Cationic liposome-mediated nitric oxide synthase gene therapy enhances the antitumor effects of cisplatin in lung cancer

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Abstract. Cisplatin is one of the most effective antitumor drugs for non-small cell lung carcinoma (NSCLC) patients. However, its efficacy has encountered a plateau due to its side effects and drug resistance. Inducible nitric oxide (NO) synthase (*iNOS*) gene therapy has been reported to have antitumor effects in several types of cancers and enhances sensitivity to cisplatin, but the effects of *iNOS* gene therapy alone or its combination with cisplatin in lung cancer remain unclear. In the current study, we evaluated the effects of cationic liposome (LP)-mediated *iNOS* gene transfection on enhancing low-dose cisplatin-mediated antitumor effects in the A549 human lung adenocarcinoma cell line *in vitro*. Furthermore, we examined whether *iNOS* gene therapy enhances the antitumor effects of low-dose cisplatin in two A549 human lung cancer cell xenograft mouse models. The results revealed that *iNOS* gene therapy may significantly enhance low-dose cisplatin-mediated inhibition of cell proliferation, invasion, migration and promotion of cell apoptosis in A549 cells. Intratumoral administration of the LP-pVAX-*iNOS* complex significantly enhanced low-dose cisplatin-mediated suppression of subcutaneous tumor growth. Moreover, intravenous injection of the LP-pVAX-*iNOS* complex greatly enhanced low-dose cisplatin-mediated inhibition of experimental lung metastasis and prolonged the life span of mice without significant organ-related toxicity in a nude mouse model of lung metastasis compared to the cisplatin alone-treated group. Furthermore, *iNOS* gene-mediated enhancement of cisplatin-mediated antitumor effects in lung cancer may be related to the attenuation of p-mTOR, MMP2 and the activation of p-p53. Thus, the combination treatment with *iNOS* gene therapy and cisplatin may be a novel and effective therapeutic strategy for lung cancer.

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Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide. Among all types of lung cancers, non-small cell lung carcinoma (NSCLC) accounts for approximately 85% of all lung cancer cases (1). As the first-line chemotherapeutic agent in lung cancer, cisplatin, has encountered a plateau due to its side effects and lack of specificity (2,3). Therefore, it is particularly urgent to discover methods by which to enhance the chemosensitivity of cisplatin.

Inducible nitric oxide (NO) synthase (*iNOS*) is a new promising cancer target gene involved in NO-mediated antitumor effects (4-7). It was first identified and characterized in cytokine-activated murine macrophages (5). The relatively lower expression of the *iNOS* gene was observed in several types of human cancers, where it is difficult to maintain high levels of NO for long periods of time (6,8,9), while full expression of the *iNOS* gene may generate high concentrations of NO for prolonged periods of time (10). Generally, cytokine stimulation, NO donor and *iNOS* gene transfer have been identified as the main methods used to upregulate the expression of *iNOS* and further produce high concentrations of NO in human cancer cells (4). Among all known methods, however, cytokine stimulation and NO donor may cause hypotension, drug resistant, toxic and other side effects, while *iNOS* gene transfer may essentially avoid the above side effects (11,12). *iNOS* gene transfer has a unique feature of a bystander effect, which would be an absolute requirement to the future success of cancer gene therapy as a contribution to the high efficiency of gene transfer (13,14).

The forced high level of NO was reported to have antitumor activities (15). In addition, clinical studies have demonstrated that overexpression of the *iNOS* gene may increase the survival of colorectal, ovarian and NSCLC patients (9,16,17). The apparent antitumor effects of a high NO level generated by *iNOS* gene transfer have been confirmed in

several types of cancers including breast, colorectal, prostate, ovarian, melanoma, kidney, thyroid cancer *in vitro* and *in vivo* (7,18-24). Thus, high concentrations of NO generated from the full expression of the *iNOS* gene may play an important role in cancer treatment.

Notably, studies have also demonstrated that high concentrations of NO generated from *iNOS* gene transfer, cytokine stimulation or NO donor may enhance the cytotoxicity of the chemotherapeutic drug cisplatin in RIF-1 tumors, ovarian cancer, leukemia, prostate cancer, colon cancer cells and lung fibroblasts (18,25-27). It is proposed that NO enhances cisplatin toxicity through inhibition of the repair enzymes that act on cisplatin-induced DNA damage (18), but the exact mechanisms of the enhanced effects of *iNOS* gene therapy and cisplatin in human cancers remain unclear. The antitumor effects of high levels of NO generated from *iNOS* gene transfer combined with cisplatin in lung cancer cells have yet to be reported.

Previous studies have demonstrated that the activity of the *iNOS* gene was observed in lung adenocarcinoma compared with normal tissues (28,29) and the *iNOS* gene expression levels are moderate in NSCLC cells as well as in other cancer cells (9). As the stages developed, the expression of *iNOS* protein gradually decreased in NSCLC cells. The intense expression of NOSs including the *iNOS* gene seems to be a favorable prognostic sign in NSCLC patients. Based on this, we hypothesize that delivering a high level of the exogenous *iNOS* gene by gene therapy in lung cancer may generate a large amount of NO and thus enhance the effects of cisplatin in lung cancer treatment.

To identify the hypothesis, in the current study, we first evaluated the effects of cationic liposome (LP)-mediated *iNOS* gene transfection on enhancing low-dose cisplatin-mediated antitumor effects in the human lung adenocarcinoma A549 cell line *in vitro*. Based on the *in vitro* results, we then aimed to demonstrate that intratumoral delivery of the LP-pVAX-*iNOS* complex enhanced low-dose cisplatin-mediated suppression of tumor growth. Meanwhile, systemic delivery of the LP-pVAX-*iNOS* complex enhanced low-dose cisplatin-mediated inhibition of experimental lung metastasis and prolonged animal survival. Apoptosis was further detected in the subcutaneous tumor tissues. Furthermore, we primarily detected several cellular targets involved in NO- and/or cisplatin-mediated signaling pathways during the antitumor procedure to understand the molecular mechanism of the enhanced effects by western blotting. This study may provide a new method for clinically effective treatment of lung cancer.

