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ORIGINAL ARTICLE

Characteristics of pathogenic microbes in lung microenvironment of lung cancer patients without respiratory infection

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Summary

Purpose: *The characteristics of pathogenic microbes are useful for understanding the microbe-driven tumorigenesis. There is a lack of studies on the lung microecology for lung cancer (LC) patients without any respiratory infection. In this work, we aimed to describe the profiles of pathogenic microbes in lung microenvironment of non-small cell lung cancer (NSCLC) patients using pathogen targeted sequencing and 16S rDNA sequencing.*

Methods: *A total of 22 NSCLC patients (13 adenocarcinomas and 9 squamous cell carcinomas) without any pulmonary infection were enrolled. Among them, we collected 15 pieces of tumor tissues, 5 pieces of peritumoral tissues, 6 blood serum samples, and 5 broncho-alveolar lavage fluid (BALF) samples. Pathogen targeted sequencingand16S rDNA sequencing was performed for microbial classification.*

Results: *The pathogen targeted sequencing results showed that 33, 14, 11, and 27 pathogenic microorganisms were detected in tumor tissues, peritumoral tissues, blood samples, and BALF, respectively. No common microorganisms were shared by four sample types. However, some common elements were shared by three sets: Streptococcus cristatus, Enterococcus, Staphylococcus haemolyticus, Corynebacterium pseudodiphtheria, Acinetobacter jungii, Haemophilus haemolyticus and Haemophilus parainfluenzae. Based on the 16S rDNA sequencing of two BALF samples, there were 104 OTUs found in one BALF sample and 127 OTUs in the other BALF sample; among them, there were 82 common ones, such as OTU1, OTU10, OTU101, OTU105, OTU106, and so on. Based on the above microbial classification and abundance, there might be enriched function in COG terms like COG1132, COG0438 and COG0745, and KEGG terms like K06147, K02029, and K09687.*

Conclusion: *This study emphasizes the role of the microbiome in LC patients without respiratory infection. These potential biomarkers of LC based on the taxonomic composition of pathogenic microorganisms might have clinical application.*

Key words: *lung cancer, pathogenic microbes, lung microenvironment, 16S RDNA sequencing, microbial classification*

Introduction

Lung cancer (LC) has a worldwide incidence with a high mortality rate. Non-small cell lung cancer (NSCLC) is the main type of LC. Previously, carcinogenic factors of LC have been widely studied [1]. However, previous studies mainly focused on genetic factors and well-known environmental factors [2].

Recently, epidemiological evidence has implied that the use of antibiotics can increase lung cancer risk [3], and lung microbial flora possibly impacts the development of LC [4-6]. Recently, studies have confirmed the variation of microecology in LC patients and that lung microbiome is different among patients with different clinicopathology [7,8]. How-

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ever, most studies supposed that there are microecology disorders and respiratory infection during LC development. There is a lack of research on lung microecology for LC patients without any respiratory infection. For those patients, the characteristics of pathogenic microbes are informative for understanding the microbe-driven tumorigenesis. Besides, most studies mainly focused on the LC tissues. From the perspective of diagnosis and monitoring multiple levels of detection, e.g., combination of blood, tumor tissues, peritumoral tissues, and broncho-alveolar lavage fluid (BALF), instead of single tumor tissues, are urgently needed to unravel the carcinogenesis process.

With the rapid development of molecular biology detection technology, the identification of pathogenic microorganisms is no longer limited to the detection of external morphological structure or physiological/biochemical characteristics. Taking advantage of the unique nucleic acid sequences, characteristics of pathogenic microorganisms can be identified with simple operation, low detection cost, and high specificity.

In this work, we applied pathogen targeted sequencing and 16S rDNA sequencing to describe the profiles of pathogenic microbes in lung microenvironment of NSCLC patients.

Methods

Patients

A total of 22 NSCLC patients (13 adenocarcinomas and 9 squamous cell carcinomas) were enrolled, including 14 males and 8 females, aged from 54 to 83 years (mean age 67.5). All these patients had stage III or IV. We collected 15 pieces of tumor tissues, 5 pieces of peritumoral tissues, 6 blood serum samples, and 5 BALF samples from these patients. Written informed consent was obtained from all participants before sampling, and all methods were carried out in accordance with hospital guidelines and regulations. This study was approved by the ethics committee of Shijiazhuang People's Hospital.

