A network study of Chinese medicine Xuesaitong Injection to elucidate a complex mode-of-action with multi-compound, multi-target and multi-pathway

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Abstract

Chinese medicine has evolved from thousands of years of empirical applications and experiences of combating diseases. It has become widely recognized that the Chinese medicine acts through complex mechanisms featured as multi-compound, multi-target and multi-pathway. However, there is still a lack of systematic experimental studies to elucidate the mechanisms of Chinese medicine. In this study, the differentially expressed genes (DEGs) were identified from myocardial infarction rat model treated with Xuesaitong Injection (XST), a Chinese medicine consisting of the total saponins from Panax Notoginseng (Burk.) F. H. Chen (Chinese Sanqi). A network based approach was developed to combine cardiovascular diseases (CVD) related DEGs with evidences from both literature mining and our experiments to investigate the mechanism of action (MOA) of XST on anti-myocardial infarction. A compound-target-pathway network of XST was constructed by connecting compounds to DEGs validated with literature evidences and the pathways that are functionally enriched. 70 potential targets of XST were identified in this study, of which 32 were experimentally validated either by our in vitro assays or by CVD-related literatures. This study provided for first time a network view on the complex MOA of Chinese medicine on anti-myocardial infarction through multiple compounds, multiple targets and pathways.
1. Introduction

Chinese medicine has evolved from thousands of years of empirical applications and experiences of combating diseases. It has been profoundly influencing the life and healthcare of Chinese throughout the history [1, 2]. Chinese medicine is becoming more widely used for preventing and curing the disease clinically and improving healthcare nutritionally [3, 4]. Much progress has been made in better understanding the chemical constituents of Chinese medicine and their therapeutic mechanisms during the last few decades [5]. Chemical constituents, especially the major constituents of some Chinese medicines have been separated and identified. Chinese medicine is usually a multi-component system whether it is an herb or a formulae consisting of several types of medicinal herbs or minerals [3, 6, 7]. The therapeutic effects of Chinese medicine rely mostly on the composition and content of the effective constituents [8]. But the effect and functional mechanism of these effective substances in the therapy of Chinese medicine is oftentimes unclear or not fully understood.

It has become more recognized that Chinese medicine produces the healing efficacy in a more holistic way [9-11]. However researchers typically focus on the mechanism of either the whole formulae or a few representative components (not necessarily effective ingredients) in single pathological model or mechanism. It is difficult to study the concrete mechanism of a whole formula as it is a mixture, while in the latter case the highly dynamic interaction between ingredients is missing. A few existing studies of multi-target property of Chinese medicine are limited to either computational predictions [12] or mechanism of the whole formulae [13].

With the finishing of human genome sequencing and development of various omics technologies, genome wide profiling has enabled systems level investigation of the mechanisms of actions (MOA) of Chinese medicine [8, 14]. Network Pharmacology aims at understanding the effect of drugs in biological system in a holistic manner providing new perspectives in understanding the complex interactions between drug components and biological molecules [15-17]. A combination of these methods may
open up new avenues for uncovering the molecular mechanisms underlying the therapeutic efficacy of Chinese medicine in the context of biological networks[18]. Xuesaitong Injection (XST) is one of the best selling prescription Chinese medicine in China [19]. It is a preparation consisting of the total saponins extracted from *Panax Notoginseng* (Burk.) F. H. Chen (Chinese Sanqi). There is a wide range of clinical efficacy of XST being extensively used for the treatment of cardio-cerebrovascular diseases such as myocardial infarction, cerebral infarction, thrombosis, coronary heart disease in China [20-27]. Notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd and ginsenoside Re have been found to be the major effective ingredients in our previous study. However their potential targets and the molecular regulatory mechanisms remain to be systematically elucidated.

In this study, we developed a network based method combining differential gene expression analysis, literature evidences as well as experimental validations to study the multi-component, multi-target, multi-pathway mechanism of XST on anti-myocardial infarction (MI). As shown in Figure 1, the genes involved in anti-MI mechanism of XST were first detected with microarray gene expression analysis. Their associations with cardiovascular diseases (CVD) were evaluated based upon information in Rat Genome Database (RGD) [28]. Considering the amount of studies on major ingredients of XST, we also collected the target information of the five major ingredients in literatures manually. If a CVD associated and differentially expressed gene is also found to be influenced directly by a major ingredient of XST in literature, it is then considered as a potential target of the compound and XST in this study. Furthermore, some of the potential targets were also experimentally validated. Finally we constructed the compound-target-pathway network on anti-MI of XST to illustrate its multi-compound, multi-target, multi-pathway mechanism.

