Characteristics of Microbiome in Lung Adenocarcinoma Tissue from Patients in Southwestern China

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Abstract

Background:

Increasing evidences have unveiled the connection between microbiome and lung cancer. This study aims to identify the characteristics of microbial communities in the lung cancer tissues from patients in southwestern China, and to compare the distinct microbial genes at different clinical stages of lung cancer for uncovering potential immunotherapy targets.

Methods:

Forty samples of primary lung adenocarcinoma tissue were performed by 16S rRNA gene sequencing. The subjects were grouped according to TNM stages (T and N group), clinical stage and smoke status. To identify the taxa composition of each sample, Operational Taxonomic Units (OTUs) were classified on the Effective Tags with 97% identity. The linear discriminant analysis effect size (LEfSe) method was utilized to compare relative abundances of all bacterial taxa between non-metastasis group and metastasis group. The Shannon index of the 97% identity OTUs was calculated to evaluate alpha diversity. Beta diversity measurement was calculated using Principal Co-ordinates Analysis (PCoA).

Results:

A total of 951 OTUs were identified in the cancer tissues, including 224 overlapping genera. No significant difference has been found in the alpha diversity within all the groups. Beta diversity was significantly different in N group, T group and clinical stage group. By LEfSe analysis, nine differential species were identified in the N group, of which the relative abundance of genus *Bifidobacterium* was $10.78\% \pm 11.59\%$ in the N0 group and $20.15\% \pm 13.44\%$ in the N+ group ($p < 0.05$). In the T1 and T2 group, the LEfSe result identified 4 phylum and 10 genera. The differential genera were *Moraxella*, *Dolosigranulum*, *Corynebacteriaceae* and *Citrobacter* in the T2 group and *Bifidobacterium*, *Alistipes*, *Akkermansia*, *Blautia*, *Lactobacillus* as well as *Facelibacterium* in the T1 group. Differential bacterial composition and abundance were also observed in the clinical stage group.

Conclusions:

In conclusion, by 16S RNA sequencing, we identified dominant species of lung cancer tissue in different groups of AD patients. *Bifidobacterium* plays important role both in lymph node metastasis and tumor progression, which could provide specific immunotherapy strategy for lung cancer.

Introduction

Microbes function in majority of the biochemical activity on the earth, and they also influence physiological homeostasis in the human body, including metabolism and immunity. Many studies have uncovered that the abundance and composition of microbial communities vary from different body habitats, such as gut, skin and vagina, which have strong links to healthy conditions as well as human

diseases[1]. In the immune system, the recognition of symbiotic microbes is the key to awake immune protection. However, the host-protective immune responses may also arise counter-protective outcomes like inflammation and neoplasia [2]. *Helicobacter pylori* is the best-known bacterial pathogen of gastric cancer (GC). By local immune activation, *H. pylori* induces pathological changes and apoptosis of stomach epithelial cells and results in tumorigenesis[3].

Although the lung was considered sterile, emerging evidences have suggested that distinct microbiome exists in the lung and was associated with malignant transformation. By molecular techniques, D'Journo XB first tested the microbial gene in the distal airways of resected lung specimens and found that CMV was in a high rate of the samples[4]. Lee SH et al. collected bronchoalveolar fluids of lung masses patients and analyzed 16S rRNA. 288 genera were identified and the results suggested that the bacterial communities were differential between patients with lung cancer and with benign lung nodules [5]. Xinmin Yan et al. assessed the diversity of salivary microbiota and explored their relationship with lung cancer, suggesting that *Capnocytophaga* and *Veillonella* could serve as potential biomarkers for lung cancer because of their high proportion in the saliva from lung cancer patients [6].

The aforementioned studies focused on the tests of saliva, sputum specimens or bronchoalveolar fluids, which may be benefit for lung cancer screening while have limited reflections of the composition and abundance of microbial communities within the lung cancer tissue. K. Leigh Greathouse et al. detected the 16S rRNA gene sequencing on the lung tissue from 33 controls and 143 cancer cases, demonstrating a lower alpha diversity in the controls and identified *Acidovorax* enriched in smokers [7].

