

# Human telomerase reverse-transcriptase promoter-controlled and herpes simplex virus thymidine kinase-armed adenoviruses for renal cell carcinoma treatment

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**Abstract:** New treatment strategies are required for renal cell carcinoma (RCC) due to its relative insensitivity to conventional radio- and chemotherapies. The promising strategy of tumor inhibition using human telomerase reverse transcriptase (hTERT)-controlled herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) in the hTERT promoter-driven *HSV-TK/GCV* suicide gene system was investigated. Tumor volume, weight, relative proliferation rate, and cell-apoptosis levels were examined in mice injected with adenovirus (Ad)-hTERT-HSV-TK and GCV. Increased cell death occurred following treatment with Ads carrying hTERT-HSV-TK/GCV or cytomegalovirus promoter-controlled (CMV)-HSV-TK/GCV for human RCC 786-0 and fibroblast MRC-5 cells. In mice, Ad-hTERT-HSV-TK/GCV more specifically inhibited tumor and RCC xenograft growth than Ad-CMV-HSV-TK/GCV ( $P < 0.05$ ). Furthermore, Ad-hTERT-HSV-TK/GCV did not significantly damage normal fibroblasts or organ systems (heart, lung, liver, brain, kidney, and spleen). Thus, Ad-hTERT-HSV-TK/GCV is an effective RCC inhibitor in human cells in vitro and in vivo mouse models, indicating potential usefulness in RCC-targeted gene therapy.

**Keywords:** hTERT promoter, HSV-TK/GCV, renal cell carcinoma, adenovirus

## Introduction

Kidney cancers account for 3% of all adult malignancies and 90%–95% of all kidney neoplasms worldwide, with incidence and mortality rates increasing as much as 2%–3% each decade.<sup>1</sup> As many as ten of every 100,000 people are affected, 25%–30% of patients develop treatment-resistant metastatic disease, and 40% develop postoperative recurrence.<sup>1,2</sup> Clear-cell renal cell carcinoma (CCRCC) is the most prominent form of kidney cancer,<sup>3,4</sup> and it commonly exhibits minimal or no sensitivity to conventional chemo- or radiotherapies.<sup>5</sup> Thus, new targeted treatments for RCC are urgently required.

Contemporary cancer gene therapies utilize immune-related, tumor-suppressor genes and drug-susceptible therapies, such as infection with herpes simplex virus thymidine kinase/ganciclovir (*HSV-TK/GCV*) suicide genes.<sup>6</sup> Such suicide gene therapies have the benefit of specifically targeting and selectively killing transformed tumor cells and simultaneously removing surrounding cells (“bystander effect”) with minimal systemic toxicity.<sup>7</sup> However, designing targeted suicide genes that properly distinguish between tumor and normal host cells without adversely affecting adjacent tissues and organ systems remains challenging.

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Suicide genes code for highly toxic, high-catalytic-activity enzymes that are either entirely absent or expressed in very low concentrations in normal host cells.<sup>8</sup> Suicide gene therapy is commonly referred to as gene directed enzyme/prodrug therapy, including both cell-based therapies, tumor-based therapies, and viral vector therapies.<sup>8,9</sup> The primary difference between these therapies is which agent (adjacent cells, the tumor itself, or a viral vector) carries the suicide gene. Due to the latency of the herpes viruses, viral TKs have been described as the ultimate target in suicide gene therapy, and numerous research studies have been conducted using the human cytomegalovirus (CMV), whose UL97 gene codes for a protein kinase that, like viral TKs, is activated by phosphorylation in various cancers.<sup>10</sup>

The *HSV-TK* gene was first proposed for use in tumor treatment by Moolten et al,<sup>11</sup> and over 40 clinical trials have since reported positive results in non-small-cell lung cancer,<sup>7</sup> brain and spinal cord malignant gliomas,<sup>12</sup> hepatocellular carcinoma,<sup>13</sup> and ovarian tumors.<sup>14</sup> GCV is a structural analogue of the second messenger cyclic guanosine monophosphate.<sup>10</sup> Notably, the HSV-TK enzyme has 1000× higher affinity for substrate GCV than the host cell TK, and HSV-TK has high affinity for various other nucleoside analogues of GCV, including acyclovir and penciclovir.<sup>15</sup> Thus, tumor cell death is caused by the conversion of GCV into a toxic metabolite at high concentrations in tumor cells but not in normal host cells, widely used in targeted cancer therapy.<sup>8</sup>