Pathogen targeted sequencing

We used the 15 tumor tissues, 5 peritumoral tissues, 6 serum samples, and 3 (out of 5) BALF samples to perform the pathogen targeted sequencing. Briefly, all samples were homogenized for nucleic acid extraction using the Zymo BIOMICS DNA/RNA Miniprep Kit (Zymo R2002), according to the manufacturer instructions. The nucleic acid products were collected for the construction of the pathogen-targeted high-throughput sequencing library. The Pathogeno One Pan-infectious Pathogen High-throughput Sequencing Library Construction Kit was used for library construction (Shanghai Bingyuan Medical Technology Co., Ltd. CatID Pathogeno 090118, Shanghai, China). In this process, two rounds of PCR were conducted. First, using the sample nucleic acid as template, 245 clinically significant microorganism-specific primers were added for amplification, which enriched the pathogenic target sequence; next, these products were purified by beads and the amplified using primers with sequencing adapters and different barcodes. After purification of the final amplified products by agarose gel electrophoresis, quality inspection and quantification were performed through the Qubit4.0 fluorometer. Normally, the library fragment size should be around 400 bp, and the library concentration should be at least 1 ng/μL. The concentration of the mixed library was re-quantified and then diluted to a final concentration of 4 nm. Next, 5 μL of the mixed library were added with 5 μL of freshly prepared NaOH (0.2 M). After vortexing and centrifuging at 280 g for 1 min, the library was placed at room temperature for 5 min. The denatured library was sequenced with Illumina MiSeq Reagent Nano Kit for high-throughput sequencing (average 0.05 M reads per library, sequencing read length=PE 60). The raw data was first identified by the linker, the reads whose doubleend length was greater than 60 bp were retained, and then low-quality filtering was performed to retain reads with Q30 >50% as high-quality data. The double-ended aligned reads were compared with the pathogen database to confirm the number of sequences (reads) in each sample. The intersection of detected pathogenic microorganisms in different samples (tumor tissue, peritumoral tissue, blood serum, and BALF) was expressed by the Venn diagram.

16S rDNA sequencing and microbial classification

We sent another two BALF samples for 16S rDNA sequencing and microbial classification. This step was performed by Sangon Biotech. Co., Ltd. (Shanghai, China). The DNA was extracted by the EZNA™ Mag-Bind Soil DNA Kit (Omega Biotech, Doraville, GA, USA), according to the manufacturer instruction. After quantification of genomic DNA using the Qubit3.0 DNA detection kit, a similar amount of DNA was added to the first step of PCR reaction: 4°C for 3 min→5 × (94°C for 30 sec→ 45°C for 20 sec→65°C for 30 sec)→20 × (94°C for 20 sec→55°C for 20 sec→72°C for 30 sec)→72°C for 5 min→10°C for 5 min. In this process, the action system was as follow: 2× Hieff® Robust PCR Master Mix 15μL, Bar-PCR primer F 1 μL, Primer R 1 μL, PCR products 10~20 ng, and H2O 9~12 μL (total volume 30 μL). The second PCR amplification was performed according to the following process: 95°C for 3 min→5×(94°C for 20 sec→55°C for 20 sec→72°C for 30 sec)→72°C for 5 min → 10°C for 5 min, and the reaction system was as follow: 2× Hieff® Robust PCR Master Mix 15 μL, Primer F 1 μL, Index-PCR Primer R 1 μL, PCR products 20~30 ng, and H2O 9~12 μL (total volume 30 μL).After electrophoresis, each library was validated by the 2% agarose gel, and the library concentration was determined using a Qubit 3.0 fluorescence quantifier. Next, the 16S rDNA sequencing was conducted. The PE reads obtained by Miseq sequencing were spliced according to the overlapping relationship, and OTU cluster analysis and species taxonomy analysis were then performed. For two BALF samples, the mean length was about 425 bp (min 354 and max 470). Based on taxonomic information, community structure was acquired teach classification level. Furthermore, the phylogenetic investigation

of communities by reconstruction of unobserved states (PICRUSt) system was used to predict flora metabolism function. In PICRUSt analysis, the flora composition data were mapped to known gene function profile databases. Here, we mainly analyzed the COG (Clusters of Orthologous Groups of proteins) and KEGG functions base on detected OTUs, and the abundance of COG and KEGG terms were presented as a heat map using the Rgplots package.

