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Metabolomics study of alcohol-induced liver injury and hepatocellular carcinoma xenografts in mice

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ABSTRACT

Alcohol abuse is one of the major causes of liver injury and a promoter for hepatocellular carcinoma (HCC). To understand the disease-associated metabolic changes, we investigated and compared the profiles of metabolites in nude mice with alcohol-induced liver injury or bearing a HCC xenograft (HCCX). Alcohol-induced liver injury was achieved by daily administration of grain liquor, and HCC xenografts were generated by subcutaneous inoculation of HepG2 cells in nude mice. Metabolites in serum samples were profiled by ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS). The acquired data was analyzed by principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) to identify potential disease-specific biomarkers. Results showed that the phosphatidylcholine (PC) levels were significantly higher in both liver injury and HCCX mice compared with the control. Interestingly, lysophosphatidylcholines (LPCs) that contain saturated or monounsaturated fatty acids were reduced in both liver injury and HCCX mice, but polyunsaturated fatty acids LPCs were elevated in liver injury mice only. These data delineated the disease-related metabolic alterations of LPCs in liver injury and HCC, suggesting that the LPC profile in serum may be biomarkers for these two common liver diseases.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide [1]. Approximately 564,000 new cases are diagnosed annually, including 398,000 men and 166,000 women [2]. Currently, the only effective treatment is surgical removal of the tumors at the early stage. Unfortunately, early stage HCC is usually not noticeable in current clinical practice. A large number of HCC diagnosed are not eligible for surgical intervention, leading to poor prognosis. Therefore, biomarkers for the diagnosis of HCC at early stages are essential for the successful management of this disease.

Excessive alcohol consumption has been recognized as a cause of chronic liver diseases, including HCC [3]. Alcohol and its metabolites stimulate lipid peroxidation [4,5] and induce release of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1) [6,7]. Inflammatory cytokines are known to increase production of hydroxyl radical (•OH), causing liver damage [8,9]. Excessive alcohol intake also increases the expression of adhesion molecules that might affect cell–cell communication and proper function of the cell [10]. Heavy alcohol consumption may enhance the development of liver cancer induced by environmental carcinogens, such as N-nitrosodimethylamine [11]. Moreover, acetaldehyde, a metabolite of alcohol, is thought to be carcinogenic [12,13]. It has been shown that people with alcohol intake of more than 80 g/day have 1.5–2.5-fold greater risk of developing liver cirrhosis and HCC compared to those who abstain from alcohol [14,15]. However, despite strong evidence that excessive alcohol consumption is an important risk factor for liver cancer, the pathogenesis and metabolic alterations of hepatocarcinogenesis induced by alcohol remain unclear.

Metabolomics, or quantitative analysis of low molecular weight metabolites [16], has been applied to disease diagnosis [17–19], drug discovery [20,21], nutrition studies [22] and toxicological investigation [23,24]. Metabolomics analyses have revealed that

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liver injury is associated with changes in the metabolism of sulfur-containing amino acids [25,26], n-acetylglutamine, n-acetylglycine and taurine [27]. Metabolomics has also been used in liver cancer research. The methodologies used for metabolomics include gas chromatography–mass spectrometry (GC–MS) [28], liquid chromatography–mass spectrometry (LC–MS) [29,30] and nuclear magnetic resonance (NMR) [31]. Several metabolites have been identified and regarded as biomarkers for this disease. However, a direct comparison of the metabolite profiles between alcohol-induced liver injury and HCC, which is critical for monitoring the carcinogenic transition of liver cells, is still lacking thus far.In this study, we compared the metabolic profiles in the serum of nude mice with alcohol-induced liver injury or bearing HCC xenografts (HCCX), using ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS). This study defined the differences of their metabolite profiles with a focus on phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs). Our data revealed a disease-related alteration of LPCs, being potential biomarkers for monitoring the tumorigenic transition of liver cells or for the diagnosis of these diseases.

