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Integrating Network Pharmacology and Experimental Models to Investigate the Efficacy of Coptidis and Scutellaria Containing Huanglian Jiedu Decoction on Hepatocellular Carcinoma

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Abstract: Unlike Western medicines with single-target, the traditional Chinese medicines (TCM) always exhibit diverse curative effects against multiple diseases through its "multi-components" and "multi-targets" manifestations. However, discovery and identification of the major therapeutic diseases and the underlying molecular mechanisms of TCM remain to be challenged. In the current study, we, for the first time, applied an integrated strategy by combining network pharmacology with experimental evaluation, for exploration and demonstration of the therapeutic potentials and the underlying possible mechanisms of a classic TCM formula, Huanglian Jiedu decoction (HLJDD). First, the herb-compound, compound-protein, protein-pathway, and gene-disease networks were constructed to predict the major therapeutic diseases of HLJDD and explore the underlying molecular mechanisms. Network pharmacology analysis showed the top one predicted disease of HLJDD treatment was cancer, especially hepatocellular carcinoma (HCC) and inflammation-related genes played an important role in the treatment of HLJDD on cancer. Next, based on the prediction by network pharmacology analysis, both *in vitro* HCC cell and *in vivo* orthotopic HCC implantation mouse models were established to validate the curative role of HLJDD. HLJDD exerted its anti-tumor activity on HCC *in vitro*, as demonstrated by impaired cell proliferation and colony formation abilities, induced apoptosis and cell cycle arrest, as well as inhibited migratory and invasive properties of HCC cells. The orthotopic HCC implantation

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mouse model further demonstrated the remarkable antitumour effects of HLJDD on HCC *in vivo*. In conclusion, our study demonstrated the effectiveness of integrating network pharmacology with experimental study for discovery and identification of the major therapeutic diseases and the underlying molecular mechanisms of TCM.

Keywords: Huanglian Jiedu Decoction (*Coptidis rhizoma*, *Scutellariae radix*, *Phellodendri chinensis cortex*, *Gardeniae fructus*); Hepatocellular Carcinoma; Network Pharmacology; Experimental Validation; Inflammation.

Introduction

Hepatocellular carcinoma (HCC) is the commonest primary hepatic malignancy and ranks third causes of cancer related death worldwide. It is a complex malignancy with complex risk factors and the mortality rate is similar to its incidence, which reflects its poor prognosis (Daher *et al.*, 2018). With advanced scientific basis in biochemical and pharmacological research, as well as clinical trials, TCM is reported to be an excellent source as complementary and alternative anti-cancer drugs. Huanglian Jiedu decoction (HLJDD) is one of the most commonly used classic TCM formulae with the actions on clearing heat and detoxifying in order to eliminate fire toxins from the body. It is composed of four heat-clearing Chinese herbs including *Coptidis rhizoma* (CR, Huang-Lian), *Scutellariae radix* (SR, Huang-Qin), *Phellodendri chinensis* cortex (PCC, Huang-Bai), and *Gardeniae fructus* (GF, Zhi-Zi). HLJDD has been reported to exert several pharmacological activities on various diseases, such as diabetes, arthritis, ischemic stroke, and liver diseases (Wang *et al.*, 2014; Zhang *et al.*, 2014). Our research group have carried out many studies on HJLDD and its isolated compounds (Wang *et al.*, 2015a,b) and demonstrated its potent liver protection and anti-HCC effects. To further optimize the application of HLJDD in the medical treatment, exploring the major disease it may act on and the underlying action of mechanism may be urgently needed.

Biomedical studies on TCM formula have been extensively conducted, but limitation is apparent. The mode of multiple components, targets, and pathways is a prominent feature of TCM. It is believed that a TCM formulae possess numerous chemical components acting on multiple targets and diseases (Ma *et al.*, 2015). The complexity of a mixture of herbal components accompanied with the limited understanding of its molecular mechanisms has restricted the use and development of TCM. Along with the rapid development of bioinformatics, network pharmacology is based on the systems biology, polypharmacology, and molecular network analysis, which provide us the information of chemical compositions, absorption, distribution, metabolism, and excretion (ADME) properties as well as compound, gene, target, and disease networks. As a state-of-the-art technique, network-based systems biology has strongly facilitated the understanding of the holistic, complementary, and synergic essence of the molecular networks of TCM (Hopkins, 2008). With the rapid increase of public biomedical data, network pharmacology provides a feasible tool for elucidation of the molecular mechanisms for TCM

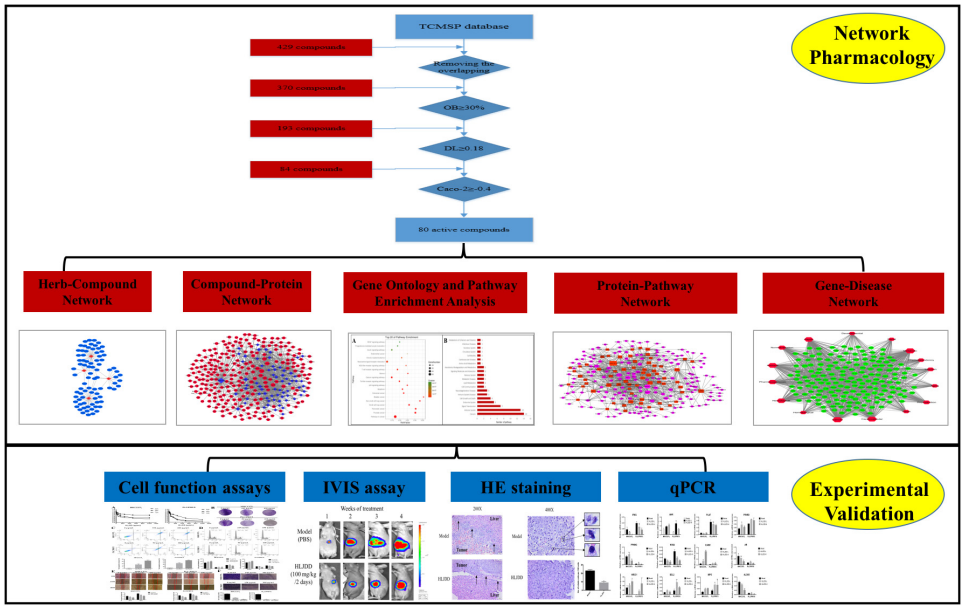

Figure 1. The detailed flow chart of the current study.

(Zhang *et al.*, 2013). In our previous studies, we have successfully applied this approach to explore the complex TCM (Huang *et al.*, 2017).

We identified partial mechanisms by which HLJDD exerted its therapeutic effect on HCC in our previous works (Wang *et al.*, 2015). To further explore and demonstrate the therapeutic potential of HLJDD, we for the first time applied a network pharmacology approach to predict the major therapeutic diseases of HLJDD and explore the underlying molecular mechanisms. At the same time, both *in vitro* HCC cell and *in vivo* orthotopic HCC implantation mouse models were established to validate the curative role of HLJDD as predicted by the network pharmacology analysis. The detailed flow chart of the current study was shown in Fig. 1.

Methods

Identification of Candidate Components

All phytochemicals of the four constituent herbs of HLJDD were retrieved from the TCM systems pharmacology database and analysis platform (TCMSP) (http://lsp.nwsuaf.edu.cn/tcmsp.php) (Ru *et al.*, 2014). The TCMSP includes data on Chinese herbal medicines and the relationships among drugs, targets, and diseases. The chemicals have the properties of oral bioavailability (OB), drug likeness (DL), intestinal epithelial permeability (Caco-2), blood-brain barrier permeability, and aqueous solubility (Ru *et al.*, 2014).

