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Suppression of hepatoma tumor growth by systemic administration of the phytotoxin gelonin driven by the survivin promoter

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Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide. However, there is currently no effective therapy strategy in the clinical practice. Recombinant phytotoxin gelonin fused to other factors have been used to treat different cancers. But there have been no reports of gelonin gene therapy. In this study, we have constructed a recombinant plasmid which contained a tumor-specific survivin promoter to drive phytotoxin gelonin (pSur-Gel). And the cytotoxicity effects of pSur-Gel in HCC were also validated both *in vitro* and *in vivo*. The expression level of survivin was detetected in different liver cancer cell lines and nomal liver cell lines by western blot analysis, and a survivin promoter-driven green fluorescent protein (GFP) expression vectors (pSur-GFP) was also tested in liver cancer cell line HepG2 and normal liver cell line LO2. Moreover, phytotoxin gelonin expression experiment and cytotoxicity experiment of pSur-Gel was performed in HepG2 cells and LO2 cells in *vitro*. Furthermore, anti-tumor effect of pSur-Gel against HepG2 xenografts and toxicity of this gene were evaluated in the mice model. Finally, LDH release assay, apoptosis assay and immunoblot analyse LC3 conversion (LC3-I to LC3-II) were tested. We found that the expression of survivin protein was higher in liver cancer cell lines compared with the normal liver cells. Further study showed that the pSur-GFP and pSur-Gel was expressed specially in liver cancer cell other than in normal liver cells. pSur-Gel plasmid could effectively inhibit the proliferation of liver cancer cells (**P*<0.05), and significantly repress the growth of HepG2 xenografts via intravenous *in vivo* (**P*<0.05). Otherwise, compared to cytomegalovirus promoter-driven gelonin expression vectors (pCMV-Gel), no significantly systemic toxicity or organ injuries had been observed in pSur-Gel treated mice. Further studies revealed that the phytotoxin gelonin induced cell death might be mediated by apoptosis and the damage of cell membrane. Taken together, treating hepatocellular carcinoma with the pSur-Gel may be a novel and interesting cancer gene therapy protocol and is worthy of further development for future clinical trials.

Key words: liver cancer, gelonin, survivin promoter, gene therapy

Hepatocellular carcinoma is the third most frequent cause of cancer-related death [1]**.** There are about 600,000 -700,000 patients with liver cancer in the world each year [2] and the incidence of liver cancer is increasing. HCC is often diagnosed at the late stage with the median survival rate of <3 months and the 5-year survival rate is only about 10% [3-5]. At present, there are a variety of treatments for liver cancer [6-8]. Liver transplantation or ablation made the patients 5-year survival rate increased to 50% [1], but donor shortage, tumour size and localisation have greatly limited its application [9-10]. In addition, HCC has poor response to radiotherapy and high resistance to conventional chemotherapy [4, 11]. Therefore, the development of more effective therapeutic tools and strategies is an urgent task for scientists.

Currently toxins have been selected to treat cancers [12-16]. Gelonin obtained from the seeds of Gelonium multiflorum is a kind of ribosome inactivating proteins (RIPs) that interacted with eukaryotic ribosomal 60S subunit to cause an irreversible inactivation, thereby blocking protein synthesis. Previous studies have shown that it does not produce the capillary leak syndrome, but also has high stability and low toxicity [17-18]. Construction of recombinant toxin fused to either growth

factors or single-chain antibodies can target solid tumor cells, tumor vasculature or hematological malignancies. All of these constructs displayed high selectivity and specificity for antigen-bearing target cells and were excellent candidates for clinical trial [19]. Moreover, recombinant gelonin protein has been used to treat cancer [19-21]. As yet, phytotoxin gelonin was rarely found in gene therapy study. A novel therapeutic approach for the treatment of HCC is warranted, which may involve the use of target-specific genes [22-23]. For cancer-specific expression of toxin, a better approach should be to use a cancer-specific promoter to limit the spectrum of cells that express the gene therapy construct [24-25].

Survivin is a new member of the inhibitors of apoptosis (IAP) family. Numerous researches have validated the specificity and utility of survivin promoter for tumor targeting therapy [26]. Survivin is over-expressed in common cancers, but not in normal adult tissues [27-29]. A series of studies confirmed that survivin mRNA was expressed in 12 human hepatoma cell lines, and survivin protein was highly positive in 70% of liver cancers. In addition, there are significant differences in the expression of survivin in hepatocellular carcinoma and adjacent tissues [30-31]. Therefore, survivin promoter is considered to be a hepatoma-specific promoter. It has potent selective transcriptional expression of therapeutic genes in hepatocellular carcinoma [32].

The object of this study was to provide a new means of plant toxins for treatment of cancer. For this purpose, we have developed a survivin promoter driven gelonin construction for gene therapy. The results showed that pSur-Gel could significantly inhibit the proliferation of liver cancer cells, and decreased the tumor volume without significantly systemic toxicity in mice models. This may offer a promising new strategy of plant toxin for cancer therapy.

Materials and methods

Cell lines and animals. LO2, HepG2, SMMC-7721, BEL-7402 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HepG2 was maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). LO2, SMMC-7721, BEL-7402 were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100U/ml penicillin G, 100 ng/ml streptomycin (Invitrogen, Carlsbad, CA, USA). All cells grew at 37°C in a humidified incubator containing 5% CO_2. BALB/c female athymic mice, 4-5 weeks old, were obtained from the Beijing HFK Bio-Technology Co. Ltd (Beijing, China).

