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ApoE-modified liposomes mediate the antitumour effect of survivin promoter-driven HSVtk in hepatocellular carcinoma

Running title: Targeted killing effect of HSVtk in hepatoma

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Abstract

Hepatocellular carcinoma (HCC) is a prevalent malignant tumour with high global morbidity and mortality associated with its multiple aetiologies, poor prognosis, resistance to chemotherapeutic drugs and high rate of recurrence. Here, we evaluated a gene therapy strategy that targets 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. Apolipoprotein E (ApoE)-modified liposomes were used for targeted gene delivery to the tumour tissue, and the survivin promoter was used to drive HSVtk expression in HCC cells. Our results showed that the survivin promoter was specifically activated in tumour cells and HSVtk was expressed selectively in tumour cells. In combination with GCV treatment, HSVtk expression resulted in the inhibition of HCC cell proliferation via enhanced apoptosis. Additionally, tail vein injection of ApoE-HSVtk significantly suppressed the growth of xenograft tumours through an apoptosis-dependent pathway and extended the survival time of tumour-bearing mice. In summary, this study illustrates an effective cancer-specific gene therapy strategy for HCC that can be further developed for future clinical trials.

Keywords: hepatocellular carcinoma; gene therapy; HSVtk/GCV; Apolipoprotein E; apoptosis
Introduction

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and the third most common cause of cancer-related mortality globally. HCC results in more than 650,000 deaths and approximately 748,300 new cases are diagnosed per year worldwide. Currently, surgical resection is the most effective clinical treatment for HCC, although many other techniques, including radiofrequency ablation, microwave therapy, percutaneous ethanol injection, radiotherapy and biotherapy, have been applied to the clinical treatment of HCC. However, less than 15% of surgically treated patients benefit from surgical resection due to the high rate of recurrence or metastasis within 5 years following cancer resection. Therefore, novel therapeutic strategies, including gene therapy, are being developed.

Gene therapy based on the transfer of therapeutic genes (such as tumour suppressor genes, suicide genes, and immunomodulatory genes) into tumour cells induces a therapeutic effect. The choice of genes and vectors is the most pivotal issue in gene therapy. The herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) system is a promising gene therapy system for malignant tumours. Tumour cells transfected with the HSV-tk gene can convert non-toxic nucleoside analogues such as GCV into toxic metabolites to selectively eliminate the tumour cells. Efficient gene transfer to tumour cells is essential for gene therapy. Currently, viral and non-viral
vectors are used for gene transfer. Non-viral vectors have been used widely since they are non-immunogenic, relatively safe and can carry a larger amount of genetic material than viral vectors.

Using specific promoters that only function in hepatocarcinoma cells but not in normal liver cells to initiate HSKtk expression is an effective strategy to avoid expressing of HSVtk in normal hepatocytes. The human alpha-fetoprotein (AFP) promoter and survivin promoter are specifically expressed in hepatoma cells and have been used in gene therapy for HCC. Another important issue in HCC gene therapy is the optimal delivery technique. Currently, liposome-mediated gene transfer is widely used in pre-clinical and clinical experiments. Furthermore, liposomes have been modified by biomaterials to bind to receptors or antigens expressed on the surface of hepatoma cells to improve gene transfer to the target.

Apolipoprotein E (ApoE) is an important component of chylomicron (CM), very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL). Studies have demonstrated that lipoproteins containing ApoE can bind specifically to the low-density lipoprotein receptor (LDLr) expressed on the membrane of hepatocyte and hepatocarcinoma cells. Therefore, we hypothesized that ApoE-modified liposomes could improve the targeting of therapeutic genes to hepatocarcinoma cells during gene transfer. In this study, we modified liposomes with ApoE and quantified the targeted
transfection efficiency in HCC cells. To ensure the specific expression of HSVtk in HCC cells, the survivin promoter was introduced to regulate the expression of the HSVtk gene. We also evaluated the antitumour effects of HSVtk in vitro using hepatocarcinoma cell lines and in vivo using an established HCC xenograft mouse model. ApoE-modified liposomes were used for the targeted transfection of the vector and delivery of the HSVtk gene specifically to HCC tumour cells because of the affinity of ApoE on the liposome to bind to the LDLr, which is highly expressed on HCC cell membranes.

**RESULTS**

**ApoE-modified liposomes have increased transfection efficiency in HCC cells and normal liver cells**

The survivin promoter was synthesized by PCR from the genomic DNA of HepG2 cells and used to replace the AFP promoter located in the pcDNA3.1-pAFP-TK plasmid and to construct the recombinant pcDNA3.1-pSurvivin-TK plasmid (Fig. 1A). DNA sequencing confirmed the insertion of the gene (data not shown). To obtain the optimal ratio of plasmid to liposomes, a plasmid-binding assay was performed. We observed that there were no strips when the mass-to-volume ratio of the pcDNA3.1-pSurvivin-TK plasmid to Lipofectamine 2000 was 1:2 (Fig. 1B). Similarly, the pcDNA3.1-pSurvivin-TK plasmid was completely encapsulated by ApoE-
Lipofectamine 2000 when the mass-to-volume ratio was 1:2.5 (Fig. 1C).

