Inhibitory action of pristimerin on hypoxia-mediated metastasis involves stem cell characteristics and EMT in PC-3 prostate cancer cells

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Abstract. The aim of the present study was to investigate whether pristimerin affects the bone metastasis, stem cell characteristics and epithelial-mesenchymal transition (EMT) of prostate cancer (PCa) PC-3 cells subjected to hypoxia. The PC-3 cells were cultured under hypoxia or normoxia for 48 h and were then treated with increasing concentrations of pristimerin from 0 to 0.8 μmol/l, under normoxia. Hypoxia-inducible factor-1α (HIF-1α) was detected by western blotting. Proliferation was assessed with the CCK-8 assay. Transwell invasion assay was used to analyze the potency of invasion. Stem cell characteristics were detected by sphere formation, colony formation assay and western blotting, including CD44, KLF4, OCT4 and AGO2, which are stem cell characteristic-related markers. EMT was confirmed by the expression changes of EMT-related markers, including N-cadherin, fibronectin, vimentin and ZEB1, which were evaluated by western blotting. The addition of pristimerin to the medium reduced the hypoxia-induced PC-3 cell proliferation in a dose-dependent manner. Pristimerin effectively inhibited hypoxia-induced invasion of the PCa cells *in vitro*. Moreover, the treatment of cells with pristimerin induced the reversal of hypoxia-induced stem cell characteristics and EMT, which was confirmed by sphere formation, colony formation assay and the expression changes of CSC- and EMT-related markers. The reversal of hypoxia-induced stem cell characteristics and EMT in the PCa cells by low-dose pristimerin was dose-dependent. These results showed that treatment with pristimerin may be a potential strategy for the suppression of hypoxia-induced metastasis through the reversal of hypoxia-induced stem cell characteristics and EMT in cancer cells, which justifies the potential use of pristimerin as a practical chemopreventive approach for patients with PCa.

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Abbreviations: PCa, prostate cancer; EMT, epithelial-mesenchymal transition; CSCs, cancer stem cells; Ago2, Argonaute 2; HIF-1α, hypoxia-inducible factor-1α

Key words: prostate cancer, bone metastasis, hypoxia, EMT, cancer stem cells

Introduction

The formation of distant metastasis is the main cause of morbidity and mortality in patients with cancer (1). Once a solid tumor spreads to other tissues and organs, curative intervention with currently available cancer drugs, surgical operation and radiotherapy are ineffective. The principal characteristic of prostate cancer (PCa) is its inclination to metastasize to the bones, which occurs in as many as 90% of the patients with advanced PCa (2). It appears that the bone microenvironment has a pivotal role in this process (3). Intratumoral hypoxia is a common pathophysiologic feature of solid tumors, from the smallest tumor of a few millimeters in diameter to the largest tumor (4). The hypoxic tumor environment results in aggressive and metastatic cancer phenotypes that are associated with resistance to radiation therapy, chemotherapy and a poor treatment outcome (5,6). Hypoxia-inducible factor-1 (HIF-1) is the key factor in this process, regulating transcription of more than 70 hypoxia-activated genes, that range in function from those that promote anaerobic metabolism to those that initiate tumor metastasis (7). HIF-1 is a heterodimeric transcription factor composed of HIF-1α and constitutively expressed HIF-1β subunits. Its biological activity depends on the amount of HIF-1α, which is tightly regulated by oxygen

tension (8). Overexpression of HIF-1α has been demonstrated in human cancers as compared with the respective normal tissues, making it a potential therapeutic target in oncologic drug discovery (9). Inhibition of HIF-1α or its genetic disruption can not only block tumor cell growth, but also inhibit tumor metastasis, so that these tumor cells would be unable to respond to the hypoxic stimulus and progress (10,11).

Emerging evidence indicates that CSCs may be the critical drivers of tumor progression and metastasis (12,13). Somatic tumors, including PCa, contain a small subset of stem-like cells, called cancer stem cells, with capacities for self-renewal, differentiation and initiation of new tumors. Cancer stem cells preferentially reside in specific hypoxic microenvironment-niches, often existing inside tumors (14,15). Meanwhile, stem-like cells are present in several established PCa cell lines, such as the PC-3 cell line (16) and under hypoxia possess greater stem-like properties (17).

