Transcriptome analysis of blood stasis syndrome in subjects with hypertension

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Abstract
OBJECTIVE: To screen for mRNAs associated with blood stasis syndrome and to explore the genetic mechanisms of blood stasis syndrome in hypertension.

METHODS: This study involved groups of patients with hypertension and blood stasis, including those with Qi deficiency, Qi stagnation, cold retention and heat retention; as well as hypertensive patients without blood stasis and healthy individuals. Human umbilical vein endothelial cells were co-cultured with the sera of healthy individuals and patients with blood stasis syndrome. Total RNA was extracted from these cells and assessed by a high-throughput sequencing method (Solexa) and digital gene expression. Differentially expressed genes among these six groups were compared using whole genome sequences, and mRNAs associated with blood stasis syndrome identified. Differences in gene use and gene ontology function were analyzed. Genes enriched significantly and their pathways were determined, as were network interactions, and encoded proteins. Gene identities were confirmed by real-time polymerase chain reactions.

RESULTS: Compared with cells cultured in sera of the blood stasis groups, those culture in sera of healthy individuals and of the non-blood stasis group showed 11 and 301 differences, respectively in stasis-related genes. Genes identified as differing between the blood stasis and healthy groups included activating transcription factor 4, activating transcription factor 3, DNA-damage inducible transcription factor 3, Tribbles homolog 3, CCAAT/enhancer binding protein-β, and Jun proto-oncogene (JUN). Pathway and protein interaction network analyses showed that these genes were associated with endoplasmic reticulum stress. Cells cultured in sera of patients with blood stasis and Qi deficiency, Qi stagnation, heat retention, and cold retention were compared with cells cultured in sera of patients with the other types blood stasis syndrome. The comparison showed differences in expression of 28, 28, 34, and 32 specific genes, respectively.

CONCLUSION: The pathogenesis of blood stasis syndrome in hypertension is related to endoplasmic reticulum stress and involves the differential expression of the activating transcription factor 4, activating transcription factor 3, DNA-damage inducible transcription factor 3, Tribbles homolog 3, CCAAT/enhancer binding protein-β, and JUN genes.

Key words: Hypertension; Blood stasis; RNA, messenger; Endoplasmic reticulum stress
INTRODUCTION

Hypertension is a highly prevalent clinical syndrome characterized by high arterial pressure that can seriously damage the heart, brain, and kidneys. In traditional Chinese medicine (TCM), blood stasis is believed to play an important role in the occurrence and development of hypertension. Current studies on the mechanism of blood stasis syndrome have been limited to observing changes in blood, focusing on the relationship between vascular changes and the initiation of blood stasis syndrome. Moreover, blood stasis biology has been associated with changes in vascular endothelial cell (VEC) function, with VEC damage closely associated with the initiation and development of blood stasis syndrome. Endoplasmic reticulum stress (ERS) has been shown to damage VECs, indicating that ERS may induce blood stasis by causing endothelial cell injury. Our research group has focused on a blood stasis syndrome model, including the effects of VEC injury and mechanisms of repair, as well as on the effects of drugs and the molecular / genetic mechanisms underlying blood stasis. We and others have found that different types of blood stasis have different biochemical foundations, but also have common pathological and physiological characteristics. Because of the complex pathogenesis of blood stasis syndrome, including the effects of various factors, screening for and identifying biomarkers associated with this syndrome may help in its diagnosis and treatment. An established model of endothelial cell injury-blood stasis syndrome was used to screen for genes associated with the initiation of hypertension-related blood stasis syndrome among groups of hypertensive patients, with and without blood stasis syndrome, and healthy individuals. The genetic pathways of identified genes were investigated to determine the molecular mechanism underlying the development of blood stasis syndrome in hypertension.

MATERIALS AND METHODS

Three groups of individuals were analyzed. The first group included 40 patients (21 males, 19 females), of mean age (66 ± 7) years, diagnosed with blood stasis syndrome with hypertension from December 2011 to June 2012 at the Second Hospital affiliated with Guangzhou Medical College. Their mean systolic and diastolic blood pressures were (157 ± 13) and (104 ± 7) mm Hg, respectively. TCM syndrome differentiation rules have identified four types of patients with blood stasis syndrome plus hypertension: blood stasis syndrome with Qi deficiency, Qi stagnation, cold retention, and heat retention. Ten patients were included in each subgroup.

