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Urinary metabolomic analysis of intrahepatic cholestasis of pregnancy based on high performance liquid chromatography/mass spectrometry

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ABSTRACT

Background: Intrahepatic cholestasis of pregnancy (ICP), a pregnancy-related liver disease, leads to complications for both mothers and fetuses. Metabolomic approach has been applied to maternal-fetal medicine. The global metabolomic alterations that are specific in ICP as yet have not been investigated.

Methods: Based on high performance liquid chromatography/hybrid quadrupole time-of-flight (HPLC/Q–TOF) mass spectrometry, the untargeted metabolomics was used to analyze the changes of urinary metabolites between ICP group and the control group.

Results: One hundred nine variables in positive model and 119 variables in negative model were significantly different ($p < 0.05$) between the ICP group and the control group, with the VIP (variable importance in the project) score > 1 by the orthogonal partial least squares discriminant analysis (OPLS-DA). 14 metabolites in positive model and 18 metabolites in negative model were selected and identified based on HMDB (human metabolome database). Most of these metabolites were involved in bile acids biosynthesis and metabolism, hormone metabolism and lipid metabolism. A metabolite panel (MG (22:5), LysoPE (22:5), L-homocysteine sulfonic acid, glycocholic acid and chenodeoxycholic acid 3-sulfate) was contrusted by the binary logistic regression analysis with high diagnostic accuracy for ICP. The area under the receiver operating characteristic curve was 0.988 with the sensitivity of 90.0% and specificity of 93.3%.

Conclusions: Urinary metabolites allow for the discrimination of ICP from the controls by orthogonal partial least squares discriminant analysis. Therefore, these findings may provide deep insights for the etiopathogenesis of ICP. Moreover, the maternal urinary metabolite panel has the potential to be used as non-invasive biomarkers for the diagnosis of ICP.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-related syndrome. The incidence varies geographically from 0.1% to 15.6% [1,2]. It is characterized by mild to severe pruritus and disordered liver function. The disease symptoms and liver dysfunction appear mainly in the late second or third trimester of pregnancy and relieve quickly after delivery. However, ICP leads to complications for both mothers and fetuses. ICP is associated with intractable pruritus and high predisposition to postpartum bleeding, which is the leading cause of maternal mortality. On the other hand, ICP results in an increased risk of spontaneous preterm labor, fetal distress and sudden intrauterine death. Currently, the exact cause of ICP is unknown. Genetic, endocrinologic, nutritional, and environmental factors are considered to be associated with the pathogenesis of the disease [1,3–5].

Because of the lack of the international uniformity, it is difficult to diagnose ICP [6]. Untargeted metabolomics, which focuses on the dynamic changes of all small molecules in response to the disturbance of the organism, can provide deep insights for the etiopathogenesis and the discovery of biomarkers for various diseases. Metabolomic approach has also been applied to maternal-fetal medicine. A growing number of metabolomic studies were reported on the complications in pregnancy including gestational diabetes mellitus [7,8], preeclampsia [9–12], small for gestational age [13] and Smith-Lemli-Opitz syndrome [14]. To our knowledge a global untargeted metabolomics of ICP has not been investigated previously. Only the bile acid profiles [15–17] and some metabolites such as hormones [18], lipid [19], and glucose [20] were measured in ICP.

Serum and urine are commonly considered as a pool of metabolites, which could express metabolic deregulation systematically in patients.

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Moreover, biomarkers in biofluids could explain the characteristics of individuals during the course of diseases. As urine is an easy approachable sample and a non-invasive source of metabolites, urinary metabolomics has been investigated in many diseases [7,11,14,21].

The commonly used platforms for metabolomics are mass spectrometry (MS) coupled with gas chromatography (GC) or liquid chromatography (LC) using a range of different ionization techniques, and nuclear magnetic resonance spectroscopy (NMR) [22]. MS was applied to characterize a large number of metabolites simultaneously in complex living systems. Furthermore, metabolomics based on LC-MS can provide sensitive, accurate and reproducible analysis for a wide range of metabolites.

2. Experimental

2.1. Subjects

We designed case-control study, including urine from 30 cases of ICP and 30 cases of healthy pregnant women to find the maternal urinary biomarkers for diagnosis of ICP. This study was performed at the First Affiliated Hospital of Chongqing Medical University, Chongqing, and designed conformed to the ethics guidelines given in the Declaration of Helsinki. Written informed consent was obtained from all subjects and all experimental protocols were approved by the ethics committee of the First Affiliated Hospital of Chongqing Medical University. Maternal urine samples were obtained from pregnancies who received prenatal care at the third-trimester ($\geq$ 28 gestational weeks) from October 2013 to August 2015. The enrollment criteria for ICP were as follows: pruritus and jaundice in the third trimester of pregnancy without signs of chronic liver diseases, skin diseases, or symptomatic cholelithiasis; elevated levels of aminotransferases and total serum bile acid; and normalization of cholestasis after delivery. For the control group, only those healthy pregnant women matched with cases on age and pregnant weeks were enrolled during the same period, excluding women with a history of gallstones or cholecystopathy, pruritus, drugs consumption, hepatitis, or any other diseases damaging hepatobiliary function. Women with ICP underwent fetal monitoring from the time of diagnosis until delivery, and received drug therapy such as ursodeoxycholic acid (UDCA) for symptom relief. Samples from the women with ICP were collected at the first visit to confirm diagnosis before drug treatment. Serum liver function tests including serum total bile acid (TBA), total bilirubin(TBIL), direct bilirubin(DBIL), alanine transaminase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) were performed on an automated Olympus Chemistry Analyzer (Olympus).

