

HSVtk/GCV system on hepatoma carcinoma cells: Construction of the plasmid pcDNA3.1-pAFP-TK and targeted killing effect

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Abstract. Previous studies demonstrated that herpes simplex virus thymidine kinase (*HSVtk*) could phosphorylate non-toxic ganciclovir (GCV) efficiently to produce phosphorylated products that result in cell apoptosis, to kill tumor cells. The present study aimed to construct a plasmid vector, pcDNA3.1-pAFP-TK, carrying the suicide gene driven by the alpha-fetoprotein (AFP) promoter, to investigate the cytotoxicity of HSVtk/GCV suicide gene system on hepatoma carcinoma cells. Reverse transcription-polymerase chain reaction and western blotting results demonstrated that the *HSVtk* gene was effectively expressed in HepG2 hepatoma carcinoma cells transfected with pcDNA3.1-pAFP-TK plasmid, whereas *HSVtk* gene expression was not detected in normal HL-7702 liver cells. In addition, MTT assays indicated that cell viability of HepG2 cells with the plasmid pcDNA3.1-pAFP-TK decreased in a dose-dependent manner following treatment with GCV for 48 h. Flow cytometry also revealed that the cell apoptosis rate and mitochondrial membrane potential reduction rate in the HepG2 cells treated with HSVtk/GCV suicide gene system were significantly higher than in the control group. Apoptosis rates in the control group and the pcDNA3.1-pAFP-TK group were (1.00±0.62%) and (38.70±6.03%), respectively. Mitochondrial membrane potential reduction rates in the control group and the pcDNA3.1-pAFP-TK group were (0.57±0.11%)

and (22.84±5.79%), respectively. Caspase-3 staining demonstrated that activated caspase-3 increased significantly in the HepG2 cells treated with HSVtk/GCV suicide gene system, whereas in the control group activated caspase-3 increase was not observed. The results of the present study, therefore, indicated that *HSVtk* suicide gene was obviously expressed in the HepG2 cells and that the HSVtk/GCV system was effective at killing HepG2 hepatoma carcinoma cells.

Introduction

Hepatocellular carcinoma (HCC), one of the most frequently diagnosed malignancies in the world, particularly in several areas of Asia and Africa, is the third largest cause of cancer-related death worldwide. Development of HCC is often associated with chronic liver disease, particularly cirrhosis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV). Imaging techniques have detected HCC at an early stage in patients with chronic HBV or HCV infection (1-4). Early detection of HCC is rare, whereas very few candidates are eligible for liver transplantation as a result of the lack of liver donors (5); thus, recurrence rates of HCC are very high following medical or surgical treatments (6,7). Therefore, the development of an effective targeted gene therapy strategy driven by the tumor-specific promoter has become an urgent requirement in treating HCC (8,9).

In recent years, gene therapy has been widely studied. The aim of gene therapy is to transfect a target gene into host cells to be specifically expressed, thus killing tumor cells. Currently, suicide gene therapy has become a promising strategy for gene therapy, especially the herpes simplex virus thymidine kinase (*HSVtk*)/ganciclovir (GCV) system (10). Previous studies have demonstrated the use of a pro-drug sensitive gene as a suicide gene, to convert a non-toxic pro-drug into a toxic product, to block the extension of DNA chains and inhibit the activation of DNA polymerase, finally leading to cell apoptosis (11-13).

At present, a variety of tumor-specific promoters have been used for HCC gene therapy, and the alpha-fetoprotein (AFP) promoter has become an ideal target (14). The regulatory

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regions and sequence of the AFP gene not only specifically activate transcription of exogenous genes but also have significant effects on tumor-specific transcriptional activity (15). Furthermore, in clinical application, AFP gene has been used as a specific marker of HCC.

Therefore, the purpose of the present study was to construct a plasmid, pcDNA3.1-pAFP-TK, to express thymidine kinase (TK) driven by the AFP promoter, to study the selective killing effect on HCC cells.

Materials and methods

Cell lines and culture. HL-7702 human liver cell line and HepG2 human HCC cell line were purchased from Shanghai Cell Bank of Chinese Academy of Science (Shanghai, China). HeLa human cervical cancer cells were purchased from Basic Medical Cell Bank of Chinese Peking Union Medical University (Beijing, China). HL-7702 cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 4 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. HeLa and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences) supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Construction of the plasmid pcDNA3.1-pAFP-TK and bacterial transformation. The pcDNA3.1-pAFP-TK plasmid (Fig. 1) was synthesized by BioVector, Inc. (Beijing, China). Chemically competent *Escherichia coli* DH5α cells were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Cells (50 μl) were thawed on ice, then 5 ng of plasmid DNA was added, mixed gently, and the mixture incubated on ice for 30 min. Cells were then transferred to a 42°C water bath for 90 sec, then placed on ice for a further 2 min. Sterile lysogeny broth (LB; 400 μl; Beijing Solarbio Science & Technology Co., Ltd.) without antibiotic was added, and the mixture was incubated for 3 h at 37°C in a shaker at 200 rpm/min to recover the cells. The mixture was then centrifuged at 1,006.2 x g for 3 min at 4°C, the supernatant was discarded, and the pellet was resuspended in 100 μl LB. Cells were plated on solid LB containing agar and 50 mg/ml ampicillin (Sangon Biotech Co., Ltd., Shanghai, China) and incubated at 37°C for 12-16 h. Single colonies were then picked into 100 ml LB and incubated at 37°C for 16 h in a shaker at 260 rpm/min.

