

Original Article

Down-regulation of microRNA-539 promotes tumor growth and invasion through regulating FSCN1 expression in non-small cell lung cancer

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Abstract: A growing volume of literature has documented that microRNAs (miRNAs) act as oncogenes or tumor suppressors in non-small-cell lung cancer (NSCLC). In this article, a significant down-regulation of miR-539 was observed in all NSCLC cell lines as compared with the normal human bronchial epithelial cell line. Gain- and loss-of-function assays revealed that overexpression of miR-539 remarkably repressed cell proliferation, migration/invasion *in vitro*, and tumor growth in xenograft nude mice, whereas knockdown of miR-539 demonstrated the opposite effect. Further investigations revealed that miR-539 suppressed the mRNA and protein expression of FSCN1, and luciferase assays confirmed FSCN1 as a *bona fide* target of miR-539. Our data provided the first evidence that miR-539 exerts onco-suppressive functions in NSCLC carcinogenesis through directly regulating FSCN1, which suggested that miR-539 can be utilized as a novel diagnostic marker or therapeutic target for NSCLC in the near future.

Keywords: Non-small-cell lung cancer, microRNA, miR-539, FSCN1, proliferation, migration, invasion

Introduction

Lung cancer remains one of the leading reasons for cancer-related deaths in the world, particularly in China [1]. At least 80% of lung cancers were non-small-cell lung cancers (NSCLC), including four main subtypes: adenocarcinoma (ADC), squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell carcinoma (LCC) [2, 3]. Despite significant improvement in the strategies for prevention, diagnosis and treatment, the overall 5-year survival rate after surgery is reported to be only 30-60% in NSCLC patients [4]. Therefore, the identification of the molecular mechanisms underlying the pathogenesis of NSCLC and potential therapeutic targets are urgent and of great importance.

Post-transcriptional regulation has been characterized as a pivotal mechanism in modulating gene expression. MicroRNAs (miRNAs) are defined as a cluster of small non-coding, single-stranded RNAs ubiquitously expressed in eukaryotic cells, which function in transcriptional and post-transcriptional regulation of gene expression through binding to complementary

sequences in the 3'-untranslated region (3'-UTR) of target mRNAs [5, 6]. It is estimated that more than 50% of the miRNAs are located in cancer-associated genomic regions or in fragile sites of chromosomes [7]. Numerous articles have documented that miRNAs functioned as oncogenes or tumor suppressors in various types of cancers, including breast cancer [8], pancreatic cancer [9], oral cancer [10] and gastric cancer [11]. Up to now, the aberrant expression of multiple miRNAs has been observed in NSCLC tissues and cell lines. For example, decreased expression of miR-320a, miR-663a, and miR-376c have been shown to contribute to the pathogenesis of NSCLC by promoting cell proliferation, invasion and metastasis through targeting the relative genes [12-14]. Accordingly, investigating the dysregulated expression pattern of miRNAs and the roles of miRNAs in NSCLC will be beneficial to a great understanding of the mechanism of NSCLC carcinogenesis and develop new approaches for NSCLC diagnosis and therapy in the near future.

MiR-539, which is initially featured to be increased in failing heart [15], has been found to be deregulated in many kinds of malignancies. The

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Table 1. The sequences of primers used for qRT-PCR

Gene name	Primer sequences	Primer length (bp)
miR-539		
Forward	5'-ACTGGAGAAATTATCCTTG-3'	19
Reverse	5'-GTGCAGGGTCCGAGGT-3'	16
FSCN1		
Forward	5'-CTACAACATCAAAGACTCCACAG-3'	23
Reverse	5'-ATGGCCACCTTGTTATAGTC-3'	20
U6		
Forward	5'-CTCGCTTCGGCAGCACA-3'	17
Reverse	5'-AACGCTTCACGAATTTGCGT-3'	20
GAPDH		
Forward	5'-ACAGTCAGCCGCATCTTCTT-3'	20
Reverse	5'-GACAAGCTTCCCGTTCTCAG-3'	20

article of Gu et al. suggested that miR-539 serves an onco-suppressor role in thyroid cancer cell migration and invasion through targeting CARMA1 [16]. Moreover, Jin and colleagues demonstrated that miR-539 represses osteosarcoma cell migration and invasion through targeting Matrix metalloproteinase-8 [17]. Therefore, it might be interesting to explore the regulatory function of miR-539 in NSCLC.

