

# Persistent anti-tumor effects via recombinant adeno-associated virus encoding herpes thymidine kinase gene monitored by PET-imaging

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**Abstract.** Despite the well-documented advantages of the recombinant adeno-associated virus (rAAV) as a gene delivery vehicle, including its non-pathogenic and long-term therapeutic gene expression, there have been very limited studies on its potential for producing persistent anti-tumor effects, particularly *in vivo*. To address this issue, we constructed rAAV vectors encoding herpes simplex virus 1-thymidine kinase (HSV-TK) or its mutant form (sc39TK) as therapeutic genes, and GFP as a control gene. Effective rAAV-mediated gene delivery was readily observed in human cancer cells using immunocytochemistry and Western blotting. Cell survival analysis following prodrug ganciclovir treatment implied that both preferential and superior cytotoxicity was achieved by rAAV-sc39TK introduction. Persistent anti-tumor effects *in vivo* were investigated in Balb/c nude mice bearing human cancer cells treated with either rAAV-sc39TK or -GFP. Severe tumor growth inhibition was clearly observed only in the case of sc39TK with ganciclovir treatment. Non-invasive micro-PET imaging using <sup>18</sup>F-FHBG directly correlated with persistent anti-tumor effects by sc39TK. Therefore, the present study provides evidence that rAAV-mediated persistent therapeutic gene expression can occur, resulting in long-term anti-tumor activities and that these events can be readily monitored using micro-PET imaging.

## Introduction

Viral vectors are advantageous due to their high transduction efficiencies and have consequently been utilized as efficient gene delivery vehicles in cancer gene therapy. Among them, recom-

binant adenovirus (rAd) has been the most commonly used viral vector (1). However, the utility of rAd is limited by its short-term gene expression, which may not be ideal for treating metastatic or recurrent malignant tumors. As opposed to rAd, recombinant adeno-associated virus (rAAV) is non-pathogenic and can also achieve long-term expression of transgenes, which lends advantages in a long-term therapeutic model (2). Thus, rAAV vectors have been employed in many clinical trials aiming for persistent therapeutic effects in cases such as cystic fibrosis, hemophilia B, and neurological diseases (3). In recent studies, rAAV vectors have even emerged as an alternative platform for cancer gene therapy (4). Among the various rAAV serotypes, rAAV2 serotype has been commonly used for cancer gene therapy because of its strong tropism towards tumor cells (5,6). rAAV2 mediates high transgene expression selectively in tumor cells by use of its tumor-associated receptors such as heparin sulfate, EGFR (7), and HGFR (8). Yet, there have been very limited studies on the potential of rAAV vectors to achieve persistent anti-tumor effects, particularly *in vivo*.

Various therapeutic genes are employed in cancer gene therapy, including tumor suppressors, anti-angiogenesis agents, and apoptosis inducers (9). Among these, herpes simplex virus 1-thymidine kinase (HSV-TK) is used as a suicide agent in combination with prodrug ganciclovir (GCV) to achieve cytotoxic therapy (10). First, HSV-TK expression is induced in tumor cells. Then tumor cells are treated with GCV, which is phosphorylated by HSV-TK. The phosphorylated GCV inhibits DNA replication and selectively induces cell death in HSV-TK expressing tumor cells. Another advantage of HSV-TK/GCV therapy is the 'bystander effect', which increases the efficiency of killing untransduced neighboring tumor cells (11). Recently, alternative mutant genes have been generated through semi-random mutagenesis of HSV-TK to preferentially improve affinity to GCV and circumvent GCV cytotoxicity (12). Mutant sr39TK is created by a five codon substitution from HSV-TK (13). Then, silent mutations are introduced to GCV-resistant spliced acceptor and donor sequences to derive the mutant sc39TK. As a splice-corrected version of sr39TK, mutant sc39TK abrogates aberrant splicing of the HSV-TK gene and increases its enzymatic activity (14).

