Network Pharmacology and Metabolomic Effects in vivo of Fufang Duzhong Jiangu Granules for the Treatment of Kashin-Beck Disease

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Keywords: Fufang Duzhong Jiangu Granules, Kashin-Beck Disease, Network Pharmacology, Metabolomic Effect

Posted Date: October 24th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3462754/v1

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Additional Declarations: No competing interests reported.
Abstract

**Background:** Fufang Duzhong Jiangu Granules (FDJG) is used clinically for treating swelling, pain and functional impairment caused by osteoarthropathy. However, the potential therapeutic mechanisms of FDJG for Kashin-Beck Disease (KBD) remain unclear.

**Objective:** Our study aims to predict the drug efficacy and molecular mechanisms of FDJG in the treatment of KBD based on network pharmacology, metabolomics and molecular docking.

**Methods:** The active ingredients and target proteins of FDJG were obtained from TCMSP database, and analyzed in conjunction with the differential genes of KBD. GO and KEGG enrichment analysis, PPI network construction and topological characteristics evaluation, molecular docking were performed to explore gene function and potential mechanisms of FDJG in the treatment of KBD. Furthermore, FDJG treatment for one month was administered to patients with KBD, and some differentially expressed genes and metabolic effects before and after treatment were measured using qPCR and nontargeted metabolomics methods. Further gene-metabolism joint pathway analysis was conducted.

**Results:** There were 151 genes which are the therapeutic targets of FDJG in the treatment of KBD. 48 core target proteins were mainly enriched in PI3K-Akt signaling pathway, TNF signaling pathway, MAPK signaling pathway, apoptosis and osteoclast differentiation. Quercetin, kaempferol and luteolin in FDJG could strongly bind to TP53, STAT3, HSP90AA1, etc., which had important anti-inflammatory and anti-apoptotic effects in the treatment of KBD. After one-month FDJG treatment, the RNA expression levels of STAT3, FOS and RELA in peripheral blood of KBD patients were significantly down-regulated. A total of 80 differential expressed metabolites were identified in the plasma of KBD patients. Drug targets and differential metabolites were co-enriched in four metabolic pathways: glycerophospholipid metabolism, inositol phosphate metabolism, phosphatidylinositol signaling system, and steroid hormone biosynthesis.

**Conclusion:** FDJG may effectively treat KBD by anti-inflammatory and regulating abnormal lipid metabolism pathway, which has great potential in the treatment of patients with KBD.

1. Introduction

Kashin-Beck disease (KBD) is a complex endemic osteoarthropathy with a primary etiologic mechanism of deep chondrocyte necrosis induced by environmental risk factors. The main feature of KBD is short stature due to multiple focal necrosis of the tubular bone growth plates, which leads to secondary, even severe, deformational osteoarthropathy\(^1,2\). Over the past few decades, a number of preventive measures have been taken, and the occurrence and development of KBD have been effectively controlled in recent decades. However, KBD in adults remains a serious problem because of its high prevalence in China in the last century. There are also no effective and special clinical measures to repair cartilage damage or defects in KBD. Patients with KBD in endemic areas still face serious disease and economic burdens, so it is important to develop targeted treatments.
Traditional treatments for KBD include non-surgical therapies, such as non-steroidal anti-inflammatory drugs, and surgical therapies, such as joint replacement for advanced pain. Emerging therapies, such as platelet-rich plasma, stem cell therapy, and extracellular vesicles, also developing\cite{3-7}. Despite the currently available therapies are various, there remains an unmet medical need for KBD treatment. For thousands of years, traditional Chinese medicine (TCM) has been widely used to prevent and treat various diseases with its remarkable curative effect and high safety. At present, more and more scientific evidence shows that a large number of TCM drugs take good effect in the treatment of osteoarthritis and reduce the occurrence of side effects\cite{8,9}. Fufang Duzhong Jiangu Granules (FDJG) is composed of 12 kinds of traditional Chinese medicine: *Radix Paeoniae Alba, Radix Angelicae Sinensis, Cortex Eucommiae, Fructus Lycii, Cortex Phellodendri, Radix Astragali, Caulis Spatholobi, Radix Achyranthis Bidentatae, Radix Ginseng, Radix Notoginseng, Radix Clematidis, Radix Dipsaci*. It has the effect of nourishing liver and kidney, nourishing blood and sinew, clearing collaterals and relieving pain. The Phase III clinical trial proved that FDJG is safe and without toxic side effects in the current oral dose and therapeutic course, and is an effective drug for the treatment of osteoarthritis of the knee joint.

