Pien Tze Huang inhibits liver metastasis by targeting TGF-β signaling in an orthotopic model of colorectal cancer

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Abstract. Metastasis is the leading cause of cancer-related mortality in almost all types of cancers, including colorectal cancer (CRC). Epithelial-mesenchymal transition (EMT) is a critical process during the metastatic cascade. This process may be a potential target for the diagnosis and treatment of CRC. Pien Tze Huang (PZH), a well-known traditional Chinese formula, has been demonstrated to be clinically effective in treating various types of human malignancies, including CRC. Our published data suggest that PZH can induce apoptosis, as well as inhibit cell proliferation and tumor angiogenesis, thus suppressing CRC growth *in vitro* and *in vivo*. We evaluated the therapeutic efficacy of PZH against CRC metastasis using a CRC liver metastasis mouse model to further explore the mechanisms underlying the antitumor action of PZH. MTT, migration, and Matrigel invasion assays were used to assess the effect of PZH on cell viability, migration and invasion. We then established an orthotopic liver metastasis model of colon cancer using microsurgical techniques. Mice were intragastrically administered 234 mg/kg/day dose of either PZH or saline for 14 days. The body and tumor weights of the mice were measured after they were sacrificed. Moreover, we examined the effect of PZH inhibition on liver metastasis. Finally, EMT-related proteins and the TGF-β signaling pathway were assessed using immunohistochemical staining (IHS). The present data revealed that PZH significantly inhibited the migration and invasion of CT-26 cells in a dose-dependent manner, which affirmed the inhibitory effect of PZH on CRC cell metastasis. No significant change was observed between the *in vivo* primary tumor growth and body weight. However, the control group had five cases of liver metastasis (5/6), whereas one case was found in the PZH group (1/6). Thus, PZH exhibited therapeutic efficacy against CRC metastasis without apparent toxicity. The inhibitory effect of PZH on EMT resulted in an increase in E-cadherin expression, as well as a decrease in N-cadherin expression. In addition, PZH significantly inhibited TGF-β, as well as the phosphorylation of Smad2/3 and Smad4 in the tumor tissues, indicating its suppressive action on TGF-β signaling. These molecular effects ultimately resulted in the inhibition of cancer cell EMT and tumor metastasis.

Introduction

Metastasis is the leading cause of cancer-related mortality in almost all types of cancers, including colorectal cancer (CRC). Although surgery, chemotherapy and radiotherapy have long been the standards of CRC treatment, the long-term survival and prognosis of patients remain quite poor due to tumor recurrence and metastasis (1,2). Over 50% of patients with CRC present with liver metastases or develop liver metastases (3). Thus, CRC remains a major global public health issue.

Metastasis is a complex, multistep and dynamic biological process, with epithelial-mesenchymal transition (EMT) being a critical step during the cascade (4). EMT refers to the morphological and molecular changes that occur when epithelial cells lose their characteristics, gain mesenchymal properties and become motile, which makes EMT a key event in tumor invasion and metastasis (5-7). EMT could thus be a potential target for CRC therapy.

Some complementary and alternative medical systems, such as traditional Chinese medicine (TCM), have various approaches to cancer treatment. TCM has the advantage of reducing cancer therapy-induced toxicity (8). The modified classic formula has been shown to further minimize the side effects of surgery, radiation and chemotherapy. This formula also increases immunity and improves survival (9-11). Therefore, novel therapeutic strategies are essential to improve the clinical management of patients with CRC.

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Pien Tze Huang (PZH) was first prescribed by a royal physician in the Ming Dynasty. PZH is a well-known traditional Chinese formulation that is popularly known in Southeast Asia. The main ingredients of PZH include Moschus, Calculus Bovis, Snake Gall and Radix Notoginseng. These products ensure that PZH has heat clearing, detoxification, blood circulation promotion, blood stasis reduction and swelling reduction effects (12). Modern pharmacological studies have proposed that PZH not only exhibits therapeutic effects on hepatocellular carcinoma and CRC in clinical trials (13,14), but also inhibits the growth of human colon carcinoma cells by activating mitochondrion-dependent apoptosis (15). Moreover, PZH reportedly suppressed the cell proliferation and tumor angiogenesis of CRC carcinoma cells *in vitro* and *in vivo* (16,17). To elucidate further the antitumor mechanism of action of PZH, we evaluated its efficacy against tumor invasiveness *in vivo* and *in vitro* and investigated its underlying molecular mechanisms.

