Antitumor Efficacy of Huqizhengxiao (HQZX) Decoction Based on Inhibition of Telomerase Activity in Nude Mice of Hepatocarcinoma Xenografts

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Abstract

Objective: Huqizhengxiao (HQZX) decoction is a mixture of traditional Chinese medicines comprising 10 herbs, with inhibitory effects on hepatocarcinoma. The aim of the study is to observe the antitumor efficacy and mechanism of HQZX decoction in nude mice with hepatocellular carcinoma xenografts. Methods: HepG2-luc subcutaneous hepatocarcinoma was established in nude mice. The mice were divided into 5 groups: control, cinobufagin, HQZXS, HQZXM, and HQZXH with doses 13.52, 27.03, and 54.06 g/kg, respectively. HQZX decoction was prepared for intraperitoneal intragastric administration for 3 weeks. Tumor growth was measured with Vernier calipers and in vivo imaging system. α-Fetoprotein (AFP) was determined by radioimmunoassay. Tumor necrosis factor–α (TNF-α) was measured with enzyme-linked immunosorbent assay (ELISA) assay. Telomerase activity was measured with polymerase chain reaction–ELISA. Nuclear mitosis and necrosis were observed with hematoxylin-eosin stain. Apoptotic proteins of caspase-3, Bcl-2, and Bax were examined by Western blot. Signaling molecules of ERK, mTOR, and STAT3 were measured with Lumines assay. Results: HQZX decoction showed good inhibition of HepG2-luc xenografts. Compared with control group, the relative tumor proliferation rate was less than 60% in the HQZXH and HQZXS. The tumor inhibition rate of HQZXH group reached 52% ± 15%. Relative average optical density values of the HQZXS and HQZXH groups decreased significantly. The mitotic index in HQZXS, HQZXM, and HQZXH groups decreased greatly. Telomerase activity of HQZXS was clearly reduced, and, the caspase-3 expression upregulated in HQZXH group. Bcl-2 expression was downregulated in HQZXS and HQZXH. The ratios of p-ERK/ERK and p-STAT3/STAT3 in HQZXS group were significantly downregulated. Conclusion: HQZX decoction can clearly inhibit the growth of hepatocellular carcinoma and induce tumor apoptosis. Its antitumor mechanism may be related to reducing telomerase activity and regulating the STAT3 and ERK signal pathway.

Keywords
telomerase activity, hepatocarcinoma, ERK, STAT3, apoptosis, Chinese herb

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Introduction

Hepatocellular carcinoma (HCC) ranks sixth in cancer incidence and second in tumor-related mortality worldwide, with more than half of the new cases and deaths occurring in China. Because of difficulties with early diagnosis and rapid progression, the survival rate of hepatocellular carcinoma is extremely low.1 Management of HCC can be maximized with the utilization of multiple treatment modalities including transplant, surgical resection, and locoregional therapies, including ablative therapies and transarterial embolotherapies.2 Commonly used new generation drugs for systemic
chemotherapy of primary HCC include sorafenib, oxaliplatin, and so on. Although they have certain curative effects, their toxicity and side effects are obvious.

Traditional Chinese medicine, specifically Chinese herbal medicine (CHM), is one of the most popular complementary and alternative medicine modalities worldwide. CHM has attracted great attention as an alternative antitumor including HCC considering its low toxicity and high activity. For patients with advanced HCC, CHM—including formulas and single herbs combined with transcatheter arterial chemoembolization or chemotherapy—is able to decrease tumor growth and toxicity and improve overall survival, quality of life, and immune function.

Huqizhengxiao (HQZX) decoction is a traditional Chinese medicine comprising a few herbs: Hu ji sheng, Huang qi, E zhu, Yu jin, San qi, Bai hua she she cao, Ku shen, Ku wei ye xia zhu, Xia ku cao (Table 1). HQZX decoction has the potential action of tonifying the liver and kidney, nourishing yin, clearing heat and detoxification, and dispersing stasis.

