with arithmetic mean (UPGMA) method.

2.6. Statistical analysis

Statistical analysis was performed using *t*-test with SPSS 19.0 software (SPSS Inc., Chicago, IL). The quantitative variables were expressed as mean \pm SD and the qualitative variables were expressed as percentage. $P < .05$ was considered to be statistically significant.

3. Results

3.1. Demographic data

The average age of the WD group was 18.0 ± 12.7 years old, the proportion of men was 57.9%, and the average body mass index was 20.54 ± 2.61 . The average age of the control group was 15.5 ± 11.8 , the proportion of men was 57.2%, and the average body mass index was 21.31 ± 2.37 . There was no significant difference in age, sex ratio and body mass index between the 2 groups ($P > .05$).

3.2. High throughput sequencing results

A total of 5,409,698 16S rRNA reads were obtained from fecal specimens by high throughput sequencing in this study, the average reads were $175,910 \pm 59,801$ in the WD group, the average reads were $134,372 \pm 80,725$ in the control group. A total of 2,548,262 sequences were obtained after the data optimization. The average sequences were $36,836 \pm 4104$ in the

WD group, and the average were $35,051 \pm 3075$ in the normal control group, there was no significant difference in the average sequence number between the 2 groups ($P > .05$).

3.3. OTU analysis

All the effective sequences are clustered, and the sequence with similarity over 97% is considered to be an OTU. OTU analysis showed that 2663 OTU were obtained in WD group, and 3271 OTU were obtained in the control group, of which 941 were common OTU.

Rank-abundance curves can be used to analyze species abundance and species evenness. In the horizontal direction,

the abundance of the species is reflected by the width of the curve, the higher the abundance is, the larger the range of the curve is on the horizontal axis. The shape of the curve (smoothness) reflects the evenness of species in the sample, the more smooth the curve is, the more uniform the species distribution is. The species accumulation curve is used to describe the increase of the species (OTU) with the increased sample size. In this study, the results of OTU analysis were shown in Figure 1. We found that the rank-abundance curve was smooth, indicating high evenness among samples. Species accumulation curves tend to be gentle, indicating that the species in the specimen will not increase significantly with the increase in the sample size. They showed that the sampling was sufficient and data analysis could be carried out.

