RESEARCH ARTICLE

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Urinary Metabolomic Profiling in Chronic Hepatitis B Viral Infection Using Gas Chromatography/Mass Spectrometry

Kanthanadon Dittharot[1], Paisan Jittorntam[1], Prapin Wilairat[2], Abhasnee Sobhonslidsuk[3]*

Abstract

Background: Chronic hepatitis B (CHB) can lead to cirrhosis and hepatocellular carcinoma. The metabolomic profiling has been shown to be associated with pathogenic mechanisms in many medical conditions including CHB. The purpose of this study was to investigate the urine metabolomic profiles in CHB patients by gas chromatography/mass spectrometry (GC/MS). **Methods:** Urine samples were collected from CHB patients (n = 20) and normal control subjects (n = 20). Metabolite profiles were assessed using GC/MS in conjunction with multivariate statistical analysis, in order to identify biomarker metabolites. Pathway analysis was performed by MetaboAnalyst 3.0 and KEGG database.**Results:** Twelve out of 377 metabolites were shown to be significantly different between the CHB and normal control groups ($p < 0.05$). These include palmitic acid, stearic acid, oleic acid, benzoic acid, butanoic acid, cholesterol, glycine, 3-heptanone, 4-heptanone, hexanal, 1-tetradecanol and naphthalene. Multivariate statistical analysis constructed using these expressed metabolites showed CHB patients can be discriminated from healthy controls with high sensitivity (95%) and specificity (85%). All the metabolic perturbations in this disease are associated with pathways of fatty acid, amino acid, bile acid and gut microbial metabolism. **Conclusion:** CHB patients have a specific urinary metabolomic profile. The abnormalities of fatty acid, amino acid, bile acid, and gut microbial metabolism lead to the development of disease progression. GC/MS-based assay is a promising tool for the metabolomic study in CHB.

Keywords: Chronic hepatitis B- metabolomic profiling- gas chromatography/mass spectrometry- Biomarkers

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Introduction

Chronic hepatitis B viral (HBV) infection continues to be a worldwide liver problem with approximately 240 million people carrying the virus (Schweitzer et al., 2015). Ten to twenty percent of patients with chronic hepatitis B (CHB) can develop liver cirrhosis in five year (Amantonico et al., 2010). Cirrhosis precedes most cases of hepatocellular carcinoma (HCC), with 70%–90% of HCC developing from the background of liver cirrhosis (Zhang et al., 2013c; Wanich et al., 2016). These data clearly indicate the critical importance of early diagnosis of liver cirrhosis. Although liver biopsy is currently recommended as the gold standard method of staging fibrosis in patients with CHB, it has several disadvantages such as invasive protocol, risks of complication, poor patient compliance and sampling error, which limits its usefulness for dynamic surveillance and follow-up. Therefore, a reliable, non-invasive diagnostic procedure to predict and assess treatment and prognosis of liver cirrhosis is needed. Metabolomic analysis has been shown to be a powerful tool for the diagnosis, treatment, and prevention of human diseases (Zhang et al., 2012; Zhang et al., 2013b).

Metabolomics, which is defined as the measurement of low molecular weight metabolites in an organism at a specified time under specific environmental conditions (Wu et al., 2009), has been shown to be an effective tool for disease diagnosis (Claudino et al., 2007; Wikoff et al., 2007), biomarker screening (Bogdanov et al., 2008; Xue et al., 2008b; Silva et al., 2011), and characterization of biological pathways (Nicholson et al., 2002). Recently, metabolomic profiling approaches have been increasingly used to elucidate significant changes in tumor metabolism and to explore candidate biomarkers from a huge number of endogenous metabolites. Blood and urine are the most common types of samples employed for exploring the systematic alteration in human metabolome. However, urine sample is more preferable as it enables non-invasive monitoring of metabolomic changing.

Metabolomic studies generally employ techniques such as nuclear magnetic resonance (NMR), fourier transform infrared (FT/IR) spectroscopy, liquid chromatography/mass spectrometry (LC/MS), and gas chromatography/mass spectrometry (GC/MS)

*[1]Office of Research Academic and Innovation, [3]Division of Gastroenterology and Hepatology, Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, [2]National Doping Control Centre, Mahidol University, Bangkok, Thailand. *For Correspondence: abhasnee.sob@mahidol.ac.th*

(Dunn et al., 2005). Amongst these, GC/MS has been described as a sensitive and reproducible method, which has been proposed as an ideal tool for metabolomic profiling of urine samples (Zhang et al., 2007; Wu et al., 2009).

