

A double suicide gene system driven by vascular endothelial growth factor promoter selectively kills human hepatocellular carcinoma cells

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Abstract. The aim of the present study was to investigate the selective killing effect on hepatocellular carcinoma (HCC) cells of an adenovirus (Ad)-mediated cytosine deaminase (CD) in combination with thymidine kinase (TK) suicide gene system, driven by the vascular endothelial growth factor promoter (VEGFp), *in vitro* and *in vivo*. A double suicide gene system with VEGFp, named Ad-VEGFp-CDglyTK, was constructed and transfected into human HCC cells (BEL-7402 or HepG2; the latter cell type is deficient in VEGF) and human umbilical vein vascular endothelial cells (HUVEC). Green fluorescent protein expression was detected by fluoroscopy to verify transfection efficiency, and *CDglyTK* gene expression was detected by reverse transcription-polymerase chain reaction (PCR). The selective killing effect of Ad-VEGFp-CDglyTK was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and flow cytometry (FCM) *in vitro* and by xenograft studies *in vivo*. PCR revealed that the transgenic *CDglyTK* gene was expressed in BEL-7402 cells and HUVEC, but not in HepG2 cells. The cell survival rate significantly decreased in line with increasing concentrations of the prodrugs, ganciclovir (GCV) alone, 5-fluorocytosine (5-FC) alone or a combination of the two, in HUVEC and BEL-7402 cells with the transfected *CDglyTK* gene, but not in untransfected HUVEC or BEL-7402 cells, or in transfected or untransfected HepG2 cells. This result was additionally confirmed by FCM. GCV and 5-FC inhibited the HUVEC and BEL-7402 cells containing the transfected *CDglyTK* gene and also inhibited adjacent unmodified cells via the 'bystander effect'. No similar results were observed

in HepG2 cells. Compared with the control group, tumors with the transfected *CDglyTK* gene were smaller and the microvessel density of the tumor tissue was significantly decreased. It was concluded that a combination TK/GCV and CD/5-FC suicide gene system driven by VEGFp may provide a promising treatment strategy for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most commonly observed malignant tumor globally, and the third leading cause of cancer-associated mortality (1-3). Surgery, chemotherapy, radiotherapy, immunotherapy and liver transplantation are typically used to treat patients with HCC (4). However, the 5-year age-standardized relative survival rates for HCC have not improved significantly over the past 10 years (5). Knowledge of the molecular mechanisms of HCC brings opportunities for therapeutic interventions against this type of cancer using novel approaches.

Suicide gene therapy, based on gene-directed enzyme prodrug therapy, is a promising alternative treatment to conventional chemotherapy. The suicide gene system makes use of an enzyme, which converts a non-toxic prodrug to its cytotoxic form (6). The combinations of the enzyme cytosine deaminase (CD) with the prodrug 5-fluorocytosine (5-FC) and of the enzyme thymidine kinase (TK) with the prodrug ganciclovir (GCV) are two of the most common suicide gene therapy systems (7,8). In each case, the prodrug (5-FC or GCV) is non-toxic to normal human cells when administered on its own, but its enzyme (CD or TK, respectively) metabolizes the prodrug into a monophosphate form that subsequently acts as a chemotherapeutic agent against cancer cells (9). However, the current low efficiency of suicide gene systems limits their application (10). A number of studies have attempted to enhance the therapeutic effect of suicide gene therapy by combining it with other gene therapies (11,12).

Vascular growth has a significant role in tumor development and metastasis (13). Anti-angiogenic therapy has been demonstrated to be safe and effective for the treatment of solid tumors (14). Vascular endothelial growth factor (VEGF), a critical proangiogenic regulator, is overexpressed in HCC, whereas it has low levels of expression in normal liver tissues (15-17). Thus, VEGF is an ideal target for HCC treatment (18).

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In the present study, a combination suicide gene system using both CD and TK was utilized, along with a VEGF promoter (VEGFp), and this was delivered into HCC cells, followed by assessment of the *in vitro* and *in vivo* effects. The present study provides an experimental basis for additional application of double suicide gene therapy strategies.

Materials and methods

Cell lines. Human HCC cell lines (BEL-7402 and HepG2) and human embryonic kidney-293 (HEK-293) cells were obtained from the Animal Experimental Center of Sun Yet-Sen University (Guangzhou, China). Human umbilical vein vascular endothelial cells (HUVEC) were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Cells were cultivated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Construction of the recombinant plasmids. A 1.3 kb gene fragment encoding the CD gene and a 1.1 kb fragment encoding the TK gene were amplified by polymerase chain reaction (PCR) from *Escherichia coli* JM109 DNA and pREP8-TK, respectively (kindly provided by Cell biology department of Southern medical University, Guangzhou, China). These two DNA fragments were then inserted into the plasmid pMD18-T (catalog no., D101A; Takara Bio, Inc., Otsu, Japan) to generate pMD18-CD and pMD18-TK. Following confirmation by sequencing (Beijing Genomics Institution, Beijing, China), the CD and TK fragments were digested and inserted into pcDNA3 (provided by Department of Cell Biology, Southern medical University) to build the plasmid pcDNA3-CDglyTK. pcDNA3-CDglyTK was then cut by the restriction enzymes *Hind*III and *Pvu*II (New England BioLabs, Inc., Ipswich, MA, USA) to generate CDglyTK-pA. The human VEGFp region was cut from pEGFP-1-SV-VEGFp (kindly provided by Professor Jiro Kishimoto, Shiseido Research Center, Yokohama, Japan) and amplified by PCR using the 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 98°C for 5 min, followed by 28 cycles at 94°C for 40 sec, 50°C for 40 sec, 72°C for 1 min and 72°C for 10 min. Subsequently, VEGFp was ligated to pAdtrack (Shanghai GenePharma Co., Ltd., Shanghai, China) to construct pAdtrack-VEGFp. Finally, CDglyTK-pA was added to generate pAdtrack-VEGFp-CDglyTK (Ad-VEGFp-CDglyTK). PCR primers are listed in Table I.

