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Research Article

Oligomer-mediated modulation of hTERT alternative splicing induces telomerase inhibition and cell growth decline in human prostate cancer cells

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Abstract. The expression of telomerase in human cells is strictly controlled by multiple mechanisms including transcription and alternative splicing of telomerase reverse transcriptase (hTERT). In this study, we demonstrated the possibility of modulating the hTERT splicing pattern in DU145 human prostate carcinoma cells through the use of 2′-O-methyl-RNA phosphorothioate oligonucleotides targeting the splicing site located between intron 5 and exon 6 in the hTERT pre-mRNA. An 18-h oligonucleotide exposure induced a decrease in the full-length hTERT transcript and a concomitant increase in the alternatively spliced transcripts, which resulted in significant inhibition of telomerase catalytic activity. Moreover, exposure to the R7 oligomer (which induced the most pronounced modulation of the hTERT splicing pattern and the greatest telomerase inhibition) caused a marked reduction in DU145 cell growth and the induction of apoptosis starting 2 days after treatment. Such data support the concept that down-regulation of hTERT expression can cause short-term effects on tumour cell growth, which are telomere-shortening independent.

Key words. Alternative splicing; hTERT catalytic subunit; 2′-O-methyl phosphorothioate oligoribonucleotide; DU145 prostate cancer cell line; apoptosis.

Human telomeres are DNA-protein structures located at the end of chromosomes, which consist of short repetitive motifs $(T_2AG_3)_n$ and associated proteins and are necessary to protect the chromosome from end-to-end fusion and degradation [1]. The ability to maintain telomere length, which provides cells with an unlimited proliferative potential, is considered one of the six hallmarks of cancer [2]. In human tumour cells, telomeres are maintained by the RNA-dependent DNA polymerase telomerase [3]. The enzyme is reactivated in 85–90% of the most common human cancers [4], and experiments have demonstrated that inhibition of telomerase affects the growth potential of cancer cells [5]. Based on these findings, interference with telomerase function appears a useful strategy to develop new anti-cancer approaches [6].

The telomerase core enzyme is composed of the catalytic subunit telomerase reverse transcriptase (hTERT), which possesses structural and functional properties similar to those of reverse transcriptases, and the RNA subunit hTR, which acts as a template for de novo synthesis of telomeric sequences [7]. Several lines of evidence indicate that expression of hTERT is the rate-limiting factor for telomerase activity [7]. The entire hTERT gene extends over ≈35 kb consisting of 16 exons and 15 introns [8], and its expression is tightly controlled by multiple regulatory mechanisms including gene amplification, transcriptional regulation mediated by several transcrip-

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tion factors, epigenetic modifications and alternative splicing [6, 7].

Alternative splicing of a gene transcript leads to different mRNAs with distinct functions and may act as a switch to turn on or turn off gene expression [9]. A variety of alternatively spliced cancer-associated genes have been identified including Bcl-2, Bcl-x, Bax, Bim, Fas, caspase 2, CD44, p73, BRCA2, Wilms' tumour suppressor gene, cathepsin B [10], HER2 [11], survivin [12] and hTERT [13]. To date, seven alternatively spliced variants of hTERT [four deletion variants (α^-, β^-, $\alpha^-\beta^-$, γ^-) and three insertion variants (1, 2 and 3)] have been identified by RT-PCR [14, 15]. The α^- transcript lacks 36 nucleotides from the 5′ end of exon 6, which codes for the A domain belonging to the reverse transcriptase motifs of the enzyme; the β^- transcript is characterised by deletion of the entire exons 7 and 8, which gives rise to a termination codon causing premature ending of translation; the $\alpha^-\beta^-$ transcript displays the combination of both deletions; the γ^- variant generates a transcript lacking exon 11, lying within RT motifs D and E. As alternative splicing removes reverse transcriptase motifs, the deletion transcripts are unlikely to code for functional proteins. Moreover, the α^- splicing variant has been recently suggested to act as a dominant negative inhibitor of telomerase activity [16].

Besides gene transcription, alternative splicing of hTERT plays an important role in the regulation of telomerase activity during embryonic development [14], and such a regulatory mechanism is also active in human tumours [17–19]. As a consequence, the possibility to interfere with hTERT alternative splicing and to modify the relative expression of the different splicing variants could represent a new selective approach for telomerase down-regulation in cancer cells. In the present study, we demonstrate for the first time the possibility to modulate the hTERT splicing pattern in human prostate carcinoma cells by using of 2′-O-methyl-RNA phosphorothioate oligonucleotides (2′-O-methyl-RNA-PTOs) targeting the splicing site located between intron 5 and exon 6 in the hTERT pre-mRNA; this resulted in significant inhibition of the catalytic activity of telomerase, which was accompanied by cell growth decline and induction of apoptosis.

Materials and methods

Cell lines

The androgen-independent DU145 human prostate adenocarcinoma cell line and the U2-OS human osteogenic sarcoma cell line (both from ATCC, Rockville, Md.), which does not express hTERT and maintains telomeres by alternative lengthening of the telomere mechanism [20], were used in this study. They were grown in RPMI-1640 and McCoy's 5A medium, respectively, supplemented with 10% fetal calf serum and 0.1% gentamycin. Cell lines were maintained as a monolayer in the logarithmic growth phase at 37 °C in a 5% CO_2 humidified atmosphere.

