Pien Tze Huang suppresses VEGF-C-mediated lymphangiogenesis in colorectal cancer

JIUMAO LIN[1,2*], JIANYU FENG[1,2*], YIYI JIN[1,2], ZHAOKUN YAN[1,2], ZIJUN LAI[1,2] and JUN PENG[1,2]

[1]Academy of Integrative Medicine, and [2]Fujian Key Laboratory of Integrative Medicine on Geriatrics,
Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China

Abstract. Colorectal cancer (CRC) is one of the most common malignancies worldwide. The majority of patients are not suitable for surgery due to the presence of metastatic disease at the time of diagnosis, which has led to a high mortality rate for patients with CRC. Lymphangiogenesis, formation of new lymphatic vessels, plays an critical role in cancer progression particularly in cancer metastasis. Vascular endothelial growth factor-C (VEGF-C) has been previously demonstrated to play a pivotal role in cancer metastasis and therefore has become an attractive target for anticancer treatments. Pien Tze Huang (PZH) is a well-known traditional Chinese formula, which has exhibited significant therapeutic effects against CRC. However, the molecular mechanisms underlying its anticancer effects, particularly in regards to antimetastasis activity, still require further elucidation. In the present study, we evaluated the effects of PZH on cell migration and VEGF-C expression using various human CRC cell lines. Moreover, using a VEGF-C-stimulated human lymphatic endothelial cell (HLEC) model, we demonstrated that PZH suppresses lymphangiogenesis by attenuating cell migration and tube formation. This indicates that PZH possesses significant antimetastatic activity. Moreover, suppression of lymphangiogenesis by PZH via the downregulation of VEGF-C may be a potential molecular mechanism by which PZH inhibits metastasis in CRC.

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

*Contributed equally

Abbreviations: PZH, Pien Tze Huang; CRC, colorectal cancer; HLEC, human lymphatic endothelial cell; LV, lymphtic vessel; VEGF-C, vascular endothelial growth factor-C

Key words: Pien Tze Huang, colorectal cancer, lymphangiogenesis, VEGF-C, metastasis

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and one of the leading causes of cancer-related death worldwide with a rising annual incidence in numerous countries except the USA (1). Conventional therapies including surgery, chemotherapy or radiotherapy have been shown to be relatively ineffective (2). The main reasons for the lack of efficacy of these therapies are regional lymph node and/or distal metastasis of CRC cells (3). Approximately one quarter of CRC patients present with metastatic disease at the time of diagnosis, and their 5-year survival rate is near 10% (4). Therefore, elucidating the mechanisms involved in the metastatic cascade underlying this malignancy is a key goal for developing therapeutic agents to reduce CRC-associated mortality.

Lymphangiogenesis or the growth of lymphatic vessels (LVs), is an important step in tumor progression that supports tumor growth and metastatic dissemination (3). Lymphangiogenesis and early regional metastasis frequently occurs in several types of malignant tumors including CRC (5). The density of LVs in or surrounding tumors is closely associated with lymph node metastasis and prognosis of CRC patients (3). Therefore, metastasis to regional lymph nodes is generally believed to be the first indication that a tumor has progressed to metastatic competence (6). Thus, tumor lymphangiogenesis may be a crucial target of antimetastatic agents.

In the past decade, there has been a dramatic increase in the number of studies examining mechanisms of tumor-associated lymphangiogenesis and lymphatic metastasis. Vascular endothelial growth factor-C (VEGF-C), a member of the VEGF family, was the first lymphangiogenic growth factor to be identified (5). Mechanistically, the binding of VEGF-C to its cognate receptor VEGFR-3, which is expressed on human lymphatic endothelial cells (HLECs) (6), promotes proliferation and migration of HLECs as well as the formation of LVs (7,8). Thus, upregulation of VEGF-C expression has been implicated in induction of tumor lymphangiogenesis and lymphatic invasion (9). Therefore, targeting VEGF-C could be a novel anti-lymphangiogenic therapy.

Genomic instability and heterogeneity of tumor cell populations can lead to a shift in the expression of angiogenic factors during tumor progression. Various tumors at their advanced stage express multiple angiogenic factors (10). During this late stage, anti-VEGF-C therapy is predicted to be ineffective

or even encounters drug resistance. Indeed, it is possible that anti-VEGF-C therapy may even allow tumors to switch on other angiogenic pathways for lymphangiogenesis. As a result, tumors can escape from anti-VEGF-C therapy after a relative long-term treatment. Additionally, drugs used in anti-VEGF-C therapy can lead to an increase in side-effects such as rash, diarrhea, hypertension, gastrointestinal perforation and arterial thromobembolic events (11). These problems highlight the urgent need for the development of more effective anticancer agents.

