

# Adenovirus-mediated herpes simplex virus thymidine kinase gene therapy combined with ganciclovir induces hepatoma cell apoptosis

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**Abstract.** The present study aimed to examine the apoptotic effects of adenovirus (ADV)-mediated herpes simplex virus thymidine kinase (ADV-TK) combined with ganciclovir (GCV) in tissues obtained from patients with hepatocellular carcinoma in order to provide a theoretical basis for the development of this gene therapy program. Apoptosis detection was conducted using the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling assay and the apoptosis index was compared between the experimental; and control groups. Furthermore, the protein expression levels of caspase-3, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated protein X (Bax) and nuclear factor (NF)- $\kappa$ B were examined in pathological specimens using immunohistochemical staining. The Bax/Bcl-2 ratio and the release of cytochrome *c* were examined using western blot analysis. Results indicated that combined ADV-TK and GCV treatment significantly increased the number of apoptotic cells compared with the control group ( $P < 0.05$ ). Immunohistological analysis revealed that ADV-TK and GCV treatment significantly increased the number of caspase-3-positive cells, reduced the Bax/Bcl-2 ratio and NF- $\kappa$ B expression levels and promoted the release of cytochrome *c* compared with the control group ( $P < 0.01$ ). In conclusion, the present results suggest that combined ADV-TK and GCV treatment exerts its effect through the apoptotic signaling pathway.

## Introduction

Hepatocellular carcinoma (HCC) is a complicated type of malignant tumor with a high global incidence rate compared with other malignant tumor types (1). The percentage of

cases of nonalcoholic steatohepatitis HCC has increased in the past; the overall survival has not. Solely 31% of patients with HCC identified via screening/surveillance received any curative treatment (2,3). Despite the reasonable progression in the understanding of the disease mechanism and its therapeutic possibilities in the past three decades, poor therapeutic outcomes have been reported in response to conventional treatments, including liver transplantation and surgical resection, and the recurrence rate remains considerably high (4,5). In addition, the presence of multifocal tumors in the liver is considered to be a notable risk factor for HCC incidence and due to tumor cell invasion and intrahepatic metastasis, alternative treatments have been described that may improve clinical outcomes, including intratumorally injected gene therapy (6).

Gene-mediated cytotoxic immunotherapy (GMCI) is a clinical intervention that forms a tumor-specific vaccine effect via intratumoral delivery of adenovirus (ADV)-mediated herpes simplex virus thymidine kinase (ADV-TK) followed by a systemic anti-herpetic prodrug, such as valacyclovir or ganciclovir (GCV), in combination with standard tumor resection surgery or radiation (7). Necrotic and apoptotic cell death, and acute inflammation form surgery activate and entice antigens, which induce immune cells and T-cell expansion (7). Stimulation of T cell proliferation and the production of inflammatory cytokines may be induced by herpes simplex virus type 1 thymidine kinase (HSV-TK) protein, which has been described to resemble a super-antigen molecule (7). This immunostimulatory environment forms a systemic anti-tumor immune response that leads to the release of autologous tumor-associated antigens (TAAs) (7). Notably, GMCI treatment towards local tumors led to protection against metastases in mouse syngeneic models (8-10). In addition, tumor growth inhibition has been presented in splenocytes from tumors treated with ADV-TK combined with GCV but not controls treated with ADV-TK plus saline (11). These findings suggest that TAA release and tumor cell death are required to induce a tumor-specific response. Therefore, GMCI may induce immune protection against recurrence from minimal residual disease after tumor debulking.

