

MicroRNA-26a regulates ANXA1, rather than DAL-1, in the development of lung cancer

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Abstract. The aim of the present study was to investigate the expression and role of microRNA-26a (miR-26a) in lung cancer, and to verify whether differentially expressed in adenocarcinoma of the lung (DAL-1) is the target protein of miR-26a. mRNA expression levels of miR-26a and DAL-1 were detected using reverse transcription-quantitative polymerase chain reaction. Protein expression levels of DAL-1 and annexin A1 (ANXA1) were evaluated by western blot analysis. Cell Counting Kit-8, Transwell and wound scratch healing assays were used to characterize the function of miR-26a in lung cancer cells. The association of DAL-1 with miR-26a or ANXA1 was determined by dual-luciferase reporter or two-dimensional gel electrophoresis assays. miR-26a revealed decreased expression levels in lung cancer tissues compared with normal lung tissues, and decreased expression levels in lung cancer cells compared with 16HBE cells. Inhibition of miR-26a promoted lung cancer cell growth, migration and invasion. The DAL-1 protein exhibited downregulated expression levels in lung cancer tissues. DAL-1 was not the direct target gene of miR-26a. The two-dimensional gel electrophoresis assay confirmed that DAL-1 and ANXA1 were associated proteins. Expression levels of the ANXA1 protein were increased following DAL-1 gene silencing. The altered expression level of miR-26a affected the expression of ANXA1, and not of DAL-1. miR-26a demonstrated decreased expression levels in lung cancer cells, and it has an important effect on the biological function of lung cancer cells. However, DAL-1 was

not a target gene of miR-26a. As a DAL-1 associated protein, ANXA1 was regulated by miR-26a.

Introduction

Lung cancer has a high incidence of tumor recurrence and metastasis, and is the most common cause of cancer-associated mortality worldwide (1). The overall 5-year survival rate among patients with lung cancer is <15%, and a high rate of metastasis is the primary cause of lung cancer-associated mortality (2). A previous study confirmed that differentially expressed in adenocarcinoma of the lung (DAL-1), a protein that belongs to the membrane-associated cytoskeleton protein 4.1 family, is an efficient suppressor of epithelial-mesenchymal transition (EMT) in lung cancer (3). EMT is a pivotal event in lung cancer metastasis progression (4-6). However, the regulators of DAL-1 in EMT remain unknown.

Recently, a novel batch of endogenous small non-coding regulatory RNAs (microRNAs; miRNAs) have received attention in the development of cancer, miRNAs bind complementary sequences in target mRNAs, resulting in selective degradation or selective inhibition of their translation (7). The present study investigated the possible miRNAs of DAL-1 using bioinformatic assays, which included miR-26b, miR-26a, miR-96 and miR-223. Among them, the regulation of DAL-1 by miR-223 has been demonstrated to serve a role in gastric cancer (8). The present study revealed that the gene silencing of DAL-1 induced annexin A1 (ANXA1) protein expression levels to increase in H460 cells. ANXA1 belongs to a family of calcium/phospholipid-binding and actin regulatory proteins (9). Dysregulation of ANXA1 has been reported in numerous types of neoplasm, suggesting that this protein may serve important roles in tumor development and progression (10). The present study aimed to profile the expression and function of miR-26a in the development of lung cancer and to verify whether miR-26a is the regulator of DAL-1.

Materials and methods

Candidate miRNAs of DAL-1 predicted using bioinformatics. microRNA.org (<http://www.microRNA.org/microRNA/home.do>)(11), TargetScan (http://www.targetscan.org/mamm_31/)(12)

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and PicTar (https://www.mdc-berlin.de/10440258/en/research/research_teams/systems_biology_of_gene_regulatory_elements/projects/pictar) (13) softwares were used in order to determine candidate miRNAs regulating DAL-1 expression in lung cancer cells.

Tissue samples and cell lines. A total of 9 non-small cell lung cancer (NSCLC) tissues and matched normal tissues were obtained from the Department of Thoracic Surgery, The First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). The aforementioned lung cancer cell lines: PAa, A549, H460, 95D, H1299, H520, PLAM, H446 and the normal lung bronchus epithelial immortalized cell line 16HBE sourced from the Central Laboratory of Guangzhou Medical University, were cultured in RPMI-1640 basic medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted by TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA yield and the ratio of absorbance at 260 to 280 nm (260/280 ratio) was measured using the Nano Drop 2000 Spectrophotometer (Nano Drop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). 1 µg RNA was reverse transcribed in a final volume of 20 µl [4 µl 5X Prime Script Buffer (for quantitative), 1 µl Prime Script RT Enzyme Mix I, 1 µl Oligo dT Primer (50 µM), 1 µl Random 6 mers (100 µM) and RNase Free dH₂O up to 20 µl] using the Prime Script[®] RT reagent kit (cat no. DRR037A; Takara Bio, Inc., Otsu, Japan) with random hexamers according to the manufacturer's protocol. The reverse transcription reaction condition was as follows: 37°C for 15 min, 85°C for 5 sec and 4°C for 2 min. RT-qPCR was performed using ABI 7500 Real-Time PCR Systems (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Green[®] Premix Ex Taq[™] II kit (cat no. DRR081A; Takara Bio, Inc.). GAPDH was used as an internal control. The specific PCR prime sequences of the genes designed by DNAClub software (Xiongfong Chen, Cornell University, Ithaca NY, USA) were as follows: DAL-1, forward 5'-acctgtgatgtagagaacgctcc-3' and reverse 5'-agtgccaagcaccactctga-3'. All primers were identified using the Gen Bank database (14) with the National Centre for Biotechnology Information blastn program (15) to ensure sequence specificity. The thermocycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec. Specificity of amplification products was confirmed by melting curve analysis. miRNA were extracted using a mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.). Specific RT primers and Taqman probe (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for the quantitative detection (16) of miR-26a (cat no. 4427012) and reference gene U6 (cat no. 4426961).