2. Materials and methods

2.1. Chemicals

Acetonitrile and methanol (HPLC grade) was purchased from Fisher (Fairlawn, NJ, USA). Formic acid (HPLC grade) was purchased from Tedia (USA). Distilled water was filtered through a Milli-Q system (Millipore, MA). DMEM was purchased from Invitrogen, USA. Fetal bovine serum was purchased from Hyclone, USA. Streptomycin sulfate and penicillin was purchased from North China Pharmaceutical, China. Trypsin was purchased from Shanghai Sangon Biological Engineering Technology & Services, China.

2.2. Cell cultures

Human hepatocellular carcinoma cells HepG2 were obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai. These cells were cultured in DMEM containing 10% fetal bovine serum, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin in a humidified incubator with 5% CO_2 at 37 °C. Cells in logarithmic growth (85% confluence) were collected with 0.25% trypsin and washed with PBS twice. Cells were then collected at $46 \times g$ for 5 min. Cell pellet was resuspended in PBS (2.5×10^7 mL^{-1}) and injected into nude mice.

2.3. Animal models

Athymic BALB/c nude mice (6–8 weeks, 20–25 g, males) were purchased from the Center of Laboratory Animal of Guangzhou University of Chinese Medicine, China. Mice were maintained under Specific Pathogen Free (SPF) conditions with room temperature at 22 ± 2 °C and relative humidity 50 ± 5%. Twenty mice were randomly divided into three groups as follows: HCCX group (8 mice), liver injury group (6 mice) and control group (6 mice). Following acclimatization for 1 week after arrival, the control group was kept under a regular condition and provided with food and water *ad libitum*. Induction of chronic liver injury in mice was performed as previously described in [32] with slight modifications. Grain liquor containing 56% (v/v) alcohol (Erguotou, Beijing, China) was diluted with water and 0.2 mL per mouse was administered daily by gastric gavaging. The procedures of alcohol administration were as follows in order: 5% (v/v) for 3 days, 10% for 4 days, 20% for 7 days, 30% for 7 days, and finally 40% for 7 days. HCCXs were generated by subcutaneous injection of 0.2 mL HepG2 cells (5×10^6/mouse). When the xenografts reached about 1 cm in diameter (about 4 weeks), mice were sacrificed, and blood samples were collected and centrifuged at $2576 \times g$ for 10 min at 4 °C. Serum (supernatants) was stored at −80 °C until use.

2.4. Sample preparation

Prior to analysis, serum samples were thawed at 4 °C. Methanol (400 μL) was added to 100 μL serum, followed by vigorous vortex for 1 min. After incubation on ice for 10 min, the mixture was centrifuged at $12{,}092 \times g$ for 10 min at 4 °C. The supernatant was taken up and dried with a stream of nitrogen. The residues were reconstituted in 1.0 mL acetonitrile/water (1:1, v/v) mix. The solution was filtered through a 0.22 μm mesh Millipore filter and injected onto the UPLC column.

2.5. Ultra-performance liquid chromatography

Ultra-performance liquid chromatography (UPLC) was performed on an Acquity™ system coupled to a Q-TOF premier (Waters Corporation, MA, USA). The chromatographic separation was carried out on a Waters Acquity™ BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm). Column temperature was maintained at 35 °C for all analyses. The flow rate was at 0.5 mL/min of a mobile phase consisting of water containing 0.1% formic acid (A) and acetonitrile (B). Elution gradient was linearly increased from 5% B to 60% B within 2 min, then to 100% B within 6 min and held for 2 min, followed by return to 5% B. Total running time was 12 min per separation. Injection volume was 10 μL.

2.6. Mass spectrometry

Mass spectrometry was performed on a Waters Q-TOF premier equipped with an electrospray ion source in the positive ion mode and V optics mode. Capillary voltage was set at 3.1 kV and cone voltage at 35 V. Cone gas flow was set at 50 L/h with an ion source temperature of 120 °C. Desolvation gas flow was set to 500 L/h with the desolvation gas temperature of 300 °C. Data were collected in the centroid mode between m/z 100 and 1000, with a scan time of 0.15 s and interscan time 0.02 s. For data accuracy and reproducibility, all analyses were carried out with an independent reference spray via the LockSpray interference. Leucine–enkephalin at 100 pg/uL in acetonitrile–water with 0.1% formic acid (50:50, v/v) was used as the lock mass (m/z 556.2771) with a flow rate of 0.05 mL/min. Lock spray frequency was set at 10 s and scan to average for correction was 10 s with the reference cone voltage at 35 V. For MS/MS analysis, the collision energies were set at 10, 20 and 30 V.