Survivin protein expression level analysis in vitro. At a confluence of 75-80%, cells were harvested by trypsin digestion and washed with PBS by centrifugation. The resultant cell pellets were lysed for 30 minutes in RIPA lysis buffer at ice. Whole-cell protein was extracted from the cell pellets and protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific Inc, USA). About 2 μg/μl protein lysate from each sample was diluted in 4×SDS loading buffer, incubated for 5 minutes at boiling water. Samples were separated by 15% SDS-PAGE and transferred to PVDF membranes, membranes were blocked for 2 h in 5% dilute nonfat dry milkin in TBST at 37°C, thereafter, the blots were hybridized with an anti-survivin antibody (Santa Cruz biotechnology, CA, USA) or β-actin (ZSGB-BIO, Beijing, China) and then incubated with relevant secondary antibodies (ZSGB-BIO, Beijing, China), after washing with TBST four times, protein expression was detected by the ECL method (Thermo Fisher Scientific Inc, Hudson, NH, U.S.A.).

Plasmid construction and preparation. The survivin promoter region 1430 bp sequence was synthesized by Genscript China Inc and amplified by PCR using the following primers: forward primer, 5'-AAGGAAAAAAGCGGCCGCAATCCCAGCACTTTGGGAGGCC-3' and reverse primer,5'-AATATATCCGGAATTCTGCCGCCGCCGCCACCTCTG-3'. Subsequently, the survivin promoter region was excised with NotI and EcoRI and subcloned into pCMV-MCS vector cut with the above enzymes to generate the plasmid pSur-MCS vector. The GFP DNA and gelonin DNA fragments were both obtained from the state key Laboratory of biotherapy, Sichuan University, China. GFP fragment was amplified by PCR from pMax-GFP plasmid using primers (forward primer, 5'-CTCGGAATTCATGGTGAGCAAGGGCGAGGAGCTGTT-3'; reverse primer, 5'-CCCAAGCTTTTACTTGTACAGCTCGTCCATGCCGA-3'), and then inserted into the HindIII/EcoRI sites of pCMV-MCS and pSur-MCS to produce pCMV-GFP and pSur-GFP plasmids. To obtain pCMV-Gel and pSur-Gel plasmids, the gelonin fragment was amplified by PCR using primers (forward primer, 5'-TACCGGAATTCATGGGCCTGGACACCGTGAGCTTTAGCACTAAAGGTGCCACTTATATT-3'; reverse primer,5'-CCGCCACCGCCCAAGCTTTTAAGCATAATCTGGAACATCATATGGATATTTAGGATCTTTATCGACG-3') and subsequently inserted into the HindIII/EcoRI sites of pCMV-MCS and pSur-MCS by ligation.

Survivin promoter activities analysis. To detect survivin promoter activities, the fluorescence imaging systems was used. About 2.5×10^5 cells/well were plated in 6-well plates and incubated overnight to reach 50-60% confluency. The cells were transiently transfected with 2 μg of pCMV-GFP or pSur-GFP using liposome (State Key Laboratory of Biotherapy, Sichuan University, Chengdu, China) per well, the mixture was removed after transfection for 4-6h, and cells were incubated with standard conditions for 72h. Then it was observed by a fluorescence microscope observation and selected different horizons (n=5) photography in each well and analyzed of fluorescence imaging data.

Phytotoxin gelonin gene, protein expression. To detect the gene expression of gelonin, 2 μg vehicle, pCMV-Gel and pSur-Gel were transfected into HepG2 cells and LO2 cells respectively. After 48h, total RNA was extracted with Trizol reagent according to the protocol described by the supplier (TakaRa, Dalian,China). cDNA of gelonin was obtained

from the total RNA by reverse transcriptase polymerase chain reaction (RT-PCR) according to the manufacture's instructions (TakaRa, Dalian,China). PCR primers were designed as following: glonin (753 bp) upstream 5'-TATATGGCCTGGACACCGTGAGCTTT-3' and downstream: 5'-GCCGCTTTAGGATCTTTATCGACGAAC-3', β-actin (132 bp): upstream 5'-CCACGAAACTACCTTCAACTCC-3' and downstream 5'-GTGATCTCCTTCTGCATCCTGT-3'. The PCR products were analysed by electrophoresis and sequencing.

To detect the protein expression of gelonin, 2 μg vehicle, pCMV-Gel and pSur-Gel were transfected into HepG2 cells and LO2 cells, separately. Whole-cell protein was extracted from the cell pellets 48 hours later. Western blot analysis was used to detect protein expression of gelonin. The protocols as mentioned above. The primary antibody of gelonin was prepared by us. To prepare anti-Gel serum, New Zealand white rabbits (Laboratory Animal Center of Sichuan University, Chengdu, China) were immunized subcutaneously 3 times with the mixture of prokaryotic expression gelonin protein/complete and incomplete Freund's adjuvant, and then, serum samples were collected and stored at -80°C.

Colony formation assay. To determine the killing effect of pSur-Gel in vitro, the viability of the cells were analyzed by crystal violet staining after transfection. In briefly, 3×10^2 cells/well were seeded on a 6-well plates, and cells were transfected with 5μg vehicle, pCMV-Gel, or pSur-Gel using liposome (n=3), 4-6 hours later, the transfection medium was replaced with the complete medium. After 7 days the cells were soaked with 70% ethanol for 30 minutes and stained with appropriate amount of crystal violet for 2 minutes, washed for 3 times with mili-Q water and air drying, and then counted the clone numbers.

Gene-therapy of liver cancer animal models in vivo. To determine the antitumor effect of pCMV-Gel and pSur-Gel in vivo, 24 female BALB/c athymic nude mice, approximately 4-6 weeks of age and average weight approximately 20 g, were used for subcutaneous xenograft mouse model. All these mice were housed and maintained in a specific pathogen-free environment, mouse were given ad libitum access to food and water in accordance with approved institutional policy and protocol. All animal procedures have been approved by the appropriate institutional review boards. The animals were injected subcutaneously on their dorsal flank with 0.2 ml of hepG2 cells suspension (1×10^7 cells) in DMEM medium without serum and antibiotic. After inoculation for two week, tumor volume reached about 30-50 mm^3, mice were randomized into four groups (n=6). Mice were injected through tail vein with 5 μg pCMV-Gel, pSur-Gel and vehicle mixed with liposome every two days for total 8 times. The NS (normal saline) group was used physiological saline with the same dose of other groups. Tumor size was measured using digital caliper every 3 days, its volume was calculated by the following formula: tumor volume (mm^3) = length (mm) × width $(mm)^2\times1/2$. The animals were sacrificed one day after the last dose treatment.

