INTRODUCTION

Multimodality molecular imaging (MMI), using positron emission tomography (PET), magnetic resonance spectroscopy (MRS) and optical (fluorescent and bioluminescent) techniques, offers powerful tools in unraveling biological processes in cancer diagnosis and treatment.1 MMI also has a crucial role in developing and optimizing gene therapy for cancer, such as gene-directed enzyme prodrug therapy (GDEPT) or ‘suicide gene therapy’.2,3 Suicide genes, such as herpes simplex virus type-1 thymidine kinase (HSV1-TK), cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT), simultaneously function as effector genes for GDEPT and marker genes for MMI. As an effector, TK converts the prodrug ganciclovir (GCV) into toxic products and, as a marker, leads to the trapping of the marker substrate 124I-FIAU (or 18F-FEAU, 18F-FHBG and so on) for PET imaging.4–6 Similarly, CD and UPRT function as effector genes, with CD converting the prodrug 5-fluorocytosine (5-FC) to the chemotherapeutic drug 5-fluorouracil (5-FU) and UPRT converting 5-FU to the cytotoxic compound (fluoronucleotides, FNuc). As marker genes they permit MRS imaging (MRSI) of the spatial distributions of 5-FC, 5-FU and FNuc, thus assessing the function and activity of the CD and UPRT genes.7–9 Individually, MRS and PET are powerful molecular imaging methods, each of which has contributed to improve cancer gene therapy. The combined use of both modalities may be even more powerful, motivating the development of combined PET/MRI units.10,11

As GDEPT employing single gene of HSV1-TK, CD, or UPRT has been demonstrated to be effective, efforts were focused on GDEPT combining two of these genes (double suicide gene therapy). Several studies have demonstrated that integrated CD/5-FC and HSV1-TK/GCV strategies is more effective with synergistic effects when compared with either strategy alone.12,13 Simultaneous expression of CD and UPRT genes could lead to synergistic effects resulting in increased sensitivity to 5-FC.14,15 Among double suicide gene therapy strategies, the combination of HSV1-TK/GCV and CD/5-FC is most widely studied and has been evaluated in clinical trials.16,17 Subsequently, a reporter gene system (the human sodium iodide symporter) was incorporated into a double suicide gene (TK/CD) adenovirus, and Na99mTcO4 single-photon emission computed tomography images demonstrated gene expression after virus injection into human prostate cancer.18 Thus, it was suggested that MMI can be used to monitor adenovirus-mediated suicide gene therapy.18

The aim of the present preclinical study is to demonstrate that the therapeutic efficacy of the triple-suicide-gene approach is improved relative to previous methods, and that MMI can be used to monitor the delivery and evaluate the distribution and function/activity of the triple suicide gene.

MATERIALS AND METHODS

Plasmids

Three plasmids were constructed: (1) pCMV-TK/enhanced green fluorescent protein (eGFP) contains the HSV1-tk and egfp fusion gene under the control of a cytomegalovirus (CMV) promoter and a neomycin-resistant gene, (2) pCMV-CD/monomeric dsred (mDsRed) containing the cd (of Saccharomyces cervisiae) and mDsRed fusion gene, and (3) pCMV-CD/UPRT/mDsRed containing the cd, uprt (of Haemophilus influenzae) and mDsRed fusion gene under the control of the CMV promoter. The latter two plasmids contain the hygromycin B resistance gene.7

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Cell cultures and stably transfected cell lines

R3327-AT rat prostate carcinoma cells were maintained in Dulbecco's Modified Eagle medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Gemini, West Sacramento, CA, USA). The cells were cotransfected with two plasmids, pCMV-CD/mDsRed and pCMV-TK/eGFP, or pCMV-CD/UPRT/mDsRed and pCMV-TK/eGFP, using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Subsequently, the transfected cells were cultured in medium containing hG418 (0.2 mg ml⁻¹; Roche, Mannheim, Germany) and G418 (0.5 mg ml⁻¹; Calbiochem, San Diego, CA, USA). Stable transfecents were selected with fluorescence activated cell sorting using a cell sorter (MoFlo; Dako, Carpinteria, CA, USA). Single-cell-derived clones with the positive mDsRed and eGFP expression were isolated, expanded, their mDsRed and eGFP expression verified by flow cytometry and were used for further experiments. All cells were grown as monolayers at 37 °C in a humidified incubator with 5% CO₂ and 95% air. Stably transfected cells were designated as TKCD cells and TKCDUPRT cells, respectively (eGFP and mDsRed were omitted for simplicity).