Metabolites are the building blocks of cellular function. Through qualitative and quantitative analysis of small molecule metabolites, metabolomics studies the dynamic changes of metabolites under intervention or disease physiological conditions. Metabolomics has a wealth of information and is considered to be the most predictive of phenotypes, which is a promising tool for disease diagnosis and prognosis. Metabolomic analysis in KBD showed that sphinganine, spermidine, and sphingosine-1P may be potential serum metabolic biomarkers of children with KBD\cite{10}. The differentially expressed metabolites among KBD grade I, II and normal control groups were involved in lipid metabolism metabolic networks\cite{11}. Additionally, disordered glycometabolism in patients with KBD was linked to the damage of chondrocytes\cite{12}.

In this study, a network pharmacology approach was used to predict the potential mechanisms of FDJG for the treatment of KBD. Drug active ingredients and disease-related genes were collected. Additionally, we conducted a non-target metabolomics study to analyze the plasma metabolic profile of KBD patients before and after taking FDJG and identify differentially expressed metabolites. Bioinformatics analysis methods were used to explore the gene function, key proteins, differentially expressed metabolites, active ingredients and the binding action of core active ingredient to core protein.

## 2. Method
### 2.1 Screening the active ingredients and targets of FDJG

The 12 TCM components (*Radix Paeoniae Alba, Radix Angelicae Sinensis, Cortex Eucommiae, Fructus Lycii, Cortex Phellodendri, Radix Astragali, Caulis Spatholobi, Radix Achyranthis Bidentatae, Radix Ginseng, Radix Notoginseng, Radix Clematidis, Radix Dipsaci*) of FDJG were separately searched in the TCMSP database (https://old.tcmsp-e.com/tcmsp.php). The collected active ingredients were screened
according to the standard of OB ≥ 30 and DL ≥ 0.18. Then, the corresponding target sites of each active ingredient were collected, which were converted into gene symbol by using Uniprot database (https://www.uniprot.org/). Cytoscape 3.9.1 was used to map the active ingredient-target network of FDJG.

### 2.2 Screening KBD-related genes

KBD-related genes were collected by searching keywords “Kashin-Beck disease” and “KBD” in the CTD database (https://ctdbase.com/), OMIM database (https://www.omim.org/) and Genecards database (https://www.genecards.org/). Then, Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) was used to analyze the duplicated genes between the targets of FDJG and the genes related to KBD.

### 2.3 Protein-protein interaction networks (PPI)

STRING (https://cn.string-db.org/) was used to analyze the protein interaction network of common genes obtained in the previous step. In the multiple proteins section, organisms were set as homo sapiens. Required score was set as highest confidence (0.900). FDR stringency was set as medium (5 percent). The results were imported into Cytoscape 3.9.1. And the core genes were screened with standard of degree, betweenness centrality, and closeness centrality criteria which were all greater than or equal to median.

### 2.4 Gene function analysis

The function of FDJG’s targets and KBD-related genes were analyzed by Metascape database (http://metascape.org), including molecular functions (MF), biological processes (BP), cellular components (CC), and KEGG pathway. Bioinformatics online tool (http://www.bioinformatics.com.cn/) was used to visualize the results of gene enrichment analysis. Cytoscape 3.9.1 was used to map the TCM-target-pathway network.

### 2.5 Molecular docking

Based on previous network pharmacology screening results, 3D structures and PDB format files of target protein receptors were retrieved and downloaded from the Protein Data Bank (https://www.rcsb.org/). Drug ligand molecular structures were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Then water and small molecule ligands were removed, and hydrogen was added using AutoDock4 software. After that, the range of receptor molecule docking was defined. With the target receptor as the center of the grid, the parameters are adjusted to ensure that the receptor is completely covered by the docking cassette. Finally, molecular docking was completed by detecting protein macromolecules and inserting tiny drug molecules. Visual analysis was performed using Pymol 2.5.4 software.

