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Original Article

Metabolomics analysis on patients with ischemic stroke based on ultra-performance liquid chromatography with quadrupole-time-of-flight mass spectrometry

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Abstract: Ischemic stroke is an important cause of death, which can severely affect the quality of life and bring the heavy burden to the society and family. To investigate the effective treatment of ischemic stroke is of great importance. The present study focused on the clinical treatment of ischemic stroke, and aimed to seek the potential biomarkers of ischemic stroke using the technique of high-performance liquid chromatography coupled with quadrapole-time-of-flight mass spectrometry (UPLC-Q/TOF-MS). 30 patients with ischemic stroke and 17 healthy controls were enrolled in the present study. The plasma samples were analyzed by UPLC-Q/TOF-MS to create the metabolomics profiles. Principal component analysis and orthogonally partial least squares discriminate analysis were used to investigate the metabolic changes related to ischemic stroke. The receiver operator characteristic curves were utilized to evaluate the specificity and the sensitivity of the obtained biomarkers. The metabolic pathways of the biomarkers were analyzed. Eight biomarkers for ischemic stroke were obtained, including sphinganine, 2-ketobutyric acid, tetradecanedioic acid, docosatrienoic acid, glutamine, phytosphingosine, lysoPE (0:0/22:0), and pyroglutamic acid. The results of the receiver operator characteristic curves indicated that, all the areas under the curve of the eight biomarkers were larger than 0.7. The specificity and the sensitivity can be high, showing a good accuracy of the early diagnosis. The results of the metabolism analysis showed that sphingolipid metabolism, D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine biosynthesis, propanoate metabolism, and glutathione metabolism were perturbed. The energy metabolism was also disturbed. The results could provide helpful targets for the treatment of ischemic stroke.

Keywords: Metabolomics analysis, ischemic stroke, UPLC-Q/TOF-MS

Introduction

During the past five years, stroke has remained the most important cause of death in China according to the China Health Statistics Yearbook in 2015, and the incidence of ischemic stroke is about 70% [1, 2]. The etiology of ischemic stroke is multi-factorial, and the pathogenesis includes oxidative stress [3], cell necrosis and apoptosis [4-7], immune response and inflammatory response [8, 9], and the interaction between the neurovascular units [10-12], etc. Although ischemic stroke has resulted in the heavy burden to the society and severely affected the people's daily life, there is still a lack of effective treatment for a long period. Therefore, the study of clinical treatments for ischemic stroke is of significant importance.

Metabolomics is a discipline that studies the set of metabolites present at a point in time *in vivo* [13], which has made great progress during the last decade in the comprehensive diagnosis of different diseases and toxicity [14, 15]. In the clinical research, metabolomics can provide multiple reliable diagnostic paths for diseases, serving as a noninvasive and holistic diagnostic technology [16-18]. Ultra-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UPLC-Q/TOF-MS) is a powerful method of analysis [19-21], with advantages of faster analysis, shorter time and increased separation efficiency compared with the other traditional instruments. It is possible to dynamically analyze plasma to identify and resolve metabolic differences in endogenous substances *in vivo* due to the high throughput, high sensitivity and high accuracy of UPLC-Q/

Table 1. General characteristics of the subjects

	IS (n=30)	HC (n=17)	*p* value
Sex (M/F)	15/15	7/10	0.560
Age (years)	71.36±4.45	70.83±5.24	0.748
Smoking (none/active/stopped)	9/16/5	6/7/4	0.782
Drinking (none/active/stopped)	20/8/2	9/5/3	0.435
Body mass index (kg/m^2)	23.00±1.87	22.56±1.98	0.491
White blood cell (*10^9/L)	7.07±2.26	7.66±4.35	0.563
Red blood cell (*10^{12}/L)	4.48±0.72	4.41±0.24	0.847
RDW (%)	13.07±0.97	12.60±0.33	0.684
Hemoglobin (g/L)	138.69±21.65	135.25±8.34	0.758
Platelet (*10^9/L)	221.34±55.20	221.20±50.66	0.991
MPV (fL)	9.56±1.31	8.88±0.61	0.315
Creatinine (μmol/L)	73.58±21.90	68.27±37.76	0.553
Glycated hemoglobin	6.70±7.67	5.32±0.42	0.080
ALT (U/L)	15.83±8.47	17.88±7.03	0.583
AST (U/L)	17.24±7.51	16.15±4.59	0.735
Cholesterol (mmol/L)	4.99±0.92	5.12±1.32	0.711
Triglyceride (mmol/L)	1.68±0.78	1.87±1.63	0.612
HDL (mmol/L)	1.17±0.30	1.35±0.41	0.134
LDL (mmol/L)	3.19±0.66	2.82±0.86	0.161
Hs-CRP (mg/L)	5.98±9.98	8.36±11.48	0.529

IS: Ischemic stroke; HC: Health control; RDW: red blood cell distribution width; MPV: mean platelet volume; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HDL: high density lipoprotein; LDL: low density lipoprotein; Hs-CRP: high-sensitivity C-reactive protein.

