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Lower Airway Microbiota and Lung Cancer

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This study was aimed at identifying the lower airway microbiota in patients with lung cancer (LC) using protected brush sampling. We enrolled 37 patients undergoing diagnostic bronchoscopy for suspected LC, 26 with LC and 11 with benign diseases. Protected brush specimens were obtained from the contralateral lung and the side of the tumor; these specimens were analyzed by 16S rRNA-based-next-generation sequencing. The results indicated that the biodiversity was not different between groups, and there were no significant differences between the proportion of microorganisms in the tumor and in the contralateral side of patients with LC. In patients with LC, there was a higher abundance of several microorganisms including *Capnocytophaga*, *Haemophilus*, *Enterococcus*, and *Streptococcus*; whereas, in individuals without LC, *Bacteroides*, *Lactobacillus*, or *Methylobacterium* were more abundant. Malignancy could be determined with an accuracy of 70% by isolating *Enterococcus*, *Capnocytophaga*, or *Actinomyces*. *Microbispora* indicated benignity with a sensitivity of 55%, specificity of 88%, and accuracy of 78%. Lower airway microbiota in patients with LC is fairly similar in both the tumor and contralateral sites. Endobronchial microbiota is different in patients with and without LC, and these differences may have a potential clinical value as diagnostic or prognostic biomarkers.

Keywords: **Biomarkers, bronchoscopy, DNA sequencing, high-throughput technologies, microbiome**

Introduction

A correlation between specific patterns of human microbiota and various diseases have been described [1] including cancer development, aggressiveness and progression [2]. In fact, microbiome has been implicated in cancer at a variety of body sites as colorectal, gastric, hepatocellular and pancreatic cancers [2, 3]. The distinct nature of the microbiome of each body niche suggests similar organ specificity to microbial effects on inflammation and carcinogenesis [4]. In particular, the contribution of an inflammation state to the pathogenesis of colorectal cancer has been correlated with microbial dysbiosis [5]. Although microbiome and microbiota are frequently used interchangeably, microbiome is the collection of all genomes of microbes in an ecosystem and microbiota refers to microbes that collectively inhabit a given ecosystem [1].

The lung microbiota is the first interface with environmental exposures including smoking, the most important risk factor for lung cancer. In this sense, the lung microbiome may play an important role in the response to carcinogens [2]. A relationship between microbes and lung cancer has been suggested. In never smokers, a significantly risk of lung cancer was associated to a history of previous pneumonia or tuberculosis [6]. *Chlamydia pneumoniae* is also associated with an increased risk of lung cancer [7]. Such observations suggest that microbial changes could be a risk for cancer [6, 8].

In addition, several experimental studies emphasized

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the potential role of microorganisms in lung cancer. Antibiotics-treated mice were shown to be more susceptible to tumor development in the lungs after inoculation with a malignant cell-line, and commensal bacteria were found to be essential for the immune activity that protects against susceptibility to lung carcinoma [9]. In a mouse model of lung cancer, Gui *et al.* [10] reported that the anti-lung cancer response was improved in presence of commensal microbiota.

However, lung cancer associated microbiome in humans has been scantily studied compared with other cancers [2]. Very few studies have reported results using high-throughput technologies [8, 11–15]. Furthermore, studies on lung cancer microbiota have been generally based on samples with risk of contamination by the upper airway microbiota [14, 16].

Our objective was to identify the lower airway microbiota in patients with lung cancer, comparing the bacterial composition and relative abundance in the site of the tumor with respect to the contralateral side, and that of patients without cancer, using protected brush sampling. This method allows the isolation of pulmonary microbial DNA avoiding the risk of oropharyngeal contamination.

Methods and Patients

Patients

We enrolled 37 patients older than 18 years of age undergoing diagnostic bronchoscopy for suspected lung cancer (pulmonary nodules or masses limited to a lobule, hemoptysis) into a prospective study. All patients had a complete study accordingly to clinical practice. Twenty-six were diagnosed of having lung cancer (Table 1) and 11 patients of benign diseases. The group of patients without lung cancer (with benign pulmonary nodules and/or hemoptysis) were 3 female (27%) and 8 male (73%), aged (mean ± SD) 50 ± 15 years, and 9 of them (82%) were smokers or ex-smokers.