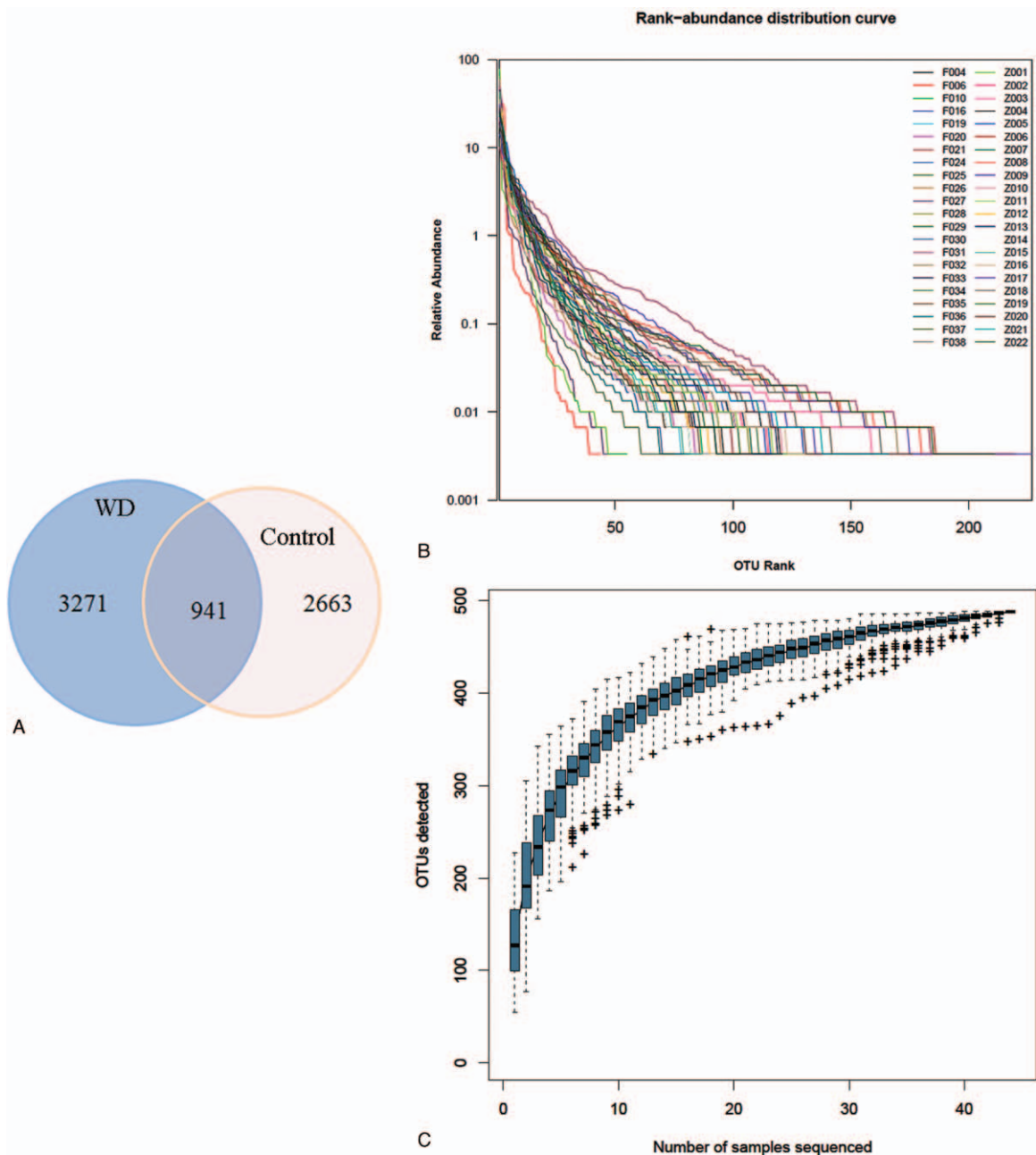


Figure 1. Venn picture (A), rank-abundance curve (B) and species accumulation curves (C) of operational taxonomic units (OTUs) in the 2 groups. The rank-abundance curve was smooth, indicating high evenness among samples. Species accumulation curves tend to be gentle, indicating that the species in the specimen will not increase significantly with the increase in the sample size. WD: WD patients; Control: healthy controls. OTUs=operational taxonomic units, WD=Wilson’s disease.

Table 1
Analysis of α diversity index between WD patients and healthy people.

Index	WD group	Control group
Shannon	2.63 ± 0.57*	2.53 ± 0.51
Simpson	0.16 ± 0.09	0.18 ± 0.12
Chao 1	160.63 ± 57.83*	178.95 ± 58.61
Ace	160.68 ± 56.09	179.23 ± 56.21
Good's coverage	0.99 ± 0.01	0.99 ± 0.01

WD = Wilson's disease.
 * $P < .05$ vs control.

3.4. α -diversity analysis

The average index of Shannon, Simpson, Chao 1, Ace and Good's Coverage was 2.63 ± 0.57 , 0.16 ± 0.09 , 160.63 ± 57.83 , 160.68 ± 56.09 , and 0.99 ± 0.01 , respectively in WD group. The average index of Shannon, Simpson, Chao 1, Ace and Good's Coverage was 2.53 ± 0.51 , 0.18 ± 0.12 , 178.95 ± 58.61 , 179.23 ± 56.21 , and 0.99 ± 0.01 , respectively in control group (Table 1). Compared with the control group, the Shannon index ($P = .011$), Chao 1 Index ($P = .029$) and Ace index ($P = .021$) of WD patients