There are few studies concerning CHB. Soga et al., (2011) applied LC/MS to analyze the serum from CHB patients and proposed γ-glutamyl threonine as a feature to discriminate CHB from other liver diseases. Zhou et al., (2012) analyzed the metabolites in serum from CHB patients and a control group by LC/MS and discovered distinctive metabolites that were involved in fatty acids, amino acids, bile acids and energy metabolism pathways. Yang et al., (2016) further investigated the serum and urine metabolic alterations of CHB by GC/MS, and they found that glycine and fatty acid metabolism may be reprogrammed in CHB patients. To date, there are still few metabolomic studies about CHB so it is a research domain that needs to be expanded.

The aims of this study were to compare metabolite profiles of urine samples from CHB patients and healthy subjects using GC/MS and to establish a diagnostic model from these metabolic biomarkers to distinguish CHB from the normal subjects.

Materials and Methods

Subjects and sample collection

The research was approved by the Committee on Human Right Related to Research Involving Human Subjects of Ramathibodi Hospital (ID 02-58-22). The study was carried out according to the Helsinki Declaration (1964) as revised in 2013. Informed consent was obtained prior to subject enrollment. Twenty CHB patients were recruited to the study from the liver clinics of Ramathibodi Hospital, Mahidol University, Bangkok, along with 20 normal control subjects. Exclusion criteria were presence of cancer, significant concomitant diseases such as congestive heart failure, renal insufficiency, respiratory failure, or those who used antiviral or herbal medication. Normal controls were healthy subjects without medical disease. Clinical information was obtained from medical records and the hospital database. Blood samples were collected for biochemical testing. Urine samples were collected and centrifuged at 3,000 rpm for 10 min at 4°C for removal of solid debris. The samples were stored at -80°C until GC/MS analysis.

Sample preparation

The urine samples were thawed at room temperature. A 10 µL l volume of internal standard (0.5 mg/ml diphenylamine in methanol) was added into 2.0 ml aliquot of urine in a 8 ml glass tube and vortex-mixed for 15 s. A 200 µL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 min at 300 rpm and centrifuged for 15 min at 2,500 rpm. The ether phase was transferred to a new glass tube and evaporated to dryness under a stream of nitrogen gas at room temperature in a fume hood. The residue was dissolved in 100 µL l TBME and transferred to the GC vial for GC/MS analysis.

GC/MS analysis

An Agilent GC/MS system (7890-5975C, Agilent Technologies, Santa Clara, CA, USA) was used in combination with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) for the analysis of all samples. A 1 µL aliquot of sample was injected in splitless mode into the GC and separated on a fused-silica capillary column HP-5MS (30 m, 0.25 mm id, 0.25 µm thickness, Agilent J and W Scientific, Folsom, CA, USA). The injector temperature was set at 250 °C. High-purity helium was used as carrier gas at a constant flow rate of 1 mL/min. The column temperature was initially kept at 80 °C for 3 min, ramped to 320 °C at 10 °C/min, and then held for 10 min. The MS quadrupole temperature was set at 150 °C and the ion source temperature at 230 °C. Ions were generated by electronic impact (EI) at 70 eV. Masses were acquired from m/z 50 to 500. The filament was turned on after a solvent delay time of 6 min. The retention time of diphenylamine (the internal standard) was set to 11.7 min with the retention time locking mode. GC/MSD ChemStation software (Agilent Technologies, Santa Clara, CA, USA) was used for acquisition of the MS data. The measured mass spectra were compared with the National Institute of Standards and Technology (NIST) mass spectra library using the ChemStation software. Peaks with mass spectra similarity index greater than 70% were assigned compound names.

Data processing and statistical analysis

After GC/MS analysis, each sample was represented by a GC/MS total ion chromatogram (TIC), and the peak areas of assigned compounds were integrated. The peak area ratio of each compound to the internal standard was calculated and used as the response. The GC/MS data were imported into the Mass Profiler Professional (MPP) software (Agilent Technologies, Santa Clara, CA, USA) for differential analysis. MPP was used for data filtering and statistical analysis, and compound identification was performed using the NIST library and Agilent MassHunter ID Browser. Independent-sample t-test statistics was used for comparison of the metabolite levels to determine the significant differences between the CHB group and the control group. Differentially expressed compounds with p-values of <0.05 were considered to be statistically significant.

