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Research Article

Evaluation of combination gene therapy with *PTEN* and antisense *hTERT* for malignant glioma *in vitro* and xenografts

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Abstract. Telomerase activation is a critical event in cell immortalization, and an increase in human telomerase reverse transcriptase (hTERT) expression is the key step in activating telomerase. The phosphatase and tensin homolog (*PTEN*) gene encodes a double-specific phosphatase that induces cell cycle arrest, inhibits cell growth, and causes apoptotic cell death. Here, we evaluated a combined *PTEN* and antisense *hTERT* gene therapy for experimental glioma *in vitro* and *in vivo*. We demonstrated that infection with antisense-*hTERT* and wild-type-*PTEN* adenoviruses significantly inhibited human U251 glioma cell proliferation *in vitro* and glioma growth in a xenograft mouse model. The efficacy of therapy was obviously higher in the tumor xenografts infected with both *PTEN* and antisense *hTERT* than in the gliomas infected with either agent alone at the same total viral dose. Consistent with these results, we showed that telomerase activity and hTERT protein levels were markedly reduced in the glioma cells following adenovirus infection. In contrast, the levels of PTEN protein expression were dramatically increased in these cells. Our data indicate that combination treatment with antisense *hTERT* and wild-type *PTEN* effectively suppresses the malignant growth of human glioma cells *in vitro* and in tumor xenografts, suggesting a promising new approach in glioma gene therapy that warrants further investigation.

Keywords. Antisense hTERT, PTEN, glioma, gene therapy, combination therapy, xenograft.

Introduction

Gliomas are the most common type of primary intracranial tumor and constitute 40–50 % of brain tumors. Patients suffering from malignant gliomas have a life-span of only 9–12 months after diagnosis [1]. Presently, the conventional treatment available for gliomas includes surgery, radiotherapy, and chemotherapy. Although a great deal of effort has been directed toward the therapy of gliomas, the prognosis for patients has not greatly improved. Therefore, developing new therapeutic strategies for malignant gliomas is a top priority.

Cellular immortality has been implicated in the development of malignant tumors, including gliomas.

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The activation of telomerase is believed to be a critical event in cell immortalization, and the up-regulation of human telomerase reverse transcriptase (hTERT) mRNA expression is the key step in the activation of telomerase [2–5]. Recent evidence showed that telomerase activity and hTERT were considerably higher in the tissues of many different cancers than in normal tissues, and that hTERT mRNA was detectable in all tumor cells possessing telomerase activity [6–9]. Higher telomerase activity and higher levels of hTERT mRNA expression correlated significantly with poor prognosis [10, 11]. The most recent and significant advances in this area demonstrated that the inhibition or down-regulation of *hTERT* expression could inhibit telomerase activity and prevent the malignant proliferation of tumor cells [12–15].

The principal reason for the poor prognosis of malignant gliomas is believed to be related to the growth pattern of glioma cells, in which the majority of the proliferative glioma cells infiltrate into the rim of the tumor or "normal brain tissue." The phosphatase and tensin homolog gene (*PTEN*), a ubiquitous tumor suppressor similar to p53, is important in this infiltration process [16–18]. PTEN is a double-specific phosphatase, exhibiting both lipid phosphatase and protein phosphatase activities. It dephosphorylates some phospholipids and proteins and thereby inhibits cell growth, promotes apoptosis, and regulates the processes of cell adhesion, migration, diffusion, and differentiation [16, 17]. Specifically, the functions of PTEN are twofold. First, PTEN regulates the activity of phosphatidylinositol 3,4,5-trisphosphate (PIP3) via its lipid phosphatase activity, subsequently inhibiting the activity of Akt/PKB and regulating cell growth and apoptosis. Second, it regulates the expression of focal adhesion kinase (FAK) via its tyrosine phosphatase activity, subsequently regulating cell adhesion, cell migration, cytoskeletal organization, and MAP kinase activation. Mutations and/or deletion of *PTEN* were shown to occur only in malignant gliomas and not in I-II gliomas [18]. More than half of the cell lines derived from glioblastoma multiforme (GBM) have mutations or deletion of the *PTEN* gene [19]. Therefore, it is likely that the mutation or deletion of *PTEN* is a critical step in the progression from I-II gliomas to GBM.

Many gene therapy strategies have been developed and used for the treatment of malignant gliomas in animal models during the last decade, but without satisfactory therapeutic effects [20–22]. This might be attributable to difficulty in selecting appropriate target genes. There are many abnormal genes related to malignancy in gliomas, and one-gene therapy can produce only limited effects. Therefore, combined gene therapy with two or more targets may improve the effectiveness of therapy. Our previous studies have demonstrated that hTERT and PTEN may play important roles in the development and progression of malignant gliomas [23, 24], thereby implicating them as potentially important gene therapy targets for the clinical treatment of malignant gliomas. Although a number of approaches have been developed to inhibit *hTERT* expression and restore *PTEN* expression in tumor cells, it has yet to be demonstrated whether *hTERT* inhibition and *PTEN* restoration can affect the proliferation and migration capacity of cancer cells in human gliomas.

In the current study, we investigated the effect of combined *PTEN* and antisense *hTERT* gene therapy on glioma growth. We used the human glioma cell line U251 as a tumor xenograft model for glioma because U251 cells have mutations of the *PTEN* gene. Furthermore, we constructed two expression adenoviral recombinants, one containing wild-type *PTEN* and the other containing *hTERT* antisense cDNA against the core sequence located adjacent to the transcriptional start point of the *hTERT* promoter, and showed significant inhibition of malignant growth, *in vitro* and *in vivo*, in glioma-derived cells co-infected with these two recombinant expression adenoviruses.

Materials and methods

Generation of recombinant adenoviruses encoding antisense *hTERT* and wild-type *PTEN*. The adenoviral recombinant encoding antisense *hTERT* (pAdeasy-hTERT) bearing enhanced green fluorescent protein (EGFP) and the 458-bp *hTERT* antisense cDNA aiming directly at the core sequence located adjacent to the transcriptional start site of *hTERT* promoter, and the adenoviral recombinant encoding wild-type *PTEN* (pAdeasy-PTEN) bearing EGFP and wild-type *PTEN* were generated in our laboratory using the Adeasy system. The procedures used for production of the adenoviral recombinants have been described previously [25]. The adenoviral recombinants pAdeasy-hTERT and pAdeasy-PTEN used for transduction were isolated from *E. coli* strain DH5α and purified with the Wizard Plus SV Minipreps Kit (Promega, Madison, WI, USA). DNA concentration and purity were measured with an ultraviolet scanning spectrophotometer. The isolated pAdeasy-hTERT and pAdeasy-PTEN were confirmed by 1% agarose gel electrophoresis after digestion with restriction enzymes. The titers of the homogeneous viruses produced after infection of 293 cells ranged from 10^{10} to 10^{11} expression-forming units (EFU) in 293 cells.

