# *In vivo* antitumor activity of liposome-plasmid DNA encoding mutant survivin-T34A in cervical cancer

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**Abstract.** The aim of the present study was to investigate the influence of liposome-plasmid encoding mutant survivin-T34A (PST34A) on tumor growth in cervical cancer *in vivo*. Liposome-plasmid DNA encoding mutant survivin-T34A was constructed and administered via an intraperitoneal injection in mice inoculated with cervical cancer cells. Following the establishment of the tumor model, the animals were randomly divided into four groups: i) The normal saline group (NS; 100 $\mu$l sterile saline once/3 days for 15 days); ii) the 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) control (100 $\mu$g DOTAP once/3 days for 15 days); iii) the plasmid PST34A (10 $\mu$g PST34A once/3 days for 15 days); and iv) the PST34A+DOTAP (10 $\mu$g PST34A+100 $\mu$g DOTAP once/3 days for 15 days). All treatments were administered via intraperitoneal injections. Tumor growth was evaluated following injection with liposome-plasmid DNA encoding mutant survivin-T34A. Apoptosis of cells in ascitic fluid was detected by flow cytometry. The expression of Ki67 and CD34 was detected by immunohistochemical staining. Administration of liposome-plasmid complexes encoding mutant survivin-T34A inhibited tumor growth, reduced the number of tumor nodules and the volume of ascitic fluid, and decreased abdomen circumference and tumor weight. The number of Ki67-positive cells was markedly reduced in the DOTAP+PST34A group compared with the remaining groups. Flow cytometry demonstrated that the number of cells in the sub-G1 phase (apoptosis) increased in the DOTAP+PST34A group compared with all other groups. In addition, tumors in the DOTAP+PST34A group exhibited lower microvessel density compared with all other groups. In the present study, liposome-plasmid DNA encoding mutant survivin-T34A could inhibit tumor growth of cervical cancer. This inhibition may be associated with an increase in the apoptosis rate of tumor cells and a reduction in angiogenesis.

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## Introduction

Cervical cancer is the fourth most common cancer diagnosed among women, with ~520,000 novel cases each year worldwide. It has been predicted that there will be 13,000 novel cases of cervical cancer diagnosed each year in the United States, with 5,000 more mortalities due to disease progression (1). Metastasis to pelvic and aortic lymph nodes is a common characteristic of cervical cancer (2). Therefore, inhibition of metastasis is essential to improve prognosis and to design effective therapeutic methods. Recently, a variety of molecular and biochemical factors have been identified to influence metastasis and the outcome of cervical cancer (3). Site-specific therapy provides a novel approach for tumor treatment; however, the associated side effects cause injury in the adjacent tissues, and limit the effectiveness and safety of this treatment method (4). Currently, more effective therapies with fewer side effects are required. Gene therapy is one approach used to treat cervical cancer, which inhibits tumor cell proliferation and also sensitizes tumor cells to chemotherapy (5).

Survivin (molecular weight, 16.5 kDa), the smallest member of the inhibitory apoptotic protein family, has demonstrated a dual role in the control of apoptosis and regulation of cell division (6). As an antiapoptotic protein, survivin is expressed in a number of human neoplasm tissues; however, it is undetectable in terminally differentiated normal tissues with the exception of the thymus, basal colonic epithelium and endothelial cells during angiogenesis (7-9). Survivin expression is associated with apoptosis, tumor aggression and recurrence, poor survival and drug resistance (10,11). The distinct expression and functional profiles of survivin in tumor tissues compared with normal tissues make it a potential target for cervical cancer treatment (12). Inhibition of survivin has been extensively investigated using different approaches, including antisense oligonucleotide, dominant negative mutants, RNA interference and cancer vaccines (13-15). Downregulation of survivin effectively prevents tumor growth and increases the

susceptibility to chemotherapy (16,17). Survivin expression is thought to be a standard method to evaluate therapeutic efficacy (18). However, whether liposome-plasmid DNA encoding mutant survivin-T34A could prevent cervical cancer growth remains to be elucidated. In the present study, a plasmid with mutant survivin-T34A was synthesized and a liposome was constructed using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). The present study aimed to evaluate the anti-cancer activity of a liposome-plasmid complex in cervical cancer *in vivo*.

