Inhibitory Effect of Biosynthetic Nanoscale Peptide *Melittin* on Hepatocellular Carcinoma, Driven by Survivin Promoter

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Hepatic resection and orthotopic liver transplantation are the only potentially curative treatments for hepatocellular carcinoma (HCC) but are indicated only in a minority of patients. Biosynthetic nanoscale peptide *Melittin* (Mel) is postulated to disrupt microbial phospholipid membranes by formation of stable or transient pores. Survivin, a member of the inhibitor of apoptosis family, is transcriptionally upregulated in most malignant tissues but not in normal tissues. It has been reported that the survivin promoter activity is tumor-specific and makes it a good candidate for construction of gene therapy vectors. In the present study, a non-viral vector (pSURV-Mel), encoding Mel gene, was developed to evaluate its anti-tumor effect in HCC cell lines and in vivo in a mouse model of human HCC xenograft tumor. Our results showed that the survivin promoter activity is specifically activated in tumor cells, and the pSURV-Mel plasmid expressed Mel selectively in tumor cells and also induced cytotoxicity. Moreover, intratumoral injection of pSURV-Mel significantly suppressed the growth of xenograft tumors. Mechanistically, pSURV-Mel induced cell death by an apoptosis-dependent pathway. All taken together, this study elucidates a relatively safe, highly effective and cancer specific gene therapy strategy for HCC. The mechanisms of non-viral vector-induced cell death which were revealed by this work will shed light on the construction of more powerful vectors for cancer therapy.

KEYWORDS: Survivin, Melittin, Hepatocellular Carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Hepatic resection and orthotopic liver transplantation are the only potentially curative treatments but are indicated only in a minority of patients.¹ Various alternative ‘sub-radical’ therapies have been introduced in the past decades, where conventional chemotherapy or radiotherapy is ineffective for HCC.²–⁴ The survival rate after the onset of symptoms is generally low, creating an urgent need for new therapeutic strategies for HCC, such as biosynthetic nanoscale antimicrobial peptides (AMPs).

Over the past decades, AMPs have been isolated and characterized. Most antimicrobial peptides have been shown to have antitumoral activity by formation of pores,⁵–⁸ such as barrel-stave pore,⁹,¹⁰ torroidal pore,¹¹ and carpet models.¹² They all differ according to the ways in which AMPs interact within phospholipid bilayers. Pores need not expand substantially because cell death can occur concomitantly as a result of membrane leakage and swelling under osmotic pressure, then AMPs can reach and bind to intracellular targets or disrupt processes that are crucial to cell viability (protein, DNA, or cell wall synthesis).¹³

Bee venom (BV) is a unique weapon in the animal kingdom and also has anti-cancer activities on several types.
of cancer cells, including lung, liver, breast, prostate, and mammary cancer cells, as well as leukemia cells. Mel, the main component of BV is an alkaline polypeptide consisting of 26 amino acids. Mel can induce cell cycle arrest, growth inhibition, and apoptosis in various tumor cells. However, to achieve a similar effect in vivo, Mel would have to be given repeatedly to maintain therapeutic levels, which may be pharmacologically unfavorable. The selective expression of the gene encoding Mel in the targeted cell type may circumvent these problems.

Local/regional gene therapy represents a new and promising therapeutic strategy that relies on the transfer of genetic material into cells in order to generate a beneficial effect against diseases. Gene transfer can be delivered by either direct injection into HCC tumors, or via the hepatic artery, from which HCCs derive the majority of their blood supplies. To facilitate cell transduction, the genetic material is packaged into vectors that can be of viral and non-viral nature. Viral vectors are the most commonly used for the treatment of HCC and have demonstrated high gene transfer efficiency. However, their pathogenicity, immunogenicity and potential of insertional mutagenesis greatly limited their clinical use. Compared with viral vectors, non-viral vectors are almost completely non-immunogenic, relatively safer, less expensive to produce, and can carry a lot of genetic materials.

