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MicroRNA-507 represses the malignant behaviors of non-small cell lung cancer *via* targeting zinc finger E-box binding homeobox 2

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is one of the most common malignancies around the world and effective therapeutic method is yet to be excavated for advance NSCLC. MicroRNA-507 (miR-507) was found to be aberrantly expressed and affected cancer cell behaviors in some types of cancers. However, the role of miR-507 in NSCLC is largely unknown. The expression, biological role, and the underlying mechanism of miR-507 in NSCLC were explored in this study.

PATIENTS AND METHODS: Quantitative real-time PCR (qRT-PCR) assay was applied for the detection of miR-507 in NSCLC tissues and cell lines. Cell Counting Kit-8 (CCK-8) and colony formation assays were carried out to assess the proliferative abilities of NSCLC cells. Cell invasive capabilities were determined by transwell assays. We used Dual-Luciferase reporter assays to verify the binding between miR-507 and zinc finger E-box binding homeobox 2 (ZEB2).

RESULTS: MiR-507 was found to be downregulated in NSCLC tissues and cell lines. Low expression of miR-507 was correlated with poor prognosis of NSCLC. Overexpression of miR-507 repressed NSCLC cell invasion and proliferation. ZEB2 was predicted to be a direct downstream molecular of miR-507 and their direct binding was verified by Dual-Luciferase reporter assays. Up-regulation of ZEB2 could significantly rescue the suppressive effects of miR-507 on NSCLC cells' invasion and proliferation.

CONCLUSIONS: MiR-507 was noticeably downregulated in NSCLC and correlated with poor prognosis of NSCLC patients. MiR-507 represses the invasion and proliferation of NSCLC via targeting ZEB2. This study indicated that miR-507 might serve as a potential therapeutic target for NSCLC.

Key Words:
NSCLC, MiR-507, ZEB2, Malignant behaviors.

Introduction

Ranking first in terms of both morbidity and mortality in all cancers, lung cancer is one of the most important malignancies that threaten people's lives and health[1]. Non-small cell lung cancer (NSCLC) is the main type of lung cancer and it accounts for about 85% among all newly diagnostic cases[2]. The main therapeutic strategies for NSCLC include surgery, chemotherapy, and radiation[3]. Even above strategies have been greatly improved in past ten years, the prognosis of NSCLC remains poor due to its highly aggressive biological behavior[4-6]. Hence, it is imperative to explore and discover new molecular therapeutic targets to improve the prognosis of NSCLC.

Non-coding RNAs are a family of RNAs, which more or less transcript into protein. MicroRNA (miRNA) is an important member of non-coding RNAs, which is unable to transcribe but regulates most genetic transcription in human cells[7]. MiRNAs could target the 3'-untranslated regions or coding sequence area of their downstream molecular[8]. The target effects would motivate translation inhibition or induce the degradation of mRNA and regulate downstream physiological or pathological processes[9]. Thus far, a number of miRNAs have been identified and some of their functions have been confirmed. In NSCLC research, it is wildly accepted that most miRNAs are aberrantly expressed in cancer cells and regulated their biological behavior. For instance, Zhang et al[10] detected the signature of miRNA-337 in a cohort of NSCLC patients and cell lines, which revealed that miRNA-337 was significantly down-regulated in NSCLC and it significantly repressed cancer cell invasion and migration by negatively regulating downstream

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molecular transcription factor 7 (TCF7). The miR-1204 was also found to be aberrantly expressed in NSCLC and it induced the apoptosis rate of NSCLC cells by affecting the Bcl2 apoptosis regulator (Bcl-2) and BCL2 associated X (Bax) expression[11]. Li et al[12] reported that the level of miR-186 was regulated by lycorine and it rescued the suppressive functions of lycorine on the proliferation of NSCLC cells by targeting cyclin dependent kinase 1 (CDK1). Until now, the features of miR-507 in NSCLC have not been clarified and we conducted this study to determine its expressive pattern and biological function in NSCLC.

Patients and Methods

Ethics Committee Approval

This investigation had been discussed by the Ethics Committees of Maoming People's Hospital. The Ethics Committees of Maoming People's Hospital had reviewed and approved this investigation, whose approval number is EC23902. Informed consent was signed by all patients involved in the experiment. The research was conducted in accordance with the World Medical Association Declaration of Helsinki.

Patient Enrollment

NSCLC tissues and adjacent normal tissues were obtained from 42 NSCLC patients who underwent surgery from March 2010 to June 2012 in Maoming People's Hospital. All the patients received no anti-cancer therapy before surgery. Among them, 21 are males and 21 are females. The patients had a median age of 51.2 years old. Pathological diagnosis of NSCLC in all specimens was confirmed by two independent pathologists. The specimen was frozen with liquid nitrogen immediately after the operation until use.

