Combinatorial gene targeting hTERT and BI-1 in CNE-2 nasopharyngeal carcinoma cell line

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Abstract. Nasopharyngeal carcinoma (NPC) is a common malignant tumor. In recent studies, we demonstrated that overexpression of the Bax inhibitor-1 (BI-1) induces cell transformation in NIH3T3 cells and that knockdown of BI-1 and human telomerase reverse transcriptase (hTERT) gene expression suppresses NPC cell proliferation and induces apoptosis. To evaluate the combination anti-tumor effects of siRNAs against hTERT and BI-1 in the CNE-2 NPC cell line, combined and separate short-hairpin (sh)RNA plasmids targeting hTERT and BI-1, respectively, were constructed. hTERT and BI-1 mRNA and protein levels were examined by real-time polymerase chain reaction (PCR) and western blot analysis. Cell proliferation, colony formation and migration ability were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), soft agar and wound healing assay. Cell apoptosis was observed by flow cytometry, Hoechst 33258 staining and caspase-3 activity. hTERT, BI-1 and combined shRNA plasmids were injected into xenograft NPC tumor tissues, and expression of hTERT and BI-1 was detected by real-time PCR and immunohistochemistry. Tumor growth was measured by tumor volume and apoptosis *in vivo* was confirmed by TdT-mediated dUTP nick end-labeling (TUNEL). Our results showed that combined shRNA specific for hTERT and BI-1 markedly suppressed hTERT and BI-1 gene expression *in vitro* and *in vivo*. In addition, CNE-2 cell proliferation was inhibited *in vitro* as well as *in vivo*. Following the knockdown of the two gene expressions, CNE-2 exhibited a decrease in colony formation and migration ability and an increase in the apoptotic rate compared to the control groups. Our *in vitro* and *in vivo* study showed that the combinative silencing of the two genes enhanced the therapeutic effect compared to the silencing of each individual shRNA. These data suggested that combinatorial gene therapy targeting hTERT and BI-1 may be beneficial as a tumor therapy strategy against human NPCs.

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Introduction

Nasopharyngeal carcinoma (NPC) is a common malignant tumor in southern China, particularly in the Guangdong population (1). Genetic as well as environmental factors are known to contribute to NPC tumorigenesis. At present, the standard treatment (the primary treatments) for NPC is radiotherapy, which in certain cases is combined with chemotherapy. However, the disease relapse and metastatic rate remain high (2). Thus, novel modalities of treatment for NPC are needed. Besides conventional therapies, target strategies aimed to reduce the expression of tumor-related genes have been investigated. Of those, small interfering RNAs (siRNAs) are potentially a curative selection.

RNA interference (RNAi) is a natural process through which the expression of a purpose gene can be knocked down theoretically with high specificity and selectivity RNAi (3). It has been used in numerous malignant cancer gene therapies. In colorectal and gastric cancers, knockdown cell division cycle associated 1 (CDCA1) and CDCA1-kinetochore associated 2 (KNTC2) suppressed cell proliferation and induced apoptosis (4). Downregulation of Wnt2 and β-catenin by siRNA suppressed malignant glioma cell growth (5). In addition, chronic lymphocytic leukemia cells induced apoptosis following the silencing of ROR1 and FMOD with siRNA (6).

Telomerase is a specific ribonucleoprotein complex that is often highly expressed in various tumors. The induction of human telomerase reverse transcriptase (hTERT) expression results in activity and is involved in the process of human carcinogenesis (7). Knockdown of hTERT by siRNA inhibits human bladder cancer cells (8), hepatocellular carcinoma cells (9) and oral cancer cell proliferation (10). In NPC, 91% of cells demonstrate hTERT expression and 85% exhibit telomerase overexpression (2). Short hairpin (sh)RNA targeted against hTERT inhibits cell viability by regulating

telomerase activity and its related protein expression in NPC cells (11).

A novel anti-apoptotic gene termed Bax inhibitor-1 (BI-1) has been found to represent a new type of regulator of cell death pathways (12). BI-1 has been found to be overexpressed in several tumors and is involved in tumor progression and malignancy due to its anti-apoptotic properties (13,14). Recently, we demonstrated that overexpression of BI-1 induces cell transformation in NIH3T3 cells, and knockdown of BI-1 and hTERT gene expression by siRNA suppresses NPC cell proliferation and induces apoptosis *in vivo* and *in vitro* (15-17).

Since cancer cells are characterized by multiple genetic alterations the single inhibition of one tumor-associated gene might not be sufficient as a therapeutic strategy. In the present study, the combination of the anti-tumor effects of siRNAs against hTERT and BI-1 were evaluated in CNE-2 NPC cell lines. Furthermore, inhibition of tumor growth was observed in subcutaneous NPC xenograft mice.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) completed medium, fetal bovine serum (FBS), Opti-MEM I medium, TRIzol reagent and Lipofectamine™ 2000 (Lipo) were purchased from Invitrogen (Guangzhou, China). Primary antibody against hTERT, BI-1 and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence substrate was purchased from Thermo Scientific (Guangzhou, China). The restriction enzyme was purchased from New England Biolabs (Beijing, China).

Design and construction of shRNA plasmids. hTERT and BI-1 RNAi target sequences were used in this study: hTERT: 5'-gacgtcttcctacgcttca-3' (GenBank no.: AF015950 Position: 2474-2492), BI-1: 5'-gatcaagattatatctggcactg-3' (GenBank no.: BC036203, Position: 738-760). The hTERT and BI-1 and scramble hairpin-like double-stranded oligo DNA was synthesized by Sangon Biotech (Shanghai, China). The restriction enzyme site *Eco*RI and *Kpn*I for hTERT and *Not*I and *Xba*I for BI-1 were designed on the ends of the oligo DNA. The oligo DNA was cloned in pcDNA3.1(+) (Invitrogen) vector and the correct insert was confirmed by DNA sequence.