Materials and methods

Materials and methods. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). E-cadherin, N-cadherin, TGF-β, Smad2/3, P-Smad2/3, Smad4 and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). All the other chemicals used, unless otherwise indicated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Preparation of PZH. PZH was obtained from and authenticated by the sole manufacturer Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd. (Zhangzhou, China; Chinese FDA, approval no. Z35020242). PZH stock solution was prepared by dissolving PZH powder in phosphate-buffered saline (PBS) to a concentration of 20 and 234 mg/ml before use. The PZH working solutions were prepared by diluting the stock solution in either culture medium or saline.

Cell culture. Murine colon carcinoma CT-26 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. The cells were grown in DMEM containing 10% (v/v) FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a 37°C humidified incubator under 5% CO_2. The cells were subcultured at 80 to 90% confluency.

Cell viability evaluation. Cell viability was assessed through an MTT colorimetric assay. CT-26 cells were seeded into 96-well plates at a density of 1×10^4 cells/well in 0.1 ml of medium. The cells were treated with various concentrations of PZH for different periods. At the end of the treatment, 100 μl of MTT (0.5 mg/ml in PBS) was added to each well. The samples were then incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO. Absorbance was measured at 570 nm using an ELISA reader (Model ELx800; BioTek, Winooski, VT, USA).

Migration assay. The migration assay was performed using Transwell cell culture chambers. Conditioned medium (500 μl

of media with 10% FBS) was added to the lower compartment of the chamber. CT-26 cells (5×10^5) in 1% FBS-containing media were added to the upper compartment of the chamber. After 24 h of incubation, the top side of the insert membrane was scrubbed with a cotton swab, whereas the bottom side was fixed with ice-cold methanol, stained with 0.01% crystal violet and scored under a fluorescence microscope (Olympus IX51 with DP70; Olympus, Center Valley, PA, USA).

Matrigel invasion assay. The invasion assay was performed using Transwell cell culture chambers (Corning Costar No. 3422; Corning, Tewksbury, MA, USA) in accordance with the manufacturer's instructions but with specific modifications. Briefly, the upper surface of polyvinylpyrrolidone-free polycarbonate filters (8.0-mm pore size; Nuclepore Corp., Pleasanton, CA, USA) was pre-coated with 15 μl of ice-cold Matrigel (BD Biosciences, Bedford, MA, USA) for 60 min at room temperature. Conditioned medium (500 μl of medium with 10% FBS) was added to the lower compartment of the chamber. CT-26 cells (5×10^5) in 1% FBS-containing media were added to the upper compartment of the chamber. After 24 h of incubation, the top side of the membrane insert was scrubbed free of cells using a cotton swab, whereas the bottom side was fixed with ice-cold methanol, stained with 0.01% crystal violet and quantified.

Animals. Male BALB/c athymic (nude) mice and BALB/c mice (with an initial body weight of 20 to 22 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with controlled temperature (22°C), humidity and a 12-h light/dark cycle. Food and water were given *ad libitum* throughout the experiment. All the animal treatments were performed in strict accordance with international ethical guidelines and the National Institutes of Health Guide Concerning the Care and the Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