### 2.6 Blood sample collection and preparation

KBD patients were diagnosed according to WS/T 207–2010 criteria, excluding patients with a history of other bone and joint diseases. Peripheral blood samples of 12 patients with KBD were collected before (control group) and one month after treatment with FDJG (treatment group). The peripheral blood
specimens were collected into an anticoagulation tube and immediately added with Trizol reagent (Invitrogen, CA, USA), and then stored at -80°C for qPCR analysis. Plasma samples were separated from peripheral blood by initial centrifugation at 1200g for 10min and secondary centrifugation at 13000 g for 2min. The metabolites were extracted by mixing the 100µl plasma sample with 400µl methanol. After centrifugation at 20000 g for 15 min, the supernatant was transferred into a new tube and dried under vacuum. The samples were dissolved with 100µl 80% methanol and stored at -80°C for LC-MS analysis.

This study was approved by the Human Ethics Committee of Xi’an Jiaotong University (approval number: 2022 – 1375), and all subjects provided informed consent.

2.7 Quantitative real-time PCR (qPCR) analysis

Total RNA in peripheral blood was extracted with TRizol reagent (Invitrogen, CA, USA) and RNA concentration was detected with NanoDropOne spectrophotometer (Thermo Fisher Scientific). Reverse transcriptions were performed using Evo M-MLVRT Mix Kit (AG11728, Accurate biology, China). SYBR Green Premix Pro Taq HS qPCR kit (AG11739, Accurate biology, China) was used in accordance with the manufacturer's instructions. Then qPCR analysis was performed using CFX-96 (Bio-Rad). The data were normalized according to the GAPDH expression level of each sample. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of genes. Primers were provided by Beijing Tsingke Biotech, as shown in Table 1.
Table 1
Primers for real time-quantitative PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| STAT3     | Forward ACCAGCAGTATAGCCGCTTC  
Reverse GCCACAATCCGGGCAATCT |
| HSP90AA1  | Forward AGGAGGTTGAGACGTTCG  
Reverse AGAGTTGATCTTGTGTTTCGG |
| AKT1      | Forward TCCTCCTCAAGAATGATGGCA  
Reverse GTGCCTGAGATGACAGTGGT |
| MAPK1     | Forward TACACCAACCTCTCGTACATCG  
Reverse CATGTCTGAAGCGCAGTGTT |
| FOS       | Forward GGGGCAAGGTGGGAACAGTTAT  
Reverse CCGCTTGGAGTGATCAGTCA |
| TP53      | Forward CAGCACATGACGGGAAGTTG  
Reverse TCATCCATAACTCCAGACG |
| RELA      | Forward CCCACGAGCTTGTAGGAAGG  
Reverse GGATTCCCAGTTCTGGAAAC |
| GAPDH     | Forward TGGTCTCCTCTGACTTCAACAGC  
Reverse CCTGTTGGCTGTAGCCAAATTCGG |

2.8 Nontargeted LC-MS-based metabolomics

Chromatographic separation was performed using UltiMate 3000 HPLC system (Thermo Scientific). Plasma samples were separated at ACQUITY UPLC BEH C18 (100mm*2.1mm, 1.8µm, Waters, UK) at 40°C. The mobile phase consisted of 1% formic acid (A) and 0.1% formic acid methanol (B), and the gradient elution procedure was 0 ~ 0.8 min, 2% B; 0.8 ~ 2.8 min, 2% ~ 70% B; 2.8 ~ 5.6 min, 70%~90% B; 5.6 ~ 6.4 min, 90% ~ 100% B; 6.4 ~ 8.0 min, 100% B; 8.0 ~ 8.1 min, 100% ~ 2% B; 8.1 ~ 10 min, 2% B. The constant flow rate was set at 0.3ml/min. MS analysis was performed using Q-Exactive (Thermo Scientific) in both positive and negative ion modes. The precursor spectra (70-1050 m/z) were collected at a resolution of 70,000 to achieve the AGC target of 3e6. The maximum injection time was set to 100ms. Fragment spectra were collected at a resolution of 17,500 to achieve the AGC target of 1e5, with a maximum injection time of 80ms. Quality control (QC) samples were prepared from 10 µl of each test sample. For QC samples, the analysis method is the same as for test samples, every 5 test samples are inserted into QC samples to check the stability and performance of the instrument.
2.9 Metabolomics data analysis

