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Sputum microbiota in severe asthma patients: Relationship to eosinophilic inflammation

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ABSTRACT

Background: Altered composition of airway microbiota has been reported in subjects suffering from asthma but its relation to eosinophilic phenotype is unclear.
Objective: To examine the relationship between sputum microbiota, asthma severity and inflammatory type in asthmatic subjects from Guangzhou, China.
Methods: Induced sputum samples were obtained from 49 non-smoking asthma patients, 25 severe and 24 non-severe, and 15 healthy subjects. Total DNA was amplified using primers specific for the V3-V5 hypervariable region of bacterial 16s rRNA and sequenced using the 454 GS FLX sequencer. Sequences were assigned to bacterial taxa by comparing them with 16s rRNA sequences in the Ribosomal Database Project.
Results: Sputum eosinophil counts were higher and FEV_1 (% predicted) was lower in severe compared to non-severe asthmatics. There were no significant differences in operational taxonomic unit (OTU) numbers at the phylum level and in diversity scores between non-severe asthmatics and severe asthmatics, and healthy subjects. At the family level, *Porphyromonadaceae* was most abundant in healthy subjects whereas *Pseudomonadaceae* and *Enterobacteriaceae* were higher in severe asthmatics compared to non-severe asthmatics ($p < 0.05$). *Actinomycetaceae* was particularly abundant in eosinophilic asthma patients compared to non-eosinophilic asthma ($p = 0.011$). *Bacteroidaceae* was positively correlated with FEV_1 in all subjects ($r = 0.335$, $p < 0.01$), whereas body mass index was negatively associated with the number of species observed ($r = -0.3$, $p < 0.05$). Principal component analysis confirmed the positive association of *Actinomycetaceae* and *Enterobacteriaceae* abundance with eosinophilic asthma.
Conclusion: Patients with asthma have an altered airway microbiota, with specific bacteria associated with severe asthma and the eosinophilic inflammatory phenotype.

1. Introduction

Asthma is a chronic inflammatory disease of the airways and its potential relationship with the lower airway microbiome continues to be of interest given the potential for microbes to influence or even cause asthma. A detailed analysis of the complete microbial community composition has been made possible by the development of culture-independent techniques such as 16S ribosomal RNA microarray which is sensitive in detecting microbial species. Using these techniques, areas of investigation have spanned from the role of early life microbial exposures and gut microbial colonization in the development of allergy or asthma [2–4] to studies of airway microbiome in established asthma [5–7].

With the increasing recognition that the lower airways, rather than being sterile, are populated by microbial populations (microbiota), there has been a change in our thinking about the role of microbes in the setting of chronic inflammatory airway disease such as asthma [6–9]. This has led to the realization that there is a complex polymicrobial-host interactions occurring in the airways which raises the possibility of potential mechanistic relationships

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to both the development and severity of asthma [1]. Alterations in the airway microbiome are now an established feature of asthma as shown in several recent studies [6–9]. These studies have shown that that the airways of moderately-severe asthmatics contain an excess of *Proteobacteria* compared to normal controls as measured in bronchsocopically-acquired samples [6,10] while microbiota measured in sputum samples in severe asthma were reported to differ from healthy controls and non-severe asthmatics by the presence of *Streptococcus* spp which was associated particularly with eosinophilia [11]. These studies therefore indicate that different types of airway dysbiosis may be related to disease severity.

These studies have been performed exclusively in Caucasian cohorts. We determined whether the airway microbiota in induced sputum samples obtained from subjects with and without asthma in the city of Guangzhou in Southern China would reveal similar findings as previously reported. We examined whether the airway microbiota diversity was related to parameters of asthma severity and/or to clinical phenotypes. We used high-throughput 454 sequencing on induced sputum samples from severe asthmatic and non-severe asthmatic subjects and control non-asthmatic subjects.

2. Methods

2.1. Subjects

49 patients with physician-diagnosed asthma of whom 24 patients were classified as non-severe asthma and 25 patients as severe asthma, and 15 healthy adults without asthma were enrolled for this study (Clinical study registration number: ChiCTR-CPC-15006555). All subjects were non-smokers, and those on maintenance oral corticosteroid therapy were excluded. Severe asthma was defined according to the ERS/ATS guidelines on severe asthma, while asthma severity was based on the GINA criteria [12,13]. Patients with non-severe asthma were at Step 2 of GINA guidelines. We divided the patients into two inflammatory phenotypes: eosinophilic asthma ($\geq$3% of total count) and neutrophilic asthma (>61% of total count) according to the granulocyte count in induced sputum. No patients were taking antibiotics at the time of the study, and all subjects were considered to be free of clinical infection. Spirometry was performed according to American Thoracic Society standard, and none of the participants had used bronchodilator therapy within 6 h of collecting sputum samples.

