Serum proteomes of hypertension patients with abundant phlegm-dampness

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Objective: To study the serum proteomes of essential hypertension (EH) patients with abundant phlegm-dampness, and try to find special proteins associated with abundant phlegm-dampness syndrome.

Methods: Fifty-nine hypertension patients were included, and the patients were divided into abundant phlegm-dampness syndrome group (39 cases) and non-phlegm-dampness syndrome group (20 cases). To find the special proteins associated with abundant phlegm-dampness, the EH patients with non-phlegm-dampness and another 30 healthy persons were regarded as control. Weak cation nano-magnetic beads were used to capture proteins in serum, and proteomic fingerprint was made by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). All the proteomic fingerprints were analyzed by Biomarker Wizard 3.1 Software. Then Biomarker Patterns Software (BPS) 5.0 was used to identify the differentiated proteins, which could induce phlegm-dampness.

Results: There were 102 differentiated protein peaks between abundant phlegm-dampness and the control group. The best markers of abundant phlegm-dampness were protein peaks with the mass to charge ratio (m/z) of 9 334.958 m/z (the expression increased), 9 280.191 m/z (the expression decreased), 8 030.794 m/z (the expression increased), and 2 941.551 m/z (the expression increased). These four protein peaks found by BPS could induce abundant phlegm-dampness. They could be used to separate the abundant phlegm-dampness syndrome from the healthy persons and the hypertension patients with non-phlegm-dampness. The sensitivity of the model was 93.103% (27/29), specificity was 92% (23/25), false positive rate was 8% (2/25), false negative rate was 6.897% (2/29) and Youden’s index was 85.103%. Blind test data indicated a sensitivity of 90% (9/10) and a specificity of 88% (22/25), and the false positive rate was 12% (3/25), false negative rate was 10% (1/10), and Youden’s index was 78%.

Conclusion: The differentiated proteins between the abundant phlegm-dampness group and the control group are the material foundation of abundant phlegm-dampness. The selected differentiated proteins can be used to distinguish the EH patients with abundant phlegm-dampness from the healthy persons and the EH patients with non-phlegm-dampness. The molecular biology diagnosis model can offer an objective and accurate way for TCM syndrome differentiation.

Keywords: proteome; molecular biology; hypertension; phlegm-dampness


高血压病痰湿壅盛证患者血清蛋白质组学研究

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目的：对高血压病痰湿壅盛证患者血清蛋白组学进行研究，试图寻找与痰湿壅盛证相关的特异蛋白。
方法：纳入59例高血压患者，将其分为痰湿壅盛组（39例）和非痰湿壅盛组（20例），并选择30例健康体检者作为正常对照。采用弱阳离子交换性微球捕获血清中的蛋白质，Ciphergen PBS-Ⅱc型蛋白质芯片阅读检测仪绘制出蛋白指纹图谱。所有蛋白指纹图谱采用Biomarker Wizard3.1分析之后用Biomarker Patterns Software 5.0识别痰湿壅盛证特异表达的蛋白质，并建立诊断模型。
结果：高血压病痰湿壅盛证（痰湿壅盛）与对照组之间共检测出有显著差异的蛋白质峰102个（P<0.05）。以质荷比（mass to charge ratio, m/z）为9 334.958（表达增加），9 280.191（表达降低），8 030.794（表达增加）和2 941.551（表达增加）4个蛋白峰组成的诊断模型能够很好地将痰湿壅盛区分出来。该诊断模型的敏感性为93.103%（27/29），特异性为92%（23/25），假阳性率为8%（2/25），假阴性率为6.897%（2/29），Youden指数为85.103%。盲法检验其敏感性为90%（9/10），特异性为88%（22/25），假阳性率为12%（3/25），假阴性率为10%（1/10），Youden指数为78%。
结论：差异表达的蛋白质很可能是高血压病痰湿壅盛证的物质基础，筛选出的差异蛋白质是该证型患者血清蛋白标记物中区别于正常人和非此证型的高血压患者的共性特异蛋白。以此建立的分子生物学诊断模型，为中医辨证提供了一种更加客观准确的辨证手段。
关键词：蛋白组学；分子生物学；高血压；痰湿