Hypoxia, a negative prognostic factor for successful treatment, is a potent driver of a multitude of molecular signal pathways that allows cells to survive and thrive in the hostile tumor microenvironment and induces epithelial-mesenchymal transition (EMT) (18). EMT can be activated by hypoxia-induced HIF-1 signaling (19), followed by a cellular switch from epithelial to mesenchymal properties (20). EMT plays a key role in tumor cell metastasis (21) and has also been identified as an important step in bone metastasis of PCa (22,23). Hypoxia also provides tumor cells with cues for maintenance of a stem-like state and may help driving the linkage between EMT and CSCs (18).

Pristimerin is a quinonemethide triterpenoid with the potential of a promising anticancer agent and induces apoptosis in PCa cells (24), yet its activity in PCa under hypoxia has not been investigated. In light of these findings, we hypothesized that pristimerin affects the hypoxia-induced HIF-1α expression and impair hypoxia-stimulated metastasis by inhibition of stem cell characteristics and EMT of PC-3 cells. To test this hypothesis, we first identified the anticancer efficacy of pristimerin in PC-3 cells under a hypoxic environment. Then, we evaluated the inhibitory effect of pristimerin on hypoxia-induced invasiveness of cancer cells. Furthermore, we investigated the reduction of the expression of HIF-1α protein by pristimerin. We detected the stem cell characteristics of PC-3 cells by sphere formation and colony formation assay, and KLF4, OCT4 and AGO2 protein expression. In addition, EMT was evaluated by detecting N-cadherin, fibronectin, vimentin, ZEB1 protein expression. Finally, our results supported that pristimerin exerted its repressive effect on the metastasis, at least in part, via restraining stem cell characteristics and EMT of PC-3 PCa cells. These findings provide further evidence that pristimerin can be a promising chemopreventive and anticancer agent in human cancer by inhibiting tumor metastasis under hypoxic stress.

Materials and methods

Reagents and antibodies. Pristimerin was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Pristimerin was solubilized in 100% dimethyl sulfoxide (DMSO) at 100 μg/ml concentration and frozen at -20°C in small aliquots until needed. The antibody, anti-HIF-1α was purchased from BD Biosciences; CD44 and β-actin were obtained from Santa Cruz Biotechnology; and KLF4, OCT4, AGO2, N-cadherin, fibronectin, vimentin and ZEB1 were purchased from Cell Signaling Technology.

Cell lines and hypoxic treatment. Human PCa PC-3 cell lines were purchased from the American Type Culture Collection (ATCC; USA) and maintained in our laboratory for the present study. For conventional cell culture, cells were seeded in culture flasks with F-12 culture medium (HyClone) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were cultured at 37°C in a humidified atmosphere and 5% CO_2 in air. For hypoxic exposure, the cells were placed in a sealed modular incubator chamber flushed with 1% O_2, 5% CO_2 and 94% N_2.

Cell proliferation assay. One day before the assay, cells were seeded under hypoxic or normoxic conditions for 48 h, and were then displaced in 96-well plates in a volume of 100 μl for 36 h and treated with increasing concentrations of pristimerin. All samples, including controls (normoxic), contained 0.1% DMSO. Viable cells were determined using CCK-8. The absorbance at 450 nm was determined using the ELx808 microplate reader (Bio-Tek, Winooski, VT, USA). Five wells were assayed at each concentration and the mean absorbance was determined. The data are expressed as mean ± SD from three independent experiments.

Cell invasion assay. The migration and invasion assays were performed in a Transwell Boyden Chamber (Corning) using a polycarbonate filter with an 8-μm pore size in a 24-well plate and the filter membranes were coated with Matrigel (BD Biosciences) as previously described (25). One day before the assay, cells were seeded under hypoxic or normoxic conditions for 48 h, and then cells were added to the inner chamber of the insert in 200 μl of serum-free medium and 500 μl of 10% FBS medium to determine the effect of pristimerin on cell invasion. Indicated concentrations of pristimerin were added to the lower chamber and all samples, including controls (normoxic), contained 0.1% DMSO. After incubation under normoxia for 48 h at 37°C, the cells were displated to the inner chamber of the insert in 200 μl of serum-free medium for 24 h, and then remained on the upper surface where they were gently removed using a cotton-tipped swab. The cells on the opposite surface of the filter membrane were stained with 0.1% crystal violet and counts were obtained from five randomly selected fields (x100 magnification) using a computer imaging system.

