miR-25 targets the modulator of apoptosis 1 gene in lung cancer

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Abstract

To determine the role of miR-25 in non-small cell lung cancer (NSCLC), we first detected miR-25 expression in clinical specimens and lung cancer cell lines by quantitative real-time polymerase chain reaction. The levels of miR-25 were elevated in the plasma of NSCLC patients and NSCLC cell lines. Transfection of A549 and 95-D cells with a miR-25 inhibitor resulted in reduced cell proliferation and enhanced apoptosis. Moreover, the modulator of apoptosis 1 (MOAP1) gene was identified as a novel target of miR-25. The ability of miR-25 to promote cell proliferation and block apoptosis is attributable to its effect on MOAP1 suppression. In addition, miR-25 antagomir significantly inhibited lung cancer growth via upregulation of MOAP1 in a mouse xenograft model. Collectively, these data demonstrate that miR-25 is an important biomarker for lung cancer, and miR-25 promotes cell proliferation and inhibits apoptosis in NSCLC cells by negatively regulating MOAP1 expression.

Introduction

MicroRNAs (miRNAs) are ~22-nt endogenous non-coding RNAs that play important gene-regulatory roles by targeting the 3′-untranslated region (UTR) of messenger RNAs (mRNAs) for mRNA degradation or translational repression (1,2). The first known miRNA, the Caenorhabditis elegans heterochronic gene lin-4 was discovered in 1993 (3). Since then, miRNAs have been discovered in all animal and plant species. The latest released version of the miRNA database in June 2014 cataloged 2588 mature miRNAs in the human genome (4). One study reported that ~50% of annotated human miRNAs are located in cancer-associated chromosomal fragile sites, which indicates that miRNAs play a crucial role in pathogenesis and progression of human cancer (5).

Lung cancer is the second most commonly diagnosed neoplasm and the leading cause of cancer-related death in both men and women in USA. Lung cancer patients have a dismal 5-year survival rate of <10% (6). Eighty percent of lung cancers are non-small cell lung cancer (NSCLC) with late disease presentation and tumor heterogeneities (7). Furthermore, our understanding of the basic biology of NSCLC is limited, resulting in poor treatment outcomes. Seeking biomarkers for accurately detecting early tumors and monitoring prognosis is a principle imperative in lung cancer research (8). In recent years, accumulating evidence has revealed that miRNAs are dysregulated in human cancers, including NSCLC and they act as oncogenes or tumor suppressors (9). For instance, miR-34, miR-138 and miR-451 are downregulated in lung cancer, and restoration of their expression promotes cell apoptosis and inhibits cell migration and angiogenesis (10–12). Overexpression of miR-17–92 and miR-21 significantly promotes proliferation and tumorigenicity of lung
cancer cells by activating oncogenic pathways (13,14). Moreover, higher levels of miR-155 and lower levels of let7a-2 are correlated with poor overall survival in lung adenocarcinoma patients (15).

miR-25 is transcribed as part of the miR-106b–25 polycistron, which is hosted by the ‘minichromosome maintenance complex component 7’ gene. Various reports have shown that miR-25 plays an oncogenic role in cholangiocarcinoma (16), Hela cells (17) and gastric cancer (18) by targeting the TNF-related apoptosis-inducing ligand, mitochondrial calcium uniporter and reversion-inducing-cysteine-rich protein with kazal motifs proteins, whereas other reports have indicated that miR-25 may function as a tumor suppressor in colon cancer and anaplastic thyroid carcinoma (19,20). Thus, whether miR-25 acts as an oncogene or a tumor suppressor may depend on the cellular context. Our previous work revealed that miR-25 directly targets the 3’-UTR of TP53 to down-regulate p53 protein levels and reduce the expression of p53 transactivation targets, resulting in a decrease in cellular apoptosis in HCT116 colon cancer cells, A549 NSCLC cells and multiple myeloma cells (21). However, our knowledge of the role of miR-25 in lung cancer remains limited.

In this study, we determined the expression of miR-25 in the plasma of NSCLC patients and NSCLC cell lines and analyzed its potential clinical significance. We also demonstrated the role of miR-25 in lung cancer cell proliferation, apoptosis and xenograft tumor growth. Additionally, we identified a new target gene for miR-25 in lung cancer cell proliferation, apoptosis and xenograft tumor growth. Additionally, we identified a new target gene for miR-25 in lung cancer cell proliferation, apoptosis and xenograft tumor growth.

