

ORIGINAL ARTICLE

Preclinical study of rAAV2-sTRAIL: pharmaceutical efficacy, biodistribution and safety in animals

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The recombinant sTRAIL has been in clinical trial for various human malignancies. However, the half-life time of sTRAIL is very short, which might be an important factor influencing its clinical efficacy for cancer therapy. We previously reported the recombinant adeno-associated virus (AAV)-encoding sTRAIL_{95–281}-mediated sTRAIL expression *in vivo* up to 8 months and suppressed tumor growth markedly in mouse xenografts. In the present study, we further evaluated the clinical potency for cancer gene therapy and the safety in mouse and non-human primates. The mouse models with HCT-116, NCI-H460 and BEL-7402 cancers were injected intraperitoneally with a single dose of 1.0×10^{11} , 1.0×10^{10} and 1.0×10^9 vg of rAAV2-sTRAIL_{95–281} virus, respectively. The cynomolgus monkeys were injected (i.m.) with a single dose of rAAV2-sTRAIL_{95–281} of 1×10^{11} , 3×10^{11} and 1×10^{12} vg, corresponding to 6-, 20- and 60-fold of intended use dosage for humans, respectively. The efficacy, pharmacology and toxicity of rAAV-sTRAIL in the animals were analyzed accordingly. The tumor inhibitory rates reached 44–76%, 48–52% and 55–74% in the three tumor models, respectively, and they had no influence on mouse spontaneous activity. Administration (s.c.) of a single dose of rAAV2-sTRAIL_{95–281} virus of 1.0×10^9 or 1.0×10^{10} vg in mice with implanted tumor led to mainly distribution in the spleen, liver, implanted tumor, blood, injected site of muscle and bone marrow. Two weeks later, there was no rAAV2-sTRAIL_{95–281} detected in blood and bone marrow, and it significantly decreased in other tissues and organs and then gradually cleared away in 4–12 weeks after administration. There was no rAAV2-sTRAIL accumulation in the animal's body and no influence on the body weights. Administration (i.v.) did not cause animal death, and no dose-related abnormal clinical symptoms were found in the mice. There were no abnormal tissue and organ found in all animals. Long-term toxicity test in cynomolgus monkeys did not cause rAAV2-sTRAIL_{95–281}-related toxic and side effects, except that anti-AAV and anti-sTRAIL antibodies were generated. In conclusion, these data demonstrated that administration of rAAV2-sTRAIL_{95–281} in mice and in cynomolgus monkeys is safe without obvious toxic and side effects to the animals, and throw light on pharmacokinetics and safety in human clinical trials for cancer gene therapy.

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INTRODUCTION

Cancer is one of the world's most common diseases. In the past years, cancer always is the leading cause of death globally, challenging human health and becoming a huge burden of patient's daily life. Cancer is a complex disease, which is usually neglected by patients in its early stage, and it becomes grave and aggressive when the patients notice its presence. The best way to face cancer is prevention and early detection. The earlier it is detected, the higher the chance to be cured. Surgery, chemotherapy and radiotherapy are the main therapies for cancer currently; however, chemotherapy and radiotherapy nonspecifically kill the cancer cells as well as the normal cells at the same time, so that serious side effects are caused. In recent years, biological therapies including targeted therapy, immunotherapy and gene therapy have increasingly been reported to efficiently cure leukemia, and certain solid tumors, which bring a new hope for human beings to stand up to cancers.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belonging to tumor necrosis factor superfamily was found in the middle of 1990s^{1,2} as a type II membrane protein. TRAIL possesses the ability to induce apoptosis in about 50% of human cancer cell lines. Apoptosis and other forms of cell death play an important

role in maintaining body homeostasis by eliminating the old and harmful cells to keep the balance between the proliferation and death and to maintain the integrity of tissues. TRAIL induces apoptosis via two signaling pathways—that is, the extrinsic pathway (death receptor pathway) and the intrinsic pathway (mitochondrial pathway). As a potential safe selective antineoplastic agent, none or little toxicity of the recombinant soluble TRAIL was found in normal tissue and cells. A couple of recombinant soluble TRAIL has been tested in phase I and/or II clinical trials; however, the very short half-life (3–5 min in rodents and 23–31 min in non-human primates) limited its therapeutic potentials.³ TRAIL derivants such as iz-TRAIL,⁴ TNC-TRAIL,⁵ HSA-TRAIL,⁶ PEG-TRAIL^{7,8}, etc., prolong the half-life and enhance stability.⁹ However, the outcomes of monodrug of rTRAIL derivants in the clinical trials for cancer therapy still were not satisfied. The further challenge we faced for TRAIL-based cancer therapy is to find the best combination therapy, strong targeting and killing activity to the cancer cells, and biotherapeutic reagents such as gene therapy, targeted therapy and immunotherapy.

The strategy of gene therapy has been tested in the clinic for more than 30 years. To date, viral vectors gained more and more attention and developed as efficient gene therapy tools, such as lentivirus, adenovirus and adeno-associated virus (AAV). Lentivirus

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as a highly efficient gene delivery vector effectively mediates the functional gene expression in the target cells, but possibly induces an immune response, so that it limits their application. Adenovirus can infect the dividing and non-dividing cells without integrating into the genome of the host, highly express the transgene in the host and therefore, become a widely used tool for gene therapy; however, it might cause a strong immune response restricting their further development. AAV mediates long-term and efficient target gene expression, infects and stably integrates into dividing and non-dividing cells, and stimulates low immune response without resulting in human diseases;¹⁰ therefore, it evokes more and more interests and usage in treating various diseases. Recently, rAAV vectors have been used to treat eye diseases,^{11,12} brain diseases,^{13,14} muscle diseases,¹⁵ blood diseases,^{16–18} cancers¹⁹ and so on.^{20–23} In particular, the European Medicines Agency (EMA) had approved the first ever gene therapy of modified adeno-associated virus AAV-LPL S447X for the clinical use to treat a rare inherited metabolic disease called lipoprotein lipase deficiency in the Western world in 2013,²⁴ which greatly stimulates the enthusiasm using AAV as the targeted gene carrier for gene therapy.

