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The microbiome of interstitial cystitis revealed by 2bRAD-M

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Abstract

Introduction: Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic, female-prone, multifactorial bladder disorder characterized by symptoms such as suprapubic pain, urinary urgency, frequency, and pelvic discomfort, often exacerbated by bladder filling and associated with urothelial dysfunction and inflammation, while the etiology of IC/BPS remains largely unknown. This study was conducted by performing 2bRAD sequencing for Microbiome (2bRAD-M) on bladder tissue samples to investigate the microbial community distribution characteristics of bladder tissues in patients with IC/BPS.

Materials and methods: The study recruited a total of 11 patients with IC/BPS. The lesion and surrounding normal tissue were collected from each patient. DNA was extracted, and the genomes of various microorganisms in the tissue were digested using IIB restriction endonucleases. The unique tags were subjected to microbial qualitative and relative quantitative analysis.

Results: A total of 118 bacteria and 2 fungi were detected in 22 samples from 11 patients diagnosed with IC/BPS. Microbial diversity was similar in bladder lesion tissue and surrounding normal tissue. The microbial composition of the bladder tissues was similar, with 3 microorganisms, namely Mycobacterium_tuberculosis, Ralstonia_sp000620465 and Klebsiella_pneumoniae, detected in all tissues. At the species level, Escherichia_coli, Bacillus_A_bombysepticus and Chlamydophila_abortus were the dominant species. 2 microorganisms (Sphingopyxis and Rhizobiaceae) were enriched in the lesion tissue, while Acetobacteraceae and Porphyromonas were enriched in the normal tissue from patients with IC/BPS.

Conclusion: This study preliminarily depicted the microbial panorama of bladder tissues from IC/BPS patients using the 2bRAD-M technique, revealing the distribution of microorganisms associated with the disease and possible differences, providing new perspectives and research directions for subsequent studies on the pathogenesis and progression of IC/BPS.

Keywords: Interstitial cystitis/bladder pain syndrome; microbiome; 2bRAD-M

1 Introduction

Interstitial cystitis (IC) / bladder pain syndrome (BPS), is a chronic condition characterized by a constellation of symptoms primarily involving the bladder and pelvic region. IC/BPS can occur in any age group and is most common in middle-aged women, with a mean age of onset of 45 years, and is very rare in children. The prevalence in women is 9:1 compared to men[1]. Common symptoms include chronic pelvic pain, pressure, or discomfort perceived to be related to the urinary bladder, accompanied by urinary urgency, frequency, and nocturia (nighttime urination), and excludes a definite infection or other distinctive pathologic features[2]. IC/BPS with long-term chronic pain has a serious impact on the quality of life of patients. Patients suffer from reduced sleep quality, avoidance of sex due to chronic moderate-to-severe pain, and a direct impact on the ability to carry out daily activities[3].Currently, the incidence of IC/BPS is increasing year by year, its etiology and pathogenesis are unclear, and patients need to undergo multiple cystoscopies and bladder tissue biopsies in clinical diagnosis, which is extremely painful for patients[4-5].

The pathogenesis of IC/BPS is currently inconclusive. Among them, various theories have been proposed, such as chronic infection, autoimmune factors, defective aminoglucan layer, genetic factors, chronic toxic effects of urine, and autonomic dysfunction[6]. However, none of these theories has fully elucidated the pathogenesis of IC/BPS. Studies have found that bladder biopsies from IC/BPS patients often have characteristic inflammatory cell infiltration; inflammation-related factors (e.g., TNF-α, IL-1β, etc.) are significantly increased in urine specimens compared with normal urine; and clinical discomfort is significantly reduced in patients with IC/BPS after receiving tetracycline antibiotic (doxycycline) treatment[5, 7-8]. The above features suggest that IC/BPS has many characteristics of pathogenic microbial infections, suggesting that there may be unidentified pathogenic microorganisms.

2bRAD-M is a microbial diversity analysis technique developed based on the 2b-RAD technology[9], which is used for the qualitative and relative quantitative analysis of microorganisms by using unique tags obtained by enzymatic cleavage of microbial genomes by type IIB restriction endonucleases. The 2bRAD-M technology has the following advantages over 16S/18S/ITS amplicon sequencing and macrogenome sequencing (Table1): Compared to 16S/18S/ITS amplicon sequencing, which generally provides classification only at the genus level, 2bRAD-M can provide precise determination at the species level. Compared to macrogenome sequencing, which is costly and requires large amounts of detectable DNA, 2bRAD-M is low-cost and has high sensitivity for trace samples[10-11]. 2bRAD-M makes it possible to accurately measure the urinary microbiota at low cost.

Table1. Comparison of 2bRAD-M Technology with Amplicon Sequencing Technology and Metagenomic Sequencing Technology

Technology	Resolution Level	Cost	Fungal Identification	Trace Sample Sensitivity
Macrogenome Sequencing	Species Level	High	Yes	Low
16S/18S/ITS Amplicon Sequencing	Genus Level	Low	Depends on Target Type	High
2bRAD-M Sequencing	Species Level	Low	Yes	High

In this study, we will examine bladder lesion and normal tissues from 11 pairs of patients with IC using the 2bRAD-M technique to investigate whether there are differences in microbiota between them, excluding urinary

tract infections that are undetectable by routine clinical cultures. It aims to characterize the microbiota and the pathogenic potential of IC/BPS, with a view to enriching the study of the etiology of the disease.

