

Telomerase reverse transcriptase interference synergistically promotes tumor necrosis factor-related apoptosis-inducing ligand-induced oral squamous cell carcinoma apoptosis and suppresses proliferation *in vitro* and *in vivo*

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is known to induce cell apoptosis in many types of cancer cells. However, some malignant cells still exhibit anti-apoptosis features induced by TRAIL; thus the underlying mechanisms that regulate sensitivity and resistance of tumor cells to TRAIL-induced apoptosis remain unclear. Human telomerase reverse transcriptase (hTERT) is overexpressed in most types of human tumors and is mostly inactive in somatic cells. The present study aimed to investigate the endogenous effects and mechanisms of hTERT inhibition and TRAIL overexpression on TRAIL-induced apoptosis of human oral squamous cell carcinoma (OSCC) cells. The effects of adeno-associated virus (AAV)-mediated TRAIL and hTERT gene silencing by RNA interference were investigated on the proliferation and apoptosis of human OSCC cells *in vitro* and *in vivo*. The present results suggest that knockdown of hTERT expression accelerated TRAIL-resistant OSCC cells to

TRAIL-induced apoptosis and impaired OSCC cell proliferation. In addition, this process is accompanied by the upregulation of caspase-3, caspase-8 and caspase-9, and downregulation of B cell lymphoma-2. Additionally, the possible mechanisms underlying the association between TRAIL expression and hTERT silencing were explored. The results demonstrated that TRAIL expression levels were elevated when the hTERT gene was silenced, and notable anti-tumor effects were observed when TRAIL upregulation and hTERT gene silencing were carried out simultaneously. The present findings provide experimental evidence for the combined use of TRAIL and hTERT as a possible gene therapy strategy in oral cancer.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common malignant cancer worldwide (1). Each year, ~500,000 new cases of OSCC are diagnosed worldwide (2,3). Although there have been many recent achievements, the 5-year survival rate for patients with OSCC has not significantly improved because of regional recurrence or distant metastases causing poor prognosis (4,5). Chemotherapy has traditionally been used as the standard treatment for OSCC, but its toxic effects and the potential for chemo-resistance impair its useful application (6,7). Therefore, novel strategies should be developed to address this disease, including more sensitive, but less toxic, therapeutic molecular targets. Gene therapy has offered a promising strategy for the treatment of OSCC (8,9).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), which is a member of the TNF superfamily, can induce tumor cell apoptosis while exerting no effect on normal human cells (10,11). Mounting evidence has demonstrated that TRAIL exerts anti-tumor effects via suppression

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of tumor proliferation and promotion of tumor apoptosis in multiple types of cancer both *in vivo* and *in vitro*; examples include: Colorectal cancer (12), breast cancer (13), esophageal cancer (14), lung cancer (15), gastric cancer (16) and hepatocellular carcinoma (HCC) (17). A treatment strategy for cancer based on TRAIL has previously been identified as a promising option (18). However, malignant tumors may become resistant to TRAIL-induced apoptosis.

Human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase, is overexpressed in ~90% of malignant tumors (19). These tumor cells have been reported to obtain immortalized features by maintaining their telomere lengths with the help of hTERT (20). Suppression of hTERT gene activity can impair tumor proliferation by shortening their replicative life span, further arresting tumor growth (21). Therefore, hTERT is another potential molecular target for tumor treatment.

Recently, combination treatment has become the prevailing approach in tumor therapy as an effective way to address cancer. For instance, the combination of cisplatin and paclitaxel has been demonstrated to induce antitumor effects of varying degrees (22,23). However, a lack of specificity, off-target activity, chemo-resistance and normal cell damage due to toxicity, markedly impaired efficacy of this treatment. A more targeted combination therapeutic strategy involving multiple genes may be a more promising solution.

In the present study, the synergistic antitumor effects of adeno-associated virus (AAV)-mediated TRAIL overexpression combined with lentivirus vector-mediated hTERT, which induces tumor apoptosis and inhibits tumor proliferation *in vitro* and *in vivo*, were investigated. The findings suggested that AAV-mediated TRAIL overexpression combined with lentivirus vector-mediated hTERT may be an effective therapy for OSCC.

Materials and methods

Ethical approval and informed consent. The present study was approved by the Ethics Committee at Sun Yat-sen Memorial Hospital (Sun Yat-sen University, Guangzhou, China). Animal experiments were approved by the Ethics Committee of Sun Yat-Sen University and were conducted following the official instructions of the laboratory animal center at Sun Yat-Sen University.

Cell lines and cell culture. Two human tongue squamous cell carcinoma cell lines, SCC25 and UMI, were used in the present study. SCC25 cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and UMI cell line was kindly supplied by Professor Wang from The First Affiliated Hospital of Sun Yat-sen University. Both cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. 293T cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

AAV-TRAIL viral preparation. Recombinant AAV2 vectors were produced using a triple transient transfection method. The

plasmid carrying TRAIL cDNA (pAAV-CMV-EGFP-TRAIL; Clontech Laboratories, Inc., Mountainview, CA, USA), pAAV-RC plasmid expressing rep/cap gene and the pHelper plasmid (Applied Viromics, LLC, Fremont, CA, USA) were mixed at a respective ratio of 15:10:10 µg and co-transfected into 293T cells with polyethylenimine (Polysciences, Inc., Warrington, PA, USA) for 72 h according to the manufacturer's protocol. The viral supernatant was collected following centrifuging at 4°C at 500 x g for 10 min and 293T cells containing packaged particles were collected, purified and concentrated by using HiTrap Heparin HP and Amicon Ultra 4 ml Filters (GE Healthcare, Chicago, IL, USA) to obtain concentrated recombinant AAV vectors. The particle titers were determined by quantitative polymerase chain reaction (qPCR), as detailed below, and are presented as genome particles/ml. To evaluate the efficiency of the recombinant AAV system on TRAIL upregulation, enhanced green fluorescence protein (EGFP) gene was inserted as a reporter gene and its expression was analyzed using Nikon Eclipse Ti fluorescence microscopy (magnification, x400; Nikon Corporation, Tokyo, Japan).

Lentivirus preparation and gene silencing. The lentivirus vector was constructed using the LV-008 plasmid (Forevergen Biosciences Co., Ltd., Guangzhou, China) with a verified short hairpin RNA against TERT (shTERT) inserted, the pMD2.G plasmid (cat. no. 12259; Addgene, Inc., Cambridge, MA, USA) expressing the vesicular stomatitis virus glycoprotein gene and psPAX2 plasmid (cat. no. 12260; Addgene, Inc.) carrying the gag/pol-gene. For viral packaging, 8 µg shRNA plasmid, 2 µg pMD2.G plasmid and 4 µg psPAX2 plasmid were co-transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) into 293T cells plated in 100-mm dishes. Transfected lentiviruses were collected 72 h following transfection and concentrated via ultra-centrifugation at 87,000 x g for 180 min at 4°C using a Beckman SW28 rotor (Beckman Coulter, Inc., Brea, CA, USA). SCC25 and UMI cells were cultured with RPMI-1640 and seeded in a six-well plate. When cells reached 50-70% confluence, cells were infected with hTERT-lentivirus or a negative control (NC)-shRNA lentivirus, respectively, in the presence of 5 µg/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). At 5 days following transfection, the knockdown efficiency was examined by reverse transcription (RT)-qPCR and western blotting. To select for stably silenced cells, puromycin (2 µg/ml; Sigma-Aldrich; Merck KGaA) was added to the medium.

