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Original Article

Analysis of the pharmacological mechanism of Roucongrong in treating osteoporosis based on a biological network module

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Abstract: Background: The network pharmacology method can be used to predict the active components, targets, and key signal transduction pathways of Roucongrong. This method has been explored in the treatment of osteoporosis. Methods: The effective ingredients and targets of Roucongrong were obtained by searching the TCMSP database. By combining OMIM, and the GeneCards database, the disease targets of osteoporosis were obtained. We determined the common goals of drugs and diseases and performed PPI network analysis on the STRING platform. The obtained data was constructed using Cytoscape software to construct a network module to perform functional annotation of GO terminology and analysis of the KEGG signal path. Results: A total of 6 active ingredients were screened and identified from Roucongrong, and 223 potential targets of the active ingredients were identified from traditional Chinese medicine. In addition, we found 1086 osteoporosis-related targets in two complete databases, and determined the intersection of disease targets and potential targets of the active ingredients; a total of 59 common targets were identified. The results show that the mechanism of Roucongrong in the treatment of osteoporosis is related to aging, response to drug, angiogenesis, positive regulation of gene expression, extracellular space, extracellular region, cytokine activity, and enzyme binding, mainly through Rheumatoid arthritis, Chagas disease (American trypanosomiasis) and Pathways in cancer to achieve the therapeutic effect. Conclusions: The mechanism of Roucongrong in the treatment of osteoporosis is directly or indirectly related to multiple signaling pathways, mainly involving aging and cytokine activity, as well as response to drug, angiogenesis, positive regulation of gene expression, extracellular space, extracellular region, and enzyme binding, etc. Systematic comprehensive intervention is used to achieve the effect of treating osteoporosis. It provides a theoretical reference for further research on the material basis and mechanism of action of Roucongrong on anti-osteoporosis.

Keywords: Roncongrong, osteoporosis, network pharmacology, signal pathway, target, module analysis

Introduction

Osteoporosis (OP) is a kind of bone disease that is characterized by decreased bone mass, abnormal bone tissue structure, increased bone fragility, and increased risk of secondary fractures [1]. The main clinical manifestations are spinal deformity, bone pain throughout the body, and easy fracture [2]. With the continuous increase of the aging population of the world, the incidence of osteoporosis and osteoporotic fractures continues to rise, which seriously affects the daily quality of life for seniors [3-5]. Some studies have shown that western medicine still has many shortcomings in the treatment of osteoporosis, such as adverse reactions after taking medicine and drug intolerance problems [6]. In recent years, with the continuous development of traditional Chinese medicine, the effectiveness of traditional Chinese medicine in the treatment of osteoporosis has also been confirmed.

According to traditional Chinese medicine, osteoporosis belongs to the category of "bone atrophy", and its disease is located in kidney and spleen meridians, in which kidney deficiency is the cause of OP. Cistanche cistanche, sci-

entific name: Cistanches Herba Ma, alias Cunyun, Cistanches Herba Y. C. Ma or Cistanche tubulosa (Schrenk), has dry fleshy stem with scales and leaves, and it has the effects of nourishing kidney yang, as well as nourishing essence, and the blood. Modern studies have found that Roucongrong is rich in 12 kinds of phenylethanol glycosides, 11 kinds of lignans, 5 kinds of Roucongrong monosaccharides, 17 kinds of amino acids such as valine, lysine, methionine, arginine, and 10 kinds of trace elements such as Mn, Fe, Cu, Zn, Se, Sr, Mo, I, Ca. At the same time, the modern pharmacological research results of Roucongrong have shown that Roucongrong can increase the serum alkaline phosphatase (ALP), osteocalcin and calcium levels in osteoporosis model of rats, and promote the expression of bone morphogenetic protein 2 (BMP2) in rat osteoblasts, as well as speed up the proliferation of bone marrow mesenchymal stem cells, and promote the synthesis of ALP by rat osteoblasts. It can be seen that Roucongrong has a certain anti-osteoporosis pharmacological basis. However, due to the complexity of the components of traditional Chinese medicine, the traditional monomer research strategy cannot fully explain the relevant mechanism of Roucongrong in the treatment of osteoporosis. Therefore, it is necessary to study the biological process of drug, disease, genes, and protein interaction at the overall level.

