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Original Article

Screening and identification of antisense accessible sites of the human telomerase reverse transcriptase gene in prostate cancer cell lines

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Abstract: Selecting optimal antisense binding sites remains difficult during application of antisense oligonucleotide techniques. A random oligonucleotide library/RNase H cutting method combined with software analysis has emerged as an effective strategy. Here, we used the method to explore therapy targeted to the human telomerase reverse transcriptase (hTERT) gene in prostate cancer. A 20 mer random oligonucleotide library was synthesized and then hybridized with total hTERT complementary RNA (cRNA) that had been transcribed in vitro. The RNA in the specific hybrid double-stranded duplex was hydrolyzed by RNase H, so the total hTERT cRNA was cut into fragments. After primer extension and autoradiography, the antisense accessible sites (AAS) of hTERT were selected. Optimal AAS with obvious stem-loop structures were identified after RNA structure software analysis. The complementary antisense oligonucleotides (AS-ODNs) of these AAS were synthesized and transferred into cells expressing high hTERT levels and the effects upon cell growth, apoptosis and expression of hTERT mRNA were determined. There were twenty-six AAS of the hTERT gene screened, and seven AAS, which had obvious stem-loop structures were selected. After transfection of complementary antisense oligonucleotides of these optimal AAS, the hTERT mRNA expression levels in the cells were significantly decreased. Cellular growth was significantly inhibited and apoptosis was detected. Screening antisense accessible sites of a target gene through random oligonucleotide library/RNase H cutting in combination with computer analysis was effective. The resulting complementary antisense oligonucleotides efficiently blocked the biological function of the hTERT gene suggesting their potential for cancer treatment.

Keywords: hTERT, random oligonucleotide library, screening, antisense accessible sites, targeted blocking, stem-loop structure

Introduction

The human telomerase is mainly composed of human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR). The hTR acts as the reverse transcription template and is complementary to telomeric repeat sequences. The hTERT is the catalytic subunit that catalyzes the reverse transcription reaction. These two components work together to maintain the length of telomeres. The expression of hTERT mRNA is related to the level of telomerase activity and this is considered to be the rate-limiting factor [1, 2]. The expression of hTERT is also associated with cellular aging and tumorigenesis, and high telomerase activity is observed in over 85% of human cancer cells [1-3], whereas normal tissues and cells have minimal expression.

Prostate cancer cell lines have been used extensively to investigate cancer in vitro. PC3 cell lines were established in 1979 from a bone metastasis of a grade IV prostate cancer [4]. These cells do not respond to androgens, glucocorticoids, or epidermal and fibroblast growth factors, but have high metastatic potential. The DU145 cell line was derived from a brain metastasis [5]. DU145 are not hormone-sensitive and do not express prostate-specific antigen

(PSA). DU145 cells have moderate metastatic potential. In both these prostate cancer cell lines telomerase activity is likely to be high because telomerase can be used as a tumor marker in the diagnosis of prostatic intraepithelial neoplasia and prostate cancer [6].

Targeting hTERT in prostate cancer cells may; therefore, provide an effective method of cancer therapy. At present research strategies to inhibit a targeted gene's mRNA expression include antisense oligodeoxynucleotide techniques, RNA interference technology, ribozyme technology, small molecule compounds, and immune therapy, but the first two techniques are the most researched and applied in the area (7). Antisense oligonucleotides have been used as effective methods of studying gene function and gene expression regulation in addition to targeted biological treatments, and telomerase has been successfully targeted with antisense by many investigations [7, 8]. These studies show the potential for antisense therapy in cancer treatment, but the efficacy of the treatment could be improved by selection of antisense oligonucleotides targeted to the optimum antisense accessible sites that most effectively shut down the target mRNA. Although antisense oligodeoxynucleotide techniques have been used for 32 years [9], selection of the most effective antisense binding site is often difficult. Early methods of designing antisense oligonucleotides were mainly based on the primary structure of RNA molecules, such as the translation initiation site. However, this leads to reduced specificity of antisense oligonucleotides due to the high homology of translation initiation sites in a wide range of genes. The effectiveness of antisense accessible site screening by the "Random shotgun" method is only 2% to 5% because the secondary structure of RNA molecules is ignored [10]. Currently, computer-assisted folding programs can forecast the secondary structure of RNA molecules using the free energy principle. From a structural molecular biology perspective, sites without intramolecular base pairing in the loop region are antisense active accessible sites, while the stems are non-active accessible sites. The acknowledged five antisense accessible sites of the hTERT gene are screened out using only a computer-assisted folding program [1]. However, for long-chain RNA, computer-assisted folding programs will form many different structures with similar free energies, which make judging the correct folding conformation very difficult. Using computer folding programs to predict antisense binding sites seems to be ineffective without also considering screening and identification in vitro.

