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MicroRNA-140's inhibition on the cell migration and invasion of non-small cell lung cancer by down-regulating Smad3 expression

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Abstract. – OBJECTIVE: To investigate the effect of miR-140 on migration and invasion of non-small cell lung cancer (NSCLC) A549 cell and its regulatory mechanism.

MATERIALS AND METHODS: The NSCLC cell lines A549, H1650, NCI-H838, and normal lung epithelial cells BEAS-2B were purchased, and the expression of miR-140 and Smad3 in cells was detected by RT-PCR. MiR-140-inhibitor, miR-140-mimincs, miR-NC, sh-Smad3, Si-Smad3, and NC were transfected into A549 cells. Quantitative Real Time-Polymerase Chain Reaction (QRT-PCR) was used to detect the expression of miR-140 and Smad3. Transwell and cell scratch assay were used to detect cell invasion and migration. Dual-Luciferase report assay was used to study the relationship between mir-140 and Smad3.

RESULTS: MiR-140 was lowly expressed and Smad3 was highly expressed in NSCLC cells. Cell researches showed that the overexpression of miR-140 can inhibit cell invasion and migration. The downregulation of Smad3 expression inhibits cell invasion and migration. Dual-Luciferase reporter assay showed that miR-140 is a Smad3 targeting site.

CONCLUSIONS: MiR-140 can inhibit the invasion and migration of NSCLC cells by regulating Smad3, and it is expected to become a potential clinical target.

Key Words:
Non-small cell lung cancer, A549 cells, MiR-140, Smad3, Migration, Invasion.

Introduction

As the most common malignant tumor with leading morbidity and mortality among cancer deaths, lung cancer brings a mounting morbidity[1]. Non-small cell lung cancer (NSCLC), the most common pathological type in lung cancer, accounts for 80%-85% of the total lung cancer cases[2]. In spite of the unclarified pathogenesis of lung cancer, microRNA has lightened a new direction for lung cancer research[3]. Many studies have pointed out the important role of miRNA, not only in the occurrence and development of tumors, but also in the biological processes, such as tumor proliferation and invasion[4], with similar function to oncogenes or tumor suppressor genes.

As a kind of miRNA that plays a key role in the development of chondrocytes and the pathological process of osteoarthritis, miR-140 receives controversy opinions about its role in the occurrence and development of tumor cells from studies[5]. For example, Iorio et al[6] showed that miR-140 expression was found to be lower in ovarian cancer than that in normal ovarian tissue, but in another study[7], which investigated the expression profiles of miRNAs in primary grade II gliomas that spontaneously progressed to grade IV secondary glioblastomas, the expression of miR-140 increased during this progression. Yuan et al[8] found that miR-140 has low expression in NSCLC, and that miR-140 can inhibit the proliferation and metastasis of NSCLC cells by targeting IGF1R, indicating the close relationship between miR-140 and NSCLC. Smad3, as a major transcription factor in the TGF-β signaling pathway, affects the biological function of cells when abnormally expressed[9]. Petersen et al[10] stated that Smad3 can promote the metastasis of breast cancer cells in the late stage. Pais et al[11] confirmed that Smad3 is a direct target of miR-140 and believed that the inhibition by miR-140 on the expression of Smad3 protein could reduce the activity of TGF-β signaling pathway.

Wondering whether miR-140 is involved in the biological processes of NSCLC by regulating the expression of Smad3, this investigation selected non-small cell lung cancer A549 cells as an experimental object and artificially regulated the

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expression of miR-140 in A549 cells, to analyze the effect of miR-140 on the migration and invasion of NSCLC cells and the relationship between miR-140 and its direct target-Smad3.

