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Utilization of Metabolomics to Identify Serum Biomarkers for Hepatocellular Carcinoma in Patients with Liver Cirrhosis

Habtom W. Ressom^{1,*}, Jun Feng Xiao¹, Leepika Tuli¹, Rency S. Varghese¹, Bin Zhou¹, Tsung-Heng Tsai¹, Mohammad R. Nezami Ranjbar¹, Yi Zhao¹, Jinlian Wang¹, Cristina Di Poto¹, Amrita K. Cheema¹, Mahlet G. Tadesse², Radoslav Goldman¹, and Kirti Shetty^{3,4}

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC 20057, USA

²Department of Mathematics and Statistics, Georgetown University, Washington DC 20057, USA

³Department of Surgery, Georgetown University Medical Center, Washington DC 20057, USA

⁴Georgetown University Hospital, Washington DC 20057, USA

Abstract

Characterizing the metabolic changes pertaining to hepatocellular carcinoma (HCC) in patients with liver cirrhosis is believed to contribute towards early detection, treatment, and understanding of the molecular mechanisms of HCC. In this study, we compare metabolite levels in sera of 78 HCC cases with 184 cirrhotic controls by using ultra performance liquid chromatography coupled with a hybrid quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS). Following data preprocessing, the most relevant ions in distinguishing HCC cases from patients with cirrhosis are selected by parametric and non-parametric statistical methods. Putative metabolite identifications for these ions are obtained through mass-based database search. Verification of the identities of selected metabolites is conducted by comparing their MS/MS fragmentation patterns and retention time with those from authentic compounds. Quantitation of these metabolites is performed in a subset of the serum samples (10 HCC and 10 cirrhosis) using isotope dilution by selected reaction monitoring (SRM) on triple quadrupole linear ion trap (QqQLIT) and triple quadrupole (QqQ) mass spectrometers. The results of this analysis confirm that metabolites involved in sphingolipid metabolism and phospholipid catabolism such as sphingosine-1-phosphate (S-1-P) and lysophosphatidylcholine (lysoPC 17:0) are up-regulated in sera of HCC vs. those with liver cirrhosis. Down-regulated metabolites include those involved in bile acid biosynthesis (specifically cholesterol metabolism) such as glycochenodeoxycholic acid 3-sulfate (3-sulfo-GCDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), taurocholic acid (TCA), and taurochenodeoxycholate (TCDCA). These results provide useful insights into HCC biomarker discovery utilizing metabolomics as an efficient and cost-effective platform. Our work shows that metabolomic profiling is a promising tool to identify candidate metabolic biomarkers for early detection of HCC cases in high risk population of cirrhotic patients.

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*Corresponding author: Habtom W Ressom, Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Suite 173, Building D, 4000 Reservoir Road NW, Washington DC 20057, USA. Phone: 202-687-2283, Fax: 202-687-0227, hwr@georgetown.edu.

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Keywords

Metabolomics; biomarkers; liquid chromatography-mass spectrometry; hepatocellular carcinoma; selected reaction monitoring

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common and one of the deadliest cancers. It has become a significant health issue with more than half a million people diagnosed with HCC per year. In particular, HCC has become the fastest-rising cause of cancer-related death in the United States [1] because of chronic alcohol use and chronic hepatitis C virus (HCV) infection [2]. About one third of HCV carriers develop liver cirrhosis. Patients with cirrhosis have an annual risk of 1–2% for developing HCC, with 80% of HCC inevitably developed from liver cirrhosis. The malignant conversion of cirrhosis to HCC is often fatal in part because adequate biomarkers are not available for diagnosis during the progression stage of HCC. Current approaches for diagnosis of HCC using liver imaging and measurement of serum alpha-fetoprotein (AFP) lack sufficient sensitivity and specificity. Determination of HCC from cirrhosis has become challenging because regenerative nodules may mimic tumors in cirrhotic livers and due to elevated serum levels of alpha-fetoprotein (AFP) in patients with cirrhosis. Therefore, there is an urgent need to identify more reliable and accurate tumor markers that would greatly improve the chances of detecting early stage HCC in high risk population of cirrhotic patients.

Metabolomics (synonymous term as “metabonomics”) is the comprehensive analysis of all metabolites in a biological system, e.g. the studies of changes in metabolic activities in response to pathophysiological stimuli or genetic modifications [3, 4]. It complements transcriptomics and proteomics as it provides a quantitative assessment of low molecular weight analytes (<1800Da). To achieve appropriate coverage of the metabolome, several different analytic platforms may be needed. Liquid chromatography coupled with mass spectrometry (LC-MS) is one of the commonly used analytical platforms for comprehensive analysis of metabolites at various levels of abundance in biological samples [5, 6]. LC-MS has shown promise in identifying metabolite-based biomarkers in various cancers studies [7–11]. LC MS signatures, alone or in combination with AFP levels, have been investigated for identification of early diagnosis biomarkers of HCC through metabolic profiling of serum, plasma, urine and fecal samples [12–18], where HCC has been successfully differentiated from patients with benign liver tumor and healthy controls. These metabolites are involved in a few key metabolic pathways, e.g. bile acid metabolism, phospholipid metabolism, fatty acids, glycolysis, urea cycle, and methionine metabolism. For example, bile acid and phospholipids metabolites were reported being altered in HCC compared to healthy individuals and patients with live cirrhosis [19]. Tan et al reported in a recent study that a small panel of marker metabolites (LPE 16:0, TCA, and LPC 22:5) can achieve up to 80% sensitivity and selectivity in discriminating HCC from pre-cancer cirrhosis and chronic hepatitis in a preclinical validation [18]. Also, it is reported that Glutathione (GSH) level and the ratios of GSH/GSSG (GSSG as GSH oxidized form) and GSH/total glutathione of the blood samples and liver tissues from HCC patients are significantly lower than those of the controls [20]. GSH works as antioxidant to protect cells from oxidative stress-induced apoptosis caused by reactive oxygen species (ROS), e.g., H₂O₂. However, discriminating early HCC from liver cirrhosis remains as the most challenging task in diagnosis of HCC partly due to lack of reliable and accurate biomarkers. Current metabolomic studies rely on global profiling using accurate mass measurement by mass spectrometers with high mass accuracy and high resolution such as quadrupole time-of-flight (QTOF), Orbitrap or Fourier-transform ion cyclotron resonance (FT-ICR). However, these platforms can only provide

