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ORIGINAL ARTICLE

MicroRNA-19a promotes proliferative and migratory abilities of NSCLC cells by inhibiting PTEN expression

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Summary

Purpose: *To investigate whether microRNA-19a can promote the proliferative and migratory abilities of non-small cell lung cancer (NSCLC) cells by target inhibition of PTEN (phosphatase and tensin homolog deleted from chromosome 10, PTEN) expression, thus leading to the development of NSCLC.*

Methods: *The expression level of microRNA-19a in NSCLC tissues, paracancerous tissues and normal lung tissues was detected by quantitative real time-polymerase chain reaction (qRT-PCR). The regulatory effects of microRNA-19a on proliferative and migratory abilities of NSCLC cells were determined by cell counting kit-8 (CCK-8), colony formation assay and Transwell assay, respectively. The binding condition between microRNA-19a and PTEN was verified by dual-luciferase reporter gene assay. PTEN expression in NSCLC cells transfected with microRNA-19a mimic or inhibitor was detected by Western blot. Rescue experiments were conducted by co-transfection of microRNA-19a mimic and pcDNA-PTEN in NSCLC cells, followed by detection of cell proliferation, colony formation and migration.*

Results: *QRT-PCR data showed higher expression of microRNA-19a in NSCLC tissues and cell lines than that of controls. Overexpression of microRNA-19a in NSCLC A549 and H1299 cell lines promoted proliferative and migratory abilities. Dual-luciferase reporter gene assay confirmed the binding site between microRNA-19a and PTEN. PTEN expression was negatively regulated by microRNA-19a both at mRNA and protein levels. Rescue experiments showed that PTEN overexpression could partially reverse the regulatory effects of microRNA-19a on promoting proliferative and migratory abilities of NSCLC cells.*

Conclusions: *Higher expression of microRNA-19a promotes proliferative and migratory abilities of NSCLC cells by target inhibiting PTEN expression.*

Key words: *MicroRNA-19a, NSCLC, proliferation, migration, PTEN*

Introduction

Lung cancer (LC) is a common cancer with a relatively high mortality. LC is pathologically divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and NSCLC is subdivided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma [1]. NSCLC accounts for about 75-80% of all LC cases, manifested with high grade of malignancy and poor prognosis. The clinical performances of NSCLC are complicated. Most of NSCLC patients are already in advanced stage at the time of diagnosis due to occult symptoms [2]. Current treatment methods for NSCLC include surgical resection, radiotherapy and chemotherapy. Although these treatments do prolong the overall survival, the therapeutic efficacy of NSCLC is still far away from satisfactory [3]. Therefore, it is of

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great significance to conduct in-depth research on the occurrence and development of NSCLC.

MicroRNAs (miRNAs), as a type of non-coding, endogenous small RNAs, contain approximately 22 nucleotides [4]. A miRNA can target more than 100 different signaling pathways by regulating target genes [5,6]. MiRNAs exert different biological effects, such as regulations on cell proliferation, migration, differentiation, necrosis, and programmed cell apoptosis. MiRNAs are expressed in many tumors, and serve as oncogenes or tumor-suppressor genes [7]. Recently, miRNAs have been used as a diagnostic and prognostic target for a variety of tumors. It is reported that some certain miRNAs are utilized as therapeutic targets on tumor treatments by regulating PTEN (phosphatase and tensin homolog deleted from chromosome 10, PTEN) [8]. Target genes that bind to miRNA possess multiple functions on tumor development, which are verified by prediction of binding site in the 3'UTR of the target gene. Relative reports pointed out that microRNA-19a promotes proliferative and migratory abilities of tumor cells by targeting PTEN, which provides new directions in tumor treatment [8,9].

MicroRNA-19a is one of the important members of the miRNA-17-92 family. The miRNA-17-92 cluster is a highly conserved gene sequence located on the C13orf25, the third intron of chromosome 13q31. This cluster consists of six basic members, namely miR-17, miR-20a, miR-18a, miR-19a, miR-19b-1 and miR-92-1 [10]. A large number of studies have confirmed the oncogenic role of microRNA-19a in a variety of tumors, such as colon cancer, NSCLC, ovarian cancer, bladder cancer and other common cancers [8,11-13]. Studies have also suggested that microRNA-19a may be a key oncogene in the miRNA-l7-92 family. To date, the specific mechanism of microRNA-19a in regulating NSCLC development has not been fully elucidated.