Extraction and identification of the pcDNA3.1-pAFP-TK plasmid. Plasmid extraction kit was purchased from Sangon Biotech Co., Ltd. and pcDNA3.1-pAFP-TK plasmid DNA was extracted from *E. coli* DH5α according to the manufacturer's protocol. Plasmid DNA was digested with *Xho*I and *Bam*HI and the products were visualized under UV transillumination following separation on a 1% agarose gel stained with fluorescence staining dye Goldviewna I (Beijing Solarbio Science & Technology Co., Ltd.), using the DNA marker DM 2000 Plus (CWBio, Beijing, China). DNA fragments were subsequently

sequenced by BioVector, Inc., and Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA) was used to analyze the homology of the *HSVtk* gene and plasmid DNA sequences.

Cell transfection. HL-7702, HeLa and HepG2 cells were transfected with Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. For MTT assays, HepG2 cells were cultured to the exponential phase of growth and then were seeded in 96 well plates at a density of 10⁴ cells per well. For flow cytometry, HepG2 cells were incubated in 6 well plates at a density of 5x10⁵ cells per well. For the transfection experiments, cells were divided into the pcDNA3.1-pAFP-TK group and the control group and were incubated overnight in 5% CO₂ at 37°C. Cells in the control group received no treatment. The following day, cells in the pcDNA3.1-pAFP-TK group were washed twice with PBS and fresh serum-free DMEM was added to each well. Transfection was conducted with 7 μl Lipofectamine 2000 and 2.5 μg plasmid DNA. Following 6 h incubation in 5% CO₂ at 37°C, the cell medium was replaced with medium containing 10% FBS and the cells were incubated for a further 48 h an additional culture of 48 h.

Reverse transcription-polymerase chain reaction (RT-PCR). mRNA expression of *HSVtk* was analyzed by RT-PCR. HL-7702, HeLa and HepG2 cells were transfected with pcDNA3.1-pAFP-TK, then total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). cDNA was reverse transcribed from the total RNA according to the manufacturer's protocol, using dNTP Mix, 5X RT buffer, HiFiScript 1st Strand cDNA Synthesis kit and RNase-free water (CWBio). PCR was then performed, using Goldviewna I (Beijing Solarbio Science & Technology Co., Ltd.) as the fluorophore, to amplify TK, with β-actin as an internal control, using the following primer sequences: TK, forward 5'-CAACAAAAAGCCACG GAAGT-3' and reverse 5'-ATGCTGCCCATTAAGGTATCG-3'; and β-actin, forward 5'-TGACGTGGACATCCGCAAAG-3' and reverse 5'-CTGGAAGGTGGACAGCGAGG-3'. The amplification products of TK and β-actin were, respectively, 446 and 205 bp in length. PCR was performed using the following DNA thermal cycler conditions: 1 cycle of 94°C for 2 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; and a final elongation step of 72°C for 5 min. The PCR products were visualized under UV transillumination following separation by 1% agarose gel electrophoresis stained with fluorescence staining dye Goldviewna I alongside a DNA marker (Takara Biotechnology Co., Ltd., Dalian, China).

Western blot. HL-7702, HeLa and HepG2 cells were treated as described. Total protein was extracted following transfection using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) at 4°C for 30 min, and the lysates were centrifuged at 4°C for 20 min at 4,360.2 x g. Protein concentration in cell lysates was determined using a bicinchoninic acid protein assay kit (Boster Systems, Inc., Pleasanton, CA, USA). Proteins were then separated by 5-12% SDS-PAGE, transferred onto polyvinylidene fluoride membranes, and blocked by incubation in 5% skim milk in

TBS containing 0.05% Tween-20 at room temperature for 2 h. Membranes were then incubated overnight at 4°C with goat polyclonal anti-HSVtk (1:800; cat no. sc-28038; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti- β -actin (1:800; cat no. TA-09; ZSGB-BIO, Beijing, China) primary antibodies, washed in TBS containing 0.1% Tween-20 (TBST) for 3x10 min, incubated at 4°C for 1 h with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat no. A0181; Beyotime Institute of Biotechnology), then the membranes washed again in TBST for 3x10 min. Finally, the expression of HSVtk and β -actin was visualized using enhanced chemiluminescence (Wuhan Boster Biological Technology, Ltd., Wuhan, China).

