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Qilian Huaji decoction exerts an anti-cancer effect on hepatocellular carcinoma by upregulating miR-122

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Abstract

Qilian Huaji Decoction (QLHJD) is a mixture of 13 herbs. The aim of this study is to observe the efficacy of QLHJD against hepatocellular carcinoma (HCC). The H22 transplanted mouse models were established to explore the effect of QLHJD on HCC in vivo. QLHJD-containing serum (QCS) was prepared using traditional Chinese medicine serum pharmacology methods. After QCS treatment, cell proliferation, cell cycle and apoptosis in HepG2 cells were assessed by MTT and flow cytometry, respectively. miRNA expression profiles were determined using qPCR arrays. miR-122 was examined by qPCR in HCC tissue samples and cells. AKT3 protein expression was detected by Western blot. We found that QLHJD significantly inhibited the growth of H22 tumor and improve immune function in vivo. In vitro, QLHJD inhibited proliferation by inducing cell cycle arrest at the S phase, and induced apoptosis in HepG2 cells. The miRNA expression profiles were altered and the reduced miR-122 levels in HepG2 cell lines were up-regulated compared with control group, accompanied by a decrease in WNT1 expression. In conclusion, QLHJD could inhibit the growth of HCC by restoring the aberrant expression of miR-122, which may be one of the mechanisms whereby QLHJD exerts its effect on HCC.

Keywords: Qilian Huaji Decoction; anti-cancer; Hepatocellular carcinoma; miR-122.

Practical Application: Qilian Huaji decoction may have anti-cancer effect on hepatocellular carcinoma.

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most universal cancers around the world. It ranks the sixth in cancer incidence and the second cancer associated mortality worldwide. In recent years, the annual death toll with 700,000 has been recorded around the global (Torre et al., 2015) and more than half of the new cases and deaths occurring in China. The low early diagnosis rate and rapid progression are the major reasons for the low survival rate of HCC (Fu & Wang, 2018). At present, multiple treatment modalities including transplant, surgical resection, and locoregional therapies, including ablative therapies and transarterial embolotherapies are widely used to relieve HCC (Lee & Khan,2017). However, not all patients are suitable for these therapy. Common utilization of new generation drugs for systemic chemotherapy of primary HCC including sorafenib (Sacco et al., 2017), oxaliplatin (Liu at al., 2016) and so on. Although they have certain curative effects, long-term drug resistance and toxic side effects are obvious. Therefore, safer and more effective treatments should be developed to improve the survival of HCC patients.

Traditional Chinese medicine (TCM), specifically Chinese herbal medicine (CHM), an important component of alternative and complementary medicine modality, has evolved over thousands of years with its own unique system of theories, diagnostics, and therapies. As an important source of novel agents with pharmaceutical potential, TCM has attracted great attention in preventing and treating cancer considering its low toxicity and high activity (Guo et al., 2012; Hu et al., 2013; Li et al., 2013, 2015; Sun, 2014). Furthermore, studies have shown that CHM, including formulas and single herbs combined with transcatheter arterical chemoembolization or chemotherapy can attenuate tumor growth and toxicity and boost immune function, improve overall survival and the quality of life for patients with advanced HCC (Wang et al., 2013; Chen et al., 2017).

Qilian Huaji (QLHJ) decoction is a traditional Chinese medicine comprising 13 herbs: Huang qi, Ling zhi, Dang gui, Bai shao, Bai zhu, Chuan lian zi, Ban xia, Ban bian lian, Ban zhi lian, E zhu, Bai hua she she cao, Hu zhang, Gan cao (Table 1). Several components of QLHJD have recently been reported to exert anti-proliferative effects in several cancer cell lines (Tsai et al., 2005; Zhang, 2017; Zhou et al., 2018). Traditional Chinese herb Ban bian lian, components of QLHJ decoction, have shown inhibitory effects on HCC xenograft tumor growth by downregulating p27 and Survivin expression in our previous study. We modified *Chinese lobelia* by adding herbs for tonifying Qi and liver, clearing away heat and toxic material, activating blood and removing stasis, which are widely used as adjuvant treatments for cancer in TCM theory. But the anticancer activity for QLHJD and the mechanistic basis for the activity is largely unknown.

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Table 1. Contents of QLHJ decoction.

Chinese Name	Botanical Name	Common Name	Dosage(g)
Huang qi	*Astragalus Mongholicus Bunge*	Radix Astragali seu Hedysari	30
Ling zhi	*Ganoderma lucidum Karst*	Reishi	10
Dang gui	*Angelica sinensis*	Radix Angelicae Sinensis	15
Bai shao	*Paeonia lactiflora Pall*	Radix paeoniae Alba	15
Bai zhu	*Atractylodes macrocephala Koidz*	Rhizoma Atractylodis Macrocephalae	15
Chuan lian zi	*Melia toosendan Sieb.et Zucc*	Melia toosendan	10
Ban bian lian	*Lobelia chinensis Lour*	Lobelia	15
Ban zhi lian	*Scutellaria barbata D.Don*	Scutellaria barbata	15
E zhu	*Curcuma Zedoaria (Christm) Rosc*	Curcuma Zedoary	10
Bai hua she she cao	*Hedytosis diffusa Willd*	Herba Hedyotis	15
Ban xia	*Pinellia ternata (Thunb.) Breit*	Rhizoma pinelliae	15
Hu zhang	*Polygonum cuspidatum Sieb.et Zucc*	Polygonum cuspidatum	10
Gan cao	*Glycyrrhiza uralensis Fisch*	Radix liquiritiae	5