Materials and Methods

Experimental Materials

The NSCLC cell lines A549, H1650, and NCI-H838 and normal lung epithelial cell BEAS-2B were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The spectrometer SpectraMax M5 was purchased from Shanghai Molecular Devices (ShanghaI, China). The real-time quantitative PCR instrument was purchased from BioRad (Bio-Rad, Hercules, CA, USA). TRIzol reagent was purchased from Applide Invi-trogen (Carlsbad, California, USA). The qRT-PCR kit and minScript reverse kit were purchased from TaKaRa (Dalian) Co., Ltd (Dalian, Liaoning, China). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Rockville, MD, USA). Fetal bovine serum (FBS) and trypsin were purchased from HyClone (South, Logan UT, USA). Transwell chamber was purchased from Corning (Corning, NY, USA). Radio Immunoprecipitation (RIPA) assay protein lysate was purchased from Beyotime Biotechnology (Shanghai, China). Smad3 antibody and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody were purchased from Beijing Zhongshan Jinqiao Biological Co., Ltd (Beijing, China). The miR-140 primer sequence, miRNA negative control, GAPDH, and U6 primer sequences were designed and synthesized by Shanghai Omicsspace Biotechnology Co., Ltd (Shanghai, China).

Cell Culture and Passage

NSCLC cells were placed in a medium containing 10% fetal calf serum (FCS), cultured in a 37°C, 5% CO_2 incubator. When the cells covered 85% of the wall, phosphate-buffered saline (PBS) was used for washing, followed by 1 ml of 25% trypsin gently shaken to digest the cells. After the digestion, 10% of the culture medium was added and cultured at 37°C, 5% CO_2 for 48 h for passage, and cells grown in log phase were selected for the experiment.

Cell Transfection

Grouping was performed before the transfection. The cells that were not transfected were selected as blank group, the cells transfected with miRNA NC were selected as negative control group, the cells transfected with miR-140 mimic were selected as up-regulated group, and the cells transfected with the miR-140 inhibitor were selected as down-regulated group. Smad3 inhibitory sequence (si-Smad3), Smad3 overexpression sequence (Sh-Smad3), negative control RNA (NC) were transfected into cells with Lipofectamine™ 2000. Specific steps of transfection: first, the cells were seeded in a 6-well plate at a density of 3×10^5 cells/well; then lipofectamine 2000 and DNA were diluted and mixed according to the instructions of Lipofectamine 2000 Transfection Kit; the mixture was kept at room temperature for 5 min, and then mixed with the cells and transfected at 37°C, 5% CO_2. 48 h after the transfection, the expression of miR-140 in transfected A549 cells was detected by qRT-PCR and the cells were collected for follow-up experiments.

QRT-PCR Detection of MiR-140 and Smad3 mRNA Expression in Each Group of Cells

In each group, approximately 2×10^5 cells were added with 1 ml of TRIzol reagent for the extraction of total RNA, and then the purity and concentration of the RNA were detected by an ultraviolet spectrophotometer, which showed a good purity since the ratio of A260/A280 was 1.9. After that, reverse transcription of 1 μg of total RNA into cDNA was performed according to the kit instructions, and the reaction parameters were: 16°C for 20 min, 42°C for 30 min, and 85°C for 5 min. The transcribed cDNA was used for PCR amplification, involving 10 μl of TaqMan PCR Master Mix II, 0.5 μl of forward primer and 0.5 μl of reverse primer, 1.5 μl of cDNA, and enough RNase-free Water to make the total volume reach up to 20 μl. U6 was used as the internal reference for miR-140 and GAPDH was used as the internal reference for Smad3 mRNA, with specific primer sequences shown in Table I. PCR reaction conditions: pre-denaturation at 95°C for 10 minutes, then 40 cycles of 95°C for 15 s, 60°C for 45 s. With U6 as the internal reference, real-time quantitative PCR detection with PCR instrument was repeated 3 times.

Western Blot Detection of Smad3 Protein Expression in Each Group of Cells

The transfected cells of each group were collected and added with the cell lysate. Following the lysis, the total protein of each group of cells

Table I. Primer sequences.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
miR-140	CTCAACTGGTGTGGTGGAGT CGGCAATTCAGTTGAGCTACGAT	ACACTGGAGCTGGGCAGTGGTTTTACCCTATG
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT
Smad3	CCATCTCCTACTACGAGCTGAA	CACTGCTGCATTCCTGTTGAC
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT

was collected and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), after that the protein was transferred to a polyvinylidene difluoride (PVDF) membrane and mixed with 5% skim milk to be blocked overnight at a temperature of 4°C. After one night of blocking, Smad3 primary antibody (1:1000), N-cadherin (1:500), E-Cadherin (1:500), vimentin (1:500), and GAPDH primary antibody (1:1000) were added and blocked at a temperature of 4°C overnight. Then, the secondary antibody was added in an incubator at 37°C. Finally, the enhanced chemiluminescence (ECL) developer was used to develop color.