Plasmids. The miR-26a mimic/inhibitor and the control were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequence for miR-26a used is as follows: 5'-UUC AAGUAAUCCAGGAUAGGCU-3'. Takara LA Taq[®] (cat no. RR02MQ; Takara Bio, Inc.) was used to amplify the 3'UTR of DAL-1. The PCR thermocycler conditions were as follows:

Pre-denaturation at 94°C for 5 min, followed by 30 cycles at 98°C for 10 s and 68°C for 100 s and posterior extension 72°C for 10 min. The 3'UTR of DAL-1 was amplified using the following primers: Sense 5'-CCGCTCGAGCCAGAGGAA TAACTTAGCTTGCACATG-3' and antisense 5'-ATAAGA ATGCGGCCCGCCAGTGCATTTGCCATTTTATTTCG-3'. The PCR product was inserted into psiCHCK2 within *Xho*I and *Not*I restriction sites (Promega Corporation, Madison, WI, USA). The mutation experiment was performed using a KOD-Plus-mutagenesis kit (Toyobo Co., Ltd., Osaka, Japan).

Cells culture and transfection. A549, H460 lung cancer cell lines and the human embryonic kidney cell line HEK 293T (supplied by the Central Laboratory of Guangzhou Medical University, Guangzhou, China), were propagated in RPMI-1640 basic medium supplemented with 10% FBS. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Transfection was performed using Lipofectamine 3000 Reagent[®] (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. A final concentration of 50 nM miR-26a mimic or 150 nM miR-26a inhibitor of their respective negative controls (NCs) were used for each transfection in proliferation, migration, invasion and wound scratch healing experiments. A further 2.5 µg DAL-1 short hairpin RNA (shRNA) was used for transfection in a two-dimensional gel electrophoresis assay. The mock group in this study is the cell that only transfected with Lipofectamine[®] 3000.

Western blot analysis. Total protein samples were extracted from primary tissues or cells using a lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with phenylmethyl sulfonyl fluoride (PMSF). Protein quantification was performed using a BCA protein content detection kit (cat no. KGP902; Nanjing Key Gen Biotech Co., Ltd., Nanjing, China). The protein samples were mixed with 6X SDS-PAGE loading buffer (cat no. P0015F; Beyotime Institute of Biotechnology) and denatured, and separated using 10% SDS-PAGE (30 µg protein was loaded per lane) prior to being transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, prior to being incubated with an anti-DAL-1 antibody (cat no. ab154071; Abcam, Cambridge, UK; 1:800 dilution), an anti-ANXA1 antibody (cat no. 32934S; Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000 dilution) or an anti-GAPDH antibody (cat no. 2118; Cell Signaling Technology, Inc.; 1:1,000 dilution) overnight at 4°C. Protein signals were quantified following incubation with an anti-rabbit immunoglobulin (Ig)G, horseradish peroxidase (HRP)-linked secondary antibody (cat no. 7074P2; Cell Signaling Technology, Inc.; 1:1,000 dilution) or anti-mouse IgG, HRP-linked secondary antibody (cat no. 7076P2; Cell Signaling Technology, Inc.; 1:1,000 dilution) for 1 h at room temperature, conjugated with peroxidase using the Super Signal[®] West Pico Chemiluminescent substrate (cat no. PH203837; Thermo Fisher Scientific, Inc.). The images of the proteins were obtained and analyzed by the Fusion Solo 6s Multifunctional Gel Imaging System (Vilber Lourmat, Marne Le-Vallée, France).

Cell proliferation assay. Cell proliferation was indirectly assayed using the Cell Counting Kit-8 (CCK-8; Beyotime

