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Basic Study

Analysis of the anti-hepatocellular carcinoma effect of Mu Ji Fang granules and their possible mechanisms associated with immune escape and apoptosis in H22 tumor-bearing mice and HepG2 cells

MJF on anti-hepatocellular carcinoma

Yi-Bing Zhang, Yong-Rui Bao, Shuai Wang, Tian-Jiao Li, He Tai, Jia-Peng Leng, Xin-Xin Yang, Bo-Cai Wang, Xian-Sheng Meng

Abstract**BACKGROUND**

Hepatocellular carcinoma (HCC) is one of the most common digestive system cancers with high mortality rates worldwide. The main ingredients in Mu Ji Fang Granules (MJF) are alkaloids, flavonoids, and polysaccharides. MJF has been used in the clinical treatment of hepatitis, cirrhosis and HCC for more than 30 years. Few previous studies have focused on the mechanism of MJF on tumor immunology in the treatment of HCC.

AIM

To explore the mechanism of action of MJF on tumor immunology in the treatment of HCC.

METHODS

The absorbable ingredients of MJF were identified using Molecule Network related to HPLC-ESI-TOF-MS, and hub potential anti-HCC targets were screened using network

pharmacology and pathway enrichment analysis. Forty male mice were randomly divided into the Blank, Model, and MJF groups (1.8, 5.4, and 10.8 g/kg/d) following 7 days of oral administration. Average body weight gain, spleen and thymus indices were calculated, tumor tissues were stained with hematoxylin and eosin, and IFN- γ , TNF- α , IL-2, AST, ALT, AFP, Fas, and FasL were measured by ELISA. Relevant mRNA expression of Bax and Bcl2 was evaluated by RT-qPCR and protein expression of TGF- β 1 and SMAD4 was assessed by Western blotting. The HepG2 cell line was treated with 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL of MJF, and another 3 groups were treated with TGF- β 1 inhibitor (LY364947) and different doses of MJF. Relevant mRNA expression of TNF- α , IFN- γ , Bax and Bcl2 was evaluated by RT-qPCR and protein expression of TGF- β 1, SMAD2, p-SMAD2, SMAD4, and SMAD7 was assessed by Western blotting.

RESULTS

It was shown that MJF improved body weight gain and tumor inhibition rate in H22 tumor-bearing mice, protected immune organs and liver function, reduced the HCC indicator AFP, affected immunity and apoptosis, and up-regulated the TGF- β 1/SMAD signaling pathway, by increasing the relative expression of TGF- β 1, SMAD2, p-SMAD2 and SMAD4 and decreasing SMAD7, reducing immune factors TNF- α and IFN- γ , decreasing apoptosis cytokines Fas, FasL and Bcl2/Bax, and inhibiting the effect of LY364947 in HepG2 cells.

CONCLUSION

MJF inhibits HCC by activating the TGF- β 1/SMAD signaling pathway, and affecting immune and apoptotic cytokines, which may be due to MJF adjusting immune escape and apoptosis.

Key Words: Mu Ji Fang granules; Hepatocellular carcinoma; TGF- β /SMAD; Immune escape; H22 tumor-bearing mice; HepG2 cells

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Core Tip: Mu Ji Fang Granules (MJF), a Chinese patent medicine, has been used in hepatitis, cirrhosis and hepatocellular carcinoma (HCC) for more than 30 years. MJF was identified with Molecule Network related to HPLC-ESI-TOF-MS, and hub potential anti-HCC targets were screened using network pharmacology and pathway enrichment analysis in H22 tumor-bearing mice and HepG2 cells. It was shown that MJF improved body weight gain and tumor inhibition rate, protected immune organs and liver function, affected immunity and apoptosis, up-regulated the TGF- β 1/SMAD signaling pathway, and inhibited the effect of TGF- β 1 inhibitor (LY364947).

INTRODUCTION

In the WHO 2018 World Cancer Report, ⁶ liver cancer is the sixth most common cancer and the fourth most common cause of cancer death worldwide, and the age-standardized rates in Asia and Africa were 2 to 3 times those in America, Europe, and Oceania^[1]. In the latest report on cancer epidemiology in China, liver cancer was the third and seventh most common malignancy in males and females, with an incidence rate of 12.74% and 5.40%, and the second and third highest mortality rate of 16.36% and 9.79%, respectively^[2].