Indeed, there has been few studies exploring the correlation between microbial communities and clinicopathology of the lung cancer. One study analyzed the lung microbiota in 165 non-malignant lung tissue samples from cancer patients and found that the lung microbiota is distinct from the microbial communities in other tissues. Besides, advanced stage patients have higher concentrations of genus *Thermus* while patients with metastases have higher *Legionella*[8]. Another study also suggested that differential genera of microbiome existed in different histologic types and metastasis conditions of bronchial washing fluid (BWF) and sputum specimens from lung cancer patients[9]. Furthermore, many researchers have demonstrated that the biological patterns, genomic signatures as well as metastasis mechanisms are differential in various clinicopathology of lung cancer[10, 11].

Inspired by these clues, we hypothesized that there are certain correlations between the taxa composition of microbiome in the lung cancer tissues and clinicopathological stages of lung carcinoma, including smoke status, tumor stages (T), lymph node and distant metastasis condition. Since 85% lung cancer cases are non-small-cell lung cancer (NSCLC), of which adenocarcinoma (ADC) is more common than other types and have an increasing trend of incidence rate, we selected ADC patients as our subjects in this study. We tend to identify the characteristics of microbial communities in the ADC tissues from patients in southwestern China. In addition, we compare the differential microbial genes at different clinical stages of lung cancer to uncover potential microbiome functions on the tumor progression.

Materials And Methods

Subjects and Samples collection

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Kunming Medical University. Forty patients diagnosed with lung adenocarcinoma were recruited, of which 20 are males and 20 are females, with a mean age of 60.1 ± 8.1 years old (45–77 years). All the subjects were classified into 3 groups (T stage group, N stage group and Clinical stage group), according to T stage (T1 and T2), N stage (N0: without lymph node metastasis; N+: with lymph node metastasis) and clinical stage (I, II, III), respectively (Table 1). The diagnosis was confirmed by histology according to the 2015 World Health Organization Classification of Tumor of the Lung. The staging was assigned by the 8th edition of the Cancer Joint Staging Manual of the American Joint Commission on Cancer (AJCC). Since antibiotics would affect the microbiome, patients who had used oral or systematic antibiotics in the past three months were not included in our study. Besides, all the enrolled patients were injected preoperative antibiotics before surgery. Finally, a total of forty lung cancer samples were collected and all the lung cancer tissues were immediately stored at -80°C then sent to the laboratory for DNA extraction.

DNA Isolation and 16S rRNA amplification

Genomic DNA of bacteria in the lung cancer samples were extracted by CTAB method. DNA purity were detected with 1% agarose gel and the DNA concentration was diluted to 1 ng/ μL in sterile water. The diluted genomic DNA was used as templates and the 16S rRNA genes of V4 region were amplified using Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs) for amplification efficiency. PCR products were subject to vertical electrophoresis on 2% agarose gels and a colloid recovery kit (Qiagen, Valencia, CA) was applied to recover the target bands. Libraries were generated by the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, USA), and the concentrations were quantitated with a Qubit fluorometer and Q-PCR. Finally, the qualified library were sequenced by NovaSeq6000 (Illumina).

Analysis of sequence data

The original Raw Tags was obtained by splicing the reads using FLASH (v1.2.7), then filtered to acquire Clean Tags using Qiime (Version 1.9.1). To identify the taxa composition of each sample, Operational Taxonomic Units (OTUs) were classified on the Effective Tags with 97% identity using Usearch (Upase v7.0.1001) software. The presentative sequence of each OUT was annotated by RDP Classifier against the Silva (SSU123)16S rRNA database using confidence threshold of 80%, obtaining taxonomic classification at the Phylum, Class, Order, Family, Genus and Species level. Multiple sequence alignment was performed using MUSCLE3.6(Version 3.8.31)to further explore the phylogenetic relationships among different OTUs. Shannon index was performed by Qiime to descript alpha diversity. To determine beta diversity, Unifrac distance was calculated by Qiime and analyzed by principal coordinate analysis (PCoA) using R (Version 2.15.3). Microbiome abundance and diversity between different groups was calculated by t-test or Wilcoxon rank sum test and drawn by R. Linear discriminant analysis (LDA) effect size

(LEfSe) analyses were performed with the online LEfSe tool (<http://huttenhower.sph.harvard.edu/lefse/>). The LDA threshold score was 4.