HQZX decoction is modified by HuQi San made by QianYing, a famous doctor of traditional Chinese medicine. Much experimental and clinical research indicates that HuQi San and its ingredients had inhibitory effects on HCC. The growth inhibition of HCC in nude mice by HQZX decoction was observed using small animal in vivo imaging systems. Regulatory effects of HQZX decoction were observed by detecting the expression of telomerase activity and proteins of the STAT, mTOR, ERK signaling pathways to further explore the antitumor mechanism, which may provide more experimental evidence for clinical application to reduce the recurrence and metastasis of HCC.

### Materials and Methods

#### Reagents

The HQZX decoction was prepared by Chinese pharmacy of Beijing You-An Hospital, Capital Medical University under good manufacturing practice, according to the preparation methods and standards of clinical medication for patients. Preparation process of HQZX is adequately guaranteed by quality control. The methods were as follows: The HQZX 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 HQZX 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. The main ingredients of HQZX decoction were detected by using liquid chromatography–mass spectrometry methods for the quality control of different batches.

Cinobufagin was purchased from Shandong Xin Qi Pharmaceutical Co Ltd. Dulbecco’s modified Eagle medium (DMEM) medium, trypsin, and fetal bovine serum were purchased from Gibco Company. Telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Roche. Dimethyl sulfoxide
was purchased from American Sigma Company. BCA protein reagent was purchased from Beijing Bomad Biotech Corp. D-fluorescein potassium was purchased from Gold Biotechnology. The primary antibodies of caspase-3, Bax, Bcl-2, and PCNA were purchased from Cell Signaling Company. α-Fetoprotein (AFP) radioimmunoassay kit was from Shanghai Bo Yu Biological Technology Co, Ltd. Tumor necrosis factor–α (TNF-α) ELISA kit was from Abcom. Total/phosph ERK MAG Kit, total/phosph STAT3 MAG Kit, total/phosph mTOR MAG Kit and tubulin MAG kit were from Merck Millipore.

Cell Line and Cell Culture

HepG2 cell line with Luciferase reporter genes was provided by the Beijing Institute of Hepatology. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified incubator with 5% CO₂.

Animals and Xenograft Experiments

Six-week-old male Balb/c nude mice were obtained from Peking University Medical Laboratory Animal Center (certification no. 11804700003171) and were maintained in individually ventilated cages (IVC) under specific pathogen free sterile conditions. The animal study protocol was approved by the Animal Welfare Committee of Capital Medical University. The 2 × 10⁶ HepG2-luc cells were inoculated subcutaneously into the right armpit of nude mice. When the tumors grew to about 800 to 1000 mm³, 2 to 3 nude mice with tumors in good condition were selected as the tumor source. The mice were sacrificed after disinfection of skin with 75% alcohol and then the tumor tissues were removed and washed with phosphate buffered saline (PBS) in precooled DMEM. Under sterile conditions, tumors were cut into about 2 × 2 × 2 mm³ size. A total of 32 Balb/c SPF nude mice (male, 6 weeks old, weight 18-22 g) were anesthetized with intraperitoneal injection of 1% pentobarbital. After anesthesia, they were cut into 0.5 cm openings near the right armpits at the ventral side of the nude mouse. A trocar was used to insert the tumor tissue under the right armpits, and then the skin was sutured. Mice were put back in the cages for rearing. When the tumors grew to about 50 to 100 mm³, the mice were randomly divided into 5 groups: control group, HQZX decoction at small dose group (HQZX5, 13.52 g/kg), HQZX decoction at middle dose group (HQZX5M, 27.03 g/kg), HQZX decoction at high dose group (HQZX5H, 54.06 g/kg), and cinobufagin group (0.41 g/kg). The grouping day was recorded as day 0. The mice were administered the required decoctions starting the next day, once daily, for 3 consecutive weeks. The grouping day was recorded as day 0. The mice of the control group were given the same volume of normal saline. The body weights and tumor sizes were measured by Vernier calipers, recorded twice a week. Tumor volume (TV) = 1/2 × a × b², where a is the long diameter and b is the short diameter.

Relative tumor volume (RTV) = Vₜ/V₀ (where Vₜ is the volume of the tumor after the intragastric administration and V₀, the volume of the tumor before the initial administration).

