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Aberrant Lipid Metabolism in Hepatocellular Carcinoma Revealed by Plasma Metabolomics and Lipid Profiling

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Abstract

There has been limited analysis of the effects of hepatocellular carcinoma (HCC) on liver metabolism and circulating endogenous metabolites. Here we report the findings of a plasma metabolomic investigation of HCC patients using ultraperformance liquid chromatography-electrospray ionization-quadrupole mass spectrometry (UPLC-ESI-QTOFMS), random forests machine learning algorithm and multivariate data analysis. Control subjects included healthy individuals as well as patients with liver cirrhosis or acute myeloid leukemia. We found that HCC was associated with increased plasma levels of glycodeoxycholate, deoxycholate 3-sulfate and bilirubin. Accurate mass measurement also indicated upregulation of biliverdin and the fetal bile acids 7 α -hydroxy-3-oxochol-4-en-24-oic acid and 3-oxochol-4,6-dien-24-oic acid in HCC patients. A quantitative lipid profiling of patient plasma was also performed using ultraperformance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UPLC-ESI-TQMS). Using this method we found that HCC was associated also with reduced levels of lysophosphocholines (LPC) and in 4/20 patients with increased levels of lysophosphatidic acid (LPA(16:0)), where it correlated with plasma α -fetoprotein levels. Interestingly, when fatty acids were quantitatively profiled by gas chromatography-mass spectrometry (GCMS), we found that lignoceric acid (24:0) and nervonic acid (24:1) were virtually absent from HCC plasma. Overall, this investigation illustrates the power of the new discovery technologies represented in the UPLC-ESI-QTOFMS platform combined with the targeted, quantitative platforms of UPLC-ESI-TQMS and GCMS for conducting metabolomic investigations that can engender new insights into cancer pathobiology.

Keywords

metabolomics; hepatocellular carcinoma; LPC; LPA; VLCFA

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Introduction

Hepatocellular carcinoma (HCC) is the fifth commonest and one of the deadliest cancers, with about 50% of cases occurring in China (1–3). The advent of HCC is accompanied by metabolic changes in the liver that may be reflected in changes in gene expression, microRNA (miRNA) profiles, together with altered circulating protein and small metabolite concentrations. A considerable number of gene expression studies have been undertaken (4–10) targeted at understanding the progression of HCC, in particular, in relation to hepatitis B and C virus infection (HBV, HCV) and also mouse models of HCC. There is virtually no mention in these studies of genes involved in metabolism of small (<1 kDa) cellular metabolites.

Metabolomics involves the global and unbiased definition of the complement of small molecules in a biofluid, tissue, organ or organism (11–14). To accomplish the goals of a metabolomic study, several different analytical platforms may be required to achieve maximum coverage of the metabolome, and these might include ultraperformance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) directly on urine or plasma, and gas chromatography-mass spectrometry (GCMS) after extraction of analytes and chemical derivatization. Each platform can produce complementary findings on the same sample sets (15, 16). Molecules bearing formal charges, such as phospholipids cannot be analyzed directly by GCMS, but UPLC-ESI-QTOFMS provides an ideal solution. Conversely, neutral lipids, such as free fatty acids and their glycerol and cholesterol esters are ideally suited to GCMS analysis after appropriate chemical derivatization. Metabolomic analyses can produce data matrices with millions of individual data points, even with modest numbers of samples. In order to separate two classes, such as HCC and controls, multivariate data analysis is employed with unsupervised principal components analysis (PCA) and supervised projection to latent structures-discriminant analysis (PLS-DA) and random forests machine learning algorithm (RF) (17). These methodologies have been used noninvasively to understand the global impact on metabolism in a mouse model of alcohol-induced liver disease (18).

A small number of metabolomic studies on HCC have been reported to date (19–21) and these have been restricted to Chinese patients. Here, we describe a metabolomic study of HCC using UPLC-ESI-QTOFMS combined with chemometric analysis and enhanced by lipid profiling using ultraperformance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UPLC-ESI-TQMS) for quantitation of lysophosphocholines and GCMS for determination of free and esterified fatty acids. A number of both up- and down-regulated molecules of interest are described for HCC that lead to enhanced understanding of the pathobiology of the disease.

