Pien Tze Huang inhibits metastasis of human colorectal carcinoma cells via modulation of TGF-β1/ZEB/miR-200 signaling network

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Abstract. Tumor metastasis, a complex process involving the spread of malignant tumor cells from a primary tumor site to a distant organ, is a major cause of failure of cancer chemotherapy. Epithelial-mesenchymal transition (EMT) is a critical step for the initiation of cancer metastasis. The processes of EMT and metastasis are highly regulated by a double-negative feedback loop consisting of TGF-β1/ZEB pathway and miR-200 family, which therefore has become a promising target for cancer chemotherapy. Pien Tze Huang (PZH), a well-known traditional Chinese formula first prescribed in the Ming Dynasty, has been demonstrated to be clinically effective in the treatment of various types of human malignancy including colorectal cancer (CRC). Our published data proposed that PZH was able to induce apoptosis, inhibit cell proliferation and tumor angiogenesis, leading to the suppression of CRC growth *in vitro* and *in vivo*. To further elucidate the mode of action of PZH, in the present study we evaluated its effects on the metastatic capacities of human colorectal carcinoma HCT-8 cells and investigated the underlying molecular mechanisms. We found that PZH significantly inhibited the migration and invasion of HCT-8 cells in a dose-dependent manner. In addition, PZH treatment inhibited the expression of key mediators of TGF-β1 signaling, such as TGF-β1, Smad2/3 and Smad4. Moreover, PZH treatment suppressed the expression of ZEB1 and ZEB2, two critical target genes of TGF-β1 pathway, leading to a decrease in the expression of mesenchymal marker N-cadherin and an increased expression of epithelial marker E-cadherin. Furthermore, PZH treatment upregulated the expression of miR-200a, miR-200b and miR-200c. Collectively, our findings in this study suggest that PZH can inhibit metastasis of colorectal cancer cells via modulating TGF-β1/ZEB/miR-200 signaling network, which might be one of the mechanisms whereby PZH exerts its anti-cancer function.

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Abbreviations: CRC, colorectal cancer; PZH, Pien Tze Huang; TCM, traditional Chinese medicine; TGF-β, transforming growth factor-β; EMT, epithelial-to-mesenchymal transition; ZEB, zinc finger E-box-binding homeobox

Key words: Pien Tze Huang, traditional Chinese medicine, cancer metastasis, TGF-β1 pathway, miRNA

Introduction

Colorectal cancer (CRC) is one of the most common cancers and a leading cause of cancer-related deaths (1,2). To date, the mainstay of anti-CRC treatment includes surgery, chemotherapy and radiotherapy. However, due to tumor recurrence and metastasis the long-term survival and prognosis of patients remains quite poor (3,4). Tumor metastasis is a complex process involving the spread of malignant tumor cells from a primary tumor site to a distant organ, which is a major cause of failure of cancer treatment (5-7). Epithelial-mesenchymal transition (EMT) is a critical step for the initiation of cancer metastasis (8,9). The processes of EMT and metastasis are highly regulated by multiple mechanisms, including TGF-β1/ZEB pathways and miRNA 200 family (10-14).

TGF-β1 is the prototypic member of transforming growth factor β superfamily. The activation of TGF-β signaling pathway is initiated by the binding of ligands to a type II receptor, resulting in the phosphorylation/activation of a type I receptor. The activated type I receptor then phosphorylates SMAD2/3 that in turn bind to SMAD4. The SMAD complex translocates to the nucleus to regulate the expression of target genes, including the ZEB (zinc finger E-box-binding homeobox) transcription factor family (15,16). Upon activation, ZEB transcription factors suppress epithelial marker gene expression

and upregulate mesenchymal gene expression, leading to the processes of EMT and cancer metastasis (17,18). MicroRNAs (miRNA) are a class of endogenous short non-coding RNAs (19-24 nucleotides), which function primarily to negatively regulate target gene expression by specifically binding to the 3'-untranslational region (3'-UTR) of target mRNAs (19-21). It has been shown that miRNAs function more likely as oncogenes or tumor suppressors to modulate multiple oncogenic cellular processes, such as cell proliferation, apoptosis and metastasis (22-24). The miR-200 family members, including miR-200a, miR-200b and miR-200c, have been proposed to act as tumor suppressors that inhibit EMT by downregulating the expression of ZEB1 and ZEB2 (11,14,25-27). However, the expression of miR-200 family is negatively regulated by TGF-β signaling, probably via TGF-β-induced DNA methylation of the miR-200 loci (12). Thus, TGF-β/ZEB/miR-200 signaling network creates a double-negative feedback loop that plays an essential role in the initiation of EMT and cancer metastasis; which therefore becomes a promising target for cancer chemotherapy (13,14).

