Adenovirus-mediated tissue-targeted expression of the CDglyTk gene for the treatment of breast cancer

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Received February 15, 2012; Accepted May 10, 2012

DOI: 10.3892/mmr.2012.925

Abstract. The aim of this study was to evaluate the selective killing efficacy of adenovirus (Ad)-mediated double suicide genes driven by the kinase domain-containing receptor (KDR) promoter in human breast cancer cells and vascular endothelial cells. Two Ad-mediated double suicide gene systems [with the two suicide genes, thymidine kinase (TK) and cytosine deaminase (CD)] with the KDR promoter (Ad-KDRP-CDglyTK) and the cytomegalovirus (CMV) promoter (Ad-CMV-CDglyTK) were established and transfected into the KDR-expressing MCF7 human breast cancer, EC304 human vascular endothelial and LS174T human colon carcinoma, which does not express KDR, cell lines. The selective killing efficiency and specificity of the double suicide gene system were measured *in vitro* by the analysis of cellular proliferation and assayed *in vivo* by subcutaneous injection of MCF7 cells into nude mice. The microvessel density (MVD) in the transplanted tumor was determined by immunohistochemical staining of CD34 cells. Our results showed that the transgenic CDglyTK genes were expressed in three cell lines (MCF7, ECV304 and LS174T) infected with Ad-CMV-CDglyTK. However, of the cells infected with Ad-KDRP-CDglyTK, the transgenic CDglyTK gene was only expressed in the KDR-expressing MCF7 and ECV304 cells, but not in the KDR-deficient LS174T cells. Cell proliferation was significantly reduced in a dose-dependent manner by pre-treatment with ganciclovir (GCV) and 5-fluorocytosine (5-FC) in MCF7 and ECV304 cells with transfected KDRP-CDglyTK genes and the three cell lines transfected with the CMV-CDglyTK genes. Similar results were not observed in the LS174T cells with transfected KDRP-CDglyTK genes. The results of this study show that the tumor-targeted expression of CDglyTK driven by the KDR promoter has a high specificity and performance. The killing effect of the CD/TK fusion gene in the target cells was significantly increased compared with the single suicide gene. The cell cycle of MCF7 and ECV304 cells transfected with KDRP-CDglyTK genes was arrested at the S phase following treatment with the prodrugs. The tumors formed by the MCF7 cells with the double suicide gene system were much smaller and the MVD of the tumor tissue was significantly decreased compared with the control. This study demonstrates that tumor-targeted expression of the CDglyTK gene driven by the KDR promotor may be a novel strategy for the gene therapy of human breast cancer.

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Key words: breast cancer, vascular endothelium, kinase domain-containing receptor promoter, CDglyTK fusion gene, gene therapy, adenovirus

Introduction

Breast cancer is a challenging disease for medical science. According to the majority of previous reports from the International Agency for Research on Cancer, in China, Singapore, the Republic of Korea and Turkey, the 5-year age-standardized relative survival rate ranges from 76 to 82% for breast cancer (1). Surgery, chemotherapy, radiotherapy and endocrine therapy are widely used to treat patients with breast cancer (2,3). However, these conventional treatment methods have not improved the prognosis of patients with advanced or metastatic breast cancer. For these reasons, novel methods are required for the treatment of patients with breast cancer. With the development of oncobiology and molecular technology, gene therapy represents a novel treatment model in cancer therapy. Suicide gene therapy is a promising option due to its high efficacy and clinically useful potential (4,5). Suicide gene therapy is a type of cancer gene therapy which transfers prodrug-activating enzyme genes that are found in viruses and bacteria, but not in mammalian cells, into cancer cells via genetic engineering, therefore expressing a number of enzymes which catalyze non-toxic prodrugs into cytotoxic substances to confer drug sensitivity to the cancer cells (6,7). It has been observed that not only suicide gene-transfected cancer cells,

but also non-transfected cells, are killed during the suicide gene therapy by means of the direct killing effect and the so-called bystander effect (8,9).

The tissue-specific expression of suicide genes for the selective killing of tumor cells could be realized by taking advantage of certain tumor-specific transcription modulating elements, including promoters and enhancers. For example, the α fetoprotein (AFP) promoter is commonly employed in suicide gene therapy for hepatic cancer and the erb2 promoter has been introduced for breast cancer (10,11). However, most of the common promoters used at present are specific for a certain type of cancer and their usage is relatively limited.

The endothelial cell type-specific tyrosine kinase domain-containing receptor (KDR) is a receptor for the vascular endothelial growth factor (VEGF). KDR is a critical regulator of endothelial cell growth and development. It has been demonstrated that KDR is expressed in the majority of solid cancer cells and neogenetic vascular endothelial cells of the neoplasma, but not in normal cells (12,13). Previous studies have revealed that the KDR promoter has the ability to overexpress genes of interest exclusively in tumor cells and neogenetic vascular endothelial cells (14).

In this study, we demonstrate the activity of the KDR promoter in breast cancer and endothelial cells and show that the KDR promoter efficiently activates the expression of the double suicide gene, CDglyTK, which includes the two suicide genes, thymidine kinase (TK) and cytosine deaminase (CD) in these cells *in vivo* and *in vitro*. The results of the present study suggest that this system selectively reduces proliferation and enhances apoptosis *in vitro* in breast cancer and endothelial cells, and reduces tumor formation *in vivo* in breast cancer.

Materials and methods

Cells and adenovirus (Ad) vector. The ECV304 human umbilical vein vascular endothelial cells, MCF-7 breast cancer and LS174T colon carcinoma cell lines were provided by the American Type Culture Collection (Manassas, VA, USA). These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37˚C in 5% CO_2.