Spheroid formation assay. Spheroid formation assay was performed as previously described (26). To investigate the effect of pristimerin treatment in preventing hypoxia-induced spheroid formation, we maintained PC-3 cells under normoxic or hypoxic conditions for 48 h, and then the cells were plated at 400 cells/well onto 6-well poly-HEMA (Sigma)-coated plates and they were grown in F12 medium (HyClone) with increasing concentrations of pristimerin for 14 days supplemented with B27 (Invitrogen), 20 ng/ml EGF (Sigma) and 20 ng/ml basic FGF (Invitrogen). After 14 days, the number of prostaspheres (tight, spherical, non-adherent masses >100 μm in diameter) were counted, and images of the prostaspheres

were captured under an inverse microscope. Sphere formation efficiency = colonies/input cells x 100%.

Colony formation assay. A colony formation assay was performed as previously described (26). To investigate the effect of pristimerin treatment in preventing hypoxia-induced colony formation, we treated PC-3 cells under normoxic or hypoxic conditions for 48 h and then treated them with indicative concentrations of pristimerin for 24 h. After that the cells were plated at 300 cells as single cells onto a 65-mm Petri dish with increasing concentrations of pristimerin for 14 days and colonies were stained with crystal violet. Plating efficiency = number of colonies ($\geq$50 cells/colony)/input cells x 100%. To determine different colony morphologies, the different colony morphologies were scored under a light microscope.

Western blot analysis. PC-3 cells were maintained under normoxic or hypoxic conditions for 48 h, and were then treated by the indication concentrations of pristimerin for 24 h and cells were lysed with buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 10 mM sodium fluoride, 20 mM β-mercaptoethanol, 250 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Sigma), and were incubated at 4°C for 30 min. The lysates were ultrasonicated and centrifuged at 14,000 x g for 15 min. The proteins were separated on a 8-10% gradient SDS-PAGE and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia, Piscataway, NJ, USA). After being blocked with skimmed milk in 5% TBST, the membranes were incubated overnight at 4°C with primary antibodies against HIF-1α, CD44, KLF4, OCT4, AGO2, N-cadherin, fibronectin, vimentin, ZEB1 or β-actin. After being washed for three times with TBST for 5 min, the membranes were incubated with secondary anti-rabbit or anti-mouse antibodies for 1 h at room temperature and were then developed by ECL.

Statistical analysis. Data are shown as the means ± SD. Multiple group comparison was performed by a one-way ANOVA for comparison of means. Comparisons between 2 groups were analyzed by the unpaired Student's t-test. P-values of <0.05 were considered to indicate a statistically significant result.

Results

Pristimerin inhibits the hypoxia-induced proliferation of PC-3 cells. Uncontrolled proliferation is one of the most distinguished traits of cancer cells. To determine the direct effect of pristimerin on hypoxic-induced cancer cell proliferation, we performed cell death assays on PC-3 cells treated with pristimerin (Fig. 1). Pristimerin treatment at a concentration of 0.4 μM for 36 h led to inhibition of proliferation in the PC-3 cells and the effect was more marked at 0.8 μM. Our findings suggest that pristimerin inhibits the hypoxia-induced proliferation of cancer cells in a dose-dependent manner.

Pristimerin prevents hypoxia-induced invasion of PC-3 cells. Hypoxia represents a physiological stimulus for tumor cell
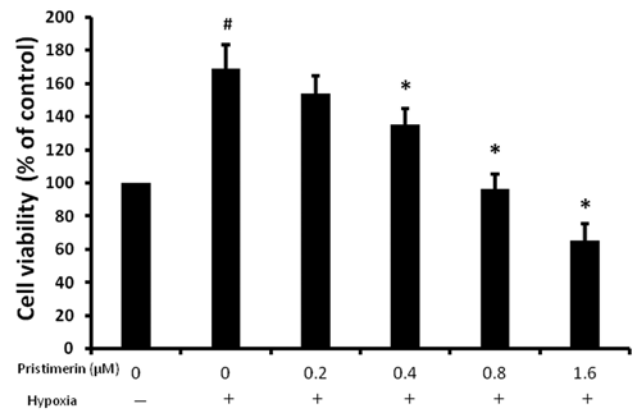