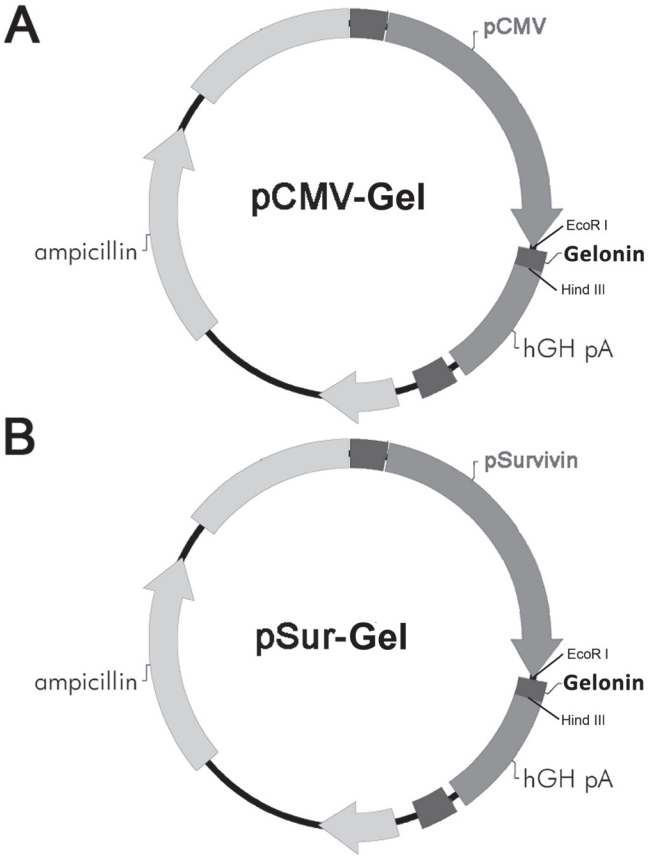

Figure 1. pCMV-Gel and pSur-Gel expression plasmid design
(A) pCMV-MCS is a common cloning vector that contain a CMV promoter. The gelonin fragment was amplified by PCR and subsequently inserted into the HindIII/EcoRI sites of pCMV-MCS to obtain pCMV-Gel. (B) For the cloning of pSur-MCS, 1430 bp survivin promoter gene was amplified and then excised with NotI and EcoRI and subcloned into pCMV-MCS vector to generate the plasmid pSur-MCS vector. The gelonin fragment inserted into the HindIII/EcoRI sites of pSur-MCS to obtain pSue-Gel.

Hematoxylin and eosin (H&E) staining. Liver tissue from tumor-bearing mice carefully dissected to evaluate the pathologic changes. The liver tissues were fixed with 10% neutral formalin, embedded in paraffin, sectioned, and stained with H&E for histopathologic examination. All specimens were evaluated using Olympus B×600 microscope and Spot Fiex camera.

Analysis of acute toxicity. Mice were injected with high dose 50 μg of plasmid/liposomes complex through tail vein administration. Blood was collected for measure at indicated time 48 h after plasmid injection. The concentration of serum alanine transaminase (ALT) was measured by automatic analyzer.

Flow cytometric and western blot analysis of apoptosis and autophagy. LO2, HepG2 cells were collected and stained with Annexin VFITC/PI apoptosis detection kit after transfection at 48h with 2μg vehicle, pCMV-Gel or pSur-Gel. LO2, HepG2