Recombinant Ad vector construction. AdEasy-KDRP-CDglyTK and AdEasy-cytomegalovirus (CMV)-CDglyTK were also constructed by homologous recombination between an expression cosmid and the parental virus genome, as described previously (15,16).

In vitro Ad infections. During the exponential growth phase, cells were plated in 6-well culture plates at a density of $5x10^5$ cells/well 24 h before the recombinant Ad infection. Immediately prior to infection, the culture medium was aspirated and the suspensions of Ad at various multiplicities of infection (MOI) (from 0 to 200 MOI) were distributed over the monolayers. Following a 24-h incubation, green fluorescent protein (GFP) expression was observed by fluorescence microscopy.

Reverse transcription-PCR (RT-PCR). Total cellular RNA was extracted from cells using TRIzol reagent (Gibco BRL, Carlsbad, CA, USA) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using the RevertAid™ First-Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) in a final volume of 20 μl containing 5 μg total RNA, 0.5 μg oligo(dT) 18 primer, 10 mmol/l deoxynucleotidetriphosphate mixture, 4 μl 5X reverse transcription buffer, 20 unitsRiboLock™ 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 of cDNA template. The newly synthesized cDNA was amplified by PCR. The 50-μl reaction mixture contained 2 μl of cDNA template, 2.5 units of Taq polymerase, 1.5 mmol/l $MgCl_2$ and 0.5 μmol/l of CDglyTK primer (forward, 5'-GGGAAGCTTAGGCTAGC AATGTCGAATAACGCT-3'; reverse, 5'-GGGTCTAGATT AGTTAGCCTCCCCCATCTC-3'). The amplified PCR product was 2.4 kb. For the β-actin primer (forward, 5'-CTTCTA CAATGAGCTGCGTG-3'; reverse, 5'-TCATGAGGTAGTC AGTCAGG-3'), the amplified PCR product was 305 bp. Amplification cycles were: 94˚C for 4 min, then 30 cycles at 94˚C for 35 sec, 54˚C for 40 sec, 72˚C for 30 sec, followed by 72˚C for 10 min. Aliquots of the PCR product were electrophoresed on 1.5% agarose gels and the PCR fragments were visualized by UV illumination (UVP Inc., Upland, CA, USA) and stained with ethidium bromide. The fluorescence intensity of β-actin fragments served as the criterion for the CDglyTK fragments.

In vitro ganciclovir (GCV)/5-fluorocytosine (5-FC) sensitivity of cells infected with recombinant Ad. The cells were seeded and cultured in 96-well plates at a density of $3x10^3$ cells in 100 μl of medium for 24 h. The culture medium was immediately removed from the wells prior to infection and the suspensions of Ad-KDRP-CDglyTK and Ad-CMV-CDglyTK at an MOI of 100 were placed onto the cell monolayers. Following an incubation for an additional 24 h, the medium containing the virus was replaced with fresh medium containing various concentrations of 5-FC (Sigma, St. Louis, MO, USA) and GCV (Roche Diagnostics, Mannheim, Germany) in combination. The cells were then cultured at 37˚C in a 5% CO_2 humidified atmosphere for another 48 h, and cell growth was then assessed by the MTT assay. Cell proliferation was proportional to the absorbance at the test wavelength (570 nm), from which the reference wavelength (620 nm) was subtracted. The results were expressed as the ratio between the number of viable cells in plates containing the drugs versus the number of viable cells in the corresponding drug-free controls.

Specimens for electron microscopy. Infected MCF7 and ECV304 cells in the exponential phase were used and cultivated with GCV (100 mg/l) and 5-FC (2,000 mg/l) for 48 h. The cells were harvested and fixed with 25 ml/l glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 2 h at 4˚C. For transmission electron microscopy (TEM) examination, cells corresponding to each population were collected in Haemoline (BioChem Pharma, Allentown, PA, USA), transferred to microcentrifuge tubes, pelleted and fixed in 1% OsO_4 (in distilled H_2O). A total of $4x10^7$ cells were sorted to collect $2x10^6$ cells representative of each of the individual populations. Following

dehydration through a series of graded alcohol and propylene oxide solutions, the cells were infiltrated with Epon (epoxy resin) and polymerized. Ultra-thin sections were cut, recovered on Formvar-coated copper grids, stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEM-1200EX, Japan Electron Optics Laboratory, Tokyo, Japan) operated at 80 kV.

Flow cytometry analysis. We used a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with a 488-nm argon ion laser. For cell-cycle analysis, asynchronous cells (500,000 cells/ml) were cultured in the presence (control group) or absence of GCV (40 mg/l) and 5-FC (250 mg/l) for 12 h. The cells were harvested and washed in PBS, fixed in 70% cold ethanol for 30 min at -20˚C and washed again in PBS. The cells were then incubated for 1 h in PBS containing 100 μg/ml RNase (Sigma) and 50 μg/ml propidium iodide (PI; Sigma) and incubated at 4˚C for 15 min. The cells were then washed with PBS and immediately analyzed using flow cytometry.

Tumor cell xenograft. To examine the therapeutic effect of the recombinant Ad *in vivo*, 4- to 6-week-old female Balb/c nu/nu athymic mice were used as hosts for MCF7 cell xenografts; $0.5x10^7$ MCF7 cells in 0.2 ml PBS were injected subcutaneously into the mammary fat pad using an 18-gauge needle and the tumors were allowed to grow for 2 weeks before randomizing the mice by size. All tumors were at least 5 mm in diameter and the mice were grouped according to the treatment regimen. The recombinant Ads ($1x10^{10}$ pfu) were injected into the tumors, followed by GCV and 5-FC treatment (intraperitoneal injection of 50 mg/kg/day and 500 mg/kg/day for 18 days, respectively).

