

Metabolomics Analysis of the Responses to Partial Hepatectomy in Hepatocellular Carcinoma Patients

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Abstract

In this study, liquid chromatography/quadrupole time of flight mass spectrometry (LC/QTOFMS) was employed for investigating the metabolome of the sera collected from hepatocellular carcinoma (HCC) patients before and 3 to 5 months after partial hepatectomy. To investigate the changes in metabolic phenotypes after the hepatic resection, principal components analysis (PCA) and support vector machine (SVM) were performed for the data grouping and classification. Based on the obtained SVM model, mass spectrometry spectra, database searching as well as the confirmation from authentic standards, several differentiating metabolites were tentatively identified. To improve visualization, z-score plot and heat map display were performed, which exhibited the changes in concentration of the metabolites. As a result, depletion of circulating carnitine, reduced amino acid biosynthesis and increased rate of lipid peroxidation were observed. Meanwhile, up-regulation of hypoxanthine indicated that purine metabolism might serve as the salvage pathway. Collectively, the results reflected metabolic responses to surgical operation in HCC patients, suggesting perturbation of energy metabolism may occur in 3 to 5 months after the partial hepatectomy.

Keywords: Metabolomics Analysis, Partial Hepatectomy, Hepatocellular Carcinoma, LC-MS

1. Introduction

Metabolite profiling was regarded to reveal metabolic changes in living systems, tissues or cell lines. The applications of the relatively new technique have been exponentially increased in numerous fields, including toxicology, pharmacology, and clinical diagnostics [1]. Studies of the identities, concentrations and fluxes on these small molecules in cells, tissues and biological fluids can enhance understanding of the mechanisms involved in pharmacology and disease pathophysiology [1-3]. Metabolomics not only involves the “quantitative determination of targeted metabolite profiling of a complex metabolic response”, but also the “qualitative determination of comprehensive or untargeted metabolite profiling” of the pathophysiological process or genetic determinants in living systems [4]. Therefore, metabolic profiling has been extended to the investigation and discover of new biomarkers for clinical diagnosis and the

evaluation of therapeutic efficacy [5-7].

Metabolomics utilizes techniques that can simultaneously quantify thousands of small molecules in a biological sample. This analytical capability must then be joined to sophisticated mathematical tools that can identify a molecular signal among millions of pieces of data [8]. A large number of metabolites occurring with very diverse physico-chemical properties and different abundance levels require the powerful analytical capacity [9]. Chromatography coupled with mass spectrometry has been demonstrated for its vast potential as a tool for this type of investigation. As a typical LC/MS analysis may involve hundreds to thousands of peaks, data extraction and analysis are important for obtaining the relevant biomarker information. The candidate peaks are then further analyzed with sophisticated mathematical tools and structurally characterized for the identification of biomarkers [10]. Treatment of the acquired mass spectrometry data from different tested groups could be con-

ducted by using multivariate data analysis. Principal components analysis (PCA), an unsupervised projection method for visualization of the dataset and display the similarity and difference, is widely applied in metabolomic analysis. Partial least squares (PLS), the regression extension of PCA such as PLS-DA, has been used as a means of data filtering and feature selection [11]. Support vector machine (SVM) as the supervised powerful machine learning can be extended to nonlinear cases with the help of kernels. SVM has been shown as a better machine learning to PLS-DA in both cases in terms of predictive accuracy with the least number of features [12-14].

More and more evidences have demonstrated that metabolomics study is a promising approach for liver diseases diagnosis. Most of the reported metabolomic studies focused on comparing liver disease with non-malignant or healthy subjects. For instance, liver cancer has been discriminated with HPLC analysis avoiding false-positive result from hepatitis and hepatocirrhosis diseases [15]. Biochemical perturbation of liver function caused by hepatitis B virus was characterized by LC/MS and GC/MS [16,17]. Low-grade human hepatocellular carcinoma tumors have been differentiated from high-grade ones based on high-resolution magic-angle spinning (MAS) ^1H nuclear magnetic resonance spectroscopy [18]. To the best of our knowledge, however, little is known about the application of metabolomics for the evaluation of prognostic metabolite profiling in HCC patients after hepatic resection. In the present study, LC/QTOFMS was conducted for non-targeted metabolomics to screen metabolic changes in the sera of HCC patients collected before and 3 to 5 months after hepatectomy. In addition to PCA and SVM introduced for pattern recognition and data mining, two other approaches were also implemented, namely hierarchical clustering heat map and z-score plot for improving visualization. The multivariate data analysis provided a powerful tool for understanding the underlying metabolic responses in HCC patients through surgical operation.

