

Mannan-modified Ad5-PTEN treatment combined with docetaxel improves the therapeutic effect in H22 tumor-bearing mice

Zhongbing Liu
Jinwei Li
Juan Li
Juan Huang
Famin Ke
Qiaona Qi
Xing Jiang
Zhirong Zhong

Luzhou Medical College, Luzhou,
Sichuan, People's Republic of China

Background: It has been reported that the tumor suppressor gene, PTEN, which is inactivated in many malignant tumors, plays an important role in apoptosis, cell cycle arrest, cell migration, and cell spread. For cancer gene therapy, one of the most important problems is low gene transfection efficiency.

Methods: In the present study, to take full advantage of adenovirus in gene expression, we prepared mannan-modified recombinant adenovirus using the PTEN gene (Man-Ad5-PTEN) and investigated the effect of Man-Ad5-PTEN combined with docetaxel (Man-Ad5-PTEN-docetaxel) on tumor growth in a murine model of hepatocellular carcinoma.

Results: Man-Ad5-PTEN effectively suppressed tumor growth and induced significant apoptosis of murine H22 hepatoma in vivo. Apoptosis levels in tumor-bearing mice treated with Man-Ad5-PTEN-docetaxel were significantly higher than those in tumor-bearing mice treated with naked Ad5-PTEN, Man-Ad5-PTEN, or docetaxel alone. Treatment with Man-Ad5-PTEN-docetaxel resulted in a significant inhibitory effect in this tumor model. Compared with the controls treated with phosphate-buffered solution, the tumor inhibition rate with naked Ad5-PTEN, docetaxel, Man-Ad5-PTEN, and Man-Ad5-PTEN-docetaxel was 48.69%, 49.98%, 75.88%, and 96.93%, respectively.

Conclusion: These results suggest that combined treatment with Man-Ad5-PTEN and other chemotherapeutic agents may be a potent adjuvant therapeutic approach for the treatment of hepatocellular carcinoma.

Keywords: cancer gene therapy, adenovirus vector, PTEN gene, mannan, docetaxel

Introduction

Viral and nonviral methods of gene delivery have been used in clinical trials, in which viral vectors such as retroviruses, adenoassociated viruses, herpes simplex viruses, and adenoviruses¹ are the most promising vectors for high transfection efficiency. Among these viral vectors, the replication-deficient adenovirus has become one of the most promising gene delivery vectors due to its many advantages, including being easily produced in high titers, an ability to transfect nonproliferating cells readily, no integration into the host genome, and broad tissue or cell tropism. However, the broad host range of the adenovirus is also a disadvantage and can lead to lack of tissue specificity and poor safety. Therefore, it is necessary to improve this therapeutic strategy by improving the cancer-targeting ability of the adenovirus vector. Previous studies have developed strategies using heterologous retargeting complexes, such as chimeric fusion proteins and bispecific antibodies, or genetic capsid modification.²⁻⁵

Correspondence: Zhirong Zhong
Luzhou Medical College, Luzhou, Sichuan
646000, People's Republic
of China
Tel +86 083 0316 2291
Fax +86 083 0316 2291
Email zhongzhirong@126.com

Hepatocellular carcinoma is an aggressive and rapidly fatal malignancy of the liver and is the third leading cause of cancer-related death worldwide. Conventional treatment for hepatocellular carcinoma includes surgery, radiotherapy, and chemotherapy. Although remarkable advances have been made in the clinical study of hepatocellular carcinoma, only a definitive subset of cases is cured, and the overall dismal outcome of patients with hepatocellular carcinoma has not changed, so it is essential that we find a more effective way to treat this disease. Gene therapy represents an exciting biotechnological advance that may revolutionize conventional cancer treatment, and combination treatment using gene therapy and chemotherapy is being paid much attention.

The phosphatase and tensin homolog deleted on chromosome ten (PTEN) gene is a tumor-suppressor gene located on human chromosome 10q23.3, originally identified by two research groups in 1997.^{6,7} Since then, a significant number of PTEN deletions and mutations have been reported in many malignant tumors, including glioblastoma, prostate cancer, melanoma, endometrial carcinoma, lung carcinoma,⁸ and hepatocellular carcinoma.⁹ PTEN has been reported to play a role in apoptosis, cell cycle arrest, proliferation, cell migration, angiogenesis, and overall survival.^{10–13} Overexpression of PTEN in cancer cells carrying mutant-type or deletion-type PTEN can inhibit cell proliferation and tumorigenicity via induction of cell cycle arrest at the G1 phase and apoptosis.^{14–16} Loss of PTEN expression has been reported to be strongly correlated with aggressive tumor behavior, eg, loss of nuclear PTEN expression was found to be associated with liver metastasis.^{17,18} Restoring PTEN expression in PTEN-deficient tumor cells has been shown to increase radiosensitivity *in vivo*.¹⁹ Furthermore, previous studies have shown that PTEN is involved in drug resistance in several types of cancer,^{20,21} and cell lines with a high expression of PTEN are sensitive to chemotherapy with 5-fluorouracil and oxaliplatin.¹⁸ Therefore, in this work, we investigated whether the PTEN gene transfected by adenovirus could effectively suppress hepatocellular carcinoma *in vivo*.

On the other hand, among those targeting strategies mentioned above, conjugating the ligand of the receptor to vectors of interest is an attractive approach. Moreover, it was reported that there is considerably high expression of the mannose receptor in liver nonparenchymal cells and endothelial sinusoidal cells, and that mannose receptors are involved in the binding and subsequent endocytosis of mannose-containing glycoproteins to and into cells.^{22–24} One study showed that plasmid DNA complexed with

mannosylated liposomes had high transfection activity due to recognition by mannose receptors both *in vitro* and *in vivo*.²⁵ Another study showed that conjugation of mannan with a recombinant antigen, mucin-1, gives rise to a high cellular immune response, leading to total resistance of experimental tumors in mice.²⁶

Putting all this information together, in the present study, to test whether the combination strategy of cancer gene therapy and chemotherapy could enhance antitumor effects, conjugates of mannan-modified Ad5-PTEN (Man-Ad5-PTEN) were prepared and used to treat H22 tumor-bearing mice, together with docetaxel, a well known chemotherapeutic agent.²⁷ After treatment, tumor volume and weight, along with apoptosis level, were investigated.

Materials and methods

Mice and cell culture

Male Kunming mice, aged 6–7 weeks, were purchased from the Laboratory Animal Center of Luzhou Medical College, Luzhou, China. The study was approved by the animal ethics committee of Luzhou Medical College, and all procedures with the animals were conducted according to the guidelines of the local animal use and care committees of Luzhou and performed according to the National Animal Welfare Law of China.