Tumor cells are characterized by their unlimited proliferative capacity, widely believed to be associated with preservation of telomeres by activated telomerase, which is composed of an enzyme protein and RNA responsible for telomerase activation.<sup>16</sup> Telomerase is inactive in normal somatic cells and benign tumors; however, upregulation of expression of human telomerase reverse transcriptase (hTERT) has been associated with telomerase activation in numerous human cancers.<sup>17</sup> Notably, Kim et al<sup>18</sup> reported telomerase activity in 100 human tumor cell lines from 18 cancer types compared to 22 normal human somatic cell lines using telomere repeat amplification protocol, revealing a 98% positive rate.

Modern therapeutic strategies for inhibition of telomerase activity primarily target telomerase RNA and hTERT, though competitive inhibition of reverse transcription has also been explored.<sup>19</sup> Recently, the hTERT promoter has been employed to control therapeutic gene expression, thus facilitating specific targeting and destruction of tumor cells by altering telomerase activation.<sup>20,21</sup> The hTERT promoter has also been used to regulate therapeutic

genes, such as B-cell lymphoma 2-associated X protein, caspase 8, and suicide genes to achieve specific expression levels in telomerase-positive tumor.<sup>22</sup> Clinically, the hTERT-driven *HSV-TK/GCV* system has inhibited tumor growth successfully in human cell lines and animal models of breast,<sup>22</sup> pulmonary,<sup>7</sup> and ovarian cancers.<sup>23</sup> Thus, selective regulation of exogenous suicide gene expression in telomerase-positive tumor cells can dramatically improve targeted therapies.

The hTERT promoter was employed to control the adenovirus (*Ad*)-*HSV-TK/GCV* suicide gene system, exploiting the proliferative character of malignant RCC cells to achieve a recombinant Ad-hTERT-*HSV-TK/GCV* with high specificity and efficacy for RCC cells both in vivo and in vitro. Consistent with previous successes in other cancer types,<sup>7,22,23</sup> this method provides a potential tool for improving RCC patient prognosis.

## Materials and methods

### Cell lines and cultures

Human (hCCRCC) 786-0 and human fibroblast (hFB) MRC-5 cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere and 0.25% trypsin (Sigma-Aldrich, St Louis, MO, USA) for cell digestion before passaging. hCCRCC 786-0 cells were cultured in 1640 medium containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 100 µg/mL ampicillin (Sigma). hFB MRC-5 cells were cultured in Eagle's minimal essential medium containing 10% fetal bovine serum (Life Technologies).

### Adenovirus construction

Replication-deficient recombinant adenoviral vectors pCA13 and pBGHE3 were used in Ad construction (Microbix Biosystems, Mississauga, ON, Canada). Briefly, pCA13-CMV-*HSV/TK* and pCA13-hTERT-*HSV/TK*; pCA13-CMV-*HSV/TK* and pCA13-hTERT-*HSV/TK*; pCA13-CMV-EGFP and pCA13-hTERT-EGFP; and pCA13-CMV-EGFP and pCA13-hTERT-EGFP were constructed by digesting plasmids pUC19-*HSV/TK* (Second Military Medical University collection); pCA13; plasmid pUC19-EGFP; and pSG-CMV-EGFP and pSG-hTERT-EGFP, respectively, with *EcoRI* and/or *XbaI*. These produced the 1143 bp *HSV/TK* fragment, which was ligated with digested pSG-CMV and pSG-TP (pSG-CMV-*HSV/TK* and pSG-hTERT-*HSV/TK*), fragments ligated with the digested pCA13 vector (pCA13-CMV-*HSV/TK* and pCA13-hTERT-*HSV/TK*), fragments ligated with digested pSG-CMV and pSG-TP (pSG-CMV-EGFP and pSG-hTERT-EGFP), and CMV-EGFP and

hTERT-EGFP fragments ligated with digested pCA13 vector (pCA13-CMV-EGFP and pCA13-hTERT-EGFP).