Western blot analysis

The sheep anti-CD polyclonal antibody was purchased from Biotrend (Cologne, Germany) but the anti-HSV1-TK monoclonal antibody was kindly provided by Dr WC Summers (Yale University, New Haven, CT, USA). The secondary antibodies were the horseradish peroxidase-labeled bovine anti-sheep IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or sheep anti-mouse IgG (Pierce, Rockford, IL, USA). The protein expression was visualized using the Supersignal chemiluminescent substrate (Pierce).

Flow cytometric analysis

The expression of eGFP and mDsRed was analyzed by flow cytometry using the cell sorter (MoFlo). Parental cells, cells constitutively expressing only TK/eGFP or CD/mDsRed, were analyzed in parallel as a negative control and eGFP-positive or mDsRed-positive controls, respectively.

Fluorescence microscopy

The cells were fixed with freshly prepared 4% paraformaldehyde for 10 min and rinsed twice with phosphate-buffered saline. The fluorescent images were acquired at 575 nm wavelength for mDsRed and at 488 nm for eGFP using a fluorescence microscope (Axiovert 200M, Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Drug cytotoxicity, radiation survival and colony-formation assay

Cells were treated with GCV (Sigma-Aldrich, St Louis, MO, USA), 5-FU (InvivoGen, San Diego, CA, USA) or 5-FU (InvivoGen) at various concentrations for 24 h. Thereafter, cells were trypsinized, counted, serially diluted and plated into 60-mm dishes. After incubation for 10–14 days, colonies were stained with crystal violet and counted. Cell survival curves were constructed by plotting the surviving fractions as a function of concentration. For radiosensitization experiments, cells were treated with GCV or 5-FU alone or in combination for 24 h, and then irradiated with graded doses using a Cs-137 unit (Mark 1 model 68; Shephard and Associates, San Fernando, CA, USA) at ~2.0 Gy min⁻¹. Cell survivals were determined by colony-formation assay and plotted as a function of radiation doses.

Animal xenografts

Animal protocols were approved by the Institutional Animal Care and Use Committee at Memorial Sloan-Kettering Cancer Center. Tumor xenografts were formed by injecting 3 × 10⁶ cells subcutaneously into the hind legs of 6- to 8-week-old male nude mice (athymic nu/nu; NCI Frederick Cancer Research Institute, Frederick, MD, USA). Each tumor was measured with digital caliper in three orthogonal dimensions (a, b and c), and tumor volume was calculated as πabc/6. Experiments were performed when the tumors reached a volume of ~ 500 mm³ for imaging study, or a volume of ~ 150 mm³ for tumor growth delay study.

19F MRS and MRS imaging

In vivo 19F MRS was performed using a Bruker 7T spectrometer. Mice were anesthetized with isoflurane and positioned in a specially designed animal holder. 19F MRS spectra were acquired and averaged over 10 min before

and after the intravenous administration of 150 mg kg⁻¹ 5-FU or 5-FC for a maximum of 1 h, using a single pulse with a time of repetition of 1 s and sweep width of 15 kHz. A glass sphere (18 μl) filled with 75 mmol l⁻¹ sodium fluoride (NaF) was used as an external reference for quantitation. The MR time domain data were analyzed using XsOs NMR (Columbia University, New York, NY, USA). The spectral area of the acquired data was obtained from a Lorentzian fit to the spectra after line broadening (30 Hz). Metabolite ratios were calculated by normalizing their resonance-peak areas to that of NaF. Graphic representation of the spectroscopic data (5-FC/NaF, 5-FU/NaF and FNuc/NaF) shows the average ± s.d. of the given ratio at each time point.

Preceding the acquisition of 124I-MRSI, T2-weighted proton images were acquired for anatomic localization of the MRSI spectra. Imaging parameters included: field of view = 30 mm × 30 mm, matrix size = 256 × 256, six averages, time of repetition = 2 s and time of echo = 30 ms. The MRSI sequence was used to acquire slice-selective two-dimensional images in the coronal plane with respect to the tumor at 7T with 13-phase encode steps at effective in-plane resolutions of 3.75 mm × 3.75 mm with a field of view = 30 mm × 30 mm, matrix size = 8 × 8, slice thickness = 4 mm, 1024 data points with a spectral width of 15 kHz, 60° flip angle, time of repetition = 1 s and 4092 scans.