Murine H22 hepatoma cells (syngeneic with the Kunming mouse strain)²⁸ and human embryonic kidney (HEK) 293 cells were sourced from the Chinese Academy of Sciences, Shanghai, China, and maintained in our laboratory. The H22 and HEK 293 cells were, respectively, cultured in RPMI 1640 medium and Dulbecco's Modified Eagle's medium (Hyclone Corporation, Logan, UH) supplemented with 10% fetal bovine serum (Hyclone Corporation), 100 units/mL penicillin, and 100 µg/mL streptomycin, and cultured at 37°C in air containing 5% CO₂.

Adenoviral vectors

Replication-deficient human type 5 adenovirus with the expressing reporter gene for β-galactosidase (Ad5-LacZ) was purchased from the Vector Gene Technology Company Limited (Beijing, China), and another adenovirus carrying the PTEN gene (Ad5-PTEN) was a gift from the National Key Laboratory of Biotherapy, Sichuan University, China. Ad5-PTEN was constructed according to a report published elsewhere.²⁹ Briefly, it was constructed by subcloning full-length PTEN cDNA from a plasmid construct (pCDNA3-PTEN) into a shuttle plasmid containing CMV promoter and SV40 poly (A) elements. The resulted PTEN-containing

plasmid was cotransfected with pJM17 into HEK 293 cells to obtain the Ad-PTEN virus. The resulting Ad5-PTEN virus was propagated in HEK 293 cells and purified by two rounds of CsCl (Amresco Inc, Solon, OH) density gradient centrifugation. The genome copy number of adenovirus stock was determined by Taqman real-time polymerase chain reaction, and the adenovirus titer was detected using a plaque-forming assay on HEK 293 cells.

Mannan and adenovirus conjugation

Mannan was conjugated to adenovirus in the following manner. Briefly, mannan (Sigma, St Louis, MO), at 25 mg/mL in 0.1 M phosphate buffer (pH 6.0) optimized by periodic acid-Schiff (PAS) stain, was oxidized with sodium periodate (0.01 M, Sigma) for 60 minutes at 4°C. The mixture was desalted by dialysis at 4°C for 5 hours against bicarbonate buffer (pH 9.0) and replaced with fresh buffer every other 30 minutes. The resulting oxidized mannan was mixed with Ad5-PTEN, incubated overnight at room temperature, and used without further purification.

In vivo animal tumor model experiment

H22 tumor cells were obtained for inoculation from in vivo passage. Briefly, the stocked murine hepatoma H22 cells were first subcultured in RPMI 1640 containing 10% fetal bovine serum and collected in blank RPMI 1640 culture medium (1×10^8 /mL). About 0.2 mL of preformed cell suspension (including 2×10^7 cells) was injected into the abdominal cavity of the mouse. After 7 days, the mouse had developed an ascites tumor and was sacrificed by nitrogen inhalation and disinfected by immersing the body in 75% ethanol. Intraperitoneal tumor cells were collected, washed twice, and resuspended in phosphate-buffered solution. Viable cells were counted by Trypan blue staining; next, 1×10^7 murine H22 hepatoma cells in 0.1 mL of phosphate-buffered saline were injected subcutaneously into the right flank of each mouse on day 0. All tumor-bearing mice were divided randomly into five groups (at least eight mice per group), and the treatments were carried out on day 6 when the tumors had grown to 100–150 mm³. At that time, 100 μ L of solution were injected intratumorally into each mouse from each group. One of the five groups was selected to be injected with phosphate-buffered saline as the control group, and the others were treated with naked Ad5-PTEN, docetaxel (Credit Pharma Co Ltd, Chengdu, China), Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel, in which the particle number of Ad5 was 1×10^9 and the amount of docetaxel was about 8 mg/kg body weight.³⁰

All the mice were monitored daily after treatment for tumor size. The tumor measurements were converted to tumor volume (V) by the formula ($L \times W^2 \times 0.52$) and expressed as the mean \pm standard deviation where L and W are the length and width, respectively. The tumors were measured with a vernier caliper every day until day 19 when the mice treated with phosphate-buffered saline began to die, and then all the mice were sacrificed by nitrogen inhalation. The tumor burden of each mouse was weighed.

H&E staining and TUNEL assay

After being treated for 2 weeks, the tumor-bearing mice were sacrificed and the tumor burdens from each mouse were weighed and immediately fixed in 4% buffered paraformaldehyde overnight for hematoxylin and eosin (H&E) staining and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

The fixed tumor tissues were dehydrated with gradient ethanol, and embedded in paraffin. Tissue sections (5 μ m) were then dewaxed and rehydrated according to the standard protocol. The sections were stained with H&E for observation. For TUNEL assay, an in situ apoptosis detection kit (Roche Applied Science, Penzberg, Germany) was used to detect apoptotic cells in the tumor tissue. Briefly, the sections were treated with 20 μ g/mL of proteinase K in distilled water for 10 minutes at room temperature. To block endogenous peroxidase, the slides were incubated in methanol containing 3% hydrogen peroxide for 20 minutes and sections were incubated with equilibration buffer and terminal deoxynucleotidyl transferase. Finally, the sections were incubated with anti-digoxigenin-peroxidase conjugate. Peroxidase activity in each tissue section was shown by the application of diaminobenzidine. Sections were counterstained with hematoxylin.

Apoptosis assay by flow cytometric analysis

The murine hepatoma H22 cells were collected for apoptosis detection by flow cytometric analysis ($n = 3$) from tumor tissues belonging to the different groups and subjected to FACS detection using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide kit (Nanjing KeyGen Biotech Co, Ltd, Nanjing, China) according to the manufacturer's instructions. Briefly, the tumors were isolated by surgery, washed with phosphate-buffered saline, cut into pieces, and squeezed through a 200-mesh sieve using a 5 mL syringe core to obtain a single cell suspension. Next, 1×10^6 single cells were collected, washed with phosphate-buffered saline, and resuspended in 500 μ L of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). After incubation, 5 μ L of

Annexin V-FITC was added into each tube. The tubes were then incubated for 30 minutes at room temperature in the dark. The cells were washed with binding buffer and resuspended in 500 μ L of binding buffer, with 5 μ L propidium iodide added. Finally, the tube was gently vortexed and incubated for another 30 minutes in the dark, and the stained cells were analyzed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) with CellQuest software within one hour.

