Assessment of α -Fetoprotein Targeted HSV1-*tk* Expression in Hepatocellular Carcinoma with *In Vivo* Imaging

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Abstract

Tumor-specific enhancer/promoter is applicable for targeting gene expression in tumors and helpful for tumor-targeting imaging and therapy. We aimed to acquire α -fetoprotein (AFP)-producing hepatocellular carcinoma (HCC) specific images using adenovirus containing HSV1-*tk* gene controlled by AFP enhancer/promoter and evaluate *in vivo* ganciclovir (GCV)-mediated therapeutic effects on AFP-targeted HSV1-*tk* expression with ¹⁸F-FDG positron emission tomography (PET). Recombinant adenovirus expressing HSV1-*tk* under AFP enhancer/promoter was produced (AdAFP-TK) and the expression levels were evaluated by RT-PCR and ¹²⁵I-IVDU uptake. GCV-mediated HSV1-*tk* cytotoxicity was determined by MTT assay. After the mixture of AdAFP-*fluc* and AdAFP-TK was administrated, bioluminescent images (BLIs) and ¹⁸F-FHBG PET images were obtained in tumor-bearing mice. *In vivo* therapeutic effects of AdAFP-TK and GCV in the HuH-7 xenograft model were monitored by ¹⁸F-FDG PET. When infected with AdAFP-TK, cell viability in HuH-7 was reduced, but those in HT-29 and SK-Hep-1 were not significantly decreased at any GCV concentration less than 100 μ M. AFP-targeted *fluc* and HSV1-*tk* expression were clearly visualized by BLI and ¹⁸F-FHBG PET images in AFP-producing HCC, respectively. *In vivo* GCV-mediated tumor growth inhibition by AFP-targeted HSV1-*tk* expression was monitored by ¹⁸F-FDG PET. Recombinant AdAFP-TK could be applied for AFP-targeted HCC gene therapy and imaging in AFP-producing HCC.

Key words: α -fetoprotein, ¹⁸F-FDG PET, ¹⁸F-FHBG PET, herpes simplex virus type 1-thymidine kinase, tumor-targeting imaging

Introduction

To enhance specificity and safety of gene therapy, therapeutic gene expression needs to be tightly confined to the target tissue. Targeted gene expression has been accomplished using tissue-specific enhancer/promoters or disease-specific enhancer/promoters.^{1,2} Enhancer/promoter driven gene expression using adenoviral vector systems plays an important role in cancer gene therapy by limiting expression of therapeutic genes to target tumor lesions and preventing deleterious effects to normal organs. α -fetoprotein (AFP) enhancer/promoter,³ carcinoembryonic antigen promoter,⁴ prostate-specific antigen promoter,⁵ tyrosinase promoter,⁶ and telomerase reverse transcriptase promoter⁷ are tumor-specific enhancer/promoters.

AFP is normally expressed up to 10 mg/mL in the fetal liver and is transcriptionally silent after birth.⁸ AFP expression levels rebound again during hepatocarcinogenesis and are used as a diagnostic and prognostic serum marker of hepatocellular carcinoma (HCC). Researchers have attempted to evaluate the targeted therapeutic effects *in vivo* and *in vitro* of adenoviral vector constructs expressing therapeutic genes including herpes simplex type 1 virus thymidine kinase (HSV1-*tk*),^{9–11} cytosine deaminase,^{12,13} or sodium iodide symporter (NIS)^{14,15} controlled by the AFP enhancer/promoter in AFP-positive HCC.