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Screening of Bioactive Components

The druggability of the bioactive compounds in HLJDD was analyzed using the criteria of OB, DL, and Caco-2 permeability (Huang *et al.*, 2014). The OB index represents the amount of medication that gets into the circulation after oral administration and the DL index is a qualitative concept used in drug design for estimating how drug-like a substance is to be suitable for drug. According to the criteria suggested by the TCMSP, an OB $\geq 30\%$ and DL index ≥ 0.18 were selected to determine the druggability of compounds. Caco-2 permeability is typically used as an coefficient *in vitro* model to study the passive diffusion of drugs across the intestinal epithelium according to the absorption rate, Caco-2 ≥ -0.4 was chosen for threshold (Huang *et al.*, 2014). A compound that satisfies all these criteria will be considered as a bioactive chemical. These parameters of all compounds were screened using the TCMSP, which provides all phytochemical information of the compounds (Ru *et al.*, 2014).

Identification of Associated Proteins and Gene

The protein targets of the compounds were retrieved from the TCMSP (http://lsp.nwsuaf.edu.cn/tcmsp.php) (Ru *et al.*, 2014). The UniProt Knowledgebase (UniProtKB) is a protein database partially curated by experts and contains 54,247,468 sequence entries. The gene names were further extracted from the UniProtKB (http://www.uniprot.org).

Identification of the Potential Pathways and Gene-Associated Diseases

The database for annotation, visualization, and integrated discovery (DAVID) bioinformatics resource comprises an integrated online biological knowledge base and analytical tools for systematically extracting biological data from large gene and protein lists and provides a comprehensive set of functional annotation tools, including chart, table, and clustering tools, for understanding the biological relevance of large lists of genes (http://david.abcc.ncifcrf.gov/) (Jiao *et al.*, 2012). To identify the compounds targeted pathways and gene-associated diseases, the obtained genes were further analyzed using DAVID. Thresholds Count ≥ 2 and EASE scores ≤ 0.05 were chosen in functional annotation clustering.

Construction of Network and Analysis

To comprehensively understand the molecular mechanisms of HLJDD, the herb-compound, compound-target, target-pathway, and target-disease networks were constructed using Cytoscape 3.3.0. In the compound-target network, the active compounds were connected with those potential targets. The target-pathway network was established by linking the targets and their potential pathways. In the target-disease network, the targets were connected with those associated diseases which were obtained by enrichment analyzing genes using DAVID. This software is a popular bioinformatics package for biological network visualization and data integration (Smoot *et al.*, 2011).

Preparation of HLJDD Aqueous Extract

HLJDD consisted of 30 g rhizomes of CR, 30 g fruits of GF, 20 g bark of PCC, and 20 g root of SR. All crude herbs were provided from the dispensary of School of Chinese Medicine, The University of Hong Kong. All crude herbs were mixed and boiled in 1000 mL distilled water for 2 h to prepare the herbal extract of HLJDD. The obtained solvent was filtered by centrifuging at 10000 rpm for 30 min. The extraction step was repeated twice and the supernatants were mixed together and then evaporated to dryness. The extract powder was re-dissolved in distilled water to 10 mg/ml and filtered with a 0.22 μm pore-size filter and stored at −20°C for further use.

Cell Culture

Human hepatocellular carcinoma MHCC97L and PLC/PRF/5 cells were selected for the following experiments. MHCC97L cells were kindly gifted by Professor Man Kwan from Department of Surgery, The University of Hong Kong and PLC/PRF/5 cells were purchased from American Type Culture Collection (ATCC; Manassa, VA, USA). All cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin and maintained in a humidified chamber with 5% CO_2 at 37°C.

Cell Viability Assay

5000 HCC cells/well were seeded in 96-well plates. After incubation overnight, the cells were treated with increasing concentrations of HLJDD (0, 7.8125, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 μg/ml) for 24, 48 and 72 h. 10 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT, 5 mg/mL; Sigma, USA) was added into each well and then cells were cultured for another 4 h at 37°C. Then the supernatants were discarded and 100 μL of DMSO was added into each well. The absorbance was measured at 595 nm using Multiskan MS microplate reader (Labsystems, Finland).

Colony Formation Assay

5000 cells/well were seeded in 6-well plates. After incubation overnight, the cells were treated with or without HLJDD (225 and 450 μg/mL for MHCC97L, 50 and 100 μg/mL for PLC/PRF/5) for continuous 10 days. After fixation with 4% paraformaldehyde for 2 h, the cells were stained with crystal violet solution for 30 min. Then the plates were rinsed with tap water and the images of colony-filled wells were taken manually.

ANNEXIN V/7-AAD Double Staining and Flow Cytometry

5×10^5 cells/well were seeded in 6-well plates. After incubation overnight, the cells were treated with or without HLJDD (225 and 450 μg/mL for MHCC97L, 50 and 100 μg/mL for PLC/PRF/5) for 24 h. Cells were collected and suspended in 100 μl binding buffer

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containing 5 µl PE-conjugated Annexin V and 5 µl 7-AAD. After incubation in the dark for 15 min at room temperature, 400 µl binding buffer was added. The apoptosis of cell samples was then analyzed on Canto II flow cytometer (BD Bioscience, USA) within 1 h.

Flow Cytometry for Cell Cycle Analysis

5×10^5 cells/well were seeded in 6-well plates. After incubation overnight, the medium was changed to serum-free DMEM for 6 h and then the cells were treated with or without HLJDD (225 and 450 µg/mL for MHCC97L, 50 and 100 µg/mL for PLC/PRF/5) for 24 h. Cells were collected and fixed with 70% ethanol at 4°C overnight. Then the cells were incubated with propidium iodide (PI, 50 µg/mL, Sigma-Aldrich, USA) in the dark for 45 min. The cell samples were tested on Canto II flow cytometer (BD Bioscience, USA) for cell cycle analysis.

Wound-Healing and Transwell Invasion Assay

For wound-healing assay, cells were seeded in 6-well plates and incubated until 100% confluence. A plastic pipette tip was used to scrap a denuded area on the cell monolayer. After wash with PBS for 3 times, the cells were treated with or without HLJDD (225 and 450 µg/mL for MHCC97L, 50 and 100 µg/mL for PLC/PRF/5). After 0, 24, and 48 h incubation, a light microscope was used to capture the cell movements into the wound area. For transwell invasion assay, 100 µl cold Matrigel (BD Biosciences, USA) was used to pre-treat the Millicell Cell Culture Inserts in a 24-well plate for 2 h at 37°C. Then 1×10^5 cells/well in 200 µl of serum-free DMEM were seeded to the chamber and incubated with or without HLJDD (225 and 450 µg/mL for MHCC97L, 50 and 100 µg/mL for PLC/PRF/5) for 36 h at 37°C. After fixation with 4% paraformaldehyde for 2 h, the invaded cells were stained with crystal violet solution for 30 min and then counted with a light microscope.

Orthotopic HCC Implantation Mouse Model

All animals received human care throughout the experiments, and the animal experimental procedures have been reviewed and approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), The University of Hong Kong (CULATR No.: 3776-15). In brief, the left waist of 5-week-old male BALB/c nu/nu athymic nude mice was subcutaneously injected with 5×10^6 luciferase-tagged MHCC97L cells to generate a subcutaneous tumor. When the subcutaneous tumor reached 1 cm in diameter, it was dissected and cut into small cubes (approximately 1 mm^3). The small tumor cube was orthotopically implanted into the left lobe of 5-week-old male BALB/c nu/nu athymic nude mice to establish orthotopic HCC implantation mouse model. One week after implantation, the growth of liver tumor was checked under *in vivo* live imaging system (IVIS Spectrum, Perkin-Elmer, USA) by injecting luciferin (i.p., 150 mg/kg) into the mice. All tumor-presenting mice were then randomly divided into model and HLJDD treatment group ($n = 5$) receiving gavage of PBS and HLJDD (100 mg powder/kg/2 day) respectively for

4 weeks. Liver tumor growth was monitored weekly. At the end point of treatment, the nude mice were humanely sacrificed to collect tissues.

