Exploration of the mechanism of Ziyin Tongluo Formula on preventing and treating postmenopausal osteoporosis based on the network pharmacology

Rong-Bin Chen
Guangdong Province Hospital of Traditional Chinese Medicine ZHUHAI BRANCH
Ying-Dong Yang
Guangzhou University of Chinese Medicine
Kai Sun
Guangzhou University of Chinese Medicine
Shan Liu
The Research Center of Basic Intergrative Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China
Wei Guo
Guangzhou University of Chinese Medicine
Jin-Xin Zhang
Guangdong Province Hospital of Traditional Chinese Medicine ZHUHAI BRANCH
Yong Li (lyzhuhai666@126.com)
Guangdong Province Hospital of Traditional Chinese Medicine ZHUHAI BRANCH
https://orcid.org/0000-0003-4257-032X

Research

Keywords: Ziyin Tongluo Formula, Postmenopausal osteoporosis, Network pharmacology

Posted Date: July 6th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-39351/v1

Abstract

Background

Postmenopausal osteoporosis (PMOP) is a global chronic and metabolic bone disease that poses huge challenges to individuals and society. Previous studies have confirmed that Ziyin Tongluo Formula (ZYTLF) has a good clinical effect in the treatment of PMOP. However, the material basis and mechanism of ZYLTF against PMOP has not been thoroughly explained.

Methods

TCMSP, TCMID, and BATMAN-TCM databases were used to identify the active ingredients and their putative targets. Genes associated with PMOP were mined from GeneCards, OMIM, DisGeNET databases, and then mapped with the putative targets to obtain overlapping target genes. A network model of "herb-active ingredient-overlapping target genes" was constructed and a protein-protein interaction network of overlapping target genes was built and the key genes were selected based on the MCC algorithm. The key genes were imported to the DAVID database to performs GO and KEGG pathway enrichment analyses.

Results

Ninety-two active components of ZYTLF corresponded to 243 targets, with 129 target genes interacting with PMOP, and 50 key genes were selected. GO analysis results showed that biological process mainly included positive regulation of transcription, negative regulation of apoptosis, and cell components were mainly nucleus, cytoplasm, and molecular functions mainly included enzyme binding, protein binding and transcription factor binding. There were two main types of significant KEGG pathways in PMOP, hormone-related signaling pathways (estrogen, prolactin, thyroid hormone) and inflammation-related pathways (TNF, PI3K-Akt, MAPK).

Conclusions

The underlying therapeutic mechanisms of ZYTLF action on PMOP maybe is that, the active ingredients such as quercetin, kaempferol, luteolin act on ESR1, TNF, IL6, MAPK8 and other key genes, which mainly enrich in estrogen, TNF, PI3K-Akt, MAPK and other signaling pathways.

Background

Postmenopausal osteoporosis (PMOP) refers to a global chronic metabolic bone disease with increased fragility and fracture susceptibility of postmenopausal women whose ovarian function declines and estrogen levels fall, resulting in bone metabolism disorders. It is characterized by greater bone resorption than bone formation, low bone mass and bone microstructure destruction. Osteoporotic fractures are one

of the main causes of post-menopausal women's disability and death. Therefore, PMOP has now become a major issue of life quality worldwide, and its prevention research is one of the hot spots of modern medical research [1–2]. The medicines currently used to treat PMOP can be divided into bone resorption inhibitors (estrogen, bisphosphonates, calcitonin), bone formation promoters (parathyroid hormone, fluoride), and bone mineralization (calcium, active Vitamin D), which has achieved good results in the clinic, but its cost is expensive, and the side effects such as endometrial hyperplasia, increased breast cancer risk [3], gastrointestinal reactions [4] are still one of the clinical problems.

Modern pharmacological experiments have confirmed that a variety of traditional Chinese medicines contain active ingredients for anti-osteoporosis, and compound Chinese medicines have stronger anti-osteoporosis effects than single medicines [5–6]. Ziyin Tongluo Formula (ZYTLF) is composed of 14 traditional Chinese medicines, *Rehmannia glutinosa* (Shudi), *Ophiopogon japonicus* (Maidong), *Ligustrum Lucidum* (Nuzhenzi), *Angelica sinensis* (Danggui), *Paeonia albiflora* (Baishao), *Achyranthes bidentata* (Niuxi), *Loranthus parasiticus* (Sangjisheng), *Caulis Millettiae* (Jixueteng), *Zaocys* (Wushaoshe), *Scolopendra subspinipes mutilans* (Wugong), *Astragalus membranaceus* (Huangqi), *Saposhnikovia divaricata* (Fangfeng), *Atractylodes macrocephala* (Baizhu), *Radix Glycyrrhizae Preparata* (Gancao). Early clinical studies have shown that Ziyin Tongluo Formula can effectively improve the clinical symptoms of PMOP, increase bone density, and it is safe and reliable [7], but its molecular mechanism of action is still unclear.

Based on the network pharmacology method, this study explores the main active ingredients of ZYTLF in the prevention and treatment of PMOP, its targets and signaling pathways, and provides directions for elucidating the material basis and mechanism of action of ZYTLF in the prevention and treatment of PMOP. (Fig. 1).

Methods

Identification of ZYTLF active ingredients

A total of 14 traditional Chinese medicines were inputted into the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP http://tcmspw.com/tcmsp.php Version 2.3) [8], BAT-MAN TCM (http://bionet.ncpsb.org/batman-tcm/) [9] and Traditional Chinese Medicine Information Database(TCMID, http://www.megabionet.org/tcmid/) [10] for searching. The certain or potential compounds of ZYTLF were obtained. The ADME (absorption, distribution, metabolism and excretion) properties of compounds play a key role in medicine discovery and development [11]. Therefore, the two most important indicators of bioinformatics to evaluate the characteristics of ADME, oral bioavailability (OB) and drug-likeness (DL) , were used as standard for screening active compounds. The TCMSP database used Obioavail 1.1 to calculate the OB value [12], and applies the Tanimoto coefficient,

, to calculate the DL value of the compound. Compounds that meet the requirement OB ≥ 30% and DL ≥ 0.18 were identified as biologically active ingredients. Because *Radix Glycyrrhizae Preparata* was used as a medicine in this formula, the standard was set as OB ≥ 60% and DL ≥ 0.36, in order to avoid

interference. Based on the above standards, the active ingredients of ZYTLF can be screened by TCMSP for subsequent analysis.

Prediction of ZYTLF targets

The next step after screening active ingredients was to predict the targets that might stimulate biological effects. The TCMSP analysis platform is loaded with prediction function of large-scale molecular network targets based on chemistry, genomics, pharmacology and system analysis technology, which is currently a commonly used target prediction platform [12]. After entering the active ingredient into the TCMSP analysis platform, the target interacting with it could be obtained. Uniprot (https: //www.Uniprot.org) [13] is a database containing accurate annotations of proteins and other substances, where the genetic information of the target can be obtained. The target information was inputted into the Uniprot database to obtain the standard gene name.

PMOP-related genes and ZYTLF-PMOP overlapping target genes

First of all, Osteoporosis and Postmenopausal were used as the key words to collect PMOP related genes from three online databases, Genecards (http://www.genecards.org) [14], OMIM (http://omim.org/) [15], and DisGenet (https://www.disgenet.org/) [16]. Next, overlapping target genes were obtained from ZYTLF target genes and PMOP-related genes, which may be potential targets for ZYTLF to prevent PMOP.

Constructing a network model of "medicine-active ingredient-overlapping target gene"

The overlapping target genes and their corresponding active ingredients and medicine-related action relationships were inputted as sources into Cytoscape 3.7.1 software [17], to build a "medicine-active ingredient-overlapping target gene" network model. CytoNCA plug-in [18] was used to conduct network topology analysis. According to the Degree Centrality (DC) and Between Centrality (BC), the key nodes in the network were screened. The higher the node's degree value was, the more important it was in the network.

