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Diminishing microbiome richness and distinction in the lower respiratory tract of lung cancer patients: A multiple comparative study design with independent validation

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ABSTRACT

Objectives: Current evidence suggests that microorganisms are associated with neoplastic diseases; however, the role of the airway microbiome in lung cancer remains unknown. To investigate the taxonomic profiles of the lower respiratory tract (LRT) microbiome in patients with lung cancer.

Materials and methods: BALF samples were collected in a discovery set comprising 150 individuals, including 91 patients with lung cancer, 29 patients with nonmalignant pulmonary diseases and 30 healthy subjects, and an independent validation set including 85 participants. The samples were assessed by metagenomics analysis. Random forest regression analysis was performed to select a diagnostic panel.

Results: In the discovery set, richness was reduced in lung cancer patients compared with that in healthy subjects, and the microbiome of patients with nonmalignant diseases resembled that of patients with lung cancer. Interestingly, *Bradyrhizobium japonicum* was only found in patients with lung cancer, whereas *Acidovorax* was found in patients with cancer and nonmalignant pulmonary diseases. A microbiota-related diagnostic model consisting of age, pack year of smoking and eleven types of bacteria was built, and the area under the curve (AUC) for discriminating the patients with cancer was 0.882 (95%CI: 0.807-0.957) in the training set and 0.796 (95%CI: 0.673-0.920) in the independent validation set.

Conclusion: Our study demonstrates that the LRT microbiome richness is diminished in lung cancer patients compared with that in healthy subjects and that microbiota-specific biomarkers may be useful for diagnosing patients for whom lung biopsy is not feasible.

1. Introduction

The risk factors for lung cancer remain poorly understood, and late diagnoses hamper the survival of these patients. Current evidence suggests that the human microbiome is associated with neoplastic diseases [1]. Oncogenic viruses (e.g., papilloma virus), bacteria (e.g., *Helicobacter pylori*), and helminths (e.g., *Schistosoma haematobium*) can be targeted by appropriate antimicrobials to prevent cancer [2]. However, the subtle relationship between the microbiome and lung cancer remains unclear.

The microbiome is defined as an “ecological community of commensal, symbiotic and pathogenic organisms that share our body space”

Abbreviations: LRT, lower respiratory tract; BALF, Bronchoalveolar lavage fluid

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[3]. Although the lungs of healthy humans have historically been considered sterile, recent studies demonstrate that the lower respiratory tract (LRT) is replete with a diverse community of microorganisms [4]. Prominent genera in the healthy lung have been reported, including *Streptococcus*, *Prevotella*, and *Veillonella* [5–8]. Moreover, an imbalanced lung ecosystem may predispose individuals to the development of respiratory diseases [8–11], such as asthma, COPD and cystic fibrosis [12–16]. Additionally, recent studies have demonstrated quantitative and/or qualitative changes in the microbiome of lung cancer patients compared with that in paired nonmalignant tissues or benign diseases [17,18], indicating that the lung microbiome may contribute to lung cancer tumorigenesis.

Regardless, previous studies have generally yielded conflicting results, and the characteristics of the LRT microbiome in lung cancer remain largely unknown. Relevant studies to date have been based on 16S rRNA gene sequencing, which can distinguish among different types of bacteria at the genus level. The aim of the current study was to explore the LRT microbiome at the species level by metagenomics analysis. Taxonomic profiles of BALF microbiomes from healthy subjects, patients with lung cancer and nonmalignant diseases were characterized via metagenomics analysis, and associations between the profiles and clinical features were evaluated.

2. Methods

2.1. Study population

We prospectively enrolled 205 patients who underwent bronchoscopy at the Bronchoscope Center, Division of Pulmonary and Critical Care Medicine, West China Hospital, Sichuan University between January 2015 and December 2016. All patients were followed up to confirm the final diagnosis. A total of 120 patients were enrolled in the discovery set, among whom 91 were pathologically diagnosed with lung cancer and 29 with nonmalignant pulmonary disease. Additionally, 85 patients were established as an independent set to validate the biomarkers found using the discovery set. Nonmalignancy was defined as negative pathological and radiologic evidence. Patients with obvious infection and other malignancies were excluded.

As a healthy control group, 30 subjects with normal CT imaging, spirometry, ECG and laboratory tests (CBC, Chemical profiles, urine and stool test) were recruited from West China-Guangan Hospital, Guangan, Sichuan. Clinical data were collected from medical records and questionnaires. This study was approved by the West China Hospital of Sichuan University Biomedical Research Ethics Committee. All patients enrolled in the study provided written informed consent.