In this study, a random oligonucleotide library/RNase H cutting method was combined with computer software analysis that has been successfully used in other studies to screen the antisense binding sites of target sequences in vitro [11]. Twenty-six antisense accessible sites of the hTERT gene were screened to select the optimal ones [10, 12, 13]. Stem-loop structures were selected as these have been shown to have higher activity than other common mRNA structures, and have been shown to have better accessibility than other mRNA structures [14, 15]. The complementary phosphorothioate antisense oligonucleotides of these antisense accessible sites were then synthesized and transferred into the prostate cancer cell lines PC-3 and DU145 that express high levels of hTERT, in order to study their influence on the biological behavior of prostate cancer cells. To our knowledge, this is the first report using the random oligonucleotide library/RNase H cutting method combined with software analysis to explore targeted therapy of the human telomerase reverse transcriptase (hTERT) gene in prostate cancer.

Materials and methods

Design and synthesis of the random oligonucleotide library

The random oligonucleotide library was synthesized according to previously described methods [16, 17]. Specific requirements were as follows: (1) the length was 20 bp; (2) the content ratio of A:C:G:T was 1.5:1.25:1.15:1.0; (3) the sequence was 5'-MMMNNNNNNNNNNNNNNNMM-3'. G, A, T, C represents the four different deoxidizing nucleotides, M represents methylation deoxycytidine, N represents random oligodeoxynucleotides. The random oligonucleotide library was synthesized and purified by the Nanjing Shengxing Biological Engineering Company (China).

In vitro transcription of the target gene

The pcDNA-3.1 (+) plasmid that contained the hTERT mRNA structure gene was gifted to us by Dr Cao Song of London University. In vitro tran-

Table 1. Antisense accessible regions of hTERT shown by primer extension analysis

Primer	Origin site of primer	Product size of primer extension (bp)	Termination site of extension	3' end of RNase H cleavage (bp)	Antisense accessible sites (bp)
P3	598	200	399	398	379~398
		70	529	528	509~528
		60	539	538	519~538
		35	564	563	544~563
P4	707	38	670	669	650~669
P5	809	100	710	709	690~709
		38	772	771	752~771
P6	940	190	751	750	731~750
		82	859	858	839~858
		70	871	870	851~870
		66	875	874	855~874
		35	906	905	896~905
P7	1148	147	1002	1001	982~1001
		35	1114	1113	1094~1113
P8	1302	90	1213	1212	1193~1212
		30	1273	1272	1253~1272
P9	1463	150	1314	1313	1294~1313
		66	1398	1397	1378~1397
		38	1426	1425	1406~1425
P10	1648	70	1579	1578	1559~1578
		26	1623	1622	1603~1622
P11	1831	210	1622	1621	1602~1621
		105	1727	1726	1707~1726
		30	1802	1801	1782~1801
		25	1807	1806	1787~1806
P12	1989	140	1850	1849	1830~1849
		30	1960	1959	1940~1959
P13	2245	30	2216	2215	2196~2215
P14	2333	82	2252	2251	2232~2251
		30	2304	2303	2284~2303
P15	2477	30	2448	2447	2428~2447
P16	2575	66	2510	2509	2490~2509
		30	2546	2545	2526~2545
P17	2760	118	2643	2642	2623~2642
P18	2946	38	2909	2908	2889~2908
P19	3160	85	3076	3075	3056~3075
		30	3131	3130	3111~3130
P20	3396	66	3331	3330	3311~3330

scription of the gene was performed by the following steps. The plasmid was digested by the restriction enzyme Xba I (Fermentas company) to linearize it, and then transcribed to complementary RNA (cRNA) using the T7 transcription kit (Fermentas company) in vitro [18]. The total reaction volume was 50 μl and was incubated for 2 hours at 37°C. RNase-free DNase I was added to a concentration of 1 U DNase/1 μg template DNA and then incubated for 15 min at 37°C to remove the DNA templates. 85 μl of diethylpyrocarbonate (DEPC)-treated water was then added with 15 μl of 3 M sodium acetate. The cRNA was extracted first with an equal volume of 1:1 phenol/chloroform mixture, and then, twice, with an equal volume of chloroform. The aqueous phase was collected and transferred into a new tube. The cRNA was then precipitated, by adding 2 volumes of ethanol and incubating at -20°C for 30 min. After centrifugation, to remove the supernatant, the pellet was rinsed with 500 μl of cold 70% ethanol. The cRNA was resuspended in 20 μl of DEPC-treated water and then stored at -70°C after its concentration was determined by UV spectrophotometry and it was identified by 1% agarose gel electrophoresis.

RNase H cleavage reaction

The random oligonucleotide library and cRNA were hybridized in a reaction system at a total volume of 150 μl. 60 μl (27.1 pmol/μL) of the random oligonucleotide library was aliquoted into a new RNase-free tube, then denatured at 95°C for 5 min and put on ice for 5 min. The RNase H cleavage was then performed according to the manufacturer's instructions (Fermentas Company).

