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Pien Tze Huang inhibits the growth of hepatocellular carcinoma cells by upregulating miR-16 expression

FEI QI[1,2*], SONGQIANG ZHOU[3*], LI LI[4], LIHUI WEI[1,2], ALING SHEN[1], LIYA LIU[1], YAODONG WANG[3] and JUN PENG[1,2]

[1]Academy of Integrative Medicine; [2]Fujian Key Laboratory of Integrative Medicine on Geriatrics, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122; Departments of [3]Hepatobiliary Surgery and [4]Disease Prevention and Healthcare, Fujian Provincial Hospital, Fujian Medical University, Fuzhou, Fujian 350001, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) is characterized by uncontrolled proliferation and the deregulation of apoptotic signaling, although its molecular pathogenesis is not fully characterized. The ability to inhibit excessive proliferation and induce the apoptosis of cancer cells are crucial characteristics of anticancer drugs. Pien Tze Huang (PZH) is a widely used traditional Chinese medicine for the treatment of various types of cancer, and has exhibited promising therapeutic effects in clinical trials of HCC. However, the underlying mechanisms for its action are unclear. In the present study, the aim was to explore the effect of PZH on the proliferation and apoptosis of the BEL-7402 HCC cell line, and the associated mechanisms. PZH treatment significantly inhibited BEL-7402 cell viability, confluence and clonogenicity, inducing cell cycle arrest and promoting apoptosis. In addition, PZH treatment suppressed the expression of the pro-proliferative genes cyclin D1 and cyclin-dependent kinase 4, and decreased the expression of the anti-apoptotic gene Bcl-2. PZH treatment also upregulated the expression of a key microRNA (miR), miR-16. The study demonstrated that PZH can effectively inhibit cancer cell proliferation and induce apoptosis in BEL-7402 HCC cells via the upregulation of the tumor suppressor miR-16.

Correspondence to: Dr Yaodong Wang, Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Fujian Medical University, 134 Dongjie, Fuzhou, Fujian 350001, P.R. China
E-mail: wangyaodongch@163.com

Dr Jun Peng, Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, 1 Qiuyang Road, Shangjie, Minhou, Fuzhou, Fujian 350122, P.R. China
E-mail: pjunlab@hotmail.com

*Contributed equally

Abbreviations: HCC, hepatocellular carcinoma; PZH, Pien Tze Huang; TCM, traditional Chinese medicine

Key words: Pien Tze Huang, hepatocellular carcinoma, proliferation, apoptosis, microRNA-16

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes for cancer-associated mortality, with one of the fastest-rising morbidity and mortality rates worldwide (1-3). Although surgical resection, systemic chemotherapy and targeted cancer therapy are widely used, the response to therapy and prognosis of patients with HCC remain suboptimal due to the development of drug resistance and severe adverse side effects (4-8). Therefore, it is essential to explore and develop novel strategies for the control and treatment of HCC.

Cancer cells are characterized by uncontrolled proliferation and the deregulation of apoptotic signaling (9). Cell proliferation is primarily regulated by cell cycle checkpoints. One of the major cell cycle checkpoints is the G1/S checkpoint; G1/S progression is regulated by the pro-proliferative cyclin D1/cyclin-dependent kinase (CDK)4 complex (10,11). The expression of cyclin D1 and CDK4 are often upregulated in various types of cancer (12-14). Bcl-2 serves a critical role in inhibiting apoptosis and is overexpressed in numerous types of cancer (15-18). The ability to inhibit excessive proliferation and induce the apoptosis of cancer cells is paramount in the development of anticancer drugs.

MicroRNAs (miRNAs/miRs) are a class of endogenous short noncoding RNAs that primarily suppress gene expression by specifically binding to the 3'-untranslated region of target mRNAs (19-21). A single miRNA can modulate the expression of hundreds of different targets and may therefore be implicated in a broad range of physiological and pathological processes (22,23). It has been demonstrated that miRNAs may function as oncogenes or tumor suppressors to modulate multiple oncogenic cellular processes, including cell proliferation, apoptosis, invasion and metastasis (24-26). miR-16 is localized at chromosome 13q14.3, and is downregulated in the majority of patients with chronic lymphocytic leukemia (CLL) (27) and HCC (28). It has been reported that the upregulation of miR-16 inhibits cell proliferation, induces cell cycle arrest and increases the rate of apoptosis by downregulating the expression of Bcl-2 in CLL, colorectal cancer

and HCC (29,30). It has also been demonstrated that miR-16 may inhibit tumor cell proliferation by targeting cyclin D1 and CDK4 to induce cell cycle arrest (31-33).