Principal component analysis (PCA) was used to differentiate the samples, using the MPP software. All of the data from the differentially expressed compounds were used for constructing PCA models. The score plots of the first three principal components allowed visualization of the data and compared samples between the CHB and control groups. Group prediction employed the partial least square discriminant analysis (PLS-DA) model. PLS-DA was constructed using the significant metabolites of filtered data using four components including auto scaling, N-fold validation type, three numbers of fold, and with ten numbers of repeats. Sensitivity and specificity were also calculated from the constructed model. Forty Samples were randomly

selected and validated through the constructed model. A portion (~75 %) of the 40 subjects (15 CHB patients and 15 healthy individuals) was used as a training set to explore the specific biomarkers associated with chronic hepatitis B. The remaining ~25 % samples (5 CHB patients and 5 healthy individuals) was used as a test set to validate the diagnostic capability of the two combined markers discovered from the training set.

Metabolic pathway and metabolite biofunction analysis was performed using the network database (KEGG PATHWAY Database, http://www.kegg.jp/kegg/pathway.html). The impact of CHB on metabolic pathways was evaluated through an online MetaboAnalyst 3.0 software suite for metabolomic data analysis (http://mirror.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml).

Results

Metabolomic profiling of urine samples

Demographic, clinical and laboratory data were collected and showed in Table 1. As expected, AST and ALT levels were significantly increased in CHB patients. Representative GC/MS total ion chromatograms (TICs) are shown in Figure 1, with large diverse sets of metabolites in the urine from a normal control group

Table 1. Clinical Characteristics and Liver Function of the Study Subjects

Samples	Control (n = 20)	CHB (n = 20)	p value
Age (years)[a]	36.5 ± 10.2	47.9 ± 13.3	0.006
Male[b]	12 (54.5)	9 (42.8)	0.251
BMI (kg/m2)[a]	22.3 ± 1.2	22.5 ± 2.3	0.278
AST (U/L)[a]	22.4 ± 4.2	63.1 ± 68.2	<0.05
ALT (U/L)[a]	27.0 ± 15.5	108.8 ± 158.9	<0.05
ALB (g/L)[a]	40.1 ± 2.2	39.7 ± 2.6	0.605
Total bilirubin (mg/dL)[a]	0.7 ± 0.2	0.8 ± 0.2	0.247
Glucose (mg/dL)[a]	91.3 ± 7.0	91.8 ± 15.6	0.943
BUN (mg/dL)[a]	10.8 ± 2.5	12.4 ± 3.1	0.110
Creatinine (mg/dL)[a]	0.87 ± 0.2	0.89 ± 0.2	0.853
Triglyceride (mg/dL)[a]	91.4 ± 25.9	115.3 ± 71.7	0.287
Cholesterol (mg/dL)[a]	196.4 ± 33.5	197.8 ± 35.2	0.911
PLT (10^3/mm^3)[a]	249.0 ± 66.0	203.1 ± 39.7	0.084

CHB, Chronic hepatitis B; BM, Body mass index; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ALB, Albumin; TB, Total bilirubin; BUN, Blood urea nitrogen; PLT, Platelet; [a]mean ± SD; [b]n (%)

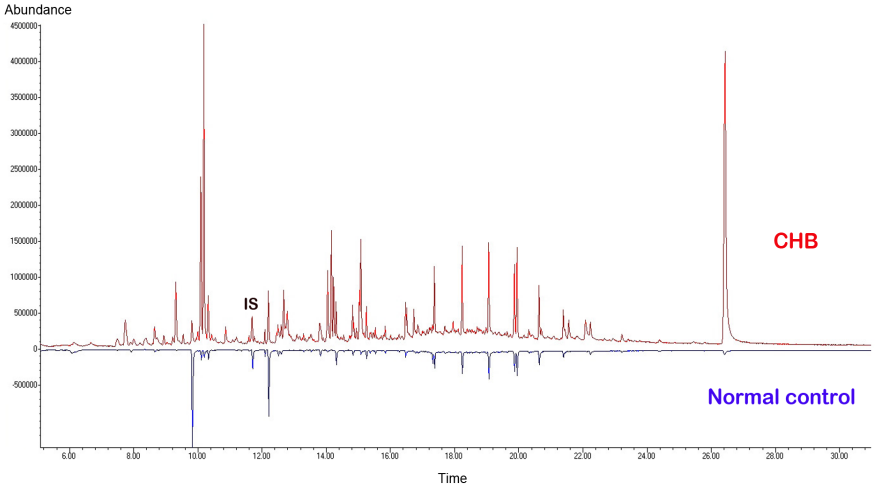

Figure 1. Comparison of Typical GC/MS Total Ion Current (TIC) Chromatograms of Urine from Chronic Hepatitis B (CHB) and Control Group (IS: Internal standard, diphenylamine)

