

Original Article

MiR-302a-5p suppresses cell proliferation and invasion in non-small cell lung carcinoma by targeting ITGA6

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Abstract: MicroRNA-302a-5p (miR-302a-5p) has been implicated in several cancers; however, its role in human non-small cell lung carcinoma (NSCLC) remains unknown. In this study, we showed that miR-302a-5p is downregulated in NSCLC tissues and cell lines. Cell Counting Kit-8 and 5-ethynyl-2'-deoxyuridine assays showed that overexpression of a miR-302a-5p mimic suppressed NSCLC cell proliferation, which was confirmed by the results of a cell cycle assay. Overexpression of miR-302a-5p also reduced the migration and invasion of NSCLC cells. Additionally, miR-302a-5p overexpression significantly inhibited NSCLC growth and metastasis in a mouse xenograft model. With regard to the underlying mechanism, integrin $\alpha 6$ (*ITGA6*) mRNA was shown to be a novel target of miR-302a-5p, and overexpression of *ITGA6* attenuated the inhibitory effects of miR-302a-5p on the proliferation and migration of NSCLC cells. In clinical NSCLC samples, miR-302a-5p expression was negatively correlated with *ITGA6* expression, which was high in the samples. Collectively, these results indicate that miR-302a-5p acts as a tumor suppressor in NSCLC by directly targeting *ITGA6* mRNA and may be useful as a theranostic biomarker of NSCLC.

Keywords: miR-302a-5p, non-small cell lung carcinoma, *ITGA6*, proliferation, motility

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma, accounts for ~85% of all lung cancers and is less sensitive to chemotherapy and radiation therapy than other lung cancer types [2]. Despite recent advances in cancer research and treatment, the prognosis of NSCLC is still poor, with a 5-year survival rate of only 15% [3]. Therefore, there is an urgent need to fully understand the pathogenesis of NSCLC and identify new treatment targets.

MicroRNAs (miRNAs) are a class of small (18-25 nucleotides) noncoding RNAs that modulate diverse biological processes by binding to the 3' untranslated region (3'-UTR) of target mRNAs, causing mRNA degradation or translational repression [4]. In tumor processes, miRNAs play crucial roles in cell proliferation, migration, and apoptosis and thus affect tumorigenesis (positively or negatively), suggesting that miRNAs are involved in tumorigenesis and

are potential targets for drug development [5, 6]. In fact, several miRNAs have been shown to be abnormally expressed during the initiation and progression of NSCLC, including miR-195, miR-361-3p, and miR-186 [7-9].

Some studies have showed that the miR-302 family, which include miR-302a, miR-302b, miR-302c, and miR-302d, exerts antitumor effects in several cancers. MiR-302a has been shown to function as a tumor suppressor by regulating diverse cellular functions. For example, miR-302a-5p and miR-367-3p suppress the malignant behavior of endometrial carcinoma cells by inhibiting *HMG2* expression [10]; miR-302a-5p targets *GAB2* to suppress cell proliferation, migration, and invasion in glioma [11]; and miR-302a, -b, -c, and -d cooperatively inhibit *BCRP* expression to increase the drug sensitivity of breast cancer cells [12]. However, the function of miR-302a-5p in the pathogenesis of NSCLC remains unclear.

In this study, we demonstrated that miR-302a-5p is downregulated in NSCLC cells and tissues and provided evidence that miR-302a-5p over-

expression inhibits the cell cycle as well as cell proliferation, migration, and invasion, suggesting that miR-302a-5p suppresses the initiation and progression of NSCLC. Moreover, ITGA6 was identified and characterized as a direct target gene of miR-302a-5p that mediates the tumor-suppressive actions in NSCLC. These findings demonstrate that miR-302a-5p suppresses the proliferation and migration of NSCLC cells by targeting ITGA6 and suggest that miR-302a-5p and/or ITGA6 may be useful molecular targets for the development of novel therapeutic strategies against NSCLC.

Materials and methods

Tissue samples and ethics statement

Thirty paired NSCLC and adjacent noncancerous lung tissue samples were obtained from patients treated at Jinshan Hospital, Fudan University. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Jinshan Hospital, in accordance with international standards. None of the patients had received preoperative radiotherapy or chemotherapy. The tissue samples were frozen and stored at -80°C.

Cell culture and transfection

Four human NSCLC cell lines (A549, SPC-A1, H1299, and SK-MES-1) and HEK293T cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). PC-9 cells and human bronchial epithelium (HBE) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The human NSCLC cell lines were cultured in DMEM or RPMI 1640 (Invitrogen, Carlsbad, CA, USA). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Oligonucleotides, plasmid construction, and cell transfection

ITGA6-coding sequences lacking the 3'-UTR were cloned into the pcDNA3.1 vector (Invitrogen) to generate an ITGA6 overexpression vector. A miR-302a-5p mimic and negative control (miR-NC) were acquired from RiboBio (Guangdong, China). Cells were transfected with the miR-302a-5p mimic and miR-NC using Lipofectamine 2000, according to the manufacturer's instructions. At 48 h after transfection,

the cells were collected for subsequent experiments.

RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted from tissues or cells with TRIzol® Reagent (Invitrogen). Complementary DNA was synthesized with the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China), and qRT-PCR analyses were performed with SYBR Premix Ex Taq II (TaKaRa). RNU6B (U6) or GAPDH was included as a control for normalization. Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method. All experiments were conducted in triplicate.

Western blotting

Cell lysates were prepared using RIPA buffer. Lysates containing equal amounts of protein were separated by SDS-PAGE on 10% gels and transferred to polyvinylidene fluoride membranes. The membranes were then blocked and probed with the following primary antibodies: anti-ITGA6 and anti-β-actin (Proteintech, Wuhan, China). Bound antibodies were detected with secondary antibodies conjugated to horseradish peroxidase (Proteintech) and visualized with an enhanced chemiluminescent substrate on an Imaging System.

Cell proliferation

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8). Transfected cells were seeded in 96-well plates at 2.0×10^3 cells per well, and cell viability was examined at different time points (1, 2, 3, and 4 days after seeding). The absorbance was measured at 490 nm on a spectrophotometric microplate reader.

A 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was performed to assess cell proliferation as the immunofluorescence of newly synthesized DNA. Briefly, cells were incubated with EdU for 2 h and then processed with the Cell-Light EdU Apollo 643 In Vitro Imaging Kit (Ribobio, Guangzhou, China). Proliferation was measured as the percentage of EdU-positive cells among the 4',6-diamidino-2-phenylindole (DAPI)-stained cells.

Cell cycle analysis

The cell cycle analysis was performed as previously described [13]. Briefly, the cells were col-

lected and fixed overnight in 75% ethanol. The fixed cells were then washed and stained with propidium iodide (PI; Kaiji Biotech, Nanjing, China) in the presence of 10 µg/mL RNase A (Sigma-Aldrich), 0.05 mM ethylene diamine tetraacetic acid, and 0.2% Triton X-100. The DNA contents were then measured on a FACSCalibur flow cytometer (BD Biosciences, NJ, USA), and the results were analyzed with ModFit software (BD Biosciences).

Cell migration and invasion assays

A wound-healing assay was performed to evaluate cell migration ability. Transfected cells were cultured in six-well plates until confluence. Then, a “scratch” was created in the monolayer with a p200 pipette tip, and the cells were cultured in serum-free medium for 24 h. Each scratch, which was marked by parallel lines on the bottom of the plates, was examined at 0 and 24 h. Each scratch was photographed, and the width of the scratch was assessed by viewing under an inverted microscope.

After A549 and H1299 cells were transfected with the miR-302a-5p mimic or a negative control (miR-NC), the cells were harvested, and a cell suspension was prepared at 10⁶ cells/ml, respectively. Then, the migration and invasion of these cells were measured by the QCM Laminin Migration Assay (ECM220; Merck Millipore, Merck KGaA, Darmstadt, Germany) and the Cell Invasion Assay kit (ECM 550, Merck Millipore), respectively. Finally, the absorbance was measured at 560 nm for migration and invasion assays according to the manufacturer's protocols.

Luciferase assay

HEK293T cells were seeded in 96-well plates and cotransfected with psiCHECK-2-ITGA6 carrying either the wild-type (wt) 3'-UTR or a mutant (mut) 3'-UTR and either the miR-302a-5p mimic or a negative control miRNA (miR-NC). After 48 h of incubation, firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

Lentivirus production and cell transduction

The pri-miR-302a-5p and negative control miRNA precursor sequences were cloned into the

pLV3 vector (GeneChem, Shanghai, China) to generate pLV3-miRNA-302a-5p and pLV3-NC, respectively. Then, the recombinant lentiviral plasmids were cotransfected into HEK293T cells with the packaging and envelope vectors. At 48 h after transfection, the lentivirus-containing medium was collected and filtered.

Animal experiments

The animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of Jinshan Hospital, Fudan University. Approximately 10⁷ A549 cells overexpressing miR-302a-5p or miR-NC were injected into 6-8-week-old female athymic nude mice. Tumor growth was monitored every 5 days, and at each time point, tumor volume was calculated as (width² × length)/2. Thirty days later, the mice were euthanized, the tumors were photographed, and the tumor weights were determined.

Immunohistochemical assay

Freshly isolated tissues from mice injected with A549 cells overexpressing miR-302a-5p or miR-NC were fixed in formalin. The fixed tissues were embedded in paraffin, sectioned (4-µm-thick), and incubated with primary antibodies against ITGA6 and Ki-67 (Proteintech, Wuhan, China) overnight. Then, the sections were incubated with the secondary antibody and stained with a 3,3-diaminobenzidine solution.

Statistical analysis

All analyses were performed using GraphPad Prism (Version 5.0; GraphPad Software, Inc, California, USA). Data are expressed as the mean ± SD and were analyzed using Student's *t* test or ANOVA. The correlation between the expression levels of ITGA6 and miR-302a-5p was assessed by Pearson's correlation analysis. Differences with *P* values less than 0.05 were considered significant.