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HSV-TK and its mutant alternatives also hold promise as a molecular imaging tool for tracking the metastasis and growth of cancer cells (15-17). Besides drug-substrate GCV, HSV-TK and its mutant alternatives can specifically phosphorylate other molecules including various radioactive probes such as  $^{18}\text{F}$ -FHBG and  $^{124}\text{I}$ -FIAU (18-20). These phosphorylated probes accumulate in cancer cells expressing HSV-TK and provide a method to visualize tumor growth through micro-PET. This non-invasive visualization method can be used to closely monitor tumor growth by multiple imaging sessions throughout a prolonged time period. HSV-TK-mediated phosphorylation of these radioactive probes provides a way to measure, characterize, and quantify anti-tumor effects. However, this imaging technology has not yet been combined with the advantages of rAAV vector systems.

Thus, we attempted to examine persistent anti-tumor effects in Balb/c nude mice bearing human tumor cells treated with rAAV2-HSV-TK and simultaneously monitored its efficacy using micro-PET scanning. As a hyperactive variant of the suicide agent HSV-TK, transgene sc39TK was also hired and combined with the drug substrate GCV to achieve cytotoxic genetic therapy selectively in tumor cells. The rAAV2 vector system explored in this study will thus prove the utility of rAAV vectors in inducing persistent anti-tumor effects and providing a valuable molecular monitoring tool for cancer development.

## Materials and methods

**Cell culture.** Human cancer cell line, HeLa cells, was purchased from the American Type Culture Collection (ATCC; Manassas, VA). These cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (50  $\mu\text{g}/\text{ml}$ ) in a 5%  $\text{CO}_2$  incubator at 37°C.

**Viral plasmid construction and recombinant adeno-associated virus (rAAV) preparation.** pSp72 rAAV2-GFP was constructed by blunt-end ligation of the 2.3-kb *PvuII*-*PvuII* fragment of pHpa-trs-SK into the *BglIII* and *HindIII* sites of the pSp72 vector, thus it contains the full length recombinant AAV2 genome with the GFP protein sequence encoded. pHpa-trs-SK was received as a gift from Dr M. McCarty (Ohio State University, USA). pSp72-scAAV2-sc39TK was constructed by subcloning the *BamHI*-*SalI* PCR fragment of sc39TK gene from pcDNA3.1(+)-sc39TK into *BamHI*-*SalI* sites in pSp72-scAAV2-GFP from which the corresponding fragment was removed. Therefore, the final product was the mutant sc39TK cassette in rAAV2 genome. The generation, preparation, and titration of the rAAV2 vector were performed according to the method outlined previously (21).

**Immunocytochemistry and Western blot analysis.** HeLa cells were seeded in 48-well and 6-well plates for immunocytochemistry and Western blot analysis, respectively. Twenty-four hours after seeding, the cells were infected with rAAV2-sc39TK at various MOIs. Subsequent experimental procedures were conducted as outlined in previous studies, generally on day 2 post-infection (22,23). For this process, the primary antibodies HSV-TK (4C8) and actin were purchased from

William Summers (Yale University, USA) and Sigma (St. Louis, MO), respectively.

**Cytotoxicity assay.** HeLa cells seeded in a 6-well plate were infected with rAAV2 vectors encoding GFP, wild-type TK, or mutant sc39TK. Twenty-four hour post-infection, cells were seeded in a 96-well plate. The GCV (Cymevene, Roche, Germany) was applied in various concentrations of 0, 0.001, 0.01, 0.05, 0.1, 0.5, and 1  $\mu\text{g}/\text{ml}$  in DMEM complete media and cells were incubated in these various GCV treatment solutions for another 5 days. Surviving cells were stained with crystal violet solution in 50% methanol for 1 h and then washed extensively with distilled water before having their absorbance read at 540 nm with a microplate spectrophotometer (Spectra MAX 340pc, Molecular Devices, Sunnyvale, CA, USA). The percentage cell survival was calculated by the following equation: % cell survival = (the absorbance in experimental group - the absorbance in blank) / (the absorbance in control group - the absorbance in blank) x100.

