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Metabolomic identification of potential phospholipid biomarkers for chronic glomerulonephritis by using high performance liquid chromatography–mass spectrometry

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Abstract

Plasma phospholipids metabolic profile of chronic glomerulonephritis was investigated using high performance liquid chromatography/mass spectrometry (LC/MS) and principal component analysis. The plasma samples of 18 patients with chronic glomerulonephritis, 17 patients with chronic renal failure (CRF) without renal replacement therapy and 18 healthy persons were collected and analyzed. It was found that combination of the LC/MS technique with PCA can be successfully applied to phospholipid profile analysis. The results showed that primary chronic glomerulonephritis and CRF had phospholipids metabolic abnormality. Nineteen phospholipid species were identified as possible biomarkers in plasma samples of chronic glomerulonephritis and chronic renal failure. Moreover, the identification of the molecular structure of the potential phospholipid markers was obtained by ESI-MS/MS experiment. It suggests that phospholipids can be used as potential biomarkers on the progress of primary chronic glomerulonephritis.

Keywords: Phospholipids; Chronic glomerulonephritis; High performance liquid chromatography; Mass spectrometry

1. Introduction

The epidemiological impact of chronic kidney disease (CKD) is now a significant problem worldwide, and the number of patients with end-stage renal disease (ESRD) has increased dramatically and partly unexpectedly [1–4]. Chronic glomerulonephritisis is one of CKDs. As the mechanism of chronic glomerulonephritis is not yet clear, its treatment and prognosis are not optimized. Although nephrologists aim to slow down the progression of chronic glomerulonephritis and limit the occurrence of ESRD by early detection and control of risk factors [5], the fact that it finally results in ESRD is certain.

Phospholipids are important constituents that form the cell membrane. Membrane phospholipids are a complex mixture of molecular species containing a variety of fatty acyl and head group compositions, such as phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), lysophosphatidylcholine (lysoPC) and sphingomyelin (SM). In addition to their structural role, some phospholipids also participate in biological processes in various pathways such as signal transduction [6], membrane trafficking and sorting [7], morphogenesis [8], and source of second messengers [9–12]. In many fields increasing attention to phospholipids has been paid, but there have been few published reports describing phospholipids metabolism in CKD.

Metabonomics is a key technology of systems biology in studies of either clinic diagnosis or pharmaceutical industry [13,14]. It is one of the latest omics technologies for measuring the relative concentrations of endogenous small molecules in biofluids and characterizing the alternation of the metabolites occurring in organisms [15]. In the application of clinical trials, searching and identification of the disease biomarkers are the primary goals of metabonomics study. In order to detect these changes and obtain better quality, information-rich techniques such as mass spectrometry coupled with high performance liquid chromatographic separation process are employed [16]. The higher the sensitivity and resolving power of the system [17–20], the more analytes detected and hence more structural

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information obtained to help identifying new biomarkers of the diseases. Metabonomics uses data from information-rich analytical techniques, which provides spectral patterns that can be evaluated using multivariate statistical methods, such as principal component analysis (PCA) [18,21].

In the paper, by combining HPLC/MS with multivariate statistical analysis, we studied plasma phospholipids metabolic profiling from patients of chronic glomerulonephritis, patients of chronic renal failure and healthy persons to discover the potential biomarkers. Product ion spectra following collisionally activated dissociation (CAD) of $[M-H]^-$ or $[M-15]^-$ ions in a tandem mass spectrometry were used to identify individual molecular composition of phospholipid biomarkers.

2. Experimental

2.1. Plasma samples

Plasma samples were collected from 18 healthy controls (aged from 23 to 61 years), 18 patients with chronic glomerulonephritis (aged from 19 to 54 years), and 17 patients with chronic renal failure (aged from 24 to 63 years) without renal replacement therapy, such as hemodialysis. To prevent platelet activation and phospholiphase activity, blood samples were collected in EDTA-containing tubes. Two milliliters of blood was collected by an EDTA-containing tube, and was centrifuged at 3000 rpm for 10 min at 4 °C. All of the plasma samples, free of any added preservatives, were kept frozen at −80 °C before lipid extraction.