Constructing PPI network of overlapping target genes and screening key genes

The overlapping target genes were inputted into the STRING database (http://string-db.org/, ver.11.0) [19]. The data analysis mode was set to "Multiple proteins" and the type was set to "Homo sapiens" (human). The minimum mutual threshold was set to "high confidence (0.700)". The remaining parameters remained unchanged, and the protein-protein interaction (PPI) network diagram was obtained. Then the information of PPI network was imported into Cytoscape 3.7.1 software. The MCC algorithm in the CytoHubba plug-in was used to perform network analysis to screen out key genes.

Biological function analysis

The DAVID (Database Visualization and Integrated Discovery system, https://david.ncifcrf.gov/) database is an online biological information database that integrates biological data and analysis tools

to provide annotation information of comprehensive biological function. Quantitative screening technology is used for biological function analysis of genes [20-21]. Key genes were inputted into the DAVID database. The species was limited to "Homo sapiens", and enrichment analysis of Gene Ontology (GO, http: //www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http: //www .genome.jp/kegg/) was performed. R software was used to draw advanced bubble diagrams and gene-pathway mechanism diagrams was obtained from the KEGG database.

Results

ZYTLF's active ingredients and targets

Through TSMSP platform, BAT-MAN TCM and TCMID database, a total of 1,738 compounds were obtained, and 92 compounds that met the standard were screened. The detailed information of active ingredients in ZYTLF are shown in Additional file 1:Table S1. There were 242 target genes for these active ingredients.

PMOP-related genes and ZYTLF-PMOP overlapping target genes

From Genecard, Disgenet and OMIM databases, a total of 1113 PMOP related genes after de-duplication were obtained. Comparing medicine target genes with PMOP-related genes, a total of 129 overlapping target genes were obtained. (Fig. 2).The detailed information of ZYTLF-genes, PMOP-genes, and ZYTLF-PMOP overlapping target genes are shown in Additional file 2:Table S2.

Network analysis

A "Herb-Active Ingredient-Overlapping Target Gene" network diagram was constructed. (Fig. 3) There were 231 nodes and 1,072 edges in the network, and the detailed information of the network are shown in Additional file 3:Table S3. Red triangle nodes represented Chinese herbs, blue square nodes were active ingredients, and green circle nodes were overlapping target genes. The results of network topology analysis showed that the active ingredients such as quercetin, kaempferol, luteolin, scutellarein, and formononetin had higher degrees, which played an important role in the network. The network showed that one active component could correspond with multiple genes, and one gene could also correspond with multiple active components. The complex interaction between active components and overlapping target genes illustrated the multi-component and multi-target features of ZYTLF.

The overlapping target genes were inputted into the STRING database, and the PPI network diagram was obtained after setting the relevant parameters. The network displayed the relevant information of the genes and the interaction relationship. (Fig. 4) Then Cytoscape 3.7.1 software was used to visually process and analyze the PPI network information. Based on the MCC algorithm of the CytoHubba plug-in, the top 50 key genes were obtained. (Fig. 5) The color of the node changeed from light yellow to dark red, indicating that the higher the MCC value was, the more significant the role it played in the network.

Biological function analysis

DAVID database was used to perform GO enrichment and KEGG pathway enrichment analysis on key genes and extract significant enrichment results (FDR < 0.05). A total of 62 GO-BP terms, 6 GO-CC terms, 11 GO-MF terms and 59 terms on the KEEG pathway were obtained. The enrichment results showed that the biological processes involved mainly included positive transcriptional regulation signals of RNA polymerase II promoter, negative regulation of apoptosis, positive regulation of transcription using DNA as a template, aging, positive regulation of gene expression, etc. (Fig. 6). Main cell components included nucleus, cytoplasm, extracellular space, etc.(Fig. 7). Regulated molecular functions mainly included enzyme binding, transcription factor binding, and protein binding. (Fig. 8).

A total of 15 signal pathways related to PMOP were screened from the significant signal pathways. (Fig. 9). The signal pathways mainly involved inflammation-related pathways, including TNF, HIF-1, PI3K-Akt, Toll-like receptors, MAPK and other signaling pathways, and hormonal pathways such as estrogen, prolactin, thyroid hormone and other signaling pathways. This indicatedthat ZYTLF could play a synergistic role through many pathways. From the KEGG database, the mechanism of action of key genes on estrogen and TNF signaling pathways was obtained. The red marks in the figure represented potential targets for possible intervention by ZYTLF. (Fig. 10 and Fig. 11).The results and detailed information on the GO terms and pathways are shown in Additional file 4:Table S4.

Discussion

At the molecular level, multiple factors participate in the development of PMOP, interacting with each other and playing a role in regulating bone metabolism in a complex regulatory network. Among them, the low level of estrogen is the main cause of PMOP, which leads to the disorder of bone metabolism. Bone resorption is greater than bone formation, and eventually osteoporosis is caused [22]. Compared with hormone replacement therapy (HRT), traditional Chinese medicine, as a viable alternative therapy, has the advantages of simplicity and low side effects. Modern research has found that traditional Chinese medicine contains a variety of biologically active ingredients, which can act on multiple targets through multiple pathways, reflecting the overall concept of traditional Chinese medicine and the theory of syndrome differentiation and treatment [23]. Compared with the “single component-single target-single pathway” research model of traditional pharmacology, network pharmacology combines the concepts of bioinformatics and multi-directional pharmacology to analyze the relationship between biological systems, medicines, and diseases from a network perspective. It has opened a new research model of traditional Chinese medicine from empirical medicine to evidence-based medicine [24].

Key active ingredient of ZYTLF for anti-PMOP

According to the network analysis results of the "herb-active ingredient-overlapping target gene", the key active ingredients of ZYTLF are mainly quercetin, kaempferol, luteolin, rhubarbin, formononetin and other flavonoid compounds. At the same time, they are also plant-like estrogen, which has two-way regulating effects. It plays the role of estrogen when the level of estrogen is low, and plays the role of anti-estrogen when the level of estrogen increases. It can improve a series of symptoms caused by the

postmenopausal low estrogen level and avoid the occurrence of tumors of the reproductive system caused by estrogen replacement therapy [25]. Quercetin with the highest degree value in the network is a typical flavonoid compound. It has multiple pharmacological effects, including free radical scavenging, anti-cancer, anti-infection, and cardiovascular protection [26-29]. Quercetin has also been proven to be an effective component against osteoporosis, with the dual effects of inhibiting osteoclastogenesis and osteoblast differentiation. Studies such as Li Zhixing's found that quercetin can relieve osteoporosis symptoms in ovariectomized rats, possibly by up-regulating ALP gene expression and inhibiting JNK, ERK, and p38 MAPK signaling pathways [30]. Quercetin can also directly or indirectly down-regulate the expression of RANKL, inhibit osteoclast differentiation, reduce bone resorption, and stimulate osteoblast activity through estrogen signaling pathway and ERK signaling pathway [31]. Kaempferol has also been shown to have bone protective effects on ovariectomized rats [32], possibly through estrogen receptor, MAPK, NF-κB and other signaling pathways [33]. Luteolin can prevent bone loss after osteoporosis by inhibiting osteoclast differentiation.