Results

Pathogenic microorganism analysis in tissues and liquid samples

First, 15 tumor tissues (11 adenocarcinomas and 4 squamous cell carcinomas) were used for pathogen targeted sequencing. Overall, 26 pathogenic microorganisms were found in these samples. For each sample, 8 sorts of pathogenic microorganisms were found in the same sample as the maximum. All pathogenic microorganisms and the detected number are presented in Table 1. Particularly, Staphylococcus haemolyticus was the most common pathogenic microorganism (found in 6 samples out of 15), followed by Enterococcus and Aerococcus viridans (in 4 samples of 15). In the 5 pieces of peritumoral tissues, 14 pathogenic microorganisms were found. Acinetobacter jungii was detected in four samples, and Streptococcus cristatus in three samples (Table 2). Other pathogenic microorganisms, including Epstein-Barr virus, Herpes simplex virus type 1, and Sphingomonas paucimobilis, were observed in 20% of the cases. Meanwhile, six blood serum samples were collected to probe the LC-associated pathogenic microorganisms in blood level. Twelve kinds of pathogenic microorganism were found (Table 3), Sphingomonas paucimobilis and Enterococcus were detected in 2 samples. Finally, we identified 18 pathogenic microorganisms in three BALF samples (Table 4). The high frequency microorganisms, including Actinomyces neseri, Peptostreptococcus, Streptococcus mitis, Enterococcus, Streptococcus, Caries acti nomycetes, Streptomyces granulosus, and Eikenella corrodans, appeared in all three samples.

Based on the above pathogenic microorganisms, the Venn diagram was generated to show the inter-

Table 1. Distribution of pathogenic microorganisms in 15 lung cancer tumor samples

Pathogenic microorganism	*Detected number*	*Min reads*	*Max reads*
Staphylococcus haemolyticus	6	5	1812
Enterococcus	4	14	45
Aerococcus viridans	4	24	266
Haemophilus haemolyticus	3	13	65
Streptococcus cristatus	3	7	1527
Streptococcus pneumoniae	3	61	1859
Actinomyces naeslundii	3	18	18
Pantoea agglomerans	2	28	71
Corynebacterium pseudodiphtheriticum	2	28	46
Human herpesvirus 7	2	15	2749
Porphyromonas gingivalis	2	81	91
Streptococcus sanguinis	2	28	51
Epstein-Barr virus	1	123	123
Acinetobacter baumannii	1	54	54
Mycobacterium smegmatis	1	121	121
Mycoplasma pneumoniae	1	1511	1511
Haemophilus parainfluenzae	1	14	14
Streptococcus gordonii	1	43	43
Moraxella catarrhalis	1	112	112
Haemophilus influenzae	1	30	30
Staphylococcus simulans	1	20	20
Acinetobacter junii	1	27	27
Abiotrophia defectiva	1	109	109
Mycobacterium tuberculosis	1	105	105
Peptostreptococcus	1	20	20
Gardnerella vaginalis	1	1051	1051

Table 2. Distribution of pathogenic microorganisms in 5 lung cancer peritumoral samples

Pathogenic microorganism	*Detected number*	*Min reads*	*Max reads*
Acinetobacter jungii	4	30	238
Streptococcus cristatus	3	8	13
Epstein-Barr virus	1	160	160
Herpes simplex virus type 1	1	602	602
Sphingomonas paucimobilis	1	98	98
Pale green balloon	1	178	178
Staphylococcus haemolyticus	1	6	6
Adenovirus	1	1199	1199
Corynebacterium pseudodiphtheria	1	11	11
Haemophilus parainfluenzae	1	19	19
Haemophilus haemolyticus	1	30	30
Mycobacterium intracellulare	1	74	74
Cryptococcus neoformans	1	17	17
Streptococcusconstellatus	1	11	11

Table 3. Distribution of pathogenic microorganisms in 6 lung cancer blood serum samples

Pathogenic microorganism	*Detected number*	*Min reads*	*Max reads*
Enterococcus	2	33	89
Sphingomonas paucimobilis	2	30	38
Epstein-Barr virus	1	29	29
Pantoea agglomerans	1	796	796
Corynebacterium pseudodiphtheriticum	1	17	17
Alternaria	1	13	13
Haemophilus influenzae	1	4	4
Actinomyces naeslundii	1	2669	2669
Acinetobacter junii	1	25	25
Abiotrophia defectiva	1	221	221
Staphylococcus haemolyticus	1	5	5
Adenovirus	1	83	83

