Original Article

Molecular network-based analysis of Guizhi-Shaoyao-Zhimu decoction, a TCM herbal formula, for treatment of diabetic peripheral neuropathy

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Aim: Guizhi-Shaoyao-Zhimu decoction (GSZ), a traditional Chinese medicine (TCM) herbal formula, has been shown effective in the treatment of diabetic peripheral neuropathy (DPN). In this study, network analysis was performed to decipher the molecular mechanisms of GSZ in the treatment of DPN.
Methods: The chemical components of the 3 herbs forming GSZ, *ie*, *Ramulus Cinnamomi* (Guizhi), *Paeonia lactiflora* (Shaoyao) and *Rhizoma Anemarrhenae* (Zhimu), were searched in Chinese medicine dictionaries, and their target proteins were identified in PubChem. DPN genes were searched in PubMed gene databases. Ingenuity Pathway Analysis (IPA) was used to build the GSZ pharmacological network and DPN molecular network. The canonical pathways between the two networks were compared to decipher the molecular mechanisms of GSZ in the treatment of DPN.
Results: Sixty-one protein targets for Guizhi, 31 targets for Shaoyao, 47 targets for Zhimu, as well as 23 genes related to DPN were identified and uploaded to IPA. The primary functions of the DPN molecular network were inflammatory response, metabolic disease, cellular assembly and organization. As far as the pharmacological network functions were concerned, Guizhi target proteins were involved in neurological disease, inflammatory disease, cellular growth and proliferation, cell signaling, molecular transport, and nucleic acid metabolism, Shaoyao target proteins were related to neurological disease, inflammatory disease, and Zhimu target proteins focused on cell death and survival, cellular movement, immune cell trafficking, DNA replication, recombination and repair, and cell cycle. In the three-herb combination GSZ, several new network functions were revealed, including the inflammatory response, gene expression, connective tissue development and function, endocrine system disorders, and metabolic disease. The canonical pathway comparison showed that Shaoyao focused on IL-12 signaling and production in macrophages, and Zhimu focused on TNFR2 signaling, death receptor signaling, ILK signaling, IL-17A in gastric cells, IL-6 signaling, IL-8 signaling, the role of JAK1, JAK2, and TYK2 in interferon signaling, IL-9 signaling, HMGB1 signaling, NO production and ROS production in macrophages, whereas GSZ focused aryl hydrocarbon receptor signaling and apoptosis signaling in addition to those pathways induced by Guizhi, Shaoyao and Zhimu.
Conclusion: Although each single herb can affect some DPN-related functions and pathways, GSZ exerts more effects on DPN-related functions and pathways. The effects of GSZ on aryl hydrocarbon receptor signaling and apoptosis signaling pathways may be the key components of its total molecular mechanisms.

Keywords: Guizhi-Shaoyao-Zhimu decoction; diabetic peripheral neuropathy; traditional Chinese medicine; *Ramulus Cinnamomi*; *Paeonia lactiflora*; Rhizoma Anemarrhenae; network pharmacology

Introduction

Traditional Chinese medicine (TCM), a healthcare-focused medical system, is characteristic of the complexity and variability of the management of all types of diseases. Because of its generally mild nature and its emphasis on maintaining balance in individuals, TCM has gained increased attention worldwide. However, it is difficult for researchers to evaluate its holistic efficacy and pharmacological mechanisms. In health care, systems biology has emerged as the paradigm of systems thinking. These insights triggered a major shift in the strategies adopted in botanical drug research from single compound drugs to multiple compound drugs. The "one drug for

one gene for one disease" model has failed because one drug often has many targets and one target is often docked by more than one drug[1, 2]. In the present study, diabetic peripheral neuropathy (DPN) was investigated through systems biology methods coupled with several public data sources such as GenBank, PubChem, and SinoMed. TCM is regarded as an effective treatment for DPN. A literature review shows that the Guizhi-Shaoyao-Zhimu decoction (GSZ), which is composed of *Ramulus Cinnamomi* (Guizhi), *Paeonia lactiflora* (Shaoyao), and *Rhizoma Anemarrhenae* (Zhimu), can be effective in treating DPN[3-5].

The GenBank sequence database is an open-access, annotated collection of all publicly available nucleotide sequences and their protein translations. Currently, the public databases in GenBank may be searched and used by every web user.

PubChem is another public database of chemical molecules and their activities against biological assays. PubChem can be accessed for free through a web user interface. Each hit provides information about synonyms, chemical properties, chemical structure, bioactivity, and other NCBI databases, such as PubMed.