Mu Ji Fang granules (MJF, also known as Fufang Mu Ji Granules), a Chinese patent medicine produced by Dandong Pharmaceutical Group Co., Ltd (Liaoning, China), derived from a folk prescription of the Manchu medicine, consists of 4 herbs including *Sophorae Tonkinensis Radix*^[3], *Cuscutae Semen*^[4], *Juglans mandshurica Maxim*^[5] and *Coriolus versicolor*^[6] (Table 1), processed into proprietary Chinese medicine, has been used in patients with hepatitis, cirrhosis and HCC for many years and has a

considerable curative effect in clinical practice^[7]. Previous studies of MJF mainly focused on its clinical efficacy in hepatitis B^[8, 9] and fundamental pharmacological effects^[10], but few reported the mechanism of action on tumor immunology in the treatment of HCC. Our research group previously established the HPLC fingerprint chromatogram of MJF, combined with UPLC-ESI-TOF-MS, and identified the main compounds in MJF to be alkaloids, flavonoids, and polysaccharides. Based on these findings we hypothesize that MJF might be able to promote tumor cell apoptosis, anti-inflammatory activity and enhance immunity. In this study, we used Molecule Network (MN) coupled with UPLC-ESI-TOF-MS to analyze the compounds in mouse plasma and a chemical profile was built based on the structures of these compounds. Combined with the compounds from MN, network pharmacology was adopted to analyze the anti-HCC pharmacological mechanisms of MJF. H22 tumor-bearing mice and HepG2 cells were introduced as experimental support for verification, ELISA, RT-qPCR, and Western blotting assays were used to evaluate the expression levels of target genes *in vivo* and *in vitro*. Our data indicated that MJF had an anti-HCC effect by regulating immune escape and promoting apoptosis.

MATERIALS AND METHODS

Establishment of H22 tumor-bearing mouse models and sample preparation

Eight-week-old ICR male mice weighing 18-22 g, (Experimental Animal Center of Liaoning Changsheng Biological Technology Co., Ltd., License No. SCXK Liao 2015-0001), were adaptively raised in a controlled-environment animal experiment room (temperature 20°C–25°C, humidity 45%–65%, light / dark cycle for 12 h / 12 h) for 7 days with free access to food and tap water. All experimental procedures were performed with the approval of the animal guidelines and protocols established by the Medicine Ethics Review Committee of Animal Experiments of Liaoning University of Traditional Chinese Medicine and The Affiliated Hospital of Liaoning University of Traditional Chinese Medicine. Mouse hepatocellular carcinoma H22 cell strain (Jiangsu

Chi Scientific Co., Ltd.) was cultured in RPMI-1640 culture medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 1% streptomycin and 10% fetal bovine serum (100 µg/mL penicillin, 100 µg/mL streptomycin) (Gibco Inc. USA) in an incubator at 37°C, 5% CO₂ and 95% of relative saturation humidity. H22 cells in the logarithmic growth period were adjusted to 1×10⁷/mL with saline for intraperitoneal injection into mice at 0.2 mL each animal. Five days later, the mice developed ascites due to the growth of H22 cells in the abdominal cavity. We extracted abdominal ascites from the mice and adjusted them to 1×10⁷/mL with saline as the first generation ascites and repeated the above intraperitoneal injection twice in healthy mice. The third generation ascites were harvested and adjusted to 1×10⁷/mL with saline as the final implant cell line. An axillary vaccination was conducted in each mouse using 0.2 mL H22 diluted ascites. Three days later, nodules were observed in the right armpit of the mouse, indicating that the established model was successful.

Forty mice were randomly divided into the Model group, CTX group (cyclophosphamide, 2.7 mg/mL), and 3 MJF groups (M-L received a low MJF dose of 1.8 g/kg, M-M received a mid MJF dose of 5.4 g/kg, M-H received a high MJF dose of 10.8 g/kg) with 8 mice in each group. A further 8 ICR male mice (weighing 18-22 g) were included in the Blank group. All mice were administered the drugs orally once a day (the Blank and Model groups received saline) for 7 days, no food was allowed 12 h before the experiment, but the animals had free access to water. Thirty minutes after the final drug administration, all mice were euthanized under deep anesthesia. Body weight was measured and blood was collected for analysis. After sacrifice, intact thymus, spleen, and tumor tissues were removed and weighed (Figure 1). The thymus index, spleen index, and tumor inhibitory rates in each mouse were calculated according to the following formulas.

