Transforming growth factor β1 is a differentially expressed candidate protein of congestive heart failure with Qi-deficiency-blood-stasis syndrome

Li Xiaqian, Huang Pinxian, Wang Shijun, Cao Xuebin, He Jiancheng

OBJECTIVE: To investigate the role of tongue coating fluid protein in regulation of congestive heart failure (CHF) in Qi-deficiency-blood-stasis syndrome.

METHODS: We studied patients with CHF (3 patients with Qi-deficiency-blood-stasis syndrome and 3 without Qi-deficiency-blood-stasis syndrome) to investigate differentially expressed proteins. We also included a control group. A biotin label-based antibody array was used for testing tongue coating fluid samples from patients. Network analysis of these differentially expressed proteins was conducted using the STRING database, which can predict the relations between differentially expressed proteins and CHF with Qi-deficiency-blood-stasis syndrome.

RESULTS: A total of seven differentially expressed proteins were identified, and among these, transforming growth factor β1 (TGF-β1) gets a particular attention for us has drawn specific attention. Network analysis showed a homologous relationship of TGF-β1 with bone morphogenetic protein 15, which is associated with myocardial fibrosis.

CONCLUSION: Occurrence and development of CHF may result from certain DE-proteins and associated signaling pathways. TGF-β1 protein may be a candidate marker for assessing the risk of CHF in Qi-deficiency-blood-stasis syndrome.

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Keywords: Congestive heart failure; Qi-deficiency blood-stasis; Transforming growth factor β1; Bone morphogenetic protein 15; Differentially expressed protein

INTRODUCTION

Congestive heart failure (CHF) is a common disease that often occurs at the final stage of various cardiovascular diseases. Traditional Chinese medicine (TCM) plays an important role in preventing and treating chronic diseases, including CHF. However, clinical intervention for CHF at the early stage remains poor by TCM and Western Medicine because of the limitation
of specific cardiovascular markers for identifying individuals who are at high risk of CHF or its Chinese medicine syndrome patterns. Inspection, auscultation and olfaction, inquiry, and palpation are the four methods of TCM diagnosis. Looking at the tongue is an important part of observation and is one of the special diagnostic methods in TCM. Through proper observation of the tongue and tongue coating, physicians can analyze the physiological and pathological conditions of the patients. Different patients with CHF might show different manifestations clinically. According to the characteristics of the tongue coating, a patient can be diagnosed with or without Qi-deficiency-blood-stasis syndrome, which is the most common syndrome pattern in CHF.

In recent years, protein chip technology with high throughput and sensitivity has been widely used in identifying specific proteins in samples.1 Our research group has made studies on whether the tongue coating fluid of different TCM syndromes with CHF contain different biological information (e.g., proteins) and whether these differences can discriminate between different syndrome patterns. Therefore, in this study, we investigated the association between tongue coating fluid proteins and CHF in patients with or without Qi-deficiency-blood-stasis syndrome.

MATERIALS AND METHODS

Clinical cases from Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Shutcm) were collected from January to March in 2015. Cases were collected according to the diagnostic criteria of CHF, inclusion and exclusion criteria, and diagnostic standards of CHF syndromes as established by our preliminary studies.1-2 We choose healthy controls from staff of Shutcm who have taken health examination. In this study, we included three cases of CHF with Qi-deficiency-blood-stasis syndrome, three with deficiency of both Qi and Yin syndromes, and four healthy controls. Patients were instructed to rinse their mouth for three to five times, and physicians obtained tongue coating fluid specimens from their tongue surface using a stainless spatula. The specimens were then dissolved in a centrifuge tube with 2 mL 0.9% normal saline. After centrifugation at 239 × g for 2 min, the supernatant was drawn into another centrifuge tube and cryopreserved at −80 °C for later assay.7

The main procedures for protein detection included protein quantification, dialysis of the sample, biotin labeling of the sample, drying the glass chip, blocking and incubation of the antibody array, and fluorescence detection. Protein quantification was determined using the Bicinchoninic Acid Kit (Biyunian Research Institute, Haimen, Jiangsu, China) for protein determination. Sample dialysis buffer (1 × phosphate-buffered saline, pH = 8.0) was dissolved in 0.6 g KCl, 24 g NaCl, 0.6 g KH2PO4, and 3.45 g Na2HPO4, in 2500 mL de-ionized water. Biotin labeling of the samples and fluorescence detection were determined using the RayBio® Biotin Label-based Human Antibody Array I (Norcross, GA USA).

Screening of DE-proteins involved identifying those with a fold change of ≥ 2.0 and a P value of ≤ 0.05. Heat map and volcano plots were performed for obtaining hierarchical clustering results on significant differentially expressed microRNAs. Genetic network analysis of differentially expressed proteins was analyzed by the STRING database. Bidirectional hierarchical clustering results showed the relationships between DE-proteins and samples.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). Results are expressed as mean ± standard deviation. Differences among three or more groups were assessed for significance using one-way analysis of variance, while differences between two groups were assessed using the least significant difference test. P < 0.05 was considered to be statistically significant.