SinoMed, a famous Chinese biomedical literature service system, is a web system for free retrieval of Chinese literature. DPN is the presence of symptoms and/or signs of peripheral nerve dysfunction in diabetic patients, diagnosed after other causes of dysfunction have been excluded[6, 7]. It is the most common quality-of-life complication of diabetes mellitus and is a tremendous hardship for diabetic people[8]. In the case of autonomic neuropathy, life can become quite dismal and the mortality rate is approximately 25%–50% within 5-10 years[9, 10]. For these reasons, the pathogenesis and diagnosis of DPN, and specific therapeutic strategies, have attracted interest from researchers, and related studies and scientific reports/literature have increased exponentially in recent years. The mass of information provides a useful opportunity to elucidate the relationship between DPN pathogenesis and prescription principles.

Materials and methods
DPN-related gene data
DPN genes were searched in the National Center for Biotechnology Information (NCBI) Gene database. The Gene database (http://www.ncbi.nlm.nih.gov/gene) integrates information from a wide variety of species. A record may include nomenclature, reference sequences, maps, pathways, variations, phenotypes, and links to genome-, phenotype-, and locus-specific resources worldwide. We searched the Gene database (until October 12, 2012) with "diabetic peripheral neuropathy" and "DPN" as keywords and filtered for genes with variations in medical significance as reported via Variation Viewer. We targeted DPN genes for Homo sapiens (Supplementary Table 1).

GSZ-related target proteins
The target proteins of the three GSZ herbs were searched in PubChem (until October 12, 2012). The PubChem (http://pubchem.ncbi.nlm.nih.gov/) database is a public knowledge

base for biological activities of small molecules and small interfering RNAs (siRNAs) that is hosted by the US National Institutes of Health (NIH). It consists of three dynamically growing primary databases: PubChem Compound, PubChem Bioassay, and PubChem Substance. All of the targeted proteins related to the active compounds in Chinese medicine can be obtained using PubChem. According to the Grand Dictionary of Chinese Medicine[11], we sought the active ingredients of the three GSZ Chinese herbs (Supplementary Tables 2–4). Each compound was searched in PubChem Compound. Because the bio-information could be cross-referenced to other NCBI databases[12], the target proteins of active compounds that were tested in bioassays could be collected in PubChem. In consideration of future research, we limited the protein category to Homo sapiens. We assumed that the GSZ protein targets were the sum of the three herbal protein targets.

Networks built and analysis
Related DPN genes were uploaded to the Ingenuity Pathway Analysis online (IPA, http://www.ingenuity.com), and the molecular gene network was built (Figure 1). Similarly, we uploaded GSZ total protein targets into IPA. The molecules we imputed into IPA were termed "focus molecules". IPA generated focus molecules into a set of networks based on different bio-functions. Molecules were represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All of the edges were supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. Nodes were displayed using various shapes that represent the functional gene product class. The networks were ranked by scores, which were calculated by IPA and represented the significance of the molecules in the network. To investigate the mechanism of GSZ against DPN, we performed a canonical pathway analysis using the IPA compare module. Using Fisher's exact test, IPA determined the significance of the association between focus molecules and the canonical pathways. Finally, we overlaid the two networks to discover the possible targets of each compound in GSZ (Figure 2).

Results and discussion
DPN related gene networks and functions
As well known, GSZ comes out of the famous classic book named "Synopsis of Golden Chamber" (Chinese name: JinGui YaoLue). Its effect is recorded in this ancient book that it was effective for the pains in joint, muscle, and nerve. Despite most existing studies are on small samples and have no control group, there are indications for potentially useful strategies of GSZ for DPN. In this study, we determined genes related to DPN and tried to elucidate the treatment mechanism of GSZ by using a complex system correlated with gene bank database and target proteins.