Materials and methods

Clinical specimens

Between February 2013 and July 2013, we enrolled 81 NSCLC patients from the Central Hospital of Wuhan, including 12 Stage I, 14 Stage II, 26 Stage III and 29 Stage IV patients. Classification was performed according to the TNM staging system for NSCLC (2009). Among these patients, 51 were diagnosed with adenocarcinoma and 30 were diagnosed with squamous cell carcinoma, and all blood samples were collected before any treatment. Additionally, 41 healthy subjects were also recruited from the same hospital. All control patients were examined for pathology, and it was confirmed that they did not have pulmonary or other cancers. Informed consent was obtained from all participants during enrollment, and this study was approved by the Ethical and Scientific Committees of the Central Hospital of Wuhan. The clinical features of NSCLC patients and control subjects were shown in Supplementary Table 1, available at Carcinogenesis Online. There was no significant difference in age and gender percentage between the normal control subjects and NSCLC patients.

Blood samples from all participants were processed within 4 h by centrifugation at 1000 g for 15 min at 4°C. Plasma was then gently transferred to a fresh RNA/DNase-free 1.5-mL EP tube (Axygen, Union City, CA) and immediately cryopreserved at −80°C.

Cells and cell culture

The NSCLC cell lines including A549, H1299, H1975 and Hcc827, as well as human embryonic kidney (HEK) 293T cells were all obtained from the American Type Culture Collection. Lung carcinoma 95-D cells were obtained from the Cell Bank of the Chinese Academy of Science. A549, 95-D, H1299, Hcc827 and H1975 cells were maintained in RPMI-1640 (HyClone, Logan, UT). HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA). Both media were supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher, Waltham, MA). All cells were incubated in a humidified incubator at 37°C with 5% CO₂.

RNA isolation and quantitative real-time polymerase chain reaction

Plasma miRNA from lung cancer patients and healthy control patients was extracted using a mirVana PARIS kit (Ambion, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA from all lung cancer cell lines was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Human pulmonary alveolar epithelial cells total RNA (PriCells, Wuhan, HB, China) as normal control. Quantitative real-time reverse transcription (qRT-PCR) was performed using the TaqMan miRNA reverse transcription kit, the TaqMan small RNA assay kit and TaqMan universal PCR master mix (Applied Biosystems, Carlsbad, CA) in the CFX96 real-time detection system (Bio-RAD, Hercules, CA). With synthesized C. elegans miRNA (cel-miR-39) as a spiked-in control during the detection of plasma miRNA (22), values were normalized to cel-miR-39 and transformed according to the 2−ΔΔCt method. With U6 serving as an internal control for miRNA detection, values were calculated according to the 2−ΔΔCt method.

Transient transfection of miRNA inhibitors

The miR-25 inhibitor and the negative control used in cell culture were purchased from Ambion. One day before transfection, A549, 95-D and H1299 cells were plated to ensure 40% cell confluence at the time of transfection. The miR-25 inhibitor was transfected at a working concentration of 100 nM using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions.

MTT assay

A549, 95-D and H1299 cells were seeded at a density of 5 × 10⁴ cells per well in 96-well plates and transfected with miR-25 inhibitor or control. After 0, 24, 48, and 72 h, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma–Aldrich, St.Louis, MO, USA) assay was used to detect cell viability. The cells were incubated with MTT at a final concentration of 0.5 mg/ml for another 4 h at 37°C. After removal of the medium, a 100 μl dimethyl sulfoxide solution (Sigma–Aldrich) was added to dissolve the formazan crystals. The absorbance was measured at 490 nm using an EnSpire multimode reader (PerkinElmer, Waltham, MA). All experiments were performed in triplicates.