2 Methods

2.1 Tissue sample acquisition

We first recruited IC/BPS patients who were admitted to Peking University First Hospital (Beijing, China). All enrolled patients were clinically diagnosed with interstitial cystitis/bladder pain syndrome (IC/BPS) according to the American Urological Association (AUA) and European Association of Urology (EAU) guidelines, presenting with chronic pelvic pain, symptoms of urinary urgency and frequency, and without urinary tract infection or other recognizable pathologic changes. The study cohort specifically referred to patients with a non-Hunner lesion phenotype (i.e., BPS without Hunner lesions) characterized by diffuse mucosal erythema, petechiae, or nodular lesions. Patients with typical Hunner lesions were excluded by cystoscopy. To minimize the bias caused by other factors affecting the microbiota of bladder tissue, we excluded these patients with urinary tract infections or who have received antibiotics or undergone catheterization within four weeks. All patients were hydrodilated and biopsied by the same treatment team. Under cystoscopic guidance, the lesion site was defined as the area showing the most prominent mucosal erythema with punctate hemorrhage. Paired “normal site” tissues are taken from areas of the bladder mucosa of the same patient that are normal to the naked eye, characterized by the absence of erythema of the mucosa and the absence of visible punctate or patchy hemorrhage. To ensure spatial separation from the diseased area, the normal tissue was sampled at least 1 cm from the border of any visually abnormal area (e.g., erythema, hemorrhage). The lesion and surrounding normal tissues excised during surgery were snap-frozen in liquid nitrogen and stored at -80℃ for subsequent microbiological component analysis.

The research had been performed in accordance with the Declaration of Helsinki. The experiments were approved by the Ethics Committee of Peking University First Hospital (Peking University First Hospital Biomedical Research Ethics Committee), and informed consent was obtained. All methods were performed in accordance with the protocol approved by the Ethics Committee of Peking University First Hospital (Peking University First Hospital Biomedical Research Ethics Committee).

2.2 DNA extraction

The genomic DNA of the lesion and normal tissue from IC/BPS patients were extracted by TIANamp Micro DNA Kit (Tiangen).

2.3 Qualitative and relative quantitative microbial analysis by the 2bRAD-M technique

2.3.1 Library construction and sequencing

The 2bRAD-M library preparation basically followed the original protocol developed by Wang et al.[9] with minor modifications. DNA (1 pg-200 ng) was digested with 4 U of the enzyme BcgI (NEB) for 3 h at 37 ℃. Subsequently, the adaptors were ligated to the DNA fragments. The ligation reaction was performed by combining 5 µl of digested DNA with 10 µl of a ligation master mix containing 0.2 µM each of two adaptors and 800 U T4 DNA ligase (NEB). Ligation was carried out at 4 ℃ for 12 h. Then, ligation products were amplified, and PCR products were subjected to 8% polyacrylamide gel. Bands of approximately 100 bp were excised from the polyacrylamide gel, and the DNA was diffused from the gel in nuclease-free water for 12h at 4 ℃. Sample-specific barcodes were introduced by PCR with platform-specific barcode-bearing primers. Each 20 µl PCR contained 25 ng of gel-extracted PCR product, 0.2 µM of each primer, 0.3 mM dNTP, 1×Phusion HF buffer and 0.4 U Phusion high-fidelity DNA polymerase (NEB). PCR products were purified using QIAquick PCR purification kit (Qiagen) and then subjected to sequencing using the Illumina Nova PE150 platform. 2bRAD-M was carried out at the Qingdao OE Biotech Co.,Ltd. (Qingdao, China). The laboratory was

managed using strict zoning and negative controls were set up in the assay session to minimize possible confounding of results by contaminants.

2.3.2 Data analysis

2.3.2.1 Identification of species-species 2bRAD-M markers from the most comprehensive genome database

Firstly, totally 173,165 microbial genomes (including bacteria, fungi and archaea) were downloaded from NCBI RefSeq database. Then, built-in Perl scripts were used to sample restriction fragments from microbial genomes by each of 16 type 2B restriction enzymes, which formed a huge 2bRAD microbial genome database. The set of 2bRAD tags sampled from each genome was assigned under the GCF number, as well as GCF's taxonomic information corresponding to the whole genome.

Finally, all 2bRAD tags from each GCF that occur once within the genome were compared with those to all the others. Those 2bRAD tags are specific to a species-level taxon (having no overlap with other species) were developed as species-specific 2bRAD markers, collectively forming a 2bRAD marker database.

2.3.2.2 Calculation of relative abundance

Firstly, to identify microbial species within each sample, all sequenced 2bRAD tags after quality control were mapped (using a built-in Perl script) against the 2bRAD marker database which contains all 2bRAD tags theoretically unique to each of 26,163 microbial species in database. To control the false-positive in the species identification, G score was derived for each species identified within a sample as below, which is a harmonious mean of read coverage of 2bRAD markers belongs to a species and number of all possible 2bRAD markers of this species. The threshold of G score for a false positive discovery of microbial species was set 5[11].

$$\mathrm{G}\ score_{\ species\ i} = \sqrt{S_i \times t_i}$$

S: the number of reads assigned to all 2bRAD markers belonging to species i

within a sample.

t: number of all 2bRAD markers of species i that have been sequenced within a sample.

Then, calculated the average read coverage of all 2bRAD markers for each species, which represent the number of individuals belonging to a species present in a sample at a given sequencing depth. The relative abundance of a given species is then calculated as the ratio of the number of microbial individuals belonging to a species against the total number of individuals from known species that can be detected within a sample.

$$Relative\ abundance_{\ species\ i} = \frac{S_i/T_i}{\sum_{i=1}^{n} S_i/T_i}$$

S: the number of reads assigned to all 2bRAD markers of species i within a sample.