Oligonucleotides and liposome-mediated transfection

Seven RNA oligonucleotides containing uniform modifications at the 2′ position of the ribose (2′-O-methyl-) and phosphorothioate linkages (PTOs), in which one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by sulphur, were used in the study. The 18-mer 2′-O-methyl-RNA-PTOs (MWG-Biotech AG, Ebersberg, Germany) were designed to be complementary to a region spanning 17 nucleotides upstream and 16 nucleotides downstream of the splicing site located between intron 5 and exon 6 in the pre-mRNA of hTERT. The oligomer sequences are shown in figure 1. A scramble

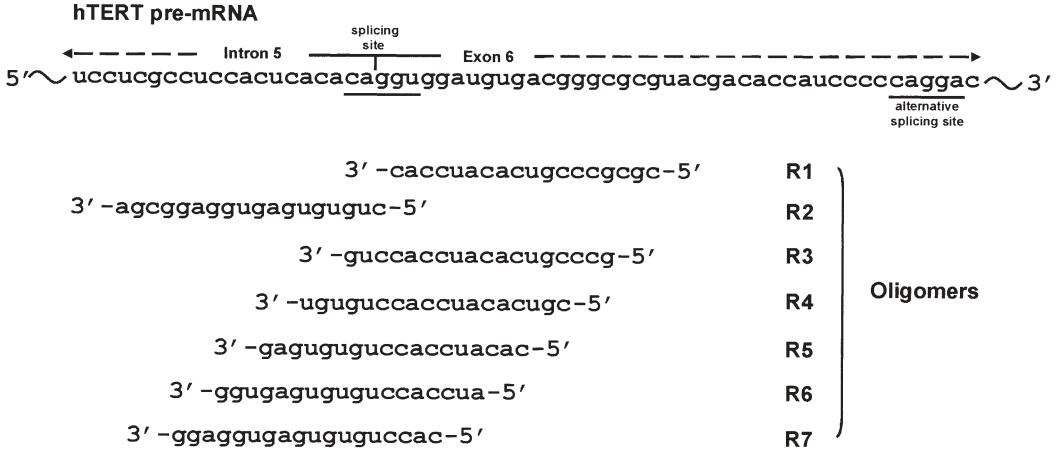

Figure 1. The splicing site located between intron 5 and exon 6 in the hTERT pre-mRNA and the sequences of the 2′-O-methyl-RNA phosphorothioate antisense oligonucleotides.

18-mer 2′-O-methyl-RNA PTO was also used as a negative control. Each oligoribonucleotide was resuspended in sterile water at the appropriate concentration and kept at –20 °C until use.

The oligomers and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP; Boehringer Mannheim, Mannheim, Germany) cationic liposomes were mixed (ratio 1:5) in 20 mM HEPES and incubated at room temperature for 15 min. For DOTAP-mediated oligomer transfection, 2×10^5 cells were seeded in 25-cm^2 flasks and allowed to attach for 24 h. Cells were then transfected for 18 h with DOTAP-oligomer complexes diluted in culture medium to obtain a final concentration of 500 nM for each oligomer and 18 µg/ml of DOTAP. At the end of the transfection, cells were washed with phosphate-buffered saline and incubated in fresh medium for 24 h, harvested and analysed for telomerase activity and hTERT RNA transcript expression.

Telomerase activity detection assay

Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) assay using the TRAPeze kit (Intergen, Oxford, UK) on 1-µg protein extracts, as previously described [17, 18].

RT-PCR analysis of hTERT splicing variant expression

Total RNA (0.5 µg), isolated from cells with TRIzol reagent (Life Technologies, Gaithersburg, Md.), was reverse transcribed using the RT-PCR core kit (Applied Biosystems, Roche Molecular Systems, Branchburg, N. J.). To measure the hTERT splicing variant expression levels, the resultant cDNA was amplified using TERT 2164S and TERT 2620AS primers [14] and by performing 35 cycles of PCR (95 °C for 30 s, 62 °C for 50 s and 72 °C for 50 s) in the presence of 1.5 µCi of [α^{32}P]deoxycytidine triphosphate (3000 Ci/mmol; Amersham Biosciences Europe, Freiburg, Germany). A fragment corresponding to β-actin was co-amplified and used as the standard of the amplification reaction. The PCR products were analysed by polyacrylamide gel electrophoresis. The densitometric value corresponding to each hTERT transcript was quantified by Image-QuanT software (Molecular Dynamics, Sunnyvale, Calif.) and normalised to that of β-actin. The relative expression level of each hTERT transcript was expressed as the ratio between the densitometric value of the specific transcript and the sum of the densitometric values of all the hTERT transcripts present in the sample.

Cell growth inhibition assay

To assess the effects of oligomers targeting hTERT splicing on cell proliferative potential, DU145 and U2-OS cells in the logarithmic growth phase were seeded in six-well plates and exposed to individual oligomers for 18 h by DOTAP-mediated transfer. At the end of treatment, adherent cells were washed with phosphate-buffered saline (PBS) and incubated at 37 °C in a 5% CO_2-humidified atmosphere for 24, 48 or 72 h. The cells were then trypsinised and counted in a particle counter (Coulter Counter; Coulter Electronics, Luton, UK). The percentages of adherent viable cells were determined by the Trypan blue dye exclusion test. Each experimental sample was run in triplicate. The results were expressed as the total number of adherent cells in oligomer-treated samples compared to control untreated samples.

Evaluation of apoptotic cells by fluorescence microscopy

Cells were harvested at different intervals (24, 48 and 72 h) after an 18-h exposure to R7 or R4, washed in PBS and stained with a solution containing 50 µg/ml propidium iodide, 50 mg/ml RNAse, and 0.05% Nonidet P40 for 30 min at 4 °C. After staining, the slides were observed by fluorescence microscopy. The percentage of apoptotic cells was determined by scoring at least 500 cells in each sample by two independent observers.

Western immunoblotting

For protein analysis, total cellular lysates were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% skimmed milk and incubated overnight with primary antibody specific for hTERT (ab 5181; Abcam, Cambridge, UK) [21]. The filters were then incubated with a rabbit anti-mouse IgM (Abcam) for 1 h and, after washing, probed for 1 h with peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences Europe). Bound antibodies were detected using the enhanced chemoluminescence Western blotting detection system (Amersham Biosciences). An anti-PCNA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) was used on each blot to ensure equal loading of protein on the gel. Results were quantified by densitometric analysis.