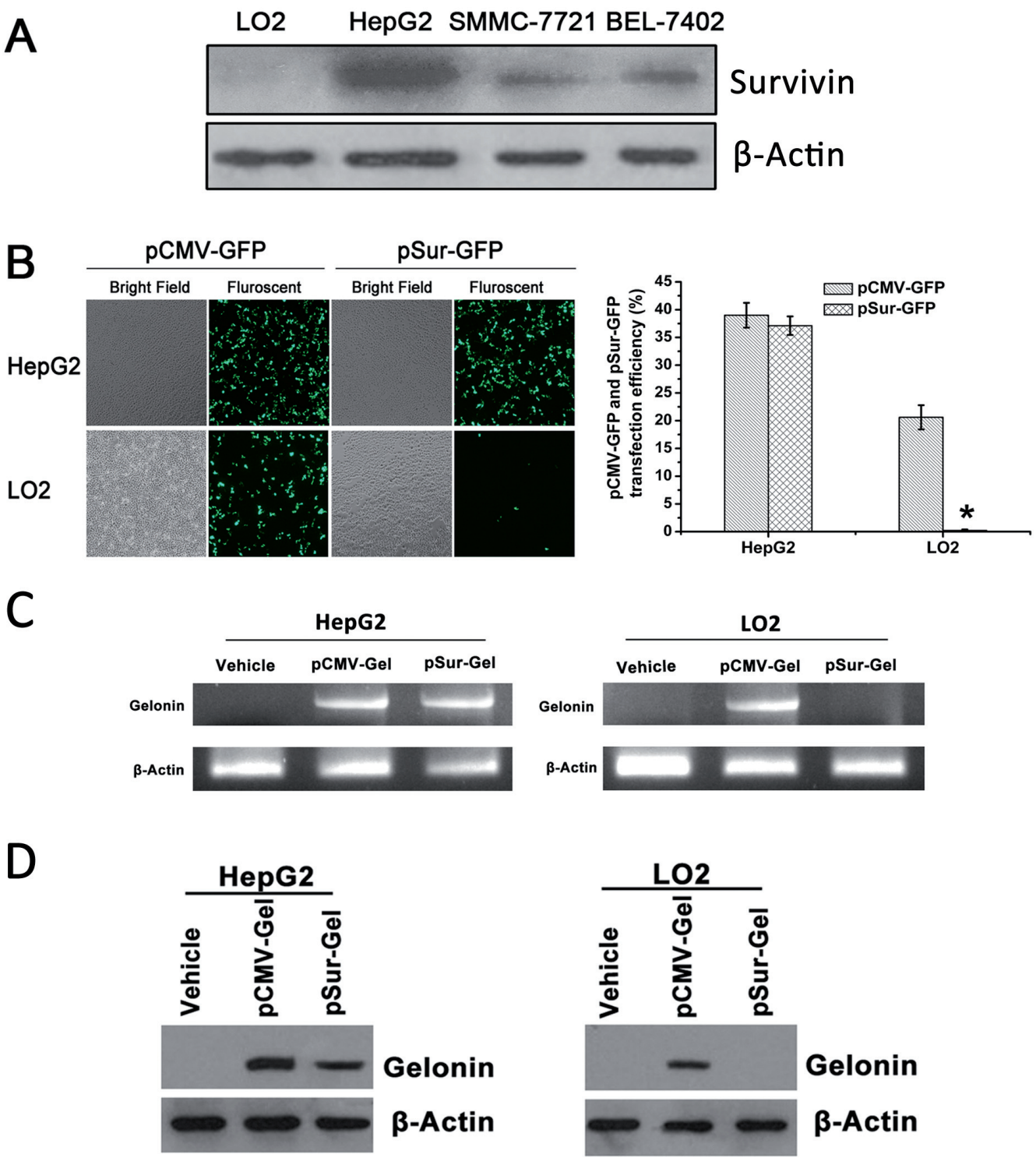

Figure 2. The expression of survivin protein in liver cell lines, and specifical expression of survivin promoter in liver cancer cells.
(A) The expression of survivin protein in human liver cancer cell lines (HepG2, BEL-7402, SMMC-7721) and in normal human liver cell lines (LO2). (B) The activities and specificities of survivin promoters. The pCMV-GFP and pSur-GFP were separately transfected into HepG2 cells and LO2 cells, after 72h, transfected cells were placed under a fluorescence microscope observation, bright field microscopy and fluorescence microscopy images were analysed. the representative images were shown on the left panel, the right histogram represented the transfection efficiency (n = 5, *P<0.05). The data shown here were representative of two independent experiments. (C) RT-PCR analysis of gelonin expression after transfected with pCMV-Gel and pSur-Gel in LO2 and HepG2. β-actin was used as control. (D) Western blot analysis gelonin protein expression in LO2 and HepG2 cells. β-actin was used as control.

cells were trypsinized and gently washed with PBS. The samples (1×10^5 cells) were centrifuged for 5 minutes at 400×g and the supernatant was discarded. The cells were then stained using Annexin V-FITC/PI apoptosis detection kit (KeyGEN Biotech, Nanjing, China) following the manufacturer's instruction. After incubation at room temperature for 15 min, the apoptotic cells were immediately analyzed by flow cytometry.

LO2, HepG2 cells were transfected with 2μg vehicle, pCMV-Gel or pSur-Gel, after 48h, whole-cell protein was extracted from the cell pellets. Western blot was performed to detect poly(ADP-ribose) polymerase (PARP), and microtubule -associated protein 1 light chain 3 beta 2 (LC3-II) expression. The protocols as mentioned above. LC3I/II and PARP antibodies were purchased from Cell Signaling (Danvers, MA, USA).

LDH release assay. Lactate dehydrogenase (LDH) release was measured as a physiological indicator of necrosis. HepG2 and LO2 cells were transfected with 2μg vehicle, pCMV-Gel or pSur-Gel**.** After 48h, LDH release from transfected cells was detected using a LDH cytotoxicity detection kit (Promega, Beijing, China). Maximum LDH release was detected by freeze-thaw lysing the transfected cells. The experiment was repeated three times.

Statistical analysis. All statistical analysis were done with SPSS 16.0 software. Data are presented as mean with s.e.m. Statistical analysis were performed by Student's t test for comparing 2 groups and by ANOVA for multiple group comparisons. *P* values <0.05 were considered statistically significant.

Results

The expression levels of survivin in liver cancer cell lines and normal cell lines. In order to detect the expression level of survivin protein in cells, protein extracts were prepared from various cells. As shown in Figure 2A, an intense 16.5-kDa survivin band was detected in HepG2 cells, a faint survivin band was detected in BEL-7402 cells and SMMC-7721 cells by western blot, but no protein traces in LO2 cells. We suspected that survivin protein was over-expressed in hepatoma cells, but not in normal liver cells.

Selective activity of survivin promoter in the cell lines. To evaluate the activity of the survivin promoter in liver cancer cells and a normal liver cells in vitro. HepG2 cells and normal hepatocyte LO2 cells were transfected with pCMV-GFP or pSur-GFP. The GFP gene transcription was activated by survivin promoter or the commonly used CMV promoter, then we compared the expression lever of GFP in the transfected cells. As shown in Figure 2B, we found that the survivin promoter activity was remarkably high in HepG2 cancer cell lines, but it remained inactive in LO2 normal cell lines by green fluorescence protein reporter assay. Contrary to the survivin promoter, the CMV promoter showed both high activity in HepG2 cells and LO2 cells. Based on the above results, the survivin promoter displayed desirable features of both liver cancer-specificity and high basal activity features in vitro. The results suggested that the survivin promoter would be an excellent candidate as an HCC-specific promoter to express specific genes and applied to gene therapy.

