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Antisense oligonucleotide-mediated inhibition of hTERT, but not hTERC, induces rapid cell growth decline and apoptosis in the absence of telomere shortening in human prostate cancer cells

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Abstract

Recent evidence points to a novel function of human telomerase reverse transcriptase (hTERT) in promoting tumour cell survival, which might be independent of the telomere-elongating activity of the enzyme. To test this hypothesis, we evaluated comparatively the effects of telomerase inhibition, accomplished through antisense oligonucleotide-mediated interference with hTERT or human telomerase RNA component (hTERC), on the proliferative potential of DU145 human prostate cancer cells. Exposure of cells to a 2′-*O*-methyl-RNA phosphorothioate oligonucleotide targeting a splicing site within hTERT pre-mRNA induced almost complete inhibition of telomerase activity as a consequence of a marked reduction of the hTERT mRNA expression level, an early decline of DU145 cell growth and apoptotic cell death without any appreciable telomere shortening. Conversely, exposure of DU145 cells to a 2′-*O*-methyl-RNA phosphorothioate oligonucleotide targeting the template region of hTERC failed to interfere with cell proliferation in spite of the almost complete abrogation of telomerase activity. These results extend and corroborate earlier evidence in favour of an enzymatic activity-independent mechanism by which hTERT maintains tumour cell survival and proliferation.

Keywords: Telomerase; hTERT; hTERC; Apoptosis; Prostate cancer

1. Introduction

Human telomeres are DNA protein structures consisting of tandem repetitive hexameric sequences (TTAGGG) located at the end of linear chromosomes [1]. They have an important protective function in preventing chromosomes from undergoing aberrant recombination and end-to-end fusion, and are involved in the senescence process, acting as a molecular clock that controls the lifespan of human cells. Whereas mortal cells shorten their telomeres during each round of replication (the end replication problem [2]), immortal and cancer cells acquire indefinite growth capacity by stabilising the length of their telomeres through the reactivation of telomerase, a unique reverse transcriptase that synthesises telomeric repeats. The telomerase core enzyme consists of an RNA component (hTERC) that provides the template for the *de novo* synthesis of telomeric DNA [3], and a catalytic subunit (hTERT) with reverse transcriptase activity [4,5]. It has been demonstrated that hTERC is expressed in all human tissues regardless of telomerase activity [6]. By contrast, the expression of the hTERT component is closely associated with the enzyme's catalytic activity in telomerase-positive cells and is therefore the primary determinant for the activity of telomerase.

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Telomerase activity has been found in a high percentage of human tumours (85–90%), while only a small fraction of cancers maintain telomeres through an alternative lengthening of telomeres recombination mechanism [7]. Telomerase activity is generally absent in normal human cell types, with a few exceptions including germ-line cells, embryonic stem cells, activated lymphocytes and cells from the basal layer of the epidermis [8–10]. The relatively tumour-restricted expression of telomerase has made the enzyme an important target for the development of new anticancer therapies. Thus far, a number of approaches have been developed to achieve telomerase inhibition in tumour cells including those based on the use of antisense oligonucleotides, peptide nucleic acids (PNA) or hammerhead ribozymes directed against the core enzyme components. Targeting hTERC resulted in delayed tumour cell growth arrest as a consequence of progressive telomere shortening when telomerase is inhibited [11]. 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 tumour cells with relatively short telomeres [12,13]. However, recent evidence indicates that the hTERT component of telomerase could be a more suitable target to induce faster antiproliferative effects in tumour cells. It has been suggested that telomeres normally exist in a capped state and that hTERT takes part in this capping process, which protects the telomere from signalling into cell-cycle arrest/apoptosis pathways [14]. As a consequence, telomerase inhibitory approaches based on the interference with hTERT expression might rapidly affect tumour cell proliferation. In this context it has been demonstrated recently that antisense-mediated down-regulation of hTERT quickly induced programmed cell death in human tumour cells [15,16], and that such an apoptotic response can be counteracted by forced expression of a hTERT dominant-negative mutant lacking catalytic activity [17]. This finding would suggest an anti-apoptotic and pro-survival role of hTERT which might be independent of the telomere-elongating activity of the enzyme.

To test this hypothesis, we evaluated comparatively the effects of telomerase inhibition, accomplished through antisense oligonucleotide-mediated interference with hTERT or hTERC, on the proliferative potential of DU145 human prostate cancer cells.

2. Materials and methods

2.1. Cell lines

The DU145 androgen-independent human prostate adenocarcinoma cell line (American Type Culture Collection, Rockville, MD), carrying a mutant p53 [18], and the U2-OS/hTERT cell line (obtained by transduction of the hTERT-negative U2-OS human osteogenic sarcoma cell line with hTERT cDNA [19]) were used in this study. The DU145 and U2-OS cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 and McCoy's 5A media, respectively, supplemented with 10% fetal calf serum and 0.1% gentamycin. Each cell line was maintained as a monolayer in the logarithmic growth phase at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Chemically stabilised oligonucleotide and transfection procedures

An 18-mer oligonucleotide (hTERT oligomer, 5′-CACCUGUGUGAGUGGAGG-3′), targeting a sequence located between intron 5 and exon 6 in the pre-mRNA of hTERT (bases 25379–25396, GeneBank Accession No. AF128893) and previously identified by us as the most efficient telomerase inhibitor within a panel of oligomers, and a 13-mer oligonucleotide (hTERC oligomer, 5′-UAGGGUUAGACAA-3′) targeting the template region of the human telomerase RNA [20] were used in the study (MWG-Biotech). Each oligomer was modified by introducing a methyl group at the 2′ position of the ribose and by substituting a phosphorothioate linkage (2′-*O*-methyl-RNA phosphorothioate) for the phosphorodiester bond. A search in the Basic Local Alignment Search Tool (BLAST) database performed for the oligomers did not reveal any sequence analogies to any known RNA other than hTERT pre-mRNA and hTERC. An additional 18-mer scrambled oligonucleotide was used as a control throughout the study. Each oligoribonucleotide was resuspended in sterile water at the appropriate concentration and kept at −20 °C until use.

Each oligomer and DOTAP (*N*-(1-(2,3 dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium methyl sulphate, Boehringer Mannheim, Mannheim, Germany) cationic liposomes were mixed in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) and incubated at room temperature for 15 min. For the DOTAP-mediated oligomer transfection, cells were seeded in 25 cm^2 flasks and allowed to attach for 24 h. Cells were then transfected for 18 h with DOTAP alone or with DOTAP-oligomer complexes, diluted in an appropriate volume of culture medium to yield the desired final concentration. At the end of the transfection, cells were washed twice with phosphate-buffered saline (PBS) and incubated in fresh medium for different time-points, harvested and subsequently analysed.