A previous study on hepatic metastasis in lung cancer has demonstrated that treatment with HSV-TK followed by ADV-TK led to significant tumor regression and prolongation of survival (12). In an interleukin (IL)-2 adenoviral vector

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expressing murine model, IL-2 treatment alone was ineffective, whereas combination therapy with HSV-TK resulted in further tumor regression and prolonged animal survival (12). In addition, a previous study indicated a similar trend was also exhibited in liver metastases in breast cancer, whereby significant tumor regression was presented in response to treatment with ADV-TK combined with GCV, which was assessed by computerized morphometric analysis towards the residual tumor (13). In addition to this result, a significant prolongation of survival was also indicated in ADV-TK and GCV-treated animals (13). Notably, recombinant ADV-TK and GCV exposure significantly suppressed the growth of SMMC-7721 liver cells *in vitro* in a previous study (14). In the examination of nonvascular invasion, recurrence-free survival and overall survival were also remarkably higher in the patients receiving liver transplantation combined with ADV-TK treatment compared with patients receiving liver transplantation only (15). Combined ADV-TK and GCV therapy was proposed as a promising treatment for HCC due to the notable anti-tumor effects observed, which included the promotion of apoptosis and inhibition of angiogenesis in a HCC model *in vivo* (16). However, to best of our knowledge, no study has reported the underlying mechanism of combined ADV-TK and GCV treatment in clinically collected tissue-based samples. The present study aimed to illustrate the therapeutic effect of ADV-TK and GCV combined with partial hepatectomy via examining the cell death and apoptosis-associated protein expression levels in patient's tissue.

## Materials and methods

**Specimens.** A total of 34 hepatocellular carcinoma tissues (1x1x0.5 cm<sup>3</sup>) were obtained from surgical specimens of hospitalized patients between January 2004 and January 2012 at the Department of General Surgery, Beijing Youan Hospital, Capital Medical University (Beijing, China). A partial liver resection was performed in all patients as previously described (17). A total of 14 patients received ADV-TK (Shenzhen Tiandaxing Gene Engineering Co., Ltd., Shenzhen, China) therapy prior to partial hepatectomy. An intratumoral dose of  $5.0 \times 10^{11}$  ADV-TK particles was administered and caused an objective response with no significant toxicity. The dose was based on a phase I dose escalation trial (15). To ensure uniform dosing, a total of  $5.0 \times 10^{11}$  ADV-TK particles in 60 ml of 0.9% saline were injected into peritoneum tissues around liver at a doses of  $1.25 \times 10^{11}$  viral particles each, including the lesser curvature of stomach, abdominal aorta side, head of the pancreas surface of the right kidney and the area under the right diaphragm. The first dose of GCV (5 mg/kg; Roche Diagnostics, Basel, Switzerland) was administered intravenously and following 24 h further doses were administered twice daily for 10 days until partial hepatectomy. Patients in control group received liver resection only and were not treated with ADV-TK/GCV. During partial hepatectomy, tumor tissue specimens were collected; necrotic and tumor junction areas were not included in the analysis. Detailed patient information is presented in Table I. Written, informed consent was obtained from all patients and the present study was approved by the Institutional Review Board of Beijing Youan Hospital, Capital Medical University. All tissues were sliced into 5- $\mu$ m sections

and were fixed with 10% neutral formaldehyde solution for 12-18 h at room temperature prior to conventional dehydration and paraffin embedding.

**Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay.** With xylene and gradient concentrated ethanol (100, 95, 90, 80 and 70%), each section was washed twice and subsequently rinsed with phosphate-buffered saline (PBS). Sections were fixed with Proteinase K solution for 20 min at 37°C and rinsed twice with PBS. Once sections were dry, 50  $\mu$ l of TUNEL reaction mixture from *in-situ* cell death kit (11684809910; Roche Diagnostics) were diluted 1:2 with PBS, added to each slice and incubated at 37°C for 1 h. All slides were washed with PBS three times, treated with 1  $\mu$ g/ml 4',6-diamidino-2-phenylindole (DAPI) working solution and incubated at 37°C for 4 min. Following, the coverslip was treated with mounting media. Images were acquired using a conventional fluorescent microscope under an excitation wavelength of 500 nm and a detection wavelength of 550 nm. Six fields of view on each slide were observed. Magnifications of x20 or x40 were used to confirmed whether the labeling was successful or not and to observe the labeled nuclei (intact or fragmented). TUNEL-positive puncta were quantified using an x10 objective. The following equation was used to calculate the apoptosis index (AI): (number of TUNEL-positive cells/total number of cells) x100%.

