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Lipidomics Analysis Based on Liquid Chromatography Mass Spectrometry for Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma

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Abstract:

Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) account for nearly all primary liver cancers. Diagnosis of ICC and HCC still remains a challenge because of the lack of sensitive diagnostic and sufficiently accurate tests. Lipidomics is a critical part of metabolomics and aims to study all the lipids within a living system. In order to have a better and noninvasive diagnostic test, we profiled the lipidomics in a total of 292 blood samples from patients and normal individuals. It revealed that four metabolites, PE(19:0/0:0), PE(18:2(9Z,12Z)/0:0), PC(14:0/0:0) and PC(18:0/0:0), were defined as "marker metabolites," which can be used to distinguish the HCC and ICC. Discrimination analysis showed that a combination of these metabolites could be used as markers for HCC and ICC with better diagnostic parameters with high sensitivity and specificity than using a single metabolite marker. Lipidomic profiling could allow us to predict disease in its early stages.

Keywords:

Hepatocellular carcinoma; intrahepatic cholangiocarcinoma; lipidomics; mass spectrometry; metabolites; biomarkers; early diagnosis

Statement of Significance

Detecting new biomarkers for early-stage in HCC and ICC patients may improve the diagnosis and prognosis of patients. PE(19:0/0:0), PE(18:2(9Z,12Z)/0:0), PC(14:0/0:0) and PC(18:0/0:0), were defined as "marker metabolites," which can be used to distinguish the HCC and ICC, may serve as a diagnostic tool for HCC and ICC biomarker detection

Introduction

Primary liver cancer comprises two major types of cancer-hepatocellular carcinoma (HCC) and cholangiocarcinoma (ICC) [1]. Both major types of liver cancer, HCC and ICC have experienced increases in incidence in the world and have become major public health problems. ICC, which originates in the epitheliallining of the biliary tract, is the second most malignant tumour in the liver after HCC [2]. The survival rate of patients with ICC is very poor, and surgical resection provides the best chance for a cure [3]. However, early detection of ICC is hampered by the absence of a sufficiently accurate and noninvasive diagnostic test. Detecting new biomarkers for early-stage biliary carcinoma in patients may improve the prognosis of patients with ICC. Late diagnosis of HCC is the key factor for the poor survival of patients [4]. To date, the only well-established tumor marker of HCC is alpha-fetoprotein (AFP), a fetal glycoprotein in plasma. However, it is not a reliable diagnostic biomarker because of its poor sensitivity and low specificity. Owing to lack of early-warning signs, HCC patients are often diagnosed late, which is one of the primary factors for the low survival of HCC patients, with a global 5-year survival of only 3-5%[5]. Therefore, an early diagnosis of HCC and ICC may greatly improve the outcome of treatment. The clinical manifestations and liver function test between the ICC and HCC diseases are too similar to distinguish[6]. Therefore it is extremely important to differentiate HCC from other ICC promptly and accurately.

Lipidomics, defined as the complete quantitative and molecular determination of lipid molecules isolated from biological samples, is a particular component of the metabolome [7]. Lipids are not only components of cell membranes but are also involved in signal transduction [8,9]. Recent lipidomic studies have shown that lipids as biomarker candidates for Alzheimer's disease [10], type 2 diabetes [11] and depression [12]. In this context, lipidomics, have become a powerful approach that opens a window to investigate how mechanistic biochemistry relates to the phenotype [13,14]. Plasma is commonly considered to be a pool of metabolites that reflect systemic metabolic deregulation in patients, and the markers in these biofluids could reflect the characteristics of the system during the course of diseases [15,16]. Nontargeted plasma lipidomic approaches are being widely used used to for the new biomarkers discovery of AD in a clinic setting [10].

The aim of this study was to evaluate the usefulness of the plasma metabolic alterations as a rapid lipidomics screening technique for HCC and ICC. Herein, we examined the plasma metabolites patterns of cancer patients and normal controls by performing LC/MS methods, and identified some potential biomarkers which might be applied to distinguish HCC patients from ICC individuals at early stage. This is the first MS-based metabolic biomarker discovery study on Chinese subjects that led to the identification of lipid metabolites that discriminate early stage HCC from patients with ICC.

