

Telomere maintenance in telomerase-positive human ovarian SKOV-3 cells cannot be retarded by complete inhibition of telomerase

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Abstract The two known mechanisms for telomere maintenance in eukaryotes are telomerase in telomerase-positive cells and alternative lengthening of telomeres (ALT) in telomerase-negative cells. We report here that telomere maintenance in the telomerase-positive human ovarian SKOV-3 cells was not affected by inhibition of telomerase. For comparison, the effect of telomerase inhibitors on telomere maintenance in another telomerase-positive cell line (i.e. human pharynx FaDu cells) and the telomerase-negative human osteosarcoma Saos-2 cells was examined. Telomerase activity was measured using a modified telomeric repeat amplification protocol and telomere length was measured using a solution hybridization-based method and fluorescence in situ hybridization. A reverse transcriptase inhibitor (3'-azido-deoxythymidine or AZT) and an antisense against a component of human telomerase RNA (antisense hTR) were used to inhibit telomerase. FaDu and SKOV-3 cells showed comparable baseline telomerase activity. Telomerase activity in both cells was inhibited about equally by AZT (maximal inhibition of ~80%) and by expression of antisense hTR (complete inhibition in SKOV-3 cells and maximal inhibition of ~80% in FaDu cells). However, treatment with telomerase inhibitors resulted in ~50% telomere shortening in FaDu cells but had no effect on SKOV-3 nor Saos-2 cells. SKOV-3 cells did not show the characteristic features of ALT (i.e. heterogeneous telomere length and promyelocytic leukemia bodies), whereas these ALT features were observed in Saos-2 cells. Collectively, these results suggest the existence of a telomerase-independent mechanism of telomere maintenance in the telomerase-positive SKOV-3 cells. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Telomerase; Telomere; Ovarian cancer; Alternative lengthening of telomeres

1. Introduction

Telomeres are DNA–protein complexes that cap the chro-

mosome ends. Telomeres have multiple functions, including preventing chromosomes from fusion and degradation (reviewed in [1]). In normal somatic cells, the telomere repeats are shortened progressively after each cell division, due to the incomplete replication at the telomeric ends. Cell senescence occurs when the telomeres reach critical length, leading to the hypothesis that telomere length is the biological clock that determines the number of divisions and life span of a cell [2]. The two known mechanisms for telomere maintenance in eukaryotes are telomerase in telomerase-positive cells and alternative lengthening of telomeres (ALT) in telomerase-negative cells.

Most germ-line and stem cells, immortalized cells, and cancer cells contain telomerase, a ribonucleoprotein DNA polymerase, that adds telomere repeats onto the 3'-ends of chromosomes. Telomerase activity is observed in >85% of cancer cell lines and human tumors [3]. Inhibition of telomerase using an antisense to human telomerase RNA component (antisense hTR) or dominant negative expression of mutant telomerase reverse transcriptase (TERT) caused telomere shortening, cell senescence, apoptosis and chromosome instability in telomerase-positive cells [4,5]. On the other hand, ectopic expression of TERT in telomerase-negative human fibroblasts, retinal epithelial cells and vascular endothelial cells results in elongation of telomere length, extension of life span and allowance of cells to bypass the senescence limitation [6–8].

About 15% of human tumors and cancer cell lines do not express detectable telomerase activity. Telomeres in these cells are maintained by mechanisms that are independent of telomerase, referred to as ALT [9]. For example, studies using telomerase RNA component-deficient mice (mTER^{-/-}) demonstrated a telomerase-independent mechanism for telomere maintenance and elongation [10]. In yeasts lacking telomerase activity and in mosquito *Anopheles gambiae*, telomeres are maintained by a homologous recombination-based mechanism [11,12]. *Drosophila melanogaster* and related *Diptera* species maintain their telomere length by transposition of a set of retrotransposons [13,14]. The characteristic features of ALT are highly heterogeneous telomere length, in a range of <5 kb to >20 kb and the presence of specific promyelocytic leukemia (PML) bodies that are donut-shaped structures in nuclei initially observed in PML cells [9,15,16]. The ALT-associated PML bodies are unique, consisting of telomeric DNA and telomere-binding proteins TRF1 and TRF2, and are universally present in ALT cells but absent in mortal cells and telo-