Thymus index = thymus weight (mg) / body weight (g) × 10

Spleen index = spleen weight (mg) / body weight (g) × 10

Tumor inhibitory rate (%) = (1 - average tumor weight in the drug administration group / average tumor weight in the Model group) × 100%

Drug and plasma sample preparation for HPLC-ESI-TOF-MS and ingredient identification

Five g MJF granules (# 20190725334, provided by Dandong Pharmaceutical Group Co., Ltd., Dandong China) were weighed and added to 30 mL anhydrous methanol (HPLC grade, Merck Co. Ltd., Darmstadt, Germany), ultrasonically extracted for 30 min, and then filtered through a micropore membrane (0.22 µm; Jinteng Corp., Tianjin, China) before use. Seventeen accurately weighed reference substances were mixed and dissolved in 10 mL of methanol to obtain a solution at the concentration of 21.00 µg/mL for protocatechuic acid, 24.54 µg/mL for ellagic acid, 18.96 µg/mL for kaempferol-3-O-rutinoside, 17.45 µg/mL for rutin, 15.09 µg/mL for hyperoside, 20.64 µg/mL for isoquercitrin, 18.44 µg/mL for astragalin, 17.42 µg/mL for kaempferol, 18.40 µg/mL for isorhamnetin, 20.62 µg/mL for marine, 23.36 µg/mL for sophocarpine, 18.43 µg/mL for gallic acid, 16.48 µg/mL for naringenin, 22.72 µg/mL for cytosine, 21.53 µg/mL for caffeate, 21.34 µg/mL for quercetin (Sichuan Victory Biological Technology Co., Ltd., Sichuan, China) and 18.92 µg/mL for ferulic acid (HPLC grade, Tianjin Kermel Chemical Co., Tianjin, China). 50 µL of thawed frozen plasma samples of MJF high dose (M-H) were placed into 1 mL centrifuge tubes, and then thoroughly mixed with 100 µL methanol and vortexed for 1 min. The solutions were centrifuged at 8000 rpm/min and 4°C for 10 min. The supernatants were filtered through a 0.22 µm membrane for HPLC-MS analyses.

The HPLC-MS analysis was performed using an Agilent 1290 HPLC system (Agilent Technologies, Inc., CA, USA) in tandem with an Agilent 6550 quadrupole-time of flight with mass spectrometry (Agilent Co., USA). The analysis system consisted of an Agilent Proshell SB-C18 column (100 mm×3 mm, 2.1 µm) (Agilent Technologies, Inc., CA, USA) and was used with the column temperature maintained at 35°C; the flow rate of the mobile phase was 0.4 mL/min and the injection volume was 0.6 µL, the mobile phase consisted at 0.1% formic acid in water (A) and acetonitrile (B), and the column was eluted with a linear gradient of 3–8% B over initial to 5.0 min, 8–15% B over 5.0–9.0

min, 15-22% B over 9.0-12.0 min, 22-55% B over 12-15 min, 55-70% over 15-20 min, 70-95% B over 20.0-24.0 min, returned to 3% B for 1.0 min and then held for 2.5 min at an eluent flow rate of 0.5 mL/min. Optimal conditions for HPLC-Q-TOF-MS analysis were as follows: ion source was Dual AJS ESI, both positive and negative ion mode detection was adopted, drying gas flow of 13 L/min, drying gas temperature maintained at 350°C, a capillary voltage of 3500 V, nebulizer pressure of 310.28 kPa, fragmentor voltage of 125 V, OCT 1RF Vpp voltage of 750 V, collision energy of 40 eV and mass spectrometric data were acquired in the mode from 1000 to 1000 m/z with an acquisition rate of 1.5 spectra/s. Data were corrected and obtained during acquisition using a correction mixed solution (Agilent Technologies, Inc., CA, USA, m/z=112.985587, m/z=1033.988109).

Molecular networking establishment of MJF and the identification of absorbable ingredients

For MN data processing, MS/MS data on MJF extraction and MJF high dose were collected and converted into mzXML format using Proteo Wizard software (www.proteowizard.sourceforge.net, Proteo Wizard, Palo Alto, CA, USA) and then uploaded separately into the GNPS platform (<https://gnps.ucsd.edu>, UCSD, San Diego, CA, USA) (accessed on 16 November 2021) (Figure 2a.). The GNPS parameters were as follows: mass error of less than 0.02 Da, matched peaks greater than 3, and cosine score greater than 0.50. After analysis, graphic format files were generated from the GNPS platform, and the files were then downloaded and imported into Cytoscape software v 3.7.0 (www.cytoscape.org, NRNB, Hill St, San Diego, CA, USA) to build the molecular network. According to Wang's study, MN of Mu Ji Fang Granules extraction and plasma after MJF oral administration were merged, and the absorbed constituents of Mu Ji Fang Granules in plasma were obtained. All constituents were identified using reference substances on HPLC-Q-TOF-MS.