2.2. Sample collection

Bronchoscopy was performed in all eligible participants before treatment. Prior to bronchoscopy, 10 to 50 ml of sterile 0.9% saline was washed through the bronchoscope and collected to serve as a negative control. The bronchoscopy was performed according to standardized procedures developed to minimize oral contamination [19]. The bronchoscope was quickly wedged into a segmental bronchus adjacent to a lesion in patients with pulmonary disease and the right middle lobe bronchus in healthy subjects without suction. A total of 50–100 ml of sterile 0.9% saline was instilled into the bronchus and then recovered by gentle hand suction. BALF was collected from bilateral bronchi in 26 patients with lung cancer. Briefly, samples were collected within 30 min and placed on ice before storing at −80 °C.

2.3. DNA isolation and metagenomics library preparation and sequencing

Microbiota data were analyzed by metagenomics. DNA was extracted from BALF samples using a TIANamp Micro DNA Kit (DP316, TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. DNA libraries were subsequently constructed, assessed and sequenced using the BGISEQ-100 platform (BGI, Shenzhen, China) [20]. Classification reference databases were downloaded from NCBI. The RefSeq database contains 1,494 bacterial genomes or scaffolds associated with human diseases.

Table 1
Baseline characteristics of patients.

Variables	Lung cancer N = 91	Non-malignant N = 29	Healthy N = 30	P
Age (range), years	57.82(40-76)	50.72(23-80)	50.57(27-72)	**< 0.001** *
Sex				0.125
Male	64(70.3%)	18(62.1%)	15(50%)	
Female	27(29.7%)	11(37.9%)	15(50%)	
Smoking status				**0.006**
Never smoking	32(36.3 %)	17(60.7%)	17(56.7%)	
Ex-smoker	28(31.8%)	6(21.4%)	0(0.0%)	
Current smokers	28(31.8%)	5(17.9%)	13(43.3%)	
Pack- year	21.19(0-180)	6.93(0-50)	8.88(0-30)	**0.016**
Using of Antibiotics				0.797
Yes	11(12.8%)	5(17.9%)	4(13.3%)	
No	75(87.2%)	23(82.1%)	26(86.7%)	
History of Cancer				0.238
Yes	12(13.6%)	7(25.0%)	3(13.3%)	
No	76(86.4%)	21(75.0%)	27(86.7%)	
History of TB	5(5.5%)	4(13.8%)	0	–
Stage			–	
I	20 (24.7%)	–	–	–
II	17 (21.0%)	–	–	–
III	22(27.2%)	–	–	–
IV	21(25.9%)	–	–	–
Histology				–
AC	49(53.8%)	–	–	–
SCC	26(31.9%)	–	–	–
SCLC	7 (7.7%)	–	–	–
Other	9 (9.9%)	–	–	–

SCC: squamous cell carcinoma; AC: adenocarcinoma; SCLC: small cell lung cancer; COPD: chronic obstructive pulmonary disease; TB: tuberculosis; Other: including non-small cell lung cancer (unclassified), adenosquamous carcinoma.

* $P < 0.05$.

2.4. Statistical analysis

To account for potential variations in sequencing efficacy, we transformed read abundances into percentages based on the total number of high-quality mapped sequences for each sample at all taxonomic levels of classification. These normalized percentages were used in all subsequent data and statistical analyses, with comparisons performed between the lung cancer, nonmalignant disease, and healthy groups. In the final analysis, we removed species for which less than 10 times the number was detected in the negative control samples The Wilcoxon rank-sum test and Kruskal–Wallis test were used to assess differences between categories. Furthermore, we performed BH correction on the P value, which is the Q value herein, and all Q values less than 0.05 were considered significant. Principal coordinate analysis (PCoA) was conducted using species abundance profiles. PERMANOVA was applied as a statistical test for PCoA. Associations between taxonomic assignments and different groups were explored using random forest regression. Detailed information on the method is presented in the supplements. The performance of the microbiome-related model was evaluated by the value of the AUC. All statistical analyses were performed using R software and SPSS 20.0. A double-sided P value < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the study participants