Transwell Assay to Detect the In Vitro Invasion Ability of Cells

The transfected cells were starved before the experiment: cells in the log phase were inoculated in a medium containing no 10% FBS and cultured in a 37°C, 5% CO_2 incubator for 24 h. After the starvation process, the cells were taken out and seeded in a 24-well plate at an adjusted cell concentration of 2×10^5 cells/ml. With 200 μl of the diluted cells in the upper chamber and 600 μl of DMEM containing 20% FBS in the lower chamber, the chamber was placed in an incubator at 37 °C, 5% CO_2 for 24 h. After that, the chamber was removed and washed with PBS. The chamber was then fixed with a 4% paraformaldehyde solution for 10 min and rinsed again with PBS. After that the cells were immersed in a 1% crystal violet solution for 20 min for coloring, they were moved out and the chamber was washed repeatedly with PBS until clarification. Finally, the invasion of the five visual fields was randomly calculated by a microscope, and the average value was calculated. The experiment was repeated 3 times.

Cell Scratch Test to Observe the Migration Ability of Cells In Vitro

The log phase cells were made into a single cell suspension, and were diluted as described above until the cell concentration reached $4x10^5$ cells/ml. Then, the liquid was added to a 6-well plate, and then transfected with different substance according to different transfection requirements in different groups. 48 h later, a cell-free area was made in the middle of the plate using 200 μl of sterile pipette tip. Then, the dissected cells were washed with PBS and cultured in a new medium. At the 0^{th} h (W0) and 24^{th} h (W24) after the cells were dissected, the width of the cell-free region was calculated by microscopy according to the scratches at three different positions in three groups. Cell migration index (M1) = (W0 - W24) / W0 × 100%.

Dual-Luciferase Reporter Assay

The target gene of miR-140 was predicted using Targetscan7.2. Smad3 -3'UTR wild type (Wt) and Smad3 -3'UTR mutant (Mut) and miR-140-inhibitor, miR-140-mimics, miR-NC were transferred into A549 cells using Lipofectamine™ 2000 kit. 48 h after transfection, luciferase activity was determined using a Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA).

Table II. Expressions of miR-140 and Smad3 mRNA in A549 cells.

Gene	Up-regulated group	Down-regulated group	Blank group	Negative control group	F	p
miR-140	2.79±0.21*	0.26±0.07*#	1.03±0.04	1.05±0.05	258.8	<0.001
Smad3 mRNA	0.86±0.13	1.33±0.04	1.02±0.07	1.04±0.08	15.42	<0.001

Note: $p<0.05$ if comparison was made between the "*" and the blank group and the negative control group, or between the "*#" and the blank group and the negative control group; $p<0.05$ if comparison was made between the "*" and the "*#".

Statistical Analysis

SPSS19.0 software [Bizinsight (Beijing) Information Technology Co., Ltd.] was used to perform statistical analysis on the collected data. The measurement data were expressed as mean ± standard deviation, and the *t*-test was used for comparison between the two groups. Differences between groups were compared using one-way analysis of variance (ANOVA) and LSD/t test was used for post hoc test. The difference was regarded as statistically significant if $p<0.05$.

Results

Comparison of Expression of MiR-140 and Smad3 mRNA in Each Group of Cells

The expression of miR-140 and Smad3 mRNA in NSCLC cell lines A549, H1650, and NCI-H838 was significantly lower than that in normal lung epithelial cells BEAS-2B, with statistically significant difference ($p<0.05$). Since miR-140 has the lowest expression in A549 cells, A549 was selected for the subsequent experiment (Figure 1).

Effect of MiR-140 on Cell Biological Function

By the regulation of miR-140 in A549 cells, it was found that the invasion and migration ability of transfected miR-140-minicis cells was significantly inhibited. Also, the ability of cells transfected with miR-140-inhibitor to invade and migrate significantly increased compared to cells transfected with miR-NC, with statistically significant difference ($p<0.05$; Figure 2).

