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Original Research

Piceatannol inhibits proliferation and induces apoptosis of bladder cancer cells through regulation of the PTEN/AKT signal pathway

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Abstract: This study was aimed to investigate the effect of piceatannol (PIC) on the proliferation and apoptosis of bladder cancer cell line EJ, and the underlying mechanism. Bladder cancer cell line EJ was incubated with different concentrations of PIC, and CCK-8 method was used to determine the effect of the treatment on cell proliferation. The effect of PIC on cell cycle, apoptosis and the expressions of related signal pathway proteins were determined using Western blotting. Flow cytometry showed that PIC inhibited the proliferation of EJ cells in a concentration- and time-dependent fashion. Moreover, EJ cells were significantly blocked in G0/G1 phase, when compared with the blank control group ($p < 0.05$). In addition, PIC enhanced apoptosis of EJ cells in a concentration-dependent manner ($p < 0.05$). Results from western blotting showed that, compared with the control group, PIC upregulated the protein expression of PTEN, but downregulated Akt protein phosphorylation, relative to control cells. PIC significantly inhibits the proliferation of EJ cells and enhances their apoptosis through a mechanism related to the activation of PTEN/Akt signaling pathway.

Key words: Bladder cancer; Piceatannol; PTEN / Akt signaling pathway.

Introduction

Bladder cancer is one of the most common malignant tumors in the world, and it results in about 150000 deaths every year (1). Once bladder cancer degenerates to muscle-invasive bladder cancer (MIBC), the risks of recurrence, progression and death increase dramatically (2). For local MIBC, radical cystectomy is routinely performed, but 25% of patients who underwent radical cystectomy showed lymph node involvement at the time of operation. In addition, more than 30% of the patients did not respond to the treatment or suffered relapse within five years. Unfortunately, 50% of the patients died of metastatic disease (3, 4). Cisplatin-based neoadjuvant chemotherapy has been shown to significantly improve overall survival, while only about 50% of MIBC patients respond to cisplatin-based chemotherapy (5). A large number of studies have shown that the pathogenesis of bladder cancer is very complex, and may involve changes in genomic regions and epigenetic modifications (6-8). However, the molecular mechanism involved in bladder tumor is still poorly understood, and new and more effective treatment strategies are urgently needed.

Piceatannol (3,3 ', 4,5' - tetrahydroxy trans stilbene; PIC) is a natural stilbene found in many plants. It is a hydroxy analogue of resveratrol, and it is produced from resveratrol through the activity of microsomal cytochrome P4501A11/2 and 1B1 (9). Like resveratrol, PIC has a wide range of health benefits, many of which are attributed to its antioxidant and anti-inflammatory properties (10, 11). It exerts anti-cancer effects against prostate, lymph, colon and breast cancers by targeting specific proteins involved in cancer cell proliferation, survival/death, invasion, metastasis and angiogenesis in the tumor microenvironment (12-15). In addition, studies have shown that PIC possesses anti-obesity, anti-diabetes, nerve protection, cardio-protection, anti-allergy and anti-aging properties (16-19). However, the effect of PIC on bladder cancer has not been reported. In this study, the effects of PIC on proliferation and apoptosis of bladder cancer EJ cells, and the underlying mechanism were investigated, so as to provide a theoretical basis for the treatment of bladder cancer.

Materials and Methods

Cell culture

Bladder cancer EJ cell line was purchased from ATCC. Fetal bovine serum (FBS) and RPMI1640 medium were products of Hyclone. The EJ cells were inoculated in a 25-ml glass flask and cultured in RPMI1640 medium containing 10% FBS and 1% penicillin/streptomycin mixture at 37°C in an atmosphere with 5% CO_2 and saturated humidity. When the cells grew to 80% confluence, they were passaged, and cells in the logarithmic growth period were used in study.