2.3. Telomerase activity detection assay

One microgram of protein extract obtained from untreated and oligomer-treated cells was used to assess

telomerase activity by the polymerase chain reaction (PCR)-based telomeric repeats amplification protocol (TRAP) assay by means of the TRAPeze kit (Intergen Co., Oxford, United Kingdom). After extension of the substrate 5′-[^{32}P]-end-labelled TS primer oligonucleotide (5′-AATCCGTCGAGCACAGAGTT-3′) by telomerase, the enzyme activity products were amplified by PCR and resolved in 10% polyacrylamide gel. Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard. Protein extracts were also incubated at 85 °C for 10 min to test their heat sensitivity. A TSR8 quantitation standard (which serves as a standard to estimate the amount of product extended by telomerase in a given extract) was included for each set of TRAP assays. Quantitative analysis was performed with the Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, USA), which allowed densitometric evaluation of the digitised image. Telomerase activity was quantified by measuring the signal of telomerase ladder bands, and the relative telomerase activity was calculated as the ratio to the internal standard using the following formula:

relative telomerase activity :

$$[(X - X_0)/C] \times [(R - R_0)/Cr]^{-1},$$

where X is the untreated sample, X_0 is the RNAse-treated sample, C is the internal control of untreated samples, Cr is the internal control of TSR8, R is the TSR8 quantitation control and R_0 is the negative control.

2.4. Reverse transcriptase (RT)-PCR analysis of hTERT mRNA expression

Total cellular RNA was isolated from untreated and oligomer-treated cells with the TRIzol reagent (Invitrogen, Gaithersburg, MD) and purified using the RNeasy kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. The polyA$^+$ mRNA was purified from total RNA with the Oligotex kit (QIAGEN GmbH, Germany) and reverse-transcribed by means of the RT-PCR core kit (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ, USA). To measure the hTERT mRNA expression levels, the resultant cDNA was amplified by performing 28 cycles of PCR (94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s) in the presence of sense (3′-GCCTTC TCACAGACCT CGTT-5′) and antisense (3′-AGGTCTGAGGCGAA-GTAGG-5′) primers (MWG Biotech). The primers 774S (5′-GGAATTCAAAACTGGAACGGTGAAGG-3′) and 775AS (5′-GGAAGCTTATCAAAGTCCTCG-GCCACA-3′) (MWG Biotech) were used to amplify a β-actin fragment, used as a standard of the amplification reaction, by performing 20 cycles of PCR under the same conditions as were used for hTERT. The resulting products were analysed through agarose gel electrophoresis and quantified by Image-QuanT software (Molecular Dynamics, Sunnyvale, CA). The densitometric value of hTERT transcript was normalised to that of β-actin.

2.5. Cell proliferation assay

For cell growth experiments, cells were seeded in each well of a 6-well plate and allowed to attach for 24 h at 37 °C. Cells were incubated for 18 h with DOTAP alone or with the DOTAP-oligomer complexes, washed twice with PBS and then incubated in fresh culture medium. Cells were trypsinised at different time-points (0, 24, 48 and 72 h from the end of treatment) and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK).

2.6. Cell cycle distribution analysis

At different time-points (0, 24, 48 and 72 h from the end of oligomer treatment) cells were harvested and fixed in 70% ethanol. Before analysis, the cells were washed with PBS and stained with a solution containing 50 μg/ml propidium iodide, 50 mg/ml RNase and 0.05% Nonidet P40 (NP-40) for 30 min at 4 °C. The fluorescence of stained cells was analysed by a FACScan (fluorescent activated cell sorter) flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). A minimum of 1×10^4 events was measured for each sample. The percentage of cells in different phases of the cell cycle was established on DNA plots by CellFit software according to the sum of broadened rectangles (SOBR) model (Becton Dickinson).

2.7. Tdt-mediated dUTP nick-end labelling (TUNEL) analysis

At different time-points (0, 24, 48 and 72 h from the end of oligomer treatment) cells were harvested and fixed in 4% paraformaldehyde for 45 min at room temperature. After rinsing with PBS, cells were permeabilised in a solution of 0.1% Triton X-100 in 0.1% sodium citrate for 2 min in ice. Samples were washed with PBS and then incubated in the TUNEL reaction mixture (Roche, Mannheim, Germany) for 1 h at 37 °C in the dark. After rinsing with PBS, cells were suspended in PBS and analysed by a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). The results were expressed as the percentage of TUNEL-positive cells in the overall cell population.

2.8. Telomere length measurement

Total DNA was isolated from untreated and oligomer-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). The mean terminal restriction fragment (TRF) length was calculated as previously reported in [21].

2.9. Statistical analysis

Student's *t* test was used to analyse differences between untreated and oligomer-treated DU145 cells in terms of telomerase activity, hTERT mRNA expression level, cell number and number of TUNEL-positive cells; all tests were two-sided. *P*-values of <0.05 were considered statistically significant.

3. Results

To evaluate the efficacy of the hTERT oligomer targeting hTERT pre-mRNA in inhibiting telomerase activity, DU145 human prostate cancer cells were exposed to different concentrations (from 0.1 to 1.5 μM)