A total of 150 participants were included in the discovery set, and

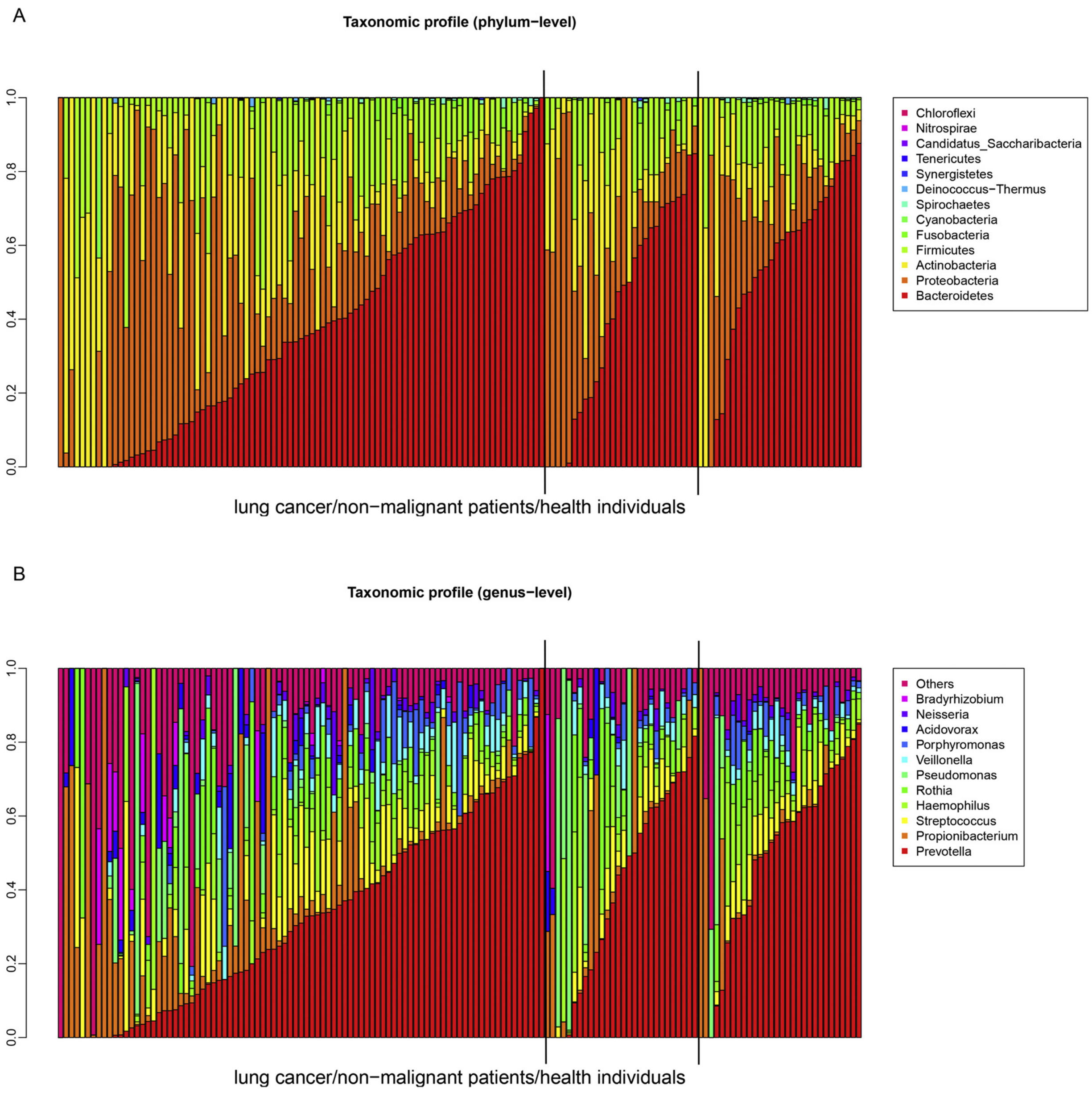

Fig. 1. Taxonomic profiles of the lung microbiota of lung cancer patients, patients with nonmalignant disease, and healthy individuals. (A) Phylum-level taxonomic profiles. (B) Genus-level taxonomic profiles. Each vertical bar represents a unique sample. Samples were ordered by different groups shown below the figure. The y-axis shows the relative abundance of each phylum/genus. Only the most common taxa are shown.

85 patients were included in the validation set. The clinical characteristics of all subjects are provided in Table 1 and supplemental E-table 1. Patients with lung cancer were older and had higher cumulative tobacco exposure compared with the nonmalignant and healthy control groups (all $P < 0.05$).

