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Plasma Lipidomic Analysis to Identify Novel Biomarkers for Hepatocellular Carcinoma

Li Yang[1,2] · Yu Bai[1] · Xiaohong Han[3,4] · Yuankai Shi[3,4] · Huwei Liu[1]

Abstract Hepatocellular carcinoma (HCC) accounts for the major primary liver cancer, and its morbidity and mortality rates are very high. Identification of novel biomarkers for the early diagnosis and treatment of this disease is of crucial importance. In this study, a continuous-flow normal-phase/reversed-phase two-dimensional liquid chromatography—quadrupole time-of-flight mass spectrometry (NP/RP 2D LC-QToF/MS) method was used to detect lipid species in human plasma from HCC patients and healthy controls. As a result, the two groups were clearly separated by principal component analysis. Besides, 23 lipid species with corrected p value <0.05 and fold change >1.5 were proposed as potential lipid biomarkers for HCC. Among them, 9 lipid species, including galactosylceramide (36:5), free fatty acid (20:4), phosphatidylethanolamine (40:6), phosphatidylethanolamine (38:6), diacylglycerol (40:5), diacylglycerol (44:2), lactosylceramide (40:3), phosphatidylcholine (40:6), and free fatty acid (22:5), exhibited excellent ability to distinguish HCC patients from healthy controls with the area under the receiver-operating characteristic curve >0.900, showing promising clinical value. Taken together, these results indicate the great potential of NP/RP 2D LC-QToF/MS method in disease diagnosis, which might be helpful in clinical practice.

Keywords Biomarker · Hepatocellular carcinoma · Lipidomics · NP/RP 2D LC-QToF/MS

1 Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world. It is also the third most frequent cause of cancer-related death with a <7% 5-year survival rate [1]. Currently, the diagnosis of HCC mainly relies on serum alpha-fetoprotein (AFP) and liver imaging, such as ultrasound (US) and computed tomography [2]. However, the sensitivity and specificity of serum AFP level are not satisfactory when predicts HCC [3], especially when the tumor is smaller than 3 cm [4]. Besides, US is also not sensitive enough to distinguish small malignant lesion from cirrhotic nodules [5]. Thus, HCC is often diagnosed at an advanced stage, when established treatment options provide limited benefit [6]. As it is still a great challenge to diagnose HCC early and accurately until now, developing noninvasive methods and investigating novel biomarkers for HCC diagnosis are urgently needed.

Lipidomics, which aims at the full characterization of lipid molecular species and of their biological roles, might provide noninvasive and effective diagnosis for diseases [7]. Currently, it has been widely used to reveal the pathogenic mechanism and identify potential lipid biomarkers associated with disease [8]. As liver plays a vital role in lipid metabolism and lipoprotein metabolism,

✉ Huwei Liu
hwliu@pku.edu.cn

1 Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, Beijing National Laboratory for Molecular Sciences, Institute of Analytical Chemistry, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

2 Department of Pharmacy, Shanxi Medical University, Taiyuan 030001, China

3 Department of Medical Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

4 Beijing Key Laboratory of Clinical Study on Anticancer Molecular Targeted Drugs, Beijing 100021, China

since it regulates their synthesis and degradation, it is believed that lipid metabolism will be disturbed once the liver disease occurs [9]. During the past decades, quite a lot of evidence has demonstrated a strong association between lipid metabolism and HCC. Total cholesterol and triglyceride in HCC patient serum were significantly lower than in controls [10]. Besides, alteration of lysophosphatidylcholine (LPC) and free fatty acid (FFA) were frequently observed in serum, plasma, or tissue from HCC patients [11–13]. However, the coverage of lipid classes or lipid species is restricted in these reports. On the contrary, normal-phase/reversed-phase two-dimensional liquid chromatography—quadrupole time-of-flight mass spectrometry (NP/RP 2D LC-QToF/MS)-based method could realize the analysis of more than 500 lipids from 17 lipid classes due to its high resolving power and sensitivity, which has been utilized for lipid analysis from atherosclerosis, breast cancer, and lacunar infarction patients in our previous studies [14–16].

In the current study, we conducted comprehensive lipid analysis in human plasma from HCC patients, as well as healthy controls, using this NP/RP 2D LC-QToF/MS method. Potential lipid biomarkers for HCC were selected based on the corrected *p* value and fold change between the two groups. Besides, we further evaluated the diagnosis potential of lipid biomarkers. This study aims to explore potential lipid biomarkers for HCC diagnosis and hopefully provide new clues for understanding its underlying mechanism.