Survivin promoter selectively driven phytotoxin gelonin expression. In order to detect the specific expression of phytotoxin gelonin genes driven by survivin promoter in HepG2 and LO2 cells, total RNA was isolated respectively and then were analyzed by RT-PCR as described in experimental procedures. As a result, the CMV promoter driven gelonin was detected in both of the HepG2 and LO2 cells, but the survivin promoter driven gelonin was expressed only in HepG2 other than LO2 cells (Figure 2C). Similar results were obtained by western blot analysis in Figure 2D.

Specifically kill cancer cells in vitro. To directly measure the selective inhibitory effect of pSur-Gel on growth of cancer cells, we performed cell viability assays in a panel of HepG2 or LO2. As mentioned above, vehicle, pCMV-Gel and pSur-Gel were separately transfected into the cells. Our results showed that HepG2 cells were much more sensitive to pCMV-Gel and pSur-Gel. The cytotoxic effect was minimal but clearly observed at 7 days after transfection. Survivin promoter exhibited low activity in LO2 cells, but the clone numbers of LO2 cells transfected with pCMV-Gel were less than pSur-Gel and vehicle (Figure 3). These results indicated that pCMV-Gel induced more prominent cytotoxicity than pSur-Gel in LO2 cells. Although the toxicity of them was significantly higher than vehicle in the HepG2 cells, we did not observe any significant differences between pSur-Gel and pCMV-Gel (Figure 3). These data consistently indicated that the survivin promoter can drive gelonin expression to inhibit growth of liver cancer cell lines but had no effect on LO2 normal liver cell lines.

pSur-Gel inhibits tumor growth in tumor xenografts. To further evaluate the feasibility and efficacy of gene therapy for hepatocellular carcinoma, tumor-bearing BALB/c nude mice were treated with different liposome/DNA complex. Mice were sacrificed at 24h after the last dose, the tumors were dissected and photographed. The volume of the macroscopic tumor mass was assessed and more significant inhibition of tumor growth was observed in the pCMV-Gel and pSur-Gel group than the vehicle and NS (normal saline) group. But the anti-tumor effects of pCMV-Gel and pSur-Gel were almost the same (Figure 4A-C). These results suggested that the pSur-Gel had an anti-tumor effect as better as pCMV-Gel.

pSur-Gel administration exerts no systemic acute toxicity in mice. An ideal gene therapy system can not only provide good therapeutic efficacy, but also retains their security with a very low or no toxicity. With this conception, analyses of serum ALT levels to assessed systemic toxic effect of pSur-Gel and pCMV-Gel in BALB/c nude mice. In Figure 5, we detected that serum ALT levels were higher ($P<0.05$) in the pCMV-Gel group after plasmid injection for 48h than other groups. The results indicated that systemic administration of pSur-Gel, compared with pCMV-Gel, had virtually no acute toxicity in normal mice (Figure 5A). The acute liver toxicity in pCMV-Gel group might be caused by gelonin non-selective expression. In summary, we concluded that pSur-Gel/liposome-mediated systemic gene

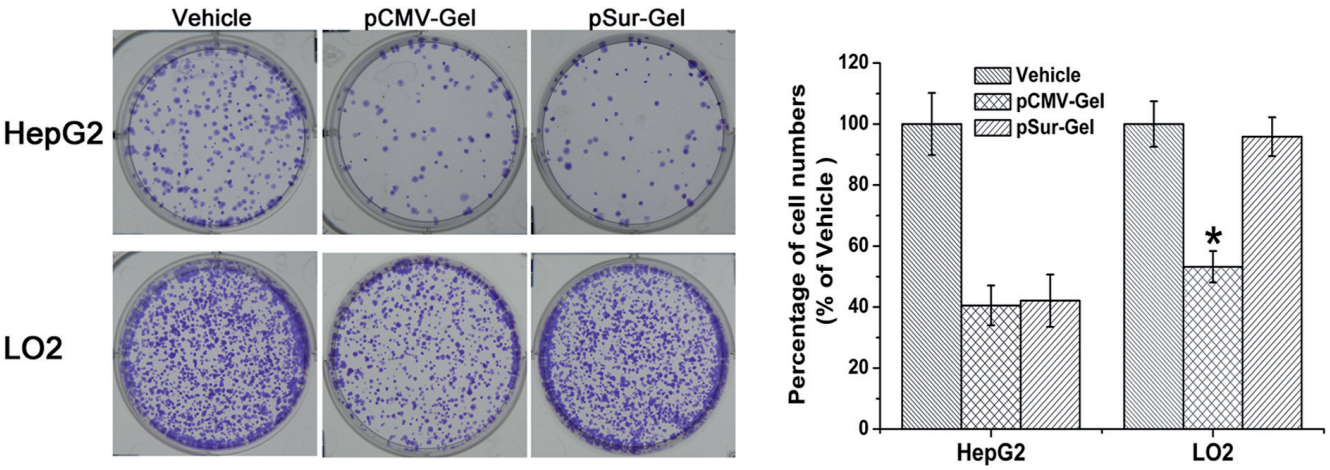

Figure 3. pSur-Gel mediated specifical tumor killing in vitro.
Survivin promoter mediated therapeutic gene gelonin effectively inhibited liver cancer cells proliferation in clone formation assay. HepG2 and LO2 cells were transfected with pCMV-Gel, pSur-Gel and vehicle as control. Clones were stained using crystal violet. The representative images were shown on the left panel. The clone numbers were quantified and illustrated as mean with s.e.m.in the right panel, (n = 3, *P<0.05). The data shown here were representative of two independent experiments.