T: the number of all theoretical 2bRAD markers of species i.

2.4 Bioinformatic analysis

Chao1 (which estimates the actual number of species in a community by counting low abundance species), Shannon (which reflects species richness and evenness of distribution) and Simpson (which reflects the level of diversity in the community) indices were calculated using the 'vegan' software package, which in turn calculates alpha diversity. Besides, the 'vegan' package was used to calculate Bray-Curtis (characterizing differences in species abundance), binary Jaccard (comparing probabilities of similarity and dispersal in a sample set), and Euclidean distance (reflecting the actual distance between the abundance of two groups of species in a multidimensional space), and thus beta diversity. Linear discriminant analysis (LDA) effect sizea (LEfSe) was used to identify taxa that differed between groups, with an LDA score threshold of 4.0[12].

2.5 Statistical analysis

Statistical analysis of the data was performed using SPSS (version 26) and R software (version 4.1.1). Wilcoxon test was used for comparison between

groups for alpha diversity, and permutational multivariate analysis of variance (PERMANOVA) was used for comparison between groups for beta diversity. Kruskal Wallis analysis was used to compare the differences in microbial communities between the lesion and normal tissues of IC/BPS patients. Wilcoxon test was used to analyze the differences between groups for functional prediction. P- value of < 0.05 was considered to be statistically significant. The data repository website was https://dataview.ncbi.nlm.nih.gov/object/PRJNA1310667?reviewer=71l0ns2aldhgrcq2f0g6lgkve8 and the accession number was PRJNA1310667.

3 Results

3.1 Microbiota annotation and quantification of IC/BPS tissue samples

A total of 11 patients were enrolled in this study. The enrolled patients were diagnosed with IC/BPS and underwent hydrodilation and biopsy. These patients were all female, with a mean age of 62 years. The mean value of the Interstitial Cystitis Symptom Index (ICSI) is 17, and the mean value of the Interstitial Cystitis Problem Index (ICPI) is 14. All included patients had not received antibiotic therapy or catheterization within four weeks. The microbiomes of 22 samples originating from 11 patients were examined, annotated as well as summarized at various taxonomic levels such as phylum, order, family, and genus, and it was found that a total of 118 bacteria and 2 fungi were detected.

3.2 Community structure distribution

Community structure refers to ecology, in a community of biological environment with direct or indirect relations between all organisms, or the sum of organisms. The various groups in the microbial community interact with each other and can coexist in a regular manner, and they have their own distinct nutritional and metabolic types. As shown in the species distribution petal map, purple represents the number of microbial species detected in lesion tissue, green represents the number of species detected in

normal tissue, and 11 patients have 3 species in common in lesion and normal tissue, namely Mycobacterium_tuberculosis, Ralstonia_sp000620465 and Klebsiella_pneumoniae (Figure 1).

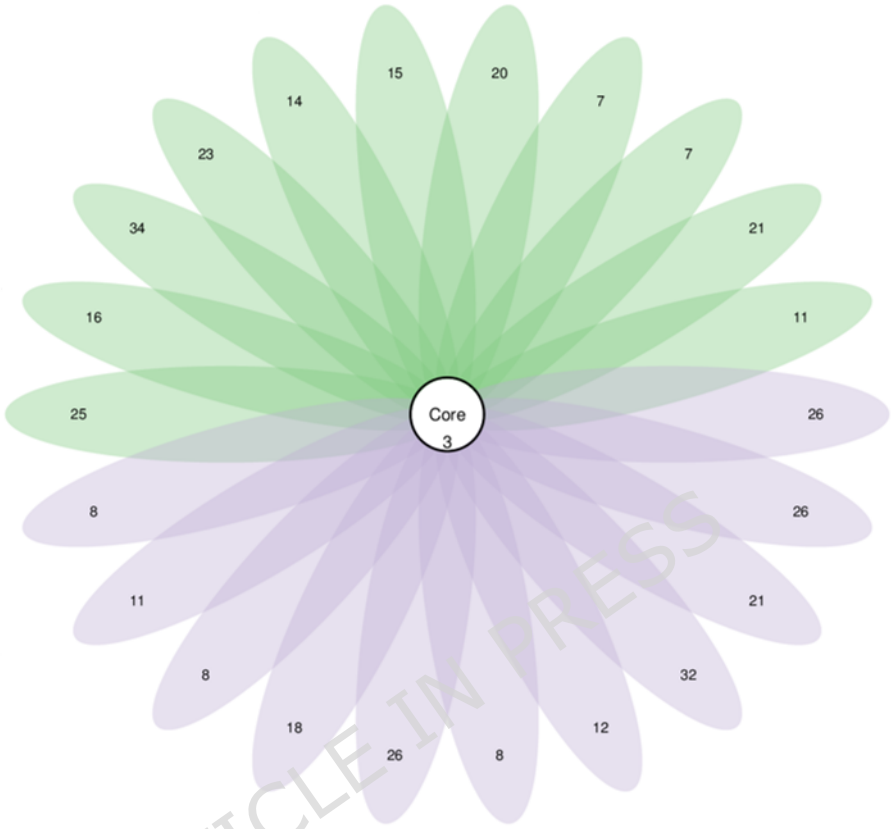

Figure 1 The species distribution petal plot of 22 bladder tissue samples from 11 patients with IC/BPS: three species that are common to all tissues (purple represents lesion tissue, green represents normal tissue)