Carboxyfluorescein diacetate succinimidyl ester labeling assay

A cell proliferation (cell division) assay was carried out by the carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) labeling method (23). CFSE covalently labels long-lived intracellular molecules with the fluorescent dye carboxyfluorescein. When a CFSE-labeled cell divides, its progeny have half the amount of fluorescence, which can thereby detected to assess the average number of cell divisions. The stock solution of CFSE was 5 mM in dimethyl sulfoxide. A549 and 95-D cells were suspended in phosphate-buffered saline (PBS) with 5 μM CFSE at 37°C for 15 min to stabilize CFSE labeling. To quench labeling, the cells were washed twice with RPMI-1640 complete medium. Labeled A549 and 95-D
cells were then plated into 12-well plates at a density of 5 × 10^3 cells/well and cultured in RPMI-1640 medium. After 24 h of growth, A549 and 95-D cells were transfected with 100 nM miR-25 inhibitor or negative control. Forty-eight hours after transfection, cells were harvested, and CFSE fluorescence was measured by flow cytometry on a FACS Aria II flow cytometer (Becton-Dickinson, BD Biosciences, Franklin Lakes, NJ). The cell proliferation index (PI) was then analyzed. The PI value is the total number of divisions divided by the number of cells prior to division.

**Nuclear staining of apoptotic cells and caspase 3/7 assay**

A549 and 95-D cells were seeded at 5 × 10^3 cells per well in 96-well plates and transfected with miR-25 inhibitor or negative control. Forty-eight hours post-transfection, cells were examined for nuclear morphology associated with apoptosis. In accord with the manufacturer’s protocol, cells were incubated with Hoechst 33342 dye (Beyotime, Nantong, China) for 15 min to enable nuclear staining, washed twice with PBS, and then visualized using a IX81 microscope (Olympus, Tokyo, Japan). Alternatively, 48 h after transfection of the miR-25 inhibitor, Caspase-Glo 3/7 reagent (Promega, Madison, WI) was used to indirectly detect caspase-3 or -7 activity according to the manufacturer’s guidelines. Luminescence values were detected using a Victor X2 multilabel reader (PerkinElmer).

**Annexin V–fluorescein isothiocyanate/propidium iodide or annexin V–PL/7-ADD double staining**

Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide staining (BD Biosciences, San Diego, CA) was performed to detect apoptotic cells. A549, 95-D, and H1299 cells were seeded as 2 × 10^4 cells per well in six-well plates. After 48 h of transfection, the cells were collected, washed twice with ice-cold PBS, and stained with annexin V–FITC/propidium iodide according to the manufacturer’s protocol. After staining for 5 to 15 min, flow cytometry analysis was performed with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. In our study, cells were transfected with expression vectors (pSIFT–GFPI-miR–25 and pCMV6–AC–GFPMOAP1). As both green fluorescent protein (GFP) and FITC were green fluorescent, there was interference in detection. Instead of using Annexin V–FITC/propidium iodide, we chose annexin V–PE/7-ADD kit (BD Biosciences) to avoid the interference in cell expressing the GFP. In cells without GFP, the Annexin V–FITC/propidium iodide kit was used.

**Luciferase reporter assay**

The full-length 5′-UTR of the miR-25 3′-UTR containing the putative miR-25 recognition elements was amplified from the genome of human A549 cells by PCR (sense, 5′-TAT CTA GAC TTA GGA AAC CAC GAG GAG-3′; antisense, 5′-ATG CGG CCG CCA ATC GGA ATG TTG ATG CTC-3′). The mutated 3′-UTR of MOAP1 was also amplified (sense, 5′-TAT CTA GAA TAC CCT AAA GGT GTA ATG AGA-3′; antisense, 5′-ATG CGG CCG CTC TGA TTT AGG AAA ATA CTG-3′). The primers for detection of MOAP1 mRNA levels were as follows: sense, TGA ACC CTC GGA AAG CG; antisense, TTA CTC GCG CTC GAG TAA GCT TCC CTG ATC AAT CTC-3′. Both wild-type and mutated PCR products were cloned downstream of the reporter gene between the XbaI and NotI sites of the pRL-TK vector (Promega). All constructs were confirmed by DNA sequencing. Luciferase reporter assays were performed in HEK-293T, A549 and H1299 cells as described previously (24), with the cells seeded into 96-well plates (1 × 10^4 cells/well) the day before transfection. In each well, the pRL-TK-MOAP1-3′-UTR or pRL-TK-MOAP1-3′-UTR mutant vectors (100 ng) and 10 ng of pGL3 control (Promega) were co-transfected into cells along with 60 ng of pSIFT–GFPI-miR–25 precursor plasmid (21) or miR-25 inhibitor (10 pmol) using Lipofectamine LTX and Plus reagent (Invitrogen) according to the manufacturer’s protocol. Luciferase activity was measured 48 h post transfection using the Dual-Glo luciferase reporter assay system (Promega).