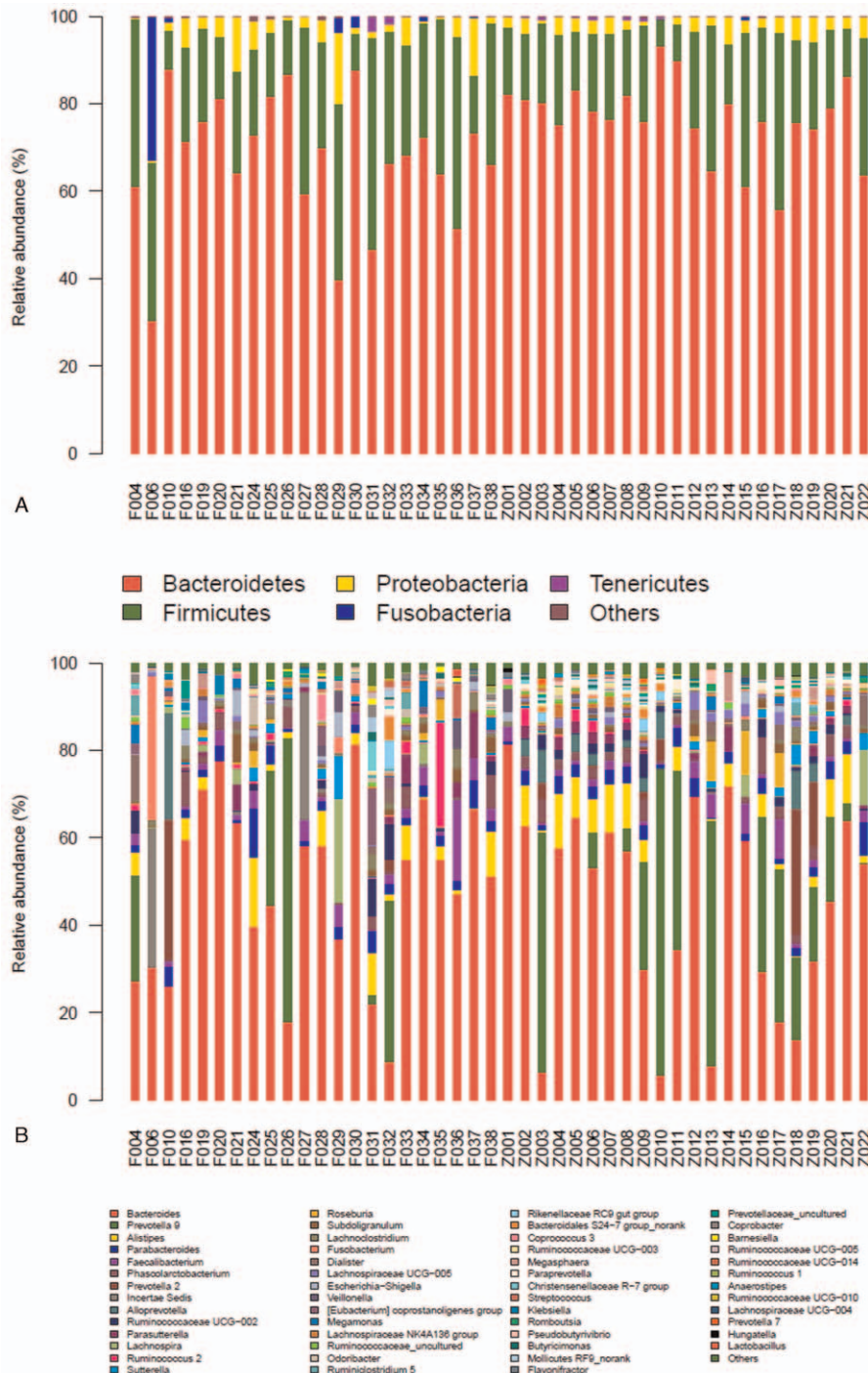


Figure 2. Composition and relative abundance of bacterial communities based 16S rDNA sequences in F (WD patients) and Z (healthy controls) groups. (A) The relative abundances of the major bacteria in phylum level. (B) The relative abundances of the major bacteria in genus level. WD=Wilson's disease.