RNA extraction and RT-qPCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and RT was performed to synthesize cDNA using a Prime Script™ RT reagent kit according to the manufacturer's protocol (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed with a SYBR Green PCR Master Mix (Takara Biotechnology Co., Ltd.). The reaction mixture consisted of a volume of 20 µl, containing 2 µl cDNA, 10 µl SYBR-Green Mix, 4 µl primer mix and 4 µl ddH₂O. The PCR cycling conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec and 50°C for 30 sec. β-actin was used as reference gene and the primers used are presented in Table I. Relative gene expression was calculated using the 2^{-ΔΔC_q} method (24).

Western blotting. Cells were lysed in sample lysis solution (50 mM Tris-HCl, pH 8.0; 1% SDS; 1 mM EDTA; 5 mM DTT; 10 mM phenylmethylsulfonyl fluoride; 1 mM NaF; 1 mM Na₃VO₄; and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), and protein concentration was determined with a bicinchoninic acid assay kit (Abcam, Cambridge, UK). Equal amounts of protein (20 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (GE Healthcare). Membranes were probed with antibodies against TRAIL (cat. no. 3219), caspase-3 (cat. no. 9662), caspase-8 (cat. no. 9746), caspase-9 (cat. no. 9504; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), β-actin (cat. no. 3700; 1:2,000; Cell Signaling Technology, Inc.), B cell lymphoma-2 (Bcl-2; cat. no. ab196495) and hTERT (cat. no. ab191523; 1:1,000; Abcam) overnight at 4°C, and subsequently with horseradish peroxidase (HRP)-conjugated secondary anti-mouse (cat. no. 7076; 1:3,000,) and anti-rabbit (cat. no. 7074; 1:3,000) antibodies (both Cell Signaling Technology, Inc.). Protein signals were visualized using an enhanced chemiluminescence kit (GE Healthcare) according to the manufacturer's instructions. Anti-β-actin was used as a control sample for protein loading. Protein expression was analyzed using ImageJ software version 14.8 (National Institutes of Health, Bethesda, MD, USA).

Immunocytochemistry. The streptavidin-peroxidase conjugated method was used to detect TRAIL, caspase-8, caspase-9, caspase-3 and Bcl-2 expression in cells. SCC25 cells were grown and fixed on coverslips in 4% paraformaldehyde (PFA) and blocked with normal goat serum (cat. no. 566380; Sigma-Aldrich; Merck KGaA) containing 0.25% Triton for 15 min at room temperature. Slides were incubated with relative primary antibodies against TRAIL (cat. no. 3219), caspase-3 (cat. no. 9662), caspase-8 (cat. no. 9746), caspase-9 (cat. no. 9504; 1:400; Cell Signaling Technology, Inc., Danvers, MA, USA) and B cell lymphoma-2 (Bcl-2; cat. no. ab196495; 1:500; Abcam), respectively, for 1 h at 37°C. The slides were then rinsed with PBS three times and subsequently incubated with a biotin-labeled anti-rabbit/mouse secondary antibody (cat. no. SP-9001/9002; 1:500; OriGene Technologies, Inc., Rockville, MD, USA) for 30 min at 37°C. HRP-conjugated streptavidin (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was added to the slides before visualization of the antigen-antibody complex with 3,3' diaminobenzidine (DAB) for 15 min at room temperature.

Apoptosis assay and cell cycle assay. Results of the apoptosis assay were observed via flow cytometry performed using an Annexin V-fluorescein isothiocyanate (FITC) kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Cells were harvested, washed and resuspended with binding buffer. Cells were subsequently incubated with Annexin V-FITC then washed with cold PBS and resuspended. Finally, propidium iodide (PI) was added to mark apoptotic cells, which were then analyzed using a flow cytometer.

Cell cycle staging was also analyzed using flow cytometry. The cell suspension was fixed with 70% cold ethanol overnight at 4°C, then washed with cold PBS three times and stained with PI solution for 30 min in the dark at 37°C prior to being analyzed with a flow cytometer, to estimate the frequency of cells in the sub-G₀/G₁ phase. Each measurement was repeated in triplicate.

Table I. Reverse transcription-quantitative polymerase chain reaction primers.

Target gene	Primer sequence
TRAIL	Forward: 5'-GTTGGCACATGCCTGTAG TCC-3' Reverse: 5'-AAACCAAGTCTCGCTCTGTCC-3'
Caspase-3	Forward: 5'-GAGCTGCCTGTAACCTTG-3' Reverse: 5'-ACCTTTAGAACATTTCCACT-3'
Caspase-8	Forward: 5'-CTGCTGGGGATGGCCACTGTG-3' Reverse: 5'-TCGCCTCGAGGACATCGCT CTC-3'
Caspase-9	Forward: 5'-TTCCCAGGTTTTGTTTCCTG-3' Reverse: 5'-CCTTTCACCGAAACAGCATT-3'
Bcl-2	Forward: 5'-CAIGTGTGTGGAGAGCGTCAA-3' Reverse: 5'-GCCGGTTCAGGTACTIONACTCAGTCA-3'
hTERT	Forward: 5'-CGTCCAGACTCCGC TTCATC-3' Reverse: 5'-GAGACGCTCGGCCCTCTT-3'
β-actin	Forward: 5'-AGGGGCCGGACTCGTCATACT-3' Reverse: 5'-GGCGGCACCACCATGTACCCT-3'

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bcl-2, B cell lymphoma-2; hTERT, human telomerase reverse transcriptase.

Cell proliferation and viability assay. To evaluate the contribution of TRAIL and hTERT to OSCC cell proliferation, an MTT assay of cell proliferation and cell viability was performed. Following infection with AAV-TRAIL or shTERT virus, OSCC cells were treated with MTT following the manufacturer's procedures. The reaction was terminated using dimethylsulfoxide. Subsequently, absorbance was measured at 570 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Each transfection was measured every day in triplicate, and the percentage of viable cells were calculated.

In vivo mice xenograft assay. BALB/c nude mice (age, 4 weeks; weight, 12-16 g) were used for tumor implantation. A total of 25 male mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experiments complied with the Regulations for the Administration of Affairs Concerning Experimental Animals (25). The mice were housed in controlled conditions of 20-22°C, relatively humidity of 50-55%, and a 12-h light-dark cycle. All mice had free access to food and water.