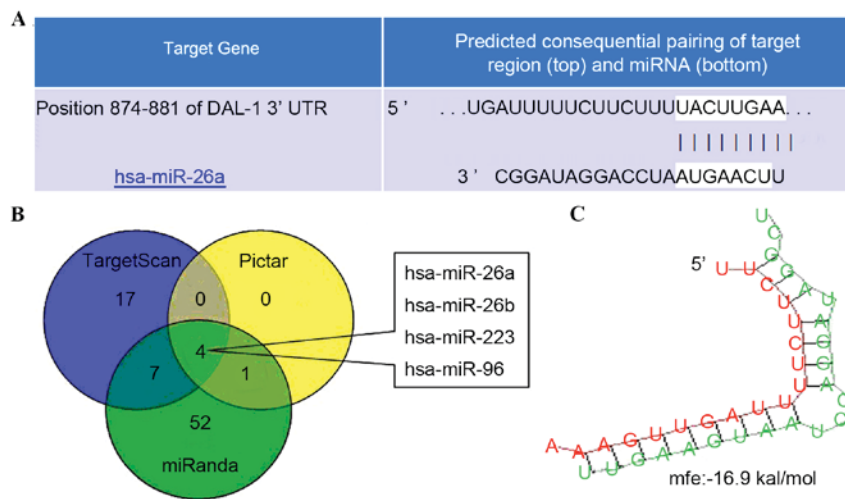


Figure 1. DAL-1 may be a target of miR-26a. (A) miR-26a-binding sites in the 3'UTR of DAL-1. (B) TargetScan, Pictar and miRanda predicted four candidate miRNAs for DAL-1. (C) The dimer structure for miR-26a combined with DAL-1. DAL-1, differentially expressed in adenocarcinoma of the lung; miRNA, microRNA; UTR, untranslated region; mfe, minimum free energy.

Institute of Biotechnology, Haimen, China), which stains living cells and this was carried out according to the manufacturer's instructions. A total of 5,000 cells per well were seeded in 96-well plates and transfected with the mimic (with a final concentration of 50 nM), inhibitor (with a final concentration of 150 nM) or their respective NC the following day, and incubated for 24 h at 37°C. The CCK-8 solution was used to determine viability once every 24 h following transfection, for 7 days. The absorbance of each well was measured with a microplate reader set at 450 nm. All experiments were performed in triplicate.

Cell migration and invasion assays. Cell migration and invasion was measured using a Transwell chamber assay (EMD Millipore, Billerica, MA, USA) with and without Matrigel (BD Biosciences, San Jose, CA, USA). For the invasion assay, 1×10^5 transfected cells were plated into the upper chambers coated with Matrigel. In the assays, cells were cultured in RPMI-1640 in the upper chambers, and 500 μ l 10% FBS-RPMI-1640 was added to the lower chamber. Following 24 h of incubation at 37°C, migrated cells were fixed with absolute methanol for 30 min at room temperature. Non-migrated cells on the upper chamber were removed using cotton swabs. Cells on the bottom surface of the membrane were subsequently stained with 0.5% crystal violet for 20 min. For each group, three cell images were randomly obtained using an inverted microscope, using a magnification of x100.

In vitro wound healing assay. A cross-hair pattern was marked on the outside surface of the undersides of the 6-well culture plates. Cells were plated in triplicate so as to reach 100% confluence after 1 day. Subsequently an ~2 cm-long and ~0.5 mm-wide wound was made by dragging a plastic 10 μ l pipette-tip along a straight edge with moderate and consistent pressure, to scratch the cell monolayer. Following this, the culture medium was replaced immediately. Cells were imaged immediately and one day subsequent to wounding, at x50 magnification at identical locations along the scratches.

Dual-luciferase reporter assay. The target sequence of DAL-1 3'UTR was cloned into the dual-luciferase reporter vector, check-2 (Promega Corporation, Madison, WI, USA) HEK-293T cells were plated into 48-well plates ($\sim 2 \times 10^4$ cell per well) with ~50-60% confluency, 24 h prior to transfection. A mixture of 50 nM miR-26a mimic and 200 ng CHEK-DAL-1 reporter vector, was transfected into cells using the Lipofectamine[®] 3000 reagent. Following 48 h, luciferase activity levels were determined using a dual-luciferase reporter assay system (Promega Corporation) and normalized by dividing firefly luciferase by *Renilla* luciferase, according to the manufacturer's instructions. Each transfection was performed in triplicate.

Two-dimensional gel electrophoresis assay. This experiment was performed as previously detailed (17). A mock group was used where cells were transfected with Lipofectamine[®] 3000 reagent alone. An NC group was used where cells were transfected with Lipofectamine[®] 3000 reagent and shRNA-NC. Finally an untreated group was used where cells were untreated.

Statistical analysis. All experiments were repeated three times. All data are presented as the mean \pm standard deviation, and were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance with a Least Significant Difference t-test was used to determine the statistical significance of the differences of miR-26a expression among nine lung cancer cell lines and a 16HBE cell line. A two-tailed Student's t-test was used to determine the statistical significance of the differences. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Candidate miRNAs of DAL-1 predicted by bioinformatics. microRNA.org, Target Scan and Pic Tar softwares were used to determine the candidate miRNA regulating DAL-1 expression in lung cancer cells. The predicted results