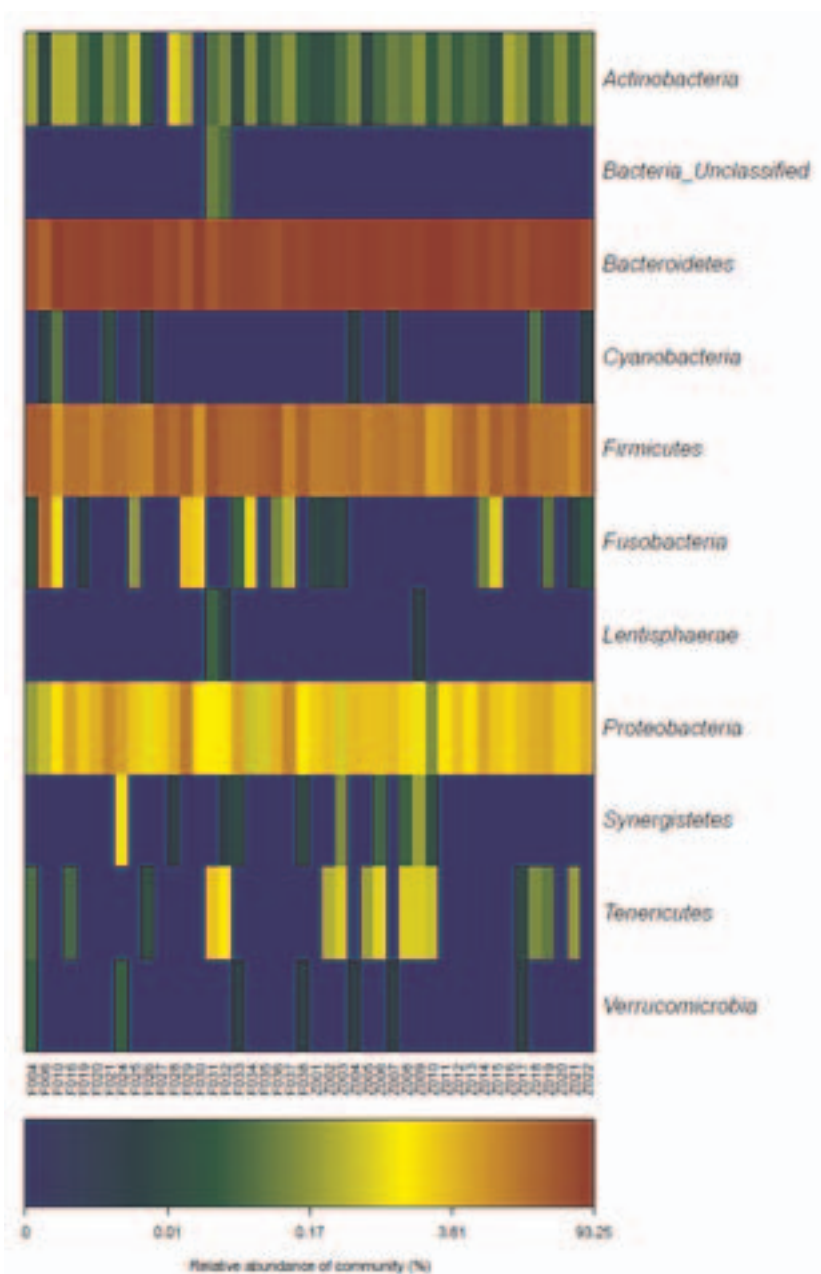


Figure 3. Heat map analysis relative abundance of bacterial communities in F (WD patients) and Z (healthy controls) groups. WD=Wilson's disease.

were significantly decreased, indicating that the diversity of intestinal flora in WD patients was lower than that of control group.

3.5. Analysis of intestinal flora structure

Colony diversity analysis showed that the intestinal flora of WD group and control group belonged to 5 phyla, they were Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, and Tenencutes, respectively. Most of them belong to Bacteroidetes and Firmicutes, accounting for about 84.1% of the total sequence (Fig. 2). In WD group, the abundance of Bacteroidetes was significantly lower than that of the control group (67.19% vs 76.75%, $P < .001$), and the abundance of Firmicutes (26.18% vs 19.83%, $P < .001$), Proteobacteria (4.31% vs 3.09%, $P < .05$),

Fusobacteria (1.88% vs 0.04%, $P < .001$) were significantly higher than that of control group. Compared with the control group at the level of the genus, the abundance of Bacteroides (4.85% vs 4.6%, $P < .05$), Faecalibacterium (2.92% vs 2.13%, $P < .05$), Megamonas (0.84% vs 0.22%, $P < .001$), Lachnospira (0.16% vs 0.09%, $P < .001$) significantly increased in WD group, while the abundance of Prevotella (1.63% vs 2.48%, $P < .001$), Roseburia (0.75% vs 1.39%, $P < .001$) and Phascolarctobacterium (1.72% vs 2.45%, $P < .001$) significantly decreased in WD group (Fig. 3).

