Diagnostic Value of the Microbiome in the Bronchoalveolar Fluid of Patients with Lung masses and its Relationship with their Clinical Features

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Research

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Abstract

**Background:** Bacterial communities were demonstrated to be correlated with patients with several respiratory diseases. Although some studies have been performed on the composition of the microbiota in lung cancer, the issue has not been fully addressed. Therefore, we characterized the microbiomes of patients with lung cancer and benign mass-like lesions and evaluated the relationship between microbiota and clinical features.

**Methods:** Bronchoalveolar fluid of patients with lung masses was collected and analyzed by 16S rRNA-based next-generation sequencing. Then, the relationships between the composition of the microbiota and clinical features were evaluated.

**Results:** The relative abundance of two genera, Megasphaera and Norank_p_Saccharibacteria, and two phyla, Firmicutes and Saccharibacteria, were significantly increased, while one phyla Proteobacteria was decreased in patients with lung cancer. The genera Atoprevotella and 1 phylum, Bacteroidetes, were increased in patients with SCLC, while 1 phylum, Chloroflexi, was more abundant in patients with NSCLC than those with SCLC. Moreover, the patients whose BALF was enriched with the genus Capnocytophaga seemed to have a better response to cisplatin-based chemotherapy.

The area under the curve of a combination of two genera (megasphaera and norank_p_Saccharibacteria) used to predict lung cancer was 0.803. The area under the curve of the genus Capnocytophaga in predicting the response to chemotherapy was 0.850.

**Conclusions:** There are differences in the composition of the microbiome of patients with lung cancer and those with benign mass-like lesions. The lung microbiota may be used as a biomarker for diagnosing lung cancer and differentiating the cancer subtype and might have an impact in the response to cisplatin-based chemotherapy among patients with lung cancer.

Background

In the past, it was thought that the lungs were sterile. However, increasing numbers of studies have revealed that there are rich and varied microbiomes in healthy lungs. Moreover, specific patterns of human microbiota are correlated with several lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis and cystic fibrosis.

Lung cancer is the most common cancer in the world, and it is the leading cause of cancer-related death worldwide. Recent studies have found that sputum samples of patients with lung cancer who had never smoked showed more predominant Granulicatella, Abiotrophia, and Streptococcus spp. and fewer OTUs relative to that of the controls.

In a previous study, the salivary microbiota was analyzed, and the levels of Capnocytophaga and Veillonella were significantly higher in the saliva from lung cancer patients. In another previous study of
bronchoalveolar lavage fluid (BALF), it was shown that the genera Veillonella and Megasphaera have the potential to become biomarkers of lung cancer. Yu et al. has demonstrated that in lung tissues, microbiota taxonomic alpha diversity increases with environmental exposures, and the genus Thermus is more abundant in tissue from advanced stage (IIIB, IV) patients, while Legionella is higher in patients who develop metastases. Moreover, the nonmalignant lung tissues have higher microbiota alpha diversity than the paired tumors. In Gomes's study, the lung cancer microbiota is enriched in Proteobacteria and more diverse in SCC than ADC, particularly in men and heavier smokers.

In our study, we characterized the profile of the microbiota of BALF in patients with benign and lung malignant masses and compared nonmalignant with lung cancer microbiota. Then, we analyzed the relationship between the microbiota and the clinical features of lung cancer patients. In addition, we evaluated the correlations of the lung microbiota with the chemotherapy response. Finally, we examined the prediction value of microbiota for lung cancer patients.

Our results indicated that specific microbiota are different from patients with benign and malignant masses, and we showed that differences exist in the bacterial communities of patients with non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) and that the genus Alloprevotella may affect the chemotherapy response.

Methods

Patients and samples collection

Patients who were admitted for the evaluation of lung masses were prospectively enrolled in Zhongshan Hospital, Fudan University, between January and May 2020. Patients were excluded if they met any of the following conditions: pregnant, had undergone any procedure other than bronchoscopy to evaluate the lung mass, treatment with antibiotics within 2 weeks, or a history of cancer of other systems.

All eligible patients underwent bronchoscopy. Prior to bronchoscopy, the patients received a topical anesthesia (lidocaine) by nebulizer. The bronchoscope was inserted for bronchoalveolar lavage (BAL). BAL was performed on the opposite side of the lung mass, and 10 ml of BALF was acquired from each patient using approximately 20–30 ml sterile 0.9 % saline.

A total of 34 patients were included in this study; 26 patients were diagnosed with lung cancer, and 8 were diagnosed with a benign mass-like lesion. Demographic and clinical data were obtained from each participant.