Methods

Screening of the active components and prediction of the targets of Roucongrong

We first searched all the chemical composition information of Roucongrong deserticola through the TCMSP (https://tcmspw.com/tcmsp.php) database [7]. According to the recommended screening threshold conditions we used, oral availability (OB) greater than 30% and drug-like properties (DL) greater than 0.18 as the limiting conditions. When OB≥30% and DL≥0.18, the key active ingredients of traditional Chinese medicine were obtained [8, 9]. Then we entered the candidate active ingredients into the PubChem (https://pubchem.ncbi.nlm.nih.gov) database to search and download the molecular structure formula, PubChem CID of the corresponding chemical compositions, targets, and obtained the 3D structure of each ingredient, and saved them into mol2 format. By combining the data in the relevant literature the obtained active ingredients of Roucongrong were analyzed. By using the UniProtKB search in the UniProt (http://www.uniprot.org/) database to enter the predicted target protein name, we set the species to Homo sapiens, and corrected the target protein to the official symbol.

Assessment of the disease targets for osteoporosis

With “osteoporosis” or “OP” as the keyword, collected and screened the target genes of osteoporosis through the comprehensive human Mendelian Inheritance Database (OMIM) (https://omim.org/) [10] and the GeneCards Database (https://www.genecards.org/) [11]. Combining the target genes of each disease database and eliminating duplicate disease target genes. Finally, the known disease targets of osteoporosis were obtained.

Constructing the database of intersecting drug-disease target genes

The herbal medicine targets in Roucongrong and the disease targets of osteoporosis were separately imported into Venny (https://bioinfogp.cnb.csic.es/tools/venny) online tools to construct drugs that intersect with Roucongrong and osteoporosis Disease target database.

Construction of the protein interaction network

The STRING [12] network database platform is used to construct the PPI network to further clarify the interactions within common target genes in the treatment of osteoporosis with Roucongrong. The protein species was set to “Homo sapiens” and the minimum interaction threshold were set to “Highest Confidence”. Then we constructed a visual network diagram to obtain protein interaction data. The data was saved in TSV format and visualized in the form of a PPI network. We imported the TSV data into Cytoscape software [13], and drew the protein interaction network, using Cytoscape tool NetworkAnalyzer for network analysis, and we set the node size and color to reflect the Degree (connection degree, the number of edges passing through the point in the network) Value, the thickness of the edge is set to reflect the size of the binding score, and finally the interaction network between the targets was obtained.

Table 1. General information of active ingredients of Roucongrong

Mol ID	Molecule Name	OB	DL
MOL000358	Beta-sitosterol	36.91	0.75
MOL005320	Arachidonate	45.57	0.20
MOL005384	Suchilactone	57.52	0.56
MOL007563	Yangambin	57.53	0.81
MOL000098	Quercetin	46.43	0.28
MOL008871	Marckine	37.05	0.69

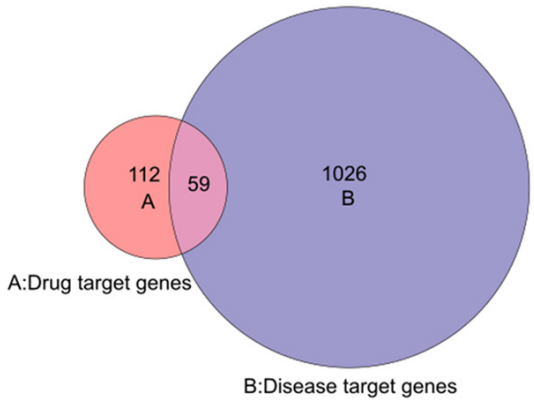

Figure 1. Number of potential targets of Roucongrong in the treatment of osteoporosis.

Biological process and pathway analysis

The GO pathway and KEGG pathway were enriched and analyzed by DAVID [14] on the target of Roucongrong. Among them, GO pathway enrichment analysis was divided into three parts: Cellular component (CC), Molecular function (MF), and Biological process (BP). We set the threshold at $P<0.05$, sorted according to the number of targets involved, and screened the biological processes with more than 10 targets and the top 10 pathways and to draw the GO pathway analysis and KEGG pathway analysis diagrams based on the results obtained.

Molecular docking

Using the above results to obtain the Degree value in the interaction network, the top 6 molecules with the Degree value and the active ingredients of Roucongrong were verified by the Sailvina software. We saved the results of molecular docking and perform a Docking Score on them to evaluate the binding activity between the active ingredients of Roucongrong and the target.