In order to explore the distribution of community structure between lesion tissues and normal tissues, we plotted the distribution histogram at phyla, class, order, family, genus, and species levels (Figure 2) and further summarized the number of detections and the top three proportions (Table 2). In lesion and normal tissues, 9 and 10 phylum were detected, respectively. The highest proportion phylum in tissues was Chlamydiota, Bacillota and Pseudomonadota. Interestingly, compared with normal tissue, Cyanobacteriota was detected in lesion tissue, while Bacillota_C and

Ascomycota were not detected. At class level, 10 and 11 class were detected respectively in lesion and normal tissues. The highest proportion class was Chlamydiia, Gammaproteobacteria and Bacilli. In lesion and normal tissues, 23 and 22 order were detected, respectively. The highest proportion order was Enterobacterales, Chlamydiales and Bacillales. In lesion and normal tissues, 28 and 24 family were detected, respectively. The highest proportion order was Enterobacteriaceae, Chlamydiaceae and Bacillaceae_G. In lesion and normal tissues, 29 and 23 genus were detected, respectively. The highest proportion genus was Streptococcus, Chlamydophila and Bacillus_A. In lesion and normal tissues, 29 and 27 species were detected, respectively. The highest proportion species was Escherichia_coli, Bacillus_A_bombysepticus and Chlamydophila_abortus.

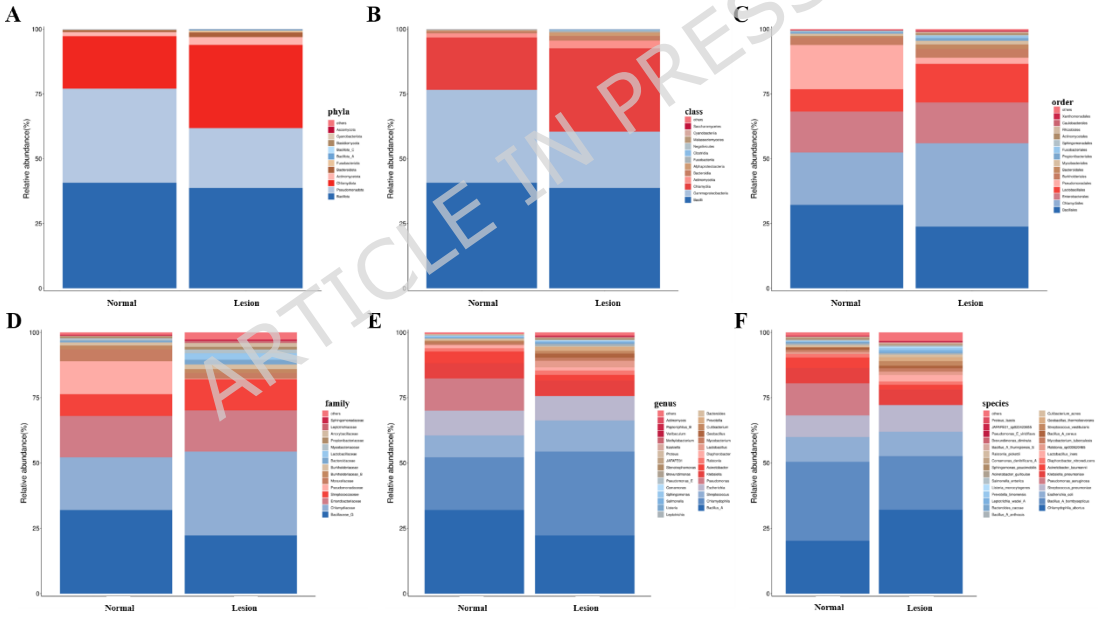

Figure 2 The distribution histogram between lesion and normal bladder tissues from IC/BPS patients at phyla (A), class (B), order (C), family (D), genus (E), and species (F) levels

Table2. The distribution of community structure between lesion and normal

bladder tissues

Taxa	No. of detections		Top three highest proportion
	lesion	normal	
phyla	9	10	Chlamydiota, Bacillota, Pseudomonadota
class	10	11	Chlamydiia, Gammaproteobacteria, Bacilli
order	23	22	Enterobacterales, Chlamydiales, Bacillales
family	28	24	Enterobacteriaceae, Chlamydiaceae, Bacillaceae_G
genus	29	23	Streptococcus, Chlamydophila, Bacillus_A
species	29	27	Escherichia_coli, Bacillus_A_bombysepticus, Chlamydophila_abortus

3.3 Alpha diversity analysis

To reflect the extent of microbial diversity in lesion tissues and surrounding normal tissues in patients with interstitial cystitis, we performed alpha diversity analysis. We calculate the diversity index of different samples and compare the diversity of samples. chao 1, shannon and simpson indexes all showed that there was no significant difference in microbial diversity between the lesion group and the normal group (Figure 3).