2 Materials and Methods

2.1 Chemicals and Reagents

Chloroform was obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). *N*-hexane, methanol and isopropanol were from Sigma-Aldrich (St. Louis, MO, USA), as well as ammonium formate (purity ≥99.995%). All the reagents were of HPLC grade. Purified water was acquired from Hangzhou Wahaha Group Co., (Zhejiang, China). Lipid standards, including FFA (17:0), LPG (17:1), PG (14:0/14:0), LPC (17:0), PC (14:1/14:1), PS (14:0/14:0), PE (14:0/14:0), MG (17:0), DG (12:0/12:0), TG (18:1/14:0/17:1), Cer (d18:1/12:0), GluCer (d18:1/12:0), GalCer (d18:1/8:0), LacCer (d18:1/8:0), and SM (d18:1/17:0), were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

2.2 Human Plasma Sample Collection

In total, we collected 20 human plasma samples in the present study, including 13 healthy controls and 7 HCC

patients. All samples were collected from the Cancer Hospital and Institute of Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Besides, every person included in this study had given verbal consent before their inclusion. However, as the plasma samples were the residual after clinical use, a written consent form was not required. This study was approved by the Institutional Review Board of Cancer Institute and Hospital, Chinese Academy of Medical Sciences.

2.3 Sample Preparation

Samples were naturally thawed at 4 °C before pretreatment. A modified Folch extraction method was employed to extract lipids from human plasma samples [17]. Specifically, 50 μL of lipid standard mixture (50 ng of each lipid standard) was added to 100 μL of plasma. Then, lipids were extracted with Folch solution (chloroform/methanol, 2:1, v/v, 6 mL). After sonicate for 3 min, insoluble portions in samples were removed by centrifugation at 10,000 rpm for 30 min (LG 10-2.4A, China). Then, 1.2 mL water was added to the supernatant. After sonicate for 3 min and centrifuging at 5000 rpm for 15 min, the organic phase was filtered by 0.22 μm PTFE filter membrane and then dried under nitrogen. Before analysis, samples were resuspended in 0.5 mL of Folch solution for injection.

2.4 Lipidomic Analysis

2.4.1 2D LC System

Lipids were separated by an NP/RP 2D LC system [14, 18] with optimized gradient. The system was built based on a ten-port, two-position valve vacuum evaporation interface. There were two interface loops (0.2 mm i.d. × 10 cm), which were kept at 50 °C and used to trap and transfer the elute from first dimension to second dimension alternately. During the analysis, 20 μL of sample was injected into the system. The first dimensional separation was performed on an Rx-SIL silica column (2.1 mm × 150 mm, 5 μm; Agilent Technologies), which was kept at 25 °C. The mobile phase was as follows: A1) hexane, B1) 5 mmol/L ammonium formate in isopropanol/water (100/2, v/v), and C1) 5 mmol/L ammonium formate in methanol/water (100/2, v/v). A Poroshell 120 EC C8 column (2.1 mm × 50 mm, 2.7 μm; Agilent Technologies) was used for the second-dimensional separation, which was kept at 25 °C. The mobile phase were as follows: A2) 5 mmol/L ammonium formate in methanol and B2) 5 mmol/L ammonium formate in water. Optimized gradient program of the NP/RP 2D LC and the valve-switching program were described in Table 1.

Table 1 Configuration of online NP/RP 2D LC system for lipid profiling

First D LC					Interface	Second D LC			
T/min	Flow (mL/min)	A1 (%)	B1 (%)	C1 (%)	Loop/position	T/min	Flow (mL/min)	A2 (%)	B2 (%)
0	0.2	80	20	0	1	0	0.3	100	0
					2	4.50	0.3	100	0
5.00	0.2	80	20	0					
						9	0.3	100	0
						9.01	0.3	70	30
					1	14.50	0.3	70	30
						21.50	0.3	100	0
						28.50	0.3	100	0
						28.51	0.3	70	30
					2	34.50	0.3	70	30
35.00	0.2	50	50	0					
						37.00	0.3	70	30
						47.00	0.3	100	0
55.00	0.2	30	70	0					
						57.00	0.3	100	0
						57.01	0.3	70	30
					1	63.00	0.3	70	30
65.00	0.2	30	70	0					
						68.00	0.3	70	30
75.00	0.2	20	80	0		75.00	0.3	100	0
						81.50	0.3	100	0
						81.51	0.3	70	30
					2	87.50	0.3	70	30
						92.50	0.3	100	0
100.00	0.2	0	60	40					
100.01	0.2	0	0	100					
						104.50	0.3	100	0
						104.51	0.3	70	30
					1	110.50	0.3	70	30
						115.50	0.3	100	0
						123.00	0.3	100	0
						123.01	0.3	70	30
					2	129.00	0.3	70	30
130.00	0.2	0	0	100					
130.01	0.4	40	60	0					
						134.00	0.3	70	30
						149.00	0.3	100	0
150.00	0.4	40	60	0					
150.01	0.4	80	20	0					
165.00	0.4	80	20	0					
165.01	0.2	80	20	0					
170.00	0.2	80	20	0		170.00	0.3	100	0