Telomere length measurement

Total DNA was isolated from treated cells using DNAzol (Invitrogen, Gaithersburg, Md.), digested with 40 U of HinfI, electrophoresed and transferred to a nylon membrane. The nylon filter was hybridised with a 5′ ^{32}P-end-labelled telomeric oligonucleotide probe $(TTAGGG)_4$ by a standard protocol. Filters were autoradiographed and the autoradiographs were scanned (ScanJet IIcx/T; Hewlett Packard, Palo Alto, Calif.). The mean telomere restriction fragment (TRF) length was calculated as previously reported [22].

Telomeric oligonucleotide ligation assay

The telomeric oligonucleotide ligation assay (T-OLA) was carried out as previously described [21, 23]. Briefly,

hybridization and ligation reactions were carried out in 20 µl of a reaction mixture containing 5 µg of native DNA, 0.5 pmol of 5′ ^{32}P-end-labelled $(AATCCC)_4$ oligonucleotide, 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 10 mM dithiothreitol and 1 mM ATP. Each reaction sample was incubated at 50 °C for 16 h. Subsequently, 20 units of Taq DNA ligase was added, and the reactions were extended for a further 5 h. Reaction products were analysed by denaturing PAGE electrophoresis. The gels were dried and exposed to autoradiography film. To ensure an equal DNA loading, 1 µg of DNA from each reaction sample was resolved on a 1% agarose gel and visualised by ethidium bromide staining.

Statistical analysis

Student's t test was used to analyse the differences between control and oligomer-treated cells in terms of hTERT transcript expression level and telomerase activity.

The association between the expression level of the different hTERT transcripts as well as between telomerase activity and the expression of specific hTERT transcripts in individual samples was assessed by means of the Spearman rank order correlation coefficient. All tests were two-sided. P values < 0.05 were considered statistically significant.

Results

Oligomer-mediated modulation of hTERT splicing

The ability of a panel of 18-mer 2′-O-methyl-RNA-PTOs targeting the splicing site located between intron 5 and exon 6 in the hTERT pre-mRNA to modulate the expression of the different hTERT transcripts was assessed by RT-PCR in DU145 cells 24 h after 18-h exposure to 500 nM of each oligomer. By using the primer pair TERT 2164S and TERT 2620AS four amplification products could be detected: the hTERT $\alpha^+\beta^+$ full-length transcript (457 bp), the α^- (421 bp), the β^- (257 bp) and the $\alpha^-\beta^-$ (239 bp) deletion splicing variants (fig. 2). In untreated control cells, the relative expression of the $\alpha^+\beta^+$ transcript was 0.616 ± 0.057 arbitrary units (a.u.), whereas in oligomer-treated cells it ranged from 0.325 ± 0.083 to 0.555 ± 0.051 a.u. (fig. 3A). A statistically significant decrease in the expression of the $\alpha^+\beta^+$ transcript was observed in cell samples exposed to R1, R3 and R7 oligomers with respect to untreated cells ($p < 0.01$, $p < 0.05$ and $p < 0.02$ by Student's t test, respectively). In particular, the percentage decrease ranged from –39.0 ± 2.6% for R1, –43.1 ± 6.4% for R3, to –48.7 ± 9.26% for R7 (fig. 3A).

With the hTERT alternative splicing variants, the relative level of the α^- deletion transcript was found to be 0.066 ± 0.017 a.u. in control cells, and superimposable or slightly enhanced expression values were observed in samples exposed to the different oligomers (from 0.059 ± 0.029 to 0.13 ± 0.079 a.u.) (fig. 3B). The relative expression of the $\alpha^-\beta^-$ transcript, which corresponded to 0.316 ± 0.048 a.u.

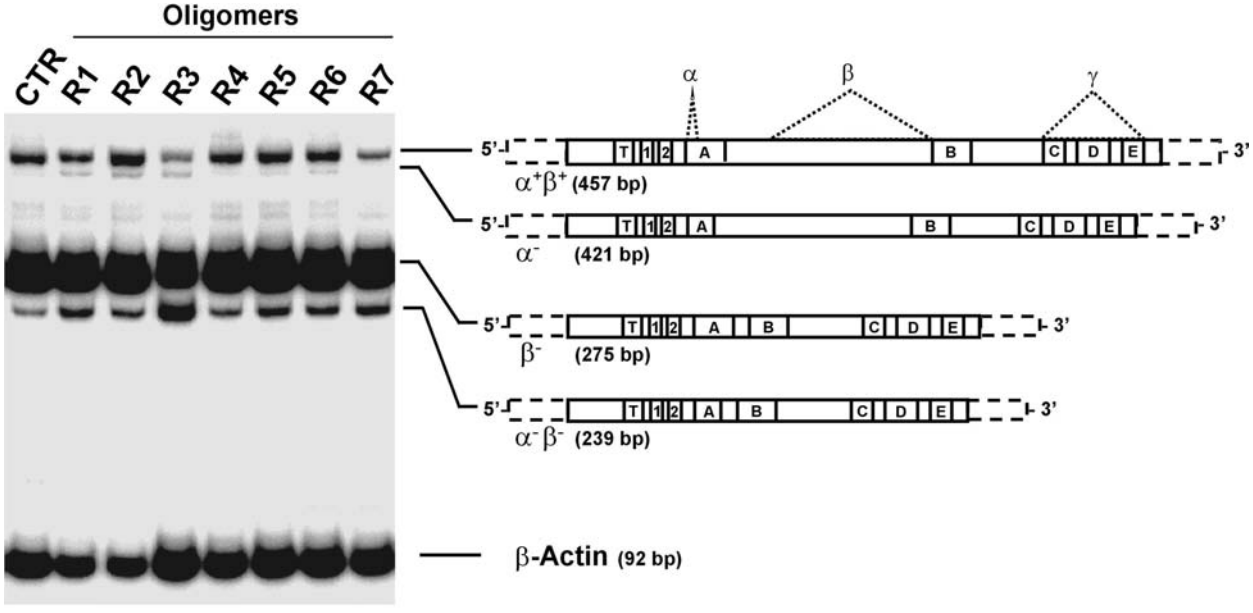

Figure 2. A representative RT-PCR experiment showing the expression of hTERT splicing variants in DU145 cells either untreated (CTR) or exposed to different oligomers (R1–R7). The position and the schematic structure of the different splicing variants are shown. Numbers in parentheses indicate the size of fragments obtained by RT-PCR; dotted lines show the location of deletion splicing sites.