The core of traditional Chinese medicine (TCM) is syndrome differentiation treatment, and syndrome is a pathological manifestation with a certain regulation. By observing the patient’s abnormal manifestation, one can grasp the nature of the disease. External pathological manifestation is bound to its material basis, which can be explained by proteomics study. After the study of proteomics relating to TCM syndrome of essential hypertension (EH) patients, the basis of the proteomics resulting from TCM syndrome of the EH patients was researched, and the syndrome-protein expression profiling was established to reveal the scientific meanings of TCM syndrome, and to further supplement the basis and methods of the objectiveness of TCM syndrome differentiation methods.

1 Clinical data and methods

1.1 Clinical data

1.1.1 Study objects From June to November 2008, 52 EH patients in Department of Cardiology, Guang’ anmen Hospital, including 39 EH patients with abundant phlegm-dampness and 20 EH patients with non-abundant phlegm-dampness were included in the study, and another 30 healthy persons were also included.

1.1.2 Diagnostic criteria The diagnosis of hypertension followed A Draft of Chinese Guidelines for Hypertension Prevention and Treatment[1]. The diagnosis of TCM syndrome followed National Standards of People’s Republic of China: Syndrome Part of Terminology of Traditional Chinese Medicine Clinical Diagnosis and Treatment[2] and Guidelines for Clinical Research on Chinese New Herbal Medicines[3]. The main common symptoms of abundant phlegm-dampness syndrome including sticky sputum, sputum expectorant, limb heaviness, stuffiness and oppression in chest and epigastrium, poor appetite, greasy taste in the mouth, white and greasy tongue coating, moderate and soggy pulse or slippery pulse. Patients meeting the hypertension diagnosis filled TCM syndrome scale, and then two associate chief physicians performed the syndrome differentiation according to the TCM scale.

1.1.3 Including criteria The patients who met with the diagnostic criteria were included, and they were asked to fill the TCM scale. The blood biochemical tests including blood glucose, blood lipid, liver and kidney function, and uric acid, and the necessary clinical examinations including electrocardiogram, ambulatory blood pressure, and carotid artery ultrasound were performed.

1.1.4 Excluding criteria 1) Patients with chronic diseases such as secondary hypertension, hyperlipidemia, diabetes, cerebral infarction, cerebral hemorrhage, angina pectoris, myocardial infarction, abnormal lipid metabolism and atherosclerosis diagnosed by carotid artery ultrasound; 2) Patients with cancer history; 3) Patients with liver and kidney dysfunction, hypothyroidism, hypoproteinaemia, alcoholism and mental diseases.

1.2 Study methods

1.2.1 Materials and instruments Weak cation-exchange (WCX) nano-magnetic beads were from SELDI Bio-engineering Technology Co., Ltd., Beijing, Tris (hydroxymethyl) aminomethane (Tris), 3-cyclohexylamino-1-propanesulfonic acid (CHA-PS), acetonitrile (ACN), dithiothreitol (DTT), and sinapic acid (SPA) were purchased from Sigma Co., Ltd. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) PBS-Ⅱc protein chip reader (Ciphergen Inc., USA), Hitachi-SCR20BA high-speed refrigerated centrifuge (Hitachi Co., Ltd., Japan), and Sanyo-MDF-U73V Ultra-low temperature refrigerator (Sanyo Electric, Japan).

