Specific antitumor effect of adeno-associated virus vector carrying TRAIL gene under the control of hTERT promoter

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Background and Objective: Adeno-associated virus (AAV) has been widely used in tumor gene therapy. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a safe and potent anti-tumor gene which could induce apoptosis of many tumor cells. This study was to use the tumor-specific promoter hTERT to construct AAV-hTERT-TRAIL, and explore the antitumor effect and mechanism of AAV-hTERT-TRAIL in vitro. Methods: Purified AAV-hTERT-TRAIL was obtained after co-transfection of HEK293 with pAAV-hTERT-TRAIL and two other help plasmids. After infection of AAV-hTERT-TRAIL into three tumor cell lines, SW620, HepG2, A549 and two normal cell lines, NHLF and MRC5, the expression of TRAIL was detected by RT-PCR, Western blot and immunohistochemistry (IHC); the influence of AAV-hTERT-TRAIL infection on cell proliferation was evaluated using MTT assay. Activation of caspase-3 and PARP was measured by western blot. Cell apoptosis was assessed using ELISA and flow cytometry. Results: AAV-hTERT-TRAIL was successfully packaged in HEK293 cells. After AAV-hTERT-TRAIL infection, specific expression of TRAIL was detected in three tumor cell lines, but not in two normal cell lines. Cell proliferation rates in SW620, A549, HepG2, NHLF and MRC5 cells were 41.55%, 44.29%, 49.95%, 84.59% and 87.22%, respectively after transfection of AAV-hTERT-TRAIL at a multiplicity of infection (MOI) of 100 for 96 h. AAV-hTERT-TRAIL activated caspase-3-dependent apoptotic pathway and induced apoptosis in tumor cell lines, but not in normal cell lines. Conclusions: hTERT increases selectivity and safety of the AAV vector. hTERT promoter controls the expression of anti-tumor genes to specifically induce the death of tumor cells.

Adeno-associated virus (AAV) is a member of the Parvoviridae family. As a gene therapeutic vector, AAV has many advantages over adenovirus and retrovirus, first of which is its low pathogenicity. AAV induces only mild immune responses. Secondly, AAV is a kind of deficient virus which can only integrate into the genome of host cells as an “incubative” infection without the help of helper viruses. Thirdly, integration of AAV at specific genomic sites makes the long-standing existence and stable expression of foreign genes possible. Fourthly, the host cells of AAV are varied from divided cells to non-divided cells.1-4 Although AAV has been widely applied in gene therapy, it poor tumor-targeting ability causes harm to normal cells. Therefore, controlling the expression of foreign genes to make them expressed specifically in tumor cells, but not in normal cells is a pressing issue.

Telomerase is a common tumor marker, which is important in immortalization, evolution and progression of tumors.5 Telomerase is inactive in most of normal cells, but highly active in about 90% of tumor cells. The activity of telomerase is found to be related to the degree of malignancy.6-8 Human telomerase reverse transcriptase (hTERT), the core component of telomerase, is the speed limit enzyme controlling telomerase activity. The expression of hTERT is mainly regulated at the transcriptional level, which is up-regulated in about 90% tumor cells, but are not detected in resting cells.9 Thus, hTERT promoter has been widely used to enhance the targeting ability of tumor gene therapy.10,11 An AAV vector with TRAIL gene controlled by the hTERT promoter (AAV-hTERT-TRAIL) was constructed in this study. TRAIL, a type of tumor necrosis factor, is a type II transmembrane protein, which can rapidly induce apoptosis of various tumor cells through both mitochondria-dependent and mitochondria-independent pathways. Moreover, TRAIL does not show significant toxicity on most normal cells. Therefore, TRAIL is considered to be a safe and promising anti-tumor agent and is widely used in gene therapy.12,13 It is reported that an AAV vector carrying a TRAIL gene could induce apoptosis of human lung adenocarcinoma cells and inhibit tumor growth in a nude mouse model.14 Oral administration or intra-splenic injection of AAV carrying TRAIL could inhibit cell growth in human hepatocellular carcinoma SMMC-7721 cell xenografts in nude mice.15 However, the hepatotoxicity induced by TRAIL is the bottleneck that prevents the application of TRAIL in clinic research.16 Due to its low activity in normal hepatic tissues, hTERT

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can be utilized to decrease hepatotoxicity of gene therapy in liver. The tumor targeting AAV vector carrying TRAIL gene under the control of hTERT promoter (AAV-hTERT-TRAIL) was constructed and assessed in this study in normal and tumor cells.