## Materials and methods

*Preparation of plasmids with survivin-T34A*. A pVITRO2 plasmid (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) expressing mutant survivin-T34A was synthesized. Briefly, cDNA clone encoding mutant survivin-T34A was a gift from Li Pan (West China Medical School, Sichuan University) and amplified by polymerase chain reaction (PCR) (DNA polymerase: cat. no. D7220; Beyotime Institute of Biotechnology, Haimen, China) with the following primers: Forward, 5'-GATCACGCGTCACCATGGGAGC-3' and reverse, 5'-GGCGGTCGACAGCATTAGGCAG-3'. The PCR protocol was as follows: 95˚C initial denaturation 5 min, 95˚C denaturation 30 sec, 58˚C annealing 16 sec, 72˚C extension 30 sec for 36 cycles. Subsequently, the clone was digested with *Sal*I/*Mlu*I and then inserted into the pVITRO2 plasmid. In a previous study, Pan *et al* (18) demonstrated that DOTAP-chol liposome encoding survivin-T34A could down-regulate survivin expression and pVITRO2 plasmid without mutant T34A (pVITRO2-null) did not affect survivin expression. Colonies of *E. coli* were cultured in Luria-Bertani broth (BW30620044; Bioworld Technology, Inc., St. Louis Park, MN, USA) containing 100 $\mu$g/ml ampicillin. Large-scale plasmid survivin-T34A was purified using a Qiagen EndoFree Plasmid Giga kit (cat. no. 12391; Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The purity (optical density 260/280) values for the prepared plasmid DNA were equal to 1.8-2.0. Plasmids were stored at -20˚C for subsequent experimentation.

*Preparation of liposome DOTAP*. Liposome was prepared according to the procedure described previously (19). DOTAP and cholesterol (1:1; both Avanti Polar Lipids, Inc., Alabaster, AL, USA) were dissolved in chloroform in a 100 ml-round-bottomed flask and rotated to obtain a thin lipid layer. Subsequently, the mixture was dried and treated with a vacuum for 2 h at 4˚C in order to remove the organic solvent. The lipid layer was rehydrated using 5% dextrose (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in water to obtain a final concentration of 10 mg/ml and eddied for 30 min at 60˚C. Finally, the film was extruded through a 100-nm polycarbonate filter using a Mini-Extruder (Avanti Polar Lipids, Inc.). Liposomes were stored at 4˚C until further use. Prior to use, the liposome-plasmid complex was freshly prepared at a ratio of 10:1 (liposome:plasmid) and incubated for 20 min at room temperature prior to injection.

*Cell culture*. The human cervical cancer cell line (HeLa) was purchased from the Cell Center, Institute of Basic Medical Sciences, Xiehe Medical University (Beijing, China). Cells were cultured at 37˚C, in an atmosphere of 5% $CO_2$ in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Hyclone; GE Healthcare, Chicago, IL, USA), 100 U/ml penicillin and 100 mg/ml streptomycin.

*Animal model*. A total of 40 female BALB/c nude mice (16±1 g; 5-6-weeks old) were purchased from Vital River Laboratories Co., Ltd. (Beijing, China). Mice were housed and maintained in specific pathogen-free conditions at a temperature of 23±2˚C, a relative humidity of 45-65%, and a controlled 12/12 h light/dark cycle. Animals had *ad libitum* access to food and water. All protocols were approved and supervised by the State Key Laboratory of Biotherapy Animal Care and Use Committee of Sichuan University (Chengdu, China). HeLa cells ($1x10^7$) were diluted in 0.2 ml PBS and administered to BALB/c nude mice by intraperitoneal injection (i.p.) on day 0. Three days following inoculation, the 40 mice were randomly divided into four groups (n=10/group): i) The normal saline group (NS; 100 $\mu$l sterile saline once/3 days for 15 days); ii) the DOTAP control group (100 $\mu$g DOTAP once/3 days for 15 days); iii) the plasmid encoding mutant survivin-T34A (PST34A) group (10 $\mu$g PST34A once/3 days for 15 days); and iv) the PST34A+DOTAP group (10 $\mu$g PST34A+100 $\mu$g DOTAP once/3 days for 15 days). The treatment was administered via i.p. injection once every three days in 100 $\mu$l volume saline; four injections were administered in total over 15 days. The general health of the mice was monitored daily. On day 15, all mice were sacrificed by dislocation of the neck and metastasis was evaluated. At the time of sacrifice, tumor tissue, ascitic fluid and the vital organs of the mice were harvested, and body weight, ascitic fluid volume, tumor weight and the number of tumor nodules were counted. Ascitic fluid, and tumor and normal tissues were used for further study. Tumor specimen were fixed by paraformaldehyde (4%, pH 7.4) at 4˚C overnight and embedded in paraffin for tissue sectioning (4 $\mu$m) vital organs (spleen, liver, kidneys, heart and lungs) were also harvested and stored at -80˚C for later assessment of tissue toxicity.