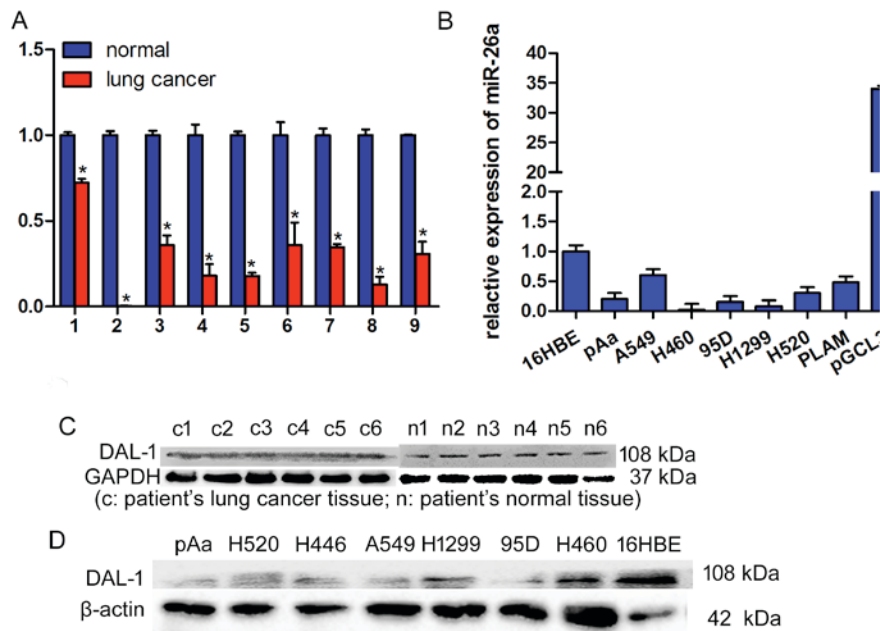


Figure 2. Expression levels of miR-26a and DAL-1 in lung cancer tissues and cell lines. (A) The expression level of miR-26a in nine paired samples from patients with lung cancer (primary cancer and non-cancerous tissues). (B) The expression level of miR-26a in nine lung cancer cell lines. (C) The expression of DAL-1 protein in six paired samples from lung cancer patients (primary cancer and non-cancerous tissues). (D) The expression level of DAL-1 protein in seven lung cancer cell lines and control 16HBE cells. * $P < 0.05$ versus the control group; DAL-1, differentially expressed in adenocarcinoma of the lung.

revealed that miR-26a, miR-26b, miR-223 and miR-96 are able to regulate DAL-1 by combining with DAL-1 mRNA 3'UTR (Fig. 1A-C).

Expression of miR-26a in lung cancer tissues and cell lines.

The expression levels of miR-26a in nine lung cancer tissues, nine matched adjacent normal lung tissues and various lung cancer cells, were determined by RT-qPCR. miR-26a expression was significantly decreased in lung cancer tissues compared with in normal tissues (Fig. 2A; $P < 0.05$). Expression levels of miR-26a in eight lung cancer cell lines, including pAa, A549, H460, 95D, H1299, H520, PLAM and H446, was significantly decreased, compared with in 16 HBE cells (Fig. 2B).

Expression levels of DAL-1 in lung cancer tissues and cell lines.

Expression levels of DAL-1 protein in lung cancer tissues, matched adjacent normal lung tissues and various lung cancer cells, were evaluated using western blot analysis. DAL-1 protein expression levels demonstrated a decrease in lung cancer tissues compared with in normal lung tissues (Fig. 2C). Additionally, expression levels of DAL-1 in lung cancer cells significantly decreased compared with 16 HBE cells (Fig. 2D; * $P < 0.05$).

miR-26a suppress lung cancer cell growth in vitro. In order to investigate the function of miR-26a in the regulation of lung cancer cell growth, A549 cells were transfected with the miR-26a inhibitor/NC, and H460 cells were transfected with the miR-26a mimic/NC (Fig. 3). Suppression of miR-26a by the miR-26a inhibitor significantly promoted the growth of A549 cells (Fig. 3E; $P < 0.05$). However, overexpression of miR-26a induced by the miR-26a mimic significantly inhibited the growth of H460 cells (Fig. 3F; $P < 0.05$).

miR-26a suppress lung cancer cell migration and invasion. In order to investigate the effect of miR-26a on the motility of lung cancer cells, the miR-26a inhibitor/NC was transfected into A549 cells, and the miR-26a mimic/NC was transfected into H460 cells (Figs. 3 and 4). Inhibition of miR-26a promoted the migration and invasion capabilities of A549 cells compared with the negative control cells (Figs. 3A and B, and 4A, B, E and F; $P < 0.05$). However, overexpression of miR-26a suppressed the migration and invasion capabilities of lung cancer cells compared with the negative control cells (Figs. 3C and D, and 4C, D, G and H; $P < 0.05$).

miR-26a is negatively associated with DAL-1 in A549 and H460 cells. The expression levels of DAL-1 and miR-26a in lung cancer cells were determined and the results revealed that the DAL-1 protein expression level in A549 cells was low, compared with H460 cells (Fig. 2C). The miR-26 mRNA expression level in A549 cells was higher compared with H460 cells (Fig. 2B).

DAL-1 is not a direct target of miR-26a in lung cancer cells. Relative luciferase activity assays demonstrated that miR-26a may not inhibit the luciferase activity of the wild type or mutation (Mut)-3'UTR of DAL-1 in 293T cells (Fig. 5A). In addition, inhibition or overexpression of miR-26a does not alter DAL-1 expression levels ($P > 0.05$; Fig. 5B and C). This result suggests that miR-26a is not the direct regulator of DAL-1.