Materials and Methods

Patients

A summary of the study groups is displayed in Table 1. Hepatocellular carcinoma (HCC) patients (n=20) were those who had been selected for transarterial chemoembolization and, with one exception, were all cirrhotics with confirmed HCC according to EASL criteria (22). They comprised seventeen males and three females with a median age of 61 (mean $\pm$ S.D. 61.1 $\pm$ 11.2) years. Six had alcohol related liver disease, three had hepatitis B virus (HBV) related disease, five had hepatitis C virus (HCV) related hepatitis, three had nonalcoholic steatohepatitis (NASH), one has NASH/alcoholic steatohepatitis (ASH), and two had hereditary hemochromatosis. The Barcelona-Clinic Liver Cancer (BCLC) staging classifications (23) were three stage A, eleven stage B, five stage C, and one stage D. The Child-Pugh scores were 5 to 6 for 16 patients, with two of 7, one of 11, and one was

noncirrhotic. Fifteen patients had ECOG scores of 0, and three of 1; two patients were not scored. They had a median number of nodules of 3 [1–6] with a mean diameter of the largest nodule of 4.7 ± 3.0 cm. Bilirubin levels were 30.6 ± 29.1 [9–120] $\mu\text{mol/l}$, trough albumin levels were 34.2 ± 4.4 [24–42] g/l, peak INR values were 1.20 ± 0.13 [1–1.54], AST of 80.2 ± 56.5 [25–237], ALT of 52.4 ± 34.1 [17–152], alkaline phosphatase of 110 ± 48 [49–233], γ -glutamyltranspeptidase of 260 ± 330 [27–1504] and median AFP concentration was 43.3 [1.3–67,725] ng/ml. Only ten HCC patients had AFP values above a threshold of 50 ng/ml.

As the first control group, patients with clinically (based on decompensation) or histologically proven liver cirrhosis (LC, $n=7$), enrolled in surveillance program for HCC and with a negative diagnosis for HCC, were enrolled. They comprised three males and four females with a median age of 48 [50.3 ± 5.7] years. Six had a Child-Pugh score of 5 and one a score of 10, similar to the HCC patient group. Their median AFP concentration was 4.0 [1.4–7.5] and all were well under the threshold of 50 ng/ml.

As a second control group, patients diagnosed with acute myelogenous leukemia (AML, $n=22$) were enrolled, representing another cancer group but without hepatic involvement. They comprised 9 males and 13 females and had a median age of 62 [57.2 ± 13.1] years. Their liver function tests were generally within normal ranges, as follows: bilirubin (10.1 ± 6.5 $\mu\text{mol/l}$), AST (37.2 ± 19.9 U/l), ALT (48.0 ± 38.0 U/l), alkaline phosphatase (161 ± 175 U/l), γ -glutamyltranspeptidase (208 ± 291 U/l).

Finally, a third control group of male healthy volunteers (HV, $n=6$) were enrolled. They had a median age of 47.5 [49.0 ± 10.6] years. ANOVA revealed no statistically significant differences in age between any of the four study groups.

All subjects provided written informed consent before enrolment into the study. The study was approved by the institutional review board and complied with the provisions of the Good Clinical Practice guidelines and the Declaration of Helsinki and local laws.

Compounds

Butylated hydroxytoluene (BHT) was obtained from Merck Schuchardt OHG (Hohenbrunn, Germany). The following lysophosphocholines (LPC) and lysophosphatidic acids (LPA) were obtained from Avanti Polar Lipids (Alabaster, AL): LPC(14:0/0:0), LPC(15:0/0:0), LPC(16:0/0:0), LPA(16:0/0:0), LPA(17:0/0:0), LPC(18:0/0:0), and LPC(18:1/0:0). The following compounds were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland): heptadecanoic acid, FAME Mix C4–C24 Unsaturates. Anhydrous organic solvents and inorganic reagents of the best available grade were ordered from Merck KGaA (Darmstadt, Germany).

Plasma metabolomics by ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS)

Serum samples were diluted with 66% aqueous acetonitrile (20 vol) containing a single deuterated internal standard, [$^2\text{H}_{35}$]1-stearoyl-*sn*-glycero-3-phosphocholine (18:0- d_{35} LPC; 5 $\mu\text{mol/l}$) and analyzed as described previously (24).

Peak alignment, data deconvolution, and random forests analysis

The raw chromatographic and spectral data were aligned, deconvoluted, and normalized (summing the total ion current to 10,000) using MarkerLynx software (Waters). A total of 1,393 features were exported from MarkerLynx. Each spectral feature was represented by a unique m/z , retention time, and peak area. Principal components analysis (PCA) and projection to latent structures-discriminant analysis (PLS-DA) were performed using

SIMCA P12+ (Umetrics, Kinnelon, NJ) to obtain an overview of the complete dataset by unsupervised (PCA) and supervised (PLS-DA) methods. Data were normalized by Pareto scaling. The random forests machine learning algorithm as previously described (25) was used to classify serum samples as either HCC or one of the three control groups -- AML, HV or LC. Random forests was chosen as it may be a more sensitive classifier (26). For the random forests analysis, age and sex were also included in the data matrix to evaluate their importance in discriminating between the four study groups.