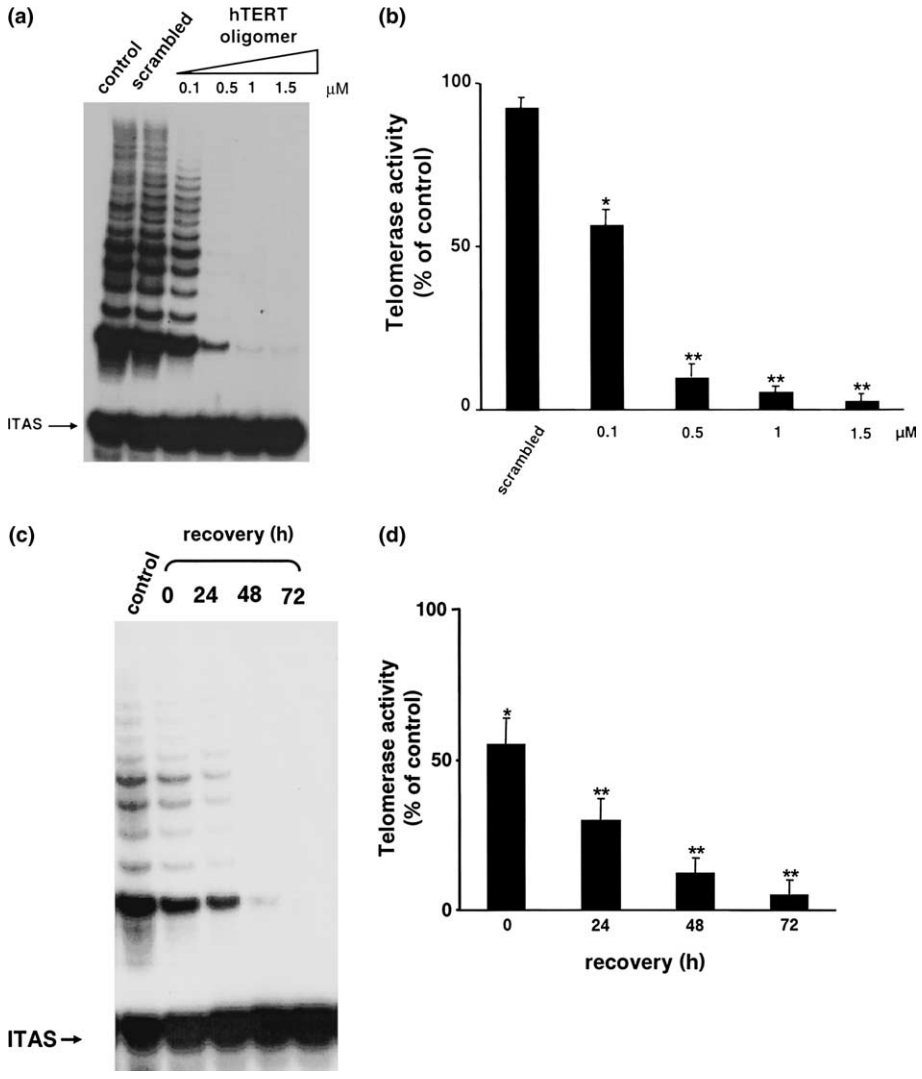

Fig. 1. Inhibition of telomerase activity by the human telomerase reverse transcriptase (hTERT) oligomer in DU145 cells. (a) A representative telomeric repeats amplification protocol (TRAP) experiment showing telomerase activity in DU145 cells either untreated (control) or at 48 h of recovery in oligomer-free medium after an 18-h exposure to 0.5 μM scrambled oligomer or different concentrations (from 0.1 to1.5 μM) of the hTERT oligomer. The location of the internal amplification standard (ITAS) is reported. (b) Quantification of telomerase activity (as a percentage of the activity observed in untreated cells) in DU145 cells treated with different concentrations of hTERT oligomer. Data represent the mean values ± standard deviation (SD) of at least three independent experiments. (*), $P < 0.05$; (**), $P < 0.01$; Student's *t* test. (c) A representative TRAP experiment showing telomerase activity in DU145 cells either untreated (control) or at different intervals of recovery (from 0 to 72 h) after an 18-h exposure to 0.5 μM hTERT oligomer. The location of the internal amplification standard (ITAS) is reported. (d) Quantification of telomerase activity (as a percentage of the activity observed in untreated cells) in hTERT oligomer-treated DU145 cells at different intervals of recovery. Data represent the mean values ± SD of at least four independent experiments. (*), $P < 0.05$; (**), $P < 0.01$; Student's *t* test.

of the oligomer for 18 h and the enzyme's catalytic activity was assessed on protein extracts from cells collected after 48 h of recovery in drug-free medium. TRAP results showed a significant and dose-dependent decrease in telomerase activity (Fig. 1(a)), ranging from $-44.0 \pm 5.1\%$ ($P < 0.05$) to $-97.2 \pm 2.3\%$ ($P < 0.01$), with respect to control cells (Fig. 1(b)). As expected no appreciable inhibition of telomerase activity was observed in DU145 cells exposed to 0.5 μM scrambled oligomer (Fig. 1). To determine the persistency of the oligomer-mediated telomerase inhibition, cells were expose to 0.5 μM oligomer for 18 h and the enzyme's catalytic activity was assessed on cells collected immediately at the end of treatment or after 24, 48 and 72 h of recovery in oligomer-free medium. TRAP results showed a significant and time-dependent decrease in telomerase activity (Fig. 1(c)), which was already appreciable at the end of oligomer exposure ($-44.8 \pm 9.0\%$ with respect to untreated cells; $P < 0.05$), increased with time, and reached its peak ($-96.3 \pm 4.3\%$; $P < 0.01$) at 72 h from the end of treatment (Fig. 1(d)).

The ability of the hTERT oligomer to downregulate hTERT mRNA was evaluated by RT-PCR on polyA$^+$ RNA obtained from DU145 cells collected immediately at the end of an 18-h exposure to 0.5 μM oligomer or after different intervals of recovery (from 24 to 72 h) in oligomer-free medium. As shown in Fig. 2(a), the hTERT oligomer induced a significant and time-dependent decrease in hTERT mRNA expression levels, ranging from $-46.2 \pm 4.7\%$ ($P < 0.05$) to $-82.1 \pm 10\%$ ($P < 0.01$) with respect to untreated cells (Fig. 2(b)). By contrast, exposure of DU145 cells to the scrambled oligomer failed to decrease the expression of hTERT mRNA (Figs. 2(a) and (b)).

To further exclude the possibility of a non-specific effect exerted by the hTERT oligomer on telomerase activity, we exposed U2-OS/hTERT cells to 0.5 μM oligomer for 18 h; such cells were obtained by transducing the hTERT-negative U2-OS human osteogenic sarcoma cell line with hTERT cDNA [19] and, as a consequence, did not express hTERT pre-mRNA. The TRAP results, obtained 48 h after treatment removal, showed that the hTERT oligomer had a negligible effect on telomerase activity, which was superimposable to that obtained with the scrambled oligomer (Fig. 3).

In a further step of the study, we evaluated the effects of oligomer-mediated hTERT downregulation and telomerase activity inhibition on the proliferative potential of DU145 cells. Eighteen-hour exposure of cells to 0.5 μM hTERT oligomer resulted in significant ($P < 0.02$) inhibition of cell growth, which was appreciable 24 h after oligomer removal ($-63.1 \pm 6.0\%$ with respect to untreated cells) and slightly increased at later intervals (approximately -78% of controls at 48 and 72 h) (Fig. 4). Conversely, exposure to the scrambled oligomer did not significantly affect DU145 cell growth at any time-point considered (Fig. 4).