1.2.2 Methods of obtaining and processing sam-
ples  Three milliliters of fasting blood were collected in the morning, contained in BD vacuum test tube without anticoagulant, stored for 1 hour at 4°C, and centrifuged at the speed of 3,000 r/min for 10 minutes, and then the blood serum was split into 5 tubes, each tube 100 μL and stored in −86°C refrigerator. For analysis, the serum out from refrigerator, melt the serum with ice-bath and then centrifuged for 10 minutes with 2,000 × g at 4°C. The serum (10 μL) was taken and added to 20 μL U9 buffer solution (9 mol/L urea, 20 g/L CHAPS, 10 g/L DTT, 50 mmol/L Tris-HCl, pH 9.0), well mixed, till centrifuged for 30 minutes at 4°C, and 100 mmol/L NaAc 370 μL (pH 4.0) was added and mixed immediately.

1.2.3 Activation of weak cation-exchange nanomagnetic beads  WCX nano-magnetic beads (10 g/L, 50 μL) was added to each PCR tube, and separated for 2 minutes on the magnetic separation plate. The supernatant part was discarded, and then 100 mmol/L NaAc 100 μL was added for activating two times, 5 minutes each time.

1.2.4 Serum protein captured by activated nanomagnetic beads  A total of 100 μL prepared serum sample was added into the activated magnetic beads, and then oscillated and incubated for 1 hour. After two-minute separation on the magnetic separation plate, the supernatant part was discarded and 100 mmol/L NaAc 100 μL was added and eluted for 2 times, 5 minutes each time. The protein was eluted and combined with the magnetic beads for 5 minutes by 5 mL/L trifluoroacetic acid (TFA) 10 μL. The protein eluent (5 μL) was mixed with 5 μL saturated SPA, and then absorbed 2 μL protein mixture spotting on the Au-chip (just as vector effect, Au-chip, Ciphergen Biosystems Inc.), dried naturally, and the protein fingerprint was detected by PBS-Ⅱe protein chip reader.

1.2.5 Chip detection  The parameters of chip reading instrument were set to the maximum detection range of 5,000, optimized scope of 2,000–10,000, laser intensity of 205, and detection sensitivity of 0.001. Before testing, adjusted the instrument using all-in-one polypeptide standard chip, and system quality deviation was less than or equal to 0.1%. The original data were processed by ProteinChip Software 3.1 in standardization and protein peak of 4,091 m/z (mass to charge ratio) was used as internal standard to correct.

1.2.6 Establishment and verification of model  Linear classification analysis was made to the peaks of differentially expressed proteins with same relative molecular masses between the abundant phlegm-dampness group and the control groups (EH patients with non-phlegm-dampness and healthy persons) by Biomarker Pattern software (BPS). With further optimization of the experiment parameters, we determined the best classification model and output original results, and then carried out cross-validation and exported the results by BPS.

The model is based on the neural network decision tree, and a best training sample was determined first. The choice of computing equation is the default choice of classification, if no logistic regression would be chosen, and the core operational idea is Bayes criteria. Classification function was used to classify and the higher score Yi function belongs to i kinds. The core operational idea of independent variable was applied for all possible set method in multiple regressions after t test. The neural network decision tree was used for drawing the decision tree after the determination of types and equation. All results were automatically formed by BPS software.

1.2.7 Perspective evaluation  Blind test (perspective evaluation) was performed to establish abundant phlegm-dampness model by testing the protein fingerprint and the sensitivity, specificity, false positive rate, false negative rate as well as Youden’s index of the model for abundant phlegm-dampness were obtained.

1.3 Statistical analysis  Statistical treatment of the protein fingerprint was performed by using Ciphergen ProteinChip software and BioMarker Wizard software. T-test was applied for the contrast between the abundant phlegm-dampness group and the control group (selected together, operated respectively and then summed up) with the data expressed as x ± s and a = 0.05 as statistical standard. After the mass spectrogram of differential protein peak was formed, BPS 5.0 was used to identify and diagnose the best marker of abundant phlegm-dampness and establish the diagnostic model of abundant phlegm-dampness. SPSS was used to perform Chi-Square and ANOVA analysis for the general information (gender, age) of the objects.

