A previously unexplored etiology for femoral head necrosis: metagenomics detects no pathogens in necrotic femoral head tissue

Abbreviations

ONFH: Osteonecrosis of the femoral head; mNGS: Metagenomic next-generation sequencing.

Key words: idiopathic; osteonecrosis of the femoral head; sequencing; pathogens

Running title

CHAO LIU et al: Unexplored etiology femoral head necrosis: metagenomics detects pathogens

Abstract

Purpose: Osteonecrosis of the femoral head (ONFH) is a frequent and refractory disease whose pathogenesis has not yet been elucidated. Infection and other factors that reduce the local blood supply can lead to bone necrosis. The aim of this study was to assess the relationship of ONFH with bone infection by use of metagenomic sequencing.

Methods: Twelve patients with idiopathic ONFH and 12 comparable controls who were undergoing hip arthroplasty were followed up in parallel. Necrotic femoral head specimens were collected for bacterial and fungal cultures using standard methods. Bone specimens were subjected to preliminary processing, and metagenomics sequencing of microorganisms was performed. A one-way analysis of variance was used to compare bacterial species in the two groups.

Results: Bacterial and fungal cultures exhibited no evidence of microbial growth in all isolated necrotic femoral head tissues. We thus performed metagenomic sequencing and classified the species as suspected pathogens or suspected background microorganisms based on known bacterial pathogenicity. There was no evidence of viruses, fungi, parasites, *M. tuberculosis* complex, or mycoplasma/chlamydia. There were also no significant differences in suspected pathogens or suspected background microorganisms (both $P > 0.05$).

Conclusions: Although we found no pathogens specific for ONFH in necrotic femoral head tissue, our research provides a foundation for future research on the metagenomics of bone pathogens.

Introduction

Osteonecrosis of the femoral head (ONFH) is a common and refractory disease characterized by the death of osteocytes and bone marrow cells, and is generally caused by an inadequate blood supply. Direct and indirect factors that reduce the local blood supply, such as femoral neck fracture, hip dislocation, long-term corticosteroid use, and excessive alcohol consumption, can lead to ONFH [1-3]. However, many patients develop idiopathic ONFH, in which there are no obvious causes or risk factors [4].

Infection is one of the various factors that can disrupt the local blood supply to the femoral head and lead to necrosis. Several specific pathogens can cause microvascular disease and lead to secondary local necrosis, such as intra-proliferative proliferative nephritis, which is often associated with type A hemolytic streptococcal infection [5,6].

Our purpose was to identify ONFH-related pathogens in the necrotic femoral head, and to examine the relationship between ONFH and infection. To our knowledge, this is the first study to examine whether pathogenic microbial infection is responsible for idiopathic ONFH in patients who do not have obvious risk factors. Our findings may have important consequences for clinical decision-making for the early intervention and follow-up treatment of ONFH.

Materials and Methods

Setting and study design

All patient data were extracted from the hospital's medical records. All included patients had diagnoses of ONFH based on clinical symptoms and imaging features, and had no known

risk factors. All patients had abnormal gaits; reduced Harris hip scores; positive results in the Thomas experiment and Patrick experiment; and X-ray features showing femoral head collapse, a crescent sign, and Ficat stage III or IV. The time from the onset of pain to diagnosis ranged from 2 months to 5 years. All patients underwent total hip arthroplasty, and all of their Harris hip scores improved after surgery. The control group, consisting of 12 patients with acute femoral neck fractures who were undergoing hip arthroplasty, were followed up in parallel.

All experimental protocols were approved by the Institutional Ethics Committee of the authors' affiliated institutions, and informed consent was obtained from all patients.

Specimen collection and processing

All specimens were collected in a thousand-level laminar flow operating room that had constant temperature and humidity to minimize contamination. In addition, bedside sampling was performed following aseptic procedures. Thus, sampling personnel wore hats, masks, and gloves, and avoided speaking to prevent possible contamination by dental bacteria. A fresh necrotic femoral head tissue sample that was about the size of a soybean (about 200 mg) was ground into pieces as small as possible using a rongeur, and equal amounts were then placed into three clean Eppendorf tubes. The first tube contained RNase-free pure water prepared for RNA sequencing, the second tube was used for DNA sequencing, and the third tube was used for bacterial and fungal cultures. The first tube was gently shaken to submerge the specimen, sealed, and immediately placed in liquid nitrogen to ensure cryopreservation of RNA. The sample in the third tube was cultured for identification of bacteria, fungi, and *Mycobacterium*, using standard methods.