124I-FIAU microPET imaging

Two to three hours after MRS measurements, 7.4 MBq of 124I-FIAU was intravenously injected with the thyroid blocked by adding SSKI (Super Saturated Potassium iodide) to their drinking water 1 day ahead. The whole-body microPET images were acquired 16–18 h afterwards at the Focus 120 microPET scanner (Concorde Microsystems, Knoxville, TN, USA) under anesthesia. General, a minimum of ~10 million events were acquired in 20–40 min, depending on the administered activity and the time post injection. The image data were corrected for nonuniformity of response, dead time count losses and physical decay. An empirically determined system calibration factor was used to convert voxel count rates (adjusted for the 124I branching ratio) to activity concentrations and the resulting data were normalized to the administered activity to yield percent of the injected dose per gram of tissue.

In vivo/ex vivo fluorescent imaging

Immediately after PET imaging, whole-body fluorescent images were acquired with a Maestro imaging system (CRI, Woburn, MA, USA) under anesthesia. The fluorescent images of eGFP and mDsRed were acquired using the 455 nm (435–480 nm) excitation per 490 nm long-pass emission filters and the 523 nm (503–548 nm) excitation per 560 nm long-pass emission filters, respectively. The images were unmixed with the tissue autofluorescence spectrum using linear least-squares optimization. After in vivo imaging, the tumors were dissected and their fluorescent images were acquired. Immediately after the ex vivo imaging, the tumors were embedded in OCT, frozen with dry ice and cut into 8-μm sections for autoradiography and fluorescent microscopy.

Autoradiography and fluorescent microscopy of tumor sections

To acquire the distributions of 124I-FIAU in the tumor sections, phosphor plate imaging was employed with 13h exposure. Digital autoradiographs were processed using a Fujifilm BAS-1800II bioimaging analyzer (Fuji Photo Film, Tokyo, Japan). Whole tumor eGFP and mDsRed fluorescent images from the same sections were acquired using a fluorescent microscope (Axiovert 200M) after the autoradiography exposure as previously described.

Tumor growth delay

The mice bearing TKCD or TKCDUPRT tumors were divided into five groups and treated with (1) phosphate-buffered saline, (2) 500 mg kg⁻¹ 5-FC plus 30 mg kg⁻¹ GCV, intraperitoneally administered (standard dose), (3) 25 mg kg⁻¹ 5-FC plus 1.5 mg kg⁻¹ GCV intraperitoneally (low dose), (4) radiation (3 Gy) or (5) radiation combined with drug treatment (25 mg kg⁻¹ 5-FC plus 1.5 mg kg⁻¹ GCV intraperitoneally, 2h before radiation). The tumors were irradiated using the Cs-137 unit (Mark 1 model 68) within a home-made animal holder. The treatments were applied once a day for 5 days. Ten mice were used in each group. For six mice, the tumor volume was measured three times a week until the average volume reached ~1500 mm³. The tumor volume relative to those at the beginning of treatments was calculated and plotted as a function of time after

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treatments. The difference in time (days) of tumor volume reaching \( \sim 1500 \text{ mm}^3 \) for treated groups relative to controlled group (relative growth delay) was calculated and compared. For other four mice, the tumors were dissected at 24 h after the last treatment, fixed with 4% paraformaldehyde, embedded with paraffin and cut into 4-\( \mu \text{m} \) sections for the immunohistochemistry staining.

Immunohistochemistry staining and imaging acquisition

The detection of proliferation and apoptosis in tumor sections was achieved by immunohistochemical staining using the rabbit anti-Ki-67 and anti-cleaved caspase-3 primary antibodies and goat anti-rabbit second antibodies, respectively (Cell Signaling Technology, Danvers, MA, USA). Finally, the sections were stained with hematoxylin and eosin. The sections were scanned on a digital histology platform (Mirax; Carl Zeiss, Göttingen, Germany) at \( \times 200 \) magnification. Composite images of sections were generated from individual microscopic images by the software Mirax viewer. Ki-67- and CCP3 staining positive cells were counted and compared between different groups.