Component-target-pathway network construction

Screening pathways that were closely related to the treatment of osteoporosis, were found by combining research data and literature search data. We obtained potential targets that were enriched in these pathways, and corresponded to the corresponding active ingredients, constructing the "components of Roucongrong for the treatment of osteoporosis-Target-pathway" multi-dimensional network relationship diagram.

Results

Active components and target selection of Roucongrong

Through searching the TCMSP database with OB>30%, DL>0.18 as the screening criteria, a total of 6 eligible active ingredients of Roucongrong were collected, including beta-sitosterol, arachidonate, suchilactone, Yangambin, quercetin, Marckineand. The main active ingredients are shown in **Table 1**. Using the TCMSP database to search for the corresponding targets of these 6 active ingredients, a total of 223 potential targets were obtained. After eliminating duplicate targets, the predicted targets of 112 herbal medicines were finally obtained.

Screening disease targets for osteoporosis

Through the OMIM and GeneCards databases, we searched and screened the known target genes which are related to osteoporosis, deleted duplicate disease targets, and obtained all 1086 potential targets. Then, the obtained Roucongrong and osteoporosis targets were imported into the Venny graph online tool. After analysis, a total of 59 treatment targets for Roucongrong for osteoporosis were obtained (**Figure 1**).

Constructing the PPI network of shared targets

Import the 59 target genes obtained in the previous step into the STRING software platform for data analysis to generate a PPI network diagram. The constructed network contains 59 protein-protein interaction targets, and 172 represent the edge of protein-protein interaction. The average node degree value in the network is 5.83, and the average central clustering coefficient is 0.463 which is showing in **Figure**