2.7. Data processing

Raw data were processed using the MarkerLynx software (version 4.1, Waters Corporation, MA, USA). MarkerLynx uses ApexTrack-peak detection package to integrate peaks in UPLC/MS data. Peaks eluting in each chromatogram were identified by retention time (RT) and mass to charge ratio (m/z) data pairs, along with their associated height intensities. Within each sample, the ion intensities were normalized to the sum of the peak height intensities in that sample and multiplied by 10,000. A three-dimensional matrix was constructed using 20 observations (sample names) with 594 variables (normalized peak intensities) each, and then exported into SIMCA-P 11.5 software (Umetrics AB, UMEÅ, Sweden) for multivariate data analyses. Pareto scaling and automatic transformation were applied to the data processing before principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) were performed. PCA is

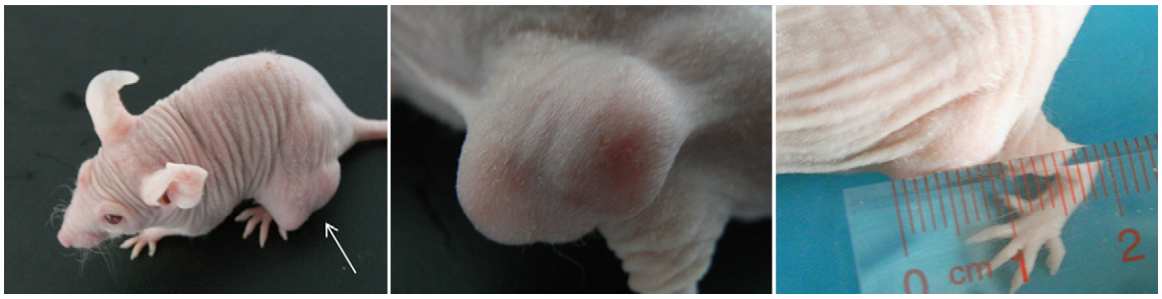

Fig. 1. HCCX in nude mouse generated with HepG2 cells.

an unsupervised multivariate statistical approach. It is used for variable reduction and separation into classes. To maximize class discrimination and biomarkers, the data were further analyzed using the OPLS-DA method. Hereon, the tested groups (i.e., control group and liver injury group, control group and HCCX group) were compared in the OPLS-DA method. S-plots were calculated to visualize the relationship between covariance and correlation within the OPLS-DA results. Variables that had significant contributions to discrimination between groups were considered as potential biomarkers and subjected to further identification of the molecular formula. Other statistical analyses used include one-way analysis of variance (ANOVA), least significant difference (LSD) test and independent sample *t*-test. They were performed with Origin 7.5 version (OriginLab, Co., MA).

2.8. Identification of biomarkers

The quasi-molecular ion ($[M+H]^+$) was found from the mass spectrometry and subsequently, accurate molecular weight was calculated. Databases of HMDB (http://www.hmdb.ca/) and KEGG (http://www.kegg.com/) were used to identify the metabolite markers by comparing molecular weights. The molecular and structural formulas of the candidate compounds were retrieved by the comparison and then confirmed by MS/MS scans for the characteristic ions and fragmentation patterns of the metabolites. Standards of metabolic interest were used to confirm the structure.

3. Results and discussion

3.1. Animal models

Liver injury and HCC-xenografts were generated in nude mice. After 4 weeks of alcohol intake, mice showed mild liver hemorrhage. This indicated alcohol-induced damage in the liver. In the HCCX group, mice inoculated with HepG2 cells developed tumors of about 1 cm in diameter at the injection site 4 weeks later (Fig. 1). Mice were sacrificed and blood samples were collected and processed for metabolomics analyses.