Cell culture and infections with adenoviruses. The human glioma cell line U251 was grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and 1% penicillin-streptomycin, and maintained at 37°C with 5% CO_2. At 1 or 2 days before infection, cells were seeded into 35-mm plates and cultured in DMEM with 10% fetal calf serum but without antibiotics. Once cells grew to 60–70% confluence, the U251 cells were infected with the adenoviral recombinants pAdeasy-hTERT and/or pAdeasy-PTEN according to the manufacturer's protocol. At 5 h after the infection, the medium was replaced with fresh DMEM containing serum but without antibiotics, and the cells were further incubated for an additional 48 h or until harvest.

Cell survival assay. The effect of antisense *hTERT* and/or *PTEN* on cell growth in the human glioma U251 cells was determined by MTT assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega), according to the manufacturer's instructions. The absorbance (A) readings were taken using a 96-well Opsys MR^{TM} Microplate Reader (ThermoLabsystems, Chantily, VA, USA) and Revelation^{TM} QuickLink Software at 490 nm. The blank control wells were used for zeroing absorbance. The percentage of cell survival was calculated using the background-corrected absorbance as follows:

$$\% \text{ cell viability} = 100 \times (1 - A_{\text{experimental well}}) / A_{\text{untreated control well}}$$

All experiments were performed at least three times with representative data presented.

Apoptosis and proliferation assays. For detection of apoptosis of glioma cells, the Apo-BrdU-IHCTM *In Situ* DNA Fragmentation Assay Kit (BioVision, Inc., USA) was used. This kit measures the amount of DNA fragmentation released into the cytoplasm. Briefly, cells were plated at a density of 1×10^5 cells/well in six-well plates in their corresponding media. After 24 h, cells were infected with pAdeasy-hTERT and/or pAdeasy-PTEN, and harvested 3 days after infection. The harvested cells were adhered to glass slides by directly placing the suspension onto the slide. Slides were pre-coated with poly-L-lysine to enhance cell adherence. Cells were permeabilized with proteinase K, and inactivation of endogenous peroxidase and end labeling reaction and detection were performed according to the manufacturer's instructions.

Proliferating cell nuclear antigen (PCNA) is a 36-kDa protein also known as cyclin that has been identified as the polymerase-associated protein, and is synthesized in early G1 and S phases of the cell cycle. PCNA was therefore measured as a parameter of cell proliferation activity. Following infection, cells were stained with PCNA antibody (1:200) via immunohistochemistry (avidin-biotin-peroxidase complex, ABC). In brief, after adhering the infected cells to glass slides, the endogenous peroxidase and nonspecific binding sites were blocked with H_2O_2 and goat serum, respectively. The cell smears were incubated overnight at 4°C with PCNA antibody. The slides were incubated next with a biotinylated secondary antibody conjugated with ABC, and finally stained with diaminobenzidine peroxidase substrate solution.

Cell cycle analysis by flow cytometry. Cells (5×10^5) were seeded in a 10-cm tissue culture dish and incubated overnight. The cells were infected with pAdeasy-hTERT and/or pAdeasy-PTEN expressing recombinant adenoviruses, maintained in medium and harvested 3 days following infection. Next, cultured cells were washed with a PBS solution and trypsinized, and then fixed in an ethanol (80 %)/PBS solution at 4°C for 30 min. Finally, the cell pellet was incubated in a solution containing 50 ng/ml propidium iodide, 0.2 mg/ml RNase, and 0.1 % Triton X-100, at room temperature for 30 min, and analyzed by flow cytometry (FACSCalibur, BD, USA).

Study of glioma growth in an *in vivo* mouse model. NOD/LtSz-Prkdc (scid)/J (NOD/SCID) mice were transplanted s.c. with 3×10^7 U251 human glioma cells. After the tumors were established, the mice were divided into five groups (n=10/group) and injected with PBS only (U251), blank adenovirus (EGFP), pAdeasy-hTERT (hTERT), pAdeasy-PTEN (PTEN), or pAdeasy-hTERT combined with pAdeasy-PTEN (combined group), respectively. Tumor dimensions were measured with calipers every 2 days. Mean tumor volume was calculated by taking width^2 × length × 0.52 [26]. All measurements were performed in a coded, blinded fashion. Mice were killed humanely 30 days later, and tumors were resected to determine telomerase activity by TRAP assay.

Western blot analysis. Cells were plated at 2×10^5 cells/well in six-well plates. Cells were infected 24 h later with the adenoviral recombinants pAdeasy-hTERT and/or pAdeasy-PTEN. PBS was used in a control group. Cell lysates or tumor tissues were prepared in a buffer containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 1 μg/ml aprotinin, 100 μg/ml PMSF, and 1 % NP40. After protein quantitation using the Lowery protein concentration assay, equal amounts of protein were separated by gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5 % BSA in PBST (PBS pH 7.5 and 0.1 % Tween 20) and then incubated with a 1:500 dilution of primary antibody (anti-hTERT or anti-PTEN) overnight at 4°C. The membrane was washed with PBST and incubated with a peroxidase-conjugated secondary antibody for 1 h after washing. Antibody binding was detected using a chemiluminescence detection system (Pierce, USA), according to the manufacturer's recommendations. Western blot films were digitized, and band net intensities were quantified using the Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA). After developing, the membrane was stripped and reprobed with an antibody against β-actin to confirm equal sample loading.