The original LC-MS data file was converted into mzXML format, and then processed by XCMS, CAMERA and metaX toolboxes implemented by R software. XCMS software was used for peak extraction and quality control of peak extraction. The extracted substances were annotated by addition and ion using CAMERA, and then identified by metaX software. Primary mass spectrum information was used for identification and secondary mass spectrum information was matched with in-house standards database respectively. KEGG (https://www.genome.jp/kegg/) and HMDB (https://hmdb.ca/) database were used to annotate metabolites on physical and chemical properties and biological function. Partial least squares discriminant analysis (PLS-DA) was performed on the normalized data using metaX software. According to the criteria of variable important for the projection (VIP > 1) and T test (P < 0.05), the metabolites with significant differences were screened.

2.10 Joint pathway analysis

The gene targets based on network pharmacological and differential metabolites from plasma metabolomics were jointly analyzed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Enrichment analysis was set as hypergeometric test, and topology measure was set as degree centrality.

3. Results

1. Active Ingredients of FDJG and Targets Network

There were totally 143 types of active ingredients in FDJG, twenty of which duplicated in different traditional Chinese medicine. The top three TCM which had the greatest number of active ingredients were Fructus Lycii, Cortex Eucommiae and Caulis spatholobi. After collecting the target information and removing the duplication, 265 targets were obtained from 143 active ingredients. Active ingredient-target network mapped by Cytoscape 3.9.1 was shown in Fig. 1, which had 416 nodes and 3411 edges. The top 10 active ingredients ranked by degree were shown in the Table 2, among which the degree value of quercetin (degree = 888) was the greatest, and the following substances with great degree were beta-sitosterol (degree = 396), kaempferol (degree = 290) and stigmasterol (degree = 240).

2. Enrichment Analysis of FDJG-KBD Identical Genes

A total of 4354 KBD-related genes were searched in three disease databases, 151 of which were targets of FDJG (Fig. 2a). KEGG and GO enrichment analysis were performed on the common genes to further understand their functions. The top 10 pathway included pathways in cancer, lipid and atherosclerosis, AGE-RAGE signaling pathway in diabetic complications, IL-17 signaling pathway, PI3K-Akt signaling pathway, etc (Fig. 2b). Some signaling pathways related to inflammation, metabolism and cell death were selected for visualization and classified according to KEGG primary classification (Fig. 2c). Biological processes of these genes were mostly enriched in response to chemical, such as cellular response to
nitrogen compound, response to inorganic substance, cellular response to organic cyclic compound, etc (Fig. 2d). Cellular components of these genes were mostly enriched in membrane raft, membrane microdomain, vesicle lumen, etc (Fig. 2e). Molecular functions of these genes were mostly enriched in kinase binding, DNA-binding transcription factor binding, protein kinase binding, etc (Fig. 2f).

3. PPI Network Construction to Obtain Core Proteins

PPI network of 151 common targets was constructed to visually predict and analyze protein-protein interaction in biological networks, which included 134 nodes and 605 edges (Fig. 3a). Then, key targets for drug treatment of disease were screened according to PPI topological characteristics. Finally, 48 core proteins were obtained with the standard of betweenness centrality \( \geq 0.0038 \), closeness centrality \( \geq 0.376 \), and degree \( \geq 7 \) (Fig. 3a). Ranked according to the degree value, the top five proteins were TP53, STAT3, HSP90AA1, AKT1, and MAPK1 (Table 3). They had more interaction with other proteins and were involved in multiple classic cell death and inflammation pathways. Figure 3b showed top 10 significantly enriched GO terms of core proteins in MF, CC, and BP. For GO-BP category, core proteins were mostly enriched in response to hormone, cellular response to nitrogen compound, positive regulation of protein phosphorylation, etc. CC of core proteins were mostly enriched in transcription regulator complex, membrane raft, membrane microdomain, etc. MF of core proteins were mostly enriched in kinase binding, protein kinase binding, cytokine receptor binding, etc. KEGG enrichment analysis revealed that core proteins were mostly enriched in pathways in cancer, lipid and atherosclerosis, C-type lectin receptor signaling pathway, Th17 cell differentiation, etc. (Fig. 3c). In classic cell death pathways, core proteins were dramatically enriched in osteoclast differentiation (involved with AKT1, FOS, PPARC, and MAPK1, etc), apoptosis (involved with AKT1, BCL2, FOS, and TP53, etc), necroptosis (involved with BCL2, STAT3, HSP90AA1, and IL1B, etc). While in classic inflammation pathways, they were primarily correlated with MAPK signaling pathway (involved with MAPK1, MAPK8, TP53, and VEGFA, etc), TNF signaling pathway (involved with TNF, PTGS2, AKT1, and IL6, etc), IL-17 signaling pathway (involved with CASP3, FOS, IL1B, and TNF, etc) (Fig. 3d). According to the comprehensive analysis of interaction between TCM, core proteins and pathways, the degree values of FDJG components Cortex Eucommiae, Radix Achyranthis bidentatae, and Caulis spatholob were the top three (Fig. 4). These findings suggested that these three drug components may have essential effect on the treatment of KBD through acting on core protein targets.
### Table 3
Connectivity of 48 core proteins