## Recombination and expansion of Ad-Her

Plasmids pCA13-CMV-HSV/TK and pCA13-hTERT-HSV/TK, pCA13-CMV-EGFP, and pCA13-hTERT-EGFP were cotransfected with pBGHE3 (containing the entire 5-Ad except 188-1339 bp) using Lipofectamine 2000 in 293 cells to obtain the recombinant virus. The viral plaque appeared after 9 days, and the recombinant adenovirus was obtained by purification (3×). DNA of the recombinant virus was extracted by the QIAamp DNA Kit (Qiagen, Venlo, Netherlands) and characterized by polymerase chain reaction, producing clones Ad-CMV-HSV/TK, Ad-hTERT-HSV/TK, Ad-CMV-EGFP, and Ad-hTERT-EGFP. The recombinant virus and the wild-type adenovirus were characterized by polymerase chain reaction.

## Virus infection

hCCRCC 786-0 cells and hFB MRC-5 cells were seeded in six-well plates at  $1 \times 10^5$ /well and  $1 \times 10^6$ /well (Thermo Fisher Scientific, Waltham, MA, USA), respectively, and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 24 hours. Cells were then placed in serum-free medium containing the nonproliferative recombinant Ad-CMV-EGFP and Ad-hTERT-EGFP with GFP genes at multiplicities of infection (MOIs) of 0, 1, 5, 10, 50, 100, and 200 for 2 hours followed by culture medium containing 5% serum. GFP expression was observed by fluorescence microscopy (TE300; Nikon, Tokyo, Japan) 2 and 7 days after infection.

Recombinant Ad effect on cells was determined by cell-viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as previously described.<sup>24</sup>

## Mouse RCC model

Forty BALB/C nude mice aged 4 and 6 weeks were purchased from the Animal Center of the Chinese Academy of Medical Sciences and housed under sterile conditions at the Third Hospital of Hebei Medical University and used in nude mice model xenograft models of RCC. Briefly,  $5 \times 10^7$ /mL (0.1 mL) of 786-0 cell suspension was subcutaneously injected into the left, front dorsa of each mouse, and all mice were killed when tumor growth reached a diameter of ~6 mm.

Mice were divided randomly into four groups: A, no treatment (control group); B, intraperitoneal injection of GCV (50 mg/kg) on days 2–18; C, Ad-hTERT-HSV-TK  $6.0 \times 10^7$ /kg tail vein injection on days 1, 7, and 13, plus

intraperitoneal injection of phosphate-buffered saline solution on days 2–18; and D, similar intravenous injection of Ad-hTERT-HSV-TK and similar intraperitoneal injection with GCV (treatment group).

Tumor diameters were measured with a vernier caliper every 3 days for 30 days. Mice were then killed, and tumor weight, pathological condition, and visceral organ condition were determined. All tissues were further subjectively assessed using visual analysis by an experienced researcher, and distinctive qualitative observations were reported.

## Immunohistochemical and pathological examinations

Excised tumors were dissected, fixed in 10% formalin, embedded in paraffin, cut into thin slices, dewaxed, stained with hematoxylin and eosin, and sealed with resin. Expression of proliferating cell nuclear antigen (PCNA) was determined by immunohistochemical staining with mouse anti-human PCNA antibody (1:100; Abcam, Cambridge, UK) or phosphate-buffered saline in negative controls. Nuclei of PCNA-positive cells (dark brown) were observed, and the proportion of PCNA-positive cells per field in ten fields was recorded (mean = PCNA proliferation index).

## TUNEL assay

Paraffin sections were dewaxed, hydrated, dried, digested with 20 µg/mL proteinase K at room temperature, fixed for 5 minutes with 4% paraformaldehyde, and incubated with terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) working solution (8.3 U TdT, 0.83 nmol Dig-dUTP, 50 µL TdT buffer) at 37°C for 60 minutes. The reaction was terminated by 2 × saline–sodium citrate. Sections were incubated with alkaline phosphatase-labeled anti-Dig Fab fragment (1:1000; Abcam) at 25°C, detected by 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium, dyed with neutral red, and sealed. Results were determined by examining positive cells (granular/diffuse brown staining) in ten randomly selected and consecutive high power (×400) fields (mean = apoptotic index).