ANXA1 is a DAL-1-associated protein regulated by miR-26a.

The results of two-dimensional gel electrophoresis assays indicated that the ANXA1 protein expression level in lung cancer was significantly increased ($P < 0.05$) following DAL-1 silencing by shRNA compared with the control (Fig. 6A and B). In addition, compared with H460-miR-26a mimic NC group,

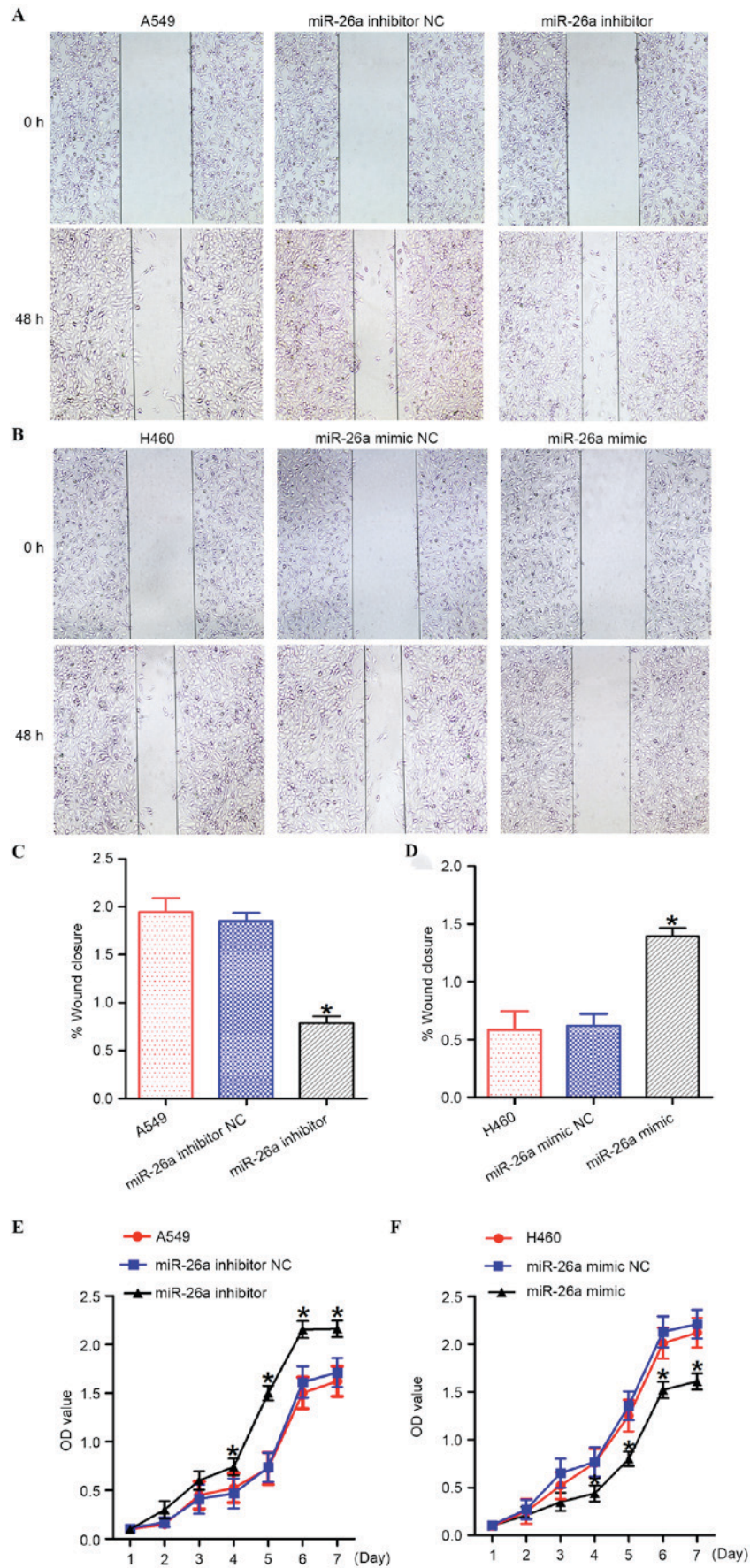


Figure 3. The effects of miR-26a on lung cancer cell proliferation and migration. (A) Downregulation of miR-26a promoted the migratory ability of A549 cells. (B) Upregulation of miR-26a inhibited the ability of the cell monolayer to gap created by a scratch in H460. (C) Downregulation of miR-26a promoted the migratory ability of A549 cells. (D) Upregulation of miR-26a inhibited the ability of the cell monolayer to gap created by a scratch in H460. (E) Downregulation of miR-26a promoted the proliferation of A549 cells. (F) Upregulation of miR-26a inhibited proliferation of H460 cells. *P<0.05 vs. the control group. NC, negative control; OD, optical density; miR, microRNA.