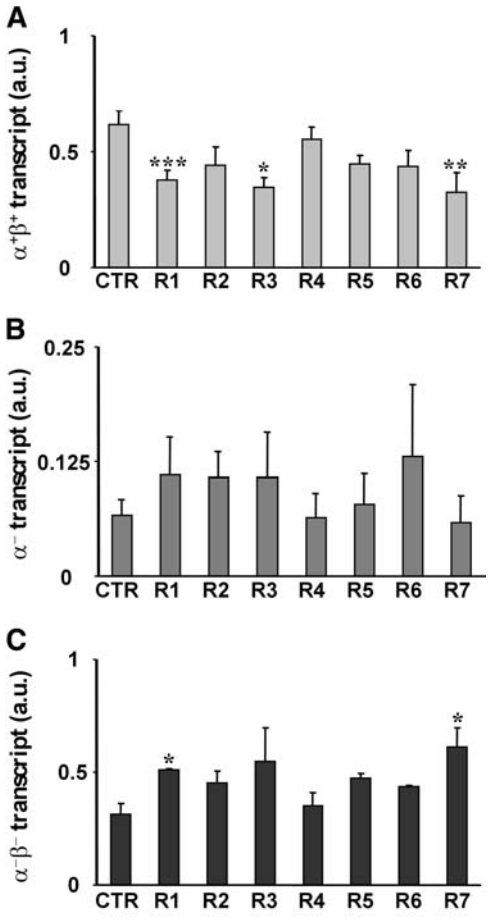

Figure 3. Quantitative analysis of the expression of hTERT $\alpha^+\beta^+$ (*A*), α^- (*B*) and $\alpha^-\beta^-$ (*C*) transcripts in DU145 cells either untreated (CTR) or exposed to different oligomers (R1–R7). Data represent the mean ± SD of at least three independent experiments; * $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$.

in control cells, was consistently increased, although to a different extent (from 0.38 ± 0.033 a.u. to 0.62 ± 0.083 a.u) after treatment of cells with the different oligomers, and significantly ($p < 0.05$ with respect to untreated cells) in cell samples treated with R1 and R7. In particular, such an enhancement reached +69.2 ± 23.9% for R1 and +96.4 ± 13.6% for R7 (fig. 3C). Conversely, we did not observe any differences between control and oligomer-treated cells in the relative expression of the β^- deletion variant, which was present at very high levels in all samples (fig. 2). In cells exposed to individual oligomers, no correlation was observed between the relative expression level of the $\alpha^+\beta^+$ transcript and that of the α^- splicing variant (Spearman rank order correlation coefficient, $r_s = -0.386$). By contrast, an inverse and statistically significant ($p < 0.01$) association was found between the relative expression level of the $\alpha^+\beta^+$ transcript and that of the $\alpha^-\beta^-$ splicing variant ($r_s = -0.905$).

Effects of modulation of hTERT splicing on telomerase activity

To assess whether oligonucleotide-based modulation of the hTERT splicing pattern influences the telomerase catalytic activity, the TRAP assay was carried out on the same cell cultures. The results showed a variable extent of telomerase inhibition (from –33.0 ± 15.2% to –72.0 ± 13.5%) in treated cells with respect to control cells, as a function of the different oligomers (fig. 4A, B). Specifically, the relative telomerase activity in control cells was 1.5 ± 0.2 a.u., whereas it ranged from 0.4 ± 0.2 to 1.0 ± 0.4 a.u. in oligomer-treated cells (fig. 4B). Such inhibition reached statistical significance in cell samples exposed to R5, R6 ($p < 0.05$) and R7 ($p < 0.01$) oligomers (fig. 4B). As expected, exposure of DU145 cells to a scramble oligomer, which was devoid of any effect on hTERT splicing (data not shown), failed to affect telomerase activity (fig. 4C).

When the extent of telomerase activity was analysed as a function of the relative expression level of the different hTERT transcripts in individual oligomer-treated samples, no significant correlation ($r_s = 0.548$) was observed between the catalytic activity of the enzyme and the expression of the $\alpha^+\beta^+$ transcript (fig. 5A). A complete lack of correlation ($r_s = 0.065$) was found between telomerase activity and α^- deletion transcript expression (fig. 5B), whereas a trend towards an inverse correlation ($r_s = -0.700$) was observed between telomerase activity and the level of the $\alpha^-\beta^-$ transcript (fig. 5C). Interestingly, a statistically significant direct correlation ($r_s = 0.810$, $p < 0.05$) was found between the enzyme catalytic activity and the ratio of the $\alpha^+\beta^+$ full-length transcript over the $[(\alpha^-) + (\alpha^-\beta^-)]$ deletion transcripts (fig. 5D).

Effects of modulation of hTERT splicing on protein expression levels

To evaluate the consequence of hTERT splicing modulation on hTERT protein expression, DU145 cells were exposed to 0.5 µM of each oligomer for 18 h and screened for hTERT expression by Western blotting 24 h later (fig. 6A). Results showed that R1–R6 oligomers induced a variable and modest inhibition of hTERT protein expression levels (which ranged from 0. 33 ± 0.07 to 0.24 ± 0.04 a.u.) with respect to untreated cells (0.35 ± 0.06 a.u.) (fig. 6B). In contrast, a significant reduction of hTERT protein levels (0.14 ± 0.04 a.u., $p < 0.05$) was observed in DU145 cells exposed to R7 oligomer (Fig. 6B). As expected, hTERT protein expression was not affected by cell exposure to a scramble oligomer (fig. 6).