Design of the extension primers

According to the mRNA sequence of the hTERT gene contained in the pcDNA-3.1 plasmid (+) (3402 bp) and the technical bulletin of the Primer Extension Kit (Promega Corporation),

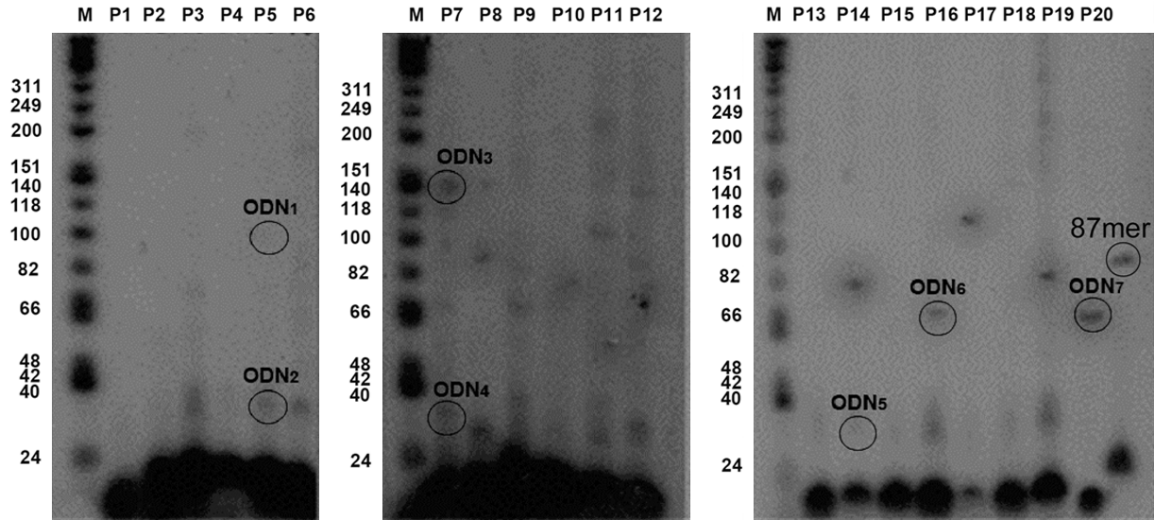

Figure 1. Seven obvious binding site regions and fragment size obtained by primer extension. Primer extension analysis for the selection of antisense accessible sites of hTERT by random oligonucleotide library/RNase H cleavage method. The seven selected sites are highlighted and labeled with their corresponding serial number. M: φX174/Hinf I DNA Marker; P1~P20: Primer extension products of P1~P20; the positive control product is an 87 mer.

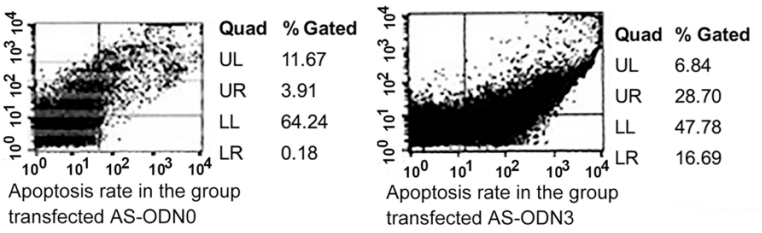

Figure 2. In vitro experiments showed a higher rate of apoptosis of antisense AS-ODN$_3$ in comparison with AS-ODN$_0$ (control) of prostate cancer cells. The right hand section of the flow cytometry graphs represents the apoptotic cell population. AS-ODN$_3$ had higher apoptosis rates than the AS-ODN$_0$ control.

we used primer premier 5.0 to design a reverse extension primer every 150 to 300 bases from the 5' to 3' end of the RNA [19]. There were a total of 20 primers designed and synthesized by Beijing Sunbiotech Co., Ltd (**Table 1**).

Primer extension reaction

The primer extension reaction involved primer labeling, marker labeling and the primer extension reaction, which were all performed by standard techniques and according to the manufacturer's guidelines (purchased from Beijing Yahui Biological Engineering Company).

Polyacrylamide gel electrophoresis and autoradiography

The primer extension products were analyzed on a 16×18 cm denaturing polyacrylamide gel containing 8% acrylamide (19:1 acrylamide:bis), 7 M urea and TBE 1×buffer.