Eighteen healthy adults from the authors' institute were chosen to be used as references. Thirty-five patients including 12 females and 23 males were from the First Affiliated Hospital of Dalian Medical University in China (from April 2003 to January 2006). Diagnoses of chronic glomerulonephritis and chronic renal failure (CRF) were made on the basis of usual clinical and laboratory findings and were confirmed by kidney biopsy. Eighteen of them are in the chronic glomerulonephritis group. The clinical diagnoses for all of them were primary chronic glomerulonephritis. Whereas, the glomerulonephritises due to systemic lupus erythematosus, allergic purpura, renal damage caused by hypertension, diabetic nephropathy and obstructive nephropathy were not in the chosen range. In the pathological diagnoses of primary chronic glomerulonephritis, there were 11 cases of mesangial proliferative nephritis and 7 cases of membranous nephropathy. The proteinuria concentration was 846.7 ± 266.8 mg/24 h, serum creatinine was 98.3 ± 26.9 μmol/L. Seventeen CRF patients did not take the renal replacement therapy (i.e. blood purification therapy). Serum creatitine was 874.4 ± 394.9 μmol/L. The basic diseases for these CRF were primary chronic glomerulonephritis.

2.2. Chemicals

The following four phospholipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA): 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (C14:0/C14:0 PE phosphatidyl ethanolamine), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (C14:0/C14:0 PC phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (C14: 0/C14:0 PS phosphatidylserine) and 1-lauroyl-2-hydroxyl-*sn*-glycero-3-phosphocholine (C12:0 lyso PC lysophosphatidylcholine). 2,6-Di-*tert*-butyl-4-methylphenol was obtained from Aldrich-Chemie (Steinheim, Germany). Formic acid and all the solvents were of HPLC grade (TEDIA, USA). Ammonia solution (25%) was of analytical grade from Lian-Bang (Shenyang, China). Deionized water was prepared by a Milli-Q reagent water system (Millipore, MA).

2.3. Pretreatment of plasma samples

The phospholipids standards were dissolved (approx. 1 mg/mL) in chloroform/methanol (2:1, v/v), and further diluted with hexane/1-propanol (3:2, v/v). For the analysis of plasma phospholipids, the samples were thawed at room temperature. The lipids were extracted in a similar procedure to literature [22], 250 μL of water was added to 210 μL of the blood sample; then 1.5 mL methanol with 0.01% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 3 mL chloroform was added and the solution was sonicated for 60 s both before and after adding chloroform. Appropriate amounts of internal standards [e.g. C14:0/C14:0 PE, C14:0/C14:0 PC, C14:0/C14:0 PS, C12:0 lysoPC] were added together into the solution. The solution was whirlmixed for 30 s and incubated for approximately 60 min at room temperature. Finally, 1.5 mL of water was added before the solution was mixed for 5 s and centrifuged at $3000 \times g$ for 10 min. The lower chloroform phase was sampled and dried by evaporation at 38 °C. Prior to analysis, the extracted samples was redissolved in 250 μL of chloroform/methanol (2:1, v/v) and then diluted 10 times with hexane/1-propanol (3:2, v/v).

2.4. High performance liquid chromatography and mass spectrometry

An HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used. The LC separation was performed on a diol column, 250 mm × 3.9 mm, i.d. × 5.0 μm (Nucleosil, 100-5 OH, Germany) at 35 °C. The total flow rate of the mobile phases (solvent mixtures A and B) was 0.4 mL/min. The flow from the LC was split using a Micro-Splitter valve such that the flow to the electrospray was approximately 0.28 mL/min. The gradient started at 32% B and 68% A, at 20 min linearly increased to 80% B, held for 13 min, then in 5 min linearly decreased to 32%, held for another 22 min [23]. Solvent mixture A was hexane/1-propanol/formic acid/ammonia solution (79/20/0.6/0.06, v/v/v/v), solvent mixture B was 1-propanol/water/formic acid/ammonia solution (88/10/0.6/0.06, v/v/v/v).

