

p53 Aerosol Formulation with Low Toxicity and High Efficiency for Early Lung Cancer Treatment

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Abstract Purpose: To develop an optimal nonviral aerosol formulation for locoregional treatment of early lung cancer.

Experimental Design: The formulation was made of polylysine/protamine combination (AND) as the carrier and *p53* gene (*p53sm*) as therapeutic agent. To estimate the aerosol deposition, the aerodynamic size of the AND-*p53sm* was measured with extrusion-precipitation method. To accurately determine the dose, the aerosol efficiency in mice was measured with a fluorescent dye. The transfection efficiency and DNA protection function of the aerosolized formulation in cultured cells and mouse lungs were detected with reporter gene assays and/or reverse transcription-PCR. The preclinical safety and efficacy of AND-*p53sm* were studied in healthy mice and mice bearing orthotopic human non-small-cell lung cancer (NSCLC) xenograft.

Results: After aerosolization, AND is 3- to 17-fold more effective than commonly used PEI or cationic lipid formulations in transfecting the NSCLC cells (relative light units, 1,494 versus 534 and 86; $P < 0.003$). Aerodynamic size of AND-*p53sm* ranged 0.2 to 3 μm is the optimal aerosol droplets for deposition in the entire human respiratory tract. Significant gene expression was detected in the lungs of mice given aerosolized AND-*p53sm* and AND-luciferase. Aerosolized AND-*p53sm* significantly prolonged the life of mice bearing orthotopic human NSCLC xenografts, and it was more effective than an optimal i.v. cisplatin chemotherapy (increased life span, 93% versus 25%; $P = 0.014$). Inhalation of AND produced low and reversible pulmonary toxicity and no systemic toxicity.

Conclusions: This optimal formulation is suitable for delivering biological materials to human lung with aerosol administration. This therapeutic strategy is an option for patients with early lung cancer and bronchoalveolar carcinoma.

Lung cancer is the number one cause of cancer-related death, causing an estimated one million deaths worldwide annually (1). Lung cancer originates in the bronchial epithelium as a result of cumulative genetic damage due to the exposure of tobacco carcinogens in at least 80% of the cases. Bronchial premalignancy is not confined to a single area of the bronchial tree; it tends to be multifocal and as a result cannot be treated by local direct injection of the therapeutic agent. Most primary tumors tend to grow as a solitary mass except for bronchoalveolar carcinoma where the primary tumor may grow and disseminate endobronchially in many cases. Both bronchial premalignancy and some cases of bronchoalveolar carcinoma are theoretically more accessible via the endobronchial space

than through the bloodstream. Locoregional approaches to treatment are therefore a logical therapeutic strategy in these cases.

Aerosol and intratracheal administration to deliver chemotherapeutic agents and particularly biological materials to premalignant or malignant tissues in direct contiguity with the endobronchial space have been explored previously. For example, gene delivery of mitogen-activated protein kinase kinase 4 (2), PTEN (3), interleukin-12 (4), interleukin-2 (5), and granulocyte macrophage colony-stimulating factor (6) have all been tested by aerosol administration to spontaneous and implanted lung cancer models in animals. Chemotherapeutic agents have also been tested by aerosol administration: aerosolized liposomal 9-nitro-20(S)-camptothecin in lung cancer patients in a phase I clinical trial (7) and gemcitabine (8), and celecoxib combined with docetaxel (9) in mice with lung metastasis and lung cancer cell lines. A uniform conclusion from all these studies is that little systemic toxicity has been observed in spite of the very high concentrations achieved locally with the different agents.

Intratumoral injection of *p53* carried by an adenovirus vector with computed tomography-guided percutaneous fine-needle injection or bronchoscopy has been tested in non-small-cell lung cancer (NSCLC) patients in clinical trials (10, 11). The basic hypothesis was that *p53* dysfunction is related to drug resistance and poor prognosis (12–14), and restoration of *p53* function may overcome these problems. However, these studies

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have shown limited efficacy because intratumoral delivery in lung parenchymal lesions is not feasible on a repeated basis, for it requires an invasive procedure for each individual lesion, and diffusion of the transfecting carrier from the injection site to the periphery of the tumor is not guaranteed.

On the other hand, the tumor suppression and therapeutic function of wild-type p53 *in vivo* has been clearly shown, showing that restoration of p53 function leads to tumor senescence, regression, and clearance in different cancer models without affecting normal tissue (15, 16). Moreover, gene delivery to the premalignant bronchial epithelium by inhalation has the advantage of a noninvasive approach, as inhalation sessions can be repeated frequently, allowing access to the whole bronchial tree to target the areas of bronchial dysplasia that are superficial and thin.

We have reported previously on an optimal liposome p53 formulation (DP3-p53) for locoregional treatment and showed efficacy by intratracheal administration against human NSCLC xenografts developed by intratracheal tumor cell inoculation (17). Direct intratracheal administration of therapeutic agents in humans is technically cumbersome and uncomfortable. Aerosol administration is much more convenient although its delivery efficiency is much lower. However, an additional problem in developing gene delivery systems for aerosol administration is destruction of DNA or the gene/transfecting vehicle complex by the shear forces generated during aerosolization. Because early NSCLC and bronchoalveolar carcinoma originate and localize with wide distribution of the malignant cells in the whole bronchial epithelium and most cases are *de novo* resistant to chemotherapy, we hypothesize that an optimal aerosol formulation and administration schedule can deliver the therapeutic gene more efficiently to the malignant cells on the respiratory tract than systemic administration and it can also reduce systemic toxicity. We report here on an optimized gene delivery formulation carrying an appropriate therapeutic gene, such as p53, which has low toxicity and high delivery efficiency in mice and is resistant to the shear forces of the aerosolization process.

Materials and Methods

Plasmids. The p53sm expression plasmid was constructed by inserting a modified human wild-type p53 cDNA driven by a cytomegalovirus promoter in a pUC19-based vector (Promega). A silent mutation in the p53 cDNA was introduced by changing one nucleotide from CGA to CCG at codon 213 without changing the amino acid on the p53 protein. This modification disrupts a restriction site of *Taq*I enzyme and therefore provides us with an opportunity to screen by reverse transcription-PCR for our therapeutic gene in patients following treatment with exclusion of patients with the polymorphism (3-4% in North Americans; refs. 18-21). We name this p53 plasmid with silent mutation as p53sm. The pUC19 vector without expression gene was prepared as vector control.