DNA extraction and PCR amplification

Microbial DNA was extracted from the BALF samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocols. The final DNA concentration and
purification were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the DNA quality was checked by 1% agarose gel electrophoresis.

The V3 to V4 regions of the 16S rRNA were amplified by a thermocycler PCR system (GeneAmp 9700, ABI, USA). The amplification was conducted using the following program: 5 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s for annealing at 55 °C, 30 s for extension at 72 °C, and a final elongation at 72 °C for 10 min. The PCR analyses were performed in triplicate in a 20 μL mixture containing 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The PCR products were extracted from a 2% agarose gel using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and QuantiFluor™-ST (Promega, USA) was used for quantifying the DNA according to the manufacturer’s protocol.

**Illumina MiSeq sequencing**

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

**Processing of the sequencing data**

Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window. (ii) Primers were exactly matched, allowing 2 nucleotide mismatching, and reads containing ambiguous bases were removed. (iii) Sequences whose overlap was longer than 10 bp were merged according to their overlap sequence.

Operational taxonomic units (OTUs) were clustered with 97% similarity cutoffs using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16SrRNA database using a confidence threshold of 70%.

**Statistical analysis**

Chao1 estimation and the Shannon diversity index were used for evaluating the richness and diversity of the samples. Random subsampling was conducted to equalize the read sizes of the samples for comparisons among samples. The overall phylogenetic distance between the two groups was estimated using Fast UniFrac and visualized by principal coordinate analysis (PCoA). Wilcoxon rank-sum and Kruskal–Wallis tests were used for differences between categories, and Spearman correlation tests were used for the associations of continuous variables. Continuous variables were compared between groups
by the Mann–Whitney test, and categorical variables were analyzed using Chi-square tests or Fisher’s exact tests. Data are shown as the median for continuous variables and number (%) for categorical variables. An adjusted p-value of < 0.05 was considered statistically significant. SPSS 19.0 was used to perform all statistical analyses in this study.

**Ethics statement**

The protocol for this prospective study was reviewed and approved by the Ethics Board of Zhongshan Hospital, Fudan University (approval number: B2020-019R). Informed consent was obtained from the patient on the day of admission to evaluate the lung mass.

**Results**

**Characteristics and taxonomic profiles of the patients with benign or malignant masses**

The median age of the study population was 61.5 years old (56 years old for the benign group vs. 63 years old for the malignant group), and 14 patients were female and 20 male. All of the patients underwent pulmonary function tests. In the lung cancer patients, 6 patients were diagnosed with small-cell lung cancer, and 20 patients were diagnosed with non-small-cell lung cancer. Of the latter, 14 patients had adenocarcinomas, 5 patients had squamous cell carcinomas and 1 patient had an adenosquamous carcinoma. Ten of the patients with NSCLC had an advanced stage, and 2 patients had an extensive type of small-cell lung cancer. Patients in this group have been examined regularly to date. Comorbidities were not significantly different between the two groups (Table 1).
### Table 1
Baseline characteristics of the study population.

| Variables                        | Patients with benign mass like lesion | Patients with lung cancer |
|----------------------------------|--------------------------------------|---------------------------|
| Age, yr                          | 56                                   | 63                        |
| Gender                           |                                      |                           |
| Male                             | 6                                    | 14                        |
| Female                           | 2                                    | 12                        |
| Smoking status(%)                |                                      |                           |
| Never smoker                     | 5                                    | 13                        |
| Ever smoker                      | 3                                    | 13                        |
| NSCLC                            |                                      | 20                        |
| Adenocarcinoma                   |                                      | 14                        |
| Squamous cell cancer             |                                      | 5                         |
| Adenosquamous carcinoma          |                                      | 1                         |
| SCLC                             |                                      | 6                         |
| Limited                          |                                      | 4                         |
| Extensive                        |                                      | 2                         |
| Benign mass                      | 8                                    |                           |

Microbial diversity, measured by the number of OTUs, ranged from 753 to 1803 with 97% similarity among the samples. The number of OTUs was not significantly different between the two groups. Meanwhile, the abundance and diversity of microbes in the two groups were not significantly different, either. By using BALF samples, we identified the phyla and genera in the benign mass-like lesion group (Fig. 1A) and the lung cancer group (Fig. 1B).