2.4.2 Mass Spectrometry

An Agilent 6530 QToF MS was used to detect and identify lipids, which was operated in both positive and negative electrospray ionization modes. The ion source parameters were set as follows: capillary voltage was set at 3500 V; fragmentor voltage was set at 190 V; skimmer voltage was set at 65 V. Dry gas temperature, dry gas flow, sheath gas

temperature, sheath gas flow, and nebulizer were set at 300 °C, 5 L/min, 350 °C, 8 L/min, 20 psi, respectively. The MS scan range was 100–2000 m/z.

2.5 Lipid Identification

Lipids were identified by the accurate m/z, retention time (RT), relative RT of lipid species in the same lipid class, and tandem mass spectra (MS/MS). Briefly, the accurate m/z of lipids was detected by Agilent 6530 QToF MS, then it was compared with exact m/z calculated based on the predicted elemental composition. The mass tolerance was less than 10 ppm. With the unique advantage of this method, all the RTs of lipid species in the same lipid class were in the same elute fraction range. In addition, within each lipid class, lipid species with the same total carbon number (CN) in their side chains were eluted in the descending order of double-bond (DB) number. Thus, we could identify that the total CN and DB number of lipid species belongs to a specific lipid class. Besides, the preliminary identification result was compared with our homemade database [14–16, 18, 19], which comprises approximately 1000 lipids currently. At last, lipids were confirmed by target MS/MS with collision energy of 40 eV.

2.6 Data Processing

MassHunter Qualitative Analysis B.04.00 software (Agilent Technologies) was applied to deal with the MS data of all samples. Peak areas of lipids measured by NP/RP 2D LC-QToF/MS were integrated from extracted ion chromatograms with ± 10 ppm m/z expansion. Besides, an internal standard normalization was performed for all lipid species in each sample. Statistical analysis of the corrected peak areas was first calculated with Mann–Whitney U test using SPSS, Version 20 (SPSS Inc., Chicago, IL), and then corrected by Benjamini–Hochberg false discovery rate (FDR) using Mass Profiler Professional (MPP) software (Agilent Technologies). Differences were considered to be significant if the corrected p value <0.05 and fold change >1.5. In addition, MPP and SPSS software were also utilized to perform principal component analysis (PCA), receiver-operating characteristic (ROC) curve, and box plots, respectively.

3 Results and Discussion

3.1 Plasma Lipid Profiling

Lipid species in human plasma from HCC patients and healthy controls were analyzed by the modified NP/RP 2D

LC-QToF/MS method as mentioned above. By taking this approach, the NPLC in the first dimension could separate different lipid classes according to their polarity. Besides, the RPLC in the second dimension could further separate different lipid species in one lipid class by the difference of their acyl chains. The representative base peak chromatograms (BPCs) of HCC patient and healthy control were illustrated in Fig. 1, indicating the differences between the two groups. Besides, monoacylglycerol (MG), diacylglycerol (DG), triacylglycerol (TG), ceramide (Cer), glucosylceramide (GluCer), galactosylceramide (GalCer), and lactosylceramide (LacCer) were characterized at positive ion mode, while FFA, phosphatidylinositol (PI), phosphatidylglycerol (PG), lysophosphatidylglycerol (LPG), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), phosphatidylcholine (PC), LPC, and sphingomyelin (SM) were characterized at negative ion mode.

3.2 Multivariate Statistical Analysis

Based on the lipid profiling data from HCC patients and healthy controls, multivariate pattern recognition analysis using the PCA model was performed. As shown in Fig. 2, the two groups were clearly distinguished at both positive ($R^2X = 89.12\%$, $R^2Y = 1.85\%$) and negative ($R^2X = 67.52\%$, $R^2Y = 7.24\%$) ion modes, indicating the existence of lipid species with significant difference between the two groups.

