Gut Microbiota Plays an Essential Role in the Onset of Membranous Nephropathy: Evidence from Multi-Center Case-Control Study and Animal Model

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Research

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Abstract

OBJECTIVE: Membranous nephropathy (MN) is one of the most common causes of nephrotic syndrome in adults. Here we aim to establish a non-invasive diagnostic model using differential gut microbiome and to explore the relationship between gut microbiome and the pathogenesis of MN in animal experiment.

DESIGN: A total of 825 fecal samples including MN patients and healthy controls (HC) were collected from Central, East and South China. We obtained crucial operational taxonomic units (OTUs) by 16S rRNA sequencing and then established a diagnostic model using five-fold cross-validation on random forest model. We subsequently validated its efficiency in cross regional cohort. MN rat model was induced by Sheep Anti-Rat Fx1A Serum and was used to explore the relationship between gut microbiome and the pathogenesis of MN.

RESULTS: The diversity and richness of gut microbiome was significantly decreased in MN patients. The diagnostic model based on 7 operational OTUs achieved excellent efficiency with an area under curve (AUC) of 98.36% in training group. The different disease states and cross regional cohort validated high diagnostic efficiency. In rat model, urinary protein was significantly relieved by intestine cleaning, while increased again by fecal microbiome transplant (FMT). Interestingly, fecal microbiome from MN patients seems to play a protective role when compared with that from healthy people.

CONCLUSION: Gut microbiome can be used as a non-invasive tool for MN diagnosis. The onset of MN depends partially on the existence of naturally colonized microbiome. Adaptive changes of gut microbiome during MN may help relieve urinary protein.

Introduction

Membranous nephropathy (MN) is one of the most common pathological types of nephrotic syndrome with high prevalence worldwide[1, 2]. Great progression has been made in diagnosis and treatment for MN in the last decade. More and more laboratory examinations including PLA2R (M-type phospholipase A2 receptor) and THSD7A (Thrombospondin type 1 domain-containing 7A) are suggestive for its diagnosis[3, 4]. Nevertheless, the sensitivity or specificity of them is not high enough. As a result, renal biopsy is still necessary especially for PLA2R-negative MN. The prognosis of MN is not optimistic, since 6%-25% MN patients failed to achieve clinical remission even after a series of treatment[5, 6]. Those who got remission still have a relapse rate up to 43%-47%[5, 6]. As a result, novel and effective ways for MN diagnosis and treatment are still urgently needed.

Recent studies have revealed that gut microbiome is closely associated with many diseases such as diabetes, systemic lupus erythematosus, hyperthyroidism and so on[7–10]. The specific changes of microbiome composition have been developed as non-invasive diagnostic tools for different diseases[11, 12]. Despite the spatial discrepancy between gut and kidney, there seems to be an “invisible” bind which is called gut-renal axis[13]. Previous researches compared constitutional differences of gut microbiome in

MN with other primary or secondary kidney diseases[14, 15]. However, characteristic changes of gut microbe in each MN stage and their cause-and-effect relationships remain unknown.

The aim of our study is to explore the key bacterial groups in MN and try to establish a model as a tool for MN diagnosis. We also attempt to explain the causal relationship between pathogenesis of MN and changes of gut microbiome, which may hopefully provide a new target for the prevention and treatment of MN.

2. Material And Methods

2.1 Study cohort

From April 2019 to January 2020, a total of 966 fecal samples from MN patients and healthy controls were collected from three medical centers in Central China (The First Affiliated Hospital of Zhengzhou University), East China (Shandong Provincial Hospital) and South China (The Third Affiliated Hospital of Sun Yat-sen University). All of the MN cases was diagnosed pathologically. The exclusion criteria were as following: (1) application of antibiotics within 3 months before enrollment, (2) obvious infection, such as oral, respiratory, digestive tract, (3) co-morbidity of other diseases such as tumor, digestive system disease, lupus, diabetes, (4) complete remission of MN (stable renal function and urine protein < 0.3g/24h), (5) repeated or incomplete information. Finally, 825 fecal samples were included for further research including 370 samples of MN patients from the First Affiliated Hospital of Zhengzhou University (Stored in the Biobank of The First Affiliated Hospital of Zhengzhou University and National Human Genetic Resources Sharing Service Platform, Grant No. 2005DKA21300), 65 samples of MN patients from Shandong provincial hospital, 44 samples of untreated MN patients from the Third Affiliated Hospital of Sun Yat-sen University and 346 samples of healthy controls from physical examination center of the First Affiliated Hospital of Zhengzhou University. None of the healthy controls had taken antibiotics or probiotics/prebiotics for at least 3 months prior to sample collection.