**Isolation of the cytosolic fraction**

Cells were washed twice with PBS, and the pellet was suspended in 200 µl of ice cold buffer (20 mM Hepes, pH 7.5, 1.5 mM MgCl₂, 10 mM KC₁, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis(aminohexylether)-tetraacetic acid, 1 mM dithiothreitol, 0.1 mM phenylmethlysulfonyl fluoride and 10 µg/ml each of leupeptin, aprotonin and pepstatin A) containing 250 mM sucrose. The cells were homogenized by a Dounce homogenizer with a sandpaper-polished pestle. After centrifugation at 10,000 rpm for 5 min at 4°C, the supernatants were then centrifuged at 100,000 g for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction (25,26).

**Western blot**

Cells were lysed in radio immunoprecipitation assay buffer (Beyotime), and the protein concentration of the cell lysates was quantified using a BCA protein assay kit (Pierce, Rockford, IL). The following primary antibodies were used: mouse monoclonal anti-MOAP1 (1:1000, Sigma–Aldrich), mouse monoclonal anti-Bax (1:500, Santa Cruz, Delaware, CA), rabbit monoclonal anti-cytochrome c (1:500, Cell Signaling Technology, Danvers, MA) and mouse monoclonal anti-p53 (1:1000, Sigma–Aldrich). Western blot analysis was carried out as described previously (24). Monoclonal anti-β-actin (1:5000, Sigma–Aldrich) was used as loading control. Labeled bands were detected using the ECL chemiluminescent kit (Thermo Fisher), and the signals were visualized using the ChemiDoc XR+ imaging system (Bio-Rad).

**MOAP1 overexpression or knockdown**

Transfection efficiency of pSIFT–GFPI-miR–25 and pCMV6–AC–GFPMOAP1 vector was checked based on GFP expression 24 h post-transfection by fluorescence microscopy. Two small hairpin RNA (shRNAs) targeting human MOAP1 and the shRNA negative control sequence were as follows: shRNA#1, TTT GGA CGG TGG ATG TTT C; shRNA#2, TTA CGT TCG ATG AAT GTC T (17); shRNA control, GGA TTT CAG TCG ATG TAC (24). The shRNA constructs were cloned into pSUPER-H2 and EcoRI site in an RNAi ready pSIREN-RetroQ vector (Clontech, Mountain View, CA), which was confirmed by sequencing. For MTI assay, A549 cells were seeded at 5 × 10^4 cells per well in 96-well plates transfected with 200 ng pSIFT–GFPI-miR–25 and/or 20 ng pCMV6–AC–GFPMOAP1 vector (Origene, Rockville, MD). For flow cytometry analyses and western blotting, cells were seeded at 2 × 10^5 cells per well in six-well plates and transfected with 2 µg pSIFT–GFPI-miR–25 and/or 0.2 µg pCMV6–AC–GFPMOAP1. In shRNA knockdown experiments, cells were seeded in six-well plates and transfected with 100 nM miR-25 inhibitor and/or 2 µg MOAP1 shRNAs. After the indicated time, the cells were collected for MTT, apoptosis assay and protein-level detection.