Clinical grade p53sm plasmid solution (1 mg DNA/mL in TE buffer) was manufactured by Qiagen, Inc. in a cyclic guanosine 3',5'-monophosphate facility.

The luciferase plasmid (luc) used is a pGL4 luciferase vector with firefly luciferase gene driven by SV40 promoters (Promega).

The p53-specific luciferase plasmid, named wwp-luc, contains a luciferase gene driven by the p21 promoter and was a gift from Dr. Guillermina Lozano (M. D. Anderson Cancer Center, Houston, TX).

Cationic polymers. Protamine sulfate, L-polylysine, and polyethyleneimine were purchased from Sigma-Aldrich.

Cell lines. H358 (with p53 deletion) and H460 (with wild-type p53) are human NSCLC cell lines from American Type Culture Collection.

Animals. Male and female ICR and athymic nude mice, 6 to 7 weeks old (Harlan), were housed in the animal facility at Albert Einstein College of Medicine. All experimental procedures were approved by the Institute for Animal Studies and in accordance with NIH guidelines.

The formulation and its stability. The aerosol nonviral gene delivery system, termed AND, was a sterile mixture of protamine sulfate and L-polylysine with a weight ratio of 1:8. The final clinical formulation was a sterile AND-p53sm complex made by mixing AND and p53sm plasmid at 5:1 polylysine to DNA weight ratio in 0.6 mmol/L Tris-HCl buffer (pH 7.8). The stability of polylysine, determined by TLC, was used to represent the stability of the AND. The stability of p53sm was determined using a spectrophotometer at 260 nm. Samples were analyzed weekly.

Detection of aerodynamic size of the aerosol. The aerodynamic diameters of aerosol droplets were determined by the extrusion-precipitation method. The detailed method is described in Fig. 2 legend.

Transfection. Transfection was done in six-well plate with different cell lines using the method described previously (17). The DNA dose was $\sim 2 \mu\text{g}/10^6$ cells.

Aerosol administration. The aerosol was generated with PARI's personal compressor and LC Star nebulizer system. The generation rate was ~ 0.25 mL/min. The nose-only exposure system (CH Technologies, Inc.) linked with PARI's aerosol system in a closed chemical hood was used for aerosol administration to mice, and the aerosol time was strictly controlled. The post-aerosol formulation or aerosolized formulation for *in vitro* transfections and *in vivo* intratracheal injections was made by collecting the condensed aerosol fog coming out of the mouthpiece of the nebulizer into a sterile tube on ice. The contents of polylysine and DNA were measured before use.

Aerosol efficiency in mice. To accurately determine the aerosol dose by directly measuring the aerosol administration time, we measured the average volume of aerosol liquid inhaled by a mouse in 1 min at different time points (related to different doses) under our experimental conditions described above. We used this as aerosol efficiency to mice. The detailed method is described in Fig. 3B legend.

Intratracheal injection to mice. The same intratracheal injection method described previously (17) was used. Briefly, the mice were anesthetized with i.p. injection of Nembutal, and then AND-DNA complex (80 $\mu\text{L}/25$ g mouse) was injected into the trachea via the mouth with a 22-gauge feeding needle.

Determination of the delivered p53 gene expression in the lung of mice. The p53 gene expression after locoregional delivery with AND-p53sm to mice was determined with reverse transcription-PCR. Briefly, the ICR mice were treated with intratracheal injections of condensed aerosolized AND-p53sm with 8 μg DNA/mouse qd $\times 4$. On day 5, each resected lung was immediately lavaged three times with 0.6 mmol/L Tris-HCl buffer to remove the nontransfected p53sm plasmid. Total RNA extracted with Trizol (Invitrogen) was treated with RNase-free DNase I (Promega). First-strand cDNA was synthesized using Invitrogen's First-Strand Synthesis kit as per manufacturer's protocol.

A 2- μL cDNA of each sample from the first-strand synthesis was used for amplification of the target cDNA under the conditions described previously (17), using human p53-specific primers (forward 5'-TATG-GAACTACTTCTCTGAAAACA-3' and reverse 5'-GTAGATTACCACTGGAGTCTTC-3'). About 1 μg of each PCR product was treated with 5 units *Taq*I restriction enzyme (Promega) at 65°C for 2 h and the digest products were electrophoresed on an agarose gel.

p53 functional assay in mice. AND was mixed with either wwp-luc or p53sm at the optimal polymer/DNA ratio. Three complexes (AND-wwp-luc, AND-p53sm, and AND-wwp-luc+AND-p53sm at 1:1 ratio) were aerosolized separately. The condensed aerosol fogs were given to ICR mice by intratracheal injection at a dose of 8 μg DNA/mouse qd $\times 2$. Mice were sacrificed 24 h after the final injection. Lungs and other organs were resected and frozen immediately. The reporter gene expression in the organs was determined with an established method (17).

Aerosol administration of AND-luc or AND-p53sm in mice. ICR mice were directly given aerosol of AND carrying the luciferase plasmid (Promega), AND-p53sm, or AND-vector (as control) for 1 h using the aerosol system described above. The reporter gene expression in the lungs was determined 24 h after using the same method above. The p53 gene expression was determined by immunohistochemical staining using a human-specific p53 antibody (Santa Cruz Biotechnology) as per manufacturer's protocol.

Dose finding and lethal toxicity. Five groups of ICR mice (five mice per group) were injected intratracheally with different doses of AND. The mice were observed for 14 days to record body weight and death. The curve of dose versus percentage death was simulated with a computer program CA-Cricket Graph III (with the best fit, $r^2 > 0.94$), and the lethal dose at different levels was calculated. The minimal dosage resulting in 100% lethality or maximal dosage causing no incidence of death in the mice was taken as LD₁₀₀ or maximum tolerated dose (MTD), respectively.

