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Targeted therapy of human laryngeal squamous cell carcinoma *in vitro* by antisense oligonucleotides directed against telomerase reverse transcriptase mRNA

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Abstract

A number of different approaches have been developed to inhibit telomerase activity in human cancer cells. In this study, the effect of antisense oligonucleotides (ODNs) by targeting human telomerase reverse transcriptase (hTERT) mRNA in a laryngeal cancer cell line (Hep-2) was investigated. A 20mer antisense oligodeoxynucleotide targeting the most open part of hTERT mRNA (anti-hTERT) and a mismatched control sequence were synthesized. Cells were treated daily with oligonucleotides for up to 72 hours. hTERT mRNA expression was measured by the reverse transcription polymerase chain reaction (RT-PCR) assay; telomerase activity by the telomerase PCR ELISA assay kit (TRAP; Boehringer Mannheim, GmbH, Mannheim, Germany). Cell viability after administration of ODNs was determined using the MTT assay. Morphological changes were examined by haematoxylin and eosin staining. The cell cycle was analyzed using flow cytometry.

It was found that antisense treatment induced a decrease in hTERT mRNA expression, telomerase activity, cell growth rate, cell viability, and an increase in apoptosis. The results suggest that inhibition of telomerase activity in Hep-2 cells by short-term antisense treatment against the mRNA of hTERT results in apoptotic cell death. The treatment with anti-hTERT may be useful as a treatment modality for laryngeal squamous carcinoma.

Key words: Telomerase; Antisense oligonucleotide; Larynx; Squamous Cell Carcinoma

Introduction

Telomeres act as protective chromosomal 'end-caps', and their length is maintained by a dynamic equilibrium between processes that shorten and lengthen telomeric DNA.[1–5] Telomere shortening is a check point for signalling the onset of cellular senescence.[6] In normal human somatic cells, telomere size reduces with each division until a critical length is reached and cell death ensues. However, the vast majority of tumour cells have overcome this cellular 'time-bomb' by expressing telomerase – an RNA protein complex that progressively elongates telomeric DNA.[7] Most normal cells (excluding germ line and stem cells) lack this enzyme and thus have a finite doubling capacity.

Genetic therapy using antisense DNA has shown promising results in the treatment of many human diseases. Antisense oligodeoxynucleotides (ODNs) traditionally consist of short stretches of DNA that are complementary to a targeted RNA molecule. Depending on the chemical composition of the antisense ODN, the RNA-DNA hybrids formed (by Watson-Crick base pairing) substrates for Rnase H, which selectively destroys the RNA component. This process prevents the expression of the targeted protein, changing the cellular phenotype.

Laryngeal cancer cells persistently express high levels of telomerase activity, while normal laryngeal epithelium rarely displays it.[8–10] Telomerase activity is mainly regulated by human telomerase reverse transcriptase (hTERT).[11] In this study, the authors explore the effect of antisense (phosphorothioated) ODN targeting hTERT mRNA in a laryngeal cancer cell line (Hep-2).

Materials and methods

Cell line and culture

Hep-2 cells were obtained as a generous gift from the Virus Research Institute, Wuhan University, China. They were incubated in RPMI 1640 medium supplemented with 10 per cent fetal calf serum, 100 u/ml penicillin G sodium, 100 μg/ml streptomycin

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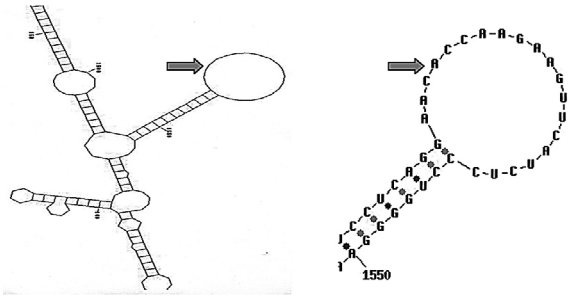

FIG. 1

Predicted MFOLD secondary structure of the human telomere reverse transcriptase (hTERT) mRNA that has a dG = 1808.73. The positions of the hTERT mRNA and the sequence used to design the antisense oligodeoxynucleotides are indicated by the arrows.

sulphate and incubated in five per cent CO_2 at 37ºC. The cells were divided into four groups for treatment: (1) antisense hTERT mRNA(ASODN); (2) mismatched ODN(MODN); (3) Lipofectamine™ reagent alone (LIP); and medium alone as a control.

