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Sequential hTERT Knockdown and Apigenin Treatment Inhibited Invasion and Proliferation and Induced Apoptosis in Human Malignant Neuroblastoma SK-N-DZ and SK-N-BE2 Cells

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Abstract Human telomerase reverse transcriptase (hTERT) plays a key role in conferring immortality to human malignant neuroblastomas. We first determined differential expression of hTERT in four human malignant neuroblastoma SH-SY5Y, SK-N-DZ, SK-N-BE2, and IMR-32 cell lines. We then used SK-N-DZ and SK-N-BE2 cell lines, which showed the highest expression of hTERT, to investigate the therapeutic effects of sequential hTERT knockdown and apigenin (APG) treatment. We performed cell invasion assay and studied alterations in expression of matrix metalloproteinases and cell cycle regulatory molecules after this combination therapy. We also investigated induction of apoptosis by using in situ Wright staining, Annexin V staining, and Western blotting. Sequential hTERT knockdown and APG treatment significantly downregulated expression of hTERT so as to cause over 90 % inhibition of cell invasion and 70 % induction of apoptosis in both SK-N-DZ and SK-N-BE2 cell lines. Western blotting demonstrated downregulation of the molecules involved in cell invasion and proliferation, but upregulation of the cell cycle inhibitor and apoptosis-inducing molecules. In conclusion, our current results clearly showed that sequential hTERT knockdown and APG treatment could be a promising therapeutic strategy for effective inhibition of invasion and proliferation and induction of apoptosis in hTERT overexpressing malignant neuroblastoma cells.

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Introduction

Malignant neuroblastoma, an extracranial solid tumor in children, is responsible for 15 % of childhood deaths in the USA (Ganeshan and Schor 2011). In spite of some advancement in the understanding on molecular mechanism of neuroblastoma pathology, an effective treatment still remains elusive. It is currently becoming evident that a specific gene-targeted therapy and a flavonoid treatment can be used as an effective combination therapy for combating the growth of human malignant neuroblastoma cells (George et al. 2010).

Human telomerase reverse transcriptase (hTERT) is the catalytic component of telomerase that regulates telomerase activity (Masutomi and Hahn 2003; Cohen et al. 2007; Carpentier et al. 2007). Telomerase plays a crucial role in conferring immortality to cancer cells through regulation of telomere length (Shay and Wright 1998; Maher et al. 2007). hTERT is upregulated in most cancers, including human malignant neuroblastomas (Hiraga et al. 1998). Upregulation of hTERT is associated with poor survival rate in neuroblastomas (Tchirkov et al. 2003; Wang et al. 2006). Furthermore, in vivo studies showed that expression of hTERT and telomerase activity correlated well with cell proliferation in meningiomas and malignant brain tumors (Cabuy and de Ridder 2001). So, modulation of the expression of hTERT may provide enormous potential for developing diagnostic, prognostic, and therapeutic strategies for various cancers (Kirkpatrick and Mokbel 2001; Shay et al. 2001). Increased telomerase activity in various tumors

including malignant neuroblastomas has been implicated in resistance to radiation and chemotherapeutic drugs. Transfection of hTERT in HeLa cells exhibited resistance to radiation and chemotherapeutic agents (Xi et al. 2006). Telomerase activity in human pancreatic carcinoma cells correlates well with their potential for migration and invasion, manifesting the aggressive behavior of the tumor cells (Sato et al. 2001). Therefore, hTERT knockdown using hTERT short hairpin RNA (shRNA) could be an effective technique to inhibit telomerase activity and thus to prevent cell cycle progression and tumor growth.

Apigenin (APG), which is a well-known flavonoid present in fruits and vegetables, induces cell death by activating extrinsic and intrinsic pathways of apoptosis in many cancers including human malignant neuroblastoma (Karmakar et al. 2009). It has been reported that APG decreases hTERT expression and telomerase activity in human cervical cancer through upregulation of the cell cycle inhibitor p27Kip1 (Lee et al. 2005). APG downregulates telomerase activity by suppressing c-Myc-mediated hTERT expression in human leukemia cell lines (Jayasooriya et al. 2012). Recent studies show that small interfering RNA (siRNA) targeting hTERT gene expression induces apoptosis and inhibits cell proliferation (Ge et al. 2011). The levels of hTERT mRNA expression in cells transfected with lower concentrations of siRNA were not much different when compared with control cells (Ge et al. 2011). These earlier studies suggest that maximum silencing of hTERT expression via siRNA technique and APG treatment can be a novel combination therapeutic strategy for inhibition of invasion and proliferation and induction of apoptosis in human malignant cells. Therefore, we thought of devising a novel therapeutic modality involving combination of hTERT shRNA transfection and APG treatment for efficient inhibition of growth of human malignant neuroblastoma cells that harbored the highest expression of hTERT.

The introduction of siRNA through a plasmid vector encoding specific shRNA (George and Tsutsumi 2007; George et al. 2009b) or specific oligonucleotide (George et al. 2009a) is an authentic procedure to knockdown expression of a target gene. The introduction of hTERT shRNA into the tumor cells can downregulate the hTERT mRNA, the cognate protein, and, thereby, the endogenous telomerase activity. An earlier investigation showed that shRNA targeted to hTERT gene decreased hTERT expression and telomerase activity, leading to significant inhibition of cell proliferation and induction of apoptosis in human gastric cancer SGC-7901 cells (Qian et al. 2008). The prime focus of our present investigation was to elucidate the molecular mechanisms of the inhibition of cell proliferation and invasion and induction of apoptosis in hTERT overexpressing human malignant neuroblastoma cells following sequential hTERT shRNA transfection and APG treatment.

Materials and Methods

Cell Cultures and Treatment Agents

The human malignant neuroblastoma SH-SY5Y, SK-N-DZ, SK-N-BE2, and IMR-32 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were maintained in RPMI 1640 medium, and SK-N-DZ, SK-N-BE2, and IMR-32 cells were grown in DMEM medium, and both media were supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin and 1 % streptomycin (Gibco/BRL, Grand Island, NY, USA). Cell lines were maintained in a fully humidified atmosphere containing 5 % CO_2 at 37 °C. The media and FBS were purchased from Mediatech (Atlanta Biologicals, Atlanta, GA, USA). APG was purchased from Sigma Chemical (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO), and stocked at −20 °C. In all experiments, control cell cultures contained the same concentration of DMSO that was used in the treatment with APG. Concentration of DMSO in all experiments was maintained at less than 0.01 %, which was not able to induce cell growth, differentiation, or death. The hTERT shRNA plasmid was procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and BIBR 1532, a pharmacological inhibitor of hTERT, was purchased from Selleck Chemicals (Huston, TX, USA).

