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Original Research

Quercetin, the Ingredient of Xihuang Pills, Inhibits Hepatocellular Carcinoma by Regulating Autophagy and Macrophage Polarization

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Abstract

Background: The key active component(s) in an anti-tumor preparation used in traditional Chinese medicine, Xihuang Pills, remains unclear. **Methods**: We used a network pharmacology analysis to construct a component-disease-target network diagram and used this to determine quercetin as a critical active ingredient in Xihuang Pills. Subsequently, human hepatocellular carcinoma (HCC) cell lines, H22 and HepG2 cells, were treated with quercetin, and BALB/c mice were injected with H22 cells and treated with different concentrations of quercetin. Tumor volume and weight were determined in these mice with and without quercetin administration. Immune and pro-inflammatory factors were measured using Enzyme Linked Immunosorbent Assay (ELISA). Macrophage polarization was assessed by western blot and flow cytometry. Finally, PD-L1, autophagy-related proteins, and the NF-κB pathway were also analyzed. **Results**: Quercetin could significantly inhibit the proliferation, migration, and invasion characteristics of HCC cells and promote apoptosis in a concentration-dependent manner *in vitro*. After quercetin treatment, tumor volume and weight significantly decreased *in vivo*. Granulocyte-macrophage and granulocyte colony-stimulating factor (GM-CSF and G-CSF, respectively) levels were blunted in response to quercetin, as well as the PD-L1 level. CD86+ cell ratio was increased, while the CD206+ cell ratio was decreased, suggesting that macrophages tend to undergo M1 polarization in response to quercetin. The expression of LC3 II/I was increased, while the expression of p62 was down-regulated. The pro-inflammatory factors TNF-α, IL-6, and IL-17A, as well as NF-κB signaling were suppressed in a quercetin concentration-dependent manner. **Conclusions**: Quercetin is a key ingredient of anti-HCC activity in Xihuang Pills by regulating macrophage polarization and promoting autophagy via the NF-κB pathway.

Keywords: hepatocellular carcinoma; Xihuang Pills; quercetin; autophagy; macrophage; NF-κB

1. Introduction

Hepatocellular carcinoma (HCC) is a common malignancy that accounts for 90% of liver tumors and is considered a global health burden [1]. Current treatment methods mainly include surgery, chemotherapy, radiotherapy and immunotherapy [2], but these treatments are only effective for 30–40% of patients. The dilemma of poor prognosis, drug resistance, and low 5-year survival rate remains [3]; therefore, it is urgent to investigate new treatment strategies for HCC.

Traditional Chinese medicine (TCM) is rich in bioactive components, and TCM is widely used to treat various cancers with few side effects [4]. Xihuang Pills are an anti-cancer TCM made of *Commiphora myrrha* Engl, *Moschus*, *Boswellia carterii* Birdwood and *Bos taurus domesticus* Gmelin [5]. The anti-tumor activity of Xihuang Pills are multiple in nature and include inhibiting the growth and invasion of tumor cells and tumor stem cells, preventing tumor invasion and metastasis, and regulating the immunosuppressive microenvironment [6,7]. Previous studies have shown that *Boswellia carterii* Birdw and *Commiphoramyrrha* Engl in Xihuang Pills can play an anti-HCC role by regulating tumor angiogenesis through EGFR-activated PI3K/Akt and MAPK signaling pathways [8]. Nonetheless, how Xihuang Pills play a regulatory role in HCC patients is still unclear.

The mechanisms by which TCM treat diseases are complex, as they often contain complex components and target multiple aspects of the disease. Network pharmacology, an important tool in traditional Chinese medicine research, can comprehensively analyze the targets, components, and diseases of multiple drugs [9]. PI3K/Akt/mTOR-

related proteins were identified as potential Xihuang Pills-related targets of prostate cancer using network pharmacology as well as other experimental approaches [10]. In previous studies, the target strategy of Xihuang Pills for the treatment of liver cancer has been explored based on network pharmacology [11]. However, the active ingredients in Xihuang Pills for the treatment of HCC have not been determined.

Autophagy, a process of degrading cellular material, is closely related to tumor development and TCM therapy [12,13]. Autophagy mediates the development of HCC via the NF-κB pathway [14,15]. On the other hand, autophagy is regulated by TCM in tumors, such as curcumin, berberine, artemisinins, etc. [16]. Furthermore, the interaction of immune cells and autophagy in the tumor immune microenvironment has an impact on tumor development [17]. The autophagy induced by tumor-associated macrophages (TAMs) regulates the resistance of HCC to oxaliplatin [18]. The role of TCM on immune regulation in cancer has also been widely reported [19]. However, whether the key components in Xihuang Pills regulate autophagy and the immune microenvironment in HCC deserves further study.

This study explored the active ingredients of Xihuang Pills and the targeted regulation of HCC through network pharmacology analysis. Both *in vitro* and *in vivo* experiments also demonstrated that quercetin, the active ingredient of Xihuang Pills, could synergistically inhibit HCC-related inflammation, autophagy, and macrophage polarization. These results may provide new insights into the disease treatment of HCC by Xihuang Pills.

2. Materials and Methods

2.1 Network Pharmacology Analysis of Xihuang Pills

The research first retrieved the compounds of *Bos taurus domesticus* Gmelin, *Boswellia carterii* Birdw, and *Commiphora myrrha* Engl through the TCMSP database [20], and the compounds in *Moschus* were obtained from the herb [21] and batman databases [22]. Oral bioavailability (OB) of $\geq$30% and drug-likeness (DL) $\geq$0.18 were used as component screening thresholds. The TCMSP database, the UniProt database [23], GeneCards database [24], NCBI gene database [25], and DisGeNET database [26] were utilized for both forecasting and screening. To construct a Protein-Protein Interaction (PPI) network, the common targets of drugs and diseases obtained from screening were inputted into the String database [26]. The PPI network was imported into Cytoscape 3.8.0 (GraphPad Software, Inc., San Diego, California, USA) [27,28]. Topological analysis was performed by the Network Analyzer tool, and genes with scores greater than average were selected as key targets by degree sorting. Further, Cytoscape 3.8.0 was adopted to construct a component/disease/target network diagram. Additionally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed.