To compare the difference in transfection efficiency between Lipofectamine 2000 and ApoE-Lipofectamine 2000, the pGenesil-1 plasmid (which expresses green fluorescent protein, Fig. 1D) and Lipofectamine 2000 or ApoE-Lipofectamine 2000 were mixed (1:2.5 mass-to-volume) and transfected into HL-7702, HepG2, HuH-7 or SW480 cells. Forty-eight hours later, the transfection efficiency was assessed by using flow cytometry. We observed that the transfection efficiency of Lipofectamine 2000 in HL-7702, HepG2, HuH-7 and SW480 cells was 26.48±2.34%, 27.29±3.50%, 26.26±2.84% and 20.37±2.48%, respectively (Fig. 2A, 2D, 2G, and 2J). The transfection efficiency of ApoE-Lipofectamine 2000 in the same cells was 37.72±3.36%, 38.17±4.49%, 36.88±3.30% and 19.46±2.34%, respectively (Fig. 2B, 2E, 2H, and 2K). These data show that the transfection efficiency of ApoE-Lipofectamine 2000 in hepatocyte and hepatocarcinoma cells was significantly higher than that of Lipofectamine 2000 (P < 0.05, Fig. 2C, 2F and 2I). Nevertheless, there was no significant difference in transfection efficiency between Lipofectamine 2000 and ApoE-Lipofectamine 2000 in SW480 cells (P > 0.05, Fig. 2L).

The TK gene was expressed in HepG2 and HuH-7 cells but not in HL-7702 and SW480 cells.

To detect the specific expression of the survivin promoter-driven TK gene in HCC but
not in normal liver cells and SW480 cells, we collected ApoE-Lipofectamine 2000/pcDNA3.1- pSurvivin-TK-transfected cells and assessed the expression of HSVtk using RT-PCR and Western blot assays. As shown in Figure 3A and 3B, HSVtk was expressed in ApoE-positive transfected HepG2 and HuH-7 hepatoma cells but not in ApoE-positive transfected HL-7702 normal liver cells and SW480 colonic cancer cells.

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

To study the effects of HSVtk driven by the survivin promoter in HCC cells, we collected the pcDNA3.1-pSurvivin-TK or pcDNA3.1 plasmid stably transfected HL-7702, HepG2 and HuH-7 cells using G418 selection and assessed cell proliferation in the presence of GCV. The transfected cells were cultured with different doses of GCV for three days, and cell proliferation was measured using the MTT assay. As shown in Fig. 4A, the HSVtk + GCV treatment of HepG2 and HuH-7 cells significantly inhibited cell growth compared with that of the vector + GCV treatment \( (P<0.01) \). Furthermore, the inhibition of HepG2 and HuH-7 cell proliferation with the HSVtk/GCV system was dose-dependent. However, the HSVtk/GCV system had no effect on cell proliferation in HL-7702 cells. Additionally, a clone formation assay also revealed that the number of clones formed in HSVtk-transfected HepG2 and
HuH-7 cells in the presence of 150 μg/mL GCV was palpably lower than that in parental vector-transfected cells. In accordance with the MTT assay, we did not observe the formation of clones in either HSVtk-transfected HL-7702 cells or vector control cells (Fig. 4B). All of these results indicate that GCV treatment inhibits the proliferation of HSVtk-transfected HCC cells but not normal liver cells.

**GCV treatment induced the apoptosis of HSVtk-transfected HCC cells but not of normal liver cells**

To investigate whether GCV administration inhibits the proliferation of HSVtk-transfected HCC cells via apoptosis in this context, we analysed the apoptosis rate of HSVtk-transfected or vector-transfected HL-7702, HepG2 and HuH-7 cells treated with 150 μg/mL GCV for 48 h using flow cytometry. As shown in Fig. 5A and 5B, the HSVtk + GCV-treated HepG2 and HuH-7 cells showed a significantly higher apoptosis rate than the vector + GCV-treated HepG2 and HuH-7 cells ($P<0.01$). Nevertheless, the effect on apoptosis of the HSVtk/GCV system was not present in HL-7702 cells. In addition, cleaved caspase 3, which serves as an indicator of apoptosis, was also analysed using Western blot. We observed that the levels of cleaved caspase 3 were increased in HSVtk + GCV-treated HepG2 and HuH-7 cells compared with those in vector + GCV-treated HepG2 and HuH-7 cells (Fig. 5C). These data indicate that GCV administration inhibits the proliferation of HSVtk-
transfected HCC cells via the promotion of apoptosis.

