Pien Tze Huang inhibits the proliferation of colorectal cancer cells by increasing the expression of miR-34c-5p

YUN WAN[1,2*], ALING SHEN[1,2*], FEI QI[1,2], JIANFENG CHU[1,2], QIAOYAN CAI[1,2], THOMAS JOSEPH SFERRA[3], JUN PENG[1,2,4] and YOUQIN CHEN[3]

[1]Academy of Integrative Medicine; [2]Fujian Key Laboratory of Integrative Medicine on Geriatrics, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China; [3]Rainbow Babies & Children's Hospital, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA; [4]Postdoctoral Workstation, Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd., Zhangzhou, Fujian 363000, P.R. China

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Abstract. MicroRNAs (miRNAs) are small, short endogenous non-coding RNA that act as oncogenes or tumor suppressors, and serve an important role in various human malignant cancers, including colorectal cancer (CRC). Evidence has indicated that miRNAs regulate the expression of various genes associated with human cancer, in particular the miR-34 family. A well-known traditional Chinese formula, Pien Tze Huang (PZH), has a significant clinical effect on CRC. Previous studies have demonstrated that PZH inhibits CRC growth *in vitro* and *in vivo* via multiple mechanisms, including the induction of apoptosis, inhibition of cell proliferation and tumor angiogenesis. To further elucidate the molecular mechanisms underlying the antitumor activity of PZH, in the present study its effects on cell proliferation and miRNA expression in human colon carcinoma (HCT)-8 cell lines was examined. It was observed that treatment with PZH inhibited cell viability and upregulated the expression of miR-34c-5p in HCT-8 cells. In addition, transfection with an miR-34c-5p mimic and treatment with PZH inhibited cell survival and arrested the cell cycle between the G0/G1 and S phase in HCT-8 cells. Furthermore, PZH treatment and transfection with miR-34c-5p downregulated the expression of cyclin-dependent kinase 4 and cMyc (a promoter of cell proliferation), and increased the expression of p53, which is a promoter of apoptosis. These results suggest that PZH may suppress proliferation in CRC cells by upregulating the expression of miR-34c-5p, which provides a novel perspective for understanding the mode of action of PZH.

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

Dr Youqin Chen, Rainbow Babies & Children's Hospital, Case Western Reserve University School of Medicine, 11100 Euclid Avenue, Cleveland, OH 44106, USA
E-mail: yxc571@case.edu

*Contributed equally

Abbreviations: CRC, colorectal cancer; PZH, Pien Tze Huang; TCM, traditional Chinese medicine

Key words: Pien Tze Huang, traditional Chinese medicine, colorectal cancer, proliferation, miR-34c

Introduction

MicroRNAs (miRNAs) are a family of small non-coding 18-22 nt RNAs, which are one of the most important epigenetic regulators that target mRNA post-transcriptionally (1). The miR-34 family (miR-34a, miR-34b and miR-34c) has been shown to exhibit antiproliferative and pro-apoptotic functions (2), in which miR-34c has an important role in the development of various malignancies, including neuroblastoma, and breast, lung and colorectal cancer (CRC) (3-6). miR-34c has two identified mature miRNAs: miR-34c-3p and miR-34c-5p. A number of studies have reported that miR-34 overexpression regulates proliferation through the inhibition of cyclin-dependent kinase (CDK) 4 and cMyc, and the induction of p53 (7,8).

CRC, a common digestive tract tumor, is reportedly the third leading cause of cancer-related mortality worldwide (9). Current therapies for CRC include surgery, radiotherapy and chemotherapy (10,11); however, these treatments have limited efficacy and toxic effects. Therefore, research in this field has been focused on investigating natural products to treat CRC, including traditional Chinese medicine (TCM), as they typically exert fewer side effects than modern medicine (12,13). A well-known traditional Chinese formula, Pien Tze Huang (PZH), has a significant clinical effect on CRC. Previous studies have demonstrated that PZH inhibits CRC growth *in vitro* and *in vivo* via multiple mechanisms, including the induction of apoptosis, inhibition of cell proliferation and tumor angiogenesis (14-23). To further elucidate the molecular mechanisms of the antitumor activity of PZH, the effects of

PZH on cell proliferation and miRNA expression in human colon carcinoma cell (HCT-8) cell lines were investigated.