We previously established a recombinant AAV-mediated soluble TRAIL_{95–281} gene expression for cancer therapy^{25,26} and demonstrated that intraportal injection via the hepatic portal veins of rAAV-TRAIL_{95–281} into the liver of an orthotopic-transplanted EL-4 mouse model mimicking liver cancer metastasis resulted in a significant suppression of tumor growth and prolonged survival, while normal hepatocyte toxicity is undetectable. Histological and biochemical analysis in tumor tissue and serum confirmed that TRAIL_{95–281} in active trimeric form was stably expressed in relatively high level in hepatocytes and secreted into the serum. Further study demonstrated that rAAV2-sTRAIL_{95–281} inhibiting tumor growth was induced by apoptosis of the tumor cells metastasizing in the livers. Furthermore, rAAV2/5-sTRAIL_{11–4281}-mediated delivery and stable expression of sTRAIL_{114–281} resulted in the presence of the trimeric form of sTRAIL_{114–281} in serum of nude mice with implanted (s.c.) or orthotopic A549 tumors.²⁷ The rAAV2/5-sTRAIL_{114–281} transduction resulted in a statistically significant reduction in tumor growth and prolonged survival of the tumor-bearing animals. Primary cell culture, histological examination of the tumors and serum analyses showed the absence of detectable TRAIL-induced toxicity in normal tissues including the liver. These data indicate a putative role of rAAV-sTRAIL in cancer therapy. In the present study, we further investigated the gene therapeutic potency of rAAV2-sTRAIL in human cancer mouse xenograft models and the pharmaceutical efficacy, tissue biodistribution and animal safety in mouse and non-human primates.

MATERIALS AND METHODS

Cell lines, animals and antibodies

Human cancer cell lines of NCI-H460 lung carcinoma, BEL-7402 hepatoma carcinoma and HCT-116 colon carcinoma were purchased from the Cell Bank of Chinese Academy of Medical Sciences (Beijing, China) or ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in RPMI-1640 medium (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% penicillin/streptomycin (North China Pharmaceutical, Shijiazhuang, China) at 37 °C in a 5% CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA). U251 neurogliocytoma cells were purchased from the Cell Bank of Chinese Academy of Medical Sciences (Beijing, China) and cultured in MEM-EBSS medium (Gibco, Waltham, MA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. Human embryo kidney (HEK293T17) cells were purchased from ATCC and cultured in DMEM medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. Human lung cancer NCI-H460 cells were purchased from ATCC and cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin.

Specific pathogen-free BALB/c nude mice (at the age of 4–6 weeks) were purchased from the Center for the Experimental Animals, National Institutes for Food and Drug Control (Beijing, China) and were raised under specific pathogen-free condition. Cynomolgus monkeys (B virus-free) with half males and half females and 2–3 kg of body weight (2–3 years old) were purchased from JingGang Experimental Animal Technology (Hainan, China. License number: SCXK (Qiong) 2004-0001) and fed with standard fare (Keaoxieli Forage, Beijing, China) after quarantine inspection. All of the animal experiments were performed in full compliance with guidelines approved by the Animal Care Committee of the Center for the Experimental Animals, National Institutes for Food and Drug Control.

Rabbit anti-human TRAIL antibody (ab65121), HRP-labeled goat anti-monkey IgG antibody (ab112767) and HRP-labeled goat anti-rabbit IgG (ab97051) were purchased from Abcam (Cambridge, MA, USA) for detecting monkey anti-human TRAIL antibody generation.

Recombinant AAV2-sTRAIL virus production

The fragment of TRAIL_{95–281} was amplified by PCR using the two oligonucleotide primers: 5'-TAGAATTCACCATGACCTCTGAGGAAACCATT-3' and 5'-CCCAAGCTTTTAGCCAACTAAAAGGCC-3', and full-length TRAIL complementary DNA as template and then inserted into AAV2 vector under the control of CAG promoter and insulin signal peptide. The subsequent vector (rAAV2-sTRAIL_{95–281}) was confirmed by agarose gel electrophoresis and DNA sequencing.²⁵ The BHK-21 cells (from Syrian baby hamster kidney) were transformed with rAAV2-sTRAIL_{95–281} vector and screened with G418 to establish a stable cell line of BHK-21-sTRAIL_{95–281}. The BHK-21-sTRAIL_{95–281} stable cell line was transfected with a helper herpes simplex virus to produce the recombinant AAV2 virus (rAAV2-sTRAIL_{95–281}). The rAAV2-sTRAIL_{95–281} virus meeting the standard of good manufacture product was produced by AGTC Gene Technology (Beijing, China).

Soluble TRAIL expression

The sTRAIL expression in the cell culture medium of HEK293T17 cells infected with rAAV2-sTRAIL_{95–281} virus at various multiplicity of infection (MOI) of 1.25 × 10⁴, 5 × 10⁴, 2 × 10⁵ and 8 × 10⁵, and with adenovirus (Ad5-null) at 50 pfu per cell as a helper virus was determined by using TRAIL/TNFSF10 ELISA kit according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). The experiments were repeated 15 times, and the data were summarized as the mean ± s.d. Statistical analysis was performed using the Excel Software (Microsoft, Redmond, WA, USA).

Killing activity

Killing activity of rAAV2-sTRAIL_{95–281} virus in various cancer cells was measured by using cell counting kit-8 (CCK-8) following manufacturer's instruction (Dojindo Molecular Technologies, Rockville, MD, USA). Four human tumor cell lines of U251 neurogliocytoma, NCI-H460 lung carcinoma, HCT-116 colon carcinoma and BEL-7402 hepatoma carcinoma were used to determine the antitumor activity of rAAV2-sTRAIL_{95–281} virus. The cancer cells were plated in 96-well plates at a density of 1 × 10⁴ cells per well and infected by rAAV2-sTRAIL_{95–281} virus at suitable MOI. The supernatant was discarded and 100 μl per well of fresh complete medium and 10 μl CCK-8 reagent were added and incubated at 37 °C and 5% CO₂ for 1–4 h. The spectrometric absorbance at a wavelength of 450 nm (reference wavelength is 630 nm) was measured with a microplate reader (Wellscan MK3, Thermo Labsystems, ISSY LES MOULINEAUX, France). Cell growth inhibition (%) was calculated by the following formula: (1-experimental absorbance of OD450/control absorbance of OD450) × 100%. The experiment was repeated 10 times, and the data were described as the mean ± s.d. Statistical analysis was performed using the Excel Software.