2. Materials and Methods

2.1. Sample Preparation

The sera were harvested from ten HCC patients pre- and post-hepatectomy in fasting conditions at Queen Mary Hospital, The University of Hong Kong. The study was approved by the Ethics Committee in Hong Kong. All patients are male with the age of 37 - 59 years old. Samples were stored at -80°C and then thawed before analysis. 400 μL acetonitrile was added to 200 μL of each serum sample and vortex vigorously. The sample mixture

was allowed to stand for 5 min and centrifuged at 13,000 rpm for 5 min at 4°C . The supernatant was lyophilized with the addition of 400 μL Milli-Q water. 50 μL of acetonitrile/water mixture (1:1) was added to the sera samples, vortex mixed and centrifuged prior to the LC/MS analysis. L-carnitine, acetylcarnitine, hypoxanthine and amino acids (proline, valine, leucine and isoleucine) were obtained from Sigma (St. Louis, MO, USA).

2.2. LC/MS and LC/MS/MS Analyses

Chromatographic separation was performed on an HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a 150 mm \times 2.1 mm Symmetry C18 reversed-phase column with particle size 3.5 μm (Milford, MA, USA). Aliquots of 3 μL each sample were injected onto the HPLC column for analysis. A linear gradient at a flow rate of 300 $\mu\text{L}\cdot\text{min}^{-1}$ was used containing solvents A (0.1% formic acid in water) and B (acetonitrile). Initial conditions were 5% B, held for 5 min, increased to 95% B from 5 to 20 min, and held for 4 min, then decreased to 5% B in 1 min and re-conditioning for 10 min to the initial conditions. The serum sample pairs were run in random order and in duplicate. To minimize the cross contamination, a blank run was inserted between the consecutive samples.

A QTOFMS (API Q-STAR Pulsar *i*, MDS Sciex, Toronto, Canada) was employed for the analysis of metabolite ions in positive mode. TurboIonspray parameters for ESI-MS were optimized as follows: ionspray voltage (IS) 4500 V, declustering potential I (DP I) 30 V, declustering potential II (DP II) 15 V, focusing potential (FP) 80 V. The mass range was from 100 to 1,000 m/z . The ion source gas I (GS I), gas II (GS II), curtain gas (CUR) and collision gas (CAD) were set at 30, 15, 30 and 3, respectively. The temperature of GS II was set at 350°C . Renin substrate tetradecapeptide at 10 $\text{pmol}\cdot\mu\text{L}^{-1}$ was used for the calibration of the mass spectrometer. Data acquisition and processing was performed based on Analyst QS software (service pack 7).

To obtain structural information of the metabolites, information-dependent acquisition (IDA) mode was used to acquire the MS/MS spectra of the metabolites in serum samples. Using the IDA in Analyst QS (Applied Biosystems/MDS Sciex), MS/MS analysis at different collision energies (CEs) were performed. For the product ion scans, the resolution of the mass resolving quadrupole (Q1) was set low (4 amu window). The ion peaks with peak intensity exceeding 50 counts in the survey scan triggered MS/MS analyses. Former target ions that had been selected for MS/MS analyses were excluded from MS/MS analyses for 30 s.

2.3. Multivariate and Univariate Statistical Analysis

The LC/MS chromatograms were imported into “Metabolomics ExportSript” (Applied Biosystems/MDS Sciex) for peak finding, alignment and filtering. LC/MS data were processed by using the criteria as described previously [19]. In the current work, nonlinear PCA as well as SVM with radial-basis-function (RBF) kernel were presented to map the classification. Here, T-test score and separation score were used in SVM model to rank each feature (metabolite ions). Moreover, the peak areas of the metabolite ions were integrated from extracted ion chromatograms prior to heat map display and z-score plot. All the scripts were performed in MATLAB R2008a software (The MathWorks, Inc., Natick, MA, USA).

The one-way analysis of variance (One-Way ANOVA) was utilized to compare the serum samples of post-hepatectomy to pre-hepatectomy for up- or down-regulated metabolites. The *P* values with Bonferroni correction were obtained by OriginPro 8 software (OriginLab, Co., MA). Human metabolome database (<http://www.hmdb.ca/>) (briefly HMDB) [20] the metlin metabolite database (<http://metlin.scripps.edu/>) [21] and KEGG database (<http://www.genome.jp/kegg/atlas/>) [22] were

used for metabolite identification and mapping.

3. Results

The schematic flowchart of the metabolomic approach performed in this study was illustrated in the **Figure 1**. Briefly, the metabolites from sera were extracted and applied to HPLC coupled with electrospray ion source in positive mode, with the QTOFMS data acquired from 100 to 1,000 *m/z*. One typical total ion chromatogram (TIC) is also inserted. The feature tables from the TICs were yielded consisting of *m/z* value, retention time, and integrated intensity using “Metabolomics ExportSript” software. Feature selection was generated from SVM model during data training for metabolite discovery. The metabolite ions were imported into PCA and SVM using MATLAB programme. For the identification of metabolites of interest, accurate mass data were searched against databases of known metabolites, such as Metlin, HMDB. A match was tested by fragmentation data from QTOFMS in comparison to authentic standards. Finally, the interpretation of metabolic signature and visualization in z-score plot and cluster heat map were performed. As a result, the metabolite ions which were statistically significant were selected as differentiating metabolites listed in **Table 1**.

