

• COLORECTAL CANCER •

Telomerase activity and cell apoptosis in colon cancer cell by human telomerase reverse transcriptase gene antisense oligodeoxynucleotide

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Abstract

AIM: To evaluate the effect of human telomerase reverse transcriptase (hTERT) gene antisense oligodeoxynucleotide (As-ODN) on telomerase activity and cell apoptosis in colon cancer cell line SW480.

METHODS: As-ODN was transfected into cells SW480 by liposomal transfection. Cultured cells were divided into three groups: ASODN (5' GGAGCGCGCGCATCGCGGG-3'), sense oligodeoxynucleotide (5' -CCCGCGATGCCGCGCTCC-3', S-ODN) and control. The concentration of oligodeoxynucleotide and liposome was 10 μ mol/L and 16 mg/L, respectively. The activity of telomerase was examined by telomeric repeat amplification protocol (TRAP)-enzyme-linked immunosorbent assay (ELISA), and cell apoptosis was observed by morphology and flow cytometry in each group.

RESULTS: Telomerase activity began to be down-regulated or inhibited when cells SW480 were treated with As-ODN for 72 h, and cell apoptosis was induced.

CONCLUSION: It is suggested that hTERT As-ODN might specially inhibit the activity of telomerase in colon cancer cells and it is further proved that the hTERT gene has a significant correlation with telomerase activity. Further evidence is needed to prove whether hTERT As-ODN is a potential tool for the treatment of colon cancer.

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INTRODUCTION

Telomeres, the distal ends of human chromosomes, are

comprised of simple, repetitive and G-rich hexameric sequences (TTAGGG) and are vital for chromosomal stability and replication. Telomerase is a ribonucleoprotein polymerase which adds telomeric sequences onto the ends of chromosomes to compensate for DNA end replication^[1,2]. Telomerase activity in humans was detected in germline and tumor tissues as well as in established cultured cell lines^[3]. In normal somatic cells, the absence or low expression of telomerase was thought to result in progressive telomeric shortening with each cell division^[4,5]. Therefore, it has been suggested that reactivation of telomerase is a critical step in tumorigenesis and that interference with the regulation of telomerase activity may serve as a basis for cancer therapy^[6,7]. Here we report the effect of SW480, a human colon cancer line, after transfected with antisense hTERT and investigated the potential value of telomerase as a target for antisense gene therapy in colon cancer.

MATERIALS AND METHODS

Cell culture

SW480 cells, a human colon cancer cell line, generously supplied by Department of Biology, Wuhan University, Wuhan, China, were maintained in RPMI 1640-10 % fetal bovine serum, supplemented with 1 mmol/L L-glutamine, 100 U/ml penicillin plus 100 μ g/ml of streptomycin at 37 °C under 5 % CO₂.

Cell counting

Cells were counted with 5 g/L of trypan blue staining.

Oligodeoxynucleotides synthesis

Oligodeoxynucleotides synthesis was designed as described by Feng L^[8,9]. Antisense oligodeoxynucleotides (As-ODN) with sequence 5' GGAGCGCGCGCATCGCGGG-3', which can recognize the RNA template region of telomerase, and sense oligodeoxynucleotides (S-ODN), with 5' CCCGCGATGCCGCGCTCC 3' were prepared on the 391 DNA synthesizer, synthesized by SBS Bio-technology Engineering Company of Beijing. The synthesized oligodeoxynucleotides were subjected to electrophoresis (PAGE) and purified (300 V, 1.5 h).

Transfection of oligodeoxynucleotides

The phosphorothiate oligodeoxynucleotides (ODNs) transfection was performed with liposomal transfection reagent DOSPER (Roche Diagnostic GmbH) according to the manufacturer's protocol. Briefly, cells were plated onto 6-well plates and incubated until the cells reached 70-80 % confluency. Before the transfection, DOSPER was diluted with serum-free medium. Then, the desired amount of ODNs was incubated for 15 minutes with diluted DOSPER. The ODNs/DOSPER mixture (100 μ l) was added dropwise in 900 μ l serum-free RPMI 1640. After incubation for 6 hours at 37 °C, 1 ml RPMI 1640 containing 20 % FBS was added into each well. Cells were harvested and analysed after 48, 72, 96 and 120 hours, respectively.