3.2. Metabolomics study and metabolite identification

Metabolites in serum samples from control, liver injury and HCCX mice were profiled by UPLC/Q-TOF MS. A typical base peak intensity (BPI) chromatogram is shown in Fig. 2.

Raw data from UPLC/Q-TOF MS were analyzed by the MarkerLynx software. RT, *m*/*z* and peak height intensities were imported into SIMCA-P software for data analysis. Multivariate data analysis was performed using the PCA method. Three principal components were created by the SIMCA-P software. The summary of the fit of

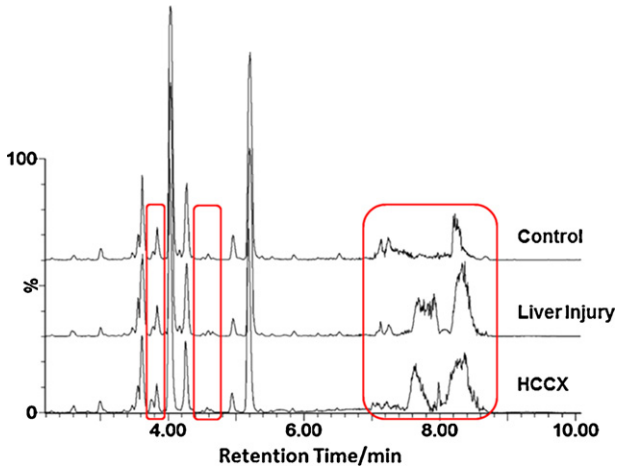

Fig. 2. BPI chromatograms obtained from the serum samples of experimental mice.

the model was displayed with R^2X (fraction of the variation of the data explained by each component) and Q^2 (cross validated R^2X) [33]. Data from the R^2X values showed that PC1 accounted for 39% of the variations in the PCA model, PC2 for 13%, and PC3 for 9%. The

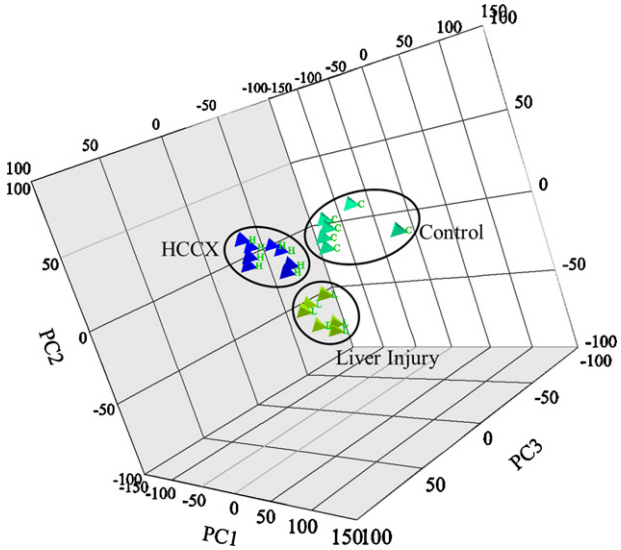

Fig. 3. Pseudo 3D-PCA score plot for the first three components (PC1 vs. PC2 vs. PC3) indicating the separation among control, liver injury and HCCX groups.

