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Short Communication

Serum metabonomics of NAFLD plus T2DM based on liquid chromatography–mass spectrometry

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ABSTRACT

Objectives: Nonalcoholic fatty liver disease (NAFLD), a main liver disease around the world, is closely associated with insulin resistance, type 2 diabetes mellitus (T2DM) and other metabolic diseases. The objective of this study is to identify distinct metabolites of NAFLD patients with or without T2DM.

Design and methods: We used a biomarker-discovery population to find distinct metabolites of NAFLD patients with or without T2DM. Then, a validation population was applied to test the model of the biomarker-discovery population. All the individuals received anthropometric and common biochemical measurements. The metabolic data were analyzed by multivariable statistical analyses using ultra-high-performance liquid chromatography/quadrupole time-of-flight-tandem mass spectrometry.

Results: There were 7, 7, 2 metabolites in the positive electrospray ionization (ESI^+) mode, which were identified between groups from both the biomarker-discovery and validation population. The NAFLD group showed higher concentrations of oleamide, L-phenylalanine, L-proline, bilirubin, L-palmitoylcarnitine, and PC (20:5) and a lower concentration of Lyso-PAF C-18 than those of control. Compared with the control group, the NAFLD + T2DM group displayed higher oleamide, L-leucine, LysoPC (14:0), bilirubin, tetradecenoylcarnitine, linoleyl carnitine, and tetradecadiencarnitine in serum. Tetradecenoylcarnitine and tetradecadiencarnitine were more elevated in patients with NAFLD + T2DM than in the NAFLD group.

Conclusions: Serum metabonomic analyses displayed great metabolic changes in patients with NAFLD and NAFLD plus T2DM. Our study is beneficial in providing a further view into the pathogenesis and pathophysiology of NAFLD and NAFLD plus T2DM, which might be useful for the prevention and therapy of NAFLD and NAFLD plus T2DM.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is described as a condition identified by imaging or liver biopsy where lipids accumulated in the liver without excessive alcohol consumption, encompassing non-alcoholic steatosis, steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma [1,2]. NAFLD can increase liver-related morbidity or mortality, and also add risks of extra-hepatic diseases, including cardiovascular disease, type 2 diabetes mellitus (T2DM), obesity, chronic kidney disease and colorectal cancer, among which, insulin resistance is the most common feature [2]. Several cohort studies have illustrated that NAFLD is positively associated with T2DM, and can increase the risk of death in patients with T2DM. The accumulation of hepatic fat has an adverse effect on peripheral insulin resistance and damages the sensitivity of β-cells in pancreas. In turn, abnormal glucose metabolism could accelerate the development of liver diseases [3]. The insulin resistance in T2DM, could increase the flux of free fatty acids to the liver, increase the release of inflammatory mediators and activate mitochondrial oxidative stress. What's more, the inflammatory mediators and adiponectins can promote the synthesis of collagen, combined with tissue growth factor, and finally give rise to fibrosis [4]. The association between NAFLD and T2DM is bidirectional, and too intricate to be explained explicitly.

Metabonomics is defined as a global and comprehensive analysis approach to identify a set of metabolites in biochemical and biological samples. Currently, ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS) has been extensively used in

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metabonomic analysis, and a combination with quadrupole time-of-flight tandem mass spectrometry (UPLC–Q-TOF-MS), makes data optimized, which can be used to investigate key metabolites and metabolic alterations in response to diseases and environmental or genetic perturbations [5]. Although a few studies have analyzed metabolic dysfunction in NAFLD, T2DM and related disorders, they focused on the discovery of non-invasive biomarkers of NAFLD [1]. Furthermore, the multiple relationships of metabolites between NAFLD and control remain unclear. The current experiments based on metabonomics are still in the infant phase, for in animal models, there are some limitations in the mimics of the development and metabolic process of NAFLD due to species differences between humans and animals. Moreover, the methods to mimic the development of NAFLD in animals vary greatly among different experiments [6,7]. In human studies, the sample size of subjects in previous studies is small and the investigated populations are Caucasian and African-American. In addition, only one study applied a validation population to independently test the biomarker-discovery model [8].

For the close link between NAFLD and T2DM from the above statements, it can be inferred that individuals with NAFLD plus T2DM have both different or joint metabolites and metabolic alterations compared with NAFLD. However, no study has focused on this point. Therefore, we used global serum metabonomics to identify metabolic changes of the pathogenesis. It might be prerequisite for understanding the pathophysiology and helpful for screening and treatment of high-risk individuals.

2. Materials and methods

2.1. Participants

500 adults aged 35 to 70 who received annual physical examinations were recruited from 2012 to 2014 at the Physical Examination Center in the Second Affiliated Hospital of Harbin Medical University (HMU). The project has passed the review of the ethics committee of HMU and all participants have signed informed consent. The patients were included based on the definition of NAFLD and T2DM previously [9,10]. The biomarker-discovery and validation populations were separately selected in 2012 ($n = 131$) and 2014 ($n = 129$). All subjects received anthropometric assessments, ultrasound tests, questionnaire investigation and venous blood collection after fasting the next morning. The methods of measurements were shown in the Supplementary data.

