Journal of Clinical and Translational Research
10.18053/Jctres/09.202303.005
ORIGINAL ARTICLE

A network pharmacology and bioinformatics exploration of the possible molecular mechanisms of Fuzheng Xiaoliu Granule for the treatment of hepatocellular carcinoma

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Article information:
Received: September 14, 2022
Revised: January 22, 2023
Accepted: April 10, 2023

Journal of Clinical and Translational Research
10.18053/Jctres/09.202303.005

Abstract

Background and aim: Hepatocellular carcinoma (HCC) is one of the ten most common malignant tumors in the world, and it is a major problem in the world. Traditional Chinese medicine (TCM) has many advantages in the prevention and treatment of HCC, but its complicated mechanism of action is difficult to clarify, which limits its research and development. The continuous development of bioinformation technology provides new methods and opportunities for the research of traditional Chinese medicine. This study uses modern network pharmacology and bioinformatic methods to explore the possible molecular mechanism of the Chinese herbal compound Fuzheng Xiaoliu Granule (FZXLG) to treat HCC, to provide a theoretical basis for their clinical application and basic research, to promote the modernization of traditional Chinese medicine and to promote its worldwide application.

Methods: The active ingredients of FZXLG were collected and screened through TCMSP, BATMAN-TCM, and other databases. The targets of FZXLG were predicted by PubChem and SwissTargetPrediction; HCC disease-related targets were obtained by GeneCards, OMIM, and other disease databases, and the potential gene targets of FZXLG for HCC treatment were screened. The "Prescription-TCMs-Ingredients-Targets" network for FZXLG for the treatment of HCC was constructed, along with the screening of core effective components. The differentially expressed genes(DEGs) of HCC tumor and non-tumor adjacent tissues combined with clinical data in the TCGA database were analyzed to obtain the prognostic genes of HCC. Then, FZXLG genes affecting HCC prognosis were screened, and further screening the core target genes. The correlation between core gene expression with prognosis, immune cell infiltration, and immunohistochemical changes in HCC patients was studied. KEGG enrichment analysis and GO enrichment analysis of the FZXLG genes affecting HCC prognosis were performed by using DAVID database. AutoDockTools software was then used for molecular docking verification.

Results: The ten core effective ingredients of FZXLG for HCC treatment included multiple flavonoids ingredients such as quercetin, luteolin, and formononetin. 11 core targets of FZXLG affecting the prognosis of HCC were screened, among which ESR1 and CAT were favorable prognostic factors, while EGF, MMP9, CCNA2, CCNB1, CDK1, CHEK1, and E2F1 were adverse prognostic factors. MMP9 and EGF were positively correlated with six TIIC subsets. The different expression levels of

CAT, PLG, AR, MMP9, CCNA2, CCNB1, CDK1, and E2F1 were correlated with the immunohistochemical staining changes in normal liver and liver cancer. KEGG pathway enrichment analysis yielded 33 pathways including cell cycle, p53, hepatitis B, and other signaling pathways. Molecular docking verified that the main core components had good binding to the protective prognostic core targets ESR1 and CAT.

Conclusions: FZXLG may treat HCC through multiple ingredients, multiple targets, and multiple pathways, affecting the prognosis, immune microenvironment, and immunohistochemical changes of HCC.

Relevance for patients: FZXLG is a Chinese herbal compound for the treatment of hepatocellular carcinoma, with significant clinical efficacy. However, the mechanism of action is unclear and lacks theoretical support, which limits its popularization application. This study preliminarily revealed its molecular mechanism, providing a theoretical basis for its clinical application, which can better guide its clinical popularization application, and also provide a new strategy for the treatment of HCC.

Keywords: Fuzheng Xiaoliu Granule; hepatocellular carcinoma; network pharmacology; bioinformatics; TCGA database; molecular mechanism.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, accounting for 8.2% of all cancer-related deaths in 2018 [1]. High-risk factors include hepatitis B and C, alcoholic liver cirrhosis, non-alcoholic fat Hepatitis, and family history of liver cancer among others [2]. In recent years, with the extensive application of local therapy, targeted drugs, and immunotherapy for HCC, the curative effect has been significantly improved. However, according to the latest global cancer burden data released by the International Agency for Research on Cancer (IARC) of the World Health Organization in 2020, liver cancer still ranks third among cancer-related deaths, posing a serious threat to the health of all mankind [3]. Traditional Chinese medicines play many important roles in the clinical treatment of HCC, such as improving clinical symptoms, enhancing curative effect, inhibiting tumor growth, reducing recurrence rate, improving patients' quality of life, prolonging survival period, and improving survival rate, which is increasingly applied in the prevention and treatment of liver cancer [4,5].

Fuzheng Xiaoliu Granule (FZXLG) is an intra-hospital agreement prescription of the First Affiliated Hospital of Henan University of CM (patent number: 201811376523.0), which can effectively relieve the clinical symptoms of HCC patients, reduce the postoperative recurrence rate, and improve patients' quality of life [6-8]. In the 13th Five-Year Science and Technology Major Project "Research on the Comprehensive Treatment Plan of Traditional Chinese Medicine for Delaying the Progress of Hepatitis B-related Liver Cancer", FZXLG has been conducted a series of national multi-center large-sample randomized double-blind clinical study, which reduced the one-year recurrence rate of stage I hepatitis B-related liver cancer by 37.33% after comprehensive minimally invasive surgery. The prescription is composed of 12 traditional Chinese medicines, including Radix Astragali (Huangqi, HQ), Smilax china L. (Baqia, BQ), Codonopsis Radix (Dangshen, DS), Atractylodes macrocephala (Baizhu, BZ), Paeoniae Radix Alba (Baishao, BS), Salvia Miltiorrhiza (Danshen, DANS), Carapax Trionycis (Biejia BJ), Concha Ostreae (Muli ML), Actinidia valvata Dunn (Maorenshen, MRS), Citri Reticulatae Pericarpium (Chenpi, CP), Radix Bupleuri (Chaihu, CH), and Glycyrrhiza uralensis (Gancao GC). Among them, HQ and BQ are “principal drugs” with the effects of tonifying qi which means tonifying all kinds of subtle substances in the body that are invisible to the naked eye and strengthening the body resistance, and the effects detoxifying and eliminating pathogens; DS, BZ, BS, and DANS invigorating spleen, tonifying qi, nourishing blood and promoting blood circulation are

"ministerial drugs"; BJ, ML, and MRS are used as adjuvants, which can dissipate mass, detoxify and disperse blood stasis; CP and CH are used as "messenger drugs", dissipating hygrosis to restore stomach and dispersing the stagnated liver-Qi; GC is an adjuvant medicine, which can invigorate qi and harmonize spleen and stomach. All medicines are used together, and they play the role of replenishing qi and reinforcing the healthy qi, dispelling blood stasis, and dispersing knots [9]. With the help of modern scientific and technological methods, it is of great significance to explore the molecular mechanism and clinical correlation of the effective components, related targets, and signaling pathways of FZXLG for the treatment of HCC, to guide its clinical application and further basic research.

This study made full use of modern scientific and technological methods, such as network pharmacology and bioinformatics, exploring the molecular mechanism and clinical correlation of FZXLG for HCC treatment from massive data information [10], and provided a theoretical basis for its clinical application and basic research.

