

MiR-188 inhibits proliferation and promotes apoptosis of lung adenocarcinoma cells by targeting SIX1 to negatively regulate ERK signaling pathway

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Abstract. – OBJECTIVE: To explore the effects of micro ribonucleic acid (miR)-188 on proliferation and apoptosis of lung adenocarcinoma (LUAD) cells, and its potential mechanism.

MATERIALS AND METHODS: The expression level of miR-188 in LUAD cell lines was detected via quantitative Real Time-Polymerase Chain Reaction (PCR). The effects of miR-188 overexpression on proliferation and apoptosis of A549 cells were detected using methyl thiazolyl tetrazolium (MTT) assay, colony formation assay, and flow cytometry. The potential targets for miR-188 were predicted using the TargetScan Human database, and the interaction between miR-188 and target gene was determined through Dual-Luciferase reporter assay. Moreover, the associations of miR-188 and sine oculis homeobox homolog 1 (SIX1) with the extracellular signal-regulated kinase (ERK) pathway were detected via Western blotting.

RESULTS: The expression of miR-188 significantly declined in LUAD cell lines ($p < 0.05$). The overexpression of miR-188 significantly reduced the proliferation rate of A549 cells and increased the percentage of apoptotic A549 cells ($p < 0.05$). Similarly, it was found in colony formation assay that the overexpression of miR-188 inhibited the colony formation ability of A549 cells most significantly ($p < 0.05$). SIX1 was a direct target for miR-188, and its mRNA and protein expressions were downregulated by the overexpression of miR-188. The remarkable downregulation of phosphorylated ERK was observed in A549 cells overexpressing miR-188, while the decline in phosphorylated ERK was reversed in A549 cells overexpressing miR-188 and SIX1.

CONCLUSIONS: The expression of miR-188 is downregulated in LUAD cell lines. The overexpression of miR-188 inhibits proliferation and promotes apoptosis of LUAD cells, whose functional mechanism may be related to its regulation on the ERK signaling pathway by targeting SIX1.

Key Words:

Lung adenocarcinoma, MiR-188, SIX1 Gene, ERK signaling pathway, Proliferation.

Introduction

Lung cancer is still one of the most malignant tumors threatening human life in the world¹, and there are about 1.82 million new cases and 1.59 million deaths of lung cancer around the world². Lung cancer is mainly classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) according to the pathological type. NSCLC, accounting for 85% in lung cancer patients, is further divided into adenocarcinoma and squamous cell carcinoma, etc.³. Currently, lung adenocarcinoma (LUAD) is the most common subtype of lung cancer⁴. Despite the rapid development of early diagnostic techniques, surgical treatment, chemotherapy, radiotherapy, and targeted therapy, the prognosis of LUAD patients remains unsatisfactory, with a 5-year survival rate of 6%. LUAD patients often lack specific clinical symptoms and they are often diagnosed with advanced cancer, almost losing the treatment opportunities⁵. Therefore, determining new cancer-specific biomarkers for LUAD patients can contribute to prompt diagnosis and guide clinical treatment.

Micro ribonucleic acids (miRNAs) are non-coding endogenous RNAs with about 22 nucleotides in length, which can regulate the gene expression by inducing cleavage of target mRNA and inhibiting translation⁶. The binding of the 5' end of miRNA (seed region) to the 3' untranslated region (UTR) of mRNA will lead to mRNA instability, exerting the