Figure 1. Pristimerin inhibits the hypoxia-induced proliferation of PC-3 cells. PC-3 cells were exposed to hypoxic conditions for 48 h, and then treated with the indicated concentrations of pristimerin. All concentrations included 0.1% DMSO as the control. After 36 h, the cells were processed for the CCK-8 proliferation assay and the OD value was measured as described in Materials and methods. #P<0.05 vs. normoxia. *P<0.05 vs. hypoxia only. DMSO, dimethyl sulfoxide; OD, optical density.

invasion and metastasis. The progression of tumors to an invasive phenotype and ultimately the formation of metastasis is associated with increased mortality (27). We examined whether pristimerin prevents the hypoxic-induced invasiveness of cancer cells by the Matrigel-coated Transwell assay. As shown in Fig. 2A and B, an increase in the baseline invasiveness of the cancer cells was observed under hypoxic conditions as compared to normoxic conditions. The number of PC-3 cells that invaded through the Matrigel membrane increased from normoxia to hypoxia. The stimulatory effect of hypoxia on the invasiveness of cancer cells was reduced by pristimerin in a dose-dependent manner. The number of invaded cells under hypoxia decreased with the addition of 0.2 μM pristimerin. These results indicate that pristimerin suppresses the hypoxia-stimulated invasion ability of cancer cells.

Pristimerin prevents hypoxia-induced spheroid formation of PC-3 cells. The ability to grow as non-adherent spheroids in sphere medium has been widely used to assess the self-renewal capability of CSCs and is one of the characteristics of prostate CSCs (28,29). To investigate the effect of pristimerin treatment in preventing hypoxia-induced spheroid formation, we exposed PC-3 cells to normoxic or hypoxic conditions for 48 h, and then the cells were pretreated with indicated concentrations of pristimerin for 24 h. Next, the PC-3 cells were plated at 400 cells/well onto 6-well polyHEMA-coated plates and they were grown in F12 medium (HyClone). As shown in Fig. 3A and B, an increase in spheroid formation was observed under hypoxic conditions as compared to normoxic conditions. The stimulatory effect of hypoxia on the spheroid formation of cancer cells was reduced by pristimerin in a dose-dependent manner. The number of hypoxic invaded spheroids was decreased with the addition of 0.2 μM pristimerin. These results indicate that pristimerin could suppress the hypoxia-stimulated spheroid formation ability of cancer cells.