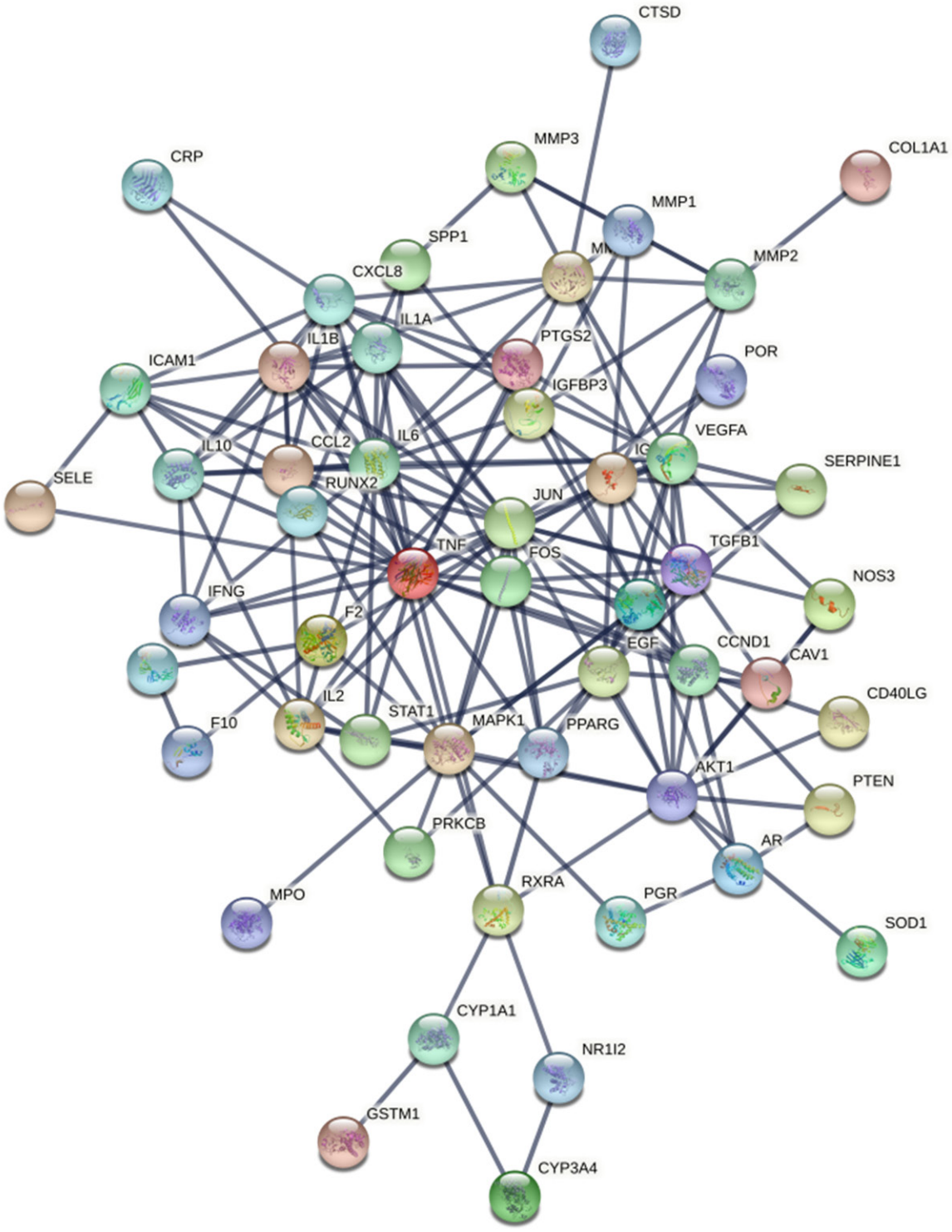

Figure 2. PPI network diagram of potential targets of Roucongrong in the treatment of osteoporosis based on String.

2. Then the data was imported into Cytoscape software, and the Roucongrong protein interaction network was simulated and comprehensively evaluated. Finally, a network module composed of 53 targets was obtained. The proteins contained in these modules may play an important role in the treatment of osteoporosis with Roucongrong which is shown in **Figure 3**.