**In vivo assay**

The chemistry of the miR-25 inhibitor/control used in mouse xenografts is based on a published report (28). The antagonist/control was chemically modified antisense oligonucleotide with a cholesterol molecule conjugated in the 3′-end to enhance cell membrane affinity (miR-Down™ antagonim, Gene Pharm Co. Ltd, Shanghai, China). Four-week-old BALB/c nude mice were purchased from Beijing HFK Bio-Techology (Beijing, China). A549 cells were resuspended with OptiMEM and a total of 1 × 10^6 cells were subcutaneously injected into the right flank of 4-week-old BALB/c nude mice. Tumor volume was calculated as: \( V = \frac{D^2 \times d}{2} \) (D, the longer diameter; d, the shorter diameter). When the tumor reached an average volume of 60 mm³, the tumor-bearing nude mice (n = 8 for each group) received intratumoral injection of saline, antagonist control or miR-25 antagonist miR-25 antagonist or the control (10 µl, 250 µl) was mixed with Lipofectamine RNAiMAX (5 µl) before administered into mice three times per week for 2 weeks. Three weeks after the first injection, the animals were killed and the tumors were then divided either for detection of miR-25 expression by qRT-PCR or fixed in formalin for immunohistochemistry. The rabbit anti-human Ki-67 antigen monoclonal antibody (1:400, Cell Signaling Technology) was used to detect nuclear Ki-67 antigen expression. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was conducted following the instructions of the In Situ Cell Death detection kit (Roche, Mannheim, Germany). A rabbit anti-human MOAP1 antigen monoclonal antibody (1:200, Abcam, Cambridge, MA) was used to determine MOAP1 expression.

**Statistical analysis**

Statistical analysis was performed with Student’s t-test (for two-group comparisons) or one-way analysis of variance (ANOVA for comparisons with more than two groups). Differences between groups with P values of less than 0.05 were considered statistically significant.
Results

Expression of miR-25 in clinical specimens and lung cancer cell lines

qRT-PCR was performed to detect miR-25 levels in the plasma of 41 healthy control subjects and 81 NSCLC patients. As shown in Figure 1A, the miR-25 levels were upregulated in the plasma of NSCLC patients compared with healthy donors. In particular, the plasma levels of miR-25 were significantly increased even in patients with early-stage (I/II) NSCLC cancer compared with healthy controls (Figure 1B). The levels of miR-25 were higher in patients with Stage III and Stage IV than those with Stage I/II but there was no significant difference between Stage III and IV. qRT-PCR was also used to determine the expression levels of miR-25 in various NSCLC cell lines and in the medium used to culture these cells. The expression of miR-25 was significantly increased (by about 2.5-fold) in five NSCLC cell lines compared with primary human pulmonary alveolar epithelial cells. Among these, 95-D exhibited the highest levels of miR-25 expression (Figure 1C). When the medium used to culture NSCLC cells were extracted for miRNA detection, the abundance of miR-25 was found to be higher (Supplementary Figure 1, available at Carcinogenesis Online).

Inhibition of miR-25 reduced cell proliferation in both A549 and 95-D cells

Cell viability was estimated using the MTT method. There was a significant decrease in the viability of A549 (at 48 and 72h) and 95-D (at 24, 48 and 72h) cells transfected with the miR-25 inhibitor (Figure 2A). Next, we used the CFSE labeling to determine cell proliferation. Figure 2B and C showed the original parental population of A549 or 95-D cells labeled with CFSE as well as the shifted populations of cells with diminished content of CFSE 48h post-transfection. There was a significant reduction in the corresponding Plin A549 cells with the miR-25 inhibitor (PI = 5.73 ± 0.18) compared with the control (PI = 16.06 ± 2.12; Figure 2D). Similarly, transfection with the miR-25 inhibitor in 95-D cells also resulted in a decrease in PI (PI = 13.27 ± 1.35) in comparison with the control (PI = 16.64 ± 1.56; Figure 2E). These results suggest that miR-25 inhibition reduces lung cancer cell proliferation.

Inhibition of miR-25 promoted cell apoptosis of A549 and 95-D cells

Using Hoechst 33342 staining, we found that A549 and 95-D cells transfected with the miR-25 inhibitor appeared to have more typical morphologic features of apoptotic cells than the controls (Figure 3A). We next performed the annexin V/propidium iodide assay to determine the impact of miR-25 inhibition on the apoptosis of A549 and 95-D cells. As shown in Figure 3B, the apoptosis rate increased from 17.8 ± 0.57% to 31.2 ± 2.98% in A549 cells with the miR-25 inhibitor. Similar effects were also observed in 95-D cells (Figure 3C). Caspase-3 and -7 are crucial apoptosis effectors, so we measured the activities of these enzymes in A549 and 95-D cells after transfection with the miR-25 inhibitor. We found a significant increase in the activities of caspase-3/7 in both A549 and 95-D cells 48h under miR-25 inhibition (Figure 3D).