Cell Lines and Culture

Human NSCLC cell lines A549, SPC-A1, H460, HCL-H359, H1299, and normal pulmonary epithelium cell line HBE were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM)/F12 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) and penicillin and streptomycin were obtained from Gibco (Invitrogen, Carlsbad, CA, USA). The cells were cultured in DMEM/F12, which were supplemented with 10% FBS and 1% penicillin and streptomycin. The cell incubator was set as 37°C and 5% CO_2. The cells were digested and passaged when they reached 80% density.

Cell Transfection

The cells were seeded in 6-well plates before transfection. The miR-507 mimic (miR-507) was purchased from GenePharma (Shanghai, China) for the overexpression of miR-507. MiRNA mimic control (miR-NC) was adopted as control vector. Lentivirus, which could express ZEB2 (len-ZEB2), was structured by Invitrogen (Carlsbad, CA, USA). Lentivirus empty vector (len-NC) was adopted as control vector. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfection assays according to manufacturer's protocol. The transfection process lasted 24 h and the transfected cells were used for subsequent experiments.

Cell Proliferation Assays

Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used to access cell proliferation. A549 and H460 cells were seeded into 96-well plates and transfected with miR-507 mimic and/or Len-ZEB2. CCK-8 reagent was added at 24 h, 48 h, 96 h, respectively and cultured for another 2 h. Microplate reader was used to measure absorbance at 450 nm. For colony formation assays, 1000 cells were seeded for 14 days, and the colonies were formed. The colony formations were stained by Giemsa (Beyotime, Shanghai, China) and counted. All experiments were conducted three times independently.

Cell Invasion Assays

The transwell and scratch tests were used for accessing cell invasion. In transwell assays, the 24-well Millipore transwell chambers with 0.8 μm aperture (Millipore, Billerica, MA, USA) were purchased and cells were put into the upper chambers. The upper chamber was added by mediums without FBS and the lower chambers added by mediums with 20% FBS. After 48 h, the cells which invaded into the lower membranes were stained and counted by microscope. In scratch test, the cells were seeded in 6-well plate and scratch was made by pipette head. After 48 h, the cell healing distances were photographed. All experiments were repeated three times independently.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from NS-

CLC tissue samples and NSCLC cell lines in accordance with manufacturer's instructions. The concentrations of RNAs were measured and reverse transcribed by PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). We applied SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) for the detection of real-time quantitative reverse transcription PCR. ABI 7500 Real Time PCR system was used to conduct the qRT-PCR. The primer sequences for ZEB2 were: forward primer 5'-CGTAGCTAGCTAGCTAGCTAG-3' and reverse primer 5'-CGTACTACGTAGCTGATCGT-3'. The primer sequences for GAPDH were: forward primer 5'-CGTACGTAGCTGACGTAGT-3' and reverse primer 5'-CGTACGTACGTAGTCGTAG-3'.

Luciferase Report Assays

The wide type (wt) sequences of ZEB2 that contain binding site with miR-507 or mutant type (mt) sequences that don't contain were synthetized. The former sequences were then inserted into pmirGLO vector (Promega, Madison, WI, USA). MiR-507 mimic or mimic control were co-transfected with the vectors into NSCLC cells and the luciferase activities were detected using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). We used *Renilla* luciferase activity as normalized control.

Western Blot Analysis

The proteins from NSCLC cells were isolated using Radio Immunoprecipitation Assay (RIPA; Beyotime, Shanghai, China), which were supplemented with 1% phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China). The bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was used to determine protein concentration in accordance with manufacture's protocol. Total protein was transferred to the polyvinylidene difluoride (PVDF; Thermo Fisher Scientific, Waltham, MA, USA) membrane after loading 12% sodium dodecyl sulfate (SDS) and polyacrylamide gel electrophoresis (PAGE). The PVDF membrane was blocked with primary antibody Recombinant Human ZEB2 (Proteintech, Wuhan, China) overnight at 4°C. The membranes were washed and added with corresponding second antibody at the following day. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Proteintech, Wuhan, China) was used as internal control and proteins were visualized by using chemiluminescence (Perkin Elmer, Waltham, MA, USA). The grayscale of all bands was calculated by Image J (Rawak Software, Inc. Germany).