2. Materials and Methods

2.1 Chemicals

Formic acid and acetonitrile were obtained from Merck (KGaA Merck, Germany). Methanol (HPLC grade) was purchased from Tedia. Distilled water was produced using a Milli-Q Reagent Water System (Millipore, Billerica, MA, United States). Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All standard [LysoPC(18:1(9Z)), lysoPC(18:0), lysoPC(14:0) and lysoPC(15:0)] preparations were purchased from Sigma-Aldrich (St. Louis, MO, United States).

2.2 Ethics statement

The study was approved by the Ethics Committee at Heilongjiang University of Chinese Medicine (approval number: HUCM-CTRP-2013-128, date of registration is 08-12, 2013).The informed consent was obtained from all subjects. All patients and all control individuals were approached using approved ethical guidelines and those who agreed to participate in this study, were required to sign consent forms. All participants provided written consent. Plasma were collected from control subjects (n = 109), 183 patients (male/female, 89/94). Of these, 85 of the patients were HCC, and 98 patients was ICC. The characteristics of the ICC and HCC patients are summarized in Table S1.

2.3 Plasma pretreatment

Four hundred microliters of acetonitrile was added to 100 μL of plasma and the mixture was vortexed for 1 minute. Then, the mixture was centrifuged for 10 minutes at a rotation speed of 13,000 rpm at 4 °C. After that, 400 μL of supernatant was transferred and lyophilized in a freeze-dryer. Finally, the dried supernatant was dissolved with 100 mL water/acetonitrile (4:1, v:v) solution *via* centrifugation at 13,000 rpm for 10 minutes at 4◦C, and then the supernatant filtered through a 0.22 μm membrane. The plasma was stored at -80 °C until analysis. Sera were thawed at room temperature before analysis. Lipids were extracted from plasma by using the method described by **ref 17**. To ensure data quality for metabolic profiling, pooled quality control (QC) samples were prepared by mixing all of the samples. During analysis of the sample sequence, one QC sample was run after every 10 injections in order to ensure the stability and repeatability of the LC system.

2.4 Lipidomic profiling analysis

2.4.1 UPLC chromatographic condition

The plasma lipidomic profiling analysis was conducted on ultra-high-performance liquid chromatography (UPLC) coupled to a hybrid Q-TOF mass spectrometry (MS) system (ACQUITY UPLC, Waters, Millford, MA, USA). The chromatographic separation was conducted on an Acquity BEH C_{18} column (100 mm x 2.1 mm, 1.7 μm). The column oven temperature was set to 45°C, injection volume at 5 μL and flow-rate at 0.5 ml/min without a split. The mobile phase consisted of phase A (water with 0.1% formic acid) and phase B (acetonitrile containing 0.1% formic acid). The gradient was as follows: 0–5 min, 1–25% B; 5–9 min, 25–50% B; 9–9.1 min, 50–99% B; 9.1–11 min, 99% B; 11–11.1 min, 99–1% B; 11.1–13 min, 1% B.

2.4.2 Accurate Mass Time-of-Flight Mass Spectrometry

MS involved use of a Waters Micromass Q-TOF micro Synapt High Definition Mass Spectrometer (Synapt HDMS,

Waters, Manchester, U.K.) equipped with electrospray ionization in positive and negative modes. Fragmentation (MS/MS) spectra were acquired as two separate positive-ion (ESI^+) and negaitive-ion (ESI^-) polarity runs for each sample. The following parameters were employed: source temperature at 110 ◦C; the desolvation temperature was set at 350◦C; capillary voltage was set at 3200 V; cone voltage at 35 V. Nitrogen was used as the dry gas, the desolvation gas flow rate was set at 500 L/h, and cone gas flow was maintained at 50 L/h. Collision energy was set at 35 eV in MS/MS mode for identification of potential metabolites. All the data were acquired using an independent reference lock mass (Leucine enkephalin) via the LockSpray™ interface to ensure accuracy and reproducibility during the MS analysis. Centroid data were collected at a rate of 1 MS spectrum per second from 50 to 1000 m/z with a scan time of 0.2 s, an inter-scan delay of 0.1 s, and a lock spray frequency of 10 s.