semi-quantitative information on metabolic changes, in which matrix effects such as ion suppression or ion enhancement inevitably affect the accurate detection of metabolites in various abundance levels with diverse chemical properties. Although adequate sample preparation prior to LC separation can be one of the solutions to alleviate this problem, the most effective way to circumvent matrix effects is to combine isotope dilution technique with selected reaction monitoring (SRM) to establish a more reliable calibration curve for absolute quantitation, and to measure real concentration of metabolites by using stable isotope-labeled analogues as internal standards. Meanwhile, the high physical-chemical similarities between stable isotope-labeled analogues and target metabolites can also compensate the effects of degradation during sample preparation and variations in instrumental response [5, 6]. Only few of recent HCC metabolomic studies have reported the combination of ultra performance liquid chromatography (UPLC) coupled with QTOF MS and chemometric analysis for global profiling followed by SRM based absolute quantitation of metabolite candidates for target profiling [17]. We believe that characterizing the metabolic changes in high risk population of cirrhotic patients can help in early detection of HCC, a better understanding of the HCC mechanism at the molecular level as well as its treatment.

This study applies LC-MS based serum metabolomics for HCC biomarker discovery using non-targeted global profiling through a semi-quantitative method followed by absolute quantitation of targeted metabolites. Specifically, we use UPLC-QTOF MS for semi-quantitative analysis of serum samples from patients with HCC and cirrhosis. Putative identifications obtained by mass-based search are verified by comparing their MS/MS fragmentation patterns with those from authentic compounds. Absolute quantitation of candidate metabolic biomarkers is performed using isotope dilution based SRM on triple quadrupole linear ion trap (QqQLIT) and triple quadrupole (QqQ) mass spectrometers, each coupled to a UPLC. The results of this study illustrate the power of accurate mass measurement by UPLC-electrospray ionization (ESI)-QTOFMS, combined with the targeted quantitative analysis by UPLC-ESI-SRM-MS for a comprehensive serum metabolomic analysis to investigate changes in metabolite levels between HCC cases and patients with liver cirrhosis.

2. Study Population

The participants in this study comprised adult patients, who were prospectively recruited from the hepatology clinics at Georgetown University Hospital (GUH), Washington, DC. All patients provided informed consent and the study was approved by the Institutional Review Board at Georgetown University. All patients were diagnosed to have liver cirrhosis on the basis of established clinical, laboratory and/or imaging criteria. Cases were diagnosed to have HCC based on well-established diagnostic imaging criteria and/or histology. Controls were required to be HCC free for at least 6 months from the time of study entry. The characteristics of the patient population in this study are summarized in Table 1. Human blood was drawn from adult patients and collected by venepuncture into 10 mL BD Vacutainer sterile vacuum tubes without the presence of anticoagulant. The blood was immediately centrifuged at 1000 ×g for 10 min at room temperature. The serum supernatant was carefully collected and centrifuged at 2500 ×g for 10 min at room temperature. After aliquoting, serum was kept frozen at -80°C until use. Primary tubes and serum aliquots were labeled using anonymous confidential code numbers with no personal identifiers. Identification codes were cross-referenced with clinical information in a pass code protected computer system.

3. Materials and Methods

Chemicals and reagents

Ammonium acetate, Sphingosine-1-phosphate (S-1-P), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), glycodeoxycholic acid (GCA), Glycodeoxycholic-2,2,4,4-d 4 acid (D4 -GDCA), Glycodeoxycholic-2,2,4,4-d 4 acid (D4-GCA) were purchased from Sigma-Aldrich (St. Louis, MO). Glycochenodeoxycholic acid 3-sulfate (3 sulfo-GCDCA) was synthesized by PharmAgra Labs (Brevard, NC). Lysophosphatidylcholine (LysoPC 16:0), LysoPC 17:0, D₃₁-LysoPC 16:0 and D₇-S-1-P were purchased from Avanti Polar Lipids (Alabaster, AL). D₅-TCA was purchased from Toronto Research Chemicals (North York, ON). Acetonitrile (ACN), methanol, isopropanol, and chloroform were from Fisher Scientific (Pittsburg, PA). All other common chemicals of analytic grade were purchased from Sigma-Aldrich.