Effect of Smad3 on the Biological Function of Lung Cancer Cells

After the regulation of Smad3 in A549 cells, the invasion and migration ability of transfected Si-Smad3 cells was significantly inhibited compared with cells transfected with miR-NC, and the ability of cells transfected with Sh-Smad3 to invade and migrate significantly increased compared to cells transfected with miR-NC, with statistically significant difference ($p<0.05$; Figure 3).

MiR-140 Gene Identification

It was found that there was a targeted binding site between Smad3 and miR-140 by predicting the downstream target gene of miR-140 with Targetscan 7.2. The results of Dual-Luciferase reporter assay showed that the Smad3-3'UT Wt luciferase activity was significantly increased after the expression of miR-140 was inhibited ($p<0.05$). When miR-140 was overexpressed, Smad3-3'UT Wt luciferase activity was significantly reduced

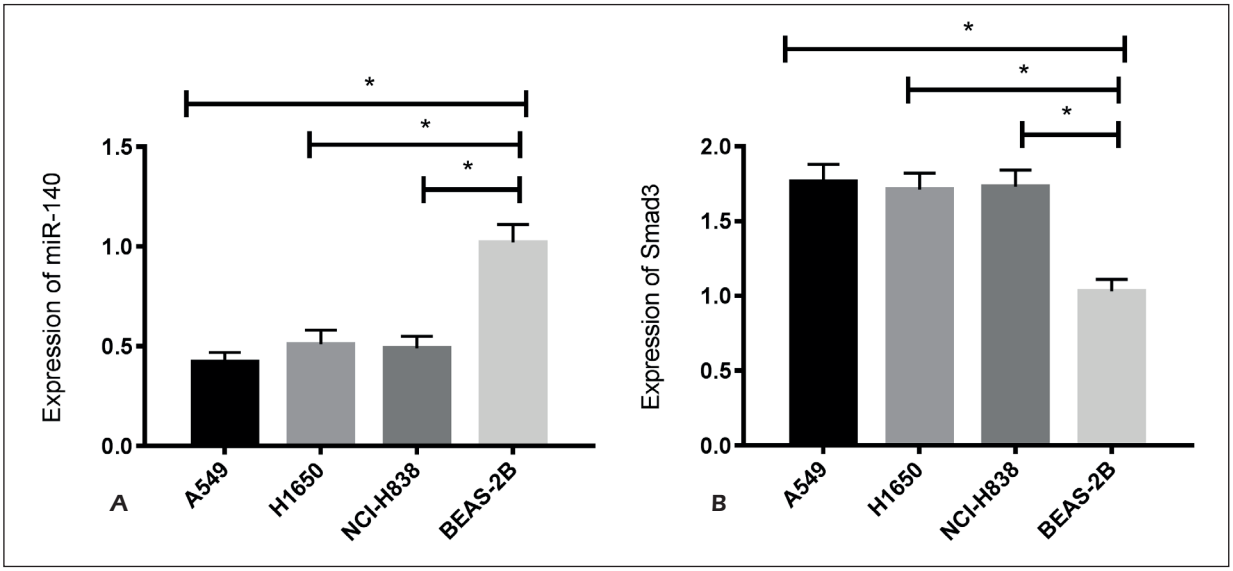

Figure 1. Effect of miR-140 on cell biological function. **A**, Expression of miR-140 in NSCLC cell lines A549, H1650, and NCI-H838 was significantly lower than that in normal lung epithelial cells BEAS-2B. **B**, Expression of Smad3 in NSCLC cell lines A549, H1650, and NCI-H838 was significantly lower than that in BEAS-2B. *$p<0.05$.

