Mechanism Exploration of Youth Ischemic Stroke by Metabolomics Analysis

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Research Article

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Abstract

The onset age of acute ischemic stroke (IS) is gradually younger, which brings great challenges and burden to the medical system. For stroke patients, time is the brain cells, so any method contributes to rapid identification or diagnosis of stroke is extremely meaningful. Metabolomics analysis provides us with new means and ideas for discovering potential stroke-related biomarkers and understanding the pathogenesis of youth IS. In this study, fifty-five patients with IS were assigned in two groups (senior or junior) according to the age (60-year-old) and were performed serum metabolomics analysis based on Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS) system. Forty differential metabolites were selected, mainly ceramides, lipids, and amino acids, which are mainly related to aging, cardiovascular and cerebrovascular diseases, and antitumor mechanisms. The pathway analysis showed that the differential metabolites were mainly enriched in sphingolipid metabolism, glycine metabolism, serine and threonine metabolism, glycerol phospholipid metabolism, biosynthesis of unsaturated fatty acids, linoleic acid metabolism, and vitamin B6 metabolism. Our study suggests that the mechanism of IS in young adults was most likely associated with elevated plasma lactose ceramide levels and abnormal sphingolipid metabolic pathway.

Introduction

Ischemic stroke (IS) is caused by cerebrovascular stenosis and occlusion that lead to the disorders of oxygen and nutrient supply for the brain tissue, which is followed by a succession of pathophysiological changes for clinical symptoms[1–2]. China is facing the biggest challenge in stroke worldwide. As the global burden of disease (GBD) 2016 stroke study estimated, the highest lifetime risk of stroke from the age of 25 years is Chinese (39.3%)[3], which means that stroke is no longer considered a disease of the elderly. In 2018, the number of deaths of stroke was 1.570 million which ranked third among the leading causes of death behind malignant tumours and heart disease[4]. Data from the Hospital Quality Monitoring System showed that IS accounts for 81.9% inpatients with stroke during 2018[4]. Previous studies had shown that smoking, hypertension, and diabetes are common risk factors for IS[5–6]. However, the reason of young patients with IS remains unclear, suggesting an insufficient understanding of the pathogenesis of youth IS[7–8].

The diagnosis of IS is mainly based on computed tomography (CT), magnetic resonance imaging (MRI), and angiographic[9], which are high sensitive, but time consuming or device-dependent[10–11]. The longer hypoxia and ischemia, the higher the risk of permanent brain injury and chronic disability. Therefore, rapid and accurate diagnostic biomarkers are needed to be well developed.

Metabolomics analysis is an emerging science devoted to the quantitative assessment of small-molecule metabolites in a given biological sample, thus to excavate the link between metabolic features and disease phenotypes[12]. It adequately addresses the problem of blood-brain barrier and disease heterogeneity[13], serving as an ideal method for discovering IS biomarker. Metabolomics analysis has shown great promise and success in predicting risk, but none marker is actually used in the clinical
diagnosis or treatment of stroke patients. This study aims to explore the biomarkers of youth IS by analysing different metabolites among junior and senior groups. As a result, pave the way to the key pathogenesis of IS.

**Methods**

**Clinical Data Collection**

Patients were eligible if they had presented clinical symptoms of acute IS within seven days and confirmed by brain imaging with CT or MRI, complying with the diagnostic criteria according to the recommendations of AHA/ASA[14]. Exclusion criteria were: patients with serious organ failure; malignancy carcinoma; autoimmune diseases; metabolic disease (e.g. hypothyroidism); patients refused to provide consent.

The national institutes of health stroke scale (NIHSS) score was calculated at admission to assess the severity of stroke. The basic clinical information including gender, age, blood pressure, medical history, and auxiliary examination.

**Sample Collection and Metabolomics Detection**

Human plasma (whole blood) samples were collected for QE-based metabolomics analysis. The samples was collected into heparin tubes by venipuncture after an overnight fasting. Plasma was immediately separated by centrifugation at 3000 rpm for 10 min, and then stored at −80°C until Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS) detection.