Results

Baseline data of all the subjects

The subjects were grouped according to TNM stages, clinical stage and smoke status. No significant difference was found in age, gender and smoking status between different groups (Table 1). The lifelong exposure to smoking (cigarette pack-years = number of packs smoked per day × number of years of smoking) was 514.3 ± 229.5 in N0 group VS. 340.0 ± 256.6 pack-years in N+ group. There were 21, 14 and 5 subjects in clinical stage I, II and III group, respectively. No patient with distant metastasis was included in our study.

	N0	N+	P value
n	28	12	
Age	61.2 ± 6.9	57.7 ± 10.3	0.09
Gender			
Female	12 (42.9%)	8 (66.7%)	0.30
Male	16 (57.1%)	4 (33.3%)	
Smoke			
No	21	9	1.00
Yes	7	3	
T stage			
T1	21	6	0.15
T2	7	6	
Distant metastasis	0	0	
(T1: tumor stage 1; T2: tumor stage 2; N0: without lymph node metastasis; N+: with lymph node metastasis)			

OTUs analyses

OUT analysis showed that a total of 951 OTUS were identified in 40 samples. The dominant (> 1% average relative abundance) classifiable OTUs belonged to 5 phyla, including Firmicutes ($32.4\% \pm 12.1\%$), Proteobacteria ($24.5\% \pm 25.1\%$), Actinobacteria ($17.3\% \pm 11.3\%$), Bacteroidetes ($16.7\% \pm 14.0\%$), and

Verrucomicrobia (3.3%±4.2%). On the genus level, 21 genera were identified (> 1% average relative abundance) and the top 10 genera are shown in Fig. 1.

Analysis of microbial diversity within groups

Shannon index was calculated to evaluate the bacterial diversity within different groups and the result showed no significant difference (Fig. 2).

Analysis of microbial composition

To compare the composition of microbial communities in different groups, we calculate and analyzed the Weighted Unifrac distances, which was presented as PCoA plot. The result showed that there was dominant separation ($p < 0.001$, Fig. 3a) and significant difference (Fig. 3b) between N0 and N+ group. Most of the samples in the T1 group were more uniform and clustered distinctly away from the T2 group (Fig. 3c) and the comparisons were significant ($p < 0.001$, Fig. 3d). The separations were also found in clinical I, II and III group (Fig. 3e) and significant differences were found in either two groups. However, based on the smoke status, no significant difference was observed between smokers and non-smokers.

Analysis of differentially abundant taxa

We identified 224 genera in total, of which the common genera were shown as heatmap in differential stages and smoke status (Fig. 4). The top genera included *Moraxella*, *Bifidobacterium*, *Lactobacillus*, *Blautia*, *Akkermansia*, *Alistipes* and *Faecalibaculum*. To further identify specific species in every group, we use LEfSe method to find the differentially abundant taxa at each level. Firstly, in the N0 and N+ group, we found 9 differential species including two classes *Actinobacteria* and *Erysipelotrichia*, three families *Bifidobacteriaceae*, *Muribaculaceae* and *Erysipelotrichales*, two orders *Bifodobacteriales* and *Erysipelotrichales*, and one genus *Bifidobacterium* (Fig. 5a). On the cladogram, we can clearly see that the main specific species mainly belong to the two classes *Actinobacteria* and *Erysipelotrichia* (Fig. 5b). All the abundance of these species was higher in N+ group. The relative abundance level of genus *Bifidobacterium* was 10.78%±11.59% in the N0 group and 20.15%±13.44% in the N+ group ($p < 0.05$). In addition, in the T1 and T2 group, the LEfSe result identified 4 phylum and 10 genera. The dominant phylum were *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Proteobacteria* (Fig. 6a). The differential genera were *Moraxella*, *Dolosigranulum*, *Corynebacteriaceae* and *Citrobacter* in the T2 group and *Bifidobacterium*, *Alistipes*, *Akkermansia*, *Blautia*, *Lactobacillus* as well as *Facelibacterium* in the T1 group (Fig. 6b).