MicroRNAs (miRNAs/miRs) are a class of small noncoding RNAs of 21-23 nucleotides that modulate ~60% of protein translation through binding to target mRNAs (Filipowicz et al., 2008). Increasing evidence has revealed that microRNAs (miRNAs) are involved in the dysregulation of oncogenes or tumor suppressor genes for human cancers and may be potential therapeutic targets for human malignancies (Oom et al., 2014; Tong et al., 2014). Among these, miR-122, an abundant miRNA in liver, has been implicated as a tumor suppressor in several types of cancer such as human glioma, gastric cancer, non-small lung cancer, bladder cancer and breast cancer (Ergün et al., 2015; Qin et al., 2015; Wang et al., 2016; Ding et al., 2018; Xu et al., 2018). Additionally, studies has revealed that miR-122 induces apoptosis in human HCC cell lines via targeting the anti-apoptosis B-cell lymphoma. Furthermore, miR-122 inhibits cell proliferation in HCC by targeting the Wnt/β catenin signaling pathway, and WNT1 gene is a direct target of miR-122 in hepatocellular carcinoma (Ahsani et al., 2017). Thus, miRNA-122 may be an anticancer therapeutic strategy against HCC.

Consistent with this concept, in this study H22 hepatocarcinoma xenograft model was established. The growth inhibition and immune function enhancement of HCC in nude mice by QLHJ decoction were observed by calculating tumor inhibitory rate, thymus index, spleen index. Regulatory effects of QLHJ decoction were detected by observing cell proliferation, cell cycle distribution and apoptosis *in vitro*. Furthermore, the potential miRNA targets were screened and verified to investigate the miRNA-mediated mechanism of QLHJD. The results of this study may lay a foundation for the development of new antitumor TCM agents.

2 Materials and methods

2.1 Preparation of Qilian Huaji decoction

QLHJD, the components of which were purchased from the TCM pharmacy of the Second Affiliated Hospital of Dalian Medical University, Liaoning Province, P.R China and identified by two pharmacognosy experts, was prepared as described previously (Ren et al., 2010). According to the standards of clinical medication for patients, the process of QLHJD is adequately guaranteed by quality control. The methods were as follows: The QLHJD components were soaked in 2-fold distilled water for 60 minutes; the aqueous mixture was heated to 100 °C for 20 minutes for the first time, and then the decoction was filtered twice. The QLHJ components were soaked and heated for a second time in the same conditions as the first time. The 2 filtrates were mixed together and reduced to the concentration required, then stored at 4 °C for usage. In order to establish the quality standards of the compound of QLHJD, the preparation of crude extracts of QLHJ were detected by using liquid chromatography–mass spectrometry methods.

2.2 Preparation of QLHJ-containing Serum (QCS)

Forty SPF (specific pathogen free) male Sprague-Dawley rats, which purchased from the laboratory animal center of Dalian Medical University, were acclimatized to laboratory condition (23 °C,12h light/12h dark, 50% humidity, adequate food and water) for two weeks prior to experimentation. Subsequently were randomly divided into 4 groups (n = 10 in each), that is, QLHJ high-dose group, QLHJ intermediate-dose group, QLHJ low-dose group, and control group, which received 48.4 g/kg/d, 24.2g/kg/d, 12.1g/kg/d QLHJ solution, and saline, respectively, by gavage twice each day for 7 days. On day 8, 1 hours after the last administration, rats were sacrificed and peripheral blood was collected and centrifuged at 2000×g for 15 minutes. For each group, the sera were pooled, filtered through a 0.22 μm cellulose membrane filter, heat inactivated at 56 °C for 30 minutes, and stored at −80 °C until use. This animal experiment protocol was approved by the Institutional Animal Care and Use Committee of Dalian Medical University, and efforts were made to minimize animal suffering.

2.3 Cell line and culture

Human HepG2 hepatoma cell line was obtained from the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in PRMI-1640 culture medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and maintained at 37°C in a humidified incubator with 5% CO_2.

2.4 Cell viability assay

Cell viability was determined using an MTT assay. Briefly, cells were seeded at a density of 4×10^3 cells/well in 96-well plates, followed by overnight incubation. On the following day, the media were removed, and the cells were treated with high-dose QLHJ drug-containing serum (20% added to serum free RPMI-1640 medium), intermediate-dose QLHJ drug-containing serum (15%) or low-dose drug-containing serum (10%) and the control group treated with a matched concentration of QCS treated with normal rat serum. After incubation of 24, 48 or 72 hours, 10% of an MTT solution (2 mg/mL, Sigma, cat.n.M2128) was added to each well, and the cells were incubated for another 4 h at 37 °C. The formazan crystals were dissolved in DMSO (100 µL/well, Sigma, cat.n.D2160) with constant shaking for 5 min. The absorbance of the plate was then read with a microplate reader at 540 nm. Three replicate wells were evaluated for each analysis.

2.5 Cell cycle and cell apoptosis assay

Cell cycle analysis was performed by flow cytometry using a BD FACS Calibur (Becton-Dickinson, San Jose, CA, USA) and propidium iodide (PI) staining. HepG2 cells were treated with indicated concentrations of QLHJD drug-containing serum. Subsequently, cells were harvested at a concentration of 1×10^5 cells/ml, and fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed twice with cold PBS and incubated for 30 min with RNase (8 µg/mL) and PI (10 µg/mL). The fluorescent signal was detected through the FL2 channel and the proportion of DNA in different phases was analyzed using ModfitLT version 3.0 (Verity Software House, Inc., Topsham, WE, USA).