Statistical analysis

Averages are presented as the mean ± s.e. Difference in cytotoxicity and radiosensitivity was determined using the Student’s \( t \)-test. The difference in Ki-67 or CCP3 staining was analyzed using the Student’s \( t \)-test, and tumor growth delay using one-way analysis of variance. A \( P \)-value of \( <0.01 \) was considered statistically significant.

Figure 1. Characteristics of the cell lines stably expressing both (i) thymidine kinase/enhanced green fluorescent protein (TK/eGFP) and cytosine deaminase/uracil phosphoribosyltransferase/monomeric dsred (CD/UPRT/mDsRed) (designated as TKCDUPRT cells), and (ii) TK/eGFP and CD/mDsRed (designated as TKCD cells). (a) Western blot. Anti-herpes simplex virus type-1 TK (HSV1-TK) monoclonal antibody (left panel) and anti-CD polyclonal antibody (right panel) was used, with re-stained \( \beta \)-actin as internal control. Protein samples from parental cells and the cells expressing CD/mDsRed (designated as CD cells), CD/UPRT/mDsRed (designated as CDUPRT cells) or TK/eGFP (designated as TK cells) were used as controls. The protein bands for CD/mDsRed (43 kDa), CD/UPRT/mDsRed (73 kDa) or TK/eGFP (65 kDa) were indicated by arrows on the right side of the western blots, respectively. (b) Flow cytometry analysis of TKCD (top panel) and TKCDUPRT (bottom panel) cells, with eGFP intensity along the \( x \) axis, and mDsRed along the \( y \) axis. The gates (dashed lines) were set to include \( <1\% \) of parental cells, cells expressing only eGFP and cells expressing only mDsRed. The \% of cells positive for both eGFP and mDsRed are given in the upper right corner. (c) Phase-contrast (upper panel) and fluorescence (eGFP—middle panel; mDsRed—lower panel) microscopic images at \( \times 200 \) magnification, of parental (left column), TKCD (middle column) and TKCDUPRT cells (right column). (d) Surviving fractions of parental (\( \bullet \)), TKCD (\( \circ \)) and TKCDUPRT (\( \triangledown \)) cells after a 24-h exposure to different concentrations of ganciclovir (GCV) (left panel), 5-fluorouracil (5-FU) (middle panel) or 5-fluorocytosine (5-FC) (right panel). Each point represents the mean ± s.e. from three experiments.
RESULTS

*In vitro* characterization of cells expressing gene multiplex.

We established rat prostate cancer cell lines, which stably express both TK/eGFP and CD/mDsRed (designated as TKCD cells), or both TK/eGFP and CD/UPRT/mDsRed (designated as TKCDUPRT cells), respectively. Expression of the transduced fusion genes were assessed by western blot, flow cytometry and fluorescent microscopy. Immunoblot with HSV1-TK antibody showed a 65-kDa band in the TKCD and the TKCDUPRT samples, and in the TK control sample (cells only expressing TK/eGFP; Figure 1a, left panel).
The expression of TK, CD, and UPRT on cell and tumor response to 5-FC, GCV, and radiation

In vitro, the simultaneous expression of these genes significantly enhances the cytotoxicity to GCV and 5-FC. Whereas TKCDUPRT cells were sensitive to GCV and 5-FC administered as single agent (Figure 1d), concurrent treatment with both GCV and 5-FC resulted in much greater cytotoxicity (Figure 3a, left panel) with significant synergistic action between the two prodrugs (Supplementary Figure 2). TKCD cells were also sensitive to combined treatment of GCV and 5-FC (Figure 3a, right panel), but ~20-fold higher 5-FC doses were needed to induce the similar levels of cytotoxicity.

To study the bystander effect, admixtures of parental and transduced cells, in the ratio of 4:1, were treated with GCV and 5-FC alone or in combination. The results (Supplementary Figures 3A and B) indicated that the admixture of parental and TKCDUPRT cells was much more sensitive than that of parental and TKCD cells for all treatment scenarios.

To provide in vivo validation of the efficacy of triple-suicide-gene approach, tumor-bearing mice were treated with the standard doses of GCV (30 mg kg\(^{-1}\)) and 5-FC (500 mg kg\(^{-1}\)), or lower doses (1.5 mg kg\(^{-1}\) GCV and 25 mg kg\(^{-1}\) 5-FC), daily for 5 days. The volumes of TKCDUPRT tumors, measured at various times, showed significant growth delay at the lower drug dose (left panel, Figure 3b, triangles) and complete tumor regression at the standard dose, with no recurrence within the 40-day observation period (left panel, Figure 3b, solid squares). Growth delay was also observed in TKCD tumors treated with GCV and 5-FC (Figure 3b, right panel), but the effect was much less relative to that of TKCDUPRT tumors.