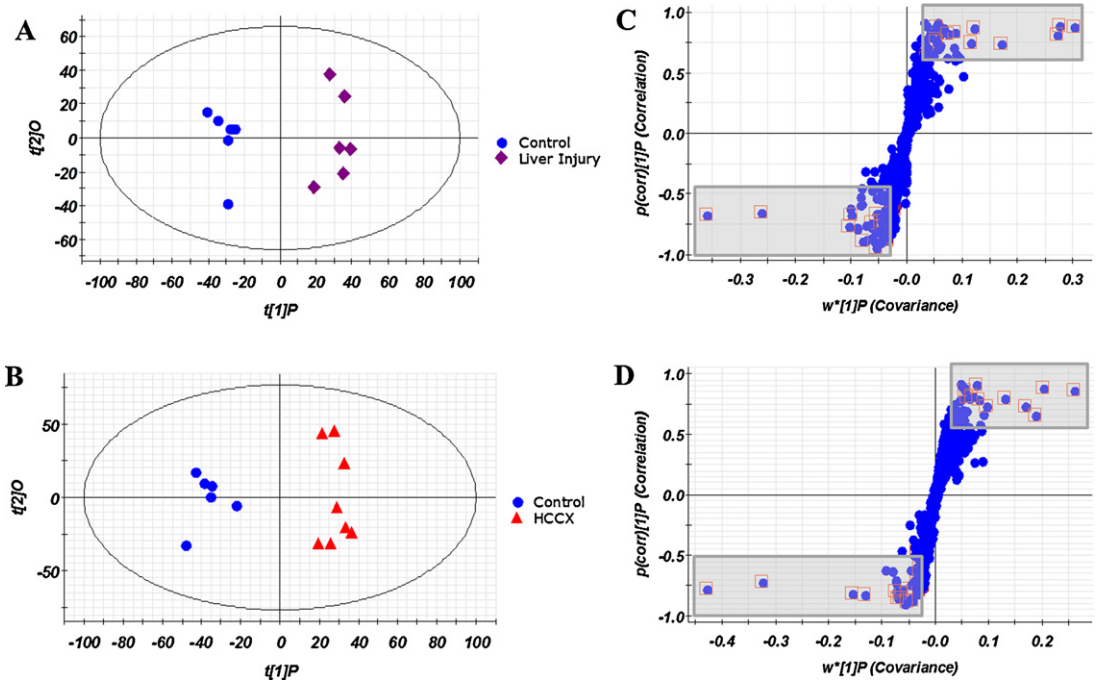

Fig. 4. OPLS-DA score plots resulting from the LC/MS spectra of serum with corresponding S-plots for three tested groups. (A) OPLS-DA score plot from control and liver injury group; (B) OPLS-DA score plot from control and HCCX group; (C) potential biomarkers in the S-plot between control and liver injury group; (D) potential biomarkers in the S-plot between control and HCCX group.

homologous Q^2 values were 31%, 6% and 4%, respectively. Overall, the model described 61% of the variations in a cumulative R^2X and 41% in a cumulative Q^2. As shown in Fig. 3, control, alcohol liver injury and HCCX mice are appreciably separated in the pseudo 3D-PCA score plot.

To highlight the metabolite differentials between the control and alcohol liver injury mice or HCCX mice, feature selections were performed using orthogonal partial least squares discriminant analysis (OPLS-DA). As shown in Fig. 4A, the score plot of OPLS-DA between control and liver injury mice are clearly separated. R^2Y (cum), the fraction of the variation of Y (all the responses) explained by the model after each components, and Q^2 (cum), the fraction of the variation of Y that can be predicted by the model according to cross-validation, were used to evaluate the OPLS-DA model [33]. The R^2Y (cum) and Q^2 (cum) are 0.979 and 0.882, respectively. These results suggest that the model explains 97.9% of the variations of Y, with a predictive ability (Q^2) of 88.2%. Values of R^2Y (cum) and Q^2 (cum) that are close to 1.0 indicate the excellence of the model. Similar OPLS-DA results between control and HCCX mice were obtained (Fig. 4B). Likewise, the values of R^2Y (cum) and Q^2 (cum) are 0.955 and 0.827, respectively. S-plot is a scatter plot which combines the covariance and correlation loading profiles arising from the predictive component of the OPLS-DA model. It was performed to visualize variables that had significant contribution to the discrimination between experimental groups. These significant variables, with high correlation and covariance values, were located in regions far away from the origin (grey areas in Fig. 4C and D). Variable importance in the projection (VIP) values were also used for the selection of biomarkers. Variables with a VIP value larger than 1 showed a higher than average influence on the classification (red squares). Those variables represented by red squares in the grey areas were eventually selected as potential biomarkers. An independent t-test indicated that these variables between the control and liver injury (including HCCX) mice were statistically significant ($p < 0.05$).