Acute toxicity and pathologic examination. Mice were treated with different doses of intratracheal injection of AND or AND-p53sm, or aerosol administration of AND, separately. Four mice in each group were euthanized on days 4, 7, and 14 after the administration. Organs, including duodenum, heart, kidney, liver, lung, spleen, and thymus, were resected and processed with standard procedure for H&E staining. The toxicity levels were determined by giving toxicity grade to each tissue sample. The grading based on the general pathology guidelines was 1 to 7; they reflect a percentage of damaged tissue of <1, 1 to 5, 5 to 10, 10 to 20, 20 to 30, 30 to 40, and 40 to 60, respectively.

Antitumor efficacy of the aerosolized AND-p53sm. Athymic nude mice were intratracheally inoculated with H358 human NSCLC cell line (2.5×10^6 cells per mouse) using the previous method (17). One week later, the mice were randomly divided into five groups (five mice per group). Three of them were treated with 6 weekly doses of the

aerosolized AND-p53sm, AND-empty vector (1 mg DNA/m²), AND (equivalent polymer dose) by intratracheal injection, respectively. Another was treated with previously optimized i.v. injections of cisplatin (3 mg/kg, weekly \times 5). A group without treatment was used as negative control. The animals were observed daily and the death was recorded. The increased life span (%) was used to measure the efficacy of each tested agent.

Statistical analysis. Differences among different groups were analyzed by two-sided log-rank assay. A difference was considered statistically significant when $P < 0.05$.

Results

Optimization of formulation

Two major nonviral gene delivery systems, cationic liposomes and cationic polymers, have been used with different administration routes, including aerosol gene delivery (2–6, 17). To date, there is little information in the literature about the optimization of aerosol formulation to prevent destruction of DNA from aerosol-shearing forces. To develop an efficient formulation, we systematically compared the aerosol gene delivery efficiency of formulations containing either cationic lipids or cationic polymers.

Premade liposome named DP3, an optimal formulation for gene delivery in lung by intratracheal injection (17), was mixed with the luciferase plasmid at different ratios (Fig. 1A) and tested for their transfection efficiency in H358 cells before and after aerosolization. Similar tests were done with the most widely used cationic polymers PEI, polylysine, and protamine sulfate solutions individually mixed with the luciferase plasmid

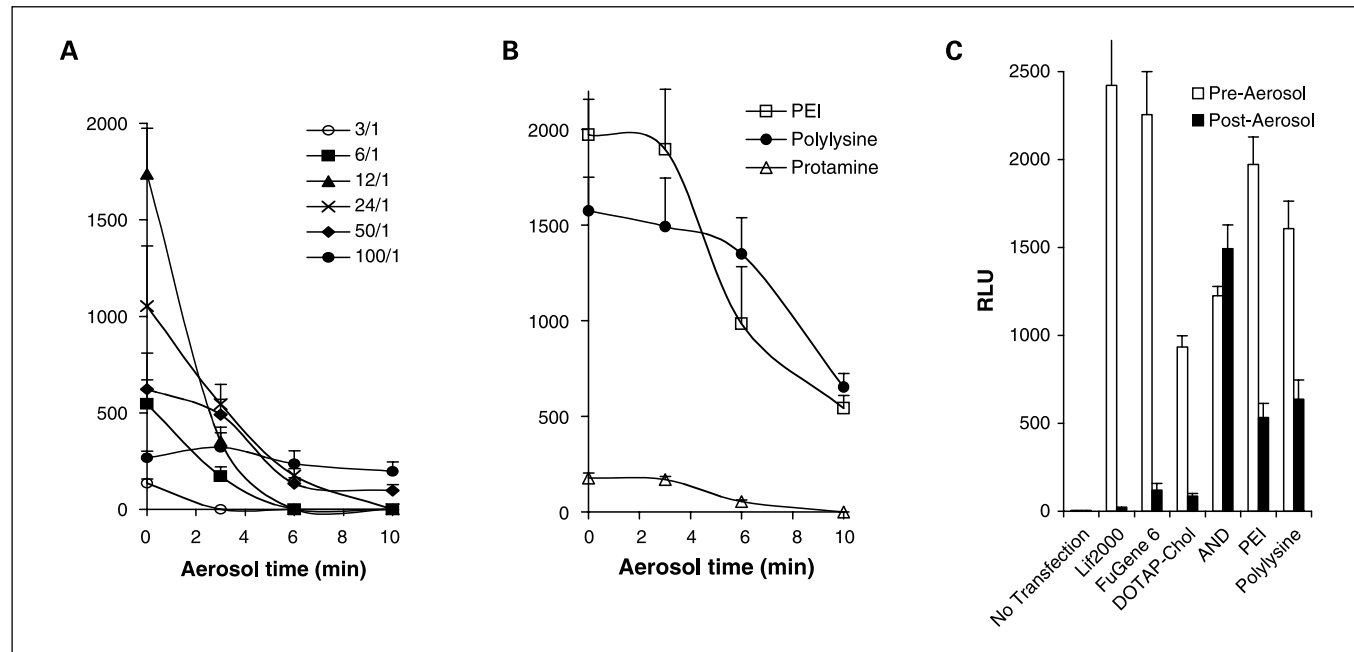


Fig. 1. Selection of the optimal aerosol formulation. Liposome-luciferase (luc) complex at different weight ratios (liposome/plasmid; A) and complexes consisting of the cationic polymers PEI, polylysine, or protamine sulfate and luc at their optimal weight ratio, respectively, (B) were aerosolized with PARI aerosol system, separately. The aerosol fogs were condensed for 0 (pre-aerosolization), 3, 6, and 10 min under sterile condition to transfect H358 cells in a six-well plate (0.2×10^6 cells per well), with the DNA dose fixed at 1 μ g/well (17). The luciferase intensity per 10^5 cells [relative light unit (RLU)] was measured at 48 h after transfection. C, comparison of AND to other transfection reagents. AND, LipofectAMINE 2000 (Lif2000), Fugene 6, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethyl sulfate-cholesterol liposome (DOTAP-Chol; ref. 23), and PEI were separately combined with luc at their optimal ratios (5:1 for AND, 9:1 for LipofectAMINE 2000, 6:1 for Fugene 6, 6.5:1 for *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethyl sulfate-cholesterol, and 2:1 for PEI). Each complex was tested for its transfection efficiency in H358 cells without (white columns) or with 10-min aerosolization (condensed fog; black columns) using the same procedures as those in (A) and (B). Points and columns, mean of three independent experiments; bars, SD.

at their optimal weight ratios (Fig. 1B). Our results indicate that after 10 min of aerosolization, most liposome/DNA complexes with low lipid/DNA ratios lost their transfection efficiency. The complexes with higher lipid/DNA ratios (>50) maintained their initial transfection efficiency before and after aerosolization (Fig. 1A); however, the efficiency was too low to lead to any significant biological function (17, 22). Under the optimal polymer/DNA ratio, the transfection efficiency of PEI and polylysine was >4-fold higher than that of the liposomal formulations. Protamine sulfate alone did not show significant transfection efficiency (Fig. 1B).