3.3 Altered Lipid Species Associated with HCC

To select lipid species that significantly altered in HCC patients, we further conducted statistical analysis and relative quantitative analysis based on the corrected peak areas of lipids in each sample. Only lipid species with corrected p value <0.05 and fold change >1.5 were considered to be potential lipid biomarkers for HCC. Consequently, a total of 23 lipid species were finally revealed, as shown in Table 2. Among them, 14 lipids, including 5 FFAs, 4 PEs, 2 PCs, 1 LacCer, 1 GalCer, and 1 TG, were up-regulated in HCC patients, while 9 lipids, including 5 DGs, 1 LPC, 1 SM, 1 GalCer, and 1 TG, were down-regulated. Besides, box plots of these potential lipid biomarkers were shown in Fig. 3, indicating their alterations in HCC patient group compared with healthy control group.

Among the 23 potential lipid biomarkers, five out of them were FFAs, including FFA (16:1), FFA (18:1), FFA (20:4), FFA (22:6), and FFA (22:5). Liver plays an important role in the metabolism of FFA [20]. The previous studies have shown that plasma FFAs were increased owing to the hypermetabolic state in cancers [21]. Besides,

Fig. 1 Base peak chromatograms of human plasma lipid extracts from a control sample and an HCC patient sample obtained by NP/RP 2D LC-QToF/MS at positive ion mode (**a**) and negative ion mode (**b**)
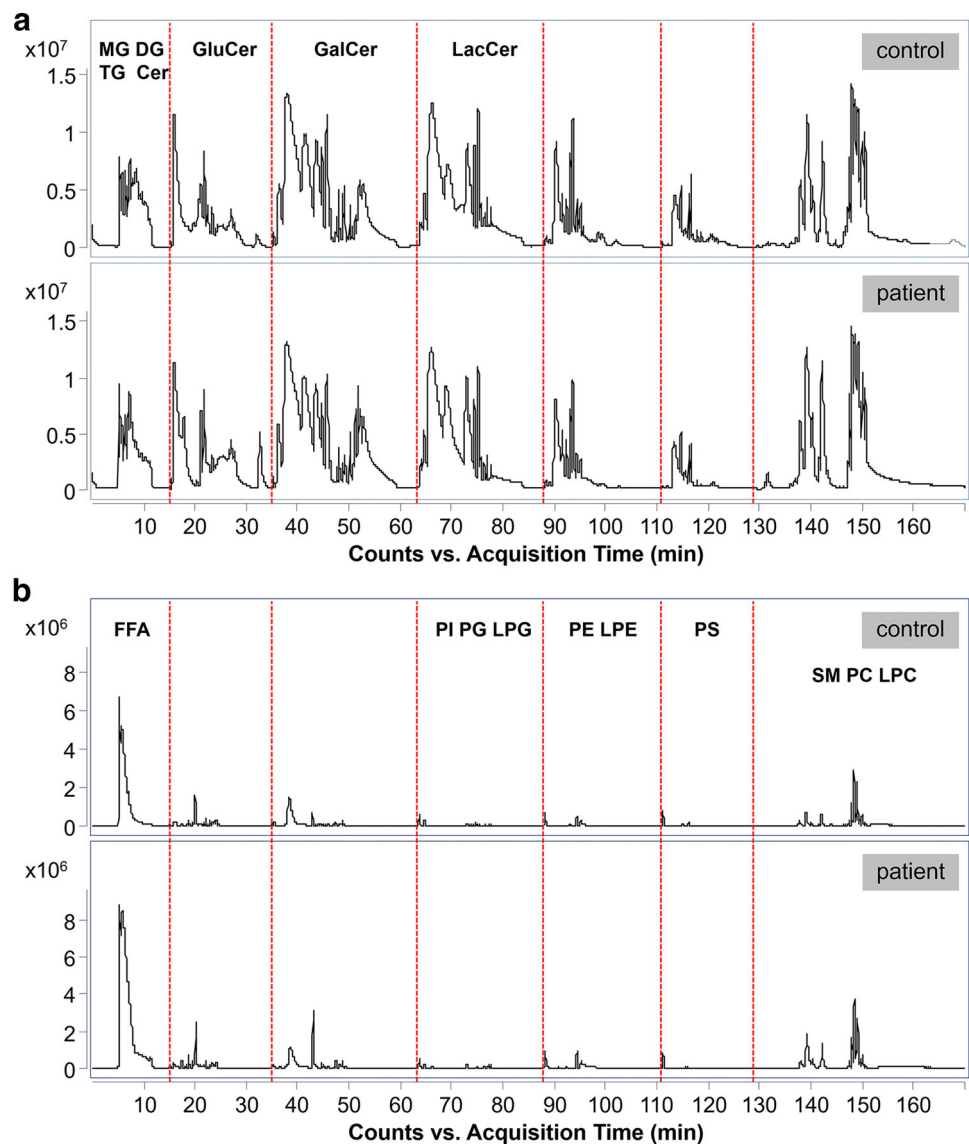
FFA could be utilized as metabolic substrates by the tumor cells [22]. Up-regulation of FFAs in HCC patients has also been revealed in other reports [23, 24], which were consistent with our findings and might be the results of energy requirement and the synthesis of cell membrane due to aggressive cell proliferation.

