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Characterization of microbiome in bronchoalveolar lavage fluid of patients with lung cancer comparing with benign mass like lesions

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ABSTRACT

Objectives: Disruption in the stability of respiratory microbiota is known to be associated with many chronic respiratory diseases. However, only few studies have examined microbiomes in lung cancer. Therefore, we characterized and compared the microbiomes of patients with lung cancer and those with benign mass-like lesions.
Materials and methods: Bronchoalveolar fluid was collected prospectively to evaluate lung masses in patients who had undergone bronchoscopies from May to September 2015. Twenty-eight patients (20 male, 8 female) were enrolled: 20 diagnosed with lung cancer and 8 diagnosed with benign diseases. Samples were analysed by 16S rRNA-based next-generation sequencing.
Results: The participants' mean age was 64 ± 11 years. Bacterial operational taxonomic units were classified into 26 phyla, 44 classes, 81 orders, 153 families, 288 genera, and 797 species. The relative abundance of two phyla (*Firmicutes* and *TM7*) was significantly increased in patients with lung cancer ($p = 0.037$ and 0.035, respectively). Furthermore, two genera (*Veillonella* and *Megasphaera*) were relatively more abundant in lung cancer patients ($p = 0.003$ and 0.022, respectively). The area under the curve of a combination of these two genera used to predict lung cancer was 0.888 (sensitivity = 95.0%, specificity = 75.0% and sensitivity = 70.0%, specificity = 100.0%; $p = 0.002$).
Conclusion: The results indicate that differences exist in the bacterial communities of patients with lung cancer and those with benign mass-like lesions. The genera *Veillonella* and *Megasphaera* showed the potential to serve as biomarkers to predict lung cancer. Thus, the lung microbiota may change the environment in patients with lung cancer.

1. Introduction

Traditionally, lungs have been thought of as a sterile space. However, recent studies have demonstrated that there are rich and varied microbiomes in healthy lungs [1]. Furthermore, diseased lungs have dominant microbiota that differ from that of healthy lungs; patients with allergies and chronic obstructive pulmonary disease (COPD) have relatively more abundant *Proteobacteria* phylum and *Hemophilus* spp. than that in the control, whereas *Prevotella* spp. were more frequently detected in controls [2].

At present, lung cancer is the most common cancer worldwide, and it is the leading cause of cancer-related morbidity and mortality both men and women [3,4]. Many previous studies have identified molecular markers (EGFR, PD-1, and ALK) related to lung cancer development. These studies have resulted in novel, tailored therapeutic drugs for patients with lung cancer [5–7].

Some microbiota are known to be associated with cancer. For example, *Helicobacter pylori* is associated with gastric cancer, and

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human papillomavirus and *Chlamydia trachomatis* are known to be associated with cervical cancer [8–10]. Although cancer-related microbiomes are not as well studied in lung cancer compared with other solid cancers, a recent meta-analysis showed pneumonia and *Mycobacterium tuberculosis* significant risk factors for lung cancer [11]. Furthermore, Meyer et al. showed that periodontitis could be associated with lung cancer [12]. These studies suggest that specific microbiota may be related to lung cancer. Additionally, a recent study 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 control [13]. Gui et al. reported that certain microbiota (*Lactobacillus*) had a therapeutic effect in a lung cancer mouse model [14].

Therefore, examining the microbiomes in patients with lung cancer is important and necessary to better understand the pathogenesis of this disease and to plan therapeutic interventions. However, until recently, there have only been a few studies on the microbiomes in patients with lung cancer. The purpose of this study was to characterize the microbiomes in patients with lung cancer compared to those with benign mass-like lung lesions using 16S rRNA-based next-generation sequencing (NGS).