Among 370 MN patients from Central China, 115 of them were first onset without medication before sample collection. They were defined as untreated MN (UMN). Other 255 patients, who had taken immunosuppressants or corticosteroids were defined as treated MN (TMN). TMNs were further divided into HPRO group (urinary protein $\geq 3.5\text{g}/24\text{h}$, $n=84$) and LPRO (urinary protein $< 3.5\text{g}/24\text{h}$, $n=171$).

2.2 Stool sample collection and DNA extraction

All stool samples collected from MN patients and healthy volunteers were immediately stored at 4°C temporarily. Then they were transferred to -80°C environment within 2 hours for further analysis. DNA extraction was completed by using E.Z.N.A.[®] Stool DNA Ki. All procedures were completed by Shanghai Mobio Biomedical Technology Co. Ltd. using the Miseq platform (Illumina Inc., USA) according to manufacturer's protocols. Detailed DNA extraction steps was shown in supplementary document 1.

2.3 OTU clustering, comparison of gut microbiome and diagnostic model construction

We classified Operational Taxonomic Unit (OTUs) based on 97% similarity and used RDP Classifier (<http://rdp.cme.msu.edu/>) against the Silva (SSU123)16S rRNA database with confidence threshold of 70% to analyze the phylogenetic affiliation of 16S rRNA gene sequence. We compared gut microbiome composition and functional alterations between different groups. Detailed statistical analyses and tests were shown in supplementary document 2.

The participants of UMNs and matched HCs were randomly divided into training group and test group. Based on the abundance profiles of OTU, we used five-fold cross-validation on random forest model to feature important OTUs (importance value>0.001) and calculated the possibility of disease (POD) values of each individual. Subsequently, we drew a receiver operating characteristic curve (ROC) and used the area under curve (AUC) to evaluate the diagnosis efficacy of gut microbiome in UMNs and HCs. AUC values were generated in R.4.0 (<http://www.R-project.org/>).

2.4 Verification of the efficiency and the application range of the diagnostic model

After the diagnostic model was established, the test group was used as internal validation cohort to verify the efficiency of the model. First onset MN patients from South China and matched HCs were used as cross regional validation cohort to evaluate the model. The diagnostic efficiency of the model was also validated in 255 TMN patients and 205 matched HCs. Sixty-five MN patients from Shandong and matched HCs were also included into the diagnostic model for cross regional validation. At last, 84 HPRO and 171 LPRO patients along with their HC groups were respectively included into the model to explore the influence of disease status on the diagnostic model.

2.5 Establishment of rat model

A total of 48 male Sprague-Dawley (SD) rats weighted 80-100g were obtained from Spaefer Biotechnology Co., Ltd. Fecal microbiome transplantation (FMT) was used on rats to explore the relationship between gut microbiome and MN. The preparation of fecal bacterial solution was shown in supplementary document 3.

The rats were randomly divided into six groups and there were 8 rats in each group: (a. Normal) normal rat group, (b. Nor+Con) normal rat, FMT from healthy human, (c. Nor+MN) normal rat, FMT from MN patients, (d. Model) MN model group, (e. Mod+Con) MN rat, FMT from healthy human, (f. Mod+MN) MN rat, FMT from MN patients. Sheep Anti-Rat Fx1A Serum (PTX-002S) was used for induction of Passive Heymann Nephritis (PHN). Modeling process and sample collection of MN rats was shown in supplementary document 4.

2.6 Clinical features, pathological changes and gut microbiome alterations in rat model

Total urinary protein/ urinary creatinine (T/Cr) was used to estimate the severity of MN. Under light microscope, hematoxylin-eosin (HE) staining, periodic Acid-Schiff (PAS) and Masson staining were used to observe glomerular histomorphology. Detailed staining process was shown in supplementary document 5. We used transparent electron microscope (Japan Electronics Co., Ltd) to observe the area of

glomerular basement membrane. The fecal samples collected from rat models underwent 16S rRNA sequencing to explore the alterations of gut microbiome in different groups and different time points.