The GenBank database identified 23 related genes linked with DPN (Supplementary Table 1). With the help of the IPA kit, the DPN molecular network was determined by importing the associated information for the 23 genes. As shown in
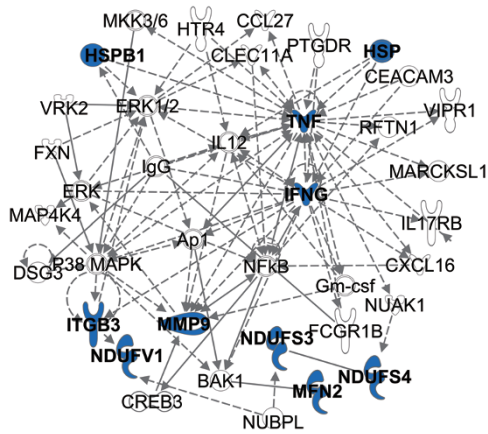
Figure 1. The molecular network of DPN. Genes related to DPN are shown in blue. Genes indirectly related to the DPN genes are shown in black.
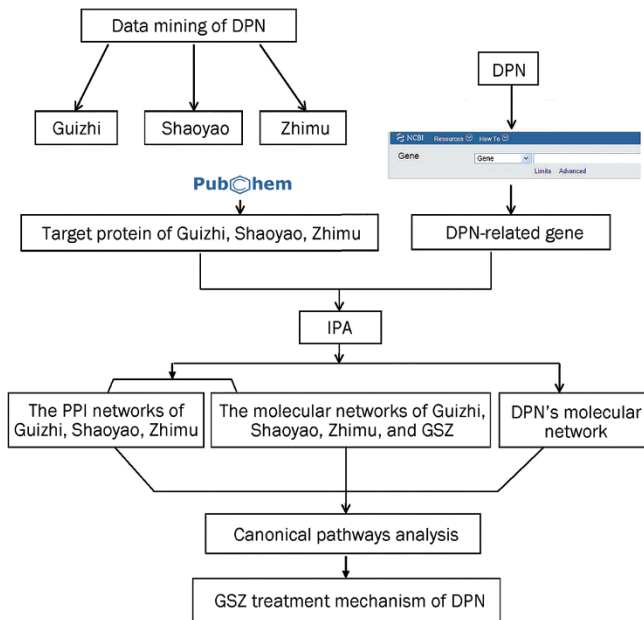

Figure 2. Frame diagram of this research. The network-based analysis of the molecular mechanism of diabetic peripheral neuropathy treatment with Guizhi-Shaoyao-Zhimu.

Figure 1, inflammatory response, metabolic disease, cellular assembly and organization were the main molecular pathways in the DPN molecular network.

GSZ-related target protein networks and functions

Modern medical research has shown that Guizhi can retard the progression of diabetes by preventing a decrease in superoxide dismutase activity or suppressing the increase of methane dicarboxylic aldehyde[13], Shaoyao can improve immune function[14], and Zhimu asphodeloides counteracts the progression of diabetic ophthalmopathy in streptozotocin-induced diabetic

rats[15].

As collected from the PubChem database, information on the drug targets of Guizhi, Shaoyao, and Zhimu are listed in Supplementary Tables 2–4. The GenInfo Identifier (GI) numbers for each protein were imported into the IPA software, and the protein-protein interaction (PPI) networks of Guizhi, Shaoyao, and Zhimu were constructed (Figure 3A–3C). To investigate their synergistic effect, we merged the three PPI networks (Figure 3D). At the highest level, the pharmacological network functions of Guizhi target proteins focused on neurological disease, inflammatory disease, cellular growth and proliferation, cell signaling, molecular transport, and nucleic acid metabolism. The pharmacological network functions of Shaoyao target proteins focused on neurological disease and inflammatory disease, and the pharmacological network functions of Zhimu target proteins focused on cell death and survival, cellular movement, immune cell trafficking, DNA replication, recombination and repair, and the cell cycle. Regarding the three-herb combination GSZ, some new pharmacological functions were identified, including inflammatory response, gene expression, connective tissue development and function, endocrine system disorders, and metabolic disease.

Comparing the two networks revealed the effects of GSZ on DPN

We hope that this research can provide the basis for a better understanding of the molecular mechanisms of GSZ-treated DPN. Both the DPN and GSZ networks, as well as the functions of related target proteins, indicated that DPN and GSZ were related to the inflammatory response, metabolic disease, and other conditions.

Metabolic disorders can cause hyperglycemia, which is inextricably linked with the development of neuropathy. Elevated blood glucose may be toxic to nerves[16] and intravenous glucose infusion can increase neuropathic pain[17]. Hyperglycemia may also injure nerves, through indirect mechanisms. Data from the Diabetes Control and Complications Trial (DCCT) demonstrated that strict control of hyperglycemia in type 1 diabetes patients without clinical neuropathy decreased DPN development in 60% of cases over 5 years of follow-up study[16, 18]. DPN occurs in both type 1 and type 2 diabetes, and systemic hyperglycemia is the most obvious symptom that these diseases have in common[19-21], suggesting that hyperglycemia is a universal trigger for DPN. Indeed, insulin treatment or treatment with insulin-sensitizing drugs to control hyperglycemia reverses some DPN symptoms and delays its progression in general[22]. The DPN and GSZ networks indicate that GSZ can decrease blood glucose and subsequently control DPN development.