Sample preparation and UPLC-QTOF data acquisition for semi-quantitative metabolomic analysis

Frozen human serum was thawed at room temperature and 25 μL serum was mixed with 1.5 mL 66% ACN containing two internal standards (debrisquinone 1 $\mu\text{g mL}^{-1}$ for positive mode and nitrobenzoic acid 10 $\mu\text{g mL}^{-1}$ for negative mode), vortexed and incubated for 10 min on ice. Mixture was then spun at 10,000 g for 10 min at 4°C. Supernatant organic solution was transferred, speed-vacuum dried at room temperature and reconstituted in 50 μL of mobile phase (2% ACN containing 0.1% formic acid) ready for injection. From each sample, 5 μL was injected onto a reverse-phase 50 $\times$ 2.1 mm ACQUITY 1.7- μm C18 column (Waters Corp, Milford, MA) using an ACQUITY UPLC system (Waters) with a gradient mobile phase consisting of 2% ACN in water containing 0.1% formic acid (A) and 2% water in ACN containing 0.1% formic acid (B). Each sample was resolved for 10 min at a flow rate of 0.5 mL/min. The gradient consisted of 100% A for 0.5 min then a ramp of curve 6 to 60% B from 0.5 min to 4.0 min, then a ramp of curve 6 to 100% B from 4.0–8.0 min, a hold at 100% B until 9.0 min, then a ramp of curve 6 to 100% A from 9.0 min to 9.2 min, followed by a hold at 100% A until 10 min. The column eluent was introduced directly into the mass spectrometer by electrospray. Mass spectrometric analysis was performed on a Q-TOF Premier (Waters) operating in both positive and negative mode. The capillary voltage was set to 3.2 kV and 3 kV, and the cone voltage of 30V and 20 V, respectively. The desolvation gas flow was set to 800 Lhr⁻¹ and the temperature was set to 350°C. The cone gas flow was 25 Lhr⁻¹, and the source temperature was 120°C. Accurate mass was maintained by introduction of LockSpray interface of sulfadimethoxine (311.0814 [M+H]⁺ or 309.0658 [M-H]⁻) at a concentration of 250 pg μL^{-1} in 50% aqueous ACN and a rate of 150 $\mu\text{L min}^{-1}$. UPLC-QTOF data were acquired in centroid mode from 50 to 850 mass-to-charge ratio (m/z) in MS scanning using the MassLynx software (Waters).

Sample preparation and UPLC-QqQLIT data acquisition for absolute quantitation of bile acid metabolites and sphingosine-3-phosphate

Frozen human serum was thawed at room temperature. 25 μL serum was spiked with 1 pmol of isotope labeled internal standards, extracted by 1.5 mL 66% ACN, vortexed and incubated for 10 min on ice. Mixture was then spun at 10,000 g for 10 min at 4°C. Supernatant organic solution was transferred, speed-vacuum dried at room temperature and reconstituted in 50 μL of mobile phase (50% methanol solution containing 0.1% NH₄OH and 10 mM NH₄OAC) ready for injection. From each sample, 10 μL was injected onto a reverse-phase 50 $\times$ 2.1 mm ACQUITY 1.7- μm C18 column (Waters) using an ACQUITY UPLC system (Waters) with a gradient mobile phase consisting of 50% methanol in water containing 0.1% NH₄OH and 10 mM NH₄OAC (A) and methanol containing 0.1% NH₄OH and 10 mM NH₄OAC (B). The whole run lasted 10 minutes at a flow rate of 0.3 mL/min.

The gradient consisted of 99% A for 1.0 min then a ramp to 60% B from 1.0 min to 6.0 min, then ramp to 100% B from 6.0–6.1 min, hold at 100% B until 8.0 min, then ramp to 100% A from 8.0 min to 8.1 min, followed by a hold at 100% A until 10 min. The column temperature was set to 50°C. UPLC-QqQLIT data were collected in the SRM mode on QTRAP 4000 (AB Sciex, Foster City, CA) operating in the negative mode with a capillary voltage of –4500 V and source temperature of 500°C. The curtain gas flow was set to 20, Sheath gas flow GS1 was set to 40.00, sheath gas GS2 was set 20.00 and exit potential was –10 V.

Sample preparation and UPLC-QqQ data acquisition for absolute quantitation of lipids

sample preparation and extraction follow the same protocol as mentioned above except that the spiked lipid isotope labeled internal standard was D₃₁-LysoPC 16:0. After serum methanol extract was speed-vacuum dried, it was reconstituted in 200 µL of ACN: chloroform: methanol=90:5:5 ready for injection. From each sample, 5 µL was injected onto a reverse-phase 50 × 2.1 mm ACQUITY 1.7-µm CSH C18 column using an ACQUITY UPLC system with a gradient mobile phase consisting of 40% ACN in water containing 0.1% formic acid and 10 mM NH₄OAC (A) and 90% isopropanol in ACN containing 0.1% formic acid and 10 mM NH₄OAC (B). The whole run lasted 10 minutes at a flow rate of 0.4 mL/min. The gradient consisted of 70% A for 1.0 min then a ramp to 50% B from 1.0 min to 5.0 min, then ramp to 99% B from 5.0–6 min, hold at 100% B until 8.0 min, then ramp to 100% A from 8.0 min to 8.1 min, followed by a hold at 70% A until 10 min. The column temperature was set to 50°C. UPLC-QqQ data were collected in the SRM mode on QqQ (Waters XEVO TQ) operating in the positive mode with a capillary voltage of 3 KV, desolvation gas flow 800 Lhr⁻¹, and desolvation gas temperature of 350°C. The cone gas flow was 25 Lhr⁻¹ and the source temperature was 120°C.