3. Results

A total of 825 fecal samples were finally included for 16S rRNA analysis. The study design and process were shown in Figure 1.

3.1 Gut microbiome profiles changed dramatically in untreated MN

We compared the composition and alteration of gut microbiome in 115 UMNs and 115 age-and-gender matched HCs. The baseline characteristics were shown in Table S1. Results showed that both α -diversity and β -diversity of microbiome in UMNs had a significant decrease compared with HCs (Figure 2A, B, Figure S1A-B, Table S2). The composition also changed obviously in UMNs (Figure S1 C). The components of PC1 and PC2 account for 79.56% proportion of total variance in two dimensions by using PCoA, suggesting enormous difference of two groups (Figure 2B and Figure S1D, E). Observed OTUs in UMNs was markedly decreased as shown by Venn diagram, rarefaction and rank abundance distribution curves (Figure S1 F-H).

At phylum level, there was a certain increase of the relative abundance of *Proteobacteria* and *Actinobacteria*, while the average abundance of *Bacteroidetes* decreased a lot in UMNs (Figure 2C, Figure S2 A-C and Table S3, S4). In terms of genus level, *Bacteroides*, *Prevotella* and *Lachnospiraceae_unclassified* decreased significantly in UMNs. However, the relative abundance of *Escherichia-Shigella*, *Subdoligranulum*, *Bifidobacterium* and *Enterobacteriaceae_unclassified* increased the most evidently in UMNs versus matched HCs (Figure 2D, Figure S2 D and Table S5, S6). The relative abundance and distribution of the key OTUs in each sample was shown in the way of heatmap (Figure 2E, Table S7). The spearman correlation analysis showed the correlation between laboratory examination and key OTUs in Figure 2F, G. The alteration of metabolic pathway of gut microbiome was also found changed in UMNs (Figure S2 E, F), including increase of osmoprotectant transport system. These results indicated that the gut microbiome composition and function had specific alteration in UMN patients.

3.2 Establishment and validation of the diagnostic model.

To explore whether the gut flora could be used to identify MN patients, 115 UMN patients and 115 matched healthy controls were randomly divided into training group (UMN=71, HC=71) and test group (UMN=44, HC=44). By means of five-fold cross-validation on random forest model, we obtained 7 crucial OTUs including OTU27 (*Lachnospira*), OTU113 (*Lachnospira*) OTU232 (*Lachnospiraceae_unclassified*) OTU559 (*Agathobacter*) OTU619 (*Roseburia*) OTU667 (*Faecalibacterium*) and OTU722 (*Lachnospiraceae_unclassified*) to establish the diagnostic model (Figure 3A, B). The POD and the ROC showed the model had a high efficiency with an AUC of 98.36% (cut-off value: 0.4988, sensitivity: 0.9306, specificity: 0.9306) in training group (Figure 3D). The test group also showed high AUC of 92.02% (cut-off value: 0.468, sensitivity: 0.9535, specificity: 0.814) (Figure 3E, F). In cross regional validation phase,

composed by 44 untreated MN from South China and 44 age and gender matched HCs, the model showed good discrimination efficiency as well, with an AUC of 95.31% (cut-off value: 0.523, sensitivity: 0.9091, specificity: 0.8958, Figure 3G H).

Previous studies reported medication could influence gut microbiome. So, we tested whether the model could be used in TMNs. Results showed POD values were markedly higher in TMNs than HCs, with an AUC of 96.44% (cut-off value: 0.6315, sensitivity: 0.9373, specificity: 0.9122, Figure 3I, J). Regional cohort of 65 MN patients (treated or untreated) from East China and matched 57 HCs showed AUC was as high as 90.24% (cut-off value: 0.4465, sensitivity: 0.9231, specificity: 0.7544, Figure 3K L). These results suggested medication did not influence the model efficiency. It could be widely used in MN patients, whether applied medication or not.