To generate the tumor implantation mouse model, SCC25 cells were infected with empty vector, AAV-TRAIL, shTERT and AAV-TRAIL + shTERT virus. The negative control (NC) group was not infected. Then, 1x10⁶ cells in 0.1 ml were subcutaneously injected into the axilla of each mouse (n=5 mice per group). Following injection, tumor appearance in mice was inspected each week by observation and palpation for 6 weeks. The greatest longitudinal diameter (a) and the greatest transverse diameter (b) were measured weekly using a digital caliper. All

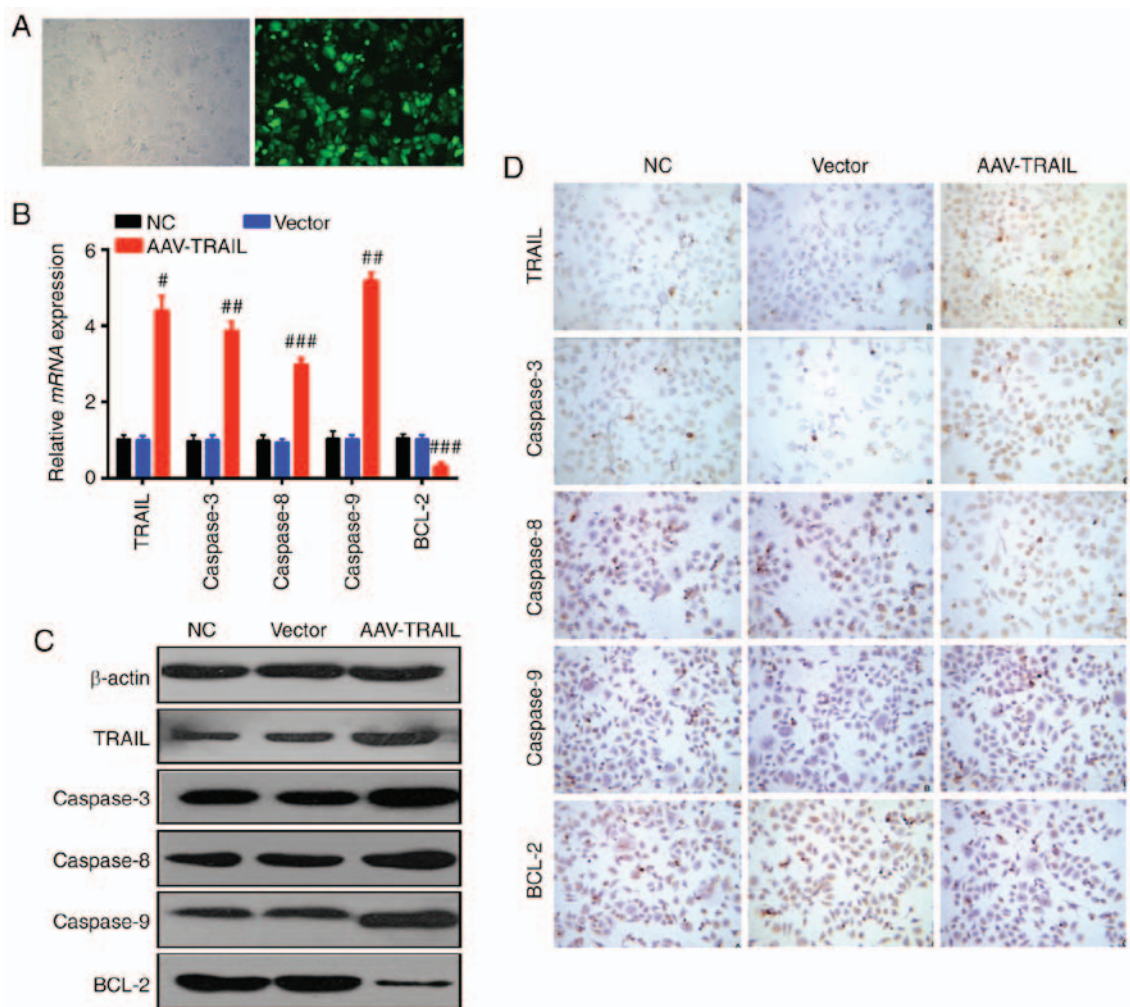


Figure 1. AAV-mediated TRAIL overexpression induced increased expression of caspase-3, caspase-8, and caspase-9, and reduced expression of Bcl-2 in OSCC cells. (A) Immunofluorescence assay was carried out to detect the infection efficiency of the recombinant AAV system in the SCC25 OSCC cell line (magnification, x400). The left panel presents light microscopy and the right field presents fluorescence microscopy. (B) Reverse transcription-quantitative polymerase chain reaction, (C) western blotting and (D) immunocytochemistry analysis (magnification, x200) were performed to measure apoptosis-related genes expression changes in SCC25 cells with upregulation of TRAIL. Data are presented as the mean \pm standard deviation. Each assay was performed independently at least three times. [#] $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs. NC. AAV, adeno-associated virus; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bcl-2, B cell lymphoma-2; OSCC, oral squamous cell carcinoma; NC, negative control.

mice were euthanized with CO₂ at the end of 6 weeks following implantation. Tumor volume was calculated using the following formula: Tumor volume = $1/2 ab^2$. Tumors were harvested, weighed and kept at -80°C for further analysis.

Immunohistochemistry. Immunohistochemistry was performed to evaluate TRAIL, caspase-3, caspase-8, caspase-9, Bcl-2 and hTERT expression in the tumors of each xenograft mice group. Tissue samples were fixed in 4% PFA at 4°C overnight, embedded in paraffin and cut into $\sim 4\text{-}\mu\text{m}$ thick sections. The slides were heated at 65°C for 30 min, deparaffinized with xylene and rehydrated with a gradient series of alcohol and rinsed in PBS, then subjected to antigen retrieval in citrate buffer (pH 6.0; 10 mM citric acid) at 95°C for 15 min in a microwave oven (Oriental Rotor Ltd., Tokyo, Japan). The slides were subsequently quenched in PBS containing 3% hydrogen peroxide at room temperature for 10 min to block endogenous peroxidase activity. Non-specific binding was blocked with PBS containing 5% BSA for 5 min at room temperature. The sections were incubated at 4°C overnight with primary

antibodies against: TRAIL, caspase-9, (1:50; Cell Signalling Technology, Inc.), caspase-3 (1:300; Cell Signalling Technology, Inc.), Bcl-2 (cat. no. AM43; 1:300; Merck KGaA), caspase-8 (cat. no. MA1-41280; 1:50; Thermo Fisher Scientific, Inc.), Ki67 (cat. no. ab16667; 1:100; Abcam), poly (ADP-ribose) polymerase (PARP; cat. no. 5625; 1:100; Cell Signalling Technology, Inc.) and hTERT (cat. no. PC563; 1:50; Merck KGaA). Slides were rinsed in PBS three times, and incubated with HRP-conjugated goat and anti-rabbit/mouse secondary antibodies with a Dako Real™ EnVision™ detection system kit (cat. no. K5007; 1:100; Dako; Agilent Technologies, Inc.). Reaction was visualized with DAB (Dako) for 5 min at room temperature and hematoxylin was used to counterstain the slides at room temperature for 1 min. The slides were observed under a light microscope (Nikon Corporation; magnification, x100).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. TUNEL staining was used for the *in situ* analysis of apoptotic cells. Tumor tissues were treated in the aforementioned manner to obtain specimen