Another group of 12 patients [6 males, 6 females, of mean age (65 ± 9) years] had hypertension but without blood stasis. Their mean systolic and diastolic blood pressures were (166 ± 8) and (104 ± 8) mm Hg, respectively. These patients were recruited from the cardiovascular department, the outpatient TCM department, and patients hospitalized at the First Affiliated Hospital of Jinan University. Patients in this group did not differ significantly from the group of hypertensive patients with blood stasis syndrome in blood pressure, sex distribution, mean arterial pressure, or average age (each P > 0.05).

The third group consisted of 30 healthy volunteers (17 males, 13 females), recruited from the Department of Traditional Chinese Medicine, School of Medicine, Jinan University. Their blood pressures were within normal ranges and differed significantly (P < 0.01) from blood pressures in hypertensive patients, both with and without blood stasis syndrome.

High blood pressure was defined according to World Health Organization / International Hypertension Alliance guidelines (1999), and blood stasis syndrome was diagnosed according to the 2011 revised criteria. Patients with target organ damage, diabetes, or severe cardiovascular, cerebrovascular, or renal disease were excluded.

Serum collection

All included individuals were instructed to fast and not take any drugs overnight prior to a blood draw. Forearm venous blood was aseptically collected into sterile, non-anticoagulation vacuum tubes, allowed to coagulate, and centrifuged at 2784 g at 4 °C for 15 min. Serum was removed, placed in an aseptic Eppendorf tube (Gibco, Guangzhou, China), and incubated in a water bath at 56 °C for 30 min to inactivate serum complement. Finally, these samples were stored at −20 °C.

Establishing a cell model

The cell model of hypertension and blood stasis involved the use of human umbilical vascular endothelial cells (HUVECs; CRL-1730), obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM); (Gibco) containing 10% fetal bovine serum at 37 °C in a 5% CO₂ incubator. To each culture flask was added serum, to a concentration of 10%, plus 90% F12K medium (Gibco), and the cells were cultured at 37 °C in a 5% CO₂ incubator for 24 h.

The cells were collected, and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed by agarose gel electrophoresis.

mRNA sequence analysis and gene screening

mRNA transcriptome sequencing was performed using second-generation Illumina Solexa sequencing technology by the Shanghai Megiddo Biotechnology Co., Ltd. (Shanghai, China). The original statistical data were assessed while retaining high-quality sequences. Statistical analyses of each fragment per kilobase of exons per million fragments mapped (FPKM), as well as the transcript value in the expressed samples, were compared.
Finally, differences in group transcripts and relative gene expression were analyzed among the six groups of samples.

**Gene function analysis**
Differentially expressed genes were analyzed using Goatools (https://github.com/tanghaibao/GOatools) in the GO database, along with a PATHWAY enrichment analysis of differential gene use with the software KO-Based Annotation System (KOBAS) (http://kobas.cbi.pku.edu.cn/home.do). False positive rates were compared by Fisher’s exact tests. A P value ≤ 0.05 was considered significant. Differences in the expression of significantly enriched genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to identify cellular pathways. The Human Protein Reference Database (http://www.hprd.org/) for network visualization. The Human Protein Reference Database (http://www.hprd.org/Release8) and the Human Protein Reference Database (http://www.hprd.org/Release9) were used to assess differential gene expression. Log ratios were determined using Cytoscape software (http://www.cytoscape.org/) for network visualization.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**
PCRs were performed according to the manufacturer’s instructions, with primers designed based on the sequences in the gene database. The 2^ΔΔCt method was used to assess differential gene expression. Log2 ratios were determined by comparing gene expression in the five hypertensive groups with that in healthy controls.

**RESULTS**

**RNA extraction**
Total RNA was extracted from HUVECs incubated with serum from each of the groups of subjects. The 28S and 18S rRNA bands were clear, with no observed residue at the dye front. The ratio of A260 to A280 was 1.8–2, showing that the quality of these total RNA samples was in full compliance with requirements for chip analysis.

**Differential gene expression of blood stasis syndrome**
Gene expression by each cell sample was assessed by differential gene expression (DGE) analysis, with a P value of < 0.01, an False Discovery Rate (FDR) of < 0.001, and a [log2Ratio] of ≥ 1 considered significant (Figure 1). The four groups with hypertension and blood stasis groups showed significant differences in mRNA expression compared with the control group. Specifically, the groups with Qi deficiency, Qi stagnation, cold retention, and heat retention showed significant differences in 76 (42 downregulated and 34 upregulated), 51 (34 downregulated and 17 upregulated), 61 (39 downregulated and 22 upregulated) and 62 (39 downregulated and 23 upregulated) mRNAs, respectively. The four groups with hypertension and blood stasis also showed significant differences compared with the group with hypertension but without blood stasis. Specifically, the groups with Qi deficiency, Qi stagnation, cold retention, and heat retention showed significant differences in 579 (117 downregulated and 459 upregulated), 475 (98 downregulated and 337 upregulated), 621 (135 downregulated and 486 upregulated) and 469 (103 downregulated and 356 upregulated) mRNAs, respectively. In addition, the group with hypertension but without blood stasis differed from the healthy group in 529 (423 downregulated and 106 upregulated) mRNAs.