Statistical analysis

The data are shown as the mean \pm standard deviation. The significance of the difference between the groups was assessed by the Student's *t*-test using SPSS version 16.0 (SPSS Inc, Chicago, IL). In all cases, there was a statistically significant difference ($P < 0.05$, $P < 0.01$, $P < 0.001$).

Results

Characterization assay on Man-Ad5-PTEN by PAS staining

Man-Ad5-PTEN was prepared under the appropriate oxidizing conditions as described earlier (Figure 1). First, mannan was oxidized by sodium periodate to form oxidized mannan (Ox-Man). The Ox-Man with its aldehyde functional groups was able to bind the amino groups anchored on the surface of the adenovirus by Schiff's base reaction, which led to formation of Man-Ad5-PTEN. The efficacy of conjugation was confirmed by PAS staining. The PAS stain is a histochemical reaction commonly used to detect glycogen and other polysaccharides in biological specimens. The reaction of periodic acid oxidizes the diol functional groups in glucose and other sugars, creating aldehydes that react with the Schiff

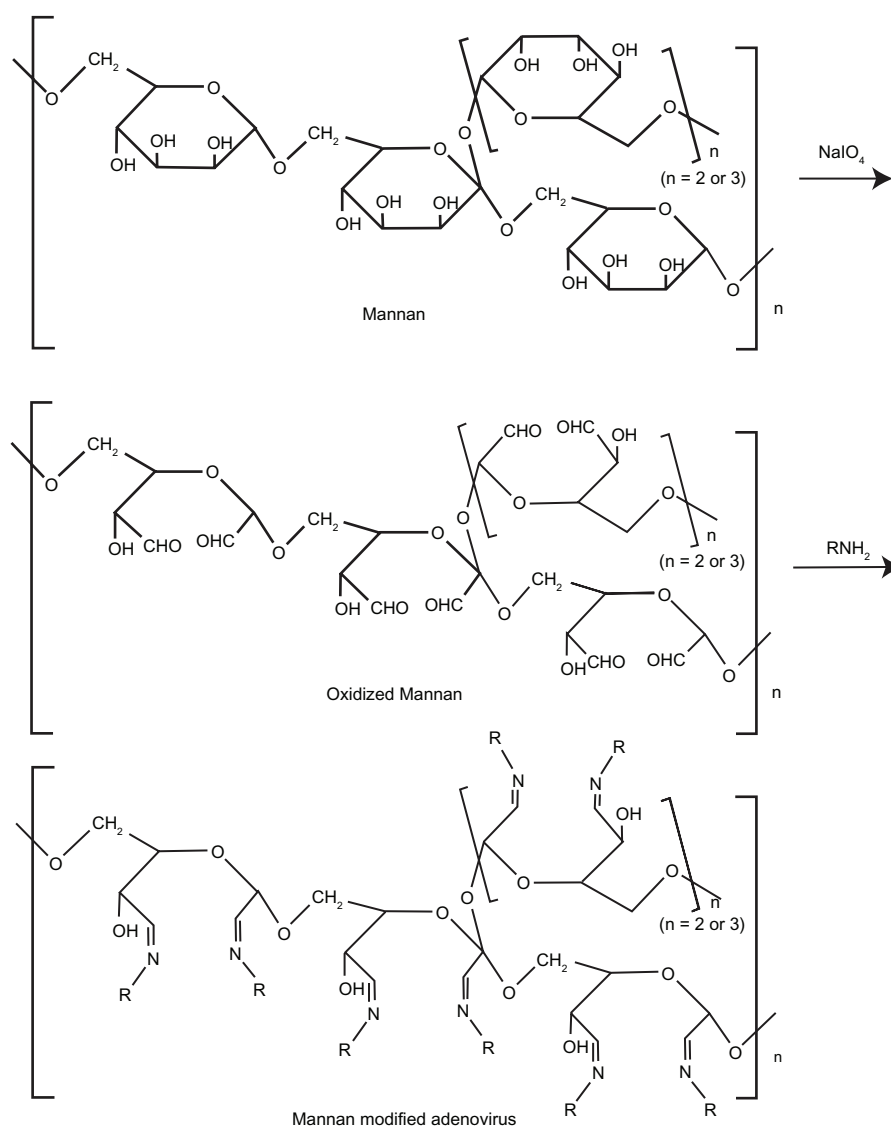


Figure 1 Synthesis scheme of conjugation strategy for mannan to adenovirus under the appropriate oxidizing conditions.

reagent to give a purple-magenta color. Therefore, after the conjugation reaction, both the Man-Ad5-PTEN and the redundant Ox-Man will react with the Schiff reagent to give positive staining, and the more the Ox-Man, the darker the color. Further, according to the result of PAS staining, we could determine the optimal concentration of mannan used to modify the adenovirus. As shown in Figure 2, both the free mannan and the conjugation groups stained positive for PAS, except for the low mannan-modified Ad5, but stained negative for the unconjugated control. The well containing

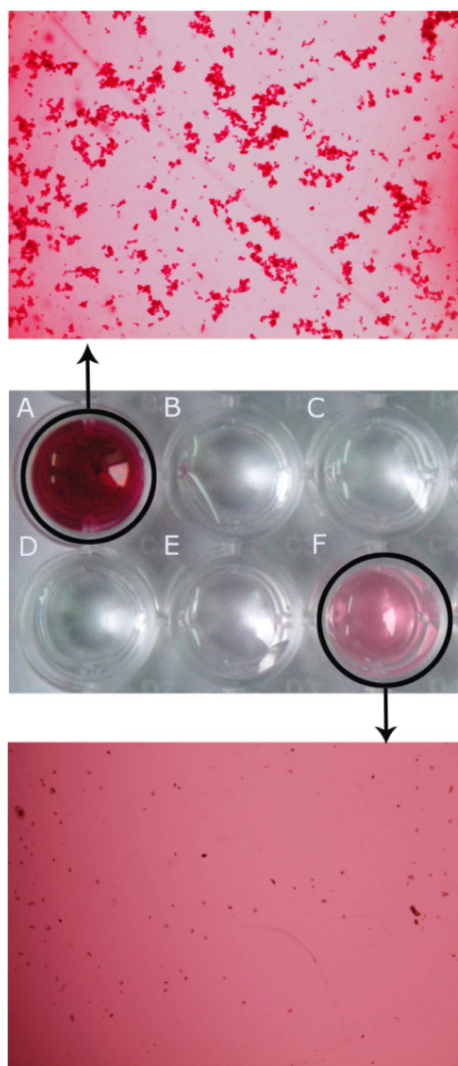


Figure 2 PAS staining to confirm the formation of mannan-modified adenovirus. The different groups subjected to PAS staining are medium-free mannan (A), PAS reagent (B), free Ad5 (C), low mannan-modified Ad5 (D), medium mannan-modified Ad5 (E), and high mannan-modified Ad5 (F), respectively. Mannan of different concentrations was conjugated to adenovirus as described in the Materials and methods section, and then the conjugates were subjected to PAS assay. At the same time, PAS reagent alone was used as the negative control. Both well (A) and well (F) were further observed under light microscopy (100 \times).