Reverse Transcription Polymerase Chain Reaction and Western Blotting to Examine the Levels of Expression of hTERT in Different Human Malignant Neuroblastoma Cells

Reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were carried out to determine the levels of expression of hTERT mRNA and protein, respectively, in four human malignant neuroblastoma (SH-SY5Y, SK-N-DZ, SK-N-BE2, and IMR-32) cell lines. The following primer sequences were used for PCR amplification of the hTERT gene (forward 5′-CAC CAA GAA GTT CAT CTC C-3′ and reverse 5′-CAA GTG CTG TCT GAT TCC-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (forward 5′-CCA CCC ATG GCA AAT TCC-3′ and reverse 5′-CAG GAG GCA TTG CTG ATG AT-3′). The expression of GAPDH was used as an internal control. Total RNA (300 ng) was used for each set of primers for transcription and amplification using a single-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) on a PCR cycler (Eppendorf, Westbury, NY, USA) with following

programming: cDNA synthesis at 50 °C for 30 min, reverse transcriptase inactivation at 95 °C for 10 min, 35 cycles of PCR amplification of transcripts (denaturation of templates at 95 °C for 45 s, annealing of primers at 52 °C for 30 s, and extension of primers at 72 °C for 1 min), and final extension of primers at 72 °C for 7 min. The RT-PCR products were resolved on 2 % agarose gels by electrophoresis, stained the gels with ethidium bromide (1 μg/ml), and destained the gel background with water, visualized on a UV (303 nm) transilluminator and photographed digitally using the UVDI Compact Digimage System (Major Science, Saratoga, CA, USA). We also followed a standard procedure (Chakrabarti et al. 2012) to perform Western blotting with an anti-hTERT polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to determine levels of hTERT protein in the cells. Both RT-PCR and Western blotting images were quantified using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

Immunofluorescence Confocal Microscopy and Flow Cytometry for Assessing Expression of hTERT in the Cells

For immunofluorescence staining, malignant neuroblastoma cells were grown on Lab-Tek chamber cover glasses (Nunc, Rochester, NY, USA), washed with phosphate-buffered saline (PBS, pH 7.4), fixed in 4 % paraformaldehyde, and permeabilized with 0.1 % Triton X-100 containing 2 % bovine serum albumin. After washing with PBS, a cocktail of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit hTERT primary IgG antibody (1:100) was applied for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole, the nuclear stain, (Thermo Scientific, Rockwood, TN, USA) which could strongly bind to A-T-rich regions in DNA, showing blue fluorescence. After mounting with the coverslips on the microscopic slide, cells were examined using the Zeiss LSM510 META laser scanning confocal microscope (Zeiss, Germany). Data sets were generated and merged using Zeiss ZEN confocal imaging software. For flow cytometry, cells were stained with FITC-conjugated goat anti-rabbit hTERT primary IgG antibody and analyzed using an Epics XL-MCL Flow Cytometer (Beckman Coulter, Brea, CA, USA), and data were presented as two-dimensional plots showing fluorescence intensity of hTERT against number of events.

Procedures for hTERT shRNA Transfection and APG Treatment

Approximately 80 % confluent cultures of SK-N-DZ and SK-N-BE2 cells, which harbored the highest expression of hTERT, were separately transfected with the plasmid vector carrying hTERT shRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions or treated with a final concentration of 100 μM APG or both agents together in serum-free medium. A set of cultures was also transfected with the plasmid vector carrying the scrambled shRNA sequence for hTERT. We selected to use 100 μM APG based on a dose–response study for cell viability, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (data not shown). Higher concentrations of APG did not significantly decrease cell viability, whereas lower concentrations were found to be less effective. After 24 h, the medium was replaced with regular serum containing medium, and the cultures were incubated for another 24 h.

RT-PCR and Western Blotting to Determine the Expression hTERT at mRNA and Protein Levels, Respectively, After Monotherapy and Combination Therapy

After hTERT shRNA transfection and/or APG treatment, malignant neuroblastoma cells were harvested to perform RT-PCR and Western blotting to determine the expression of hTERT mRNA and protein levels, respectively, following the same procedures as we described above. Inhibition of expression of hTERT was also studied using BIBR 1532 (a pharmacological inhibitor of hTERT) in course of APG treatment.

Invasion Assay

Transwell (10.5 mm diameter, 8 μm pore size) inserts from BD Biosciences (Franklin Lakes, NJ, USA) were coated with 3.5 mg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to form a thin continuous layer and allowed to gel overnight at 37 °C. After hTERT shRNA transfection and/or APG treatment, human malignant neuroblastoma cells were harvested and seeded (5×10^4 cells) into each insert (upper chamber) in serum-free medium, and complete medium was placed in the lower chamber to act as a chemoattractant. Then, cells were incubated for 18 h to allow cell invasion through the Matrigel layer. Cells on the upper surface of insert membrane were then removed by wiping with a cotton swab, and cells that migrated to the lower surface were fixed with 10 % formaldehyde and stained in crystal violet. The number of control cells migrated through the Matrigel was counted and compared with the number of treated cells.

Determination of Residual Cell Viability Using the MTT Assay

The residual cell viability was determined by the MTT assay after hTERT shRNA transfection and/or APG treatment. Briefly, human malignant neuroblastoma cells were seeded

into 96-well microculture plates at 1×10^4 cells/well and allowed to attach overnight. The medium was removed, and cells were transfected with hTERT shRNA plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and incubated for 12 h. Then, old medium was replaced with fresh medium. After hTERT shRNA transfection and/or APG treatment, the cells were subjected to the MTT assay and washed twice in PBS (pH 7.4); formazan crystals were dissolved by adding isopropyl alcohol, and absorbance was measured at 570 nm wavelength using a multiwell plate reader (BioTek, Winooski, VT, USA). Residual cell viability was presented as percentage of viable cells in total population.

Western Blotting for Molecules Involved in Cell Proliferation, Invasion, and Apoptosis

Both human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines were subjected to hTERT shRNA transfection and/or APG treatment prior to extraction of protein samples. Monoclonal primary IgG antibodies against GAPDH and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used to monitor the equal loading of proteins in course of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were resolved by SDS-PAGE and analyzed by Western blotting using also the primary IgG antibodies against matrix metalloproteinase-2 (MMP-2), MMP-9, N-Myc, proliferating cell nuclear antigen (PCNA), cyclin-dependent kinase-2 (CDK-2), CDK-4, cyclin D1, p21Waf1, caspase-8, tBid, survivin, calpain, caspase-3, and spectrin break down product (SBDP). Primary IgG antibodies and the horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary IgG antibody were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blots were incubated with enhanced chemiluminescence detection reagents (Amersham Pharmacia, Buckinghamshire, UK) and exposed to X-OMAT AR films (Eastman Kodak, Rochester, NY, USA) for autoradiography. The autoradiograms were scanned on an EPSON Scanner using Photoshop software (Adobe Systems, Seattle, WA, USA).

In Situ Wright Staining for Detection of Morphological Features of Apoptosis

At the completion of transfection and/or treatment, both adherent and nonadherent cells were forced to attach onto the dishes by centrifugation at 2,500 rpm for 10 min and then washed twice with PBS (pH 7.4) before being fixed with 95 % (*v*/*v*) ethyl alcohol. The cells were allowed to dry and then subjected to in situ Wright staining according to the manufacturer's instructions (Fisher Scientific, Kalamazoo, MI, USA). Finally, the morphological features of apoptosis in the cells ($n=300$) were observed under the light microscope. The morphological features of apoptotic cells included at least one of such characteristics as cell shrinkage, chromatin condensation, and membrane-bound apoptotic bodies.

