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肿瘤靶向腺相关病毒携带干扰素 β 和 TRAIL 对 A549 肺癌移植瘤的增强治疗效应

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摘 要：干扰素 β(IFN-β) 和肿瘤坏死因子相关凋亡诱导配体 (TRAIL) 是有效抗癌药物。腺相关病毒(AAV) 为目前最有应用前景的基因转移载体之一。利用 AAV 携带 *IFN-β* 和 *TRAIL* 基因并置于 hTERT 启动子控制下分别构建成肿瘤靶向病毒 AAV-hTERT-*IFN-β* 和 AAV-hTERT-*TRAIL*，且单个 *IFN-β* 或 *TRAIL* 基因治疗发挥了一定的抗癌效果。将 AAV-hTERT-*IFN-β* 和 AAV-hTERT-*TRAIL* 进行联合，旨在研究其对 A549 肺癌细胞体内外的生长抑制效应。ELISA 法检测了 AAV-hTERT-*IFN-β* 感染 A549 细胞后分泌型 *IFN-β* 的表达；MTT 法检测 AAV-hTERT-*IFN-β* 联合 AAV-hTERT-*TRAIL* 对肿瘤细胞的生长抑制作用；凋亡细胞染色和流式细胞仪分别检测了 AAV-hTERT-*IFN-β*、AAV-hTERT-*TRAIL* 及其联合对 A549 细胞的凋亡效应；进一步评价了联合 AAV-hTERT-*IFN-β* 和 AAV-hTERT-*TRAIL* 对 A549 裸鼠移植瘤的抑癌效果。结果显示，联合治疗优于任一单独治疗并且导致了增强的肿瘤细胞毒性和凋亡诱导效应。更进一步显示，联合 AAV-hTERT-*IFN-β* 和 AAV-hTERT-*TRAIL* 治疗发挥了重要的抑制裸鼠移植瘤效果甚至消除全部移植瘤，为探究 IFN-β 和 TRAIL 联合抗癌的分子机制奠定了基础。

关键词：干扰素 β，*TRAIL*，腺相关病毒，肺癌，抗肿瘤效应

Enhanced antitumor effect of combining interferon β with TRAIL mediated by tumor-targeting adeno-associated virus vector on A549 lung cancer xenograft

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Abstract: Interferon β (IFN-β) and TNF-related apoptosis-inducing ligand (TRAIL) are effective anticancer agents. Adeno-associated virus (AAV) is one of the current most promising gene delivery vectors. Previously, we constructed tumor-targeting AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* by inserting *IFN-β* or *TRAIL* gene into AAV controlled by hTERT promoter. The studies showed that either single *IFN-β* or *TRAIL* gene therapy exhibited a certain extent anticancer effect. Here, we report their inhibitory effects on A549 lung cancer cell growth *in vitro* and *in vivo* by combined AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL*. Expression of secreted IFN-β in lung cancer A549 cells infected by AAV-hTERT-*IFN-β* was detected by enzyme-linked immunosorbent assay (ELISA). The growth-suppressing effect of AAV-hTERT-*IFN-β* in combination with AAV-hTERT-*TRAIL* on several cancer cell lines was assessed by MTT assay. Apoptosis of A549 cancer cells infected by AAV-hTERT-*IFN-β* alone, AAV-hTERT-*TRAIL* alone, and their combination was evaluated by apoptotic cell staining and flow cytometry (FCM), respectively. The antitumor effect of the combination of AAV-hTERT-*IFN-β* with AAV-hTERT-*TRAIL in vivo* was further evaluated through A549 lung cancer xenograft in nude mice. The results showed that the combinational treatment was superior to any alone and presented intensified tumor cytotoxic and apoptotic effect on A549 cancer cells. Most importantly, the combination of AAV-hTERT-*IFN-β* with AAV-hTERT-*TRAIL* exhibited significant antitumor effect and eliminated all tumor masses in nude mice, which lay a foundation for exploring the molecular mechanisms of combined IFN-β and TRAIL anti-tumor activity.

Keywords: *IFN-β*, *TRAIL*, adeno-associated virus, lung cancer, antitumor effect

Introduction

Adeno-associated virus (AAV) is a member of the *Parvovirdae* family, which presently has been deemed to be one of the most promising gene therapy vectors. The characteristic of AAV mainly includes few or null immune response, non-pathogenicity, wide tissue or cell tropisms, long-term and stable transgene expression etc [1]. Currently, among all used AAV serotype, AAV2 was the most usual gene delivery vector. However, the wide host range made AAV2 short of tissue or cell specificity in cancer gene therapy. To improve the targeting cancer therapeutic effect of AAV, there are mainly two approaches to be attempted, including transcriptional targeting strategy and transduction targeting strategy, which is the use of tumor-specific promoters and the tropism modification of AAV capsids, respectively [2]. Several data has showed that the application of human telomerase reverse transcriptase (hTERT) could effectively improve the therapeutic effect in targeting cancer therapy [3-4].

