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Effects of silencing PTTG expression by small interference RNA

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Abstract. – OBJECTIVE: We investigated the effects of small interference RNA (siRNA) on the cell proliferation inhibition, sensitivity to radiotherapy effects and cell apoptosis. The siRNA used here was specific to the pituitary tumor transforming gene (PTTG).

MATERIALS AND METHODS: Vectors containing the specific functional siRNAs for PTTG were designed and constructed. Cells were divided into four groups: (I) blank control group; (II) radiotherapy group: cells were exposed to X-ray radiation; (III) Group PTTG siRNA: transfected with PTTG siRNA; (IV) PTTG siRNA+ radiotherapy group: transfected with PTTG siRNA and then were exposed to X-ray radiation. HEC-1A cells were transfected by the specific interfering plasmids using Lipofectamine 2000 transfection reagent. The PTTG protein expression levels were analyzed using Western blot Cell proliferation was examined by MTT assay and the HEC-1A cell line apoptosis was evaluated by flow cytometry.

RESULTS: Recombinant small interference RNA (siRNA) expression vectors targeting PTTG were successfully constructed. The results of MTT showed that the growth of the HEC-1A cell was negatively influenced after cells were transfected with PTTG siRNA. Furthermore, PTTG siRNA combined with radiotherapy demonstrated more powerful inhibitory effects. Cell apoptosis rates were significantly increased in the radiotherapy group and the PTTG siRNA transfection group when compared to the control group. A more pronounced cell apoptosis rate was observed in the group that was treated with PTTG siRNA combined with radiotherapy.

CONCLUSIONS: Recombinant small interference RNA (siRNA) expression vector targeting PTTG successfully inhibited the cell proliferation and induced apoptosis in endometrial carcinoma cells and increased the cancer cells vulnerability to the effects of radiation.

Key Words
siRNA, Endometrial carcinoma, Pituitary tumor transforming gene, Radio therapeutic sensitivity.

Introduction

In recent years, we have witnessed a significant surge in the number of endometrial carcinoma cases. Currently, the main treatment for this type of cancer is surgical resection. Radiotherapy is also an important tool in the adjuvant therapy. Despite all these treatments, the recurrence rate for endometrial carcinoma is still high. Researchers are working hard in order to come up with an effective method, which can produce a better outcome for the uterine endometrial carcinoma cases. In this study, we tried a treatment method using the siRNAs.

We constructed a PTTG specific siRNA vector to interfere with the expression of pituitary tumor transforming gene, which is known to play a key role in the proliferation of the cancer cells. This vector was employed either alone or along with radiotherapy on HEC-1A cells in endometrial carcinoma. Subsequently, we explored the effects of our specific siRNAs on the proliferation of endometrial cancer cells. MTT was used for the detection of cell apoptosis while flow cytometry was employed to measure the cell proliferation.

Materials and Methods

To conduct our study we used the following materials: endometrial carcinoma cell lines HEC-1A (Research Institute of Cell Biology, Shanghai, China), interference plasmid pSilencer3.0-Hl (Biological Engineering Technology Co. Ltd., Wuhan Jingsai, China), Trizol reagent, BamH I, Hind III, Sal I digestion, TaqDNA polymerase, T4 DNA ligase, Lipofectamine 2000 transfection reagent (Santa Cruz Co., Santa Cruz, CA, USA), mouse anti-human PTTG monoclonal antibody (Santa Cruz Co., Santa Cruz, CA, USA), MTT, DMSO, Annexin V-PI (Zhongshan Biological Engineering Company, Beijing, China), fresh calf serum, low mel-

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ting point glucose agar, cell lysates, DMEM cell culture medium, trypsin (Boster Biological Engineering Company, Wuhan, China).

The PTTG specific siRNA was designed according to the cDNA sequence available on the Gen Bank PTTG gene (NM_004129). We employed the TaKaRa Company's online design software (Otsu, Shiga, Japan) to complete our designing process. Subsequently, the designed sequence was synthesized by Shanghai MST Biological Engineering Company. For plasmid amplification, we transformed DH5a *E. coli* cells using calcium chloride method. After amplification, the pSilencer3.0-Hl plasmids were purified.