Methods

Sample collection

50 cases of NSCLC tissues and paracancer tissues (5 cm away from the tumor edge) were harvested from NSCLC patients admitted in Sichuan Provincial People's Hospital from June 2014 to August 2017. NSCLC tissues were pathologically confirmed by H&E staining. Besides, paracancer tissues were confirmed without tumor infiltration and inflammatory cell infiltration. Enrolled patients did not receive preoperative tumor treatments. Tissues were immediately preserved in liquid nitrogen. This study was approved by the ethics committee of Sichuan Provincial People's Hospital. Signed informed consents were obtained from the patients and/or their guardians before study entry.

Cell culture and transfection

NSCLC cell lines A549, H1299, SK-MES-1, 95D, and NCI-H520 and normal lung cell line HELF were incubated in Roswell Park Memorial Institute 1640 medium (RPMI-1640, HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 0.1% streptomycin. Cells were incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed until 85% of cell confluence. Culture medium was replaced every 3 days. Transfection was performed following the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). MicroRNA-19a mimic, inhibitor and pcDNA-PTEN were provided by GenePharma (Shanghai, China).

RNA extraction and quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). RNA concentration was detected using spectrometer. QRT-PCR was then performed based on the manufacturer's instructions of SYBR Premix Ex Taq TM (TaKaRa, Tokyo, Japan). The relative gene expression was calculated using $2^{-\Delta Ct}$ method. Primers used in the study were as follows: MicroRNA-19a F: 5'-TGTGCAAATCTATGCAAA-3', R: 5'-ACGUGACACGUUCGGAGAATT-3'; PTEN F: 5'-TGGATTCGACTTAGACTTGACCT-3', R: 5'-GTGCAGGGTCCGAGGTATTC-3'; GAPDH F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'; U6 F: 5'-CTCGCTTCGGCAGCACACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'.

CCK-8 (cell counting kit-8) assay

Transfected cells were seeded into 96-well plates at a density of 1×10^6/μL. 10 μL of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) were added in each well after cell culture for 0, 1, 2 and 3 days, respectively. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony formation assay

Transfected cells were collected and 1000 cells were seeded in 60 mm culture dish. After culture for 2-3 weeks, cells were washed with phosphate buffered saline (PBS) and fixed in 2 mL of methanol for 20 min. After PBS wash for three times, colonies were stained with 0.1% crystal violet for 20 min. Colonies were captured in a light microscope (Olympus, Tokyo, Japan).

Transwell assay

After 48 h of transfection, cells were digested and resuspended in serum-free medium. Cell density was adjusted to 1×10^5/mL. Transwell chambers containing Matrigel were placed in 24-well plates. 200 μL of cell suspension and 500 μL of medium containing 10% FBS were added in the upper and lower chamber, respectively. After cell culture for 48 h, cells were fixed with 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min. Inner cells were carefully cleaned.

Penetrating cells were captured in 5 randomly selected fields of each sample for cell counting.

Dual-luciferase reporter gene assay

The binding site of microRNA-19a and PTEN was predicted to construct wild-type and mutant-type PTEN. Cells were seeded in 12-well plates and co-transfected with 50 pmol/L microRNA-19a mimic or inhibitor and 80 ng wild-type or mutant-type PTEN for 48 h, respectively. Cells were then washed with PBS and incubated with 1×PLB for complete lysis. Luciferase activity was finally detected according to the relative commercial kit instructions.

Western blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Protein sample was separated by gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence method.

Statistics

SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. The quantitative data were presented as mean±standard deviation (x±s). The two-paired t-test was used for comparing differences between the two groups. P<0.05 was considered statistically significant.