Besides, five DGs, including DG (34:0), DG (36:0), DG (38:0), DG (40:5), and DG (44:2), were down-regulated in HCC patients. It has been reported that DG signaling pathway played a key role in the progression of cancer via protein kinase C (PKC) [25]. Alterations of particular DG molecules may affect the activation of PKC, thereby influencing the regulation of cell proliferation [26]. Our results might provide more clues for revealing the role of this pathway in this disease. Besides, no matter in the biosynthesis or degradation of TG, DGs are important intermediates [27]. In addition, both of them are mainly

biosynthesized through the sn-glycerol-3-phosphate pathway, which is predominant in liver [28]. Abnormal TG metabolism has been revealed to be associated with the cancer development, including HCC [29, 30]. In our work, TG (50:2) was increased in HCC patient group, while TG (60:2) was decreased, indicating abnormal TG synthesis or hydrolysis occurred during HCC.

Apart from these, 4 PE lipid species, including PE (34:1), PE (38:6), PE (40:5), and PE (40:6), were increased in patients, which represents the third leading disturbed lipid class. PEs are generated from the Kennedy pathway and CDP-DAG pathway [31]. It has been demonstrated that PEs were significantly increased in HCC tissue [29, 32], which is consistent with our results. Besides, another two kinds of glycerophospholipids, namely PC and LPC, were also altered in HCC patients. PC and LPC have important biochemical and biophysical functions [33]. As PCs are the
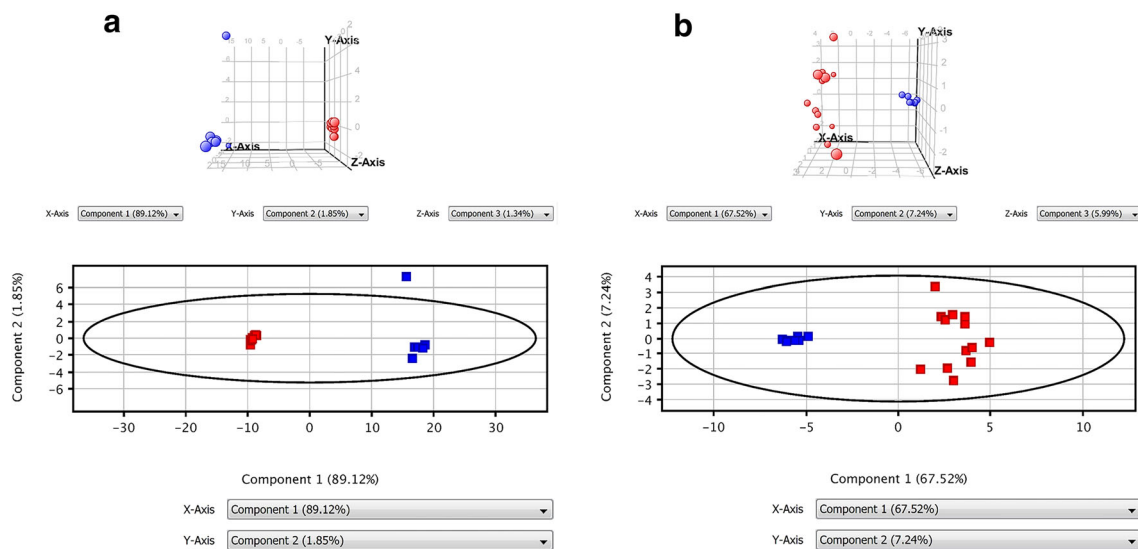
Fig. 2 2D and 3D score plots of principal component analysis based on healthy control group (*red*) and HCC patient group (*blue*) at positive ion mode (**a**) and negative ion mode (**b**)