Abbreviations: PAS, periodic acid Schiff; Man, mannan; Ad5, recombinant adenovirus using the PTEN gene; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

a medium concentration of free mannan (Figure 2A) was a darker magenta color, whereas the well containing the product resulting from a medium concentration of mannan conjugated with adenovirus showed an impalpable clear color when stained with PAS (Figure 2E). In contrast, the well containing a high concentration of mannan-modified Ad5 showed a moderate color (Figure 2F). The well containing free Ad5 (Figure 2C) and the well containing a low concentration of mannan-modified Ad5 (Figure 2D) showed no color. The inconspicuous light color observed in the well shown in Figure 2E indicates that the redundant Ox-Man is relatively less likely to react with the PAS reagent. Meanwhile, in our preliminary experiment (data not shown), we used mannan at different concentrations to react with Ad5, and the resulting products were subjected to PAS staining. The absorbance of the mixture was read at 544 nm using a microplate reader (model-550, BioRad, Hercules, CA) after PAS staining. The results indicate that when the concentration of mannan was less than 25 mg/mL, the PAS stain (Figure 2D) was negative and the absorbance in each mixture showed no differences compared with the negative control of PAS reagent alone. Increasing the concentration of mannan to 25, 30, 35, 40, 45, and 50 mg/mL, the absorbance of the mixture also increased and Figure 2F is a representative image. Therefore, we took the corresponding amount as the optimal concentration of mannan conjugated with adenovirus. For visualization, the wells shown in Figure 2A and F were also observed under light microscopy (AL and FL). All the results confirmed the formation of mannan-adenovirus conjugates.

Combination treatment significantly decreased tumor volume

To determine the effects of naked Ad5-PTEN, docetaxel, Man-Ad5-PTEN, and Man-Ad5-PTEN-docetaxel on tumor development in mice, we constructed a mouse tumor model and a single injection of each formulation was administered intratumorally. Among them, one control group was treated with 0.1 mL of phosphate-buffered saline, and the other four groups of mice were treated with Man-Ad5-PTEN, docetaxel, or Man-Ad5-PTEN-docetaxel in a total amount of 0.1 mL.

As shown in Figure 3, there was no therapeutic effect in the control group receiving phosphate-buffered saline, which was consistent with the untreated group (data not shown). Tumor growth in mice treated with Man-Ad5-PTEN-docetaxel was significantly inhibited compared with the other groups of animals treated with phosphate-buffered saline, naked Ad5-PTEN, or docetaxel alone, respectively ($P < 0.01$). Tumor volume

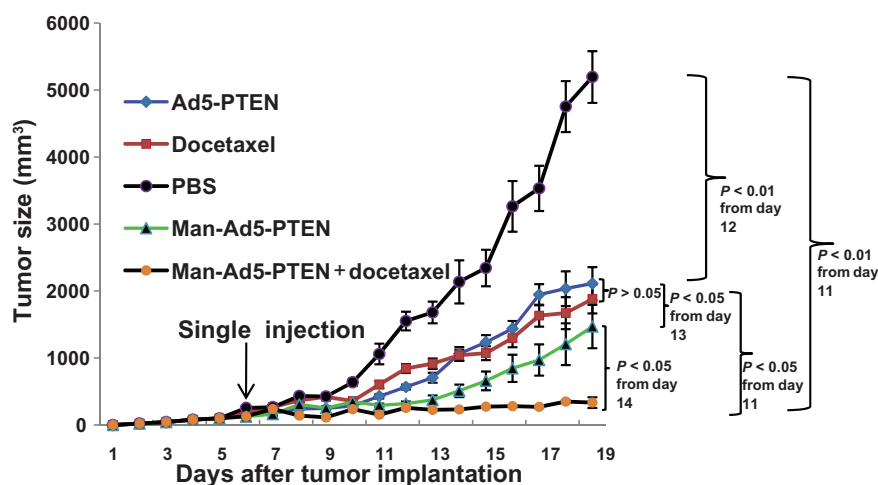


Figure 3 Antitumor effect of combined therapy on suppression of pre-existing subcutaneous mice tumors.

Notes: The tumor model was established by subcutaneous injection of murine H22 hepatoma cells (1.0×10^7) into the right mouse flank. When the mean tumor size reached 100–150 mm³, the mice were divided into five groups (at least $n = 8$) and injected intratumorally with phosphate-buffered solution, naked Ad5-PTEN, docetaxel alone, Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel. Arrow indicates the time point of single injection. Tumor volumes were monitored every day and were estimated as: tumor volume V (mm³) = $0.52 \times \text{length (mm)} \times \text{width (mm)}^2$, which was compared in tumor-bearing mice after treatment with either phosphate-buffered solution, naked Ad5-PTEN, docetaxel alone, Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel. The significance of the differences was evaluated using the Student's *t*-test. Data are shown as the mean \pm standard deviation for five mice in each group.

Abbreviations: Man, mannan; Ad5, recombinant adenovirus using the PTEN gene; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

was suppressed by both Man-Ad5-PTEN and Man-Ad5-PTEN-docetaxel. Compared with the group treated with Man-Ad5-PTEN-docetaxel, the mice treated with Ad5-PTEN or docetaxel appeared to be moderately inhibited in tumor growth ($P < 0.05$). There was no difference between the groups treated with docetaxel or Ad5-PTEN ($P > 0.05$). A clear difference was observed between the Man-Ad5-PTEN-docetaxel and Man-Ad5-PTEN treated groups from day 14 post-injection. When the Ad5-PTEN or docetaxel groups were compared with the combination group, significant differences were also observed ($P < 0.05$).

These results indicate that combination treatment of Man-Ad5-PTEN-docetaxel was the most effective of these formulations, suggesting that Man-Ad5-PTEN can synergize with docetaxel to improve the antitumor effect in H22 tumor-bearing mice after intratumoral administration.

Combination strategy significantly decreases tumor weight

To demonstrate the tumor growth inhibition effect more clearly, the H22 tumor-bearing mice were first given the formulation of Ad5-PTEN, docetaxel, PBS, Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel. Then, after two weeks, the mice were euthanized by nitrogen inhalation, the tumors were removed and weighed, and the mean tumor weight was calculated. The tumor inhibition rate was calculated using the following formula:

$$\text{Tumor inhibition rate} = \frac{(\text{mean tumor weight of control group} - \text{mean tumor weight of treated group}) / \text{mean tumor weight of control group} \times 100\%.$$

The results are shown in Figure 4I and II.