3.2. Taxonomic profiles of the LRT microbiome in all participants

Taxonomic analyses were conducted at the phylum, genus and species levels based on relative abundance. The dominant lung microbiota is defined as taxa with a relative abundance over 0.05. At the phylum level, Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes predominated in the LRT microbiome of all participants (Fig. 1A). At the genus level, the dominant lung microbiota in patients with lung cancer was Prevotella, belonging to Bacteroidetes (Fig. 1B). The relative abundances of the genera *Propionibacterium*, *Streptococcus*, *Rothia*, *Haemophilus* and *Pseudomonas* were greater than 0.05. *Prevotella intermedia*, *Prevotella melaninogenica*, and *Propionibacterium acnes* were the dominant species in patients with lung cancer.

The dominant species in the healthy and nonmalignant groups were similar to those in the patients with lung cancer. The phyla and genera that predominated in each group are presented in E-table 2.

3.3. Decreased richness of the LRT microbiome in patients with lung cancer

Of all the bacteria noted herein, 77 (32.1%) species in patients with lung cancer, 24 (10.0%) in healthy individuals and 8 (3.3%) in patients with nonmalignant pulmonary disease were present in the LTR microbiome (Fig. 2).

Patients with lung cancer showed significantly reduced LRT richness compared to healthy individuals ($P = 0.018$, Fig. 3A) but a significantly higher Shannon evenness index ($P = 0.011$). Furthermore, less LRT richness was observed in patients with nonmalignant pulmonary disease compared to healthy patients ($P = 0.024$).

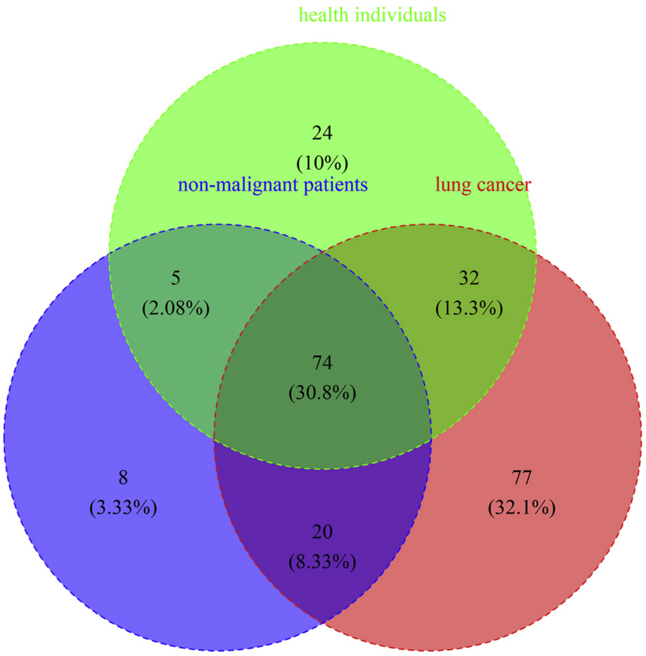

Fig. 2. Venn diagram of the three groups: lung cancer patients, patients with nonmalignant disease, and healthy individuals. A total of 240 types of bacteria were identified, as follows: 203 types of bacteria were detected in lung cancer, 158 in nonmalignant patients and 135 in healthy individuals. Among all the bacteria, 77 (32.1%) distinctive species were found in lung cancer, 8 (3.33%) distinct species were found in patients with nonmalignant disease, and 24 (10%) distinct species were found in healthy individuals.

The levels of *Bacteroides*, *Cryptobacterium* and *Fusobacterium* and 11 types of bacteria were significantly different between the three groups (all $Q < 0.05$). Furthermore, *B. japonicum* was only found in patients with lung cancer. Interestingly, *Acidovorax* sp. JS42 and *Acidovorax ebreus* were enriched only in patients with pulmonary disease, and neither was detected in healthy subjects. More information on the differential bacterial species is provided in E-table 3.

At the phylum level, Bacteroidetes, Fusobacteria, and Spirochaetes were less abundant in the cancer group than in the healthy group (all $Q < 0.05$, Fig. 3B). The abundances of 8 genera were reduced in patients with cancer compared with healthy individuals (all $Q < 0.05$, Fig. 3B). Prevotella, the predominant microbe in healthy individuals, was significantly reduced in abundance in patients with lung cancer ($Q = 0.038$). At the species level, the relative abundances of 15 types of bacteria were significantly decreased in the lung cancer patients (E-Table 4, Fig. 3D). In addition, beta diversity was significantly different between patients with lung cancer and healthy subjects at the species level (Fig. 3C, $P = 0.004$), with *Haemophilus influenzae* showing the greatest difference between the two groups ($Q = 0.004$).