Antitumor efficacy of rAAV2-TRAIL in mouse xenografts

The xenografts of human HCT-116 colon cancer, NCI-H460 lung cancer and BEL-7402 hepatoma cancer were established in BALB/c (nu/nu) nude mice. Briefly, BALB/c (nu/nu) nude mice were subcutaneously injected at the right axillary space with 0.1 ml of cell suspension containing 2 × 10⁶ tumor cells. When tumors were grown up to about 300 mm³, the animals were killed. The tumors were excised and cut into small pieces with about 2 mm diameter, and then subcutaneously inoculated at the right axillary space of another group of mice. When the size of xenografts reached about 100 mm³, a single dose of rAAV2-TRAIL virus at 1 × 10⁹, 1 × 10¹⁰ and

1×10^{11} vg in 100 μ l phosphate-buffered saline (PBS) was administered (s.c., $n = 6$). *cis*-diaminedichloroplatinum (CDDP) (4 mg kg^{-1} , intraperitoneally) was as a positive control and PBS as a negative control. The tumor sizes were monitored for 19–24 days depending on the animal life conditions. At the end of the experiment, the mice were killed, and the tumors were excised and weighed. Relative tumor volume (RTV) was calculated based on the formula (V_t/V_0) (V_t is the tumor volume measured on the corresponding day, and V_0 is the tumor volume measured on day 0). T/C% was calculated based on the formula ($T_{\text{RTV}}/C_{\text{RTV}} \times 100\%$) (T_{RTV} is RTV of the experimental group, and C_{RTV} is RTV of the control group). T/C% > 60% was considered as inefficacy, and T/C% ≤ 60 and $P < 0.05$ were accepted. Data were presented as mean \pm s.d. Statistical comparisons were made by *t*-test. The accepted level of significance was $P < 0.05$.

General pharmacological study in mice

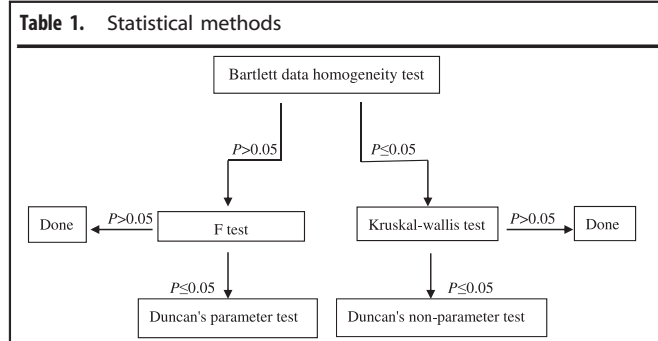
To study the spontaneous activity of mouse, BALB/c mice (male 20 and female 20) were evenly divided into four groups ($n = 10$) and injected (i.m.) with 0.2 ml of PBS, 2×10^{10} , 1×10^{11} and 2×10^{11} vg of rAAV2-TRAIL per mouse, corresponding to 2-, 10- and 20-fold of pharmacokinetic curative dosage (1×10^{10} vg per mouse), respectively. The mouse autonomic activities were determined on day 0, 2 h of day 1, day 7, day 14 and day 21 after administration in the morning between 0900 and 1200 hours, and the times of spontaneous activity in 5 min for 20 male and 20 female mice were recorded by Autonomic Activity Monitoring System (MPU-8A, MUROMACHI KIKAI, TOKYO, Japan). The data were statistically compared between groups with non-parametric method.

To study mouse pharmacokinetics, BALB/c nude mice of specific pathogen-free grade implanted with ACC-2 human adenoid cystic carcinoma (> 100 mm^3) were injected (i.m.) with a single dose of rAAV2-sTRAIL at 1.0×10^9 vg (the low-dose group) and 1.0×10^{10} vg (the high-dose group) per mouse, respectively. PBS was used as control. After 24 h and 2, 4 and 12 weeks of administration, the mice were killed, and blood and various organs (such as testicle/uterus, parastata/ovary, spleen, small intestine, mesenteric lymph node, liver, stomach, lung, heart, brain, marrow, tumor and so on) were collected, and the tissue genome DNA was extracted. The fluorescent quantitative PCR (ABI PRISM 7000) was performed to detect the copy number of target genes TRAIL_{95–281} to assess the biodistribution of rAAV2-sTRAIL in the blood and various organs. Mouse β -actin gene was used as control. The copy number of sTRAIL_{95–281} DNA representing the rAAV2-sTRAIL_{95–281} existence in mouse tissue or organ was calculated by the following formula: copy number of rAAV-sTRAIL/mouse genome = copy number of sTRAIL/copy number of β -actin gene $\times 2$.

To study the acute toxicity of rAAV2-sTRAIL_{95–281}, 7-week-old BALB/c mice of specific pathogen-free grade were injected (i.v.) with a single dose of rAAV2-sTRAIL at 5×10^{10} (low dose), 1.5×10^{11} (middle dose) and 5×10^{11} vg (high dose) per mouse ($n = 12$), corresponding to 150-, 450- and 1500-fold of intended use dosage for human (1×10^{12} vg), respectively. PBS was used as control. After administration, carriage, reactive state, nervous activity, appetite, clothing, hair, eyes, ear, mouth, nose, limbs, anapnea, dejecta, urea and clinical symptoms were continuously observed for 28 days. On day 29, mouse body weights were weighed, and all the animals were anesthetized by using pentobarbital sodium and grossly anatomized for general pathological examination.

Safety in cynomolgus monkey

Long-term toxicity of rAAV2-sTRAIL virus was evaluated in B virus-free cynomolgus monkey. Briefly, 2- to 3-year-old cynomolgus monkeys were divided into four groups ($n = 3$) and injected (i.m.) with a single dose of rAAV2-sTRAIL of 1×10^{11} , 3×10^{11} and 1×10^{12} vg, corresponding to 6-, 20- and 60-fold of the intended use dosage for humans (1×10^{12} vg), respectively. PBS was used as control. Then, the animal's clinical symptoms were continually observed twice a day for 3 months. Body temperature, electrocardiogram, hematology, blood chemistry, urea chemistry, sTRAIL expression in serum, generation of monkey antibody against AAV and human sTRAIL, and toxicokinetics were also examined regularly. At the end of a 3-month experiment, the animals were killed, and gross pathology anatomy and histopathology for all tissues and organs were carried out. The data statistics was in accordance with the following schematic diagram (Table 1). Briefly, the data of body weight, hematology index, serum chemistry index and organ weight were first calculated for the homogeneity with Bartlett test, then F-test was taken if $P > 0.05$; otherwise, Kruskal–Wallis test was taken if $P \leq 0.05$. Second, Duncan's



parameter test for multiple range comparison was taken if $P \leq 0.05$ in F-test; otherwise, the statistics was done if $P > 0.05$ in F-test. Third, Duncan's non-parameter test for the multiple range comparison was taken if $P \leq 0.05$ in Kruskal–Wallis test; otherwise, the statistics was done if $P > 0.05$ in Kruskal–Wallis test.