2 Results

2.1 Baseline data  There was no significant difference in general information between the abundant phlegm-dampness group and the control group (P > 0.05). Detailed information and clinical grouping are listed in Table 1. To find out the special protein in the EH patients with abundant phlegm-dampness, the EH patients with non-phlegm-dampness and another group of healthy persons were combined as the control.

2.2 Fingerprint of proteins and the establishment of abundant phlegm-dampness model  A total of 102 significantly differentiated protein peaks were tested in the abundant phlegm-dampness group and the control group (2,000–5,000 m/z), among which 20 kinds of proteins were higher in the abundant phlegm-dampness group than in the control group, while the other 82 kinds of proteins were lower in the abundant-dampness group than in the control group (P < 0.05). The information was shown in Table 2.

2.3 Best marker of abundant phlegm-dampness  Differential protein peaks obtained from abundant
phlegm-dampness modeling group and control modeling group were analyzed by BPS software to select the best marker for diagnosis of abundant phlegm-dampness. The results showed that the best markers of abundant phlegm-dampness were protein peaks of 9 334. 958 m/z (the expression increased), 9 280. 191 m/z (the expression decreased), 8 030. 794 m/z (the expression increased), and 2 941. 551 m/z (the expression increased). The information was shown in Figure 1. The model formed by these four protein markers (Figure 2) can classify the EH patients with abundant phlegm-dampness clearly, and the sensitivity of the model was 93. 103% (27/29), specificity was 92% (23/25), false positive rate was 8% (2/25), false negative rate was 6. 897% (2/29) and Youden’s index was 85. 103%. Receiver operator characteristic (ROC) curve can be seen in Figure 3.

<table>
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<th>Group</th>
<th>n</th>
<th>Male/Female (Cases)</th>
<th>Age (Years)</th>
<th>Average age (x ± s, years)</th>
<th>Modeling (Cases)</th>
<th>Blind text (Cases)</th>
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<td>16-59</td>
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<td>Abundant phlegm-dampness</td>
<td>39</td>
<td>11/9</td>
<td>19-59</td>
<td>50.68±10.05</td>
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* : These 20 kinds of proteins were higher in the abundant phlegm-dampness group than in the control group, and the other 82 kinds of proteins were lower in the abundant phlegm-dampness group than in the control group (P <0.05).

2.4 Blind test Blind test was performed on establishing diagnostic model of abundant phlegm-dampness over 35 samples of protein fingerprint. The results showed that the sensitivity of the model was 90% (9/10), the specificity was 88% (22/25), false positive rate was 12% (3/25), false negative rate was 10% (1/10), and Youden’s index was 78%.