Materials and methods

Cell cultures, chemotherapeutic drug and animals. Human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was grown in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified 5% CO_2 atmosphere. Cisplatin was obtained from the West China Hospital pharmacy. Female athymic BALB/c nude mice were purchased from the Shanghai Laboratory Animal Centre (SLAC, Shanghai, China). The mice were housed in laminar flow cabinets under specific pathogen-free conditions. All animal experiments were performed in accordance with the institutional guidelines established for animal care and use.

Construction of iNOS gene expression vector and preparation. According to the *iNOS* cDNA coding sequence (GeneBank serial no., BC130283.1), the CDS sequence of the *iNOS* gene was cloned from *iNOS*-pCR4-TOPO plasmid (Open Biosystems, USA) with a PrimeSTAR™ HS PCR kit (Takara, Dalian, China) and connected to the pVAX plasmid vector (Invitrogen Life Technologies, Carlsbad, CA, USA). Pure pVAX and pVAX-*iNOS* plasmids were prepared using an EndoFree™ Plasmid Giga kit (Qiagen, Chatsworth, CA, USA), measured for concentration using a spectrophotometer and diluted to 1 μg/μl of DNA.

Preparation of plasmid LP-DNA complexes for cell transfection and animal treatment. Preparations of plasmid LP-DNA complexes were previously described (30). Briefly, pVAX/pVAX-*iNOS* plasmid and LPs diluted in equal volumes of RPMI-1640 were mixed to form LP-DNA complexes (LP-pVAX and LP-pVAX-*iNOS*) according to their molecular weight ratio (1:6). Cells were transfected with the complexes for 4 h and replenished with RPMI-1640 medium supplemented with 10% FBS and *iNOS* gene co-factor tetrahydrobiopterin (BH_4) (Sigma, St. Louis, MO, USA) ($1x10^{-5}$ M) if cells were transfected with LP-pVAX-*iNOS* and incubated for another 48 h (18,19,21,22). The transfection efficiency was evaluated by a parallel transfection with an equal amount of enhanced green fluorescent protein-expressing plasmid vector p-EGFP-N1 (Clontech, Beijing, China) in each of the cell lines.

For the animal experiments, pVAX/pVAX-*iNOS* plasmid and LPs diluted in equal volume of 5% glucose were mixed to form a final concentration of 20 μg DNA-4 mM LPs in 200 μl final volume (weight ratio 1:7). Particle size and ζ-potential of plasmid LP-DNA complexes were measured by a Zeta Nano series (Malvern Instruments, Herrenberg, Germany) at room temperature. The average particle size of the complexes was limited from 200 to 350 nm and the average ζ-potential of the complexes was limited from 20 to 35 mV.

Cell viability assay. To assess the sensitivity of the A549 cells to cisplatin, the 3-(4,5)-dimethylthiahiazo(-z-yl)-3,5-di-phenytetrazoliumromide (MTT) (Sigma) assay was used. Cells grown in 96-well plates were transfected with LP-DNA complex using the method described above. The transfection media were replaced with fresh medium containing cisplatin with serial concentrations (0.0-15.0 μM) and incubated for another 48 h. Twenty microliters of MTT in each well together with 180 μl RPMI-1640 were added and incubated for another 4 h. Absorbance at 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). Both the IC_{50} and IC_{20} values of cisplatin were calculated using the Origin 8 software (OriginLab Corporation, Northampton, MA, USA; www.OriginLab.com). To detect the inhibition of A549 cell proliferation in the different treatment groups, cells seeded in 96-well plates were treated with LPs-DNA and/or an IC_{20} dose level of cisplatin. The cell viabilities after a 48-h treatment were quantified by MTT assay as described above. IC_{20} dose level of cisplatin was used in all of the following *in vitro* experiments.

Cell apoptosis assay. To determine the apoptosis rates of A549 cells following different treatments, Hoechst 33258 staining and an Annexin V-FITC Apoptosis Detection kit (KeyGEN, Nanjing, China) and flow cytometry [fluorescence-activated cell sorting (FACS)] were used according to the manufacturer's instructions. A549 cells following different treatments were stained with Hoechst 33258 as previously described (31). Additionally, cells with the same treatments were rinsed with pre-chilled PBS 3 times, trypsinized with EDTA-free trypsin and then rinsed with PBS supplemented with 2% BSA. Cells were then labeled by Annexin V-PI reagent and analyzed with the aid of FACS for cell apoptosis analysis.

Cell invasion and migration assays. Evaluation of the invasion and migration abilities was performed using cell invasion and migration assays. After treatment with LPs-DNA and/or low-dose cisplatin for 24 h, cells were harvested and $4x10^4$ cells of each treatment in RPMI plus 1% FBS were replaced in the upper chamber. To assess cell invasion, membranes of the Boyden cell system were coated with BD Matrigel™ (all were from BD Biosciences, Franklin Lakes, NJ, USA). The bottom chamber was filled with RPMI containing 10% FBS as a chemoattractant. After being incubated for another 24 h, the attached cells in the lower section of the chamber were stained with 0.1% crystal violet solution (Sigma). The number of invading cells was manually counted as the sum of 3 randomly selected fields at a x20 magnification. The same experimental design was used for the migration experiments except that the membranes were not coated with BD Matrigel; RPMI plus 1% FBS were placed in the lower chamber of Millicell systems (Millipore, Billerica, MA, USA).