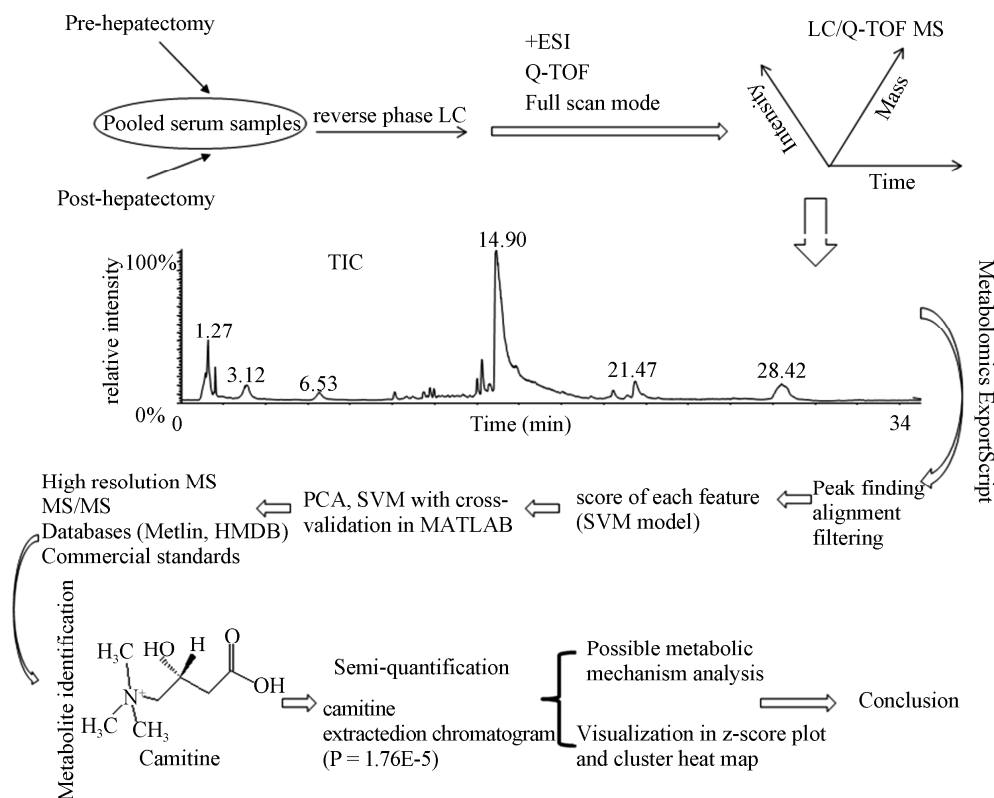


Figure 1. Schematic flowchart of the reported metabolomic study.

Table 1. Statistical differentiating metabolites.

No.	m/z ^a (RT, min)	productions, m/z	metabolites	Fold-change	P value
1	203.0503 (1.25)		Un-known	2.82↓ ^b	7.19E-5
2	162.1146 (1.32)	103, 85, 60	carnitine	3.03↓	1.76E-5
3	204.1170 (1.35)	85, 60	acetylcarnitine	4.06↓	0.0071
4	116.0680 (1.28)	70	proline	1.78↓	0.033
5	118.0821 (1.28)	72	valine	1.83↓	0.0089
6	132.0738 (1.28)	86	leucine/isoleucine	2.37↓	0.0012
7	137.0409 (1.35)	119, 110	hypoxanthine	2.35↑	0.015
8	494.3157 (19.83)	104,184	LysoPC ^c (16:1)	2.11↑	0.011
9	496.3260 (21.45)	104,184	LysoPC (16:0)	1.26↑	0.031
10	468.3114 (19.4)	104, 184, 450	LysoPC (14:0)	2.23↑	0.0062
11	520.3364 (20.5)	104, 184, 502	LysoPC (18:2)	2.25↑	3.97E-4
12	522.3429 (21.88)	104, 184	LysoPC (18:1)	1.55↑	0.0072
13	546.3537 (22.20)	104, 184	LysoPC (20:3)	2.26↑	0.0026
14	782.5476 (25.05)	184	PE ^d (22:2/18:1)/ isomers	1.49↓	0.0139
15	806.5580 (25.40)	184	PC ^e (18:2/20:4)/ isomers	1.29↓	0.014

^aPositive ion mode; ^bpost-hepatectomy versus pre-hepatectomy up-regulation (↑) down-regulation (↓); ^cLysoPC: lysophosphatidylcholine; ^dPE: phosphatidylethanolamine; ^ePC: phosphatidylcholine.