At the histological level, in TKCDUPRT tumors treated with the standard dose cell proliferation was significantly attenuated (Figure 4a, upper panel) and extensive apoptosis was observed (Figure 4b, upper panel). In quantitative analysis the Ki-67-positive

Figure 3. Effect of coexpression of thymidine kinase (TK), cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT) on cellular and tumor response to ganciclovir (GCV) and 5-fluorocytosine (5-FC). (a) Surviving fractions of TKCDUPRT (left panel) or TKCD (right panel) cells treated with various dose of GCV (●), 5-FC (○) or GCV + 5-FC (▲) for 24 h. (b) Tumor growth kinetics in mice bearing TKCDUPRT tumors (left panel) or TKCD tumors (right panel): ( ■) untreated, ( □) treated with five daily standard dose of GCV (30 mg kg\(^{-1}\)) and 5-FC (500 mg kg\(^{-1}\)), ( △) treated with five daily lower dose of GCV (1.5 mg kg\(^{-1}\)) and 5-FC (25 mg kg\(^{-1}\)). Six mice were used for each group.

Multimodality and multiparametric molecular imaging

When transplanted into nude mice, the TKCDUPRT cells form tumors, which allow multimodality and multiparametric imaging studies of transgene expression/function, all in the same tumor as illustrated in the schematics of Figure 2a.

As CD catalyzed the conversion of 5-FC to 5-FU, and UPRT evince the function and activity of the HSV1-TK, CD and UPRT enzymes in the transduced cells.

As an alternative approach of in vivo biodistribution of TKCDUPRT tumors were assessed for their constitutive expression of TK and CD in the TKCD and TKCDUPRT cells, enhanced the cellular sensitivity to GCV and 5-FC compared with that of the parental cells. Similarly, the expression of UPRT in the TKCDUPRT cells enhanced their sensitivity to 5-FU and 5-FC, relative to that of the TKCD cells (Figure 1d). These observations evince the function and activity of the HSV1-TK, CD and UPRT enzymes in the transduced cells.

After the NMR studies, the same TKCDUPRT tumors were used for each group.

At the histological level, in TKCDUPRT tumors treated with the standard dose cell proliferation was significantly attenuated (Figure 4a, upper panel) and extensive apoptosis was observed (Figure 4b, upper panel). In quantitative analysis the Ki-67-positive positive...
counts in 5-FC + GCV-treated and control tumors was 90.7 ± 7.2 and 328.9 ± 12.8 (Figure 4a, right panel, \( P < 0.01 \)), respectively; and the CCP3-positive index in treated and control tumors was 152.3 ± 15.5 and 26.1 ± 3.1 (Figure 4b, right panel, \( P < 0.01 \)), respectively. In treated TKCD tumors, although cellular proliferation was decreased (Figure 4b, lower panel) the Ki-67-positive index in 5-FC + GCV-treated and control tumors was 122.3 ± 7.7 and 210.9 ± 14.8 (Figure 4a, right panel, \( P < 0.01 \)), respectively. In addition, the apoptotic index were slightly increased (Figure 4a, lower panel) with CCP3-positive index in 5-FC + GCV-treated and control tumors of 37.3 ± 3.5 and 16.9 ± 1.5 (Figure 4b, right panel, \( P > 0.01 \)), respectively.

As was previously shown, expression TK, CD and UPRT led to enhanced response to produrg treatment. Here we present data showing that cells and tumors expressing TK, CD and UPRT, and treated with prodrugs are more radiosensitive. As shown in Figure 5a, TKCD and TKCDUPRT cells have similar radiosensitivity without produrg. Single-agent treatment with GCV or 5-FC slightly enhanced the radiation response, but combined treatment with both 5-FC and GCV resulted into greater radiosensitization. In addition, the degree of radiosensitization is much higher in TKCDUPRT cells than TKCD cells, such that the 5-FC dose needed is only 1/50 to achieve the same radiosensitivity. A similar effect was observed for cell mixture of 80% parental and 20% TKCDUPRT cells (Supplementary Figure 2C). In growth delay assay, radiation (3 Gy per fraction per day for 5 days) alone slightly delayed the tumor growth, but when combined with low-dose treatment of GCV and 5-FC it produced significant tumor growth delay (Figure 5b). Again, the effect was greater in TKCDUPRT tumors. The relative growth delay (days) due to 5-FC + GCV and 5-FC + GCV + radiation treatments was much longer in TKCDUPRT tumors than that in TKCD tumors (\( P < 0.01 \)).