miRNA targeting function⁷. The abnormal expression and dysfunction of miRNAs are involved in many processes of cancer cells, including differentiation, proliferation, development, death, and metabolism⁸. It is reported that miR-188 is significantly downregulated and acts as an inhibitor in many cancer types. Li et al⁹ studied the effect of miR-188 on glioma and explored its related mechanism and found that the expression of miR-188 is remarkably downregulated in glioma tissues and cell lines. Silencing β -catenin can reproduce the cellular and molecular effects under miR-188 overexpression, indicating that miR-188 inhibits the proliferation of glioma cells by targeting β -catenin, which is an effective therapeutic strategy for glioma. There are many reports evidencing that miR-188 serves as a key inhibitor during cancer development. However, the functional mechanism of miR-188 in LUAD remains unclear. Therefore, the present study aims to explore the potential role of miR-188 in LUAD.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, and fetal bovine serum (FBS; HyClone, South Logan, UT, USA), miRNeasy FFPE kit (QIAGEN, Hilden, Germany), miRNA first-strand complementary deoxyribose nucleic acid (cDNA) synthesis kit, SYBR Premix Ex TaqTM II kit, and PrimeScriptTM RT kit (TaKaRa, Otsu, Shiga, Japan), TRIzol reagent (Invitrogen, Carlsbad, CA, USA), horseradish peroxidase (HRP) substrate (Merck, Billerica, MA, USA), apoptosis assay kit (CWBIO, Beijing, China), Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), pcDNA-sine oculis homeobox homolog 1 (SIX1) expression plasmid and methyl thiazolyl tetrazolium (MTT) kit (Beyotime, Shanghai, China), psiCHECK-2 plasmid and Dual-Luciferase assay kit (Promega, Madison, WI, USA), SIX1, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphorylated (p)-extracellular signal-regulated kinase (ERK) antibodies (Cell Signaling Technology, Danvers, MA, USA), quantitative Real Time-Polymerase Chain Reaction (RT-qPCR) system (Applied Biosystems, Foster City, CA, USA), and flow cytometer (BD FACSCantoTM II, Franklin Lakes, NJ, USA).

Cell Culture

Human LUAD A549, PC9, and H1299 cells, and human bronchial epithelioid cells 16HBE

were purchased from the Cell Bank, the Chinese Academy of Sciences (Shanghai, China). LUAD cells were cultured in the DMEM supplemented with 10% FBS under 5% CO₂ at 37°C. 16HBE cells were cultured in the RPMI-1640 medium under the same conditions.

MiRNA Transfection

NC, miR-188 mimic, and miR-188 inhibitor were designed and supplied by RiboBio (Guangzhou, China). When 75% of cells were fused in the 6-well plate, they were transfected according to the instructions of Lipofectamine 3000. The transfection efficiency was detected *via* RT-qPCR.

RT-qPCR

In the detection of miR-188, the total RNA was isolated from cell lines using the miRNeasy FFPE kit, and reversely transcribed into cDNA. Then, the mature miRNAs were quantified using the SYBR Premix Ex TaqTM II kit on the ABI PRISM 7500 RT-PCR system, with U6 as an internal reference control. In the detection of SIX1 mRNA, the total RNA was extracted using the TRIzol reagent, and also reversely transcribed into cDNA using the PrimeScriptTM RT kit, followed by RT-qPCR on the same system using the SYBR Premix Ex TaqTM II kit. GAPDH was amplified as the internal reference control. The RT-qPCR conditions for miRNA and mRNA detection are as follows: 95°C for 30 s, 95°C for 5 s, and 60°C for 35 s (a total of 40 cycles). The primer sequences are as follows: miR-188 F: 5'-CACATCCCTTGCATGGTGGAG-3', R: 5'-GCAAACCCTGCATGTGGGAG-3'. U6 F: 5'-CTCGCTTCGGCAGCA-CA-3', R: 5'-ACGCTTCACGAATTTGCGT-3'. SIX1 F: 5'-AAGGAGAAGTCGAGGGGTGT-3', R: 5'-TGCTTGTGGAGGAGGAGTT-3'. GAPDH F: 5'-CTGGGCTACACTGAGCACC-3', R: 5'-AAGTGGTCGCGTTGAGGGCAATG-3'. The relative expressions of miRNA and mRNA to U6 and GAPDH were calculated using the 2^{- $\Delta\Delta C_t$} method.