Annexin V-Fluorescein Isothiocyanate/Propidium Iodide Double Staining and Flow Cytometric Detection of a Biochemical Feature of Apoptosis

Cells were harvested from each treatment group ($n=3$) and washed twice with PBS (pH 7.4) before being fixed with 70 % (*v*/*v*) ethyl alcohol for 15 min on ice. Subsequently, the cells were centrifuged to obtain pellets, and residual ethyl alcohol was aspirated. Cells were then exposed to DNase-free RNase A (2 mg/ml) for 30 min at 37 °C and subjected to Annexin V-FITC/propidium iodide (PI) double staining as per manufacturer's instructions (BD Biosciences, San Diego, CA, USA) and then analyzed on an Epics XL-MCL Flow Cytometer (Beckman Coulter, Fullerton CA, USA). Cells that were Annexin V negative and PI positive were considered as mechanically injured (quadrant A1), cells that were both Annexin V and PI positive (quadrant A2) were considered as late necrotic, cells that were both Annexin V and PI negative cells (quadrant A3) were considered as normal, and cells that were Annexin V positive and PI negative were considered as early apoptotic (quadrant A4). Flow cytometry detected Annexin V positive cells that experienced externalization of membrane phospholipid, an early biochemical feature of apoptosis. The Annexin V-stained apoptotic cells were analyzed for statistical significance.

Statistical Analysis

We analyzed results from various experiments using Minitab® 16 statistical software (Minitab, State College, PA, USA) and compared using one-way analysis of variance with Fisher's post hoc test. All data were presented as means ± standard error of mean of independent experiments ($n\geq3$). Difference between control and monotherapy or combination therapy was considered significant at a p value less than 0.05.

Results

Estimation of Relative Expression of hTERT in Four Human Malignant Neuroblastoma Cell Lines

We examined the relative expression of hTERT mRNA and protein by RT-PCR and Western blotting, respectively, in

four human malignant neuroblastoma SH-SY5Y, SK-N-DZ, SK-N-BE2, and IMR-32 cell lines (Fig. 1). Among the four cell lines, SK-N-DZ and SK-N-BE2 cell lines showed the highest levels of expression of hTERT mRNA (Fig. 1a) and protein (Fig. 1b). Further, we employed immunofluorescence confocal microscopy and flow cytometry for assessing and confirming the levels of expression of hTERT in the neuroblastoma cell lines (Fig. 2). Confocal microscopy studies using anti-hTERT antibody confirmed qualitatively the highest expression of hTERT protein in SK-N-DZ and SK-N-BE2 cell lines (Fig. 2a). Flow cytometry was then used for quantification of expression of hTERT protein in all four cell lines (Fig. 2b). We found that more than 89 % of SK-N-DZ and SK-N-BE2 cells were hTERT positive, whereas 64 % of SH-SY5Y and 47 % of IMR-32 cells showed expression of hTERT (Fig. 2b). Because both SK-N-DZ and SK-N-BE2 cell lines showed the highest expression of hTERT, we selected to use these cell lines for further studies for downregulation of hTERT for controlling their growth.

Downregulation of hTERT mRNA and Protein by hTERT shRNA Transfection and APG Treatment

Sequential transfection of SK-N-DZ and SK-N-BE2 cells with a plasmid encoding for hTERT shRNA and treatment of the cells with APG resulted in a marked decrease in expression of hTERT mRNA and protein (Fig. 3). Compared with untreated control cells, there was no alteration in hTERT mRNA or protein level when cells were transfected with scrambled shRNA plasmid. Although monotherapy with hTERT shRNA transfection or APG treatment reduced the expression of hTERT to some extent, combination therapy drastically inhibited expression of hTERT mRNA and protein in both cell lines (Fig. 3a). We used expression of GAPDH and β-actin as loading controls in RT-PCR and Western blotting, respectively. We estimated the levels of hTERT mRNA and protein following monotherapy and combination therapy (Fig. 3b). Combination therapy resulted in 70 % inhibition of hTERT mRNA expression and 40–50 % inhibition of hTERT protein expression in both cell lines (Fig. 3b). It appeared to be highly notable that transfection with hTERT shRNA plasmid was much more effective than treatment with BIBR 1532, the pharmacological inhibitor of hTERT, in accomplishing knockdown of expression of the hTERT gene in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells (Fig. 3).

Sequential hTERT Knockdown and APG Treatment Resulted in Remarkable Reduction in Cell Invasion

We examined the effects of hTERT knockdown and/or APG treatment on the capability of SK-N-DZ and SK-N-BE2 cells in invasion and alterations in expression of the molecular factors such as MPP-2 and MMP-9 in this process (Fig. 4). The staining of cells that invaded through the Transwell membrane demonstrated that the capability of cell invasion was reduced due to hTERT shRNA transfection and/or APG treatment, when compared with scrambled shRNA-transfected control cells

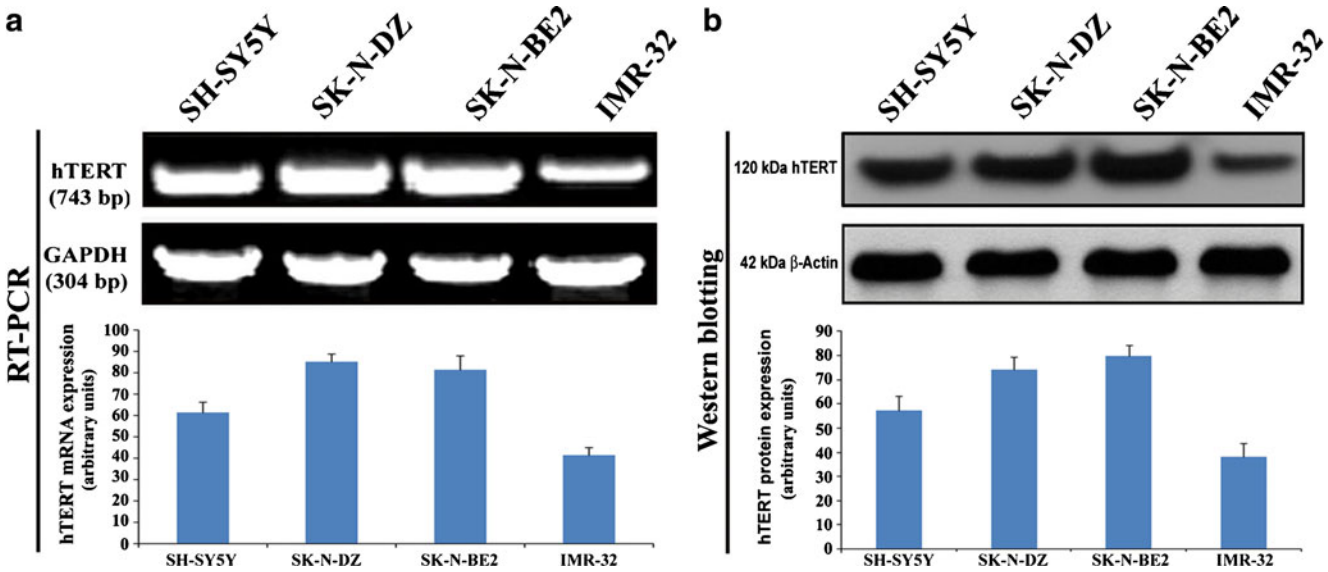

Fig. 1 RT-PCR and Western blotting to examine levels of expression of hTERT in four human malignant neuroblastoma cell lines. **a** RT-PCR to determine relative expression of hTERT mRNA in four human malignant neuroblastoma cell lines. **b** Western blotting to determine relative expression of hTERT protein in four human malignant neuroblastoma cell lines