## Statistical analysis

All statistical analyses were performed using SPSS version 11.0 (IBM, Armonk, NY, USA). All measured data were expressed as means ± standard deviation. Differences between groups were compared by *t*-tests or variance analyses, and unpaired *t*-tests were used to compare data between cell lines. Tumor weight, apoptosis, and proliferation were compared with controls using  $\chi^2$  and least significant difference *t*-tests. Rates were

compared with  $\chi^2$  and rank-sum tests.  $P$ -values less than 0.05 were considered statistically significant ( $P < 0.05$ ).

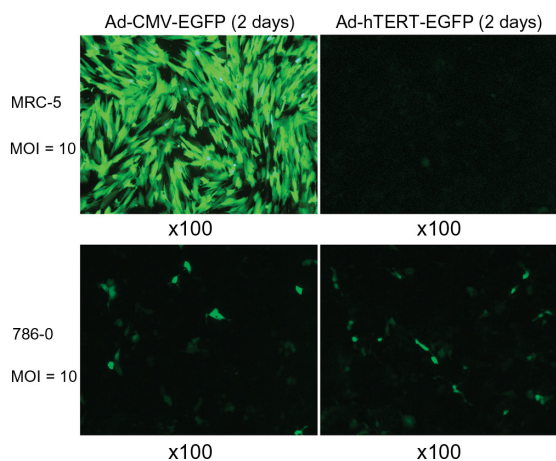
## Results

### Characteristics of viral infection

Efficient infection in MRC-5 and 786-0 cells was achieved using the recombinant Ad-CMV-EGFP at an MOI of 10. Expression of GFP was highest after postinfection day 1, demonstrating expressions in excess of 65% of MRC-5 and 786-0 cells. MRC-5 cells were better infected. For Ad-hTERT-EGFP at an MOI of 10, only 786-0 was appreciably infected. Highest GFP expression was observed on postinfection day 2, demonstrating expressions in excess of 55% of 786-0 cells. No infection was observed in MRC-5 cells using Ad-hTERT-EGFP at MOI values of 1, 5, 10, 20, 30, 50, and 100 (Figure 1).

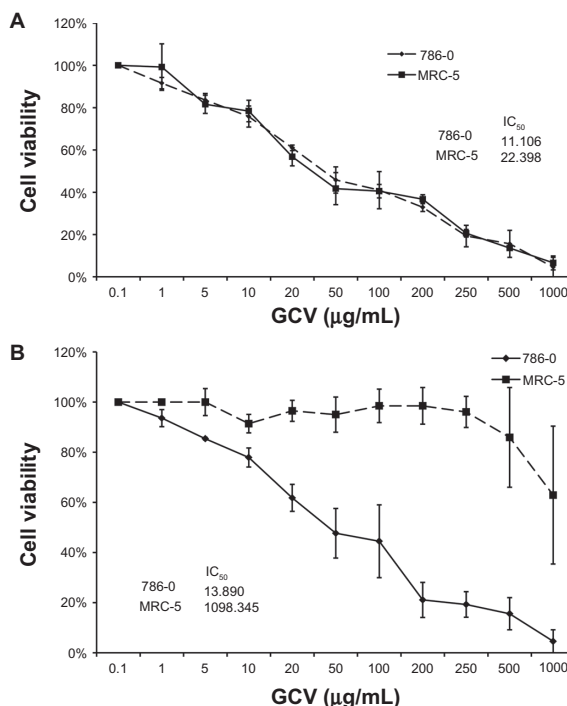
### Ad-hTERT-HSV-TK/GCV-mediated specific cytotoxicity to RCC cells in vitro

At similar MOIs of Ad-CMV-HSV-TK, half-maximal inhibitory concentration ( $IC_{50}$ ) values of GCV in in vitro cell lines 786-0 and MRC-5 were not significantly different (11.106  $\mu\text{g/mL}$  and 22.398  $\mu\text{g/mL}$ , respectively;  $P > 0.05$ ) (Figure 2A). At similar MOIs of Ad-hTERT-HSV-TK, however,  $IC_{50}$  values of GCV for 786-0 and MRC-5 cells were significantly different (13.890  $\mu\text{g/mL}$  and 1098.345  $\mu\text{g/mL}$ , respectively;  $P < 0.05$ ) (Figure 2B), indicating specific cytotoxicity of Ad-hTERT-HSV-TK/GCV for RCC cancer cells.