HSV1-*tk* is a therapeutic gene applied with ganciclovir (GCV) and is also an imaging reporter gene with various radiolabeled thymidine analogs such as ¹²⁴I or ¹⁸F-labeled 2'-deoxy-2'-fluoro-5-iodo-1-[β]-D-arabinofuranosyluracil (FIAU),

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^{18}F -labeled 2'-fluoro-5-ethyl-1- $[\beta]$ -D-arabinofuranosyluracil (FEAU), and ^{18}F -labeled 9-(4-[^{18}F]fluoro-3-hydroxymethyl-butyl) guanine (FHBG).¹⁶⁻¹⁸

The advantageous features of the various molecular imaging methods, including noninvasiveness, repeatability, and quantification, lend themselves to the evaluation of location, duration, and expression levels of the therapeutic gene.¹⁹⁻²¹ Because some therapeutic genes cannot be imaging reporters, simultaneous expression constructs with therapeutic genes and reporter genes including fusion forms or bi-cistronic forms have been developed. The location and magnitude of HSV1-tk expression can be monitored repeatedly by positron emission tomography (PET) with ^{18}F -FHBG²² or ^{124}I -FIAU²³; this approach has translated to clinical practice. The first human PET image of HSV1-tk expression in a glioma patient was performed with ^{124}I -FIAU before and after HSV1-tk gene transfer.²⁴ Exogenous HSV1-tk activity was successfully visualized by ^{18}F -FHBG PET after intratumoral injection of adenovirus expressing HSV1-tk in HCC patients.²⁵

^{18}F -FDG PET is considered to be a useful imaging technique for diagnosis, staging, and monitoring of therapeutic responses in oncology.²⁶⁻²⁸ ^{18}F -FDG PET additionally provides information on metabolic activity before the actual reduction of tumor volume.

In this study, we simultaneously acquired AFP-producing HCC-targeted images by ^{18}F -FHBG PET and bioluminescent image (BLI) on using mixtures of two adenoviruses expressing HSV1-tk gene and firefly luciferase (*fLuc*) gene controlled by the AFP enhancer/promoter, respectively. We also utilized ^{18}F -FDG PET to evaluate the therapeutic effect of *in vivo* GCV-mediated cytotoxicity on AFP-targeted HSV1-tk expression in AFP-producing HCC models.

Materials and Methods

Cell culture

Human HCC cell line (HuH-7) was obtained from the Japanese Collection of Research Bioresources Cell Bank. Other human HCC cell lines (HepG2 and SK-Hep-1) and human colorectal adenocarcinoma cell line (HT-29) were obtained from the American Type Culture Collection. HuH-7 and HepG2 are AFP-producing HCC cell lines, SK-Hep-1 is AFP-nonproducing HCC cell line, and HT-29 is AFP-nonproducing colorectal adenocarcinoma cell line. HuH-7, SK-Hep-1, and HT-29 were cultured in Dulbecco's Modified Eagle's Medium and HepG2 was grown in Minimum Essential Medium and supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 250 ng/mL amphotericin B in an atmosphere containing 5% CO_2 at 37°C.

Construction of recombinant Ad vectors

AdAFP-fLuc was prepared as previously described²⁹ and fLuc expression of AdAFP-fLuc was controlled by human AFP enhancer/promoter. AdCMV-TK for positive control and AdCMV-LacZ for negative control in which expression of HSV1-tk and LacZ was controlled by the CMV promoter were kindly provided by Professor Lee (Dankook University, Yongin, Korea). To specifically produce adenoviral vector, HSV1-tk in AFP-producing HCC, the *fLuc* gene of pGL3-

AFPfLuc²⁹ was replaced by HSV1-tk gene prepared by PCR from pMOD-HSV1tk (Invivogen), using the primer: 5'-acc aag atc tcc acc atg gct tct tac cct gga cac-3' (forward) and 5'-cca tct aga tta gtt ggc ctc tcc cat etc cc-3' (reverse), through *Bgl*III and *Xba*I digestion and ligation. The DNA fragment containing AFP enhancer/promoter and HSV1-tk from the plasmid above (pAFP-TK) was cloned into pShuttle vector (Agilent Technologies, Inc.) (pShuttle-AFPTK). AdAFP-TK (HSV1-tk expressed by AFP enhancer/promoter) was made by recombining the pShuttle construct with the adenoviral backbone using the AdEasy Adenoviral Vector Systems (Agilent Technologies, Inc.) in accordance with the manufacturer's instructions. All recombinant adenoviral vectors were amplified in 293 cells and purified by double cesium chloride density gradient ultracentrifugation. Titers were determined by the TCID₅₀ method and the value obtained was converted to plaque-forming units (pfu)/mL.

