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Keywords
Apoptosis; Gene therapy; GP73; Hepatocellular carcinoma; HSVtk/GCV

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Golgi membrane protein GP73 modified-liposome mediates the antitumor effect of survivin promoter-driven HSVtk in hepatocellular carcinoma

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Abstract
Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide, and there is currently no effective therapeutic strategy in clinical practice. Gene therapy has great potential for decreasing tumor-induced mortality but has been clinically limited because of the lack of tumor-specific targets and insufficient gene transfer. The study of targeted transport of therapeutic genes in HCC treatment seems to be very important. In this study, we evaluated a gene therapy approach targeting HCC using the herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) suicide gene system in HCC cell lines and in an in vivo human HCC xenograft mouse model. GP73-modified liposomes targeted gene delivery to the tumor tissue, and the survivin promoter drove HSVtk expression in the HCC cells. Our results showed that the survivin promoter was specifically activated in tumor cells and HSVtk was expressed selectively in tumor cells. Combined with GCV treatment, HSVtk expression resulted in suppression of HCC cell proliferation via enhancing apoptosis. Moreover, tail vein injection of GP73-HSVtk significantly suppressed the growth of xenograft tumors through an apoptosis-dependent pathway and extended the survival of tumor-bearing mice without damaging the mice liver functions. Taken together, this study demonstrates an effective cancer-specific gene therapy strategy using the herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) suicide gene system for HCC that can be further developed for future clinical trials.

Keywords: Hepatocellular carcinoma; Gene therapy; HSVtk/GCV; GP73; Apoptosis

1 Introduction
Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and the sixth of most common malignant tumor type. HCC is the second leading cause of cancer-related mortality worldwide and is a major public health problem [1]. Because
HCC has reached advanced stages in more than 70% patients at the time of diagnosis, utilizing standard treatments, such as curative hepatic resection, for solid tumors frequently results in recurrence and poor survival [2]. Additionally, HCC is highly refractory to chemotherapy and other systemic treatments. Thus, novel strategies are urgently needed to improve the traditional therapeutic approaches for HCC.

Recently, gene therapy has rapidly developed as a novel strategy for cancer using various viral and non-viral vectors [3]. Among therapeutic genes used in this strategy, suicide genes, such as the herpes virus thymidine kinase gene (HSVtk), are the classic choice [4]. The HSVtk gene encodes the thymidine kinase enzyme, which metabolizes ganciclovir (GCV) into a toxic compound that interferes with DNA replication and results in cell apoptosis [5]. In addition, molecular targeting therapy using specific promoters to initiate the expression of a suicide gene in tumor cells is a desirable strategy for cancer therapy. At present, analpha-fetoprotein (AFP) promoter is one of the hepatoma cell-specific promoters and it can help achieve a HCC-targeted gene therapy in vitro and in vivo [6,7]. However, the possibility of other specific promoters could also be explored.

Survivin is a member of the inhibitor of apoptosis (IAP) family and it plays a key role in the regulation of apoptosis and cell division [8]. Studies have shown that survivin is over-expressed in common cancers, especially highly positive in 70% of liver cancers [9-11]. Moreover, survivin is highly and uniquely expressed in hepatocellular carcinoma but not in adjacent tissues [12,13]. The expression difference of survivin makes survivin promoter as a good candidate for constructing the vectors for cancer gene therapy for HCC [11,14]. Interestingly, several studies have shown that survivin promoter-driven oncolytic adenovirus exhibited tumor selective cytotoxicity in vitro and in vivo [15,16].

The inability to target delivery of a suicide gene to the tumor tissue restricts the application of gene therapy, although it has been shown to be effective in vivo studies. The GP73 protein is significantly expressed in tumor tissues and isolated from HCC patients but rarely in normal human hepatocytes. GP73 is secreted from the Golgi apparatus and localizes to the cell membrane and has been regarded as a promising biomarker for HCC [17,18]. In the present study, we quantified the inhibition of HCC cell growth induced by transfection with survivin promoter driven-HSVtk. We also modified the liposome with the anti-GP73 antibody and evaluated the anti-tumor effects of HSVtk in vivo using an established HCC xenograft mouse model. To ensure the specific expression of HSVtk in HCC cells, the survivin promoter was used to control the HSVtk gene. The GP73-modified liposome was used to deliver the HSVtk gene and target the transfection of the vector specifically to the HCC tumor cells by discernment of the GP73 protein, which was highly expressed on the tumor cell membrane. In addition, doxorubicin (DOX) treatment was used as the positive control in our experiments since DOX induces cancer cell apoptosis which has been widely used systemically or locally for the treatment of HCC [19].
2 Materials and methods

2.1 Cells, mice and ethics statement

HepG2 (hepatic carcinoma cell line), Bel-7402 (hepatic carcinoma cell line) and HL-7702 cells (normal hepatic cell line) were purchased from the Shanghai Cell Bank of the Chinese Academy of Science (China) and were cultured in DMEM and RPMI 1640 with 10% fetal bovine serum (FBS, HyClone) in 5% CO₂ at 37°C. Male BALB/c nude mice (8–10 weeks old) were purchased from the Animal Center for SIAS (Shanghai, China) and maintained under pathogen-free conditions on a 12-h light–dark cycle with food and water provided ad libitum. Investigation has been conducted in accordance with the regulations in the Guide for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of the People’s Republic of China. The protocol was approved by the Animal Ethics Committee of Shanxi Medical University.

2.2 Screening of the expression rates of GP73 on the cell surfaces of HepG2, Bel-7402 and HL-7702 cells

The density of cells were adjusted to 1×10^6/ml and divided into two groups: the experimental group and the control group. The GP73 primary antibody (anti-GOLPH2) was diluted (1:1000) and added to the experimental group. The corresponding primary antibody-derived animal serum (mouse serum) was added to the control group. Then, cells were incubated at room temperature for 30min in the dark, centrifuged at 1000rpm for 5min, and washed with PBS. The FITC-labeled secondary antibody was diluted (1:2000) and added to both the experimental group and the control group. Cells were then incubated at room temperature for 20min in the dark, centrifuged at 1000rpm for 5min, and resuspended with PBS. Flow cytometry was applied to determine the expression rates of GP73 on the surfaces of liver cancer cells and normal liver cells.

