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Jianpi-Huayu Formula Inhibits Development of Hepatocellular Carcinoma by Regulating Expression of miR-602, Which Targets the *RASSF1A* Gene

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Abstract

The traditional Chinese medicine formula Jianpi-Huayu (JPHY) has been reported to be effective in the treatment of hepatocellular carcinoma (HCC). However, its underlying mechanism remains unclear. In this article, we employed an orthotopic transplantation model in nude mice to explore whether JPHY could inhibit the development of HCC by regulating miR-602, which targets the Ras association domain-containing protein 1A (RASSF1A) pathway. HCC SMMC-7721 cells were treated with JPHY to test whether the *RASSF1A* gene as mediated by miR-602 affected the proliferation and apoptosis of tumor cells. We subsequently detected miR-602, RASSF1A, and tumor cell apoptosis–related markers in cells and liver tumor tissues. We observed that mice treated with JPHY had smaller tumors and higher survival rates than untreated ones. Similarly, JPHY-treated SMMC-7721 cells exhibited alterations in morphology and higher cytotoxicity compared with the control group. Furthermore, we found that JPHY decreased overexpression of miR-602 and increased protein expression levels of the *RASS1A* gene, which in turn altered protein expression levels of tumor cell apoptosis–related genes in the cells and liver tumor tissues of drug-treated mice. These results indicated that JPHY could potentially be used to treat HCC by targeting miR-602, which targets the *RASSF1A* gene, which in turn plays a major role in HCC pathogenesis.

Keywords

Jianpi-Huayu formula, miR-602, RASSF1A, hepatocellular carcinoma, SMMC-7721, apoptosis

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Introduction

Hepatocellular carcinoma (HCC) is a common malignancy with high morbidity and mortality rates and a 5-year overall survival rate of about 50%,[1] thereby placing a heavy burden on patients and their families. As recent research into micro–ribonucleic acids (miRNAs, a noncoding RNA family) has progressed, the supposition has developed that miRNAs play indispensable roles in various pathophysiological processes, including HCC development.[2-5] Ras association domain-containing protein 1A (RASSF1A) affects various cellular functions such as migration, proliferation, and apoptosis; induces cell cycle arrest and senescence; and

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promotes cell microtubule stability.[6-9] Its expression level and function are of great importance to HCC pathogenesis.[10,11] The theory that overexpression of miR-602 is related to the occurrence and development of HCC has been confirmed by several studies.[12,13] In addition, it has been reported that the potential mechanism by which miR-602 promotes hepatitis B virus (HBV)–related HCC is inhibiting the expression of its target gene, *RASSF1A*.[14]

Jianpi-Huayu formula (JPHY), a traditional Chinese medicine (TCM) compound, consists of Bai Shu (*Atractylodes macrocephala* Koidz [AM], rhizome), E Shu (*Curcuma zedoaria* [Christm.] Roscoe, rhizome), Fo Shou (*Citrus medica* L. var. *sarcodactylis* Swingle, fruit), Fu Ling (*Poria cocos* [Schw.] Wolf, sclerotium), She She Cao (*Hedyotis diffusa*, whole grass with roots), and Ku Shen (*Sophora flavescens* Aiton, radix). In our previous clinical and experimental studies, we found that JPHY had significant curative effects in clinical practice, could negatively regulate miR-570 and the B7-H1/programmed death-ligand 1 pathway,[15] and could affect Jagged1/Notch pathway–mediated angiogenesis of HCC cells in vitro.[16] However, the potential antitumor mechanism of JPHY remains unclear.

This study aimed to determine whether JPHY could inhibit tumors via miRNA 602 (miR-602), which targets the *RASSF1A* gene, a critical tumor suppressor mediator that can influence the expression of downstream cellular components such as dual leucine zipper kinase, mitogen-activated protein kinase kinase kinase 1-4, mixed-lineage kinase 2/3, and apoptosis signal-regulating kinase 1/2. We employed an in vivo orthotopic transplantation model[17] by treating mice with high doses (24.96 g/kg/d) of JPHY. We also treated HCC SMMC-7721 cells with 3 different JPHY doses (1.25, 2.5, and 5 mg/mL) in vitro. Finally, we explored its effects on expression of miR-602, *RASSF1A*, and tumor cell apoptosis–related markers in cells and liver tumor tissues. We hypothesized that the mechanism by which JPHY acts on HCC might be via miRNA-602 and the *RASSF1A* pathway.

Methods and Materials

Cell Culture

The human HCC cell line SMMC-7721 was kindly donated by the Tropical Medicine Institute of Guangzhou University of Chinese Medicine (GUCM; Guangzhou, China). We cultured the cells in Roswell Park Memorial Institute-1640 medium (GIBCO [Thermo Fisher Scientific, Waltham, MA]) supplemented with 10% fetal bovine serum (GIBCO), 100 units/mL penicillin, and 100 µg/mL streptomycin (GIBCO). Next, we incubated the cells at 37°C in a 95% humidified atmosphere containing 5% CO_2 in the laboratory of the Institute of Clinical Pharmacology at GUCM. The culture medium was routinely changed.

Establishment of an Orthotopic HCC Model

The animal study was approved by the Ethics Review Committee of the GUCM's Laboratory Animal Center (Approval Number 20181205011). We purchased 4- to 7-week-old nude mice from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and maintained them in a specific pathogen-free environment. All mice were treated according to GUCM's Laboratory Animal Center care guidelines. The SMMC-7721 cells in logarithmic growth phase were digested by trypsinase and resuspended in Roswell Park Memorial Institute-1640 medium. Cell density was adjusted to 1×10^7 cells/injection site. We prepared a 2% sodium pentobarbital solution by dissolving 2 g pentobarbital sodium powder (Sigma-Aldrich, St Louis, MO) in 100 mL sterile physiological saline (Li Tai Pharmaceutical Co, Ltd, Guangzhou, China). We then anesthetized the mice via intraperitoneal injection of this solution at a dose of 80 mg/kg. After iodophor (Shandong Li'Erkang Disinfecting Technology Co, Ltd, Shangdong, China) disinfection, we exposed the left lobe of the liver along the midline to the xiphoid process and controlled the incision at about 0.8 cm. Cells were extracted using a sterile micro-sampler and slowly injected into the liver. The lower lobe of the liver was compressed with a cotton swab (Guangzhou Yongyi Medical Equipment Co, Ltd, Guangzhou, China) for 30 seconds, then the abdominal cavity was closed. On awakening, the mice were returned to their cage for feeding. We routinely observed mice daily for heterogeneous abdominal masses. On the seventh day, we randomly selected 2 mice and reexposed the liver per the above-mentioned methods. Heterogeneous tumors were detected, and the abdomen was closed after examination.

Preparation of JPHY Formula

We purchased the TCM herbs that compose JPHY from GuangDong Zhi Sun Chinese Pharmaceutical Co, Ltd (Guangdong, China) The 6 raw herbs, mixed at a ratio of 1:1:1:1.67:1.67:1.67, were soaked in pure water (8× volume) for 30 minutes and decocted at 90°C to 100°C for 1.5 hours. We then poured out the extracts, filtered them into a beaker, concentrated them using a rotary evaporator (IKA RV10, Germany), calculated the concentration of the final extract at 2.4 g/mL, and stored it at −20°C.

