Antitumor efficacy of human telomerase reverse transcriptase gene antisense oligonucleotide in pancreatic cancer cells

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Abstract. Human telomerase reverse transcriptase (hTERT) is the key component of telomerase catalytic activity, and is associated with tumorigenesis. Recent evidence suggests that the down-regulation of hTERT could rapidly induce an antiproliferative effect in tumor cells, independent of the telomere-elongating function of the enzyme. To test the immediate antitumor efficacy of this down-regulation, antisense oligodeoxynucleotides (AS-ODN) targeting hTERT mRNA were transfected into two human pancreatic cancer cell lines in vitro. In both cell lines, single transfection with AS-ODN decreased the level of hTERT mRNA expression in a dose-dependent manner (from 0.05 to 0.2 μM), while transfection with 0.2 μM hTERT AS-ODN for 24 h achieved the maximum down-regulation of hTERT mRNA. Additionally, 0.2 μM AS-ODN significantly reduced telomerase activity in the cell lines. However, after the first transfection with 0.2 μM AS-ODN, almost no inhibition of cell proliferation was observed in either of the lines, while multiple consecutive transfections with the same concentration of AS-ODN resulted in a continuous reduction in cell viability, the significant inhibition of colony formation ability and increased cell apoptotic rates. Cell cycle analysis indicated that hTERT AS-ODN mainly arrested the cell cycle at the G0/G1 phase in the cells. The data validate an antisense oligonucleotide approach to hTERT inhibition therapy in pancreatic cancer cells.

Introduction

Pancreatic cancer is one of the most common causes of cancer-related mortality in the world, and the incidence of the disease has been steadily increasing. Surgery is the only viable treatment option; however, the cancer is usually diagnosed at a late stage, making tumor resection possible in less than 20% of patients (1,2). Less than 5% of all patients diagnosed with pancreatic cancer can expect to survive over 5 years (2,3). Although a great deal of attention has been directed towards the therapeutic treatment of pancreatic cancer, including various adjuvant, neo-adjuvant, locoregional or radio-chemotherapy strategies, the prognosis for patients has not improved significantly, and developing new therapeutic strategies for the treatment of the disease is a top priority.

Telomerase is a ribonucleoprotein enzyme responsible for adding telomeric repeats to the 3' ends of chromosomes. The activation of telomerase is thought to be essential to the stabilization of telomere length and for overcoming replicative senescence, which may lead to cellular immortality (4). Telomerase is activated in the vast majority of cancer cells (5,6), including those of pancreatic cancer (7), but is usually repressed in most normal somatic tissue. This suggests that telomerase activation may be a critical step in the progression to cellular immortality and carcinogenesis. The relative tumor selective expression of telomerase has made the enzyme an appropriate target for the development of new anticancer therapies. Telomerase consists of a protein reverse transcriptase, hTERT, and an RNA component, hTR. hTTR is ubiquitously expressed in normal cells, while it is the presence of hTERT that confers telomerase activation (8). Without the expression of hTERT, no telomerase activation takes place. Consequently, the elongation and capping of telomeres does not occur. Uncapped or critically shortened telomeres cause cell cycle arrest and/or apoptosis (9). Thus, strategies targeting hTERT may be a more attractive approach to the inhibition of telomerase activity and gene therapy of cancer. A number of methods have been developed to achieve the inhibition of telomerase and cell proliferation in tumor cells, including those based on the use of antisense oligonucleotides (10-14), RNA interference (15-18), peptide nucleic acids (19) or hammerhead ribozymes directed against hTERT (20).

In this study, sequence-specific antisense oligonucleotides targeting the coding region of the protein component...
of human telomerase were designed to examine whether
telomerase activity and hTERT mRNA could be inhibited, and
to test the antitumor efficacy of this inhibition in pancreatic
cancer-derived cells that exhibit different sensitivities to
common chemotherapeutics.