The mass spectrometric detection was conducted on a QTRAP LC/MS/MS system from Applied Biosystems/MDS Sciex (USA) equipped with a turbo ion spray source. The combination of highly selective triple quadrupole MS–MS scans and high sensitivity ion trap product scans on the same instrument platform provides rapid identification of phospholipids in extracted blood samples. The detection of phospholipids

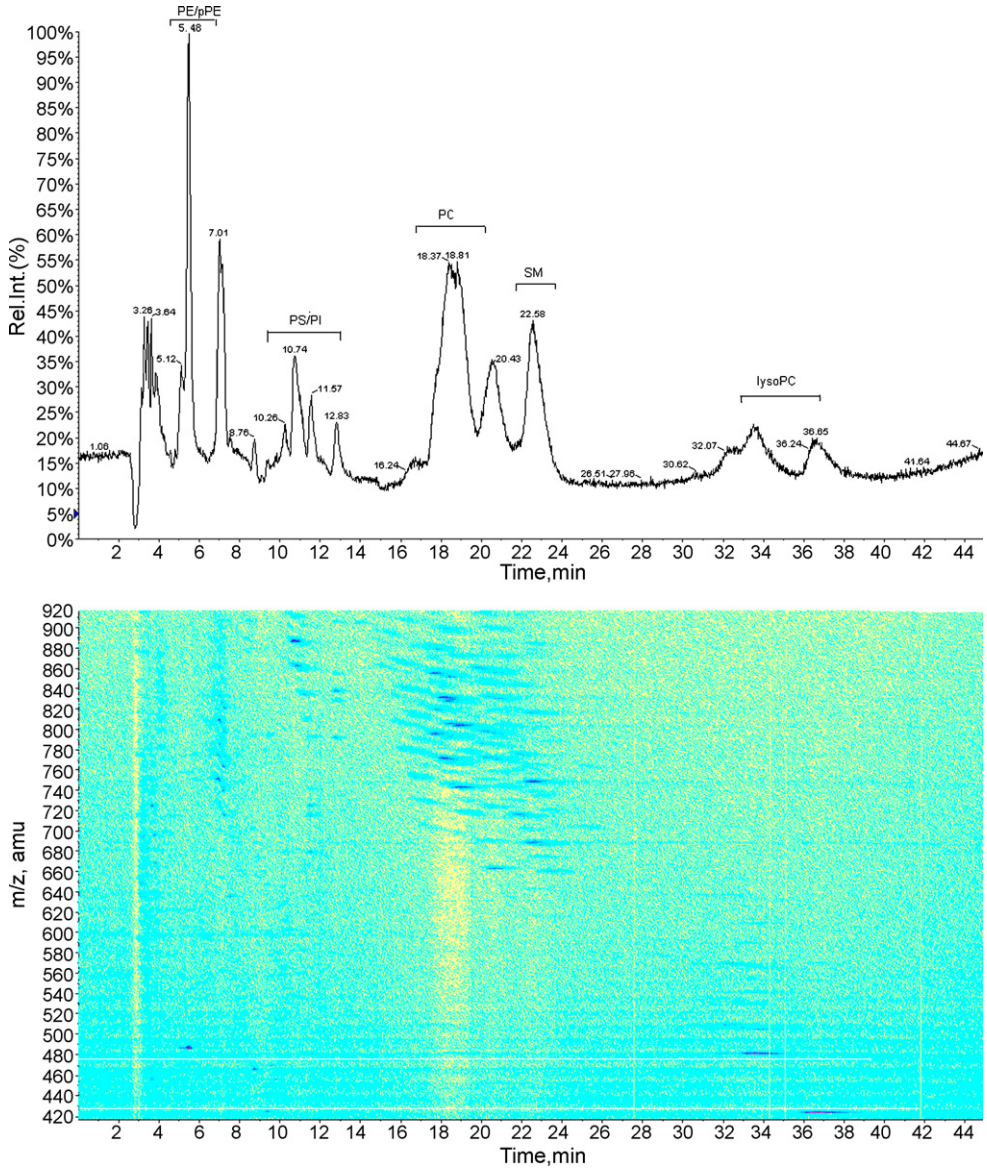

Fig. 1. Total ion current chromatogram of phospholipid mixture obtained from a blood sample of primary chronic glomerulonephritis by LC/ESI-MS analysis showing both total ion current chromatogram (top) and a two-dimensional plot including retention times and *m/z* values (bottom). PE was eluted at first, followed by PS (PI), PC, SM and lysoPC successively. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; LysoPC, lysophosphatidylcholine; SM, sphingomyelin.

