Abstract. – OBJECTIVE: To observe the effects of pheophorbide a-mediated photodynamic therapy (Pa-PDT) on the in vitro proliferation, apoptosis, invasion and metastasis of human prostate cancer PC-3 cells and to investigate its possible mechanism.

MATERIALS AND METHODS: Pa-PDT in gradient concentrations (0 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, and 4 μM) were used to act on PC-3 cells; the cell proliferation in each group was detected via methyl thiazolyl tetrazolium (MTT) assay and clone formation assay, and the cell apoptosis was detected via Hochst33258 staining and Annexin V/propidium iodide (PI) double labeling. Moreover, the effects of Pa-PDT on invasion and proliferation of PC-3 cells were observed via wound healing assay and transwell chamber assay. Finally, the expressions of apoptosis-related proteins, epithelial-mesenchymal transition (EMT)-related proteins and matrix metalloproteinases (MMPs) in each group were detected after treatment by Western blotting.

RESULTS: MTT and clone formation assays showed that Pa-PDT could inhibit the proliferation of PC-3 cells in a dose-dependent manner. The results of apoptosis assay revealed that Pa-PDT could significantly promote the apoptosis of PC-3 cells, obviously up-regulate the expressions of pro-apoptotic proteins, such as B-cell lymphoma-2-associated X protein (BAX), Caspase-3 and poly adenosine diphosphate-ribose polymerase (PARP), and inhibit the expression of Bcl-2. Besides, the wound healing assay and Transwell chamber assay showed that Pa-PDT could inhibit the invasion and metastasis capacities of PC-3 cells, whose relevant mechanisms were related to the fact that Pa-PDT inhibited the EMT process and down-regulated the expressions of MMPs in PC-3 cells.

CONCLUSIONS: Pa-PDT can inhibit the proliferation and promote the apoptosis of PC-3 cells. Moreover, it can also inhibit the invasion and metastasis capacities of PC-3 cells via inhibiting the EMT process and down-regulating the expressions of MMPs.

Key Words: Prostate cancer, Photodynamic therapy, Pheophorbide a, Apoptosis, Invasion.

Introduction

Prostate cancer (PCa) is a kind of common malignant tumor of urinary system. In the United States, PCa has become the malignant tumor with the highest incidence rate in males. There were about 161,360 new cases in 2017, while about 26,730 people died of PCa. In Europe, the number of deaths from PCa ranks third in male tumors, and about 72,600 people died of PCa in 2015. Currently, the conventional therapeutic methods are mainly radical surgery and endocrine therapy, supplemented by radiotherapy and chemotherapy, which can achieve better clinical treatment effects. However, there are still some patients who lose the operation opportunity and are not sensitive to endocrine therapy, radiotherapy and chemotherapy, which remain to be resolved. Therefore, searching for effective treatment means of PCa is one of the hot topics of anti-PCa.

Photodynamic therapy (PDT) is a new treatment method of malignant tumors, whose main principle is that the tumor tissues can selectively take in photosensitizer and produce the singlet oxygen upon the excitation of specific-wavelength light, thereby killing the tumor cells. Compared with traditional tumor therapies, this new technique is characterized by high specificity and few complications, so it is widely used in the treatment of a variety of tumors. Pheophorbide a (Pa) is a major degradation product of chlorophyll a, which has good photosensitization. Studies have shown that Pa-PDT has an anti-tumor effect on a variety of tumors. However, there are few
reports on Pa-PDT in PCa. In view of the advantages of Pa-PDT, the effects of Pa-PDT on the proliferation, invasion, and metastasis of PCa PC-3 cells and its mechanism were investigated in this study, so as to provide new and more superior treatment means for PCa.

**Materials and Methods**

**Materials**

Anti-B-cell lymphoma-2-associated X protein (BAX), anti-Bcl-2, anti-Caspase-3, anti-poly adenosine diphosphate-ribose polymerase (PARP), anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-matrix metalloproteinase (MMP)-2, anti-MMP-9 and anti-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Hoechst and Annexin V/propidium iodide (PI) staining kits were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Pa and other experimental reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Cell Culture**

PC-3 cells were purchased from American Type Cell Culture (ATCC, Manassas, VA, USA), and they were cultured in the Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% non-essential amino acid in an incubator with 5% CO$_2$ at 37°C.