Dunn, W.B. et al and Want, E.J. et al had previously described the detailed accounts of LC-MS detection principle[15-16]. Samples were slowly thawed at 4°C and then were placed in a centrifuge at 4,000 rpm for 5 min. After mixing 200μL of the supernatant liquid with 600μL of methanol, the mixture was exposed to ultrasonics for 30 min. After a second centrifugation, the new supernatant liquid was dried under a mild flow of auxiliary gas, then added to 200μL of pure methanol for redissolve and transferred into the sample vial after adding the internal label (1 mg/ml of dichloro-phenylalanine, 5μL). Plasma metabolomics data was collected on a column (ACQUITY UPLC HSS T3 (2.1*100mm 1.8 μm)) combined with LC-MS (Waters, UPLC; Thermo, Q Exactive) analysis platform in the following parameters: heater temperature, 300°C; sheath gas flow rate, 45 arb; auxiliary gas flow rate, 15 arb; sweep gas flow rate, 1 arb; capillary temperature, 350°C. The spray voltage was 3.0 KV in the electron spray ionization (ESI)+ modes and that was 3.2 KV in the ESI- modes; S-Lens RF level was 30% and 60%, respectively.

The Quality Control (QC) samples, a mixture of equal volume of the trial samples, were used for LC-MS analysis before, between, and after the trial samples. The detection process that adding one QC sample per 10 trail samples was taken to ensure the reliability and reproducibility of the analysis results.

**Statistical Analysis**
Continuous variable was expressed as mean±SD, median and interquartile range according to its distribution. Student t test or nonparametric test were used to examine the difference between two groups. Categorical variable was shown as percentage, and the Chi-square test was used to examine the difference between groups. Statistical analyses were performed using IBM SPSS 18.0. The reported P-value was two-tailed, and P<0.05 was considered to be statistically significant.

Metabolomics data analysis was based on the LC-MS system combined quadrupole Orbitrap mass spectrometer (Q Exactive Orbitrap, Thermo Fisher Scientific, USA)[17-18], including ESI+ model and ESI- model. Initial data from LC-MS detection were import into the Compound Discoverer3.1 (CD) software for spectrograph-progress and database-search to obtain the qualitative and quantitative results of the metabolites.

After data management of single-peak filtration, missing value recording, and normalization, data formatting and modeling were performed for the Multivariate Analysis (MVA) by SIMCA-P software (Umetrics AB, Umea, Sweden)[19]. Principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA)[20] were taken to discover more reliable differential metabolites between two groups. Traditional univariate analysis (UVA) methods (e.g. Student t-test) pay more attention to independent changes of metabolite level while MVA methods concentrate on the relationship in biological processes (promotion / antagonistic) between metabolites. Combining two type of methods for filter can help to avoid false positive errors or model overfitting caused by using only one class of statistical analysis methods[21-22].

Substances that met the next two criteria were selected as differential metabolites: P value of Student's t test was less than 0.05 and Variable Importance in the Projection (VIP) of the OPLS-DA model was more than 1.0. The results of differential metabolites was visualized in the form of volcano plot. In addition, commercial databases including Kyoto Encyclopedia of Genes and Genomes(KEGG) (http://www.genome.jp/kegg/) and MetaboAnalyst(http://www.metaboanalyst.ca/) were utilized to search for the meaningful pathways of metabolites[23].

The whole experimental process is shown in Figure 1.

**Results**

**Basic Characteristics of Patients**

Fifty-five patients who met the trial criteria were included from November 30,2020 to March 27,2021. Patients were divided into junior group (age ≤60 years old, N=24) and senior group (age ≥60 years old, N=31). The baseline demographic and disease characteristics are balanced between groups (Table 1). There are no significant differences between the junior and the senior group in sex, medical history, principle auxiliary examination, systollc blood pressure, rate of thrombolysis and NIHSS score.
Table 1. Descriptive Statistics of Study Population

