Immunotherapy for SV40 T/t antigen-induced breast cancer by recombinant adeno-associated virus serotype 2 carrying interleukin-15 in mice

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Abstract. Human interleukin-15 (hIL15) exerts anticancer effects through the activities of lymphokine-activated killer (LAK) cells. However, its short half-life hinders its clinical application. Recombinant adeno-associated virus serotype 2 (rAAV2) is used for hIL15 gene transfer vectors, because of its low immunogenicity and long-term gene expression in human clinical trials. SV40 T/t antigens are related with many human epithelial cancers and are generally found in human breast cancer. In order to demonstrate the anticancer effects of hIL15 on SV40 T/t antigen-induced breast cancer, rAAV2-hIL15 was constructed and an SV40 T/t antigen-induced transgenic mouse breast cancer model was established. Our study showed that rAAV2-hIL15 could express the hIL15 protein with anticancer bioactivity. In addition, rAAV2-hIL15 could activate the cytotoxic activity of LAK cells *in vivo*. Furthermore, the rAAV2-hIL15 successfully delayed cancer growth and eventually led to cancer cell death in SV40 T/t antigen-induced breast cancer transgenic mice. In summary, our study indicates that rAAV2-hIL15 may be applied for cancer immunotherapy of SV40 T/t antigen-induced breast cancer.

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Introduction

Breast cancer is a multifactorial disease frequently found in women with a developmental period that can span decades. Although breast cancer is complex and highly variable in its clinical course, studies have indicated that SV40 T/t antigens are related to the biological behavior and prognosis of breast cancer (1-3). Previous studies have indicated that interleukins (IL1, IL2, IL12, IL15, IL18, IL24), tumor necrosis factor-α (TNF-α), and the interferon-γ (IFN-γ) can control the immune response and play an important role in breast cancer immunotherapy (4-6). In order to demonstrate whether human interleukin-15 (hIL15) can display an anticancer effect on SV40 T/t antigen-associated breast cancer, SV40 T/t antigen-induced transgenic mice were successfully established in this study.

hIL15 can induce cell proliferation and differentiation of T and B cells, and induce activation of NK cells. That is, hIL15 plays a principal role in the innate immune defense system to kill pathogenically-infected cells and cancer cells (7-9). Previous studies also showed that IL15 is able to induce antitumor cytotoxic activities of cytotoxic T cells (CTL) and NK cells. These types of lymphocytes can further develop into lymphokine-activated killer (LAK) cells (10,11). Many years ago, LAK cells were widely used in cancer immunotherapy, including breast, ovarian and pancreatic cancers in phase II clinical trials (12). Although the hIL2 has a similar function with hIL15 in cancer immunotherapy (13-16), many studies have suggested that systemic administration of high-dose hIL2 has significant toxicity (17,18). Because of the features of hIL15 in the immune response, it was considered a potential candidate for cancer immunotherapy (8,19).

To date, many clinical studies have used hIL15 administration for cancer immunotherapy through repeated multi-dose daily injections (three to four doses/day) because of its short

biological half-life (20-23). The method of repeated daily injections is inconvenient for human cancer treatments. Hence, using gene therapy to replace multi-dose daily injections for cancer immunotherapy was proposed (18,24,25). To date, several studies have indicated that recombinant adeno-associated virus serotype 2 (rAAV2) is a good gene transfer system for gene therapy because of its nonpathogenicity and low immunogenicity (26-28). In addition, rAAV2 mediates gene transfer into both dividing and quiescent cells and may induce a long-term gene expression in many cells (29-32). Furthermore, rAAV2 has successfully been applied in clinical treatment (33-36). For these reasons, in this study, rAAV2-hIL15 was produced for cancer immunotherapy.

Recently, we have successfully produced rAAV-hIL15 to inhibit cell growth of human cervical cancer and xenografted JC breast cancer (4,37). In order to study the anticancer effects of hIL15 on SV40 T/t antigens-induced breast cancer, we successfully established a SV40 T/t antigen-induced breast cancer mouse model and produced rAAV2-hIL15. In our current study, our experimental results showed that rAAV2-hIL15 could inhibit SV40 T/t antigen-induced breast cancer cells effectively. These studies indicated that rAAV-2-hIL15 may be a potential treatment method for clinical T/t antigen-induced breast cancer in the future.