High-Performance Liquid Chromatography (HPLC) Analysis

We qualitatively analyzed JPHY via HPLC. An ACQUITY UPLC I-Class-Xevo G2-XS QTof Agilent 7890B system (Waters Corp, Milford, MA), equipped with an evaporative light scattering detector, a quaternary-solvent delivery system, a column temperature controller, and an autosampler,

was used for chromatographic analysis. We separated JPHY and the mixed standard solution on an ACQUITY UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm) at 52°C. The mobile phase was composed of water (A) and acetonitrile (B). We performed programmed gradient elution as follows: 0 to 8 minutes, 5% to 10% B; 8 to 11 minutes, 90% to 100% B; and 11 to 30 minutes, 5% to 10% B. The flow rate was 1 mL/min. Monitoring was performed at 254 nm with a photodiode array detector. We recorded and processed the chromatographic data with Allchrom Plus client/server software.

Dose Determination

According to a previous study,[16] the equivalent dose ratio calculated based on body surface area (humans, 70 kg; mice, 0.020 kg) is 0.0026. According to the conversion formula, the equivalent dose for mice is 12.48 g/kg (96 g × 0.0026/0.020 kg = 12.48 g/kg). Therefore, we set the high dose of JPHY in this study at 24.96 g/kg.

Administration Method

Mice in the JPHY group received the high dose of the JPHY extract intragastrically; administration volume was 10.4 mL/kg. We treated the mice for 21 consecutive days. Mice in the control group were given equal volumes of saline.

Collection of Liver Cancer Tissues and Tumors

All mice were anesthetized and decapitated immediately after administration. After laparotomy, we removed the liver, carefully separated HCC tumors and the tissues adhering to them, and rapidly stored all liver cancer tissues and tumors in liquid nitrogen.

Preparation of JPHY for Cell Administration

We diluted the JPHY extract to 10 mg/mL and filtered it to remove impurities. The final working solution was stored at 4°C until use.

Methyl Thiazolyl Tetrazolium (MTT) Cell Viability Assay

We prepared the MTT solution by adding 50 mg MTT powder (Sigma-Aldrich) to 10 mL culture medium. The cultured SMMC-7721 cells were collected to prepare single-cell suspensions under aseptic conditions, and the suspensions were adjusted to a density of 8×10^4/mL. We seeded the cells in 96-well plates (Corning, Inc, Corning, NY) at a volume of 100 μL per well. After the cells were attached, we added JPHY to the 96-well plate at a concentration gradient of 10, 5, 2.5, 1.25, and 0.625 mg/mL. After culturing for 48 hours, we discarded the drug, added 10 μL MTT solution (concentration: 5 mg/mL) to each well, and continued the culture for 4 hours. Then, the supernatant was discarded, and 100 μL dimethyl sulfoxide (Chemical Reagent Co, Ltd, Guangzhou, China) was added to each well. After shaking the plate for 10 minutes, we measured optical density at a wavelength of 490 nm with a microplate reader (Thermo Fisher).

Determination of Drug Concentrations Administered to Cells

According to MTT results, we determined the drug concentration corresponding to the half-maximal inhibitory concentration (IC_{50}) to be the JPHY medium dose, the concentration corresponding to the three-fourth maximal inhibitory concentration (IC_{75}) inhibition rate to be the JPHY high dose, and the concentration corresponding to the one-fourth maximal inhibitory concentration (IC_{25}) to be the JPHY low dose.

Administration of JPHY to Cells

We collected the cultured SMMC-7721 cells to prepare a single-cell suspension under aseptic conditions, and the suspension was adjusted to a density of 3×10^5/mL. The cells were seeded in a 6-well plate (Corning) at a volume of 3 mL per well. After the cells had attached to the bottom of each well, we added the drugs to the plate at high, medium, and low concentrations. The control group received an equal amount of basal medium. After 48 hours culture, we discarded the supernatant and collected the cells in each well.

Hematoxylin and Eosin (H&E) Staining of Liver Cancer Tissues

We fixed liver cancer tissues in 4% paraformaldehyde (Biosharp, Beijing, China) for 2 to 3 days, dehydrated them using an automated tissue processor (Leica Microsystems, Wetzlar, Germany), embedded them in paraffin (Leica), serially sectioned them, and dried them overnight in an oven at 37°C. For staining with H&E (Beyotime Bioinstitute of Technology, Ltd, Jiangsu, China), we deparaffinized the sections using 2 changes of dimethylbenzene for 15 minutes and rehydrated them through a decreasing ethanol gradient (100%, 90%, and 80%) and then with water. Next, we stained the sections with hematoxylin for 8 minutes and rinsed them in tap water. Sections were differentiated using 0.5% acid alcohol fast-differentiation solution for 5 seconds, rinsed in tap water, stained with eosin for 5 minutes, and then washed in tap water. Finally, we dehydrated the

sections through an ascending alcohol gradient and with Histo-Clear (Atlanta, GA) before mounting. The images were analyzed using a light microscope and Leica QWin Plus software (both Leica).

4′,6-Diamidino-2-phenylindole (DAPI) Analysis of Cell Lines

Cells in logarithmic growth phase were evenly inoculated into a 6-well plate at a density of 5×10^5 cells/well and then cultured for 24 hours to allow the cells to adhere to the bottom of the well. We treated the cells with different concentrations of JPHY (0, 5, 1.24, and 2.5 mg/mL). After 48 hours of continuous culture in an incubator, we discarded the supernatant. DAPI (Solarbio, Beijing, China) staining was performed after fixing the SMMC-7721 cells in 4% formaldehyde for 10 minutes. After 3 washes with phosphate-buffered saline, we stained the cells with DAPI in the dark for 15 minutes at room temperature (RT) and then washed them 3 times with tap water. Images were obtained using a fluorescence microscope (20 × 10; Leica).

Annexin V–Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI)Apoptosis Assay

We purchased an Annexin V–FITC/PI double-staining kit from KeyGen Biotech Co, Ltd (Nanjing, China). Cells were treated as described above. After 48 hours of continuous culture in an incubator, we collected and centrifuged cells for 10 minutes at 1500 rpm, discarded the supernatant, and added precooled phosphate-buffered saline to resuspend the cells in each tube. Approximately 500 μL buffer, 5 μL Annexin V–FITC, and 5 μL PI were added to stain the cells for 30 minutes, with light shielding, followed by analysis using an Elite flow cytometer (FCM; Beckman Coulter, Brea, CA).

Quantitative Reverse-Transcription Polymerase Chain Reaction

We extracted total RNA from the SMMC-7721 cells and liver cancer tissues using an RNA extraction kit (Beyotime) per manufacturer's protocol. Concentration of total RNA was measured using a NanoDrop Spectrophotometer (Thermo Fisher). We used a Goldenstar RT6 complementary deoxyribonucleic acid synthesis kit (TsingKe, Guangzhou, China) for reverse transcription. Primer sequences used for reverse transcription of miR-602 were 5′-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG CGG GCC GCA-3′, and for the control U6, Oligo. We determined RNA expression via real-time polymerase chain reaction using SYBR Green Master Mix (TsingKe). Results were normalized to the expression of U6. The primer sequences used for miR-602 were F: 5′-TCG GCA GGG ACA CGG GCG ACA G-3′, R: 5′-CTC AAC TGG TGT CGT GGA-3′; for RASSF1A, F: 5′-GAA GTC ATT GAG GCC CTG CT-3′, R: 5′-ATC ATC CAA CAG CTT CCG CA-3′; and for the control U6, F: 5′-CTC GCT TCG GCA GCA CA-3′, R: 5′-AAC GCT TCA CGA ATT TGC GT-3′, with product lengths of 60, 188, and 96 bp, respectively.