ApoE-liposome delivery of HSVtk followed by GCV administration inhibited tumour growth in HCC xenograft models

To evaluate the role of ApoE-liposome-delivered HSVtk in the presence of GCV, we established an HCC xenograft tumour model induced by subcutaneous inoculation of HepG2 cells into nude mice. First, we measured HSVtk expression in tumour and normal (lung and brain) tissues using RT-PCR and Western blot. HSVtk mRNA and protein was not detected in the vector group; HSVtk mRNA and protein was expressed in tumour tissues, and the expression levels were significantly higher in the ApoE-HSVtk group than in the HSVtk group. However, HSVtk expression was not detected in lung and brain tissues in either the HSVtk group or the ApoE-HSVtk group (Fig. 6A and 6B). We also found that HCC xenograft tumour volume and weight were decreased before and after euthanasia in the HSVtk and ApoE-HSVtk transfection groups compared with those of the vector control group. Moreover, the inhibition effect on tumour size and weight in the ApoE-HSVtk transfection group was more remarkable than that of the HSVtk transfection group (Fig. 6C, 6D). Meanwhile, we also found that the body weights of tumour-beared mice in the ApoE-HSVtk transfection group were obviously lighter than those of the HSVtk or vector transfection group (Table 1).
ApoE-liposome-mediated delivery of HSVtk combined with GCV treatment promoted HCC xenograft tumour cell apoptosis and increased animal survival

We then assessed the effect of ApoE-liposome-mediated HSVtk expression and GCV treatment on the apoptosis of tumour cells in vivo via TUNEL staining assay and Western blot. As shown in Fig. 7A and 7B, liposome delivery of HSVtk followed by GCV treatment augmented tumour cell apoptosis in comparison with the effects of the vector control (P < 0.01). Furthermore, tumour cell apoptosis was significantly increased in the HSVtk combined with GCV treatment group compared with that in the vector and HSVtk groups (P < 0.01).

Caspase 3 is well-known executors of apoptosis. Cleaved caspase 3 is acknowledged indicators of apoptosis. We next analysed the levels of cleaved caspase 3 in HCC xenograft tumour tissues. In accordance with the TUNEL staining assay, we observed that the levels of cleaved caspase 3 was increased in the liposome-mediated delivery of HSVtk followed by GCV treatment group compared with those in the vector control group. Moreover, ApoE-liposome-mediated delivery of HSVtk combined with GCV treatment dramatically increased the levels of cleaved caspase 3 compared with the vector only and HSVtk only controls (Fig. 7C).

ApoE-liposome-mediated delivery of HSVtk followed by GCV treatment increased animal survival
To further assess the protective role of HSVtk delivered by liposomes followed by GCV treatment, tumour-bearing mice from each group were followed for 70 days to evaluate their survival. The data showed that mice receiving the HSVtk gene by liposome-mediated delivery followed by GCV selection lived longer than the animals receiving the empty vector \((P < 0.05)\). Fascinatingly, we observed that mice receiving the HSVtk gene by liposome-mediated delivery followed by GCV selection lived longer than the mice receiving the empty vector and mice receiving HSVtk with no GCV \((P < 0.01; \text{Fig. 8A})\). Next, the liver-specific enzymes alanine transaminase (ALT) and aspartate transaminase (AST) and the inflammatory marker C-reactive protein (CRP) were detected to evaluate the safety of the liposome-HSVtk/GCV system \textit{in vivo}. The results showed that the levels of ALT, AST and CRP were not different among the treatment groups (Fig. 8B and 8C). These data demonstrate that drug delivery is feasible and safe \textit{in vivo}.

**DISCUSSION**

HCC is one of the most frequent neoplasms worldwide and has become a major cause of cancer-related death globally, due to its poor prognosis and low survival rates \(^{14,15}\). Although liver transplantation or resection is the first line of treatment \(^{16}\), HCC treatment is challenging since only 25% of patients are eligible for curative resection, and this therapy frequently results in recurrence \(^{17}\). Scientists have been working on
the alternative therapeutic technology such as gene therapy which kills tumour cells via transfection with plasmid DNA (pDNA) containing a therapeutic gene that produces a protein to induce the apoptosis of cancerous cells\textsuperscript{17, 19}. Cell death is triggered by either toxin gene therapy, in which genes for a toxic protein are transduced into tumour cells, or enzyme-activating prodrug therapy. The latter involves the direct delivery of a gene into the tumour for the expression of a heterologous enzyme followed by the administration of a prodrug that can be converted to a cytotoxic drug by the heterologous enzyme\textsuperscript{20}.

Suicide gene therapy systems have been developed as a promising treatment approach\textsuperscript{21}. At present, several enzyme-prodrug systems, such as carboxyl esterase (CE)/irinotecan, carboxypeptidase A (CPA)/MTX-a-peptide, carboxypeptidase G2 (CPG2)/CMDA and HSV-TK/GCV systems, have been developed for suicide gene therapy\textsuperscript{22, 23}. The HSV-TK/GCV system is the preferred choice in gene therapy since it encodes a thymidine kinase enzyme, which metabolizes ganciclovir (GCV) into a toxic compound that blocks DNA replication and induces apoptosis\textsuperscript{24}. Additionally, the HSV-tk gene also has a "bystander effect" that results in the death of adjacent non-transfected tumour cells\textsuperscript{4}. Importantly, it has been reported that HSV-TK/GCV therapy induces antitumour immunity and regression of brain tumours\textsuperscript{25, 26}. HSV-tk gene therapy has been widely used in the treatment of various tumours, such as liver
cancer, colon cancer, oesophageal cancer, glioma, melanoma and non-small cell lung
cancer 27, 28, 29. Nevertheless, a serious drawback is its low gene delivery efficiency,
which is one of the major barriers for the use of suicide gene therapy in clinical
practice. Therefore, increasing the efficiency of the targeted delivery and expression
of the HSV-TK gene in tumour cells/tumour tissues has become an important issue.