**Figure 1** Expression of adenovirus cytomagalovirus-enhanced green fluorescent protein (Ad-CMV-EGFP) and Ad-human telomerase reverse transcriptase (hTERT)-EGFP in MRC-5 and 786-0 cells.

**Notes:** Photographs of human clear-cell renal cell carcinoma 786-0 cells and hFB MRC-5 cells infected with Ad-CMV-EGFP or Ad-hTERT-EGFP at multiplicities of infection (MOIs) of 10 and 30 at postinfection day 2.



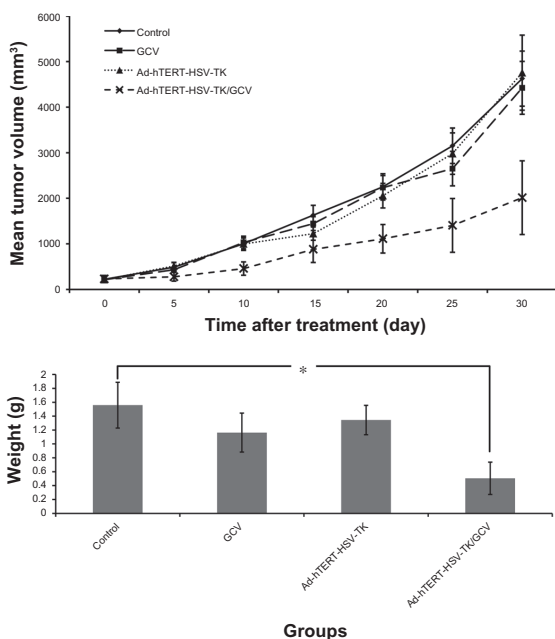
**Figure 2 (A and B)** Cell viability comparison of MRC-5 and 786-0 cell lines infected by either adenovirus human telomerase reverse-transcriptase herpes simplex virus thymidine kinase/ganciclovir (Ad-hTERT-HSV-TK/GCV) or Ad-cytomegalovirus (CMV)-HSV-TK/GCV. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure the cell viability after 7 days. Half-maximal inhibitory concentration ( $IC_{50}$ ) was compared using unpaired  $t$ -tests.  $P$ -values less than 0.05 were considered statistically significant. **(A)** Cell-viability comparison of MRC-5 and 786-0 cell lines infected by Ad-CMV-HSV-TK/GCV. **(B)** Cell-viability comparison of MRC-5 and 786-0 cell lines infected by Ad-hTERT-HSV-TK/GCV.

### Ad-hTERT-HSV-TK/GCV inhibited RCC tumor growth and prolonged survival in vivo

In in vivo mouse models, injection with recombinant Ad-hTERT-HSV-TK/GCV produced significantly smaller mean tumor volume on postinjection days 13, 17, 21, 25, and 30 in the treatment group than in the other 3 groups as controls ( $P < 0.05$ ) (Figure 3A). At postinjection day 30, the tumor inhibition rate reached 76.98% in Ad-hTERT-HSV-TK/GCV-treated mice (<26.49% in all other groups) (Figure 3A). Tumor weights in Ad-hTERT-HSV-TK/GCV-treated mice were significantly lower than those in all other groups (Figure 3B). By postinjection day 14, surviving mice numbered six (control group), four (GCV group), and five (Ad-hTERT-HSV-TK group). Conversely, all Ad-hTERT-HSV-TK/GCV-treated mice survived ( $P < 0.05$ ).

### Pathological characteristics

Treatment-group specimens exhibited drags and coke-like necrosis upon pathological assessment, while no obvious necrosis and more neovascularization was observed in

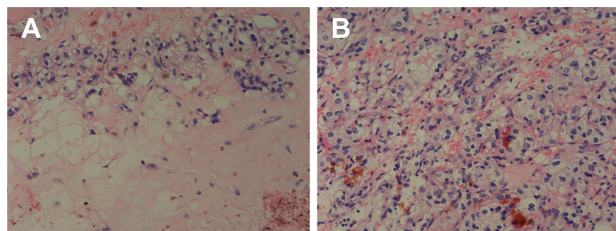


**Figure 3 (A and B)** Comparison of tumor weight and volume in a nude mice model of renal cell carcinoma (RCC). **(A)** Growth curve of nude mice RCC. **(B)** Tumor weight of nude mice RCC.

