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Investigation of volatile organic metabolites in lung cancer pleural effusions by solid-phase microextraction and gas chromatography/mass spectrometry

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ABSTRACT

Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography/mass spectrometry (GC/MS) method was applied for the investigation of low molecular weight volatile organic metabolites (VOMs) in pleural effusion samples. Three important HS-SPME experimental parameters that influence extraction efficiency (fiber coating, extraction time and temperature of sampling) were optimized by a univariate optimization design. The highest extraction efficiency was obtained when sampling was performed at 50 °C for 10 min under agitation using a carboxen/polydimethylsiloxane (CAR/PDMS) fiber. A total of 36 volatile metabolites belonging to nine distinct chemical classes were identified in 40 pleural effusion samples (20 malignant effusions from lung cancer patients and 20 benign effusions from inflammatory patients). Ketones, alcohols, and benzene derivatives were the main chemical classes for the metabolomic profile of malignant effusions. The average peak areas of ketones and alcohols were much higher in malignant group compared to benign group. Together with phenols, they exhibit significant differences ($P < 0.05$) between the two groups. Particularly, the average peak areas of cyclohexanone and 2-ethyl-1-hexanol in malignant effusions were significantly higher than those in benign ones. Furthermore, of the 36 identified metabolites, 5 compounds including cyclohexanone and 2-ethyl-1-hexanol were found to be statistically different (Student's *t*-test, $P < 0.05$) between the two groups by statistical analysis based on the peak areas of all identified metabolites. Among them, cyclohexanone and 2-ethyl-1-hexanol might be considered as candidate biomarkers of lung cancer to differentiate malignant from benign effusions. The results show that HS-SPME-GC/MS is a simple, rapid, sensitive and solvent-free method for the determination of VOMs in pleural effusion samples. Pleural effusion is a valuable sample source for observation of changes in VOMs for differentiation between lung cancer patients and inflammatory individuals.

1. Introduction

Lung cancer is a devastating disease and a leading cause of cancer-related death. The WHO estimated that 18 percent of all cancer-related death was due to lung cancer. Each year, lung cancer is considered to be responsible for the death of about 1.3 million people worldwide [1]. Pleural effusion is the pathological accumulation of fluid in the pleural cavity that surrounds the lung, caused by a variety of diseases including lung cancer and inflammatory diseases [2]. Therefore, a pleural effusion case can be benign or malignant based on its definitive pathologies or cytology diagnoses. Benign effusions are caused by non-malignant conditions such as tuberculosis, pneumonia, congestive heart failure and cirrhosis. When malignant cells or tissues are found in the pleural fluid or pleural biopsy samples, the effusions are diagnosed as malignant [3]. The most common cancer associated with malignant effusions is lung cancer, responsible for approximately 30% of malignant effusions [4]. The presence of a malignant effusion, regardless of the type of originating cancer, indicates a poor prognosis with a median survival of about 4 months. Conversely, patients with non-malignant effusions often need additional testing to identify the cause and may require immediate treatment for underlying conditions such as pneumonia or tuberculosis. Since the two types of effusion have completely different clinical treatments, follow-up, and patient outcomes, it is important to elucidate their precise etiologies to distinguish malignant from benign effusions. Although cytological analysis of pleural fluid is the gold standard for the differential diagnosis, the differentiation between benign

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and malignant pleural effusions is still a clinical challenge due to its poor sensitivity.

Recently, metabolomics has developed rapidly following the proteomic approach. It reveals the dynamic change of the overall organism under the specific pathophysiologic conditions, and reflects quantitative change of small molecule metabolites resulting from inner genes, proteins and enzymes [5]. The metabolites might reflect physiological function and pathological characteristics in more detail, because the metabolome is the endpoint of the omics cascade. Low molecular weight volatile organic metabolites (VOMs) are a class of biomarkers thought to have potential for the detection of malignancy. Thousands of VOMs were present in trace amounts in human breath [6–13], serum [14–16] and urine samples [17–20]. Several studies have shown that VOM profiles of patients with cancer can be discriminated from those of healthy subjects. For example, Phillips and his group first reported in 1999 that 22 VOMs in breath had been regarded as candidate markers of lung cancer [8], and in the following 10 years they updated the volatile biomarkers continuously [9,11,21]. In 2004, Deng et al. investigated VOMs in lung cancer blood and found that the concentrations of hexanal and heptanal in lung cancer blood were much higher than those in control blood [14]. In addition, a recent study has demonstrated that nine urinary VOMs from lung cancer patients were identified as elevated than those in control group [20].