Materials and methods

Design and synthesis of antisense phosphorothiate oligomers.
Based on the hTERT gene cDNA sequence (4015 nt; accession
no. AF01 5950), the antisense oligonucleotide was designed
to be complementary to the translation initiation region of
hTERT mRNA. The antisense oligodeoxynucleotide sequence
(AS-ODN) was 5'-GGAGGCGCGGATCGCGGG-3', and the
control non-specific oligodeoxynucleotide sequence
(NS-ODN) was 5'-CATTTCTTGCTCTCCACGG-3', having
the same base composition as the antisense oligonucleotide but
with a different sequence. The oligodeoxynucleotides were
fully phosphorothioated, and were synthesized by Invitrogen
(Carlsbad, CA, USA). Their lack of significant interfering
homology was validated using BLAST analysis.

Cell culture and transfection. The relatively sensitive
pancreatic cancer cell line BxPC-3 and the relatively resistant
pancreatic cancer cell line panc-1 were kindly provided by
the Center Laboratory of The Second Changzhou People's
Hospital, China, and were routinely incubated in DMEM
(Gibco BRL), 4 mM glutamine, 50 U/ml penicillin and 50
μg/ml streptomycin. Cells were grown at 37˚C in a humidified
channel containing 95% air and 5% CO₂. The transfection
of the oligonucleotides was performed using Oligofectamine™
Reagent (Invitrogen) according to the manufacturer's
instructions. Briefly, the cells were seeded the day before the
experiment in different culture plates at various densities per
well. On the day of the experiment, they had reached 30-50% confuence and were transfected with 0.2 μM oligofecta-
mamine and 0.2 μM of oligonucleotides in serum-free DMEM,
icubated at 37˚C for 4 h, and then added to various volumes of
growth medium containing 3x the normal concentration of
serum according to the different culture plates without the
removal of transfection mixture. Cells were transfected once
or every 2-3 days.

Quantitative RT-PCR. The pancreatic cancer cell lines BxPC-3
and panc-1 were harvested with trypsin, washed with PBS
and collected by centrifugation at 1,000 rpm for 5 min. Total
RNA was extracted using the SV Total RNA isolation system
(Promega, Madison, WI, USA) following the manufacturer's
protocol. The purity and quality of the total RNA were
measured by the Bio-visible spectrophotometer (Eppendorf,
Germany), and 1% agarose gel electrophoresis was used to
assess the integrity of the obtained RNA. cDNA was a total
volume of 20 μl was synthesized using a reverse transcription
system containing reverse transcriptase (Promega, Madison,
WI, USA) according to the manufacturer's protocol. Real-time
quantitative PCR of the target hTERT gene with β-actin as
an internal control was carried out on an iycler iQ Multi-
color real-time PCR Detection System (Bio-Rad Laboratories
Inc.). The 20-μl PCR reaction mixture was composed of
1x primers and probe mixture (Applied Biosystems, Foster
city, CA. Assay IDs: hTERT, Hs99999022_m1; β-actin,
Hs9999903_m1), and 1x Absolute QPCR Mix (ABgene,
Surrey, UK). PCR conditions were 50˚C for 2 min and 95˚C
for 15 min, followed by 45 cycles at 95˚C for 15 sec and 60˚C
for 1 min. Relative gene expression quantifications were
calculated using the comparative Ct method with β-actin as
an endogenous control and untreated cells as calibrator. The
final results were determined using the 2-ΔΔCT formula (21).

Telemetric repeat amplification protocol-silver staining assay.
Improved telemetric repeat amplification protocol (TRAP)-
silver staining assay, based on the protocol introduced by Kim
et al (5), was used to assess telomerase activity. Briefly, 20 μl
PCR products were loaded on 12.5% polyacrylamide gel and
resolved by electrophoresis at 180 EV for 1-2 h. After electro-
phoresis, the gel was immobilized and rinsed in 10% glacial
acetic acid for 25 min, washed in deionized water for 10 min
and soaked in 0.2% AgNO3 for 15 min, then washed in
denized water for 5 min, immobilized and rinsed in 5%
glacial acetic acid for 10 min, and again washed in deionized
water for 5 min. Quantitative analysis was performed using a
gel imaging system (Bio-Rad, USA) that allowed densitometric
(A) evaluation of the digitized image. The amplified telomerase
products were of heterogeneous length and created a ladder
pattern of bands, each representing the addition of a hexa-
nucleotide teleric repeat by telomerase. Telomerase activity
was quantified by measuring the total density signal of the
telomerase ladder bands (A) and the formula: relative telo-
merase activity=sample A/untreated sample A.