<table>
<thead>
<tr>
<th>Variables</th>
<th>junior</th>
<th>senior</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>24</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td><strong>Demographic characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age,y,mean(SD)</td>
<td>53.4±5.2</td>
<td>72.1±7.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female,%</td>
<td>9(37.5)</td>
<td>14(45.2)</td>
<td>0.568</td>
</tr>
<tr>
<td><strong>Medical history</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension,%</td>
<td>14(58.3)</td>
<td>16(51.6)</td>
<td>0.620</td>
</tr>
<tr>
<td>Diabetes melitus,%</td>
<td>8(33.3)</td>
<td>7(22.6)</td>
<td>0.375</td>
</tr>
<tr>
<td>Smoking,%</td>
<td>5(20.8)</td>
<td>6(19.4)</td>
<td>1.000</td>
</tr>
<tr>
<td>Drinking,%</td>
<td>4(16.7)</td>
<td>6(19.4)</td>
<td>1.000</td>
</tr>
<tr>
<td>Previous cardiovascular and cerebrovascular diseases,%</td>
<td>4(16.7)</td>
<td>9(29.0)</td>
<td>0.284</td>
</tr>
<tr>
<td><strong>Auxiliary examination</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid atherosclerosis,%</td>
<td>15(62.5)</td>
<td>21(67.7)</td>
<td></td>
</tr>
<tr>
<td>Fasting blood-glucose,mean(SD)</td>
<td>6.6±2.2</td>
<td>7.2±4.5</td>
<td>0.547</td>
</tr>
<tr>
<td>TG,mean(SD)</td>
<td>1.7±1.2</td>
<td>1.7±1.1</td>
<td>0.959</td>
</tr>
<tr>
<td>TC,mean(SD)</td>
<td>4.6±0.6</td>
<td>4.4±1.0</td>
<td>0.352</td>
</tr>
<tr>
<td>LDL,mean(SD)</td>
<td>2.8±0.7</td>
<td>2.6±1.1</td>
<td>0.401</td>
</tr>
<tr>
<td><strong>Blood pressure characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure mmHg,mean(SD)</td>
<td>157.5±22.8</td>
<td>151.1±19.8</td>
<td>0.276</td>
</tr>
<tr>
<td>Diastolic blood pressure mmHg,mean(SD)</td>
<td>94.3±13.9</td>
<td>85.6±10.9</td>
<td>0.013</td>
</tr>
<tr>
<td>Thrombolysis,%</td>
<td>5(20.8)</td>
<td>12(38.7)</td>
<td>0.155</td>
</tr>
<tr>
<td>NIHSS,mean(SD)</td>
<td>6.88(5.1)</td>
<td>6.84(6.3)</td>
<td>0.982</td>
</tr>
<tr>
<td>0-1,%</td>
<td>3(12.5)</td>
<td>3(9.7)</td>
<td></td>
</tr>
<tr>
<td>2-4,%</td>
<td>6(25.0)</td>
<td>10(32.3)</td>
<td></td>
</tr>
<tr>
<td>5-15,%</td>
<td>14(58.3)</td>
<td>14(45.2)</td>
<td></td>
</tr>
<tr>
<td>16-20,%</td>
<td>1(4.2)</td>
<td>2(6.5)</td>
<td></td>
</tr>
<tr>
<td>21-42,%</td>
<td>0(0.0)</td>
<td>2(6.5)</td>
<td></td>
</tr>
</tbody>
</table>
**Multivariate Statistic Analysis**

The PCA score plots show no significant difference between the group junior and senior, with $R^2X[1]=0.09$ and $R^2X[2]=0.082$ in ESI+ mode(Figure 2a) and $R^2X[1]=0.129$ and $R^2X[2]=0.078$ in ESI- mode(Figure 2b). The quality control samples are closely gathered together in both the ESI+ and ESI- modes, indicating that the analysis system and the results are stable and reliable.

The statistical method of OPLS-DA is used to model the relationship between metabolite expression and sample category for the prediction of sample category. Both the permutation test results of OPLS-DA model in ESI+ and ESI- mode show the significant discrepancy between two groups with the samples all within the 95% confidence interval (hotelling's t-squared ellipse). $Q^2=-0.97$ in ESI+ mode(Figure 2c), and $Q^2=-1.03$ in ESI- mode(Figure 2d). The results reveal a good robustness of the model with no overfitting existence.

