

Evaluation of Metabolite Biomarkers for Hepatocellular Carcinoma through Stratified Analysis by Gender, Race, and Alcoholic Cirrhosis

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Abstract

Background: The effects of hepatocellular carcinoma on liver metabolism and circulating metabolites have been subjected to continuing investigation. This study compares the levels of selected metabolites in sera of hepatocellular carcinoma cases versus patients with liver cirrhosis and evaluates the influence of gender, race, and alcoholic cirrhosis on the performance of the metabolites as candidate biomarkers for hepatocellular carcinoma.

Methods: Targeted quantitation of 15 metabolites is performed by selected research monitoring in sera from 89 Egyptian subjects (40 hepatocellular carcinoma cases and 49 cirrhotic controls) and 110 U.S. subjects (56 hepatocellular carcinoma cases and 54 cirrhotic controls). Logistic regression models are used to evaluate the ability of these metabolites in distinguishing hepatocellular carcinoma cases from cirrhotic controls. The influences of gender, race, and alcoholic cirrhosis on the performance of the metabolites are analyzed by stratified logistic regression.

Results: Two metabolites are selected on the basis of their significance to both cohorts. Although both metabolites discriminate hepatocellular carcinoma cases from cirrhotic controls in males and Caucasians, they are insignificant in females and African Americans. One metabolite is significant in patients with alcoholic cirrhosis and the other in nonalcoholic cirrhosis.

Conclusions: The study demonstrates the potential of two metabolites as candidate biomarkers for hepatocellular carcinoma by combining them with α -fetoprotein (AFP) and gender. Stratified statistical analyses reveal that gender, race, and alcoholic cirrhosis affect the relative levels of small molecules in serum.

Impact: The findings of this study contribute to a better understanding of the influence of gender, race, and alcoholic cirrhosis in investigating small molecules as biomarkers for hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*; 23(1); 64–72. ©2013 AACR.

Introduction

Hepatocellular carcinoma is a significant worldwide health problem with as many as 500,000 new cases diagnosed each year (1). In the United States, the rate of hepatocellular carcinoma has increased by 70% over the

last two decades (2) and the increasing incidence of hepatocellular carcinoma has been associated with hepatitis C virus (HCV) infection (3–5). Chronic hepatitis develops in about 80% of those infected with HCV. Over the course of 20 years or more, 10% to 30% of HCV carriers develop cirrhosis; patients with cirrhosis have an annual risk of 1% to 2% for developing hepatocellular carcinoma (6). HCV has been reported as the main risk factor for hepatocellular carcinoma in both Egypt and the United States (7). The prognosis for patients with hepatocellular carcinoma remains extremely poor. Survival rates of patients with hepatocellular carcinoma can be improved significantly if the diagnosis were made at earlier stages, when treatment is more effective (8). Current diagnosis of hepatocellular carcinoma relies on clinical information, liver imaging, and measurement of serum α -fetoprotein (AFP). However, elevated AFP levels may be seen in patients with cirrhosis or chronic hepatitis (9). Thus, the reported sensitivity (41%–65%) and specificity (80%–94%) of AFP are not sufficient for early diagnosis and additional markers are needed (9, 10).

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Characterizing metabolites in human samples can facilitate the discovery of biomarkers that may reveal early signs of cancers when the chances for cure are highest. Metabolomics provides a simultaneous assessment of numerous metabolites allowing for the quantification of individual metabolites and the characterization of phenotypic profiles based on a combination of metabolites (11–14). Metabolomic studies are typically carried out through two complementary methods: untargeted and targeted. Although untargeted metabolomics can be used to generate new hypotheses for further tests by analyzing the ion abundances of a large number of unknown metabolites, targeted approaches focus on quantitation of the levels of known metabolites to detect true differences between distinct biologic groups. Liquid chromatography coupled with mass spectrometry (LC/MS) has been applied for both untargeted and targeted quantitation of metabolites. Various LC/MS based untargeted metabolomic studies have led to the identification of significant differences of bile acids, phospholipids, fatty acids, glycolysis, urea cycle, and methionine metabolism in serum, plasma, urine, and fecal samples of patients with hepatocellular carcinoma compared with benign liver tumor or healthy subjects (9, 12–20). We previously reported results from our untargeted metabolomic study, in which we observed downregulation of metabolites of bile acids and long chain carnitines and upregulation of phospholipids and amino acids in sera of hepatocellular carcinoma cases versus cirrhotic controls (15, 16). Targeted quantitation of these relevant metabolites using isotope dilution by selected reaction monitoring (SRM) on triple quadrupole (QqQ) or quadrupole linear ion trap (QLIT) mass spectrometers allows more sensitive and accurate quantitation to identify candidate biomarkers. Recent quantitative analysis of lipids in plasma by ultraperformance liquid chromatography (UPLC)–ESI–QqQ MS showed aberrant lipid metabolism of lysophosphocholines (LPC) and lysophosphatidic acid in hepatocellular carcinoma (17). Also, targeted analysis of taurocholic acid (TCA), lysophosphoethanolamine (LPE) 16:0, and LPC 22:5 by LTQ Orbitrap helped evaluate these metabolites as serum biomarkers distinguishing the different stages of hepatocarcinogenesis (18).

