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Research Article

Cancer Prevention Research

Metabolomics Profiles of Hepatocellular Carcinoma in a Korean Prospective Cohort: The Korean Cancer Prevention Study-II

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Abstract

In the prospective Korean Cancer Prevention Study-II (KCPS-II), we investigated the application of metabolomics to differentiate subjects with incident hepatocellular carcinoma (HCC group) from subjects who remained free of cancer (control group) during a mean follow-up period of 7 years with the aim of identifying valuable metabolic biomarkers for HCC. We used baseline serum samples from 75 subjects with incident HCC and 134 age- and gender-matched cancer-free subjects. Serum metabolic profiles associated with HCC incidence were investigated via metabolomics analysis. Compared with the control group, the HCC group showed significantly higher serum levels of aspartate aminotransferase (AST), alanine aminotransferase, and γ-glutamyl transpeptidase. At baseline, compared with the control group, the HCC group showed significantly higher levels of 9 metabolites, including leucine, 5-hydroxyhexanoic acid, phenylalanine, tyrosine, arachidonic acid, and tauroursodeoxycholic acid (TUDCA), but lower levels of 28 metabolites, including oleamide, androsterone sulfate, L-palmitoylcarnitine, lysophosphatidic acid (LPA) 16:0, LPA 18:1, and lysophosphatidylcholines (lysoPC). Multiple linear regression revealed that the incidence of HCC was associated with the levels of tyrosine, AST, lysoPCs (16:1, 20:3), oleamide, 5-hydroxyhexanoic acid, androsterone sulfate, and TUDCA (adjusted $R^2 = 0.514$, $P = 0.036$). This study showed the clinical relevance of the dysregulation of not only branched amino acids, aromatic amino acids, and lysoPCs but also bile acid biosynthesis and linoleic acid, arachidonic acid, and fatty acid metabolism. In addition, tyrosine, AST, lysoPCs (16:1, 20:3), oleamide, 5-hydroxyhexanoic acid, androsterone sulfate, and TUDCA were identified as independent variables associated with the incidence of HCC. *Cancer Prev Res; 11(5); 1–10. ©2018 AACR.*

Introduction

Hepatocellular carcinoma (HCC), the most prevalent form of liver cancer, is difficult to diagnose and has limited treatment options, resulting in a low survival rate. In particular, the 5-year overall survival rate for patients with HCC is less than 5% (1). Liver cirrhosis and infection with hepatitis B or C virus (HBV and HCV, respectively) are common causes of HCC. Notably, HBV is specifically a major risk factor for HCC in Asia. Although tremendous efforts have been made to discover novel biomarkers for the early diagnosis of HCC in clinical practice over the past few decades, recent approaches to HCC diagnosis involving measurement of serum α-fetoprotein and liver imaging lack sufficient specificity and sensitivity (2). Improving both understanding of HCC etiology and early detection of the disease is an important first step toward designing effective prevention strategies aimed at early diagnosis and reduction of HCC incidence.

Metabolomics systematically analyzes all small molecules in a biological sample, and this technology is being widely used for the detection of metabolic biomarkers (3, 4). Metabolic profiling of HCC is currently underway, but the results of studies of prediagnostic metabolic biomarkers are contradictory. In addition, little information has been derived from prospective settings, in which biological samples are collected prior to disease diagnosis (5–7). Fages and colleagues reported for the first time the metabolic patterns in Europeans that are associated with HCC risk and involved in the dysregulation of fatty acid

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oxidation and carbohydrate, amino acid, and lipid metabolism within a short time period prior to clinical diagnosis (8). The levels of endogenous metabolites associated with the risk of HCC can vary with not only changes in lifestyle and metabolic status but also differences in the equipment used for metabolic profiling. Moreover, a longer study period would generally be more accurate in predicting the risk of HCC development. Therefore, in the prospective Korean Cancer Prevention Study-II (KCPS-II), which enrolled a cancer-free cohort at baseline, we applied metabolomics to differentiate individuals with incident HCC (HCC group) from age- and gender-matched controls who remained free of cancer (healthy controls) during a mean follow-up period of 7 years. Our objective was to identify baseline metabolic biomarkers with value in discriminating between these two groups using data derived from the serum metabolic profiles of the HCC and healthy control groups.

Materials and Methods

Subjects

We used data from the Korean Metabolic Syndrome Research Initiative in Seoul, which was initiated in 2005, and we labeled this study as the "Korean Cancer Prevention Study-II (KCPS-II)," as noted above. A detailed description of the KCPS-II is provided in our previous publications (9, 10). Study subjects were recruited from among participants in routine health assessments at health promotion centers in Seoul and Gyeonggi Province, Korea, between 2004 and 2013. From an initial study population comprising 270,514 individuals, we acquired written informed consent for both study participation and publication from 156,701 participants. The Institutional Review Board of Yonsei University (Seoul, Korea) reviewed and approved the study protocol, which complied with the Declaration of Helsinki. Among these individuals, age- and gender-matched subjects were included in our study at a ratio of approximately 1:2 for a total of 75 HCC patients and 134 healthy subjects.

Questionnaire and anthropometric measurements

Each participant was interviewed using a structured questionnaire to collect the individual's smoking history (never smoker, ex-smoker, or current smoker) and alcohol drinking history. Participant height and weight were measured while the participants were wearing light clothing, and body mass index (BMI) was calculated by dividing body weight (kg) by height squared (m^2). In addition, systolic and diastolic blood pressures were measured after a rest period of at least 15 minutes, and waist circumference was measured midway between the lower rib and the iliac crest.

Blood collection and biochemical analyses

For clinical chemistry assays, serum was separated from the peripheral venous blood obtained from each participant after a minimum fasting period of 12 hours and was stored at −70°C until further analysis. The levels of fasting glucose, total cholesterol, triglyceride, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP) were measured using automatic analyzers. Each laboratory measurement was performed according to internal and external quality control procedures according to the Korean Association of Laboratory Quality Control. The agreement for each biomarker across individual hospitals was high (correlation coefficients ranging from 0.96 to 0.99; ref. 11).