**Methyl Thiazolyl Tetrazolium (MTT) Detection**

PC-3 cells were cultured in vitro till the logarithmic growth phase, and digested with 0.25% trypsin to prepare the single-cell suspension; they were followed by counting, centrifugation and re-suspension. The cell concentration was adjusted to $8 \times 10^4$/mL. Cells were inoculated into a 96-well plate (100 μl/well), and cultured in an incubator with 5% CO$_2$ at 37°C for 12 h. Then, Pa in corresponding concentrations (final concentration of 0 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, and 4 μM) was added into each group for incubation for 6 h; the supernatant was discarded and the original medium was replaced with the fresh medium containing 10% serum. After the radiation of laser with a wavelength of 664 nm and energy density of 2.0 J/cm$^2$ for 15 min, the 96-well plate was placed into an incubator for incubation for 24 h, followed by MTT detection.

**Clone Formation Assay**

The cells in the logarithmic growth phase were taken and inoculated into a 6-well plate (400/well). After incubation for 24 h, Pa in corresponding concentrations (final concentration of 0 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, and 4 μM) was added for incubation for 6 h; the original medium was replaced with the fresh medium containing 10% serum. After the radiation of laser with a wavelength of 664 nm and energy density of 2.0 J/cm$^2$ for 15 min, cells were incubated for another 12 d, followed by washing with phosphate buffered saline (PBS), fixation via 10% formaldehyde, Giemsa staining and photograph. The cell colony with more than 50 cells was one clone. The experiment was repeated for three times.

**Hoechst 33342 Staining**

Cells in the logarithmic growth phase were taken and inoculated into the 6-well plate. After adherence, Pa (final concentration of 0 μM, 0.5 μM, 1 μM, and 2 μM) was added and cells were exposed to laser following the steps above. After that, the culture solution was replaced for incubation for 12 h. The supernatant was discarded, and cells were washed twice with PBS, added with 1 mL Hoechst 33242 fluorescent dyes and incubated for 15 min at 37°C; the fluorescent dyes were discarded and cells were washed again with PBS, followed by observation under inverted fluorescence microscope and photograph. The experiment was repeated for three times.

**Apoptosis Detection**

After Pa-PDT treatment, cells were incubated with Annexin V-fluorescein isothiocyanate (FITC) and PI for 15 min in a dark place at room temperature, and detected using flow cytometer to obtain the cell scatter plot consisting of four quadrants. The results of flow cytometry were analyzed using the software, and the experiment was repeated for three times.

**Cell Migration Test**

600 μL fresh culture solution containing 10% FBS was added into a 24-well plate and the Transwell chamber was placed into the corresponding 24-well plate. 200 μL cell suspension ($1 \times 10^5$ cells) was added into the Transwell chamber, and the chamber was transferred into an incubator. After incubation for 12 h, the culture solution with different concentrations of Pa was added for incubation for 6 h, after which it was replaced with fresh culture solution. After laser irradiation for
15 min, cells continued to be incubated in the incubator for 24 h. Then, the chamber was taken out, and the cells which were not passing through the inner face of chamber were gently wiped with cotton swabs, fixed with 95% alcohol for 15 min, stained using hematoxylin for 15 min, rinsed with fresh water, observed, and photographed. The experiment was repeated for three times.

**Cell Invasion Assay**

Matrigel was diluted at 1:20 using DMEM, and 100 μL Matrigel was taken to cover the bottom of Transwell chamber and air-dried sterilely for standby application. The remaining operations were the same as those in cell migration assay. The experiment was repeated for three times.