Western Blot Analysis of Cells and Liver Cancer Tissues

We purchased primary antibodies against anti-RASSF1A from Abcam (Cambridge, UK). B-cell lymphoma 2 (Bcl-2), Caspase-8, and Caspase-9 antibodies were purchased from Cell Signaling Technology, Inc, (CST; Danvers, MA). Anti–Bcl-2-associated X protein (anti-BAX) antibody was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX), and anti–β-actin was purchased from Sigma-Aldrich. All secondary antibodies (horseradish peroxidase–conjugated anti-rabbit immunoglobulin G [IgG] and anti-mouse IgG) were purchased from CST. All other reagents were of the highest grade available commercially. We homogenized the SMMC-7721 cells and mouse liver cancer tissues and lysed them in sample buffer (0.5 M Tris/HCl, pH 6.8, 50% glycerol, 10% sodium dodecyl sulfate [SDS], 1:100 inhibitor protease/phosphatase cocktail). The lysate was centrifuged at 12 000*g* for 10 minutes at 4°C and then denatured by boiling at 100°C with 1:4 loading buffer. We fractionated the same amount of protein (50 μg) using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred it onto polyvinylide fluoride membranes, which we blocked with 7% skim milk dissolved in Tris-buffered saline–Tween-20 for 2 hours at RT. Membranes containing the protein were incubated with the anti-RASSF1A, anti–Caspase-8, anti–Caspase-9, anti–Bcl-2, anti-BAX, and mouse anti–β-actin antibodies overnight at 4°C. Then, the membrane was incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG for 2 hours at RT. We monitored protein load using an electrochemiluminescence reagent (Merck KGaA, Darmstadt, Germany).

Statistical Analysis

Experimental values are expressed as mean ± standard deviation. Statistical comparisons between 2 groups were evaluated using a 2-tailed unpaired *t* test. We applied a 1-way analysis of variance to analyze differences in biochemical parameter data among different groups, followed by Dunnett's post hoc test for pairwise multiple comparisons. A P value <.05 was considered statistically significant ($^*P < .05$, $^{**}P < .01$, $^{***}P < .001$). Statistical analyses were performed using SPSS statistical software version 23.0 (IBM, Armonk, NY).

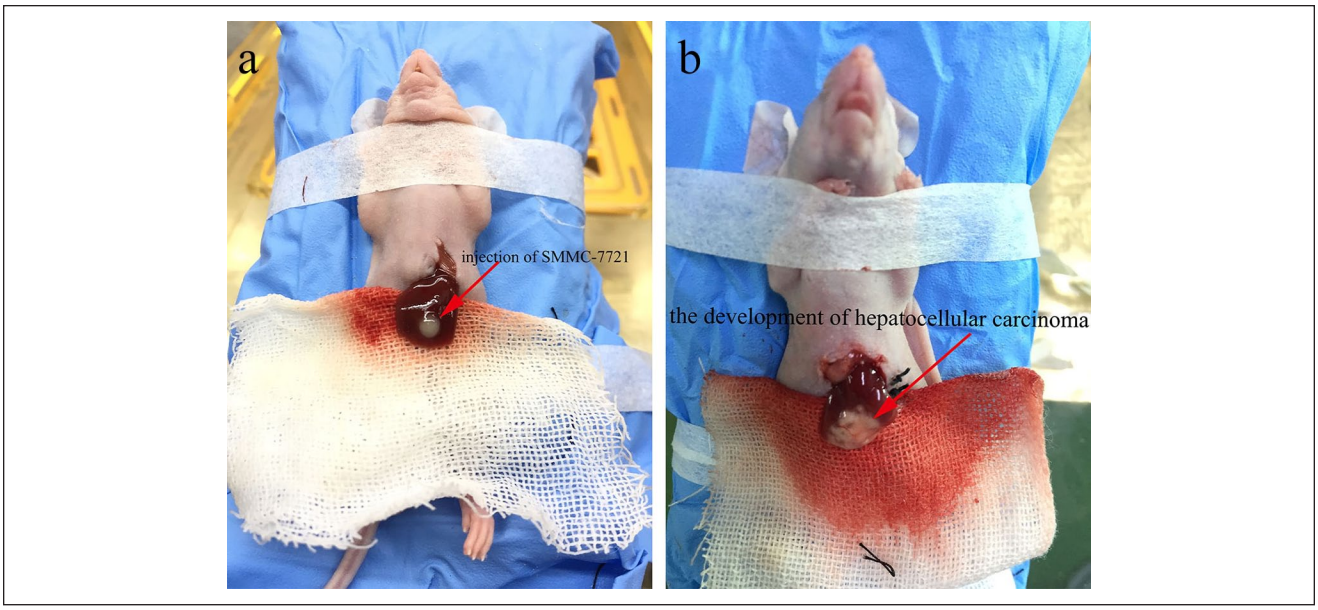


Figure 1. (a) SMMC-7721 cells were injected into the lower margins of hepatic lobes of the mice, ensuring that no leakage occurred. (b) Seven days later, we observed the liver tissue injected with HCC cells via laparotomy to assess HCC tissue transformation, indicating success in constructing a model.

Results

Successful Establishment of Orthotopic HCC Model In Vivo

Disease models are essential for more in-depth research investigations. Our research team has made extensive efforts to construct such models (Figure 1a and b).

HPLC Analysis of JPHY

We chemically characterized JPHY's components using HPLC. Figure 2 shows that 17 compounds were identified as major peaks in the HPLC chromatogram, which included galactosamine (Peak 1), stachydrine (Peak 2), isomatrine (Peak 3), 7,11-dehydromatrine (Peak 4), sophoranol (Peak 5), formononetin (Peak 6), Ar-curcumene (Peak 7), shizukanolide A (Peak 8), (10E)1,10-heptadeca-diene-4,6-diyne-3,8,9-triol (Peak 9), murragatin (Peak 10), 23-acetate alisomalactone (Peak 11), 24-acetate alisol F (Peak 12), sanjoinine (Peak 13), 11α-O-(2-methylbutyryl)-12β-O-acetyltenacigenin B (Peak 14), chelerythrine (Peak 15), (E)-hexadecyl-ferulate (Peak 16), and chebuloside II (Peak 17).