Traditional Chinese medicine (TCM) has been used in China for thousands of years and may provide treatment with multi-target and multi-level intervention against various types of cancer, with relatively few side effects (34,35). Pien Tze Huang (PZH), a well-known TCM formula that originated in the Chinese Ming Dynasty >450 years ago, has been widely used in China and Southeast Asia as a remedy for various diseases, including cancer (36). It was previously demonstrated that PZH may inhibit colon cancer growth via multiple mechanisms (37-49) and PZH has exhibited promising therapeutic effects in clinical trials regarding HCC (50,51). However, the effect of PZH on HCC, including on miR-16 expression level, has not been evaluated; therefore, the present study aimed to explore the effect of PZH on the proliferation and apoptosis of the HCC BEL-7402 cell line.

Materials and methods

Materials and reagents. RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A Hoechst staining kit was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). A BD Pharmingen™ Cell Cycle kit was obtained from BD Biosciences (San Jose, CA, USA). An RNAiso Plus for Total RNA kit, an RNAiso for microRNA kit, a PrimeScript™ RT reagent kit and a SYBR® PrimeScript™ miRNA RT-PCR kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). SYBR® Select Master Mix was purchased from Thermo Fisher Scientific, Inc. Antibodies against CDK4 (cat no. 2906S), cyclin D1 (cat no. 2978S), Bcl-2 (cat no. 15071S) and β-actin (cat no. 4967S), horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G, (cat no. 7074P2) and HRP-conjugated horse anti-mouse IgG (cat no. 7076S) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Preparation of PZH. PZH was obtained from and authenticated by Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd. (Zhangzhou, China; Chinese Food and Drug Administration approval no. Z35020242). PZH was prepared by dissolving in PBS to a stock concentration of 20 mg/ml, which was stored at -20˚C. Dissolving the stock solution in RPMI-1640 to varying concentrations produced the working concentrations of PZH.

Cell culture. Human HCC BEL-7402 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium containing 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained in a humidified incubator at 37˚C with 5% CO_2.

Evaluation of cell viability by MTT assay. Cell viability was assessed by an MTT assay. BEL-7402 cells were seeded in 96-well plates at a density of $8x10^3$ cells/well in 100 μl medium. Cells were incubated overnight and treated with various concentrations (0, 0.25, 0.5 and 0.75 mg/ml) of PZH for 24, 48 or 72 h. An MTT assay was subsequently performed by the addition of 100 μl MTT reagent (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and 0.5 mg/ml PBS into each well, followed by incubation for 4 h at 37˚C. The resulting purple-blue MTT formazan precipitate was dissolved in 100 μl DMSO. The optical density (OD) at 570 nm was measured with an ELISA reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was determined using the following formula: Cell viability = (absorbance of the experimental samples/absorbance of the control samples) x100%.

Observation of cell confluence. BEL-7402 cells were seeded into 6-well plates at a density of $2.5x10^5$ cells/well and treated with 0, 0.25, 0.5 or 0.75 mg/ml PZH for 24 h. Cell confluence was observed using a phase-contrast microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were captured at x200 magnification.

Cell cycle analysis. Cell cycle analysis was performed by flow cytometry using a FACSCalibur system (Becton-Dickinson, San Jose, CA, USA). Following treatment with 0, 0.25, 0.5 or 0.75 mg/ml PZH for 24 h, BEL-7402 cells were collected at a final concentration of $1x10^6$ cells/ml, then fixed in 70% ethanol at 4˚C overnight. The cells were subsequently washed twice with ice cold PBS and incubated with propidium iodide (10 μg/ml) and a BD Pharmingen™ Cell Cycle kit, which contained DNase (8 μg/ml; BD Biosciences) for 30 min. The fluorescence signal was observed in the FL2 channel and the proportion of DNA in each phase was analyzed using Modfit LT software version 3.0 (Verity Software House, Inc., Topsham, ME, USA).

Colony formation assay. BEL-7402 cells were seeded into 6-well plates at a density of $2.5x10^5$ cells/well and treated with 0, 0.25, 0.5 or 0.75 mg/ml PZH for 24 h. The cells were subsequently reseeded into 6-well plates in RPMI-1640 without PZH at a density of $1x10^3$ cells/well. Following incubation for 8 days, cell colonies were fixed with 4% paraformaldehyde for 10 min at room temperature, stained with 0.1% crystal violet for 15 min at room temperature and observed using phase-contrast microscopy. The number of colonies per plate were counted.