2.2 Ultra Performance Liquid Chromatography-Mass Spectrometer (UPLC-MS) of Xihuang Pills

We evaluated the core components of Xihuang Pills via UPLC-MS using the Shimadzu Prominence UPLC system (SCIEX, ExionLC AD) coupled with the QTRAP MS (SCIEX, QTRAP 5500). The flow rate, the injection tray temperature, and the column temperature was 0.3 mL/min, 4 ℃, and 40 ℃, respectively. The column used was a Waters HSS T3 column (100 $\times$ 2.1 mm, 1.7 μm) with (A) H_2O (0.1% FA) and (B) Acetonitrile. The liquid gradient conditions are shown in **Supplementary Table 1**. Retention time was compared with that of a quercetin standard.

2.3 Cell Culture and Grouping

H22 and HepG2 cell lines were purchased from Procell Life Science & Technology Co., Ltd (Wuhan, China). The H22 and HepG2 cell lines were cultured in 1640 medium (Sigma, Saint Louis, R8758, USA) and Dulbecco's minimum essential medium (DMEM) (Sigma, D5796, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, 10099141, USA), respectively. The cells were divided into four groups: a control group, a low-dose group, a medium-dose group, and a high-dose group [29], which were treated with quercetin (MedChemExpress, New Jersey, HY-18085, China) at concentrations of 0, 25 μM, 50 μM and 100 μM, respectively.

2.4 Cell Counting Kit-8 (CCK8) Detection of Cell Proliferation

After digestion, H22 and HepG2 were counted and divided into four groups. Each well was seeded with 500 μL of cell suspension, the number of cells/well was adjusted to 5×10^4, and each group was seeded in triplicate. After cell adherence, cells were treated with quercetin for 24, 48, and 72 h. After removing the drug-containing medium, 10% CCK8 (DOJINDO, Kumamoto, NU679, Japan) was added to each well and after incubating for four hours, the optical density (OD) value was measured at 450 nm.

2.5 Transwell Assay to Analyze Cell Invasion Ability

Matrigel (354262, Becton Dickenson, Franklin Lakes) was diluted and placed in the upper chamber and medium containing 10% FBS was placed in the lower chamber. HepG2 cells were treated with different concentrations of quercetin for 72 h, digested into single cells, and then resuspended at 2×10^6/mL in serum-free medium. 100 μL/well of cell suspension was added to the upper chamber and the plate incubated at 37 ℃ for 48 h. After this, the upper chamber was removed and the cells in the lower chamber were fixed with 4% paraformaldehyde for 30 minutes, stained with 0.1% crystal violet for 5 minutes, placed on a glass slide, and photographed under a microscope (DSZ2000X, Beijing Zhongxian Hengye Instrument Co., Ltd., China). After destaining, the OD value was obtained at 550 nm.

2.6 Flow Cytometry Apoptosis Assay

The cells (H22 and HepG2) were collected and stained with 5 μL Annexin V-APC and 5 μL Propidium Iodide (Keygen Biotech, Jiangsu, KGA1030, China). Binding buffer (500 μL) was used to resuspend cells and apoptotic cells were detected by flow cytometry (Beckman, Fullerton, USA).

2.7 Scratch Test to Detect Cell Migration Ability

Approximately 5 $\times$ 10^5 HepG2 cells were added to each well of a 6-well plate. After the cells reached confluence, a pipette tip was used to scratch the cell monolayer. Scratches at 0 h were photographed, and three fields of view were photographed from each group. The scratches were subsequently re-photographed after culturing cells at 37 ℃ for 24 h and 48 h.

2.8 Western Blot Detection of Protein Expression

The cells (H22 and HepG2) and tissue were washed twice with PBS, and the protein supernatant was obtained by lysing with RIPA buffer and clearing the lysate by centrifugation. Proteins were separated by SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membrane. Then, the membranes were blocked with 5% non-fat milk for 90 min. The membrane was incubated with primary antibodies to MMP-2, MMP-9, CD86, CD206, PD-L1, LC3, P62, IκBα, p-IκBα, P65, p-P65, and β-actin overnight at 4 ℃. After washing in triplicate, membranes were incubated with diluted secondary antibodies for 90 min at room temperature. Finally, membranes were incubated for 1 min using Enhanced Chemiluminescence (ECL) solution (Abiowell, Changsha, AWB0005, China) and then imaged by a gel imaging system (CLINX, Shanghai, China). The antibodies used are provided in **Supplementary Table 2**.

2.9 Homologous Tumor Transplantation Experiment

A total of 32 four weeks old male BALB/c mice were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Animals were given free access to water and food under standard animal rearing conditions (temperature 25 $\pm$ 2 ℃, relative humidity 50–60%). Animals were maintained on a 12 h/12 h dark/light cycle throughout the experiments. After a week of adaptive feeding, the mice were subcutaneously injected with H22 cells (1 $\times$ 10^6) in the left armpit and the tumor was measured twice a week. The mice were divided into four groups, the control group, low-dose group (25 mg/kg quercetin), medium-dose group (50 mg/kg quercetin), and high-dose group (100 mg/kg quercetin). Mice were given drug intervention by gavage, and the control group was given the same volume of normal saline once a day for 21 days [30,31]. At the end of the experiment, peripheral blood was obtained from mice after anesthesia, mice were subsequently sacrificed and the tumor tissue was dissected and weighed.

2.10 Histological Observation

Tumor tissues were fixed in 4% paraformaldehyde. The sections were deparaffinized and stained according to instructions provided with the Hematoxylin-Eosin (H&E) staining kit (Abiowell, Changsha, China). The sections were observed and photographed under a light microscope.

2.11 Enzyme Linked Immunosorbent Assay (ELISA)

First, peripheral blood samples and tumor tissue homogenates were prepared. Subsequently, the concentration of granulocyte-macrophage (GM-CSF, KE10015) and granulocyte colony-stimulating factor (G-CSF, KE10025) in cancer tissue homogenates, and TNF-α (KE10002), IL-6 (KE10007), and IL-17A (KE10020) in peripheral blood were determined according to the instructions provided with the ELISA kit (Proteintech, Chicago, USA).

2.12 Flow Cytometry Analysis of CD86+ and CD206+ Cells

After resuspending the cells, CD86 antibody (eBioscience, San Diego, 12-0862-82, USA) and CD206 antibody (eBioscience, 12-0114-82, USA) were added and incubated with the cells in the dark for 30 min. The cells were subsequently resuspended with PBS, filtered through a nylon mesh, and analyzed by a flow cytometry (Beckman, USA).