Recently, traditional Chinese medicines (TCM) have received great interest in the field of anticancer treatment since they have fewer adverse effects as compared to modern chemotherapeutics and have been used in China for thousands of years as important alternative remedies for various diseases including cancer (28,29). Pien Tze Huang (PZH) is a well-known TCM formula that was first prescribed >450 years ago in the Ming Dynasty. The main ingredients of PZH include *Moschus, Calculus Bovis, Snake Gall* and *Radix Notoginseng*. These products together confer PZH properties of heat clearing, detoxification, dissipation of hard mass, detumescence and analgesia (30). Traditionally, PZH has been used to clinically treat traumatic injuries and a variety of inflammatory diseases, particularly hepatitis (30-32). More importantly, PZH has also been used in China and Southeast Asia for centuries as a folk remedy for treatment of various types of human cancer. We recently reported that PZH can inhibit colon cancer growth through the promotion of cancer cell apoptosis, the inhibition of cell proliferation and tumor angiogenesis, which is probably mediated by modulation of multiple signaling pathways (33-40). To further elucidate the mode of action of PZH, in the present study we evaluated its effects on the metastatic capacities of human colorectal carcinoma HCT-8 cells and investigated the underlying molecular mechanisms.

Materials and methods

Materials and reagents. Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, were obtained from Life Technologies Corp. (Grand Island, NY, USA). N-cadherin and E-cadherin antibodies were purchased from Abcam (HK) Ltd. (Hong Kong, China). TGF-β1, SMAD2/3, SMAD4, ZEB1, ZEB2 and β-actin antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Cell Signaling Technology (Beverly, MA, USA). Transwell chambers were obtained from Corning Life Sciences (Tewksbury, MA, USA). BD BioCoat Matrigel Invasion Chamber was purchased from BD Bioscience (San Jose, CA, USA). PrimeScript RT reagent kit, RNAiso for Small RNA kit and SYBR Premix Ex Taq II kit were provided by Dalian Takara Biotechnology Co., Ltd. (Dalian, Liaoning, China). All the other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

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

Cell culture. Human colorectal carcinoma HCT-8 cells were obtained from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, Jiangsu, China). Cells were grown in RPMI-1640 medium containing 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 37˚C humidified incubator with 5% CO_2. The cells were subcultured at 80-90% confluency.

Evaluation of cell migration by wound-healing assay. Migration of HCT-8 cells was examined by wound-healing assay. Cells were seeded into 6-well plate at a density of $1x10^6$ cells/well in 2 ml medium. After 24 h of incubation, cells were scraped away vertically in each well by using a P100 pipette tip. Three randomly selected views along the scraped line were photographed on each well using a phase-contrast inverted microscope (Leica, Germany) at a magnification of x100. Cells were then treated with indicated concentrations of PZH for 24 h, and another set of images were taken using the same method. A reduction in the scraped area indicates a sign of migration.

Measurement of cell migration and invasion by transwell assay. Migration assay was performed using transwell cell culture chambers with 8-μm pore filters (Corning Life Sciences, USA). After treatment with various concentrations of PZH for 24 h, HCT-8 cells were trypsinized and resuspended in serum-free RPMI-1640. A total of $5x10^4$ cells in 200 μl of serum-free RPMI-1640 were plated in the upper chambers. RPMI-1640 media containing 10% (v/v) FBS was used in the lower chambers as a chemoattractant. Cells were allowed to migrate for 12 h, and the non-migrated cells were removed from the upper surface of transwell membranes by a cotton swab. Membranes were then stained with crystal violet. For quantification, the average number of migrating cells per field was assessed by counting 3 random fields under a phase-contrast microscope (Leica) at a magnification of x200. For cell invasion assay, the procedure was the same as that of above-described migration analysis, except that the upper chambers were coated with Matrigel Matrix (BD Biosciences, USA).