Human interferon β (IFN-β) is type I IFNs and one of the cytokines earliest for clinical cancer therapy. Presently, IFN-β has been widely used as a clinical anticancer agent on malignant glioma, melanoma, colorectal cancer, renal cell carcinoma, etc [5-6]. The pleiotropic antitumor mechanisms are involved in the immunmodulatory activity to tumor through upregulation of MHC class I expression, activation of Natural Killer cell and cytotoxic T lymphocyte [7-9], the inducement of tumor cell apoptosis and inhibition of tumor angiogenesis et al [10-11]. Many studies showed *IFN-β* has a significant antitumor effect both *in vitro* and *in vivo* via gene therapy methods or recombinant protein [11-12]. TNF-related apoptosis-inducing ligand (TRAIL) is one member of tumor necrosis factor (TNF) superfamily and currently is under development as a potential therapeutic agent because it can specifically induce apoptosis of various cancer cells through two pathways including dependent on or undependent on mitochondria, while no significant toxic side effects to the vast majority of normal cells [13]. The tumor targeting virus AAV-hTERT-*IFN-β* or AAV-hTERT-*TRAIL* previously constructed by us, which using the hTERT promoter to control AAV-mediated *IFN-β* or *TRAIL* gene expression, resulted in cancer-specific cell killing effect and inhibited the xenograft growth of lung cancer, colon cancer and hepatocellular carcinoma in nude mice model [14-15]. However, the single gene therapy has no significant antitumor effect [16]. Some studies reported that the two genes of different mechanisms can play a synergistic or complementary antitumor effect [17]. In the treatment of melanoma, the combined treatment of *IFN-β* and *TRAIL* gene led to more apoptosis and improved inhibitory effect of tumor growth than the alone[18], which suggested that they have a synergistic mechanism of action. Up to date, there is no report available that combines tumor targeting AAV-mediated dual gene (*IFN-β* and *TRAIL*) therapy of cancer.

This study is based on the construction of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL*, and explored their inhibitory effect on lung cancer cell growth *in vitro* and *in vivo* by combined *IFN-β* and *TRAIL*. The results showed that the combinational antitumor effect by AAV-hTERT-*IFN-β* and

AAV-hTERT-*TRAIL* is more evident than any the alone in both tumor cell lines and an animal model of lung cancer xenografts, and it lay a foundation for exploring the antitumor activity and the molecular mechanisms of *IFN-β* and *TRAIL* dual gene.

1 Materials and methods

1.1 Cell lines and culture

HEK293 cell line (human embryonic kidney containing the E1 region of Ad5) was obtained from Microbix Biosystems Inc. (Toronto, Canada). Human lung fibroblast cell line MRC5, human lung cancer cell line A549, human hepatocellular carcinoma cell line BEL7404 and human cervical cancer cell line HeLa were purchased from Shanghai Cell Collection (Shanghai). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide) and Hoechst33258 apoptosis detection kit were purchased from Sigma company (St. Louis, MO, USA) and Beyotime (Nantong), respectively. The MRC5 and HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL). The A549, BEL7404 and HeLa cell lines were grown in DMEM supplemented with 5% heat-inactivated FBS at 37°C in a humidified atmosphere with 5% CO_2 in air.

1.2 Vector construction and rAAV production

AAV vector plasmids were constructed with hTERT promoter (from Dr. Fang, University of Texas M.D.) to control transgene expression instead of CMV promoter to form pAAV-hTERT-Genes. The enhanced green fluorescent protein (*EGFP*) gene, *IFN-β* gene, and *TRAIL* gene were inserted into the vector plasmid pAAV-hTERT-Genes for respective construction of pAAV-hTERT-*EGFP*, pAAV-hTERT-*IFN-β* and pAAV-hTERT-*TRAIL*, which were preserved in our labortary[15-16]. For AAV production, the packaging plasmid pDG were employed. All virus preparations were purified by CsCl gradients to ensure purity and titers of AAV virus vectors were determined by PCR according to previous studies[16]. The titration of AAV-hTERT-*EGFP*, AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* is 5×10^{12} v.g/mL (virus genomes/mL), 2×10^{12} v.g/mL and 2×10^{12} v.g/mL, respectively.