RESULTS

Soluble TRAIL expression and bioactivity

We previously had constructed a recombinant AAV2 expression vector encoding soluble TRAIL_{95–281}. BHK-21 cells were transfected with the vector and screened with G418 to establish a stable cell line (BHK-21-sTRAIL). BHK-21-sTRAIL was then transfected with a helper herpes simplex virus to produce recombinant AAV virus. We demonstrated that the rAAV2-sTRAIL virus infection significantly suppressed tumor growth and prolonged survival of mice implanted with human liver cancer or leukemia, while toxicity to normal tissue and cells was undetectable.^{25,26}

To further evaluate the safety and potential use in clinical trials for cancer, validation and safety test were carried out in this study. First, the existence of sTRAIL_{95–281} complimentary DNA fragment in BHK-21-sTRAIL cells was identified by PCR and agarose gel electrophoresis. As shown in Figures 1a, a 570 bp fragment with the same size as the sTRAIL_{95–281} in the positive control was observed. To test TRAIL_{95–281} protein expression, enzyme-linked immunosorbent assay was carried out and as shown in Figure 1b, the sTRAIL protein secreted into the HEK293T17 cell culture was up to about 5 ng ml^{-1} while the cells were infected with rAAV2-sTRAIL_{95–281} virus at MOI of 1.25×10^4 – 8×10^5 vg per cell ($R^2 > 0.98$), and the expression of sTRAIL reached the peak at MOI of 2×10^5 vg per cell.

To determine the cytotoxicity of rAAV2-sTRAIL in the human cancer cells, four cancer cell lines of U251 neurogliocytoma, NCI-H460 lung carcinoma, HCT-116 colon carcinoma and BEL-7402 hepatoma carcinoma were infected with rAAV2-sTRAIL virus, and the viability was determined by the CCK-8 assay. As shown in Figure 1c, infection of rAAV2-sTRAIL virus caused marked cell growth inhibition. In the tested U251, NCI-H460, HCT-116 and BEL-7402, the inhibition rates reached about 56.78, 14.39%, 22.33% and 18.45%, respectively.

While the cells were infected with rAAV2-sTRAIL virus, adenovirus (Ad5-null) was also added into the cells at MOI < 50, since it can help AAV infection. To get rid of affection of Ad5-null as well as AAV2-null virus on the cytotoxicity, Ad5-null and AAV2-null viruses were used as negative controls. It was shown that neither Ad5-null nor AAV2-null virus possessed an influence on the tested tumor cell growth (data not shown), indicating that the cytotoxicity mediated by rAAV2-sTRAIL virus infection was caused by AAV-mediated sTRAIL expression, not by the AAV2-null or Ad5-null helper virus.

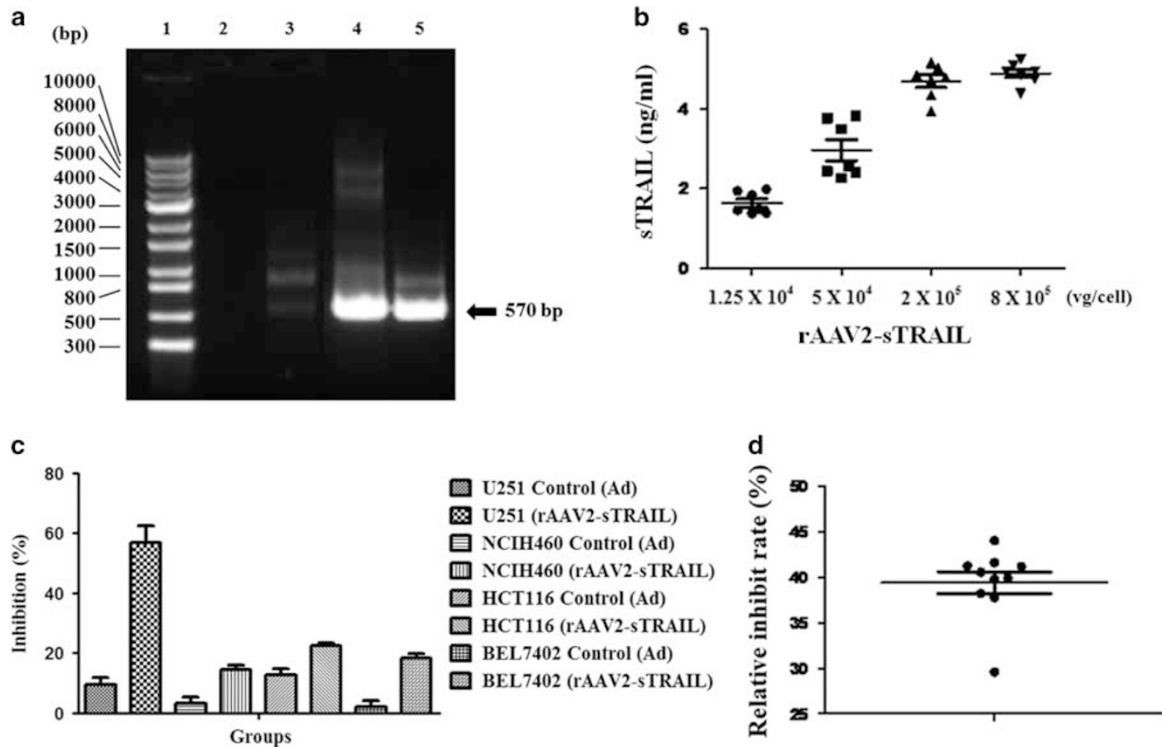


Figure 1. Soluble TRAIL expression in HEK293T7 cells infected with rAAV2-sTRAIL virus and its tumoricidal activity in various cancer cells. **(a)** Identification of the cDNA encoding the sTRAIL by PCR using the total DNA extracted from BHK-21-sTRAIL cells as template and agarose gel electrophoresis. Line 1, 1 kb plus DNA marker (300–10 000 bp); Line 2, negative control; Line 3, PCR product of the total DNA extracted from BHK-21-sTRAIL cells; Line 4, 5, TRAIL_{95–281} PCR product from vector as positive control. **(b)** The sTRAIL expression in the culture supernatant of HEK293T17 cells infected with rAAV2-sTRAIL at MOIs of 1.25×10^4 , 5×10^4 , 2×10^5 and 8×10^5 vg per cell, respectively, and determined by ELISA. **(c)** Cytotoxicity of rAAV2-sTRAIL in the human cancer cells of U251 neurogliocytoma, NCI-H460 lung carcinoma, HCT-116 colon carcinoma and BEL-7402 hepatoma carcinoma. The cell viability was determined by the CCK-8 assay. **(d)** Repeatability of the cytotoxicity of rAAV2-sTRAIL in U251 neurogliocytoma cells. The experiments were repeated at least for three times.