Amplification and identification of recombinant adenovirus. The vector (Ad-VEGFp-CDglyTK) was transfected into HEK-293 cells and purified using cesium chloride gradient ultracentrifugation at 10,000 x g. Viral production and amplification were monitored with the aid of green fluorescent protein (GFP), and the titer of the purified recombinant adenoviruses was as high as 2.0x10¹² pfu/ml. The recombinant adenovirus was verified by PCR.

Transfection efficiency. To measure the efficiency of transfection with Ad-VEGFp-CDglyTK, HUVEC and the human

HCC cell lines BEL-7402 and HepG2 (the latter is deficient in VEGF) were used. Cells were plated into six-well culture plates (Guangzhou RiboBio Co., Ltd., Guangzhou, China) at a density of 2x10⁵ cells/well, and transfected with Ad-VEGFp-CDglyTK using various multiplicities of infection (MOI; 0, 10, 20, 50, 100, and 200 pfu/cell). Fluorescence microscopy (GFM600 microscope, Leica Microsystems GmbH, Wetzlar, Germany) was used to assess GFP expression following 24 and 48 h of incubation at 37°C.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells using TRIzol[®] reagent according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.) and digested with RNase-free DNase (Promega Corporation, Madison, WI, USA) to clear residual genomic DNA. The reverse transcription reaction was performed using the RevertAid[™] First-Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) in a final volume of 20 µl containing 2 µg total RNA, 1 µg oligo (dT)₁₈ primer, 10 mmol/l deoxynucleotidetriphosphate mixture, 4 µl 5X reverse transcription buffer, 20 units RiboLock[™] Ribonuclease inhibitor, diethylpyrocarbonate-treated water and 200 units RevertAid[™] M-Mulv reverse transcriptase. Following incubation at 42°C for 60 min, the reverse transcription reaction was terminated by heating at 70°C for 10 min. The reaction contained 2 µl cDNA template. The cDNA was amplified by PCR using the 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 25 µl reaction mixture contained 2.5 units of *Taq* polymerase (Promega Corporation), 1 µl of cDNA template (Promega Corporation), 1.5 mmol/l MgCl₂ (Promega Corporation) and 0.5 µmol/l CDglyTK primers. The CDglyTK primers were synthesized by Beijing Genomics Institution (forward, 5'-GGGAAGCTTAGGCTAGCAATGTCTGAATAACGCT-3' and reverse, 5'-GGGTCTAGATTAGTTAGCCTCCCCCATCTC-3'; generating a DNA fragment of 2,400 bp). Expression was normalized to the glyceraldehyde-3-phosphate dehydrogenase gene (forward, 5'-CTCAGACACCATGGGGAAGGTGA-3' and reverse, 5'-ATGACTTGAGGCTGTTGTCATA-3'; generating a DNA fragment of 450 bp; Beijing Genomics Institute, Beijing, China). The reaction conditions were as follows: Preliminary denaturation at 94°C (5 min), followed by 30 cycles of denaturation at 94°C (40 sec), annealing at 58°C (60 sec) and extension at 72°C (90 sec), with a final extension step at 72°C for 10 min. The PCR products were run on a 1.5% agarose gel, and examined on a CX2000 UV illuminator (UVP Inc., Upland, CA, USA) and photographed using a Canon EOS 60D camera (Canon, Inc., Tokyo, Japan). This experiment was performed three times.

In vitro study

Cell proliferation assay. To investigate the biological effect induced by suicide gene systems, cytotoxicity (the effect on cell viability) was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA] assay. HUVEC, BEL-7402, and HepG2 cells were seeded into 96-well plates (Guangzhou RiboBio Co., Ltd.) at a density of 5x10³ cells/well for 24 h. Following incubation at 37°C for 24 h, the culture medium was removed, and cells were infected with Ad-VEGFp-CDglyTK

Table I. Polymerase chain reaction primer sequences.

Gene	Size, bp	Primers
CD	1,300	Forward 5'-GGGAAGCTTAGGCTAGCAATGTCGAATAACGCT-3' Reverse 5'-GGGGGATCCCTCCACGTTTGTAAATCGATGGCTTC-3'
TK	1,100	Forward 5'-GGGGGATCCGGCGGGGGCGGTGGAGGAGGGGGTATGGCTTCGTAC-3' Reverse 5'-GGGTCTAGATTAGTTAGCCTCCCCCATCTC-3'
VEGFp	572	Forward 5'-TCACCGCCTCGGCTTGTACATCT-3' Reverse 5'-ATGAACCTTCTGCTGTCTTGGGTG-3'

CD, cytosine deaminase; TK, thymidine kinase; VEGFp, vascular endothelial growth factor promoter.

(100 pfu/cell) and incubated for 24 h at 37°C. Uninfected cells incubated at 37°C for the same duration served as a control. Following incubation, the medium was replaced with various concentrations of GCV (0, 5, 10, 50, 100, or 200 µg/ml; Roche Diagnostics GmbH, Mannheim, Germany) or 5-FC (0, 20, 40, 60, 80, or 100 µg/ml; Sigma-Aldrich), or a combination of the two. The cells were subsequently cultured for 48 h and incubated with 10 µl MTT (10 mg/ml; Sigma-Aldrich). The medium was removed and the remaining purple-blue sediment was dissolved in 150 µl dimethyl sulfoxide (Sigma-Aldrich) for 10 min. The relative optical density (OD) of each well was determined at the test wavelength (490 nm) using a Bio-Rad 2550 EIA Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Viability of the cells was calculated using the following equation: Cell survival rate (%) = (OD value of experimental group / OD value of control group) x 100%.