Tumor-specific expression of a tumor-killing molecule or a nontoxic prodrug converting enzyme by non-viral vectors has emerged as a potentially important approach. Such selective expression can be achieved transcriptionally by placing a tumor-specific promoter or tumor-specific 5'-UTR at the 5' end of the transgene. Survivin is a member of inhibitor of apoptosis (IAP) family, which plays a key role in the regulation of apoptosis and cell division. The most significant feature of survivin is its differential expression in tumor tissues versus normal tissues. Studies have shown that survivin promoter-driven oncolytic adenovirus exhibited tumor-selective cytotoxicity in vitro and in vivo. This makes it a good candidate for constructing the vectors for cancer gene therapy.

In the present study, a recombinant non-viral vector, encoding Mel gene and driven by survivin promoter, was constructed. Its antitumor effect on HCC was also evaluated by in vitro and in vivo experiments.

**MATERIALS AND METHODS**

**Ethics Approval**

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanjing Medical University. All efforts were made to minimize mice suffering. All surgeries were performed under anesthesia with pentobarbital (2 mg/kg). Mice were euthanized with an intraperitoneal (i.p.) injection of 0.05 mg/g sodium pentobarbital.

**Cell Culture**

Human HCC cell lines (HepG2) was obtained from American Type Culture Collection (ATCC) and normal human liver cell lines (L02) was bought from KeyGEN Biotech. Cells were grown in Dulbecco’s modified Eagle’s medium (GIBCO-BRL) plus 10% heat-inactivated fetal bovine serum (FBS) (GIBCO-BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained in a humidified incubator with 5% CO₂ atmosphere at 37 °C.

**Plasmid Construction**

To construct the luciferase expression plasmid, under the control of the survivin promoter (pSURV-Luc), a pair of primers was designed according to DNA sequence of the human survivin promoter gene (nucleotides 1824–2800, GenBank Accession Number U75285). The survivin promoter was then amplified by polymerase chain reaction (PCR), using human bacterial artificial chromosome libraries (ResGen, Inc.) as template. The 977 bp segment of PCR products, by Bgl II and Hind III digestion, was sub cloned into the Bgl II–Hind III site of pGL3-basic vector (Promega). To make the green fluorescent protein (GFP) and Mel constructs, named as pSURV-GFP and pSURV-Mel, the pSURV-Luc luciferase gene was replaced with GFP and Mel gene, respectively by Nco I and Xba I digestion. The gene encoding GFP was amplified by using pEGFP-N1 (Takara) as template. Oligonucleotides (171 bp) encoding two Mel genes (GenBank accession No. X02007) connected with a linker (GGGGS) were artificially synthesized (Fig. 1(A)). Control vector pSURV was constructed by deleting the pSURV-Luc luciferase gene. These plasmids were confirmed by restriction endonuclease analysis (Nco I and Xba I) and further sequencing analysis.

**In Vitro Survivin Promoter Activity**

Cells were plated into 6-well plates at a density of 3 × 10⁵ cells per well and incubated overnight. Cells were then transfected with pSURV-GFP using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection, the pSURV-GFP transfection efficiency in the HepG2 and L02 cells was either directly evaluated under a DM2500 fluorescence microscopy (Leica) or assessed by flow cytometry (FCM) using a FACScan flow cytometer (Becton Dickinson).

**Mel Expression Analysis**

Mel expression was evaluated by real-time RT-PCR on total RNA isolated from pSURV-Mel transfected HepG2 and L02 cells. Briefly, HepG2 and L02 cells were plated in triplicate into 6-well plates at a density of 3 × 10⁵ cells/well and cultured routinely for 24 h. Cells were then transfected with or without pSURV-Mel using Lipofectamine™ 2000. The cells were harvested 48 h later by trypsin digestion. Total cellular RNA was extracted.
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Figure 1. Construction and identification of recombinant vectors. (A) Schematic diagram of GFP and Mel constructs, as driven by survivin promoter. (B) Restriction endonuclease digestion analysis of constructed plasmids, as detected by agarose gel electrophoresis. Lane 1, 2, 3, 4, 5 and 6 represent pSURV, pSURV-GFP, pSURV-Mel, digested-pSURV, digested-pSURV-GFP and digested-pSURV-Mel, respectively. (C) DNA sequencing result of pSURV-Mel.

