

Original Article

Study on application of photodynamic therapy for the treatment of cervical cancer

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Abstract: Objective: To evaluate the inhibition function of Photodynamic Therapy mediated by Tetrakis(4-methylpyridinium)porphyrin (TMPyP4-PDT) in cervical cancer cell lines (Hela and Caski) in vitro and its effects on the expression levels of c-myc and hTERT, analyzing the advantages of TMPyP4-PDT for the treatment of cervical cancer. Methods: The cervical cancer cell lines (Hela and Caski) in vitro irradiated by photosensitizer (TMPyP4) at various concentrations and laser energy densities were classified into the normal control group, the PDT group, the TMPyP4 group and the TMPyP4-PDT group in terms of treating methods. The inhibition effects of PDT on the proliferation of the cervical cancer cell lines were detected by MTT colorimetry. Meanwhile, the apoptosis rates of the cell lines were detected by flow cytometry and the expression of c-myc and hTERT in the cell lines by the Western blot method. Results: With the increase in TMPyP4 concentration and laser energy density, the inhibition rate and apoptosis rate of cervical cancer cell lines (Hela and Caski) also increased. At the same laser energy density, some statistically significant differences ($P < 0.05$) were found in cell inhibition rate and apoptosis rate between Hela cell lines and Caski ones at different TMPyP4 concentrations; at the same concentration of TMPyP4, some statistically significant differences ($P < 0.05$) were also found in cell inhibition rate and apoptosis rate between Hela cell line and Caski cell line mediated by TMPyP4 at different laser energy densities. With the same concentration of TMPyP4 and the same laser energy density, the inhibition rate of Caski cells was higher than that of Hela cells, without statistically significant difference ($P < 0.05$); while the apoptosis rate of Caski cells was higher than that of Hela cells with statistically significant difference ($P < 0.05$). The expression levels of c-myc and hTERT in the cervical cancer cell lines Hela and Caski detected by the Western blot method decreased. Conclusion: TMPyP4-PDT has inhibitory effects on cervical cancer cell lines (Hela and Caski) in vitro and induces their apoptosis dependent on concentration of photosensitizers. The possible mechanism of TMPyP4 maybe related with degrading the expression levels of hTERT and c-myc mRNA.

Keywords: Cervical cancer, photodynamic therapy, TMPyP4

Introduction

Cervical cancer is the most common malignant tumor in women, second in incidence rate and mortality rate. The symptoms of early cervical cancer are not obvious. A majority of patients have been in the intermediate or advanced stage of cancer at treatment. Within five-year survival rate, most patients may have tumor recurrence after the treatment [1, 2]. Since the mid 1970s, photodynamic therapy (PDT) has become an effective scheme for the clinical treatment of tumor after it was applied in treating genital herpes (GH). The principle of PDT is

to transform photochemical reactions into molecular internal energy, destroy the structure of tumor cells, and promote the apoptosis of tumor cells, so as to inhibit the proliferation and growth of tumor cells [3, 4]. With the advantages of less trauma, less toxicity, repeated treatment and protecting organs and fertility function of patients, photodynamic therapy becomes an alternative treatment for some young non-fertility cervical cancer patients who have a strong desire for fertility and for some older patients with advanced cervical cancer who have difficulty in tolerating adverse reactions of chemotherapy and radiotherapy.

C-myc gene is a proto-oncogene of nuclear transcription factors and its products may promote the proliferation and progression of malignant tumor cells, which plays an important role in the occurrence and development of tumor. RNA gene (hTERC) of human chromosome telomerase is a vital component of telomerase, whose products can prevent the apoptosis of cells, even leading to the occurrence of tumor. Some observations showed that hTERC and c-myc genes are overexpression in cervical cancer [5, 6]. PDT is a kind of cold photochemical reaction, the effects of which are associated with specific photosensitizer types, cell types and irradiation conditions. Photosensitizers play a crucial role in PDT. In this study we used four methyl pyridine porphyrin photosensitizer (5,10,15,0-tetra-(N-methyl-4-pyridyl), TMPyP4), a new type of water-soluble photosensitizer. There are few reports on the studies of the effects of TMPyP4-PDT on cervical cancer cells [7]. The main purpose of this study is to observe the cytotoxic effects of TMPyP4 with different laser energy density and different concentrations on cervical cancer cell lines (Hela and Caski) *in vitro*, and the effects of TMPyP4-PDT on the expression levels of c-myc and hTERC. The findings of this study will be the basis of experimenting TMPyP4-PDT in the treatment of cervical cancer.