Statistical Analysis

SPSS 22.0 software (IBM, Armonk, NY, USA) was used to perform statistical assay. The difference was determined by Student's *t*-test (2-tailed), Pearson's correlation analysis, and Analysis of Variance (ANOVA) followed by Dunnett's test. The data were shown as mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

Results

MiR-507 Was Down-Regulated in NSCLC and Associated With Prognosis

To uncover the role of miR-507 on NSCLC, a total of 42 patients, who underwent surgery in our hospital, were enrolled in our study. We collected postoperative specimens, including tumor tissues and adjacent normal tissues. QRT-PCR was applied to evaluate the expression of miR-507 in these specimens and follow-up was made. The expression of miR-507 was noticeably down-regulated in NSCLC samples compared with the adjacent normal samples (Figure 1A). The patients were further divided into two groups based on postoperative pathological stage. It was found that the level of miR-507 was remarkably reduced in patients with advanced stage than not (Figure 1B). Kaplan-Meier analysis revealed that patients with lower miR-507 expression exhibited shorter survival time, whereas those with higher miR-507 expression exhibited longer survival time (Figure 1C). Further analysis of miR-507 in NSCLC cell lines and normal pulmonary epithelium cells revealed that miR-507 was down-regulated in cancer cell lines (Figure 1D). These results indicated that miR-507 might play a suppressive role in NSCLC development.

MiR-507 Repressed Cell Invasion and Proliferation of NSCLC

To explicate the role in NSCLC development, we performed functional experiments on NSCLC cells. A549 and H460 cell lines were chosen for transfection with miR-507 for their low endogenous level. The overexpression of miR-507 was verified by qRT-PCR (Figure 2A). Scratch experiments and transwell assays were applied to evaluate the invasive abilities of NSCLC cells. As showed, it was found that overexpression of miR-507