TOF-MS. The achieved results can provide clinical guidance in the treatment of disease mechanisms.

In this study, the method of metabolomics based on the UPLC-Q/TOF-MS platform was utilized to screen and evaluate the diagnostic biomarkers of ischemic stroke. First, the UPLC-Q/TOF-MS technique was used to generate a metabolomics profile of plasma samples from patients with ischemic stroke and the healthy controls. Second, principal component analysis (PCA) and orthogonally partial least squares discriminate analysis (OPLS-DA) were performed to identify metabolite perturbations. Next, the classification performance (specificity and sensitivity) was verified by using the receiver operator characteristic (ROC) curves. The metabolic pathways of the metabolites were analyzed by Metabolomics Pathway Analysis (MetPA). The present study aimed to seek the potential biomarkers of ischemic stroke. The results could assist in the clinical treatment of ischemic stroke.

Material and methods

Subjects

30 eligible patients with ischemic stroke (Group IS) were recruited consecutively in the period from January 1 to August 31, 2016 at the Second Hospital of Tianjin Medical University, China. The inclusion criteria were as follows: (1), the age from 60 to 80 years old; (2), the first attack of ischemic stroke has been confirmed via medical history, physical examination, laboratory examination, computed tomography and magnetic resonance imaging; (3), onset within 48 hours. Please note that, patients, who have hemorrhage history, myocardial infarction, atrial fibrillation, diabetes mellitus, immune diseases, acute infectious disease, liver or renal insufficiency, tumor or the recent history of trauma, have been excluded from this group. Neurological defects were assessed using the National Institute of Health Stroke Scale (NIHSS). In addition, 17 healthy control individuals (Group HC) with the general characteristics (**Table 1**) were also enrolled into the subjects.

This study protocol was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University (No. KY2015K004), and all the participants have signed the informed consent.

Reagents and materials

In the present study, formic acid (high-pressure liquid chromatography HPLC grade) was obtained from ROE (USA), and acetonitrile (HPLC grade) was from Oceanpak (Gothenburg, Sweden). Purified water bought from Wahaha Company (Hangzhou, China) was used for the solution preparation. The ALLLEGRATM-64R high-speed centrifuge was purchased from Beckman (USA), and the KQ-300DV ultrasonic cleaner was produced in Kunshan Ultrasonic

Instrument Co., Ltd. (Jiangsu, China). The rapid mixer was supplied by Xinkang Medical Equipment Co., Ltd. (Jiangsu, China). The Acquity UPLC liquid chromatograph, the Xevo G2 Q/TOF MS mass spectrometer, and the ACQUITY UPLC HSS C18 chromatography column (2.1× 100 mm, 1.8 μm) that played a pivotal role in the experiments were all manufactured by Waters (USA).

Sample preparation

Venous blood (3 mL) was drawn from the overnight-fasted subjects and then transferred into heparin-sodium anti-coagulation vacutainers. Within 2 hours, the blood was centrifuged for 15 min at 3,000 rpm and 4°C. The obtained supernatant was centrifuged for another 8 min at 3,500 rpm and 4°C. Finally, the supernatant samples were stored in the 1.5 mL labeled centrifuge tubes at -80°C. Before UPLC-Q/TOF-MS analysis, the samples were centrifuged for 10 min at 10,000 rpm and 4°C after thaw at room temperature. In this process, we should try to reduce the repeated freezing and thawing in order to avoid the unpreferable change of metabolic profile. Then the supernatant (200 μL) was mixed with the cold acetonitrile (600 μL) by vigorous vortex for 2 min aiming to protein precipitation. The mixer was ultrasonic cleaned for 10 min in the cold water, and then centrifuged for 15 min at 13,000 rpm and 4°C. The supernatant was prepared for UPLC-Q/TOF-MS analysis.

With consideration of the quality control (QC), five samples (30 μL for each) from each group, which carried all the metabolic information, were used to maintain the precision of the instrument, the repeatability of the operation, and the system stability by periodically sampling.