2.5 Plasma metabolite analyses

All the LC-MS raw files were converted to TransOmics program (this software enables peak identification and quantification using an in-house LIPID MAPS database[**18**]), and subsequently the converted files were calculated for generation of alignment, peak picking, deconvolution, filter data, identify compounds (**Fig. S1**). The metabolites were expressed as ‘‘concentration’’ or as a ‘‘ratio’’ and were exported in EXCEL format or exported to EZinfo sofware for compound statistics (PCA, and OPLS-DA), correlation analysis and compound validation. The variable importance for projection (VIP) plot derived from the OPLS-DA analysis was carried out to select distinct variables as potential markers. Besides the multivariate approaches, one univariate method, the Student’s t test, was selected to measure the significance of each metabolite in separating patients from healthy controls. The classification performance of the selected metabolites was assessed using area under the AUC of receiver operating characteristic curve (ROC). The AUC measure of an ROC plot is a measure of predictive accuracy.

2.6 Statistical analysis

PermutMatrixEN software version 1.9.3.019 was used to identify clusters among groups. Heatmap was performed by the open-source R software package. ROC was applied to the plasma data using GraphPad Prism Version 5.00 for Windows (GraphPad Software, San Diego, California, USA). For the significantly changed metabolites, pathway analysis was carried out using Metaboanalyst tool based on KEGG pathway database (http://www.genome.jp/kegg/). Correlation network was constructed using the TransOmics software (Waters, Millford, MA, USA). Differences in the amounts of molecules between groups were analysed using Student’s unpaired t-test from SPSS Statistics 19.0 (SPSS, Chicago, IL, USA). The p values less than 0.05 were considered significant.

3. Results and Discussion

3.1 Plasma metabolite profiles

In this study, a total of 292 plasma samples were collected and analyzed both by LC-Q-ToF-MS. **Fig. S2** in supplementary materials presents the basic peak ion (BPI) chromatograms of the normal, ICC and HCC in negative

mode. BPI exhibited the ideal separation result under the optimized gradient elution procedure and plasma metabolomic profile for each sample, indicating that the sample analysis sequence had satisfactory stability and repeatability. Validations of method, including linearity, precision, stability, and recovery, were also carried out, which indicates that this plasma lipidomics method is reliable.

3.2 Differential metabolites between controls and patients

In this study TransOmics showed the detailed information for each individual feature including statistics, extracted ion chromatograms, spectrum details, and putative identifications. The alignment algorithm will generate 'compound ions' in the 2D ion intensity map (**Fig. S3**), which consisted of approximately 748 chromatographic peaks. Correlation analysis for all the compounds in plasma samples collected from normal and patient groups was shown in **Fig S4**. Raw data from UPLC/MS were analyzed by the TransOmics was imported into EZinfo 2.0 software for data analysis. Multivariate data analysis was performed using the score plot of PCA, and there is an obvious separation between the clustering of the patient and control groups **(Fig. 1A**), suggests that biochemical perturbation significantly happened in patient group. It suggests that the metabolic profiles between ICC and HCC were similar, but they were significantly different from that of controls.

3.3 Selected metabolites for diagnosing HCC and ICC

For further analysis of the metabolic differences between the HCC and ICC group, all the significant ions from the positive ion and negative ion modes were merged and imported into the Ezinfo package. The detailed information for each individual feature was then exporting to EZinfo for compound statistics and compound validation (**Table S2**). The OPLS-DA model was applied to investigate the altered metabolites in HCC by comparison with ICC samples. The score plot (**Fig. 1B**) showed well separation between the HCC and ICC samples, indicating the metabolites were significantly different between the two types of liver cancer. Loading-plot of metabolomic profiling of serum samples collected from HCC and ICC patients was shown in **Fig. 1C**. The VIP value of each metabolic feature was then calculated to indicate its contribution on the samples classification in the score plot; the p value was calculated by Paired t-test to quantify the statistical significance of each metabolic feature (**Fig1D**). Finally, a total of 14 lipidomic features responsible for the class separation between HCC and ICC were identified based on VIP >1.40, and with p value <0.01 (**Table 1**). The quasimolecular ions were confirmed. Then, the MS/MS spectra were also analyzed to verify the structure of the identified metabolites. Subsequently, 14 lipid metabolites were identified, and were further confirmed using authentic standard samples.

