Paeoniflorin prevents hypoxia-induced epithelial–mesenchymal transition in human breast cancer cells

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Abstract: Paeoniflorin (PF) is a monoterpene glycoside extracted from the root of Paeonia lactiflora Pall. Previous studies have demonstrated that PF inhibits the growth, invasion, and metastasis of tumors in vivo and in vitro. However, the effect of PF on hypoxia-induced epithelial–mesenchymal transition (EMT) in breast cancer cells remains unknown. Therefore, the objective of this study was to investigate the effect of PF on hypoxia-induced EMT in breast cancer cells, as well as characterize the underlying mechanism. The results presented in this study demonstrate that PF blocks the migration and invasion of breast cancer cells by repressing EMT under hypoxic conditions. PF also significantly attenuated the hypoxia-induced increase in HIF-1α level. Furthermore, PF prevented hypoxia-induced expression of phosphorylated PI3K and Akt in MDA-MB-231 cells. In conclusion, PF prevented hypoxia-induced EMT in breast cancer cells by inhibiting HIF-1α expression via modulation of PI3K/Akt signaling pathway. This finding provides evidence that PF can serve as a therapeutic agent for the treatment of breast cancer.

Keywords: paeoniflorin, breast cancer, hypoxia, epithelial–mesenchymal transition, PI3K/Akt signaling pathway

Introduction
Breast cancer is the leading malignancy in females worldwide and the incidence rates have been increasing annually.1 Although various treatments for breast cancer have been used and have been improved recently, the clinical outcome of patients remains unsatisfactory.2–4 Thus, development of effective chemotherapeutic adjuvants is important to increase cure rates in these patients.

Epithelial–mesenchymal transition (EMT) is a crucial step in tumor progression and has an important role during cancer invasion and metastasis.5–7 Mounting evidence shows that the hypoxic environment in most of the rapidly growing solid tumors results from the poor development of angiogenic vessels which leads to insufficient supply of oxygen.8,9 It was demonstrated that hypoxia might stimulate EMT in breast cancer cells. Hypoxia increased the expression of Slug and Snail in breast cancer cells, which in turn inhibited E-cadherin expression.10,11 Therefore, inhibiting hypoxia-induced EMT can be used as a promising target for developing new and effective anticancer therapy.

The root of Paeonia lactiflora Pall. (family Ranunculaceae) has long been used in oriental medicine for pain, muscle spasm, inflammation, menstrual dysfunction, and degenerative disorders. Paeoniflorin (PF) is a monoterpene glycoside extracted from the root of P. lactiflora Pall. Increasing evidences have reported that PF possesses a variety of physiological properties, including immune regulation, anti-inflammation, and...
protection from myocardial infarction. Furthermore, PF has been reported to inhibit the growth, invasion, and metastasis of tumors in vivo and in vitro. However, the effect of PF on hypoxia-induced EMT in breast cancer cells remains unknown. Therefore, the objective of this study was to investigate the effect of PF on hypoxia-induced EMT in breast cancer cells, as well as characterize the underlying mechanism.

Materials and methods

Reagents

PF (99% purity) was obtained from Nanjing Institute for Drug Control (Nanjing, People’s Republic of China). The anti-E-cadherin, anti-cadherin, anti-vimentin, anti-HIF-1α, anti-PI3K, anti-p-PI3K, anti-Akt, anti-p-Akt, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology Inc., (Dallas, TX, USA). All other chemicals and reagents were purchased from Sigma-Aldrich Co., (St Louis, MO, USA).

Cell lines and cell culture conditions

Human breast cancer cell lines (MDA-MB-231 and MCF-7) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich Co.) in a humidified 5% CO2 atmosphere at 37°C. The protocol and procedure of the experiment were approved by the Ethics Committee of Huaihe Hospital.