Materials and methods

Materials and reagents. RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA, MirVanaTM miRNA mimics of miR-34c-5p and scramble control, Opti-MEM, Lipofectamine RNAiMAX transfection reagent and Trypan Blue solution were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Antibodies against cMyc (cat. no. 13987S), CDK4 (cat. no. 12790S) and β-actin (cat. no. 4970S) and horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. 7074) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-p53 antibody (cat. no. ab131442) was purchased from Abcam Hong Kong, Ltd., (Hong Kong, China). Cell cycle assay kit (KGA512) was purchased from Nanjing KeyGen Biotechnology Co., Ltd., (Nanjing, China). PrimeScript RT Reagent kit and SYBR *Premix Ex Taq* II kit were purchased from Takara Biotechnology Co., Ltd (Dalian, China). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparations of PZH. PZH was obtained from and authenticated by Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd., (Zhangzhou, China) (Chinese FDA approval no. Z35020242). Stock solutions of PZH were prepared by dissolving PZH powder in phosphate-buffered saline (PBS) to a concentration of 20 mg/ml. Diluting the stock solution with RPMI 1640 culture medium made the working concentrations of PZH.

Cell culture. HCT-8 cells were purchased from Nanjing KeyGen Biotechnology Co., Ltd. (Nanjing, China) and cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS at 37˚C in a 5% CO_2 humidified environment. Cells were digested using trypsin-EDTA when the confluence was 80-90%.

Measurement of cell viability. Cell viability was determined using an MTT assay. Briefly, cells at the logarithmic growth phase were seeded into 96 well plates at a density of 8,000 cells/well in 0.1 ml RPMI 1640 medium, and allowed to adhere overnight. Subsequently, cells were treated with PZH (0, 0.25, 0.5 and 0.75 mg/ml) for 24 h, after which 100 μl MTT (0.5 mg/ml in PBS) was added to each well, followed by incubation for 4 h at 37˚C. Purple-blue MTT formazan precipitate was dissolved in 100 μl dimethyl sulfoxide. Absorbance was measured at 570 nm using an ELISA reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA).

Transfection of miR-34c-5p mimic. To elucidate the effect of miR-34c-5p on the proliferation of HCT-8 cells, cells were transfected with an miR-34c-5p mimic or scrambled control, according to the manufacturer's protocol. Briefly, cells were seeded into 12 well plates at $2x10^5$ cells/well in 1 ml RPMI 1640 medium and allowed to adhere overnight until 30-50% confluency. Cells were washed, placed in Opti-MEM and transfected with oligonucleotides using Lipofectamine RNAiMAX Transfection Reagent, according to the manufacturer's instructions. Transfection efficiency (>95%) was confirmed with the use of the Silencer 6-carboxy-fluo-rescine (FAM)-labeled Negative Control. After 6 h, the RPMI 1640 medium was changed to RPMI 1640 medium supplemented with 10% (v/v) FBS and cells were cultured at 37˚C in 5% CO_2.

Observation of cell morphology and calculation of live cells. HCT-8 cells were treated with the indicated concentrations of PZH or transfected with an miR-34c-5p mimic for 24 h. Cell morphology was observed using a phase-contrast microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were captured at a magnification of x200. Trypan Blue exclusion test was used to calculate the live cell numbers using Countstar, according to the manufacturer's protocol (Inno-Alliance Biotech, Inc., Wilmington, DE, USA).

Colony formation assay. HCT-8 cells were treated with the indicated concentrations of PZH or transfected with an miR-34c-5p mimic for 24 h. Subsequently, 1,000 collected cells from each group were seeded in 6 well plates and cultured for an additional 10 days in RPMI 1640 supplemented with 10% FBS. The formed colonies were fixed with 4% formaldehyde, stained with 0.01% crystal violet and counted. Cell survival was calculated by normalizing the survival of the control cells as 100%.