**Western Blotting**

PC-3 cells were washed with pre-cooled PBS and fully cleaved using radioimmunoprecipitation assay (RIPA) cell lysis buffer. After centrifugation, the supernatant was taken and quantified. Next, 30 g total protein was taken and mixed with 5 × sodium dodecyl sulfate (SDS) protein loading buffer, followed by denaturation at 100°C for 5 min, loading in SDS polyacrylamide gel electrophoresis (SDS-PAGE); after that, the gel and activated polyvinylidene difluoride (PVDF) membrane were placed in the membrane transfer frame for membrane transfer under constant current for 2 h. The PVDF membrane was removed and sealed in 5% skim milk powder for 1 h; the corresponding primary antibody was added for incubation at 4°C overnight. The membrane was washed with Tris-buffered saline and Tween 20 (TBST) and added with the corresponding horseradish peroxidase (HRP)-labeled secondary antibody for incubation at room temperature for 1 h, followed by washing with TBST. The development and analysis of gray value were conducted using ImageJ software (MD, USA). The relative expression level of target protein was presented as target protein/actin. The experiment was repeated for three times.

**Statistical Analysis**

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparisons between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). Percentage (%) was used to express the enumeration data and chi-square test was used for data analysis. p values < 0.05 were considered statistically significant.

## Results

**Pa-PDT Inhibited the PC-3 Cell Proliferation in a Dose-Dependent Manner**

To investigate the inhibitory effect of Pa-PDT on PC-3 cells, PC-3 cells were first incubated with different concentrations of Pa (0 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, and 4 μM) in a dark place for 6 h. PC-3 cells received the radiation of laser with a wavelength of 664 nm and energy density of 2.0 J/cm² for 15 min and they were incubated for 24 h, followed by MTT detection. The results (Figure 1A) showed that Pa-PDT had a significant inhibitory effect on the proliferation of PC-3 cells, which was significant when the concentrations of Pa were 2 μM and 4 μM. The experiment suggested that Pa-PDT has a certain dose-dependent characteristic.

**Pa-PDT Promoted the PC-3 Cell Apoptosis**

Studies have shown that Pa-PDT can promote the apoptosis of a variety of tumor cells, thus inhibiting tumors. In order to further explore the mechanism of Pa-PDT in inhibiting the PC-3 cell proliferation, the effect of Pa-PDT on apoptosis was demonstrated. First, the apoptotic rates of PC-3 cells after treatment with different doses of Pa-PDT were detected via flow cytometry. Pa-PDT could significantly promote the PC-3 cell apoptosis (Figure 2A). With the gradual increase in the dose of Pa, the apoptotic rate was also increased significantly. At the same time, Hoechst 33342 staining also showed the consistent results: Pa-PDT promoted the PC-3 cell apoptosis in a dose-dependent manner (Figure 2E). Next, in order to further study the pro-apoptotic mechanism of Pa-PDT, Western blotting was used to detect the changes in expressions of apoptosis-related proteins after treatment with Pa-PDT. The expressions of BAX, Bcl-2, Caspase-3 and PARP in PC-3 cells were detected after photodynamic therapy mediated by different concentrations of Pa (0 μM, 0.5 μM, 1 μM, and 2 μM).

In the experiment, BAX and Bcl-2 in the Bcl-2 protein family were firstly detected. Bcl-2 belongs to the anti-apoptotic protein, while BAX belongs to the pro-apoptotic protein, and both of them regulate each other. The results of this investigation (Figure 2C) showed that Pa-PDT could significantly up-regulate the expression of BAX and down-regulate the expression of Bcl-2 protein in a dose-dependent manner. The higher
the Pa concentration was, the more significant its effect was. The downstream molecules of the mitochondrial apoptotic pathway, Caspase-3 and PARP, were further detected. The results consistently showed that Pa-PDT could significantly up-regulate the expressions of cleaved Caspase-3 and cleaved PARP in a dose-dependent manner. In conclusion, Pa-PDT can promote the apoptosis of PC-3 cells, and its mechanism is related to the fact that Pa-PDT promotes the mitochondrial apoptosis pathway.