Results

MicroRNA-19a was highly expressed in NSCLC

The expression level of microRNA-19a in NSCLC tissues, paracancer tissues and normal lung tissues was detected by qRT-PCR. The results showed that microRNA-19a was highly expressed in NSCLC tissues compared with that of paracancer tissues and normal lung tissues (Figure 1A,1B). The relative ratio of microRNA-19a expression in NSCLC tissues to paracancer tissues is shown in Figure 1C. Similarly, microRNA-19a was highly expressed in NSCLC cell lines (A549, H1299, SK-

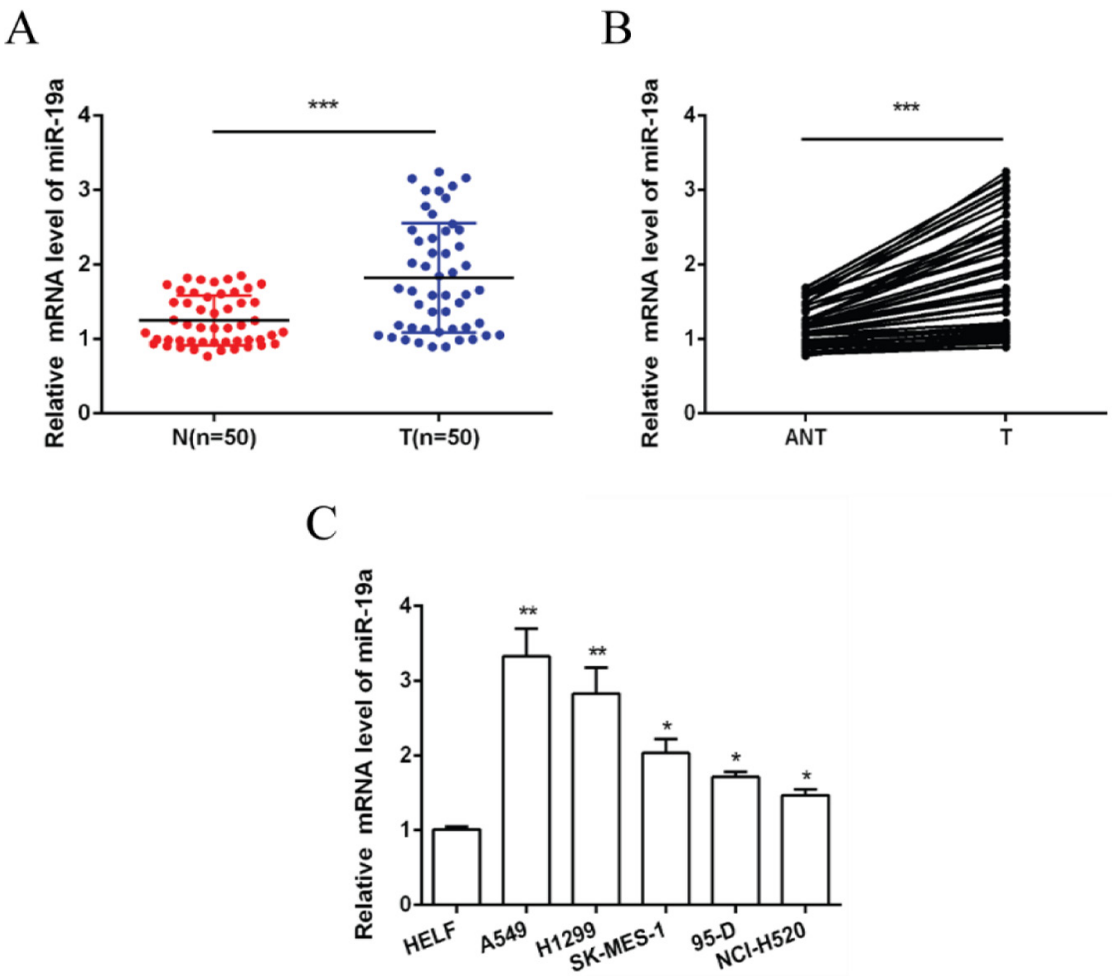

Figure 1. MicroRNA-19a was highly expressed in NSCLC. **A:** MicroRNA-19a was highly expressed in NSCLC tissues compared to normal lung tissues. **B:** MicroRNA-19a was highly expressed in NSCLC tissues than in paracancer tissues. **C:** MicroRNA-19a was highly expressed in NSCLC cells than in normal lung cells (*p<0.05, **p<0.01, ***p<0.001).

