Serum Metabolomic Characteristics of Patients with Liver Cirrhotic Ascites

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Key Words
Metabolomics · Cirrhotic ascites · Amino acids · Bile acids · Monoamine neurotransmitter · Ultra-high-performance liquid chromatography-tandem mass spectrometry method

Abstract
Chronic hepatitis B is a common and frequently encountered disease in our country, the final outcome of which develops into liver cirrhosis and primary liver cancer. It was the aim of this study to provide a theoretical basis for the early diagnosis and treatment of liver cirrhosis. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) is used to analyze endogenous bioactive substance change in healthy controls and patients with liver cirrhotic ascites. A metabolic fingerprint spectrum was established for the analysis. The results show that metabolic profiling of the serum indicates significant differences between the controls and the patients. Except for the tyrosine content which was decreased in the serum, the other 12 amino acids and 8 conjugated bile acids were significantly increased compared to controls (p < 0.01). Additionally, the 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) of serum were significantly decreased in the patients with liver cirrhotic ascites. In conclusion, the lysophosphatidylcholines C18:0, C18:2 and C16:1 are potential biomarkers. Moreover, the bile acid metabolism, amino acid metabolism and 5-HT as well as 5-HIAA metabolites are significantly changed in patients with cirrhotic ascites. These endogenous metabolites are potential biomarkers used for the diagnosis and treatment of liver fibrosis and cirrhosis.

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Introduction

Liver cirrhosis marks the end stage of many chronic liver diseases. Ascites is the most frequent complication of liver cirrhosis which results in patient hospitalization and carries a poor prognosis [1]. Cirrhotic ascitic fluid accumulation results from portal hypertension, albumin synthesis reduction and sodium retention [2]. The development of ascites is associated with poor quality of life, increased risk of infections, renal failure and poor long-term outcomes [3]. The diagnosis of ascites is considered in cirrhotic patients with a given constellation of clinical and laboratory findings and ultimately confirmed with insight into etiology by imaging and paracentesis procedures [4]. The management of cirrhotic ascites usually calls for comprehensive measures: the primary treatment includes a sodium-restricted diet, diuretics application and albumin infusion [5]. Traditional Chinese medicine (TCM) has a long history and rich experiences in treating cirrhotic ascites and, nowadays, is widely applied in clinical practice as a complementary and alternative approach. For example, Xiaozhang Tie – an adjuvant to the primary therapy of cirrhotic ascites – is safe and shows a remarkable efficacy on relieving abdominal distention [6].

Metabolomics is defined as ‘the quantitative measurement of the dynamic multiparametric responses of a living system to pathophysiological stimuli or genetic modification’ [7]. It is a powerful analytical platform that allows for the assessment of global metabolic profiles in easily accessible biofluids and biomarker discovery in order to distinguish between diseased and non-diseased status information [8] as well as for the evaluation of drug toxicity and gene function [9]. A number of new analytical technologies have been employed for metabolomics analysis, including $^1$H-NMR [10], ultra-high-performance liquid chromatography quadrupole-time-of-flight/high-sensitivity mass spectrometry (UPLC-Q-TOF/H SMS) [11], UPLC-electrospray ionization/MS (UPLC-ESI/MS) [12], gas chromatography/MS [13] and others.

In this article, UPLC-MS-based metabolomics was used to investigate the metabolic profiles of amino acids and bile acids in patients with cirrhotic ascites. We used this approach in an attempt to explore the possible biomarkers involved in cirrhotic ascites.

Materials and Methods

Design, Setting and Participants

A total of 60 subjects (30 patients with cirrhotic ascites and 30 healthy controls) were enrolled in this cross-sectional case-control study, conducted at the Department of Shuguang Hospital Affiliated to Shanghai University of TCM over a period of 6 months (July to December, 2012). The study was approved by the Ethics Committee of Shuguang Hospital Affiliated to Shanghai University of TCM. After written consent, subjects were counseled, and the objectives of the study were explained to them by a qualified medical doctor. Their detailed personal history was taken using a standard questionnaire. The participants were volunteers and received no payment.

Patients aged from 18 to 80 years were diagnosed with cirrhotic ascites based on criteria established by the Chinese Medical Association Society of Infectious and Parasitic Diseases and the Society of Liver Diseases in 2000 [14]. They all suffered from cirrhosis of the decompensated stage (B/C grade of Child-Pugh classification), and their ascites were certified by abdominal ultrasound. None of the patients had had prior treatment for ascites. Laboratory assessment of liver function, renal function and electrolytes was also performed. Patients with psychosis, cancer (including liver tumors without hemorrhagic ascites), renal insufficiency, diabetes with unstable blood glucose or any serious diseases of the cardiovascular, respiratory, nervous or hematologic systems were not eligible. Lactating and pregnant women were also excluded.
Whole-blood samples (1 ml) were withdrawn from the forearm vein after an overnight fast with sodium heparin as anticoagulant. After 2 h at 4 °C, the serum was separated by centrifugation at 4,000 g for 10 min at 4 °C and stored at –80 °C until analysis.

