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Quantitative breath analysis of volatile organic compounds of lung cancer patients

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ABSTRACT

Due to state-of-art analytical techniques, non-invasive exhaled volatile organic compounds (VOCs) analysis has become a potential method for early diagnosis of lung cancer. We collected breath samples from 43 patients with non-small cell lung cancer (NSCLC) and 41 normal controls using Tedlar® gas bags. The VOCs were extracted with solid phase micro-extraction (SPME) and analyzed by gas chromatography (GC)/mass spectrometry (MS). The number of VOCs detected in each breath sample ranged from 68 to 114. Among the VOCs 1-butanol and 3-hydroxy-2-butanone were found at significantly higher concentrations in breath of the lung cancer patients compared to the controls. VOCs levels were not significantly different between early stage lung cancer patients and late stage lung cancer patients. Lung adenocarcinoma was significantly related to higher VOCs concentrations in the breath. Our data showed that 1-butanol and 3-hydroxy-2-butanone in breath could possibly be taken as useful breath biomarkers for discerning potential lung cancer patients and VOCs analysis could be used as a complementary test for the diagnosis of lung cancer.

1. Introduction

Lung cancer is the most common kind of cancer in the world. In China, lung cancer has an incidence of 26/100,000, and compared to gastric, liver and breast cancer, it is the leading cause of cancer death [1,2]. The 5-year survival rate for the patients with lung cancer is disappointing. To improve the overall survival, many screening methods including chest radiography, sputum cytology, low-dose spiral computer tomography, fluorescence bronchoscopy and positron emission tomography have been used [3–6], while these procedures seem to be complicated and expensive.

In 1971, Pauling et al. [7] reported a new method to analyze the VOCs in human breath which shed light on developing new methods of early diagnosis. VOCs are the products of oxidative stress, which can cause peroxidative damage to protein, polyunsaturated fatty acids and DNA in the cells [8–10]. The peroxidative damage to DNA may be carcinogenic [11,12]. In some cancers, oxidative stress seems to be increased [13]. VOCs derived from the blood are excreted through the lung by diffusing across the pulmonary alveolar membrane. Some VOCs were identified as markers for the diseases, such as hexane, methylpentane, isoprene and benzene [14,15]. Nevertheless, breath analysis has taken a long time to be a useful tool because of the lack of standardized breath collecting methods and the low concentrations of VOCs which are present in nanomolar (10^{-9} mol) or picomolar (10^{-12} mol) [16]. Phillips et al. [17–20] developed a breath collecting apparatus to determine VOCs in breath and selected nine VOCs to distinguish patients with lung cancer and normal controls. As a new, simple pre-concentration technique, SPME was developed and widely used in breath analysis [21–23]. So far, many VOCs have been identified in breath samples of normal human and patients with lung cancer, and a combination of some VOCs is able to discriminate patients. Nevertheless, these VOCs confirmed in above studies were not constant, and the published studies did not concern the relationship of the VOCs concentrations of patients with the histology of lung cancer, such as squamous cell carcinoma and adenocarcinoma.

Previous studies showed that breath analysis might improve the sensitivity and specificity of lung cancer diagnosis in Europe and USA, while the lung cancer susceptibilities and genetic mutations in East Asian were much different from those in the West. For example, the genotype CYP1A1 MSPIC might be one of the susceptibly genetic markers of lung cancer [24,25] and mutations of the epidermal growth factor receptor gene EGFR were found more frequently in East Asian patients [26,27]. Study should be done for lack of statistical analysis of VOCs concentrations in the breath of lung

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Table 1
Histology and stage of lung cancer.

Histology	Number
Squamous cell carcinoma	24
Adenocarcinoma	19
Stage	
I	13
II	7
III	6
IV	17

cancer patients in China. In the present work, we tried to apply the methodology of SPME with GC/MS to the breath analysis of lung cancer patients in China. The differences of VOCs levels in the lung cancer patients with different stages or histology were also investigated.

2. Materials and methods

2.1. Reagents and instruments

The standards (purity >99.5%) of 1-butanol and 3-hydroxy-2-butanone were purchased from Guangfu Research Institute, Tianjin, China. Solid phase micro-extraction manual holder and the fiber of 75 μm carboxen-polydimethylsiloxane (CAR-PDMS) were ordered from Ampel Company, Shanghai, China. 4-l Tedlar® gas bags were bought from Delin Company, Dalian, China. GC/MS-QP 2010 Puls equipped with NIST 05 library was the product of Shimadzu, Japan. The Rxi™–5MS column (30 m, 0.25 mm i.d., 0.25 μm thick) was made by Restek, USA.