Bystander effect assay. Cells infected with recombinant virus were mixed with uninfected cells in various ratios (5:95, 10:90, 20:80, 40:60, 60:40, and 80:20) and seeded into 96-well plates at 1x10⁴ cells/well. Cells were treated with GCV (100 mg/l) and 5-FC (80 mg/l) together for 48 h and analyzed by MTT assay, as described above.

Flow cytometry analysis. To determine the effect of recombinant viruses on apoptosis, Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA) were used for flow cytometry (FCM) analysis to assess apoptosis. Cells infected with recombinant virus and uninfected cells were treated with GCV (100 mg/l), 5-FC (80 mg/l) or a combination of the two for 24 h. The harvested cells were washed twice with phosphate-buffered saline (PBS), and adjusted to a concentration of 5x10⁵ cells/ml by dilution with PBS. The cell suspension (200 µl) was added to each labeled tube, followed by 5 µl of Annexin V-FITC and 10 µl PI. The tube was incubated for at least 10 min at room temperature in the dark. Cells were assayed using a FACSCalibur™ Flow Cytometer (BD Biosciences). Apoptotic cells were defined as the population that were Annexin V-FITC positive and PI-negative.

In vivo study

Tumor cell xenograft. Animal experiments were approved by the Institutional Animal Care and Use Committee of Southern Medical University (Guangzhou, China). In total, 20 male

nude mice (4-6 weeks of age, 18-20 g) were purchased from the Laboratory Animal Center of Sun Yet-Sen University. The mice were kept in a specific-pathogen-free room at 26-28°C in a 10 h light and 14 dark cycle, and had free access to food and water. The mice were randomly divided into four groups with 5 mice in each group. The BEL-7402 cell suspension (either infected with Ad-VEGFp-CDglyTK or uninfected) was injected subcutaneously (0.5x10⁷ cells in 200 µl serum-containing medium). Mice were observed daily to ensure that the injection site was healthy. GCV (200 mg/kg/day) and 5-FC (100 mg/kg/day) were injected into the tumor daily for 10 days. At 1 week subsequent to injection, tissue samples and other biological data (tumor weight, and long and short diameter) were collected. The tumor volume was calculated using the following formula: Volume=(axb²)/2 mm³ (a, longer diameter of the tumor; b, shorter diameter of the tumor) (19). The rate of inhibition of tumor growth was subsequently calculated as the tumor inhibition rate as follows: (1-tumor weight of treatment group/tumor weight of control group)x100%.

Microvessel density assay. To assess tumor angiogenesis, cluster of differentiation 34 antibody was used to determine microvessel density (MVD). Tumor tissue was collected and fixed in 10% formaldehyde (Shanghai Chemical Reagent Co., Ltd., Shanghai, China) for 24 h. The tumor tissue was embedded in paraffin and 4-µm sections were cut using the RM2245 microtome (Leica Microsystems GmbH). To block endogenous peroxidase activity, 3% H₂O₂ (Shanghai Chemical Reagent Co., Ltd.) was added for 10 min. Following antigen retrieval with sodium citrate (0.01 mmol/l, pH 6.0; Shanghai Chemical Reagent Co., Ltd.) for 10 min, rabbit anti-human cluster of differentiation 34 monoclonal antibody (catalog no., sc-19621; 1:150 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was incubated with samples overnight at 4°C, followed by incubation with the secondary biotin-labeled goat anti-mouse immunoglobulin G antibody (1:500 dilution; catalog no., A0286; Beyotime Institute of Biotechnology, Haimen, China) at 37°C for 30 min. Following staining with 3,3'-diaminobenzidine (OriGene Technologies, Inc., Beijing, China) and hematoxylin (Shanghai Chemical Reagent Co.), MVD was assessed under a microscope (TS100; Nikon Corporation, Tokyo, Japan), as described previously by Weidner *et al* (20). Under x100 magnification, five areas were identified with the highest vascular density ('hot spots'), and the number of vessels in each of these regions was counted

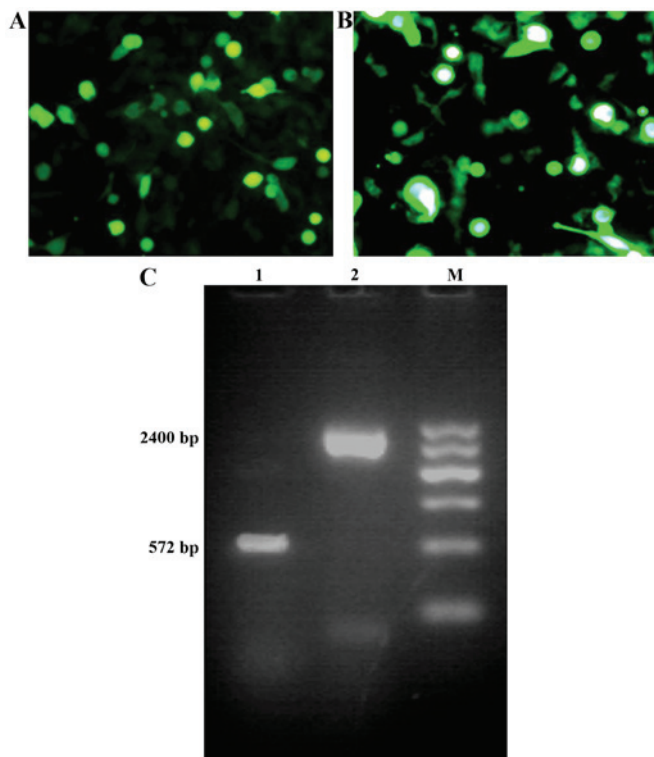


Figure 1. Transient expression of the recombinant GFP genes was observed by fluorescence microscopy (A) 24 h and (B) 48 h post-transfection (MOI, 100 pfu/cell; magnification, x200). (C) Reverse transcription-polymerase chain reaction was used to confirm the recombinant adenovirus in HEK-293 cells. Lane M, Marker; Lane 1, transfected Ad-VEGFp-CDglyTK HEK-293 cells revealing the presence of the 572 bp target band (VEGFp); Lane 2, transfected Ad-VEGFp-CDglyTK HEK-293 cells showing the presence of the 2400 bp target band (CDglyTK). VEGFp, vascular endothelial growth factor promoter; CD, cytosine deaminase; TK, thymidine kinase; Ad, adenovirus.

under x200 magnification. Microvessels were counted by two independent observers, and the mean value was used for analysis.