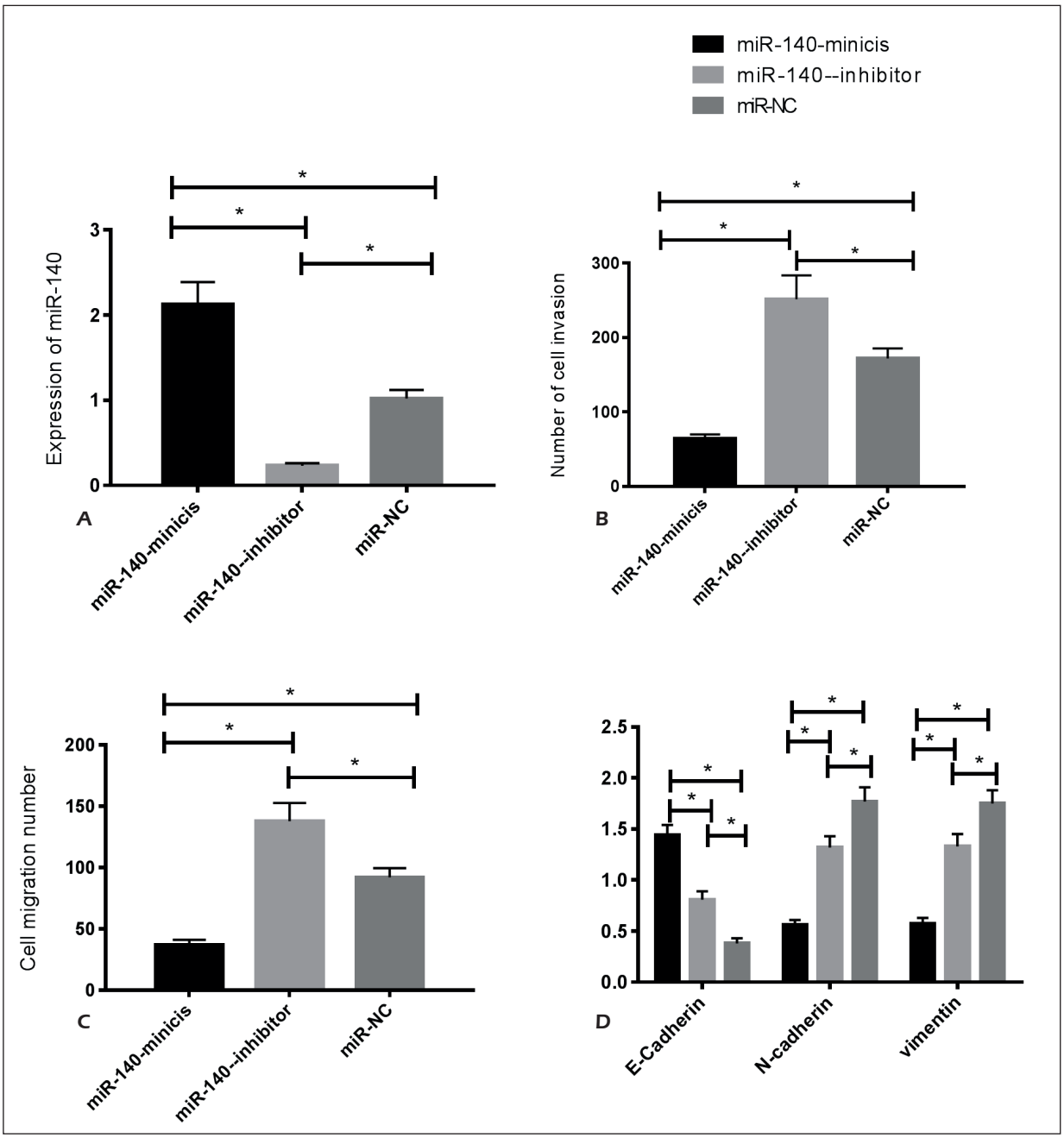

Figure 2. Effect of miR-140 on cell biological function. **A**, Expression of miR-140 in transfected cells. **B**, Invasive ability of transfected miR-140-minicis cells was significantly inhibited; the invasive ability of cells transfected with miR-140--inhibitor was significantly increased compared with cells transfected with miR-NC. **C**, Migration ability of transfected miR-140-minicis cells was significantly inhibited, and the migration ability of cells transfected with miR-140--inhibitor was significantly higher than that of cells transfected with miR-NC; **D**, Transfection of miR- After 140-mimics, the expression of N-cadherin and vimentin was significantly decreased, the expression of E-Cadherin protein was significantly increased, and the expression of N-cadherin and vimentin in transfected miR-140-inhibitor cells was significantly increased. The expression of E-Cadherin was significantly decreased. *p<0.05.