1.3 Transduction assay of reporter gene in vitro

Cells were plated at a density of 10^5 cells in 6-well plates and infected with AAV-hTERT-*EGFP* at a multiplicity of infection (MOI) of 10^5 v.g/cell. After infection for 24 h, 48 h, 72 h, respectively, EGFP expression was detected by fluorescence microscope with a digital camera apparatus DP70 (Olympus, Japan).

1.4 Cell viability assay

Three tumor cell lines A549, BEL7404, HeLa and normal cell line MRC5 were seeded on 96-well plates at a density of 1×10^4 per well. When cells adhered, they were infected with AAV-hTERT-*EGFP*, AAV-hTERT-*IFN-β*, AAV-hTERT-*TRAIL* and the combination with AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL*. MTT assay was used to measure the cell viability. Briefly, 20 μL MTT (5 mg/mL in PBS) was added to each well 72 h after infection. After 4 h at 37°C, media and MTT were lightly removed and 150 μL DMSO was added. Absorbance was read at 595nm with an ELISA reader (Molecular Devices, Sunnyvale, CA).

1.5 Detection of IFN-β expression by enzyme-linked immunosorbent assay (ELISA)

The expression of secreted IFN-β in media was measured using human IFN-β ELISA Kit (Biomedical Laboratories, Piscataway, NJ). Tumor cells and normal cells were infected with AAV-hTERT-*IFN-β* at a MOI of 10^3, 10^4, and 10^5 v.g/cell. Diluted standards and supernatants containing IFN-β protein were added at 100 μL/well in 96-well plates coated with anti hIFN-β antibody. All procedures complied with the manufacture's instruction and the optical density (OD) of the plates was measured at a wavelength of 450 nm.

1.6 Flow cytometry analysis

Cells were seeded on 6-well plates at a density of 5×10^5 cells/well. Virus infection was performed with a MOI of 10^5 v.g/cell. Cells were harvested 72 h after infection and washed once with complete medium. Aliquots of cells were resuspended in 500 μL binding buffer and stained with fluorescein isothiocyanate (FITC)-labeled annexin V (BioVision, Palo Alto, CA) according to the manufacturer's instructions. A fluorescence-activated cell-sorting (FACS; Becton Dickinson) assay was performed immediately after staining.

1.7 Apoptotic cell staining

Cells seeded in 24-well plates were treated with various groups. After 72 h of treatment, the cells were incubated with Hoechst 33258 for 30 min, washed with PBS twice, and observed and took photos under a fluorescence microscope.

1.8 Animal experiments

All animals used in the experiments were

maintained at institutional facilities in accordance with the regulations and standards of U.S. Department of Agriculture and National Institutes of Health. Female BALB/c nude mice (4−5 weeks of age), obtained from Animal Research Committee of the Institute of Biochemistry and Cell Biology (Shanghai), were used in all of the experiments. Aliquots of A549 cells (5×10^6, suspended in 100 μL PBS) were subcutaneously inoculated into the lower right flank of the nude mice. When the tumors were 100−150 mm^3 in size after 2 weeks of tumor implantation, mice were randomized into five groups and were administrated with AAV-hTERT-*EGFP*, AAV-hTERT-*IFN-β*, AAV-hTERT-*TRAIL* and AAV-hTERT-*IFN-β* plus AAV-hTERT-*TRAIL* at a dose of 2×10^{12} v.g/kg respectively or PBS via intratumoral injection every second day for three times. Tumor growth was monitored using calipers every week. Tumor volume (V) was calculated by using the formula: tumor volume V (mm^3) = 1/2×Lenth (mm) ×Width $(mm)^2$. At the end of the experiment, tumor tissues were harvested for additional analyses as described below. Differences in tumor growth were tested for statistical significance.

1.9 HE staining and immunohistochemistry assay

Tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin. Tissue sections (5 μm) were then dewaxed and rehydrated according to a standard protocol. For Hematoxylin and Eosin (HE) analysis, sections were stained with hematoxylin and eosin. For Immunohistochemistry (IHC) assay, the sections were washed with PBS, treated with 3% H_2O_2 at room temperature, blocked at 37°C for 20 min and then followed by incubation with human monoclonal anti-IFN-β antibody (diluted 1:200; Chemicon Inc. Single Oak Drive-Temecula) overnight. After incubation with rabbit anti-human secondary antibody, expression of intracellular IFN-β was detected with diaminobenzidine (DAB; Sigma) by enhancement with an avidin-biotin reaction ABC kit (Bio-Genex laboratories, CA). The slides were then counterstained with hematoxylin.