To further test its efficiency in different disease states, we divided TMN into high protein group (HPRO, n=84) and low protein group (LPRO, n=171). With equal number of age-and-gender matched HCs respectively, these two cohorts were recruited into the diagnostic model. Results showed AUCs were 96.02% (cut-off value: 0.6195, sensitivity: 0.9643, specificity: 0.881) in HPRO group (Figure 3M, N) and 96.5% (cut-off value: 0.615, sensitivity: 0.9357, specificity: 0.9181) in LPRO group (Figure 3O, P), indicating the model was also suitable across MN states.

3.3 Medication of MN did not influence gut microbiome

To further explore the influence of medication on gut microbiome, we compared them among 78 UMNs, 108 TMNs and 100 HCs based on the matched age and gender. Urine protein, serum creatinine, albumin, triglyceride and total cholesterol were also matched in these UMNs and TMNs. Clinical data of three groups was shown in Table S8. Observed OTUs were decreased in either UMNs or TMNs compared with HCs (Figure 4A). PCoA showed the gut microbiome composition was similar between UMNs and TMNs, while quite different from HCs (Figure 4B, Figure S3A, B). Similar results were obtained by Plsda and ANOSIM analyses (Figure 4C, D, Table S9).

The analysis of relative abundance also suggested similar changes of gut microbiome in UMNs and TMNs compared with HCs. Microbial composition at phylum level showed *Proteobacteria* increased mostly while *Bacteroidota* decreased significantly either in UMNs or TMNs compared with HCs (Figure 4E, F, G, Figure S3B, Table S10). Similar pattern was obtained at genus level (Figure S3C and Table S11), suggesting synchronized microbial changes in UMNs and TMNs. The Venn diagram and heatmap indicated UMNs and TMNs were more alike while quite different from HCs (Figure S3D, Figure 4H and Table S12). KEGG pathways also showed great differences of UMNs or TMNs when compared with HCs (Figure S3E, F). The specific of functional pathway changed a lot, including increase of human diseases in both UMNs and TMNs. And it might be related to the pathogenesis of MN. These findings were quite different from our previous expectation, since the microbiome seems not to be affected much by medication.

3.4 The changes of gut microbiome remained stable across different disease states of MN.

Composition of gut microbiome between HPROs and LPROs were compared (clinical data was shown in Table S13). Interestingly, observed OTUs and α -diversity were similar in HPROs and LPROs and it was not affected by increasing of sample size as shown in rarefaction curves (Figure 5A). ANOSIM analysis suggested there was no statistical difference between HPROs and LPROs (Figure 5B and Table S14). PCoA analysis also suggested the gut microbiome of HPROs resembled with that in LPROs (Figure 5C, Figure S4A, B, C). Venn diagram showed 797 out of 897 OTUs were shared between both groups (Figure S4 D). The similarity of OTU composition was further shown in way of heatmap (Figure 5E and Table S15). It was hard to found the differences in the relative abundances of microbial composition in neither phylum nor genus level (Figure S4 E, F and Table S16-S19).

We further divided these individuals into training group (HPRO=56, LPRO=114) and test group (HPRO=28 LPRO=57). With five-fold cross-validation on random forest model, 8 key OTUs were obtained for the new model establishment including OTU48 (*[Ruminococcus]_torques_group*), OTU90 (*Ruminococcaceae_Incertae_Sedis*), OTU144 (*Butyricicoccus*), OTU178 (*Saccharimonadaceae*), OTU251 (*Parabacteroides*), OTU292 (*Lachnospiraceae_UCG-010*), OTU301 (*Peptococcus*) and OTU843 (*Oscillospiraceae_uncultured*) (Table S20). Nevertheless, the model did not suggest good discriminative ability (Figure S4G-J). These results implied that the changes of gut microbiome might not be associated with the disease states of MN.