Statistical analysis. Each assay was performed at least three times, and data are presented as the mean \pm standard deviation. Analysis of variance was used to determine the significance of differences in multiple comparisons. All data were analyzed with SPSS (version 13.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Recombinant viruses. At 24 h following transfection of recombinant virus plasmids into HEK-293 cells, a stronger GFP fluorescence signal was observed in the cells transfected with Ad-VEGFp-CDglyTK, while the majority of the remaining HEK-293 cells expressed GFP protein at 48 h post-transfection. The recombinant adenoviruses were confirmed by PCR and DNA electrophoresis, producing bands of 572 bp (same size as VEGF) and 2,400 bp (same size as the CDglyTK gene fragment; Fig. 1).

Transfection efficiency of Ad-VEGFp-CDglyTK in various cell lines. To determine the infection efficiency of the adenoviral

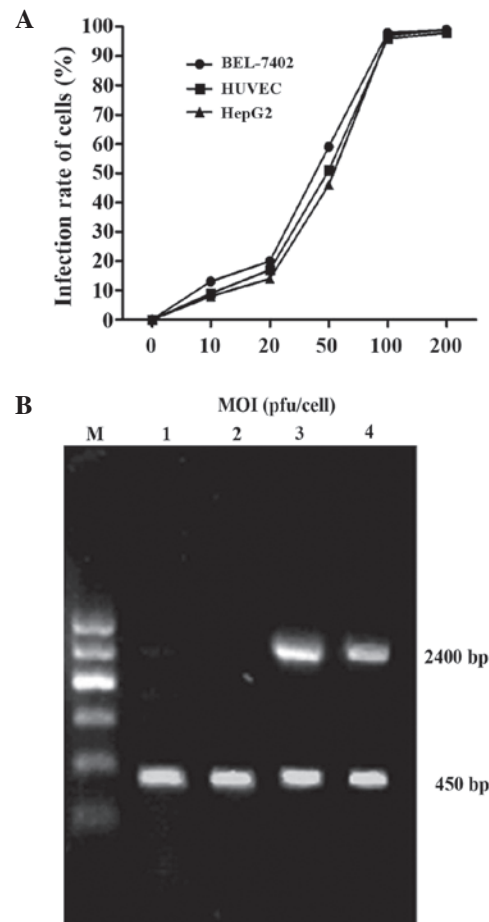


Figure 2. Recombinant adenovirus transfer efficiency and CDglyTK production in various cell lines. (A) Transfer efficiencies of recombinant adenoviruses in various cell lines. When the MOI was 100 pfu/cell, the transfer efficiency was 95% in all three cell lines. (B) Reverse transcription-polymerase chain reaction analysis of the expression of CDglyTK lane (DNA fragment of 2400 bp). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (DNA fragment of 450 bp). Lane M, Marker; Lane 1, blank control group; Lane 2, no fragments of CDglyTK gene detected in HepG2 cells; Lane 3, CDglyTK gene detected in BEL-7402 cells; Lane 4, CDglyTK gene detected in HUVEC. MOI, multiplicity of infection; CD, cytosine deaminase; TK, thymidine kinase; HUVEC, human umbilical vein vascular endothelial cells.

vector, the BEL-7402, HUVEC and HepG2 cells were infected with the Ad-VEGFp-CDglyTK at a MOI ranging from 10-200 pfu/cell. At a MOI of 10 pfu/cell, only a small number of cells expressed GFP, whereas at a MOI of 100 pfu/cell, >95% of cells were GFP-positive without demonstrating any marked adenoviral toxicity (Fig. 2A). To investigate expression of the CDglyTK gene in the infected cells, RNAs were analyzed by RT-PCR. It was observed that the CDglyTK fusion gene was expressed in the BEL-7402 cells and HUVEC, but not in HepG2 cells (Fig. 2B).

Cytotoxicity analysis of the Ad-VEGFp-CDglyTK on transfected cells in vitro. To investigate the biological effect induced by Ad-VEGFp-CDglyTK, cytotoxicity was assessed in HUVEC, BEL-7402, and HepG2 cells infected with Ad-VEGFp-CDglyTK and treated with GCV, 5-FC or GCV+5-FC. As shown in Fig. 3A, HUVEC and BEL-7402 cells were highly sensitive to the prodrugs, but HepG2 cells were