Pristimerin prevents hypoxia-induced colony formation of PC-3 cells. To investigate the effect of pristimerin treatment in
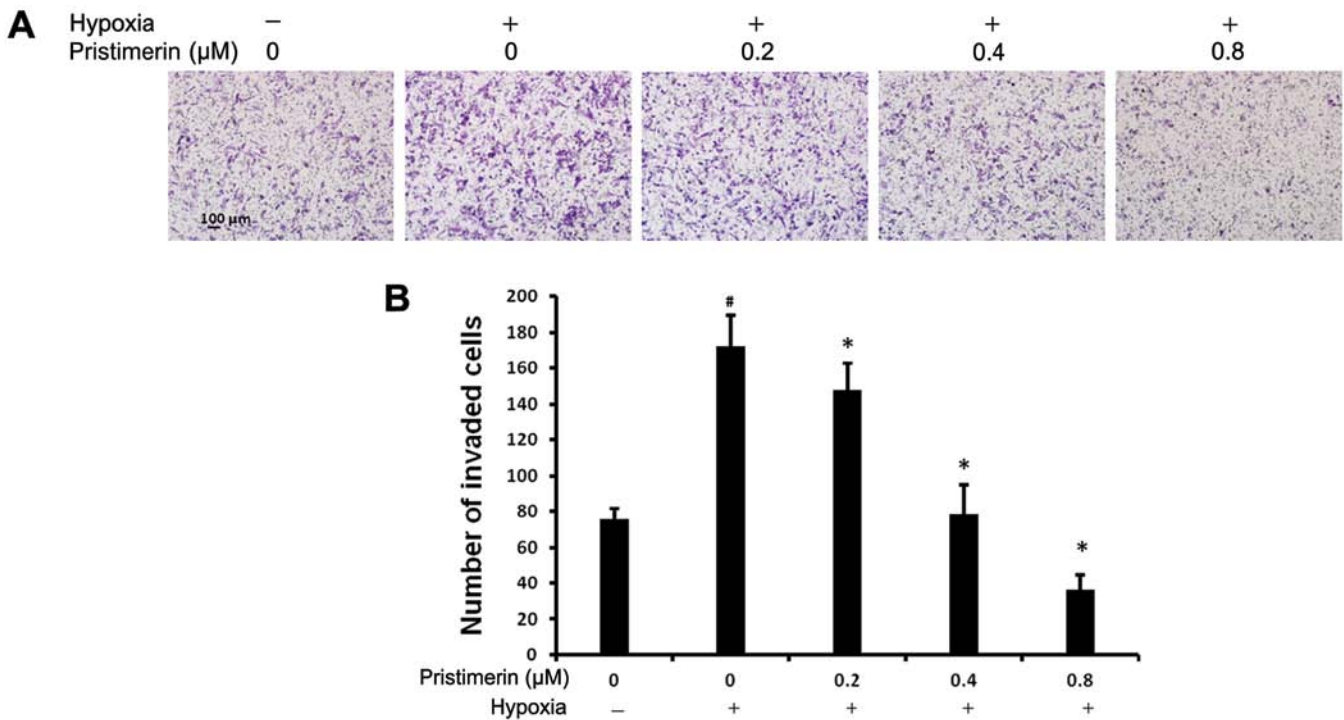
Figure 2. Pristimerin inhibits hypoxia-induced invasion of PC-3 cells. Pristimerin dose-dependently inhibited the invasion of PC-3 cells. (A) Images of invaded PC-3 cells (x100 magnification). (B) The PC-3 cells that had invaded to the lower chamber were counted in four randomly selected microscopic fields (x100 magnification). Values represent means ± SD, n=3. [#]P<0.05 vs. normoxia. [*]P<0.05 vs. hypoxia only.
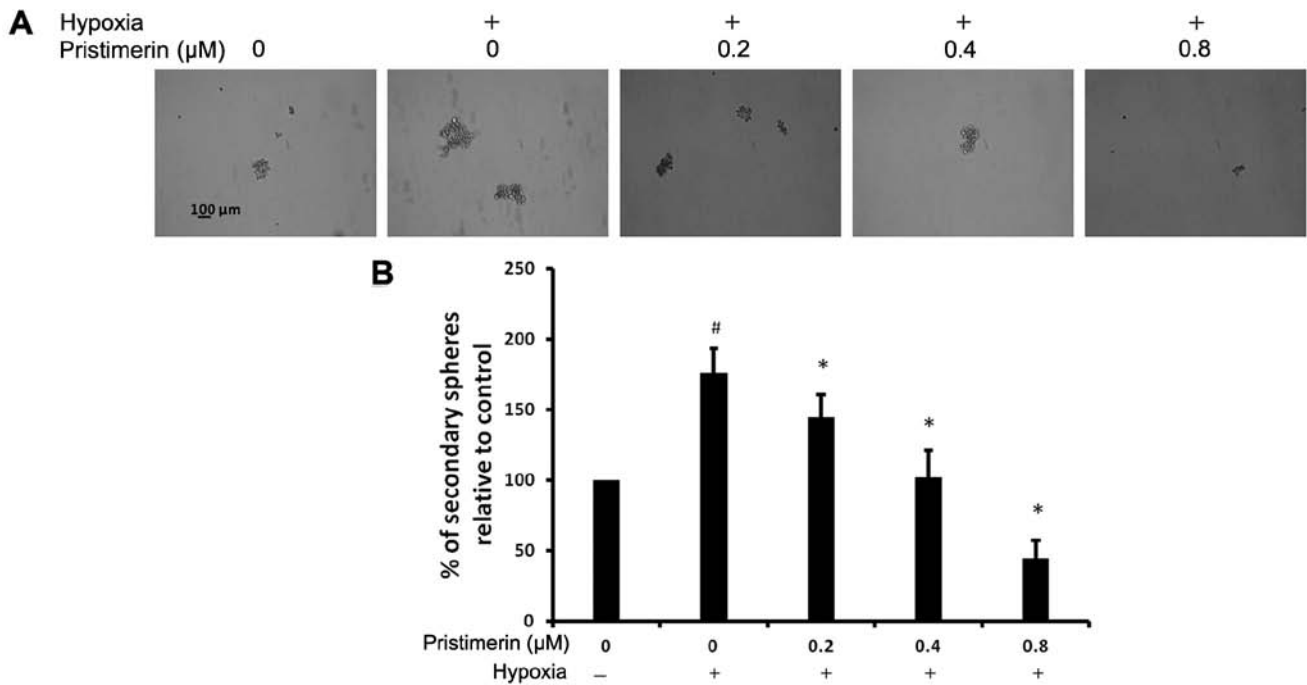