The relative average normalized quantities of the identified differential metabolites in the HCC group compared to those signatures in the ICC group were plotted in a Heatmap (**Fig. 1E**) using the R software package. To visualize the relationship between these metabolites, correlation analysis of hierarchical clustering was used to arrange the 14 lipid metabolites based on their relative levels across samples (**Fig. S5**). And it found that 4 lipid metabolites including PE(19:0/0:0), PE(18:2(9Z,12Z)/0:0), PC(14:0/0:0) and PC(18:0/0:0) were similar the abundance profiles of each other

and enabling the grouping of compounds together (**Fig. S5**). With the use of binary logistic regression, the top 4 metabolites were selected as the best subset for case prediction. It was found that, among them, these metabolites (Fasting concentrations) PE(19:0/0:0) (*P*= 0.00019), PC(14:0/0:0) (*P*= 0.00024) and PC(18:0/0:0) (*P* = 0.00025) were significantly upregulated and PE(18:2(9Z,12Z)/0:0) (*P*< 0.00015) was significantly downregulated (Fig. 2A and Table 1). The results suggest that the levels of PE(19:0/0:0), PC(14:0/0:0) and PC(18:0/0:0) significantly increased in the HCC group; the levels of PE(18:2(9Z,12Z)/0:0) was markedly reduced compared to those in the ICC group.

3.4 Metabolic pathway analysis

To gain insight into the metabolic mechanism of HCC and ICC, metabolic pathways of the significantly altered metabolites were analyzed using the ‘‘pathway analysis’’ module within the MetaboAnalyst software. We identified a total of 2 distinct metabolic pathways that were significantly altered in the plasma samples from HCC and ICC group (**Table S3**). MetPA asigned a total of feature compounds in two pathways which were identified together are important for the host response to HCC and ICC (**Fig. S6**). The predominant hits were pathways involved in glycerophospholipid metabolism, primary bile acid biosynthesis with higher score had yield satisfactory results, and clearly help us to better understand the underlying mechanisms.

3.5 Diagnostic potential of differential metabolites

Clinical estimation of markers with ROC analysis was determined in plasma samples from 292 patients as the validation set to evaluate the metabolite profile for diagnosing patients. There was no statistical significance observed between male and female patients (P > 0.05) in the clinical tests employed. Therefore, we investigated the plasma diagnostic potentials of these significantly different metabolites. We examined the plasma samples from the participants for untargeted lipidomic analysis. A total of 14 lipidomic features responsible for the class separation between HCC and ICC were identified based on VIP >1.40, and with p value <0.01 (**Table 1**). From the 14 differentially expressed metabolites, a combination four metabolites gave the best discriminating power. We measured the four metabolites of interest in an independent replication sample from the HCC and ICC study. Box plots show the changes of relative signal intensities for significantly changed metabolites among HCC and ICC groups (**Fig 2**).

To identify more sensitive diagnostic biomarkers for HCC and ICC, we used ROC analysis to evaluate the diagnostic capabilities of those differential metabolites identified in plasma. The ROC analysis revealed PE(19:0/0:0), PE(18:2(9Z,12Z)/0:0), PC(14:0/0:0) and PC(18:0/0:0) to be potent discriminators of the between HCC and ICC groups (Fig.3). According to the data in patients with HCC versus ICC diseases, the ROC curve of four individual metabolites yielded an area under the curve (AUC) of 0.92. For the group classification, the PE(19:0/0:0), PE(18:2(9Z,12Z)/0:0), PC(14:0/0:0) and PC(18:0/0:0) yielded a robust AUC of 0.918 (**Fig.3**A), 0.833 (Fig.3B), 0.807 (Fig.3C) and 0.784 (Fig.3D). More valuably, the accuracy rate of a panel of 4 metabolites attained a diagnostic accuracy of 99.7% both for patients with HCC and ICC. As shown in Fig. 3E, the 4 marker metabolites significantly

increased the specificity and the diagnostic performance of the metabolic markers, gave better diagnostic results than a single marker.