Antisense binding site selection and the design of control antisense oligonucleotides

RNA structure software was used to predict the full length secondary structure of hTERT mRNA [20]. Combined with the results of the in vitro random oligonucleotide library/RNase H digestion and screening, the best significant stem-loop structures of the region were designated as the best antisense binding sites. We based these best binding sites on the stem-loop structures because we expect these to be easily accessible because the activity of a stem-loop structure is higher than that of other common mRNA structures. Seven of the best antisense accessible sites were screened and named AS-ODN$_1$~AS-ODN$_7$ respectively. A selected region (1468~1487 bp), which had an obvious stem-loop structure according to the computer analysis, but did not have cleavage sites in the random oligonucleotide library/RNase H digest method was used as a control antisense accessible site (see **Figure 2**) and the synthesized complementary control antisense oligonucleotide was named AS-ODN$_0$, the sequence was 5'-GAGATGAACTTCTTGGTG-

TT-3'. Another control was the most efficient hTERT antisense oligonucleotide of the five that were published by AOBase, the sequence was 5'-TTGAAGGCCTTGCGGACGTG-3' and this was named AS-ODN$_{AOBase}$. This control sequence was one used previously by Kraemer et al. [3] and was listed as ASt2315 in their study to reflect its location start point of 2315 in the mRNA sequence.

Cell culture and gene transfection

Human prostate cancer cell lines, androgen-independent PC-3 cells [21] and DU145 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium for PC-3 cells or DMEM for DU145 cells, with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere and passaged once every 3 days. At logarithmic growth phase the PC-3 cells were seeded in culture plates, following the protocol of the liposome Oligofectamine™ Reagent kit (Invitrogen Company, Cat. No. 12252-011) for the transfection of the AS-ODNs (200 nM). To avoid oligonucleotide decomposition in the cells, three bases at both ends of oligonucleotides were phosphorothioated at the time of synthesis [22]. The transfection efficiency was between 57.7% and 58.1%.

MTT assay for detecting cell growth activity

100 μl PC-3 prostate cancer cells were seeded in each well of 96-well plates at a density of 5×10^3/100 μl. The wells were set as the untransfected control group, control group (AS-ODN$_0$), AOBase control group (AS-ODN$_{AOBase}$) and AS-ODN$_1$~AS-ODN$_7$ experimental groups, each oligonucleotide was also set at 200, 400 and 600 nmol/L concentrations. Each group had five duplicate wells. 10 μl of 5 mg/ml MTT (pH = 7.4) was added after transfection, for 12, 24 and 36 hours. The cells were continued in culture for 4 hours and the supernatant was removed after centrifuging at 4000 r/min for 10 minutes. 100 μl DMSO was added and left at room temperature for 10 minutes to fully dissolvethecrystals. A microplate reader was used to detect the absorbance ($A_{490, 630}$ dual-wavelength method). The cell growth inhibition rate (%) = (1-the average $A_{490, 630}$ value of experimental group/the average $A_{490, 630}$ value of control group)×100% [23].

Detection of apoptosis

DU145 prostate cancer cells were seeded in 24-well plates at a density of 5×10^3/100 μl with each well inoculated with 500 μl. The conditions were set as the untransfected control group, control group (AS-ODN$_0$), AOBase control group (AS-ODN$_{AOBase}$) and AS-ODN$_1$~AS-ODN$_7$ experimental groups, each oligonucleotide was also used at 200, 400 and 600 nmol/L concentrations. Each group used five duplicate wells. Annexin V-FITC and PI double staining flow cytometry was used to detect cell apoptosis after transfection at 12, 24 and 36 hours [24].

Detection of hTERT expression levels

Cultured cells were collected 12-24 h after transfection and Trizol reagent was used to isolate total RNA. This was reverse transcribed into cDNA. PCR primers were synthesized according to references, P1: 5'-CGGCTTTTGTTCAGATGCC-3' (sense), P2: 5'-AGCACACATGCGTGAAACCT-3' (antisense). The product length was expected to be 301 bp (30). The PCR internal reference was GAPDH, 5'-GAAGGTGAAGGTCGGAGTC-3' (sense), 5'-GAAGATGGTGATGGGATTTC-3' (antisense), the product length was expected to be 226 bp. The PCR reaction system was: 1 μl DNA template, 0.5 μl 10 mM dNTPs, 2.5 μl 10×PCR buffer, 1.5 μl 25 mM $MgCl_2$, 1 μl of two 10 mM primers, 1 μl 25 U/μl Taq enzyme, 16.5 μl ddH_2O to a total volume of 25 μl. The cycling conditions were a first predenaturation at 95°C for 5 min, then 94°C for 40 sec, 60°C for 45 sec and 72°C for 60 sec for 30 cycles and a final extension step at 72°C for 10 min. The products were electrophoresed on 2.5% agarose gels and observed under a UV lamp and photographed. The band density was measured using ImageJ and calculated for each group: gray value of hTERT/GAPDH. hTERT expression inhibition rate (%) = [1-the average (hTERT/GAPDH) value of experimental group/the average (hTERT/GAPDH) value of untransfected control group]×100%.