MTT assay. HepG2 cells transfected with pcDNA3.1-pAFP-TK (100 μ l transfected cells) were seeded in 96-well plates at a density of 10^4 cells per well and incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then treated with 0, 1, 5, 10, 20, 40, 60 or 80 μ g GCV and incubated in 5% CO₂ at 37°C. After 4 days, cells were observed under the microscope. MTT substrate (20 μ l) was added to each well and the plates were incubated in 5% CO₂ at 37°C for a further 4 h. The medium was then discarded and 150 μ l DMSO were added to each well at room temperature for 10 min. A microplate reader was used to measure the absorbance at 490 nm (A₄₉₀), and the inhibition rate was calculated as: $[1-(A_{490} \text{ of pcDNA3.1-pAFP-TK group}/A_{490} \text{ of the control group})] \times 100\%$.

Detection of cell apoptosis by flow cytometry. Cell apoptosis was detected with an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Invitrogen; Thermo Fisher Scientific, Inc.). pcDNA3.1-pAFP-TK was transfected into HepG2 cells. Cells in the control group received no transfection and no further treatments. Following 48 h incubation, the pcDNA3.1-pAFP-TK group was treated with 150 μ g/ml GCV for 2 days. Cells were harvested with 0.25% trypsin then sedimented by centrifugation at 335.4 x g for 3 min at room temperature. The supernatant was then discarded, cells were washed twice with PBS, then 100 μ l binding buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was added to resuspend the cells. Cell suspension (100 μ l) was then added to the flow tube, along with 5 μ l Annexin V-FITC at a final concentration of 1 μ g/ml and 10 μ l (250 ng) of PI. The cells were mixed and incubated for 15 min at room temperature in the dark. Finally, 400 μ l binding buffer was added and flow cytometry was used to detect cell apoptosis, using the BD FACSCalibur™ flow cytometer and the BD FACStation™ software (BD Biosciences, Franklin Lakes, NJ, USA).

Detection of mitochondrial membrane potential by flow cytometry. The mitochondrial membrane potential apoptosis detection kit was purchased from Beijing ComWin Company (Beijing, China). HepG2 cells were transfected with pcDNA3.1-pAFP-TK, whereas the control group received no intervention. Following 48 h incubation, the pcDNA3.1-pAFP-TK group was treated with 150 μ g/ml GCV for 2 days. The culture media was then discarded, the cells were washed once with PBS, then 1 ml cell culture medium and 1 ml JC-1 staining solution was added and the cells were incubated in a cell culture incubator at 37°C for 20 min. The supernatant was then removed and the cells were washed

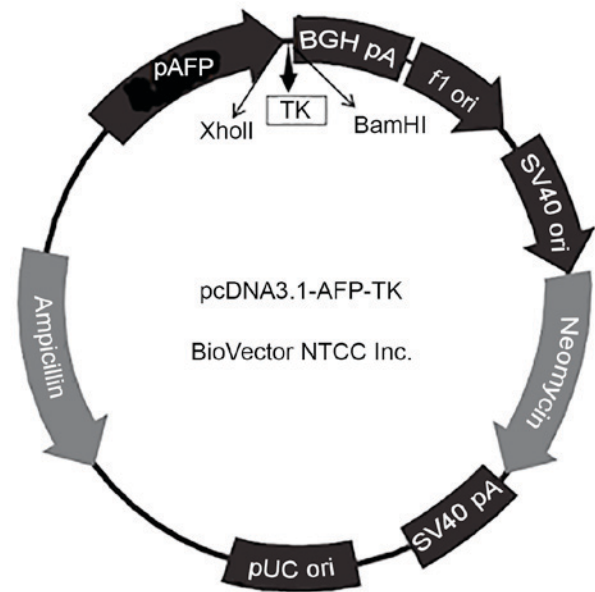


Figure 1. Plasmid map of pcDNA3.1-pAFP-TK. TK, thymidine kinase; pAFP, alpha-fetoprotein-producing promoter.

twice with 1X JC-1 staining buffer (Abcam, Cambridge UK). Finally, 2 ml of cell culture medium was added to the cells and fluorescence was observed under a fluorescence microscope, to reflect cell apoptosis. Three slides were observed and >10 fields of view/slide were assessed.