Western blot analysis. HCT-8 cells were seeded into 25 cm^2 flasks at a density of $1.5x10^6$ cells/flask in 5 ml medium. After incubation for 24 h, the cells were treated with the indicated concentrations of PZH for 24 h. The treated cells were lysed with mammalian cell lysis buffer containing protease and phosphatase inhibitor cocktails. Total protein concentrations

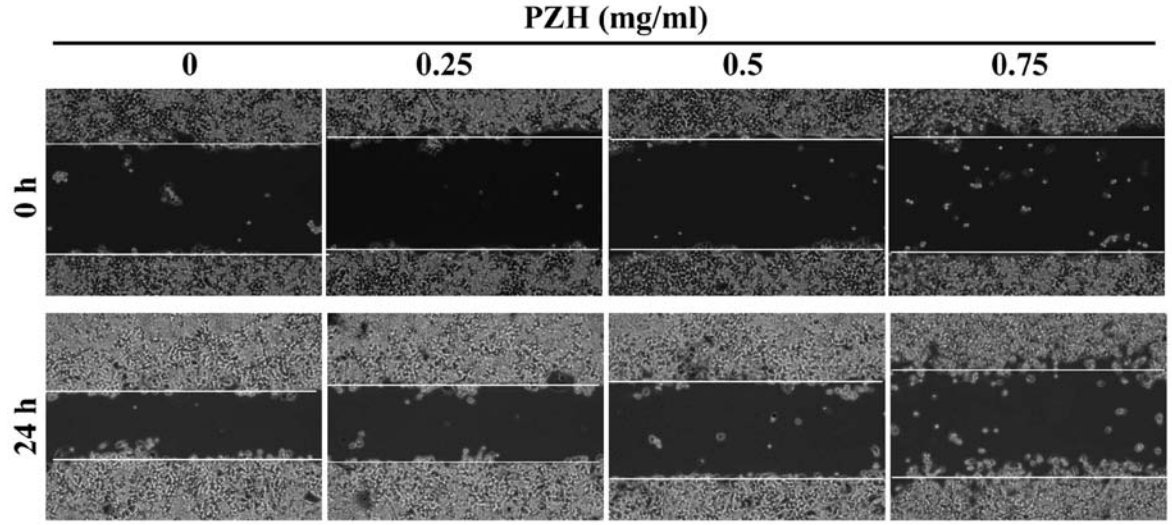

Figure 1. Effect of PZH on migration of HCT-8 cells by wound-healing assay. Following treatment with the indicated concentrations of PZH for 24 h, the migration pattern of HCT-8 cells was observed using phase-contrast microscopy. Images were captured at a magnification of x100. Images are representative of three independent experiments.

were determined by BCA assay. Equal amounts of total proteins were resolved in 12% SDS-PAGE gels and electroblotted. The PVDF membranes were blocked with 5% skimmed milk and probed with primary antibodies N-cadherin, E-cadherin, TGF-β1, SMAD2/3, SMAD4, ZEB1, ZEB2 and β-actin overnight at 4˚C and subsequently with the appropriate HRP-conjugated secondary antibody followed by enhanced chemiluminescence detection.

Q-PCR analysis. Total small RNA from HCT-8 cells was isolated with RNAiso for Small RNA kit. Total small RNA (500 ng) was reverse-transcribed with SYBR PrimeScript miRNA RT-PCR kit according to the manufacturer's instructions. The obtained cDNA was used to determine the miRNA amount of miR-200a, miR-200b and miR-200c, U6 was used as an internal control. The primers of miR-200a (DHM0178), miR-200b (DHM0179), miR-200c (DHM0180) and U6 (D356-03) were obtained from Dalian Takara Biotechnology Co., Ltd. Quantitative PCR was performed using SYBR Premix Ex Taq II in an ABI 7500 Fast instrument. Q-PCR reactions were carried out following the manufacturer's protocol. miRNA expression values were determined as ΔCt=Ct (sample)-Ct (U6) and relative quantities between different samples were determined as ΔΔCt=ΔCt (sample 1)-ΔCt (sample 2), the values were expressed as $2^{-\Delta\Delta Ct}$. All Q-PCR reactions were conducted in triplicate.

Statistical analysis. The data are presented as the means of three determinations and was analyzed using the SPSS package for Windows (Version 18.0). Statistical analysis of the data was performed with Student's t-test and ANOVA. Differences with $P<0.05$ were considered statistically significant.

