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MicroRNA-374b accelerates the development of lung cancer through downregulating PTEN expression via activating PI3K/Akt pathway

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Abstract. – OBJECTIVE: To elucidate whether microRNA-374b could participate in the development of lung cancer (LC) through downregulating PTEN (gene of phosphate and tensin homolog deleted on chromosome ten) expression via activating PI3K/Akt pathway.

PATIENTS AND METHODS: Expression levels of microRNA-374b and PTEN in LC tissues and adjacent normal tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Moreover, the expression level of microRNA-374b in LC cell lines was detected as well. The microRNA-374b inhibitor was constructed and transfected to downregulate microRNA-374b expression in A549 and H358 cells. The regulatory effects of microRNA-374b on migratory and proliferative capacities of LC cells were explored by wound healing and cell counting kit-8 (CCK-8) assay, respectively. After co-transfection of microRNA-374b inhibitor and si-PTEN in LC cells, expression levels of PTEN/PI3K/Akt were determined by qRT-PCR and Western blot.

RESULTS: QRT-PCR results showed that microRNA-374b expression was higher, while PTEN expression was lower in LC tissues than adjacent tissues. Identically, microRNA-374b was also highly expressed in LC cell lines. PTEN expression was negatively correlated with microRNA-374b expression in LC. The downregulation of microRNA-374b in A549 and H358 cells inhibited their migratory and proliferative potentials. Subsequently, we verified that microRNA-374b could bind to PTEN through dual-luciferase reporter gene assay. MicroRNA-374b could inhibit PTEN expression and activate the PI3K/Akt pathway. Furthermore, PTEN knockdown enhanced migratory and proliferative abilities of LC cells, which were attenuated by co-transfection of microRNA-374b inhibitor.

CONCLUSIONS: MicroRNA-374b promotes the development of LC by downregulating PTEN expression through activating PI3K/Akt pathway.

Key Words:
Lung cancer, MicroRNA-374b, PTEN, Migration, Proliferation.

Introduction

LC (lung cancer) is a major malignancy leading to death throughout the world. The tumorigenesis of LC involves multiple environment-gene factors and progresses. Current researches have shown that smoking is a major risk factor for LC[1]. The incidence and mortality of LC in China remain high, accounting for about 20-25% of all malignant tumors[2]. Among several subtypes of LC, non-small cell lung cancer (NSCLC) accounts for about 80% of LC cases. Tumor invasion, metastasis, and drug resistance to chemotherapy or radiotherapy are the leading causes of poor prognosis of NSCLC[3,4]. Due to the lack of early diagnosis, many NSCLC patients have missed the optimal therapeutic opportunity as they have already progressed into the advanced stage at the time of diagnosis[5]. Al-

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though therapeutic approaches have been greatly improved in recent years, the survival rate of LC patients, especially those in the advanced stage, is still very low. In particular, metastatic LC leads to unsatisfied outcomes of affected patients[6-8]. It is of great significance to develop novel and efficient therapeutic approaches for LC.

Tumor metastasis is regulated by a variety of factors. Accumulating evidence has proved that microRNAs are key factors in regulating invasion and metastasis of tumor cells, which can be served as targets for tumor therapy. MicroRNAs are a class of non-coding small RNAs with 19-22 nucleotides in length. They participate in the development and progression of tumors through degrading or inhibiting translation of target mRNAs[9,10]. A large number of researches have showed the involvement of microRNAs in the occurrence and progression of tumor diseases. For example, miR-31 can promote the formation and growth of LC11. MiR-210 is highly expressed in LC and involved in the disease development[12]. MiR-145 inhibits tumor growth, invasion and metastasis of breast and rectal cancer as a tumor suppressor[13-15].

MicroRNA-374b was firstly discovered in the semen of infertility males16. However, the role of microRNA-374b varies a lot in different tumors. MicroRNA-374b is lowly expressed in prostate cancer and is considered to be an independent prognostic indicator of recurrence-free survival of prostate cancer[17]. On the contrary, the microRNA-374b expression is highly expressed in gastric cancer, which promotes the migratory and invasive capacities of gastric cancer cells[18]. At present, the mechanism of microRNA-374b in regulating the development of LC is still unclear, which is fully elucidated in this study.