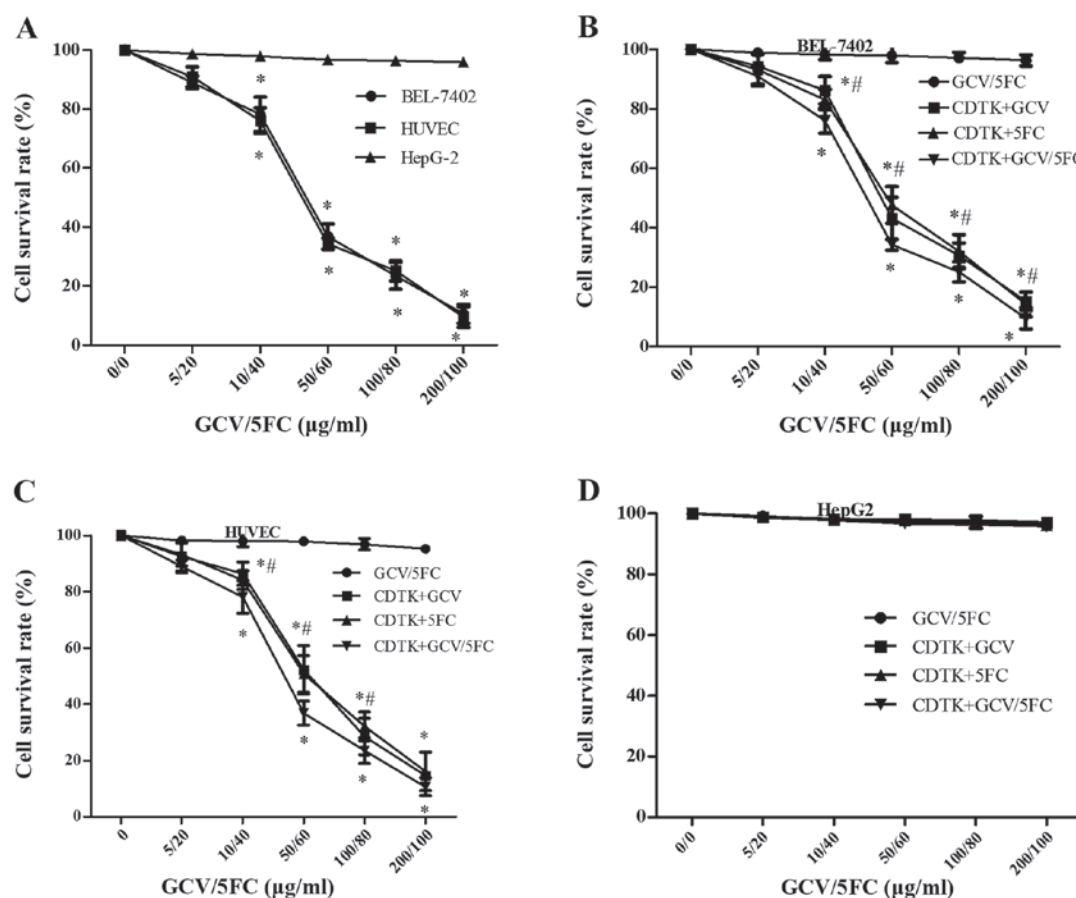


Figure 3. Effect of the recombinant adenoviruses on the cell survival rate of various transgenic cells. (A) Influence on survival rate of HUVEC, BEL-7402 and HepG2 cells of various concentrations of GCV combined with 5-FC. * $P < 0.05$ compared with HepG2 cells. (B) Sensitivity of BEL-7402 cells and BEL-7402 cells transfected with *CDglyTK* cells to various concentrations of GCV, 5-FC or a combination of the two. * $P < 0.05$ compared with the untransfected group; # $P < 0.05$ GCV or 5-FC alone compared with GCV+5-FC. (C) Sensitivity of HUVEC and HUVEC transfected with *CDglyTK* cells to various concentrations of GCV, 5-FC or a combination of the two. * $P < 0.05$ compared with the untransfected group; # $P < 0.05$ GCV or 5-FC alone compared with GCV+5-FC. (D) Sensitivity of HepG2 cells and HepG2 cells transfected with *CDglyTK* cells to various concentrations of GCV, 5-FC or a combination of the two. HUVEC, human umbilical vein vascular endothelial data; GCV, ganciclovir; 5-FC, 5-fluorocytosine; CD, cytosine deaminase; TK, thymidine kinase.

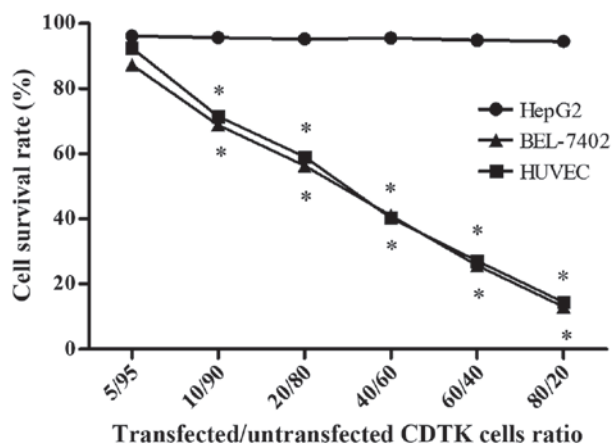


Figure 4. Bystander effects of prodrugs (ganciclovir+5-fluorocytosine) on BEL-7402 cells, HUVEC and HepG2 cells with various proportions of transfected and untransfected cells. * $P < 0.05$ compared with HepG2 cells. HUVEC, human umbilical vein vascular endothelial data; CD, cytosine deaminase; TK, thymidine kinase.

not sensitive to the prodrugs ($P = 0.0006$). Cell survival rates significantly decreased in line with increasing concentrations of the prodrugs in the GCV+5-FC group and in the

individual prodrug treatment groups ($P = 0.027$). The sensitivity of *CDglyTK*-transfected cells to GCV+5-FC was greater compared with their sensitivity to GCV or 5-FC alone in the BEL-7402 and HUVEC groups ($P = 0.013$; Fig. 3B and C); however, there was no significant difference in the sensitivity of HepG2 cells to either of the single drugs or to the combination treatment ($P = 0.923$; Fig. 3D).