The microbiome of patients with nonmalignant disease generally resembled that of patients with lung cancer, and we noted no significant differences in diversity between the two groups (Fig. 3A). Indeed, no species differed significantly between the two groups (E-Table 5). There was also no significant difference in diversity or relative abundance at the phylum level between the healthy controls and patients with nonmalignant disease.

Among 26 bilateral BALF samples from patients with lung cancer, taxonomic analysis showed that the microbiome was similar between two sides at the phylum and genus levels. Additionally, alpha diversity did not differ between bilateral BAL samples.

3.4. Associations between demographic and clinical characteristics and the LRT microbiome in patients with lung cancer

Several clinical characteristics were associated with the microbiome of the LRT in patients with lung cancer, such as sex, smoking status, history of cancer and tuberculosis, histology, stage and use of antibiotics. However, there were no significant associations between these clinical characteristics and the diversity of the LRT microbiome. The abundance of *A. ebreus*, which was enriched only in patients with pulmonary disease, was higher in female compared with male lung cancer patients (male vs. female: 0.25% and 1.20%, respectively; $P = 0.017$). Furthermore, the abundance of *H. influenzae*, which was one of the biomarkers, increased in patients who were current smokers compared with other patients ($P = 0.039$). However, the high proportion of *Bradyrhizobium* sp. BTAi1, which belongs to the same genus as *B. japonicum*, increased in those with early-stage disease ($P = 0.024$). Although we noted no differences in the abundances of *B. japonicum* between tumors of different histological types in those with cancer ($P = 0.607$), patients with squamous cell carcinoma and adenocarcinoma showed a higher level of this species than did patients with small cell lung cancer. Furthermore, increased levels of *B. japonicum* were observed in patients with early-stage tumors compared with those in patients with advanced-stage tumors (I & II vs. III & IV: 1.5% vs. 0.87%; $P = 0.162$).

3.5. Bacterial markers that identify patients with lung cancer

In the discovery set, a diagnostic model was built based on age, pack year of smoking and 11 types of bacteria (*Prevotella melaninogenica, Streptococcus* sp. I-P16, *Corynebacterium urealyticum, Acidovorax* sp. KKS102, *Pseudomonas aeruginosa, Streptococcus sanguinis, H. influenzae, Streptococcus pseudopneumoniae, Bacteroides salanitronis, Campylobacter concisus,* and *B. japonicum*) though random forest regression. We then performed receiver operating characteristic (ROC) analysis to evaluate the preclinical utility of several potential biomarkers for the diagnosis of lung cancer (Fig. 4), and the AUC was 0.882 (95%CI: 0.807-0.957). Fifty-seven patients with lung cancer and 28 with nonmalignant pulmonary diseases were subsequently enrolled in the validation cohort. The AUC for the validation cohort was 0.796 (95%CI: 0.673-0.920), confirming that the microbiota-targeted biomarkers may be useful in identifying patients with cancer. Detailed information on the contribution of different variables in the model is provided in the supplement (sFig. 2). Furthermore, *B. japonicum* was only found in patients with lung cancer, and *Acidovorax* sp. JS42 and *A. ebreus* were only detected in healthy participants.

4. Discussion

The intrinsic relationship between the microbiome and pulmonary diseases is currently under investigation. Novel relevant works in this field have revealed that the distal airways of humans harbor several bacterial species [21,22]. Most studies have been performed on the gut microbiome of gastrointestinal cancer [23–26]. Results to date indicate that microbes present at mucosal sites may become part of the tumor microenvironment of aerodigestive tract malignancies and that intratumoral microbes may affect cancer development and progression in many ways. In contrast, the microbiota of healthy humans may function by producing various detoxifying compounds, maintaining a balance between host cell proliferation and death and reducing inflammation to regulate the immune system.

The current study explored the profiles of the LRT microbiome in patients with lung cancer and nonmalignant pulmonary diseases and healthy individuals and assessed differences between the profiles by metagenomics analysis. Our results demonstrate the structure of the LRT microbiome in lung cancer and reveal that richness was diminished in patients with lung cancer compared with that in healthy individuals.