2.13 Statistical Analysis

GraphPad 8.0 software (GraphPad Software, Inc., San Diego, California, USA) was used for statistical analysis, and three independent experimental data points were expressed as mean $\pm$ standard deviation (SD). Student's *t*-test or one-way ANOVA was used to analyze differences between two or more groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Network Pharmacology Analysis of Xihuang Pills

189 potential drug targets and 2998 liver cancer-related genes were acquired through database screening and 135 common targets were obtained (Fig. 1A). JUN, MAPK1, HSP90AA1, and AKT1 could be found to be highly correlated targets in the PPI network (Fig. 1B). Through the network diagram of ingredients/diseases/targets (Fig. 1C), we observed a complex interaction relationship among components, such as *Bos taurus domesticus* Gmelin, *Moschus*, *Commiphora myrrha* Engl and *Boswellia carterii* Birdw, liver cancer, and corresponding targets. These common targets were principally enriched in responses to lipopolysaccharide, responses to molecules of bacterial origin, cellular responses to chemical stress, epithelial cell proliferation, responses to oxidative stress, lipid and atherosclerosis, chemical carcinogenesis-receptor activation, hepatitis B, human cytogenetic infec-

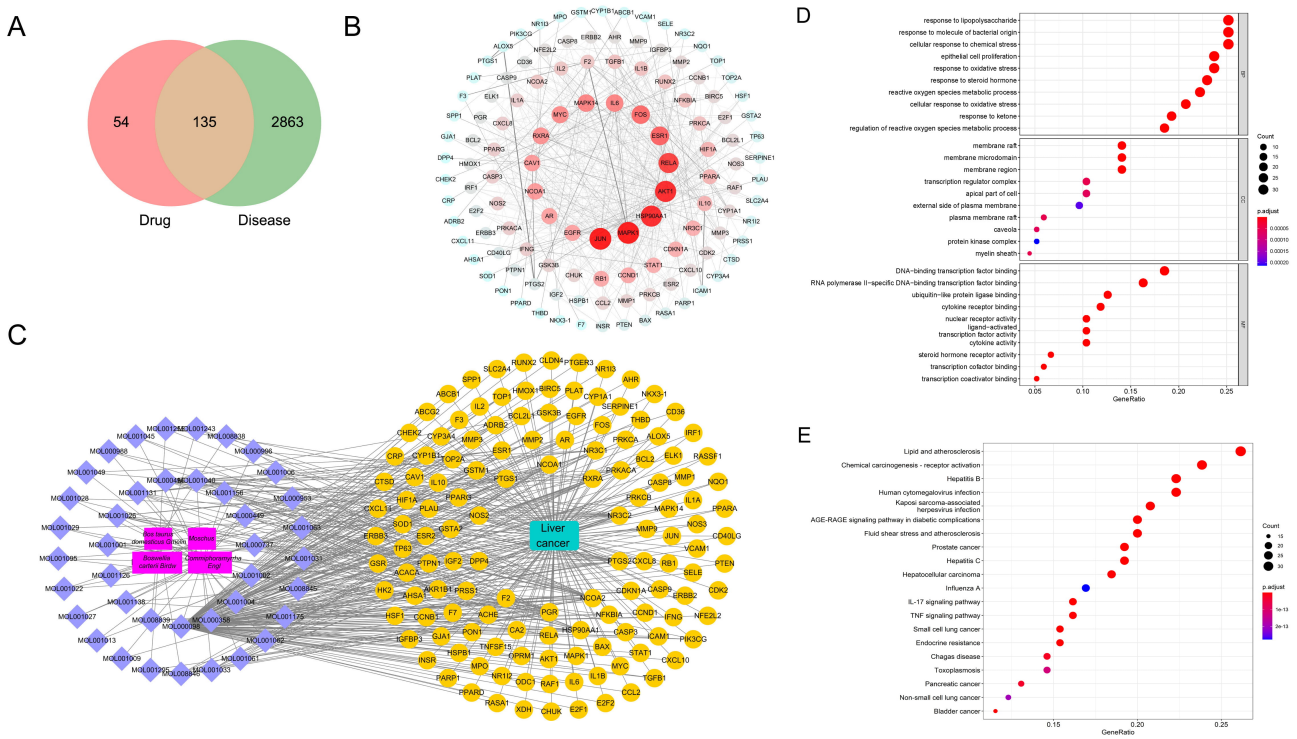

Fig. 1. Network pharmacological analysis of Xihuang Pills. (A) Venn diagram shows the targets of Xihuang Pills and liver cancer. (B) PPI network shows the interaction of common targets of drug diseases. (C) The component-disease-target network diagram in Xihuang Pills. The main components, the ID of active ingredients, liver cancer-related targets, related pathways and liver cancer are marked with red, purple, yellow and green, respectively. (D, E) Bubble chart of GO and KEGG analysis.

tion, Kaposi sarcoma-associated, herpesvirus infection and other processes (Fig. 1D,E).

From the ingredients/diseases/pathways/targets network diagram of Xihuang Pills, we found that the principal active ingredients of Xihuang Pills were quercetin, beta-sitosterol, pelargonidin, ellagic acid, morin, stigmasterol, and 3-methoxyfuranoguaia-9-en-8-one. Among these, quercetin had the highest degree score (Fig. 2A and Table 1). The core components of Xihuang Pills were identified via UPLC-MS and it was confirmed that quercetin was one of the key components of the Xihuang Pills (Fig. 2B) and thus we focused our investigation on quercetin action in HCC.

3.2 Quercetin Inhibits the Proliferation, Invasion, and Migration of HCC

To verify whether quercetin has an anti-cancer effect, we first explored the effect of quercetin on HCC *in vitro*. CCK8 was used to detect cell proliferation and we observed that the proliferation ability of cells treated with quercetin decreased compared to the control group, and that this was proportional to the quercetin concentration (Fig. 3A). After 72 h of quercetin treatment, the average inhibition rate was the highest, thus this time point was adopted for all subsequent cell experiments. Invasion characteristics of HepG2 were detected by transwell migration assays. Data gathered (Fig. 3B) indicates that with increasing quercetin concentrations, reduced cell migration was observed, suggesting that quercetin inhibits the cell invasion ability. We found that quercetin could promote the apoptosis of HCC cells while inhibiting the migration in a concentration-dependent manner, as shown in Fig. 3C,D. The expression of MMP-2 and MMP-9 in HCC cells gradually decreased with increased quercetin concentration (Fig. 3E). These results suggest that quercetin could significantly inhibit HCC invasiveness.