**Generation of tumor models and GCV treatment.** Each Balb/c nude mice (male, 7 weeks) was subcutaneously inoculated in the right and left forelimbs with  $5 \times 10^6$  of rAAV2-sc39TK-infected HeLa cells and sham-treated HeLa cells, respectively. With the approval of the University committee on the use and care of animals, mice were maintained in an optimal temperature and humidity-controlled room with a 12 h light/dark cycle. Sham-treatment included mock-treated tumors or GFP-expressing tumors. Tumor size was measured using a caliper, and tumor volume was calculated according to the formula  $(\pi/6) \times (a) \times (b) \times (c)$ ; (a) tumor dimensions including the largest diameter, (b) the perpendicular diameter, and (c) the vertical diameter. When the tumors had reached a volume of  $\sim 40 \text{ mm}^3$  (9 days post inoculation), half of the mice were intraperitoneally injected with GCV 10 mg/kg/day for 16 days. The other half was intraperitoneally injected with equivalent amounts of saline. Tumor size and volume were measured every 2 to 3 days up until day 45 post infection.

**Micro-PET image acquisition.** When the tumors had reached a volume of  $\sim 100 \text{ mm}^3$  (14-15 days post inoculation), the small animal scanner micro-PET was used to acquire tomographic whole-body images of the biodistribution of  $^{18}\text{F}$ -FHBG (R4, Concord Microsystems, Knoxville, TN, USA). After mice were anesthetized, they were injected with approximately 200  $\mu\text{Ci}$  ( $\sim 3.7 \text{ MBq}$ ) of  $^{18}\text{F}$ -FHBG via the tail vein about 1 h prior to the start of micro-PET data acquisition. Prior to positioning the mice for PET scans, their bladders were emptied as much as possible. Imaging data for each mouse was acquired during 10 min. For image reconstruction, list-mode data was sorted into three-dimensional sonograms. Region of interest (ROI) analyses of reconstructed images were done by vendor software (ASI Pro 1.1, Concord Microsystems) (24).

## Results

**In vitro cytotoxicity by rAAV2-mediated TK expression and GCV treatment.** Various AAV2 vectors were constructed bearing wild-type HSV-TK (rAAV2-TK), mutant sc39TK (rAAV2-sc39TK), and GFP (rAAV2-GFP) as control (Fig. 1A),