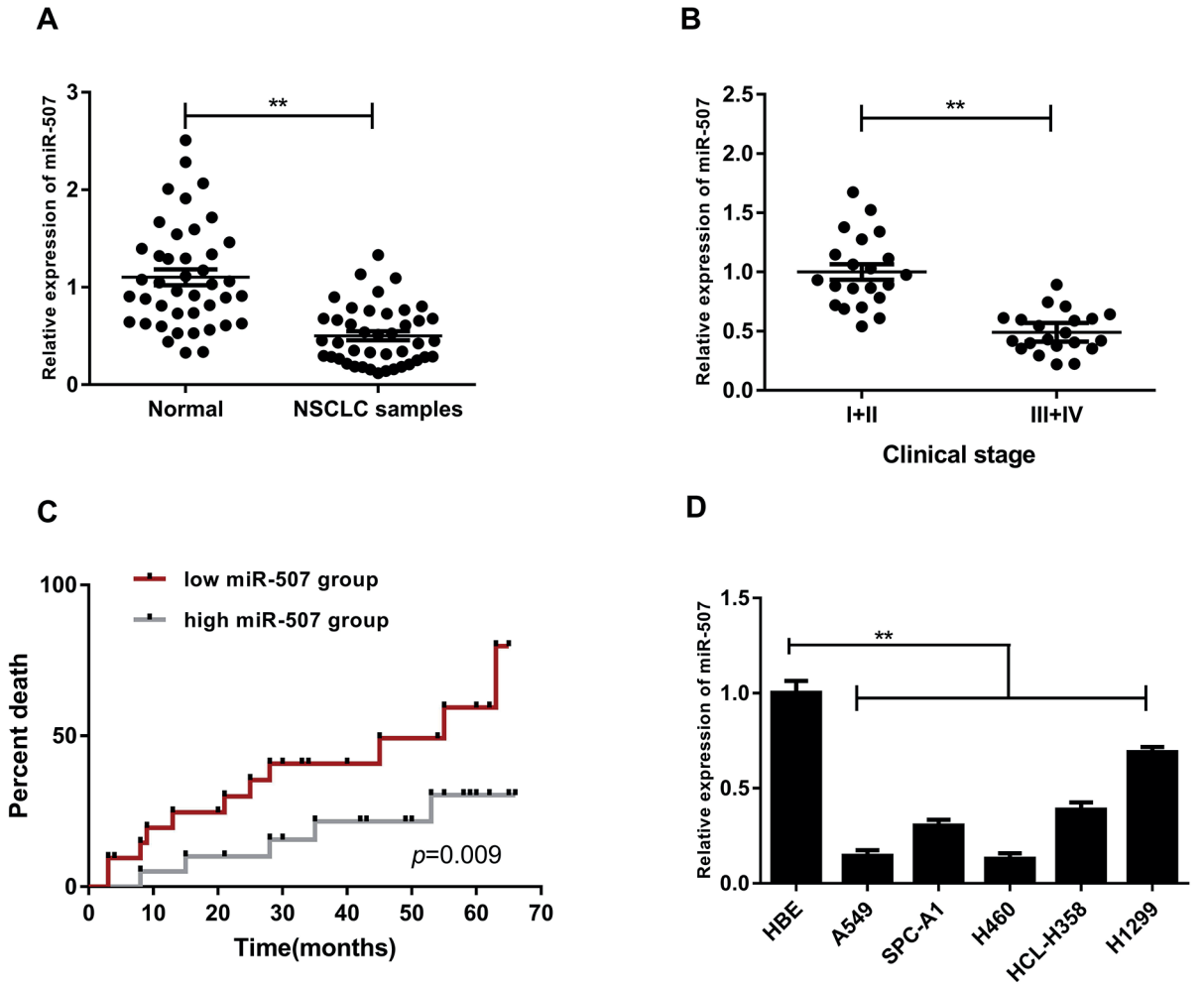

Figure 1. miR-507 was noticeably down-regulated in NSCLC. **A,** Expression of miR-507 was significantly down-regulated in NSCLC tissues than normal tissues. **B,** MiR-507 was remarkably down-regulated in III+IV stage patients than I+II. **C,** Lower miR-507 expression exhibited shorter survival time than higher miR-507 expression patients. **D,** Level of miR-507 was determined in NSCLC cell lines and normal human bronchial epithelial cell line. (**$p<0.01$).

significantly repressed the healing rates of scratch (Figure 2B-2C). MiR-507 could also repress the invasive abilities of A549 and H460 cells (Figure 3A-3B). CCK-8 assays and colony formation assays were used to access the impact of miR-507 on cell proliferation. It was found that overexpression of miR-507 significantly inhibited the OD values of A549 and H460 cells (Figure 2D-2E). The colony formation capabilities were also inhibited by miR-507 noticeably (Figure 3C-3D). In general, these results demonstrated that miR-507 repressed NSCLC cells' invasion and proliferation.

ZEB2 Is a Direct Target of MiR-507 in NSCLC Cells

To elucidate the mechanisms of how miR-507 affects NSCLC progression, we made bioinformatics analysis of miR-507 targets by TargetScan 7.2 database. It was predicted that zinc finger E-box binding homeobox 2 (ZEB2) might be a potential direct molecular of miR-507 (Figure 4A). We then synthesized wt or mt ZEB2 luciferase vectors according to the binding site between 3'-UTR region of ZEB2 and miR-507. Luciferase reporter assays showed that the activities could be reduced by miR-507 in wt groups but not in mt groups or miR-NC groups (Figure 4B-4C). Moreover, qRT-PCR found that the ZEB2 mRNA level could be noticeably decreased by miR-507 (Figure 4D). In addition, we measured the expression of ZEB2 in clinical specimens and found that ZEB2 level was negatively correlated with miR-507 (Figure 4E). Accordingly, these data demonstrated that ZEB2 was a target of miR-507, which was negatively regulated by miR-507.

Overexpression of ZEB2 Alleviated the Effects of MiR-507 in Invasion and Proliferation

To verify whether ZEB2 mediated the effects of miR-507 in invasion and proliferation of NSCLC cells, we transfected the NSCLC cells with len-ZEB2 to increase the ZEB2 level. The cells were co-transfected with miR-507 and len-ZEB2. As shown in the result of Western blot, transfection of miR-507 inhibited the ZEB2 protein expression, but these effects could be reversed by co-transfection of len-ZEB2 (Figure 5A-B). Then, the cell invasion and proliferation capabilities were evaluated. Transwell assays demonstrated that the cell invasive abilities could be decreased by miR-507 but reversed by ZEB2 overexpression (Figure 5C). Moreover, colony formation experiments and CCK-8 assays revealed that ZEB2 overexpressing abolished the promotive effect of the miR-507 on cell proliferation (Figure 5D-E). These results suggested that miR-507 facilitated the invasion and proliferation of NSCLC cells by directly targeting ZEB2.

Discussion

Increasing evidence shows that miRNAs involve in most pathological and physiological processes. It is believed that 60% or more human genes are regulated by specific miRNAs[13,14]. The aberrant expressive miRNAs, however, could significantly affect physiological change and even promote the progression of NSCLC[15]. Hence, it is reasonable to identify the expressive pattern of miRNAs in disease and explore their mechanism. It is also widely accepted that most miRNAs were aberrantly expressed in cancer cells and affected cell behaviors. For example, it was found that miRNA-23a-3p was largely down-regulated in melanoma and associated with the prognosis[16]. Song et al[17] revealed that the knockdown of miR-454-3p restrained cervical cancer cell proliferation and apoptosis via regulating tripartite motif-containing 3. Calsina et al[18] discovered a cluster of six miRNAs that were associated with metastatic risk and progression in pheochromocytomas and paragangliomas. Further mecha-