2. Materials and methods

2.1 Materials

Database used in this study

Table 1. Related databases

No.	Database	Website
1	TCMSP	https://tcmsp-e.com/
2	BATMAN-TCM	http://bionet.ncpsb.org.cn/batman-tcm/
3	Compound Reference Database, Chinese Academy of Sciences	http://www.chemcpd.csdb.cn/cmpref/default.htm
4	CNKI	http://www-cnki-net-s.vpn1.hactcm.edu.cn/
5	SwissADME	http://www.swisstargetprediction.ch/
6	PubChem	https://pubchem.ncbi.nlm.nih.gov/
7	Uniprot	https://www.uniprot.org/
8	SwissTargetPrediction	http://www.swisstargetprediction.ch/
9	GeneCards	https://www.genecards.org/
10	OMIM	https://omim.org/
11	DrugBank	https://go.drugbank.com/
12	STRING	https://cn.string-db.org/
13	DAVID	https://david.ncifcrf.gov/tools.jsp
14	PDB	https://www1.rcsb.org/
15	TCGA	https://portal.gdc.cancer.gov
16	Ensembl	http://asia.ensembl.org/index.html
17	TIMER（tumor immune estimation resource）	https://cistrome.shinyapps.io/timer/
18	HPA（human protein atlas）	https://www.proteinatlas.org/
19	bioinformatics	https://www.bioinformatics.com.cn/

2.2 Methods

2.2.1 Collecting and screening of active ingredients of FZXLG

The active ingredients of TCMs contained in FZXLG were collected through the TCMSP database; and then based on the pharmacokinetic parameters of absorption, distribution, metabolism, and excretion (ADME) [11], the active ingredients with human oral bio-availability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18 were screened. The traditional Chinese medicine whose ingredients were not retrieved continued to be searched in BATMAN-TCM, Chinese Academy of Sciences chemical composition database, CNKI, and other databases to collect their active ingredients; then using the SwissADME online platform the active ingredients which satisfied the condition that GI (gastrointestinal, GI) absorption as "High" group and the drug-likeness which accorded with at least three items included were filtered and screened out.

2.2.2 Searching for the potential targets of FZXLG

The target proteins corresponding to the active components of traditional Chinese medicine in FZXLG were collected through Uniprot, PubChem, SwissTargetPrediction, and other databases, and then the corresponding target genes were predicted [12]. All target genes were summarized and sorted out to obtain the potential target genes for FZXLG prescription.

2.2.3 Obtaining HCC disease gene targets

Through GeneCards, OMIM, DrugBank, and other disease gene target databases, taking "Hepat ocellular Carcinoma" as the keyword and restricting the search conditions to "gene" and "hom o sapiens", HCC disease gene targets were gathered and screened.

2.2.4. Screening potential gene targets of FZXLG for HCC treatment and constructing a "Prescription-TCMs-Ingredients-Targets" network

Screening the shared targets between HCC disease gene targets and the potential target genes of FZXLG, the potential gene targets and corresponding active ingredients of FZXLG for HCC treatment were obtained. Then Cytoscape 3.8.0 "NetworkAnalyzer" app was applied to analyze the data

including traditional Chinese medicines, potential target genes, and effective ingredients of FZXLG for HCC treatment, and the "Prescription-TCMs-Ingredients-Targets" network was constructed. According to the more connections between drug components and gene targets in the network, the higher the degree value was, the more important their roles played; 10 core active ingredients were screened.

2.2.5 Screening out the prognosis-related genes of HCC

First, retrieve the TCGA-LIHC Data set from the TCGA DATA online database, select the transcriptome profiling data file by the Data Category, and then choose “data type: Gene Expression Quantification”. Finally, add all files to the cart, as well as clinical, and biospecimen metadata among other data, and download them. For TCGA-LIHC_ MRNA data was preprocessed to remove outliers based on the Spearman correlation coefficient (cor. cut=0.6). The gene id in the downloaded transcriptome data was extracted into the matrix file with Perl software, and the number of cases in the normal group and tumor group was calculated. The gene id in the matrix file was converted into the gene symbol by using the human gene annotation set data file downloaded from the Ensemble gene database. The EDASeq package was used to standardize expression matrix data, and low expression genes (qnt. cut=0.25) were filtered out. The edgeR package was used to analyze and screen DEGs of tumors and adjacent normal tissues, setting the conditions: foldchange=1 (difference multiple $\geqslant$ 2), FDR(false discovery rate) < 0.05. The data were uploaded to the online platform of "bioinformatics" to draw volcano plot of HCC DEGs. Then the R language univariate Cox package was utilized to perform univariate Cox survival analysis, screening the genes affecting the prognosis of HCC patients.

2.2.6 Screening potential gene targets of FZXLG affecting the prognosis of HCC

The common genes between the potential gene targets of FZXLG for HCC treatment and Cox prognostic genes (Section 2.2.5) were screened, that is, the potential gene targets of FZXLG affecting the prognosis of HCC.

2.2.7 Protein-protein interaction (PPI) network analysis and screening of core targets

The potential gene targets of FZXLG affecting the prognosis of HCC were uploaded to the STRING database for protein-protein interaction network (PPI) analysis (limiting species to human, setting

"medium confidence: 0.400", hiding free nodes, and other parameters remained default). In the network, nodes represent gene targets, and edges connecting nodes represent interactions between targets. The node degree value represents the number of edges connected to the node. The larger the value, the more nodes are connected, which indicates that nodes are more important in the network and play key roles in the whole network. According to this, the data was imported into the software Cytoscape 3.8.0, and a "core targets PPI network" was constructed to screen the core targets.

2.2.8 Clinical correlation analysis of core targets

The correlation between the expression of core targets with the prognosis, immune cell infiltration, and immunohistochemical changes was investigated by using R language, TIMER (tumor immune estimation resource), and human protein atlas (HPA) database.

2.2.9 KEGG and GO enrichment analysis

The potential gene targets of FZXLG which affect HCC prognosis obtained (Section 2.2.6) were uploaded to the DAVID 6.8 online database for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and gene ontology (GO) enrichment analysis; FDR was used to perform multiple test corrections on results. GO enrichment analysis principally comprises three categories, namely Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The results obtained were visualized by using the R language.

2.2.10 Molecular docking

The PubChem database and OpenBable 2.4.1 software were used to retrieve the mol2 format file of the core ingredients, and the protein 3D structure as a PDB format file corresponding to the core targets was downloaded from the PDB database. (Protein 3D structure screening conditions [13] are: 1) The source is limited to "Homo sapiens"; 2) The protein structure is obtained by X-RAY analysis; 3) The resolution of protein is high (resolution $\leqslant$ 3 Å); 4) The protein structure reported in the molecular docking literature is preferred.) AutoDockTools 1.5.7, MGLTools-1.5.7, and Python 2.7.11 were utilized to perform molecular docking; the results were visualized through PyMol 2.2.0 software.

3. Results

3.1 Active ingredients of FZXLG

A total of 217 active ingredients of FZXLG were obtained, including 16 for HQ, 11 for BQ, 17 for DS, 4 for BZ, 8 for BS, 5 for CP, 13 for CH, 59 for DS, 3 for BJ, 11 for ML, 5 for MRS, 88 for GC. There were 16 ingredients shared by two or more traditional Chinese medicines.

3.2 Potential targets of FZXLG

Predicting the target genes of 217 active ingredients of FZXLG, 501 potential action targets of FZXLG were obtained.

3.3 HCC disease gene targets

The HCC disease gene targets collected from disease databases such as GeneCards (relevance score ≥ 4.07), OMIM, and DrugBank were screened and merged. A total of 2173 HCC disease gene targets were obtained.