**Difference between lung tumor and nonmalignant tissue microbiotas**

To examine the composition of the specific bacteria between the benign and malignant mass groups, we evaluated the relative abundance of bacteria according to the mass type. We detected 34 phyla (Fig. 2A) and 616 genera (Fig. 2B) in all patients. Relatively dominant phyla (%) and genera (%) are presented in this figure. Bacteroidetes, Firmicutes, and Proteobacteria were the most common phyla, and Prevotella, Streptococcus, Veillonella and Neisseria were the most common genera in the study samples. The relative abundance of two genera, megasphaera (p = 0.014) and norank_p_saccharibacteria (p = 0.022);
and three phyla, Firmicutes (p = 0.018), Proteobacteria (p = 0.037), and Saccharibacteria (p = 0.02), were significantly increased in patients with lung cancer.

To evaluate the similarities of the microbiota profiles, a Fast Unifrac Analysis was performed (Fig. 3A, B). Figure 3 shows a 3(A) and a PCoA plot (B) of the microbiota from all patients according to mass type. There was no difference in β-diversity between the two groups.

**Association between microbiota and clinical features of lung cancer patients**

We have observed that microbiota differed significantly between NSCLC and SCLC (Fig. 4A). One genera Atoprevotella (p = 0.005) and 1 phylum Bacteroidetes (p = 0.049) were abundant in patients with SCLC, while 1 phylum Chloroflexi (p = 0.041) was enriched in patients with NSCLC. Furthermore, compared with lung cancer patients who had a CR/PR response to cisplatin-based chemotherapy, the relative abundance of one genera (Capnocytophaga, p (Bonferroni) = 0.033) and one phylum Bacteroidetes, p (Bonferroni) = 0.045 were found to be decreased in those whose best response was non-PR (Fig. 4B). However, no major differences were observed by metastatic status (Fig. 4C). At the genus level, the relative abundance of Capnocytophaga differed significantly based on the smoking histories of the lung cancer patients (p = 0.024) (Fig. 4D).

**The prediction value of microbiota for lung cancer patients**

To better evaluate the prediction value of microbiota for the differentiation of lung benign and malignant masses, we constructed receiver operating characteristic (ROC) curves with norank_p_Saccharibiotera and/or Megasphaera. The area under the ROC (AUC) value of Megasphaera was 0.793 (p = 0.013) and that of norank_p_Saccharibiotera was 0.769 (p = 0.023). The combination of the two genera showed a higher AUC value than either alone (AUC = 0.803, p = 0.011) (Fig. 5A). Meanwhile, the AUC values of Firmicutes, Saccharibiotera and proteobacteria were 0.779, 0.764 and 0.750, respectively (Supplementary Fig. 1A, B). The combination of the Firmicutes and Saccharibiotera showed a higher AUC value (AUC = 0.909, p = 0.001) than either alone or a combination of the three phyla (AUC = 0.894, p = 0.001) (Supplementary Fig. 1C).

Subsequently, to better understand the role of the microbiota in lung cancer treatment, we evaluated the prediction value of microbiota for the response to cisplatin-based chemotherapy. The AUC value of the genus Capnocytophaga was 0.854 (p = 0.028) (Fig. 5B).

**Discussion**

In previous studies, lung microbiota were examined in patients with asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, and lung transplantation. It was shown that the lung
microbiota was unique and different from the digestive tract microbiota. It may have an influence on the immune system and metabolism. Although several studies have been performed on the composition of the microbiota in lung cancer, it still remains quite unclear.

In this study, we characterized the microbiota in BALF samples from 34 patients with benign or malignant mass-like lesions and analyzed the correlation between several clinical features and specific microbiota. Our results revealed that the composition of the microbiota of BALF samples from lung cancer is different from that of benign mass-like lesions and could be used as biomarkers in the diagnosis of lung cancer and prediction of the chemotherapy response.

The predominant bacterial phyla in the lung microbiota of healthy adults include Firmicutes, Proteobacteria and Bacteroidetes. Several studies have shown that the Firmicutes phylum is predominant in lung tissues and the sputum of COPD patients compared with that of healthy adults. Our results confirmed that the abundance of the Firmicutes phylum is increased in lung cancer patients, consistent with the results of Lee et al. Moreover, an increase in the phylum Saccharibacteria (TM7) was observed in both COPD and lung cancer cases, indicating that Firmicutes and Saccharibacteria might play a potential role in the transformation of COPD into lung cancer, since COPD is one of the risk factors for lung cancer.

Furthermore, the level of Proteobacteria in the lower airway was demonstrated to be correlated with asthma. Patients with COPD have relatively more abundant Proteobacteria than controls, whereas Millares et al. showed that the abundance of Proteobacteria was elevated in COPD patients. Until now, no studies have reported the relationship between Proteobacteria and lung cancer. Our results revealed that the level of Proteobacteria was reduced in lung cancer patients. Therefore, the relationship between Proteobacteria and lung cancer needs further confirmation. Recently, the genera Megasphaera and Veillonella were reported to be diagnostic markers for lung cancer. Similar to a previous study, we have confirmed that the abundance of Megasphaera was increased in lung cancer patients. However, Veillonella was observed to be increased in lung cancer patients but had no significance.