Cell apoptosis was analyzed by flow cytometer using an Annexin V-FITC Apoptosis Detection Kit (BD FACS Calibur, Becton Dickinson Co., Franklin Lakes, NJ, USA). In brief, after treatment as described previous assay, cells in 6-well plates were harvested and resuspended in 1 ×binding buffer at a concentration of 1×10^6 cells/mL. Then, 5 µL of Annexin V-FITC and 10 µL of propidium iodide were added to 100 µL of the cell suspension. The cells were incubated for 15 min in the dark before 400 µL of 1×binding buffer was added. The samples were analysed by flow cytometry within 1 hours and the percentage of apoptosis was calculated with CellQuest software.

2.6 MiRNA PCR array

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturers instructions. Contaminating DNA in the RNA preparations was removed with DNase I, and the RNA was purified using an RNeasy MinElute Cleanup Kit (Qiagen, Germany). The RNA quantity and purity were assessed using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States), and RNA integrity was examined by denaturing agarose gel electrophoresis.

The expression of mature miRNAs was detected using ExiLENT SYBR Green master mix (Exiqon, Denmark) and the microRNA Ready-to-Use PCR, Human panel I+II (V4.M) (Exiqon, Denmark), according to the manufacturers instructions. Briefly, the template RNA was reverse transcribed using a Universal cDNA Synthesis Kit (Exiqon, Denmark), and the reverse transcription products were then amplified and detected on a 7900 Real-Time PCR System (Applied Biosystems, United States) using the following thermocycler conditions: denaturation at 95°C for 10 min, followed by 40 cycles of 95 °C for 10s and 60 °C for 1 min.

Data analysis was performed with the GenEx qPCR analysis software (www.exiqon.com/mirna-pcranalysis). U6 snRNA was used as an endogenous control. The fold change for each miRNA was calculated as $2^{-\Delta\Delta Ct}$. We filtered out raw data for which the cycle threshold values were greater than 30 from the 372 total human miRNAs, and the differentially expressed miRNAs with a fold-change≥1.5 in at least one cell line were included in the further analyses.

2.7 Quantitative recerse transcription PCR (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen-Life Technologies). The RNA concentration and purity were assessed using NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, United States). Single-strand cDNA was synthesized by reverse transcription of 2µg total RNA using SuperScrip IIreverse transcriptase (Promega, Madison, WI, USA) and Oligo (dT) or special RT-miR-122 primer according to the manufacturers instructions. The mRNA levels of miR-122 was determined with RT-qPCR and performed on Axygen 96-well reaction plates with the Applied Biosystems 7500 Real-time PCR System following the SYBR Premix Ex Taq kit (Takara Bio, Inc.), according to the manufacturers protocol. U6 was used as the internal control for miR-122. The mRNA expression was quantified by comparing the cycle threshold (Ct) values. The experimental data were analyzed using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate.

2.8 Animals and xenograft experiments

Six-week–old male SPF Balb/c nude mice (22-25 g) were purchased from Peking University Medical Laboratory Animal center (Beijing, China). The animal study protocol was approved by the Animal Welfare Committee of Dalian Medical University. Mice were inoculated subcutaneously with 5×10^6 H22 hepatocellular carcinoma cell suspension on the back. When the tumors were grew to about 50 to 100mm^3, the mice were randomly divided into 5 groups (n = 10 for each group), that is, vehicle group, QLHJ decoction at low dose group (QLHJL, 12.1 g/kg/d), QLHJ decoction at middle dose group (QLHJM, 24.2g/kg/d), and QLHJ decoction at high dose group (QLHJH, 48.4 g/kg/d) and 5-FU group. Mice in QLHJ group were given

QLHJ decoction intragastrically and those in 5-FU group were given 5-FU 20mg/kg/d, the mice in the vehicle group were given the same volume of saline. The grouping day was recorded as day 0. The mice were administered the required decoctions starting the next day, once daily, for 21 consecutive days. The body weights and tumor sizes were measured by Vernier caliper, recorded every two days. Tumor volume (TV) = $1/2 \times a \times b^2$, where a is the long diameter and b is the short diameter.

At day 21, all mice were sacrificed, spleens, thymuses and tumor tissues were harvested. Then weighed and tumor inhibition ratio (Equation 1), ratio of tumor weight to body weight (Equation 2), spleen index (Equation 3), thymus index (Equation 4) were calculated.

$$\text{The tumor inhibition ratio} = \left[\begin{pmatrix} \text{mean tumor} \\ \text{weight of} \\ \text{control group-mean} \\ \text{tumor weight of} \\ \text{experiment group} \end{pmatrix} / \text{mean tumor weight of control group} \right] \times 100\% \quad (1)$$

$$\text{Ration of tumor weight to body weight } (\text{T/W}) = \begin{pmatrix} \text{tumor weight} \\ /\text{body weight} \end{pmatrix} \times 100\% \quad (2)$$

$$\text{Spleen index} = \text{spleen weight} / \text{body weight} \times 10 \quad (3)$$

$$\text{Thymus index} = \text{thymus weight} / \text{body weight} \times 10 \quad (4)$$