**Differential Metabolites Analysis**

Under the filter strategy of Univariate Analysis combined with Multivariate Analysis, 40 differential metabolites are selected (VIP>1.0, P<0.05), including 18 from ESI+ mode and 22 from ESI- mode which are visualized by volcano plots(Figure 3a, 3b). The differential metabolites are mainly ceramides, lipids, and amino acids, which are mainly related to aging, cardiovascular diseases, cerebrovascular diseases, and antitumor mechanisms.

Fourteen differential metabolites with VIP> 2 are shown in Table 2, including seven from both ESI+ and ESI- mode. In the ESI+ mode, C16 lactosyl ceramide, hexadecanamide, and acetyl-L-carnitine are up-regulated in the junior group, while dihydrosphingosine, 2-amino-1,3,4-octadecanetriol, arachidoyl ethanolamide, and choline are down-regulated compared to the senior group. In the ESI- mode, testosterone sulfate is up-regulated, while carnitine hydrochloride, tretinoin, phosphatidylinositol, (R)-2-hydroxystearic acid, eicosatrienoic acid, and linoleic acid are down-regulated.
Table 2. Results of Differential Metabolites (VIP>2)

<table>
<thead>
<tr>
<th>MS2 NAME</th>
<th>CHANGE</th>
<th>VIP</th>
<th>P-VALUE</th>
<th>FOLD CHANGE</th>
<th>ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrosphingosine</td>
<td>↓</td>
<td>2.71961</td>
<td>0.001949</td>
<td>0.671091886</td>
<td>+</td>
</tr>
<tr>
<td>2-Amino-1,3,4-octadecanetriol</td>
<td>↓</td>
<td>2.70002</td>
<td>0.002341</td>
<td>0.617616494</td>
<td>+</td>
</tr>
<tr>
<td>C16 Lactosyl Ceramide (d18:1/16:0)</td>
<td>↑</td>
<td>2.62468</td>
<td>0.025526</td>
<td>3.529571713</td>
<td>+</td>
</tr>
<tr>
<td>Hexadecanamide</td>
<td>↑</td>
<td>2.60350</td>
<td>0.004725</td>
<td>1.415260653</td>
<td>+</td>
</tr>
<tr>
<td>Arachidoyl Ethanolamide</td>
<td>↓</td>
<td>2.56377</td>
<td>0.004757</td>
<td>0.606560793</td>
<td>+</td>
</tr>
<tr>
<td>Acetyl-L-carnitine</td>
<td>↑</td>
<td>2.53171</td>
<td>0.009356</td>
<td>2.740594559</td>
<td>+</td>
</tr>
<tr>
<td>Choline</td>
<td>↓</td>
<td>2.18583</td>
<td>0.011085</td>
<td>0.855065369</td>
<td>+</td>
</tr>
<tr>
<td>Carnitine (Dl) Hydrochloride</td>
<td>↓</td>
<td>2.90806</td>
<td>0.002970</td>
<td>0.611185518</td>
<td>-</td>
</tr>
<tr>
<td>Tretinoin</td>
<td>↓</td>
<td>2.73664</td>
<td>0.004504</td>
<td>0.59221435</td>
<td>-</td>
</tr>
<tr>
<td>Testosterone sulfate</td>
<td>↑</td>
<td>2.49990</td>
<td>0.001125</td>
<td>2.078929089</td>
<td>-</td>
</tr>
<tr>
<td>Phosphatidylinositol lyso 18:0</td>
<td>↓</td>
<td>2.32769</td>
<td>0.043117</td>
<td>0.831578852</td>
<td>-</td>
</tr>
<tr>
<td>(R)-2-hydroxystearic acid</td>
<td>↓</td>
<td>2.26779</td>
<td>0.002748</td>
<td>0.775020864</td>
<td>-</td>
</tr>
<tr>
<td>Eicosatrienoic acid</td>
<td>↓</td>
<td>2.18754</td>
<td>0.011764</td>
<td>0.760404101</td>
<td>-</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>↓</td>
<td>2.15654</td>
<td>0.003687</td>
<td>0.57008088</td>
<td>-</td>
</tr>
</tbody>
</table>