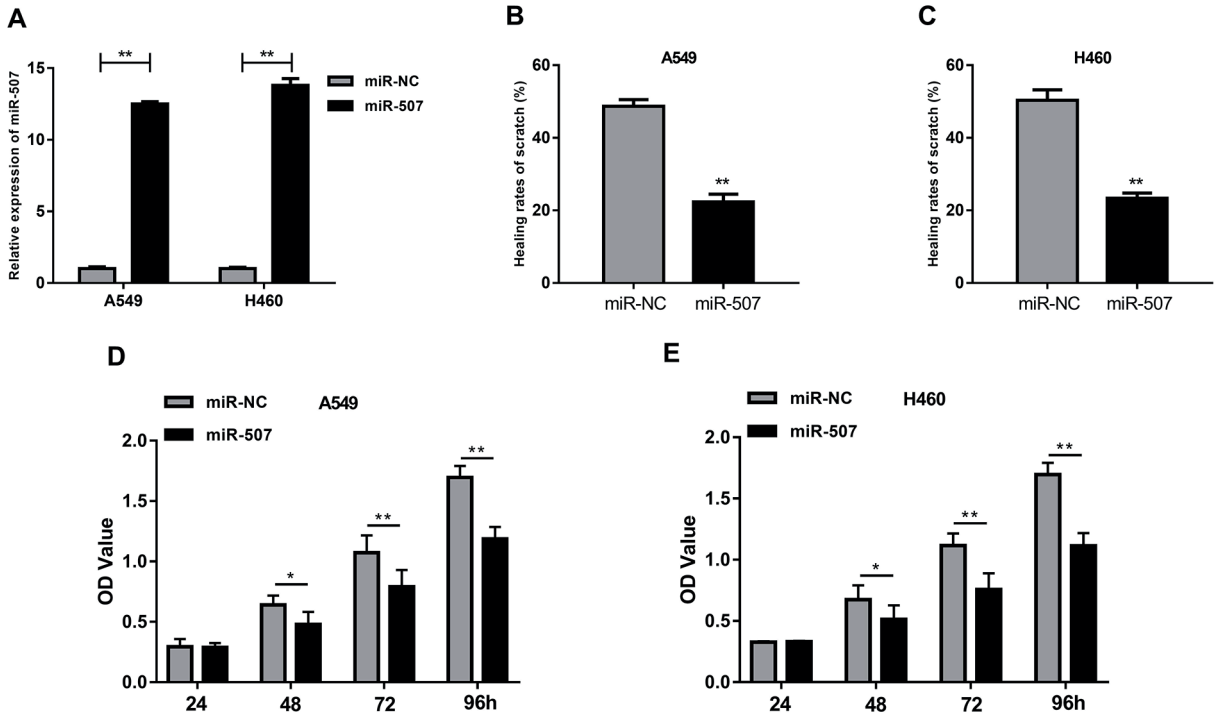

Figure 2. MiR-507 repressed cell scratch healing rates and proliferation of NSCLC. **A**, Transfection of miR-507 mimic successfully induced miR-507 overexpression in A549 and H460 cell lines. **B-C**, miR-507 overexpression significantly suppressed scratch healing rates in A549 and H460 cell lines. **D-E**, CCK8 assays indicated that miR-507 could repress NSCLC cells' proliferation. (**p<0.01).