2.3 Construction of recombinant plasmid pBI-CMV2-pSurvivin-TK

The coding region of HSVtk (1137 bp) was prepared via polymerase chain reaction (PCR) using the pAFP-HSVtk-IRES2-EGFP plasmid DNA template [20]. The forward primer was 5′- CGCGGATCCATGGCTTCG TACCCCTGC -3′ and the reverse primer was 5′- CCCAAGCTTTCAG TTAGCCTCCCCCATC-3′, which contained BamHI and HindIII restriction sites (underlined), respectively. The fragment was cloned into the pBI-CMV2 vector (BioVector Science Lab, Inc., Beijing, China) to create pBI-CMV2- HSVtk.

The survivin promoter (910 bp) was amplified via PCR from the genomic DNA of HepG2 cells. The forward primer was 5′- CCGCTCGAGCTGGCCATAGAACCAGAGAA -3 ′, and the reverse primer was 5′- CGCGGATCCGCAGCGCTCTTAGGCG -3′, which contained XhoI and BamHI restriction sites (underlined), respectively. PCR was performed according to
2.4 Liposomes conjugation and cell transfection

Lipofectamine 2000 (100 μl) was activated by adding 24 μg distearoyl phosphatidyl ethanolamine a polyethylene glycol maleimide (DSPE-PEG-MAL) and incubated at 60°C for 1 h. Next, activated Lipofectamine 2000 (100 μl) was conjugated with anti-GP73 antibody (8 μg/ml) at room temperature for 4 h under nitrogen. Finally, glycine and iminothiolane hydrochloride (2-IT) (1:2 M ratio) were incubated at room temperature for 1 h. Next, 100 μl GP73-Lipofectamine 2000 was purified by adding 1 μl of the glycine and 2-IT mixture and incubated at room temperature for 1 h to prepare the GP73-conjugated Lipofectamine 2000 reagent.

For cell transfection, HL-7702, HepG2 or Bel-7402 cells were seeded in six-well plates at a density of 5.0 × 10^4 cells per well and incubated overnight. Before transfection, 2 μg pBI-CMV2-pSurvivin-HSVtk or pBI-CMV2 plasmid and 4 μl GP73-conjugated Lipofectamine 2000 were incubated in 100 μl Opti-MEM at room temperature for 5 min. Next, plasmid DNA and GP73-conjugated Lipofectamine 2000 were mixed and allowed to incubate for 20 min. Cells were washed twice with phosphate-buffered saline (PBS) and new culture medium without FBS was added. GP73-lipo-DNA complex (400 μl) was added to each well, and cells were incubated for 6 h. Next, the serum-free medium was replaced with a medium containing 10% FBS, and the cells were incubated for another 48 h. The efficiency of transfection was assessed by observing green fluorescence and measured using a fluorescence microscope. The expression of HSVtk was detected by reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis.

2.5 RT-PCR analysis

Total RNA was extracted using TRIzol reagent from transfected or untransfected cells according to the manufacturer’s instructions (Invitrogen, USA). First-strand cDNA was synthesized and the HSVtk gene was amplified using the GoldScript one-step RT-PCR kit (Invitrogen) according to the manufacturer’s protocols. HSVtk-specific primers (sense primer: 5’-AGCAAGAAGCCACGGAAGT-3’; anti-sense primer: 5’-CGATATGAGGAGCCAGAACG-3’) were used to generate a 330 bp PCR product. The housekeeping gene β-actin was amplified using specific primers (sense primer: 5’-TGACGTGGACATCCGCAAAG-3’; anti-sense primer: 5’-CTGGAAGGTGGACAGCGAGG-3’) were used to generate a 205 bp PCR product. The PCR products were evaluated by electrophoresis on a 1% agarose gel.

2.6 Western blot analysis
Proteins were extracted from transfected or untransfected cells with RIPA buffer and quantified using the BCA protein assay reagent (Boster Biotechnology, Wuhan, China). Protein samples were separated by 10% SDS-PAGE and transferred onto a 0.2μm PVDF membrane (Millipore, USA). Membranes were blocked in 5% skim milk at room temperature for 1h, followed by incubation at 4°C overnight with the primary antibody. Primary antibodies were detected with their corresponding HRP-IgGs (ZSGB-Biotechnology, China) using an ECL blot detection system (Transgene, Beijing, China). TK and β-actin primary antibodies were purchased from Santa Cruz Biotechnology (USA). Survivin, Cleaved poly(ADP-ribose) polymerase (PARP) and cleaved Caspase-3 primary antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA).

2.7 Flow cytometry

Flow cytometry was performed with the BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). To determine the efficiency of transfection and to collect the transfected cells, cells were diluted into test tubes containing sheath solution and GFP fluorescence was measured. The green fluorescence signal from 1.0×10^6 cells was excited with a 488-nm blue laser, emission was detected through a 530/30-nm bandpass (GFP) filter, and the positive cells were harvested for further analysis.

For analysis of cell apoptosis, pBI-CMV2-pSurvivin-HSVtk-transfected or pBI-CMV2-transfected HL-7702, HepG2 and Bel-7402 cells were treated with 150μg/ml GCV for 48h, then washed twice with PBS, digested with 0.25% trypsin without EDTA and centrifuged at 1000rpm for 3min. The cells were collected, washed three times with PBS and the cell concentration was adjusted to 1×10^6 cells/ml. Next, 5μl AnnexinV-FITC was added to 100μl cells and incubated at room temperature for 10min in the dark. Next, 10μl propidium iodide (PI) staining solution was added to the AnnexinV-FITC stained cells and analyzed by flow cytometry to determine the percentage of the apoptotic cells. 0.75μM DOX (Sigma, USA) were added to HepG2 cells for 48h as a positive control [21]. Experiments were repeated three times for each group.

