Network Pharmacology-Based Prediction of Mechanism of Shenzhuo Formula for Application to DN

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ABSTRACT

Background: Shenzhuo formula is a traditional Chinese medicine (TCM) prescription which has significant therapeutic effects on diabetic nephropathy (DN). However, its mechanism remains unknown. Therefore, this study aimed to explore the underlying anti-DN mechanism of shenzhuo formula.

Methods: The active ingredients and targets of shenzhuo formula were obtained by searching TCMSP, TCMID, SwissTargetPrediction and HIT. The DN target was identified from TTD, DrugBank and DisGeNet. The potential targets were obtained and PPI network were built after mapping the disease and drug targets. The key targets were screened out by network topology and the “drugs - DN - key targets” network was constructed by Cytoscape. GO analysis and KEGG pathway enrichment analysis were performed using DAVID, and the results were visualized using the Omicshare Tools.

Results: We obtained 182 potential targets and 30 key targets. Ulteriorly, “drugs - DN - key targets” network were constructed, and results showed that nodes like M51, M21, M5, M71, M28, EGFR, MMP9, MAPK8, PIK3CA and STAT3 had a higher degree. GO analysis results mainly involved in positive regulation of transcription from RNA polymerase II promoter, inflammatory response, lipopolysaccharide-mediated signaling pathway and other biological processes. The results of KEGG showed that DN-related pathways like TNF signaling pathway, PI3K-Akt signaling pathway were at the top of the list.

Conclusion: This article reveals the possible mechanism of shenzhuo formula in the treatment of DN through network pharmacology research, and lays a foundation for further studies.

Keywords: Network pharmacology, Chinese medicine, Shenzhuo formula, Diabetic nephropathy, Mechanism
1. Introduction

Diabetic nephropathy (DN) is one of the most common chronic microvascular complications of diabetes. It may be caused and shaped by the interaction of many factors such as endoplasmic reticulum dysfunction, high sugar-mediated generation of terminal advanced glycation endproducts (AGE), increased activation of the renin angiotensin aldosterone system, increased generation of reactive oxygen species (ROS), and activation of extracellular matrix (ECM) and protein kinase C[1-2]. It is reported that the incidence of DN is about 40% in the diabetic population[3]. Furthermore, with the increasing incidence of diabetes, the incidence of DN is increasing yearly[4]. Therefore, it is important to intensify studies of the pathogenesis of DN and the search for effective intervention targets.

Shenzhuo formula as a traditional Chinese medicine (TCM) prescription has certain advantages in the treatment of DN[5]. It is created by Tong Xiaolin, an academician at the Chinese Academy of Sciences, and his research team. This formula was based on the pathogenesis of qi deficiency blood stasis, and the classic prescription of Didang decoction. Years of clinical practice have shown the effectiveness of shenzhuo formula where it can increase the glomerular filtration rate, reduce 24-hour urinary protein and kidney damage, and reverse kidney disease when used early[5-6]. However, due to the diversity of TCM compounds and complexity of in vivo processes, the systematic mechanism research of shenzhuo formula has been hindered.

Recently, network pharmacology aiming to predict pharmacological mechanisms has been developed rapidly with the use of multiomics, high-throughput screening, network visualization and analysis, or other techniques. It intuitively reveals the network structure of drug action[7], providing possibilities for exploring the mechanism of action of TCM compounds. Therefore, we planned to adopt network pharmacology
method to preliminarily explore the mechanism of shenzhuo formula in preventing DN.

2. Methods

2.1. Research tools

The Chinese Traditional Medicine System Pharmacological Database Analysis Platform (TCMSP, http://lsp.nwu.edu.cn/tcmsp.php)[8], Traditional Chinese Medicine Integrated Database (TCMID, http://www.megabionet.org/tcmid/)[9], SwissTargetPrediction (http://www.swisstargetprediction.ch/)[10] and HIT (http:lifecenter.biosino.org/hit/)[11] were used to access to shenzhuo formula ingredients, targets. (2) The Therapeutic Target Database (TTD, http://bidd.nus.edu.sg/group/cjttd/)[12], DrugBank (https://www.drugbank.ca/)[13], and DisGeNet (http://www.disgenet.org/)[14] were used to get the target protein of DN. (3) The protein-protein interaction (PPI) network was obtained online using STRING (http://string-db.org)[15]. Compositional software Cytoscape 3.2.1 (http://www.cytoscape.org/)[16] was used to carry out network topology analysis and construct drug - DN - key target networks. The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.ncifcrf.Gov)[17] was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The Omicshare Tools (https://www.Omicshare.com/) were used for visual analysis of GO and KEGG results.

