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·*Original Article*·

Inhibition of telomerase with human telomerase reverse transcriptase antisense increases the sensitivity of tumor necrosis factor-α-induced apoptosis in prostate cancer cells

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Abstract

Aim: To investigate the effect of inhibition of telomerase with human telomerase reverse transcriptase (hTERT) antisense on tumor necrosis factor-α (TNF-α)-induced apoptosis in prostate cancer cells (PC3). **Methods:** Antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) was synthesized and purified. Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP) and polymerase chain reaction enzyme-linked immunoassay (PCR-ELISA). hTERT mRNA was measured by reverse transcription PCR (RT-PCR) assay and gel-image system. hTERT protein was detected by immunochemistry and flow cytometry. Cell viability was detected by 3-(4, 5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT) assay. Cell apoptosis was observed by morphological method and determined by flow cytometry. **Results:** The telomerase activity decreased with time after hTERT AS PS-ODN treatment. The levels of hTERT mRNA decreased with time after hTERT AS PS-ODN treatment, which appeared before the decline of the telomerase activity. The percentage of positive cells of hTERT protein declined with time after hTERT AS PS-ODN treatment, which appeared after the decline of hTERT mRNA. There was no difference in telomerase activity, hTERT mRNA and protein levels between hTERT sense phosphorothioate oligodeoxynucleotide (S PS-ODN) and the control group. The cell viability decreased with time after hTERT AS PS-ODN combined with TNF-α treatment. The percentage of apoptosis increased with time after hTERT AS PS-ODN combined with TNF-α treatment. There was no difference in cell viability and the percentage of apoptosis between hTERT S PS-ODN and the control group. **Conclusion:** hTERT AS PS-ODN can significantly inhibit telomerase activity by downregulating the hTERT mRNA and protein expression, and inhibition of telomerase with hTERT antisense can enhance TNF-α-induced apoptosis of PC3 cells. *(Asian J Androl 2007 Sep; 9: 697–704)*

Keywords: human telomerase reverse transcriptase; antisense phosphorothioate oligodeoxynucleotide; telomerase; prostate cancer cells; tumor necrosis factor-α

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1 Introduction

In developed countries, prostate cancer is a main cause of cancer-related deaths in men. Androgen ablation is the main treatment for advanced prostate cancer.

This therapy is very effective in androgen-dependent cancer; however, as a result of the emergence of androgen-independent cells, tumors have become insensitive to this kind of treatment, rendering anti-androgen therapy ineffective [1]. Amplification of the androgen receptor gene is the most common cause by which prostate tumors have become androgen independent. Approximately 30% of tumors exhibiting androgen independence after ablation therapy show overexpression of the androgen receptor [2]. Therefore, we should find new targets for treatment of androgen-independent prostate cancer.

Telomeres are specialized heterochromatin structures that protect the ends of chromosomes. Studies have shown that telomerase activity is found in 85%–90% of all human tumors, but not in their adjacent normal cells [3]. This makes telomerase a good target not only for cancer diagnosis, but also for the development of novel therapeutic agents. Telomerase is composed of at least three subunits: human telomerase RNA component (hTR), human telomerase-associated protein (TEP1) and human telomerase catalytic subunit (hTERT). The RNA subunit and the catalytic subunit are the essential components for telomerase activity. The RNA subunit of telomerase serves as the template for addition of short sequence repeats to the chromosome 3' ends. The catalytic subunit, telomerase reverse transcriptase, is the most important component in telomerase complex, which is responsible for catalytic activity of telomerase. The expression of hTERT correlates with the presence of telomerase activity [4].

The *hTERT* gene seems to be regulated by androgens [5, 6]. Administration of androgens to androgen-sensitive prostate cancer cells activates the hTERT promoter, whereas androgen ablation leads to a decrease in hTERT expression accompanied by a concomitant reduction in telomerase activity which, in turn, is reversed by the subsequent administration of androgens. Therefore, hTERT is a good target for gene therapy to prostate cancer.