3.1. Multivariate Statistics

The separation from kernel PCA was obtained from the first two components. In **Figure 2**, PC1 accounts for 89.61% of the total variation and PC2 for 6.65%. The result showed that the partial hepatectomy had a small effect in such a short time and four post-hepatectomy samples (namely post-1, -3, -4 and -9) had not been separated from the pre-hepatectomy group. Therefore, we introduced the supervised learning method further. SVM with RBF kernel was performed for data training in **Figure 3**, then feature selection was also finished based on the model. It was well-known that the evaluation for the classification accuracy was of great importance. Two parameters (C and γ) were used for determination by using the grid search strategy [24]. Because there are only two independent parameters for parallel searching rather than iterative processes, the computational time required would be not much more than that by other advanced methods (e.g. walking along a path). Consequently, the grid search on C and γ using cross-validation was also performed shown in **Figure S1**. The parameters (C and γ) in SVM construction were chosen with the cross-validation rate 85%. It should be noted that when the cross-validation rate is higher, the prediction accuracy is higher. Considering metabolomic variations, such personalized responses to a particular therapy-partial hepatectomy, was displayed in z-score plot (**Figure S2**) and cluster heat map (**Figure S3**). Carnitine as the most significantly decreased metabolite in post-hepatectomy data set was highlighted in red box in the figures.

4. Discussion

By using LC/QTOFMS, the sera from ten HCC patients pre- and post-surgery were investigated for highlighting the differentiating metabolites via multivariate data analysis. Multivariate data analysis in PCA and SVM models was discussed. Upon finishing the capture of systems-level examination of features, identification of metabolites was performed for indication of the underlying metabolic mechanisms with the improved visualization using z-score plot and cluster heat map.

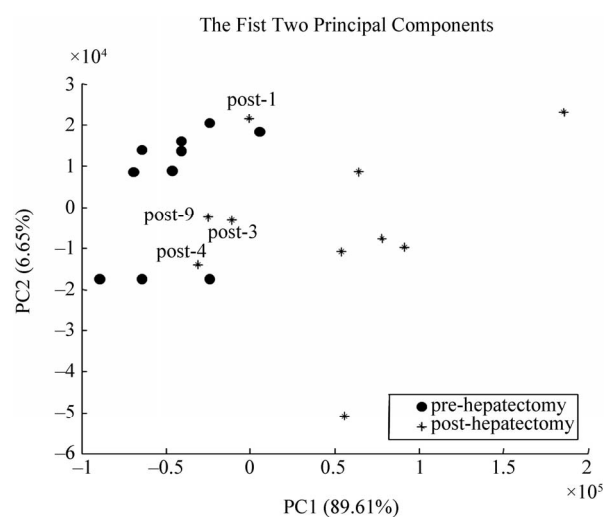


Figure 2. The analysis of PCA for pre-hepatectomy and post-hepatectomy groups. Four samples in post-hepatectomy group, namely post-1, post-3, post-4 and post-9, were labelled.

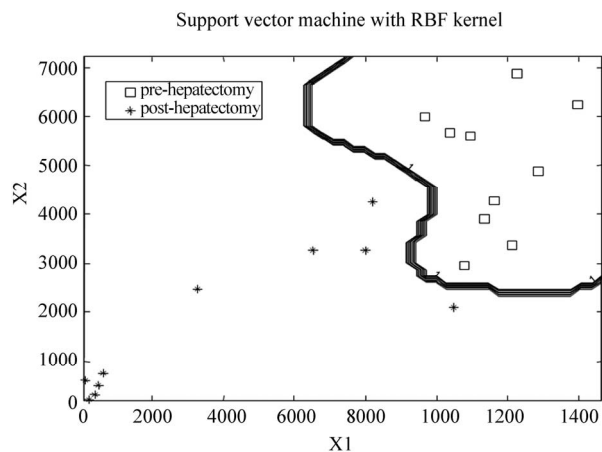


Figure 3. The clustering result of support vector machine with RBF kernel for pre-hepatectomy and post-hepatectomy groups.

4.1. Identification of Differentiating Metabolites

Identification of differentiating metabolites listed in **Table 1** was mostly based on the comparison of the obtained chromatography retention time and MS/MS frag-

mentation pathway with those from database record or reference standard. Ions at m/z 162 and m/z 204 were detected with the relatively high scores from the SVM data training in these metabolite ions. Their identifications were carried out by using reference standard through retention time in liquid chromatography and MS/MS fragmentation. It should be noted that the fragment ions at m/z 60 and m/z 85 were the characteristic ions from acylcarnitine [23]. Thus, the ion at m/z 162 yielded its fragment ions at m/z 60 and m/z 85, corresponding to carnitine (**Figure 4(a)**). Similarly, the ion at m/z 204 was identified to be acetylcarnitine. **Figure 4b** shows the MS/MS spectrum of the ion at m/z 520 in positive ion mode. Because the fragment ion at m/z 184 as a characteristic choline moiety from lysophosphatidylcholine (LysoPC) [24,25] was detected, the ion at m/z 520 was identified to be LysoPC (18:2). Similarly, other LysoPCs as well as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were tentatively identified in positive ion mode. Additionally, identification of hypoxanthine and amino acids was carried out by comparison to the obtained MS/MS spectra with those of authentic standards.