Results

miR-302a-5p is downregulated in NSCLC cell lines and tissues

To explore the role of miR-302a-5p in NSCLC, the expression of miR-302a-5p was analyzed in

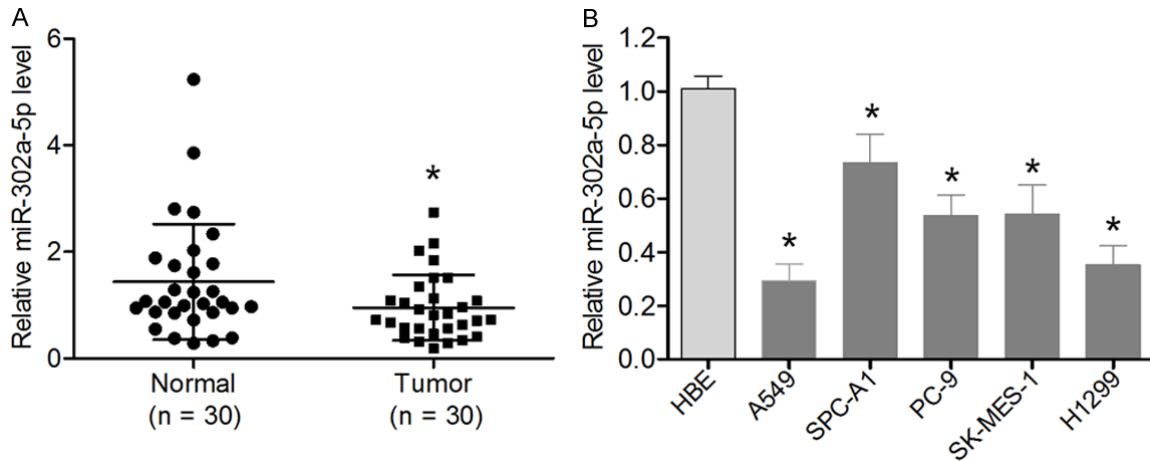


Figure 1. MiR-302a-5p is downregulated in NSCLC tissue samples and cell lines. A. Quantification of miR-302a-5p expression in 30 paired human NSCLC samples and adjacent noncancerous lung tissue samples. B. Quantification of miR-302a-5p expression in NSCLC cell lines (A549, SPC-A1, PC-9, H1299 and SK-MES-1) and normal HBE cells. *P < 0.05 vs. expression levels in HBE cells.

30 paired NSCLC and adjacent non-cancerous lung tissue samples by qRT-PCR. miR-302a-5p expression was significantly lower in the NSCLC tissues than in adjacent noncancerous lung tissues (**Figure 1A**). Moreover, the expression of miR-302a-5p was lower in the NSCLC cell lines (A549, SPC-A1, PC-9, SK-MES-1, and H1299) than in HBE cells (**Figure 1B**). These results suggest that overexpression of miR-302a-5p inhibited the progression of NSCLC.

MiR-302a-5p inhibits NSCLC cell proliferation and the cell cycle in vitro

The miR-302a-5p mimic or a negative control (miR-NC) was transfected into two NSCLC cell lines (A549 and H1299), and the transfection efficiency was determined by qRT-PCR (**Figure 2A**). The CCK-8 assay showed that the proliferation of NSCLC cells transfected with the miR-302a-5p mimic was obviously suppressed when compared to the proliferation of cells transfected with the negative control miRNA (**Figure 2B**). Similarly, the EdU assays suggested that overexpression of miR-302a-5p decreased the numbers of EdU-positive nuclei in A549 and H1299 cells compared with the numbers in the miR-NC-transfected cells (**Figure 2C**). We also assessed whether miR-302a-5p affected cell cycle progression in A549 and H1299 cells. As determined by flow cytometry, overexpression of miR-302a-5p increased the number of cells in G1 phase and decreased the number of cells in S phase (**Figure 2D**). These

results indicate that miR-302a-5p inhibited NSCLC cell proliferation and cell cycle progression in vitro.

MiR-302a-5p inhibits NSCLC cell migration and invasion in vitro

Next, we examined the effects of miR-302a-5p on the migration and invasion of NSCLC cells in vitro. The wound-healing assay showed that the migratory ability of A549 and H1299 cells transfected with the miR-302a-5p mimic was lower than that of cells transfected with miR-NC (**Figure 3A**). Consistent with these results, the migration assay confirmed that transfection of the miR-302a-5p mimic remarkably inhibited the migratory ability of A549 and H1299 cells (**Figure 3B**). In addition, the invasion assay revealed that transfection of the miR-302a-5p mimic significantly decreased the invasive ability of A549 and H1299 cells compared with that of the cells transfected with miR-NC (**Figure 3C**). These data suggest that overexpression of miR-302a-5p reduces the migratory and invasive ability of NSCLC cells in vitro.