eluted from the chromatographic column was performed in the "enhanced MS" (EMS), single quadrupole mode (so called "survey scan") where ions were accumulated and then filtered in the Q3-linear ion-trap. The structures of phospholipids are elucidated according to the tandem mass spectra acquired under the "enhanced product ion scan" (EPI) mode.

The split HPLC effluent entered the MS through a steel ES ionization needle set at −4500 V (in negative ion mode) and the ion source temperature set at 250 °C. The ion source and ion optical parameters were optimized with respect to the negative $[M-H]^-$ or $[M-15]^-$ ions of the phospholipids standards. Both the nebulizing gas and turbo gas were set at 40 psi, the curtain gas was set at 30 psi. The declustering potential was set at 80 V. The other parameters were as follows: EMS as survey scan (mass range *m/z* 450–950, scan speed 1000 Da s^{-1}, fill time 20 ms) and EPI as dependent scan (scan speed 1000 Da s^{-1}, fill time 150 ms, collision energy set from −50 to −35 eV in the negative-ion mode).

In the selected conditions, the phospholipid standards were analyzed for six times. Reproducibility of HPLC–MS was evaluated. The RSDs (%) of retention time and peak intensity ratio were <0.5% and <5%, respectively.

2.5. Data analysis

Negative-ion LC/MS chromatograms were inspected for profiling the phospholipid species in plasma. Negative-ion mode ESI-MS was chosen because it gave a more information-rich data than positive-ion ESI. Masses corresponding to the quasi-molecular anions $[M-H]^-$ (for PE, PS, and PI species) or

$[M-15]^-$ (for PC, SM and lyso-PC species) for each phospholipid species were plotted against elution time. From LC/MS profile of a plasma sample, more than 80 phospholipid species were discovered and the peaks constantly occurring in all plasma samples were collected to form a data array. The peak intensities in the extracted ion chromatography were normalized by that of internal standard using a home-made software, and were used for principal component analysis (PCA) [21] with the SIMCA-P software (Version 10.0 DEMO, Umea, Sweden).

The concentration of the plasma phospholipid species (responsible for separation between classes) of healthy controls, patients with chronic glomerulonephritis and CRF were expressed as the mean ± SD, compared with the Student' *t*-test for paired or unpaired data. Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. LC/MS analysis

The full scan of phospholipid species in plasma was taken in the negative-ion mode because under such mode most of the phospholipid species have relatively high sensitivity. The two-dimensional projection method is applied to the elution mode of plasma phospholipids. One-dimensional projection setting: retention time, another dimension setting: *m/z*. Fig. 1 illustrates the complexity of phospholipid species in plasma samples of chronic kidney disease patients based on the negative-ion HPLC-ESI/MS. Under our separation conditions, PE was eluted at first, followed by PS (PI), PC, SM and lysoPC successively for phospholipids containing a given fatty acyl composition. Since different molecular species within a given class have the same polar head, their retention times only have minor differences. However, the retention time difference of compounds within the same class is less than that in two different classes, which can be used to align the retention times of phospholipid species in the extracted ion chromatography. In the negative-ion spectra, molecular species of PE, PS and PI mainly give the $[M-H]^-$ ions, $[M-15]^-$ ions for PC, SM and lysoPC species. The internal standards (C14:0/C14:0 PE, C14:0/C14:0 PS, C14:0/C14:0 PC and C12:0 lysoPC) were selected based on their solubility and the lack of any demonstrated endogenous molecular ions in that region, which was verified by acquiring a mass spectrum