1.10 Statistical analysis

All data are expressed as $\bar{x}\pm s$ (standard deviation) values. Student's *t*-test was applied to study the relationship between the different variables. The animal survival percentage was assessed by the Kaplan-Meier. Statistical significance was taken at $P<0.05$.

2 Results

2.1 Tumor-selective killing effect by different viruses controlled by hTERT promoter

The tumor targeting AAV-hTERT-gene system was constructed for this study. The human *IFN-β* gene and *TRAIL* gene were inserted into pAAV-hTERT-*gene* to form pAAV-hTERT-*IFN-β* and pAAV-hTERT-*TRAIL*, respectively. The construction of pAAV-hTERT-*EGFP* was used as control. The packaging and purification of recombinant AAV2 were generated as described previously [15-16], and the titer of rAAV were presented as virus genomes (v.g)/mL. First of all, in order to prove that rAAV-mediated gene expression driven by hTERT promoter has tumor targeting ability, we detected tumor-specific expression of IFN-β protein by ELISA analysis in cultured cell line. The results showed that the expression of IFN-β is very obvious in A549 cells after transduced with AAV-hTERT-*IFN-β* but seldom in normal MRC5 cells (Fig. 1). The secreted concentration of IFN-β protein was dose-dependent, and its expression in A549 cells infected by 10^5 v.g/cell of AAV-hTERT-*IFN-β* was the maximum and reached 1866 pg/mL compared with 255 pg/mL in normal MRC5 cells. The specific EGFP expression was also detected in tumor cell lines. As shown in Fig. 2, green fluorescence can be observed in A549 cells but seldom in MRC5 cells after transduced with AAV-hTERT-*EGFP*, and was time-dependent. These results suggested that hTERT promoter which controlled gene expression mediated by rAAV had the

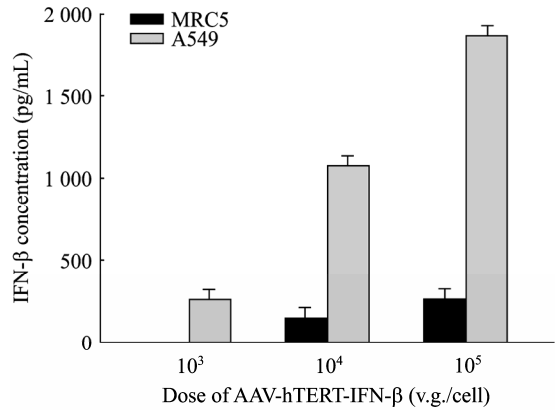

Fig. 1 Tumor-specific IFN-β expression in A549 lung cancer cells transduced by AAV-hTERT-*IFN-β*. The normal cell line MRC5 and tumor cell line A549 were infected with AAV-hTERT-*IFN-β* at a gradient of MOI 10^3, 10^4, and 10^5 v.g/cell, respectively. The expression of secreted IFN-β protein was detected by ELISA assay after 72 h. The results were represented as $\bar{x}\pm s$. Data were summarized from three experiments and expressed as histogram to reflect the secreted IFN-β concentration after transduction of different dose AAVs.

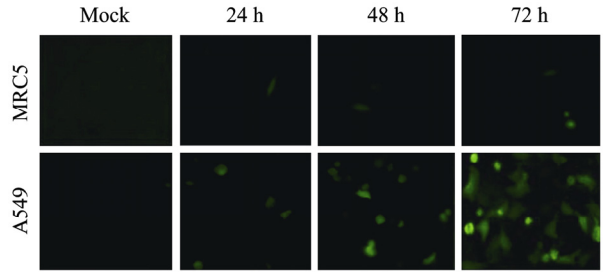

Fig. 2 Tumor-specific EGFP expression by AAV-hTERT-*EGFP* transduction. The normal cell line MRC5 and tumor cell line A549 were infected with AAV-hTERT-*EGFP* at a MOI of 10^5 v.g/cell. The green fluorescent cells were observed under fluorescence microscopy after 24 h, 48 h and 72 h, respectively.

potential tumor targeting ability.

