Inhibiting hTERT antisense oligodeoxynucleotide changes proliferation and telomerase activity of HL-60 cells☆

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Abstract

Objective. To investigate the inhibition of hTERT antisense oligodeoxynucleotide (ASODN) on the proliferation and telomerase activity in HL-60 cells and to explore the relationship between the telomerase activity and the expression of human telomerase reverse transcriptase (hTERT) gene in HL-60 cells. *Methods.* hTERT expression was detected by RT-PCR after treated with ASODN, the morphological changes of HL-60 cells was observed with inverted microscopy, the cell proliferation was measured by MTT method, and the telomerase activity was determined with TRAP-ELISA and TRAP-PAGE. *Results.* Treated with ASODN for 72 hours, the hTERT gene was significantly inhibited, the cell growth was depressed and the ability of proliferation was decreased, and the effect was specific in sequence and showed concentration-dependent and time-dependent. $OD_{450\text{-}690}$ values were 1.504 ± 0.47, 1.223 ± 0.39, 0.944 ± 0.16 respectively, as the cells were treated with 10, 20, 30 μmol/L ASODN for 72 hours. The difference was significant when the 10, 20, 30 μmol/L groups were compared with untreated group (2.648 ± 0.42) respectively ($P < 0.05$), but the difference was of no significance when 20 μmol/L SODN group (2.376 ± 0.65) was compared with untreated group ($P > 0.05$). TRAP-PAGE detection revealed that the telomerase activity in ASODN groups was decreased compared with SODN groups, and decreased the most was in the group of 30 μmol/L ASODN. *Conclusion.* The hTERT ASODN may inhibit the proliferation and down-regulate the telomerase activity in HL-60 cells by sealing the expression of hTERT gene. [Life Science Journal. 2007; 4(3): 17 – 21] (ISSN: 1097 – 8135).

Keywords: HL-60 cells; hTERT gene; antisense oligodeoxynucleotide; telomerase activity

1 Introduction

Eukaryotic chromosomes are capped with repeated telomere sequences that protect the ends from damage and rearrangements. Telomere length decreases along with the increase of cell cycles. Telomere shortening has therefore been proposed to play a role in cellular senescence. Telomere repeats are synthesized by telomerase, a RNA-protein complex, which elongates the end of pre-existing telomeres by synthesizing TTAGGG sequences, using an internal RNA molecule as template. Telomerase counteract telomere shortening in cell division, therefore, maintains telomere length and make cell immortalization[1]. Previous researches showed that high telomerase activity was correlate closely with tumor development including acute leukemia[2]. In general, telomerase activation is prerequisite for the unlimited proliferation of immortal tumor cells. Thus, restraining telomerase activity can inhibit tumor proliferation and induce tumor cells apoptosis. Human telomerase reverse transcriptase (hTERT) gene has direct association with telomerase activity[3]. The present research would transfect hTERT antisense oligodeoxynucleotide (ASODN) into human myelogenous leukemia cell strain HL-60 with antisense technology, to investigate the effect of hTERT ASODN on the proliferation and telomerase activity in leukemic cells.

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2 Materials and Methods

2.1 Cell culture

HL-60 cell was incubated at 37 ºC in a humidified 5% CO_2 incubator, and cultured in RPMI1640 (Invitrogen, USA) supplemented with 10% inactivated fetal calf serum (Invitrogen, USA) and antibiotics. Cell growth state and morphological changes were observed with inverted microscopy.

2.2 Designing and synthesis of antisensenucleic acids

Targeting at hTERT mRNA translation initiator, thio-modified hTERT ASODNs were synthesized by Sangon (Shanghai, China). Another thio-modified plus sense oligonucleotides (SODN) was designed as control. ASODN: 5'-GGAGCGCGCGGCATCGCGGG-3'; SODN: 5'-CCCGCGATGCCGCGCGCTCC-3', antisense oligodeoxyribonucleotide had no homology with other foregone human gene, which was confirmed by computer retrievating.

