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Identification of essential hypertension biomarkers in human urine by non-targeted metabolomics based on UPLC-Q-TOF/MS

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ABSTRACT

Background: In recent years, using metabolomics technology to study hypertension has made some progress. However, in actual clinical studies, there are few studies on hypertension related metabonomics with human urine as samples. In this study, the urine samples of patients with essential hypertension (EH) were studied by comparing with healthy people to explore the changes of urine metabolites between hypertensive patients and healthy people in order to find potential biomarkers and metabolic pathways.

Methods: An ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) technology was used to analyze the urine metabolites of 75 cases of essential hypertension group (EH) and 75 cases of healthy control group (HC).

Results: According to the PLS-DA pattern recognition analysis, substances with significant differences ($P < .05$) between the EH group and the HC group were screened out, including 10 potential biomarkers such as L-methionine. The metabolic pathways involved were amino acid metabolism, fatty acid metabolism steroid hormone, biosynthesis and oxidative stress.

Conclusion: The non-targeted metabolomics based on UPLC-Q-TOF/MS technology can effectively identify the differential metabolites of potential biomarkers in the urine of essential hypertensive patients and provide a theoretical basis for the treatment of clinical hypertension.

1. Introduction

Hypertension, is a chronic disease characterized by increased blood pressure in systemic circulation arteries, which is prone to complications and is the main risk factor for cardiovascular and cerebrovascular diseases [1]. On the one hand, due to changes in lifestyle and diet structure, overweight and obesity are not controlled, resulting in atherosclerosis, which further increases the incidence of hypertension. On the other hand, the serious aging of population leads to an increase in the incidence of hypertension [2]. By 2000, the prevalence of hypertension was 26.4%, and it is forecast to rise to 29.2% by 2025 [3]. Despite the high prevalence rate, only 53.7% of hypertensive patients were treated satisfactorily [4], and the control of hypertension was only 8.1% [5]. Hypertension is a genetic disease with complex pathogenesis, multiple factors involved, high incidence, great harm and genetic correlation, but the pathogenesis of hypertension is still unclear. We call hypertension that is not yet known to be essential hypertension (EH) [6].

Metabonomics is a new science that aims to quantitatively describe the dynamic changes of many metabolites in organisms. It is an important field of systems biology. The metabolites of small molecules in the body are the material basis of the life and biochemical metabolism of the system. Some specific metabolites are different in different individuals, reflecting the metabolic characteristics of the individual and the inherent manifestations of the disease, metabolomics is to quantitatively detect the level of small molecule compounds in vivo and the corresponding data processing methods in order to study the changes in the body's basal metabolism, to provide direct data for exploring the pathogenesis and pathogenesis of metabolic diseases [7].

In recent years, some advances have been made in the study of EH using metabolomics techniques. However, in the actual clinical studies, due to the complexity of organisms and the influence of individual differences, the study of hypertension-related metabonomics has remained at the stage of basic research on animal models, a small part of human-centered research also uses blood as a specimen to search for biomarkers to explore its pathogenesis, such as Ameta et al. [6] used

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800 MHz nuclear magnetic resonance (NMR) spectroscopy to analyze filtered serum samples from 64 patients with EH and 59 healthy controls, and found related metabolites such as alanine, arginine and methionine. Yang et al. [8] selected 113 patients with EH and 15 healthy subjects to perform metabolomics examination of venous blood by using metabolomics MRM-MS technique. They found that 47 metabolites have significant changes in hypertensive patients and were closely related to the pathophysiological mechanisms of the cardiovascular system. Li et al. [9] used metabolomics to study the metabolic mechanisms of EH and its traditional Chinese medicine subtypes, including "Yin-deficiency and Yang-hyperactivity syndrome" (YDYHS) and the "Yin-Yang deficiency syndrome" (YYDS). Plasma samples from 22 healthy volunteers, 31 YDYHS hypertensive patients and 29 YYDS hypertensive patients were analyzed by H-NMR spectroscopy and gas chromatography-mass spectrometry (GC-MS). It is concluded that abnormal glucose metabolism may be the main common pathway from YDYHS to YYDS. In recent years, there are few experiments on human urine as samples, but urine is the best matrix for the systematic study of the final product of human metabolism [10], which has the advantages of easy collection, non-injury and large sample size. Therefore, this study prospectively applied metabonomics to analyze potential biomarkers in urine of hypertensive patients.

2. Materials and methods

2.1. Clinical trial registration and ethics statement

The trial is registered with the China Clinical Trial Registration Center, registration number is ChiCTR1800016074, and the registered unit is Tianjin University of Traditional Chinese Medicine. The relevant experimental design can be found on the platform. This experimental study follows the Helsinki Declaration and was approved by the Ethics Committee of Tianjin University of Traditional Chinese Medicine by written consent, the approval number TJUTCM-EC20180004 was examined.