We hypothesize that miR-539 might play a pivotal role in the initiation and progression of human NSCLC. In the present study, we discovered that miR-539 expression level was drastically decreased in human NSCLC cells. *In vitro* and *in vivo* functional assays were performed to confirm the anti-tumor effects of miR-539 in NSCLC by directly targeting FSCN1, which is overexpressed in multiple cancers. Thus, our results suggested that miR-539 might play critical functions in NSCLC progression and act as a promising therapeutic target for treatment of NSCLC.

Materials and methods

Cell culture and transfection

A normal human bronchial epithelial cell line (16HBE), three NSCLC cell lines (A549, H1299 and NCI-H520), and a human embryonic kidney cell line (HEK293T) were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc. Rockville, MD, USA) and anti-

biotics in a humidified atmosphere with air supply containing 5% CO₂ at 37°C.

MiR-539 mimics (miR-539), miR-539 negative control (miR-NC), miR-539 inhibitor (anti-miR-539) and miR-539 inhibitor negative control (anti-miR-NC) were designed and synthesized by RiboBio (Ribobio Co., Guangzhou, China). A549 cells were cultured to about 80% confluence in 6-well plates and were transfected using Lipofectamine™2000 transfection kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. 48 hours after transfection, the cells were harvested for further testing.

RNA extraction and qRT-PCR analyses

Total RNA was isolated from cells with TRIzol reagent (Invitrogen). For investigation of miR-539 expression, complementary DNA (cDNA) synthesis was performed using the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), then were quantified using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany). To quantify FSCN1 expression, cDNA was synthesized using PrimeScript RT reagent Kit (Takara, Dalian, China), then was quantified using FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). The sequences of primers were listed in **Table 1**. All qRT-PCR reactions were run in an ABI 7900 Real-time PCR system (Applied Biosystems). U6 small nuclear RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as endogenous controls for miRNAs and mRNAs, respectively; the 2^{-ΔΔCt} method was used to calculate the relative expression levels of miR-539 and FSCN1 [18].

3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation was determined by MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (KyeGEN BioTECH, Nanjing, China). The transfected A549 cells were seeded into 96-well culture plates at densities of 5000 cells per well in media containing 10% FBS and incubated for 0, 24 h, 48 h, 72 h, 96 h. On the indicated days, cells were treated with 10 μl MTT solution. About 4 h later, the medium was replaced with 100 μl dimethyl sulfoxide (DMSO). The absorbance was measured for

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each well at the wavelength of 490 nm using a microplate reader (TECAN, Switzerland).

Cell apoptosis assay

Cell apoptosis was evaluated by flow-cytometric analysis using an Annexin V-FITC Apoptosis Detection Kit (KyeGEN BioTECH). The transfected A549 cells were harvested and washed with PBS twice, incubated in a dark room with Annexin V-FITC and propidium iodide (PI) for 15 min. The cells were analyzed immediately by flow cytometry (BD FACSCalibur, BD Bioscience, San Diego, CA, USA) and analyzed using FlowJo software (FlowJo, Ashland, OR, USA).

Caspase-3 activity assay

The activity of caspase-3 was investigated using the Caspase-3 activity kit (Beyotime, Haimen, China). Protein was extracted from the transfected A549 cells using ice-cold RIPA buffer (Santa Cruz, Santa Cruz, CA, USA) containing protease inhibitors. Proteins were quantified using a Bradford protein assay kit (Galen Biopharm International Co., China). Assays were performed on 96-well culture plates by incubating 10 μ L protein lysates per well in 80 μ L reaction buffer containing 10 μ L caspase-3 substrate. Following 4 h of incubation at 37°C, samples were measured at an absorbance of 405 nm.

Cell migration and invasion assays

For the migration assays, 5×10^4 A549 cells in 200 μ L serum-free medium were seeded into the upper Transwell chamber (Corning, Tewksbury, MA, USA) containing 8 μ m pores. For the invasion assays, cells in 200 μ L serum-free medium were placed into the upper chamber of an insert coated with Matrigel (BD Biosciences). RPMI-1640 medium containing 10% FBS was added to the lower chamber as a chemo-attractant. After 24 h of incubation, the cells remaining on the upper membrane were scraped off with a cotton swab, whereas the successfully translocated cells were then fixed with 4% paraformaldehyde, stained with crystal violet, photographed and counted at five randomly selected fields using a light microscope.