2.9 Western blot analysis

Cells were washed with PBS and total proteins were extracted using lysis buffer, which contains 50 mmol/L Tris HCl, pH7.4, 150mmol/L NaCl, 5mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.1% SDS and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). The supernatant was collected by centrifugation at 12000g for 20min at 4 °C and the samples were subsequently stored at -80 °C. Protein concentration was measured using a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Subsequently equal amounts of proteins (25μg) were mixed with Laemmli sample buffer, resolved on SDS/PAGE (10%) and blotted onto NC membranes (Millipore). Non-specific protein interactions were blocked by incubation with 5% non-fat dry milk in 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% Tween 20 at room temperature for 1 h. Membranes were incubated with primary antibodies against AKT3 and β-actin (at 1:1000 dilution) overnight at 4 °C followed by HRP-conjugated secondary antibodies at 1:2000 dilution at room temperature for 1h. Detection was performed using an ECL kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression was normalized to an endogenous reference (β-actin) and was relative to the control.

2.10 Statistics analysis

All experiments were repeated three times. The results were expressed as the mean±standard deviation (SD). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). The *P*-values were calculated using the unpaired two-tailed *t*-test, one-way analysis of variance (ANOVA) or multiple *t*-tests. $P < 0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 Qilian Huaji decoction inhibits the tumor growth of HepG2 Xenograft

There was no significant difference in weight of mice of each QLHJD group, while the weight of mice of 5-FU group decreased significantly compared with vehicle group ($P < 0.05$) (Figure 1A). Similarly, no significant differences in the daily consumption of diet and drinking water by the mice and the mental states of the mice in each QLHJD group were better and more active, indicating that the mice in each group could tolerate the doses of QLHJ decoction. Using Vernier calipers to measure the length and diameter of tumors of nude mice every 2 days, we found that tumor volumes in the treatment groups were smaller than those in the vehicle group. At the end of the intragastric administration, the tumor volumes of the QLHJL ($433.43 \pm 102.03 mm^3$), QLHJM ($309.8 \pm 128.52 mm^3$), 5-FU groups ($302.09 \pm 155.10 mm^3$), and QLHJH ($182.5 \pm 96.41 mm^3$) decreased significantly compared with the vehicle group ($846.93 \pm 134.23 mm^3$) ($P < 0.05$ and $P < 0.01$). (Figure 1B). At the end of the experiment, the subcutaneous tumors, spleens, thymuses were removed. The tumor, spleen, thymuses and body weights were determined. The tumor inhibition rates of treatment groups were all above 30%; inhibition rate of the QLHJH was up to 56.45%±0.6% (Figure 1C). The ratios of tumor weight to body weight of QLHJD and 5-FU groups were significantly smaller than those of the vehicle group ($P < 0.05$) (Figure 1D). Additionally, thymus index and spleen index in QLHJD groups were markedly higher than those of the vehicle and 5-FU groups ($P < 0.05$) (Figure 1E). The above results showed that the QLHJ decoction could inhibit the growth of hepatocellular carcinoma and has the function of immunological enhancement.

3.2 QLHJ-Containing Serum (QCS) inhibits the viability of HepG2 cells in vitro

Cell viability was assessed by MTT. As shown in Figure 2, compared with the control group, cell viabilities were inhibited by QLHJD-containing serum in dose- and time-dependent manners. The results indicated that serum contained ingredients and/or metabolites of QLHJ that could effectively inhibit the proliferation of HepG2 cells.

3.3 QLHJ-Containing Serum (QCS) induces cell cycle arrest in the G0/G1 phase

Cell cycle analysis was performed by Flow cytometry (FCM). Results demonstrated that, compared with the control group, the percentage of HepG2 cells in the QLHJ group was significantly decreased at the S phase and a marked accumulation at the G0/G1 phase (Figure 3). These results indicated that QLHJ-containing serum (QCS) inhibited HepG2 cell proliferation by inducing cell cycle arrest at the G0/G1 phase.

