SPANDIDOS PUBLICATIONS

Inhibition of cell proliferation, migration and invasion by DNAzyme targeting MMP-9 in A549 cells

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Abstract. Matrix metalloproteinases (MMPs) have been regarded as major critical molecules assisting tumor cells during angiogenesis and metastasis. Enhanced expression of matrix metalloproteinase-9 (MMP-9) is associated with human non-small cell lung cancer (NSCLC) invasion and metastasis. DNAzyme is a single-stranded DNA catalyst that can be engineered to bind to its complementary sequence in the target gene and cleave the mRNA. In this study, DNAzyme targeting MMP-9 was designed and synthesized. We found it strongly inhibited MMP-9 mRNA and protein expression in the NSCLC cell line A549. Inhibition of cell proliferation, adhesion, migration and invasion was also demonstrated. Thus, DNAzyme targeting MMP-9 may be a promising anti-invasion and anti-metastasis strategy for cancer gene therapy in NSCLC.

Introduction

Lung cancer is the most common cause of cancer-related death in the world. Non-small cell lung cancer (NSCLC) constitutes about 80% of all lung cancers. The primary reason for the difficulty in treating NSCLC is the development of metastasis (1). Matrix metalloproteinases (MMPs) are a family of structurally related zymogens capable of degrading the extracellular matrix, including the basement membrane. They are considered to be critically involved in the tumor invasion and metastasis of various cancers (2,3). Among the many MMPs that have been identified, MMP-9 is thought to be key enzyme because it can degrade type IV collagen, the main component of epithelial-mesenchymal transition (EMT). Increased expression of MMP-9 was shown to correlate with an invasive phenotype of cancer cells (4,5). MMP-9 was found to be significantly associated with survival in NSCLC patients and giving an inverse prognostic effect (6-9), thus suggesting MMP-9 as an interesting target for adjuvant anticancer therapy in operable NSCLC using specific inhibitors of MMP-9.

DNAzyme is a DNA residue-based molecule capable of specific cleavage of complementary mRNA. This catalyst has emerged as a potential new class of nucleic acid-based drug because of its relative ease and low cost of synthesis, high stability, and flexible rational design features (10,11). These agents have been used in a number of applications *in vivo* to inhibit the expression of their target genes. Their capacity to block the development of diverse stages of pathologies in animal models suggested a potential use for DNAzyme as a therapeutic tool (12-14). The purpose of this study was to evaluate the effect of DNAzyme targeting MMP-9 on cell proliferation, adhesion, migration and invasion in human NSCLC A549 cells.

Materials and methods

Cell culture and transfection. A549 is a NSCLC cell line (ATCC CCL-185). Cells were cultured in RPMI-1640 (Gibco-BRL), supplemented with 10% FBS (Invitrogen), 10 μg/ml streptomycin and 10 IU/ml penicillin, incubated in a humidified atmosphere of 5% CO_2 at 37˚C.

One day before transfection, cells were plated in 6-wells of growth medium without antibiotics so that cells would be 30-50% confluent at the time of transfection. The transfection was performed with the manufacturer's protocol for oligofectamine reagent (Invitrogen). Briefly, 4 μl of oligofectamine reagent was mixed with 17 μl of RPMI-1640, then incubated with a mixture of 4 μl DNAzyme (500 μM, the final concentration is 2 μM (15) and 175 μl of RPMI-1640 for an additional 20 min at room temperature. After 800 μl RPMI-1640 was added, 1000 μl of DNAzyme and oligofectamine complexes was applied to each well, then incubating for 4 h. Then 500 μl of growth medium containing 3-fold the normal concentration of serum was added without removing the transfection mixture. Cells were usually assayed 24 h after transfection.

DNAzyme synthesis. Based on the analysis of sequences, thermodynamics and site distribution within the MMP-9

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gene, the DNAzyme targeting MMP-9 mRNA was designed and synthesized (10,15), purified by high-pressure liquid chromatography. The Control oligo (antisense equivalent of DNAzyme, mutated the catalytic domain) served as a control. To increase the resistance against nuclease degradation, the phosphorothioate modifications were incorporated in the first and last two phosphodiester linkages. The sequences were the following: DNAzyme: 5'-aggcgcccaGGCTAGCTACA ACGActccgcggc-3'; Control oligo: 5'-aggcgcccaGGGATCG TACAACGActccgcggc-3'.