Antitumor effect and toxicity of recombinant Ads and prodrugs in vivo. The perpendicular tumor diameter was measured with a sliding caliper at 3-day intervals and tumor weight (W) was calculated using the formula: $W=(AxB^2)/2$ mg (where A is the longer diameter and B is the shorter diameter) (17), then the tumor growth inhibition rate was calculated and the growth curves of the tumors were drawn. Moreover, the hematoxylin and eosin (H&E) staining of the tumor tissues was performed for histological examination. In addition, the systemic toxicities of recombinant Ad and prodrugs were determined by examining histological changes in the heart, lung, kidney, liver and small intestine of the nude mice following treatment.

Microvessel density (MVD) assay. To assess tumor angiogenesis, MVD was determined by immunohistochemical staining of CD34. The tumorous tissues were obtained and fixed in a sufficient amount of 10% neutral-buffered formaldehyde for 24 h. The tumorous tissues were embedded in paraffin and 4-μm sections were made. After blocking the endogenous peroxidase reaction with 3% H_2O_2 and following sodium citrate antigen retrieval (0.01 mmol/l, pH 6.0), rabbit anti-human CD34 monoclonal antibody (1:150 dilution) was allowed to react on the serial sections and incubated overnight at 4˚C. Biotin-labeled IgG was added to the sections and incubated at 37˚C for 30 min. SP complex was added and then diaminobenzidine was used as a substrate for horseradish peroxidase in the developmental step. MVD was assessed according to the report by Weidner *et al* (18). A single CD34-positive cell, clusters of endothelial cells clearly separated from adjacent microvessels and other connective tissue elements were considered to be vessels. The stained sections were screened at x100 magnification under a light microscope to identify the five regions of the section with the highest vascular density. The vessels were counted in the five regions at x200 magnification, and the average number of microvessels was recorded. The MVD of each tumorous tissue was expressed as the mean number of microvessels counted in five high power fields. The microvessels were counted by two observers and the mean value was used for analysis (19).

Statistical analysis. All data are presented as the means ± standard deviation (SD). The data were evaluated by one-way ANOVA followed by the least significant difference (LSD) test as a post-hoc test. $P<0.05$ was considered to indicate a statistically significant result.

Results

Ad-mediated gene transfer efficiency and CDglyTK production in various cell lines. To examine the Ad-mediated gene transfer efficiencies, MCF7 breast cancer, ECV304 human vascular endothelial and LS174T human colon carcinoma cell lines were transfected with Ad-KDRP-CDglyTK and Ad-CMV-CDglyTK at various MOI. When the MOI was 10, only a few cells expressed GFP. When the MOI was 100, 95% of the cells expressed GFP. When the MOI was 200, almost all the cells expressed GFP (Fig. 1A and B). CDglyTK mRNA expression was detected in all the transfected cells with the exception of in the LS174T cells transfected with Ad-KDRP-CDglyTK (Fig. 1C).

Specificity and efficiency of Ad-KDRP-CDglyTK in KDR-expressing cells. To analyze the specificity and efficiency of the KDR promoter induced by CDglyTK gene expression, KDR-expressing cells (MCF7 and ECV304) and KDR-deficient cells (LS174T) were infected with Ad-KDRP-CDglyTK or Ad-CMV-CDglyTK at an MOI of 100 and were exposed to various concentrations of the prodrugs GCV/5-FC for 48 h. As shown in Fig. 2, compared with the control group, the cell survival rate of the ECV304 and MCF7 cells infected with Ad-CMV-CDglyTK and Ad-KDRP-CDglyTK and LS174T cells infected with Ad-CMV-CDglyTK were significantly decreased following exposure to the prodrugs GCV and 5-FC (all $P<0.05$). The survival rates of the sensitive cells decreased gradually with the increase in the prodrug concentration. Compared with the control group, the LS174T cells infected with Ad-KDRP-CDglyTK were insensitive to the two prodrugs ($P>0.05$). It was verified that the specific and high-performance killing effect of Ad-KDRP-CDglyTK was present in the KDR-expressing cells.

Sensitivity of CDglyTK-expressing cells to different prodrugs. Compared with the 250 mg/l 5-FC group or 40 mg/l GCV group, the survival rates of the ECV304 cells infected with Ad-KDRP-CDglyTK were significantly decreased by treatment with a combination of GCV and 5-FC ($P<0.05$). A similar result was observed in the MCF7 cells infected with Ad-KDRP-CDglyTK. However, this was not observed in the LS174T cells infected with Ad-KDRP-CDglyTK (Fig. 3). This

