PLASMA METABOLIC BIOMARKERS FOR SYNDROME OF PHLEGM AND BLOOD STASIS IN HYPERLIPIDEMIA AND ATHEROSCLEROSIS

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OBJECTIVE: To explore the plasma metabolite profiles in patients with the syndrome of phlegm and blood stasis in hyperlipidemia and atherosclerosis (As), and to search for the metabolic biomarkers of the syndrome.

METHODS: The plasma metabolite profiles of 31 patients with the syndrome of phlegm and blood stasis in hyperlipidemia and As, 6 patients with syndromes without phlegm and blood stasis, and 10 healthy subjects were analyzed by gas chromatography-mass spectrometry (GC-MS). Partial least squares-discriminant analyses (PLS-DA) were used to carry out the pattern-recognition analyses of the data. The plasma metabolic biomarkers of patients were obtained by variable importance plot value (VIP value) and Student's t-test. The structures of biomarkers were defined by the National Institute of Standards and Technology (NIST) database.

RESULTS: PLS-DA score plots of plasma metabolomes did not show overlap between the phlegm-blood stasis syndrome group and syndromes without phlegm and blood stasis group, whereas significant differences in the concentrations in the plasma of 5 metabolites were found (P < 0.05). They were identified as urine, isoleucine, glucuronic acid, palmitic acid and glycerol by searching in NIST database. The concentrations of four metabolites in the plasma of patients with syndrome of phlegm and blood stasis were higher than those with syndromes without phlegm and blood stasis, whereas the glycerol concentration was lower.

CONCLUSION: Compared with patients with syndromes without phlegm and blood stasis, five metabolites showed abnormal levels in patients with the syndrome of phlegm and blood stasis. These metabolites could be diagnostic and prognostic biomarkers.

Keywords: Hyperlipidemias; Atherosclerosis; Metabolism; Biological markers; Phlegm and blood stasis

INTRODUCTION

Metabolomics is the study of temporal and spatial changes of small-molecule metabolites after an organism is stimulated or disturbed. From the viewpoint of systems biology, a "syndrome" in Traditional Chinese Medicine (TCM) might be a specific deviated state af-
ter "disturbances" made to protein networks and gene regulatory networks. Endogenous substances secreted into blood and the uterine system have been shown to be a group that contribute to TCM syndrome. Metabolomics is able to solve these kind of problems. On the basis of the pattern of the association of syndrome and disease, by using a metabolomic approach and computational mathematics, we explored the metabolic marker group for the phlegm-blood stasis syndrome of hyperlipemia and atherosclerosis (As). We also identified the endogenous substances contributing to the syndrome, and provided a basis and guidance for criteria and treatment for the phlegm and blood stasis syndrome of hyperlipemia and As.

**MATERIALS AND METHODS**

### Diagnostic criteria

The diagnostic criteria for hyperlipemia and As were as described previously. The diagnostic criteria for TCM syndromes were: phlegm syndrome, blood stasis syndrome, syndrome of phlegm-blood stasis, syndrome of spleen-Qi deficiency, and syndrome of kidney-Qi deficiency. The inclusion criteria were: patients with the diagnostic criteria described above set forth by Western Medicine and with phlegm-blood stasis syndrome and syndromes without phlegm and blood stasis prescribed by TCM. The exclusion criteria were patients: 1) who did not fit the TCM diagnosis; 2) with nephrotic syndrome, kidney and liver disease, diabetes mellitus, endocrine disorder and hematopoietic system disease; 3) with acute myocardial infarction, severe accidental damage, or major surgery to cerebral vessels in the previous 6 months; 4) who had been administered drugs for lipid regulation, promotion of blood circulation or anti-As, or for other diseases; 5) at pregnancy and lactation; 6) with hyperlipemia induced by drugs.

