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BRIEF COMMUNICATION

Differential Cytokine Pattern in the Exhaled Breath of Patients with Lung Cancer

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Abstract Tumour cells may alter the protein pattern of biological samples resulting in specific differences that may aid diagnosis and treatment. In this pilot study we tested the cytokine pattern of exhaled breath condensate of patients with lung cancer. Breath condensates collected from 50 smoking patients with lung cancer and 25 smokers without clinical or radiological sign of a pulmonary tumour but having co-morbidities with similar severity as those with lung cancer were pooled for antibody microarray analysis testing 120 cytokines in parallel. Every cytokine on the array gave a signal in both groups. Nine cytokines including eotaxin, FGFs, IL-10 and MIP-3 were present with more than two-fold difference between the two groups. Large number of cytokines is present in the exhaled breath. Further analysis of specific differences associated with lung cancer may have clinical importance.

Keywords Lung cancer · Proteomics · Exhaled breath condensate · Microarray · Biomarker

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Abbreviations

EBC	exhaled breath condensate
FVC	forced vital capacity
FEV1	forced expiratory volume in 1 s

Introduction

Lung cancer is the leading cause of cancer death in developed countries. To date, surgery is the only possible curative treatment of the disease. Extirpation of the tumour can only be performed at its early stage. Current screening techniques are either not sensitive enough (X-ray) or too expensive such as computer tomography. An effective screening method would therefore have great clinical importance.

Protein microarray is a new method to detect cytokine pattern of different biological samples [1]. By the simultaneous evaluation of five selected serum cytokine markers non-small cell lung cancer patients could be separated from healthy control subjects with 90% specificity and sensitivity [2]. However, specificity of the results obtained in serum for pulmonary tumours is questionable as elevation of serum cytokine levels could be the consequence of inflammatory conditions and/or malignancies other than lung cancer.

Exhaled breath condensate (EBC) collection is a non-invasive sampling technique of the lungs. EBC samples contain various cytokines as demonstrated by immunoassays [3]. Profiling EBC cytokines by protein microarray may be more specific for lung cancer. Protein microarray has already been used for the profiling of EBC of asthmatic patients [4]. In this pilot study we compared the cytokine

Table 1 Tumour stage and histology at the time of EBC collection

Stage		Histology	
I	5	Planocell.	24
II	5	Adenocell.	17
IIIa	4	Microcell.	9
IIIb	11		
IV	25		

pattern in the pooled exhaled breath condensate of patients with lung cancer to that of healthy smokers.

Low protein content of the samples is a major drawback when using EBC to study cytokines [5]. Pooling samples collected from several individuals not only circumvents this difficulty but offers a solution to individual variability as well. Sample pooling can help obtain appropriate protein concentration but only reveals differences in signalling molecules when changes are pronounced and uniform at the group level. The pooled samples used in this study permit no stratification of results according to the stage and histological subtypes of lung cancer cases. It does not reveal small but possibly important alterations at the individual level and yields no information about molecular signatures specifically linked with cancer subtypes.

Methods

Patients

Fifty smoking patients were enrolled at the time of diagnosis of primary lung cancer (mean age: 61 years, mean pack year: 48, mean FVC: 80%, mean FEV_1: 71%, mean FEV_1/FVC: 69%, mean FENO: 7.0 ppb, all tumours were central endobronchial lesions, for stage and histology see Table 1). Twenty five smokers without clinical or radiological sign of a pulmonary tumour (mean age: 52 years, mean pack year: 35, mean FVC: 100%, mean FEV_1: 88%, mean FEV_1/FVC: 71%, mean FENO: 6.9 ppb) served as controls. Some lung cancer patients as well as some control smokers had ongoing chronic obstructive pulmonary disease (COPD). Based on lung function parameters and the degree of airway inflammation (as suggested by the exhaled NO level) COPD severity was similar in the two groups. The study was approved by the local ethics committee and participants gave their written consent.

EBC Collection

EBC was collected for 15 min in TWEEN-20 treated vials (EcoScreen, Jaeger, Würzburg, Germany). Persons were breathing through the mouth in their normal rhythm while wearing a nose clip. Samples were stored at −70°C in the presence of protease inhibitors (Buffer A, Sigma). 1 ml of each tumour sample and 2 ml of each control sample was concentrated by lyophilization, resuspended in deionised water and pooled for antibody microarray analysis.