2.2 Collection of major chemical constituents

This study relies on TCMSP, TCMID database and literatures mining to search for the chemical constituents of shenzhuo formula (Hedysarum Multijugum Maxim, Radix Salviae, Hirudo and Radix Rhei Et Rhizome).

2.3. Screening of active compounds

As we all know, TCM drugs reach into human body and take effect through absorption, distribution, metabolism, and excretion (ADME) processes. Among them,
Oral bioavailability (OB) and drug similarity (DL), the key parameters of ADME components, were used as the screening criteria for active ingredients in this study. In this section, we operated it with TCMSP for which the ADME properties of each active molecule were collected in that data platform. And then the chemical constituents that meet the requirements of OB ≥ 30%, DL ≥ 0.18 were selected as potential active ingredients.

2.4. Prediction of targets

The SwissTargetPrediction and HIT web tools were used to collect the drug targets. In addition, TTD, DrugBank and DisGeNet databases were used to search for DN targets by entering the key words of diabetic kidney disease and diabetic nephropathy. Further, we matched the drug and DN targets to obtain the target database of "shenzhuo formula - DN - target".

2.5. Network construction and analysis

Based on the "shenzhuo formula - DN - target" database, the PPI network was obtained online using STRING. Furthermore, The PPI network topology analysis was carried out using Cytoscape 3.2.1 software and then key targets were obtained. To explore the sophisticated interactions between the active ingredients and their related targets at a system level, a “drugs - DN - key targets” network was constructed by Cytoscape3.2.1 software.

2.6. GO and KEGG analysis

The GO is widely used for gene function classification in the field of biology and mainly describes the molecular function (MF), biological processes (BP), and cellular components (CC) of genes. In this step, we use the DAVID tool for GO and KEGG pathway analysis. Then we used Omicshare Tools for visual display.
3. Results

3.1. Screening of candidate components in shenzhuo formula

Through TCMSP, TCMID database, 87 species of compound Hedysarum Multijugum Maxim, 210 species of Radix Salviae, 35 species of Hirudo, 92 species of Radix Rhei Et Rhizome were obtained. Then the collected chemical constituents were calculated by ADME (OB≥30%, DL≥0.18) for further screening of active constituents (because the Hirudo could not be queried in TCMSP database, its ADME parameters could not obtained). After screening, one hundred and one active molecules were found, respectively 20 Hedysarum Multijugum Maxim, 65 in Radix Salviae, 16 in Radix Rhei Et Rhizome. In addition, through literature mining, another 4 active molecules were obtained, respectively 2 in Hedysarum Multijugum Maxim[18-19], 1 in Radix Salviae[20], 1 in Radix Rhei Et Rhizome[21].

3.2. Target prediction

Matching the targets of shenzhuo formula with 567 DN genes, a total of 182 potential targets of shenzhuo formula were obtained (Table 1).

Table 1
Potential anti-DN effects on gene targets of active component of shenzhuo formula (50 of 182 targets)