Table 2 Lipids selected as potential biomarkers for HCC

Lipid species	ESI+			ESI−		
	m/z	*p* value[a]	Fold change[b]	*m/z*	*p* value[a]	Fold change[b]
FFA (16:1)				253.2173	4.11E−02	1.80
FFA (18:1)				281.2486	2.72E−02	1.51
FFA (20:4)				303.2330	1.26E−02	1.88
FFA (22:6)				327.2330	2.51E−02	2.26
FFA (22:5)				329.2486	1.61E−02	1.89
PE (34:1)				716.5236	4.11E−02	1.70
PE (38:6)				762.5079	1.26E−02	1.77
PE (40:6)				790.5392	1.26E−02	1.80
PE (40:5)				792.5549	3.50E−02	1.53
PC (40:6)				878.5917	1.45E−02	1.90
PC (O−40:7)				862.5968	2.51E−02	1.63
LPC (18:2)				564.3307	2.33E−02	−1.66
SM (39:1)				817.6439	2.51E−02	−1.51
LacCer (40:3)	942.6876	3.50E−02	2.15			
GalCer (36:5)	720.5409	2.48E−02	3.44			
GalCer (36:3)	724.5722	4.11E−02	−1.90			
DG (34:0)	614.5718	2.72E−02	−1.94			
DG (36:0)	642.6031	3.30E−02	−1.88			
DG (38:0)	670.6344	4.11E−02	−1.95			
DG (40:5)	688.5875	1.26E−02	−2.30			
DG (44:2)	750.6970	1.33E−02	−2.65			
TG (50:2)	848.7702	2.81E−02	1.57			
TG (60:2)	988.9267	3.50E−02	−3.10			

[a] *p* values were determined by the Mann–Whitney *U* test and corrected by the Benjamini–Hochberg FDR correction

[b] Fold change was calculated from the arithmetic mean values of the HCC patients and healthy controls. Fold change with a positive value indicates a relatively higher concentration in HCC patient, while a negative value indicates a relatively lower concentration compared with the control subjects
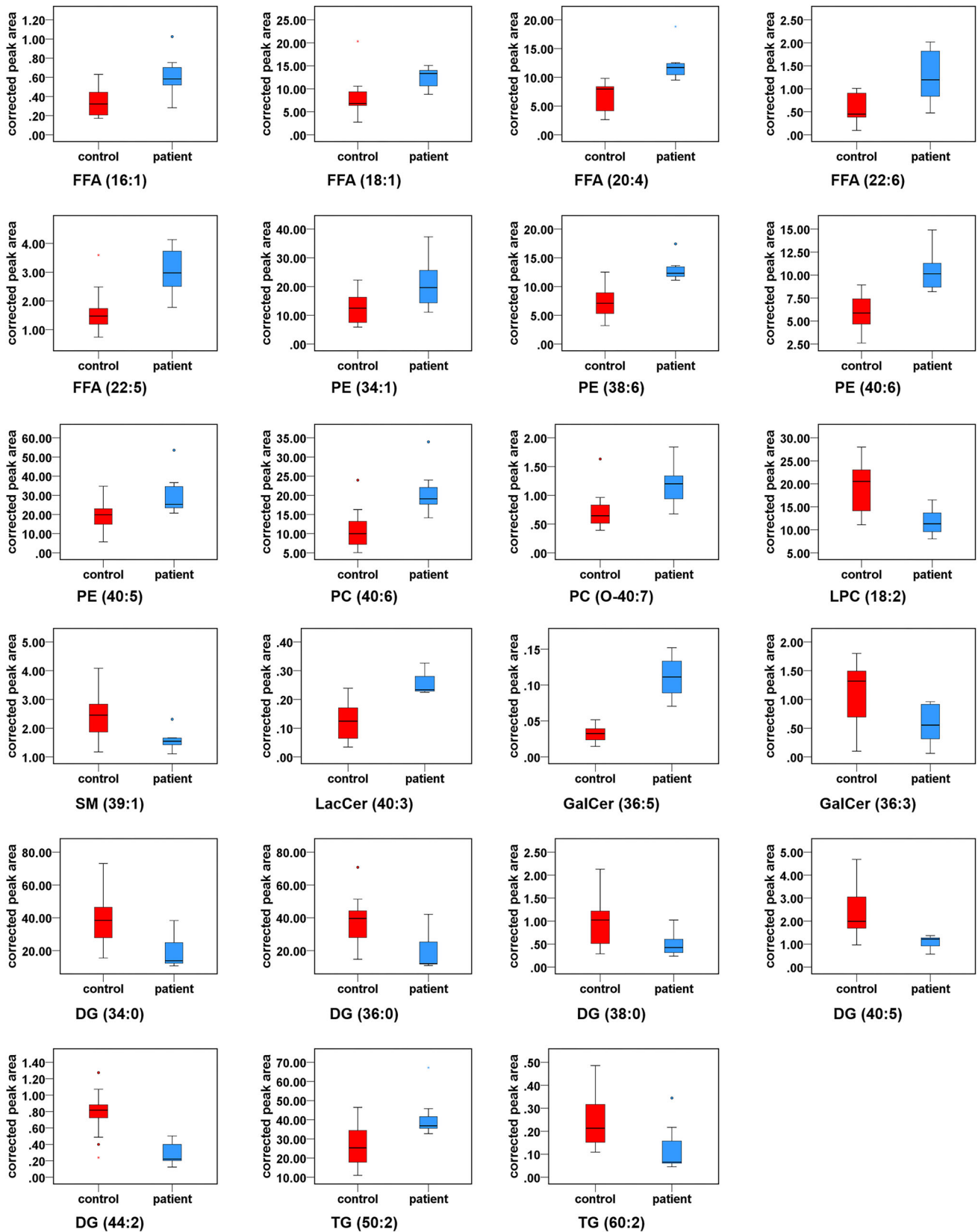
Fig. 3 Box plots of the 23 potential lipid biomarkers between HCC patient group and healthy control group