^{125}I -IVDU and ^{18}F -FHBG synthesis

Radiolabeling was performed as previously described³⁰ with some modifications. ^{125}I -NaI (Perkin Elmer, Inc.) was added to a solution of precursor (2'-Deoxy-5-(trans-2-trimethylsilylvinyl)uridine; FutureChem Co.) of 5-(2-Iodo-vinyl)-2'-deoxyuridine (IVDU) dissolved in ethanol, followed by the addition of 30% hydrogen peroxide/acetic acid (1:3, v/v; Sigma-Aldrich, Inc.). Aqueous saturated sodium metabisulfate was added to quench the reaction; the reaction mixture was then loaded onto a C18 Sep-Pak cartridge system (Waters). After quenching with sodium metabisulfite, the radioiodinated IVDU was isolated on a C-18 Sep-Pak cartridge (Waters), and eluted with ethanol. The ethanol was evaporated and the radioiodinated IVDU was formulated with a cell culture medium or saline. ^{18}F -FHBG and ^{18}F -FDG was supplied from the Korea Institute of Radiological and Medical Sciences.

In vitro test of HSV1-tk expression

HuH-7, HepG2, and HT-29 were plated in six-well plates (5×10^5 cells per well). After 24 hours in culture, cells were infected with AdAFP-TK and AdCMV-TK. Infected cells were used for RT-PCR and uptake assay. Total RNA was extracted using the CellLytic™-M cell Lysis reagent (Sigma-Aldrich) and reverse transcribed to cDNA with a Superscript III reverse transcriptase (Invitrogen). PCR amplifications of individual cDNAs were performed using specific primers for HSV1-tk and β -actin in a GeneAmp PCR System (Applied Biosystems) for 30 cycles of denaturation (95°C for 30 seconds), annealing (HSV1-tk, 55.5°C; β -actin, 60°C for 30 seconds), and extension (72°C for 120 seconds): HSV1-tk, 5'-atg cca acc ctg ctg agg-3' (forward) and 5'-ttc tgg gag cac tgc ctg-3' (reverse); β -actin, 5'-ctg gga cga cat gga gaa aa-3' (forward) and 5'-aag gaa ggc tgg aag agt gc-3' (reverse).

^{125}I -IVDU uptake was measured by incubating the cells with 74 KBq of ^{125}I -IVDU in 2 mL serum-free media per well at 37°C for 120 minutes. After incubation, the cells were quickly washed twice with 4 mL ice-cold phosphate-buffered saline (PBS) buffer. The cells were detached with 500 μL trypsin and the radioactivity was measured with a gamma counter (1480 WIZARD; PerkinElmer, Inc.). ^{125}I -IVDU uptake was expressed as the percentage of the injected dose (%ID). Data represented the mean $\pm$ standard deviation (SD) of three wells.

In vitro cytotoxicity assay

HuH-7, SK-Hep-1, and HT-29 cells were cultured in 96-well plates in triplicate at a density of 1×10^4 cells per well. After 24 hours, cultured cells were infected with AdCMV-LacZ, AdCMV-TK, or AdAFP-TK, respectively, in medium supplemented with 2% FBS. The next day, cell culture media was replaced with media containing different concentrations of GCV (0–100 μ M) and incubated for 2 or 5 days. The cell cytotoxicity was measured using the In Vitro Toxicology assay Kit, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide (MTT) based (Sigma-Aldrich, Inc.) according to the manufacturer's instructions. Absorbance was measured in 595 nm single filter by an ELISA reader (Bio-rad Laboratories, Inc.).