Lipid profiling for LPCs in plasma by ultra-performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UPLC-ESI-TQMS)

Plasma samples were prepared and chromatographed by UPLC as described above. The eluant was introduced by electrospray ionization in a triple quadrupole mass spectrometer (Waters Xevo TQ). LPCs were detected by multiple reaction monitoring (MRM). The LPCs and their respective MRMs are as follows: 14:0 (468→184, 104), 16:0 (496→184, 104), 16:1 (494→184, 104), 18:0 (524→184, 104), 18:0-d₃₅ (559→184, 104), 18:1 (522→184, 104), 18:2 (520→184, 104), 18:3 (518→184, 104) and 20:4 (544→184, 104). TargetLynx was used for targeted quantitation.

Lipid profiling for 16:0 lysophosphatidic acid (LPA(16:0)) in plasma by ultra-performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UPLC-ESI-TQMS)

LPA(16:0) was profiled by modification of a previously described method (27). Briefly, plasmas were diluted 1:10 with buffer (50 µmol/l ammonium acetate with 0.5% trimethylamine and 90% methanol), centrifuged at 4°C to remove precipitated protein and other debris, and the supernatants transferred to autosampler vials. Samples were analyzed on a Xevo TQ. LPA(17:0) was used as an internal standard. MRMs were as follows: LPA(16:0) 409→153 and LPA(17:0) 423→153. TargetLynx was used for targeted quantitation.

Lipid profiling for free and esterified fatty acids in plasma by gas chromatography-mass spectrometry (GCMS)

Frozen samples were defrosted at ambient temperature, vortex mixed, and placed on ice. The entire sample preparation was performed under nitrogen and all solvents and solutions degassed before use. For the analysis of free and esterified fatty acids, plasma samples were analyzed in triplicate with three independent sample preparations. Plasma (100 µl) was combined in 10 ml glass tubes with internal standard solution (100 µl) composed of heptadecanoic acid (200 µg/ml in hexane containing 0.01% BHT), vortex mixed for 5 s, and then reduced to dryness under a gentle stream of nitrogen at room temperature. The residues were dissolved in a mixture of anhydrous methanol and toluene 2:1 (2.0 ml) containing BHT (0.01 %) and the samples placed on ice. To this solution, ice-cold acetyl chloride (200 µl) was added slowly to avoid splashing. After vortex mixing for 10 s, the samples were transferred to 3 ml Reacti-Vials (Thermo Fisher Scientific (Schweiz) AG, Basel, Switzerland) and incubated for 1 hour at 100°C in a heating block. Thereafter, the samples were transferred to 10 ml screw top glass tubes after cooling to room temperature and combined slowly with 6% potassium carbonate solution (5 ml). After thorough vortex mixing for 1 min, the solutions were centrifuged at 780 × g for 10 minutes at room temperature and the organic upper phase carefully transferred to clean glass tubes containing approximately 200 mg of anhydrous sodium sulfate to eliminate trace amounts of water. After 1 hour at room temperature and repeated brief vortex mixing, the samples were centrifuged at 780 × g for 5 minutes at room temperature and the solutions transferred to glass tubes and dried under a humble stream of nitrogen at 50°C. Thereafter, the residues were reconstituted in hexane (200 µl) containing BHT (0.01%), vortex mixed, then briefly

centrifuged to spin down insoluble precipitates, and finally an aliquot transferred to autosampler vials and subjected to GCMS analysis. The rest of the samples was stored at -80°C under nitrogen. Samples were analyzed by GCMS as previously described (28). For quantitation of fatty acids, calibration curves were prepared with heptadecanoic acid as internal standard (see above) and a fatty acid concentration range of 0–1,000 $\mu\text{mol/l}$. For all fatty acids, the calibration curves were linear in this range ($r>0.99$) and with coefficients of variance in the range 1–4%.

Statistical analysis

Significances were determined by Student's *t* test or ANOVA with Bonferroni correction for multiple comparisons using GraphPad Prism (GraphPad Software Inc., LaJolla, CA). Pearson correlation analysis was used to assess relationships between the various clinical and demographic parameters and the measured lipids.