2.8 Cell proliferation assay

1×10^4 pBI-CMV2-pSurvivin-HSVtk-transfected or pBI-CMV2-transfected HL-7702, HepG2 and Bel-7402 cells/well were cultured in 96-well plates in triplicate and treated with different doses of GCV (0, 1, 5, 10, 20, 40, 60, 80,100 and 150μg/ml) for 72 h. Next, 10μl CCK-8 (Cell Counting Kit-8; Beijing Dojindo, China) reagent was added to each well on day 4, and cells were cultured for 4h at 37°C under 5% CO₂. The results are expressed as the cell proliferation percent, which is the ratio of the OD₄₅₀ of pBI-CMV2-pSurvivin-HSVtk-transfected cells to the OD₄₅₀ of pBI-CMV2-transfected cells.

2.9 Clone formation assay
pBI-CMV2-pSurvivin-HSVtk-transfected or pBI-CMV2-transfected HL-7702, HepG2 and Bel-7402 cells were seeded in 6-well plates with $5 \times 10^2$ cells per well and cultured with 150 $\mu$g/ml GCV for 14 days. The cell colonies were fixed with 4% formaldehyde for 10 min and stained with 0.5% crystal violet for 3 min (Sigma-Aldrich). Individual colonies with more than 50 cells were counted under a microscope. Clone formation ratio (%) = (number of clones/number of seeded cells) $\times$ 100%. 0.75 $\mu$M DOX (Sigma, USA) were added to HepG2 cells for 14 days as a positive control [21].

2.10 In vivo mouse tumor model

Single-cell suspensions of HepG2 cells ($1.0 \times 10^7$ cells in 100 $\mu$l PBS) were injected subcutaneously into the right anterior flank of BALB/c nude mice, and the mice were randomly divided into four groups (5 mice per group). At the 29th and 31st day after injection, the mice received Lipofectamine 2000 mixed with pBI-CMV2 plasmid DNA (250 $\mu$g/kg body weight, vector group), Lipofectamine 2000 mixed with pBI-CMV2-pSurvivin-HSVtk plasmid DNA (250 $\mu$g/kg body weight, HSVtk group) or GP73-conjugated Lipofectamine 2000 mixed with pBI-CMV2-pSurvivin-HSVtk plasmid DNA (250 $\mu$g/kg body weight, GP73-HSVtk group) via tail vein injection. At the 32nd day after injection, the mice in the HSVtk and GP73-HSVtk groups were intraperitoneally injected with GCV (15 $\mu$g/mouse in 200 $\mu$l 0.9% NaCl). Meantime, DOX was administered (3 mg/kg, intraperitoneal injection, weekly, for 2 weeks) as a positive control group [22]. All of the mice were monitored daily for 40 days. The body weight which served as an indicator of toxicity of drug delivery was measured every 4 days for each mouse. Once the mice showed obvious suffering, such as struggling or whining, they were anesthetized by inhaling ether and euthanized. Tumors were removed and tumor size was measured with calipers. Formalin-fixed paraffin-embedded xenograft tumors were analyzed using the in situ terminal deoxynucleotidyl transferased UTP nick-end labeling (TUNEL) assay.

2.11 Animal survival and tumor volume measurement

After the mice succumbed to the tumor or were sacrificed, calipers were used to measure the maximum diameter (a) and minimum diameter (b) of the tumors removed from the anesthetized mice. The tumor volume (V, mm$^3$) was calculated using formula $V = \frac{ab^2}{2}$. The five mice in each group were observed for 70 days for survival analysis.

2.12 Measurement of the liver function

Just after the final measurement of anti-tumor activity, blood samples were drawn from the eye ground venous plexus, centrifuged at 3000 rpm for 15 min at 4°C then the serum was kept at $-80^\circ$C for assessing alanine transaminase (ALT), aspartate transaminase (AST) and C-reactive protein (CRP). ALT and AST levels were determined as described protocols [23] using an automated analyzer (Model 200; Toshiba, Tokyo, Japan) and expressed as international units per liter (U/l). Serum CRP was measured using mouse
CRP ELISA Test Kit (Life Diagnostics, West Chester, PA) as manufacturer’s instruction. ALT, AST and CRP levels were determined in two independent assays.

2.13 TUNEL assay

The tumor samples were fixed in 10% formalin for 24 h and were embedded in paraffin and sliced in 5-μm-thick sections for apoptosis analysis. The TUNEL protocol was based on the TUNEL detection Kit (Roche, Switzerland). The 5-μm-thick sections were deparaffinized by washing in xylene and a descending ethanol gradient. The sections were subsequently incubated with 20μg/ml proteinase K at 37°C for 30 min and endogenous peroxidase was inactivated by incubation with 3% H₂O₂ in methanol for 10 min. Next, 50μl of TUNEL reaction mixture was added to the sections, and samples were incubated at 37°C for 1 h followed by the addition of 50μl converter-POD to the samples and incubation at 37°C for 30 min in a wet box. For color development, 50μl DAB substrate was added to the sections and allowed to incubate at room temperature for 5 min to detect labeled nuclei. Next, sections were counter stained with hematoxylin, washed with distilled water, dehydrated, made transparent, mounted and photographed under a microscope. Cells were considered to be positive if they showed brown particles in the nuclei. Ten fields of view (200×) were randomly selected in each slide, and the number of positive and negative cells was counted. The apoptosis rate was calculated as the percentage of TUNEL-positive cells.