*Flow cytometry*. Ascitic fluid from the NS, DOTAP, PST34A and PST34A+DOTAP treatment groups was collected. A total of 5 ml normal saline solution was injected into abdomen cavity and withdrawn from mice with ascitic fluid. All specimens were washed with PBS, resuspended in propidium iodide/RNase A solution (5 ml; Beyotime Institute of Biotechnology) and incubated in the dark at 4˚C for 15 min. Samples were analyzed by flow cytometry using the NovoCyte® Flow Cytometer System which included the analysis software (ACEA Biosciences, San Diego, CA, USA).

*Hematoxylin and eosin (H&E) and immunohistochemical staining*. Immunohistochemical analyses of proliferation marker protein Ki-67 (Ki67) and microvessel density (MVD) were determined using rabbit anti-human Ki67 (cat. no. NB500; Novus Biologicals, LLC, Littleton, CO, USA) and rabbit anti-mouse CD34 (cat. no. ab81289; Abcam, Cambridge, MA, USA) antibodies using the labeled streptavidin-biotin method. Briefly, sections (4 $\mu$m) were sliced from paraffin-embedded tumor tissue and deparaffinized by sequential washing with

xylene, and 100, 95, 85 and 75% ethanol. Endogenous peroxide activity was blocked by 3% $H_2O_2$ for 10 min at room temperature. The sections were stained with H&E for 20-30 sec at room temperature. Representative images were captured under a light microscope (magnification, x400) in at least 5 random selected fields.

For immunohistochemical staining, antigen retrieval was conducted by heating the slices in a steam cooker (100˚C) in 10 mM sodium citrate buffer (pH 6.0). The sections were blocked in 5% goat serum (Hyclone; GE Healthcare) for 2 h at room temperature. Following washing with PBS, slices were incubated with the primary antibodies (both antibodies in 1:300) overnight at 4˚C. Following washing with PBS for three times, peroxidase conjugated goat anti-mouse IgG (1:100; cat. no. TA130004; OriGene Technologies, Inc., Beijing, China) was added and incubated for 2 h at room temperature. This was followed by staining using a 3,3'-Diaminobenzidine substrate kit (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) for 30-45 sec at room temperature. Cells were counterstained with H&E for 20-30 sec at room temperature. Control slices were exposed to the secondary antibody alone and did not demonstrate specific staining (data not shown). Representative images were captured under a light microscope (magnification, x400) in at least 5 randomly selected fields.

All slices were observed and counted by two pathologists (Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan, China) in a blind manner. CD34 is as a cell surface antigen selectively expressed on hematopoietic progenitor cells and vascular endothelial cells. Weidner's method was used to determine the density of the tumor-associated microvasculature (19). Microvessel density within tumors was calculated under a light microscope in 10 randomly selected fields. Ki67-positive cells were counted.

