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Exploring the relationship between 5′AMP-activated protein kinase and markers related to type 2 diabetes mellitus

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ABSTRACT

The importance of 5′AMP-activated protein kinase (AMPK) in regulating glucose and fatty acid metabolism is increasing. Thus, it is regarded as a new pharmacological target for treatment of obesity, insulin resistance and type 2 diabetes mellitus (T2DM). In order to explore the relationships between AMPK and diabetes mellitus, urines samples from four groups of C57 mice, i.e., the normal male and female C57 mice, female C57-AMPK gene knocked-out mice, and male C57-AMPK gene knocked-out mice, were studied by coupling GC/MS with a powerful machine learning method, random forest. The experimentation has been designed as two steps: firstly, the normal male and female mice were compared with male and female C57-AMPK gene knocked-out mice, respectively; then the differences between male C57-AMPK gene knocked-out mice and female C57-AMPK gene knocked-out mice were further detected. Finally, not only the differences between the normal C57 mice and C57-AMPK gene knocked-out mice were observed, but also the gender-related metabolites differences of the C57-AMPK gene knocked-out mice were obviously visualized. The results obtained with this research demonstrate that combining GC/MS profiling with random forest is a useful approach to analyze metabolites and to screen the potential biomarkers for exploring the relationships between AMPK and diabetes mellitus.

1. Introduction

Type II diabetes mellitus (T2DM) is a worldwide health problem of the public with the rising of people's living standard. It deserves much more attentions due to the increasing prevalence of this condition in the whole world, especially western countries. Numerous human, even children, are suffering from it in the form of various acute complications, such as diabetic ketoacidosis, kidney failure, stroke amputations, and so on [1]. T2DM is considered to be a complex heterogeneous disease, a typical disease of metabolic syndrome and imbalance of energy metabolism. Previous researches have demonstrated that carbohydrate metabolic block is the most prominent characteristic of T2DM, and carbohydrate, especially the glucose has been widely used as biochemical marker for diagnosis, screening, and evaluation of glycemia control of the disease [2]. Recent data collected in several laboratories indicated that 5′AMP-activated protein kinase (AMPK) plays a key role in regulation of carbohydrate and fat metabolism, serving as a metabolic master switch in response to alterations in cellular energy charge [3–5]. Therefore, AMPK is emerging as a potentially interesting drug target for treatment of diabetes [6].

The AMPK is a multi-substrate serine/threonine protein kinase that is ubiquitously-expressed and functions as an intracellular fuel sensor activated by depletion of high energy phosphor compounds [7]. Activation of AMPK initiates a complex series of signaling events, causing an increase in uptake and oxidation of substrates for adenosine tri-phosphate (ATP) synthesis concurrent with decreasing ATP consuming biosynthetic processes such as protein, lipid, and glycogen synthesis [8]. Furthermore, activated AMPK is associated with dramatic changes in the control of glucose and fatty acid metabolism. Work by several groups during the past decade have revealed that AMPK probably serves as a key metabolic sensor in both insulin-sensitive and other tissues that is capable of responding to metabolic stresses (and in particular depletion of intracellular ATP) by shutting down the synthesis of fatty acids and cholesterol, two major energy-consuming pathways [8–12]. The dysfunction of AMPK activity would confuse many metabolic pathways and resulting in the metabolites perturbation. Therefore, monitoring the variations in metabolites could provide many evidences for exploring the relationship between AMPK and diabetes mellitus.

High-throughput metabolomics have been widely used in the biomedical sciences and have been proved to be a powerful

Abbreviations: 5′AMP-activated protein kinase, AMPK; Type II diabetes mellitus, T2DM; Adenosine tri-phosphate, ATP; Random forest, RF; Multidimensional scaling, MDS.

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approach in diagnosis of human diseases [13], physiological evaluations [14], elucidation of biomarkers [15,16], and drug toxicity [17]. In these researches, most samples were blood, serum, and urine. Compared with other matrixes, urine samples are non-invasive, easily obtained and have large sample volume, so it would be the first choice to perform such researches. To date, many analytical techniques have been applied to the metabolomic analysis of urine, including gas chromatography/mass spectrometry GC/MS [18–21], high-resolution nuclear magnetic (NMR) [22–25], ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) [26]. GC/MS, as one of the most widely used techniques, can generate metabolic data and simultaneously provides quantitative, qualitative and structural information on a wide range of biological molecules. For its favorable stability, reproducibility, sensitivity and better separation of compounds [27], GC/MS is considered to be one of the most effective tools to perform the research of metabolomics.

In the present study, we constructed models by using random forest (RF) to visually discriminate C57-AMPK gene knocked-out mice from healthy controls and to investigate the metabolic differences between male and female C57-AMPK gene knocked-out mice. Firstly, urines samples from normal mice, female C57-AMPK gene knocked-out mice and male C57-AMPK gene knocked-out mice were profiled by GC/MS. Then, the qualitative and the quantitative work were carried out by using heuristic evolving latent projections (HELP) and selected ions analysis (SIA). Finally, RF was employed to visualize the differences among four groups by assembling enough classification and regression trees. Some informative metabolites or potential biomarkers have been successfully discovered by means of variable importance ranking in random forest program. Using the obtained sample proximity matrix, not only the differences between the normal C57 mice and C57-AMPK gene knocked-out mice were observed, but also the gender-related metabolites differences of the C57-AMPK gene knocked-out mice were obviously visualized.