main components of cell membrane, up-regulation of PCs, including PC (40:6) and PC (O-40:7), is probably to meet the demands of rapid proliferation of liver cancer cells [34]. In addition, down-regulation of LPC (18:2) in HCC patients has been observed in many other reports [11, 12, 35]. The disturbance of PC and LPC might attribute to the alteration of lysolecithin acyltransferase activity, the inhibition of phospholipase 2, the overexpression of lysophospholipase D/autotoxin, and the excess consumption of LPC in the inflammatory response [36–38].

Last but not least, four sphingolipids, including SM (39:1), LacCer (40:3), GalCer (36:5), and GalCer (36:3), were also altered in HCC patient group. Studies have shown that dysregulation in sphingolipid homeostasis contributed to cancer pathogenesis [39]. In the sera and plasma of HCC patients, SMs were decreased [29, 30], as well as in the present study, which might attribute to the deactivation of SM synthases [40]. Besides, up-regulation of LacCer was also observed in HCC patients from another study [41]. However, the relationship between GalCer and HCC is few till now, which needs further investigation in the future.

3.4 ROC Analysis Based on Potential Lipid Biomarkers for HCC

After selecting potential lipid biomarkers for HCC, we further evaluated the diagnosis potential of the 23 lipids by ROC curve. The results of ROC analysis based on the 23 lipid species were shown in Table 3. As we could see from the ROC analysis results, the area under the curve (AUC) values of 9 lipid species, including GalCer (36:5), FFA (20:4), PE (40:6), PE (38:6), DG (40:5), DG (44:2), LacCer (40:3), PC (40:6), and FFA (22:5), were larger than 0.900. The ROC curve of these 9 lipids was shown in Fig. 4. These lipids might play a crucial role in the diagnosis of HCC. Besides, they could act as the complement of the existing diagnosis tools for HCC, such as AFP and liver imaging. To validate these novel lipid biomarkers, more verification with a larger and heterogeneous population is needed in the further.

Table 3 ROC analysis results of the 23 potential lipid biomarkers

Lipid species[a]	AUC	Standard error	95% CI		Sensitivity	Specificity
			Lower	Upper		
GalCer (36:5)	1	0.000	1.000	1.000	1.000	1.000
FFA (20:4)	0.978	0.028	0.923	1.000	0.857	1.000
PE (40:6)	0.967	0.037	0.894	1.000	1.000	0.923
PE (38:6)	0.956	0.047	0.864	1.000	1.000	0.923
DG (40:5)	0.945	0.056	0.836	1.000	0.923	1.000
DG (44:2)	0.934	0.053	0.830	1.000	0.769	1.000
LacCer (40:3)	0.933	0.074	0.789	1.000	1.000	0.900
PC (40:6)	0.923	0.062	0.801	1.000	0.857	0.923
FFA (22:5)	0.912	0.065	0.784	1.000	1.000	0.769
LPC (18:2)	0.890	0.073	0.746	1.000	0.692	1.000
FFA (22:6)	0.868	0.091	0.689	1.000	0.714	1.000
PC (O-40:7)	0.868	0.083	0.706	1.000	0.714	0.923
SM (39:1)	0.868	0.082	0.707	1.000	0.846	0.857
FFA (16:1)	0.857	0.098	0.665	1.000	0.857	0.833
FFA (18:1)	0.857	0.088	0.685	1.000	1.000	0.692
DG (34:0)	0.857	0.087	0.687	1.000	1.000	0.571
TG (50:2)	0.846	0.088	0.673	1.000	1.000	0.692
DG (36:0)	0.835	0.098	0.642	1.000	1.000	0.571
PE (40:5)	0.824	0.094	0.641	1.000	0.857	0.769
TG (60:2)	0.810	0.125	0.565	1.000	1.000	0.714
PE (34:1)	0.791	0.102	0.591	0.992	0.857	0.692
GalCer (36:3)	0.791	0.102	0.590	0.992	0.692	1.000
DG (38:0)	0.791	0.101	0.593	0.989	0.692	0.857