(p<0.05), but Smad3-3'UTR Mut luciferase activity was not affected (p>0.05). Western blot (WB) assay showed that the expression of Smad3 protein in A549 cells was significantly increased after transfection with miR-140--inhibitor, while the expression of Smad3 protein in A549 cells was significantly decreased after transfection with miR-140-mimicis (p<0.05; Figure 4).

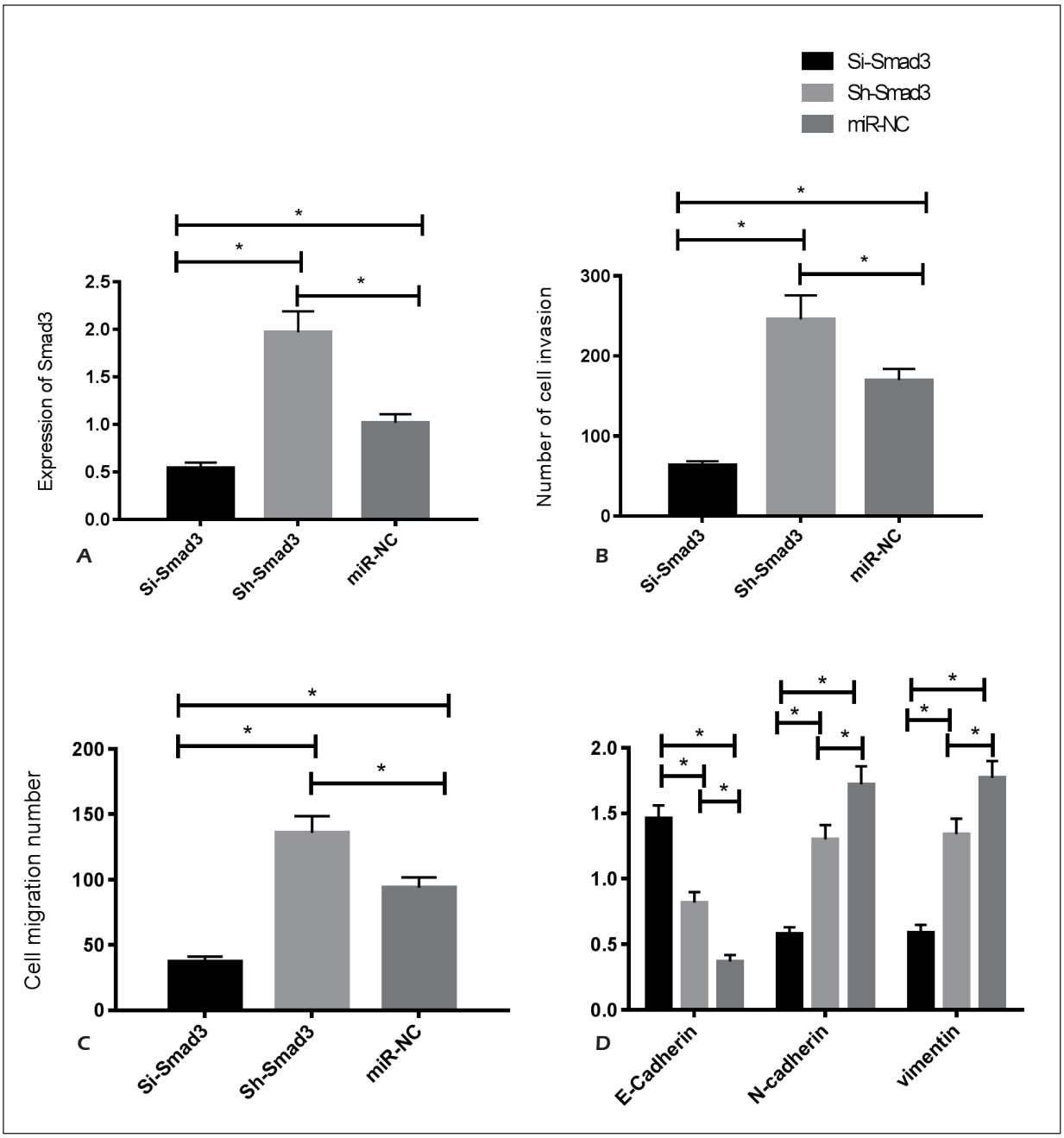

Figure 3. Effect of Smad3 on the biological function of lung cancer cells. **A**, Expression of Smad3 in cells after transfection. **B**, Invasive ability of cells transfected with Si-Smad3 was significantly inhibited, and the invasive ability of cells transfected with Sh-Smad3 was significantly increased compared with cells transfected with miR-NC. **C**, Migration ability of transfected Si-Smad3 cells was significantly inhibited, and the migration ability of cells transfected with Sh-Smad3 was significantly higher than that of cells transfected