Materials and methods

Materials

Cervical cancer cell lines (Hela and Caski) (provided by the laboratory), MTT (purchased from Sigma, USA), TMPyP4 (purchased from Merck, Germany), Normal CO₂ incubator (purchased from Heraeus, USA), NucleoCounter and centrifuge (purchased from Beckman, USA), Laser therapeutic apparatus (wavelength 630 nm, power 800 W) was provided by this laboratory. Flow cytometry (purchased from BD, USA), automatic fluorescence inverted microscope (purchased from Olympus, Japan).

Methods

Cell culture and grouping: Cervical cancer cell lines (Hela and Caski) were incubated in DMEM culture medium containing 10% fetal bovine serum in culture incubators (5% CO₂ at 37°C) and subculture every 3-4 days respectively. After cell fusion reached 80%, the cells were

trypsinized into single cell suspension which was then inoculated in cell culture plates, and incubated in the CO₂ incubator in TMPyP4 at different concentrations (0 mmol/L, 0.1 mmol/L, 0.5 mmol/L, 1.0 mmol/L, 2.5 mmol/L) for 24 h. Three repeated holes were set. The cells were incubated for 4 h without exposure to light. After that, the cells were taken out of the medium containing TMPyP4 and vertically irradiated by a laser therapeutic apparatus with a wavelength of 630 nm and powered at 800 W (laser energy density, 0, 2, 4, 8 J/cm²). The cells in the TMPyP4 group were only incubated in TMPyP4 at different concentrations, the cells in the PDT group were only irradiated by laser at different energy densities without photosensitizers, a normal control group without photosensitizer nor laser.

MTT colorimetry to detect cell proliferation: The Hela and Caski cells were inoculated in 96-well plates (5*10³/hole), then incubated in the 5% CO₂ culture incubator for 2 days at 37°C, adding 20 µl MTT solution at the concentration of 5 g/L, continuing to culture for another 4 hours. Next, we removed the solution, added DMSO, and mixed them up. 15 minutes later, we observed the dissolution of crystalline substance and detected absorbance (OD) of culture holes in every group by the full-automatic microplate reader.

Flow cytometry to measure apoptosis: The cells in each group were collected by the trypsinization method, and then washed twice in PBS at the concentration of 1*10⁶/ml. The cells were incubated for 15 minutes without exposure to light in the cell suspension with 5 µL Annexin V-FITC and PI staining solution. The apoptosis rate of cells was detected by flow cytometry according to the instructions.

The expression levels of c-myc and hTERC protein were detected by the Western blot method

We collected the cells from each group, from which some protein were extracted and quantified, and then added samples based on the results of quantification. We performed SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then we transferred the protein to the PVDF membranes closed by 5% skimmed milk powder. After some mouse anti-human c-myc antibody solution or mouse anti-human hTERC antibody solution were added, the protein was incubated at 4°C for overnight, and then incuba-

Table 1. Inhabitation rate of HeLa cells and Caski cells at different photosensitizer concentrations and different laser energy densities in TMPyP4-PDT group

Photosensitizer concentration (mmol/L)	Laser energy density of HeLa cells (J/cm ²)			Laser energy density of Caski cells (J/cm ²)		
	2	4	8	2	4	8
0.1	21.58%	31.67%	63.65%	27.54%	34.67%	68.54%
0.5	25.47%	37.57%	71.464%	28.68%	39.69%	75.51%
1.0	27.46%	43.58%	75.44%	31.46%	47.53%	76.87%
2.5	34.46%	55.45%	90.48%	37.66%	57.67%	92.64%

ted in Goat anti-mouse secondary antibody at the room temperature for 1 h. The protein was scanned and photographed after being incubated in enhanced luminescence reagent (ELC) for 1-2 min, with β -actin as the internal reference.