Detection of activated caspase-3 by caspase-3 staining. The Active Caspase-3 Staining kit was purchased from BioVision, Inc. (Milpitas, CA, USA). HepG2 cells in 6 well plates were transfected with pcDNA3.1-pAFP-TK using Lipofectamine2000 (Invitrogen; Thermo Fisher Scientific, Inc.), alongside an untreated control group. The pcDNA3.1-pAFP-TK group was treated with 150 μ g/ml GCV for 48 h, then all the cells were collected by centrifugation at 4°C at 335.4 x g for 3 min. Cells were then resuspended in 300 μ l cell culture medium with 1 μ l Red-DEVD-FMK and incubated for 1 h at 37°C with 5% CO₂. Cells were then centrifuged at 335.4 x g for 3 min, the supernatant was discarded, and cells were resuspended in 100 μ l water buffer provided in the kit. Finally, a drop of the cell suspension was placed onto a microslide and covered with a coverslip. Red fluorescence was observed under a fluorescence microscope, to evaluate the levels of activated caspase-3. Three slides were observed and >10 fields of view/slide were assessed.

Statistical analysis. Data was expressed as the mean \pm standard deviation, and all assays were performed in triplicate. Statistical differences were evaluated by Student's t-test using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Detection of the pcDNA3.1-pAFP-TK plasmid. Following digestion of pcDNA3.1-pAFP-TK was digested with XhoI and BamHI, a 1131-bp fragment was detected by agarose gel electrophoresis (Fig. 2), which demonstrated that the HSVtk gene was successfully inserted into the plasmid pcDNA3.1-pAFP-TK.