Determination of the cell cycle by fluorescence-activated cell sorting (FACS). Cell cycle analysis was performed by flow cytometry using a BD FACSCalibur (Becton-Dickinson, San Jose, CA, USA) and propidium iodide (PI) staining. HCT-8 cells were treated with indicated concentrations of PZH or transfected with an miR-34c-5p mimic for 24 h. Subsequently, cells were harvested at a concentration of $1x10^5$ 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).

Reverse transcription (RT)-quantitative polymerase chain reaction (qPCR) analysis of miRNA. Mature miRNAs were isolated and purified using TRIzol reagent for small RNA (Takara Biotechnology Co., Ltd.), according to the manufacturer's instructions. Small RNA (1 μg) was tailed and reverse transcribed using a SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. qPCR was performed using miRNA specific primers. hsa-miR-34c-5p (DHM0302) and U6 (D356-03) qPCR primers were purchased from Takara Biotechnology Co., Ltd. The PCR reaction included the following constituents: SYBR premix Ex Taq II (10 μl), PCR forward primer (10 μM; 0.8 μl), Uni-miR qPCR primer (10 μM; 0.8 μl), ROX reference dye II (50X; 0.4 μl), cDNA (2 μl) and dH2O (6 μl). An initial denaturation step was performed at 95˚C for 30 sec, 95˚C for 3 sec and then annealing at 60˚C for 30 sec. This was repeated for 40 cycles. All miRNA Cq values were normalized to small nuclear RNA U6. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression levels of the miRNAs (24).

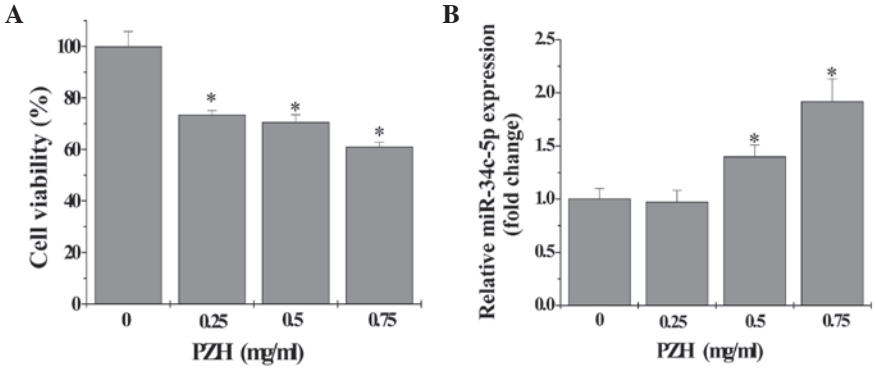

Figure 1. Effect of PZH on the suppression of cell viability. (A) HCT-8 cells were treated with the indicated concentrations of PZH for 24 h. Cell viability was determined using an MTT assay. (B) miRNA was extracted from HCT-8 cells, and the expression of miR-34c-5p was assayed by reverse transcription-quantitative polymerase chain reaction and normalized to a control. Data are expressed as the mean ± standard deviation of three independent experiments. PZH, Pien Tze Huang; HCT-8, human colorectal cancer-8. *P<0.05 vs. control (0 mg/ml).

RT-qPCR analysis of mRNA. Total RNA from HCT-8 cells following treatment with PZH or transfection with an miR-34c-5p mimic for 24 h were isolated with RNAiso and were reverse transcribed using PrimeScript II 1st Strand cDNA Synthesis kit, according to the manufacturer's protocol. cDNA was used to determine the expression of CDK4, cMyc and p53 mRNA, and GAPDH was used as an internal control. qPCR was performed using SYBR Premix Ex Taq II in an ABI 7500 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR reactions were performed according to the manufacturer's protocol. mRNA expression values were determined as ΔCt = Ct (sample) - Ct (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 performed in triplicate. Primers used for amplification were as follows: CDK4 forward, 5'-TGTGGAGTGTTGGCTGTATC-3' and reverse, 5'-AGGCAGAGATTCGCTTGTG-3'; cMyc forward, 5'-CGGAAACGACGAGAACAGT-3' and reverse, 5'-AGACTCAGCCAAGGTTGTG-3'; p53 forward, 5'-ACAATCAGCCACATTCTAGGTAG-3' and reverse, 5'-CCAGCAGAGACTTGACAACT-3'; and GAPDH forward, 5'-CTGCCTTCTCTTGTGACA-3' and reverse, 5'-TGTAGACCATGTAGTTGAGG-3'.