Cell culture and transfection. Poorly differentiated human CNE-2 NPC cell line was established and provided by Professor Yi Zeng (18). This cell line was cultured in DMEM completed medium supplemented with 10% FBS, penicillin (100 U/l) and streptomycin (100 μg/l) (Invitrogen Guangzhou, China) at 37˚C in a humid incubator with 5% CO_2. Using the Lipofectamine™ 2000 reagent (Invitrogen), CNE-2 cells were transfected with (plasmids pcDNA3.1(+) empty vector, sh-hTERT, sh-scramble hTERT, sh-BI-1, sh-scramble BI-1, sh-hTERT-BI-1 and sh-scramble-hTERT-BI-1), according to the manufacturer's protocol.

Real-time reverse transcription polymerase chain reaction (RT PCR). Primers used in this study were: hTERT: 5'-agtgtctg gagcaagttgcaaag-3' and 5'-cacgacgtagtccatgttcacaatc-3', BI-1: were 5'-atcattgtaaccaatcctgccagac-3' and 5'-agcctcgctctgttgatg tgaa-3', β-actin: 5'- tggcacccagcacaatgaa-3' and 5'-ctaagtca tagtccgcctagaagca-3. After 48 h of transfection, total RNA was prepared with TRIzol reagent. Quantitative real-time PCR was performed using One Step SYBR® PrimeScript® RT-PCR kit II (Takara Biotechnology Co., Ltd., Dalian, China), following the manufacturer's instructions on ABI 7500 HT real-time PCR detection system. The results were normalized for the expression of β-actin.

Western blot analysis. The transfected cells were harvested in RIPA buffer and the protein concentration was determined by Bradford reagent (Bio-Rad, Guangzhou, China). Total protein (100 μM) was separated on SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane (Millipore China Ltd., Guangzhou, China). The membrane was incubated overnight at 4˚C with primary antibodies (1:200 for hTERT and BI-1, respectively, and 1:500 for β-actin). The primary antibody for hTERT (sc-7212), BI-1 (sc-12393) and horseradish perodidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. The primary antibody (BSAP0060) for β-actin was purchased from Bioworld Technology (Shanghai, China). The protein bands were visualized using the enhanced chemiluminescence substrate kit (Thermo, Guangzhou, China). The percentage reduction in band intensity was calculated based on the untreated samples and normalized to β-actin.

Cell proliferation and apoptosis analysis. CNE-2 cells ($5x10^3$) were seeded in 96-well plates and incubated at 12 h for transfection. At 48 h after transfection, each well was treated with 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution [10 mg/ml in phosphate-buffered saline (PBS)] and incubated sequentially at 37˚C. Four hours after incubation, 100 μl of dimethyl sulfoxide (DMSO) were added to dissolve the crystals. The plate was oscillated for 10 min at room temperature and absorbance was measured at 570 and 630 nm. The apoptotic level was performed as previously described (16). Briefly, for Hoechst 33258 fluorescence staining, CNE-2 cells were seeded in 24-well plates and treated with 5 μl 20 μg/ml Hoechst 33258 for 30 min in the dark. The morphological change of nuclei in CNE-2 cells was analyzed and counted under a fluorescent microscope (magnification, x200) in five different fields to discriminate normal and apoptotic cells. Cells were photographed and the images were processed using the Adobe Photoshop software, version 7.0 (Adobe, CA, USA).

Flow cytometry followed an Annexin V-FITC Apoptosis Detection Kit (Nanjin KeyGen Biotech Co., Ltd., Nanjin, China). Briefly, after 48 h of plasmid transfection, cells were collected and washed with PBS three times. The cells were suspended in 500 μl binding buffer and stained with 5 μl Annexin V-FITC and 5 μl propidium iodide for 15 min at room temperature in the dark. The cells were examined using a BD FACSCanto II flow cytometer and analyzed using BD FACSDiva software 6. Caspase-3 activity was determined using a caspase assay kit according to the manufacturer's instructions (Clontech, Mountain View, CA, USA). The samples were read at 405 nm in a microplate reader.