3.5 Successful construction of MN rat model depends largely on gut microbiome

Patient samples indicated relationship between gut microbiome and MN onset. To further explore the role of gut microbiome in the pathogenesis of MN, we established MN rat model. The grouping and process of model construction was shown in figure 6A. The dynamic changes of average urine protein in each group were shown by means of point-fold line chart in Figure 6B. Scatter plots showed that at baseline, each group had no statistical differences in urinary protein (Figure 6C). What interested us was that the use of antibiotics significantly prohibited fully presentation of MN phenotype (Figure 6D, Group Mod+Con, Mod+MN verse Group Model). What's more, even in control rats, the use of antibiotics also significantly lowered the urinary protein levels (Figure 6D, Group Nor+Con, Nor+MN verse Group Normal). This effect quickly disappeared when antibiotics discontinued (FMT for 1 week, Figure 6B E, Group d-f). Compared with Model group, FMT groups (group e, f), both from healthy people and MN patients, showed delayed and reduced presentation of phenotype (Figure 6B). Contrast to expectations, FMT using MN feces hardly had any effect in aggravating urinary protein excretion until sacrifice. On the contrary, healthy feces could partially replicate the MN model (Figure 6B, E-G). This phenomenon indicates that naturally existed microbiome is essential for the pathogenesis of MN. Lack of natural microbiome prevents MN development, while restoring it, but not MN microbiome, could at least partially reconstruct MN phenotype.

In terms of histological changes, HE, PAS and Masson staining showed significant glomerular damages in Model rats at the end of the study. Consistent with urinary protein, FMT using MN feces showed more mild glomerular damages than that using healthy feces (Figure 6H). Electronic microscope showed more

details at the basement membrane which further verified what we got using light microscope (Figure 6I). Histological results confirm that the naturally existed gut microbiome plays as a prerequisite condition in MN pathogenesis, while changed gut microbiome in MN patients is more likely to play a protective role.

3.6 Analysis of rat fecal microbiome confirmed effective elimination and transplantation of microbe

The fecal samples of rats at different time points were collected and underwent 16S rRNA sequencing. Since the excretion of urinary protein after model establishment was largest at 1 week, we compared gut microbiome among these three points, which were defined as Nor (fecal collection at 0w in Normal group), ME (fecal collection at 0w when Model was Established in Model group) and AM (fecal sample collection at 1w After Model was established in Model group). Gut microbiome of MN model altered from control rats at the point of 0w. These changes got more significant at 1w, even when the urinary protein excretion began to relieve at this point (Figure 7A, Figure S5A and Table S21-S24), indicating a delay in microbiome adaptation. The composition of gut microbiome differed from each other analyzed by Plsda among three groups of fecal samples. And heatmap showed the key OTU composition also changed obviously when compared with Nor in both ME or AM group (Figure 7B, Figure S5B, Table S25, S26). Observed OTUs of microbiome were markedly decreased after using antibiotics (AB, fecal collection at 0w in group e), as shown by cloudplot, PCoA and ANOSIM analyses (Figure 7C, D E, Figure S5C-E and Table S27), indicating success in intestine cleaning. Composition of FMT groups (group Mod+Con and group Mod+MN) differed from each other (Figure 7F, G). Each of them differed greatly from control or Model group as shown by Plsda (Figure 7H and Table S28), suggesting successful transplantation of different feces. Differences in composition of gut microbiome between these four groups were also shown in heatmap and PCoA diagrams (Figure S5E, F Table S29). Analysis of rat feces confirms gut microbiome was successfully eliminated and transplanted in different groups. Together with physiological and pathological results, it demonstrates natural gut microbiome is the crucial factor responsible for MN development.

Discussion

In this multi-center study, a total of 825 fecal samples were included. Results indicated significant dysbiosis of MN patients. The diagnostic model based on key OTUs demonstrated excellent identification capability and was validated by multi-center external cohort. The existence of natural gut microbiome played a very important role in promoting disease onset, which was proved by rat model.

The composition of gut microbiome changed quickly from the point of MN onset. However, the typical change maintains stable even after patients got treated or as disease alleviated. It is different from previous results. Ren et al. found gut microbiome changed gradually along with the development of chronic kidney disease[13]. In children with acute lymphoblastic leukemia, patients with schizophrenia and mice with rheumatoid arthritis, application of drugs altered the gut microbial composition[16–18]. To confirm our assumption, we analyzed data using different method. However, the pattern of microbiome changed little across different status of MN, or whether drugs were applied or not. It seems as gut

microbiome can promptly sense the MN onset and adapt itself throughout the whole course. Another possible reason is the composition of microbiome changed so dramatically compared with healthy control that changes between different stages was smoothed out.