Table 1 Effect of telomerase activity in SW480 cells by ODNs ($\bar{x} \pm s$)

Groups	Active duration				
	24 h	48 h	72 h	96 h	120 h
As-ODN 10 $\mu\text{mol/L}$	0.648 \pm 0.057	0.324 \pm 0.029	0.283 \pm 0.072	0.189 \pm 0.093	0.172 \pm 0.114
S-ODN 10 $\mu\text{mol/L}$	1.082 \pm 0.249	1.272 \pm 0.372	1.204 \pm 0.190	0.902 \pm 0.193	0.909 \pm 0.146
Positive control	1.336 \pm 0.231	1.376 \pm 0.238	1.354 \pm 0.186	1.298 \pm 0.172	1.246 \pm 0.169
Negative control	0.339 \pm 0.181	0.312 \pm 0.139	0.283 \pm 0.086	0.072 \pm 0.039	0.057 \pm 0.028

Telomerase assay

Polymerase chain reaction enzyme linked immunosorbent assay (PCR-ELISA) was performed following the instructions from Roche Diagnostics GmbH of Germany. Briefly, 2×10^6 cells were isolated, mixed with 200 μL protein extraction buffer, and left on ice for 30 minutes. 175 μL supernatant was collected after centrifugation (16 000 \times g, 20 minutes, 4 $^{\circ}\text{C}$). PCR was performed in 50 μL supernatant containing 25 μL transfer reaction mixture, 2 μL protein extract and 2 μL primers, and added to 23 μL nuclease-free water. The PCR condition was as follows: at 25 $^{\circ}\text{C}$ for 30 minutes for primer elongation, and at 94 $^{\circ}\text{C}$ for 5 minutes for telomerase inactivation. Amplification for 30 cycles under the condition at 94 $^{\circ}\text{C}$ for 30 s for denaturation, at 50 $^{\circ}\text{C}$ for 30 s for annealing, and at 72 $^{\circ}\text{C}$ for 90 s for polymerization. Five μL of amplified product and 20 μL denaturated reagent were incubated at room temperature, 225 μL hybridization buffer was then added and mixed, and 100 μL of them was distributed in the wells of a microtitering plate. After 2 hours of incubation (37 $^{\circ}\text{C}$, 300 rpm), the anti-DIG-POD 100 μL working solution was added and incubated for another 30 minutes followed by adding 100 μL TMB substrate solution, and 100 μL stop reagent was added at last. The OD value in each well was read at the wave length of 450 nm and 655 nm on a microtiter plate reader (Bio-RAD Model 550 microplate reader). The results $\text{OD}_{450} - \text{OD}_{655} > 1.5$ unit using a protein extract from immortalized telomerase-positive human embryonic kidney cell (293 cells) were judged as a positive control. The negative control was considered as $\text{OD}_{450} - \text{OD}_{655} < 0.2$ unit by reading the protein extract pretreated with RNase A at 65 $^{\circ}\text{C}$ for 10 minutes. Telomerase activity was considered positive when the value of $\text{OD}_{450} - \text{OD}_{655}$ of a sample was at least 0.2 units higher than that of the negative control, otherwise it would be negative. Each sample was examined for more than twice. The final value was presented as $\bar{x} \pm s$ after a statistical treatment by using *t* test.

Cytologic morphology

Cytologic morphological changes were observed under the Olympus optical microscope and Hitach transmission electron microscope.

Detection of apoptotic cells by flow cytometry

Cells were fixed and stained with propidium iodide (PI, Sigma product). The DNA content of each cell was analyzed by a FACSORT flow cytometry (Becton Dickinson). Briefly, cells were trypsinized, washed once in ice-cold PBS, and incubated with annexin-V- fluorescein/PI, and then analyzed immediately with FACSORT flow cytometry. All data were analyzed using Cell Quest software.

Statistical analysis

Results were expressed as the means \pm SD. Statistical analyses were carried out with the software package SPSS10.0. A *P* value < 0.05 was considered statistically significant.