Patients and Methods

Sample Collection

We collected 40 cases of LC and adjacent tissues in our hospital. Tissues were immediately placed in liquid nitrogen and transferred to a -80°C refrigerator for long-term storage. Enrolled patients and their families signed the informed consent prior to the experiment. The study was approved by the Research Ethics Committee of Beijing Chest Hospital.

Cell Culture and Transfection

Human bronchial epithelial cell line (16HBE) and LC cell lines (A549, H358, H226) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) containing 10% FBS (Gibco, Grand Island, NY, USA) and placed in a 5% CO2 humidified incubator at 37°C. The microRNA-374b inhibitor, PTEN (gene of phosphate and tensin homolog deleted on chromosome ten) siRNA and the corresponding negative controls were purchased from GenePharma (Shanghai, China).

When the cell density reached 50%, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 4-6 h of incubation. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to verify the transfection efficacy at 48 h.

RNA Extraction

Tissues or cells were lysed in 500 μL of TRIzol (Invitrogen, Carlsbad, CA, USA). After maintenance for 5 min, 200 μL of chloroform was added, mixed and stand at room temperature for 5 min. The supernatant was transferred into a new RNase-free centrifuge tube after centrifugation at 4°C, 12000 r/s for 15 min. Isopropanol with the same volume of the supernatant was added for harvesting RNA precipitate by centrifugation. The extracted RNA was air dried, quantified and dissolved in 15-50 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China).

QRT-PCR

RNA was subjected to a reverse transcription system using a PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan) to obtain a cDNA. The reverse transcription conditions were reverse transcription at 37°C for 15 min and inactivation of reverse transcriptase at 85 °C for 5 s. The reverse transcript template was diluted in RNase-depleted water to a final concentration of 10 ng/μL. QRT-PCR was carried out in accordance with the instruction of SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan). The total qRT-PCR system was 10 μL and performed as pre-denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50-65°C for 30 s, and extension at 72°C for 60 s. Primer sequences were as follows: MicroRNA-374b, F: 5'-ATATAATACAACCTGCTAAGTG-3', R: 5'-GTGCAGGGTCCGAGGTATTC-3'; PTEN, F: 5'-TGGTGAGGTTTGATCCGCATA-3', R: 5'-CCCAGTCAGAGGCGCTATG-3'; PI3K, F: 5'-AACGAGAACGTGTGCCATTTG-3',

R: 5'-AGAGATTGGCATGCTGTCGAA-3'; Akt, F: 5'-TGAGCGACGTGGCTATTG-3', R: 5'-CAGTCTGGATGGCGGTT-3'; β-actin, F: 5'-CACCCGCGAGTACAACCTTC-3', R: 5'-CCCATACCCACCATCACACC-3'; U6, F: 5'-AGAGAAGATTAGCATGGCCCCTG-3', R: 5'-ATCCAGTGCGGGTCCGAGG-3'.

Dual-Luciferase Reporter Gene Assay

The transcript 3'UTR sequence of PTEN was cloned into the vector pGL3 containing the luciferase reporter gene, which was the Wt PTEN 3'UTR group. Mut PTEN 3'UTR group was constructed by mutating the core binding sequences using a site-directed mutagenesis kit. Cells were co-transfected with microRNA-374b mimics or negative control and Wt PTEN 3'UTR or Mut PTEN 3'UTR, respectively. At 24 hours, cells were lysed and centrifuged at 10,000 g for 5 min. 100 μL of the supernatant was collected for determining the luciferase activity.

Wound Healing Assay

Cells were seeded into 6-well plates with 1×106 cells per well. An artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. The images were taken at 0 and 24 h using an inverted microscope, respectively.

Cell Proliferation Assay

Cells were digested and inoculated into 96-well plates at a density of 5×104/mL. After culture for 6, 24, 48, and 72 h, respectively, 20 μL of cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was supplied in each well. After incubation in the dark for 2 h, absorbance at 450 nm was recorded by a microplate reader.