using Trizol reagent (Invitrogen). The primer pair used for Mel was 5'-GACTGCCCGCTCTGATTCC-3' (sense) and 5'-TTGCTGTCTTTTCTCTTGAGAG-3' (antisense), for β-actin was 5'-TGACGTGGACATCCGCAAAG-3' (sense) and 5'-CTGGAAGGTGGACAGCGAGG-3' (antisense), respectively. The Mel mRNA level was analyzed by one-step real-time reverse transcription polymerase chain reaction (RT-PCR) with RNA-direct™ SYBR Green Real time PCR Master Mix (Toyobo), according to the manufacturer’s instructions. Cycling conditions were: 90 °C for 30 s, 61 °C for 20 min, 95 °C for 60 s, then 40 cycles at 95 °C for 15 s, 60 °C for 1 min. The Mel mRNA level from each sample was normalized to that of the β-actin mRNA. The amplification was monitored on an ABI prism 7500 real-time PCR apparatus (Applied Biosystems).

Cytotoxicity Assay

HepG2 and L02 cells were seeded at 5 × 10⁴ cells per well density in triplicate into 96-well plates. Cells were then transfected, after 24 h, with pSURV-Mel or pSURV, using Lipofectamine™ 2000. The cells in the control group were treated with Lipofectamine™ 2000 only. Cell survival was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma). Briefly, 48 h after transfection, 50 µL of MTT substrate was added into each well and the plates were returned...
to standard tissue incubator conditions for an additional 4 h. The medium was then removed, cells solubilized in 150 μL of dimethyl sulfoxide, and colorimetric analysis performed at 490 nm wavelength using an ELx800 micro plate reader (Bio-Tec Instruments). The percentage of viable cells was calculated as: Viability (%) = 100% × (A − B)/(C − B), where A is the absorbance of pSURV-Mel and pSURV transfected cells and B is the absorbance of blank well. C is the absorbance from control cells cultured in medium without any treatment.

Cytology and Ultra Structure Analysis
HepG2 and L02 cells were plated into a 6-well plate at 3 × 10⁵ cells per well density. 24 h later, cells were transfected with pSURV-Mel or pSURV while the untreated cells were used as control. The change of morphology and ultra structure was observed by phase contrast microscopy (PCM) and transmission electron microscopy (TEM), respectively. After being incubated for 48 h in Dulbecco’s modified Eagle’s medium containing 10% FBS, the cells were examined under a DM IRB phase contrast microscope (Leica). For TEM analysis, cells were fixed with 25 g/L glutaraldehyde in 0.1 mol/L of sodium cacodylate buffer, osmicated with 10 g/L osmium tetroxide, then cell block stained, dehydrated in graded ethanol, infiltrated with propylene oxide, embedded overnight and incubated in a 60 °C oven for 48 h. Silver sections were cut with an Ultracut E microtome, collected on a carbon-coated grid, stained with uranyl acetate and Reynold’s lead citrate, and then examined under a TEM-1200EX TEM (JEOL).

Cell Apoptosis Analysis
For Flow Cytometry (FCM), the annexin-V-fluorescein isothiocyanate (Annexin V-FITC) apoptosis detection kit (Becton Dickinson) was used. Briefly, after transfection with pSURV-Mel, cells were harvested, washed twice with phosphate buffer saline (PBS) and adjusted to a concentration of 5 × 10⁵ cells/mL with PBS. 200 μL of suspensions were added to each labeled tube. 5 μL of annexin V-FITC and 10 μL (20 μg/mL) propidium iodide (PI) were then added into the labeled tube and incubated for 15 min at room temperature in the dark. Cells were then run on a FACScan flow cytometer.

For DNA fragmentation, cells were harvested from the 6-well plate 48 h after transfection and re-suspended in PBS. Genomic DNA was extracted using the QIAamp DNA blood mini kit (Qiagen), according to the manufacturer’s instructions. After precipitation with ethanol, DNA samples were dissolved in Tris-EDTA buffer, separated on 1% agarose gel and visualized by an ultraviolet transilluminator by ethidium bromide staining.

For detection of apoptosis and proliferation signaling, cells were harvested from the 6-well plate 48 h after transfection and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 100 μg/mL Phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100) for 30 min on ice. Cell lysates were then collected after 12,000 rpm centrifugation for 5 min at 4 °C. 20 μg of lysate proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transblotted onto polyvinylidene difluoride membranes (PVDF) membranes (Pall Corporation). Western blots were performed as previously described. Rabbit polyclonal antibodies to cleaved caspase-3 (Cell signaling), poly(ADP-ribose) polymerase (PARP) (Cell signaling) and proliferating cell nuclear antigen (PCNA) (Santa Cruz) were employed for antigen detection, while rabbit polyclonal antibody to GAPDH (Xianzhi) was used as control. Immunodetection was carried out with horseradish peroxidase-coupled secondary antibodies to mouse (Sigma) or rabbit (Santa Cruz) antibodies.