Effects of modulation of hTERT splicing on in vitro growth of DU145 cells

To investigate whether the modulation of hTERT splicing affects the proliferative potential of tumour cells, we comparatively assessed the consequences of an 18-h ex-

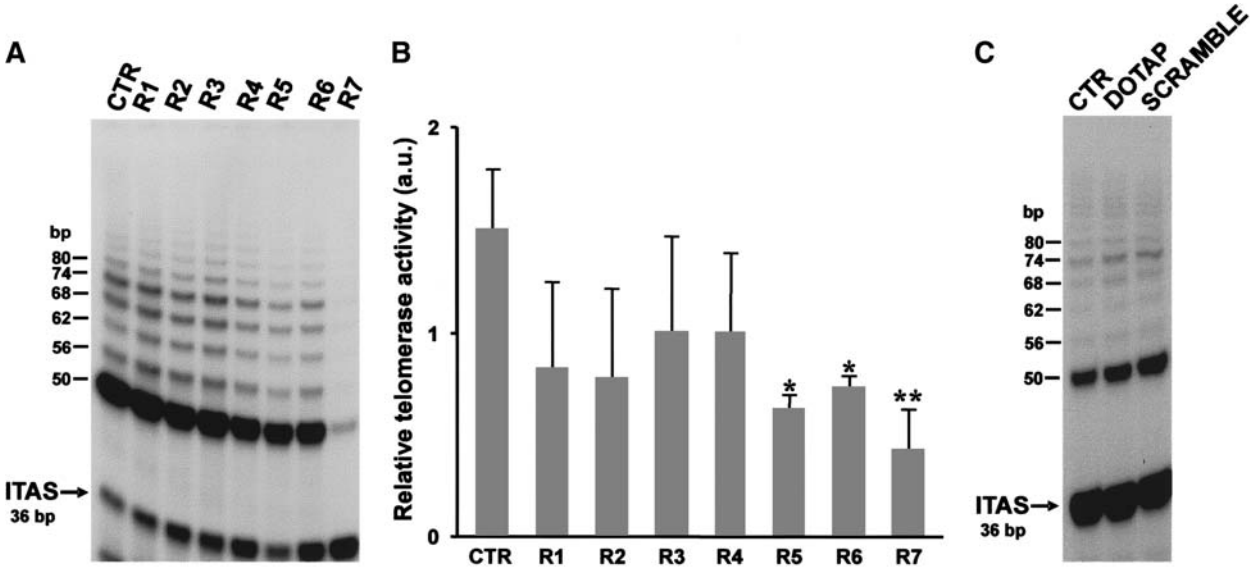

Figure 4. (*A*) A representative TRAP experiment showing telomerase activity in DU145 cells either untreated (CTR) or exposed to different oligomers (R1–R7). The 36-bp internal amplification control band is indicated by an arrow. (*B*) Quantification of telomerase activity in untreated (CTR) and oligomer-treated (R1–R7) DU145 cells. Data represent the mean ± SD of at least three independent experiments; $*p < 0.05$, $**p < 0.01$. (*C*) A representative TRAP experiment showing telomerase activity in DU145 cells untreated (CTR) or exposed to DOTAP alone or to a scrambled oligomer. The 36-bp internal amplification control band is indicated by an arrow. (*A*, *C*) Numbers to the left of each gel indicate the size, expressed as base pairs (bp), of the typical TRAP products.

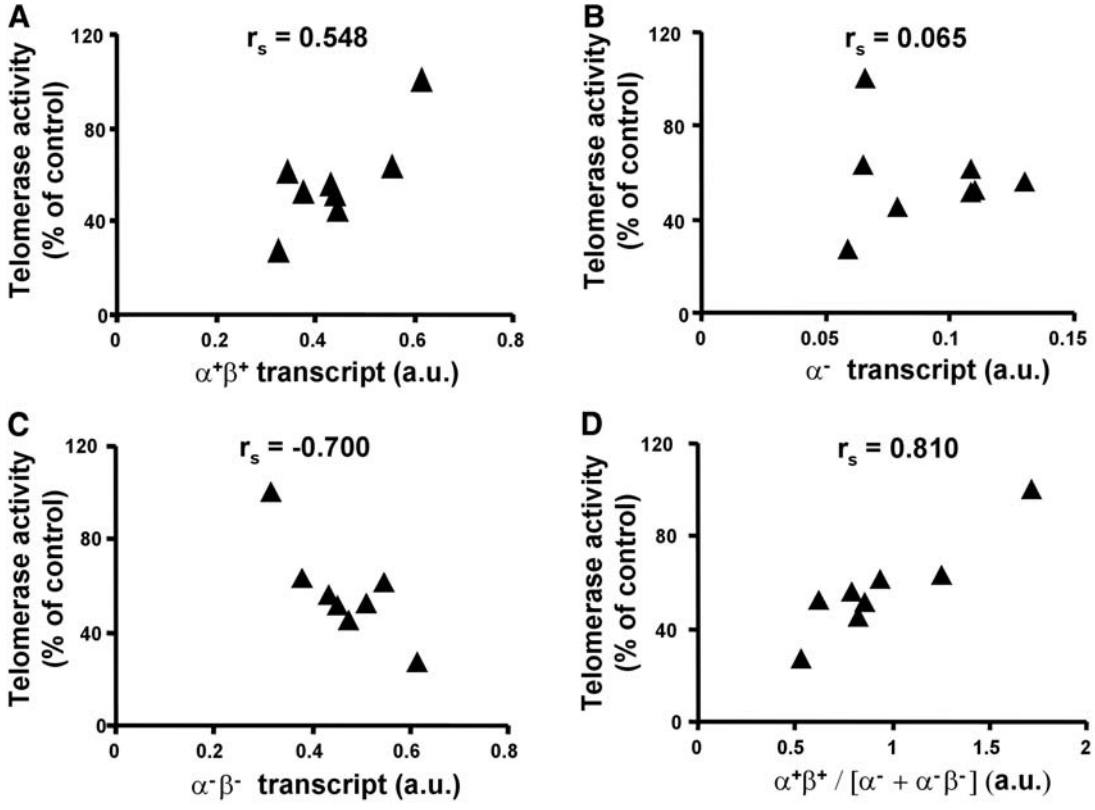

Figure 5. Correlation between telomerase activity and expression of hTERT $\alpha^+\beta^+$ (*A*), α^- (*B*), $\alpha^-\beta^-$ (*C*) or the ratio of the $\alpha^+\beta^+$ over the $[(\alpha^-) + (\alpha^-\beta^-)]$ (*D*) transcripts in DU145 cell samples exposed to the different oligomers. The mean values of transcript expression (see fig. 3) and telomerase activity (see fig. 4B) are reported. The Spearman rank order correlation coefficient (r_s) is indicated.