**Immunohistochemical staining.** For the immunohistochemical staining of targeted proteins, HCC tissue sections were deparaffinized and dehydrated. Once sections were washed with distilled water, all sections were soaked in methanol with 3.0% hydrogen peroxide for 15 min at room temperature in order to block the peroxidase in tissues and further washed with distilled water. Antigens were retrieved with citric acid buffer by being heating samples at 100°C for 20 min prior to cooling for 20 min at room temperature. Once sections were washed with PBS five times, the slides were incubated with primary rabbit polyclonal antibody against nuclear factor (NK)- $\kappa$ B (1:1,000; ab86299; Abcam, Cambridge, UK) and caspase-3 (1:500; ab2302; Abcam); and primary mouse monoclonal antibodies against B-cell lymphoma (Bcl-2; 1:1,100; ZA-0611; OriGene Technologies, Inc., Beijing, China) and B-cl2-associated protein X (Bax; 1:200; ZA-0611; OriGene Technologies, Inc.) overnight at 4°C. The slides were subsequently washed five times with PBS and then incubated with horseradish peroxidase (HRP)-conjugated goat secondary antibody (1:500; TA130005; OriGene Technologies, Inc.) for 10 min at room temperature. Slides were washed with PBS and treated with diaminobenzidine substrate from a kit (OriGene Technologies, Inc.) for 5 min at room temperature to visualize the reaction between antigen and antibody. Sections were counterstained at room temperature with Mayer's hematoxylin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 sec, dehydrated, cleared and mounted. Class- and species-matched irrelevant antibodies and incubations were used as controls. Sections were observed under an Olympus BX53 fluorescence microscope (magnification, x10; Olympus Corporation, Tokyo, Japan).

**Western blot analysis.** For assessing cytochrome *c* release, subcellular fractions were separated using the technique

Table I. General clinical data of all patients.

Characteristics	Experimental (14 cases)	Control (20 cases)	P-value
Age (years)	51.14±12.64	60.40±9.80	0.109 <sup>a</sup>
Sex			
Male	10/14	2/20	
Female	4/14	18/20	0.354 <sup>a</sup>
AFP before surgery (ng/ml)	2227±5474	101±161	0.232 <sup>a</sup>
Neoplasm stage			
Phase I	10 cases	10 cases	
Phase II	2 cases	6 cases	
Phase III	2 cases	4 cases	0.439 <sup>b</sup>
Postoperative pathological grades			
High differentiation	2 case	6 cases	
Moderate differentiation	4 cases	8 cases	
Poor differentiation	8 cases	6 cases	0.274 <sup>b</sup>

<sup>a</sup>Independent sample t-test; <sup>b</sup>rank sum test. AFP, alpha-fetoprotein.

indicated by Ott *et al* (18). Liver tissues were homogenized in radioimmunoprecipitation assay buffer (Sigma Aldrich; Merck KGaA). Cell lysates were mixed with Laemmli sample buffer and boiled for 3 min. Protein concentrations were determined by bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). Equal amounts of proteins (20 µg) were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk with TBS for 1 h at room temperature and incubated with anti-Bax antibody (1:500; ab32503; Abcam), anti-Bcl-2 antibody (1:200; ab196495; Abcam) and anti-cytochrome *c* antibody (1:200; ab13575; Abcam) overnight at 4°C. Mitochondrial and cytosolic fractions (20 µg each) were resolved in 1-mm thick 12% Novex Tris-glycine polyacrylamide gel and immunoblotted as described above. Subsequently, the membranes were incubated with a HRP-conjugated secondary antibody (1:500; ZB-2306; OriGene Technologies, Inc.) at room temperature for 1 h. Protein bands were visualized with an ECL system (Clinx Scientific Instruments, Shanghai, China). The relative band intensity was determined using a gel image analysis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Densitometry was performed using Quantity-One image analysis Software (Version 4.6.9; Bio-Rad Laboratories, Inc.). Protein expression levels in each sample were quantified and the ratio of protein to GAPDH (1:200; ab8245; Abcam) was defined as the protein expression.