CRC liver metastasis animal model. CT-26 cells were grown in culture and then detached through trypsinization. Thereafter, the cells were washed and resuspended in serum-free DMEM. Then, 1.5×10^6 of cells mixed with Matrigel (1:1) were subcutaneously injected into the right flank area of athymic nude mice, the growth of which was monitored regularly. After 16 days of xenograft implantation, tumors were selected for implantation to other nude mice using a 20-inoculating needle at an average size of 1 cm^3. The tumor tissues of 4-6 generations were chosen for orthotopic transplantation into the cecum surface. Mice were anesthetized with an i.p. injection of Nembutal (pentobarbital; Abbott Laboratories, Abbott Park, IL, USA) at a dose of 70 mg/kg. After shaving, a small incision was made through their skin over the cecum under a dissecting microscope and by using microsurgical techniques. The cecum was exteriorized through a small midline laparotomy, and a piece of tumor tissue that was derived from CT-26 cells was sutured to the cecal surface with a single Maxon 11/0 suture, which left the tumor tissue buried in a 'pouch' that consisted of a double cecal wall on each side. After implantation, the abdominal wall was closed in two layers with Dexon 5/0. Food and water
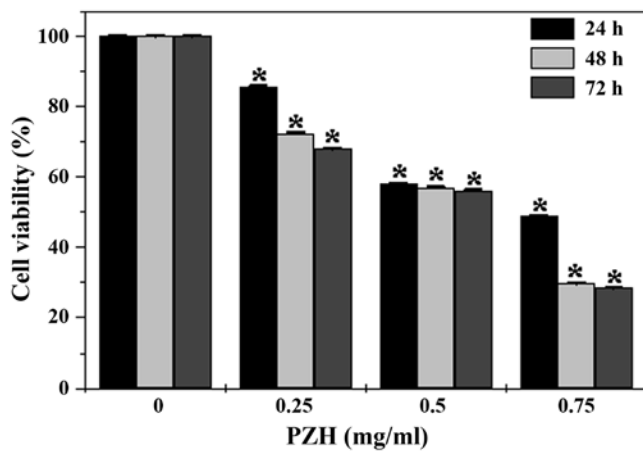
Figure 1. Effect of PZH on the viability of CT-26 cells. The cells were treated with various concentrations of PZH for the indicated time-periods. Cell viability was determined using an MTT assay. The data were normalized to the viability of the control cells (100%, treated with 0.5% DMSO vehicle). Data are presented as the mean ± standard deviation from 3 independent experiments. *P<0.05 vs. the control cells. PZH, Pien Tze Huang.

were given *ad libitum*. A few days after the cecal implantation of the colon tumor, the groups of mice were randomly assigned to receive one of two treatments (n=6/group). The groups of mice were intragastrically administered with a 234 mg/kg/day dose of either PZH or saline daily. The animals were sacrificed 14 days after tumor implantation. The tumor tissues were removed and weighed. A portion of each tumor was fixed in 10% buffered formalin. The remaining tissues were snap-frozen in liquid nitrogen and stored at -80°C. We recorded all the macroscopic tumor deposits and abnormalities in the liver. The number of liver metastases was calculated. All neoplasms were identified through H&E staining.

Histological examination by H&E staining. The metastasized liver tissues were fixed with 10% buffered formalin for 24 h. Samples were then paraffin-embedded, sectioned and stained with H&E. Histopathological changes were observed under a light microscope.

Immunohistochemistry. Immunohistochemical staining for E-cadherin, N-cadherin, TGF-β, Smad2/3, P-Smad2/3 and Smad4 was performed as follows. Briefly, after being fixed with 10% formaldehyde for 12 h, tumor samples were processed for paraffin-embedded tumor slides. The slides were subjected to antigen retrieval, and the endogenous peroxidase activity was quenched with hydrogen peroxide. After blocking nonspecific proteins with normal serum in PBS (0.1% Tween-20), the slides were incubated with rabbit polyclonal antibodies against E-cadherin, N-cadherin, Smad2/3, P-Smad2/3, Smad4, TGF-β, ZEB1 and ZEB2 (all in 1:200 dilution). After being washed with PBS, the slides were incubated with a biotinylated secondary antibody followed by conjugated HRP-labeled streptavidin (Dako) and then washed with PBS. The slides were then incubated with diaminobenzidine (DAB, Sigma Chemicals) as the chromogen, followed by counterstaining with diluted Harris' hematoxylin (Sigma Chemicals). After staining, five 5-power fields (400x) were randomly selected for

each slide. The average proportion of the positive cells in each field was quantified using the True Color Multi-Functional Cell Image Analysis Management System (Image-Pro Plus; Media Cybernetics, Rockville, MD, USA). To rule out any non-specific staining, PBS was used to replace the primary antibody as a negative control.