Key Genes of ZYTLF for Preventing PMOP

Combined with the analysis of PPI network and key gene network, it was found that among the key genes of ZYTLF for anti-PMOP, JUN, MAPK8, AKT1, IL-6, MMP9, PTGS2, TNF, MAPK1, CASP3, etc. were all related to the inflammatory response process. Studies have found that the reduction of estrogen levels in postmenopausal women can stimulate the immune system to produce a large number of osteoclastogenic factors, including TNF-α, RANKL, IL-17A, etc., which in turn activates related signaling pathways, further aggravating bone loss [33]. Li Zha and other scholars have found that postmenopausal women with osteoporosis have significantly increased levels of TNF-α. In-vitro experiments have found that TNF-α and RANKL synergistically enhance bone resorption of osteoclasts through NF-κB and PI3K/Akt signaling pathways [35]. TNF-α and IL6 play an important role in the immune response and bone metabolism, mainly affecting the differentiation and proliferation of osteoclasts by regulating complex mechanisms, and they are important pathogenic factors for immune-mediated bone diseases [36]. Studies have shown that increased levels of inflammatory factor TNF-α in the serum of postmenopausal women may be one of the important causes of osteoporosis. TNF-α can activate the RANK/RANKL signaling pathway and induce the formation of osteoclasts [37]. The author speculates that elevated TNF-α level in the serum of postmenopausal women with osteoporosis may be related to estrogen deficiency.

The signal pathways ZYTLF in preventing PMOP

The enrichment analysis of KEGG pathways for the key genes of ZYTLF in preventing PMOP showed that 15 signaling pathways were related to the occurrence and development of PMOP, including hormone pathways and inflammation-related pathways. Hormones in postmenopausal women, including estrogen [38], parathyroid hormone [39], and prolactin, if not normally secreted could affect bone metabolism [40]. Estrogen bound with the estrogen receptor in osteoblasts and osteoclasts to act on the OPG/RANK/RANKL signaling pathway, which further promoted OPG secretion, down-regulated the

expression of RANKL, and inhibited the formation of osteoclasts [41]. After the combination of estrogen and estrogen receptor, it could also regulate the expression of various target genes through the estrogen signaling pathway, thereby activating downstream PI3K/Akt, MAPK, WNT and other signaling pathways to promote the differentiation and proliferation of osteoblasts [42-43]. The TNF signaling pathway had the largest number of enriched genes, which might be the key signaling pathway for ZYTLF to prevent PMOP. The TNF signaling pathway was mainly opened by TNF-α and interacted with multiple signaling pathways to synergistically inhibit osteoclast differentiation and bone resorption function [44].

Conclusions

Quercetin, kaempferol, luteolin and other active ingredients in ZYTLF may be enriched in estrogen signaling pathways and inflammation related TNF, PI3K/Akt signaling pathways through ESR1, TNF, IL6, MAPK8, STAT3, MMP9, PTGS2, JUN, MAPK, inhibiting osteoclast formation, promoting osteoblast differentiation, regulating bone metabolism, and treating PMOP. The shortcomings of this study lie in that the results of online database analysis may not be consistent with the actual situation for reasons: 1. The compatibility and dosage of the formula are not taken into consideration; 2. The screened active components are different from the actual components in blood; 3. There may be errors in the prediction results of targets and signal pathways, therefore, further experiments need to be conducted combining with Western Blot, Rt-PCR and multi-omics technology. However, the network pharmacology methodology can save time and energy by predicting the active ingredients, targets and signal pathways, providing directions for further experimental research.

Abbreviations

PMOP:postmenopausal osteoporosis; ZYTLF:Ziyin Tongluo Formula; TCMSP: traditional Chinese medicine system pharmacology analysis platform; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: biological processes; CC: cellular components; MF: molecular functions; OB: oral bioavailability; DL: drug-likeness; ADME: absorption, distribution, metabolism, excretion; IL-6: Interleukin 6; TNF:Tumor necrosis factor.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and 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 study was supported by grants from Traditional Chinese Medicine Bureau Of Guangdong Province for Arranged Project of Experiential prescription (No. 20194010). Funder provided financial support for the study.

Authors' contributions

YL and RBC conceived and designed the study. RBC and YDY drafted the manuscript. JXZ and SL collected the data. YDY. KS and WG performed the data analysis. KS, SL, and WG provided advice during the study and manuscript preparation. All authors read and approved the final manuscript.

Author details

[1]GuangDong Province Hospital Of Traditional Chinese Medicine ZHUHAI BRANCH, Zhuhai 519015, Guangzhou University of Chinese Medicine, Guangzhou 510006, China. [2]Guangzhou University of Chinese Medicine, Guangzhou 510006, China.

Acknowledgements

Not applicable

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Figures

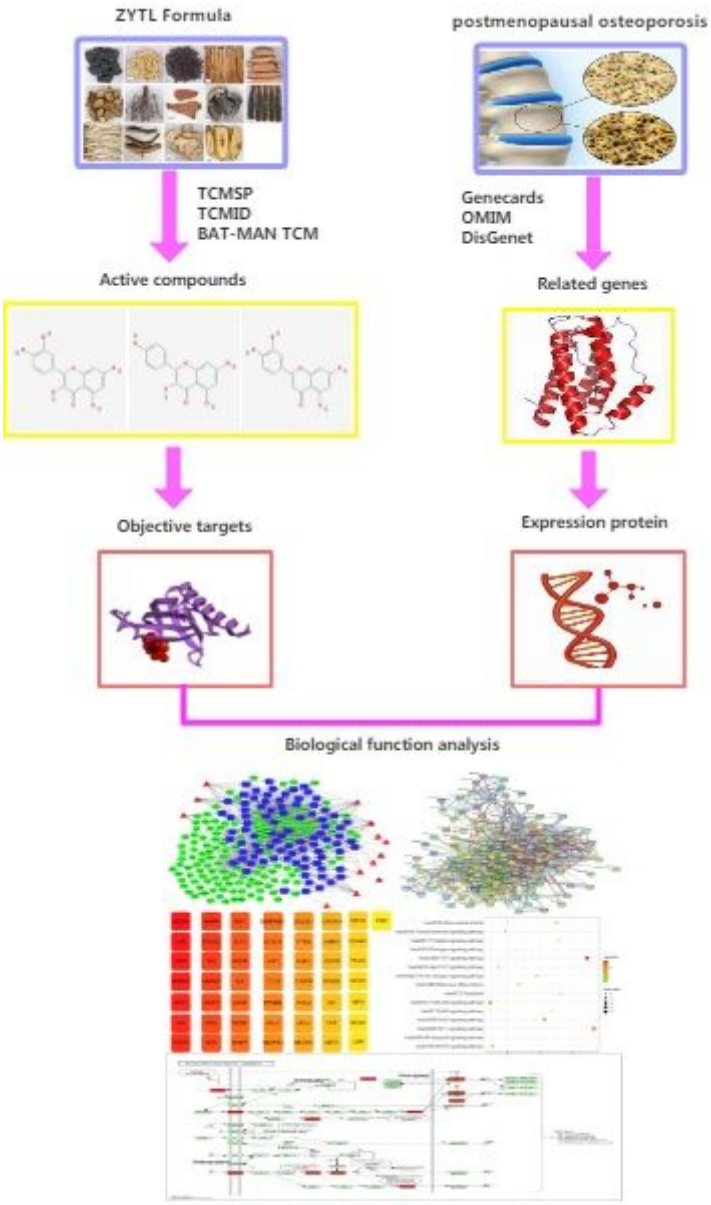

Figure 1

Flowchart showing the network pharmacology approach for determining the molecular mechanisms of action of ZYTLF on PMOP.

