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Serum metabolic profiling study of lung cancer using ultra high performance liquid chromatography/quadrupole time-of-flight mass spectrometry☆

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A B S T R A C T

Lung cancer is currently the leading cause of cancer-related mortality worldwide. It is, therefore, important to enhance understanding and add a new auxiliary detection tool of lung cancer. In this work, serum metabolic characteristics of lung cancer were investigated with a non-targeted metabolomics method. The metabolic profiling of 23 patients with lung cancer and 23 healthy controls were analyzed using ultra high performance liquid chromatography/quadrupole time of flight mass spectrometry (UPLC/Q-TOF MS). Partial least squares discriminant analysis (PLS-DA) model of the metabolic data allowed the clear separation of the lung cancer patients from the healthy controls. In total, 27 differential metabolites were identified, which were mostly related to the perturbation of lipid metabolism, including choline, free fatty acids, lysophosphatidylcholines, etc. Choline and linoleic acid were defined as one combinational biomarker using binary logistic regression, which was supported by the validation with a smaller sample-set (9 patients and 9 healthy controls). These findings show that LC/MS-based serum metabolic profiling has potential application in complementary identification of lung cancer patients, and could be a powerful tool for cancer research.

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1. Introduction

Lung cancer is currently the leading cause of cancer-related death worldwide. It accounts for more than one million deaths annually in the world, and its incidence is increasing in China [1]. Lung cancer results from environment–gene interaction [2,3]. Cigarette smoking is one of the most common risk factors, chemical carcinogens, diet and family history are closely related to the incidence of lung cancer [4,5]. Computed tomography (CT) and magnetic resonance imaging (MRI) are two main general diagnostic techniques of lung cancer. Biomarkers offer non-invasive and cost-effective methods to assist disease detection, which are being paid more and more attention. Now some of biomarkers have been used in screening and diagnosis of lung cancer.

Metabolomics, which focuses on changes of small molecules to reveal what has happened in a biological system [6], could provide an alternative to gain insight into the progression of lung cancer from a different angle. Previously, metabolomics based on the extensive analysis of differential metabolites has been used in research on the underlying mechanisms of lung cancer, the classification of cancer subtypes and the discovery of biomarkers for diagnosis [7–9]. Rocha and co-workers found that HDL, VLDL + LDL, several amino acids and lactate are changed significantly in lung cancer patients [10]. Hori et al. reported a marked change in 23 serum metabolites, which may represent the differences between disease stages or histological subtypes in lung cancer [11]. The metabolomics techniques that are commonly applicable to lung cancer are nuclear magnetic resonance analysis (NMR) [12,13], gas chromatography/mass spectrometry (GC/MS) [14] and liquid chromatography/mass spectrometry (LC/MS) [15,16]. Especially, LC/MS is increasingly used for analysis of complex samples, such as serum or plasma.

In this study, we investigated serum metabolites from patients with lung cancer and healthy controls using ultra high performance liquid chromatography/quadrupole time of flight mass

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spectrometry (UPLC/Q-TOF MS) and multivariate statistical analysis, aiming to identify metabolites differentially regulated in lung cancer and to find potential biomarkers for the discrimination of lung cancer patients from healthy controls, thereby providing a complementary method for cancer detection.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol (HPLC grade) were purchased from Merck (Germany). Ammonium bicarbonate and formic acid (HPLC grade) were from Fisher Scientific (USA). Distilled water was filtered through the Milli-Q system (Millipore, MA, USA). Leucine–enkephalin and all the standard samples were from Sigma–Aldrich (Germany).

2.2. Sample collection and preparation

All serum samples were collected from fasting subjects and provided by the Second Affiliated Hospital of Dalian Medical University, Dalian. The study was approved by the Ethics Committee of the hospital. All subjects got an informed consent before the study. The inclusion criteria of patients were as follows: patients were newly diagnosed with lung cancer, no previous surgery, no radiotherapy or chemotherapy, no cancer metastasis. Cancer diagnosis was performed by the histopathological analysis of tissue specimens. The healthy volunteers whose age and sex were equivalent to those of the patients enrolled. They were in health state, without known chronic or major diseases and no medicine treatment. One batch of serum samples from 23 lung cancer patients (6 small cell lung cancer and 17 non-small-cell lung cancer) and 23 healthy controls were collected at the discovery phase. The age and sex information is provided in Table 1. Another batch of serum samples from 9 lung cancer patients and 9 healthy controls were at the validation phase. The two batches of samples shared the same preparation and analysis method. After collection, serum samples were kept at −80 °C until use.