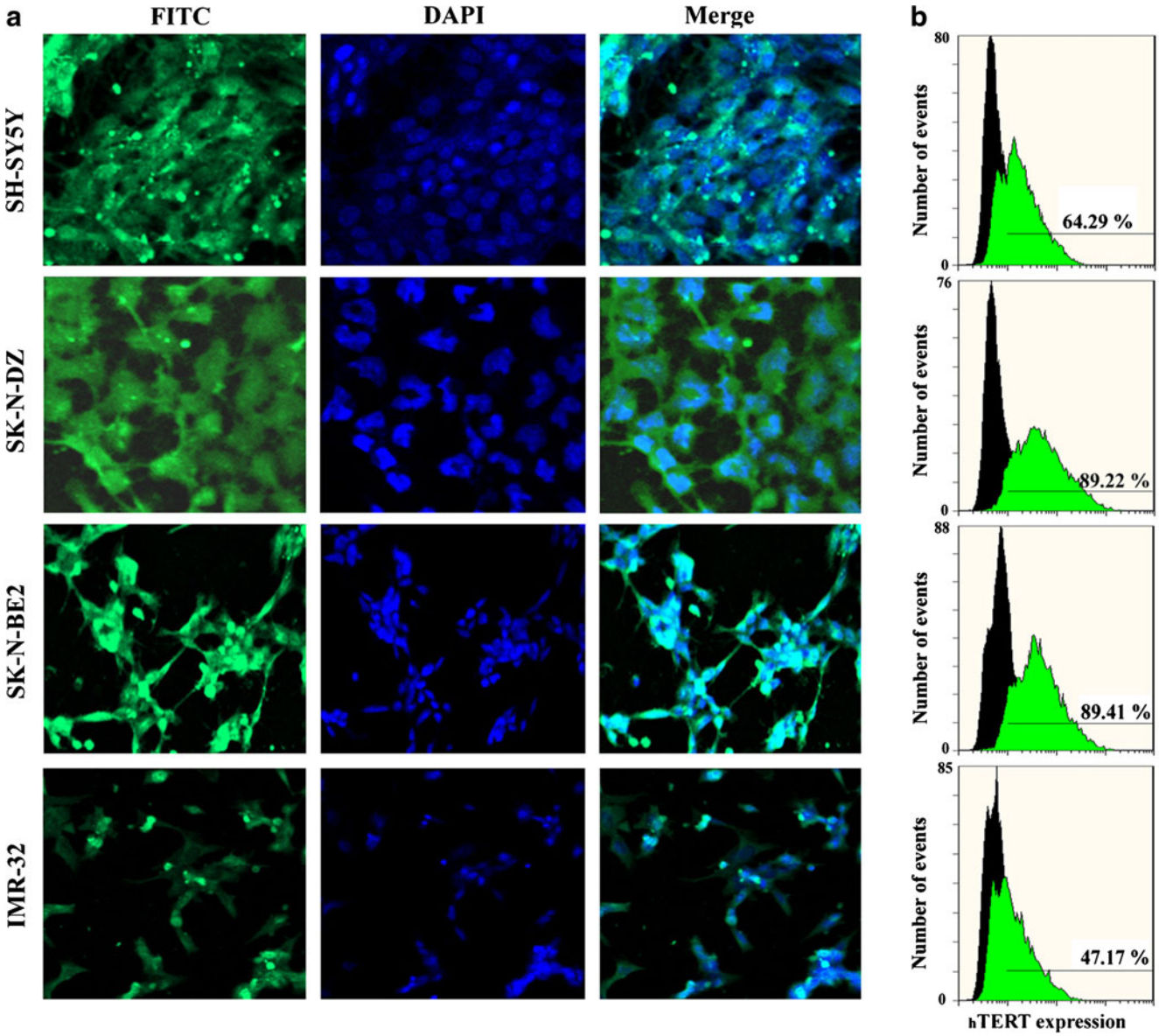

Fig. 2 Immunofluorescence confocal microscopy and flow cytometric analysis to examine the levels of expression of hTERT in four human malignant neuroblastoma cell lines. **a** Immunofluorescence confocal microscopy for qualitative expression of hTERT in four human malignant neuroblastoma cell lines. **b** Flow cytometric analysis for quantitative expression of hTERT in four human malignant neuroblastoma cell lines

(Fig. 4a). Combination therapy was much more effective than monotherapy in reducing cell invasion. We performed quantitative analyses of the cell invasion data for the percentages of tumor cells invaded through the Transwell membrane following monotherapy and combination therapy (Fig. 4b). Combination therapy showed the highest efficacy in almost completely inhibiting the capability of cell invasion (Fig. 4b). Further, our Western blotting data showed downregulation of MMP-2 and MMP-9 (the molecules involved in cell invasion) after sequential hTERT knockdown and APG treatment in both SK-N-DZ and SK-N-BE2 cell lines (Fig. 4c).

Sequential hTERT Knockdown and APG Treatment Reduced Cell Viability Through Modulation of Proliferation and Cell Cycle Regulatory Molecules

We examined the effects of hTERT shRNA transfection and APG treatment on cell viability and expression of cell cycle regulatory molecules (Fig. 5). Monotherapy with hTERT shRNA was more effective than monotherapy with APG in reducing cell viability in both neuroblastoma SK-N-DZ and SK-N-BE2 cell lines (Fig. 5a). Combination therapy with hTERT shRNA and APG was more effective than combination therapy with BIBR 1532 and APG in reducing cell