Statistical analysis. All data are the means of 3 determinations and were analyzed using SPSS Package for Windows (Version 11.5). Statistical data analysis was performed using Student's t-test and ANOVA. Differences with P<0.05 were considered statistically significant.

Results

PZH inhibits the viability of CT-26 cells. The viability of the CT-26 cells was examined using an MTT assay to compare the relative number of cells in the PZH-treated monolayers with that in the untreated controls. As shown in Fig. 1, treatment with 0.25 to 0.75 mg/ml of PZH for 24, 48 and 72 h, respectively reduced cell viability by 14.3 to 51.1%, 27.4 to 60.3%, and 32.1 to 71.5% compared with the untreated control cells (P<0.05). These data indicate that PZH inhibited CT-26 cell growth and proliferation in a dose- and time-dependent manner.

PZH inhibits migration and invasion of CT-26 cells. To examine the effects of PZH on cell migration, we performed Transwell migration assays with CT-26 cells. As demonstrated in Fig. 2 (upper panels), PZH effectively inhibited cell migration of CT-26 cells in a dose-dependent manner. Furthermore, a Transwell invasion assay was used to determine the invasive activity of the tumor cells across the basement membrane (Fig. 2, lower panels). Overall, the results revealed that PZH significantly decreased the invasive potential of CT-26 cells in a dose-dependent manner.

PZH inhibits tumor liver metastasis in the orthotopic CRC mouse model. The antitumor effect on liver metastasis of PZH *in vivo* was determined by examining the tumor weight and the number of liver metastases in the orthotopic CRC mouse model. The adverse effects were evaluated by measuring body weight changes. As shown in Fig. 3A and B, PZH treatment significantly reduced the number of tumor liver metastases as compared with the control (P<0.01). After H&E staining, histological changes in the liver samples of CRC mice were observed under light microscopy. PZH treatment was observed to have no effect on the changes in the tumor and body weights of the mice (Fig. 3C). Thus, PZH effectively suppressed CRC liver metastasis *in vivo*, but did not significantly affect CRC growth and caused no apparent signs of toxicity.

PZH inhibits EMT in the orthotopic CRC mouse model. E-cadherin and N-cadherin, which are markers of EMT, not only mediate cell migration and invasion properties, but also contribute to metastasis (18,19). Therefore, we further investigated E-cadherin, N-cadherin, and ZEB1 expression to explore further the anti-metastatic activity of PZH. We performed immunohistochemical staining (IHS) analyses to examine the protein expression of both E-cadherin and N-cadherin
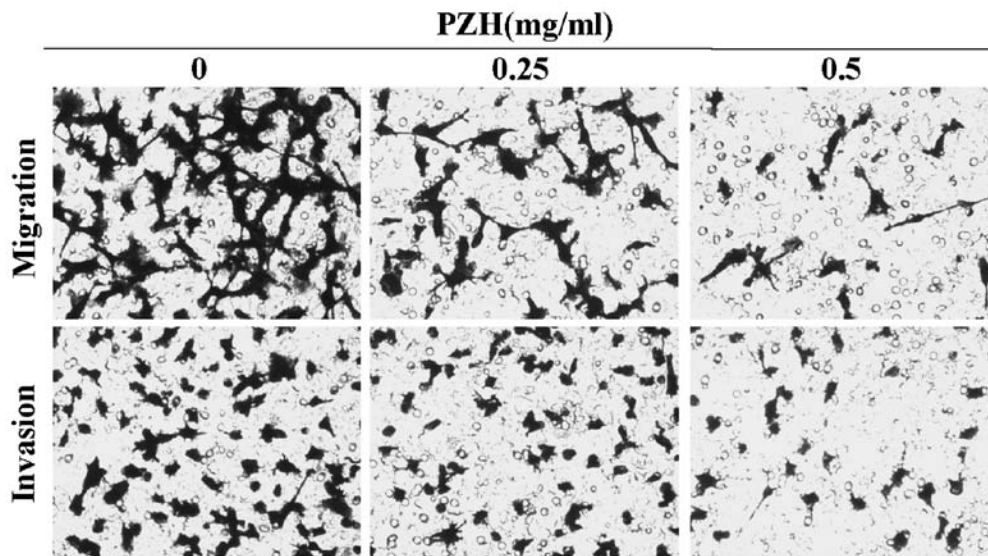
Figure 2. Effect of PZH on CT-26 cell migration and invasion. CT-26 cells were treated with the indicated concentrations of PZH for 6 h. Cell migration was determined using Transwell® cell culture chambers. Cell invasion was determined using Transwell cell culture chambers with membranes coated with Matrigel™. Cells were fixed and stained with crystal violet, and images were captured at a magnification, x200. The average number of migrated cells was determined from three randomly selected fields. Data are presented as the mean ± standard deviation from three independent experiments. PZH, Pien Tze Huang.