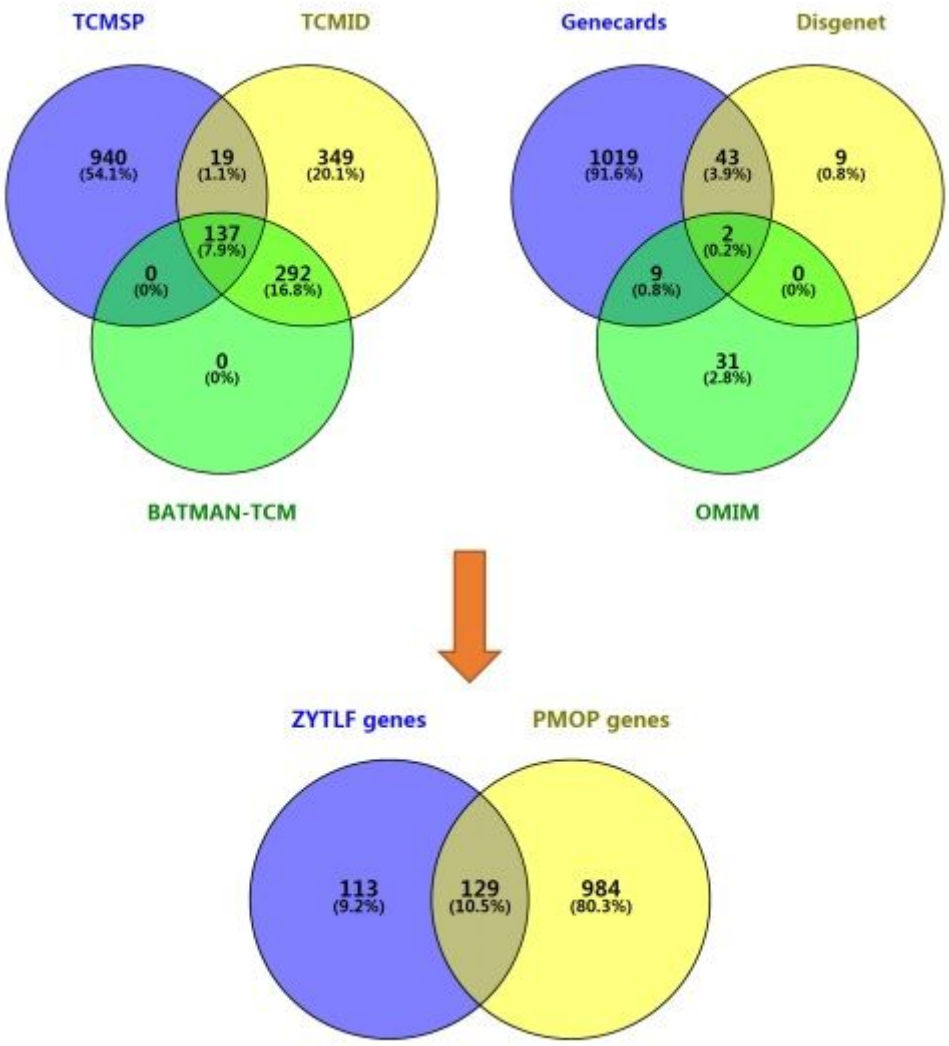

Figure 2

Venn diagram

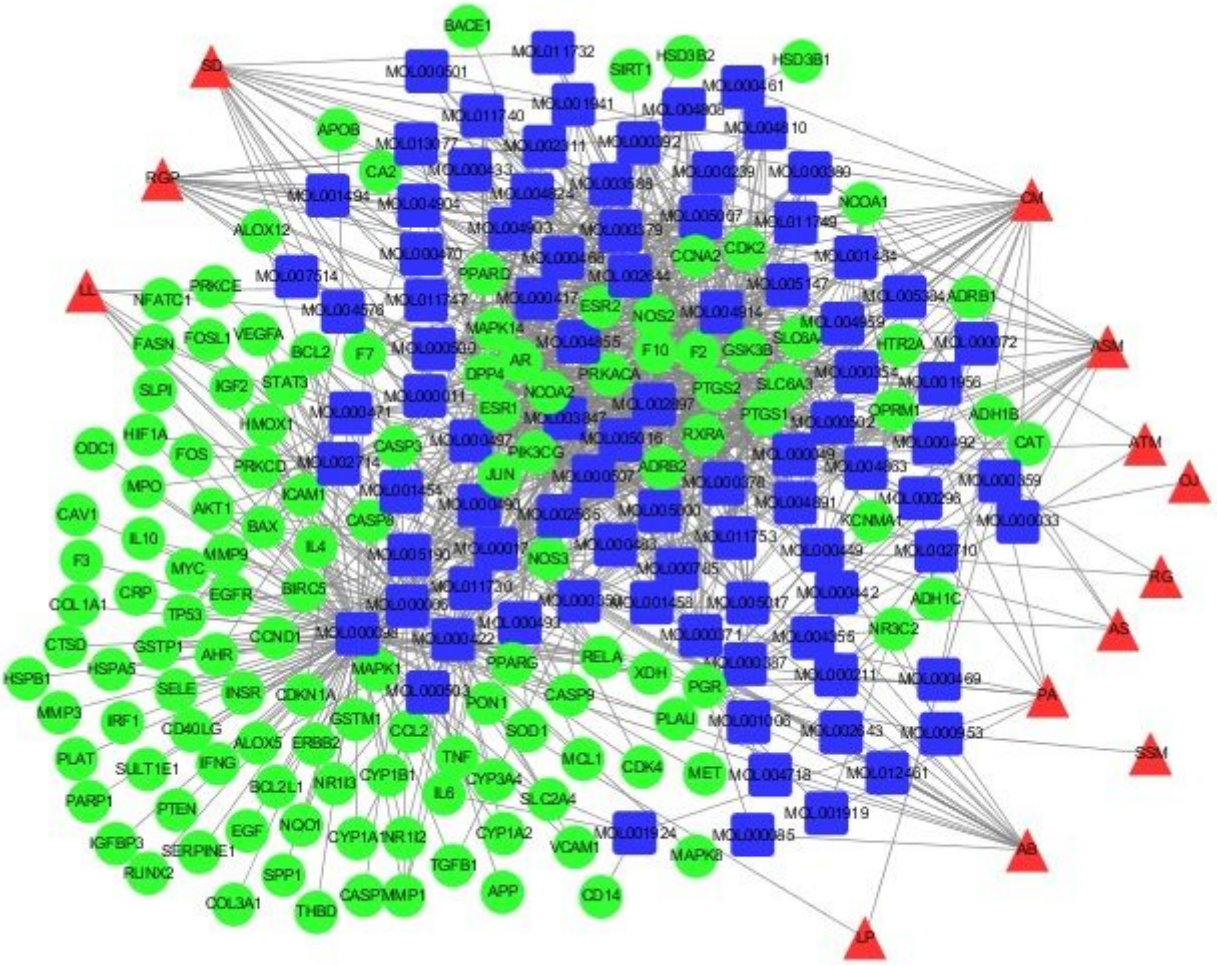

Figure 3

"Medicine-active ingredient-overlapping target gene" network

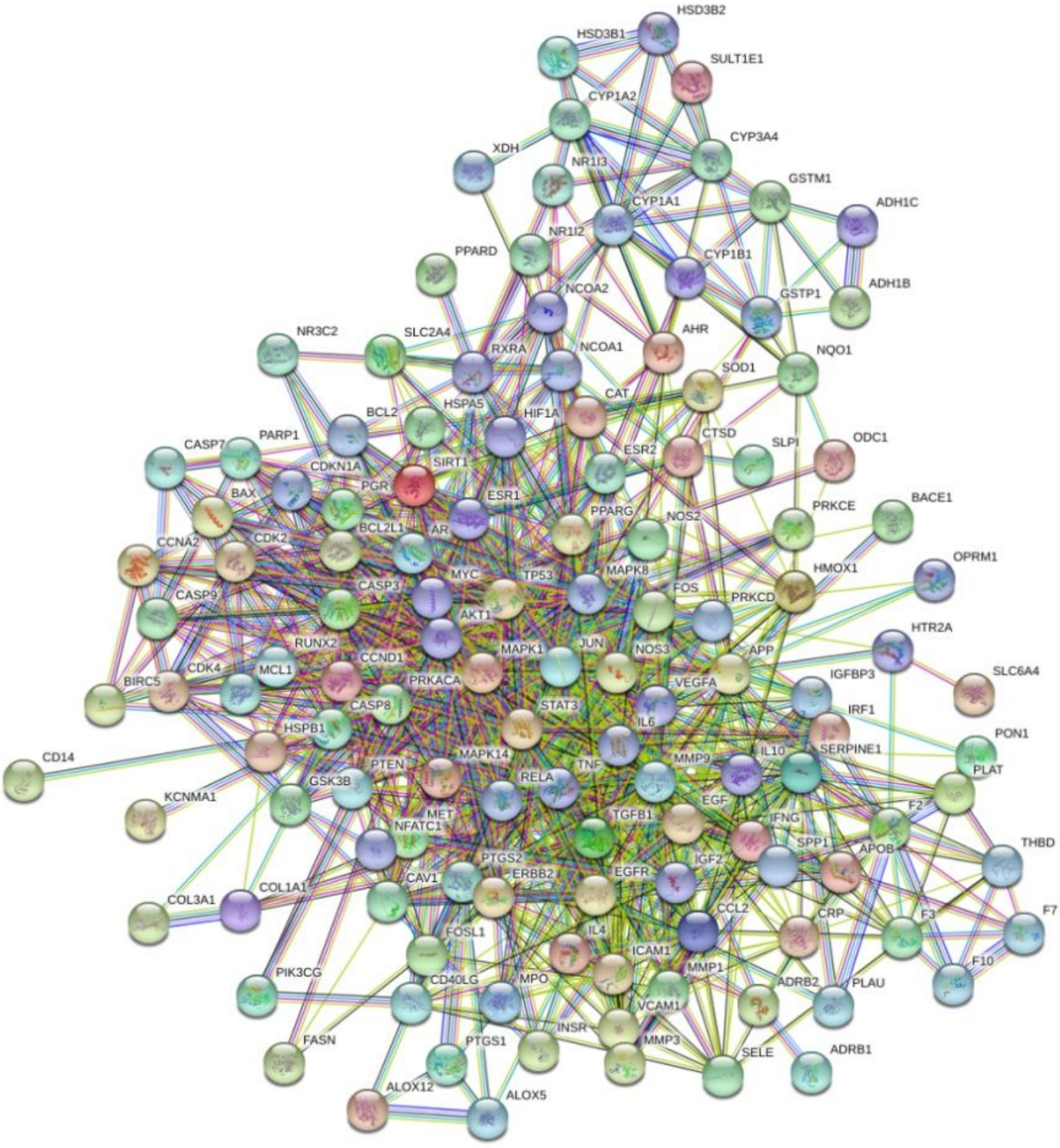

Figure 4