Table 4. Distribution of pathogenic microorganisms in 3 lung cancer BALF samples

Pathogenic microorganism	*Detected number*	*Min reads*	*Max reads*
Enterococcus	3	2427	4823
Streptococcus mitis	3	951	1891
Streptococcus	3	1057	2746
Actinomyces naeslundii	3	1	171
Granulicatella adiacens	3	8	18
Actinomyces odontolyticus	3	352	1929
Peptostreptococcus	3	234	1718
Eikenella corrodens	2	2	21
Streptococcus pneumoniae	2	5	128
Streptococcus cristatus	2	2	13
Abiotrophia defectiva	2	72	121
Streptococcus constellatus	2	1	4
Candida albicans	1	1	1
Haemophilus parainfluenzae	1	8	8
Streptococcus gordonii	1	50	50
Haemophilus influenzae	1	2	2
Human herpesvirus 7	1	3	3
Haemophilus haemolyticus	1	4	4

section of our sample types (Figure 1). All detected microorganisms were put in each sample type. No common microorganisms were shared by four sample types. However, the following common elements were shared by three: Enterococcus, Actinomyces naeslundii, Haemophilus influenza and Abiotrophia defective were the 4 common elements in the tumor, BALF and blood serum, 2 common elements in the tumor, peritumor and blood serum were Staphylococcus haemolyticus and Epstein-Barr virus, and 3 common elements in tumor, peritumor and BALF were Haemophilus haemolyticus, Streptococcus cristatus, and Haemophilus parainfluenzae.

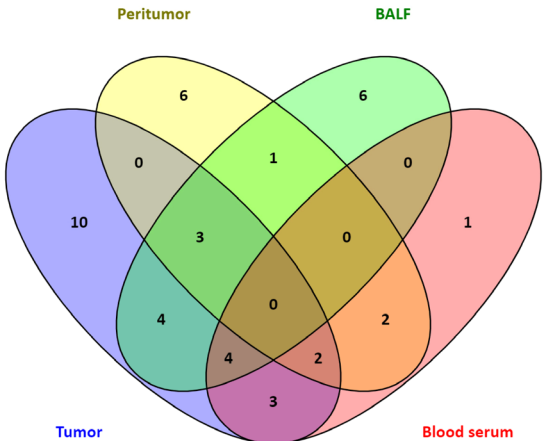

Figure 1. The Venn diagram of the intersection of four sample types of lung cancer (LC): tumor, peritumor, blood serum and bronchoalveolar lavage fluid (BALF). No common microorganisms were shared by four sample types. However, some common elements shared by three sets were as follows: 4 common elements shared by the tumor, BALF and blood serum, 2 common elements shared by the tumor, peritumor and blood serum, and 3 common elements in the tumor, peritumor and BALF.

16s rDNA sequencing and microbial classification in LC BALF

Based on the 16S rDNA sequencing of two BAFL samples, there were 104 OUTs found in one BALF sample and 127 OUTs in the other BALF sample; between them, there were 82 common ones, such as OTU1, OTU10, OTU101, OTU105, and

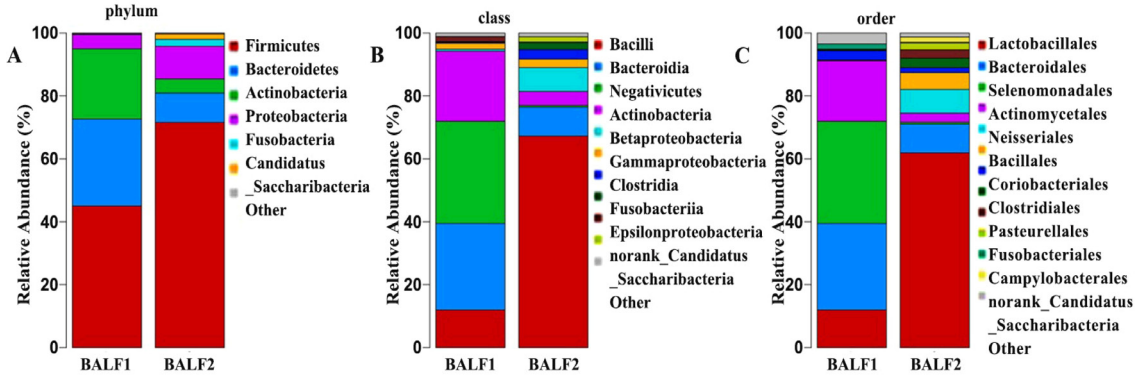

Figure 2. The relative abundance bar plot of16SrDNA sequencing of two BAFL samples, presented at the phylum **(A)**, class **(B)**, order **(C)** levels.