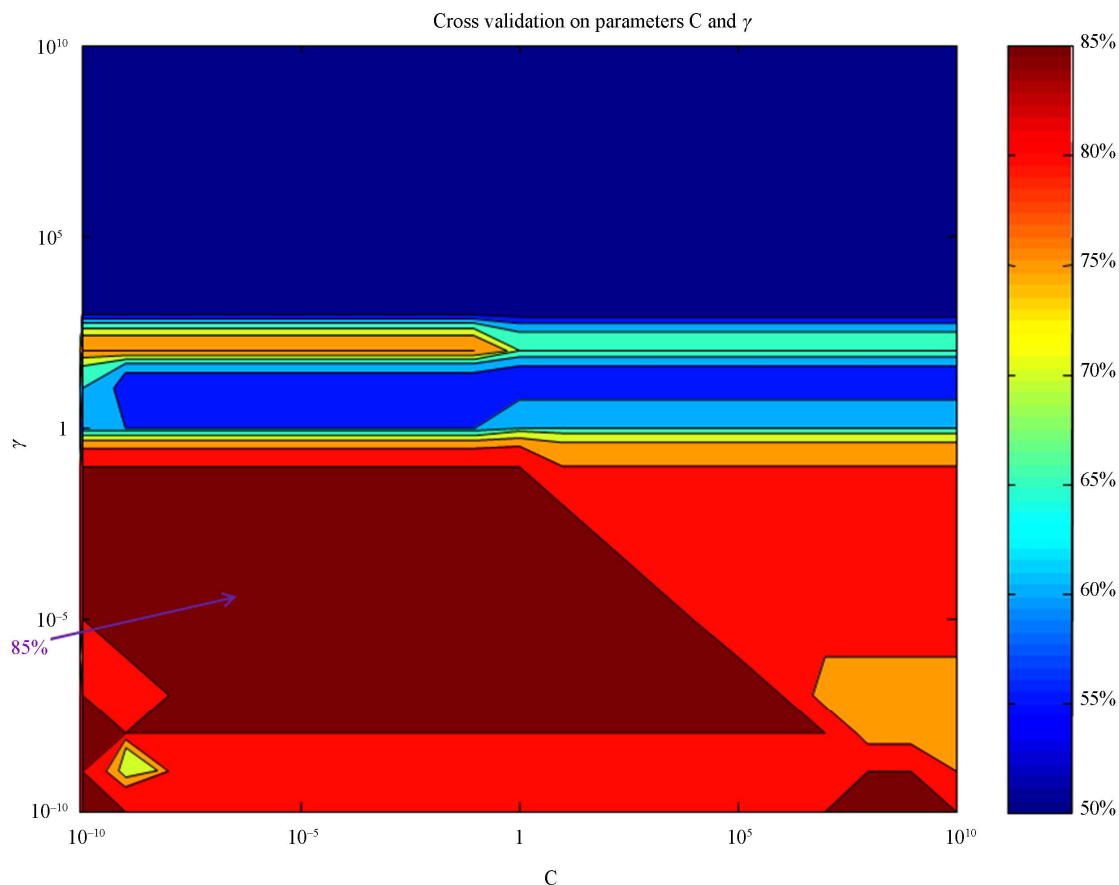


Figure S1. Cross-validation for performance in SVM model.