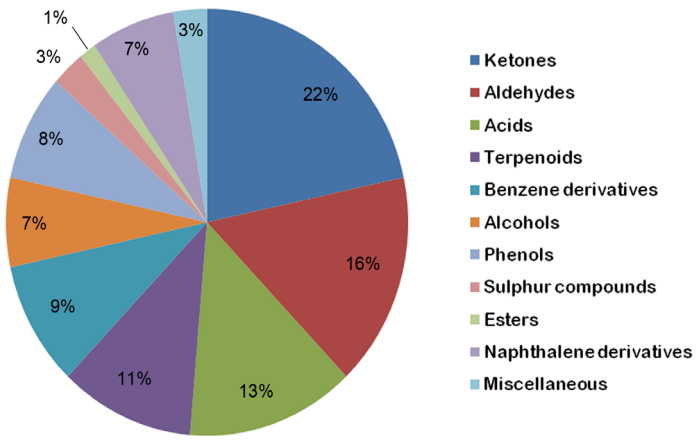

Figure 2. Distribution of Identified Urine Metabolites of Chronic Hepatitis B (CHB) and Control Groups According to Classes of Compounds

Table 2. Twelve High Abundance Compounds Identified Using the Mass Profiler Professional (MPP) Software and Filtered Using t-test

Metabolites	FC ([CHB] vs [Control])	Regulation	p value
3-Heptanone	1.3996902	up	2.05×10^{-6}
Stearic acid	1.7362183	up	4.69×10^{-5}
Palmitic acid	5.1711727	up	1.04×10^{-3}
1-Tetradecanol	1.2326117	up	8.04×10^{-4}
Naphthalene	1.7234368	up	1.16×10^{-6}
4-Heptanone	2.2869997	up	1.76×10^{-5}
Glycine	1.9506816	up	6.89×10^{-3}
Hexanal	3.8837343	up	1.96×10^{-7}
Oleic acid	1.4874096	up	2.55×10^{-2}
Butanoic acid	1.2648753	up	7.09×10^{-4}
Benzoic acid	2.2584724	up	1.52×10^{-5}
Cholesterol	1.3778079	up	3.95×10^{-3}

CHB: Chronic hepatitis B; FC: Fold change

and a CHB patient. The difference between urinary GC/MS profiles of normal control subject and CHB patient were observed. In all, 377 metabolites were presumptively identified in urine samples of the normal control and CHB groups. These include a variety of chemical compounds that were identified to be involved in multiple biological functions. The 377 metabolites were subsequently classified into ketones, aldehydes, acids, alcohols, terpenoids, benzene derivatives, sulfur compounds, phenols, esters, naphthalene derivatives and miscellaneous Figure 2.

Pattern recognition and function analysis

Twelve compounds with high abundances were identified using MPP and all of them were found to be present at significantly higher levels in the CHB group. These are palmitic acid, stearic acid, oleic acid, benzoic acid, butanoic acid, cholesterol, glycine, 3-heptanone, 4-heptanone, hexanal, 1-tetradecanol and naphthalene Table 2. A PCA model was constructed using the marker metabolite intensities as variables. The PCA scores plot showed that the samples are scattered into two regions Figure 3. Hierarchical cluster analysis (HCA) was performed to produce a dendrogram for clustering of samples groups using normalized intensities of 12 significant metabolites. HCA of these 12 metabolites again divided the samples into CHB and control groups as shown in Figure 4.

Class prediction model and test

Using the 12 statistically significant metabolites, PLS-DA algorithm was used to classify the samples into discrete classes. A clear separation was observed between the CHB and control groups in the PLS-DA scores plot (Figure 5). Sensitivity and specificity were also calculated for the constructed model. The sensitivity and specificity were 95% and 85%, respectively, and the overall accuracy of the model was 90% (Supplemental Table 1). The model was used to validate an independent or blind-test set of 10 urinary samples (5 healthy controls and 5 CHB patients). PLS-DA classifier correctly predicted the presence of CHB in 5 out of 5 patients, and healthy control in 4 out of 5 samples, resulting in 100% sensitivity and 80% specificity, respectively.