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Abbreviations: ALT, alternative lengthening of telomeres; AZT, 3'-azido-deoxythymidine; FISH, fluorescence in situ hybridization; hTR, human telomerase RNA component; IPTG, isopropyl-β-D-thiogalactopyranoside; NHEJ, non-homologous end joining; PML, promyelocytic leukemia; TERT, telomerase reverse transcriptase; TRAP, telomeric repeat amplification protocol

merase-positive cell lines or tumors [15–17]. ALT in human cells was surmised from their ability to maintain the telomere length in the absence of telomerase and from the presence of telomeres with highly heterogeneous lengths [9]. Recently, an ALT mechanism of telomere-to-telomere recombination was found in GM847 cells derived from human skin fibroblasts [18].

Zaffaroni and colleagues reported that partial inhibition of telomerase by either peptide nucleic acids or a hammerhead ribozyme targeting the hTR did not result in telomere shortening in the telomerase-positive human melanoma JR8 and M14 cells, and postulated the emergence of activation of the ALT mechanism as a response to telomerase inhibition [19,20]. However, these cells did not show the characteristics of ALT before or after treatments with telomerase inhibitors. It is also not known whether the residual telomerase activity was sufficient for telomere maintenance in these cells.

We report here that telomere maintenance in the telomerase-positive human ovarian SKOV-3 cells was not affected by complete inhibition of telomerase. Another telomerase-positive cell line (i.e. human pharynx FaDu cells) was used as the positive control, and the telomerase-negative human osteosarcoma Saos-2 cells which use the ALT mechanism [9] was used as the negative control. A reverse transcriptase and telomerase inhibitor, 3'-azido-deoxythymidine or AZT [21] and antisense to hTR, were used to inhibit telomerase.

2. Materials and methods

2.1. Chemicals and reagents

Sulforhodamine B, streptolysin O, *p*-phenylenediamine and bicinchoninic acid kit for protein determination were purchased from Sigma (St. Louis, MO, USA); polynucleotide kinase, restriction endonucleases (*Hinf*I, *Hae*III and *Hha*I), DNA molecular weight markers III and V, hygromycin B, G418, isopropyl- β -D-thiogalactopyranoside (IPTG), cell proliferation ELISA kit (BrdU) and cell death detection ELISA kit from Roche Molecular Biochemicals (Indianapolis, IN, USA); cefotaxime sodium from Hoechst-Roussel (Somerville, NJ, USA); gentamicin from Solo Pak Laboratories (Franklin Park, IL, USA); minimum essential medium, RPMI1640 medium, fetal bovine serum (FBS), Lipofectamine[®], and colcemid from Life Technologies (Grand Island, NY, USA); γ -³²P-ATP from ICN (Costa Mesa, CA, USA); and Advantage cDNA Polymerase Mix from Clontech (Palo Alto, CA, USA). All chemicals and reagents were used as received.

2.2. Cell culture

Human ovarian SKOV-3, pharynx FaDu and osteosarcoma Saos-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). SKOV-3 cells were cultured in McCoy's medium and FaDu and Saos-2 cells in minimum essential medium. The media were supplemented with 9% heat-inactivated FBS, 0.1% 10 mM non-essential amino acids, 2 mM L-glutamine, 90 μ g/ml gentamicin and 90 μ g/ml cefotaxime sodium. Cells in exponential growth phase were used for experiments.

SKOV-3, FaDu and Saos-2 cells were continuously treated with AZT for up to 9 weeks. Each week, the medium was exchanged and cells were harvested for analysis of telomerase activity and telomere length.