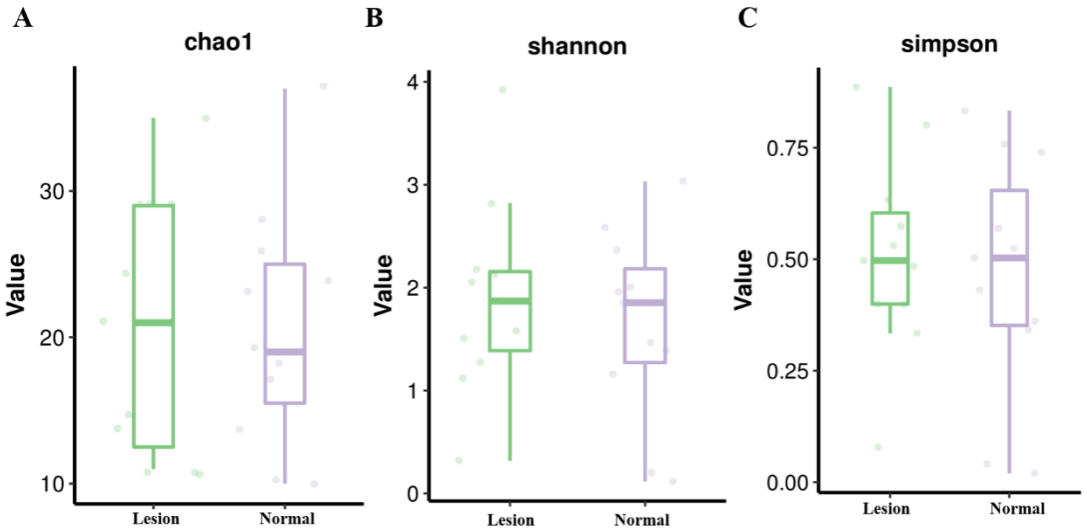

Figure 3 Comparison of alpha diversity [Chao1 index (A), Shannon index (B) and Simpson index (C)] between lesion and normal bladder tissues of IC/BPS patients

3.4 Beta diversity analysis

In addition, in order to compare the differences of samples from different groups, we conducted beta diversity analysis, and the results of PCoA analysis showed that among the three distance algorithms (Binary Jaccard distance, Bray-Curtis distance and Euclidean distance), samples from the lesion group showed no significant difference in microbial diversity between the samples from the normal group (Figure 4).

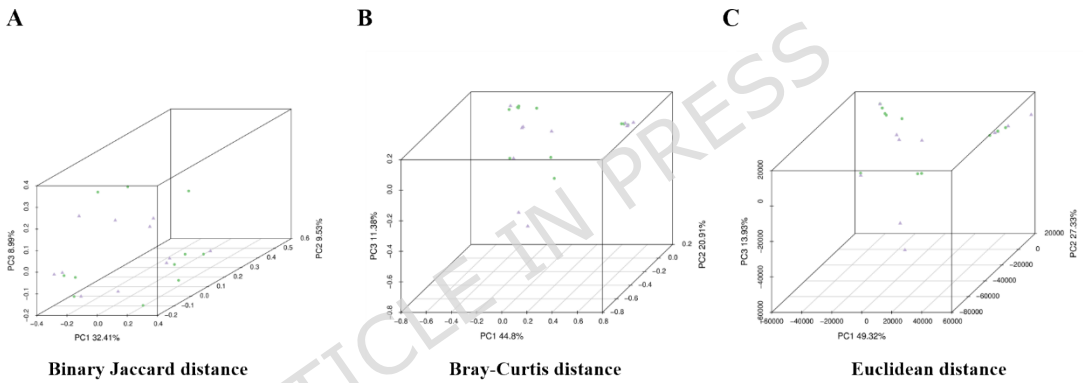

Figure 4 3D-PCoA plots comparison of beta diversity [(Binary Jaccard distance (A), Bray-Curtis distance (B), and Euclidean distance (C)] between lesion and normal bladder tissues of IC/BPS patients (each point in the plot represents a sample and the same color for the same group)

3.5 Statistical analysis of microbial differences between lesion and normal groups

In order to explore whether there were differences in the microorganisms detected between the lesion group and the normal group, we used Wilcoxon analysis to conduct a statistical summary of the differences between the two groups. The analysis results showed that at the order level, there was Acetobacterales in normal tissues, but not in lesion tissues, and the

difference was statistically significant. At the family level, Rhizobiaceae was detected in lesion tissue, and Acetobacteraceae and Porphyromonadaceae were detected in normal tissue, and the difference between the two groups was statistically significant. At the genus level, Sphingopyxis was present in lesion tissues, and Porphyromonas was detected in normal tissues. At the species level, Sphingopyxis_sp022701295 was detected in lesion tissues, but not in normal tissues, and the difference was statistically significant (Table 3).

Table 3. Comparison of the microbiota abundance of the lesion and normal tissues at the order, family, genus and species levels, respectively.

Taxa	Name	Lesion-mean	Normal-mean	P value
order	*Acetobacterales*	0	<0.001	0.052
family	*Rhizobiaceae*	<0.001	0	0.050
	Acetobacteraceae	0	<0.001	0.046
	Porphyromonadaceae	0	<0.001	0.038
genus	*Sphingopyxis*	0.001	0	0.021
	Porphyromonas	0	<0.001	0.039
species	*Sphingopyxis_sp022701295*	0.001	0	0.024

In addition, we performed LEfSe (linear discriminant analysis coupled with effect size measurements) analyses to reveal the composition of species that differed in the biome between the lesion and normal groups. LEfSe analysis showed that, at the genus level, Sphingopyxis was enriched in the lesion group relative to the normal group.