with miR-NC; **D**, After transfection of Si-Smad3 the expression of N-cadherin and vimentin was significantly decreased, and the expression of E-Cadherin protein was significantly increased. The expression of N-cadherin and vimentin in transfected Sh-Smad3 cells was significantly increased, and the expression of E-Cadherin was significantly decreased. *$p<0.05$.

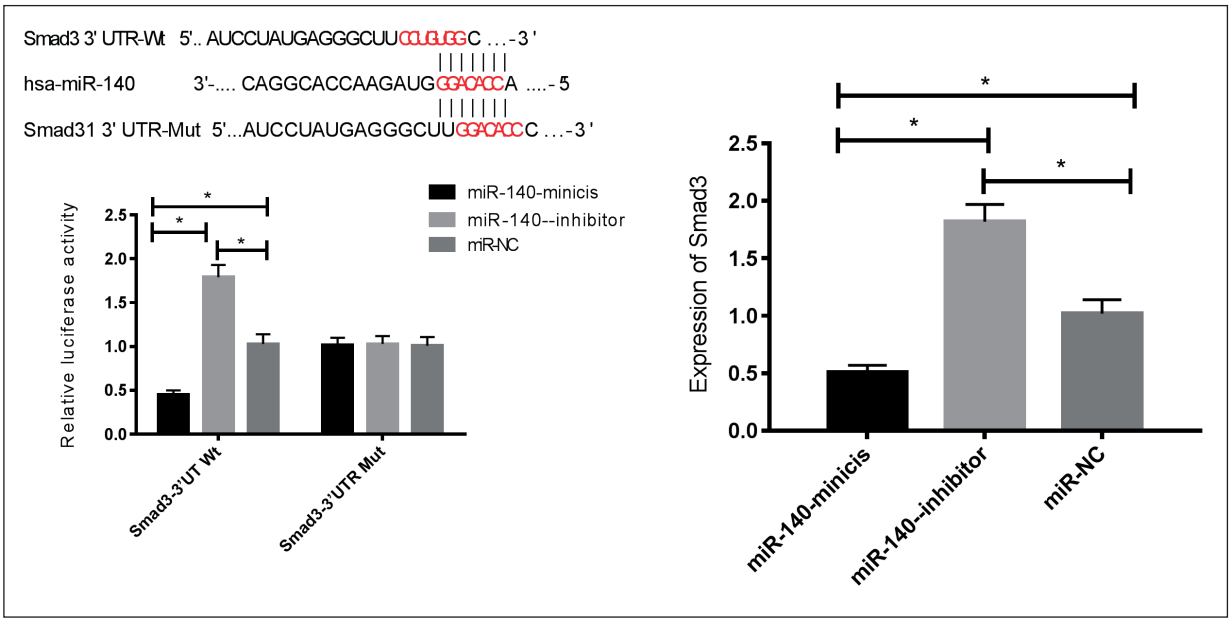

Figure 4. Dual-Luciferase reporter assay. **A**, Smad3-3'UT Wt Luciferase activity was significantly increased after miR-140 expression was inhibited. When miR-140 was overexpressed, Smad3-3'UT Wt Luciferase activity was significantly reduced. However, Smad3-3'UTR Mut Luciferase activity was not affected. **B**, Smad3 protein expression was significantly increased in A549 cells after transfection with miR-140-inhibitor, and Smad3 protein expression was significantly decreased in A549 cells after transfection with miR-140-minicis. *$p<0.05$.