Gut microbiome is suggested to have relations with more and more diseases recent years and even can be used as non-invasive tools for many diseases such as COVID-19[11, 12, 19]. In our study, diagnostic model was successfully constructed using 7 crucial OTUs by means of five-fold cross-validation on random forest model. The discrimination model showed good ability of identification in both training and test groups. And the subsequent validations in East China, South China, high urinary protein and low urinary protein cohorts also reached more than 90% of the diagnostic efficiency. The multi-center and grouping verification of patients with different disease states suggested good discrimination capability to all MN patients. It might provide a new non-invasive tool for MN diagnosis. In another way, high diagnostic efficacy of the model in different cohorts also indicated that the typical microbiome change was hardly affected by drug treatment or disease states.

Whether the key OTUs are related with the pathogenesis of MN is not known now. But the metabolism of these microbe may somewhat affect the physiology of the host. According to previous reports, *Lachnospiraceae* was thought to be related to metabolic disturbance, including glucose and/or lipid metabolism[20, 21]. *Agathobacter* are found as a novel species of a new genus in the family of *Lachnospiraceae*, and the main products of this genus are butyrate, acetate, hydrogen, and lactate[22]. *Roseburia* and species of *Faecalibacterium* can ferment various sugars into short-chain fatty acids (SCFA)[23–25]. These SCFAs have great benefits to human health while abnormal state of SCFAs might lead to diseases[26–28]. Disorder of gut microbiome, particularly increase of pathogenic bacteria and the decrease of SCFA producing bacteria, may be the important factors to cause or aggravate primary MN. But the detailed effect of these metabolism on MN still needs focused on.

The MN rat model was established to explore causal relationship between pathogenesis of MN and alteration of gut microbiome. The formation rate of rat model was decreased after intestinal clearance. The reason might be related with decreased levels of proinflammatory cytokines and fibrosis markers when gut microbiota was depleted [29]. In accordance, after FMT with fecal samples from HCs or MNs, the urinary protein level had an obvious increase in gut purge MN rat models. These results implied that gut microbiome was the necessary condition for MN. Interestingly, after FMT for one week, rat models transplanted control microbe had a much severe disease state than Model group, suggesting normal gut microbiome, instead of disordered gut microbiome, might be the reason for MN. We surmised that the gut flora of MN patients would have adaptive changes due to the disease onset, helping the host preventing disease progression. It could help to explain why the urinary protein level of Mod + MN group was the lowest among three MN rat groups. Another interesting phenomenon was the unparalleled clinical manifestation and gut dysbiosis. Although Model group had much severe clinical symptoms at 0 week, the microbial differences were more significant after FMT for 1 week. Similar phenomenon was also observed in hepatitis B virus infected mice[30]. This might due to the relatively delayed changes of the

gut microbiome compared with alterations of clinical symptoms. Also, once again, it gave credence to the conjecture that the gut flora was an adaptive change of MN.

There are several innovations and advantages in our study. First of all, the sample size was large. Secondly, the diagnostic model based on gut microbiome provides non-invasive and broadly applied tool for clinical diagnosis of MN. Most importantly, we proved natural existed gut microbiome is essential for MN pathogenesis, which might be developed as a novel therapy in the future.

Our study also has some limitations. The molecular mechanism of how gut microbiome affect MN pathogenesis remains unknown. The difference of gut microbiome between species is another point that should not be neglected. Therefore, results of rat model need to be further validated clinically.

Conclusion

Gut microbiome of MN patients varies greatly from healthy ones. The typical changes could be used as a non-invasive tool for MN diagnosis. What's more, the onset of MN depends on the existence of natural gut microbiome and the subsequent changes of gut microbiome in MN patients seems to play as a protective factor, which needs to be focused on in the future.