Animal studies

The care, maintenance, and treatment of animals in these studies followed protocols approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences. For established HCC-bearing mouse model, subcutaneous xenografts were produced in 5-week-old female Balb/c-nu/nu mice (Nara Biotech.) by subcutaneous injection of 1×10^7 tumor cells in serum-free cell culture medium into the right foreleg. The mixture of AdAFP-fLuc (5×10^8 pfu) and AdAFP-TK (5×10^8 pfu) suspended in saline was injected into the tumor after allowing tumor growth (~ 500 mm³). BLI and ¹⁸F-FHBG PET images were acquired a day after administration of adenovirus.

In vivo imaging studies

All BLIs were acquired using an IVIS imaging system series 200 (Xenogen) to detect the bioluminescence signals in the HCC xenograft model ($n=6$). Each model was intraperitoneally injected with 3 mg of D-luciferin (Molecular Imaging Products Company) in PBS and the BLIs were acquired for 1 minute at 6 minutes after injection. The bioluminescent signals are expressed in units of photons per cm² per second per steradian (p/cm²/s/sr).

The mice were anesthetized by 2% isoflurane in oxygen and then injected with 7.4 MBq of ¹⁸F-FHBG or ¹⁸F-FDG into the tail vein and then kept in a chamber with 0.5% isoflurane for 1 hour. PET images were acquired using an Inveon small animal PET/SPECT/CT system (Siemens Medical Solutions). During the acquisition of the PET images, the mice were anesthetized using 2% isoflurane. The level of radioactivity in tumor was measured as standard uptake values (Cutoff value of SUV_{max} was 5.0), which normalizes activity for body weight and injected activity. Reconstruction of data sets and image analysis were done using Inveon Research Workplace software (Siemens Medical Solutions).

In vivo therapeutic efficacy studies

Two days after injection of AdAFP-TK (1×10^9 pfu/tumor) into tumors in HCC models, mice began receiving intraperitoneal injections of prodrug GCV (50 mg/kg) every day for 5 days.^{31,32} Control mice with HCC were treated with saline. Tumor growth was monitored by calculating the tumor volume from caliper measurements. Tumor volumes

were calculated according to the formula: (tumor volume; mm³) = (major axis; mm) $\times$ (minor axis; mm)²/2.

Statistical analysis

Results were expressed as means $\pm$ SD. Statistical analysis was conducted by the Student's *t*-test for comparisons between two groups using the GraphPad Prism 5.00 (GraphPad Software, Inc.), and a *p*-Value less than 0.05 was considered statistically significant.

Results

AFP-targeted HSV1-tk expression in vitro

HSV1-tk expression controlled by AFP enhancer/promoter in AdAFP-TK was confirmed by RT-PCR analysis (Fig. 1A) and cell uptake of ¹²⁵I-IVDU (Fig. 1B, C). RT-PCR analysis of infected cells including HuH-7 and HepG2 as AFP-producing HCC exhibited HSV1-tk expression by

FIG. 1. Analysis of AFP-targeted HSV1-tk gene expression by ¹²⁵I-IVDU uptake assay in adenovirus-infected cell lines. (A) RT-PCR analysis of HSV1-tk gene expression 24 hours after infection of adenovirus. (B, C) *In vitro* ¹²⁵I-IVDU uptake for validation of HSV1-tk activity targeted to AFP. HuH-7, HepG2, and HT-29 cells were infected with 0, 5, 10, 30, and 50 MOI of AdAFP-TK (B) and AdCMV-TK (C). Twenty-four hours after infection, cells were incubated with ¹²⁵I-IVDU for 2 hours and radioactivity was measured by gamma counter. ¹²⁵I-IVDU uptake was expressed as the percentage of the injected dose (%ID). AFP, α -fetoprotein.