Morphological observation

Cell growth and morphological changes were observed under an inverted microscope before and after transfection. Prostate cancer cells were collected after transfection and fixed with 2.5% glutaraldehyde for 30 minutes. After con-

Table 2. Antisense accessible sites and their complementary oligonucleotide sequences selected by random oligonucleotide library/RNase H cleavage combined with RNA structure analysis

Serial number	Base sequence	Origin-end sites	Base no.	MTT assay inhibition	Apoptosis results	RT-PCR results
ODN_1	AAGTCTGCCGTTGCCCAAGA	690~709	20			
AS-ODN_1	TCTTGGGCAACGGCAGACTT			35.3±1.9%	30.9±1.3%	28.6±1.4%
ODN_2	CTGCCGTTGCCCAAGAGGCC	752~771	20			
AS-ODN_2	GGCCTCTTGGGCAACGGCAG			33.9±1.3%	21.2±0.9%	32.5±2.3%
ODN_3	ACCAAGCACTTCCTCTACTC	982~1001	20			
AS-ODN_3	GAGTAGAGGAAGTGCTTGGT			40.6±1.0%	35.4±1.2%	35.8±1.2%
ODN_4	TCTGGGTTCCAGGCCCTGGA	1094~1113	20			
AS-ODN_4	TCCAGGGCCTGGAACCCAGA			1.4±0.2%	19.2±1.0%	12.3±3.8%
ODN_5	ACGTCTCTACCTTGACAGAC	2284~2303	20			
AS-ODN_5	GTCTGTCAAGGTAGAGACGT			20.3±3.4%	18.7±0.6%	11.2±2.1%
ODN_6	GGCTCCATCCTCTCCACGCT	2490~2509	20			
AS-ODN_6	AGCGTGGAGAGGATGGAGCC			0.4±0.05%	19.5±0.8%	17.9±0.6%
ODN_7	GTCGGAAGCTCCCGGGGACG	3311~3330	20			
AS-ODN_7	CGTCCCCGGGAGCTTCCGAC			17.3±0.9%	16.4±0.6%	15.2±1.0%
AS-ODN_0	GAGATGAACTTCTTGGTGTT'	1542~1561		9.7±1.7%	13.2±1.0%	9.4±1.1%
AS-ODN_{AOBase}	TTGAAGGCCTTGCGGACGTG	2315~2334		23.5±1.2%	20.5±0.6%	20.6±0.9%

ventional embedding and slicing, the changes in the cell's ultrastructure were observed under a transmission electron microscope.

Statistical analysis

SPSS11.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis and the outcomes were expressed as mean ± standard error. Differences were evaluated using two independent samples with Student's t test, a p value of <0.05 was considered statistically significant.

Results

hTERT mRNA primer extension reaction and autoradiography

As shown in **Figure 1**, P1 and P2 located in the non-coding region of hTERT had no clear extension product that could be seen after the primer extension reaction and autoradiography. There were 26 antisense binding sites selected after analysis of the extension products of P3~P20. The size of the fragment obtained from the primer binding with antisense and the start and end sites of the product are shown in **Table 1**. These are the primer initiation site, extension of the product size, RNase H 3' end cleavage site and the corresponding antisense binding sites.

RNA structure analysis

Analysis of the secondary structure of hTERT mRNA, showed that among the 26 antisense accessible sites, seven sites had obvious stem-loop structures as forecast by RNA structure software. The 7 antisense accessible sites of the hTERT gene that were considered to be the best because of the high chance of being a stem-loop are represented as ODN1-7 in **Table 2**, and were located in the 690 bp~709 bp, 752 bp~771 bp, 982 bp~1001 bp, 1094 bp~1114 bp, 2284 bp~2303 bp, 2490 bp~2509 bp, and 3311 bp~3330 bp areas of the hTERT mRNA. The seven complementary antisense oligonucleotide sites were AS-ODN_1~AS-ODN_7 (shown in **Table 2**).

Results of the MTT assay

Cell growth was investigated as a measure of the antisense oligonucleotide's potential for anti-cancer therapy. After transfection with 200 nmol/L of AS-ODN_0, AS-ODN_{AOBase}, and AS-ODN_1~AS-ODN_7 for 12-36 h, the growth of the PC-3 cell growth slowed down, each to a different extent. Each group exhibited the most significant inhibition after 24 h transfection, and the inhibition rates were 9.7±1.7%, 23.5±1.2%, 35.3±1.9%, 33.9±1.3%, 40.6±1.0%, 1.4±0.2%, 20.3±3.4%, 0.4±0.05%, and