Cell Proliferation Analysis

After transfection with miR-188 mimic or NC for 24 h, A549 cells were inoculated into a 96-well plate (2×10³ cells/well) and cultured for 24 h, 48 h, and 72 h. Then, 15 μ L of MTT dye was added into each well for incubation under 5% CO₂ at 37°C for 3 h. 100 μ L of dimethyl sulfoxide (DMSO) was added into each well, and the absorbance was measured at 570 nm using a spectrophotometer. The clone ability was evaluated using the colony

formation assay: after transfection for 24 h, A549 cells were inoculated into the 6-well plate (300 cells/well), and the culture medium was replaced every 4 d for about 2 weeks. Finally, the formed colonies were fixed, stained, and counted.

Apoptosis Analysis

A549 cells transfected for 48 h were suspended using binding buffer at a density of 1×10^6 cells/mL, added with Annexin V/FITC (fluorescein isothiocyanate) and propidium iodide (PI) and incubated at room temperature in the dark for 15 min. Then, the apoptotic cells were analyzed using the flow cytometer.

Western Blotting

After transfection for 72 h, A549 cells were collected and lysed on ice to extract the total protein. After separation *via* 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protein was transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), sealed with 5% bovine serum albumin for 1 h, incubated with the primary antibody at 4°C overnight and with the secondary antibody again at room temperature for 1 h, with GAPDH as the internal reference. Finally, the protein was visualized *via* enhanced chemiluminescence (ECL) using the horse radish peroxidase (HRP) substrate.

Luciferase Activity Assay

First, the wild-type and mutant-type fragments of SIX1 containing the putative binding sites of miR-188 were synthesized and integrated into psiCHECK-2 Dual-Luciferase vectors, forming the psiCHECK-2-SIX1-3'-UTR-wild type (SIX1-wt) or psiCHECK-2-SIX1-3'-UTR-wild type (SIX1-mut) reporter vectors. Then, A549 cells (1×10^5 cells/plate) were co-transfected with the Luciferase reporter plasmids and miR-188 mimic or miR-NC. After transfection for 48 h, firefly Luciferase activity was measured and normalized into the Renilla signal.

Statistical Analysis

At least three independent repeated experiments were performed. All data were expressed as mean $\pm$ standard deviation using GraphPad Prism 5 software (La Jolla, CA, USA), and the intergroup differences were calculated using Chi-square test or One-way analysis of variance followed by post-hoc test (Least Significant Difference). $p < 0.05$ suggested the statistically significant difference.

Results

Expression of MiR-188 was Downregulated in LUAD Cell Lines

To clarify the expression level of miR-188 in LUAD, the relative expression level of miR-188 in human LUAD cells (A549, PC9, and H1299) to that in human bronchial epithelioid cells (16HBE) was analyzed *via* RT-qPCR. It was found that the expression of miR-188 was significantly downregulated in human LUAD cell lines (A549, PC9, and H1299) compared with that in human bronchial epithelioid cells (16HBE) (Figure 1, $p < 0.05$), indicating that the decline in miR-188 expression may be closely related to the occurrence and development of LUAD.

Effect of MiR-188 Overexpression on Proliferation of A549 Cells

Based on the miR-188 expression in LUAD cell lines, A549 cell lines with a relatively lower expression of miR-188 were selected for further study. A549 cells were transfected with miR-188 mimic or miR-NC. The transfection efficiency was first detected *via* RT-qPCR, and it was found that transfection with miR-188 mimic significantly raised the endogenous miR-188 expression compared with transfection with miR-NC (Figure 2A, $p < 0.05$). Besides, the cell proliferation was determined using MTT assay, and the results showed that the overexpression of miR-188 significantly reduced the proliferation rate of A549 cells (Figure 2B, $p < 0.05$). The effect of miR-188 overexpression on the colony formation of A549 cells was further detected using colony formation