According to the initial paradigm for telomerase inhibitors, telomerase inhibitors should initially decrease telomerase activity without affecting the growth rate. Decreased proliferation should only be observed when the telomeres reach a critically short length. The lag phase between the times at which telomeres shorten sufficiently to produce detrimental effects on cancer cells is a serious obstacle for the application of anti-telomerase strategy, especially in cancer cells with long telomeres. Moreover, lengthy exposure to anti-telomerase agents can lead to the development of resistant tumor cells through overexpression of telomerase activity or reactivation of alternative telomere-lengthening mechanisms [1]. Therefore, the combination of anti-telomerase strategy with conventional drugs might improve the response of prostate cancer. One way to overcome this limitation might be to combine telomerase inhibition with DNA-damaging chemotherapeutic drugs. There is a possibility that inhibition of telomerase activity with hTERT antisense might increase susceptibility of prostate cancer cells (PC3) to immunotherapy drug-induced apoptosis.

In the present paper, an *in vitro* study was performed to examine if inhibition of telomerase activity with hTERT mRNA antisense can increase tumor necrosis factor-α (TNF-α)-induced apoptosis of PC3 cells.

2 Materials and methods

2.1 Design and synthesis of antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN)

Oligodeoxynucleotide synthesis was designed as described previously [7]. Based on the *hTERT* gene cDNA sequence, from upstream 6-base and downstream 11-base at start code, 20-antisense oligomers were synthesized, purified and modified by phosphorothioate from Shanghai Institute of Biochemistry (Shanghai, China). The AS PS-ODN sequence is 5'-GGAGCGCGCGGCATCGCGGG-3', which can recognize the catalytic subunit template region of telomerase. The sense phosphorothioate oligodeoxynucleotide (S PS-ODN) sequence is 5'-CCCGCGATGCCGCGCGCTCC-3', as a control. Through examination of the Blask soft from the Internet (http: //www.ncbi.nlm.nih.gov/BLAST/), there is no homologue for the antisense sequence with other genes except hTERT cDNA.

2.2 Cell culture

Human prostate cancer cell lines were kindly provided by the Research Institute of the Second Hospital, Lanzhou University (Lanzhou, China). PC3 were incubated in RPMI 1640 medium with 10% newborn bovine serum containing 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C under 5% CO_2. The experiments were divided into three groups: AS PS-ODN, S PS-ODN and normal control group, each group consisting of three wells on 24-well plates. Oligomers were added into the wells with a concentration of 10 μmol/L. Cell telomerase activity was measured 24, 48, 72 h later.

2.3 Detection of telomerase activity by the telomeric repeat amplification protocol (TRAP) and telomerase polymerase chain reaction enzyme-linked immunoassay (PCR-ELISA)

Telomerase activity was detected using the TRAP and the PCR-ELISA kit (Kaiji Bioengineering, Nanjing, China). Briefly, the cell extract was prepared at different time points of treatment. The negative control group was established in each experiment. Cell extract was heated to 65ºC for 10 min as a negative control.

2.3.1 Qualitative analysis by TRAP assay

For qualitative analysis by TRAP, 25 μL reaction mixture was transferred into a tube suitable for PCR amplication, and then 2 μL cell extract and sterile water were added to the final volume of 50 μL. The PCR condition was as follows: the telomerase reaction was carried out at 25ºC for 30 min, followed by PCR amplification of 30 cycles: at 94ºC for 30 s for denaturation, at 50ºC for 30 s for annealing of primers, at 72ºC for 90 s for polymerization and at 72ºC for 10 min for balance. The PCR products were revealed by 12% polyacrylamide no denaturing gel electrophoresis-silver staining.