UPLC-QTOF datasets, data analysis methods, and software tools

We generated UPLC-QTOF MS data from sera of 78 HCC cases and 184 cirrhotic controls in three separate experiments (Exp. 1, Exp. 2, and Exp. 3). The three experiments were conducted in May 2010, July 2010, and March 2011, respectively. Each serum sample was run in duplicate in both positive and negative modes. In addition to these serum samples, we analyzed other samples together for quality control purpose. These quality control samples include sera from five healthy volunteers and aliquots of serum pooled from a subset of the HCC and cirrhotic patients. The raw data were converted into Network Common Data Form (NetCDF) format using the MassLynx software (Waters). We used the XCMS package (Scripps Center for Metabolomics, La Jolla, CA) to preprocess each of the three UPLC-QTOF MS datasets separately. The first step in XCMS is to detect the peaks. The peak detection algorithm first cuts the UPLC-QTOF data into slices, a fraction of a mass unit wide, and then applies a model peak matched filter on those individual slices over the chromatographic time domain. After detecting peaks in individual samples, the peaks are matched across samples to allow calculation of retention time (RT) deviations and relative ion intensity comparison. This is accomplished using a grouping method that uses kernel density estimation to group peaks in the mass domain. The peak matching algorithm in XCMS takes into account the two-dimensional anisotropic nature of data. These groups are then used to identify and correct drifts in RT from run to run.

Following data preprocessing, ion annotation is conducted to identify derivative ions originating from the same compound in the forms of adducts, isotopes, and in-source fragments. Adducts of a molecule are formed during the ionization process in ESI, e.g. sodium and ammonium adduct. Ions of molecular isotopes are detected with distinct m/z values during MS analysis and the peak with the lowest m/z is defined as the monoisotopic peak. In-source fragments are formed during ionization such as ion fragments of [M+H-

$\text{H}_2\text{O}]^+$ or $[\text{M}-\text{H}-\text{H}_2\text{O}]^-$ through neutral loss of water molecule. Different adducts/isotopes/water-loss products of the same compound theoretically share the same retention time in chromatograms. As long as the scan rate is properly adjusted and enough scanning points are acquired to define the chromatographic peaks, the ions from the same compound share similar-shaped elution profiles which can be represented by their extracted ion chromatograms (EICs). Thus, ion annotation can be performed by clustering similar elution profiles together. Recognition of such metabolites, often represented by multiple peaks with distinct m/z values at similar retention times can facilitate metabolite identification in LC-MS based metabolomics. We used the R-package CAMERA [21] for ion annotation.

For difference detection, in-house developed MATLAB (Natick, MA) and R scripts were used on the basis of parametric and non-parametric statistical tests (t-test and the Wilcoxon rank-sum test). In both statistical tests, we calculated q-values using the Storey method to control the false discovery rate (FDR) [22]. To select ions with significant and consistent changes between HCC and cirrhotic patients in multiple experiments, we used an ion annotation assisted approach depicted in Figure 1. This approach is designed to determine overlapping ions among multiple experiments by taking into consideration the presence of a large number of derivative ions such as isotopes, adducts, and fragments. This is accomplished by using a combination of two ion annotation-assisted methods (M_1 and M_2). M_1 searches for common ion masses shared by the three experiments and it uses the ion annotation to create a list of monoisotopic masses. This helps the selection of ions with the same mass and retention time that may have been missed, if annotated as different monoisotopes when ion annotation is performed separately for each experiment. In M_2 , separate ion annotation is utilized to group the list of ion masses obtained from each experiment. This facilitates the comparison of ions selected across multiple experiments by allowing us to compare their monoisotopic mass instead of their individual masses. The monoisotopic masses from M_1 and M_2 are merged prior to mass-based identification. A combination of these two methods leads to an inclusive list of ions where ion annotation is performed prior or after comparing ions from the three experiments. Thus, the ion annotation-assisted approach helps eliminate wrong identifications by clustering together ions that share the same monoisotopic masses. Also, it helps capture overlapping monoisotopic ion masses that may be missed by the traditional method due to the lack of recognition of derivative ions shared by multiple experiments.

Putative identifications for the resulting ion list were obtained through mass-based search against four databases: the Human Metabolite DataBase (HMDB) [23], Metlin [24], Madison Metabolomics Consortium Database (MMCD) [25], and LIPID MAPS [26]. The mass tolerance in the database search was set to 10 ppm. The m/z values of annotated isotopes/adducts/in-source fragments peaks were converted to the corresponding neutral mono-isotopic masses before searching them against the databases. Identities of a subset of the putative metabolite identifications were verified by comparing their MS/MS fragmentation patterns and RT with those of authentic standard compounds. Absolute quantitation of metabolite candidates was performed using isotope dilution technique by UPLC-SRM-MS analysis on the QTRAP 4000 and XEVO TQ instruments.

3. Results

Data preprocessing

Data matrices of ion intensities were obtained by preprocessing each of the three UPLC-QTOF MS datasets separately. In Exp. 1, Exp. 2, and Exp. 3, we detected 1587, 3231, and 613 ions in the positive mode, respectively. In the negative mode, 942, 1210, and 392 ions were detected in Exp. 1, Exp. 2, and Exp. 3, respectively.

Ion annotation

Using CAMERA software, we annotated ions detected in the three data matrices in two steps. In the first step, the detected ions with similar RT are roughly grouped together using a sliding RT window. In the second step, within each group, the ions are clustered into smaller groups on the basis of the correlation between their extracted ion chromatograms (EICs). The m/z difference between each ion pair within a group is calculated and compared to known m/z relationships between various ion formations. Two ions are considered to come from the same metabolite if their m/z difference can be explained by one of the known m/z relationships.