[a] Based on their AUC values, lipid species were sorted in descending order
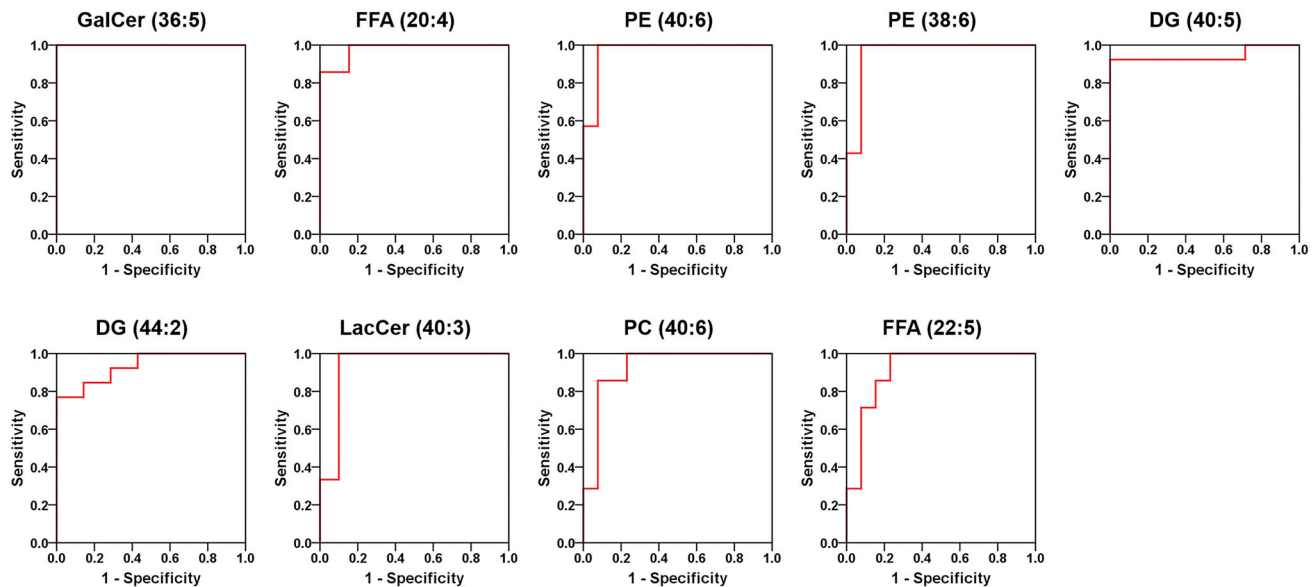
Fig. 4 ROC curves of the nine potential lipid biomarkers with AUC values larger than 0.900

4 Conclusions

In the present study, we employed an NP/RP 2D LC-QToF/MS-based lipidomic method to analyze lipids in HCC patients and healthy controls. After comprehensive lipid profiling and data processing, 23 lipid species, including 5 FFAs, 5 DGs, 4 PEs, 2 PCs, 2 TGs, 2 GalCers, 1 SM, 1 LPC, and 1 LacCer, were suggested as potential lipid biomarkers for HCC. Among them, GalCer (36:5), FFA (20:4), PE (40:6), PE (38:6), DG (40:5), DG (44:2), LacCer (40:3), PC (40:6), and FFA (22:5) showed excellent diagnostic ability to differentiate HCC patients from healthy controls, which might be helpful for the diagnosis of HCC. These results contribute to providing valuable clues for the clinical diagnosis and molecular mechanism of HCC. In the further, more validations are still needed to make these potential biomarkers become routine and robust tests for clinical use.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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