Discussion

As the research on miRNA goes further, many studies[12] have shown that abnormal miRNA expression regulation is closely related to tumor occurrence and development. The ability of tumor cells to invade and metastasize, as a biological behavior of malignant tumor cells, also plays an important role in the treatment and prognosis of tumors[13]. Thus, the role of miRNAs in the invasion and metastasis of tumor cells has attracted increasing attention[14]. Overexpression of miR-137 in lung cancer has an inhibitory effect on the invasion and migration of NSCLC cells[15]. Overexpression of miR-493 may promote the invasion and migration of NSCLC cells[16]. Despite the important role of miR-140 in the development and proliferation of cartilage, it has been found by some studies that it is able to inhibit the invasion and migration of colorectal cancer cells by down-regulating Smad3, which suggests a relationship between miR-140 and the biological function of tumor cells[17].

This research artificially upregulated and down-regulated the expression of miR-140 in non-small cell lung cancer A549 cells to explore the effects of changes in miR-140 expression on cell invasion and migration. The results showed that miR-140 overexpression led to an inhibition on the invasion and migration of A549 cells, while the down-regulated miR-140 expression brought an enhanced invasion and migration, which indicated that miR-140 can significantly inhibit the invasion and migration of NSCLC A549 cells. Although little is known on the biological function of lung cancer cells by miR-140, it was found that there is a targeting relationship between miR-140 and Smad3 through online website prediction. Therefore, the role of Smad3 in lung cancer was explored, and the targeting relationship between the two was confirmed by the dual fluorescein reporter assay. Kai et al[18] reported that miR-140 can inhibit the invasion and migration of tongue squamous cell carcinoma by targeting the ADAM10 gene, confirming the conclusions of this study from the side.

As a key protein at the downstream of the TGF-β signaling pathway, Smad3 can directly form a heterodimerization with p-Smad2 and then recombined into Smad4 to form a new complex when phosphorylated by the serine/threonine kinase of the type I TGF-β receptor and activated into p-Smad3, and form the TGF-β/Smads signaling pathway[19]. Do et al[20] discovered that Smad3 can accelerate the decomposition of extracellular matrix components by downregulating E-cadherin, up-regulating N-cadherin, promoting epithe-

lial mesenchymal transition, and inducing high expression of MMPs, which arouse promoted invasion and metastasis of tumor cells. That is consistent with our results. Pais et al[11] observed that Smad3 is a direct target of miR-140, and that Smad3 protein expression is down-regulated in C3H10T1/2 cells transfected with miR-140 mimetic, but up-regulated in cells transfected with miR-140 inhibitor. This study analyzed the relationship between the inhibitory ability of miR-140 on invasion and migration of NSCLC cells and Smad3-the target of miR-140. According to the detection of Smad3 mRNA in this study, the Smad3 mRNA in upregulated group was lower than that in blank group and negative control group, the Smad3 mRNA in down-regulated group was higher than that in blank group and negative control group, but the differences were not statistically significant ($p>0.05$). According to the detection of Smad3 protein, up-regulated group had significantly lower expression of Smad3 protein ($p<0.05$) and weaker ability of invasion and migration of the cells than blank group and negative control group, while down-regulated group had significantly higher expression of Smad3 protein and stronger ability of invasion and migration of the cells than blank group and negative control group ($p<0.05$). Such results indicate that miR-140 may also inhibit the invasion and migration of NSCLC cells by down-regulating the expression of Smad3 protein. Zhao et al[21] showed that the inhibitory effect of miR-140 on invasion and migration of colorectal cancer cells is also achieved by the regulation on the Smad3, and that miR-140 mainly regulates the expression of transcribed Smad3, which also explains and demonstrates the conclusions of this study from the side.

Conclusions

In summary, upregulated miR-140 expression leads to lower expression of Smad3 protein and weaker ability of the invasion and migration of NSCLC cells, whereas down-regulated miR-140 expression arouses higher expression of Smad3 protein and stronger ability of the invasion and migration of NSCLC cells. Therefore, it is possible that miR-140 achieved its inhibition on the migration and invasion of NSCLC cells by down-regulating Smad3. Capable of being a new target of NSCLC metastasis diagnosis and treatment, miR-140 sparks a new idea for the treatment of NSCLC after metastasis.

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Availability of Data and Materials

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

Authors' Contributions

PH wrote the manuscript. PH and XW performed PCR and Western blot. YH was responsible for cell scratch assay and transwell assay. GC contributed to observation indexes analysis. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The investigation was approved by the Ethics Committee of Linyi People's Hospital.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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