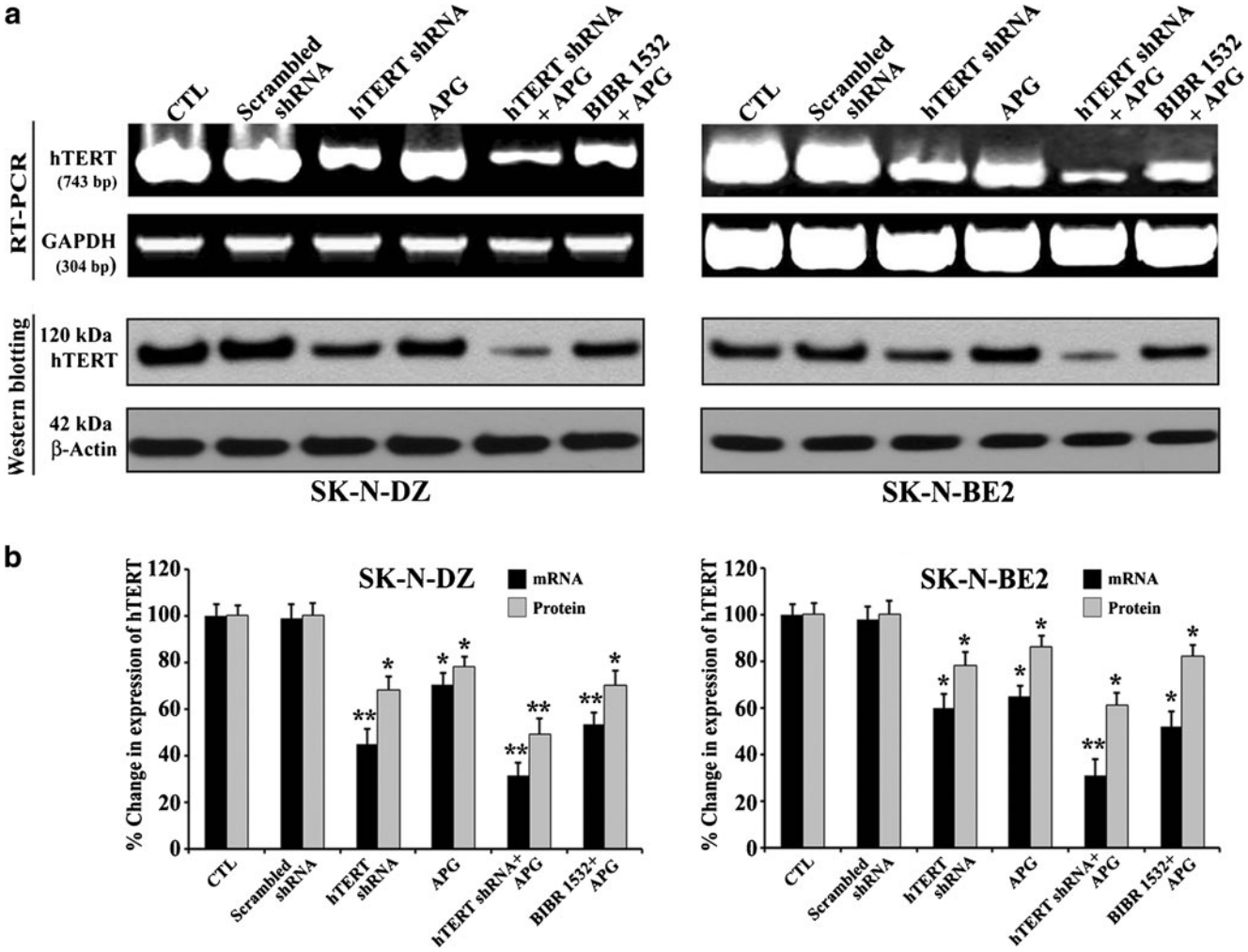

Fig. 3 Alterations in expression of hTERT in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines after sequential hTERT knockdown and other treatments. Treatments: untreated control (CTL), scrambled shRNA (1 μg/ml) for 12 h, hTERT shRNA (1 μg/ml) for 12 h, 100 μM APG for 24 h, hTERT shRNA (1 μg/ml) for 12 h+ 100 μM APG for 24 h, and 1 μM BIBR 1532+100 μM APG for 24 h. **a** RT-PCR and Western blotting to examine changes in expression of hTERT mRNA and protein, respectively. **b** Quantitative evaluation of expression of hTERT mRNA and protein based on RT-PCR and Western blotting, respectively. Difference between CTL and a monotherapy or a combination therapy was considered significant at $*p<0.05$ or $**p<0.01$

viability in these cell lines (Fig. 5a). Then, our Western blotting showed that the expression of the oncoprotein N-Myc and the cell proliferation marker PCNA was downregulated in both neuroblastoma cell lines after hTERT shRNA transfection and APG treatment (Fig. 5b). This combination therapy also reduced expression of the cell cycle regulatory molecules such as CDK-2, CDK-4, and cyclin D1 (Fig. 5b). Our Western blotting also showed that the major CDK inhibitor p21Waf1 was dramatically upregulated after combination therapy (Fig. 5b) for prevention of cell proliferation. There was no apparent difference in the expression of all these molecules in the cells transfected with a plasmid encoding scrambled hTERT shRNA compared with the untreated cells (data not shown), and therefore, cells treated with scrambled hTERT shRNA were considered as the treated control. Reprobing of Western blots for expression of GAPDH demonstrated equal loading of protein samples in all lanes.

Induction of Morphological and Biochemical Features of Apoptosis After Combination Therapy with Sequential hTERT shRNA Transfection and APG Treatment

The morphological and biochemical features of apoptosis were examined using in situ Wright staining and flow cytometric analysis, respectively, after monotherapy and combination therapy (Fig. 6). In situ Wright staining showed the morphological features in apoptotic cells after monotherapy and combination therapy in both cell lines (Fig. 6a). Characteristic morphological features of apoptosis included shrinkage of cell volume, chromatin condensation, and