Western blot analysis. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (50 mmol/l Tris, 0.5% NP-40 and 0.01% SDS; pH 7.4) supplemented with protease inhibitors (Roche Applied Science, Mannheim, Germany). The supernatant was collected by centrifugation at 20,139xg at 4˚C for 20 min 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). Total protein (50 μg) was separated by 12% sodium dodecyl sulfate-polyacrylamide electrophoresis and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Non-specific protein interactions were blocked by incubation with 5% non-fat milk in Tris-buffered saline with Tween 20 (50 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20; pH 7.6) at room temperature for 2 h. Membranes were incubated with primary antibodies against cMyc, CDK4, p53 and β-actin (all at 1:1,000 dilution) overnight at 4˚C followed by incubation with an HRP-conjugated secondary antibody (1:5,000 dilution) at room temperature for 2 h. Proteins were detected using a chemiluminescence detection system (Pierce Biotechnology, Inc.) and were visualized using enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression was normalized to an endogenous reference (β-actin) and was relative to the control.

Statistical analysis. Data were expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS for Windows (version 18.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare quantitative data among groups. The Bonferroni post-hoc test was used if ANOVA indicated statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

PZH suppresses the viability of HCT-8 cells. Infinite proliferation is the primary characteristic and pathogenic factor of various malignant tumors, including CRC (25). To evaluate the effect of PZH on proliferation, $8x10^4$ HCT-8 cells were seeded in 96-well plates and treated with various concentrations of PZH for 24 h. Cell viability was determined using an MTT assay. As presented in Fig. 1A, treatment with PZH (0.25, 0.5 and 0.75 mg/ml) significantly suppressed the viability of HCT-8 cells in a dose-dependent manner, as compared with untreated control cells (P<0.05), suggesting that PZH treatment significantly suppressed the viability of CRC cells.

PZH upregulates the expression of miR-34c-5p in HCT-8 cells. As miR-34c has been demonstrated to have antiproliferative and pro-apoptotic functions (26,27), whether PZH could regulate the expression level of miR-34c-5p in HCT-8 cells was investigated. After HCT-8 cells were treated with different doses of PZH for 24 h, miR-34c-5p expression levels were detected by qPCR. As indicated in Fig. 1B, 0.25 mg/ml PZH had no significant effect on the expression of miR-34c-5p. However, 0.5 and 0.75 mg/ml PZH significantly upregulated the expression of miR-34c-5p (P<0.05), suggesting that the upregulation of miR-34c-5p expression might be a mechanism by which PZH exerts an inhibitory effect on the growth of CRC cells.