The designed sequence was a hairpin stem structure and the intermediate loop sequence was TTCAAGAGA. Here we present the sequences of three specific siRNAs along with the negative control sequence:

(I) PTTG siRNA-1

- positive-sense strand:
  5'GATCCACAAAGAACTAGACAGAAATTCAAGAGATTTCTGTCTAGTTCTTTGTTTTTTTGGAAA3'
- antisense strand:
  5'AGCTTTTCCAAAAAAACAAAGAACTAGACAGAAATCTCTTGAATTTCTGTCTAGTTCTTTGTG3'

PTTGsiRNA-2

- positive-sense strand:
  5'GATCCGCAAAGATCTGGAGGAGCATTCAAGAGATGCTCCTCCAGATCTTTGCTTTTTTGGAAA3'
- antisense strand:
  5'AGCTTTTCCAAAAAAGCAAAGATCTGGAGGAGCATCTCTTGAATGCTCCTCCAGATCTTTGCG3'

(III) PTTGsiRNA-3

- positive-sense strand:
  5'GATCCGATAATGAGTGGAGAAAGATTCAAGAGATCTTTCTCCACTCATTATCTTTTTTGGAAA3'
- antisense strand:
  5'AGCTTTTCCAAAAAAGATAATGAGTGGAGAAAGATCTCTTGAATCTTTCTCCACTCATTATCG3'

(IV) negative control NC

- positive-sense strand:
  5'GATCCGACTTCATAAGGCGCATGCTTCAAGAGAGCATGCATGCGCCTTATGCTTTTTTGGAAA3';
- antisense strand:
  5'AGCTTTTCCAAAAAAGCATAAGGCGCATGCATGCTCTCTTGAAGCATGCGCCTTATGAAGTCG3'.

Construction of siRNA Expression Vector

Generation of a double stranded cDNA to be inserted in pSilencer3.0-Hl vectors: Each of the above-mentioned fragments was mixed with annealing buffer and then transferred to a 94°C water bath. After 4 minutes they were removed from the bath and were placed at room temperature. The final concentration of annealed DNA oligonucleotide template chain was 100 mol/L. We then prepared our pSilencer 3.0-Hl vectors using BamH I and Hind III double digestion. The annealed fragments were then cloned with a 3:1 of mol ratio. After the antibiotic screening, the positive clones were selected and sequenced, and the recombinant plasmids obtained were labeled as: (i) PTTG siRNA-1, (ii) PTTG siRNA-2, (iii) PTTG siRNA-3, and (iv) negative control NC.

Plasmid T ransfection

The cells were grouped as follows: (I) blank control group (HEC-1A cells group without transfection), (II) PTTG siRNA-1 group, (III) PTTG siRNA-2 group, (IV) PTTG siRNA-3 group and (V) NC group (siRNA negative sequence). Lipofectamine 2000 was used as our transfection reagent for transient transfection. Twenty-four hours before the transfection, we initiated the endometrial carcinoma HEC-1A cell cultures (10^5 per hole) in culture plates and cells were transfected when the cell density reached 70%-90%. Two μg plasmid, 100 μL of 0.01 M phosphate buffer solution (PBS) and 8 μg Lipofectamine 2000 were added to each hole in cell culture plates, and plates were then placed at room temperature for 10-15 minutes. The supernatant was removed and Lipofectamine was added.

Determination of Transfection Efficiency

The endometrial carcinoma HEC-1A cells were spread on the six-well plates covered with a coverslip and the inoculation density was about 5×10^5 per hole. Media was changed after 24 hours. Using Lipo2000, cells were transfected with pSilencer 3.0-Hl vector and the corresponding plasmids containing fluorescent light gene green fluorescent protein (EGFP). After 6-hour, the media was changed to DMEM containing 10% fetal bovine serum (FBS). Subsequently, 48 hours after transfection, the cells were collected and examined by fluorescence microscopy to determine transfection efficiency.