2.14 Statistical analysis

Experiments were performed in triplicate and data are presented as the mean±standard deviation (SD). Student’s t-test was used for statistical analysis of two groups, and data from more than two groups were analyzed by a one-way ANOVA in the SPSS Statistics 16.0 package. Kaplan-Meier method was used to calculate mouse survival data and Log-rank test was used to analyze the survival rate. The results were considered to be significant when P < 0.05.

3 Results

3.1 The expression rates of GP73 on the cell surfaces of liver cancer cells were higher than normal liver cells

We first screened the expression rates of GP73 on the cell surfaces of HepG2 (HCC cell line), Bel-7402 (HCC cell line) and HL-7702 (normal liver cell line). The experimental group was incubated with GP73 antibody, and the control group was incubated with corresponding serum. As shown in Fig. 1A and B, the expression rates of GP73 on the cell surfaces of HepG2 and Bel-7402 cells were higher than that on the HL-7702 cells. Specifically, the expression rates of GP73 in the HepG2, Bel-7402 and HL-7702 cells were 28.6±0.73%, 25.42±0.48% and 2.27±0.16%, respectively. Taken together, the
expression rates of GP73 on the cell surfaces of HCC cell lines were higher than normal liver cells ($P < 0.001$). Furthermore, we determined the expression level of survivin in the above three cell lines. As shown in Fig. 1C, the expression level of survivin in HL-7702 is higher than the other two HCC cell lines.

3.2 The transfection efficiency of liver cancer cells by the pBI-CMV2-pSurvivin-HSVtk + GP73 liposome group was higher than the pBI-CMV2-pSurvivin-HSVtk + liposome group

We then determined the transfection efficiency of GP73 liposome group. The liposome and GP73 antibody-modified liposome were mixed with plasmid pBI-CMV2-pSurvivin-HSVtk in the optimal binding ratio (4μl: 2μg), respectively, and were transfected into HepG2, Bel-7402 and HL-7702 cells. According to Fig. 2A and B, due to the high
expression level of GP73-specific antigen on the cell surfaces of HepG2 and Bel-7402 liver cancer cells, the GFP expression rates of plasmid pBI-CMV2-pSurvivin-HSVtk mediated by GP73 liposomes were higher than those mediated by ordinary liposomes ($P < 0.01$). In addition, there is no selective specificity for the two HCC cell lines when transfected with ordinary liposomes. Furthermore, as shown in Fig. 2A and B, due to the low expression rates of GP73 on the cell surfaces of HL-7702 cells, the fluorescence rates of the cells transfected with GP73 liposomes and ordinary liposomes were lower than HCC cell lines. Surprisingly, the transfection efficiency of HL-7702 cells mediated by GP73 liposomes was lower than that by ordinary liposomes, which reduced the killing effects of therapeutic gene to normal cells. Furthermore, we found that the transfection efficiency of HepG2, Bel-7402 and HL-7702 cells without the targeting GP73 was $21.57 \pm 2.83\%$, $19.17 \pm 0.71\%$ and $12.56 \pm 3.1\%$, and the difference was statistically significant ($P < 0.05$). It can be suggested that the transfection efficiency of HL-7702 is lower than HCC cell lines, which is in line with previous reports that HL-7702 human hepatocytes were resistant to the liposomal treatment [24,25].
The comparison of transfection efficiency between the pBI-CMV2-pSurvivin-HSVtk + liposome group and pBI-CMV2-pSurvivin-HSVtk + GP73 liposome group in three cell lines. (A) pBI-CMV2-pSurvivin-HSVtk + GP73 liposome and pBI-CMV2-pSurvivin-HSVtk + liposome were transfected into HepG2, Bel-7402 and HL-7702 cells, respectively. (B) Comparative analysis of the transfection efficiency between HSVtk + liposome group and HSVtk + GP73 liposome group in HepG2, Bel-7402 and HL-7702 cells. **P < 0.01 indicates significant differences between the groups as shown. (HepG2 liposome group vs HepG2 GP73 liposome group, 21.57 ± 2.83% vs 30.54 ± 1.41%, P < 0.01; Bel-7402 liposome group vs Bel-7402 GP73 liposome group, 19.17 ± 0.71% vs 24.4 ± 1.52%, P < 0.01).

3.3 TK gene was expressed in hepatoma cells rather than normal liver cells

The HSVtk gene was synthesized by PCR and cloned into the pBI-CMV2 vector. The pBI-CMV2-pSurvivn-TK recombinant plasmid was confirmed by BamHI and HindIII restriction enzyme digestion and the results showed a 1137bp fragment presented in the recombinant plasmid but not presented in the control vector (Fig. 3A). The cytomegalovirus (CMV) 1 promoter was removed and replaced with a 0.9-kb survivin promoter synthesized by PCR (Fig. 3B). The survivin promoter ensures that the HSVtk
gene is only expresses in liver cancer cells. DNA sequencing confirmed insertion of the genes (data not shown).

To detect the specific expression of the survivin promoter driven TK gene in hepatoma cells but not in normal cells, we first detected the transfection efficiency of GP73-pBl-CMV2-pSurvivin-TK plasmid into HCC and liver cells using flow cytometry. As shown in Fig. 3C, the transfection efficiency of the pBl-CMV2-pSurvivin-TK plasmid into HL-7702, HepG2 and Bel-7402 cells was 12.88%, 29.86% and 22.56%,
respectively. We collected the transfected cells using flow sorting and assessed the expression of HSVtk using RT-PCR and Western blot assays. As shown in Fig. 3D and E, HSVtk was expressed in GP73-positive transfected Bel-7402 and HepG2 hepatoma cells but not in GP73-positive transfected HL-7702 normal liver cells, un-transfected Bel-7402 and HepG2 hepatoma cells.