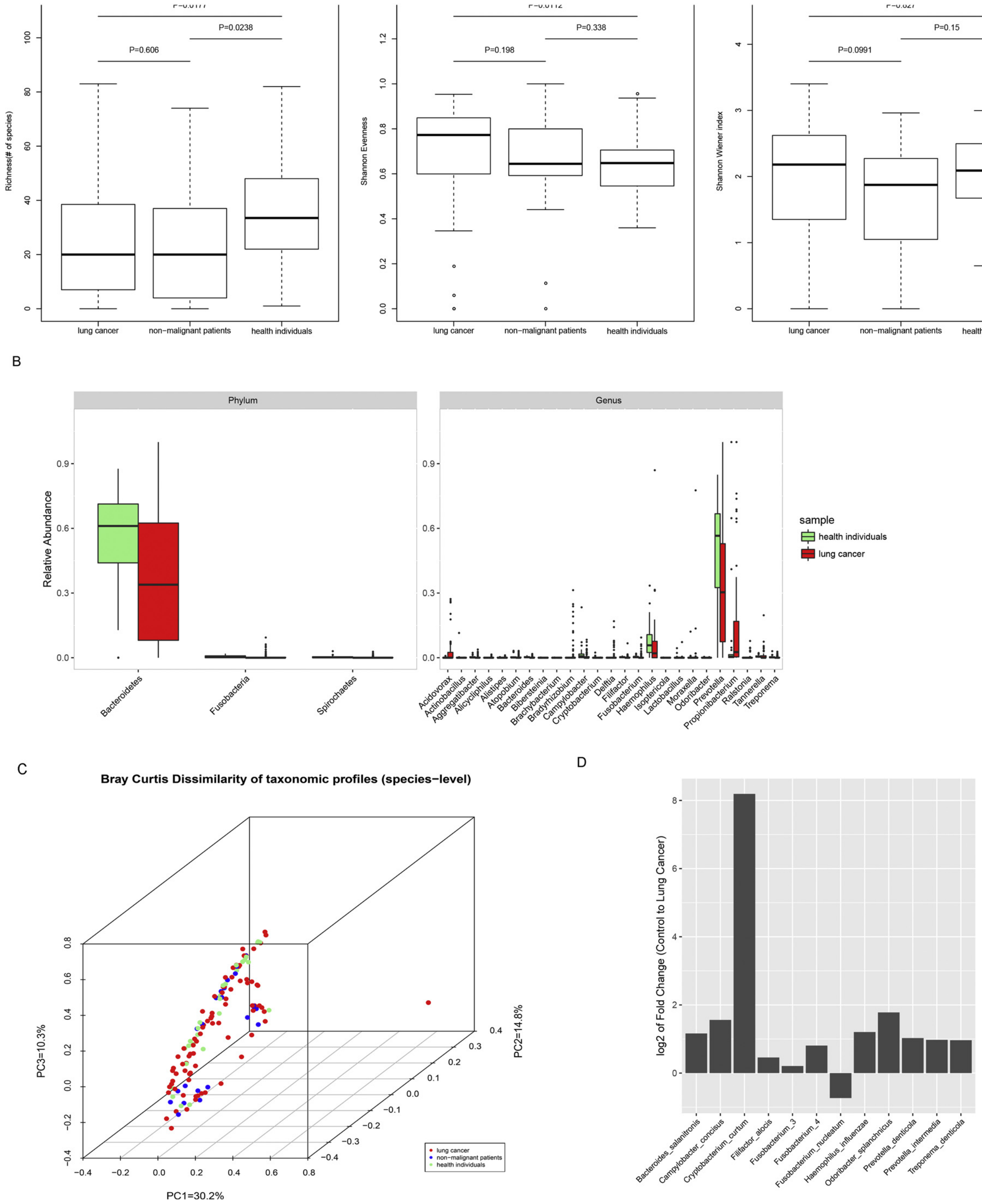

Fig. 3. Comparison of the microbiota in BALF from lung cancer patients, patients with nonmalignant disease and healthy individuals (A). Diversity indices in the three groups: lung cancer, lung cancer patients, patients with nonmalignant disease and healthy individuals. Each p-value obtained through the Kruskal-Wallis test is reported along with the name of the index. Boxes denote the interquartile range (IQR) between the 25th and 75th percentiles (first and third quartiles); the inner line represents the median. (B) Differentially abundant phyla and genera in lung cancer patients ($n = 91$) and healthy individuals ($n = 30$). Green and red represent healthy controls and patients with lung cancer, respectively. Only significant species in the two groups are shown for clarity (Wilcoxon rank-sum test, p-value $<$ 0.05). Boxes denote the interquartile range (IQR) between the 25th and 75th percentiles (first and third quartiles); the inner line represents the median. (C) Comparison of microbiota from lung cancer patients, patients with nonmalignant disease and healthy individuals. Principal coordinate analysis (PCoA) of Euclidean distance of species-level taxonomic profiles. The proportion of variance explained by