2.2. Sample preparation and analysis

A mixture of 720 μL methanol and 360 μL serum per aliquot was vortex-mixed and centrifuged for 10 min at 12,000 rpm. The supernatant was shifted to an autosample vial and evaporated under N_2. A 360 μL mixture of acetonitrile and ultrapure water (2:1) was added into each tube. Finally 5 μL of the solution was injected into the system. The lock mass utilized L-leucine enkephalin in 200 pg/mL (556.2771 in ESI^+) over 10 scans to ensure accuracy and reproducibility. MassLynx software (version 4.1 SCN 714) was used to analyze raw data for peak detection and alignment. Data were exported to the EZinfo software for supervised partial least squares-discriminant analysis (PLS-DA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA). The conditions of peak analyses and data collection were shown in the Supplementary data.

The serum differential variables with variable importance in the projection (VIP) values >1.5 from the OPLS-DA model were assessed by Student's *t*-test or Wilcoxon (Mann–Whitney) to test the significance. Metabolites were firstly identified based on accurate mass and MS spectra online, including Metlin (http://metlin.scripps.edu/) and HMDB (http://www.hmdb.ca/). Further, the joint metabolites from both populations were affirmed by comparison of the retention time and fragmentation pattern with authentic standards.

3. Results and discussion

3.1. Characteristics of subjects and data quality control

The biochemical and anthropometric parameters of the biomarker-discovery and validation populations were manifested in Supplementary Tables 1a and 1b. The repeatability and precision of the present method was evaluated by quality control (QC) samples. The QC samples, mixed by equal aliquots of serums from different groups, were injected into every 10th sample throughout the analytical workflow. The results showed that the method was excellent for both biomarker-discovery and validation samples (Supplementary Table 2).

3.2. UPLC–Q-TOF-MS analysis of metabolites of NAFLD

The typical based peak intensity (BPI) chromatograms of serum samples from three groups in the biomarker-discovery and validation populations were shown in Supplementary Fig. 1. There were clear separations between the 3 groups (NAFLD vs Con, NAFLD + T2DM vs Con, NAFLD + T2DM vs NAFLD) from the biomarker-discovery and validation populations by PLS-DA and OPLS-DA analyses (Fig. 1 and Supplementary Fig. 2). The values of $R2_{(cum)}$ and $Q2_{(cum)}$ in PLS-DA and OPLS-DA models were all over 0.5, thus the models were considered proper. In the validation plots, all permuted R2-values and Q2-values on the left were lower than the original point values on the right, and the Q2 regression line had a trend to intersect the R2 regression line (Fig. 1 and Supplementary Fig. 2). To avoid over-fitting of supervised models, 100 random permutation tests were carried out by SIMCA-P version 11.5 software (Umetrics, Umeå, Sweden) (Supplementary Fig. 3).

The metabolites with a VIP value >1.5 and *P* value <0.05 in the OPLS-DA models were selected as the distinct factors for class differentiation. To obtain reliable, duplicated and explicit models of differential metabolites, we used a validation population to determine the joint metabolites between 3 groups. The joint metabolites differing from the 3 groups are shown in Table 1.

3.3. Differential metabolites from both populations

In contrast with the control group, the concentrations of L-proline, L-phenylalanine, oleamide, bilirubin, L-palmitoylcarnitine, and PC (20:5) were higher in patients with NAFLD, while, Lyso-PAF C-18 was lower in patients with NAFLD. The metabolites separating the control group from patients with NAFLD + T2DM were L-leucine, oleamide, LysoPC (14:0), bilirubin, tetradecenoylcarnitine, linoleyl carnitine and tetradecadiencarnitine, which were elevated in patients with NAFLD + T2DM. Compared with the NAFLD group, tetradecenoylcarnitine and tetradecadiencarnitine ascended in patients with NAFLD + T2DM. Compared with Con, oleamide and bilirubin were both higher in patients with NAFLD and NAFLD + T2DM. There were joint significant metabolites in NAFLD vs Con and NAFLD + T2DM vs NAFLD, and bilirubin, tetradecenoylcarnitine and tetradecadiencarnitine increased in patients with NAFLD and patients with NAFLD + T2DM. There were no joint metabolites in NAFLD vs Con and NAFLD + T2DM vs NAFLD. We used standard samples to confirm these joint significant metabolites.

Significant metabolites were classified into amino acids, lipids, acylcarnitines and bilirubin. For the biomarkers of amino acid, we found that serum L-phenylalanine concentration was nearly two-fold higher in NAFLD patients than that in control, which was consistent with previous studies [6]. However, we are the first to show the multiple relationships between the NAFLD and control groups. In a similar study, Julian Swierczynski et al. found that L-phenylalanine concentration was positively correlated with ALT concentration in obese subjects [11]. L-Phenylalanine in circulation might be the symbol of liver