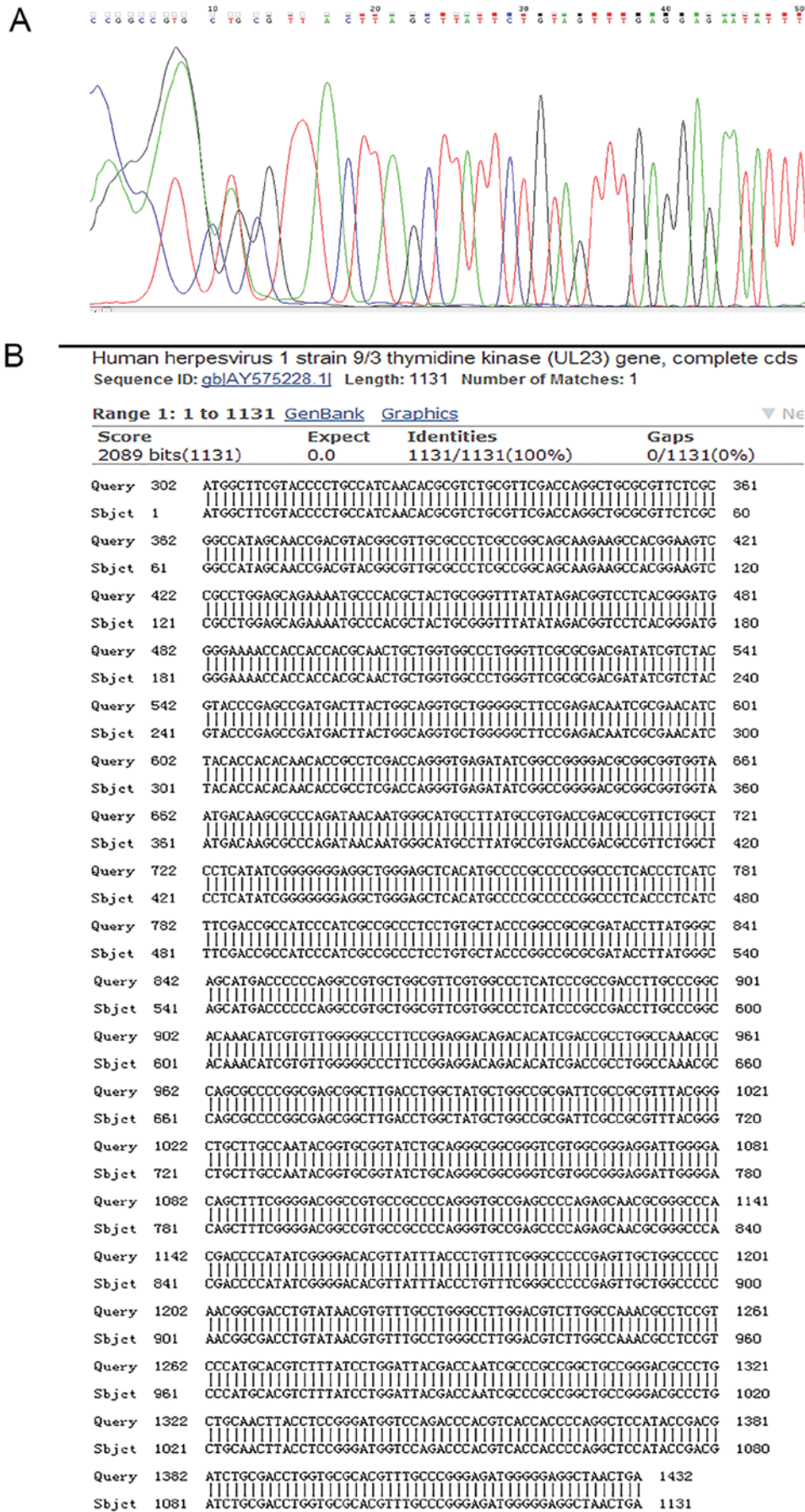


Figure 3. The result of the plasmid pcDNA3.1-pAFP-TK sequencing. (A) The pcDNA3.1-pAFP-TK plasmid fragment following DNA sequencing. (B) Analysis of fragment homology of DNA sequences by Basic Local Alignment Search Tool. Subject sequence, 1,131-bp fragment of the pcDNA3.1-pAFP-TK plasmid, confirmed as the insertion of the *HSVtk* gene; query sequence, *HSVtk* gene.

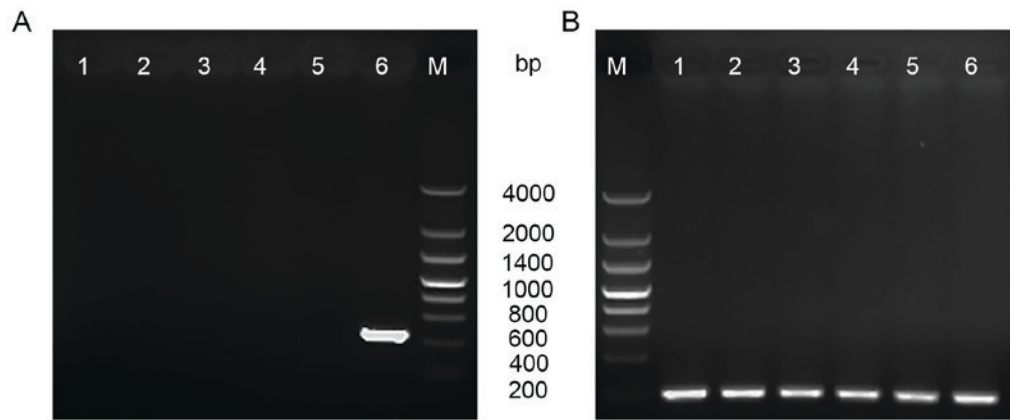


Figure 4. Expression of *HSVtk* mRNA examined by reverse transcription polymerase chain reaction. (A) Expression of the 446 bp *HSVtk* gene and (B) the 205 bp β -actin gene. Lane 1, HL-7702; Lane 2, HL-7702/pcDNA3.1-pAFP-TK; Lane 3, HeLa; Lane 4, HeLa/pcDNA3.1-pAFP-TK; Lane 5, HepG2; Lane 6, HepG2/pcDNA3.1-pAFP-TK; Marker, 200 bp ladder.

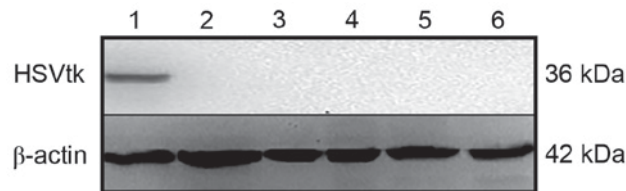


Figure 5. Expression of *HSVtk* protein expression examined by western blotting. Lane 1, HepG2/pcDNA3.1-pAFP-TK; Lane 2, HepG2; Lane 3, HeLa/pcDNA3.1-pAFP-TK; Lane 4, HeLa; Lane 5, HL-7702/pcDNA3.1-pAFP-TK; Lane 6, HL-7702.