To further increase the transfection efficiency, we combined these polymers by grouping two or three polymers with different ratios. We found that the best combination was polylysine and protamine at weight ratio of 8:1 (data not shown). Comparing this formulation with the most frequently used nonviral transfection agents on human NSCLC cell lines, we found that our polymer combination was the only formulation that tolerated the aerosolization and yielded significant transfection efficiency (Fig. 1C). In contrast, the efficiency of other formulations was reduced >70%. Similar results were found in another NSCLC cell line H460 (data not shown). We named this efficient aerosol nonviral gene delivery system as AND and prepared it following Food and Drug Administration's Good Laboratory Practice standards for clinical development.

To avoid unnecessary interactions between the delivery system AND and the p53 plasmid (p53sm) during storage, they were stored separately and mixed right before use. Their stability was also determined separately. We found that after 2 years of storage at room temperature, there was no detectable change in polylysine and protamine amount, but p53sm was only stable at lower temperatures (e.g., 4°C or -20°C); at room temperature, degradation was found after 7 weeks (data not shown).

Aerodynamic size of AND-p53sm

Aerodynamic size directly affects the deposit site of aerosol-delivered drug. It has been suggested that droplets <5 μm, particularly <3 μm, deposits were most frequently in the lower airways and, thereby, appropriate for pharmaceutical inhalation aerosols in humans (24, 25).

The results shown indicate that under our experimental conditions, ~97% (weight) of droplets measured between 0.2 to 5 μm in diameter and ~48% (weight) between 1 to 3 μm (Fig. 2A). Therefore, approximately 48% to 97% of the droplets should deposit in the distal airway in humans (24).

Aerosol efficiency in mice

Accurately measuring the aerosol dose administered in animals is necessary for determining the dose response in a preclinical trial. However, there is little information available about this method, particularly in mice. Here, we developed a method (see Fig. 2B legend) to accurately measure aerosol dosage by controlling the time of administration. Under our experimental conditions, the aerosol efficiency was similar when the mice received ≤200-min aerosol. Therefore, we can use the average aerosol efficiency to calculate the dose. As shown in Fig. 2B, the average aerosol efficiency of AND from 2 to 200 min was 0.132 ± 0.006 μL/mouse lung/min (versus 0.082 ± 0.041 μL/mouse lung/min at 220 min; $P = 0.0002$).

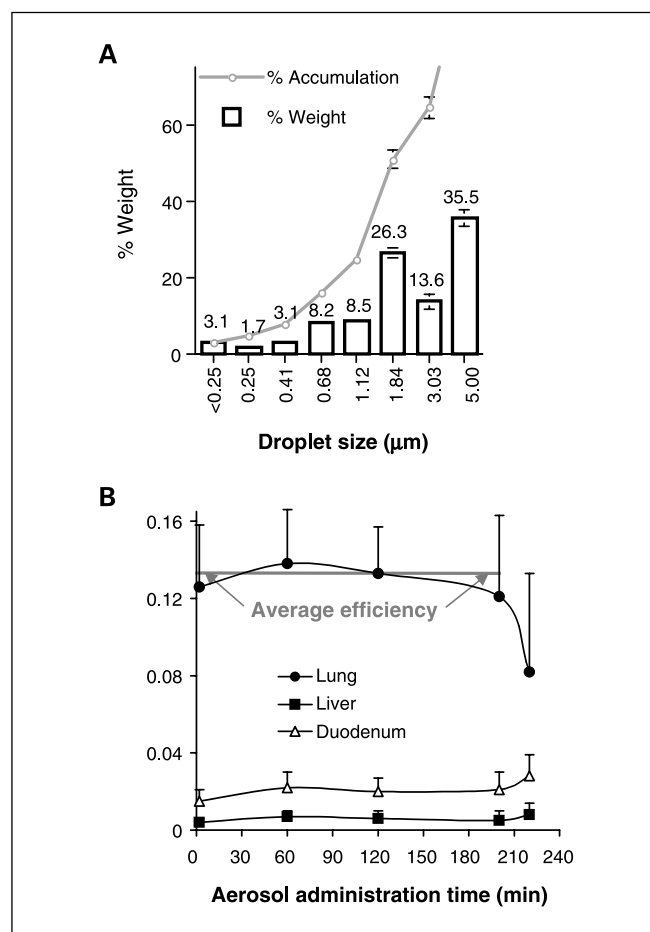


Fig. 2. A, aerodynamic size of AND-p53sm. The aerodynamic diameters of aerosol droplets were determined with extrusion-precipitation method using a seven-stage cascade impactor (In-Tox Products) linked to PARI's personal compressor and LC Star nebulizer system. AND-p53sm suspension (4 mL) was aerosolized under the airflow rate of 5 L/min. The condensed aerosol samples were collected at three different periods, from 1 to 1.5 min, from 3 to 3.5 min, and from 5 to 5.5 min. Aerodynamic size and fraction of aerosol with a particular size range were measured and calculated as per manufacturer's protocol. Mean of the aerodynamic size based on weight (columns) and accumulative weight (points) of three independent experiments; bars, SD. B, aerosol efficiency. Five groups of mice (six per group) were exposed to the aerosol of AND-vector with our aerosol system described in Materials and Methods. At each designed time point (related to different dose; e.g., 2, 60, 120, 200, and 220 min), one group of the animals was switched to calcein (20 μmol/L) aerosol for 60 s, and then the lungs, livers, and duodenum were resected immediately and the calcein levels in the organs were quantitatively determined by a fluorescence spectrophotometer. The proportion of calcein amount in each organ in contrast to the total calcein amount sprayed out from the PARI's aerosol generation system at each time point. Points, mean of six mice; bars, SD.