Diagnosis of HCC and HBV/HCV infection

Data regarding the incidence of HCC were obtained from the records of the National Cancer Registry as well as from the hospitals where the participants had been treated. Although Korea has a National Cancer Registry, the reports were not complete during follow-up; therefore, hospital admission records were used to identify the initial admission events leading to HCC diagnosis. HCC was considered as present based on a positive report either from the National Cancer Registry or upon hospital admission for cancer diagnosis. According to the International Classification of Diseases, Tenth Revision (ICD-10), HCC was coded as C22 (12). HBV surface antigen (HBsAg) was detected in serum using enzymatic assays (Boehringer Mannheim) on a Hitachi 737 autoanalyzer. Serum HBsAg was tested by a radioimmunoassay or reverse passive hemagglutination in hospital laboratories (Ausria II, Abbott). HCV antibodies were detected using the ECLIA (Elecsys Cyclosporine; Roche Diagnostics GmbH; ref. 13).

Nontargeted metabolic profiling of serum

The detailed methods for sample preparation and analysis using ultraperformance liquid chromatography (UPLC)-linear-trap quadrupole (LTQ)-Orbitrap mass spectrometry (MS), data processing, and putative identification of serum metabolites were reported in a previous study (14).

Statistical analysis

Statistical analysis was performed using SPSS version 23.0 (IBM/SPSS). Logarithmic transformation was performed on skewed variables. For descriptive purposes, mean values are presented as untransformed values. Overall, the results are expressed as the mean ± SE. A two-tailed P value <0.05 was considered statistically significant. Independent t tests were used to compare parameters between the two groups, and Pearson correlation coefficient was used to examine relationships between variables. FDR-corrected q-values were computed using the R package "fdrtool," and q-values less than 0.05 were considered to indicate significance. Stepwise regression analysis was also

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Table 1. Clinical characteristics of cancer-free controls and incident cases of HCC

	Control group ($n = 134$)	HCC group ($n = 75$)	P^a	P^b
Age (years)	52.7 ± 0.81	52.8 ± 1.18	0.968	0.983
Male/female *n*, (%)	121 (90.3)/13 (9.7)	67 (89.3)/8 (10.7)	0.824	0.793
Current smoker *n*, (%)	42 (33.3)	31 (45.6)	0.093	
Alcohol drinker *n*, (%)	103 (82.4)	48 (71.6)	0.083	
Alcohol intake (g/day)[c]	18.2 ± 3.24	22.3 ± 7.24	0.235	0.359
Weight (kg)	69.2 ± 0.89	69.7 ± 1.17	0.744	0.892
Height (cm)	168.0 ± 0.61	168.5 ± 0.88	0.604	0.601
Waist circumference (cm)	84.9 ± 0.88	86.8 ± 1.07	0.154	0.305
BMI (kg/m^2)	24.5 ± 0.25	24.6 ± 0.38	0.868	0.966
Systolic blood pressure (mmHg)	122.8 ± 1.3	121.5 ± 1.59	0.552	0.730
Diastolic blood pressure (mmHg)	77.4 ± 1.00	76.5 ± 1.18	0.590	0.803
Glucose (mg/dL)[c]	96.8 ± 1.78	98.7 ± 2.70	0.546	0.623
Triglyceride (mg/dL)[c]	135.1 ± 7.20	120.4 ± 9.24	0.133	0.064
Total cholesterol (mg/dL)[c]	188.6 ± 2.76	179.6 ± 3.41	0.049	0.068
HDL-cholesterol (mg/dL)[c]	50.6 ± 0.93	51.0 ± 1.36	0.871	0.803
LDL-cholesterol (mg/dL)[c]	114.2 ± 2.44	106.8 ± 3.52	0.063	0.101
AST (IU/L)[c]	24.1 ± 0.63	42.9 ± 4.08	<0.001	<0.001
ALT (IU/L)[c]	27.5 ± 1.56	49.7 ± 8.95	<0.001	<0.001
γ-GTP (U/L)[c]	42.9 ± 4.98	90.7 ± 15.4	<0.001	<0.001
hs-CRP (mg/L)[c]	0.21 ± 0.03	0.22 ± 0.06	0.509	0.325
HBV-positive result (%)	5.7	70.5	<0.001	
HCV-positive result (%)	0.9	0.0	0.521	

NOTE: Mean ± SE.
[a]*P* values derived from independent *t* test.
[b]*P* values adjusted for smoking and drinking.
[c]Tested by logarithmic transformation.

performed to determine the independent effects of variables on the incidence of HCC. On the basis of the results of the stepwise regression analysis, the optimal cut-off value for each biochemical characteristic was determined using ROC curve analysis. In addition, heatmaps were created to visualize and evaluate relationships among metabolites and biochemical measurements in the study population. Metabolic pathway analysis was performed using MetaboAnalyst 3.0 (http://metaboanalyst.ca). A detailed method for pathway analysis is provided in Supplementary Table S1.

The spectrometric data were exported to SIMCA-P+ 14.0 (Umetrics, Inc.) for multivariate analysis. Prior to in-depth analysis, Pareto scaling was applied to all data. We used a supervised classification tool, namely orthogonal projection to latent structures-discriminant analysis (OPLS-DA), to analyze our models. The validity of the models was also assessed using R^2Y and Q^2Y parameters and cross-validation-analysis of variance (CV-ANOVA). Next, we performed total ion chromatography (TIC) normalization using SIEVE software (Thermo Fisher Scientific).