3 Discussion

In recent years, with the rapid development of proteomics technology, proteomics study methods have been applied to clinical research[4, 5]. A growing number of scholars carried out research on the nature of TCM syndrome by using the technology of proteomics. Xiao et al.[6] carried out the research for the serum protein in 23 hypertension cases of hyperactivity of liver yang syndrome, contrasted with the normal. A total of approximately 500 protein spots were detected, and there were 16 significantly differentiated proteins by group comparison. Then peptide mass fingerprinting (PMF) was made by using MALDI-TOF-MS. It was found that n-methyl-aspartyl acid receptor, serum ceruloplasmin, transferrin, vitamin D-binding protein, apolipoprotein were up-regulated in hyperactivity of liver yang group, and the expression of glucoprotein reduced. Xiong et al.[7] studied the serum protein differences between liver-yang transforming into wind and hyperactivity of liver-yang of hypertensive cerebral hemorrhage. Forty cases of hypertensive cerebral hemorrhage were divided into 17 cases of liver-yang transforming into wind and 23 cases of hyperactivity of liver-yang syndrome. The images were processed by using 2-DE electrophoresis, scanner and PDQuest V7.3.1 software, and the average protein points of serum map were 113 103 and 94 in liver-yang transforming into wind and hyperactivity of liver-yang syndrome of hypertensive cerebral hemorrhage as well as healthy persons respectively. Five proteins were preliminary identified, which were serum amyloid precursor, ceruloplasmin, vitamin D-binding protein, apo lipoprotein C III and transferrin. These 5 proteins might be related to liver-yang transforming into wind syndrome of hypertensive cerebral hemorrhage.
Figure 1  Mass spectrum of modeling protein peaks
The left stands for abundant phlegm-dampness group, and the right stands for control group.
In this study of the serum proteomics in the hypertension patients with abundant phlegm-dampness, we found that there were 102 differential protein peaks between the abundant phlegm-dampness group and the control group (2 000 - 50 000 m/z), among which 20 kinds of proteins in the abundant phlegm-dampness group were higher than those in the control group, and the other 82 kinds of proteins in the abundant phlegm-dampness group were lower than those in the control group (P<0.05). The diagnostic model set up by four protein peaks of 9 334.958 m/z (the expression increased), 9 280.191 m/z (the expression decreased), 8 030.794 m/z (the expression increased), 2 941.551 m/z (the expression increased) could distinguish the hypertension patients with abundant phlegm-dampness syndrome from the hypertension patients with other TCM syndromes and normal. The sensitivity of the diagnostic model was 93.103%, specificity was 92%, false positive rate was 8%, and false negative rate was 6.897%. By blind test, the sensitivity was 90%, specificity was 88%, false positive rate was 12%, and false negative rate was 10%. The manifestation of TCM syndrome is based on the expression of specific protein. The molecular biology diagnostic model established by differentially expressed protein combined with artificial intelligence decision tree, had higher sensitivity and specificity, and provided the theoretical support and new syndrome differentiation method for TCM.

The discovery of specific protein provides the evidence for further study of the nature of TCM syndrome. The material basis of syndrome is the basis for correspondence of syndrome and treatment, and correspondence of prescription and syndrome.

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第四届全国中西医结合围手术期医学研讨会征文
暨全国中西医结合围手术期研究新进展学习班报名通知

为了促进中西医结合围手术期领域的学术发展和经验交流，推动本领域临床实践和研究的不断深入，由中国中西医结合学会围手术期专业委员会主办、江苏省中医院（南京中医药大学附属医院）承办的“第四届全国中西医结合围手术期医学研讨会暨全国中西医结合围手术期研究新进展学习班”将于2009年9月在江苏省南京市举办。现将有关事宜通知如下。

1 征文内容 （1）围手术期中西医结合治疗的理论探讨；（2）围手术期中西医结合治疗的临床研究；（3）围手术期中西医结合治疗的实验研究；（4）围手术期中西医结合治疗的经验总结；（5）围手术期中西医结合治疗的研究进展；（6）其他与围手术期医学有关的内容。

2 征文要求 （1）论文内容真实、可靠，具有科学性、先进性、实用性，未公开发表过；（2）全文3000字左右，文稿须附中文摘要、关键词；（3）寄全文、摘要的打印版、电子版由E-mail发送；（4）来稿请务必注明作者姓名、工作单位、通讯地址、邮政编码和联系电话；（5）征文截稿日期：2009年8月20日。

3 会议形式 （1）专家专题演讲（会议将邀请国内著名专家进行专题讲座）；（2）大会交流；（3）参观交流、手术演示等。

4 会议时间 2009年9月，具体时间请见第2轮会议通知。

5 会议地点 江苏省南京市，具体会议地址请见第2轮通知。

6 其他事项 会务费800元/人，食宿由会议统一安排，费用自理。授予国家级继续教育学分。

7 联系方式 联系人：马朝群、曹仕兵，杨飞朋；联系电话：025-86617141-70816，025-86617141-60252，13584003725；E-mail：zhuyk8888@126.com；地址：南京市汉中路155号江苏省中医院外科办公楼；邮政编码：210029。

中国中西医结合学会