Animal studies

In vivo lung metastasis nude mouse model. Lung metastatic tumors were established via tail vein injection of $2x10^6$ A549 cells in a volume of 200 μl of RPMI-1640 into female BALB/c athymic nude mice (3-4 weeks old). On Day 12 after cell injection, animals were randomly divided into 6 treatment groups (5 mice/group) including glucose, LP-pVAX, cisplatin, LP-pVAX plus cisplatin, LP-pVAX-*iNOS* and LP-pVAX-*iNOS* plus cisplatin groups. Gene therapy was administered through tail vein injection at a dose of 20 μg/mouse of the LP-DNA complexes. Twenty-four hours later and immediately prior to i.p. injection of low-dose cisplatin (2 mg/kg/mouse), all mice (including the controls) were injected i.p. with 200 μl of 10^{-3} M BH_4 as previously described (18,19,21,22). Mice were treated every 3 days and a total of 4 injections were administered to all mice. On the 14th day after the last treatment, all mice were anesthetized and their lungs were filled with India ink to count the number of metastases. In addition, the same lung metastasis mouse model (5 mice/group) was established to analyze the effect of the combination treatment on mice survival. After the same administration, mice were fed until all mice in the glucose group were sacrificed. Mice survival curves were assessed according to the Kaplan-Meier method.

In vivo subcutaneous tumor nude mouse model. A subcutaneous tumor nude mouse model was obtained by intradermal injection with $5x10^6$ A549 cells in a volume of 100 μl of RPMI-1640 in the right flank of female BALB/c athymic nude mice (5-6 weeks old) as previously described (32). When the tumor volume reached ~80 mm^3, the same administration procedures for 6 treated groups (5 mice/group) as described above were used in this experiment except that mice were treated by the administration of intratumoral injection at a dose of 20μg/mouse of the LPs-DNA. Tumor size was measured with callipers in 3 dimensions twice every week. The tumor volume was calculated using the following formula: Volume (mm^3) = 0.52 x length (mm) x width (mm) x width (mm). When the average tumor volume reached ~1,000 mm^3 or the tumors were necrotic, all mice were sacrificed and the tumors were peeled off and fixed in formalin. Growth curves were plotted for each group. Efficiency of the combination treatment was also assessed by the time required for tumors to reach 3 times their volume from the commencement of treatment.

Analysis of subcutaneous tumor cell apoptosis and histology. To evaluate apoptosis, the fixed subcutaneous tumors were evaluated by *in situ* TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) staining using an *In Situ* Cell Death Detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions (33). The number of apoptotic bodies was counted from tumor tissues at x200 magnification in 20 randomly selected fields. The hematoxylin and eosin (H&E) double staining was applied to detect the histology of the tumor tissue and organic tissue paraffin sections as previously described (34).

Western blot analysis. Lung cancer cells following different treatments were collected and lysed in RIPA buffer supplemented with Protease Inhibitor Cocktail Set I (Merck KGaA, Germany). Equal amounts of lysate proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels, electrotransferred onto PVDF membranes (Millipore) and probed with anti-*iNOS*, anti-MMP2 (Abcam, Cambridge, MA, USA), anti-phosho-p53, anti-p53, anti-phospho-mTOR, anti-mTOR (Cell Signaling Technology, Inc., Beverly, MA, USA) and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were detected using an enhanced ECL system (GE Healthcare Life Sciences, Piscataway, NJ, USA).

Statistic analysis. SPSS13.0 software was used. Each experiment was performed at least 3 times. The data are expressed as the means ± SD and one-way ANOVA and an unpaired Student's t-test were used to determine the significant differences of all the results. $P<0.05$ was considered to indicate a statistically significant difference.

Results

High expression level of iNOS protein on the sensitivity of A549 cells to cisplatin. In this study, the expression of *iNOS* protein in the A549 cell line after LP-pVAX-*iNOS* transfection was first detected by western blotting. The moderate expression level of *iNOS* protein was demonstrated in A549 cells, while LP-pVAX-*iNOS* transfection induced high expression of the *iNOS* protein in A549 cells (Fig. 1A). To evaluate whether enforced high expression of the *iNOS* gene sensitizes the response of A549 cells to