Bystander effect of Ad-VEGFp-*CDglyTK*. The bystander effect of the *CDglyTK* gene was assessed by mixing transfected and untransfected cells in various ratios. In the BEL-7402 and HUVEC groups, the survival rate decreased significantly as the proportion of Ad-VEGFp-*CDglyTK*-transfected cells increased ($P = 0.041$; Fig. 4); however, the HepG2 cells did not exhibit this phenomenon ($P = 0.718$). In addition, cell survival rates were all markedly lower in the transfected cells compared with the untransfected cells for HUVEC and BEL-7402 cells, but not for HepG2 cells. The cell survival rate was ~81% at a ratio of 95% untransfected to 5% transfected BEL-7402 cells, but when the proportion of untransfected cells was reduced to 90%, the cell viability fell to 69%. These results indicate that GCV and 5-FC are able to kill transfected cells, but are additionally able to kill untransfected cells via a bystander effect.

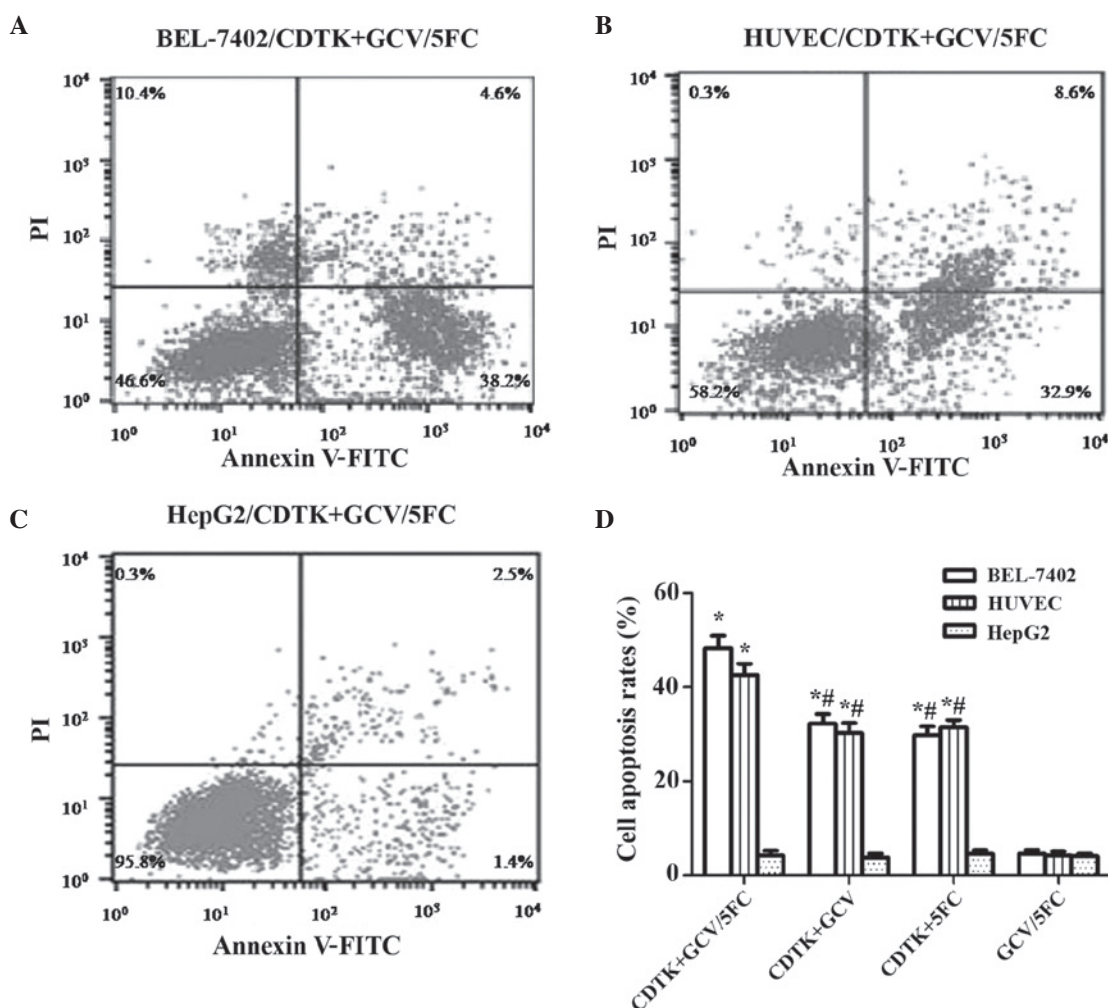


Figure 5. Flow cytometric analysis of the effect of GCV combined with 5-FC on cell apoptosis. Cell lines transfected with Ad-VEGFp-CDglyTK; (A) BEL-7402; (B) HUVEC and (C) HepG2 cells. (D) Cell apoptosis rate induced by the prodrugs GCV and/or 5-FC in the cell lines BEL-7402, HUVEC and HepG2. * $P < 0.05$ compared with the untransfected group; # $P < 0.05$ GCV or 5-FC alone compared with GCV+5-FC. GCV, ganciclovir; 5-FC, 5-fluorocytosine; VEGFp, vascular endothelial growth factor promoter; CD, cytosine deaminase; TK, thymidine kinase; HUVEC, human umbilical vein vascular endothelial data; PI, propidium iodide; FITC, fluorescein isothiocyanate; Ad, adenovirus.

Flow cytometric analysis. To additionally analyze the effect of Ad-VEGFp-CDglyTK on tumor cells, cell apoptosis was assessed by FCM. As demonstrated in Fig. 5, apoptosis rates were significantly increased ($P < 0.0001$) in the Ad-VEGFp-CDglyTK-transfected BEL-7402 cells and HUVEC compared with the HepG2 cells. The apoptosis rate was additionally higher in the recombinant virus-transfected group compared with the untransfected groups for the BEL-7402 cells and HUVEC ($P = 0.012$), but not for the HepG2 cells ($P = 0.872$). The combination of GCV+5-FC exerted a stronger effect than either drug alone ($P = 0.023$; Fig. 5D).