Materials and methods

Cell cultures. HT1080 (human fibrosarcoma), HEK293 (human embryonic kidney cell), HT2 (murine IL2/IL15 dependent cell) and YAC-1 (a murine T-lymphoma cell sensitive to NK-cell) cells were obtained from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). HT1080, HEK293 cells, and YAC-1 cells were cultured in DMEM media, and HT2 cells were cultured in RPMI-1640 medium. These media were supplemented with 10% fetal bovine serum, and 100 IU/ml penicillin/streptomycin. Additionally, HT2 cells were supplemented with IL15 protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to maintain their survival. Culture media (DMEM and RPMI-1640), fetal bovine serum, L-glutamine, penicillin/streptomycin, sodium pyruvate and non-essential amino acids were purchased from Invitrogen (Carlsbad, CA).

Plasmid construction. The rAAV2 helper-free system (containing pAAV-MCS plasmid, pAAV-RC plasmid and pHelper plasmids) was purchased from Strategene (La Jolla, CA). The plasmid containing human IL15 gene (hIL15) was kindly provided by Dr Liao K.W. (National Chiao Tung University, Taiwan). The hIL15 containing *Eco*RI and *Bam*HI sites was amplified using the PCR method. Primers containing *Eco*RI and *Bam*HI sites were synthesized as follows: sense primer, 5'-GAATTC AAA GAA TTC ATG TAC AGG ATG CAA CTC CT, and antisense primer, 3'-GGATCC AAA GGA TCC TTA AGA AGT GTT GAT GAA CAT TTG G. The amplified hIL15 cDNA was inserted between the *Eco*RI and *Bam*HI sites of pAAV-MCS to yield pAAV-hIL15 plasmid.

Production and purification of rAAV2. The rAAV2-hIL15 and rAAV2-empty vectors were produced using the helper-free system as described according to its manufacturer's instructions. Briefly, for rAAV2-hIL15 production, HEK293 cells were cultured on fifty 15-cm-dishes and co-transfected with calcium chloride solution containing 2 mg pAAV-hIL15, 2 mg pAAV-RC and 2 mg pAAV-Helper plasmids. For rAAV2-empty vector production, HEK293 cells were cultured on fifty 15-cm-dishes and co-transfected with calcium chloride solution containing 2 mg pAAV-MCS, 2 mg pAAV-RC and 2 mg pAAV-Helper plasmids. After 65 h of transfection, rAAV2-hIL15 and rAAV2-empty vectors were produced in 293 cell culture medium. Then rAAV2-hIL15 and rAAV2-empty vectors were purified with heparin column using a single-step column purification (SSCP) method, concentrated with an Amicon Ultra-15 centrifugal filter (Millipore, Billerica, MA) and stored at -80˚C as described in a previous study (38). Finally, the titer of rAAV2 (rAAV2-hIL15 and rAAV2-empty vector) was determined by a real-time PCR method (39). The purified rAAV2-hIL15 and rAAV2-empty vectors were observed by loading 10^{10} viral particles on 10% SDS gel as described in a previous study (37,38).

IL15 expression and bioactivity of rAAV2-hIL15. To determine the IL15 protein expression of rAAV2-hIL15, the culture media from 10^{13} viral particles/ml rAAV2-hIL15-infected and rAAV2 empty vector-infected HT1080 cells ($1.5x10^5$ cells/well) were collected for 3 days and used for an ELISA assay. For IL15 expression of rAAV2-hIL15, the ELISA assay (Biosource, Camarillo, CA) was executed. In brief, the culture media from rAAV2-hIL15-infected and rAAV2 empty vector-infected HT1080 cells were added in 96-well plates coated with hIL15 antibody. After a 4-h reaction, the plates were measured at an optical density of 450 nm. For the rAA2-hIL15 bioactivity assay, the viability of HT2 cells was examined. HT2 cells are IL15 dependent cells cannot survive without IL15. The culture media from rAAV2-hIL15-infected or rAAV2-empty vector infected HT1080 cells were obtained to treat HT2 cells ($6x10^3$ cells/well) for 16 h, and the HT2 cell viability was determined using the MTS assay (Promega, Madison, WI). Media containing purified recombinant hIL15 (1 μg/ml) (Santa Cruz Biotechnology, Inc.) were added to HT2 cells which then served as the positive control.