**Statistical analysis.** All statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation for normal distribution, whereas the median was representative of skewed distribution. When data were satisfactory for qualification of normal distribution, the independent sample Student's t-test was performed. When data did not meet this qualification, the Kruskal-Wallis test was then used.  $\chi^2$  test was used for enumeration data. One-way analysis of variance was used for multiple group comparison followed by a Bonferroni post-hoc

test.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

*ADV-TK combined with GCV significantly induced apoptosis.* TUNEL assay examination revealed that few TUNEL-positive apoptotic cells and limited DAPI staining of the same tissue section was indicated in the control group. Conversely, the number of TUNEL-positive cells was markedly increased in the experimental group (Fig. 1A). In addition, DAPI staining in the experimental group revealed multiple condensed and fragmented nuclei, suggesting the cells were going through apoptosis in the identical tissue sections subjected to combined ADV-TK and GCV treatment. In the experimental group, the AI in HCC tissues was significantly elevated compared with the control group ( $P<0.01$ ; Fig. 1B). These findings suggest that treatment with ADV-TK combined with GCV may lead to significantly increased apoptosis.

*ADV-TK combined with GCV induces apoptosis through alternating the protein expression of caspase-3 and NF-κB, the Bax/Bcl-2 expression ratio and cytochrome *c* release.* As indicated in Fig. 2A, the protein expression levels of Bax, Bcl-2, caspase-3 and NF-κB was detected by immunohistochemistry. Brown particles revealed positive protein expression, which was primarily located in the cell membrane, cell nucleus and cytoplasm. The positive staining was diffuse and focal distribution was exhibited. Notably, the number of caspase-3-positive cells was significantly elevated in the experimental group compared with the normal control ( $P<0.05$ ; Fig. 2B). By contrast, the Bax/Bcl-2-positive cell ratio was significantly increased in the experimental group compared with the normal control group ( $P<0.01$ ; Fig. 2C). Furthermore, a significantly increased number of NF-κB-positive cells was observed in the control group compared with the experimental group ( $P<0.01$ ; Fig. 2D). In order to confirm the effect of combined ADV-TK