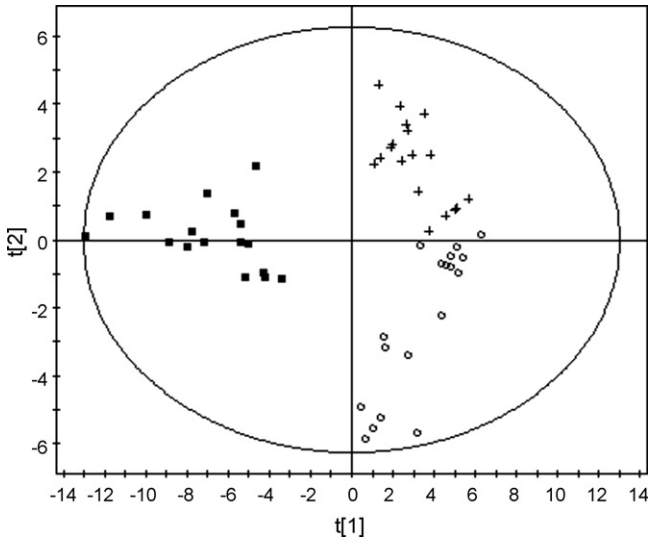

Fig. 2. Scores plot from PCA classifying patients with primary chronic glomerulonephritis, CRF patients and healthy controls. (◯): Healthy controls; (+): primary chronic glomerulonephritis; (●): CRF.

Table 1
Comparison of phospholipid species in plasma samples from healthy controls, patients with chronic nephritis, and chronic renal failure[a]

Class	Mass	Comparison between groups		
		Chronic nephritis and healthy controls	CRF and healthy controls	Chronic nephritis and CRF
PI	833	<0.05	<0.001	<0.001
	857	>0.05	<0.001	<0.001
	861	<0.01	<0.001	<0.001
	863	<0.001	<0.001	<0.001
	883	<0.05	<0.001	<0.001
	885	<0.001	<0.001	<0.001
	887	>0.05	<0.001	<0.001
	909	>0.05	<0.001	<0.001
	911	<0.05	<0.001	<0.001
	913	>0.05	<0.001	<0.001
PS	786	<0.01	<0.001	<0.001
	788	<0.05	<0.001	<0.01
	790	>0.05	<0.001	<0.001
	808	>0.05	<0.001	<0.001
	810	<0.01	<0.001	<0.001
	832	<0.05	<0.001	<0.001
	834	<0.05	<0.001	<0.001
	836	<0.001	<0.001	<0.001
	838	>0.05	<0.001	<0.001

[a] The concentrations of 19 phospholipids were significantly elevated in CRF patients ($P < 0.001$). Compared with the healthy controls, phospholipid species in chronic glomerulonephritis except the ions of 857, 887, 909, 913, 790, 808 and 838 have significant changes.

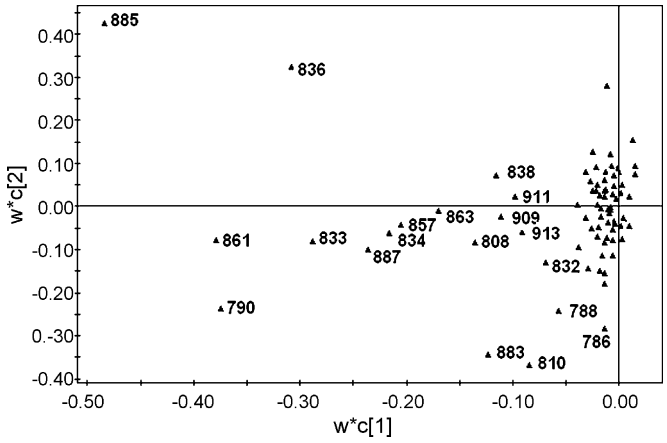

Fig. 3. Corresponding loadings plot from PCA, the data in the loading figure represent the *m/z* value.

without internal standards. These phospholipid species were quasi-quantified by the comparison of the individual ion peak intensity with that of corresponding internal standard. For PI and SM species, due to the lack of commercial internal standards, their retention times were close to those of PS and PC species, and their quasi-quantification was based on PS and PC internal standards (i.e. C14:0/C14:0 PS and C14:0/C14:0 PC, respectively).