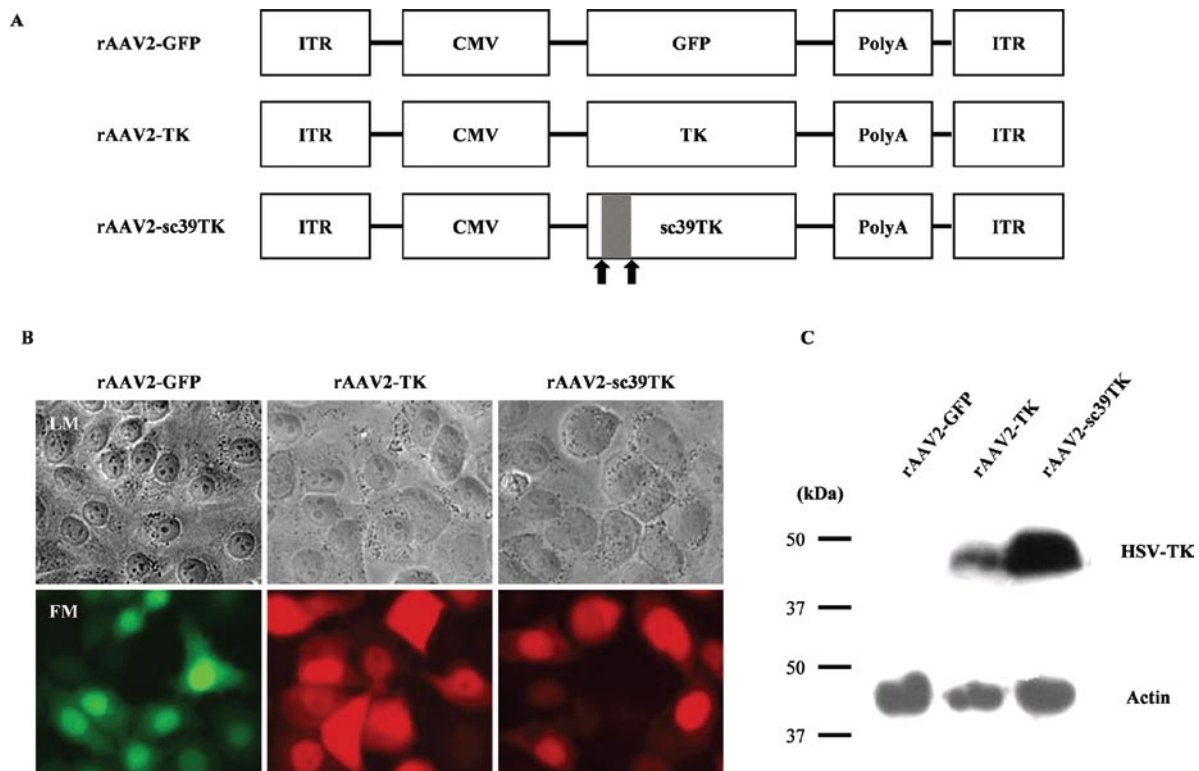


Figure 1. Features of rAAVs in the present study and TK-based transgene expression in HeLa cells. HeLa cells were infected with rAAV2-wild-type TK, rAAV2-sc39TK, or rAAV2-GFP at MOI=1,000. At 48 h post-infection, the cells were recovered to evaluate transduction efficiencies. The expression of transgenes was confirmed by immunocytochemistry and Western blotting. (A) Schematic representations of rAAV2 vectors. The sc39TK is derived by introducing two silent point mutations in the cryptic splicing donor and acceptor sites of the gene (indicated by arrow heads) (14). (B) GFP expression images under FM and immunocytochemistry images specific to TK. (C) Western blot analysis for TK. The data shows efficient expression of rAAV2-delivered GFP, TK, and sc39TK in the cells. ITR, inverted-terminal repeat; CMV, CMV promoter; LM, light microscopy; FM, fluorescent microscopy.

and transduction efficiencies were determined in HeLa cells following virus treatment. Immunocytochemistry and Western blotting of TK expression, along with GFP expression, suggested effective rAAV2-mediated delivery of all transgenes into the cells (Fig. 1B and C). At MOI 1000, almost entire cell populations were transduced.

In cytotoxicity assays, GFP-expressing cells experienced no significant cytotoxicity while sc39TK-expressing HeLa cells suffered from dramatic cytotoxicity that further increased with higher GCV doses (Fig. 2A and B). LD<sub>50</sub> in sc39TK-expressing HeLa cells was 0.01 GCV concentration. LD<sub>50</sub> in HSV-TK was 1.0 GCV concentration, which was 100 times less sensitive than that by sc39TK (data not shown). Crystal violet staining taken at GCV concentration of 0.01 μg/ml clearly showed drastically greater cell death in sc39TK-expressing cells in comparison to GFP-expressing cells (Fig. 2C). Further experiments conducted in a different human cancer cell line, SK-Hep1, also demonstrated successful transgene delivery and cytotoxicity to cancer cells by both rAAV2-TK and sc39TK with GCV treatment. Again, selective cytotoxicity by sc39TK was much greater over HSV-TK in SK-Hep1 (data not shown).

In a long-term study to verify persistent transgene expression by rAAV2, significant levels of rAAV2-mediated transgene expression in HeLa cells was detected over a month post infection (data not shown). Thus, these *in vitro* results support the persistent therapeutic potential of rAAV2-sc39TK to achieve

long-term transgene expression and cytotoxicity in various human cancer cell lines.

*Persistent tumor growth inhibition by rAAV2-sc39TK/GCV treatment in vivo.* To examine anti-tumor effects by rAAV *in vivo*, nude mice were implanted with rAAV2-sc39TK or sham-treated HeLa cells as demonstrated in Fig. 1, and intra-peritoneally administered with GCV (n=4) or saline (n=4). As expected, severe tumor growth retardation was observed in all sc39TK-expressing tumors in the presence of GCV (Fig. 3A). In one tumor out of these four cases, complete regression was observed. No significant growth inhibition was observed in sc39TK-expressing tumors without GCV treatment. In contrast, sham-treated tumors showed no significant growth inhibition not only without GCV but with GCV treatment as well. Accordingly, H&E staining indicated that massive necrotic region resided only in the sc39TK-GCV group (Fig. 3B). Taken all together, these data demonstrated that sc39TK/GCV cyto-toxic therapy via rAAV2 could achieve persistent anti-tumor effects *in vivo*.