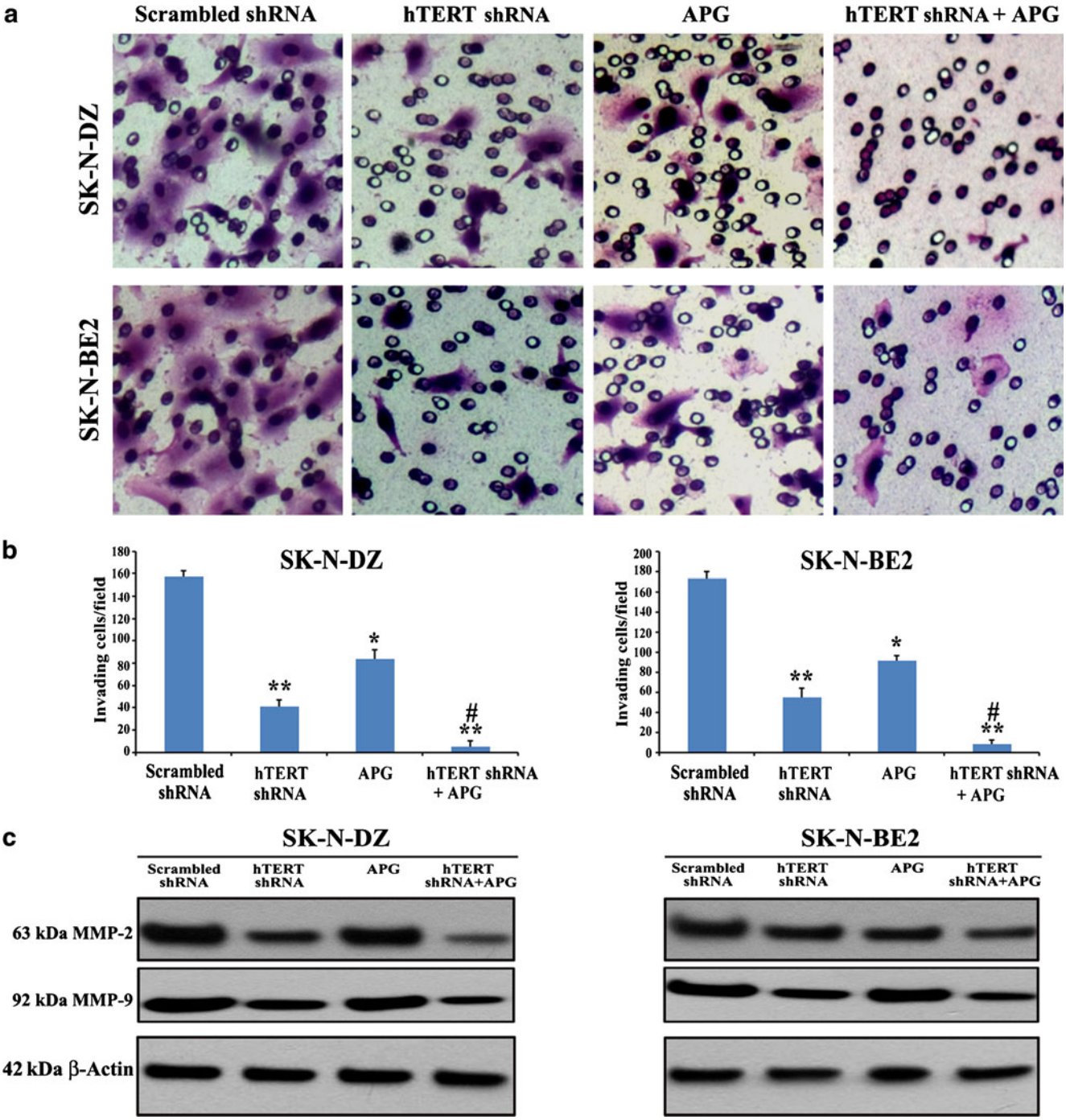

Fig. 4 Alterations in cell invasion and expression of MMPs in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells after sequential hTERT knockdown and APG treatment. Treatments: scrambled shRNA (1 μg/ml) for 12 h, hTERT shRNA (1 μg/ml) for 12 h, 100 μM APG for 24 h, and hTERT shRNA (1 μg/ml) for 12 h+100 μM APG for 24 h. **a** Cell invasion assay. A reduction in the number of invaded cells indicated the decrease in invasive potency of the cells due to hTERT shRNA transfection and/or APG treatment. **b** Quantitative evaluation of reduction in cell invasion. **c** Western blotting to examine changes in expression of MMPs in the cells. Difference between scrambled shRNA transfection and hTERT shRNA transfection and/or APG treatment was considered significant at $*p<0.05$ or $**p<0.01$. Difference between hTERT shRNA transfection or APG treatment and combination therapy was considered significant at $^{\#}p<0.05$

membrane-bound apoptotic bodies that appeared prominently following combination therapy with sequential hTERT shRNA transfection and APG treatment (Fig. 6a). Based on in situ Wright staining and light microscopy, we determined the percentages of apoptosis in SK-N-DZ and SK-N-BE2 cells (Fig. 6a). Further, flow cytometric analysis showed huge increase in population of Annexin V positive cells in A4 area indicating occurrence of apoptosis, which was very

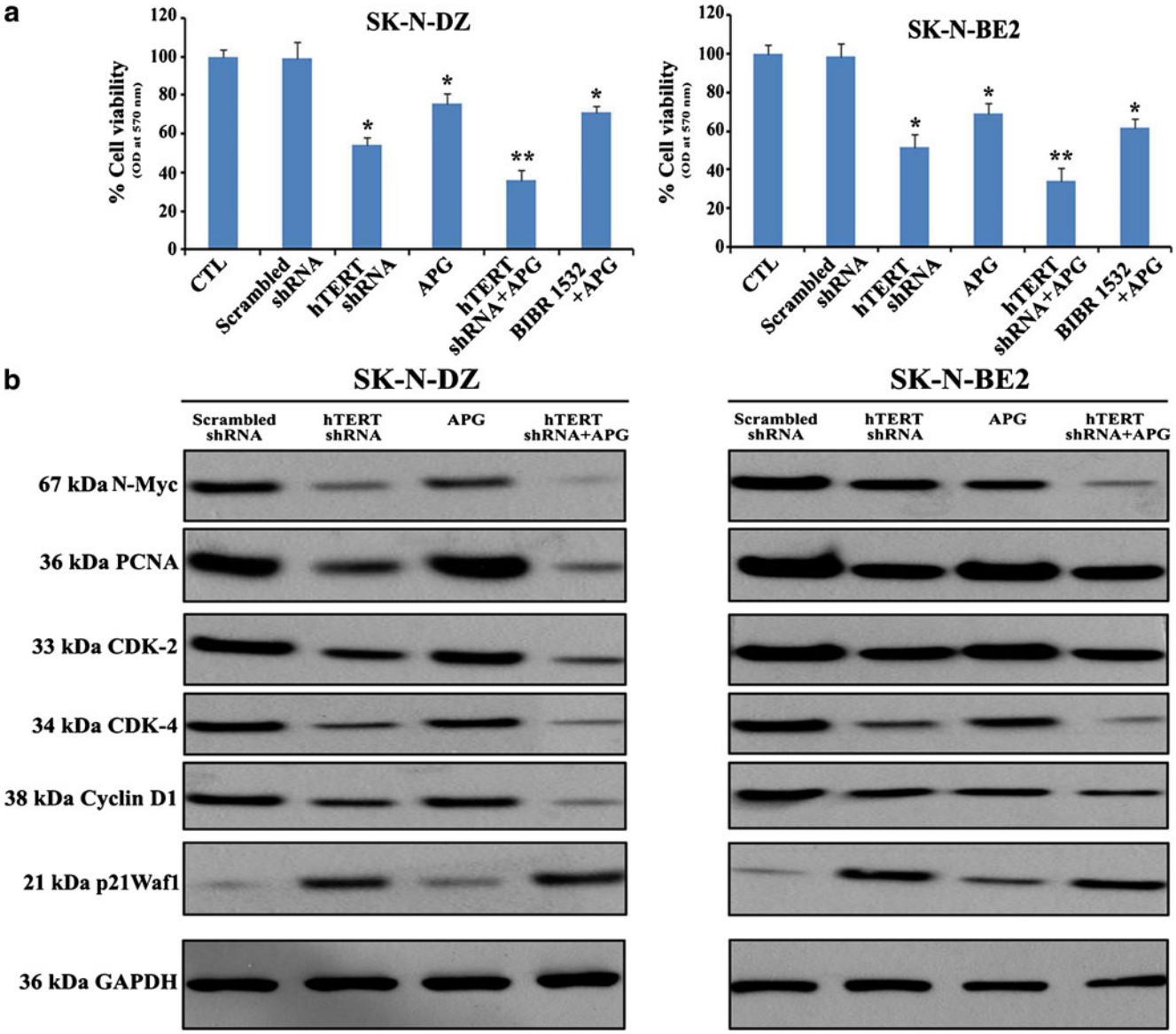

Fig. 5 Determination of residual cell viability and alterations in expression of the proliferation and cell cycle regulatory molecules in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells. Treatments: untreated control (CTL), scrambled shRNA (1 μg/ml) for 12 h, hTERT shRNA (1 μg/ml) for 12 h, 100 μM APG for 24 h, hTERT shRNA (1 μg/ml) for 12 h+100 μM APG for 24 h, and 1 μM BIBR 1532+100 μM APG for 24 h. **a** Determination of residual cell viability after sequential hTERT knockdown and APG treatment. All experiments were conducted in triplicates and the results were analyzed for statistical significance. Difference between CTL and a monotherapy or a combination therapy was considered significant at $*p<0.05$ or $**p<0.01$. **b** Western blotting to examine alterations in expression of the proliferation and cell cycle regulatory molecules after sequential hTERT knockdown and APG treatment

effective in both SK-N-DZ and SK-N-BE2 cells, following combination therapy with sequential hTERT shRNA transfection and APG treatment (Fig. 6b). Increase in Annexin V positive cells is known to be an early biochemical feature of apoptosis. Based on flow cytometric analysis, we determined the percentages of apoptosis in the cells. Combination therapy with sequential hTERT shRNA transfection and APG treatment very significantly enhanced the percentages of the Annexin V positive populations in both SK-N-DZ and SK-N-BE2 cell lines (Fig. 6b).