Telomerase activity assay. Telomerase activity was measured by the polymerase chain reaction (PCR)-based telomeric repeats amplification protocol (TRAP) assay [27] using the TRAPeze Kit (Intergen Co., Oxford, UK). In brief, after extension of the substrate [5′-^{32}P]-end-labeled 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. The TRAP reaction was as follows. For primer elongation, 25 μl reaction mixture was transferred into a tube suitable for PCR amplification, and 2 μl cell extract was added with sterile water to a final volume of 50 μl; the tubes were transferred to a thermal cycler and one cycle at 37°C for 20 min was performed. For telomerase inactivation, one cycle was performed at 85°C for 5 min. The reaction mixture, 50 μl, containing dNTP, Taq polymerase, the ^{32}P-labeled TS primer, and RP primer (5'-GCGCGGCTTACCCTTACCCT-TACCCTAACC-3') was amplified for 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, polymerization at 72°C for 45 s, and finally 72°C for 5 min. Quantitative analysis was performed with the Image-QuaNT software (Molecular Dynamics), which allowed densitometric evaluation of the digitalized 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 formula: $[(X-X_0)/C] \times [(R-R_0)/Cr]^{-1} \times 100$, which represents the total product generated (TPG), where X is the heat-untreated sample, X_0 is the heat-treated sample, C is the internal control of heat-untreated samples, Cr is the internal control of TSR8, R is the TSR8 quantitation control, and R_0 is the negative control. An extract of the 293 cell line that had telomerase activity was used as a positive control (provided by the kit).

Statistical analysis of data. Data were analyzed with SPSS 10.0, followed by variance analysis for comparison among different groups. The Student's t-test was also used to analyze the statistical significance of the differences between control and treatment groups. A value of $p<0.05$ was considered statistically significant.

Results

Cell survival and proliferation were decreased in U251 human glioma cells following infection with pAdeasy-hTERT and pAdeasy-PTEN. We set out to determine the survival rates of U251 glioma cells infected with pAdeasy-hTERT and pAdeasy-PTEN recombinant adenoviruses. As shown in Figure 1a, the infection with pAdeasy-hTERT and/or pAdeasy-PTEN adenoviruses reduced cell survival significantly, as assessed by an MTT assay. The survival rate was 47.10 % on day 5, and the lowest rate of survival, 37.61 % on day 6 (not shown) compared with the controls, was observed in the combined group, in which U251 cells were

infected with both adenoviruses expressing antisense *hTERT* and wild-type *PTEN* (Fig. 1a). Additionally, the population of apoptotic cells was increased remarkably in the U251 cells infected with both antisense-*hTERT* and *PTEN* adenoviruses. The percentages of apoptotic cells on day 3 were 8.38%, 5.88%, and 10.10% in the hTERT, PTEN, and combined groups, respectively. Similar data for apoptotic cell death were obtained in the glioma cells from an *in vivo* tumor xenograft model after adenovirus infection (Fig. 1b). Using PCNA as a biomarker for cell proliferation, we examined the effect of antisense *hTERT* and wild-type *PTEN* on U251 cell proliferation *in vitro* by observing PCNA immunohistochemical staining. As shown in Figure 1c, the proliferation of U251 glioma cells was inhibited significantly following infection with the recombinant adenoviruses, resulting in proliferation activities (PCNA-positive rate) of 52.69%, 58.64%, and 45.94% in the hTERT, PTEN, and combined groups, respectively, on day 3; these values were significantly lower than those in the U251 and EGFP control groups, in which U251 glioma cells were not infected or were infected with the adenovirus containing only EGFP. Similar PCNA-positive rates were also found in the glioma cells from an *in vivo* mouse model (Fig. 1c), indicating a suppressive effect of *PTEN* and antisense *hTERT* on U251 cell growth and proliferation both *in vitro* and *in vivo*.

Effect of antisense *hTERT* and wild-type *PTEN* on cell cycle control in human U251 glioma cells. We then determined whether the inhibitory effect of antisense *hTERT* and wild-type *PTEN* on U251 cell proliferation was mediated, at least in part, through blocking cell cycle progression. We analyzed the cell cycle by flow cytometry and found that U251 cells were arrested at the G0/G1 phase after the cells were infected with pAdeasy-hTERT and/or pAdeasy-PTEN adenoviruses (Fig. 2). The proliferative index (PI) values were 33.7, 31.5, and 9.3 in the hTERT, PTEN, and combined groups, respectively, and the S-phase fractions (SPF) were 16.8, 19.3, and 5.7 in the respective groups. Thus, the proliferation rate was diminished by antisense *hTERT* and *PTEN* in glioma cells, with the maximal effect observed in the cells infected with both recombinant adenoviruses. The cell cycle was not significantly altered in the EGFP control group (G0/G1, 54.7%; S, 32.9%; and G2/M, 12.5%).

Effect of antisense *hTERT* and wild-type *PTEN* on glioma growth in an *in vivo* tumor xenograft model. Given that cell survival and proliferation were reduced in U251 human glioma cells *in vitro* following infection with adenoviruses expressing antisense