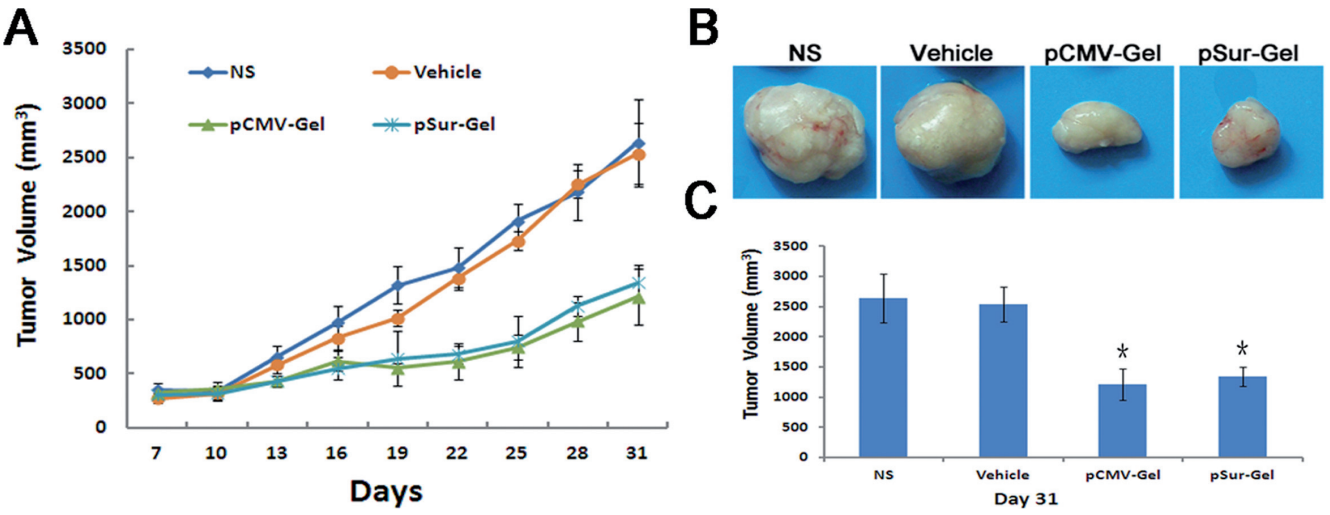

Figure 4. pSur-Gel can inhibit tumor growth in mouse model and have similar effect to pCMV-Gel.
(A) Nude Balb/c mice were injected with 1× 10^7 HepG2 cells subcutaneously in the flank on day 0. When the tumors volume reached about 30-50 mm^3, mouse were intravenously injected with 5 μg vehicle, pCMV-Gel and pSur-Gel plasmid coating with liposome every two days. The normal control group received the same amount of normal saline instead of plasmid. Tumor volumes were measured every 3 days for 4 weeks. One day after the last treatment, all of the mice were sacrificed and the tumors were separated and photographed. (B) Representive images of tumors from each group. (C) The data were represented as mean with s.e.m. (n = 6, *P<0.05).

therapy provided an effective and safe therapeutic approach in liver cancer animal models, as well as provided a scientific basis for consideration of future clinical trials.

Histopathologic examination. Liver tissues were collected on the one day after the last treatment. The tissues were embedded in paraffin, and stained with Hematoxylin eosin. It is obvious that large areas of liver tissue in the pCMV-Gel group showed blood sinus dilation and cell atrophy. But in contrast, no pathological change was observed in the groups subjected to pSur-Gel, vehicle or NS therapy (Figure 5B).

Mechanisms of cytotoxic effects. To investigate the mechanisms of cytotoxic effects mediated by pCMV-Gel and pSur-Gel, the studies related to apoptosis, autophagy and necrosis were performed.

Annexin V-FITC/PI staining and flow cytometric measurement were used to quantify the apoptotic effect after

transfected with 2μg vehicle, pCMV-Gel, pSur-Gel for 48h. As shown in figure 6A, the results indicated that there were both very few PI-positive and Annexin-V-positive cells in the HepG2 and LO2 cells after transfected with vehicle. pCMV-Gel caused more apoptosis in both HepG2 and LO2 cells, the apoptotic percentage were 17.88% and 12.68% respectively. However, pSur-Gel can increase more apoptosis in HepG2 (about 11.09%), but cause lower apoptosis in LO2 (about 5.70%). Then, we detected the cleavage of the caspase substrate PARP by western blot. As shown in figure 6B, the PARP was obviously cleaved in HepG2 transfected with pCMV-Gel or pSur-Gel, and in LO2 transfected with pCMV-Gel. These results further confirmed the cytotoxic effects of gelonin might be induced via cell appoptosis. Besides, the results from autophagy analysis showed that the ratio of LC3-II formation was not increased (Figure 6C).

What's more, to assess whether the cell necrosis was induced, we examined lactate dehydrogenase (LDH) release. The results showed that the percentage of LDH release were significantly increased in HepG2 cells after transfected with pCMV-Gel or pSur-Gel, and in LO2 cells after transfected with pCMV-Gel compared with vehicle (Figure 6D).