2.3 Transfection and drug sensitivity test

(1) According to Oligofectamine™Reagent (Invitrogen, USA) instruction, dilute ASODN and SODN with RPMI1640 to 5, 10, 20, 30 μmol/L of each group. (2) Dilute 2.5 μl each concentration of ASODN and SODN to 42.5 μl as A solution with serum free RPMI1640, and 1.5 μl Lipofectamine™ 2000 to 7.5 μl as B solution. (3) Mix A and B and incubate the mixed solution for 20 minutes at room temperature. (4) Put 200 μl (1.5×10^5) log phase growth cells in 24-well plate, add the mixed solution to each well. Incubate the cells at 37 ºC in a CO_2 incubator for 4 hours. (5) After incubation for 4 hours, add 125 μl fresh RPMI1640 medium (30% serum) to each well and incubate the cells at 37 ºC in a CO_2 incubator right along. Set three repeat wells in each concentration. Meanwhile, set untreated group as control.

After disposing for 24 hours, 48 hours, 72 hours, 96 hours respectively, growth inhibition of treated cells were detected with MTT reduction assay. Growth inhibition ratio = (indisposed group OD_{490} – disposed group OD_{490}) / indisposed group OD_{490}.

2.4 Cell proliferation activity detection

Log phase growth HL-60 cells (5×10^4/ml) were transfected with 20 μmol/L ASODN (ASODN group) and SODN (SODN group) respectively. Set three repeat wells in each group. HL-60 cells were dyed with 4 g/L trypan blue and counted 24, 48, 72, 96 hours later.

2.5 Detection of hTERT mRNA expression in transfected cells

Total RNA was extracted with Trizol Reagent. Expression of hTERT mRNA was evaluated by RT-PCR. The primers of hTERT gene: up-stream: 5'-CTGGGTGGCACGGCTTTTGTTC-3'; down-stream: 5'-GGGCTGCTGGTGTCTGCTCTCG-3'. The expected PCR product is 705 bp. The β-actin primers: up-stream: 5'-TCCTGTGGCATCCACGAAACT-3', down-stream: 5'-GAAGCATTTGCGGTGGACGAT-3'. The product is 315 bp. Reaction condition: 95 ºC for 4 minutes; then 30 cycles (94 ºC for 30 seconds, 58 ºC for 40 seconds, 72 ºC for 60 seconds); and 72 ºC for 7 minutes. Gene expression (hTERT/β-actin) were evaluated by scanning grey scale with Gel Imaging System.

2.6 Telomerase activity assays of transfected cells

2.6.1 Sample extraction. Samples for telomerase activity assays were extracted following standard protocols of Telo*TAGGG* Telomerase PCR ELISA^{PLUS} Kit (Roche, Germany). Positive control was cell extract prepared from immortalized telomerase-expressing human kidney cells (293 cells). Inactivated samples and lysis buffer served as negative controls.

2.6.2 RT-PCR amplification. Took 20 μg cell extract per tube, transfered 25 μl reaction mixture, then supplemented with H_2O to 50 μl. Telomerase elongation conditions were 25 ºC for 30 minutes and 94 ºC for 5 minutes. PCR cycling conditions: 94 ºC for 30 seconds, 50 ºC for 30 seconds, and 72 ºC for 90 seconds, 30 cycles and then 72 ºC for 10 minutes.

2.6.3 TRAP-ELISA assays. TRAP-ELISA assays were operated according to the protocol of Telo*TAGGG* Telomerase PCR ELISA^{PLUS} Kit. All telomerase assays were performed in triplicate and were repeated at least three times.

2.6.4 Polypropylene gel electrophoresis and silver staining. Referred to the book of *Molecular Coning*, telomerase PCR amplification products were detected with the methods of polypropylene gel electrophoresis and silver staining.

2.7 Statistical analysis

Data were reported as $\bar{x} \pm s$ and analyzed by SPSS 11.0 software. Two samples comparison was analyzed by *t* test, and multi-samples comparisons by ANOVA. Set $\alpha = 0.05$ as significant.

3 Results

3.1 Effect of hTERT ASODN on the growth of HL-60

cells

All the concentrations of ASODN have inhibitory effect on the growth of HL-60 cells. The effect was concentration-dependent and time-dependent, and correlation coefficient were 0.951, 0.959, 0.960, 0.958 ($P < 0.05$) 24 hours, 48 hours, 72 hours, 96 hours later respectively. The strongest inhibitory action was at the 72nd hour then decreased. Compared with SODN group, the inhibitory rate of ASODN were increased significantly ($P < 0.05$) (Table 1).