Antisense oligonucleotides

Based on the hTERT gene cDNA sequence, the analysis of hTERT mRNA using the MFOLD program revealed the most open part to be between residues 1523 and 1542 (Figure 1).[12] An antisense sequence was designed against this part and the oligonucleotide was synthesized, purified and modified by the Dalian TaKaRa Company Limited. The ASODN is 5'-GAG ATG AAC TTC TTG GTG TT 3'. A 20mer MODN was synthesized as a control with the sequence 5'-GAC ATC AAG TTG TTC GTC TT 3'. No homologous sequence, except for the hTERT cDNA, was found by searching the internet with BLAST.

Detection of hTERT mRNA expression by reverse transcription polymerase chain reaction (RT-PCR)

Forty-eight hours after administration of ODNs, 200 to 300 ng of total RNA was reverse transcribed in a 20 µl reaction containing 1 × reverse transcriptase reaction buffer (Gibco-BRL, USA), 200 µM deoxyribonucleoside triphosphates (dNTPs), a 10 ng random hexamer primer (Gibco), 40U RNasin® and 100U Moloney murin leukaemia virus (M-MLV) reverse transcriptase (Gibco) for 45 minutes at 42ºC, and then heated for 10 minutes at 72ºC. After heat inactivation of the reverse transcriptase at 94ºC for four minutes, 2 µl of the reverse transcriptase reaction and 48 µl of the PCR reaction mixture were mixed and amplified with PCR. The primers for the hTERT gene to be amplified were as follows: the hTERT upstream primer was 5'-CGG AAG AGT GTC TGG AGC AA-3'; the hTERT downstream primer was GGA TGA AGC GGA GTC TGG A-3'; the β-actin upstream primer was 5'-GTG GGG CGC CCC AGG CAC CA-3' and the β-actin downstream primer was 5'-GTC CTT AAT GTC ACG CAC GAT TTC-3'(primers from Shanghai Institute of Biochemistry). The condition of the PCR test were: 30 seconds at 90ºC for denaturation; one minute at 60ºC for annealing; one minute at 72ºC for extension, and 30 cycles. PCR products were then electrophoresed on a two per cent agarose gel containing ethidium bromide.

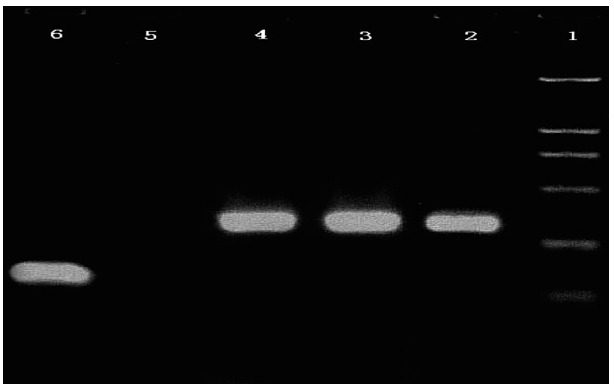

FIG. 2

Expression of hTERT mRNA in Hep-2 cells after treatment. Lanes 1: Marker: 2: Control: 3: LIP: 4: MODN: 5: ASODN: 6: β–actin

Telomerase activity assays

A commercial telomerase PCR ELISA kit (Boehringer Mannheim, GmbH, Mannheim, Germany) was used to determine telomerase activity in all specimens according to the manufacturer's protocol using 20 PCR cycles.[13] Protein extraction and measurement was performed as described.[14,15]

Analysis of cell growth and viability

Cell viability after administration of ODNs was determined using the thiazoyl blue tetrazolium bromide (MTT) method. On the evening of day 0, cells were plated at 1×10^4cell/well (100 µl) in 96-well plates. On the morning of days one to three, at 24 hour intervals, the ODNs were added in the following manner. The culture medium was removed, and 10 µl 5 µM ODN with 0.3 µl Lipofectamine reagent (Gibco-BRL, USA) was added to the appropriate well and incubated for one minute. Medium (90 µl) was then added to each well to make a final ODN concentration of 0.5 µM.[11] After 20 hours, culture medium was removed and dimethylsulphoxide (DMSO) (0.5 mg/mL, Sigma, USA) 20 µl was added to the wells. Four hours later, absorbency was measured by an enzyme immunoassay analyzer at 490 nm.