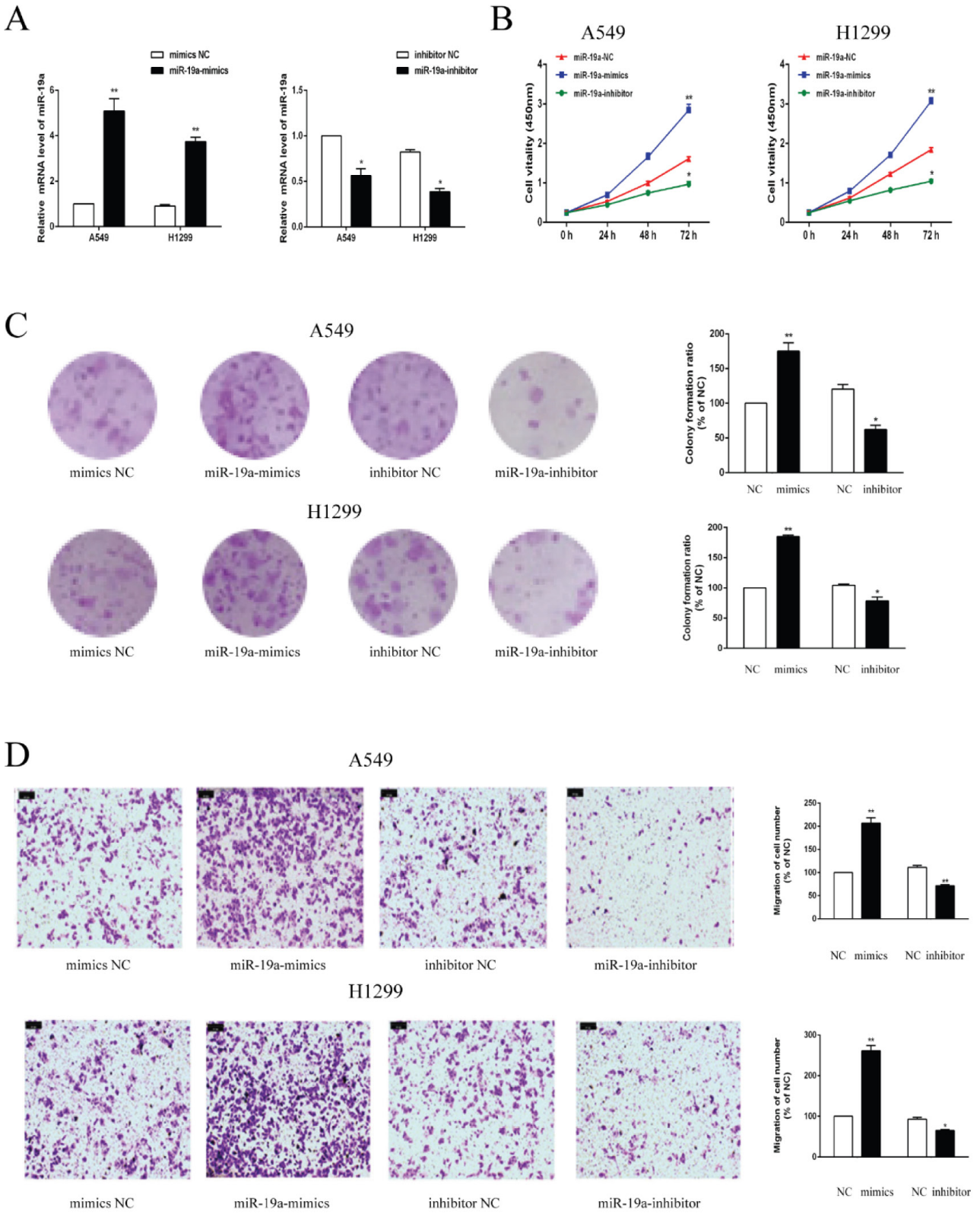

Figure 2. MicroRNA-19a promoted the proliferative and migratory abilities of NSCLC cells. **A:** Transfection efficacies of microRNA-19a mimic and microRNA-19a inhibitor in A549 and H1299 cells. The qRT-PCR showed that transfection of microRNA-19a mimics significantly increased microRNA-19a expression, whereas transfection of microRNA-19a inhibitor decreased microRNA-19a expression. **B:** CCK-8 assay detected the proliferation ability of NSCLC cells. This assay showed microRNA-19a overexpression promoted cell proliferation and microRNA-19a knockdown obtained the opposite result. MicroRNA-19a overexpression promoted cell proliferation, and microRNA-19a knockdown obtained the opposite result. **C:** MicroRNA-19a overexpression promoted colony formation ability and microRNA-19a knockdown obtained the opposite result. **D:** MicroRNA-19a overexpression promoted cell migration and microRNA-19a knockdown obtained the opposite result (*p<0.05, **p<0.01).

MES-1, 95D, and NCI-H520) compared with normal lung cell line HELF (Figure 1D). Among them, expression difference of microRNA-19a was most pronounced in A549 and H1299 cells, which were selected for the following experiments.

MicroRNA-19a promoted proliferative and migratory abilities of NSCLC cells

A549 and H1299 cells were transfected with miR-negative control (NC), microRNA-19a mimic or microRNA-19a inhibitor to verify their transfection efficacies. Transfection of microRNA-19a mimic remarkably increased microRNA-19a expression, whereas transfection of microRNA-19a inhibitor decreased microRNA-19a expression (Figure 2A). The proliferative ability of NSCLC cells was then assessed by CCK-8 assay. It was found that microRNA-19a overexpression markedly promoted the proliferative ability, while knockdown of microRNA-19a showed the opposite result (Figure 2B). Similarly, microRNA-19a overexpression promoted the colony formation ability in NSCLC cells as well (Figure 2C). Subsequently, Transwell assay was conducted to determine the regulatory effect of microRNA-19a on cell migration. The migratory ability of NSCLC cells increased after microRNA-19a overexpression (Figure 2D).

Targeted degradation of PTEN expression by microRNA-19a

PTEN expression in NSCLC tissues and normal lung tissues was examined by qRT-PCR. The results showed that mRNA level of PTEN was lower in NSCLC tissues than normal lung tissues (Figure 3A). The expression level of microRNA-19a in NSCLC tissues was negatively correlated with