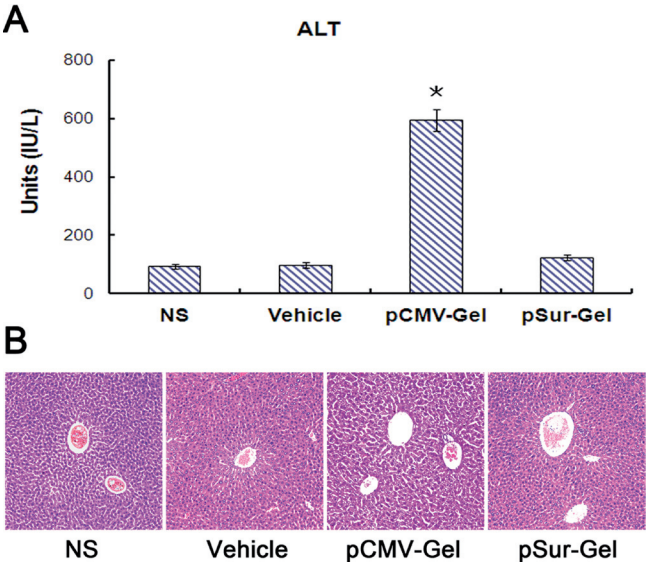

Figure 5. pSur-Gel administration exerted no obviously systemically toxicity compared with pCMV-Gel in mice.
(A) The 8 weeks old of BALB/c mice were received a single dose of normal saline (NS) and liposome delivery 50 μg vehicle, pCMV-Gel or pSur-Gel by tail vein injection. Blood was collected at indicated time and serum was separated for further analysis of the levels of alanine aminotransferase (ALT). The data were represented as mean with s.e.m. (n=6, *P<0.05). (B) To evaluate the pathological changes of pSur-Gel treated mice, liver tissues were stained with H&E. Original magnification×200.

Discussion

Hepatocellular carcinoma is a highly aggressive and metastatic disease. Unfortunately, until recently there has been no promising therapy for it. Gelonin is a plant toxin that can make the ribosome inactive and mostly is used for recombinant protein to tumor therapy. An immunotoxin of anti-EGF receptor single-chain antibody fusion gelonin can inhibit EGF receptor-positive cancer cells proliferation *in vitro* [33]. We once constructed similar immunotoxin that can effectively inhibited tumor growth in mouse non-small cell lung cancer models and reflected the anti-tumor activity[18]. Recently MA *et.al* [19] focused on some studies of fusion constructs composed of gelonin toxin recombinant either growth factors or single-chain antibodies can target solid tumor cells, tumor vasculature or hematological malignancies and were excellent clinical trial candidates.

Unfortunately, immunogenicity, vascular leak syndrome, toxicity to nontumor tissue and poor penetration into solid tumors were the major challenges of immunotoxin treatment, which limited the therapy efficacy in clinical trails [34-36]. Targeted gene therapies which use toxin to fight cancer might be a good strategy. Polymeric nanoparticles to deliver DT-A encoding DNA combined with transcriptional regulation to target gene expression to ovarian tumor cells, this strategy suppressed tumor growth more effectively than treatment with clinically relevant doses of cisplatin and paclitaxel [37]. However, no one take phytotoxin for gene therapy before. Organization or cancer specific promoter could regulate gene expression that is a very attractive strategy and has been extensively tested. In previous studies, Xu *et. al* [38] constructed a novel adenovirus vector Ad.SPDD containing survivin promoter, which mediated targeted gene hepatocellular carcinoma suppressor 1 expression to specifically induce apoptosis in cancer cells, enabling them to significantly improve the safety and efficacy of oncolytic-mediated gene therapy for liver cancer. In this experiment, we developed a tumor-specific survivin promoter-driven phytotoxin protein expression vector for systemic liver cancer gene therapy. Our data demonstrated the superiority of our engineered pSur-Gel system had nearly no toxicity in normal tissues, but could effectively inhibited tumor growth [37].

Consequently, it is shown that the specific promoter was joined to extend the application of the toxin. CMV as a common promoter was often used as a gene therapy. It can mediate gene expression in both normal tissue and cancer tissue [39]. The survivin promoter was superior to other promoters, additionally it exhibited very low activities in major organs [40]. It mediated genes targeted therapy that had been detected in breast cancer [26], lung cancer [41-42] , gastric cancer [43] and glioma [40].

So far, the studies of survivin promoter for liver cancer therapy were very rarely. In this study, we found that survivin protein showed high expression in liver cancer cells especially in the HepG2 cells, but no expression or low expression in LO2 cells (Figure 2A). This suggested that the survivin promoter may be able to apply to treatment of liver cancer. We found that the CMV promoter can induce overexpression of GFP in both