2.2. Preparation of samples

The urine samples were obtained and processed within 2 h. Processing involved centrifugation at 3000*g* for 10 min at 4 °C, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C to remove particulates. The urinary supernatant was stored at − 80 °C until analysis. Before analysis, the urine samples were thawed in a 4 °C water bath. The urinary supernatant containing 0.5 μmol creatinine was extracted and then purified water was added to make a final volume to 400 μl. Quality control (QC) samples were prepared by mixing 20 μl of each diluted urine sample of the whole sample set and the pooled urine was aliquoted into 20 μl. The QC samples were stored at − 80 °C until analysis. Five microliters of the diluted urine sample and QC samples were injected to the LC-MS system.

2.3. Chemicals

Water, acetonitrile, formic acid and ammonium hydroxide were from Sigma-Aldrich. Acetonitrile and water were of liquid chromatographic mass grade. All other chemicals were of analytical grade (Nanjing Chemical Reagent Co.).

2.4. Metabolomic analysis

Metabolomic analyses were performed using a HPLC system (Dionex UltriMate 3000; Thermo Scientific) connected to an ultra-high resolution time-of-flight MS (maXis4G; Bruker) equipped with an electrospray ionization source in both of the positive-ion model and the negative-ion model. Sodium trifluoroacetate was continuously introduced as the internal calibration. Mass operation parameters of positive-ion scan model were as the following: capillary voltage, − 4500 V; end plate voltage, − 500 V; nebulizer pressure, 0.7 Bar; drying gas flow rate, 7 l/min; drying gas temperature, 200 °C. The operation parameters of negative-ion scan model were as the following: capillary voltage, 3500 V; end plate voltage, 500 V; and other parameters were then same as positive ion model. TOF MS accurate mass spectra were recorded across the range 50 to 1000 *m/z* at 3 × 1.0 Hz. A 6-way valve with 3 segment time (0–0.1 min to waste, 0.1–0.3 min to source, 0.3 to no limit to waste) was used to continuously introduce the internal solution.

Reversed phase chromatographic separation was performed on a Waters Xelect CSH C18 column (2.1 mm × 150 mm, 3.5 μm) equipped with a Waters Xselect CSH C18 guard cartridge (2.1 mm × 1.0 mm, 3.5 μm) maintained at 35 °C. The mobile phase was using as phase A (water with 0.1% formic acid for positive scan model and with 0.05% ammonium hydroxide solution for negative scan mode), and as phase B (acetonitrile with 0.1% formic acid for positive scan model and with 0.05% ammonium hydroxide solution for negative scan mode). Elution was performed at a flow rate of 0.25 ml/min and the following gradient program: 2% -60% B for 0–15 min, 60–100% B for 15–16 min and held for 9 min., 2–20%B in 4–6 min, 20–100%B in 6–10 min, 100% B in 10–12 min. The gradient then returned to 2%B for 25–26 min and held for 4 min.

To test the reproducibility of the sample preparation procedure and LC-MS analyze, QC samples were injected at the beginning of the run and after every eight real samples. Each of the prepared QC samples was analyzed only once.

2.5. Data processing

The raw data were calibrated by internal calibration of sodium trifluoroacetate and converted to the mass format of CDF using the software of DataAnalysis (Bruker). The CDF files were processed with XCMS, an open-source package written in the platform-independent programming language R (www.r-project.com). The processes were as follows: filtering and identification peaks, matching peaks across samples, retention time correction, matching peaks across samples again, filling in missing peak data and statistical analysis. The language of processes was listed in appendix (Table S1). And then XCMS software was used to output the data, including a table of *m/z*, retention time and peak areas for all detected peaks. The total area normalization for each sample was performed by dividing the integrated area of each analyte by the sum of all peak areas of analytes present in the sample. The normalized data were used to perform a multivariate statistical analysis.

2.6. Statistical analysis

The orthogonal partial least squares discriminant analysis (OPLS-DA) was applied to investigate the global metabolic changes between the ICP group and the control group using SIMCA-p 11.5 software (Umetrics AB, Umea) [23]. Potential biomarkers were ascertained when the value of variable importance in the project (VIP) was > 1 [24] (by SIMCA-p) and the two-sided *p* value was < 0.05 calculated on R platform in the step of statistical analysis. The characteristics of participants