Table 1. The inhibition of hTERT ASODN to the growth of HL-60 cells

Group	Dose (μmol/L)	Inhibitory rate (%)			
		24 hours	48 hours	72 hours	96 hours
SODN	20	2.0 ± 0.2	2.9 ± 0.4	3.4 ± 0.2	3.1 ± 0.4
ASODN	5	6.9 ± 0.7*	8.4 ± 1.3*	11.8 ± 1.4*	10.7 ± 1.6*
	10	10.9 ± 1.3*	17.1 ± 1.6*	25.3 ± 2.5*	24.1 ± 3.4*
	20	14.7 ± 2.3*	25.1 ± 2.6*	43.0 ± 4.3*	41.3 ± 5.0*
	30	16.1 ± 2.5*	28.9 ± 2.9*	49.2 ± 4.7*	46.6 ± 4.8*

$F_{time} = 111.924$, $P < 0.05$; $F_{group} = 263.400$, $P < 0.05$. *: *vs.* SODN group, $P < 0.05$.

3.2 Detection of cell proliferation activity

HL-60 cells in untreated group were log growth at 24 – 96 hours, and cell doubling time was 20 – 24 hours. The inhibition of 20 μmol/L ASODN began at the 24th hour. From that time the cell doubling time was obviously prolonged to 24 – 28 hours. But the cell doubling time changed little in 20 μmol/L SODN group. Compared with untreated group, cell population decreased slightly, and cell doubling time had no change (Figure 1).

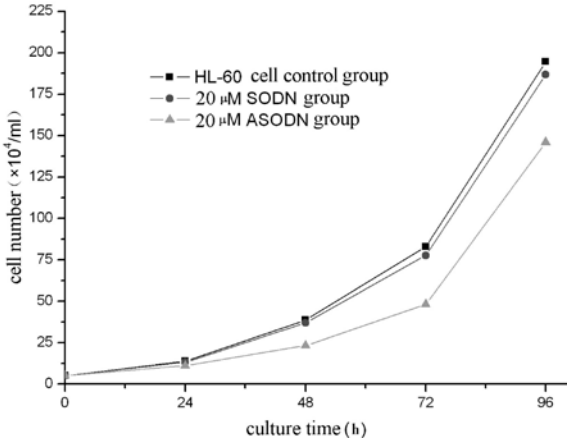

Figure 1. Growth curve of HL-60 cells in each group.

3.3 Morphological changes of transfected cells

After transfecting HL-60 cells with ASODN for 72 hours, we observed cell growth state and morphological change with inverted microscopy and found that cell growth was prosperity in half adherence state, and the cell outline was distinct in SODN and untreated groups. However, cells colony diminished, granules were found in cells and cell debris appeared in ASODN treated group. (Figure 2).