2.3. Telomere length analysis and detection of PML bodies

Telomere length was measured by two methods. The first method used a solution hybridization-based analysis to detect the mean length of the terminal restriction fragments. This method measured both the amount and length of telomere (i.e. telomere amount and length assay, TALA) [22]. In brief, genomic DNA was isolated and 10 μ g of DNA was digested at 37°C overnight with 10 U each of *Hinf*I/*Cfo*II/*Hae*III. The oligonucleotide probe (TTAGGG)₄ was labeled by γ -³²P-ATP with polynucleotide T4 kinase. 3 ng of the probe was added to 2.5 μ g of DNA solution. After denaturation at 98°C for 5 min, hy-

bridization was performed at 55°C overnight. The samples were electrophoresed on 0.7% agarose gel. After drying under vacuum without heating, the gel was exposed to phosphorimage screen and the result was analyzed by ImageQuaNT software from Molecular Dynamics (Sunnyvale, CA, USA).

The second method for telomere length analysis was fluorescence in situ hybridization (FISH), which detected telomere signal in individual cells, as described previously [22]. FISH was also used to detect PML bodies. The fluorescein-labeled peptide nucleic acid (PNA) probe (CCCTAA)₃ was synthesized by PerSeptive Biosystems (Framingham, MA, USA). We evaluated the telomere length in interphase and M phase cells. Briefly, cells were treated with 0.1 μ g/ml colcemid for 4 h and then harvested. After treatment with hypotonic solution and fixation with acetic acid and methanol, cells were dropped onto slides, air-dried and stored at -20°C. Cells were denatured at 80°C for 2 min and hybridized to the PNA probe at room temperature for 2 h. Slides were then washed at room temperature with 70% formamide and phosphate-buffered saline (PBS). The chromosomes were counterstained with propidium iodide and examined under a fluorescence microscope. The digital images were analyzed by Scion Image software (NIH Image for PC).

2.4. Measurement of telomerase activity

Telomerase activity in intact cells was measured using a modified quantitative telomeric repeat amplification protocol (TRAP) [23]. Briefly, cells were washed twice with PBS solution and reversibly porated by streptolysin O (5 U/ml) in the presence of TS primer (2 μ M), and dNTP (50 μ M) in 100 μ l of serum-free medium at room temperature for 5 min. The culture medium (900 μ l) containing 10% FBS was added to stop the permeabilization process. The TS primer that penetrated the cells was extended in situ by intracellular telomerase by incubation at 30°C for 30 min. The extended TS primer was then extracted by CHAPS-based lysis buffer, purified using phenol/chloroform extraction to eliminate polymerase chain reaction (PCR) inhibitors. The resulting primers were used as the template for PCR amplification as described above. The PCR products were separated by polyacrylamide gel electrophoresis, followed by ethidium bromide staining and quantification by GPTools gel analysis software (Biophotonics, Ann Arbor, MI, USA).

2.5. Inhibition of telomerase by antisense hTR

To construct a recombinant expression vector containing antisense or sense hTR, a 185 bp hTR fragment, including the telomere template sequence in hTR, was obtained by RT-PCR with the primers 5'-CAGCTGACATTTTGTGCTCTA-3' and 5'-GGGTTGCGGAGGGTGGGCCT-3'. The fragment was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The recombinant plasmid was digested using *Not*I and the resulting 219 bp DNA fragment was subcloned into the *Not*I site of the expression vector pOPRSV/MCS (LacSwitch[®] II Inducible Mammalian Expression System, Stratagene, La Jolla, CA, USA). The clones that expressed antisense and sense hTR were confirmed by sequencing with T7 or T3 promoter primer (fmol[®] DNA Sequencing System, Promega). SKOV-3 cells were transfected with the Lac repressor vector, pCMVLac (LacSwitch[®] II Inducible Mammalian Expression System), using Lipofectamine. The transfected cells were selected by hygromycin B treatment. Cells were then transfected with the recombinant operator vector