Luciferase reporter assay

The miR-539 target genes were predicted by using computer-aided algorithms, namely TargetScan (<http://www.targetscan.org/>), Pictar

(<http://pictar.bio.nyu.edu/>), and miRanda (<http://www.microrna.org>). Dual luciferase reporter gene system was used to further confirm whether FSCN1 was a direct target for miR-539. The 3'-UTR fragment of the FSCN1 mRNA containing a putative miR-539-binding site, was amplified and subcloned into the downstream multiple cloning sites of the pLUC Luciferase vector (Ambion, Austin, TX, USA). Site-directed mutagenesis of the miR-539 target site in the FSCN1-3'-UTR was performed using the Quick-change mutagenesis kit (Stratagene, Heidelberg, Germany).

50 nM miR-539 mimics or negative control oligonucleotides were cotransfected into A549 cells with 100 ng of firefly luciferase reporter and 20 ng of pRL-TK (Promega, Madison, WI, USA) using Lipofectamine 2000. Cells were collected 48 hours post-transfection, and luciferase activity was detected through a dual-luciferase reporter gene assay kit (Promega). *Renilla* luciferase activities were used for normalization.

Western blotting

Protein lysates were separated by 10% SDS PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and then transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked in 5% non-fat dry milk, and immunostained with FSCN1 (Abcam, UK), and GAPDH (Cell Signaling Technology, USA) primary antibodies. Next, membranes were probed with corresponding horseradish peroxidase-labeled secondary antibodies. Protein bands were detected with an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.) and analyzed using BandsScan software (Glyko, USA). Protein levels were normalized to those of GAPDH.

In vivo assays in nude mice xenograft model

A total of 24 BALB/C nude mice (4-6 weeks), purchased from the SLAC Laboratory Animal Ltd., Co. (Shanghai, China), were bred under SPF conditions at room temperature with a 12 h light/dark cycle and humidity of 60-70%, and provided with food and water *ad libitum*. The experiments were approved by Animal Care and Use Committee of Three Gorges University.

NSCLC xenografts were established by subcutaneously injecting 1×10^7 A549 cells in the dorsal flanks of the nude mice. When palpable

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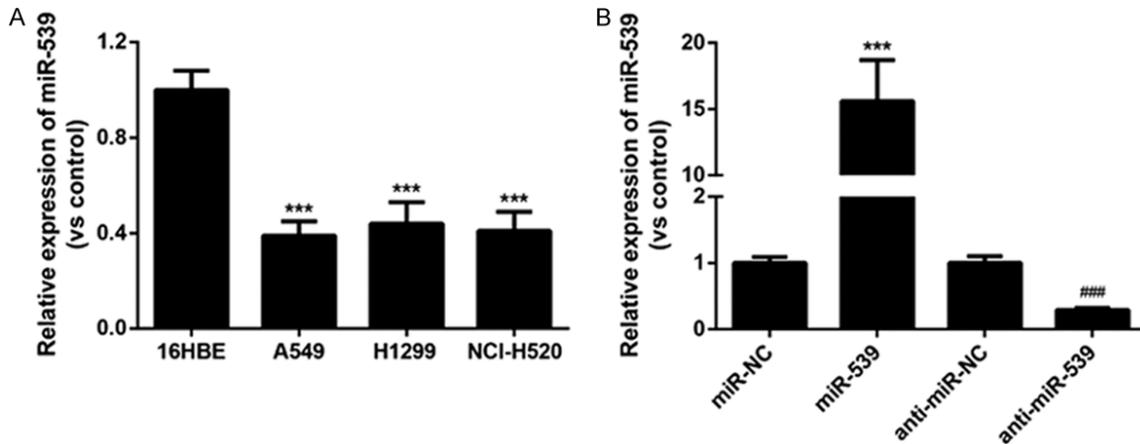


Figure 1. qRT-PCR analysis of miR-539 in NSCLC cell lines. A. Analysis of miR-539 expression levels in three NSCLC cell lines (A549, H1299 and NCI-H520) compared with the normal bronchial epithelial cell line (16HBE) by qRT-PCR. B. Analysis of miR-539 expression following treatment of A549 cells with miR-539 (miR-NC) or anti-miR-539 (anti-miR-NC) by qRT-PCR. Data are presented as the mean \pm SD by at least 3 independent experiments. *** P <0.001 vs. 16HBE cells or miR-NC group; ### P <0.001 vs. anti-miR-NC group.