SV-40 antigen-induced transgenic mice and breast cancer induction. SV40 T/t genes were kindly provided by Dr Yin-Jeh Tzeng (Tzu Chi University). The NMRI mice were purchased from the National Laboratory Animal Center (Taiwan). The SV40 T/t antigen-induced transgenic mice were established as previously described (39). Using a microinjection method SV40 T/t genes were transfected into the oocytes of female NMRI mice. Then the fertilized oocytes were implanted into pregnant agent mice. After the birth of the fetal mice, the SV40 T/t genes were verified by RT-PCR. About 30% of the SV40 T/t antigen-induced transgenic mice could be obtained successfully. In order to observe the anti-cancer activity of rAAV2-hIL15, induction of breast cancer was required. In the SV40 T/t antigen-induced transgenic mouse model, the breast cancer cells could be induced and grew rapidly after mating for more than 2-3 days.

LAK cell cytotoxicity assay. rAAV2-hIL15 (10^{13} viral particles/kg), rAAV2-empty vector (10^{13} viral particles/kg) or PBS were injected into the quadricep muscle of the hind limb of

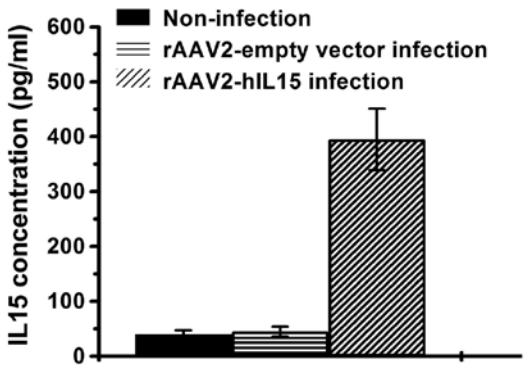

Figure 1. rAAV2-hIL15 expresses the IL15 protein. For the IL15 production assay, culture media obtained from rAAV2-hIL15-infected, rAAV2-empty vector-infected and non-infected HT1080 cells were collected and measured by the ELISA method. Data were obtained from four independent quadruplicate experiments and presented as mean ± SD.

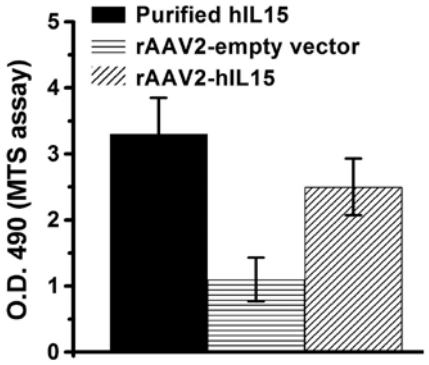

Figure 2. rAAV2-hIL15 has bioactivity *in vitro*. Culture media obtained from rAAV2-hIL15-infected, rAAV2-empty vector-infected HT1080 cells were collected and added to the HT2 cell culture for the bioactivity assay. HT2 cell viability was determined by the MTS assay. Purified hIL15 protein was used as a positive control. Note that there is higher cell viability in the purified hIL15 and rAAV2-hIL15 group. Data were obtained from four independent quadruplicate experiments and presented as mean ± SD.

experimental mice. After 4 weeks, these experimental animals were sacrificed and their LAK cells were obtained and separated from dead cells and red blood cells by ACK buffer (0.15 mM NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3). The cytotoxic activity of LAK cells was examined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) as described previously (4). Briefly, YAK-1 cells (target cells) which can be killed by activated LAK cells were co-cultured with LAK cells (effector cells) in a total volume of 200 μl RPMI-1640 containing 10% FCS in 96-well round-bottom microtiter plates. Various cell densities were prepared to achieve effector-to-target (E/T) ratios (1:1, 12.5:1, 25:1 and 50:1) for 4 h at 37˚C. The cytotoxic percentage of YAK-1 cells was calculated as follows: (OD490 nm of sample - OD490 nm with spontaneous release of LDH from target cells - OD490 nm with spontaneous release of LDH from effector cells) x100/(OD490 nm with maximum release of LDH from target cells - OD490 nm with spontaneous release of LDH from effector cells).

Animal studies and cancer observation. SV40T/t antigen-induced transgenic mice (6 mice/group) were infected with rAAV2-hIL15 (10^{13} viral particles/kg) by intramuscular injection over the quadriceps muscle of the hind limb, 4 weeks prior to breast cancer induction. The control groups were infected with rAAV2-empty vector (10^{13} viral particles/kg) PBS at the same time. All procedures were performed in compliance with the standard operating procedures of the Laboratory Animal Center of Tzu Chi University. After mating (induction of breast cancer), cancer growth of SV40T/t antigen-induced transgenic mice was determined with the caliper every two days and the cancer volume was calculated as 1/2 x length x width x height. When cancer volume reached to about 2,000-3,500 mm^3, the animals were sacrificed, and the cancerous tumors were removed and further observed. Finally, the cancer cells were fixed with 10% formalin, dehydrated, and dissected. Subsequently, the cancer sections were stained with hematoxylin and eosin (H&E) and observed under a light microscope.