Antitumor efficacy in mouse xenografts of human cancers

To explore the antitumor efficacy of rAAV2-sTRAIL virus infection, when the size of the xenograft of HCT-116, NCI-H460 and BEL-7402 cancer cells reached about 100 mm³, a single dose of rAAV2-sTRAIL virus (1×10^9 , 1×10^{10} and 1×10^{11} vg) was injected (s.c.), respectively. Then tumor growth was monitored for 19–24 days depending on the mouse life conditions. After 19 days of administration, it was shown that the RTV in HCT-116 colon cancer models injected with a single dose of rAAV2-sTRAIL virus at 1×10^9 , 1×10^{10} and 1×10^{11} vg was 16.44 ± 5.69 , 13.51 ± 4.00 and 9.57 ± 2.38 ($P < 0.01$), respectively, compared with the CDDP (4 mg kg^{-1} , RTV = 11.77 ± 5.15 , $P < 0.05$) positive control (Figure 2a). The corresponding percentages of tumor over control (T/C%) were 76%, 63%, 44% and 55%, respectively. These data indicate that changes of RTV and T/C% were in a dose-dependent manner in the HCT-116 tumor model administered with rAAV2-sTRAIL virus, and the best tumor inhibitory efficacy occurred in the 1×10^{11} vg dose group.

Similar results were observed in the NCI-H460 lung cancer model (Figure 2c) injected with a single dose of 1×10^9 , 1×10^{10} and 1×10^{11} vg of rAAV2-sTRAIL virus. The RTVs after 24 days after administration were 26.71 ± 7.10 ($P < 0.05$), 23.96 ± 11.73 ($P < 0.05$) and 20.00 ± 5.63 ($P < 0.01$), respectively, compared with the RTV of 30.58 ± 8.36 in the positive control group (CDDP, 4 mg kg^{-1}). It was notable that the RTV in the group with a single dose of 1×10^9 vg plus CDDP (4 mg kg^{-1}) was 18.38 ± 3.36 ($P < 0.01$), suggesting that this combination synergized the antitumor efficacy. Corresponding T/C% were successively 69.8%

(1×10^9 vg group), 62.6% (1×10^{10} vg group), 52.3% (1×10^{11} vg group), 48.0% (1×10^9 vg plus 4 mg kg^{-1} CDDP) and 79.9% (4 mg kg^{-1} CDDP), respectively, in the NCI-H460 tumor model. These data indicate that administration of rAAV2-sTRAIL virus well suppresses NCI-H460 lung cancer growth, and the combination with CDDP synergistically inhibits the cancer cell growth.

In BEL-7402 liver cancer model (Figure 2e), the RTVs on day 21 in the animal models with a single dose administration of 1×10^9 , 1×10^{10} and 1×10^{11} vg of rAAV2-sTRAIL virus were 20.91 ± 5.98 ($P < 0.05$), 17.17 ± 5.50 ($P < 0.01$) and 15.45 ± 5.01 ($P < 0.01$), respectively, compared with the RTV in the positive control group (CDDP, 4 mg kg^{-1}) of 14.95 ± 1.40 ($P < 0.01$). The T/C% was successively 74% (1×10^9 vg group), 61% (1×10^{10} vg group), 55% (1×10^{11} vg group) and 53% (4 mg kg^{-1} CDDP), respectively. These data suggest that BEL-7402 human liver cancer growth in the animal was also suppressed markedly by rAAV2-sTRAIL administration.

At the end of the experiment, the mean weights of the excised tumors in the animals were 2.00 ± 0.71 g (1×10^9 vg group), 1.55 ± 0.44 g (1×10^{10} vg group, $P < 0.01$) and 1.23 ± 0.45 g (1×10^{11} vg group, $P < 0.01$) when comparing with the negative control group 2.71 ± 0.97 g and the positive control group 1.23 ± 0.57 g ($P < 0.01$), respectively, in the HCT-116 model (Figure 2b). In the NCI-H460 models, the tumor weights were 2.04 ± 0.17 g (the low-dose group, $P < 0.05$), 1.77 ± 0.16 g (the middle-dose group) and 1.34 ± 0.26 g (the high-dose group, $P < 0.01$), respectively (Figure 2d), compared with the negative group 2.60 ± 0.49 g, the CDDP-positive control group 1.83 ± 0.32 g