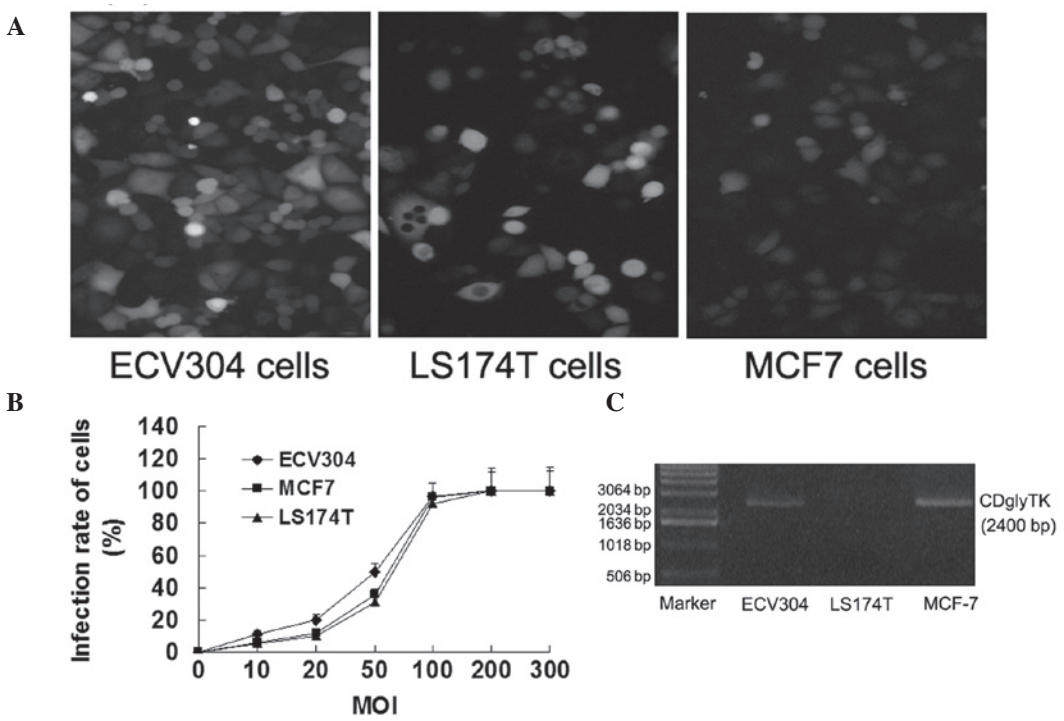

Figure 1. Ad-mediated gene transfer efficiency and CDglyTK production in various cell lines. (A) Green fluorescent protein (GFP) expression of cells infected with the recombinant Ad. (B) The Ad-mediated gene transfer efficiencies. Data are expressed as the means ± SD, n=3. RT-PCR analysis of the CDglyTK fusion gene in various cells. CD, cytosine deaminase; TK, thymidine kinase; Ad, adenovirus; RT-PCR, reverse transcription-PCR; MOI, multiplicity of infection.

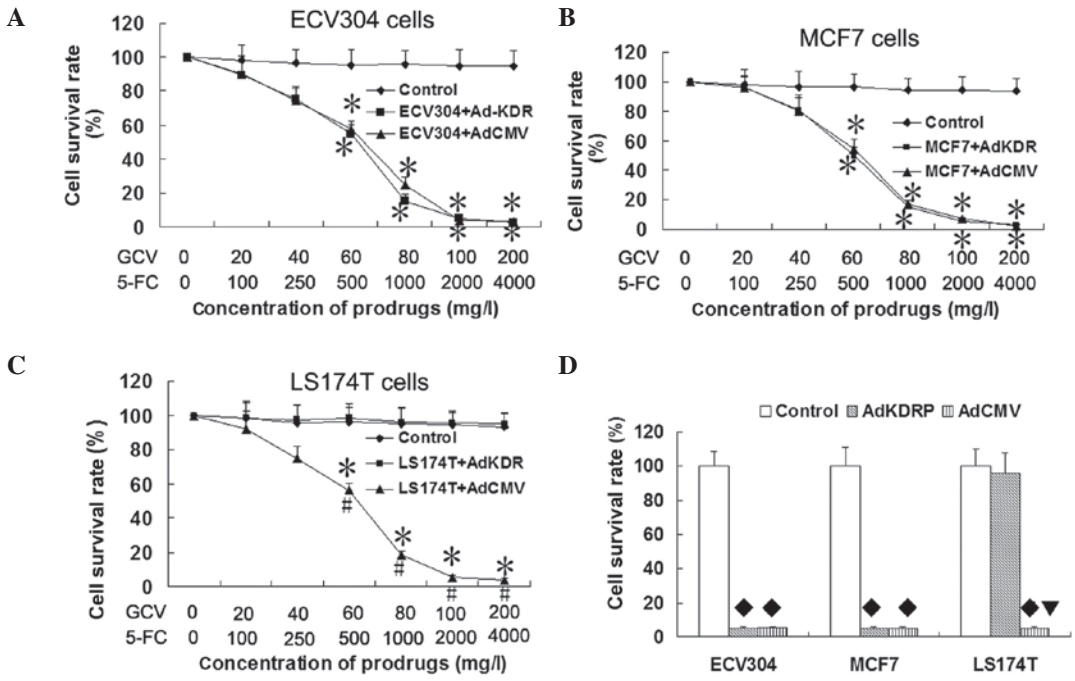

Figure 2. Effect of the two recombinant Ads on the cell survival rate on various transgenic cells. KDR-expressing cells (MCF7 and ECV304) and KDR-deficient cells (LS174T) were infected with Ad-KDRP-CDglyTK or Ad-CMV-CDglyTK at an MOI of 100 and exposed to various concentrations of GCV/5-FC for 48 h. The survival ratio of cells was detected by the MTT method. (A) ECV304 cells; (B) MCF7 cells; (C) LS174T cells; (D) corresponding drug-free controls. Data are expressed as the means ± SD, n=3. *P<0.05 compared with the control group; $^{\#}$P<0.05 compared with the LS174T + AdKDR group; $^{\bullet}$P<0.05 compared with the ECV304 cells group; $^{\blacktriangledown}$P<0.05 compared with the LS174T + AdKDR group. CD, cytosine deaminase; TK, thymidine kinase; MOI, multiplicity of infection; Ad, adenovirus; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

demonstrates that the effect of the double suicide gene was much stronger than that of each single suicide gene.