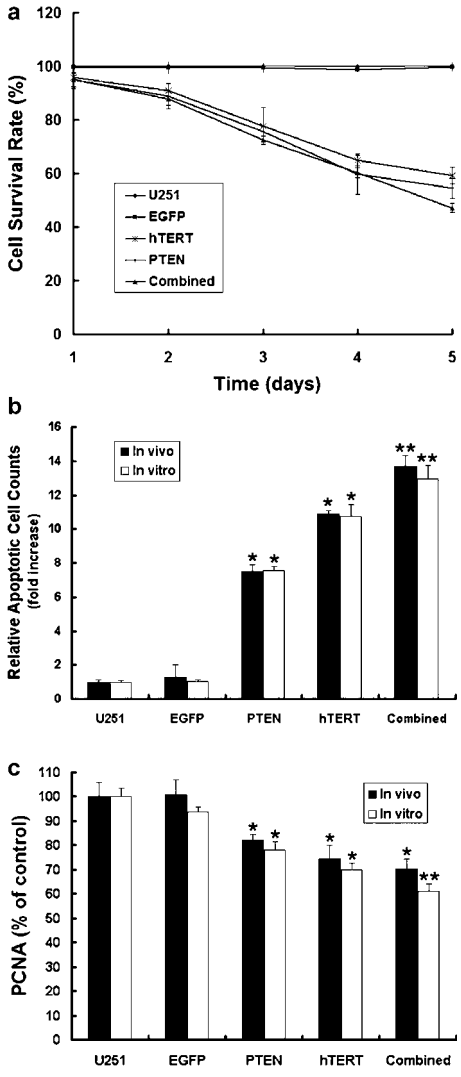

Figure 1. Survival, apoptosis and proliferation of human U251 glioma cells infected with antisense human telomerase reverse transcriptase (*hTERT*) and wild-type *PTEN* adenoviruses *in vitro* and *in vivo*. (*a*) U251 cells were plated at a density of 1×10^5 cells/well in six-well plates. After 24 h, cells were infected with the blank adenovirus (EGFP), pAdeasy-hTERT (hTERT), pAdeasy-PTEN (PTEN), and both recombinant adenoviruses (Combined), respectively. Non-infected cells served as controls (U251). Cell survival was assessed by MTT assay, as described in the Materials and methods. Cell growth values are expressed relative to those of the U251 control cells (control value, 100%). The results represent the means of at least three independent experiments. $p<0.05$ for the combined group vs. the hTERT group at day 5; $p<0.001$ for the combined group vs. the U251 group. (*b*) U251 glioma cell infection was performed using adenoviruses as outlined above, or the *in vivo* tumor model was injected with the adenoviral recombinants, as described in the Materials and methods. Apoptosis of the glioma cells, *in vitro* and *in vivo*, was detected using the Apo-BrdU-IHCTM *In Situ* DNA Fragmentation Assay Kit at 3 days after adenovirus infection. *$p<0.001$ vs. the U251 group; **$p<0.05$ for the combined group vs. the hTERT or PTEN group. (*c*) U251 glioma cell infection was performed *in vitro* using adenoviruses, or the *in vivo* tumor model was injected with the adenoviral recombinants, as mentioned above. Following infection, U251 cells were stained with PCNA antibody (1:200), and PCNA was measured by immunohistochemistry, as described in the Materials and methods. *$p<0.001$ vs. control (the U251 group); **$p<0.05$ for the combined group vs. the hTERT or PTEN group.

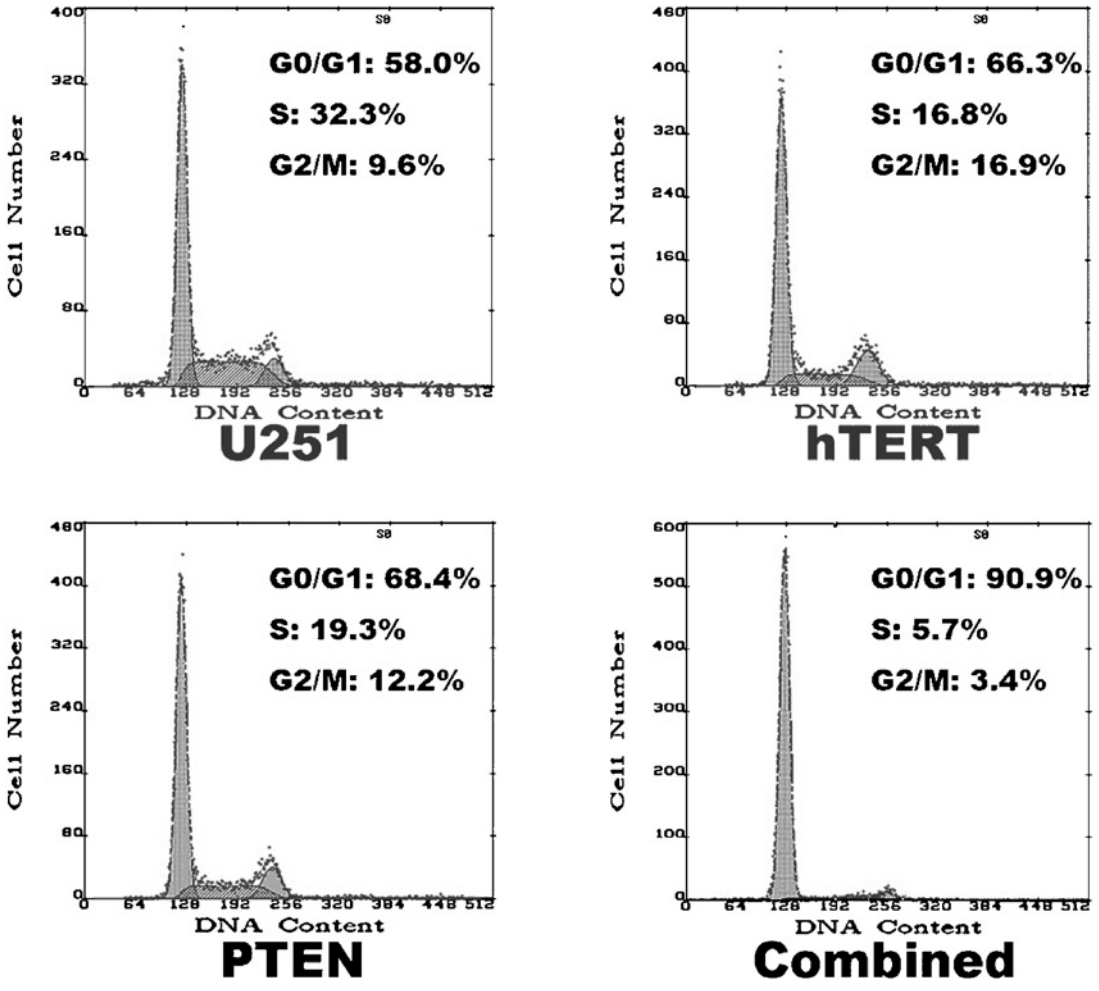

Figure 2. Cell cycle analysis by flow cytometry in U251 human glioma cells following infection with adenoviruses expressing antisense *hTERT* and wild-type *PTEN*. U251 cells were infected with pAdeasy-hTERT (hTERT), pAdeasy-PTEN (PTEN), and both recombinant adenoviruses (Combined), respectively. Non-infected cells were used as controls (U251). The cells were harvested and stained, and the cell cycle distribution of the propidium iodide-labeled cells was analyzed by flow cytometry, as described in Materials and methods. The data are representative of three separate experiments. $p<0.05$ for the G0/G1 population in the combined group vs. the hTERT or PTEN group.

hTERT and wild-type *PTEN*, we next evaluated the effect of antisense *hTERT* and wild-type *PTEN* on glioma growth in an *in vivo* mouse model. The tumors in the hTERT and/or PTEN groups grew very slowly *in vivo*, with dimensions considerably smaller than those in the U251 and EGFP control groups. There was a substantial difference in tumor size between the hTERT/PTEN groups and the control groups even on day 14 of the study; at the termination of the study, the differences in tumor mass between the hTERT/PTEN groups and the control groups were marked ($p<0.001$). The best antitumor effect was observed in the combined group. As shown in Figure 3, the average glioma volumes on day 30 were 3034.60, 3399.67, and 1689.91 mm^3 in the hTERT, PTEN, and combined groups, respectively; the tumor volumes in U251 and EGFP control groups were 7171.78 and 6940.97 mm^3, respectively (Fig. 3).