Morphological changes

Cells were plated at 5×10^5cell/well (1 ml) in 24-well plates. On the morning of days one to three, at 24 hour intervals, the ODNs were added in the following manner. The culture medium was removed, and 100 µl of 50 µM ODN with 3 µl Lipofectamine reagent (Gibco-BRL) was added to the appropriate well and incubated for one minute. Medium (900 µl) was then added to each well to make the final ODN concentration 5 µM. The

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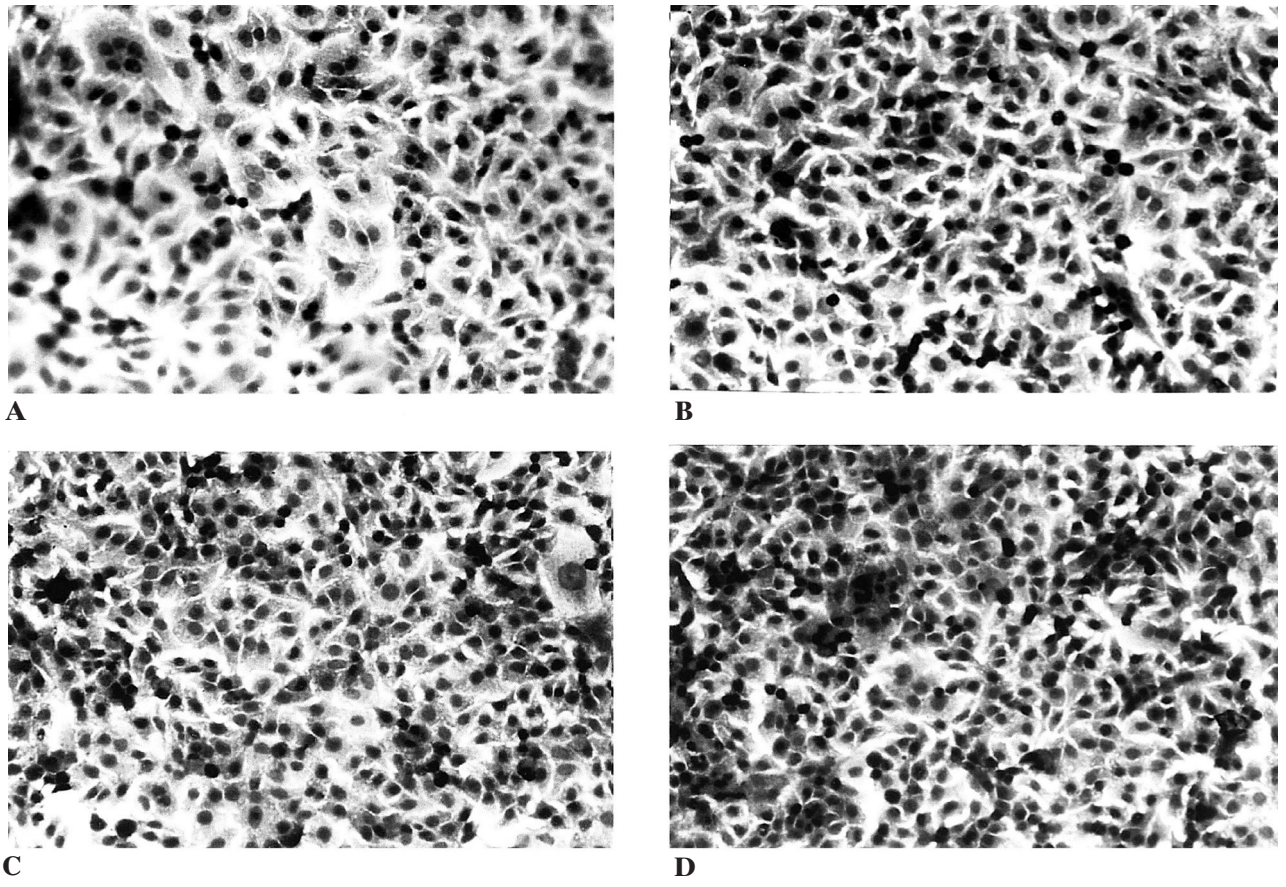

FIG. 3
Morphological changes of transfected and untransfected cells (H & E; ×200).
A: ASODN; B: MODN; C: LIP; D: Control

growth condition of cells was examined by inverse-microscopy everyday. At 72 hours, photographs were taken after haematoxylin and eosin staining.