Animal Studies
Female BALB/c nude mice (4–5 weeks of age) were purchased from the Experimental Animal Center of Nanjing Medical University. All procedures were performed according to institutional guidelines and conformed to the National Institutes of Health guideline on the ethical use of animals. Tumor xenografts were established by subcutaneous injections with 1 × 10⁶ HepG2 cells in serum free media (100 μL) mixed with an equal volume of matrigel in the right flank of nude mice. These nude mice were monitored everyday and the tumors were detected every 3 d. These nude mice were used for further study when tumors reached a mean size of 5 mm in diameter (about 10 d after injection).

To demonstrate in vivo survivin promoter activity, three mice were injected intratumorally with 100 μL of the mixture containing 20 μg of pSURV-GFP, pSURV-Mel or pSURV and cationic lipids from 3 directions, respectively. The control mice were injected with PBS. Mice were sacrificed and tumors removed 3 d later. Tissues dissected from tumors injected with pSURV-GFP, pSURV or PBS were embedded with optimum cutting compound (OCT) and prepared in 5-μm-thick sections. These sections were then fixed with 4% paraformaldehyde, stained with 4',6-diamidino-2-phenylindole and visualized using a fluorescence microscope. Mel expression was detected by RT-PCR in total RNA isolated from tissues dissected from tumors injected with pSURV-Mel, pSURV or PBS, using the same primer pairs and procedure as mentioned above.

For detection of apoptosis undergoing in tumor tissue after Mel expression, the Apo-BrdU in situ DNA Fragmentation Assay Kit (BioVision) was used at single cell level, based on labeling of free 3′-OH terminal in DNA strand breaks. Tissues dissected from tumors were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Five-micrometer sections were then prepared. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) staining was performed according to the manufacturer’s instructions and

photographed by a fluorescent microscope. The percentage of apoptotic cells was analyzed by randomly selecting 4 fields.

For determining the in vivo anti-tumor effectiveness of pSURV-Mel, another 24 nude mice bearing the tumor xenografts were randomly divided into three groups (8 animals per group). The mice in the pSURV-Mel and pSURV groups were directly injected with 100 μL of the mixture containing 20 μg of each plasmid and cationic lipids. The injections were given into the growing tumor from 3 directions, twice a week, for 3 weeks. Tumor sizes were measured using sliding calipers every week. The tumor volume was calculated using the following formula: 

\[ V = \frac{1}{2} \times \text{length} \times \text{width}^2 \]

The mice in the PBS group were injected with PBS only. Survival was monitored and plotted against time after plasmid injections.

**Statistical Analysis**

All data were expressed as mean ± SD. All experimental observations were repeated 3 times. Student’s t-test and Analysis of Variance (ANOVA) were used to determine the significance of differences in multiple comparisons. \( P < 0.05 \) was considered significant.

**RESULTS**

**Construction and Characterization of Recombinant Vectors**

As a promising candidate, the survivin promoter has been frequently used for the construction of tumor-specific gene therapy vectors. The non-viral vector pSURV-Mel was designed to be driven by the survivin promoter and harbor the antitumor Mel gene (Fig. 1(A)). By restriction endonuclease digestion and sequencing analysis, it was confirmed that the survivin promoter was successfully cloned into the Sac I–Hind III site of pGL3-basic vector to generate pSURV-luc vector (data not shown). The GFP gene and the artificially synthesized Mel gene (target segments) were then correctly inserted into corresponding site of the pSURV-luc vector, respectively. The sequences of the two inserted fragments in the recombinant vectors were respectively identical to published sequences as proved by restriction endonuclease digestion (Fig. 1(B)) and sequencing (Fig. 1(C)).

