Mechanism of Xianling Gubao Capsule in the Treatment of Steroid Induced Osteonecrosis of Femoral Head

LINA LIU AND S. Y. LEI[1]*

Department of Plastic Surgery, [1]Department of Joint and Motor Surgery, The Second Affiliated Hospital of the University of South China, Hengyang, Hunan 421001, China

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This study aimed to obtain the main active components of Xianling Gubao capsule and identify their pharmacological characteristics and mechanism of action in treatment of steroid-induced femoral head necrosis. The chemical constituents and direct targets of Xianling Gubao capsule were obtained by the system pharmacology platform of traditional Chinese medicine. GeneCards database and gene expression omnibus database were used to obtain pathogenic genes related to steroid-induced femoral head necrosis. Through Venn diagram software, the intersection of direct drug targets and disease-related genes was obtained to obtain the potential drug targets of Xianling Gubao capsule. In the Cytoscape 3.7.1 software, the drug component-target and protein-protein interaction networks were visualized and analyzed by network analyze plugin and molecular complex detection plugin, the degree values of nodes were analyzed to screen the core genes. Finally, the database for annotation, visualization and integrated discovery database was used for gene enrichment analysis. We obtained 76 chemical components of Xianling Gubao, 583 pathogenic related genes of steroid-induced femoral head necrosis and 38 potential drug targets of Xianling Gubao capsule for the treatment of steroid-induced femoral head necrosis. A drug component-targets network with 1001 nodes and 3661 edges, a protein-protein interaction network with 31 nodes and 67 edges were constructed. 15 core genes were obtained by network topology analysis and enrichment analysis of potential drug targets showed that they were mainly concentrated in chemokine signaling pathway. Finally, 5 optimal drug targets of Xianling Gubao capsule for steroid-induced femoral head necrosis were obtained, respectively. Xianling Gubao capsule can prevent or treat steroid-induced femoral head necrosis by promoting the reconstruction of new capillaries after necrosis, improving intravascular coagulation, accelerating the formation of callus and regulating the differentiation and direction of bone marrow stem cells.

Key words: Xianling Gubao capsule, steroid-induced necrosis of femoral head, chemokine receptor, network pharmacology

Steroid-Induced Femoral Head Necrosis (SONFH) is a special style of femoral head aseptic necrosis. It is due to the excessive accumulation of glucocorticoids in the body due to the large amount of glucocorticoids which leads to pathological changes such as lipid metabolism disorder, increased blood viscosity, microthrombosis and abnormal osteogenic differentiation of bone marrow stem cells in the body, and finally leads to necrosis of the femoral head[1]. Most of the patients had early atypical clinical symptoms, showing only pain after hip joint fatigue. Most SONFH patients have already progressed to stage III-IV by the time they come to the clinic due to their lack of awareness of the disease[2]. Therefore, early prevention of the disease is very important.

Xianling Gubao capsule contains six kinds of Chinese Traditional medicine, including Yin Yang Huo, Bu Gu Zhi, Xu Duan, Danshen, Zhimu, Dihuang. It has the effect of nourishing liver and kidney, activating blood circulation and degrading collaterals, and strengthening tendons and bones[3]. Mainly used in the treatment of osteoporosis, fracture, osteoarthritis and bone aseptic necrosis and other disease.

This study intends to analyze the main target and

*Address for correspondence
E-mail: leisaiyunqu@163.com

mechanism of Xianling Gubao capsule in the treatment of SONFH through network pharmacology and bioinformatics and to explore the clinical application value of Xianling Gubao capsule as a preventive drug for patients using a large number of glucocorticoids to alleviate or block the pathogenesis of SONFH.

MATERIALS AND METHODS

Screening of effective chemical compositions of Xianling Gubao capsule:

The Chinese traditional medicine contained in Xianling Gubao capsule was obtained by literature retrieval. In the search box of Traditional Chinese Medicine (TCM) and chemical components in the chemical database, the names of six Chinese traditional medicines were respectively entered to obtain the corresponding chemical composition, molecular formula and Simplified Molecular Input Line Entry System (SMILES) ID. SMILES ID of chemical components were input into Swiss Absorption, Distribution, Metabolism and Excretion (ADME) database, through the screening of oral bioavailability and drug similarity, the effective chemical components of Xianling Gubao capsule were obtained.

To predict the direct target protein of Xianling Gubao capsule:

We input the above SMILES ID of Xianling Gubao capsule effective chemical composition into SwissTargetPrediction database and obtained its corresponding component target database file. Finally, the target protein of Xianling Gubao capsule was obtained.

Construction of Chinese traditional materials-component-target network:

Firstly, according to the above search results, the TCM component target network file and attribute file are constructed in Excel software. Secondly, the TCM-component-target network is obtained by importing the network file and properties file into Cytoscape 3.7.1 software. Finally, the properties box is adjusted to beautify the network.

Screening the genes related to SONFH:

Enter the keywords "SONFH" or "Steroid-induced femoral head necrosis" into the search box of Gene Expression Omnibus (GEO) database and download the series of matrix data and chip platform data.

Differential expression analysis was performed on the data by Excel software. According to $p<0.05$ and $|log2FC|>1$, screening of SONFH and non-SONFH differentially expressed genes as SONFH pathologic genes, in which log2FC>0 indicated that the gene was upregulated in SONFH and log2FC<0 indicated that the gene was downregulated in SONFH. Sangerbox mapping software was used to draw the differentially expressed genes heatmap and volcano map.

Construction of Protein-Protein Interaction (PPI) network:

First of all, we use Venn diagram to analyze the differentially-expressed genes of SONFH and direct target protein of Xianling Gubao capsule, and obtain the potential drug targets of Xianling Gubao capsule in the treatment of SONFH. Secondly, the relationship between potential drug targets was analyzed in Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, and the "node to node" network file was downloaded. Then, the "node to node" relationship was analyzed in Excel software and the degree value of each node was obtained. Finally, we import the "node to node" network file and "node degree" properties file into the Cytoscape 3.7.1 software, and construct PPI network. We further obtained the core genes and modules by network analyze tool and Molecular Complex Detection (MCODE) plugin.

Gene enrichment analysis:

The Database for Annotation, Visualization and Integrated Discovery (DAVID) is the effective software for us to analyze the gene function and signaling pathway. Firstly, we enter the potential drug targets into "enter gene list" search box. Then, we import *Homo sapiens* into the "select species" search box. Finally, we choose "official_gene_symbol" choice and "gene list" choice. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment results were obtained. We further screened the Biological Process (BP), Molecular Function (MF), Cellular Component (CC) and signaling pathway with $p<0.01$ in Excel software.

Key targets and pharmacological mechanism of Xianling Gubao capsule in the treatment of SONFH:

The core genes in the PPI network and the genes enriched in the signaling pathway were analyzed by Venn diagram software and the key targets and biological effects were analyzed by KEGG mapper. Finally, the key target and the main mechanism of

the drug were obtained.