2.3.2 Quantitative analysis by PCR-ELISA assay

For quantitative analysis by PCR-ELISA assay, 5 μL amplified product and 20 μL denaturated reagent were incubated at room temperature, and then 225 μL hybridization buffer was added into the mixture. After 100 μL mixture was distributed in the wells of a microtiter plate at 37ºC for 2 h, 100 μL anti-DIG-POD (peroxidase) working solution was added and incubated for 30 min. Finally, 100 μL 3,3'-5,5'-tetramethyl benzidine substrate solution was added and incubated for 10 min at room temperature for color development, and then 100 μL stop reagent was added to stop the reaction. Absorbance (A) values were determined at 450–655 nm to calculate $A = A_{450}–A_{655}$.

2.4 Analysis of human telomerase reverse transcriptase (hTERT) mRNA by reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from the AS PS-ODN, S PS-ODN or media-treated cells with the Trizol RNA kit (Shenggong Bioengineering, Shanghai, China) and RT-PCR reaction was performed with the One Tube RT-PCR kit (Shenggong Bioengineering, Shanghai, China). hTERT up-stream primer is 5'-CGGAAGAGTGTCTGGAGCAA-3' and hTERT down-stream primer is 5'-GGATGAAGCGGAGTCTGGA-3'. Beta-actin up-stream primer is 5'-GTGGGGCGCCC CAGGCAGGCACCA-3' and β-actin down-stream primer is 5'-GTCCTTAATGTCACGCACGATTTC-3' (Shenggong Bioengineering, Shanghai, China). PCR consisted of one cycle at 40ºC for 30 min, at 94ºC for 2 min, followed by PCR amplification of 35 cycles: at 94ºC for 15 s for denaturation, at 55ºC for 30 s for annealing of primers and at 72ºC for 8 min for extension. PCR products were assayed on a 2.0% agarose gel, visualized by ethidium bromide staining and analyzed with gel-image system.

2.5 Determination of hTERT protein by flow cytometry

The levels of hTERT protein were determined using an immunochemical assay kit (Zhongshan Golden Biotechnology, Beijing, China). Cells were collected and fixed with 70% formaldehyde at 4ºC for 15 min. After being washed with phosphate buffer solution (PBS) twice, the cells were incubated with 50 μL hTERT protein antibody at 4ºC for 1 h, and then 50 μL FITC-IgG of rabbit anti-goat was added and incubated at 4ºC for 30 min. Finally, the mixture was washed twice with the PBS and hTERT protein was determined by flow cytometry.

2.6 Treatment of cells by AS PS-ODN combined with tumor necrosis factor-α and detection of cell viability by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium (MTT) assay

The experiments were divided into six groups: normal control, AS PS-ODN, S PS-ODN, TNF-α, TNF-α/AS PS-ODN and TNF-α/S PS-ODN. Each group consisted of three repeat wells on 24-well plates. The AS PS-ODN was added into the wells with a concentration of 10 μmol/L. After the cells had been treated with AS PS-ODN for 24 h, 4 μg/mL TNF-α was added into the wells. The cell viability was detected by MTT assay (Baiao Bioengineering, Beijing, China) at 24, 48, 72 and 96 h. Briefly, the MTT solution (5 g/L) was added to each well and incubated at 37ºC for 4 h, and then culture medium was removed and 100 μL dimethyl sulfoxide was added to dissolve the formazan. Finally, the density of each well was detected at 590 nm using a microplate reader. The inhibition of cell viability was calculated using the following formula: (1–average A value of experimental group/average A value of control group) × 100%.

2.7 Observation of morphological feature of apoptosis

by inverted microscope

After the cells had been inhibited with 10 μmol/L AS PS-ODN for 24 h, 4 μg/mL TNF-α was incubated with PC3 cells for 48 h, and then the cells were observed under the inverted microscope and photos were taken.

2.8 Determination of apoptotic cells by flow cytometry

Cells were collected by low centrifugation and washed with ice-cold PBS, and then recollected by centrifugation. After being washed with the PBS twice, the cells were incubated in 10 μL Annexin V-FITC (fluorescein isothiocyanate) and 5 μL propidium iodine at 4ºC for 30 min using the Annexin V-FITC apoptosis assay kit (Baiao Bioengineering, Beijing, China). Finally, the cells were analyzed within 60 min by flow cytometry.