Results

Plasma metabolomics by UPLC-ESI-QTOFMS and random forests

UPLC-ESI-QTOFMS plasma analysis yielded PCA and PLS-DA scores plots (Fig. 1A–B, respectively) and multidimensional scaling plots (MDS) from the random forests analysis for HCC vs. HV (Fig. 1C), HCC vs. LC (Fig. 1D), and HCC vs. AML (Fig. 1E). In the PCA scores plot, it can be seen that the HV and AML controls cluster together and are separated from the HCC and LC groups, which also clustered together. This unsupervised PCA analysis gives a view of the internal structure of the data and reveals that supervised analyses must be used to generate the maximum separation between the classes HCC vs. HV, HCC vs. LC, and HCC vs. AML. PLS-DA, a supervised analysis tool, was chosen and the scores plot is shown in Fig. 1B. Compared with PCA (Fig. 1A), a better clustering and separation of the four classes was obtained with PLS-DA, with some slight overlap remaining between HCC and LC (Fig. 1B). Supervised analysis using random forests yielded 100% classification accuracy for HCC vs. HV (Fig. 1C), 96.3% classification accuracy for HCC vs. LC (Fig. 1D), and 97.6% classification accuracy for HCC vs. AML (Fig. 1E). A single LC patient was misclassified as an HCC patient by random forests (Fig. 1D). It is of considerable interest to note that this LC patient was the only one with a Child-Pugh score of 10 (see Table 1) while all other LC patients had a score of 5. It is possible that this patient, with metabolomic hallmarks closer to HCC than LC, was in transition to HCC. Additionally, a single HCC patient was classified as an AML patient by random forests (Fig. 1E). No explanation is offered for this observation.

Random forests analysis produced a ranked list of ions responsible for the separation between each class of samples analyzed. It should be noted that PLS-DA analysis identified similar metabolites as the random forests analysis (Supplemental Fig. 1). The principal upregulated and downregulated plasma markers in HCC versus each of the three control groups, AML, LC, and HV, are presented in Table 2. It is noteworthy that age and gender did not influence the results since after their inclusion in the random forests analysis, neither of these variables appeared in the top 100 variables (data not shown) identified in any of the comparisons described below.

Hepatocellular carcinoma versus acute myelogenous leukemia—This control group (AML) was chosen because it represented a large tumor cell mass that probably had little direct influence upon hepatic metabolism. Their liver function tests were, in general, within normal ranges (Table 1).

Two 3-oxo- Δ^4 -bile acids, that are intermediates in the synthesis of chenodeoxycholic acid from cholesterol and generally considered to be fetal bile acids (29–33), 7 α -hydroxy-3-oxochol-4-en-24-oic acid and 3-oxochol-4,6-dien-24-oic acid, were upregulated in HCC relative to AML (Table 2). Based upon ion intensity, both of these bile acids were upregulated approximately 4-fold in HCC plasma relative to AML plasma. In addition, three lysophosphocholines (LPC(16:0), LPC(18:0), and LPC(18:3)) were downregulated in HCC versus AML. Bilirubin was also upregulated in HCC relative to AML plasma (Table 2) and this is confirmed by clinical chemistry data in Table 1 ($t = 3.24$; $P = 0.0024$).

Hepatocellular carcinoma versus liver cirrhosis—This control group (LC) was chosen because all but one of the HCC tumors occurred within a cirrhotic liver (Table 1) and it was important to determine metabolomic markers that were associated with HCC, rather than the underlying cirrhosis. Two unconjugated heme pigment metabolites, bilirubin and biliverdin, were upregulated in HCC relative to LC plasma. Based purely upon ion intensity, unconjugated bilirubin was ~3-fold upregulated and unconjugated biliverdin was ~10-fold upregulated. In addition, three downregulated LPCs, LPC(14:0), LPC(20:3), and LPC(22:6), were found for this comparison of patient classes by random forests. Based upon ion intensity, these LPCs were downregulated in HCC versus LC plasma between 2- and 10-fold.

Hepatocellular carcinoma versus healthy volunteers—This control group (HV) was chosen in an attempt to uncover metabolomic markers that separated health from disease. Two conjugates of the bile acid deoxycholic acid, namely, glycodeoxycholic acid and deoxycholic acid 3-sulfate, were highly upregulated in HCC relative to HV plasma. Based purely upon ion intensity, this upregulation was of the order of 30- to 50-fold. Additionally, seven lysophosphocholines, LPC(14:0), LPC(16:0), LPC(18:1), LPC(20:2), LPC(20:3), LPC(20:4) and LPC(20:5), were downregulated in HCC plasma relative to HV plasma (Table 2). Based upon ion intensity, the extent of downregulation of these ions in HCC plasma versus HV plasma was between 2- and 10-fold.