3.4 "Prescription-TCMs-Ingredients-Targets" network of FZXLG for HCC treatment

The shared targets of HCC disease gene targets and the FZXLG potential targets were screened, and 205 potential gene targets of FZXLG for HCC treatment and 204 corresponding effective ingredients were obtained. The data information was imported into Cytoscape 3.8.0 software; the "Prescription-TCMs-Ingredients-Targets" network of FZXLG for HCC treatment was constructed (see Figure 1). Then 10 core active ingredients were screened according to the degree value ⩾ 22 (see Table 2).

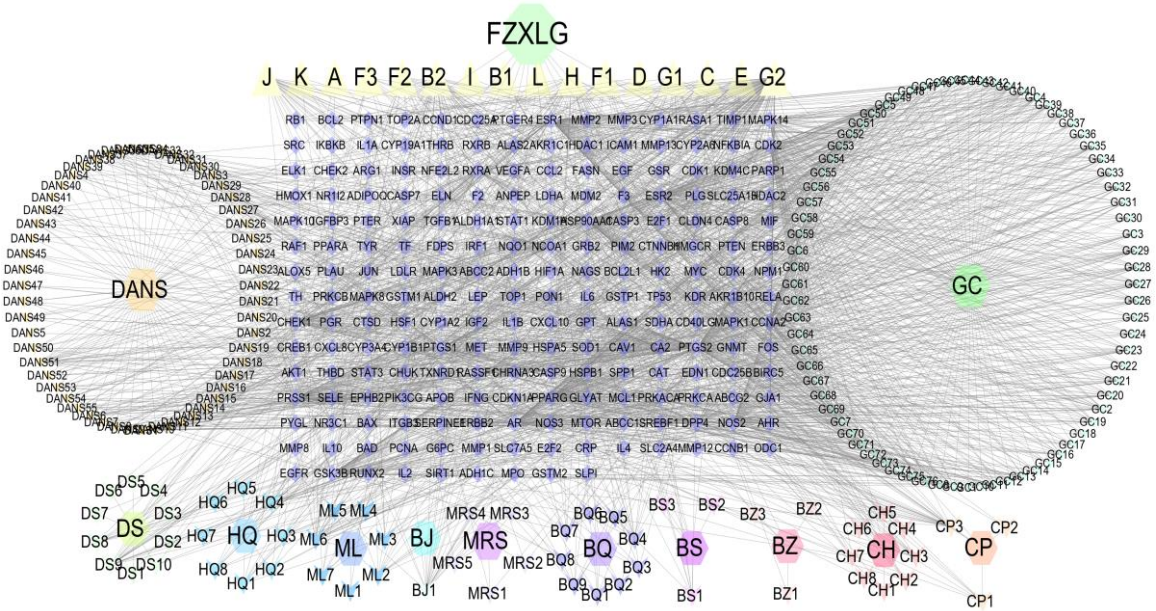

Figure 1. "Prescription-TCMs-Ingredients-Targets" network of FZXLG for HCC treatment

Note: octagon: FZXLG; hexagons: TCMs; triangles: the 16 ingredients shared by two or more TCMs; diamonds: targets.

Table 2. Core ingredients of FZXLG for HCC treatment.

sort	name	degree
1	quercetin	111
2	luteolin	52
3	kaempferol	44
4	glycine	30
5	naringenin	30
6	nobiletin	24
7	isorhamnetin	24
8	tanshinone IIA	23
9	formononetin	23
10	licochalcone A	22

3.5 HCC prognosis-related genes

374 cases of the HCC tumor group and 50 cases of the adjacent normal group were retrieved from the TCGA-LIHC database; the data was analyzed and the DEGs volcano map was drawn (see Figure 2),

and 9607 DEGs (up-regulated 8023, down-regulated 1584) were obtained. Then, combined with the clinical data of 369 HCC patients, univariate Cox survival analysis was performed, and 2378 prognosis-related genes of HCC were screened out ($P < 0.05$).

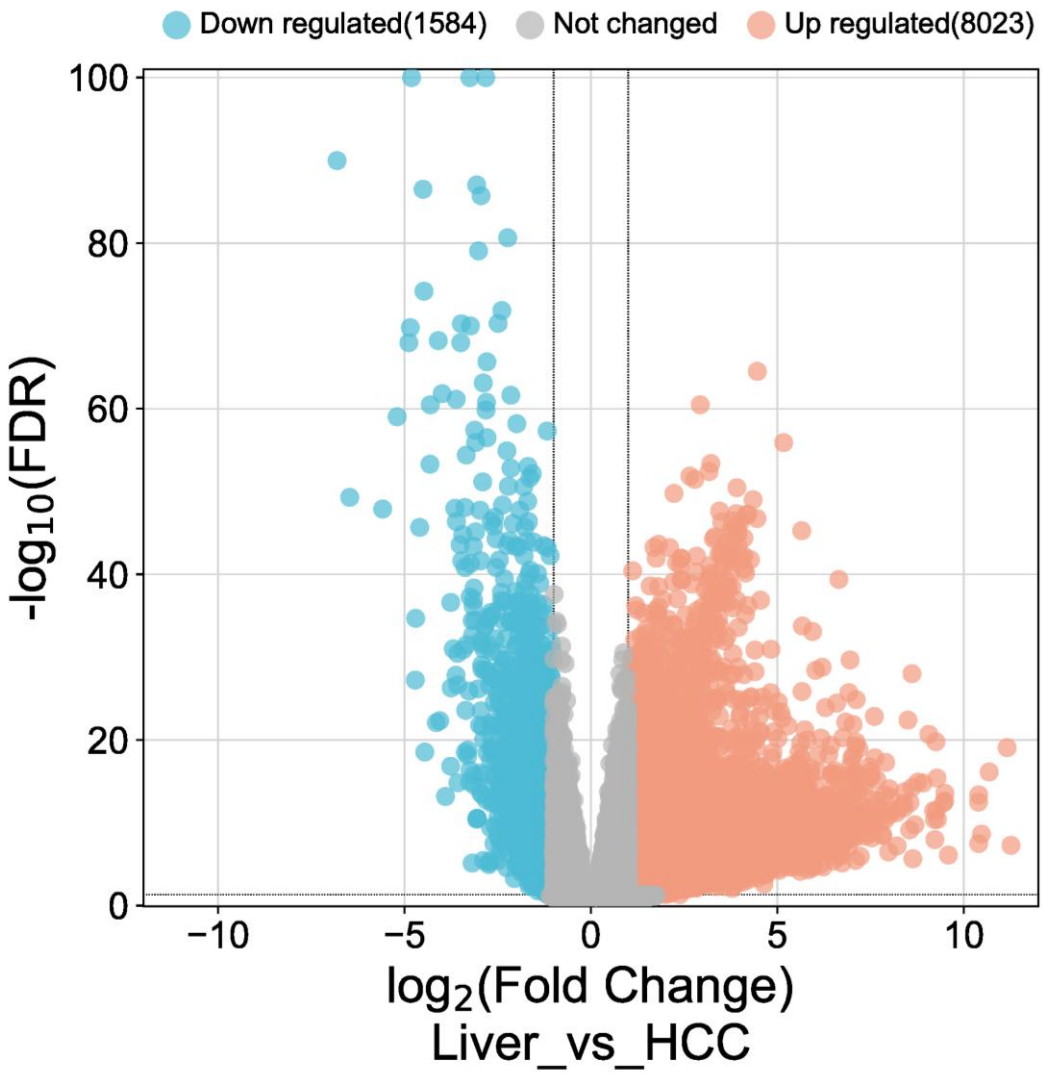

Figure 2. Volcano map of DEGs of HCC and tumor-adjacent tissues.