3.6. Principal component analysis

According to unweighted UniFrac and Bray-Curtis distance matrices analysis of 16S rRNA sequence, the first PCoA, second

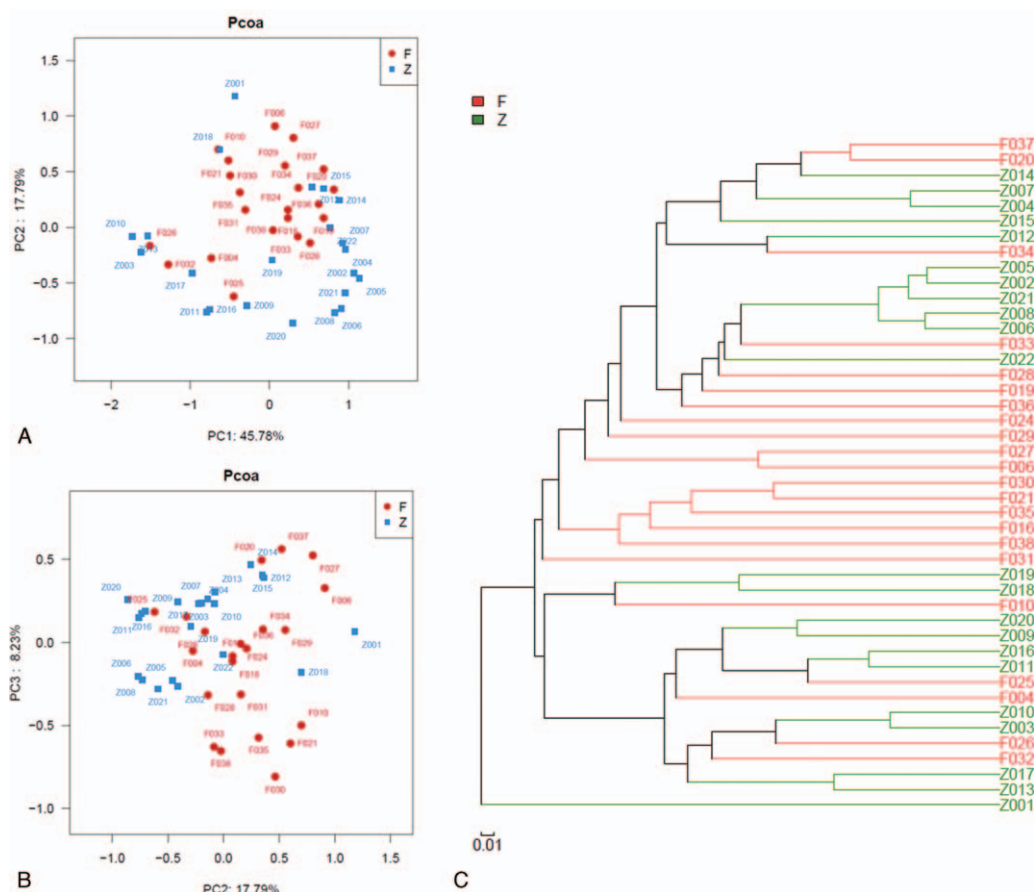


Figure 4. The principal coordinates analysis (PCoA) (A) and UPGMAM method of Beta diversity index (B) of groups F (WD patients) and Z (healthy controls). WD = Wilson's disease.

PCoA, and third PCoA contribution rate of the difference between WD group and control group were 45.78%, 17.79% and 8.23%, respectively. PCoA and UPGMA tree analysis showed that there were significant differences of gut microbial compositions between the 2 groups (Fig. 4).