Table 1. Characteristics of patients with lung cancer.

Number of patients	26
Female (%)	6 (23%)
Age (year ± SD)	71 ± 8
Smokers of ex-smokers	22 (85%)
Histology	5, Small cell carcinoma 7, Adenocarcinoma 9, Squamous cell carcinoma 5, non-small cell carcinoma

Sample collection

The flexible bronchoscopy was performed as usually in clinical practice. Patients were premedicated intravenoulsy with midazolam and topical anesthesia (lignocaine). The first step was to perform a protected brush specimen (PBS) in the lung contralateral to the side with the tumor, at the equivalent level. The PBS was advanced to the chosen position; after dislodging the distal plug to obtain the airway secretions, the brush was retracted into de inner cannula and the whole unit was removed from the bronchoscope. Then, with another PBS the manoeuvre was repeated in the side with the tumor (visible during the bronchoscopy or in the anatomic subsegmental division according to radiological findings). In the control group, was similarly obtained consecutively in both lungs. PSB brush was cut sterilely into a tube containing 1 ml of RNAlater solution (Thermo Fisher Scientific, USA) and immediately sent to laboratory and frozen at -80℃.

DNA extraction

DNA was isolated from frozen bronchoscopic brushing samples by direct centrifugation of the brushes in the ATL Tissue Lysis Buffer (Qiagen, Germany) followed by digestion with 2 µg/µl of proteinase K (Qiagen) at 56℃ for 24 h. The DNA was purified using the E.Z.N.A Forensic DNA Kit. (Omega bio-tek) by adding 1 µg/µl RNA carrier of (Qiagen) to mix with the BL buffer and sample, then the procedure was performed according to the manufacturer's instructions. The concentration of isolated DNA was analyzed by Quantus Fluorometer (Promega, Germany).

Sequencing analysis

16S rDNA gene amplicons were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol. The gene-specific sequences used in this protocol target the 16S rDNA gene V3-V4 region (459 bp). Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences. The primers are selected from those proposed by Klindworth *et al.* [17]. The full length primer

sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are: 16S rDNA gene Amplicon PCR Forward Primer: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG 16S rDNA gene Amplicon PCR Reverse Primer: 5' GTCTCGTGGGCTCGGAGAT-GTGTATAAGAGACAGGACTACHVGGGTATCTAATC.

Microbial genomic DNA (5 ng/ul in 10 mM Tris pH 8.5) was used to initiate the protocol. After 16S rDNA gene amplification, the mutiplexing step was performed using Nextera XT Index Kit (FC-131-1096). We run 1 ul of the PCR product on a Bioanalyzer DNA 1000 chip to verify the size, the expected size on a Bioanalyzer trace is 550bp. After size verification the libraries were sequenced using a 2x300 pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) on a MiSeq Sequencer according to manufacturer's instructions (https://support.illumina.com/downloads/16s_metagenomic_sequencing_ library_preparation.html).

Bioinformatics analysis

Sequencing reads were quality-assessed and trimmed using PRINSEQ software [18]. Paired-end reads were joined using Fastq-join from the ea-tools suite [19]. Chimera sequences were excluded using Usearch [20]. Taxonomic assignments were performed using the RDP Classifier from the Ribosomal Database Project [21]. After sequencing by IlluminaMiseq the sequences obtained were processed using Qiime v1.9.0 (Quantitative Insights Into Microbial Ecology) [22]. Chimeric, low quality and/or short sequences (less than 150 bp) were discarded for the microbial community analysis by Qiime split libraries script. After quality filtering the remain sequences were pair-end assembled and only sequences with length 400–450 bp were used for taxonomic assignment by Qiime v1.9.0 using the "Ribosomal Database Project" database [23]. The Operational Taxonomic Units (OTU) picking method was established with a cut-off threshold of 97% of identity to describe each sample OTU microbiome composition. Total of assigned reads were estimated for each patient and also relative abundances were determined at each OTU level for the clinical samples individually and for the clinical study groups "Tumor", "Contralateral" and "Control" samples, only OTUs with a relative abundance over 0.01 were included in the study. Also richness index as Shannon-Wiener diversity and Chao1 richness index were determined for each sample and clinical study group [24].