Detection of apoptosis with Hoechst staining. BEL-7402 cells were seeded into 12-well plates at a density of $1x10^5$ cells/well, and treated with 0, 0.25, 0.5 or 0.75 mg/ml PZH for 24 h. Cells were subsequently washed with PBS, fixed with 4% polyoxymethylene for 10 min at room temperature and washed twice more in PBS. Cells were then incubated in Hoechst 33258 for 10 min in the dark at 37˚C and observed using a phase-contrast fluorescence microscope (Leica Microsystems GmbH). Images were captured at x200 magnification.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. BEL-7402 cells were seeded into 6-well plates at a density of $2.5x10^5$ cells/well in 2 ml medium. The cells were treated with 0, 0.25, 0.5 or 0.75 mg/ml PZH for 24 h.

Analysis of Bcl-2, CDK4 and cyclin D1 expression. Total RNA was isolated with RNAiso Plus reagent, and 1 μg of total RNA

was reverse-transcribed with the PrimeScript™ RT reagent kit according to the manufacturer's protocol. The produced cDNA was used to determine the mRNA expression of Bcl-2, CDK4 and cyclin D1 by qPCR with the SYBR® Select Master Mix using the ABI 7500 Fast instrument (both from Thermo Fisher Scientific, Inc.) under the following thermocycling conditions: 50˚C for 2 min, 95˚C for 2 min and 40 cycles at 95˚C for 1 sec and 60˚C for 30 sec. GAPDH was used as an internal control. Primer sequences are listed in Table I.

Analysis of miR-16 expression. Total miRNA was isolated with the RNAiso for microRNA kit. Total miRNA (1 μg) was reverse-transcribed with the SYBR® PrimeScript™ miRNA RT-PCR kit according to the manufacturer's protocol. The resulting cDNA was used to determine the expression of miR-16 by qPCR; U6 was used as an internal control. The primers for U6 (cat no. D356-03) and miR-16 (cat no. DHM0135) were obtained from Takara Biotechnology Co., Ltd. qPCR was performed with the SYBR® Premix Ex Taq II using the ABI 7500 Fast instrument under the following thermocycling conditions: 95˚C for 30 sec, 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec.

Quantification of qPCR results. The mRNA or miRNA expression levels were determined as ΔCq = Cq (sample) - Cq (U6 or GAPDH) and relative quantities between different samples were determined as ΔΔCq = ΔCq (sample 1) - ΔCq (sample 2); values were expressed as $2^{-\Delta\Delta Cq}$. All qPCR reactions were conducted in triplicate.

Western blot analysis. BEL-7402 cells were seeded into 25 cm^2 culture flasks at a density of $2.5x10^5$ cells/ml and treated with 0, 0.25, 0.5 or 0.75 mg/ml PZH for 24 h. Cells were lysed with cell lysis buffer (CWBIO, Beijing, China) containing protease and phosphatase inhibitor cocktails, and the resulting total protein concentration was determined by a bicinchoninic acid assay. Proteins (50 μg/lane) were resolved on 10% SDS-PAGE gels and electroblotted onto polyvinyl difluoride membranes. The membranes were blocked for 1 h at room temperature with blocking buffer (Beyotime Institute of Biotechnology) and probed with primary antibodies for Bcl-2, CDK4, cyclin D1 and β-actin (dilution, 1:1,000) overnight at 4˚C, then incubated with an appropriate HRP-conjugated secondary antibody for 1 h at room temperature (dilution, 1:5,000). Protein bands were subsequently detected using Thermo Scientific™ SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.).

Statistical analysis. Statistical analysis was performed using one-way analysis of variance with Bonferroni's multiple comparison test using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as the mean of three individual experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

PZH inhibits the proliferation of BEL-7402 cells. The effect of PZH on the proliferation of BEL-7402 cells was evaluated using phase-contrast microscopy. PZH treatment decreased the confluence and cell density of BEL-7402 cells in a dose-dependent manner (Fig. 1A). Furthermore, the MTT assays demonstrated that PZH treatment significantly decreased the cell viability of BEL-7402 cells in a dose- and time-dependent manner (Fig. 1B; P<0.05). In addition, a colony formation assay indicated that PZH treatment significantly reduced the clonogenicity rate of BEL-7402 cells (Fig. 1C; P<0.05).