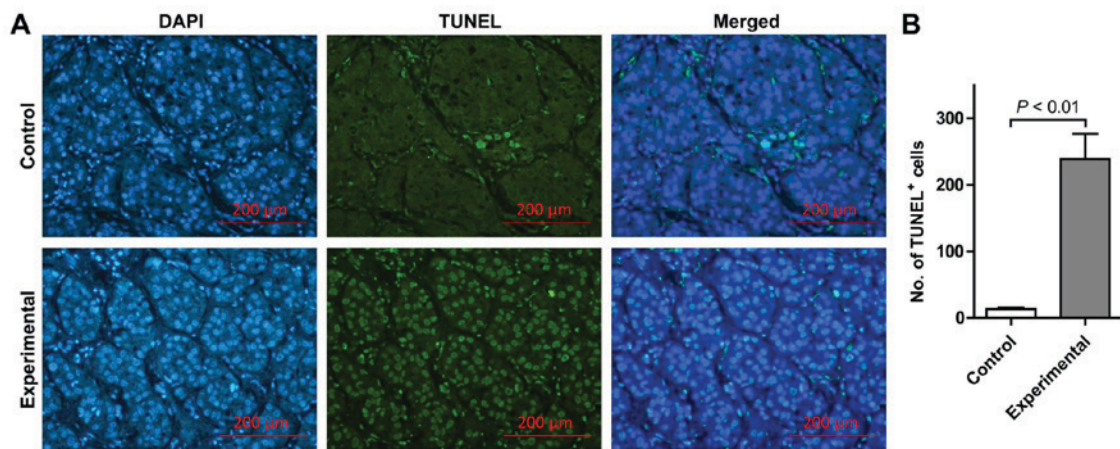


Figure 1. Combined adenovirus-mediated herpes simplex virus thymidine kinase and ganciclovir treatment induces hepatic cell apoptosis. (A) A TUNEL assay revealed apoptotic-positive hepatic cells marked by green staining. The blue DAPI staining marked intact DNA (magnification,  $\times 400$ ). (B) The apoptotic index was calculated. Data are presented as the mean  $\pm$  standard deviation. TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.

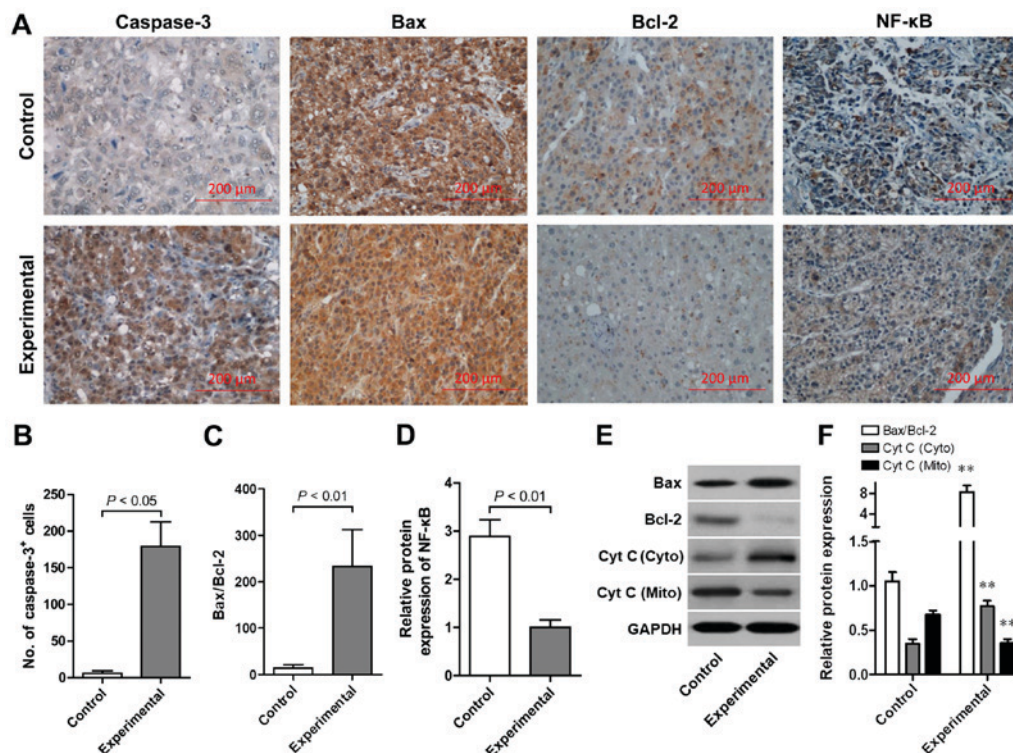


Figure 2. Expression of apoptosis-associated proteins in tissues of patients with hepatocellular carcinoma detected by immunohistochemical staining and western blot analysis. (A) Image of positively stained cells and their localization in the combined ADV-TK and GCV treatment group and control groups. Analyses of (B) caspase-3, (C) Bax/Bcl-2 ratio and (D) NF-κB were performed in the combined ADV-TK and GCV treatment group and control group. (E) Representative images of western blot analysis. (F) Histogram of relative protein expression analysis. Data are presented as the mean  $\pm$  standard deviation. \*\* $P < 0.01$  vs. control. ADV-TK, adenovirus-mediated herpes simplex virus thymidine kinase; GCV, ganciclovir; NF-κB, nuclear factor-κB; Bcl-2, B-cell lymphoma; Bax, B-cell lymphoma-associated protein X; Cyt C, cytochrome c; Cyto, cytosol; Mito, mitochondria.