3.6 Differences in predicted function between lesion and normal groups

We used PICRUSt2 software for functional prediction of the identified microbiota and Wilcoxon test for comparative analysis. We identified 34 COGs that differed significantly between bladder lesion tissues and normal tissues from patients with interstitial cystitis, of which the top 10 COGs were COG2207 (AraC-type DNA-binding domain and AraC-containing proteins), COG1280 (Threonine/homoserine/homoserine lactone efflux protein), COG0665 (Glycine/D-amino acid oxidase (deaminating)), COG1522 (DNA-binding transcriptional regulator, Lrp family), COG0183 (Acetyl-CoA acetyltransferase), COG0600 (ABC-type nitrate/sulfonate/bicarbonate transport system, permease component), COG0841(Multidrug efflux pump subunit AcrB),COG0654(2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases), COG5006(2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases) and COG1902 (2,4-dienoyl-CoA reductase or related NADH-dependent reductase, Old Yellow Enzyme (OYE) family) (Figure 5A). Besides, the top 5 KEGG pathways with significant differences between bladder diseased and normal tissues included: fatty acid metabolism, citrate cycle, porphyrin and chlorophyll metabolism, valine，leucine and isoleucine degradation and biotin metabolism (Figure 5B).

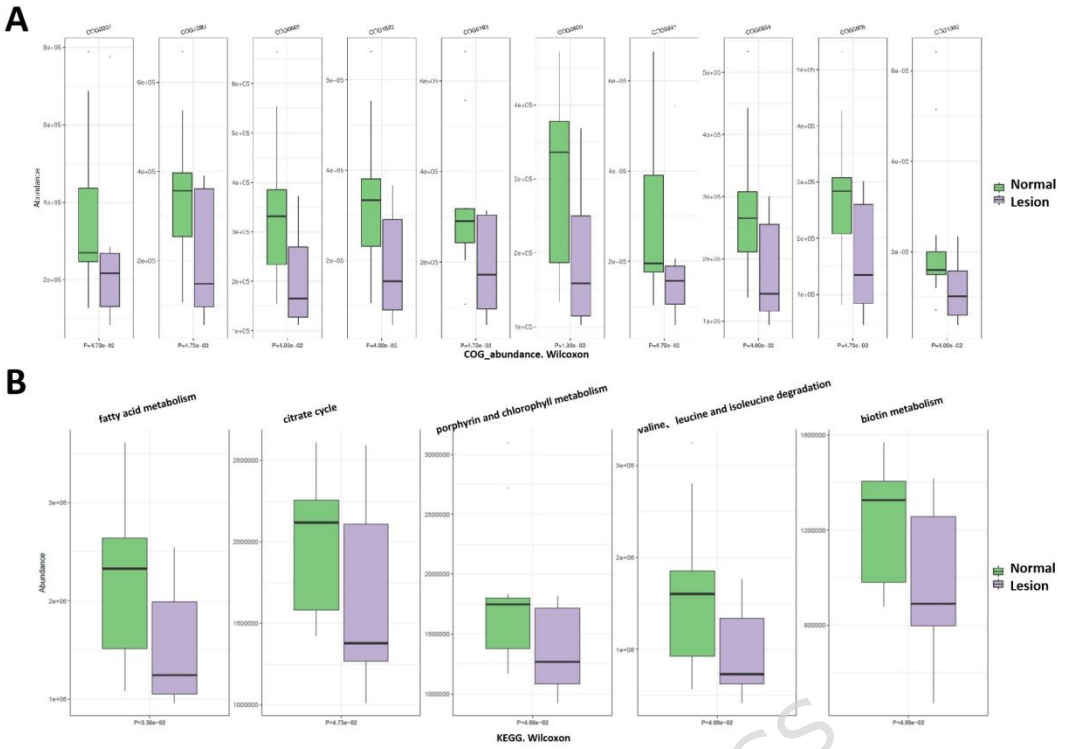

Figure 5 Microbial function differences between lesion and normal bladder tissues of IC/BPS patients. (A) The top 10 of COG function prediction results. (B) Predicted the top 5 of KEGG function. The plot was generated using the ggplot2 R package based on data from the KEGG database (http://www.kegg.jp/)

4 Discussion

IC/BPS is a group of conditions characterized by chronic bladder-related pelvic pain, accompanied by urinary frequency and urgency, which belongs to the category of chronic pelvic pain defined by the EAU. It has been 100 years since Hunner first reported it. However, due to the uncertainty of the etiology, the diversity and heterogeneity of the symptoms, the diagnosis and treatment of this disease have always been clinically difficult, and patients suffer from persistent and recurrent pelvic or generalized pain episodes, dysuria, and decreased quality of life caused by depression and anxiety[13]. The pathogenesis of IC/BPS is still unclear, and there are many different opinions. At present, it is generally believed that the occurrence of this

disease is related to infection, immunity, mast cell infiltration, defective amino glucan (GAG) layer, neurogenic factors, microbial factors, etc. In recent years, with the development of microbial detection technology, the application of high-throughput sequencing of the 16s rDNA gene and the improvement of bacterial culture technology such as EQUC, some studies have shown that microbial factors may play a role in the pathogenesis of IC/BPS. One study found that the anaerobic bacteria in the fecal flora of patients with IC/BPS differed significantly from those of healthy people[14], with decreased abundance of E. sinensis, C. aerofaciens, F. prausnitzii, O. splanchnicus, and L. longoviformis in the stool samples of patients with IC/BPS compared with those of normal people[15]. In another study, high-throughput sequencing of the urinary flora of female patients with IC/BPS and healthy females showed a significant decrease in the microbial diversity of urine and a significant increase in the abundance of Lactobacillus in patients with IC/BPS[16], while another study showed that patients with IC/BPS had lower levels of Corynebacterium and higher levels of Lactobacillus in their urine[17]. In order to gain a more accurate and in-depth understanding of the role of microbiota in the pathogenesis of IC/BPS, we used 2bRAD-M technology to detect and analyze the bladder lesion tissues and surrounding normal tissues of IC/BPS patients. 2bRAD-M is characterized by high resolution, high accuracy and high sensitivity to trace samples compared with sequencing technologies such as 16s and macro genomes, which makes it suitable for clinical testing.