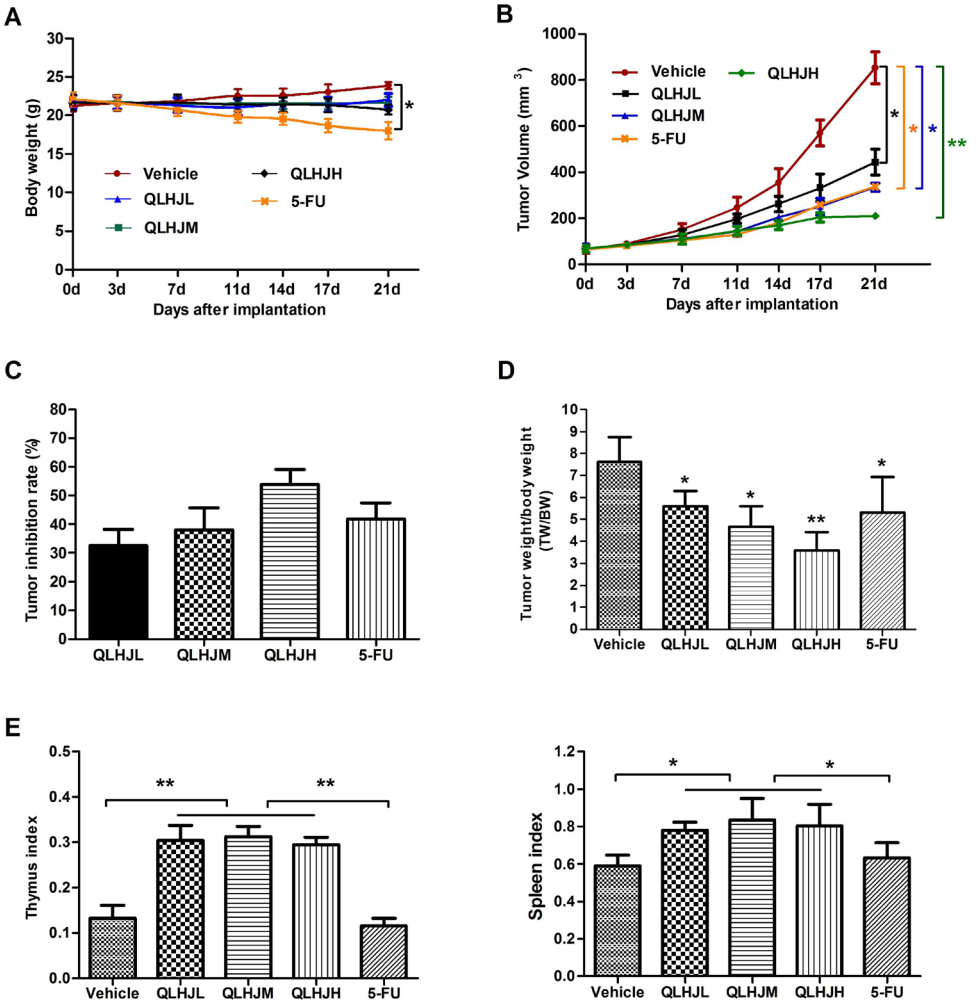

Figure 1. Inhibition of QLHJ decoction on the tumor growth of H22 xenograft. (A) Influence of QLHJ decoction on body weights of nude mice with hepatocellular carcinoma xenograft; (B) Influence of QLHJ decoction on tumor volumes of nude mice with hepatocellular carcinoma xenograft; (C) Influence of QLHJ decoction on tumor inhibition rate; (D) Influence of QLHJ decoction on the ratios of tumor weight to body weight (TW/BW). (E) Influence of QLHJ decoction on the thymus index and spleen index. *P < 0.05 and **P < 0.01, compared with vehicle group or 5-FU group.

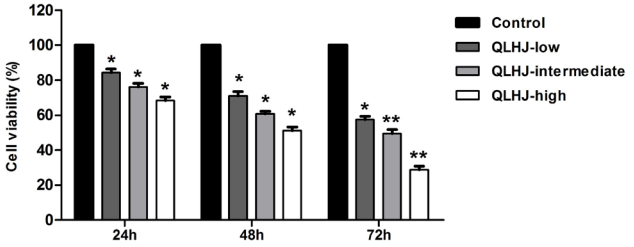

Figure 2. The effect of Qilian Huaji (QLHJ)-containing serum on HepG2 cell viability. HepG2 cells were treated with QLHJ-containing serum or control normal rat serum for 24, 48 and 72 hours. The cell viability was determined by MTT assay. Percentage cell survival in the control group was assumed 100. *P < 0.05, **P < 0.01, compared with the control.

3.4 QCS induces apoptosis of HepG2 cells in Vitro

Apoptotic cells were evaluated by Annexin V-FITC/PI double staining. As shown in Figure 4. The early apoptosis ratio and the late apoptosis ratio were both increased in the presence of QLHJ-containing serum in a dose-dependent manner. And the percentages of total apoptotic cells in the QLHJ-low group, QLHJ-intermediate group and QLHJ-high group were all notably increased compared with the control group.

3.5 QLHJD Regulates the miRNA expression Profile in Hepatoma cells

To explore the molecular mechanism involved in QLHJD-induced anticancer activity, we employed qPCR arrays to determine the changes in the miRNA expression profile in HepG2 cells treated with QCS. The expression levels of a large number of miRNAs were altered. Using a recommended cut-off of 30 Ct, we identified 36 differentially expressed miRNAs in HepG2 cells. 33 of which were up-regulated and 3 of which were down-regulated (Table 2). Notably, miR-122 expression was altered the most significantly.