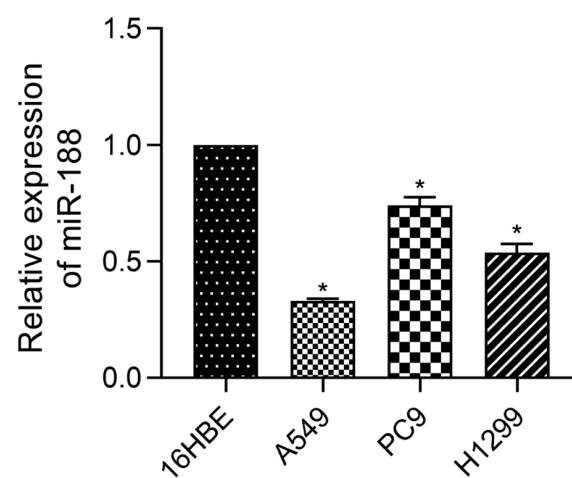


Figure 1. Expression level of miR-188 in LUAD cells analyzed *via* RT-qPCR (* $p < 0.05$).

assay. Similarly, it was observed that miR-188 overexpression inhibited the colony formation ability of A549 cells most significantly (Figure 2C, $p<0.05$).

Effect of MiR-188 Overexpression on Apoptosis of A549 Cells

The effect of miR-188 overexpression on apoptosis of A549 cells was determined using flow cytometry. The results revealed that the overexpression of miR-188 could evidently increase the percentage of apoptotic A549 cells (Figure 3, $p<0.05$).

SIX1 was the Direct Target for MiR-188

To clarify the molecular mechanism of miR-188 in regulating proliferation and apoptosis of LUAD cells, the potential targets for miR-188 were predicted using TargetScan Human 7.2 database. It was found that miR-188 had binding sites to the SIX1 3'-UTR (Figure 4A). Then, the predicted results were confirmed via Dual-Luciferase reporter assay. The cells were co-transfected

with SIX1-wt and SIX1-mut and miR-188 mimic or miR-NC, and the Luciferase activity was determined. The results manifested that the Luciferase activity of the cells co-transfected with miR-188 mimic and SIX1-wt evidently declined compared with that of the cells co-transfected with miR-NC and SIX1-wt (Figure 4B, $p<0.05$). However, the Luciferase activity had almost no change among cells transfected with miR-188 mimic and miR-NC in the case of mutation of binding sites of SIX1 3'-UTR (Figure 4B). Furthermore, the effects of miR-188 overexpression on the mRNA and protein expressions of SIX1 in A549 cells were verified. The results showed that the overexpression of miR-188 reduced the mRNA and protein expressions of SIX1 (Figure 4C and 4D).

Associations of MiR-188 and SIX1 with ERK Pathway

The phosphorylation of ERK protein in A549 cells overexpressing miR-188 was detected through Western blotting. The significant down-