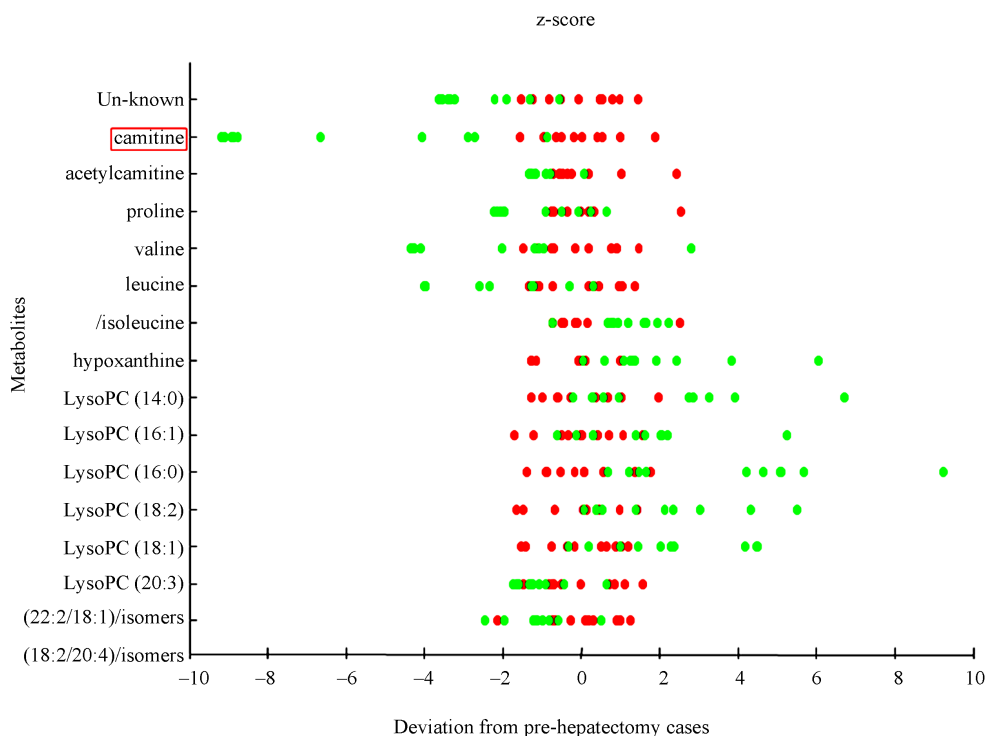


Figure S2. Z-score plot for all the potential biomarkers. Red dots indicated that pre-hepatectomy whereas green dots indicated that post-hepatectomy sample sets. (LysoPC: lysophosphatidyl-choline; LysoPE: lysophosphatidylethanolamine; PC: lysophosphatidylcholine; PE: phosphatidylethanolamine). (z-score range: -10 to 10).

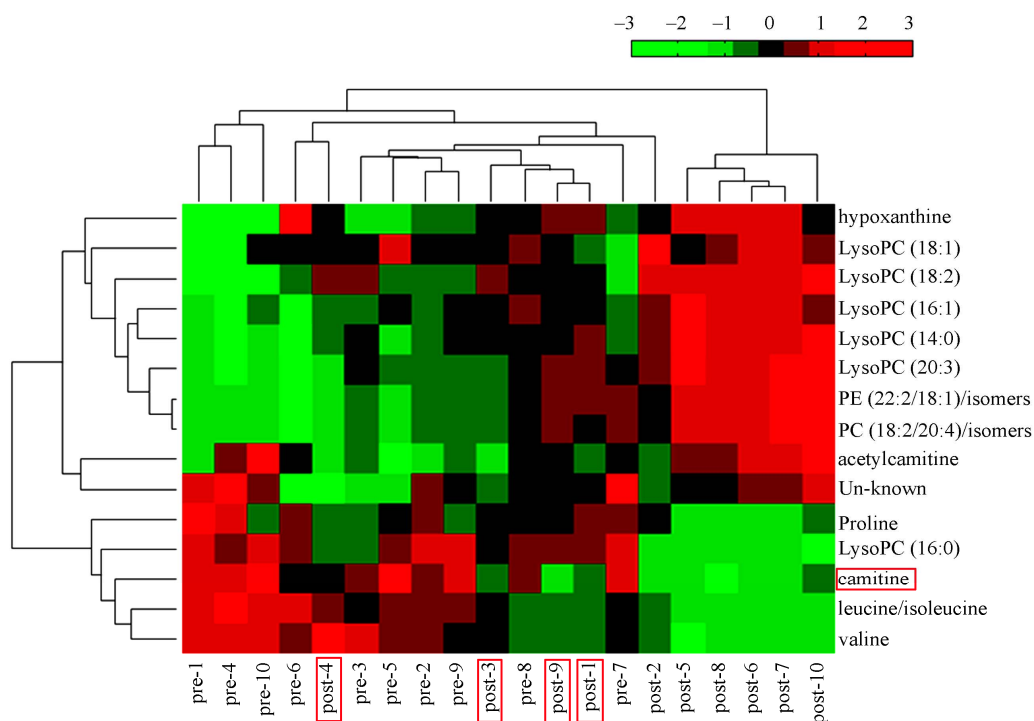


Figure S3. Cluster heat map. Six post-hepatectomy samples are clearly separated from the pre-hepatectomy group, but distribution of other four samples in post-hepatectomy group (namely post-1, post-3, post-4 and post-9) could not be recognized, consistent with kernel PCA result. (LysoPC: lysophosphatidyl-choline; LysoPE: lysophosphatidylethanolamine; PC: lysophosphatidylcholine; PE: phosphatidylethanolamine). (Fold-change range: -3 to 3).