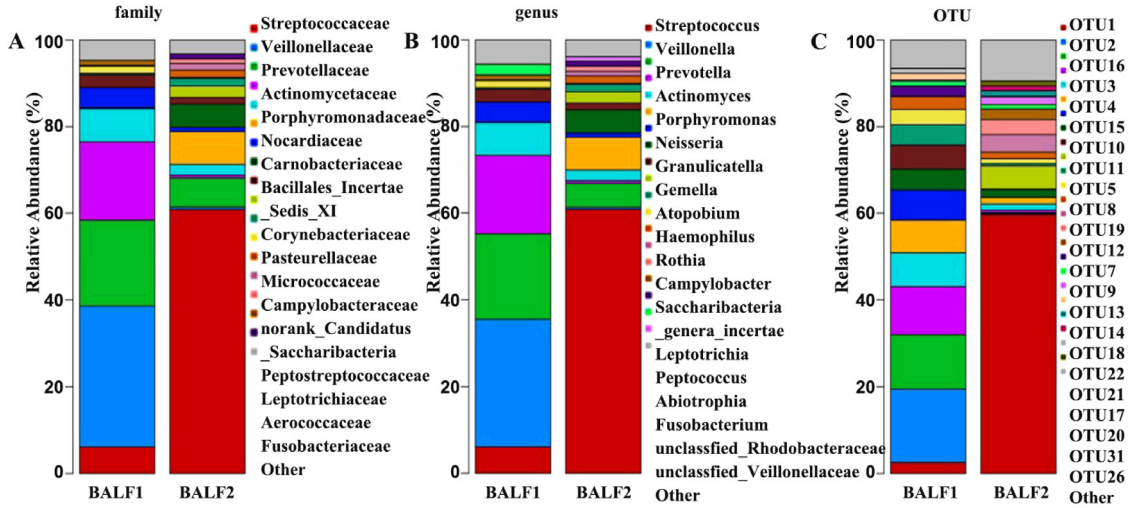

Figure 3. The relative abundance bar plot of16SrDNA sequencing of two BAFL samples, presented at the family **(A)**, genus **(B)**, and OTU **(C)** levels.

OTU106. Atthephylum, class, order, family, genus and OUT levels, and their relative abundances are shown in Figures 2 and 3, respectively. Overall, Firmicutes and Bacteroidetes (at the phylum level), Bacteroidia and Bacilli (at the class level), Streptococcaceae and Prevotellaceae (at the phylum level), Streptococcus and Prevotella (at the genus level), and OTU1, 3, 4, and 10 (at the OUT level) were abundant in the LC BALF. Based on the above microbial classification and abundance, COG function enrichment and KEGG enrichment were analyzed. The functional abundance heatmap is drawn (Figures 4 and 5): in the LC BALF, there might be the rich function in COG terms like COG1132 (ABC-type multidrug transport system, ATPase and permease components),COG0438 (Glycosyltransferase) and COG0745 (Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain) and KEGG terms like K06147 (ATP-binding cassette, subfamily B, bacterial), K02029 (polar amino acid transport system permease protein), and K09687 (antibiotic transport system ATP-binding protein). These functional abundances might be involved in the development of LC driven by pathogenic microorganism reasons.

Discussion

Recently, there has been growing interest in understanding the lung microbiome, and accumulating evidence has suggested that the onset of LC may be related to the changes of pathogenic microorganism composition. Metagenomics tools have provided a broad-scale analysis of microbial species, distribution, metabolic properties, and their potential impact on disease. Known pathogenic microorganisms were related to LC, including Haemophilus influenzae, Streptococcus pneumoniae, Acidotrophic bacteria, lebsiella, Moraxella catarrhalis, and Mycobacterium tuberculosis, etc [9-11]. Moreover, among individuals without a history of smoking, LC patients had more abundant Granulicatella, Abiotrophia and Streptococcus in the sputum samples [12-14]. In this field, the preliminary cognition was mainly regarding the modulatory role of the microbiome in chronic inflammation, which drives the development of LC. A recent study showed that the pulmonary symbiotic flora could cause inflammation related to lung adenocarcinoma by activating the γT cells residing in the lungs, and this is mediated by Myd88-dependent IL-1β and