2.2. Atopy

Atopy was assessed by means of skin prick testing. Subjects were characterised as atopic if they had at least one positive of common aeroallergen (cat, dog, house dust mites, grass pollen, tree pollen and a mixture of moulds).

2.3. Collection and processing of induced sputum

Sputum was induced from each subject by inhalation of successively increasing concentrations of nebulized saline solutions at 3%, 4% and 5% respectively for a 10-min period each. First, the mouth was rinsed with saline before sputum induction in order to minimize oral contamination. The subject then spat out the saliva, takes 2 deep inspirations of saline aerosol, and then coughed up sputum into a cup. Each procedure was repeated until sufficient sputum has been obtained for analysis. Peak flow was monitored throughout the procedure. Within an hour of collection, the sputum sample was processed. Two volumes of dithiothreitol solution were added to one part of the sputum, and the sample was mixed vigorously on a plate shaker for 15 min to solubilize mucus. The sputum sample was centrifuged and cell counts performed on the cell pellet. Differential cell counts were performed on cytospin preparations. Sputum aliquots were collected and stored at −80 °C.

2.4. DNA extraction and sequencing

The sputum sample was washed twice with TE buffer (10 mMTris-HCl, 1 M EDTA, pH 8.0). Bacterial DNA was isolated and purified using a Wizard Genomic DNA Purification Kit, according to the manufacturer's instructions (Promega, Madison, USA) [14]. A standard concentration of 10 ng/ml of DNA was prepared for each individual sample for all PCR assays. After doing a sample quality test, we used those quantified DNA samples to construct a library. The bacterial DNA was amplified for the V3-V5 hypervariable region of bacterial 16s rRNA. PCR was amplified with fusion primer, then short fragments were removed with Ampure beads. Finally, the quantified library was used for sequencing with the 454 GS FLX, according to the manufacturer's instructions. The DNA extraction and sequencing were performed at the Beijing Genome Institute, Shenzhen, China.

2.5. Data analysis

After sequencing, the reads were preprocessed to remove any that did not match the barcode or the primer sequences. Unique reads were pre-clustered by scripts of software Mothur (Version 1.31.2,http://www.mothur.org/) within 2 mismatch similarity. Then, operational taxonomic units (OTU) numbers were calculated through scripts of the Mothur software based on 97% similarity [15]. Firstly, unique reads were aligned to Ribosome Database Project database (http://www.mothur.org/wiki/RDP_reference_files) using scripts of software Mothur and annotated. Then, OTUs were classified to one species if more than 51% contained unique reads were annotated to this species. Taxonomic analysis of OTU was done at the higher level (such as from genus to family), and the Mothursoftware was used to summarize all OTU analysis results in one sheet. Numbers of reads in each OTU were also calculated to obtain abundance information in the samples. Scripts of software QIIME (Version1.50) were used to analyze distribution of all annotated species or OTU in different taxonomic levels (phylum, class, order, family, genus) among different samples, summarized in the profiling tables [16,17]. The Chao1 estimator and ACE were used to estimate the species richness sequences and ecological diversity was estimated by using the Shannon-Wiener and Simpson indices [15]. The relationship between phenotypic variables and abundance of bacteria were explored using principal components analysis (PCA). Statistical analyses were performed using the SPSS statistics software package (version 18.0). A p value of <0.05 was considered significant. Data are presented as mean ± standard deviation or median (interquartile range). p values were determined using the Mann-Whitney *U* test, chi-square or Student's t-test.

3. Results

3.1. Characteristics of subjects

Compared to non-severe asthmatics, severe asthmatics had higher percentage of atopy (64% vs. 50%) with 46.9% with eosinophilic inflammation and 18.4% with neutrophilic inflammation. They were on a higher dose of inhaled beclomethasone dipropionate and oral corticosteroids, had a longer duration of asthma, worse lung function and lower ACT score. There were no significant differences in gender, body mass index (BMI), and sputum neutrophils between the three groups (Table 1).