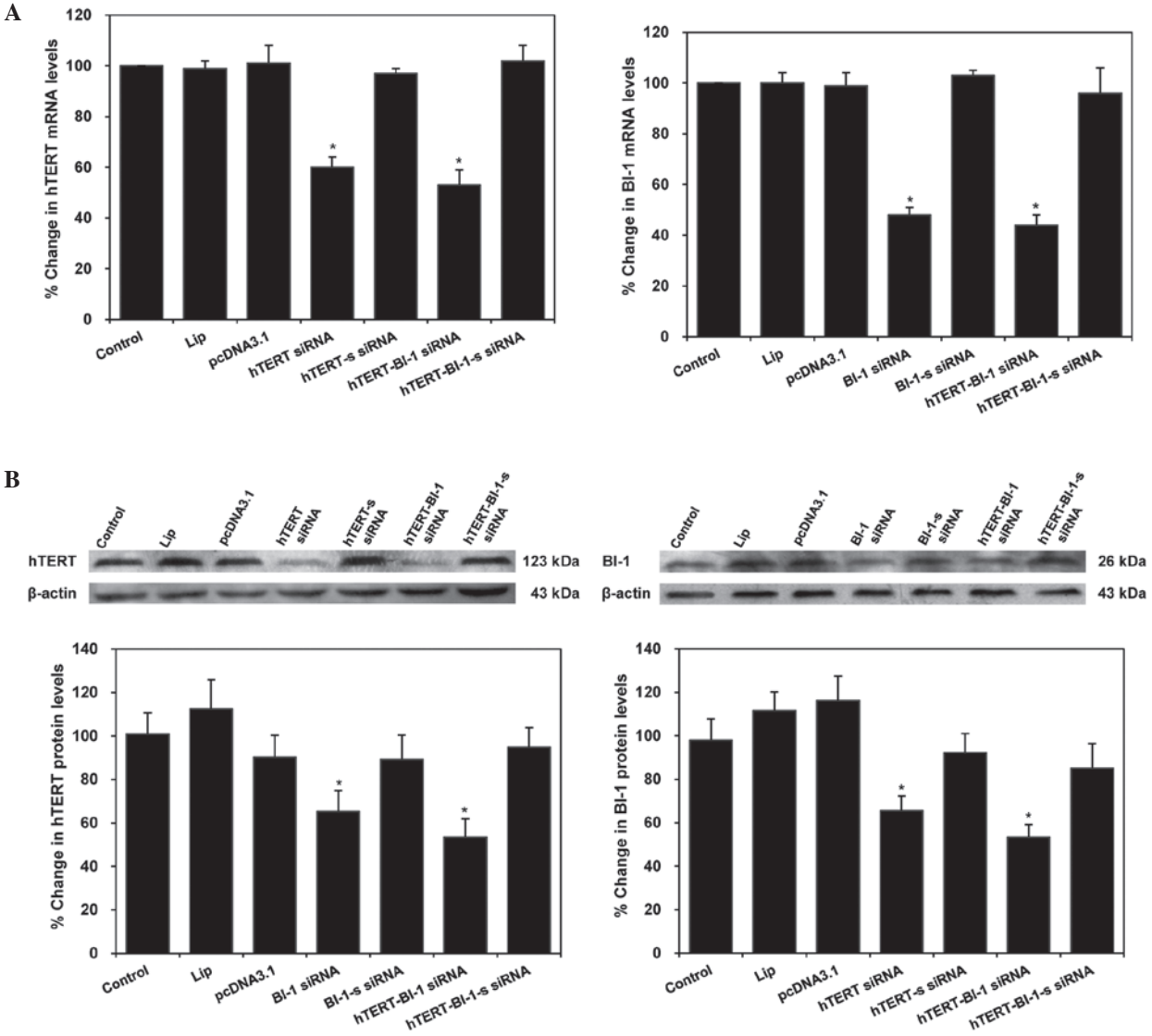

Figure 1. Downregulation of human telomerase reverse transcriptase (hTERT) and Bax inhibitor-1 (BI-1) gene expression after shRNA transfection is shown. hTERT and BI-1 gene expression in CNE-2 cells was suppressed following transfection with sequence-specific short-hairpin (sh)RNA against hTERT, BI-1 and a combination of the two gene plasmids. Untreated, transfection reagent Lipofectamine™ 2000, empty vector pcDNA3.1(+) and vector carrying scrambled shRNA sequence was used as the negative control. After transfection CNE-2 cells were collected and used for RNA and protein isolation, respectively. (A) hTERT and (B) BI-1 mRNA and protein levels were analyzed using real-time PCR and western blot analysis. Results of the analysis are shown in the bar graph. The labels for the bars are: control, untreated, lip transfection reagent Lipofectamine™ 2000, pcDNA3.1: empty vector pcDNA3.1(+), hTERT or BI-1 siRNA: hTERT- or BI-1-specific shRNA-contained plasmids, hTERT-BI-1 siRNA: hTERT-BI-1 combination shRNA-contained plasmids, hTERT-s, BI-1-s or hTERT-BI-1-s siRNA: hTERT, BI-1 or hTERT-BI-1 scramble shRNA-containing plasmid. The western blot analysis was stripped and re-probed with β-actin antibody to check for equal loading of total protein. Data are shown as the mean ± SD of three independent experiments. *P<0.05 vs. the parental group.

Wound healing assay. Cells ($2x10^5$) were seeded in a 6-well plate. After forming a confluent monolayer, the cells were transfected with plasmids and scratched using a 100 μl tip. The culture medium was replaced with fresh complete medium. The closure of scratch was analyzed under the microscope and images were captured at 0, 24 and 48 h after incubation.

Colony formation assay. Cells ($1x10^4$) were plated in triplicate in 6-cm diameter plates with 0.6% base agar and 0.3% top agar and incubated for 21 days. The colonies were counted in 10 randomly chosen microscope fields.

In vivo antitumor assay. Twenty BALB/c nude mice (SPF grade, 6-8 weeks of age) were purchased from the Animal Experimental Center of the Guangdong Medical College. CNE-2 cells ($2x10^6$) in 100 μl serum-free medium were injected into the right flank of each mouse. When the tumors reached 3-5 mm in diameter (~6 days), the mice were randomized into four groups. The hTERT, BI-1, hTERT-BI-1, scrambled shRNA plasmid DNA (20 μg) were respectively injected directly into the tumor, which had been mixed with 40 μl Lipo. This treatment was performed three times on days 9, 12 and 15 following CEN-2 cell injection. Tumor size was measured every three days and tumor volume was calculated as $V=ab^2/2$, where a is the length and b is the width of tumor. At day 18 after cell injection, the animals were sacrificed and the tumors were frozen immediately for future assays. Tissue sections were fixed in 4% paraformaldehyde and embedded

in paraffin. Tissue sections (3 μM) were prepared and stained with hematoxylin and eosin. The expression levels of hTERT and BI-1 were examined by real-time RT PCR and immunohistochemical staining, performed according to the ImmunoCruz™ Staining Systems manufacturer's instructions (Santa Cruz Biotechnology, Inc.). Briefly, paraffin sections of tumor tissues were immersed in the primary antibody to hTERT and BI-1 (Santa Cruz Biotechnology, Inc., 1:100) at 4˚C overnight and detected by a biotinylated secondary antibody (Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Subsequently, sections were incubated in the HRP-streptavidin complex (Santa Cruz Biotechnology, Inc.) for 30 min. Sections were then incubated with three drops of peroxidase substrate for 12 min and counterstained with hematoxylin for 5-10 sec, washed, dehydrated in ascending grades of ethanol, and coverslipped with Depex. The sections were examined and microphotographed by light microscope (magnification,x200). Cell apoptosis was determined using the TUNEL method. The animal experiments were approved by the Experimental Animal Care and Use Committee in Guangdong Medical College.