*Micro-PET imaging of tumors expressing TK via rAAV2.* To non-invasively monitor anti-tumor effects by rAAV, <sup>18</sup>F-FHBG, a radioactive probe that interacts with sc39TK (data not shown), was systematically injected in the mice as described in Fig. 3. Micro-PET imaging was then conducted from 1 h up to 4 weeks post tumor implantation. In micro-PET images at

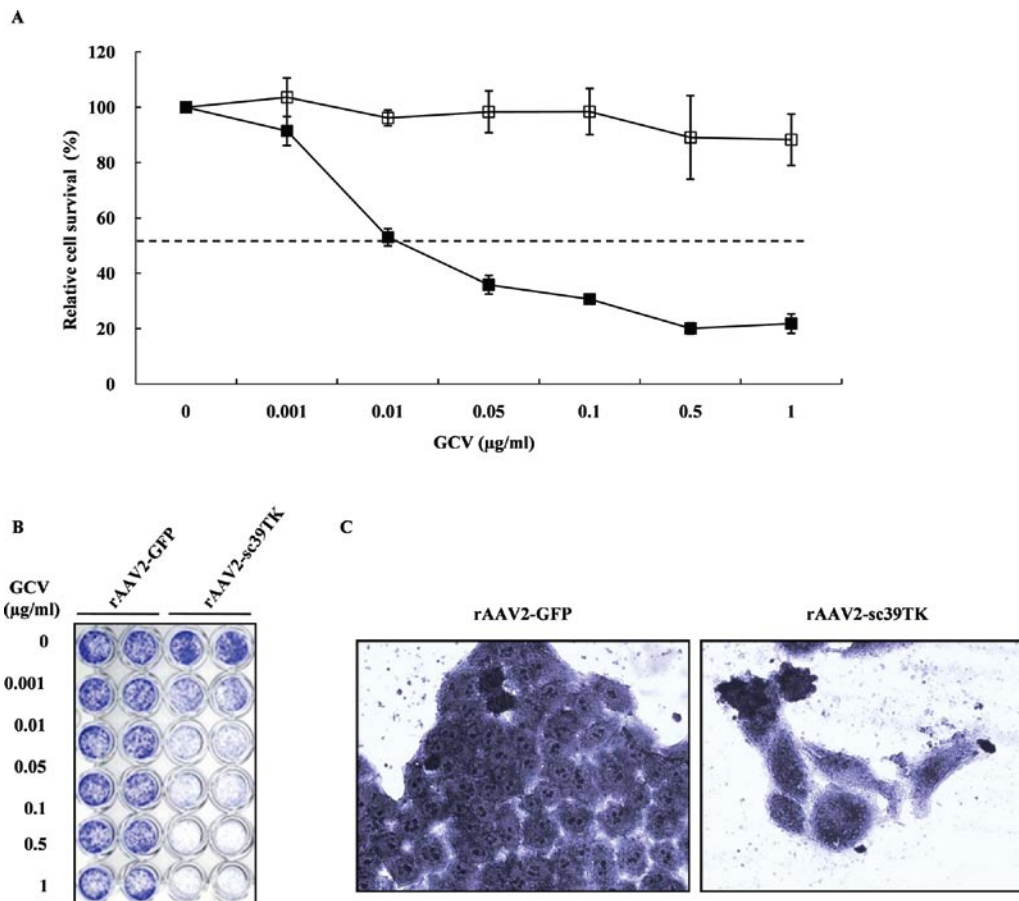


Figure 2. Enhanced cytotoxicity of rAAV2-sc39TK in HeLa cells. (A) Cells were infected with rAAV2-sc39TK at MOI 1,000 (■). As control, cells were infected with rAAV2-GFP at the same MOI (□). sc39TK with GCV treatment showed significant cytotoxicity effects, whereas control GFP showed no significant cytotoxicity effects regardless of GCV treatment. (B) Crystal violet-stained image of surviving cells in a 96-well. (C) Crystal violet-stained cells at a higher magnification under GCV 0.01 µg/ml. The data depict the increasing cytotoxicity efficacies of sc39TK with increasing GCV concentrations.