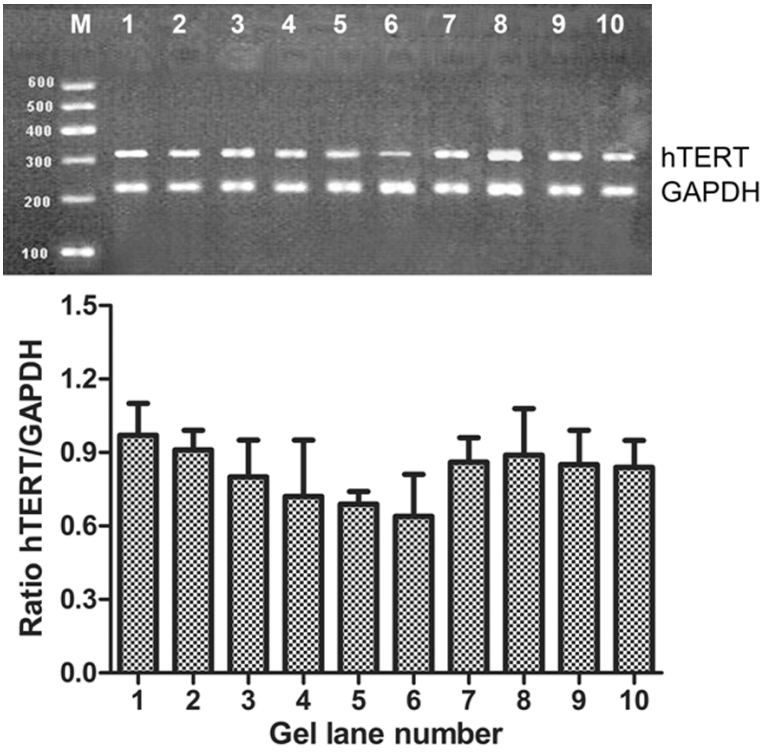

Figure 3. After seven antisense oligonucleotides (AS-ODN$_n$) and control group were transfected into PC-3 cells, hTERT was blocked as detected by RT-PCR electrophoresis. RT-PCR detection of the blocking effects of antisense oligonucleotides on hTERT mRNA expression of PC-3 cells: 1. not transfected control. 2. AS-ODN$_0$. 3. AS-ODN$_{AOBase}$. 4. AS-ODN$_1$. 5. AS-ODN$_2$. 6. AS-ODN$_3$. 7. AS-ODN$_4$. 8. AS-ODN$_5$. 9. AS-ODN$_6$. 10. AS-ODN$_7$. M: PCR marker (100 bp~600 bp); GAPDH as an inner control (226 bp). The experiment was repeated six times and the mean values are presented in the graph.

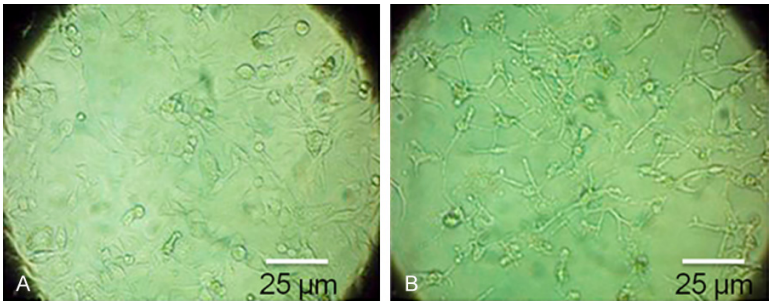

Figure 4. Antisense (AS-ODN$_3$) and the control group were observed 24 hours after transfection of PC-3 cells in cell morphology. A: The cell morphology before antisense oligonucleotide was transfected. B: The cell morphology 24 h after 200 nmol/L AS-ODN$_3$ was transfected.

17.3±0.9% respectively. The difference was statistically significant between the groups (P<0.05) so different oligonucleotides had different efficiencies at targeting cell growth and AS-ODN$_3$ was most effective. The three different concentrations of oligonucleotides had no significant difference in the inhibition of growth (P>0.05).

Detection of apoptosis

After transfection with 200 nmol/L of AS-ODN$_0$, AS-ODN$_{AOBase}$, and AS-ODN$_1$~AS-ODN$_7$ for 12-36 h, the cells in each group showed the most obvious apoptosis after 24 h. The apoptosis rates were 13.2±1.0%, 20.5±0.6%, 30.9±1.3%, 21.2±0.9%, 35.4±1.2%, 19.2±1.0%, 18.7±0.6%, 19.5±0.8% and 16.4±0.6% respectively. The difference was statistically significant between the groups (P<0.05) so different oligonucleotides had different efficiencies at causing apoptosis and AS-ODN$_3$ was most effective. The three different concentrations of oligonucleotides showed no significant differences in the rate of apoptosis (P>0.05) (**Figure 2**).