3.4 GCV treatment inhibited cell proliferation of HSVtk-transfected HCC cells but not normal liver cells

The effect of HSVtk driven by the survivin promoter in HCC cells has not been studied. In this study, we evaluated the inhibition of cell growth induced by GCV in cultured HepG2, Bel-7402 and HL-7702 cells transfected with the survivin promoter-driven HSVtk plasmid or a control vector. The transfected cells were sorted by flow cytometry and cultured with different doses of GCV for three days, and cell proliferation was measured using the CCK-8 assay. We found that the HSVtk + GCV treatment of HepG2 and Bel-7402 cells significantly inhibited cell growth compared with the vector + GCV treatment (P<0.01). The inhibition of HepG2 and Bel-7402 cell proliferation with the HSVtk/GCV system had a dose dependent manner. However, the HSVtk/GCV system had no effect on cell proliferation in HL-7702 cells (Fig. 4A). In addition, clone formation assay also revealed that the number of clones formed in HSVtk-transfected HepG2 and Bel-7402 cells treated with 150 μg/ml GCV was obviously lower than that in parental vector-transfected cells. The formed clones were not observed in both HSVtk-transfected HL-7702 cells and vector control cells (Fig. 4B). All of these data suggested that GCV treatment inhibited cell proliferation of HSVtk-transfected HCC cells but not normal liver cells. We also tested the cell viability of HSVtk-transfected HepG2, Bel-7402 and HL-7702 cells without GCV treatment. As shown in Fig. 4C, the cell viability of HSVtk-transfected HepG2 and Bel-7402 cells without GCV treatment was higher than cells treated by GCV. However, GCV treatment had almost no effect on cell viability of HL-7702 cells. Then, We tested whether the GP73 antibody binding may cause any cellular effects. We incubated HepG2, Bel-7402 and HL-7702 cells with GP73 antibody overnight. Then the GP73 antibody-modified liposome were mixed with plasmid pBI-CMV2-pSurvivin-HSVtk in the optimal binding ratio (4 μl: 2 μg), respectively, and were transfected into HepG2, Bel-7402 and HL-7702 cells. The transfected cells were sorted by flow cytometry and cultured with 150 μg/ml GCV for three days, and cell proliferation was measured using the CCK-8 assay. As shown in Fig. 4D, the cell viability of GP73 antibody-incubated HepG2 and Bel-7402 cells was higher than cells without GP73 antibody treatment.
GCV treatment promotes an inhibitory effect on cell growth of HSVtk-transfected hepatocellular carcinoma cells. The HSVtk or vector transfected HepG2, Bel-7402 and HL-7702 cells were treated with different doses of GCV. The cell proliferation was assessed using a CCK-8 assay (A) (HepG2-vector vs HepG2-HSVtk, 150 μg/ml GCV, 1.633 ± 0.021 vs 0.921 ± 0.033, *P* < 0.01; Bel-7402-vector vs Bel-7402-HSVtk, 150 μg/ml GCV, 1.542 ± 0.012 vs 0.897 ± 0.003, *P* < 0.01) and cell clone formation assay (B) (HepG2 vector + GCV vs HepG2 HSVtk + GCV, 53.4 ± 13.6% vs 35.9 ± 12.5%, *P* < 0.01; Bel-7402 vector + GCV vs Bel-7402 HSVtk + GCV, 42.9 ± 14.5% vs 28.1 ± 15.2%, *P* < 0.01; HL-7702 vector + GCV vs HL-7702 HSVtk + GCV, 16.1 ± 4.9% vs 18.2 ± 4.5%, *P* > 0.05). Each assay was repeated three times with similar results. **P* < 0.01 indicates significant differences between the groups as shown. 0.75 μM DOX treatment served as a positive control. (C) The HSVtk transfected HepG2, Bel-7402 and HL-7702 cells were treated with 150 μg/ml GCV or without GCV for 72 h. Then the cell proliferation was assessed using a CCK-8 assay. (HepG2 HSVtk + GCV vs HepG2 HSVtk, 1.00 ± 0.10 vs 1.60 ± 0.05, *P* < 0.01; Bel-7402 HSVtk + GCV vs Bel-7402 HSVtk, 1.00 ± 0.05 vs 1.52 ± 0.04, *P* < 0.01). (D) The
3.5 GCV treatment promoted the apoptosis of HSVtk-transfected HCC cells but not normal liver cells

To investigate whether GCV treatment inhibits the proliferation of HSVtk-transfected HCC cells through apoptosis, we analyzed the apoptosis rate of HSVtk-transfected or vector-transfected HL-7702, HepG2 and Bel-7402 cells treated with 150 μg/ml GCV for 48 h using flow cytometry. We found that the HSVtk + GCV treated HepG2 and Bel-7402 cells had a significantly higher apoptosis rate than that of vector + GCV treated HepG2 and Bel-7402 cells (P < 0.05). Nevertheless, the HSVtk/GCV system had no effect on apoptosis in HL-7702 cells (Fig. 5). These results suggest that GCV treatment inhibits the proliferation of HSVtk-transfected HCC cells by promoting apoptosis.
GCV treatment promotes the cell apoptosis of HSVtk-transfected hepatocellular carcinoma cells. The HSVtk or vector transfected HepG2, Bel-7402 and HL-7702 cells were treated with 150 μg/ml GCV for 48 h, and apoptosis was analyzed by flow cytometry. Each assay was repeated three times with similar results. **P < 0.01 indicates significant differences between the groups as shown. (HepG2 vector + GCV vs HepG2 HSVtk + GCV, 7.8 ± 0.5% vs 23.4 ± 1.8%, P < 0.01; Bel-7402 vector + GCV vs Bel-7402 HSVtk + GCV, 6.5 ± 0.8% vs 21.2 ± 2.2%, P < 0.01). 0.75μM DOX treatment served as a positive control.
3.6 GP73-liposome delivery of HSVtk followed by GCV treatment synergistically increased HCC xenograft tumor cell apoptosis *in vivo*

We established an HCC xenograft tumor model induced by subcutaneous inoculation of HepG2 cells into nude mice. To assess the effect of GP73, we detected HSVtk expression in tumor tissue and normal tissue (lung) by Western blot assay (Fig. 6A). The HSVtk protein was not detected in the vector group and its levels were significantly higher in the GP73-HSVtk group than the HSVtk group.