4. Discussion

According to our knowledge, there is no study on the intestinal flora diversity in WD patients. In this study, we analyzed the high throughput sequencing results of 16S rRNA V4 variable region of the intestinal flora in the WD patients and the normal population. We found that there were significant differences in intestinal flora diversity and intestinal flora composition between WD patients and normal people. Microbial diversity is a new health marker, it is important to maintain the stability and performance of the ecosystem. The decrease of intestinal flora diversity is related to many diseases, such as active inflammatory bowel disease, cirrhosis, obesity, diabetes, child autism, depression, Alzheimer's disease, and so on.^[6–13,16] The Shannon index, Chao 1 index, and Ace index of intestinal flora in WD patients were significantly lower than those in healthy people, which indicating that the diversity of intestinal flora in WD patients was significantly lower than that in the control group. Therefore, these results suggested that WD may be associated with the decrease in intestinal flora diversity.

The decrease of intestinal flora diversity may directly affect the physiological function of intestinal microbes. The diversity of intestinal flora in obese patients was significantly lower than that

in normal population.^[17] The decrease of intestinal flora diversity was also associated with insulin resistance, dyslipidemia, and inflammation.^[18–20] The athletes have more intestinal microbes than the healthy people.^[21] The structure of intestinal flora in identical twins is surprisingly similar, but there are significant differences between the fraternal twins. This indicates that the structure of intestinal flora is regulated by the host gene.^[22] The results of this study also suggested that WD, an autosomal recessive hereditary disease, may also be associated with intestinal flora.

The abundance of Bacteroidetes in WD patients was significantly higher than that in the control group, while the abundance of Firmicutes, Proteobacteria, and Fusobacteria was significantly lower than that of the control group. Bacteroidetes phylum mainly includes Bacteroides genus and Prevotella genus. They play an important role in maintaining the homeostasis of mucosal T cells and establish a symbiotic relationship with the host.^[23,24] An increase in Prevotella abundance may promote inflammatory responses in the host, which is associated with the incidence of rheumatoid arthritis.^[25] The metabolite of Faecalibacterium has anti-inflammatory effect to prevent NF- κ B activation and secretion of IL-8.^[26] There were immune disorders in WD patients, their T lymphocytes were lower than normal people, while B lymphocyte, NK cell, and circulating immune complex IgM were higher than normal people.^[27] In this study, Prevotella abundance increased and Faecalibacterium abundance decreased in WD patients, indicating that changes in intestinal flora in WD may be related to immune dysfunction.

In this study, we also found that the abundance of intestinal flora Proteobacteria in WD patients decreased significantly, and the decrease was positively related to the dyskinesia of WD patients. It is not clear whether the decrease of the abundance of Proteobacteria in the WD is associated to the neurologic disorders for the limited activity in the brain function area of the competent exercise. Firmicutes plays a crucial role in obesity. In this study, there was no significant difference in BMI between WD patients and controls, but the abundance of Firmicutes in WD patients was significantly reduced, indicating that this may be related to WD.

In a word, this study firstly clarified that intestinal flora diversity and flora structure in WD patients were significantly different from those of the normal population, the immune disorder and nervous system symptoms of WD may be related to the structure of intestinal flora. More research is needed to elucidate whether there is a causal relationship between WD and intestinal flora diversity.

Author contributions

Conceptualization: Nan Cheng.

Data curation: Hao Geng, Jianjian Dong, Chenchen Xu, Yongsheng Han, Renmin Yang.

Formal analysis: Shan Shu, Hai Li, Jiyuan Hu.

Investigation: Nan Cheng, Hao Geng, Shan Shu, Jianjian Dong.

Methodology: Hao Geng, Shan Shu, Chenchen Xu, Yongzhu Han.

Project administration: Nan Cheng.

Resources: Yongsheng Han.

Software: Jianjian Dong, Hai Li, Yongsheng Han, Yongzhu Han.

Validation: Hai Li, Yongzhu Han.

Visualization: Jiyuan Hu, Renmin Yang.

Writing – original draft: Hao Geng, Shan Shu.

Writing – review & editing: Nan Cheng.