Figure 3. Pristimerin inhibits hypoxia-induced sphere formation of PC-3 cells. Pristimerin dose-dependently inhibited the sphere formation of PC-3 cells. (A) Images of sphere formation in PC-3 cells (x100 magnification). (B) The PC-3 cell sphere formation was counted in four randomly selected microscopic fields (x100 magnification). Values represent means ± SD, n=3. [#]P<0.05 vs. normoxia. [*]P<0.05 vs. hypoxia only.

preventing hypoxia-induced colony formation, we subjected the PC-3 cells to normoxic or hypoxic conditions for 48 h, and then the cells were pretreated with the indicated concentrations of pristimerin for 24 h. Next, they were plated at 300 cells as single cells onto a 6-well plate with increasing concentrations of pristimerin for 14 days. As shown in Fig. 4A and B, an increase in colony formation was observed under hypoxic conditions as compared to normoxic conditions. The stimula-
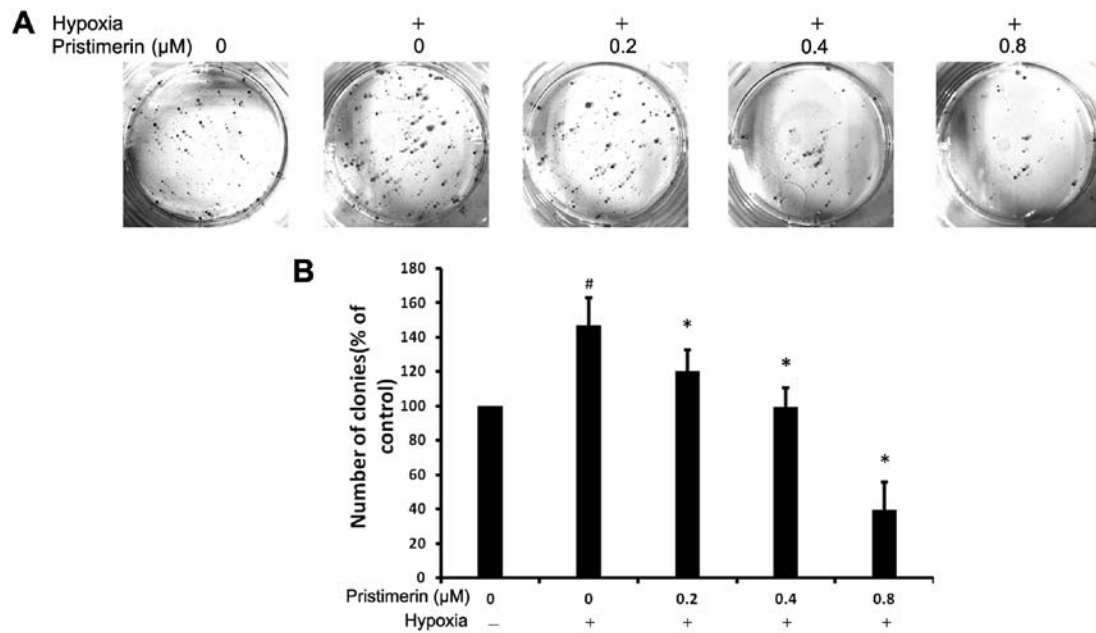
Figure 4. Pristimerin inhibits hypoxia-induced colony formation of PC-3 cells. Pristimerin dose-dependently inhibited the colony formation of PC-3 cells compared with control (normoxia). (A) Images of invaded PC-3 cells (x100 magnification). (B) The PC-3 cell colony formation was counted in four randomly selected microscopic fields (x100 magnification). Values represent means ± SD, n=3. #P<0.05 vs. normoxia. *P<0.05 vs. hypoxia only.
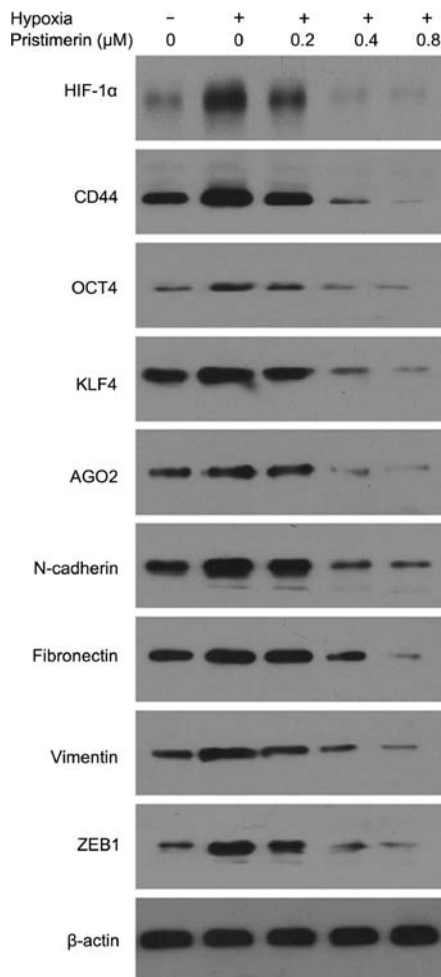