2.9 Statistical analysis

Results were expressed as mean ± SD and statistically compared using the Kruskal–Wallis H-test. Statistical analyses were carried out with the software package SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at $P < 0.05$.

3 Results

3.1 Effect of AS PS-ODN on the telomerase activity of prostate cancer cells

To evaluate the effect of AS PS-ODN on the telomerase activity of PC3 cells, the telomerase activity was measured using the TRAP assay and the telomerase PCR-ELISA assay. There was little effect of 10 μmol/L AS PS-ODN on telomerase activity from 24 to 48 h; the telomerase activity was significantly repressed after 48 h. There was no effect of 10 μmol/L S PS-ODN on telomerase activity of PC3 cells ($P < 0.05$; Figures 1 and 2). These findings suggest that this inhibitory action was sequence specific in a time-dependent manner.

3.2 Effect of AS PS-ODN on the levels of hTERT mRNA in prostate cancer cells

To clarify the relationship between telomerase activity and hTERT mRNA, the levels of hTERT mRNA were measured by RT-PCR and gel-image system. The levels of hTERT mRNA were significantly decreased after PC3 cells had been treated with 10 μmol/L AS PS-ODN for 24 h, which occurred before the decline of the telomerase activity. However, there was no effect of 10 μmol/L S PS-ODN on the levels of hTERT mRNA in PC3 cells ($P < 0.05$; Table 1, Figure 3).

3.3 Effect of AS PS-ODN on the expression of human hTERT protein in prostate cancer cells

To make further elucidate the relationship between

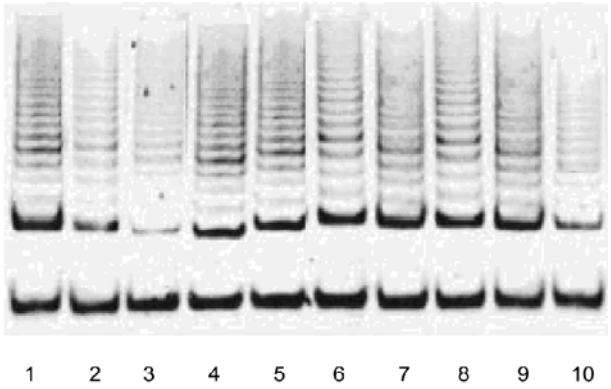

Figure 1. Telomeric repeat amplification protocol products (TRAP) were revealed by 12% polyacrylamide no denaturing gel electrophoresis-silver staining in prostate cancer cells (PC3). Lanes 1, 2 and 3 are human telomerase reverse transcriptase (hTERT) antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN)-treated cells for 24, 48 and 72 h, respectively; lanes 4, 5 and 6 are hTERT sense phosphorothioate oligodeoxynucleotide (S PS-ODN) treated cells for 24, 48 and 72 h, respectively; lanes 7, 8 and 9 are untreated cells for 24, 48 and 72 h, respectively; lane 10 is the PC3 cells lysate heat-treated at 65ºC for 10 min prior to the TRAP.

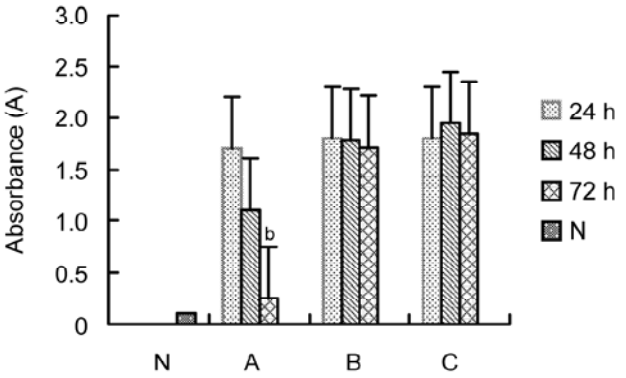

Figure 2. Effect of human telomerase reverse transcriptase (hTERT) antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) on the telomerase activity of PC3 cells by polymerase chain reaction enzyme-linked immunoassay (PCR-ELISA). A, AS PS-ODN; B, sense phosphorothioate oligodeoxynucleotide (S PS-ODN); C, normal control; N, negative control. Data were expressed as mean ± SD. $^bP < 0.05$, compared with S PS-ODN or control group, respectively.