### Clinical materials

Most of the patients in the present study had visited the Cardiology Department of Dongzhimen Hospital (affiliated to the Beijing University of Chinese Medicine, Beijing, China) for the treatment of hyperlipemia and As. We recruited 37 patients: 31 cases of phlegm-blood stasis syndrome and 6 of syndromes without phlegm and blood stasis. We also recruited a further 10 healthy subjects as the control group. Patients voluntarily signed the informed consent form. Patients in the two groups and healthy subjects were comparable with respect to sex and age (both $P>0.05$). Two milliliters of blood anticoagulated with ethylenediamine tetra-acetic acid (EDTA) was extracted from the cubital vein of each subject. Blood was centrifuged (3500 rpm for 15 min at room temperature); the upper portion (plasma) of the blood was transferred to an Eppendorf tube and stored at -80°C.

### Reagents and Instruments

Acetonitrile (chromatographically pure; Sinopharm Chemical Reagent, Beijing, China), pyridine (chromatographic grade, Tianjin Dingguang Institute of Fine Chemicals, Tianjin, China), marginal acid (Sigma-Aldrich, St. Louis, MO, USA), methoxamine (Sigma-Aldrich), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Sigma-Aldrich), trimethylchlorosilane (TMCS) (chromatographically pure, Sigma-Aldrich) and normal heptane (analytically pure, Sinopharm Chemical Reagent) were purchased. A QP2010 gas chromatography-mass spectrometry (GC-MS) from Shimadzu (Tokyo, Japan) was employed.

### Plasma metabolomic detection

Plasma was removed from storage at -80°C and defrosted. One-hundred microliters of plasma was transferred to a centrifuge tube, 20 μL of marginic acid (1 mg/mL) was added to the tube as the internal standard, and 900 μL of methanol and water (9:1) added to the solution. The solution was vortex-mixed for 30 s, ultrasonicated in an ultrasonic ice bath for 10 min, and then centrifuged (15 000 rpm, 15 min, 4°C). Supernatant (200 μL) was transferred and evaporated to dryness in a vacuum. Then, 30 μL (15 mg/mL) of methoxypruvine was added to the dried supernatant, and the solution stored at room temperature for 16 h. One hour after MSTFA (99:1 TMCS, v/v) had been added, 200 μL of normal heptane was added to the solution. The solution was vortex-mixed for 30 s, and then 10 μL of supernatant transferred as an injection to be used in GC-MS analyses. The same sample was used for six continuous injections, and the relative standard deviation (RSD) of each relative peak area computed to reflect inter-assay accuracy.

### GC-MS conditions

The temperature was maintained at 100°C for 3 min, increased to 300°C at 8°C/min, then maintained for 2 min; the injection volume was 1.0 μL and the split ratio 10:1. The temperature at the injection port was 280°C, the temperature at the interface was 250°C, and the ion source temperature was 200°C. High-purity helium was used as the carrier gas at flow rate of 1.0 mL/min. Electron impact ionization was adopted with an electron energy of 70 eV and scanning range of 35-800 m/z.

### Statistical analyses

Common peaks (peaks common to each chromatogram) were selected according to the retention time of each peak in the GC-MS total ion chromatograms. Each peak area and internal standard peak area was then calculated, and the metabolite concentration represented by the relative peak area (ratio of each peak area to internal standard peak area). Partial least square
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RESULTS

Total ion chromatograms (TICs) of metabolomes in each group

The same sample had six continuous injections, and the RSD of relative peak area of each peak ranged between 2.05% and 6.75%. GC-MS TICs of plasma metabolites in each group are shown in Figure 1. The Shimadzu Chromatographic Station found that each TIC contained several substances, which were identified to be 73 chemicals by the spectral library of the National Institute of Standards and Technology (NIST; Beijing, China). These 73 substances included amino acids (e.g., alanine, glycine, valine, pyroglutamic acid) carbohydrates (e.g., galactose mannose) fatty acids (e.g., palmitic acid linoleic acid) and other small-molecule metabolites (e.g., urea, phosphoric acid).