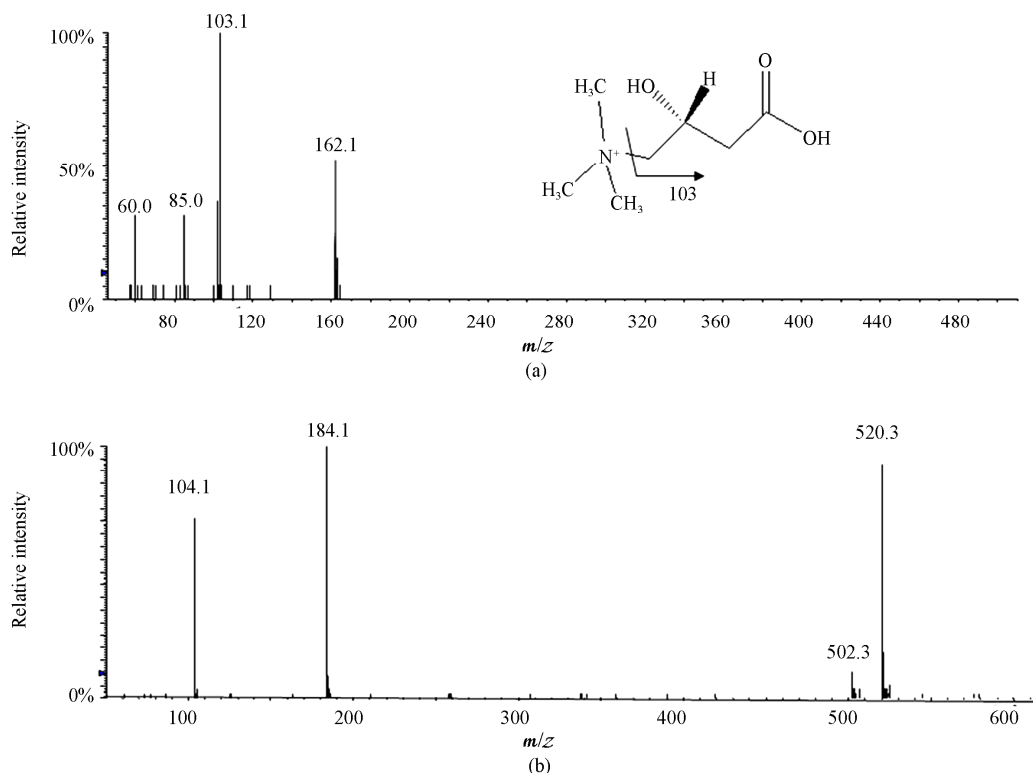


Figure 4. MS/MS spectra of carnitine (a) and LysoPC (18:2) with the collision energy of 20 eV.

4.2. Multivariate Analysis and Annotation of Metabolic Shifts

In **Figure 2**, PCA was applied for grouping pre- and post-hepatectomy in HCC patients. The obtained data showed that discrimination of the two groups was not clear. Furthermore, SVM with RBF kernel as the supervised machine learning was applied to examine the effectiveness of the classification function and feature selection (**Figure 3**). Cross-validation was also performed to evaluate the performance of SVM model (**Figure S1**). The parameter pairs (C and γ) were tested and the one with the best cross-validation accuracy was picked. A wide range from 10^{-10} to 10^{10} for the parameters C and γ was selected in construction, and the best region on the grid was obtained. The cross-validation rate was much less than 100%, probably resulted from the small number of cases and variation of the personalized status. Nevertheless, SVM model with RBF kernel could classify two groups for feature selection.

After the metabolites have been identified, the possible metabolic regulation could be used for the interpretation of biochemical changes induced by partial hepatectomy. Carnitine, a trimethylated amino acid, was reported to exhibit deficiency in some individuals following a strict vegetarian diet [26]. On one hand, carnitine deficiency observed in our study might indicate that en-

ergy metabolism was disturbed by partial hepatectomy in the HCC patients because carnitine has functioned to reduce the availability of lipid peroxidation by transporting fatty acids into the mitochondria for beta-oxidation to generate adenosine triphosphate (ATP) energy. On the other hand, the increase of serum carnitine could be considered a new index of improved liver function [27], suggesting that L-carnitine could inhibit hepatocarcinogenesis via protection of mitochondria in another investigation [28]. Taken together, sharp down-regulation of carnitine implied that reduced energy production may occur in 3 to 5 months after partial hepatectomy. Notably, decreased amino acid biosynthesis after the surgical operation might play a key role in the HCC patients, because carnitine is synthesized endogenously from the essential amino acids. The pronounced decrease of proline, valine and leucine/isoleucine may contribute to carnitine deficiency [29]. Therefore, we speculated that partial hepatectomy could cause reduced energy production through decreased amino acid synthesis and carnitine deficiency.

Interestingly, LysoPCs in post-hepatectomy were up-regulated significantly compared to pre-hepatectomy. LysoPCs levels previously described in hepatitis patients were down-regulated compared to the healthy control, but the mechanisms remain to be explored [16]. Nevertheless, the decreased concentrations of PC and PE were

also observed, indicating that lipid peroxidation occurred after hepatectomy in HCC patients [30]. One of the possible correlations was highlighted in **Figure 5 (a)**. Lipid peroxidation could be controlled by carnitine, thus the increased rate of lipid peroxidation as the feedback revealed depletion of carnitine could cause metabolic shifts in relative pathways. Similarly, two kinds of LysoPCs (16:0 and 18:2) were found with increased concentrations but carnitine with decreased concentration in the intestinal fistulas patients [31]. Furthermore, It was elevated hypoxanthine in purine metabolism that was observed in post-hepatectomy groups from **Table 1**, suggesting that the purine biosynthesis could be substituted by the salvage pathway after the operation [32]. The possible metabolic pathways were simply delineated in **Figure 5(b)**.