HE Staining

Tissues from the orthotopic HCC implantation mice were collected and fixed in 4% formalin buffer. Then the paraffin-embedded blocks were prepared and sections at 4 μm thickness were cut and stained with haematoxylin and eosin for histological examination.

RT-PCR

5×10^5 HCC cells/well were seeded in 6-well plates. After incubation overnight, the cells were treated with or without HLJDD of 225 and 450 μg/mL for MHCC97L, 50 and 100 μg/mL for PLC/PRF/5. The cells were harvested using a micro-scraper (Corning) after 24 h treatment. Total RNA was isolated using RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. Firstly, the strand cDNA was prepared from the total RNA (1 μg) with RT2 First Strand Kit (Qiagen, Germany). Then the cDNA was amplified in SYBR Green I reagent (Takara, Japan) with specific primers. All the assays were conducted on LC480 platform (Roche, USA). The sequences of the primers for particular genes were as follows: TCAGTTCTTAGGCTCAGCGTC (forward) and AGTTATC CTGGCCTCCGTTT (reverse) for H-AHR, CTCAAGCAACACCGACGTAAA (forward) and CCTTGTGGCATTTGGCATCG (reverse) for H-ALOX5, CCTGGCTTCCG CAACTTACAC (forward) and GGACTTGTGCATGCGGTACTCA (reverse) for H-AR, TCGCCCTGTGGATGACTGA (forward) and CAGAGACAGCCAGGAGAAATCA (reverse) for H-BCL2, GGAGCTTCGTGTCCTGTATGGC (forward) and CAGTGAT-GATGACAATCTCATACCG (reverse) for H-ICAM1, AGAGCATCCAAAAGAGT GTGGAG (forward) and TGGCGACAGTTCAGCCATCACT (reverse) for H-IFNG, TCCTTCGTCACTGGCGTCA (forward) and ATGCAGTCGGCTTGGTTCTT (reverse) for H-MPO, TCCGAGGCAAACAGCACATTC (forward) and GGGTTGGGGG TGTGGTGATGT (reverse) for H-NOS2, ATGAGACCAGATGTAAGCTC (forward) and AATGCCATAAGAAACATCCA (reverse) for H-NR3C1, TGGTGCTACGTCTT-TAAGGCGG (forward) and GCTGACCCATTCCCAAAGTAGC (reverse) for H-PLAT, CACATCTACAATGCCTACCT (forward) and CTTCTCTGCCTGCCACAATGTCT (reverse) for H-PPARD, CATTCTGGCCCACCAACTTTGG (forward) and TGGA-GATGCAGGCTCCACTTTG (reverse) for H-PPARG, CCAGAGGCGTACAGGGATAG (forward) and CCAACCGCGAGAAGATGA (reverse) for H-ACTIN.

Statistical Analysis

Statistical analysis was performed with Prism 6 software. Data were expressed as the mean ± SD and analyzed using Student's *t*-test. Differences between groups were considered to be statistically significant if values of $P < 0.05$.

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Results

Identification of Potential Bioactive Compounds in Huanglian Jiedu Decoction

A total of 429 compounds were retrieved from HLJDD (Supplementary Table 1), 48 of which belong to CR, 143 to SR, 140 to PCC, and 98 to GF. 370 compounds were obtained from TCMSP after eliminating the overlapping compounds. Among these 370 compounds, 193 (approximately 52.2%) met the OB threshold ($\geq 30\%$). Furthermore, 84 compounds (approximately 22.7%) among these 193 constituents satisfied the DL index criterion (≥ 0.18), and 80 compounds (21.6%; with both high OB and DL indexes) met the Caco-2 permeability threshold (≥ -0.40). The screening process was illustrated in Fig. 1. The herb-compound network of potential bioactive constituents was conducted (Fig. 2), and these 80 potential bioactive compounds were further analyzed.

Target Identification of Huanglian Jiedu Decoction

In total, 64 out of 80 compounds from HLJDD were associated with 1208 target proteins. After eliminating the overlapping proteins, 247 associated proteins were obtained. The detailed information of the obtained target proteins is described in Supplementary Table 2. A compound-protein network was constructed on the basis of the 64 bioactive compounds and their targets. As shown in Figure 3, the network is composed of 311 nodes (64 bioactive compounds and 247 targets). Notably, this network includes some compounds with multiple targets, particularly the high-degree compounds MOL034 (quercetin, degree $= 150$), MOL320 (kaempferol, degree $= 63$), MOL 052 (wogonin, degree $= 45$), MOL095 (beta-sitosterol, degree $= 38$), MOL058 (baicalein, degree $= 36$), MOL229 (isocorypalmine, degree $= 36$), and MOL249 [(S)-canadine, degree $= 32$]. The predicted compounds were observed to be pharmacologically bioactive (Table 1). TCM is a multi-component complex system and one component might act on multiple targets and act synergistically to treat diseases. For example, quercetin may have a wide range of biological actions including anticancer, anti-cardiovascular, anti-inflammatory, and so on. In addition, there are 19 inflammation-related proteins in all 247 proteins including NOS2, AR, RXRA, HSP90AA1, PPARG, AKR1B1, BCL2, ICAM1, PLAT, THBD, IFNG, ALOX5, MPO, AHR, PPARD, CHUK, NR3C2, NR3C1, and IKBKB (Zhang *et al.*, 2015). 47 out of 80 compounds from HLJDD have effects on the above mentioned anti-inflammatory proteins (Supplementary Table 3).

Determination of Potential Pathways

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the functional annotation tool of DAVID Bioinformatics Resources 6.7 (Huang da *et al.*, 2009). In total, 68 pathways were observed to be significantly associated with the input set of genes (Supplementary Table 4). The top 20 pathways obtained from pathway enrichment analysis were shown in Fig. 4A. Subsequently, these pathways were