Quantitative real-time RT-PCR. For quantitative mRNA expression analysis, a SYBR real-time RT-PCR protocol was applied using an ABI5700 (Applied Biosystems) instrument. Quantification of the copy number of MMP-9, was performed by serial dilutions of cDNA fragments (10^3 up to 10^6 cDNA copies), which form the T-vector pGEM (Promega) with MMP-9. Total RNA was isolated with TRIzol Reagent (Invitrogen) and reverse transcriptase reaction was performed using 2 μg total RNA by Superscript II enzyme (Gibco-BRL). The primers of MMP-9 were the following: 5'-tcctcg ccctgaacctg-3' (forward) and 5'-cttcccatccttgaacaaatac-3' (reverse). Reactions were performed in a 20 μl volume with 0.5 μM primers, 5 mM $MgCl_2$, Nucleotides, Taq DNA polymerase, and buffer were included in the DNA Master SYBR-Green I mix (Applied Biosystems). Cycling conditions were: 95˚C 10 min, followed by 30 cycles at 95˚C for 15 sec, 58˚C for 30 sec, and 72˚C for 30 sec. Specificity of amplification products was confirmed by melting curve analysis.

Western blot analysis. After transfection for 24 h, cells were lysed in the lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2% SDS, 5 mM DTT, 10 mM PMSF, proteinase inhibitors mix), and then the protein quantified was applied by the BCA Assay Reagent (Pierce Chemical). Total protein was resolved on 10% polyacrylamide SDS gel and transferred onto a nitrocellulose membrane by electroblotting. The membrane was incubated in blocking buffer (TBS containing 5% skim-milk and 1% Tween-20) for 2 h, followed by incubation with the primary antibody. Blots were then incubated with peroxidase-conjugated secondary antibodies. Protein expressions were determined using a super signal chemiluminescence system (ECL, Pierce), followed by exposure to autoradiographic film. The study employed antibodies against MMP-9 (sc-21733, Santa Cruz Biotechnology), and Actin (sc-8432, Santa Cruz Biotechnology). Relative protein levels were quantified by the use of ImageJ software (NIH) on scanned film.

Cell proliferation assay. A549 cells transfected with mock, Control oligo or DNAzyme in 96-well plates were incubated for 24-48 h. After incubation, MTT dye (0.5 mg/ml) was added to each well for further 4 h incubation. The reaction was stopped by the addition of DMSO, and the absorption was determined at 570 nm on a multiple-well plate reader (Bio-Tek). The survival rate of cells was expressed as *A*/*B* 100%, where *A* was the absorbance value from the experimental cells and *B* was that from the control cells, which were not treated.

In vitro cell adhesion assay. Twenty-four-well plates were incubated with 150 μl/well of fibronectin (Gibco) (50 μg/ml) for 45 min and blocked with 1% bovine serum album. A549 cells after transfected for 24 h with mock, Control oligo or DNAzyme were harvested for the cell adhesion assay. Cell mixture (100 μl) (10^5 cells/ml) was added to each well and allowed to attach for 2 and 4 h at 37˚C. After three times of washing with PBS to remove non-adherent cells, the number of adherent cells was counted. Each assay was performed in triplicate in at least two independent experiments.

Wound-healing/cell migration assay. The assay was performed on 6-well plates coated with the extracellular matrix protein, fibronectin (Gibco). A549 cells after transfection were grown to confluent monolayers in a 6-well plate for 24 h, then the monolayers were disrupted by scraping them with a sterile P200 micropipette tip, and cultured for 24 h, the migration ability of the cells was evaluated by measuring the width of the wounds (measured at x400 magnification). Each assay was performed in triplicate in at least two independent experiments.