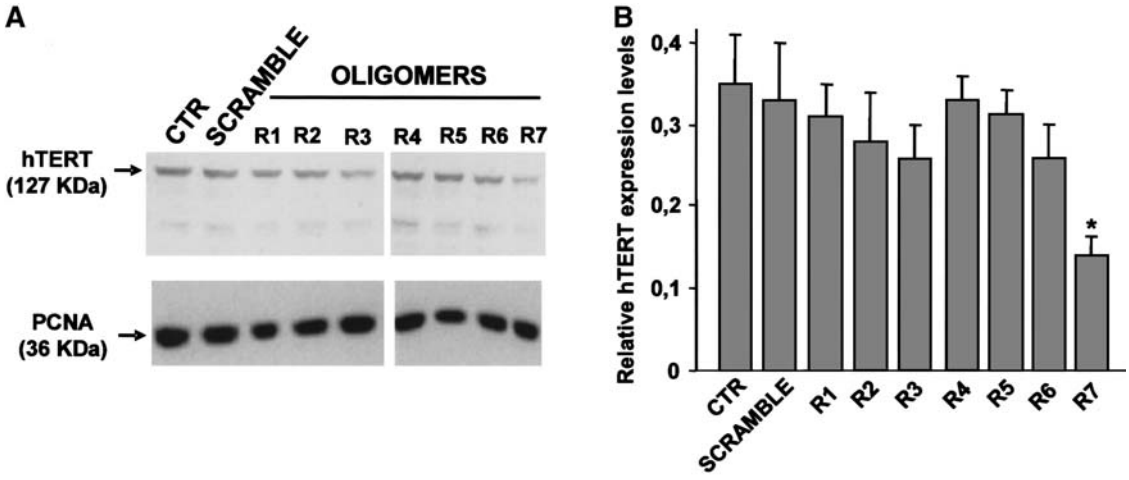

Figure 6. hTERT protein expression in DU145 cells exposed to oligomers. (*A*) A representative Western blotting experiment illustrating the expression of hTERT protein in untreated and oligomer-exposed DU145 cells. Proliferating cell nuclear antigen (PCNA) was used as control for protein loading. (*B*) Quantification of hTERT expression levels. Densitometric signals corresponding to hTERT bands were normalised to that of PCNA and the resulting values were used as relative hTERT expression units for each sample. Data represent means ± SD of three independent experiments; * $p < 0.05$.

posure to two oligomers – one able to significantly modify the hTERT splicing pattern (fig. 3A, C) and inhibit hTERT protein expression and telomerase activity (fig. 4B, 6B), R7; and one devoid of any appreciable effect on these two cellular functions (figs 3A, C, 4B, 6B), R4 – on the in vitro growth of DU145 cells. Exposure to R7 resulted in a decrease in DU145 cell proliferation, which was appreciable starting 24 h after the end of treatment, and the extent of growth inhibition increased as a function of time from treatment (fig. 7). Conversely, a negligible interference with DU145 cell proliferation was observed at all time points after exposure of cells to R4 (fig. 7). No appreciable interference with DU145 cell growth was observed after exposure to other oligomers (R1–R6) or the scramble oligomer, with a reduction in the cell number ranging from 0 to 21% with respect to untreated controls, as detected 72 h from the end of treatment.

To exclude the possibility of an off-target effect exerted by the R7 oligomer, we exposed the hTERT-negative U2-OS human osteogenic sarcoma cell line to 0.5 µM R4 or R7 oligomer for 18 h. Results showed that the R7 oligomer did not affect U2-OS cell proliferation at any time point considered compared to untreated and R4-treated cells (fig. 7).

To determine whether oligomer treatment interferes with the susceptibility of DU145 cells to undergo programmed cell death, we examined the presence of cells with a nuclear apoptotic morphology by fluorescence microscopy (fig. 8A) in cell samples stained with propidium iodide at different intervals after the end of treatment. In control, untreated DU145 cells, a negligible rate of spontaneous apoptosis (ranging from 1 to 3% of the overall cell popu-

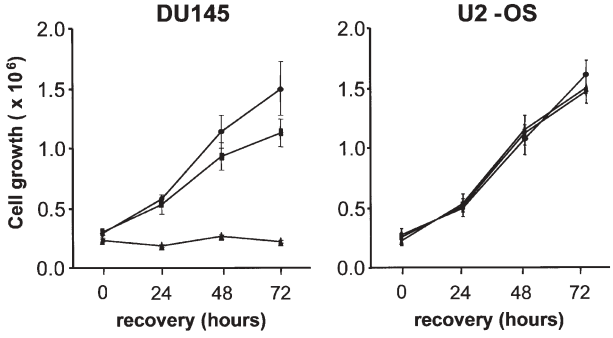

Figure 7. Cell growth curves of DU145 and U2-OS cells untreated (●) and after an 18-h exposure to R4 (■) or R7 (▲) oligomer. Data represent means ± SD of three independent experiments.

lation) was observed during the time course of the experiment. Such a percentage markedly increased over time in cells exposed to the R7 oligomer, reached a maximum at 48 h (40%), and was still present at an appreciable level (30%) at 72 h (fig. 8B). In contrast, in R4-treated cells, the fraction of apoptotic cells was similar to that of control cells, ranging from 1.0% immediately after oligomer removal to 4.0% 72 h after the end of treatment (fig. 8B). No appreciable variation in telomere length was detected by Southern blot analysis in oligomer-treated cells at any time point considered, with mean telomere restriction fragment values around 3.8 kb in control, R7- and R4-treated cells (data not shown). Moreover, no difference in the telomere G-rich single-stranded 3′ overhang was detected by T-OLA in DU145 cells exposed to R7 oligomer compared to untreated cells, as revealed by the presence of the same pattern of ligated fragments (fig. 9).