The first and second tubes were stored vertically in dry ice (–80 °C) and sent to Huada Gene Company Tianjin Branch within 4 h after collection. These specimens were received within 48 h, and subjected to further homogenization using previously described ortexing/sonication methods[7]. The eluted specimens were initially tested for nucleic acid content to assure they met standard requirements, and were then subjected to sequencing.

Nucleic acid extraction and construction of DNA libraries

Each tissue sample, with 0.5 mm glass beads, was added to a microcentrifuge tube with a lysis buffer and centrifuged at 3000 rpm for 30 min. Then a 0.3 mL aliquot was transferred into a new microcentrifuge tube, and DNA was extracted using the TIANamp Micro DNA Kit (Tiangen Biotech) according to the manufacturer's recommendations. The same procedures were used for RNA extraction using the QIAAmp Viral RNA Mini Kit (52904#, Qiagen), and then cDNA was generated from an RNA template by reverse transcription. The DNA was then fragmented, end-repaired, and a ligated adapter and PCR amplification were used to construct DNA libraries. The Agilent 2100 Bioanalyzer was used for quality control, and eligible libraries were sequenced as described below[8].

Metagenomic sequencing and bioinformatic analysis

Metagenomic next-generation sequencing (mNGS) was performed using the BGISEQ-50 gene sequencer (Huada Gene Company). Data quality was achieved by removing low quality and short (<35 bp) reads. Then, the Burrows-Wheeler transformation was used to perform alignment to the reference genome (hg19). By aligning four microbial genome databases (for bacteria, viruses, fungi, and parasites), the rest of the data were analyzed, except for low-complexity reads. The classification reference databases were from NCBI

(ftp://ftp.ncbi.nlm.nih.gov/genomes/). RefSeq contains 1424 genomic sequences of DNA viruses, 2637 genomic sequence of RNA viruses, 2406 genomic sequences of bacteria, 83 genomes or scaffolds of *Mycobacterium*, and sequences of 199 fungi, 135 parasites, and 41 mycoplasma/chlamydia that are related to human infections. The detection limit was 100 to 1000 copies/mL for microbial nucleic acids; the detection specificity for microorganisms with copy numbers greater than the detection limit was greater than 99%; and the repeatability was greater than 99%.

Statistics

All data analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY). A one-way analysis of variance was used to determine statistically significant differences. A *P* value below 0.05 was considered significant ($P < 0.05$).

Results

Basic characteristic

Twelve patients with idiopathic ONFH were enrolled (Table 1). The median patient age was 61 years (range: 48–69, mean: 58, standard deviation: 7). There were 7 patients with left femoral necrosis, 3 with bilateral femoral necrosis, and 2 with right femoral necrosis. Three patients had comorbidities that were unrelated to ONFH (lumbar disc herniation, lumbar disc herniation and lumbar spinal stenosis, prostatic hyperplasia and bronchitis).

Culture results

Aerobic bacteria and anaerobic bacteria cultures were negative on the third day, fungal cultures were negative for 56 days, and tuberculosis liquid cultures were negative for 42 days.

These results indicate the need to perform culture-independent analysis, such as mNGS.

Sequencing results

According to the results of the mNGS, we cannot make final conclusion regarding the causative pathogens without further clinical assessments. Nonetheless, we used a method to rank the microbes detected from the samples. First, we compared microbes detected with background microbes in the database, and labeled them as "background" microbes. Second, we compared the microbes detected using negative controls; if the number of reads was less than 50, the difference was considered to be "more than 5-fold" and if the number of reads was more than 50, the difference was considered to be "more than 3-fold". For bacteria, we reported the top 5 genera and the 2 major species in each genus; for fungi and parasites, we reported the top 5 genera and the 1 major species in each genus; for viruses, we reported all detected species; each species has two types and each type has two subtypes.

批注 [A1]: Confusing to me. Does this refer to the species of viruses?

We sorted the microbial sequencing results according to the number of sequences, and then classified detected bacterial sequences as suspected pathogens or suspected background microorganisms, based on known common clinical pathogens. The suspected pathogens in the patients and controls were *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Enterococcus cecorum*, and *Pseudomonas putida* (Figure 1 and Supplementary Table 1). The major suspected background microorganisms in the patients and controls were *Ralstonia insidiosa*, *Propionibacterium acnes*, and *Burkholderia ubonensis*, and several other species (Figure 2 and Supplementary Table 2). There was no evidence of viruses, fungi, parasites, *M. tuberculosis* complex, or mycoplasma/chlamydia. Statistical analysis indicated there were no significant differences in suspected pathogens or suspected background microorganisms (both

P > 0.05, Supplementary Table 3).