Pien Tze Huang (PZH) is a well-known traditional Chinese formulation that was first prescribed 450 years ago by a royal physician in the Ming Dynasty. The main active ingredients of PZH include: Moschus, Calculus Bovis, Snake Gall and Radix Notoginseng. These ingredients together confer PZH properties of heat-clearing, detoxification, promotion of blood circulation and removal of blood stasis (12). Since in the Chinese medicine system accumulation of toxic dampness and heat is one of the major causative factors in the pathogenesis of cancers, PZH is thought to be an effective anticancer agent. In fact, PZH has long been used as an alternative treatment strategy for cancers in China and Southeast Asia. Previously, we reported that PZH can inhibit CRC growth *in vivo* and *in vitro* via promotion of apoptosis and suppression of proliferation of cancer stem cells (CSCs) as well as inhibition of angiogenesis and reversal of multidrug resistance through multiple pathways while causing minimal side-effects (13-17). Recently, we found that PZH also prevents metastasis by inhibiting the progression of epithelial-mesenchymal transition (EMT) (18,19), but the underlying mechanism of the antimetastasis effects of PZH are still unknown. To further elucidate the mechanism underlying the anticancer activity of PZH, in the present study, we evaluated the effects of PZH on tumor metastasis and lymphangiogenesis using different CRC cell lines and the VEGF-C-stimulated HLEC model.

Materials and methods

Materials and reagents. Roswell Park Memorial Institute (RPMI)-1640 medium (C11875500BT), Dulbecco's modified Eagle's medium (DMEM) with high glucose (C11995500BT), fetal bovine serum (FBS; #10099-141), penicillin-streptomycin (SV30010), 0.25% trypsin-EDTA (#25200-072), Pierce RIPA buffer (#89901), Pierce BCA Protein Assay kit (#23227) and SuperSignal™ West Pico Chemiluminescent Substrate (#34080) were all purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS; G5430) was provided by Promega Corporation (Madison, WI, USA). The exogenous VEGF-C (CYT-527) was purchased from ProSpec-Tany TechnoGene Ltd. (East Brunswick, NJ, USA). Annexin V-FITC apoptosis detection kit (KGA108) was obtained from KeyGen Biotech Co., Ltd. (Jiangsu, China). Transwell chambers (8.0 μm; #3422) were obtained from Corning Life Sciences (Corning, NY, USA). The *In Vitro* Angiogenesis assay kit (ECM625) and nitrocellulose (NC) membrane (0.45 μm; HATF00010) were purchased from Millipore (Billerica, MA, USA). Rabbit polyclonal antibody against VEGF-C (#2445), VEGFR-3 (#2485) and β-actin (#4967) were obtained from Cell Signaling Technology

(Beverly, MA, USA). Rabbit polyclonal antibody against MMP-2 (ab37150) and MMP-9 (ab38898) were purchased from Abcam (Cambridge, MA, USA). HRP-conjugated goat anti-rabbit secondary antibody (E030120) was purchased from EarthOx Life Science (Millbrae, CA, USA). Culture flask and plates were purchase from NEST Biotechnology Co., Ltd. (Wuxi, Jiangsu, China). All the other chemicals used, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Preparation of PZH. PZH was obtained from and authenticated by Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd. (Zhangzhou, China) (Chinese FDA approval no. Z35020242, lot no. 1009039). Stock solutions of PZH were prepared just before use by dissolving the PZH powder in phosphate-buffered saline (PBS) 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 metastatic CRC cell lines HCT-8, HCT-116 and SW620 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The HLEC was purchased from the JENNIO Biological Technology Co., Ltd. (Guangzhou, China). HCT-8, HCT-116 cells and HLECs were grown in RPMI-1640; SW620 cells were grown in DMEM. All cell media were supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and cultured at 37°C with 5% CO_2 in a humidified incubator (Forma 3110; Thermo Fisher Scientific, Inc.).

Treatment of PZH and exogenous VEGF-C. CRC cells were seeded into 6-well plates for 12 h, which was followed by PZH treatment for the indicated period of time. HLECs were first grown in complete RPMI-1640 (10% FBS) until ~60% confluency, and then continuously cultured in FBS-free medium overnight. The medium was replaced with RPMI-1640 with 2% FBS and cells were treated with 5 ng/ml VEGF-C and/or various concentrations of PZH for the indicated period of time.

Observation of morphological changes. Morphology of HLECs was observed using a phase-contrast microscope (FMIL/DFC295; Leica, Wetzlar, German) after PZH and/or exogenous VEGF-C treatment for 24 h. The images of five randomly selected views were captured at a magnification of x200.

Cell viability evaluation. Cell viability was assessed using MTS assay. CRC cells and HLECs were seeded in 96-well plates. After PZH and/or exogenous VEGF-C treatment for 24 or 48 h, 10 μl MTS was added to each well after which the samples were incubated for 1 h at 37°C. The resulting absorbance was measured at 490 nm using an ELISA reader (Model ELx800; BioTek, Winooski, VT, USA).