3.3 Quercetin Inhibits HCC Proliferation by Regulating the Polarization of Macrophages

In order to evaluate whether quercetin also plays a role in inhibiting HCC *in vivo*, we employed animal modeling. The experimental results obtained were consistent with the results obtained from cultured cells. The tumor volume and weight in the quercetin treatment group were significantly reduced (Fig. 4A–C). With increased quercetin concentration, its inhibitory effects on tumor development were measurably stronger. Similarly, after quercetin treatment, the density of cells decreased, and the nuclei appeared pyknotic and fission. The highest quercetin concentration group displayed the most significant results on tumor development (Fig. 4D).

GM-CSF and G-CSF are important factors regulating the immune microenvironment [32,33]. Therefore, the GM-CSF and G-CSF were measured by ELISA in HCC tissue homogenates. The concentrations of GM-

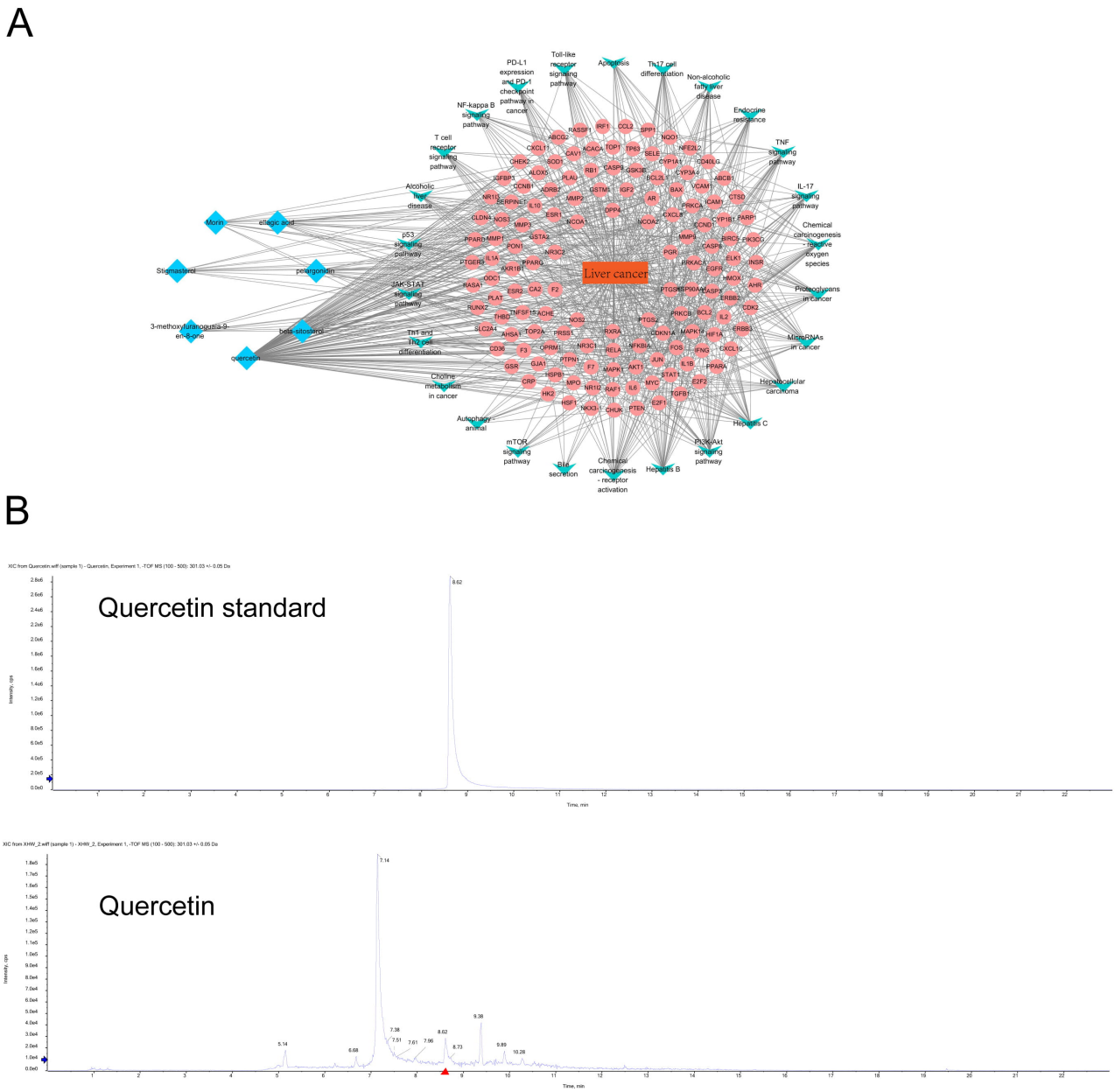

Fig. 2. The identification of drug components in Xihuang Pills and the network analysis of liver cancer targets and pathways. (A) The network diagram of ingredients-diseases-pathways-targets in Xihuang Pills. The key active ingredients, liver cancer-related targets, related pathways and liver cancer are marked with blue, pink, green and orange, respectively. (B) HPLC was used to identify the core active ingredients of Xihuang Pills (quercetin).

Table 1. Key ingredient information of Xihuang Pills.

MOL ID	Name	Average shortest path length	Betweenness centrality	Closeness centrality	Degree
MOL000098	quercetin	1.469274	0.324187	0.680608	118
MOL000358	beta-sitosterol	2.564246	0.014017	0.389978	19
MOL001004	pelargonidin	2.597765	0.009671	0.384946	16
MOL001002	ellagic acid	2.642458	0.008912	0.378436	15
MOL000737	Morin	2.877095	0.011215	0.347573	13
MOL000449	Stigmasterol	2.642458	0.005531	0.378436	12
MOL001156	3-methoxyfuranoguaia-9- en-8-one	2.675978	0.003721	0.373695	10

The main active ingredient information of Xihuang Pills was analyzed by network pharmacology.