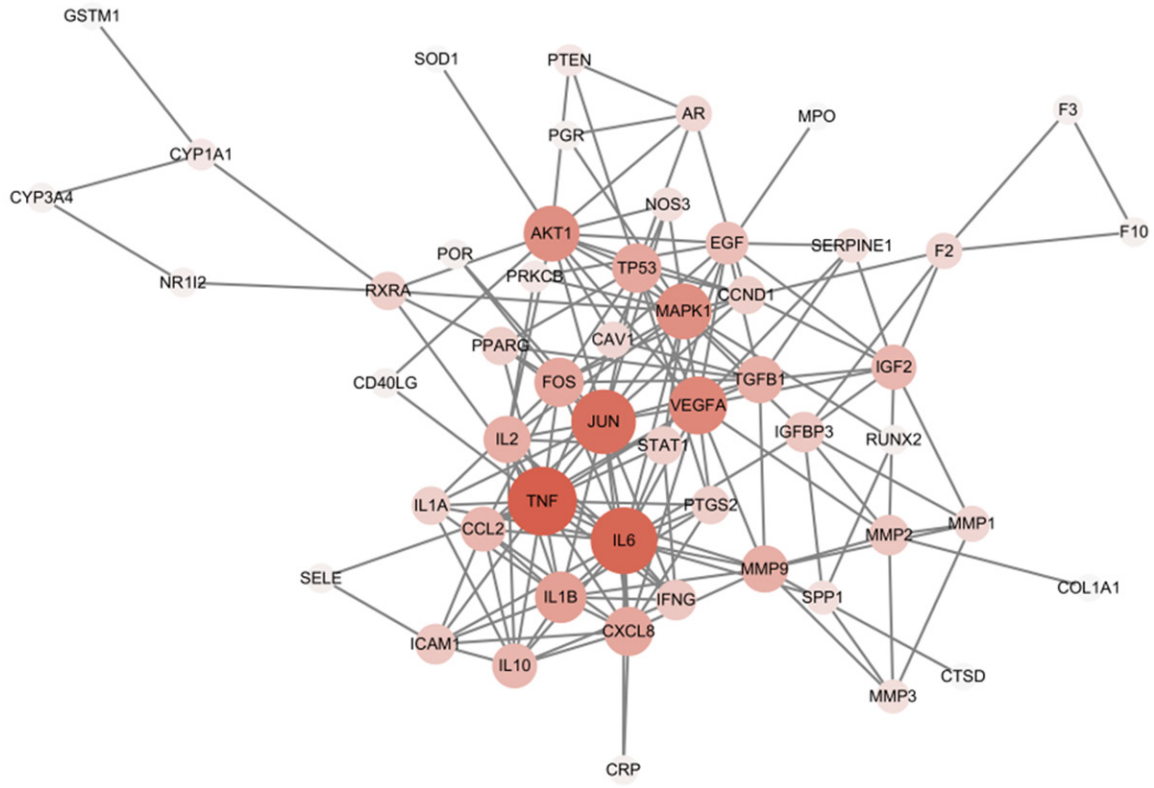

Figure 3. Active component target network treated by Cytoscape

GO biological processes of common target genes and KEGG pathway analysis

The 66 common target genes screened above were mapped for enrichment analysis of the GO terms and KEGG pathways displayed in the chart shown in **Figure 4**. GO biological process analysis showed that aging, response to drug, angiogenesis, and positive regulation of gene expression in BP, extracellular space, the extracellular region in CC, and cytokine activity and enzyme binding in MF play an important role in the biological process of Roucongrong in the treatment of osteoporosis. KEGG signaling pathway analysis shows that Rheumatoid arthritis, Chagas disease (American trypanosomiasis), and Pathways in cancer play an important role in the treatment of osteoporosis, as shown in **Figure 5**.

Discussion

Research results suggest that Roucongrong may affect the onset of osteoporosis through multiple components, multiple targets, and multiple pathways. The PPI protein interaction network also shows that the target protein is a process of multi-channel, mutual regulation, and mutual action, rather than a separate mechanism of action. The five core genes of Roucongrong in the treatment of osteoporosis are TNF, JUN, IL6, MAPK1, VEGFA, AKT1. Tumor necrosis factor-alpha (TNF-α) is a multifunctional cytokine produced mainly by monocytes-macrophages, B cells, NK cells, T cells, endothelial cells, etc. It is also the key to bone metabolism and remodeling and regulatory factors [15]. It can directly regulate the proliferation and differentiation of osteoclasts and osteoblasts, and affect bone metabolism; it can also damage the renal blood vessel-basement membrane barrier, resulting in increased vascular permeability, and decreased absorption of calcium and phosphorus by renal tubules. Impairment of calcium and phosphorus transport, leads to bone calcium and phosphorus metabolism disorders and decreased renal hydroxylase activity, and affects bone metabolism [16, 17]. It can also affect the bone microenvironment and aggravate the oxidative stress response by regulating the metabolic activity of