Abbreviations

MN: Membranous nephropathy; HC: healthy controls; OUT: operational taxonomic units; FMT: fecal microbiome transplant; PLA2R: M-type phospholipase A2 receptor; THSD7A: Thrombospondin type 1 domain-containing 7A; UMN: untreated membranous nephropathy; TMN: treated membranous nephropathy; HPRO: treated membranous nephropathy with high urinary protein level; LPRO: treated membranous nephropathy with low urinary protein level; POD: possibility of disease; ROC: receiver operating characteristic curve; AUC: area under curve; Nor: normal group; Mod: model group; PHN: Passive Heymann Nephritis; T/Cr: Total urinary protein/ urinary creatinine; HE: hematoxylin-eosin; PAS: periodic Acid-Schiff; COVID-19: Coronavirus Disease 2019; SCFA: short-chain fatty acids; PCoA: principal coordinate analysis; CV: coefficient of variation; ANOSIM: analysis of similarities; Plsda: Partial least squares discrimination analysis. ME: model establishment; AM: after model establishment for 1 week; PICRUST: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; KEGG: Kyoto Encyclopedia of Genes and Genomes; KO: KEGG orthologous groups. LDA: Linear Discriminant Analysis.

Declarations

Acknowledgment

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Authors' contributions

Jin Shang, Zhigang Ren, Zhanzheng Zhao and Jing Xiao designed the research and experiments. Yiding Zhang and Ruixue Guo wrote the manuscript. Wenli Liu and Jun Zhang collected the samples. Ge Yan, Feng Wu, Wen Cui, Peipei Wang, Xuejun Zheng and Ting Wang finished the experiments. Suying Ding, Xinxin Gao, Yijun Dong, Jing Zhao, Li Wang, Hongyan Ren and Chao Liu participated in data statistical analysis. All authors participated in the revision of the manuscript.

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Availability of data and materials

Raw Illumina read data for all samples were deposited in the European Bioinformatics Institute European Nucleotide Archive database (PRJNA752445).

Ethics approval and consent to participate

The First Affiliated Hospital of Zhengzhou University Ethics Review Committee granted ethical approval for the study and the ethics review approval ID was "2019-KY-361". The ethics review approval ID in Shandong Provincial Hospital was "SWYX: NO. 2020-077". All the participants included in this project provided written informed consents. The ethics review approval ID of animal experiments was "ZZU-LAC20210115[02]".

Consent for publication

Not applicable.

Competing interests

All author declared no conflict of interest.

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Figures

Figure 1

Study design and flow diagram: the profile of inclusion exclusion and grouping of patients. Follow rigorous inclusion and exclusion criteria, a total of 825 fecal samples were collected including 370

samples of MN patients from Central China, 65 samples of MN patients from East China, 44 samples of MN patients from South China and 346 samples of HCs from Central China. Gut microbiome was compared. Then 115 UMNs and matched 115 HCs were randomly divided into training group and test group to develop and validate diagnostic model based on key OTUs. Samples from different areas of China were included as cross regional cohort. Patients were divided further into different subgroups to test the influence of drugs and disease severity on intestinal flora. MN, Membranous nephropathy; UMN, untreated membranous nephropathy; TMN treated membranous nephropathy; HC healthy control; HPRO, high urine protein; LPRO, low urine protein.

Figure 2

The composition and alteration of gut microbiota in UMN and HCs. (A) The relationship between number of observed OTUs and numbers of samples. Cloudplot showed α -diversity in UMN group and HC group by ace index (B) PCoA analysis showed visualized β -diversity by unweighted UniFrac algorithm along PC1 and PC2. (C) Wilcoxon rank sum test showed the distribution of gut microbiome in UMN and HCs at phylum level. (D) Average compositions and relative abundances of the bacterial communities in

UMNs and HCs at genus level. (E) Heatmap showed the similarity and difference of key OTUs selected by random forest model in each participant of UMNs and HCs. (F) Spearman correlation analysis showed the correlation between different OTUs and clinical indicators in heatmap (all $p < 0.001$). (G) Spearman correlation analysis was used to analyze the degree of correlation between key OTUs and clinical indicators (Solid line, $p < 0.01$, dotted line, $0.01 < p \leq 0.05$). OTU, operational taxonomic units; PCoA, principal coordinate analysis; POD, possibility of disease; ROC, receiving operational curve. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Figure 3