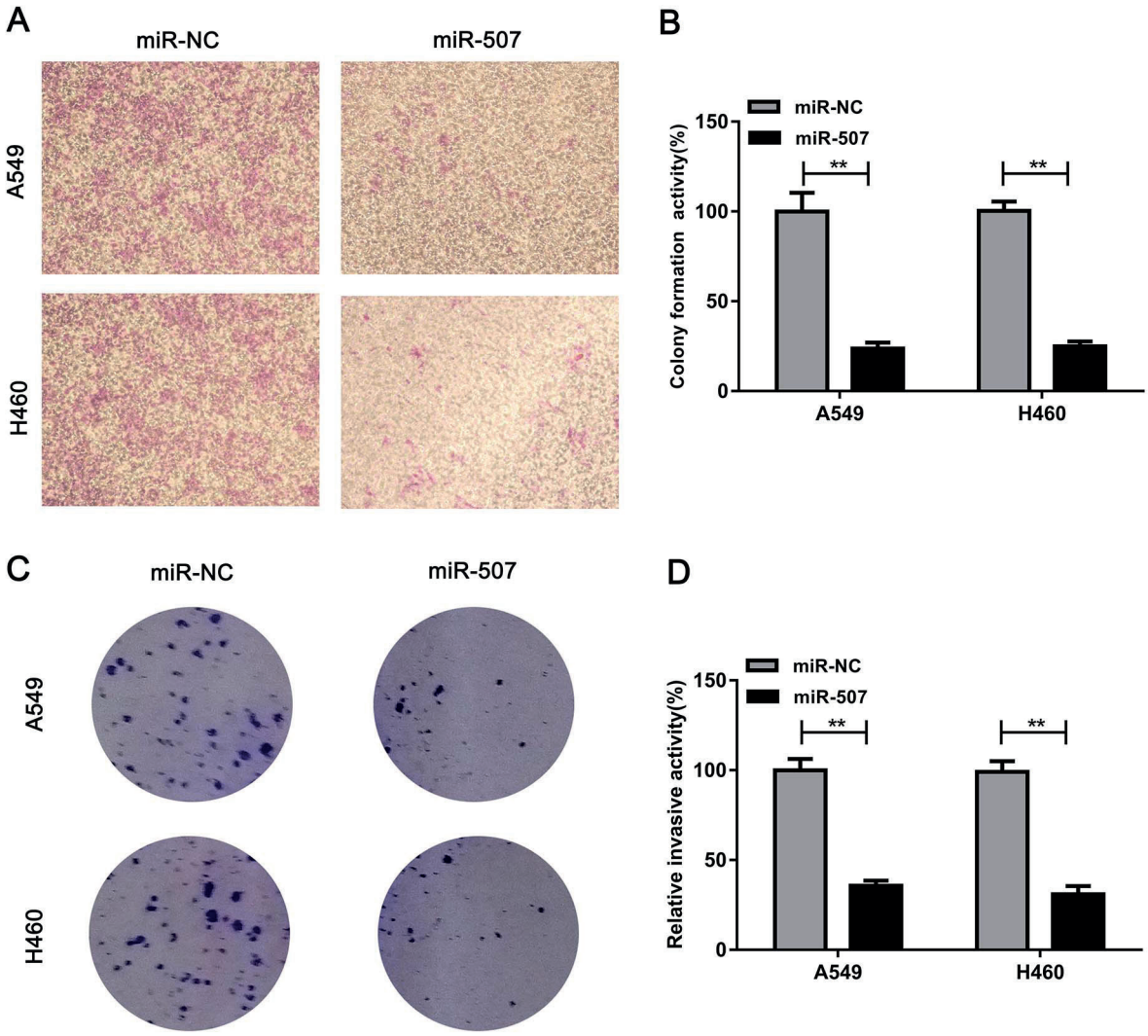

Figure 3. MiR-507 repressed cell invasion and colony formation of NSCLC. **A**, Representative image of transwell assays in NSCLC cells transfected with miR-507 (magnification: 200x). **B**, Quantitative analysis suggested that overexpression of miR-507 in NSCLC obviously repressed cell invasion. **C**, Representative image of colony formation assays in NSCLC cells transfected with miR-507 (magnification: 40x). **D**, Quantitative analysis demonstrated that miR-507 overexpression inhibited NSCLC cell colony formation. (**p<0.01).

nism analysis demonstrated that miR-21-3p/TSC complex subunit 2/mTOR signal shaft might contribute to the pheochromocytoma and paraganglioma metastasis.

The specific miRNA in this study, miR-507, was reported to be aberrantly expressive in some types of cancers, including esophageal squamous cell carcinoma, chondrosarcoma, and hepatocellular carcinoma[19-21]. However, no research excavates its expression and function in NSCLC. Our group firstly measured the expressive signature of miR-507 in NSCLC clinical samples and cell lines. We firstly observed that miR-507 was down-regulated in NSCLC samples when comparing with adjacent normal tissues. The qRT-PCR also indicated that miR-507 was reduced in all NSCLC cell lines. Additionally, the level of miR-507 was correlated with different clinical stage, which suggested the potential tumor promoting role of miR-507 in NSCLC. Functional experiments, including scratch healing assays, transwell invasion assays, CCK-8 assays, and colony formation assays unveiled that miR-507 overexpression dramatically repressed the NSCLC cell invasion and

proliferation. Thus, miR-507 was down-regulated in NSCLC and might play a suppressive role in NSCLC development.