Apoptosis

Quantification of apoptosis was determined using flow cytometric analysis of cells labelled with propidium iodide (PI) (Sigma, USA). Cells (5×10^5 /well) were plated in 24-well dishes and again treated daily with ODNs at a final concentration of 5 μM. After 72 hours initial ODN treatment, cells were harvested, washed and fixed. Examination of apoptotic cells was performed using a FACScalibur flow cytometer (Becton, Dickinson and Company, USA) equipped with CellQuest software.

Statistics

The one-way analysis of variance (ANOVA) test was employed to determine whether the percentage of viability values differed among the ASODN, MODN, LIP and the control groups. A standard p value of 0.05 was considered statistically significant.

Results

Expression of hTERT mRNA in Hep-2 cells

At 48 hours, after administration of ASODN, the expression level of cell hTERT mRNA was significantly downregulated or inhibited (Figure 2).

Telomerase activity

The telomerase activity decreased significantly after ASODN treatment but remained unaltered after the other treatments. The results are summarized in Table I.

Cell growth and viability

The MTT assay showed that the viability of the ASODN-treated cells was significantly lower than any other group (Table II).

Morphological changes

After administration of MODN, LIP or normal medium, as examined under the inverse-microscope,

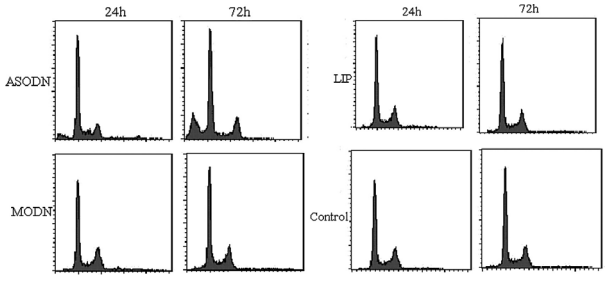

FIG. 4
Flow cytometric analysis: ASODN transduction induced. G_1 arrest and cells in pre-G_1 phase (Apoptosis cells).

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Hep-2 cells grew as normal. In the ASODN group, at 24 hours, some cells became rounder. At 48 hours, this phenomenon was more noticeable. Their volume diminished, chromatin concentrated and density increased and granules appeared. The degree of refraction decreased and cells became detached. The cells became smaller and the nucleolus deflated as seen by haematoxylin and eosin staining. At the same time, cells grew more sparsely (Figure 3).

Apoptosis

In the ASODN group, flow cytometry showed that the percentage of cells in the S phase decreased and that the percentage in the G_0 phase increased. Before the G_0/G_1 phase, there was an apoptotic wave, composed of cells containing a low concentration of DNA (Figure 4).

Discussion

Telomerase is a RNA-dependent DNA polymerase that synthesizes telomeric repeats at the 3'end of the leading DNA strand. Its activity is detected in 85 to 95 per cent of human tumours,[16–18] and is probably critical for sustained tumour proliferation. Maintenance of telomerase length in tumour cell lines can be prevented by the expression of antisense RNA complementary to hTR, leading to cell crisis; supporting telomerase inhibition as a viable strategy for the suppression of tumour growth. Many research experiments have shown that telomerase may be a new target for cancer treatment.[19,20]

It is currently unclear how telomerase is activated or regulated in human tumour cells. The human telomerase complex is comprised of multiple components, but hTERT is the key component for the control of telomerase activity. Specifically, normal human somatic cells can acquire the ability to maintain telomeres and replicate well beyond the Hayflick limit by stable enforced expression of TERT.