Results

PZH inhibits migration and invasion of HCT-8 cells. We first performed a wound-healing assay to evaluate the effect of PZH on the migration of HCT-8 cells. As shown in Fig. 1, after post-wounding for 24 h, untreated HCT-8 cells migrated into the clear area, whereas PZH treatment dose-dependently inhibited HCT-8 cell migration. We further verified these results using transwell assay; and the data showed that treatment with 0.25-0.75 mg/ml of PZH for 24 h dose-dependently reduced cell migratory rate of HCT-8 cells by 44.4-85.8%, as compared to untreated cells (Fig. 2, $P<0.05$). We next determined the effect of PZH on the invasion capacity of HCT-8 cells using the transwell assay. As shown in Fig. 3, compared with untreated cells (100%), the invasion rate of HCT-8 cells following treatment with 0.25, 0.5 or 0.75 mg/ml of PZH was 46.0±8.4, 29.6±3.0 or 19.1±4.0%, respectively ($P<0.05$). Taken together, these data suggest that PZH can inhibit metastasis of human colorectal cancer cells.

PZH modulates the activation of TGF-β1 pathway and the expression of EMT-regulatory genes in HCT-8 cells. To determine the PZH effect on the activation of TGF-β1 signaling, we examined the protein expression of several key mediators of this pathway using western blot analysis. As shown in Fig. 4, the protein expression levels of TGF-β1, Smad2/3 and Smad4 were downregulated by PZH treatment in a dose-dependent manner. Moreover, PZH treatment suppressed the expression of TGF-β1 target genes ZEB1 and ZEB2, leading to the downregulation of expression of mesenchymal marker N-cadherin as well as an increase in the expression of epithelial marker E-cadherin (Fig. 4). Therefore, the inhibitory effect of PZH on cancer cell metastasis might be mediated by the suppression of TGF-β1 pathway and the process of EMT.

PZH upregulates the expression of miR-200a, miR-200b and miR-200c in HCT-8 cells. To further explore the mechanism of anti-metastasis activities of PZH, we determined the expression of miR-200 family in HCT-8 cells using Q-PCR assay. As shown in Fig. 5, PZH treatment significantly and dose-dependently increased the expression of miR-200a,

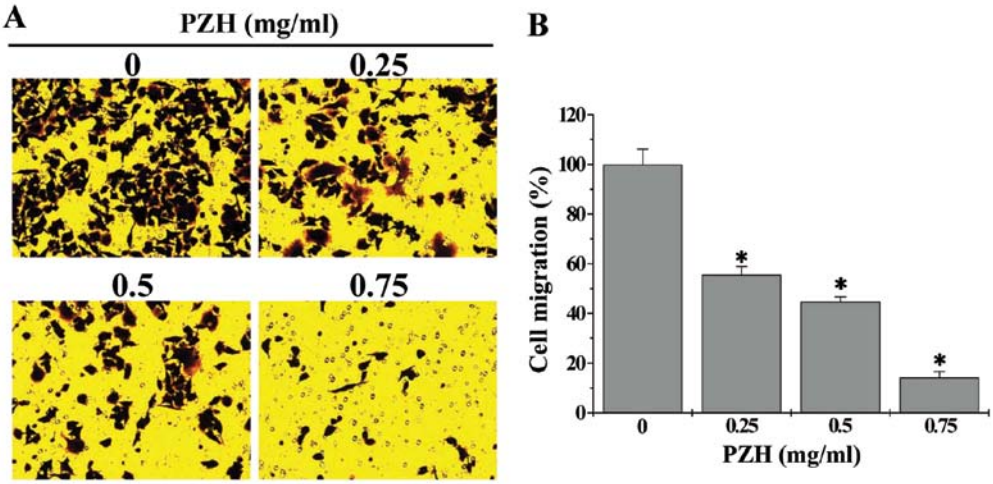

Figure 2. Effect of PZH on the migration of HCT-8 cells by transwell assay. HCT-8 cells were treated with indicated concentrations of PZH for 24 h. (A) The migration of HCT-8 cells was determined using transwell cell culture chambers. Cells were stained with crystal violet; the photographs were taken at a magnification of x200. (B) The average number of migrated cells was counted in 3 randomly selective fields. The data were normalized to the migration of untreated HCT-8 cells (100%). Data are averages with SD (error bars) from three independent experiments. *P<0.05, versus untreated HCT-8 cells.