Statistical analysis

The data was statically analyzed by the SPSS 17.0 statistical software, among which, the measurement data were shown by mean \pm standard and the count data were compared by the χ^2 test. The mean between two groups were compared by the T-test while the mean among multiple groups by the one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Inhibition rate of HeLa and Caski cells at different photosensitizer concentrations and different laser energy densities

In the experimental group, the inhibition rate of cervical cancer cell lines (HeLa and Caski) increased with the rise in the photosensitizer concentration and in the laser energy density, showing a clear laser-energy-dose-dependent effect. On the condition of 2.5 mmol/L photosensitizer concentration and 8 J/cm² laser energy density, the inhibition rate of HeLa and Caski cells showed 90.48% and 92.64% respectively. There were no significant effects on the proliferation of HeLa and Caski cells in the PDT group, the TMPyP4 group and in the normal control group. At the same laser energy density, some statistically significant differences ($P < 0.05$) were found in the inhibition rate of the cells at different TMPyP4 concentrations; at the same concentration of TMPyP4, some statistically significant differences ($P < 0.05$) were

also found in the inhibition rate of the cells at different energy densities. At the same concentration of TMPyP4 and the same laser energy density, the inhibition rate of Caski cells was higher than that of HeLa cells without statistically significant difference ($P < 0.05$), indicating there were similar sensitivity to TMPyP4-PDT between

HeLa cells and Caski ones, as shown in **Table 1** and **Figure 1**.

Apoptosis rate of TMPyP4-PDT HeLa cells and Caski cells at different photosensitizer concentrations and different laser energy densities

In the experimental group, the apoptosis rates of HeLa and Caski cells increased with the rise in the concentration of photosensitizer and laser energy density, showing a significant laser-energy-dose-dependent effect. On the condition of 2.5 mmol/L photosensitizer concentration and 8 J/cm² laser energy density, the apoptosis rate of HeLa and Caski cells showed 44.58% and 55.44% respectively. There were no significant effects in the apoptosis of HeLa cells and Caski cells among the PDT group, the TMPyP4 group and the normal control group. At the same laser energy density, statistically significant differences ($P < 0.05$) were found in the apoptosis rate of the cells at different TMPyP4 concentrations; and at the same concentration of TMPyP4, some statistically significant differences ($P < 0.05$) were also found in the apoptosis rate of the cells at different energy densities. At the same concentration of TMPyP4 and the same laser energy density, the apoptosis rate of Caski cells was higher than that of HeLa cells with statistically significant difference ($P < 0.05$), indicating that Caski cells were more sensitive to TMPyP4-PDT than HeLa cells, as shown in **Table 2** and **Figure 2**.

Expression level of c-myc protein and hTERT protein detected by the Western blot

After TMPyP4-PDT treated HeLa and Caski cell lines, the expression level of c-myc protein decreased. There were statistically significant differences ($P < 0.05$) in the ratios of the expression of internal reference β -actin

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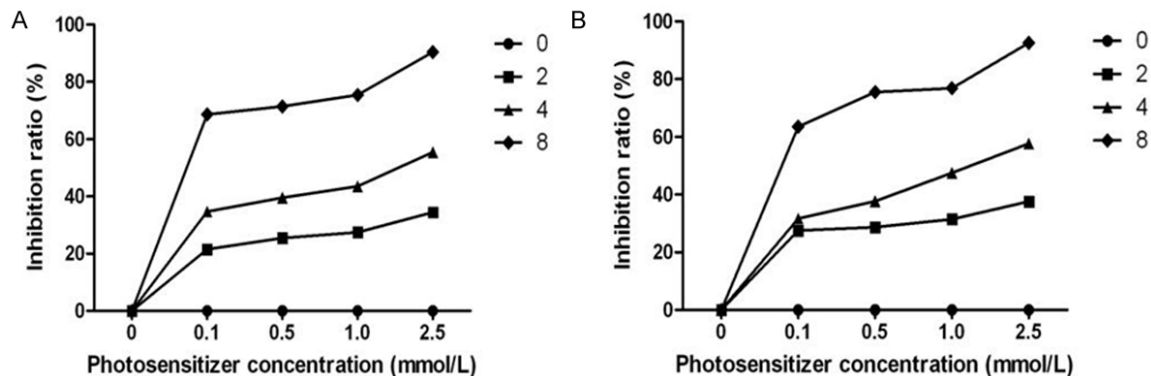


Figure 1. Inhibitory effects of TMPyP4-PDT on cervical cancer cells in TMPyP4-PDT group. A: HeLa cells; B: Caski cells.