Statistical analysis

Samples for comparisons were classified as 'Tumor' (microbiota obtained in the site of the lung cancer), 'Contralateral' (the opposite side in patients with cancer) and 'Control' (patients without cancer). We conducted differential abundance analysis at the genus level. Since the variables (taxa) showed a non-normal distribution, non-parametric Mann-Whitney test and Kruskal-Wallis test were used. Individual microorganism presence in patients with and without lung cancer was evaluated in terms of sensitivity, specificity and accuracy. A p value < 0.05 was considered statistically significant.

Ethics statement

The protocol of the study was reviewed and approved by the local ethics committee. Written informed consent was obtained from each patient before any research procedure.

Results

Three groups of samples were evaluated in terms of bacterial composition and relative abundance. These groups were designated as (1) tumor, the microbiota obtained in the side of the lung cancer, (2) contralateral, the microbiota obtained in the lung not affected by the tumor in patients with cancer, and (3) control, the endobronchial microbiota in patients eventually without cancer.

Next generation sequencing and biodiversity

A total of 5,071,120 reads ranging with a mean length of 443 bp were obtained after quality filtering and taxonomically assigned. The samples reads distribution by clinical study groups category were as follow: 1,995,494 reads in "Tumor" group samples, 2,564,019 reads in "Contralateral" group samples and 511,607 reads in "Control" group samples (mean reads per clinical study groups were 76,749, 98,616, and 46,509, respectively). Biodiversity (Shannon and Chao1 indices) was not statistically different between groups (Table 2).

Table 2. Biodiversity indices between groups.

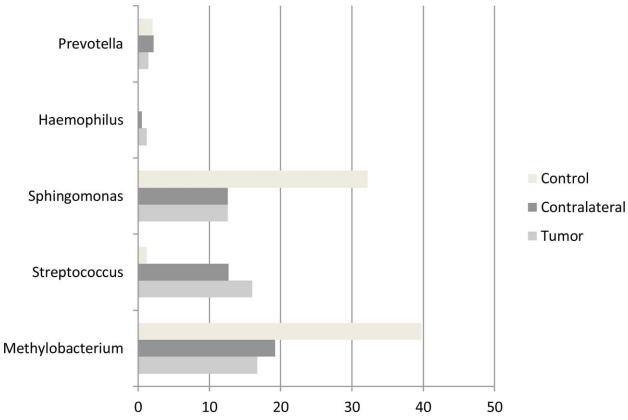
	Tumor	Contralateral	Control	*p* value
Shannon	2.92 (2.45-3.12)	2.88 (2.65-3.12)	2.92 (2.65-3.32)	0.721
Chao1	13 (11-23)	13 (12-23)	13 (13-55)	0.412

Values are median (25th-75th percentile)

Differential abundance

There were no statistically significant differences between the proportion of abundance of microorganisms in the tumor side and in the contralateral side of patients with lung cancer. The individual values of each taxon identified in the tumor group and in the control group are detailed in Table 3 (median and 25^{th}–75^{th} percentile values). This table includes the bacteria with potential clinical interest, *i.e.* those with statistically different values between groups, those with a relative abundance higher than 1% and those that were reported of possible interest in previous studies using high-throughput technologies [8, 11–15]. The relative abundance of main microorganism is graphically represented in Fig. 1 (taxa with at least 1% of abundance in median values).