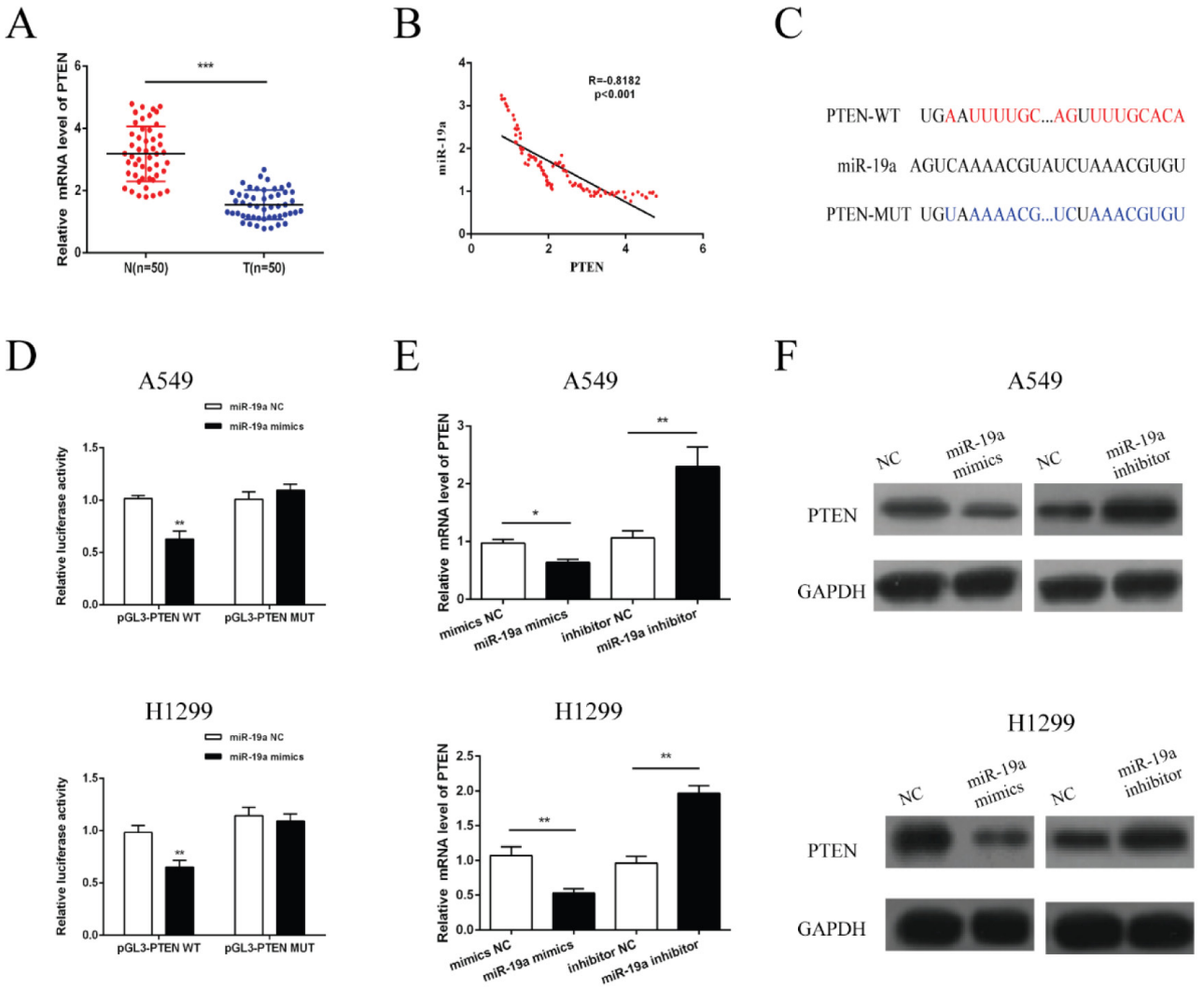

Figure 3. Targeted degradation of PTEN expression by microRNA-19a. **A:** PTEN was lowly expressed in NSCLC tissues. **B:** PTEN expression was negatively correlated to microRNA-19a. **C:** Binding site between microRNA-19a and PTEN predicted by bioinformatics. **D:** Luciferase activity in each group. **E, F:** Protein and mRNA levels of PTEN were negatively regulated by microRNA-19a (*p<0.05, **p<0.01).

PTEN expression (Figure 3B). We predicted the target gene of microRNA-19a through bioinformatics and PTEN was screened out (Figure 3C). Dual-luciferase reporter gene assay showed that the luciferase activity decreased in NSCLC cells transfected with PTEN-WT 3'UTR compared with PTEN-MUT 3'UTR (Figure 3D), indicating that PTEN could bind to microRNA-19a. The mRNA expression of PTEN in A549 and H1299 cells transfected with miR-NC, microRNA-19a mimics or microRNA-19a inhibitor was detected and it was found that PTEN expression decreased in NSCLC cells transfected with microRNA-19a mimic, whereas it increased after transfection of microRNA-19a inhibitor (Figure 3E). Western blot results also found that protein expression of PTEN was negatively regulated by microRNA-19a expression (Figure 3F).

PTEN overexpression reversed the regulatory effects of microRNA-19a on cell proliferation and migration

We transfected pcDNA-NC or pcDNA-PTEN into A549 and H1299 cells, followed by detection of PTEN expression. It was found that pcDNA-PTEN transfection significantly increased mRNA and protein levels of PTEN (Figure 4A, 4B). We also detected cell proliferation after NSCLC cells