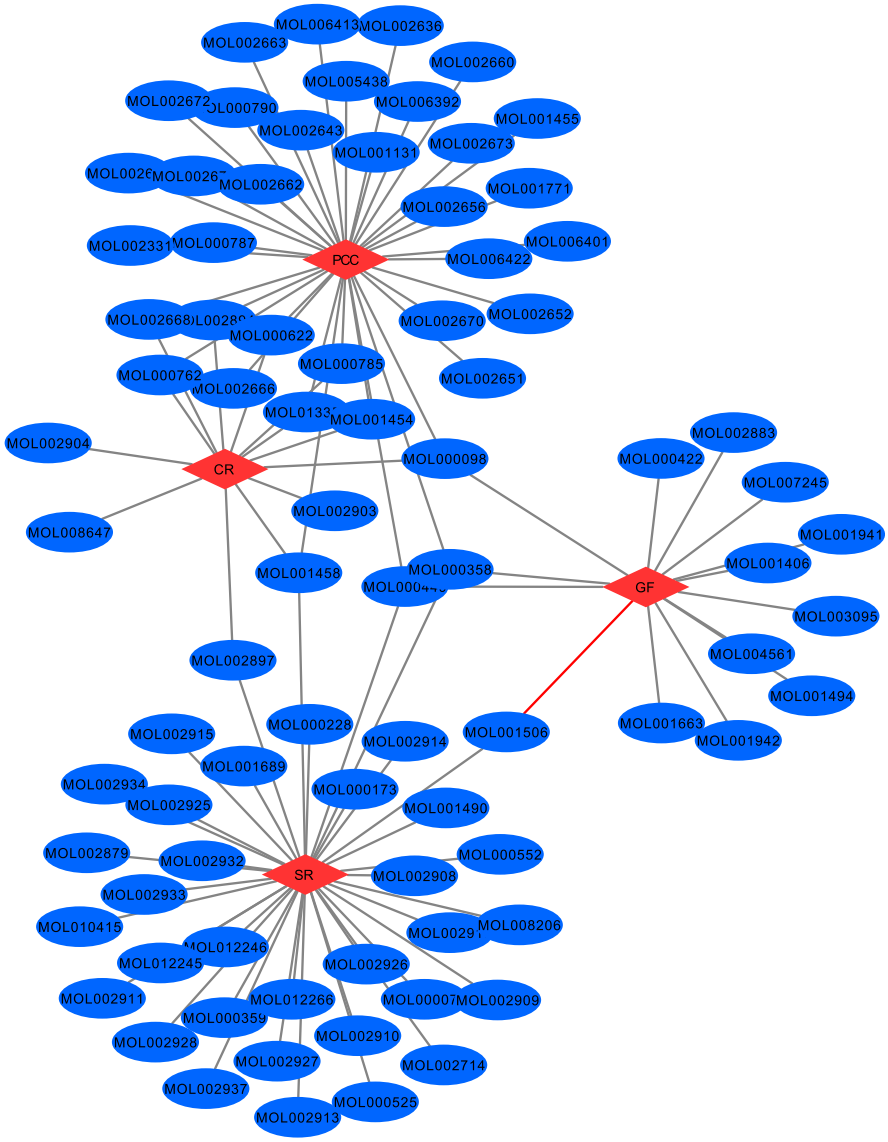

Figure 2. Herb-Compound network of potential bioactive constituents for HLJDD. The red nodes represent herbs, and the blue nodes represent of potential bioactive compound.

sub-divided into 20 categories according to their functions (Fig. 4B). The KEGG pathway enrichment of the 64 compounds revealed the close association of the 68 pathways with cancers (14, 21.875%), the immune system (13, 20.3125%), signal transduction (7, 10.9375%), the endocrine system (5, 7.8125%), cell growth and death (4, 6.25%), as well as immune related diseases (3, 4.6875%). To further elucidate the protein-pathway mechanisms, a protein-pathway network based on all proteins and their corresponding

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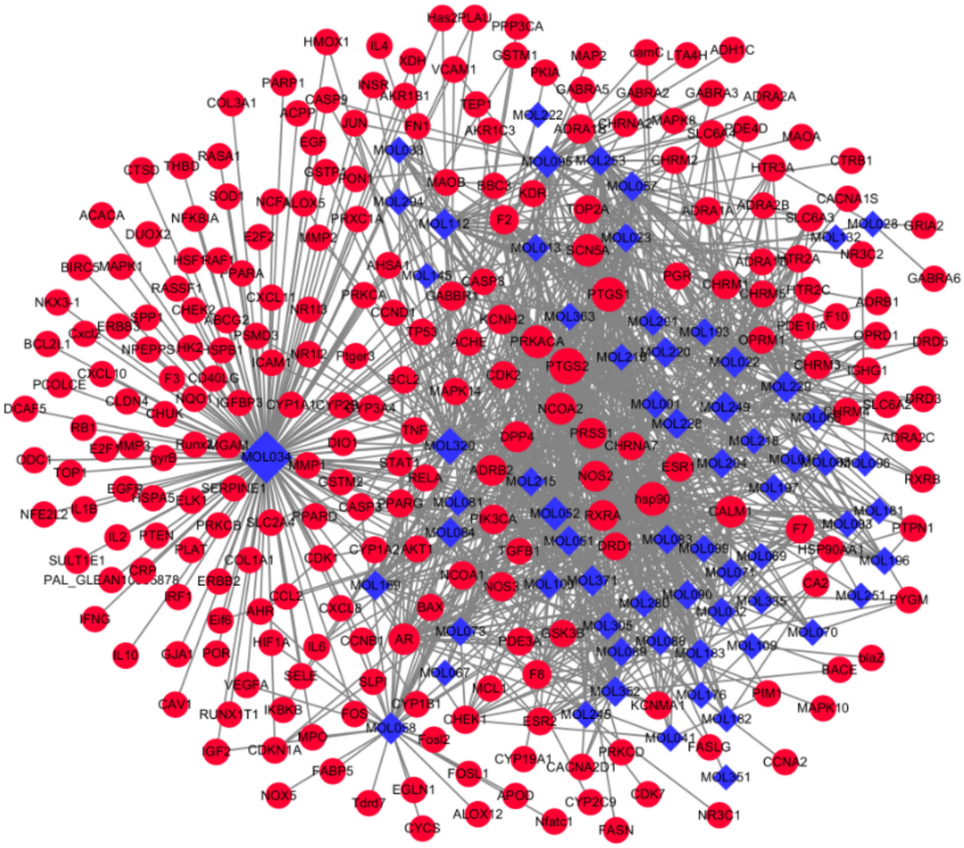


Figure 3. Compound–Protein network for HLJDD. The red nodes represent potential protein and the blue nodes represent compounds. The edges represent the interaction between them and nodes size are proportional to their degree.

Table 1. Bioactivity of Potential Active Compounds

Number	Compounds Name	Bioactivity
MOL 001	Berberine	Hepatocellular carcinoma (Wang *et al.*, 2010)
MOL 011	Obacunone	Prostate cancer (Murthy *et al.*, 2015)
MOL 013	Berberrubine	Antidiabetics
MOL 016	Epiberberine	Anticholesterol metabolism
MOL 023	Berlambine	Anti-lung cancer (Hou *et al.*, 2014)
MOL 032	Palmatine	Prostate cancer (Hambright *et al.*, 2015)
MOL 034	Quercetin	Hepatocellular carcinoma (Maurya and Vinayak, 2015)
MOL 038	Coptisine	Breast cancer (Zhang *et al.*, 2014)
MOL 051	Acacetin	Non-small cell lung cancer (Chien *et al.*, 2011)
MOL 052	Wogonin	Malignant cells (Fas *et al.*, 2006)
MOL 062	Baicalein	Hepatocellular carcinoma (Han *et al.*, 2015)

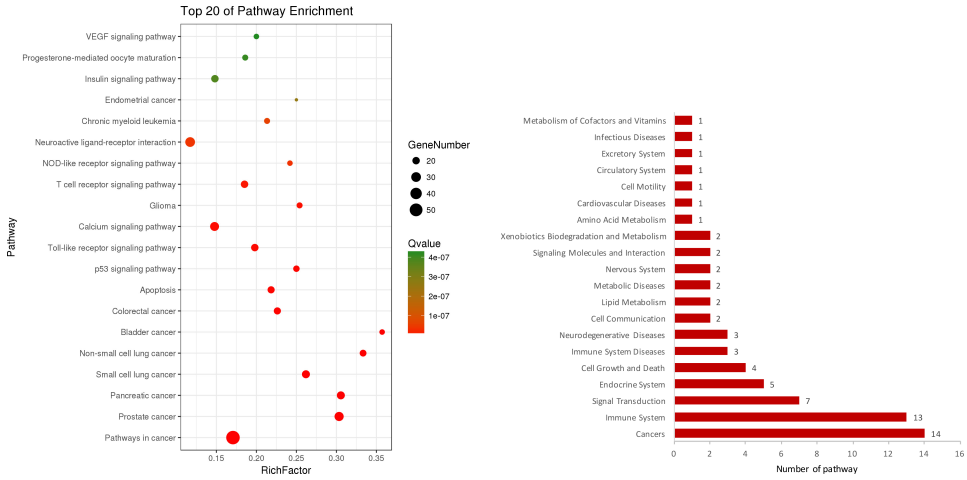
Table 1. (*Continued*)