Diabetes mellitus is related to inflammation

Type 1 diabetes mellitus is a chronic autoimmune disease with a strong inflammatory component. Polymorphisms in inflammatory cytokines, such as IL-1, IL-6, and TNF-α, play an important role in diabetes mellitus[23]. Inflammation seems to be important in diabetes mellitus pathogenesis and progression[23]. Diabetic patients have increased levels of soluble
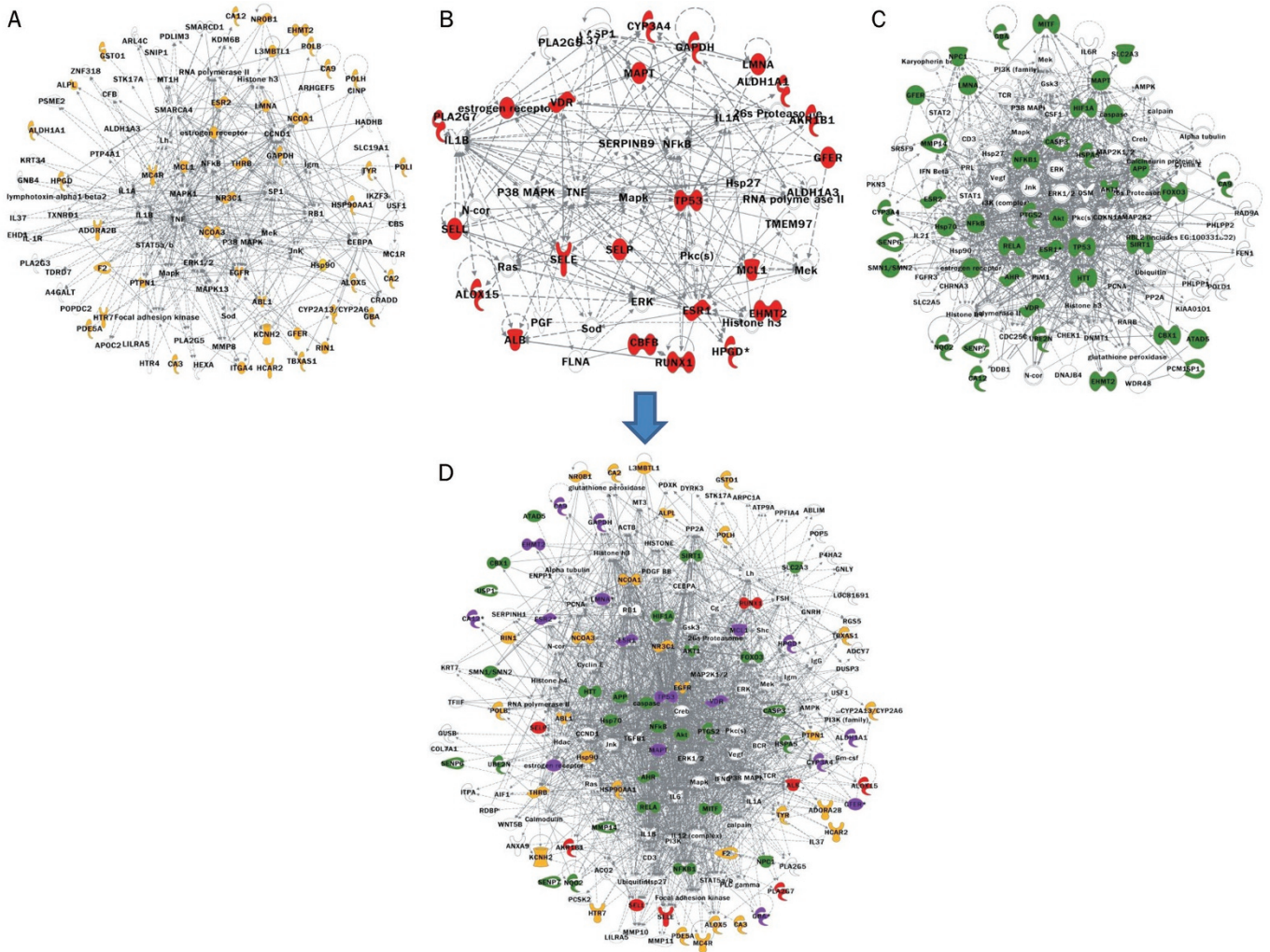
Figure 3. The molecular network of Guizhi, Shaoyao, Zhimu and GSZ. The molecular networks of Guizhi, Shaoyao, Zhimu, which are merged, are shown, as is the molecular network of GSZ. The target proteins are identified in the networks: yellow for Guizhi, red for Shaoyao, green for Zhimu, and purple for those shared by two or more proteins (details are listed in Supplementary Table 1).