The relative tumor proliferation rate (T/C%) = TRTV/CRTV × 100, where TRTV is the relative tumor volumes of treatment groups and CRTV is the relative tumor volumes of control group.

At the end of the experiment, the tumor tissues were taken and the tumor inhibition ratio and ratio of tumor weight to body weight were calculated.

The tumor inhibition ratio = [(mean tumor weight of control group – mean tumor weight of experiment group)/ mean tumor weight of control group] × 100%.

Ratio of tumor weight to body weight (T/W) = [tumor weight/body weight] × 100%.

Detection With Small Animal In Vivo Imaging System

After administration on days 0, 7, 14, and 21, each mouse was injected intraperitoneally with 200 μL luciferase at 15 mg/mL. Carestream living imaging system was used to detect the tumor fluorescence of each mouse. Data on the bioluminescence image were obtained and analyzed.

Detection of Serum Level of AFP and TNF-α

Blood samples were collected from the orbits of the mice, and the serum was separated. The contents of AFP in the serum of nude mice were measured by radioimmunoassay kit. The contents of TNF-α in serum of nude mice were detected by ELISA kit.

Histological Observation

The tumor tissues were fixed in 4% paraformaldehyde solution for conventional hematoxylin and eosin (H&E) staining. H&E slices were observed under a microscope and photographed at 200 times magnification. Nuclear mitotic index (NMI) was calculated in 3 slices of each animal. NMI = number of mitotic cells/1000 cells.

Detection of Telomerase Activity

The telomerase activity was measured by telomere repeat amplification protocol (TRAP). The processes of preparation of telomerase extracts, amplification of telomeric repeats and hybridization and ELISA procedure were according to the Roche kit.
Western Blot Analysis

Proteins of tumor tissues were conventionally extracted. The protein concentrations of lysates were measured with the bicinchoninic acid method. Lysates were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane. Membranes were blocked with 5% bovine serum albumin and then incubated overnight at 4°C with primary antibodies (1:1000). Membranes were washed 3 times with TBS/T and incubated for 1 hour at room temperature with the appropriate secondary antibody (1:2000). Membranes were then washed and immunoreactive bands were developed with ECL and visualized by autoradiography. Protein loading was normalized using GAPDH antibody.

Luminex Assay

The protein concentrations of sample tissues were diluted below 2 μg/mL. Processing of samples was according to the kits (Total/phosph ERK MAG Kit, phosph/Total STAT3 MAG Kit, and Total/Phosph mTOR MAG Kit). The mean fluorescence intensity was measure with FLEXMAP 3D system (Austin, TX, USA).

Results

Inhibition of HQZX Decoction on the Tumor Volume of HepG2 Xenograft

There was no significant difference in weight of mice of each group (Figure 1A). The mental states of the mice in each HQZX group were better and more active, indicating that the mice in each group could tolerate the doses of HQZX decoction. Using Vernier calipers to measure the length and diameter of tumors of nude mice every 3 days, we found that the absolute tumor volumes in the treatment groups were smaller than those in the control group. At the end of the intragastric administration, the tumor volumes of the HQZXS (524.43 ± 261.33 mm³), HQZXH (412.5 ± 136.41 mm³), and cinobufagin groups (542.09 ± 255.10 mm³) decreased significantly compared with the control group (866.93 ± 304.23 mm³) (*P < .05). There was no significant difference in tumor volumes between the treatment groups (Figure 1B). The relative volume of tumor results showed that growth of tumor volumes in the HQZXS (27.93 ± 9.25), HQZXH (25.31 ± 13.16), and cinobufagin (32.51 ± 14.91) groups were significantly slower than those in the control group (47.85 ± 17.12) (Figure 1C). At the end of the intragastric administration, the relative tumor proliferation rate of the tumor was less than 60% in the HQZXH,
HQZXS, and the cinobufagin groups. The HQZX decoction at different doses showed obvious antitumor effect, and the tumor inhibition rates of different doses of HQZX were all >40%. HQZXH group reached 52% ± 15% at the endpoint of the experiment (Figure 1D). The above results showed that the HQZX decoction could inhibit the growth of subcutaneous xenografts of HepG2 cells.