Materials and Methods

Materials. Reagents. The following agents were obtained commercially: the restriction endonuclease and T4 DNA ligase (MBI Company), KOD enzyme (TOYOBA Company), and rTaq (TaKaRa Company). The DNA purification kit, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and Trizol were purchased from the Gibco BRL Company. MMLV cDNA single chain synthesis kit was purchased from Shanghai Sangon Company; Bradford Protein Assay Kit was purchased from Beyotime Company; the other reagents were all analytical reagents obtained in China or abroad. The primers were synthesized at Shanghai Sangon Company.

**Bacteria and plasmids.** DH5α were preserved at our laboratory. AAV Helper-free system, including pAAV-MCS (ITR containing vector), pAAV-RC (carrying AAV-2 replication and capsid genes), and pHelper (carrying adenovirus-derived genes) were gifted by Professor Huizhen Sheng of the Developmental Biology Laboratory of the Chinese Academy of Sciences. The pAd/hTERT-TRAIL containing hTERT 5′ end and TRAIL cDNA sequences, pCA13 and pEGFP–C1 were stored at our lab.

**Cell lines and cell culture.** The human embryonic kidney cell line HEK293, containing the Ad5 E1 region, was purchased from the ATCC Company, and was maintained in DMEM containing 10% FBS. The cell lines below were all purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology. The human colon cancer cell line SW620, human hepatocellular carcinoma cell line HepG2, and human lung cancer cell line A549 were maintained in DMEM containing 2.5% FBS; the human lung embryonic cell line MRC5, and human lung fibroblasts NHLF were maintained in DMEM containing 10% FBS. All cells were cultured at 37°C with 5% CO2.

**Methods. Construction of the AAV vector.** The hTERT-TRAIL fragment was amplified by PCR using pAd/hTERT-TRAIL as the template. The 5′ end sequence of hTERT and the 3′ end sequence of TRAIL were used to amplify hTERT-TRAIL. Then the fragment was cloned into double-enzyme digested pAAV-MCS to obtain pAAV-hTERT-TRAIL. The EGFP was amplified by PCR from the template of pEGFP–C1. The sequence of TRAIL was deleted by double-enzyme digestion from pAAV-hTERT-TRAIL to obtain pAAV-hTERT. pAAV-hTERT-EGFP was constructed by inserting EGFP obtained after double-enzyme into pAAV-hTERT. All the constructed plasmids were identified by sequencing.

**AAV packaging.** AAV packaging was performed following the protocol below. Three million HEK293 cells were plated in 100 mm dishes and incubated in 10 mL complete medium in an atmosphere containing 5% CO2 at 37°C for two days until the cell confluence reached approximately 80–90% and were ready for transfection. pAAV-hTERT-TRAIL/pAAV-hTERT-EGFP, pAAV-RC and pHelper were diluted to 1 mg/mL with sterilized TE buffer (pH7.5). Ten micrograms of each of the above three plasmids was added to a 15 mL centrifuge tube, mixed gently with 1 mL of 0.3 mol/L CaCl2; 1 mL of 2×HBS was added to another 15 mL centrifuge tube, gently mixed with gradually added 1.03 mL DNA/CaCl2 Solution. The above mixture was immediately added to HEK293 cells, and then the dish was shaken gently to distribute the mixture evenly. After cells were incubated in the incubator at 37°C for 6 h, medium was replaced with 10mL fresh medium and incubated at 37°C for 66–72 h.