3.6 Potential targets of FZXLG affecting the prognosis of HCC

42 genes that were the potential gene target of FZXLG affecting the prognosis of HCC were screened out.

3.7 PPI network and core targets

The 42 potential prognostic gene targets (Section 3.6) were uploaded to the STRING database for PPI

analysis; then the analysis result was imported into Cytoscape 3.8.0 software NetworkAnalyzer, and according to the target degree value $\geqslant$ 11, 11 core targets were screened out(See Table 3) and the PPI network was drawn (see Figure 3).

Table 3. Core targets of FZXLG treatment for HCC.

gene name	degree
ESR1	23
MMP9	15
EGF	15
CCNB1	14
CCNA2	14
CAT	13
CDK1	13
PLG	12
E2F1	11
AR	11
CHEK1	11

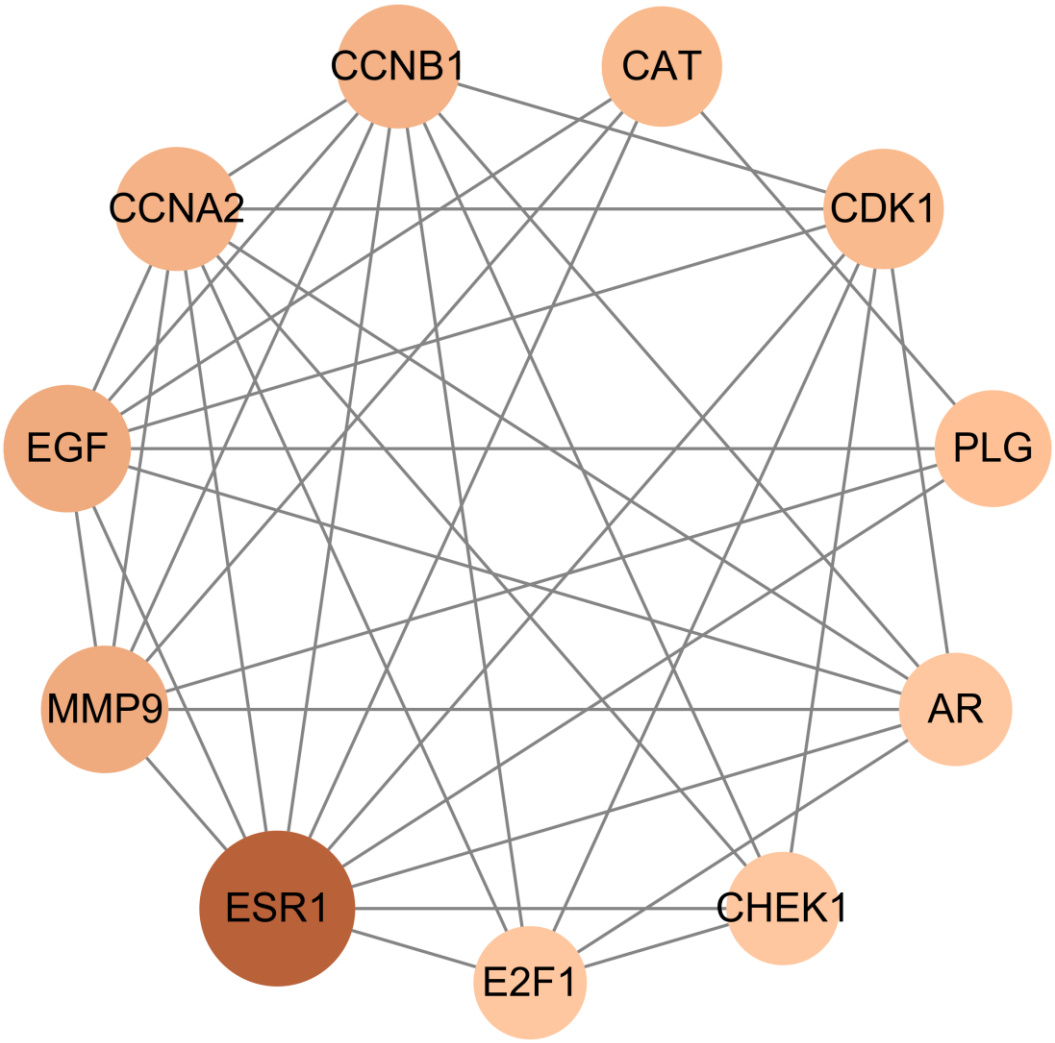

Figure 3. Core targets PPI network.

Note: The size and color depth of the nodes are proportional to the degree value. The larger and darker the node, the more important it is in the network.

3.8 Clinical correlation analysis of core targets

3.8.1 Relationship between core targets and prognosis of HCC patients

The relationship between the 11 core targets obtained (Section 3.7) and the survival prognosis of HCC patients were analyzed. The hazard ratio (HR) of core targets was obtained based on univariate Cox prognostic analysis (See Table 4). ESR1, CAT, PLG, and AR (HR<1) were low-risk factors for the prognosis of HCC patients, while EGF, MMP9, CCNA2, CCNB1, CDK1, CHEK1, and E2F1 (HR>1) were high-risk factors. Kaplan-Meier survival analysis was performed and survival curves were drawn

(see Figure 4) by using the R language "survival package"; as shown in the figure, except for PLG and AR ($P > 0.05$), the other 9 core targets were significantly correlated with the prognosis of HCC patients ($P < 0.05$). The survival rate of ESR1 and CAT high expression group was better than that of the low expression group, while the survival rate of MMP9, EGF, CCNA2, CCNB1, CDK1, CHEK1, and E2F1 low expression group was better than that of the high expression group.

Table 4. HR of the core targets.