Development and validation of diagnostic models. (A) Mean decrease accuracy and mean decrease gini showed the contribution values of 7 selected OTUs in the diagnostic model. (B) Random forest model showed the optimal number of vars is 7. (C) Diagrams of POD value based on microbial markers showed significant difference between UMN and HCs in training group. (D) ROC curve based on obtained microbial markers showed discrimination rate in training group. (E) Diagrams of POD value based on microbial markers in test group. (F) ROC curve showed discrimination rate in test group. (G) Diagrams of POD value of the validation cohort from South China. (H) ROC curve of the validation cohort from South China. (I) Diagrams of POD value in TMNs and HCs. (J) ROC curve showed discrimination rate in TMNs and HCs. (K) Diagrams of POD value in the validation cohort from East China. (L) ROC curve showed discrimination rate in the validation cohort from East China. (M) Diagrams of POD value in HPROs and HCs. (N) ROC curve showed discrimination rate in HPROs and HCs. (O) Diagrams of POD value showing difference between LPROs and HCs. (P) ROC curve showing discrimination rate in LPROs and HCs. POD, possibility of disease; CV, coefficient of variation; ROC, receiving operational curve.

Figure 4

The composition and alteration of gut microbiota in 78 UMNs, 108 TMNs and 100 HCs. (A) Rarefaction curve showed the relationship between number of observed OTUs and numbers of samples among three groups. (B) PCoA analysis showed visualized β -diversity of TMNs, UMNs and HCs by unweighted UniFrac algorithm along PC1 and PC2. (C) Plsda axis showed the different composition among HCs, UMNs and TMNs. (D) ANOSIM analysis among three groups. (E) Average compositions and relative abundances of

the bacterial communities among three groups at phylum level. (F) The relative abundance of Proteobacteria increased while Bacteroidota decreased significantly both in UMN and TMNs compared with HCs. (G) Average compositions and relative abundances of the bacterial communities of each sample in three groups at phylum level. (H) Heatmap showed relative abundance of key OTUs selected by random forest model were more similar between UMN and TMNs than HCs. ANOSIM, analysis of similarities; Plsda, Partial least squares discrimination analysis.

Figure 5

Comparison of gut microbiome between HPROs and LPROs. (A) Rarefaction curve showed the relationship between number of observed OTUs and numbers of samples between two groups. The cloudpolt showing α -diversity calculated by ace indexed was similar between two groups. (B) ANOSIM showed there was no difference between groups or within groups in HPROs and LPROs. (C) PCoA analysis showed visualized β -diversity of HPRO group and HC group by unweighted UniFrac algorithm (D) Heatmap showed the similarity and difference of top 50 most abundant OTUs in each participant of HPROs and LPROs.

Figure 6

Clinical features and pathological changes in MN rat model. (A) Flow chart of rat experiment. (B) Line chart showed the alteration of T/Cr in 6 groups from baseline to 3 weeks after FMT. (C) Scatter plot of rat urine protein at baseline. (D) Scatter plot of rat urine protein after intestine cleaning. (E) Scatter plot of rat urinary protein after 1 week of FMT. (F) Scatter plot of rat urinary protein after 2 weeks of FMT. (G) Scatter plot of rat urinary protein after 3 weeks of FMT. (H) HE, PAS and Masson staining of glomerulus

in different groups. (I) Electron microscopy showed detailed changes in glomerular basement membrane area in each group. T/Cr, Total urinary protein/ urinary creatinine; HE, hematoxylin-eosin staining; PAS, periodic acid-schiff staining. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, No statistical difference.

Figure 7

The rat fecal microbiome analysis showed effective elimination and transplantation of microbe. (A) Diagram of average compositions and relative abundances of the bacterial communities at phylum level

showed the changes were more obvious at AM than ME. (B) Plsda axis showed the different composition at different time points in MN group. (C) The cloudplot showed observed OTUs were markedly decreased after using antibiotics. (D) PCoA diagram showed obvious change in gut microbial composition after intestines cleaning. (E) Diagram of ANOSIM showed significant difference after using antibiotics. (F) Diagram of ANOSIM showed microbial composition is different after FMT using feces of Cons or MNs for 1 week. (G) Diagram of PCoA showed composition of microbe was significantly different after FMT with feces from Cons or MNs for 1 week. (H) Plsda axis showed different microbial composition in different groups. ME, model establishment; AM, after model establishment for 1 week; FC, FMT with feces from Con in Mod+Con group; FM FMT with feces from MN in Mod+MN group.

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