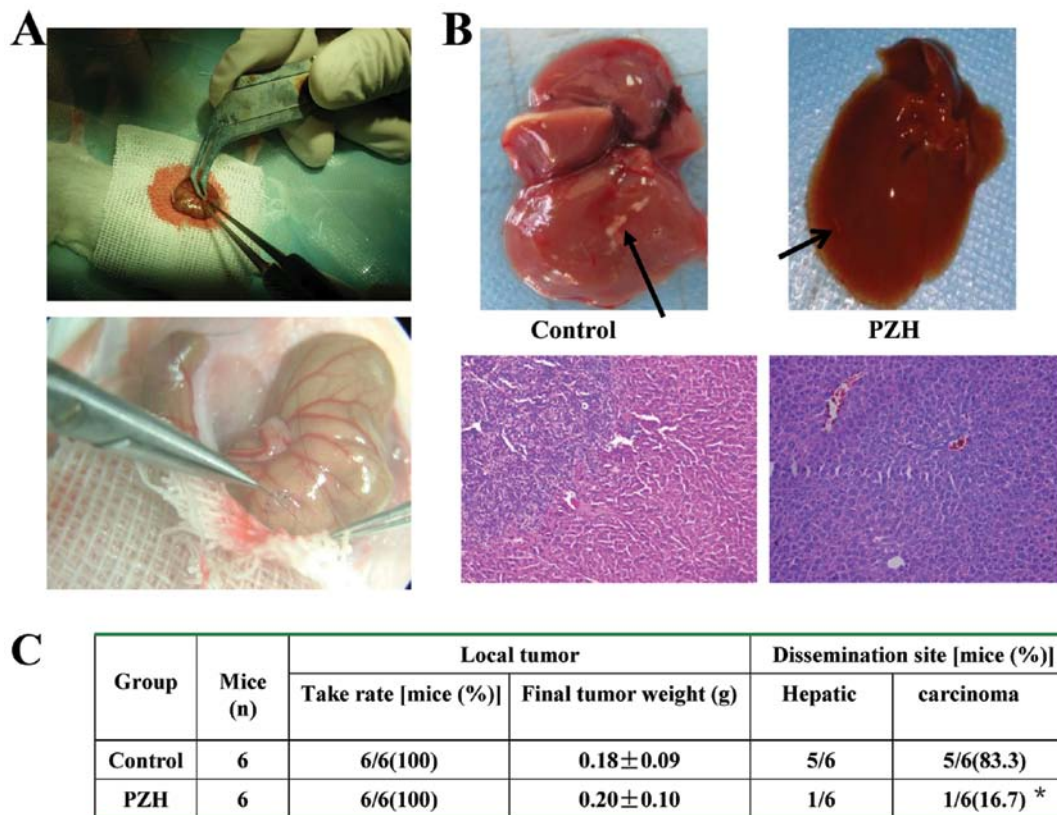| Group	Mice (n)	Local tumor		Dissemination site [mice (%)]	
		Take rate [mice (%)]	Final tumor weight (g)	Hepatic	carcinoma
Control	6	6/6(100)	0.18±0.09	5/6	5/6(83.3)
PZH	6	6/6(100)	0.20±0.10	1/6	1/6(16.7) *