**DISCUSSION**

In previous efforts, we constructed expressing vectors of CDUPRT fused with red fluorescent protein (mDsRed) and HSV1-TK fused with eGFP, and generated tumor models in which expression of the respective gene complexes can be monitored with MRS/optical or PET/optical imaging, respectively. 7,19 In the present study we report for the first time the triple-suicide-gene approach, using TK, CD and UPRT. Importantly, all three genes function simultaneously as effectors for GDEPT and markers for MMI. It is demonstrated that MMI can evaluate the distribution and...
function/activity of the triple suicide gene. The concomitant expression of these genes significantly enhances prodrug cytotoxicity and radiosensitivity in vitro and in vivo. Our data convincingly demonstrate in proof-of-principle that integrating MMI and triple suicide gene therapy can significantly improve the efficacy of combinatorial cancer treatment.

Both PET and MRI techniques are important in developing and optimizing gene therapy, initially in laboratory setting and then translated into clinical studies. Jacobs et al. employed 19F-MRSI PET to monitor liposomal vector-mediated HSV1-TK gene expression in five patients with recurrent glioblastomas. Their preliminary findings showed that FIAU PET imaging of HSV-1-tk expression in patients is feasible and that vector-mediated gene expression may predict the therapeutic effect. Quantitative MRSI of 5-FU pharmacokinetics in patients has been reported previously.

Clinical trials of TKCD cancer gene therapy suggest that MRI and PET monitoring of gene therapy with MRI and PET could have immediate implications for the investigation of gene therapy strategies. Our data clearly showed that the prodrug activation in TKCDUPRT-expressing tumors could be measured with 19F-MRSI (for CDUPRT). As hybrid PET/MRI scanner has been developed from the preclinical prototype to clinical platforms, visualization and quantification of triple suicide gene expression/function in a single system will become available.

There has been steady progress in applying GDEPT or suicide gene therapy to cancer treatment, from single suicide gene therapy (for example, using HSV1-TK with GCV, or CD with 5-FC) to the combined use of two suicide genes (for example, CD and HSV1-TK, or CD and UPRT), resulting in improved tumoricidal effect for different tumors. Both the in vitro and in vivo data in the present study (Figures 3–5 and Supplementary Figure 2) clearly demonstrated that the triple-suicide-gene therapy approach is more effective in in vivo tumors than double suicide gene therapy employing HSV1-TK/GCV and CD/5-FC, which has been tested in clinical trials. The potential advantage of suicide gene therapy relevant for clinical application is its radiosensitization effect, as demonstrated in our study. We clearly showed that the coexpression of HSV1-TK, CD and UPRT triple genes not only significantly increased the sensitivity of GCV and 5-FC but also improved the radiosensitizing effect of GCV and 5-FC, relative to that induced by double suicide gene HSV1-TK and CD strategy (Figure 5). In this regard, it has been shown that HSV1-TK/GCV gene therapy may inhibit the repair of radiation-induced sublethal DNA damage. Others have suggested that the radiosensitization in CD/5-FC and CD/UPRT/5-FC approaches is mediated through the inhibition of thymidylate synthase by 5-FC/LMPT, resulting in the depletion of deoxythymidine-phosphate pools and increased DNA strand break, as well as redistribution of cells to the radiosensitive early S phase. Previously, we have demonstrated that CDUPRT/5-FC approach has greater radiosensitization effect than the CD/5-FC system. Therefore, it is not surprising that significant tumor control effects were found as triple-suicide-gene approach combined with radiation.

The difficulty in achieving efficient gene delivery to all cells in a tumor mass in vivo is a major limitation for successful cancer gene therapy. Therefore, the bystander effect is an important feature of suicide gene therapy, whereby the surrounding but untransduced cells are inactivated by the diffusion of toxic metabolites derived from the transduced cells. Our data clearly demonstrated that a strong bystander effect was associated with the triple-suicide-gene approach. Mixed cell populations in which only 20% of cells expressing TK, CD and UPRT were much more sensitive to GCV and 5-FC treatments than cells expressing TK and CD (Supplementary Figure 2A). More importantly, an enhanced radiosensitizing effect was observed in the cell population that consisted only 20% cells expressing TK, CD and UPRT after treatment with GCV plus 5-FC (Supplementary Figure 2B).