Levels of hTERT and PTEN in U251 glioma cells infected with pAdeasy-hTERT and pAdeasy-PTEN. We examined the levels of PTEN and hTERT in the human glioma cells exhibiting significantly decreased growth and survival after infection with the antisense-*hTERT* and *PTEN* adenoviruses. After infecting U251 glioma cells with pAdeasy-hTERT, pAdeasy-PTEN, or both recombinant adenoviruses, we determined the protein expression levels of PTEN and hTERT in these cells by Western blot analysis. As shown in Figure 4a, the levels of hTERT protein were considerably lower in U251 cells infected with the antisense-*hTERT* adenovirus alone or with the combination of antisense-*hTERT* and *PTEN* adenoviruses in comparison with the levels in the U251 and EGFP controls. The hTERT protein level was also moderately reduced in U251 cells following infection with the *PTEN* adenovirus (Fig. 4a, c). By contrast, the PTEN protein

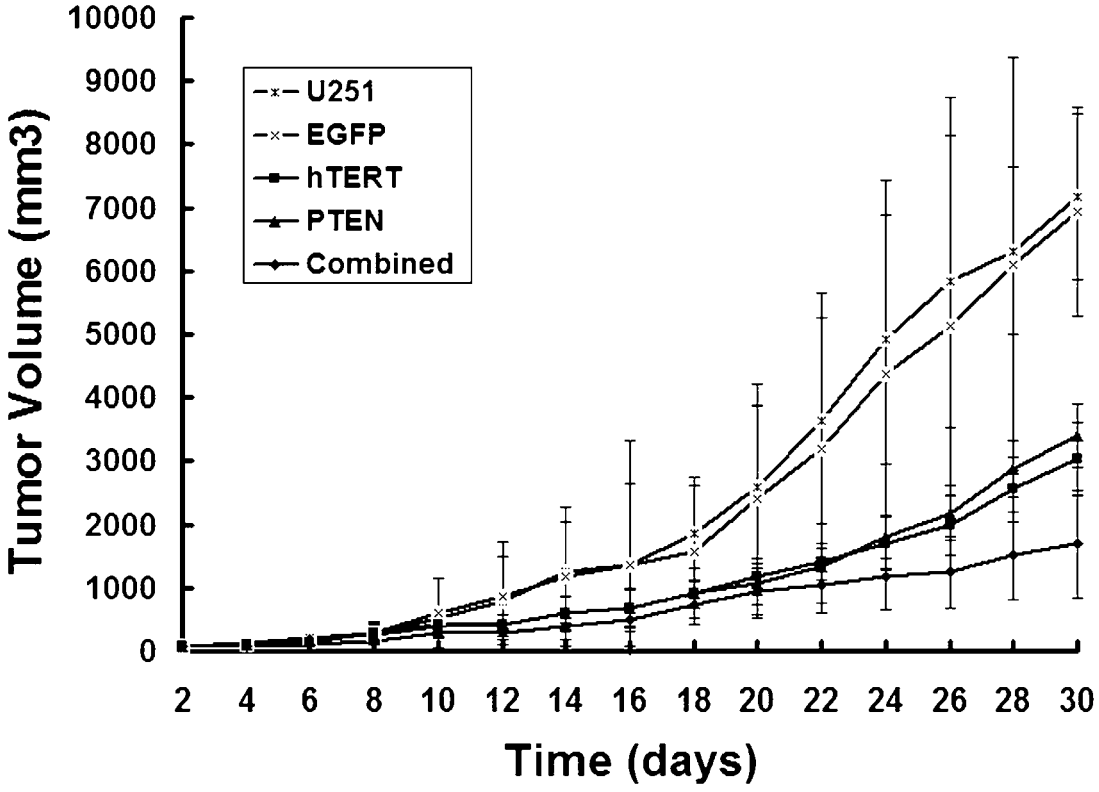

Figure 3. Antitumor effect of antisense *hTERT* and wild-type *PTEN* on the growth of human U251 glioma in nude mice. A total of 3×10^7 U251 glioma cells was transplanted s.c. into NOD/SCID mice. After the tumors were established in the mice, each of five groups was injected with PBS only (U251), the empty adenovirus (EGFP), the antisense-*hTERT* adenovirus (hTERT), the wild-type-*PTEN* adenovirus (PTEN), or the combination of antisense-*hTERT* and *PTEN* adenoviruses (Combined), respectively. The ability of antisense *hTERT* and *PTEN* to inhibit tumor growth, *in vivo*, was determined by measuring the tumor volume, as described in Materials and methods. The tumor dimensions were measured with calipers every 2 days. The mean tumor size (mm^3) was calculated as $width^2 \times length \times 0.52$. All measurements were performed in a coded, blinded fashion. $p<0.001$ for the combined group vs. the hTERT or PTEN group at day 30.

levels were dramatically elevated in U251 glioma cells infected with the *PTEN* adenovirus alone or with both the antisense-*hTERT* and *PTEN* adenoviruses (Fig. 4b, c).

Telomerase activity was reduced in U251 glioma cells infected with pAdeasy-hTERT and pAdeasy-PTEN. We reasoned that the demonstrated ability of antisense *hTERT* and *PTEN* to inhibit human U251 glioma cell proliferation and tumor growth *in vitro* and *in vivo* might be attributable to their interference with telomerase activity. We therefore assessed telomerase activity in glioma cells in which the protein levels of PTEN and hTERT were changed by antisense *hTERT* and wild-type *PTEN*. For these experiments, U251 glioma cells were infected with the adenoviruses, and the *in vivo* tumor model was injected with the adenoviral recombinants, as described in the Materials and methods. Telomerase activity in glioma cells from both the *in vitro* and *in vivo* studies was measured using the TRAP method. Our results revealed that the telomerase activity in U251 cells was strikingly decreased by antisense *hTERT* and *PTEN* as compared with the activity in the control cells. As shown in Figure 5a and b, the telomerase activities were 63.4, 131.6, and 28.8 TPG *in vitro*, and 105.4, 187.4, and 57.2 TPG *in vivo*, in the hTERT, PTEN, and combined groups, respectively. In contrast, the telomerase activity ranged from 237.0 to 310.8 TPG *in vitro*, and from 314.2 to 261.4 TPG *in vivo*, in the U251 and EGFP control groups. These data suggest that the inhibitory effect of antisense *hTERT* and *PTEN* on the survival and growth of human glioma cells *in vitro* and *in vivo* may occur partly through the down-regulation of *hTERT* expression and the suppression of telomerase activity.