Metabolite profiling for plasma lysophosphocholines using UPLC-ESI-TQMS

Because of the consistent pattern of downregulated LPCs in HCC plasma observed above, it was decided to determine individual LPC plasma concentrations in all samples using UPLC-ESI-TQMS. Eight LPCs were determined by this method and the findings are shown in Fig. 2. No correlations between any of the quantitated LPCs and the Child-Pugh scores were found in either the HCC or LC groups, separately or combined.

The metabolomic study above had indicated that LPC(14:0) was downregulated in HCC versus LC and the profiling study confirmed this and also showed that this LPC was downregulated in HCC versus HV (Fig. 2). A negative correlation with AST ($P < 0.05$) was identified by Pearson's correlation analysis. LPC profiling revealed statistically significant downregulation of LPC(16:0) in HCC relative to AML ($P < 0.001$) and HV ($P < 0.05$). When the metabolomic and profiling data were considered together, LPC(16:0) was downregulated in HCC relative to both AML and HV control groups. The mean concentrations of LPC(16:1) were similar and tightly grouped in each study group and showed no statistically significant differences. The plasma concentration of LPC(18:0) in HCC was significantly ($P < 0.05$) lower than in AML and very significantly ($P < 0.01$) lower than in HV, using stringent statistical evaluation. LPC(18:1) in HCC plasma showed a statistically significant ($P < 0.05$) lower plasma concentration than in HV plasma was determined in this quantitative assay and also a statistically significant ($P < 0.05$) downregulation in HCC relative to LC. The plasma concentration of LPC(18:2) in HCC was highly statistically significantly ($P < 0.001$) lower in HCC than in HV controls. A negative correlation with ALT ($P < 0.05$) was identified

by Pearson's correlation analysis. The plasma concentration of LPC(18:3) in HCC was highly significantly ($P<0.001$) lower in HCC plasma compared to AML plasma and significantly ($P<0.05$) lower than in HV plasma. The plasma concentration of LPC(20:4) in HCC was statistically significantly lower than in HV controls ($P<0.05$) and in LC controls ($P<0.05$).

Overall findings on LPCs using UPLC-ESI-TQMS—For seven of the eight LPCs measured, plasma levels in HCC were lower than in HV. For three of the eight LPCs, the HCC plasma levels were lower than in LC. For three of the eight LPCs measured, the HCC plasma level lower than in AML plasma.

Metabolite profiling for plasma fatty acids using GCMS

Because of the provocative findings of lower LPCs in HCC plasma, it was decided to profile the total fatty acid concentration of the same plasma samples using GCMS. The analytical method employed methylates free fatty acids and transesterifies fatty acid esters such as mono-, di- and tri-glycerides, so that the total fatty acid content of plasma, free and esterified, can be determined (34). A total of 13 fatty acids were determined in plasma by GCMS as $\mu\text{mol/l}$ concentrations derived from calibration curves using heptadecanoic acid (FA(17:0)) as an internal standard. The coefficients of variation for the determination of these fatty acids by GCMS were all in the range 1–4%. Their mean $\pm$ SD concentrations in HCC plasma versus AML, LC, and HV plasma is given in Supplemental Table 1 and for lignoceric acid (FA(24:0)) and nervonic acid (FA(24:1)) are shown in Fig. 3A.

Tetracosanoic acid (lignoceric acid, 24:0)—The plasma concentration in HCC was virtually zero for all patients and showed a very statistically significantly ($P<0.01$) lower concentration than LC plasma and a highly statistically significantly ($P<0.001$) concentration that HV plasma. Remarkably, the AML plasma concentrations were similarly extinguished (Fig. 3A).

Tetracosenoic acid (nervonic acid, 24:1)—Similar to its unsaturated counterpart lignoceric acid, this fatty acid was virtually absent from all HCC and AML plasmas. The plasma concentration of nervonic acid in HCC was very statistically significantly ($P<0.01$) lower concentration than LC plasma and highly statistically significantly ($P<0.001$) below the concentration in HV plasma.

Overall findings on fatty acids using GCMS—Of the 13 fatty acids profiled in plasma by GCMS, five (FA(15:0), FA(20:4), FA(22:6), FA(24:0) and FA(24:1)), showed lower plasma concentrations in HCC than certain controls (Supplemental Table 1; Fig. 3A). The most interesting finding to emerge from the fatty acid profiling of HCC and control plasma was the highly statistically significant quenching of the two VLCFAs lignoceric acid (24:0) and nervonic acid (24:1). These very low levels in plasma may be due to enhanced peroxisomal β -oxidation of VLCFAs under the control of the peroxisome proliferator activated receptor alpha (PPAR α) and as a result of enhanced lignoceryl-CoA ligase activity (35) or as a result of activation of liver X receptor α (LXR α) (36). There is evidence that PPAR α is upregulated and related to the etiology of HCC in model systems (37, 38). This concept was therefore further examined in these patient samples. There is also evidence that LXR α activation may be involved in both HBV and HCV lipogenesis and hepatocarcinogenesis (39, 40).