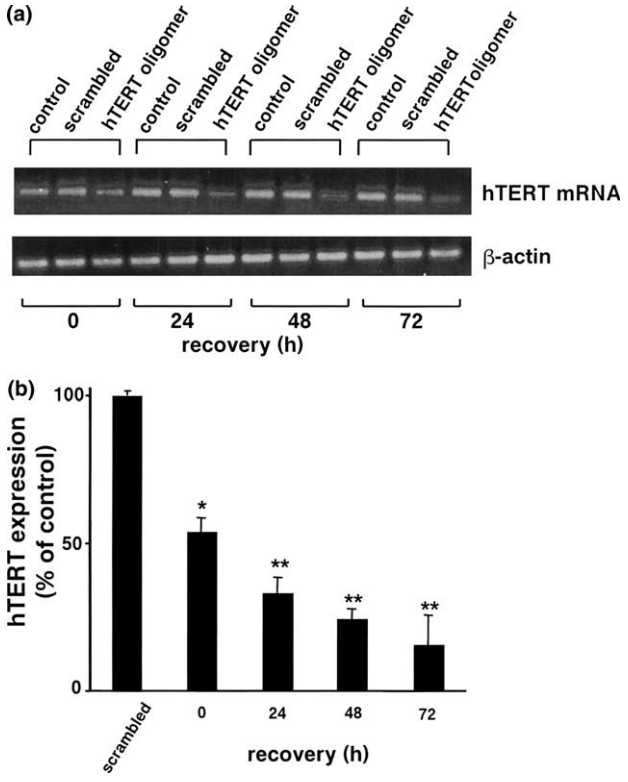

Fig. 2. Downregulation of hTERT mRNA by the hTERT oligomer in DU145 cells. (a) A representative reverse-transcription-polymerase chain reaction (RT-PCR) experiment showing hTERT mRNA in DU145 cells untreated (control) or at different intervals of recovery (from 0 to 72 h) in oligomer-free medium after an 18-h exposure to 0.5 μM scrambled or hTERT oligomer. β-actin was used as a control for the amplification. (b) Quantification of hTERT mRNA expression levels (as a percentage of the expression observed in untreated cells) in DU145 cells exposed to scrambled or hTERT oligomer. Data represent the mean values ± SD of at least three independent experiments. (*), $P < 0.05$; (**), $P < 0.01$; Student's t test.

Flow cytometric analysis of DU145 cell distribution in the different cell cycle phases showed that exposure to the hTERT oligomer induced an increase in the percentage of cells accumulated in the G_1 compartment with respect to untreated controls. Such a G_1-phase cell block was already appreciable at the end of treatment and still present, to a comparable extent, after 72 h of recovery in oligomer-free medium. In parallel, an hTERT oligomer-induced decrease in the number of S-phase cells was observed at all time-points. Conversely, the percentage of cells in the G_2/M compartment was not affected by the hTERT oligomer (Fig. 5). Exposure to scrambled oligomer did not interfere with the distribution of DU145 cells in the different cell cycle phases at any time-point considered (Fig. 5).

TUNEL analysis of nuclear DNA fragmentation showed that exposure of DU145 to hTERT oligomer

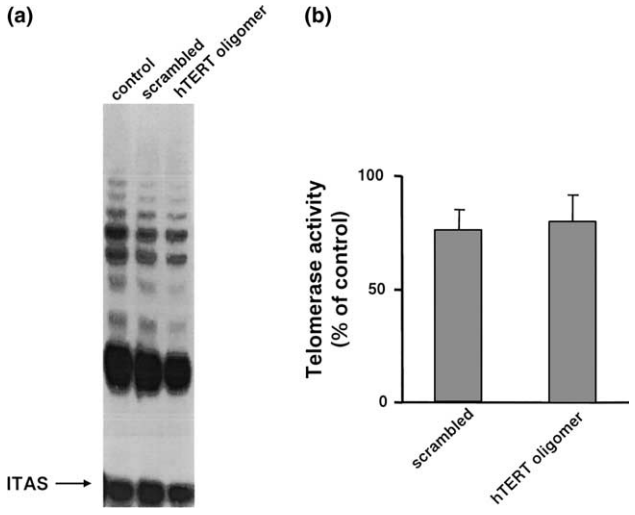

Fig. 3. Lack of interference of hTERT oligomer with telomerase activity in U2-OS/hTERT cells (obtained by transduction of the hTERT-negative U2-OS human osteogenic sarcoma cell line with hTERT cDNA [19]). (a) A representative TRAP experiment showing telomerase activity in U2-OS/hTERT cells untreated (control) or at 48 h of recovery in oligomer-free medium after an 18-h exposure to 0.5 μM of scrambled or hTERT oligomer. (b) Quantification of telomerase activity (as a percentage of the activity observed in untreated cells) in DU145 cells exposed to scrambled or hTERT oligomer. Data represent the mean values ± SD of at least three independent experiments.

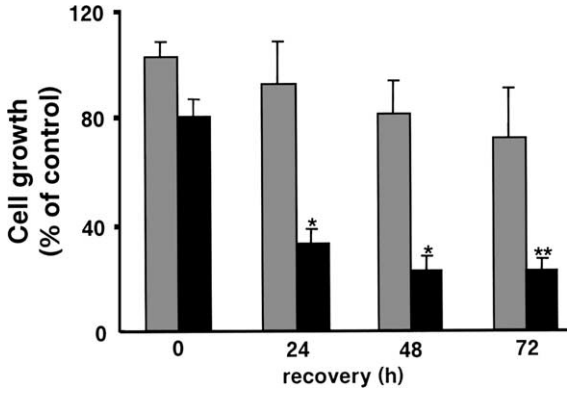

Fig. 4. Cell growth inhibition induced by the hTERT oligomer in DU145 cells. The relative cell growth (as a percentage of the growth observed in untreated cells) of DU145 cells at different intervals of recovery (from 0 to 72 h) in oligomer-free medium after an 18-h exposure to 0.5 μM of scrambled (grey column) or hTERT (black column) oligomer is reported. Data represent the mean values ± SD of at least three independent experiments. (*), $P < 0.02$; (**), $P < 0.01$; Student's t test.

markedly increased the fraction of apoptotic cells (Fig. 6). Specifically, the percentage of TUNEL-positive cells in untreated samples as well as in samples exposed to scrambled oligomer was always lower than 5% of the overall cell population at all time-points considered. Conversely, the percentage of apoptotic cells significantly increased with time in hTERT oligomer-treated samples, reaching its peak at 48 h (40 ± 3.6%; $P < 0.01$). Although to a lesser extent, a significant fraction of apoptotic cells (29 ± 1.9%; $P < 0.05$) was still present at 72 h after hTERT oligomer removal (Fig. 6(a)).