UPLC-Q/TOF-MS configuration

ACQUITY-HSS-C18 column (2.1×100 mm, 1.8 μm) was applied for the chromatographic analysis, and the column temperature was kept stationary at 40°C. The binary solvent separation system was consisted of phase A (0.1% formic acid in water) and phase B (0.1% formic acid in acetonitrile), and the flow rate was 0.3 mL/min. The gradient elution process was set as follows: 0-0.5 min, 99-99%A; 0.5-2 min, 99-50%A; 2-9 min, 50-1%A; 9-10 min, 1-1%A; 10-10.5 min, 1-99%A; 10.5-12 min, 99-99%A.

The quadrupole-time-of-flight mass spectrometry (Q/TOF-MS) system in tandem with the UPLC system was set in ESI+ mode (electrospray ionization with positive ion). The capillary ionization voltage was set at 3.1 kV, and the collision voltage was at 20-30 kV. The temperature of the dry gas was 325°C with 10 mL/min flow rate. The high-purity nitrogen used as the auxiliary spray ionized gas was 350°C in temperature and 10 mL/min in the flow rate. As the desolvation gas, the flow rate of the nitrogen was maintained at 600 L/h. Atomizing gas pressure was 310 kPa, and the counter-blowing gas was set at 50 L/h. The scanning range of the quadrupole was 50-1000 m/z. In the experiments, each QC sample was parallel tested for six times to verify the performance of the instrument according to the measurements of the retention time, the mass-to-charge ratio, and the peak area. In addition, the QC samples were repeatedly detected every 5 hours to guarantee the system stability.

Data processing and statistical analysis

The data was processed in three steps as follows:

First, the raw data of each plasma sample generated by the MassLynx (Version 4.1, Waters Corporation, USA) was recorded according to the mass-to-charge ratio and the retention time, and then filtered to suppress the noise signal and baseline drift, etc. After the extraction and comparison of ion pair, peak matching, and peak intensity correction, the data matrix can be obtained, which can be used for the principal component analysis (PCA).

Next, as an unsupervised recognition method, principal component analysis (PCA) in SIMCA-P^+ 11.5 (Umetrics, Sweden) was conducted to show the main contradiction between Group IS and Group HC. Consequently, according to the variable importance in the projection (VIP) and the confidence interval of the first principal component, the metabolites were selected by the orthogonally partial least-squares discriminant analysis (OPLS-DA) performed on the supervised data. The large value of the VIP indicates that the component contributes a great deal of difference to distinguish the two groups of samples, and the fold change value is the binary logarithm of the ratio between the mean of the two groups. In this case, the value of VIP was set to be larger than 1.5, so that the under-