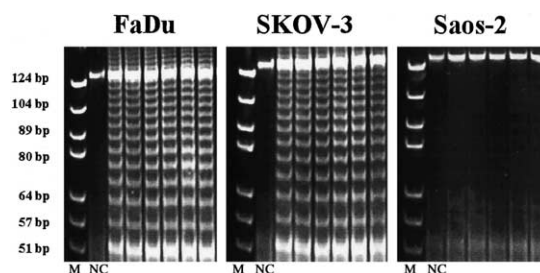


Fig. 1. Telomerase activity in SKOV-3, FaDu and Saos-2 cells. Telomerase activity (2.0 μ g protein per sample) was detected by the modified TRAP. Six replicates for each cell line. M: pBR322/*Hae*III DNA molecular weight marker. NC: negative control using cell lysis buffer instead of lysate.

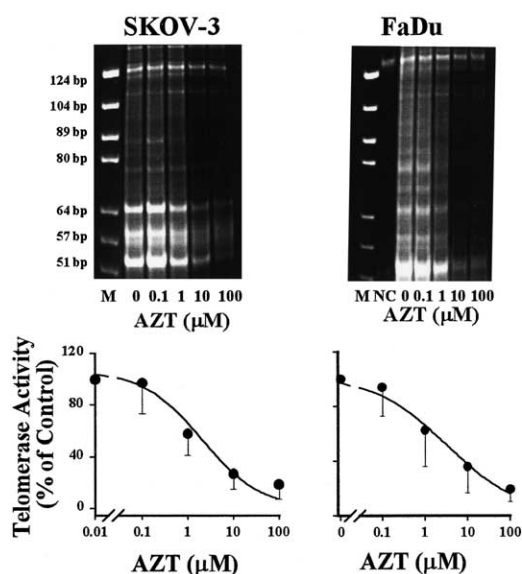


Fig. 2. Inhibition of telomerase in SKOV-3 and FaDu cells by AZT. Top panels: Results of intracellular TRAP. M: pBR322/*Hae*III DNA molecular weight marker, NC: negative control obtained using the permeabilization solution without TS primer. Bottom panels: Drug concentration–response curves.

pOPRSVI/MCS which expressed either antisense or sense hTR. The transfected cells were selected by treatment with hygromycin B and G418. The expression of antisense and sense hTR was induced by IPTG (3 mM). To ascertain the functionality of the antisense hTR and to rule out the effect of transfection and IPTG treatment, we used three controls, i.e. parent cells, cells that expressed sense hTR, and cells transfected with antisense hTR that was not expressed (i.e. without IPTG induction).

2.6. Analysis of AZT concentrations

Changes in AZT concentrations (starting concentration of 100 μM) in culture medium over time, in the absence or presence of tumor cells, were analyzed using an enzyme-linked immunosorbent assay (Neogen, Lexington, KY, USA). Briefly, 50 μl each of medium (pre-diluted 625-fold with the extraction buffer), AZT antibody, and horseradish peroxidase-conjugated AZT were added to the wells precoated with protein A and incubated at room temperature for 1 h. After washing three times, 150 μl of substrate solution containing tetramethylbenzidine and hydrogen peroxide was added to each well and allowed to stand for 30 min. The absorbance at 650 nm was measured using a microplate reader. The concentration of AZT was calculated according to the standard curve constructed by adding known amounts of AZT to culture medium. The standard curve was linear between 2 and 200 ng/ml AZT.

3. Results

3.1. AZT concentrations in culture medium

Analysis of AZT in the culture medium showed that AZT was stable under the experimental conditions. The AZT concentrations, after incubation in the absence or presence of FaDu, Saos-2 and SKOV-3 cells for 3 and 7 days, ranged from $96 \pm 9\%$ to $105 \pm 16\%$ of the starting concentrations.