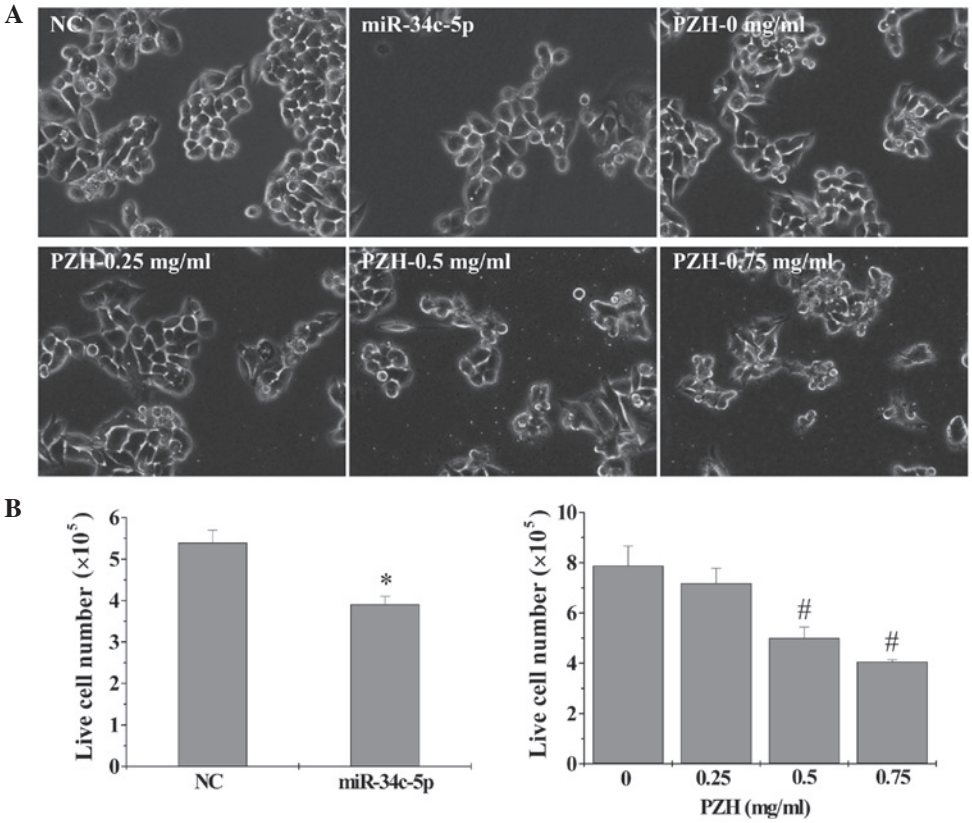

Figure 2. Effect of miR-34c-5p transfection and PZH treatment on the growth of human colorectal cancer-8 cells. (A) The morphology of cells treated with PZH or transfected with miR-34c-5p were observed using a phase-contrast microscope (magnification, x200). (B) Live cells treated with PZH or transfected with miR-34c-5p were analyzed using Trypan Blue staining by Countstar. Data are expressed as the mean ± standard deviation of three independent experiments. PZH, Pien Tze Huang; NC, normal cells. *P<0.05 vs. NC; #P<0.05 vs. control.

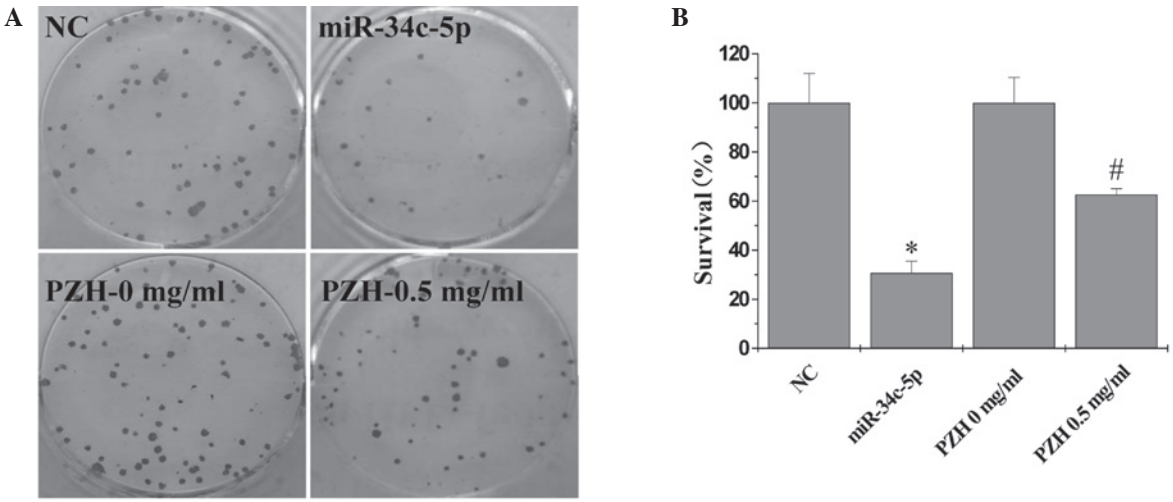

Figure 3. Effect of miR-34c-5p transfection and PZH treatment on the survival of human colorectal cancer-8 cells. (A) Cells were treated with 0.5 mg/ml PZH or transfected with an miR-34c-5p mimic (20 nm) for 24 h and cultured for 10 days. Colony formation analysis was employed to detect cell survival. Images are of three independent experiments. (B) Quantification of colony formation analysis. Data are expressed as the mean ± standard deviation of three independent experiments. PZH, Pien Tze Huang; NC, normal cells. *P<0.05 vs. NC; #P<0.05 vs. control.