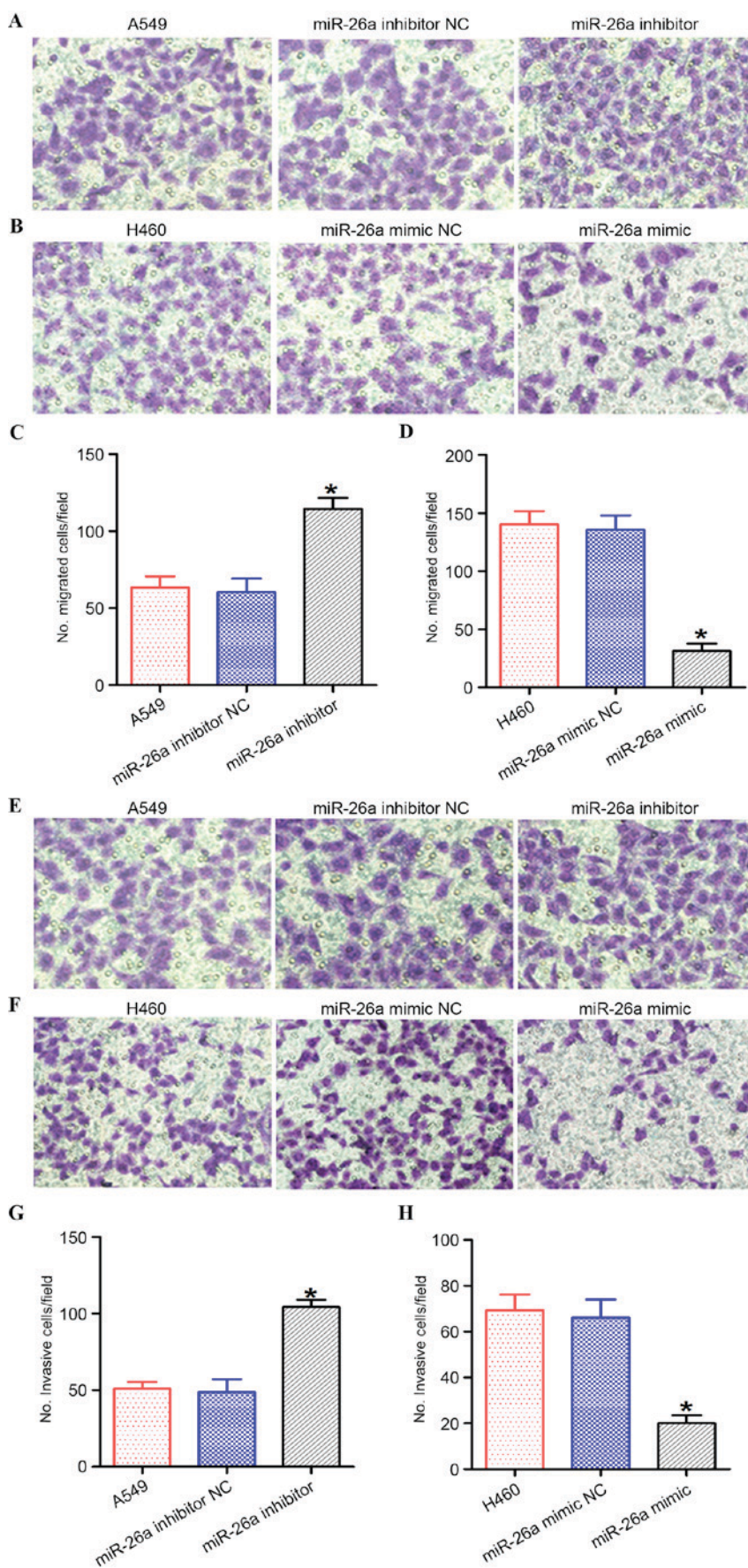


Figure 4. The effects of miR-26a on lung cancer cell migration and invasion determined by Transwell assay. (A) Downregulation of miR-26a promoted the migration in A549 cells. (B) Downregulation of miR-26a promoted the migration in H460 cells. (C) Upregulation of miR-26a inhibited the migration in A549 cells. (D) Upregulation of miR-26a inhibited the migration in H460 cells. (E) Downregulation of miR-26a promoted the invasion in A549 cells. (F) Downregulation of miR-26a promoted the invasion in H460 cells. (G) Upregulation of miR-26a inhibited the invasion in A549 cells. (H) Upregulation of miR-26a inhibited the invasion in H460 cells. All images are presented at magnification $\times 100$, * $P < 0.05$ vs. the control group. NC, negative control; miR, microRNA.