Statistics. Experimental data are indicated as mean ± SEM. Statistical significance was analyzed by the Student's t-test. The survival analysis was performed using the Kaplan-Meier method where $P<0.05$ was considered to be statistically significant.

Results

IL15 protein is expressed from rAAV2-hIL15 and has bioactivity in vitro. In this study, we were able to produce and purify rAAV2-hIL15 and rAAV2-empty vector successfully with a helper-free system and SSCP methods (38). We further determined the IL15 expression and bioactivity after rAAV-hIL15 infection. The IL15 protein titer was measured by an ELISA method. There was about 400 ng/ml IL15 protein in rAAV2-hIL15-infected HT1080 cells (Fig. 1). In relation, there was <50 ng/ml IL15 protein in rAAV2-hIL15 infected or non-infected HT1080 cells. These results indicated that rAAV2-IL15 can express IL15 protein. Next, we examined the bioactivity of IL15 protein from rAAV2-IL15-infected HT1080 cells by using the HT2 cell viability assay. HT2 cell viability is dependent on IL15 protein. Culture media obtained from the rAAV2-hIL15-infected or rAAV2-empty vector-infected HT1080 cells were used for HT2 cell culture. Then HT2 viability was determined using the MTS assay. The HT2 cells treated with the media from rAAV2-hIL15 infected HT1080 cells have a higher viability than from the rAAV2-empty vector-infected HT1080 cells (Fig. 2). The purified hIL15 was used as a positive control. Taken together, our study showed that rAAV2-hIL15 can express IL15 protein and has bioactivity *in vitro*.

Cytotoxic activity of LAK cells is activated by rAAV2-hIL15 in vivo. In order to demonstrate that rAAV2-hIL15 can activate LAK cells *in vivo*, the SV40 T/t antigen-induced transgenic mice were infected with rAAV2-hIL15 or rAAV2-empty vector. One month post-infection, LAK cells were collected from the sacrificed mice and co-cultured with YAC-1 cells (target for LAK cells). The cytotoxic activity of LAK cells was studied using the lactate dehydrogenase (LDH) release assay with the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit as described in a previous study (4). In this study, our data

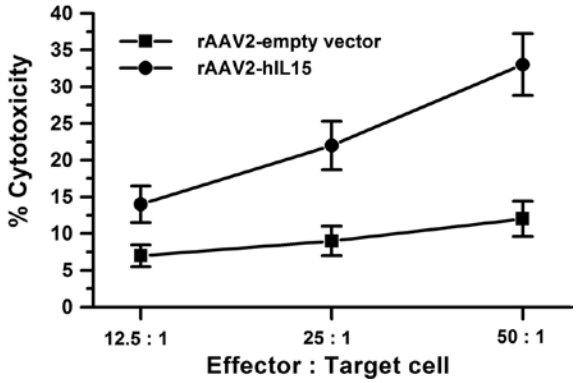

Figure 3. rAAV2-hIL15 induces the cytotoxic activities of LAK cells. LAK cells were obtained from splenocytes of rAAV2-hIL15-infected and rAAV2-empty vector-infected mice and co-cultured with their target cells (YAK-1 cells) for the cytotoxic activity assay. The cytotoxic activity was determined using lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxic Assay kit; Promega, Madison, WI). Note that there is significant cytotoxicity activity in the rAAV2-hIL15 group.

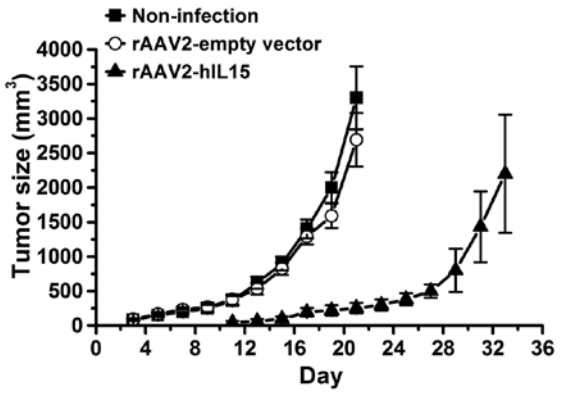

Figure 4. rAAV2-hIL15 delays breast cancer growth. Mice were injected with rAAV2-hIL15 and rAAV2-empty vector, respectively. After 4 weeks, breast cancer cells were induced and observed. Tumor size was determined every 2 days and calculated as 1/2 x length x width x width. The non-infection group was used as negative control.