Western Blotting Confirmed Induction of Intrinsic and Extrinsic Pathways of Apoptosis in Human Malignant Neuroblastoma Cells After Combination Therapy

Our Western blotting showed activation of the extrinsic pathway of apoptosis in generation of active caspase-8 and tBid after combination therapy in both SK-N-DZ and SK-N-BE2 cells (Fig. 7). This combination therapy also very effectively reduced expression of survivin, an important inhibitor of caspases of the intrinsic pathway of apoptosis. Combination

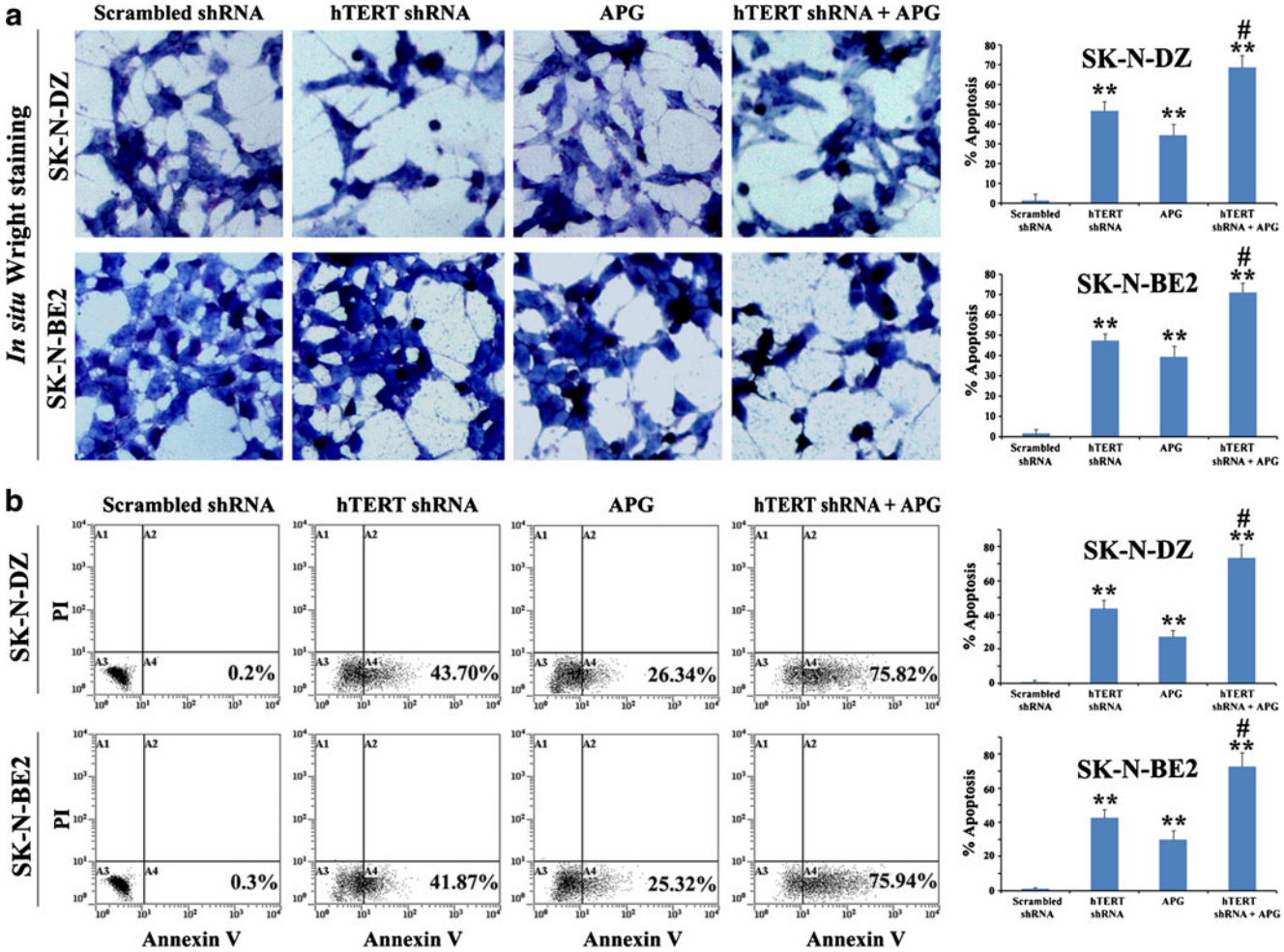

Fig. 6 Determination of apoptosis in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells after sequential hTERT silencing and APG treatment. Treatments: scrambled shRNA (1 μg/ml) for 12 h, hTERT shRNA (1 μg/ml) for 12 h, 100 μM APG for 24 h, and hTERT shRNA (1 μg/ml) for 12 h+100 μM APG for 24 h. **a** In situ Wright staining to examine morphological features of apoptosis and determination of amounts of apoptosis. **b** Annexin V-FITC/PI staining and flow cytometric analysis for an early biochemical feature of apoptosis and determination of apoptotic populations. Difference between scrambled shRNA and hTERT shRNA transfection and/or APG treatment was considered significant at $*p<0.05$ or $**p<0.01$. Difference between hTERT shRNA transfection or APG treatment and combination therapy was considered significant at $^{\#}p<0.05$

therapy very effectively promoted activation of both calpain and caspase-3 that cleaved the key cytoskeletal protein α-spectrin at specific sites to generate calpain-specific 145 kDa SBDP and caspase-3-specific 120 kDa SBDP, respectively, in course of apoptosis (Fig. 7). Reprobing of Western blots for expression of GAPDH provided evidence of equal loading of protein samples in all lanes.

Discussion

Our present study demonstrated that combination of sequential hTERT shRNA transfection and APG treatment effectively inhibited cell invasion and proliferation and induced apoptosis in human malignant neuroblastoma cells that harbored overexpression of hTERT. We selected human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines, which harbored the highest expression of hTERT, to evaluate the efficacy of combination of sequential hTERT knockdown and APG treatment in inhibition of invasion and proliferation and induction of apoptosis. Sequential hTERT knockdown and APG treatment remarkably modulated the levels of expression of invasion, cell cycle, and apoptosis regulatory molecules for inhibiting the growth of SK-N-DZ and SK-N-BE2 cell lines. We did not observe any significant difference between these two cell lines with regard to the effects of various treatments.