Network pharmacology construction and pathway enrichment analysis of MJF

1232 HCC-related genes were extracted from DisGeNET (<https://www.disgenet.org>) (Piñero *et al* 2020) and two liver cancer databases Liverome^[11] (<http://Liverome.kobic.re.kr/index.php>) and OncoDB. HCC^[12] (<http://oncodb.hcc.ibms.sinica.edu.tw>). Validated and predicted targets of 17 ingredients screened from MN of Mu Ji Fang granules were collected from Swiss Target Prediction^[13] (<http://www.swisstargetprediction.ch>), an online tool to predict the macromolecular targets (proteins from human, mouse, and rats) of small bioactive molecules. All the ingredient-related genes were mapped to HCC-related genes to obtain a total of 221 shared targets, which were then uploaded to the STRING database^[14] (<https://string-db.org>) to acquire the protein-protein interaction (PPI) network. Cytoscape software and the Network Analyzer App were employed for topological analysis of the PPI network, and the medians of each index were calculated. Nodes of a degree over 19, betweenness centrality over 0.00283, and closeness centrality over 0.51485 were gathered as predicted targets and were uploaded to the Metascape database^[15] (<https://metascape.org/>) for possible GO and KEGG pathways enrichment. The final results of the ingredient-pathway-target network were demonstrated by Cytoscape.

Histopathological analysis and biochemical analysis

Mouse tumor tissues were immediately fixed in 4% paraformaldehyde (Beijing Solaibao Technology Co., Ltd., Beijing, China). Following paraffin embedding, the tissues were cut into 4 μ m slices, stained with hematoxylin and eosin (HE), and morphological changes were observed with a microscope ($\times 200$ high power visual field).

Plasma samples from all five groups were centrifuged at 8000 rpm/min and 4°C for 10 min. The supernatants were then used for IFN- γ , TNF- α , IL-2, AST, ALT, AFP, Fas, and FasL analyses by ELISA in accordance with the kit instructions (Shanghai Langton Biotechnology Co., Ltd., Shanghai, China), respectively.

HepG2 cell culture and drug administration

The mouse hepatocellular carcinoma H22 cell strain (Jiangsu Chi Scientific Co., Ltd, Jiangsu, China) was cultured in DMEM culture medium containing 1% streptomycin and 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) (100 µg/mL penicillin, 100 µg/mL streptomycin) in an incubator at 37°C 5% CO₂ and 95% of relative saturation humidity. Cells at the density of 1×10^5 /mL were inoculated into 6-well plates, and after culture for 24 h the medium was removed. 4.0 g MJF was added to 20 mL 50% methanol and treated with ultrasonic extraction for 20 min (700 W, 40 kHz), and the liquid was filtered and dried. 1.0 g Mu Ji Fang Granules were mixed with 0.5 mL DMSO, diluted with DMEM culture medium to 10 mg/mL, 20 mg/mL, 30 mg/mL, and 40 mg/mL, and then added to a 6-well plate with 1 mL of MJF in each well (M1, M2, M3, and M4). The TGF-β1 inhibitor Ly364947 (# HY-13462, MedChem Express Co., Ltd., NJ, USA) was adjusted to 1 µM and 2.5 µM, respectively, and added to 6-well plates with 1 mL of LY364947 in each well. M2+LY-1 and M4+LY-2 groups contained 20 mg/mL and 40 mg/mL of MJF each and 1 µM Ly364947. 1 mL culture medium was added to the Blank group. All groups were cultured for 8 h.

Quantitative RT-qPCR analysis

Tumor tissue was placed in TRIzol reagent to obtain a homogenate, and total RNA was extracted according to the manufacturer's instructions. The reverse transcription kit (Trans Script One-Step gDNA Removal and cDNA Synthesis Super Mix, Beyotime Biotechnology Co., Ltd., Shanghai, China) was used to reverse transcribe the extracted RNA into cDNA and processed as previously described^[16]. The total RNA in HepG2 cells in each group was processed in the same way. The primer sequences for RT-qPCR of tumor tissue and HepG2 cells are listed in Tables 2 and 3.