Pathway analysis

The biological pathways involved were analyzed using the online MetaboAnalyst 3.0 software Figure 6. All matched pathways are shown according to p-values from the pathway enrichment analysis (y-axis) and pathway impact values from pathway topology analysis (x-axis), with the most impacted pathways colored in red (Xia and Wishart, 2011). Consequently, eight pathways were considered closely related to CHB as shown in Figure 6. These include fatty acid biosynthesis, fatty acid elongation in mitochondria, the biosynthesis of primary bile acid and steroid hormone and the metabolism of cyanoamino acid, thiamine, glutathione and phenylalanine. The altered metabolic pathways were generated using the reference map by searching KEGG database, as shown in supplemental materials

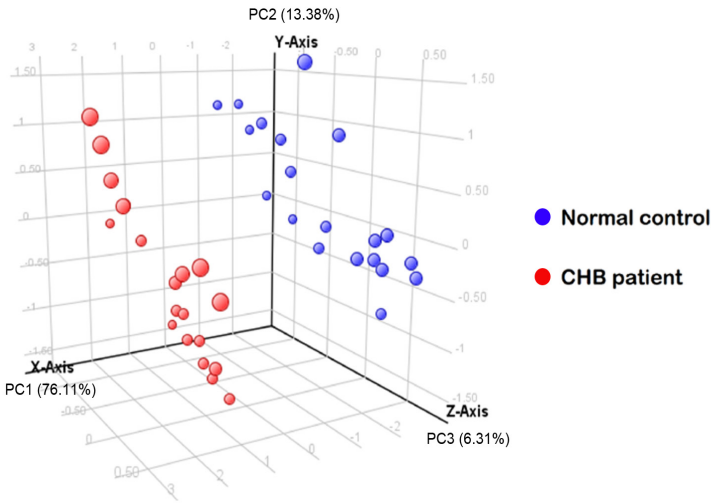

Figure 3. Sample Pattern Recognition Using Principal Component Analysis (PCA). PCA Analysis of the Twelve Significantly Abundant Compounds Results in two Distinctive Groups of Samples. The Blue and Red Circles Indicate Healthy Volunteers (n=20) and Chronic Hepatitis B (CHB) Patients (n=20), Respectively

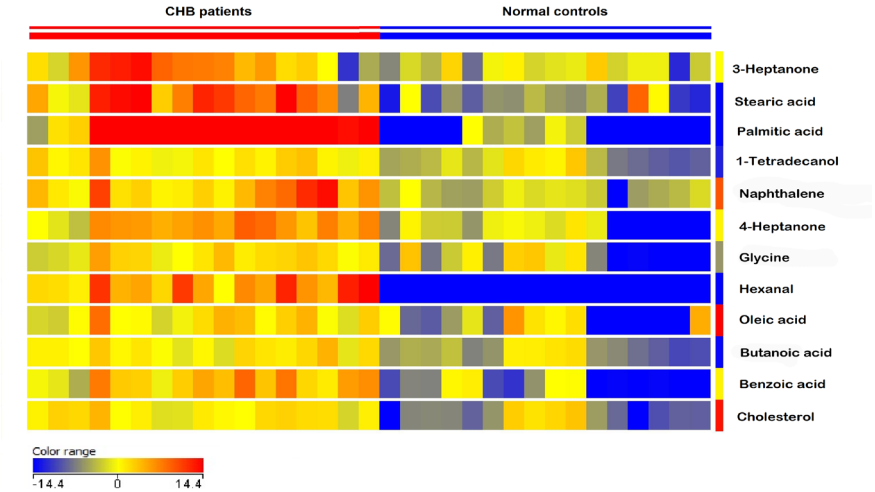

Figure 4. Hierarchical Cluster Analysis of Urine Metabolic Profile for Distinguishing Chronic Hepatitis B (CHB) Patients from Healthy Controls