3.2. Multivariate analysis

A single LC/MS of phospholipids metabolite profile in plasma samples of the chronic kidney disease patients can yield many components. This provides a wealth of information to be interpreted and leads to significant challenges in processing the data. Thus, applying multivariate statistical methods to the analysis of phospholipids data was to identify the characteristics of phospholipids metabolite profile for chronic kidney disease in different stages of renal function by comparison with the plasma samples of healthy persons. More than 80 variables (phospholipid molecules) from 18 normal healthy persons, 18 chronic glomerulonephritis patients and 17 CRF patients were set to be the row of X matrix, 53 plasma samples were set to be the column of X matrix. After that, they were applied to PCA analysis.

It can be seen from Fig. 2 that PCA can separate the healthy controls from the kidney disease population. It indicates that patients with renal disease have abnormal phospholipids metabolism. Meanwhile, it can also be seen that the phospholipids metabolism of uremia and chronic nephritis were different from the failure of renal function.

3.3. Potential biomarkers

The loading plot indicates that the most influential ions are responsible for separation between sample classes: the ions having the greatest influence in the PCA score plot are those farthest away from the main cluster of ions. In turns, these compounds might be the candidates for biomarkers. Fig. 3 indicates that the possible biomarkers are the ions with *m/z* 833, 857, 861, 863, 883, 885, 887, 909, 911, 913, 786, 788, 790, 808, 810, 832, 834, 836 and 838 in the negative-ion mode.

Nineteen phospholipid species as possible biomarkers of healthy controls, patients with chronic nephritis and patients with chronic renal failure are listed in Table 1. To evaluate the significance of variations between groups, a Student's *t*-test was

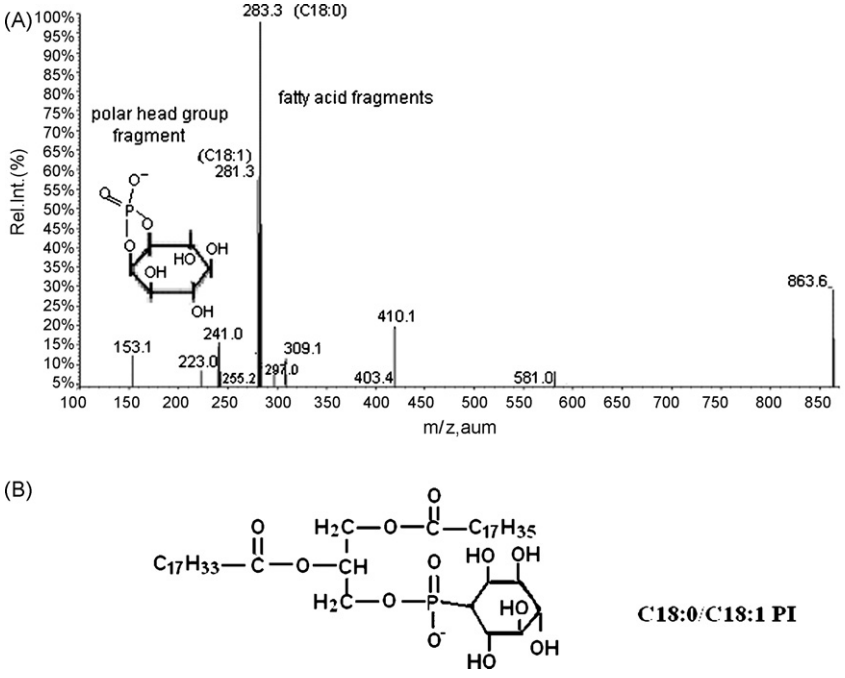

Fig. 4. EPI and structure of PI. (A) Enhanced product ion (EPI) spectrum of *m/z* 863.6 representing the $[M-H]^-$ ion of PI from extract primary chronic glomerulonephritis patient plasma samples. (B) Structure of phosphatidylionsitol molecule (*m/z* 863.6 representing the $[M-H]^-$ ion of PI). The *m/z* 241.0 is polar head group fragment, both *m/z* 283.3 and 281.3 respond to fatty acid moieties of C18:0 and C18:1.