Various viral and non-viral vectors have rapidly been developed as efficacious gene
carriers. Considering the potential risks of viral vectors, including pathogenicity,
immunogenicity and insertional mutagenesis, non-viral vectors, which have almost no
immunogenicity, low production costs and can carry more genetic material than viral
vectors, have received increasing attention 30,31,32. Among them, the liposome system
can deliver oligonucleotides with increased biocompatibility. Moreover, many
protocols focus on tumour-targeting liposomes modified with specific ligands, such as
transferrin, and peptide- or antibody-linked liposomes, to improve the specificity of
the interaction between liposomes and tumour cells 33, 34, 35. These modified liposomes
can selectively recognize and combine the overexpressed receptors on tumour cells to
improve the targeting efficiency 7, 36, 37. ApoE is primarily synthesized by the liver,
recognized by the LDL receptor (LDLR) and mediates the recognition and recycling
of circulating LDL 38,39. LDLR is expressed in HCC tissues and hepatoma cell lines
and is involved in cell proliferation 40. Therefore, we hypothesized that ApoE-
modified liposomes would improve the specificity of gene delivery to HCC cells or
HCC tissues.

High survivin expression levels have been observed in HCC, and several studies
have shown that survivin promoter-driven nanoscale peptide expression or survivin
promoter-driven phytotoxin gelonin expression is selectively cytotoxic to tumours in
vitro and in vivo \cite{41,42}. These data indicate that survivin promoter-driven gene
expression is a promising approach for the construction of vectors for cancer gene
therapy. In this study, we constructed a recombinant pcDNA3.1-pSurvivin-TK
plasmid containing a survivin promoter, driving the expression of HSVtk (Fig. 1A).

Lipofectamine 2000 is a type of liposome that can encapsulate pDNA to form
liposome-DNA complexes that bind the cell membrane and enter cell endosomes
through direct fusion or endocytosis of membranes. DNA released from endosomes
can be directly used for the expression of biologically meaningful proteins through
transcription and translation processes \cite{43}. To improve the transfection efficiency and
target specificity, Lipofectamine 2000 was modified with ApoE protein to form ApoE-
Lipofectamine 2000 liposomes. In addition, we optimized the mass-to-volume ratio of
plasmid DNA to Lipofectamine 2000 to completely encapsulate plasmid DNA with
Lipofectamine 2000 (Fig. 1C). The flow cytometric analysis illustrated that the
transfection efficiency in the present study was higher than that in our previous study.
In particular, ApoE-modified Lipofectamine 2000 has a greater affinity for hepatocyte and hepatocarcinoma cells and has a higher transfection efficiency in HCC and hepatocyte cells than in other cells.

To analyse cell- or tissue-specific transcriptional regulation, the pcDNA3.1-pSurvivin-TK vector was transfected into HCC cells (HepG2 and HuH-7), normal liver cells (HL-7702) and colonic cancer cells (SW480). RT-PCR and Western blot assays showed that TK was expressed in HCC cells but not in normal liver cells and colonic cancer cells (Fig. 3). These results demonstrate that the survivin promoter has cell- or tissue-specific transcriptional regulation.

The balance of cellular proliferation and apoptosis is vitally important for the development of normal cells. Once this balance is disrupted, normal cells will transform into tumour cells. Uncontrolled cellular proliferation plays a crucial role in the progression of human tumours. Accordingly, inducing apoptosis to inhibit cancer cell proliferation has become a common therapeutic strategy for cancer. We then assessed the HCC-killing effect of HSVtk in the presence of GCV and observed that the proliferation of HepG2 and HuH-7 cells was significantly inhibited, whereas this impairment of cell proliferation was not seen in HL-7702 cells (Fig. 4). Furthermore, we evaluated whether the inhibition of the proliferation of HCC cells was a result of the enhancement of apoptosis. Flow cytometry and Western blot assays
revealed that the apoptosis rate increased significantly in HepG2 and HuH-7 cells but not in HL-7702 cells after pcDNA3.1-pSurvivin-TK transfection and GCV treatment (Fig. 5).

Using different strategies such as ultrasound-targeted or GP73-modified liposomes improves the targeting of HSVtk delivery and enhances its tumour cell killing effect when combined with GCV in vivo. In this study, we verified that ApoE-modified liposomes could increase the gene transfer efficiency into HCC cells in vitro.