AdAFP-TK. However, HSV1-tk expression in AFP-non-producing cell (SK-Hep-1 and HT-29) was not observed on AdAFP-TK infection with the same MOI (MOI=10). Therefore, HSV1-tk was specifically expressed at the transcription level in AFP-producing cells infected by AdAFP-TK (Fig. 1A). Functional HSV1-tk activity infected by AdAFP-TK or AdCMV-TK was determined by cell uptake of ^{125}I -IVDU. Cells infected by AdCMV-TK exhibited increased ^{125}I -IVDU uptake activities according to increasing virus MOI. HSV1-tk activity of AdCMV-TK infected HuH-7 and HepG2 was higher than that of HT-29, due to the presence of a CAR receptor in liver cells.³³ ^{125}I -IVDU uptake in HuH-7 and HepG2 infected by AdAFP-TK (MOI=10) was 16 and 20-fold higher than HT-29, respectively and increased with increasing virus MOI (HuH-7 and HepG2 was 53 and 161-fold higher than HT-29, respectively, at 50 MOI). These results suggested that HSV1-tk expression by AdAFP-TK infection was specifically targeted to AFP-producing cells.

In vitro cytotoxicity with AFP-targeted HSV1-tk expression and GCV

Cytotoxicity induced by HSV1-tk expression was evaluated at various GCV concentrations (0–100 μM) (Fig. 2). Cytotoxicity by AdCMV-TK and GCV was clearly demonstrated independent of AFP production by the target cells. Cell viability in AdCMV-TK (MOI=10) infected HuH-7, SK-Hep-1, and HT-29 was reduced by increasing GCV concentrations. However, cell viability in AdAFP-TK (MOI=10) infected HuH-7 was definitely reduced but not HT-29 and SK-Hep-1 at any GCV concentration up to 100 μM , similar to AdCMV-LacZ infection. Thus, GCV-mediated cytotoxic effect induced by AdAFP-TK infection was evident only in AFP-producing HCC.

In vivo bioluminescent and ^{18}F -FHBG PET imaging

To confirm the expression of HSV1-tk or *fLuc* gene by AdAFP-TK or AdAFP-fLuc infection in AFP-producing HCC using molecular imaging methods, a mixture of AdAFP-fLuc and AdAFP-TK was administrated via intratumoral injection into HepG2 and HuH-7 xenograft models. BLI and ^{18}F -FHBG PET images were obtained 24 hours after infection. As shown in Figure 3, fLuc and HSV1-tk expression, driven by AFP enhancer/promoter, were clearly visualized by BLI and ^{18}F -FHBG PET images in AFP-producing HCC. The tumor to muscle ratio was measured 3.46 and 5.27 in AdAFP-TK-injected HepG2 ($n=2$, Fig. 3B) and HuH-7 ($n=4$, Fig. 3C) xenograft model, respectively, while nonvirus-injected HuH-7 tumor ($n=4$, Fig. 3A) measured 1.16 in ^{18}F -FHBG PET imaging.

In vivo assessment of GCV-mediated tumor growth retardation and therapeutic effects by AFP-targeted HSV1-tk expression with ^{18}F -FDG PET

The therapeutic effects of HSV1-tk expression under the control of AFP enhancer/promoter were verified in the HuH-7 xenograft model as an AFP-producing HCC. *In vivo* GCV-mediated tumor growth retardation ($n=5$) after intratumoral injection with AdAFP-TK was apparent in tumor volume (Fig. 4A). HuH-7 tumors treated with saline ($n=3$) continued

FIG. 2. *In vitro* GCV-mediated cytotoxicity by AFP targeted HSV1-tk expression. Dose-dependent cytotoxicities of GCV with AdCMV-LacZ (◆), AdCMV-TK (Δ), and AdAFP-TK (□) were determined by MTT assay in HuH-7 (A), SK-Hep-1 (B), and HT-29 (C) cells. (A) AFP-producing HuH-7 cells showed dose-dependent cytotoxicity to GCV with AdAFP-tk similar to AdCMV-tk. (B, C) AFP-non-producing cells, SK-Hep-1, and HT-29, showed dose-dependent decrease of survival rate to GCV with AdCMV-tk alone. Cells were infected with adenovirus at MOI of 10. GCV, ganciclovir.

to grow. GCV treatment showed significant difference ($p<0.05$) in tumor size compared with the control group from the fourth day after treatment. Therapeutic effects with AdAFP-TK and GCV were determined by ^{18}F -FDG PET. As shown in Figure 4B, ^{18}F -FDG uptake in tumor lesion was scarcely changed in the AdAFP-TK and GCV-treated group, but consistently increased in the saline-treated group over the progression of time. The quantification of ^{18}F -FDG uptake in tumor was analyzed as SUV_{max} and presented in Figure 4C. Generally, SUV_{max} patterns showed a decreasing tendency after treatment with AdAFP-TK and GCV. The therapeutic effects of AdAFP-TK and GCV treatment in AFP-producing HCC could thus be monitored by ^{18}F -FDG PET.