*Statistical analysis*. All data are presented as the mean ± standard deviation with 10 repeats. Statistical analysis was performed by SPSS version 19.0 (IBM Corp., Armonk, NY, USA) using one-way analysis of variance followed by Bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*General observation of anti-tumor activity of liposome-PST34A in vivo*. Mice in all groups survived the experimental period, although mice with tumors are not in healthy condition (ascites, reduced activity, no eating). Following sacrifice, tumor tissues, ascitic fluid and vital organs of mice were harvested. Administration of DOTAP+PST34A significantly inhibited tumor growth when compared with the other groups (NS, DOTAP alone and PST34A alone; all $P<0.05$; Fig. 1). There were no statistical differences in body weight among the different groups (NS, 18.03±1.37 g; DOTAP, 17.89±2.04 g; PST34A, 19.02±2.72 g; DOTAP+PST34A, 16.98±1.55 g). Liposome-plasmid complex significantly decreased the number of tumor nodules to 5.33±3.80 compared with the other groups (NS, 50.19±25.78; DOTAP, 56.89±20.13; PST34A, 80.92±30.12; all $P<0.05$; Fig. 1B). The mean volume of ascitic fluid collected from mice in the DOTAP+PST34A group (0.21±0.09 ml) significantly decreased compared with the other groups (NS, 1.31±0.03 ml; DOTAP, 1.29±0.79 ml; PST34A, 2.03±1.21 ml; all $P<0.05$; Fig. 1C). Furthermore, a decrease in abdomen circumference and tumor weight were observed in the DOTAP+PST34A group compared with all other groups [(NS, 7.03±0.49 cm; DOTAP, 6.22±0.98 cm; PST34A, 7.38±0.74 cm; DOTAP+PST34A, 5.64±0.13 cm; all $P<0.05$; Fig. 1D) and (NS, 1.60±0.69 g; DOTAP, 1.19±0.89 g; PST34A, 2.10±0.64 g; DOTAP+PST34A, 0.05±0.02 g; all $P<0.05$; Fig. 1E), respectively].

*Evaluation of apoptosis in the ascitic fluid*. Flow cytometry was performed to detect the rates of apoptosis in ascitic fluid following the administration of the liposome-plasmid complex. DOTAP+PST34A significantly increased the number of cells in the sub-G1 phase (apoptotic cells; 10.21±3.43%) compared with all other treatments (NS, 5.83±1.51%; DOTAP, 4.96±0.98%; PST34A, 6.78±1.39%; all $P<0.05$; Fig. 2).

*Results of H&E and immunohistochemical staining*. Morphology of tumor tissue was observed in the present study. Characteristic morphological alterations, including cell shrinkage, chromatin condensation and nuclear fragmentation were observed in the DOTAP+PST34A group compared with other groups (Fig. 3). Apoptotic cell and certain lymphatic cell infiltrations were observed in the DOTAP+PST34A group. By contrast, there were more tumor cells with apparently enlarged atypical nuclei in the NS group. There were no significant pathological alterations in the heart, liver, spleen, lung and kidney samples following treatments (data not shown).

In the present study there were significantly fewer (59±6) CD34-positive cells in the DOTAP+PST34A treated group, compared with the 89±31 CD34-positive cells in the NS, 110±42 in the DOTAP and 101±39 in the PST34A groups (all $P<0.05$; Fig. 4). Treatment with DOTAP+PST34A resulted in significant inhibition of angiogenesis, compared with the control group (all $P<0.05$). The effects of DOTAP+PST34A treatment on cervical tumor cell proliferation were determined by Ki67 staining. The positive/negative expression rate of Ki67 in the DOTAP+ST34A group was 23±9%, which was significantly lower compared with the other treatments (NS, 70±17%; DOTAP, 45±13%; PST34A, 68±14%; all $P<0.05$; Fig. 5).

## Discussion

Survivin expression is associated with inhibition of apoptosis, increased tumor aggressiveness and poor survival (20). Survivin gene repression can induce tumor cell apoptosis and increase sensitivity to radiotherapy and chemotherapy in human cervical cancer lines (21). In the present study, liposome-plasmid DNA encoding mutant survivin-T34A was constructed and revealed that DOTAP+PST34A exhibited antitumor abilities in cervical cancer cells. CD34 staining also revealed that DOTAP+PST34A treatment markedly decreased MVD, which indicated that the plasmid together with the liposome could result in inhibition of tumor growth by preventing the formation of novel vessels. Furthermore, decreased level of Ki67 staining following treatment with the plasmid-liposome complex, demonstrated that the plasmid-liposome complex could inhibit the proliferation of tumor cells.