Subsequently, we generated an HCC xenograft BALB/c mouse model induced by human HepG2 cells and administered the HSVtk gene driven by the survivin promoter and ApoE protein-modified liposomes to mice through hydrodynamic tail vein injection. The TK gene was only expressed in tumour tissues and not in normal (lung and brain) tissues (Fig. 6A, B). In the presence of GCV treatment in vivo, the TUNEL assay and Western blot analysis showed apoptosis-related DNA fragmentation and apoptosis-related proteins, such as cleaved caspase 3, in the ApoE-HSVtk-treated tumour tissue compared with that in the HSVtk- or vector-treated tumour tissue (Fig. 7). Additionally, we found that ApoE-HSVtk treatment significantly reduced tumour volume, tumour weight and prolonged the survival of the tumour-bearing mice (Fig. 6C, D and E).

To assess the safety and practicability of the ApoE-modified liposome system, we
measured the serum ALT, AST and CRP levels because ALT and AST are indicators of liver function \(^{45}\), and CRP is a marker of inflammation \(^{46}\). We observed that the levels of serum ALT, AST and CRP were unaltered in mice in the vector group, HSVtk group and ApoE-HSVtk group during the experiment (Fig. 8). These data demonstrate that ApoE-liposome delivery of HSVtk \textit{in vivo} is safe and feasible.

In conclusion, we modified liposomes using the ApoE protein to form a novel gene packaging system and constructed a pcDNA3.1-pSurvivin-TK non-viral recombinant vector encoding TK driven by the survivin promoter. We demonstrated the antitumour activity of this gene therapy system in an orthotopic HCC model of HepG2 cells and clarified its therapeutic effect based on the repression of HCC cell proliferation by inducing apoptosis in vitro and in vivo. This study strongly indicates that combining a tumour-selective promoter with tissue-specific gene delivery could greatly promote the antitumour effect of gene therapy and minimize its toxicity.

Materials and Methods

Cell lines, mice and ethics statement

HepG2, HuH-7 (hepatic carcinoma cell lines), HL-7702 (normal hepatocyte cell line) and SW480 (colonic cancer cell line) were purchased from the Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and Iscove’s Modified Dulbecco’s medium.
(IMDM) supplemented with 10% foetal bovine serum in 5% CO$_2$ at 37°C, respectively. Eight to ten-week-old, male BALB/c nude mice were purchased from the Animal Center for SIAS (Shanghai, China) and housed in pathogen-free conditions on a 12 h light-dark cycle with food and water provided *ad libitum*. Experimental procedures were conducted in accordance with ethical standards, the Declaration of Helsinki and national and international guidelines and were approved by the Animal Ethics Committee of Shanxi Medical University.

**Construction of the recombinant pcDNA3.1-pSurvivin-TK plasmid**

The survivin promoter (910 bp) was PCR-amplified from the genomic DNA of HepG2 cells. The forward primer sequence was 5'-CCCAAGCTTCCGCTCGAGCTGGCCATAGAACCAGAA-3', and the reverse primer sequence was 5'-CCGCTCGAGCGCGGATCCGCACGCCCTCTTAGGCG-3, which contain *Hind* III and *Xho* I restriction sites (underlined), respectively. PCR was performed according to a conventional protocol. The PCR products and the recombinant pcDNA3.1-pAFP-TK plasmid were digested with *Hind* III and *Xho* I. The digested products were purified from agarose gel using a gel extraction kit (COWIN Biotechnology Company, Beijing, China). The recombinant pcDNA3.1-pSurvivin-TK plasmid was constructed by inserting the digested amplified fragments into digested pcDNA3.1-pAFP-TK via the *Hind* III and *Xho* I sites and confirmed by
DNA sequencing.

**ApoE modification of liposomes**

A total of 125 μL of Lipofectamine 2000 (Invitrogen, USA) was activated by adding 30 μg distearoyl phosphatidylethanolamine, which is a polyethylene glycol maleimide (DSPE-PEG-MAL) (Avanti Corporation, Alexandria, USA), and incubated at 60°C for 1 h. ApoE protein (Peprotech Corporation, Rocky Hill, USA) dissolved in phosphate-buffered saline (PBS) and iminothiolane hydrochloride (2-IT) (1:2 molar ratio) were incubated at room temperature for 1 h. Next, activated Lipofectamine 2000 (125 μl) was conjugated to ApoE (1.24 μl) at room temperature for 4 h under nitrogen. Finally, glycine and 2-IT (1:2 molar ratio) were incubated at room temperature for 1 h. Then, 125 μl of ApoE-Lipofectamine 2000 was purified by adding 1 μl of the glycine and 2-IT mixture, and the two were incubating together at room temperature for 1 h to prepare the ApoE-conjugated Lipofectamine 2000 reagent.