Results

Clinical characteristics at baseline

Over a mean follow-up period of 7 years, 75 subjects developed HCC among 156,701 participants aged 20 to 84 years. Among those 75 subjects, the mean diagnostic time after inclusion in the study was 4.4 years. We used the 75 patients with incident HCC as the HCC group and 134 age- and gender-matched controls who remained free of cancer as the control group. After adjusting for smoking and drinking, no significant differences in baseline characteristics, including BMI and systolic and diastolic blood pressures as well as serum levels of glucose, triglyceride, total cholesterol, HDL cholesterol, LDL cholesterol, and hs-CRP, were observed between the age- and gender-matched control group (free of cancer, $n = 134$) and the HCC group ($n = 75$; Table 1). However, the HCC group showed significantly lower leukocyte numbers and significantly higher levels of AST ($P < 0.001$), ALT ($P < 0.001$), and γ-glutamyl transpeptidase (γ-GTP; $P < 0.001$). There was no evidence that the elevated serum enzyme levels were biased toward those cases diagnosed within 1 or 2 years of baseline (Supplementary Table S2). Serum blood samples of the HCC group were more likely to test positive for HBV infection (70.5% in the HCC group vs. 5.7% in the control group). In addition, only one control subject tested positive for HCV infection (Table 1).

Serum metabolic profiling using UPLC-LTQ-Orbitrap MS

Nontargeted metabolic pattern analysis. The total ion chromatography data (from both positive and negative ion modes) obtained at baseline for serum metabolites were analyzed using an OPLS-DA score plot. In particular, OPLS-DA was conducted to compare baseline metabolite levels between the control group (free of cancer both at baseline and at 7 years of follow-up; $n = 134$) and the HCC group (free of cancer at baseline but developed incident HCC during the 7 years of follow-up; $n = 75$) in positive ion mode (Fig. 1A). The quality of the OPLS-DA was examined using the R^2 and Q^2 values to confirm that the models were not overfitted and to evaluate the predictive ability of each

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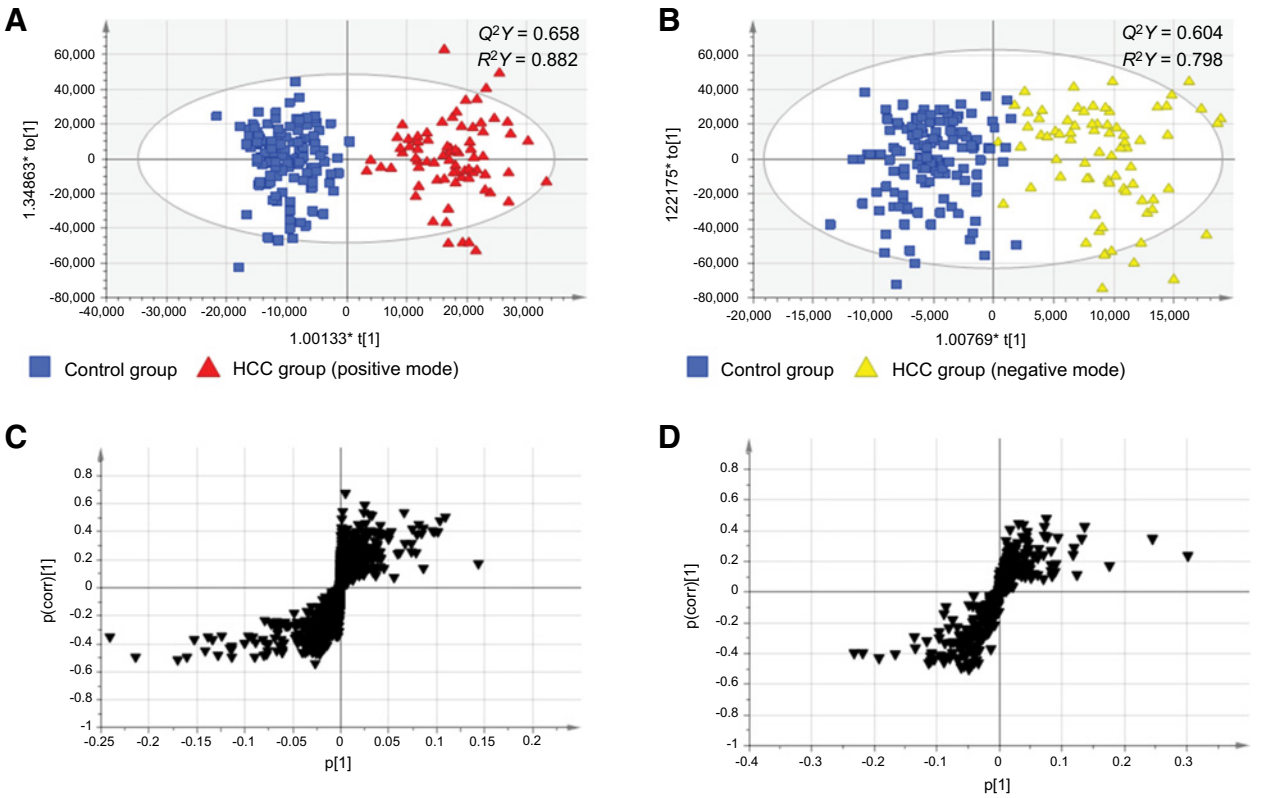

Figure 1.