![Fig. 6](image)

GP73-Liposome delivery of HSVtk followed by GCV treatment shows no toxicity on mice. (A) The HSVtk expression in tumor tissues from the vector group, HSVtk group and GP73-HSVtk group were measured via a Western blot assay. β-actin served as a loading control. Each assay was repeated three times with similar results. **P < 0.01** indicates significant differences between the groups as shown. (HSVtk + GCV vs GP73 + HSVtk + GCV, 16.5 ± 2.2% vs 30.1 ± 3.1%, P < 0.01) (B) The body weights of mice in vector group, HSVtk group and GP73-HSVtk group were recorded every 4 days after drug injection. (C and D) The serum ALT, AST and CRP in vector group, HSVtk group and GP73-HSVtk group were measured before the euthanasia of mice. Error bars indicate the mean value ± SD (n = 5 per group). DOX treatment served as a positive control.

To monitor the toxicity of GP73-liposome delivery of HSVtk, the body weight of each mouse was measured. The average body weight of mice in vector group, HSVtk group and GP73-HSVtk group showed no significant difference (Fig. 6B). Furthermore, liver-specific enzymes alanine transaminase (ALT) and aspartate transaminase (AST) and inflammatory marker C-reactive protein (CRP) were detected. We found that the levels of ALT, AST and CRP remain unchanged among these groups (Fig. 6C and D). These data suggest that the drug delivery is feasible and safe.

To assess whether GP73-liposome mediated HSVtk transfection followed by GCV treatment could increase apoptosis of tumor cells *in vivo*, TUNEL staining of HCC
xenograft tumor sections was performed. We found that liposome delivery of HSVtk followed by GCV treatment increased tumor cell apoptosis compared with the vector control \((P < 0.01)\). Moreover, GP73-liposome delivery of HSVtk combined with GCV treatment significantly increased tumor cell apoptosis compared with the vector and HSVtk groups \((P < 0.01)\) (Fig. 7A). Cleaved (activated) caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) were considered as an acknowledged indicator of apoptosis [26]. Therefore, we analysed the levels of cleaved caspase 3 and cleaved PARP in HCC xenograft tumor tissues. In line with TUNEL staining assay, we found that liposome delivery of HSVtk followed by GCV treatment increased the levels of cleaved caspase-3 and cleaved PARP compared with the vector control. Furthermore, GP73-liposome delivery of HSVtk combined with GCV treatment dramatically increased the levels of cleaved caspase-3 and cleaved PARP compared with the vector and HSVtk groups (Fig. 7B).

### 3.7 GP73-liposome delivery of HSVtk followed by GCV treatment synergistically increased animal survival

Tumor-bearing mice from each group were followed for 70 days to assess their survival. The results demonstrated that mice receiving liposome-mediated delivery of the HSVtk
gene followed by GCV lived longer compared with animals in the vector group ($P<0.05$). In addition, we also found that mice receiving GP73-liposome-mediated delivery of the HSVtk gene followed by GCV lived longest compared with animals in the vector and HSVtk groups ($P<0.01$; Fig. 8A). Next, the HCC xenograft tumor size was measured. The data showed that tumors in the vector control group were larger than those in the HSVtk and GP73-HSVtk transfection groups (Fig. 8B and C).

![Fig. 8](image)

GP73-Liposome delivery of HSVtk followed by GCV treatment synergistically increasing animal survival. (A) Liposome delivery of HSVtk significantly prolonged survival of tumor-bearing mice compared to the vector control. GP73-Liposome delivery of HSVtk produced the longest survival (GP73 + HSVtk + GCV vs vector + GCV, 49.3 ± 0.5% vs 18.8 ± 1.7%, $P < 0.01$; GP73 + HSVtk + GCV vs HSVtk + GCV, 49.3 ± 0.5% vs 31.5 ± 1.5%, $P < 0.01$; HSVtk + GCV vs vector + GCV, 31.5 ± 1.5% vs 18.8 ± 1.7%, $P < 0.05$). (B) Tumors (encircled) were induced in nude mice by subcutaneous inoculation of HepG2 cells and (C) GP73-Liposome delivery of HSVtk followed by GCV treatment, which significantly inhibited tumor growth (n = 5 per group) (GP73 + HSVtk + GCV vs vector + GCV, 20 d, 534.92 ± 0.03 vs 1471.32 ± 30.05, $P < 0.01$; GP73 + HSVtk + GCV vs HSVtk + GCV, 20 d, 534.92 ± 0.03 vs 1195.25 ± 95.73, $P < 0.01$; HSVtk + GCV vs vector + GCV, 20 d, 1195.25 ± 95.73 vs 1471.32 ± 30.05, $P < 0.05$). DOX treatment served as a positive control. (**$P < 0.01$; #$P < 0.05$).

### 4 Discussion

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor worldwide and the second most common cause of cancer-related mortality [27]. The most effective therapy to date is surgical resection, although less than 15% of patients benefit from this treatment because of the presence of multiple tumor nodules [28]. In the past several years, several innovative approaches based on therapeutic gene transfer, also known as gene therapy, have been studied for treating HCC [29–31].
The efficiency of gene therapy depends on the choice of therapeutic gene, vector and route of gene delivery. The best characterized therapeutic gene is the suicide gene therapy system - the herpes simplex virus type thymidine kinase (HSVtk). This gene encodes the thymidine kinase enzyme, which metabolizes ganciclovir (GCV), an anti-viral drug that normally does not show anti-tumor effects, into a toxic compound that interferes with DNA replication and results in apoptosis. This system provides conditional toxicity based on the presence of the sensitizing gene and its substrate. Moreover, nearby unmodified tumor cells are also killed by pro-apoptotic signals and enzymes that diffuse from cells expressing the HSVtk gene via intercellular communication, termed the “bystander effect.” In vivo, this system additionally induces a strong immune response directed against tumor cells [4,32,33]. Both the transfection efficiency and bystander effect are essential factors for the success of the anti-tumor efficacy of the HSVtk and prodrug GCV suicide gene therapy system.