**The Survivin Promoter is Active Only in HCC Cells**

The pSURV-GFP plasmid was utilized to determine whether the survivin promoter could function in HCC cells and also to determine its activity specificity. The promoter activity was determined from the GFP expression in conditioned medium from transiently transfected HepG2 cells. As shown in Figure 2(A), the GFP gene, under the control of survivin promoter, displayed high expression level in the HepG2 cells and the GFP-positive HepG2 cells accounted for approximate 36.2% of whole cells, as determined by FCM analysis (Fig. 2(B)). By contrast, GFP expression was not observed in normal liver LO2 cells, a line of normal human hepatic cells.
The survivin promoter could consistently induce abundant expression of Mel gene in HepG2 cells transfected with pSURV-Mel (Fig. 2(C)), whereas the Mel gene expression was barely detected in the pSURV-Mel transfected L02 cells.

Expression of pSURV-Mel Induced Cytotoxicity and Morphological Changes in HepG2 Cells

MTT assay was adopted to evaluate the in vitro cytotoxicity of recombinant non-viral vector pSURV-Mel on HepG2 cells. HepG2 cells showed significant decrease in cellular viability 48 h after pSURV-Mel transfection (about 40% reduction) compared with pSURV-transfected (p < 0.01) and control HepG2 cells (p < 0.01). There was no discernable cytotoxicity from pSURV-Mel transfection on normal L02 cells (Fig. 3(A)). Some HepG2 cells displayed slight morphologic changes (data not shown) 24 h after pSURV-Mel transfection. A large number of HepG2 cells displayed dramatic morphological changes under the light microscopy 48 h later. As shown in Figure 3(B), roundish and serried cells (control group) became polygonal, detached or sparse, and wizened (pSURV-Mel group). The ultrastructural apoptotic characteristics, including chromatin condensation and nuclear fragmentation, were observed by electron microscopy in the pSURV-Mel-transfected group. There were no evident morphological and ultrastructural changes in the HepG2 cells from the other two HepG2 cells groups or in the L02 cells after all treatments (Fig. 3(B)). These findings suggest that expression of pSURV-Mel specifically induces apoptosis in the cancerous HepG2 cells.

Expression of pSURV-Mel Induced Cellular Apoptosis in HepG2 Cells

The above study findings strongly suggest that pSURV-Mel transfection specifically kills tumor cells and the anti-tumor effect might be mediated by the apoptosis pathway. To confirm this, FCM analysis was performed to examine whether apoptosis was induced by pSURV-Mel transfection in the HCC HepG2 cell line and the L02 normal cell line, the untreated cells serving as normal control. The pSURV-Mel transfection induced much more apoptotic and necrotic cells than pSURV-transfection or normal control HepG2 cells (p < 0.01). No significant difference in apoptosis was noted between L02 cells transfected with pSURV-Mel and those with pSURV (Fig. 4(A)). To further investigate whether the basic apoptosis machinery was involved in this process, DNA fragmentation, apoptosis and proliferation signaling were detected. DNA fragmentation is marked by the separation or breaking of DNA strands into pieces. Apoptotic DNA fragmentation is a natural process that cells perform in apoptosis. As shown in Figure 4(B), pSURV-Mel transfection could induce DNA fragmentation in the HepG2 cells. No obvious DNA fragmentation was observed in the normal HepG2 cells or those transfected with pSURV, as well as in the L02 cells. Apoptosis related signaling transducers including caspase-3 and PARP and proliferation related molecules like PCNA were detected by Western-blot analysis. As shown in Figure 4(C), caspase-3 and PARP were all activated, accompanied by obvious induction of the cleaved caspase-3 and PARP, while PCNA expression was inhibited in the pSURV-Mel-transfected HepG2 cells. No obvious changes of cleaved caspase-3, PARP and PCNA expression were observed in the HepG2 and L02 cells from other groups.
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**Figure 4.** pSURV-Mel transfection induced cellular apoptosis in HepG2 cells. (A) Apoptosis in the HepG2 and L02 cells after pSURV-Mel transfection, as detected by FCM. * indicates p value < 0.01. (B) DNA fragmentation in the HepG2 and L02 cells after pSURV-Mel transfection, as detected by agarose gel electrophoresis. (C) Western-blot analysis of apoptosis and proliferation signaling in HepG2 and L02 cells after pSURV-Mel transfection.