gene name	HR
ESR1	0.88
CAT	0.81
PLG	0.91
AR	0.90
EGF	1.07
MMP9	1.08
CCNA2	1.23
CCNB1	1.38
CDK1	1.30
CHEK1	1.41
E2F1	1.18

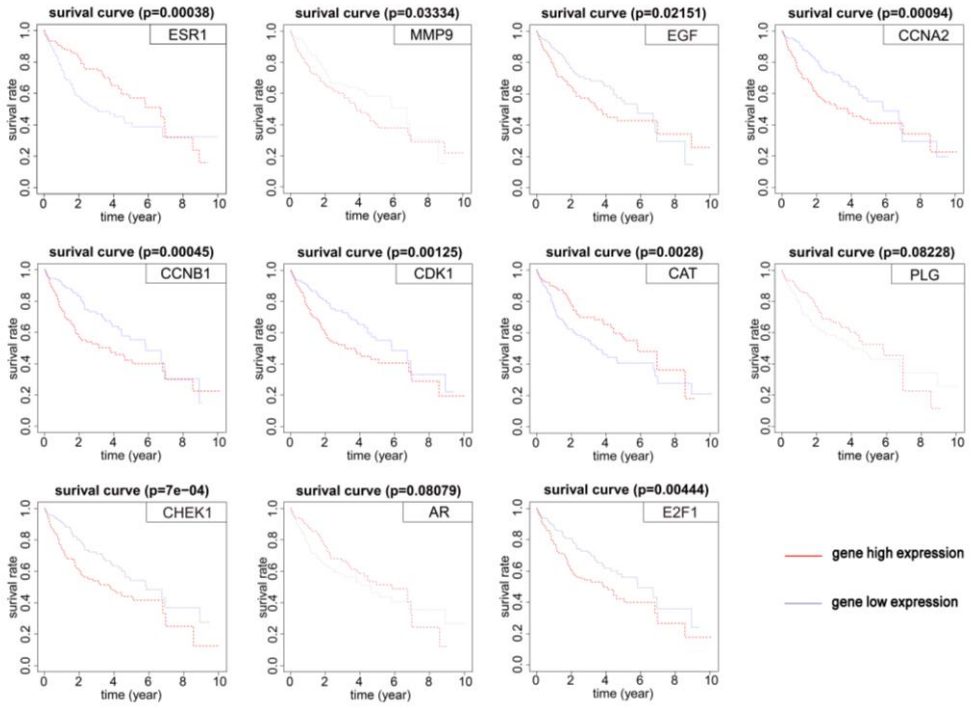

Figure 4. KM survival curve of core targets affecting the prognosis of HCC patients.

3.8.2 Correlation between core targets and immune cell infiltration in HCC tissues

The correlation between 11 core targets and tumor purity, 6 types of tumor-infiltrating immune cells (TIICs) (B cells, CD8+T cells, CD4+T cells, macrophages, neutrophils, and dendritic cells) in HCC were analyzed by TIMER database. Scatter plots of correlations between 11 core targets and tumor purity, 6 TIICs in HCC were shown in Figure 5 (the blue curve represented the trend of gene expression level and immune correlation). As shown in the figure, ESR1, MMP9, and EGF are negatively correlated with tumor purity among which MMP9 and EGF are positively correlated with six TIIC subsets, while CCNA1, CCNB2, CDK1, CAT, CHEK1, and E2F1 were positively correlated ("partial correlation" is the purity-corrected partial Spearman's correlation, cor<0 represents negative correlation, cor>0 represents positive correlation).

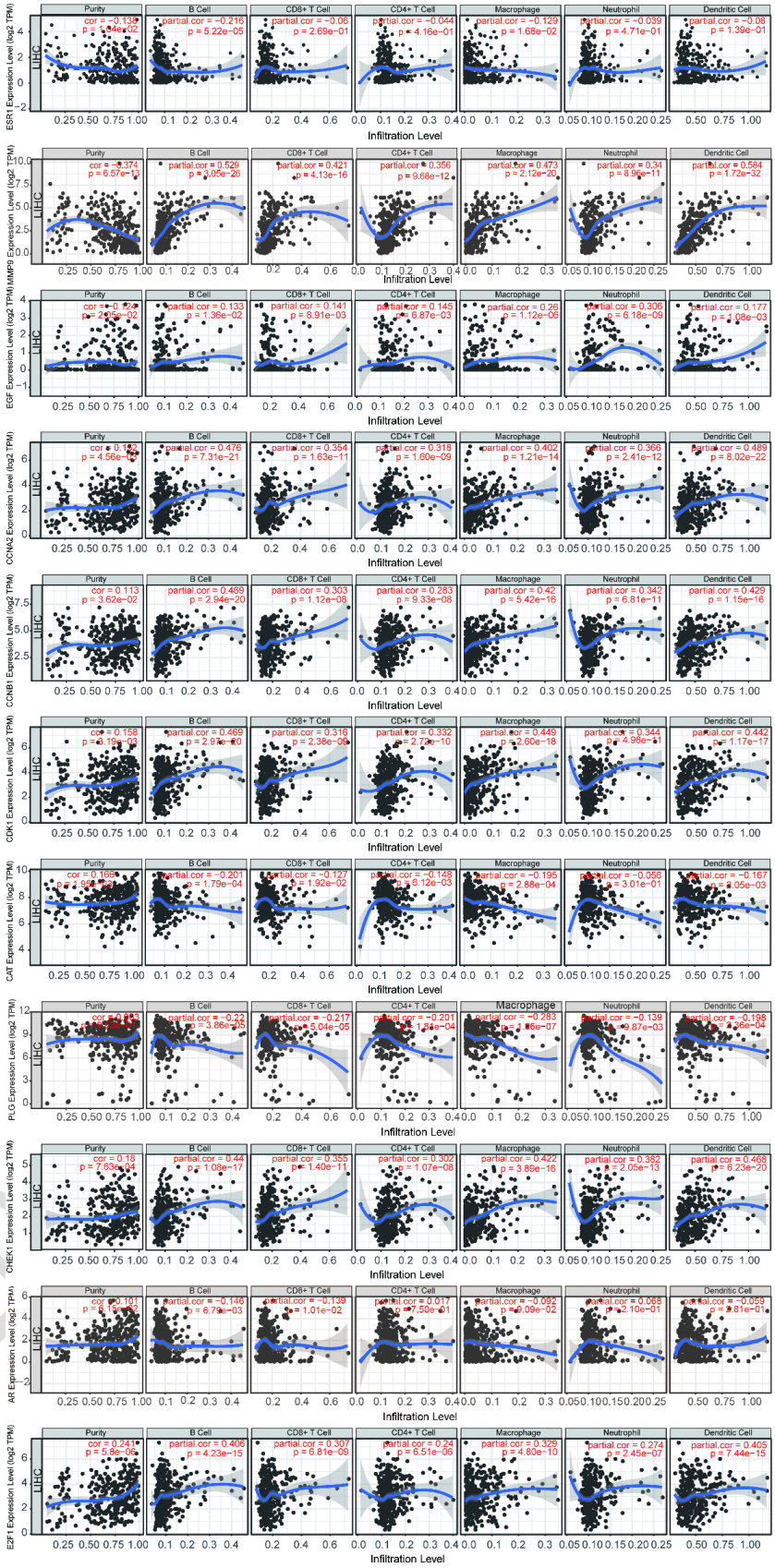

Figure 5. Scatter plots of correlation between core genes and tumor purity and six TIIC subsets in HCC.

3.8.3. Correlation between core target expression and immunohistochemical changes

We further studied the correlation between the protein expression of core target genes in the h uman protein map and immunohistochemistry and showed the representative characteristics im munohistochemical map of their different expression levels in Figure 6. Except that there are no relevant images of CHEK1 and EGF; as shown in the figure, there was no significant differe nce in the expression of ERS1 in liver and HCC. The different expression levels of the other eight core targets CAT, PLG, AR, MMP9, CCNA2, CCNB1, CDK1, and E2F1 were correlate d with the immunohistochemical staining changes in normal liver and liver cancer (identified by eyeballing, no formal statistical test).