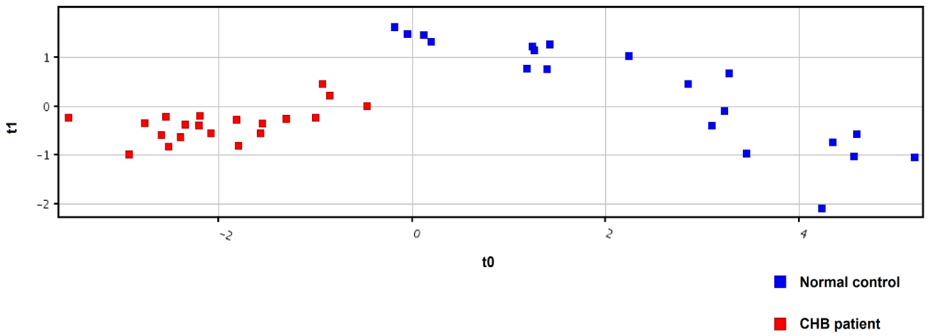

Figure 5. PLS-DA Scores Scatter Plots Discriminating Controls and Chronic Hepatitis B (CHB) Patients Based on the Twelve Significant Metabolites. The Blue and Red Squares Indicate Healthy Volunteers (n=20) and Chronic Hepatitis B (CHB) Patients (n=20), Respectively

Supplemental Figure 1-8.

Discussions

GC/MS-based urine metabolomics coupled with multivariate statistical analysis clearly differentiated CHB patients from normal subjects with high sensitivity (95%) and specificity (85%) and able to identified the metabolite biomarkers.

In this study, we have carried out the analysis of the clinical and biochemical indicator. There was no significant difference in mean age, sex, BMI, ALB,

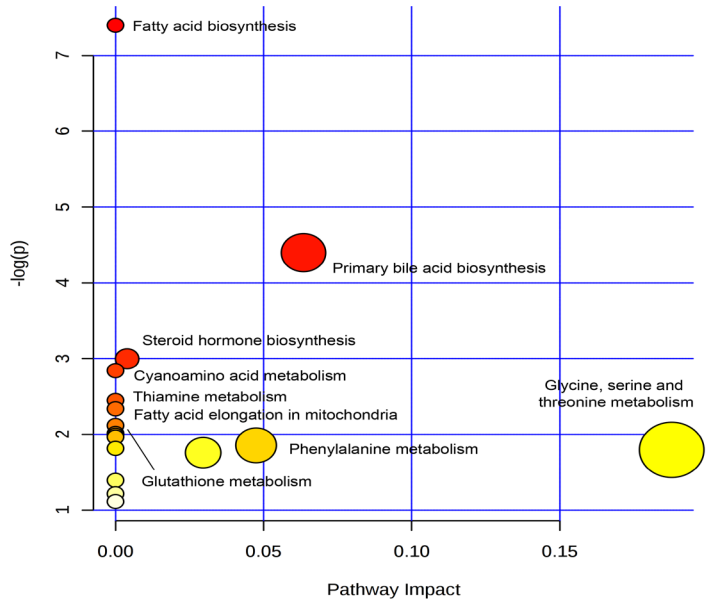

Figure 6. Summary of Aberrant Pathways in the Chronic Hepatitis B (CHB) Group, as Analyzed by MetaboAnalyst

TB, BUN, creatinine, glucose, triglyceride, cholesterol and PLT among these two groups. On the contrary, a significant increase in the activities of AST and ALT was observed in patients with CHB, indicating considerable hepatocellular injury (Ganem and Prince, 2004). According to the GC/MS TIC chromatograms displayed in Figure 1, the majority of the peaks in the chromatograms were identified by NIST mass spectra library, including ketones, aldehydes, fatty acids, amino acids, organic acids, and so on. These metabolites are known to be involved in multiple biochemical processes, especially in energy and lipid metabolism (Postic et al., 2004; Wu et al., 2009). In this study, we constructed a PCA, HCA, and PLS-DA model to obtain metabolomic profiling. A clear separation was observed between the CHB and control groups. These results indicate that the models had a good ability of explaining and predicting.