Previous studies showed that either AAV-hTERT-*IFN-β* or AAV-hTERT-*TRAIL* specifically suppressed tumor cell growth in vitro. Based on them, to further improve the ability for killing tumor cells, we tested the effect on tumor cell viability by combined use of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL*. Tumor cell lines (BEL7404, HeLa, and A549) and normal cell line (MRC5) were infected with various viruses at a dose of 10^4 v.g/cell, and cell viability was analyzed by MTT assay. As shown in Fig. 3, the cell viability in tumor cell lines treated with a combination of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* decreased to approximately 60%−80%, compared with AAV-hTERT-*EGFP*, AAV-hTERT-*IFN-β*, or AAV-hTERT-*TRAIL*. About 79% of A549 cells and 69% of BEL7404 cells were killed by the combinational treatment after 72 h, but such a phenomenon could not be observed in other treatments. Among three tumor cell lines, A549 cell line is the most sensitive with only 21% survival cells. No significant changes were observed for normal MRC5 cells viability. Moreover, the cytotoxic effect of AAV-hTERT-*EGFP* was less apparent in the tested cell lines than other treatments, suggesting the virus itself is safe. These data indicated *IFN-β* and *TRAIL* armed with AAV controlled by hTERT promoter exhibited the additive tumor-specific killing effect and had the potential antitumor effect in animal experiments.

2.2 Induction of apoptosis by AAV-hTERT-*IFN-β* and/or AAV-hTERT-*TRAIL*

AAV-hTERT-*IFN-β* or AAV-hTERT-*TRAIL* could efficiently induce the apoptosis of tumor cells, respectively, as shown in previous study [15-16]. To evaluate the effect of apoptosis by the combination of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL*, we first observed the apoptotic morphological changes of tumor cells by Hoechst33258 staining after each treatment using a fluorescence microscope. More tumor cells treated by the combination of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* showed obvious apoptosis features including chromatin condensation and nuclear fragmentation (arrows indicate the apoptotic cells), compared with other groups. However, normal MRC5 cells had no significant apoptotic morphological changes after treatment of different viruses (Fig. 4). Further, we performed flow cytometry analysis to detect the percentage of apoptotic cells by annexin V staining. The results were consistent with the morphological changes. The combined treatment of AAV-hTERT-

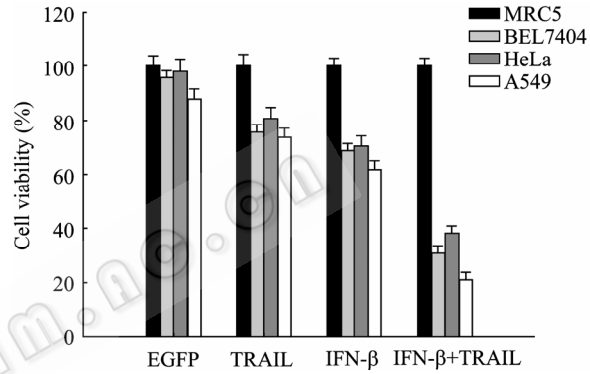

Fig. 3 Tumor cell viability after different AAVs transductions *in vitro*. The normal cell line MRC5, three tumor cell lines BEL7404, HeLa, and A549 seeded in 96-well plate were infected with AAVs at a MOI of 10^4 v.g/cell. Cells with PBS treatment were used as control. Cells were treated with MTT after 72 h. Experiments were repeated by 3 times and data expressed as histogram to reflect the cell viability after different AAV transductions. Transduced with AAV-hTERT-*EGFP* (*EGFP*), AAV-hTERT-*TRAIL* (*TRAIL*), AAV-hTERT-*IFN-β* (*IFN-β*), and the combination of AAV-hTERT-*TRAIL* and AAV-hTERT-*IFN-β* (*IFN-β+TRAIL*), respectively.

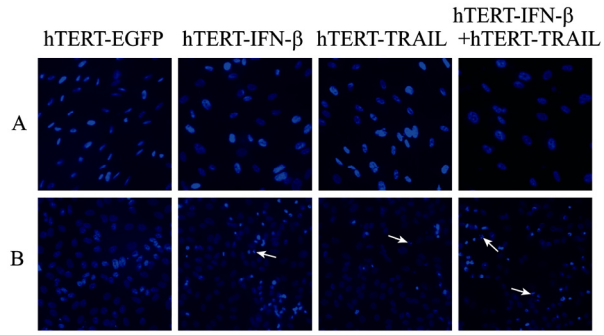

Fig. 4 Observation of apoptotic morphological changes by Hoechst33258 staining. MRC5 cells (A) and A549 Cells (B) were infected by AAV-hTERT-*EGFP*, AAV-hTERT-*TRAIL*, AAV-hTERT-*IFN-β*, and the combination of AAV-hTERT-*TRAIL* with AAV-hTERT-*IFN-β* at a MOI of 10^4 v.g/cell for 48 h. Apoptotic change of tumor cells was observed by fluorescence microscopy, as arrows indicate.