Western Blot Detects the Expression Level of PTTG Protein

The blank control group and HEC-1A cells were collected at 24 h, 48 h, 72 h after the transfection of each group. Cells were washed three times with cold PBS, added into cell lysate for protein extraction, and protein concentration was determined using a protein quantification kit.

Western blot: The samples were separated by SDS-PAG, and transferred to a PVDF membrane. 5% skim milk-TBST (Tris-Buffered Saline and Tween) was added and mixed at room temperature for 1 h. The primary antibody was added and the membranes were stored at 4°C for overnight. Membranes were then washed three times by TBST (5 mins each time). The secondary antibody was added to the membrane, and after 1 h at 37°C, membranes were rinsed by TBST three times (5 min each time). The results were analyzed by the grayscale scanner, and the gray value was calculated as the ratio of the objective strip and horizontal strip β-actin, and the level of targeted gene protein expressed. The experiment was repeated three times.

The Detection of PTTG siRNA's Effect on HEC-1A Cell Line Proliferation Activity by MTT (methyl thiazol tetrazolium) method

Cell culture and groups

We selected the most potent interfering plasmid and using this plasmid we transfected endometrial cancer HEC-1A cells. Transfection was performed using Lipofectamine 2000 on HEC-1A cells in DMEM cell media containing 10% calf serum (penicillin and streptomycin each 100 u/ml), 5% CO_2 incubator, while cells were in their logarithmic growth phase cells. Cells were divided into four groups: (I) blank control group (without any treatment); (II) radiotherapy group: cells were exposed to 6MV X-ray radiation (dose: 2Gy, dose rate: 100 Mu/min, irradiation area: 10 cm × 10 cm, isocenter irradiation); (III) Group PTTG siRNA: transfected by 0.2 μg PTTG siRNA; (IV) PTTG siRNA+ radiotherapy group: transfected by 0.2 μg PTTG siRNA and then were exposed to 6MV X-ray radiation (dose: 2Gy, dose rate: 100 Mu/min, irradiation area: 10 cm × 10 cm, isocenter irradiation). We used six replicas per group.

HEC-1A detected cell proliferation activity by MTT method

Ninty-six holes culture plates were used to determine the cell proliferation. We started with 1×10^5 cells per hole. The cells were cultured at 37°C and cells OD values were measured after 48h. Subsequently, 100 μl medium with 10 μl (5 mg/ml) MTT solution was added to each hole and incubated at 37°C for 4 h. Then, the cell cultures were terminated and the supernatants were discarded. Each hole was then filled with 150 μl (dimethyl sulfoxide) DMSO, oscillated for 10 min, in order to achieve complete crystal melt. The OD absorbance was measured and calculated. The inhibitory rate of cell growth (%) = (control group OD value-experimental group OD value/control group OD value) × 100%.

HEC-1A cell apoptosis was detected by flow cytometry

Cells grouping and cultures were the same as described in 1.7.1 section. Annexin V and PI double staining were used to detect apoptosis. The method employed here can be summarized in the following steps: (a) Cells were washed the twice with PBS (1600 rpm, centrifugation 5 min). (b) Cells were collected (1×10^4) and cell suspensions were prepared. Each group 3 tubes, each tube containing 500 μl Binding k Buffer suspension cell; (c) 5 μl Annexin V and mixk was added, followed by 5 μl Propidium Iodide, (d) All reagents were mixed at room temperature in dark; (e) After 5 to 15 min reaction they were examined by flow cytometry.

Statistical Analysis

Data were expressed by the mean±standard deviation ($\bar{\chi} \pm s$). Data were analyzed by SPSS10.0 (SPSS Inc., Chicago, IL, USA) statistical software. We used paired *t*-test for the selection and comparison of two groups. More than two groups of data were used by analysis of variance and $p<0.05$ was considered as statistically significant.

Results

The recombinant plasmid DNA sequencing identification

The recombinant plasmids were identified by sequencing, and the sequencing results were consistent with the design.