CCK-8 assay for cell proliferation

Cells at logarithmic growth period were collected after trypsin digestion. After counting, the cell concentration was adjusted to 5×10^4 - 8×10^4 cells/ml with complete cell culture medium containing 10% serum and 1% penicillin/streptomycin mixture. The EJ cells were inoculated in 96-well plates (100uL single cell suspension per well) overnight at 37°C in a 5% CO_2 and saturated humidity incubator. When the cells were attached to the wall, they were treated with PIC at concentrations of 5, 10, 20, 40 and 80 μM, each at a volume of 100uL. Triplicate wells were up for each concentration. Then, 10 μL of CCK-8 was added to each well after 12, 24 and 48 h, and the wells were incubated at 37°C in a 5% CO_2 saturated humidity incubator for 4h. Thereafter, the absorbance (OD) of each well was read at 450 nm in a multifunctional enzyme instrument.

Detection of apoptosis by flow cytometry

The apoptosis was determined using flow cytometry combined with Annexin V-PE / AAD. Cells at logarithmic growth stage were inoculated in 6-well plate at a density of 2×10^5 cells/well cells. The cells were divided into groups according to different PIC concentrations and digested and collected using trypsin in line with the instructions on the kit manual. The cells were washed twice with cold PBS, and the density was adjusted to about 1×10^9 cells/ L with 400 μL Annexin V binding solution. Then, 5 μL AnnexinV-PE staining solution was added, followed with gentle mixing at 2-8°C. After incubation in the dark for 15 min, 10 μL AAD staining solution was added with gentle mixing at 2 -8°C. After incubating in the dark for 5 min, apoptosis was analysed with flow cytometry (20).

Determination of cell cycle with flow cytometry

After incubation with different concentrations of PIC for 48 h, the cells were prepared at a density of 2×10^6 cells/ml cell suspension, and washed twice with PBS, followed by centrifugation at 1000 rpm for 5 min at 4°C. The cells were fixed in cold 70% ethanol for 30 min, followed by digestion with ribonuclease. Then, the cells were stained with propidium iodide (PI) for 30 min, and the cell cycle was detected with flow cytometry.

Western blot analysis

Cells at logarithmic growth stage were seeded into 6-well plate at a density of 2×10^5 cells/well. When the cell growth density reached about 80%, the cells were treated with PIC at the same concentrations as described earlier. Then, the cells in each well were lysed on ice with 150 μL of enhanced RIPA lysing solution containing 1 mM protease inhibitor and PMSF. Thereafter, total protein was extracted and quantified using bicinchoninic acid (BCA) protein assay kits. Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene membrane. Non-specific binding was blocked by incubating the membrane with 5% skimmed milk powder at room temperature for 1 h. Then, the membrane was incubated overnight at 4°C with primary antibodies for PTEN, Akt, p-Akt and β-actin (dilutions of 1:1000-1:2000), with GAPDH as internal reference. Then, the membrane was washed thrice with TBST, each wash for 10 min, followed by incubation with secondary antibody at room temperature for 1 h. After washing thrice with TBST, enhanced chemiluminescence was used to detect the immunoblots. Gray analysis was done with ImageJ.

Statistical Analysis

Data are expressed as mean ± SD. One-way ANOVA was used to compare differences among groups, while *t*-test was used to compare two groups. All statistical analyses were done with SPSS 19.0 statistical software.

Results

Effect of PIC on EJ cell viability

Results from CCK-8 experiments showed that PIC significantly and dose-dependently reduced the viability of EJ cells (Figure 1). When PIC concentration was 20 μM, cell viability was not statistically different when compared with cell viabilities at PIC concentrations of 40 μM and 80 μM after 48 h of cell culture. Therefore, 20 μM PIC was used in the subsequent experiments. The cells were cultured in 20 μM PIC for 48 h.