ITGA6 is a direct target of miR-302a-5p

To elucidate the molecular mechanisms through which miR-302a-5p exerts its tumor-suppressive functions, we searched publicly available databases to identify functionally relevant targets of miR-302a-5p. ITGA6 was identified as a target gene of miR-302a-5p, as it contains

miR-302a-5p suppresses NSCLC by targeting ITGA6

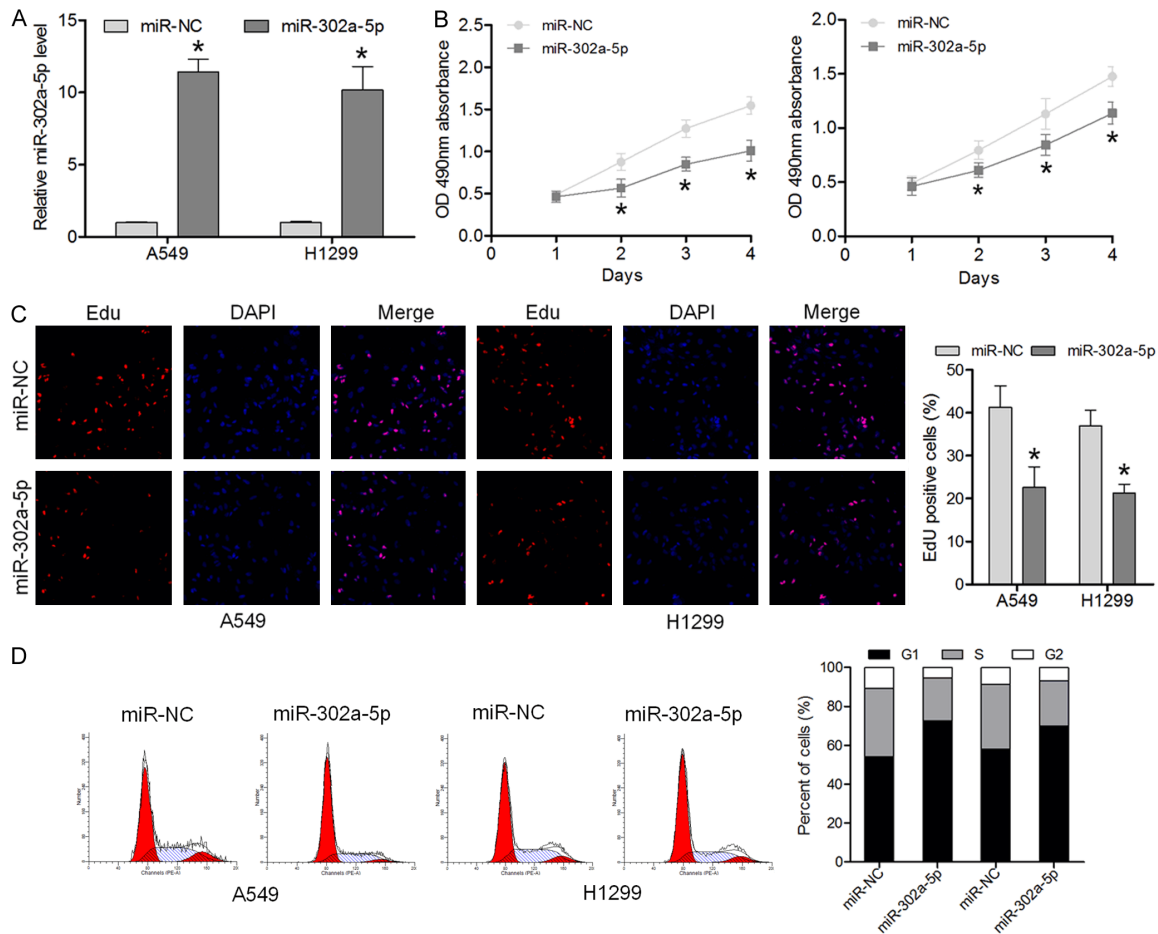


Figure 2. Overexpression of miR-302a-5p suppresses NSCLC cell proliferation and the cell cycle. A. Quantification of miR-302a-5p expression after transfection of a miR-302a-5p mimic or control miRNA (miR-NC) into A549 and H1299 cells. B. Proliferation of these cells was measured by the CCK-8 assay. C. An EdU incorporation assay was performed on miR-NC- or miR-302a-5p-transfected A549 and H1299 cells. D. miR-302a-5p mimic or miR-NC-transfected A549 and H1299 cells in each cell cycle phase were quantified by a flow-cytometric assay. *P < 0.05 vs. miR-NC-transfected cells.

a putative target sequence (**Figure 4A**). Therefore, we constructed luciferase reporter vectors that contained either the wt or a mut miR-302a-5p target sequence in the ITGA6 3'-UTR. The luciferase reporter assays showed that the luciferase activity of the wt 3'-UTR reporter was significantly decreased by miR-302a-5p overexpression, whereas the activity of the mutant reporter was not affected by miR-302a-5p overexpression (**Figure 4B**). qRT-PCR and western blot analyses indicated that miR-302a-5p overexpression significantly reduced the mRNA and protein levels of ITGA6 (**Figures 4C, 4D** and **S1**). Moreover, we found that ITGA6 expression was higher in NSCLC tissues than in adjacent non-cancerous lung tissues, and its expression was inversely correlated with miR-302a-5p expression in the NSCLC tissue samples (**Figure 4E**

and **4F**). These results indicated that miR-302a-5p can directly suppress the expression of ITGA6 in NSCLC cells by directly targeting the 3'-UTR of ITGA6 mRNA.