adhesion molecules (soluble intercellular adhesion molecule (sICAM)-1, sICAM-2, soluble vascular cell adhesion molecule (sVCAM)-1, soluble E-selectin, soluble L-selectin, and soluble P-selectin), which are thought to be integral inflammatory state components. The functions of the DPN and GSZ networks are related to inflammation, and we believe that GSZ can reduce inflammation and control DPN development.

The canonical pathway of DPN and GSZ networks as analyzed by IPA

To elucidate the mechanism, we selected the pathway with columns that were higher than the threshold both in GSZ and DPN. There were two types of columns: one was related to apoptosis, cellular growth, proliferation and development (Figure 4), and its pathway is shown in Figure 5; the other was related to the cell immune response, cytokine signaling, and the humoral immune response (Figure 6), and its pathway is

shown in Figure 7. The canonical pathway comparison indicated that Shaoyao focused on IL-12 signaling and production in macrophages; Zhimu focused on TNFR2 signaling, death receptor signaling, ILK signaling, IL-17A in gastric cells, IL-6 signaling, IL-8 signaling, the role of JAK1, JAK2, and TYK2 in interferon signaling, IL-9 signaling, HMGB1 signaling, nitric oxide and reactive oxygen species production in macrophages; and GSZ focused on aryl hydrocarbon receptor signaling and apoptosis signaling in addition to the pathways that were induced by Guizhi, Shaoyao and Zhimu.