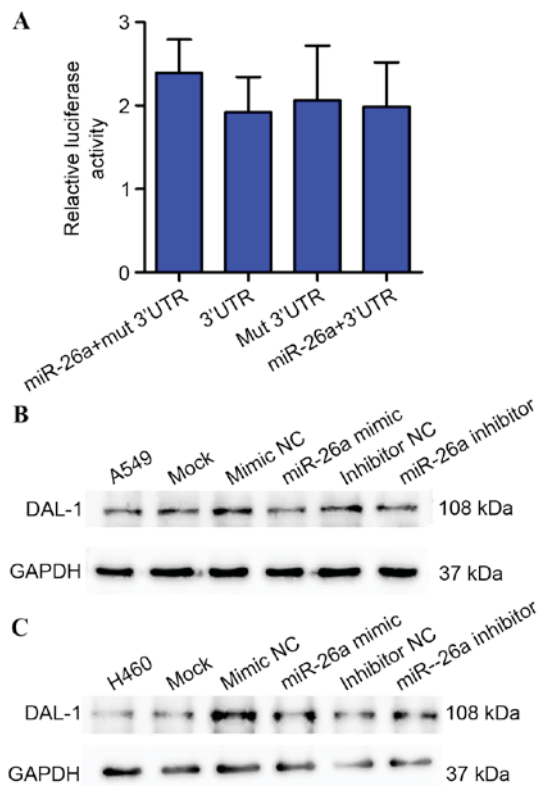


Figure 5. miR-26a did not regulate DAL-1 in lung cancer. (A) Luciferase reporter assay with co-transfection of miR-26a mimic/mimic NC and WT or Mut 3'UTR of DAL-1 in 293T cells. (B) Western blotting results of DAL-1 protein in A549 cells transfected with miR-26a mimic, miR-26a inhibitor and their respective NC at 48 h. (C) Western blotting results of DAL-1 protein in H460 cells transfected with a miR-26a mimic, miR-26a inhibitor and the respective NC at 48 h. DAL-1, differentially expressed in adenocarcinoma of the lung; NC, negative control; miR, microRNA; mut, mutant; UTR, untranslated region.

the overexpression of miR-26a decreased the expression level of ANXA1; and compared with A549-miR-26a inhibitor NC group, the inhibition of miR-26a increased the expression level of ANXA1 (Fig. 6C and D).

Discussion

The initiation of lung cancer is a complicated process with numerous genes and stages of development (18). A previous study demonstrated that DAL-1 is a fundamental tumor suppressor gene and serves an important role in the development of lung cancer (19). A previous study revealed that the expression level of DAL-1 was decreased in lung cancer tissues and the silencing of the DAL-1 gene may enhance the growth, migration and invasion capabilities of lung cancer cells (20). DAL-1 directly associates with the E-cadherin promoter and the subsequent regulation of its expression may induce impairment of EMT and decrease cell migration and invasion (3). The present study utilized shRNA to silence the DAL-1 gene by using a two-dimensional gel electrophoresis assay to identify DAL-1 associated proteins. There are five main upregulated proteins (stathmin, ANXA1, chaperonin containing TCP1 subunit 2, heterogeneous nuclear ribonucleoprotein H1 and moesin) and four main downregulated proteins (peroxiredoxin I, peroxiredoxin II, peroxiredoxin III and

mitochondrial antioxidant manganese superoxide dismutase). DAL-1 may regulate the expression level of ANXA1 protein and participate in the progress of lung cancer, as demonstrated by the results of the present study. However, the detailed role of DAL-1 in lung cancer remains poorly understood.

Previously, tumor suppressive miR-26a has been studied in numerous types of human cancer, and it was revealed to serve a key role as a cancer suppressor gene in liposarcoma (21), thyroid carcinoma (22) and breast cancer (23). miR-26a was demonstrated to be upregulated in glioma, and may enhance cancer cell growth and colony formation (24). The function of miR-26a in lung cancer remains to be elucidated. miR-26a was selected in the present study for investigation as it was suggested that it may combine with DAL-1 mRNA 3'UTR and regulate DAL-1 gene expression, according to the bioinformatics predictions presented in Fig. 1.

The present study investigated the function of miR-26a in the progression of lung cancer. The validation experiments revealed that the mature form of miR-26a was downregulated in lung cancer tissues and lung cancer cells. The biological behavior-inhibitory effects of miR-26a on two NSCLC cell lines, A549 and H460, were also investigated. *In vitro* experiment results demonstrated that miR-26a significantly inhibited the proliferation, migration and invasion capabilities of NSCLC cells. These results suggest that miR-26a serves an important role in NSCLC and may be exploited for adjuvant therapeutic application.

Furthermore, to confirm the association between the expression levels of miR-26a and DAL-1 in lung cancer tissues and cells, the expression of DAL-1 was decreased in lung cancer tissues and various lung cancer cell lines. This result indicated that a decreased expression level of DAL-1 may promote the development of lung cancer. Furthermore, the present study utilized shRNA to silence the DAL-1 gene using a two-dimensional gel electrophoresis assay to identify DAL-1 associated proteins. Congruent with a previous study (25), it was revealed that DAL-1 may regulate the expression levels of ANXA1 protein, and ANXA1 may also participate in the lung cancer process.

It has previously been revealed that the expression level of ANXA1 protein is decreased or not present in head and neck cancer (26), esophageal squamous cell carcinoma (27) and prostate cancer (28), whereas it was revealed to be upregulated in breast cancer (25), glioma (29) and oropharyngeal cancer (30). The molecular mechanisms underlying ANXA1 modulation of cellular responses have not been fully determined. The previous study revealed that ANXA1 upregulation in lung cancer, and suppressing ANXA1 gene expression by the lentiviral vector LV-ANXA1-RNAi, may inhibit the proliferation, migration and invasion capabilities of lung cancer cells (31).