Profiling for LPC(24:0) by UPLC-ESI-TQMS

Because of the observations above of very low 24:0 and 24:1 fatty acids in HCC and AML, it was decided to profile the corresponding LPC of 24:0, LPC(24:0) by UPLC-ESI-TQMS.

Fig. 3B shows the plasma concentrations in $\mu\text{mol/l}$ for this low abundance LPC. Of considerable interest was the finding that HCC plasma displayed a statistically significant ($P < 0.05$) elevated concentration compared to HV plasma. This suggests that the prior findings were due to the free and esterified fatty acid compartment and not the phospholipid fraction.

Profiling for LPA(16:0) by UPLC-ESI-TQMS

Reduced levels of circulating LPCs may be due to their enhanced conversion by extracellular lysophospholipase D (autotaxin) to lysophosphatidic acids (LPA) (41). To test this hypothesis, we profiled LPA(16:0) concentrations in HCC and control plasma by UPLC-ESI-TQMS (27). The distribution of LPA(16:0) plasma concentrations in the four study groups is shown in Fig. 3C. All groups had similar levels, except for four HCC samples, which displayed 5- to 10-times higher concentration. Inspection of the data set failed to reveal a common etiologic path for these four subjects, they each having different predisposing factors. However, it was noted that these four patients had very high AFP concentrations. Both parametric linear regression and nonparametric Spearman rank correlation showed that LPA(16:0) levels and AFP concentrations well significantly correlated ($r = 0.60$; $P = 0.005$) (Fig. 3D).

Evidence for a role of PPAR α in HCC from clinical plasma data

If PPAR α is activated specifically in the livers of HCC patients relative to LC patients and healthy volunteers (HV), one would expect to observe in those former patients a decline in plasma uric acid, triglycerides, LDL cholesterol, and perhaps total cholesterol, accompanied by a rise in HDL cholesterol (25). Supplemental Fig. 2 shows no evidence for the operation of PPAR α in these HCC patients relative to the control groups. It is therefore likely that, if PPAR α plays a role in these HCC cases, it does so very specifically, for example, by upregulating peroxisomal lignoceryl-CoA ligase activity (35).

Overall pattern of up- and down-regulated molecules in hepatocellular carcinoma patients

A summary of the total molecular profile identified in hepatocellular carcinoma patients by metabolomic and lipid profiling procedures is given in Table 2. Perusal of this table reveals that there are no upregulated or downregulated molecules that are common to all three comparator groups (healthy volunteers [HV], liver cirrhosis [LC], and acute myelogenous leukemia [AML]). There are seven molecules downregulated with respect to two comparator classes, five LPCs and two fatty acids. The perturbations in bile acid disposition (bile acid conjugates relative to HV) and fetal bile acids (relative to AML) are of interest because the two comparator groups involved (AML and HV) are the two groups without liver disease. In addition, bilirubin was found to be elevated in HCC compared to AML and LC and biliverdin in HCC compared to LC only.

The most obvious change in small plasma molecules was displayed by the two fatty acids lignoceric acid (24:0) and nervonic acid (24:1), which were virtually extinguished in both HCC and AML. Whether or not these molecules are similarly reduced in the plasma of other cancer patients falls beyond the scope of this report.

Discussion

Metabolomic analysis of intermediate stage hepatocellular carcinoma (23) using plasma and employing UPLC-ESI-QTOFMS based metabolomic protocols and random forests machine learning algorithm (26, 42) was employed for the identification of metabolic intermediates in plasma that are altered in HCC. These studies were augmented by metabolite profiling for LPC and LPA by UPLC-ESI-TQMS and fatty acids by GCMS. The accumulated data were

analyzed using nonparametric statistics and tests for multiple comparisons. This stringency was thought to eliminate spurious metabolomic findings. Three comparator groups were used and random forests readily clustered and classified HCC from each comparator group. Healthy volunteers (HV) and acute myelogenous leukemia (AML) were taken as control groups with no liver disease. Liver dysfunction is a rare complication of AML (43) and in one study of AML and acute lymphoblastic leukemia in Nepal (44), abnormal liver function tests (LFTs) were recorded in a subset of the patients. LFTs were within normal ranges for the 22 AML patients described in this study.