The DPN and GSZ networks had common pathways in cellular immune responses. The cellular immune system and T-cell-mediated autoimmune responses are involved in the pathogenesis of diabetes mellitus[24]. Both CD4 and CD8 T cells are required for diabetes mellitus activation in an animal model[25]. CD8 T cells are directly responsible for islet destruction and clinical diabetes development[26]. Furthermore, IL-6
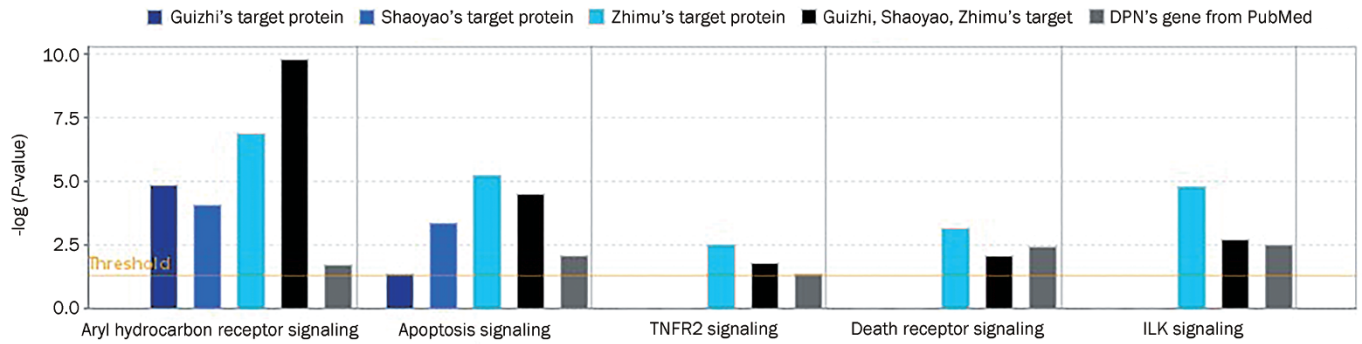
Figure 4. Comparison of GSZ target proteins and related genes in apoptosis, cellular growth, proliferation and development. Not all of the columns related to the herbs are shown for all pathways. In total, five columns of herbs and DPN are shown in the same pathway, which indicates that these herbs may treat the disease in that pathway (details are listed in Supplementary Table 2).
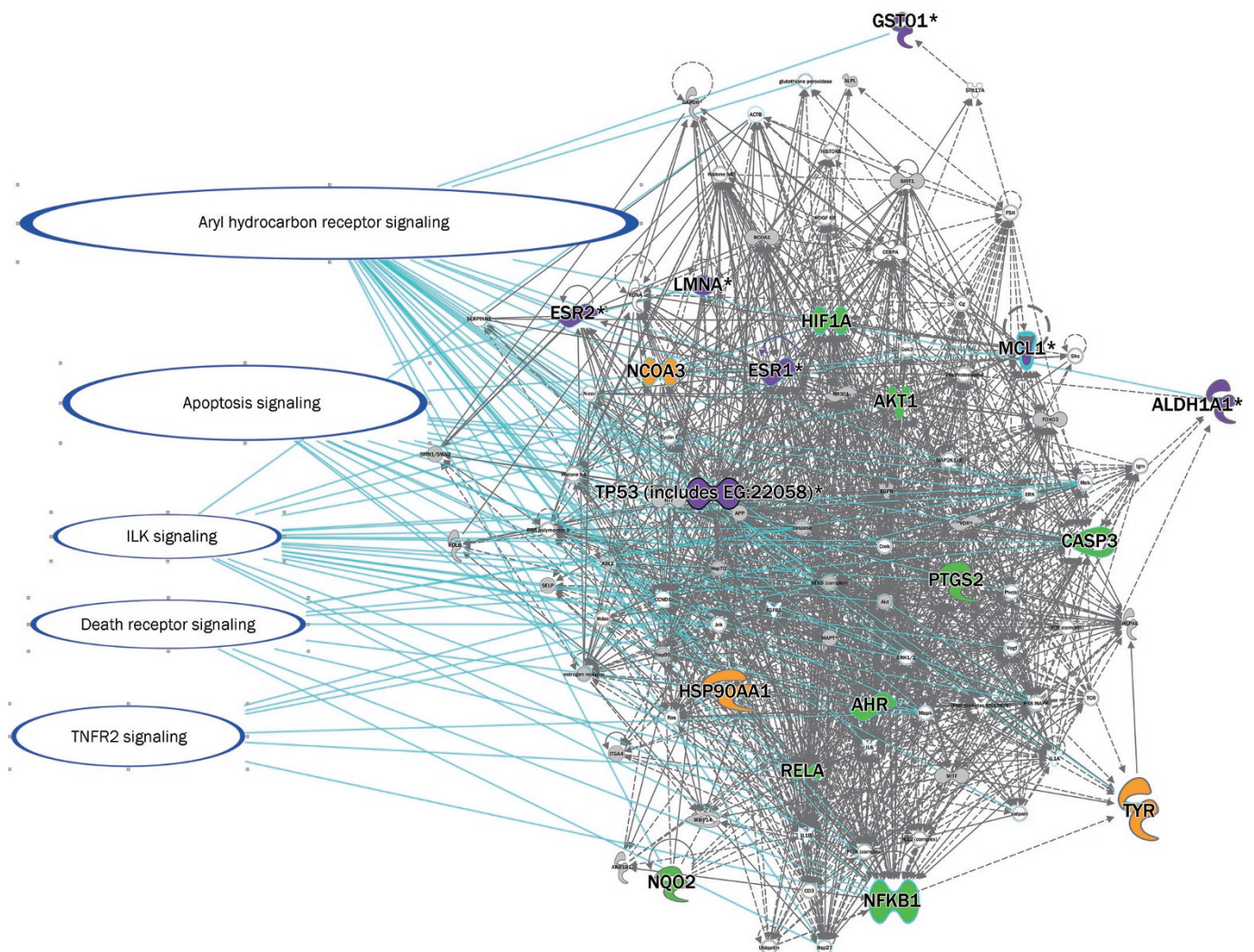