Discussion

People considered that the lung wasn't sterile until some researchers demonstrated that chronic obstructive pulmonary disease (COPD) was associated with lung microbiome [12]. Afterwards, evidences were unveiled between microbiome and lung cancer[4, 5, 8]. However, due to the difficulty of getting lung

cancer tissue samples, previous studies were always focus on the sample tissues from BWF and sputum specimens. In addition, as the oral cavity is connected with lung by air and upper respiratory tract, the oral microbiome is likely to affect and contaminate the lung microbiome[13]. Herein, studies directly on the cancer tissues are extremely pressing and important. Some researchers described associations between specific microbial diversity patterns and epidemiological exposures as well as associations between stages of disease with microbial composition, raising interesting mechanistic hypotheses[7–9]. In this study, based on stages and smoking status, we analyzed microbial diversity, composition and abundance on the tissue samples of lung adenocarcinoma. Because some investigators found that administration of antibiotics may reflect the inflammatory effects of repeat infections and changes in the composition of the lung microbiome[14], we excluded the subjects who had used oral or systematic antibiotics in the past three months to minus the antibiotic's effects.

Smoke is considered associated with both SCC and AD lung cancer while the association was stronger in SCC[15]. K. Leigh Greathouse et al. identified a specific taxa, *Acidovorax*, which was enriched in smokers with SCC[7]. Additionally, in a study of non-malignant lung tissue (n = 152), they observed a significant increase in alpha diversity with higher number of pack years of smoking[8]. In our AD samples, we did not find differential microbial diversity and composition between smokers and non-smokers, which may contribute to the different carcinogenesis of AD and SCC. Nevertheless, because the sample size was not big enough (30 in non-smoker group and 10 in smoke group), we need expand the sample size to further validate these results.

In our stage groups, we found no difference in the alpha diversity, indicating that the microbial diversity was uniform in our early AD tissues. Some researchers indicated that the non-malignant lung tissues had higher microbiota alpha diversity than the paired tumors, which also been previously shown for tumor/non-malignant samples from colorectal cancer patients[8, 16]. Unfortunately, we did not collect normal lung tissue to confirm whether difference exists between normal and lung tissues. It will be important to explore if the microbiota in lung tissue plays a role in non-malignant area and influence tumor progression or on the contrary.

In the lymph node metastasis and non-metastasis group, we identified two classes *Actinobacteria* and *Erysipelotrichia* and one genera *Bifidobacterium* (*Actinobacteria*) by LEfSe analysis. *Bifidobacterium* is common commensal in human oral, intestinal tract and vagina and is always believed to be nonpathogenic bacteria and beneficial to the host. However, increasing evidences demonstrated that *Bifidobacterium* is possible causing invasive human infection[17]. Especially, patients with positive *Bifidobacterium* blood culture always accompanied by gastrointestinal disease and/or impaired immunity[18]. In our study, the higher abundance of *Bifidobacterium* in the lymph node metastasis group may be related to the impaired immunity of cancer patients, which causing proliferation of this bacteria in the blood and lymph and further inducing lymph node metastasis of lung cancer cells. To verify this hypothesis, we need collect blood samples detecting abundance of *Bifidobacterium* from lung cancer patients in our future study. Interestingly, one study demonstrated that *Bifidobacterium* could enhance

host antitumor immunity by increasing CD8⁺ T cell and promote anti-PD-L1 efficacy, which provides us unique carcinogenesis and immunotherapy strategy of lung cancer[19].