Statistical analysis. Data were shown as the mean ± standard deviation (SD) and were analyzed using the commercially available statistical software package, SPSS 13.0 (SPSS, IL, USA). One-way analysis of variance (ANOVA) test was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Specific knockdown of hTERT and BI-1 in CNE-2 NPC cell line. hTERT- and BI-1-specific inhibited and scrambled shRNA fragments were cloned to mammalian cell expression vector pcDNA3.1(+). The constructs were identified using DNA sequencing and the sequences were correct (data not shown). Plasmid DNA was transfected to CNE-2 cells. After 48 h, the hTERT and BI-1 mRNA and protein expression were detected using real-time PCR and western blot analysis, respectively (Fig. 1). Suppression of mRNA and protein expression was observed (Fig. 1). The combined hTERT/BI-1 shRNA vector simultaneously inhibited hTERT and BI-1 gene expression with the same efficiency as single hTERT or BI-1 shRNA carrying vectors. Untreated, transfection reagent, empty vector and vector-carrying scrambled shRNA sequence was used as the negative controls.

Simultaneous knockdown of hTERT and BI-1 inhibited CEN-2 cell growth and migration. Inhibition of the proliferation and migration of CNE-2 cells by hTERT, BI-1 and combined hTERT/BI-1 shRNA was tested through cell viability, soft agar colony formation ability and healing wound assay (Fig. 2). Using the MTT assay, we analyzed *in vitro* cell growth following hTERT, BI-1 and combined hTERT/BI-1 shRNA-mediated knockdown (Fig. 2A). Significant suppression of cell proliferation was observed in CNE-2 cells at 48 h. The double knockdown group showed better inhibitory effect following reduction of the cell growth rate to 49.00±3.75%. Results of the colony formation test also showed that cell growth inhibition of CNE-2 cells transfected with hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmids (Fig. 2B). The colony formation rate was 9.25±1.05, 8.95±1.17 and 6.72±1.00% for the hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmid transfection groups, respectively. Inhibition of the migration ability for CNE-2 cells was tested following hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmid transfection by healing wound assay. The wound was almost completely repaired after 48 h in the control group, while the hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmid transfection groups were still in the process of completing the repair (Fig. 2C). Compared to the single knockdown groups, the double-gene knockdown group inevitably showed a decrease in repairing ability.

Induction of apoptosis following shRNA treatments. CNE-2 cells treated with control or hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmids for 48 h were evaluated for apoptosis. The apoptosis was determined using Hoechst 33258 fluorescence staining, flow cytometry and caspase-3 activity. Our results showed that treatment of CNE-2 cells with hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmids for 48 h resulted in apoptotic bodies and fragmentation of nuclei, as shown by Hoechst 33258 fluorescence staining (Fig. 3A). Flow cytometry showed (Fig. 3B), that the apoptotic rate of the hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmid transfection groups to be 17.20±2.40, 16.73±2.30 and 21.90±1.87% respectively, which was significantly higher compared to the control group (3.53±2.44%). A more quantitative apoptotic manner was measured by caspase-3 activity, while transfection with hTERT, BI-1 and combined hTERT/BI-1 shRNA plasmids resulted in a 2.7±0.4-, 2.4±0.3- and 3.7±0.3-fold increase in caspase-3 activity (Fig. 3C). Compared with hTERT and BI-1 single blocked groups. The double hTERT/BI-1 blocked group showed an increased apoptotic rate. These findings are consistent with our previous findings and support the hypothesis that double-gene silencing is better than single gene silencing in inducing CNE-2 cell apoptosis.

Proliferation inhibition of NPC BALB/c xenograft tumors with hTERT, BI-1 and combined hTERT/BI-1 shRNA in vivo. After shRNA was injected three times, tumor growth was significantly reduced in hTERT-, BI-1- and combined hTERT/BI-1 shRNA-treated NPC tumors, while no obvious effects were observed on the control group (Fig. 4A). The difference of the average size between the tumors from the hTERT-, BI-1- and combined hTERT/BI-1 shRNA-treated groups and the tumors from the control groups was statistically significant on day 18. The average tumor size for the untreated, hTERT-, BI-1- and combined hTERT/BI-1 shRNA-treated groups was 1570.70±108.50, 1119.10±142.72, 1070.32±160.24 and 908.52±234.16 mm^3, respectively (Fig. 4B). The inhibition rate for the hTERT shRNA-treated group was 28.75±2.55%, the BI-1 shRNA-treated group was 31.86±2.99% and the combined hTERT/BI-1 shRNA-treated group was 42.16±5.15% (Fig. 4C). Morphological similarity of the xenograft to the human NPC was evident on paraffin-embedded sections stained with HE (Fig. 4D).

hTERT, BI-1 and combined hTERT/BI-1 shRNA inhibited hTERT and BI-1 expression and induced apoptosis in vivo.