Gender differences have been previously observed in the onset of hepatocellular carcinoma; the risk of hepatocellular carcinoma is 2 to 7 times higher in males than in females, although this ratio varies across the world (21, 22). In addition to the speculation that men could have higher rates of environmental exposure to liver carcinogens (such as smoking or alcohol) and hepatitis virus infections, the risk difference could be attributed to estrogen and androgen effects (23, 24). Although few studies have addressed racial/ethnic differences in survival for patients with hepatocellular carcinoma, African Americans have persistently shown poor survival rate. Also, it has been reported that the sensitivity of AFP for the diagnosis of hepatocellular carcinoma in African Americans with HCV infection is less than that of patients of all

other racial groups combined (25). Alcohol is one of the major risks of liver injury and has been considered as a promoter for hepatocellular carcinoma (26). It has been found that patients with alcoholic cirrhosis and bearing two copies of the RANTES G-403 variant (2G-403 genotype) had a higher rate of hepatocellular carcinoma occurrence compared with patients carrying at least one RANTES A-403 allele (27). AFP has been reported to be higher in patients with hepatocellular carcinoma arising from chronic viral conditions compared with those with alcoholic liver disease (28).

In this study, we evaluate the levels of metabolites in sera of hepatocellular carcinoma cases and patients with liver cirrhosis. This is accomplished by targeted quantitation of selected metabolites in sera from two cohorts (Egypt and the United States) using isotope dilution on a QLIT MS coupled with an UPLC (UPLC–QLIT–MS). The metabolites are chosen from candidates discovered by our previous untargeted analysis of metabolites in the Egyptian cohort (15). To address the challenge in detecting true differences between hepatocellular carcinoma cases and cirrhotic controls due to the heterogeneity of the patients and their disease, we propose to compare differences among homogeneous subpopulations through stratification of the subjects by gender, race, alcoholic cirrhosis, and tumor stage. This offers the opportunity to identify subgroup-specific biomarkers for accurate detection of hepatocellular carcinoma. We present our observations about the influence of gender, race, and alcoholic cirrhosis on the performance of candidate biomarkers for hepatocellular carcinoma.

Materials and Methods

Study cohorts

The samples in this study were obtained from participants recruited in Egypt and the United States. The Egyptian cohort consists of adult patients with hepatocellular carcinoma or cirrhosis recruited from the outpatient clinics and inpatient wards of the Tanta University Hospital (Tanta, Egypt). The participants consist of 89 subjects (40 hepatocellular carcinoma cases and 49 patients with liver cirrhosis). The characteristics of the patient population in this study are summarized in Table 1. All participating patients provided informed consent before taking part in the study. The study protocol was approved by the Tanta University Ethical Committee. Patients were diagnosed to have cirrhosis on the basis of established clinical, laboratory and/or imaging criteria with ultrasound examination. Cases were suspected to have hepatocellular carcinoma if they had focal lesion in ultrasound examination and/or elevated AFP above 200 ng/mL, but only diagnosed to have hepatocellular carcinoma based on well-established diagnostic imaging criteria with spiral CT scanning and/or histopathology examination of liver biopsy. Clinical stages for hepatocellular carcinoma cases were determined on the basis of the tumor–node–metastasis (TNM) staging system. Suspected cases that were