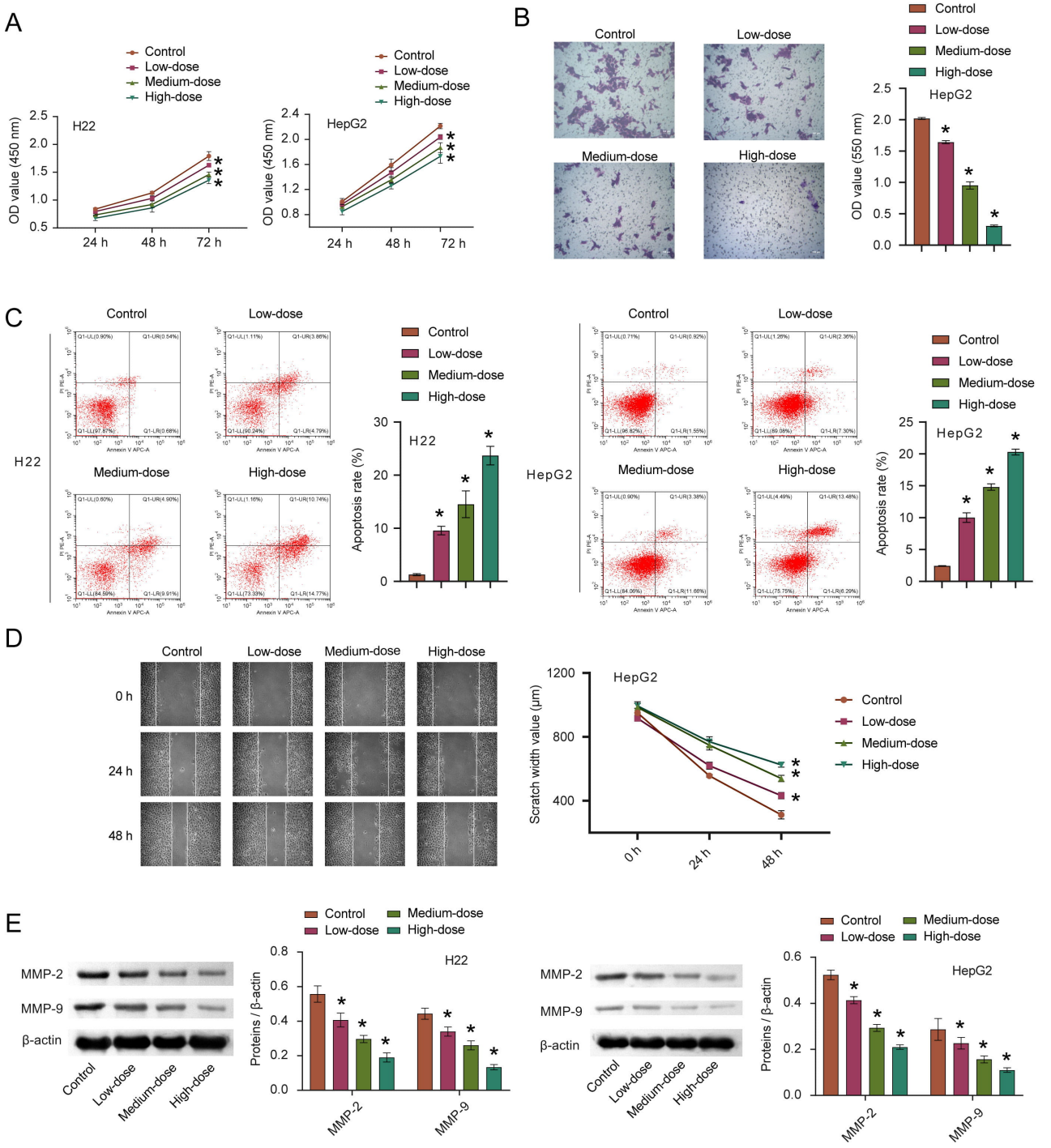

Fig. 3. The effects of quercetin on the proliferation, apoptosis, invasion and migration of HCC. (A–D) The proliferation, invasion, apoptosis, and migration of HCC cells after quercetin treatment were measured by CCK8, transwell, FCM, and scratch test, respectively. (E) The MMP-2 and MMP-9 expression were detected via WB. *$p < 0.05$, vs. the control.

CSF and G-CSF after quercetin treatment were lower than those in the control group (Fig. 4E). In addition, flow cytometry was used to analyze the percentages of M1 macrophages (CD86+) and M2 macrophages (CD206+). After quercetin treatment, the abundance of M1 macrophages increased, while the abundance of M2 macrophages decreased (Fig. 4F). At the protein level, CD86 and CD206 levels were consistent with flow cytometry results (Fig. 4G), suggesting that macrophages in the HCC tissue were induced to polarize towards the M1 phenotype after quercetin treatment. PD-L1 is significantly locally upregulated in the tumor microenvironment of HCC [34] and it was observed that PD-L1 was obviously down-regulated in the low, medium, and high-