RESULTS

Effect of telomerase activity in SW480 cells by antisense hTERT ODNs

As-ODN (10 $\mu\text{mol/L}$) and S-ODN (10 $\mu\text{mol/L}$) were transfected into SW480 cells, and the cells were collected at 24, 48, 72, 96 and 120 hours after transfection, respectively. Telomerase activities were measured by TRAP-ELISA. Results were as the followings. (1) Telomerase activity of SW480, transferred with As-ODN, was greatly inhibited when compared with that in the S-ODN. (2) Telomerase activity of SW480, transferred with As-ODN, at 72 and 96 hours after transfection was significantly lower than that at 24 hours, respectively. (3) Telomerase activity of SW480, transferred with As-ODN, was significantly lower than that in the positive control, as shown in Table 1. These findings suggested that this inhibitory action was sequence specific and in a time-dependent manner.

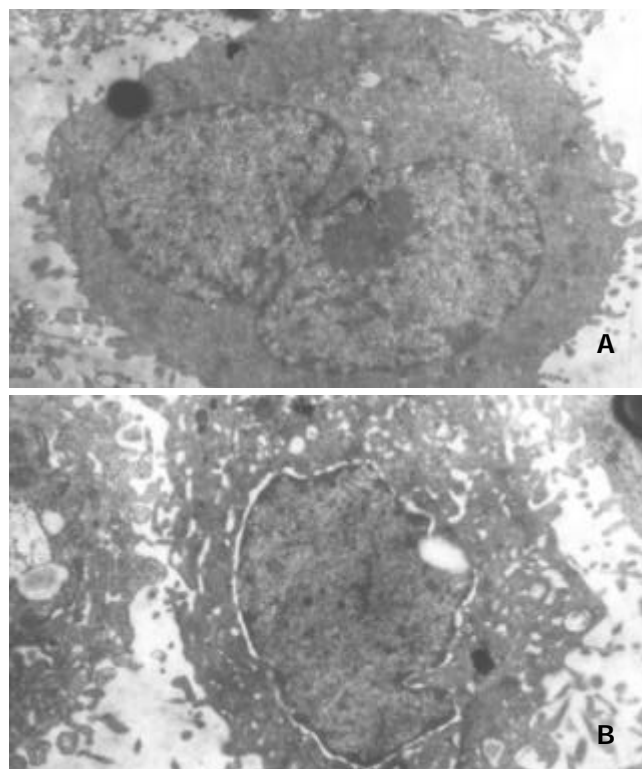


Figure 1 Morphologic observation under transmission electron microscope. A: cell had no apoptotic features; and B: cell rounded up off the plastic, exhibiting cytoplasmic blebbing, fragmentation and chromatin condensation, features of apoptosis.

Effect of antisense hTERT ODNs on induction of SW480 cells apoptosis

Cytologic morphological changes Cytologic morphology of SW480, transferred with 10 $\mu\text{mol/L}$ As-ODN for 3 days was observed under the Olympus optical microscope and Hitachi

transmission electron microscope. It was found that cells rounded up off the plate, exhibiting cytoplasmic blebbing, fragmentation and chromatin condensation, and features of apoptosis. No apoptotic features (normal morphology) were observed in SW480 transfected with 10 $\mu\text{mol/L}$ S-ODN (Figure 1).

Detection of apoptotic cells To determine the apoptotic rate, SW480 was treated with 10 $\mu\text{mol/L}$ As-ODN and S-ODN for 2 days. After permeabilization, cells were stained with propidium iodide and analysed with flow cytometry. The apoptotic rate of SW480 cells transfected with As-ODN increased (at 48 hours, 4.82 ± 0.39 ; 72 hours, 8.76 ± 0.14 ; and 96 hours, 9.25 ± 0.37 , respectively, $P < 0.001$), but no significant changes of apoptosis were observed in SW480 cells transfected with 10 $\mu\text{mol/L}$ S-ODN as shown in Table 2, which indicated that this apoptotic induction was sequence specific and in a time-dependent manner.