RESULTS AND DISCUSSION

The effective ingredients and direct targets of Xianling Gubao capsule were discussed below. Six kinds of Chinese Traditional medicine, Yin Yang Huo, Bu Gu Zhi, Xu Duan, Danshen, Zhimu, Dihuang were input into the retrieval box of chemical database and their corresponding molecular formula of chemical components and SMILES ID were obtained. In Swiss ADME, 76 active ingredients were obtained, including 42 active ingredients in Danshen, 8 in Dihuang, 7 in Yin Yang Huo, 12 in Bu Gu Zhi, 2 in Xu Duan and 5 in Zhimu.

In SwissTargetPrediction, we import SMILES ID of effective ingredients into search box and obtained the direct targets of each ingredient. After removing repeated targets and targets with probability=0.918

direct targets were finally obtained.

The gene microarray Genomic Spatial Event (GSE) 123 568 was obtained from the GEO database, containing 40 samples (10 non-SONFH samples and 30 SONFH samples), and came from GPL15207 ([PrimeView] Affymetrix Human Gene Expression Array) platform. We downloaded the series of matrix file and gene probe file, and proposed the data in Excel software (including substitution gene name, get rid of duplicate genes, data standardization). In accordance with condition of $p<0.05$ and $|log2FC|>1$, 583 differentially expressed genes were obtained, among then, 335 upregulated genes and 238 down regulated genes were obtained.

In Sangerbox software, we imported the differentially-expressed genes file, and the heatmap and volcano figure were constructed. The results are shown in fig. 1 and fig. 2.
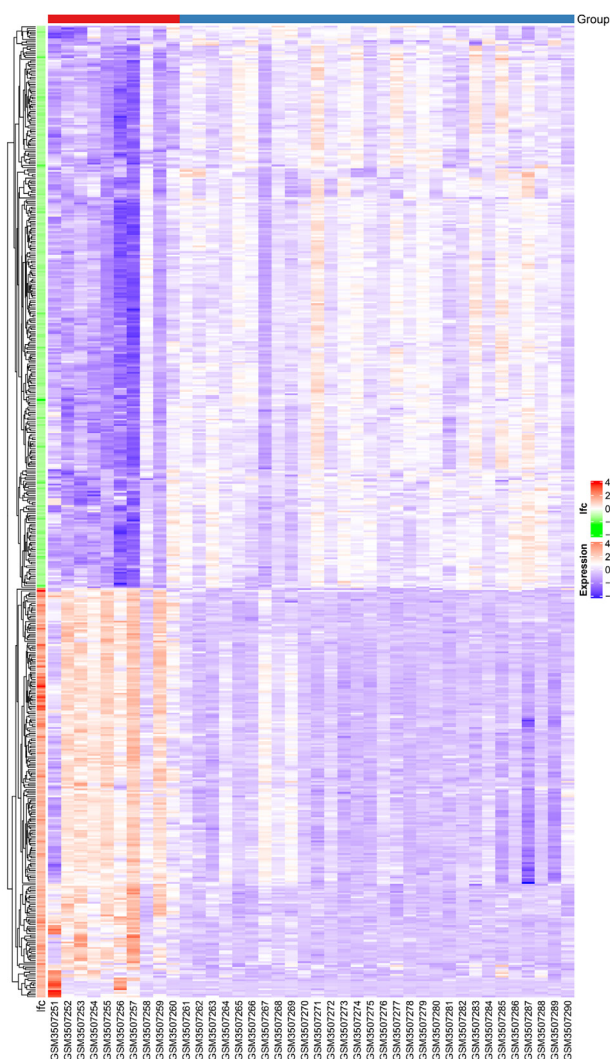

Fig. 1: Non-SONFH to SONFH heatmap of differentially expressed genes, (■) Non-SONFH group and (■) SONFH group
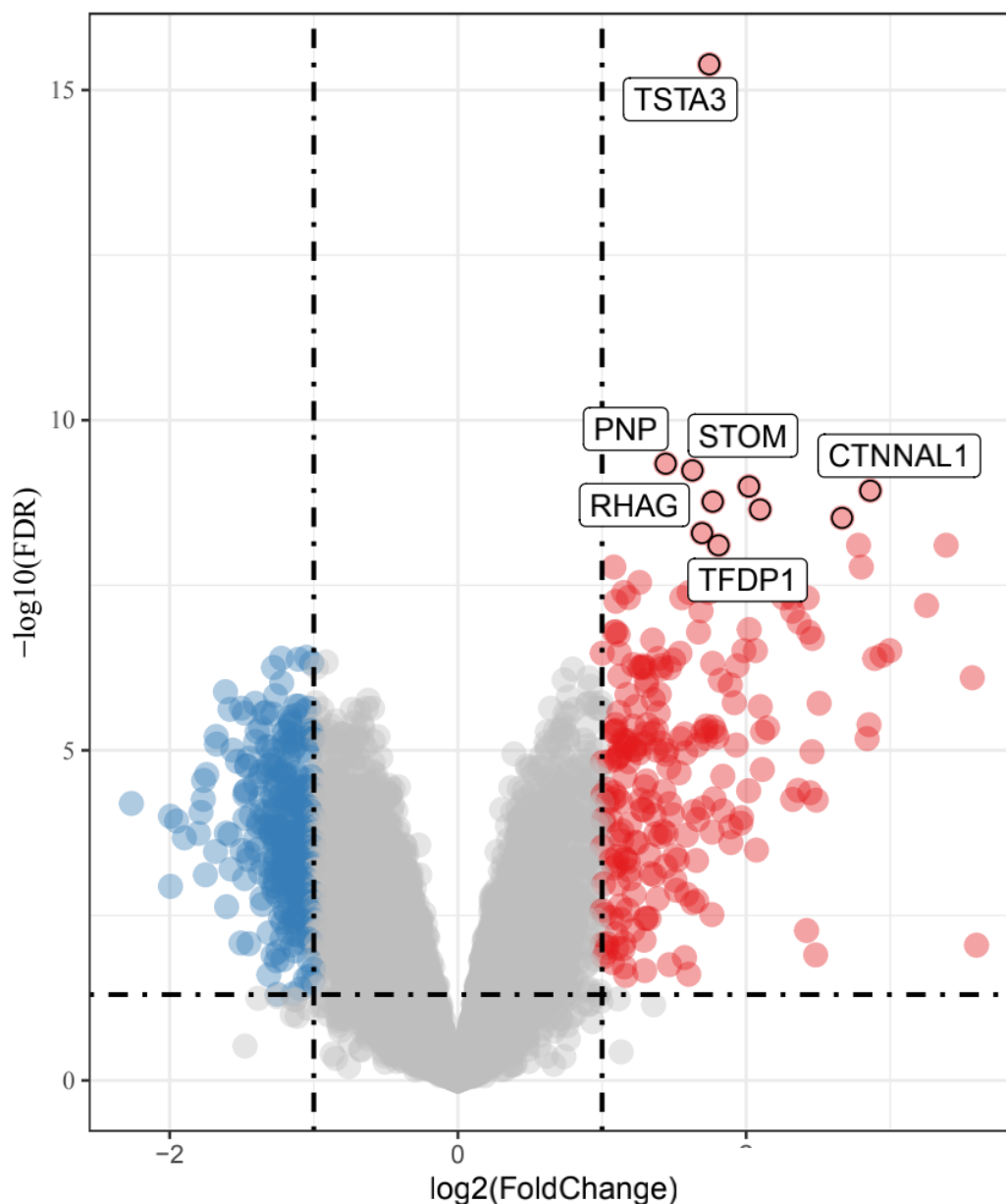
Fig. 2: Non-SONFH to SONFH volcano map of differentially expressed genes, (🔵) Down-regulated genes; (⚪) None and (🔴) Up-regulated genes