Cell invasion assays. The cell invasion assay was conducted using BD Biocoat Matrigel 24-well invasion chambers with filters coated with Matrigel on the upper surface (BD Biosciences, Bedford, MA) with 8.0 μm pores. A549 cells were transfected with mock, Control oligo or DNAzyme. After 24 h, the cells were trypsinized, resuspended in serum-free medium, seeded $1x10^5$ cells in 0.2 ml medium to the upper chamber, and 0.5 ml 10% FBS medium was added to the lower chamber, after incubation at 37˚C with 5% CO_2 for 24 h, the cells were stained with 0.1% Crystal violet solution, and the cells and Matrigel on the top surface of the filter were carefully removed with a cotton swab. The invasive cells attached to the bottom surface of the filter were quantified under a light microscope (x200). The data are presented as the average number of cells from randomly chosen fields. Each treatment condition was assayed using triplicate filters and filters were counted in five areas.

Statistical analysis. Statistical analyses were carried out by Student's t-test (SPSS.V11.0). The p-values ≤ 0.05 were considered significant.

Results

DNAzyme inhibits MMP-9 mRNA expression in A549 cells. As shown in Fig. 1, the DNAzyme had obvious inhibition effect on MMP-9 mRNA levels in the A549 cells, whereas the Control oligo had no effect ($p<0.01$). The copies of MMP-9 of the mock, Control oligo and DNAzyme was $3.94\pm0.75 \times 10^5/\mu$g RNA, $3.86\pm0.69 \times 10^5/\mu$g RNA, $6.25\pm1.06 \times 10^2/\mu$g RNA (means ± SE), respectively. These results indicated that the targeting MMP-9 DNAzyme inhibited the MMP-9 mRNA expression in A549 cells.

Effect of DNAzyme blockade on MMP-9 protein expression. DNAzyme was transfected into A549 cells and assayed for its effect on the MMP-9 expression at the protein level using Western blotting. As shown in Fig. 2, the expression of