Figure 3. Effect of PZH on tumor liver metastasis in the orthotopic CRC mouse model. At the end of the experiment, tumor tissues from the control and the PZH-treated group were measured, and the number of tumor liver metastases was calculated. (A and B) PZH treatment significantly reduced the number of tumor liver metastases (arrows) as compared with the control. Tumor liver metastasis tissues were fixed with 10% buffered formalin for 24 h. Samples were then paraffin-embedded, sectioned, and stained with H&E. Histopathological changes were observed under a light microscope. Images are representative images captured at a magnification, x200. (C) Data are presented as the mean ± standard deviation from six individual mice in each group. *P<0.01 vs. controls. CRC, colorectal cancer; PZH, Pien Tze Huang.

in the orthotopic CRC mouse model. As shown in Fig. 4, the percentages of E-cadherin-positive cells in the PZH-treated and control mice were 37.50±5.16 and 27.17±5.42% (P<0.01), respectively. By contrast, the proportion of cells expressing
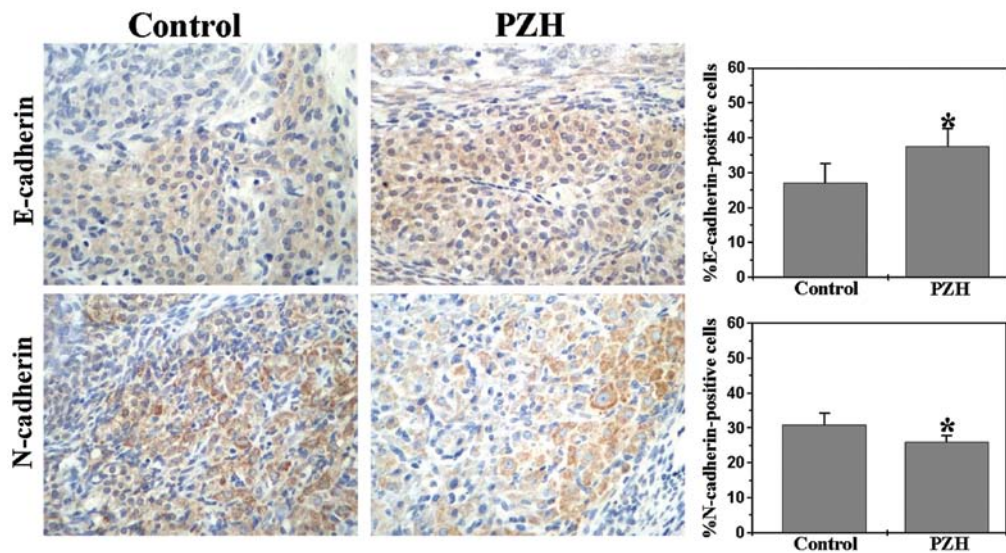