tumors developed, the tumor volume was measured using a digital caliper every 3 days, using the formula: $(\text{mm}^3) = 1/2 \text{ width}^2 \times \text{length}$. Once the tumor volume reached 75-100 mm^3 , the mice were randomized into four groups (six mice/group). These mice were then treated with 200 pmol miR-539 (miR-NC) or anti-miR-539 (anti-miR-NC) in 10 μl Lipofectamine 2000 through a local injection of the xenografts at multiple sites. The mice were sacrificed on 15 days subsequent to inoculation, and the tumors were removed and weighed.

Statistical analysis

The data were presented as mean \pm standard deviation (SD) from a minimum of 3 independent experiments. Student's *t*-test (two-tailed) was used to determine the significant differences between NC and treatment groups. Statistical analysis was conducted using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). For all analyses, a P <0.05 is regarded as the level of statistically significant.

Results

MiR-539 expression is reduced in human NSCLC cells

We performed qRT-PCR analysis to first determine the expression of miR-539 in three NSCLC

cell lines (A549, H1299 and NCI-H520) and a normal bronchial epithelial cell line 16HBE. After normalization to U6 expression levels, the expression levels of miR-539 were remarkably decreased in three NSCLC cell lines compared to that in normal bronchial epithelial cell line, 16HBE (all P <0.001, **Figure 1A**). Subsequently, A549 cells were transfected with miR-539 or anti-miR-539 and their matched negative controls to further confirm whether miR-539 actually exerted onco-suppressive functions in NSCLC cells. Transfection efficiency was validated through qRT-PCR. As demonstrated in **Figure 1B**, miR-539 expression was increased 15.6-fold in the miR-539 group when compared with the miR-NC group (P <0.001), whereas miR-539 expression was decreased 3.4-fold in the anti-miR-539 group when compared with the anti-miR-NC group (P <0.001).

MiR-539 induces apoptosis and attenuates invasion in NSCLC cells

To further examine whether miR-539 is involved in NSCLC progression, *in vitro* functional analyses were conducted. As shown in **Figure 2A**, compared with control, overexpression of miR-539 resulted in a significant elevation in cell apoptosis rate (P <0.001). In contrast, inhibition of miR-539 significantly inhibited cell apoptosis (P <0.001). To further determine the mechanisms of miR-539-induced apoptosis, we investigated the levels of apoptosis-related caspase family protein. We measured the caspase-3