Table 2 Effect of ODNs on induction of SW480 cells apoptosis ($\bar{x} \pm s$)

Groups	Active duration		
	48 h	72 h	96 h
As-ODN 10 $\mu\text{mol/L}$	4.82 ± 0.39	8.76 ± 0.14	9.25 ± 0.37
S-ODN 10 $\mu\text{mol/L}$	2.08 ± 0.38	2.96 ± 0.47	2.19 ± 0.29
hTERT blank	1.47 ± 0.14	1.49 ± 0.21	1.44 ± 0.19

DISCUSSION

Three major components of telomerase, i.e. human telomerase RNA component (hTR), human telomerase-associated protein (TEP1), and human telomerase catalytic subunit (hTERT), have been identified^[8,10-14]. hTR, functioning as a template for telomere elongation by telomerase, has been cloned from humans and mice^[8,15] and expressed both in cancer and normal tissues, although its level was inclined to increase with tumor progression^[16]. Human telomerase-associated protein (TEP1), as a human homolog of the Tetrahymena telomerase p80, was expressed ubiquitously and did not correlate with levels of telomerase activity^[10,12]. hTERT, the catalytic protein subunit of human telomerase, contained the reverse transcriptase motif^[12,13]. High levels of hTERT expression were observed in telomerase positive cell lines but not in telomerase negative primary fibroblasts^[12]. hTERT was the limiting component necessary for the restoration of telomerase activity in normal diploid cells which was expressed transiently^[17,18]. Recent studies have demonstrated telomerase activity was significantly associated with hTERT mRNA expression but not with hTR or TEP1 mRNA expression. These findings provide strong evidences that the expression of hTERT was a rate-limiting determinant of the enzymatic activity of human telomerase and that the up-regulation of hTERT expression might play a critical role in human carcinogenesis^[19]. Previously, many studies^[20-23] demonstrated that antisense oligonucleotides against human telomerase RNA resulted in inhibition of telomerase activity and induction of apoptosis in ovarian cancer cells and prostate cancer cells.

In our study, we found that transfection of SW480 with antisense-hTERT ODNs at a concentration of 10 $\mu\text{mol/L}$ significantly inhibited the telomerase activity and induce apoptosis, when compared with positive control cells. The S-ODN with sense sequence did not affect the telomerase activity and induce apoptosis of transfected cells, indicating that this inhibitory and inducing action was sequence specific and in a time-dependent manner.

Recently, it has been shown that telomerase activity was the dominant mechanism providing telomere maintenance to

human immortalized cells. The findings that the ability of cells with long telomeres to proliferate in the absence of telomerase demonstrated that telomerase activity did not require basic replicative functions of these cells, instead, telomerase activity appeared to be required to maintain a minimum telomere length^[24]. However, the exact mechanisms of how telomerase activity is regulated in tumor cells remain poorly understood. Some researchers have shown that telomerase activity was correlated with the growth rate of immortal cells^[25-27], whereas others found no significant association between telomerase activity and proliferative index in the tissue specimens from breast carcinoma^[28], gastric carcinoma^[29], and Wilm's tumor^[30]. Based on the recent findings, we did not observe any correlation between telomerase activity and proliferative index.

Some other studies, have shown that cells expressing high telomerase activity were more resistant to apoptosis than those with low telomerase expression^[22,31,32], and that treatment with antisense telomerase inhibited the telomerase activity and subsequently induced either apoptosis or differentiation, and that regulation of these two distinct pathways might depend on the expression of ICE (Interleukin-1 β -converting enzyme) or CDKIs (Cyclin-dependent kinase inhibitors)^[20]. Interestingly, inhibition of telomerase with an antisense telomerase expression vector not only decreased telomerase activity but also increased the susceptibility to cisplatin-induced apoptotic cell death in cisplatin-resistant U251-MG cells^[22].

Inhibition of telomerase activity has been proposed as a potential approach to the treatment of human malignancy. It is suggested that telomerase inhibition may serve as an effective tool for eliminating tumor cells that have short telomeres. Such tumors may provide reasonable targets for the agents that inhibit telomerase. These experiments await the development of specific inhibitors for the components of the telomerase complex.

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