Detection of apoptosis. After incubation with various concentrations of PZH and/or exogenous VEGF-C for 24 h, apoptosis of HLECs was determined by FACSCalibur and Annexin V-FITC apoptosis detection kit, as previously described (14). Staining was performed according to the manufacturer's instructions. In the present study,
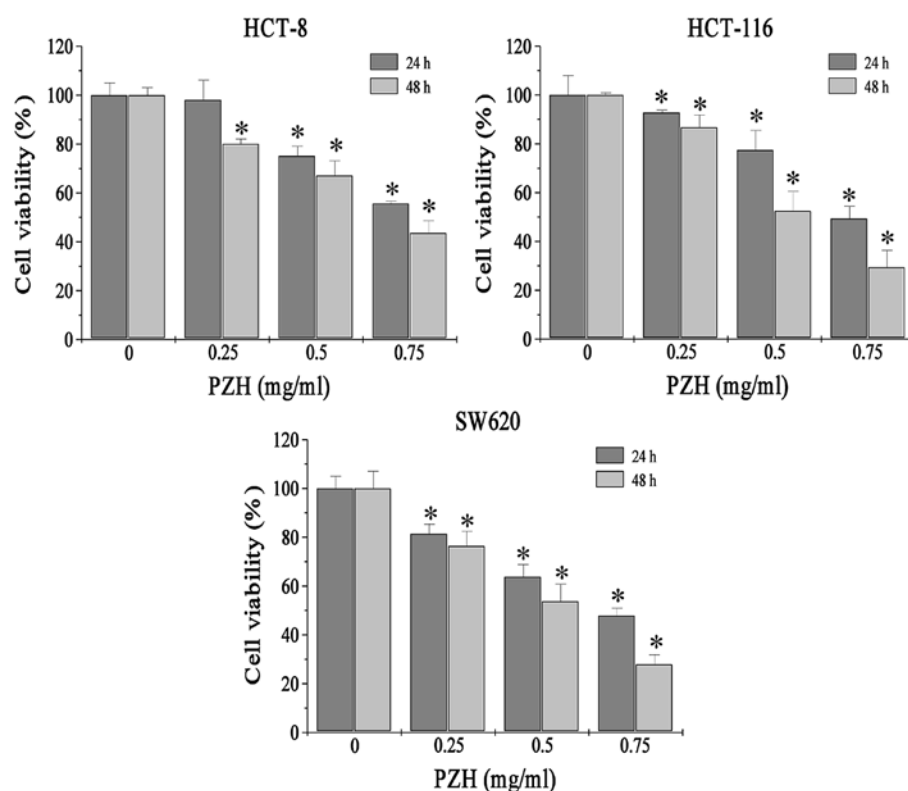
Figure 1. PZH reduces the viability of CRC cell lines. Subsequent to treatment with 0, 0.25, 0.5 and 0.75 mg/ml of PZH for 24 or 48 h, cell viability of human metastatic CRC cell lines HCT-8, HCT-116 and SW620 was determined by MTS assays. The data were normalized to the viability of the control cells. Data are averages with SD (error bars) from at least three independent experiments; *P<0.05, vs. the untreated control cells.

Annexin V/PI double-negative population (labeled as LL in the FACS diagram) indicates viable cells; Annexin V-posi-tive/PI-negative or Annexin V/PI double-positive population (labeled as LR or UR in the FACS diagram) represents cells undergoing early or late apoptosis, respectively.

Migration assays. Migration ability of the CRC cell lines was evaluated by wound-healing, as previously described (15). Briefly, CRC cells were seeded into 6-well plates (10^6 cells/well in 2 ml culture medium). After 12 h of incubation, cells were scraped away vertically in each well using a P200 pipette tip. Five randomly selected views along the scraped line were photographed from each well using a phase-contrast inverted microscope at a magnification of x100. Cells were then treated with the indicated concentrations of PZH for 24 h and another set of images were captured in the same way. A reduction in the scraped area indicates migration of cells. For HLECs, the migration assay was performed using Transwell cell culture chambers as previously described (18). The inserts were placed within a 24-well chamber containing 0.7 ml RPMI-1640 with 10% FBS to serve as a chemoattractant. As before, HLECs were seeded into 6-well plates and treated with different concentrations of PZH and/or exogenous VEGF-C for 24 h. Cells ($5x10^4$ cells) were seeded into the inserts suspended in 0.2 ml of serum-free RPMI-1640. The cells were incubated at 37°C with 5% CO_2 for 12 h before the upper surface of the filter was scraped to remove nonmigratory cells. Migrated cells were then fixed and stained with crystal violet. For quantification, the average number of migrating cells/field was assessed by counting five random fields under a phase-contrast microscope at a magnification of x200.

Tube formation assays. The HLEC tube formation was assessed using the *In Vitro* Angiogenesis assay kit as previously described (15). Briefly, HLECs were treated with PZH and/or exogenous VEGF-C for 24 h, harvested and diluted $2x10^5$ cells/well in 200 μl medium individually, were seeded into 1:1 ECMatix gel (v/v) coated 48-well plate, and incubated for 8 h. The series of tube-like structures were photographed using a phase-contrast microscope at a magnification of x40.