Table 1. Effect of antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) on the levels of human telomerase reverse transcriptase (hTERT) mRNA in prostate cancer cells by reverse transcription polymerase chain reaction (RT-PCR). Data were expressed as (mean ± SD). $^{b}P < 0.05$, compared with the control, $^{e}P < 0.05$, compared with S PS-ODN. The value in the table is ratio of gel bands scanned with gel image system. Data are expressed as a ratio of hTERT/β-actin.

Time (hour)	Control	S PS-ODN	AS PS-ODN
24	0.68 ± 0.15	0.63 ± 0.14	0.29 ± 0.07^{b,e}
48	0.75 ± 0.30	0.60 ± 0.13	0.07 ± 0.04^{b,e}

Table 2. Effect of antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) on the percentage of positive cells of human telomerase reverse transcriptase protein (hTERT) in prostate cancer cells by flow cytometry (mean ± SD). $^{b}P < 0.05$, compared with the control, $^{e}P < 0.05$, compared with S PS-ODN.

Time (hour)	Control	S PS-ODN	AS PS-ODN
24	84.20 ± 3.80	87.50 ± 4.80	81.26 ± 2.36
48	85.60 ± 5.53	86.40 ± 2.56	70.51 ± 4.36^{b,e}
72	84.56 ± 2.42	85.15 ± 4.78	42.18 ± 5.07^{b,e}

the hTERT mRNA and hTERT protein, the expression of hTERT protein was determined by flow cytometry. The percentage of positive cells of hTERT protein was significantly declined compared to that of S PS-ODN after PC3 cells had been treated with 10 μmol/L AS PS-ODN for 48 h, which occurred after the decline of hTERT mRNA ($P < 0.05$; Table 2).

3.4 Effect of AS PS-ODN on the cell viability of prostate cancer cells

The inhibition of cell viability was detected by MTT assay. The results showed that there was a significant decrease in the cell viability of PC3 cells treated with 10 μmol/L AS PS-ODN combined with 4 μg/mL TNF-α. There was no significant decrease in the cell viability of PC3 cells treated with 10 μmol/L S PS-ODN combined with 4 μg/mL TNF-α ($P < 0.05$; Figure 4).

3.5 Effect of AS PS-ODN on the morphological feature of apoptosis in prostate cancer cells

The morphological features of apoptosis in PC3 cells were observed under a microscope. Many apoptotic cells were discovered after the cells had been treated with 10 μmol/L AS PS-ODN combined with 4 μg/mL TNF-α for 48 h. No apoptotic features were observed in PC3 cells treated with 10 μmol/L S PS-ODN combined with 4 μg/mL TNF-α (Figure 5).

3.6 Effect of AS PS-ODN on the percentage of apoptosis in prostate cancer cells

The percentage of apoptosis was determined by flow cytometry. There was a significant increase in the percentage of apoptosis in PC3 cells treated with 10 μmol/L AS PS-ODN combined with 4 μg/mL TNF-α for 48 h, but no significant increase in the percentage of apoptosis