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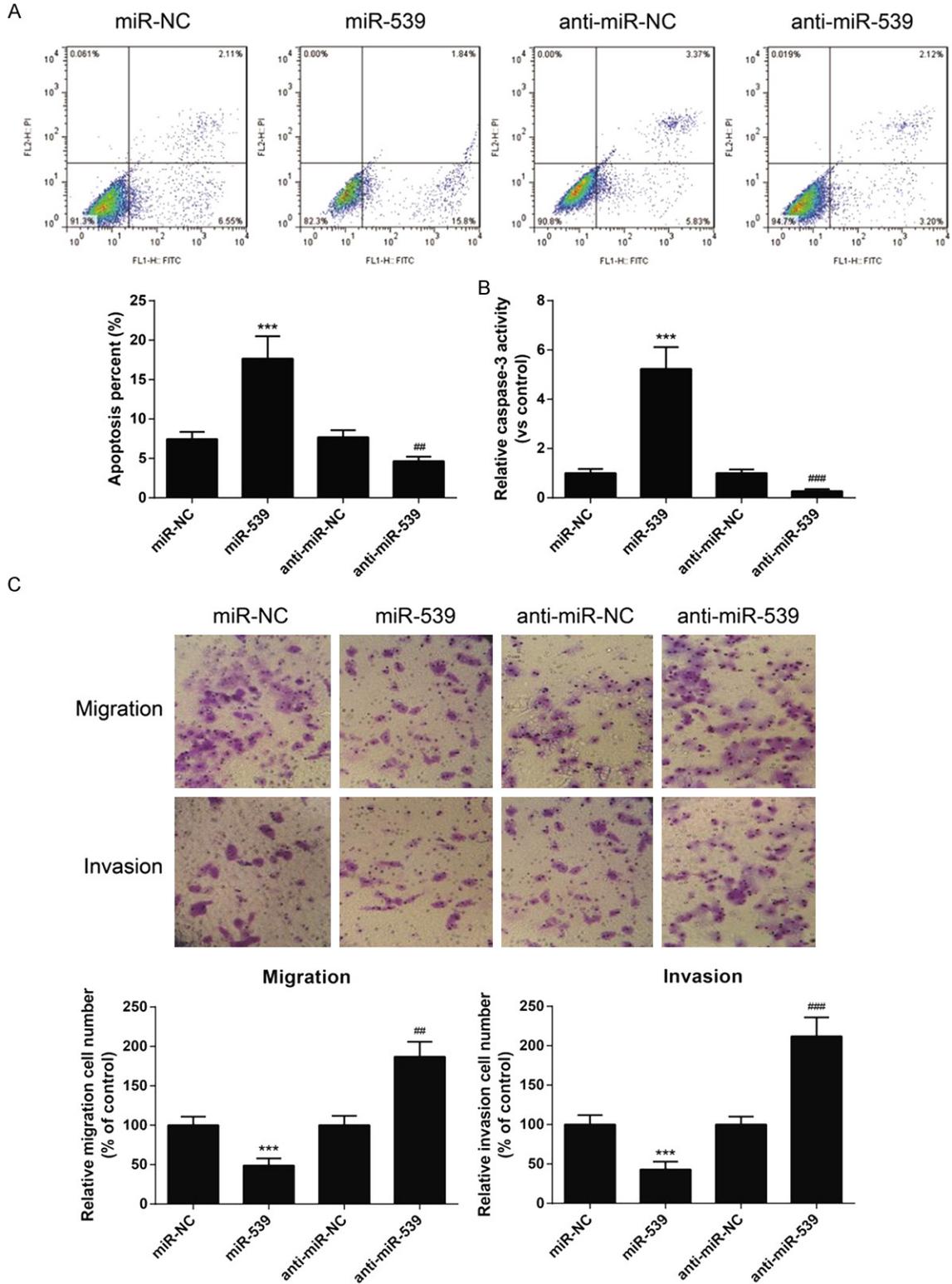


Figure 2. MiR-539 induces apoptosis and attenuates invasion in NSCLC cells. A. The apoptosis of A549 cells was measured by Annexin V staining and flow cytometry. B. Quantitative representation of caspase-3 activity in A549 cells after transfection for 48 h. C. Transwell assays were performed for the migration and invasion capacities of A549 cells. Data are presented as the mean \pm SD by at least 3 independent experiments. *** P <0.001 vs. miR-NC group; #### P <0.001 vs. anti-miR-NC group; ## P <0.01 vs. anti-miR-NC group.