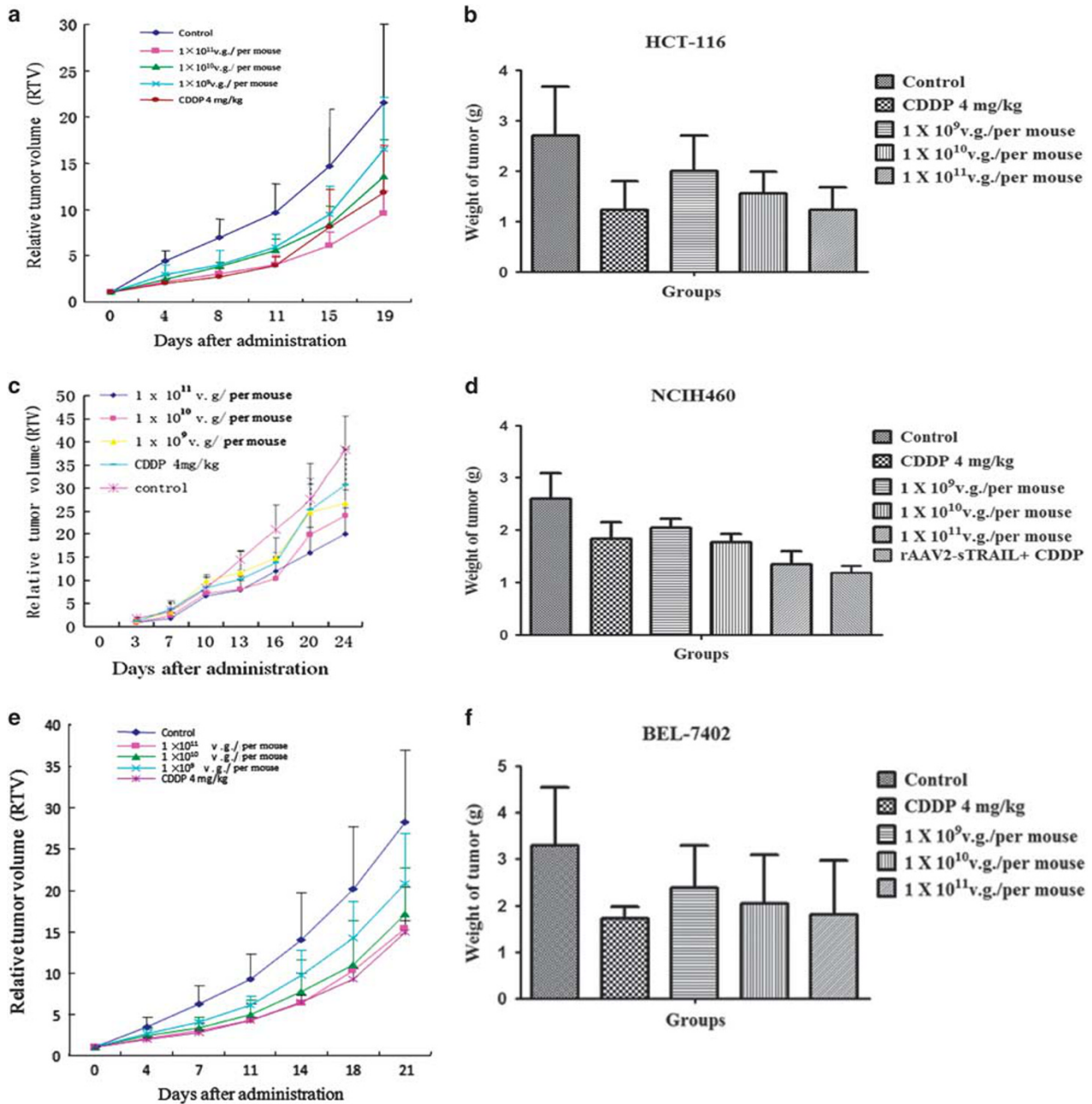


Figure 2. Antitumor efficacy of rAAV2-sTRAIL virus in mouse xenografts of human cancer. Nude mice with established xenografts were administrated with a single dose of rAAV2-sTRAIL (1×10^9 , 1×10^{10} and 1×10^{11} vg in 100 μ l volume, s.c.), CDDP (4 mg kg^{-1} , i.p.) as positive control and PBS (100 μ l) as negative control. The tumor size was monitored for 19–24 days depending on the animal life conditions. At the end of the experiment, the mice were killed, and the tumors were excised and weighed. RTV, T/C% and tumor weight were calculated. (a, b): HCT-116 colon cancer; (c, d): NCI-H460 lung cancer; (e, f): BEL-7402 liver cancer.

($P < 0.01$), and the combination group (CDDP plus low dose of the rAAV2-sTRAIL group) 1.19 ± 0.13 g ($P < 0.01$), respectively. Similarly, in BEL-7402 liver cancer model, tumor weights were 2.38 ± 0.91 g (the low-dose group), 2.04 ± 1.06 g (the middle-dose group) and 1.81 ± 1.17 g (the high-dose group, $P < 0.05$), respectively. Tumor weights in the negative and positive control groups were 3.29 ± 1.26 g and 1.72 ± 0.25 g (CDDP, $P < 0.01$), respectively (Figure 2f). These data confirm that rAAV2-sTRAIL effectively suppresses tumor growth in the animals, and the combination with CDDP results in a synergic advantage in cancer treatment.

Effect of rAAV2-sTRAIL administration on mouse spontaneous activity

Observation of the spontaneous activities of BALB/c mice injected (i.m.) with rAAV2-TRAIL at 2×10^{10} , 1×10^{11} and 2×10^{11} vg per mouse showed that the mean autonomic activity of the low-dose group (male) was 1009.6 ± 189.3 times on day 0 and 1264.2 ± 91.4 times on the day of killing the animals; the mean autonomic activity of the middle-dosage group (male) was 906.6 ± 297.6 times on day 0 and 1287.4 ± 272.5 times on the day of killing the animals; the mean autonomic activity of the high-dose group

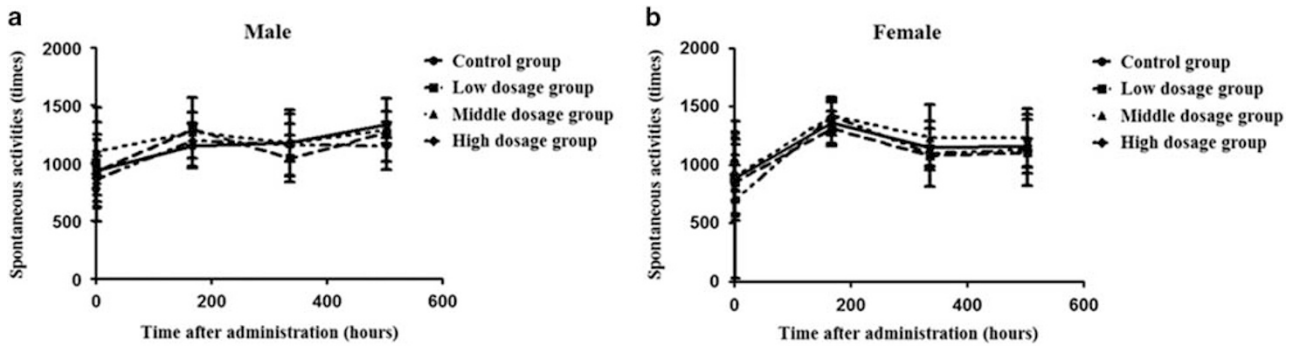


Figure 3. Effect of rAAV2-sTRAIL administration on mouse spontaneous activity. A total of 40 mice (male and female 20 each) were divided into four groups ($n=10$ per sex) and injected (i.m.) with 0.2 ml of PBS (control), low dose (1×10^{11} vg ml^{-1}), middle dose (5×10^{11} vg ml^{-1}) and high dose (1×10^{12} vg ml^{-1}) of rAAV2-sTRAIL. Then the mouse spontaneous activity was determined at 0 and 2 h of day 1, day 7, day 14 and day 21 after administration in the morning between 09:00 and 12:00 by the Autonomic Activity Monitoring System. Data were statistically compared between groups with non-parametric method. (a) Male. (b) Female.