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Degree</th>
<th>Betweenness Centrality</th>
<th>Closeness Centrality</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>38</td>
<td>0.134</td>
<td>0.504</td>
</tr>
<tr>
<td>STAT3</td>
<td>36</td>
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<td>0.491</td>
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Page 10/29
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Degree</th>
<th>Betweenness Centrality</th>
<th>Closeness Centrality</th>
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</tr>
</tbody>
</table>

4. Molecular Docking Analysis

Molecular docking is a common method used in drug discovery. It is used to study the interactions between molecules and to predict their binding patterns and affinities. Based on the above results, we screened the three most important compounds (quercetin, kaempferol and luteolin), and performed molecular docking with the top ten core proteins in terms of degree value (TP53, STAT3, HSP90AA1, AKT1, MAPK1, etc.). The docking fractions of the compounds to the proteins were shown in the Table 4.
Meanwhile, Fig. 5 showed the 2D and 3D visualization of the optimal docking between quercetin and HSP90AA1, kaempferol and STAT3, luteolin and TP53. As the lower the binding energy, the higher the affinity of the receptor to the ligand and the more stable the conformation. It is generally accepted that binding energies of less than $-5$ kcal/mol indicate good binding activity between ligand and receptor. Molecular docking results showed that these binding energies were all less than $-5$ kcal/mol, indicating that quercetin, kaempferol and luteolin had a high affinity with the core proteins (TP53 (PDB ID: 2G3R), STAT3 (PDB ID: 5AX3), HSP90AA1 (PDB ID: 5J80), etc.).

<table>
<thead>
<tr>
<th>Compound/Protein (PDB ID)</th>
<th>Quercetin</th>
<th>Kaempferol</th>
<th>Luteolin</th>
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</thead>
<tbody>
<tr>
<td>TP53 (2G3R)</td>
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<td>-8.4</td>
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<td>HSP90AA1 (5J80)</td>
<td>-8.4</td>
<td>-8.0</td>
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<td>AKT1 (1H10)</td>
<td>-6.5</td>
<td>-6.1</td>
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<td>MAPK1 (8A0J)</td>
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<td>-7.5</td>
<td>-7.5</td>
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<td>RELA (6NV2)</td>
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<td>-7.8</td>
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<td>TNF (5UUI)</td>
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<td>FOS (1A02)</td>
<td>-8.7</td>
<td>-10.0</td>
<td>-8.2</td>
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5. Differential Expression Validation of Seven Core Genes in Vivo

Seven core targets, including STAT3, HSP90AA1, AKT1, MAPK1, FOS, TP53, RELA, were chosen for experimental validation to further validate the predicted results of network pharmacology. After taking FDJG for one month, the RNA expression of seven core genes in peripheral blood of KBD patients showed a decreasing trend, in which the expressions of STAT3, FOS and RELA were significantly down-regulated ($P<0.05$) (Fig. 6).