Discussion

Idiopathic osteonecrosis is a painful disorder that mainly affects individuals who are 30 to 40 years-old [9]. The basic features of ONFH pathogenesis are impaired blood circulation that ultimately leads to necrosis. Some studies attributed the disease to a combination of metabolic factors, genetic susceptibility, and insufficient local blood supply [1]. ONFH can progress to symptomatic osteoarthritis of the hip joint and collapse, or even destruction of the femoral head [10,11]. Microscopic or macroscopic disruption of the blood supply to the femoral head are considered the hallmarks of this condition, because they lead to necrosis of bone forming cells [6,12]. Many pathogenic bacteria and viruses can release damaging toxins or cause inflammation of small blood vessels, thereby resulting in ischemia, infarction, and tissue necrosis. For example, *Helicobacter pylori* can cause gastritis, hepatitis B virus can cause chronic hepatitis, and streptococcal infection can lead to acute glomerulonephritis [13]. Furthermore, infectious pathogens can cause a local immune response, leading to microemboli in the arteries. However, our sequencing results provided no evidence that idiopathic ONFH is associated with infections by any particular microorganisms. Traditional culturing methods also have not identified the causative pathogens. mNGS provides enhanced capabilities by offering culture-independent, comprehensive diagnostic assessment of the microbial composition of clinical samples [14,15]. This motivated our use of mNGS to investigate the presence of microorganisms in the necrotic femoral head of patients with ONFH.

One of the limitations of the present study is that we only examined a relatively small number of cases. Our sequencing results indicated that the femoral head tissues of the ONFH and control groups had no significant differences in pathogenic microorganisms or background microorganisms. Thus, we have no evidence that idiopathic ONFH is associated with any specific femoral head pathogens, indicating there is still a need to identify the cause of ONFH in these patients. Another limitation is that mNGS can lead to false-positive and false-negative results. In particular, the presence of background bacteria can interfere with the results. In fact, we identified several background bacteria in both groups (*R. insidiosa*, *P. acnes*, and *B. ubonensis*). These background bacteria often appear because they are common in the laboratory and other environments, and are occasionally associated with patient infections. Moreover, our culturing of necrotic femoral head tissue indicated no bacterial growth.

Some studies have reported that HIV infection is a risk factor for ONFH [16,17], although there is no reliable evidence of a causative relationship. Patients infected with HIV often receive radiotherapy, chemotherapy, and corticosteroid treatments, and these may favor the development of ONFH. Thus, it is difficult to identify the specific causes of ONFH in patients infected with HIV [18]. Some studies support the hypothesis that the protease inhibitors used by HIV-infected patients promotes the development of osteonecrosis because they cause hyperlipidemia [19].

Although our results were negative, our proof-of-concept study indicated there are still many other possible routes for examining the metagenomics of pathogens in ONFH. The present study was apparently limited by the relatively small sample size and by our detection

of a small number of suspected pathogens (*Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Enterococcus cecorum*, and *Pseudomonas putida*). In addition, our mNGS results detected no microbial growth in isolated necrotic femoral head tissues of some patients, a finding that complicates interpretation of differential gene expression profiling. Therefore, future multicenter randomized and prospective studies with larger sample sizes are ongoing to validate the specificity and sensitivity of mNGS for the diagnosis of idiopathic ONFH. We also plan to use robust genetic classifiers in this population to distinguish specific pathogens from non-infectious microvascular diseases in idiopathic ONFH.

In summary, this is the first study to use mNGS to identify specific pathogens in the femoral heads of patients who have idiopathic ONFH with no obvious risk factors. Although we found no differences in the ONFH and control groups, we demonstrated that mNGS can detect microbes in femoral head tissues and has potential for use in association with the analysis of transcription factors related to the host immune response and the microbiome.

Declarations

Acknowledgements

Not applicable.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contribution

Study concept and design: DW and FP; study supervision: DW; administrative, technical, and material support: FP; analysis and interpretation of data: CL, and WL; acquisition of data: CZ; drafting of the manuscript: CL; critical revision of the manuscript for important intellectual content: CZ, FP, and CL; statistical analysis: DS and QX. All authors read and approved the final manuscript

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with ethical standards of Institutional Ethics Committee of Liaocheng People's Hospital (reference number 2018010). Informed consent was obtained from all participants in this study.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. Characteristics of patients with idiopathic ONFH.