Number	Compounds Name	Bioactivity
MOL 083	Skullcapflavone II	Anti-allergic asthma
MOL 084	Oroxylin a	Breast cancer (Wei *et al.*, 2015)
MOL 090	Neobaicalein	Prostate carcinoma (Bonham *et al.*, 2005)
MOL 093	Dihydrooroxylin	Antifungal
MOL 095	Beta-sitosterol	Mast cell-mediated allergic diseases
MOL 099	Norwogonin	Anti-EV71 infections
MOL 112	Stigmasterol	Anti-inflammatory
MOL 197	Phellopterin	Neuroprotective
MOL 215	Rutaecarpine	Immunosuppressive
MOL 218	Chelerythrine	Malignant tumors (Cao *et al.*, 2016)
MOL 291	Crocetin	Cancer (Gutheil *et al.*, 2012)
MOL 294	Ammidin	Leukodermia
MOL 320	Kaempferol	Breast cancer (Kim *et al.*, 2016)
MOL 371	Isoimperatorin	Mycobacterium tuberculosis

significant signaling pathways was constructed (Fig. 5). As shown in Fig. 5, this network is composed of 241 nodes (68 pathways and 173 proteins) and 941 interactions.

Identification of Gene-Associated Diseases

In deeper analysis, diseases related to the pathways identified in gene enrichment analysis were analyzed using DAVID Bioinformatics Resources 6.7 (Sherman *et al.*, 2007). GENETIC_ASSOCIATION_DB_DISEASE_CLASS was selected as the functional annotation clustering tool for searching of significant diseases associated with the input genes, which were statistically verified using the Fisher exact test and the DAVID platform (Huang da *et al.*, 2009). The modified Fisher exact p value ranged from 0 to 1. $p \leq 0.05$ was considered to be significant enrichment in the annotation categories. A total of 16 classes of diseases were observed to be strongly associated with the input genes (Supplementary Table 5). A gene-disease network was constructed on the basis of all proteins and corresponding diseases (Fig. 6). The network is composed of 195 nodes (16 diseases and 179 genes) and 941 interactions. Most diseases obtained from this gene-disease network were in concordance with the results of the series of pharmacological function studies on HLJDD (Table 1). The five main diseases were cancer (degree = 102), followed by metabolic (degree = 98), cardiovascular (degree = 92), immune (degree = 86), and psychological (degree = 79) diseases (Fig. 5). Thus we identified the top one predicted disease of HLJDD treatment as cancer. Especially, most of the high-degree compounds including quercetin, baicalein, and berberine in the compound-protein network exerted therapeutic effects on HCC (Table 1). Therefore, in this study, we exampled HCC as the typical and primary disease which HLJDD may act on. In addition, there are 30 common targets involved in the 5 major diseases: cancer, metabolic, cardiovascular, immune, and psychological diseases. These 30 common targets genes included PTGS2, CRP, PPARG,



(A) (B)

Figure 4. The top 20 pathways of pathway enrichment analysis (A) and the classification of potential pathways by functions (B).

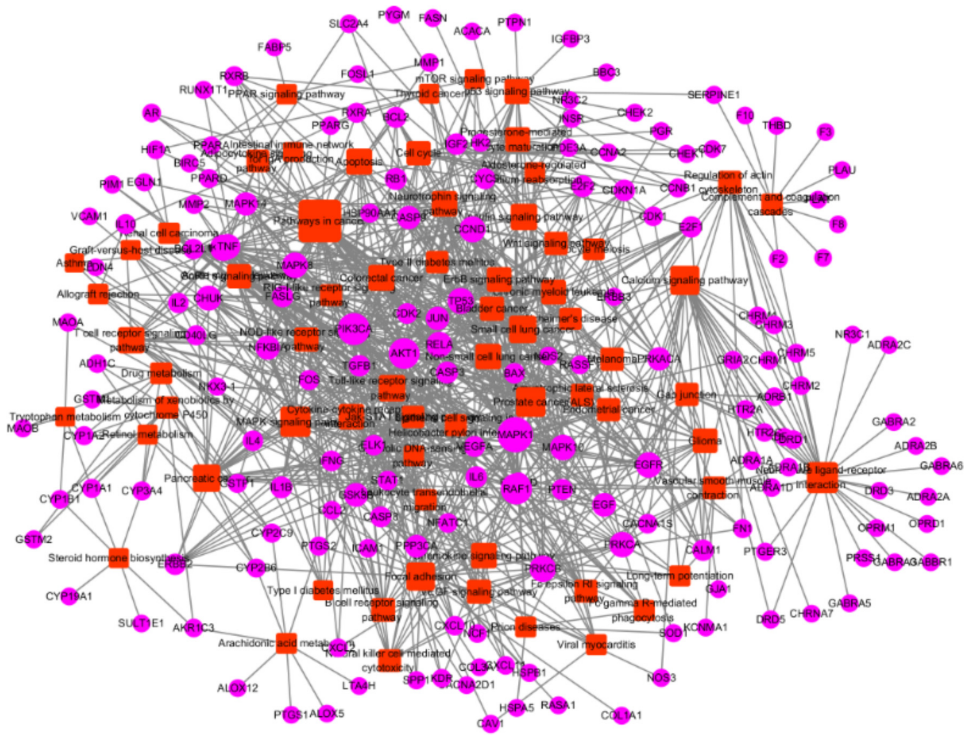


Figure 5. Protein–Pathway network for HLJDD. The red nodes represent pathway and the pink nodes represent protein. The edges represent the interaction between them and nodes size are proportional to their degree.

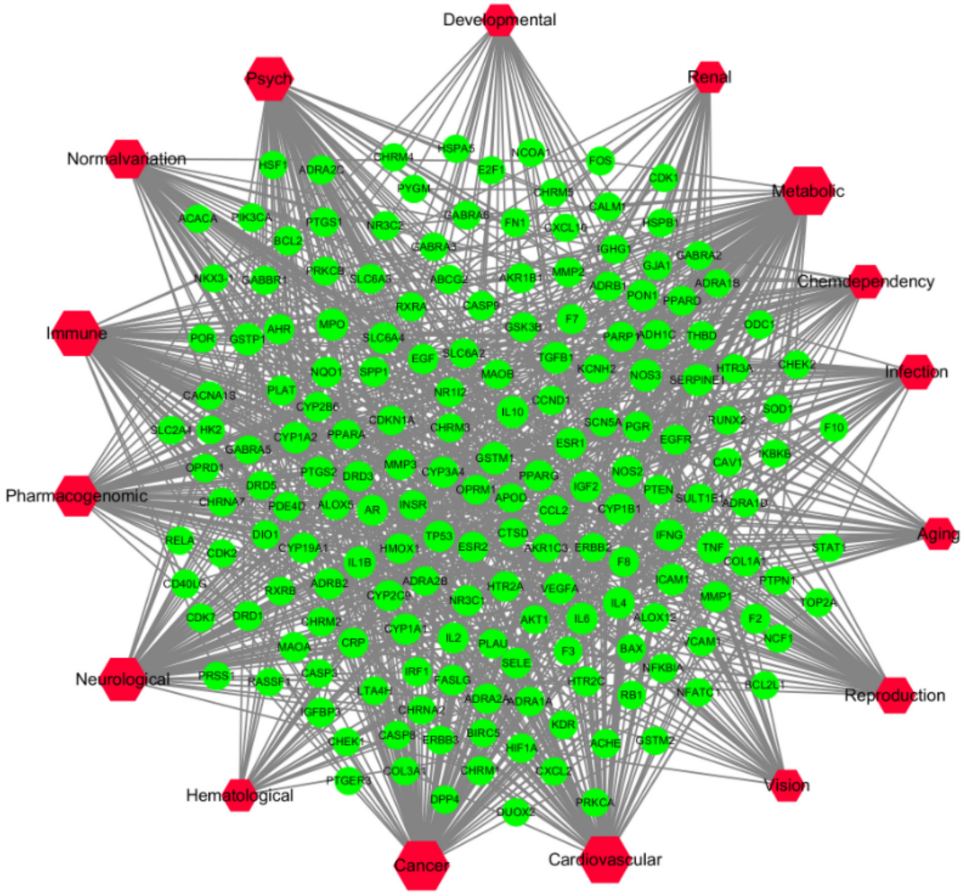


Figure 6. Gene–Disease network for HLJDD. The red nodes represent diseases and the green nodes represent genes. The edges represent the interaction between them and nodes size are proportional to their degree.