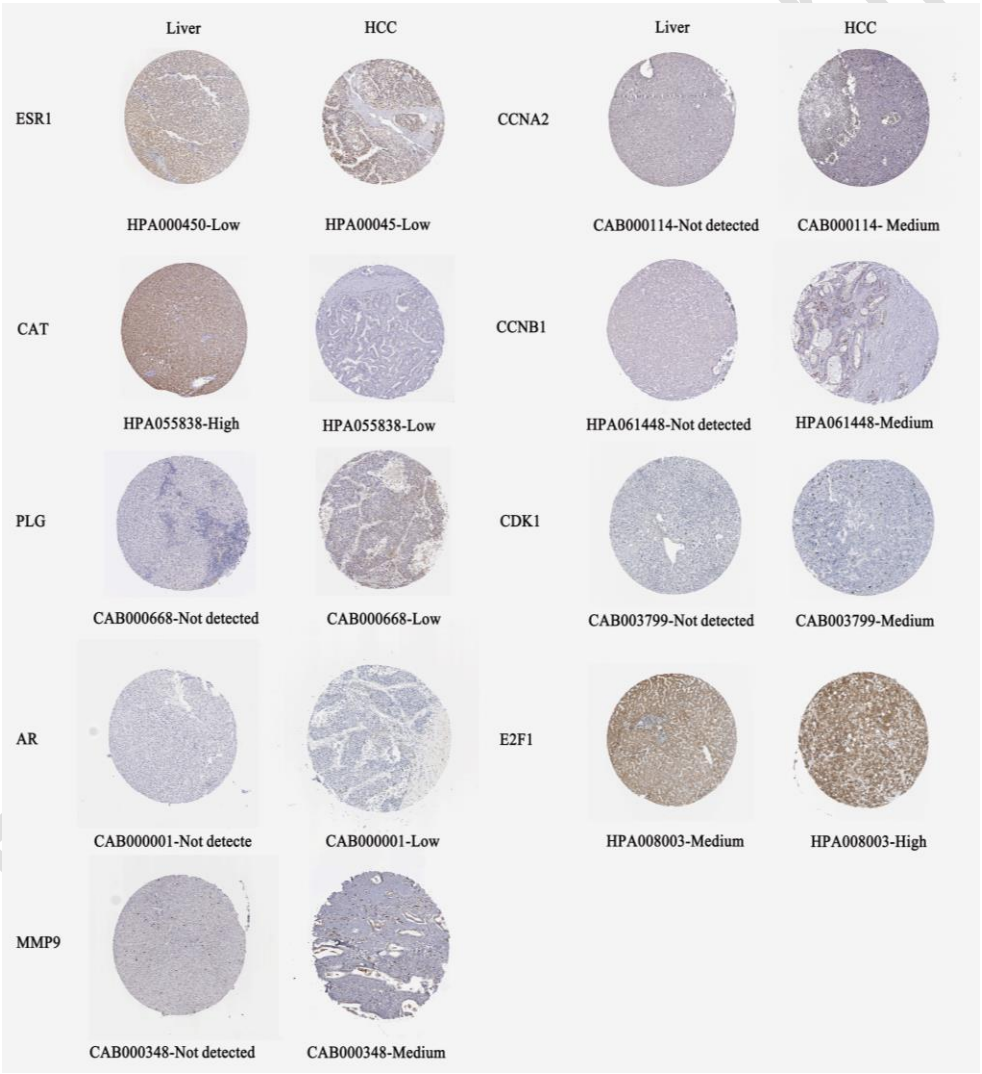

Figure 6. Representative immunohistochemistry images of different protein expression levels of core targets in HCC tissues and normal liver tissues.

3.9. KEGG and GO enrichment analysis

The DAVID database was used for the enrichment analysis of 42 potential prognostic gene targets. The KEGG pathway enrichment analysis mainly involved 33 pathways, such as cell cycle, Hepatitis B, and p53 signaling pathway; the result data was uploaded to "bioinformatics" for visualization (see Figure 7). GO enrichment analysis results included biological processes (BP) such as G2/M transition of mitotic cell cycle, positive regulation of fibroblast proliferation, cell division among others, cellular components (CC) for example cyclin B1-CDK1 complex, mitochondrial matrix, chromatin, molecular functions (MF) such as enzyme binding, sequence-specific DNA binding, endopeptidase activity; the top 5 results of BP, CC, and MF which were screened out according to the FDR from small to large were uploaded to "bioinformatics" for visualization (see Figure 8).

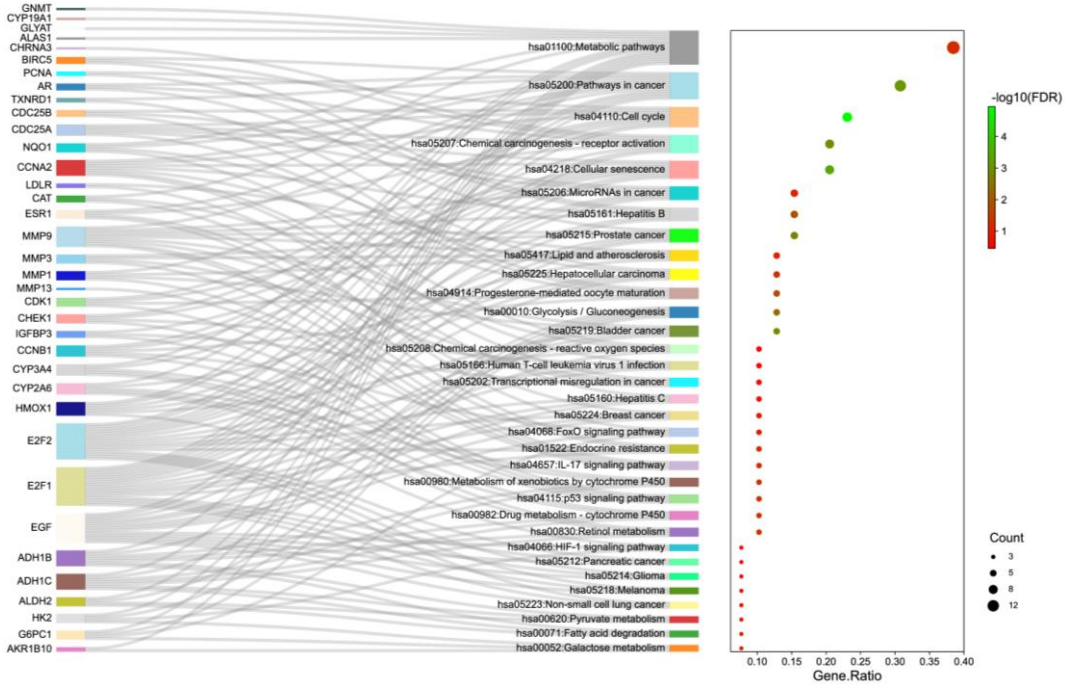

Figure 7. Sankey dot KEGG pathway enrichment. Note: The left side of the picture is the gene corresponding to the pathway, and the right side is the pathway enrichment analysis enrichment dot bubble.