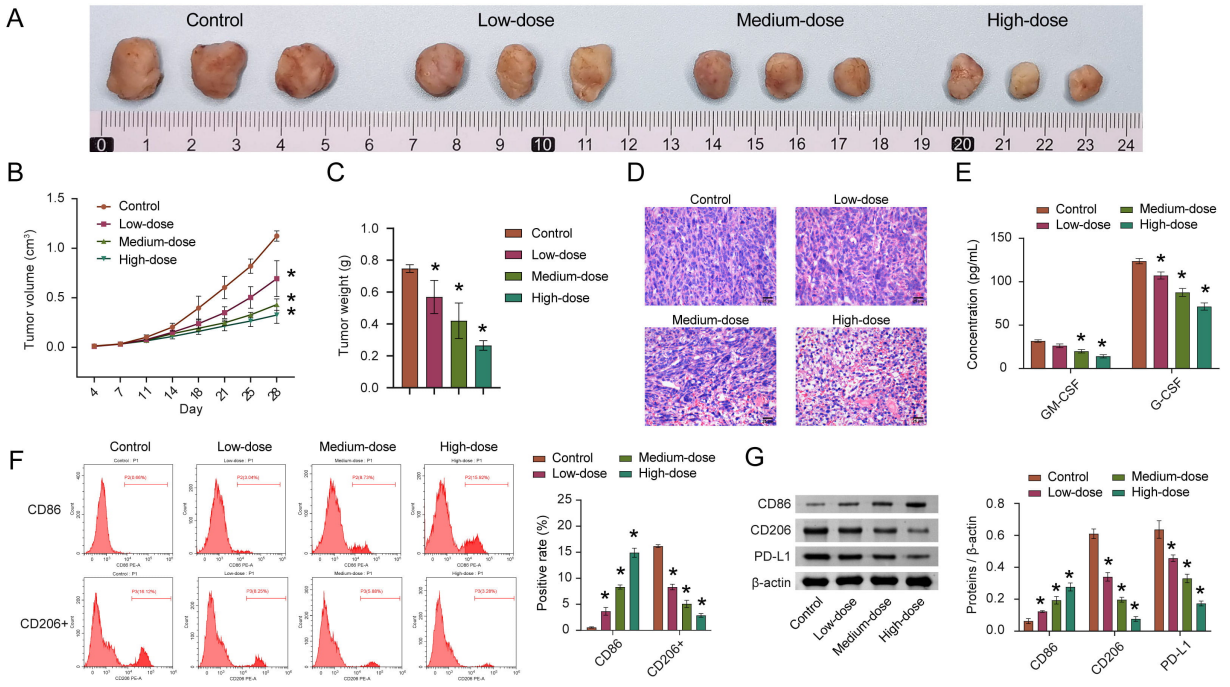

Fig. 4. Quercetin inhibits the development of HCC by inducing M1 polarization of macrophages. (A) Representative pictures of tumor morphology. (B,C) Quantitative statistics of changes in tumor volume and weight. (D) Representative images in the pathological changes. (E) The concentration of GM-CSF and G-CSF in cancer tissue homogenate was detected via ELISA. (F) The percentage of CD86 and CD206+ macrophages in tumor tissue were analyzed by FCM. (G) The CD86, CD206, and PD-L1 levels were measured by WB. n = 8 mice/group. *$p < 0.05$, vs. the control.

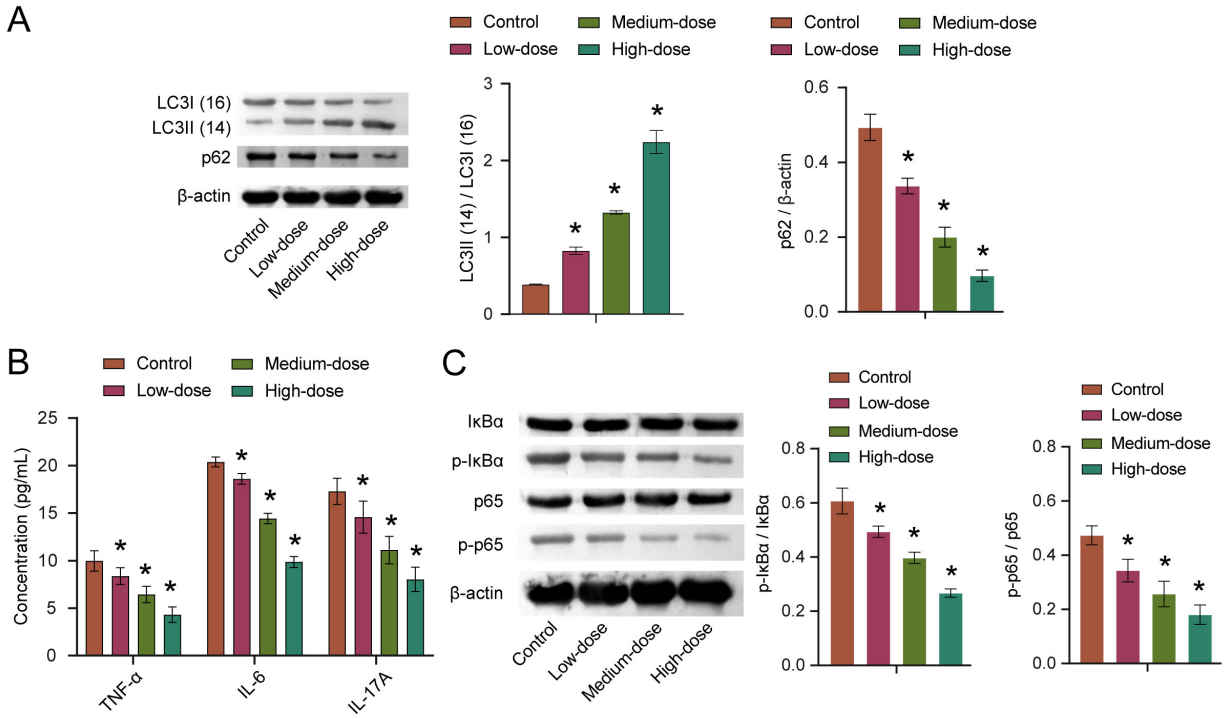

Fig. 5. Quercetin affects autophagy through the NF-κB pathway. (A) The expression levels of autophagy-related proteins (LC3 and p62) were analyzed by WB in tumor tissue. (B) ELISA was used to detect the concentration of TNF-α, IL-6, and IL-17A in peripheral blood. (C) The expression levels of NF-κB pathway proteins (IκBα, p-IκBα, p65 and p-p65) were elucidated by WB. n = 8 mice/group. *$p < 0.05$, vs. the control.