**Differential mRNA expression analysis**
Integrated analyses of differential mRNA expression in the six groups are shown in Table 1.

![Figure 1 Numbers of mRNAs differentially expressed between pairs of groups](image-url)

**Figure 1** Numbers of mRNAs differentially expressed between pairs of groups. QDQS: Qi deficiency and blood stasis; QSBS: Qi stagnation and blood stasis; CBS: cold retention and blood stasis; HBS: heat retention and blood stasis; NC: healthy controls. Compare with NC group, the groups with QDQS, QSBS, CBS, and HBS showed significant differences in 76 (42 downregulated and 34 upregulated), 51 (34 downregulated and 17 upregulated), 61 (39 downregulated and 22 upregulated) and 62 (39 downregulated and 23 upregulated) mRNAs, respectively. Compared with NBS group, the groups with QDQS, QSBS, CBS, and HBS showed significant differences in 579 (117 downregulated and 459 upregulated), 475 (98 downregulated and 337 upregulated), 621 (135 downregulated and 486 upregulated) and 469 (103 downregulated and 366 upregulated) mRNAs, respectively. In addition, the group with NBS differed from NC group in 529 (423 downregulated and 106 upregulated) mRNAs.
Patients with blood stasis and heat retention, cold retention, Qi deficiency, and Qi stagnation were compared with the group with hypertension but without blood stasis. GO enrichment analysis demonstrated that differentially expressed genes are related to multiple stress responses, cell differentiation, apoptosis, and autophagy in VECs. These genes are also immune related (P < 0.05) (Figure 2). Five of the 20 pathways identified were directly associated with endoplasmic reticulum stress (ERS), including PERK-regulated gene expression, chaperone activation of IRE1alpha, chaperone activation by activating transcriptional factor 6 (ATF6)-alpha, and the release of eIF4E and the ATF-2 transcription factor network. Other pathways included the interleukin (IL)-6 and IL-2 inflammatory pathways, the p53 and p38 apoptosis pathways, the mammalian target of rapamycin (mTOR) and vascular endothelial growth factor (VEGF) pathways, the mitogen-activated protein kinase (MAPK) signaling pathway, and signal transduction and amino acid metabolism pathways (Table 2).

**Protein expression and blood stasis syndrome**

Comparisons of gene expression by cells incubated with sera from patients with hypertension alone and those with hypertension and blood stasis revealed evidence of differential expression of specific genes in protein networks. Genes associated with ERS included activating transcription factor 4 (ATF4), activating transcription factor 3 (ATF3), DNA-damage inducible transcription factor 3 (DDIT3), Tribbles homolog 3 (TRIB3), CCAAT / enhancer binding protein-β (CEBPB), JUN, Jun dimerization protein 2 and Early growth response 1 (Figure 3).

**Table 1 Specific gene statistics in blood stasis syndrome**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>mRNA Difference</th>
</tr>
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<tbody>
<tr>
<td>Qi deficiency and blood stasis syndrome vs normal and vs hypertension alone</td>
<td>22</td>
<td>10 12</td>
</tr>
<tr>
<td>Qi stagnation and blood stasis syndrome vs normal and vs hypertension alone</td>
<td>19</td>
<td>11 8</td>
</tr>
<tr>
<td>cold retention and blood stasis syndrome vs normal and vs hypertension alone</td>
<td>14</td>
<td>7 7</td>
</tr>
<tr>
<td>heat retention and blood stasis syndrome vs normal and vs hypertension alone</td>
<td>14</td>
<td>7 7</td>
</tr>
<tr>
<td>All four groups with blood stasis syndrome vs hypertension alone</td>
<td>301</td>
<td>239 62</td>
</tr>
<tr>
<td>All four groups with blood stasis syndrome vs normal</td>
<td>11</td>
<td>2 9</td>
</tr>
<tr>
<td>Qi deficiency and blood stasis syndrome in endemic hypertension</td>
<td>28</td>
<td>14 14</td>
</tr>
<tr>
<td>Qi stagnation and blood stasis syndrome in endemic hypertension</td>
<td>28</td>
<td>8 20</td>
</tr>
<tr>
<td>cold retention and blood stasis syndrome in endemic hypertension</td>
<td>32</td>
<td>10 22</td>
</tr>
<tr>
<td>Heat retention and blood stasis syndrome in endemic hypertension</td>
<td>34</td>
<td>9 25</td>
</tr>
<tr>
<td>Qi deficiency and blood stasis syndrome</td>
<td>124</td>
<td>67 57</td>
</tr>
<tr>
<td>Qi stagnation and blood stasis syndrome</td>
<td>81</td>
<td>34 47</td>
</tr>
<tr>
<td>cold retention and blood stasis syndrome</td>
<td>161</td>
<td>83 78</td>
</tr>
<tr>
<td>heat retention and blood stasis syndrome</td>
<td>69</td>
<td>38 31</td>
</tr>
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</table>