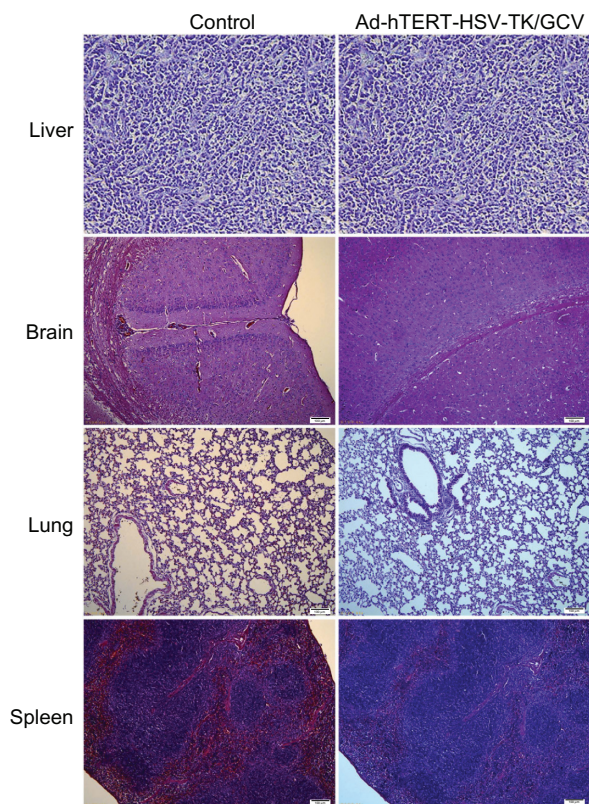
**Notes:** Weight of the tumor was compared with control using the  $\chi^2$  test followed by the least significant difference t-test. *P*-values less than 0.05 were considered statistically significant.

**Abbreviation:** Ad-hTERT-HSV-TK/GCV, adenovirus human telomerase reverse-transcriptase herpes simplex virus thymidine kinase/ganciclovir.

control-group specimens. Furthermore, pathological examination revealed that tumor cells treated with Ad-hTERT-HSV-TK/GCV displayed a unique pattern of scattered, sparse, and flaky necrosis, and tumor vessels evidenced significant reduction, disorder was observed in tumor structure, and replacement of neoplastic parenchyma with fibrous tissue was apparent. In some tumor cells, cytoplasmic vacuolar degeneration, reduced nuclei numbers, loosened chromatin, lightly stained cytoplasm, and nuclear pyknosis were observed (Figure 4). Notably, the heart, lung, liver, brain, kidney, and spleen tissues evidenced no significant morphological differences following Ad-hTERT-HSV-TK/GCV treatment (Figure 5).



**Figure 4 (A and B)** Histological slices of infected tumor tissues. Infected tissues with **(A)** adenovirus human telomerase reverse-transcriptase herpes simplex virus thymidine kinase/ganciclovir or **(B)** control.



**Figure 5** Histological slices of tissues from brain, lung, spleen, and liver in control mice (left panel) and in mice infected by adenovirus human telomerase reverse-transcriptase herpes simplex virus thymidine kinase/ganciclovir (Ad-hTERT-HSV-TK-GCV, right panel).

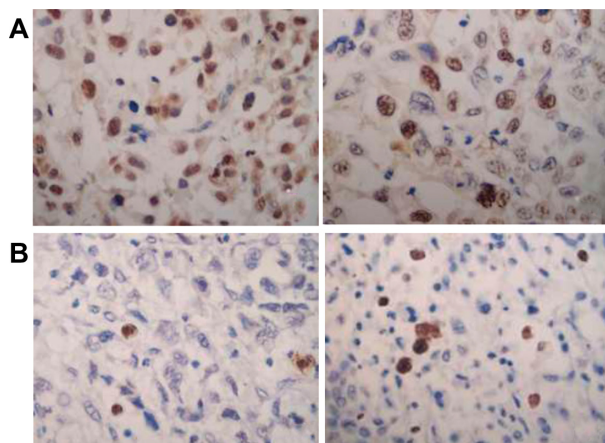
### Ad-hTERT-HSV-TK/GCV inhibits proliferation and induces apoptosis in RCC cells