Western blot analysis. Cells were treated with PZH and/or exogenous VEGF-C for 24 h after which they were lysed using Pierce RIPA buffer containing protease inhibitor and phospha-tase inhibitor cocktails. The lysates were then centrifuged at 14,000 rpm for 20 min, and the resulting protein concentra-tions were determined using BCA protein assay reagent kit. A total of 50 μg of protein for each sample was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electropho-resis (SDS-PAGE) and resolved using 20 V for 10 min→ 80 V for 30 min→ 120 V for 1 h, then transferred onto nitrocel-lulose membranes. Following blocking with 5% non-fat dry milk, the membranes were incubated with antibodies against VEGF-C, VEGFR-3, MMP-2, MMP-9 and/or β-actin (1:1,000 dilution) overnight at 4°C and subsequently incubated with HRP-conjugated anti-rabbit secondary antibodies (1:5,000 dilution) for 1 h at room temperature. The membranes were then exposed with enhanced chemiluminescence (ECL)
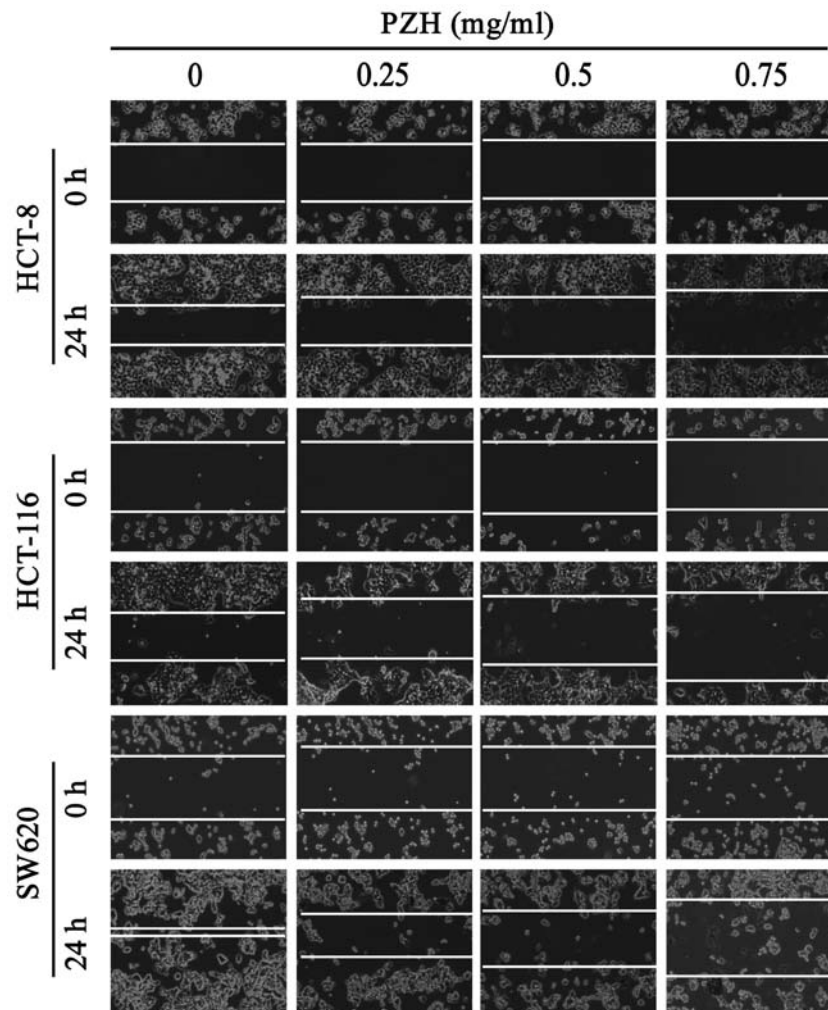
Figure 2. PZH reduces migration of CRC cell lines. HCT-8, HCT-116 and SW620 cells were treated with the indicated concentrations of PZH for 24 h. The images of wound healing are representative images of three independent experiments, and were captured at a magnification of x200.

detection using SuperSignal™ West Pico Chemiluminescent Substrate. Image Lab™ Software (version 3.0) was used for densitometric analysis and quantification of western blot analyses.

Statistical analysis. All data were collected based on the mean of three experiments. Statistical analysis was performed using the SPSS software (version 17.0) for Windows (SPSS, Inc., Chicago, IL, USA) using one-way ANOVA. P<0.05 was considered to indicate a statistically significant result.

Results

PZH reduces viability and migration of CRC cell lines. We first determined the effect of PZH on the viability of various CRC cell lines using MTS assay. PZH treatment at 0.25, 0.5 and 0.75 mg/ml for 24 or 48 h significantly reduced cell viability in the HCT-8, HCT-116 and SW620 cells (P<0.05) in a dose- and time-dependent manner (Fig. 1). We next evaluated the effect of PZH on migration of various CRC cell lines using a wound-healing assay. Twenty-four hours post-wounding, untreated HCT-8, HCT-116 and SW620 cells migrated into the wounded (clear) area of the cell monolayer, whereas PZH

treatment dose-dependently inhibited migration of all these three cell lines (Fig. 2).

PZH downregulates the expression of VEGF-C in CRC cell lines. To explore a potential mechanism underlying the anti-metastasis activity of PZH, we performed western blotting to examine the protein expression of VEGF-C in HCT-8, HCT-116 and SW620 cells. We found that PZH treatment profoundly and dose-dependently reduced the expression of VEGF-C (Fig. 3).