Southern blot hybridisation carried out 72 h after exposure of DU145 cells to scrambled or hTERT oligomer showed that the TRF length ranged from approximately 2.0–6.5 kb, with a mean value of approximately 3.8 kb in both cell cultures (Fig. 7), thus indicating that hTERT oligomer treatment did not affect telomere length.

To establish whether cell growth arrest and apoptotic cell death observed in DU145 cells exposed to hTERT oligomer were due to attenuation of telomerase activity or reduction of the hTERT mRNA expression level, we decided to assess the effects of telomerase inhibition, accomplished through antisense oligonucleotide-mediated interference with the telomerase RNA component hTERC, on the proliferative potential of DU145 cells. For this purpose, we evaluated the ability of a 13-mer oligonucleotide directed against the hTERC template (hTERC oligomer) to inhibit telomerase activity in

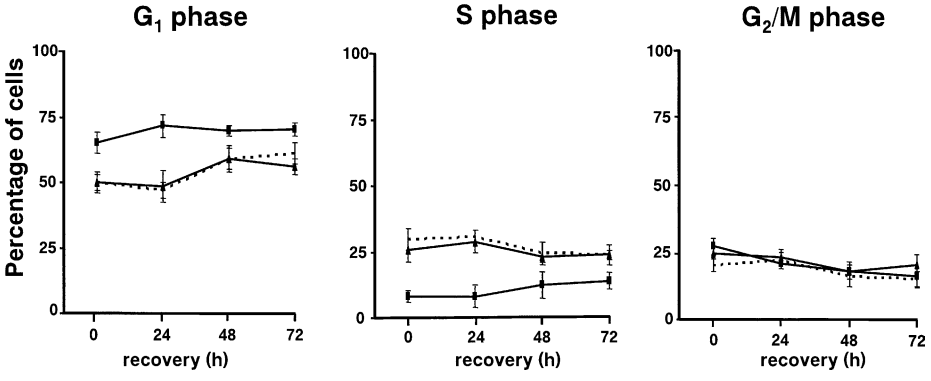

Fig. 5. Cell cycle perturbations induced by the hTERT oligomer in DU145 cells. The percentage of cells in the different cycle phases observed in DU145 cells untreated (broken line) or at different intervals of recovery (from 0 to 72 h) in oligomer-free medium after an 18-h exposure to 0.5 μM of scrambled (△) or hTERT (□) oligomer is reported. Data represent the mean values ± SD of at least three independent experiments.

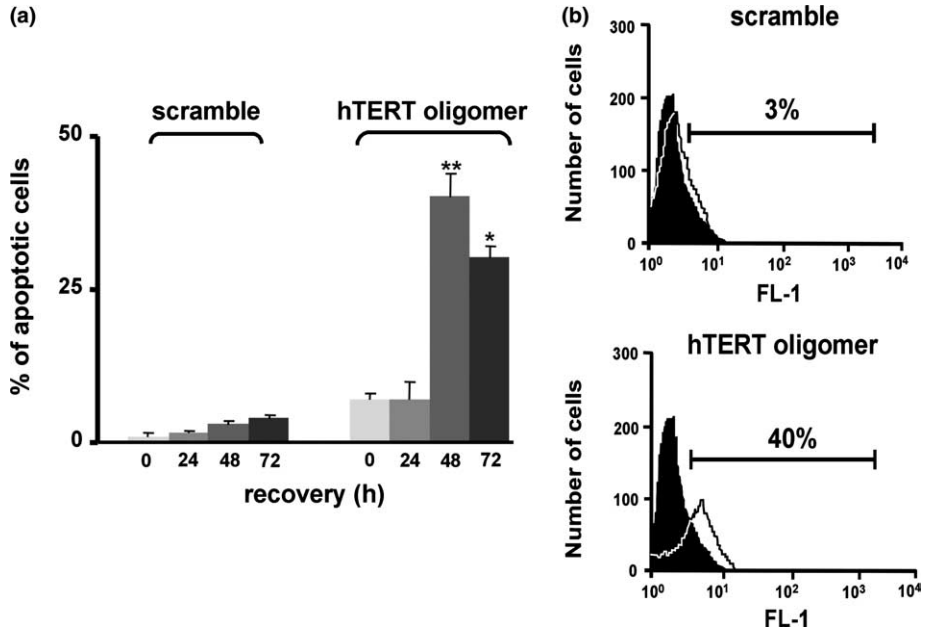

Fig. 6. Induction of apoptosis by the hTERT oligomer in DU145 cells. Tdt-mediated dUTP nick-end labelling (TUNEL) analysis of apoptosis was performed in DU145 cells at different intervals (from 0 to 72 h) of recovery in oligomer-free medium after an 18-h exposure to 0.5 μM of scrambled or hTERT oligomer. (a) Quantification of TUNEL-positive cells after exposure to scrambled or hTERT oligomer. Data represent the mean values ± SD of at least three independent experiments. (*), $P < 0.05$; (**), $P < 0.01$; Student's t test. (b) Representative TUNEL histograms obtained from DU145 cells at 48-h of recovery after an 18-h exposure to 0.5 μM of scrambled or hTERT oligomer. Solid histograms represent the negative controls incubated in the absence of terminal transferase; open histograms represent the test samples incubated with TUNEL reaction mixture. The percentage of TUNEL-positive cells in each sample is reported.

DU145 cells. TRAP results showed that an 18-h exposure to the hTERC oligomer induced a dose-dependent attenuation of the enzyme's catalytic activity, as detected 48 h after treatment (Fig. 8(a)). Specifically, exposure to 5 μM hTERC oligomer induced almost complete abrogation of telomerase activity (−92.0 ± 2.5% with respect to untreated cells; $P < 0.01$) (Fig. 8(b)), which was superimposable to that obtained at the same time-point following exposure of DU145 cells to 0.5 μM hTERT oligomer (Fig. 1(b)). A time-course experiment indicated that, similar to what was observed with the hTERT oligomer, significant inhibition of telomerase activity was already appreciable at the end of hTERC oligomer exposure (−43.0 ± 9.0%; $P < 0.05$), reached its peak at 24–48 h, and was still appreciable at 72 h (−81.2 ± 3.9%; $P < 0.01$) (Figs. 8(c) and (d)). As expected, exposure of DU145 cells to the hTERC oligomer did not modify the expression of hTERT mRNA (Fig. 9).