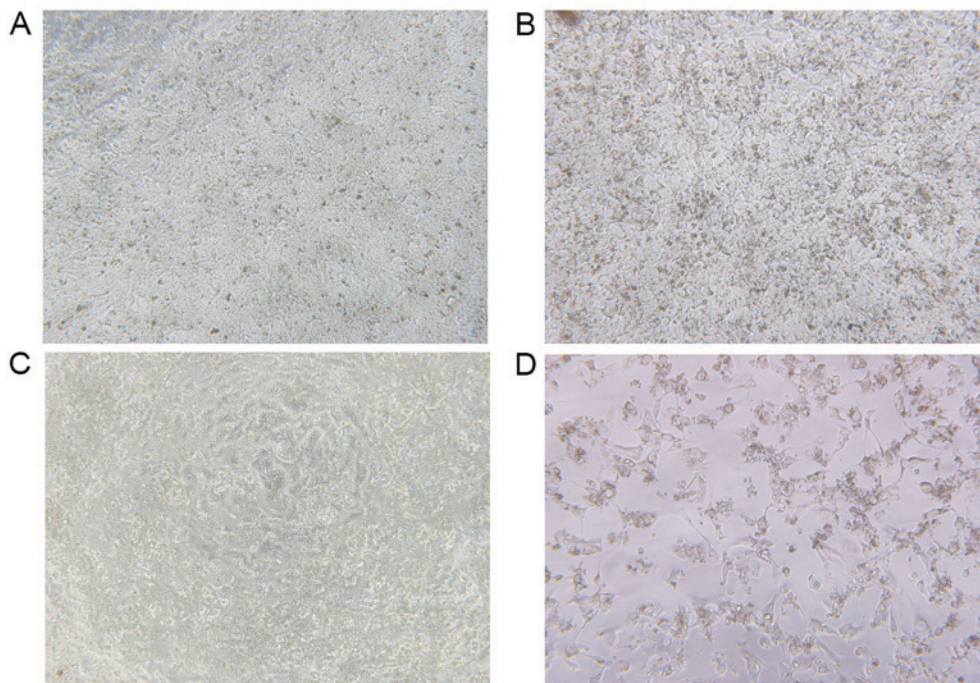


Figure 6. Cell morphology in the untransfected control group and pcDNA3.1-pAFP-TK (original magnification, $\times 100$). (A) Control with 0 μ g GCV; (B) Control with 80 μ g GCV; (C) pcDNA3.1-pAFP-TK group with 0 μ g GCV; (D) pcDNA3.1-pAFP-TK group with 80 μ g GCV. GCV, gancyclovir.

Detection of mitochondrial membrane potential by flow cytometry. As demonstrated in Fig. 9, the mitochondrial membrane potential reduction rate in the pcDNA3.1-pAFP-TK group ($22.84 \pm 5.79\%$) was significantly higher than the rate in the control group (0.57 ± 0.11 ; $P < 0.01$).

Detection of activated caspase-3 by fluorescence microscopy. Caspase-3 staining was used to detect activated caspase-3 in the HepG2 cells. The results demonstrated that compared with control cells (Fig. 10A), HepG2 cells interfered with *HSVtk*/GCV suicide gene system emitted a brighter red

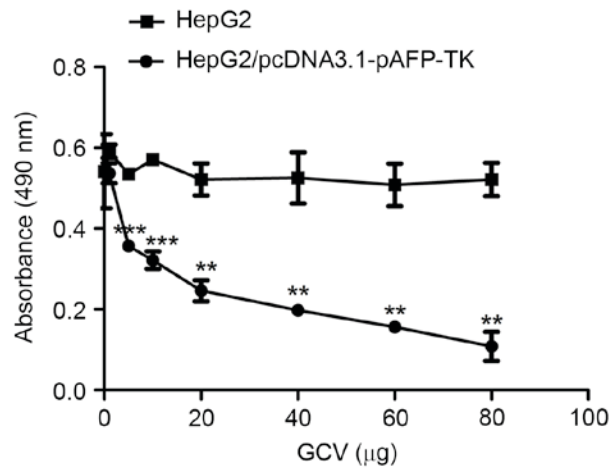


Figure 7. Cell viability in the control group and pcDNA3.1-pAFP-TK group, in response to GCV. Significant growth inhibition was observed in the pcDNA3.1-pAFP-TK group compared with the control group. **P<0.01 and ***P<0.001 vs. control group (n=3). GCV, gancyclovir.