MOAP1 is a direct target of miR-25

Among many predicted target genes of miR-25 in miRNA database (Version 21), MOAP1 encodes the MOAP1 protein, which binds to Bax to mediate caspase-dependent apoptosis (29). There are two putative binding sites for miR-25 in the 3'-UTR of MOAP1 (Figure 4A). To identify the potential relationship between miR-25 and MOAP1, we measured MOAP1 mRNA levels in NSCLC cell lines by qRT-PCR and found that they were downregulated (Figure 4B). Additionally, as measured by western

![Figure 1](https://academic.oup.com/carcin/article-abstract/36/8/925/1851116)

**Figure 1.** miR-25 levels in clinical specimens and lung cancer cell lines. (A) qRT-PCR assay for miR-25 in plasma from NSCLC patients (n = 81) and normal control subjects (n = 41). Values were normalized to cel-miR-39. In the Whisker-box plot, boxes indicate the 25th and 75th percentile; the lines inside the boxes indicate the 50th percentile, whisker caps indicate the first and the 100th percentile. (B) Analysis of miR-25 in the plasma of normal controls (n = 41) and patients with stage I/II (n = 26), stage III (n = 26) and stage IV (n = 29) lung cancer. (C) qRT-PCR for miR-25 in NSCLC cell lines (95-D, A549, H1299, H1975 and Hcc827) and normal human pulmonary alveolar epithelial cells, with U6 as an internal control. *P < 0.05, **P < 0.01, ***P < 0.001.
blotting, inhibition of miR-25 not only upregulated MOAP1, but also elevated the expression of MOAP1-associated Bax and mitochondria-released cytosolic cytochrome c (cyto-c) in both A549 and 95-D cells (Figure 4C). Furthermore, we performed luciferase assays in HEK293T, A549 and H1299 cells by placing either the wild-type MOAP1 3′-UTR or a mutant lacking both miR-25 binding sites (Figure 4A) downstream of the luciferase gene. We found that when miR-25 was introduced, luciferase activities were reduced in cells carrying the luciferase gene with the wild-type 3′-UTR, but not in those with the mutant 3′-UTR (Figure 4D). Conversely, when miR-25 was inhibited, there was a significant increase in luciferase activity in all three lines with the wild-type 3′-UTR but not in cells with the mutant 3′-UTR (Figure 4D, F and G).

The tumor suppressor TP53 was reported as a target of miR-25 (21). We determine whether miR-25 inhibition triggered apoptosis was p53 dependent. We transfected a p53-null lung cancer cell line H1299 with the miR-25 inhibitor. As shown in Figure 4H, miR-25 inhibition elevated MOAP1, Bax and cytosolic cyto-c protein levels. The effects of miR-25 inhibition on proliferation and apoptosis in H1299 (Figure 4I and J) were similar as those on A549 and 95-D. Thus, miR-25 inhibitor mediated apoptosis is likely independent of p53.

The ability of miR-25 to promote cell proliferation and block apoptosis is attributable, in significant part, to MOAP1 suppression

We modulated the expression of miR-25 or MOAP in A549 cells by transfection with pSIF–GFP–miR-25 and...
pCMV6-AC–GFP–MOAP1, and we observed >30% transfection efficiency based on GFP expression (Figure 5A). As measured by western blotting, miR-25 introduction inhibited p53 and MOAP1 expression (Figure 5B). The MTT assay showed that there was a significant increase in the viability of miR-25-overexpressing A549 cells (48 and 72 h) compared with the control group (Figure 5C). Additionally, the apoptosis rate decreased from 13.38 ± 1.73% to 9.15 ± 1.13% with miR-25 overexpression (Figure 5D and E). In contrast to miR-25 overexpression, MOAP1 overexpression had opposite effects on MOAP1 protein level, cell proliferation, and apoptosis of A549 cells. MOAP1 overexpression had no effect on p53 expression. We co-transfect A549 cells with both MOAP1 and miR-25 constructs and found that the phenotype of miR-25 overexpression was partially offset by MOAP1 overexpression (Figure 5B–E). These data suggest that in lung cancer cells with miR-25 overexpression, a decrease in
MOAP1 expression is required for increased cell proliferation and decreased apoptosis.