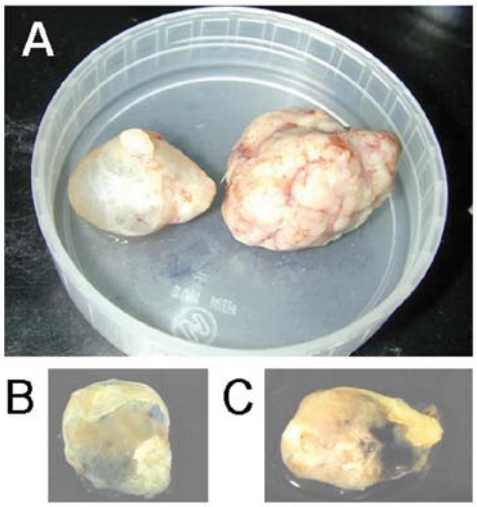

Figure 5. rAAV2-hIL15 can destroy breast cancer cells. After mice were sacrificed, the breast cancer tumors were removed and observed. (A) After cancer induction, breast cancer cells were observed to grow in tumors from the rAAV-hIL15-infected group at day 33 (left) and from the rAAV2-empty vector-infected group at day 21 (right). (B) The inside portion of the breast cancer tumor is hollow in the rAAV2-hI15-infected group. (C) The breast cancer tumor is solid in the rAAV2-empty vector-infected group. The data indicate that rAAV2-hIL15 destroys breast cancer cells.

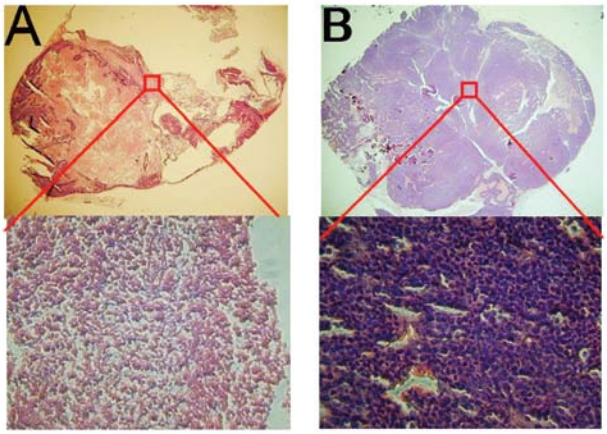

Figure 6. Cell types observed with H&E staining. (A) The breast cancer cells obtained from AAV2-hIL15-infeted group display a dark-red color. This indicates that most of the remaining cells having a low nuclear/cytoplasm ratio, are non-malignant cells. (B) Breast cancer cells obtained from AAV2-empty vector-infected group show a blue-purple color and indicate that most of the remaining cells have a high nuclear/cytoplasm ratio and are malignant cells.

indicated that rAAV2-hIL15 can induce a significant cytotoxic activity of LAK cells as compared with the rAAV2-emptpy vector (Fig. 3). Therefore, rAAV2-hIL15 certainly can induce LAK cell activity to kill their target cells *in vivo*.

rAAV2-hIL15 inhibits cell growth of SV40 T/t antigen-induced breast cancer. After mating, breast cancer cells were found on day 3 in SV40 T/t antigen-induced transgenic mice without rAAV2 infection. The breast cancer cells grew rapidly and the tumor size exceeded 3,000 mm^3 at day 21 (Fig. 4). Similarly, breast cancer appeared on day 3 and their size exceeded 2,500 mm^3 on day 21 in SV40 T/t antigen-induced transgenic mice with rAAV2-empty vector pre-infection for 4 weeks (Fig. 4). That is, the rAAV2-empty vector did not significantly influence the cell growth of SV40 T/t antigen-induced breast cancer. However, it is noted that mammary tumors grew slowly in SV40 T/t antigen-induced transgenic mice with rAAV2-hIL15 pre-infection for 4 weeks. The cancer cells appeared on day 11 and their size did not reach 2,500 mm^3 on day 33 (Fig. 4). Based on the observation from the results mentioned above, our study demonstrated that rAAV2-hIL15 can delay the growth of breast cancer and inhibit the increase of the tumor size.