"ZYTLF-PMOP overlapping target gene PPI" network diagram

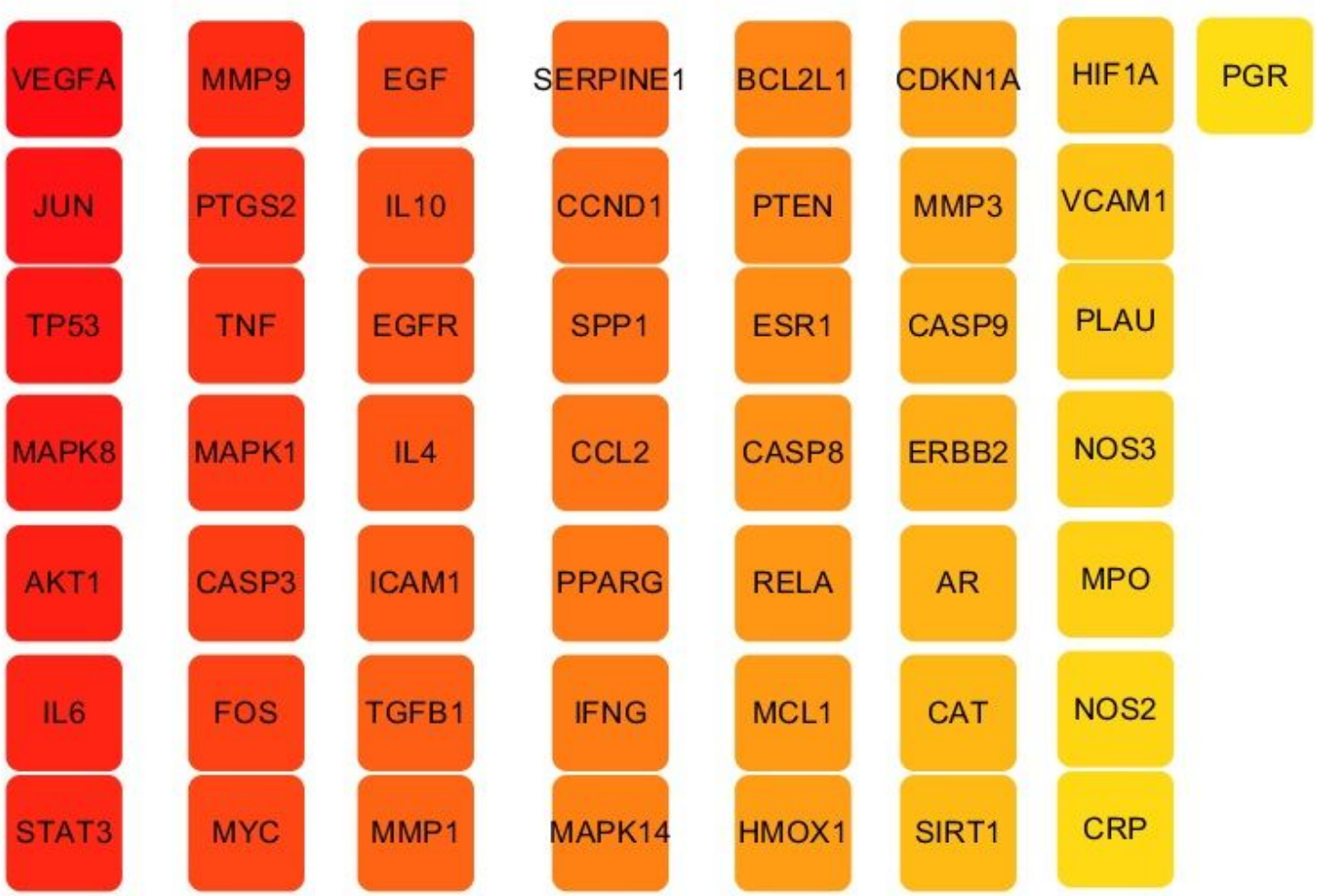

Figure 5

Key genes in overlapping target genes

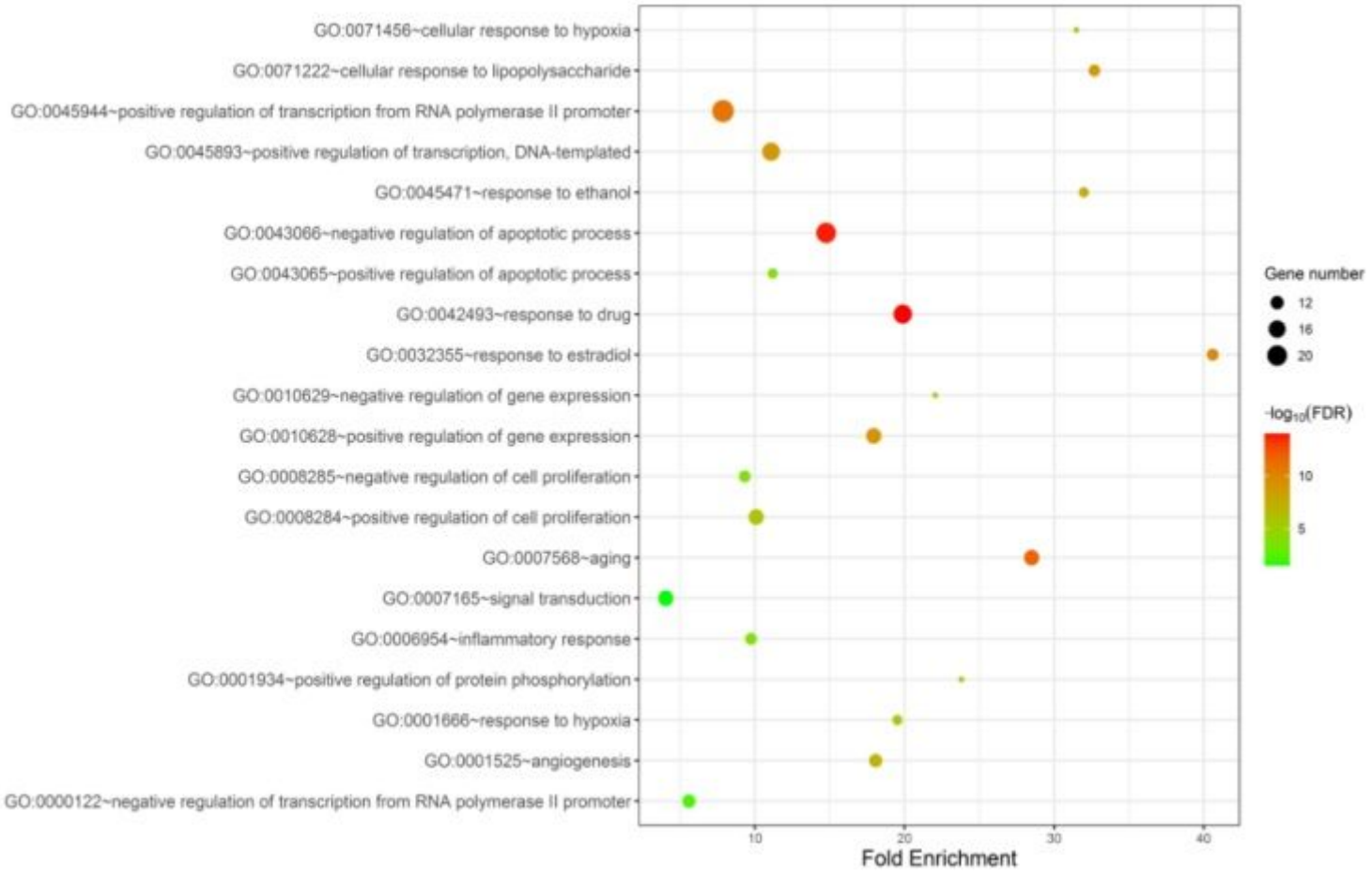

Figure 6

Biological process of Key gene diagram