| Serial number | Target                                               | Common name          | Uniprot ID |
|---------------|------------------------------------------------------|----------------------|------------|
| 1             | Aldose reductase                                      | AKR1B1               | P15121     |
| 2             | Acyl coenzyme A:cholesterol acyltransferase          | CES1                 | P23141     |
| 3             | Signal transducer and activator of transcription 3   | STAT3                | P40763     |
| 4             | Protein-tyrosine phosphatase 1C                       | PTPN6                | P29350     |
| 5             | Vascular endothelial growth factor receptor 2        | KDR                  | P35968     |
| 6             | Epidermal growth factor receptor erbB1               | EGFR                 | P00533     |
| 7             | PI3-kinase p110-alpha subunit                        | PIK3CA               | P42336     |
| 8             | c-Jun N-terminal kinase 1                            | MAPK8                | P45983     |
| 9             | LXR-alpha                                            | NR1H3                | Q13133     |
| 10            | Estrogen receptor alpha                              | ESR1                 | P03372     |
| 11            | Testis-specific androgen-binding protein             | SHBG                 | P04278     |
|   | Protein Name                                      | Gene Symbol | GeneID |
|---|--------------------------------------------------|-------------|--------|
|12 | Cytochrome P450 2C19                             | CYP2C19     | P33261 |
|13 | Protein-tyrosine phosphatase 1B                  | PTPN1       | P18031 |
|14 | Butyrylcholinesterase                           | BCHE        | P06276 |
|15 | Vitamin D receptor                               | VDR         | P11473 |
|16 | Glucose-6-phosphate 1-dehydrogenase             | G6PD        | P11413 |
|17 | Peroxisome proliferator-activated receptor alpha | PPARA       | Q07869 |
|18 | Peroxisome proliferator-activated receptor delta | PPARD       | Q03181 |
|19 | Peroxisome proliferator-activated receptor gamma | PPARG       | P37231 |
|20 | UDP-glucuronosyltransferase 2B7                  | UGT2B7      | P16662 |
|21 | 11-beta-hydroxysteroid dehydrogenase 2          | HSD11B2     | P80365 |
|22 | NADPH oxidase 4                                  | NOX4        | Q9NPH5 |
|23 | Tyrosine-protein kinase SYK                      | SYK         | P43405 |
|24 | Glycogen synthase kinase-3 beta                 | GSK3B       | P49841 |
|25 | Matrix metalloproteinase 9                       | MMP9        | P14780 |
|26 | Matrix metalloproteinase 2                       | MMP2        | P08253 |
|27 | Matrix metalloproteinase 12                      | MMP12       | P39900 |
|28 | ATP-binding cassette sub-family G member 2       | ABCG2       | Q9UNQ0 |
|29 | P-glycoprotein 1                                 | ABCB1       | P08183 |
|30 | Arachidonate 12-lipoxygenase                     | ALOX12      | P18054 |
|31 | Cyclooxygenase-2                                 | PTGS2       | P35354 |
|32 | Insulin-like growth factor I receptor            | IGF1R       | P08069 |
|33 | Myeloperoxidase                                 | MPO         | P05164 |
|34 | Matrix metalloproteinase 3                       | MMP3        | P08254 |
|35 | Serine/threonine-protein kinase AKT              | AKT1        | P31749 |
|36 | Beta-secretase 1                                 | BACE1       | P56817 |
|37 | Tyrosine-protein kinase receptor UFO             | AXL         | P30530 |
|38 | NUAK family SNF1-like kinase 1                  | NUAK1       | O60285 |
|39 | Aldehyde reductase                              | AKR1A1      | P14550 |
|40 | Plasminogen                                     | PLG         | P00747 |
|41 | PI3-kinase p110-delta subunit                    | PIK3CD      | O00329 |
|42 | PI3-kinase p110-gamma subunit                    | PIK3CG      | P48736 |
|43 | Hematopoietic prostaglandin D synthase          | HPGDS       | O60760 |
|44 | Serine-protein kinase ATM                        | ATM         | Q13315 |
|45 | Cytochrome P450 24A1                             | CYP24A1     | Q07973 |
|46 | Mineralocorticoid receptor                       | NR3C2       | P08235 |
|47 | Cannabinoid receptor 1                          | CNR1        | P21554 |
|48 | Hepatocyte nuclear factor 4-alpha               | HNF4A       | P41235 |
|49 | C-C chemokine receptor type 1                   | CCR1        | P32246 |
|50 | Histone-lysine N-methyltransferase EZH2         | EZH2        | Q15910 |

Note: organism: *Homo sapiens*. Only 50 potential targets information was shown here, and the whole was in the appendix A.
3.3. Construction and analysis of network maps

The PPI network of the 182 potential targets was obtained online using STRING (Fig. 1). Then we used Cytoscape 3.2.1 to obtained 30 key targets by network topology analysis with the inclusion criteria of “degree≥2 times of the median, closeness centrality≥median, betweenes centrality≥median” (Table 2). Next we constructed a “drugs - DN - key targets” network by Cytoscape 3.2.1 software (Fig. 2).