FIG. 3. *In vivo* bioluminescent and ^{18}F -FHBG PET imaging after co-injection of the same titer of AdAFP-TK and AdAFP-fLuc via intratumoral injection. HuH-7 (A, C) or HepG2 (B) cells were implanted in the right forelegs of BALB/c nude mice. (A) Nonviral-infected HuH-7 xenograft model. (B, C) A mixture of AdAFP-TK (5×10^8 pfu) and AdAFP-fLuc (5×10^8 pfu) was injected into the tumor. ^{18}F -FHBG PET images (left panel) were obtained after bioluminescent image (right panel) acquisition. PET images were acquired for 20 minutes, 1 hour after intravenous administration of 7.4 MBq (200 μCi) of ^{18}F -FHBG by small animal PET. Arrows indicate HuH-7 tumors (A, C) and HepG2 tumors (B). PET, positron emission tomography; pfu, plaque-forming units.

Discussion

HCC is the most common malignancy of the liver³⁴ with $\sim 750,000$ new patients diagnosed each year; it is the third most lethal cancer.³⁵ Therefore, early diagnosis and effective therapeutics of HCC is pivotal for prolongation of the lifetime of patients. Since its initial identification about 50 years ago, AFP is a widely used HCC-marker although not satisfactory for early diagnosis because of poor sensitivity and specificity.^{8,36}

Despite safety issues, gene therapy is still a valuable therapeutic approach for treating cancers. Adenovirus is the most common expression vector system for therapeutic genes³⁷ and may be manipulated for targeted expression by using enhancer/promoter elements of tumor-specific marker proteins. Because of the limited duration of gene expression, adenoviral vector is a more desirable tool to treat cancer rather than to treat hereditary diseases. HCC treatment using an adenovirus system expressing HSV1-tk under AFP enhancer/promoter has already been tried by some research

groups, *in vivo* and *in vitro*^{9–11} and its efficacy has been evaluated by measuring the xenografted tumor size. Monitoring the accuracy of expression of the tumor targeted therapeutic genes with AFP enhancer/promoter system is important to ensure successful HCC therapy with avoiding adverse influence on normal organs. *In vivo* imaging provides real-time assessment of gene expression in living subjects.^{19,20} HSV1-tk has the dual function of imaging reporter genes with radioisotope-labeled nucleoside and therapeutic genes with GCV. In this study, after coinjection of AdAFP-TK and AdAFP-fLuc, fLuc and HSV1-tk expression driven by AFP enhancer/promoter were clearly visualized by BLI and ^{18}F -FHBG PET images in both HepG2 and HuH-7 xenograft tumor models (Fig. 3). The tumor to muscle ratio was significantly higher measurements as 3.46 and 5.27 in AdAFP-TK injected HepG2 ($n=2$) and HuH-7 ($n=4$) tumors, respectively, compared with nonvirus-injected HuH-7 tumor (1.16, $n=4$). This is the first report that visualized HSV1-tk expression by AdAFP-TK infection in AFP-producing HCC xenograft and