**AAV purification and titer quantification.** When HEK293 cells became round, detached from the dish, floated in the medium, and the medium changed from orange to yellow, the AAV was ready to be purified. Briefly, the HEK cells and medium were collected and frozen at -70°C for 10 min, warmed and vortexed at 37°C for 5 min. These freezing and thawing procedures were repeated three times, then the supernatant was collected by centrifugation at 1000 x g for 5 min at 4°C. An equal volume of chloroform was added and mixed by vortex. NaCl powder was added to reach the final concentration of 1 mol/L. The supernatant was collected by centrifugation at 11,000 x g for 15 min at 4°C, then PEG8000 was added to reach the final concentration of 10%. After incubation on ice for 1 h, the mixture was centrifuged at 11,000 x g for 15 min at 4°C. The supernatant was discarded, and the pellet was then re-suspended in 100 µL AAV store buffer (50 mmol/L HEPES; 150 mmol/L NaCl pH 7.4, filtered) with addition of DNase and RNase (1/10 V, final concentration: 1 µg/mL). After incubation at room temperature for 30 min, the mixture was extracted with an equal volume of chloroform, then centrifuged at 11,000 x g for 15 min at 4°C. The supernatant collected under aseptic conditions was the purified virus liquid. The virus liquid was stored at -70°C for later use. The virus liquid was digested with protease K at 50°C for 1 h and inactivated at 95°C for 20 min. The virus titer was quantified by real-time PCR analysis. PCR primers were as follows: (sense) 5′-CCG AAG GTA ACT GGC TTC AGCAG-3'; (antisense) 5'-GCT GTA GGT ATC TCA GTT CGGTG-3'. The amplification conditions were 50°C for 2 min, 95°C for 10 min (one step), 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec (three steps), in total 44 cycles. A serial of two-fold-diluted pAAV-MCS were adopted as the standard. The Opticon MonitorTM analysis software was used to analyze the data after the reaction.

**RT-PCR.** The total RNA was extracted with Trizol according to the protocol below. After cells were infected with virus for about 48 h, the medium was aspirated, and then 1 mL Trizol was added per million cells. The purified RNA was dissolved in DEPC-H2O and quantified. Two micrograms of RNA were used to perform reverse transcription to cDNA. The RT-PCR product was analyzed by 1% agarose gel electrophoresis. After staining with EB, the gel was placed under a UV lamp and photos were taken.

**Western blot.** After being infected by the virus, cells were collected and washed twice with PBS, then lysed with NP40 lysis buffer; the protein was quantified with Bradford quantification kit. Loading buffer (6×SDS) was added and mixed. Each well was loaded with 20 µg protein sample to run the SDS-PAGE and protein was transferred to PVDF membrane afterwards. After being blocked, the membrane was incubated with the primary antibody for 2 h, then washed with PBST three times and incubated with the secondary antibody for 1 h. After being washed with PBST three times, the membrane was incubated with ECL reagent (Pierce Company). Anti-TRAIL (Santa Cruz Company, 1:500), anti-caspase (Santa Cruz Company, 1:1000), anti-PARP (Santa Cruz Company, 1:1000) were used.
**Immunohistochemistry.** SW620 cells were seeded in a six-well plate coated with glass slides. The cells were stained using the ABC immunohistochemical method after infection of virus at a multiplicity of infection (MOI) of 100 for 48 h. The cells were fixed with 4% paraformaldehyde, washed twice with PBS, then blocked with the inhibitor of endogenous peroxide enzyme (200 μL) for 10–20 min. After being washed with PBS for three times, cells were incubated with serum for 60 min. After a rinse with PBS for three times, cells were incubated with the primary antibody at room temperature for 90 min, then with biotinylated secondary antibody for 30 min. After another wash, cells were incubated with AP labeled streptavidin for 20 min. Subsequently, the slides were developed and counterstained with hematoxylin, sealed and photographed. All the reagents were purchased from Shanghai Xitang Technologies Company.

**MTT assay.** NHLF, MRC5, SW620, HepG2, A595 were seeded on 96-well plates at 5000 cells/well. After cells were infected by virus for 24, 48, 72, 96 h, 40 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h, followed by addition of 150 μL cell lysis buffer (10% SDS, 50% N,N'-dimethylformamide). The absorbance value \((A_{595\text{nm}})\) was detected using an enzyme-labeling instrument 450 (Bio-Rad) at 595 nm, and the blank well was used to adjust zero. Proliferation ratio = \(\frac{A_{595\text{value of cells infected by virus}}}{A_{595\text{value of untreated cells}}} \times 100\%\).