From each sample, 20 μL sera were mixed and divided into several aliquots as the quality controls (QCs). The QCs were used to test the instrument state, equilibrate the UPLC/Q-TOF MS system before sample injection and indicate the stability of system during the analytical sequence [17].

For the pretreatment of the serum samples, 800 μL acetonitrile was added to 200 μL serum. Then, the mixture was vortexed and centrifuged at 15,000 × g for 10 min at 4 °C to remove protein [18,19]. Next, 900 μL of the supernatant was lyophilized. The lyophilized sample was redissolved in 200 μL acetonitrile/water (20:80, v/v) for injection.

2.3. UPLC/Q-TOF MS analysis

The sample analysis was performed on a Waters ACQUITY Ultra High Performance liquid chromatography system (Milford, MA) coupled with a Waters Q-TOF Micromass system (Manchester, UK) in both positive and negative ionization modes. In the positive ionization mode, mobile phases A and B were 0.1% aqueous formic acid and acetonitrile, respectively. The gradient started with 10% B, increased linearly to 100% B during 24 min and remained at 100% B for 4.5 min. In negative ionization mode, mobile phases C and D were 6.5 mM NH_4HCO_3 in water and 6.5 mM NH_4HCO_3 in methanol/water (95:5, v/v), respectively. The gradient started at 10% D, increased linearly to 100% D during 22 min and remained at 100% D for 5.5 min. The column was a 2.1 nm × 100 nm, 1.8 μm, HSS T3 column (Waters, USA) and the temperature was maintained at 55 °C. The flow rate was 0.35 mL/min. The injection volume was 10 μL.

The capillary voltage was set to 3000 V for the positive mode and 2500 V for the negative mode. The cone voltage was 40 V. The nebulization gas was maintained at a flow of 600 L/h and a temperature of 300 °C. The cone gas flow was set to 50 L/h, and the source temperature was set to 110 °C. The MCP detector voltage was set to 2500 V. The scan range was from 80 to 1000 *m*/*z*. Data were acquired at 0.48 s intervals with a 0.1 s scan delay in the centroid mode. The mass accuracy calibration was performed with the lock mass, leucine–enkephalin at 2 ng/L and 10 μL/min, the data acquisition frequency was set at 20 s.

2.4. Data analysis

The raw data were imported to the MarkerLynx program (Waters, Manchester, UK) for peak alignment to obtain a peak list containing the retention time, *m*/*z*, and peak area of each sample. According to the 80% rule [20], the background and non-biologically relevant information were eliminated, and only variables having more than 80% of the nonzero measurement values in the healthy or lung cancer groups were kept in the peak list. Then, the peak list was imported into the SIMCA-P 11.0 software (Umetrics AB, Umea, Sweden) for principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA) and orthogonal signal correction partial least-squares discriminant analysis (OSC-PLS-DA) to acquire clustering information and important variables between the lung cancer group and the control group. Student's *t*-test was performed between the two groups, and variables with a low *p*-value (<0.05) were selected as biomarker candidates for further statistical analysis using SPSS (Statistical Package for the Social Sciences) version 11.0 (SPSS Inc., USA). All differential metabolites were randomly chosen for combination using the binary logistic regression model. Meanwhile, receiver operating characteristic (ROC) curve was used to evaluate sensitivity and specificity of the combinational biomarker.

3. Results and discussion

3.1. Serum metabolic profiling

Typical total ion current (TIC) chromatograms of serum metabolic profiles analyzed using UPLC/Q-TOF MS in the positive mode are shown in Fig. 1. There were obvious differences between the cancer and control groups, mainly in the region from 18 min to 28 min. Our earlier studies of serum and plasma samples showed that this region in the RPLC system consisted of medium-polar and nonpolar compounds. TIC chromatograms in negative mode are not shown. And they also directly showed differences in the medium-polar and nonpolar compounds.

After eliminating zero values using the "80% rule," 988 peaks from the positive mode and 436 peaks from the negative mode were obtained for the subsequent data analysis. The QC samples were used to appraise the system stability and data quality. It was found from the first principal component in the PCA of the time series analysis [18] that all QC samples were totally included in two fold RSD for the QC samples versus time analyzed in the positive and negative modes, which demonstrated the stability of our analytical system and the reliability of the data. The metabolic profiling data could be used to classify and gain more knowledge of lung cancer.

3.2. Multivariate statistical analysis

To obtain a comprehensive and complete understanding of the metabolic profiling data, PCA was used after unit variance (UV) scaling. The PCA score plots of the patients with lung cancer and the

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