JPHY Inhibited Tumor Development and Improved the Survival Rate of Mice

To investigate whether JPHY could inhibit tumor growth, we monitored mouse body weight, measured the sizes of tumors protruding from the body surface, and recorded mouse mortality rate. The weight of the JPHY group was higher than that of the control group in the first 18 days, though not significantly so ($P > .05$), but it became significantly higher at 21 days ($P < .001$; Figure 3a). On the sixth day, after the tumor protruded from the body surface, we measured tumor volume according to the following formula:

$$V = (\text{Width})^2 \times \text{Length}/2\ (\text{mm}^3).$$

The results showed that the tumor volume of the JPHY group did not significantly differ from that of the control group in the first 12 days ($P > .05$), but on the 15th, 18th, and 21st days, it significantly decreased ($P < .05$, $P < .05$, $P < .01$, respectively; Figure 3b). At the same time, we observed that some mice in the control group exhibited cachexia (malnutrition, weight loss, and muscle atrophy) compared with the mice in the JPHY group (Figure 3c). After the mice were sacrificed, liver cancer tissues and tumors were carefully peeled off, and the tumors were weighed. The results showed that the tumor weight of the JPHY group was significantly lower than that of the control group ($P < .001$; Figure 3d and e). H&E staining of tumor tissues revealed 1 to 2 cancer nodules in the JPHY group. The tumor cells were arranged in a gland-like manner, with a small amount of infiltrating inflammatory cells, showing the central vein and a hepatic-lobule structure. However, in the control group, we observed multiple cancer nodules; the tumor cells showed invasive growth accompanied by infiltration of a great number of inflammatory cells, which

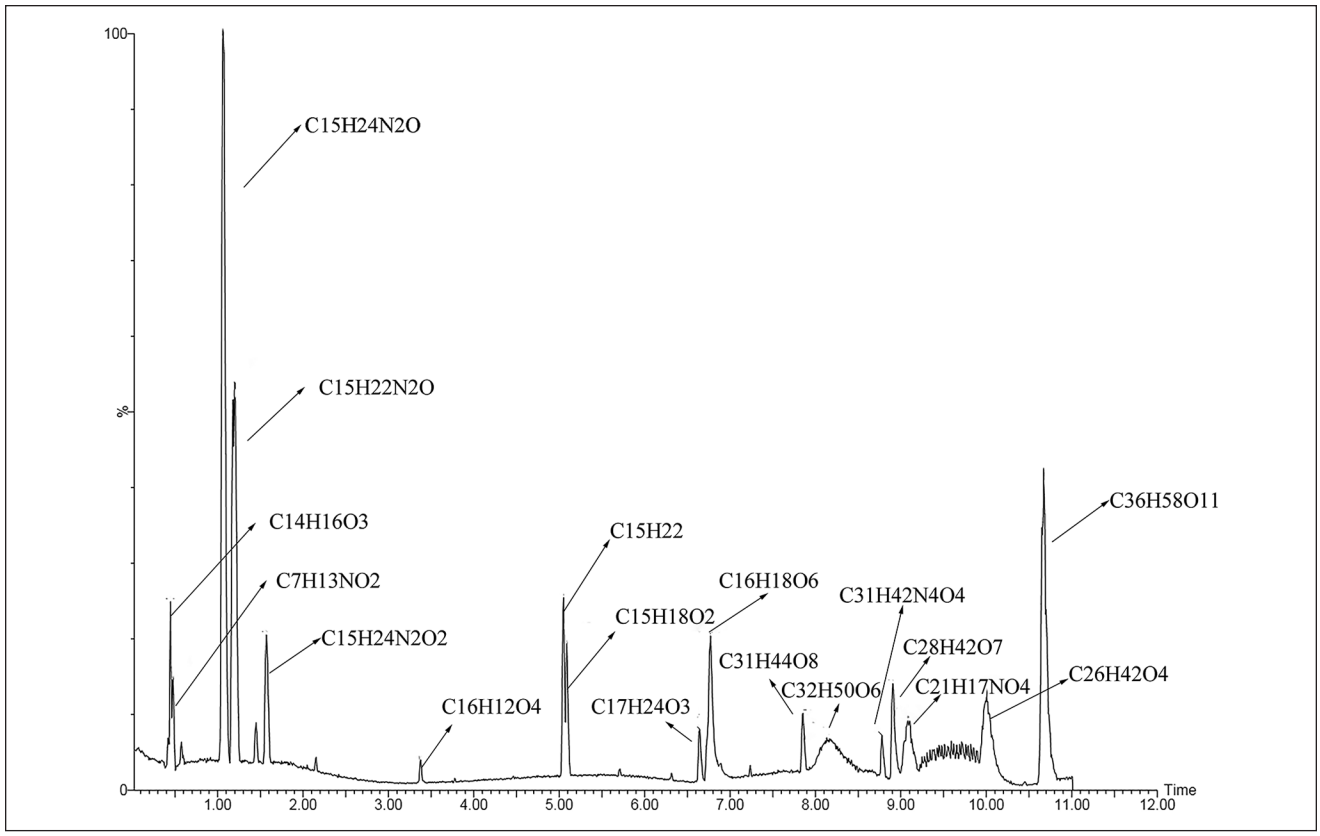


Figure 2. Chemical standardization of JPHY as analyzed by HPLC.

destroyed the normal hepatic-lobular structure and resulted in rupture of the sinusoids (Figure 3f). These findings implied that treatment with JPHY was beneficial for suppression of HCC tumors.

JPHY Caused Morphological Changes in SMMC-7721 Cells and Inhibited Their Proliferation In Vitro

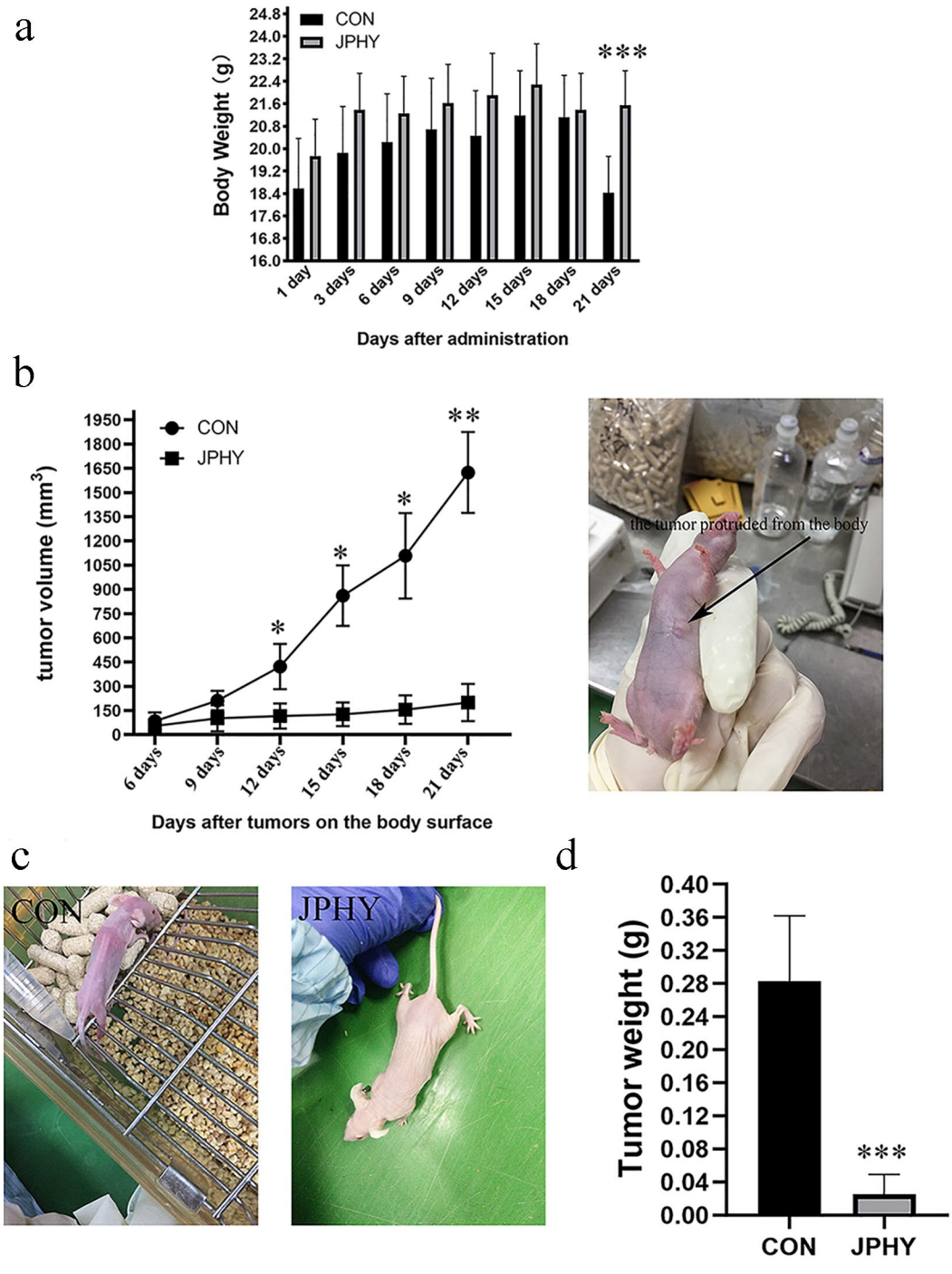
Approximately 48 hours after administering JPHY to SMMC-7721 cells, we performed light microscopy, which revealed irregular morphology of the cells and disintegration of the cellular membrane. These changes were concentration dependent (Figure 4a). We assessed SMMC-7721 cell viability using an MTT assay with 5 mg/mL JPHY as the high dose, 2.5 mg/mL as the middle dose, and 1.25 mg/mL as the low dose (Figure 4b). The results suggested that JPHY could inhibit the proliferation of SMMC-7721 cells.