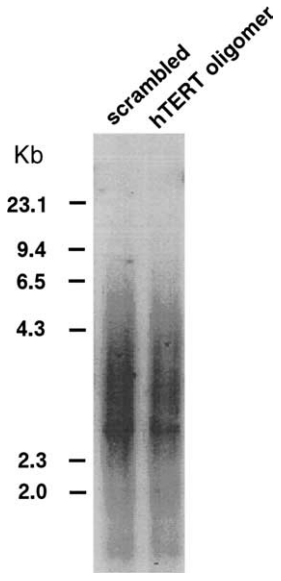

Fig. 7. Lack of telomere shortening in DU145 cells exposed to hTERT oligomer. Telomere length of DU145 cells at 72 h of recovery in oligomer-free medium after an 18-h exposure to 0.5 μM of scrambled or hTERT oligomer, as determined by Southern blot hybridisation.

In spite of the marked decrease in telomerase activity, the hTERC oligomer did not appreciably interfere with DU145 cell proliferation at any time-point considered (Fig. 10). Furthermore, TUNEL analysis revealed that exposure of DU145 cells to the hTERC oligomer failed to induce programmed cell death. Specifically, the percentage of TUNEL-positive cells was superimposable to that observed in untreated cells and always lower than 5% of the overall cell population during the time-course of the experiment (data not shown).

4. Discussion

Telomerase activation and subsequent maintenance of telomeres are required for the survival and proliferation of the large majority of tumour cells [22]. As a

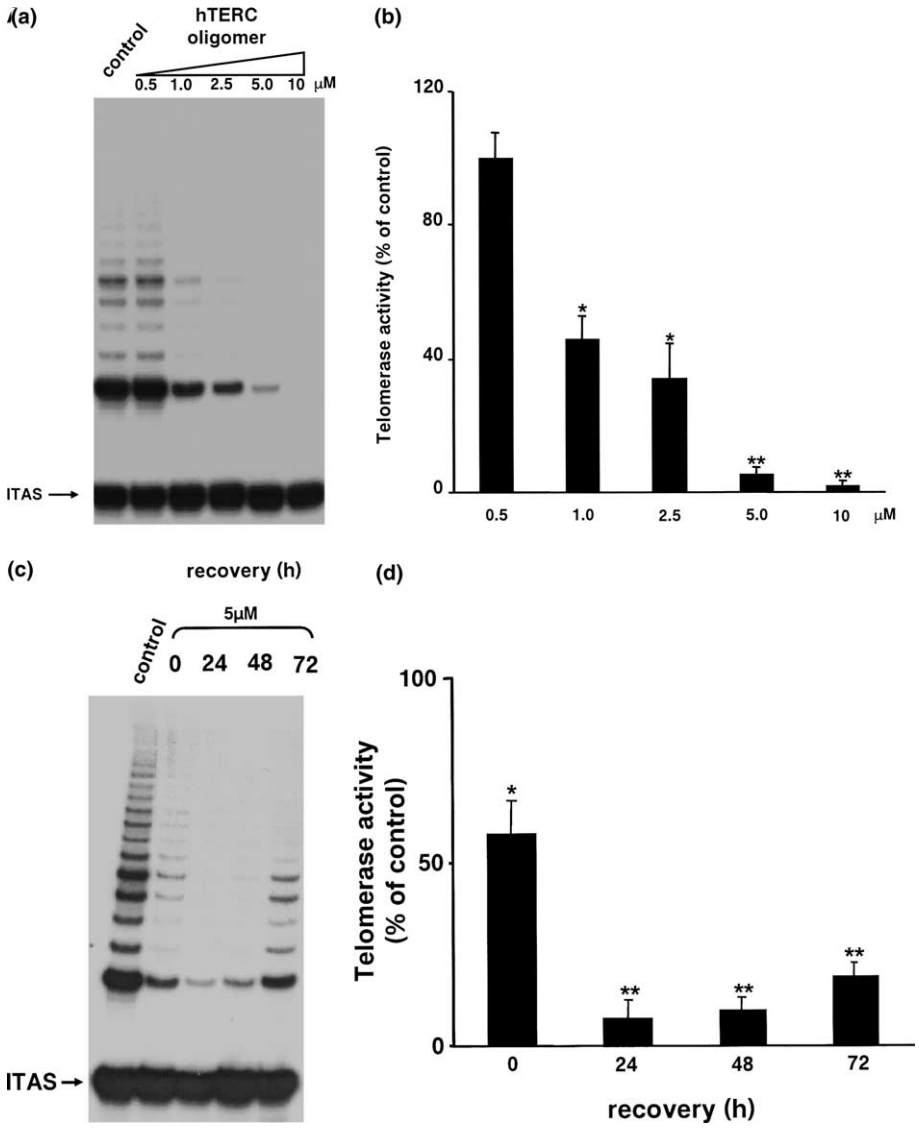

Fig. 8. Inhibition of telomerase activity by the hTERC oligomer in DU145 cells. (a) A representative TRAP experiment showing telomerase activity in DU145 cells either untreated (control) or at 48 h of recovery in oligomer-free medium after an 18-h exposure to different concentrations (from 0.5 to 10 μM) of the hTERC oligomer. The location of the internal amplification standard (ITAS) is reported. (b) Quantification of telomerase activity (as a percentage of the activity observed in untreated cells) in DU145 cells exposed to different concentrations of the hTERC oligomer. Data represent the mean values ± SD of at least three independent experiments. (*), $P < 0.05$; (**), $P < 0.01$; Student's t test. (c) A representative TRAP experiment showing telomerase activity in DU145 cells untreated (control) or at different intervals of recovery (from 0 to 72 h) after an 18-h exposure to 5 μM hTERC oligomer. The location of the internal amplification standard (ITAS) is reported. (d) Quantification of telomerase activity (as a percentage of the activity observed in untreated cells) in hTERC oligomer-treated DU145 cells at different intervals of recovery. Data represent the mean values ± SD of at least three independent experiments. (*), $P < 0.05$; (**), $P < 0.01$ Student's t test.

consequence, telomerase is considered a promising target for new anticancer interventions, and different inhibitory approaches have already been used to efficiently downregulate the enzyme [11,23,24]. However, it has not yet been clarified how telomerase inhibition affects the proliferative capacity of tumour cells. In several experimental studies (which were mainly based on the use of antisense oligonucleotides targeting hTERC, inhibitors of hTERT reverse-transcriptase activity and molecules able to stabilise G-quadruplex structure in telomeric DNA), it was shown that telomerase inhibition had to be maintained for a long time before it resulted in tumour cell growth arrest [13,25,26]. Moreover, telomere shortening and a positive correlation between the initial telomere length of the cell population and the number of rounds of cell division before growth arrest was reported. Conversely, in recent reports dealing with the use of antisense oligonucleotides [16] and ribozymes [15] targeting hTERT, cell growth decline and induction of apoptotic cell death was observed after a few days of treatment; this probably did not depend on telomere erosion, since the cells