In the grouping, red represents non-SONFH samples, blue represents SONFH samples; log2FC>0 represents non-SONFH up-regulated gene relative to SONFH, log2FC<0 represents non-SONFH down-regulated gene relative to SONFH; 238 non-SONFH differentially up-regulated genes and 335 non-SONFH differentially down-regulated genes are expressed in fig. 1.

The "node to node" network file and node attribute file were established among Chinese traditional medicine, drug ingredient and drug target, and the above files were input in the Cytoscape software to obtain a network diagram of Chinese traditional medicine-ingredient-target. In the style attribute box, the nodes and edges

can be set. In the layout, the drug targets can be set to "Grid Layout", the drug ingredient corresponding to same Chinese traditional medicine can be set to "Attribute Circle Layout". The network diagram of Chinese traditional medicine-ingredient-target is shown in fig. 3.

In fig. 3, square icon represents the direct targets of drugs; circular icon represents the chemical components corresponding to different Chinese herbal medicines; peach red, orange, yellow, green, blue and purple are used to represent the chemical components of *Salvia miltiorrhiza*, sequestration, *Anemarrhena*, *Epimedium*, *Psoralea* and *Rehmannia* respectively.
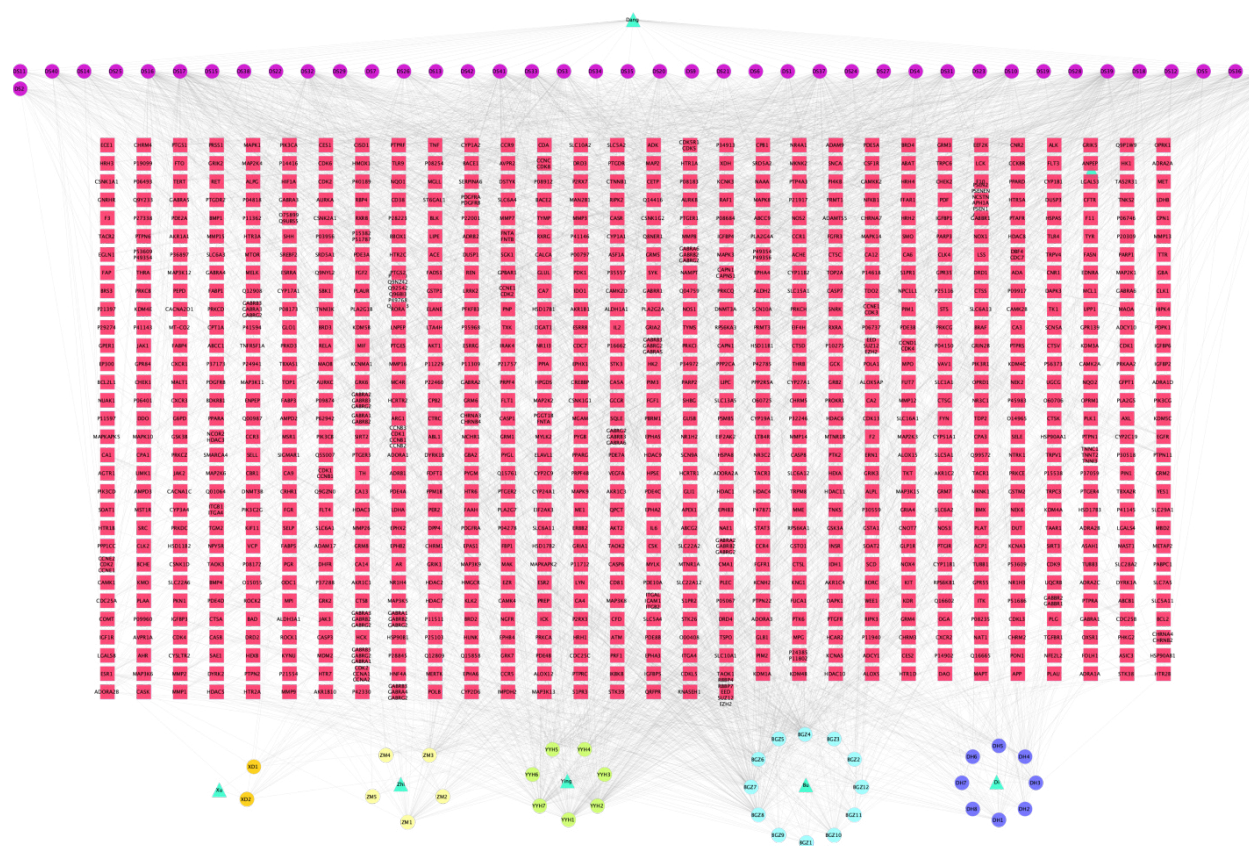
Fig. 3: Network diagram of TCM-ingredient-target

The direct targets of Xianling Gubao capsule and genes related to SONFH were analyzed by Venn diagram software, a total of 38 potential drug targets (27 upregulated and 11 downregulated) were obtained, the results are shown in fig. 4 where green represents differentially down-regulated genes in SONFH; blue represents differentially up-regulated genes in SONFH and red represents the direct targets of Xianling Gubao capsule.

We input above potential drug targets in STRING database, analyzed the interaction between potential drug targets and downloaded the "node to node" network file. In Excel software, we can analyze and obtain the "degree value" attribute file.

The "node to node" network file and "degree value" attribute file were imported into Cytoscape 3.7.1 software, and the PPI network was constructed. The result is shown in fig. 5, where the size of the icon represents the degree value of potential drug targets. The red icon represents up-regulated genes and the blue icon represents down-regulated genes. The edges color represents the combine score; the darker the color, the greater the value of combine score.

