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Research Article

Therapeutic Efficacy Evaluation and Underlying Mechanisms Prediction of Jianpi Liqi Decoction for Hepatocellular Carcinoma

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ABSTRACT

Objective: The aim of this study was to assess the therapeutic effects of Jianpi Liqi decoction (JPLQD) in hepatocellular carcinoma (HCC) and explore its underlying mechanisms.

Methods: The characteristics and outcomes of HCC patients with intermediate stage B who underwent sequential conventional transcatheter arterial chemoembolization (cTACE) and radiofrequency ablation (RFA) only or in conjunction with JPLQD were analysed retrospectively. The plasma proteins were screened using label-free quantitative proteomics analysis. The effective mechanisms of JPLQD were predicted through network pharmacology approach and partially verified by ELISA.

Results: Clinical research demonstrated that the Karnofsky Performance Status (KPS), traditional Chinese medicine (TCM) syndrome scores, neutropenia and bilirubin, median progression-free survival (PFS), and median overall survival (OS) in HCC patients treated with JPLQD were superior to those in patients not treated with JPLQD (all $P<0.05$). The analysis of network pharmacology, combined with proteomics, suggested that 52 compounds targeted 80 potential targets, which were involved in the regulation of multiple signaling pathways, especially affecting the apoptosis-related pathways including TNF, p53, PI3K-AKT, and MAPK. Plasma *IGFBP3* and *CA2* were significantly up-regulated in HCC patients with sequential cTACE and RFA therapy treated with JPLQD than those in patients not treated with JPLQD ($P<0.001$). The AUC of the *IGFBP3* and *CA2* panel, estimated using ROC analysis for JPLQD efficacy evaluation, was 0.867.

Conclusion: These data suggested that JPLQD improves the quality of life, prolongs the overall survival, protects liver function in HCC patients, and exhibits an anticancer activity against HCC. *IGFBP3* and *CA2* panels may be potential therapeutic targets and indicators in the efficacy evaluation for JPLQD treatment, and the effective mechanihsms involved in the regulation of multiple signaling pathways, possibly affected the regulation of apoptosis.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor, and the fourth leading cause of cancer-related deaths globally [1]. According to the Barcelona Clinic Liver Cancer (BCLC) staging system, the conventional transcatheter arterial chemoembolization (cTACE) is recognized as the first-line treatment for intermediate stage HCC patients, with a median survival time of < 20 months, and a 5-year survival rate of 6-19% by combining the effect of targeted chemotherapy with ischaemic necrosis, by arterial embolization. However, this could result in acute hypoxia, leading to the increase of VEGF, which might induce tumor revascularization and local recurrence [2-5]. Radiofrequency ablation (RFA) is an effective treatment for recurrent HCC [6]. Individually, cTACE and RFA have limitations and are complementary to each other. cTACE can block blood flow in the hepatic artery, reducing heat loss, and at the same time, target undetected satellite lesions contributing to the expansion of the necrosis area caused by RFA. Furthermore, the effect of anticancer chemotherapeutic agents on cancer cells during cTACE treatment can be enhanced by hyperthermia [7].

The combined minimally invasive procedures (sequential cTACE and RFA) have been reported to be a more effective treatment to slow tumor progression and improve survival in HCC patients [8]. The 1-, 3-, and 5-year overall survival rates were 94%, 69%, and 46%, respectively, and the corresponding recurrence-free survival rates were 80%, 45%, and 40% for the sequential treatment group [6]. However, the rates of cure, drug resistance, and side effects have remained the major limitations in the treatment of HCC.

As a complementary and alternative medicine, traditional Chinese medicine (TCM) is playing an increasingly important role in the treatment of malignant tumors by improving clinical symptoms and prolonging survival rate [9]. One study demonstrated that after sequential TACE and RFA, combined with TCM treatment of middle and advanced primary HCC, the complete tumor necrosis rate (76.92%) and KPS score were higher than those of the TACE group or TACE + RFA group. In addition, the first recurrence rate (7.69%) and the incidence of adverse reactions, nausea and vomiting, were significantly lower than those in the other two groups [10]. With the advantages of minimal invasiveness, rapid recovery, fewer complications, and high safety, sequential cTACE and RFA combined with TCM therapy is an effective, safe, and reliable comprehensive therapy, which may become the developmental direction of non-surgical therapy in HCC.

JPLQD is an ideal TCM formula, commonly used in the treatment of primary liver cancer, and has been proved effective both in animal experiments and clinical trials [11]. JPLQF combined with transcatheter arterial chemoembolization (TACE) improved quality of life, clinical TCM symptoms, and liver function in patients with HCC [12]. However, the therapeutic effects of JPLQF combined with TACE on survival in patients with HCC and its molecular mechanisms are still unclear. Network-based systems biology provides an encouraging methodological strategy for a better understanding of the holistic, complementary and synergic essence of TCM and the overall regulatory mechanism of CHM, and it has been applied in the studies on the therapeutic effect mechanisms in liver cancer and colorectal cancer and so on [13-16].

Here, we investigated the therapeutic effect of JPLQD combined with minimally invasive procedures in HCC, and performed an analysis of network pharmacology, combined with proteomics, to predict the effective mechanisms of JPLQD. Furthermore, the predicted targets were verified for as potential indicators of efficacy evaluation.

Materials and Methods

I JPLQD Preparation and Quality Control

JPLQD was composed of *Codonopsis pilosula* (Dangshen, 15g), *Atractylodes macrocephala* (Baizhu, 10 g), *Poria cocos* (Fuling, 15 g), five leaf Akebia fruit (Bayuezha, 15 g), and *Hedyotis diffusa* (Baihuasheshecao, 15 g), and the decoction was manufactured at the Fudan University Shanghai Cancer Center as described previously [12].