Figure 5. The pathways, including apoptosis, cellular growth, proliferation and development, are overlaid in the GSZ molecular network. The molecules are identified in different colors on the network: yellow for Guizhi, red for Shaoyao, green for Zhimu, and purple for those shared by two or more proteins (details are listed in Table 3).
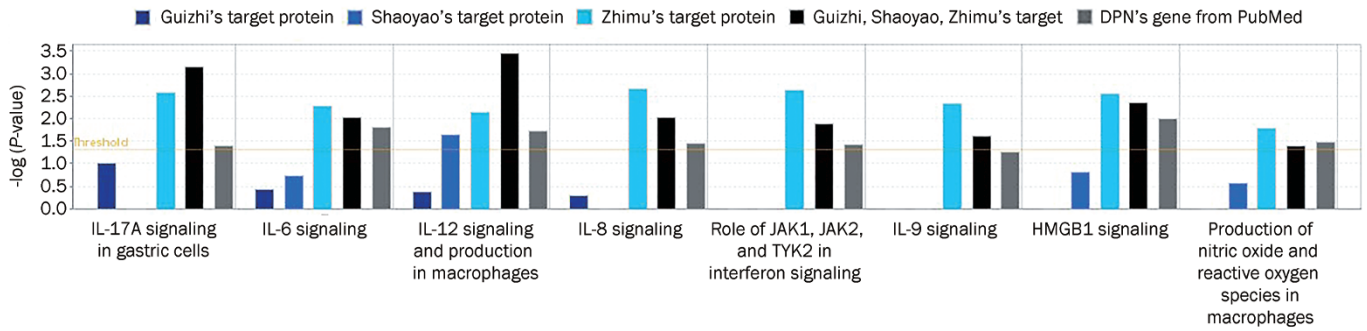
Figure 6. Comparison of the GSZ target proteins and related genes in the cell immune response, cytokine signaling, and the humoral immune response. In total, eight columns of herbs and DPN are shown in the same pathway (details are listed in Supplementary Table 2).
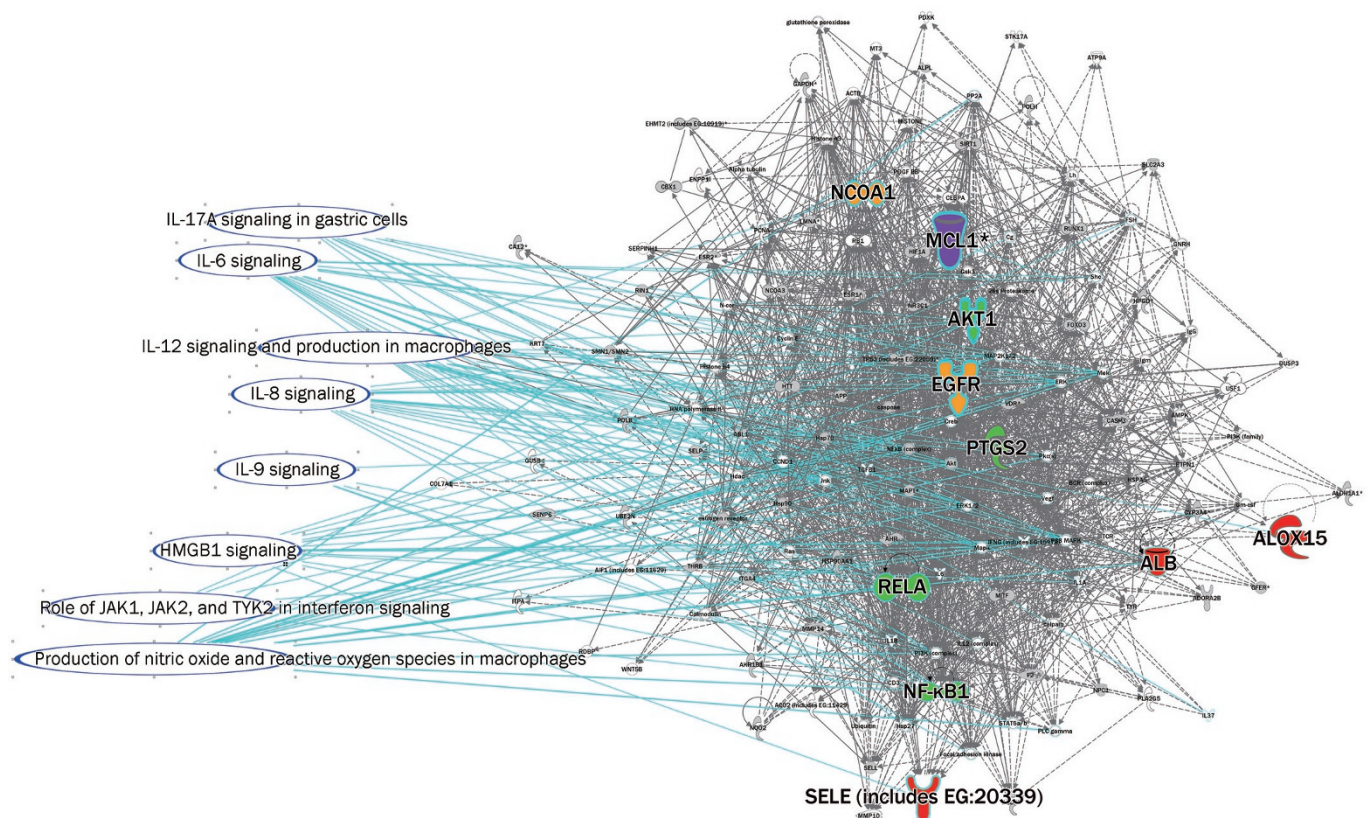