**Pa-PDT Inhibited the Migration and Invasion Capacities of PC-3 Cells**

As everyone knows, the tumor recurrence and metastasis are often the main reasons for the failed treatment of tumor patients. Many studies have shown that PDT has an effect of inhibiting tumor metastasis\(^\text{14,15}\). In order to avoid the effect of significant proliferation inhibition on experimental results, Pa-PDT in sub-lethal doses (0.5 μM and 1 μM) was selected for wound healing assay. After treatment with Pa-PDT, the wound healing degrees were observed after 24 h and 48 h. The results (Figure 3A) showed that Pa-PDT could significantly inhibit the migration of PC-3 cells. After 48 h, the wound was almost completely healed in control group, while the healing capacity in Pa-PDT treatment group was significantly inhibited; the inhibitory effect in 1 μM group was more obvious than that in 0.5 μM group. Similar to the wound healing assay, Pa-PDT also had a significantly inhibitory effect in cell migration and invasion assays. The results (Figure 3C) showed that the inhibitory effect in 1 μM group was more significant than that in 0.5 μM group. The above results indicated that Pa-PDT can inhibit the migration and invasion capacities of PC-3 cells in dose- and time-dependent manners.

**Pa-PDT Inhibited the EMT Process and MMP-2 and MMP-9 Expressions.**

Invasion and metastasis are the characteristics of tumors, and its mechanism is closely related to EMT. E-cadherin, as a key molecule of EMT, is used to maintain the stability of normal intercellular connections, and its expression level is negatively correlated with the occurrence of EMT and invasion capacity of tumors\(^\text{16}\). EMT process is often accompanied by low expression of E-cadherin and high expressions of N-cadherin and vimentin. In this w, Western blotting was used to detect the changes in expressions of EMT-related proteins in PC-3 cells after Pa-PDT. The results (Figure 4) showed that the expression of E-cadherin was significantly up-regulated, while the expressions of N-cadherin and vimentin were significantly down-regulated after treatment with Pa-PDT. It is well known that the extracellular hydrolases are necessary for tumor cells to pass through the
basement membrane. The extracellular matrix degradation is needed when tumor cells invade the peripheral tissues, pass through the vessel wall and form the distant metastasis. Besides, the extracellular matrix degradation requires the involvement of hydrolases, among which MMPs are the most important enzymes and are thought to play core roles in the development of tumors. In this study, the effects of Pa-PDT on expressions of MMP-2 and MMP-9 were investigated. The results (Figure 4) revealed that Pa-PDT could also down-regulate the expressions of MMP-2 and MMP-9 in PC-3 cells, suggesting that Pa-PDT can inhibit the invasion and metastasis of PC-3 cells from different pathways.

Discussion

In recent years, the incidence rate of PCa has shown an upward trend; many PCa patients have already been in the middle and advanced stage when diagnosed; although the traditional radiotherapy, chemotherapy and other adjuvant therapies have a certain effect on prolonging the survival time, they have poor selectivity to tumors, many adverse reactions and unsatisfactory efficacy. PDT, with its good tumor specificity and less adverse reactions, is re-recognized, which is a therapeutic method emerged quickly in the past 2 decades for multi-system and various malignant tumors. It has been proven to be effective against
a variety of malignant tumors. It is recognized that the mechanism of PDT in killing tumor cells is the singlet oxygen. The photosensitizer absorbs the energy of photons and transmits the energy to the oxygen at the excitation of specific wavelength of light, produces the singlet oxygen with electrophilicity that can efficiently oxidize the biomacromolecule, causing the inactivation of lipid peroxidase and protein denaturation, cross-linking and structural changes, thus damaging the structure and function of cells. Due to the limitations of light permeability of early PDT, it is mainly used for the body surface and intraluminal tumors, but there is less research on the treatment of PCa. At present, the commonly-used photosensitizer, haematoporphyrin, and its derivatives are mostly the complex porphyrin mixture with uncertain chemical composition, so its mechanism research and quality control are difficult. In this research, a new type of photosensitizer, Pa, was used, which is a derivative of chlorophyll a. Related studies have shown that Pa-PDT can promote

![Image of Figure 3](image-url)