Gene delivery to mice with aerosolized AND

We first used reverse transcription-PCR to determine p53sm gene expression in the lungs of mice receiving intratracheal injections of aerosolized AND-p53sm. Significant amount of p53 mRNA, transcribed from the AND-delivered p53sm lacking the *TaqI* restriction site, was detected (Fig. 3A1 and A2). The method was confirmed by determining the AND-p53sm-transfected p53-null NSCLC cell line H358 (see Supplementary Fig. S1).

Next, to see if the aerosolized AND-delivered p53sm was functional *in vivo*, we intratracheally coinjected a p53-dependent luciferase reporter, AND-wvp-luc, with aerosolized AND-p53sm to mice. As shown in Fig. 3B1, a >5-fold higher luciferase

expression was found in the lungs of mice receiving both genes compared with mice receiving AND-wwp-luc alone ($P < 0.001$), which could be due to activation by mouse endogenous wild-type p53. These results indicate that AND-delivered p53sm had a significantly higher expression and function levels than that of endogenous p53 in the mouse lung.

Finally, we directly treated mice with aerosol administration of AND-luc and AND-p53sm. Significantly higher luciferase gene expression (1.6-fold; $P < 0.008$) was found in the lungs of mice receiving aerosol AND-luc than that of mice injected intratracheally with an equal dose of DP3-luc, a previously described efficient liposomal gene delivery system for intratracheal injection (17), indicating that AND was an efficient system for aerosol gene delivery (Fig. 3B2). In a similar experiment, the mice received 1-h AND-p53sm administration, the immunohistochemical staining (Fig. 4A) showed that the majority epithelial cells in the respiratory tract were transfected and the lung parenchyma cells away from the airway were transfected less.

Lethal dose level and acute toxicity of AND and AND-p53sm

MTD and acute toxicity are essential data for transferring a preclinical pharmaceutical to a clinical trial. Because intratracheal injection can be dosed more rapidly and accurately, we used intratracheal injections of different doses of AND (carrier) alone and AND-p53sm (final formulation for administration)

for the acute toxicity studies. We also determined the toxicity of aerosol AND alone. These results could provide an estimate of the toxicity range of aerosol AND-p53sm. We determined the MTD and acute toxicity of AND and AND-p53sm in mice, using standard pathologic examination combined with daily observation. The average toxicity grade of the lung or other organs of each mouse (see Materials and Methods) at each time point was used to describe the dose response.

MTD of intratracheal AND. In a lethal toxicity test, the intratracheal AND was fitted with linear dose response curve: % death = $0.47 \times \text{dose} - 2.64$ ($r^2 > 0.94$). The lethal dose levels were calculated based on the simulated curves. The maximum dose causing no incidence of death (LD_0) in animals based on this curve was 5.62 mg/m^2 , which we used as MTD in further studies. The LD_{10} , LD_{50} , LD_{90} , and LD_{100} was 26.9, 112, 197, and 218 mg/m^2 , respectively.

Acute toxicity of intratracheal AND. Four doses of AND, from 0.9 to 112 mg/m^2 (LD_{50}), were used to treat the mice by intratracheal injection. The toxicity in the lungs and other organs at days 4, 7, and 14 was determined, and the summarized toxicity grade of the lungs is presented in Fig. 4B. There was no detectable pulmonary or systemic toxicity when the mice received 0.9 or 4.5 mg/m^2 AND via intratracheal injection (pathology data not shown). When the dose increased to 22.4 mg/m^2 (25-fold), lung toxicity was

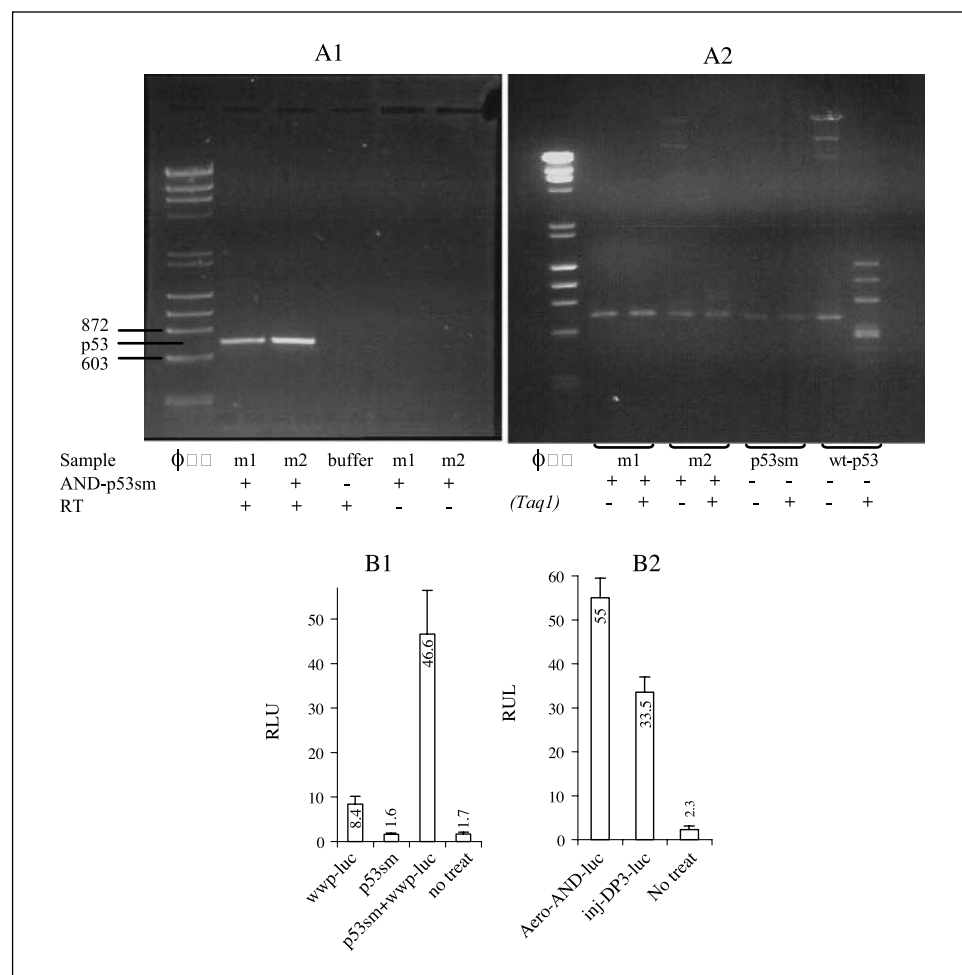


Fig. 3. A, reverse transcription-PCR detection of expression of transfected p53 in lung of mice (A1 and A2). B, functional assay of aerosolized AND-p53sm in mice, using a p53-dependent luciferase reporter (B1). Aerosol-delivered luciferase (B2). Columns, mean luciferase intensity of the lung of mice 1 day after the final intratracheal injection ($n = 3-6$); bars, SD.