IFN-β and AAV-hTERT-*TRAIL* led to a higher percentage of cell apoptosis in A549 cells and reached almost 50% compared with AAV-hTERT-*IFN-β* alone and AAV-hTERT-*TRAIL* alone (Fig. 5). Together these, our results confirmed that the inhibition of tumor cell growth mediated by dual tumor targeting virus AAV-hTERT-*IFN-β* plus AAV-hTERT-*TRAIL* was involved with the apoptotic process.

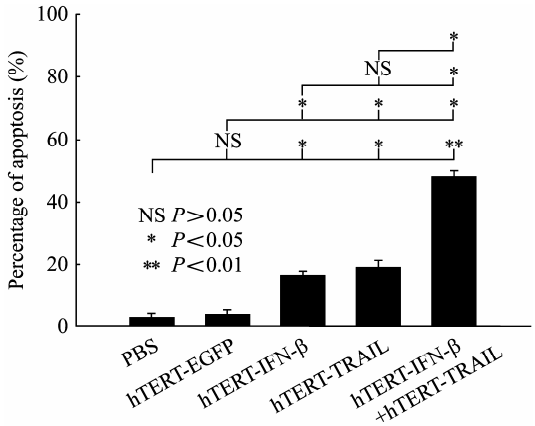

Fig. 5 Percentage of apoptosis was measured by Annexin V staining. A549 cells were harvested after infection as the same with MTT analysis. Infected cells were stained with Annexin V-FITC and immediatedly followed by flow cytometry for apoptosis assay. The percentage of apoptotic cells was calculated with CellQuest software. Each value represents the mean of 3 wells.

2.3 Antitumor efficacy of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL in vivo*

Both IFN-β and TRAIL are recognized as potent antitumor agent for their pleiotropic antitumor mechanisms. IFN-β can induce apoptotic cell death in lymphoma cells or ovarian carcinoma cells through TRAIL, which imply that the combined treatment of IFN-β and TRAIL caused better therapeutic effect on tumor growth than the alone[18-20]. To determine whether AAV-hTERT-*IFN-β* plus AAV-hTERT-*TRAIL* can induce enhanced antitumor activity *in vivo*, we established subcutaneously transplanted mice model with A549 lung cancer cells. Tumors were administrated by different viruses or PBS, and tumor growth curves were plotted to compare the difference of their antitumor efficacy. The results indicated that there was a rapid decrease in mean tumor volume in receiving intratumoral injections of AAV-hTERT-*IFN-β*, AAV-hTERT-*TRAIL*, and their combination compared with those receiving injections of PBS, AAV-hTERT-*EGFP* alone (Fig. 6A). Tumor growth was significantly inhibited after receiving intratumoral injection of AAV-hTERT-*IFN-β* plus AAV-hTERT-*TRAIL*, and the inhibition was more profound than that of AAV-hTERT-*IFN-β* alone or AAV-hTERT-*TRAIL* alone. The tumor volume in the combinational group only was 52 mm^3 after 10 weeks of treatment than 287 mm^3 of AAV-hTERT-*IFN-β* and 294 mm^3 of AAV-hTERT-*TRAIL*. Moreover, intratumoral injection of AAV-hTERT-*IFN-β*, AAV-hTERT-*TRAIL* or their combination resulted in an improved survival rate compared with PBS, AAV-hTERT-*EGFP* groups (Fig. 6B). Only one mouse died 63 days after treatment with AAV-hTERT-*IFN-β*, whereas all established lung tumor xenografts were almost eliminated, and all nude mice survived until euthanasia on day 91 after receiving a combined injection of AAV-hTERT-*IFN-β* with AAV-hTERT-*TRAIL*. The results proved that their combination has significant enhanced antitumoral potential *in vivo*.