Comparison of serum metabolites between the control and HCC groups. **A,** Score plots from OPLS-DA models classifying baseline profiles in positive ion mode. **B,** Score plots from OPLS-DA models classifying baseline profiles in negative ion mode. **A** and **B,** Comparison between the baseline levels in the control group (free of cancer at baseline and follow-up; $n = 134$) and the baseline levels in the HCC group (free of cancer at baseline and incident HCC at follow-up; $n = 75$). **C,** *S*-plots for covariance (p) and reliability correlation [p(corr)] from OPLS-DA models in positive ion mode. **D,** *S*-plots for covariance (p) and reliability correlation [p(corr)] from OPLS-DA models in negative ion mode.

model. This analysis revealed that the model shown in Fig. 1A displayed a goodness of fit of 88.2% ($R^2Y = 0.882$) and a predictive ability of 65.8% ($Q^2Y = 0.658$), showing that the OPLS-DA model was well fitted and displayed acceptable predictability. OPLS-DA was also conducted to compare baseline metabolite levels between the control group and the HCC group in negative ion mode with the statistical parameters $R^2Y = 0.798$ and $Q^2Y = 0.604$ (Fig. 1B). The presented results suggest that this model could discriminate between the groups based on differences in metabolite abundances. The permutation plots (Supplementary Fig. S1) for the OPLS-DA model strongly indicate that the original model is valid. The criteria for validity are as follows: All blue Q^2 values to the left are lower than the original values to the right or the blue regression line of the Q^2 values intersects the vertical axis (on the left) at or below zero (15, 16). Moreover, CV-ANOVA (Supplementary Table S3) showed lower *P* values, implying that the separation between the groups is significant. To extract potential variables contributing to the detected differences, *S*-plots of $p(1)$ and $p(\text{corr})(1)$ were generated for the OPLS-DA models using Pareto scaling in both positive ion mode (Fig. 1C) and negative ion mode (Fig. 1D). The *S*-plots revealed that metabolites with higher or lower $p(\text{corr})$ values were more relevant in discriminating between the two groups. Furthermore, the Q^2Y values for HBV-positive and HBV-negative results in both the positive and negative ion modes were less than 0.5 (data not shown). Models with $Q^2Y \geq 0.5$ are considered to have good predictive capability; thus, this measure did not provide an estimate of the predictive ability of the model.

Putative identification of serum metabolites. Among 1,960 and 516 variables obtained in positive and negative ion mode, respectively, the peaks (variables) that played an important role in the separation between the groups were selected according to the parameter of variable importance in the projection (VIP), and VIP values >1.5 indicated high relevance to the difference between sample groups.

When comparing baseline metabolite levels between the control group (free of cancer both at baseline and at 7 years of follow-up; $n = 134$) and the HCC group (free of cancer at baseline but developed incident HCC during the 7 years of follow-up; $n = 75$), a total (both positive and negative ion

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Table 2. Putative identification of upregulated serum metabolites in the control and HCC groups

M/Z [M+H][a] [M−H][b]	Molecular formula	Putative identified metabolite	VIP	*t*-test		Cohen *d*
				P	*q*	
132.102	$C_6H_{13}NO_2$	L-Leucine[a]	3.682	<0.001	1.84E−04	0.591
133.086	$C_6H_{12}O_3$	**5-Hydroxyhexanoic acid[a]**	1.631	0.008	7.47E−03	0.461
166.086	$C_9H_{11}NO_2$	L-Phenylalanine[a]	3.353	<0.001	9.22E−05	0.615
182.081	$C_9H_{11}NO_3$	**L-Tyrosine[a]**	3.844	<0.001	3.24E−09	1.192
205.097	$C_{11}H_{12}N_2O_2$	L-Tryptophan[a]	2.608	0.776	0.287	0.041
253.217	$C_{16}H_{30}O_2$	Palmitoleic acid[b]	2.871	0.180	0.098	0.220
279.232	$C_{18}H_{32}O_2$	Linoleic acid[b]	4.466	0.098	0.062	0.267
295.226	$C_{18}H_{30}O_3$	9-HOTE[a]	1.961	0.145	0.084	0.255
295.227	$C_{18}H_{32}O_3$	Alpha-dimorphecolic acid[b]	2.116	0.116	0.071	0.302
301.216	$C_{20}H_{30}O_2$	Eicosapentaenoic acid[b]	1.968	0.302	0.142	0.157
303.232	$C_{20}H_{32}O_2$	Arachidonic acid[b]	4.243	0.032	2.51E−02	0.373
305.248	$C_{20}H_{34}O_2$	8,11,14-Eicosatrienoic acid[b]	2.401	0.005	5.23E−03	0.502
319.227	$C_{20}H_{32}O_3$	15(S)-HETE[b]	2.321	0.235	0.119	0.227
325.216	$C_{22}H_{28}O_2$	Etonogestrel[a]	1.713	0.850	0.306	0.027
327.232	$C_{22}H_{32}O_2$	Docosahexaenoic acid[b]	4.127	0.117	0.071	0.260
329.247	$C_{22}H_{34}O_2$	Docosapentaenoic acid[b]	1.537	0.189	0.101	0.211
343.227	$C_{22}H_{32}O_3$	8-HDoHE[b]	2.557	0.271	0.132	0.209
448.306	$C_{26}H_{43}NO_5$	Glycoursodeoxycholic acid[b]	4.341	0.001	1.24E−03	0.941
464.300	$C_{26}H_{43}NO_6$	Glycocholic acid[b]	2.171	0.013	1.18E−02	0.759
498.288	$C_{26}H_{45}NO_6S$	**Tauroursodeoxycholic acid[b]**	2.320	<0.001	3.55E−04	1.126

NOTE: Bold type indicates the metabolic markers that are independently associated with HCC incidence based on a stepwise regression analysis. *P* values were derived from independent *t* tests between the control group ($n = 134$) and the HCC group ($n = 75$). The *q*-value is an adjusted *P* value that controls for the FDR. Cohen *d* is an effect size for a comparison between two means; differences between two means were divided by a pooled SD and defined as "small, $d = 0.20$," "medium, $d = 0.50$," or "large, $d = 0.80$." The trend in change was determined through a comparison of the peak intensities of the metabolites in the HCC group compared with the control group.

[a]Metabolites obtained in ESI-positive ion mode (*M/Z* [M+H]).