Table 2
Phospholipid molecular species identified as potential biomarkers for classifying chronic kidney diseases from healthy controls

Class	Ion	Mass (*m/z*)	Molecular species
PI	$[M-H]^-$	833	C16:0–C18:2
		857	C16:0–C20:4, C18:0–C18:4
		861	C18:0–C18:2, C18:1–C18:1
		863	C18:0–C18:1
		883	C18:1–C20:4
		885	C18:0–C20:4
		887	C18:0–C20:3
		909	C18:0–C22:6
		911	C18:0–C22:5
		913	C18:0–C22:4
PS	$[M-H]^-$	786	C18:0–C18:2, C18:1–C18:1
		788	C18:0–C18:1
		790	C18:0–C18:0
		808	C18:1–C20:4
		810	C18:0–C20:4
		832	C18:1–C22:6
		834	C18:0–C22:6
		836	C18:0–C22:5
		838	C18:0–C22:4

used. Samples from healthy persons were chosen as a control group to indicate the selectivity of phospholipid species from patients with chronic glomerulonephritis and patients with CRF. Clearly, 19 phospholipid species were significantly elevated in CRF patients ($P < 0.001$). Compared with the healthy controls, phospholipids species in chronic glomerulonephritis except the ions of 857, 887,909, 913 790, 808 and 838 have significant changes.

Moreover, recognition of molecular structures of phospholipids was carried out by the enhanced product ion (EPI) spectrum experiment under the anion module [24]. For example, the negative ion EPI mass spectra of PI species were obtained by ESI-MS/MS. The EPI spectrum of the $[M-H]^-$ ion of PI at *m/z* 863.6 is shown in Fig. 4A. The carboxylate anion fragment ions at *m/z* 283.3 and 281.3 respond to fatty acid moieties of C18:0 and C18:1, respectively. The *m/z* 241.0 is polar head group fragment. In this paper we adopted that the phospholipids isolated from animals most often contain a saturated fatty acid at sn-1 position and an unsaturated fatty acid at sn-2 position [25]. Therefore, it is supposed that the $[M-H]^-$ ion at *m/z* 863.6 is identified as C18:0/C18:1 diacyl PI. Fig. 4B shows the possible structure of this kind of phospholipids molecule. Similarly, the recognition of other biomarkers of phospholipids molecules was also carried out by the EPI experiment of negative ion (Table 2).

3.4. Abnormal phospholipids metabolism in chronic glomerulonephritis

The application of LC/MS coupled with PCA makes it possible to show the fact that the abundances of phospholipid molecules (especially for PI and PS species) of patients with chronic glomerulonephritis are obviously different from those of the healthy persons. Phospholipids are the main constituents of biological membrane. The results suggest that chronic glomerulonephritis has metabolic abnormality of phospholipids.

At present, the relationship of the increase of phospholipids and chronic glomerulonephritis is still not very clear. The possible mechanism is that the intracellular PI metabolism is active, and it generates IP_3 and DAG after hydrolysis by activating phosphatidylinositol with specific phospholipase C (PI-PLC) [26]. IP_3 induces sarcoplasmic reticulum to release Ca^+ and increase the concentration of Ca^+ in cytochyma [27,28]. Ca^+, PS and DAG all together participate in the activation of PKC signal transfer path. The activated PKC can increase the space between endothelial cells [29] by changing the structure and function of phosphorylated actin, weakening the connections between endothelial cells and between endothelial cell and matrix [30,31]. So, the activation of PKC leads to the injury of endothelial cell barrier of renal glomerular capillary, in turns, the increase of the permeability of vessel. It results in the leakage of protein and erythrocytes, further to proteinuria and microscopic hematuria. At the same time, PKC can also lead to the increase of activity of angiotensin [32], induces hypertension, which aggravates chronic glomerulonephritis.

4. Conclusions

Combining the LC/MS technique with multivariate statistical analysis suggests that primary chronic glomerulonephritis and CRF had abnormality in phospholipids metabolism. At the same time, chronic glomerulonephritis and CRF also had changes in phospholipids metabolites. Moreover, the recognition of the structure of potential phospholipids molecular markers was realized by ESI-MS/MS experiment. These results suggest that phospholipids can be used as a potential biomarker in the progress of primary chronic glomerulonephritis.

Acknowledgements

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