JPHY Promoted Apoptosis of SMMC-7721 Cells In Vitro

We assessed apoptosis of SMMC-7721 cells as compared with the control group by FCM and DAPI staining (Figure 5a). The apoptosis rate of SMMC-7721 cells in the high-, middle-, and low-dose JPHY groups significantly increased ($P < .05$, $P < .01$, $P < .001$, respectively; Figure 5b). These findings indicated that JPHY promoted the apoptosis of HCC cells.

Antitumor Effect of JPHY Might Involve the Pathway of miR-602, Which Targets RASSF1A

Studies have reported that miR-602 levels are elevated in liver cancer patients,[12-14] which in turn inhibits expression of the tumor suppressor gene *RASSF1A*, thereby promoting the development of liver cancer.[14] Our previous studies have confirmed that JPHY exerts a regulatory effect on multiple signaling pathways.[15,16] Therefore, in this study we hypothesized that JPHY inhibited expression of miRNA-602 and increased that of *RASSF1A*. To test this hypothesis, we examined the expression of miRNA-602 and *RASSF1A* in mouse liver cancer tissues, as well as that of tumor apoptosis–related genes such as *Caspase-8* and *-9, BAX*, and *Bcl-2*. The results showed that compared with the control group, the expression of miRNA-602 ($P < .01$; Figure 6a), and *RASSF1A* ($P < .001$; Figure 6b and c) in the JPHY group decreased, that of *Caspase-8* and *-9* and of *BAX* was upregulated, whereas *Bcl-2* expression was downregulated ($P < .001$, $P < .05$, $P < .001$, $P < .001$, respectively; Figure 6d


a
Body Weight (g)
CON
JPHY
***
Days after administration
b
tumor volume (mm3)
CON
JPHY
Days after tumors on the body surface
the tumor protruded from the body
c
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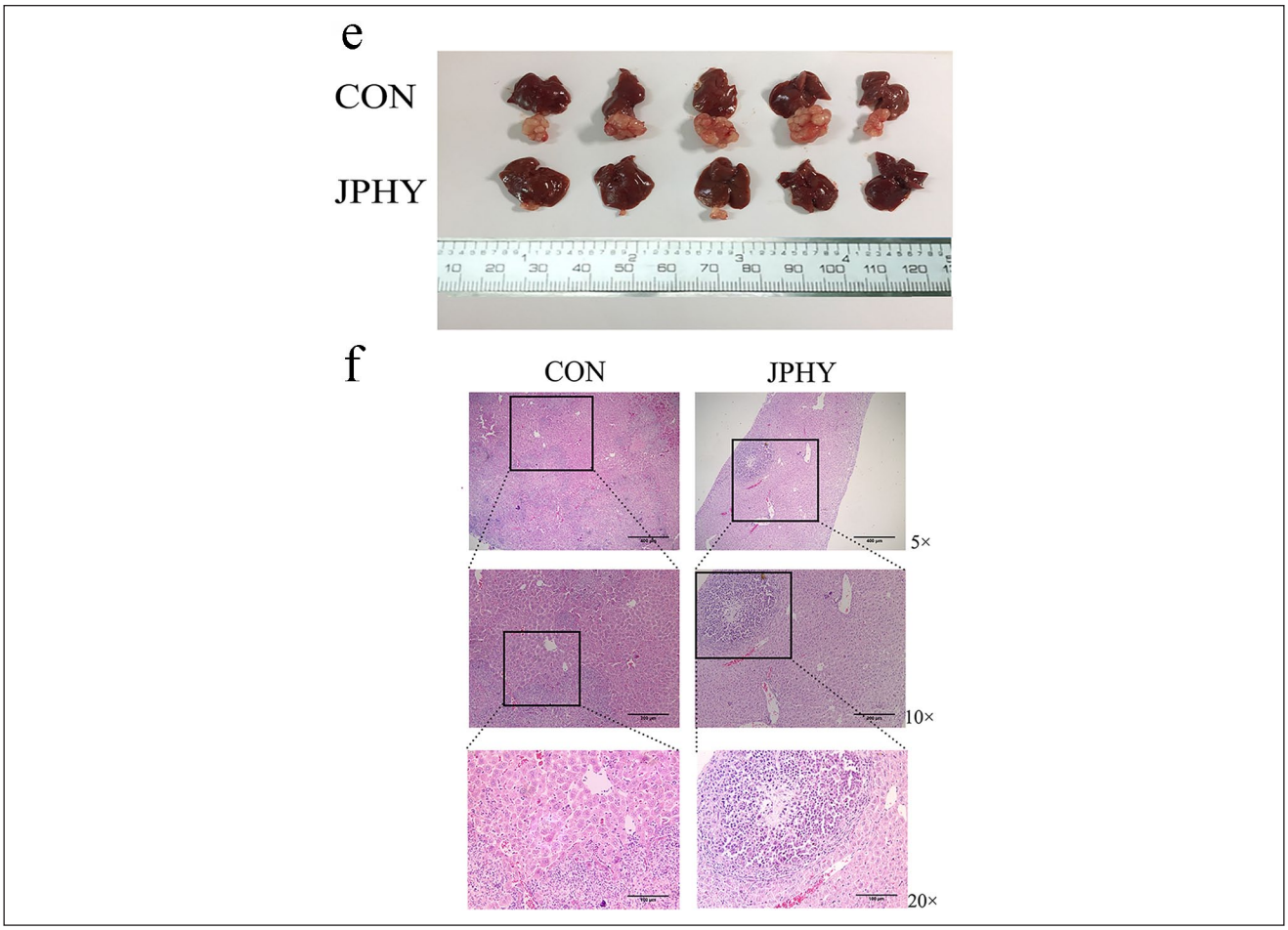