The principal issue that needs to be addressed is what do these small molecules that are either elevated and attenuated in plasma of HCC patients teach us about HCC? It is pertinent to first examine their potential origins in order to gain more insights into what mechanisms may be at play in HCC. There were six main findings of this study in relation to HCC versus controls, (1) that two bile acid conjugates and two fetal bile acids were elevated, (2) that bilirubin and biliverdin were elevated, (3) that 11 lysophospholipids were attenuated, (4) that two very long-chain fatty acids were attenuated, (5) that LPA(16:0) was considerably elevated in 4/20 HCC patients, and (6) that in the HCC group as a whole LPA(16:0) was strongly correlated with plasma AFP concentration.

Regarding bile acid homeostasis, an early study recognized that fasting serum glycocholate concentrations were elevated in most patients with liver disease, but especially in LC (97.1% of cases) and HCC (94.7%) (45). Overall, the literature provides a basis for the observation of elevated bile acid conjugates in HCC in this study. Regarding the elevated plasma levels of 7 α -hydroxy-3-oxocholesterol-4-en-24-oic acid and 3-oxocholesterol-4,6-dien-24-oic acid, these are so-called 3-oxo- Δ^4 bile acids (30, 33) that are rarely encountered in the plasma of adults and usually considered to be fetal bile acids (33), that spill over into the urine in infants with cholestatic disease (30). There is a report that these bile acids are increased in both plasma and urine of HCC patients (33), where it was proposed that the appearance of fetal bile acids in the serum of HCC patients is the result of a metabolic process in the liver, rather than cholestasis (33).

The largest number of molecules of interest that were altered in the plasma of HCC patients were in the phospholipid group of lysophosphocholines. A total of 11 LPCs were attenuated in HCC plasma. This finding strongly suggests that liver cirrhosis contributes greatly to the decline in LPCs in HCC plasma. As mentioned above, this interpretation is supported by the work of Yin *et al.* (21), whereby HCC and HBV-induced LC shared a common decline in four LPCs, three of which (LPC(18:0), (18:2) and (18:3)) are reported in this study. LPCs are metabolized by phospholipase D, generating lysophosphatidic acids (LPAs), potent mitogens that act through LPA1-5 receptors (46). LPA are also produced from LPC extracellularly by lysophospholipase D (LPD) first described in rat plasma and that converted polyunsaturated LPC to their saturated LPA counterparts (47). In the preceding 25 years LPD has been shown to be identical to autotaxin (ATX), and that ATX is strongly suspected of being involved in hepatocarcinogenesis (41). It was therefore decided to profile the HCC and control plasmas for LPA(16:0), a product of LPD/autotaxin. Indeed, 4/20 HCC patients had inordinately high LPA(16:0) plasma levels and that LPA(16:0) strongly correlated with plasma AFP concentrations. It is of interest to note that both LPA (41) and AFP (48) may play a role in progression of HCC.

Two very long-chain fatty acids (VLCFA), lignoceric acid (24:0) and nervonic acid (24:1) were virtually extinguished in both HCC and AML plasmas. Lignoceric acid underwent a 7.5-fold decline in HCC patients relative to LC ($P < 0.01$). Similarly, nervonic acid fell 3.4-fold in HCC relative to LC ($P < 0.001$). Note that, in both cases, plasma levels in HV were

similar to those in LC. Whether or not other malignancies share this phenomenon with HCC and AML is not known and beyond the scope of this report.

In conclusion, we have undertaken a plasma metabolomic study of hepatocellular carcinoma, in which control groups of healthy volunteers, liver cirrhosis, and acute myelogenous leukemia were used. Upregulated molecules of interest in HCC included two conjugated bile acids, two bile pigments, and two fetal 3-oxo- Δ^4 bile acids. Downregulated molecules of interest included 11 lysophosphocholines, and two VLCFAs, lignoceric acid, and nervonic acid. All these molecular changes in the plasma of HCC patients provide new insights into the pathobiology of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Multivariate data analysis and random forests analysis of ions from HCC plasmas and three control groups

Panel A, Principal components analysis (PCA) showing clustering and partial resolution of the four study groups; Panel B, Projection to latent structures-discriminant analysis (PLS-DA) showing improved clustering and resolution of the four study groups; Panel C, Separation by random forests analysis of HCC and HV with 100% classification accuracy; Panel D, Separation of HCC and LC by random forests analysis with 96.3% classification accuracy; Panel E, Separation of HCC and AML patients by random forests analysis with 97.6% classification accuracy.