Transfection efficiency

Using fluorescent microscopy, we detect the expression of EGFP in the cell 4 h post transfection. The number of EGFP expressing cells gradually increased and peaked at 48 h. As it is presented in Figures 1-A and 1-B, the result of fluorescence expression for HEC-1A cells in the

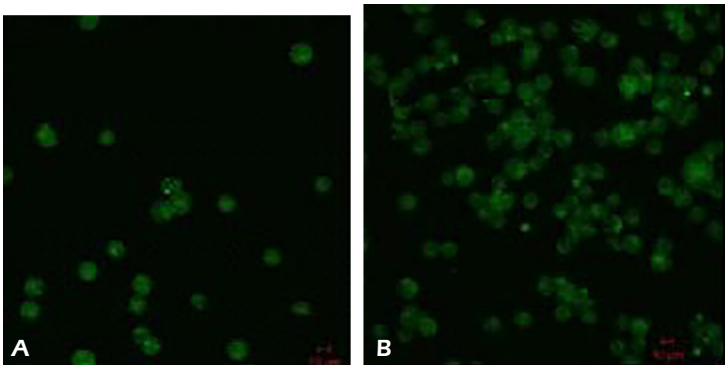

Figure 1. The transfection result detected by fluorescent microscopy. **A**, **B** were the transfection result detected by fluorescent microscopy in normal control group and experiment group respectively.

negative control group was about 6.2%. For transfection group, the fluorescence expression rate was 76.8%. The transfection efficiency was about 70.6%, which was deemed to meet the needs of our study.

The expression level of PTTG protein

Western blot analyses on PTTG siRNA-1, PTTG siRNA-2, PTTG siRNA-3, NC groups, revealed that the expression of PTTG in HEC-1A cells was markedly down-regulated in PTTG siRNA-2 group, 48 hours post transfection (Table I, Figure 2). Therefore, the strongest interference plasmid PTTG siRNA-2 was screened for other experiments.

Cell proliferation

Our results showed that the cell growth rate in the radiotherapy and the PTTG siRNA transfection groups were significantly lower than that of the control group ($p<0.01$). We detected a more pronounced cell proliferation inhibition for the group that was treated with PTTG siRNA combined with radiotherapy. The difference was statistically significant with $p<0.01$ (Table II). These results suggested that the proliferation of endometrial cancer cells could be inhibited by our PTTG siRNA. Moreover, PTTG siRNA could meaningfully increase the cancer cells vulnerability to the effects of radiation. The inhibition rates for the radio therapy group, the PTTG siRNA group and the PTTG siRNA combined with radiotherapy group were (31.39±5.62)%, (38.37±4.48)%, and (54.65±6.27)%, respectively.

Apoptosis rate

Cell apoptosis rates were significantly increased in the radiotherapy group and the PTTG siRNA transfection group when compared to the control group ($p<0.01$). A more pronounced cell apoptosis rate was observed in the group that was treated with PTTG siRNA combined with radiotherapy. The difference was statistically significant ($p<0.01$). The results suggested that PTTG siRNA can induce apoptosis in endometrial cancer cells and can increase the sensitivity of cells to radiotherapy. The apoptosis rate at 48 h for the control group, the radiotherapy group, the PTTG siRNA group, and the PTTG siRNA combined with radiotherapeutic group were (6.53±0.80)%, (32.72±4.56)%, (38.96±4.37)%, and (64.76±6.53)%, respectively in the flow cytometry analyses.

Discussion

We designed siRNAs specific to PTTG gene. The cDNA of the designed siRNA sequence was inserted into a plasmid vector. We selected PTTG siRNA-2 to transfect HEC-1A endometrial carci-

Table I. The expressions of PTTG protein detected by Western blot ($\bar{\chi} \pm S$).

Group	24h	48h	72h
Blank control group	0.97±0.04	0.98±0.05	0.97±0.03
PTTG siRNA-1 group	0.92±0.05	0.69±0.04*	0.83±0.04
PTTG siRNA-2 group	0.85±0.05	0.28±0.04**	0.41±0.03**
PTTG siRNA-3 group	0.87±0.06	0.45±0.04*	0.54±0.05*
NC group	0.96±0.06	0.98±0.03	0.93±0.04

*Compared to the blank control group, $p<0.01$ for all;** Compared to PTTG siRNA-1(PTTG siRNA-3) $p<0.01$ for all.