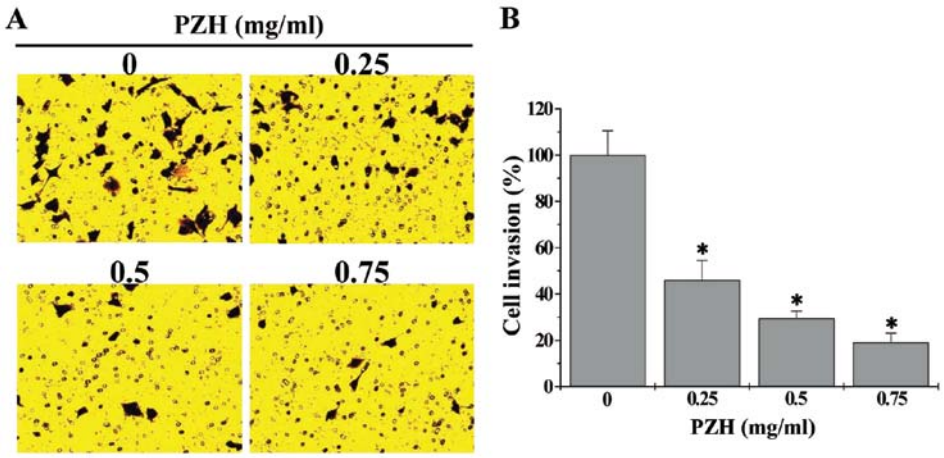

Figure 3. Effect of PZH on the invasion of HCT-8 cells. HCT-8 cells were treated with indicated concentrations of PZH for 24 h. (A) The invasion of HCT-8 cells was determined using transwell cell culture chambers with membranes coated with Matrigel matrix. Cells were stained with crystal violet; the photographs were taken at a magnification of x200. (B) The average number of invaded cells was counted in 3 randomly selective fields. The data were normalized to the invasion of untreated HCT-8 cells (100%). Data are averages with SD (error bars) from three independent experiments. *P<0.05, versus untreated HCT-8 cells.

miR-200b and miR-200c, consistent with the observations that PZH inhibited the TGF-β1 pathway and expression of ZEB transcription factors (Fig. 4).

Discussion

Drug resistance and intrinsic cytotoxicity against normal cells profoundly limit the long-term use of currently-used chemotherapeutic regimens and thereby their therapeutic effectiveness (41,42), emphasizing the need for the development of novel antitumor drugs. Due to the relatively higher safety and the long history of pharmacological applications, traditional Chinese medicines (TCM) have attracted great interest in the field of cancer treatment (28,29). TCM formula is a complex combination of many natural products, each of which contains numerous chemical compounds. Therefore, TCM formulas are considered to be multi-component and multi-target agents exerting their therapeutic function in a more holistic way; and discovering naturally-occurring agents could be a promising approach of cancer treatment. Pien Tze Huang (PZH) is a well-known TCM formula that has been used in China and Southeast Asia for centuries as a folk remedy for various types of cancer. We recently reported that PZH can inhibit colon cancer growth through the promotion of cancer cell apoptosis, the inhibition of cell proliferation and tumor angiogenesis, which is probably mediated by modulation of multiple signaling pathways (33-40). These data demonstrate that PZH possesses a broad range of anticancer activities due to its ability to affect multiple intracellular targets, suggesting that PZH could be a novel multi-target anticancer agent.

Tumor metastasis is a complex process involving the spread of malignant tumor cells from a primary tumor site to a distant organ, which is a major cause of failure of clinical cancer chemotherapy and therefore has become an important focus for anticancer therapies (5-7,13,14). To further elucidate the mode of action of PZH, in the present study we evaluated