3. Discussion

HCC and ICC is the most common primary liver cancer globally, representing 25-50% of primary hepatic malignancies worldwide, and its incidence is increasing. Early diagnosis allows for timely therapeutic intervention, which in turn results in longer survival and better quality of life. Metabolomics suggests that there is a great potential for candidate metabolite discovery, metabolite signatures may also have the potential to be used as diagnostic biomarkers. As generally known, with recent advances in metabonomics, plasma metabolites could be used as a novel diagnostic indicator for disease. A marker for early diagnosis would meet the following requirements: first, it should achieve high accuracy, which would increase the probability of a diagnosis; second, specimen should be easily collected for detecting the marker; and third, the cost-effectiveness should be considered. Mark Mapstone et al. had reported that plasma metabolomics may serve as a diagnostic tool for AD biomarker detection [16]. In this study, high-throughput UPLC/ESI-Q/TOF-MS and multiple data processing method analysis could provide a powerful approach to clearly differentiate patients with HCC and ICC and identify the potential biomarkers. PCA revealed a statistically significant separation between the HCC and ICC samples. Interestingly, 14 differential metabolites were identified in HCC and ICC subjects compared to controls. Four metabolites were identified as potential biomarkers of HCC and ICC. The levels of PE(19:0/0:0), PC(14:0/0:0) and PC(18:0/0:0) significantly increased while the levels of PE(18:2(9Z,12Z)/0:0) significantly reduced comparing with the control groups.

Lipids are a diverse class of biological molecules that play a central role as structural components of biological membranes, energy reserves, and signaling molecules. The diversity of lipids in all organisms is a challenge for the qualitative as well as quantitative lipid analysis, so called “lipidomics”, which, however has dramatically gained importance in many fields of biosciences. Lipidomics could be a promising new approach for the identification of new biomarkers for monitoring or predicting disease states and/or drug responsiveness. Therefore identification and characterization of these metabolites offers a unique opportunity to devise therapeutic strategies to prevent or reverse these pathological states. Lipidomics as a field aims at characterization of lipid molecular species and their biological roles with respect to the expression of proteins involved in lipid metabolism and function including gene regulation. Achieving successful screening is critically important as early diagnosis can potentially provide curative opportunities. Once cancer is advanced, there are multiple therapeutic venues, but most eventually fail, therefore developing new targeted diagnosis may provide greater chance for effective therapies [19]. UPLC-MS based combined with pattern recognition analysis approach could be an advanced tool to help us find metabolites with regards to its capacity of processing large datasets, and classifying of sample groups, as well as its indiscriminative nature of biomarker and metabolite identification [20-23]. Clinical putative diagnostic biomarkers were further validated in an independent

sample set, which comprises 292 cases, with similar demographic and clinical characteristics when compared to the training set. By using our lipidomics platform, furthermore, a panel of 4 candidate markers was found to differentiate the HCC and ICC in the test cohort. Using a mass spectrometry-based metabolite profiling platform, we identified a panel of 4 metabolites whose fasting concentrations at a routine examination diagnosed the HCC and ICC. Further study of these metabolites may facilitate the development of non-invasive biomarkers and more efficient therapeutic strategies for HCC and ICC. These findings yield a valuable tool that can engender new insights into the pathophysiology and advance the early diagnosis and monitor the progression of HCC and ICC. In addition, further research into the pathways related to these metabolites would provide new clinical targets for cancer therapy.

4. Conclusions

HCC and ICC are fatal cancer with a global increasing trend in recent years. In view of this, it remains a need for more reliable diagnostic biomarkers. Lipidomics has the potential to be developed into a clinically useful diagnostic tool, and could also contribute to a further understanding of disease mechanisms. In this study, A LC/MS-based plasma lipidomic approach integrated analytical method was developed in this study to identify the characteristic metabolites which have the capability to distinguish HCC patients from ICC individuals. 14 altered metabolic biomarkers could cause more severe disturbances in glycerophospholipid metabolism, primary bile acid biosynthesis, *etc*. A panel of 4 candidate marker has a better ROC curve than single metabolite; it is useful for distinguishing HCC from ICC. We also demonstrated that marker metabolites could be a potential early-warning biomarker of HCC and ICC. This research showed that plasma lipidomics has the potential as a promising screening technology for novel biomarkers with potential diagnostic and prognostic value.

Acknowledgments

We thank Andrew D. Patterson for many helpful discussions. We are also specifically grateful to Jack Lawler for helpful suggestions. In addition, we thank BGI for excellent technical assistance. This work was supported by grants from the Key Program of Natural Science Foundation of State (Grant No. 81470196, 81302905), Natural Science Foundation of Heilongjiang Province of China (H2015038).