Figure 7. The pathways, including the cell immune response, cytokine signaling, and the humoral immune response, are overlaid in the GSZ molecular network. The molecules are identified in different colors on the network: yellow for Guizhi, red for Shaoyao, green for Zhimu, and purple for those shared by two or more proteins (details are listed in Supplementary Table 3).

signaling lies in the common pathway in the network, and IL-6 is involved in diabetes mellitus pathogenesis. There is a significant correlation between adipose IL-6 mRNA expression and insulin resistance[27]. High circulating IL-6 levels have been associated with insulin resistance and a greater risk of type 2 diabetes[28]. Cytokine-induced inflammation is important in diabetes mellitus pathogenesis and progression[23]. Thus, inflammation associated with cytokines such as IL-6 was important in biological activities common to diabetes

mellitus. Some molecules are involved in the pathway, such as RELA and AKT. The serine-threonine kinase AKT, also known as protein kinase B (PKB), is an important effector for phosphatidylinositol 3-kinase signaling initiated by numerous growth factors and hormones[29]. AKT/PKB is expressed in classic insulin target tissues, such as liver, muscle, and adipocytes[30-33]. Mice rendered deficient in AKT/PKB exhibit normal glucose homeostasis as ascertained by glucose and insulin tolerance tests. AKT may participate in GSZ treatment of dia-

betes mellitus and DPN.

According to the IPA analysis, apoptosis is a common pathway of DPN and GSZ. Apoptosis is a key mechanism that regulates tissue composition and homeostasis. Endothelial cell apoptosis may be highly significant in diabetes development[34]. Studies from the past ten years have suggested that the neonatal wave of β-cell apoptosis provide the autoantigens necessary for triggering β-cell-directed autoimmunity[35]. As a common pathway of DPN and GSZ, apoptosis may play an important role in GSZ-treated DPN. Many molecules are involved in the pathway, including nuclear factor-κB (NF-κB) and TP53. NF-κB regulates the transcription of many genes involved in the immune response, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis[7]. NF-κB and NF-κBI gene polymorphisms are related to the development of common inflammatory diseases, including ulcerative colitis, Crohn's disease (CD), rheumatoid arthritis, and type 1 diabetes. Inactivation of the tumor-suppressor gene TP53 was observed in pancreatic cancer, and approximately 80% of pancreatic cancer patients were identified as having concomitant diabetes with a poor prognostic factor[36]. Another study determined that the polymorphous marker Pro72Arg of the TP53 gene was associated with DPN in Russian patients with Type 1 diabetes mellitus living in Moscow[37]. These molecules may participate in the action of GSZ in DPN treatment.

GSZ focused the aryl hydrocarbon receptor signaling in addition to the pathways induced by Guizhi, Shaoyao and Zhimu. The aryl hydrocarbon receptor is a ligand-activated transcription factor in the Per-Arnt-Sim (PAS) protein superfamily[38]. The PAS domain of the aryl hydrocarbon receptor, which mediates heterodimerization with a structurally related protein known as the aryl hydrocarbon receptor nuclear translocator (ARNT), affects DNA recognition, ligand binding and chaperone interactions[39-41]. ARNT is thought to be a transcription factor that can regulate the metabolic phenotype of pancreatic β-cells and is reduced in islets obtained from type 2 diabetic patients[42]. Data indicate that ARNT may play a critical role in maintaining the normal secretion competence of β-cells[43]. These findings suggest that GSZ could treat DPN via aryl hydrocarbon receptor signaling.

Conclusions

In this work, we analyzed two molecular networks and their functions by IPA: one network identified genes related to DNP, and the other detailed GSZ target proteins. Although each herb could affect certain DPN-related functions and pathways, GSZ had greater effects on those functions and pathways, and the effects on aryl hydrocarbon receptor signaling and apoptosis signaling pathways might be important for elucidating the molecular mechanism of GSZ as a whole. In future work, we will seek to improve this network-analysis method because the target proteins available from the chemical components of TCM herbs are limited, which restricts the analysis for the herb. We will attempt to solve this difficult problem in the future.

Author contribution

Ai-ping LU and Ning ZHAO designed the research; Jian LI, Li LI, Xu-yan NIU, Ning ZHAO, Miao JIANG, Xiao-juan HE, Zhao-xiang BIAN, Ge ZHANG performed the research; Ning ZHAO and Jian LI drafted the paper.

Supplementary information

Supplementary information is available at Acta Pharmacologica Sinica's website.

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