II Patients and Treatment Evaluation

Between April 2010 and April 2018, 190 HCC patients with intermediate stage B who underwent sequential cTACE and RFA as their primary treatment were enrolled, including 99 patients who additionally received 2 consecutive months or more of JPLQD orally, twice daily, and were divided into the Chinese herbal medicine (CHM) group. The No CHM group only received monotherapy. The Seldinger's technique of arterial embolization was administered as the standard cTACE procedure, and 3 weeks later, RFA was performed using the standard procedure, as previously described [6].

Patient eligibility criteria was as previous report, included the following: i) pathologically or clinically proven HCC; ii) age between 18 to 81 years; iii) Karnofsky Performance Status (KPS) score higher than 60; iv) Child-Pugh class A or B; v) BCLC B stage; vi) without severe acute and chronic diseases such as cardiovascular and cerebrovascular disease, diabetes; vii) no refractory ascites, or organ dysfunction, or other contraindications of TACE and RFA; viii) no previous anticancer therapy; and ix) complete medical records [12].

Patients were matched with respect to age, gender, liver function test, tumor size and number and AFP etc. The TCM syndrome scores were assessed according to the symptom grading scale. After treatment, the TCM syndrome scores decreased by more than 70% were defined as obviously improved, and scores decreased by more than 30% were defined as partly improved. Tumor viability was assessed using the modified response evaluation criteria in solid tumor (mRECIST) criteria by CT or MR imaging, or contrast-enhanced US. The primary end point was overall survival (OS), defined as the time from first date of first treatment on the study to the date of death by any cause, or the last follow-up. The secondary end point was progression-free survival (PFS), defined as the interval between the time of initial treatment and the time of disease progression being confirmed, or patient death if disease progression was not evident and the tertiary end point were side effects of the treatment. Patients lost to follow-up were censored at the date of the last registration. The toxicity was assessed according to the WHO criteria for acute and subacute toxic reactions of anticancer drugs.

This retrospective study was supported by the Human Research and Ethics Committee of the Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine and the Fudan University Cancer Center, with written informed consent obtained from all patients and healthy donors.

III Plasma Collection

Peripheral blood samples (1 mL) were collected from all subjects in the fasting state, in the morning, in EDTA-containing tubes, centrifuged at 4°C and 12,000 g for 20 min. The supernatant was collected, and 200 μL aliquots were stored at -80°C until assay.

IV Label-Free Analysis

Plasma samples (100 μL) from 8 pre-treatment HCC patients and 6 healthy donors respectively were used for detection. The major experimental steps were as follows: i) each sample was concentrated by 10 KDa ultrafiltration membrane and centrifuged at 4°C and 12,000 g for 20 min; ii) Protein was quantified using the Bradford method; iii) Next, 8 M urea and 0.1 m Tris-HCl (pH 8.0) was added to 200 μg concentrated sample for denaturation; iv) Protein samples were reduced with DTT (final concentration 10 mM) at 56°C for 1 h, then alkylated with IAA (final concentration 25 mM) in the dark at room temperature for 1 h; v) After calculating the amount of protein per sample, trypsin was added to each aliquot for digestion at an enzyme–protein ratio of 1:25 (w/w) at 37°C for 12 h, followed by the addition of 1% FA to terminate the enzymatic hydrolysis reaction at 1:10 (v/v); vi) An appropriate amount of the sample was used to make the total amount of protein 0.5 μg, 100 fmol internal standard (GluFib, Sigma) was added, and nanoLC-MS^E analysis was carried out. According to the internal scalars, the accurate amount of enzyme digestion in the samples was determined, and the injection volume was adjusted so that the amount of samples in the column was 0.5 μg, and each sample evaluation was repeated three times; vii) We searched the databases and combined all results of quantitative and qualitative protein analysis.

V Data Preparation

The chemical compounds of JPLQD were collected from TCMSP, CancerHSP, TCM Database@Taiwan, TCMID, HIT, TCMGeneDIT, BATMAN-TCM, chemDB (Shanghai Institute of Organic Chemistry of CAS. Chemistry Database), and screened for ADMET (Absorption, Distribution, Metabolism, Elimination, Toxicology) properties or drug-likeness value (oral bioavailability (OB) ≥ 30%; drug-likeness (DL) ≥ 0.18) in TCMSP. We also collected the constituents in plasma after oral administration of JPLQD by text mining. The active compound related targets were obtained from TCMSP, TCMID, HIT, and BATMAN-TCM database. The drug-related targets of HCC were identified using the keywords "Hepatocellular Carcinoma" by searching DrugBank, Therapeutic Target Database (TTD), PharmGKB, CIViC and Comparative Toxicogenomics Database (CTD).

VI Network Construction and Bioinformatics Analysis

The active compounds-targets network of JPLQD was constructed using the Cytoscape software (Version 3.4.0) to visualize the interaction network. The network topology analysis was performed using the Network Analyser plugin in Cytoscape. A Venn analysis was conducted, and the overlap was presumed to be the putative targets of JPLQD for the treatment of HCC. The gene ontology and pathway enrichment analysis of the putative targets were performed by OmicsBean.

VII Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma samples (100 μL) from HCC patients with a CHM group (n=80), a No CHM group (n=80) before and after interventions were used for assays. *IGFBP3* and *CA2* concentrations in all patients were measured using Human *IGFBP-3* ELISA Kit (MultiSciences, China) and Human Carbonic Anhydrase II (*CA2*) ELISA Kit (BlueGene, China) according to the manufacturer's instructions.