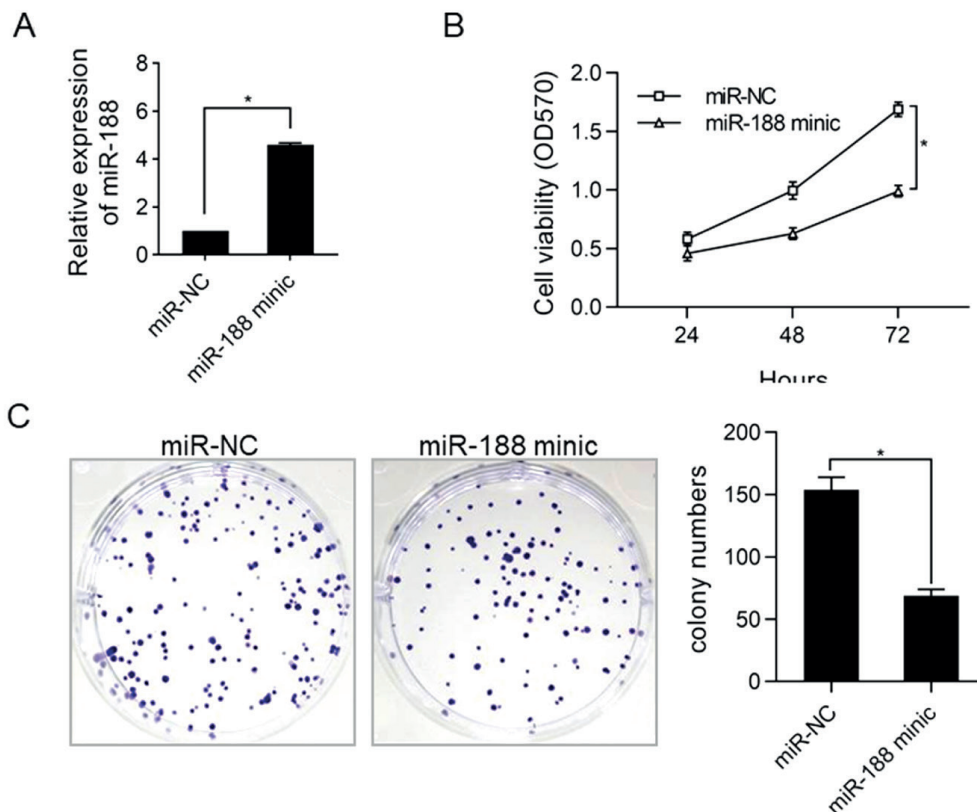


Figure 2. Effect of miR-188 overexpression on proliferation of A549 cells. **A**, The overexpression effect of miR-188 is detected via RT-qPCR. **B**, The effect of miR-188 overexpression on the proliferation of A549 cells is detected via MTT assay. **C**, The effect of miR-188 overexpression on the colony formation ability of A549 cells is detected via colony formation assay (magnification: 40×) (* $p<0.05$).

regulation of p-ERK was observed in A549 cells overexpressing miR-188 (Figure 5A), suggesting that the expression of miR-188 is related to the ERK pathway. Then, whether the regulation of miR-188 on the ERK pathway is mediated by SIX1 was further explored. The results showed that the decline in p-ERK was reversed in A549 cells overexpressing miR-188 and SIX1 (Figure 5B), which demonstrates that miR-188 can regulate the ERK signaling pathway by targeting SIX1.

Discussion

Lung cancer has a high mortality rate mainly due to the strong ability of invasion and metastasis¹⁰. With the improvement of surgery and treatment means, its mortality rate is decreasing, but its 5-year survival rate is still only about 18%¹¹. LUAD is one of the subtypes of NSCLC, and the targeted molecular therapy is a new treatment method for LUAD, which can improve the efficacy and prognosis of patients.

Over the past two decades, miRNAs have been considered as key regulators of various biological processes, including cell differentiation, growth, and apoptosis⁷. Dysregulation of miRNAs in cells has been reported in many human cancers. MiR-188, located on the human X chromosome, was first discovered in 2003¹², which acts as a tumor suppressor in various cancers, including hepatocellular carcinoma, oral squamous cell carcinoma, and prostate cancer^{9,13}. Downregulation of miR-188-5p is an independent factor for the poor over-

all and recurrence-free survival of prostate cancer patients¹³. A unique miRNA molecular profile of miR-188 in the diagnosis and prognosis of lung cancer was found by Yanaihara et al¹⁴. According to previous studies, miR-188 has displayed its potential predictive role and therapeutic potential in cancer therapy. However, the molecular function of miR-188 in lung cancer is poorly understood. In this study, the expression level of miR-188 in LUAD was first clarified, and it was found that miR-188 was downregulated in LUAD cell lines. Then, the effects of overexpression of miR-188 on the proliferation and apoptosis of LUAD cells were detected. The results revealed that the overexpression of miR-188 inhibited proliferation and promoted apoptosis of A549 cells.