**Influence of HQZX Decoction on the Optical Density of HepG2 Xenograft**

In vivo imaging results showed the mean optical density of different groups increased rapidly at the first week; the average optical intensity of the control group was significantly higher than those of the treatment groups and reached the peak. The average optical intensity increased in the treatment groups, reaching a peak at the second week. At the third week, relative average optical density values of the HQZXS (31.12 ± 17.57) and HQZXH (33.76 ± 36.30) groups were significantly lower than those of control group (49.76 ± 33.48) (P < .05), which further demonstrated that HQZX decoction could significantly inhibit the growth of HepG2 xenograft (Figure 2A and B).

**Influence of HQZX Decoction on the Ratios of Tumor Weight to Body Weight in HepG2 Xenograft**

At the end of the experiment, the subcutaneous tumors were removed. The tumor and body weights were determined. The tumor sizes of the cinobufagin, HQZXS and HQZXH groups were smaller than those of control group (Figure 3A). The tumor inhibition rates of treatment groups were all above 30%; inhibition rate of the HQZXS was up to 50.55% ± 0.40% (Figure 3B). The ratios of tumor weight to body weight of HQZXS (4.68 ± 2.07), HQZXM (4.72 ± 1.51), HQZXH (4.22 ± 2.33), and cinobufagin (4.22 ± 2.33) groups were smaller than those of the control group (3.87 ± 1.92) (Figure 3C). These results also illustrated that HQZX could suppress the growth of HepG2 hepatocellular carcinoma.

**Influence of HQZX Decoction on Histopathologic Changes of HepG2 Xenograft**

H&E stain results showed that the mitotic cells existed in tumor tissues of different groups. Compared with control group (53.33 ± 8.35), the mitotic index in HQZXS (29.56 ± 4.36), HQZXM (26.56 ± 5.29), HQZXH (28.78 ± 9.68), and cinobufagin (25.33 ± 5.02) group decreased greatly, while tumor tissues of the treatment groups had different degrees of necrosis (Figure 4).

**Influence of HQZX Decoction on Serum TNF-α and AFP Level of HepG2 Xenograft**

Using radioimmunoassay for the detection of AFP level in serum, the results suggested that there was no significant difference among different groups. The level of TNF-α in serum was detected by ELISA method. The results showed increased trends of TNF-α in each treatment group. Compared with the control group (95.24 ± 21.01 pg/mL), TNF-α in HQZXM group (198.78 ± 50.12 pg/mL) increased greatly (Figure 5A and B).

**Influence of HQZX Decoction on Telomerase Activity of HepG2 Xenograft**

We detected telomerase activity of tumors with ELISA-PCR method. We found that the positive rates of telomerase
in control, HQZXS, HQZXH, and cinobufagin groups were 50%, 33.3%, 50%, 42.8%, and 42.8%, respectively. While the average optical density value of control group (0.35 ± 0.30) were larger than HQZXH group (0.19 ± 0.09) and cinobufagin group (0.26 ± 0.11). The above results suggest that the HQZX decoction could inhibit the telomerase activity of HepG2 xenograft (Figure 6A and B).

**Influence of HQZX Decoction on Apoptosis Proteins of HepG2 Xenograft**

We detected apoptosis proteins with the Western blot method (Figure 7A). Compared with the control group (1 ± 0.14), the fold changes of Bcl-2 in HQZXS (0.25 ± 0.06), HQZXH (0.05 ± 0.02) and cinobufagin (0.07 ± 0.01) group decreased greatly. Compared with the group (1 ± 0.19), the fold changes of caspase of HQZXH (1.42 ± 0.14) and cinobufagin (1.79 ± 0.19) group increased greatly (Figure 7B). There were no significant difference between the different groups in Bax protein.