RESULTS

Patients’ characteristics

The age range of the 10 subjects was 54-80 years, with an balanced sex distribution. Patients with Qi-deficiency-blood-stasis syndrome were grouped as G1, deficiency of both Qi and Yin as G2, and healthy controls as G3. The underlying diseases of G1 and G2 included coronary heart disease, with a cardiac function grade ranging from 2-3. In G1, the mean level of brain natriuretic peptide (BNP) was 246.90 pg/mL and that in G2 was 141.24 pg/mL.

Differentially expressed proteins screening between G1 and G3

Patients with syndrome of Qi deficiency and blood stasis had seven differentially expressed proteins compared with healthy controls (Table 2).

Differentially expressed proteins screening between G2 and G3

There were eight differentially expressed proteins between G2 and G3 in (Table 3).

Differentially expressed proteins screening between G1 and G2

There were three differentially expressed proteins between G1 and G2 (Table 4).

Hierarchical clustering of differentially expressed proteins

The hierarchical clustering results of differentially ex-
compared with G2. In G1, all differentially expressed proteins were upregulated.

Notes: BMP-15: bone morphogenetic protein 15; CXCR4: chemokine (C-X-C motif) receptor 4; FGF-13: fibroblast growth factor 13; TGF-β1: transforming growth factor β1. In G1/G3, six differently expressed proteins were upregulated, except for CXCR4. P value is the result of G1 compared with G3.

**Table 2** Differentially expressed proteins between G1 and G3

<table>
<thead>
<tr>
<th>Differentially expressed protein</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-15</td>
<td>7.92</td>
<td>0.018</td>
</tr>
<tr>
<td>CD30</td>
<td>2.49</td>
<td>0.029</td>
</tr>
<tr>
<td>CXCR4</td>
<td>0.03</td>
<td>0.008</td>
</tr>
<tr>
<td>Follistatin</td>
<td>3.26</td>
<td>0.037</td>
</tr>
<tr>
<td>FGF-13 1B</td>
<td>2.05</td>
<td>0.028</td>
</tr>
<tr>
<td>S100A10</td>
<td>20419.43</td>
<td>0.012</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>2.37</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Notes: BMP-15: bone morphogenetic protein 15; CXCR4: chemokine (C-X-C motif) receptor 4; FGF-13: fibroblast growth factor 13; TGF-β1: transforming growth factor β1. In G1/G3, six differently expressed proteins were upregulated, except for CXCR4. P value is the result of G1 compared with G3.

**Table 3** Differentially expressed proteins between G2 and G3

<table>
<thead>
<tr>
<th>Differentially expressed protein</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR7</td>
<td>25.47</td>
<td>0.003</td>
</tr>
<tr>
<td>Glut3</td>
<td>13.45</td>
<td>0.048</td>
</tr>
<tr>
<td>Hepassocin</td>
<td>4.23</td>
<td>0.018</td>
</tr>
<tr>
<td>IL-1 R6</td>
<td>0.00</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.92</td>
<td>0.012</td>
</tr>
<tr>
<td>Insulin</td>
<td>14.28</td>
<td>0.021</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2.83</td>
<td>0.037</td>
</tr>
<tr>
<td>MMP-11</td>
<td>9.30</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Notes: CCR7: CC-chemokine receptor 7; IL: interleukin; MCP-1: monocyte chemotactic protein 1; MMP-11: matrix metalloproteinase-11. In G2/G3, seven DE-proteins were upregulated, except for IL-1 R6. P value is the result of G2 compared with G3.

**Table 4** Differentially expressed proteins between G1 and G2

<table>
<thead>
<tr>
<th>Differentially expressed protein</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD30</td>
<td>10.71</td>
<td>0.005</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5.36</td>
<td>0.014</td>
</tr>
<tr>
<td>S100A10</td>
<td>21358.92</td>
<td>0.033</td>
</tr>
</tbody>
</table>

Notes: GM-CSF: granulocyte-macrophage colony-stimulating factor. In G1/G2, all differently expressed proteins were upregulated. P value is the result of G2 compared with G3.

Gene network analysis of differentially expressed proteins

Gene network analysis is a method of determining the correlation of specific proteins. In network analysis of STRING, the network node represented a protein, and networking was performed on behalf of the forecast relation function.3

**Different colored lines represent different associations**

We found a functional association of differentially expressed proteins of CHF in Qi-deficiency-blood-stasis syndrome. There was an association in text mining with follistatin, bone morphogenetic protein 15 (BMP-15), TGF-β1, and chemokine (C-X-C motif) receptor 4 (CXCR4), and homology between BMP15 and TGF-β1 (Figure 2).