SLC6A4, IL10, GSTM1, SERPINE1, IL1B, NOS3, NOS2, ICAM1, AR, ESR1, TP53, CYP1A2, ESR2, ADRB2, VEGFA, GSTP1, CCL2, TNF, NR3C1, EGF, CYP19A1, IL4, IL6, CYP2C9, IGF2, PON1, and MPO. Further analysis showed there are 12 inflammation-related genes in 102 cancer genes including IFNG, AHR, PLAT, PPARD, PPARG, NOS2, ICAM1, AR, NR3C1, BCL2, MPO, and ALOX5.

HLJDD Suppressed in vitro HCC Cell Growth

Based on the analysis of network pharmacology, we exampled HCC as the typical and primary disease HLJDD may act on. To verify the efficacy of HLJDD on HCC, the cell viability assay of different HCC cells was firstly performed. As shown in Fig. 7A, HLJDD exhibited a time- and dose-dependent effects on HCC cell viability when the cells were

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exposed to increasing concentrations (7.8125–1000 μg/ml) of HLJDD at different time points (24 h, 48 h and 72 h). The IC50 value for 48 h treatment of HLJDD was about 450 μg/ml for MHCC97L cells and 100 μg/ml for PLC/PRF/5 cells. The results were consistent with the previous study (Wang *et al.*, 2015). Besides, HLJDD treatment for 10 days also showed a dose-reduced colony formation in MHCC97L and PLC/PRF/5 cells (Fig. 7B). ANNEXIN V/7-AAD double staining was conducted to assess cell apoptosis in MHCC97L and PLC/PRF/5 cells treated with HLJDD. As shown in Fig. 7C, HLJDD treatment could obviously induce early and late apoptosis in a dose-dependent manner. Cell cycle analysis were also conducted to assess the effects of HLJDD treatment to cell cycle progression of MHCC97L and PLC/PRF/5 cells. PI staining of cellular DNA suggested a delay of the G1/S transition in MHCC97L and PLC/PRF/5 cells after HLJDD treatment (Fig. 7D). Remarkably, HLJDD treatment further exhibited a dose-reduced migratory and invasive activities in MHCC97L and PLC/PRF/5 cells, as shown in Figures 7E and 7F. Taken together, these results suggest that HLJDD could suppress *in vitro* HCC cell growth in various aspects.

HLJDD Inhibited Orthotopically Implanted HCC Tumor Growth in vivo

To further demonstrate the antitumor effects of HLJDD on HCC, an orthotopic HCC implantation mouse model was established using nude mice. Firstly, MHCC97L cells were transfected with luciferase reporter gene and subcutaneously injected into the left waist of nude mice to generate a small cube of solid tumor. After that, the tumor cubes were orthotopically implanted into the left lobe of other nude mice to establish orthotopic HCC implantation mouse model. The oral treatment of HLJDD throughout the experiments suppressed the orthotopic growth of implanted HCC, as indicated by luciferase reporter-dependent live animal imaging (Fig. 8A). The reduced signal intensity indicated smaller tumor size in HLJDD-treated mice. As shown in Fig. 8B, the end-point tumor size in HLJDD group was remarkably smaller than that in the model group, which further verified the inhibitory effect of HLJDD on HCC. Of note, there was no significant difference in body weight between these two groups, indicating no observational toxicity of HLJDD treatment (Fig. 8C). There was an irregular and invasive edge at the boundary between the tumor and normal liver tissue in model group, which indicated that the orthotopically implanted HCC cells remarkably invaded into the normal liver tissue (Fig. 8D). As opposed to model group, there was a clear and well-defined edge at the boundary of hepatic tumor in HLJDD group of mice. Additionally, oral treatment of HLJDD significantly decreased the mitotic event index of tumor, as shown in Fig. 8E. All these observations confirmed the remarkable inhibitory activities of HLJDD on the *in vivo* growth of HCC.

Anti-Inflammatory Mechanism May Play an Important Role in the Inhibitory Effects of HLJDD on HCC

Based on the network pharmacology analysis, 12 inflammation-related genes including IFNG, AHR, PLAT, PPARD, PPARG, NOS2, ICAM1, AR, NR3C1, BCL2, MPO, and