Competing financial interests

The authors declare no competing financial interests.

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Figure legends

Fig. 1. Metabolomic profiling derived from UPLC-TOFMS. A, PCA score plots of serum samples collected from patients (red) and controls (black); B, PCA score plots of metabolomic profiling of serum samples collected from HCC (black) and ICC patients (red); C, Loaing-plot of metabolomic profiling of serum samples collected from HCC and ICC patients; D: The combination of VIP-score plots constructed from the supervised OPLS-DA analysis of serum samples collected from HCC and ICC patients; E, Heatmap (cluster identification) of the 14 significantly changed metabolites (left side) among the cluster groups (top) HCC and ICC groups. The colors from green to yellow indicate the relative contents of metabolites in the HCC compared with those in the ICC group.

Fig 2. Box charts showing fluctuations in integrated intensities of the significantly changed serum differential metabolites.

Fig. 3 ROC analysis for potential biomarkers in diagnosing patients with HCC versus ICC. (A–D) Plots of ROC results from the targeted analysis of the four metabolites (PE(19:0/0:0), PE(18:2(9Z,12Z)/0:0), PC(14:0/0:0) and PC(18:0/0:0)) in the independent validation phase; (E) the application of the four-metabolite panel developed from the targeted discovery phase in the independent validation phase.

Table 1. TransOmics detailed identification data of serum lipid metabolites in the HCC compared with those in the ICC group.

No	Retention time (min)	m/z	Adducts	Formula	Mass Error (ppm)	Compound	Anova (p)	Trend	ROC-AUC
1	4.40	468.31	M+H	C22H46NO7P	-1.51	PC(14:0/0:0)	0.00024	Down	0.807
2	4.62	518.32	M+H	C26H48NO7P	-2.11	PC(18:3(6Z,9Z,12Z)/0:0)	0.05639	Down	0.687
3	4.80	482.32	M+H	C23H48NO7P	-2.49	PC(7:0/O-8:0)	0.08912	Down	0.635
4	5.04	496.34	M+H	C24H50NO7P	-1.47	PE(19:0/0:0)	0.00019	Up	0.918
5	5.17	478.29	M+H	C23H44NO7P	-2.11	PE(18:2(9Z,12Z)/0:0)	0.00015	Up	0.833
6	5.42	508.34	M+H	C25H50NO7P	-2.09	PC(17:1(10Z)/0:0)	0.00542	Down	0.630
7	5.74	313.27	M+H	C19H36O3	-3.55	3-oxo-nonadecanoic acid	0.06293	Down	0.711
8	5.87	544.34	M+H	C28H50NO7P	-3.57	PC(0:0/20:4(5Z,8Z,11Z,14Z))	0.04483	Down	0.737
9	6.76	524.37	M+H	C26H54NO7P	-0.13	PC(18:0/0:0)	0.00025	Up	0.784
10	6.96	546.35	M+H	C28H52NO7P	-2.97	PC(20:3(8Z,11Z,14Z)/0:0)	0.90619	Up	0.625
11	7.43	538.39	M+H	C27H56NO7P	-4.14	PC(19:0/0:0)	0.01456	Up	0.617
12	8.12	780.55	M+H	C44H78NO8P	-1.50	PE(20:5(5Z,8Z,11Z,14Z,17Z)/19:0)	0.04977	Down	0.692
13	8.24	780.56	M+H	C44H78NO8P	2.65	PE(22:4(7Z,10Z,13Z,16Z)/17:1(9Z))	0.05197	Up	0.731
14	8.62	552.40	M+H	C28H58NO7P	-2.31	PC(20:0/0:0)	0.08883	Down	0.674