Metabolite identification was conducted with high resolution MS and MS/MS fragments, as well as database analyses. To illustrate the identification of metabolites, we took the ion at 5.20_524.3705 (retention time 5.20 min, m/z 524.3705) as an example to be described below. In positive ion electrospray ionization mass spectrum, three ions at m/z 524.3705, 546.3566 and 562.3336 were found at retention time 5.20 min. We inferred that the quasi-molecular ion was m/z 524.3705 ($[M+H]^+$). The other two ions were the adducts $[M+Na]^+$ and $[M+K]^+$, respectively. Therefore, the monoisotopic weight (MW) of the metabolite at m/z 524.3705 ($[M+H]^+$) was determined to be 523.3627 Da. According to the HMDB database, the metabolite was tentatively identified as LPC (18:0/0:0) or (0:0/18:0). In line with MS/MS scan supplemented mass fragments of the metabolite (Fig. 5), fragments 184 Da and 104 Da were thought to be diagnostic ions of PC and LPC [34,35], which could not be differentiated under the current conditions. Similarly, other metabolites were identified and shown in Fig. 6A (control vs. liver injury mice) and Fig. 6B (control vs. HCCX mice).

3.3. Analysis of potential metabolite biomarkers

Liver injury and HCCX mice clearly shared some common metabolites (Fig. 6A and B), significantly different from the control animals. A one-way ANOVA, followed by a LSD test, was used to analyze the statistically significant differences of these metabolites among the three groups. The results are listed in Table 1. Liver injury and HCCX mice shared five metabolic pathways changes. The first one was phenylalanine and tyrosine metabolism. It included two metabolites, i.e. phenylpyruvic acid and phenylalanine. Leucine and tryptophan belong to leucine degradation and tryptophan metabolism, respectively. These biomarkers related to amino acid metabolism showed similar changing tendency. The serum concentrations of these biomarkers in control group were highest among the three groups, followed by HCCX and then liver

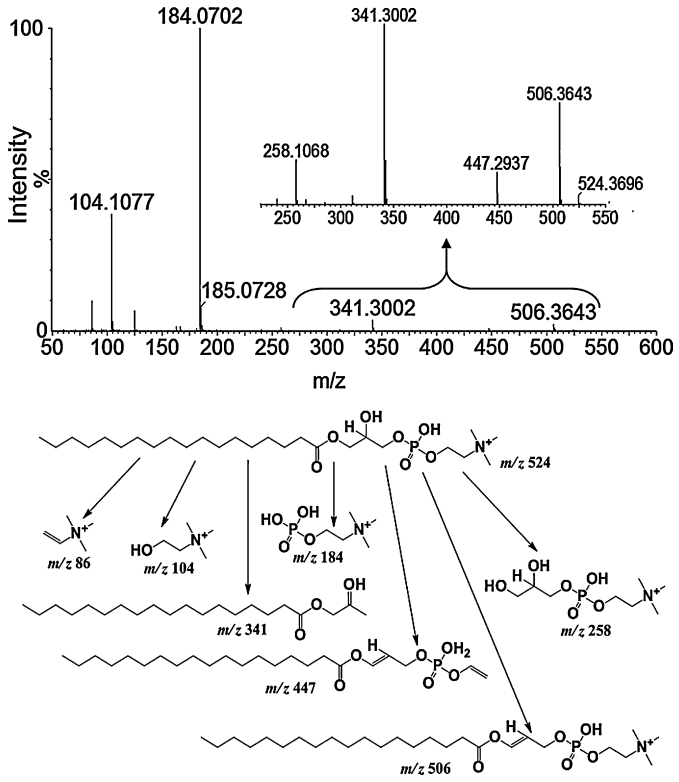

Fig. 5. MS/MS spectra of the metabolite at *m/z* 524.3705 and its fragmentation behavior.

