Aloperine prevents hypoxia-induced epithelial-mesenchymal transition in bladder cancer cells through regulating the mTOR/p70S6K/4E-BP1 pathway

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Abstract

Background: Aloperine (ALO), a novel active alkaloid extracted from *S. alopecuroides*, has been reported to possess anti-tumor effect. However, its potential effect on bladder cancer remains unknown. Therefore, the objective of this study was to investigate the effect of ALO bladder cancer cells under hypoxia condition.

Methods: Human bladder cancer cell line T24 cells were treated with different concentrations of ALO and maintained in hypoxic condition for 12, 24, or 48 h. MTT assay was performed to detect cell viability. Transwell assay was performed to detect cell migration and invasion. Epithelial-mesenchymal transition (EMT) was evaluated by detecting the expression levels of E-cadherin, N-cadherin, and vimentin using western blot. The mRNA and protein levels of HIF-1 α , snail, slug, and twist1 *were measured using* qRT-PCR and western blot. The expression levels of mTOR/p70S6K/4E-BP1 pathway-related proteins were detected using western blot.

Results: Our results showed that ALO inhibited the cell viability of T24 cells cultured in hypoxia condition. ALO also attenuated hypoxia-induced migration and invasion of T24 cells. We also found that ALO treatment caused a significant increase in E-cadherin expression and decreases in N-cadherin and vimentin expressions. Besides, ALO dose-dependently inhibited the expressions of EMT inducers including snail, slug, and twist1 both in mRNA and protein levels in T24 cells induced by hypoxia. Furthermore, ALO significantly inhibited HIF-1 α protein synthesis and phosphorylation of mTOR, as well 4E-BP1 and p70S6K in hypoxia-induced T24 cells.

Conclusion: These results indicated that ALO exerted anti-tumor effect on bladder cancer *in vitro* via inhibiting the activation of mTOR/p70S6K/4E-BP1 pathway.

1. Background

Bladder cancer (BC) is the most common malignancy in the urinary tract worldwide and is associated with significant morbidity and mortality [1]. Established risk factors for developing bladder cancer include Age, gender, smoking, medications, diet, radiation and genetic factors [2]. Bladder cancer can be clinically categorized into 2 groups: nonmuscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) [3]. NMIBC accounts for approximately 70–80% of bladder cancer patients and patients with NMIBC exhibit high risk of recurrence and a variable risk of progression, which may be attributed to the metastasis [4].

Epithelial-mesenchymal transition (EMT) is an important cellular process that is characterized by loss of cell adhesion and increased cell mobility [5]. The EMT is a key progression mechanism of cancer invasion and metastasis [6]. Hypoxic condition is always present in tumor environment due to the fast growth of tumor cells and inadequate blood supply [7]. There is increasing evidence that this condition may influence the production of hypoxia inducible factor (HIF), which is a helix transcription factor involved in carcinogenesis and tumor growth [8]. The HIF signaling may regulate the cancer-related genes

and thereby affect multiple biological mechanisms including EMT [9]. Hence, targeting the hypoxia-induced EMT may provide new approach for clinical treatment of cancers.

Sophora alopecuroides (S. alopecuroides) is a traditional Chinese herb with multiple properties [10]. Aloperine (ALO), a novel active alkaloid extracted from S. alopecuroides, has been proven to show broad pharmacological activities, such as anti-inflammatory, anti-viral, anti-microbial, anti-apoptotic, anti-oxidative, and anti-tumor effects [11–14]. Therefore, increasing experiments have confirmed the therapeutic potential of ALO in many kinds of diseases. Notably, ALO has been demonstrated to have therapeutic potential in various cancers, including thyroid cancer, breast cancer, prostate cancer, myeloma, hepatocellular carcinoma, glioma, and colon cancer [13, 15–18].

However, its potential effect on bladder cancer remains unknown. Therefore, the objective of this study was to investigate the effect of ALO on hypoxia-induced EMT in bladder cancer cells. We found that ALO inhibited hypoxia-induced EMT in bladder cancer cells through regulation of mTOR/p70S6K/4E-BP1 pathway.

2. Materials And Methods

2.1. Cell culture and treatment

Human bladder cancer cell line T24 cells were obtained from ATCC (Manassas, VA, USA). The T24 cells were maintained in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% FBS (Gibco). For the normoxic culture, cells were cultured in a humidified incubator with 5% CO₂ and O₂ (21%). For hypoxic culture, the cells were cultured in a modular incubator flushed with O₂ (1%), CO₂ (5%), and N₂ (94%) for different hours.