2 weeks, prior to GCV administration, the sc39TK-expressing tumors showed significant signaling of  $^{18}\text{F}$ -FHBG compared to the sham-treated tumors (Fig. 4). Subsequently, with GCV treatment, tumors expressing sc39TK showed significantly decreased signaling of  $^{18}\text{F}$ -FHBG. These observations of specific reduction in  $^{18}\text{F}$ -FHBG signaling, along with HeLa tumor growth retardation in the sc39TK-GCV group, demonstrated that tumor growth inhibition following GCV treatment can be monitored via non-invasive imaging. Contrary to the absence of signaling in sham-treated tumors at the initial stages of tumor development, some degree of non-specific signaling were observed in the subsequent weeks as the tumor sizes became greater. These data thus indicated that the interactions of sc39TK and  $^{18}\text{F}$ -FHBG, captured by micro-PET, can serve as an effective imaging tool to monitor the anti-tumor effects of sc39TK/GCV treatment, with some limitation.

## Discussion

Here, we explored i) the potential of rAAV as a gene delivery tool driving persistent anti-tumor effects by hiring mutant HSV-TK and ii) the feasibility of TK/ $^{18}\text{F}$ -FHBG-based micro-PET imaging for non-invasively monitoring the anti-tumor effects. By delivering rAAV2-sc39TK and GCV together to human cancer cells, we demonstrated that long-term anti-tumor

effects can be obtained both *in vitro* and *in vivo*. These preferential anti-tumor effects were readily captured repeatedly using non-invasive micro-PET.

When sham- and rAAV2-sc39TK-treated HeLa tumors were implanted in Balb/c nude mice, dramatic GCV-induced cell death was observed in sc39TK-expressing tumors with one case of total tumor regression. Anti-tumor effects of rAAV-mediated sc39TK expression and GCV were monitored and confirmed for several weeks post tumor implantation and affirmed the expected advantage of employing persistent transgene expression by rAAV. Other studies have also employed rAAV to establish various long-term therapeutic strategies. For example, in one study, rAAV-mediated dopamine expression was induced in MPTP-lesioned primate brains as a potential therapeutic method for Parkinson patients. Consequently, rAAV-mediated transgene expression was sustained for 6 years *in vivo* and successfully achieved long-term improvement in clinical rating scores (25). In a more recent study, rAAV-mediated microRNA delivery to hepatocellular carcinoma cells in an *in vivo* model successfully produced stable transgene expression and tumor-suppressant activity (26). The results of these previous studies in addition to our own support the advantages of rAAV mediated long-term therapeutic effects and the potential of rAAV as a promising and important tool for gene therapy.