We next employed two MOAP1-shRNA constructs that effectively downregulated MOAP1-mRNA and protein levels (Figure 5F and G). The knockdown of MOAP1 by transfection with either shRNA in A549 cells produced comparable changes in cell proliferation and apoptosis to that of miR-25 overexpression (Figure 5H and I). Furthermore, MOAP1-shRNA and the miR-25 inhibitor were co-transfected into A549 cells. p53 expression was moderately increased after the co-transfection (Figure 5J). We noted that inhibition of miR-25 reduced cell proliferation and promoted cell apoptosis through upregulation of MOAP1, Bax, and cytosolic cyto-c protein levels in H1299 cell 48 h post-transfection. (I and J) Proliferation and apoptosis analysis of H1299 cells with the miR-25 inhibitor. Results are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

miR-25 antagomir inhibited lung cancer growth and promoted apoptosis in vivo

To determine whether inhibition of miR-25 attenuates tumor growth and promotes apoptosis in vivo, we injected A549 cells into BALB/c nude mice subcutaneously. Xenografted tumors were treated with saline, the antagomir control or miR-25 antagomir for 2 weeks. We found that the antagomir significantly inhibited tumor growth and resulted in smaller tumors (Figure 6A and B). The expression of miR-25 was markedly decreased in tumor tissues treated with miR-25 antagomir (Figure 6C). Tumor size and miR-25 expression showed no significant differences in mice treated with saline or the antagomir control. Additionally, as shown in Figure 6D, immunohistochemistry analysis showed that the number of Ki-67-positive cells was much lower in tumors treated with miR-25 antagomir than the controls. TUNEL assays of tumor tissues revealed that miR-25 antagomir-treated tumors showed more TUNEL-positive cells than the controls. Furthermore, MOAP1 was significantly upregulated in tumors treated by miR-25 antagomir compared with the controls. These

Figure 4. MOAP1 as a target gene of miR-25. (A) The predicted miR-25 binding sites in the 3′-UTR of MOAP1 are indicated. MOAP1 3′-UTR mutants with a deletion in the miR-25 binding site are also shown. (B) qRT-PCR assay for MOAP1 mRNA expression in NSCLC cell lines (95-D, A549, H1299, H1975 and Hcc827) and normal human pulmonary alveolar epithelial cells. β-actin mRNA is used as endogenous control and each sample is analyzed in triplicate. (C) Western blot analysis of MOAP1, Bax, and cytosolic cyto-c protein levels in miR-25 inhibitor-transfected (100 nM) A549 and 95-D cells or the negative control at 48 h. β-actin is used as a loading control. (D and E) HEK-293T cells are co-transfected with pRL-TK carrying a wild-type or mutant 3′-UTR sequence and the miR-25 precursor (60 ng) or the miR-25 inhibitor (10 pmol), and the luciferase activity is measured at 48 h. (F and G) Luciferase reporter assays with A549 and H1299 cells. (H) Western blot analysis of MOAP1, Bax and cytosolic cyto-c protein levels in H1299 cell 48 h post-transfection. (I and J) Proliferation and apoptosis analysis of H1299 cells with the miR-25 inhibitor. Results are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
data suggest that miR-25 inhibition reduces tumor growth and increases apoptosis via MOAP1 upregulation.

Discussion
Xu et al. (30) reported that miR-25 expression in female lung adenocarcinoma patients might be associated with tumor progression and had prognostic implications. miR-25 was also reported to be overexpressed in the gemcitabine-resistant NSCLC cell line (31). miR-25 is one of the NSCLC-specific serum miRNAs (32). Circulating miRNAs are stable and specific to tissues or biological stages and have been proposed as novel biomarkers amenable to clinical diagnosis in translational medicine (33,34). Our previous research demonstrated that miR-25 adversely affects apoptotic cell death, cell cycle arrest and cellular senescence in lung cancer cells by targeting p53, suggesting that miR-25 might act as an oncogene (21). In the present study, we measured the levels of circulating miR-25 in both NSCLC patients and healthy subjects. We found that the plasma levels of miR-25 were significantly increased in NSCLC patients compared with normal
subjects, and miR-25 upregulation was significantly correlated with the TNM stages of NSCLC. miR-25 expression was elevated in five NSCLC cell lines; miR-25 abundance was also increased in the medium to culture A549, H1299 and 95-D cells. Furthermore, inhibition of miR-25 in both A549 and 95-D cells suppressed cell proliferation and induced cell apoptosis. Our results in combination with published reports, indicate that circulating miR-25 is an important biomarker for lung cancer diagnosis.