2.2. Cell viability assay

T24 cells were seeded on 96-well plate at a density of 5,000 cells per well. After incubation for 24 h at 37 °C, T24 cells were added with 100 µl MTT (1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) and then cultured for 4 h. Then the cells were added with 100 µl of DMSO to dissolve the formazan. Finally, the value of absorbance was measured at a wavelength of 490 nm using a microplate reader (Tecan, Männedorf, Switzerland).

2.3 Cell migration and invasion assays

Transwell assays were applied to measure cell migration and invasion ability using Transwell chambers (8 µm pores) coated with or without Matrigel (30 µg; BD Biosciences, Bedford, MA, USA). In brief, 1×10^5 T24 cells were resuspended in serum-free culture medium and planted in the upper chambers. Culture medium containing 10% FBS was added to the lower chamber. After 24 h culturing, migrated or invaded cells were fixed in methanol and stained with crystal violet. Five random fields were photographed, and the cell number was counted using AIS software (Imaging Research Inc., St Catherine's, Canada).

2.4. Real-time quantitative PCR analysis

Total RNA of T24 cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then the cDNA was prepared using a PrimeScript RT reagent kit (Takara, Dalian, China) according to the manufacturer's protocols. The obtained cDNA was used for PCR analyses with a SYBR Green PCR Master Mix (Takara) under the following condition: 95 °C 10 min; 40 cycle; 95 °C 10 sec, 60 °C 30 sec, 72 °C 30 sec; and 72 °C 10 min. The expression levels of target genes including HIF-1 α , snail, slug, twist1 were calculated relative to β -actin.

2.5. Western blotting

T24 cells were lysed by RIPA lysis buffer and the total protein was collected for the separation by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then gel was used for the western blot analysis. The proteins on the gels were transferred onto PVDF membranes, followed by blocking in 5% nonfat milk buffer. Subsequently, the membranes were subjected to overnight blotting at 4 °C with primary antibodies anti-E-cadherin (Abcam, Cambridge, MA, USA), anti-N-cadherin (Abcam), anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-snail (Santa Cruz Biotechnology), anti-slug (Santa Cruz Biotechnology), anti-twist1 (Santa Cruz Biotechnology), anti-HIF-1 α (Cell Signaling Technology, Boston, MA, USA), anti-p-mTOR (Sigma-Aldrich, St. Louis, MO, USA), anti-mTOR (Sigma-Aldrich), anti-p-p70S6K (Cell Signaling Technology), anti-p70S6K (Cell Signaling Technology), anti-p-4E-BP1 (Abcam), anti-4E-BP1 (Abcam) or anti- β -actin (Santa Cruz Biotechnology). Then the membranes were probed with appropriate goat anti-rabbit or goat anti-mouse secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The bands were visualized with an ECL kit (Thermo Fisher Scientific, Waltham, MA, USA) and the intensity was quantified with the ImageJ software (National Institutes of Health, NIH, Bethesda, MD, USA).

2.6. Statistical analysis

The data were analyzed by SPSS 19.0 software and shown as means $\pm$ SD. Comparisons among different groups were analyzed with one-way ANOVA. The results were considered statistically significant if $p < 0.05$.

3. Results

3.1. ALO inhibits the viability of bladder cancer cells cultured in hypoxia

First, we evaluated the effect of hypoxia on cell viability. As indicated in Fig. 1A, exposure to hypoxia condition for 48 h caused significant reduction in cell viability of T24 cells. Thus, T24 cells were exposed to hypoxic condition for 24 h in the subsequent experiments. Then, we found that ALO significantly suppressed cell viability both in normoxic and hypoxic conditions, especially in hypoxic condition (Fig. 1B).

3.2. ALO prevents hypoxia-induced migration and invasion of bladder cancer cells

Next, we investigated the effects of ALO on migration and invasion under hypoxic condition. Transwell assay demonstrated that hypoxia caused significant increase in migration and invasion ability of T24 cells. However, treatment with ALO significantly prevented the hypoxia-induced migration and invasion ability (Fig. 2A-2B).

3.3. ALO inhibits hypoxia-induced EMT in bladder cancer cells

We then investigated the effects of ALO on the EMT of bladder cancer cells through detection of the expression levels of E-cadherin, N-cadherin, and vimentin. The expression level of E-cadherin was obviously decreased, while the N-cadherin and vimentin expression levels were markedly increased in T24 cells under hypoxia condition. However, the decreased E-cadherin expression and the increased N-cadherin and vimentin expression were attenuated by ALO treatment (Fig. 3).