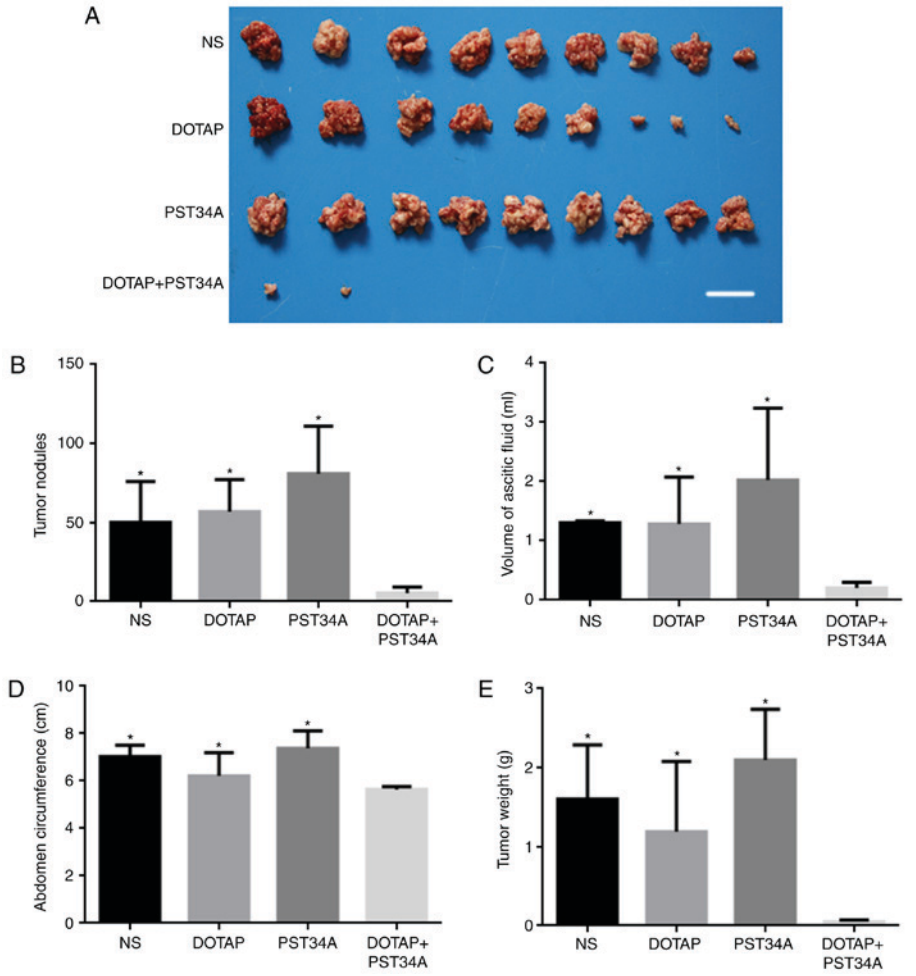


Figure 1. Antitumor effects of liposome-PST34A. (A) Representative images of tumor tissues. Scale bar, 10 mm. (B) The number of tumor nodules, (C) the volume of ascites fluid, (D) the abdomen circumference and (E) tumor weight were analyzed. Data are presented as the mean ± standard deviation. *P<0.05 vs. the DOTAP+PST34A group. NS, normal saline; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PST34A, plasmid encoding mutant survivin-T34A.