Case	Sex/ Age(years)	Site	Comorbidities	Physical examination	X-ray features	Ficat stage	Time from pain onset to admission	Surgical approach	Outcome
1	M/50	Bilateral	Lumbar disc herniation	Limb gait, Harris score: 25 (left), 19 (right), left hip joint mobility: flexion 90°, extension 0°, abduction 15°, adduction 10°, external rotation 0°, internal rotation 10°, right hip joint mobility: flexion 90°, extension 0°, abduction 10°, adduction 0°, external rotation 0°, internal rotation 0°, Thomas experiment (+), Patrick experiment (+).	Bilateral femoral head collapse, crescent sign, joint space narrowing, with hip osteoarthritis	IV (left) IV (right)	2 years	Bilateral artificial total hip arthroplasty	Harris score: 48 (left), 47 (right)
2	F/62	Left	None	Limb gait, Harris score: 35, hip joint mobility: flexion 90°, extension 5°, abduction 20°,	Femoral head collapse	III	7 months	Left artificial total hip	Harris score: 52

				adduction 15°, external rotation 15°, internal rotation 5°, Thomas experiment (+), Patrick experiment (+).	occurs, crescent sign			arthroplasty	
3	F/68	Left	None	Limb gait, Harris score: 35, hip joint mobility: flexion 70°, extension 0°, abduction 10°, adduction 10°, external rotation 10°, internal rotation 10°, Thomas experiment (+), Patrick experiment (+).	Femoral head collapse occurs, crescent sign	III	2 months	Left artificial total hip arthroplasty	Harris score: 54
4	F/69	Right	None	Limb gait, Harris score: 17, hip joint mobility: flexion 60°, extension 0°, abduction 0°, adduction 0°, external rotation 0°, internal rotation 5°, Thomas experiment (+), Patrick experiment (+).	Femoral head collapse occurs, crescent sign	IV	15 months	Right artificial total hip arthroplasty	Harris score: 48

5	M/48	Bilateral	None	Limb gait, Harris score: 24 (left), 37 (right), left hip joint mobility: flexion 90°, extension 0°, abduction 40°, adduction 10°, external rotation 15°, internal rotation 10°, right hip joint mobility: flexion 110°, extension 0°, abduction 60°, adduction 15°, external rotation 15°, internal rotation 15°, Thomas experiment (+), Patrick experiment (+).	Femoral head collapse occurs, crescent sign (left); No femoral head collapse, appear cystic (right)	Ⅲ (left) Ⅱ (right)	15 months	Left artificial total hip arthroplasty	Harris score: 56 (left), 37 (right)
6	F/63	Left	Lumbar disc herniation and lumbar spinal stenosis	Limb gait, Harris score: 32, hip joint mobility: flexion 70°, extension 0°, abduction 25°, adduction 10°, external rotation 10°, internal rotation 10°, Thomas experiment (+), Patrick experiment (+).	Femoral head collapse, joint space narrowing	Ⅳ	3 years and 6 months	Left artificial total hip arthroplasty	Harris score: 49
7	M/61	Left	Prostatic	Limb gait, Harris score: 45, hip	Femoral	Ⅲ	5 years	Left	Harris

			hyperplasia, bronchitis	joint mobility: flexion 100°, extension 0°, abduction 20°, adduction 10°, external rotation 15°, internal rotation 10°, Thomas experiment (+), Patrick experiment (+).	head collapse occurs, crescent sign			artificial total hip arthroplasty	score: 55
8	M/56	Right	None	Limb gait, Harris score: 30, hip joint mobility: flexion 80°, extension 10°, abduction 15°, adduction 5°, external rotation 0°, internal rotation 5°, Thomas experiment (+), Patrick experiment (+).	Femoral head collapse occurs, crescent sign	Ⅳ	16 months	Right artificial total hip arthroplasty	Harris score: 51
9	F/62	Left	None	Limb gait, Harris score: 40, hip joint mobility: flexion 90°, extension 10°, abduction 15°, adduction 5°, external rotation 10°, internal rotation 15°, Thomas experiment (+), Patrick experiment	Femoral head collapse occurs, crescent sign	Ⅲ	5 months	Left artificial total hip arthroplasty	Harris score: 57