Figure 4 indicates that the average tumor weight of the mice treated with phosphate-buffered saline (Figure 4IA), docetaxel (Figure 4IB), Ad5-PTEN (Figure 4IC), Man-Ad5-PTEN (Figure 4ID), and Man-Ad5-PTEN-docetaxel (Figure 4IE) was 4.8827 ± 0.3942 g, 2.4424 ± 0.2860 g, 2.5053 ± 0.2274 g, 1.1779 ± 0.2008 g, and 0.1499 ± 0.0123 g, respectively. There is a significant difference between Figure 4IA and IB ($P < 0.05$), Figure 4IA and IC ($P < 0.05$), Figure 4IA and ID ($P < 0.01$), and Figure 4IA and IE ($P < 0.001$). Compared with the control group receiving phosphate-buffered saline, the tumor inhibition rate in Figure 4IIB, IIC, IID, and IIE was 49.98%, 48.69%, 75.88%, and 96.93%, respectively. These results demonstrate that combination treatment with Man-Ad5-PTEN and docetaxel significantly decreased the tumor weight in H22-tumor bearing mice.

Effect of combination treatment on tumor issues analyzed by FACS

We found that all four treatments could suppress tumor growth to varying degrees compared with the control group receiving phosphate-buffered saline, so we wanted to study the likely mechanism of suppression. To investigate

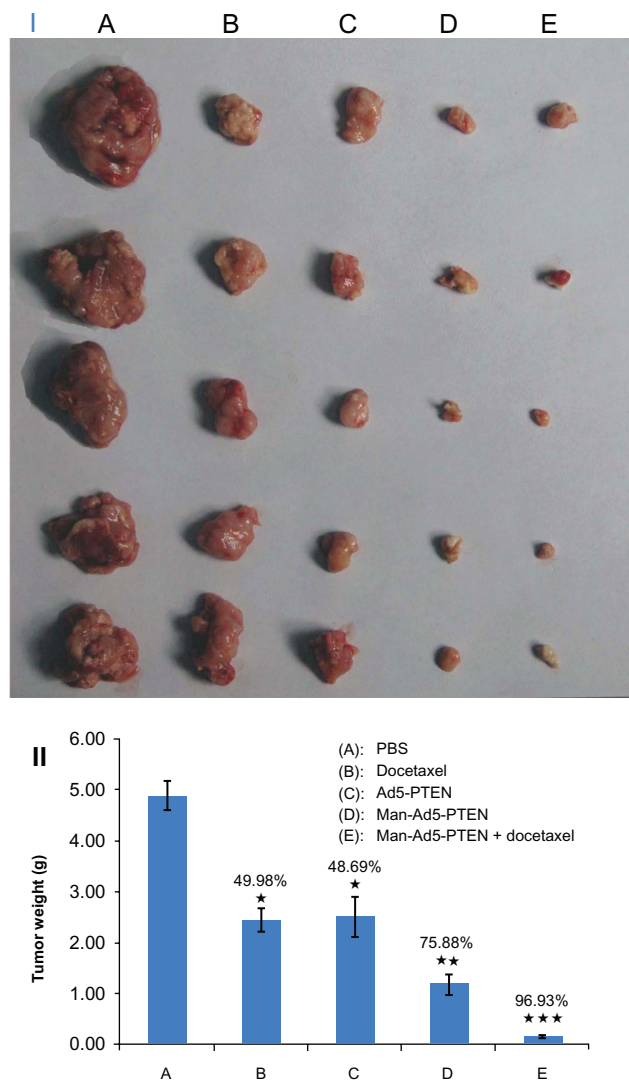


Figure 4 Effect of combination treatment on tumor weight. The H22-tumor bearing mice in each group were given the different formulations, respectively. Two weeks later, the mice were euthanized by nitrogen inhalation. Tumor images were taken by Nikon D70 digital camera (I) and the weight was also examined (II). The tumor weight was compared between phosphate-buffered solution (A) and docetaxel (B), Ad5-PTEN (C), Man-Ad5-PTEN (D), and Man-Ad5-PTEN-docetaxel (E).

Notes: The significance of the differences was evaluated using the Student's *t*-test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). The tumor inhibition rate was calculated and normalized to the phosphate-buffered solution group. Data are shown as the mean \pm standard deviation in each group.

Abbreviations: Man, mannan; Ad5, recombinant adenovirus using the PTEN gene; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

quantitatively whether the treatments induced apoptosis in H22 tumor cells, we also performed double-staining flow cytometry with Annexin V-FITC and propidium iodide. A single-cell suspension obtained from tumor tissue was prepared as described earlier.

As shown in Figure 5, compared with the control group treated with phosphate-buffered solution, all the other treatment groups, including Ad5-PTEN (Figure 5A, *P* < 0.05), docetaxel (Figure 5B, *P* < 0.05), Man-Ad5-PTEN

(Figure 5D, *P* < 0.001), and Man-Ad5-PTEN-docetaxel (Figure 5E, *P* < 0.001) showed elevated levels of apoptosis, as measured by the percentage of Annexin V-positive cells. Of note, combined treatment with Man-Ad5-PTEN and docetaxel led to more obvious apoptosis of tumor cells from tumor-bearing mice compared with treatment using Man-Ad5-PTEN or docetaxel alone.

Effect of combination on tumor tissues analyzed by H&E and TUNEL assay

To investigate qualitatively whether the treatments induced cell necrosis and apoptosis in H22 tumor cells, all the groups of tumor-bearing mice were treated intratumorally on day 6. Two weeks later, mice were euthanized by nitrogen inhalation, and the tumors were removed and cut into 5 μ m slices. The slices were then prepared for subsequent H&E staining and TUNEL assay. The H&E staining results (Figure 6A and B) indicate that tumor cells from the treated mice showed the typical morphology of apoptosis, ie, nuclear fragmentation, chromatin condensation, and loss of membrane asymmetry. They also showed that the treated groups had increased apoptotic and necrotic cells compared with the control group treated with phosphate-buffered solution. Both the apoptotic and necrotic cell populations in the Ad5-PTEN and docetaxel groups were much smaller than those in the Man-Ad5-PTEN and Man-Ad5-PTEN-docetaxel groups. Moreover, there were fewer apoptotic and necrotic cells in the Man-Ad5-PTEN group than in the group receiving Man-Ad5-PTEN-docetaxel.