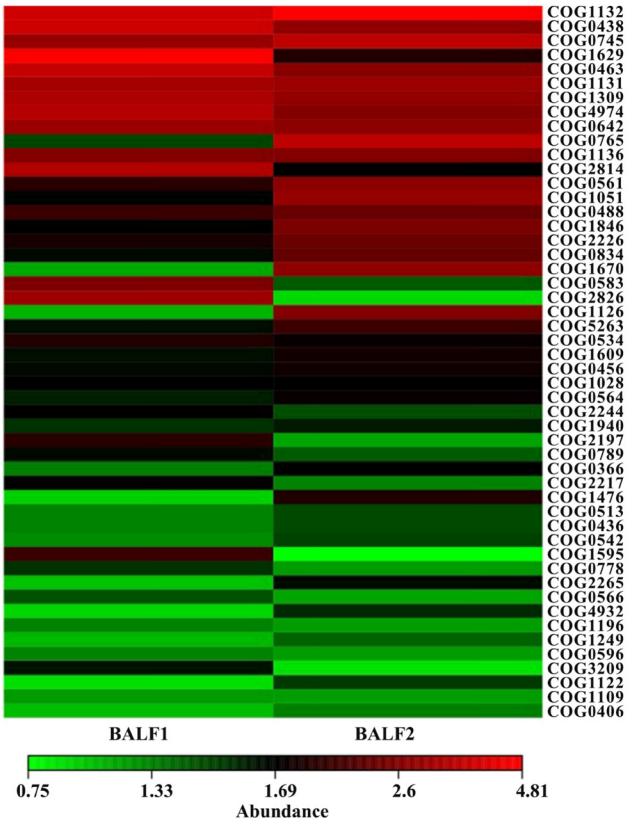

Figure 4. The functional abundance heatmap of COG function enrichment and KEGG enrichment based on microbial classification and abundance in the16S rDNA sequencing of two BAFL samples.

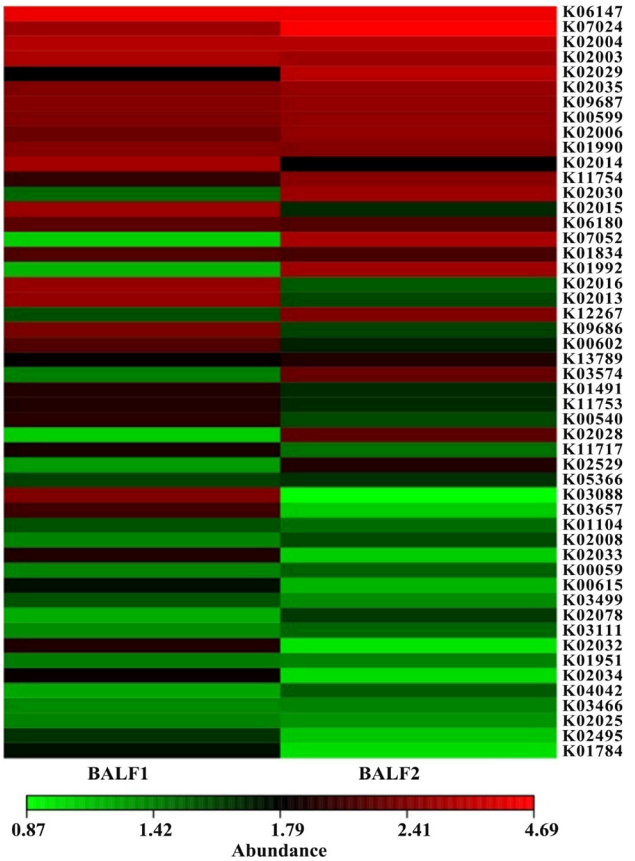

Figure 5. The functional abundance heatmap of COG function enrichment and KEGG enrichment of two BAFL samples.