				(+).					
10	M/46	Bilateral	None	Limb gait, Harris score: 26 (left), 28 (right), left hip joint mobility: flexion 85°, extension 10°, abduction 35°, adduction 15°, external rotation 10°, internal rotation 10°, right hip joint mobility: flexion 90°, extension 10°, abduction 25°, adduction 10°, external rotation 10°, internal rotation 15°, Thomas experiment (+), Patrick experiment (+).	Bilateral femoral head collapse, crescent sign	III (left) III (right)	3 years	Bilateral artificial total hip arthroplasty	Harris score: 46 (left), 48 (right)
11	F/55	Left	None	Limb gait, Harris score: 40, hip joint mobility: flexion 95°, extension 15°, abduction 15°, adduction 20°, external rotation 10°, internal rotation 10°, Thomas experiment (+), Patrick experiment (+).	Femoral head collapse occurs, crescent sign	III	9 months	Left artificial total hip arthroplasty	Harris score: 58

12	M/61	Left	None	Limb gait, Harris score: 22, hip joint mobility: flexion 65°, extension 0°, abduction 5°, adduction 0°, external rotation 5°, internal rotation 0°, Thomas experiment (+), Patrick experiment (+).	Femoral head collapse occurs, crescent sign	Ⅳ	4 years	Left artificial total hip arthroplasty	Harris score: 46

Figure legends

Figure 1. Number of cases in the ONFH and control groups who had different suspected pathogens.

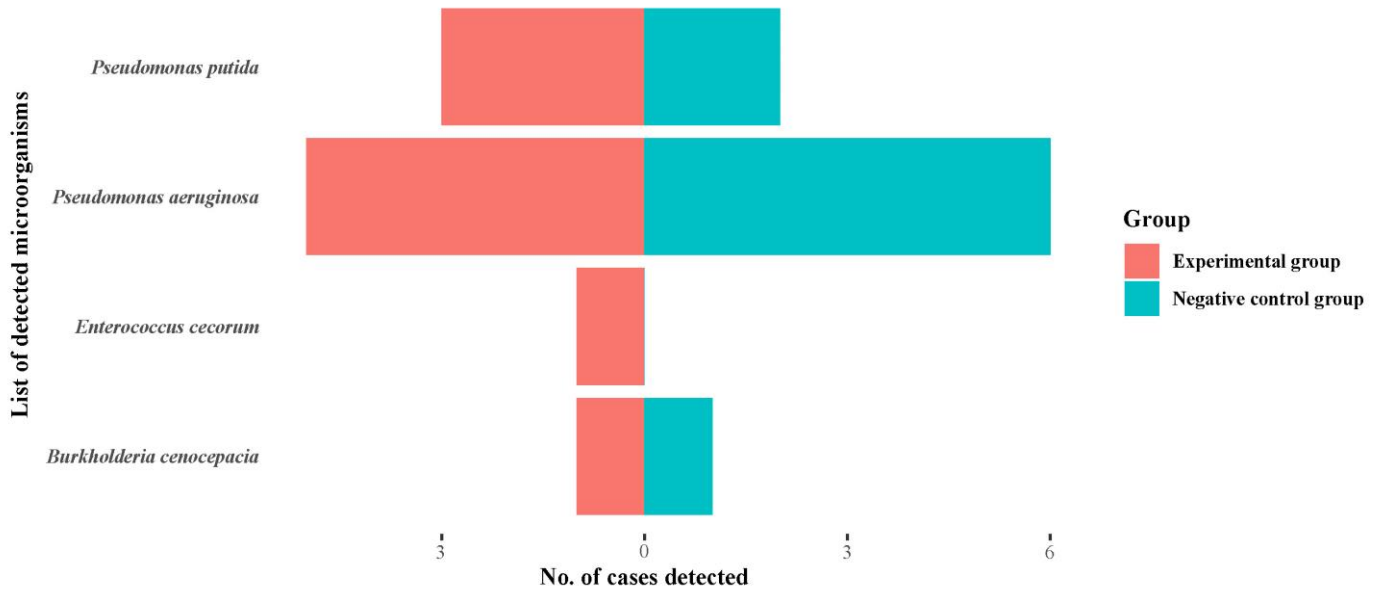
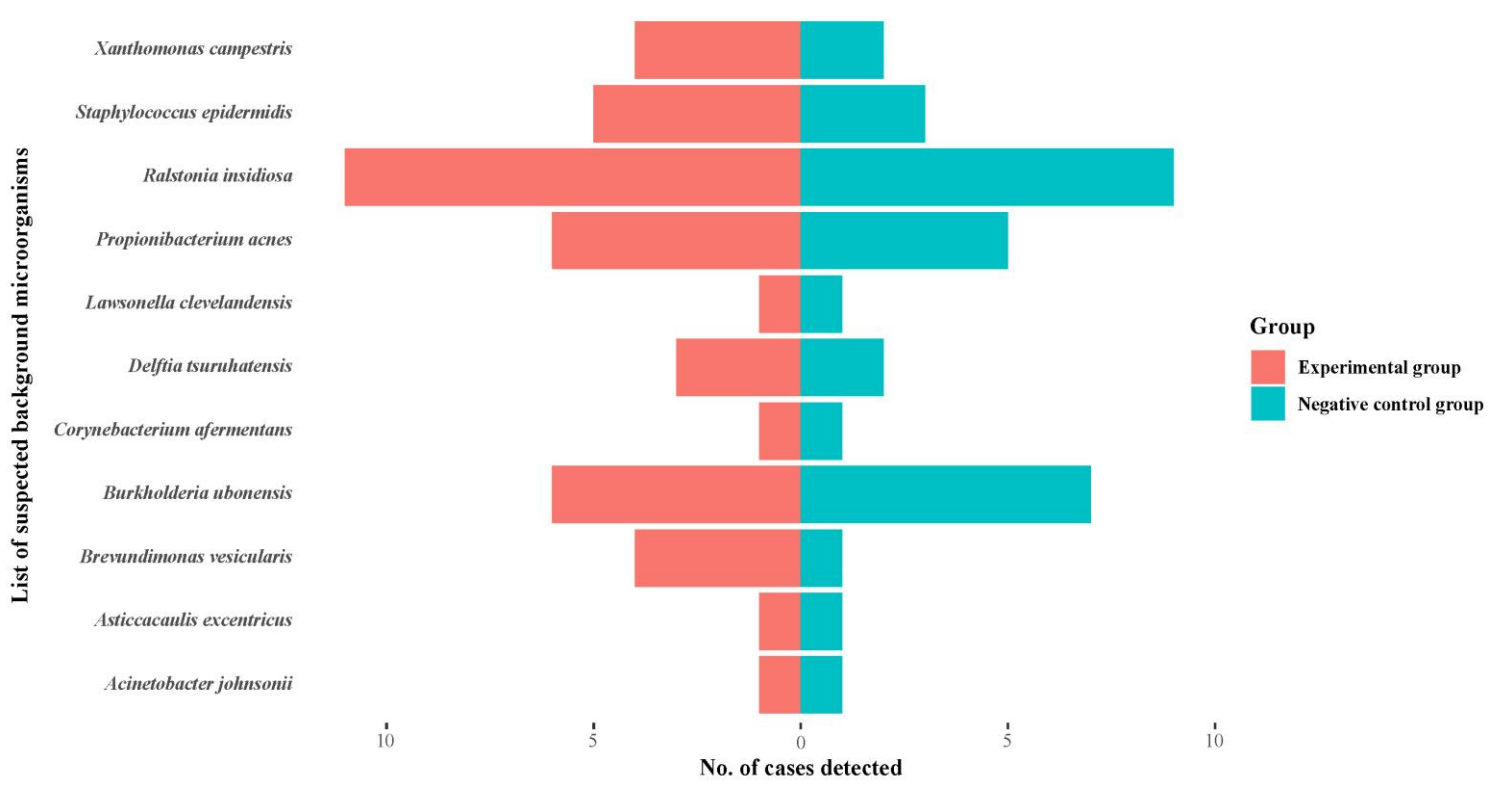