Discriminatory metabolites were significantly identified as palmitic acid, stearic acid, oleic acid, benzoic acid, butanoic acid, cholesterol, glycine, 3-heptanone, 4-heptanone, hexanal, 1-tetradecanol and naphthalene Table 2. The present study adds evidence to support the previous results of metabolomic study that the levels of some fatty acids and amino acids are altered in CHB patients (Zhang et al., 2013a; Yang et al., 2016). Previous reports have shown significant increases in levels of glycine, benzoic acid and butanoic acid in CHB patients compared with the control groups (Zhao et al., 2013). Lu et al., (2015) have also reported increased levels of glycine, stearic acid, oleic acid and cholesterol, in a study performed by LC/MS and GC/MS, when comparing the urinary metabolomic profiles of CHB patients and control groups. Moreover, a recent GC/MS-base metabolomics study of CHB patients identified the metabolites glycine, cholesterol, palmitic acid, stearic acid and oleic acid as discriminatory metabolites which corroborate the finding in this study (Yang et al., 2016). In our study, we observed increased levels of saturated fatty acids, such as palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and cholesterol in CHB patients, which suggests that chronic HBV infection may interfere with fatty acid metabolism. In addition, Lu et al., (2015) have reported increased levels of fatty acids in hepatitis B related HCC that might be the results of energy requirement and cell membrane synthesis due to aggressive cell proliferation.

Moreover, the pathway analysis of our study confirmed the alteration of cholesterol, bile acid and bile salt metabolism, as shown in Supplemental Figure 3. Bile acids are steroidal amphipathic molecules, derived from the catabolism of cholesterol. Bile acids are involved in signal transduction pathways that regulate apoptosis (St-Pierre et al., 2001). Previous clinical observations showed increased conjugated bile acid as well in other HBV-induced liver diseases such as liver failure (Nie et al., 2014) and cirrhosis (Wang et al., 2016). Furthermore, a recent report revealed that the alterations of bile acid metabolism could characterize the different stages of CHB progression (Huang et al., 2016).

The urine level of glycine, a non-essential amino acid, was significantly increased in CHB patients compare with the control group, which suggested the abnormality of amino acid metabolism (Zhao et al., 2013). From a previous study, an increased urinary glycine level was also observed in hepatitis B related HCC, which may be attributed to the increased glycolysis in tumor (Yang et al., 2007; Wu et al., 2009). Glycine is involved in glutathione (GSH) metabolism and bile synthesis (Lu, 1999). Additionally, glycine exerts anti-inflammatory, cytoprotective, and immunomodulatory properties and recues liver injury through attenuation of oxidative stress, and apoptosis (Chen et al., 2013).

Interestingly, the changes of benzoic acid and butanoic acid are associated with gut microflora. Benzoic acid is the metabolite from phenylalanine metabolism in bacteria. It is mainly produced when gut bacteria obtains polyphenols from food and aromatic amino acids from food protein decomposition (Rechner et al., 2004). Butanoic acid is a short-chain fatty acids mainly generated from dietary cellulose, starch and other undigested substances under microbial fermentation in the cecum or colon (Mao et al., 2012).

In addition, the levels of hexanal, heptanone and naphthalene were significantly increased in CHB patients comparing with control subjects. Hexanal is a breakdown product of lipid peroxidation that is formed as a result of oxygen free radical activity. The specificity of this marker for different cancers is under investigation (Xue et al., 2008a). The origin of 3-heptanone and 4-heptanone is still unknown but is derived probably from an exogenous source (Mills and Walker, 2001). It has been reported that 4-heptanone is produced from the in vivo metabolism of plasticizers in man (Walker and Mills, 2001). Moreover, the source of the metabolites identified to derive from naphthalene is not yet known; they may be the degradation products of steroids (Turfitt, 1948; Annweiler et al., 2000).

There are some limitations of our study that need to be addressed, i.e. it was a cross-sectional study with a small sample size, and conducted in a single center. Further studies with larger population and multi-analytical techniques are required to confirm the findings.

In summary, GCMS-based urine metabolomics profiling coupled with multivariate statistical analysis is able to identify metabolite biomarkers which can significantly differentiate CHB patients from normal subjects with high sensitivity and specificity. The metabolic signature of CHB patients comprised metabolite changes associated with the metabolism of fatty acid, bile acid, amino acid and gut microbiomes. CHB-related alteration included palmitic acid, stearic acid, oleic acid, benzoic acid, butanoic acid, cholesterol, glycine, 3-heptanone, 4-heptanone, hexanal, 1-tetradecanol and naphthalene metabolites. Our study indicates that GC/MS-based metabolomics is a promising tool that could provide insights into the metabolomics study in CHB. Further study of these metabolites may facilitate the development of non-invasive biomarkers and more efficient therapeutic strategies for CHB patients.

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