Ad-hTERT-HSV-TK/GCV-treated mice exhibited large numbers of apoptotic cells and cells with vacuolar degeneration, while few or no apoptotic cells were observed in tumors from other groups (Figure 6A). The apoptotic index of the Ad-hTERT-HSV-TK/GCV treatment, control, and GCV and Ad-hTERT-HSV-TK groups was  $3.2\% \pm 0.3\%$ ,  $0.1\% \pm 0.1\%$ ,  $0.2\% \pm 0.1\%$ , and  $0.2\% \pm 0.1\%$ , respectively ( $P < 0.05$ ) (Table 1).

The PCNA proliferation index of the Ad-hTERT-HSV-TK/GCV treatment, control, and GCV and Ad-hTERT-HSV-TK groups were  $27.57\% \pm 9.36\%$ ,  $71.2\% \pm 13.1\%$ ,  $69.8\% \pm 11.6\%$ , and  $72.4\% \pm 12.3\%$ , respectively. Ad-hTERT-HSV-TK/GCV significantly inhibited the proliferation of RCC cells compared to effects observed in the other 3 groups as controls ( $P < 0.01$ ) (Figure 6B and Table 1).

### Discussion

Ad-hTERT-HSV-TK/GCV can not only inhibit the growth of RCC xenografts in mice but it can also prolong



**Figure 6 (A and B)** Histology of apoptosis and proliferation in nude mice renal cell carcinoma xenografts from adenovirus human telomerase reverse-transcriptase herpes simplex virus thymidine kinase/ganciclovir (Ad-hTERT-HSV-TK-GCV)-infected or control mice. **(A)** Histology of apoptosis cells from Ad-hTERT-HSV-TK-GCV-infected (left panel) or control mice by terminal deoxynucleotidyl transferase dUTP nick-end labeling (right panel). **(B)** Histology of proliferative cells from Ad-hTERT-HSV-TK-GCV-infected (left panel) or control mice by nuclear antibody staining (right panel).

the survival time of tumor-bearing mice. Furthermore, Ad-hTERT-HSV-TK/GCV therapy was shown to produce no significant tissue damage in nude mice in adjacent cells, indicating a limited bystander effect. Ad-hTERT-HSV-TK/GCV caused selective tumor cell death in vitro and also reduced the volume and weight of mice RCC xenografts by increasing apoptosis and reducing proliferation of the tumor cells. This novel suicide gene therapy for RCC may thus provide an effective future clinical method for improving patient outcomes.

Currently, HSV-TK/GCV suicide therapy has been successfully applied in experimental research and phase I clinical trials,<sup>13</sup> but no studies of RCC have been previously reported. Leveraging the unique proliferative property of

malignant tumors, the current study employed an Ad carrying the *HSV-TK* gene under the regulation of the hTERT promoter to limit the expression of *HSV-TK* in telomerase-positive tumors. Mehle et al<sup>25</sup> detected telomerase activity in 71 cases of kidney cancer, reporting a positive rate of 78.9% in RCC tumor tissues and 0% in adjacent tissues. These results identify hTERT expression as a specific characteristic of RCC cells, a finding consistent with the current results. In both RCC 786-0 cells and normal human fibroblast MRC-5 cells of the present study, efficient Ad-CMV-EGFP infection was achieved at an MOI of 10 and 30, respectively. These findings indicate CMV vector-mediated, high-level, nonselective expression of the exogenous gene. Ad-hTERT-EGFP infected 786-0 cells effectively at the same MOI, but did not infect MRC-5 cells at any MOI, suggesting that the hTERT-mediated gene expression is highly tumor-specific. These findings are consistent with the results published by previous reports.<sup>26,27</sup>