Western blotting assay for target protein detection

Tumor tissue proteins from the mice in each group were extracted with RIPA reagent. Proteins were separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Beyotime Biotechnology Co., Ltd., Shanghai, China). The membranes were

washed, blocked with BSA blocking solution for 2 h, and then incubated overnight with anti- β -actin (# 20536-1-AP), anti-TGF- β 1 (# 21898-1-AP), anti-SMAD4 (# 10231-1-AP), anti-SMAD7 (# 25840-1-AP) (Proteintech Group, Inc., Rosemont, IL, USA), anti-SMAD2 (# 5339S), and anti-phosphorylated-SMAD2 (# 3108S, Cell Signaling Technology, Danvers, MA, USA) at 4°C. After washing with TBST 3 times for 10 min each time, the membranes were incubated with secondary antibodies (Goat Anti-Rabbit IgG H+L) (# SA00001-2, Proteintech Group, Inc., Rosemont, IL, USA) for 2 h at room temperature. Immunoreactivity was determined using ECL glow (Beyotime Biotechnology Co., Ltd., Shanghai, China). Data analysis was conducted *via* Image J 1.8.0 software (National Institutes of Health, Bethesda, MD, USA). Protein in HepG2 cells from each group was processed using the same steps as above.

Statistical analysis

Statistical analyses were performed with SPSS 25.0 software (IBM Corporation, Armonk, NY, USA) using one-way ANOVA and data were expressed as the mean \pm SD. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered very significant. RT-qPCR data were analyzed by the $2^{-\Delta\Delta C_t}$ algorithm^[17].

RESULTS

MJF ingredient identification in plasma after oral administration

A comprehensive MN based on LC-MS/MS spectra in order to reveal absorbable ingredients in MJF was performed ⁷ as shown in Figure 1b. The MN map contained 933 precursor ions, including 137 clusters (nodes > 2) and 352 single nodes. A total of 17 main prototype components (P1-17) were screened out by the GNPS MS database, based on the mass measurements and fragmentation patterns confirmed by reference substances. These compounds were identified as cysteine, gallic acid, matrine, sophocarpine, protocatechuic acid, caffeate, hyperoside, quercetin, isoquercitrin, rutin, kaempferol-3-o-rutinoside, astragalin, naringenin, kaempferol, ellagic acid, ferulic acid, and isorhamnetin (Figure 2, Table 3). These compounds were of three main types:

polyphenol acids, flavonoids, and alkaloids, all of which were selected and used for the following target prediction in network pharmacology analysis.

Network pharmacology construction of the anti-HCC targets of MJF and pathway enrichment analysis

Seventeen ingredients related 1232 genes to HCC related genes were mapped, a total of 166 targets were obtained and an ingredients-anti-HCC-target network was established. The degree of each node was measured by the Network Analyzer App in Cytoscape (Figure 3a). The degree of one node indicates the number of nodes that have direct interactions with it, the higher degree one node has, the more biological processes it participates in, and the more biological importance it possesses.

From the topological analysis of 177 ingredients-anti-HCC-targets through the PPI network generated by the STRING database, 72 predicted targets were acquired. By uploading all these predicted targets into the Metascape database, we obtained the GO and KEGG pathways to enrich the analysis. Figure 3b lists the top 5 GO pathways to enrich the analysis indicating that MJF could respond to inorganic substances and xenobiotic stimulus *etc.* of Biological Processes (BP), kinase binding and transcription factor binding *etc.* of Cellular Components (CC), and membrane raft, vesicle lumen *etc.* of Molecular Functions (MF). Among the top 15 KEGG pathways which enriched the analysis (Figure 3b), only 6 of them were related to HCC including pathways in cancer, hepatitis B, proteoglycans in cancer, hepatitis C, HCC, and the TGF-beta signaling pathway. From the heatmap in Figure 3b, 14 genes with the most significantly different abundance related to the 6 KEGG pathways of HCC were clustered, including FAS, BCL2, TNF, IL2, BAX, IFNG, TGFB1, SMAD4, MMP9, SRC, EGF, STAT1, IGF2, and IGF1R. A comprehensive predicted ingredient-anti-HCC-pathway-target network is shown in Figure 3c, where 17 potentially effective compounds related to the 6 pathways of HCC, from which, we clustered 8 hub-targets had a greater degree (above the median of 34.763) from the topological analysis of the network including TNF, BAX, BCL2, TGFB1, IFNG, FAS, IL2 and SMAD4 (Figure 3d).