Table I. Polymerase chain reaction primer sequences.

Gene	Primers (5' to 3')
Bcl-2	F: CAGCTGCACCTGACGCCCTT R: GCCTCCGTTATCCTGGATCC
Cyclin D1	F:TGGATGCTGGAGGTCTGCGAGGAA R: GGCTTCGATCTGCTCCTGGCAGGC
Cyclin-4 dependent kinase	F: CATGTAGACCAGGACCTAAGC R: AACTGGCGCATC AGATCCTAG
GAPDH	F: CGACCACTTTGTCAAGCTCA R: AGGGGTCTACATGGCAACTG

F, forward; R, reverse.

PZH inhibits G1/S transition in BEL-7402 cells. Cell cycle analysis was then performed using flow cytometry. There was a significant increase in the number of BEL-7402 cells in G0/G1 phase following treatment with 0.75 mg/ml PZH, whereas the percentage of cells in S phase was decreased, compared with untreated control cells (Fig. 2; P<0.05).

PZH induces the apoptosis of BEL-7402 cells. The rate of BEL-7402 cell apoptosis following PZH treatment was determined using Hoechst staining. PZH-treated cells exhibited the typical morphological features of apoptosis, including chromatin condensation and nuclear fragmentation, at a greater rate compared with the untreated control cells, which exhibited more homogenous staining of the nuclei (Fig. 3).

PZH modulates the expression of Bcl-2, cyclin D1, CDK4 and miR-16 in BEL-7402 cells. In order to determine the underlying mechanisms for the pro-apoptotic and anti-proliferative activity of PZH, the expression of key factors associated with cell cycle regulation and apoptosis was examined using RT-qPCR and western blot analysis. As demonstrated in Fig. 4A, miR-16 expression was significantly upregulated following treatment with PZH in a dose-dependent manner (P<0.05). In addition, PZH treatment decreased the mRNA (Fig. 4A; P<0.05 for doses ≥0.5 mg/ml) and protein expression levels (Fig. 4B) of Bcl-2, cyclin D1 and CDK4.

Discussion

HCC is one of the leading causes of cancer-associated mortality worldwide (1-3). Current treatment strategies for HCC include surgery, chemotherapy and targeted drug therapy (4-7). However, there are significant limitations in the effectiveness of current treatment options due to the development of drug