Figure 3. Changes in body weight (a) and protruding body surface tumors (b) in mice. Comparison of mouse body conditions at later stages (c), tumor size (d, e), and H&E-stained liver cancer tissues of mice (f).

and e). Our hypothesis was also confirmed in cell experiments. We detected the expression of the above-mentioned genes in SMMC-7721 cells, and the results coincided with our in vivo findings. Compared with the control group, expression levels of miR-602 in the low-, middle-, and high-dose groups of JPHY were downregulated ($P < .001$, $P < .001$, $P < .001$, respectively; Figure 6f), but that of *RASSF1A* was significantly upregulated in the high-dose group only ($P < .001$, $P < .05$; Figure 6g and h). Similarly, when we detected protein levels of apoptosis-related markers, low, middle, and high doses of JPYH had regulatory effects on the markers *Caspase-8* ($P < .01$, $P < .001$, $P < .01$, respectively), *Caspase-9* ($P < .001$, $P < .001$, $P < .001$, respectively), *BAX* ($P < .001$, $P < .05$, $P < .001$, respectively), and *Bcl-2* ($P < .01$, $P < .001$, $P < .05$, respectively), but not dose-dependently (Figure 6i and j).

Discussion

HCC is a serious threat to human health. Its occurrence and development are complex, involving several intricate mechanisms such as activation of proto-oncogenes and immune escape. Since HCC involves multiple mechanisms and targets, multicomponent and multitarget drugs such as those used in TCM might be helpful for its treatment. Although there are many methods of treating HCC clinically, these methods have some limitations.[18-20] Several studies have proven that TCM or extracts thereof inhibit the development of HCC, as well as reduce the occurrence of adverse effects such as those associated with the long-term use of radiotherapy, chemotherapy, and targeted drugs.[21]

This study showed that JPHY has superior ability to inhibit tumors. We evaluated its antitumor effects in an orthotopic HCC mouse model and its proapoptotic effect in SMMC-7721 cells. Treatment with JPHY significantly inhibited the growth of tumors and improved the survival rate in vivo. We also verified the proapoptotic effect of JPHY in this study. JPHY induced changes in cell morphology and promoted apoptosis in vitro. In our mechanism study, we observed that JPHY inhibited the expression of miR-602 and increased that of *RASSF1A*. This experimental evidence showed that JPHY could be potentially used for the treatment of HCC.

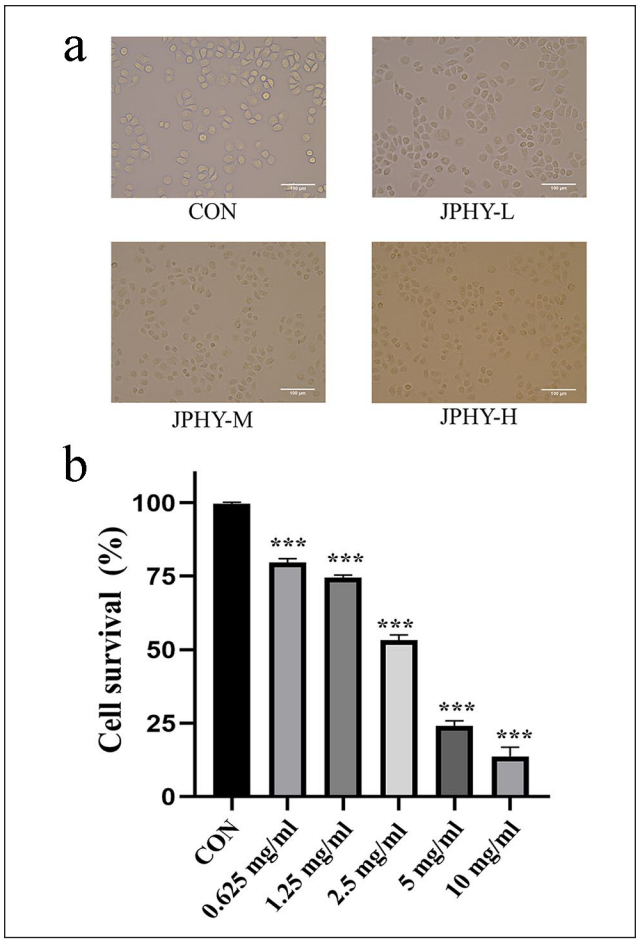


Figure 4. Changes in cell morphology using different JPHY concentrations (after 48 hours of treatment) (a). Effects of different JPHY concentrations on cell proliferation as measured by MTT assay (b).