Figure 2. Number of cases in the ONFH and control groups who had different suspected background microorganisms.



Supplementary Table

Supplementary Table 1. Microorganismsidentified by metagenomic sequencing of patients with idiopathic ONFH

Number	Suspected pathogens (reads number, relative abundance)	Suspected background microorganisms (reads number, relative abundance)	Fungus	Virus	Parasite	*Mycobacterium tuberculosis* complex	Mycoplasma/Chlamydia
1-RNA	*Pseudomonas aeruginosa* (9, 0.68)	*Ralstoniainsidiosa* (505, 71.31)	None	None	None	None	None
1-DNA	*Burkholderiacenocepacia* (4, 1.75); *Pseudomonas aeruginosa*(3, 1.05)	*Ralstoniainsidiosa* (28, 18)	None	None	None	None	None
2-RNA	*Enterococcus cecorum* (3, 8.46)	*Ralstoniainsidiosa* (25, 31.96)	None	None	None	None	None
2-DNA	-	*Propionibacterium acnes* (25, 3.91); *Burkholderiaubonensis* (21, 8.46); *Staphylococcus epidermidis*(15, 2.45)	None	None	None	None	None
3-RNA	-	-	None	None	None	None	None
3-DNA	-	*Ralstoniainsidiosa* (11, 30.38)	None	None	None	None	None
4-RNA	*Pseudomonas aeruginosa* (22, 11.96)	*Ralstoniainsidiosa* (23, 18.43)	None	None	None	None	None
4-DNA	*Pseudomonas aeruginosa*(7, 1.46)	*Ralstoniainsidiosa* (14, 9.48); *Delftiatsuruhatensis* (6, 4.14); *Propionibacterium acnes* (5, 3.52);*Burkholderiaubonensis* (4, 3.93)	None	None	None	None	None
5-RNA	*Pseudomonas aeruginosa* (9, 1.51)	-	None	None	None	None	None
5-DNA	-	*Ralstoniainsidiosa* (4, 9.52)	None	None	None	None	None
6-RNA	*Pseudomonas putida*(4, 1.84)	*Propionibacterium acnes* (16, 11.28); *Ralstoniainsidiosa* (10, 28.15); *Corynebacteriumafermentans*(5, 3.96); *Staphylococcus epidermidis* (3, 1.25)	None	None	None	None	None
6-DNA	-	*Xanthomonascampestris* (8, 8.23);	None	None	None	None	None