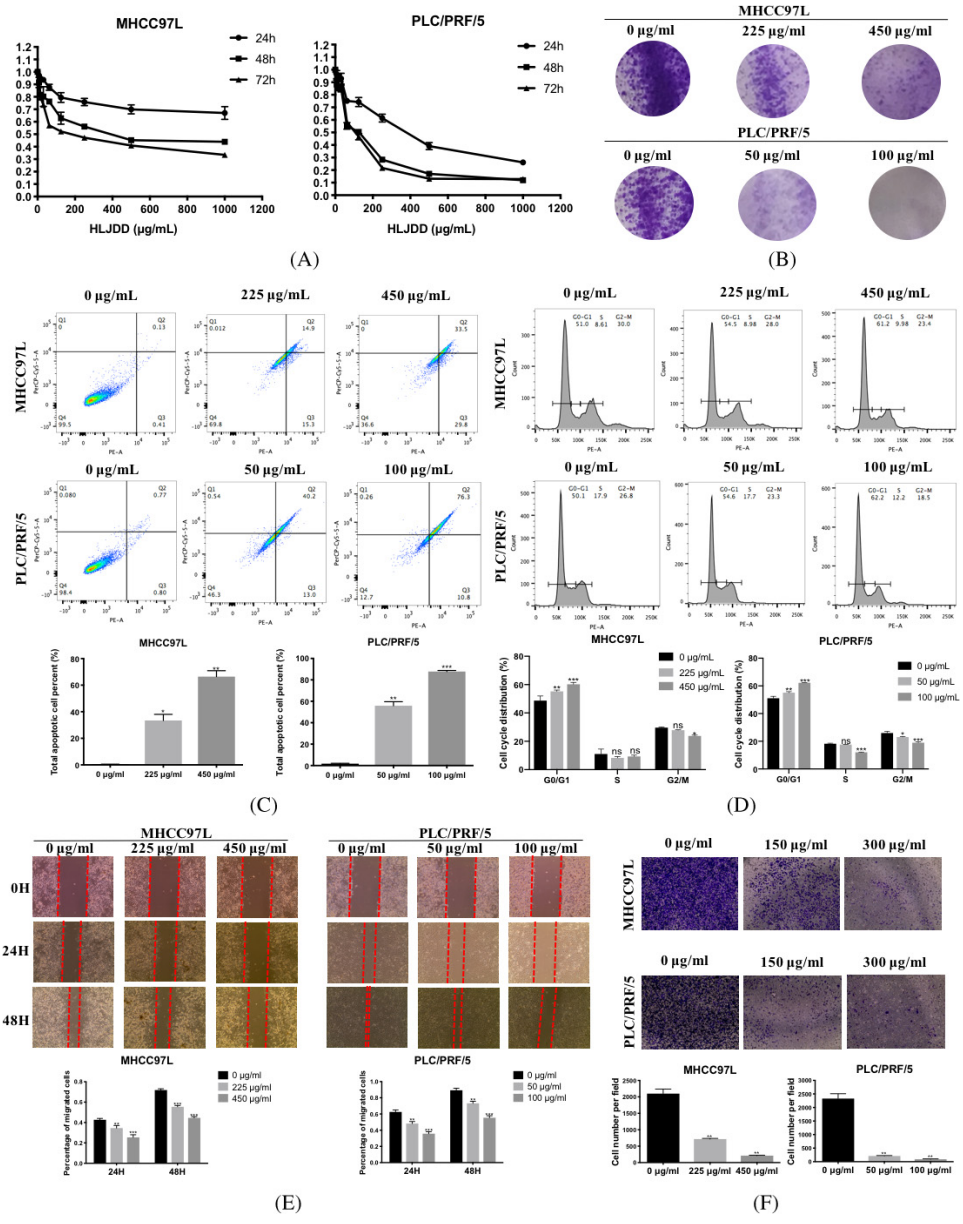

Figure 7. HLJDD suppressed *in vitro* HCC cell growth. (A) Time- and dose-dependent effects of HLJDD treatment on the viability of MHCC97L and PLC/PRF/5 cells. (B) HLJDD treatment showed a dose-reduced colony formation in MHCC97L and PLC/PRF/5 cells. (C) HLJDD treatment induced early and late apoptosis of MHCC97L and PLC/PRF/5 cells in a dose-dependent manner. (D) HLJDD treatment delayed the G1/S transition of MHCC97L and PLC/PRF/5 cells in a dose-dependent manner. (E) Time- and dose-dependent effects of HLJDD treatment on the migratory activities of MHCC97L and PLC/PRF/5 cells. (F) HLJDD treatment inhibited the invasive activities of MHCC97L and PLC/PRF/5 cells in a dose-dependent manner. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the non-treated group.

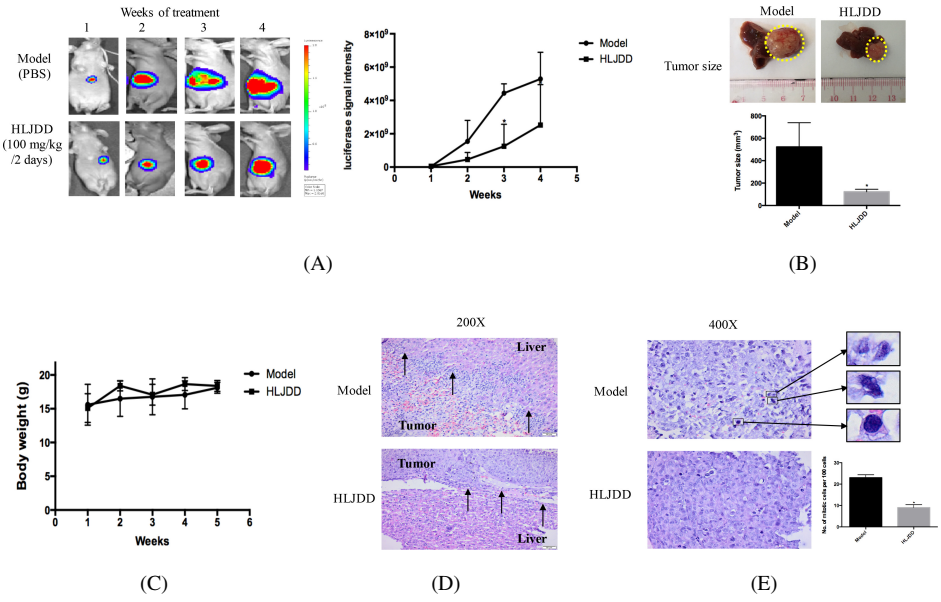

Figure 8. HLJDD showed an antitumor effect in orthotopic HCC implantation mouse model *in vivo*. (A) The representative images and statistical graph of luciferase reporter-dependent signal of animals throughout the oral treatment. (B) The representative images and statistical graph of tumor size at the end of experiment. (C) The body weight of animals throughout the oral treatment. (D) The representative images of HE staining of the boundary of tumor and normal liver. (E) The representative images of HE staining of the tumor and statistical graph of mitotic index. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the non-treated group.

ALOX5 were identified as the primary drug candidate targets for the treatment of HLJDD on cancer. These targets were then validated by qPCR. As shown in Fig. 9, the relative mRNA expressions of NR3C1, MPO, ICAM1, PPARD, AHR and BCL2 were significantly increased while the relative mRNA expressions of IFNG, PLAT, PPARG, NOS2, AR, and ALOX5 were significantly decreased in MHCC97L cells (225 and 450 μg/mL) and PLC/PRF/5 cells (50 and 100 μg/mL) after treatment with HLJDD. These results revealed that anti-inflammatory mechanism may play an important role in the inhibitory effects of HLJDD on HCC.

Discussion

In our study, a total of 80 out of 429 compounds were identified using our drug prediction method. Notably, many compounds have been reported to exhibit significant pharmacological bioactivity (Table 1). Based on the potential targets that 80 compounds act on, 68 associated pathways and 16 classes of diseases that are associated with the targets were obtained. The KEGG pathway enrichment analysis revealed the close association of the 68 pathways with cancers, the immune system, signal transduction, the endocrine system, cell growth and death, as well as immune related diseases. The five main diseases HLJDD may