2.2. Reagents and materials

Acetonitrile (Sigma-Aldrich); formic acid (ROE); pure water (Hangzhou Wahaha Group Co., Ltd.); Waters Acquity UPLC (Waters Co., Milford); chromatographic column: Acquity UPLC BEH C_{18} (2.1 mm × 100 mm, 1.7 μm, Waters).

2.3. Subjects

This study was conducted at the General Hospital of Tianjin Medical University. We designed a case-control study with 75 hypertensive patients (EH group) and 75 healthy controls (HC group).

2.4. Standard of the study subject

2.4.1. Diagnostic criteria for the study subject

The diagnostic criteria for hypertension refer to *Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Synopsis of the 2017 American College of Cardiology/American Heart Association Hypertension Guideline*. The guideline has made important amendments to the classification of blood pressure (normal < 120/80 mmHg, elevated 120–129/ < 80 mmHg, Hypertension Stage 1, 130–139/80–89 mmHg, and Stage 2, ≥ 140/≥ 90 mmHg).

2.4.2. Inclusion and exclusion criteria for research subjects

The inclusion criteria for the study subjects were: between the ages of 20–80 y old, in line with the Western medical diagnostic criteria for EH; although taking antihypertensive drugs, but have stopped for > 2 weeks, and blood pressure levels are still in line with diagnostic criteria. Exclusion criteria were: patients with a history of chronic illness including severe chronic diseases such as coronary heart disease, diabetes, malignancy, nephropathy, rheumatic immune diseases, hyperuricemia, hypertension, hyperlipidemia, hypothyroidism, hyperthyroidism, depression, etc.; major surgery such as hysterectomy, thyroidectomy, malignant tumor, pituitary tumor, ventricular septal defect, breast cancer, etc.; AIDS, syphilis; patients with abnormal liver and kidney function; pregnant or lactating women.

2.5. Clinical sample collection

The subjects were collected fasting morning urine, and centrifuged at 4 °C, 1370 ×*g* for 15 min to remove the supernatant within 2 h, and then the supernatant was removed and centrifuged at 1000 ×*g* for 8 min at 4 °C. The sample after centrifugation was added with sodium azide in a volume ratio of 100:1 for preservation at − 80 °C. All samples were used for the metabonomic analysis.

2.6. Metabolomics analysis

2.6.1. Instruments and reagents

Data acquisition was performed on an UPLC-Q-TOF/MS system (Waters). UPLC analysis was performed in a Water Acquity UPLC system. Urine samples (10 μl) were injected into an Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm, Waters). The column temperature was set at 45 °C, and the flow rate was 0.3 ml/min. The gradient system consisted of 0.1% formic acid in water in mobile phase A and 0.1% formic acid in acetonitrile in mobile phase B (0–8.5 min, A, 99–75%; 8.5–11 min, A, 75–50%; 11–13 min, A, 50–10%; 13–15 min, A, 10–1%; 15–17 min, A, 1%; 17–18.5 min, A, 1–99%; 18.5–20 min, A, 99%).

MS was performed on a Waters Micro mass Q/TOF micro Synapt High Definition Mass Spectrometer. Electrospray ionization source (ESI source) was used for mass spectrometric detection in positive ionization mode. The MS analysis parameters were as follows: a capillary voltage of 3.0 kV, drying gas temperature of 325 °C, drying gas flow of 10 ml/min, desolvation gas flow of 600 l/h, source temperature of 120 °C, desolvation temperature of 350 °C and cone gas flow of 50 l/h. The reference ion ($[M + H]^+ = 556.2771$) was used to ensure accuracy in the spectral acquisition and the quadrupole scan range was *m/z* 50–1000 Da.

2.6.2. Processing and preparation of samples

Each group of samples was thawed at room temperature. After the samples were completely thawed, 300 μl of each was taken in a centrifuge tube and centrifuged at 4 °C and 11,180 ×*g* for 10 min. After that, 150 μl of the supernatant was added and 150 μl of distilled water was added to mix 1:1. Vortex with a vortexer for 1 min. After centrifugation at 18,890 ×g for 15 min, 200 μl of the supernatant was analyzed in the vial for metabolomic analysis. In addition, 100 μl of each urine sample from each group was pipetted into a centrifuge tube to prepare quality control (QC) samples to validate the experimental precision, repeatability and stability.

2.7. Experimental methodology

Before sample analysis, the QC samples were injected to validate the experiment including precision, repeatability and stability.

2.7.1. Instrument precision test

A QC sample was injected 6 times consecutively. Twenty chromatographic peaks were selected randomly, and RSD values of the areas and retention times of these peaks were calculated.

2.7.2. Method repeatability test

Six QC samples were injected consecutively. Twenty chromatographic peaks were selected randomly, and the RSD values of the areas

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