**Analysis of the packaging efficiency of the liposomes**

Different volumes of Lipofectamine 2000 or ApoE-Lipofectamine 2000 were mixed with 1 μg of pcDNA3.1-pSurvivin-TK plasmid DNA and incubated at room temperature for 20 min. The mixture was subjected to 1% agarose gel electrophoresis. The optimum proportion between liposomes and plasmids was analysed according to gel electrophoresis.
Cell transfection

For transient cell transfection, HL-7702, HepG2, HuH-7 or SW480 cells were seeded in 3.5 cm dishes at a density of $5.0 \times 10^4$ cells and incubated overnight. Before transfection, 1 μg of pcDNA3.1-pSurvivin-TK mixed with 1 μg of pGenesil-1 and 5 μl of Lipofectamine 2000 or ApoE-conjugated Lipofectamine 2000 was incubated in 100 μl Opti-MEM at room temperature for 5 min. Subsequently, the plasmid DNA and Lipofectamine 2000 or ApoE-conjugated Lipofectamine 2000 were mixed and allowed to incubate for another 20 min. The cells were washed with PBS, and 1 ml of Opti-MEM was added. Two hundred microliters of the ApoE-lipo-DNA complex or lipo-DNA complex was added to each dish, and the cells and liposome complexes were incubated for 6 h. Finally, the Opti-MEM was replaced with medium containing 10% FBS, and the cells were incubated for another 48 h. The efficiency of the transfection was assessed by observing and measuring the amount of green fluorescence emitted by the cells using a fluorescence microscope.

For stable transfection, at 48 h post-transient transfection, cells were cultured in selection medium with 600 μg/ml (HL-7720 cells) or 800 μg/ml (HepG2, HuH-7 cells) G418 for 2 weeks. Then, drug-resistant cell clones were transferred to a 10 cm dish for further amplification in the presence of the selection medium. The expression of HSVtk was detected by RT-PCR and Western blot analysis.
Flow cytometry

To determine the transfection efficiency, flow cytometry was performed with a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Transfected cells were diluted in test tubes containing sheath solution, and GFP fluorescence was detected. The green fluorescence signal from $1.0 \times 10^6$ cells was excited with a 488-nm blue laser, and the emission was detected through a 530/30-nm bandpass (GFP) filter. The transfection rate was calculated as follows: transfection rate ($\%$) = (the number of cells expressing green fluorescence / the number of total cells) $\times$ 100.

For the analysis of apoptosis, pcDNA3.1-pSurvivin-HSVtk-transfected or pcDNA3.1-transfected HL-7702, HepG2 and HuH-7 cells were treated with 150 $\mu$g/ml GCV for 48 h, harvested with 0.25% trypsin and centrifuged at 1000 rpm for 3 min at room temperature. The cells were suspended, washed three times with PBS and adjusted to $1 \times 10^6$ cells/ml. Next, 5 $\mu$l of Annexin V-FITC was added to 100 $\mu$l of cell suspension for 10 min in the dark, and then 10 $\mu$l of propidium iodide (PI) was added before the percentage of apoptotic cells was measured by flow cytometry. Experiments were repeated three times for each group.

RT-PCR analysis

Total RNA from transfected or non-transfected cells or nude mouse tissues was extracted using TRIzol reagent according to the manufacturer’s protocols (Invitrogen).
RT-PCR was carried out using the GoldScript One-Step RT-PCR kit (Invitrogen). β-actin served as an internal control. A total of 2 μl of the RT product was used as a template, and HSVtk and β-actin were amplified together. The HSVtk primer sequences were 5'-AGCAAGAAGCCACGGAAGT-3' (sense) and 5'-CGATATGAGGAGCCAGAACG-3' (antisense). The β-actin primer sequences were 5'-TGACGTGGACATCCGCAAAG-3' (sense) and 5'-CTGGAAGGTGGACAGCGAGG-3' (antisense). The expected amplification fragment lengths of the HSVtk and β-actin genes were 330 and 205 bp, respectively. PCR was carried out as follows: pre-denaturation at 95°C for 5 min, 32 amplification cycles (denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 45 s), and a final extension at 72°C for 5 min. The PCR products were evaluated by electrophoresis on a 1% agarose gel.

**Western blot analysis**

Protein samples from transfected, non-transfected cells or nude mouse tissues were prepared using 1 × SDS lysis buffer, which contained 50 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% SDS. Cell lysates were boiled at 95°C for 5 min and then centrifuged at 13,000 rpm for 15 min at room temperature and then quantified using the BCA protein assay reagent (Pierce, USA). Protein samples (25 μg) were separated by 12% SDS-PAGE and transferred to a 0.2 μm PVDF membrane (Millipore,
Massachusetts, USA). The membranes were blocked in 5% BSA for 2 h at room
temperature, followed by a 4°C overnight incubation with primary antibodies. The
primary antibodies were visualized with their corresponding HRP-IgGs (ZSGB-
Biotechnology, China) using an ECL blot detection system (CWBIO, Beijing, China).
The experiments were performed in triplicate. TK (sc-56967) and β-actin (sc-58673)
primary antibodies were purchased from Santa Cruz Biotechnology. Cleaved caspase
3 primary antibody (Catalog Number: 9661) was purchased from Cell Signaling
Technology.