3.4. ALO represses expressions of EMT-inducing transcription factors in bladder cancer cells

The snail, slug, and twist1 are three important transcription factors that can induce EMT. The mRNA and protein levels of snail, slug, and twist1 were measured using qRT-PCR and western blot. The results demonstrated that ALO dose-dependently inhibited the expressions of snail, slug, and twist1 both in mRNA and protein levels induced by hypoxia (Fig. 4A-4D).

3.5. ALO suppresses hypoxia-induced HIF-1 α expression in bladder cancer cells

To elucidate the molecular mechanism involved in the effects of ALO on T24 cells under hypoxia condition, we examined the mRNA and protein levels of HIF-1 α . The qRT-PCR results showed that the mRNA level of HIF-1 α was increased in hypoxia-induced T24 cells compared with the T24 cells cultured under normal condition. Meanwhile, western blot analysis indicated that the protein level of HIF-1 α was also induced by hypoxia. ALO treatment efficiently suppressed the HIF-1 α expression in both mRNA and protein levels (Fig. 5A and B).

3.6. ALO inhibits the activation of mTOR/p70S6K/4E-BP1 pathway in bladder cancer cells in response to hypoxia

It has been shown that p70S6K and 4E-BP1 are two effectors of mTOR, which is involved in HIF-1 α protein synthesis at the translational level. Thus, we aimed to explore whether the mTOR/p70S6K/4E-BP1 pathway was involved in the effects of ALO. As shown in Fig. 6, ALO treatment inhibited the protein levels of p-mTOR, p-p70S6K, and p-4E-BP1 in a dose-dependent manner under hypoxia. While as, ALO exhibited no effect on the total protein levels for mTOR p70S6K, and 4E-BP1.

4. Discussion

Hypoxia microenvironment has attracted increasing interest in the recent research of malignant tumors [19]. It has been reported that the hypoxia microenvironment is considered as a significant factor contributing to the progression of cancer because it is associated with tumor aggressiveness and metastasis, poor prognosis, post-treatment recurrence and resistance to radiation therapy [9]. Peixoto et

al. [20] proved that hypoxia enhances the malignant nature of bladder cancer cells. A 24-gene hypoxia signature has strong and independent prognostic and predictive value for muscle-invasive bladder cancer patients [21]. The hypoxia microenvironment is being therapeutic target for the treatment of bladder cancer. Sulforaphane inhibits NMIBC cells proliferation and glycolytic metabolism in hypoxia [22]. Scutellarin inhibits hypoxia-induced cell migration, invasion and EMT in bladder cancer cells, as well as suppresses metastasis in vivo [23].

Recent studies have demonstrated that ALO possesses anti-tumor activity in various cancers. For instance, ALO exhibits anti-tumor activity on human thyroid cancer, as proven by its inhibitory effects on cell growth in human anaplastic thyroid cancers and multidrug-resistant papillary thyroid cancers [16]. ALO inhibits proliferation, migration and invasion and induces apoptosis in human breast cancer cells [24]. ALO executes anti-tumor effects on prostate cancer through the induction of apoptosis and cell cycle arrest both in vitro and in vivo [15]. Our results showed that ALO inhibited the viability of bladder cancer cells cultured in hypoxia. ALO attenuated hypoxia-induced migration and invasion of bladder cancer cells. We also found that ALO mitigated the hypoxia-induced EMT process due to the increased E-cadherin expression and the decreased N-cadherin and vimentin expression. EMT can be induced by several transcription factors, such as snail1, slug and twist [25]. Our data showed that ALO dose-dependently inhibited the expressions of snail, slug, and twist1 both in mRNA and protein levels in T24 cells induced by hypoxia.

HIF-1 α is a key stress-responsive transcription factor to low oxygen and correlated with oncogenic activation or loss of tumor suppressor function [26, 27]. Therefore, HIF-1 α is connected with mechanisms of tumor invasion capacity, angiogenesis, radiotherapy, and chemotherapy resistance. Targeting HIF-1 α is a promising strategy for the treatment of cancer. In the present study, we demonstrated that ALO significantly inhibited HIF-1 α protein synthesis in hypoxia-induced bladder cancer cells. It has been shown that the PI3K/Akt/mTOR pathway plays a crucial role in the hypoxia microenvironment and is involved in HIF-1 α protein synthesis at the translational level [28–30]. The mTOR is a serine/threonine kinase and carries out its functions by two distinct complexes, namely mTORC1 and mTORC2 [31]. The mTORC1 regulates protein synthesis through at least two downstream targets 4E-BP1 and p70S6K [32]. In the present study, we revealed that treatment with ALO suppressed the phosphorylation of mTOR, as well 4E-BP1 and p70S6K. The results indicated that ALO inhibited the activation of mTOR/p70S6K/4E-BP1 pathway in hypoxia-induced T24 cells.