ITGA6 reverses the effects of miR-302a-5p on NSCLC cells

To determine whether the inhibition of NSCLC cell proliferation, migration, and invasion induced by miR-302a-5p is mediated by ITGA6, A549 and H1299 cells were cotransfected with the miR-302a-5p mimic and the ITGA6 overexpression plasmid or the miR-302a-5p mimic and an empty vector. As shown in **Figures 5A-C** and **S1**, overexpression of ITGA6 significantly attenuated miR-302a-5p-induced suppression of NSCLC cell proliferation, invasion, and migra-

miR-302a-5p suppresses NSCLC by targeting ITGA6

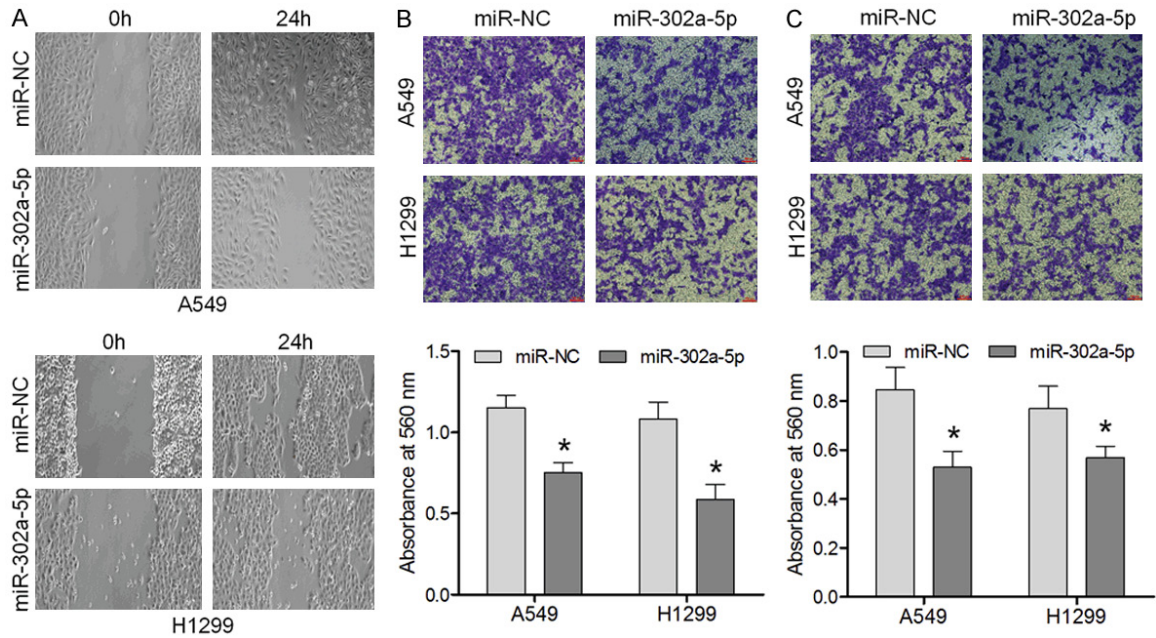


Figure 3. MiR-302a-5p inhibits NSCLC cell migration and invasion *in vitro*. A. Representative images of a wound-healing assay using A549 and H1299 cells transfected with either the miR-302a-5p mimic or a negative control (miR-NC). B. Migration assays of A549 and H1299 cells transfected with either the miR-302a-5p mimic or miR-NC. C. Invasion assays using A549 and H1299 cells transfected with either the miR-302a-5p mimic or miR-NC. *P < 0.05 vs. miR-NC-transfected cells.