The most common mechanism that miRNA comes into play is miRNA-mRNA sponging. Some direct sponging targets of miR-507 have been identified. Wei et al[22] identified forkhead box M1 (FOXM1) was a direct target of miR-507 in melanoma. Wang et al[23] found that miR-507 could directly target ETS transcription factor ELK3 (ELK3) and mediated the stemness and chemoresistance of colorectal cancer[23]. Jia

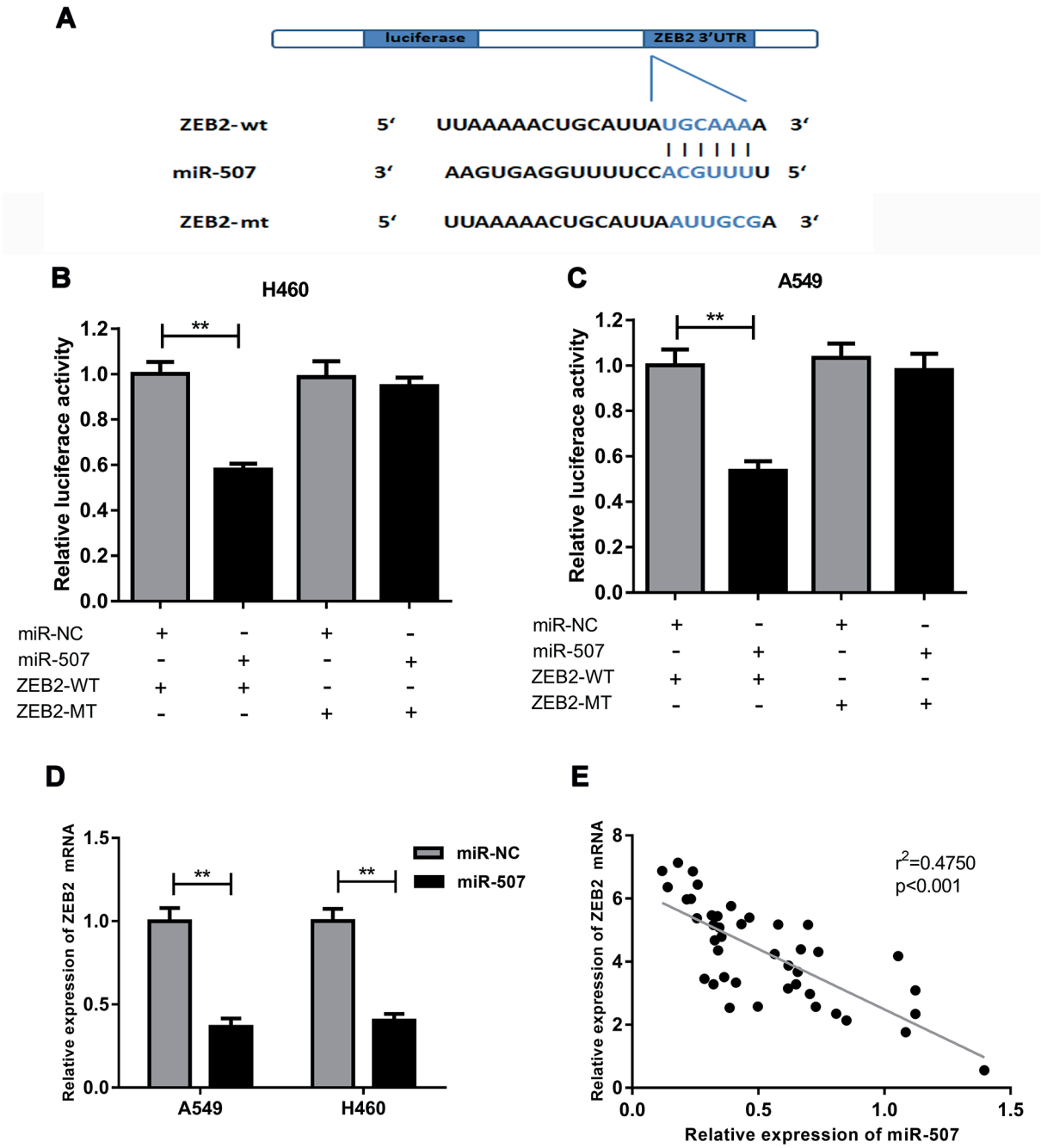

Figure 4. MiR-507 targeted ZEB2 directly in NSCLC. **A**, Predicted interacting sites between miR-507 and ZEB2 were showed including wilt type and mutant type. **B-C**, Luciferase reporter activities of wild type ZEB2 vectors were decreased by miR-507 but not miR-NC or mutant ZEB2 vectors in NSCLC cells. **D**, ZEB2 mRNA expression was largely decreased by miR-507 overexpression. **E**, Level of ZEB2 level was negatively correlated with miR-507 in NSCLC samples. (**p<0.01).