Table 1. Characteristics of the Egyptian study cohort

		Hepatocellular carcinoma (N = 40)	Cirrhosis (N = 49)	P
Age	Mean (SD)	53.2 (3.9)	53.8 (7.6)	0.3530
Gender	Male	77.5%	67.3%	0.3474
HCV serology	HCV Ab ⁺	100.0%	100.0%	1.0000
HBV serology	HbsAg ⁺	0.0%	6.1%	0.2492
MELD	Mean (SD)	18.6 (7.7)	18.9 (7.1)	0.1328
	MELD ≤ 10	20.0%	12.2%	0.3863
AFP	Median (IQR)	275.9 (1,244.3)		
Hepatocellular carcinoma stage	Stage I	72.5%		
	Stage II	15.0%		
	Stage III	5.0%		
	Unknown	7.5%		

Abbreviations: MELD, model for end-stage liver disease; IQR, interquartile range.

not confirmed by CT or biopsy were excluded from both groups of this study.

The U.S. participants in this study comprised of adult patients, recruited from the hepatology clinics at MedStar Georgetown University Hospital (Washington, DC). The cohort consists of 110 participants (56 hepatocellular carcinoma cases and 54 patients with liver cirrhosis). The characteristics of the cohort are summarized in Table 2. All patients provided informed consent and the study was approved by the Institutional Review Board at Georgetown University (Washington, DC). All patients were diagnosed to have liver cirrhosis on the basis of established clinical, laboratory, and/or imaging criteria. Cases

were diagnosed to have hepatocellular carcinoma based on well-established diagnostic imaging criteria and/or histology. Clinical stages for hepatocellular carcinoma cases are determined on the basis of the TNM staging system. Controls were required to be hepatocellular carcinoma free for at least 6 months from the time of study entry.

Chemicals and reagents

Vacutainer sterile vacuum tubes were purchased from Becton Dickinson and Company. We purchased from Sigma-Aldrich LC check solutions (mixture of dodecanamide, 11-deoxycorticosterone, cortisone, thyroxine,

Table 2. Characteristics of the U.S. study cohort

		Hepatocellular carcinoma (N = 56)	Cirrhosis (N = 54)	P
Age	Mean (SD)	59.7 (7.4)	58.2 (8.4)	0.3489
Gender	Male	75.0%	63.0%	0.2168
Ethnicity	Caucasian	55.4%	59.3%	
	African American	30.4%	25.9%	0.8926
	Others	14.3%	13.0%	
HCV serology	HCV Ab ⁺	51.8%	35.2%	0.0392
	HCV RNA ⁺	55.4%	33.3%	0.6356
HBV serology	Anti-HBC ⁺	14.3%	7.4%	0.2893
	HbsAg ⁺	28.6%	20.4%	0.6777
Alcoholic cirrhosis	Alcoholic cirrhosis ⁺	28.6%	33.3%	0.6811
MELD	Mean (SD)	11.0 (6.4)	14.0 (6.0)	0.0002
	MELD ≤ 10	44.6%	14.8%	0.0008
AFP	Median (IQR)	14.5 (103.6)	4.8 (13.6)	0.0002
Hepatocellular carcinoma stage	Stage I	53.6%		
	Stage II	23.2%		
	Stage III	7.1%		
	Stage IV	1.8%		
	Unknown	14.3%		

epitestosterone, and N-benzoyl-D-phenylalanine), ammonium acetate (NH₄OAC), ammonium bicarbonate (NH₄HCO₃), glycolic acid (GCA), taurochenodeoxycholic acid (TCDCa), glycodeoxycholic acid (GDCA), glycodeoxycholic-2,2,4,4-d₄ acid (D₄_GDCA), glycodeoxycholic-2,2,4,4-d₄ acid (D₄_GCA), Phe-Phe, 2C13_Phe-Phe, 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid, and oleoyl carnitine. LPC 16:0, LPC 17:0, D35_LPC 18:0, LPE 18:0, D54_PE 14:0 were purchased from Avanti Polar Lipids. Glycochenodeoxycholic acid 3-sulfate (3sulfo-GCDCA) was synthesized by PharmAgra Labs. D5_sulfo-GCDCA was synthesized by Toronto Research Chemicals. Acetonitrile, methanol, isopropanol, and chloroform were from Fisher Scientific. Metabolomics MS System Test Mix (theophylline, caffeine, hippuric acid, 4-nitrobenzoic acid, nortriptyline HCl) was purchased from Waters. All other common chemicals of analytic grade were purchased from Sigma-Aldrich.