**ELISA.** Cell Death Detection ELISA PLUS was purchased from ROCHE Company (Cat. No. 1192685001) to detect apoptosis. After SW620 cells were infected, the medium was discarded and cell lysis buffer was added to lyse cells. Samples (20 μL) were added into anti-histone antibody coated plates, and incubated with HRP labeled anti-DNA antibody. After incubation at room temperature for 2 h, the plate was washed three times and ABTS was added to develop color. The absorbance value \((A_{405\text{nm}})\) was detected using enzyme-labeling instrument 450 at 405 nm. The blank well was used to adjust zero.

**Flow cytometry.** The treated cells were collected and washed with PBS, and then fixed in 70% ethanol overnight at 4°C. The fixation buffer was discarded after centrifugation. After PBS wash, the cells were re-suspended and 100 μL RNase (0.5 mg/mL) was added to obtain a final concentration of 50 μg/mL. Cells were incubated at 37°C for 30 min, then 400 μL PI staining buffer (100 μg/mL PI, 1% Triton-X100, 0.9% NaCl) was added to obtain a final concentration of 50 μg/mL. Cells were incubated away from light for 30 min before FACS assay. The standard program was set on FACS detected the intracellular apoptosis. CellQuest (Version: Mac V1.01) was applied to analyze data.

**Results**

**Plasmid construction and virus packaging.** The genomic structure of pAAV-hTERT-TRAIL and pAAV-hTERT-EGFP are shown in Figure 1A. The constructed plasmids were identified by PCR. A 798bp fragment amplified from pAAV-hTERT-EGFP, and a 846bp fragment amplified from pAAV-hTERT-TRAIL are shown in Figure 1B.

The HEK293 cells were transfected with the above identified plasmids, pAAV-RC and pHelper to package the virus. The cells became round, detached from the bottom, and some of which even floated in the medium after 66–72 h. The medium color also changed from orange to yellow (Fig. 2). The cells and supernatants were collected to purify the recombinant AAV virus. The virus titer was quantified with real-time PCR.

![Figure 1](image1.png)

![Figure 2](image2.png)
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Tumor specific expression of TRAIL mRNA. Normal cell lines NHLF, MRC5 and tumor cell lines SW620, A549, and HepG2 were infected with 100MOI AAV- hTERT-TRAIL in the experimental groups, while the same MOI of AAV- hTERT-EGFP was used in the control groups. After transfection for 48 h, the mRNA expression of TRAIL was obviously appeared in the three tumor cell lines, but not in normal cell lines (Fig. 3).

Tumor specific expression of TRAIL protein. Normal cell lines NHLF, MRC5 and tumor cell lines SW620, A549, and HepG2 were infected with 100MOI AAV- hTERT-TRAIL in experimental groups, and with AAV- hTERT-EGFP in control groups. After infection for 48 h, TRAIL protein remarkably appeared in SW620, A549, and HepG2 cells, but not in normal cell lines (Fig. 4). This result was further confirmed by immunohistochemistry in SW620 cells at 48 h. No TRAIL expression appeared in cells infected with AAV-hTERT-EGFP (Fig. 5).

Tumor specific cytotoxicity. As shown in Figure 6, AAV- hTERT-EGFP infection did not induce cell death in either tumor or normal cells, indicating that recombinant AAV was a safe vector. AAV- hTERT-TRAIL infection significantly inhibited the proliferation of tumor cells in a time-dependent manner, but did not apparently inhibit the proliferation of normal cells. After 96 h of infection with AAV- hTERT-TRAIL, the proliferation rates of SW620, A549, HepG2, NHLF and MRC5 were 41.55%, 44.29%, 49.95%, 84.59% and 87.22% respectively comparing with untreated cells, indicating that the cytotoxicity induced by AAV- hTERT-TRAIL was tumor specific.