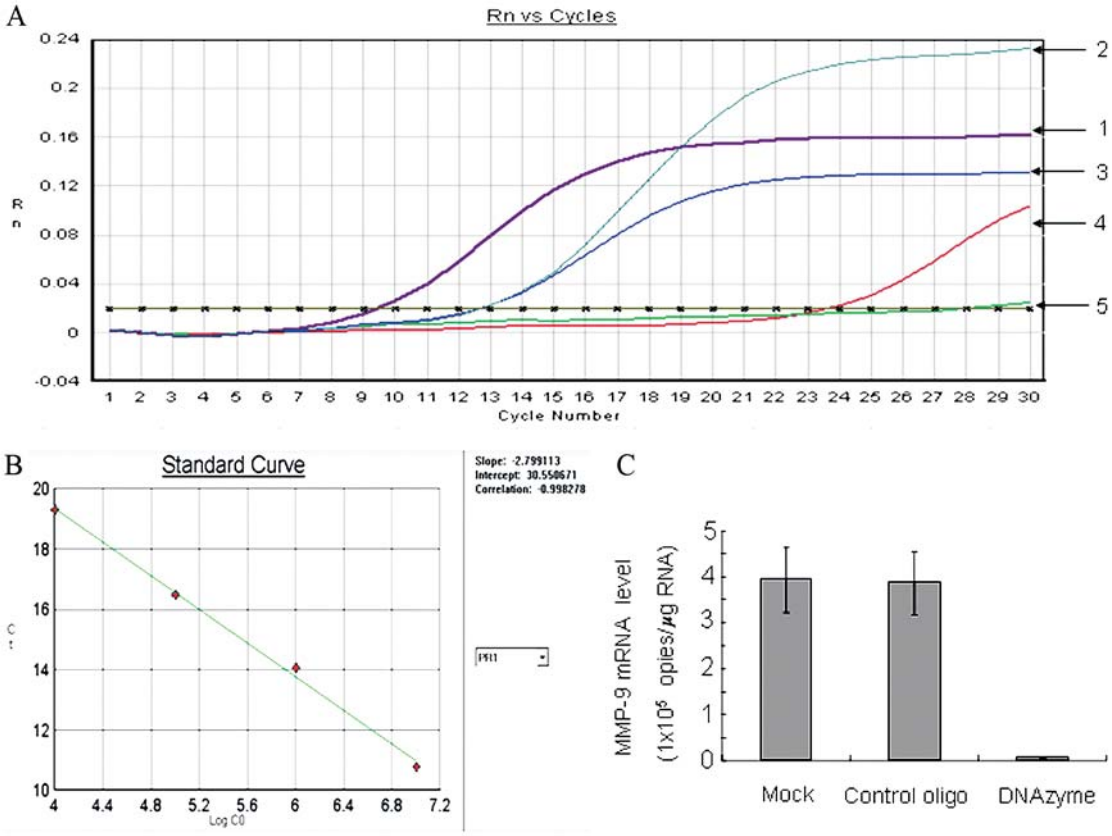

Figure 1. The MMP-9 mRNA was analyzed by SYBR real-time RT-PCR. (A) The detection curves of real-time RT-PCR of MMP-9 mRNA. 1, positive control; 2, mock; 3, control oligo; 4, DNAzyme; 5, negative control. (B) The standard curve for MMP-9 obtained by SYBR-Green PCR using plasmid DNA as template. (C) p<0.01 (relative to those in the control group). Columns, means; bars, ± SE. The experiment was repeated three times.

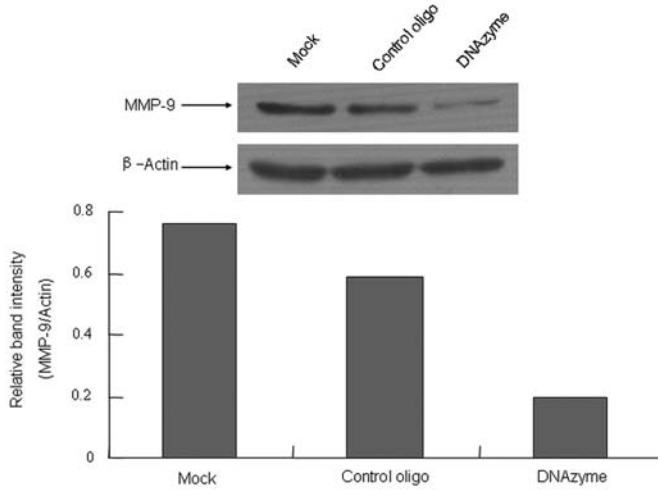

Figure 2. Blockade of MMP-9 protein expression in A549 cells by DNAzyme. Expression level of each protein was estimated by densitometry and presented as a ratio to the loading control Actin. p<0.05 (relative to those in the control group).

MMP-9 was strongly decreased in A549 cells transfected with DNAzyme compared with Control oligo and mock (p<0.05), the relative band intensity units of DNAzyme, Control oligo and mock were 0.201 0.759, 0.588, respectively.

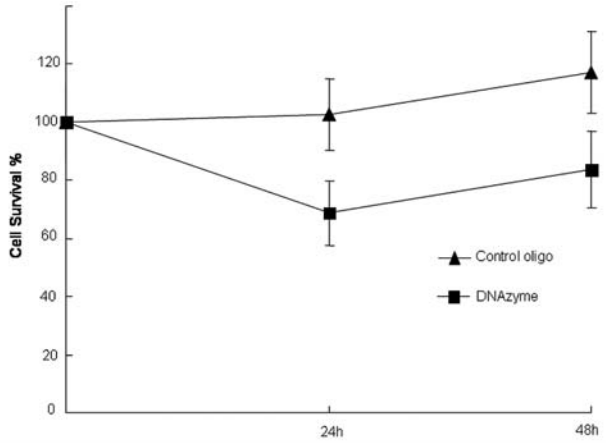

Figure 3. The A549 cell proliferation assessed by MTT assay. n=6, p<0.05 (relative to those in the control group). Means; bars, ± SE.

Inhibition of A549 cell growth by targeting MMP-9 DNAzyme. The A549 cell proliferation was assessed by MTT assay. A549 cell proliferation was suppressed by treatment with DNAzyme compared with the treatment of Control oligo (n=6, p<0.05) (Fig. 3). These findings demonstrated that the growth of A549 cells could be inhibited by DNAzyme targeting MMP-9.