Different from other studies, the results of our study showed the enrichment of norank_p_Saccharibacteria in the BALF of patients with lung cancer. Additionally, the area under the curve (AUC) of a combination of these two genera used to predict lung cancer was 0.803, and the AUC of a combination of the phyla Saccharibacteria and Firmicutes was 0.909. Therefore, a combination of these two genera or two phyla both showed significantly high AUC values in predicting lung cancer and could serve as biomarkers for lung cancer or microbial therapeutic targets for the disease.

Several studies have reported diverse pulmonary complications and enhanced death rates after treatment of lung cancer patients carrying potential pathogenic microorganisms in their airways with chemotherapy. We evaluated the relationship between microbiota and the response to chemotherapy. The results showed the patients whose responses were PR (partial recovery)/CR
(complete recovery) have enrichment in Capnocytophaga at the genus level and Bacteroidetes and Nitrospirae at the phylum level compared with patients with SD (stable disease) /PD (progression of disease). Although we were unable to rigorously address the impact of microbiota in cancer treatment, our findings suggest the need for a differentiated medical intervention in these patients.

For some patients, it is hard to differentiate non-small-cell lung cancer (NSCLC) from small-cell lung cancer (SCLC). In our study, we found that the genus Alloprevolla as well as the phylum Bacteroidetes were enriched in patients with NSCLC rather than SCLC, indicating that these microbial differences could be characteristic of NSCLC. The detection of these two microbiota might help to diagnose the subtype of lung cancer.

In Gomes's study, it was shown that high frequencies of Proteobacteria were found to discriminate a major cluster, and the lung cancer microbiota was more diverse in SCC and ADC, particularly in men and heavier smokers\(^1\). Additionally, in Yu's study, higher phylogenetic diversity with an increased relative abundance of Thermus and a decreased relative abundance of Ralstonia was observed in adenocarcinoma compared with squamous cell carcinoma, implying that the microbiota might be correlated with cancer histology, while Legionella was highly abundant in metastasis cases\(^9\). However, in our study, the microbiota of patients with lung cancer did not differ in terms of cancer histology and metastatic status.

Additionally, our study showed there were significant differences in the relative abundance of specific microbiota in lung cancer patients according to their smoking histories. This might mean that environmental factors, such as smoking, can increase the risk of lung cancer by altering the microbial composition.

The current study has some limitations. First, the size of the study sample was too small to validate the correlation between specific microbiota and the clinical features of lung cancer patients. Large-scale clinical trials are needed to confirm the influence of these microbiota on patients with lung cancer. Second, in our study, lung function, daily diet and BMI were not considered. Those factors may have effects on the composition of the microbiota.

**Conclusion**

Our study showed that there are differences in the composition of the microbiome of patients with lung cancer and those with benign mass-like lesions. Our study also showed that the genus Megasphaera and norank_p_Saccharibacteria might serve as biomarkers in the diagnosis of lung cancer, and the genus Atoprevotella could serve as a biomarker for differentiating NSCLC from SCLC. The genus Capnocytophaga might be used as a biomarker for the response to chemotherapy. Further large-scale studies are needed to validate the role of the microbiome in patients with lung cancer.

**List Of Abbreviations**
chronic obstructive pulmonary disease (COPD)
bronchoalveolar lavage fluid (BALF)
non-small-cell lung cancer (NSCLC)
small-cell lung cancer (SCLC)
Operational taxonomic units (OTUs)
principal coordinate analysis (PCoA)

Declarations

Ethics approval and consent to participate

The protocol for this prospective study was reviewed and approved by the Ethics Board of Zhongshan Hospital, Fudan University (approval number: B2020-019R). Informed consent was obtained from the patient on the day of admission to evaluate the lung mass.

Consent for publication

The human participants involved in this research consent to publish the manuscript and all the authors agree with the submission and publication of this manuscript.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Fundings

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Authors' contributions

MSY, JZ and SDW collected the samples from the patients and performed the major experimental work. JRX helped with the analysis of the data. SW helped to draft the manuscript. YS and LT helped to revised the manuscript. XZ and NX participated in the design of the study, supervised the laboratory work. All authors read and approved the final manuscript.

Acknowledgement

The authors declare that they have no competing interests.

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