Discussion

We evaluated the effect of a combined *PTEN* and antisense *hTERT* gene therapy on tumor growth in a human U251 glioma cell line *in vitro* and in an

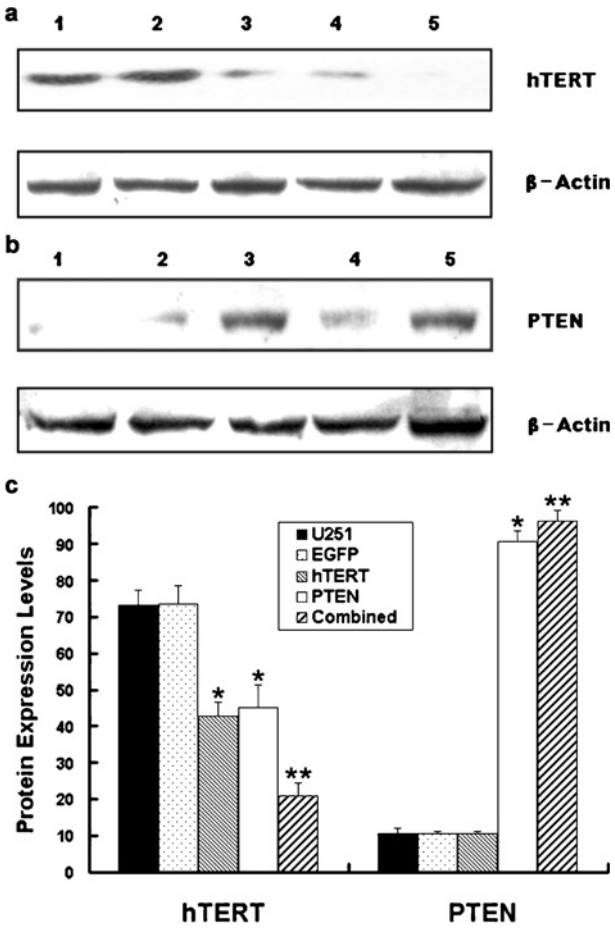

Figure 4. Western blot analysis of hTERT and PTEN protein levels in human glioma cells infected with the *PTEN* and antisense-*hTERT* adenoviruses. U251 cells were infected, *in vitro*, with the adenovirus bearing only EGFP (EGFP), pAdeasy-hTERT (hTERT), pAdeasy-PTEN (PTEN), and both pAdeasy-hTERT and pAdeasy-PTEN (Combined), respectively, or were left non-infected as a control (U251). Cellular protein was extracted from the glioma cells, and the protein levels of hTERT (*a*) and PTEN (*b*) were analyzed by Western blotting. Shown are Western blot data representative of those obtained from three separate experiments. Lane 1, U251; lane 2, EGFP; lane 3, PTEN; lane 4, hTERT; lane 5, combined. The numeric data are shown in (*c*). *$p<0.001$ vs. the U251 group. **$p<0.05$ for the combined group vs. the hTERT group.

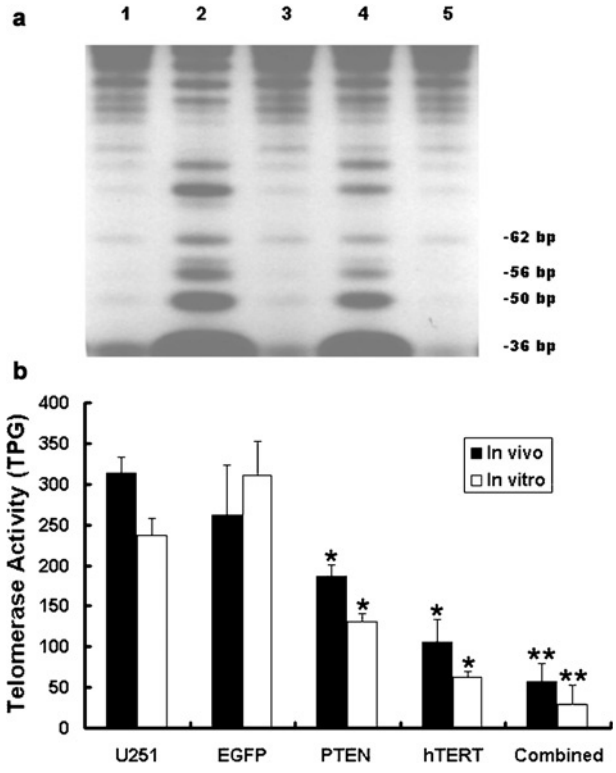

Figure 5. Telomerase activity in U251 glioma cells infected with antisense *hTERT* and wild-type *PTEN* adenoviruses *in vitro* and tumor xenografts. (*a*) U251 cells were infected, *in vitro*, with the adenovirus bearing only EGFP (EGFP), pAdeasy-hTERT (hTERT), pAdeasy-PTEN (PTEN), and both pAdeasy-hTERT and pAdeasy-PTEN (Combined), respectively, or were left non-infected as a control (U251). Telomerase activity was measured by the TRAP assay, as described in Materials and methods. A DNA ladder of 6-bp septa is a mark of telomerase activity. Shown are clear DNA ladders of 6-bp septa in both the U251 (lane 2) and EGFP control group cells (lane 4). By contrast, the DNA ladders are obviously weaker in the glioma cells of the combined (lane 1), PTEN (lane 3), and hTERT groups (lane 5). The results shown are representative of three independent experiments. (*b*) U251 glioma cell infection was performed using adenoviruses as mentioned above, or the *in vivo* tumor model was injected with the adenoviral recombinants; the telomerase activity in glioma cells from both the *in vitro* and *in vivo* studies was measured by the TRAP method. *$p<0.001$ vs. the U251 group. **$p<0.05$ for the combined group vs. the hTERT or PTEN group.