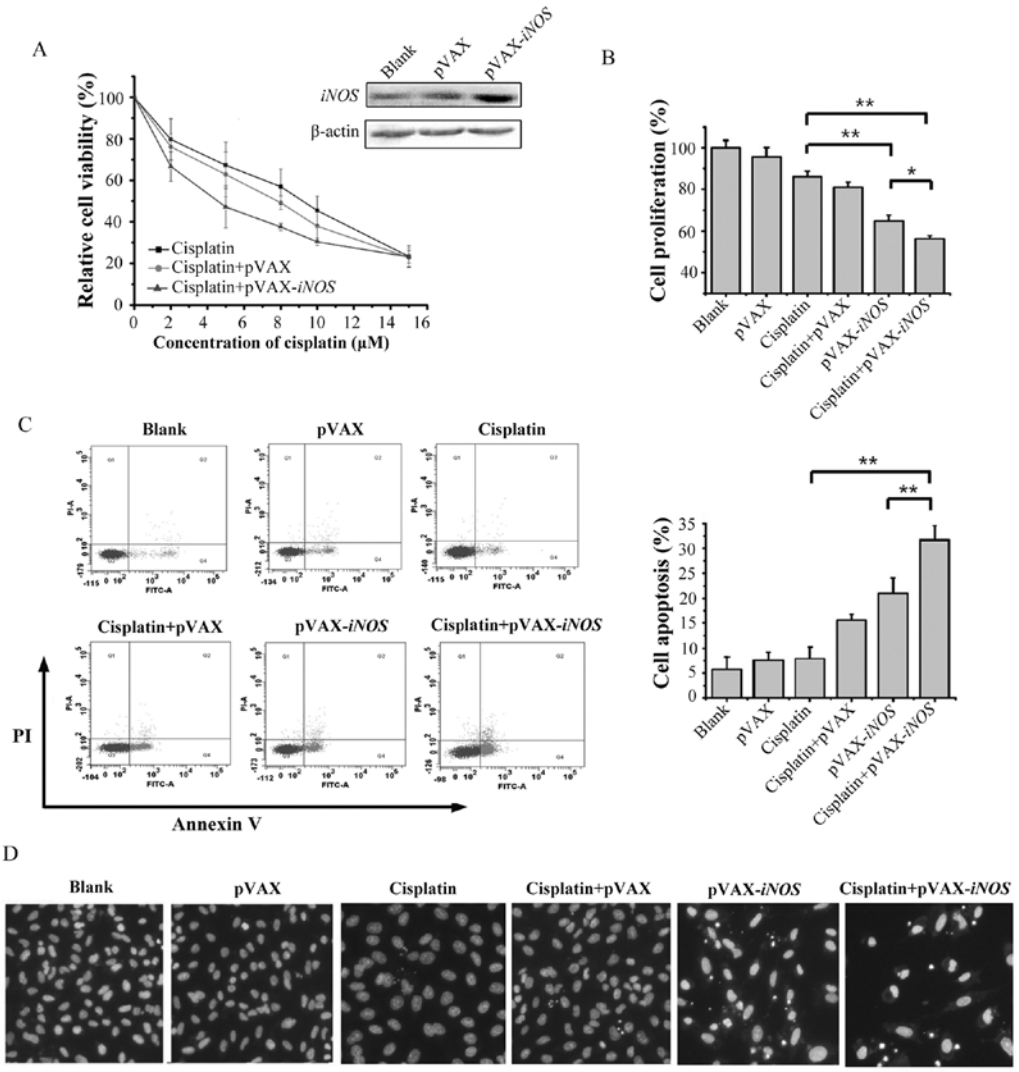

Figure 1. Effect of exogenously enforced high expression of *iNOS* on cisplatin sensitivity and low-dose cisplatin-mediated cell proliferation inhibition and cell apoptosis promotion in A549 cells. (A) The expression of *iNOS* protein in A549 cells transfected with LP-pVAX-*iNOS* was detected. Meanwhile, the sensitivity of A549 cells with the combination treatment of LP-pVAX-*iNOS* and a series of cisplatin concentrations for 48 h was analyzed by MTT assay. (B) The proliferation of A549 cells following the combination treatment of LP-pVAX-*iNOS* and cisplatin at an IC_{20} dose for 48 h was analyzed by MTT assay. (C) Cell apoptosis following different treatments was detected by FCM. (D) Apoptosis following different treatments was detected by Hoechst 33258 staining. Columns, the means of 3 individual experiments; bars, SD. *P<0.05; **P<0.01; Blank, untreated group.

cisplatin, we analyzed the cell viability of the A549 cells following cisplatin treatment alone or the combination treatment with LP-pVAX-*iNOS* transfection and cisplatin by MTT assay. The IC_{50} value of cisplatin in the A549 cell line was decreased from 8.80±1.95 μM in the cisplatin alone group to 5.08±0.73 μM in the combination treatment group. Therefore, the results suggest that exogenously enforced high expression of the *iNOS* gene significantly enhances the sensitivity of A549 cells to cisplatin.

Enhanced proliferation inhibition following the combination treatment of LP-pVAX-iNOS and low-dose cisplatin. To examine whether the exogenously enforced high expression of the *iNOS* gene enhances cisplatin-mediated cell proliferation inhibition, the IC_{20} dose level (3.05±1.25 μM) of cisplatin in A549 cells was adopted in the following experiments. The cell viability of A549 cells following different treatments was assessed by MTT assay. There was >30 and 15% of an average decrease in cell viability at 48 h in the combination treatment group compared with the low-dose cisplatin alone or *iNOS* gene alone groups (P<0.01) (Fig. 1B). The results indicated that the exogenously enforced high expression of the *iNOS* gene significantly enhances the proliferation inhibition of low-dose cisplatin in A549 cells.

Enhanced induction of apoptosis following combination treatment of LP-pVAX-iNOS and low-dose cisplatin. To evaluate whether the enforced high expression of the *iNOS* gene is implicated in the reactivity of cells to cisplatin-induced apoptosis, both Hoechst 33258 and Annexin V-PI staining by flow cytometry were used. Nuclear condensation, cleavage fragments and additional apoptotic bodies appeared in the A549 cells following the combination treatment, while these were rarely noted in the low-dose cisplatin alone or *iNOS* gene alone treated groups (Fig. 1D). Moreover, the results of Annexin V and PI double staining demonstrated