Regulatory effects of MJF on H22 tumor-bearing mice

As displayed in Figure 4a, except for the Model group, the average body weight in the other groups all showed varying rapid gain. Compared with the Blank group, H22 tumor-bearing mice in the Model group had a much higher spleen index and lower thymus index ($P<0.05$), due to H22 bearing tumors causing splenomegaly and thymic atrophy in mice. The CTX and MJF groups (M-L, M-M and M-H) revealed a significantly reduced spleen index and increased thymus index relative to the Model group, and in the MJF groups these levels progressed with increased dose ($P<0.05$, $P<0.01$). Figure 4b indicates that the concentration of AFP (HCC indicator), ALT and AST (liver function indicators), and TNF- α , IFN- γ , and IL-2 (immune factors) in the Model group markedly increased compared with the Blank group ($P<0.05$), which confirmed that H22 tumors may affect immune action and inflammation in the liver and reduce liver function in mice. Compared with the Model group, CTX and MJF effectively reduced the concentration of AFP, ALT, AST, TNF- α and IFN- γ , and increased the concentration of IL2. With an increase in dose, the MJF mid and high dose groups (M-M and M-H) showed significant enhancement in adjusting the concentration of the above indicators ($P<0.05$, $P<0.01$).

¹ As shown in Figure 4c, the size of the tumor in the Model group was larger and the color was darker, blood and vessels were clearly observed on the surface, while the tumors in the other groups were pale, and the size of the tumor and the average inhibitory rate in the 3 MJF groups (M-L, M-M and M-H) gradually changed with increased dose, respectively. Figure 4d shows the microscopic images and local enlarged images of HE stained sections of tumor tissue from each group. The tumor cells in the Model group were densely arranged with high cell density and large nuclei, and necrotic cells and cytoplasm were rarely seen. In the CTX group, tumor cells were loosely arranged, necrotic cells showed nucleus necrosis and rupture, and the cytoplasm was condensed and shrunken. In contrast, the three MJF groups all had different degrees of necrosis and apoptotic cell areas. The cytoplasm was highly

agglutinated and condensed. In the MJF high dose group (M-H), the number of dead cells and round apoptotic bodies were more numerous, the size of the cells varied, and the connective tissue was more obvious than in the other two MJF groups (M-L and M-M).

We evaluated the concentration of the cell apoptotic factors Fas and FasL, and the mRNA expression of Bax and Bcl2, two cytokines that can form homologous or heterologous dimers to regulate apoptosis, in order to determine the effect of MJF in improving the apoptosis of cancer cells. As shown in Figure 5a, the concentrations of Fas and FasL in the Model group were markedly increased relative to the Blank group, whereas, CTX and MJF (M-L, M-M and M-H) reduced the concentrations, respectively ($P<0.01$). In addition, CTX and MJF also up-regulated the relative mRNA expression of Bax and down-regulated that of Bcl2 compared to the Model group ($P<0.01$) (Figure 5b). Moreover, Western blot analysis was performed to determine the protein level of TGF- β 1 and SMAD4, and it was demonstrated that CTX and MJF significantly increased the expression of TGF- β 1 and SMAD4, and the expression levels in the MJF mid and high dose groups were even higher than that in the CTX group ($P<0.01$) (Figure 5c).

MJF modulated the TGF- β 1/SMAD pathway, immune and apoptotic cytokines in HepG2 cells

Western blot analysis was used to determine the protein expression of TGF- β 1, SMAD2, p-SMAD2, SMAD4, and SMAD7, and we evaluated the effectiveness of MJF in regulating immune escape factors and promoting apoptosis in HepG2 cells, as the TGF- β 1/SMAD signaling pathway plays an essential role in the development and invasion of HCC. The results showed that MJF (10 mg/mL to 40 mg/mL) effectively increased the expression of TGF- β 1, SMAD2, p-SMAD2 and SMAD4, and reduced the expression of SMAD7 protein relative to the levels in the Control group (MJF 0 mg/mL), respectively ($P<0.05$) (Figure 6a). Furthermore, the results also showed that, compared with the Control group (MJF 0 mg/mL), mRNA expression of the immune cytokines, TNF- α and IFN- γ , in MJF (10 mg/mL to 40 mg/mL) was markedly decreased, and mRNA

expression of the apoptosis cytokine, Bax in MJF (10 mg/mL to 40 mg/mL) was significantly increased and that of Bcl2 was reduced ($P<0.05$) (Figure 6b).