Blood collection

Human blood was drawn from the participants in the Egyptian and U.S. cohorts and collected by venipuncture into 10 mL BD Vacutainer sterile vacuum tubes without the presence of anticoagulant. The blood was immediately centrifuged at 1,000 \times g for 10 minutes at room temperature. The serum supernatant was carefully collected and centrifuged at 2,500 \times g for 10 minutes 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 passcode protected computer system.

Metabolite extraction

Metabolites were extracted using a 96-well filter plate that allows high throughput serum sample preparation. Briefly, serum samples from 96 hepatocellular carcinoma cases and 103 cirrhotic controls representing the two cohorts (Egypt and the United States) were thawed and vortexed in separate storage boxes. Six samples randomly selected from the hepatocellular carcinoma groups and 6 from the cirrhosis group (50 μL each) were simultaneously pipetted into odd numbered wells and even numbered wells, respectively, in each row of the 96-well filter plate through a multiple channel pipettor. This was followed by in-well protein precipitation by adding 200 μL chilled methanol containing 1% NH₄HCO₃ and a mixture of isotope-labeled internal standards. After in-well agitation on an oscillating table, vacuum was applied to filtrate and remove the precipitated proteins. The methanol filtrate of approximate 200 μL was then ready for LC/MS analysis.

Internal standards and QC runs for quality assessment

To evaluate the quality of metabolite extraction and the impact of matrix effects, a mixture of internal standards

(0.1 pmol/ μL), including 2C13_Phe-phe, D₄_GCA, D₄_GDCA, D5_3sulfo-GCDCA, D35_LPC 18:0, and D54_PE 14:0, was spiked into serum. In addition, we ran six serum quality control (QC) samples along with the hepatocellular carcinoma cases and cirrhotic controls to evaluate the reproducibility of the analytical methods. The QC samples were pooled from both the hepatocellular carcinoma cases and cirrhotic controls.

Target metabolites

Target metabolites were chosen from a list of candidate metabolite biomarkers discovered by our previous hepatocellular carcinoma biomarker discovery study using the serum samples from Egyptian cohort (15). The list includes bile acids (GDCA, GCA, TCDCa, 3sulfo-GCDCA, 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid), phospholipids (LPC 17:0, LPC 16:0, and LPC 18:0 and LPE 18:0, LPE 16:0, LPE 20:4, LPE 22:6, and Phe-Phe), long chain carnitines of oleoyl carnitine and linoleidyl carnitine. SRM-based quantitation of these 15 metabolites was done by UPLC-QqQLT in sera from hepatocellular carcinoma cases and cirrhotic controls in the Egyptian and the U.S. cohorts as outlined in the next section.

Quantitation of target metabolites by UPLC-QqQLT

From each sample, 10 μL was injected onto a reverse-phase 50 \times 2.1 mm ACQUITY 1.7 μm CSH C18 column using a Waters ACQUITY UPLC system with a gradient mobile phase consisting of 40% acetonitrile solution containing 10 mmol/L NH₄OAC and 0.1% formic acid (FA) (A) and 10% acetonitrile in isopropanol containing 0.1% FA and 10 mmol/L NH₄OAC (B). The whole run lasted 11 minutes. The gradient consisted of 100% A for 1.0 minutes at a flow rate of 0.25 mL/min then a ramp to 50% B from 1.0 to 5.0 minutes at a flow rate of 0.25 mL/min, then ramp to flow rate of 0.4 mL/min and 100% B from 5.1 to 9.1 minutes, return to 0.25 mL/min flow rate and 100% A from at 9.2 minutes, followed by a hold at 100% A until 11 minutes. The column temperature was set to 50 $^{\circ}\text{C}$. UPLC-QqQLT data were collected in the SRM mode on the AB Sciex QTRAP 4000 MS operating in the positive mode with a capillary voltage of 4,500 V and source temperature of 450 $^{\circ}\text{C}$. The curtain gas flow rate was set to 20, sheath gas flow rate GS1 was set to 40.00, sheath gas GS2 was set to 20.00 and exit potential was 10 V. Each target metabolite was monitored by two SRM transitions: one transition works for identification and the other transition for quantitation shown in Table 3. Quantitation of each metabolite was performed by calculating the ratio of the MS signal of the metabolite to its corresponding isotope-labeled internal standard. To obtain accurate and reproducible SRM quantitation data, dwell time and scheduled SRM detection were customized for each target metabolite, allowing us to acquire enough data points (>15 points) across the chromatographic elution profile.