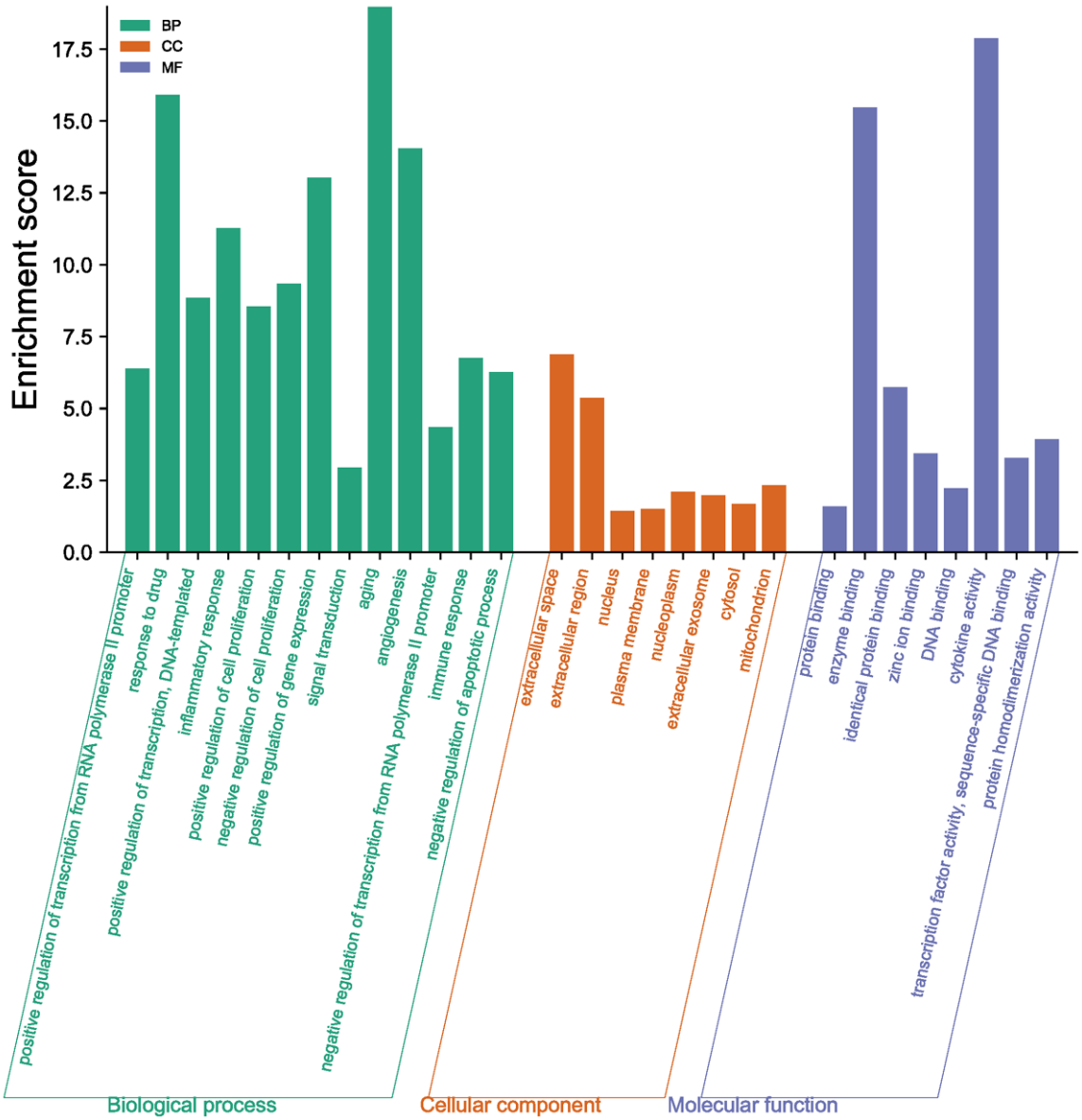

Figure 4. Go enrichment analysis of Roucongrong in the treatment of osteoporosis.

other tissues and promoting the synthesis and release of other cytokines [18]. JUN gene, as a kind of rapid response gene, acts as a transcription factor by forming a homodimer with its JUN protein or forming a dimer AP-1 with FOS protein. It is known that many osteogenic-related genes, such as TGF-β, IGF-1, IGF-II, BMP, ALP, type I collagen, etc., have AP-1 sites. As an important part of AP-1, JUN protein is an activator that promotes cell differentiation, so it also plays an important role in bone formation and regulating the expression of osteoblast-specific genes [19, 20]. The structure of the JUNB gene is very similar to JUN. JUNB can also directly activate the transcription of cyclin A to promote the growth of osteoblasts and chondrocytes [21]. IL6 is a multi-effect cytokine, which was first discovered to promote the differentiation and maturation of osteoblasts. Related research has pointed out [22] that endogenous cytokines (RANKL and IL6) can directly inhibit the proliferation and differentiation of osteoblasts, and can also stimulate the formation of osteoclasts. Other reports [23] also pointed out that under the co-culture condition of osteoblasts and osteoclast precur-

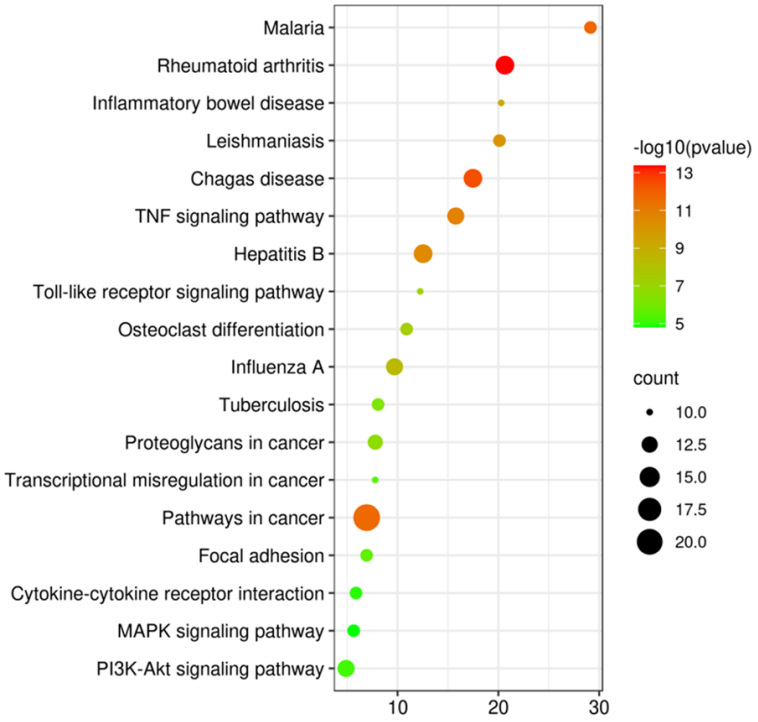

Figure 5. KEGG signaling pathway analysis of Roucongrong in the treatment of osteoporosis.