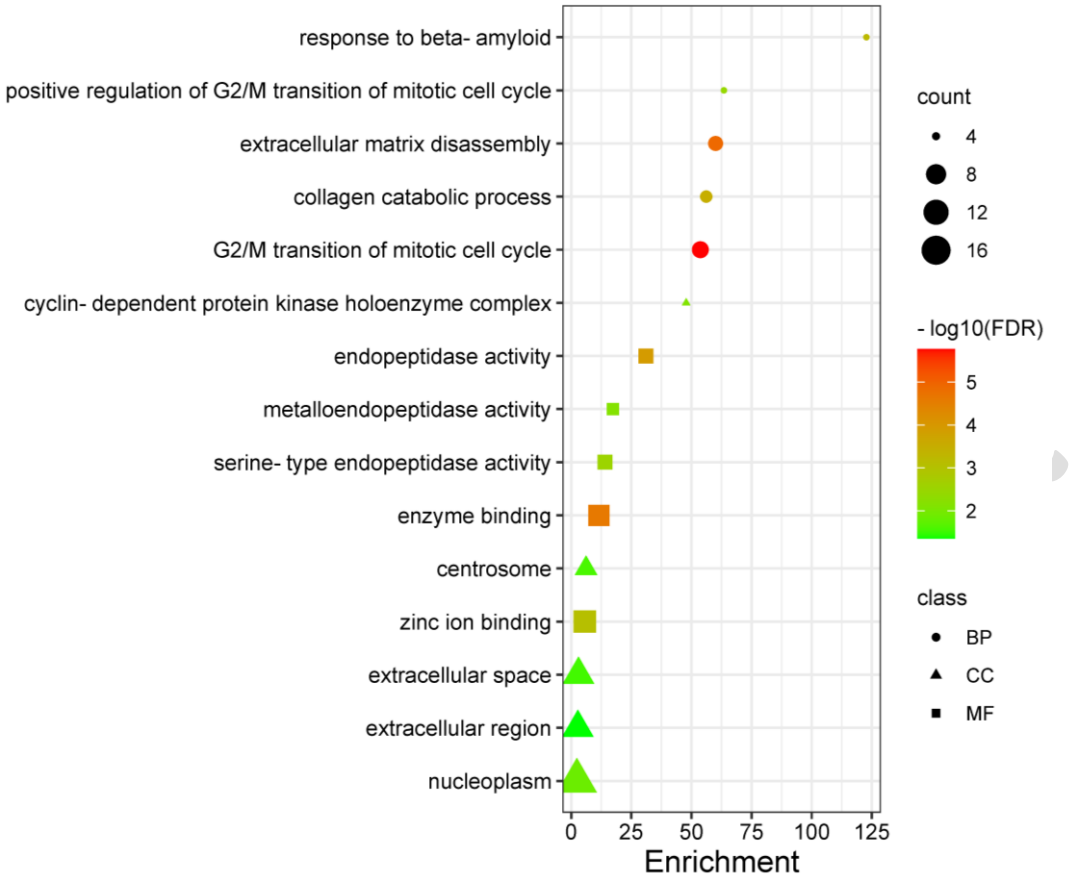

Figure 8. GO enrichment dot bubble.

3.10. Molecular Docking

According to the above results, using AutoDockTools 1.5.7 software, ESR1, and CAT, the core targets of protective prognosis were respectively selected as the receptors, and 10 core ingredients were respectively used as ligands for molecular docking verification, and the optimal binding energy for molecular docking was selected (see Table 2). Studies have shown that the binding energy of the ligand and the receptor is less than 0, which indicates that they can bind spontaneously. The lower the binding energy (affinity), the better the docking effect [14]. The core targets ESR1 and CAT had no docking site with glycine, and their binding energies with other 9 core ingredients such as quercetin, luteolin, and kaempferol were all less than -5.0kJ·mol-1, showing good binding properties. The results were visualized with PyMol 2.2.0 software (see Figures 9 and 10).

Table 5. Molecular docking binding energies (kJ·mol^{-1}) of ESR1 and CAT with core ingredients of FZXLG.

Ingredient	ESR1	CAT
quercetin	-23.14	-28.53
luteolin	-22.64	-28.83
kaempferol	-22.18	-26.44
naringenin	-25.86	-27.03
nobiletin	-19.46	-25.44
isorhamnetin	-21.84	-26.74
tanshinone IIA	-29.29	-26.36
formononetin	-27.2	-26.78
licochalcone A	-26.65	-29.66

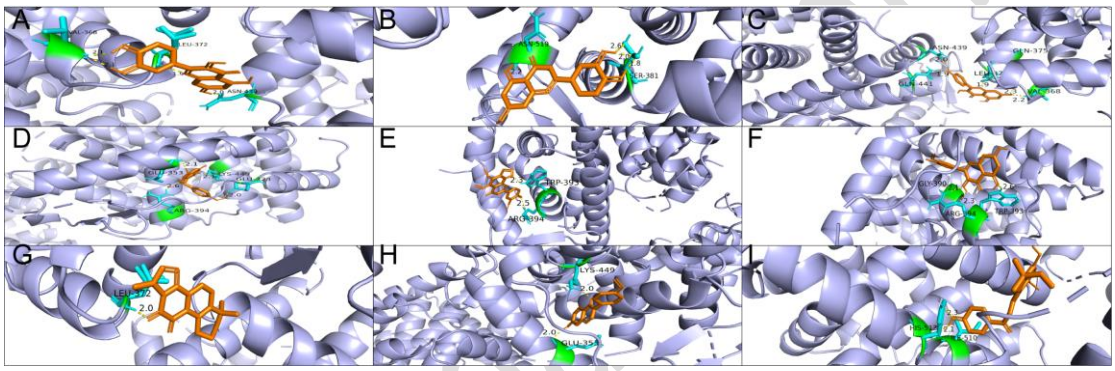

Figure 9. Diagram of the molecular docking patterns of ESR1 with the core ingredients

A: ESR1-quercetin; B: ESR1-luteolin; C: ESR1-kaempferol; D: ESR1-naringenin; E: ESR1-nobiletin; F: ESR1-isorhamnetin; G: ESR1-tanshinone IIA; H: ESR1-formononetin; I: ESR1-licochalcone A.

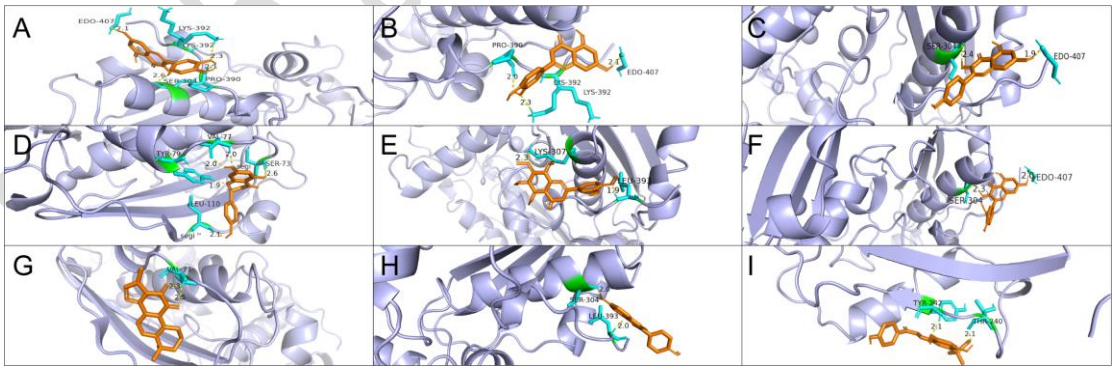

Figure 10. Diagram of the molecular docking patterns of CAT with the core ingredients

A: CAT-quercetin; B: CAT-luteolin; C: CAT-kaempferol; D: CAT-naringenin; E: CAT-nobiletin; F: CAT-isorhamnetin; G: CAT-tanshinone IIA; H: CAT-formononetin; I: CAT-licochalcone A.

4. Discussion

Our team had previously conducted some studies on Fuzheng Xiaoliu Granule in the treatment of liver cancer, the results of which showed its effectiveness in liver cancer treatment. FZXLG could not only inhibit the growth of liver cancer in nude mice by down-regulating the expression of FGFR4, MMP2, Cyclin D1, and Bcl-xL proteins [15]; but also inhibit the proliferation and induce apoptosis of liver cancer HepG2 cells by down-regulating Bcl-2 and Survivin proteins and up-regulating the expression of p53 protein [9]. However, its molecular mechanisms, including effective components, targets, and signaling pathways are still unclear. Therefore, this research used bioinformatics methods to explore the molecular mechanism and clinical correlation of FZXLG in the treatment and prognosis of HCC.