MiR-539 inhibits NSCLC by targeting FSCN1

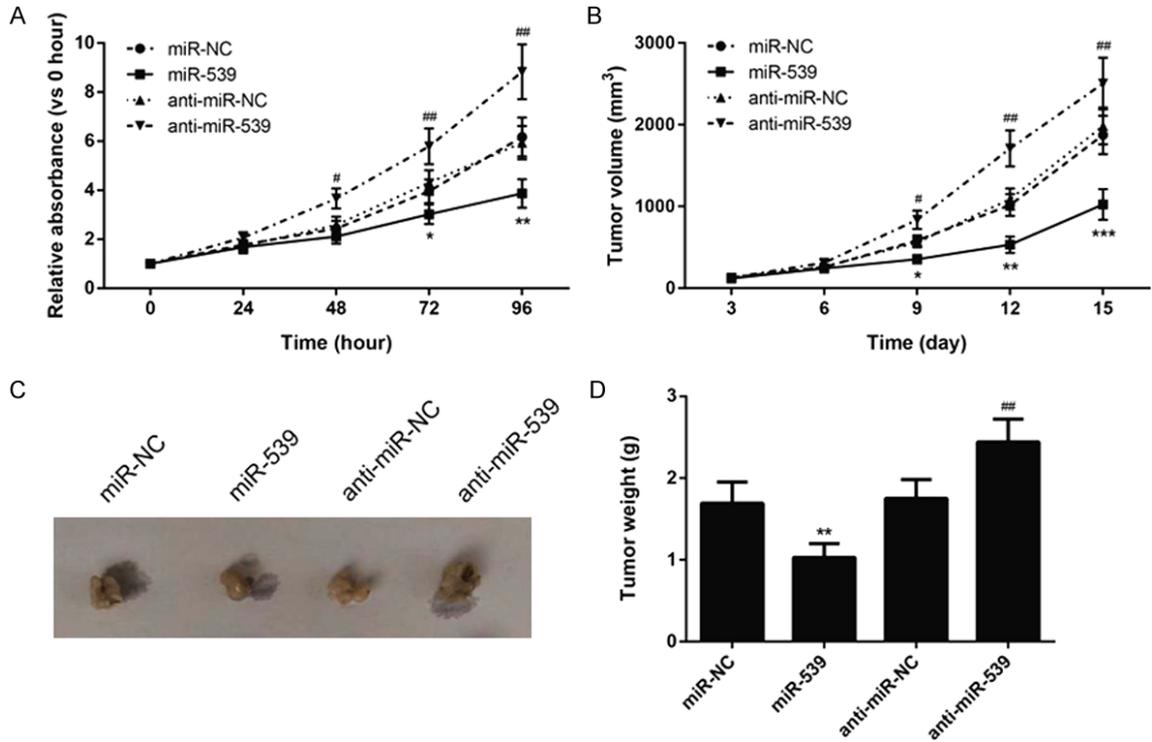
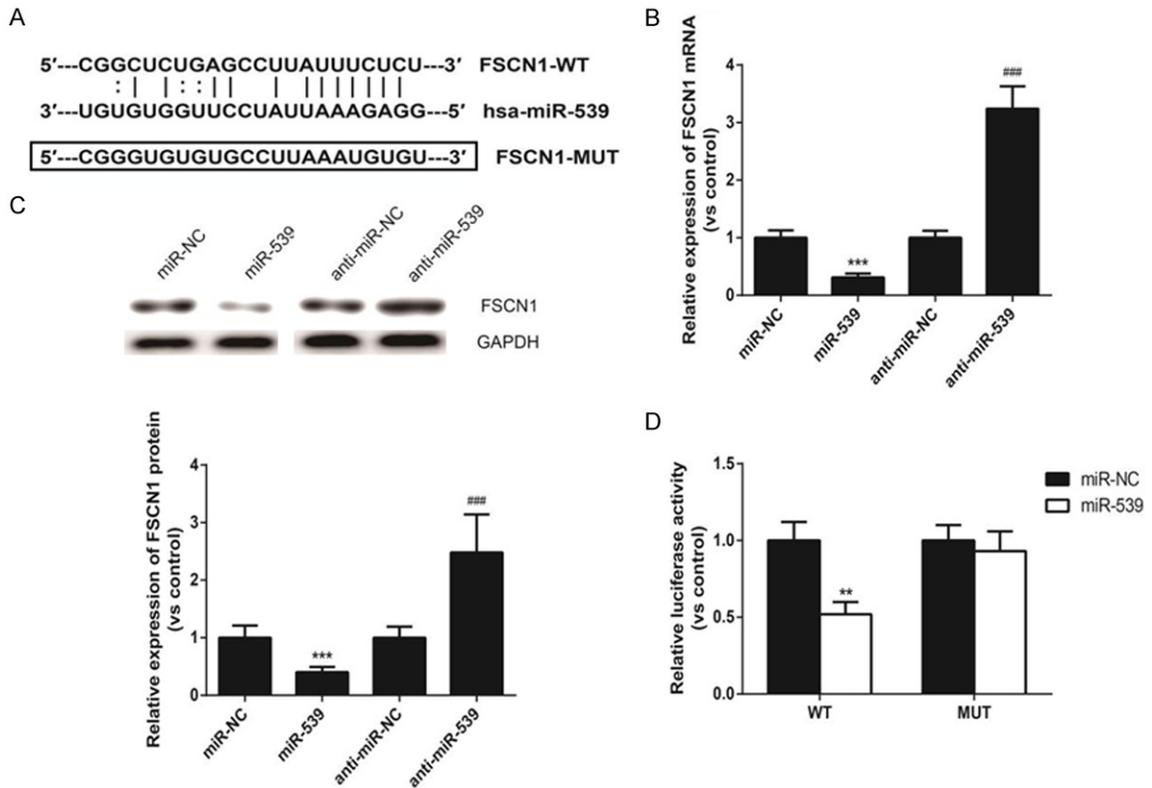