7-RNA	-	*Burkholderiaubonensis* (6, 6.91); *Acinetobacterjohnsonii* (6, 6.89); *Brevundimonasvesicularis* (6, 7.05);*Asticcacaulisexcentricus*(3, 4.21) *Propionibacterium acnes* (17, 14.66); *Xanthomonascampestris* (7, 8.24); *Ralstoniainsidiosa* (6, 6.63); *Lawsonellaclevelandensis* (4, 4.15)	None	None	None	None	None
7-DNA	*Pseudomonas putida* (10, 8.19)	*Xanthomonascampestris* (32, 25.74); *Ralstoniainsidiosa* (5, 4.39); *Brevundimonasvesicularis* (5, 4.37);*Burkholderiaubonensis* (4, 2.33); *Staphylococcus epidermidis* (3, 1.38)	None	None	None	None	None
8-RNA	-	*Xanthomonascampestris* (12, 9.96); *Burkholderiaubonensis* (4, 5.11); *Brevundimonasvesicularis* (3, 4.01)	None	None	None	None	None
8-DNA	-	*Xanthomonascampestris* (19, 30.08); *Staphylococcus epidermidis* (8, 18.43); *Ralstoniainsidiosa* (4, 2.69)	None	None	None	None	None
9-RNA	-	*Xanthomonascampestris* (29, 45.81); *Ralstoniainsidiosa* (15, 29.62); *Brevundimonasvesicularis* (6, 10.58)	None	None	None	None	None
9-DNA	*Pseudomonas aeruginosa*(8, 9.61)	*Xanthomonascampestris* (36, 50.12); *Staphylococcus epidermidis* (13, 15.28)	None	None	None	None	None
10-RNA	-	*Ralstoniainsidiosa* (19, 22.05)	None	None	None	None	None
10-DNA	-	*Ralstoniainsidiosa* (33, 20.14)	None	None	None	None	None
11-RNA	*Pseudomonas putida* (12, 8.13)	*Burkholderiaubonensis* (14, 9.08); *Ralstoniainsidiosa* (12, 8.27);	None	None	None	None	None

		Propionibacterium acnes (8, 6.97)					
11-DNA	-	*Ralstoniainsidiosa* (10, 5.31); *Delftiatsuruhatensis* (6, 2.78)	None	None	None	None	None
12-RNA	*Pseudomonas aeruginosa*(6, 15.98)	*Delftiatsuruhatensis* (12, 20.47); *Propionibacterium acnes* (7, 17.79)	None	None	None	None	None
12-DNA	-	*Propionibacterium acnes* (15, 9.54)	None	None	None	None	None

Supplementary Table 2. Microorganismsidentified by metagenomic sequencing of controls (without ONFH).

Number	**Suspected pathogens (reads number, Relative abundance)**	**Suspected background microorganisms (reads number, Relative abundance)**	**Fungus**	**Virus**	**Parasite**	***Mycobacterium tuberculosis* complex**	**Mycoplasma/Chlamydia**
1-RNA	-	*Propionibacterium acnes* (12, 30.21); *Ralstoniainsidiosa* (9, 22.45); *Lawsonellaclevelandensis* (5, 10.33)	None	None	None	None	None
1-DNA	-	*Ralstoniainsidiosa* (7, 4.14); *Burkholderiaubonensis* (5, 3.52)	None	None	None	None	None
2-RNA	-	-	None	None	None	None	None
2-DNA	*Pseudomonas aeruginosa* (11, 20.49)	*Ralstoniainsidiosa* (9, 15.47)	None	None	None	None	None
3-RNA	*Pseudomonas aeruginosa* (17, 9.51)	*Ralstoniainsidiosa* (19, 10.62)	None	None	None	None	None
3-DNA	*Pseudomonas aeruginosa*(15, 17.83)	*Delftiatsuruhatensis* (16, 18.39); *Propionibacterium acnes* (15, 16.4);*Burkholderiaubonensis* (6, 2.6)	None	None	None	None	None
4-RNA	-	*Ralstoniainsidiosa* (19, 18.01)	None	None	None	None	None
4-DNA	-	*Ralstoniainsidiosa* (31, 22.13)	None	None	None	None	None
5-RNA	*Pseudomonas aeruginosa*	*Ralstoniainsidiosa* (65,	None	None	None	None	None