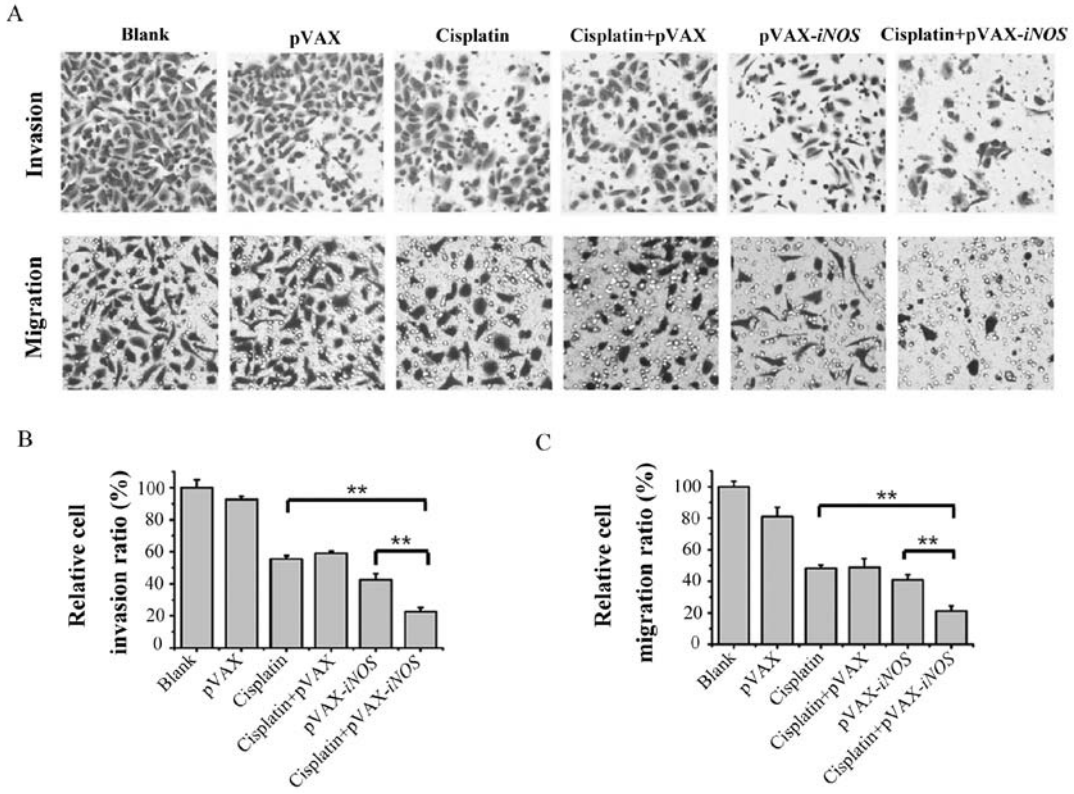

Figure 2. Cell invasion and migration of A549 cells following LP-pVAX-*iNOS* and/or low-dose cisplatin treatment. (A) Photomicrographs of A549 cells in the lower section of the Boyden cell and Millicell chamber stained with crystal violet. (B) The relative cell invasion ratio of A549 cells under different treatment conditions. (C) The relative cell migration ratio of A549 cells under different treatment conditions. Columns, the means of 3 individual experiments; bars, SD. **P<0.01. Blank, untreated group.

that the early apoptosis rate in cells following treatment of low-dose cisplatin alone or *iNOS* gene alone was an average of 8.00 or 20.97%, while significantly enhanced induction of early apoptosis was observed (an average of 31.7%) in the combination treatment group (Fig. 1C). Taken together, these results implied that the exogenously enforced high expression of the *iNOS* gene may significantly enhance low-dose cisplatin-mediated cell apoptosis (P<0.01).

Enhanced inhibition of cell invasion and migration abilities by the combination treatment with LP-pVAX-iNOS and low-dose cisplatin. As distant metastasis is responsible for the failure of lung cancer treatment, assessment of cell invasion and migration ability is significantly important for studying cancer treatment. Moderate inhibition (an average of 44.4 and 57.3%, respectively) of invasion was observed in the A549 cells treated with low-dose cisplatin or the *iNOS* gene alone, while the combination treatment resulted in significant inhibition (an average of 77.4%) of invasion in the A549 cells (P<0.05) (Fig. 2A and B). Similar results were obtained in the Boyden Millicell assay. As shown in Fig. 2A and C, the changes in the inhibition of migration (an average of 51.8 and 59.1% respectively) were also considered modest in the A549 cells treated with low-dose cisplatin or the *iNOS* gene alone, but more significant suppression (~78.8%) was observed in the cells following combination treatment with LP-pVAX-*iNOS* and low-dose cisplatin (P<0.05). These results indicated that the *iNOS* gene may significantly enhance the low-dose cisplatin-mediated inhibition of cell invasion and migration in A549 lung cancer cells.

Enhanced tumor growth inhibition and apoptosis induction by the combination treatment with LP-pVAX-iNOS and low-dose cisplatin in vivo. As the enhanced antitumor activity in A549 cells in the combination treatment was observed *in vitro*, we hypothesized that the same effects may emerge *in vivo*. To verify this assumption, we established a human A549 lung cancer metastasis mouse model and a subcutaneous tumor xenograft mouse model to evaluate the effects of the *iNOS* gene on cisplatin-induced tumor regression. In the human A549 lung cancer metastasis mouse model, as shown in Fig. 3A and C, consistent with the *in vitro* experiment, the combination treatment significantly inhibited tumor growth with an average reduction of 92.37% compared with an average reduction of 37.25 and 79.67%, respectively, in low-dose cisplatin- or *iNOS* gene-mediated tumor growth inhibition (P<0.01). Meanwhile, we evaluated the combination treatment on animal survival in the human A549 lung cancer metastasis mouse models. The combination treatment resulted in a significant and prolonged survival (mean survival time, 200.8±11.2 days) compared to the group treated with low-dose cisplatin alone (mean, 133.6±22.2 days) or treated with LP-pVAX-*iNOS* alone (mean, 181.2±8.5 days) (Fig. 3B).

In addition, the A549 cell subcutaneous tumor xenograft mouse model was also established to explore the effects of the *iNOS* gene on enhancing the cisplatin-induced antitumor

Table I. Time (days) required for tumors to grow to three times their volume from the day of treatment.

Treatment	Glucose	pVAX	Cisplatin	Cisplatin + pVAX	pVAX-*iNOS*	Cisplatin + pVAX-*iNOS*
Time to reach 3 times treatment volume (days)	8.53±1.04	13.30±3.76	13.53±0.94	15.18±0.88	18.04±1.72	20.34±0.88