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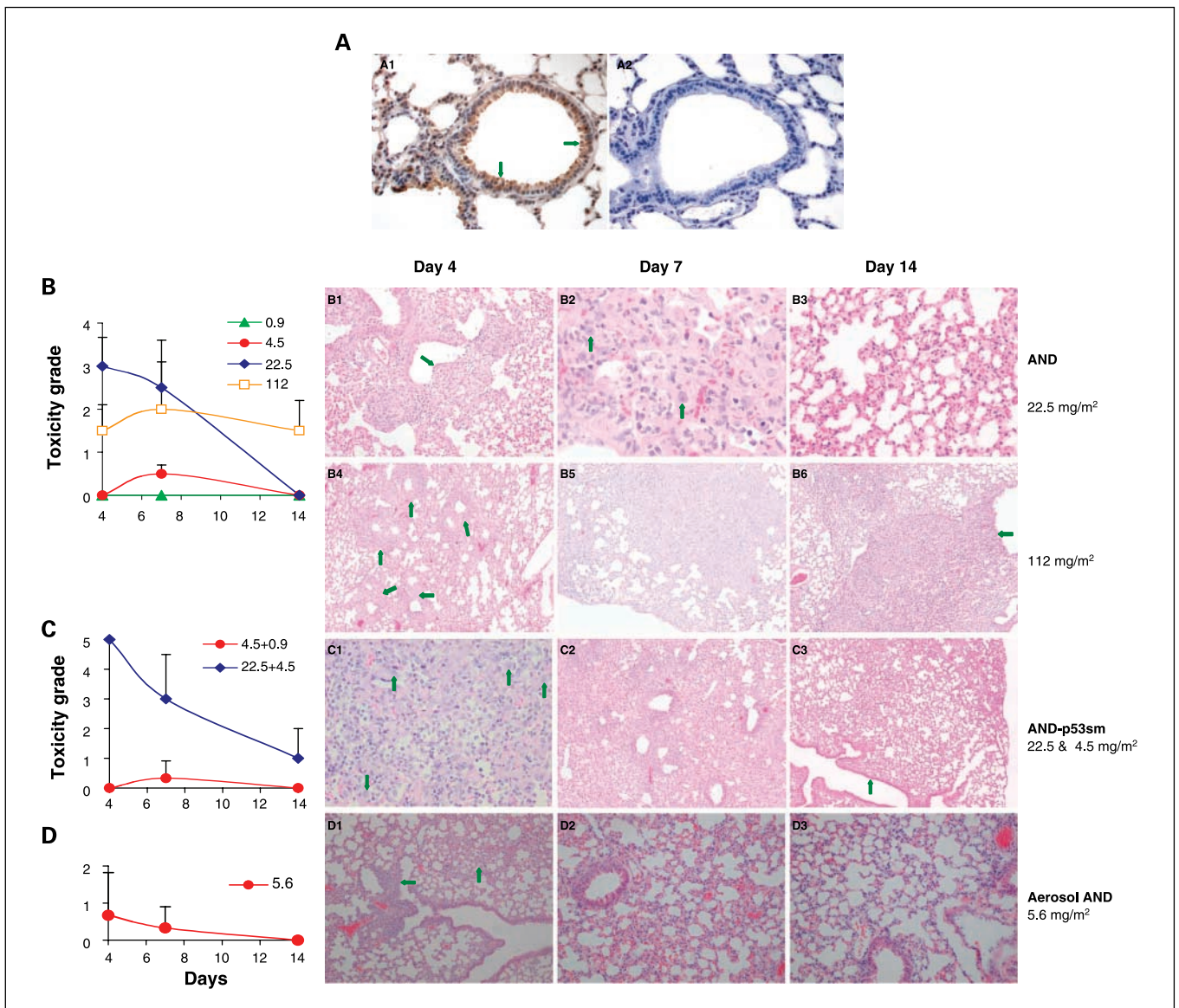


Fig. 4. *A*, p53 gene expression in mouse lung. Aerosol-delivered human p53 gene expressed (brown color) in majority epithelial cells of mouse respiratory tract (arrows, A1). The human-specific p53 antibody was used to specifically detect the human p53 transfected by AND-p53sm aerosol administration. The lung of mouse receiving aerosol-administered AND-vector was used as negative control (A2). *B*, four groups of mice were intratracheally injected with AND at doses of 0.9, 4.5, 22.5, and 112 mg polylysine/m², respectively. The curve of dose versus toxicity grade (B) and the standard H&E staining of lung tissues (B1-B6) from four mice/group/time point were used to present kinetically the dose response (toxicity). Lungs of mice received intratracheal AND at 22.5 mg/m² (B1-B3). The major toxicity was peribronchial inflammatory infiltrate with lymphocytes, histiocytes, reactive pneumocytes, and partially destroyed bronchial wall (arrow) on day 4 (grade 3, B1). Magnification, $\times 10$. The toxicity (grade 5) on day 7 involved alveolar parenchyma. Arrows, the inflammatory cells and the reactive pneumocytes (B2). Magnification, $\times 100$. The toxicity fully recovered on day 14 (grade 0, B3). Magnification, $\times 20$. The lungs of mice that received the highest dose of intratracheal AND (LD₅₀ = 112 mg/m²; B4-B6). Magnification, $\times 5$. On day 4, patchy areas of inflammation (grade 4, arrows, B4); on day 7, extensive areas of coalescence inflammation and consolidation (grade 7, B5); and on day 14, the toxicity reduced, but large area of inflammation and consolidation (arrow) was still being observed (grade 5; B6). Points, mean toxicity grades of the lungs versus time after injection; bars, SD. Representative pathologic images with higher dose of AND-p53sm (4.5 mg/m²; B1-B3). *C*, two groups of mice with 12 in each were treated with intratracheal injection of AND-p53sm complex at doses 0.9 and 4.5 mg p53 plasmid/m², respectively. On day 4, the typical toxicity (grade 5) was the lung inflammatory infiltrate with lymphocytes, histiocytes, and reactive pneumocytes (arrows, C1). Magnification, $\times 40$. On day 7, the inflammation-associated edema reduced to grade 4 (C2). Magnification, $\times 5$. On day 14, the damage fully recovered [grade 0, normal alveoli (top of the image) and normal bronchi (arrow), C3]. Magnification, $\times 5$. *D*, six ICR mice received 200-min continuous aerosol administration of AND. The estimated aerosol dose was 5.6 mg/m² (AND, 2 mg/mL; average body weight of mice, 28.1 \pm 2.7 g; aerosol efficiency, 0.013 μ L/min/lung). Points, mean toxicity grades versus days after aerosol administration; bars, SD. Representative pathologic images of the lungs (D1-D3). Magnification, $\times 10$. Low-level inflammation (grade 2) in bronchi (horizontal arrow) and alveoli (vertical arrow) was found on day 4 (D1), and it was reduced to minimal peribronchial inflammation on day 7 (grade 1, D2). The toxicity fully recovered on day 14 (grade 0, D3).