The targets of degree value>mean degree value were screened out and 15 core genes (HCK Proto-Oncogene, Src Family Tyrosine Kinase (HCK), B-cell lymphoma 2-like protein 1 (BCL2L1), C-X-C Motif Chemokine Receptor 2 (CXCR2), Matrix Metallopeptidase 8 (MMP8), C-X-C Motif Chemokine Receptor 1 (CXCR1), LYN Proto-Oncogene, Src Family Tyrosine Kinase (LYN), Cathepsin S (CTSS), C-C Motif Chemokine Receptor 1 (CCR1), Protein Tyrosine Phosphatase Non-Receptor Type 6 (PTPN6), Thymidine Kinase 1 (TK1), Caspase 1 (CASP1), C-C Motif Chemokine Receptor 3 (CCR3), Sphingosine-1-Phosphate Receptor 3 (S1PR3), Synuclein Alpha (SNCA), Arginase 1 (ARG1)) were obtained, which were used to construct sub network. The results are shown in fig. 6 where 15 potential drug targets with degree greater than the average value were extracted from PPI network diagram to construct subnetwork; icon size represents the degree value of potential drug targets; red icon represents down-regulated genes and green icon represents up-regulated genes. In the MCODE plugin, we can obtain 2 modules, which are shown in fig. 7 where icon size represents the degree value. The red icon represents up-regulated genes and the blue icon represents down-regulated genes.
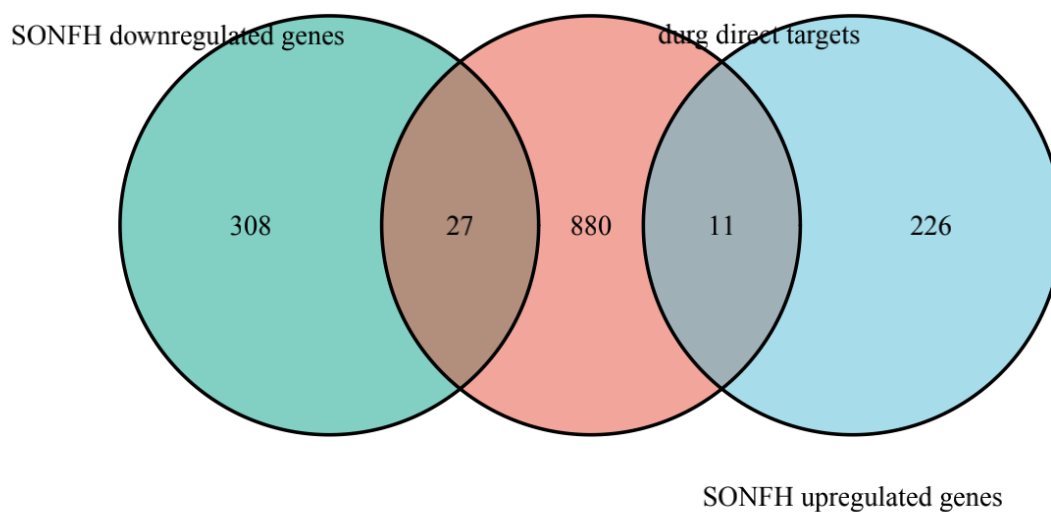
Fig. 4: The common genes of Xianling Gubao direct targets and SONFH related genes
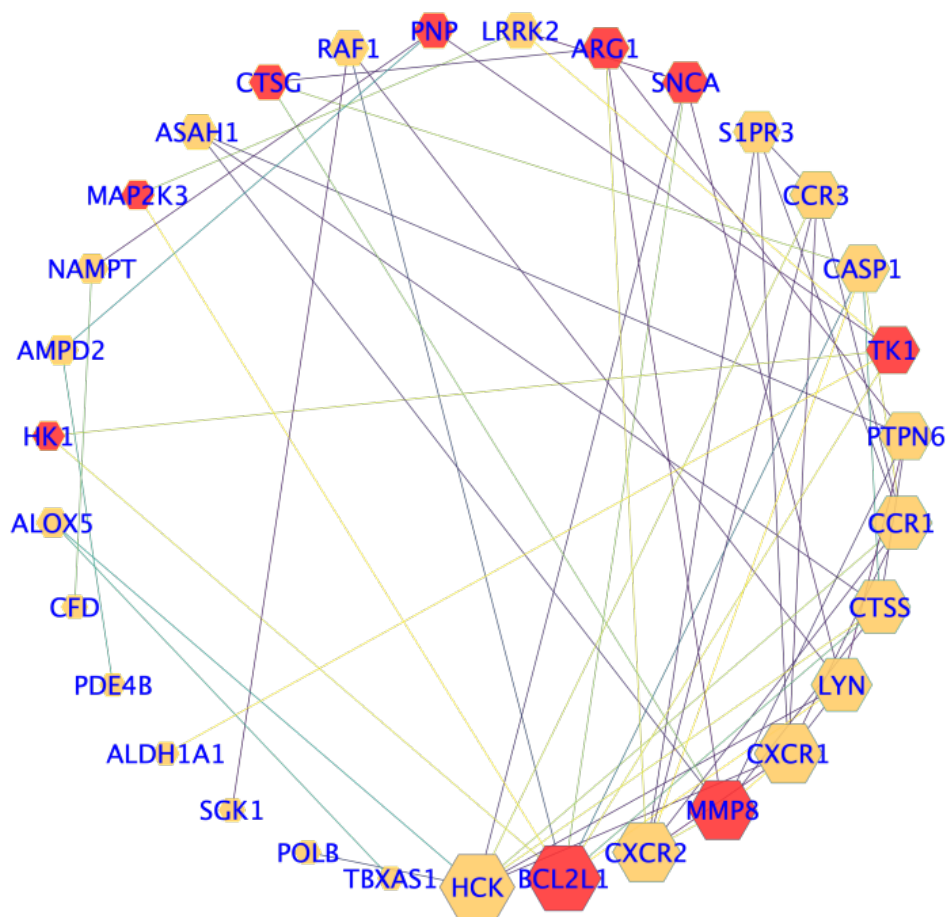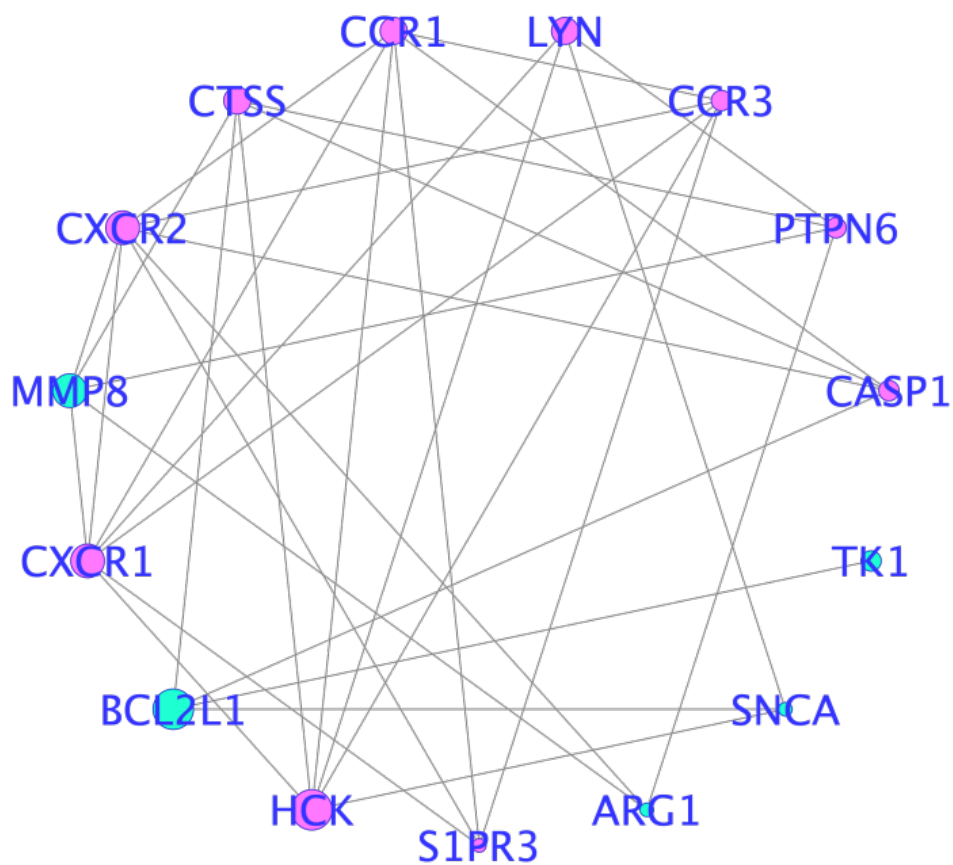