Ultrastructural features of apoptotic MCF7 and ECV304 cells. After exposure to GCV and 5-FC for 48 h, the ultrastructure of the cells transfected with KDRP-CDglyTK was

Table I. Effect of prodrugs on the cell cycle in MCF7 cells transfected with KDRP-CDglyTK.

Groups	G1 phase (%)	S phase (%)	G2 phase (%)
Control	54.50±4.27	33.90±2.96	11.58±0.75
Transgene	42.43±3.65[a]	57.20±5.14[a]	0.43±0.06[a]

The MCF7 cells transfected with the KDRP-CDglyTK were treated with GCV (40 mg/l) and 5-FC (250 mg/l) for 12 h, the cell cycle was tested by flow cytometry. The percentage of cells in each phase was calculated. Data are expressed as the means ± SD, n=3. [a]P<0.05 compared with the control group. KDRP, kinase domain-containing receptor promoter; CD, cytosine deaminase; TK, thymidine kinase; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

Table II. Effect of prodrugs on the cell cycle in ECV304 cells transfected with KDRP-CDglyTK.

Groups	G1 phase (%)	S phase (%)	G2 phase (%)
Control	48.80±2.65	34.83±1.74	16.48±1.12
Transgene	37.22±2.01[a]	62.60±4.37[a]	0.20±0.08[a]

The ECV304 cells transfected with KDRP-CDglyTK were treated with GCV (40 mg/l) and 5-FC (250 mg/l) for 12 h, the cell cycle was tested by flow cytometry. The percentage of cells in each phase was calculated. Data are expressed as the means ± SD, n=3. [a]P<0.05, compared with the control group. KDRP, kinase domain-containing receptor promoter; CD, cytosine deaminase; TK, thymidine kinase; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

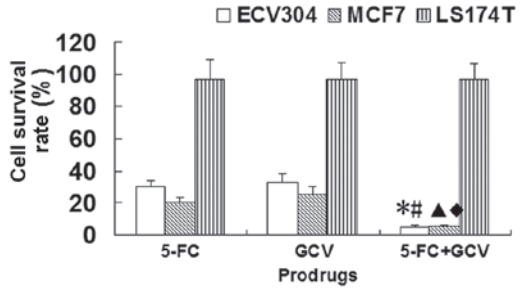

Figure 3. Effect of different prodrugs on the cell survival rate in transgenic cells. KDR-expressing cells (MCF7 and ECV304) and KDR-deficient cells (LS174T) were infected with Ad-KDRP-CDglyTK at an MOI of 100 and exposed to 100 mg/l GCV and or 2,000 mg/l 5-FC for 48 h. The rate of cell survival was detected by MTT method. Data are expressed as the means ± SD, n=3. *P<0.05 compared with the ECV304 + 5-FC group; #P<0.05 compared with the ECV304 + GCV group; ▲P<0.05 compared with the MCF7 + 5-FC group; ◆P<0.05 compared with the MCF7 + GCV group. CD, cytosine deaminase; TK, thymidine kinase; KDR, kinase domain-containing receptor; MOI, multiplicity of infection; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

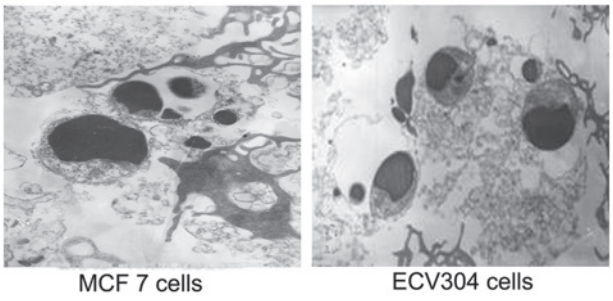

Figure 4. Ultrastructural features of apoptotic MCF7 and ECV304 cells. MCF7 and ECV304 cells were infected with Ad-KDRP-CDglyTK at an MOI of 100 and exposed to 100 mg/l GCV and or 2,000 mg/l 5-FC for 48 h. The ultrastructural characterization of cells infected with Ad-KDRP-CDglyTK was observed by transmission electron microscope (TEM). The original magnification was x6,000. CD, cytosine deaminase; TK, thymidine kinase; KDRP, kinase domain-containing receptor promoter; MOI, multiplicity of infection; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

observed using TEM. Cell shrinkage, chromatin gathering along the nuclear membrane, cell budding, chromatin condensation, chromatin fragmentation and apoptotic bodies were observed in some infected cells and the other cells exhibited necrosis (Fig. 4).

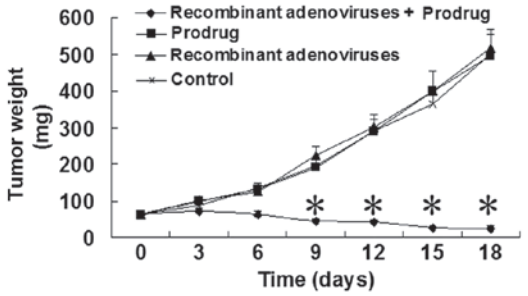

Figure 5. Effect of the recombinant Ad containing fusion suicide gene on the growth curve of human breast tumors. MCF7 human breast tumor cells were injected into the nude mice by subcutaneous injection. The tumors, of 50 mm mean diameter, were established 12 days after MCF7 cells were injected. The recombinant Ad ($1x10^{10}$ pfu) were injected into the tumor, followed by GCV (50 mg/kg/day) and 5-FC (500 mg/kg/day) treatment by intraperitoneal injection for 18 days. The tumor was weighed. Data are expressed as the means ± SD, n=5. *P<0.05 compared with the control group. Ad, adenovirus; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