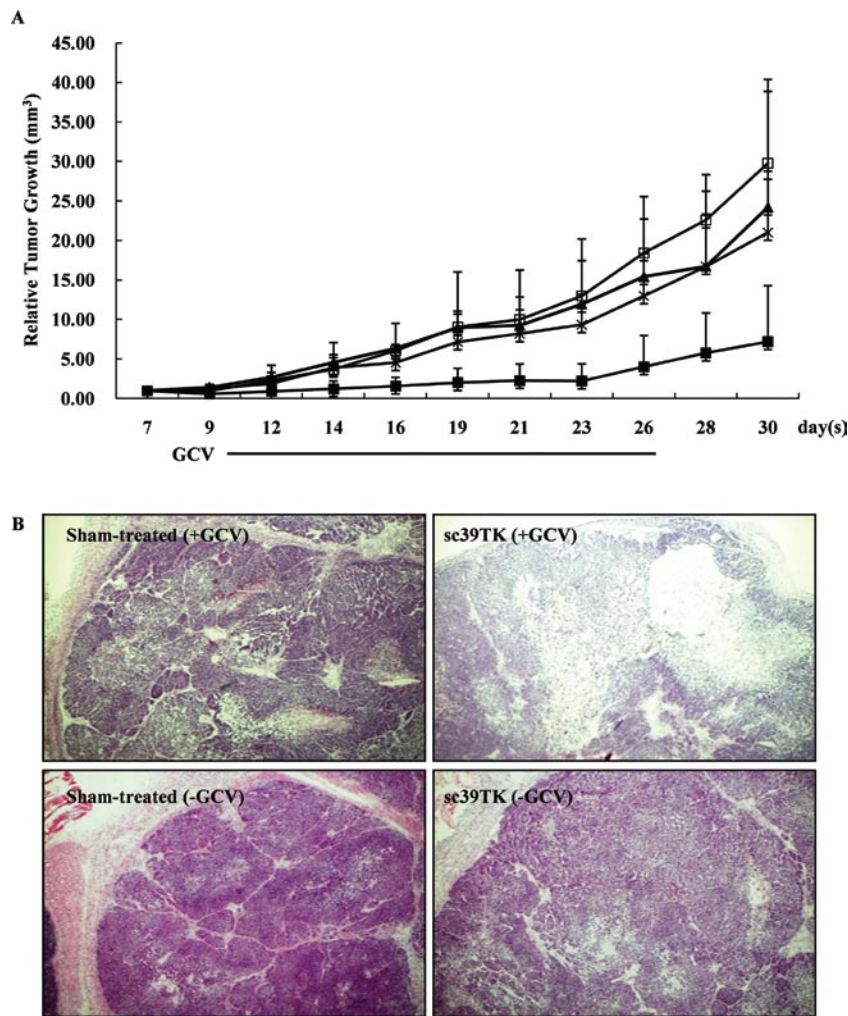


Figure 3. HeLa tumor growth suppression by sc39TK expression and GCV treatment in mice. (A) After being implanted with rAAV2-treated cells, the Balb/c nude mice were intraperitoneally treated with GCV. The size of tumors expressing sc39TK was significantly reduced following GCV treatment. By contrast, tumors with sham-treatment showed no significant inhibition of growth. (B) H&E staining of tumors harvested from mice at 5 weeks post tumor implantation. -□-, rAAV2-sc39TK treated without GCV; -■-, rAAV2-sc39TK treated with GCV; -▲-, sham-treated without GCV; -x-, sham-treated with GCV.