VIII Statistical Analysis

Clinical data were retrieved from the electronic medical system of the hospital, determining the sample size of this study. Statistical analysis was performed using SPSS version 23.0 (IBM, Armonk, NY, USA), and GraphPad Prism 6.0 (San Diego, California, USA) was used to generate the statistical charts. The comparisons between the two groups were performed using the student's t-test or non-parametric Mann-Whitney U test, as appropriate for continuous data, χ^2 test or Fisher's exact test for categorical data, and rank sum test or Ridit analysis for ranked data. The survival curves were estimated using the Kaplan-Meier method and compared with the log-rank test. All patients were followed up until mortality, or until December 8, 2018. The differentially expressed proteins (DEPs) between HCC and normal plasma were identified using the t-test and volcano plot filtering and analysed with the OmicsBean data analysis tool with a threshold of |log of fold change| > 1 and $P < 0.05$. The predicted probability of the JPLQD therapeutic efficacy was used as potential indicator to construct a receiver operating characteristic (ROC) curve and the corresponding area under the curve (AUC) was estimated. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

I Characteristics of the Patients

The baselines of patient demographics are summarized in (Table 1). There were no significant differences in the patients' age, gender, Child-Pugh score (A/B), diagnostic type, serum AFP levels (≤400 μg/L/> 400 μg/L), and antiviral therapy (yes/no) between the CHM and No CHM groups.

II KPS Scores

There were no significant differences in KPS scores between the two groups before treatment (86.67±5.53 vs 87.80±5.12). After treatment, the KPS score of the CHM group (90.00 ±7.42) was considerably higher than that of the No CHM group (87.91±5.27) ($P < 0.05$), showing a marked improvement ($P < 0.001$). However, no significant improvement in the No CHM group was observed (Figure 1A).

Table 1: Baseline Characteristics of the Study Patients.

Characteristics	CHM (n=91)	No CHM (n=99)	*P*
Age(years) *	56.8±9.5	55.0±10.7	0.220
Female/Male	16/75	14/85	0.516
Child-Pugh Class(A/B)	83/8	97/2	0.050
Clinical/Cytological diagnosis	47/44	46/53	0.475
Antiviral (Yes/No)	18/73	31/68	0.069
AFP (ng/mL) (≤400/>400)	54/37	56/43	0.699

Except where indicated, data are numbers of patients.

*Data are means ± standard deviations.

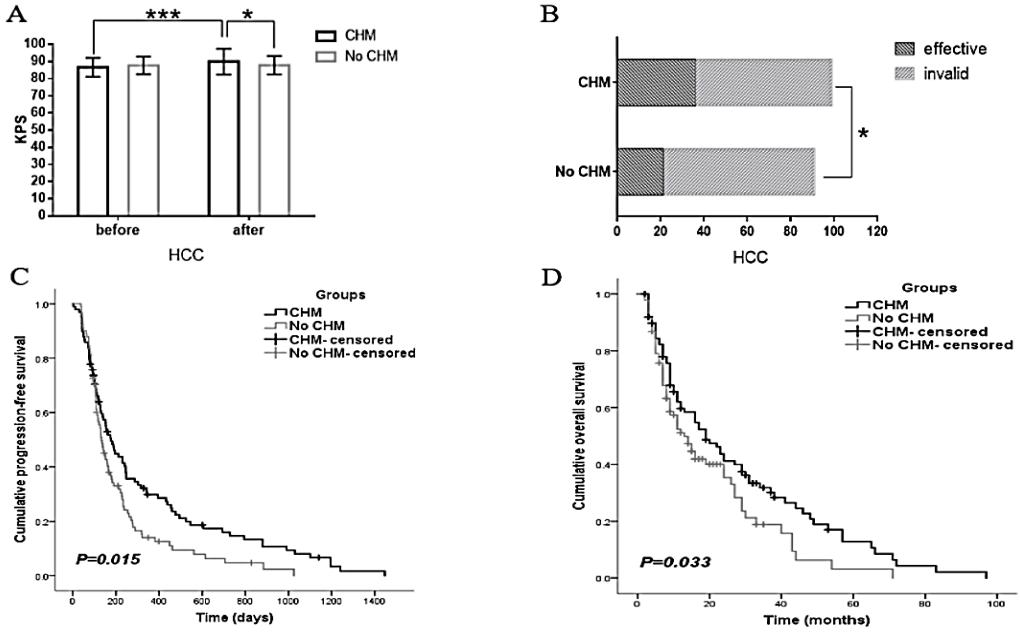

Figure 1: A) Comparison of KPS scores between two groups of patients with HCC. ***$P < 0.001$ vs. after treatment; *$P < 0.05$ vs the No CHM group after treatment. **B)** Comparison of TCM syndrome scores between two groups of patients with HCC. *$P < 0.05$ when compared with the No CHM group. **C & D)** Survival curves for HCC patients with and without JPLQD treatment. C) Progression-Free Survival curves. D) Overall Survival curves.

III TCM Syndrome Scores

After treatment, 30 cases showed significant improvement in the TCM syndrome score and 6 cases showed partial improvement in the CHM group. Additionally, 17 cases significantly improved, and 4 cases partially improved in the No CHM group. There was a significant difference in the total effective rates of TCM syndromes in the two groups [36.4% and 23.1%, respectively; (P<0.05)] (Figure 1B).

IV Clinical Efficacy

In the CHM group, there were 8 cases of partial remission (PR), 50 cases of stable disease (SD), 41 cases of progression diseased (PD) with the response rate (RR) being 8.1%, while in the No CHM group, there were 7 cases of PR, 41 cases of SD, 43 cases of PD with the RR being 7.7%. The treatment efficacy of the CHM group was similar to the No CHM group (P>0.05).

V Tumor Markers

The two groups were compared before and after treatment, regarding the levels of AFP, CEA, and CA199. Before treatment, the difference was not statistically significant. After treatment, the level of CEA in the CHM group was significantly lower than that in the No CHM group, with a remarkable decline compared to before treatment ($P < 0.05$). However, no significant change in the level of AFP, CEA, and CA199 were observed in the No CHM group.