Effect of prodrugs on cell cycle of MCF7 and ECV304 cells transfected with KDRP-CDglyTK. The MCF7 and ECV304 cells transfected with KDRP-CDglyTK were treated with GCV (40 mg/l) and 5-FC (250 mg/l) for 12 h, the cell cycle was tested by flow cytometry. As shown in Table I, the percentages of cells in the G1 and G2 phase were significantly decreased and the percentage of cells in the S phase was significantly increased compared with the control group following treatment with GCV (40 mg/l) and 5-FC (250 mg/l) for 12 h in MCF7 cells transfected with KDRP-CDglyTK (P<0.05). Similar results were observed in ECV304 cells infected with KDRP-CDglyTK (P<0.05; Table II). These results suggest that the cell cycle was arrested at the S phase following exposure to GCV (40 mg/l) and 5-FC (250 mg/l) for 12 h in MCF7 and ECV304 cells transfected with KDRP-CDglyTK.

Antitumor effect of the recombinant Ads containing fusion suicide gene in vivo. MCF7 human breast tumor cells were injected into the corresponding syngeneic nude mice by subcu-

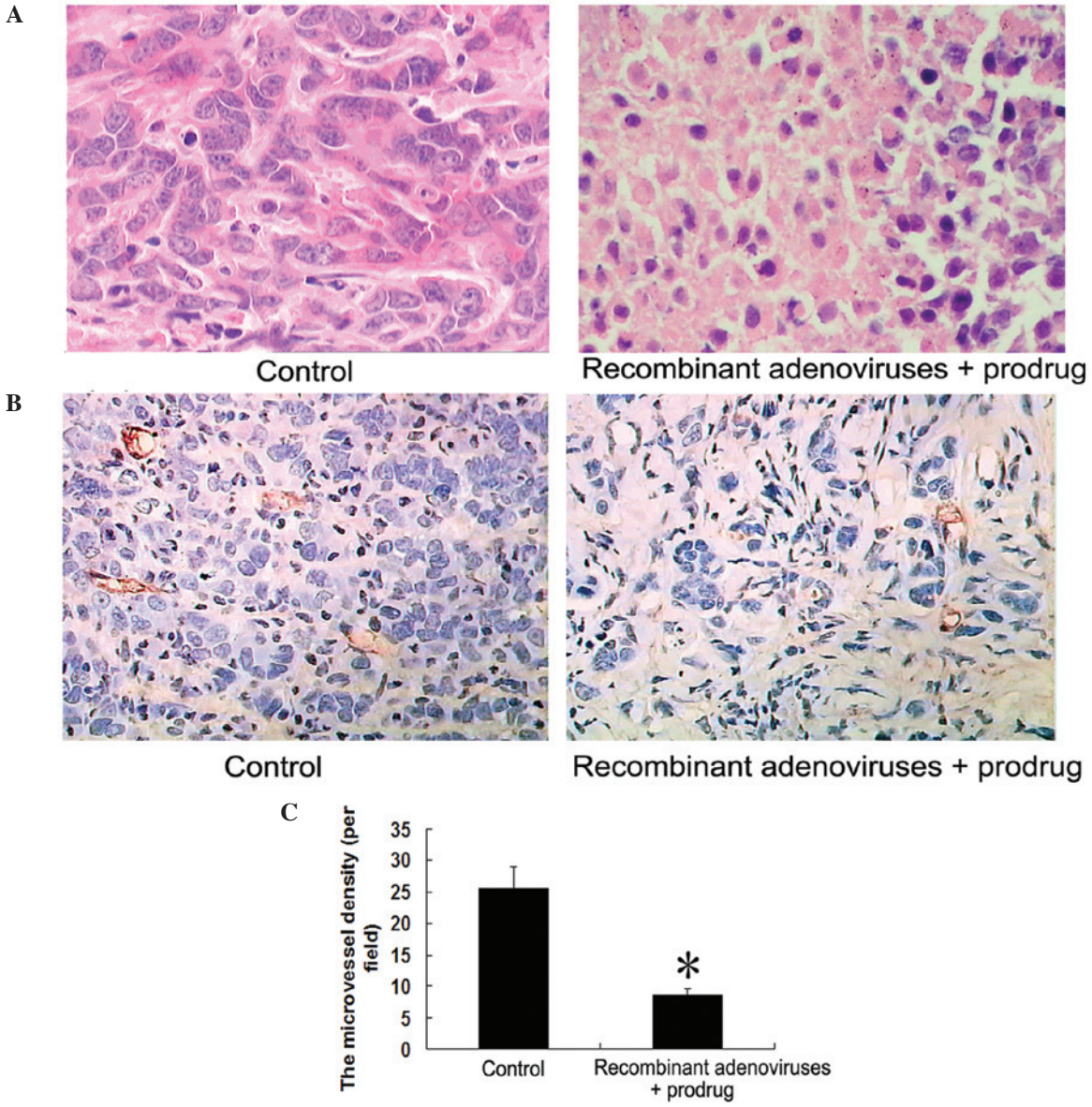

Figure 6. Effect of the recombinant Ad containing fusion suicide gene on the histology and microvessel density (MVD) of human breast tumors in nude mice. MCF7 human breast tumor cells were injected into the nude mice by subcutaneous injection. The tumors, of 50 mm mean diameter, were established 12 days after MCF7 cells were injected. The recombinant Ad ($1x10^{10}$ pfu) were injected into the tumor, followed by GCV (50 mg/kg/day) and 5-FC (500 mg/kg/day) treatment by intraperitoneal injection for 18 days. (A) The tumors were excised and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was x400. (B and C) MVD of tumor tissue was assessed using immunohistochemistry with CD34 monoclonal antibody. The original magnification was x400. Data are expressed as mean ± SD, n=5. *P<0.05, compared with the control group. Ad, adenovirus; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