To gain insight into the molecular mechanisms underlying the role of miR-25, we identified a new miR-25 target gene, MOAP1. MOAP1 is a Bax-associating protein containing a BH3-like (BH, Bcl-2 homology) motif mediating caspase-dependent apoptosis in mammalian cells when overexpressed (29). miR-1228 was reported to suppress cellular apoptosis through targeting MOAP1 in Hela cells (35). Since MOAP1 was found in 2000, there were no in-depth studies on MOAP1 function, beyond an article showing MOAP1 binding to tumor suppressor RASSF1A to activate BAX (36–38). Thus, we select MOAP1 in this project for investigations. We demonstrate that MOAP1 is a bona fide target gene of miR-25 using luciferase reporter assays, mutagenesis and western blotting. Re-expression of MOAP1 in miR-25-overexpressing A549 cells or knockdown of MOAP1 in A549 cells with the miR-25 inhibitor reversed the effects of miR-25 on cell proliferation and apoptosis. Therefore, the ability of miR-25

Figure 6. miR-25 antagomir inhibits lung cancer growth and promotes apoptosis in mouse xenografts. (A) Representative images of nude mice bearing tumors after treatment with saline, the antagomir control or miR-25 antagomir. (B) Time course of tumor volumes from mice treated with saline, the antagomir control or miR-25 antagomir. Mean ± SD is shown. **P < 0.01, ***P < 0.001. (C) miR-25 expression in tumors was detected by qRT-PCR, with U6 as the endogenous control. Values are normalized by the 2^(-△△Ct) method. **P < 0.01. (D) Representative images of immunohistochemical analysis for Ki-67, MOAP1 and the TUNEL assay in tumors (original magnification, 400×). Blue represents nuclear staining by haematoxylin for cellular localization; in IHC, brown represents the target protein (Ki-67 or MOAP1); in TUNEL, brown represents nicked DNAs in apoptotic cells.
to promote cell proliferation and block apoptosis is attributable, in significant part, to its suppression of MOAP1. We further evaluated the impact of miR-25 inhibition in lung cancer using mouse xenografts. Immunohistochemical analysis of Ki-67 and the TUNEL assay revealed that decreased tumor growth in nude mice was, in part, due to lower proliferation and elevated cell apoptosis caused by miR-25 inhibition. Moreover, tumors treated with miR-25 antagonist exhibited an increase in MOAP1 protein levels.

Based on bioinformatics analyses using TFSEARCH, JASPAR and ConSite, there are multiple putative transcription factors binding sites in the promoter of miR-25, including E2F1, c-Myc, STATs, NF-kB, CEBPB and AP-1. MYC is a major oncogene and is amplified in lung cancer (39); MYC regulates the expression of the mir-106b-25 cluster (40). E2F1 also regulates the expression of the mir-106b-25 cluster (41); in addition, E2F1 can activate MYC transcription and vice versa, and they cooperatively modulate the expression of miRNAs (42–44). We speculate that miR-25 upregulation in lung cancer is the result of MYC overexpression and plan to investigate the underlying mechanisms of miRNA dysregulation in lung cancer. Yet, this is out of the scope of the current work, which centers upon MOAP1 as a novel target of miR-25.

In conclusion, the present study demonstrates the diagnostic value of circulating miR-25 in lung cancer. Upregulated miR-25 in lung cancer promotes cell proliferation and inhibits cell apoptosis by suppressing MOAP1 and subsequently Bax-related mitochondrial-released cytosolic cyto-c. miR-25 may act as an oncogene in lung cancer and is a promising biomarker and a drug target in lung cancer.

Supplementary material

Supplementary Figure 1 and Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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