VI Progression-Free Survival and Overall Survival

The 3-, 6- and 12-month PFS rates of CHM group were 74.7%, 43.4%, and 24.2% respectively, and the median PFS was 176 days. In the No CHM group, the 3-, 6-, and 12-month PFS rates were 76.9%, 31.9%, and 11.0%, respectively, and the median PFS was 132 days. The survival curve for the CHM group was significantly better than that for the No CHM group (Figure 1C, $P < 0.05$). The 1-, 3-, and 5-year OS rates were 52.5%, 19.2%, and 5.7% for the CHM group and 46.2%, 6.6%, and 1.1% for the No CHM group, respectively. The median OS of the CHM group was 19 months, surpassing the 13 months of the No CHM group (Figure 1D, $P < 0.05$).

VII Adverse Events

As for adverse events, 8 patients experienced grade 3/4 toxicity in the CHM group, while 11 patients experienced grade 3/4 toxicity in the No CHM group. Ridit analysis demonstrated significant differences in side effects such as neutropenia and elevated bilirubin between the two groups ($P < 0.05$) (Table 2).

Table 2: Adverse reaction.

Laboratory indicators	CHM					No CHM				
	0	I	II	III	IV	0	I	II	III	IV
WBC	75	20	4	0	0	68	12	11	0	0
Neutrophil*	86	13	0	0	0	69	15	6	1	0
Platelets	75	8	14	1	1	61	16	12	2	0
Haemoglobin	85	10	2	2	0	77	6	4	3	1
Alanine transaminase (ALT)	77	17	3	0	2	74	13	2	2	0
Total bilirubin (TBIL)*	84	11	2	2	0	70	18	1	2	0
Urea nitrogen	98	1	0	0	0	90	0	1	0	0
Creatinine	98	1	0	0	0	89	2	0	0	0

Data are numbers of patients. *$P < 0.05$, CHM group compared with the No CHM group.

VIII DEPs of HCC

A total of 739 proteins were detected by label-free analysis. After data preprocessing, the obscuring variations in the raw data were normalized. The results of the partial least square discriminant analysis (PLS-DA) identified the significant differences in proteins between HCC patients and healthy donors. The results of DEPs analysis demonstrated 36 differential proteins between the two groups, among which 26 proteins were up-regulated, and 10 proteins were down-regulated.

IX Drug-Related Targets of HCC

In this study, the above 5 drug-related databases were searched, repeated targets were deleted, finally 287 known drug-related targets for the treatment of HCC were retrieved.

X Compounds and Targets of JPLQD

The chemical compounds of each herb in JPLQD were collected from 8 databases, and associated literatures. There were 195 compounds in *Hedyotis diffusa*, 309 compounds in *Atractylodes macrocephala*, 508 compounds in *Codonopsis pilosula*, 113 compounds in *Poria cocos*, and 83 compounds in the five leaf Akebia fruit. In order to discover the active compounds of JPLQD, we selected ingredients meeting the requirements of both OB ≥ 30% and DL ≥ 0.18. To elucidate potential targets of active compounds, present in the JPLQD, we searched the TCMSP database etc., and eliminated those with no target information. Finally, 833 targets corresponding to 52 chemical constituents in the JPLQD were obtained after the removal of duplicates. Specifically, 9 active compounds in *Hedyotis diffusa*, 4 active compounds in *Atractylodes macrocephala*, 21 active compounds in *Codonopsis pilosula*, 14 active compounds in *Poria cocos*, and 8 active compounds in the five leaf Akebia fruit, with beta-Sitosterol and kaempferol being common components of *Hedyotis diffusa* and five leaf Akebia fruit, and stigmasterol was shared by *Hedyotis diffusa*, five leaf Akebia fruit and *Codonopsis pilosula*.

XI Putative Targets of JPLQD for the Treatment of HCC

The active compounds-targets interaction network was visualized by Cytoscape after the evaluation of the network parameters. There were 890 nodes and 2011 edges in the network, of which 52 nodes were active compounds and 833 nodes were drug targets. The node sizes represented the degree values, the larger the degree, the more important the node was in the network (Figure 2A).

We overlapped the DEPs of HCC, drug-related targets of HCC and targets of active compounds in JPLQD using the online tool Venny 2.1, and reported 2 targets (*IGFBP3*, *CA2*) were present in all 3 modules. The 80 targets that the compounds of JPLQD targeted to the DEPs of HCC and to the drugs for the treatment of HCC were presumed to be the putative targets of JPLQD for the treatment of HCC and were selected for further analysis (Figure 2B).

To identify relevant pathways and functions, we conducted the KEGG pathway and GO enrichment analyses for these putative targets. As a result, these targets are significantly related to many apoptosis-related biological processes, including signaling pathways of PI3K-AKT (17 targets), TNF (10 targets), MAPK (10 targets), NF-kappa B (6 targets), and p53 (9 targets) (Figure 3A and Supplementary Table 1). These results demonstrated that they were located mainly in the membrane-bounded vesicle and extracellular space, such as membrane-bounded organelles, exosomes and vesicles, where they significantly enriched responses of multiple biological processes to chemical stimulus, organic substances and oxygen-containing compounds, participating primarily in positive regulation of cellular and biological processes. For the molecular function categorization, the targets were mainly connected with protein binding, enzyme binding, macromolecular complex binding, receptor binding, etc. (Figure 3B and Supplementary Table 2). Further analysis of the 46 KEGG pathways revealed that among the 80 potential targets, with the strongest correlation involved in the enrichment of these pathways, were *FOS*, *TGFB1*, *CTNNB1*, *IL6*, *MET*, *MTOR*, *MMP9*, *MMP2*, *CDK4*, *BCL2L1*, *RAC1*, *IGFBP3*, *PTEN*, and *PIK3CA* (Figure 3C). This suggested that apoptosis played a significant role in the molecular mechanisms of HCC, and many of the HCC-related biomarkers were directly regulated by apoptosis.