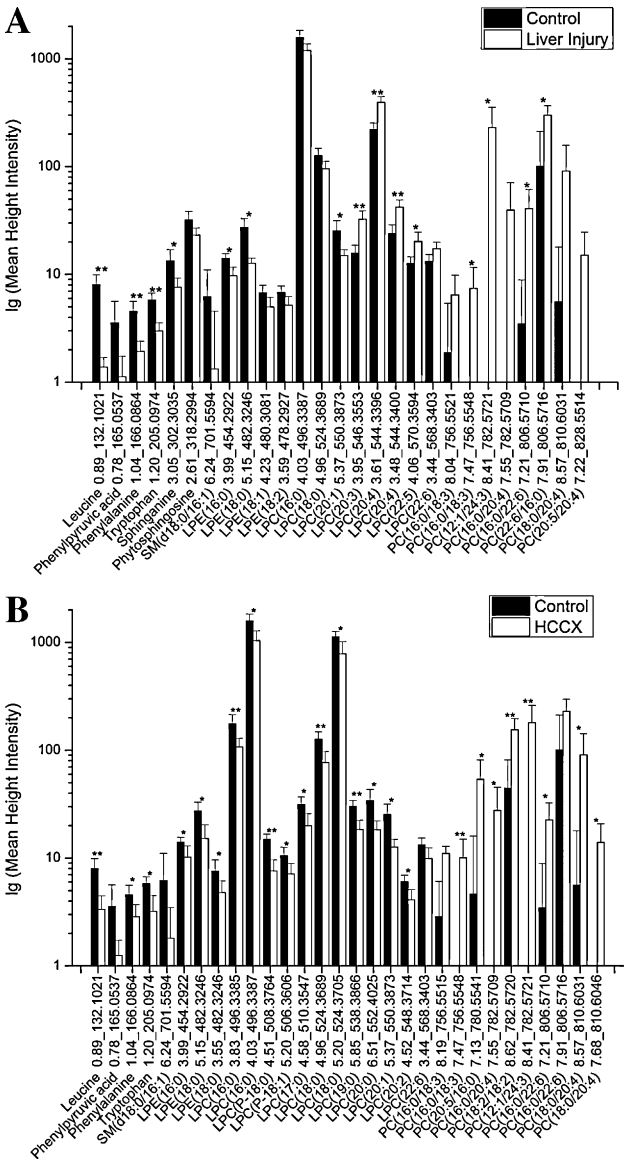

Fig. 6. The relative mean height intensity of different metabolites of liver injury and HCCX. (A) Liver injury; (B) HCCX. Y-axis is logarithmic scale. $^{*}p < 0.01$; $^{**}p < 0.001$; others $p < 0.05$.

injury. Sphingolipid metabolism pathway was also affected. Sphingomyelin [SM (d18:0/16:1)] was found to be reduced both in liver injury and cancer groups, but sphingolipid metabolites, phytosphingosine and sphinganine were reduced in liver injury mice only.

Glycerophospholipid metabolites [PC, LPC, and lysophosphatidylethanolamine (LPE)] were significantly increased in the serum of liver injury and HCCX mice compared to the control. PC and LPC are related metabolites and were analyzed in more details in this study. It has been reported that PCs synthesis is enhanced by alcohol stimulation and plays a protective role against alcohol liver injury [36]. Heavy alcohol consumption also decreases the activity of lecithin cholesterol acyltransferase (LCAT) and enzymes of the lipolytic transformation of lipoproteins, leading to serum PCs increase [37]. Our data showed that serum PCs were also increased in HCCX mice compared to the control. PCs are the main components of cell membrane. The increases were probably to meet the demands of rapid proliferation of liver cancer cells [38]. In malignant tissues, enhanced synthesis of PCs may be attributed to the increase of lysophosphatidylcholine acyltransferase (LPCAT) activity, leading to fatty acid remodeling via the deacylation–reacylation cycle [39].