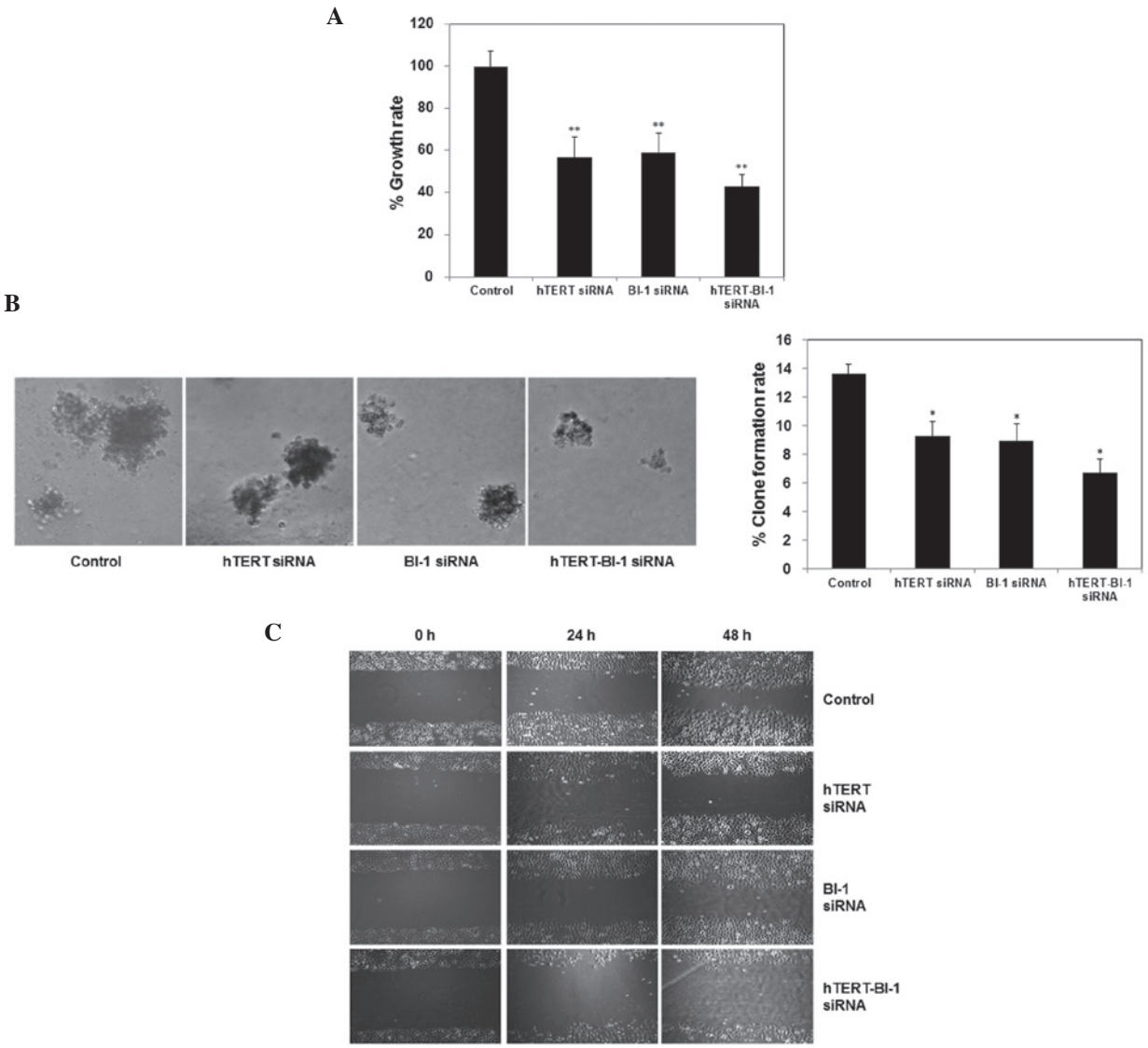

Figure 2. Simultaneous knockdown of human telomerase reverse transcriptase (hTERT) and Bax inhibitor-1 (BI-1) inhibited CEN-2 cell growth and migration (A) Cell viability of CNE-2 cells following transfection with shRNA against hTERT, BI-1 or hTERT-BI-1, using MTT assay. The values are normalized to the control (100%). (B) Colony formation ability of CNE-2 cells following transfection with various plasmids was assayed in soft agar. At day 21, cell colonies were counted in 10 randomly chosen microscope fields and the colony formation rate was calculated. (C) Effect of hTERT, BI-1 or hTERT-BI-1 combinative shRNA on CNE-2 cell migration is shown. CNE-2 cells were seeded in 6-well plates and wounded the following day. Images were captured at 0, 24 and 48 h, respectively, after the wound was made. Data were normalized to NS control and are the means ± SD of triplicates. Asterisks are representative of significant differences compared to the control. (*P<0.05, **P<0.01).

After treatment was administered three times, the mice were sacrificed and the tumor tissues were isolated to detect alterations of hTERT and BI-1 expression. The results (Fig. 5A and B) demonstrated that a significant decrease in the expression of hTERT and BI-1 in tumor tissues treated with hTERT, BI-1 and combined hTERT/BI-1 shRNA compared to the control group. There was no detectable difference in the expression among the lip, empty vector and hTERT, BI-1 hTERT-BI-1 scramble shRNA treatment groups (data not shown).

Cell apoptosis in tumor samples derived from the control and treated mice was examined using the TUNEL method. Results showed that the number of apoptotic cells was markedly increased in the tumor specimens treated with hTERT (17.34±2.48%), BI-1(16.01±2.01%) and combined hTERT/BI-1 (28.46±3.28%) shRNA, as compared to those in tumor specimens from control (2.87±1.74%) or scramble shRNA-treated mice (Fig. 5C).