Anti-tumor effect of Ad-VEGFp-CDglyTK *in vivo*. Based on the results of cytotoxicity and FCM analysis, BEL-7402 cells, with or without the Ad-VEGFp-CDglyTK system, were injected subcutaneously into nude mice. As demonstrated in Fig. 6, the tumor cells transfected with recombinant virus formed smaller tumors compared with the untransfected cells, and the volumes of the tumors were significantly smaller for the transfected group compared with the untransfected group ($P = 0.0003$). The inhibition rate was increased for the Ad-VEGFp-CDglyTK cells treated with GCV+5-FC group

compared with the untransfected group ($P = 0.003$). Therefore, combination of the recombinant virus with the prodrugs (GCV, 5-FC or GCV+5-FC) was able to suppress the growth of HCC cells significantly *in vivo*. The GCV+5-FC combination exerted a stronger tumor-suppressor effect than either drug alone ($P < 0.001$). It is notable that this difference was more marked in the *in vivo* experiment compared with the *in vitro* studies.

Effect of Ad-VEGFp-CDglyTK on MVD of HCC in nude mice. The MVD of the tumor tissue was assessed by cluster of differentiation 34 immunohistochemistry. The MVD of the tumor tissue was decreased more significantly ($P = 0.0008$) in the group transfected with Ad-VEGFp-CDglyTK and treated with prodrugs (GCV, 5-FC, or GCV+5-FC) compared with the control group (Fig. 7). The MVD was slightly but significantly ($P = 0.012$) lower in the GCV+5-FC group compared with the groups treated with either drug alone.

Discussion

Chemotherapy is an established and successful therapy for retarding the growth of a variety of tumor types (4). However,

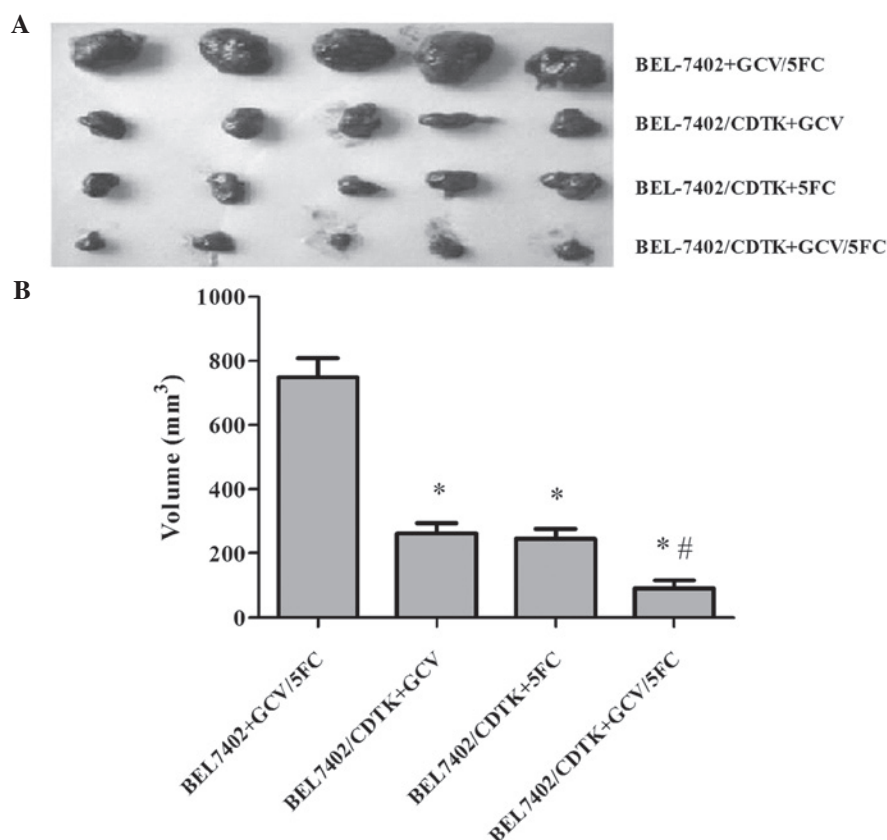


Figure 6. Tumor formation in BEL7402 cells was treated with 5-FC, GCV or a combination of the two for 14 days. Tumors in the suicide gene transfection group were significantly (A) smaller in size and (B) lower in volume compared with those in the untransfected group. * $P < 0.05$ transfected group compared with the untransfected group; # $P < 0.05$ GCV or 5-FC alone compared with GCV+5-FC. 5-FC, 5-fluorocytosine; GCV, ganciclovir; CD, cytosine deaminase; TK, thymidine kinase.

the majority of chemotherapy drugs are not cancer-specific and may cause off-target organ toxicity (21). Suicide gene therapy, using enzymes that are not toxic to healthy tissues but that produce highly toxic metabolites from a much less toxic prodrug, is a safe and efficient therapeutic option for cancer (22). As reported in several studies, TK/GCV or CD/5-FC are the longest established of these suicide gene therapy systems (23-25). Single suicide gene systems (using one prodrug and one enzyme) are able to inhibit tumor development, but a number of studies have demonstrated that TK/GCV combined with CD/5-FC has a higher efficacy for the treatment of solid tumors compared with single suicide gene systems (26,27). However, the suicide gene systems investigated in the present study (TK/GCV and CD/5-FC) have demonstrated little efficacy in clinical practice, due to their low targeting and poor gene-transfer efficiencies in tumors (28). Therefore, a gene that is able to improve the targeting and gene-transfer efficiencies of these systems is urgently required.