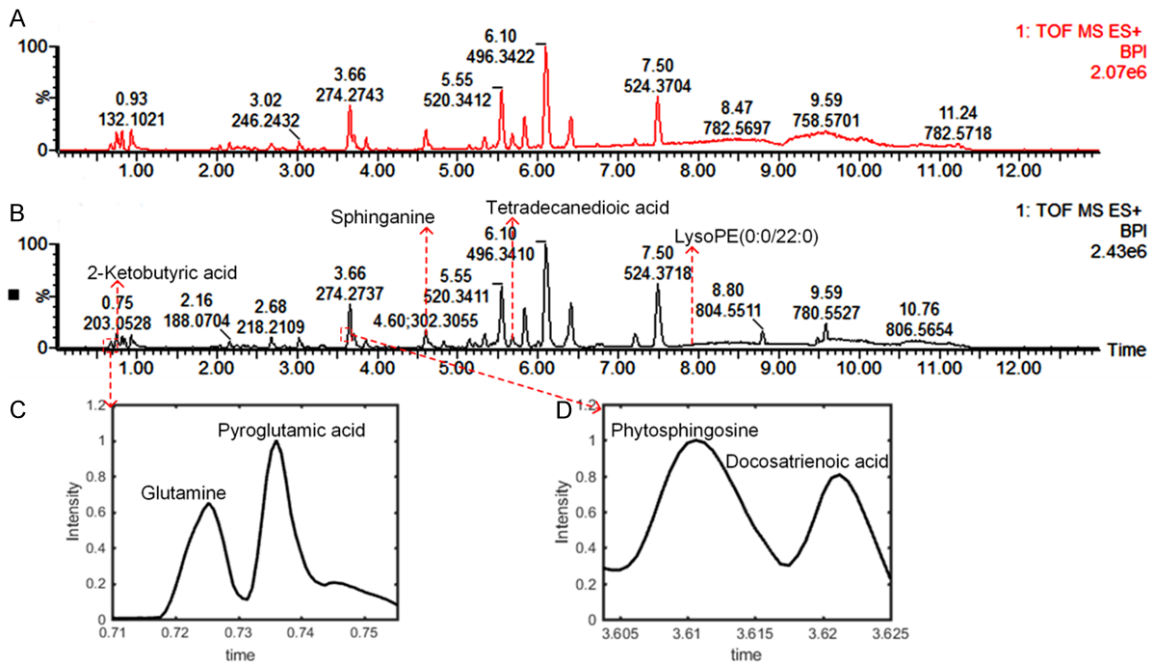

Figure 1. Base peak ions chromatogram of the human plasma samples by UPLC-Q/TOF MS. A: Group HC; B: Group IS; C: Details from 0.71 to 0.755 min; D: Details from 3.605 to 3.625 min.

lying metabolites can be highlighted, which will be used as the identification subjects. Considering the OPLS-DA, both the higher values of R^2Y and Q^2 can ensure that the results are reasonable. R^2 represents the ability of the principal components of the model to explain the global variation, and the Q^2 indicates the predictive ability of the principal components to the model variation. The Student's t test was then used to analyze the differences between the two groups, so as to find out the differences of the metabolic profiles among the plasma of each group, and to find out the different metabolites.

Finally, the obtained metabolites were identified. The steps include the prediction of the elements based on the exact molecular mass, the retrieval of the obtained metabolites, and the confirmation of the obtained metabolites by using the standard samples and the secondary information of mass spectrometry provided by the Human Metabolome Database (HMDB, www.hmdb.ca) and the Mass Bank (www.massbank.jp). The metabolic pathways of the confirmed metabolites were analyzed by Metabolomics Pathway Analysis (MetPA, www.metaboanalyst.ca) and Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg).

In the statistical analysis of the basic clinical data, Student's t test, Mann-Whitney U test and Pearson Chi-square test were used via IBM SPSS Statistics 22 (IBM Co., New York, NC, USA). The clinical data were presented as mean ± standard derivation for the continuous variables, and we used the exact number to represent the categorical variables. The *p* value less than 0.05 was considered as the significant differences statistically. The receiver operator characteristic curves were utilized to evaluate the specificity and the sensitivity of the obtained biomarkers.

Results

General characteristics of the subjects

The general characteristics of the subjects, and the age, the gender, and the other aspects affecting the basal metabolism are at the same level (**Table 1**).

Metabolomics analysis of plasma

There are obvious differences between the metabolic fingerprints of patients with ischemic stroke and healthy controls (**Figure 1**). Several different peaks can be observed from the typical base peak intensity (BPI), which are actually