![Fig. 1. PPI network of the 182 potential targets](image)

| serial number | node | Degree | Closeness centrality | Betweenes centrality |
|---------------|------|--------|----------------------|----------------------|
|               |      |        |                      |                      |
|   | Gene   | Total Count | Log2 Fold Change | p Value |
|---|--------|-------------|------------------|---------|
| 1 | PIK3CA | 40          | 0.49508197       | 0.09370214 |
| 2 | STAT3  | 40          | 0.5              | 0.0863086  |
| 3 | AKT1   | 35          | 0.49025974       | 0.15311921 |
| 4 | KNG1   | 33          | 0.44023324       | 0.06128185 |
| 5 | VEGFA  | 33          | 0.49185668       | 0.06953442 |
| 6 | JUN    | 32          | 0.48089172       | 0.07229449 |
| 7 | MAPK3  | 30          | 0.4617737        | 0.02240476 |
| 8 | MAPK1  | 30          | 0.4689441        | 0.06714477 |
| 9 | EGF    | 27          | 0.4617737        | 0.0336672  |
| 10| EDN1   | 27          | 0.46604938       | 0.05180077 |
| 11| EGFR   | 26          | 0.44023324       | 0.02254905 |
| 12| JAK1   | 26          | 0.44940476       | 0.02254905 |
| 13| IL6    | 26          | 0.45209581       | 0.02532622 |
| 14| CXCL8  | 25          | 0.43768116       | 0.03191743 |
| 15| RELA   | 24          | 0.45757576       | 0.04035241 |
| 16| FN1    | 23          | 0.4351585        | 0.01464828 |
| 17| JAK2   | 23          | 0.44940476       | 0.01620852 |
| 18| CTNNB1 | 23          | 0.45481928       | 0.06488997 |
| 19| TNF    | 22          | 0.44281525       | 0.0272631  |
| 20| TGFB1  | 21          | 0.44281525       | 0.03270724 |
| 21| MMP9   | 20          | 0.40921409       | 0.03200512 |
| 22| CXCR4  | 19          | 0.41032609       | 0.01402652 |
| 23| TIMP1  | 19          | 0.41712707       | 0.00798146 |
| 24| MAPK14 | 19          | 0.44411765       | 0.01628416 |
| 25| BDKRB1 | 19          | 0.3994709        | 0.00725308 |
| 26| PIK3CB | 18          | 0.40921409       | 0.00732155 |
| 27| MAPK8  | 18          | 0.42296919       | 0.03099697 |
| 28| ITGB3  | 18          | 0.42296919       | 0.01050834 |
| 29| CCR5   | 16          | 0.39841689       | 0.00592392 |
| 30| PLG    | 16          | 0.40266667       | 0.02168017 |
Fig. 2. “Drugs - DN - key targets” network. The nodes were visualized with degree. The larger and the redder the node, the higher the degree it was. M1-75 stand for the active ingredients which specific names were shown at appendix B.

3.4. GO and KEGG analysis

The DAVID tool was used to do the GO analysis. And the GO terms were constructed by the Omicshare Tools (Fig. 3). The GO analysis results showed that the targets were mainly involved in positive regulation of transcription from RNA polymerase II promoter, inflammatory response, lipopolysaccharide-mediated signaling pathway, positive regulation of peptidyl-serine phosphorylation and other biological processes. As the top 20 GO enrichment items listed, suggesting that DN is relevent to scores of BP in body abnormalities, shenzhuo formula is likely to regulate these items and then play an anti-DN role.
Fig. 3. Top 20 enrichments in GO analysis

The DAVID online tool was used to conduct the KEGG pathway enrichment analysis of the screened 30 key target proteins, and a total of 104 enrichment results were obtained. Fig. 4 is the top 20 enrichment analysis of KEGG pathway for predicting the anti-DN effect of shenzhuo formula. It showed that 20 targets of key targets were involved in the Pathways in cancer (20/30, 66.7%), 15 targets were involved in Hepatitis B (15/30, 50.0%), 15 targets were involved in Influenza A (15/30, 50.0%), 15 targets involved in Proteoglycans in cancer (15/30, 50.0%), 13 targets involved in TNF signaling pathway (13/30, 43.3%) and 13 targets involved in PI3K-Akt signaling pathway (13/30, 43.3%).
4. Discussion

Previous studies have suggested that shenzhuo formula has a good therapeutic effect on DN. However, the potential mechanisms of shenzhuo formula treating in DN have not been fully explained. In this study, we mainly applied network pharmacology method to explore it. To be more specific, 140 potential active compounds and their related 182 potential targets were obtained after pharmacokinetic screening and DN-related target mapping, which will be contribute to the further research of this formula. Then, we constructed two networks, including the PPI of 182 potential targets network.

Fig. 4. Top 20 KEGG pathway enrichments
and drug - DN - key target network, and applied GO and KEGG enrichment analysis to explore the regulation of shenzhuo formula for treating DN.