Western blot

Total protein was extracted using the cell lysate for determining protein expression. The protein sample was quantified by bicinchoninic acid (BCA; Abcam, Cambridge, MA, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhancedchemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The t-test was used for comparing differences between the two groups. Differences among multiple groups were analyzed by one-way one-way ANOVA, followed by Least Significant Difference as the post-hoc test. Correlation analysis was conducted using GraphPad Prism 7 (La Jolla, CA, USA). $|R|>0.3$, $p<0.05$ was considered statistically significant.

Results

MicroRNA-374b Expression Was High in LC and Promoted Migratory and Proliferative Capacities of LC Cells

QRT-PCR was performed to detect the expression level of microRNA-374b in 40 cases of LC tissues and adjacent tissues. MicroRNA-374b was remarkably upregulated in LC tissues compared with adjacent tissues (Figure 1A). Meanwhile, the expression level of microRNA-374b was also higher in LC cells A549, H358, and H226 than normal human bronchial epithelial cells 16HBE (Figure 1B). Here, A549 and H358 cells were utilized for subsequent studies. Transfection of microRNA-374b inhibitor effectively downregulated microRNA-374b expression in A549 and H358 cells, showing a sufficient transfection efficacy (Figure 1C). We thereafter observed the regulatory effect of microRNA-374b on migratory and proliferative potentials of LC cells. Through wound healing assay, we found that microRNA-374b knockdown markedly decreased the migratory rate in A549 and H358 cells (Figure 1D). CCK-8 results showed that cell proliferation was also significantly inhibited after downregulation of microRNA-374b expression in A549 and H358 cells (Figure 1E).

MicroRNA-374b Targeted PTEN in Lung Cancer

PTEN expression in 40 cases of LC tissues and adjacent tissues was also determined by qRT-PCR, which was lowly expressed in tumor tissues (Figure 2A). Besides, PTEN expression was negatively correlated to microRNA-374b expression (Figure 2B). A potential binding site between microRNA-374b and PTEN was predicted by bioinformatics (Figure 2C). After constructing Wt PTEN 3'UTR and Mut PTEN 3'UTR, dual-luciferase reporter gene assay showed decreased luciferase activity in Wt PTEN 3'UTR group. However, no