6. Differential Metabolite Identification and Pathway Analysis

PLS-DA was used to partition the metabolic profiles of the control group and the treatment group. Based on cross-validation analysis, PLS-DA model presented satisfactory explanations and predictions, with
results for R2 (0.966) and Q2 (0.231). The result of the permutation test showed that the longitudinal intercept of Q2 was −0.346 (Fig. 7b). The differential metabolites between control group and treatment group were screened according to the criteria of VIP value > 1.0, |log$_2$FC| > 0.58 and $P< 0.05$. There were 80 differentially expressed metabolites (S1), of which 24 were up-regulated and 56 were down-regulated (Fig. 7c). They were most enriched in phosphatidylinositol signaling system, cAMP signaling pathway, inositol phosphate metabolism, metabolic pathways, glycerophospholipid metabolism (Fig. 7d).

7. Joint Pathway Analysis of Targets and Metabolites

To explore the joint metabolic pathways of drug targets and metabolites, we imported 151 drug targets and 80 differentially expressed metabolites into MetaboAnalyst 5.0. As shown in Fig. 7e and Table 5, based on pathway impact > 0.1, a total of 19 pathways were enriched, including glycerophospholipid metabolism, drug metabolism - cytochrome P450, retinol metabolism, arachidonic acid metabolism, synthesis and degradation of ketone bodies, tryptophan metabolism, linoleic acid metabolism, inositol phosphate metabolism, etc. Among these pathways, 4 metabolism pathways enriched both targets and metabolites, including glycerophospholipid metabolism, inositol phosphate metabolism, phosphatidylinositol signaling system, and steroid hormone biosynthesis.
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene</th>
<th>Metabolite</th>
<th>P value</th>
<th>Pathway Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerophospholipid metabolism</td>
<td>ACHE</td>
<td>LysoPC 18:4; LysoPE 18:3; PG 36:4; PG (18:2/18:2); PI 32:1; PI (16:0/16:1); LysoPI 16:0</td>
<td>0.0002</td>
<td>0.1647</td>
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<td>Drug metabolism - cytochrome P450</td>
<td>CYP3A4; CYP1A2; GSTP1; GSTM2; ADH1C; MAOB; ADH1B</td>
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<td>0.0008</td>
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<td>Retinol metabolism</td>
<td>CYP3A4; CYP1A2; ADH1C; ADH1B; CYP1A1</td>
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<td>0.0018</td>
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<td>Metabolism of xenobiotics by cytochrome P450</td>
<td>CYP3A4; CYP1A2; GSTP1; GSTM2; ADH1C; ADH1B; CYP1A1</td>
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<td>0.0063</td>
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<td>Arachidonic acid metabolism</td>
<td>PTGS1; PTGS2; ALOX5; LTA4H; PTGES</td>
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<td>Synthesis and degradation of ketone bodies</td>
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<td>(R)-3-Hydroxybu, tyric acid</td>
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<td>Tryptophan metabolism</td>
<td>CYP1A2; CYP1A1; MAOB; CAT</td>
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<td>Linoleic acid metabolism</td>
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<td>Arginine biosynthesis</td>
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<td>Inositol phosphate metabolism</td>
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<td>Ubiquinone and other terpenoid-quinone biosynthesis</td>
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<td>Caffeine metabolism</td>
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<td>Phosphatidylinositol signaling system</td>
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<td>Steroid hormone biosynthesis</td>
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<td>Porphyrin and chlorophyll metabolism</td>
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<td>Fatty acid biosynthesis</td>
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<td>0.7394</td>
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**Discussion**

Based on the method of network pharmacology, we analyzed the potential mechanism of Fufang Duzhong Jiangu Granules in treating KBD. We found 143 types of active ingredients in FDJG by using public database. There are 151 genes which are the therapeutic targets of FDJG in the treatment of KBD. A total of 48 core genes are screened by using PPI network topological characteristics evaluation and calculation of node degree. These genes are enriched in PI3K-AKT signaling pathway, MAPK signaling pathway, TNF signaling pathway, Toll-like receptor signaling pathway, p53 signaling pathway, osteoclast differentiation, etc. The RNA expression levels of STAT3, FOS and RELA in peripheral blood of KBD patients in the treatment group were significantly down-regulated, which further verified the prediction results of network pharmacology. Mapping the drug-target-pathway network, we found that for TCM, the degree values of *Cortex Eucommiae*, *Radix Achyranthis Bidentatae*, and *Caulis spatholob* were much higher than that of other medicines in the prescription, indicating that they may be the main effective medicines in FDJG acting on KBD. By combining metabolomics with network pharmacology, we found 8 key metabolites (LysoPC 18:4, LysoPE 18:3, PG 36:4, PG (18:2/18:2), PI 32:1, PI (16:0/16:1), LysoPI 16:0, Estrone) and 4 related pathways (glycerophospholipid metabolism, inositol phosphate metabolism, phosphatidylinositol signaling system, steroid hormone biosynthesis).