PIC affects EJ cell cycle

After 48-h incubation with PIC, the percentages of cells in G0/G1 phase, S phase and G2/M phase in the blank control group were 54.2 ± 3.2, 25.1 ± 1.9 and 22.7 ± 1.5, respectively. However, after 5 μM PIC treatment,

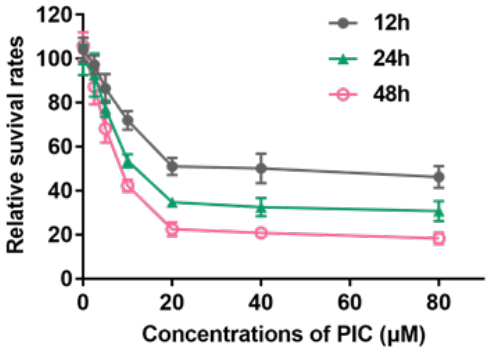

Figure 1. Effect of PIC on the viability of bladder cancer EJ cells. Values are mean ± SD (n =3).

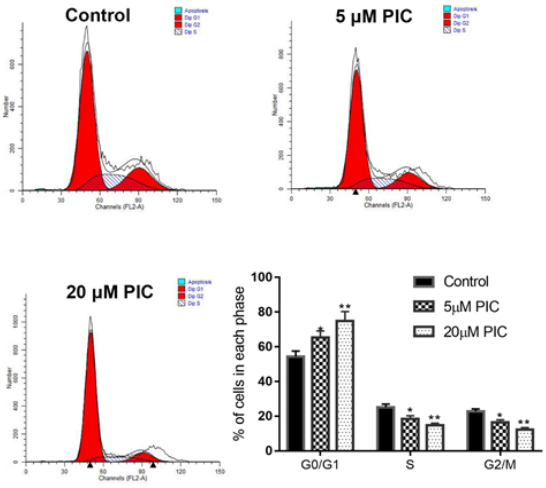

Figure 2. Effect pf PIC on the cell cycle of EJ cells. * $P < 0.05$, ** $p < 0.01$, compared with the control group. Data are presented as mean ± SD (n =3).

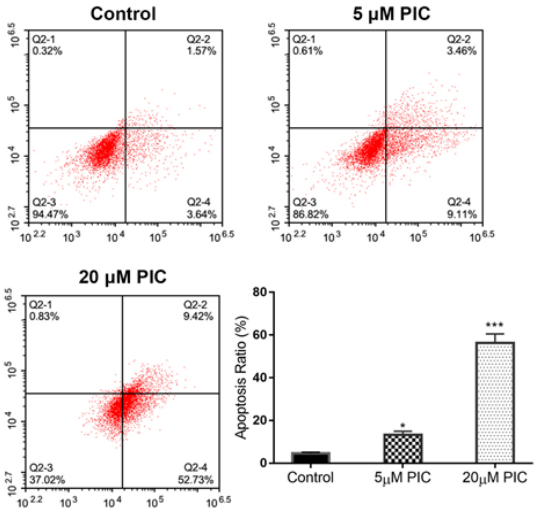

Figure 3. Apoptosis ratio of EJ cells treated with PIC; $^{**}p< 0.01$, $^{***}p< 0.001$, vs control group.

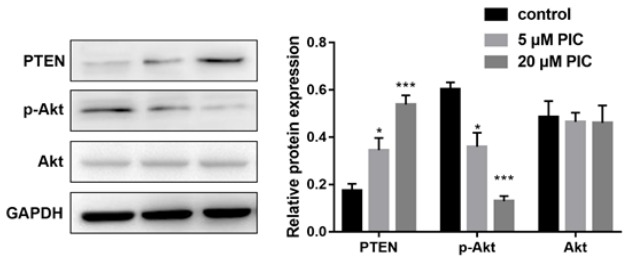

Figure 4. Effect of PIC on the protein expressions of PTEN，p-Akt，and Akt in the EJ cells．Results are presented as mean ± SD (n = 3). $^{*}P <0.05$，$^{*}p <0.001$, vs control group.

the percentages of cells in G0/G1 phase, S phase and G2/M phase were 63.3 ± 5.4, 18.3 ± 1.9 and 16.3 ± 1.6, respectively, while the corresponding values after 20 μM PIC treatment were 74.9 ± 3.8%, 14.8 ± 1.2% and 12.3 ± 1.0%, respectively. Compared with the control group, the proportion of cells in G0/G1 phase increased significantly, while the proportion of cells in S phase and G2/M phase decreased significantly, both concentration-dependently ($p < 0.05$). These results are shown in Figure 2.