taneous injection. Tumors with a mean diameter of 50 mm were established 12 days after the MCF7 cells were injected. The recombinant Ads ($1x10^{10}$ pfu) were injected intratumorally, followed by GCV (50 mg/kg/day) and 5-FC (500 mg/kg/day) treatment by intraperitoneal injection for 18 days. As shown in Fig. 5, the tumor weights from the nude mice were significantly decreased in the recombinant Ad + prodrug group compared with the control group at 9, 12, 15 and 18 days after the recombinant Ads were injected. The tumor weights were significantly different between the prodrug, recombinant Ad and control groups.

Effect of recombinant Ads containing fusion suicide gene on the histology and MVD of human breast tumors in nude mice. The tumors were excised and analyzed by H&E staining. H&E sections showed lamellar necrosis of tumor cells and leukocyte infiltration in the recombinant Ad + prodrug group (Fig. 6A).

The MVD of the tumor tissue was assessed using immunohistochemistry with CD34 monoclonal antibody. Compared with the control group, the MVD of the tumor tissue was significantly decreased in the recombinant Ad + prodrug group ($P<0.05$; Fig. 6B and C).

Histology of heart, liver, lung, kidney and small intestine. To observe the systemic toxicity of the recombinant Ads and prodrugs, the liver, heart, lung, kidney and small intestine of the nude mice transplanted with human breast tumors, injected

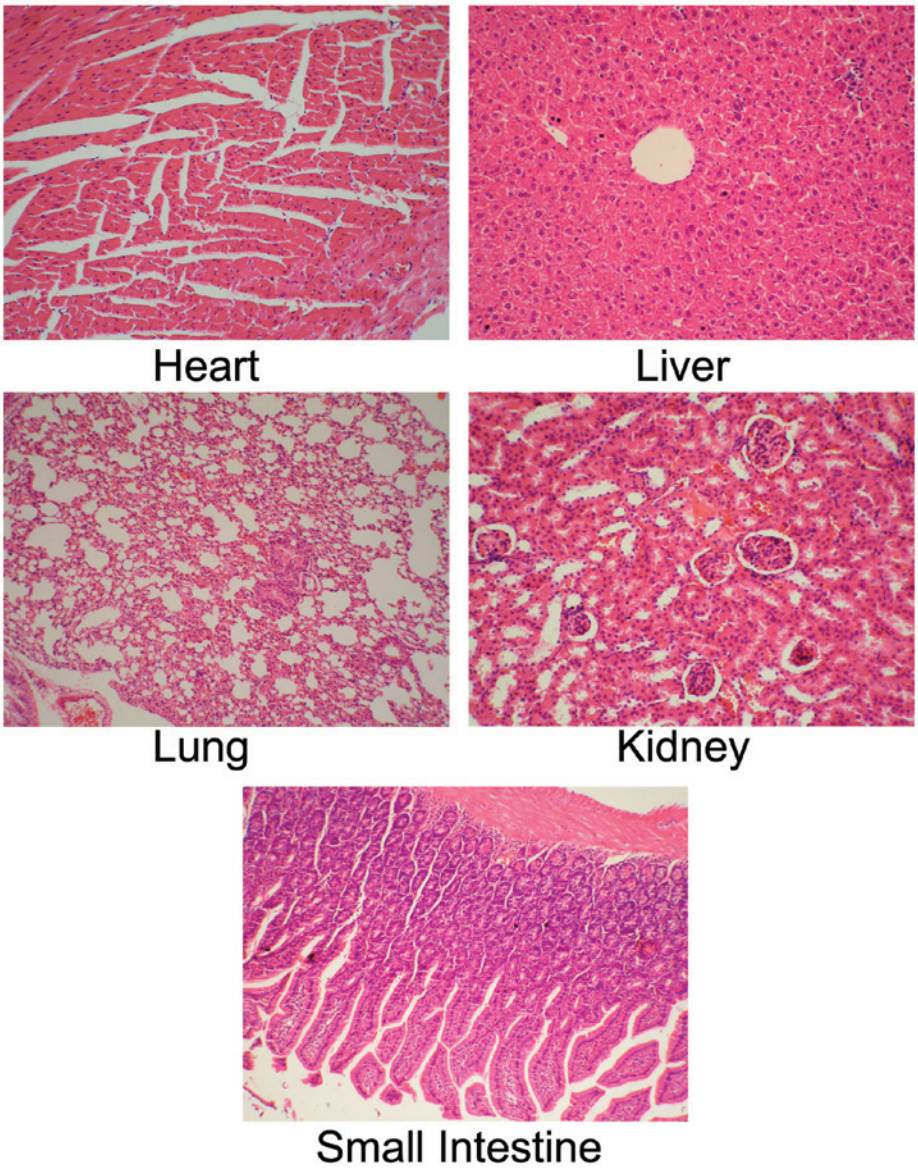

Figure 7. Histology of heart, liver, lung, kidney and small intestine in nude mice transplanted with human breast tumors and injected with the recombinant Ad and treated with GCV and 5-FC. Human breast tumor cells MCF7 were injected into the nude mice by subcutaneous injection. The tumors, of 50 mm mean diameter, were established 12 days after MCF7 cells were injected. The recombinant Ad ($1x10^{10}$ pfu) were injected into the tumor, followed by GCV (50 mg/kg/day) and 5-FC (500 mg/kg/day) treatment by intraperitoneal injection for 18 days. The heart, liver, lung, kidney and small intestine of nude mice were excised and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was x400. Ad, adenovirus; GVC, ganciclovir; 5-FC, 5-fluorocytosine.

with the recombinant Ads and treated with GCV and 5-FC were examined by H&E staining. There was no abnormal histology in the heart, liver, lung, kidney and small intestine of the nude mice transplanted with human breast tumors and injected with the recombinant Ads and treated with GCV and 5-FC (Fig. 7).