Discrimination between groups

By carrying out partial least squares-discriminant analyses (PLS-DA) for the 73 metabolites in plasma, all groups were discriminated from each other (Figure 2). Figure 2A, with each dot representing one specimen, demonstrated all specimens to be scattered in the two areas within the oval scatter plot. There was no overlap between the hyperlipemia and As group and control group, indicating that plasma metabolites could be...
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Figure 2 PLS-DA score plots of plasma metabolomes in each group

PLS-DA: partial least squares-discriminant analyses; A: control group and hyperlipemia and As group. (●) control group; (■) hyperlipemia and As. B: Phlegm-blood stasis syndrome group and syndromes without phlegm and blood stasis group. (●) phlegm-blood stasis syndrome group; (■) syndromes without phlegm and blood stasis group.

Figure 3 PLS-DA loading plots of the phlegm-blood stasis syndrome group and syndromes without phlegm and blood stasis group

PLS-DA: partial least squares-discriminant analyses.

Table 1 Comparison of plasma metabolite concentrations in the control group and different syndrome groups (x̄ ± s)

<table>
<thead>
<tr>
<th>Endogenous metabolites</th>
<th>Control group</th>
<th>Syndromes without phlegm and blood stasis group</th>
<th>Phlegm-blood stasis syndrome group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>1.519±0.714</td>
<td>1.220±0.344</td>
<td>1.814±0.332</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.110±0.064</td>
<td>0.008±0.005 *</td>
<td>0.069±0.037 *</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>0.055±0.020</td>
<td>0.015±0.008 a</td>
<td>0.040±0.015 a</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>0.185±0.103</td>
<td>0.102±0.017 a</td>
<td>0.134±0.076</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.016±0.009</td>
<td>0.190±0.060 b</td>
<td>0.082±0.043 b</td>
</tr>
</tbody>
</table>

Note: Compared with the control group, *P<0.05 and **P<0.01; Compared with the syndromes without phlegm and blood stasis group, *P<0.05. Used to discriminate the control group from patients with metabolic disorders. Similarly, Figure 2B did not show overlap between the phlegm-blood stasis syndrome group and syndromes without phlegm and blood stasis group. This finding indicated that plasma metabolites could be used to discriminate the phlegm-blood stasis syndrome group from syndromes without phlegm and blood stasis group. The difference was greater between the syndrome groups and control group than that between the two syndrome groups.
This finding suggested that the model we established was reasonable. Furthermore, the metabolites that contributed to the differences mentioned above were identified (Figure 3). A PLS-DA loading plot ranked the 73 metabolites according to their contribution to grouping. We then calculated the VIP value of metabolites in the model, and found 15 metabolites to have a VIP value >1. Student’s t-test demonstrated that, of the 15 metabolites, 5 metabolites had different concentrations between groups (P<0.05). The NIST spectral library search identified the 5 metabolites to be urea, isoleucine, glucuronic acid, palmitic acid and glycerol. The glycerol concentration in plasma in the phlegm-blood stasis syndrome group was significantly less than in the syndromes without phlegm and blood stasis group, and the concentrations of the other 4 metabolites in the phlegm-blood stasis syndrome group were significantly higher than in the syndromes without phlegm and blood stasis group (Table 1). These 5 metabolites could be important metabolic markers in plasma that can be used to discriminate between the two syndromes.