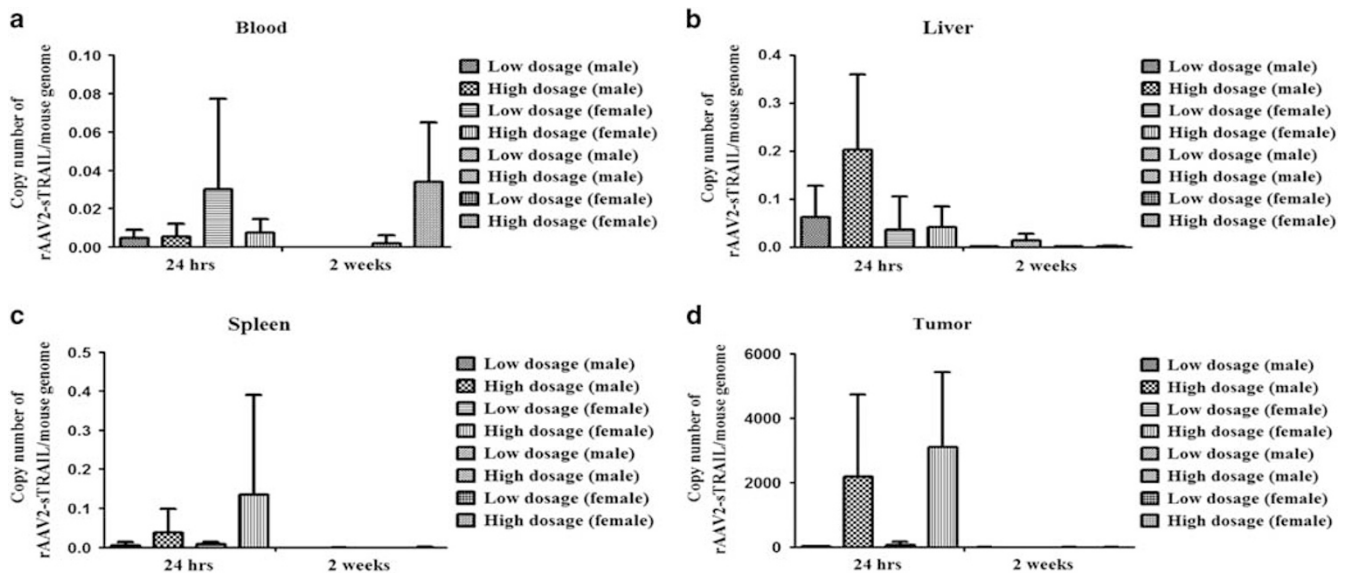


Figure 4. Analysis of distribution and elimination of rAAV2-sTRAIL in mouse organ. BALB/c nude mice of SPF grade with implanted ACC-2 human adenoid cystic carcinoma ($> 100 \text{ mm}^3$) were injected (i.m.) with a single dose of rAAV2-sTRAIL at 1.0×10^9 vg (low dose) per mouse and 1.0×10^{10} vg per mouse (high dose) ($n=5$). PBS was used as control. After 24 h, 2, 4 and 12 weeks of administration, the mice were killed, and tumor, blood and various organs were collected and the tissue genome DNA extracted. The fluorescent quantitative PCR was performed to detect the copy number of target genes TRAIL to assess the biodistribution of rAAV2-sTRAIL in the tumor and various organs. Mouse β -actin gene was used as control. The copy number of sTRAIL DNA representing the rAAV-sTRAIL existence in mouse tissues or organs was calculated by following formula: copy number of rAAV-sTRAIL gene/mouse genome = copy number of sTRAIL/copy number of β -actin gene $\times 2$. (a) Blood. (b) Liver. (c) Spleen. (d) Implanted tumor.

(male) was 910.6 ± 189.2 times on day 0 and 1148.0 ± 203.1 times on the day of killing, compared with the mean autonomic activity of the control group (female) being 1028.8 ± 238.8 times on day 0 and 1160.4 ± 232.0 times on the day of killing the animals. The mean autonomic activities of the low-dose group (female) were 881.8 ± 204.2 times on day 0 and 1106.4 ± 123.7 times on the day of killing. The mean autonomic activity of the middle-dosage group (female) was 895.0 ± 337.0 times on day 0 and 1231.6 ± 251.4 times on the day of killing the animals. The mean autonomic activities of the high-dose group (female) was 923.4 ± 363.4 times on day 0 and 1130.2 ± 305.6 times on the day of killing (Figure 3). Statistical analysis showed that there was no significant difference in the spontaneous activities of mice in all groups. In summary, administration of rAAV2-sTRAIL (i.m.) at 2×10^{10} – 2×10^{11} vg per mouse, corresponding to 2- to 20-fold of

intended use dose in humans, has no influence on mouse spontaneous activity.

Biodistribution of rAAV2-sTRAIL virus in mouse

The biodistribution study by fluorescent quantitative PCR showed that injection of rAAV2-sTRAIL virus of 1.0×10^9 or 1.0×10^{10} vg in muscles of BALB/c nude mice with ACC-2 human adenoid cystic carcinoma xenografts ($> 100 \text{ mm}^3$) leading to the virus was mainly distributed in the animal's spleen, liver, implanted tumor, blood (Figure 4), injected site of muscle and bone marrow, and also in the small intestine, mesenteric lymph node and lung of certain mice in the first 24 h after administration (data not shown). Two weeks later, there was no rAAV2-sTRAIL detected in blood and bone marrow, and it significantly declined in other tissues and

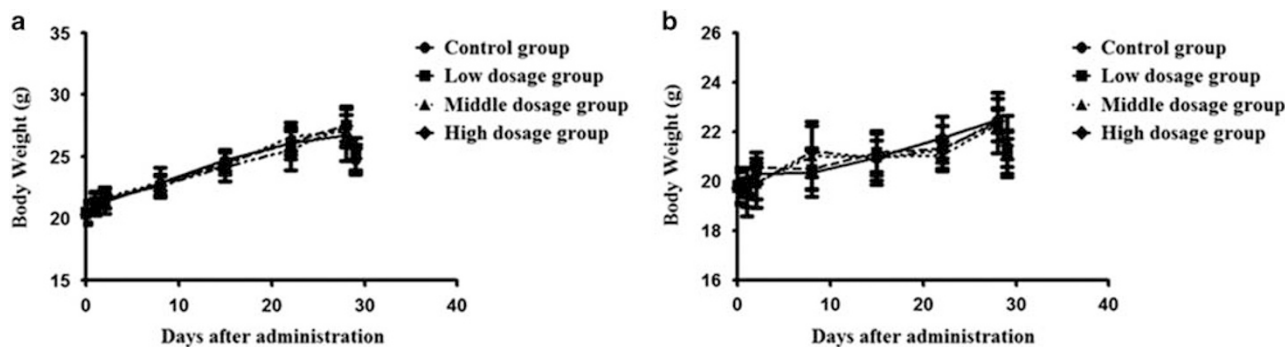


Figure 5. Acute toxicity of rAAV2-sTRAIL. BALB/c mice were injected through tail vein (i.v.) with a single dose of rAAV2-sTRAIL of 5×10^{10} (low dosage), 1.5×10^{11} (middle dosage) and 5×10^{11} vg (high dosage) per mouse ($n = 5$), corresponding to 150-, 450- and 1500-fold of intended use dosage for human (1×10^{12} vg), respectively. PBS was used as control. Animal clinical symptoms were continuously observed for 28 days. On day 29, body weights of the mice were weighed, and all animals were anesthetized by using pentobarbital sodium and grossly anatomized for general pathological examination. Data were presented as mean \pm s.d. (a) Male. (b) Female.