Detection of hTERT expression levels

After transfection with 200 nmol/L of AS-ODN$_0$, AS-ODN$_{AOBase}$, and AS-ODN$_1$~AS-ODN$_7$ for 12-36 h, the hTERT/GAPDH ratio in each group decreased most significantly after 24 h. The RT-PCR detection is shown in **Figure 3**. The inhibition rates of the hTERT mRNA expression levels were 9.4±1.1%, 20.6±0.9%, 28.6±1.4%, 32.5±2.3%, 35.8±1.2%, 12.3±3.8%, 11.2±2.1%, 17.9±0.6%, and 15.2±1.0%, respectively. The difference was statistically significant between the groups (P<0.05) so different oligonucleotides had different efficiencies at inhibition of hTERT and AS-ODN$_3$ was most effective. The effect of different concentrations of oligonucleotides was not significant on the inhibition of hTERT mRNA's expression (P>0.05).

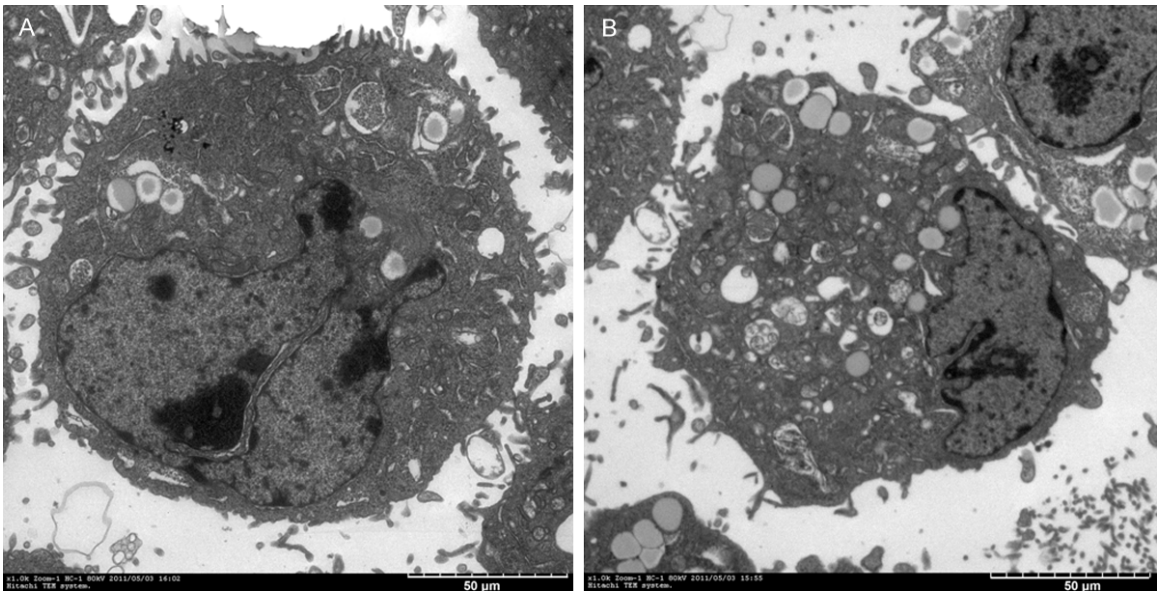

Figure 5. Antisense (AS-ODN$_3$) and the control group were observed 24 hours after transfection of PC-3 cells in cell ultrastructure. A: Control group (×1.0 k). The nucleus is large and microvilli are dense. B: 24 h after 200 nmol/L AS-ODN$_3$ was transfected (×1.0 k). The nucleus was condensed, nuclear membrane had shrunk, chromatin was marginalized and budding phenomenon appeared.

Morphological observation

Under an inverted microscope, the prostate cancer cells in the control group were found to be polygonal in shape. After the cells were transfected with the antisense oligonucleotides, the cell volume decreased and the cell morphology was irregular (**Figure 4**). Most of the cells showed changes typical of apoptosis under electron microscopy after transfection, including condensed nucleus, shrunk nuclear membrane, and marginalized and budded chromatin, compared to control cells with large nucleus and dense microvilli (**Figure 5**).

Discussion

The aim of this study was to use a random oligonucleotide library/RNase H cutting method combined with software analysis to explore targeted therapy of the hTERT gene in prostate cancer. The results showed that targeting seven optimal antisense accessible sites decreased hTERT mRNA expression levels in the prostate cancer cell lines and significantly inhibited cell growth producing detectable levels of apoptosis. An alternative method for targeted anticancer therapy is RNA interference (interference RNA, RNAi) which induces sequence-specific posttranscriptional gene silencing by use of double-stranded small interfering RNA (SiRNA) is characterized by strong inhibition, high stability, and easier cellular uptake. This method has been used successfully to study gene function and in anti-tumor research [25, 26]. However, SiRNA has some disadvantages; it cannot be used to bind anti-cancer drugs because of its double-stranded structure which has lower specificity than antisense oligodeoxynucleotides and has a less efficient transfer into cells, decreased specificity due to energy diffusion in later stage silencing, and poor stability in the body, also, it has not been widely used in mammalian systems [27]. For these reasons, we used antisense therapy in this study.