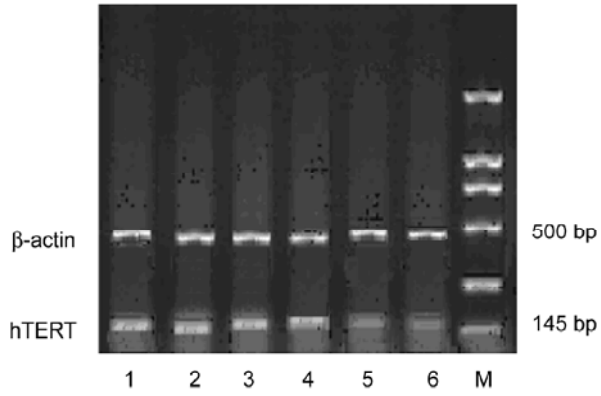

Figure 3. Effect of human telomerase reverse transcriptase (hTERT) antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) on the levels of hTERT mRNA in prostate cancer cells by reverse transcription polymerase chain reaction (RT-PCR). M, PBR322 DNA/Mspl Markers. Lanes 1 and 2 are untreated for 24, 48 and 72 h, respectively; lanes 3 and 4 are hTERT S PS-ODN treated cells for 24 and 48 h, respectively; lanes 5 and 6 are hTERT AS PS-ODN treated cells for 24 and 48 h, respectively.

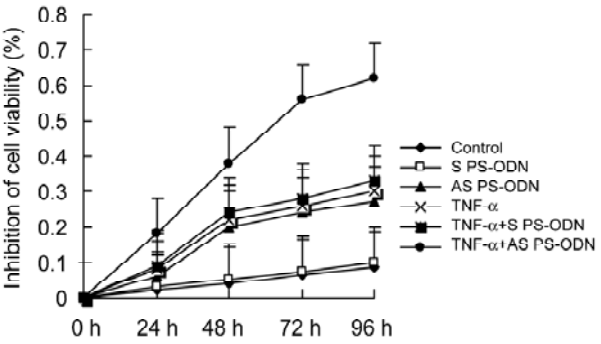

Figure 4. Effect of antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) combined with tumor necrosis factor-α (TNF-α) on the cell viability of prostate cancer cells by the MTT assay. Data were expressed as mean ± SD. $^{b}P < 0.05$, compared with S PS-ODN or control group, respectively.

in PC3 cells treated with 10 μmol/L S PS-ODN combined with 4 μg/mL TNF-α, which indicates that this apoptotic induction was sequence-specific in a time-dependent manner ($P < 0.05$; Figures 6 and 7).

4 Discussion

Promising approaches that directly target either telomerase or the telomerase-associated regulatory mechanisms are reported in the present paper. Strategies targeting telomerase-positive cells are means of directly killing tumor cells [8]. Among three major components of telomerase, hTERT, as the essential component in telomerase complex, plays an important role in telomerase activity. Recent studies demonstrate that telomerase activity is significantly associated with hTERT mRNA expression but not with hTR or TEP1 mRNA expression. These findings provide strong evidence that the expression of hTERT is a rate-limiting determinant of the enzymatic activity of human telomerase and that the upregulation of hTERT expression might play a critical role in human carcinogenesis [9]. Many studies demonstrate that antisense oligonucleotides against human