FIG. 4. *In vivo* GCV-mediated cytotoxicity on AFP targeted HSV1-tk expression and therapeutic assessment by ^{18}F -FDG PET. After xenografted HuH-7 tumors in the right shoulder reached to $\sim 500 \text{ mm}^3$, mice were given an intratumoral injection of 5×10^8 pfu of AdAFP-TK. Mice were daily treated with 50 mg/kg of GCV ($n=5$, $\square$) or saline ($n=3$, $\blacklozenge$) for 5 days by intraperitoneal injection. (A) Tumor volume ratio was calculated by dividing the tumor volume measured after treatment by the tumor volume measured before treatment (on day 0). Black arrows indicate GCV treatment day. $*p < 0.05$. (B) ^{18}F -FDG PET images were acquired at day 0, 3, 5, and 7 after GCV treatment. The upper panel and the lower panel show saline treatment group and AdAFP-TK and GCV treatment group, respectively. H and white arrows indicate heart and tumor, respectively. (C) The quantification of ^{18}F -FDG uptake in (B) was analyzed as SUV_{max} .

simultaneously acquired BLI with AdAFP-fLuc and PET images with ^{18}F -FHBG in an identical model.

GCV-mediated therapeutic effects by AFP targeted HSV1-tk expression could be longitudinally evaluated by measuring the tumor size and ^{18}F -FDG PET, as shown in Figure 4. Although the study had a relatively short-term investigation of 7 days, ^{18}F -FDG PET images showed a clearly decreased pattern in the AdAFP-TK and GCV-treated groups. ^{18}F -FDG PET appears to be of value in the therapeutic monitoring of tumors; but there is a limitation to the applicability of this imaging modality for all therapeutic regimens based on action mechanism of therapeutic drug and imaging acquisition time point post-therapy.²⁷ Tumor-specific therapy could be evaluated by measuring the reduction in tumor size or monitoring histopathological changes measured at the end point of treatment by obtaining the tumor tissues.³⁸ However, ^{18}F -FDG PET is able to monitor and compare treatment responses at any point during the treatment course. Wang et al. have reported that ^{18}F -FDG PET can be used as an additional surrogate biomarker for monitoring tumor responses to prodrug activation gene therapy with HSV1-tk and GCV in HSV1-tk-transduced sarcoma models; and ^{123}I -5-iodo-2'-fluoro-1- β -D-arabinofuranosyluracil (^{123}I -FIAU) SPECT as the most reliable imaging reagent for prediction of tumor response to therapy.³⁹ There is some

problem associated with longitudinal assessment by ^{18}F -FHBG of GCV tumor treatment on HSV1-tk expression, due to competition between GCV and ^{18}F -FHBG for the identical nucleoside transporter or HSV1-tk. Yaghoubi et al. reported that GCV treatment was halted 48 hours prior to scanning with ^{18}F -FHBG to avoid competition between GCV and ^{18}F -FHBG as substrates for HSV1-sr39TK enzyme for PET imaging of the HSV1-tk and GCV suicidal gene therapy in living subjects.⁴⁰ Therefore, to monitor the HSV1-tk and GCV therapeutic effect, ^{18}F -FDG PET would be more suitable than ^{18}F -FHBG PET during the GCV treatment.

AdAFP-fLuc could be efficiently targeted to an AFP-producing HCC xenograft model and a diethylnitrosamine-treated HCC model via intravenous injection route as in our previous study.²⁹ We assessed the targeted efficiency of intravenous injection of AdAFP-TK with ^{18}F -FHBG PET and found a lower targeting efficiency than that of AdAFP-fLuc (data not shown). Therefore, AdAFP-TK was introduced by intratumoral injection to increase *in vivo* therapeutic efficiency. Kaneko et al. likewise obtained AFP targeted *in vivo* therapy by direct injection of AdAFP-TK.¹⁰ The authors were unable to elucidate some factors that caused the difference in target gene expression between AdAFP-TK and AdAFP-fLuc and proposed to clarify the mechanism to further increase target gene expression.

The results obtained from this study showed that a recombinant adenoviral vector expressing HSV1-*tk* gene under the AFP enhancer/promoter (AdAFP-TK) was successfully produced and could be applied for AFP-targeted HCC gene therapy and imaging in AFP-producing HCC *in vivo*. In conclusion, HSV1-*tk* expression within the accurate lesion and longitudinal therapeutic efficiency could be evaluated by ^{18}F -FDG PET.

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