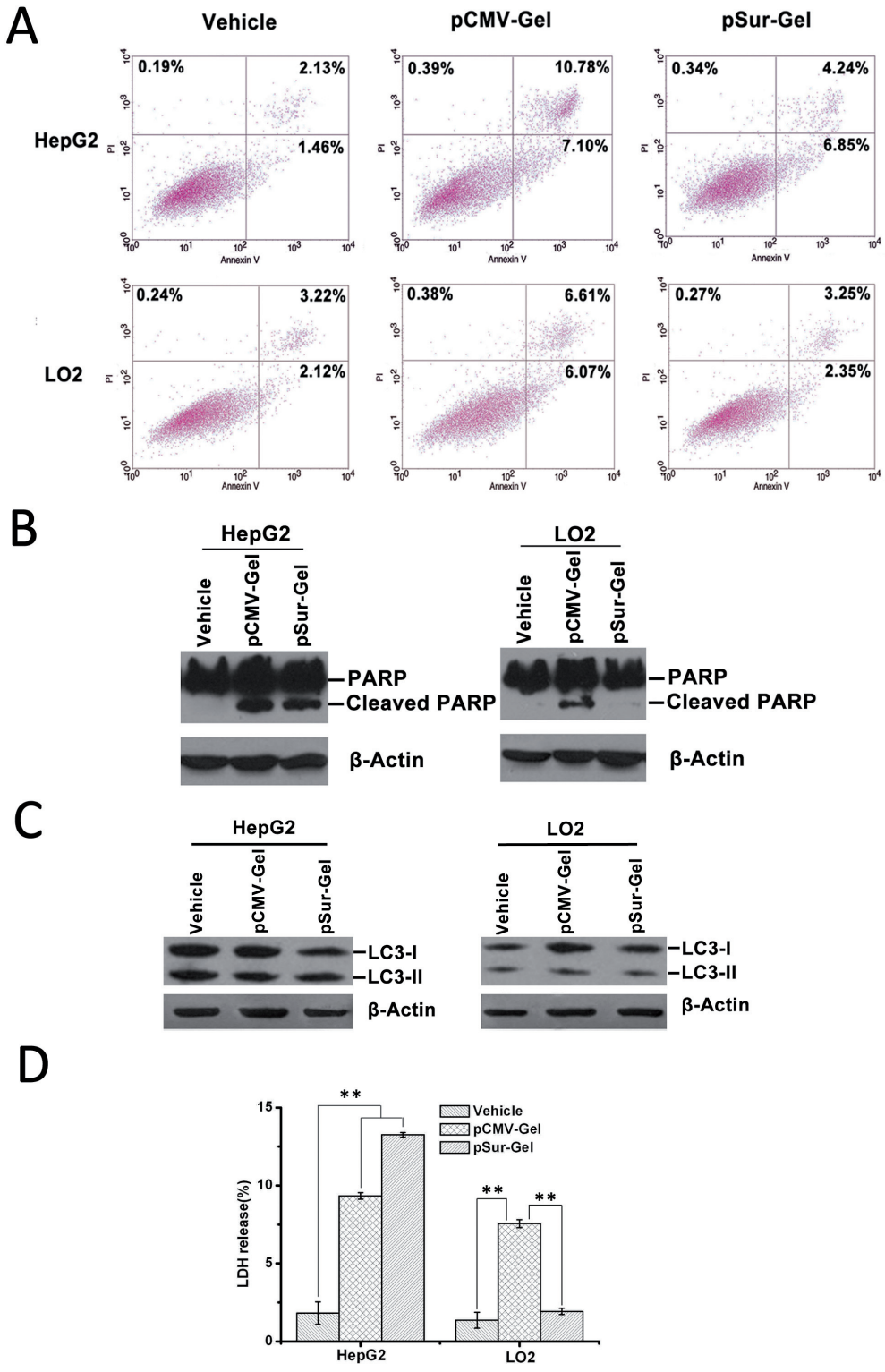

Figure 6. Cytotoxicity mechanism of pCMV-Gel and pSur-Gel.
(A) After HepG2 and LO2 cells were transfected with 2 μg vehicle, pCMV-Gel or pSur-Gel for 48 h. The apoptosis was quantified by flow cytometric analysis of Annexin V-FITC and PI staining. Cells in the lower right quadrant indicate Annexin-positive, early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI-positive, late apoptotic cells. (B) Western blot analysis of PARP cleavage in HepG2 and LO2 cells after transfected for 48 h. (C) Western blot analysis of LC3-II in HepG2 and LO2 cells after transfected for 48 h. (D) After HepG2 and LO2 cells were transfected for 48h. Cytotoxicity was then determined by the LDH release assay. The data were represented as mean with s.e.m. (n = 3, **P<0.01)

HepG2 and LO2 cells transfected with pCMV-GFP. However, the survivin promoter showed a good selective activity, GFP was overexpressed only in HepG2 cells (Figure 2B). Further experiments showed that survivin promoter can induce the gelonin expression in HepG2 cells (Figure 2C-D). The proliferation of HepG2 cells was inhibited after transfected with pSur-Gel, whereas, pSur-Gel almost had no effect on LO2 cells (Figure 3). The pCMV-Gel obviously inhibited the growth of HepG2 and LO2 cells (Figure 3). In vitro experiments showed survivin promoter had a good selectivity and promoted the phytotoxin gene specifically expression to inhibit the growth of hepatoma cell. In vivo experimental, data showed that promoter mediated gelonin gene can inhibit the growth of liver tumor, similar results were observed in the pCMV-Gel and pSur-Gel group (Figure 4A-C). However, liver pathologic changes were found only in pCMV-Gel treatment group by H&E staining (Figure 5B). Toxin-induced liver damage includes vascular damage, induction of liver tumors and development of liver cirrhosis [44]. These pathological phenomenas may be due to the toxin-induced vascular damage, hepatic sinusoid congestion leading to cellular hypoxia, resulting in liver cell atrophy. A small dose of the medication was injected and the low toxicity of the plant toxin gelonin may be the reasons why we have not found liver cell necrosis in pCMV-Gel group. In acute toxicity assessment test, we found that the ALT was obvious increased after injected high dose of the pCMV-Gel (Figure 5A), it may cause damage or necrosis of hepatocytes or other certain tissues. But the pSur-Gel group had nearly no changes, which was confirmed its security again.

Although gelonin toxins have been used to treat malignant cancers many years, studies using different cell types have shown distinct mechanisms underlying the induction of cell death [45-47]. Also the mechanism of targeted gelonin gene therapy has no detailed reports. In this study, we clearly showed that pCMV-Gel and pSur-Gel could induce cell apoptosis and necrosis. Then, the flow cytometric analysis revealed that the inhibitory effect of gelonin maybe partly benefit from apoptosis (Figure 6A). In addition, the results of western blot analysis indicated that the cleaved caspase substrate PARP were increased in HepG2 and LO2 cells after transfected with pCMV-Gel or pSur-Gel (Figure 6B). However, the autophage marker LC3-II had no changes (Figure 6C). Subsequently, to assess whether the necrotic cell death was induced, we examined lactate LDH release, which is a marker of abrupt membrane lysis [46].The results showed that pCMV-Gel or pSur-Gel significantly increaseed the LDH release in HepG2 cells, and pCMV-Gel also significantly increased the LDH release in LO2 cells, but low in LO2 cells treated with pSur-Gel (Figure 6C), which indicated that the observed cytotoxicity may be also a result of necrosis.

In summary, we have designed a new cancer therapy strategy. Using constitutively active phytotoxin gelonin with tumor-specific survivin promoter can provide a safe and effective gene therapy systerm that can be adapted for other phytotoxins. That might offer a promising new tactic of plant toxins for the cancer therapy.

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