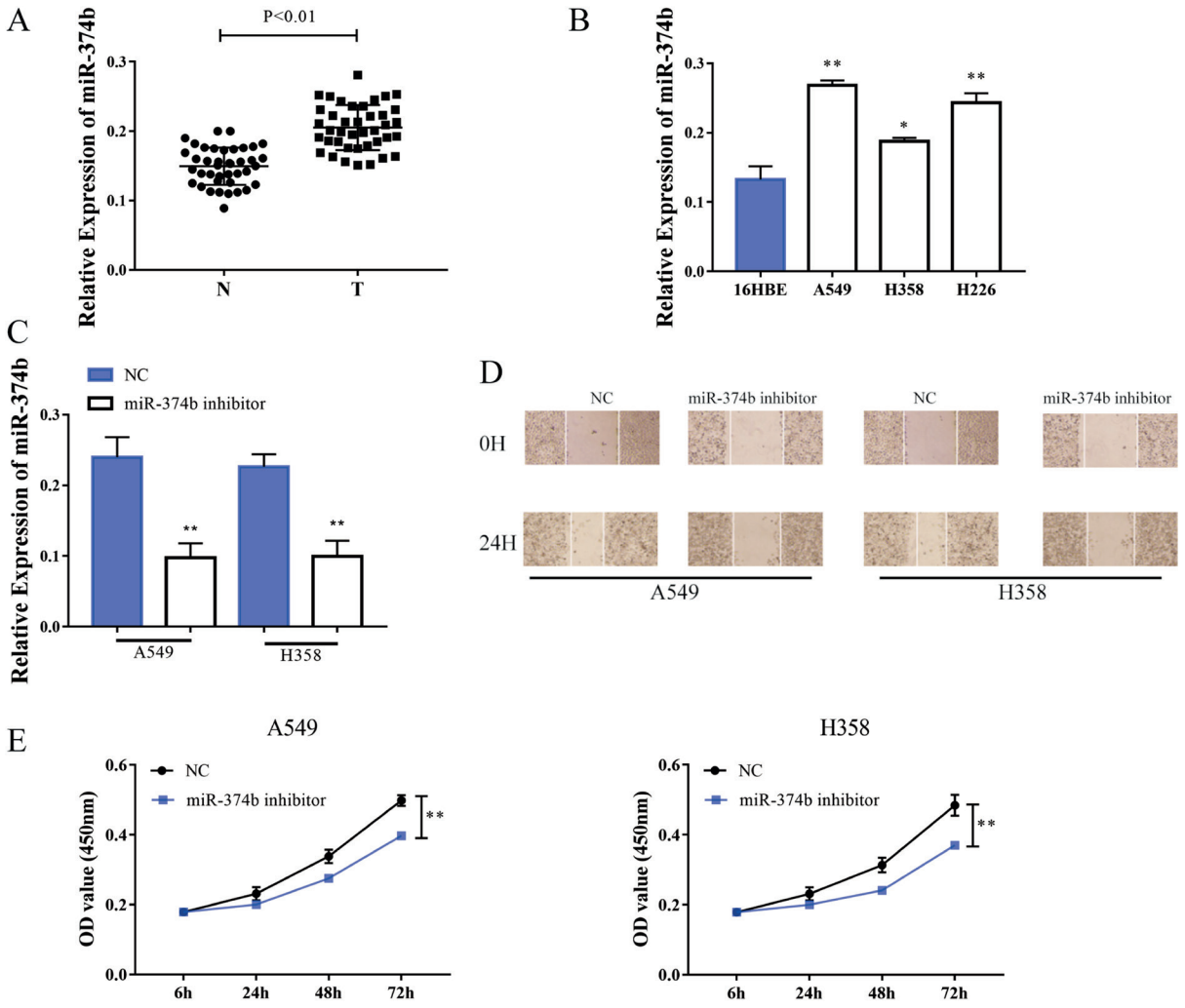

Figure 1. MiR-374b expression was high in lung cancer and promoted migratory and proliferative capacities of lung cancer cells. ***A,*** MiR-374b expression was remarkably upregulated in lung cancer tissues compared with adjacent tissues. B, The expression level of miR-374b was higher in lung cancer cells A549, H358, and H226 than normal human bronchial epithelial cells 16HBE. ***C,*** Transfection of miR-374b inhibitor effectively downregulated miR-374b expression in lung cancer cells. ***D,*** Wound healing assay indicated that miR-374b knockdown markedly decreased the migratory rate in A549 and H358 cells. ***E,*** CCK-8 results showed that cell proliferation was significantly inhibited after downregulation of miR-374b expression. **p*<0.05, ***p*<0.01.

significant change in luciferase activity was observed in Mut PTEN 3'UTR group (Figure 2D).

MicroRNA-374b Activated PI3K/Akt Pathway Through Downregulating PTEN Expression

We found that PTEN expression was upregulated in LC cells transfected with microRNA-374b inhibitor (Figure 3A). To further explore the potential role of PTEN in regulating behaviors of LC cells, si-PTEN was constructed and its transfection efficacy was verified. QRT-PCR data revealed a remarkable decrease in PTEN expression after transfection of si-PTEN in A549 and H358 cells (Figure 3B), while microRNA-374b expression was upregulated (Figure 3C). Furthermore, mRNA levels of PI3K and Akt decreased by the microRNA-374b knockdown. On the contrary, PTEN knockdown upregulated mRNA levels of PI3K and Akt, which were reversed by co-transfection of microRNA-374b and si-PTEN (Figure D). Similar results were obtained at their protein levels as Western blot indicated (Figure 3E). It is concluded that microRNA-374b exerted its function in regulating behaviors of LC cells by inhibiting PTEN expression via activating the PI3K/AKT pathway.

Downregulation of PTEN Promoted Migratory and Proliferative Capacities of LC Cells

To further investigate how microRNA-374b participated in influencing the development of LC, we tested the growth and metastasis of LC cells transfected with si-PTEN. Wound healing results showed that PTEN knockdown enhanced migratory ability, which was reversed by co-transfection of si-PTEN and microRNA-374b inhibitor (Figure 4A). Similarly, CCK-8 assay revealed that PTEN knockdown enhanced proliferative ability, which was reversed by co-transfection of si-PTEN and microRNA-374b inhibitor (Figure 4B).