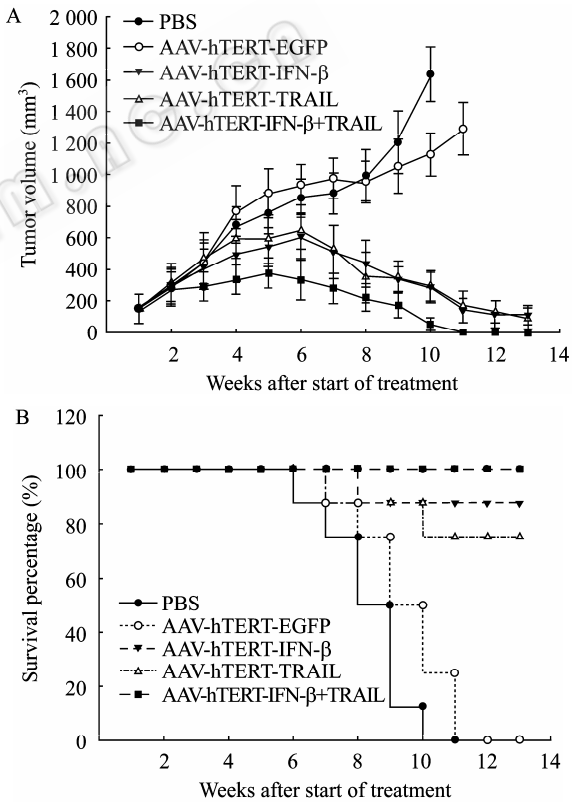

Fig. 6 Antitumor effect of different AAV injections on A549 tumor xenografts *in vivo*. Tumors were established by injecting A549 cells (5×10^5) subcutaneously into nude mice. (A) Tumor volume was observed for 13 weeks in mice with tumors after intratumoral injection with PBS, AAV-hTERT-*EGFP*, AAV-hTERT-*TRAIL*, AAV-hTERT-*IFN-β*, and their combinations, respectively. Each point represents the $\bar{x}\pm s$ of each group (n=8). Statistical significance: $P<0.001$ for the combinational treatment to AAV-hTERT-*IFN-β*; $P<0.01$ for the combinational treatment to AAV-hTERT-*TRAIL*. (B) Kaplan-Meier survival curves of animals after the same treatments as above. Pair-wise log-rank test was used to analyze survival rates in different groups.

2.4 Detection of tumor cell death and Immunohistochemistry of IFN-β in tumor tissues

To further verify the possibility that antitumor effect of AAV-hTERT-*IFN-β* was due to the IFN-β overexpression in tumor xenografts, the presence of human IFN-β was examined by IHC staining using anti-human IFN-β antibody. It is evident that there was a strong expression of IFN-β in all xenografts with the injection of AAV-hTERT-*IFN-β* and that the highest expression of IFN-β was observed in tumor sections that was received combined injections of AAV-hTERT-*IFN-β* with AAV-hTERT-*TRAIL*. IFN-β staining was also seen in tumor tissue receiving AAV-hTERT-*TRAIL* injection alone (Fig. 7B). In contrast, no IFN-β expression was observed in tumors received PBS or AAV-hTERT-*EGFP*. It suggests that antitumor effect of AAV-hTERT-*IFN-β* on lung cancer may be a consequence of IFN-β overexpression. To understand the mechanism underlying IFN-β-induced tumor growth suppression, tumor sections were further analyzed for cell death by HE staining. The results showed that infections of AAV-hTERT-*IFN-β*, AAV-hTERT-*TRAIL*, and their combination caused profound cell death and necrosis in tumor mass, whereas the effect of combinational treatment is more obvious. No cell death was found in tumor tissue receiving PBS or AAV-hTERT-*EGFP* injection (Fig. 7A).

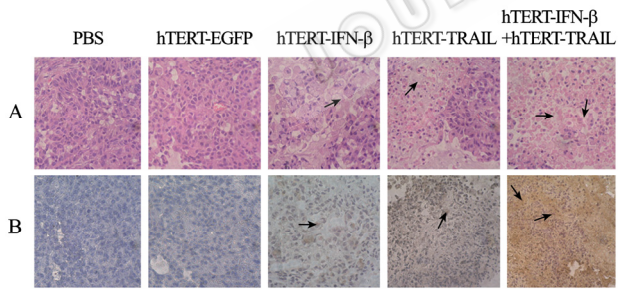

Fig. 7 Detection of cell death and IHC staining for IFN-β in tumor tissue *in vivo*. (A) H-E staining. Tumor sections were excised, fixed, dewaxed, followed by HE staining. Lung tumor in nude mice injected with various viruses or PBS. Tumor tissues treated with the combination showed more cell death than others groups. The arrow denotes the death of tumor cells (200×). (B) Representative micrographs of IHC staining. Tumor sections were processed as described in Materials and Methods and incubated with primary antibodies against human IFN-β. Representative images of at least 3 experiments. The expression of IFN-β in tumors as indicated with the arrows (200×).

3 Discussion

We recently demonstrated that treatment with AAV-hTERT-*IFN-β* not only caused tumor-specific cytotoxic effect in a panel of tumor cell lines but also effectively suppressed xenograft tumor growth either colorectal cancer or lung cancer in nude mice. Our findings suggested that *IFN-β* meditated by AAV under the control of hTERT promoter could inhibit tumor cell growth by inducing apoptosis[16]. Others recently have also showed that AAV-mediated *IFN-β* can efficaciously lead to tumor regression both in murine neuroblastoma models and even in clinical glioblastoma growth by intracerebroventricular (ICV) injection [21-22]. In this study, we characterized the expression of IFN-β and inducement of apoptosis by AAV-hTERT-*IFN-β* on A549 lung cancer cells and MRC5 normal cells, and found that tumor cells were more sensitive to AAV-hTERT-*IFN-β*-induced apoptosis and expressed IFN-β than that of normal cells. Moreover, a combined treatment of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* resulted in a profound tumor cytotoxic effect and apoptosis phenomenon in tumor cells.