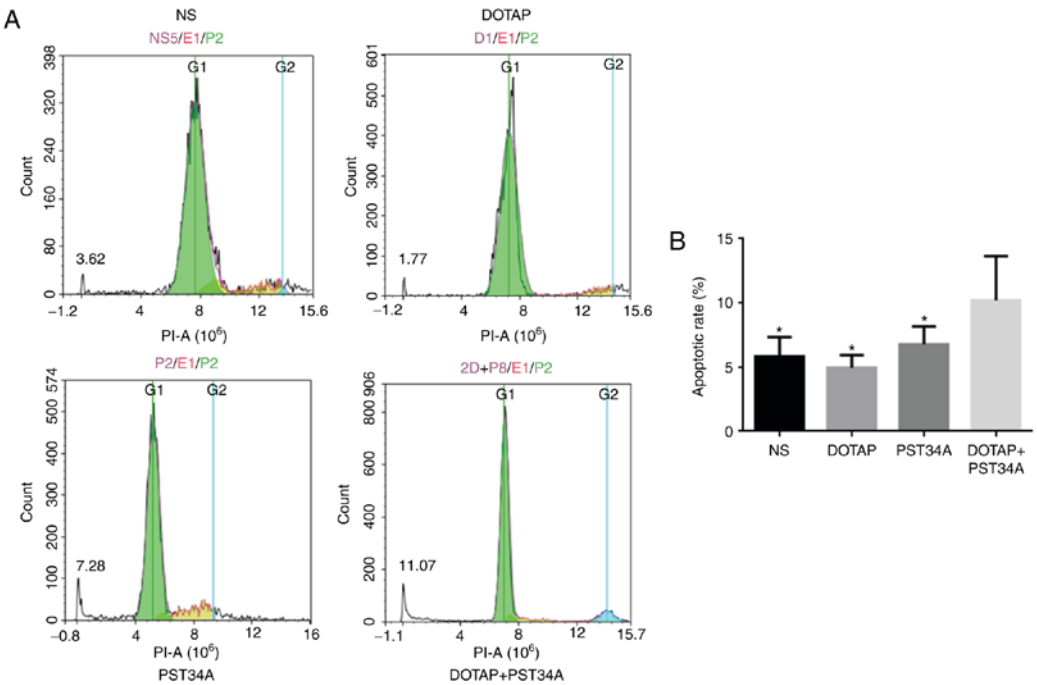


Figure 2. Liposome-PST34A induces apoptosis in ascitic fluid. (A) Flow cytometry was performed to analyze (B) the apoptotic rate across the different groups. Data are presented as the mean ± standard deviation. *P<0.05 vs. the DOTAP+PST34A group. NS, normal saline; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PST34A, plasmid encoding mutant survivin-T34A; PI, propidium iodide.

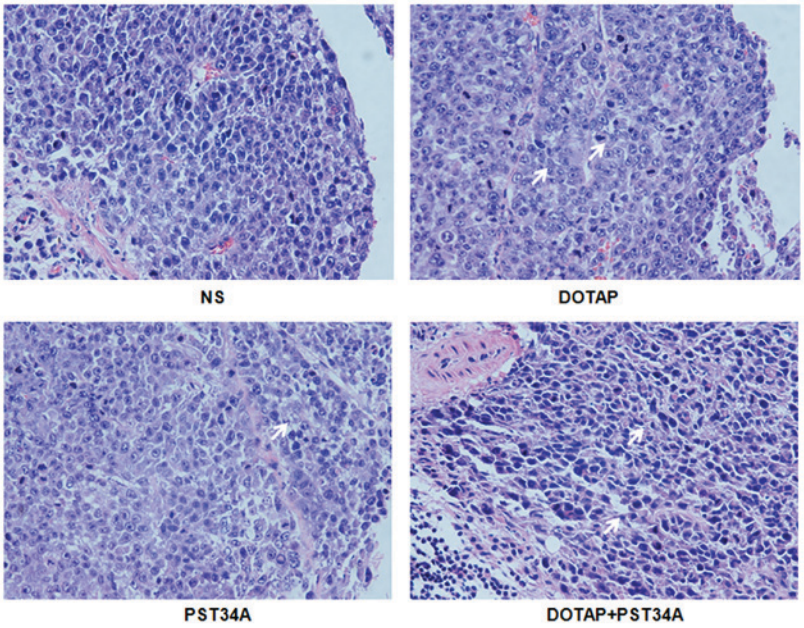


Figure 3. Hematoxylin and eosin staining of the tumor tissues. Morphological alterations, including cell shrinkage, chromatin condensation and nuclear fragmentation (arrows), were observed in the DOTAP+PST34A group (magnification, x400). NS, normal saline; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PST34A, plasmid encoding mutant survivin-T34A.