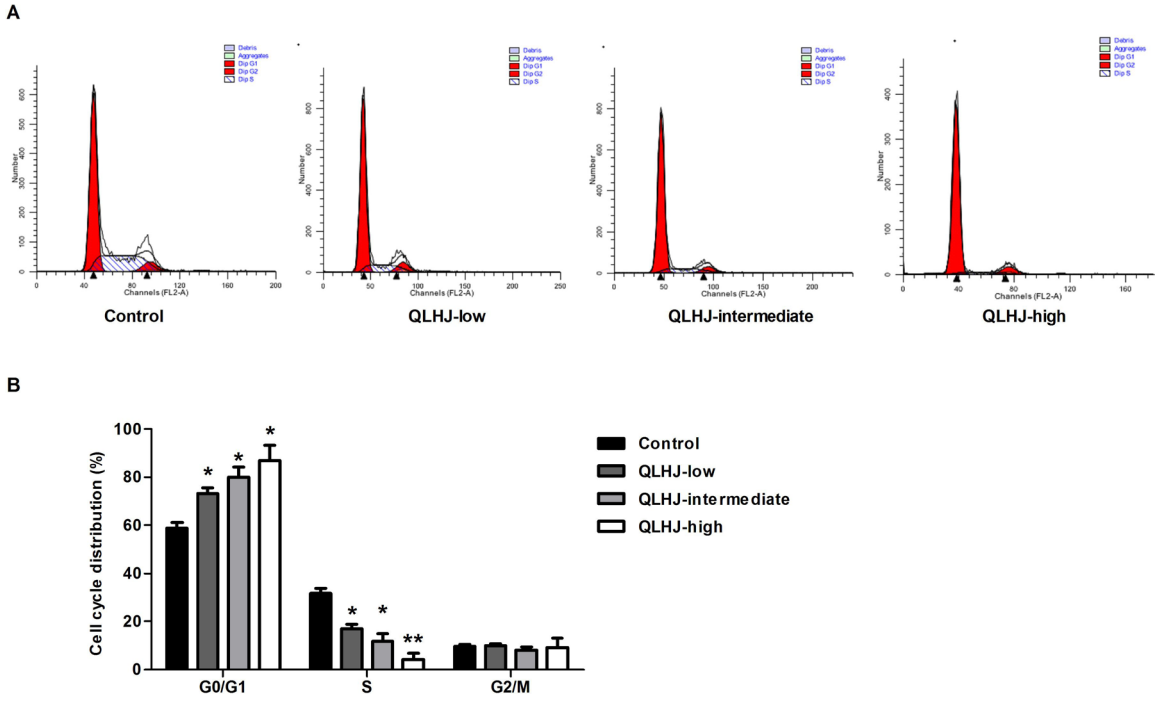

Figure 3. Effects of QCS on cell cycle distribution. (A) Proportion of cells in various phases of the cell cycle in the Control group and each QLHJ group; (B) Cell cycle analysis of QLHJ and Control group. *P < 0.05, **P <0.01.

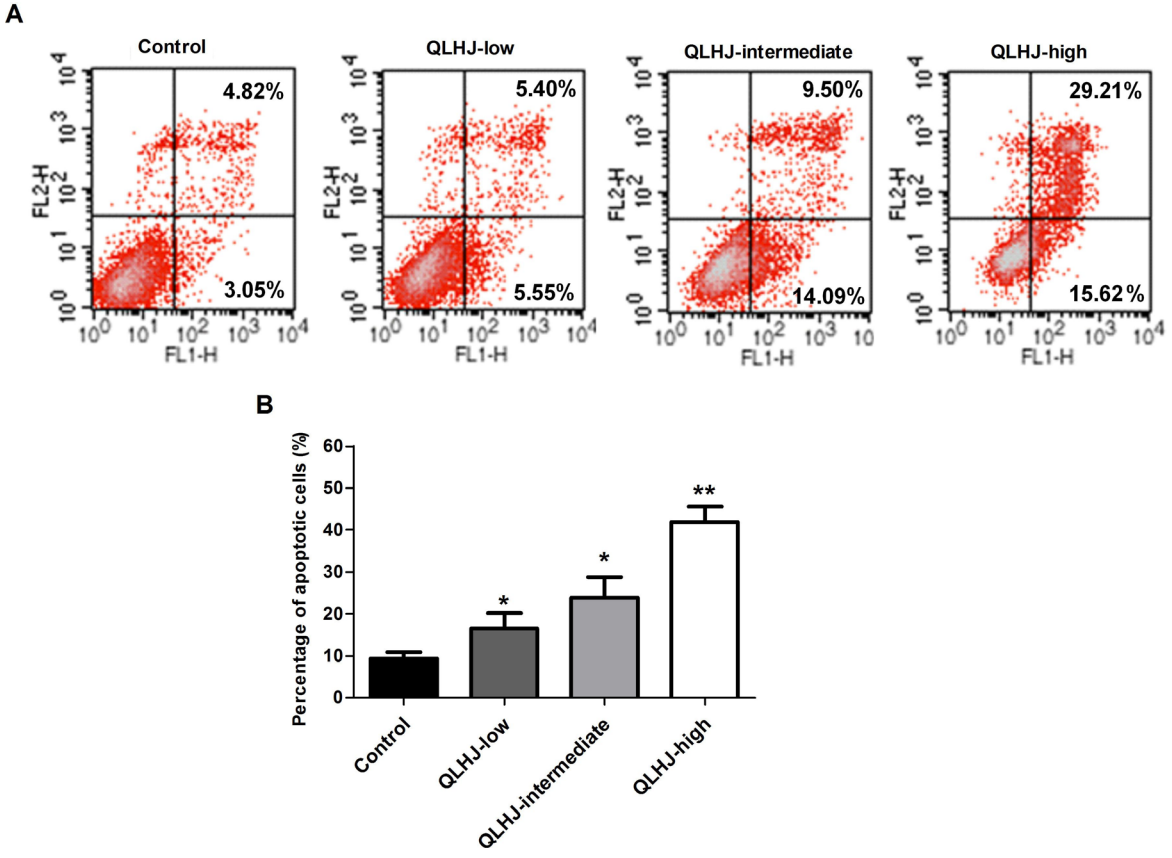

Figure 4. Effects of Qilian Huaji decoction (QLHJ)-containing serum on apoptosis of HepG2 cells. The cell apoptotic rate was determined by flow a cytometer using Annexin V–fluorescein isothiocyante (FITC)/propidium iodide (PI) staining. (A) The early apoptotic portion (right lower quadrant) and the late apoptotic portion (right upper quadrant) were both increased in a dose-dependent manner; (B) The statistical result of the percentages of total apoptotic cells in QLHJ group with different dose and the control group. *P < 0.05, **P <0.01.