Fig. 5: PPI network of potential drug targets

Fig. 6: Subnetwork of potential drug targets
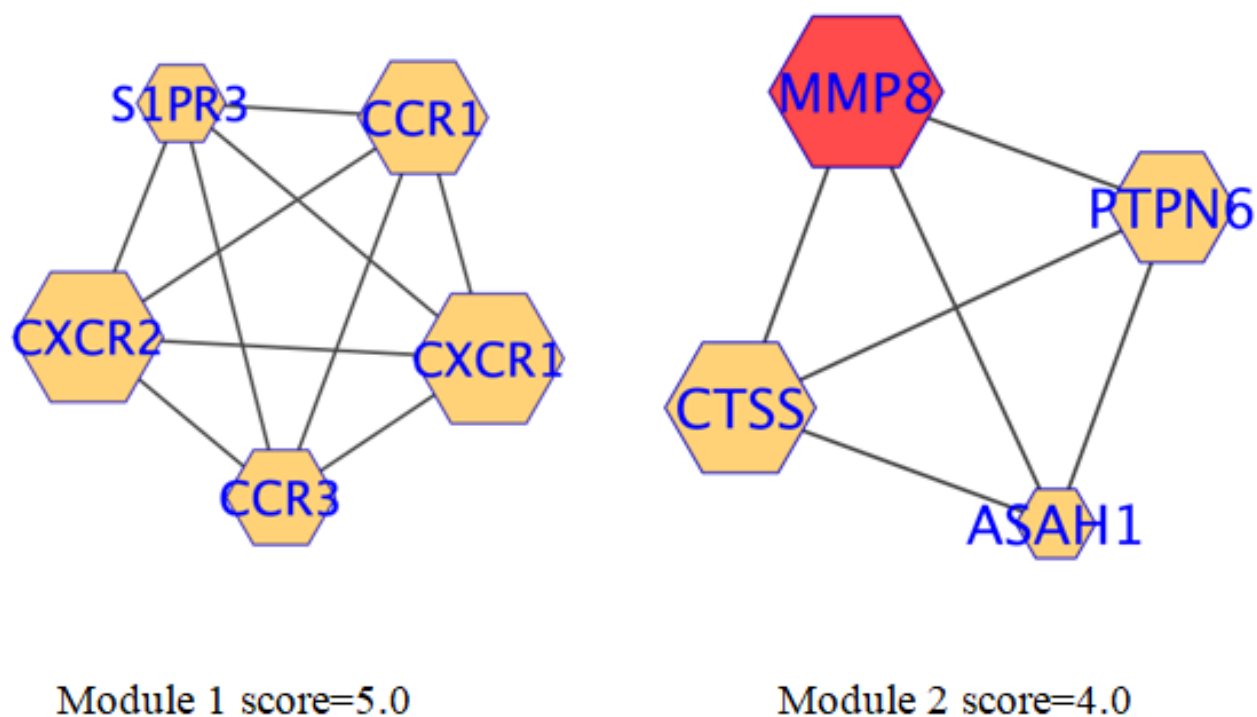

Module 1 score=5.0

Module 2 score=4.0

Fig. 7: MCODE of potential drug targets

We can analyze the potential drug targets in the DAVID database. The GO enrichment analysis results contain GO_BP, GO_CC and GO_MF. GO_BP is mainly enriched in inflammatory response, protein phosphorylation, chemotaxis, positive regulation of cell proliferation and chemokine-mediated signaling pathway. GO_CC is enriched in "cytosol", "mitochondrial outer membrane", "extracellular space", "cell" and "lysosome". GO_MF is enriched in "chemokine receptor activity", "protein serine/threonine kinase activity", "Adenosine Triphosphate (ATP) binding", "interleukin-8 receptor activity" and "interleukin-8 binding". The KEGG signaling pathway enrichment analysis results is mainly enriched in "chemokine signaling pathway" and the genes enriched in this signaling pathway are LYN, CCR1, CCR3, CXCR2, CXCR1, Raf-1 Proto-Oncogene, Serine/

Threonine Kinase (RAF1). The results of GO and KEGG enrichment analysis bubble chart are shown in fig. 8 and Table 1.