2. Materials and Methods

2.1. Chemicals and reagents
Chloral hydrate was purchased from Tianjin Kemiou Chemical Reagent Co. (Tianjin, China). The XST lyophilized powder, one of the major types of XST Injection in
clinical practices (Batch No. s120425-1) was manufactured by Heilongjiang Zhenbaodao pharmaceutical Co. Ltd. (Heilongjiang, China). Ginsenoside Rg1 (Batch No. W13-5-1) was purchased from Zhongxin Innova Laboratories (Tianjin, China). Ginsenoside Rb1 (Batch No. 120420), notoginsenoside R1 (Batch No. 120325), ginsenoside Rd (Batch No. 120507) and ginsenoside Re (Batch No. 120510) were purchased from Ronghe Pharmaceutical Technology Development Co. Ltd. (Shanghai, China). Lipopolysaccharide (LPS) and Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The primary antibody for iNOS was obtained from Abcam (Cambridge, UK), β-actin was obtained from Beyotime Institute of Biotechnology (Jiangsu, China) and all the secondary antibodies were obtained from Shuji biotechnology (Shanghai, China). Penicillin and streptomycin were purchased from BIO BASIC INC (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and 0.25% trypsin-EDTA for the cell culture were purchased from GIBCO (USA). RIPA lysate and PMSF was obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets were purchased from Roche Diagnostic GmbH (Mannheim, Germany).

2.2. Genome-wide transcriptomic experiment

2.2.1. Myocardial infarction rat model

Male Sprague-Dawley rats (230-295g) used in this experiment were purchased from Weitong-Lihua Experimental Animal Co. Ltd. (Beijing, China). Myocardial infarction was produced by occlusion of the left anterior descending coronary artery. Rats were randomly assigned to three groups: control group (the ligation suture was placed in the heart, but without ligation), myocardial infarction group (MI), XST treatment group (MI +XST). 5% ethanol-saline solutions (v/v) of XST (150 mg /kg body wt) were given to XST treatment group by intravenous injection once daily and consecutively for 7 days, respectively. 5% ethanol-saline solutions (v/v) were given to control group and MI group. The administration procedure for rats in this study is in accordance with clinical use. On the eighth day, rats were anaesthetized by intraperitoneal injection of chloral hydrate (12%, 360 mg/kg body wt). Then the
infarcted zone in rat heart was collected and stored in liquid nitrogen.

2.2.2. Microarray experiment

Total RNA was extracted using TRIZOL Reagent (Life technologies, Carlsbad, CA, US) following the manufacturer’s instructions and amplified, labeled and purified using GeneChip 3′IVT Express Kit (Affymetrix, Santa Clara, CA, US) following manufacturer’s instructions to obtain biotin labeled cRNA. Array hybridization and wash was performed using GeneChip® Hybridization, Wash and Stain Kit (Affymetrix, Santa Clara, CA, US) in Hybridization Oven 645 (Affymetrix, Santa Clara, CA, US) and Fluidics Station 450 (Affymetrix, Santa Clara, CA, US) following manufacturer’s instructions. Slides were scanned by GeneChip® Scanner 3000 (Affymetrix, Santa Clara, CA, US) and Command Console Software 3.1 (Affymetrix, Santa Clara, CA, US) with default settings. Raw data were stored in ArrayTrack 3.5.0 [29], a java-based microarray analysis tool developed by US FDA.

2.2.3. Gene expression data analysis

Global scaling normalization was performed with Median Scaling Normalization in ArrayTrack 3.5.0 using a target median value of 1000. Genes in RGD associated with CVD were selected for further analysis since we focused on the effect of XST on anti-rat cardiac ischemic injury. Reverse rate (RR) and fold change (FC) were applied to select the differentially expressed genes (DEGs) using a RR > 0.5 and a FC threshold of 1.1. RR was calculated with Eq (1) to evaluate the effect of XST in reversing the changes of gene expression induced by MI modeling

\[
RR = \frac{M_i - X_i}{N_i - C_i}
\]  

(1)

Where \(C_i\), \(M_i\), \(X_i\) are the average expression of gene i in control group, MI group, XST treatment group, respectively.