2. Materials and methods

2.1. Chemicals, reagents and preparation of standard solutions

Adonitol, pyridine, *o*-methylhydroxylamine hydrochloride, urease and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) were all obtained from Sigma-Aldrich (Shanghai, China). Methanol was of analytically pure grade. Adonitol, as internal standard, was prepared in methanol at a concentration of 2 mg/mL. A 2 mg/mL urease solution was freshly prepared by dissolving urease powder in deionized water that produced by a ultra-pure water system (Molecular, Shanghai) and *o*-methylhydroxylamine hydrochloride pyridine solution with the concentration 15 mg/mL was prepared.

2.2. Acquisition of mice urine samples

Urine samples were collected from normal C57 mice (eight male mice and eight female mice) and other two groups of C57 mice, whose AMPK gene were knocked out, say C57-AMPK gene knocked-out mice of different gender (female: $n=47$ and male: $n=39$ respectively). Normal C57 mice and the C57-AMPK gene knocked-out mice were provided by Experimental Zooprophylactic Institute of Chinese Academy of Medical Sciences (CAMS) (Beijing, China). Until the urine samples were collected, the experimental mice were raised by institute of laboratory animal science in CAMS. The treated mice were raised at the temperature between 20 and 6 °C, and other ambient conditions were as follows: relative humidity was 40–70%, ventilation rate was 10–15 per hour, illuminance was 150–300 lx, and the alternation of light and shadow was 12 h per each. Food (standard mouse diet) was available. Samples were taken at the same time each morning and were stored at −80 °C prior to analysis.

2.3. Preparation of the metabolites extraction and derivatization

Aliquots of 500 μL mice urine was centrifuged at 4 °C, 16,000 rpm/min for 5 min, and 150 μL supernatant added with 100 μL, 2 mg/mL urease solution was incubated at room temperature for 30 min to remove and decompose excess urea present in it. The resulted solution was protein precipitated using 800 μL methanol, and then 2 mg/mL adonitol–methanol was added to it as the internal standard. The mixture was vigorously eddied for 30 s, followed by reaction at room temperature for 10 min. Next, the solution was centrifuged at 4 °C, 16,000 rpm ($17800\times g$) for 10 min, 150 μL supernatant was taken out and evaporated to dryness in a vacuum oven. The dried extractive was derivatized to its (MeOx-) TMS-derivatives through reaction with 40 μL of 15 mg/mL methoxyamine hydrochloride solution in pyridine at 70 °C for 30 min. After methoximation reaction, the samples were trimethylsilylated for another 30 min by adding 60 μL BSTFA-1% TMCS as catalyst. At last, the solution was vigorously eddied for 30 s again before GC–MS analysis.

2.4. GC–MS data acquisition

Following protein precipitation and derivatization, gas chromatography-mass spectrometry (Shimadzu GC/MS-QP2010, Japan) was used to analyze the metabolic profiling of all urine samples. The instrument is equipped with a 30 m × 0.25 mm ID, fused silica capillary column, which was chemically bonded with 0.25 mm DB-5ms stationary phase (Agilent, USA). The helium carrier gas flow rate was 1.0 mL/min. The column initial temperature was kept at 70 °Cfor 5 min. Then the temperature was ramped at a rate of 20 °C/min to 160 °C, 4 °C/min to 180 °C and 10 °C/min to 300 °C, and held for 1.5 min at 300 °C. 1 μL of the metabolite derivative solution with the derivatization reagent was run through the gas chromatograph-mass spectrometer with a 10:1 split throughout. The injector temperature was 280 °C, the septum purge flow rate was 3 mL/min, and the purge was turned on all the time. The total GC run time was 28 min. The interface temperature was 250 °C and ion source temperature was 200 °C. Ionization was achieved by a 70 eV electron beam. Masses were acquired in a full scan mode, over the range from m/z 35 to 800, with a scan speed of 0.2/s when the 0.9 kV of detector voltage was turned on after a solvent delay of 5 min.

2.5. Data handling and analysis

First, a chromatogram that has the greatest number of peaks was selected as the referential chromatogram. The identification of its specific metabolites and internal standard was based on the search results in NIST 05 mass chromatography library in the GC–MS Postrun Analysis software (Shimadzu), the characteristic ions according to the literature and retention time of authentic standards and those of online available data. For quantitative analysis of pure chromatographic peaks, areas were obtained directly by the GC–MS Postrun Analysis software (Shimadzu). As for some overlapped peaks, two chemometric resolution methods, say HELP [28,29] and SIA [30] were utilized to extract the pure mass spectra, and calculate the corresponding area. After the referential chromatogram was processed, those chromatograms from the other samples were compared with the referential chromatogram based on retention times. If a peak in the referential chromatogram was