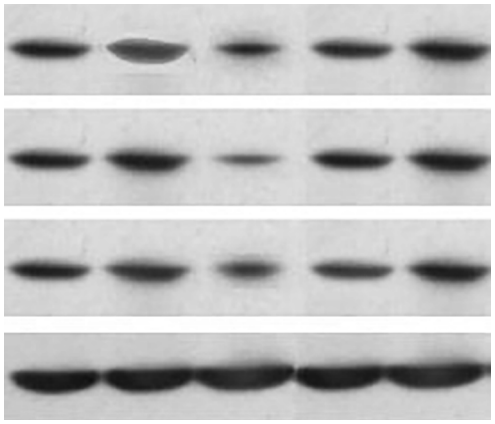

Figure 2. PTTG protein expression detected by Western blot. Note: 1 is the blank control group; 2 is PTTG siRNA-1 group; 3is PTTG siRNA-2 group; 4 is PTTG siRNA-3 group; 5 is NC group.

noma cells, and observed the effects on uterine endometrial carcinoma cells proliferation and its effects of radiotherapeutic cell sensitivity. By combining the PTTG siRNA with radiotherapy, we achieved better results in cancer cells proliferation inhibition and cell apoptosis rate. Although PTTG siRNA combined with radiotherapy demonstrated a more enhanced effect on inhibiting proliferation and inducing apoptosis, the PTTG siRNA alone also produced promising results.

Endometrial cancer is a form of endometrial epithelial malignant tumors accounting for 7% of all female malignant tumors. Currently, the primary method for the treating endometrial carcinoma is surgery, radiotherapy, and chemotherapy. In the early stages, based on the pathological results and the existence of the recurrence factors, the physician would select the adjuvant therapy. In the more advanced stages, comprehensive treatment of surgery, radiation, and drugs would be the best choice of the treatment. However, there is still a lack of ideal and effective treatment for this type of cancer.

Presently, gene therapy on the malignant tumors has become a hotspot of the international research. The occurrence of the endometrial cancer is a process in which we see the involvement of several genes in many steps. Pituitary tumor transforming gene also known as PTTG is a proto-oncogene, which is highly expressed in many tumors. Some researches detected high levels of PTTG expression in endometrial carcinoma[1-3]. PTTG is located on chromosome 5q33 and contains five exons and four introns. Some researches[4-8] detected high levels of PTTG expression in testes, thymus, liver as well as pituitary adenoma, gastric cancer, thyroid cancer, breast cancer, lung cancer and other tumors. PTTG is closely related to the tumor pathologic stage, the degree of differentiation and the prognosis. PTTG also plays an important role in cell proliferation and transformation and tumor formation. *In vitro* experiments demonstrated that the PTTG overexpression can result in: (i) cell proliferation, (ii) cell transformation *in vitro*, and (iii) tumorigenicity in nude mice. PTTG can also inhibit sister chromatid monomer separation; regulate cell mitosis[9], stimulate the expression and secretion of fibroblast growth factor[10], induce tumor angiogenesis, and regulate cell proliferation[11]. PTTG can also encode[12] a protein blocking p53. Down-regulation of the PTTG expression can significantly inhibit tumor cell proliferation[13].

RNA interference or RNAi can inhibit the expression of its target gene and is highly specific and can be used to find targets related to proliferation and invasion[14,15]. At present, there are five methods for the preparation siRNAs: 1 chemical synthesis; 2 *in vitro* transcription; 3 Feature of dsRNAs by RNase class III (such as Dicer, *E. coli*, RNase III) degradation method; 4 siRNA expression of frame method; 5 siRNA expression by plasmid or viral vectors. In our study, we used the fifth method, which is the most popular method currently used by most researchers. This method can be used after transfecting and screening and is suitable for a long time research on interference RNAs.