Through the drug - DN - key target network, we can know that most ingredients are linked to no less than one target, which tells us that TCM ingredients have a property of multitargets. The result of the drug - DN - key target network analysis has suggested that different active compounds from different herbs can act on the same one target, which might demonstrate that shenzhuo formula has a synergistic effect during treating DN. In addition, the results of the drugs - DN - targets network topology analysis showed that there are 8 active ingredients which degrees were greater than 2 times of the average. Interestingly, 3 of these have been experimentally proven to have kidney protection effect. For example, quercetin liposomes has renal protective effects by reducing oxidative stress, attenuating AGE expression, and delaying the progression of DN[22]. Luteolin attenuates DN mainly via suppression of inflammatory response and oxidative response[23]. Ursolic acid alleviated renal damage in type 2 diabetic db/db mice by downregulating proteins in the angiotensin II type 1 receptor-associated protein / angiotensin II type 1 receptor signaling pathway to inhibit extracellular matrix accumulation, renal inflammation, fibrosis and oxidative stress[24]. These results accord closely with our predictions, which suggested that the ingredients with higher degree might play an important role in the treatment of DN. Meanwhile, we discovered five ingredients (M5, M27, M28, M60 and M70) are likely to have renal protection effect but haven’t been verified up to now.

Moreover, the results of the drugs - DN - targets network topology analysis also showed that there are 5 targets which degrees were greater than 2 times of the average. Particularly, 3 of these have been experimentally proven to be in contact with DN. For instance, EGFR activation has a significant role in activating pathways that mediate podocyte injury and loss in diabetic nephropathy[25]. Down-regulated expression of
MMP-9 can promote the process of DN[26]. STAT3 inhibition can hinder the development and progression of DN in diabetic patients[27].

As shown in the GO enrichment analysis results, the potential targets of shenzhuo formula acting on DN were mainly associated with various biological processes, such as lipopolysaccharide-mediated signaling pathway, inflammatory response, positive regulation of cyclase activity, protein kinase B signaling, positive regulation of MAP kinase activity, response to estradiol, which have a strongly direct correlation with the pathogenesis of DN[28-34].

Similarly, the results of KEGG enrichment analysis showed that shenzhuo formula takes an effect in the treatment of DN through multiple pathways. Through further research, we found that these pathways have been experimentally confirmed, such as TNF signaling pathway[35], HIF-1 signaling pathway[36], Toll-like receptor signaling pathway[37], FoxO signaling pathway[38], Focal adhesion[39], NOD-like receptor signaling pathway[40] and other ways to exert anti-DN potential. These results were also consistent with the result predicted by the network analysis. In addition, the KEGG enrichment analysis suggested that shenzhuo formula may have potential therapeutic effects on diseases such as cancer, hepatitis, influenza, leishmaniasis, pertussis and tuberculosis. As is reported that different diseases have common or similar pathological changes and can be treated with the same prescription[41], the above results suggest that shenzhuo formula concentrates more on the systematicness of the body when treating DN. In other words, shenzhuo formula possibly regulates the body to reach the balance state, then reaching the aim of treatment.

5. Conclusion

In conclusion, this study based on the network pharmacology, has preliminary explained the anti - DN mechanism of shenzhuo formula from the perspective of
multi-active ingredients, multi-targets, multi-pathway. And it also provides reference for further research and might be benefit for shenzhuo formula's clinical application to some extent.

Supplementary Materials
Appendix A: Table. S1. A total of 182 potential targets.
Appendix B: Table. S2. The code information for active ingredients

Abbreviations
TCM: traditional Chinese medicine; DN: diabetic nephropathy; AGE: advanced glycation endproducts; ROS: reactive oxygen species; ECM: extracellular matrix; TCMSP: the Chinese Traditional Medicine System Pharmacological Database Analysis Platform; TCMD: Traditional Chinese Medicine Integrated Database; TTD: Therapeutic Target Database; PPI: protein-protein interaction; DAVID: Database for Annotation, Visualization and Integrated Discovery; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; ADME: absorption, distribution, metabolism and excretion; OB: Oral bioavailability; DL: drug similarity; MF: molecular function; BP: biological processes; CC: cellular components

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Patient consent for publication
Not applicable.

Authors’ contributions
XT and LZ designed the study; XW and LH wrote the paper; LZ, XW, HZ and HY performed the study and analyzed the data; SD and HW supervised the study and
revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The data used to support the results of this study can be obtained from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interest**

There is no conflict of interest declared.

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