Table 2. Apoptosis rate of HeLa cells and Caski cells at different photosensitizer concentrations and different laser energy densities in TMPyP4-PDT group

Photosensitizer concentration (mmol/L)	Laser energy density of HeLa cells (J/cm ²)			Laser energy density of Caski cells (J/cm ²)		
	2	4	8	2	4	8
0.1	6.58%	11.67%	16.62%	17.89%	20.05%	23.62%
0.5	7.47%	13.84%	19.77%	18.79%	27.96%	35.61%
1.0	9.46%	19.54%	25.72%	21.89%	37.79%	46.67%
2.5	14.47%	25.43%	44.58%	29.76%	47.86%	55.44%

between the control group and the TMPyP4-PDT group (9.254 ± 0.312 and 2.674 ± 0.275 respectively) after the HeLa cells were treated by the TMPyP4-PDT at the photosensitizer concentration of 2.5 mmol/L and laser energy density of 8 J/cm² for 48 hs; and there were also statistically significant differences ($P < 0.05$) in the ratios of the expression of internal reference β -actin between the control group and the TMPyP4-PDT group (8.914 ± 0.379 and 2.176 ± 0.453 respectively) after the Caski cells were treated by the TMPyP4-PDT at the photosensitizer concentration of 2.5 mmol/L and laser energy density of 8 J/cm² for 48 hs, as shown in **Figure 3**.

After HeLa and Caski cell lines were affected by TMPyP4-PDT, the expression level of hTERT protein decreased. Some statistically significant differences ($P < 0.05$) were found in the ratios of the expression of internal reference β -actin between the control group and the TMPyP4-PDT group (8.624 ± 0.262 and 2.165 ± 0.226 respectively) after the Caski cells were treated by the TMPyP4-PDT at the photosensitizer concentration of 2.5 mmol/L and laser energy density of 8 J/cm² for 48 hs; and there

were also statistically significant differences ($P < 0.05$) in the ratios of the expression of internal reference β -actin between the control group and the TMPyP4-PDT group (8.578 ± 0.312 and 1.824 ± 0.381 respectively) after the Caski cells were treated by the TMPyP4-PDT at the photosensitizer concentration of 2.5 mmol/L and laser

energy density of 8 J/cm² for 48 hs, as shown in **Figure 4**.

Discussion

Photodynamic therapy (PDT), a new medical technology developed in recent years, has become an effective technology besides surgery, radiotherapy and chemotherapy for the treatment of malignant tumors. It is reported that photodynamic therapy has killing effects on skin cancer, carcinoma of prostate, breast carcinoma, etc. [8]; however, there are no large-scale studies about the application of PDT in the treatment of cervical cancer. Some studies have shown that photosensitizer is a crucial component in PDT. It was accumulation and photochemical reaction of photosensitizers in malignant tumor tissues that killed the lesion tissues. PDT has little cytotoxicity and adverse reaction to the human normal tissues [9, 10].

As a newly-developed photosensitizer, TMPyP4 is an artificial tetravalent cationic porphyrin compound which can induce and stabilize the formation of G-quadruplex. It is reported that TMPyP4 has an attribute of combining photo-

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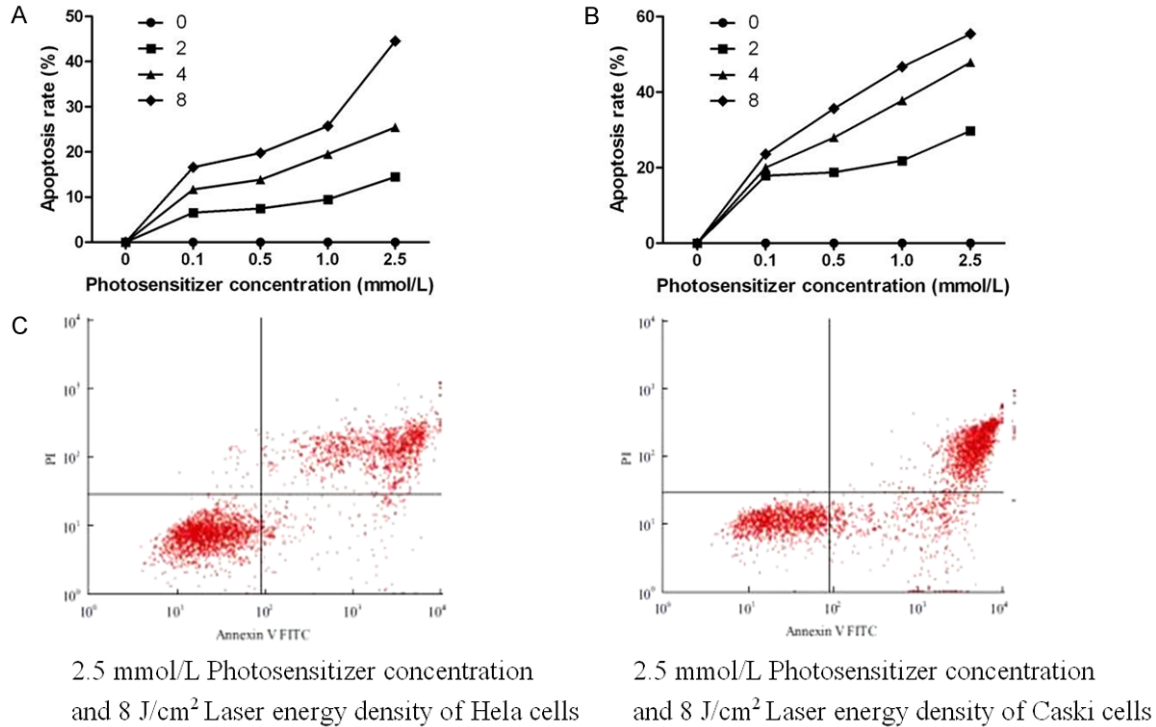