Difference detection

The six data matrices obtained after data preprocessing were used to select ions with significant differences between HCC cases and cirrhotic controls. Each matrix was analyzed separately using parametric and non-parametric statistical methods (t-test and the Wilcoxon rank-sum test) to select the most significant ions. Ions with q -values < 0.1 by either t-test or the Wilcoxon rank-sum test were selected.

Ion annotation-assisted analysis of overlapping ions

We compared the ions selected by each experiment to identify those selected by more than one experiment. This is accomplished by using a combination of two ion annotation-assisted methods (M_1 and M_2) (Figure 1). The monoisotopic molecular mass is used to compare the ions across the three experiments. In searching for common ions, we used a tolerance of 15 seconds for RT and a tolerance of 10 ppm for m/z . Also, we required overlapping ions to have the same fold change direction across the three experiments. We found 40 monoisotopic ions (27 in the positive mode and 13 in the negative mode) overlapping in at least two out of three experiments, of which two monoisotopic ions overlap in all three experiments. There were three monoisotopic masses whose putative identifications were repeated, indicating that these metabolites were selected in both positive and negative ionization modes. Thus, 37 unique monoisotopic ions were found in total, of which putative identifications were found for 23 ions by searching against mass-based databases. Table 2 presents the number of overlapping monoisotopic masses and the number of ions with putative identifications.

Metabolite ID verification

From the list of significant monoisotopic ions with putative identifications, we verified seven metabolites by comparing their TOF MS/MS spectra against their corresponding synthetic compounds. Specifically, we ran an authentic compound for each candidate and a patient sample side by side and compared the fragmentation patterns and their LC retention time. Although 3-sulfo-GCDCA and GCA were found significant ($q < 0.1$) in only one out of the three experiments, we verified their identity and included them in the subsequent targeted metabolite quantitation. The reason for including these two metabolites is the presence of other related metabolites or fragments (e.g., GCDCA, GDCA, TCA, and fragment of GCA) that were statistically significant in at least two out of the three experiments. Figure 2 depicts the fragmentation patterns for bile acid metabolites 3-sulfo-GCDCA, TCA, S-1-P and LysoPC 16:0. Table 3 presents average peak intensities within the HCC and cirrhosis groups for candidate metabolites selected in this study. These include nine verified metabolites, two fragments of verified metabolites, and six putative identifications. The metabolites are categorized as several classes of compounds according to the chemical taxonomy: glycerophospholipid, sphingophospholipids and cholesterol metabolites. Most of the metabolites verified in this study represent cholesterol metabolism, phospholipid catabolism and sphingolipid metabolism pathway. Verification of the identities

of other putative identifications in Table 3 and in the supplementary table is underway. The supplementary table presents a list of verified metabolites, putative identification, and unidentified peaks along with their average peak intensities and standard deviations within the HCC and cirrhosis groups and their statistical significance in distinguishing HCC cases from cirrhotic controls. The raw LC-MS data are deposited in MetaboLights (Accession # MTBLS17) along with all peaks detected, fold change values, statistical analysis results, and a list of verified metabolites.

Metabolite quantitation

Following ID verification, candidate metabolites were quantified using isotope dilution by UPLC-SRM-MS, where a stable isotope analogue of the analyte compound is used as an internal standard (IS). Because of the high physiochemical similarities between the labeled IS and the analyte, degradation during sample preparation, variations in instrumental response, and ion suppression effects in LC-ESI-MS can be compensated. Absolute quantitation of metabolites can be achieved by correlating the ratio of the peak area of the analyte detected by SRM to that of the corresponding isotope-labeled IS. We spiked serum sample with IS of D₄-GCA, D₄-GDCA, D₅-TCA, D₃₁-LysoPC 16:0 and D₇-S-1-P for absolute quantification of GCA, GDCA, TCA, TCDCA, LysoPC 16:0, LysoPC 17:0, LysoPC 15:0, LysoPC 22:6 and S-1-P. Two SRM transitions were selected for each compound for both quantitation and ID confirmation. We ran each standard solution twice for constructing the calibration plots of each analyte prior to quantitation of the analytes in the serum samples. Blank controls were tested after the injections of high concentrations of standards (> 10 pmol on column) to monitor the carry over. Also, they were run after the injection of every four serum samples to remove the contaminants buildup in the system. Figure 3 shows the detection of GCA in serum and its IS (1pmol on column) by SRM under one transition. Through linear regression analysis, calibration plots were constructed for each analyte. Two calibration plots are shown in Figure 4 as examples. The UPLC-SRM-MS analysis is fast (lasts 10 minutes) and is sensitive in detection with limit of detection of 2 fmol for each analyte. The dynamic range is four orders of magnitude and has a linearity of 0.999 in correlation coefficients. Quantitation of these metabolites in serum samples of 20 patients (10 samples from the HCC group and 10 samples from the cirrhosis group) confirmed the preliminary discovery results we previously obtained using UPLC-QTOF, in terms of their fold change directions. These samples were randomly selected and the sample size was adequate to have appropriate statistical power to detect the differences observed in UPC-QTOF. Figure 5 presents dot plots for six of these metabolites that exhibit statistically significant or marginally significant differences between HCC and cirrhosis. As shown in the figure, GCA, GDCA, TCA, and TCDCA are significantly down-regulated in sera of HCC vs. those with cirrhosis, while LysoPC 17:0 and S-1-P show a marginally significant up-regulation.