The biological processes of 151 corresponding genes are more enriched in the cellular response to the compounds contained in the drug. Among the top 10 key active ingredients, quercetin, kaempferol, luteolin and wogonin are all flavonoids with a variety of pharmacological activities, including anti-apoptotic, antioxidant and anti-inflammatory activities in vitro and in vivo. Our molecular docking analysis also suggested that quercetin, kaempferol and luteolin can strongly bind with core protein targets. Quercetin suppresses inflammation and apoptosis of chondrocytes by regulating the SIRT1/AMPK signaling pathway\(^{[13]}\), the p38 MAPK signaling pathway\(^{[14]}\), and the polarization of synovial macrophages to M2 macrophages\(^{[15]}\). Kaempferol protects chondrocytes from inflammatory damage by downregulating miR-146a\(^{[16]}\). In addition, kaempferol inhibits the proliferation of synovial fibroblasts, as well as the production of MMPs, COX-2 and PGE2, which are involved in arthritis\(^{[17]}\). Luteolin can not only
inhibit IL-1β-induced inflammation in rat chondrocytes, but also reduce the progress of osteoarthritis by activating AMPK-Nrf2 signaling to protect chondrocytes from H₂O₂-induced oxidative damage\(^{18,19}\). The anti-inflammatory effect of Wogonin is mediated by inhibiting the expression of iNOS and COX-2, as well as suppressing the activation of AP-1\(^{20,21}\). Moreover, Wogonin plays a protective role in human OA cartilage through the suppression of molecular events involved in oxidative stress, inflammation and matrix degradation via the Nrf2/ARE pathway\(^{22}\). In addition to flavonoids, alkaloids in FDJG also play an important role. The active ingredients of *Radix Achyranthis bidentatae* and *Cortex Phellodendr* contain berberine, which has many novel bioactivities including antidiabetic, anticancer, neuroprotective, anti-inflammatory, and therapeutic potential in the treatment of common orthopedic disorders such as osteoporosis and osteoarthritis\(^{23}\). In our study, NOS2, PTGS1, PTGS2, RXRA, HSP90AA1 were the targets of berberine in the treatment of KBD. PGE2 is extremely high in the synovial fluid and peripheral blood of patients with KBD\(^{24}\). Non-steroidal anti-inflammatory drugs, such as ibuprofen, are often used to relieve joint pain, but they have serious side effects especially in digestive system. Apparently, traditional Chinese medicine could achieve the same function without such side effects.

Most of the 48 core genes enrichment pathways play important role on the maintenance of cartilage proliferation, chondrocytes differentiation and death, including PI3K-AKT signaling pathway, p53 signaling pathway, MAPK signaling pathway, TNF signaling pathway, JAK/STAT and so on. PI3K-Akt signaling pathway and P53 signaling pathway contribute to the development of KBD via regulating chondrocyte apoptosis and cell death\(^{25,26}\). P53 pathway is essential for the regulation of cell cycle, DNA damage and apoptosis. The expression level of p53 is upregulated in KBD chondrocytes compared with NC\(^{27,28}\). P38 MAPK is usually phosphorylated during chondrogenesis and positively regulate chondrogenesis and chondrocyte proliferation and hypertrophy\(^{29–31}\). Pro-inflammatory cytokines, such as TNF-α and IL-1β, induce activation of the JAK/STAT signaling, leading to chondrocyte apoptosis and upregulation of matrix metalloproteinase gene expression\(^{32}\). FDJG may play a role in the treatment of KBD by regulating the above multiple signaling molecules and pathways.