rAAVV2-hIL15 can remove breast cancer cells in SV40 T/t antigen-induced transgenic mice. Compared with the rAAV2-empty vector pre-infection group, breast cancer cell growth was delayed and the tumor size was small in the rAAV2-hIL15 pre-infection group (Fig. 4). In addition, the tumors were hard in the rAAV2-empty vector pre-infection group and soft in the rAAV2-hIL15 pre-infection group. Hence, we excised the breast cancer tumors for rAAV2-empty vector and rAAV2-hIL15 pre-infection groups, and compared their differences

(Fig. 5A). After dissection of these tumors, it was observed that the inside portion of the tumors in the rAAV2-hIL15 pre-infection group (Fig. 5B) was hollow while it was solid in the rAAV2-empty vector pre-infection group (Fig. 5C). The cell types were examined next using H&E staining assay. We observed that the cells displayed a dark red type in the rAAV2-hIL15 pre-infection groups while it was dark blue type in the rAAV2-empty vector pre-infection groups (Fig. 6). These data indicated that non-cancer cells were found in the rAAV2-hIL15 pre-infection groups and cancer cells were found in the rAAV2-empty vector pre-infection groups. Taken together, the study showed that rAAV2-hIL15 can destroy breast tumors in SV40 T/t antigen-induced transgenic mice.

Discussion

Breast cancer is a multifactorial disease and is highly variable in its clinical course. SV40 T/t antigens have been shown to be related to the biological behavior and prognosis of breast cancer (1-3). In this study, we successfully established SV40 T/t antigen-induced breast cancer transgenic mice. In this transgenic mouse model, breast cancer can be induced and may start to form on day 3 to 4 after mating. Compared with the xenografted JC breast cancer model (4), the SV40 T/t antigen-induced breast cancer has a more rapid growth rate. Previous studies have demonstrated that IL15 can inhibit breast cancer growth (4,40-43). However, there is less evidence to show the anticancer effect of IL15 on SV40 T/t-related breast cancer. In this study, we are able to show that rAAV-hIL15 can express IL15 protein, can activate LAK cells and inhibit SV40 T/t-antigen-induced breast cancer. We thus suggest that IL15 may be a good candidate for SV-40 T/t antigen-related cancer therapy.

A previous study showed that IL15 has a short half-life and repeated daily multiple injections are required in clinical trials (20-23). Gene therapy is currently used as a method for cancer immunotherapy. AAV, lentivirus and adenovirus are commonly used as gene transfer systems (44,45). Many studies have indicated that the rAAV2 vector is more safe and potent than other viral delivery systems (27-33). We chose rAAV2 to carry the hIL15 gene to treat SV40 T/t antigen-induced breast cancer. Our data demonstrate that rAAV2-hIL15 can express IL15 protein with bioactivity *in vivo* and activate the activity of LAK cells *in vivo*. These experimental data are similar to those of previous studies (4,37). In addition, our previous studies have demonstrated that rAAV2-hIL15 has anticancer activity in human cervical cancer (37) and xenografted JC breast cancer (4). Today, we further demonstrate that rAAV2-hIL15 displays an anticancer effect on SV40 T/t antigen-induced cancer cells. Together with the above-mentioned studies, rAAV2-hIL15 can inhibit cancer cell growth *in vivo*. We therefore, consider that rAAV2-hIL15 may be a useful cancer immunotherapy for many types of cancer.

As shown in Fig. 4, rAAV2-hIL15 can delay SV40 T/t antigen-induced breast cancer growth. On the other hand, a hollow growth is observed in SV40 T/t antigen-induced breast cancer tumors with AAV2-hIL15 pre-infection (Fig. 5). That is, rAAV2-hIL15 can destroy breast cancer tumors. Taken together, these data indicate that not only can rAAV2-hIL15 inhibit breast cancer growth, but it can also eradicate breast cancer tumors in SV40 T/t antigen-induced transgenic mice. Previous studies showed that rAAV2-hIL15 can only delay cancer growth in human cervical cancer and xenografted JC breast cancer (4,37). Compared with those studies, rAAV2-hIL15 seems more effective in treating SV40 T/t antigen-induced breast cancer.

In summary, we successfully established SV40 T/t antigen-induced transgenic mice and produced rAAV2-hIL15. Our experimental results demonstrate that rAAV2 can delay breast cancer cell growth and remove breast cancer cells in SV40 T/t antigen-induced transgenic mice. These studies provide valuable information for SV40 T/t antigen-related cancer immunotherapy.

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