4. Discussion

We analyzed 262 serum samples from patients with HCC and cirrhosis in three experiments using UPLC-QTOF-MS under positive and negative detection modes. The data were first preprocessed to detect peaks, match the peaks, and correct RT drifts. The most relevant ions in distinguishing HCC cases from patients with cirrhosis were selected by parametric and non-parametric statistical methods. Specifically, an ion-annotation-assisted approach is used to identify ions with significant and consistent changes between HCC and cirrhosis in three experiments. This approach was designed to determine overlaps among the three experiments by considering the presence of a large number of derivative ions such as isotopes, adducts, and in-source fragments. We found 37 unique monoisotopic ions with statistically significant difference in metabolite levels between HCC and cirrhotic controls in at least two out of three experiments. Putative identifications for these ions were obtained by

searching the selected significant monoisotopic ion masses against mass-based databases. A subset of these putative identifications was verified. The verified metabolites are involved in primary bile acid biosynthesis and glycerophospholipid metabolism. It has been known that the abnormal levels of bile acid and lysophospholipid are associated with liver cirrhosis and hepatitis [13, 14, 16]. In our study population, 82% HCC patients and 50% cirrhotic patients have hepatitis (HBV and HCV). Previous study reported the up-regulation of bile acid of GCDCA (or GDCA), GCA, TCA, CDCA and down-regulation of LPC (18:0), LPC (18:1), LPC (16:0) in serum of patients with hepatitis B versus healthy controls [13]. Another study also reported the down regulation of bile acid and up regulation of LPCs in HCC versus liver cirrhosis [14], which is consistent with the findings of our study. GDCA, GCA, 3 sulfo-GCDCA, TCA and TCDCA are conjugated bile acids that enable absorption of lipids, cholesterol, and fat-soluble vitamin. The sulfation of lipophilic secondary bile acids, such as GDCA was reported to play a key role in the detoxification of endogenous and exogenous lipophilic compounds [27]. In addition, decreased bile acid in serum has been associated with the accumulation of toxic and even carcinogenic bile acid in liver that may be caused by the alteration of the bile acid transport pathway [28]. Prevention of the synthesis of bile acids can cause the accumulation of cholesterol and liver dysfunction (cholestasis) [29]. This pathway is associated with cholestatic liver disease. There are also indications from in vitro experiments that bile acids stimulate growth of adenoma cells and invasion of carcinoma cells [30]. At high physiologic concentrations, bile acids can cause oxidative/nitrosative stress, DNA damage, apoptosis, and mutation. Evidence show that bile acids play an important role in carcinogenesis in different organs of the gastrointestinal tract [31]. GCDCA has been reported as an inducer of apoptosis and necrotic cell death in human hepatocyte [32]. Dysfunction of bile acid biosynthesis might lead to liver cancer development and progression [14, 33]. It is yet to be investigated whether there is a causal effect between the down-regulation of bile acid in sera and HCC development.

Recent metabolic profiling studies indicated the marked difference of liver lipid profiles during liver disease process such as alcoholic induced liver damage [34]. Recent study by Patterson et al. showed that HCC is associated with dysregulation of LPC in the plasma compared to liver cirrhosis and healthy controls [17]. It is known that LPCs regulate a variety of biological processes including cell proliferation, tumor cell invasion, and inflammation. Both LPC and PC have been reported as markers of diseases such as Parkinson's disease, glomerular inflammation, and Alzheimer's disease. The up-regulation of lysophosphatidylethanolamine (LPE) in our study can be explained by the fact that phospholipids take part in the metabolism of lipids. The balance of phospholipids constituting cellular membrane were kept by enzymes, such as PE N-methyltransferase 2 (PEMT2) that catalyzes the conversion of PE to PC in the liver when dietary choline supply is inadequate. However, the expression of PEMT2 mRNA has been reported to be reduced or absent in HCC and to potentially cause the accumulation of PE or LPE, partly accounting for the high level of circulation PE or LPE [18, 35].

Metabolism of sphingolipids was reported to have potential relation to mucosal protection, inflammation and carcinogenesis [36, 37]. Phytosphingosine and dihydrosphingosine have been reported in a previous metabolomic study to be down-regulated in HCC compared to patients with cirrhosis [14]. For the first time, we confirmed that S-1-P, the conversion product of these two compounds, is up-regulated in HCC vs. cirrhosis. S-1-P has been well-recognized as a potent messenger molecule involved in regulating cell growth, and apoptosis [38] and an immune modulator for regulating lymphocyte flow from the thymus, bone marrow to blood and peripheral tissue involved in development and progression of cancer [14]. However, because blood serum or plasma is a metabolite pool derived from the organs and tissues of the human body, it can be influenced by many exogenous and endogenous factors.

Following verification of their identities, we chose several metabolites for absolute quantitation by UPLC- SRM-MS using isotope dilution technique. Quantitation of these metabolites in 20 serum samples (10 HCC and 10 cirrhotic controls) confirmed that S-1-P and LysoPC 17:0 are up-regulated in sera of HCC vs. those with cirrhosis, while GCA, GDCA, TCA, and TCDCA are down-regulated. While some of these metabolites have been reported previously, recent work by Wang et al. has shown the discovery of down-regulation of GCDCA and its fragments (m/z 414.3) in serum of patients with HCC, and up-regulations of LPC (16:0), LPC (18:0) and other PCs [19], supporting our previous interpretation. However, Wang et al. did not further confirm their results through absolute quantitation of the above metabolites. Also, to our knowledge, this study reports for the first time 3-sulfo-GCDCA, S-1-P, and LysoPC 17:0 as potential biomarkers of HCC. Since metabolites are regulated by diverse intrinsic or extrinsic factors, it is important to examine the specificity of metabolic markers. One possible approach to achieve higher specificity is to resort to the combined use of AFP and metabolic markers.