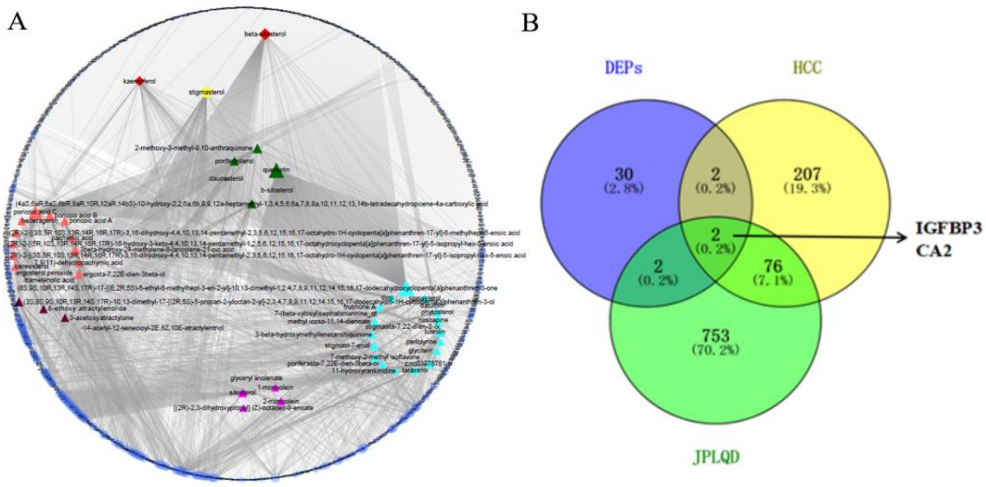

Figure 2: A) The active Components-Targets network. The blue nodes in the outer circle represent 833 targets of JPLQD. The nodes in the inner circle represent 52 active compounds in JPLQD. The deep green, brown, light green, light red and pink triangles represent unique active compounds in *Hedyotis diffusa*, *Atractylodes macrocephala*, *Codonopsis pilosula*, *Poria cocos*, five leaf Akebia, respectively. The red diamonds represent the common compounds of *Hedyotis diffusa* and five leaf Akebia fruit, and the yellow hexagonal represent the common compounds of *Hedyotis diffusa*, five leaf Akebia fruit and *Codonopsis pilosula*. The size of these nodes is positively related to their degrees in the network. **B)** Venn diagram of putative targets of JPLQD for the treatment of HCC.

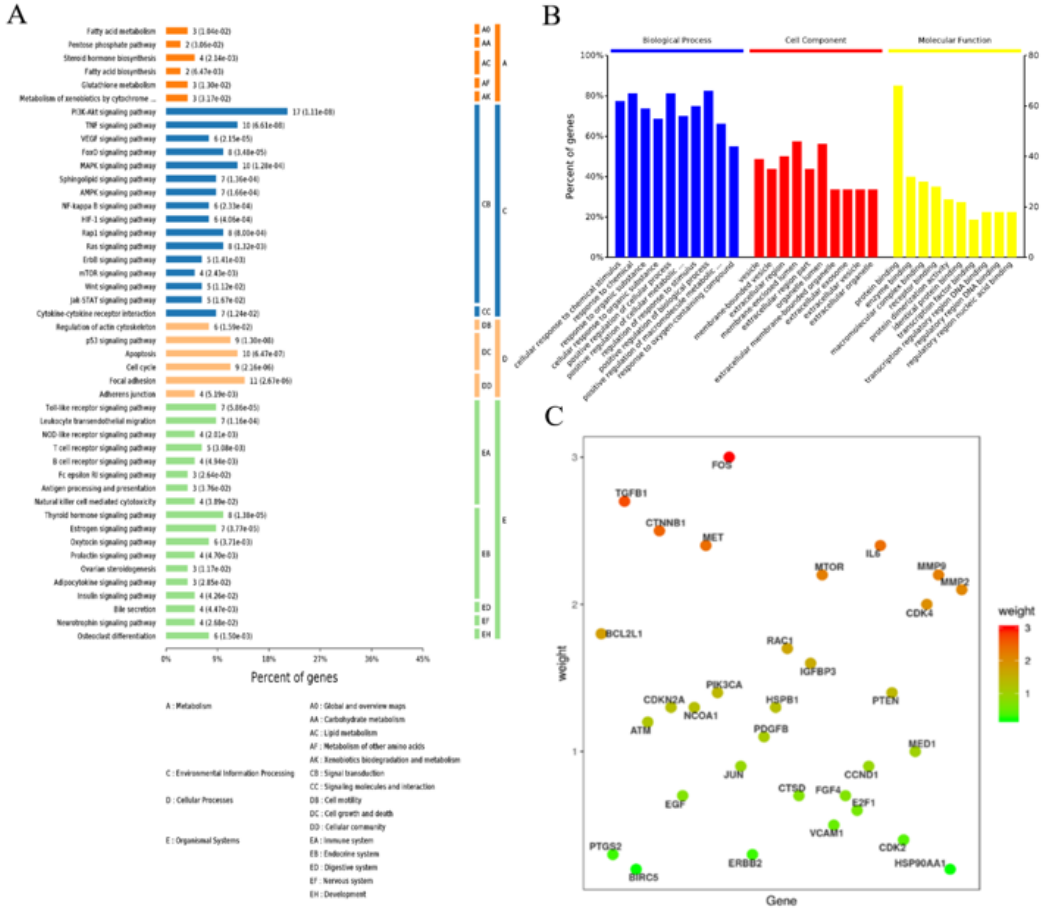

Figure 3: Pathway and function enrichment analysis for potential targets of JPLQD. **A)** The main pathways of potential targets. The *y*-axis shows significantly enriched pathway categories of the targets, the *x*-axis shows the enrichment scores of these terms and the numbers at the end of the bars represent the counts of targets (the *p*-value of pathway enrichment analysis). **B)** The GO enrichment analysis of the potential targets. Contain: Molecular Function Group (MF), Biological Process Group (BP), and Cellular Component Group (CC). In the figure, GO items are aligned from left to right according to their *p* value from low to high. **C)** Bubble map of important targets involved in the pathways. The order of importance was ranked from top to bottom.