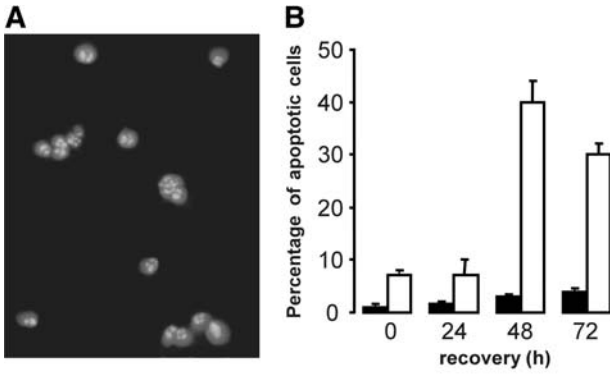

Figure 8. (*A*) Propidium iodide staining of apoptotic DU145 cells 48 h after exposure to R7. A representative field is shown. (*B*) Quantification of apoptosis in DU145 cells at different intervals (from 0 to 72 h) after an 18-h exposure to R4 or R7 oligomer. Data represent means ± SD of three independent experiments.

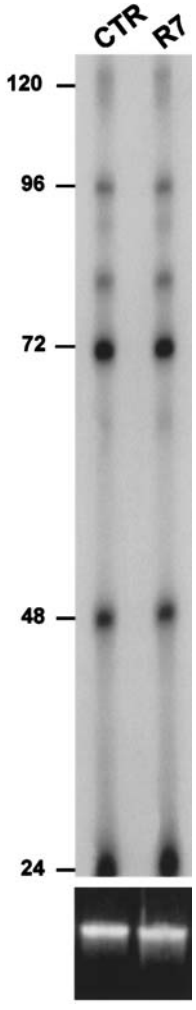

Figure 9. A representative experiment showing a T-OLA. Upper panel: ladder of products obtained on a denaturing polyacrylamide gel after a T-OLA performed in the presence of a specific probe $(AATCCC)_4$ for the G-rich single-strand 3′ overhang in DU145 cells untreated or exposed to R7 oligomer. Lower panel: ethidium bromide staining of agarose gel carried out to ensure equal DNA loading.

Discussion

Alternative splicing is one of the mechanisms exploited by mammalian cells to regulate gene expression at the post-transcriptional level [9]. Many tumour-associated genes undergo alternative splicing, and their expression leads to the production of multiple splicing variants with distinct, and sometimes antagonistic, activities [10]. Therefore, the possibility to modify the ratio of splicing variants of a particular gene could represent an interesting approach to interfere with its function in tumour cells. The use of chemically modified antisense oligonucleotides (such as 2′-O-methyl-PTOs, which form duplexes with target RNAs that are resistant to degradation by RNase H and other nucleases) directed against sites involved in the splicing processes of pre-mRNAs has been investigated in the context of genetic diseases to prevent aberrant splicing of genes such as β-globin [24], cystic fibrosis transmembrane conductance regulator and dystrophin [10]. With cancer-related genes, the oligonucleotide-mediated approach has only been exploited to redirect the splicing of the Bcl-x pre-mRNA from the anti-apoptotic variant Bcl-xL to the pro-apoptotic variant Bcl-xS, which resulted in an increase in spontaneous and drug-induced apoptosis in cancer cells [25].

In the present study, we demonstrated that the splicing pattern of the telomerase catalytic subunit hTERT can be modulated by an oligomer-mediated approach and, as a consequence, the telomerase catalytic activity in human prostate carcinoma cells can be attenuated. hTERT alternative transcripts are well-known to play a role in the regulation of the enzyme activity [6]. Ectopic expression of hTERT α^-, β^-, $\alpha^-\beta^-$ deletion variants did not reconstitute telomerase activity in normal human fibroblasts [26]. However, when overexpressed in telomerase-positive tumour and immortal cells, hTERT α^- was able to inhibit the endogenous enzyme activity, to progressively shorten telomeres and to induce apoptosis or a senescence-like state [16]. Thus far, such a dominant-negative effect has only been demonstrated for the hTERT α^- variant; the β^- and $\alpha^-\beta^-$ splicing variants did not show any phenotype when transfected into telomerase-positive tumour cell lines [26]. However, due to the lack of specific antibodies against the N-terminal half of hTERT it was not possible to detect the presence (if any) of the products of these splicing variants within tumour cells, or to determine whether they had accumulated at sufficient levels to alter endogenous telomerase activity [26].

Our results indicate that the exposure of DU145 human prostate carcinoma cells to 2′-O-methyl-RNA-PTOs targeted to the splicing junction located between intron 5 and exon 6 of the hTERT pre-mRNA redirected to some extent the splicing pattern of hTERT by inducing a decrease in the $\alpha^+\beta^+$ transcript and a concomitant increase in the alternatively spliced transcripts α^- and, mainly,

$\alpha^-\beta^-$. In our experimental model, the α^- transcript, which was probably generated early during treatment, may have been almost completely converted into a $\alpha^-\beta^-$ transcript, as revealed by the significant increase in this transcript in oligomer-treated samples. Conversely, we did not observe any treatment-mediated modulation of the β^- transcript, which was present at very high expression levels in control and treated cell samples. This finding is not surprising since the maintenance of high expression levels of the β^- splicing variant in the presence of telomerase inhibition was previously observed in HaCaT human skin keratinocytes following exposure to transforming growth factor-β1, which almost completely abrogated the enzyme activity and induced a reduction of the $\alpha^+\beta^+$ and α^- transcripts without affecting the expression of the β^- transcript [27].