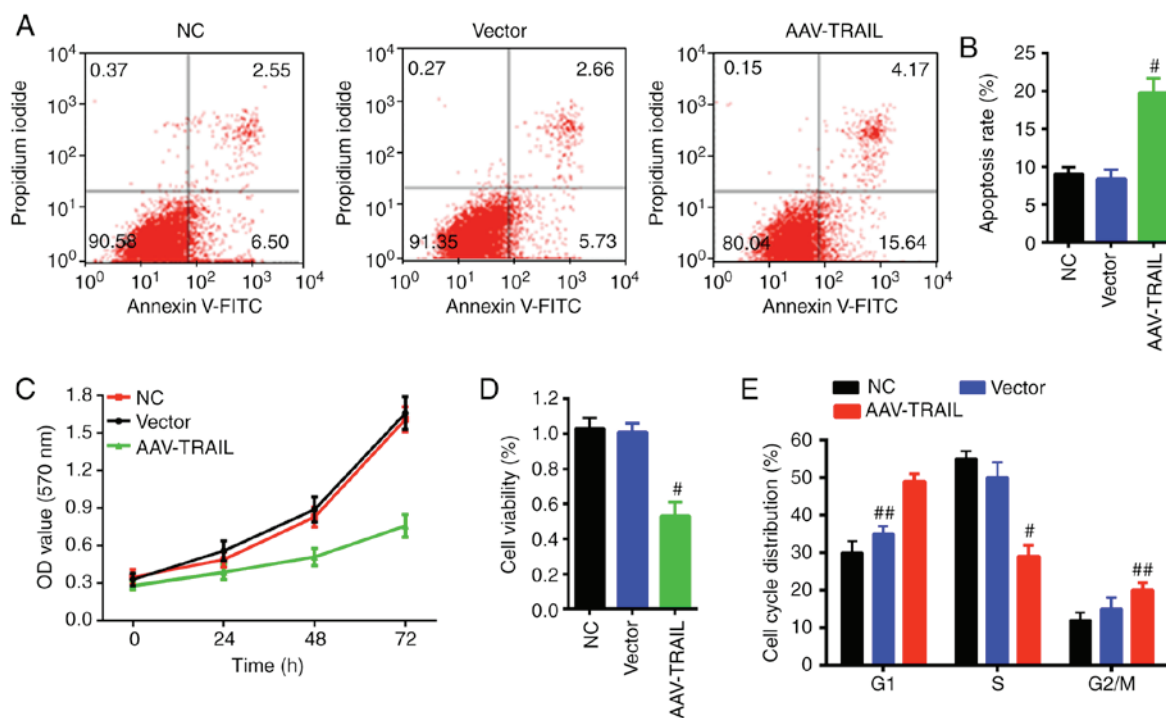


Figure 2. AAV-mediated TRAIL upregulation promoted tumor cell apoptosis, proliferation prohibition and cell cycle arrest. (A) Cell apoptosis was examined by flow cytometry in SCC-25 cells following infection with AAV-TRAIL and control. (B) The rate of apoptosis was calculated. (C) MTT assay was performed to measure the cell proliferation ability following transfection with AAV-TRAIL. (D) Cell viability and (E) cell cycle distribution were calculated to evaluate the effect and function of TRAIL. Data are presented as the mean \pm standard deviation. Each assay was performed independently at least three times. [#]P<0.05, ^{##}P<0.01 vs. NC. AAV, adeno-associated virus; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; NC, negative control; FITC< fluorescein isothiocyanate; OD, optical density.

slides from each group. TUNEL staining was performed with an *In Situ* Cell Death Detection kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. In brief, formalin-fixed, paraffin-embedded tumor tissues were cut into 5- μ m thick sections. Following heating at 65°C for 30 min, the sections were deparaffinized in xylene and rehydrated through graded ethanol. The tissue sections were rinsed in PBS and antigen retrieval was performed in sodium citrate solutions. Sections were further incubated with TUNEL reaction mixture at 37°C for 1 h in humid conditions. Coverslips were then washed in PBS and counterstained with DAPI (Sigma-Aldrich; Merck KGaA) at room temperature for 1 min. The slides were observed under the Nikon E600 fluorescence microscope (Nikon Corporation; magnification, x100).

Statistical analysis. All data are expressed as the mean \pm standard deviation, and the differences between groups were analyzed by one-way analysis of variance with Tukey's post hoc test using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Pearson's χ^2 test was also employed to compare difference among groups in univariate analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

AAV-mediated TRAIL overexpression induces OSCC expression of caspase-3, caspase-8, and caspase-9, as well as Bcl-2 suppression. As presented in Fig. 1A, a high level of EGFP expression was detected in SCC25 cells, indicating that the virus vectors constructed for the present study effectively

infected cells. To confirm the effect of TRAIL overexpression on the AAV system, RT-qPCR was carried out. Results demonstrated that in the AAV-TRAIL group, the TRAIL, caspase-3, caspase-8 and caspase-9 mRNA were increased, whereas BCL-2 mRNA was decreased, compared with the NC and vector groups (Fig. 1B). TRAIL has been well characterized as inducing tumor cells apoptosis (8,9). The expression level of several genes closely associated with apoptosis including caspase-3, caspase-8, caspase-9 and Bcl-2, were monitored by western blotting and immunocytochemistry assays. As presented in Fig. 1C, overexpression of TRAIL resulted in marked upregulation of caspase-3, caspase-8 and caspase-9, and downregulation of Bcl-2, an apoptosis suppressor gene, in comparison with the NC and vector groups. This phenomenon was also confirmed by immunocytochemistry assay (Fig. 1D).

AAV-mediated TRAIL upregulation promotes tumor cell apoptosis and suppresses cell proliferation and cell cycle arrest. To further explore the function of TRAIL in OSCC cells, an apoptosis assay was performed on SCC25 cells by flow cytometry. The apoptosis rate of the cells infected with AAV-TRAIL was significantly increased compared with the NC group (Fig. 2A and B). In addition, cell proliferation ability and viability of SCC-25 cells transduced with AAV-TRAIL were evaluated using the MTT assay. The results demonstrated that the cell proliferation and viability were prohibited with exogenous overexpression of TRAIL by the AAV system, compared with the NC group (Fig. 2C and D).