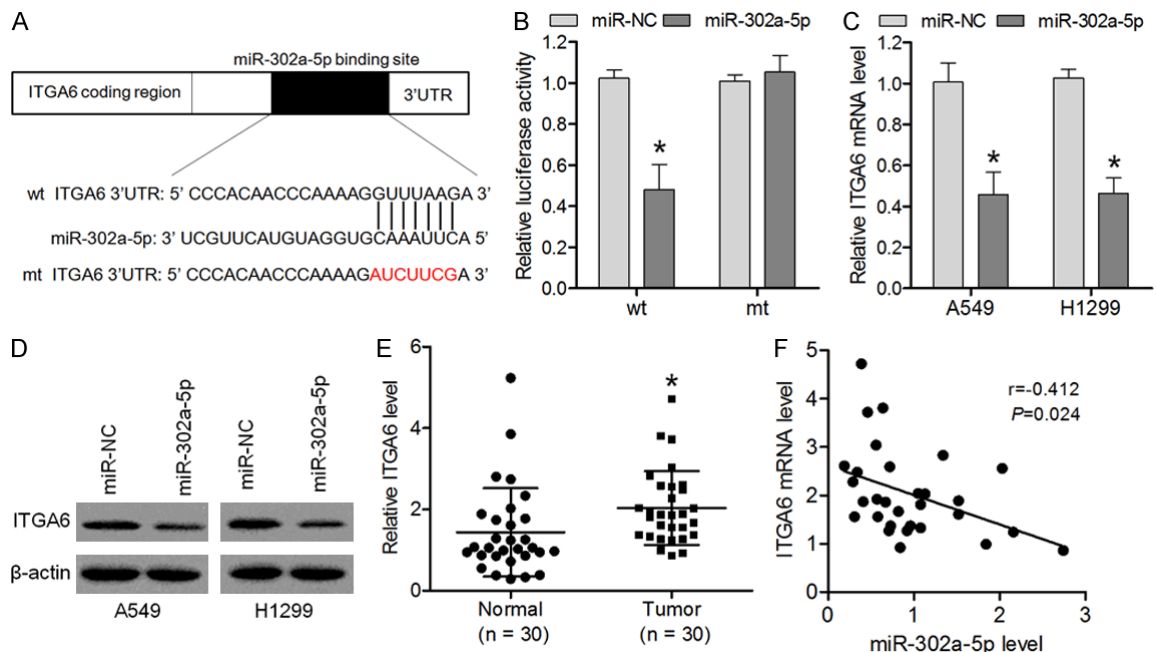


Figure 4. ITGA6 is a target of miR-302a-5p. A. Predicted miR-302a-5p-binding sites in the wt and mutant (mt) ITGA6 3'-UTR. B. Relative luciferase activity of HEK293 cells cotransfected with either the wt or mt ITGA6 3'-UTR reporter along with either the miR-302a-5p mimic or miR-NC (control). C. Quantification of ITGA6 mRNA expression in A549 and H1299 cells transfected with the miR-302a-5p mimic or miR-NC by qRT-PCR analysis. D. Detection of ITGA6 protein expression in A549 and H1299 cells transfected with the miR-302a-5p mimic or miR-NC by western blotting. E. Quantification of ITGA6 expression in 30 paired human NSCLC and noncancerous lung tissue samples. F. The correlation between miR-302a-5p expression and ITGA6 expression in NSCLC tissue samples. *P < 0.05 vs. miR-NC-transfected cells.

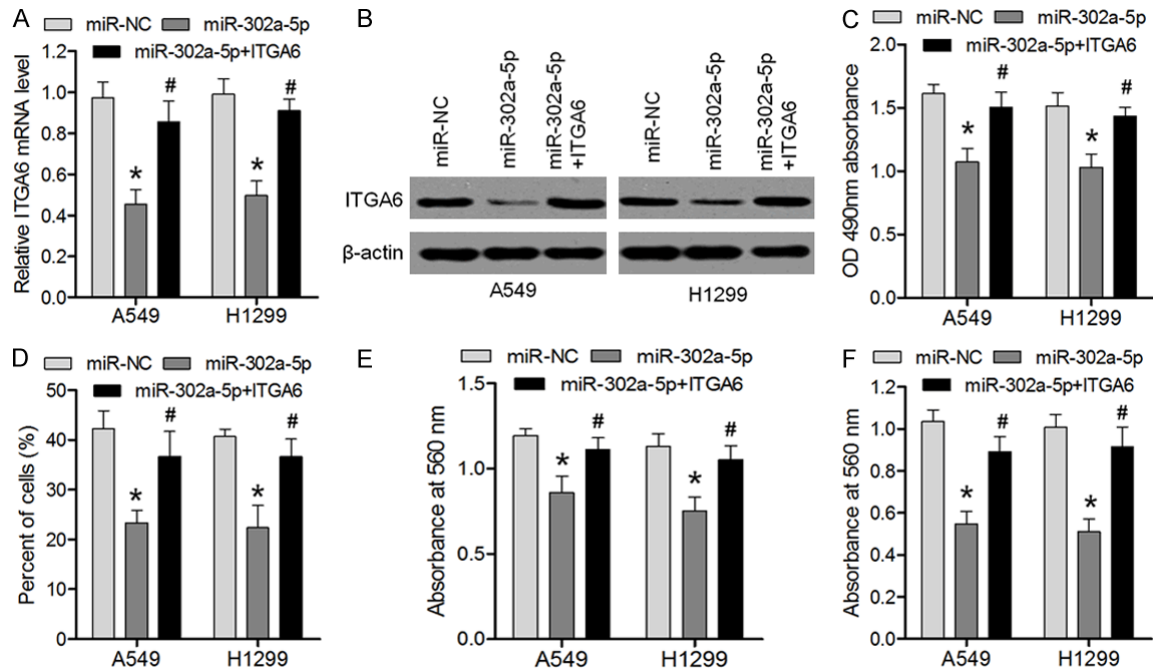


Figure 5. Expression of ITGA6 reverses the effects of miR-302a-5p on NSCLC cell proliferation, migration, and invasion. A549 and H1299 cells were transfected with either the miR-302a-5p mimic or a negative control (miR-NC) or cotransfected with the miR-302a-5p mimic and ITGA6. A. Quantification of ITGA6 mRNA expression in transfected A549 and H1299 cells by qRT-PCR analysis. B. Evaluation of ITGA6 protein expression in transfected A549 and H1299 cells by western blotting. C. Assessment of cell proliferation by the CCK-8 assay. D. Measurement of EdU incorporation in transfected A549 and H1299 cells. E, F. Migration and invasion assays of A549 and H1299 cells. * $P < 0.05$ vs. the miR-NC group; # $P < 0.05$ vs. the miR-302a-5p group.