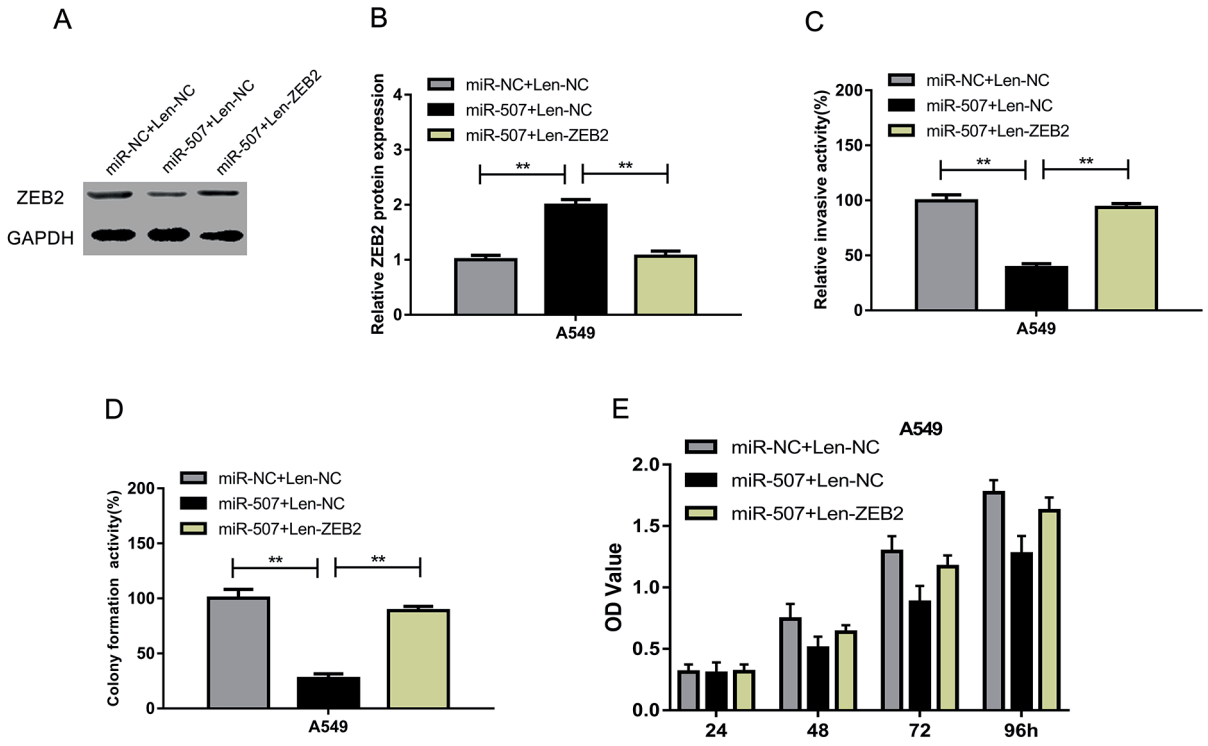

Figure 5. ZEB2 alleviated the suppressive effects of miR-507 in NSCLC cells. **A**, Representative image of ZEB2 protein expression in NSCLC cells transfected with miR-507 and/or len-ZEB2. **B**, ZEB2 protein expression could be repressed by miR-507 but rescued by len-ZEB2. **C**, Invasive abilities of NSCLC cells were inhibited by miR-507 but alleviated by ZEB2. **D**, ZEB2 alleviated the suppressive effects of miR-507 on in NSCLC cells' colony formation. **E**, Cell proliferation of NSCLC were repressed by miR-507 but reversed by ZEB2. ZEB2 alleviated the suppressive effects of miR-507 on in NSCLC cells' proliferation. (**p<0.01).

et al[24] verified that fms related tyrosine kinase 1 (Flt-1) was also a target of miR-507 in human breast cancer cells. Herein, by applying online bioinformatic prediction and Dual-Luciferase reporter assays, we firstly predicted and verified a new direct target of miR-507, ZEB2. ZEB2 belongs to the Zeb family, which is two-handed zinc-finger/homeodomain protein and acts actively as transcription factor[25]. It is a core molecule that regulates epithelial-mesenchymal transition and cancer cell invasion and metastasis[26]. ZEB2 could interact with E-cadherin and thus induced epithelial-mesenchymal transition process, which accelerated the cancer development and metastasis[27]. Former researches[28,29] also identified the role of ZEB2 in NSCLC and potential interacting network. Herein, we firstly uncovered that ZEB2 was a direct target of miR-507 and it reversed the tumor suppressive role of miR-507 in NSCLC, which might help for a better understanding of NSCLC development and therapy in the future.

Conclusions

In sum, our research demonstrated that miR-507 was remarkably down-regulated in NSCLC tissues and cell lines. Moreover, it was found that miR-507 acted as tumor suppressor in NSCLC and it significantly repressed NSCLC malignant behaviors. Importantly, we verified the miR-507/ZEB2 axis in NSCLC, which provided a new insight into the mechanisms underlying NSCLC progression, and suggested potential therapeutic targets for NSCLC therapy in the future.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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