Metabolic Pathway Analysis

All differential metabolites were performed KEGG annotation analysis and comprehensive metabolic pathway analyses (including enrichment analysis and topological analysis) for searching key pathways which were the highest correlation with metabolite differences. The result show that in ESI+ mode(Figure 4a), differentially expressed compounds mainly concentrate on the metabolic pathways of sphingolipid metabolism, glycine, serine and threonine metabolism, and glycerophospholipid metabolism. In ESI-mode(Figure 4b), the differences are mainly enriched in pathway of biosynthesis of unsaturated fatty acids, linoleic acid metabolism, and vitamin B6 metabolism.

Discussion

The incidence of IS in young adults is increasing gradually, which occurred with a considerably wider range of risk factors than older patients. More than 150 causes of early-onset IS has been identified, but the specific pathogenesis remains unclear[24]. This study intends to explore molecular mechanism and screen biomarkers of youth stroke via metabolomics data analysis based on LC-MS/MS system. Totally,
40 differential metabolites were selected, including 14 with VIP value > 2 and Student's t-test P value < 0.05, which mainly were ceramides, lipids, and amino acids. After database search and article analysis, elevated plasma level of lactose ceramide and abnormal sphingolipid metabolism pathway were considered the most likely mechanism associated with the onset of stroke in young patients.

Experimental evidence is accumulating which suggesting a crucial role of sphingolipids in the pathogenesis of IS. Glycosphingolipids (GSLs) are important structural molecules constituting the cell membrane and signal transduction regulators producing a variety of different biological functions[25]. Ceramides has been proved not only to regulate cell proliferation, differentiation, senescence and apoptosis, but also to participate in cellular stress responses[26]. In particular, several different animal models of ischemia/reperfusion injury have shown that ceramide accumulation in ischemic tissue might be a crucial trigger of apoptosis.

Endogenous ceramides are generated by three different biochemical pathways[27]: 1) Ceramides can be produced via the de novo synthesis involving several catalytic steps which finally N-acylation of sphinganine to convert ceramide; 2) Ceramide can also be formed via the salvage pathway by re-acylation of sphingoid long chain bases, such as sphingosin; 3) Ceramides can be generated through hydrolysis of complex sphingolipids. In our study, dihydrosphingosine and 2-amino-1,3,4-octadecanetriol (phytosphingosine), both synthetic precursors of ceramide[28], were significantly reduced in young patients, whereas C16 lactosyl ceramide was significantly increased. It indicates more sphingolipid precursors might be used to synthesize lactoceramide (LacCer) in junior group than senior group, which may contribute to the onset of youth IS.

Edsfeldt, Andreas et al. analyzed several GSLs in homogenates from 200 human carotid plaques using mass spectrometry and discovered that glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and sphingosine-1-phosphate (S1P) were all significantly increased in symptom-associated plaques compared with plaques from asymptomatic patients[29]. Their study also revealed that increased level of ceramides were correlated with inflammatory cytokines and served as histological markers of plaque instability. However, unstable plaques were considered to be highrisk and prone to thrombotic complication, such as cerebral emboli caused ischemic events[30].

It has already been shown that critical effects of ceramides in various neurodegenerative and inflammatory diseases are physiological condition-exerted and chain length-specific[31]. Alterations of long chain ceramides (≧C16), as well as their respective dihydro-ceramides and precursors, were regulators in apoptotic cell death[32]. Elevated plasma levels of ceramides were predictors of both risk and severity at admission in IS patients, which was confirmed by the study of 202 age and sex matched control patients[33]. Moreover, the serum level of LacCer was strong correlated with arterial stiffness and could be used to indicate vascular dysfunction. Rabbits fed high fat and high cholesterol diet showed a marked increase of LacCer accompanied by extensive atherosclerosis, which was prevented by treatment of the glycosphingolipid glycosyltransferase inhibitor D-PDMP[34]. Kim, Minjoo et al. revealed LacCer were independent predictors of increased arterial stiffness in middle-aged individuals[35].
Further study has elucidated that LacCer stimulates the GTP load on the Ras to initiate cell proliferation via a series of signal transductions which results in the phosphorylation of MAPK p44 [36]. In human smooth muscle cell (SMC), oxidative LDL (ox-LDL) could dose-dependently activate the synthesis of LacCer by rapid phosphorylation of LacCer synthetase[37]. It was reported that D-PDMP reduced ox-LDL guided SMC multiplication[38], indicating that the activation of LacCer synthetase and the increased of LacCer were essential to ox-LDL induced arterial SMC proliferation.