experimental xenograft mouse model for glioma. We selected wild-type *PTEN* and the *hTERT* sequence located adjacent to the transcriptional start point of the promoter as the targets of gene therapy and constructed two expression recombinant adenoviruses, one encoding wild-type *PTEN* (pAdeasy-PTEN) and the other encoding antisense *hTERT* (pAdeasy-hTERT). We demonstrated that U251 glioma cell growth was suppressed significantly *in vitro* and *in vivo* following infection with pAdeasy-hTERT or pAdeasy-PTEN and that a greater inhibitory effect occurred in cells co-infected with both recombinant adenoviruses, in comparison with cells infected with either adenovirus alone. In addition, we found that the levels of hTERT protein expression and telomerase activity in these cells were substantially decreased, whereas the PTEN protein expression level was significantly increased. These observations suggest that the anti-proliferative effect and anti-neoplastic activity of the combination *PTEN* and antisense *hTERT* therapy in human glioma are mediated through the suppression of *hTERT* expression and the down-regulation of telomerase activity. These findings also provide the rationale and framework for developing the combination of *PTEN* and antisense *hTERT* as a potentially effective gene therapy for

patients with malignant gliomas and other brain tumors.

The main function of the ribonucleoenzyme telomerase is to lengthen and cap the ends of linear chromosomes, the telomeres. Telomerase activation and the subsequent maintenance of telomeres are required for the survival and proliferation of the large majority of tumor cells. Uncapped or critically shortened telomeres cause cellular responses such as cell cycle arrest and apoptosis. hTERT is essential for telomerase function, and an increase of hTERT mRNA expression is the key to activating telomerase. hTERT expression was found solely in tumor cells [5, 7–9]. Therefore, targeting hTERT represents a promising approach for diminishing telomerase activity in tumor cells without causing substantial side effects in telomerase-negative somatic cells. Several recent reports described telomerase inhibition via anti-hTERT treatment, in which anticancer effects on tumor cell growth in different types of solid tumors were seen after weeks or months of treatment [12–15]. For example, Yokoyama and coworkers [28] used a hammerhead ribozyme targeting the 5′-end of hTERT mRNA to suppress telomerase activity. Fu et al. [29] used a combination of telomerase antisense oligonucleotides, simultaneously targeting human telomerase RNA (hTR) and hTERT, to transfect human colon cancer cells *in vitro*. They found that telomerase activity was suppressed and that cancer cell growth was inhibited via the induction of apoptosis and retardation of the cell cycle.

Although the regulation of hTERT mRNA expression has been studied extensively during the past decade, no method of specifically regulating *hTERT* expression has been reported. A review of previous reports revealed that a region of about 250 base pairs located adjacent to the transcriptional start site of the *hTERT* promoter is the "core sequence" regulating *hTERT* expression. A variety of factors are believed to regulate the expression of *hTERT* through interactions at this region. Any sequence mutation or deletion occurring in this region may cause a significant change in the gene expression of *hTERT* [30, 31]. Therefore, we chose this region as a target of antisense gene therapy in the present study.

Many studies in recent years have also provided evidence that PTEN regulates telomerase activity, probably through its known effects on the PI3K/Akt pathway. For example, Zhou and colleagues [32] transfected *PTEN* cDNA into *PTEN*-null Ishikawa endometrial cancer cells via adenovirus-mediated gene transduction and found inhibition or reduction of cell growth, Akt phosphorylation, and telomerase activity, as well as hTERT mRNA level in these cells. Kang et al. [33] also showed that Akt may directly phosphorylate and increase the catalytic activity of the hTERT protein. Two putative Akt kinase phosphorylation sites were identified exclusively in the hTERT subunit, and none were found in any other components of the telomerase enzyme [33]. Thus, PTEN may modulate telomerase activity through inhibition of Akt activation and subsequent decrease in phosphorylation of the hTERT protein [33]. In addition, PTEN can reduce telomerase activity by transcriptional and post-transcriptional regulation of hTERT mRNA and protein levels through several mechanisms. First, PTEN down-regulates hTERT expression levels directly through suppression of the transcription factors NF-κB, HIF-1, c-Myc and Sp1 via a PI3K/Akt cascade or other mechanisms [31, 34–39]. Binding of these transcription factors to the region of *hTERT* core promoter activates transcription of *hTERT* gene. Thus, down-regulation of these critical transcription factors or inhibition of the association of these transcription factors with *hTERT* may, at least in part, contribute to telomerase inactivation during *PTEN* cancer gene therapy. Second, recent evidence showed that insulin-like growth factor I (IGF-I) stimulates telomerase activity in cancer cells through a dual mode of action, including early rapid effects involving phosphorylation of hTERT by Akt and later up-regulation of hTERT mRNA and protein expression [40]. There may be other growth factors utilizing the PI3K/Akt pathway to activate hTERT expression [31]. Therefore, PTEN may reduce the levels of hTERT indirectly through inhibition of the IGF-I or other growth factor-induced hTERT expression via the PI3K/Akt cascade. Third, several studies reported that rapamycin, a potent inhibitor of the serine/threonine kinase mammalian target of rapamycin (mTOR), suppressed telomerase activity by decreasing hTERT mRNA levels in different cancer cell lines [12, 31, 41]. Given that mTOR is a downstream target of the PI3K/Akt pathway and that PTEN loss leads to an increase in mTOR signaling [41], it seems logical that PTEN would also play a role in the modulation of telomerase activity and hTERT mRNA levels. Lastly, some differentiation-inducing agents can repress telomerase activity in a variety cancer cells, probably through indirect action via the induction of cellular differentiation [31, 42, 43]. For example, treatment of cancer cells with vitamin D and retinoic acids leads to a decrease in *hTERT* promoter activity, as well as inhibition of both hTERT mRNA expression and telomerase activity. Furthermore, we and others showed that reconstitution of wild-type *PTEN* in the cancer cell lines containing endogenously mutated *PTEN* causes tumor cell differentiation and apoptotic cell death; both effects of PTEN would certainly lead to reduction in the total hTERT level and telomerase

activity within the tumor masses. In fact, a gene therapy strategy targeting the *PTEN* gene has been used to treat malignant gliomas. Davies et al. [44] transfected wild-type *PTEN* into U251 human glioma cells in which *PTEN* was mutated and found that the level of Akt phosphorylation was reduced, and that the rate of apoptotic cells was increased significantly. Furnari and coworkers [45] demonstrated that, after transfection with *PTEN*, the telomerase activity was decreased in U87 glioma cells, and that tumor cell growth was slowed. Cheney et al. [46] showed that U87 human glioma cells could not form tumors in nude mice following transfection with *PTEN*. These observations suggest that PTEN may be a good candidate target for anticancer gene therapy of human gliomas.