Figure 4. Effect of PZH on EMT in the orthotopic colorectal cancer mouse model. At the end of the experiment, tumor tissues from the control and the PZH-treated group were processed for immunohistochemical staining for E-cadherin and N-cadherin. The photographs are representative images captured at a x400 magnification. Quantification of the immunohistochemical assay is represented as the percentage of the positively stained cells. Data are presented as the mean ± standard deviation from six individual mice in each group. *$P<0.01$ vs. controls. PZH, Pien Tze Huang.
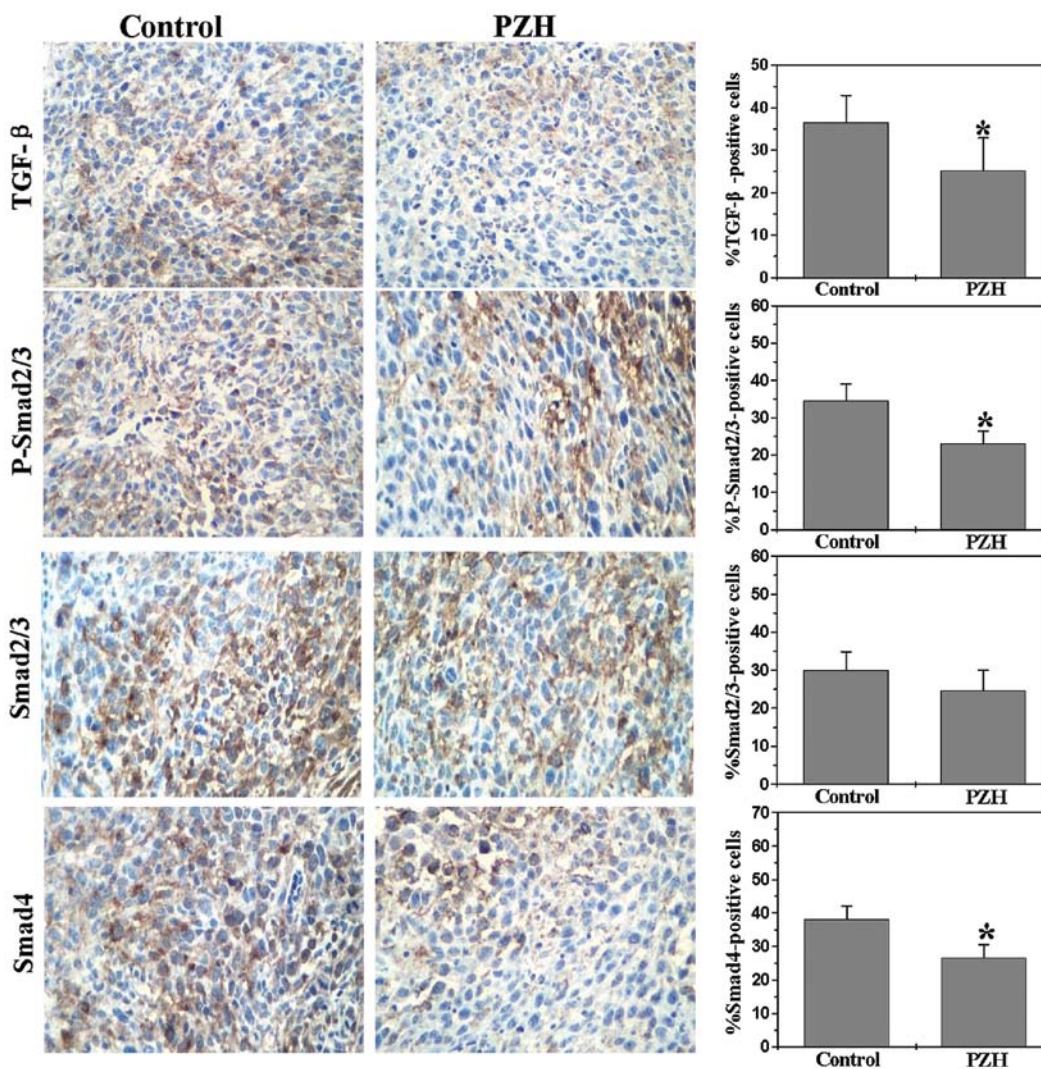

Figure 5. Effect of PZH on the TGF-β/Smad pathway in the orthotopic colorectal cancer mouse model. Tumor tissues from the control and the PZH-treated group were also processed for immunohistochemical staining for TGF-β, Smad2/Smad3, P-Smad2/Smad3 and Smad4. The photographs are representative images captured at a x400 magnification. Quantification of the immunohistochemical assay is represented as the percentage of positively stained cells. Data are presented as the mean ± standard deviation from six individual mice in each group. *$P<0.01$ vs. controls. PZH, Pien Tze Huang.