Figure 5. Pristimerin inhibits stem cell characteristics and EMT protein expression of PC-3 cells. PC-3 cells were exposed to normoxic or hypoxic conditions for 48 h and then treated with or without pristimerin for 24 h. Pristimerin inhibited HIF-1α, CD44, OCT4, KLF4, AGO2, N-cadherin, fibronectin, vimentin and ZEB1 protein levels.

tory effect of hypoxia on the colony formation of cancer cells was reduced by pristimerin in a dose-dependent manner. The number of hypoxic-induced colonies was decreased with the addition of 0.2 μM pristimerin. These results indicate that pristimerin could suppress the hypoxia-stimulated colony formation ability of cancer cells.

Pristimerin prevents hypoxia-induced stem cell characteristics and EMT protein expression in PC-3 cells. To investigate the effect of pristimerin treatment in preventing hypoxia-induced stem cells, we subjected PC-3 cells to normoxic or hypoxic conditions for 48 h, and then the cells were pretreated with the indicated concentrations of pristimerin for 24 h and CD44, KLF4, OCT4, AGO2, N-cadherin, fibronectin, vimentin and ZEB1 levels were tested by western blotting. As shown in Fig. 5, an increase in CD44, KLF4, OCT4, AGO2, N-cadherin, fibronectin, vimentin and ZEB1 expresssion was observed under hypoxic conditions as compared to normoxic conditions. The stimulatory effect of hypoxia on the expression of these proteins was reduced by pristimerin at a very low concentration (0.2 μM). Hence, we showed that pristimerin treatment decreased CD44, KLF4, OCT4, AGO2, N-cadherin, fibronectin, vimentin and ZEB1 levels in a dose-dependent manner under hypoxia.

Discussion

In the present study, we found that pristimerin suppressed HIF-1α expression and hypoxia-induced metastasis via blocking the proliferation, as well as the invasion ability of cancer cells. Furthermore, pristimerin repressed hypoxia-induced sphere formation, colony formation, expression of CSC markers and stemness factors including CD44, KLF4, OCT4, AGO2 and expression of EMT markers including N-cadherin, fibronectin, vimentin, ZEB1 in PC-3 cells.

These findings demonstrate that pristimerin can inhibit bone metastasis, hypoxia-induced stem cell characteristics and EMT of tumor cells. More importantly, CSCs and EMT may be the critical drivers of tumor progression and metastasis. Thus, our results suggest that the ability of pristimerin to inhibit bone metastasis is mediated partly by targeting hypoxia-induced stem cell characteristics and EMT of tumor cells.