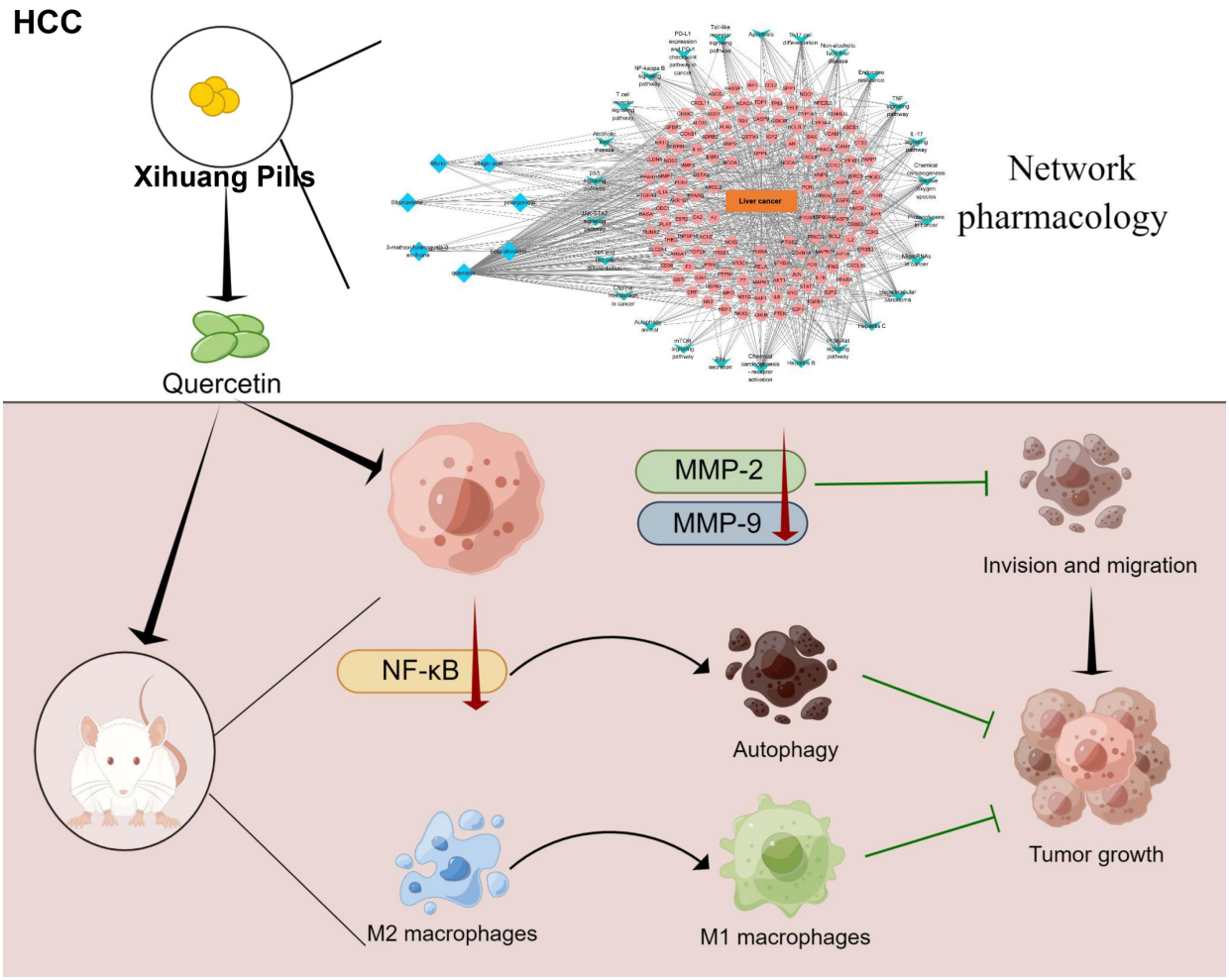

Fig. 6. Schematic diagram of this study. Quercetin, the key active ingredient of Xihuang Pills, was screened out by network pharmacology. Next, quercetin was used to treat mice and cells *in vivo* and *in vitro*. *In vitro* experiments showed that quercetin might inhibit cell invasion and migration via the MMP-2/MMP-9 pathway. *In vivo* experiments clarified that quercetin might blunt HCC development by inhibiting the NF-κB pathway, promoting autophagy, and regulating macrophage polarization.

quercetin groups (Fig. 4G). We speculate that these results indicate that quercetin may inhibit tumor growth by regulating macrophage polarization.

3.4 Quercetin Inhibits HCC via Affecting Autophagy and the NF-κB Pathway

Autophagy is critical for antigen presentation and homeostasis in immune cells and the tumor microenvironment [17]. To explore the effects of quercetin on autophagy, we detected the expression of autophagy-related proteins LC3 and p62. The results showed that the LC3II/LC3I ratio was increased with the increase of quercetin concentration, while the p62 expression was decreased with the increase of quercetin concentration. Moreover, both findings were statistically significant (Fig. 5A). The levels of TNF-α, IL-6, and IL-17A in peripheral blood were also assayed and these pro-inflammatory factors were significantly reduced by quercetin (Fig. 5B).

NF-κB can influence HCC progression by regulating inflammation and downstream metabolic pathways [35]. In addition, the NF-κB pathway is a classical pathway that regulates autophagy [36]. We speculated that the effects of quercetin on autophagy might be related to the NF-κB pathway and to test this expression of IκBα, p-IκBα, p65, and p-p65 in HCC tissue were analyzed. Results showed that the phosphorylation levels of IκBα and p65 were significantly reduced (Fig. 5C). Further, these results indicate that quercetin might promote autophagy in HCC by regulating the NF-κB pathway.

In sum, our findings indicate that quercetin inhibited HCC progression by inhibiting migration and invasion, promoting apoptosis and M1-type polarization (Fig. 6).

4. Discussion

A previous study revealed that Xihuang Pills inhibited tumor growth in a Lewis lung cancer (LLC) mouse model by combining network pharmacology with metabolomics [37]. Another study, also using network pharmacology

analysis, found that VEGFA and EGFR may be potential therapeutic targets of Xihuang Pill in the treatment of liver cancer [11]. While different from the above studies, we specifically analyzed the key components of Xihuang Pills for HCC treatment. Our study found that quercetin might be a key anti-HCC component in this TCM, based on network pharmacology and UPLC-MS. Following-up on these findings, the effects of quercetin on HCC and the potential mechanisms for these effects were analyzed through cultured cell and animal experiments. Quercetin inhibits the growth factor-induced migration of HCC cells by inhibiting AKT signaling [38]. Quercetin may also restrain the progress of HCC by inhibiting human glutamate dehydrogenase 1 to regulate mitochondrial function and metabolism [39]. Quercetin inhibits HCC cell migration and invasion, and promotes HCC apoptosis and autophagy by regulating JAK2 and STAT3 pathways [40]. Similarly, our study revealed that quercetin could inhibit the invasion and migration of H22 and HepG2 cells, thereby potentially inhibiting the progression of HCC.