Table 3. Experimental parameters for SRM quantitation of target metabolites

Compound	[M+H] ⁺ + m/z	SRM	Internal standard	CE (V)	RT (min)	DP	CXP
GDCA	450.5	450.5 → 90.9	D4-GCDCA	109	5.0	91	4
		450.5 → 414.3		23		91	26
GCA	466.4	466.4 → 412.3	D4-GCA	27	3.15	101	26
		466.4 → 337.2		33		101	20
TCDCA	500.4	500.4 → 464.2	D5-TCA	23	3.20	106	6
		500.4 → 126.1		55		106	6
3Sulfo-GCDCA	547.3	547.3 → 414.2	D5-3sulfo GCDCA	31	2.30	76	24
3β,6β-Dihydroxy-5β-cholan-24-oic acid	393.3	[M+NH ₄] ⁺ 547.3 → 432.3	D4-GCDCA	19	5.5	76	26
		393.3 → 357.3		13		66	4
LPC 17:0	510.5	393.3 → 81.1	D35-LPC18	63	6.90	66	4
		510.5 → 184.2		37		101	10
LPC 16:0	496.5	510.5 → 86.2	D35-LPC18	69	6.63	101	4
		496.5 → 184.2		37		101	10
LPC 18:0	524.4	496.5 → 86.2	D35-LPC18	69	7.04	101	4
		524.4 → 184.2		37		101	10
LPE 18:0	482.3	524.4 → 86.2	D54 PE14	69	7.44	101	4
		482.3 → 341.3		29		86	20
LPE 16:0	454.28	482.3 → 62.2	D54 PE14	35	6.67	86	10
		454.3 → 313.3		29		86	20
LPE 20:4	502.3	454.3 → 62.2	D54 PE14	35	6.36	86	10
		502.3 → 361.3		29		86	20
LPE 22:6	526.3	502.3 → 62.2	D54 PE14	35	6.30	86	10
		526.3 → 385.3		29		86	20
Phe-Phe	313.0	526.3 → 62.2	2C13Phe-phe	35	1.32	120	10
		313.0 → 120.1		53		120	10
Oleoyl carnitine	426.3	313.0 → 103.1	D3-Palmitoyl carnitine	53	6.60	96	4
		426.3 → 85.0		55		96	2
Linoleaidyl carnitine	424.3	426.3 → 62.4	D3-Palmitoyl carnitine	63	6.30	96	4
		424.3 → 85.0		55		96	4
		424.3 → 62.4		63		96	2

Abbreviations: CE, collision energy; DP, de-clustering potential; CXP, collision cell exit potential.

Quality assessment of the quantitation data

The quantitation performance was evaluated on the basis of the linearity of the standard curves [e.g., coefficient of determination (R^2)], signal reproducibility (e.g., SD; coefficient of variation, CV), dynamic range of detection, analyte recovery (percentage of extraction efficiency), accuracy, and ESI matrix effects. Each standard curve was created by at least five points of standard solutions and each point had triplicate injections at the beginning, in the middle and at the end of run. The majority of the target metabolites achieved more than 80% recovery except Phe-Phe (67%). This could be due to the hydrophilic property of Phe-Phe that causes the sample loss during extraction and the poor retention on the C18 column. The coeluting interferences from serum sample cause ion enhancement on Phe-Phe and GCA and ion suppression on the rest of target metabolites during the ionization process, of which LPE 18:0 has the worst ion suppression effect of 31%. However, the presence of spiking isotope-labeled internal standards enables us to correct the above detection varia-

tions with remarkable linearity ($R^2 > 0.999$) and reproducibility (CV < 10% and accuracy > 85%). The six serum QC samples that were analyzed along with the other serum samples yielded a relative SD < 15%.