Discussion

For NPC, platinum-based doublet chemotherapy is the standard treatment, while targeted approaches are currently being developed (19). Human telomerase is a ribonucleoprotein complex composed of at least the reverse catalytic transcriptase hTERT and the RNA component hTR. The enzyme stabilizes telomere length, thereby contributing to unlimited cell proliferation. Reactivation of telomerase activity during carcinogenesis is a common hallmark in most human tumor types. Previous

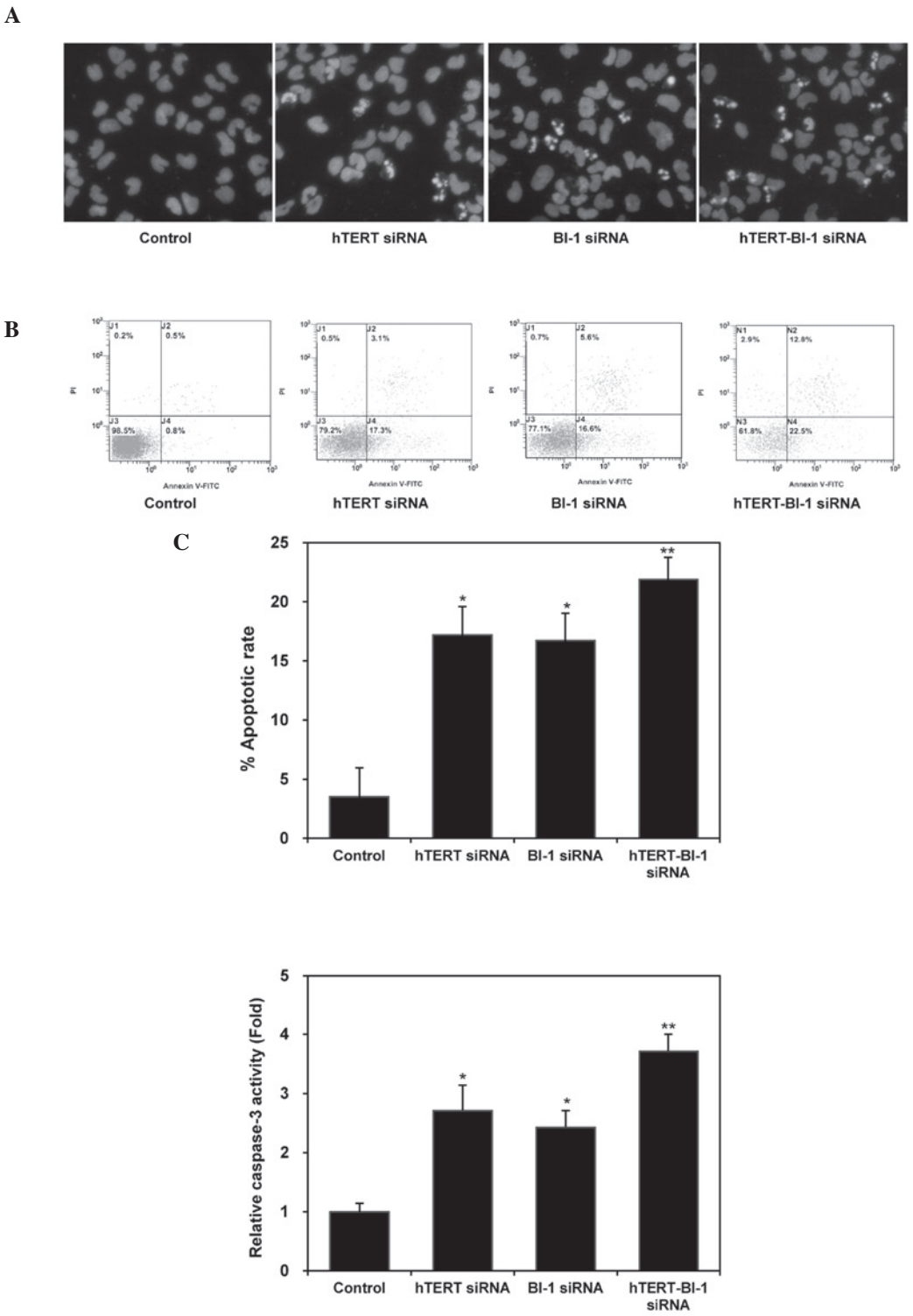

Figure 3. Induction of apoptosis following short hairpin (sh)RNA treatments is shown. (A) Cellular apoptosis was observed using Hoechst 33258 fluorescence staining to detect chromosomal condensation and nuclear fragmentation. (B) Early CNE-2 cells apoptosis rate was analyzed by flow cytometry. (C) Caspase-3 activity was measured and described as a percentage of change in the mean values derived from three separate experiments compared to the control group. Data were normalized to NS control and represent the means ± SD of triplicates. Asterisks are representative of statistically significant differences compared to the control. (*P<0.05, **P<0.01).

findings have shown that the downregulation of hTERT can lead to a decrease in cell proliferation and/or induction of apoptotic cell death in several types of cancer cells. siRNA inhibited telomerase-induced apoptosis in Barrett's adenocarcinoma cells (20). Similar results were obtained on the human colorectal HT29 cell line (21). In Capan-2 human pancreatic cancer cell knockdown hTERT suppresses cell growth via the inhibition of Bcl-2 and COX-2 expression (22).