**Confirmation of differential mRNA expression by qRT-PCR**

qRT-PCR analysis comparing mRNA expression in the four groups with hypertension and blood stasis syndrome and the group with hypertension alone and the control group confirmed findings on the differential expression of ATF4, ATF3, DDIT3, TRIB3, CEBPB, and JUN mRNAs (Figure 4).

**DISCUSSION**

Hypertension is a polygenic disease resulting from different genetic and environmental factors and can enhance the risk of cardiac and cerebrovascular diseases. Hypertensive patients with blood stasis syndrome show increases in hemorheology, hemodynamics, and microcirculation. ERS can damage VECs by promoting the expression of p38 mitogen activated protein kinase (MAPK) and its downstream molecules, along with oxidative stress. These findings suggest that ERS may be caused by endothelial cell injury resulting from blood stasis, consistent with results showing that ERS inhibition is effective in treating vascular-related diseases. The ER is important for protein synthesis and folding, Ca²⁺ storage, and lipid synthesis. Modifications of secreted, transmembrane, and ER resident proteins can help them fold into their native conformations, eventually forming functional proteins. Changes in the inner cellular environment, such as hypoxia, Ca²⁺ homeostasis, oxidative stress, or abnormal glycosylation, can result in the accumulation of unfolded or misfolded proteins in the ER, resulting in ER dysfunction or ERS. Protein accumulation leads to a series of follow-up reactions, called an unfolded protein response (UPR). Reducing the amount of protein in the ER re-
results in the repair of misfolded proteins and the degrada-
tion of unfolded and misfolded proteins in the ER, restor-
ing ER function and ensuring the release of mature pro-
teins. Overexposure to the altered cellular in-
volve ment may overwhelm UPR capacity, leading to
apoptosis. Stress and UPR can activate three tran-
scription factors, inositol enzyme (IRE-1), pancreatic
endoplasmic protein kinase (PERK), and activating
transcription factor 6 (ATF6), resulting in three steps
to induce cell apoptosis, involving PERK, ATF4, and
the phosphorylation of eukaryotic translation initiation
factor 2α (eIF2α). PERK pathway activation occurs
by inhibiting protein during early ERS, resulting in the
phosphorylation of eIF2α and ATF4 activation; this in
turn promotes the activation and expression of
CCAAT-enhancer-binding protein homologous pro-
tein (CHOP) and induces cell apoptosis. ATF4 is a
key molecule increased in ERS-induced
apoptosis. The transcription factor CEBPB is
in volved in many biological processes, including cell
differentiation, proliferation, metabolism, tumors, in-
flammation, and apoptosis. The C/EBP family of tran-
scription factors. Its high expression may affect protein folding in the ER
and trigger an unfolded protein response and apoptosis
induced by a specific ER cell pathway network, causing
cell death. This protein plays an important role in in-
duced cell cycle arrest, promoting cell differentiation
and apoptosis. ATF3 is a member of the ATF/CERB
transcription factor family that inhibits transcription
by binding to the gadd153/CHOP promoter and cor-
responding sites. ATF3 plays an important role in the
transcriptional regulation of cell apoptosis, with any
factor that upregulates ATF3 expression inducing cell
apoptosis. The v-Jun sarcoma virus 17 gene homo-
logue JUN binds to the promoter regions of many
genes, regulating the expression of many genes in vari-
ous malignant tumors. TRIB3 is a serine/threonine pro-
tein kinase and a member of a newly discovered protein
gene family that can inhibit mitosis. Overexpress-
sion of TRIB3 can inhibit the MAPK pathway, adjust
the NF-κB pathway, and induce apoptosis. Thus, all
of these genes and ERS are closely related to cell apop-
tosis.