Cell cycle distribution was also analyzed to assess the effect of TRAIL on cell cycle. The results demonstrated that

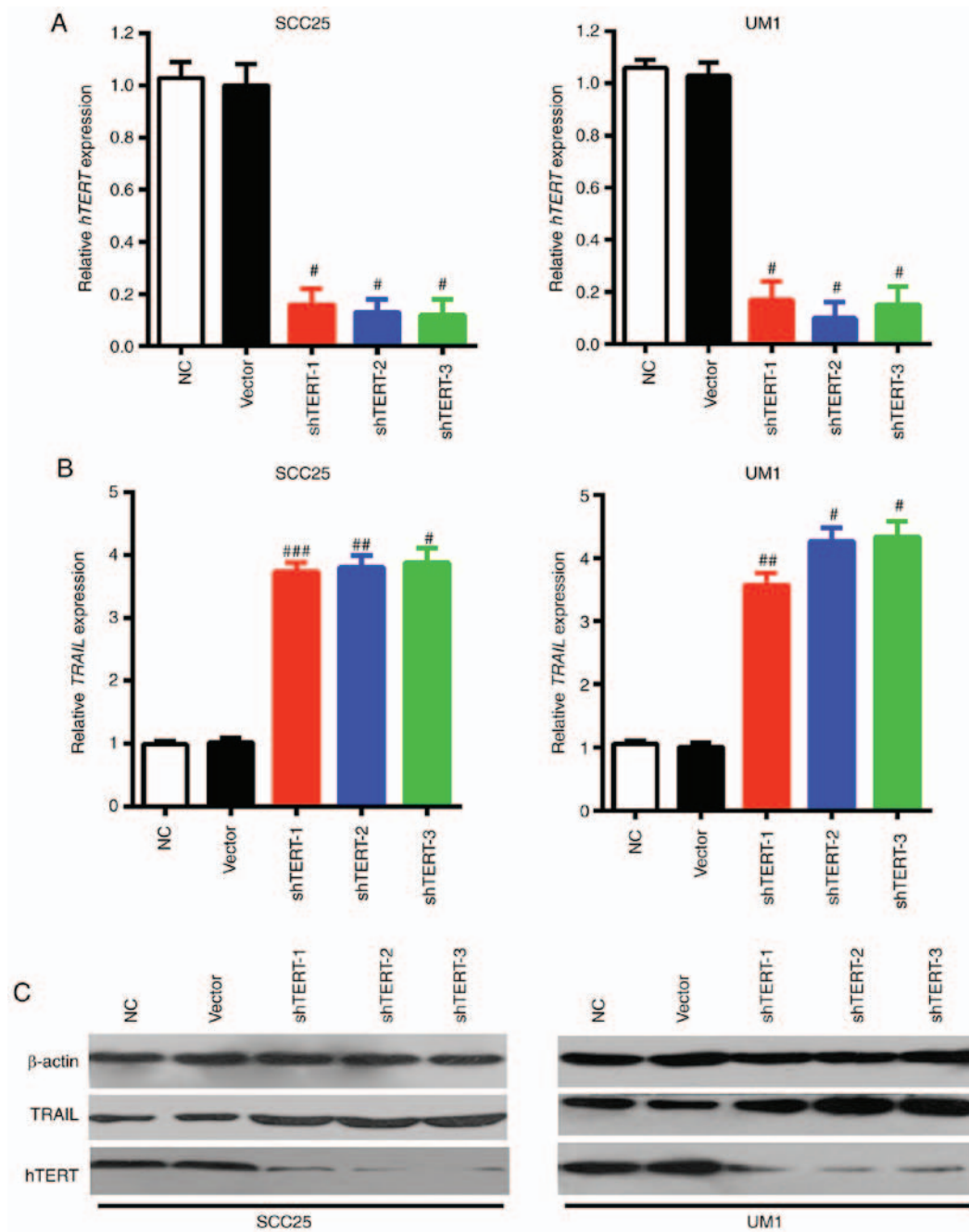


Figure 3. Lentivirus-mediated hTERT interference upregulates TRAIL expression. (A) RT-qPCR was performed to measure the lentivirus interference efficiency against hTERT in SCC25 and UM1 cells. (B) TRAIL mRNA expression changes were examined in SCC25 and UM1 cells using RT-qPCR. (C) Western blotting was performed to measure the TRAIL protein expression level changes following hTERT interference. Data are presented as the mean \pm standard deviation. Each assay was performed independently at least three times. [#] $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs. NC. hTERT, human telomerase reverse transcriptase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; sh, short hairpin RNA.

the proportion of cells in G₁ phase in the AAV-TRAIL group was higher than that of the NC and vector groups; whereas the proportion of cells in the S phase in the AAV-TRAIL group was significantly lower than that of the NC group (Fig. 2E). These results suggest that overexpression of TRAIL promotes OSCC cell apoptosis, inhibits proliferation and arrests the cell cycle.

Lentivirus-mediated hTERT interference upregulates TRAIL expression. It has been reported previously that the aberrant expression of hTERT can promote cancer progression; there

is a negative correlation between TRAIL and hTERT in multiple cancer types (26). As such, the present study aimed to clarify the association between TRAIL and hTERT in OSCC. Initially, three lentivirus vectors targeted to different fragments of hTERT were constructed to interfere with hTERT gene expression. Then, the interference efficiencies were measured in SCC25 (epithelial) and UM1 (mesenchymal) cells, via RT-qPCR and western blotting. Results of these experiments revealed that all three shRNAs significantly inhibited hTERT expression, when compared with the NC group, in SCC25 and UM1 cells (Fig. 3A). TRAIL expression changes were further