**Figure 3.** Effects of Pa-PDT treatment on the invasion and migration ability of PC3 prostate cancer cells. (A, B) Cell migration ability was assessed by the wound-healing assay. The linear scratch was made with a 10-μl sterile pipette tip, followed by the Pa-PDT (0 μM 0.5 μM, 1 μM, respectively). After 24 h and 48 h, scratch wound healing was observed and analyzed. (C, D) Migration and invasion of PC3 cells were detected by the transwell assay 24 h after Pa-PDT (0 μM 0.5 μM, 1 μM, respectively). Data shown are representative of 3 independent experiments. Data are presented as means ± SD (n = 3). *p < 0.05, **p < 0.01 vs. control group.
the reactive oxygen species (ROS) production in PCa LNCaP cells, thereby killing LNCaP cells. However, the specific way to kill cells remains unclear. In view of the limitations in the treatment of PCa and the advantages of PDT, we further investigated the inhibitory effects of Pa-PDT on proliferation, invasion and metastasis of PC-3 cells and its potential mechanism. We found that Pa-PDT could inhibit the proliferation of PC-3 cells in a dose-dependently manner, and its mechanism was related to the fact that Pa-PDT activated the mitochondrial apoptosis pathway, causing apoptosis. The results of Western blotting showed that Pa-PDT could up-regulate the expressions of pro-apoptotic proteins (BAX, Caspase-3 and PARP), and down-regulate the expression of anti-apoptotic protein (Bcl-2). Yoon et al.10 studied and found that Pa-PDT can kill the human skin tumor cells through promoting the autophagy. More studies have found that Pa-PDT can promote the apoptosis of a variety of tumor cells, and its mechanism involves different apoptotic signaling pathways10,12,17.

Tumor invasion and distant metastasis are the leading causes of treatment failure and death in tumor patients. Tumor invasion and metastasis are a multi-factor and multi-step dynamic process that involve cancer cells shedding from the primary lesion, invading adjacent tissues and organs, entering the blood vessels or lymphatic vessels, reaching the distant end, proliferating to be new metastases. EMT is one of the key steps of epithelium-derived malignant tumor invasion and metastasis, as well as a process in which epithelial cells with polarities are converted into cells that are active and capable of free movement between cell matrices, which involves the changes in the expressions of a variety of key molecules. Cavalaro et al.18 proposed the cadherin-switch theory that the interstitial cell phenotype transformation is accompanied by the loss of E-cadherin and expression of N-cadherin, the former of which is one of the most important molecules affecting the tumor invasion and metastasis. Numerous studies have shown that maintaining stable cell connections requires E-cadherin. The down-regulation of E-cadherin expression is often found in interstitial cell phenotype transformation and carcinogenesis of epithelial cell, which can initiate EMT and lead to tumor invasion and metastasis. Of note, it was found in transwell assay that the sub-lethal dose of Pa-PDT could significantly inhibit the invasion and metastasis capacities of PC-3 cells. In order to explore its underlying cause, the changes in the expressions of E-cadherin and N-cadherin after the treatment with different doses of Pa-PDT were detected. The results showed that after action of Pa-PDT, the expression of E-cadherin was significantly increased and the expression of N-cadherin was down-regulated. Vimentin is a major mem-

Figure 4. Effects of Pa-PDT treatment on the proteins expression related to migration and invasion of PC3 prostate cancer cells. (A) The expression levels of target proteins (E-cadherin, N-cadherin, Vimentin, MMP-2 and MMP-9) were measured by Western blot 24 h after Pa-PDT (0 μM 0.25 μM 0.5 μM, 1 μM, respectively). (B) Data shown are representative of 3 independent experiments. Data are presented as means ± SD (n = 3). *p < 0.05, **p < 0.01 vs. control group.
Pa-PDT in the treatment of PCa.

Conclusions

We showed that Pa-PDT can promote the apoptosis and inhibit the invasion and metastasis capacities of PC-3 cells. The mechanism of Pa-PDT in inhibiting the invasion and metastasis is related to the fact that Pa-PDT inhibits the EMT process and the expressions of MMPs in tumor cells. However, the detailed mechanism of intermolecular interactions of the above effects of Pa-PDT still needs further research. In conclusion, we provide a theoretical basis for the application of Pa-PDT in the treatment of PCa.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

Pheophorbide a-mediated photodynamic therapy in prostate cancer