Targeting MMP-9 DNAzyme inhibit the adhesion of A549 cells. The attachment rate after 2 h of A549 cell transfection

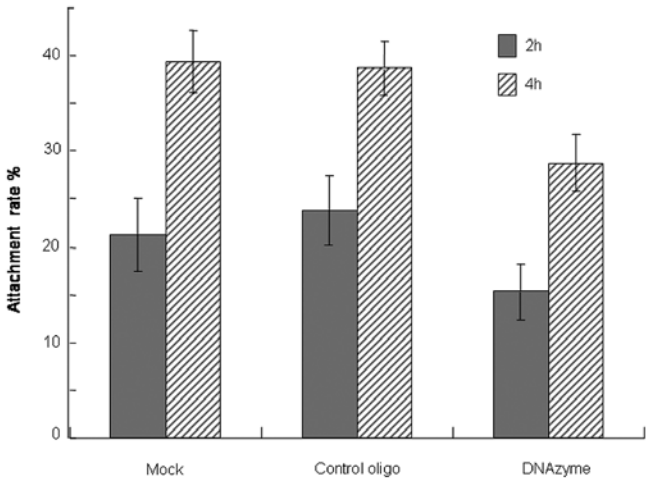

Figure 4. Inhibition of A549 cell adhesion by targeting MMP-9 DNAzyme. $p<0.05$ (relative to those in the control group). Columns, means; bars, ± SE. Each sample was assayed in triplicate, and at least two independent experiments.

with DNAzyme to fibronectin was 15.34±2.86 (means ± SE, %). It was lower than the cells treated with Control oligo and mock ($p<0.05$) (Fig. 4). The attachment rates were 21.28±3.81, 23.80±3.65, respectively. The result was similar to the attachment rate after 4 h, which was 28.70±3.09, 39.32±3.25, 38.64±2.98, respectively.

Inhibition of A549 cell migration by targeting the MMP-9 DNAzyme. To provide further support for the effect of the targeting of MMP-9 by DNAzyme, *in vitro* scratch wound healing assay was performed. The results are shown in Fig. 5. The migration rate of treatment with DNAzyme was 3.34±0.52 (means ± SE, μm/h), being significantly inhibited compared to cells treated with Control oligo and mock ($p<0.05$), which was 5.32±0.86, 5.21±0.92, respectively. Therefore, the DNAzyme targeting of MMP-9 inhibited the migration of A549 cells.

Targeting of MMP-9 DNAzyme decreases A549 cell invasiveness. The invasive potential of the A549 cells after transfection with targeting MMP-9 DNAzyme was determined using the Matrigel invasion assay. Fig. 6C shows decreased staining of the invaded cells through the Matrigel with targeting MMP-9 DNAzyme transfected compared with the Control oligo and mock controls (Fig. 6A and B). Quantitative analysis indicated that the targeting of MMP-9 by DNAzyme decreased 50% the invasiveness of the A549 cells compared with controls ($p<0.05$) (Fig. 6D). These data suggest that the targeting of MMP-9 by DNAzyme remarkably inhibited the invasive capacity of the A549 lung cancer cells.

Discussion

In recent years, much attention has been focused on anti-metastatic agents constructed by genetic engineering techniques for the therapy of malignant tumor cells. Down-