MiR-34c-5p transfection and PZH treatment inhibit the growth of HCT-8 cells. HCT-8 cells were transfected with a miR-34c-5p mimic in order to investigate the effect of miR-34c-5p transfection on the morphology and growth of HCT-8 cells. As presented in Fig. 2A, following PZH treatment or transfection with an miR-34c-5p mimic, HCT-8 cells became enlarged, irregularly shaped and exhibited vacuolated changes in the cytoplasm. In addition, the cell number significantly decreased in a dose-dependent manner, as compared with untreated cells (Fig. 2B; P<0.05). These results further confirmed that PZH treatment and miR-34c-5p overexpression each markedly suppressed the growth of HCT-8 cells.

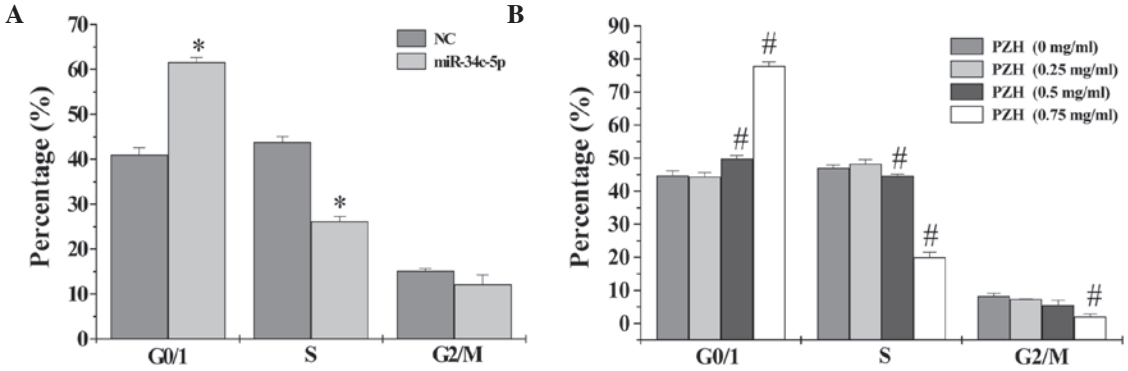

Figure 4. Effect of miR-34-5p transfection and PZH treatment on cell cycle progression. Human colorectal cancer-8 cells were (A) transfected with an miR-34c-5p mimic and (B) treated with indicated concentrations of PZH for 24 h, stained with propidium iodide (PI) and analyzed by flow cytometry. Data are expressed as the mean ± standard deviation from three independent experiments. PZH, Pien Tze Huang; NC, normal cells. *P<0.05 vs. NC; #P<0.05 vs. control.