In the present study, we constructed two adenoviral recombinant vectors (pAdeasy-hTERT and pAdeasy-PTEN) and assessed their effects on human U251 glioma cell growth *in vitro*, and in a xenograft mouse model. The growth of U251 cells was inhibited significantly in a time-dependent manner, *in vitro* and *in vivo*, following infection with antisense-*hTERT* adenovirus, *PTEN* adenovirus, or both, with the greatest effect seen in the cells co-infected with both adenoviruses. Furthermore, the inhibition of glioma cell growth, *in vitro* and *in vivo*, was associated with reduced levels of telomerase activity and hTERT protein expression, but increased PTEN protein levels. Finally, we demonstrated that the population of apoptotic cells was increased remarkably, but that cell proliferation was decreased significantly in adenovirus-infected gliomas, as indicated by flow cytometry results showing cell cycle arrest at G1/G0 and significantly decreased PI and SPF values in the U251 cells co-infected with the *PTEN* and antisense-*hTERT* adenoviruses. Although the mechanism underlying the combination anticancer therapy with *PTEN* and antisense *hTERT* is not clearly defined at this point, these results have potential relevance for a better understanding of the effect of antisense *hTERT* and *PTEN* on the proliferative potential of tumor cells.

One possible mechanism for this combination therapy may be that the down-regulation of the hTERT level by antisense *hTERT* and *PTEN* causes a reduction in telomerase activity, which leads to growth inhibition of the glioma cells. The expression of wild-type *PTEN* in our tumor cells with endogenous mutant *PTEN* may also exert other actions in the system, such as inhibiting Akt activity, inducing G1 cell cycle arrest, suppressing cell proliferation, and causing apoptotic cell death. Interestingly enough, it was shown that knock-down of *hTERT* enhances PTEN protein level by a presently unknown mechanism, possibly through regulating the transcription of *PTEN* gene and/or the stability of PTEN mRNA, suggesting that hTERT and PTEN interplay in the cell to control cell growth and cell death in normal and cancer cells. Although previous studies have shown that antisense *hTERT* or *PTEN* alone could significantly inhibit the growth of human glioma cells, to our knowledge, this is the first study to demonstrate the superior inhibitory effect of a combination of antisense *hTERT* and *PTEN* on the growth of human glioma cells. The mechanisms supporting the combination of *hTERT* and *PTEN* gene therapy are twofold: (i) antisense *hTERT* and wild-type *PTEN* may act collaboratively to inhibit the malignant growth of tumor cells *in vitro* and in xenografts, as compared to the single gene therapy administered with either agent alone; and (ii) both agents may interplay synergistically to control cell proliferation and cell survival, as evidenced by the findings that introduction of *PTEN* decreases both hTERT activity and hTERT level, while down-regulation of *hTERT* increases PTEN level in *PTEN*-deficient U251 cells. Further work is needed to elucidate the mechanisms by which PTEN modulates telomerase activity and hTERT levels through the PI3K/Akt pathway and the mechanisms by which PTEN and hTERT interplay coordinately to control cell growth and apoptosis leading to inhibition of malignant glioma progression.

An increasing understanding of the molecular mechanisms that cause human disease has rationalized gene transfer as an approach for the treatment of diseases resistant to more conventional therapies. Gene therapy aims at the transfer of genes for correction of either genetic or somatic disease phenotypes or for expression of molecules within target cells for therapeutic effect. The ultimate objects of cancer gene therapy are the suppression of tumor growth and the eradication or inhibition of distant metastatic foci. Vehicles for gene transfer include both nonviral and viral vectors, such as adenovirus, retrovirus, herpes simplex virus, and adeno-associated virus. Nonviral gene transfer is most commonly based on plasmid DNA, particle bombardment, or cationic liposomes. Viral gene delivery has already been optimized by evolution and is therefore generally more effective. Adenoviruses are among the most commonly used vectors for gene therapy [47, 48]. Adenovirus vectors have certain advantages such as high transduction efficiency for a broad range of cell types, including both quiescent and dividing cells and high levels of short-term expression to provide therapeutic benefits [47, 48]. Thus, adenovirus vectors have been used extensively in preclinical and clinical studies for gene delivery applications [47]. In this project, we found that adenovirus is a useful gene transfer vector for

delivery of wild-type *PTEN* and antisense *hTERT* genes to U251 glioma cells. The expression levels of PTEN were dramatically increased, while the levels of hTERT were markedly decreased in the glioma cells infected with the wild-type-*PTEN* and/or antisense-*hTERT* recombinant adenoviruses. However, caution should be taken during glioma gene therapy with adenovirus vector administration because studies demonstrated that higher systemic doses of adenovirus vectors invariably lead to hepatotoxicity and initiation of acute inflammatory responses, mainly due to activation of innate immunity by vector particles.

In summary, our work suggests that gene transfer may be a potentially useful approach for the treatment of brain tumors. We have provided evidence that the introduction of *PTEN* or antisense *hTERT* into the *PTEN*-null U251 cells resulted in effective suppression of malignant growth of the glioma cells *in vitro* and *in vivo*. More importantly, the therapy efficacy was significantly higher in the tumor cells and xenografts infected with both *PTEN* and antisense *hTERT* than in the gliomas infected with either agent alone at the same total viral dose. In addition, our results showed that the inhibitory effect of the combination treatment on glioma growth and survival occurs through G1 cell cycle arrest and also through the down-regulation of the hTERT level and telomerase activity. Considering the current lack of effective medical treatment options for malignant gliomas – surgery, radiotherapy and chemotherapy being the mainstays of management, we advocate the combination gene therapy with *PTEN* and antisense *hTERT* as an attractive new approach for the treatment of gliomas that cannot be cured by conventional therapies. Further investigation on rat models will pave the way for future glioma gene-therapy clinical trials and allow the realization of gene therapy with two targets as a viable non-surgical option for this deadly brain tumor, either alone or as an adjuvant to existing treatment modalities.

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