[b]Metabolites obtained in ESI-negative ion mode (*M/Z* [M−H]).

modes) of 180 variables were selected on the basis of VIP values >1.5. In all, 55 of these variables had been previously identified, whereas the remaining variables were novel. Tables 2 and 3 show putative identification of serum metabolites in the control and HCC groups. Compared with the control group, the HCC group showed significantly higher peak intensities for 9 metabolites. In addition, compared with the control group, the HCC group showed a trend toward an increase in the level of linoleic acid (Table 2). However, compared with the control group, the HCC group showed significantly lower peak intensities for 28 metabolites (Table 3). There was no evidence that the metabolites measured in this metabolomics study were biased toward those cases diagnosed within 1 or 2 years of baseline (Supplementary Table S4).

Metabolic pathways relevant to the pathogenesis of HCC in healthy subjects. To identify the most relevant pathways associated with the selected metabolites, a metabolic pathway analysis was performed using MetaboAnalyst 3.0, a web-based analysis module (Fig. 2; ref. 17) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Through pathway analysis, we mapped 11 correlated metabolic pathways with the identified 55 metabolites. Enrichment analysis was conducted for the 55 metabolites (VIP > 1.5), and all 11 pathways had low *q*-values (FDR < 0.05). These pathways also had high pathway impacts (over 0) obtained from topology analysis (Supplementary Table S5). Through the pathway analysis, L-tyrosine was the most significantly overrepresented metabolite in tyrosine metabolism ($q = 1.85E{-}18$), phenylalanine metabolism ($q = 4.16E{-}18$), and phenylalanine, tyrosine, and tryptophan biosynthesis ($q = 6.39E{-}16$). In addition, the linoleic acid metabolism pathway exhibited a high score in topology analysis (0.656), indicating that significant metabolites in this pathway have a high degree of interaction with other molecules in the same pathway.

Correlations among the clinical parameters and major serum metabolites according to the incidence of HCC

We generated a correlation matrix containing clinical characteristics and major metabolites according to the incidence of HCC for the 75 patients with incident HCC during the 7 years of follow-up and the 134 age- and gender-matched controls, who remained free of cancer (Supplementary Fig. S2). To determine the independent effects of different variables on the incidence of HCC, stepwise regression analysis of the following variables was performed: leukocyte number; AST levels; ALT levels; γ-GTP levels; and 37 metabolites, including isovaleraldehyde, 4-hydroxynonenal, azelaic acid, 3-hydroxysebacic acid, 3-hydroxydodecanedioic acid, 3-hydroxytetradecanedioic acid, oleamide, phenylalanyl-phenylalanine, dehydroepiandrosterone sulfate, androsterone sulfate, L-palmitoylcarnitine, LPA 16:0, LPA 18:1, lysophosphatidylcholines (lysoPC; 14:0, 15:0, 16:1, 16:0, 17:0, 18:4, 18:3, 18:2, 18:1, 20:5, 20:4, 20:3, 22:6, 22:5), leucine, 5-hydroxyhexanoic acid, phenylalanine, tyrosine, arachidonic acid, 8,11,14- eicosatrienoic acid, glycoursodeoxycholic acid,

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Table 3. Putative identification of downregulated serum metabolites in the control and HCC groups

M/Z [M+H][a] [M−H][b]	Molecular formula	Putative identified metabolite	VIP	*t* test		Cohen *d*
				P	*q*	
87.080	$C_5H_{10}O$	Isovaleraldehyde[a]	1.547	<0.001	1.22E−04	−0.540
157.122	$C_9H_{16}O_2$	4-Hydroxynonenal[a]	1.585	<0.001	1.32E−04	−0.536
189.112	$C_9H_{16}O_4$	Azelaic acid[a]	1.590	<0.001	7.39E−05	−0.554
219.122	$C_{10}H_{18}O_5$	3-Hydroxysebacic acid[a]	2.118	<0.001	6.91E−05	−0.551
227.201	$C_{14}H_{28}O_2$	Myristic acid[b]	1.571	0.093	0.060	−0.244
229.143	$C_{12}H_{20}O_4$	Traumatic acid[a]	3.256	<0.001	6.85E−05	−0.544
247.154	$C_{12}H_{22}O_5$	3-Hydroxydodecanedioic acid[a]	3.515	<0.001	6.94E−05	−0.546
255.232	$C_{16}H_{32}O_2$	Palmitic acid[b]	5.146	0.735	0.276	−0.049
275.185	$C_{14}H_{26}O_5$	3-Hydroxytetradecanedioic acid[a]	3.790	<0.001	1.12E−04	−0.525
277.216	$C_{18}H_{30}O_2$	Gamma-linolenic acid[b]	1.725	0.270	0.131	−0.160
281.248	$C_{18}H_{34}O_2$	Oleic acid[b]	5.867	0.938	0.327	−0.011
282.279	$C_{18}H_{35}NO$	**Oleamide**[a]	2.817	<0.001	2.17E−05	−0.580
283.263	$C_{18}H_{36}O_2$	Stearic acid[b]	3.144	0.356	0.161	−0.133
301.216	$C_{20}H_{28}O_2$	All-trans-retinoic acid[a]	2.211	0.904	0.319	−0.017
313.154	$C_{18}H_{20}N_2O_3$	Phenylalanylphenylalanine[a]	3.472	<0.001	5.30E−10	−0.893
367.157	$C_{19}H_{28}O_5S$	Dehydroepiandrosterone sulfate[b]	2.382	<0.001	4.26E−04	−0.548
369.173	$C_{19}H_{30}O_5S$	**Androsterone sulfate**[b]	2.784	<0.001	1.08E−06	−0.780
400.342	$C_{23}H_{45}NO_4$	L-Palmitoylcarnitine[a]	1.518	<0.001	1.26E−05	−0.624
409.235	$C_{19}H_{39}O_7P$	LPA (16:0)[b]	4.089	<0.001	4.64E−07	−0.810
435.250	$C_{21}H_{41}O_7P$	LPA (18:1)[b]	2.338	<0.001	4.18E−06	−0.731
468.307	$C_{22}H_{46}NO_7P$	LysoPC (14:0)[a]	4.560	<0.001	3.22E−10	−1.051
480.344	$C_{24}H_{50}NO_6P$	LysoPC (P-16:0)[a]	2.151	0.820	0.299	−0.033
482.323	$C_{23}H_{48}NO_7P$	LysoPC (15:0)[a]	2.860	<0.001	3.77E−08	−0.865
494.323	$C_{24}H_{48}NO_7P$	**LysoPC (16:1)**[a]	5.700	<0.001	1.81E−10	−1.066
496.339	$C_{24}H_{50}NO_7P$	LysoPC (16:0)[a]	9.992	<0.001	4.62E−06	−0.728
510.355	$C_{25}H_{52}NO_7P$	LysoPC (17:0)[a]	3.721	<0.001	2.26E−06	−0.760
516.308	$C_{26}H_{46}NO_7P$	LysoPC (18:4)[a]	2.067	<0.001	3.88E−08	−0.821
518.323	$C_{26}H_{48}NO_7P$	LysoPC (18:3)[a]	4.068	<0.001	8.63E−09	−0.942
520.338	$C_{26}H_{50}NO_7P$	LysoPC (18:2)[a]	7.483	<0.001	1.41E−08	−1.070
522.354	$C_{26}H_{52}NO_7P$	LysoPC (18:1)[a]	6.090	<0.001	2.09E−05	−0.766
542.323	$C_{28}H_{48}NO_7P$	LysoPC (20:5)[a]	5.908	<0.001	3.32E−11	−1.117
544.338	$C_{28}H_{50}NO_7P$	LysoPC (20:4)[a]	5.182	<0.001	5.67E−09	−0.958
546.354	$C_{28}H_{52}NO_7P$	**LysoPC (20:3)**[a]	4.878	<0.001	1.97E−08	−0.812
568.338	$C_{30}H_{50}NO_7P$	LysoPC (22:6)[a]	4.158	<0.001	6.43E−08	−0.875
570.354	$C_{30}H_{52}NO_7P$	LysoPC (22:5)[a]	1.931	0.001	1.11E−03	−0.494