Statistical analysis

A univariate logistic regression model (Eq. 1) was used to evaluate the log-transformed quantitation data for the 15 metabolites in terms of distinguishing hepatocellular carcinoma cases from cirrhotic controls.

$$\log\left(\frac{\Pr(Y = 1)}{1 - \Pr(Y = 1)}\right) = \alpha + \beta X, \quad (1)$$

where Y is the binary response variable with $Y = 1$ indicating that the sample is from the hepatocellular carcinoma group, X is the metabolite expression level, the exponential of β is the OR of a subject coming from the hepatocellular carcinoma group by increasing one unit of the metabolite expression level. Our interest is in testing the null hypothesis $H_0 : \beta = 0$ against the alternative

$H_1 : \beta = \beta^*$, where $\beta^* \neq 0$, that the metabolite is related to the binary response variable. In addition, a proportional odds model (Eq. 2) was applied to evaluate the metabolite effects on two subgroups based on hepatocellular carcinoma stage: early (stage I) and late (stages II, III, and IV).

$$\log\left(\frac{\Pr(Y = j)}{\Pr(Y = 0)}\right) = \alpha_j + \beta_j X, \quad j = 1, 2 \quad (2)$$

where Y is the multinomial distributed response variable with $Y = 0$ indicating that the sample is from the cirrhotic control group, $Y = 1$ denotes that the sample is from the early-stage hepatocellular carcinoma group, and $Y = 2$ indicates that the sample is from the late-stage hepatocellular carcinoma group. Finally, the logistic regression model in Eq. 1 was applied separately to subcohorts stratified by gender, race, or alcoholic cirrhosis to investigate the influence of these factors. This analysis helped us to discover gender, race, or alcoholic cirrhosis-specific biomarkers. For the U.S. cohort, the AFP values were available for both hepatocellular carcinoma cases and cirrhotic controls. So, we considered three logistic regression models with the following predictors: (i) gender and AFP; (ii) gender and selected metabolites; and (iii) gender, selected metabolites, and AFP. Area under the receiver operating characteristic (AuROC) curve was used to evaluate the performance of each model. Model cross-validation was performed 500 times by randomly choosing 35% of the samples from each group. The remaining 65% are used for model training.

Results

Supplementary Table S1 presents the results of the statistical analysis based on the log-transformed metabolite levels measured by SRM in 40 hepatocellular carcinoma cases and 49 cirrhotic controls in the Egyptian cohort. The hepatocellular carcinoma cases are stratified by stage into two groups. The first group consist of 29 stage I cases. The second group has 8 cases of stages II and III combined. For each metabolite, the statistical significance levels (P values) are presented on the basis of the univariate logistic regression model in distinguishing hepatocellular carcinoma from cirrhosis and the univariate proportional odds model for the subgroups stratified by tumor stage. We observe that the metabolites with significant and consistent discrimination between hepatocellular carcinoma cases and cirrhotic controls are primarily involved in bile acid biosynthesis, long-chain carnitine, and small peptide metabolism. Deregulation of bile acids (GCDCA, GDCA, GCA, and TCDCA etc.) in serum has been reported in association with liver diseases, for example, liver cirrhosis and hepatitis (11, 19, 20). Previous hepatocellular carcinoma studies also indicated the downregulation of those bile acids metabolites in serum compared with liver cirrhosis (15, 16, 20, 29). Meanwhile the abnormal expression of conjugated bile acids and sulfation product was investigated in injured liver during hepatocellular carcinoma carcinogenesis that could lead to deteriorating detoxification of endogenous and exogenous

lipophilic compounds (20, 30, 31). As shown in Supplementary Table S1, GCA, GDCA, and TCDCA significantly discriminate stage II and III hepatocellular carcinoma cases from cirrhotic controls ($P < 0.05$). Downregulation of these three metabolites was observed in the progression of the disease from cirrhosis to early-stage and to late-stage, indicating their metabolic deregulation during the process of hepatocellular carcinoma tumorigenesis. Although GCA, TCDCA, and 3sulfo-GCDCA do not significantly distinguish early-stage hepatocellular carcinoma cases from cirrhotic controls, we observe consistent pattern and statistically significant discrimination of stage I cases and stage II and III cases from cirrhosis for GDCA.

Supplementary Table S2 presents statistical analysis results for the U.S. cohort with 56 hepatocellular carcinoma cases and 54 cirrhotic controls. Two metabolites (3sulfo-GCDCA and 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid) significantly distinguish hepatocellular carcinoma cases from cirrhotic controls. The early-stage group consists of 30 stage I hepatocellular carcinoma cases. The late-stage group has 18 hepatocellular carcinoma cases of stages II, III, and IV combined. Consistent downregulation patterns and statistically significant discrimination of early-stage hepatocellular carcinoma from cirrhosis are observed for these two metabolites. LPC 17:0 can significantly discriminate early-stage from cirrhotic controls with an upregulation pattern. These three metabolites may reveal early signs of hepatocellular carcinoma in patients with liver cirrhosis. Of note, 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid can distinguish late-stage hepatocellular carcinoma from cirrhotic controls as well.