Cellular apoptosis in tumor samples derived from the control and treated mice was also examined using the in situ TUNEL method. The results are shown in Figure 6C, and indicate that the number of apoptotic cells was dramatically increased in tumors treated with Man-Ad5-PTEN and Man-Ad5-PTEN-docetaxel when compared with those in tumors treated with the control phosphate-buffered solution. The number of TUNEL-positive cells was not different between tumors treated with naked Ad5-PTEN and those treated with docetaxel. These results were consistent with those from H&E staining. All the results were consistent with the tumor growth effect in the different groups, suggesting that the apoptotic effect led to tumor growth inhibition.

Discussion

The success of gene therapy in vivo relies on development of a vector that achieves targeted cell-specific, efficient, and prolonged transgene expression after its application. Currently, recombinant adenovirus vectors represent one of the most efficient delivery vehicles for gene therapy in cancer.

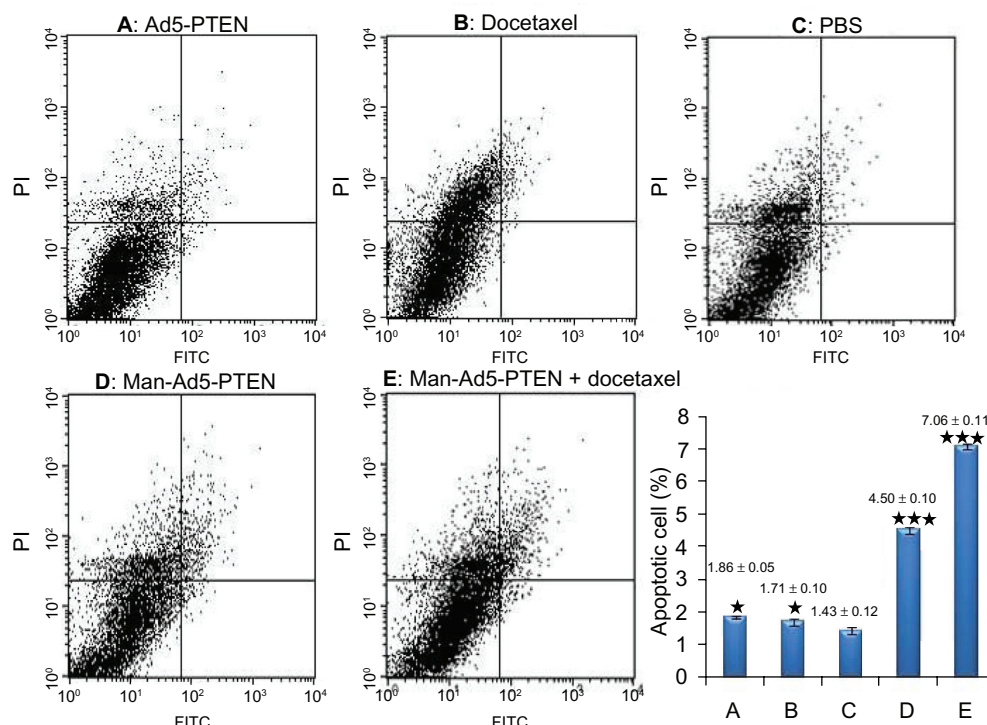


Figure 5 Flow cytometry analysis of cell apoptosis in murine hepatoma H22 cells obtained from tumor-bearing mice. The different groups of mice were treated with Ad5-PTEN, docetaxel, phosphate-buffered solution, Man-Ad5-PTEN, and Man-Ad5-PTEN-docetaxel, respectively. The single cell solution was prepared as described in the Materials and methods section and then stained with Annexin V and propidium iodide. The samples were analyzed by FACSCalibur within one hour. The percent of apoptotic cells was compared between the phosphate-buffered solution group (C) and Ad5-PTEN (A), docetaxel (B), Man-Ad5-PTEN (D), and Man-Ad5-PTEN-docetaxel (E).

Notes: The significance of the differences was evaluated using the Student's *t*-test (**P* < 0.05; ****P* < 0.001). Data are expressed as the mean ± standard deviation (*n* = 3) in each group.

Abbreviations: FITC, fluorescein isothiocyanate; Man, mannan; Ad5, recombinant adenovirus using the PTEN gene; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

Their translocation from the cell surface to the nucleus is extremely rapid and far more efficient than nonviral gene delivery vectors. However, adenoviral vectors show strong antigenicity as a result of their capsid domains, leading to decreased expression of the target gene constructed in the adenoviral vector, along with a need for increasing frequency of administration. Moreover, transfer of adenovirus has limited tissue specificity.

Numerous strategies have been developed to address the abovementioned issues, including second-generation and third-generation adenoviral vectors, fiber-mutated or pentone-mutated recombinant adenoviral vectors, conjugated adenovirus, and some ligands to modify adenoviral tropism and reduce vector-related toxicity. On the other hand, active targeting delivery systems have become a focus in cancer therapeutics, and some targeting ligands, such as aptamer, transferrin, antibody, and folate, are used to modify the vectors of interest for binding to particular receptors overexpressed by tumor cells or tumor vasculature.³¹ In this work, in order to block native tropism, extend blood residence time, and enhance tumor targeting, we conjugated adenovirus with mannan under appropriate oxidizing conditions to enable

formation of a mannan-modified adenovirus based on the fact that mannose receptors are extensively expressed in nonparenchymal liver tissue. Thus, introduction of mannose residues may lead to enhancement of endocytosis mediated by these receptors. Compared with naked Ad5-PTEN, Man-Ad5-PTEN significantly suppressed growth of H22 tumors, indicating that the mannan and adenovirus conjugation strategy indeed played an important role. The cytotoxicity of Ad5-PTEN or preformed Man-Ad5-PTEN at a variable multiplicity of infection (80, 40 and 20 plaque-forming units/cell)³² was assessed by MTT viability assay in normal Chang hepatocytes (data not shown). No cytotoxicity was observed in the group treated with naked Ad5-PTEN or in the group treated with Man-Ad5-PTEN, even at the highest multiplicity of infection (80 plaque-forming units/cell), taking the phosphate-buffered solution group as the control. This result suggests that both Ad5-PTEN and Man-Ad5-PTEN may not be cytotoxic to normal cells *in vivo*. To ensure that chemical modification did not damage the gene expression mediated by Ad5, we investigated transgene expression *in vitro* using the LacZ reporter gene. The experiment was carried out according to our previous report³³ whereby murine