PZH reduces cell confluency and viability of VEGF-C-stimulated HLECs. As seen from the cell morphology, VEGF-C stimulation increased the confluency of HLECs, while PZH treatment decreased cell confluency in a dose-dependent manner (Fig. 4A). Moreover, we found that cell viability of HLECs increased to 105.56% at 24 h and 113.56% by 48 h (P<0.05) after VEGF-C stimulation compared to control cells that did not receive VEGF-C, using an MTS assay. Treatment with 0.0625-0.5 mg/ml of PZH for 24 h decreased the viability of VEGF-C-stimulated cells from 97.94 to 35.07% and from 81.01 to 24.10% after 48 h (P<0.05 vs. PZH-untreated cells) (Fig. 4B).
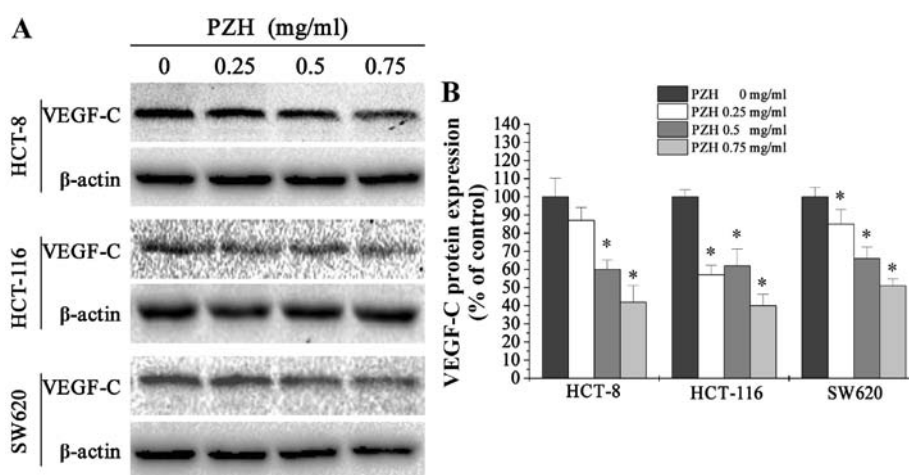
Figure 3. PZH downregulates the expression of VEGF-C in CRC cell lines. The protein expression levels of VEGF-C were analyzed by western blotting. β-actin was used as the internal control. (A) Data are representative of three independent experiments. (B) Quantification of western blot analysis; *P<0.05, vs. the untreated control cells.
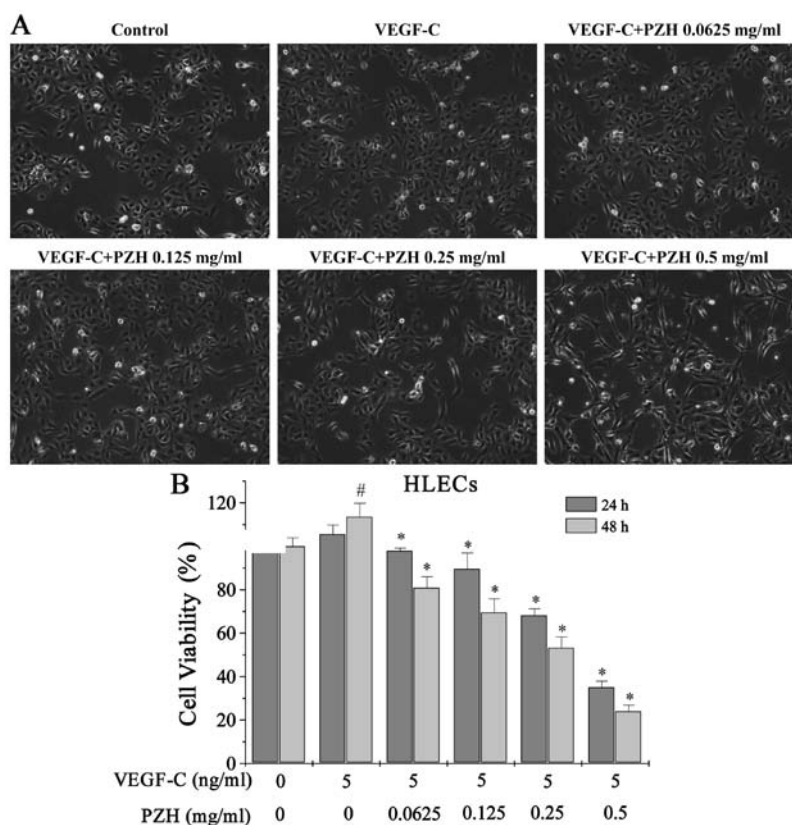

Figure 4. PZH reduces the cell confluency and viability of VEGF-C-stimulated HLECs. (A) HLECs were treated with exogenous VEGF-C or the indicated concentrations of PZH for 24 h; cell confluency and morphology are representative images of three independent experiments, captured at a magnification of x200. (B) Cell viability was determined by MTS assays. The data were normalized to the viability of the control cells. Data are averages with SD (error bars) from at least three independent experiments; $^\#$P<0.05, vs. the control cells; *P<0.05, vs. the PZH-untreated cells.

PZH does not affect the cell apoptosis of VEGF-C-stimulated HLECs. The effect of PZH on apoptosis in the VEGF-C-stimulated HLECs was determined by Annexin V/PI staining followed by FACS analysis. The results indicated that the percentage of cells undergoing apoptosis after treatment with 0.0625, 0.125 or 0.25 mg/ml of PZH (including the early and late apoptotic cells) was not significantly different when

compared with the control cells that did not receive PZH treatment (Fig. 5).