**pSURV-Mel Based Gene Therapy Induced Tumor Cell Apoptosis in a Mouse Model of Human HCC Xenograft Tumor**

To determine the survivin promoter activity from the vectors in vivo, pSURV-GFP, pSURV-Mel, pSURV or PBS were intratumorally injected into tumors when tumors reached a mean size of 5 mm in diameter. As shown in Figure 5(A), strong green fluorescence was observed in the frozen sections prepared from pSURV-GFP-injected tumors, proving the high level expression of GFP in vivo under control of the survivin promoter. Mel expression was also demonstrated in the pSURV-Mel-injected tumor by the RT-PCR analysis (Fig. 5(B)). The in situ TUNEL assay was used to assess in vivo apoptosis-related DNA fragmentation. Treatment with pSURV-Mel led to remarkable apoptosis in the tumor tissues compared to pSURV or PBS treated tumors (Fig. 5(C)). As shown in Figure 5(D), pSURV-Mel treatment significantly induced high percentage of TUNEL positive cells compared to pSURV and PBS treatment (p < 0.01).
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Figure 5. pSURV-Mel transfection induced tumor cell apoptosis in a mouse model of human HCC xenograft tumor. (A) In vivo survivin promoter activity in liver tumor xenografts after pSURV-GFP injection, as observed under fluorescence microscopy. (B) RT-PCR analysis of in vivo Mel expression in liver tumor xenografts after pSURV-Mel injection, as detected by agarose gel electrophoresis. (C) In vivo apoptosis in liver tumor xenografts after pSURV-Mel injection as detected by TUNEL assay and quantified % of apoptotic cells from 4 fields. (D) * indicates p value < 0.01.

pSURV-Mel Based Gene Therapy Substantially Reduced Tumor Burden and Improved Survival in a Mouse Model of Human HCC Xenograft Tumor

The in vivo anti-tumor effect was evaluated in a murine model of liver tumor xenografts. pSURV-Mel injection exhibited significant suppression of tumor growth compared with control PBS and pSURV-treated animals (Fig. 6(A)). The average tumor volume after treatment with pSURV-Mel was about 0.38 cm³ at 5 weeks compared with the average tumor volumes of 1.38 and 1.34 cm³ after PBS (p < 0.01) and pSURV treatments, (p < 0.01) respectively, which was in agreement with a partial inhibition of HepG2 human liver tumor growth (Fig. 6(B)). As shown in Figure 6(C), the survival ratio was also significantly improved in pSURV-Mel-treated mice. 7 weeks post-injection of plasmids, 7 of 8 mice in the pSURV-Mel-treated group were still alive, in contrast to 0 of 8 animals in the PBS and pSURV-treated groups. These results suggest that pSURV-Mel has a significant in vivo antitumor effect.

DISCUSSION

The development of HCC is strongly associated with chronic liver disease. Advanced imaging technologies and serum a-fetoprotein (AFP) assays have been used to diagnose HCC at an early stage. However, HCC is a refractory disease for which current treatment modalities are ineffective even in patients for whom HCC could be diagnosed at an early stage. Moreover, clinical observations have shown that tumor recurrence rates are very high in patients with HCC who receive medical or surgical treatments. It is thus, imperative to develop better therapeutic strategies to treat HCC.

The strategies that have been tested to treat HCC in animal models include oncolytic virus, apoptosis-mediated cell death, genetic prodrug activation, inhibition of angiogenesis, siRNA-mediated gene silencing, and genetic
immunotherapy. Most of these strategies were carried out using recombinant adenoviruses because they are efficient vectors for gene transfer. However, the major risk in using these modified adenovectors is a possible recombination with wild-type adenovirus. In addition, the mounted antiviral immune response prevents repeated administrations of the vectors. We report herein a novel strategy that associates an antimicrobial peptide, possessing anticancer activities and using a non-viral delivery system, which may allow repeated administrations.