In this study, we detected a total of 118 bacteria and 2 fungi in 22 bladder tissue samples from 11 patients with IC/BPS. The analysis of the mapping of species distribution petals showed that 11 patients have 3 species in common in lesion and normal tissue, namely Mycobacterium_tuberculosis, Ralstonia_sp000620465 and Klebsiella_pneumoniae. Mycobacterium tuberculosis is known to be the causative agent of tuberculosis (TB). Urinary tract tuberculosis (UTB) ranks

second among extrapulmonary tuberculosis after lymph node tuberculosis. UTB can be complicated in 2% to 20% of patients with pulmonary tuberculosis[18-19]. Clinically, BCG has been recognized as the most effective immunologic agent for preventing recurrence of bladder tumors and is indicated for postoperative perfusion therapy of superficial, noninvasive bladder tumors[20-21]. However, the species distribution and pathogenicity of Mycobacterium tuberculosis in bladder tissues of interstitial cystitis is unknown and warrants further study. Ralstonia is a group of aerobic Gram-negative, oxidase-positive, non-fermenting bacilli that are widely found in water and soil. Interestingly, recent studies have shown that Ralstonia_sp000620465 was the predominant species detected in bladder cancer tissues[22]. Besides it has been detected in ovarian tissues and urinary tract stones[23-24]. Klebsiella pneumoniae belongs to the Enterobacteriaceae family and usually colonizes the mucosal surfaces of the gastrointestinal tract and nasopharynx of patients, and when spread to other tissues and organs, it can cause pneumonia, urinary tract infections, bloodstream infections, and sepsis[25]. Klebsiella pneumoniae helps pathogenic bacteria to resist host defense mechanisms by forming biofilms, which can inhibit or evade phagocytosis by host cells due to the production of a large number of sticky polysaccharides coated with pods, further inducing the maturation of dendritic cells, weakening the antimicrobial activity of the body's immunity, and triggering inflammation[26]. In summary, the discussion of the above studies speculates that we believe that Mycobacterium_tuberculosis, Ralstonia_sp000620465 and Klebsiella_pneumoniae may be the three bacteria co-existing in the bladder tissue of patients with interstitial cystitis, constituting the microbial distribution pattern of the bladder tissue background.

In order to explore the distribution of community structure between lesion tissues and normal tissues, we plotted the distribution histogram at phyla, class, order, family, genus, and species levels. As the microbial species

classification order element progresses step by step, more and more species are being detected, both in the bladder lesion group and the normal group. In phylum level, 9 and 10 phylum were detected in lesion and normal tissues. In species level, 29 and 27 species were detected, respectively. The highest proportion species was Escherichia_coli, Bacillus_A_bombysepticus and Chlamydophila_abortus. As a resident bacterium in the host colon, Escherichia_coli maintains a mutual balance and harmonious symbiosis with the host organism, and can be isolated from the upper respiratory tract and the genitourinary system of healthy hosts, and some strains of the bacterium can trigger infections in the urinary system, which may be related to the special structures and associated proteins associated with pathogenesis[27]. Bacillus bombysepticus is a gram-positive bacillus that primarily infects silkworms (Bombyx mori), causing septicemia[28]. Chlamydia is a specialized intracellular parasitic Gram-negative prokaryotic microorganism with a unique biphasic developmental cycle that infects a wide range of eukaryotic hosts from protozoa to invertebrates and mammals[29]. Chlamydophila_abortus is an important pathogen causing zoonotic diseases mainly through the oral and nasal routes and sexually transmitted infections[30], which brings serious economic losses to the livestock industry[31].

In order to study the extent of microbial diversity in lesion tissues and their surrounding normal tissues in patients with interstitial cystitis, we performed alpha diversity analysis. Also, to compare the differences between samples from different groups, we performed a beta diversity analysis. The results showed that there were no significant differences in microbial diversity within and between the lesion and normal groups.

Next, we analyzed the differences between the microorganisms detected in the lesion group and the normal group, and the results of this analysis showed that Sphingopyxis and Rhizobiaceae was enriched in the lesion group relative to the normal group. Besides, Acetobacteraceae and Porphyromonas

were enriched in the normal group. The genus Sphingopyxis was reported in 2001 and contains 20 validly published species. Species of Sphingopyxis have been isolated from a wide range of ecological niches, including agricultural soils, marine and freshwater, caves, activated sludge, hot springs, oil and pesticide contaminated soils, and sites contaminated with heavy metals. Species of the genus Sphingopyxis are of interest not only for their ability to survive in extreme environments, but also for their ability to degrade a wide range of exogenous substances and other environmental pollutants that pose serious threats to human health[32]. The Rhizobiaceae family contains several species of rhizobia and Agrobacterium. Rhizobia are symbiotic bacteria of legumes that fix nitrogen within the rhizomes, while Agrobacterium tumefaciens is a pathogen that causes crown gall disease in a variety of plants[33]. Acetobacteraceae, commonly associated with fermented food products, have been shown to produce polymers with a wide range of structurally and functionally distinct profiles. Some strains are capable of producing industrially important homopolysaccharides and can also form acetan-like heteropolysaccharides that are highly similar in structure to xanthan[34]. Of interest, Porphyromonas is a genus of Gram-negative, specialized anaerobes with proteolytic properties and no saccharolytic capacity. Porphyromonas gingivalis, a typical species of the genus, is the main causative agent of adult periodontitis, destroying periodontal tissues by secreting gingival proteases and regulating plaque biofilm development[35]. The genus can migrate to the lungs, kidneys, and other organs through the blood circulation, leading to the progression of systemic pathologies such as lung infections, chronic kidney disease, and tumors[36-38]. The relationship between differential microorganisms and bladder pathology requires further study and validation.