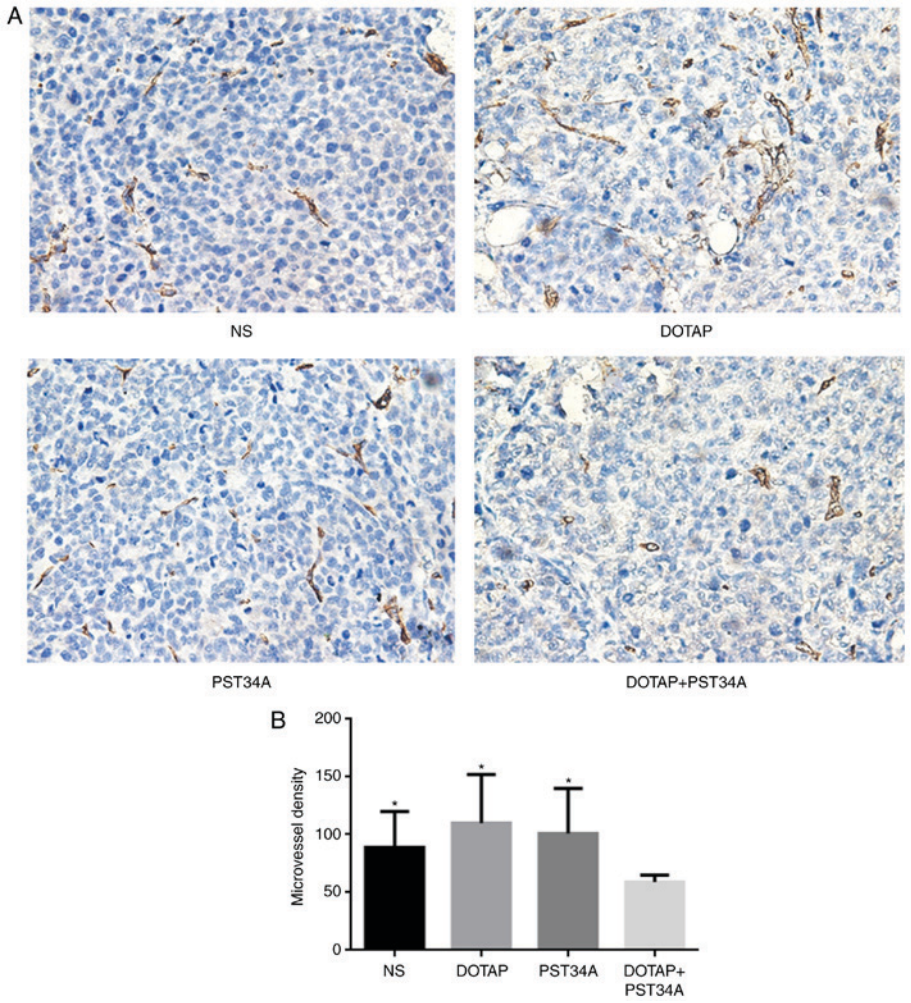


Figure 4. Liposome-PST34A inhibits angiogenesis assayed by CD34 staining. (A) Microvessel density was detected using an antibody against CD34 under a light microscope (magnification, x400). (B) Quantification of angiogenesis was performed by calculating the number of CD34-positive cells. Data are presented as the mean ± standard deviation. *P<0.05 vs. the DOTAP+PST34A group. NS, normal saline; DOTAP, 1,2-Dioleoyl-3-trimethylammonium-propane; PST34A, plasmid encoding mutant survivin-T34A.