Tumor-associated macrophages (TAM) play an important role within the inflammatory microenvironment and promote the progression of HCC by promoting M1 and M2 polarization. TAM with M2 polarization induce cell proliferation, angiogenesis, and epithelial-mesenchymal transition in HCC [41]. Although it has been reported that M1-polarized TAM may enhance the viability of HCC through the NF-κB/FAK pathway [42], it is generally believed that M2 TAM can promote tumor growth, while M1 TAM inhibit tumor growth [43,44]. Using mouse models of HCC, we used different concentrations of quercetin to treat tumor-bearing mice. It was found that M2-polarized TAMs were reduced and M1 TAMs increased, thereby inhibiting tumor cell proliferation and metastasis. The overexpression of PD-L1 in HCC is also associated with TAM. Blocking the PD-1/PD-L1 signaling pathway *in vivo* can improve the phagocytosis of macrophages, reduce tumor growth, and prolong the survival time in tumor-bearing mice [45]. Transcriptome analysis of tissue biopsies from the tumor microenvironment of HCC also revealed the presence of significant local upregulation of PD-1 and PD-L1 [34]. In the present study, the expression of PD-L1 was significantly decreased after quercetin treatment. Therefore, it is likely that quercetin can reduce the expression of PD-L1 and induce the polarization of TAMs in HCC tissue toward the M1 phenotype, thus improving the phagocytosis of macrophages and inhibiting tumor growth.

Autophagy is a catabolic pathway that regulates hepatocyte function and affects non-parenchymal cells such as endothelial cells, macrophages, and hepatic stellate cells [46]. Emerging evidence suggests that autophagy can induce type II programmed cell death in cancer cells and acts as a potential tumor-suppressive mechanism [47]. P62, an important autophagic receptor, is also known as Sequestosome 1 protein and is involved in a variety of cell signal transduction, oxidative stress response, and autophagic processes [48]. During the process of autophagy, the LC3II/I ratio can estimate the level of autophagy, while the level of p62 protein is inversely proportional to the level of autophagy. Using western blotting, LC3II/I was found to increase with the concentration of quercetin, and p62 was coordinately down-regulated in this study. Data gathered indicated that quercetin could improve the autophagic response in HCC. NF-κB is considered a core factor linking inflammation and cancer [48], and exogenous intervention may activate the NF-κB pathway in TAM by affecting the level of p62 and activating autophagy [49]. Given this, our study also detected the expression of IκBα, p-IκBα, p65, and p-p65 in HCC tissues and we found that IκBα and p65 proteins did not change significantly, but their phosphorylation levels were decreased. Thus, these findings demonstrated that quercetin might induce autophagy by regulating the NF-κB pathway.

TCM have multi-component and multi-target pharmacological characteristics [50]. Our study found that the anti-tumor effects of quercetin might be related to the promotion of autophagy and M1-type polarization of macrophages through the NF-κB pathway. However, there may be other active ingredients in Xihuang Pills that also have anti-HCC effects. For example, it has been found that *Boswellia carterii* Birdw and *Commiphora myrrha* Engl can down-regulate the inflammatory microenvironment in HCC, thereby restoring the anti-tumor immune response [51]. Among these, *Boswellia carterii* Birdw can also regulate signal transduction through various mechanisms such as cell cycle arrest and proliferation, angiogenesis, inhibition of tumor cell invasion and metastasis, and has potential therapeutic effects [52]. However, due to funding constraints, we have not yet studied these possibilities. A planned follow-up study will examine other active ingredients of Xihuang Pills, such as pelargonidin, ellagic acid, and morin, and their effects on HCC.

Teng *et al*. [5] used ultra performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) analysis to identify 12 other compounds excluding quercetin. Unlike the study by Teng *et al*. [5], our study used UPLC-MS to analyze the presence of quercetin in Xihuang Pills. Therefore, we speculate that the difference in experimental results may likely be due to the difference in analytical methods employed in these two studies. In the current study, network pharmacological analysis suggested that quercetin might be the key active ingredient in Xihuang Pills that targets HCC. UPLC-MS of Xihuang Pills, an anti-cancer TCM made of *Commiphora myrrha* Engl, *Moschus*, *Boswellia carteri* Birdwood and *Bos taurus domesticus* Gmelin [5], was conducted. The extract of Saudi *Commiphora opobalsamum* L has been shown to contain quercetin by MS and NMR spectra analysis [53]. The presence of polyphenols with possible anti-cancer effects in *Commiphora leptophloeos* extracts, such

as rutin, vitexin, and quercetin diglycosides, was demonstrated by thin layer chromatography (TLC) and other analyses [54]. Similar to the above study, our results indicated the presence of quercetin in Xihuang Pills.

H22 are suspension cells, and HepG2 are adherent cells. Compared with HepG2 cells, H22 cells can rapidly increase the number of cells required for tumorigenesis in a short time without digestion and passage. In view of the time and funding constraints, we chose to use H22 cells for our tumorigenesis studies. Our study confirmed the effect of drugs on H22 and HepG2 cells *in vitro*, providing a theoretical basis for the application of drugs. However, we did not explore the effects of quercetin on HepG2 cells tumorigenesis *in vivo*, which is a study limitation.

5. Conclusions

In sum, this study uncovered a key active ingredient in Xihuang Pills is quercetin by using network pharmacology. Cell and animal experiments have proved that quercetin could inhibit the development of HCC. At the same time, it was found that the anti-tumor function of quercetin might be through the regulation of macrophage polarization in coordination with the NF-κB pathway to regulate autophagy. This experimental study will provide new experimental evidence and molecular mechanism for the treatment of HCC with Xihuang Pills.

Abbreviations

HCC, hepatocellular carcinoma; ELISA, Enzyme Linked Immunosorbent Assay; FCM, Flow cytometry; GM-CSF, Granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; TCM, Traditional Chinese medicine; TAMs, tumor-associated macrophages; OB, Oral bioavailability; DL, drug-likeness; PPI, Protein-Protein Interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; UPLC-MS, Ultra performance liquid chromatography-mass spectrometer; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; CCK8, Cell Counting Kit-8; FCM, Flow Cytometry; WB, Western blot; PVDF, polyvinylidene fluoride; ECL, Enhanced Chemiluminescence; H&E, Hematoxylin-eosin; SD, standard deviation; LLC, Lewis lung cancer; TAM, Tumor-associated macrophages.

Availability of Data and Materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Author Contributions

RW and XT designed the research study. RW, TZ, JX, ZZ, ST, and XT performed the research. YW and JC analyzed the data. RW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

According to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, animal experiments were conducted and approved by the Ethics Committee of the First Hospital of Hunan University of Chinese Medicine (No. ZYFY20210320).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2712323.

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