The studies were done in nude mice and eliminated the acquired immune response, which has been shown to be active, at least in the HSV1-tk therapy. It suggests that our approach might work better in intact animals. Although this study involved the use of stably transfected cell lines and associated tumor models with different transgene expression, drug sensitivity and growth rates, future studies plan to translate this strategy to clinical application. Specifically, we are creating adenovirus vectors armed with TK, CD and UPRT genes to deliver them into in vivo tumor models and to test whether triple gene transduction following GCV and 5-FC treatment will improve the efficacy of radiotherapy. As shown in present study, molecular imaging using PET and MRI can be used to monitor the function/activity of a triple suicide gene. Although the clinical utility of molecular imaging in suicide gene therapy must await clinical trials, we can illustrate its potential use with the following example. Suppose in a particular patient, PET and/or MRI imaging suggest heterogeneity in the expressions of the triple suicide gene. Clinical studies will be needed to evaluate this approach in cancer patients.

Figure 5. Effect of coexpression of thymidine kinase (TK), cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT) on cellular and tumor response to graded doses of radiation, by itself, or in combination with ganciclovir (GCV) and 5-fluorocytosine (5-FC). (a) Surviving fractions of TKCDUPRT cells (left panel) or TKCD cells (right panel) treated with radiation alone (●) or in combination with GCV (○), 5-FC (▲), or both GCV and 5-FC (△). Mean ± s.e. were calculated from three experiments. (b) Growth kinetics of TKCDUPRT tumors (left panel) or TKCD tumors (right panel): (○) untreated, (●) radiation only (3 Gy per day for 5 days), (△) treated with lower doses of GCV (1.5 mg kg\(^{-1}\)) and 5-FC (25 mg kg\(^{-1}\)), (▲) radiation (3 Gy per day for 5 days) after lower dose of GCV (1.5 mg kg\(^{-1}\)) and 5-FC (25 mg kg\(^{-1}\)). Six mice were used for each group. Time of tumor volume reaching ~1500 mm\(^3\) relative to the controlled group was compared among groups (one-way analysis of variance). Error bars represent the s.e. of six mice. &: P < 0.01, #: P > 0.01.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES
1 Frangioni JV. New technologies for human cancer imaging. J Clin Oncol 2008; 26: 4012–4021.
2 Rome C, Couillaud F, Moonen CT. Gene expression and gene therapy imaging. Eur Radiol 2007; 17: 305–319.
3 Bhaukik S. Advances in imaging gene-directed enzyme prodrug therapy. Curr Pharm Biotechnol 2011; 12: 497–507.
4 Yaghoubi SS, Barrio JR, Namavari M, Satyamurthy N, Phelps ME, Herschman HR et al. Imaging progress of herpes simplex virus type 1 thymidine kinase suicide gene therapy in living subjects with positron emission tomography. Cancer Gene Ther 2005; 12: 329–339.
5 Gambhir SS, Barrio JR, Phelps ME, Iyer M, Namavari M, Satyamurthy N et al. Imaging adeno-associated reporter gene expression in living animals with positron emission tomography. Proc Natl Acad Sci USA 1999; 96: 2333–2338.
6 Tjvajev JG, Finn R, Watanabe K, Joshi R, Oku T, Kennedy J et al. Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a potential method for monitoring clinical gene therapy. Cancer Res 1996; 56: 4087–4095.
7 Xing L, Deng X, Kotedia K, Ackerstaff E, Ponomarev V, Ling CC et al. Noninvasive molecular and functional imaging of cytosine deaminase and uracil phosphoribosyltransferase fused with red fluorescence protein. Acta Oncol 2008; 47: 1211–1220.
8 Hamstra DA, Lee KC, Tychewick JM, Scheepkin VO, Moffat BA, Chen M et al. The use of 19F spectroscopy and diffusion-weighted MRI to evaluate differences in gene-dependent enzyme prodrug therapies. Mol Ther 2004; 10: 916–928.