Gene transfer can be delivered by either direct injection into the target tissue (tumor) or via the hepatic artery, which is the major blood supply for hepatic tumor cells [34]. To facilitate cell transduction, therapeutic genes are packaged into vectors that possess either a viral or non-viral nature [35,36]. Viral vectors are the most commonly used owing to their natural property to infect human cells. However, the vectors’ pathogenicity, immunogenicity and potential of insertion mutagenesis greatly limit their clinical use. In contrast, non-viral vectors are almost completely non-immunogenic, relatively safe, and less expensive to produce and can carry a great amount of genetic material [37].

Tumor-specific expression of tumor-killing molecules is necessary for gene therapy. The best way is a “robust” molecular targeting system (a promoter driving the gene of interest within the vector itself) specific for cancer cells. Survivin is a member of the inhibitor of apoptosis (IAP) family, which is differentially expressed in tumor tissues compared with normal tissues and plays a key role in the regulation of apoptosis [8,38]. A high level of survivin expression has been observed in HCC, and several studies have shown that survivin promoter-driven nanoscale peptide or phytotoxin gelonin exhibited selective tumor cytotoxicity in vitro and in vivo [11,15] This finding makes the approach a promising candidate for constructing vectors for cancer gene therapy.

In the present study, we cloned the survivin promoter into the pBI-CMV2 vector. Next, we constructed a recombinant plasmid, pBI-CMV2-pSurvivin-TK, which contained both the cytomegalovirus immediate early gene (CMV) promoter driving the expression of green fluorescent protein (GFP) and the survivin promoter driving the expression of HSVtk (Fig. 3B). To detect the tissue and cell specificity of transcriptional regulation, the pBI-CMV2-pSurvivin-TK vector was transfected into HCC cells (Bel-7402 and HepG2) and normal liver cells (HL-7702). RT-PCR and Western blot assays showed that TK was expressed in HCC cells and not in normal cells (Fig. 3D and E). These data indicate that
the survivin promoter has the property of tissue- or cell-specific transcriptional regulation.

Cellular proliferation and apoptosis maintain a balance in normal tissues. Normal cells transform into tumor cells once this balance is disrupted [39]. Therefore, a common therapeutic strategy for cancer is inducing apoptosis to inhibit the proliferation in cancer cells. We detected the HCC-killing effect of HSVtk in the presence of GCV and observed that the proliferation of HepG2 and Bel-7402 cells was significantly impaired, but this inhibition of cell growth was not seen in HL-7702 cells (Fig. 4). We further assessed whether the proliferation inhibition of HCC is a result of enhancement of apoptosis. Flow cytometry analysis showed that the proportion of apoptotic cells significantly increased after pBI-CMV2-pSurvivin-TK transfection followed by administration of GCV in HepG2 and Bel-7402 cells but not in HL-7702 cells (Fig. 5).

Our previous study showed that human α-fetoprotein (AFP) promoter-driven HSVtk expression in HCC cells inhibited cell proliferation when combined with GCV treatment in vitro and in vivo [23]. Interestingly, several reports have shown that GP73 is expressed on HCC cell membranes earlier than AFP, indicating that GP73 has higher sensitivity and specificity for early diagnosis of liver cancer [40,41]. We modified liposomes with a GP73 antibody in order to enhance targeted gene delivery in HCC treatment. Using a human HepG2-induced xenograft tumor model, we delivered the HSVtk gene driven by the survivin promoter and with GP73 surface antigen by hydrodynamic tail vein injection. We observed that the TK gene was only expressed in tumor tissue and not in normal (lung) tissue (Fig. 6A). It is well known that serum ALT and AST are markers for liver function [42] and serum CRP is an indicator of inflammation [43]. Our data showed that the levels of serum ALT, AST and CRP remain unchanged in mice in the vector group, HSVtk group and GP73-HSVtk group during the experiment. Meanwhile, the body weights of mice were also unaffected by different treatment (Fig. 6B). All of these data suggested that GP73-liposome delivery of HSVtk in vivo is safe and feasible. Combined with GCV treatment in vivo, the TUNEL assay and Western blot analysis showed remarkable apoptosis-related DNA fragmentation and apoptosis-related protein such as cleaved caspase 3 and cleaved PARP in the GP73-HSVtk-treated tumor tissue compared with HSVtk or vector treatment (Fig. 7A and B). Additionally, we found that GP73-HSVtk treatment significantly reduced tumor volume and extended the survival of tumor-bearing mice (Fig. 8).

In the present study, we chose the doxorubicin as the positive control and found that it had an advantageous anti-tumor effect in vitro and in vivo. However, the doxorubicin can cause some side effects such as cardiotoxicity [44]. Besides, we also found that the doxorubicin also bring the liver damage indicated by an increase in the levels of biochemical markers including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine (CRE). All of these side effects limit the use of the
doxorubicin in clinical trials. Compared with doxorubicin, the GP73-HSVtk treatment can induce tumor cell apoptosis without liver damage.