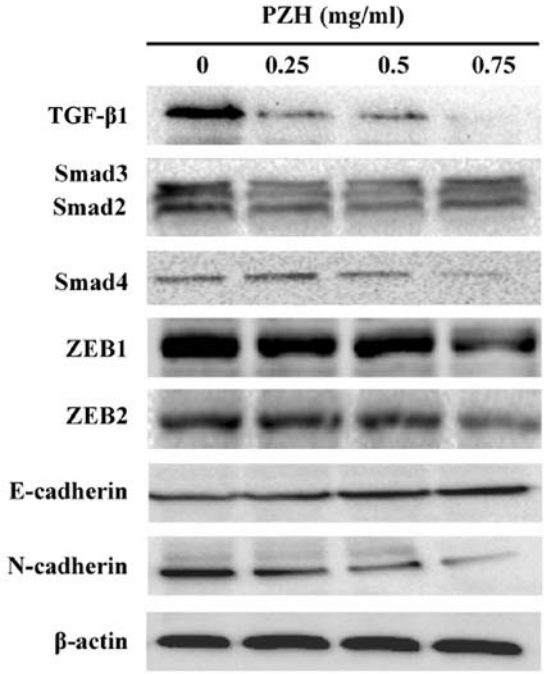

Figure 4. Effect of PZH on the expression of relative genes involved in TGF-β1 pathway in HCT-8 cells. HCT-8 cells were treated with indicated concentrations of PZH for 24 h. The protein expression levels of E-cadherin, N-cadherin, ZEB1, ZEB2, TGF-β1, SMAD2/3 and SMAD4 in HCT-8 cells were determined by western blot analysis. β-actin was used as the internal control. Images are representatives of three independent experiments.

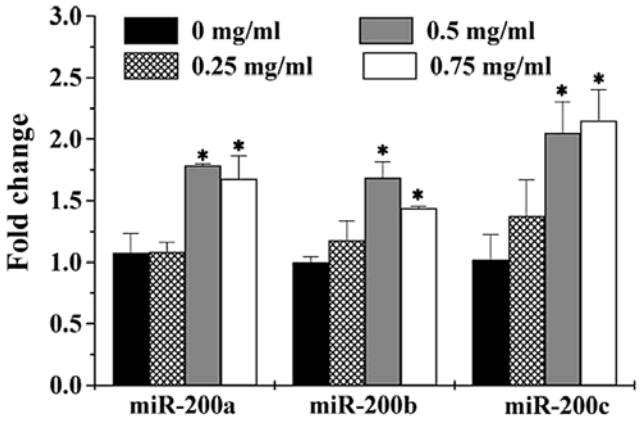

Figure 5. Effect of PZH on the expression of miR-200 family in HCT-8 cells. HCT-8 cells were treated with indicated concentrations of PZH for 24 h. The expression miR-200a, -b, and -c in HCT-8 cells were determined by Q-PCR analysis. U6 was used as the internal control. *P<0.05, versus untreated HCT-8 cells.

its effects on cancer metastasis. Using wound healing and transwell assays we found that PZH treatment significantly inhibited the migration and invasion of human colorectal carcinoma HCT-8 cells in a dose-dependent manner, demonstrating the inhibitory activity of PZH on the metastatic capacities of colorectal cancer cells. Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells lose their polarity and cell-cell adhesion, and acquire migratory and invasive properties of mesenchymal cells (8,9,14,15). After acquiring a mesenchymal phenotype through the process of EMT, carcinoma cells invade adjacent tissues, break through the basement membrane, and eventually enter the bloodstream leading to cancer metastasis (8,9,14-17). Therefore, EMT is an essential step for the initiation of cancer metastasis. Using western blot analysis we found that PZH treatment reduced the protein expression of mesenchymal marker N-cadherin but increased that of epithelial marker E-cadherin, indicating that the anti-metastasis activity of PZH was associated with its inhibitory effect on EMT. The processes of EMT and metastasis are highly regulated by multiple mechanisms, including TGF-β1/SMAD pathways and miRNA 200 family (10-14). The activation of TGF-β1 signaling enhances the expression of ZEB transcription factors, which in turn modulates the expression of EMT-regulatory genes resulting in the initiation of EMT (15,16). Interestingly, the expression of ZEB transcription factors can be downregulated by miR-200 family members (11,14,25-27); but miR-200 family expression is negatively regulated by TGF-β1 signaling (12), forming a double-negative feedback loop to control the processes of EMT and metastasis (13,14). Data from western blot and Q-PCR analyses indicated that PZH suppressed the activation of TGF-β1 pathway and the expression of ZEB1 and ZEB2, whereas the expression of miR-200a, miR-200b and miR-200c was upregulated after PZH treatment.

In conclusion, here we report that PZH can inhibit the metastatic capacity of human colorectal carcinoma cells via modulating TGF-β1/ZEB/miR-200 signaling network, which might be one of the mechanisms whereby PZH exerts its anti-cancer function.

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