sors, the IL6/gp130/Jak-Stat3 pathway can promote the production of RANKL in osteoblasts, and RANKL binds to its receptor RANK to activate NFκB, Erk1/2, p38/MAP kinase, and other signals to promote the differentiation and activity of osteoclast cell lines. *In vivo* experiments have found that IL6 activation of gp130 can not only enhance the promotion of osteoclast differentiation and maturation of osteoclasts but also inhibit the signal transduction pathway of osteoclast precursors to osteoclast differentiation [24]. MAPK is a type of serine/threonine-protein kinase in cells that can regulate cell growth and differentiation. Experiments have shown that activating the MAPK signaling pathway can promote the mineralization of osteoblasts and the expression of ALP and BMP-2, thereby promoting bone formation. When ERK, a downstream factor of MAPK, is inactivated, it can inhibit the formation of osteoclasts [25]. Vascular endothelial growth factor (VEGF) is the primary factor for the growth and differentiation of vascular endothelial cells, which is very critical in the process of angiogenesis. Usually, VEGF refers to VEGF-A, and its binding receptors include vascular endothelial growth factor receptor 1 (VEGFR1) and receptor 2 (VEGFR2) [26]. Relevant studies have shown that, compared with patients without osteoporosis, postmenopausal patients with osteoporosis show lower VEGFA levels [27]. AKT1 is a member of the AKT (protein kinase B) gene family. AKT1 is identified as a unique signal transduction intermediate in osteoblasts, which can simultaneously control osteoblast and osteoclast damage [28]. Studies have found that after the AKT1 gene knockout treatment in rats, rats have restricted bone development, suggesting that AKTI may play a role in promoting bone growth in bone development [29].

The KEGG pathway enrichment analysis results show that the mechanism of Roucongrong in the treatment of osteoporosis is related to aging, response to drug, angiogenesis, positive regulation of gene expression, extracellular space, extracellular region, cytokine activity, and enzyme binding, mainly through Rheumatoid arthritis, Chagas disease (American trypanosomiasis) and Pathways in cancer to achieve the therapeutic effect. Studies also show that the Toll-like receptor signaling pathway has a certain role in the treatment of osteoporosis with Cistanche. The toll-like receptor is a pattern recognition receptor in the body's innate immune system that recognizes exogenous and endogenous ligands. TLR4 is a subtype and an important member of the Toll protein. Studies have found that the TLR4 signaling pathway is divided into My D88-dependent and non-dependent signaling pathways, which require the participation of MD2, TLR4, and CD14 to activate [30, 31]. Related studies have found that the TLR4 pathway can inhibit the differentiation of bone marrow mesenchymal stem cells into osteoblasts by activating the ERK pathway, and inhibit the expression of the SP7 gene through the My D88-dependent pathway, exerting an inhibitory effect on the early phase of osteoblast differentiation [32]. The TLR4 pathway can also reduce important markers in the process of osteoblast

mineralization by activating the downstream ERK pathway, such as ALP, OPN, and other important factors that affect the mineralization of osteoblasts, and ultimately affect the mineralization of osteoblasts [33, 34].

Conclusions

In summary, this paper studied the relevant therapeutic mechanism of Roucongrong in the treatment of osteoporosis from the biological process, signal pathway, protein, and other aspects through the network pharmacology and biological modules. In addition, we also explored the correlation between the efficacy of Roucongrong in the treatment of osteoporosis and the key signal pathways related to diseases in this study. The development of traditional Chinese medicine has been integrated with modern network pharmacology research technology. Analyzing the basic mechanism and related methods of traditional Chinese medicine efficacy through modern visualization technology will also become a new direction for in-depth exploration of traditional Chinese medicine. Through accurate and in-depth exploratory research, it will also provide more reliable reference values for subsequent clinical experiments and animal experiment verification to ensure the correct direction of exploratory research.

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Disclosure of conflict of interest

None.

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