**Cell proliferation assay**

HL-7702, HepG2 or HuH-7 cells (1×10⁴/well were transfected with pcDNA3.1-
pSurvivin-HSVtk or pcDNA3.1 plasmid, cultured in 96-well plates in triplicate and
treated with different doses of GCV (0, 1, 5, 10, 20, 40, 60, 80, 100 and 150 μg/ml)
for 72 h. On day 4, 20 μl of MTT substrate (BOSTER, Wuhan, China) was added to
each well, and the cells were cultured for 4 h at 37°C and 5% CO₂. Next, the medium
was discarded, and 150 μl of DMSO was added to each well at room temperature for
10 min. The absorbance at 490 nm was measured, and the proliferation rate was
expressed as the cell proliferation percentage, i.e., the ratio of the OD490 of
pcDNA3.1-pSurvivin-HSVtk-transfected cells to the OD490 of pcDNA3.1-
transfected cells. The experiments were performed in triplicate and the averages were
used for express the cell proliferation.

**Clone formation assay**

pcDNA3.1-pSurvivin-HSVtk-transfected or pcDNA3.1-transfected HL-7702, HepG2 and HuH-7 cells were seeded at 300–500 cells per well in 6-well plates containing complete medium in the presence of 150 μg/ml GCV for 14 days. On day 14, cells were fixed with 4% formaldehyde for 15 min and stained with 1% crystal violet (Sigma-Aldrich) before quantification. Individual colonies containing more than 50 cells were microscopically counted. The experiments were performed in triplicate, and the clone formation ratio was calculated using the following formula: clone formation ratio (%) = (number of clones/number of seeded cells) × 100%.

**Xenograft Model of HCC in Nude Mice**

Single-cell suspensions of HepG2 cells at a density of 1.0 × 10^7 cells in 100 μl of PBS were inoculated subcutaneously into the right armpit of BALB/c nude mice, and the mice were randomly divided into three groups (5 mice per group). On the 29th and 31st days after the injection, the tumour-bearing mice were randomly divided into three groups and received Lipofectamine 2000 mixed with pcDNA3.1 plasmid DNA (250 μg/kg body weight, vector group), Lipofectamine 2000 mixed with pcDNA3.1-pSurvivin-HSVtk plasmid DNA (250 μg/kg body weight, HSVtk group) or ApoE-conjugated Lipofectamine 2000 mixed with pcDNA3.1-pSurvivin-HSVtk plasmid
DNA (250 µg/kg body weight, ApoE-HSVtk group) via tail vein injection. On the 32nd day after HepG2 cell injection, the mice in the HSVtk and ApoE-HSVtk injected groups were intraperitoneally injected with GCV (15 µg in 200 µl of 0.9% NaCl). All of the mice were monitored daily for 70 days. In the experimental period, the mice were anaesthetized by ether inhalation and euthanized when they showed obvious signs of suffering (struggling or whining). At the end of the experiment, the tumours were removed, and tumour size and tumour weight were measured with callipers and an electronic balance. Then, the tumour specimens were divided into two parts: one part was used for Western blot analysis, and the other part was used for haematoxylin and eosin (H&E) staining and the \textit{in situ} terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay.

\textbf{Animal survival, tumour volume and tumour weight measurement}

Vernier callipers were used to measure the maximum diameter (a) and minimum diameter (b) of the removed tumours when the mice succumbed to the tumour or were sacrificed. The tumour volume ($V$, mm$^3$) was calculated according to the formula $V = \frac{ab^2}{2}$. The tumour weight was measured, and the tumour growth inhibition rate was calculated using the following formula: tumour growth inhibition rate (%) = (the tumour weight of the control group - the tumour weight of the treatment group) / the tumour weight of the control group $\times$ 100%. The eight mice in each group were
observed for 70 days for survival analyses.

**Serum ALT and AST detection**

Before the allogeneic tumours were removed from mice, blood samples drawn from the eyeground venous plexus and serum were isolated via centrifugation at 3000 rpm for 15 min at 4°C. Serum alanine transaminase (ALT) and aspartate transaminase (AST) were analysed using commercial kits (Catalogue numbers C009-2 and C010-2, Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocols.

**HE staining and TUNEL assay**

Tumour tissue was fixed in 4% formaldehyde for 24 h, embedded in paraffin and then sectioned (5.0 mm). Sections were stained with haematoxylin and eosin for histological analysis. The TUNEL assay was performed with an In Situ Cell Death Detection kit (Solarbio, Beijing, China) according to the manufacturer’s protocols. Five fields of view (200×) were randomly selected on each slide, and the number of total cells and TUNEL-positive cells were counted. The apoptosis rate was calculated based on the formula: the apoptosis rate (%) = the number of TUNEL-positive cells/the number of total cells ×100%.

**Statistical analysis**

Experiments were performed in triplicate, and the data are expressed as the mean ±
standard deviation (SD). Student’s $t$-test was used to compare the differences between
two groups, and a one-way ANOVA in the SPSS Statistics 16.0 package was used for
the statistical analysis of the data from more than two groups. $P < 0.05$ was
considered to indicate a statistically significant difference.

Conflict of Interest

The authors declare no conflict of interest.