We conducted stratified analyses to evaluate the impact of gender on the ability of the metabolites in distinguishing hepatocellular carcinoma cases and cirrhotic controls in both the Egyptian and the U.S. cohorts, as well as the impact of race and alcoholic cirrhosis in the U.S. cohort because it has been known that such cohort compositions can affect the relative concentration of small-molecule metabolites in the serum. Supplementary Table S1 shows that all except GCA, 3sulfo-GCDCA, and Phe-Phe are significant in the male Egyptian group (31 hepatocellular carcinoma cases and 33 cirrhotic controls), whereas only Phe-Phe shows significance in the female Egyptian group (9 hepatocellular carcinoma cases and 16 cirrhotic controls). Supplementary Table S2 indicates that for the U.S. cohort, the two significant metabolite candidates are significant in the male group (42 hepatocellular carcinoma cases and 34 cirrhotic controls) but insignificant in the female group (14 hepatocellular carcinoma cases and 20 cirrhotic controls). Oleoyl carnitine is significant in the female group but not in the male group. The U.S. cohort includes 63 Caucasians (31 hepatocellular carcinoma cases and 32 cirrhotic controls) and 31 African Americans (17 hepatocellular carcinoma cases and 14 cirrhotic controls). We observe that none of the two-metabolite candidates can differentiate hepatocellular carcinoma cases

from cirrhotic controls among African Americans, but can differentiate hepatocellular carcinoma cases in the Caucasian group. Though oleoyl carnitine does not seem to be significant in considering all participants in the U.S. cohort, it shows marginal significance in discriminating hepatocellular carcinoma cases from cirrhotic controls among African American patients. As female participants account for only about 30% in both cohorts and African Americans account for less than 30% in the U.S. cohort, the observations about gender and race could be merely due to lack of adequate statistical power. Also, we observed that in patients with alcohol-induced cirrhosis (16 hepatocellular carcinoma cases and 18 cirrhotic controls), 3sulfo-GCDCA distinguishes hepatocellular carcinoma cases from cirrhotic controls, whereas in patients with nonalcoholic cirrhosis (40 hepatocellular carcinoma cases and 36 cirrhotic controls), 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid distinguishes the two groups.

Because 3sulfo-GCDCA and 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid significantly discriminate hepatocellular carcinoma cases from cirrhotic controls in the U.S. cohort and are marginally significant in the Egyptian cohort, we evaluated the diagnostic capability of these two metabolites combined with gender in a panel. Table 4 presents the evaluation of the model through AuROC curve. A model trained using the U.S. cohort with gender, 3sulfo-GCDCA, and 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid as predictors yielded an AuROC of 0.74 in distinguishing the hepatocellular carcinoma cases from cirrhotic controls. An AuROC of 0.70 was obtained in evaluating the model by cross-validation. The model resulted in an AuROC of 0.66 when the Egyptian cohort is used as an independent testing set. Another model trained using the Egyptian cohort yielded an AuROC of 0.69. The model resulted in an AuROC of 0.63 in cross-validation and 0.70 when using the U.S. cohort as a testing set. A model built using the combination of the U.S. and Egyptian cohort as a training set yielded an AuROC of 0.71 and an AuROC of 0.69 in cross-validation. Because AFP measurements were unavailable for the cirrhotic controls in the Egyptian cohort, we compared the performance of these metabolites together with AFP for the U.S. cohort only. Gender and AFP alone resulted in an AuROC of 0.69 using the U.S. cohort as a training set and an AuROC of 0.68 in cross-validation. The combination of gender, AFP and the two metabolites increased the AuROC to 0.79 during training, and increased the AuROC to 0.74 in cross-validation. For further evaluation, we split the U.S. cohort into two groups based on the time of sample collection that is the samples that were collected before March 2012 (US1) and those collected after March 2012 (US2). US1 includes 20 hepatocellular carcinoma cases and 20 cirrhotic controls, whereas US2 includes 36 hepatocellular carcinoma cases and 34 cirrhotic controls. These two sets are used as training and testing sets interchangeably to evaluate the performance of the metabolites with and without AFP. The results indicate that improved diagnosis of hepatocellular carcinoma in high-risk population of cirrhotic