Discussion

As the main type of cancer that causes death, LC seriously threatens human health. About 80% of LC cases pathologically belong to NSCLC19,20.

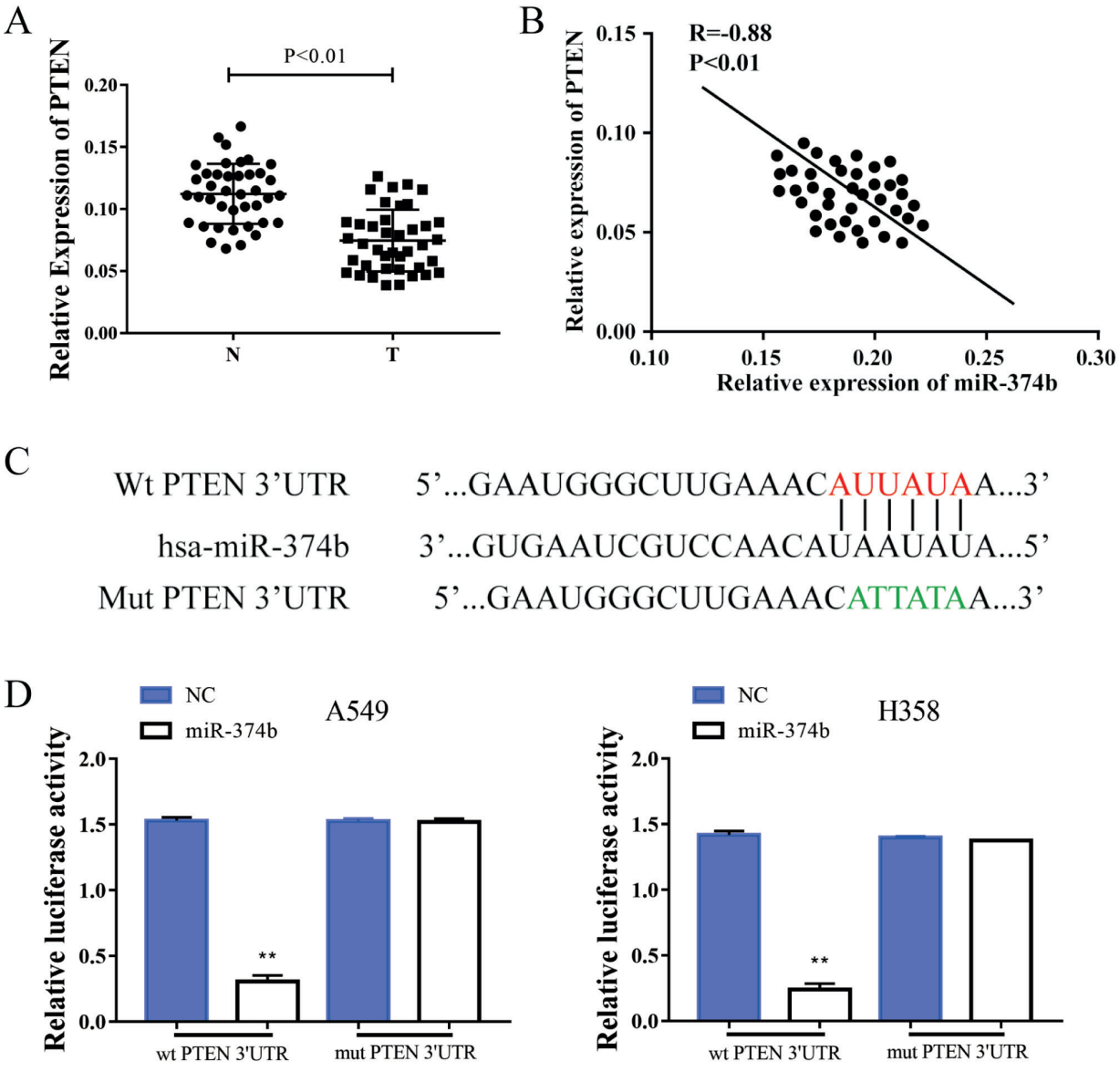

Figure 2. MiR-374b targeted PTEN in lung cancer. ***A,*** PTEN expression was remarkably downregulated in lung cancer tissues compared with adjacent tissues. B, PTEN expression was negatively correlated to miR-374b expression (R=-0.88, $p<0.01$). ***C,*** A potential binding site between miR-374b and PTEN. ***D,*** Dual-luciferase reporter gene assay confirmed that miR-374b could bind to PTEN. $^{**}p<0.01$.