IL-23, and their the promoted inflammation [15]. However, there are still many challenges to be unraveled. The influence of lung flora on the onset, progression, and drug resistance of LC are largely unclear, and many deep mechanisms are still to be elucidated. Additionally, no studies have parallelly observed the lung microbiome or the common pathogenic microbes in different types of LC samples. Furthermore, given the pulmonary infection and LC may impact each other, and the causal relationship between them has not been fully elucidated. This descriptive study, for the first time, used four types of samples from the LC patients without respiratory infection and revealed a link between the characteristics of pathogenic microbes in lung microenvironment and LC development. When the pulmonary infection is controlled, the variation derived from acute pathogenic microbial infection can be excluded. Therefore, the characteristics of pathogenic microbes in this study are more typical of the LC microenvironment. In this study, we observed some common pathogenic microorganisms shared by three sets, such as Enterococcus, Staphylococcus haemolyticus, Epstein-Barr virus, Corynebacterium pseudodiphtheria, Acinetobacter jungii, Haemophilus haemolyticus, Streptococcus cristatus, and Haemophilus parainfluenzae. Theoretically, these elements may have close links with NSCLC development. In comparison with previous studies, our results not only show consistency but also provide direct support. In 2017, a pilot study using metagenomic sequencing of the sputum microbiome suggested that the abundance of Enterococcus can be a potential bacterial biomarker for LC [16]. Another study focusing on the gut microbiome reported LC patients with elevated levels of enterococcus in the gut microbiota [17]. Besides, Cameron et al demonstrated that genera such as Streptococcus and Acinetobacter were significantly abundant in LC samples [16]. Additionally, never-smoking LC patients have differing sputum microbiota than controls, and a feature of these sputum samples was significantly varied Streptococcus abundance [18]. A retrospective analysis of the microbiological spectrum of pneumonia in Turkish LC patients showed an obvious difference from the Chinese patients; however, they also demonstrated that Haemophilus had significant genus difference in LC patients [19]. Moreover, referencing the 16S rDNA sequencing and microbial classification in LC BALF, Streptococcus, and Haemophilus are truly among the most abundant genera in LC samples. For example, OTU1 and OTU19 belong to Streptococcus, and OTU13 belongs to Haemophilus. However, there has been no existing evidence about the role of Staphylococcus Haemolyticus, Corynebacterium pseudodiphtheria, and Streptococcus cristatus in LC development. The oncogenic role of these pathogenic microorganisms is highly worth understanding. Mechanically, it is reasonable that pathogenic microbes (such as Haemophilus, Streptococcus, Acinetobacter, and Corynebacterium) can stimulate both innate and adaptive immune responses, and therefore can contribute to the pathogenesis of chronic lung diseases, even LC. Collectively, the changed microbes, in particular, Enterococcus, Haemophilus, and Streptococcus are closely related to the occurrence and development of LC.

In COG and KEGG enrichment analysis, a noteworthy finding is that some ATP-associated functions are enriched in LC samples. The enrichments in COG1132 (ABC-type multidrug transport system, ATPase and permease components), K06147 (ATP-binding cassette, subfamily B, bacterial) and K09687 (antibiotic transport system ATP-binding protein) suggest that LC development is closely linked to the ATP system. The associated mechanisms are as follows: An enhanced internalized ATP level may be a carcinogenic factor, which can induce epithelial-mesenchymal transition and early metastatic activities in LC [20-23]. Moreover, some ATP-binding cassette multidrug resistance transporter (e.g., ABCC2) may contribute to the development of various human cancers [24]. Conversely, ATP competitive tyrosine kinase inhibitor (e.g., Crizotinib) can target c-MET, ROS1, and ALK, which plays an anti-LC role [25], and ATP synthase blockade induces cancer cell death [26,27]. In future research, ATP-related signals are worthy of in-depth exploration in LC occurrence driven by pathogenic microorganisms and LC treatment.

However, the current study still has some limitations. First, this is a descriptive but not a comparative study. Due to the limited time and the difficulty of multi-type sampling, it is highly impracticable to compare the difference between normal individuals without lung infection and NSCLC patients without lung infection. Besides, the sample size of 16sDNA sequencing in this pilot study was small. We have tested another two tumor tissues and two peritumoral tissues, but no significant results were acquired (only BALF had adequate lung microbiome). In these two BALF samples, the common dominant flora needs to be further confirmed by more evidence. Moreover, due to the limited sample size, only two pathological types were included, and there were very few samples in some subtypes. Therefore, we have neither observed difference in microbiome composition among different clinicopathologies of NSCLC nor between adenocarcinomas and squamous cell carcinomas.

Conclusion

This novel study has expanded our knowledge of the microbiome in LC patients without respiratory infection. Some potential biomarkers of LC based on the taxonomic composition of pathogenic microorganisms could have clinical application. Further studies are needed to confirm the suggestive relationships between characteristics of pathogenic microbes in lung microenvironment and LC development/histology, as well as the underlying mechanisms.

Conflict of interests

The authors declare no conflict of interests.

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