NOTE: Bold type indicates the metabolic markers that are independently associated with HCC incidence based on a stepwise regression analysis. *P* values were derived from independent *t* tests between the control group ($n = 134$) and the HCC group ($n = 75$). The *q*-value is an adjusted *P* value that controls for the FDR. Cohen *d* is an effect size for a comparison between two means; differences between two means were divided by a pooled SD and defined as "small, $d = 0.20$," "medium, $d = 0.50$," or "large, $d = 0.80$." The trend in change was determined through a comparison of the peak intensities of the metabolites in the HCC group compared with the control group.

[a]Metabolites obtained in ESI-positive ion mode (*M/Z* [M+H]).

[b]Metabolites obtained in ESI-negative ion mode (*M/Z* [M-H]).

glycolic acid, and TUDCA. In all subjects, the incidence of HCC was associated with the levels of AST and 7 metabolites, including tyrosine, oleamide, lysoPC 16:1, lysoPC 20:3, 5-hydroxyhexanoic acid, androsterone sulfate, and TUDCA (adjusted $R^2 = 0.514$, $P = 0.036$). On the basis of the results, we produced a correlation matrix including AST and the 7 major metabolites according to the HCC incidence (Fig. 3). In light of the results of the stepwise regression analysis, we determined the optimal cut-off value for each variable via ROC curve analysis. The area under the ROC curve (AUROC) was 0.79 ± 0.03 ($P < 0.001$) for L-tyrosine, 0.78 ± 0.04 ($P < 0.001$) for AST levels, 0.61 ± 0.04 ($P = 0.010$) for 5-hydroxyhexanoic acid levels, and 0.77 ± 0.04 ($P < 0.001$) for TUDCA, and the AUROC was below 0.5 for the remaining variables (lysoPC 16:1, lysoPC 20:3, oleamide, and androsterone sulfate; Supplementary Fig. S3; Supplementary Table S6). The sum of those four variables' prediction probability (88%, $P < 0.001$) was greater than that when the variables were considered independently (Supplementary Fig. S3; Supplementary Table S6).

Discussion

This prospective study showed the clinical relevance of deregulation of tyrosine metabolism; phenylalanine metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; valine, leucine, and isoleucine degradation; valine, leucine, and isoleucine biosynthesis; tryptophan metabolism; glycerophospholipid metabolism; linoleic acid metabolism; primary bile acid biosynthesis; and fatty acid metabolism. The data presented here specifically suggest that the dysregulation of these metabolic processes may be a key mechanism underlying HCC progression and development. In addition, AST, tyrosine, oleamide, lysoPC 16:1, lysoPC 20:3, 5-hydroxyhexanoic acid, androsterone

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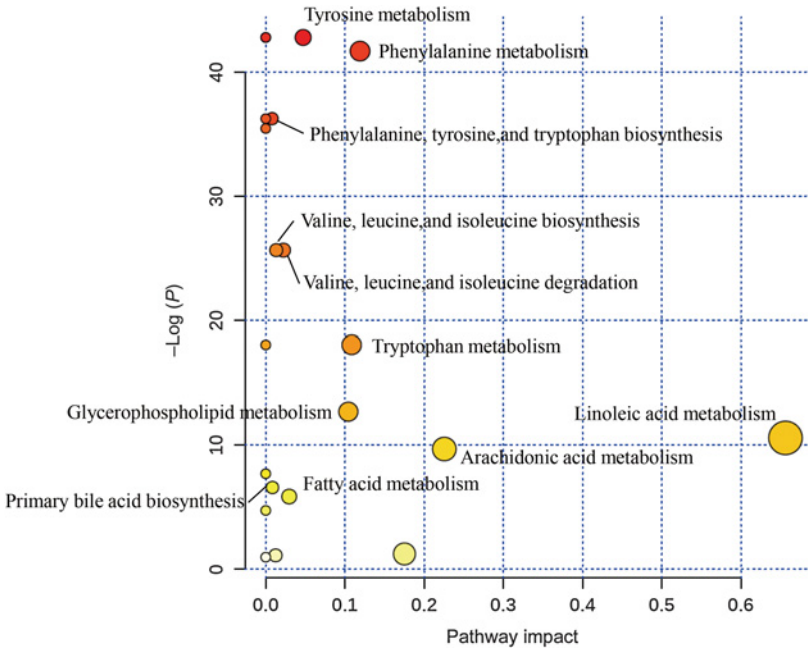

Figure 2.
Overview of metabolic pathway analysis. The "metabolome view" presents pathways arranged according to the scores based on enrichment analysis (*y*-axis) and topology analysis (*x*-axis). The color and size of each circle are based on *P* values and pathway impact values, respectively.

sulfate, and TUDCA levels were independently associated with the incidence of HCC.