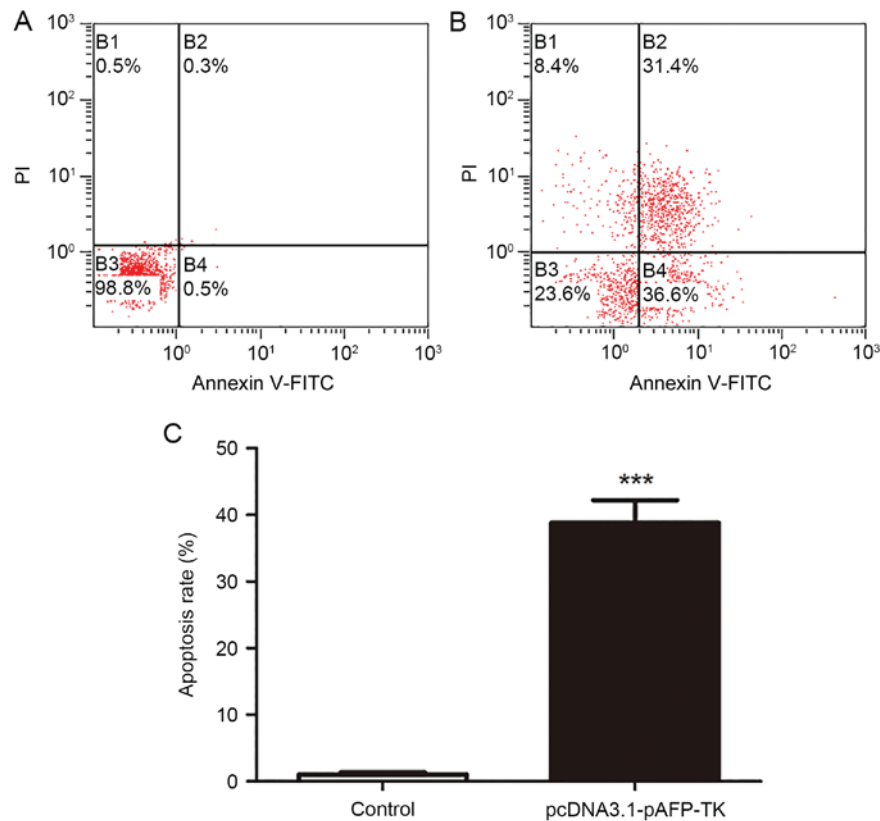


Figure 8. Detection of HepG2 cell apoptosis by flow cytometry in the (A) control group and (B) pcDNA3.1-pAFP-TK group. (C) Comparison of apoptosis rates between the pcDNA3.1-pAFP-TK group and the control group. ***P<0.001 vs. control group (n=3). PI, propidium iodide; FITC, fluorescein isothiocyanate.

signal (Fig. 10B), indicating increased levels of activated caspase-3. Activated caspase-3 played an important role in the early stage apoptotic cells, therefore, HSVtk/GCV suicide gene system had a significant killing effect on the HepG2 cells.

Discussion

The worldwide incidence and mortality rates of HCC appear to be increasing year by year, the incidence of which ranked

the fifth among all cancer cases (16). HCC represents the third leading cause of cancer-associated mortality worldwide (17,18). Based on the cellular and molecular levels, targeted therapy for combining drugs with the specific target could kill tumor cells, but rarely threatened normal tissues and cells. Gene therapy aims to insert exogenous normal genes into target cells to compensate for genetic defects and disease-related abnormalities. The combination of targeted therapy with gene therapy acts as a powerful target gene for killing human hepatoma carcinoma cells.

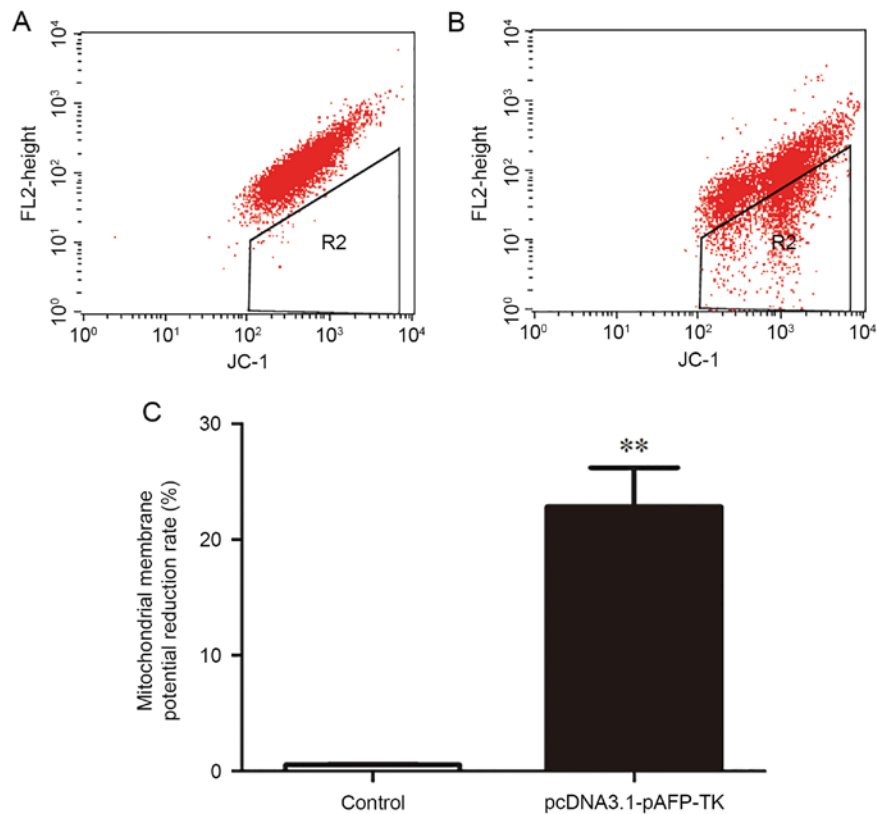


Figure 9. Flow cytometric detection of HepG2 cell mitochondrial membrane potential in the (A) control group and (B) pcDNA3.1-pAFP-TK group. (C) Comparison of rate of reduction of mitochondrial membrane potential between the pcDNA3.1-pAFP-TK group and the control group. ** $P < 0.01$ vs. control group ($n=3$).