Intratumoral hypoxia is a common characteristic feature of solid tumors and it is the driving force for tumor metastasis. Activation of hypoxia-inducible factor-1 (HIF-1) signaling is observed in a broad range of human cancers due to tumor hypoxia and promotes physiological changes associated with therapeutic resistance. It also includes the inhibition of apoptosis and senescence, as well as the activation of drug efflux and cellular metabolism, while approaches to inhibit HIF-1 signaling are primarily focused on reducing HIF-1α protein levels (30). As a result, targeting HIF-1 represents an attractive strategy to enhance the efficacy of current therapies, as well as reduce resistance to chemotherapy in tumors (30). In the present study, pristimerin inhibited hypoxia-induced HIF-1α protein levels. Therefore, our findings suggested that pristimerin may possess a similar function to hypoxia-induced progression and metastasis.

CSCs cells have been proposed to play a critical role in cancer metastasis as demonstrated in several human malignancies (31). With the affirmation of the CSC concept, cancer biology and cancer drug discovery have attained a new avenue to target cancer. CSCs are considered to be responsible for tumor initiation, metastasis and resistance to conventional radiotherapy and chemotherapy. Therefore, different approaches to targeting these tumorigenic and rare cells are urgently needed in order to improve the efficacy of anticancer therapy (32). In the present study, pristimerin inhibited hypoxia-induced stem cell characteristics and pristimerin directly inhibited the proliferation, sphere formation and colony formation of hypoxic PC-3 cells and impaired hypoxia-induced expression of CD44, KLF4, OCT4, AGO2 in hypoxic cancer cells. Therefore, our findings suggested that pristimerin may possess an inhibitory function against progression and metastasis by inhibited hypoxia-induced stem cell characteristics of PC-3 cells.

Increasing evidence emphasizes a critical role of EMT during PCa progression and malignant transformation, endowing the incipient cancer cells with invasive and metastatic properties (33-35). Thus, EMT could be a very promising therapeutic target, and the inhibition of EMT may prevent or restrain the invasion and metastasis of PCa. We found that pristimerin could diminish the hypoxia-induced expression of N-cadherin, fibronectin, vimentin, ZEB1 which showed that pristimerin changes the predominant mesenchymal phenotype. Therefore, our findings suggested that pristimerin may possess an inhibitory function against progression and metastasis by inhibition of EMT in PC-3 cells.

Due to the complexity of a tumor model, a single drug targeting a particular oncogene is unlikely to be entirely effective for cancer therapy. In this respect, pristimerin which contains various phytochemicals targeting multiple dysregulated pathways in cancer cells, may provide an alternative/complementary way to treat cancer. The utility of antitumor strategies for cancer control is strongly compromised by hypoxia-driven phenotypic changes, which make cancer cells more invasive and more prone to give rise to metastasis. In the present study, we showed that pristimerin decreased the expression of HIF-1α, CD44, KLF4, OCT4, AGO2, N-cadherin, fibronectin, vimentin, ZEB1 in a dose-dependent manner under pre-treatment hypoxic conditions. Thus, it is evident that concurrent measures for halting hypoxia-induced phenotypic changes may be required if pristimerin therapy is ever to achieve its goal of providing long-term cancer control and improved survival.

In conclusion, we demonstrated that several activities of pristimerin may account for its suppressive effect on cancer metastasis under hypoxia. Firstly, pristimerin markedly inhibited the stimulatory effect of hypoxia on the proliferation and invasion ability of cancer cells. Secondly, pristimerin could suppress stem cell characteristics, which has been shown to regulate multiple steps in the complex process of metastasis. Thirdly, pristimerin decreased levels of the predominant mesenchymal phenotype, which is overexpressed in a variety of tumor types and associated with increased metastases and poor prognosis.

To the best of our knowledge, this is the first documentation that pristimerin exerts its broad spectrum of antimetastatic effects through its potent inhibition of stem cell characteristics and EMT, in the context of tumors under hypoxia, a common feature of most solid cancers. These results provide new insight into the mechanisms of the antitumor action of pristimerin.

Acknowledgements

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