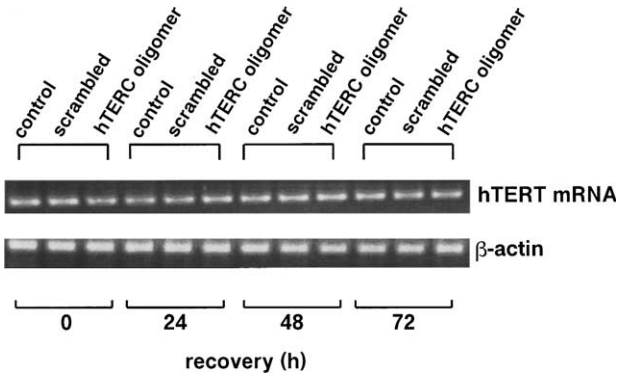

Fig. 9. Lack of interference of hTERC oligomer with hTERT mRNA expression in DU145 cells. A representative RT-PCR experiment showing hTERT mRNA in DU145 cells untreated (control) or at different intervals of recovery (from 0 to 72 h) in oligomer-free medium after an 18-h exposure to 5 μM scrambled or hTERC oligomer. β-actin was used as a control for amplification.

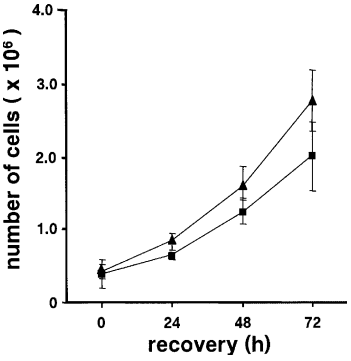

Fig. 10. Lack of interference of hTERC oligomer with DU145 cell proliferation. Cell growth curves of DU145 cells untreated or at different intervals (from 0 to 72 h) after an 18-h exposure to 5 μM of scrambled (△) or hTERC (□) oligomer. Data represent the mean values ± SD of at least three independent experiments.

would not have undergone enough divisions to significantly shorten their telomeres. Overall, these data suggest that the impairment of cell growth by telomerase inhibition can be sustained by two pathways: (a) telomere shortening as a result of prolonged inhibition of the telomere lengthening activity of the enzyme; and (b) loss of the hTERT-mediated capping function of telomerase.

In the present study, we analysed the effects of telomerase inhibition, accomplished through oligomer-mediated downregulation of hTERT, on the proliferative potential of DU145 human prostate cancer cells. Specifically, the hTERT oligomer targeting a splicing site within hTERT pre-mRNA was able to induce a marked reduction of the hTERT mRNA expression level, which resulted in almost complete inhibition of telomerase activity. Moreover, hTERT oligomer treatment interfered with the hTERT splicing pattern in DU145 cells, inducing a decrease in the full-length hTERT transcript and a concomitant accumulation of the α^- and $\alpha^-\beta^-$ alternative splicing variants [27]. It is possible that such oligomer-mediated modulation of the hTERT splicing pattern also contributed, at least partially, to telomerase inhibition. It is well known that alternative splicing plays a role in the regulation of the enzyme's activity during embryonic development [28], and that such a regulatory mechanism is also active in human tumours [29–31]. As alternative splicing removes reverse transcriptase motifs, it is unlikely that deletion transcripts code for functional proteins. Moreover, the α^- splicing variant has been recently suggested to act as a dominant-negative inhibitor of telomerase activity [32,33].

In DU145 cells exposed to the hTERT oligomer, we observed a marked reduction of cell growth and induction of p53-independent apoptosis within a few days of treatment. This decline in cell survival appeared to be a direct consequence of oligomer-mediated interference with hTERT expression, since exposure of DU145 cells to the hTERC oligomer targeting the RNA template of the holoenzyme failed to affect cell growth, in spite of its ability to almost completely abrogate telomerase's catalytic activity. These results have potential relevance for a better understanding of the effect of the interference with telomerase core enzyme components on the proliferative potential of tumours cells, since, for the first time, they have been obtained from a head-to-head comparison of the effects induced on the same tumour cell model by specific inhibition of hTERT and hTERC.

Since no appreciable telomere shortening was detected in DU145 cells exposed to the hTERT oligomer before the onset of apoptosis, we presume that the immediate cell loss observed in oligomer-treated cells could be related to telomere destabilisation. There is substantial evidence suggesting that telomeres normally exist in a capped state, due to the formation of a large-tailed loop, the T-loop, which is thought to provide a structure that hides the chromosomal ends, preventing them from resembling DNA double-strand breaks [34,35]. It has been hypothesised that hTERT plays a role in regulating telomere loop closing and in protecting telomeres in unclosed loops [36]. It is therefore possible that disruption of a single or a few T-loops, as a consequence of reduced hTERT expression, might signal cell cycle arrest and apoptosis in DU145 cells. This is consistent with the growth inhibitory effect we recently observed in the same cell line two days after an 18-hour exposure to a photochemically internalised PNA targeting hTERT [37]; it is also in agreement with results reported by Kraemer and colleagues [16], who showed an immediate and continuous reduction of the growth of EJ28 human bladder cancer cells after exposure to antisense oligonucleotides targeting hTERT. These data are in keeping with previous findings by Saretzki and colleagues [15], who

demonstrated massive cell death in four human ovarian cancer cell lines a few days after transduction with an adenoviral vector carrying a ribozyme sequence directed against hTERT mRNA, independent of the initial telomere length of the tumour models and without telomere shortening. Moreover, Cao and colleagues [17] recently reported that downregulation of hTERT expression quickly induced programmed cell death in human breast cancer cells and that such an apoptotic response could be counteracted by the expression of an hTERT mutant lacking telomerase activity. Altogether such findings have conferred on hTERT a putative pro-survival and anti-apoptotic role, which could be partially independent of its telomere-elongating activity. Additional novel functions of telomerase, which are distinct from its telomere-maintenance activity and might have potentially important consequences in tumour cells, are related to the ability of hTERT to cross-link telomere and enhance genomic stability and DNA repair [38]. Moreover, it has been suggested that hTERT is involved in the maintenance of cell survival and proliferation via enzymatic activity-independent intermolecular interactions involving p53 and poly(ADP-ribose) polymerase [17]. As a consequence, it appears that approaches aimed at downregulating hTERT expression, which result in the onset of apoptosis within days, could have greater therapeutic relevance than telomerase inhibitory approaches that rely on telomere shortening. In fact, the former should allow us to overcome a possible constraint to the clinical application of telomerase inhibition as anticancer therapy, mainly in tumours with long telomeres, which is related to the lag period needed to obtain cell growth impairment as a consequence of telomere attrition and during which telomerase inhibitors have to be given continuously for months, possibly in conjunction with other treatment modalities. A better understanding of the functions of hTERT that are distinct from its telomere-elongating role should spawn a new generation of telomerase inhibitors that have great potential to develop into an effective and fast way to control tumour cell growth.