5. Conclusion

In this study, we characterized the metabolic changes pertaining to HCC in patients with cirrhosis using both semi-quantitative and absolute quantitative approaches by UPLC-MS/MS. The candidate biomarkers we identified in this study include: (1) Bile acid related and liver-specific metabolites down-regulated in HCC vs. cirrhosis; and (2) Metabolites such as S-1-P and LysoPC 17:0 up-regulated in HCC vs. cirrhosis. The accumulation of conjugated bile acids in cirrhosis or chronic hepatitis could be an indicator of liver dysfunction. Up-regulation of S-1-P and LysoPC 17:0 could be attributed to the development and progression of cancer or tumor cell invasiveness and inflammation. Since these metabolites are not specific to liver, they could be derived from cancer tissues, liver, muscle, immune systems and other tissues [14]. Overall, the results obtained in this study provide useful insights into HCC biomarker discovery utilizing LC-MS-based metabolomics, which provides an efficient and cost-effective platform.

Since the three UPLC-QTOF-based untargeted metabolomic experiments presented in this paper were conducted at different times, the average absolute peak intensities of the same analyte within the HCC and cirrhosis groups varies significantly across the three experiments, as shown in Table 3. This is due to differences in sample preparation and experimental settings among the experiments. Reliable normalization and alignment methods are needed in order to combine the data from the three experiments. In our study, we chose to combine the results from these experiments after statistical analysis on the basis of significant differences of peak intensities within the individual experiments. Specifically, we first identified ions with statistically significant difference between HCC cases and cirrhotic controls in individual experiments. From these ions, we selected those that showed consistent pattern across the experiments in terms of up-regulation and down-regulation. Although the absolute peak intensities measured by the UPLC-QTOF cannot be readily combined across experiments without proven normalization and alignment methods, we strongly believe that the consistency in terms of statistical significance of analytes across experiments is a valuable alternative method to compare diverse experiments.

Given the limited cases of cirrhotic HCC at present, further studies with more samples are required to confirm these findings. The effect sizes for the metabolites selected in our study follow a mixture distribution, with about 80% of the effects centered around 2.7 and the remaining 20% centered around 9.8. We estimate that similar effect sizes can be detected in future experiments designed to compare HCC and cirrhosis patients with reasonably large sample sizes. Based on the peak intensity variability observed in our data, when 90% of the overall peaks arise from the null distribution and the FDR is controlled at a 0.05 level, a

power of 90% can be reached with n=57 samples in each group. In addition, some follow-up studies on the same patients are important to understand the process of pathological development. Our future studies will focus on: 1) investigating reliable normalization and alignment methods that would allow us to gain statistical power by combining LC-MS data from multiple experiments; 2) comparing the bile acid metabolites in sera of patients with early stage vs. end stage of cirrhosis and HCC to investigate their regulation pattern in relation to the progression of liver disease; 3) performing absolute quantitation of candidate biomarkers including PE and LPE to confirm the results from our discovery study; and 4) conducting statistical analysis following stratification of the samples into homogeneous and biologically meaningful groups on the basis of etiology and disease stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

We analyzed sera from HCC and cirrhotic patients by LC-MS in three experiments.

Metabolites with significant and consistent changes in HCC vs. cirrhosis were selected.

Verification of the identities of selected metabolites was performed by MS/MS.

Quantitation of candidate metabolites was conducted using isotope dilution by SRM.

Figure 1.

Ion-annotation-assisted approach for analysis of UPLC-QTOF MS data from three experiments. M_1 : Overlapping significant ions selected based on ion masses without the ion annotation information. M_2 : Overlapping significant ions selected on the basis of monoisotopic masses obtained from ion annotation information.

Figure 2.

Verification of metabolite IDs. A1, B1, C1, and D1 are MS/MS spectra of authentic compounds TCA, 3 sulfo-GCDCA, LysoPC 16:0, and S-1-P, respectively. A2, B2, C2, and D2 are MS/MS spectra of the ions (m/z values 514.3, 528.3, 496.3, and 378.3, respectively) obtained in serum samples.

Figure 3.
Detection of GCA in patient sample and its IS (1pmol on C18 column) by UPLC-SRM-MS.

Figure 4. Calibration plots of GCA (top) and S-1-P (bottom) for absolute quantification. Each standard solution was spiked with 1pmol of isotope labeled internal standards.

Figure 5. Quantitation of candidate metabolites in 20 serum samples (10 HCC, 10 cirrhosis) by UPLC-SRM-MS using isotope dilution technique.

Table 1

Characteristics of the study population.