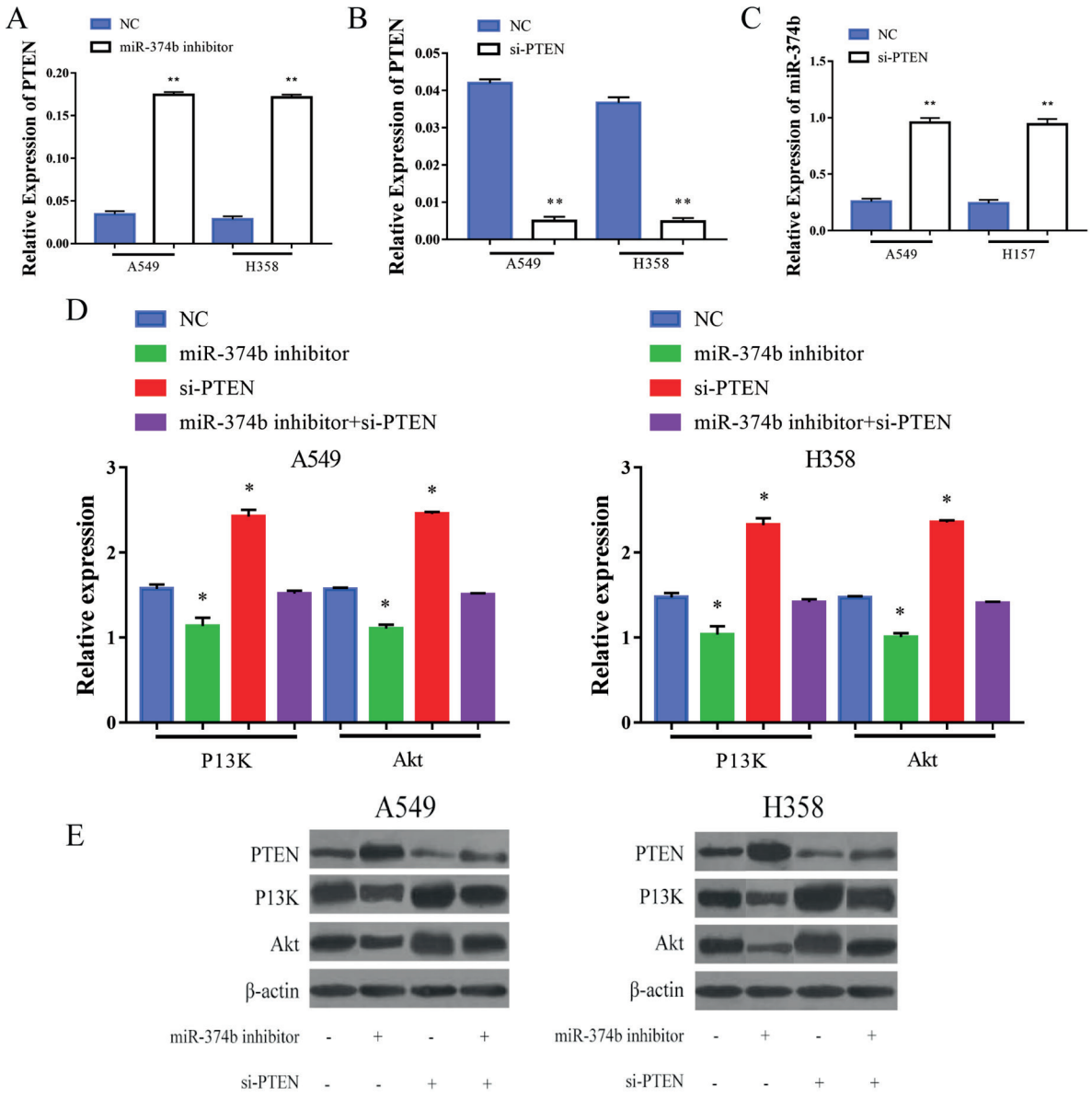

Figure 3. MiR-374b activated PI3K/Akt pathway through downregulating PTEN expression. ***A,*** PTEN expression was upregulated in lung cancer cells transfected with miR-374b inhibitor. ***B,*** Transfection of si-PTEN remarkably downregulated PTEN expression in A549 and H358 cells. ***C,*** Transfection of si-PTEN remarkably upregulated miR-374b expression in A549 and H358 cells. ***D,*** MiR-374b knockdown downregulated mRNA levels of PI3K and Akt. PTEN knockdown upregulated mRNA levels of PI3K and Akt, which were reversed by co-transfection of miR-374b and si-PTEN. ***E,*** MiR-374b knockdown downregulated protein levels of PI3K and Akt. PTEN knockdown upregulated protein levels of PI3K and Akt, which were reversed by co-transfection of miR-374b inhibitor and si-PTEN. *$p<0.05$, **$p<0.01$.

At present, many researchers have made great progresses in developing tumor markers and targeted therapy of NSCLC. However, NSCLC patients still face the predicament of poor prognosis and high recurrence rate. Some studies[21-23] on microRNAs bring hopes for NSCLC treatment. MicroRNA is a kind of relatively conservative non-coding, small RNA. It exerts various biological functions by regulating expressions of target genes. Differentially expressed microRNAs are very crucial in the occurrence and development of tumors, indicating that microRNAs can precisely prevent or accelerate the malignant progression of tumors based on their different functions.

As a tumor-suppressor gene that dephosphorylates lipids, PTEN is widely expressed in