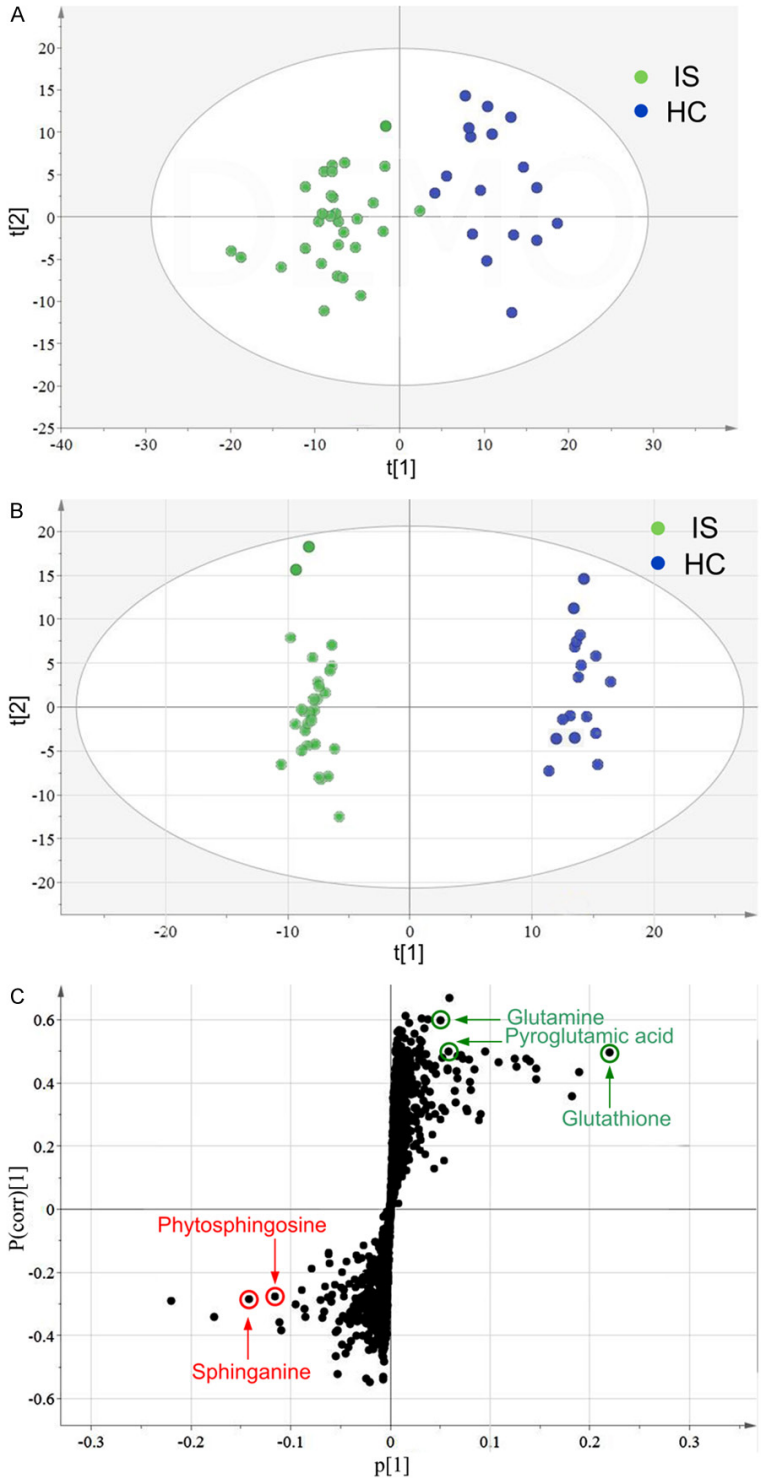

Figure 2. A: Scoring plots of PCA of the plasma samples, R^2X=0.27, Q^2=0.26; B: Scoring plots of OPLS-DA, R^2Y=0.989, Q^2=0.859; C: S-plot obtained from the metabolites. Red words indicate the increased biomarkers, and green words represent the decreased biomarkers.

the metabolite differences between the two sets of data.

To roughly distinguish the two groups, PCA was performed on all the individuals in the two groups (**Figure 2A**), and the results were R^2X=0.27, Q^2=0.26. Further, OPLS-DA was carried out to finely analyze the obtained metabolites (**Figure 2B**, **2C**) (R^2Y=0.989, Q^2=0.859). It can be found that the model is sufficiently reasonable with higher values of R^2Y and Q^2. Consequently, the metabolites were filtered following the rules of VIP > 1.5 and P < 0.05 to obtain the different metabolites which have the significant difference.

In this step, the obtained metabolites, i.e., the potential biomarkers, were identified. The potential biomarkers were analyzed by HMDM in the modes of $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$, while the molecular weight tolerance was 0.01. Then, the potential biomarkers were verified based on the demonstration and the source of the substance. After the Student's t test and the filtering rule, 8 biomarkers of the ischemic stroke were finally screened (**Table 2**).

The levels of sphinganine and phytosphingosine increase obviously, and the levels of 2-ketobutyric acid and glutamine decrease significantly. The concentrations of tetradecanedioic acid and docosatrienoic acid increase softly, and the levels of lysoPE (0:0/22:0) and pyroglutamic acid decrease slightly (**Table 2**).

To examine the sensitivity and the specificity of the 8 biomarkers, IBM SPSS Statistics 22 was used to generate the ROC curves corresponding to the biomarkers (**Figure 3**). 1-specificity and sensitivity locate at

Table 2. Biomarkers of ischemic stroke

No.	t_R (min)	Obsd m/z	Calcd m/z	Error (ppm)	Metabolite	Formula	Variance	Pathway
1	4.5	302.3055	302.3059	-1.32	Sphinganine	$C_{18}H_{39}NO_2$	↑**	Sphingolipid metabolism
2	0.7	103.0397	103.0395	1.94	2-Ketobutyric acid	$C_4H_6O_3$	↓**	Valine, leucine and isoleucine biosynthesis, propanoate metabolism
3	5.6	259.1908	259.1909	-0.36	Tetradecanedioic acid	$C_{14}H_{26}O_4$	↑*	
4	3.6	335.2966	335.295	4.77	Docosatrienoic acid	$C_{22}H_{38}O_2$	↑*	
5	0.7	169.0581	169.0589	-4.73	Glutamine	$C_5H_{10}N_2O_3$	↓**	D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism
6	3.6	318.3003	318.3008	-1.57	Phytosphingosine	$C_{18}H_{39}NO_3$	↑**	Sphingolipid metabolism
7	7.9	538.39	538.3873	5.01	LysoPE (0:0/22:0)	$C_{27}H_{56}NO_7P$	↓*	
8	0.7	130.0505	130.0504	0.77	Pyroglutamic acid	$C_5H_7NO_3$	↓*	D-glutamine and D-glutamate metabolism glutathione metabolism

*: P < 0.05; **: P < 0.01; IS: Ischemic stroke; HC: Health control.