In the tumor stage groups, the LEfSe result identified 4 phylum and 10 genera, of which genus *Bifidobacterium*, *Alistipes*, *Akkermansia*, *Lactobacillus* and *Facelibacterium* were existed mainly in the T2 group. The abundance of *Bifidobacterium* was also the highest among the dominant genera, suggesting the possible pathogenic mechanism between *Bifidobacterium* with tumor progression. *Akkermansia* specializes in mucin degradation and functions between mucosal layer of large intestine and host immunity. Decreased *Akkermansia* is associated with some disease like IBD and acute appendicitis[20]. Besides, some studies believe that *Akkermansia muciniphila* has an anti-tumor effect, especially in gastroenteric tumor[21]. Fecal microbiota transplantation (FMT) of *Akkermansia* could modulate efficacy of PD-1 immunotherapy[22]. However, in our study, we find decreased *Akkermansia* in the T1 group, which is contrary to its protective function in the gastrointestinal tract thus the pathogenic mechanism of *Akkermansia* needs to be explored in following study. *Alistipes*, belongs to *Bacteroidetes* phylum, has contrasting function on human metabolism and disease. It can protect host from diseases like liver fibrosis and colitis as well as promoting the incidence of colorectal cancer and metal depression[23]. Like *Akkermansia*, the abundance of *Alistipes* is higher in T2 group. These results suggest that microbiome in the lung tissues may promote the tumor progression despite some of which have protective function in intestinal diseases. Alternatively, tumor progression could also affect the microenvironment and microbiota of a larger surrounding area.

Some researchers identified specific species in the advanced stage of lung cancer, indicating that the differential microbiome function in the tumor development[8]. We also observed differential bacterial composition and abundance in the clinical stage group. Unfortunately, the sample size in the III stage group was only 5 and no tumor tissues of IV stage were collected, which could not represent the microbiome status in the advanced AD. We will enroll more cancer patients with advanced stage to demonstrate our guess.

In conclusion, by 16S RNA sequencing, we identified dominant species of lung cancer tissue in different groups of AD patients. *Bifidobacterium* plays important role both in lymph node metastasis and tumor progression, which could provide specific immunotherapy strategy for lung cancer.

Declarations

Acknowledgments

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Availability of data and materials :

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate:

All the subjects have signed informed consent for inclusion before participating in the study. The study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of The First Affiliated Hospital of Kunming Medical University Kunming.

Conflicts of Interest:

The authors declare no conflict of interest.

Funding:

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Authors' contributions

Sinuo Zhu and Xiaobo Chen designed the study and drafted the manuscript. Yunping Zhao and Yanan Bao collected the patients' samples and clinical information. Xuan He and Mengxiao Ye performed the 16S rRNA sequencing. Yue Cui and Xingming Zhu analyzed the OTU data. All the authors read and approved the final manuscript.

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Figures

Figure 1

Relative abundance of dominant genera in all the samples.

Figure 2

Taxonomic alpha diversity of lung cancer microbiomes within samples in different groups. (a) Comparison of Shannon index between N0 and N+ group. (b) Comparison of Shannon index between T1 and T2 group. (c) Comparison of Shannon index between clinical I, II and III group. (d) Comparison of Shannon index between smokers and non-smokers. No significant difference was found in all the groups.

Figure 3

Comparison of bacterial community structure (a) PCoA plot of N0 and N+ group. (b) Weighted Unifrac distance between N0 and N+ group with significant difference. (c) PCoA plot of T1 and T2 group. (d) Weighted Unifrac distance between T1 and T2 group. Significant difference was found between the two groups. (e) PCoA plot of clinical I, II and III group. (f) Weighted Unifrac distance between clinical I, II and III group. The differences were significant in every two groups. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4

Heatmap of common genera in differential stages and smoking status.

Figure 5

Differential taxa in the N stage group. (a) The result of LEfSe analysis between N0 and N+ group. Nine species were identified based on the LDA score and the abundance of which were higher in N+ group. (b) Means of abundance level on differential taxa between N0 and N+ group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6

Differential taxa in the T stage group. (a) Cladogram of differential taxa between T1 and T2 group. Dominant taxa were shown in red color as T1 group and green color as T2 group. (b) T-test results of relative abundance between T1 and T2 group. T1 was shown in orange column and T2 shown in blue column. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.