The SIX family first discovered in *Drosophila melanogaster* is evolutionarily conserved in *Drosophila melanogaster* and human. SIX1, an important member of the SIX family, is also highly conserved in many organisms¹⁵, which plays an important role in the early development of many tissues according to reports. For example, SIX1 maintains neurodevelopment by facilitating proliferation of neuroblast precursors in the optic neural ectoderm¹⁶. SIX1 is also well-known as an oncogene overexpressed in many types of cancer, and the high expression of SIX1 has an association with the poor prognosis of patients. In addition to its important role in the tumor growth, SIX1 is also widely involved in regulating the occurrence and development of tumor. For example, SIX1 can regulate proliferation, invasion, epithelial-mesenchymal transition, and apoptosis¹⁷. In this study, in order to clarify the molecular

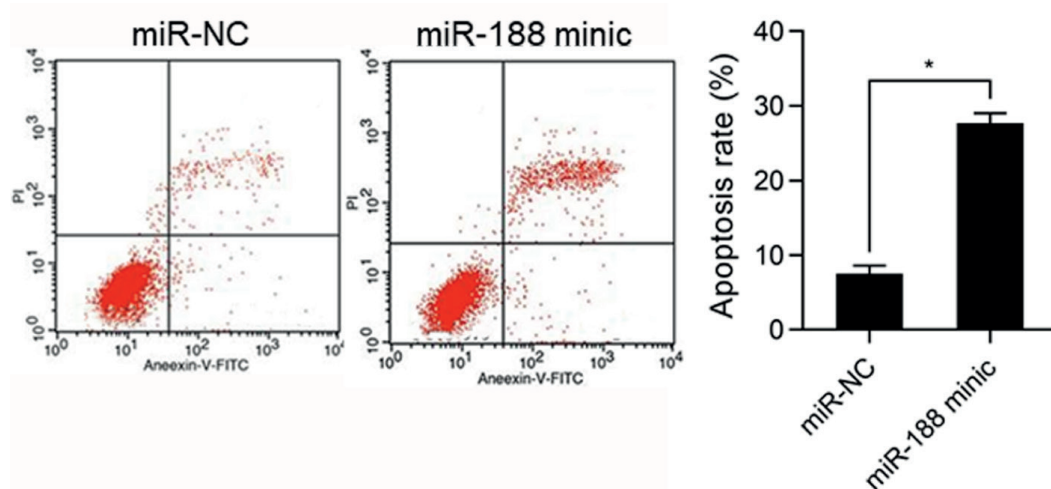


Figure 3. Effect of miR-188 overexpression on apoptosis of A549 cells determined using flow cytometry (* $p < 0.05$).