Both Ad-CMV-HSV-TK/GCV and Ad-hTERT-HSV-TK/GCV demonstrated the ability to limit the proliferation of RCC tumor cells in the current study. The variant  $IC_{50}$  values for GCV observed in tumor cells versus normal cells suggests that introduction of the hTERT promoter reduced nonspecific cytotoxicity to normal cells and improved the efficacy/toxicity ratio of the recombinant adenovirus. Furthermore, Ad-hTERT-HSV-TK/GCV inhibited the growth of tumor cells in vivo in mice, without excessive damage to adjacent tissues and organs through the bystander effect. Notably, this effect has been associated with gap-junctional intercellular communication, which allows toxic metabolites of GCV to surrounding cells despite nonexpression in these cells both in vitro and in vivo.<sup>28</sup> The reason for minimization of this effect is currently undetermined, though the notably low level of adjacent tissue damage merits further investigation and may offer insights into possible molecular strategies for enhancing the extent of tumor cell death without producing excessive bystander effects. Although Ad-CMV-HSV-TK/GCV mediated higher expression levels of HSV-TK, thereby producing stronger inhibition of tumor cell proliferation, its main deficiency was a lack of specific targeting. Thus, further study of appropriate targeting mechanisms must be explored before clinical implementation is considered.

The unique degenerative characteristics of tumor cells treated with Ad-hTERT-HSV-TK/GCV, including visible necrosis upon pathological examination and in vivo reductions in tumor growth and cell proliferation, indicate that this treatment may act in both direct and indirect mechanisms,

**Table 1** Index of apoptosis and proliferation in renal cell carcinoma xenograft

Group	Apoptosis index by TUNEL	Proliferation index by PCNA
Control	0.1 ± 0.1 (%)	71.2 ± 13.1 (%)
GCV	0.2 ± 0.1 (%)	69.8 ± 11.6 (%)
Ad-hTERT-HSV-TK	0.2 ± 0.1 (%)	72.4 ± 12.3 (%)
Ad-hTERT-HSV-TK/GCV	3.2 ± 0.3 (%)*	27.57 ± 9.36 (%)**

**Notes:** Comparison was studied using the  $\chi^2$  test followed by the least significant difference *t*-test. *P*-values less than 0.05 were considered statistically significant. \*Compared with other groups (*P* < 0.05); \*\*compared with other groups (*P* < 0.01).

**Abbreviations:** TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PCNA, proliferating cell nuclear antigen; Ad-hTERT-HSV-TK/GCV, adenovirus human telomerase reverse-transcriptase herpes simplex virus thymidine kinase/ganciclovir.

as suggested by previous studies of other cancer cell lines.<sup>28–30</sup> Notably, these mechanisms exhibited no harmful effects on major organ systems, such as the heart, lung, liver, kidney, and spleen, in the current study. These findings further indicate the safety of Ad-hTERT-HSV-TK/GCV treatment. While previous studies have shown that intratumoral injection of Ad-CMV-HSV-TK may be effective, these studies have still reported severe toxicity associated with treatment.<sup>31</sup> The current route of administration, however, produced limited systemic toxicity.

In the current study, tumors treated with Ad-hTERT-HSV-TK or GCV showed no statistically significant reduction in size compared to controls, further confirming that isolated Ad-hTERT-HSV-TK or GCV treatments exhibit no antitumor effects. Zhang et al<sup>7</sup> found that the exogenous gene expression peaked 3 days after the intratumoral injection of the adenovirus Ad-hTERT-E1A-TK, suggesting a time-sensitive parameter associated with GCV administration. Further study, however, will be required to assess the effects of GCV injection timing on outcomes. The relationship between Ad distribution in tumors and exogenous gene-expression levels should also be further clarified prior to clinical implementation. Furthermore, future work should carefully examine the high immunogenicity induced by anti-Ad antibodies immediately after intravenous administration, which may compound the findings of the current study.

The therapeutic antitumor effects of Ad-hTERT-HSV-TK/GCV for the treatment of RCC were apparent in the current study, the first reported application of HSV-TK/GCV for RCC treatment. Tumor-specific regulation of the hTERT promoter was able to ensure cancer-specific expression of *HSV-TK* in malignant RCC cells, with minimal harm to surrounding normal cells and organ systems. Because systemic toxicity is limited in this treatment strategy, future clinical strategies may be designed to limit damage to organ systems through the bystander effect and improve overall patient quality of life. The current study provides valuable preliminary data for the clinical application of Ad-hTERT-HSV-TK/GCV suicide gene therapy for RCC treatment.

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## Disclosure

The authors report no conflicts of interest in this work.

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