2.2.4. Verification with literature evidence

The DEGs were differentially expressed due to the whole formulae of XST, and there is a lack of evidence to explain the multi-compound, multi-target action of XST. We verified the DEGs through mining existing literatures manually. We downloaded and read abstract of all articles related to notoginsenoside R1, ginsenoside Rg1,
ginsenoside Rb1, ginsenoside Rd and ginsenoside Re in PUBMED (as of April 10, 2013). If a selected DEG gene is found directly affected by a certain ingredient in literature, it was considered as a potential target of the ingredient and XST. The frequency it appears and its CVD relevancy was recorded. The detailed information of literatures used in this study is listed in supplemental Table S1.

2.3. Experimental validation

The literature information was collected from various sources with some reported in diseases other than CVD. Thus we are interested in validating these results in CVD with in vitro experiments.

2.3.1. Cell culture

RAW 264.7 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured at 37 °C in 5% CO2 in DMEM containing 10% heat-inactivated FBS, 50 U/mL penicillin, and 50 μg/ml streptomycin. In all experiments, cells were grown to 80–90% confluency and subjected to no more than 15 cell passages.

2.3.2. Western blot analysis

The RAW 264.7 cells were plated in 60 mm culture dishes (2×10^6 cells). Twenty four hours later, cells were incubated with LPS (200ng/ml) and 50μM different ingredients of XST or different concentrations of ginsenoside Rd (1, 10, 25, 50μM). In our experiments, all ingredients were dissolved in DMSO as 100 mM stocks. LPS were dissolved in sterile water at 1 mg/ml. All final cell-culture volumes were 5.0 mL and the cells were incubated at 37 °C for twenty four hours after addition of stimulus. The cells were lysed on ice for 10 min in RIPA lysate with 1 mM PMSF and protease inhibitor and phosphatase inhibitor. The cell lysate solutions were transferred into 1.5 mL polypropylene tubes and the samples centrifuged for 10min at 12000 rpm at 4 °C. Cell lysate proteins were quantified with the BCA assay (Beyotime Institute of Biotechnology, Jiangsu, China). 20μl protein solutions from each culture were electrophoresed into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred onto PVDF membranes by iBlot Western Blotting System (Invitrogen). The β-actin content of each sample was
determined to ensure equal protein loading. The membrane was blocked with 5% skim milk solution in tris-buffered saline (150mM NaCl, 10 mM Tris-HCl, pH 7.4) with 0.1% Tween 20 (TBST) buffer for 1.5 hour at room temperature (RT). After blocking, the membrane was incubated with primary antibodies against iNOS (1: 200 diluted in TBST containing 5% skim milk) and β-actin (1: 1000 diluted in TBST containing 5% skim milk) for 3 hours at RT or overnight at 4 °C. The membrane was then washed with TBST and incubated with anti-rabbit (iNOS) or anti-mouse (β-actin) horseradish peroxidase (HRP)-conjugated immunoglobulin G secondary antibodies (1: 5000 diluted in TBST containing 5% skim milk) for 1.5 hours at RT. The specific proteins were detected using a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific Inc., Bremen, Germany). Digital images were collected using a Bio-Rad Universal Hood II gel documentation system and the quantitation of protein was evaluated with Quantity One software (Bio-Rad).

2.4. Network construction and network analysis
The associated targets of XST and the individual ingredients were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [30] enrichment analysis using ArrayTrack v.3.5.0. The pathways closely related to MI or CVD were selected for further analysis. Based on these results, we constructed the compound-target-pathway network and compound-pathway network using Cytoscape version 2.8.2 [31]. If the interaction between a compound and a target was described in more than one CVD-related literature (including one), the connection between the component and target was marked as solid line. Otherwise it was marked as a dotted line. The thickness of the lines was proportional to the numbers of related literatures.