	HCC (N=78)	Cirrhosis (N=184)
Age mean (SD)	58.8 (7.8)	54.0 (10.3)
Male (%)	84%	57%
Ethnicity (%)		
Caucasian	53%	52%
African American	24%	36%
Hispanic	5%	5%
Asian	18%	5%
Other (American Indian)	0%	1%
Etiology (%)		
HCV	67%	47%
HBV	15%	3%
Alcoholism	29%	34%
NASH	13%	9%
Cryptogenic	8%	5%
Autoimmune	3%	7%
PBC	0%	5%
PSC	0%	9%
Other	0%	4%
Stage (%)		
Stage I	44%	
Stage II	27%	
Stage III	14%	
Stage IV	3%	
MELD mean (SD)	11.0 (4.0)	15.1 (5.9)
MELD 10	46%	23%
AFP mean (SD)	932.4 ngmL ⁻¹ (5395)	17.5 ngmL ⁻¹ (64.8)

Note: The MELD scores are missing for two HCC and three cirrhosis cases. AFP measurements are unavailable for one HCC and three cirrhotic patients. For one cirrhotic patient, the ethnicity and etiology information are missing. Tumor stage information is unavailable for 10 HCC cases.

Table 2

Significant monoisotopic ions in at least two out of three (2/3) experiments and those found significant in all three (3/3) experiments. The number of ions with corresponding putative identifications is shown in parenthesis.

Mode	M ₁		M ₂		Selected Ions		
	2/3	3/3	2/3	3/3	2/3	2/3	3/3
Positive	27 (17)	1 (1)	16 (12)	2 (2)	27 (17)	2 (2)	2 (2)
Negative	12 (8)	0	13 (9)	0	13 (9)	0	0
Total	36 (22)	1 (1)	26 (18)	2 (2)	37 (23)	2 (2)	2 (2)

Table 3

Average peak intensities within the HCC and cirrhosis groups for candidate metabolites selected in this study.

Verified Metabolites	Biological Pathway	m/z	RT (s)	Mode	Exp 1			Exp 2			Exp 3		
					HCC Mean (SD)	Cirrhosis Mean (SD)	HCC Mean (SD)	Cirrhosis Mean (SD)	HCC Mean (SD)	Cirrhosis Mean (SD)	HCC Mean (SD)	Cirrhosis Mean (SD)	
Fragment of GDCA	cholesterol metabolism	414.300	227.2	POS	293 (111)	398 (108)	-	-	132 (51)	-	2642 (92)	-	
Fragment of GCA *	cholesterol metabolism	432.308	200.9	POS	2849 (189)	9150 (202)	-	-	-	-	-	-	
GDCA	cholesterol metabolism	448.306	227.0	NEG	50346 (33926)	56887 (29862)	-	-	629 (394)	-	9701 (8772)	-	
GDCA	cholesterol metabolism	450.321	227.0	POS	11461 (918)	16066 (829)	10188 (153)	22995 (192)	364 (12)	-	7003 (331)	-	
TDCA	cholesterol metabolism	516.300	184.9	POS	-	-	645 (521)	1756 (569)	89 (75)	-	967 (86)	-	
3-sulfo-GCDCA *	cholesterol metabolism	528.264	202.6	NEG	3716 (4183)	7056 (14509)	-	-	-	-	-	-	
GCA *	cholesterol metabolism	464.302	201.8	NEG	-	-	-	-	103 (77)	-	1475 (1087)	-	
TDCA	cholesterol metabolism	498.290	205.9	NEG	-	-	35724 (231)	98303 (232)	1165 (1270)	-	17259 (20014)	-	
TDCA	cholesterol metabolism	500.306	206.9	POS	-	-	121 (152)	420 (136)	91 (22)	-	1106 (398)	-	
S-P	sphingolipid metabolism	378.241	261.4	NEG	3042 (1370)	2239 (1443)	-	-	182 (18)	-	104 (42)	-	
S-P	sphingolipid metabolism	380.256	261.4	POS	511 (354)	428 (234)	-	-	275 (74)	-	154 (66)	-	
LysoPC (16:0)	phospholipid catabolism	496.340	298.9	POS	230263 (160)	197037	-	-	44164 (64)	-	21684 (13)	-	
LysoOC (17:0)	phospholipid catabolism	510.357	318.2	POS	4823 (195)	3762 (169)	-	-	763 (23)	-	234 (31)	-	
LysoPC (18:0)	phospholipid catabolism	524.371	338.6	POS	4150 (734)	3588 (523)	-	-	17893 (584)	-	8801 (464)	-	
Unverified Metabolites													
LysoPC (15:0)	phospholipid catabolism	482.324	280.5	POS	2608 (440)	2176 (448)	-	-	463 (30)	-	297 (22)	-	
LysoPE (22:6)	phospholipid catabolism	524.279	281.4	NEG	3128 (2260)	1967 (1691)	-	-	257 (135)	-	40 (42)	-	
LysoPE (20:4)	phospholipid catabolism	500.279	282.7	NEG	3101 (1416)	2397 (1307)	-	-	251 (71)	-	95 (70)	-	
LysoPE (20:3)	phospholipid catabolism	504.310	282.3	POS	372 (678)	298 (484)	-	-	540 (7)	-	88 (5)	-	
LysoPC (22:6)	phospholipid catabolism	568.341	283.2	POS	3693 (315)	2593 (222)	-	-	460 (10)	-	114 (16)	-	
PS (O-18:0/0:0)	phospholipid catabolism	512.335	271.3	POS	1255 (208)	1032 (166)	-	-	122 (10)	-	77 (134)	-	

Note: 3 sulfo-GCDCA and GCA (designated by *) were found significant ($q < 0.1$) in one out of the three experiments.

Average intensities are left blank (-) for those that did not reach the desired statistical significance level ($q < 0.1$).