XII Putative Effective Mechanisms of JPLQD

As shown in (Figure 4), putative major signaling pathways of JPLQD were constructed based on the internal relationship of the network connected by the targets. It was suggested that TNF, p53, PI3K-AKT, and MAPK signaling pathways were regulated by multiple compounds in JPLQD. Further investigation demonstrated that the compound that was repeatedly associated with these pathways was quercetin, which regulated multiple targets, such as TNF, *CASP8*, *CTSD*, *BIRC5*, *CDKN2A*, *IGFBP3*, *BCL2L1*, *IL6*, *PTEN*, *EGF*, *and TGFB1*. It is worth noting that one of the overlapping targets, *IGFBP3* was involved in p53 signaling pathway, inducing apoptosis. Therefore, these data provided theoretical evidence that JPLQD in HCC is possibly linked with the apoptosis pathway.

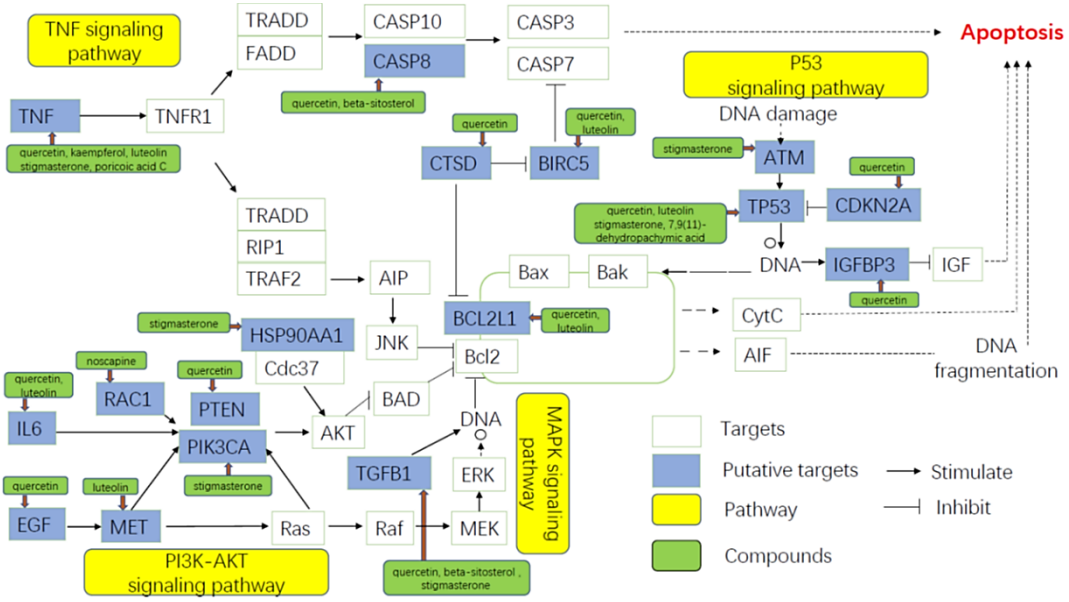

Figure 4: Putative pharmacological mechanisms of JPLQD.

XIII ELISA Verification Analysis

To examine whether the overlapping targets played important roles in the efficacy of JPLQD in HCC, we performed ELISA to measure the levels of *IGFBP3* and *CA2* expressions using the plasma samples from the 80 HCC patients before and after sequential cTACE and RFA treatment, with or without JPLQD. As shown in (Figure 5A), before treatment, the difference in *IGFBP3* was not statistically significant between groups. After treatment, the levels of *IGFBP3* expression were up-regulated in the CHM group ($P < 0.001$), and significantly higher than that of the No CHM group ($P < 0.001$), however, *IGFBP3* in No CHM group sharply decreased ($P < 0.001$). Additionally, the expression of *CA2* demonstrated an opposite trend in the two groups after treatment, significantly increased in CHM group than in the No CHM group ($P < 0.001$) (Figure 5B). To evaluate the ability of *IGFBP3* and *CA2* to evaluate the clinical efficacy of JPLQD comprehensive therapy, the ROC curves were determined for IGFBP3 and *CA2*, both of which were measured before and after JPLQD treatment with sequential cTACE and RFA. ROC analysis showed that these proteins were suitable to assess the clinical efficacy of JPLQD comprehensive therapy, with an area under curve of 0.804, 0.829 for *IGFBP3*, *CA2*, respectively. Comparisons of AUC revealed the significant superiority of protein panel with *IGFBP3* and *CA2* (AUC = 0.867), and the logit model was 0.078*IGFBP3+0.887*CA2-11.039 (Figure 5C).

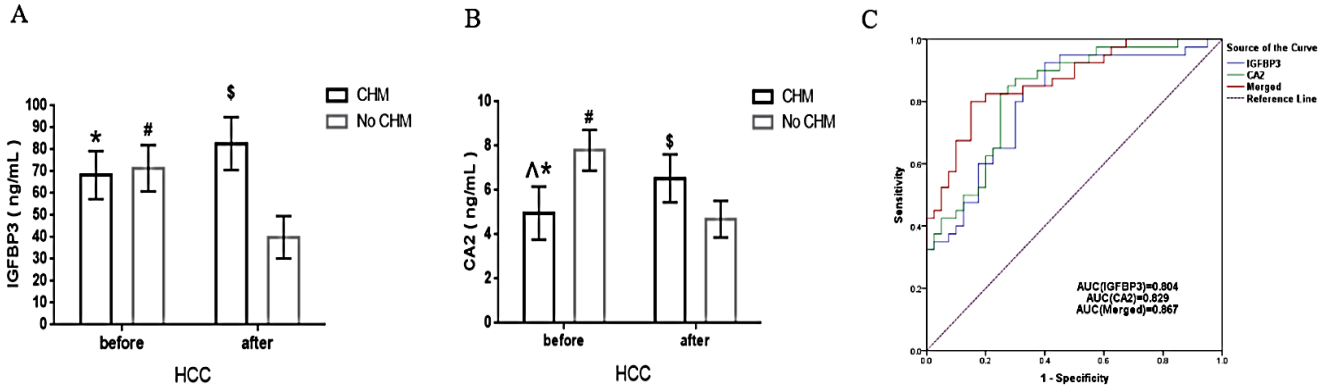

Figure 5: A & B) Comparison of plasma protein levels in HCC before and after treatment with or without JPLQD. A) The expression of IGFBP3. B) The expression of CA2. *$P < 0.001$ vs after treatment in CHM group, $^{\#}P < 0.001$ vs after treatment in No CHM group, $^{\wedge}P < 0.001$ vs the No CHM group before treatment, $^{\$}P < 0.001$ vs the No CHM group after treatment. **C)** ROC curves for *IGFBP3* and *CA2* in CHM group.