Compared to the control group, changes of relative amounts of LPCs in HCCX group were different from that of liver injury group. Serum LPCs were decreased in HCCX mice compared to the control, and all LPCs detected were saturated or monounsaturated fatty acids except for LPC (20:2) and LPC (22:6). The decrease of serum LPCs in HCCX mice is owing to increased autotaxin activity within the serum, resulting in the rapid conversion of LPCs into lysophosphatidic acids (LPAs) [40]. Serum LPCs in liver injury mice varied largely. Saturated or monounsaturated fatty acids LPCs [LPC (16:0), LPC (18:0), and LPC (20:1)] in serum were decreased, whereas polyunsaturated fatty acids LPCs [LPC (20:3), LPC (20:4), LPC (22:5), and LPC (22:6)] were increased. The discriminating changes of LPCs among the three groups may root in different mechanisms. LPCs are generated in liver tissues from hydrolysis of sn-2 fatty acyl bond of phospholipids by phospholipase A2 (PLA2). PLA2 activity is increased in liver cancer [41,42]. Therefore, one may expect increased hydrolysis of phospholipids by PLA2 and subsequent increase of serum LPCs. However, the opposite data was found. Enhanced conversion of LPCs to LPAs by lysophospholipase D may reduce serum saturated or monounsaturated fatty acid LPCs [43,44]. In alcohol-injured liver, PLA2 activity is increased [45]. This may explain the increase of polyunsaturated fatty acids LPCs. Further studies are needed to elucidate whether increased conversion by lysophospholipase D of saturated or monounsaturated fatty acids to lysophosphatidic acids or the substrate discrimination of PLA2 to this type of lipids contribute to this variation of LPCs. Nevertheless, this differential change of the serum LPCs in liver injury and HCC cancer may serve as metabolite markers for distinguishing one disease from the other.

Table 1
Metabolite changes and statistical significance in mice with liver injury or HCCX, compared to control animals.

RT	*m*/*z*	Changes compared with the control		One-way ANOVA *p*-value	Compounds
		Liver injury	HCCX		
0.89	132.1021	↓↓	↓	<0.0001[a,a]	Leucine
0.78	165.0537	↓↓	↓	<0.01[a,a]	Phenylpyruvic acid
1.04	166.0864	↓↓	↓	<0.001[a,a]	Phenylalanine
1.20	205.0974	↓↓	↓	<0.001[a,a]	Tryptophan
3.99	454.2922	↓↓	↓	<0.01[a,a]	LPE (16:0)
5.15	482.3246	↓↓	↓	<0.0001[a,a]	LPE (18:0)
4.03	496.3387	↓	↓↓	<0.01[a,a]	LPC (16:0)
4.96	524.3689	↓	↓↓	<0.001[b,a]	LPC (18:0)
5.37	550.3873	↓	↓	<0.0001[a,a]	LPC (20:1)
3.44	568.3403	↑	↓	<0.001[a,b]	LPC (22:6)
7.47	756.5548	↑	↑↑	<0.001[a,a]	PC (16:0/18:3)
8.41	782.5721	↑↑	↑	<0.001[a,a]	PC (12:1/24:3)
7.55	782.5709	↑↑	↑	<0.05[a,b]	PC (16:0/20:4)
7.21	806.5710	↑↑	↑	<0.001[a,b]	PC (16:0/22:6)
7.91	806.5716	↑↑	↑	<0.01[a,b]	PC (16:0/22:6)
8.57	810.6031	↑	↑	<0.01[a,a]	PC (18:0/20:4)
6.24	701.5594	↓↓	↓	<0.05[b,b]	SM (d18:0/16:1)

The superscript letters in the one-way ANOVA *p*-value column represent the *p*-value of LSD tests. The first letter corresponds to liver injury group, the other one denotes HCCX group. RT, retention time; HCCX, hepatocellular carcinoma xenograft; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, Sphingomyelin.

[a] $p < 0.01$.

[b] $p < 0.05$.

4. Conclusions

Metabolite differences in serum of nude mice with alcohol-induced liver injury or bearing HCCX were unraveled using UPLC/Q-TOF MS and multivariate data analysis. Five metabolic pathways were identified, namely: phenylalanine and tyrosine metabolism, leucine degradation, tryptophan metabolism, sphingolipid metabolism and glycerophospholipid metabolism. Serum LPCs showed disease-specific changes, probably reflecting metabolic difference between liver injury and HCCX in nude mice. This difference of serum LPCs may denote their potential as metabolite biomarkers for differentiating liver injury and HCC. Monitoring changes of serum LPCs may predict the transition of noncancerous liver injury to cancer.

Acknowledgements

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