The BI-1 family is a highly preserved family of small transmembrane proteins located mostly in the endoplasmic reticulum belonging to the regulatory proteins that determine the fate of a cell regarding death or survival (13). In

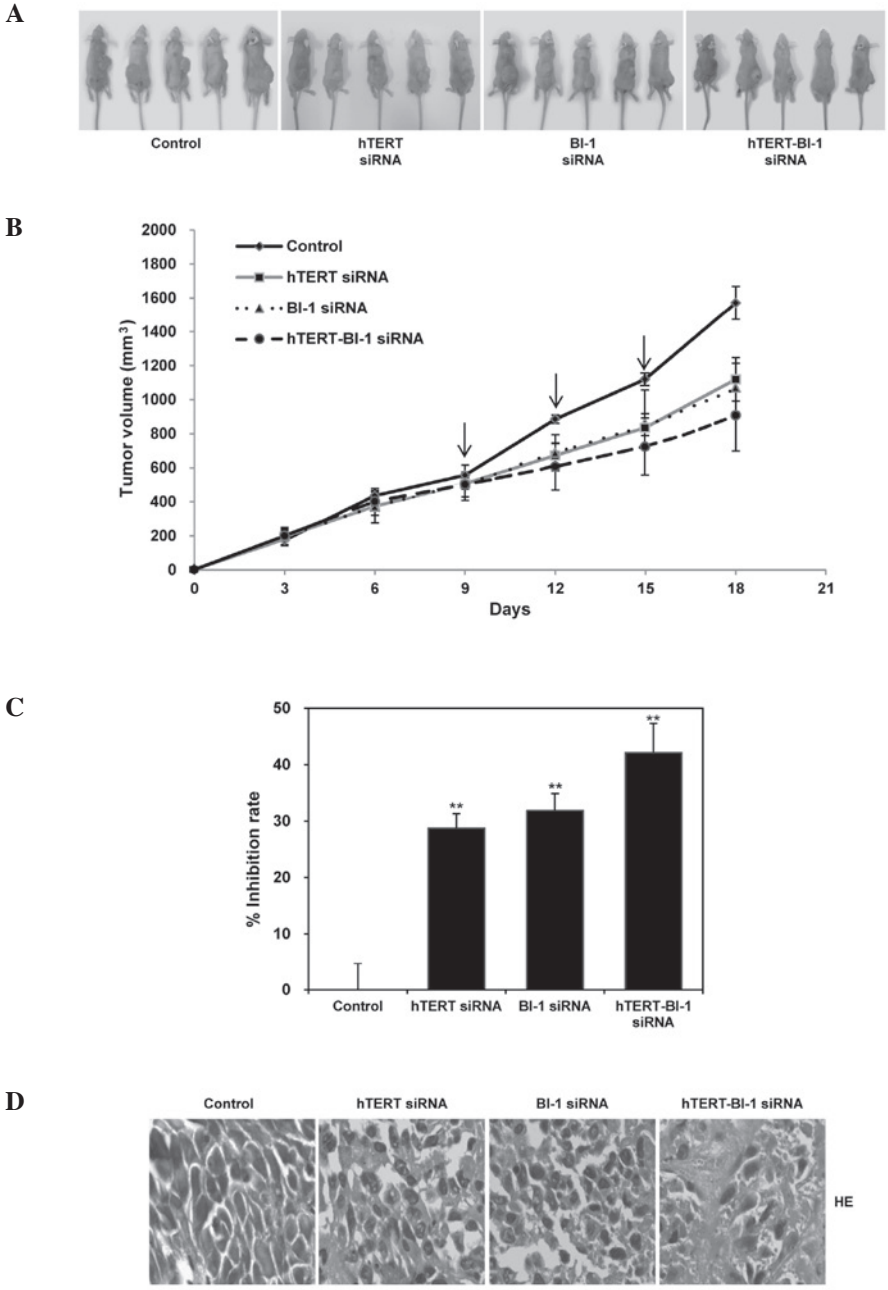

Figure 4. Therapeutic effect of shRNA on the tumor obtained from inoculated CNE-2 cells in nude mice is shown. (A) The appearance of the xenograft subcutaneous nasopharyngeal carcinoma (NPC) in the control and various treatment groups is shown. The xenograft tumors treated with specific targeting short-hairpin (sh)RNA were markedly smaller compared to the control group of mice. (B) Growth curves of subcutaneous NPC in nude mice with various treatments demonstrates that tumor growth was markedly retarded compared to the control group. Compared to the single targeting group, the combinative shRNA-treated group showed enhanced therapeutic effects. (C) Inhibition rate of the control and various treatment groups is shown. As compared to the control group, the inhibitory rate of the three shRNA treatment groups was markedly increased and the combinative shRNA treatment group showed a higher inhibitory effect ($^{**}P<0.01$). (D) Hematoxylin and eosin (HE) staining results of the tumors formed in nude mice, which are typical nasopharyngeal squamous cell carcinomas.

the lung cancer cell lines, HT1080 and B16F10, BI-1 altered mitochondrial function, which in turn altered glucose metabolism and activated sodium-hydrogen exchanger to enhance cancer metastasis. Similar to previous studies, results of our previous studies demonstrated that BI-1 mediated apoptotic resistance in human NPC cells (16). Since tumor cells may bypass the inhibition of a single gene by changing their expression profile, the combined antisense-mediated inhibition of tumor-promoting genes is a promising strategy for cancer treatment.

Multitarget siRNA inhibition of antiapoptotic genes in bladder cancer cells reduced cell growth and sensitized cells to subsequent chemotherapy (23). Recently, Song *et al* (24) constructed a shRNA plasmid containing VEGF-, C-myc-, survivin- and hTERT-inhibited siRNA sequences and found that the multiple gene shRNA more markedly induced cell apoptosis compared to each individual shRNA, respectively. The combinative silencing of these four genes had an enhanced inhibitory effect on xenograft tumors compared to the silencing of each individual shRNA. The RNA polymerase III promoters