Figure 2. Apoptosis effects of TMPyP4-PDT on cervical cancer cells in TMPyP4-PDT group. A: Apoptosis rate of HeLa cells; B: Apoptosis rate of Caski cells; C: Flow chart of cervical cancer cells

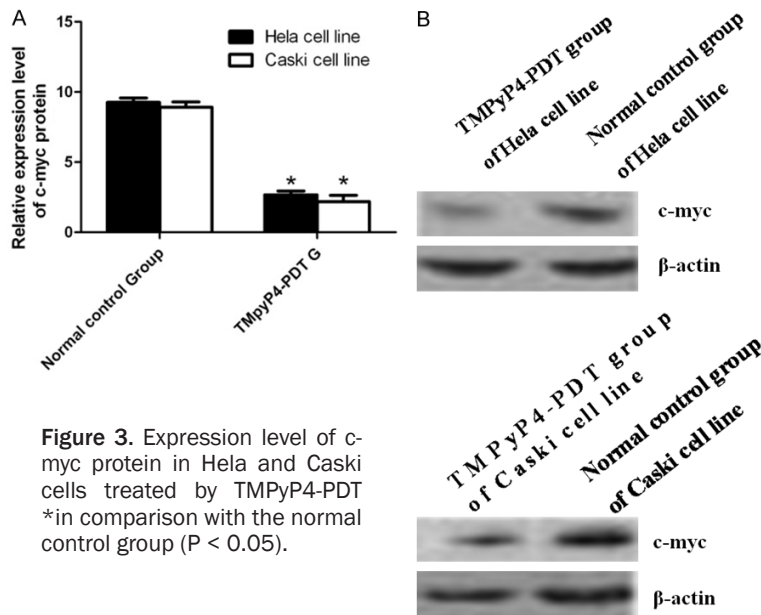


Figure 3. Expression level of c-myc protein in HeLa and Caski cells treated by TMPyP4-PDT *in comparison with the normal control group ($P < 0.05$).

while 5-ALA does not have these benefits. Obviously, compared with 5-ALA, the killing effect of TMPyP4 on tumor cells is more target-oriented.

In this study, the cervical cancer cell lines (HeLa and Caski) were taken as the research subjects, with the application of PDT. The results show that TMPyP4-PDT has an effective antitumor effect, especially in inhibiting the production of tumor cells and inducing the apoptosis of cells. And the effects of photodynamic therapy were determined by the concentration and laser energy density of photosensitizers, which is consistent with

sensitizer with G-quadruplex ligand, destroying tumor cells in various ways [11, 12]. The comparison of the effects between TMPyP4 and 5-ALA (the second generation photosensitizer) on leukemia shows that [13, 14] TMPyP4 is located in the nucleus and can damage DNA,

previous studies [15, 16]. When we chose the photosensitizer at 2.5 mmol/L concentration and 8 J/cm² laser energy density, the inhibition rates of HeLa cells and Caski cells were 90.48% and 92.64% respectively and the apoptosis rates were 44.58% and 55.44% respectively. At