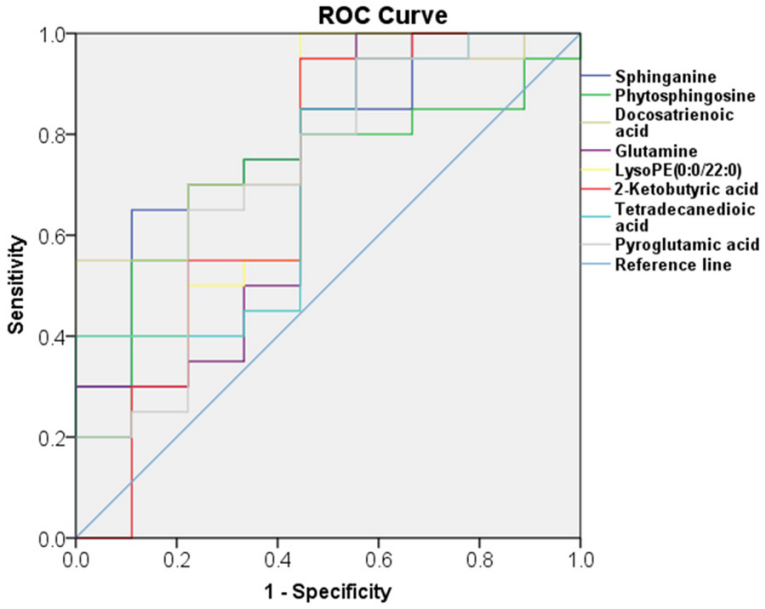

Figure 3. Receiver operating characteristic curve analysis for the 8 biomarkers.

the x axis and y axis, respectively. The ROC curves are close to the upper left. Hence, the specificity and the sensitivity of the test are reasonable, and the rates of misdetection are relatively low. Quantitatively, the results show that the area under the curve (AUC) of 6 metabolites was larger than 0.75, which means 6 important biomarker candidates were identified (**Table 3**).

In the present study, MetPA was used to perform the pathway analysis of the biomarkers. The overview of pathway impact of the biomarkers was obtained (**Figure 4**), and the x axis is the impact of the pathways. The pathway could be related to ischemic stroke, when the value of pathway impact is larger than 0. Therefore, sphingolipid metabolism, D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine biosynthesis, propanoate metabolism, and glutathione metabolism could be perturbed in patients with ischemic stroke. Finally, the disturbed metabolic pathways obtained by using UPLS-Q/TOF-MS analysis were analyzed (**Figure 5**).

Discussion

The present research investigated the metabolic variations in Ipatients with ischemic stroke using technique of UPLC-Q/TOF-MS. A clear metabolic difference was found between Group IS and Group HC. The levels of sphinganine, phytosphingosine, tetradecanedioic acid, and docosatrienoic acid increased, and the levels of 2-ketobutyric acid, glutamine, lysoPE (0:0/22:0), and pyroglutamic acid decreased in patients with ischemic stroke compared with the healthy controls.

Increased neurotoxicity

As a direct and indirect precursor, glutamine plays an important role in the balance between glutamate and γ-aminobutyric acid (GABA). Glutamate is the main excitatory neurotransmitter in the central nervous system, and with

Table 3. Results of ROC curves

	AUC	Cutoff value	Standard error	Significance	95% CI of AUC		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
					Upper limit	Lower limit					
Sphinganine	0.837	18487.55	0.080	0.008	0.613	0.925	75.00	53.80	75.00	53.80	67.57
2-Ketobutyric acid	0.732	9.82715	0.080	0.006	0.653	0.965	87.00	61.50	77.27	53.33	67.57
Tetradecanedioic acid	0.756	27.2611	0.068	0.001	0.704	0.969	95.80	46.20	76.67	85.71	78.38
Docosatrienoic acid	0.809	2.60785	0.094	0.048	0.522	0.891	90.90	40.00	83.33	69.23	78.37
Glutamine	0.776	226.3595	0.085	0.006	0.608	0.943	87.50	61.50	80.77	72.73	78.38
Phytosphingosine	0.769	15885.28	0.101	0.022	0.534	0.931	50.00	92.30	92.31	50.00	64.86
LysoPE (0:0/22:0)	0.707	18.0033	0.083	0.011	0.595	0.918	82.60	50.00	74.07	60.00	70.27
Pyroglutamic acid	0.753	795.1929	0.090	0.012	0.577	0.929	95.80	46.20	75.86	75.00	75.68

ROC: receiver operator characteristic; PPV: positive predictive value; NPV: negative predictive value; CI: confidence interval.