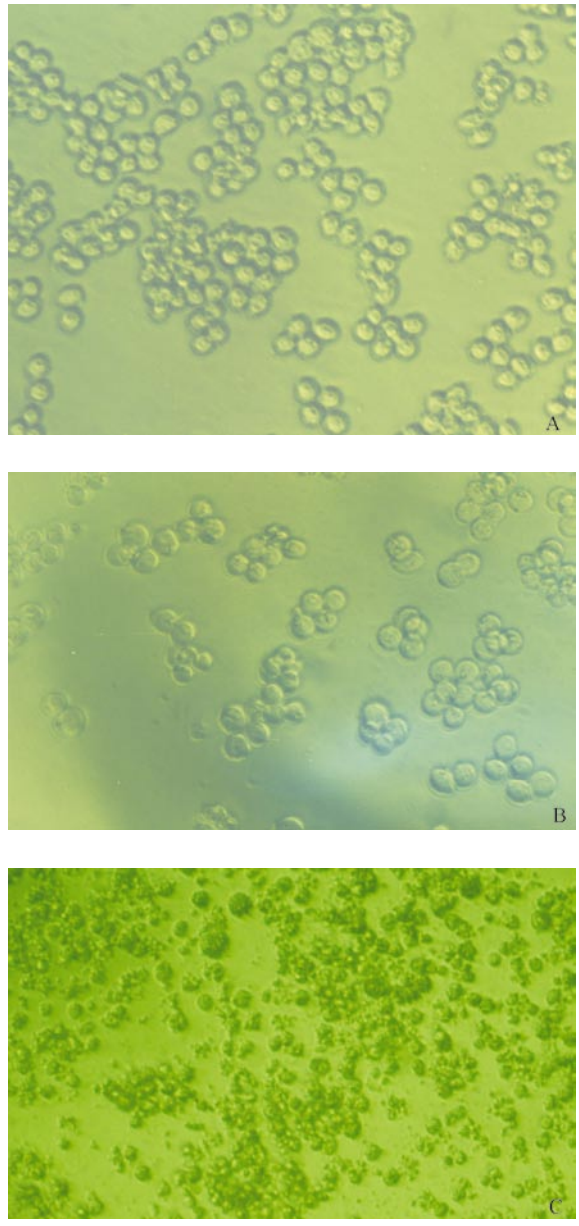

Figure 2. Morphology changes of HL-60 cells after transfected with hTERT ASODN (× 200). A: untreated group; B: SODN group; C: ASODN group.

3.4 Detection of hTERT mRNA expression in transfected cells

RT-PCR results showed that the amplification product of β-actin (confer) was 315 bp. The product of hTERT was 705 bp, and the electrophoresis band weakened along with ASODN concentration increasing. Seventy-two hours later the grey scale of hTERT/β-actin with 20 μmol/L (0.34 ± 0.07) ASODN decreased obviously compared with 20 μmol/L SODN (0.97 ± 0.14) and untreated group (1.01 ± 0.16). Especially, hTERT/β-actin with 30 μmol/L ASODN decreased the most (0.13 ± 0.03) (P < 0.01) (Figure 3).

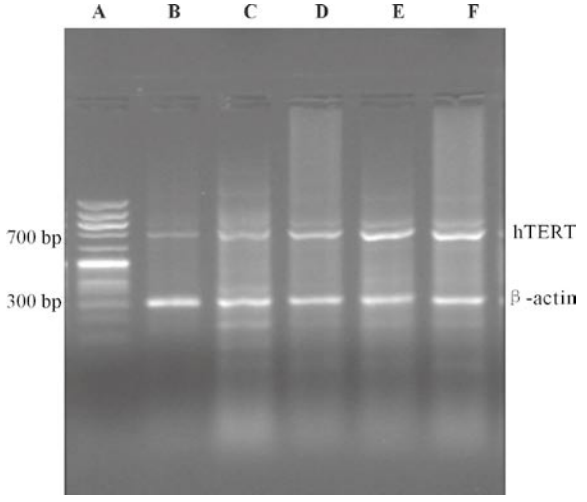

Figure 3. The detection of hTERT mRNA expression in transfected cells. A: GeneRuler™ 100 bp DNA ladder; B, C, D: group of 30, 20, 10 μmol/L ASODN; E: group of 20 μmol/L SODN; F: untreated group.

3.5 Quantitative analysis of telomerase activity

Seventy-two hours later with hTERT ASODN, the detected telomerase activity of HL-60 cells decreased with ASODN concentration increasing. The difference was significant when ASODN treated groups compared with SODN group and untreated group respectively (P < 0.05); there was no statistical significance between SODN treated group and untreated group, P > 0.05 (Table 2).

3.6 Qualitative analysis telomerase activity

The TRAP-PCR products of different groups were separated by polypropylene gel electrophoresis. After silver staining, telomerase ladder bands decreased with ASODN concentration increasing. Meanwhile, compared with SODN group, telomerase ladder bands of ASODN treatment group lessened apparently (Figure 4).

Table 2. The inhibition of hTERT ASODN with different dose to telomerase activity of HL-60

Group	Dose (μmol/L)	$OD_{450-690}$	t	Inhibitory rate (%)
untreated		2.648 ± 0.42		0
ASODN	10	1.504 ± 0.47*	4.0584	43.2
	20	1.223 ± 0.39*	5.5595	53.8
	30	0.944 ± 0.16*	8.4777	64.4
SODN	20	2.376 ± 0.65Δ	0.7859	10.3

*: *vs.* SODN and untreated group respectively, P < 0.05; Δ: *vs.* untreated group, P > 0.05.