Survivin was identified by hybridization screening of human genome. Survivin can bind to and inactivate cellular caspases 3 and 7, thus preventing cellular apoptosis. A serial analysis of gene expression (SAGE) analysis found that survivin transcripts are the fourth most frequently overexpressed transcripts in common human cancers when compared to levels in normal cells, suggesting that survivin may be a potential target for cancer therapy. Increased survivin activity is transcriptionally controlled which indicates that the survivin promoter may control transgene expression in a tumor-specific manner. Survivin-mediated oncolytic adenovirus could induce non-apoptotic cell death in lung cancer cells and show antitumoral activity in vivo. In a non-viral vector carrying the BikDD gene, the survivin promoter was found to drive a specific cytotoxic effect on lung cancer growth. In the present study, to assess whether the survivin promoter can selectively drive transgene expression in HCC cells, a non-viral vector was successfully constructed by inserting the survivin promoter into pGL3-basic vector and used to drive GFP expression. Results from the in vitro and in vivo transfection experiments and FCM analysis demonstrate that the survivin promoter is specifically activated only in HCC cells but not in L02 cells, indicating that survivin is a tumor-specific promoter.

A growing number of studies have shown that some of the antimicrobial peptides (e.g., BMAP, Cecropin, Hcap-18) are toxic to bacteria but not to normal mammalian cells and exhibit a broad spectrum of cytotoxic activity against cancer cells. Mel, a nanoscale peptide isolated from honeybee Apis mellifera, is known to disturb a variety of membrane perturbing effects through nanopore formation, such as hemolytic and antimicrobial activity. Mel-based gene therapy for human cancers is also a possibility, as demonstrated by the adenovirus-mediated transfer of the Mel gene under the control of α-fetoprotein promoter to human HCC cells BEL-7402, which resulted into a dramatic in vitro and in vivo inhibition of the HCC cells growth. In this study, two Mel genes, connected with a linker (GGGGS), were cloned into the non-viral vector pSURV, driven by the survivin promoter. The Mel gene obtained efficient in vitro and in vivo expression as demonstrated by RT-PCR, indicating pSURV is a suitable vehicle for Mel expression.

Cellular proliferation and apoptosis in normal tissues maintain a balance. The ability to induce apoptosis is frequently diminished in the majority of human tumors, suggesting that disruption of apoptotic function contributes significantly to the transformation of normal cells into tumor cells. So, inhibiting proliferation by inducing apoptosis in cancer cells is a new therapeutic target for cancer. Our results from the MTT assay showed significant inhibition of proliferation in the pSURV-Mel transfected HepG2 cells as compared to pSURV-transfected and untreated HepG2 cells. Transfection of pSURV-Mel into the HepG2 cells resulted into obvious morphological changes and apoptosis in a large number of HepG2 cells. The apoptosis-related ultrastructural characteristics, including chromatin condensation and nuclear fragmentation, were also observed in the pSURV-Mel-transfected HepG2 cells. We then assessed whether the basic apoptosis driving machinery was involved in this process. FCM analysis showed the proportion of apoptotic HepG2 cells significantly increased after pSURV-Mel transfection. Transfection of pSURV-Mel also induced DNA fragmentation and increased the cleaved caspase-3 and PARP in the HepG2 cells but not in the L02 cells, whereas, the PCNA expression was inhibited in the pSURV-Mel-transfected HepG2 cells. In vivo TUNEL assay also demonstrated remarkable apoptosis-related DNA fragmentation in the pSURV-Mel-treated tumor tissue. These findings suggest that the pSURV-Mel anti-tumor effect may be, at least, partially dependent on apoptosis pathway, since Mel can reach...
and bind to intracellular targets such as protein, DNA, or cell wall syntheses.9

In summary, we constructed a novel non-viral vector pSURV-Mel that encodes Mel, when driven by the survivin promoter, for HCC-specific gene therapy. The pSURV-Mel specifically expressed Mel in tumor cells and killed tumor cells. The basic apoptotic machinery was involved in the tumor cell cytolysis process. Growth of xenograft tumors in nude mice was efficiently suppressed by the proposed pSURV-Mel based gene therapy. However, although the findings from results presented here are promising, a number of problems remain to be solved. First, s.c. tumor models using an HCC cell line may not fully mimic HCCs in human patients. Second, challenges, including transfection efficiency and delivery way for the non-viral vector, need to be resolved before it can be applied clinically. Nevertheless, our study’s findings suggest that the pSURV-Mel based gene therapy may potentially serve as a promising therapeutic strategy for HCC. In last two decades, nanotechnology has found more and more application in biomedical scopes47–79 we are carrying out this research in depth with the help of biomedical nanotechnology.

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