Angiogenesis, the formation of new blood vessels from existing vasculature, is an important process in numerous malignancies, including HCC (29). VEGF is a critical proangiogenic factor that has a significant role in the invasion and metastasis of HCC (30). A number of studies have verified that VEGF expression levels are increased in vascular endothelial cells of HCC compared with those of normal tissues (31,32). A meta-analysis additionally revealed that the

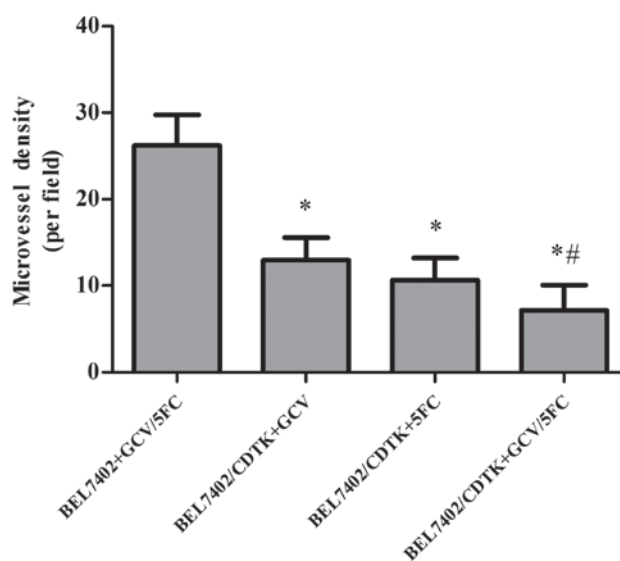


Figure 7. Effect of Ad-VEGFp-CDglyTK on the microvessel density of human hepatocellular carcinoma tumors in nude mice. * $P < 0.05$ transfected group compared with the untransfected group; # $P < 0.05$ GCV or 5-FC alone compared with GCV+5-FC. VEGFp, vascular endothelial growth factor promoter; CD, cytosine deaminase; TK, thymidine kinase; GCV, ganciclovir; 5-FC, 5-fluorocytosine; Ad, adenovirus.

serum VEGF level is associated with prognosis for patients with HCC (33). Inhibiting angiogenesis by using anti-VEGF has been proposed as a potential anticancer strategy (34).

Therefore, the VEGF promoter has been utilized to express target genes in HCC due to its tumor-specific expression (32). In the current study, the results demonstrated that VEGFp was able to direct the *CDglyTK* gene in VEGF-high expressing cells (BEL-7402 and HUVEC), but the transgenic *CDglyTK* double suicide genes were not expressed in HepG2 cells owing a deficiency in VEGF. Therefore, this suggests that when the VEGFp-driven suicide gene is systemically administered, the systemic toxicity to normal cells may be significantly reduced compared with that of traditional chemotherapy.

In the present cytotoxicity experiment, BEL-7402 cells and HUVEC with high levels of *CDglyTK* expression were highly sensitive to the prodrugs used (GCV, 5-FC and a combination of the two). These prodrugs significantly decreased the survival rate of BEL-7402 cells and HUVEC, and the cytotoxic effect increased in line with increasing drug dose. However, such inhibition was not observed in HepG2 cells, which have low *CDglyTK* expression. Previous studies indicated an increased killing efficiency of a combination suicide gene system compared with any single suicide gene system, due to the synergetic cytotoxicity of the combined gene system (6,35). Furthermore, Su *et al* (8) indicated that the effect of double suicide genes was much stronger than that of individual suicide genes in breast cancer cells. Although the present study used different genes and cancer cells, the experiments of the current study also revealed that although each single prodrug (GCV or 5-FC) was able to kill tumor cells in the transgenic *CDglyTK* BEL-7402 cells and HUVEC, the combination of these two prodrugs exerted a more powerful killing effect ($P < 0.05$). Thus, combination treatment may reduce the drug dose required and additionally decrease the toxic side effects of suicide gene systems on other organs.

The bystander effect is the primary driving force of the suicide gene therapy strategy (36,37). The results of the present study demonstrated that GCV+5-FC was not only able to kill the transfected cells but was additionally able to kill neighboring untransfected cells. Therefore, the bystander effect greatly amplifies the efficacy of suicide gene therapy for cancer.

Apoptosis is an important biological phenomenon in tumor treatment. In the present cell apoptosis experiment, 5-FC and/or GCV treatment significantly decreased cell viability in HUVEC and BEL-7402 cells, but not in HepG2 cells. Treatment with 5-FC combined with GCV induced a more marked decrease in cell viability compared with either prodrug alone.

In order to observe the antitumor effect *in vivo*, HCC nude mouse models were established using human BEL-7402 cells. The results of the present study revealed that compared with the untransfected groups, tumors consisting of BEL-7402 cells expressing the *CDglyTK* gene were significantly suppressed by GCV and/or 5-FC treatment *in vivo*.

Angiogenesis is closely correlated with tumor growth, invasion and metastasis (38). MVD protein, which is released by tumor and stroma cells, has a significant role in angiogenesis (39). A number of proteins, including cluster of differentiation 31, cluster of differentiation 34, Factor VIII and cluster of differentiation 105, are markers of angiogenesis (40). As cluster of differentiation 34 is more sensitive and specific compared with other markers used for staining endothelial cells induced by tumor neovascularization (41), the present

study assessed MVD by the presence of cluster of differentiation 34. The results of the present study revealed that the MVD of tumors was decreased by treatment with prodrugs (GCV, 5-FC or GCV+5FC) in the Ad-VEGFp-*CDglyTK*-transfected group, but not in the untransfected group. This result confirmed that the double suicide genes regulated by VEGFp are able to suppress tumor growth and angiogenesis *in vivo*.

In conclusion, the results of the present study indicate that the VEGFp-mediated double suicide gene system is able to effectively inhibit human HCC cells and vascular endothelial cells *in vitro* and *in vivo*. Expression of the *CDglyTK* gene under the control of the VEGFp may represent a promising gene therapy approach for the treatment of HCC, aiming to improve long-term patient survival rates.

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