In our investigation, we first employed four human malignant neuroblastoma cell lines (SH-SY5Y, SK-N-DZ, SK-N-BE2, and IMR-32) and then selected two cell lines (SK-N-DZ and SK-N-BE2) that harbored the highest expression of hTERT at mRNA and protein levels. Sequential hTERT

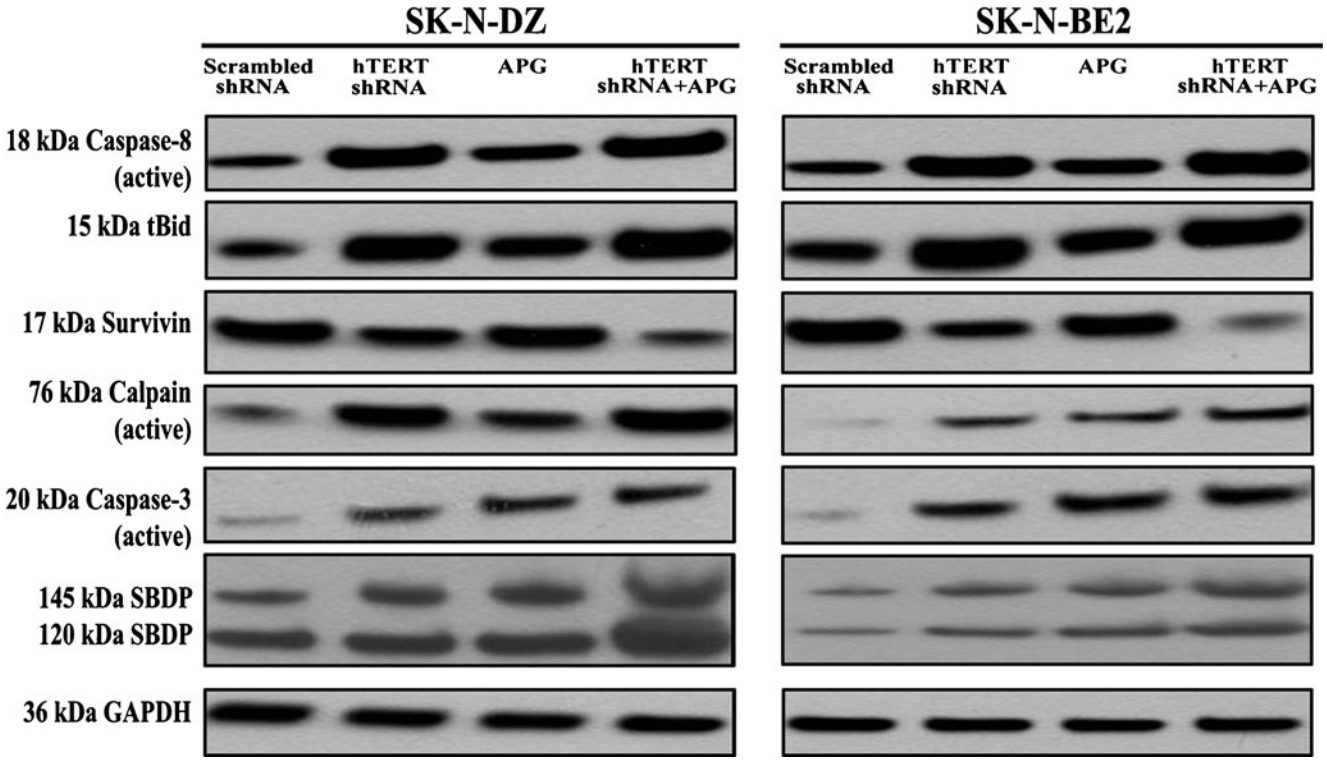

Fig. 7 Western blotting to examine alterations in expression of apoptosis regulatory molecules in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells after sequential hTERT silencing and APG treatment. Treatments: scrambled shRNA (1 μg/ml) for 12 h, hTERT shRNA (1 μg/ml) for 12 h, 100 μM APG for 24 h, and hTERT shRNA (1 μg/ml) for 12 h+100 μM APG for 24 h. Western blotting showed alterations in expression of apoptosis regulatory molecules and induction of proteolytic activities of calpain and caspase-3 for completion apoptotic process

shRNA transfection and APG treatment resulted in drastic suppression of expression of hTERT at mRNA and protein levels. The knockdown of hTERT was correlated with reduction in cell invasion and proliferation and induction of apoptotic cell death. After combination therapy, downregulation of MMP-2 and MMP-9 indicated the inhibition of cell invasion due to lack of degradation of the connective tissue matrix. Inhibition of expression of these pro-invasive factors was correlated with the downregulation of N-Myc, which, otherwise, could activate a number of genes associated with aggressiveness in neuroblastoma. Combination therapy inhibited expression of PCNA that could be directly involved in cell multiplication and DNA replication. Targeted downregulation of CDK-2 by RNA interference technique could induce apoptosis in N-Myc-amplified neuroblastoma cell lines (Molenaar et al. 2009). In our study, we found that CDK-2 expression was remarkably repressed in the N-Myc-amplified neuroblastoma cell lines (SK-N-DZ and SK-N-BE2) after combination therapy. So, interruption of the lethal relationship between CDK-2 and N-Myc using combination of hTERT shRNA and APG can provide an enormous potential in selective therapy for the N-Myc-amplified neuroblastomas. Our results also suggests that downregulation of cyclin D1 and its kinase partner CDK-4 probably leads to cell cycle arrest at G1 phase and, thereby, growth inhibition in human malignant neuroblastoma cells. Upregulation of p21Waf1 after the combination therapy also strengthened our stance because p21Waf1 inhibits CDKs at G1 phase and thus blocks cell cycle progression from G1 to S phase.

Our in situ Wright staining and Annexin V-FITC/PI binding results showed that combination therapeutic approach using hTERT shRNA and APG very effectively and successfully inhibited the survival advantages in the N-Myc-amplified neuroblastoma cell lines leading to induction of apoptosis. We performed Western blotting to unveil the molecular mechanisms for induction of apoptosis after combination therapy. We observed activation of both the intrinsic and extrinsic pathways of apoptosis. The extrinsic pathway of apoptosis was triggered by activation of caspase-8, which cleaved Bid to tBid in both neuroblastoma K-N-DZ and SK-N-BE2 cell lines. Our results showed that reduction in expression of survivin (a potent inhibitor of apoptosis protein in intrinsic pathway) after combination therapy could activate caspase-3 and induce apoptosis. We further confirmed that increases in activation of both calpain and caspase-3 cleaved α-spectrin to generate calpain-specific 145 kDa SBDP and caspase-3-specific 120 kDa

SBDP, respectively. So, our therapeutic approach using sequential hTERT knockdown and APG treatment could effectively block the survival strategy of the N-Myc-amplified neuroblastoma cells (SK-N-DZ and SK-N-BE2) through induction of both the extrinsic and intrinsic pathways of apoptosis.

In conclusion, we found that expression of hTERT was highest in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines, and sequential hTERT knockdown and APG treatment very effectively inhibited cell invasion and proliferation and induced apoptosis in these cell lines through efficient modulation of expression or activity of the molecules involved in these processes. So, sequential use of a specific gene targeted shRNA therapy and a flavonoid treatment, which may circumvent current issues of drug resistance and adverse side effects, can be a promising combination therapeutic strategy for controlling the growth of human malignant neuroblastoma cells.

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