3.2. Baseline telomerase activity

FaDu and SKOV-3 cells showed comparable baseline telomerase activity (Fig. 1); the activity in SKOV-3 cells was $86.5 \pm 10.5\%$ of the activity in FaDu cells ($P=0.12$). In agreement with a previous report [9], Saos-2 cells did not show the characteristic ladder TRAP products. The background signals obtained from Saos-2 cells were $9.5 \pm 5.8\%$ of the signals in FaDu cells.

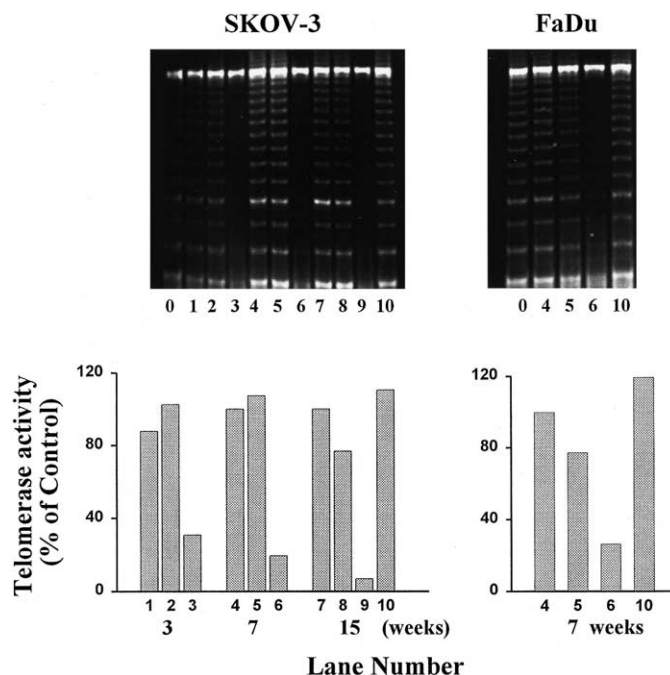


Fig. 3. Inhibition of telomerase in SKOV-3 and FaDu cells by antisense hTR. Lane 0: control cells before transfection. Lanes 1–3, 4–6 and 7–10: cells cultured for 3, 7 and 15 weeks, respectively. Lanes 1, 4, 7: non-transfected control cells. Lanes 2, 5, 8: cells expressing sense hTR (i.e. with IPTG induction). Lanes 3, 6, 9: cells expressing antisense hTR. Lane 10: cells transfected with antisense hTR without IPTG. Data are expressed as % of non-transfected control cells harvested at the beginning of the experiment (i.e. data shown in Lane 0).