Cell viability assay. Cytotoxicity was determined by the
CellTiter 96 AQueous One Solution Cell Proliferation Assay
kit (Promega). Briefly, tumor cells growing in log-phase were
trypsinized and seeded at 2x10⁵ cells/well in 96-well plates
and allowed to attach overnight. The medium in each well
was replaced with fresh medium or medium with various
concentrations of the drug in at least 6 replicate wells every 2
days. On the second day following the first to fifth trans-
fections, one-fifth of the value of CellTiter 96 AQueous
One Solution was added to each well and incubated for an
additional 3 h. Absorbance was determined with a microplate
reader (Bio-Rad) at 490 nm. The blank control wells were used
for zeroing absorbance. Each experiment was allocated ten
wells containing drug-free medium as the control. The percen-
tage of cell survival was calculated using background-corrected
absorbance as follows: % cell viability = 100 x Aexperimental well/
Auntreated control well. Each experiment was performed in triplicate,
with representative data presented.

Colony-forming cell assay. Pancreatic tumor cells were
transfected 3 times consecutively with AS- or NS-ODN at
0.2 μM. Subsequently, the transfected and parental cells
(300 cells/well) were plated in triplicate in 60-mm Petri dishes.
On day 7, the plates were fixed in 70% methanol and treated
with Giemsa stain. Clonogenic survival was determined by
counting the macroscopically visible colonies.

Apoptosis assay. Cell quantification of apoptotic cells was
performed using an Annexin-V-fluorescein isothiocyanate
Cell cycle analysis. Analysis of the cell cycle was performed using a flow cytometer (FACScalibur, Becton-Dickinson). The percentage of cells in different phases of the cell cycle was analysed using a flow cytometer (FACScalibur, Becton-Dickinson, Franklin Lakes, NJ, USA).

Briefly, on the second day after the third transfection with AS-ODN, cells were harvested, washed twice and stained with 25 μg/ml propidium iodide solution. The fluorescence of the stained cells was analysed using a flow cytometer (FACScalibur, Becton-Dickinson). A minimum of 1x10⁴ events was measured for each sample. The percentage of cells in different phases of the cell cycle was established on DNA plots using CellFit software according to the sum of broadened rectangles model (Becton-Dickinson).

Statistical methods. Values are expressed as the means ± standard deviations. Statistical comparison was performed using the Student's t-test. p<0.05 was considered statistically significant.

Results

hTERT antisense oligodeoxynucleotides down-regulate hTERT mRNA expression and telomerase activity in pancreatic cancer BxPC-3 and panc-1 cell lines. We first examined the level of hTERT mRNA expression and telomerase activity in BxPC-3 and panc-1 using quantitative RT-PCR and TRAP-silver staining assay. The expression level of hTERT mRNA was found to be 2.4-fold higher in panc-1 cells compared to BxPC-3 cells. (t=5.05, p=0.007). According to the results of quantitative RT-PCR, the level of telomerase activity was also higher in panc-1 parental cells compared to BxPC-3 parental cells (p<0.05). We further examined whether hTERT AS-ODN could down-regulate the expression level of hTERT mRNA in the cell lines. As shown in Fig. 1, hTERT mRNA expression was decreased by AS-ODN in the cell lines in a dose-dependent manner (from 0.05 to 0.2 μM). Treatment with 0.2 μM hTERT AS-ODN for 24 h achieved the maximum down-regulation of hTERT mRNA: 28.76 and 35.02% in BxPC-3 and panc-1 cells, respectively, relative to the oligofectamine-treated control. Further dose escalation to 0.25 μM did not result in more pronounced down-regulation. Thus, 0.2 μM AS-ODN was selected for subsequent analysis. The NS-ODN control sequence was not found to down-regulate hTERT mRNA expression levels in either of the cell lines. Additionally, we examined the effect of suppressing hTERT mRNA on telomerase activity. According to the results of quantitative RT-PCR, NS-ODN control cells exhibited significant telomerase activity, equal to that of the oligofectamine-treated control cells, whereas 0.2 μM AS-ODN clones expressed significantly decreased levels of telomerase activity in the cell lines (Fig. 2) at 24 h as indicated by the TRAP-silver staining assay.