Discussion

HCC is a type of epithelial carcinoma originating from hepatocytes, demonstrating a rise in global incidence due to the complexity, postoperative recurrence, metastasis and heterogeneity, and a persistently high mortality rate [17]. It is estimated that nearly half of the all diagnosed cases of HCC and reported mortalities occurred in China [18]. Unfortunately, due to low rates of early diagnosis, many HCC patients are not eligible for potential treatment options, such as surgery, percutaneous ablation, and liver transplantation. TACE is considered a well-developed treatment for HCC patients with stage B [2]. Local ablation has the characteristics of small trauma, rapid response, strong reproducibility, and accurate effects on tumor tissues [19]. Some case-control studies support the combination of these two therapeutic interventions, as jointly these therapies can overcome their respective shortcomings. However, the strong side effects and low response rates, along with the emergence of drug resistance, present limitations in the treatment of HCC, seriously affecting the quality of life in HCC patients. Recently, research on the treatment of HCC in the middle stage has been focused on effective, and low toxicity drugs.

The main purpose of TCM treatment is to protect liver, inhibit tumor growth, extend survival and improve the quality of life in patients. Previous studies provided evidence that JPLQD could alleviate the postembolization syndrome caused by TACE, protect liver function, and improve the long-term effects of TACE, but the therapeutic effect was still not satisfactory [20]. Hence, there is an urgent need to develop new treatment strategies and therapeutic targets. Combination therapy provides a potential solution to tumor heterogeneity and drug resistance by utilizing several mechanisms of action of a variety of treatments [21]. Therefore, we investigated whether minimally invasive surgery combined with JPLQD could achieve better therapeutic effects than monotherapy. In this study, we evaluated the efficacy of sequential cTACE and RFA, combined with JPLQD in HCC in the intermediate stage B. Our results demonstrated that JPLQD comprehensive therapy could significantly improve the clinical symptoms of patients, reduce the side effects, improve the quality of life, and prolong OS and PFS. These results confirmed the clinical effectiveness of JPLQD. However, its anti-tumor mechanisms are elusive.

TCM compounds are a complex system, in which herbs are always used to prevent and treat diseases by incorporating multiple levels, multiple targets and synergistic interventional effects of a large number of bioactive compounds. In order to identify the major compounds and elucidate the effective mechanisms of JPLQD on HCC, we used network pharmacology combined with label-free protein technique, detected 36 HCC DEPs, collected 287 drug targets for HCC, and predicted 833 corresponding targets of 52 active compounds of 5 the drugs in JPLQD, overlapped 80 potential targets, and conducted an enrichment analysis to find the potential GOs and signal pathways of the anti-HCC effect of JPLQD. Network pharmacology analysis reported that one component of JPLQD acted on a variety of targets, such as quercetin on *TNF*, *CASP8, CTSD, BIRC5, CDKN2A, IGFBP3, BCL2L1, IL6, PTEN, EGF and TGFB1*, stigmasterone on *PIK3CA, ATM, TGFB1, TP53, TNF* and *HSP90AA1*, and luteolin on *TP53, IL6*, TNF, *BIRC5* and *BCL2L1*. A target could be affected by a variety of compounds, such as *TNF* by quercetin, kaempferol, luteolin, stigmasterone, and poricoic acid C; *TP53* by quercetin, luteolin, stigmasterone, and 7, 9(11)-dehydropachymic acid; and *TGFB1* by quercetin, beta-sitosterol and stigmasterone. The anti-HCC effect of JPLQD involved the regulation of 46 pathways, including PI3K-AKT, focal adhesion, TNF, apoptosis, MAPK, p53 signaling pathways and the cell cycle. These revealed the synergistic characteristics of the multi-compounds, multi-targets and multi-pathways of JPLQD in the treatment of HCC. Furthermore, we constructed a putative major signaling pathway of JPLQD, and predicted that JPLQD might induce apoptosis, by regulating apoptosis-related pathways to improve the survival rate in HCC patients. Notably, previous studies in HCC confirmed that JPLQD could induce apoptosis through the up-regulation of *bax* in HAC cells, inhibition of proliferation, and inducing apoptosis of SMMC 7721 cells through the up-regulated expression of *p53* and $p21^{WAFI/\ CIP1}$ [22, 23].

Natural compounds extracted from CHM have increasingly been proven to be potential candidates in HCC. It has been reported that quercetin has multiple effects and plays an important role in the occurrence and development of HCC. Quercetin inhibited the growth of HCC cells in a dose-and time-dependent manner *in vivo* and *in vitro*, and inhibited the proliferation, and induced cell cycle arrest by regulating the expression and function of *p53*, ROS, *p21, p27, p16*, cyclinB1, *SP1*, and the PI3K/PKC and MEK/ERK pathways [24, 25]. Quercetin was found to induce apoptosis, associated with some biochemical changes in HCC cell lines, such as ROS production, caspase activation, *Bcl-2* regulation, down-regulation of *Sp1*, reduction of *p16*, PI3K/Akt and ERK inhibition, and *p53* stability by inhibiting the AKT/mTOR pathway, activating the MAPK signaling pathway, and abrogating the JAK2/STAT3 pathway to activate autophagy [26]. The combination of quercetin with cisplatin reported a synergistic inhibitory effect on cell growth and apoptosis. Additionally, quercetin could enhance the apoptosis of HCC cells induced by paclitaxel [24, 25]. The effects of JPLQD on apoptosis-related pathways could be derived from the synergistic effect of the 9 major compounds, including quercetin. As a mixture of several active compounds, JPLQD may affect the HCC cells through a variety of signaling pathways, which could complement the single target model of traditional chemotherapy. Additionally, the characteristics of the multi-target model of TCM was beneficial in avoiding chemotherapy resistance.