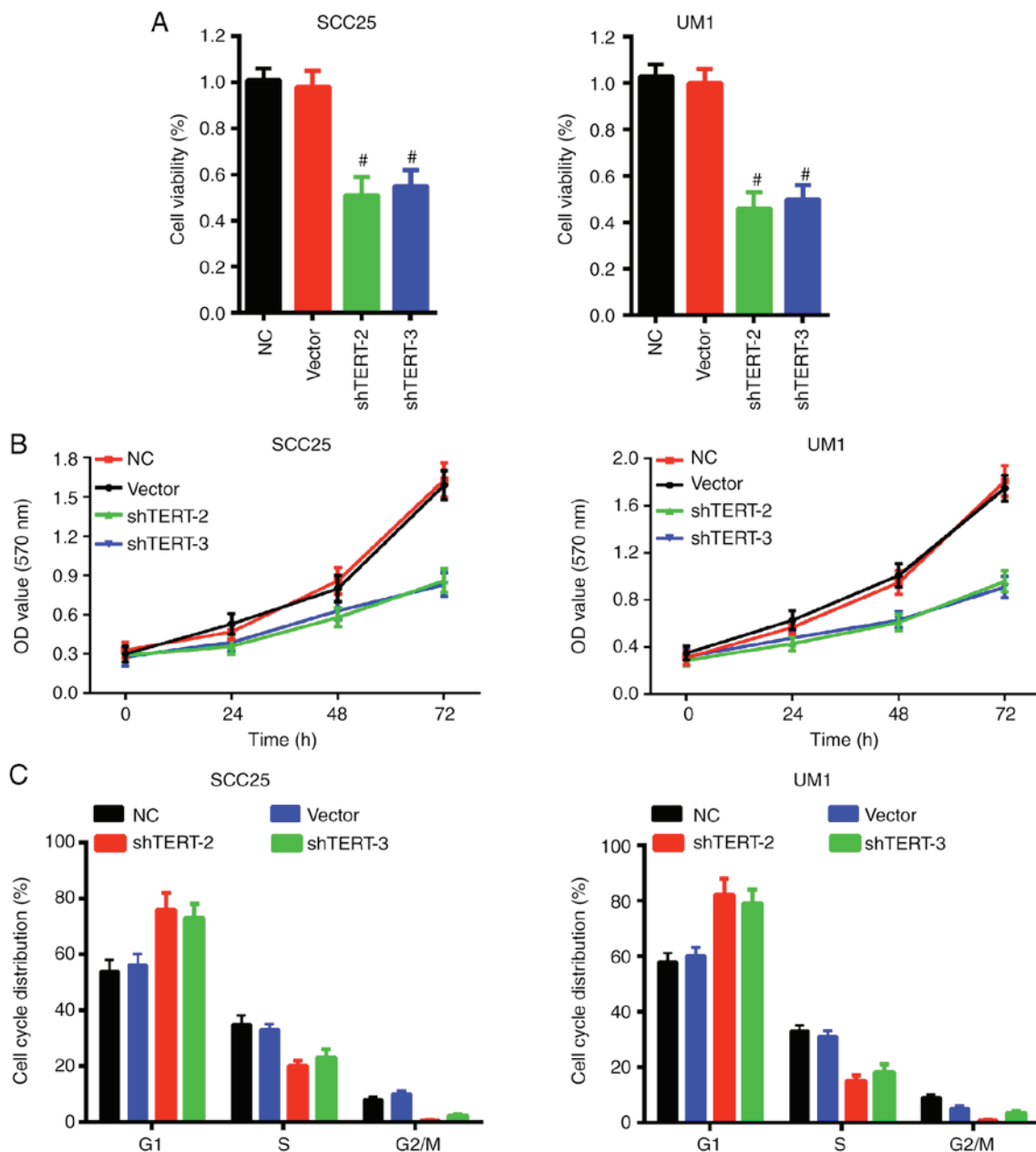


Figure 4. Silencing of hTERT expression impaired oral squamous cell carcinoma cell viability, proliferation and cell cycle arrest. (A) Cell viability was determined by MTT assay following silencing of hTERT in SCC-25 and UM1 cells. (B) Cell proliferation ability was measured by MTT assay following silencing of hTERT in SCC25 and UM1 cells. (C) Cell cycle distribution was analyzed following silencing of hTERT in SCC-25 and UM1 cells. Data are presented as the mean \pm standard deviation. Each assay was performed independently at least three times. [#] $P < 0.05$ vs. NC. hTERT, human telomerase reverse transcriptase; NC, negative control; sh, short hairpin RNA; OD, optical density.

detected via silencing hTERT through shRNAs with RT-qPCR and western blotting. The results demonstrated that mRNA and protein expression of TRAIL was elevated following knocking down hTERT expression in both SCC25 and UM1 cells (Fig. 3B and C). These results suggest that attenuation of hTERT expression upregulates TRAIL expression.

Silencing of hTERT expression impairs OSCC cell viability, proliferation and cell cycle arrest. We further investigated cell viability and proliferation ability with lentivirus-mediated hTERT silencing using flow cytometry and MTT assay. The results indicated that silencing hTERT significantly impaired cell viability in SCC25 and UM1 cells (Fig. 4A). The MTT

assay also demonstrated that knockdown of hTERT expression prohibited OSCC cell proliferation in SCC25 and UM1 cells (Fig. 4B). Furthermore, the cell cycle was also analyzed to evaluate the hTERT silencing effect. The results demonstrated that knockdown of hTERT markedly increased the proportion of cells in the G₁ phase, but decreased the proportion of cells in the S phase in SCC25 and UM1 cells (Fig. 4C). These results indicate that inhibition of hTERT activity regulates cell cycle distribution, inhibits the proliferation of tumor cells and promotes apoptosis.

Combination of AAV-TRAIL and hTERT-shRNA suppresses OSCC xenograft growth in BALB/c nude mice. A BALB/c nude