Table 4. Performance evaluation of three logistic regression models using different predictors: (i) AFP and gender; (ii) two metabolites and gender; (iii) AFP, two metabolites, and gender

Metabolite	Egypt (TRN) and US (TES)															
	Egypt and US (TRN)				US (TRN) and Egypt (TES)				US1 (TRN) and US2 (TES)				US2 (TRN) and US1 (TES)			
	TRN	CRV	TRN	CRV	US	US1	US2	TRN	CRV	TES	TRN	CRV	TES	TRN	CRV	TES
AFP	-	-	-	-	-	-	-	0.69	0.68	-	-	-	-	0.75	0.75	0.70
AuROC	-	-	-	-	-	-	-	(0.59, 0.80)	(0.50, 0.86)	-	-	-	-	(0.59, 0.90)	(0.48, 0.98)	(0.56, 0.83)
95% CI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(0.43, 0.91)
Metabolite	0.71	0.69	0.63	0.63	0.70	0.78	0.65	0.74	0.70	0.66	0.81	0.74	0.63	0.71	0.66	0.73
AuROC	(0.63, 0.78)	(0.57, 0.82)	(0.58, 0.80)	(0.43, 0.83)	(0.60, 0.80)	(0.64, 0.93)	(0.51, 0.78)	(0.64, 0.83)	(0.53, 0.87)	(0.54, 0.77)	(0.67, 0.94)	(0.47, 0.98)	(0.49, 0.98)	(0.58, 0.84)	(0.43, 0.89)	(0.57, 0.90)
95% CI	-	-	-	-	-	-	-	0.79	0.74	-	0.89	0.81	0.68	0.74	0.67	0.75
Metabolite	-	-	-	-	-	-	-	(0.69, 0.88)	(0.58, 0.91)	-	-	-	-	(0.79, 0.99)	(0.58, 0.99)	(0.54, 0.82)
AuROC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(0.60, 0.91)
95% CI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(0.42, 0.92)

NOTE: The metabolites are 3sulfo-GCDCA and 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid.

Abbreviations: CI, confidence interval; CRV, cross-validation; TES, the cohort used for testing; TRN, the cohort used for training.

patients can be achieved by combining the metabolites with AFP and gender information.

Discussion

In this study, we evaluated the levels of targeted metabolites in sera of hepatocellular carcinoma cases and cirrhotic controls from two cohorts (Egypt and the United States) by SRM on UPLC-ESI-QLITMS. We found that hepatocellular carcinoma in Egyptian cohort is associated with dysregulated levels of bile acids, LPCs, LPEs, sphingophospholipid, long-chain carnitines, and amino acids. Hepatocellular carcinoma in the U.S. cohort is associated with abnormal bile acids metabolism. We found that two metabolites (3sulfo-GCDCA and 3 β ,6 β -dihydroxy-5 β -cholan-24-oic acid) are relevant for both the Egyptian and the U.S. cohorts. Together with gender and AFP, these two metabolites led to significant improvement in AuROC compared with gender and AFP only (AuROC 0.66–0.81 vs. 0.66–0.75 based on cross-validation and independent testing). This indicates the potential of these two candidate biomarkers in combination with AFP and gender information for improved diagnosis of hepatocellular carcinoma in high-risk population of cirrhotic patients. Thus, following appropriate clinical validation, the metabolic perturbations of these candidates can be used for early detection of hepatocellular carcinoma or to monitor the progression of cirrhosis to hepatocellular carcinoma. The proportional odds model discovered candidate biomarkers that are specific to the tumor stage level. This improves the ability to detect early-stage hepatocellular carcinoma in high-risk population of liver cirrhosis when treatment is more effective. Our stratified statistical analysis reveals that gender, race, and alcoholic cirrhosis play

a decisive role in affecting the relative concentration of small molecules in the serum. These findings will not only contribute to a greater understanding of the implications of health disparities in hepatocellular carcinoma, but also improve the ability to accurately diagnose hepatocellular carcinoma through gender, race, and alcoholic cirrhosis-specific biomarkers.

Disclosure of Potential Conflicts of Interest

K. Shetty has received commercial research grant support from Bayer Pharmaceuticals and has honoraria from speakers' bureau from Onyx. No potential conflicts of interest were disclosed by the other authors.

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