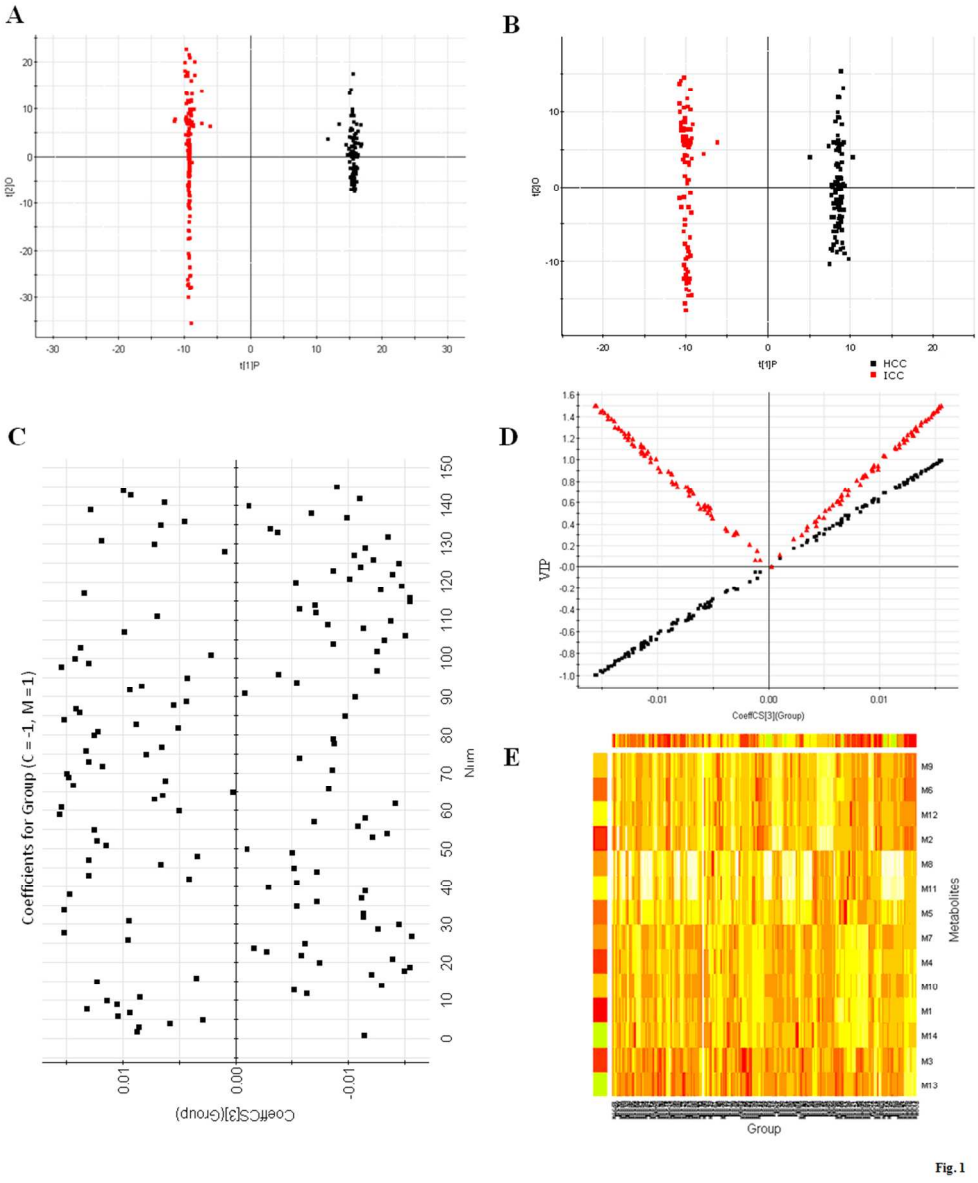

Fig. 1

290x345mm (96 x 96 DPI)