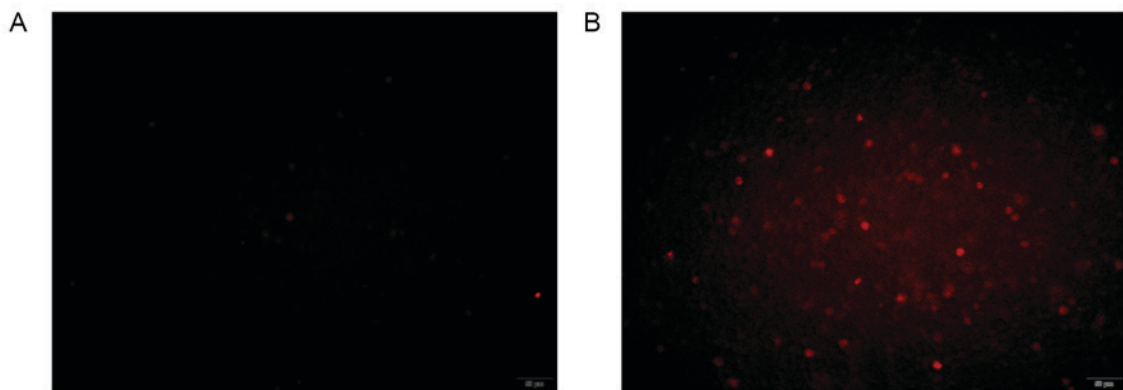


Figure 10. Detection of activated caspase-3 in HepG2 cells by caspase-3 staining (original magnification, x200). (A) Control group and (B) pcDNA3.1-pAFP-TK group.

The two most widely used suicide gene systems are the CD/5-FC system and the HSVtk/GCV system (19). In the CD/5-FC system, the cytosine deaminase gene (*CD*) of some bacteria and fungi generates the CD enzyme, which converts cytosine to uracil; non-toxic 5-fluorocytosine is transformed into cytotoxic 5-fluorouracil (5-FU), thereby killing tumor cells (20). *HSVtk* efficiently phosphorylates non-toxic gancyclovir (GCV) to produce phosphorylated products that lead to an arrest of DNA synthesis and cell death (21,22). However, normal cells could be killed as a result of lack of specific suicide gene, therefore, improved efficiency of targeted gene therapy is essential. It was, therefore, imperative to construct a novel recombinant vector,

to be transfected into tumor cells with a targeted ability to kill cells. Previous studies have demonstrated that some biochemical markers were tied to hepatoma carcinoma cells, such as AFP, vascular endothelial growth factor (VEGF) (23,24).

AFP is a major serum protein produced by fetal hepatocytes. It is not detectable in normal adult cells, however, in HCC cells, the AFP gene is highly expressed (25,26). Therefore, AFP sequences could be used to regulate expression of cytotoxic genes, as in the HSVtk/GCV system, through use of the AFP promoter; the system has been reported to have a limited effect on hepatoma cells (27). The HSVtk/GCV system was a successful suicide gene therapy strategy for HCC (28,29).

The results of the present study demonstrated that the novel plasmid pcDNA3.1-pAFP-TK, driven by the AFP promoter, was constructed successfully. The plasmid pcDNA3.1-pAFP-TK was transfected into HL-7702, HeLa, and HepG2 cells. RT-PCR and western blot demonstrated that *HSVtk* was effectively expressed in HepG2 cells transfected with the plasmid pcDNA3.1-pAFP-TK, whereas *HSVtk* gene expression was not detected in HL-7702 and HeLa cells. Furthermore, MTT assays indicated that, with increasing of GCV doses, the HepG2 cells viability significantly decreased; cell viability was significantly affected in HepG2 cells transfected with pcDNA3.1-pAFP-TK by 5 μ g GCV, and when GCV was increased to 80 μ g, it was evident that that cell viability was severely suppressed. In addition, flow cytometry demonstrated that in HepG2 cells treated with HSVtk/GCV suicide gene system, cell apoptosis rates and mitochondrial membrane potential reduction rates were increased dramatically in comparison with the control group. Caspase-3 staining demonstrated that activated caspase-3 increased significantly in the HepG2 cells with the HSVtk/GCV suicide gene system, supporting the cell apoptosis results, whereas increased activated caspase-3 was not observed in the control group.

Therefore, the present study has constructed a novel plasmid vector driven by the human AFP promoter, which may has become an effective approach in overcoming the restrictions of current technologies.

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