Analysis of Cytokine Expression

One hundred twenty cytokines in duplicate were parallel screened for the two pools using Human Cytokine Antibody Array VI+VII (Chemicon International LTD Hampshire, UK) according to the manufacturer's instructions. Signal intensities were recorded using a chemiluminescence imaging system and analyzed with Genetools software (Syngene, UKFrederich, MD, US)

The raw intensity level detected at each individual cytokine spot was background corrected and then normalized using the average intensity at the six positive control spots. Relative intensities were calculated by dividing the background corrected and normalized intensity of each tumour sample spot with that of the corresponding control spot. A difference in cytokine expression was only considered significant when the relative intensity of both parallel spots was above 2 or below 1/2.

Results

All 120 cytokines present on the array were detectable in both groups indicating a sufficiently high concentration of cytokines in the concentrated pool of samples to fall within the detection limits of microarray analysis. Three cytokines exhibited at least a two-fold increase and six exhibited at least a two-fold decrease in the exhaled breath condensate

Table 2 Cytokines showing over two-fold change in the pooled breath condensate of lung cancer patients compared to healthy smokers

Cytokine	Change in relative level of individual markers		Biological role
	Decrease	Increase	
eotaxin-2	2.55		Eosinophil chemoattractant
eotaxin-3	2.43		Eosinophil chemoattractant
FGF-6	3.16		Fibroblast growth factor
FGF-7	2.52		Fibroblast growth factor
Flt-3 lig.	2.25		Haematopoietic growth factor
IL-10	2.49		General inhibitory cytokine
CCL-28		3.08	Lymphocyte chemoattractant
GROα		2.35	Monocyte adhesion molecule
MIP-3		2.00	Granulocyte activator

The numbers indicate the fold of decrease or increase in the relative level of the individual markers. The main biological role of each cytokine is listed

of patients with lung cancer as compared to the control pool. A lymphocyte chemoattractant, CCL-28 showed the greatest increase (3.08-fold) and a fibroblast growth factor (FGF-6) the greatest decrease (3.16-fold).

Table 2 shows the relative cytokine levels in the pooled exhaled breath condensate of patients with lung cancer as compared to the control pool. Only changes over two-fold with variability of less than 25% between pairs are listed.

Discussion

We found the cytokine profile of exhaled breath of patients with lung cancer to be different from that of controls with a similar smoking habit, lung function and airway inflammation.

Changes in EBC cytokine levels have been demonstrated by immunoassays and flow cytometry in a number of inflammatory airway diseases (Table 2) [3, 5–8]. A recent study using antibody microarray has found a 1.5–2.7 fold increase in the level of certain cytokines in asthmatic patients as compared to healthy controls [4].

This is the first study that employs antibody microarray for the analysis of EBC of lung cancer patients. The observed changes in cytokine level between lung cancer patients and controls are higher than those described for asthmatics. While some of the differences found are well in line with previous studies in serum (CCL-28 is increased in colorectal tumours), others are in contrast (FGFs are increased in prostate cancer) or have not been described in cancer patients (eotaxin, GRO-α) [9, 10]. Differences in eotaxin and IL-10 bring attention to the immunological behaviour of lung cancers. Importantly, pooled samples were used in our study to compare EBC from lung cancer patients with those from controls precluding stratification of the results according to stage and histological subtypes. Further work is required to compare exhaled molecular fingerprint with genetic or morphological characteristics at the level of the individual. COPD is frequent in patients with lung cancer and this co-morbidity is associated with well-characterized morphological and pathophysiological changes in the lung [11] as well as changes in EBC biomarkers [3]. To rule out the potential confounding effect of smoking and COPD, current smokers some with known COPD were enrolled as controls in the study.

The feasibility of breath testing for lung cancer screening has been demonstrated by measuring various volatiles using methods such as gas chromatography/mass spectrometry and electronic nose [12, 13]. Among other technologies, the development of genomics and proteomics have been suggested to play a role in clinical decision making in cancer screening, diagnosis and monitoring [14, 15]. Application of protein microarray to exhaled breath condensate may provide a new perspective for the assessment of different types of lung cancer. Whether the measurement of selected cytokine markers will be reliable to predict pulmonary tumour in individual samples needs further investigation.

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