Our results, using gene chip technology, showed differ-
ences in mRNA expression among patients with hyper-
tension plus blood stasis, hypertension alone, and nor-

Figure 2 GO gene protein analysis related to blood stasis syndrome
A: Biological processes; B: molecular functions, C: cellular components. The ordinate indicates significant enrichment using the
GO term; the abscissa represents the enrichment factor; GO: gene ontology.
and JUN mRNAs were more highly expressed in the mal controls. ATF3, CEBPB, DDIT3, TRIB3, and JUN mRNAs were more highly expressed in the blood stasis groups. GO and PATHWAY analysis showed that multiple genes and a signal pathway were

### Table 2 Identification of gene pathways and involved genes differentiating patients with hypertension plus blood stasis syndrome and those with hypertension alone

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway</th>
<th>Number of genes</th>
<th>Background gene number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PERK regulated gene expression</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>Amino acid and oligopeptide SLC transporters</td>
<td>8</td>
<td>341</td>
</tr>
<tr>
<td>3</td>
<td>Activation of Chaperones by IRE1alpha</td>
<td>4</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>Amino acid transport across the plasma membrane</td>
<td>6</td>
<td>190</td>
</tr>
<tr>
<td>5</td>
<td>Serine biosynthesis</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>Signaling mediated by p38-alpha and p38-beta</td>
<td>5</td>
<td>343</td>
</tr>
<tr>
<td>7</td>
<td>IL6-mediated signaling</td>
<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>Axonal growth stimulation</td>
<td>2</td>
<td>18</td>
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<tr>
<td>9</td>
<td>Activation of Chaperones by ATF6-alpha</td>
<td>2</td>
<td>26</td>
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<tr>
<td>10</td>
<td>p53 signaling pathway</td>
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<td>691</td>
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<tr>
<td>11</td>
<td>Vitamin B6 metabolism</td>
<td>2</td>
<td>42</td>
</tr>
<tr>
<td>12</td>
<td>Release of eIF4E</td>
<td>2</td>
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<tr>
<td>13</td>
<td>Disease</td>
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<tr>
<td>14</td>
<td>mTOR signaling pathway</td>
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<td>553</td>
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<td>15</td>
<td>ATF-2 transcription factor network</td>
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<td>16</td>
<td>IL12 and STAT4 dependent signaling pathways in Th1 development</td>
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<td>17</td>
<td>Neurophilin interactions with VEGF and VEGFR</td>
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<td>18</td>
<td>Circadian clock</td>
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<tr>
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<td>VEGF signaling pathway</td>
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<tr>
<td>20</td>
<td>MAPK signaling pathway</td>
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</table>

Notes: PERK: pancreatic endoplasmic reticulum kinase; SLC: solute-carrier; IRE1: inositol-requiring protein 1; IL-6: interleukin-6; ATF6: activating transcriptional factor 6; eIF4E: eukaryotic initiation factor 4E; mTOR: mammalian target of rapamycin; ATF2: activating transcription factor-2; IL-12: interleukin-12; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; MAPK: mitogenactivated protein kinase.

### Figure 3 Correlations between blood stasis and gene expression network interactions

A: Qi stagnation and blood stasis (QSB5) vs hypertension without blood stasis (NBS); B: Qi deficiency and blood stasis (QDBS) vs NBS; C: cold retention and blood stasis (CBS) vs NBS; D: heat retention and blood stasis (HBS) vs NBS. ATF4: activating transcription factor 4; ATF3: activating transcription factor 3; JUN: Jun proto-oncogene; CEBPB: CCAAT/enhancer binding protein-β; DDIT3: DNA-damage inducible transcription factor 3; TRIB3: Tribbles homolog 3; CEBPG: CCAAT/enhancer binding protein gamma; TSC22D3: TSC22 domain family member 3; JDP2: Jun dimerization protein 2; MYC: v-myc avian myelocytomatosis viral oncogene homolog; RASSF1: Ras association (Ral GDS/AF-6) domly member 1; EGR1: Early growth response 1.
related to ERS. Determining the relationships of ATF4, ATF3, DDIT3, TRIB3, CEBPB, and JUN, as well as of ERS, with the pathogenesis of blood stasis in hypertension requires additional in vitro and in vivo studies.

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