For hypoxic conditions, cells were seeded in a six-well plate at a density of 1×105 cells/well and cultured in hypoxic conditions (5% CO2 and 1% O2 [v/v]), balanced with N2 gas, at 37°C for 12, 24, and 48 hours. For normoxic conditions, cells were exposed to normoxic (21% O2, at room temperature) conditions. The medium was then replaced with new medium supplemented with various concentrations of PF (12.5–50 μM).

Determination of cell viability

The number of viable cells was determined by the trypan blue exclusion assay. In brief, MDA-MB-231 and MCF-7 cells were seeded at a density of 1×103 cells/well in six-well plates. After reaching a confluency of 90%, the cells were cultured in serum-free medium for 24 hours and then stimulated with various concentrations of PF (12.5–50 μM) for 24 hours. Only dimethyl sulfoxide (DMSO) was added for the control group. After trypsinization, the cells were stained with 40 μL trypan blue (Sigma-Aldrich Co.). The number of viable cells was determined using a hemocytometer (Abcam, Cambridge, UK).

Cell migration and invasion assays

In vitro transwell migration assay was performed in modified Boyden chambers with 8 mm pore filter inserts in 24-well plates (BD Biosciences, San Jose, CA, USA). Briefly, MDA-MB-231 and MCF-7 cells (1×105 cells/well) were harvested and suspended in serum-free Roswell Park Memorial Institute (RPMI) 1640 medium. After that, cancer cells were plated into the top chamber of the transwell chambers. The lower chamber was filled with RPMI 1640 medium containing 10% FBS. After 24 hours, cells on the upper membrane surface were removed with cotton-tipped swabs, and those that had migrated to the bottom side of the filter were subsequently fixed in methanol for 15 minutes and stained with 0.05% crystal violet in phosphate-buffered saline for 15 minutes. The number of cells per four high-power fields was counted under a microscope in order to determine the average number of cells that had migrated.

The invasion assay was performed using BD BioCoat Matrigel invasion chambers (BD Biosciences; 8 mm pore size). The same procedures as described earlier were used, except that the filters were precoated with 100 mL Matrigel.

Real-time quantitative polymerase chain reaction analysis

Total RNA from breast cancer cells was prepared with Trizol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Then, cDNA was synthesized using a two-step reverse transcription reaction kit (Takara, Dalian, People’s Republic of China). The levels of HIF-1α gene mRNA transcripts were analyzed by using specific primers and SYBR Green I reagent and the real-time polymerase chain reaction kit, according to the manufacturer’s instructions, using the Bio-Rad iQ5 Quantitative PCR System (Takara). The specific primers for HIF-1α were forward: 5′-AGCCGAGGAAGAACTATGAAC-3′, reverse: 5′-ATTTGATGGGTGAGGAATGGG-3′; HIF-1α forward: 5′-GAGCTACGAGCTGCCTGACG-3′, reverse: 5′-CCTAGAAGCATTTGCGGTGG-3′. The cycling conditions included a holding step at 95°C for 10 minutes, and 35 cycles at 95°C for 25 seconds, 59°C for 30 seconds, and 69°C for 30 seconds. Quantitative real-time PCR analysis was performed using an ABI 7500 Sequence Detector (ABI, Warrington, UK), and the results were analyzed by the method of 2-ΔΔct.

Western blotting

Total protein was extracted from breast cancer cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Nantong, People’s Republic of China) according
to the manufacturer’s instructions. Equal amounts of protein (30 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Whatman Schleicher & Schuell, Middlesex, UK). After blocking in Tris-buffered saline (TBS) buffer (50 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) containing 5% nonfat milk, the membrane was immunoblotted with primary antibodies (anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-HIF-1α, anti-PI3K, anti-p-PI3K, anti-Akt, anti-p-Akt, and anti-GAPDH antibodies; Santa Cruz Biotechnology Inc.) overnight at 4°C, followed by three washes in TBS with Tween-20 (TBST) for 10 minutes. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After washing, the sites of antibody binding were visualized by chemiluminescence (Boehringer Mannheim, Mannheim, Germany).

siRNA knockdown of HIF-1α
MDA-MB-231 cells were transfected with siRNA against HIF-1α and scramble siRNA (negative control) using LipofectamineTM2000 (Thermo Fisher Scientific), according to the manufacturer’s protocols. Following transfection, cells were incubated at 37°C in a CO2 incubator for 24 hours before being harvested for the assays described earlier.