tion. Therefore, ITGA6 mRNA is a direct target of miR-302a-5p through which NSCLC cell proliferation, migration, and invasion are suppressed.

miR-302a-5p inhibits tumor growth in vivo

A xenograft model of NSCLC was established to determine the effects of miR-302a-5p on tumor growth in vivo. A549 cells transduced with an miR-302a-5p-expressing or miR-NC lentivirus were subcutaneously injected into nude mice. Tumors derived from miR-302a-5p-overexpressing cells were significantly smaller than tumors derived from miR-NC lentivirus-transduced control cells (Figure 6A). Overexpression of miR-302a-5p dramatically suppressed tumor growth, resulting in reduced tumor weight (Figure 6B and 6C). We noted higher expression of miR-302a-5p in tumors derived from miR-302a-5p-overexpressing cells in comparison to the expression in miR-NC-transduced cells (Figure 6D). The immunohistochemistry results showed lower protein expression of ITGA6 and the cell proliferation marker Ki-67 in the miR-302a-5p group than in the miR-NC

group (Figure 6E and 6F). These results suggested that miR-302a-5p inhibited the growth of NSCLC in vivo.

Discussion

Accumulating evidence indicates that miRNAs with deregulated expression in tumors can function as oncogenes or tumor suppressors [14]. In our study, we showed that miR-302a-5p is downregulated in NSCLC tissue samples and cell lines. Exogenous overexpression of miR-302a-5p suppressed NSCLC cell proliferation, invasion, and migration in vitro and inhibited tumor growth in vivo. Furthermore, we demonstrated that ITGA6 mRNA is a direct target of miR-302a-5p, and overexpression of ITGA6 antagonized the inhibitory effects of miR-302a-5p on the proliferation and motility of NSCLC cells. These results show that miR-302a-5p functions as a tumor suppressor in NSCLC by directly targeting ITGA6 mRNA and may serve as a theranostic biomarker of NSCLC.

An increasing number of miRNAs have been found to be abnormally expressed in NSCLC

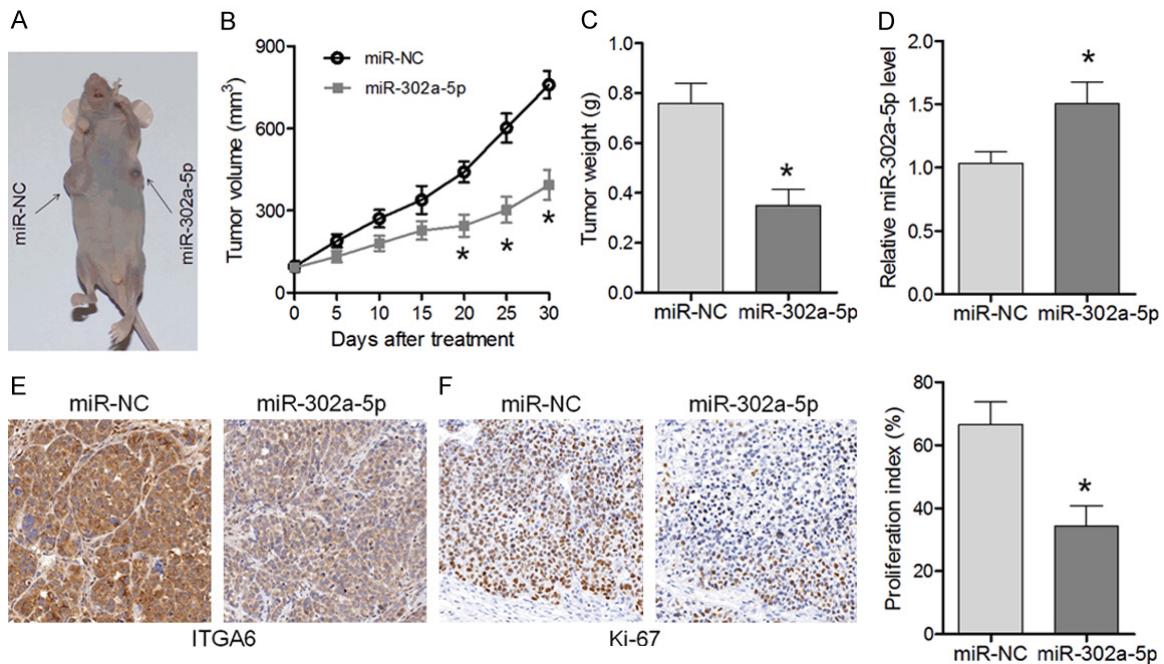


Figure 6. MiR-302a-5p inhibits tumor growth in vivo. A. Images of excised xenograft tumors from miR-NC group and miR-302a-5p group. B. Tumor volume curves of two groups were measured. C. Tumor weight of tumors from two groups were determined. D. Quantification of miR-302a-5p expression in mice injected with cells transfected with the miR-302a-5p mimic or miR-NC. E, F. Evaluation of ITGA6 and Ki-67 protein levels in the tumors by immunohistochemical staining. *P < 0.05 vs. miR-NC-transfected cells.