Table 2. Differentially expressed microRNAs in HepG2 cells treated with Qilian Huaji decoction-containing serum.

miRNA ID	Fold change (QLHJD/Control)
has-miR-7-3p	**1.55**
has-miR-21-5p	**1.65**
has-miR-22-3p	**1.56**
has-miR-22-5p	**1.66**
has-miR-122	**3.76**
has-miR-105-3p	**1.58**
has-miR-215-3p	**1.74**
has-miR-215-5p	**1.62**
has-miR-25-3p	**1.62**
has-miR-25-5p	**2.01**
has-miR-26b-3p	**1.63**
has-miR-27a-3p	**1.71**
has-miR-27b-3p	**1.71**
has-miR-30a-3p	**1.67**
has-miR-29-5p	**1.73**
has-miR-30b-5p	**1.98**
has-miR-30d-5p	**1.83**
has-miR-30e-5p	**1.96**
has-miR-32-3p	**1.56**
has-miR-374a-5p	**1.51**
has-miR-214-5p	**-1.52**
has-miR-133	**-2.94**
has-miR-92b-3p	**1.60**
has-miR-98-5p	**1.65**
has-miR-105-5p	**2.06**
has-miR-134-3p	**1.97**
has-miR-139-5p	**1.83**
has-miR-212-3p	**2.18**
has-miR-215-5p	**1.74**
has-miR-181-5p	**1.61**
has-miR-192-5p	**1.72**
has-miR-452-3p	**1.56**
has-miR-484	**-2.27**
has-miR-193b-3p	**1.57**
has-miR-224-3p	**2.23**
has-miR-454-3p	**1.56**

miRNAs with a fold change≥1.5 are listed. QLHJD: Qilian. Huaji decoction; miRNAs: MicroRNAs.

3.6 QLHJD regulates the expression of miR-122 expression in HepG2 cells

We performed qRT-PCR to detect miR-122 expression in HCC cell lines with different backgrounds. Significant down-regulation of miR-122 expression was found in SMMC-7721, HepG2, HL-7702 and Bel-7402 cells ($P < 0.01$ or $P < 0.001$) (Figure 5A). Next, the miR-122 expression in HepG2 cells treated with QCS was further verified. As shown in Figure 5B, the levels of miR-122 were significantly up-regulated ($P < 0.01$). We then conducted Western blot analysis to determine the AKT3 expression. The WNT1 protein expression was lower in the QLHJD groups than in the Control groups (Figure 5C and D). These results indicate that QLHJD increased the expression of miR-122 and decreased WNT1 protein expression in HepG2 cells.

4 Discussion

To the best of our knowledge, Hepatocellular carcinoma (HCC) is one of most commonly diagnosed malignancies worldwide with poor prognosis. Although many treatment modalities including transplant, surgical resection, ablative therapies and transarterial embolotherapies have certain curative effects, the five-year survival rate of HCC patients remains poor. A number of chemotherapy drugs such as sorafinib, 5-FU and so on contain intrinsic cytotoxicity to normal cells, which limits their long-term use and therapeutic efficacy (Alvarez-Cabellos et al., 2007; Sanoff et al., 2016), emphasizing the need to develop novel anticancer drugs.

Traditional Chinese medicines (TCM), especially Chinese herbal medicines (CHM), which have been used for thousands of years, have relatively higher safety compared with typical anticancer agents and have attracted great attention for advanced stage cancer treatment (Gordaliza, 2007; Ji et al., 2009). According to the medicine compatibility, TCMs consist of numerous natural products, including principal, assistant, adjuvant and dispatcher herbs in specific formula, which have the function of improved therapeutic efficacy, synergistic action, targeting specific lesion, treatment of complex diseases, and reducing side effects (Bian et al., 2014).

Hepatocellular carcinogenesis is strongly linked to cirrhosis and infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) etc, suggesting its complex pathology and multi-factor crosstalk effect. Similarly, in TCM theory, HCC is a sophisticated disease with multiple factors working together, resulting from internal deficiency and relative pathogenic factors, including toxin, phlegm, and blood stagnation (Lin et al., 2014). Accordingly, Fuzheng Guben herbs and Qingre Jiedu herbs are widely used as adjuvant treatments for cancers. Fuzheng Guben means to replenish vital energy, nourish yin and tonify kidney. Qingre Jiedu means to clear heat and detoxify the body. In TCM, heat and toxin are considered to be an important causative factor of malignant tumors. Pharmacological studies demonstrate that both Fuzheng Guben and Qingre Jiedu herbs contain ingredients that can inhibit tumor proliferation and induce cell apoptosis, such as *Ganoderma lucidum, Astragalus membranaceus, Lobelia chinensis, Sculellaria barbata and so on*. QLHJ decoction is a self-made Chinese herbal which contains 2 Fuzheng Guben herbs (*Ganoderma lucidum, Astragalus membranaceus,*) and 6 Qingre Jiedu herbs (*Lobelia chinensis-Lour, Sculellaria barbata, Curcuma zedoary, Oldenlandia, Pinellia ternata, Polygonum cuspidatum*) and we modified them by adding herbs for promoting Qi and blood circulation (*Angelica sinensis, Atractylodes macrocephala rhizoma, Radix paeoniae alba, Szechwan chinaberry fruit*). However, whether QLHJD has an effect on HCC, and the exact mechanism of this, remains uncovered.