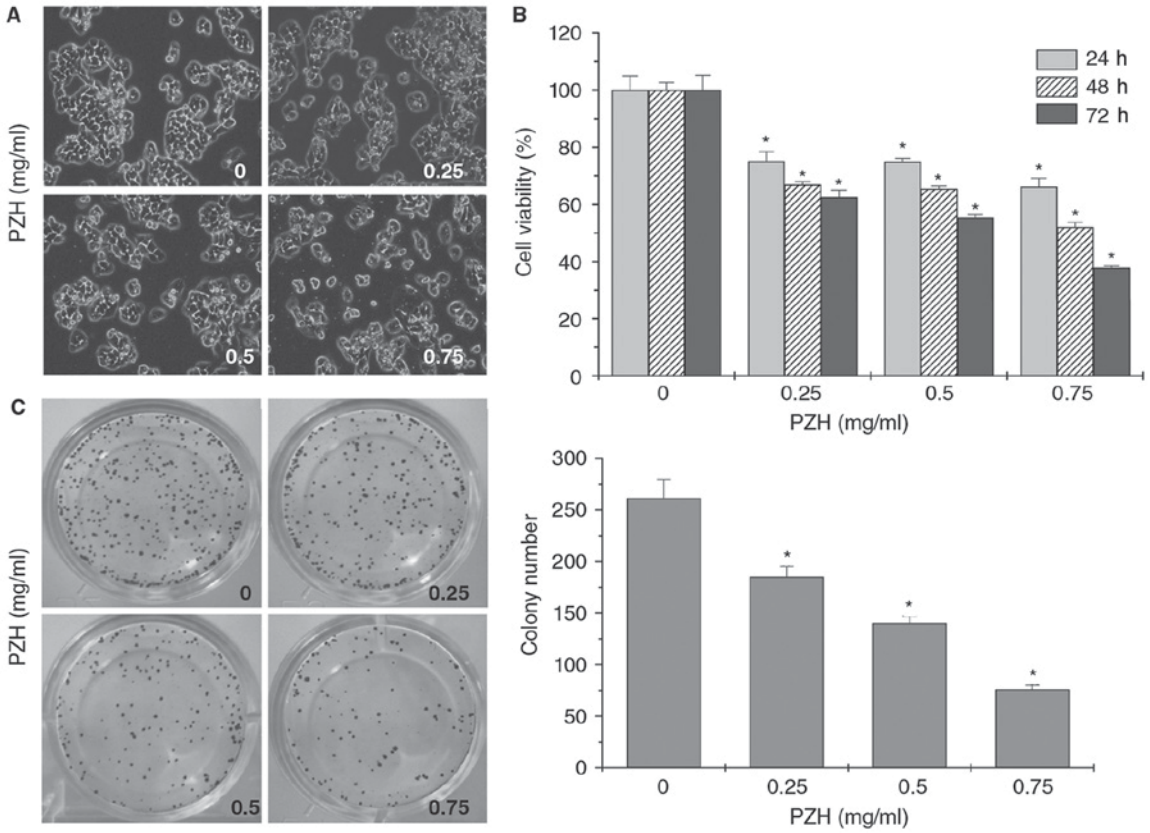


Figure 1. Effect of PZH on the proliferation of BEL-7402 cells. (A) Representative images of the confluence of BEL-7402 cells as observed using phase-contrast microscopy. Magnification, x200. (B) Relative viability of BEL-7402 cells, as determined using MTT assays. (C) Cell clonogenicity as determined by a colony formation assay, with representative images. Data are represented as the mean ± standard deviation from 3 independent experiments. *P<0.05 vs. respective control group. PZH, Pien Tze Huang.

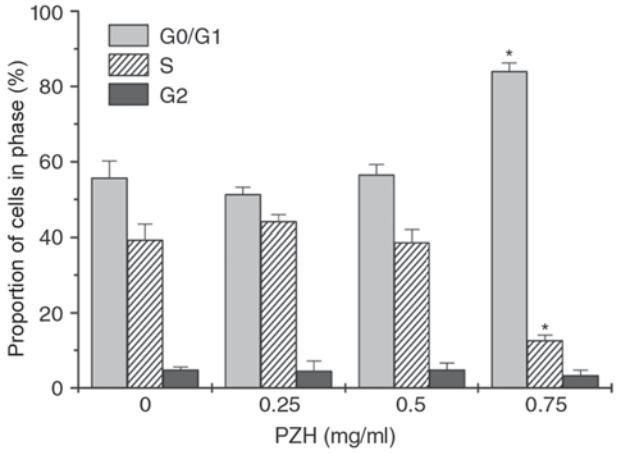


Figure 2. Effect of PZH on the cell cycle distribution of BEL-7402 cells. Following treatment with PZH, cells were stained with propidium iodide and analyzed by flow cytometry. The proportion of DNA in the G0/1, S and G2 phases was calculated using Modfit LT software. Data are represented as the mean ± standard deviation from 3 independent experiments. *P<0.05 vs. respective control group. PZH, Pien Tze Huang.

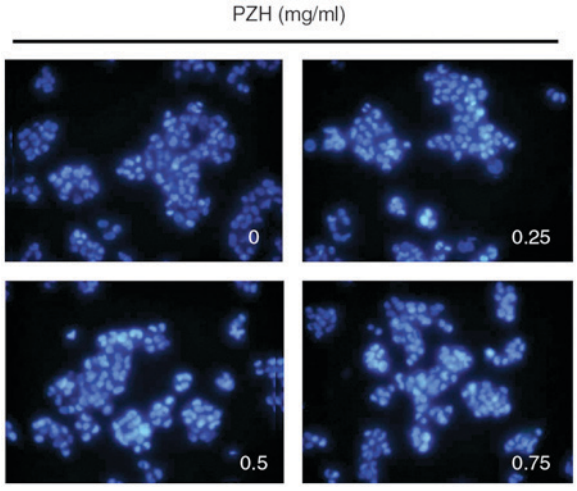


Figure 3. Effect of PZH on the apoptosis of BEL-7402 cells. The extent of apoptosis was evaluated with Hoechst staining. Stained images were recorded using a phase-contrast fluorescence microscope. Images are representative of 3 independent experiments. Magnification, x200. PZH, Pien Tze Huang.