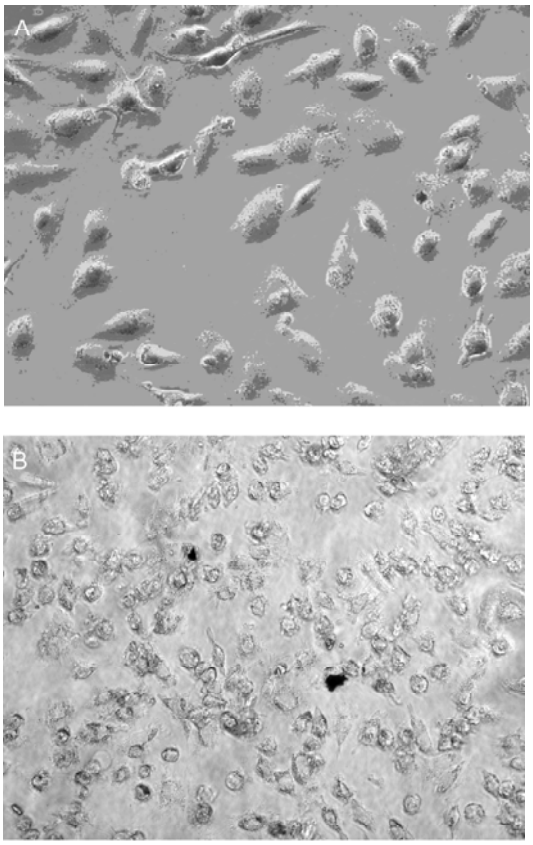

Figure 5. Effect of antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) combined with tumor necrosis factor-α (TNF-α) on the morphological feature of apoptosis of prostate cancer cells (PC3) by inverted microscope. (A): Control cells without any treatment. (B): Cells treated with human telomerase reverse transcriptase AS PS-ODN and TNF-α combination for 48 h; some cells show the morphological feature of apoptosis in PC3 cells. Magnification: 40 ×.

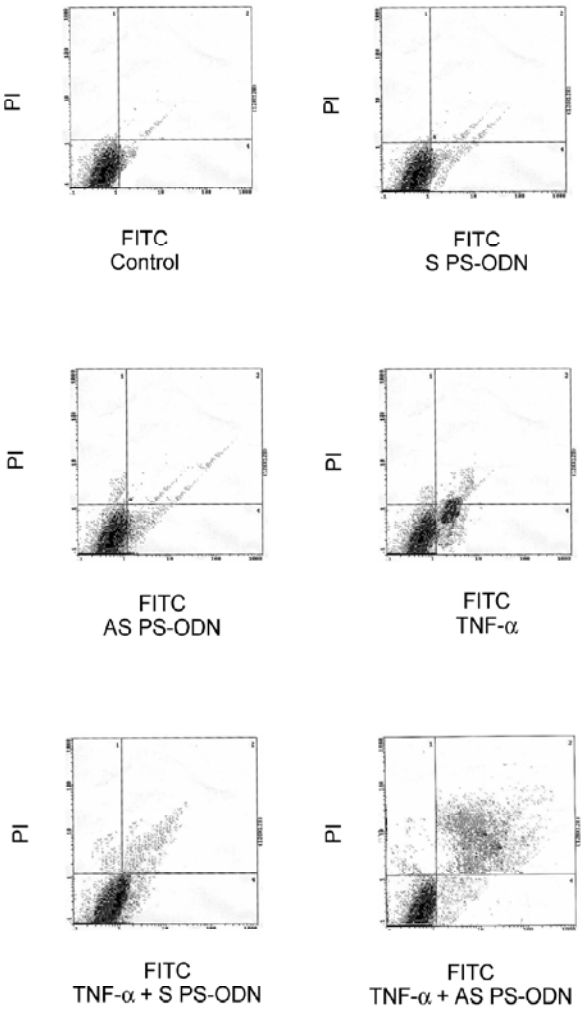

Figure 6. Effect of antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) combined with tumor necrosis factor-α (TNF-α) on the percentage of apoptotic cells of prostate cancer cells by Annexin V-FITC apoptosis assay; the representative profile are shown. FITC, fluorescein isothiocyanate; PI, propidium iodine.

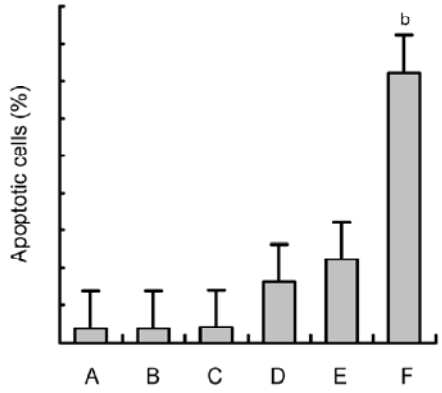

Figure 7. Effect of antisense phosphorothioate oligodeoxynucleotide (AS PS-ODN) combined with tumor necrosis factor-α (TNF-α) on the percentage of apoptotic cells of prostate cancer cells by Annexin V-FITC apoptosis assay. A, control; B, S PS-ODN; C, AS PS-ODN; D, TNF-α; E, TNF-α+S PS-ODN; F, TNF-α+AS PS-ODN. Data were expressed as mean ± SD. $^bP < 0.05$, compared with S PS-ODN and control group, respectively.

telomerase RNA results in inhibition of telomerase activity and induction of apoptosis in ovarian cancer cells and prostate cancer cells [10].