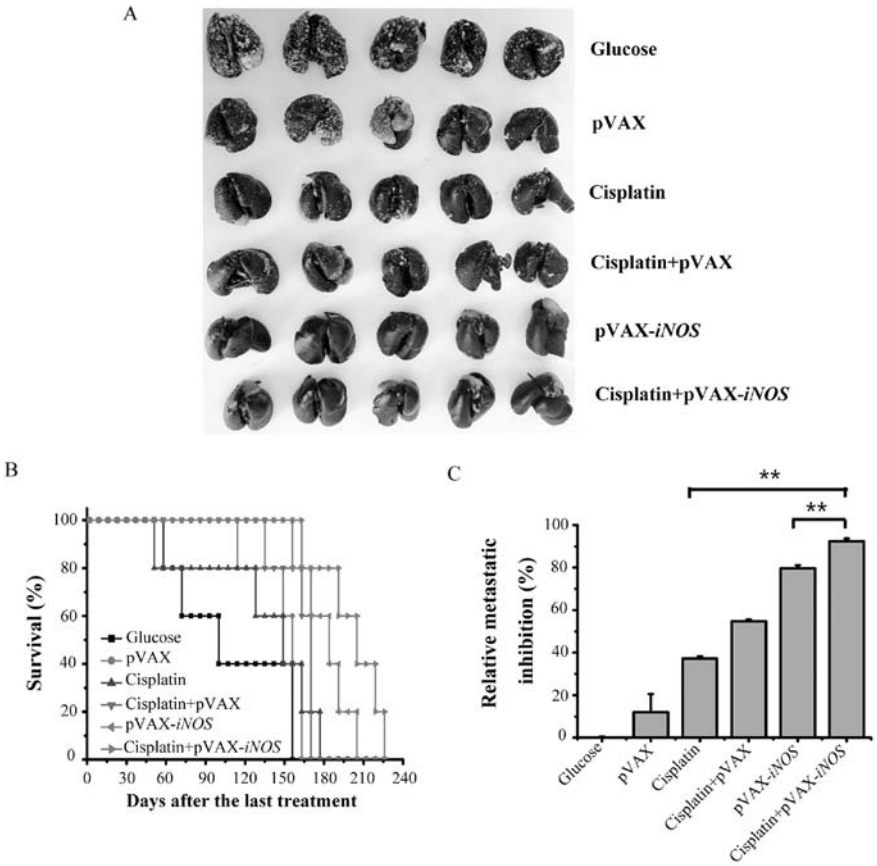

Figure 3. Effect of the combination treatment with LP-pVAX-*iNOS* and low-dose cisplatin on tumor growth and the life span of mice in the lung metastasis nude mouse model. (A) Metastatic tumors in the lungs of the 6 treatment groups. On the 14th day after the last treatment, mice were anesthetized and their lungs were filled with India ink to examine the number of metastases. (B) Combination treatment prolonged the life span of the mice. Mouse survival curves were plotted according to the Kaplan-Meier method. (C) The percent of metastatic inhibition in each group. Columns, the means of 3 individual experiments; bars, SD. **P<0.01.

effects. Combination treatment significantly inhibited tumor growth with an average reduction of 71.99% compared with a tumor growth reduction of 19.65 and 51.71%, respectively following treatment with a low-dose cisplatin- or the *iNOS* gene alone (P<0.01) (Fig. 4A, B and D). The tumors in the glucose-treated groups increased 3 times in volume on day 8.53±1.04 after treatment while a single injection of 2 mg/kg cisplatin or LP-pVAX-*iNOS* slowed this growth in the tumor volume to 13.53±0.94 or 18.04±1.72 days, respectively (Table I). Moreover, the combination treatment significantly slowed tumor growth. Tumor growth 3 times the original volume in the combination treatment group was not reached until 20.34±0.88 days (P<0.05).

The peeled off subcutaneous tumor tissues were fixed in formalin and further used by TUNEL staining to analyze the apoptosis of tumor tissues. Histological analysis was also applied to detect necrosis/apoptosis in the formalin-fixed tumor tissues. As shown in Fig. 4C and E, the tumor tissues in the combination treatment groups displayed more TUNEL-positive nuclei and visible necrotic/apoptotic regions compared to that in the *iNOS* gene or low-dose cisplatin-treated groups. Histological analysis of the various organs demonstrated no significant treatment-related toxicity. Our results further demonstrated that the increased apoptosis in tumor tissues following the combination treatment may be responsible for the enhancement of low-dose cisplatin-induced antitumor effects *in vivo*.

Induction of p-p53 overexpression and suppression of p-mTOR and MMP2 expression by combination treatment with LP-pVAX-iNOS and low-dose cisplatin in vitro. Phosphorylation of p53 plays important roles in both cisplatin-