Statistical analysis
All results were reported as mean ± standard deviation (SD). The differences between groups were compared by Dunnett’s test subsequent to analysis of variance (ANOVA). P<0.05 was considered to be significant.

Results
PF inhibits the viability of breast cancer cells cultured in hypoxia
First, we determined the effect of hypoxia on cell viability. As shown in Figure 1A, the cell viability was not significantly affected with an exposure to hypoxia for 24 hours, while the viability was decreased by 28.2%±3.1% after treatment with hypoxia for 48 hours. Thus, MDA-MB-231 cells were exposed to hypoxia for 24 hours in the subsequent experiments. Next, to examine the effect of PF on cell viability under hypoxia
conditions, MDA-MB-231 cells were treated with PF and incubated under normoxic and hypoxic conditions for 24 hours. As shown in Figure 1C, the inhibitory effect of PF on viability was significantly higher on MDA-MB-231 cells grown under hypoxia than those cultured under normoxia. Similar results were observed in MCF-7 cells (Figure 1B and D).

**PF prevented the hypoxia-induced migration and invasion of breast cancer cells**

Hypoxia has been reported to induce invasion and migration of cancer cells. So, we examined the effect of PF on hypoxia-induced cell migration and invasion. As shown in Figure 2A, compared with untreated MDA-MB-231 cells, hypoxia significantly increased the number of cells migrating in Boyden chambers. However, the hypoxia-induced migration of MDA-MB-231 cells decreased by 29% ($P<0.05$ vs hypoxia controls) in PF-treated cells. Similarly, hypoxic PF-treated cells showed a 31% reduction ($P<0.05$ vs hypoxia controls) in the invasive capacity of MDA-MB-231 cells compared to the hypoxia-only controls (Figure 2B). In addition, we also found that PF prevented the hypoxia-induced migration and invasion of MCF-7 cells (Figure 2C and D).

**HIF-1α is involved in the prevention of hypoxia-induced EMT by PF**

HIF-1 is the best regulator under hypoxia exposure and is involved in the process of EMT. So, we investigated whether the inhibiting effect of PF against hypoxia-induced EMT involves modulation of the HIF-1α expression. As shown in Figure 4A, PF treatment suppressed the hypoxia-induced HIF-1α expression. To further verify the role of HIF-1α in hypoxia-mediated EMT, a lentivirus-mediated siRNA-HIF-1α was designed and transfected into the MDA-MB-231 cells. As shown in Figure 4B, knockdown...
PF prevents hypoxia-induced EMT in breast cancer cells

**PF-mediated blockade of HIF-1α and EMT involved PI3K/Akt pathway**

Previous studies showed that PI3K/Akt signaling controls the levels of HIF-1α. Therefore, we postulated that PF might be involved in the regulation of HIF-1α expression via inhibiting PI3K/Akt signaling. As shown in Figure 5, hypoxia treatment significantly increased the expression of phosphorylated PI3K and Akt in MDA-MB-231 cells. When treated with PF, the increase in levels of phosphorylated PI3K and Akt was reversed, whereas total levels of PI3K and Akt remained unaffected.

**Discussion**

The results presented in this study demonstrate that PF blocks the migration and invasion of breast cancer cells by repressing EMT under hypoxic conditions. PF also significantly attenuated the hypoxia-induced increase of HIF-1α level. Furthermore, PF prevented the hypoxia-induced expression of phosphorylated PI3K and Akt in breast cancer cells.