The plasma differential metabolites of KBD patients before and after one month of FDJG treatment mainly included fatty acyls and glycerophospholipids. Glycerophospholipids are the most abundant phospholipids, and participate in the formation of biofilms, cell membrane recognition of proteins and signal transduction recognition. Glycerophospholipid metabolism was the main metabolic pathway associated with the differential IL-6 expression in rheumatoid arthritis patients\(^{33}\). Lysophosphatidylcholine (Lyso PC) is associated with the development of inflammatory diseases, can increase the production of pro-inflammatory cytokines, induce oxidative stress, and promote apoptosis\(^{34}\). Researchers found that activation of the phosphatidylcholine to lysophosphatidylcholine pathway is associated with osteoarthritis knee cartilage volume loss. The serum lysoPC 18:2 to PC44:3 ratio is highly associated with a greater risk of cartilage volume loss of the knee\(^{35}\). Phosphoinositides (PIs) have a huge impact on cell regulation, which not only induce cytoskeletal changes and actin remodeling, but also play an important role in cell proliferation, survival and metabolism related to Akt.
signal transduction\cite{36}. Compared with normal controls, the metabolism of lipid and lipid-like molecules were most significantly different in serum metabolites of KBD patients\cite{11}. In our study, enrichment of lipid metabolism pathway was dominant, suggesting that FDJG may effectively treat KBD by regulating abnormal lipid metabolism pathway.

Although our research has many new findings, there are limitations in it. The composition of traditional Chinese medicine is complicated, and the proportion of traditional Chinese medicine is not considered. The process of drug interaction in the organism is complex, and the real efficacy of drugs needs to be verified by relevant experiments in vivo and in vitro and larger population trials. In addition, due to the different degrees of research on TCM, many active ingredients probably have not been identified.

In summary, through the combination of network pharmacology and metabolomics, the multi-compound, multi-target and multi-mechanism of Fufang Duzhong Jiangu Granules in the treatment of KBD were systematically clarified. It may be effective in treating KBD by regulating abnormal lipid metabolism pathway and anti-inflammatory. The drug has great potential in the treatment of patients with KBD.

**Abbreviations**

FDJG  
Fufang Duzhong Jiangu Granules  
KBD  
Kashin-Beck disease  
BP  
biological processes  
CC  
cellular components  
MF  
molecular functions  
OB  
oral bioavailability  
DL  
drug likeness  
TCM  
traditional Chinese medicine, PPI, protein-protein interaction networks.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**
Not applicable.

**Availability of data and material**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

This work was supported by National Key Research and Development Program of China (2022YFC2503100).

**Authors' contributions**

X.D., H.N., Q.Z., W.L., J.W. and Y.Z. collected data. X.D. and H.N. analyzed the data and drafted the manuscript. C.W., X.G. and H.L. designed the experiment and revised the manuscript. F.Z. provided financial support. All authors read and approved the final manuscript.

**Acknowledgements**

This work was funded by National Key Research and Development Program of China (2022YFC2503100). We appreciate LC-Bio Technologies (Hangzhou) Co., Ltd. for LC-MS analysis.

**References**


**Tables**

Table 2 is available in the Supplementary Files section.

**Figures**
Figure 1

TCM-Target Network.

Figure 2

GO and KEGG Analysis of TCM-KBD related Identical Genes.

Figure 3

PPI Network and Enrichment Analysis of Core Proteins.

a. PPI network of 151 intersection genes and core proteins. The core proteins were screened with standard of degree, betweenness centrality, and closeness centrality criteria which were all greater than or

Figure 4

TCM-Target-Pathway Network.

Comprehensive analysis of herb in FDJG, target genes and pathway network maps. The trend in shape size is consistent with the value of the degree.

Figure 5

The Binding Modes.
optimal binding modes of luteolin and TP53 ($\Delta G = -8.4$ kcal/mol). Left: two-dimensional image; Right: three-dimensional image.

**Figure 6**

Box plots of relative RNA expression.

Relative expression of STAT3, HSP90AA1, AKT1, MAPK1, FOS, TP53, RELA in peripheral blood of KBD patients in control group and treatment group (n = 12 per group). The wilcoxon test was used for statistical analysis.
Figure 7

Metabolomics analysis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table2.docx
- SupplementaryTable1.xlsx