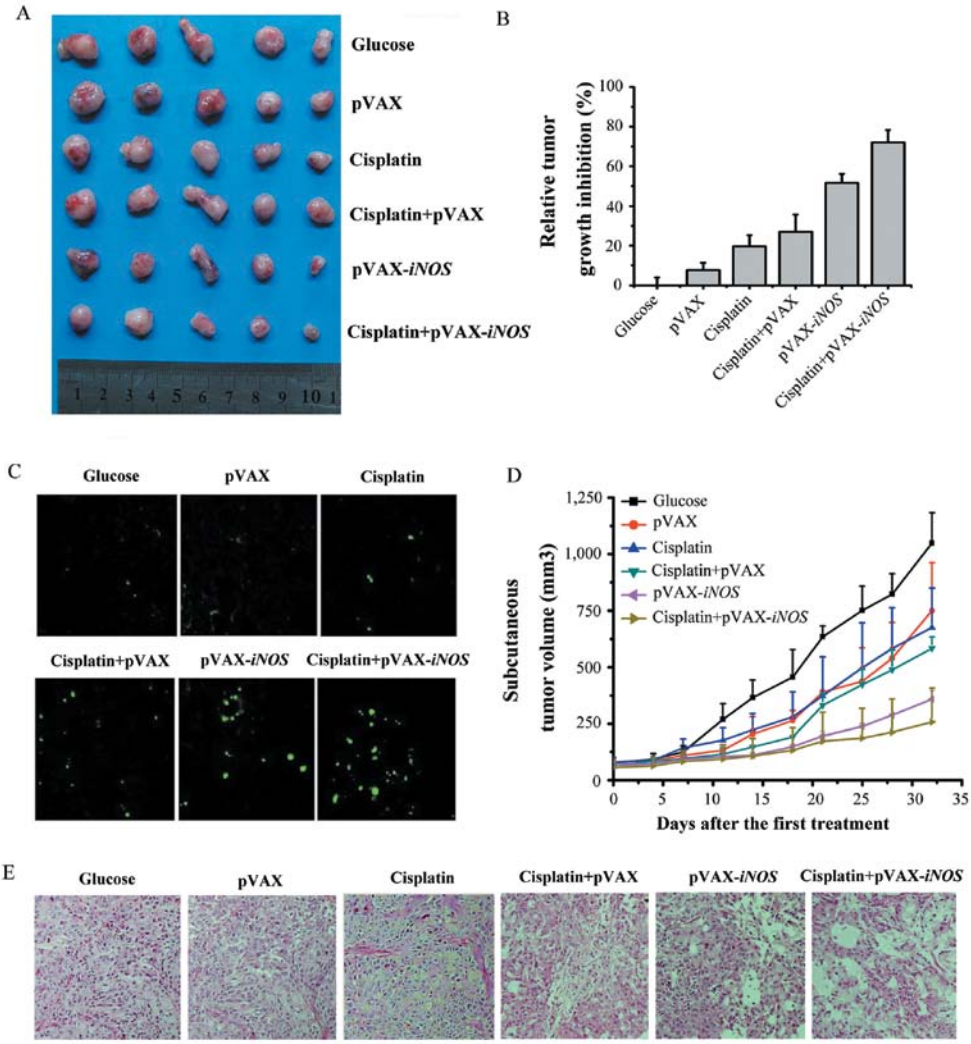

Figure 4. Effect of the combination treatment with LP-pVAX-*iNOS* and low-dose cisplatin on tumor growth and apoptosis in subcutaneous tumor nude mouse model. (A) Images of the subcutaneous tumor tissues peeled off from the nude mice in 6 groups. (B) The growth inhibition ratio of the subcutaneous tumor in each group. (C) The induction of apoptosis for the subcutaneous tumor tissues. After TUNEL staining of subcutaneous tumor tissues, discrete TUNEL fluorescence signals were observed. (D) The growth curves of each treatment group. Tumor volumes of the subcutaneous lung tumors were recorded at different time periods. (E) Histological analysis of the subcutaneous tumor tissues. The paraffin blocks embedded with subcutaneous mouse tumor tissues were used with H&E staining to analyze the general pathological changes with tumor tissues. Columns, the means of 3 individual experiments; bars, SD. $^{**}P<0.01$.

and NO-induced cell apoptosis (4,35,36). To evaluate whether phosphorylation of p53 was implied in the enhanced effects of the combination treatment, we detected the expression levels of p-p53 protein in the different treatment groups by western blotting. The expression of p-p53 protein was increased in cells treated with the *iNOS* gene or low-dose cisplatin alone, but a dramatic upregulated level of p-p53 protein was detected in cells following the combination treatment (Fig. 5).

mTOR is vital in mediating cisplatin sensitivity (37-39). However, the relationship of mTOR and NO in antitumor processes has not been reported. To examine whether a correlation exists between mTOR and NO, which may participate in the reactivity of cells to low-dose cisplatin after LP-pVAX-*iNOS* treatment, we analyzed the expression of phosphorylated mTOR protein in the differently treated cells by western blotting. Only a slight downregulation of p-mTOR was detected in cells treated with the *iNOS* gene or low-dose cisplatin alone, but a dramatic downregulation of p-mTOR was observed in cells after the combination treatment (Fig. 5).

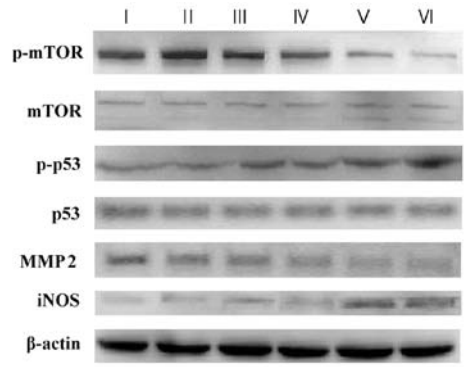

Figure 5. The *in vitro* expression of related proteins in the various treated cell groups by western blotting. After treatment with LP-pVAX-iNOS and/or cisplatin, A549 cells were collected and lysated. The total cellular lysates were analyzed by western blotting with special antibodies including, iNOS, p-mTOR, mTOR, p-p53, p53, MMP2 and β-actin (an internal control). Lane I, untreated group; lane II, pVAX treated group; lane III, cisplatin-treated group; lane IV, cisplatin plus pVAX-treated group; lane V, pVAX-*iNOS*-treated group; lane VI, cisplatin plus pVAX-*iNOS*-treated group.

MMP2 is also an important kinase in the process of cell invasion. To explore whether MMP2 protein was involved in the enhanced effects of the combination treatment, we also detected the expression levels of the MMP2 protein in the different treatment groups by western blotting. The expression level of the MMP2 protein in A549 cells after the combination treatment was also significantly downregulated compared with that in cells treated with the *iNOS* gene or low-dose cisplatin alone (Fig. 5).

Discussion

Cisplatin is one of the first-line chemotherapeutic drugs used in the clinical treatment of lung cancer patients. However, the side effects and drug resistance restrict its wide use. The important task of cancer therapy is to seek a suitable method to enhance the sensitivity of existing chemotherapeutic agents (25). Thus, refinement for enhancing the sensitivity of cisplatin is highly required.

As previous studies reported, high NO levels generated from *iNOS* gene transfer, cytokine stimulation or NO donor may have antitumor effects and even enhance the cytotoxicity of the chemotherapeutic drug cisplatin in RIF-1 tumors, ovarian cancer, leukemia, prostate or colon cancer cells (18,26,27). Among all the methods used for high concentrations of NO production, *iNOS* gene transfer may be accepted as a superior way due to its marked 'bystander' effect and safety (11,12,14). Only one article previously demonstrated that *iNOS* gene transfer treatment may increase the cytotoxicity and cause a delay in the growth of cisplatin-treated RIF-1 tumors, prostate and colon cancer cells (18). However, whether this combined efficacy is observed in other types of tumors remains unknown. Moreover, whether the combination treatment affects the invasion and metastasis of cancer cells, which is important in cancer treatment, remains unclear.