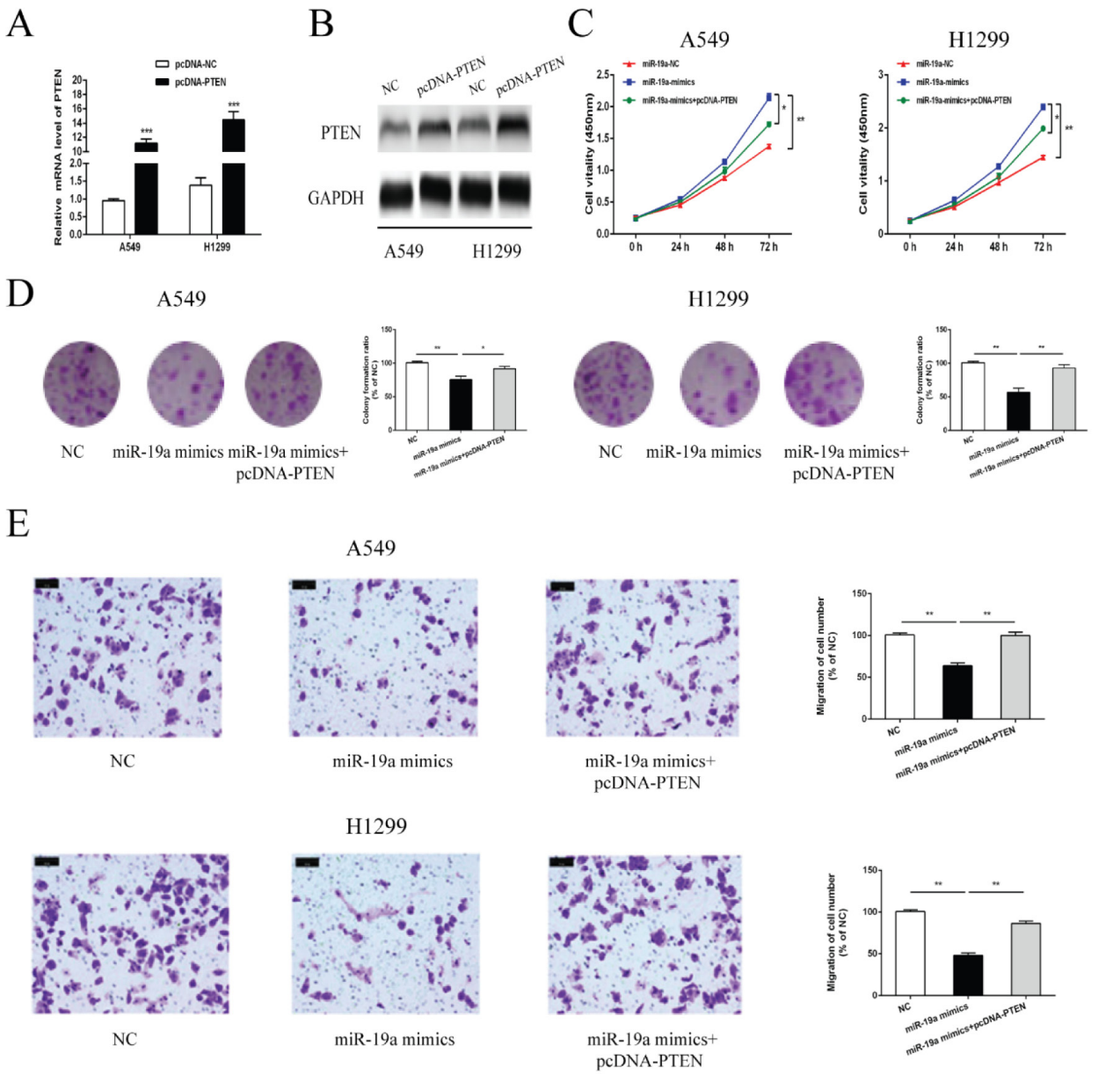

Figure 4. PTEN overexpression reversed the regulatory effects of microRNA-19a on cell proliferation and migration. **A, B:** Transfection of pcDNA-PTEN in A549 and H1299 cells significantly increased mRNA and protein levels of PTEN. **C:** PTEN overexpression partially reversed the enhanced cell proliferation by microRNA-19a. **D:** PTEN overexpression partially reversed the enhanced colony formation ability by microRNA-19a. **E:** PTEN overexpression partially reversed the enhanced cell migration by microRNA-19a (*p<0.05, **p<0.01).

were co-transfected with microRNA-19a mimic and pcDNA-PTEN. The data showed that increased proliferative ability and colony formation ability induced by microRNA-19a overexpression were partially reversed by PTEN overexpression (Figure 4C,4D). Similarly, PTEN overexpression partially reversed the enhanced cell migration by microRNA-19a (Figure 4E). These results elucidated that microRNA-19a can promote proliferative and migratory abilities of NSCLC cells by inhibiting PTEN expression.