Figure 2. Plasma concentrations of eight lysophosphocholines in hepatocellular carcinoma and three control groups

Plasma concentrations are expressed in $\mu\text{mol/l}$. HCC=hepatocellular carcinoma (20 cases), AML=acute myelogenous leukemia (22 cases), LC=liver cirrhosis (7 cases), HV=healthy volunteers (6 subjects). Horizontal lines represent group means. Because of unequal variances the nonparametric Kruskal-Wallis test was used with Dunn's multiple comparisons test. Levels of statistical significance are $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***). n.s. indicates $P>0.05$.

Figure 3. Results of lipid profiling by TQMS and GCMS for selected fatty acids, LPC and LPA
 Panel A, Concentrations (μmol/l) of lignoceric acid (FA(24:0)) and nervonic acid (FA(24:1)) in hepatocellular carcinoma (HCC; 20 cases), acute myelogenous leukemia (AML; 22 cases), liver cirrhosis (LC; 7 cases) and healthy volunteers (HV; 6 subjects). Horizontal lines represent group means. Because of unequal variances, the nonparametric Kruskal-Wallis test was used with Dunn's multiple comparisons test. Levels of statistical significance are $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***); Panel B, Concentration (μmol/l) of LPC(24:0); Panel C, Relative concentration (normalized against internal standard LPA(17:0)) of LPA(16:0) in HCC, AML, LC and HV; Panel D, Correlation between α -fetoprotein (AFP) plasma concentration (log ng/ml) and relative plasma concentration of LPA(16:0) in 20 cases of HCC.

Table 1

Demographics and clinical measurements of study groups.

	HCC	LC	AML	Healthy Controls
Age (years)	61.1±11.2	50.3±5.7	57.2±13.1	47.0±10.7
Gender	17M/3F	3M/4F	9M/13F	6M
Child-Pugh (# of patients)	5 (10) 6 (6) 7 (2) 11(1) noncirrhotic (1)	5 (6) 10 (1)	–	–
AFP (ng/ml) [range]	3663±15087 [1.3–67,725]	4.0±2.0 [1.4–7.5]	–	–
ALT (U/l)	52.4±34.1	44.0±31.1	48.0±38.0	–
AST (U/l)	80.2±56.5	59.0±37.7	37.2±19.9	–
ALP (U/l)	110±48	130±127	161±175	–
GGT (U/l))	260±330	126±111	208±291	–
Bilirubin (μmol/l)	30.6±29.1	17.0±14.3	10.0±6.5	–

All values are expressed as the average ± standard deviation. – indicates measurement was not conducted. M, male; F, female. AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase.

Summary of upregulated and downregulated plasma metabolomic markers of hepatocellular carcinoma

Table 2

↑ means upregulated, ↓ means downregulated, LPC means lysophosphocholine, FA means fatty acid, AML means acute myelogenous leukemia, LC means liver cirrhosis, HV means healthy volunteers. TQMS means triple quadrupole mass spectrometry.

Molecular changes	Identification Status	Quantified	COMPARATOR GROUP			
			AML	LC	HV	
LPC(14:0)	Tandem MS	TQMS		↑	↑	
LPC(16:0)	Tandem MS w/Standard	TQMS	↑		↑	
LPC(18:0)	Tandem MS w/Standard	TQMS	↑		↑	
LPC(18:1)	Tandem MS w/Standard	TQMS			↑	
LPC(18:2)	Tandem MS	TQMS			↑	
LPC(18:3)	Tandem MS	TQMS	↑		↑	
LPC(20:4)	Tandem MS	TQMS			↑	
LPC(24:0)	Tandem MS w/Standard	TQMS			↑	
FA(24:0)	Tandem MS w/Standard	GC-MS		↑	↑	
FA(24:1)	Tandem MS w/Standard	GC-MS		↑	↑	
Bilirubin	Accurate Mass (m/z)	Clinical Chemistry	↑	↑		
LPC(20:2)	Accurate Mass (m/z)				↑	
LPC(20:3)	Accurate Mass (m/z)			↑	↑	
LPC(20:5)	Accurate Mass (m/z)				↑	
LPC(22:6)	Accurate Mass (m/z)			↑		
Biliverdin	Accurate Mass (m/z)			↑		
7α-Hydroxy-3-oxochol-4- en-24-oic acid	Accurate Mass (m/z)		↑			

Molecular changes	Identification Status	Quantified	COMPARATOR GROUP		
			AML	LC	HV
3-Oxachola-4,6-dien-24- oic acid	Accurate Mass (m/z)		↖		
Glycodeoxycholic acid	Tandem MS w/Standard				↖
Deoxycholic acid 3-sulfate	Tandem MS w/Standard				↖