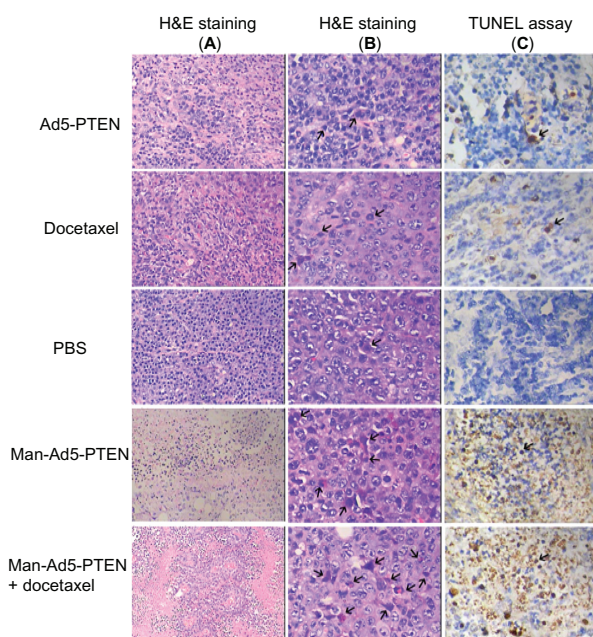


Figure 6 In vivo tumor cell death study of Man-Ad5-PTEN in combination with docetaxel. The H22 tumor-bearing mice were treated as mentioned earlier with Ad5-PTEN, docetaxel, phosphate-buffered solution, Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel. Tumor sections were excised, fixed, dewaxed, and followed by standard H&E staining for necrosis (**A**, 200 \times) and apoptosis (**B**, 400 \times). As the arrow indicates, condensation and fragmentation of nuclei were observed, which are the hallmarks of apoptosis. Detection of apoptotic cells were also carried out by TUNEL assay (**C**, 400 \times).

Abbreviations: Man, mannan; Ad5, recombinant adenovirus using the PTEN gene; PTEN, phosphatase and tensin homolog deleted on chromosome ten; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

H22 hepatoma cells were treated with naked Ad5-LacZ, Man-Ad5-LacZ, and Man-Ad5-LacZ-docetaxel, respectively. The results show that the average amounts of β -galactosidase in murine H22 hepatoma cells transduced by Man-Ad5-LacZ or Man-Ad5-LacZ-docetaxel were $(3.80 \pm 0.12) \times 10^5$ and $(3.83 \pm 0.25) \times 10^5$ pg β -galactosidase/ μ g protein, respectively. Their expression levels were about 8-fold higher than those of β -galactosidase in cells transduced by naked Ad5-LacZ, ie, $(4.12 \pm 0.05) \times 10^4$ pg β -galactosidase/ μ g protein. There was no difference in gene expression between Man-Ad5-LacZ and Man-Ad5-LacZ-docetaxel, indicating that docetaxel 10 μ M did not affect gene expression when combined with Man-Ad5-LacZ. These results suggest that neither mannan modification nor the presence of docetaxel affected gene expression mediated by Ad5. Moreover, mannan-modified Ad5 could enhance LacZ gene expression in murine H22 hepatoma cells. The results of this in vitro experiment laid a good foundation for the subsequent in vivo experiments.

Hepatocellular carcinoma is the fifth most common cancer worldwide and the third most common cause of cancer mortality. The conventional therapeutic strategy for

hepatocellular carcinoma is surgery, chemotherapy, and radiotherapy. However, the cancer recurs and metastasizes in more than one third of patients after conventional therapy. Currently, combined traditional treatment such as chemotherapy and radiation with vaccines has attracted increasing attention. Vaccination can be quite effective in protecting naive mice from tumor challenge and for delaying tumor growth in mice bearing minimal residual disease, but is less effective when given to mice with established tumors. On the other hand, it has been reported that the cancer-suppressing gene, PTEN, plays a critical role in controlling tumorigenesis and regulating cell spread and migration,³⁴ and adenovirus-mediated PTEN gene therapy is an attractive strategy. However, the ability of single gene therapy alone to suppress tumor growth is limited. To achieve an improved chemotherapeutic effect, a combination of two or more different agents according to their individual mechanisms of action has already been demonstrated to be a popular and effective therapeutic strategy. In a previous study, we reported that combining carmustine chemotherapy with adenovirus-mediated stromal cell-derived factor-1 α gene therapy could be successful in the treatment of the murine B16 melanoma.³³ Thus, in this study, we developed a mannose receptor-mediated chemoadenovirus-PTEN therapy strategy and combined this with docetaxel, a conventional chemotherapy agent, and PTEN, a tumor suppressor gene.

To investigate the inhibitory effect on tumor growth directly in an empirical manner, we developed a tumor model in vivo and administered Ad5-PTEN, docetaxel, Man-Ad5-PTEN, or Man-Ad5-PTEN-docetaxel by the intratumoral route. To ensure uniform distribution of the formulation in tumor tissue, a single intratumoral injection was carried out in a multipoint manner, whereby pinpoint with fluid does not quit the tumor tissue during one single injection. The results indicate that treatment with Ad5-PTEN and with docetaxel alone could only moderately inhibit tumor growth in H22-bearing mice. However, a combination of Man-Ad5-PTEN and docetaxel dramatically suppressed tumor growth and induced high levels of apoptosis.

The present study is, to our knowledge, the first to combine adenovirus-mediated PTEN gene therapy with docetaxel for cancer treatment. However, the results we obtained are still far from our goal of complete inhibition of tumor growth. Even now, the antitumor mechanism of this combination treatment is still not very clear and requires further investigation. In future studies, we will further optimize the ratio of Ad5-PTEN particle numbers and docetaxel, and also

investigate the use of combination strategies in hepatocellular carcinoma and other tumors by intravenous administration and elucidate clearly the mechanism of combination therapy. Meanwhile, in the present study, Man-Ad5-PTEN was injected intratumorally and showed obvious tumor growth inhibition. In the next study, we plan to use this approach for in situ liver cancer by intravenous administration. High targeting efficiency mediated by the mannose receptor and strong antitumor activity are expected.

Conclusion

Overall, this study demonstrated that Man-Ad5-PTEN significantly enhanced the antitumor effect in a murine model of hepatocellular carcinoma in vivo. Combination treatment of Man-Ad5-PTEN and docetaxel had a better antitumor effect than either strategy used alone. Because of its simplicity, this method may be used to target therapy in other kinds of tumors by intratumoral or intravenous administration. It can be concluded that the combined use of Man-Ad5-PTEN and docetaxel may be a powerful and effective tool for future exploration in tumor therapy.