In the present study, the data show that telomerase activity was significantly downregulated or inhibited. (Figures 1 and 2). The levels of hTERT mRNA decreased significantly, which occurred before the decline of telomerase activity (Table 1, Figure 3). The expression of hTERT protein was declined significantly, occurring after the decline of hTERT mRNA (Table 2). These results suggest that AS PS-ODN regulated the telomerase activity of PC3 cells by modifying the hTERT mRNA level and hTERT protein, which consistent with similar research from another laboratory [11]. In these experiments, we did not find non-sequence-specific ribonucleic acid enzyme H (RNaseH) inhibition of S PS-ODN [12]. Our findings suggest that this inhibitory action was sequence specific in a time-dependent manner.

TNF-α has shown cytotoxity *in vitro*. Different tumor cells have different sensitivities for TNF-α-induced apoptosis. Approximately 40% of tumor cells have growth inhibition or cell lysis [13]. The results show that hTERT AS PS-ODN combined with TNF-α could significantly decrease the cell viability of PC3 cells (Figure 4) and increase the percentage of apoptosis in PC3 cells (Figures 5–7). hTERT S PS-ODN combined with TNF-α could not induce apoptosis of PC3 cells, indicating that this inhibitory and inducing action is sequence specific in a time-dependent manner.

The mechanism of apoptosis of sensitive cells induced by TNF-α is supported by the fact that TNF-α can trigger the signal transduction and initiate apoptosis after combining with the specific TNF-α receptor (TNFRI) in target cell membrane [14]. TNFRI is a death receptor, which can activate the endogenous nuclease (DNase) and caspase family by combining with TNF-α [15]. hTERT inhibition leads to a gradual reduction in telomere length followed by growth arrest or apoptosis; moreover, researchers have found that suppression of hTERT expression abrogates the cellular response to DNA double strand breaks. Loss of hTERT does not alter short-term telomere integrity, but instead affects the overall configuration of chromatin. Under the downregulation of telomerase and the tumor cells lacking hTERT, the tumor cells diminish capacity for DNA repair, fragment chromosomes and impair the DNA damage response; cells lacking hTERT exhibit an increased susceptibility to apoptosis-induced agents [16]. Hence, TNF-α can easily divide DNA cells into fractions by the function of endogenous nuclease. hTERT inhibition sensitizes PC3 cells to TNF-α-induced apoptosis, which might improve clinical cytokine therapy for prostate cancer.

Other studies show that overexpression of hTERT protected a maturation-resistant acute promyelocytic leukemia (APL) cell line from apoptosis induced by TNF-α. The cells expressing high telomerase activity were more resistant to apoptosis than those with low telomerase expression [17]. Treatment with antisense telomerase inhibited the telomerase activity and, subsequently, induced either apoptosis or differentiation. The regulation of these two distinct pathways might depend on the expression of interleukin-1 β-converting enzyme or cylin-dependent kinase inhibitors [18].

Results in these experiments are some similar to those of Zhang *et al*. [19]. However, their experiments aim to construct *hTERT* gene negative dominant mutation vectors and to transfer them into tumor cells so that cell telomerase activity is lowed or inhibited. It is easy to consider problems regarding virus-vector safety. However, it is hard to eliminate the possibility of virus vectors contaminating human genetic materials, which could produce serious problems. Use of antisense oligonucleotides to develop a new genetic drug might remove this problem.

In conclusion, the present study shows, for the first

time, that hTERT inhibition sensitizes PC3 cells to TNF-α-induced apoptosis. AS PS-ODN is a promising treatment strategy for prostate cancer with telomerase activity.

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