	(6, 7.26)	89.04);*Burkholderiaubonensis* (7, 8.81)					
5-DNA	*Burkholderiacenocepacia* (9, 10.48); *Pseudomonas aeruginosa*(7, 7.96)	*Ralstoniainsidiosa* (39, 50.24)	None	None	None	None	None
6-RNA	-	*Xanthomonascampestris* (19, 23.57); *Brevundimonasvesicularis* (9, 11.03)	None	None	None	None	None
6-DNA	*Pseudomonas aeruginosa*(12, 40.29)	*Xanthomonascampestris* (16, 55.23); *Staphylococcus epidermidis* (9, 36.02)	None	None	None	None	None
7-RNA	-	-	None	None	None	None	None
7-DNA	-	*Ralstoniainsidiosa* (16, 12.83)	None	None	None	None	None
8-RNA	-	*Delftiatsuruhatensis* (12, 9.2); *Propionibacterium acnes* (7, 8.13)	None	None	None	None	None
8-DNA	*Pseudomonas aeruginosa*(19, 11.92)	*Burkholderiaubonensis* (14, 8.35); *Propionibacterium acnes* (11, 6.7)	None	None	None	None	None
9-RNA	*Pseudomonas putida* (15, 9.84)	*Ralstoniainsidiosa* (19, 12.36); *Propionibacterium acnes* (15, 9.29)	None	None	None	None	None
9-DNA	-	*Ralstoniainsidiosa* (16, 10.09)	None	None	None	None	None
10-RNA	-	*Xanthomonascampestris* (15); *Burkholderiaubonensis* (9)	None	None	None	None	None
10-DNA	*Pseudomonas aeruginosa* (12, 17.01)	*Staphylococcus epidermidis* (12, 16.87); *Ralstoniainsidiosa* (7, 5.24)	None	None	None	None	None
11-RNA	-	*Ralstoniainsidiosa* (14, 34.79)	None	None	None	None	None
11-DNA	-	*Propionibacterium acnes* (19, 7.62); *Burkholderiaubonensis* (12, 5.58)	None	None	None	None	None
12-RNA	*Pseudomonas putida*(12, 2.45)	*Ralstoniainsidiosa* (17, 6.17); *Corynebacteriumafermentans*(15, 5.56); *Staphylococcus epidermidis* (9, 2.83)	None	None	None	None	None
12-DNA	-	*Burkholderiaubonensis* (11, 5.4);	None	None	None	None	None

	Acinetobacterjohnsonii (9, 4.81);*Asticcacaulisexcentricus*(6, 2.27)

Supplementary Table 3. Comparisons of suspected pathogens and suspected background microorganisms in the OHFH and control groups

		ONFH group		Control group		*P**
		negative	positive	negative	positive	
Suspected pathogens	*Pseudomonas aeruginosa*	7	5	6	6	1.00
	Burkholderiacenocepacia	11	1	11	1	1.00
	Enterococcus cecorum	11	1	12	0	1.00
	Pseudomonas putida	9	3	10	2	1.00
Suspected background microorganisms	*Ralstoniainsidiosa*	1	11	3	9	0.59
	Propionibacterium acnes	6	6	7	5	1.00
	Burkholderiaubonensis	6	6	5	7	1.00
	Staphylococcus epidermidis	7	5	8	3	0.67
	Delftiatsuruhatensis	9	3	10	2	1.00
	Corynebacteriumafermentans	11	1	11	1	1.00
	Xanthomonascampestris	8	4	10	2	0.32
	Acinetobacterjohnsonii	11	1	11	1	0.76
	Brevundimonasvesicularis	8	4	11	1	0.32
	Asticcacaulisexcentricus	11	1	11	1	1.00
	Lawsonellaclevelandensis	11	1	11	1	1.00

**P* values were determined using Fisher's exact text.