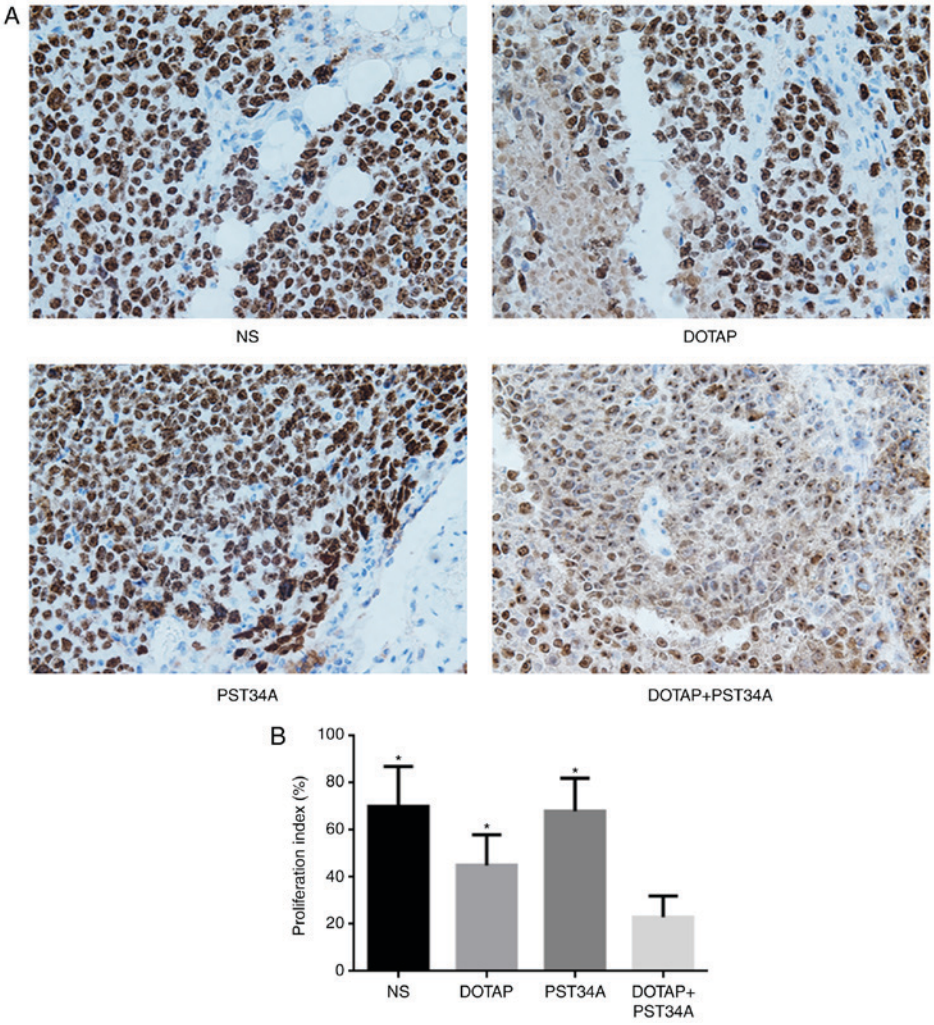


Figure 5. Liposome-PST34A inhibits cell proliferation determined by Ki67 analysis. (A) Representative images of tumor tissues (magnification, x400). (B) Proliferative index was expressed as the percentage of Ki67 positive to negative cells in tumor slices across the different groups. Data are presented as the mean ± standard deviation. *P<0.05 vs. the DOTAP+PST34A group. NS, normal saline; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PST34A, plasmid encoding mutant survivin-T34A.

Survivin is an inhibitor of apoptosis that regulates cell proliferation. To exert its function, its threonine 34 (Thr34) residue is phosphorylated by the cyclin-dependent kinase p34-cyclin B1 protein complex (22). Loss of Thr34 phosphorylation results in dissociation of the caspase-9-survivin protein complex, leading to caspase-dependent apoptosis (21). Adenoviruses encoding mutant survivin-T34A can promote spontaneous apoptosis in breast carcinoma (23). Furthermore, liposome-plasmid DNA PST34A can sensitize Lewis lung carcinoma cells to cisplatin-based chemotherapy and to radiation (24,25). The present study demonstrated that i.p. injection with liposome-plasmid DNA PST34A could inhibit the tumor growth of cervical cancer *in vivo*. By contrast, PST34A did not affect the viability of normal human cells, including fibroblasts, endothelium and smooth muscle cells (21). The above observations together implicate that liposome-plasmid DNA PST34A is a potential candidate for cancer therapy. Phosphorylated Thr34 and p34 kinase have not been previously detected in normal tissues, though they were upregulated in the advanced stage cancer tissues (26). Survivin-dependent apoptosis could improve the efficacy of a number of agents used to treat cancer. The above results supported the previously reported apoptosis-inducing ability of PST34A (27). The present study also demonstrated that inhibition of tumor growth was associated with suppression of angiogenesis as previously demonstrated (28,29).

Similar to other targeted therapies used in recent years, there remain several limitations for gene therapies. The relatively low efficiency of integration is a disadvantage. In order to improve the efficiency, cationic liposome delivery system was used in the present study, which represents the most common tool in gene therapy (30). The other limitation may result from a diversity of mutations in tumor cells and a variety of histological types of tumors. Additionally, an empty vector control was not used in the present study and this is a limitation of the study, as the background effects of the pVITRO2 plasmid could not be ascertained. In the future study, a pVITRO2 plasmid should be included in the study to confirm the specific function of PST34A delivered by DOTAP.

In conclusion, the present study demonstrated that the liposome-plasmid complex PST34A can efficiently inhibit the growth of cervical cancer *in vivo*. The mechanism potentially involves two aspects: Induction of apoptosis of tumor cells and inhibition of tumor-associated angiogenesis. In the present

study, the targeted strategy using survivin dominant negative mutant mediated by liposome represents an alternative approach to survivin gene therapeutic strategies.

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