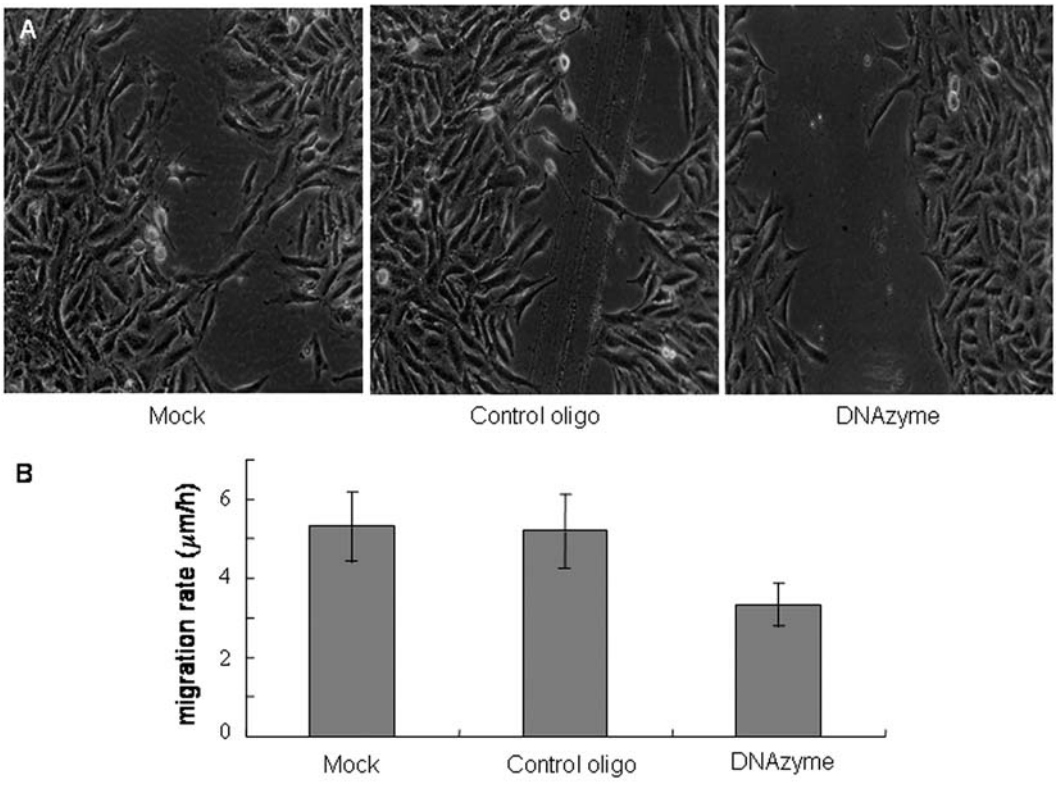

Figure 5. DNAzyme targeting MMP-9 inhibits A549 cell migration *in vitro*. $p<0.05$ (relative to those in the control group). Columns, means; bars, ± SE. Measured at x400 magnification. Panel A shows the results from single experiments, in which each sample was assayed in triplicate, and are representative of at least two independent experiments.