Collectively, perturbation of the differentiating metabolites might reveal reduced energy production from our results associated with partial hepatectomy in HCC patients, which was consistent with the evidence from altered protein and energy metabolism in chronic liver disease associated with malnutrition and the report in primary carcinogenesis and generation of metastasis [33,34]. Therefore, partial hepatectomy might lead to liver function deterioration instead of amelioration in terms of whole body level.

Finally, to improve the visualization, z-score plot (**Figure S2**) and cluster heat map (**Figure S3**) were performed. They were plotted for each of fifteen metabolites

with emphasis on depletion of carnitine. The z-score plot displayed metabolic alterations between pre-hepatectomy and post-hepatectomy (z-score range: -10 to 10). The heat map represented the unsupervised hierarchical clustering of the data grouped by sample type (fold-change range: -3 to 3). It also visualized the up- or down-regulation of each metabolites and distribution of four samples (namely post-1, 3, 4 and 9) among them could not be recognized, revealing the discrimination from the metabolite clustering as well as sample grouping. Carnitine, as the amino acid derivate, was classified with three amino acids (proline, valine and leucine/isoleucine) in the same cluster.

It is important to note firstly that the number of patient cohort was small but still may be scientifically important, and bear further investigation in a large population. Secondly, the sera were collected from pre- and 3 to 5 months post-partial hepatectomy in HCC patients, and the result indicated the resection might induce liver function deterioration. Hereby, we would follow up for a longer time to investigate their liver functions. Thirdly, lifestyle factors greatly influence metabolism, making it difficult to disentangle their effects from operation-related outcomes. Nevertheless, the reported metabolomics study involving LC/QTOFMS and multivariate data analysis might provide a promising tool for understanding the underlying metabolic responses because little is known currently about metabolic perturbation in HCC patients through the surgical operation.

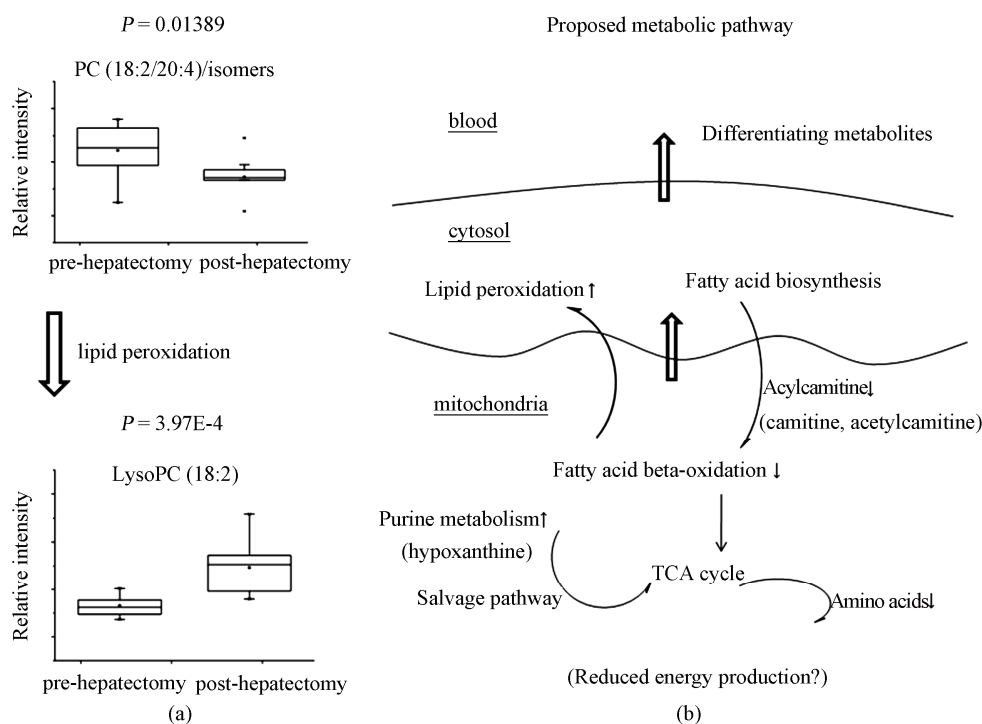


Figure 5. Lipid peroxidation (a) and proposed metabolic pathways (b).

5. Conclusions

Among fifteen statistically significant metabolites, amino acids and acetylcarnitine decreased significantly and down-regulation of PC and PE was accompanied with pronounced increase of LysoPCs. Carnitine, a necessary factor in the utilization of long chain fatty acids to produce energy, was down regulated sharply by partial hepatectomy. The elevated hypoxanthine might play a vital role in salvage pathway for reduced energy production. Metabolomic approach with highly efficient classification was performed to distinguish the two groups of data sets and delineate the effects of partial hepatectomy in metabolic shifts, reflecting that hepatic resection might induce liver function deterioration in 3 to 5 months.

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7. References

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