In order to discover potential therapeutic targets and indicators of efficacy evaluation, we performed ELISA and ROC analysis on the overlapping targets. The ELISA results demonstrated that the levels of *IGFBP3* and *CA2* increased significantly after JPLQD comprehensive treatment. ROC curve analysis revealed that *IGFBP3* and *CA2* panel performed well, with an AUC of 0.867 for efficacy evaluation of JPLQD combined with sequential cTACE and RFA therapy.

IGFBP3 is the most abundant *IGFBP* in circulation. It is synthesized in many tissues, including the liver, mainly produced by Kupffer, endothelial cells and hepatic stellate cells. *IGFBP3* binds to IGFs and the acid labile subunit (ALS) to form the 150 kDa ternary complex. *IGFBP3* function can be either dependent or independent of its ability to bind IGFs and is an effective IGF antagonist [27]. With the exception of a few studies, most relevant studies have shown that the mRNA and protein expression of *IGFBP3* in HCC was significantly down-regulated. The expression of *IGFBP3* in HCC decreased gradually, from well-

differentiated HCC to poorly differentiated HCC. Aishima *et al.* showed that serum *IGFBP3* levels correlated with tissue *IGFBP3* protein levels [28]. Aleem *et al.* demonstrated that the serum level of *IGFBP-3* decreased with the progression of liver dysfunction [29]. In addition, serum *IGFBP3* is known to be a better marker for liver synthesis capacity than serum albumin or cholinesterase [30].

The OS rate in patients with low expression of *IGFBP3* was significantly lower than in patients with high expression of *IGFBP3*. The decreased expression of *IGFBP3* significantly correlated with the clinicopathological characteristics and the early incidence of recurrence of HCC. This indicated that *IGFBP3* might serve as an independent molecular marker to evaluate the prognosis of HCC patients [31]. In terms of mechanism, *IGFBP3* acted as a key mediator of the effects on hepatoma cell growth and migration [32]. Anti-proliferation and pro-apoptotic activity were reported in HCC cells [28]. Huynh *et al.* indicated that loss of autocrine/paracrine *IGFBP3* loops may lead to HCC tumor growth, and the addition of exogenous *IGFBP3* to HCC cells could significantly reduce cell proliferation and induce apoptosis by inhibiting IGF-1-induced activation of IGF-1R, ERK, and Akt proteins [33]. Han *et al.* proved that the overexpression of *IGFBP3* could induce apoptosis and reduce colony formation in HUH7 cells [34]. It was also thought that either *p53* or *TGFβ1* induced apoptosis [35]. As *IGFBP3* functions like a tumor suppressor gene, it may be the most promising therapeutic target in HCC [36]. Our data also showed that after treatment with JPLQD, *IGFBP3* levels were significantly increased in HCC patients. This indicated that *IGFBP3* was not only involved in apoptosis induction in HCC but may also be a therapeutic target and an indicator of the effectiveness of JPLQD.

CA2, the cytoplasmic high-activity isozyme, is the most widely distributed member in the *CA* gene family. It regulates ion transport and pH balance [37]. In addition to the physiological functions, *CA2* has been abnormally expressed in many types of human cancers and is a tumor vessel endothelium–associated antigen [38]. The effect of *CA2* on cancer cells may be cell type specific, and *CA2* with prognostic features in several tumor diseases, including HCC. Additionally, *CA2* serves as a suppressor of HCC metastasis [39, 40]. *CA2* might play a role as a tumor suppressor gene in HCC development, and progression. Kuo *et al.* observed that *CA2* expression is reduced in HCC tumor areas, especially in poorly differentiated HCC, which might promote tumor cell motility, contributing to tumor growth and metastasis [41]. These results were consistent with previous study, which *CA2* inhibited tumorigenesis and metastasis in HCC, and increasing *CA2* expression significantly improved OS [39]. Zhang *et al.* also reported that the down-regulation of *CA2* in HCC significantly correlated with the clinical characteristics (AFP, microvascular invasion, differentiation degree and tumor stage) and prognosis of HCC, and elevated *CA2* increases DFS and OS in HCC [40]. In the current study, *CA2* levels were significantly increased after JPLQD treatment, indicating that *CA2* may also be a potential therapeutic target and indicator of efficacy evaluation for JPLQD treatment in HCC.

Conclusion

In summary, JPLQD prolonged survival, improved the quality of life and reduced the side effects in HCC patients with cTACE and RFA treatment, indicating that it may be an anti-HCC and liver protective complementary medicine. The underlying mechanism of JPLQD was the regulation of multi-compounds, multi-targets and multi-signaling pathways including apoptosis induction, which should be further examined. Moreover, *IGFBP3* and *CA2* panels may be potential indicators of efficacy evaluation for JPLQD in HCC.

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Author Contributions

XC drafted the manuscript; XC, PW, YQL and MY recruited patients, collected the clinical samples and information; XC and WZ carried out the detection; XC, YYL and JC performed the data collection, analysis and interpretation; SS, PW, YQL and ZM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Availability of Data and Materials

All the data used to support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

The study was approved by the Ethics Committee of Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Certificate No. 2014-345-41-01).

Consent to Participate

All patients provided written informed consent.

Consent for Publication

Not applicable.

Competing Interests

None.

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