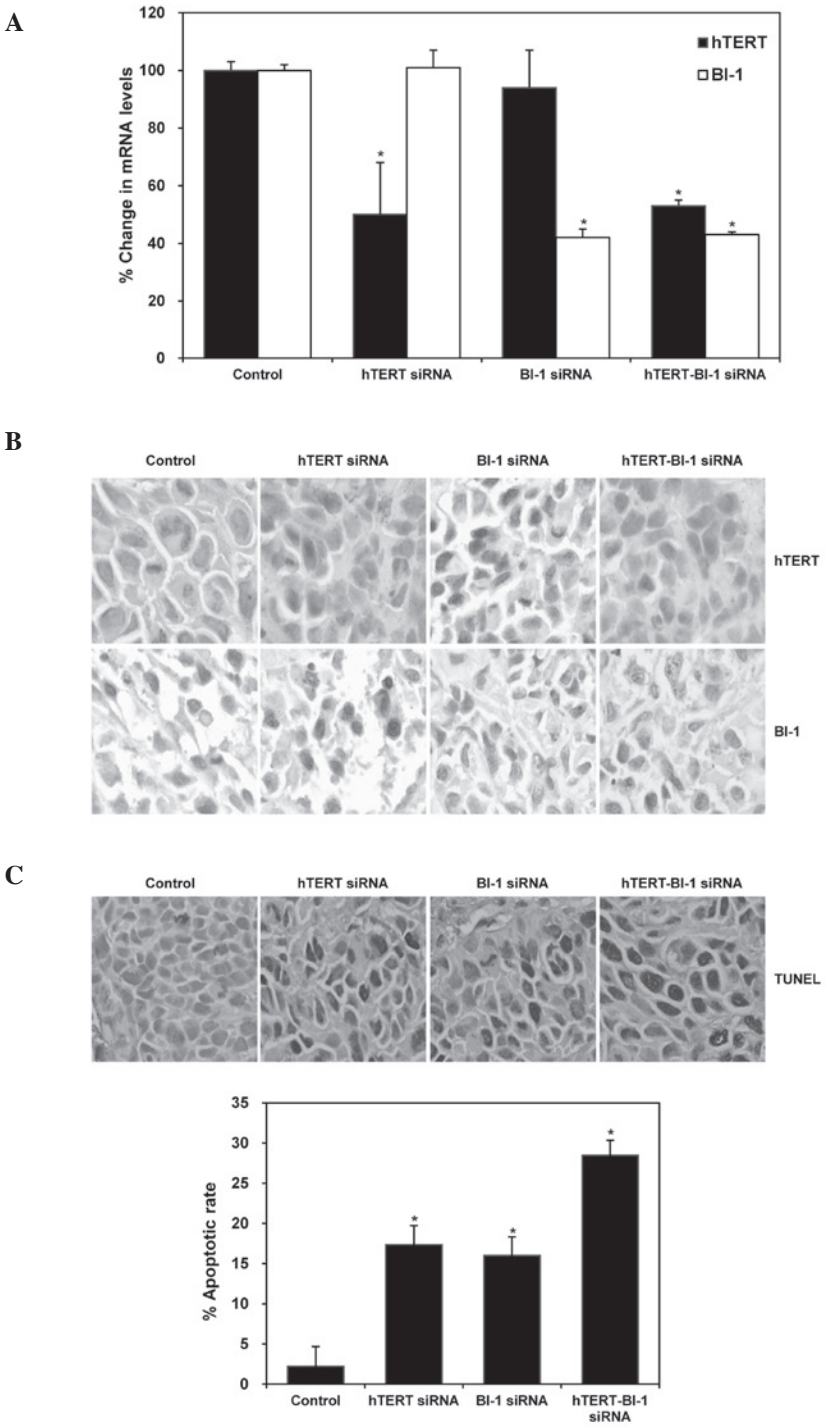

Figure 5. Specific short-hairpin (sh)RNA downregulated hTERT and BI-1 gene expression and induced apoptosis in tumor tissue are shown. (A) Expression of hTERT and BI-1 in subcutaneous nasopharyngeal carcinomas (NPCs) treated with shRNA targeting hTERT or BI-1 was suppressed, as shown by results of real-time RT-PCR. (B) Similar results were also obtained by immunohistochemistry (magnification, x200) (*P<0.05). Compared to the single knockdown group, combinative shRNA simultaneously suppressed hTERT and BI-1 gene expression. (C) Apoptotic cells were increased in subcutaneous NPCs that were treated with shRNA targeting hTERT, BI-1 or combined hTERT/BI-1 detected by the TUNEL (TdT-mediated dUTP nick end-labeling) method (magnification, x200) Combinative shRNA-treated group demonstrated a higher apoptotic rate (*P<0.05).

contained plasmids that synthesized shRNAs and elicit RNAi efficiently, while lacking cell specificity and rendering the monitoring of shRNA expression levels difficult. An alternative method to deliver RNAi is to use RNA polymerase II through direct synthesis of shRNA (25). In this study, we demonstrated that shRNA ligation into vector pcDNA3.1(+) against hTERT and BI-1 led to a notable reduction of gene expression *in vitro* and *in vivo* (Fig. 1). The specific suppression of hTERT and BI-1 expression induced a significant decrease in CNE-2 cell proliferation, colony formation and migration (Fig. 2). It also enhanced CNE-2 apoptosis as assessed by flow cytometry, caspase-3 activity and Hoechst 33258 fluorescence staining (Fig. 3). Furthermore, we observed an effective suppression of hTERT and BI-1 expression in the nude mice model. After

the knockdown of hTERT and BI-1 gene expression simultaneously or respectively by direct neoplasm injection, the xenograft tumor showed retarded growth. Compared to the single knockdown group, the combined knockdown demonstrated a higher inhibition rate (Fig. 4). Moreover, the same effect of apoptosis was confirmed on the xenograft tumor using the TUNEL method (Fig. 5).

In conclusion, to the best of our knowledge, we have demonstrated for the first time that the simultaneous knockdown of hTERT and BI-1 reduced NPC cell proliferation and migration and induced apoptosis *in vitro* and *in vivo*. The combination inhibition may greatly improve the outcome of NPC treatment and contribute towards a better clinical prognosis. Therefore, further studies are required to apply these targeted therapies in patients to improve the outcome of NPC treatment.

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