The core genes and genes enriched in chemokine signaling pathway were intersected to obtain 5 key targets of Xianling Gubao capsule in the treatment of SONFH, which were CCR1, CCR3, CXCR2, CXCR1 and HCK. In KEGG mapper, the interactions between key targets and their ligands are reached and the biological effects of key targets are searched (the result is shown in fig. 9). The results showed that the key targets of Xianling Gubao in the treatment of SONFH were mainly involved in promoting the growth of vascular endothelial cells, inhibiting the apoptosis of vascular endothelial cells, inducing the formation of new capillaries and the expression of vascular endothelial growth factor.
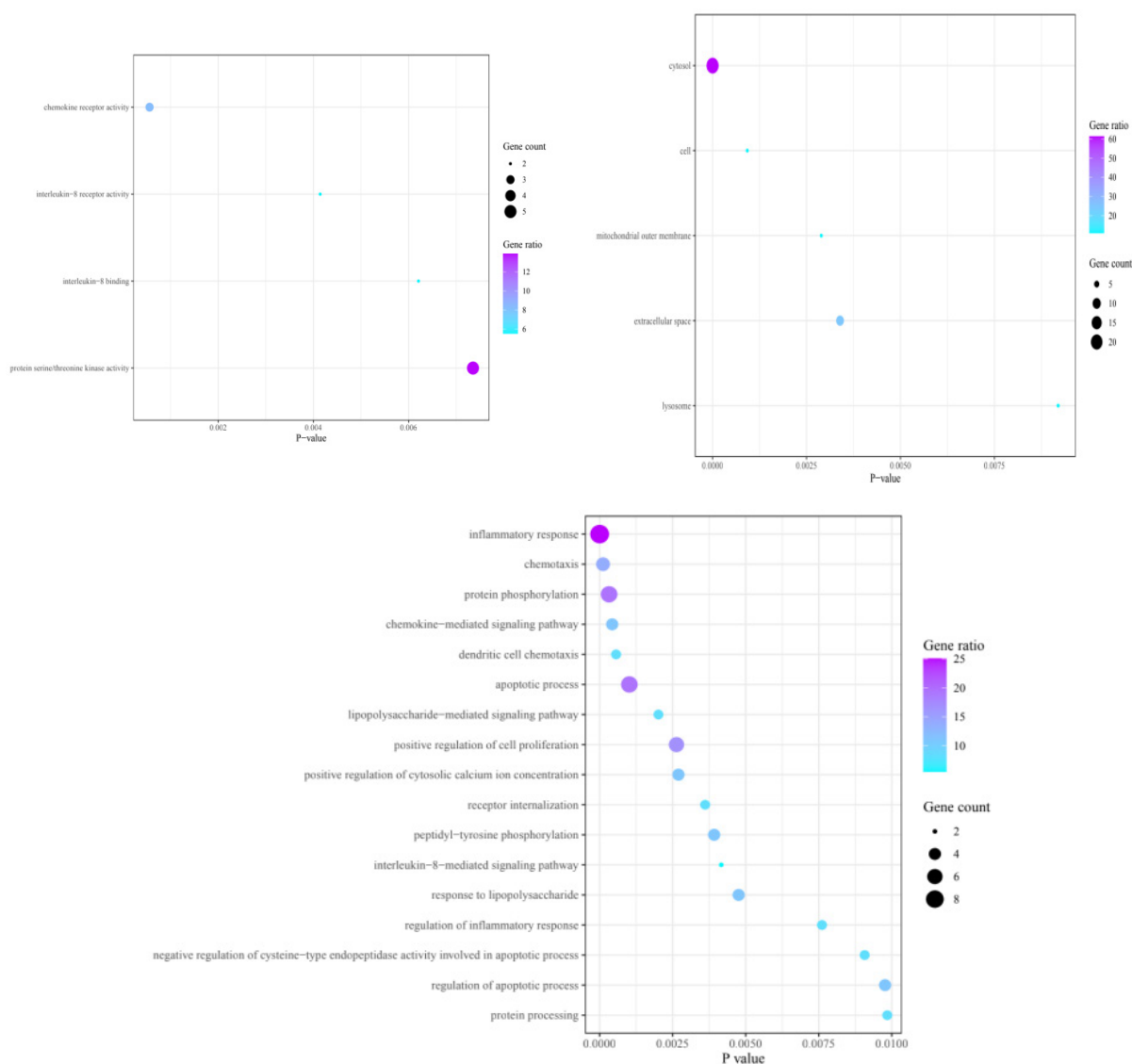

Fig. 8: GO enrichment analysis results, (A) Shows the result of GO_MF analysis; (B) Shows the result of GO_CC and (C) Shows the result of GO_BP analysis

TABLE 1: THE RESULTS OF GO AND KEGG ENRICHMENT ANALYSIS

Category	Description	Gene count	Gene ratio	p value	Genes
GO _BP	Inflammatory response	9	25	8.64E-07	LYN, CCR1, POLB, HCK, CXCR1, CXCR2, PTAFR, S1PR3, CCR3
	Chemotaxis	5	13.8889	1.17E-04	CCR1, CXCR1, CXCR2, PTAFR, CCR3
	Protein phosphorylation	7	19.4444	3.23E-04	LYN, RPS6KA3, HCK, CTSG, MAP3K8, RAF1, SGK1
	Chemokine-mediated signaling pathway	4	11.1111	4.30E-04	CCR1, CXCR1, CXCR2, CCR3
	Dendritic cell chemotaxis	3	8.33333	5.63E-04	CCR1, CXCR1, CXCR2
	Apoptotic process	7	19.4444	0.00102	RPS6KA3, CASP1, PTPN6, MAP3K8, RAF1, SGK1, SNCA
	Lipopolysaccharide-mediated signaling pathway	3	8.33333	0.00201	LYN, HCK, PTAFR
	Positive regulation of cell proliferation	6	16.6667	0.00263	LYN, HCK, CXCR2, PTPN6, S1PR3, BCL2L1
	Positive regulation of cytosolic calcium ion concentration	4	11.1111	0.0027	CCR1, CXCR2, S1PR3, CCR3
	Receptor internalization	3	8.33333	0.00361	CXCR1, CXCR2, SNCA
	Peptidyl-tyrosine phosphorylation	4	11.1111	0.00392	LYN, MAP2K3, HCK, PTPN6
	IL-8-mediated signaling pathway	2	5.55556	0.00416	CXCR1, CXCR2
	Response to lipopolysaccharide	4	11.1111	0.00476	RPS6KA3, CASP1, CTSG, SNCA
	Regulation of inflammatory response	3	8.33333	0.00761	LYN, HCK, CASP1
	Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	3	8.33333	0.00907	RPS6KA3, RAF1, SNCA
	Regulation of apoptotic process	4	11.1111	0.00977	CASP1, RAF1, SGK1, BCL2L1
	Protein processing	3	8.33333	0.00985	CASP1, CTSG, CTSS
GO _CC	Cytosol	22	61.1111	4.47E-08	LYN, MAP2K3, EGLN1, ARG1, AMPD2, HK1, HCK, RPS6KA3, CA1, PNP, CA2, ALOX5, ALDH1A1, CASP1, PDE4B, MAP3K8, PTPN6, TK1, RAF1, SGK1, BCL2L1, SNCA
	Cell	4	11.1111	9.24E-04	EGLN1, TBXAS1, CXCR2, HK1
	Mitochondrial outer membrane	4	11.1111	0.00289	ARG1, RAF1, BCL2L1, HK1
	Extracellular space	9	25	0.00339	CFD, ASAH1, CA2, ARG1, ALOX5, CTSG, MMP8, CTSS, SNCA
	Lysosome	4	11.1111	0.0092	HCK, ASAH1, CTSS, SNCA
GO_MF	Chemokine receptor activity	3	8.33333	5.57E-04	CCR1, CXCR1, CCR3
	IL-8 receptor activity	2	5.55556	0.00414	CXCR1, CXCR2
	IL-8 binding	2	5.55556	0.00621	CXCR1, CXCR2
	Protein serine/threonine kinase activity	5	13.8889	0.00735	MAP2K3, RPS6KA3, MAP3K8, RAF1, SGK1
KEGG_pathway	Chemokine signaling pathway	7	19.4444	2.58E-04	LYN, CCR1, HCK, CXCR1, CXCR2, RAF1, CCR3

Fig. 9: Research hypothesis map

We further analyzed the relationship between the key targets and the components of Xianling Gubao, and found that the direct targets are mainly enriched in 3 alpha (α)-hydroxytanshinone IIA (DS32), Isotanshinone II (DS41), Tanshinone diphenol (DS18), Dihydroflavone of *Psoralea corylifolia* (BGZ8), Dihydrodehydrogenisolone (YYH1), quercetin (YYH2). Among the five key targets, DS32 and DS41 can act on CCR1; BGZ8 can act on CCR3; DS18 and DS41 on CXCR2; DS18, DS41, YYH1 and YYH2 can act on CXCR1 and BGZ8 can act on HCK (Table 2).