Figure 3. MiR-539 significantly suppressed proliferation of NSCLC cells. A. A549 cell proliferation was analyzed at the indicated time points via the MTT assay. B. An *in vivo* xenograft model was established. The tumor volumes were calculated every 3 days after implantation and growth curves were plotted from tumor volumes. C. Representative images showing the tumor xenografts 15 days after implantation. D. Tumor tissues weight was measured. Data are presented as the mean \pm SD by at least 3 independent experiments. *** P <0.001 vs. miR-NC group; ** P <0.01 vs. miR-NC group; * P <0.05 vs. miR-NC group; ### P <0.01 vs. anti-miR-NC group; # P <0.05 vs. anti-miR-NC group.



MiR-539 inhibits NSCLC by targeting FSCN1

Figure 4. FSCN1 is a target of miR-539 in NSCLC cells. A. Bioinformatics tools were used to predict the binding region of miR-539 in the FSCN1-3'-UTR. B. qRT-PCR was performed to detect the expression of FSCN1 mRNA expression in A549 cells transfected with miR-539 (miR-NC) or anti-miR-539 (anti-miR-NC). C. Western Blot analysis was performed to detect the expression of FSCN1 protein expression in A549 cells transfected with miR-539 (miR-NC) or anti-miR-539 (anti-miR-NC). D. A549 cells were co-transfected with miR-539 (miR-NC) and pLUC vector with FSCN1 3'-UTR-WT or MUT. After 24 hours, the luciferase activity was measured. Values are presented as relative luciferase activity after normalization to *Renilla* luciferase activity. Data are presented as the mean \pm SD by at least 3 independent experiments. *** $P < 0.001$ vs. miR-NC group; ** $P < 0.01$ vs. miR-NC group; ### $P < 0.001$ vs. anti-miR-NC group.

activity in A549 cells following transfection with miR-539 (miR-NC) or anti-miR-539 (anti-miR-NC), and data demonstrated that overexpression of miR-539 significantly increased the caspase-3 activity in A549 cell lysate compared to that of in NC cells ($P < 0.001$, **Figure 2B**). In contrast, down-regulation of miR-539 significantly decreased the caspase-3 activity ($P < 0.001$).

Cell migration and invasion abilities are strongly associated with cancer metastasis. Subsequently, we evaluated whether miR-539 was able to suppress the migration and invasion of NSCLC cells through transwell assays. As shown in **Figure 2C**, down-regulation of miR-539 significantly promoted the migration of A549 cells. Similarly, the invasion of A549 cells was dramatically reduced by miR-539 overexpression. The above results indicated that miR-539 potently inhibits the migratory and invasive phenotypes of NSCLC cells *in vitro*.

MiR-539 significantly suppressed proliferation of NSCLC cells

MTT assays were employed to investigate the proliferation of NSCLC cells. As shown in **Figure 3A**, ectopic expression of miR-539 significantly inhibited cell proliferation while down-regulation of miR-539 remarkably promoted cell proliferation *in vitro*. To further confirm the above findings, an *in vivo* xenograft model was established. Growth curves plotted from tumor volumes showed that, throughout the tumorigenic period, tumors in mice receiving A549 cells transfected with miR-539 grew significantly slower in comparison with tumors in mice receiving A549 cells transfected with miR-NC (**Figure 3B**). 15 days subsequent to inoculation, the tumor xenografts were dissected and weighed, and the tumor weight of mice receiving A549 cells transfected with anti-miR-539 was greatly up-regulated than that of mice receiving A549 cells transfected with anti-miR-539 (**Figure 3C** and **3D**). These results

revealed that miR-539 might serve as a potential tumor suppressor in NSCLC carcinogenesis.