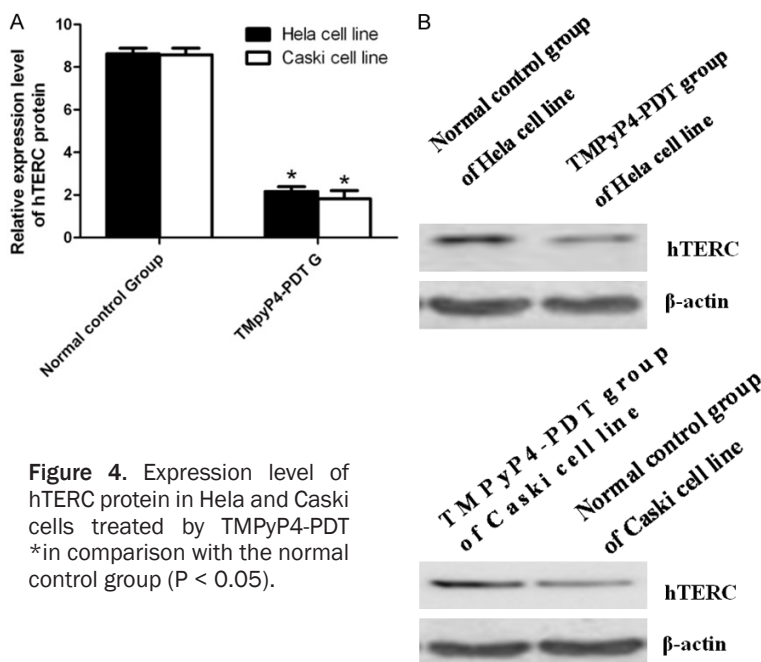


Figure 4. Expression level of hTERC protein in HeLa and Caski cells treated by TMPyP4-PDT *in comparison with the normal control group (P < 0.05).

the same laser energy density, some statistically significant differences (P < 0.05) were found in inhibition rate and apoptosis rate between HeLa cell lines and Caski ones at different TMPyP4 concentrations. At the same concentration of TMPyP4, some statistically significant differences (P < 0.05) were also found in the inhibition rate and apoptosis rate between HeLa cells and Caski ones at different laser energy densities. At the same concentration of TMPyP4 and the same laser energy density, the inhibition rate of Caski cells was higher than that of HeLaones, indicating that Caski cells are more sensitive to TMPyP4-PDT than HeLa cells.

The hTERC gene, whose gene mutation may contribute to changes in the function of telomerase, is an important component of telomerase. The amplification of hTERC genes can prevent apoptosis of cells, even contributing to the occurrence of tumors. The C-myc gene is a member of the myc oncogene family. The amplification and over expression of c-myc genes play a vital role in regulating malignant transformation, proliferation and differentiation of cells. A large number of studies confirmed that amplification and overexpression of hTERC and c-myc appeared in cervical cancer and its precancerous lesions [17, 18]. It is reported that amplification of hTERC and c-myc genes increase with levels of cervical intraepithelial

neoplasia, and the difference was statistically significant [19]. And other studies showed that there is a binding site of c-myc in the E-box, the core promoter 5-flanking region of Telomerase Reverse Transcriptase (hTERT), where the c-myc genes can be bound to the DNA sequence of hTERT genes, regulating hTERT transcription and activating telomerase so as to influence its stability and promote immortalization of cells [20, 21]. Thus, this study detected the changes of the relative expression of hTERC and c-myc after TMPyP4-PDT affected the cervical cancer cell lines (HeLa and Caski). The results showed that, compared with

that in the control group, hTERC and c-myc considerably decreased, and the difference was statistically significant. Thus, TMPyP4-PDT may inhibit activity of telomerase by inhibiting the expression levels of c-myc and hTERC, thereby promoting the apoptosis of cervical cancer cell lines and inhibiting their growth.

In summary, TMPyP4-PDT considerably inhibited proliferation of Caski cells and HeLa cells and induced their apoptosis, but both of them had different reactions to photosensitizers. In the PDT individualized treatment, quantification was made in every stage of treatment according to different targeted cells. Currently, the PDT of cervical cancer remains to be further studied and it is necessary to establish an excellent system regulating clinical work, exploring the best conditions for PDT and determining the range of each parameter. Furthermore, we have many problems and difficulties in applying PDT in the treatment of cervical cancer. However, we are sure that, with the development and progression of medical research and continuous improvement of medical technologies, PDT as a promising treatment will be widely used and play a greater role in the treatment of cervical lesions.

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Disclosure of conflict of interest

None.

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