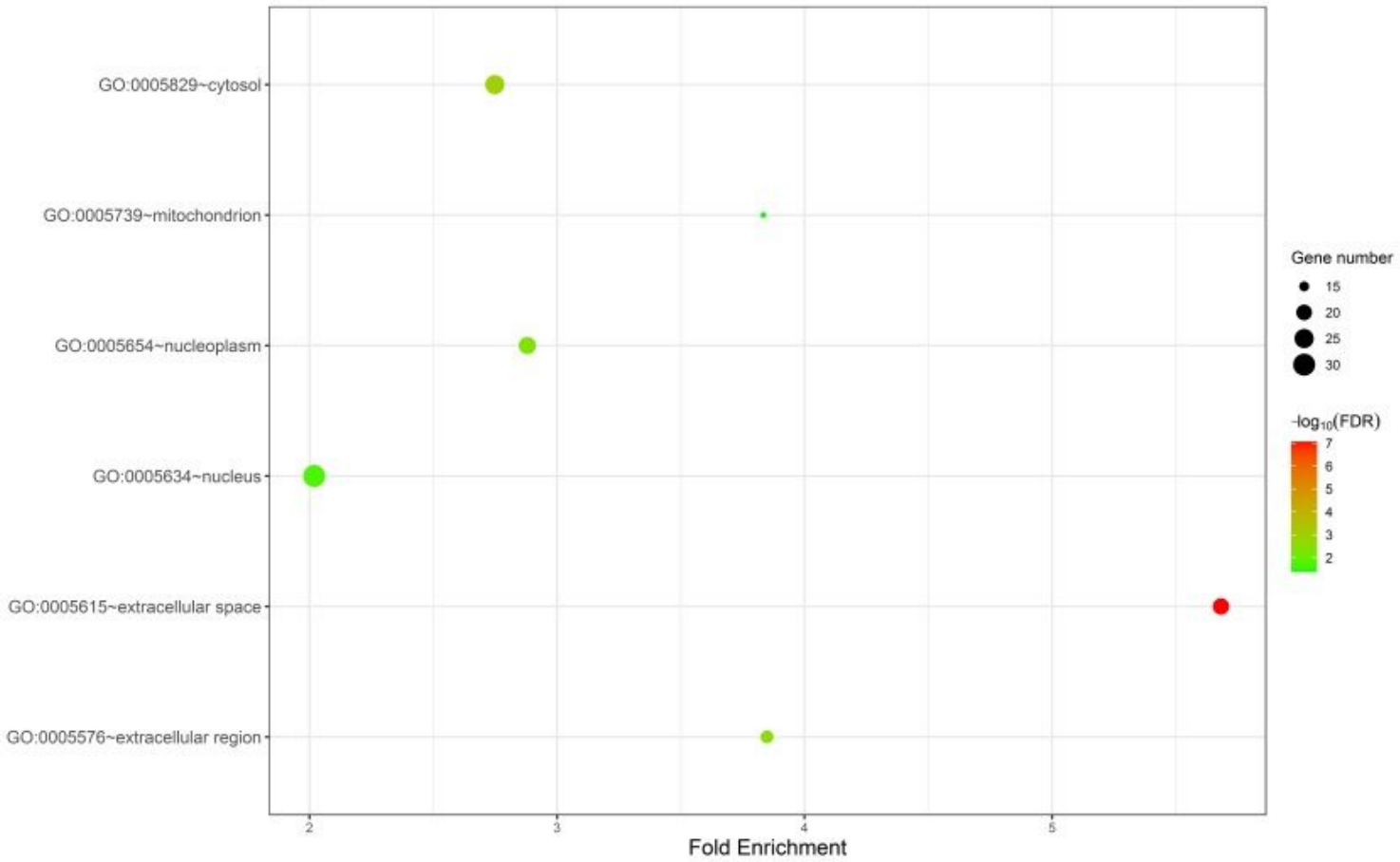

Figure 7

Cell component of key genes diagram

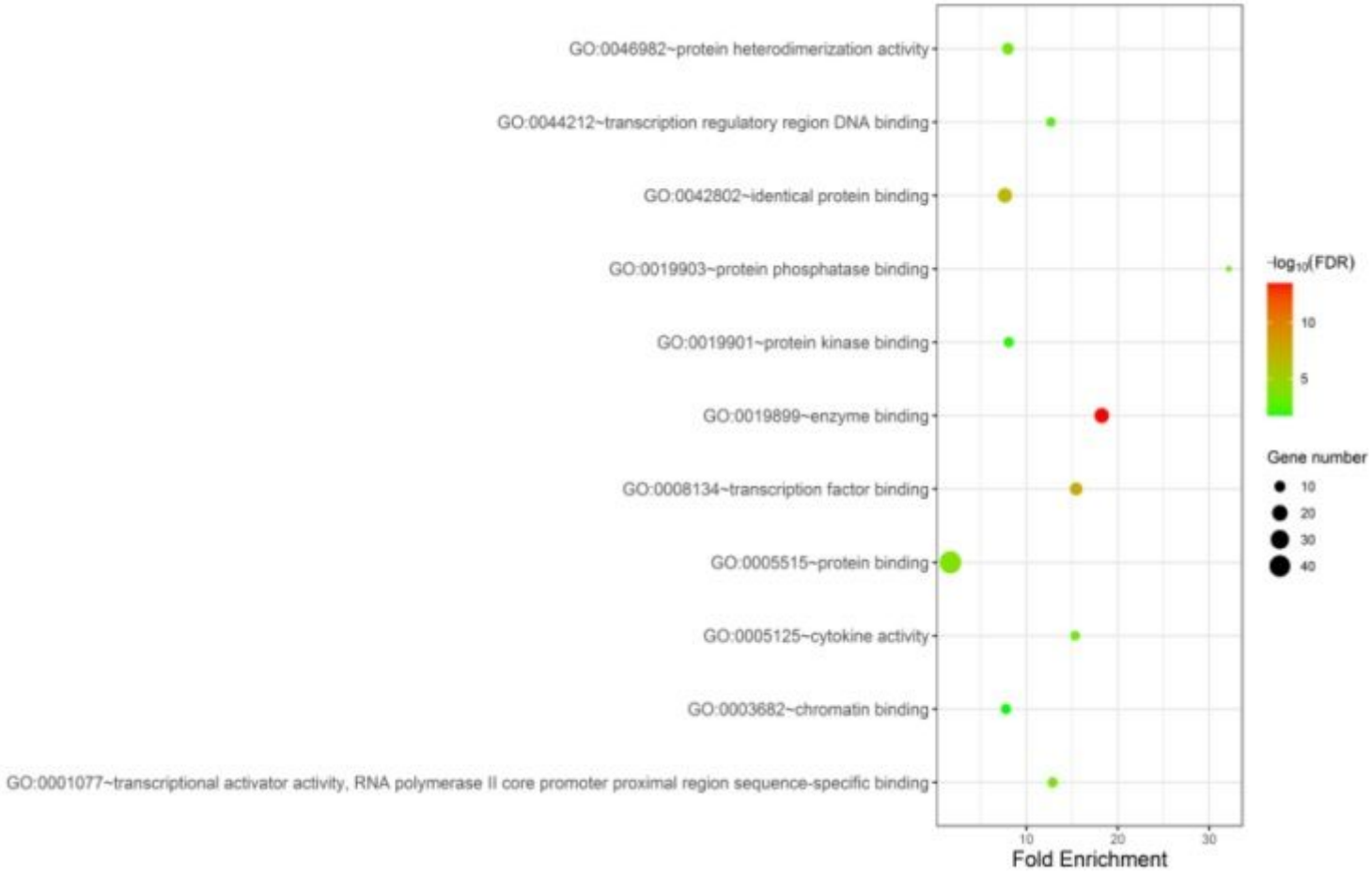

Figure 8

Molecular function of key genes diagram

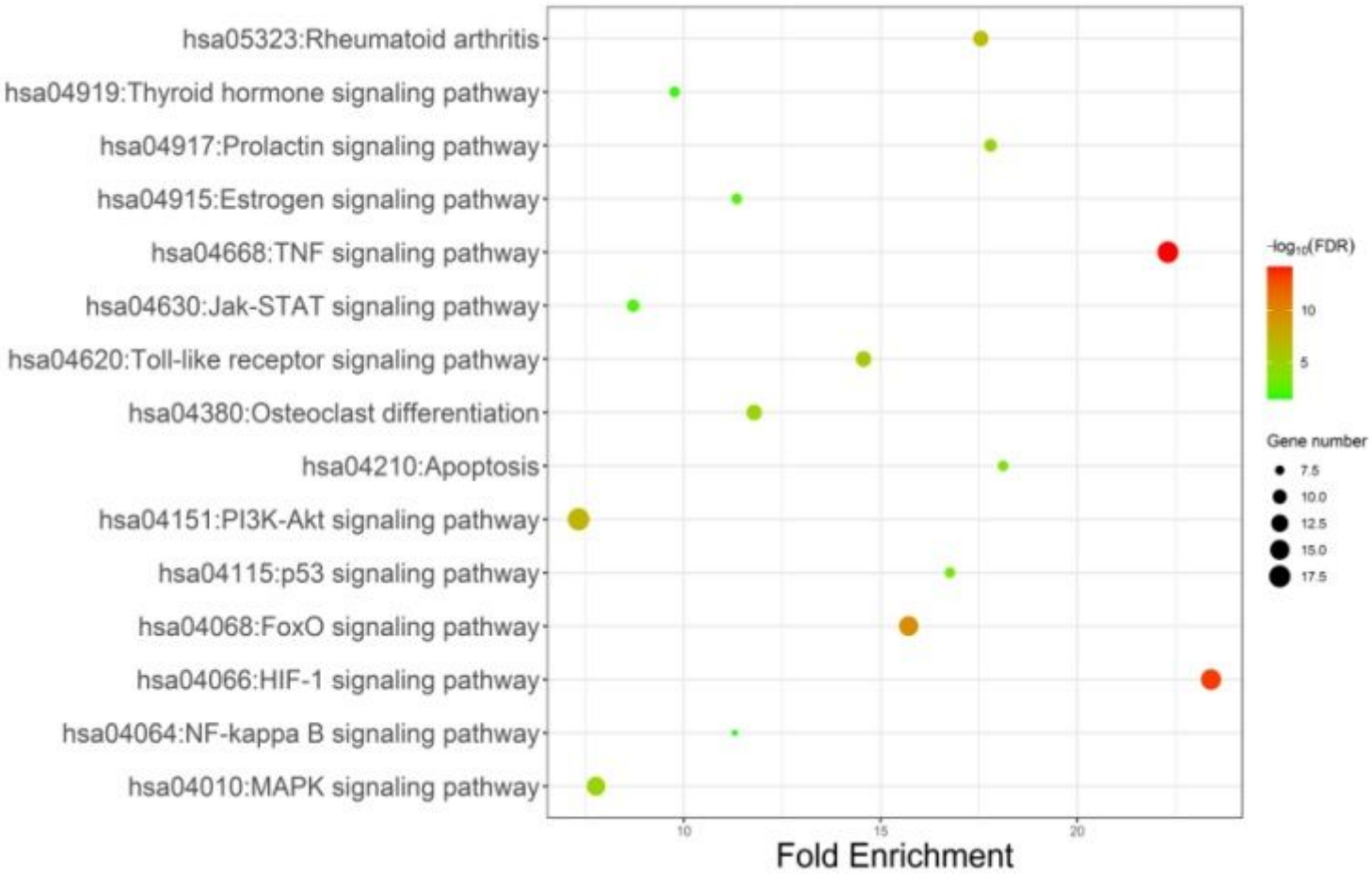

Figure 9