9 Gade TP, Koutcher JA, Spees WM, Beattie BJ, Ponomarev V, Dobrovin M et al. Imaging transgene activity in vivo. Cancer Res 2008; 68: 2878–2884.
10 Judenhofer MS, Wehrl HF, Newport DF, Catana C, Siegel SB, Becker M et al. Simultaneous PET-MRI: a new approach for functional and morphological imaging. Nat Med 2008; 14: 459–465.
11 Catana C, Wu Y, Judenhofer MS, Qi J, Pichler BJ, Cherry SR. Simultaneous acquisition of multislice PET and MR images: initial results with a MR-compatible PET scanner. J Nucl Med 2006; 47: 1968–1976.
12 Aghi M, Kromm CM, Chou TC, Breakefield XO, Chiocca EA. Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. J Natl Cancer Inst 1998; 90: 370–380.
13 Boucher PD, Im MM, Freytag SO, Shewach DS. A novel mechanism of synergistic cytotoxicity with 5-fluorocytosine and ganciclovir in double suicide gene therapy. Cancer Res 2006; 66: 3230–3237.
14 Erbs P, Regulier E, Kintz J, Leroy P, Poitevin Y, Exinger F et al. In vivo cancer gene therapy by adenovirus-mediated transfer of a bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene. Cancer Res 2000; 60: 3813–3822.
15 Xing L, Sun X, Deng X, Kotedia K, Urano M, Koutcher JA et al. Expression of the bifunctional suicide gene CDUPRT increases radiosensitization and bystander effect of 5-FC in prostate cancer cells. Radiother Oncol 2009; 92: 345–352.
16 Freytag SO, Movsas B, Aref I, Stricker H, Peabody J, Pegg J et al. Phase I trial of replication-competent adenovirus-mediated suicide gene therapy combined with IMRT for prostate cancer. Mol Ther 2007; 15: 1016–1023.
17 Freytag SO, Stricker H, Pegg J, Pailidi D, Pradhan DG, Peabody J et al. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. Cancer Res 2003; 63: 7407–7506.
18 Barton KN, Stricker H, Brown SL, Elshaikh M, Aref I, Lu M et al. Phase I study of noninvasive imaging of adenovirus-mediated gene expression in the human prostate. Mol Ther 2008; 16: 1761–1769.
19 He F, Deng X, Wen B, Liu Y, Sun X, Xing L et al. Noninvasive molecular imaging of hypoxia in human xenografts: comparing hypoxia-induced gene expression with endogenous and exogenous hypoxia markers. Cancer Res 2008; 68: 8597–8606.
20 Jacobs A, Voges J, Reszka R, Lercher M, Gossmann A, Kracht L et al. Positron-emission tomography of vector-mediated gene expression in gene therapy for gliomas. Lancet 2001; 358: 727–729.
21 Li CW, Negendank WG, Padavic-Shaller KA, O’Dwyer PJ, Murphy-Boesch J, Brown TR. Quantitation of 5-fluorouracil catabolism in human liver in vivo by three-dimensional localized 19F magnetic resonance spectroscopy. Clin Cancer Res 1996; 2: 339–345.
22 Boss A, Bisadas S, Kolb A, Hofmann M, Ennemann U, Claussen CD et al. Hybrid PET/MRI of intracranial masses: initial experiences and comparison to PET/CT. J Nucl Med 2010; 51: 1198–1205.
23 Qiu Y, Peng GL, Liu QC, Li FL, Zou XS, He JX. Selective killing of lung cancer cells using carcinoembryonic antigen promoter and double suicide genes, thymidine kinase and cytosine deaminase (pCEA-TV/CD). Cancer Lett 2007; 255: 18–27.
24 Freytag SO, Kim JH, Brown SL, Barton K, Lu M, Chung M. Gene therapy strategies to improve the effectiveness of cancer radiotherapy. Expert Opin Biol Ther 2004; 7: 1475–1777.
25 Hwang HS, Davis TW, Houghton JA, Kinsella TJ. Radiosensitivity of thymidine synthase-deficient human tumor cells is affected by progression through the G1 restriction point into S-phase: implications for fluoropyrimidine radiosensitization. Cancer Res 2000; 60: 92–100.
26 Khatri A, Zhang B, Doherty E, Chapman J, Ow K, Pwint H et al. Combination of cytotoxic deaminase with uracil phosphoribosyltransferase leads to local and distant bystander effects against RM1 prostate cancer in mice. J Gene Med 2006; 8: 1086–1096.

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