When we tried to correlate the extent of oligomer-induced telomerase inhibition with the level of modulation of hTERT splicing variant expression, we failed to find any significant association between enzyme catalytic activity and the expression of the $\alpha^+\beta^+$ transcript. Moreover, a complete lack of correlation was found between telomerase activity and the α^- transcript. This finding would suggest that the slight increase in α^- message expression observed in oligomer-treated cells was not sufficient to inhibit telomerase by itself. A trend towards an inverse association between telomerase activity and the expression level of the $\alpha^-\beta^-$ deletion transcript was observed. However, the strongest and statistically significant correlation was found between enzyme catalytic activity and the overall modulation of the hTERT splicing pattern expressed as the ratio between the expression level of the $\alpha^+\beta^+$ transcript and that of the $[(\alpha^-) + (\alpha^-\beta^-)]$ deletion variants. This finding is in keeping with previous data we obtained in clinical melanoma and breast cancer specimens [17, 18]. Specifically, we demonstrated that tumours expressing telomerase activity were characterised by the presence of the $\alpha^+\beta^+$ transcript alone or in association with one or more alternatively spliced transcripts. However, in these lesions, the full-length hTERT transcript was more abundant than or as abundant as the alternatively spliced variants [17]. Conversely, in melanomas lacking telomerase activity, we detected the presence of the β^- spliced transcript alone, or considerably higher levels of alternatively spliced species, compared to that of the $\alpha^+\beta^+$ transcript [17]. Similarly, in telomerase-negative breast cancers, the $\alpha^+\beta^+$ message was either completely absent or present at a lower level than that of the spliced variants, or, alternatively, only the β^- deletion transcript was expressed [18]. The data reported in the present study demonstrate for the first time that oligomer-mediated modulation of the splicing pattern of hTERT pre-mRNA represents a suitable strategy to achieve telomerase inhibition in human cancer cell lines by promoting the production of non-functional or dominant-negative types of alternative splicing variants. Such an approach could be helpful to gain a better understanding of the role of the hTERT splicing variants in the regulation of telomerase expression and activity in human normal and cancer cells.

In our experimental model, some oligonucleotides appeared devoid of any biological effect, in terms of hTERT splicing modulation and telomerase activity inhibition. Such a lack of biological activity could reside in a different sequence-dependent affinity of these oligonucleotides for their target site compared to biologically active oligomers. Moreover, since most oligonucleotides used thus far to modulate cancer-related gene expression are 15- to 25-mer [10, 25, 28], in this study we used 18-mer 2′-O-methyl RNA phosphorothioates. Such a length would guarantee a sufficient affinity (needed to produce a biological effect) of the oligomers for their target site and reduce the occurrence of secondary and/or tertiary intramolecular structures [29]. However, the possibility that shorter or longer oligonucleotides directed against the same target molecule could be more effective in redirecting the splicing of hTERT pre-mRNA and, as a consequence, in inhibiting telomerase activity cannot be excluded.

When we analysed the effects of the oligomer, R7, that induced the most pronounced modulation of the hTERT splicing pattern, the most marked reduction of hTERT protein expression and the greatest telomerase inhibition on the proliferative potential of DU145 cells, we observed a significant reduction of cell growth and the induction of apoptosis starting 2 days after treatment. Such a decline in cell survival appeared to be a direct consequence of R7-mediated interference with hTERT, since exposure of DU145 to the R4 oligomer, which was devoid of any effect on hTERT splicing and telomerase activity, failed to affect cell growth. Moreover, no interference with the proliferative potential of the hTERT-negative U2-OS human osteogenic sarcoma cell line following R7 exposure was observed.

The impairment of cell growth by specific hTERT targeting can be sustained by two pathways: (i) telomere shortening as a result of prolonged inhibition of the telomere-lengthening activity of telomerase, and (ii) loss of the hTERT-mediated capping function of telomerase. Since no appreciable telomere shortening was detected in DU145 cells exposed to R7, the immediate cell loss observed in oligomer-treated cells could be based on the latter mechanism and related to telomere destabilisation, although no difference in the G-rich single-stranded 3′ overhang, which is thought to be important for t-loop formation at the end of telomeres [30], was detected in R7-treated cells compared to controls.

There is substantial evidence which suggests that telomeres normally exist in a capped state but may switch to an uncapped state. Uncapped chromosome ends are gen-

erally thought to be at great risk of degradation, recombination, or fusion by DNA repair systems. In response to dysfunctional or damaged telomeres, cells can undergo apoptosis and die. The appropriate response to the uncapping of a telomere is action by telomerase to protect the telomere from signalling into cell cycle arrest/apoptosis pathways [1]. Based on these suggestions, when there is marked inhibition of telomerase activity, the enzyme may no longer be able to protect the telomere and cells can die through a mechanism independent of telomere length. Such a possibility is also consistent with the growth inhibition effect we recently observed in DU145 cells 2 days after an 18-h exposure to a photochemically internalized peptide nucleic acid targeting hTERT [31]. It is also in accord with results reported by Saretzki et al. [32], who demonstrated massive cell death in four human ovarian cancer cell lines 3 days after transduction with an adenoviral vector carrying a ribozyme sequence directed against hTERT mRNA, and those obtained by Kraemer et al. [28], who showed an immediate and continuous reduction of the growth of EJ28 human bladder cancer cells after exposure to antisense oligonucleotides targeting hTERT. Moreover, the attenuation of hTERT expression levels has been reported to quickly induce programmed cell death in human breast cancer cells and such an apoptotic response could be counteracted by the expression of an hTERT mutant lacking telomerase activity [33]. Together, such findings have conferred on telomerase a putative pro-survival and anti-apoptotic role, which could be independent of its telomere-elongating activity. However, the role of telomerase in tumorigenesis, beyond the classical mechanism of telomere lengthening, needs to be further investigated to provide a better rationale for the use of anti-telomerase-based therapies in the clinical setting.

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