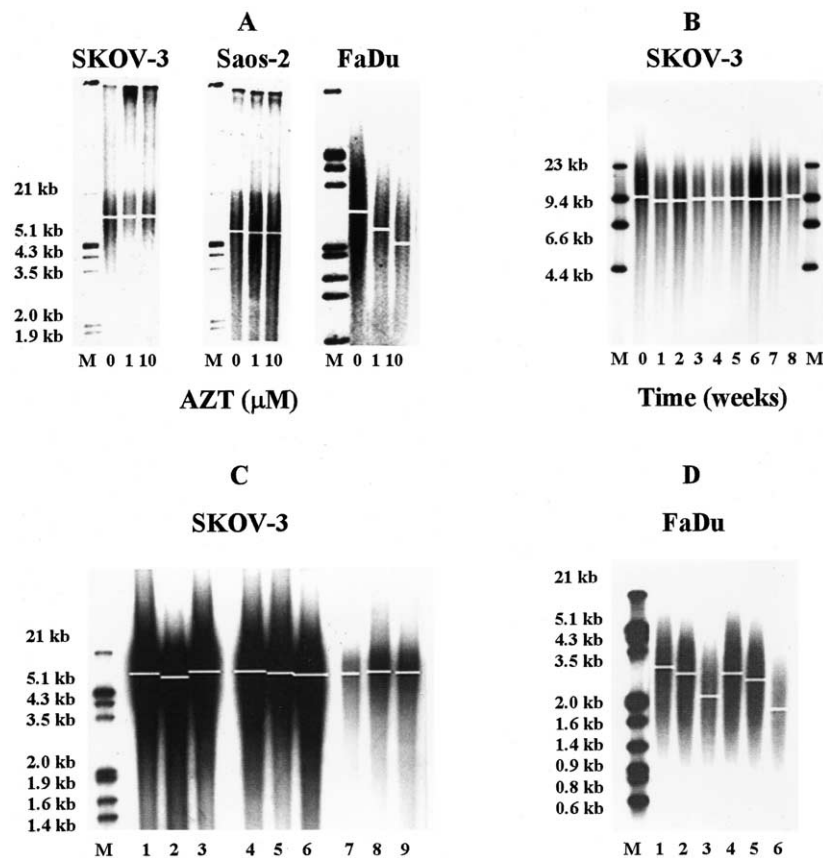


Fig. 4. Effect of AZT and antisense hTR on telomere length in SKOV-3, Saos-2 and FaDu cells. A: Telomere length in cells treated with AZT for 7 weeks. B: Effect of AZT (100 μ M) on telomere length in SKOV-3 cells, as a function of treatment time. Lane 0, control (before AZT treatment). Lanes 1–8 correspond to AZT treatment for 1–8 weeks. C: Effect of antisense hTR in SKOV-3 cells. Lanes 1–3, 4–6 and 7–10 for samples treated for 3, 7 and 15 weeks, respectively. Lanes 1, 4, 7: non-transfected control cells. Lanes 2, 5, 8: cells expressing sense hTR (i.e. with IPTG induction). Lanes 3, 6, 9: cells expressing antisense hTR. D: Effect of antisense hTR in FaDu cells. Lanes 1–3 and 4–6 for samples treated for 3 and 7 weeks, respectively. Lanes 1, 4: control FaDu cells. Lanes 2, 5: cells transfected with sense hTR. Lanes 3, 6: cells transfected with antisense hTR. M: Molecular weight markers: λ DNA/*Eco*RI and *Hind*III for (A), (C) and (D); λ DNA/*Hind*III for (B). Horizontal bars depict the locations of the average telomere length.

3.3. Inhibition of telomerase by AZT

AZT is converted intracellularly to AZTTP, which inhibits telomerase. Hence, we examined the effect of AZT only in intact cells. Fig. 2 shows the concentration-dependent telomerase inhibition by AZT in SKOV-3 and FaDu cells. In both cell types, the maximal inhibition was $\sim 80\%$ and was achieved at 100 μ M AZT, the highest concentration used in the experiment. Similar IC_{50} values were found for SKOV-3 and FaDu cells (2.2 ± 1.5 μ M and 2.1 ± 1.3 μ M, respectively).

3.4. Inhibition of telomerase by antisense hTR

Fig. 3 shows the effect of antisense hTR on the telomerase activity in SKOV-3 and FaDu cells. In both cells, inhibition of telomerase was observed in cells expressing antisense hTR (i.e. cells transfected with antisense hTR and induced by IPTG) treatment, whereas no changes were observed in cells expressing sense hTR or cells transfected with antisense hTR but without IPTG induction of antisense expression.

Inhibition of telomerase activity in SKOV-3 and FaDu cells by antisense hTR progressed with time and reached maximal levels at 15 weeks. The maximal inhibition in SKOV-3 cells was greater than in FaDu cells (93.3% vs. 74.1%). The resid-

ual telomerase activity in SKOV-3 after expressing the antisense hTR for 15 weeks was less than the background signal found in Saos-2 cells, indicating complete telomerase inhibition in SKOV-3 cells

3.5. Effect of telomerase inhibition on telomere length

Fig. 4 shows the effect of telomerase inhibition on telomere length. Treatment with AZT for up to 9 weeks or with antisense hTR for up to 15 weeks had no effect on the telomere length in SKOV-3 cells nor Saos-2 cells. In contrast, the same treatments resulted in significant telomere erosion in FaDu cells (i.e. 27.3% by antisense hTR and 18.4% by 10 μ M AZT in 7 weeks).