It has been reported that pleural effusion, which may be more specific for lung disorders than other body fluids due to its close correlation with the affected organ, contains plasma proteins as well as proteins released by inflammatory, epithelial or even cancer cells [22,23]. Using the proteomic approach, Tyan et al. analyzed the global protein composition of human malignant pleural effusions [24,25], and Wang et al. further identified proteins differentially expressed in malignant effusions from lung adenocarcinoma and benign inflammatory effusions [26]. Pleural effusion may be a valuable sample source for observation of changes in VOMs, which can be used to differentiate malignant from benign pleural effusions. However, no reference of metabolomic investigations and comparisons of volatile metabolites between benign and malignant pleural effusions appears to exist so far.

The aim of this study was to investigate and confirm the existence of VOMs in pleural effusions, and preliminarily identify VOMs differentially expressed in malignant effusions from lung cancer and benign inflammatory effusions from pneumonia and tuberculous pleurisy by headspace solid-phase microextraction (HS-SPME) combined with gas chromatography/mass spectrometry (GC/MS). Several parameters related to HS-SPME extraction efficiency (fiber coating, extraction temperature and extraction time) were optimized. The proposed method was applied to comparative analysis of VOMs in malignant effusions and benign effusions. At the same time, the peak areas of all volatile metabolites were submitted to statistical analysis to obtain the specific volatile metabolites that are able to differentiate lung cancer patients from inflammatory individuals.

2. Materials and methods

2.1. Chemicals and samples

The standard compounds of methanol, acetone, toluene, hexanal, cyclohexanone, heptanal, phenol, decane, 2-ethyl-1-hexanol, acetophenone, 1, 2, 4, 5-tetramethyl benzene, dodecane, 2-methylnaphthalene and 2, 6-di(*tert*)butyl-4-methylpnenol were purchased from Aladdin (Shanghai, China).

The explored subjects were divided into two groups: malignant group (20 patients with lung cancer) and benign inflammatory group (20 benign inflammatory patients with pneumonia or tuberculous pleurisy). The malignant group was seventeen patients with non-small cell lung cancer (NSCLC) and three patients with small cell lung cancer (SCLC). The benign inflammatory group was fifteen patients with tuberculous pleurisy and five patients with pneumonia, who had no history of cancer. The average age of these two groups was 73 and 63.6, respectively. The characteristics of these two groups were described in Table 1. Lung cancer patients were confirmed by histopathology, and all the patients never received drug therapy, radiotherapy and chemotherapy or surgery.

Table 1
The characteristics of the patients.

	Malignant group (NSCLC/SCLC)	Benign group (tuberculous pleurisy/pneumonia)
Number	20 (17/3)	20 (15/5)
Age (mean/range)	73/53–85	63.6/44–86
Sex (male/female)	16/4	14/6

All pleural effusion samples were collected by thoracentesis in sterile tubes at Zhejiang Provincial People's Hospital, Hangzhou, China. The samples were stored at −80 °C until further analysis.

A standard test solution containing five compounds (toluene, cyclohexanone, heptanal, 2-ethyl-1-hexanol and octane) at 0.2 mM was prepared by dissolving them into 50 mL mixture solution of water and methanol (20/1(v/v)). Furthermore, other diluent samples were prepared by spiking appropriate amount of the standard test solution into mixture solution of water and methanol (20/1(v/v)). The optimization of SPME extraction conditions was investigated by using 2 mL of standard test solution with a concentration of 0.2 mM. Repeatability and the limit of detection were detected with 2 mL of samples at concentration of 0.02 mM and 0.04 μM, respectively.

2.2. Instrumentation

A GC/MS system (trace DSQ; Thermo Finnigan, Austin, TX, USA) was used for sample analysis. A capillary column from Frontier Lab. Ltd., (Ultra ALLOY-5, Fukushima, Japan) with 30 m long, 0.25 mm i.d. and 0.25 μm film, was used for separation.

A manual SPME holder (57330U) and three commercial SPME fibers, *i.e.*, 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane/divinyl benzene (PDMS/DVB) and 75 μm carboxen/polydimethylsiloxane (CAR/PDMS), were purchased from Supelco (Bellffone, PA, USA). A magnetic stirrer with heating function was purchased from IKA Works (Guangzhou, China).

2.3. Extraction

The SPME fibers were conditioned for 30–60 min following the manufacturer instructions before they were used for the first time. Then, the fibers were conditioned for only 10 min at 250 °C in GC injector before analysis.

The samples were completely thawed at room temperature, then the supernatant (2 mL) was transferred into a glass headspace vial (15 mL) with a silicone septum after centrifuged for 10 min. The SPME fiber was inserted into the headspace of the vial and was exposed for 10 min at 50 °C, at a stirring rate of 800 rpm. After sampling, the fiber was injected into the GC/MS for analysis.

2.4. GC/MS measurements

The adsorbed compounds on the fiber were desorbed at 250 °C in GC injector for 3 min. Then the thermally desorbed trace components were separated by capillary column with a helium flow at