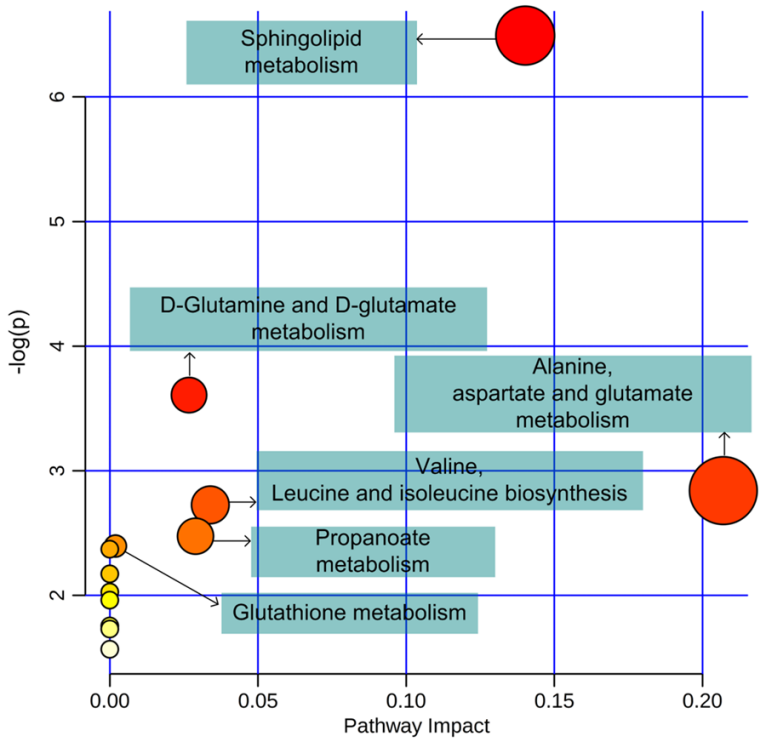

Figure 4. Overview of pathway analysis of the biomarkers. Colors varying from yellow to red represent that the metabolites are with different levels of importance.

the highest levels in the cerebral cortex, cerebellum and striatum. In general, glutamate is converted to glutamine under the catalysis of specific glutamine synthetase after entering into the astrocytes. Then, glutamine is converted to glutamate in the neurons. The cerebral ischemia and hypoxia can directly inhibit the activity of the sodium-potassium-ATPase, and the extra cellular potassium is increased significantly leading to the neuronal depolarization. The release of glutamate is promoted, while the neuronal depolarization can inhibit the uptake of glutamate, and the arachidonic acid is increased. Therefore, the glutamate-glutamine cycle is disordered [22]. Generally, the glial cells can convert the excessive glutamate into glutamine, which can help to weaken the effect of excitotoxicity [23]. The previous studies have reported that one of the earliest changes that occur during cerebral ischemia is an increase of glutamate, which is also accompanied by decreases in the amounts of glutamine [24-26]. In the present study, the level of glutamine is decreased significantly in the plasma of the patients with ischemic stroke, showing a good agreement with the previous reports. The glutamate-glutamine cycle may be disordered, resulting in the decompensation of glutamine metabolism. Thus, glutamine can be used as a biomarker of the ischemic stroke.

Pyroglutamic acid is a cyclized derivative of L-glutamate, which is formed by L-glutamate, glutamine, and γ-glutamylated peptides. It can be also produced by the action of γ-glutamyltransferase [27]. In the present study, we consider that the decreased level of pyroglutamic acid may be associated with the glutamine metabolism in the patients of ischemic stroke.