It can be challenging finding the optimum antisense accessible sites in the target gene and there are many different methods for selecting them, including oligonucleotide arrays [28], reverse transcription with random oligodeoxynucleotide libraries [29], mRNA accessible site tagging [30] serial analysis of antisense binding sites [31], and mRNA antisense-accessible sites library using native mRNA [32]. A random oligonucleotide library/RNase H cutting method combined with computer software analysis has been used successfully elsewhere and in this study we referred to the experience of Ho et al. [16] and Lloyd et al. [33], and synthesized

an oligonucleotide library which contained all possible random 20 mer sequences with a number of methylated bases on both ends. The sites identified for the oligonucleotides hybridized with the hTERT template chain sites, also referred to antisense accessible sites. In this study, 26 antisense accessible sites were screened in the hTERT gene, 7 of which were in significant stem-loop regions suggesting better accessibility when combined with RNA structure computer software analysis, so these were considered to be the best antisense accessible sites. Synthesis of the complementary strands of the best antisense accessible sites were designated as AS-ODN, we transfected the AS-ODN into prostate cancer cells and this significantly inhibited their growth. The highest inhibition rate was with AS-ODN_3 (40.6±1.0%). Apoptosis was highest for the AS-ODN_3 group (35.39±1.2%), the hTERT mRNA expression level was also significantly inhibited and by the highest amount with AS-ODN_3 (35.8±1.2%). In this study, of the 7 selected binding sites of the antisense oligonucleotides targeted against hTERT, AS-ODN_3 was most effective at shutting down hTERT expression. Each oligonucleotide was used at three different concentrations 200, 400 and 600 nmol/L but we found that there were no significant differences in the slowing of cell growth, apoptosis rates or expression levels between the different concentrations used. The fact that these concentrations are quite low and that the lowest concentration had similar effects to the highest one, suggests that these oligonucleotides are acting in a sequence specific manner rather than by general nonspecific inhibition. The most effective time point was apparently 24 hours in terms of cell growth inhibition, apoptosis and hTERT expression levels. We investigated at 12, 24 and 36 hours and so this is slightly surprising as the longest time period might be expected to show greater effects.

The Beijing Institute of Nuclear Medicine has built a database of different antisense oligonucleotides that have been identified in published literature. The database uses an English interface and is free of charge. We used this to inquire into different antisense oligonucleotides [34] (http://www.bioit.org.cn/ao/aobase). The database contains five antisense oligonucleotides targeted at hTERT mRNA. These five are all located in different areas to the seven best locations we discovered using the RNase H method in combination with computer software. We selected the most efficient antisense oligonucleotide of the five as an experimental control. We also selected an obvious stem-loop structure on the basis of the hTERT mRNA secondary structure prediction by the RNA structure software, which does not have cleavage sites in the region of the random oligonucleotide library/RNase H cutting method as another control. Ultimately, these results show that these two control AS-ODNs have no significant difference in efficiency, and are lower than the experimental screening of the AS-ODN_3. An important point to consider is the time course of these studies. We found effects at very short time periods when compared to previous studies where effects were seen at 15 days [7]. It is probably to be expected that a longer time period would be needed to fully shutdown the telomere's influence in cancer cells because they are usually very long and take time to degrade [35].

This study has some limitations, we have considered the secondary and tertiary structures that the RNA is likely to adopt, but in vivo the RNA will form a complex with proteins, these may interfere with access to these sequences. Before the efficacy of the different AS-ODNs can be fully understood, they need to be tested in vivo and over longer time periods. We therefore intend to undertake further studies in cell culture and animals.

Screening for optimum antisense accessible sites is beneficial for antisense oligonucleotide design. This study into inhibiting the expression of the hTERT gene using antisense oligonucleotides has suggested several application prospects: 1. These antisense oligonucleotides are expected to be developed as an anticancer drug; 2. The activity of both siRNA and AS-ODN aimed at the same locus have been shown to be significantly correlated, while highly activated antisense nucleic acid had better activity than siRNA [36]; therefore, screening for optimum antisense accessible sites also provides an important reference for RNA interference; 3. These hTERT-specific antisense oligonucleotides can bind anticancer drugs at the 3' terminal end, to provide both gene shutdown and drug treatment, reducing the systemic side effects of antitumor drugs. This has the potential

for the dual function treatment of tumors by targeted therapy combined with anti-cancer drugs, such as curcumin and theprubicin as a prodrug and trigger for the catalytic release of a drug in a highly sequence specific manner. This approach could potentially be used to selectively kill prostate cancer cells. However, this would not be possible with siRNA because of its double stranded nature [37].

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Disclosure of conflict of interest

None.

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