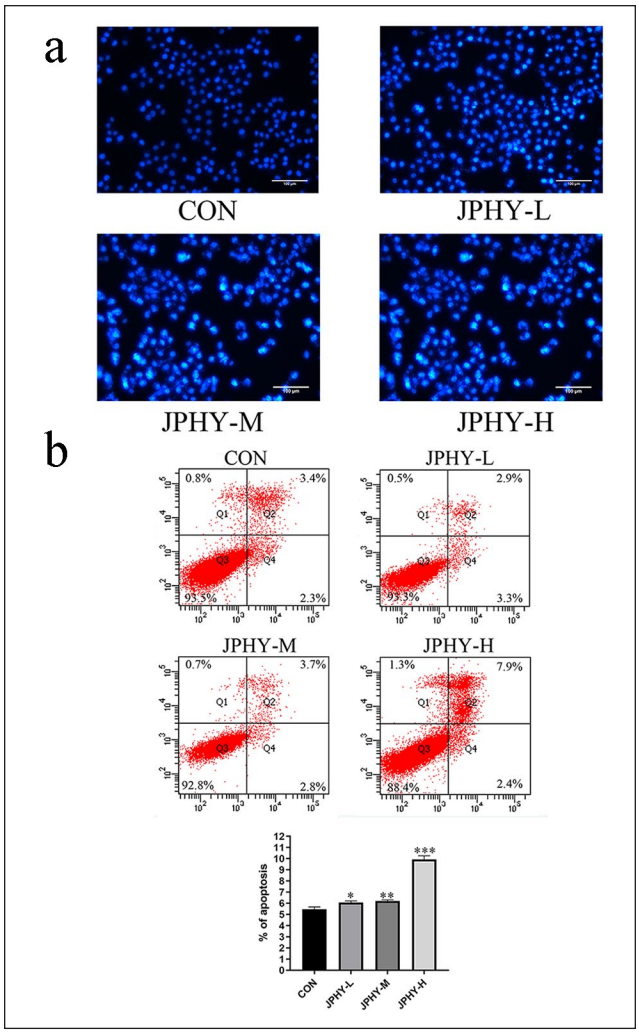
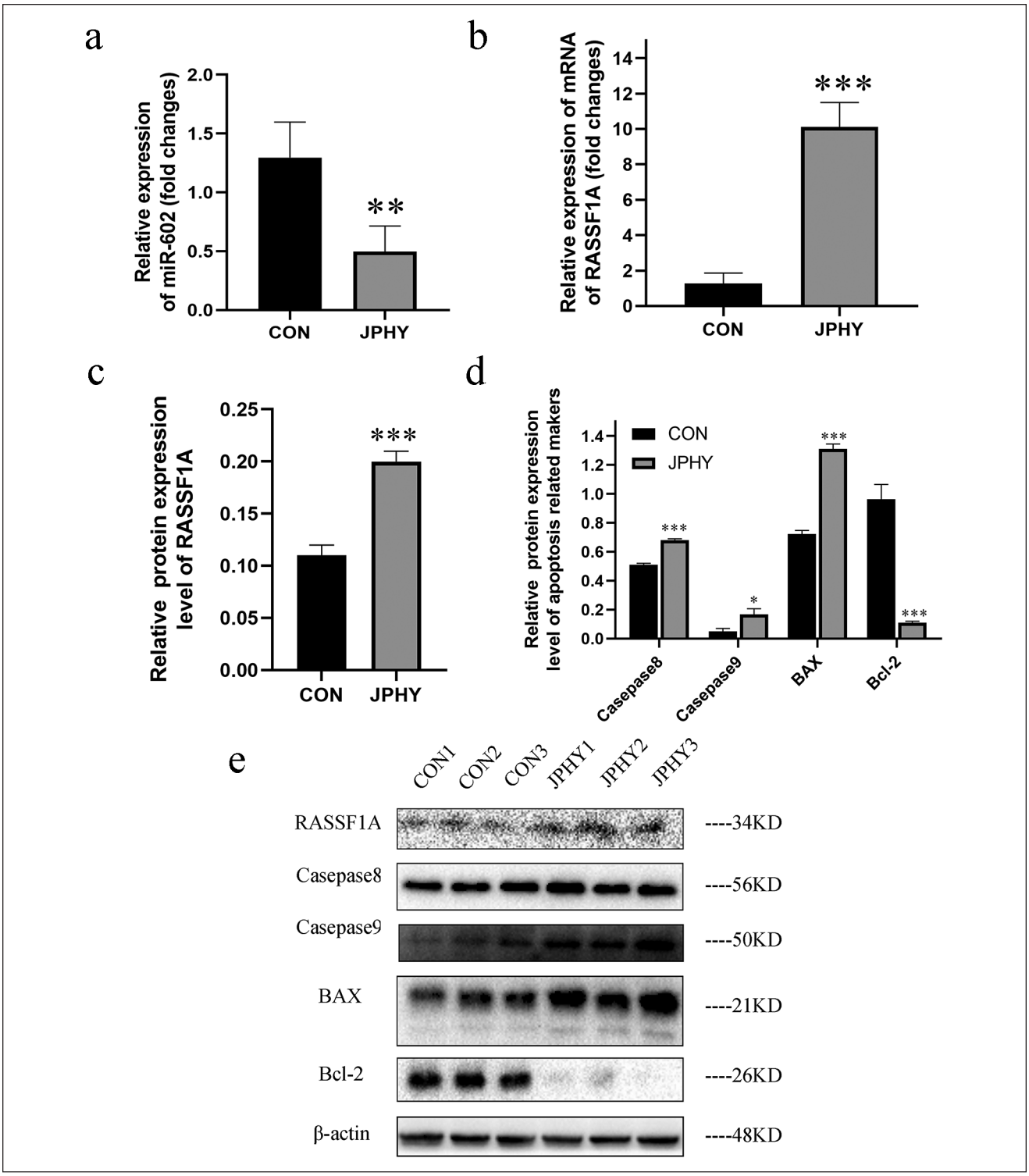


Figure 5. Effect of different JPHY concentrations on apoptosis as measured by DAPI (a) and FCM (b).

JPHY contains a variety of complex components, which we therefore detected. We found that it contains more than 200 components. Fraxinellone, stachydrine, isomatrine, 7,11-dehydromatrine, sophoranol, formononetin, Ar-curcumene, shizukanolide A, (10E)1,10-heptadeca-diene-4,6-diyne-3,8,9-triol, murragatin, and 23-acetate alisomalactone, 24-acetate alisol F, sanjoinine, 11α-O-(2-methylbutyryl)-12β-O-acetyltenacigenin B, chelerythrine, (E)-hexadecyl-ferulate, and chebuloside II were identified as the most abundant monomer compounds in JPHY. Some of these small molecules have been shown to trigger apoptosis,[22,23] ameliorate anoxia/reoxygenation,[24] induce tumor cell cycle arrest,[25] reverse chemoresistance,[26] inhibit tumor metastasis,[27] and exhibit antioxidant, anti-inflammatory, and antinociceptive activity.[28] We speculate that these components are what render JPHY's antitumor effects, but we have not analyzed all components of JPHY nor reviewed their interactions. Therefore, the details of JPHY's mechanism require further investigation.

In vivo, we injected human HCC SMMC-7721 cells into the livers of mice to establish an orthotopic HCC model. Our experimental results showed that JPHY significantly inhibited the growth of HCC. In vitro, our MTT results also showed that JPHY had cytotoxic effects on HCC cells and could inhibit their proliferation. FCM and DAPI staining analyses indicated that the mechanism by which JPHY inhibited tumors possibly involved induction of apoptosis in tumors. *RASSF1A* is a well-known protective gene that can inhibit tumor development by triggering apoptosis in tumors.[29] It has been proven that miR-602 can inhibit expression of the *RASSF1A* gene. This study explored whether JPHY induced apoptosis of HCC cells by regulating the miR-602/*RASSF1A* pathway, ultimately affecting the development of HCC. Our in vivo and in vitro experiments showed that under JPHY intervention, expression of miR-602 was downregulated and that of *RASSF1A* was upregulated in HCC tissues and cells. Further research on


a
Relative expression of miR-602 (fold changes)
2.0
1.5
1.0
0.5
0.0
**
CON
JPHY
b
Relative expression of mRNA of RASSF1A (fold changes)
14
12
10
8
6
4
2
0
***
CON
JPHY
c
Relative protein expression level of RASSF1A
0.25
0.20
0.15
0.10
0.05
0.00
***
CON
JPHY
d
Relative protein expression level of apoptosis related makers
CON
JPHY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
*
***
***
Casepase8
Casepase9
BAX
Bcl-2
e
CON1
CON2
CON3
JPHY1
JPHY2
JPHY3
RASSF1A
----34KD
Casepase8
----56KD
Casepase9
----50KD
BAX
----21KD
Bcl-2
----26KD
β-actin
----48KD

(cotinued)