each principal component is denoted in the corresponding axis label. Red, blue and green represent lung cancer patients, patients with nonmalignant disease and healthy individuals, respectively. PERMANOVA was used as a statistical test. (D) Significant changes in the abundance of 16 types of species in healthy individuals and lung cancer patients. Fold-differences were calculated from the relative abundance, and only those with a p-value of < 0.05 (Wilcoxon rank-sum test) are charted. Significant differences in species, at both higher and lower levels, were found in the lung cancer samples compared with the controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

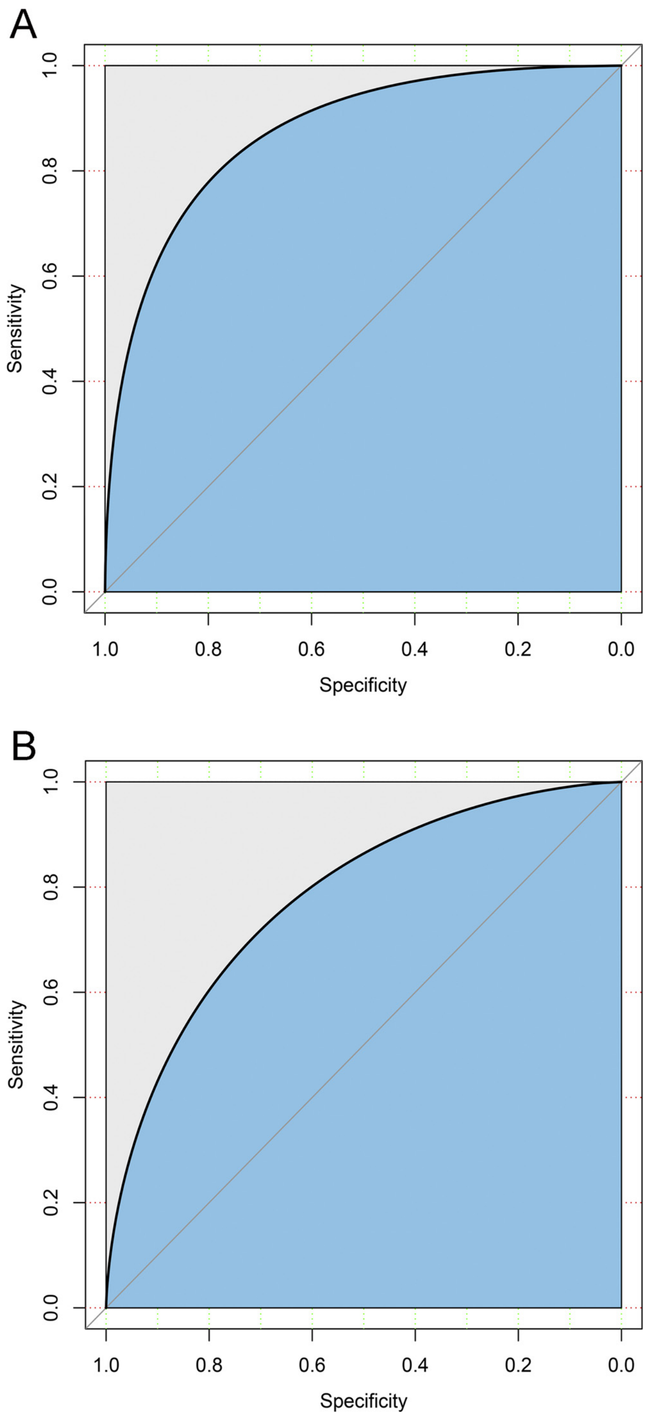

Fig. 4. Microbiota-based classification of lung cancer patients, patients with nonmalignant disease and healthy individuals. The AUC plot is shown for the random forest regression model in the training set and validation set (A, training set. B, validation set). The model was trained using variables including age, pack year of smoking and relative abundance of bacteria in the samples. The area under the receiver operating curve (AUC) and 95%CI for the training set and validation set were 0.882 (0.807-0.957) and 0.776 (0.673-0.920), respectively.