detected at median levels (average toxicity grade, ≤ 3). The damage was mainly peribronchial inflammatory infiltrate with lymphocytes, histiocytes, and reactive pneumocytes, which resolved within 14 days (Fig. 4B1-B3). Only when the dose increased to LD₅₀ (112 mg/m², 125-fold) the lung was signifi-

cantly damaged with severe inflammation and consolidation (toxicity grade up to 7), and the toxicity did not resolve in 2 weeks (Fig. 4B4-B6); this could be the major reason for lethality in the mice receiving high-dose AND. There was no systemic toxicity observed at this dose (data not shown).

Acute toxicity of intratracheal AND-p53sm. Two p53 doses were selected, 0.9 and 4.5 mg DNA/m², which corresponded to 4.5 and 22.4 mg/m² AND at the optimal polymer/DNA ratio. We found that AND-p53sm had similar toxicity pattern as AND alone. The median dose (0.9 mg DNA) caused minimal toxicity (average toxicity grade, <1) and it was reversible (pathology data not shown). The higher dose (4.5 mg DNA/m²), close to the LD₁₀ of intratracheal AND, caused median level of lung inflammation associated with mild edema, but this damage resolved in 2 weeks (Fig. 4C1-C3). The AND-empty vector was tested as a nonspecific AND-DNA control at the same dose range, the similar toxicity was found (see Supplementary Fig. S2), which indicate that the toxicity was mainly from the nonspecific inflammation caused by AND, but not the DNA.

Acute toxicity of aerosol AND. Because of the limited aerosol efficiency in small animal and the low toxicity of AND, we decided to use maximum givable dose to test the acute toxicity of aerosol-administered AND in mice. Using the average aerosol efficiency of 0.13 μL/min for a 20 to 30 g mouse for 200-min aerosol administration (the aerosol efficiency dramatically reduced after 200 min; see Fig. 2B) of 2 mg/mL, AND solution would give each mouse 52 μg AND. In this experiment, the average body weight of mice was 28.1 ± 2.7 g; therefore, the average aerosol dose was 5.6 mg/m², equivalent to MTD of intratracheally injected AND. The results indicate that (a) aerosol AND resulted in very low-level bronchi and alveoli inflammation only (average grade, <1) and the lung recovered fully within 2 weeks (Fig. 4D1-D3) and (b) compared with the similar dose of intratracheal injection of AND or AND-p53sm (e.g., 4.5 mg AND/m² or 0.9 mg DNA + 4.5 mg AND/m²; Fig. 4B and C, red circles), the toxicity of aerosol AND was observed early and toxicity levels were similar.

Antitumor efficacy

The antitumor activity of AND-p53sm was tested in an orthotopic xenograft of human NSCLC in nude mice. This model partially mimicked human lung cancer: the intratracheally inoculated human NSCLC cells initially grow on the surface of airway epithelium, subsequently invade lung parenchyma, and finally cause death of the animal in 2 months approximately (17, 22). We used intratracheal injection of condensed aerosol fog of AND-p53sm to treat the tumor-bearing mice (1 mg DNA/m² weekly × 6). A previously optimized schedule for multiple i.v. injections of cisplatin was used to mimic current chemotherapy in clinic. As shown in Fig. 5, the optimal chemotherapy by i.v. cisplatin had limited improvement of median survival of mice by 23% versus no treatment control ($P < 0.05$). The aerosolized AND-p53sm significantly increased the median survival by 1.7- and 2.1-fold compared with cisplatin treatment and the no treatment control ($P < 0.008$), respectively. The increased life span of aerosolized AND-p53sm was 68% and 93% higher than that of cisplatin and no treatment groups (Table 1). The AND alone and the AND carried empty vector had no antitumor effect in this model.

Discussion

The cure for NSCLC remains low because of lack of efficient methods for early detection and suboptimal therapies for locally advanced or metastatic disease. Most NSCLC cases are

due to tobacco exposure, which allows for an easy identification of populations at a high risk of developing the disease. New radiographic and molecular methods of early detection are being actively studied in these patient populations. As progress is made in this area, the number of patients diagnosed with early, localized disease will increase. These patients remain at risk for a second lung cancer after surgical operation. New approaches for the localized therapy for these patient populations are needed in anticipation of such advances in the diagnostic area.

The specific purpose of this study was to identify and develop an aerosol-compatible p53 gene therapy formulation that could be developed clinically. We selected the p53 gene as a model because its antiproliferative and proapoptotic function is frequently lost in severe dysplastic lesions or carcinoma *in situ* as a result of mutations, particularly in patients with a smoking history, and also because it is thought to play a role in the intrinsic lack of sensitivity of many NSCLC to cytotoxic therapy and radiotherapy (26–29). Therefore, successful transfection and restoration of p53 function in premalignant or malignant cells should result in apoptosis, tumor regression, or increased sensitivity to cytotoxic therapy. This basic hypothesis is supported by the most recent research (published in *Nature*), which clearly showed that restoration of p53 function leads to tumor senescence, regression, and clearance in different cancer models without affecting normal tissue (15, 16).