MJF alleviated the modulation of TGF- β 1 inhibitor (LY364947) on the TGF- β 1/SMAD pathway, and immune and apoptotic cytokines in HepG2 cells

To evaluate the regulation of MJF on the TGF- β 1/SMAD signaling pathway and both immune and apoptotic cytokines in HepG2 cells, LY364947, a TGF- β 1 inhibitor, was added to the cell culture. As demonstrated in Figure 7a, relative to the Control group (LY364947 0 μ M and MJF 0 mg/mL), LY364947 effectively inhibited the expression of TGF- β 1, SMAD2, p-SMAD2 and SMAD4 protein and promoted that of SMAD7 (LY364947 2.5 μ M and MJF 0 mg/mL), while, MJF (LY364947 2.5 μ M, MJF 20 and 40 mg/mL) reduced the effect of LY364947 by improving the expression of TGF- β 1, SMAD2, p-SMAD2 and SMAD4, and down regulating SMAD7 ($P<0.05$).

With regard to the immune cytokines, TNF- α and IFN- γ , compared to the Control group (LY364947 0 μ M and MJF 0 mg/mL), LY364947 significantly increased mRNA expression of TNF- α and IFN- γ (LY364947 2.5 μ M and MJF 0 mg/mL), and MJF (LY364947 2.5 μ M, MJF 20 and 40 mg/mL) showed a marked decrease in these two cytokines ($P<0.05$) (Figure 7b). Moreover, compared with the Control group LY364947 0 μ M and MJF 0 mg/mL), the expression of Bax mRNA was inhibited and that of Bcl2 was improved after treatment with LY364947 (LY364947 2.5 μ M and MJF 0 mg/mL), yet, MJF (LY364947 2.5 μ M, MJF 20 and 40 mg/mL) weakened this effect by increasing Bax and reducing Bcl2, respectively ($P<0.05$) (Figure 7c).

DISCUSSION

Hepatocellular carcinoma is a malignant tumor with high morbidity and mortality worldwide^[18]. At present, the main clinical treatments for malignant liver tumors are surgery, radiation, and chemotherapy^[19]. Common chemotherapy drugs have side effects such as cytotoxicity, multiple drug resistance, *etc.*, and have different degrees of impact on liver function and the immune system^[20]. Traditional Chinese Medicine

(TCM) has a particular function in cancer treatment and prevention due to its high multi-target biological activity and low toxicity^[21]. The long history and extensive study of TCM indicate its potential advantages in alleviating symptoms and improving quality of life in different patterns and stages of the disease^[22]. As a Chinese patent medicine, MJF has been used in the treatment of liver cancer since the 1980s, with the exception of HCC, and has also been proved to have anti-inflammatory effects, enhances immunity, and improves liver function. Thus, in the present study, we mainly focused on exploring the mechanism of action of MJF.

In a recent study, the MN technique associated with LC-MS/MS was employed in studies of natural products derived from plants, microorganisms, marine organisms, and other biological sources^[23]. As a visual computational strategy, MN can be intuitively implemented based on a comparison of the theoretical MS/MS spectra to test MS/MS spectra and establish the relative network by clustering similar structures with similar mass spectra for compound identification^[24]. In this study, we imported this recent approach, and established the MN of MJF solution and plasma after oral administration, and by merging the two MNs, the shared compounds were thought to be the absorbable constituents of MJF. To verify this, we also used reference substances to identify the 17 ingredients obtained from MN.

We then introduced network pharmacology to establish an image of the function and behavior of the biological network of MJF, identify potential targets and pathways of the drugs related to the disease using topology and computational methods, and explored the topology parameters of the 17 ingredients and their related targets in anti-HCC among all the ingredients-anti-HCC-pathways-targets. As a consequence, targets including TGF β 1, SMAD4, TNF, IFN α 1, IL2, BAX, BCL2, and FAS were screened out.