PZH attenuates cell migration and tube formation ability of VEGF-C-stimulated HLECs. Transwell assays were performed to determine the effects of PZH on migration of HLECs. VEGF-C stimulated cells had enhanced migratory capacity
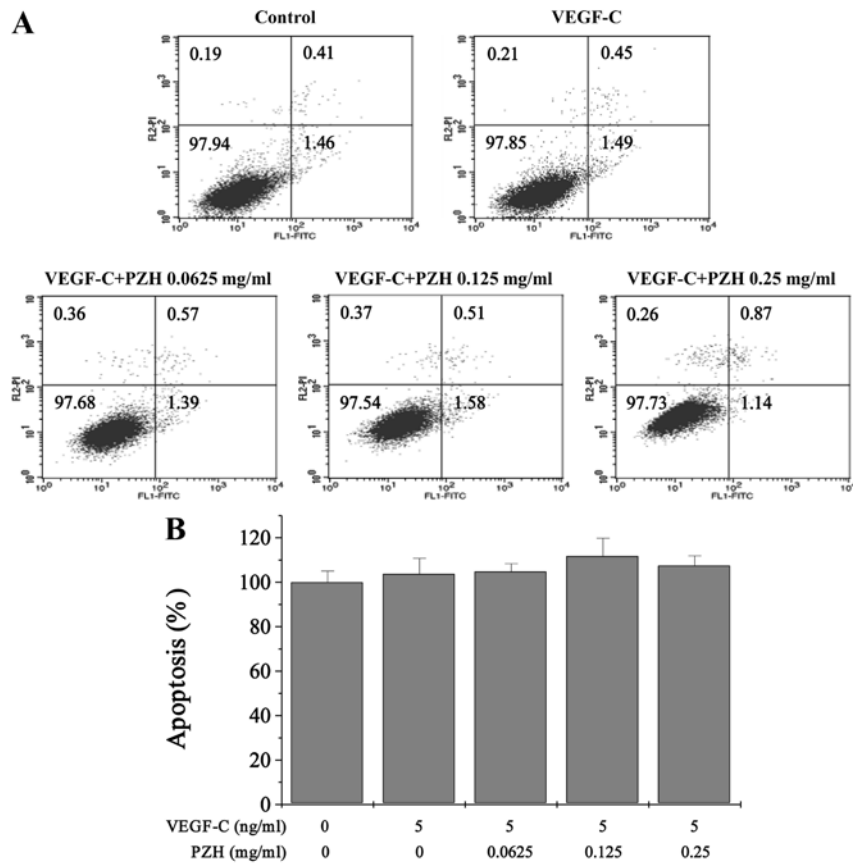
Figure 5. PZH not affects cell apoptosis of VEGF-C stimulated HLECs. (A) FACS analysis with Annexin V/PI staining. (B) Quantification of FACS analysis.
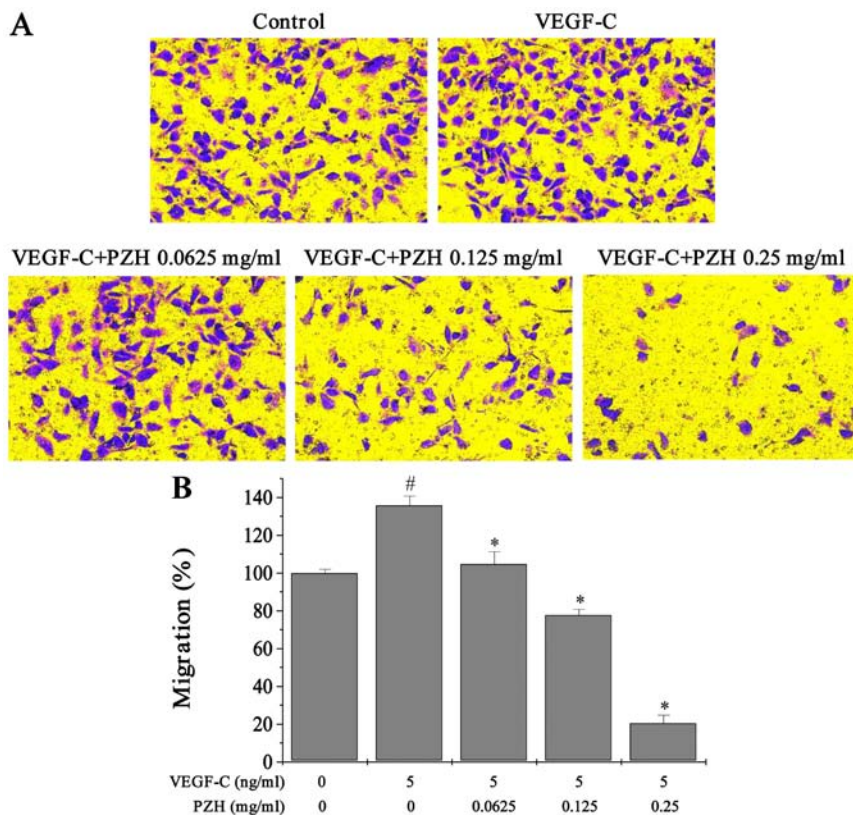