KEGG pathway enrichment of key genes

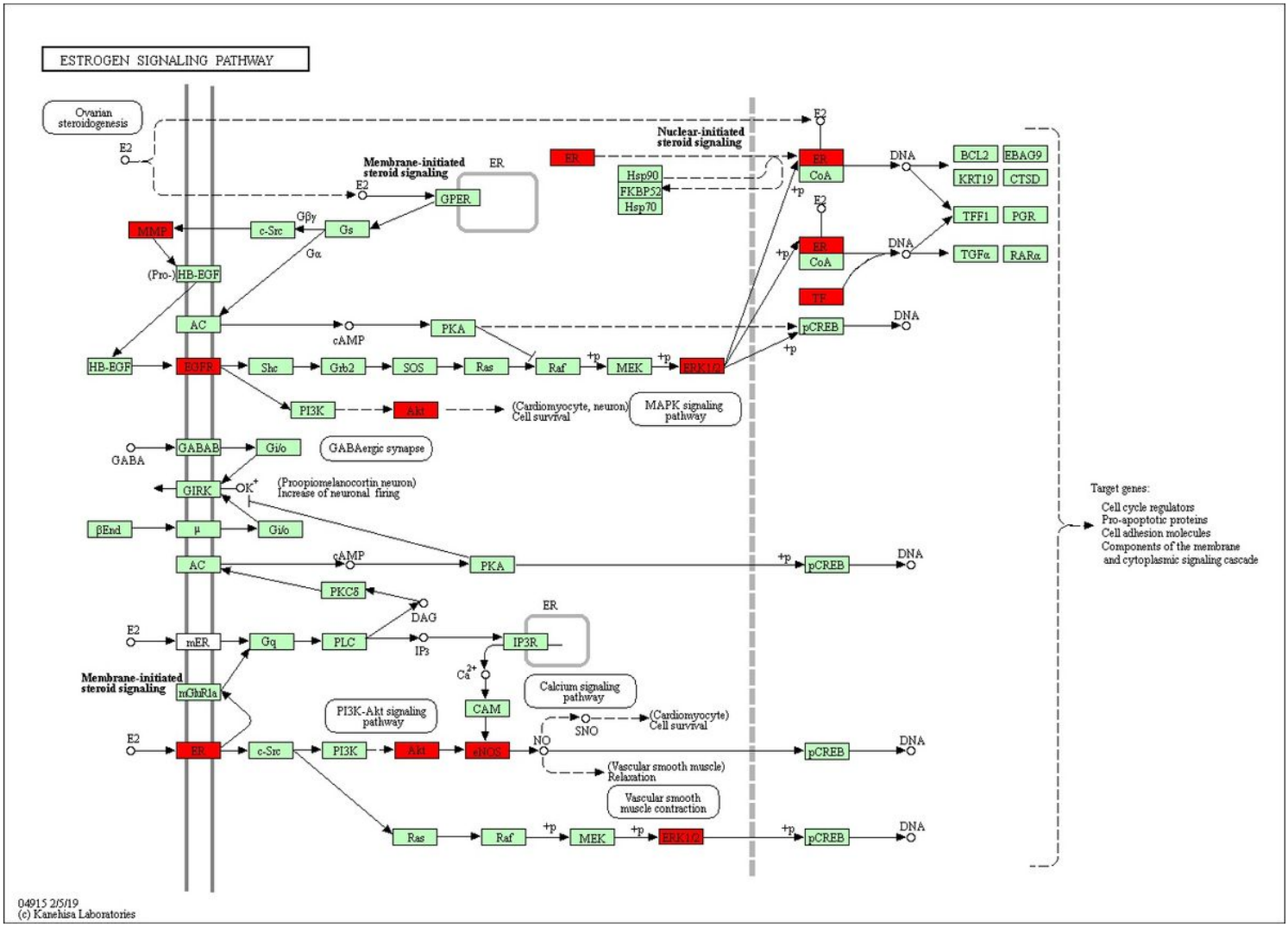

Figure 10

Specific mechanism of gene enrichment in estrogen signaling pathway

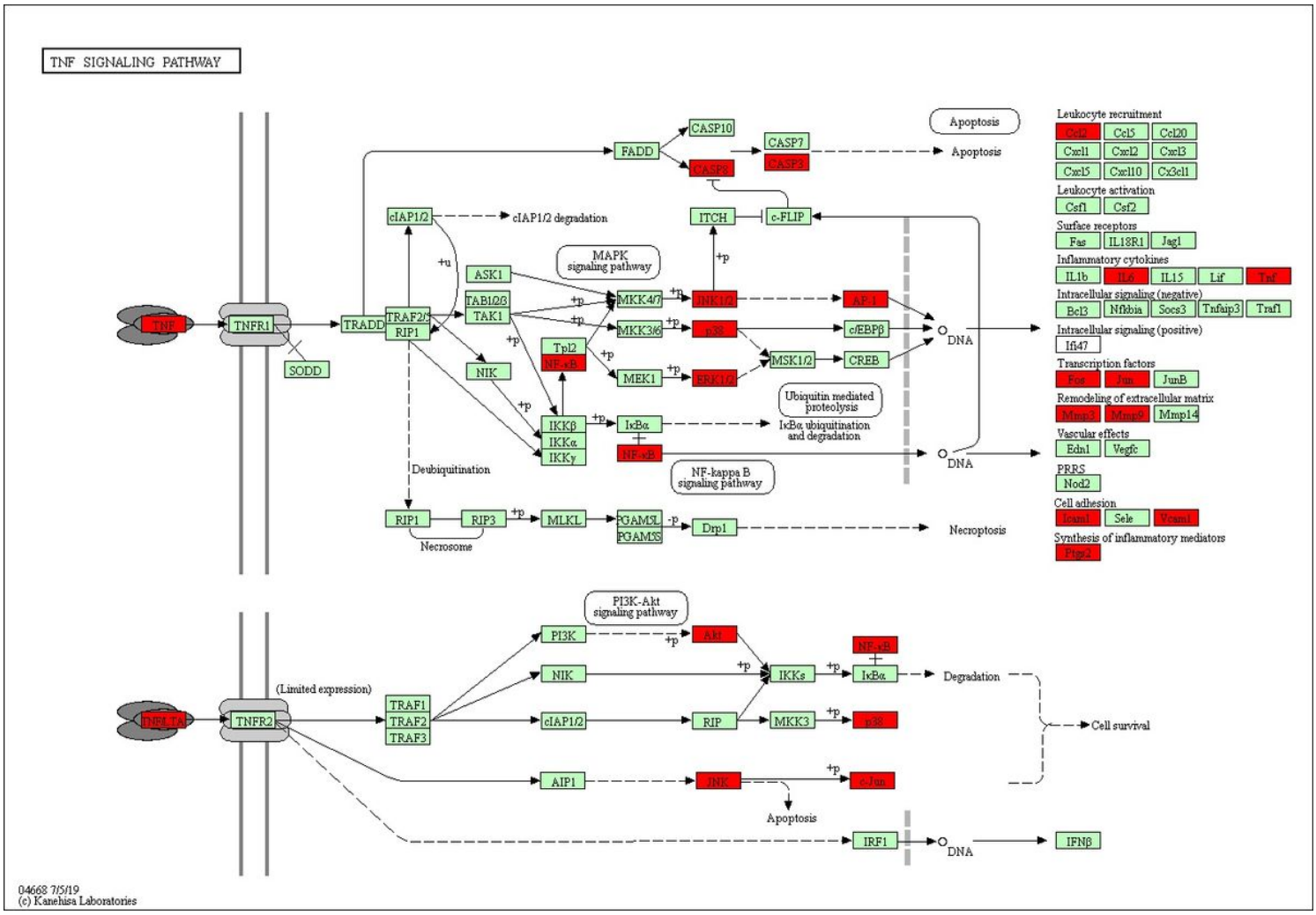

Figure 11

Specific mechanism of gene enrichment in TNF signaling pathway

Supplementary Files

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

- Additionalfile1TableS4.xls
- Additionalfile1TableS3.xls
- Additionalfile1TableS2.xls
- Additionalfile1TableS1.xls