Conflict of interest statement

None declared.

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References

1. Blackburn EH. Structure and function of telomeres. *Nature* 1991, **350**, 569–573.
2. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol* 1973, **41**, 181–190.
3. Feng J, Funk WD, Wang SS, *et al*. The RNA component of human telomerase. *Science* 1995, **269**, 1236–1241.
4. Harrington L, Zhou W, McPhail T, *et al*. Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev* 1997, **11**, 3109–3115.
5. Harrington L, McPhail T, Mar V, *et al*. A mammalian telomerase-associated protein. *Science* 1997, **275**, 973–977.
6. Avilion AA, Piatyszek AA, Gupta J, *et al*. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res* 1996, **56**, 645–650.
7. Reddel RR. Alternative lengthening of telomeres, telomerase, and cancer. *Cancer Lett* 2003, **194**, 155–162.
8. Kim NW, Piatyszek MA, Prowse KR, *et al*. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994, **266**, 2011–2015.
9. Wright WE, Piatyszek MA, Rainey WE, *et al*. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet* 1996, **18**, 173–179.
10. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997, **33**, 787–791.
11. Folini M, Pennati M, Zaffaroni N. Targeting human telomerase by antisense oligonucleotides and ribozymes. *Curr Med Chem Anti-Cancer Agents* 2002, **2**, 605–612.
12. Keith WN, Evans TRJ, Glasspool RM. Telomerase and cancer: time to move from a promising target to a clinical reality. *J Pathol* 2001, **195**, 404–414.
13. Corey DR. Telomerase inhibition, oligonucleotides, and clinical trials. *Oncogene* 2002, **21**, 631–637.
14. Blackburn EH. Telomere states and cell fates. *Nature* 2000, **408**, 53–56.
15. Saretzki G, Ludwig A, von Zglinicki T, *et al*. Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells. *Cancer Gene Ther* 2001, **8**, 827–834.
16. Kraemer K, Fuessel S, Schmidt U, *et al*. Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. *Clin Cancer Res* 2003, **9**, 3794–3800.
17. Cao Y, Li H, Deb S, *et al*. TERT regulates cell survival independent of telomerase enzymatic activity. *Oncogene* 2002, **21**, 3130–3138.
18. Carroll AG, Voeller HJ, Sugars L, *et al*. p53 oncogene mutations in three human prostate cancer cell lines. *Prostate* 1993, **23**, 123–134.
19. Vonderheide RH, Hahn WC, Schultze JL, *et al*. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity* 1999, **10**, 673–679.
20. Norton JC, Piatyszek MA, Wright WE, *et al*. Inhibition of human telomerase activity by peptide nucleic acids. *Nat Biotechnol* 1996, **14**, 615–619.
21. Villa R, Folini M, Perego P, *et al*. Telomerase activity and telomere length in human ovarian cancer and melanoma cell lines: correlation with sensitivity to DNA damaging agents. *Int J Oncol* 2000, **16**, 995–1002.
22. Hahn WC, Meyerson M. Telomerase activation, cellular immortalization and cancer. *Ann Med* 2001, **2**, 123–1299.
23. Autexier C. Telomerase as a possible target for anticancer therapy. *Chem Biol* 1999, **6**, 299–303.

24. Zaffaroni N, Villa R, Pennati M, *et al*. Approaches for the inhibition of human telomerase based on the use of peptide nucleic acids and hammerhead ribozymes. *Mini Rev Med Chem* 2003, **3**, 51–60.
25. Kelland LR. Telomerase: biology and phase I trials. *Lancet Oncol* 2001, **2**, 95–102.
26. Mergny J-L, Riou J-F, Mailliet P, *et al*. Natural and pharmacological regulation of telomerase. *Nucleic Acids Res* 2002, **30**, 839–865.
27. Brambilla C, Folini M, Gandellini P, *et al*. Oligomer-mediated modulation of hTERT alternative splicing induces telomerase inhibition and cell growth decline in prostate cancer cells. *Cell Mol Life Sci* 2004, **61**, 1764–1774.
28. Ulaner GA, Hu J-F, Vu TH, *et al*. Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res* 1998, **58**, 4168–4172.
29. Ulaner GA, Hu JF, Vu TH, *et al*. Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium. *Int J Cancer* 2000, **85**, 330–335.
30. Villa R, Della Porta C, Folini M, *et al*. Possible regulation of telomerase activity by transcription and alternative splicing of telomerase reverse transcriptase in human melanoma. *J Invest Dermatol* 2001, **116**, 867–873.
31. Zaffaroni N, Della Porta C, Villa R, *et al*. Transcription and alternative splicing of telomerase reverse transcriptase in benign and malignant breast tumours and in adjacent mammary glandular tissues: implications for telomerase activity. *J Pathol* 2002, **198**, 37–46.
32. Colgin LM, Wilkinson C, Englezou A, *et al*. The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity. *Neoplasia* 2000, **2**, 426–432.
33. Yi X, White DM, Aisner DL, *et al*. An alternate splicing variant of the human telomerase catalytic subunit inhibits telomerase activity. *Neoplasia* 2000, **2**, 433–440.
34. Griffith JD, Comeau L, Rosenfield S, *et al*. Mammalian telomeres end in a large duplex loop. *Cell* 1999, **97**, 503–514.
35. de Lange T. Protection of mammalian telomeres. *Oncogene* 2002, **21**, 532–540.
36. Chan SWL, Blackburn E. New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. *Oncogene* 2002, **21**, 553–563.
37. Folini M, Berg K, Millo E, *et al*. Photochemical internalization of a peptide nucleic acid targeting the catalytic subunit of human telomerase. *Cancer Res* 2003, **63**, 3490–3494.
38. Sharma GG, Gupta A, Wang H, *et al*. hTERT associates with human telomeres and enhances genomic stability and DNA repair. *Oncogene* 2003, **22**, 131–146.