**Reagents**

Alanine (Ala), valine (Val), leucine (Leu), phenylalanine (Phe), tryptophane (Trp), methionine (Met), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), asparagine (Asn), glutamine (Gln), histidine (His), arginine (Arg), glutamic acid (Glu), glycochenodeoxycholic acid (G-CDCA), taurocholic acid (T-CA), glycocholic acid (G-CA), taurochenodeoxycholic acid (T-CDCA), taurodeoxycholic acid (T-DCA), glycochenodeoxycholic acid (G-DCA), glycourso-deoxycholic acid (G-UDCA), chenodeoxycholic acid (CDCA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were analytical grade and were supplied by the China National Pharmaceutical Group Corporation (Shanghai, PR China). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific Co. (Santa Clara, Calif., USA). HPLC-grade water (>18 mΩ) was obtained from a Milli-Q water purification system (Millipore, Milford, Mass., USA). Formic acid (HPLC grade) was obtained from Merck (Darmstadt, Germany). The other chemicals were all analytical reagents.

**Instrumentation and Conditions**

The separation was performed on a Waters-ACQUITY™ UPLC system (Waters Corp., Milford, Mass., USA) using an Agilent Zorbax SB-C\textsubscript{18} column (150 × 4.6 mm, i.d. 3.5 μm) maintained at 30 °C. The mobile phase was composed of water (A) and acetonitrile (B) at a flow rate of 0.3 ml/min. A gradient program was used as follows (time, min/A%): 0/90, 6/40, 10/20, 16/10, 16.5/90. The injection volume was 5 μl in partial loop mode. ESI/MS was operated in negative/positive mode under the following operating parameters: capillary voltage, 3.5 kV; cone voltage, 35 V; source temperature, 120 °C; desolvation temperature, 300 °C; desolvation gas (nitrogen), 600 liters/h, and cone gas (nitrogen), 50 liters/h. The calibration curves, precision, stability and recovery of each amino acid and bile acid in serum samples are within the acceptable range according to the guidance of the Food and Drug Administration.

**Sample Preparation**

To a 100-μl aliquot of serum samples, 50 μl of IS solution (0.2 μg/ml) and 850 μl of acetonitrile were added into a 1.5-ml polypropylene microcentrifuge tube. The mixture was vortex mixed for approximately 1 min and centrifuged at 12,000 g for 10 min. Then the supernatant (900 μl) was carefully removed and transferred to another clean test tube and evaporated to dryness under nitrogen at 40 °C. The dried residue was reconstituted in 100 μl of 20% acetonitrile followed by vortex mixing for 1 min. After centrifuging at 12,000 g for 15 min, a 3-μl aliquot of the supernatant was injected into the UPLC-MS system for determination of amino acids and bile acids in the serum.

**Statistical Analysis**

The results were presented as the mean ± standard deviation (SD) and compared between two different groups at the same phase by the t test. All data analyses were performed with SPSS version 15.0 (SPSS, Chicago, Ill., USA). A p value <0.05 was considered as statistically significant. The resulting data were exported into Microsoft Excel, and the peaks were normalized to the total sum of spectrum prior to multivariate analyses. The resulting data were analyzed by principal component analysis and partial least squares discriminate analysis (PLS-DA) using SIMCA-P 11.5 software (Umetrics, Umea, Sweden) after undertaking a unit variance procedure.
Results

Metabolomic Characteristics of Patients with Cirrhotic Ascites

In order to screen the metabolomic characteristics of serum in patients with cirrhotic ascites, the sample was analyzed by UPLC-MS/MS techniques. The total-ion chromatograms of serum in patients with cirrhotic ascites are shown in figure 1. The spectral data were recorded in the m/z range of 100–1,000. To obtain fragmentation patterns of compounds from serum samples, tandem MS spectra of seven reference compounds were first analyzed by direct infusion. On the basis of full-scan results, specified precursor ions in each MS scan were selected in turn and subjected to tandem MS analyses. The metabolic fingerprint spectrum at a certain range was carried on the computations by segmental integrals, and then, normalization processing is carried out according to the total integrals of the metabolic fingerprint spectrum. These integral data were applied to the analysis using SIMCA-P 11.5 software (Umetrics). A multidimensional PLS-DA classification method was subsequently used to maximize metabolite variations and identify the metabolites responsible for such variations in an attempt to improve the classification of the controls and the patients with cirrhotic ascites. The samples in the model and the control group were clearly separated using this procedure, as shown by score plots of the first two principal components [R2X (cum) = 0.50, Q2 (cum) = 0.70] (fig. 2, 3). Based on the result of the PLS-DA score plot, three metabolites related to group separation, for which the parameter VIP (variable importance in the projection) was >1, were selected as potential biomarkers (p < 0.05, Student’s t test), including lysophosphatidylcholines C18:0, C18:2 and C16:1. Each of these potential biomarkers was further identified using the reference compounds available and the commercial compound libraries.