In addition, *B. japonicum* was only observed in patients with lung cancer, whereas *Acidovorax* was found only in patients with lung disease.

In general, the current study included a relatively large number of participants with detailed medical histories and performed metagenomics analysis, which can both identify unknown microbes and also classify components at the species level. Overall, our findings provide new insight into the potential role of a reservoir of bacterial pathogens in detecting lung cancer. However, due to the limited data about the microbiota we acquired, we cannot conduct further analysis of bacterial function or relative pathways. The role of these bacteria in the tumorigenesis of lung cancer is largely unknown and requires further investigation.

Our results are similar to those of previous studies showing that microbiota alpha diversity is decreased in tumor tissues compared with in healthy tissues [17]. However, the microbial composition of the LRT varies between studies on patients with lung cancer. For example, Yan, X et al. investigated the salivary microbiota of lung cancer patients and found *Capnocytophaga*, *Selenomonas*, *Veillonella*, and *Neisseria* to be the predominant taxa [27]. Lee, S. H et al. explored the microbiome using BALF samples and found that two genera, *Veillonella* and *Megasphaera*, were more abundant in patients with lung cancer than in patients with benign disease [18]. In the above studies, PCR amplification of bacterial 16S rRNA was used. In contrast, the current study was based on metagenomics analysis, which can identify unknown microbes and classify the microbiome at the species level. Additionally, differences between this study and previous studies may be due to environmental and genetic factors, as microbiome diversity is affected by exposure to environmental elements [17]. The air quality in China may also contribute to the microbiome characteristics of its population as well as the development of lung diseases.

Interestingly, *B. japonicum* was only observed in patients with lung cancer, and this genus was noted in previous studies on inflammatory bowel disease [28,29]. The endotoxic activities and immunostimulant capability of lipopolysaccharides isolated from this bacterium are extremely low compared with those of *Salmonella* and *Typhimurium* [30]. Moreover, *Acidovorax*, which is a genus of Proteobacteria, was noted only in patients with lung disease. *Acidovorax* is reportedly enriched in smokers with squamous cell carcinoma, and its abundance is associated with TP53 mutations [31]. In general, differences in the microbiota may contribute to tumorigenesis or secondary tumor development. Nonetheless, this was a cross-sectional observational study, and rigorous studies on the causality between these two genera and lung cancer are essential.

We also examined the abundance of specific types of bacteria in patients with tumors with different clinical variables, but no strong relationships were observed between these clinical characteristics and the diversity of the LRT microbiome. Regarding the three important species mentioned above, patients with early-stage tumors appeared to have an increased level of *B. japonicum* compared to those with advanced-stage tumors, and those with squamous cell carcinoma and adenocarcinoma showed a higher level than did patients with small cell lung cancer. *Staphylococcus haemolyticus* and *H. influenzae* were significantly more abundant in patients who were current smokers than in patients with a prior history of smoking and in those who had never smoked before, in agreement with the genus-level study by Lee, S. H [18]. Additionally, we found that short-term antibiotic use did not greatly influence the microbiome profile in patients with lung cancer.

The combination of age, pack year of smoking and 11 types of bacteria, which were different from those in previous studies [18,27], may be used to distinguish patients with lung cancer from those with another disease with high specificity in a training set. This diagnostic test will be helpful for diagnosing patients who are not candidates for lung biopsy due to comorbidities. Microbiota-specific biomarkers may also provide clinicians with new methods for monitoring and preventing lung cancers. Further large-scale studies are needed to validate these microbial biomarkers for lung cancer.

5. Conclusion

Our findings found that the microbiome richness is diminished in the lower respiratory tract of lung cancer patients. The multi-factorial predictive model accounting for both the microbiota and demographics may provide a new method for the detection of lung cancer.

Author contributions

(I) Conception and design: WM Li, HL Wu. (II) Administrative support: J Jin, D Liu, HY Liu. (III) Provision of study materials or patients: YC Gan, TB Deng, YZ Zhou. (IV) Collection and assembly of data: YY Zhu, H Zhu, S Yang. (V) Data analysis and interpretation: JY Yuan, ZR Wang, W Shen. (VI) Manuscript writing: all authors. (VII) Final approval of manuscript: all authors.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.08.022.

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