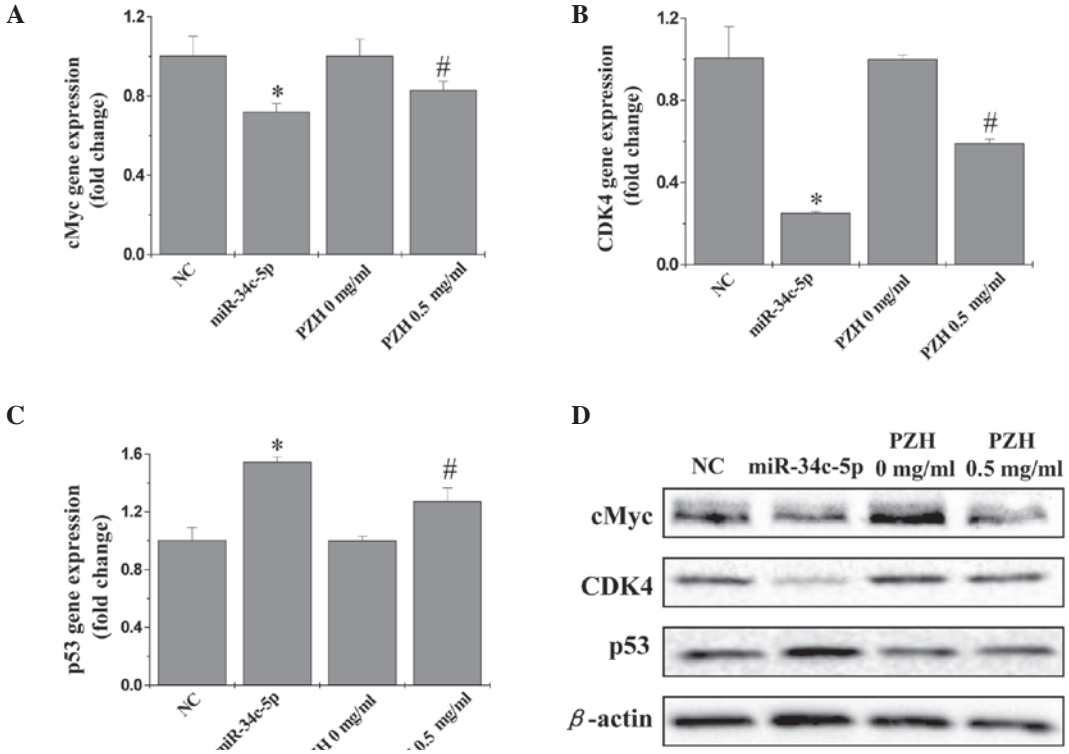

Figure 5. Effects of PZH treatment and miR-34c-5p transfection on the expression of CDK4, cMyc and p53 in human colorectal cancer-8 cells. (A) cMyc (B) CDK4 and (C) p53 mRNA expression levels were determined by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. NC; #P<0.05 vs. control. (D) Protein expression levels were determined by western blot analysis. The results shown are representative of three independent experiments. PZH, Pien Tze Huang; NC, normal cells; cMyc, cyclin-dependent kinase 4.

PZH and miR-34c-5p decrease the clone formation rate of HTC-8 cells. To evaluate the effect of PZH treatment and miR-34c-5p transfection on HCT-8 cell survival, a colony-forming assay was performed. HCT-8 cells were exposed to various concentrations of PZH or transfected with 20 nm miR-34c-5p mimic and cultured for 14 days. As shown in Fig. 3, PZH treatment (0.5 mg/ml) and miR-34c-5p transfection for 24 h significantly decreased the number of colonies in HCT-8 cells (P<0.05). These experiments revealed that PZH treatment, as well as miR-34c-5p overexpression markedly suppressed the survival in HCT-8 cells.

PZH and miR-34c-5p induced G1/S arrest of HCT-8 cells. As autonomous cell proliferation is a hallmark of cancer cells, cell cycle arrest has become a primary indicator of anticancer effects (28). In order to explore the underlying mechanism of PZH inhibition on the proliferation of HCT-8 cells, flow cytometry was performed to evaluate the direct effects of PZH on the cell cycle. It was observed that there was a significant decrease in the S phase (P<0.05) and a significant increase in the G0/1 phase (P<0.05) following transfection with a miR-34c-5p mimic compared with the negative control group (Fig. 4A). In addition, PZH at doses of 0.5 and 0.75 mg/ml induced a significant increase in the G0/1 phase, in comparison with the control group (P<0.05; Fig. 4B), suggesting that PZH treatment and miR-34c-5p overexpression each significantly arrested the cell cycle at the G0/G1 phase.