and GCV therapy on the apoptotic signaling pathway, western blot analysis was performed to further assess the Bax/Bcl-2 ratio and cytochrome *c* release. Representative western blot analysis images were demonstrated (Fig. 2E). Results indicated that the Bax/Bcl-2 protein ratio was significantly increased with combined ADV-TK and GCV treatment compared with the control ( $P < 0.01$ ). Furthermore, the release of cytochrome *c* from the mitochondria was significantly increased with

combined ADV-TK and GCV treatment compared with the control ( $P < 0.01$ ; Fig. 2F).

## Discussion

The present study was performed to assess the therapeutic effects of ADV-TK and GCV treatment combined with partial hepatectomy by comparing the biological activities in tissues

from patients with HCC. The present results demonstrated that increased apoptotic cell death was observed in patients with HCC who underwent ADV-TK and GCV treatment combined with partial hepatectomy compared with those who were only subjected to partial hepatectomy. Furthermore, the expression levels of apoptosis-associated proteins were assessed in the present study. Notably, the protein expression levels of caspase-3, the ratio of Bax/Bcl-2 protein and the release of cytochrome C were significantly increased, whereas the protein expression levels of NF- $\kappa$ B were decreased in HCC tissues obtained from patients treated with combined ADV-TK and GCV therapy compared with the control.

Suicide gene therapy using HSV-TK gene transduction in combination with GCV is a therapeutic strategy that has been used in a wide variety of cancer treatments, and its application has been assessed in over 17 clinical trials in the United States (19). Phosphorylation of GCV results in its conversion into a non-diffusible nucleoside analogue, which terminates DNA synthesis and consequently results in cell death (20). Another benefit of this approach is the 'bystander effect', in which the cytotoxic GCV-triphosphate molecule is taken up by untransduced cells through gap junctions, which ultimately enhances tumor cell death (21).

The role of HSV-TK and GCV in the promotion of cell death has been demonstrated in the treatment of diverse types of cancer *in vitro* and *in vivo* (22-24). One of the mechanisms involved in combined ADV-TK and GCV-induced cell-death is apoptosis (25,26). A study on colorectal cancer with combined ADV-TK and GCV treatment suggested that its cytotoxic effect may be dependent on the tumor cell type, as evidenced by early apoptosis in the G1 phase of cell cycle and late apoptotic or necrotic cell death at the sub-G1 phase with DNA fragmentation (27). Cell death in ADV-TK and GCV transduced oral carcinoma has been revealed to be mediated through the apoptotic signaling pathway (28). By contrast, it was proposed that nonapoptotic biological activity may be a central manifestation in cell death induced by combined ADV-TK and GCV therapy (29). The present results demonstrated combined ADV-TK and GCV treatment significantly increased the number of apoptotic cells according to TUNEL analysis, which suggested that combined ADV-TK and GCV treatment induced apoptotic cell death in HCC tissues.

Caspase-3 is one of the key effector caspases in the cell. Inactive procaspase is cleaved into an active molecule with a lower molecule weight (~17KD), which activates other proteins to trigger the apoptotic process (30). In ADV-TK and GCV transduced NT8e cells, cleaved caspase could not be detected (31). Similarly, it has also been reported that no cleaved or activated caspase-3 band was observed following combined HSV-TK and GCV treatment (32). These findings suggest that the biological activity serving a role in the cytotoxic effect of HSV-TK and GCV treatment on NT8e cells other than apoptosis requires further investigation. The results in the present study suggested that combined ADV-TK and GCV treatment increased the caspase-3 protein expression levels and was subsequently responsible for the activation of the early stage apoptosis signaling pathway.