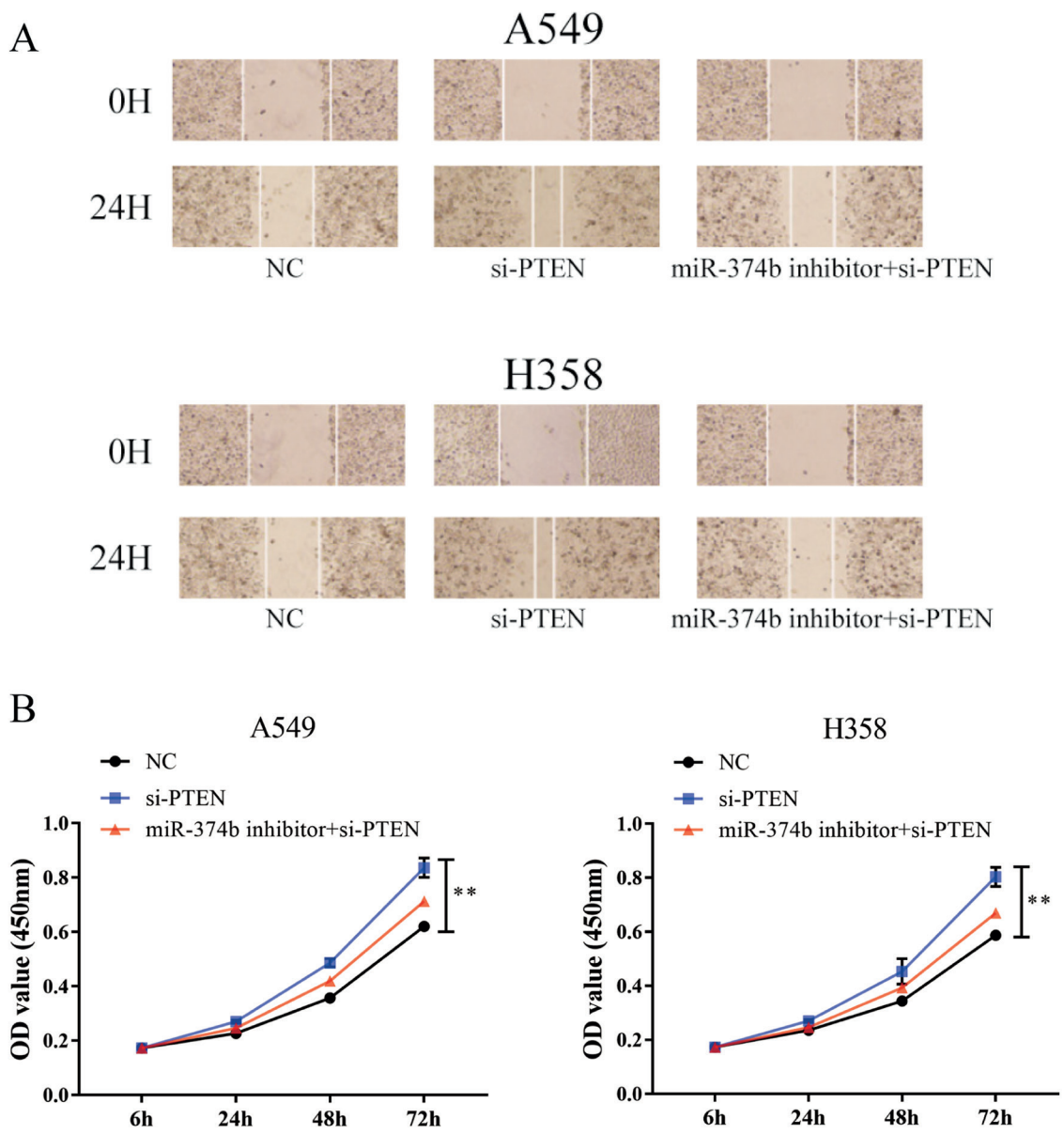

Figure 4. Downregulation of PTEN promoted migratory and proliferative capacities of lung cancer cells. ***A,*** Wound healing results showed that PTEN knockdown enhanced migratory ability, which was reversed by co-transfection of si-PTEN and miR-374b inhibitor. ***B,*** CCK-8 assay revealed that PTEN knockdown enhanced proliferative ability, which was reversed by co-transfection of si-PTEN and miR-374b inhibitor. $^{**}p<0.01$.

tissues and cells. The major function of PTEN is to antagonize PI3K, which is achieved by activating PI3K/Akt pathway. Hence, it is also known as PTEN-PI3K/Akt pathway. Abnormal activation of the PI3K/Akt pathway is thought to be important for mediating apoptosis escape, abnormal proliferation, and tumor angiogenesis of malignant tumor cells[24]. PI3K is an intracellular protein kinase that is involved in multiple cellular activities, such as proliferation and differentiation, apoptosis, and glucose molecule transport. PI3K also presents a close relationship with the occurrence of various human tumors[25]. Akt is one of the key downstream genes of PI3K. It is a highly conserved serine/threonine protein kinase composed of approximately 480 amino acid residues[26]. Activated PI3K stimulates Akt transfer from the cytoplasm to the cell membrane. The activated phosphorylated Akt catalyzes into the cytoplasm or nucleus, and subsequently, it leads to proliferation acceleration, anti-apoptosis, and chemotherapy tolerance through pho-

sphorylating a series of substrates[27]. Studies have found the crucial role of the PI3K/Akt pathway in the growth, proliferation, adhesion, and activation of adhesion molecules in liver cancer[28]. Activation of the PI3K/Akt pathway is closely related to the development of LC as well[29].

In this study, we found that microRNA-374b was highly expressed in LC tissues compared with adjacent tissues. Identically, the microRNA-374b expression also remained high in LC cell lines. Knockdown of microRNA-374b in LC cells attenuated migratory and proliferative capacities. Moreover, PTEN was found to be lowly expressed in lung cancer, which could bind to and was negatively regulated by microRNA-374b. Knockdown of microRNA-374b in LC cells upregulated PTEN expression, but inhibited PI3K/Akt pathway. On the contrary, PTEN knockdown upregulated microRNA-374b expression and activated PI3K/Akt pathway. More importantly, PTEN knockdown enhanced migratory and proliferative abilities of LC cells, which were attenuated by co-transfection of si-PTEN and microRNA-374b inhibitor. Thus, we believed that microRNA-374b may promote the development of LC by downregulating PTEN through activating PI3K/Akt pathway.

Conclusions

We showed that microRNA-374b promotes the development of LC by downregulating PTEN expression through activating PI3K/Akt pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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