Based on the network pharmacology analysis, the "Prescription-TCMs-Ingredients-Targets" network of FZXLG in treating HCC was obtained, and the relationship network among TCMs, effective ingredients, and gene targets of FZXLG in treating HCC was revealed, providing a basis for the study of its molecular mechanism. Furthermore, 10 core ingredients that played key roles in the relationship network were screened out, among which 9 ingredients, such as quercetin, luteolin, and formononetin are flavonoids except for glycine. Studies have found that flavonoids such as quercetin, luteolin, kaempferol, and naringenin can inhibit the development of liver cancer, and they were of great value in the prevention and treatment of HCC [16,17]. Quercetin can not only block the cell cycle of liver cancer and inhibit its proliferation [18] but also control the migration and invasion of cells [19], thus exerting an anti-HCC effect. Modern pharmacological studies have shown that luteolin [20], naringenin [21], isorhamnetin [22], and tanshinone IIA [23] can induce apoptosis of liver cancer cells and have the potential to treat HCC. Jiao Wenpeng et al. [24] found that formononetin could reduce the expression levels of COX-2 and cyclin D1 in liver cancer tissues and cells, block the cell cycle, inhibit cell proliferation, and affect the occurrence and development of liver cancer.

The purpose of this study was to explore the molecular mechanism of FZXLG in the treatment of HCC, mainly to study the correlation between the expression of the FZXLG target gene and the prognosis of HCC patients, without involving gender, age, and other factors, so univariate Cox survival analysis and KM survival analysis were chosen. The results of our study screened out the core genes of FZXLG which influenced the prognosis of HCC, and analyzed their relationship with clinical survival of HCC; among them, the high expression of ESR1 and CAT were positively correlated with the prognosis, which were favorable prognostic factors. It is well known that the incidence of HCC is significantly higher in men than in women, and it is a sexually dimorphic cancer. Because liver cells

express estrogen receptor (ESR1) and androgen receptor (AR), gender has a significant impact on HCC incidence and survival [25]; Bhat et al. [26] found that ESR1 was highly expressed in HCC and had a protective correlation with the prognosis of HCC patients, which was more obvious in women. The catalase encoded by the CAT gene is a key antioxidant enzyme in the body's defense against oxidative stress; Li et al. [27] found through experimental studies that catalase (CAT) and sorafenib could synergistically regulate liver cancer hypoxia and immunosuppressive microenvironment to improve the efficacy of hepatic arterial chemoembolization for HCC. The high expressions of EGF, MMP9, CCNA2, CCNB1, CDK1, CHEK1, and E2F1 were negatively correlated with the prognosis of HCC, which were adverse prognostic factors. Epithelial-mesenchymal transformation (EMT) is a necessary transformation process for the progression of many malignancies, including HCC. MMP-9 is one of the MMPs commonly cited in the EMT of HCC, and also plays an important role in many other basic functions such as angiogenesis [28]. CCNA2, CCNB1, CDK1, CHEK1, and E2F1 are all positively correlated with the regulation of the KEGG pathway cell cycle; and their high expression promoted the occurrence and development of HCC, while their down-regulation inhibited the progress of HCC, which were the potential therapeutic targets of HCC [29-35]. EGFR (epidermal growth factor receptor) is the major receptor of EGF, which is involved in the inhibition of tumor cell proliferation, angiogenesis, tumor invasion and metastasis, and apoptosis [36]. Currently, tumor-targeted drugs targeting EGFR have been widely used in clinical treatment with remarkable effects. The drugs suitable for liver cancer mainly include cetuximab, erlotinib, and gefitinib among others [37]. Our results were consistent with literature studies, indicating that FZXLG may inhibit the development of HCC through the above 9 core target genes, and can be used to treat HCC and affect the prognosis of patients.

The tumor microenvironment includes not only tumor cells but also infiltrating immune cells. The immune microenvironment of liver cancer cells is an important factor affecting their growth, invasion, metastasis, and drug resistance [38]. Therefore, intervention in the tumor immune microenvironment is of great significance for guiding the treatment of liver cancer. genes that are highly expressed in the microenvironment are negatively correlated with tumor purity, while genes that are highly expressed in tumor cells are the opposite expected genes highly expressed in the tumor cells [39]. The results showed that MMP9 and EGF were negatively correlated with tumor purity, and positively correlated with six TIIC subsets, suggesting that MMP9 and EGF were highly expressed in the

microenvironment, which may play an important role in the immunology of FZXLG in the treatment of HCC. Most immune cell types are negatively correlated with tumor purity, but were not consistent with some of the results of this study. We also found that the protein expression levels of core genes CAT, PLG, AR, MMP9, CCNA2, CCNB1, CDK1, and E2F1 were related to immunohistochemical changes in normal liver and HCC, which further verified the clinical correlation between core targets and HCC. The results of all genes seem to be driven by a small number of samples, so the results were for reference only and need further study.

The results of KEGG pathway enrichment analysis showed that FZXLG could act on cell cycle signal pathway; all the above-mentioned core targets played roles in regulating the cell cycle, which indicated that FZXLG played an anti-HCC effect and affected its prognosis by regulating cell cycle pathways through cell cycle-related genes. The p53 signaling pathway affects cell cycle progression, apoptosis, tumor angiogenesis, and metabolism, and has tumor suppressive effects [40]; Yu et al. [41] found that three core genes in the p53 signaling pathway may play an important role in the occurrence and development of HBV-related HCC through bioinformatics analysis and experimental studies. Currently, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are still the main risk factors for HCC worldwide, and antiviral therapy targeting both can significantly reduce the incidence of HCC [42]. The results of KEGG pathway enrichment analysis also involved the hepatitis B and hepatitis C pathways, indicating that FZXLG could prevent the occurrence and development of HCC from etiology, and improve its prognosis. FZXLG also acted on multiple cancer-related pathways, multiple viral infection pathways, and metabolism-related pathways. The results of molecular docking showed that the binding energy between the core components of FZXLG and the protective core targets ESR1 and CAT had good binding, which verified the reliability of the results of network pharmacology, and confirmed that FZXLG played anti-HCC roles through multiple components, target genes, and pathways.

In conclusion, based on network pharmacology and bioinformatics methods, our study explored the molecular mechanism of FZXLG for HCC treatment: the main core effective components included flavonoids core active ingredients such as quercetin, luteolin, formononetin; prognosis of HCC patients were affected by protective prognostic factors ESR1 and CAT, and risk prognostic factors MMP9, CCNA1, CCNB2, CDK1, E2F1, and these genes were also associated with the immune microenvironment and pathological changes of HCC. FZXLG affected the prognosis of HCC by

acting on the cell cycle, p53, hepatitis B, and other pathways. Our research was expected to provide a bioinformatics basis for the clinical application and basic research of FZXLG for the treatment of HCC. However, the results need further experimental verification.

Acknowledgments

This work was supported by the National Science and Technology Major Special Project (Minkou) Project (2018ZX10303-502), the Second Batch of Discipline Construction Project for Traditional Chinese Medicine, a Featured Backbone Discipline in Henan Province (STG-ZYX01-202101) (STG-ZYX03-202126), and 2023 Henan Province Key R&D and Promotion Special Project (Science and Technology Research) (232102310188).

Conflicts of Interest

All authors declare no conflicts of interest.

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