Finally, we predicted functional differences between the lesion tissues and their surrounding normal tissues in patients. Predictive analyses showed that there were significant functional differences in the microbiome between

diseased and normal tissues. It is suggested that the microbiota influences the inflammatory state of bladder tissues through interaction with the host, which in turn affects the disease process. The top five KEGG pathways with significant differences in functional prediction analysis between bladder diseased and normal tissues, namely fatty acid metabolism, citric acid cycle, porphyrin and chlorophyll metabolism, valine/leucine/isoleucine degradation, and biotin metabolism-related pathways, are closely related to microbial life activities. For pathogenic bacteria, conditionally pathogenic bacteria and commensal bacteria associated with disease development, these pathways are not only the basis for their survival, but also closely related to their adaptation to the host microenvironment, evasion of immunity and pathogenicity. Porphyromonas gingivalis can induce disease by altering fatty acid metabolism. Porphyromonas gingivalis has been found to infect mice to induce periodontitis and promote the development of oral cancer. Porphyromonas gingivalis significantly increased the levels of free fatty acids in the tongue tissue and serum of mice, altering the fatty acid profile and inducing fatty liver formation[39]. Further studies are needed to investigate the role of the predictive pathway in the progression of interstitial cystitis and the mechanism of its interaction, and to seek targets for intervention studies.

It is worth noting that Walton's review[40] states that the main conclusion of the studies based on voided or catheterized samples is that there is no conclusive evidence of a unique urinary microbiome in IC/BPS. This study differed in both sample type (bladder tissue) and methodology (2bRAD-M technique). The microorganisms we detected in bladder tissue (e.g., Mycobacterium, Pseudomonas) may not be easily shed into or detected in urine, which may represent different ecological niches. Thus, our data do not necessarily contradict urine-based studies, but rather suggest that the bladder tissue microbiota may constitute a separate ecosystem worthy of special study. The absence of differences between the lesion tissues and

their surrounding normal tissues is consistent with the concept of diffuse lesions; however, the presence of a stable group of tissue-resident flora provides a new hypothesis for their potential role in mucosal homeostasis or low-grade inflammation.

As a preliminary exploratory study, a control group consisting of healthy bladder tissue and other bladder pathology tissues (e.g., bacterial cystitis, bladder cancer) was lacking. This absence limited our ability to determine whether the identified microbial communities (e.g., Mycobacterium tuberculosis, Ralstonia_sp000620465) were specifically present in interstitial cystitis/bladder pain syndrome (IC/BPS), representative of the baseline state of the microbiome of the bladder tissues, or common to any etiologically triggered inflammatory state of the bladder. It is therefore not possible to clarify whether these microorganisms are causative agents, consequences of disease, or simply symbionts. Also, longer antibiotic histories or possible alterations in colonization were unmeasured confounders. Future validation studies with better matched controls are needed to clarify the context of these findings. Besides, it is important to acknowledge a key limitation regarding our definition of normal tissue. Given the potential for diffuse microscopic alterations in IC/BPS, the normal-appearing tissue designated by our macroscopic criteria may still harbor undetected biological abnormalities. Therefore, our comparison essentially reflects differences between areas of overt pathology and areas of relative normality within a potentially diffusely affected organ. The finding of small microbial differences between these sites provides both preliminary confirmation of the hypothesis of the presence of diffuse bladder lesions in non-Hunner-type IC/BPS and microbiologic data to support this hypothesis.

5 Conclusion

In summary, in this study, the microbial environment in the lesion site tissues and surrounding normal tissues of the bladders of patients with

IC/BPS was characterized and meticulously analyzed for the first time in terms of microbial taxonomy using the novel technique 2bRAD-M. Overall, the microbial diversity and composition of the lesion site tissues and surrounding normal tissues of bladders visible to the naked eye from patients with interstitial cystitis were similar, suggesting that the disease may be a diffuse lesion. Among them, Mycobacterium_tuberculosis, Ralstonia_sp000620465 and Klebsiella_pneumoniae were the microorganisms coexisting in the tissues. Escherichia_coli, Bacillus_A_bombysepticus and Chlamydophila_abortus were the dominant microbial species. Significantly higher abundance of Sphingopyxis and Rhizobiaceae was observed in the lesion tissues, whereas in the surrounding normal tissues Acetobacteraceae and Porphyromonas had higher abundance. We hypothesize that some characteristic microorganisms are present in bladder tissue and are associated with IC/BPS. Further in-depth investigation of the causative microorganisms is still needed.

Author contributions

Ying Gan, Jingjun Zhang and Yang Yang performed sample preparation, DNA extraction, data analysis and the paper writing. Kaifeng Yao, Rui Jiang, Zheng Li collected tissue samples. Yang Yang was responsible for the study design and supervision. Yang Yang and Ying Gan provided financial support for the project. All authors contributed to the article and approved the submitted version.

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Data availability

The data repository website was https://dataview.ncbi.nlm.nih.gov/object/PRJNA1310667?reviewer=71l0ns2aldhgrcq2f0g6lgkve8 and the accession number was PRJNA1310667.

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