2. Methods and patients

2.1. Study subjects and sample collection

Patients who were admitted for evaluation of lung masses were prospectively enrolled in this study at a 2500-bed tertiary university medical centre in Seoul, South Korea between May and September 2015. Patients were excluded if they met any of the following conditions: less than 20 years of age, pregnant, or had undergone any procedure other than bronchoscopy to evaluate the lung mass. All eligible study population underwent bronchoscopy, and bronchoalveolar fluid (BALF) was collected from each patient. Prior to bronchoscopy, subjects received a topical anaesthesia (lidocaine) by nebulizer and then were sedated with midazolam and fentanyl. The bronchoscope was inserted and wedged into the mouth for the bronchoalveolar lavage (BAL). BAL was performed following a standardized protocol on the opposite side of the lung mass, and 10 cc of BALF was acquired from each patients using about 30 ml sterile 0.9% saline. If a patient had a lung mass on the right upper lobe, BAL was performed on the left upper lobe. A total of 28 patients were included in this study; 20 patients were diagnosed with lung cancer, and 8 were diagnosed with a benign mass-like lesion. Demographic and clinical data, including age, gender, body mass index (BMI), pulmonary function, smoking status, smoking amount, comorbidities, and final diagnosis were obtained from each participant.

2.2. PCR amplification and pyrosequencing

DNA was extracted from a BALF sample the same day as bronchoscopy using a standard protocol. This extraction was then shipped to a sequencing centre. Sequencing was performed using an Illumina HiSeq 2500. The V1 to V3 regions of the 16SrRNA were also amplified by PCR. Amplifications were performed by the following sequences: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final elongation step at 72 °C for 5 min. Through the 2% agarose gel electrophoresis and visualized using a Gel Doc System (BioRad, Hercules, CA, USA), PCR products were confirmed. A QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) were used for purifying the amplified products. Equal concentrations of purified products were pooled together, and AMpure Beads kit (Agencourt Bioscience, MA, USA) was used for short fragments (non-target products). The product size and quality were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Mixed amplicons were subjected to emulsion PCR and then deposited on Picotiter plates. According to the manufacturer's instructions, sequencing was carried out at Chunlab, Inc. (Seoul, Korea) with a GS Junior System (Roche, Branford, CT, USA).

2.3. Pyrosequencing data analysis

Reads obtained from the different samples were sorted by unique barcodes associated with each PCR product. The sequences of the barcode, linker, and primers were removed from the original sequencing reads. Following conditions were discarded; (1) any reads containing two or more ambiguous nucleotides, (2) a low quality score (average score <25), or (3) reads shorter than 300 bp. Potential chimeric sequences were detected by the Bellerophon method, which compares the BLASTN search results from the forward half and reverse half sequences [15]. Each read was assigned a taxonomic classification in the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) after removing chimeric sequences, which contains 16S rRNA gene sequences of type strains with valid published names and representative species level phylotypes from either cultured or uncultured entries in the GenBank database with complete hierarchical taxonomic classification from the phylum to the species level [16]. Chao1 estimation and Shannon diversity index were used for the richness and diversity of samples. Random subsampling was conducted to equalize the read sizes of samples for comparison among samples. The overall phylogenetic distance between two groups was estimated using Fast UniFrac and visualized by principal coordinate analysis (PCoA) [17].

2.4. Statistical analysis

Continuous variables were compared between groups by Mann-Whitney test, and categorical variables were analysed using Chi-square test or Fisher's exact test. Data are shown as the median [quartile] for continuous variables and number (%) for categorical variables. An adjusted p-value of <0.05 was considered statistically significant. All statistical analyses were carried out using SPSS Version 22 (SPSS, Chicago, IL, USA).

2.5. Ethics statement

The protocol for this prospective study was reviewed and approved by the Institutional Review Board of Yonsei University Health Service, Severance Hospital, Seoul, Korea (IRB approval number: 4-2014-1014). Informed consent was obtained mainly from the patient him/herself on the day of admission to evaluate the lung mass.

3. Results

Table 1 shows the baseline characteristics of the two groups. The median age [quartile] of the study population was 59.5 [56.0, 74.5] years. Eight patients were female, and 20 patients were male. BMI was significantly higher in patients with benign mass-like lesions than in patients with lung cancer ($p = 0.012$). Only one patient performed a pulmonary function test in benign lung disease group, because almost patients with benign mass-like lesions did not show respiratory symptoms. There was no significant difference in smoking status and smoking amount between the two groups ($p = 1.000$ and $p = 0.854$). In lung cancer patients, two patients were diagnosed with small cell lung cancer, and the others ($n = 18$) were diagnosed