5. Conclusion

In summary, we investigated the anti-tumor effect of ALO on bladder cancer in vitro. We found that ALO reduced cell viability and inhibited migration, invasion, and EMT of T24 cells under hypoxic condition. Further analysis demonstrated that ALO suppressed the expression of HIF-1 α and activation of mTOR/p70S6K/4E-BP1 pathway in T24 cells. Our results suggested that ALO could serve as a potential therapeutic agent for bladder cancer.

Declarations

Authors' contributions

Xiaoyong Song designed the study, Qian Liu and Jihong An conducted the experiments, analyzed the data and prepared the manuscript. Weiling Lv edited the manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgement

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the Ethic Committee of Huaihe Hospital of Henan University the Declaration of Helsinki Principles.

Written informed consents were obtained from each participant involved in this study.

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Figures

A **B**

Figure 1

Effect of ALO on cell viability of T24 cells in hypoxic condition. (A) T24 cells were maintained in normoxia or hypoxic condition for 12, 24 or 48 h. MTT assay was performed to detect cell viability. (B) T24 cells were treated with different concentrations of ALO (5 and 10 μ M) and maintained in hypoxic condition for 24 h. MTT assay was performed to detect cell viability. *p < 0.05, compared with normoxia group.

Figure 2

Effect of ALO on migration and invasion of hypoxia-induced T24 cells. T24 cells in control group were maintained in normoxia condition. T24 cells in hypoxia group were maintained in hypoxia condition. T24 cells in ALO treatment groups were treated with 5 and 10 μ M ALO and maintained in hypoxia condition for 24 h. (A and B) Transwell assay was performed to detect cell migration and invasion. * $p < 0.05$, compared with normoxia group. # $p < 0.05$, compared with hypoxia group.

Figure 3

Effect of ALO on EMT in hypoxia-induced T24 cells. T24 cells in control group were maintained in normoxia condition. T24 cells in hypoxia group were maintained in hypoxia condition. T24 cells in ALO treatment groups were treated with 5 and 10 μ M ALO and maintained in hypoxia condition for 24 h. (A) The protein expression levels of E-cadherin, N-cadherin and vimentin were evaluated using western blot. (B-D) Quantification analysis of E-cadherin, N-cadherin and vimentin. * $p < 0.05$, compared with normoxia group. # $p < 0.05$, compared with hypoxia group.

Figure 4

Effect of ALO on EMT-inducing transcription factors in hypoxia-induced T24 cells. T24 cells in control group were maintained in normoxia condition. T24 cells in hypoxia group were maintained in hypoxia condition. T24 cells in ALO treatment groups were treated with 5 and 10 μ M ALO and maintained in hypoxia condition for 24 h. The mRNA and protein levels of snail, slug, and twist1 were measured using qRT-PCR (A-C) and western blot (D). * $p < 0.05$, compared with normoxia group. # $p < 0.05$, compared with hypoxia group.

Figure 5

Effect of ALO on HIF-1α expression in hypoxia-induced T24 cells. T24 cells in control group were maintained in normoxia condition. T24 cells in hypoxia group were maintained in hypoxia condition. T24 cells in ALO treatment groups were treated with 5 and 10 μM ALO and maintained in hypoxia condition for 24 h. The mRNA and protein levels of HIF-1α were measured using qRT-PCR (A) and western blot (B). * $p < 0.05$, compared with normoxia group. # $p < 0.05$, compared with hypoxia group.

Figure 6

Effect of ALO on activation of mTOR/p70S6K/4E-BP1 pathway in hypoxia-induced T24 cells. T24 cells in control group were maintained in normoxia condition. T24 cells in hypoxia group were maintained in hypoxia condition. T24 cells in ALO treatment groups were treated with 5 and 10 μ M ALO and maintained in hypoxia condition. (A) The expression levels of mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1, and p-4E-BP1 were detected using western blot. (B) The ratio of p-mTOR/mTOR. (C) The ratio of p-

p70S6K/p70S6K. (D) The ratio of p-4E-BP1/ 4E-BP1. *p< 0.05, compared with normoxia group. #p< 0.05, compared with hypoxia group.