**Influence of HQZX Decoction on Signaling Molecular of HepG2 Xenograft**

STAT3, ERK, and mTOR are key regulatory signaling pathways that are involved in many biological effects such as proliferation, differentiation, metastasis, invasion, and metabolism of tumor cells. The effects of HQZX on STAT3, ERK, and mTOR pathway proteins were observed with Luminex method. The results showed that HQZX could significantly affect the STAT3 and ERK signaling pathways, and the ratios of p-ERK/ERK (0.58 ± 0.06) and p-STAT3/STAT3 (0.58 ± 0.16) in the HQZXS group were significantly downregulated, compared with control group (1 ± 0.12, 1 ± 0.04). These results suggested that the inhibition of HQZX decoction might be mediated by the STAT3 and ERK signaling pathways (Figure 8).

**Discussion**

Telomerase is an enzyme that elongates one chain of the telomeric DNA and compensates for its shortening during replication. Telomerase activation is the main pathway for cells to gain immortality. It is also an important step in malignant proliferation of the tumor, which is observed in more than 85% of all known human tumors and is considered a promising tumor marker.

Some researchers found that Huqi San, total alkaloids of mistletoe and mistletoe polysaccharides reduced telomerase activity in SMMC-7721 hepatocellular carcinoma cells, and decreased the telomerase activity in rat hepatic precancerous lesion. The main herbs of the HQZX decoction were same as those of Huqi san and had obvious inhibitory effects in patients with HCC. We speculated that the HQZX might inhibit the HCC based on influencing telomerase activity.

To observe the efficacy of HQZX decoction on tumors, we used the HepG2 subcutaneous xenograft model in nude mice. We found the absolute tumor volumes, relative tumor volumes, relative tumor proliferation rates, relative average optical densities, ratios of tumor weight to body weight and mitotic index of HQZX groups were deceased greatly relative to those of the control group. These results suggested that HQZX decoction regressed the growth of HepG2 tumors.

To explore the inhibitory mechanism of HQZX decoction on liver cancer, we used PCR-ELISA method to detect telomerase activity in tumor tissue. This method was based...
on the telomeric repeat amplification protocol (TRAP) assay. The TRAP assay included 3 steps: extension, amplification, and detection of telomerase products. In the extension step, telomeric repeats (TTAGGG) were added to the telomerase substrate (which is actually a nontelomeric oligonucleotide labeled with Biotin) by telomerase. In the amplification step, the extension products are amplified by the PCR using specific primers and in the detection step, the presence or absence of telomerase is analyzed by ELISA method. The results showed that HQZX decoction could reduce telomerase activity, which suggested that inhibition of telomerase activity might be one of the anticancer mechanisms of HQZX decoction.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor, which in humans is encoded by the STAT3 gene. It is a member of the STAT protein family. STAT3 is phosphorylated by receptor-associated Janus kinases (JAK), from homo- or heterodimers, and translocate to the cell nucleus where they act as transcription activators and play a key role in many cellular processes such as cell growth and apoptosis. STAT3 activates several other genes involved in cell cycle progression such as Fos, Cyclin-D, CDC25A, c-Myc, or Pim1 and upregulates antiapoptotic genes such as Bcl-2 (B-Cell CLL/Lymphoma-2), BCLXL, and Beta2-macroglobulin. Extracellular regulated protein kinases (ERK), a member of
Yu et al. have established that members of the MAPK family, have been major participants in the regulation of cell growth and differentiation, but when improperly activated contribute to malignant transformation. ERK1 and 2 form a central component in the MAPK cascade. Some research indicated that ERK is another protein that regulates Bcl-2 expression.

Mammalian target of rapamycin (mTOR) is associated with normal proliferation and differentiation, and its alteration is detectable in cancer cells that exploit the normal mechanisms to overcome apoptosis. In this study, compared with control group, p-STAT3/T-STAT3 and p-ERK/T-ERK of HQZXS group decreased greatly. Bcl-2 expression in HQZXS group decreased, while caspase-3 expression increased. The mTOR pathway was not influenced by HQZXS decoction.

**Conclusion**

In summary, HQZXS decoction can inhibit the growth of hepatocellular carcinoma and induce tumor apoptosis. Its...
antitumor mechanism may be related to reducing telomerase activity and influencing expressions of apoptosis genes by regulating the STAT3 and ERK signal pathways.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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