resistance and non-specific cytotoxicity. The TCM treatment of cancer has received widespread attention due to its therapeutic efficacy and relatively few side effects. Previous studies have demonstrated that the commonly used TCM formula PZH can inhibit the growth and metastasis of colorectal cancer cells (34-46). However, its effects on HCC and the underlying mechanisms remain unclear.

miRNAs may serve crucial functions in liver tumorigenesis by regulating various proliferation and apoptosis-associated pathways. Previous studies have reported that miR-16 upregulation inhibited cell proliferation and induced cell

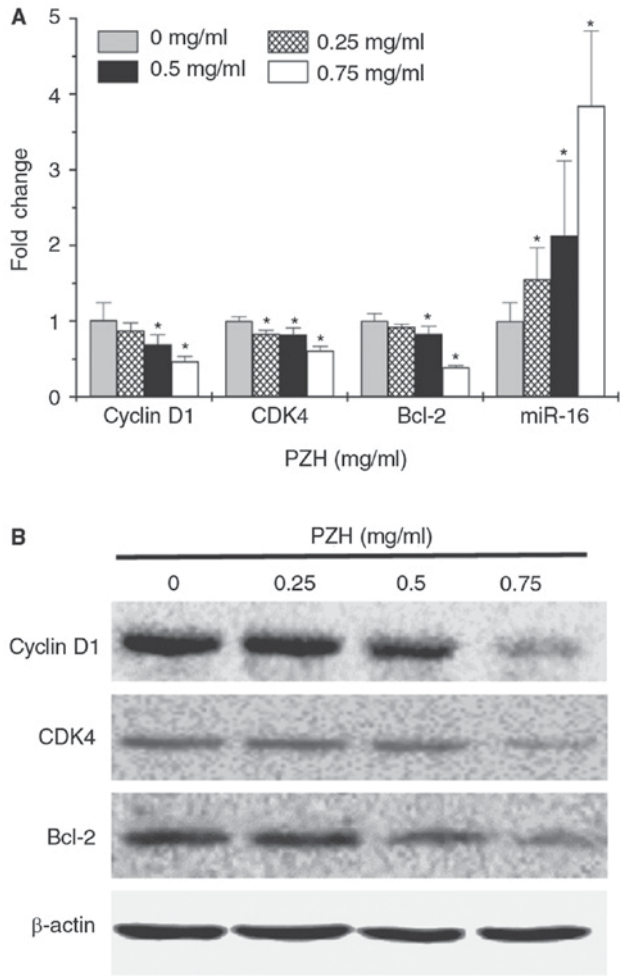


Figure 4. Effect of PZH on the expression of proliferation and apoptosis-associated genes in BEL-7402 cells. (A) The mRNA expression levels of cyclin D1, CDK4, Bcl-2 and miR-16 in BEL-7402 cells were analyzed by reverse transcription-quantitative polymerase chain reaction. GAPDH and U6 were used as the internal control for mRNA and miRNA, respectively. Data are represented as mean ± standard deviation from ≥3 independent experiments. *P<0.05 vs. control group. (B) The protein expression levels of cyclin D1, CDK4 and Bcl-2 in BEL-7402 were analyzed by western blotting. β-actin was used as the internal control. Images are representatives of three independent experiments. PZH, Pien Tze Huang; CDK4, cyclin-dependent kinase 4; miR/miRNA, microRNA.

cycle arrest (29,30). Thus, determining the effect of miRNAs, particularly miR-16, in HCC and the effect of PZH treatment on miR-16 expression may be of critical importance. The aim of the present study, was to demonstrate the inhibitory effect of PZH treatment on the proliferation and apoptosis of the BEL-7402 HCC cell line and explore the underlying mechanisms. It was identified that PZH treatment significantly decreased the viability and survival of BEL-7402 cells in a dose- and time-dependent manner. In addition, higher doses of PZH increased the percentage of cells in the G0/G1 phase and prevented G1/S cell cycle progression. Consistent with these data, PZH treatment significantly downregulated the expression of the anti-apoptotic gene Bcl-2, and the pro-proliferative factors cyclin D1 and CDK4. In addition, the miR-16 expression level in BEL-7402 cells was significantly upregulated following PZH treatment.

To conclude, the data of the present study demonstrate that PZH suppresses HCC growth through inhibiting cancer cell proliferation and inducing apoptosis. Modulating the expression of miR-16 and its target genes may be a possible mechanism for PZH's anti-tumor action.

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