Discussion

Although great progress has been done in the study of tumor markers and targeted therapies, NSCLC still displays high recurrence rate and poor prognosis [14]. The lack of early diagnostic methods and limited understanding of tumor biology are important reasons for the difficulty in treating NSCLC. MiRNAs are a class of highly conserved non-coding small RNAs that perform a variety of biological functions on post-transcriptional regulation of target genes. Some certain miRNAs have been confirmed to be greatly involved in the occurrence and development of NSCLC [15-17]. Studies have shown that more than 31% of miRNAs in solid tumors are differentially expressed compared with normal tissues, suggesting that miRNAs can precisely regulate the tumor development as oncogenes or tumor-suppressor genes [18].

Studies have shown that many miRNAs are involved in the pathogenesis of NSCLC. For example, miR-34 is downregulated in a variety of tumors, including lung cancer [19]. Members of miR-34 (a, b and c) are regulated by p53 and downregulated in p53-mutant tumors [20]. MiR-34 could regulate cell cycle, apoptosis and cellular aging through specific targeting of the BCL-2, MYC, and MET [21]. The expressions of miR-34a and miR-34c are downregulated in NSCLC cells, which are negatively correlated to the expressions of PDGFR-α/β [22]. Let-7 is one of the earliest tumor-suppressor miRNAs found in LC, and its expression level is negatively correlated to the disease prognosis [23]. In addition, let-7 regulates several oncogenic genes. For example, cell cycle oncogene KRAS is negatively regulated by let-7 [24,25], which is often associated with lung adenocarcinoma. MYC and HMGA2 are proved to be regulated by let-7 in LC as well [26]. Overexpression of let-7 in lung adenocarcinoma cell line A549 inhibits cell growth and slows down cell cycle progression by acting on CDC25A, CDK6, and cyclin D2. In addition, let-7 in LC shows potential of radiotherapy resistance. The miRNA-29 family (29a, 29b, and 29c) expressed in LC tissues is associated with DNA methyltransferases DNMT-3A and DNMT-3B, which are important enzymes for DNA methylation [27]. Upregulation of miR-29 family members in LC cells restores the normal pattern of DNA methylation, increases the expressions of tumor-suppressor genes, such as FHIT and WWOX, and thus inhibits tumorigenesis. These findings all suggested the significant role of miR-29 in NSCLC, providing a theoretical basis for miRNA intervention in the treatment of LC [28].

Phosphatase and tensin homolog deleted from chromosome 10, PTEN) was discovered in 1997. Previous studies have shown the important role of PTEN in tumor development. It is located in chromosome 10q23.3 and consists of nine exons. As a tumor-suppressor gene, PTEN shows dual specific phosphatase activity [29]. PTEN can inhibit the proliferative and migratory activities of tumor cells. Moreover, PTEN can inhibit tumor invasion and metastasis by enhancing the adhesion ability and inhibiting the degradation of extracellular matrix and tumor angiogenesis. The mutated PTEN gene is involved in the development and progression of many malignant tumors.

This study explored the cellular function of microRNA-19a in NSCLC and its possible mechanisms. We found that microRNA-19a expression was significantly elevated in NSCLC tumor tissues. Similar results were further verified in the NSCLC cell lines, which suggested that microRNA-19a is closely related to certain biological functions of NSCLC. Based on the above studies, we selected A549 and H1299 cell lines for functional studies of microRNA-19a. The results showed that high expression of microRNA-19a promotes the proliferative and migratory abilities of A549 and H1299 cells. Our studies predicted that PTEN is a downstream target of microRNA-19a, which was subsequently verified by dual-luciferase reporter gene assay. PTEN expression was negatively regulated by microRNA-19a at mRNA and protein levels. Furthermore, rescue experiments confirmed that PTEN could reverse the regulatory effects of microRNA-19a on biological performances of NSCLC cells.

Conclusions

Higher expression of microRNA-19a promotes proliferative and migratory abilities of NSCLC cells by target inhibiting PTEN expression. Our results provide new directions in the clinical treatment of NSCLC.

Conflict of interests

The authors declare no conflict of interests.

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