3.6. Characteristic features of ALT

Fig. 5 compares the morphology of Saos-2 and SKOV-3 cells. Note the presence of donut-shaped PML bodies in the interphase Saos-2 cells and the presence of heterogeneous strength of telomere signals in the M phase Saos-2 cells. The results indicate the variable telomere length. In contrast, both M phase and interphase SKOV-3 cells showed relatively homogeneous telomeric signals with no PML bodies in the nucleus.

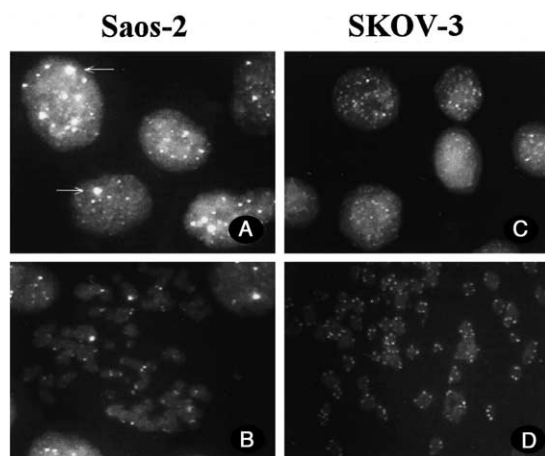


Fig. 5. Detection of telomeres and PML bodies by FISH. Panels A and C: interphase cells. Arrows indicate PML bodies. Panels B and D: M phase cells.

4. Discussion

Results of the present study show that in the telomerase-positive human ovarian SKOV-3 tumor cells, inhibition of telomerase by a reverse transcriptase inhibitor AZT or antisense to hTR did not lead to telomere erosion. Because the telomerase activity in SKOV-3 cells was completely inhibited by antisense hTR, the absence of telomere shortening could not be due to residual telomerase activity. This is further confirmed by the finding that AZT and antisense hTR treatments resulting in the same or lower extent of telomerase inhibition caused significant telomere erosion in other telomerase-positive human pharynx FaDu cells, which showed about the same baseline telomerase activity compared to SKOV-3 cells. SKOV-3 cells also did not show the characteristics of ALT, i.e. highly heterogeneous telomere length and presence of PML bodies, as found in the telomerase-negative Saos-2 cells. The data of AZT stability in culture media rule out AZT instability as a reason that AZT failed to reduce the telomere length in SKOV-3 cells.

There is evidence that telomere maintenance may involve molecules that participate in DNA repair. For example, mutations of *ATM* homologs that are DNA damage sensors, i.e. *MEC1* and *TEL1*, in budding yeast resulted in telomere shortening and senescence phenotype similar to a telomerase-deficient strain [24,25]. The involvement of DNA damage sensors in telomere maintenance raises the possibility that the uncapped telomeres may transiently resemble double strand breaks in DNA and thereby invoke the repair process [26]. The two repair processes for double strand breaks are the homologous recombination which has been observed in ALT cells and the non-homologous end joining (NHEJ) which involves the binding of Ku heterodimers to DNA breaks that acts to protect DNA from degradation and to stabilize the lesion [26,27]. The Ku protein is present in telomere DNA-protein complex [28]. The loss of *MEC1* and *TEL1* functions may affect the ability to recruit Ku from telomeric heterochromatin to the chromosome terminus, which in turn affects the chromosome end processing [27].

In addition to Ku, *RAD50*, *XRS2* and *MRE11* are also involved in NHEJ in yeast, and mutations in these proteins lead to telomere shortening, even in the presence of functional telomerase [26]. It is not known whether NHEJ plays a role in the telomere maintenance in SKOV-3 cells.

In summary, results of the present study suggest the existence of a telomerase-independent mechanism for telomere maintenance in the telomerase-positive SKOV-3 cells.

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