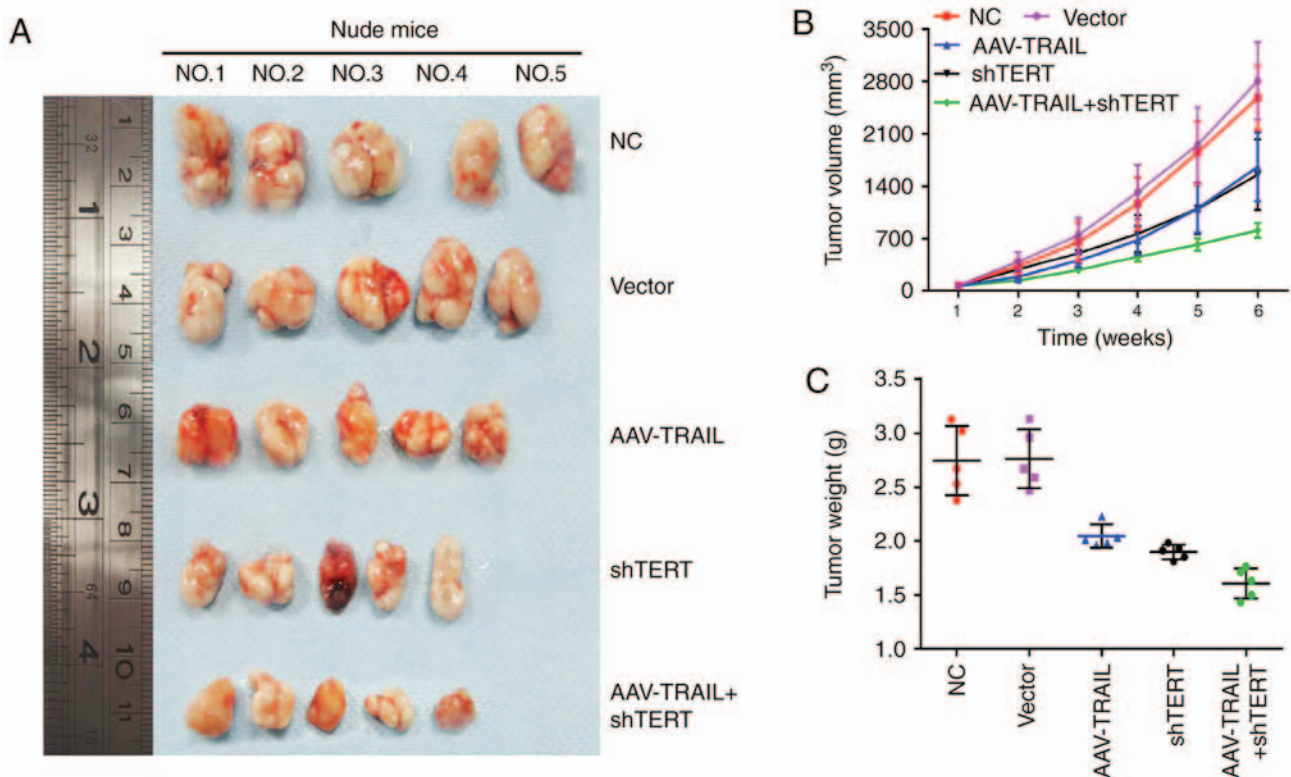


Figure 5. Combination treatment with AAV-TRAIL and hTERT-shRNA suppressed oral squamous cell carcinoma xenografts growth in nude mice. SCC25 cells transfected with negative control, empty vector, AAV-TRAIL, shTERT and AAV-TRAIL+shTERT were inoculated into the axilla of BALB/c nude mice (n=5 per group). (A) Images of the tumors harvested from all mice in each group. (B) Tumor volume and (C) tumor weight were analyzed. Data are presented as the mean \pm standard deviation. Each assay was performed independently at least three times. AAV, adeno-associated virus; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; hTERT, human telomerase reverse transcriptase; shRNA/sh, short hairpin RNA; NC, negative control.

mouse xenograft model was used as an *in vivo* model to verify the tumor suppression effects of AAV-mediated TRAIL overexpression combined with lentivirus vector-mediated hTERT silencing in OSCC. The five groups of SCC25 cell lines (NC, vector, AAV-TRAIL, shTERT and AAV-TRAIL+shTERT) were subcutaneously implanted in the axilla of the mice. Xenograft growth curves were plotted according to the tumor volume changes (Fig. 5A and B). As presented on the respective plots, there was a marked reduction in tumor size and tumor weight in the AAV-TRAIL and shTERT group, compared with the NC and vector groups (Fig. 5B and C). Notably, there was a marked suppression of tumor growth in the AAV-TRAIL+shTERT combination group, compared with the NC and vector groups (Fig. 5).

Combination of AAV-TRAIL and hTERT-shRNA promotes OSCC xenograft apoptosis in BALB/c nude mice. To confirm these *in vivo* results, immunohistochemistry was used to detect the combination effect on apoptosis in mouse tumor tissues from each subgroup. The results demonstrated that compared with the NC and vector groups, AAV-TRAIL group has a higher level of TRAIL and PARP expression, as well as lower expression of hTERT and Ki67. The shTERT group also exhibited higher expression of TRAIL and PARP, and lower expression of hTERT and Ki67, compared with the negative control and vector groups. Notably, the TRAIL+hTERT group exhibited the highest expression of TRAIL and PARP, and the lowest expression of hTERT and Ki67. These results suggest

that silencing hTERT upregulates TRAIL expression and results in promotion of apoptosis and inhibition of proliferation in OSCC. It is also clear that shTERT has a synergistic effect on TRAIL-induced OSCC apoptosis enhancement and proliferation suppression in a trial of combined AAV-TRAIL and shTERT treatment (Fig. 6A). Furthermore, this effect was further verified via TUNEL assay in xenograft tissues (Fig. 6B).

The present findings indicate that lentivirus-mediated TERT inhibition synergistically promotes TRAIL-induced OSCC apoptosis enhancement and proliferation suppression *in vitro* and *in vivo*.

Discussion

In the present study, TRAIL expression was upregulated and hTERT expression was inhibited experimentally using a stable AAV-mediated system and a lentivirus-mediated interfering system in OSCC cells. The upregulation of TRAIL activated apoptosis, suppressed proliferation and induced cell cycle arrest in OSCC cells *in vitro*. Additionally, silencing hTERT expression triggered elevation of TRAIL expression and displayed the same effects. Finally, the upregulation of TRAIL and suppression of hTERT potentially inhibited the growth of xenograft OSCC tumors in BALB/c nude mice; with a combination of TRAIL overexpression and hTERT silencing exhibiting the most potent suppressive effect on tumor growth.