PZH and miR-34c-5p downregulate the expression of CDK4 and cMyc and upregulated the expression of p53. To further elucidate the mechanism by which miR-34c-5p participates in the antitumor effects of PZH, the expression of the target genes of miR-34c-5p (CDK4, cMyc and p53) were investigated. These genes are associated with the cell proliferation cycle (29-31). PCR and western blot analysis demonstrated that, following treatment with 0.5 mg/ml PZH or transfection with a miR-34c-5p mimic for 24 h, the mRNA and protein expression levels of CDK4 and cMyc were significantly decreased in HCT-8 cells, as compared with untreated cells ($P<0.05$). In addition, the mRNA and protein expression levels of P53 were significantly increased following treatment with 0.5 mg/ml PZH or transfection with a miR-34c-5p mimic for 24 h (Fig. 5). for 24h (Fig. 5). These findings indicated that PZH treatment and miR-34c-5p overexpression each significantly downregulated the expression of c-myc and CDK4, while upregulating the expression of p53, suggesting regulation of the expression of these genes may be a mechanism by which PZH and miR-34c-5p suppress HCT-8 cell growth.

Discussion

CRC is a multistage process involving genetic and epigenetic alterations (32). In the pathogenesis of CRC, excessive abnormal cell proliferation results in tumorigenesis. Recently, epigenetic studies have provided novel molecular evidence to better categorize CRC subtypes and predict clinical outcomes, such as miRNA (33,34). miRNAs are implicated in normal biological functions, including proliferation, differentiation, homeostasis and apoptotic cell death (35). A number of microRNAs have critical roles in colorectal tumorigenesis by regulating the functional pathways of proliferation (36,37). The family of miR-34, including miR-34a, b and c, has been reported to regulate numerous cellular events, including the cell cycle, cell migration and apoptosis (38,39). miR-34c has two identified mature miRNAs: miR-34c-3p and miR-34c-5p (40,41). Previous studies have indicated that miR-34c has tumor-suppressive effects, and suppresses the cell cycle primarily by induction of G1 cell cycle arrest (42).

Studies have demonstrated that natural products, including curcumin, Tectorigenin and *Trametes robiniophila murris*, can regulate the expression of numerous miRNAs, which increase the sensitivity of cancer cells to conventional agents and thereby effectively suppress tumor cell proliferation (43-45). PZH, as a TCM formula, has been demonstrated to have cancer activity via a number of mechanisms, including the stimulation of apoptosis, inhibition of proliferation and regulati multiple downstream cancer-related signaling molecules in CRC cells. Previous studies have identified that PZH can regulate a number of miRNAs, such as the miR-200 family (19).

In the present study, it was observed that treatment with PZH inhibited cell viability and upregulated the expression of miR-34c-5p in HCT-8 cells. In addition, transfection with a miR-34c-5p mimic and treatment with PZH inhibited cell survival and arrested the cell cycle between the G0/G1 and S phase in HCT-8 cells. Furthermore, PZH was demonstrated to inhibit the proliferation of HCT-8 cells by regulating miR-34c-5p.

Emerging evidence suggests that p53 acts as a transcription factor to increase the expression of miR-34 family members, which, in turn, modulate cell cycle progression, senescence and apoptosis, and the inhibition of invasion and migration (46,47). Upregulation of miR-34 has been shown to induce cell-cycle arrest, inhibition of invasion and migration, and p53 induced apoptosis (48-50). In the present study, the mRNA and protein expression level of p53 was significantly upregulated in HCT-8 cells following transfection with a miR-34c-5p mimic. Treatment with PZH (0.5 mg/ml) induced a significant increase in p53 mRNA expression levels, although no significant difference was identified at the protein level, which may suggest that p53 protein synthesis is delayed. This must be addressed in future studies.

cMyc is a well-known oncogene that has an important role in neoplastic transformation and apoptosis, and can be negatively regulated by p53 (51). CDK4 is an important regulator of the G0/G1 phases of mammalian cell cycle progression (52). It was demonstrated in the present study that the mRNA and protein expression levels of cMyc and CDK4 were significantly decreased in HCT-8 cells following transfection with a miR-34c-5p mimic or treatment with PZH. These results demonstrate that miR-34c-5p suppresses the proliferation of HCT-8 cells, at least partially via the regulation of the expression of p53, CDK4 and cMyc.

In conclusion, PZH may suppress proliferation in HCT-8 cells via the upregulation of miR-34c-5p, which provides a novel perspective for understanding the antitumor effects of PZH.

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