By contrast, a large number of studies demonstrated that miRNAs plays an important role in hepatocellular carcinoma and serves as a diagnostic marker or target of therapeutics for HCC.

In this study, we demonstrated that QLHJ decoction inhibited the hepatocellular growth via inducing cell cycle arrest, promoting cell apoptosis and inhibiting cell proliferation, which was mediated by activation of miR-122 and likely suppression

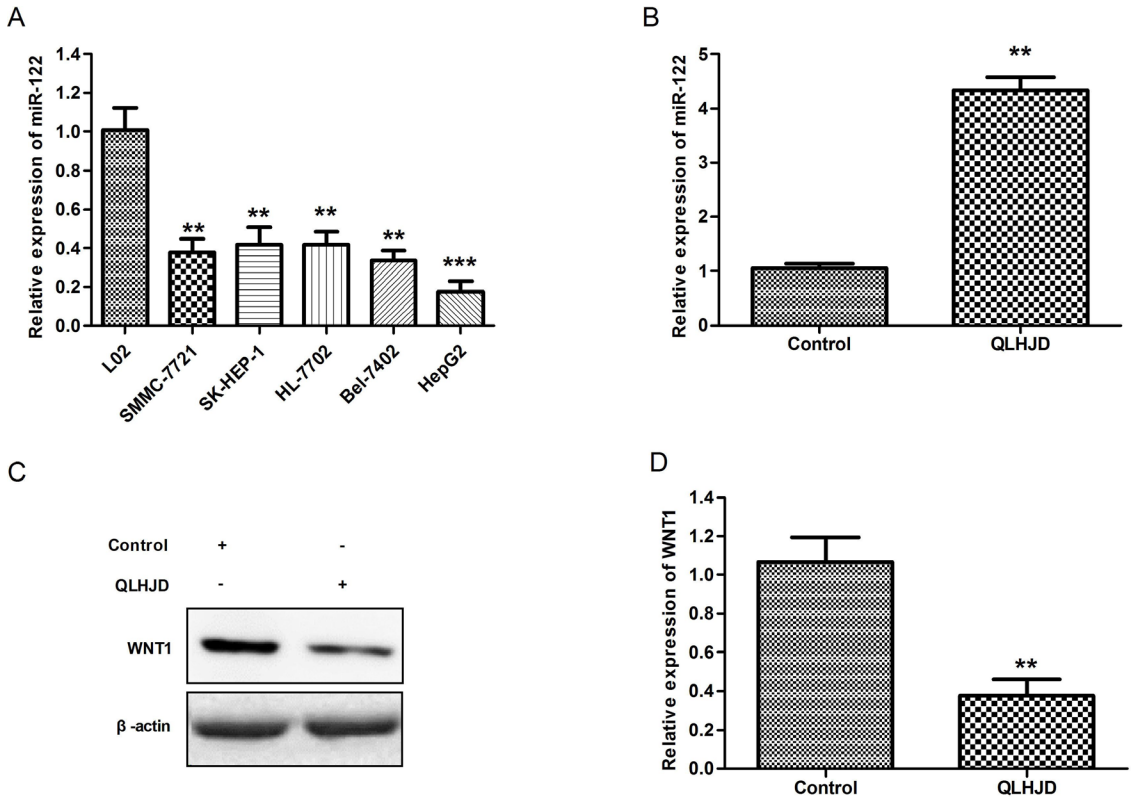

Figure 5. Effects of Qilian Huaji decoction on miR-122 and WNT1 expression in hepatoma carcinoma cells. (A) Down-regulation of miR-122 expression in Hepatoma carcinoma cells; (B) HepG2 cells were treated with 20% QCS or 20% normal rat serum for 48 h, and quantitative RT-PCR was performed to determine the expression levels of miR-122 (C), WNT1 expression was measured by Western blot ; D: Statistical analysis of Western blot data. **P < 0.01 vs the control group. ***P < 0.001 vs the control group. QCS: QLHJD-containing serum; QLHJD: Qilian Huaji decoction.

of AKT3 expression. Our findings also indicated that QLHJ decoction has significant anti-tumor effect on transplanted H22 tumor in nude mice and has the function of immunological enhancement. Therefore, QLHJ decoction might be applied to the clinical treatment of hepatic carcinoma. However, since the results from this study were based on *in vitro* assays of hepatoma carcinoma HepG2 cell lines and *in vivo* assays in nude mice xenograft model, clinical data will be necessary. Future studies will be focused on clinical trials in the patients treated with QLHJ decoction. The prognosis data can be used as evidence to suggest the possibility of QLHJ decoction as a therapeutic agent for the treatment of hepatic carcinoma.

Abbreviations

QLHJD, Qilian Huaji decoction; HCC, hepatocellular carcinoma; TCM, traditional Chinese medicine; CHM, Chinese herbal medicine; QCS, Qilian Huaji decoction containing serum; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-dephenyl-2-H-tetrazolium; AKT 3, protein kinase B; HCV, hepatitis C virus; HBV, hepatitis B virus; FCM, flow cytometry; QCS, QLHJ-containing serum; TCM, Traditional Chinese medicines; CHM, Chinese herbal medicines; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HCI, hydrochloric acid; NaCI, sodium chloride; $MgCI_2$, magnesium chloride; EDTA, ethylenediaminetetraacetic acid.

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