Effects of hTERT antisense oligodeoxynucleotides on cell proliferation in pancreatic cancer BxPC-3 and panc-1 cell lines. We next investigated whether the down-regulation of hTERT mRNA and telomerase activity could affect cell proliferation in the cell lines. Multiple consecutive transfection cycles (up to five) were performed. After the first transfection, almost no inhibition of cell proliferation was observed. On the second transfection, a reduction in cell viability was achieved in BxPC-3 cells in comparison with the oligofectamine treated control (p<0.05), compared to an only moderate inhibition in panc-1 cells. Multiple consecutive transfections with 0.2 μM hTERT AS-ODN resulted in further inhibition of cell proliferation. This suggests that the down-regulation of hTERT mRNA or telomerase activity could result in the inhibition of cell proliferation.

Effects of hTERT antisense oligodeoxynucleotides on colony formation ability in pancreatic cancer BxPC-3 and panc-1 cell lines. To further investigate the effect of hTERT AS-ODN on cell growth, colony formation assays were performed in the two cell lines. The ability of isolated cells to proliferate and generate colonies was clearly reduced in the cells after three transfections (Fig. 4). In particular, BxPC-3 cells transfected with AS-ODN showed a remarkable decrease of ≥50% in the relative colony number. Furthermore, colonies arising from
AS-ODN-treated cells were smaller than colonies originating from NS-ODN-treated cells.

**Discussion**

In the present study, we evaluated the effect of antisense hTERT gene therapy on tumor growth in the human BxPC-3 and panc-1 pancreatic cancer cell lines *in vitro*. We initially demonstrated that the level of hTERT mRNA expression and telomerase activity was higher in panc-1 cells than in BxPC-3 cells, and that an antisense oligodeoxynucleotide complementary to the translation region of hTERT mRNA inhibited the expression of hTERT mRNA and telomerase activity in both cell lines. Subsequently, we showed that antisense hTERT inhibited cell proliferation in a relatively short period of time, mainly arresting the cell cycle at the G0/G1 phase. BxPC-3 cells were more sensitive than panc-1 cells to hTERT AS-ODN. These results suggest that the rapid anti-proliferative effect of antisense hTERT therapy in human pancreatic cancer may be independent of telomere shortening.

The activation of telomerase is a critical event in cell immortalization and tumorgenesis, and the up-regulation of human telomerase reverse transcriptase (hTERT) mRNA expression is in turn a key step in the activation of telomerase (5,22,23). Previous studies have indicated that telomerase activity is strongly correlated with an abundance of hTERT mRNA but not of hTER (23-25), and that ectopic expression
of hTERT in somatic cells is sufficient to restore telomerase activity (8,26-28). Other reports suggest that the down-regulation of hTERT expression or the expression of dominant-negative hTERT could inhibit telomerase activity (13,29-33). For the present experiments, we selected two pancreatic cancer cell lines, BxPC-3 and panc-1, which are respectively relatively sensitive and resistant to gemcitabine, the first line reference treatment for pancreatic cancer patients. Apparently, the higher the level of hTERT mRNA and telomerase activity, the more resistant the cancer cells were to gemcitabine (data not shown). We then demonstrated that treating the pancreatic cancer cells with hTERT AS-ODN decreased the level of hTERT mRNA expression in the cell lines in a dose-dependent manner (from 0.05 to 0.2 μM), and that transfection with 0.2 μM hTERT AS-ODN for 24 h achieved the maximum down-regulation of hTERT mRNA in both lines. According to the results of quantitative RT-PCR, single transfection with 0.2 μM AS-ODN was capable of down-regulating telomerase activity by 0.3- and 0.47-fold in BxPC-3 and panc-1 cells, respectively, relative to the oligofectamine-treated control. This suggests that hTERT AS-ODN is an appropriate agent for the down-regulation of telomerase activity in cancer cells.