Apoptosis induced by AAV- hTERT-TRAIL. The main substrate of Caspase 3 is 116kD poly-ADP-ribose polymerase (PARP), which is cleaved into two fragments (31kD and 85kD) when apoptosis starts. After infection with AAV- hTERT-TRAIL, pro-Caspase 3 was gradually weakened in SW620 cells, with the appearance of the 85kD subunit of PARP at 48 h (Fig. 7).

Another feature of apoptosis is the appearance of nucleosome, the compact complex formed by DNA and histones, which can be detected with the ELISA method using the monoclonal antibodies against DNA and histone. Results showed that AAV- hTERT-TRAIL, but not AAV- hTERT-EGFP induced apoptosis in SW620 cells in a dose-dependent manner, indicated by the increase of the absorbance value (Fig. 8).

FACS data also showed that, after treating SW620 at MOI 100 for 48 h, AAV- hTERT-EGFP did not induce apoptosis apparently, while AAV-hTERT-TRAIL induced 28.95% of cell apoptosis (Fig.9).

Discussion

When viruses are widely applied in gene therapy, the safety becomes the major concern. There are two methods to enhance the specificity of the use of the virus. One is to change the tropism of the virus through altering its coat protein, so that it can target at different cell surface receptors; the other one is to use the specific promoter/ enhancer systems to regulate the specific expressions of foreign genes carried by the virus. This study shows that hTERT effectively mediated TRAIL gene to be specifically expressed in tumor cells, but not in normal cells. TRAIL gene carried by AAV induced tumor specific cytotoxicity in a time- and dose-dependent manner, while the vector was safe. Moreover, apoptosis was the main pathway that the virus killed tumor cells.

The reasons why we chose TRAIL are as follows: (1) TRAIL could induce apoptosis of various tumor cells; (2) TRAIL could also induce the bystander effect, which could result in the death of not only the infected cells, but also those surrounding, uninfected cells, thus to increase the tumor killing effect.18 This study adopted the tumor specific promoter-hTERT to regulate the TRAIL expression, which could theoretically avoid the hepatotoxicity of TRAIL and enhance the safety of gene therapy. Further experiments are needed to address this issue. One of our problems is that we could not obtain enough purified viruses that could be used for animal experiments. If this problem could be solved, the safety of AAV- hTERT-TRAIL would be evaluated in vivo.
Specific antitumor effect of adeno-associated virus vector carrying TRAIL gene under the control of hTERT promoter

Figure 6. Tumor specific cytotoxicity in five cell lines after transfection of AAV plasmids measured by MTT assay. All cells were infected with the AAV-hTERT-TRAIL at an MOI of 100. Results are represented as mean ± SD of three independent experiments.

Figure 7. Activation of caspase 3 and PARP upon infection of AAV-hTERT-TRAL in SW620 cells. Cells were infected with AAV-hTERT-TRAL at an MOI of 100 for 24, 48 and 72 h.

Figure 8. Apoptosis of SW620 cells induced by AAV-hTERT-TRAL detected by ELISA assay. Results are represented as mean ± SD of three independent experiments.

Figure 9. Apoptosis of SW620 cells upon virus infection detected by flow cytometry. (A) Control; (B) AAV-hTERTEGFP; (C) AAV-hTERT-TRAIL
Recently, gene therapy researchers have been inclined to study the double gene therapy. Two genes with either the complementary or the synergistic effect are used together to kill tumor cells more effectively. Higher efficacy has been achieved when combining TRAIL with other genes. It has been shown that TRAIL and Smac possess synergistic effects in gene therapy. \(^{19,20}\) The combination of TRAIL with other tumor therapy genes, such as k5 and p53 has also achieved good efficacy. \(^{21,22}\) Our future work will use the tumor specific promoter hTERT to construct the tumor targeting AAV vector carrying double genes in gene therapy.

In conclusion, this research used AAV as the vector and utilized the tumor specific promoter hTERT to successfully construct AAV-hTERT-TRAIL, and did preliminary studies on the anti-tumor effect of AAV-hTERT-TRAIL in tumor and normal cell lines. AV-hTERT-TRAIL could induce tumor specific gene expression and in tumor cells, but not in normal cells, which lays a foundation for exploring new methods for tumor therapy.

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