The TGF- β 1/SMAD signaling pathway plays an important role in the development and invasion of HCC^[25, 26]. Cytoplasmic protein SMAD is the most critical and important signal transduction factor in the TGF β signaling pathway. When the TGF- β pathway is activated, phosphorylated SMAD2 combines with SMAD4 to form a complex, and then enters the nucleus to activate the expression of downstream target

genes. SMAD 7 is a TGF- β signaling inhibitory factor, which is localized in the cell nucleus, and can regulate the activity of TGF- β ^[27]. IL-2 is an important cytokine which regulates immune function, ¹ promotes the proliferation and activation of T cells, and stimulates the proliferation of natural killer cells^{[28],[29]}. TNF- α and IFN- γ are essential cellular immune molecules, which affect the immune escape of cells. Clinical studies have shown that disordered TNF- α and IFN- γ levels have a significant impact on the stability of the body's internal environment, leading to abnormal immune function in patients, and inducing the occurrence and progression of tumors^[30]. Tumor cells can avoid apoptosis by up-regulating Bcl2 and down-regulating Bax^[31]. Bax and Bcl2 antagonize each other in apoptosis regulation of HCC cells. With the decrease or deletion of Bax expression, apoptosis of HCC cells is gradually weakened, which may be an important reason for the rapid growth and enlargement of tumors. Fas is a cell surface death receptor and an apoptosis signaling molecule. Fas which binds to FasL activates and transmits apoptosis signals, which is an important pathway in inducing tumor cell apoptosis. Previous research showed that the apoptosis of cancer cells mediated by Fas and FasL is an important part of the body's immune surveillance^[32], and the abnormal expression of Fas and FasL can lead to disordered apoptosis, which results in massive cell proliferation due to escaping the killing effect of cytotoxic T cells^[33, 34].

In this study, we evaluated the impact of MJF on HCC using both *in vivo* (H22 tumor-bearing mice) and *in vitro* (HepG2 cells) models (Figure 8). The results indicated that MJF could prevent HCC by improving body weight gain and tumor inhibition rate, protecting immune organs and liver function, affecting immunity and apoptosis, and up-regulating the ⁵ TGF- β 1/SMAD signaling pathway, by increasing the relative expression of TGF- β 1, SMAD3, p-SMAD2 and SMAD4 and decreasing SMAD7, reducing immune factors TNF- α and IFN- γ , and decreasing the apoptosis cytokines Fas, FasL and Bcl2/Bax. In addition, to verify these findings, we treated HepG2 cells with the TGF- β 1 inhibitor LY364947, which inhibited the TGF- β 1/SMAD signaling pathway, increased LAMA4, TNF- α , IFN- γ , Fas, FasL, and Bcl2/Bax expression. However, MJF

significantly inhibited the effect of LY364947. This suggested that MJF has potential effects on HCC by regulating the TGF- β 1/SMAD pathway, immune and apoptotic cytokines.

Based on the identification of absorbable ingredients in mouse serum, we mainly focused on the fundamental study of potential anti-HCC targets of MJF, and intensive research on the mechanism of tumor cell apoptosis and immunology are still required. Moreover, other absorbable ingredients also need to be identified.

CONCLUSION

Our results demonstrated that MJF inhibits hepatocellular carcinoma by activating the TGF- β 1/SMAD signaling pathway, immune and apoptotic cytokines, which may be due to the mechanisms of MJF in adjusting immune escape and apoptosis.

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ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is one of the most common digestive system cancers with high mortality rates worldwide.

Research motivation

Considerable effort has been expended in understanding the mechanism of MJF on tumor immunology in the treatment of HCC.

Research objectives

Our study explored the mechanism of MJF adjusting immune escape and apoptosis.

Research methods

We conducted Molecule Network related to HPLC-ESI-TOF-MS to identify the absorbable ingredients of MJF, and hub potential anti-HCC targets were screened using network pharmacology and pathway enrichment analysis. Both *in vivo* and *in*

vitro experiments were demonstrated, and multiply processes including Histopathological analysis, ELISA, RT-qPCR, Western blotting assays were to adopted to explore the function MJF inhibits HCC.

Research results

We found that MJF improved body weight gain and tumor inhibition rate in H22 tumor-bearing mice, protected immune organs and liver function, reduced the HCC indicator AFP, affected immunity and apoptosis, and up-regulated the TGF- β 1/SMAD signaling pathway, by increasing the relative expression of TGF- β 1, SMAD2, p-SMAD2 and SMAD4 and decreasing SMAD7, reducing immune factors TNF- α and IFN- γ , decreasing apoptosis cytokines Fas, FasL and Bcl2/Bax, and inhibiting the effect of LY364947 in HepG2 cells.

Research conclusions

Our findings suggested that MJF inhibits HCC by activating the TGF- β 1/SMAD signaling pathway, and affecting immune and apoptotic cytokines.

2

Research perspectives

Considerable effort has been expended in understanding the anti-hepatocellular carcinoma mechanism of MJF, all of which due to MJF's particular participate in adjusting immune escape and apoptosis.

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