DISCUSSION

TCM categorizes hyperlipidemia into phlegm syndrome and blood stasis. Lipids and lipoprotein metabolic disorders are believed to contribute to phlegm in blood, whereas platelet activation, thrombosis, endothelial injury and atheromatous plaques are thought to be caused by blood stasis. On the basis of TCM theory, clinical practice and many studies, we believe that, as an important pathogenic factor to many diseases, phlegm is the upstream cause of stasis, toxins, vessel injury, and phlegm-blood stasis. Small-molecule substances such as inflammatory factors and free radicals might have a role in the interaction between phlegm, stasis and toxins. Phlegm-blood stasis is pathological feature of the late phase of several chronic diseases, and phlegm-blood stasis syndrome is the major syndrome type in hyperlipidemia and As. By utilizing gas chromatography/time of flight-mass spectrometry (GC/TOF-MS), we investigated the plasma metabolomes in the control group, phlegm-blood stasis syndrome group and syndromes without phlegm and blood stasis group. Not only could syndrome groups be discriminated from the control group, but the two syndrome groups could be typed well from each other. This finding indicated that small-molecule endogenous chemicals (the study subjects of metabolomics) could be utilized for discriminating between TCM syndromes. Significant differences between the groups were noted with regard to the concentrations of endogenous metabolites. This finding suggested that patients with hyperlipemia and As had changes in the relevant biological metabolite pathways, and that the two syndrome groups also had different metabolic pathways. Our previous study revealed that 9 metabolic markers in patients with hyperlipemia and As were involved in lipid metabolism, oxidative stress, energy metabolism, and glycol-metabolic pathways, indicating that hyperlipemia and As are relevant to changes in several functional systems. The findings of the present study demonstrated that the concentrations of urea, isoleucine, glucuronic acid, and palmitic acid increased, and that the concentration of glycerol decreased. Hence, these compounds were specific markers helping to discriminate phlegm-blood stasis syndrome and syndromes without phlegm and blood stasis in hyperlipidemia and As.

Plasma urea concentration in the phlegm-blood stasis syndrome group was higher than that in the syndromes without phlegm and blood stasis group and control group. This increase in urea concentration indicated the increase in the concentration of nitrogen metabolites, which are toxic and produced during the catabolism of proteins and amino acids. The increase in urea concentration suggested a metabolic disorder of proteins and amino acids in patients with phlegm-blood stasis syndrome.

Isoleucine is a branched-chain amino acid that participates in glutamine generation. Isoleucine concentrations in the two syndrome groups were less than those in the control group, indicating worsened injury to cell membranes, decreased immunity, and more damage to the body by free radicals. Furthermore, isoleucine participates in the energy supply during long-term continuous energy consumption. Isoleucine concentrations in the phlegm-blood stasis syndrome group were higher than in the syndromes without phlegm and blood stasis group and control group. This may be because: 1) further liver damage in the phlegm-blood stasis syndrome group led to a decreased capacity of absorption and endogenous proteins reserved for energy supply, and 2) necrocytosis and cell rupture caused liver cells to release amino acids into the blood. These two phenomena would result in higher concentrations of amino acids in blood.

Glucuronic acid is relevant to glycometabolism in vivo, and glucuronic acid concentration in two syndrome group was different, indicating that the two syndromes had different glycometabolism. The concentrations of palmitic acid in the two syndrome groups were less than those in the control group, and this might be because decreased synthesis of lipoproteins in hepatic tissue affected the secretion and transport of lipids. Plasmic acid concentrations in the phlegm-blood stasis syndrome group were higher than in the syndromes without phlegm and blood stasis group and control group, and this finding might be associated with a higher blood fat level in the phlegm-blood stasis syndrome group.

Glycerol concentrations in the plasma of the two syndrome groups were higher than those in the control group. This result might have been caused by higher
blood fat levels in patients with hyperlipidemia and As. Plasma glycerol concentrations in the phlegm-blood stasis syndrome group were lower than those in the syndromes without phlegm and blood stasis group. This finding indicated that the two syndromes had different glycolipid metabolism, to which glycerol is very relevant. The present study revealed a specific marker group for phlegm-blood stasis syndrome in hyperlipidemia and As. This finding could provide a metabolomic basis for endogenous substances and the treatment of phlegm-blood stasis syndrome in hyperlipidemia and As.

REFERENCES