We have reported previously on the use of intratracheal gene restoration therapy in models of endobronchial human NSCLC in nude mice (17, 22). In our previous work, we used cationic liposomes as a model of gene delivery system. The rationale behind this work was since human NSCLC arises in the bronchial epithelium and remains confined within the respiratory wall for a long period and the malignant cells distributed widely in the airway surface; therefore, there are at least two keys to improve the therapy: (a) direct administration by aerosolization of agents intended to correct genetic lesions involved in the lung carcinogenesis process should be technically an easier task than trying to develop a systemic gene therapy approach and (b) the agent administered must be able to distribute widely to the surface of respiratory tract; an optimal

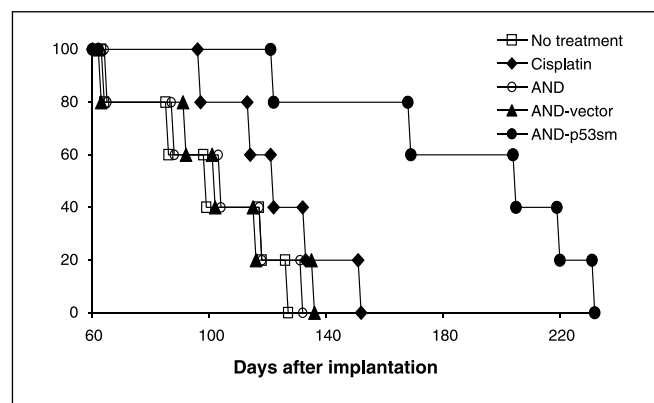


Fig. 5. Efficacy of AND-p53sm. Five groups of nude mice, intratracheally inoculated with H358 human NSCLC cell line, treated with 6 weekly doses of the condensed aerosolized AND-p53sm at 1 mg DNA/m² (●), equivalent dose of AND-vector (▲) or AND alone (○), optimal doses of cisplatin (3 mg/kg, weekly × 5; ◆), or without treatment (□), were observed daily and their death was recorded.

Table 1. Antitumor efficacy of aerosolized AND-p53sm

	No treatment	Cisplatin	AND	AND-vector	AND-p53sm
Median survival (d)	98	121	103	101	204
Average survival (d)	98 ± 25	123 ± 21	100 ± 26	101 ± 27	189 ± 45*
ILS (%)	0	25	2	3	93

Abbreviation: ILS, increased life span.

**P* = 0.003 and 0.014 compared with no treatment and cisplatin group, respectively.

aerosol should be the best choice. Therefore, the long-term goal of our efforts was the development of a gene delivery system that could be used clinically in an aerosol form. Aerosolization not only has the advantage of an enhanced therapeutic effect in early bronchial lesions, small metastases from other distant primaries, or diffuse forms of NSCLC like bronchoalveolar carcinoma but also offers the likely possibility of a much-decreased systemic toxicity due to lower systemic exposure (4, 5, 8, 10) and a higher distribution into the intrathoracic lymphatic system.

Early-stage development of gene delivery has been focused on nonviral systems in several laboratories (3, 17, 22, 30–32) because of its obvious advantages over viral carriers. However, few reports have shown in detail the optimization efforts carried in terms of selection of materials and composition. We realized that gene delivery systems composed of cationic lipids or the cationic polymer PEI alone are very sensitive to the shearing forces produced during the aerosolization process, thus leading to DNA destruction and lack of efficacy. Additionally, we also found that a 6-min long aerosolization step could reduce by >70% the transfection efficiency of most current reported nonviral gene delivery systems made of cationic lipids or single cationic polymer (e.g., PEI; refs. 3, 30–32). Therefore, developing an optimized formulation that is not affected by the aerosol shearing forces was a crucial initial step.

To determine the optimal aerosol dose, we tested the efficiency of aerosol delivery to the lungs in mice. It was 0.13 µL/min/mouse (20–30 g). We also found a detectable amount of dye in the duodenum (16% of that in lung) and liver (5% of that in lung) after aerosol administration. It may be due to the small diameter of the murine airways, causing high resistance to the aerosol fog and promoting condensation of large amounts of aerosol in the mouth and nose, which is subsequently swallowed by the animal.

The aerodynamic size of AND-p53sm ranged from 0.25 to 5 µm. The formulation developed was optimized for human use and may not be optimal for mice, which may also contribute negatively to the low efficiency in mice. However, the human airways are much larger, the cross-section area being about 240- to 400-fold larger than that of mice, so the formulation and aerosol system should be more efficient in humans.

After confirming that the functional *p53* gene (*p53sm*) can be transfected in mouse lung by our formulation and aerosol method, we tested the efficacy using aerosolized AND-p53sm to treat the orthotopic human NSCLC xenograft model that closely mimicked human early lung cancer. The aerosolized *p53* gene therapy significantly prolonged the life of tumor-bearing mice and the efficacy was significantly higher than the positive control mimicking the optimal clinical chemotherapy. In the toxicity studies, we found lung inflammation that was dose dependent after intratracheal injection of aerosolized AND and AND-p53sm, but we did not find detectable toxicity in any other organs. In a parallel experiment, the toxicity of an equal dose of AND administered by aerosol was lower than that of intratracheally injected AND, and the lower toxicity was reversible after 1 to 2 weeks. This suggests that aerosol-administered AND-p53sm at a therapeutic dose (e.g., 0.9–4.5 mg DNA/m²) might have minimal and resolvable lung toxicity and no systemic toxicity. Because of the high antitumor efficacy and low toxicity, application of this method is not restricted only to single-agent therapy for localized tumors but also as adjuvant therapy after surgery or to sensitize lung cancer to chemoradiation or radiation therapies. We believe that this method will have a wider application compared with invasive adenoviral *p53* therapy because the delivery system is nonviral and the administration is noninvasive. Furthermore, although *p53* was selected as the model gene, the technology and strategy described should be easily applicable to the delivery of other plasmids and oligonucleotides.

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