PIC promoted apoptosis of EJ Cells

As shown in Figure 3, PIC significantly enhanced apoptosis of EJ Cells in a concentration-dependent manner ($p < 0.05$).

Effect of PIC on protein expressions of PTEN /Akt pathway in EJ Cells

The expressions of PTEN, Akt and p-Akt were measured with Western blotting after EJ cells were treated with different concentrations of PIC for 48 h. As shown in Figure 4, compared with the control group, PTEN protein expression was increased, while p-Akt protein expression was decreased in the PIC treatment group ($p < 0.05$). However, Akt protein expression in the two groups was comparable ($p > 0.05$).

Discussion

Bladder cancer is one of the malignant tumors that affect the urogenital tract (21, 22). A major difficulty posed by this disease is that even if it is superficial in diagnosis, the current treatment method is intravesical administration of BCG after transurethral resection, which is not completely effective in preventing tumor recurrence and progression. Therefore, several investigations have focused on a variety of natural products from plant secondary metabolism in an effort to find a new and better treatment for bladder cancer.

The biological effects of PIC are similar to those of resveratrol, being that it is a well-known metabolite of 1,2-stilbene resveratrol, although the relative effect of PIC varies with animal model and dose (23). Piceatannol (PIC) has a catechol component. Thus, it can be redox-cycled to produce an electrophilic quinone metabolite that can interact with the cell nucleophilically (24). Despite several studies on PIC effects, the basic molecular mechanism is still poorly understood. Hsu and the co-workers have founded that the induction of p21/WAF1 and activity of the Fas/mFasL apoptotic system may participate in the antiproliferative activity of piceatannol in bladder cancer cells.(25) In this study, it was found that PIC inhibited cell proliferation and induced cell death by inhibiting PTEN/Akt signaling pathway. Results from CCK8 showed that PIC inhibited the proliferation of EJ cells in a time- dependent and concentration-dependent manner. In addition, after incubation of EL cells with PIC, the proportion of EJ cells in the G0/G1 phase increased significantly and concentration-dependently, indicating that cell proliferation was inhibited. The results also showed that PIC enhanced apoptosis of EJ cells.

In tumor cells, including pancreatic cancer, liver cancer, breast cancer and gastric cancer, the PTEN/Akt signaling pathway is widely involved in cell proliferation, differentiation and metastasis (26-28). The activation of PTEN/Akt pathway is involved in the inhibition of proliferation of bladder cancer cells (29, 30). In this study, results from western blotting showed that PIC significantly upregulated the expression of PTEN protein, but inhibited the phosphorylation of Akt protein in bladder cancer EJ Cells, indicating that PTEN/Akt signaling pathway is involved in the PIC-induced effects on the proliferation and apoptosis of EJ cells. It is not clear whether other signaling pathways are involved in this process. This will be investigated in subsequent studies.

This study has demonstrated that PIC significantly inhibited the proliferation of EJ cells and promoted their apoptosis, through a mechanism related to inhibition of the activation of PTEN/Akt cell signaling pathway. These findings are of clinical relevance in the treatment bladder cancer.

Acknowledgements

None.

Conflict of Interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Wen-sheng Zhang;

Xun-gang Li, Wen-sheng Zhang, Xiao-ping Yan collected and analysed the data; Xun-gang Li wrote the text and all authors have read and approved the text prior to publication.

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