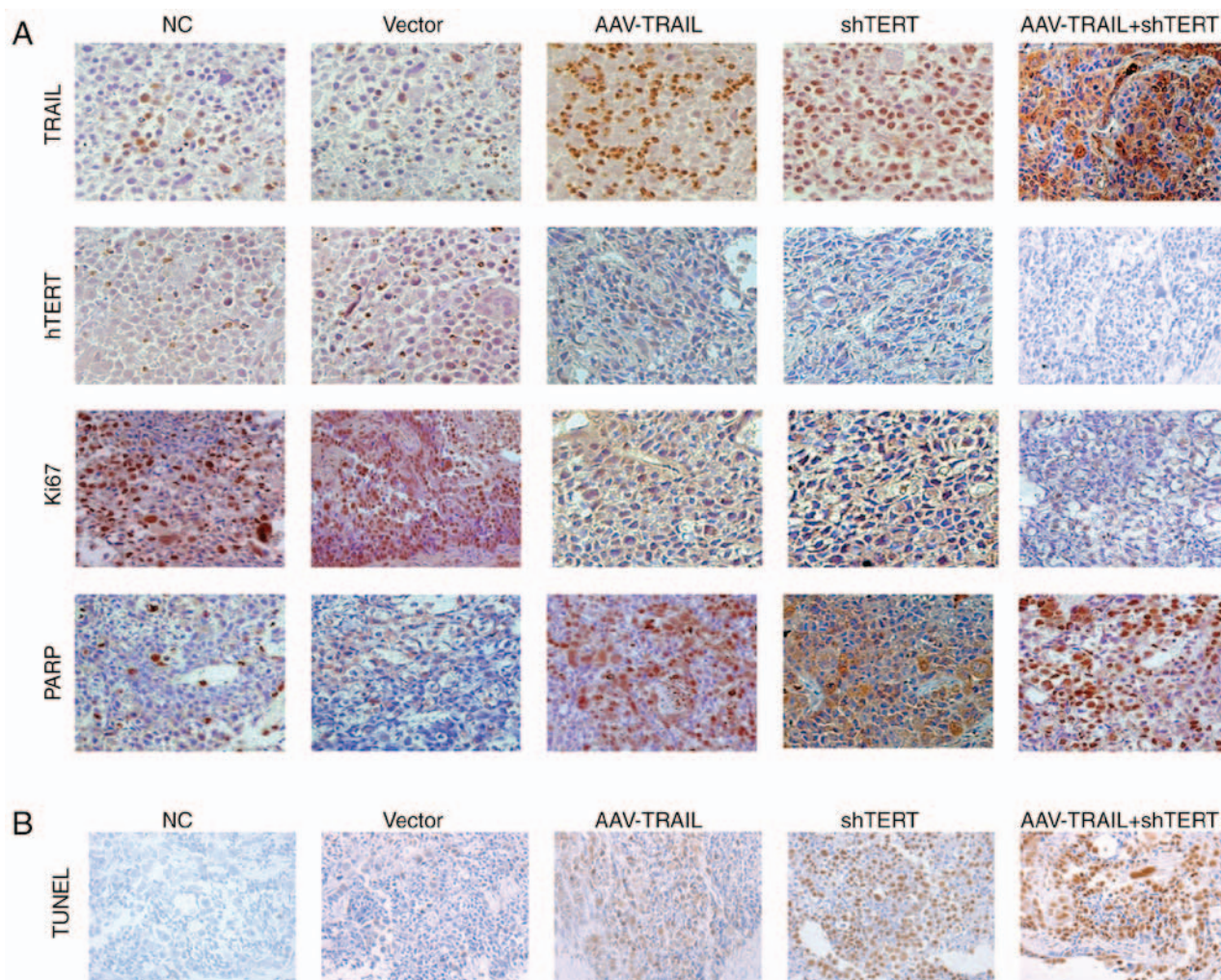


Figure 6. Combination treatment with AAV-TRAIL and hTERT-shRNA promoted oral squamous cell carcinoma xenograft apoptosis in BALB/c nude mice. (A) Immunohistochemistry assay was performed to analyze the apoptosis and proliferation related gene expression changes in mice xenograft tumors (magnification, x400). (B) TUNEL assay was also used to measure the apoptosis alternation in xenograft mice (magnification, x200). AAV, adeno-associated virus; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; hTERT, human telomerase reverse transcriptase; shRNA/sh, short hairpin RNA; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; NC, negative control; PARP, poly (ADP-ribose) polymerase.

Dysregulation of apoptosis-related genes is a pivotal event during carcinogenesis and has been identified as a critical mechanism in cancer development (27). The TRAIL gene efficiently induces apoptosis in multiple types of tumor cells while sparing normal cells, making TRAIL an ideal candidate for cancer gene therapy (28,29). TRAIL induces malignant tumor cell apoptosis by binding to the TRAIL receptors, such as death receptors DR4 and DR5 (15,30). This interaction activates the signal pathway for apoptosis, including Bcl-2, PARP and the caspase cascade (31). Caspase-8 activation, initiated via cleaving of pro-apoptotic protein, induces a downstream expression cascade caspase-9 and caspase-3. Caspase-3 is the ultimate effector protein that directly triggers apoptotic process (32). The downstream signal of the Bcl-2 pathway accompanied with upregulation of cleaved PARP activates caspase-3 to directly induce apoptosis in certain types of malignant tumors, such as HCC (33,34). In the present study, the expression of TRAIL triggered the apoptosis process and pathway, including the upregulation of caspase-8, caspase-9 and caspase-3, as well as the inhibition of Bcl-2.

The catalytic subunit of telomerase, hTERT, can reverse the telomere shortening process. The abnormal elevation of hTERT expression and activation is a common event that causes chemoresistance and increased proliferative ability in tumor cells (35-37). Previous large studies have demonstrated that hTERT has a pivotal role in cancer tumorigenesis, growth, migration and invasion (38,39). The upregulation of hTERT is positively correlated with metastasis and poor prognosis in many types of malignant tumors, including HCC, ovarian cancer and colorectal cancer (37,40,41). Therefore, hTERT is a potential therapeutic target for cancer treatment, and elucidating the underlying regulatory mechanisms of hTERT is essential.

In previous studies, TRAIL has been administered in combination with chemotherapy drugs, such as doxorubicin or flavonoids (42-44). Several studies have suggested an association between hTERT and TRAIL, which has demonstrated that downregulation of hTERT by RNA interference (RNAi) technology significantly improves the sensitivity of HCC cells to TRAIL treatment, including TRAIL-resistant cells (35,45-47). An underlying mechanism is that knockdown

of hTERT can increase cell apoptosis in TRAIL-treatment cells via the mitochondrial type II apoptosis pathway and telomerase-dependent pathway (47,48). In the present study, a regulatory association between hTERT and TRAIL was demonstrated; that lentivirus-mediated interference of hTERT expression can significantly upregulate the mRNA and protein expression of TRAIL. In addition, the present study provided, to the best of our knowledge, the first evidence that downregulated hTERT and overexpressed TRAIL cells exhibit reduced cell viability and increased cell apoptosis *in vivo* and *in vitro*, indicating that interfering with hTERT expression may benefit the therapeutic effect of TRAIL in OSCC. Another previous study regarding the association between hTERT and TRAIL revealed that AAV-mediated TRAIL gene expression driven by hTERT promoter has an inhibitory role in HCC growth in mice, which revealed the clinical application of hTERT promoter-driven TRAIL gene therapy (26). In contrast with this therapeutic strategy, the present study suggested a combination use of hTERT knock-down and TRAIL overexpression to enhance the effect of OSCC treatment.

Consistent with previous results, the present results also confirmed that silencing of hTERT increases TRAIL expression and promotes the anti-tumor effect induced by TRAIL in OSCC cells (49). This effect was also verified via *in vivo* xenograft nude mice experimentation. The combination of AAV-TRAIL and shTERT demonstrated a synergistic effect in the suppression of tumor growth and overall tumor apoptosis compared with groups treated with a single gene therapy target.

The present results also provide a rationale for a combined virotherapy strategy to treat OSCC. The downregulation of induced myeloid leukemia cell differentiation protein Mcl-1 has been demonstrated to sensitize hTERT-adenovirus in TRAIL-mediated apoptosis in HCC (50). Another previous study reported that hTERT RNAi overcomes TRAIL resistance via the mitochondrial type II apoptosis pathway and the telomerase-dependent pathway (47,48). Therefore, the main limitation of the present study is that the interaction between TRAIL and hTERT was not evaluated, and the molecular mechanism that regulates OSCC cell apoptosis promotion and growth inhibition was not elucidated. Accordingly, further investigation must be performed to clarify the underlying interaction mechanisms between TRAIL and hTERT that synergistically regulate OSCC progression *in vitro* and *in vivo*.

Together, the present results indicate that AAV-mediated TRAIL expression and lentivirus-mediated hTERT suppression display anti-tumor effects induced by TRAIL, including apoptosis promotion, proliferation inhibition and cell cycle arrest in OSCC both *in vitro* and *in vivo*. In addition, hTERT interference was demonstrated to effectively upregulate TRAIL expression. Furthermore, treatment with a combination of TRAIL overexpression and hTERT interference has enhanced anti-tumor effects, inhibiting tumor cell proliferation and increasing apoptosis in a xenograft model. This suggests that there is a synergistic effect between TRAIL and hTERT. The present results indicate that the delivery of AAV-TRAIL and shTERT in combination may be an effective treatment strategy for OSCC gene therapy.

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Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

CZ and JS carried out the viral vector construction and data analysis. XinZ and ZL performed animal experiments. SZ carried out the reverse transcription-quantitative polymerase chain reaction and western blotting analyses. XinZ wrote the manuscript. JW and XiaZ conceived the present study and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee at Sun Yat-sen Memorial Hospital (Sun Yat-sen University, Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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