The mitochondrial apoptosis-induced channel is responsible for cytochrome *c* release in the early stage of apoptosis (33). It has been reported that the combined ADV-TK and GCV system

effectively inhibited the proliferation of non-small cell lung cancer cells *in vitro* and *in vivo* via the potent induction of apoptosis, in which the release of the apoptosis initiator cytochrome *c* was increased (34). The therapeutic mechanism of suicide gene therapy was further investigated by Beltinger *et al* (26). Their study suggested that treatment with ADV-TK and GCV led to mitochondrial perturbations, including loss of the mitochondrial membrane potential and release of cytochrome *c* from the mitochondria into the cytosol, as well as caspase activation and nuclear fragmentation. As major members of the Bcl-2 family, Bcl-2 and Bax serve a crucial role in the inhibition of the intrinsic apoptotic signaling pathway and tumor progression triggered by mitochondrial dysfunction (35). In a previous study, the expression level of Bcl protein was significantly lower, whereas Bax protein expression level was significantly higher following 70-HSV-TK and GCV administration combined with  $Mn_{0.5}Zn_{0.5}Fe_2O_4$  nanoparticles for HCC treatment *in vitro* and *in vivo* (36). A study suggested that the balance between Bcl and Bax proteins is important in assessing the sensitivity of tumor cells to GCV (37). Following the induction of glioma cell death, it was suggested that cytosine deaminase/5-fluorocytosine- and ADV-TK/GCV-induced apoptosis does not require cell death receptors or p53, but gathering at a mitochondrial pathway caused by different mechanisms of Bcl-2 modulation (38). A previous study suggested that downregulation of Bcl-2 and increased caspase-3 expression was a possible apoptosis mechanism in BIU87 cells induced by a HSV-TK/GCV system combined with allitride (39). Along with the present analysis of caspase-3 activity and apoptosis suggest that combined ADV-TK and GCV treatment induces apoptosis in host tumor cells by increasing the Bax/Bcl-2 ratio and inducing the release of cytochrome *c* from mitochondria to the cytosol.

NF- $\kappa$ B-inducing kinase serves an important role in promoting the processing of p100/NF- $\kappa$ B2, which is defined as the non-canonical NF- $\kappa$ B signaling pathway (40). Furthermore, NF- $\kappa$ B was overexpressed in various types of cancer and the inhibition of NF- $\kappa$ B was implied to reduce cell growth and enhance cell apoptosis (41,42). A previous study indicated that apoptosis is the central mechanism in cell death and tumor progression, whereby latent membrane protein 1 regulated the NF- $\kappa$ B-positive cells upon their treatment with the pVLTR-TK and GCV system (43). However, to the best of our knowledge, no study has ever reported the NF- $\kappa$ B-associated mechanism in combined ADV-TK and GCV-induced apoptosis. The present results demonstrated that combined treatment with ADV-TK and GCV could significantly reduce the expression of NF- $\kappa$ B protein, suggesting that NF- $\kappa$ B may be associated with ADV-TK and GCV-induced apoptosis in HCC. However, further investigations are required to fully elucidate this potential mechanism.

In the present study, a simple approach was applied to understand the cell death modulation in tissues from patients with HCC who had received combined ADV-TK and GCV therapy. The results concluded that combined ADV-TK and GCV treatment promoted cell death in HCC tissues. Furthermore, the present findings suggested that cell death was primarily mediated by the apoptotic signaling pathway.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

NL conceived and designed the study. HTZ and LQ performed experiments, and were major contributors in writing the manuscript. CLL and JYJ made significant contributions to acquisition and analysis of data. LBS and XFZ performed the statistical analysis. HTZ, LQ and LBS were responsible for biological samples collection. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Beijing Youan Hospital, Capital Medical University. Written informed consent was obtained from each patient.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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