3. Results and discussion
571 genes (721 probe sets) in RGD database were found differentially expressed in this study with RR > 0.5 and FC > 1.1 after the treatment of XST. The gene list can be found in supplementary Table S2. These genes were treated as the potential targets of XST on anti-MI associated with CVD. The genes were further filtered with literature evidences. As a result, 70 potential targets were affected by at least one
compound as found in literature (supplementary Table S3). Among them, TNF-α and iNOS were influenced by all the five ingredients and associated with anti-inflammatory activity. IκB, eNOS, caspase-3, JNK, IL-4, SOD, IL-1beta, COX-2 were affected by four compounds. Eight targets were affected by three compounds and thirteen targets were influenced by two compounds. The remaining thirty nine were affected by only one compound.

Among the 70 targets of XST, iNOS was affected by all five compounds with four of them reported in non-cardiovascular diseases. We selected iNOS as an example to validate its involvement in the mechanism of XST treating MI. LPS-stimulated RAW264.7 macrophage cell is a commonly used cell model of inflammation, which produces numerous pro-inflammatory mediators and cytokines and significantly promotes the expression of iNOS protein upon induction. The effects of ginsenoside Rg1, ginsenoside Rb1, notoginsenoside R1, ginsenoside Rd and ginsenoside Re on iNOS protein expression in RAW264.7 cells were examined with Western blot analysis. As shown in Figure 2, the expression of iNOS protein was significantly up-regulated in response to LPS (200ng/ml), ginsenoside Rb1, Rd and notoginsenoside R1 showed significant effects in inhibiting its expression (P<0.05) and ginsenoside Rg1 and Re also showed a decreasing tendency. Furthermore, we studied the dose dependent effect of ginsenoside Rd on iNOS which has not been reported before in CVD related literatures. We found that ginsenoside Rd dose-dependently down-regulated the expression of iNOS as shown in Figure 2. These results indicated a novel compound-target interaction between ginsenoside Rd and iNOS in CVD. Therefore combining evidences from microarray gene expression analysis and literature survey provided an effective way for finding novel compound-target connections.

Based on the results of gene chip and literatures, we generated the compound-target-pathway network (Figure 3) which was consisted of 93 nodes (5 compounds, 70 targets and 18 pathways) and 238 edges, and compound-pathway network (Figure 4) which was consisted of 22 nodes (5 compounds and 17 pathways) and 55 edges. Among the eighteen KEGG pathways enriched within all targets of
XST, apoptosis and p53 signaling pathway is a critical pathway in regulating cell death. It is known in literature that p53 signaling pathway and apoptosis pathway mediates cardiomyocyte apoptosis and plays a decisive role in the progression of pathological remodeling and heart failure following MI [32, 33]. In addition, many inflammatory response related pathways were also enriched, including complement and coagulation cascades, Fc epsilon RI signaling pathway, hematopoietic cell lineage, leukocyte transendothelial migration, NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway, Toll-like receptor signaling pathway. It suggested that XST modulates inflammatory responses in treating ischemia, which has been found preventing damages of the inflammatory factors to cardiac cells [34-37]. The influencing in complement and coagulation cascades pathway indicated that XST and its constituent compound notoginsenoside R1 are functional in modulating the coagulation process in MI, which is one of the most common clinical risk factors [32-35].

Focal adhesion, tight junction and adherens junction pathway plays important roles in maintaining tissue architecture, cell polarity, cell proliferation and cell death. It may affect the pathologic remodeling of cardiovascular tissues in myocardial infarction [38-40]. Interestingly, adherens junction was not influenced by any component individually but enriched by the all targets in combination. It suggests that XST might modulate adherens junction pathway through a synergistic action, a typical phenomenon of Chinese medicine.

ErbB signaling pathway, MAPK signaling pathway, VEGF signaling pathway, Wnt signaling pathway may exert pleiotropic effects on cardiovascular cells, regulating cell growth, fibrosis and inflammation [41-46]. Adipocytokine signaling pathway, Insulin signaling pathway are two pathways involved in energy metabolism such as fatty acid oxidation and glucose uptake, glycogen synthesis, the influence of which could regulate the energy supply in cardiac ischemia. Ginsenoside Rg1 is likely the most important one due to its comprehensive involvement in modulating various targets and pathways.