Antisense technology acting as a gene seal can specifically block or suppress the expression of targeted mRNA or DNA. Many types of mRNA may be folded on itself in many sites and form its secondary structure, which is called the barrette formula. However, the quantity, length, sequence, magnitude and relative position of the barrette formula varies greatly. There is no common orderliness about secondary structures of RNA. In this experiment, the antisense oligonucleotide was designed according to the most open part of the secondary structure of hTERT mRNA. It might improve the chances of the antisense oligonucletides combining with the targeted mRNA.

In the present research, the authors have used the hTERT ASODN to inhibit its expression so as to influence telomerase activity. From the results shown, the authors have succeeded in downregulating or inhibiting hTERT gene expression, thus reducing the telomerase activity in the Hep-2 cells. In the ASODN group, absorbency (A) was significantly reduced suggesting that some Hep-2 cells had died and some cells had stopped growing. It was found that under the same conditions Hep-2 cell growth was reduced. Under the light microscope cells transfected with ASODN were larger with abundant cytoplasm and decreased nuclear division; the ratio of nuclei to cytoplasm increased as compared with their parent cells. This suggested that the hTERT antisense gene may promote differentiation of Hep-2 cells.

Flow cytometry analysis of cells showed that the number of cells in the S phase was lower and that the number in the G_1 phase was higher in the ASODN group. Before the G_0/G_1 phase there was an apoptotic wave. The authors speculate that ASODN reduced Hep-2 cell growth by suppressing telomerase activity and, as a result, induced programmed cell death. These effects were not found in the MODN and LIP group, showing the specificity against telomerase. In addition, Lipofectamine may be toxic to cells, so it was used as a control in order to observe the real effect of ASODN on Hep-2 cells.

- **Human telomerase reverse transcriptase (hTERT) is a protein complex present in tumour cells, preventing cell death caused by chromosomal shortening**
- **Laryngeal cancer cells express high levels of telomerase activity**
- **Treatment with anti-hTERT may be useful as a treatment modality for laryngeal squamous carcinoma**

Alterations of cell cycle proteins contribute to the development and biological behaviour of malignant tumours.[21,22] Telomerase activity is a sign of malignant neoplasm related to many oncogenes. It was found that *myc* oncogene directly activates the hTERT gene and Sp1 seems to be required for this action of *myc*.[23–27] The relationship between cell cycle proteins and hTERT is still unknown. In this work, the authors examined only the viability of cells by MTT assay after they were

TABLE I

CHANGES OF TELOMERASE ACTIVITY IN CELLS OF DIFFERENT TREATMENTS ($\bar{x}\pm s$)

	n	Telomerase activity
ASODN	6	0.05 ± 0.01
MODN	6	0.14 ± 0.02
LIP	6	0.16 ± 0.02
Control	6	0.17 ± 0.03

*Compared with any other group: $p<0.01$

TABLE II

CHANGES OF CELLS PROLIFERATION IN DIFFERENT TREATMENTS (ABSORBENCY ($\bar{x}\pm s$)

	24h	48h	72h
ASODN	0.63 ± 0.09[1]	0.73 ± 0.16[1]	1.23 ± 0.18[3,4]
MODN	0.83 ± 0.12	1.59 ± 0.12	2.06 ± 0.35
LIP	0.86 ± 0.05	1.45 ± 0.072[3])	2.02 ± 0.36
Control	0.92 ± 0.09	1.75 ± 0.17	2.52 ± 0.39

[1] Compared with any other group: $p<0.001$
[2] Compared with control group: $p<0.05$
[3] Compared with MODN and LIP: $p<0.01$
[4] Compared with control group: $p<0.001$

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transferred for 24, 48, and 72 hours and measured the telomerase activity at 72 hours. Therefore, research is still needed on the effect of ASODN on Hep-2 cells in the longer term, on telomere length and on other oncogene expression.

More and more research has proved that suppressing telomerase RNA and telomerase reverse transcriptase mRNA can both reduce telomerase activity and restrain the growth of cancer cells. hTERT is more specific to malignant neoplasm than telomerase.[28–30] However, it is unclear whether there is another telomerase activator and whether by merely suppressing hTERT gene expression can telomerase activity be suppressed in the long term. Therefore, further investigations on the mechanisms by which telomerase can be suppressed are needed.

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