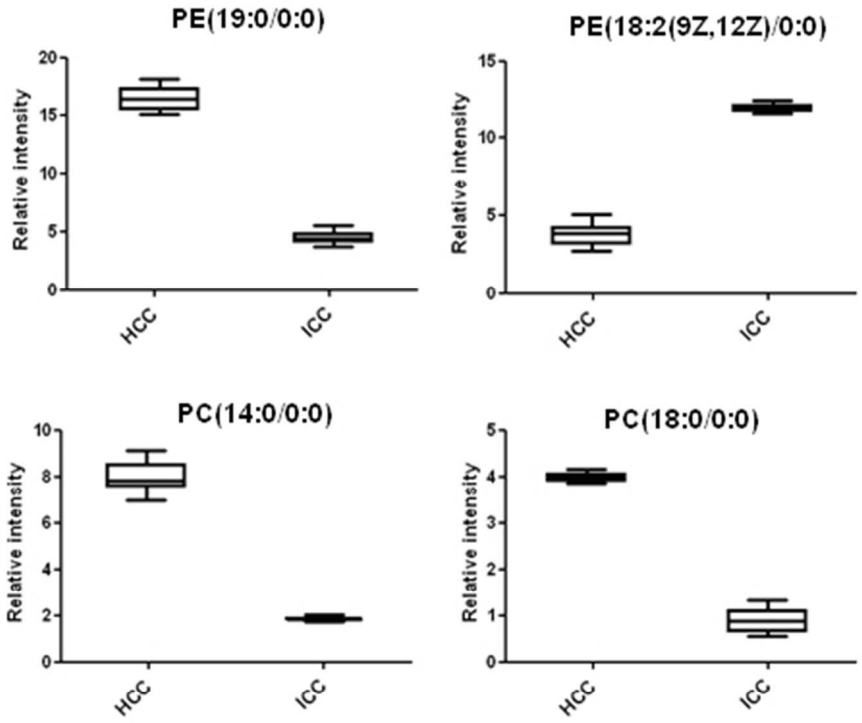

Fig 2

137x111mm (96 x 96 DPI)

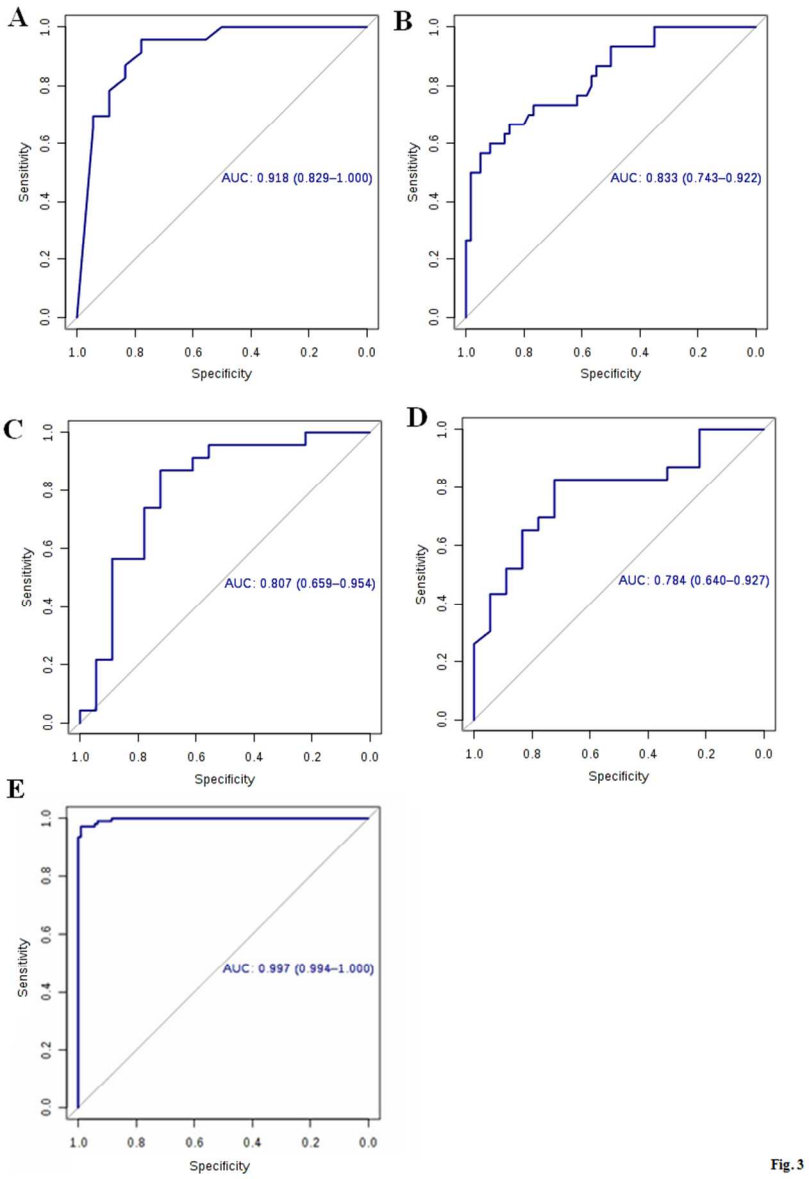

Fig. 3

218x314mm (96 x 96 DPI)