Deguchi, Hiroshi et al. reported a new mechanism for cross-talk between sphingolipid metabolism and thrombin generation[39], which implied sphingosine disrupted interactions between factor Va and the Gla domain of factor Xa in the prothrombinase complex. Thus, certain sphingolipids might be bioactive lipid mediators of thrombin generation to modulate cell growth and death, blood coagulation, and inflammation. Certain factors induced hypercoagulability were high risk factors of thromboembolic diseases[40]. The role of down-regulated sphingosine in coagulation state and in onset of youth IS need to be further studied.

Generally, the onset of youth IS maybe caused by up-regualted lactoceramide and abnormal sphingolipid metabolism, which are correlate with plaque instability, arterial stiffness, cell proliferation, high sensitive of ox-LDL and hypercoagulability. Our result indicates a potential biomarker for the early onset of IS, but the potential mechanism needs more further studies for confirmation.

Limitation

The present study has some limitations. The sample size was relatively small, which might limit the generalizability of the results. This study is a prospective cohort study of single-center and the current findings are preliminary, which needs further validation in more control cohort studies. The relationship between lactose ceramide and the mechanism of young stroke still needs to be verified by further precise studies.

Abbreviations

IS
Ischemic Stroke
LC
Liquid Chromatography
MS
Mass Spectrometry
ESI
Electron Spray Ionization
QE
Quadrupole Exactive Orbitrap
QC
Quality Control
Conclusion

There are still many spaces need to be explored about the pathogenesis of IS, and the development of untargeted metabolomics provides us a better method to further investigate relevant mechanisms. This study analysed differential plasma circulation metabolites in 24 young and 31 elderly stroke patients and revealed that the disorder metabolism of lactose ceramide and the abnormal sphingolipid metabolic pathway is most likely to be associated with the mechanism of early onset of stroke.

Declarations

Ethics approval

This study was conducted in accordance with the principles of the Declaration of Helsinki and had been approved by the Ethics Committee of Changhai Hospital.

Consent to participate

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

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no relevant financial or non-financial interests to disclose and have no conflict of interest.

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Authors' contributions

Jian Zhou and Jian Dong contributed to the study conception and design. Material preparation, data collection and analysis were performed by Fude Wang, Pengcheng Du, Zilin Lu and Sheng Chang. The first draft of the manuscript was written and polished by Xianfei Liu and Shuangshuang Li, and all
authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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References


Figures

Figure 1

Workflow Chart of Experimental Procedure
Figure 2

Results of the Multivariate Analysis. PCA score plot (a. ESI+, b. ESI-). Permutation test result of OPLS-DA model for group junior vs senior (c. ESI+, d. ESI-), R² measures the goodness of fit and Q² measures the predictive ability of the model. The criterion for model validity is that the regression line of the Q²-points (blue dotted line) intersects the vertical solid line (on the left) below zero.
Figure 3

Volcano plot of different metabolites for group junior vs senior (a. ESI+, b. ESI-). Significantly up-regulated metabolites are shown in red, significantly down-regulated metabolites in blue, and indifference metabolites in gray. The color tone indicates P-value: a dark color indicates a small P-value. The circle radius indicates the VIP value of corresponding peak features.

Figure 4

Bubble plot of pathway analysis, as identified using KEGG pathway enrichment analysis. The abscissa and size of the bubble indicate the influence of the pathway, the bigger the more impact. The color of the bubble indicate the ln P-value of the enrichment analysis, the darker of the color indicating the smaller of the P-value and the more significant of the enrichment. (a)results in ESI+ mode. (b)results in ESI- mode.