As shown in Table 1, there were a number of targets influenced by XST and its
constituent compounds were not confirmed in CVD-related literatures. They can serve as targets of priority for experimental validation in the future, and iNOS is such an example validated in this study.

4. Conclusion
In this study, we presented a network-based approach to illustrate the multi-compound, multi-target and multi-pathway mechanism of Chinese medicine. In a case study of XST, for the first time, we dissect a complex MOA of anti-myocardial infarction through multiple compounds, multiple targets and pathways with sound experimental evidences. The compound-target-pathway network was constructed to illustrate its multi-compound, multi-target and multi-pathway regulatory mechanism on anti-MI. 70 potential targets of XST, of which 32 were experimentally validated either by our in vitro assays or by CVD-related literatures, were identified in this study. Specifically, ginsenoside Rb1, Rd and notoginsenoside R1 were experimentally validated to exert repressive regulatory effects on iNOS in CVD specific context indicating their anti-inflammatory roles. The dose dependent relation between ginsenoside Rd and iNOS was also validated in this study. Our results indicated that the major ingredients of XST might modulate numerous different targets and pathways involved in inflammation, adhesion, cell proliferation, apoptosis, and energy supply.

Conflict of Interests
The authors claim no conflict of interests.

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with rat experiment and also appreciate Dr. Huasheng Xiao (National Engineering Center for Biochip at Shanghai, China) for his help with Microarray experiment.
References:


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Figure legend

Figure 1: A framework of network pharmacology study of Chinese medicine. The genes involved in anti-MI mechanism of XST were first detected with microarray gene expression analysis and Rat Genome Database (RGD). The target information of the major ingredients was collected from literatures manually and validated experimentally in vitro. The compound-target-pathway network and compound-pathway network on anti-MI of XST were constructed to illustrate the multi-compound, multi-target and multi-pathway mechanism of XST.

Figure 2: The effect of ingredients of XST on the protein expression of iNOS in LPS-stimulated RAW 264.7 cells. (A) The cells were incubated with LPS (200ng/ml) and ginsenoside Rg1, ginsenoside Rb1, notoginsenoside R1, ginsenoside Rd and ginsenoside Re at 50 μM for twenty-four hours. (B) Cells were incubated with LPS (200ng/ml) and indicated concentrations of ginsenoside Rd for twenty-four hours. The whole cell extracts were prepared and the expression level of iNOS was determined by Western blot analysis. The values are expressed as the mean±S.D. from three independent experiments. Statistical significance: *p<0.05 and **p<0.01 vs. LPS-stimulated cells and # p<0.05 and ##p<0.01 vs. control (non-stimulated cells).

Figure 3: The compound-target-pathway network of XST. The hexagon nodes represent the compounds; the circular nodes represent the targets and the rounded square nodes represent the pathways. The node size was proportional to the number of interactions between nodes. The line width was proportional to the number of related literatures.

Figure 4: The compound-pathway network of XST. The hexagon nodes represent the compounds. Pathways in different classes were differently colored and shaped. The node size was proportional to the number of interactions between nodes.
Figures

Figure 1. A framework of network pharmacology study of Chinese medicine.
Figure 2. The effect of ingredients of XST on the protein expression of iNOS in LPS-stimulated RAW 264.7 cells. The values shown are means±SD of three tests.
Figure 3 Compound-target-pathway network

- **Compounds**: Represented by orange circles.
- **Pathways**: Represented by green circles.
- **Targets confirmed in CVD related literatures**: Represented by cyan circles.
- **Targets confirmed in non CVD related literatures**: Represented by green circles with a white outline.
- **Interactions in CVD related literatures**: Represented by solid lines.
- **Interactions in non CVD related literatures**: Represented by dashed lines.
Figure 4 Compound-pathway network
### Tables

Table 1. The number of targets and pathways of XST and the major ingredients. The pathways represent the number of CVD-related pathways enriched by the all targets of XST or the major ingredients.

<table>
<thead>
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<th>Targets</th>
<th>Confirmed in All literatures</th>
<th>Confirmed in CVD-related literatures</th>
<th>Confirmed in non CVD-related literatures</th>
<th>Pathways</th>
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