Radiotherapy as primary treatment for endometrial cancer, at present, has made significant progress, but the clinical effects are still not ideal. Kim et al[16] found that RNAi technology can significantly inhibit the proliferation of rectal cancer cells. Wang et al[17] employed RNA to interfere and

Table II. The result of inhibitory rate in endometrial carcinoma cells (OD) ($\bar{\chi} \pm S$).

Control group	Radiotherapy group	PTTG siRNA group	PTTG siRNA+ Radiotherapy group
OD value 0.86±0.06	0.59±0.04*	0.53±0.05*	0.39±0.04**
Inhibition ratio (%)	31.39±5.62	38.37±5..48	54.65±6.27

*Compared to the control group, $p<0.01$ for all; **Compared to PTTG siRNA and radiotherapy group, $p<0.01$ for all.

Table III. The apoptosis rate of endometrial carcinoma cells ($\bar{\chi} \pm S$) (%).

Groups	Control	Radiotherapy	PTTG siRNA	PTTG siRNA+ Radiotherapy
Apoptosis rate	6.53 ±0.80	32.72±4.56*	38.96±4.37*	64.76 ±6.53**

*Compared to the control group, $p<0.01$ for all; **Compared to PTTG-3 group and radiotherapy group, $p<0.01$ for all.

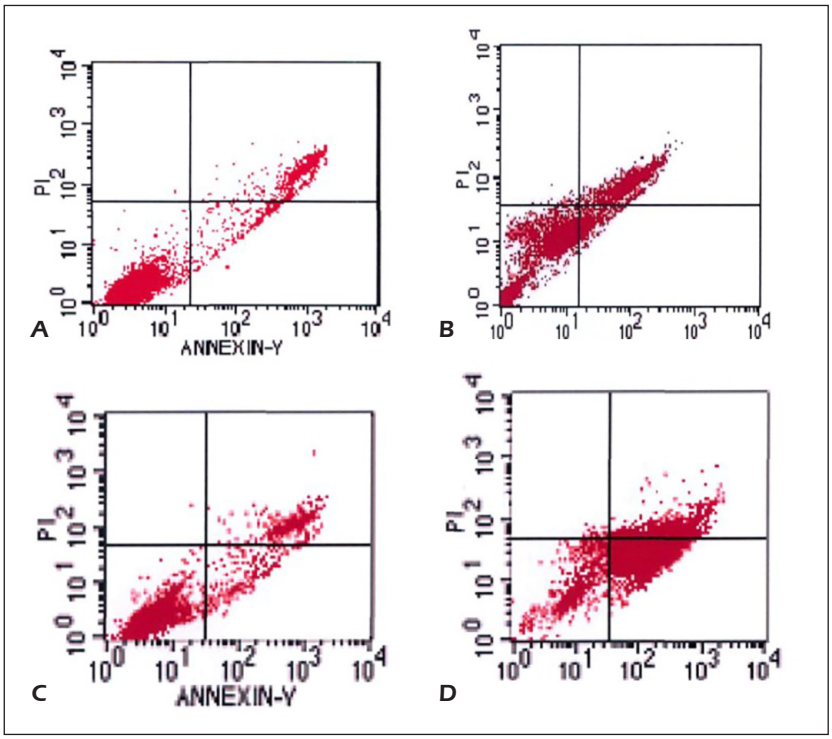

Figure 3. Cell apotosis detected by flow cytometry. **A**, **B**, **C**, **D** were the control group, radio therapeutic group, PTTG siRNA group and PTTG siRNA combined with radio therapeutic group respectively.

silent the expression of cervical cancer cells. In that study, cell proliferation decreased and the cell sensitivity to radiotherapy was amplified. Vandersickel et al[18], reported that down-regulation of mammary epithelial cells Ku70 expression by using the RNAi technology significantly increased the cell sensitivity to radiotherapy.

Conclusions

In this paper we employed the siRNA technology to silence the PTTG in endometrial carcinoma, we applied siRNA PTTG combined with the radiotherapy to increase proliferation inhibition and showed that the specific siRNA could improve the radiotherapy sensitivity. We believe RNA interference technology has an important application value in the endometrial cancer gene therapy and PTTG gene might be a worthy target in endometrial cancer gene therapy.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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