FSCN1 is a target of miR-539 in NSCLC cells

It has been extensively reported that miRNAs can function post-transcriptionally through targeting certain types of genes [19]. To fully understand the mechanisms by which miR-539 executed its function in NSCLC cell proliferation and invasion, we adopted the bioinformatic algorithms (MiRanda, TargetScan, and PicTar) for target gene prediction. According to miRanda, we identified the complementary sequence of miR-539 in the 3'UTR of the FSCN1 mRNA transcript, as demonstrated in **Figure 4A**. Further, qRT-PCR analysis revealed that up-regulation of miR-539 in A549 cells led to decreased expression of endogenous FSCN1 mRNA compared with NC ($P < 0.001$, **Figure 4B**). Additionally, western blot analysis showed that FSCN1 protein expression was clearly up-regulated following transfection of A549 cells with anti-miR-539 ($P < 0.001$, **Figure 4C**). To further verify the direct interaction between miR-539 and its binding site within 3'UTR of FSCN1, we created pLUC-WT-FSCN1-3'UTR plasmids and pLUC-MUT-FSCN1-3'UTR. Luciferase reporter assays demonstrated that over-expression of miR-539 caused a remarkable decrease of luciferase activity of pLUC-WT-FSCN1-3'UTR by approximately 50% when compared to the miR-NC transfected cells ($P < 0.01$, **Figure 4D**). These results indicate that miR-539 directly modulate FSCN1 expression by binding 3'UTR of FSCN1.

Discussion

Currently, roles of miRNAs, a family of short non-protein coding RNAs extensively involved in cancer progression and metastasis, have attracted more attention than ever before. miRNA interacts with 3'-UTR of mRNA specifically to cause mRNA degradation, and transla-

tional repression [20]. An increasing amount of evidence notes that aberrant expression profiles of specific miRNA can be significantly associated with the development and progression of cancers [21, 22]. As a type of malignant tumor involved in multi-factorial physiological and pathological events, NSCLC initiates from multiple processes of genetic and epigenetic alterations. Accordingly, experimental validations were performed to identify a novel kind of miRNA associated with NSCLC to provide a reference for the future NSCLC research and a potential molecular target for the diagnosis and clinical treatment of NSCLC.

In the present work, we focused on miR-539, which was previously revealed to be down-regulated and function as a tumor suppressor in a variety of malignant tumors, such as osteosarcoma [17], nasopharyngeal carcinoma [23], and prostate cancer [24]. Our results indicated that miR-539 was significantly lower in three NSCLC cell lines compared with that of normal human bronchial epithelial cells. Furthermore, we studied the effect of miR-539 on the biological function of NSCLC cells through gain- and loss-of function experiments. Ectopic expression of miR-539 in NSCLC cells significantly inhibited cell proliferation, migration and invasion ability. Furthermore, we performed flow-cytometric analysis to test the effects of miR-539 on apoptosis levels and observed that overexpression of miR-539 induced apoptosis through activating the caspase-3. In accordance with our *in vitro* findings, overexpression of miR-539 significantly repressed tumorigenesis in murine model of NSCLC xenograft. Collectively, these findings supported that miR-539 might function as a tumor suppressor in NSCLC tumorigenesis and progression, similar to the functions of other reported miRNAs, including miR-124 and miR-1976, in NSCLC [25, 26].

MiRNA could affect malignant phenotypes of cancer cells through directly targeting oncogene or tumor suppressive gene [27]. Intriguingly, according to predicted results from bioinformatics database, we observed that miR-539 would bind with the 3'UTR of FSCN1 mRNA, and further validations confirmed that FSCN1 is a *bona fide* target of miR-539. Fascin actin-bundling protein 1 (FSCN1) is a 55-kDa evolutionarily conserved actin-binding protein that organizes F-actin into well-ordered, tightly

packed parallel bundles in a variety of cells [28], and it facilitates tumor cell motility, migration and invasion. Deregulation of FSCN1 has been observed in a wide variety of human tumors [29-32]. In NSCLC, FSCN1 also might function as an oncogene and the high level of FSCN1 was significantly correlated with aggressive pathological factors and poor overall survival in NSCLC patients [33, 34]. Therefore, the modulation of FSCN1 expression by miR-539 may explain why the down-regulation of miR-539 can promote cancer progression during NSCLC carcinogenesis.

Taken together, we have demonstrated for the first time that miR-539 expression was down-regulated in NSCLC cell lines, indicating that its down-regulation might be a potential diagnostic factor for NSCLC patients. Overexpression of miR-539 exerted tumor-suppressive functions through repressing cell proliferation, migration and invasion in NSCLC cells. Furthermore, miR-539 mediated the tumor-suppressive effects is partially through its regulation of FSCN1. Our findings deepened the understanding of NSCLC pathogenesis, and accelerate the development of microRNA-directed diagnostics and therapeutics against NSCLC.

Disclosure of conflict of interest

None.

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