In summary, for HCC-specific gene therapy, we designed a novel strategy using a GP73 antibody to modify a reformed non-viral vector, pBI-CMV2-pSurvivin-TK, encoding TK driven by the survivin promoter. This gene therapy system is a promising therapeutic strategy for HCC since it can suppress the proliferation of HCC by inducing cell apoptosis in vitro and in vivo. Although these results are encouraging, we recognize that subcutaneous tumor models using an HCC cell line may not fully represent HCC in human patients. We also recognize that the lower transfection efficacy of the present gene delivery system as its performance in vitro experiments will limit its further application. Therefore, we will focus on improving the transfection efficiency of therapeutic gene into target cells or tissues in our future study. Furthermore, in vivo delivery of ordinary cationic transfection agents like lipo2000 is associated with several drawbacks such as immune system response and cytotoxicity. Accordingly, we plan to develop other safer and targeted delivery system in the future and modify them with anti-GP73 antibody to reduce the side effects in vivo, such as ultrasound-targeted microbubble destruction (UTMD) or exosomes. UTMD is a non-invasive, efficient, targeted and safe transfection technique that can deliver plasmids to specific organs and tissues. UTMD-mediated gene therapy may be more useful than conventional gene delivery approaches because it is non-invasive and can target gene to a specific tissue with limited toxicity or immunogenicity [45]. Our previous study showed that UTMD delivery of Timp3 and HSVtk synergistically inhibited HCC xenograft tumor growth and increased animal survival [29]. In the future, we plan to modify UTMD with anti-GP73 antibody to enhance its targeted efficiency and reduce the side effects. On the other hand, exosomes can evade rapid clearance by mononuclear phagocytes and show great stability in blood, with almost no harmful immune response [46]. With 70–120nm in diameter, exosomes can make use of the EPR effect and infiltrate into the tumor tissues selectively [47]. Exosomes can be purified from the engineered dendritic cells (DCs) and loaded with the plasmid and the anti-GP73 antibody.

In the experiment, we found that the transfection efficiency of HepG2, Bel-7402 and HL-7702 cells without the targeting GP73 was 21.57±2.83%, 19.17±0.71% and 12.56±3.1%, respectively, and the difference was statistically significant (P<0.05). In addition, the transfection efficiency of HepG2, Bel-7402 and HL-7702 cells with the targeting GP73 was 30.54±1.41%, 24.40±1.52% and 8.43±3.5%, respectively, and the difference was also statistically significant (P<0.05). It can be concluded that the transfection efficiency of HL-7702 is lower than HCC cell lines, which is in line with previous reports that HL-7702 human hepatocytes were resistant to the liposomal treatment [24,25]. One of our previous paper also get similar results that there is no TK expression in HL-7702 normal liver cells [48]. Till now, we speculated that the difference in survivin expression and the transgene expression in our construct has something to
do with the liposome and the transfection efficiency. We planed to further determine the promoter driving survivin expression in HCC cell lines and normal liver cells, which will be excellent next steps in this project.

GP73 is widely expressed in normal epithelial cells in many human tissues and is a crucial factor for maintaining normal survival of animals and the development of the liver and kidney [49-52]. Therefore, off-target expression of GP73 should be carefully managed. The expression patterns of GP73 in many other cells or tissues are to be further explored in the future.

Survivin is overexpressed in most cancer cells and embryonic tissues, but only barely detectable in terminally differentiated normal tissues [53]. Survivin expression may be activated transcriptionally [54]. Therefore, the survivin promoter was reported to control the transgene expression in a cancer-specific manner, which was more active in cancer cell lines than in normal cells [55,56].

Both survivin and GP73 expression can be upregulated in liver cirrhosis prior to HCC development. Liver cirrhosis is marked by widespread fibrous scarring and is the irreversible terminal stage of chronic liver disease. Serious complications of liver cirrhosis include hepatic dysfunction, esophageal varices, ascites and liver cancer [57]. To date, gene therapy strategies is not applicable to liver cirrhosis, because (1) transducing a tissue with substantial fibrosis is rather difficult; (2) the use of the vector and/or transgene may aggravate the existing damage of the target organ [58]. Therefore, using this combination clinically, where most HCC develop in a setting of cirrhosis, could be problematic. HSVtk expression in these already compromised livers may not be a viable clinical option. However, the search for efficient ways of vector administration to cirrhotic livers is still an urgent field to be developed.

HCC is very heterogeneous with high expression variability in vivo and in vitro. Targeting the molecules which may not be expressed throughout the tumour or in the tumour at all may be problematic. Therefore, the expression patterns of GP73 in many other cells or tissues are to be further explored in the future. Clinically, suicide gene (TK) has been proved to be effective in the treatment of gynecological [59,60] and gastrointestinal tumors [61,62], as well as hepatocellular carcinoma patients with the tumor diameter less than 5cm [63]. It was reported that 54 HCC patients’ overall survival rates were significantly improved and the tumor recurrence rates were obviously reduced after gene therapy when the tumor diameter was less than 5cm compared with 23 HCC patients receiving traditional surgical resection, which indicated that gene therapy was worthy of being popularized [63]. Clinically, however, HCC is often not biopsied so the genetic makeup of the tumour is often not known. Then, how would this genetic approach to targeting the tumour fit in with clinical reality is another question waiting to be answered.
Author contributions

Conceived and designed the experiments: Baofeng Yu, Jun Xu, Jun Xie and Lumei Wang. Performed the experiments: Chang Liu, Chaochao Wen, Xi Wang, Yan Wei, Chunyang Xu and Xiuli Mu. Analyzed the data: Lina Zhang, Xuan Wang, Jiubo Tian, Peiyuan Ma, Fanxiu Meng, Qi Zhang and Na Zhao. Contributed reagents/materials/analysis tools: Tao Gong, Rui Guo, Hailong Wang, Jun Xie, Gongqin Sun, Gaopeng Li, Hongwei Zhang, Qin Qin, Xiushan Dong and Lumei Wang. Wrote the paper: Hailong Wang and Baofeng Yu. Interpreted results of experiments: Gongqin Sun and Baofeng Yu.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2019.111496.

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[51]


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Appendix A Supplementary data
The following is the Supplementary data to this article:

Multimedia Component 1

Multimedia component 1

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