N-cadherin was 26.01±1.78% in the PZH-treated mice and 30.83±3.43% in the controls. These data collectively suggest that the antitumor liver metastasis function of PZH may be attributed to the inhibition of EMT of CRC cells.

PZH inhibits activation of the TGF-β pathway in the orthotopic CRC mouse model. Numerous pathways have been implicated in colorectal carcinogenesis. Among these pathways is the TGF-β/Smad signaling pathway, which is responsible for TGF-β-mediated cell growth inhibition and apoptosis. TGF-β induces angiogenesis, inflammation and EMT, thus providing a beneficial environment for tumor progression and metastasis (20). Therefore, we assessed the effect of PZH on the expression of key mediators of the TGF-β/Smad pathway using IHS analyses. As shown in Fig. 5, PZH treatment significantly reduced the protein expression of TGF-β, P-Smad2/Smad3, Smad2/Smad3, and Smad4 in the tumor tissues. The percentages of TGF-β-, P-Smad2/Smad3-, Smad2/Smad3- and Smad4-positive cells in the control group were 36.5±6.41, 34.5±4.55, 30.0±4.63 and 38.17±3.68%, respectively. By contrast, the percentages of TGF-β-, P-Smad2/Smad3-, Smad2/Smad3- and Smad4-positive cells in the tumor tissue of PZH-treated mice were 25.2±7.69, 23.0±3.40, 24.67±5.32 and 26.67±3.82%, respectively (P<0.01). These data suggest that the *in vitro* inhibitory effect of PZH on tumor metastasis could be mediated by the suppression of the TGF-β/Smad pathway.

Discussion

The occurrence of metastases due to tumor progression causes the vast majority of cancer-related deaths. Metastatic progression is a multi-step process that includes the detachment of cancer cells from the primary tumor mass, migration and invasion, thus enabling the re-establishment of malignant cells at distant sites. TGF-β is a multifunctional cytokine and is a potent inducer of EMT (21,22). Moreover, TGF-β can serve as a tumor suppressor by inhibiting cell proliferation in the early stages of tumor development while promoting metastasis in various cancer models (23,24). CRC is associated with deregulated levels of TGF-β, which predicts poor prognosis and limited recurrence-free survival. A growing number of *in vivo* studies have recently shown that the inhibition of TGF-β signaling and transcription reduces the metastatic and/or invasive properties of various experimental cancers, including CRC, presumably by preventing the induction of migration and invasion in cancer cells (25-27).

The function of the TGF-β signaling pathway depends on the binding of ligands to cell membrane receptors, activating cytoplasm mediators into the nucleus, and regulating expression of their target gene. The Smad pathway is a major transducer of TGF-β signaling (28). Smad2 and Smad3, which are known as receptor-regulated Smads, are both phosphorylated by the type 1 TGF-β receptor and form complexes with Smad4 (29). These complexes accumulate in the nucleus of the cell and regulate the transcription of target genes, thus serving critical functions in controlling cell proliferation, differentiation, apoptosis and cell migration.

E-cadherin and N-cadherin are target genes of the TGF-β signaling pathway. These genes are important for control-

ling cell migration. The malignancy of carcinoma cells is characterized by the loss of both cell-to-cell adhesion and cellular differentiation, which have been repeatedly reported to correlate with E-cadherin downregulation and N-cadherin upregulation (30,31). Dysfunction or loss of E-cadherin and increase in N-cadherin could be attributed to somatic mutations in some tumor types, which include CRC and are associated with the development of invasive carcinoma, metastatic dissemination and poor clinical prognosis. Therefore, the deregulation of E-cadherin and the upregulation of N-cadherin expression may contribute to tumorigenesis.

In conclusion, the present study is the first to demonstrate that PZH inhibits the invasiveness of CRC by suppressing the TGF-β/Smad pathway, by promoting the expression of E-cadherin and by suppressing the expression of N-cadherin. Our findings suggest that PZH may be a potential novel therapeutic agent for the treatment of cancers owing to its inhibition of CRC metastasis.

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