In patients with lung cancer there was significantly more abundance of several microorganism including *Capnocytophaga, Haemophilus, Enterococcus* and *Streptococcus*. Comparing with these patients, in individuals without lung cancer there was a significantly higher abundance of *Bacteroides*, *Lactobacillus* or *Methylobacterium* among others (Table 3).



	Tumor	Contralateral	Control
Methylobacterium	16,7	19,2	39,7
Streptococcus	16	12,7	1,2
Sphingomonas	12,6	12,6	32,2
Haemophilus	1,2	0,5	0
Prevotella	1,4	2,2	2

Fig. 1. Relative abundance: median value of main genera in each group.

Presence and absence of microorganisms

There were no microorganisms exclusively present in malignant samples or exclusively present in benign samples. Consequently, there were no microorganisms capable of discriminating between these groups. However, several microorganisms were more frequently found in the samples from malignancy and more frequently absent in benign ones. The isolation of *Enterococcus, Capnocytophaga* or *Actinomyces* was able to establish malignancy with an accuracy of 70% (Table 4). On the other hand, *Microbispora* was much more frequently isolated in benign samples, and indicated benignity with a sensitivity of 55% (95% confidence interval CI: 23–83), specificity 88% (CI: 70–98) and accuracy of 78% (CI: 62–90).

Discussion

Our study describes the lower airway microbiota in patients with lung cancer, comparing the findings in the tumor site and in the contralateral side of the same patients. In addition, we report the lower airway microbiota in a group of patients with pulmonary signs or symptoms of suspicion for lung cancer (hemoptysis or pulmonary nodules) but in whom malignancy was ruled out eventually. The major findings of our study are that the lower airway microbiota in lung cancer patients is similar in the tumor site and in the contralateral site; and different to that of individuals with benign pulmonary nodules or hemoptysis. This study employs the best possible methodological approach (protected brush sampling) a method that minimizes the potential risk of oro-

Table 3. Differences between tumor and control: Taxa with potential clinical interest.

Microorganism	Tumor	Control	*p* value
Abiotrophia	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.515
Atopobium	0.02 (0.00-0,60)	0.00 (0.00-0.24)	0.086
Bacteroides	0.32 (0.06-0.74)	1.17 (0.28-1.53)	0.018*
Blautia	0.15 (0.04-0.41)	0.67 (0.25-0.83)	0.033*
Bulleidia	0.09 (0.00-1,00)	0.00 (0.00-0.00)	0.041*
Capnocytophaga	0.06 (0.00-0.45)	0.00 (0.00-0.04)	0.048*
Enterococcus	0.00 (0.00-0.02)	0.00 (0.00-0.00)	0.007**
Granulicatella	0.37 (0.00-1.21)	0.00 (0.00-0.36)	0.064
Haemophilus	1.22 (0.01-2.96)	0.02 (0.00-0.48)	0.058
Lactobacillus	1.35 (0.49-4.13)	5.64 (1.56-6.71)	0.043*
Lysinibacillus	0.18 (0.10-0.59)	1.07 (0.33-1.86)	0.005**
Megasphaera	0.00 (0.00-0.73)	0.00 (0.00-0.35)	0.116
Methylobacterium	16.7 (4.35-37.0)	39.7 (20.0-42.0)	0.033*
Microbacterium	0.23 (0.05-0.60)	0.64 (0.16-1.13)	0.054
Microbispora	0.00 (0.00-0.00)	0.00 (0.00-0.05)	0.007**
Mogibacterium	0.01 (0.00-0.02)	0.00 (0.00-0.00)	0.020**
Nesterenkonia	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.006**
Oscillospira	0.64 (0.24-2.31)	1.99 (0.74-4.25)	0.063
Phascolarbacterium	0.04 (0.01-0.17)	0.21 (0.14-0.39)	0.010*
Porphyromonas	0.46 (0.01-1.47)	0.00 (0.00-0.25)	0.022*
Prevotella	1.43 (1.05-6.60)	1.95 (1.19-2.23)	0.894
Ralstonia	0.18 (0.04-0.41)	0.62 (0.14-0.70)	0.026*
rc4.4	0.00 (0.00-0.00)	0.01 (0.00-0.04)	0.042*
Ruminococcus	0.32 (0.13-1.00)	1.42 (0.44-1.64)	0.014*
Selenomonas	0.01 (0.00-0.16)	0.00 (0.00-0.00)	0.058
Sphingomonas	12.6 (2.53-26.2)	32.2 (14.9-33.1)	0.017*
Staphylococcus	0.16 (0.07-0.36)	0.14 (0.01-0.23)	0.153
Streptococcus	16.1 (1.27-23.3)	1.18 (0.26-12.1)	0.075
Vagococcus	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.042*
Variovorax	0.57 (0.10-1.53)	1.61 (0.55-1.91)	0.063
Veillonella	0.55 (0.18-6.24)	0.02 (0.00-3.83)	0.158