2.2. Subjects

The study included the following two groups: patients with NSCLC and healthy volunteers. We enrolled 43 patients from the First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China. The diagnosis of lung cancer was confirmed in each patient according to bronchoscopy and biopsy findings. The tumor was staged by the tumor, node, metastasis (TNM) system for lung cancer on the basis of AJCC (American Joint Committee on Cancer) cancer staging manual, the 6th edition (2002). The histological diagnoses and stages of lung cancer were shown in Table 1. Patients with acute respiratory tract infection were excluded from the study. Patients who had a history of previously diagnosed cancer at any site or history of diabetes, liver disease, alcohol abuse were not eligible for the study. The history of smoking was obtained from all subjects, and the ex-smokers were defined as the ones who had stopped smoking for at least 1 year. 11 patients classified as stage III or IV received 1 cycle chemotherapy, and the breath samples were collected 4 weeks after the previous treatment. The other patients never received surgery, radiation or chemotherapy. The controls were 41 non-smokers who had no pulmonary symptoms or a history of pulmonary disease. All the subjects gave their willingness to cooperate with the breath collecting procedure. The characteristics of the subjects were shown in Table 2.

Table 2
The characteristics of subjects.

	Patients with lung cancer	Normal controls
Number	43	41
Mean age (S.D.)	58.1 (8.1)	47.9 (6.5)
Sex (male/female)	34/9	34/7
Current smokers	0	0
Ex-smokers	21	0

2.3. Breath collection and preparation of gas standards

After the subjects had fasted overnight, they deeply breathe in Tedlar® gas bags which trapped 4 l breath gas through a disposable mouthpiece. Mixed expiratory samples were collected, with no restriction on a particular part of breath. Fifteen environmental gas samples were collected from the rooms where the subjects performed the breath collection.

The gas samples with concentration of 0.66 μg/ml for 1-butanol and concentration of 4.91 μg/ml for 3-hydroxy-2-butanone were prepared. Calibration gas of 1-butanol in different levels were made by adding 5 μl, 10 μl, 25 μl, 50 μl and 100 μl gas samples into 50 ml glass bottle filled with pure nitrogen gas, respectively. Calibration gas of 3-hydroxy-2-butanone in different levels were made by adding 0.5 μl, 1 μl, 2.5 μl, 5 μl, 20 μl and 45 μl gas samples into 50 ml glass bottle filled with pure nitrogen gas, respectively.

2.4. Extraction and analysis

The VOCs in breath, environmental gases and calibration gases were extracted by SPME. The SPME fiber was pretreated in the injection port of GC at 250 °C for 20 min to clean it before extraction. In extracting procedure, SPME fiber was inserted into Tedlar® bag or glass bottle for 30 min at room temperature to extract VOCs.

After extraction, VOCs were thermally desorbed and analyzed. The analysis was performed using GC/MS. The carrier gas was helium with flow rate of 1 ml/min. Splitless mode was used. The GC injection port temperature was 250 °C. The column temperature programs were: initial temperature of 35 °C for 5 min, then increase to 100 °C at 4 °C/min, finally increase to 260 °C at 20 °C/min. The ion source temperature of mass spectrometer was 200 °C. Data were analyzed by NIST 05 library.

The method was validated by investigating the precision and linearity. The precision was expressed by the R.S.D. Six replicate measurements of standard gases of 1-butanol and 3-hydroxy-2-butanone in constant concentration were performed to obtain the peak area values. Precision was calculated as R.S.D. of the values. The linearity was established by analyzing the relationship between concentrations of standard gases and peak areas. Five different concentrations for 1-butanol and six different concentrations for 3-hydroxy-2-butanone were measured to obtain standard curves. Replicate two analyses were performed for each concentration.

2.5. Statistical analysis

The significances of the differences between the groups were obtained from Wilcoxon rank sum test. A P value of <0.05 was considered significant. The discriminative power was assessed by ROC curve. SPSS 13.0 was used for the statistical analyses.

3. Results

3.1. Precision and linearity

The method validations including precision and linearity were investigated in the study. The R.S.D. ($n = 6$) values for 1-butanol and 3-hydroxy-2-butanone were 5.04% and 3.25%, respectively. The linearity and regression equation for 1-butanol are $y = 5.22 \times 10^4 x - 4.17 \times 10^4$ (y: the peak area of 1-butanol; x: the concentration of 1-butanol), $r^2 = 0.996$, respectively. The linearity and regression equation for 3-hydroxy-2-butanone are $y = 5.81 \times 10^4 x - 1.81 \times 10^5$ (y: the peak area of 3-hydroxy-2-butanone; x: the concentration of 3-hydroxy-2-butanone), $r^2 = 0.988$, respectively.