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Urinary metabonomic study of patients with acute coronary syndrome using UPLC-QTOF/MS

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ABSTRACT

This urinary metabonomic study aimed to identify the potential metabolic biomarkers in acute coronary syndrome (ACS) patients. Ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) was used to analyze the urine samples from ACS patients and healthy controls. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to characterizing the endogenous metabolites and potential biomarker, respectively. Among twenty biomarkers that functioned in nine metabolic pathways, nine biomarkers were found up-regulated significantly, including of isobutyryl-L-carnitine, 3-methylglutarylcarnitine, cinnavalininate, L-tryptophan, 3-methyldioxyindole, palmitic acid, *N*4-acetylaminobutanal, 3-sulfino-L-alanine and *S*-adenosyl-L-homocysteine. The other eleven biomarkers were showed down-regulated, including of L-lactic acid, trigonelline, nicotinuric acid, L-alanine, D-alanyl-D-alanine, creatine, *N*4-acetylaminobutanoate, glutathionyl spermidine, 5-methoxytryptamine, kynurenic acid and xanthurenic acid. This study also implied that fatty acid metabolism, fatty acid β-oxidation metabolism, amino acid metabolism and TCA cycle played important roles in ACS. Therefore, urinary metabolomics may improve the diagnosis efficacy of ACS and make it more accurate and comprehensive for ACS diagnosis.

1. Introduction

Coronary artery disease (CAD), one of the most serious causes of death in the world, is the result of metabolic disorders [1]. Furthermore, as a serious type of CAD, the acute coronary syndrome (ACS) is usually associated with atherosclerotic plaque rupture, thrombus formation [2] which could lead to arrhythmia, heart failure or even sudden death in severe cases [3].

In clinical diagnosis, electrocardiograms and traditional cardiac biomarkers, such as troponin, are often used to determine the patient's prevalence [4]. However, there are many similarities in terms of clinical symptoms between ACS and some diseases such as Takotsubo syndrome (TTS). TTS patients were often misdiagnosed as “ACS” [5] in consequence of the similar electrocardiographic performances and the changes of myocardial necrosis markers. Thus, it was of great importance to develop a fast, convenient and accurate approach to distinguish ACS patients from other non-acute diseases by identifying the key factors which lead to progression of atherosclerosis and incidence of acute coronary syndrome.

Metabolomics, a newly developed discipline, is widely used in diagnosing variety of diseases, such as neuropsychiatric disorders [6], liver fibrosis [7], hepatocarcinoma [8], lung cancer [9], pneumonia [10], diabetes [11] and so on, because for its qualitative and quantitative analysis for all low-molecular-weight metabolites of organisms or cells produced in physiological or pathological conditions [12]. Simultaneously, the application of metabolomics in cardiovascular diseases like ACS, hyperlipidemia [13], atherosclerosis [14] and hypertension [15] has also gradually attracted people's attention [16].

Due to its high sensitivity, high resolution and rapid separation, UPLC/MS has become a commonly used analytical method in

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metabolomics analysis [17,18]. At the same time, UPLC/MS-based metabolomics analysis plays an important role in the diagnosis of cardiovascular disease [19–21].

Urine samples have plenty of small molecule metabolites. At the same time, the specialties of non-invasive [22] and easy acquisition [23] make it widely used in cardiovascular disease diagnosis of metabolomics research [24–26]. In this study, urine metabolomics based on UPLC/MS was applied to investigate potential biomarkers and metabolic profiles in ACS group and the changes in related metabolic pathways will assist in the prediction and diagnosis of ACS in the future.

2. Material and method

2.1. Chemicals

LC-MS grade acetonitrile and formic acid were purchased from Fisher Scientific (Massachusetts, USA). Pure water was purchased from Watsons (Hong Kong, China).

2.2. Sample collection and preparation

Participants over 18 years old with complete information on medical history and clinical and biochemical parameters were recruited between November 2015 and January 2017 from Fuwai Hospital, the National Center for Cardiovascular Diseases of China. Thirty-six ACS patients and thirty healthy volunteers were finally enrolled in this study. The study was conducted according to the guidelines of the Helsinki Declaration and was approved by the Ethics Committee of Fuwai Hospital. Written informed consent was obtained and urine samples were collected for each participant. Participants without pathological changes in heart, brain, liver, kidney, lung and other major organs were recruited in the control group. Detailed data about patients and controls were presented in Table S1, and the general data of all participants were compared and a two-sided P value of < 0.05 was considered to indicate statistical significance. All urine samples were thawed at room temperature before sample preparation and 200 μL of urine was taken out followed by addition of 600 μL acetonitrile. The mixed samples were centrifuged at 14,000 rpm for 20 min at 4 °C. Then, the 600 μL of supernatants per sample were analyzed using UPLC-Q-TOF/MS. The quality control (QC) samples were prepared by mixing 20 μL of each urine sample before the analysis.