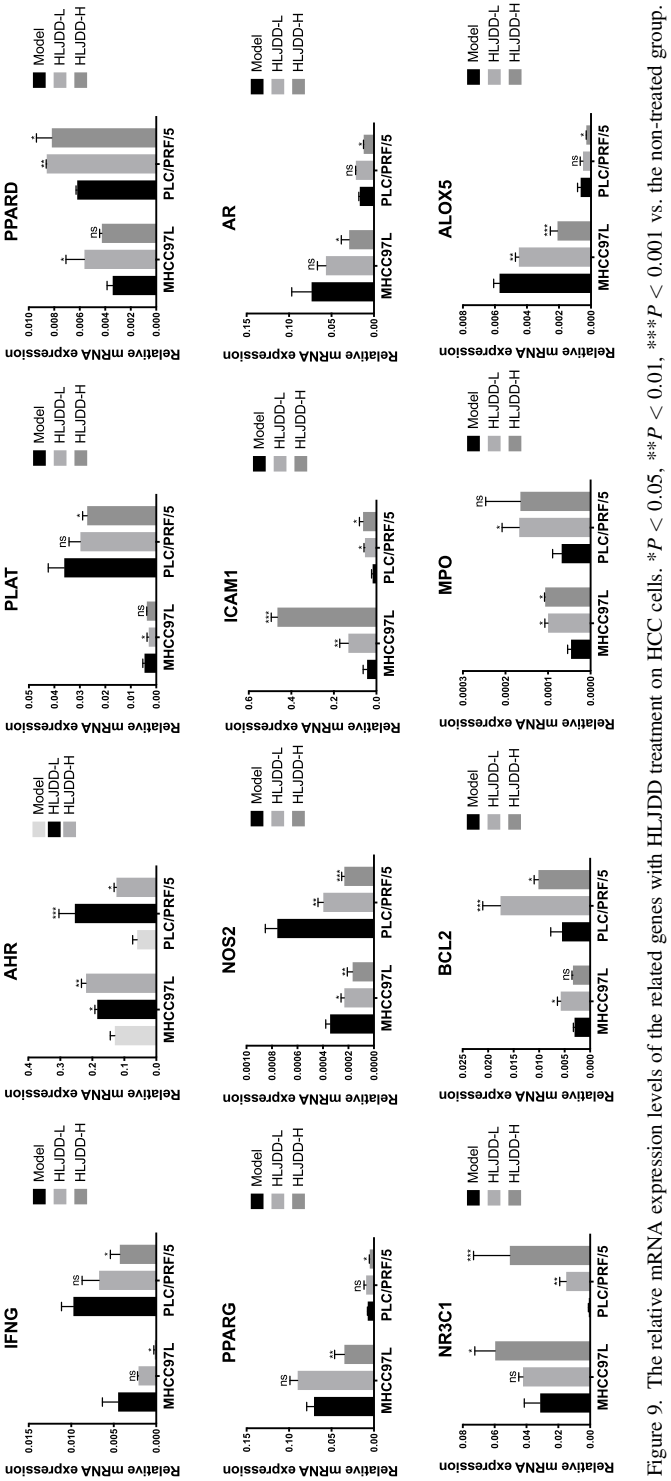

Figure 9. The relative mRNA expression levels of the related genes with HLJDD treatment on HCC cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the non-treated group.

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act on based on the gene-disease network analysis were cancer, followed by metabolic, cardiovascular, immune, and psychological diseases. As a result, cancer was predicted as the primary disease HLJDD may act on. Thus we identified the top one predicted disease of HLJDD treatment as cancer. The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors (Hanahan and Weinberg, 2000, 2011). So TCM which has multiple target therapy mechanism will be new means to treat human cancer. HLJDD has been mainly shown to possess anticancer properties through inducing cell-cycle arrest and apoptosis (Hsu *et al.*, 2008) and activating AMPK signaling pathway (Wang *et al.*, 2015). Our previous study showed that HLJDD, CR aqueous extract and berberine showed potent antiliver cancer effects (Zhu *et al.*, 2011; Wang *et al.*, 2014). Other compounds which we predicted also show a wide range of anticancer effects (Table 1).

Of note, we exampled HCC as the typical and primary disease HLJDD may act on as most of the high-degree compounds listed in Table 1 including quercetin, baicalein, and berberine exerted therapeutic effects on HCC. Both HCC cell and orthotopic HCC implantation mouse models were established to verify the curative role of HLJDD on HCC as predicted by the network pharmacology analysis. In detail, HLJDD exhibited a time- and dose-dependent effect on MHCC97L and PLC/PRF/5 cell viability when the cells were exposed to increasing concentrations of HLJDD at different time points. Besides, HLJDD treatment for ten days also showed a dose-reduced colony formation in MHCC97L and PLC/PRF/5 cells. HLJDD treatment could obviously induce early and late apoptosis and delay the G1/S transition in a dose-dependent manner. Remarkably, HLJDD treatment further exhibited a dose-reduced migratory and invasive activities in MHCC97L and PLC/PRF/5 cells. The oral treatment of HLJDD throughout the experiments could suppress the orthotopic growth of implanted HCC and decrease the mitotic event index of tumors. All these observations confirmed the remarkable antitumor activities of HLJDD on the *in vitro* and *in vivo* growth of HCC.

Cancer is associated with immunity and inflammatory microenvironment (Coussens and Werb, 2002). Indeed, inflammation is a complex pathophysiological process, which is known to promote and exacerbate malignancy (Mantovani *et al.*, 2008). The common pathways between cancer and inflammatory included Apoptosis, MAPK signaling pathway, NOD-like receptor signaling pathway, VEGF signaling pathway, Fc epsilon RI signaling pathway, and toll-like receptor (TLR) signaling pathway. It has been reported that HCC is a typical example of inflammation-related cancer because more than 90% of HCCs arise from the background of hepatic injury and inflammation (Nakagawa and Maeda, 2012). Targeting inflammation has great potential in both chemoprevention and therapy of HCC (Stauffer *et al.*, 2012). In our study, 47 out of 80 compounds from HLJDD may act on the anti-inflammatory proteins (Supplementary Table 3). For example, quercetin is a flavonoid derived from HLJDD component herbs including CR, PCC, and GF. Quercetin nanoparticles showed antitumor activity against HCC via COX-2 mediated anti-inflammation (Ren *et al.*, 2017). Berberine, as a bioactive alkaloid, is derived from HLJDD component herbs including CR and PCC. Berberine might suppress inflammation and angiogenesis via p38MAPK/ERK-COX2 pathway to prevent non-alcoholic steatohepatitis-

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derived HCC (Luo *et al.*, 2019). Of note, based on the network pharmacology analysis, 12 inflammation-related proteins targeted by these 47 active compounds including IFNG, AHR, PLAT, PPARD, PPARG, NOS2, ICAM1, AR, NR3C1, BCL2, MPO and ALOX5 were identified as the potential anti-inflammatory proteins for the treatment of HLJDD on HCC, which were then validated by qPCR. It was found the relative mRNA expression levels of NR3C1, MPO, ICAM1, PPARD, AHR, and BCL2 were significantly increased while the relative mRNA expression levels of IFNG, PLAT, PPARG, NOS2, AR, and ALOX5 were significantly decreased when HCC cells were pre-treated with HLJDD. HLJDD is a TCM preparation with anti-inflammatory properties. Anti-inflammatory therapy is important for the treatment of HLJDD on HCC. With conceptual progress in the last decade, the inflammation can be gradually considered as the core hallmark of Cancer (Hanahan and Weinberg, 2011). Our previous study found HLJDD can directly target HCC cells to exert its therapeutic effect on HCC. In detail, HLJDD was found to reduce the activity of translation elongation regulator eEF2 in HCC cells by inducing the Th56 phosphorylated-inactivation of eEF2 and also inhibit the nascent protein synthesis for HCC inhibition. However, our current study preliminarily delineated that anti-inflammatory mechanism may also play an important role in the inhibitory effects of HLJDD on HCC. Traditionally, TCM is featured to treat cancer in a relative and holistic point of view. Except directly targeting on cancer cells, HLJDD may also regulate the tumor immune microenvironment to exert its antitumor effects. Further detailed studies on how inflammation regulates the curative effects of HLJDD on HCC will be performed in our next study.

TCM includes numerous bioactive compounds, and one herbal compound is associated with many targets, and multiple herbal compounds can treat one disease. Therefore, it is difficult to determine which modern diseases TCM may work, and how it works by using a one-target and one-drug model. Overall, network pharmacology can help to predict the primary diseases, target profiles and pharmacological actions of herbal compounds (Li and Zhang, 2013). Although network pharmacology has become a hot spot in TCM study, there are still some essential technical issues to be addressed. First, the most common challenge is the data collection on TCM. The current databases for TCM remain limited and incomplete. Second, how to build a more dynamic network modelling between compounds, corresponding targets, genes and pathways is still a great challenge for current network pharmacology research. Last but not least, most current network pharmacology study are based on computational data mining and experimental verification are strictly needed. To address these issues, more informative database and new methods in data mining as well as combined network pharmacology with other multi-omics technologies will be developed.

Conclusion

In the current study, an integrated strategy by combining network pharmacology with experimental evaluation for the discovery and prediction of HLJDD therapeutic potentials was developed. Network pharmacology analysis showed that HLJDD primarily targeted on cancer, especially HCC and inflammation-related genes played an important role in the

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treatment of HLJDD on cancer. The *in vitro* HCC cell and *in vivo* orthotopic HCC implantation mouse models demonstrated that HLJDD could remarkably suppress *in vitro* and *in vivo* HCC cell growth in various aspects. In summary, the integrated strategy developed in our study provided novel insights into the therapeutic potential and mechanism study of complex TCM such as HLJDD.

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