Values are median (25th-75th percentile). Mann-Whitney U test. *$p < 0.05$, **$p < 0.001$

pharyngeal contamination [25]. Very few studies have evaluated the microbiota in patients with lung cancer, and most of them with methods that do not avoid the contamination by upper airway microbes confidently [8, 11–15]. Only Liu *et al.* [15] very recently reported to employ a technique identical to ours.

We found no differences in biodiversity between the evaluated groups. Very few studies have addressed this question. Yu *et al.* [14] used fresh frozen lung tissue samples from surgical resections. They compare 31 tumor samples with non-malignant tissue (paired samples) and concluded that alpha biodiversity (mean of species diversity) was higher in non-malignant tissue. However, the same study describes no definitive differences in microbiota between malignant and benign samples using other tests. Interestingly, Liu *et al.* [15] using protected brush samples, reported, in agreement with our findings, no differences in biodiversity between the microbiome in the tumor site and in the contralateral side. However, they observed some differences in the bio-

Table 4. Microorganisms for detecting malignancy.

Microorganism	Sensitivity	Specificity	Accuracy
Enterococcus	65 (44-83)	82 (48-98)	70 (53-84)
Capnocytophaga	69 (48-86)	73 (39-94)	70 (53-84)
Actinomyces	77 (56-91)	55 (23-83)	70 (53-84)
Selenomonas	62 (41-80)	82 (48-98)	68 (50-82)
Mogibacterium	54 (33-73)	91(59-100)	65 (47-80)
Atopobium	58 (37-77)	73 (39-94)	62 (45-78)
Vagococcus	28 (12-49)	100 (72-100)	50 (33-67)

Values are percentage and 95% confidence interval.

diversity between tumor samples and controls [15]. It should also be taken into account that diversity may increase in relation to several exposures, such as air particulates or tobacco [14].

We have not found significant differences (in terms of composition or relative abundance) between the microbiota of the tumor site and the microbiota in the contralateral healthy lung. This is different to that reported in other organs. The microbiota of tumor and nearby healthy tissue is different in patients with colorectal cancer [26], and a similar finding has been demonstrated in breast cancer [27], where there exists a distinct breast tissue microbiota in benign and malignant disease. Similarly to our results, the study by Liu *et al.* [15] found minor differences between tumor and paired non tumor samples in the same individual.

It may be more interesting to consider differentiating microbiota in lung cancer patients and in subjects without cancer. The association of specific microorganisms and lung cancer has been suggested in several studies. With conventional microbiological methods, Laroumagne *et al.* [28] described that almost half of bronchial aspirates in patients with lung cancer had microbial colonization, mainly gram negative bacilli including *Enterobacter* spp. However, a more accurate method, high-throughput technology, has been used in very few studies. In these studies, different associations between microbiota and lung cancer have been reported.