Figure 3. Effects of hTERT antisense oligonucleotides on the cell viability of (A) BxPC-3 and (B) panc-1 cells. Cell viability was dependent on the number of consecutive transfections. The relative cell viability normalized to the oligofectamine-treated controls is displayed. Data represent the mean values ± standard deviation of at least three independent experiments. *P<0.05 vs. the oligofectamine-treated control.

Figure 4. Colony formation ability in (A) BxPC-3 and (B) panc-1 cells. The AS-ODN-transfected cells showed much fewer colonies than the NS-ODN-transfected cells. The relative colony formation ability normalized to the oligofectamine-treated control is displayed. Data represent the mean values ± standard deviation of at least three independent experiments. *P<0.05 vs. the oligofectamine-treated control.

Figure 5. Changes in the cell-cycle distribution of (A) BxPC-3 or (B) panc-1 cells. An analysis of cell cycle distribution revealed that the number of cells in the S phase was decreased and the number of cells in the G0/G1 phase was increased in AS-ODN-transfected cells.
Recent studies suggest that hTERT can maintain cell survival and proliferation by means of a telomere-elongating (8) or telomere-capping process of the enzyme (34,35). As a consequence, telomerase inhibitory approaches based on interference with hTERT expression might affect tumor cell proliferation by inhibiting one or both of the above functions. As for the inhibition of telomere-elongating function, most studies have demonstrated that cancer cell death was observed only after many cell divisions over a long lag-phase before the telomeres were reduced to critical lengths (14,36). While it has also been demonstrated that the down-regulation of hTERT by treatment with antisense oligonucleotides or hammerhead ribozyme rapidly induces programmed cell death in human tumor cells, independent of the telomere-elongating function (13,37,38), these rapid effects appear to be caused by deficient telomere capping (9,34,35,37,38). In the present study, after the first transfection with 0.2 μM AS-ODN, almost no inhibition of cell proliferation was found in either of the cell lines, while multiple consecutive transfections with the same concentration of AS-ODN resulted in a continuous reduction in cell viability and the significant inhibition of colony formation ability. In particular, BxPC-3 cells transfected with AS-ODN showed a remarkable decrease of ≥50% in the relative colony number. Multiple tranfections with hTERT AS-ODN further increased apoptosis rates in both cells.

Our results are partially consistent with those of the abovementioned studies, though we did not observe the immediate inhibition of cell proliferation in the AS-ODN-transfected cells. It can be speculated that one single transfection with AS-ODN was not enough to interfere with the telomere elongating function or capping process of hTERT in the cells. However, the observed efficient inhibition of cell proliferation over a relatively short time period may be mediated by the telomere uncapping process, not by telomere shortening. In fact, as human cells reduce their telomere length by 50-100 base pairs per cell division, a long lag phase is required before growth arrest can be obtained, even in tumor cells with relatively short telomeres (39). The consecutive monitoring of telomere length or telomere state will be the subject of our next study.

It is well known that cells in the S phase possess the highest proliferating capacity, while cells in G0/G1 phase possesses the lowest one. Telomerase activity is also regulated in a cell cycle-dependent manner (40). For example, telomerase activity is maximally detected in the S phase, but is barely detectable in the G2/M phase (41). The analysis of cell cycle distribution in our study showed that AS-ODN mainly arrested the cell cycle in the G0/G1 phase. Thus, we speculate that the inhibition of cell proliferation using hTERT AS-ODN in our study may be mediated by the down-regulation of telomerase through the arresting of the cell cycle in the G0/G1 phase.

In conclusion, our results indicate that hTERT AS-ODN down-regulates the expression of hTERT mRNA and telomerase activity, and arrests cancer cell growth over a relatively short period of time. The most likely mechanism behind this is the telomere uncapping process, which arrests cell cycle progression and results in the inhibition of cell proliferation or apoptosis. These findings validate an antisense oligonucleotide approach to hTERT inhibition therapy in pancreatic cancer cells, and should be further explored in vivo.
References