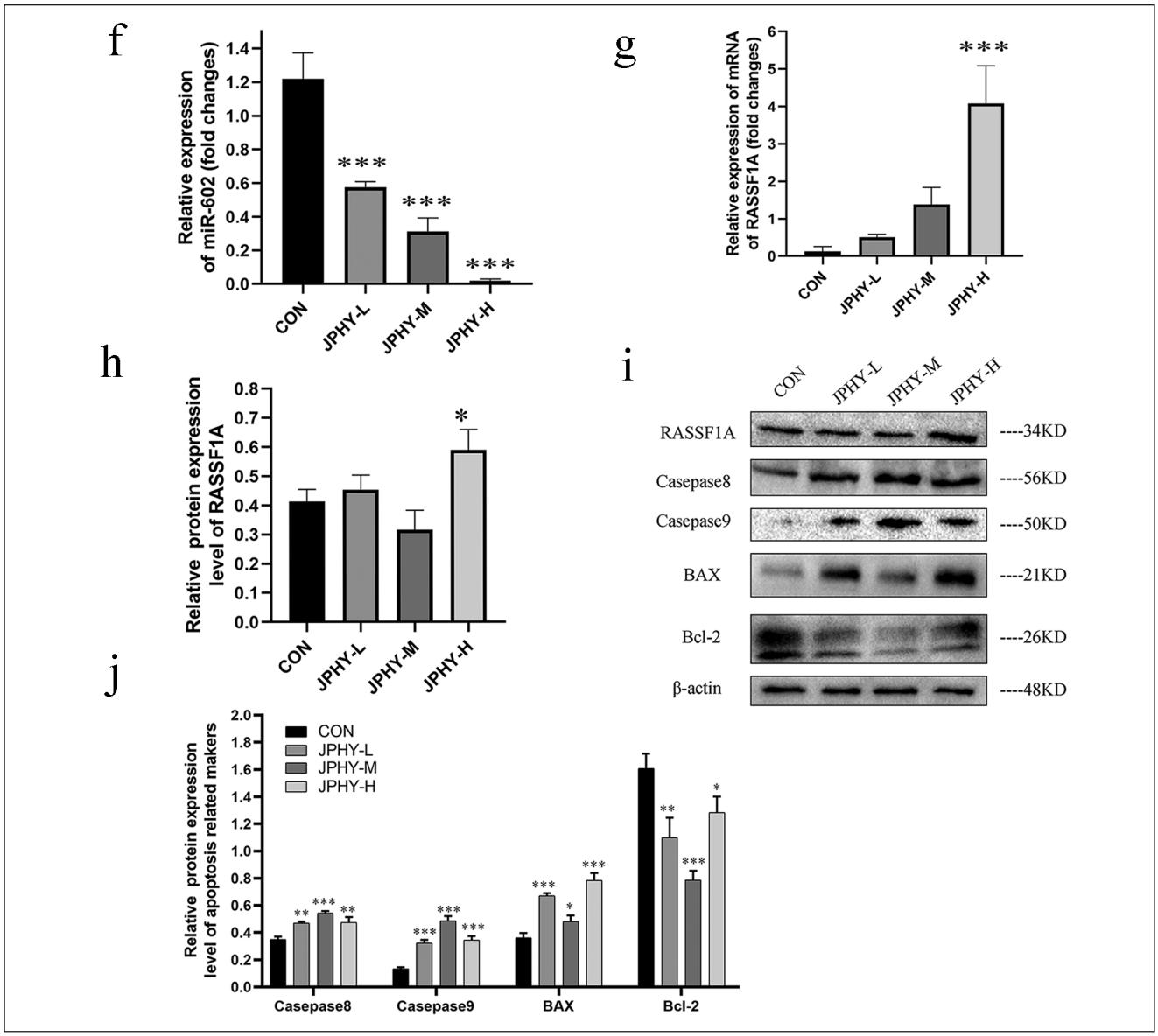


Figure 6. In vivo effects of JPHY on expression of miR-602 (a), RASSF1A (b, c), and tumor apoptosis–related markers (d, e) in mouse liver cancer tissues. The in vitro effects of low, medium, and high doses of JPHY on the expression of miR-602 (f), RASSF1A (g, h), and tumor apoptosis–related markers (i, j) in SMMC-7721 cells.

the *RASSF1A* gene in relation to HBV-related HCC is thus warranted.[14] Although our research confirmed that JPHY affected the development of HCC by regulating the miR-602/*RASSF1A* pathway, it was independent of HBV-related HCC. Based on the results of this study, we were prompted to determine whether miR-602 could still inhibit the *RASSF1A* gene in non–HBV-related HCC. Our research group has conducted relevant research; the current data show that miR-602 is inversely related to *RASSF1A* in SMMC-7721 cells.

Apoptosis is an autonomous, gene-regulated process that plays an important role in organism development and survival. Apoptotic defects are an important etiological basis of tumorigenesis. Caspase is a member of the interleukin-1 β-converting enzyme family and plays a key role in apoptosis. Caspase-8 and -9 are apoptotic initiators located upstream of the cascade reaction. Their roles lie in cell surfaces[30] and mitochondria.[31] They can self-activate with the participation of other proteins and recognize and activate downstream Caspase. Via western blot analysis, we found that JPHY upregulated the expression of Caspase-8 and -9. Bcl-2 and BAX are 2 typical antiapoptotic and proapoptotic proteins; they regulate cell apoptosis by forming homologous or heterodimers. They also directly determine the permeability of

various channels of mitochondrial extracorporeal membrane and thus cell survival.[32] We found that JPHY increased the expression level of BAX and decreased that of Bcl-2, resulting in a decrease in the Bcl-2:BAX ratio.

Although there have been numerous investigations into the antitumor mechanisms of TCM herbal compounds or monomers, our study is the first to explore the axis of TCM herbal compounds, miR-602, and *RASSF1A*. Compared with other research drugs, TCM herbal compounds exhibit a greater relationship between effect and concentration. We found that JPHY could promote apoptosis of cancer cells, but the effect was not concentration dependent. The reason could be that other studies feature 10× or even 100× differences between high- and low-dosage groups of TCMs; the differences between our concentrations were not huge and thus did not result in any significant difference between the high- and low-dosage groups. Another possible explanation is that different drug concentrations have various regulatory effects on genes, so that expression of Caspase-8 and -9 in the high-dose JPHY group was lower than that in the middle-dose group. However, the expression profiles of Bcl-2 and BAX showed the opposite.

There were many shortcomings in this study. First, we did not use a positive drug group. It is impossible to observe the advantages and disadvantages of JPHY and positive drugs during treatment, or whether JPHY has fewer side effects than a positive drug. Second, due to limitations in experimental conditions, it is impossible to conduct dynamic observation of animal tumors. The use of positron emission tomography-computed tomography might better illustrate the inhibitory effect of JPHY on tumors. Third, we did not measure the level of RASSF1A methylation in this study because RASSF1A expression is inactivated in most tumors due to methylation,[33] resulting in low expression levels, and some samples cannot even be detected. Fourth, this study did not detect apoptosis-executing molecules such as Caspase-3, -6, and -7, or inflammatory mediators such as Caspase-1, -4, -5, and -11; nor did we study the configuration of BAX, because BAX-mediated apoptosis involves oligomerization.[34]

These shortcomings must be considered in future research. In addition, p38 MAPK can promote apoptosis through phosphorylation of p53,[35] participation in Fas/Fas ligand (FasL)–mediated apoptosis,[34] and induction of BAX translocation.[36] RASSF1A can promote cell apoptosis by upregulating apoptosis signal-regulating kinase 1[6] of which p38 MAPK is the downstream kinase. Therefore, we hypothesize that expression of the *RASSF1A* gene activates the p38 MAPK signaling pathway, thereby affecting the development of HCC. This hypothesis, and the question of whether JPHY plays a regulatory role in the *RASSF1A*/p38 MAPK pathway, will be the focus of our future research.

Author's Note

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Author Contributions

YH designed, analyzed the data, and wrote the manuscript. CW and QW were involved in the writing of the manuscript. CZ, YX, HW, CY, JL, XH, XL, CY, YM, and YM performed the experiments. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

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Ethical Approval

Animal experiments were conducted in accordance with the guidelines of the Animal Research Ethics Board of Guangzhou University of Chinese Medicine. This ethics board also approved the animal studies.

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