PF is reported to have antitumor effect. Hung et al reported that PF inhibited the proliferation of non-small-cell
lungs cancer A549 cells by blocking cell cycle progression in the G(0)/G(1) phase.\textsuperscript{21} PF also inhibited the tumor invasion and metastasis in human hepatocellular carcinoma cells.\textsuperscript{22} Furthermore, hypoxia was shown to induce cell migration and invasion in many types of cancers. In the current study, we found that the inhibitory effect of PF on viability was significantly higher on MDA-MB-231 and MCF-7 cells grown under hypoxic than in the normoxic conditions. We also found that PF prevented the hypoxia-induced migration and invasion of MDA-MB-231 and MCF-7 cells.

The EMT is an important molecular step in cancer progression. Reduction or a loss of E-cadherin expression has a crucial role in tumor progression to invasive cancer and is also one of the well-established hallmarks of EMT.\textsuperscript{5,23} In addition, it has been reported that hypoxic microenvironment plays a key role in the progression of EMT.\textsuperscript{11,24,25} In the current study, we found that PF prevented the hypoxia-induced EMT in breast cancer cells. These results suggest that PF blocks the migration and invasion of breast cancer cells by repressing EMT under hypoxic conditions.

HIF-1 is a transcription factor that is critical for tumor adaptation to microenvironmental stimuli, and consists of HIF-1α and HIF-1β subunits. It was reported that HIF-1α was an important upstream regulator of the EMT process. In addition, hypoxia induces HIF-1α expression, which induces the expression and activity of major transcription factors including TWIST, Snail, Slug, SIP1, and ZEB1, leading to the suppression of E-cadherin and induction of vimentin expression.

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**Figure 4** HIF-1α is involved in the prevention of hypoxia-induced EMT by PF.

**Notes:** MDA-MB-231 cells grown under normoxia and hypoxia were treated with 25 µM PF for 24 hours. (A) PF inhibited the hypoxia-induced expression of HIF-1α. (B) Knockdown of HIF-1α inhibited hypoxia-induced HIF-1α expression. (C) Knockdown of HIF-1α restored hypoxia-induced reduction of E-cadherin expression. Each bar represents the mean ± SD. The results were reproduced in three independent experiments. *P < 0.05 vs normoxia group, #P < 0.05 vs hypoxia group.

**Abbreviations:** PF, paeoniflorin; EMT, epithelial–mesenchymal transition; SD, standard deviation.
in breast cancer cells.26–28 Consistent with these results, in the current study, we found that hypoxia-induced HIF-1α expression and knockdown of HIF-1α clearly inhibited hypoxia-induced E-cadherin expression; however, PF treatment suppressed the hypoxia-induced HIF-1α expression. Our results suggest that PF prevented the hypoxia-induced EMT by inhibiting HIF-1α.

Previous studies have shown that PI3K/Akt signaling pathway is involved in HIF-1α expression in tumor cells.29–31 For example, one study reported that a PI3K-specific inhibitor (LY294002) or overexpression of dominant-negative Akt significantly blunted HIF-1α and NF-κB induction in response to hypoxia in prostate cancer cells.32 Furthermore, PF inhibits human gastric carcinoma cell proliferation through upregulation of microRNA-124 and suppression of PI3K/Akt and STAT3 signaling.33 In the current study, we found that hypoxia treatment significantly increased the expression of phosphorylated PI3K and Akt in MDA-MB-231 cells. When treated with PF, the increase in the levels of phosphorylated PI3K and Akt was reversed. These results suggest that PF prevented hypoxia-induced EMT in breast cancer cells by inhibiting HIF-1α expression via modulation of PI3K/Akt signaling pathway.

**Conclusion**

In conclusion, PF prevented hypoxia-induced EMT in breast cancer cells by inhibiting HIF-1α expression via modulation of PI3K/Akt signaling pathway. This finding provides evidence that PF can serve as a therapeutic agent for the treatment of breast cancer.

**Disclosure**

The authors report no conflicts of interest in this work.

**References**