The characteristic of SONFH is osteoblast death and articular surface collapse due to the interruption of blood supply of subchondral bone[4]. This destructive disease has become an increasing prominent problem in the world, mainly involving young and middle-age people, mostly bilateral, with a wide range of necrosis. If not treated properly, the disease is likely to develop to the advanced stage (Association Research Circulation Osseous classification (ARCO) III-IV).

The pathological mechanism of this disease is not clear, among them, the theory of mechanism are as follows: The theory of lipid metabolism disorder[5], blood hyper viscosity state[6], intravascular coagulation[7], osteoporosis[8] and vasculitis caused by immune complex antibody deposition[9], etc. The main pathogenesis is glucocorticoid deposition leading to blood viscosity, hemodynamic changes, vascular endothelial cells damaged at the shunt or turning point and microthrombosis is gradually formed[10]. At the same time, bone marrow stem cells are induced by glucocorticoid to differentiate into pathological adipocytes, adipocytes accumulate in the bone marrow cavity, the increased pressure leads to the obstruction of venous and accelerates the process of femoral head ischemia[11,12]. In addition, osteoporosis leads to the decrease of mechanical bearing capacity of bone trabecular and eventually avascular necrosis of the femoral head. Therefore, ischemia is the key to the disease, and the prevention and treatment of SONFH are based on the prevention and treatment of ischemia.

According to TCM, SONFH falls with the ambit of bone erosion, arthralgia of bone and bone fistula[13]. Glucocorticoid is pungent hot property. People who use glucocorticoids for long term were prone to chills, weakness, pale, loss of appetite and other manifestation. According to TCM syndrome (Yin-Yang deficiency)[14], kidney yin deficiency leads to warm and incongruous, wasting sperm and injuring bone marrow, kidney yang deficiency leads to slow and weak pulse which lose nourishment and obstruction of the blood circulation. Thus kidney deficiency and blood stasis appear. The principle of treatment should be in a way of tonifying kidney and strengthening bone, improving blood circulation and dispersing stasis[15]. The effectiveness of Xianling Gubao capsule is mainly nourishing liver and kidney, activating blood circulation, strengthening tendons and bones, and it is consistent with the treatment of SONFH[16,17]. Yin Yang Huo is good at invigorating kidney, strengthening Yang, strengthening tendons and bones, dispelling wind and removing dampness, which is a gentle medicine. Xu Duan is bitter and pungent but slightly warm, which is good at tonifying liver and kidney, strengthening waist and knee, and promoting blood circulation. Bu Gu Zhi is bitter and pungent but warm and dry, which is good at warming spleen and kidney. The two drugs are used together to help the monarch to nourish liver and kidney, strengthen tendons and bones, and promote blood circulation. Therefore, Xu Duan and Bu Gu Zhi are ministerial drug. Danshen is good in purifying blood, promoting blood circulation and removing stasis, degrading tendons and relieving pain. Dihuang tastes sweetness and bitterness, which is good at "getting rid of blood brain barrier and promoting bone marrow dryness".

TABLE 2: DOCKING TABLE OF TRADITIONAL CHINESE DRUG-INGREDIENT-TARGET

Traditional Chinese drug	Ingredient/PubChem CID	Ingredient name	Target
Salvia/Danshen	DS32/14610644	3α-hydroxytanshinone IIA	CCR1
	DS41/44425166	Isotanshinone II	CCR1/CXCR2/CXCR1
	DS18/11011966	Tanshinone diphenol	CXCR 2/CXCR1
Psoralea/Bu Gu Zhi	BGZ8/5321775	Psoralen dihydroflavonoids	CCR3/HCK
Epimedium/Yin Yang Huo	YYH1/71599997	Dihydrodehydroconiferyl alcohol	CXCR1/HCK
	YYH2/5280343	Quercetin	CXCR1

The combination of 3 d can increase the function of nourishing liver and kidney, strengthening the tendons and strengthening the bones and also inhibiting the dryness of Yin Yang Huo and Bu Guzhi. Therefore, these three drugs are adjuvants.

In this study, CCR1, CCR3, CXCR1, CXCR2 and HCK are the core targets of Xianling Gubao capsule in the treatment of SONFH. In gene enrichment analysis, these core targets were enriched in the chemokine signaling pathway (fig. 9). CCR1, CCR3, CXCR1 and CXCR2 are chemokine receptors. They are important members of G Protein-Coupled Receptor (GPCR) superfamily. They contain a short acidic N-terminal, seven helical transmembrane structures, three intracellular and extracellular hydrophilic rings and a C-terminal containing serine and threonine residues[18]. It is usually expressed on the membrane of neutrophils, immune cells and endothelial cells[19]. The extracellular domain of chemokine receptor contains an active pocket that can interact with chemokines. After being stimulated by the corresponding chemokines, serine, threonine in the intracellular domain activates the downstream active factors inside the cell to play a cascade reaction, regulate the biological effects of cytoplasm or nucleus and affect cell division, proliferation, apoptosis and production of cytokines and oxygen free radicals, the process of birth and so on[20]. Therefore, GPCR is an important target for disease treatment and 40 % of the drugs in the market target GPCR[21].

CXCR1 and CXCR2 are also known as Interleukin-8 Receptor Alpha (IL-8RA) and Interleukin-8 Receptor Beta (IL-8RB), which are the receptor of IL-8 (C-X-C Motif Chemokine Ligand 8 (CXCL8)). CXCL8 is mainly secreted by macrophage. It can also be secreted by epithelial cells and endothelial cells when stimulated[22]. CXCL8 combines with the receptors of CXCR1 or CXCR2 to participate in the chemotaxis of centrioles and has a strong role in promoting angiogenesis at the same time[23]. Yang et al. found that IL-8 can activate Matrix Metalloproteinases (MMPs), Protein kinase B (Akt) and other downstream signaling pathways by binding CXCR1/CXCR2, and participate in the process of tumor cell proliferation and tumor angiogenesis[24]. Li et al. found that the disorder of CXCL6/CXCR2 system in low hypoxia leads to inhibition of vascular remodeling and aggravate the process of vascular injury[25]. Yu et al. found that CXCL5/CXCR2 can aggravate the ischemia-reperfusion injury of human microvascular endothelial cells[26].