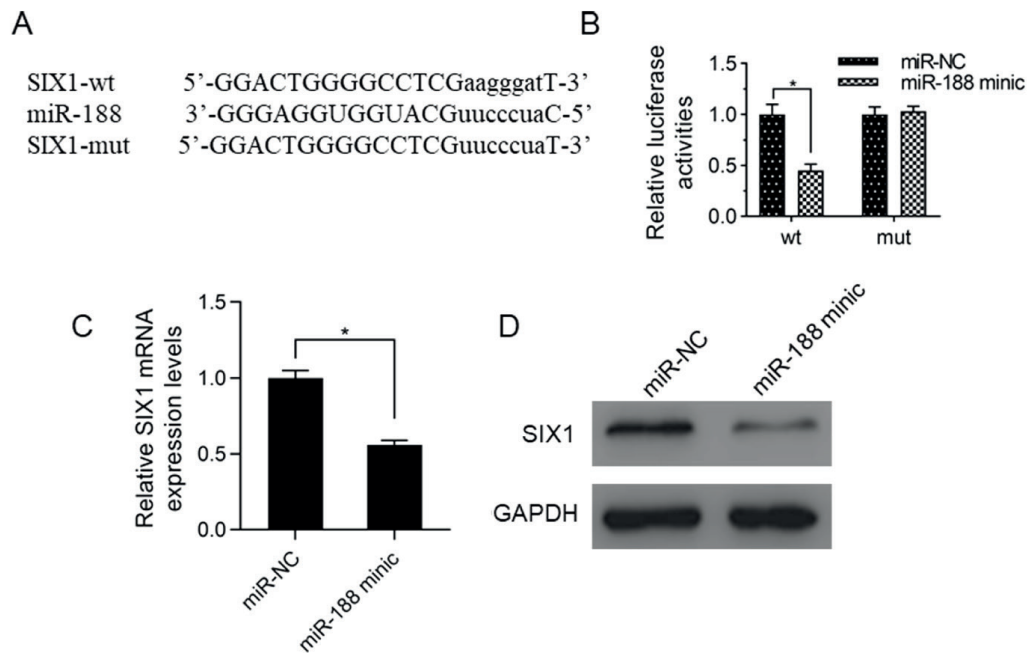


Figure 4. SIX1 is a direct target for miR-188. **A**, Binding sites between miR-188 and SIX1 3'-UTR predicted. **B**, The interaction between miR-188 and SIX1 is determined via Luciferase reporter assay. **C**, and **D**, The effects of miR-188 overexpression on the mRNA and protein expressions of SIX1 are determined using RT-qPCR and Western blotting. (* $p < 0.05$).

mechanism of miR-188 in regulating proliferation and apoptosis of LUAD cells, it was predicted and confirmed that SIX1 was a target for miR-188.

It is reported in many studies that the down-regulation of SIX1 remarkably weakens the proliferation of cancer cells, including prostate cancer, oral squamous cell carcinoma, NSCLC, and hepatocellular carcinoma cells. SIX1 serves as a transcription factor regulating cell cycle, promoting cell proliferation and migration, and in-

hibiting apoptosis¹⁸. Moreover, SIX1 protein can promote proliferation of progenitor cells before cell differentiation, thus playing a crucial role in the development and differentiation of many tissues and organs¹⁹. According to reports, there is an association between SIX1 and ERK. In this study, the correlations of miR-188 and SIX1 with the ERK pathway were explored. As a result, the remarkable downregulation of p-ERK was observed in A549 cells overexpressing miR-188,

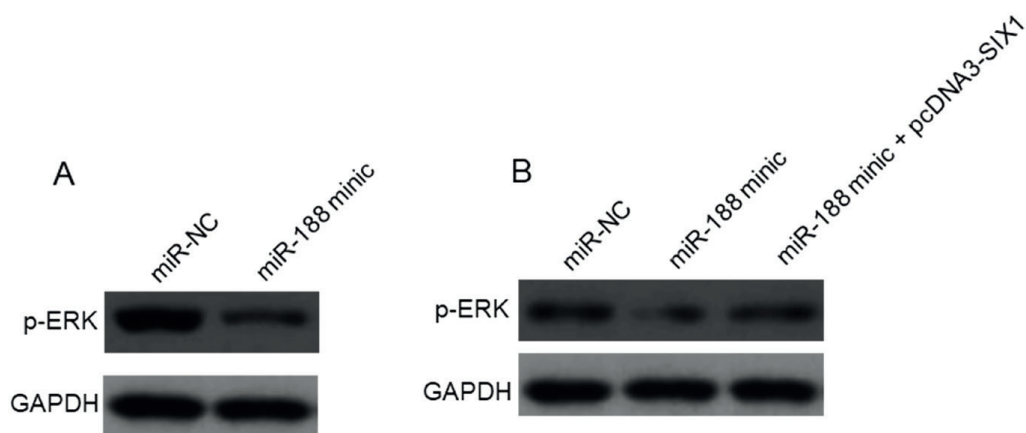


Figure 5. Associations of miR-188 and SIX1 with ERK pathway. **A**, The phosphorylation of ERK protein in A549 cells overexpressing miR-188 is detected through Western blotting. **B**, The phosphorylation of ERK protein in A549 cells overexpressing miR-188 and SIX1 is detected through Western blotting.

while the decline in p-ERK was reversed in A549 cells overexpressing miR-188 and SIX1, which demonstrates that miR-188 can regulate the ERK signaling pathway by targeting SIX1.

Conclusions

The expression of miR-188 is downregulated in LUAD cell lines. The overexpression of miR-188 inhibits proliferation and promotes apoptosis of LUAD cells, whose functional mechanism may be related to its regulation on the ERK signaling pathway by targeting SIX1.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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