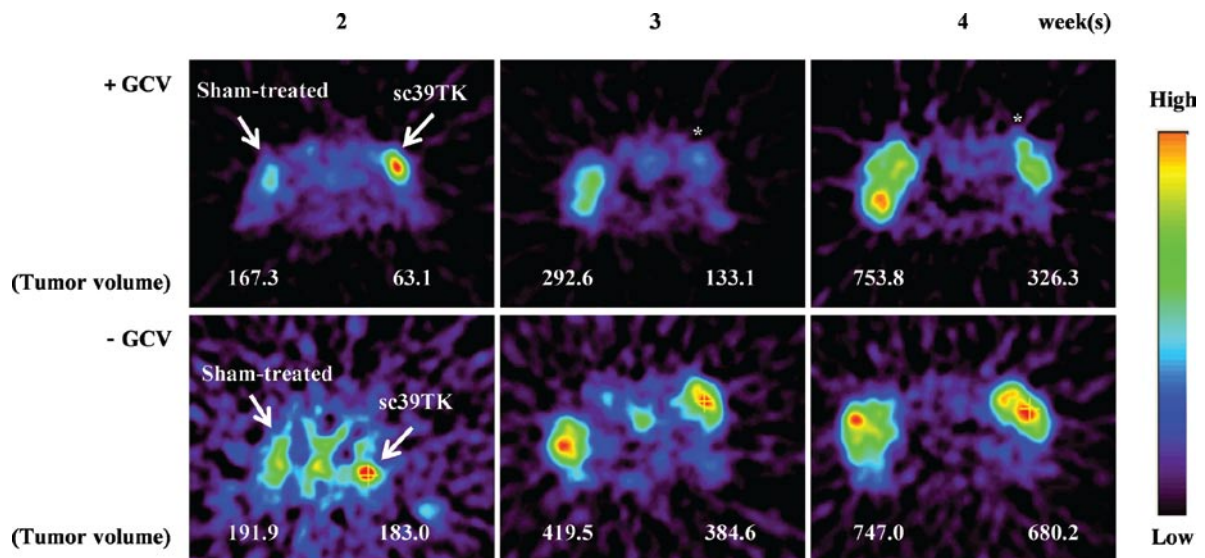


Figure 4. Non-invasive micro-PET analysis of rAAV2-treated tumors. PET-scans were performed on nude mice after intravenous injection of <sup>18</sup>F-FHBG. Multiple images were obtained from the same mice representing each experimental group. Imaging data indicate that the signal of <sup>18</sup>F-FHBG correlated well with the degree of tumor growth retardation in sc39TK-GCV tumors (\*).

*In vitro* functional studies provided clear evidence that effective transgene expression and significant cytotoxicity can be achieved by rAAV-mediated sc39TK expression and GCV treatment in human cancer cells. sc39TK, the mutant form of HSV-TK was reported as a more optimal alternative because it could further induce greater anti-tumor cytotoxicity with GCV by retaining higher enzymatic activity due to not generating aberrant splicing forms (14). To confirm whether sr39TK is indeed invulnerable to spontaneously generating alternative splicing forms, we infected various human cancer cell lines with rAAV2-sr39TK or -TK, including FADU, U251, U81-MG, MDA-MB345, TE3, A541, Baxpc3, and SK-Hep1. RNAs extracted from these cells were amplified by RT-PCR using TK-specific primers that allowed the generation of various splicing forms. Both agarose gel analysis and DNA sequencing analysis suggested that only enzymatically functional splicing forms were produced by both genes (data not shown) (27). However, an *in vivo* situation might lend different results and our own study nevertheless demonstrated the potential of mutant sc39TK for effective GCV-induced gene therapy.

Presently, we also explored the application of rAAV for the first time in micro-PET imaging as a non-invasive, efficient method to monitor tumor development. Micro-PET imaging using <sup>18</sup>F-FHBG also provided further visual evidence of the tumoral growth inhibition by sc39TK/GCV treatment (28). Radioactive probe <sup>18</sup>F-FHBG was delivered and its interactions with rAAV-mediated sc39TK expression were captured via micro-PET imaging, which visualized the anti-tumor effects by sc39TK and GCV over multiple imaging sessions. In addition to the probe used in this study, there are a wide variety of probes that have been developed, such as <sup>18</sup>F-FEAU (29), and <sup>123</sup>I-FIAU (30), that vary in their safety and their selectivity and affinity to host kinases. In our study, we employed <sup>18</sup>F-FHBG for its high selectivity for HSV-TK. However, as further investigations of radioactive probes continue, it would be beneficial to keep in mind the various options to optimize the specificity and efficacy of micro-PET imaging techniques.

Our study was limited by the small number of *in vivo* models which prevented significant quantitative analysis of micro-PET images. Thus, further investigation with additional tumor xenograft experiments using micro-PET and <sup>18</sup>F-FHBG tracer with larger *in vivo* experimental groups could provide additional quantitative evidence for rAAV-sc39TK/GCV therapy. Eventually, long-term *in vivo* models employing rAAV2-sc39TK could be developed to address the possibility of therapeutic efficacy for metastatic tumors. The anti-metastasis efficacy achieved by rAAV vectors encoding other anti-angiogenic transgenes, such as TIMP1 (31), endostatin (32-34), VEGFR antibody, and vasostatin has already been shown in past experiments (35). Thus, future studies investigating rAAV2-sc39TK in long-term *in vivo* models would be the next important step to support both the anti-tumor and anti-metastatic effects of sc39TK/GCV therapy.

In conclusion, our study demonstrated that rAAV vectors bearing sr39TK can be employed as efficient tools for anti-tumor efficacy in long-term cancer gene therapy and for monitoring tumor development using micro-PET imaging. As afore-mentioned, future studies of rAAV-sc39TK in long-term *in vivo* metastatic models with rigorous statistical analyses of micro-PET imaging would provide even greater support for

rAAV and sc39TK/GCV as promising therapeutic tools in cancer gene therapy.

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