Consistent with the above-mentioned study, we also showed that the IC_{50} of cisplatin was reduced in A549 cells after transfection with the *iNOS* gene. The combination treatment significantly inhibited the growth of subcutaneous tumors. Most importantly, we further aimed to clarify that *iNOS* gene therapy may significantly enhance the antitumor effects of cisplatin through the promotion of cell apoptosis, as well as effective inhibition of proliferation, invasion and migration abilities *in vitro* and *in vivo*. In order to simulate the metastatic characteristics of lung cancer cells *in vivo*, an A549 lung metastatic tumor- bearing mouse model was established. The combination treatment significantly suppressed the formation of lung metastases via systemic administration of tail vein injection of LP-pVAX or LP-pVAX-*iNOS* (20 μg DNA/mouse) and/or i.p. injection of low-dose cisplatin (2 mg/kg/mouse) and dramatically prolonged the life spans of tumor-bearing mice with no significant organ-related toxicity. Systemic administration of the LP-DNA complex is a novel drug delivery method that has been shown to deliver genes effectively to the lungs when administered intravenously (40). More importantly, systemic administration is more suitable for lung cancer patients in clinical treatment procedure. To the best of our knowledge, this is the first report demonstrating that exogenously enforced high expression of the *iNOS* gene by cationic liposome (LP)-mediated *iNOS* gene transfer in lung cancer A549 cells increases the cisplatin sensitivity and significantly enhances the antitumor effects of cisplatin in human lung cancer A549 cells both *in vitro* and *in vivo*. The *iNOS* gene significantly enhanced the cisplatin-mediated inhibition of lung cancer cell migration and invasion. Tumor metastasis is responsible for approximately 90% of lung cancer-related death (41). The combination treatment with *iNOS* gene therapy and low-dose cisplatin would be a novel and potential strategy for lung cancer treatment (24).

The mechanisms of cisplatin- or NO-based antitumor activity have been basically reported (35-37,42,43). Studies suggest that both p53 and mTOR signaling pathways are important in cisplatin-mediated antitumor activity (35,37,42). Meanwhile, the antitumor effects of NO generated by *iNOS* gene transfer were reported to be at least partly dependent on the phosphorylation of p53 and MMP2 (36,44). However, the associated mechanisms of the combined effects of *iNOS* gene therapy and cisplatin in human cancers have not been reported. Thus, to reveal the molecular mechanisms of *iNOS* involved in enhancing low-dose cisplatin-mediated antitumor activities, we further detected the expression levels of these key proteins in NO-mediated and/or cisplatin-mediated signaling pathways by western blotting.

p53 is an important marker in the process of cell apoptosis (4), which is the main mode of cisplatin-induced cell death. Cisplatin sensitivity is closely related with the presence of the pro-apoptotic protein p53 (35). Meanwhile, studies have demonstrated that a high level of NO resulting from the high expression of the *iNOS* gene may promote cell apoptosis in melanoma, renal cell cancer and their adjacent cells (7,44). Cook *et al* (36) reported that the pro-apoptotic mechanisms of NO generated by *iNOS* gene transfer involved in the death signaling pathway were at least partly dependent on the phosphorylation of p53. When p53 was knocked out, the combination treatment of *iNOS* gene therapy and radiotherapy reduced the incidence of tumor cell apoptosis and antitumor effects in colon cancer cells. Consistent with previous studies, we also observed that *iNOS* gene therapy may significantly enhance the low-dose cisplatin-mediated apoptosis of human lung cancer A549 cells. Similarly, the dramatic upregulation of p-p53 protein expression was observed in the combination treatment group, while a slight upregulation of p-p53 protein expression was noted in the *iNOS* gene or cisplatin alone treatment group. The results indicated that *iNOS* gene therapy enhancing the antitumor effects of low-dose cisplatin in lung cancer may be partly related to the upregulated expression of p-p53 protein.

mTOR is a serine/threonine protein kinase, which plays an important role in the regulation of cell functions including cell proliferation, cell cycle, biosynthesis and cell migration (42). Meanwhile, mTOR protein is one of the widely studied kinases involved in the main signaling pathway of cisplatin. A high degree of intracellular phosphorylation of mTOR was often noted in several cisplatin-resistant cancer cells, including NLCLC cells (37,38), which indicates that inhibition of mTOR activity may enhance cancer cell sensitivity to cisplatin (37,39). Currently, the relationship between NO-mediated antitumor effects and p-mTOR protein expres-

sion has not been reported. To the best of our knowledge, our study first discovered that the expression of p-mTOR protein was significantly diminished in the combination treatment group compared with the *iNOS* gene or cisplatin alone-treated group. The results suggested that the downregulated expression of p-mTOR protein may be another probable reason for *iNOS* enhancing low-dose cisplatin-mediated inhibition of metastasis and invasion in lung cancer.

Metastasis is responsible for the poor effect of clinical treatment in lung cancer. Matrix metalloproteinases (MMPs) play a key role in the process of tumor metastasis (45). Karam *et al* (46) reported that cisplatin may inhibit the invasion and migration of human ovarian cancer cells by downregulating the expression of MMP2, TIMP1 and TIMP2. Another study also discovered that an increased amount of cisplatin resulted in a time- and dose-dependent decreased level of the MMP2 protein in transformed rat thyroid cancer cells (47). Meanwhile, *iNOS* gene activity was inversely related to the metastasis of tumor cells (4,7). NO may affect the invasion of mouse mammary adenocarcinoma through breaking the balance between MMP2 and its inhibitors, including tissue inhibitor of metalloproteinase (TIMP2 and TIMP3) (43). When the high concentration of NO occurred, the expression levels of MMPs gradually decreased (48). In our study, we discovered that *iNOS* gene therapy significantly reduced the low-dose cisplatin-mediated invasion and migration capacity in lung cancer A549 cells and the expression levels of the MMP2 protein were downregulated in the combination treatment group compared with the *iNOS* gene or cisplatin alone treated groups. The results indicated that *iNOS* gene therapy enhancing the antitumor effects of low-dose cisplatin in lung cancer may occur through the downregulation of MMP2 protein expression.

In conclusion, our study confirmed that the combination treatment with cationic LP-mediated *iNOS* gene therapy and low-dose cisplatin may significantly enhance cisplatin-mediated cell apoptosis and inhibition of cell proliferation, metastasis and invasion in human lung adenocarcinoma A549 cells *in vitro* and *in vivo*. The enhanced antitumor effects of low-dose cisplatin by *iNOS* gene therapy in lung adenocarcinoma is associated with the upregulation of p-p53 expression and the downregulation of MMP2 and p-mTOR protein expression. Therefore, the combination treatment of *iNOS* gene therapy and cisplatin is an effective strategy for the treatment of lung cancer.

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