It is well known that increased levels of liver enzymes, including AST, ALT, and γ-GTP, are observed in individuals with liver disease (18). In the current study, the HCC group showed significantly higher levels of AST, ALT, and γ-GTP than the control group even though these levels were measured in the baseline assessment, prior to the incidence of HCC. Similarly, Hann and colleagues (18) prospectively observed the incidence of HCC. They demonstrated that individuals with increased baseline levels of AST or γ-GTP showed a significantly higher HR for HCC risk compared with individuals with normal levels of these liver enzymes. Moreover, they reported that the cumulative incidence of HCC in accordance with baseline levels of liver enzymes, and particularly AST or γ-GTP, was significantly higher in

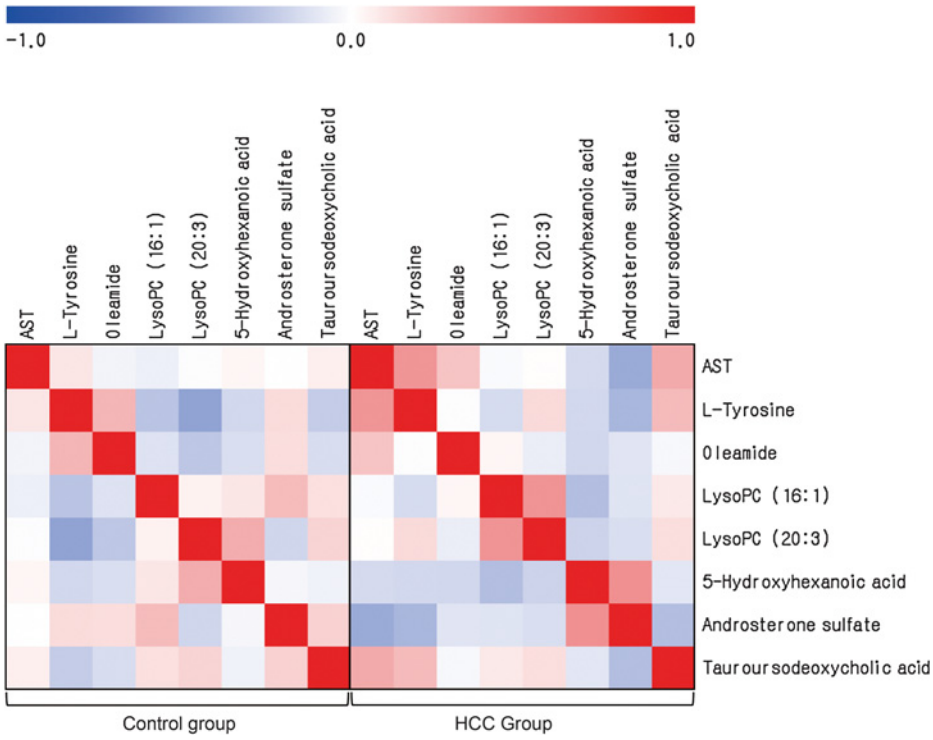

Figure 3.
Correlation matrix including AST and 7 major metabolites in the control and HCC groups. Correlations were obtained using Pearson correlation coefficient. Red, positive correlation; blue, negative correlation.

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individuals with elevated levels of the liver enzymes than in those with levels in the normal range during a median follow-up period of 7.5 years. In particular, according to our stepwise regression analysis, which was performed to determine which variables are independently involved in HCC incidence, AST was one of the independent markers associated with HCC occurrence. Thus, those three liver enzymes, and particularly AST, may be valuable markers for predicting HCC risk.

Similarly, previous metabolomics studies examined the involvement of certain pathologic pathways in HCC. In this context, AAAs, BCAAs, glycerophospholipid, and bile acid have been proposed as potential metabolic markers for HCC (8, 19, 20). Specifically, an amino acid imbalance has been recognized in the serum of HCC and liver cirrhosis patients, showing a decrease in BCAAs and an increase in AAAs, and particularly tyrosine (8, 21). However, the current metabolomics study showed that the serum levels of both BCAAs and AAAs were significantly higher at baseline in the HCC group (cancer free at baseline but developed incident HCC during the 7-year follow-up period) than in the control group (cancer free at both baseline and the 7-year follow-up visit). These findings support the following pathologic progression of HCC: initially increased biosynthesis of BCAAs and AAAs and eventual increased degradation of BCAAs, partly reflecting elevated levels of serum carnitine, which oxidizes BCAAs (22).

The differential serum levels of carnitine between HCC patients and healthy controls seemed to exhibit a specific pattern, with mostly increased levels of long-chain acylcarnitines. Long-chain acylcarnitines are formed intracellularly for the purpose of energy production (4, 23, 24). In contrast, in the current study, the level of palmitoylcarnitine was lower in the HCC group than in the cancer-free control group. Interpretation of these contradictory findings should be performed with caution, as carnitine levels can be influenced by many factors, such as diet, renal dysfunction, and altered biosynthesis rates. Furthermore, blood acylcarnitine levels may be influenced by hepatic fatty acid metabolism, that is, decreased acylcarnitine levels may reflect the decreased production of acyl groups in the liver or other tissues. Indeed, palmitoylcarnitine and palmitic acid are associated with fatty acid metabolism, and this group displayed an impact factor of 0.030 based on metabolic pathway analysis in the current study.