CCR3 is the C-C style receptor of chemokine, which can combine with C-C Motif Chemokine Ligand 5 (CCL5) and adjust the differentiation process of osteoblasts and osteoclasts. A large number of studies have shown that CCR3 deficiency is closely related to tumor bone metastasis. The main reason is that CCR3 deficiency leads to the increase of osteoclast activity, imbalance of osteogenic and osteoclast differentiation and bone loss. Rosendahl et al. found that CCR3 deletion is associated with bone loss after bone metastasis of non-small cell lung cancer[27]. Yamaguchi et al. found that CCR3 deletion can affects the bone metastasis of breast cancer which leads to the increase of osteoclast activity and the decrease of cortical bone mass[28]. Rosendahl et al. found that CCR3 has closely relationship with the decrease of bone loss in mice[27]. At the same time, CCR3 can promote angiogenesis in combination with other chemokines. Liu et al. found that CCR3 can promote the angiogenesis of pterygium[29]. Nagai et al. found that CCR3 has closely relationship with choroidal neovascularization and vascular permeability[30].

CCR1 also belongs to chemokine C-C receptor, which can bind to a variety of chemokines. Binding with CCL7 and CCL13 mainly plays the role of chemotaxis to inflammatory cells, while binding with CCL3 (Macrophage Inflammatory Protein-1 Alpha (MIP-1α)) can affect the differentiation and survival of cells. MIP-1α is an important osteoclast activating factor, MIP-1α can activate osteoclast differentiation of bone marrow stem cells, resulting in the decrease of bone density[31]. In addition, MIP-1α can also act on CCR1 binding on erythroid precursor cells and inhibit the generation of erythroid cells[32].

The protein which is encoded by recombinant Hematopoietic Cell Kinase (HCK) gene is a member of tyrosine kinase Src family. It is mainly expressed in myeloid cells and this protein is mainly involved in the process of cellular hematopoiesis. As a downstream key protein of chemokine receptor, Src can active Ras/Raf, Phosphoinositide-3 kinase (PI3K)/Akt and the process, and regulate cytokine, cell proliferation, differentiation and apoptosis[33]. It can also regulate actin cytoskeleton and promote the chemotaxis of inflammatory cells (fig. 9)[34]. But beyond that, HCK may also participate in the development of chondrocyte. Matsuura et al. found that HCK activated Akt, Extracellular Signal-Regulated Kinase (ERK), Signal Transducers and Activators of Transcription (STAT) and promoted chondrocyte proliferation and inhibited its apoptosis mainly through Wnt and Hedgehog signaling pathways[35]. The ligands and mechanism of action of the five key targets are

shown in Table 3.

At the stage of SONFH, the expression levels of CCR1, CCR3, CXCR1, CXCR2 and HCK reduce to lower levels. The decreased expression of chemokine receptors (CCR1, CCR3, CXCR1 and CXCR2) results in the obstruction and activation of chemokine signaling pathway. The expression levels of HCK decreased, the downstream Mitogen-Activated Protein Kinase (MAPK) and PI3K/Akt pathways were blocked. The obstruction of chemokine signaling pathway leads to the difficulty of neovascularization, osteogenic differentiation of bone tissue, accumulation of metabolites and accumulation of adipocytes, which will accelerate the progress of SONFH.

At present, Xianling Gubao capsule is commonly used in the adjuvant treatment of facture, osteoporosis and femoral head necrosis, which mainly plays the role of promoting blood circulation and removing blood stasis. Xianling Gubao capsule can be used in the treatment of SONFH, promote the reconstruction of injured blood vessels, bone marrow stem cells, improve the local oxygen partial pressure hypoxia microenviroment and accelerate the circulation of metabolites[36-38]. Zheng *et al.* found that Xianling Gubao capsule can prevent the occurrence and progress of SONFH, but the specific mechanism is not clear[39].

Through this study, we found that Xianling Gubao capsule, a compound drug of TCM, contains six kinds of Chinese herbal medicines and 76 active ingredients. Among them, six chemical components from *Salvia miltiorrhiza*, *Psoralea corylifolia* and *Epimedium* play a role in the treatment of steroid induced osteonecrosis of the femoral head. These chemical components are mainly used as five potential drug targets, CCR1, CCR3, CXCR1, CXCR2 and HCK, which are involved in the regulation of inflammatory response, the production of cytokines, cell differentiation, proliferation and apoptosis, microcirculation reconstruction and many other physiological changes, due to the complex chemical composition and mechanism of action of TCM.

Direct experimental research direction is not clear and will produce a large consumption. Through network pharmacology analysis and bioinformatics analysis, the effective chemical components of Xianling Gubao capsule were screened out, including DS32, DS41, DS18, BGZ8, YYH1 and YYH2. We can gradually study the synergistic or antagonistic effects of various chemical components, adjust the dose ratio, in order to develop a more reasonable drug for the treatment of steroid induced osteonecrosis of the femoral head.

TABLE 3: LIGANDS OF KEY TARGETS AND THEIR MAIN MECHANISMS OF ACTION

Receptor	Ligand	Mechanism
CCR1	CCL7 (MCP-3), CCL13 (MCP-4), CCL3 (MIP-1α)	Chemotaxis of inflammatory cells; osteoclast differentiation and activation; inhibition of erythroid differentiation of progenitor cells; invasion and metastasis of tumor cells
CCR3	CCL7 (MCP-3), CCL13 (MCP-4), eotaxin-1,2, CCL5 (RANTES)	Chemotaxis of inflammatory cells; osteoclast differentiation and activation; promote bone metastasis of tumor; promote the formation of new capillaries; invasion and metastasis of tumor cells
CXCR1	CXCL1 (IL-8RA), CXCL6 (GCP-2)	Inflammation; immunoreaction; cell growth; metastasis; regulation the process of vascular remodeling; invasion and metastasis of tumor cells
CXCR2	CXCL2 (IL-8RB), CXCL6 (GCP-2), CXCL3, CXCL5 (ENA-78), CXCL7 (NAP-2), LIX	Inflammation; immunoreaction; cell growth; metastasis; regulation the process of vascular remodeling; regulation the process of organs or tissues after ischemia-reperfusion injury; invasion and metastasis of tumor cells
HCK	G protein alpha i subunit (Gαi)	Cytokine production; cellular growth and differentiation; cell survival; migration; apoptosis; cellular shape changes; Reactive Oxygen Species (ROS) production

In conclusion, Xianling Gubao capsule acts on CCR1, CCR3, CXCR2, CXCR1 and HCK targets in chemokine signaling pathway, and plays a role in prevention or treatment of SONFH by promoting the reconstruction of new capillaries after necrosis, improving intravascular coagulation, accelerating cells formation, and regulating the differentiation and direction of bone marrow stem cells.

Conflict of interests:

The authors declared no conflict of interest.

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