organs (Figure 4). Four and 12 weeks after administration, rAAV2-sTRAIL had been eliminated in all organs except a trace amount at the injected site of muscle (data not shown). There was no rAAV2-sTRAIL detected in the genital system and other physiological life-critical organs (for example, heart, data not shown), indicating that rAAV2-sTRAIL virus was gradually cleared away with the time-lapse after administration, and there was no rAAV2-sTRAIL accumulation in the animal's body and there was no influence on the animal body weights, demonstrating that administration of rAAV2-sTRAIL virus at 1.0×10^9 or 1.0×10^{10} vg per mouse is metabolically safe.

Acute toxicity of rAAV2-sTRAIL virus in mouse

Acute toxicity study showed that no died or dying animals occurred during the study, and no rAAV2-sTRAIL dosage-related abnormal clinical symptoms were found, except that the injection site turned red, which disappeared in 3 days after administration (data not shown), suggesting that it was caused by injection stimulus.

Mouse body weights of all animals were increased gradually over time during the experiment, and there was no statistical difference found between the experimental group and the control group (Figure 5).

Gross anatomy showed that there were no abnormal tissues and organs in both the experimental group and the control group of animals. This result lets us make a clue that intravenous injection of 5×10^{11} vg of rAAV2-sTRAIL virus per mouse, corresponding to 1500-fold of intended dosage use for human, is safe without obvious toxic and side effects to the animal.

Long-term toxicity of rAAV2-sTRAIL virus in cynomolgus monkey

No abnormal clinical symptoms were found in the cynomolgus monkeys during the study. No changes related to rAAV2-sTRAIL virus administration were observed in the animal body temperature, electrocardiogram, hematology, serum biochemistry, uroscopy, general pathological examination and gross anatomy (data not shown).

After 2 weeks of administration, anti-AAV antibody was detected in certain experimental groups of the monkeys. After 4 weeks of administration, the anti-AAV antibody was found in all monkeys of experimental groups, and the antibody existed constantly till the end of experiment (data not shown).

The sTRAIL expression could be detected in the liver of monkey. Monkey anti-human sTRAIL antibody was detected in few animals in the treated groups 2 weeks after administration of rAAV2-sTRAIL virus, and, 12 weeks after administration, the

anti-human sTRAIL antibody was detected in 5/6, 6/6 and 4/6 of animals in the low-dose group, the medium-dose group and the high-dose group, respectively (data not shown).

In conclusion, there was no side effect detected in the monkeys administrated with the rAAV2-sTRAIL virus.

DISCUSSION

It has been shown that a single administration (i.v.) of recombinant AAV virus can result in an extensive transduction in dividing and non-dividing cells, depending on the different serotypes of AAV,²⁸ and it mediates carrier gene long-term expression,²⁹ thus exhibiting its potential in a range of clinical applications of cancer gene therapy. In fall 2012, the EMA approved the first ever gene therapy of the modified adeno-associated virus AAV-LPL S447X for the clinical use for lipoprotein lipase deficiency in the Western world. It greatly stimulates the enthusiasm using AAV as a targeted gene carrier for gene therapy. There is now a large body of data relating to the use of recombinant AAV-mediated gene therapy for various diseases such as eye diseases,³⁰ brain diseases,³¹ tumors,¹⁹ blood diseases³² and so on.³³ Soon, additional data will become available on clinical trials of rAAV-mediated gene therapy to treat eye diseases, hemophilia B, Alzheimer's disease, Parkinson's disease and so on.

We previously demonstrated that intraportal administration of rAAV2-TRAIL_{95–281} in an orthotopic mouse model of lymphoma (EL-4) mimicking liver cancer metastasis suppressed tumor growth by 95% and enhanced median survival by 92%, an effect of induced apoptosis in the tumor cells metastasizing to the liver, while hepatocyte toxicity was undetectable.²⁵ Meanwhile, Shi *et al.*²⁷ in our group reported that transduction of rAAV2/5-TRAIL_{114–281} resulted in the expression of the trimeric form of TRAIL_{114–281} in sera of nude mice with s.c. or orthotopic A549 tumors and a statistically significant reduction in tumor growth and prolonged survival of the tumor-bearing animals. More interestingly, we demonstrated that oral or intraperitoneal administration of rAAV-TRAIL resulted in an effective suppression of tumor growth without toxicity to normal hepatocytes since AAV is more resistant to acid and alkali, suggesting that oral administration of rAAV-TRAIL might be an important alternative route with practical significance for cancer gene therapy.²⁶ Yoo *et al.*³⁴ also reported that intratumor injection of rAAV2 encoding soluble TRAIL to A549 lung adenocarcinoma mouse models (s.c.) led to 62% of reduced tumor size, and systemic delivery before implantation of A549 cells lowered the frequency of tumor occurrence to 43%, compared to 100% in untreated mice. These

data strongly suggest that it is possible to increase AAV-mediated sTRAIL expression as a novel therapeutic strategy for cancer therapy.

The present study is the first preclinical study to evaluate the potency of AAV-mediated soluble TRAIL expression for cancer gene therapy and the safety in mouse as well as in non-human primates. The data from this study displayed a well therapeutic potential use of rAAV2-sTRAIL for the treatment of liver, lung and colon cancers. Especially combination of rAAV2-sTRAIL gene therapy with CDDP chemical therapy resulted in a synergic advantage in the treatment of NCI-H460 lung cancer and BEL-7402 liver cancer. And the rAAV2-sTRAIL virus delivered to mouse and cynomolgus monkey demonstrated a favorable safety profile without obvious toxic and side effects on the animals, proving that rAAV2-sTRAIL-mediated gene therapy could be a novel antineoplastic therapeutic strategy. These data and our previous data strongly support further development of rAAV2-sTRAIL for human clinical trials to treat cancers. Administration of rAAV2-sTRAIL_{95–281} in mice and cynomolgus monkeys is safe to the animals without obvious toxic and side effects. This study throws light on pharmacokinetics and safety in human clinical trials for cancer gene therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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