IFNs are the multifunctional regulatory cytokine, and were discovered on the basis of their potent antiviral activities since the late 1950s [23]. Currently, all IFNs can be classified into two groups, type I (IFN-α, IFN-β, and IFN-ω) and type II (IFN-γ) according to their different cell surface receptors. Besides the antiviral effects, IFNs have become increasingly recognized for their pleiotropic biological activities, including antitumor and immunomodulatory effects in tumor therapy [9,24]. Despite acquired interesting preclinical results, there exist some limitations including short half life and systemic toxicity at high doses in the antitumor efficacy of type I IFNs[25-26]. Hence, some studies explored that virus-mediated delivery of type I IFNs was able to efficiently circumvent the pitfall and achieve long-term and significant antitumor effect [11-12]. Among these used viral vectors, AAV-mediated *IFN-β* delivery exhibited efficacious suppression action of tumor growth. In order to improve its tumor targeting ability, we previously used hTERT promoter to control AAV-mediated *IFN-β* and obtained tumor-specific IFN-β expression and antitumor effect in tumor cells and xenografts in nude mice [16].

TRAIL recently is developed as a potential therapeutic agent because it kills various tumor cells via an apoptotic cascade while spares normal cells [27]. The function of TRAIL in suppression of tumor cells and induction of apoptosis had been studied in detail by others [28]. Previous study showed that AAV-

mediated *TRAIL* by hTERT promoter (AAV-hTERT-*TRAIL*) induced the tumor-specific cell apoptosis and TRAIL expression [15,29]. We displayed in this study that AAV-hTERT-*TRAIL* transduction enhanced tumor cytotoxic and apoptotic effect of AAV-hTERT-*IFN-β* (Fig. 3, Fig. 5), suggesting TRAIL has intimate linkage with IFN-β. Some reported TRAIL is a key target in S-phase slowing-dependent apoptosis induced by IFN-β in cervical carcinoma cells and strongly boosted the apoptotic response of IFN-β by deregulating cell cycle[30]. Others also showed the interaction of IFN-β and TRAIL was able to induce apoptosis in ovarian carcinoma, lymphoma, multiple sclerosis, and colorectal cell through different way such as STAT1-demendent [20,31-32]. However, the synergy mechanism of the apoptotic induction and growth inhibition of tumor cells by the combination of IFN-β with TRAIL was not clear and needs to be investigated in the future studies.

It is known that the multiple, effective and tumor-specific therapeutic effect is necessary for an antitumor agent. Our preclinical study indicated that AAV-mediated *IFN-β* or *TRAIL* expression driven by hTERT promoter exhibited the remarkable antitumor efficacy. But the therapeutic effect is not ideal especially in lung cancer, and that lung cancer currently became one of the most common cause of cancer-related death, and more and more lung cancer occurred in recent years due to the environmental destroy and aggravating contamination [33]. To be interesting, the tumor-targeting strategy used here proved to be highly efficacious. We showed here that a combined injection of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* in A549 lung cancer xenograft resulted in a profound inhibition in tumor growth and almost eliminated all tumor masses in nude mice (Fig. 6A), and unreported before *in vivo*. These findings imply the great clinical application of AAV-hTERT-*IFN-β* in combination with AAV-hTERT- *TRAIL* in addition to currently available chemotherapy or radiotherapy due to their different mechanisms.

In conclusion, we showed that the IFN-β overexpression transduced by AAV-hTERT-*IFN-β*, and the combination of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* inhibited tumor cell growth both *in vitro* and *in vivo*. The inhibitory ability of AAV-hTERT-*IFN-β* and AAV-hTERT-*TRAIL* is consistent with the apoptotic effect induced by them. The results demonstrated that either AAV-hTERT-*IFN-β* or AAV-hTERT-*TRAIL* exhibited potent antitumor activity in lung cancer subcutaneous xenograft animal model, and even their combined intratumoral injection completely suppressed the growth of tumor xenografts and remarkably improved animal survival. Consequently, these findings provided the novel tumor targeting strategy of dual gene therapy approach, which might be a potential way for clinical cancer therapy.

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