and to be involved in the pathogenesis of NSCLC. Zhang *et al.* reported that miR-411 promotes NSCLC carcinogenesis by directly targeting the mRNAs of the tumor suppressor genes SPRY4 and TXNIP [15]. Liu *et al.* reported that miR-661 promotes tumor invasion and metastasis by directly inhibiting RB1 expression in NSCLC [16]. Gu *et al.* demonstrated that miR-383 inhibits cell viability and induces apoptosis via the Wnt- β -catenin signaling pathway in NSCLC [17]. Liu *et al.* reported that miR-1253 suppresses the proliferation and invasion of NSCLC cells by targeting *WNT5A* mRNA [18]. MiR-302a-5p was shown to be downregulated in several malignancies, including endometrial, gastric, and breast cancers [10, 19, 20]. However, the biological function and molecular mechanism underlying the action of miR-302a-5p in NSCLC were unknown. In our study, we demonstrated that miR-302a-5p is upregulated in NSCLC cells and tissue samples and that overexpression of miR-302a-5p significantly suppressed the proliferation, migration, and invasion of NSCLC cells *in vitro* and inhibited tumor growth *in vivo*. These results suggest miR-302a-5p as a tumor suppressor

and a critical factor regulating NSCLC cell proliferation, migration, and invasion.

To elucidate the mechanisms underlying the miR-302a-5p-induced inhibition of NSCLC cell proliferation, migration, and invasion, we used publicly available prediction algorithms and identified ITGA6 as a direct target of miR-302a-5p in NSCLC cells. ITGA6 is a member of the integrin family that performs a key function in the interactions between many cell types and is involved in several biological processes, including cell proliferation and invasion [21]. ITGA6 has been shown to play oncogenic roles in various malignancies, including colorectal cancer, breast cancer, glioblastoma, and neck squamous cell carcinoma [22-25]. For example, p53-induced miR-30e-5p inhibited colorectal cancer invasion and metastasis by targeting ITGA6 and ITGB1 [22]. In addition, ITGA6 was directly regulated by hypoxia-inducible factors and promotes cancer stem cell activity and invasion in metastatic breast cancer models [23]. Kruppel-like factor 9 inhibited glioblastoma stemness through global transcriptional repression and inhibition of ITGA6 [24]. Silencing of the ITGA6 gene significantly inhibited

the migration and invasion of neck squamous cell carcinoma cells [25]. In our study, we verified ITGA6 as a direct target of miR-302a-5p in NSCLC through a luciferase reporter gene assay, which showed that miR-302a-5p overexpression significantly suppressed ITGA6 mRNA and protein expression in NSCLC cell lines. In addition, ITGA6 expression was upregulated in NSCLC tissues and negatively correlated with miR-302a-5p expression. Moreover, overexpression of ITGA6 reversed the inhibitory effects of miR-302a-5p on the proliferation and migration of NSCLC cells, thereby revealing the important contribution of ITGA6 to the responses triggered by miR-302a-5p.

In conclusion, we provided evidence that miR-302a-5p functions as a tumor suppressor by decreasing ITGA6 expression in NSCLC and inhibiting the tumorigenesis of these cells. MiR-302a-5p was underexpressed in NSCLC tissues and cell lines at baseline, and overexpression of miR-302a-5p suppressed in vitro and in vivo tumor growth by directly inhibiting ITGA6 in NSCLC cells. The newly identified miR-302a-5p-ITGA6 axis provides new insight into the pathogenesis of NSCLC and suggests novel targets for therapeutic interventions in NSCLC.

Disclosure of conflict of interest

None.

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miR-302a-5p suppresses NSCLC by targeting ITGA6

Fig. 4D

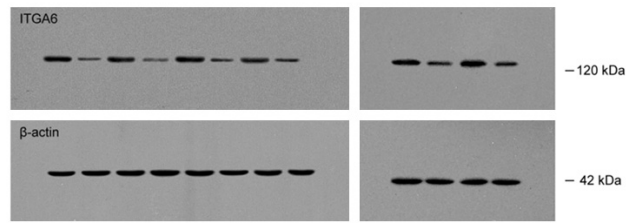


Fig. 5B

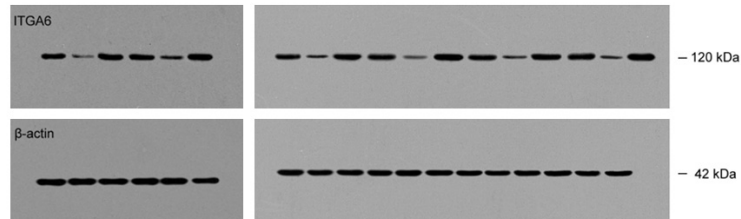


Figure S1. Uncropped images of all the western blotting data.