Figure 6. PZH attenuates cell migration ability of VEGF-C-stimulated HLECs. (A) The cell migration ability of HLECs was evaluated using Transwell cell culture chambers. Cells were stained with crystal violet; the images were captured at a magnification of x200. (B) The average number of migrated cells was counted in five randomly selective fields. The data were normalized to the migration of the control cells (100%). Data are averages with SD (error bars) from three independent experiments; [#]P<0.05, vs. the control cells; [*]P<0.05, vs. the PZH-untreated cells.
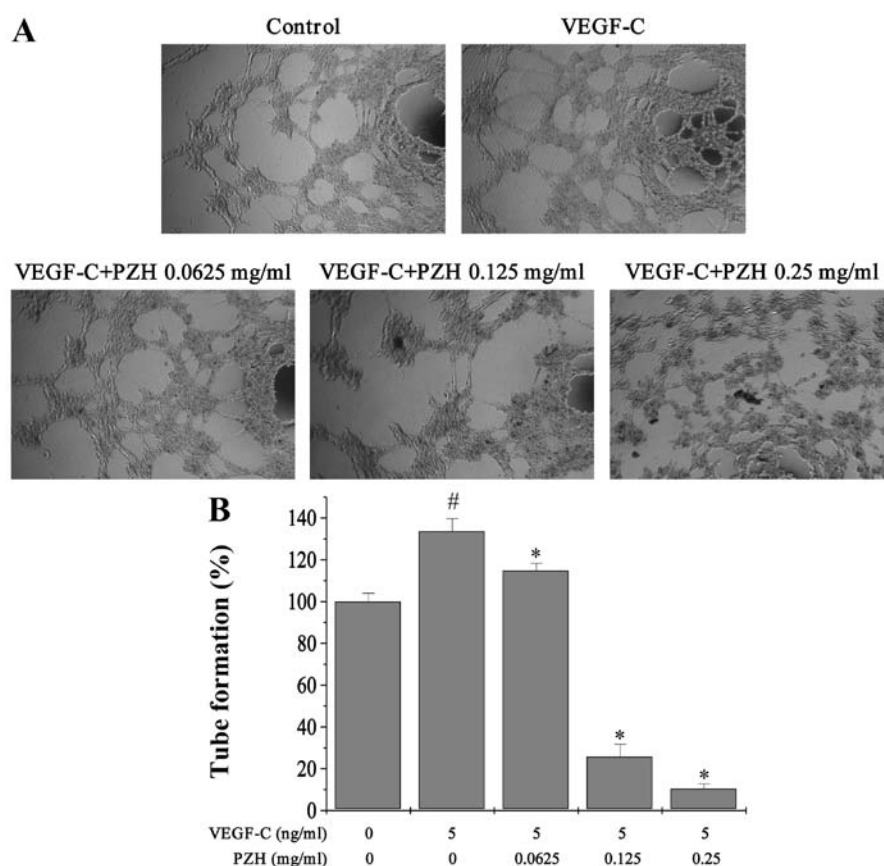
Figure 7. PZH attenuates tube formation ability of VEGF-C-stimulated HLECs. (A) The tube formation ability of HLECs was evaluated using the *In Vitro* Angiogenesis Assay kit. The images were captured at a magnification of x40. (B) The average number of formed tubes was counted in five randomly selective fields. The data were normalized to the formed tubes of control cells (100%). Data are averages with SD (error bars) from three independent experiments; #P<0.05, vs. the control cells; *P<0.05, vs. the PZH-untreated cells.
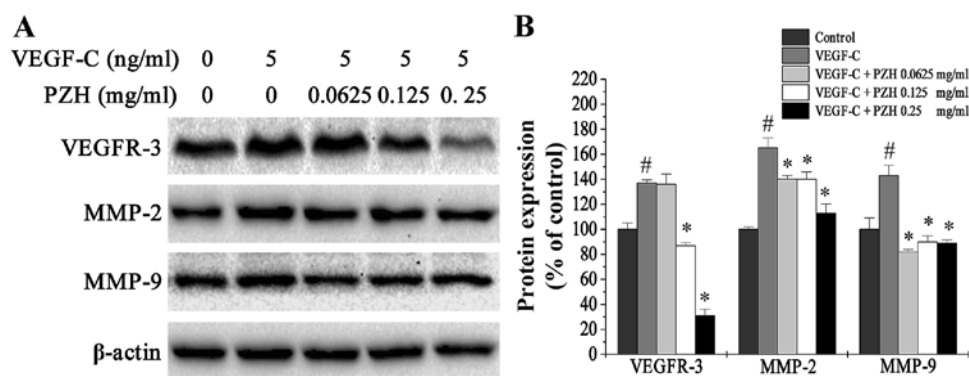

Figure 8. PZH downregulates the expression of VEGFR-3, MMP-2 and MMP-9 in VEGF-C-stimulated HLECs. The protein expression levels of VEGFR-3, MMP-2 and MMP-9 were analyzed by western blotting. β-actin was used as the internal control. (A) Data are representative of three independent experiments. (B) Quantification of western blot analysis; #P<0.05, vs. the control cells; *P<0.05, vs. the PZH-untreated cells.

by ~35.74% (P<0.0327). Treatment with 0.0625-0.25 mg/ml of PZH dose-dependently reduced the cell migratory ability by 104.81-20.49% (P<0.05) (Fig. 6). Similarly, exogenous VEGF-C stimulation led to a 33.69% (P<0.0218) increase in tube formation of HLECs, compared to control cells that did not receive VEGF-C, which in turn was decreased by PZH treatment by 114.81-10.49% (P<0.05) (Fig. 7).