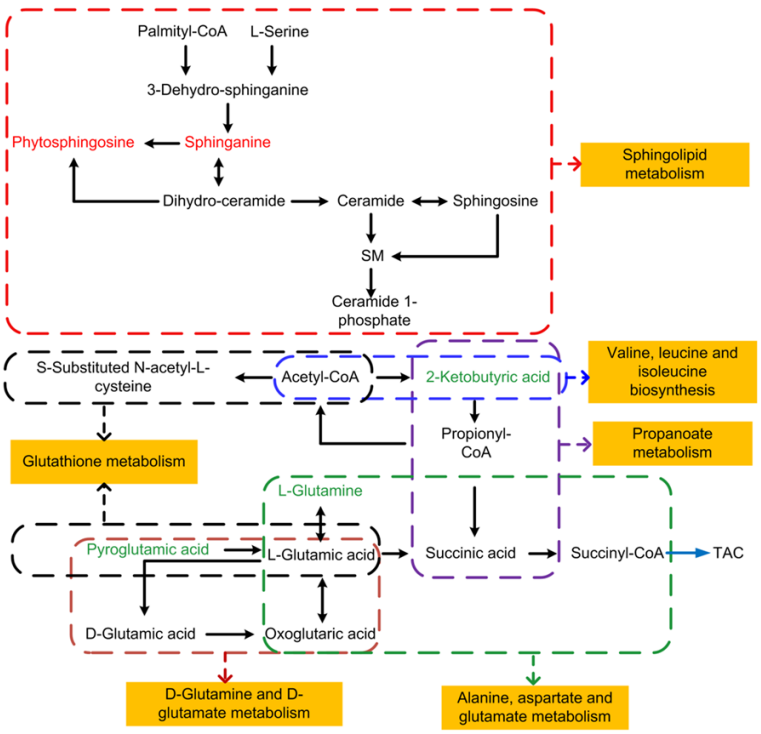

Figure 5. Schematic diagram of the disturbed metabolic pathways detected by UPLC-Q/TOF-MS.

Sphingolipid metabolism disturbance

Significant changes of sphingomyelin were found in the present study, including sphinganine and phytosphingosine. Sulfolipid metabolites are the important components of the cell membrane, and can participate in many biological processes, such as cell growth, differentiation, aging and apoptosis [28]. The synthesis and metabolic pathways of the sphingolipid metabolites in the human body are shown in **Figure 5**. The serine and palmitoyl-CoA generate sphinganine under the action of serine palmitoyltransferase. The sphinganine is further combined with the fatty acyl to form dihydroceramide, which is converted into ceramide with a trans-4,5-double bond. Ceramide can be phosphorylated or glycosylated to produce sphingomyelin (SM) and glycospholipid, and generate sphingosine with the action of hydrolase. Sphinganine is of great importance in the process of sphingomyelin metabolism, and is the basic component of various sphingolipids [29]. Ceramide is a cytokine, which is a potential endogenous regulator of programmed disease deaths caused by environmental stress inducers [30]. As a lipid compound with immune-enhanced activity, phytosphingosine has a variety of physiological activities, which can induce cell apoptosis. Previous investigation reported that the cell apoptosis was one of the pathogenesis of ischemic stroke [31]. In the present study, the levels of sphinganine and phytosphingosine were significantly increased, which may cause the brain cell apoptosis and further lead to ischemic stroke.

Energy metabolism disturbance

Under the normal circumstances, the production of the brain energy is mostly dependent on the own oxidative phosphorylation process, and the maintenance of the brain function requires the sufficient amount of cerebral blood perfusion. In the case of ischemic stroke, the brain tissue metabolism can change. 2-ketobutyric acid is a metabolic substrate for many amino acids, such as glycine, methionine, valine, leucine, serine, threonine, and isoleucine, etc. As shown in **Figure 5**, 2-ketobutyric acid can be converted to propionyl-CoA, and then to methyl malonate mono-CoA. The methyl malonate mono-CoA can be further converted to succinyl-CoA in the tricarboxylic acid cycle (TAC), to provide the energy for the body. In the present study, the level of 2-ketobutyric acid is decreased. Therefore, the energy metabolism may be perturbed in the patients with ischemic stroke. The previous reports have indicated that TAC has been reduced in the patients with ischemic stroke [32], which shows a good consistency with our results. Hence, the decreased level of 2-ketobutyric acid and the decreased level of TAC could be related to the mechanism of ischemic stroke.

In conclusion, a UPLC-Q/TOF-MS method was developed to identify the metabolites in the patients with ischemic stroke, compared with the healthy controls. The patients with ischemic stroke were significantly distinguishable

from the healthy controls based on the plasma metabolism. 8 biomarkers were successfully identified. ROC curves were used to evaluate the sensitivity and specificity. The pathways of the obtained biomarkers were analyzed, indicating that, sphingolipid metabolism, glutamate-glutamine cycle, and energy metabolism were perturbed in the patients with ischemic stroke. These results are of great importance in understanding the pathological mechanism of ischemic stroke, and may help to provide new method for the treatment of ischemic stroke. Future work will focus on validating the results on a larger scale of plasma samples.

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Disclosure of conflict of interest

None.

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