Several free fatty acid species have been reported as discriminatory in serum between HCC patients and healthy controls (23–25). In the current study, at baseline, free fatty acid metabolism, including the metabolism of arachidonic acid and linoleic acid, was relevant to the pathogenesis of HCC based on metabolic pathway analysis. In addition, the level of arachidonic acid was higher in the HCC group than in the cancer-free control group. Arachidonic acid may play important roles in the progression of HCC, as this compound has been described as an important player in endothelial cell migration, that is, vascularization processes (26). This finding is also consistent with the observation that hypervascularization is a frequent feature of HCC.

In the current study, oleamide was another serum lipid compound demonstrated as discriminative between HCC and healthy controls. More specifically, the HCC group showed lower levels of oleamide than the controls did, and the incidence of HCC was independently associated with the oleamide level. Oleamide, the amide of oleic acid, exhibits a variety of neuropharmacologic effects, including increased food intake (27) and relaxation of blood vessels (28). Because oleamide has not been associated with cirrhotic liver diseases (29), this compound may represent a specific metabolic marker for HCC.

Recently, Zhang and colleagues (30) showed that decreased levels of lysoPCs are observed in HCC patients; this result is in agreement with our results. In the current study, not only the levels of lysoPC 16:1 and lysoPC 20:3 but also the levels of other lysoPC species, except for lysoPC P-16:0, were significantly decreased in the HCC group. Polyunsaturated lysoPCs, such as lysoPC 16:1 and lysoPC 20:3, have an antagonistic effect on arachidonic acid (23), which induces a proinflammatory cascade (31). Thus, the decreased levels of these polyunsaturated lysoPCs and the increased arachidonic acid levels observed in the HCC group in our study may be involved in acceleration of the inflammatory response that induces cancer and tumor development (32). In addition, in individuals with a tumor diagnosis, phosphatidylcholine turnover is rapidly increased, so downregulation of lysoPC occurs (33). As this finding corresponds with our result, it would be reasonable to assume that rapid membrane phosphatidylcholine turnover may arise before cancer development.

TUDCA, which is endogenously produced at very low levels in humans, is a hydrophilic bile acid (34). This bile acid has antiapoptotic properties against unfolded protein response (UPR) signaling, which is activated by endoplasmic reticulum stress as well as liver diseases, such as fatty liver disease, alcohol-induced liver disease, viral hepatitis, and HCC (35). Under severe stress, UPR signaling activates apoptosis via elevation of the levels of proapoptotic transcription factors (35), and chronically increased apoptosis of hepatocytes is carcinogenic (36). Indeed, our study showed a significant increase in serum ALT levels, which is a marker for hepatocyte apoptosis (36), in the HCC group compared with the control group. Therefore, the increased TUDCA levels in the HCC group before the incidence of HCC might have been a compensatory mechanism to reduce UPR-induced hepatocyte apoptosis.

In the current study, the incidence of HCC was independently associated with the level of 5-hydroxyhexanoic acid, a normal dicarboxylic acid degradation product of fatty

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acids (37), and androsterone sulfate, the most abundant 5-α–reduced androgen metabolite in serum (38). Unfortunately, no studies have explored the potential associations of 5-hydroxyhexanoic acid and androsterone sulfate with HCC. In addition, MetaboAnalyst 3.0, a web-based analysis module used to identify the most relevant pathways associated with the selected metabolites, does not include 5-hydroxyhexanoic acid and androsterone sulfate, which were detected at higher and lower levels, respectively, in the HCC group than in the control group at baseline. Thus, it is difficult to discuss relevant pathways and the relationship between these pathways and the incidence of HCC. To identify the roles of 5-hydroxyhexanoic acid and androsterone sulfate in HCC development, further studies are therefore required.

Several studies have utilized metabolomics to identify candidate biomarkers for HCC and used a prospective setting in different populations for the early detection of HCC (8, 19–21). Nuclear magnetic resonance (NMR) and MS, the two most commonly used analytic technologies in metabolomics, generate different metabolic profiles from the same sample; thus, currently, the vast majority of metabolomics studies use either NMR or MS separately (39). In general, the metabolic profile of a biological specimen is affected by numerous factors, such as diet, age, ethnicity, drugs, or lifestyle, and these factors need to be either controlled or deconvoluted to obtain information specific to the disease (40, 41). Therefore, metabolic profiling in different ethnic and lifestyle populations may provide personalized treatment during HCC development. Furthermore, a longer follow-up period could be more effective for detecting HCC in its early stage. In conclusion, our study provides useful in-depth information about HCC incidence.

Notably, in the current study, the follow-up period was long (7 years), although the exposure assessment was performed at a single time point, that is, baseline. Despite this limitation, similar to the results of previous metabolomics studies, the clinical relevance of dysregulation of not only BCAAs, AAAs, and lysoPCs but also primary bile acid biosynthesis and linoleic acid, arachidonic acid, and fatty acid metabolism was observed in our study. In addition, the serum levels of AST, oleamide, 5-hydroxyhexanoic acid, and androsterone sulfate were found to be independent variables associated with the incidence of HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.H. Jee, J.H. Lee
Development of methodology: Minjoo Kim, K.J. Jung
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.H. Jee, Minjoo Kim, K.J. Jung, J.H. Lee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.H. Jee, Minjoo Kim, Minkyung Kim, H.J. Yoo, H. Kim, K.J. Jung, J.H. Lee
Writing, review, and/or revision of the manuscript: S.H. Jee, Minjoo Kim, Minkyung Kim, H.J. Yoo, H. Kim, K.J. Jung, S. Hong, J.H. Lee
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Minjoo Kim, H.J. Yoo, H. Kim, S. Hong, J.H. Lee
Study supervision: J.H. Lee

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