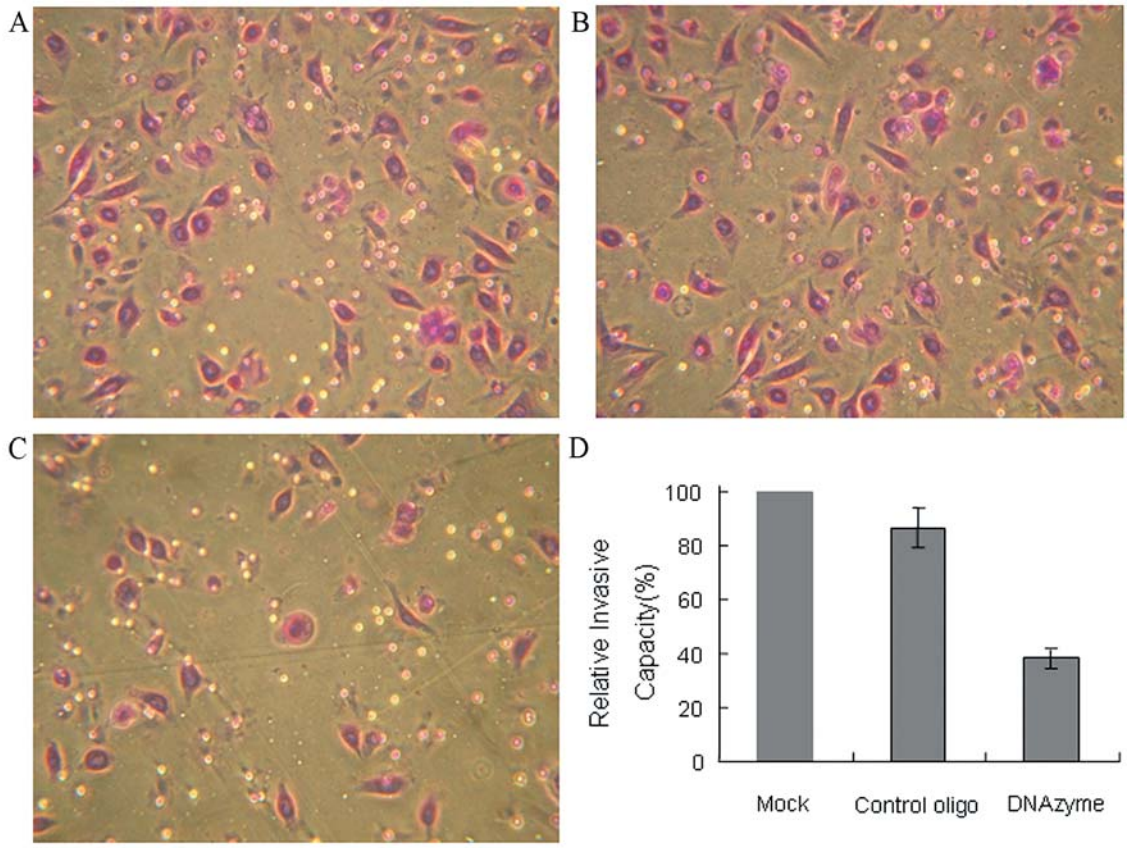

Figure 6. Targeting MMP-9 DNAzyme inhibits lung cancer cell invasiveness through Matrigel. (A-C) Representative images of the cells on the lower side of a membrane 24 h after plating for the invasion assay. Measured at x200 magnification. (D) Invasive activity was determined as the percent invasion through Matrigel matrix and membrane relative to controls. Columns, mean of at least two independent experiments; bars, ± SE.

regulation of MMP-9 by RNAi or antisense DNA constructs has been used to diminish production of MMP-9 in tumor cells (16-20). Target silencing of MMP-9 by RNAi in human Ewing's sarcoma indicated that MMP-9 constitutes a trigger for the switch between the adhesive and migratory states of tumor cells by regulating E-cadherin-mediated cell adhesion, extracellular matrix (ECM)-mediated cell spreading and paxillin signaling (16). Downregulation of MMP-9 in a prostate carcinoma xenograft model with specific antisense construct inhibited tumor growth (17). Further study implicated an anti-apoptotic role of MMP-9 shown to render tumor cells resistance to apoptosis (18). The cysteine protease cathepsin B and the MMP-9 played important roles in tumor progression. The migration and invasion of meningioma cells were decreased after treatment with siRNA constructs for cathepsin B and MMP-9. Furthermore, the abrogation of cathepsin B and MMP-9 expression decreased the activation of major proteins involved in MAP kinase and PI3 kinase pathways (19). In addition, MMP-9 were interrelated with the uPA/uPAR system in metastasis, the functional role of MMP-9/uPAR was highlighted in experiments of downregulation of MMP-9 and uPAR in lung carcinoma cells with antisense constructs. The downregulation of MMP-9 efficiently inhibited tumor cell invasion, angiogenesis and tumor growth, and even lung metastasis (20). Taken together, these studies implicated multiple functional roles of MMP-9 in the development of secondary metastasis, and strongly indicated of that MMP-9 could modify the microenvironment of the tumor either leading to enhanced or diminished tumor cell dissemination and metastasis formation.

Recent studies have shown the potential of DNAzyme can serve as drugs both in cell-based assays and preclinical models of cancer. The DNAzymes could offer the advantages of catalytic activity with inherent stability and low cost of synthesis (21,22). However, most important in gene therapy of DNAzyme is how to improve their stability in the body. In order to enhance the stability of DNAzyme, many scholars have tried to modify the DNAzyme, including phosphorothioate, 3' terminl reverse, N3'-P5' phosphorus amide modified, 2'-O-methylation, LNAzyme modified by LNA (Locked nucleic acid), and so on (22). It is anticipated that with the development of smart delivery systems for DNAzymes, better pharmacokinetics and pharmacodynamics will be possible (10).

In this study, we explored the potential of using DNAzyme as an anti-metastasis strategy for cancer gene therapy in NSCLC by targeting the MMP-9 gene. This study indicated that the targeting of MMP-9 by DNAzyme downregulated the MMP-9 expression at the mRNA and protein level, and inhibited the cell proliferation, adhesion, migration and invasion in the NSCLC cell line A549. In conclusion, DNAzyme targeting of the MMP-9 may serve as useful anti-metastasis agent in NSCLC.

Acknowledgments

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