References

- Petrukhin K, Lutsenko S, Chernov I, et al. Characterization of the Wilson disease gene encoding a P-type copper transporting ATPase: genomic organization, alternative splicing, and structure/function predictions. *Hum Mol Genet* 1994;3:1647–56.
- Litwin T, Gromadzka G, Czlonkowska A. Gender differences in Wilson's disease. *J Neurol Sci* 2012;312:31–5.
- Frydman M. Genetic aspects of Wilson's disease. *J Gastroenterol Hepatol* 1990;5:483–90.
- Li K, Reichmann H, Ziemssen T. Recognition and treatment of autonomic disturbances in Parkinson's disease. *Expert Rev Neurother* 2015;15:1189–203.
- Li WJ, Wang JF, Wang XP. Wilson's disease: update on integrated Chinese and Western medicine. *Chin J Integr Med* 2013;19:233–40.
- Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Lett* 2014;588:4223–33.
- Abrahamsson TR, Jakobsson HE, Andersson AF, et al. Low gut microbiota diversity in early infancy precedes asthma at school age. *Clin Exp Allergy* 2014;44:842–50.
- Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 2012;490:55–60.
- Jie Z, Xia H, Zhong SL, et al. The gut microbiome in atherosclerotic cardiovascular disease. *Nat Commun* 2017;8:845.
- Maeda Y, Takeda K. Role of gut microbiota in rheumatoid arthritis. *J Clin Med* 2017;6:
- Scher JU, Ubeda C, Artacho A. Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease. *Arthritis Rheumatol* 2015;67:128–39.
- Bienenstock J, Kunze W, Forsythe P. Microbiota and the gut-brain axis. *Nutr Rev* 2015;73(suppl 1):28–31.
- Mertsalmi TH, Aho VTE, Pereira PAB, et al. More than constipation-bowel symptoms in Parkinson's disease and their connection to gut microbiota. *Eur J Neurol* 2017;24:1375–83.
- Mach N, Clark A. Micronutrient deficiencies and the human gut microbiota. *Trends Microbiol* 2017;25:607–10.
- LaVaute T, Smith S, Cooperman S, et al. Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. *Nat Genet* 2001;27:209–14.
- Claesson MJ, Jeffery IB, Conde S. Gut microbiota composition correlates with diet and health in the elderly. *Nature* 2012;488:178–84.
- Nicolucci AC, Hume MP, Martinez I, et al. Prebiotics reduce body fat and alter intestinal microbiota in children who are overweight or with obesity. *Gastroenterology* 2017;153:711–22.
- Rajkumar H, Kumar M, Das N, et al. Effect of probiotic *Lactobacillus salivarius* UBL S22 and prebiotic fructo-oligosaccharide on serum lipids, inflammatory markers, insulin sensitivity, and gut bacteria in healthy young volunteers: a randomized controlled single-blind pilot study. *J Cardiovasc Pharmacol Ther* 2015;20:289–98.
- He K, Hu Y, Ma H, et al. Rhizoma *Coptidis* alkaloids alleviate hyperlipidemia in B6 mice by modulating gut microbiota and bile acid pathways. *Biochim Biophys Acta* 2016;1862:1696–709.
- Roselli M, Devirgiliis C, Zinno P, et al. Impact of supplementation with a food-derived microbial community on obesity-associated inflammation and gut microbiota composition. *Genes Nutr* 2017;12:25.
- Clarke SF, Murphy EF, O'Sullivan O, et al. Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* 2014;63:1913–20.
- Org E, Parks BW, Joo JW, et al. Genetic and environmental control of host-gut microbiota interactions. *Genome Res* 2015;25:1558–69.
- Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 2008;453:620–5.
- Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011;334:105–8.
- Pianta A, Arvikar S, Strle K, et al. Evidence of the immune relevance of *Prevotella copri*, a gut microbe, in patients with rheumatoid arthritis. *Arthritis Rheumatol* 2017;69:964–75.
- Sokol H, Pigneur B, Watterlot L, et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* 2008;105:16731–6.
- Zhirnova IG, Kuznetsova AV, Rebrova OY, et al. Logical and statistical approach for the analysis of immunological parameters in patients with Wilson's disease. *Russ J Immunol* 1998;3:173–84.