Hosgood *et al.* [11] studied oral and sputum samples from eight never-smoker Chinese women and controls found that *Granulicatella*, *Abiotrophia* and *Streptococcus* were enriched in sputum of lung cancer patients. In our groups with endobronchial samples, both *Granulicatella* and *Streptococcus* were more abundant in the tumor group with p values close to statistical significance (0.06 and 0.07, respectively). Yan *et al.* [12] analyzed the salivary microbiota of 20 patients with cancer and 10 controls. In this study, the isolation of *Veillonella* and *Capnocytophaga* was a useful tool for distinguishing between patients with and without lung cancer. In our experience, both microorganisms were also more abundant in malignancy, the former with statistically significant difference. Lee *et al.* [13] used bronchoalveolar lavage for obtaining samples from the lower airway in 20 patients and 10 controls. Four genera, *Veillonella, Megasphaera, Atopobium,* and *Selenomonas* were more abundant in lung cancer patients.

Cameron *et al.* [8] in a pilot study of sputum microbiome including 10 patients (four with lung cancer) reported *Streptococcus viridians* was more abundant in cancer patients, and several other microorganisms differ between groups. In the study by Yu *et al.* [14], the authors suggest that microbiome may be different depending on the tumor histology (squamous vs adenocarcinoma); however, the sample size of our study does not allow the exploration of this possibility.

Most of the findings reported in the aforementioned studies generally agree with ours. Nevertheless, it must be taken into consideration that these studies use methods that can be interfered by upper airway contamination. Only Liu *et al.* [15] reported results with a study very similar to ours, with protected bilateral brushing. They obtained that *Streptococcus* was significantly more abundant in cancer cases than in controls, whereas *Staphylococcus* was more abundant in the controls. In our sample, *Streptococcus* tended to be more abundant in malignant samples, but *Staphylococcus* was similar in the groups. This difference would be due to the effect of

geographic variation in lung microbiome. In healthy volunteers of Western Europe and North America this variation was not observed, but it is possible that it occur when compared with other populations [29].

Very recently, Tsay *et al.* [30] reported that lower airway of lung cancer patients was enriched for *Streptococcus* and *Veillonella*, and these microorganisms were associated with transcriptomic changes of airway epithelial cells, including the ERK/PI3K pathways relevant to lung cancer [30, 31]. In our series, both *Streptococcus* and *Veillonella* tend to be more abundant in malignant samples.

The importance of infection in cancer progression is another aspect to consider. Pulmonary infections with gram-negative bacteria have also been suggested to contribute to lung metastasis. Acute lung infection models induced by either infection with *Escherichia coli* or administration of LPS increased cancer cell homing to the lung and enhanced lung metastasis [32].

It certainly is exciting to identify bacteria able to differentiate malignant from benign samples, and consequently, be employed as a biomarkers. However, to date, the possible candidates have exhibited only a moderate classification potential [29]. In our experience, *Enterococcus, Capnocytophaga* and *Actynomices* have an accuracy of 70% for malignancy, and *Microbispora* an accuracy of 78% for excluding malignancy.

We present herein a descriptive study on the composition and differences of endobronchial microbiota in patiens with lung cancer. Their main limitation (as occurs in this sort of studies) is the relatively small number of subjects included. All the studies are cross-sectional and with moderate sample sizes, further large studies are needed to evaluate and validate microbial biomarkers in lung cancer patients [29]. On the other hand, our control group was not healthy individuals, but clinically relevant control samples. Additionally, it is necessary to ascertain as to whether geographical or environmental differences influence the distinctive endobronchial microbiota in these patients. This study is a contribution to increase the knowledge in this understudied field.

In conclusion, our study shows that lower airway microbiota in patients with cancer is fairly similar in the tumor side and in the contralateral site, and the endobronchial microbiota is different in patients with and without lung cancer. In patients with lung cancer there was a significantly higher abundance of several microorganisms including *Capnocytophaga, Haemophilus, Enterococcus* and *Streptococcus*. In individuals without lung cancer *Bacteroides*, *Lactobacillus* or *Methylobacterium* were more abundant. In addition, and with potential clinical interest as biomarkers, the isolation of several microorganisms was useful for establishing malignancy, with an accuracy of 70%; or to rule out malignancy with a specificity of 88%.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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