PZH downregulates the expression of VEGFR-3, MMP-2 and MMP-9 in VEGF-C-stimulated HLECs. VEGFR-3 is

the cognate receptor of VEGF-C that is expressed in HLECs. Binding of VEGF-C to VEGFR-3 results in proliferation of HLECs as well as the formation of LVs. Moreover, activation of the VEGF-C/VEGFR-3 signaling pathway can lead to the upregulation of matrix metalloproteinases (MMPs). Expression of MMP-2 and MMP-9 has been associated with the migration ability of VEGF-C-stimulated cells and lymphangiogenesis (20). To further explore the anti-lymphangiogenesis properties of PZH, we used western blot analysis to determine the relative expression of these proteins after PZH

treatment. We found that VEGFR-3, MMP-2 and MMP-9 were upregulated after VEGF-C stimulation and downregulated with PZH treatment (Fig. 8).

Discussion

The growth and metastatic spread of malignant tumors is critically dependent on the development of new vasculature. Lymphangiogenesis is strongly associated with tumor metastasis and has therefore been evaluated in various types of tumor including colon malignancies (5), esophageal carcinoma (21) and breast cancer (22). Recently, numerous studies have cast new light on the process of lymphangiogenesis and potential molecular mechanisms underlying tumor regional lymph node metastasis. One such mechanism is tumor-induced lymphangiogenesis. Evidence of intratumoral LVs has raised the possibility that tumor cells themselves contribute to lymphatic metastasis through the induction of lymphangiogenic processes (23). It is well known that tumor cells enter the lymphatic vasculature by eliciting lymphangiogenesis via growth factor production. Moreover, lymphangiogenic growth factors produced by tumor cells stimulate growth and dilation of the tumor-induced LVs as well as facilitate tumor regional lymph node metastasis.

VEGF-C, a lymphangiogenic growth factor, is a key regulator of lymphangiogenesis and tumor metastasis (24). Early studies have suggested that VEGF-C can promote the growth of new LVs and regional metastasis by binding to their receptor tyrosine kinase VEGFR-3, which was found to be densely expressed in lymphatic endothelial cells (25,26). Studies with human or animal tumor models have demonstrated that malignant tumor cells themselves can secrete high levels of VEGF-C (27), and this overexpression of tumor-derived VEGF-C may play an important role in the occurrence of intratumoral lymphangiogenesis leading to the dissemination of tumor cells to regional lymph nodes (28-30).

Targeting lymphangiogenesis and VEGF-C may be a new treatment strategy for CRC. However, gene therapy targeting VEGF-C or the use of VEGFR-3 receptor antagonist has many disadvantages in clinical practice and can lead to drug-resistance or other side-effects. For this reason, the advantage of traditional Chinese medicine (TCM) has been fully recognized. TCM formulas consist of a combination of many natural products, each of which contains numerous chemical compounds. Therefore, TCM prescription is often considered to have multi-component and multi-target effect. Due to the broad range of therapeutic functions of TCM, it has long been used to treat various diseases. One such prescription is Pien Tze Huang (PZH), which exhibits specific anticancer activities.

In the present study, we demonstrated that PZH significantly reduced viability and migration in different CRC cell lines, which indicated that PZH possesses markedly antimetastasis ability (Figs. 1 and 2). To better understand its underlying mechanism, we determined the expression of VEGF-C in different CRC cell lines and found that PZH markedly downregulated VEGF-C expression. This suggests a potential mechanism, that the inhibition of VEGF-C expression is associated with the lymphangiogenesis-suppressive effects of PZH (Fig. 3). Since VEGF-C secreted from CRC cells can lead to the growth

of HLECs and the subsequent formation of lymph vessels, we used exogenous VEGF-C stimulation of HLECs to establish a model of these processes *in vitro*. As shown in the results, 5 ng/ml of exogenous VEGF-C stimulation significantly enhanced cell confluency, viability and the ability of migration and tube formation, while PZH treatment attenuated all of these effects (Figs. 4 and 6). Notably, we found no change in cell apoptosis in response to PZH treatment (Fig. 5), which suggests that PZH suppresses lymphangiogenesis mainly by the inhibition of cell proliferation, migration and tube formation but not directly by promoting apoptosis of HLECs. Next, to further investigate the mechanisms of the anticancer effects of PZH on VEGF-C stimulated HLECs, we determined its effect on the expression of related proteins. VEGFR-3 is the cognate receptor to VEGF-C and is expressed in HLECs. PZH treatment notably downregulated the expression of VEGFR-3, which was increased by exogenous VEGF-C stimulation. Moreover, the VEGF-C/VEGFR-3 signaling pathway can lead to the activation of downstream transcription factors, resulting in the upregulation of MMPs, which is important for cell migration. Particularly, the increase in MMP-2 and MMP-9 can lead to the breakdown of extracellular matrix (ECM) in the surrounding tumor tissue and allow for the migration of HLECs. However, MMP-2 and MMP-9 can trigger the release of VEGF-C resulting in the proliferation of HLECs and the formation or remodeling of LVs (31,32). We found that PZH prominently downregulated the expression of MMP-2 and MMP-9, which was increased by exogenous VEGF-C stimulation. Overall, these results demonstrate that PZH suppression of lymphangiogenesis via the downregulation of VEGF-C may be a molecular mechanism by which PZH inhibits metastasis in CRC.

In conclusion, PZH exerts its antimetastatic activities through the suppression of VEGF-C-mediated lymphangiogenesis, and thus may be developed as a promising multi-potent anticancer agent for the clinical treatment of CRC.

Acknowledgements

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