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Construction.
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**Figure legends**

Figure 1. Map of the pcDNA3.1-pSurvivin-TK plasmid and plasmid binding assay. (A) Map of the pcDNA3.1-pSurvivin-TK plasmid. (B) The results of Lipofectamine 2000

Figure 2. The transfection efficiency of Lipofectamine 2000 - or ApoE-Lipofectamine 2000 - pGenesil-1 plasmid into HL-7702 (A, B and C), HepG2 (D, E and F), HuH-7 (G, H and I) and SW480 (J, K and L) cells using flow cytometry analysis. Each assay was repeated three times with similar results. *P < 0.05 indicates significant differences between the groups as shown.

Figure 3. TK gene expression in hepatoma cells but not in normal liver cells. (A) The expression of HSVtk in HCC, normal liver cells and SW480 colonic cancer cells was analyzed by RT-PCR. Lane 1-4 were pcDNA3.1 plasmid DNA transfected HL-7702 cells, HepG2 cells, HuH-7 cells and SW480 cells, respectively; Lanes 5-8 were
pcDNA3.1-pSurvivin-TK plasmid DNA transfected HL-7702 cells, HepG2 cells, HuH-7 cells and SW480 cells, respectively. β-actin served as a loading control. Lane M: DNA Ladder. (B) The expression of HSVtk in HCC and normal liver cells was analyzed by Western blotting. Lane 1-4 were pcDNA3.1 plasmid DNA transfected HL-7702 cells, HepG2 cells, HuH-7 cells and SW480 cells, respectively; Lanes 5-8 were pcDNA3.1-pSurvivin-TK plasmid DNA transfected HL-7702 cells, HepG2 cells, HuH-7 cells and SW480 cells, respectively. β-actin served as a loading control. Each assay was repeated three times with similar results.

Figure 4. GCV treatment induces an inhibitory effect on cell proliferation of HSVtk-transfected hepatocellular carcinoma cells. The HSVtk or vector transfected HL-7702, HepG2 and HuH-7 cells were treated with different doses of GCV. The cell proliferation was evaluated using a MTT assay (A) and cell clone formation assay (B). Each assay was repeated three times with similar results. **P < 0.01 indicates significant differences between the groups as shown.

Figure 5. GCV treatment promotes the cell apoptosis of HSVtk-transfected hepatocellular carcinoma cells. The HSVtk or vector transfected HL-7702, HepG2 and HuH-7 cells were treated with 150 μg/ml GCV for 48 h, and apoptosis was
analyzed by flow cytometry (A and B) and Western blot assay (C), respectively. 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.

Figure 6. ApoE-Liposome delivery of HSVtk followed by GCV treatment
synergistically resulted in human liver tumour xenograft growth arrest. (A, B) HSVtk
expression in tumour tissues from the vector, HSVtk and ApoE-HSVtk groups were
measured via a RT-PCR and Western blot assay, respectively. β-actin served as a
loading control. Each assay was repeated three times with similar results. M: DNA
Ladder. (C) Representative photograph of tumour-bearing mice from each group are
shown and the tumour size was illustrated using red circles. (D) Tumour volume was
measured every 7 days after HSVtk first injection. After sacrifice on day 70, tumours
were excised and are shown above. (E) Average weight of the excised tumours. Values
are given as mean ± SEM. *p < 0.05 compared with vector group, #p < 0.05 compared
with HSVtk group, $p < 0.01 compared with vector group.

Figure 7. ApoE-liposome delivery of HSVtk combined with GCV treatment
synergistically increased HCC xenograft tumour cell apoptosis. (A, B) Terminal
transferase dUTP nick-end labeling staining showed tumour cell apoptosis (arrows;
**P < 0.01 indicates significant differences between the groups as shown. (C)

The apoptosis-related protein cleaved caspase 3 in tumour tissues from the vector group, HSVtk group and ApoE-HSVtk group were analyzed using a Western blot assay. β-actin served as a loading control. Each assay was repeated three times with similar results.

Figure 8. ApoE-Liposome delivery of HSVtk followed by GCV treatment synergistically increasing animal survival (A) Liposome delivery of HSVtk significantly prolonged survival of tumour-bearing mice compared to the vector control. ApoE-Liposome delivery of HSVtk produced the longest survival (\(^*P < 0.01\) vs vector, \(^#p < 0.01\) vs HSVtk + GCV, \(^$p < 0.01\) vs vector + GCV). (B and C) The serum ALT, AST and CRP in vector group, HSVtk group and ApoE-HSVtk group were measured before the euthanasia of mice. Error bars indicate the mean value ± SD (n= 5 per group).
Table 1 the changes of body weight during the various treatments

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<th>Vector + GCV n=5</th>
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<th>ApoE-HSVtk-GCV n=5</th>
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<td>Body weight (g)</td>
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</table>

mean ± SEM (g) 29.962 ± 0.318 28.624 ± 0.460 * 26.836 ± 0.472 *$

Data are expressed as the mean ± SEM (for n = 5 per treatment group). Statistical analysis was performed using one-way ANOVA (*p < 0.05 compared with vector group, *p < 0.05 compared with HSVtk group, $p < 0.01 compared with vector group).