Discussion

The KDR gene is specifically expressed in certain tumor and vascular endothelial cells; therefore the KDR promoter has been used to express target genes in certain tumors due to its tumor-specific expression (20-22). In the present study, we report a potential treatment modality for breast cancer. This strategy involves the use of the tumor and tumor blood vessel double targeting tissue-specific promoter, KDR, incorporated into recombinant Ad vectors, to target the expression of the CDglyTK fusion gene transcriptionally to breast cancer cells and neogenetic vascular endothelial cells. The Ad-mediated and KDR promoter-driven CD/TK double suicide gene system was successfully established and transfected into the MCF7 human breast cancer and the ECV304 endothelial cell lines, which expressed endogenous KDR, and the colon carcinoma cell line, LS174T, which did not express endogenous KDR. The CD/TK gene was expressed in MCF7 and ECV304 cells, but was not expressed in LS174T cells. These results revealed that the CDglyTK gene was stably expressed in the cells with a higher level of endogenous KDR.

The results of this study suggest that the double suicide genes were functionally activated in the three cell lines (FCF-7,

ECV304 and LS174T cells) infected with Ad-CMV-CDglyTK. However, in the cells infected with Ad-KDRP-CDglyTK, the double suicide genes were functionally activated only in the MCF7 and ECV304 cells which expressed endogenous KDR *in vitro*. Therefore, treatment with 5-FC, GCV and 5-FC + GCV did not influence the cell survival rate in LS174T and LS174T-CDglyTK cells. These results show that the transgenic CDglyTK double suicide genes are not expressed in the LS174T cells due to the inactivity of the KDR promoter. The cell survival rate was significantly decreased by treatment with 5-FC, GC or 5-FC + GCV in the MCF7 and ECV304 cells transfected with the CDglyTK double suicide genes. The results of the present study demonstrate that the tumor-targeted expression of CDglyTK driven by the CMV promoter has a high performance but does not have specificity and that the tumor-targeted expression of CDglyTK driven by the KDR promoter has a high specificity and performance.

A previous study revealed that the killing efficiency of the combined suicide gene system is higher compared with any single system in human lung cancer cells (14). Rogulski *et al* demonstrated that neuroglioma cells transfected with the CDglyTK double suicide gene are easily inhibited and that double suicide gene therapy augments the antitumor activity of a replication-competent lytic Ad via enhanced cytotoxicity and radiosensitization (23). Qiu *et al* reported that the recombinant plasmid, pCEA-TK/CD, containing a carcinoembryonic antigen (CEA) promoter and the double suicide genes, TK and CD, decreased the half maximal inhibitory concentration of the prodrugs and increased apoptosis and cyclomorphosis in the presence of the prodrugs, 5-FC and GCV, in lung cancer cells (24). The TK/GCV and CD/5-FC suicide gene therapy induced cell death via the mitochondrial pathway triggered by the modulation of Bcl-2 proteins in glioma cells (25). Our study indicates that the effect of double suicide genes is much stronger than that of individual suicide genes. The combined treatment of 5-FC and GCV resulted in a lower cell survival rate than the single prodrug treatment, which suggests that the killing effect of the CDglyTK double suicide genes combined with prodrug treatment was enhanced. The results of the present study also showed that the CDglyTK-transduced MCF7 and ECV304 cells were arrested at the S phase following treatment with the prodrugs. Moreover, apoptosis and necrosis of cells were exhibited in the CDglyTK-transduced MCF7 and ECV304 cells.

In order to observe the antitumor effect of Ad-KDRP-CDglyTK *in vivo*, breast cancer nude mouse models from human MCF7 were established. The results from these models showed that the tumors from the breast cancer cells were significantly suppressed and that the MVD of tumors was decreased by the systemic treatment of the prodrugs, 5-FC and GCV, in the breast cancer nude mouse models with the CDglyTK gene. Therefore, Ad-KDRP-CDglyTK has both tumor cell-targeting and tumor vessel blood-targeting effects. Abnormal histology of liver, heart, lung, kidney and small intestine was not observed in the breast cancer nude mouse models with the CDglyTK gene. These results suggest that the recombinant Ads and prodrugs do not exert systemic toxicity to nude mice.

In conclusion, our study suggests that the KDR promoter is capable of regulating a double suicide gene system in human breast cancer cells and vascular endothelial cells, thus providing evidence for the development of a gene therapy approach to treating breast cancer. Our research indicates that the expression of CDglyTK genes under the control of the KDR promotor represents a new strategy for the effective gene therapy of breast cancer.

Acknowledgements

This study was supported by a grant from the National High Technology Research and Development Program of China (2001AA217171), Natural Science Foundation of Guangdong Province (No. 013072) and the Program of Health Bureau of Xiamen City (WSZ0609).

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