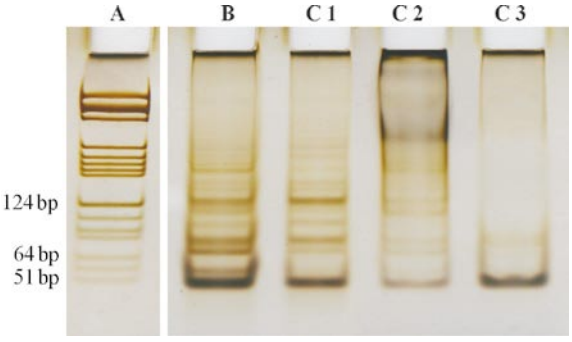

Figure 4. The telomerase activity in HL-60 cells with the inhibition of hTERT ASODN. A: pBR322DNA/*Bsu*RI (*Hae*III) Marker; B: group of 20 μmol/L SODN; C1, C2, C3: groups of 10, 20, 30 μmol/L ASODN.

4 Discussion

For the past few years, the correlation between telomerase and tumor cells was concerned. The human telomerase is a RNA-protein complex and composed of template RNA components (hTR) and two proteins, telomerase-associated protein-1 (TP1) and telomerase reverse transcriptase (hTERT)[4]. Telomerase activity is the basis of infinite proliferation of malignant tumor cells[5]. Telomerase is specifically activated in most malignant tumors but is usually inactive in normal somatic cells[6]. Finite lifespan cells exposed to hTERT have been rendered immortal, suggesting that telomerase plays an important role in cellular immortalization and tumorigenesis[7].

Most of malignant tumors in hematopoietic system have been detected activated telomerase, but the expression levels are not the same in different tumors. The highest expression level exists in acute leukemia[2]. Xu measured the telomerase of sixty-six acute myelocytic leukemia patients, 87% of which was positive[8]. Kubuki

thought that telomerase activity and telomere length are correlated with prognosis of adult T-cell acute lymphatic leukemia[9]. Seol's results showed that the telomerase activity of all the acute myelocytic leukemia patients was positive, which was present heterogeneity[10].

Some research data showed that the telomerase in normal somatic cells are activated when introduced hTERT gene, and the cells having no telomerase expression did not express hTERT gene either. The hTERT mRNA is often up-regulated in cells with activated telomerase[11, 12]. There is evidence that human myelocytic leukemia cell line express hTERT highly[13]. A previous study from our laboratory showed that hTERT also expressed at high level in myelocytic leukemia cells.

Based on our previous studies, HL-60 cells were transfected with hTERT ASODN in the present research.The morphological change were observed with inverted microscopy and found that cells colony diminished, granules in cells and cells debris increased in ASODN treated group. Growth inhibiting of treated cells were also detected with MTT reduction assay. All the ASODN groups showed inhibitory action on the growth of HL-60 cells. RT-PCR results showed that hTERT gene expression in different ASODN groups decreased obviously comparing with SODN and untreated groups. There was direct correlation between inhibitory action and ASODN treated concentration. It demonstrated that the inhibitory action of ASODN on HL-60 cell proliferation was specific in sequence and concentration-dependent and time-dependent. The essay results showed restraining hTERT gene expression can inhibit HL-60 cells proliferation.

Telomerase activity quantitative analysis showed that after 72 hours with hTERT ASODN, the telomerase activity of HL-60 cells decreased obviously comparing with SODN group. Telomerase activity qualitative analysis also showed that telomerase ladder bands decreased with ASODN concentration increasing. The results above demonstrated that hTERT ASODN could inhibit effectively telomerase activity of HL-60 cells, furthermore, telomerase activity and hTERT mRNA transcriptional level kept at equal place. Our results concord with Braunstein's study in which telomerase activity would lessened or vanished if amino acids sequence of hTERT protein had been changed[14]. It demonstrated that hTERT is the telomerase subunit and thought to be the rate-limiting component[15], and repression of hTERT expression can directly result in telomerase inactivation[16].

5 Conclusion

The suppression of the telomerase activity and cell proliferation by hTERT ASODN was mainly through down-regulation of hTERT gene expression to repress the leukemic cells. These findings should be helpful for investigating antileukemic mechanisms of hTERT ASODN and developing novel gene drug targeting telomerase for leukemic treatment.

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