Fig. 1. Total-ion chromatograms of the serum samples of healthy controls and patients with cirrhotic ascites in the positive-ion mode.
Serum Metabolomic Profile of Amino Acids

The contents of amino acids measured by UPLC-MS in the serum of controls and patients are shown in figure 4. Compared with controls, the tyrosine content was significantly increased in patients with cirrhotic ascites ($p < 0.05$), while the contents of alanine, valine, leucine, phenylalanine, tryptophane, proline, serine, threonine, asparagine, glutamine, histidine and arginine significantly decreased ($p < 0.01$). Additionally, the ratio of branched chain amino acid/aromatic amino acid (BCAA/AAA) was $1.16$ in controls and $0.5$ in patients with cirrhotic ascites.

Serum Metabolomic Profile of Bile Acids

The contents of bile acids in the serum of controls and patients are listed in figure 5. Eight bile acids were significantly increased in patients with cirrhotic ascites compared to controls ($p < 0.01$), including glycochenodeoxycholic acid, taurocholic acid, glycocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, glycochenodeoxycholic acid, glycoursodeoxycholic acid, and chenodeoxycholic acid.

The Content of 5-HT and 5-HIAA

The content of 5-HT and 5-HIAA in the serum of controls and patients is listed in figure 6. Compared with controls, the contents of 5-HT and 5-HIAA were significantly decreased ($p < 0.01$) in the patients with cirrhotic ascites.
Fig. 4. Contents of acetic acids measured by UPLC-MS in the rat serum of healthy controls and patients with cirrhotic ascites. Values are represented as means ± SD; significant differences between the controls and patients are based on the two-tailed Student t test (* p < 0.05; ** p < 0.01).

Fig. 5. Contents of bile acids measured by UPLC-MS in the serum of healthy controls and patients with cirrhotic ascites. Values are represented as means ± SD; significant differences between the controls and patients are based on the two-tailed Student t test (** p < 0.01).

Fig. 6. Contents of 5-HT and 5-HIAA measured by UPLC-MS in the serum of healthy controls and patients with cirrhotic ascites. Values are represented as means ± SD; significant differences between the controls and the patients are based on the two-tailed Student t test (** p < 0.01).
Discussion

In this study, the metabolic serum profiles of patients with ascites were successfully investigated by the UPLC-MS method. Based on the results of the statistical analysis, lysophosphatidylcholines C18:0, C18:2 and C16:1 were screened out as potential biomarkers. Moreover, the contents of amino acids had significantly changed in patients compared to controls. Furthermore, the value of BCAA/AAA was notably decreased. With regard to the ratio, it is closely related to the degree of liver damage, with a lower ratio indicating the emergence of hepatic encephalopathy. Therefore, the value is helpful for the diagnosis and prognosis of cirrhotic ascites. On the other hand, the results of bile acids indicate that a close connection also exists between glycine- and taurine-conjugated bile and cirrhotic ascites. The concentration of bile acids is increased in different periods of hepatic cirrhosis, especially in the periods of advanced hepatic cirrhosis. Even if the other liver function index is restored, the concentration of bile acid still keeps going up when the active hepatic cirrhosis is kept at a minimum status. Therefore, the level of serum bile acid is an important biological marker of inactive hepatic cirrhosis. Additionally, the content of 5-HT and 5-HIAA has been found to be significantly decreased (p < 0.01) in patients with cirrhotic ascites. In view of the tryptophane which could be transformed into 5-HT and 5-HIAA in the body, the content of 5-HT and 5-HIAA could be changed with changing the contents of tryptophane.

Conclusion

There are considerable differences in the serum metabolomic characteristics between controls and patients with cirrhotic ascites. The bile acid metabolism, the amino acid metabolism and 5-HT as well as the 5-HIAA metabolites are significantly changed in patients with cirrhotic ascites. These endogenous metabolites, including 3 lysophosphatidylcholines, 13 amino acids and 8 bile acids, are potential biomarkers for the early diagnosis and treatment of liver fibrosis and cirrhosis.

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