The present study demonstrated an inverse correlation between the expression levels of miR-26a and DAL-1 in A549 and H460 cells. However, the relative luciferase activity in 293T cells co-transfected with miR-26a mimics did not reveal a significant decrease following 48 h or subsequent to transfection with the miR-26a mimics or inhibitor, significant changes were also not observed in DAL-1 protein levels. These results suggest that DAL-1 may not be the target gene of miR-26a. However, the present study demonstrated that ANXA1 protein

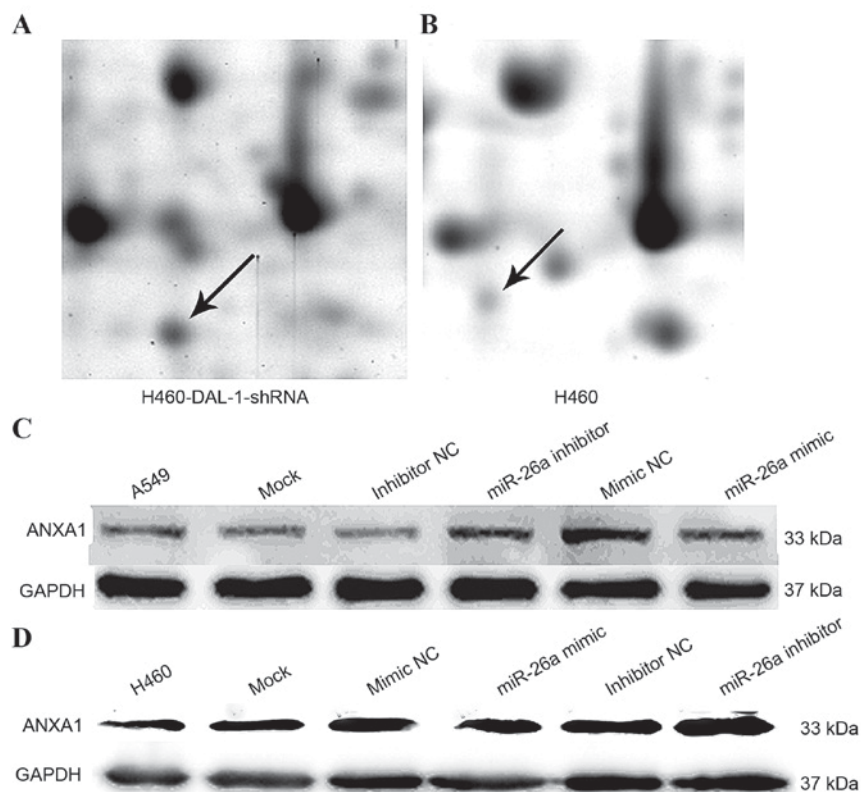


Figure 6. ANXA1 was regulated by miR-26a in lung cancer cells. (A and B) The expression level of ANXA1 protein was significantly regulated by DAL-1 using the two-dimensional electrophoresis assay (with arrows indicating differentiated proteins). (C) A549 cells transfected with miR-26a mimic/NC or miR-26a inhibitor/NC, and western blot analysis was used to detect the protein levels of ANXA1. (D) H460 cells transfected with miR-26a mimic/NC, or miR-26a inhibitor/NC, and western blot analysis was used to detect the protein levels of ANXA1. DAL-1, differentially expressed in adenocarcinoma of the lung; NC, negative control; ANXA1, annexin 1; shRNA, short hairpin RNA; miR, microRNA.

levels were decreased following transfection of the miR-26a mimic into A549 and H460 cells, and increased subsequent to transfection with the miR-26a inhibitor.

miR-26a is decreased in numerous types of human cancer and has anti-oncogenic roles. A previous study demonstrated that miR-26a targets the bone morphogenetic protein (BMP)/mothers against decapentaplegic homolog 4 pathway and regulated neovascularization (32). miR-26a was revealed to suppress enhancer of zest homolog 2 (33), myeloid cell leukemia sequence 1 (23) and fibroblast growth factor 9 (34), which inhibited cancer cell migration and invasion. miR-26a regulated the proliferation and growth of cancer cells by targeting chromodomain helicase DNA binding protein 1 (35), growth regulation by estrogen in breast cancer 1 (35) and β -catenin (36). A number of studies demonstrated that the expression levels of miR-26a decreased in various types of tumor (23,37,38), whereas ANXA1 protein was overexpressed (39-41). Combined with the results of the present study suggesting that the miR-26a mimic may reduce the expression levels of ANXA1, a negative correlation was identified between miR-26a and ANXA1 in numerous tumors. Furthermore, the present study revealed that overexpression of miR-26a or suppression of ANXA1 may inhibit the ability of growth, invasion and migration of lung cancer cells. The aforementioned results suggest that miR-26a may regulate ANXA1 in lung cancer.

In conclusion, miR-26a expression was decreased in lung cancer and the altered expression level of miR-26a

may affect various biological processes in lung cancer cells, including proliferation, migration and invasion. miR-26a regulated the expression levels of ANXA1 and not DAL-1. miR-26a may function as a biomarker for lung cancer, providing a novel strategy for the treatment of lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and TC conceived and designed the study. TC, HW, JL, YF, XG and XX performed the experiments. TC and XG wrote the paper. YZ, TC and XG reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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