2.3. UPLC/MS measurement of urine samples

The separation of urine samples was performed using a Waters Acquity™ Ultra Performance Liquid Chromatography system (Waters Corp., Milford, MA, USA), with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm, Waters Corp., Milford, MA, USA). To ensure the quality and stability of samples, the temperature of autosampler was maintained at 4 °C during the analysis. And, Column temperature was maintained at 40 ± 0.5 °C in the process of analysis. 2 μL samples were injected and elution was performed using the gradient mobile consisting phase A (0.1% formic acid aqueous solution) and phase B (acetonitrile with 0.1% formic acid). The optimum chromatographic separation was achieved with a flow rate of 500 μL/mL and the following gradient elution profile: The solvent gradient system was performed as follow: 0–1 min, 99% A; 1–3 min, 99%–85% A; 3–5 min, 85%–80% A; 5–13 min, 80%–50% A; 13–14 min, 50%–5% A; 14–15 min, 5% A; 15–15.1 min 5%–99% A; 15–17 min, 99% A.

The UPLC system was coupled to a Xevo™ G2 Q/TOF (Waters Corp., Milford, MA, USA) which was equipped with an electrospray ionization source and operated in either positive or negative ionization mode. The profile data from 50 to 1200 m/z were recorded. The capillary voltage was set at 3.0 kV in positive and 2.5 kV in negative mode, respectively. The sampling cone voltage was set at 40, and the source offset voltage was set at 80. The desolvation gas was set at a flow rate of 800 L/h and a temperature of 500 °C, the cone gas was set at a flow rate of 50 L/h, and the source temperature was set at 120 °C. All of the data accuracy and reproducibility were ensured by Lock Spray. Leucine–enkephalin was used as lockmass at a concentration of 1500 ng/mL and a flow rate of 5 μL/min. Data were collected in continuum mode, the Lock Spray's frequency was set at 60 s, and the lockmass's data were averaged over 10 scans for correction. All of the acquisition and analysis of the data were controlled by Waters MassLynx v4.1 software (Waters Corporation, Milford, MA, USA).

2.4. Data acquisition

In order to check the initial state of the instrument, ten QC samples were continuously analyzed before the start of the experiment. During the operation, a QC sample was analyzed every seven samples to monitor the stability of the instrument, and a blank sample (acetonitrile) was analyzed every three samples to ensure that there were no residual samples on the column.

2.5. Data processing and statistical analysis

The raw data was acquired through Waters Masslynx v4.1 software, and all of the raw data were imported into Progenesis QI software (Waters Corp., Milford, MA, USA) for peak extraction, peak alignment and normalization. In order to perform PCA and OPLS-DA, this data was transferred into EZinfo 2.0 software (Waters Corporation, Milford, MA, USA). PCA was used to visually observe the separation of samples from different groups while OPLS-DA was used to find the separated metabolites which had significant difference in the content between ACS group and control group. Those metabolites were considered to be potential biomarkers with variance importance for projection (VIP) value above 1.0 and P value below 0.05. All the statistical analyses were performed by SPSS Statistics ver18.0.0, with a $P < 0.05$ considered statistically significant. The potential biomarkers were identified by following databases: HMDB (http://www.hmdb.ca/), ChemSpider (http://www.chemspider.com/) and Progenesis MetaScope. Pathway analysis was found through Integrated Molecular Pathway Level Analysis (IMPaLA) base on the potential biomarkers.

3. Results

3.1. Demographics and clinical characteristics

There was no significant difference in gender, age, Body Mass Index (BMI), total cholesterol, HDL and LDL both the groups based on SPSS analysis ($P > 0.05$). The level of troponin I, isoenzyme of creatine kinas and triglycerides in ACS group was higher than that of the controls ($P < 0.05$) (Table S1). Therefore, the findings cannot be attributed to demographic factors.

3.2. Analysis of urine samples by LC–MS

Typical positive and negative base peak intensity (BPI) chromatograms of representative urine samples from the control group and ACS group were shown in Fig. 1. According to the BPI chromatograms, significant differences were found in the peak height and peak area of many peaks which had the same retention time between two groups. From the results above, it was proved that the content of some small molecular metabolites in the body changed due to different disease states. In addition, the two groups of BPI chromatograms were also significantly different in the positive and negative ionization mode. Using positive and negative ionization modes could detect different substances and made the detection range more comprehensive.