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Disclosure

The authors report no conflicts of interest in this work.

References

- Hatefi A, Cappello J, Ghandehari H. Adenoviral gene delivery to solid tumors by recombinant silk-elastin-like protein polymers. *Pharm Res*. 2007;24(4):773–779.
- Yao XL, Nakagawa S, Gao JQ. Current targeting strategies for adenovirus vectors in cancer gene therapy. *Curr Cancer Drug Targets*. 2011;11(7):810–825.
- Yao X, Yoshioka Y, Morishige T, et al. Tumor vascular targeted delivery of polymer-conjugated adenovirus vector for cancer gene therapy. *Mol Ther*. 2011;19(9):1619–1625.
- Korokhov N, Mikheeva G, Krendelshchikov A, et al. Targeting of adenovirus via genetic modification of the viral capsid combined with a protein bridge. *J Virol*. 2003;77(24):12931–12940.
- Zeng Q, Han J, Zhao D, Gong T, Zhang Z, Sun X. Protection of adenovirus from neutralizing antibody by cationic PEG derivative ionically linked to adenovirus. *Int J Nanomedicine*. 2012;7:985–997.
- Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*. 1997;275(5308):1943–1947.
- Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet*. 1997;15(4):356–362.
- Zhang C, Kang C, Wang P, et al. MicroRNA-221 and -222 regulate radiation sensitivity by targeting the PTEN pathway. *Int J Radiat Oncol Biol Phys*. 2011;80(1):240–248.
- Villanueva A, Newell P, Chiang DY, Friedman SL, Llovet JM. Genomics and signaling pathways in hepatocellular carcinoma. *Semin Liver Dis*. 2007;27(1):55–76.
- Weng LP, Brown JL, Eng C. PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. *Hum Mol Genet*. 2001;10(6):599–604.
- Weng L, Brown J, Eng C. PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and -independent pathways. *Hum Mol Genet*. 2001;10(3):237–242.
- Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science*. 1998;280(5369):1614–1617.
- Dahia PL. PTEN, a unique tumor suppressor gene. *Endocr Relat Cancer*. 2000;7(2):115–129.
- Li DM, Sun H. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc Natl Acad Sci U S A*. 1998;95(26):15406–15411.
- Furnari FB, Huang HJ, Cavenee WK. The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells. *Cancer Res*. 1998;58(22):5002–5008.
- Davies MA, Lu Y, Sano T, et al. Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits Akt activation and induces anoikis. *Cancer Res*. 1998;58(23):5285–5290.
- Sawai H, Yasuda A, Ochi N, et al. Loss of PTEN expression is associated with colorectal cancer liver metastasis and poor patient survival. *BMC Gastroenterol*. 2008;8:56.
- Hsu CP, Kao TY, Chang WL, Nieh S, Wang HL, Chung YC. Clinical significance of tumor suppressor PTEN in colorectal carcinoma. *Eur J Surg Oncol*. 2011;37(2):140–147.
- Pappas G, Zumstein LA, Munshi A, Hobbs M, Meyn RE. Adenoviral-mediated PTEN expression radiosensitizes non-small cell lung cancer cells by suppressing DNA repair capacity. *Cancer Gene Ther*. 2007;14(6):543–549.
- Steelman LS, Navolanic PM, Sokolosky ML, et al. Suppression of PTEN function increases breast cancer chemotherapeutic drug resistance while conferring sensitivity to mTOR inhibitors. *Oncogene*. 2008;27(29):4086–4095.
- Mellinghoff IK, Cloughesy TF, Mischel PS. PTEN-mediated resistance to epidermal growth factor receptor kinase inhibitors. *Clin Cancer Res*. 2007;13(2 Pt 1):378–381.
- Magnusson S, Berg T. Extremely rapid endocytosis mediated by the mannose receptor of sinusoidal endothelial rat liver cells. *Biochem J*. 1989;257(3):651–656.
- Yeeprae W, Kawakami S, Yamashita F, Hashida M. Effect of mannose density on mannose receptor-mediated cellular uptake of mannosylated O/W emulsions by macrophages. *J Control Release*. 2006;114(2):193–201.
- Magnusson S, Berg T. Endocytosis of ricin by rat liver cells in vivo and in vitro is mainly mediated by mannose receptors on sinusoidal endothelial cells. *Biochem J*. 1993;291(Pt 3):749–755.
- Kawakami S, Sato A, Nishikawa M, Yamashita F, Hashida M. Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. *Gene Ther*. 2000;7(4):292–299.
- Apostolopoulos V, Pietersz GA, Loveland BE, Sandrin MS, McKenzie IF. Oxidative/reductive conjugation of mannan to antigen selects for T1 or T2 immune responses. *Proc Natl Acad Sci U S A*. 1995;92(22):10128–10132.

27. Liu Q, Li R, Zhu Z, et al. Enhanced antitumor efficacy, biodistribution and penetration of docetaxel-loaded biodegradable nanoparticles. *Int J Pharm.* 2012;430(1–2):350–358.
28. Lin C, Wei W, Zhang J, Liu S, Liu Y, Zheng D. Formyl peptide receptor-like 1 mediated endogenous TRAIL gene expression with tumoricidal activity. *Mol Cancer Ther.* 2007;6(10):2618–2625.
29. Stewart AL, Mhashilkar AM, Yang XH, et al. PI3 kinase blockade by Ad-PTEN inhibits invasion and induces apoptosis in RGP and metastatic melanoma cells. *Mol Med.* 2002;8(8):451–461.
30. Liu Q, Li RT, Qian HQ, et al. Gelatinase-stimuli strategy enhances the tumor delivery and therapeutic efficacy of docetaxel-loaded poly(ethylene glycol)-poly(ϵ -caprolactone) nanoparticles. *Int J Nanomedicine.* 2012;7:281–295.
31. Zhong Z, Wan Y, Han J, Shi S, Zhang Z, Sun X. Improvement of adenoviral vector-mediated gene transfer to airway epithelia by folate-modified anionic liposomes. *Int J Nanomedicine.* 2011;6: 1083–1093.
32. Zhong Z, Shi SJ, Han JF, Zhong ZR, Sun X. Anionic liposomes increase the efficiency of adenovirus-mediated gene transfer to coxsackie-adenovirus receptor deficient cells. *Mol Pharm.* 2010;7(1):105–115.
33. Zhong Z, Wan Y, Shi S, Han J, Zhang Z, Sun X. Co-delivery of adenovirus and carmustine by anionic liposomes with synergistic anti-tumor effects. *Pharm Res.* 2012;29(1):145–157.
34. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A.* 1999;96(8):4240–4245.

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