**DISCUSSION**

In this preliminary study, we found that syndrome of Qi deficiency and blood stasis was the major pattern of CHF. Syndrome of Qi deficiency and blood stasis usually results in a worse prognosis of CHF and higher mortality rate.4,5 A circulating biomarker would be convenient for early diagnosis or treatment of CHF. In this study, we screened and found seven differentially expressed proteins from the coating liquid on the tongue in patients with Qi-deficiency-blood-stasis syndrome. Among them, TGF-β1 was an upregulated differentially expressed protein, and it is an important cytokine associated with myocardial fibrosis. TGF-β1 may induce increased expression of connective tissue growth factor, leading to the occurrence and development of myocardial fibrosis.6 Additionally, in our network analysis, we

![Figure 2](image-url)
found a text mining association of follistatin, BMP-15, TGF-β1, and CXCR4, and homology between BMP-15 and TGF-β1, which indicated network associations for TGF-β1 with other proteins.
At present, proteomics is widely used in many cardiovascular diseases, including CHF. Researchers have studied the pathogenesis from the protein level, and predicted protein markers of these diseases.\(^7\) In clinical practice, approximately 100 types of cardiac proteins in pathological states have been identified.\(^8\) Some other researchers have found that normal myocardial cells can be separated to $1232 \pm 56$ protein spots. Among them, 11 types of proteins got qualitative and quantitative change after stress, and these DE-proteins might be involved in cardiovascular stress reactions and the process of stress injury.\(^9\) Moreover, an increasing amount of studies have focused on biomarkers in non-invasive examinations.\(^10\) The protein biochip technique plays an important role in screening prognostic information from samples.

TCM plays a major role in preventing and treating CHF.\(^11\) Currently, studies on the material basis of TCM syndromes have become the focus of research. In TCM, information regarding a disease is obtained by inspecting a patient’s tongue. However, there is no study focused on the biological basis of using tongue coating liquid in syndrome of Qi deficiency and blood stasis with CHF, and a protein biomarker still remains unclear.

In our study, we found seven differentially expressed proteins in the tongue coating liquid of patients with syndrome of Qi deficiency and blood stasis. Among them, TGF-β1 protein was significantly upregulated. To the best of our knowledge, this is the earlier report to analyze the association between protein levels in tongue coating in TCM syndromes of CHF. Most importantly, we found homologous and text mining relationships of TGF-β1 with BMP-15, and BMP-15 is associated with myocardial fibrosis and cardiac remodeling. TGF-β1 is the most widely expressed subtype in the TGF-β family.\(^12\) The TGF-β1 signaling pathway is one of the key pathways affecting cardiac remodeling in hypertension or myocardial infarction by activating and promoting the occurrence and development of cardiac remodeling.\(^13\) TGF-β1 may promote Smad protein phosphorylation by combining its receptors, and then combine with auxiliary type Smad proteins into complex one. This in turn interacts with transcription factors in the nucleus, promoting proliferation of myocardial fibroblasts and hypertrophy of myocytes.\(^14\) In the cardiovascular system, TGF-β1 can promote synthesis of the extracellular matrix and is considered as one of the most important pro-myocardial fibrosis factors, and is closely related to myocardial fibrosis in CHF.\(^15\)

Studies have shown that TGF-β1 is a bidirectional regulator of myocardial cells. TGF-β1 protects myocardial cells and promotes their repair, maintaining normal activity of myocardial structure and function. However, overexpression of TGF-β1 may lead to deposition of the extracellular matrix and aggravate myocardial fibrosis and remodeling.\(^16\) BMP is a member of the TGF-β superfamily, and it plays an important role in differentiation of stem cells into cardiac stem cells, as well as in different stages and locations in cardiovascular system development.\(^17\) In this study, upregulation of TGF-β1 and BMP-15 protein suggested that the TGF-β1 signaling pathway might be activated in these CHF patients with syndrome of Qi deficiency and blood stasis. This change may be attributed to myocardial fibrosis in the process of CHF. The underlying mechanism for syndrome of Qi deficiency and blood stasis to mediate upregulation of TGF-β1 protein requires further investigation. However, upregulated TGF-β1 protein might be a useful biomarker for predicting progression of CHF.

In summary, our study shows that occurrence and development of CHF with syndrome of Qi deficiency and blood stasis might result from certain differentially expressed proteins that participate in several signaling pathways. Additionally, tongue coating liquid contains information of CHF. Upregulation of TGF-β1 protein is closely associated with CHF; and TGF-β1 protein might act as a candidate marker for predicting the risk of Qi-deficiency-blood-stasis syndrome in CHF.

REFERENCES