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MiR-376b-3p functions as a tumor suppressor by targeting KLF15 in non-small cell lung cancer

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Abstract. – OBJECTIVE: The aim of this study was to explore the regulatory effects of micro ribonucleic acid (miR)-376b-3p on proliferation and apoptosis of non-small cell lung cancer (NSCLC) cells by targeting Kruppel-like factor 15 (KLF15) and its mechanism of action.

PATIENTS AND METHODS: The expression of miR-376b-3p in NSCLC and para-carcinoma normal tissues, as well as NSCLC cell lines, was detected via quantitative Polymerase Chain Reaction (qPCR). The effects of miR-548-3p on the proliferation, cycle distribution, and apoptosis of NSCLC cells were detected *via* colony formation assay, flow cytometry, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, respectively. The interaction between miR-376b-3p and KLF15 was determined using Dual-Luciferase reporter gene assay. *In vivo* tumorigenic ability of NSCLC cells was studied using nude mouse tumorigenicity assay. Furthermore, the expression of Ki67 in tumor in nude mice was detected via immunohistochemistry.

RESULTS: The expression of miR-376b-3p was significantly downregulated in NSCLC tissues when compared with para-carcinoma normal tissues ($p<0.05$). MiR-376b-3p was lowly expressed in NSCLC cells as well ($p<0.05$). After overexpression of miR-376b-3p, the proliferation ability of NSCLC cells remarkably declined ($p<0.05$). The apoptosis rate rose, and cell cycle was arrested in the G1/G0 phase. Dual-Luciferase reporter gene assay confirmed that miR-376b-3p could specifically bind to KLF15 3'UTR to regulate the expression activity of KLF15. After overexpression of miR-376b-3p, tumor volume and weight were significantly reduced in tumor-bearing mice ($p<0.05$).

CONCLUSIONS: MiR-376b-3p plays an important role in the occurrence and development of NSCLC, which affects the proliferation and apoptosis of NSCLC cells by targeting KLF15.

Key Words:
Non-small cell lung cancer (NSCLC), MiR-376b-3p, Proliferation, Apoptosis, KLF15.

Introduction

Lung cancer is one of the most common causes of cancer-related morbidity and mortality rates around the world[1]. It can be generally divided into two types, including: small cell lung cancer (SCLC, about 20%) and non-small cell lung cancer (NSCLC, about 80%)[2]. Currently, the morbidity rate of NSCLC continuously declines. However, the 5-year survival rate of NSCLC patients is still far from satisfactory due to the lack of early detection methods[3]. Therefore, the exploration of the occurrence and development of NSCLC is of great significance in accurate diagnosis and treatment strategies of lung cancer patients.

Micro ribonucleic acids (miRNAs) are a class of endogenous small non-coding RNAs with 18-25 nucleotides discovered in eukaryotes. Their aberrant expression may lead to messenger RNA (mRNA) degradation or translational inhibition of functional protein[4]. MiRNAs play important roles in regulating cell development, differentiation, proliferation, metastasis, invasion, and apoptosis[5,6]. Therefore, abnormal changes in miRNAs are critical for the development of diseases, including cancer. MiR-15a, by regulating the AKT signaling pathway, promotes the proliferation and inhibits apoptosis of papillary thyroid carcinoma[7]. Meanwhile, miR-221-5p promotes invasion and metastasis of breast cancer *via* regulating the expression of E-cadherin[8].

Abnormal expression of miR-376b-3p induces the occurrence of malignant tumors, such as gastric cancer, kidney cancer, glioma, and prostate cancer[9-12]. However, the biological function and molecular mechanism of miR-376b-3p in NSCLC remain unclear. In this study, *in vitro* and *in vivo* experiments demonstrated that miR-376b-3p was downregulated in NSCLC tissues and cells. Furthermore, the overexpression of miR-376b-3p significantly in-

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hibited proliferation and promoted apoptosis of NSCLC cells.

Patients and Methods

Tissue Samples

A total of 46 pairs of NSCLC tissues and para-carcinoma tissues were obtained from patients diagnosed with NSCLC in Linyi People's Hospital in 2016. After excision, the collected tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Signed written informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of Linyi People's Hospital. All collected samples were classified according to the classification criteria of the World Health Organization.

Experimental Reagents and Instruments

Lipofectamine 2000 and miR-376b-3p mimics were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China), TRIzol from Ambion (Austin, TX, USA), Kruppel-like factor 15 (KLF15) antibody from Abcam (Cambridge, MA, USA), Polymerase Chain Reaction (PCR) kit from TaKaRa (Otsu, Shiga, Japan), Luciferase activity assay kit from Promega (Madison, WI, USA), and the Luciferase reporter vector was synthesized by Promega (Madison, WI, USA).

Cell Culture

H1299, H466, A549, H1650, and 16HBE cell lines were obtained from the cell bank of China Center for Type Culture Collection (Shanghai, China), the Chinese Academy of Sciences. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 Ul/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in an incubator with 5% CO_2 at 37°C.

RNA Extraction and Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was isolated from NSCLC tissues or cells using TRIzol reagent. Subsequently, extracted RNA was synthesized into cDNA using reverse transcriptase (TaKaRa, Otsu, Shiga, Japan) and oligonucleotide (dT) according to the manufacturer's instructions. QRT-PCR was performed using the SYBR Premix Ex Taq™ II kit under the following conditions: 94°C for 10 s, 94°C for 5 s, annealing at 52°C for 30 s, 72°C for 15 s, for a total of 40 cycles. The primers used in this experiment were as follows: miR-376b-3p F 5'-GGGATGAGCGAGATGGCGA-3', R 5'-GGATGCGGATGGGAGCGGGAC-3', U6 F 5'-CATC ACCATCAGGAGAGTCG-3', R 5'-TGACGCTTGCCCACAGCCTT-3', KLF15 F 5'-TCTCGC TGGAGGCCAGG-3', R 5'-TCCATCACCCGGCAGGA-3'.

Colony Formation Assay

The cells were first inoculated into a 60 mm cell culture dish (1×10^3 cells/mL). After 10 d of culture, the cells were stained with crystal violet dye. Colonies containing ≥20 cells were counted. The difference in the number of cell clones was detected, and representative colonies were photographed and counted under an inverted microscope. This experiment was repeated for 3 times.

Cell Cycle

Transfected cells were first collected from each group. Subsequently, the cells were fixed with 70% cold ethanol at 4°C overnight and stained with propidium iodide (0.05 g/L) and RNase (2 g/L) at room temperature for 30 min. Flow cytometry was performed to analyze cell cycle. The percentage of cells in different cell cycles was finally calculated using the Cell Lab Quanta SC software (LabX, Midland, ON, Canada).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL)

The cells were treated as described above, followed by staining, according to the instructions of TUNEL kit (Beyotime, Shanghai, China) and then, photographed.

Dual-Luciferase Reporter Gene Assay

The potential targets for miR-376b-3p were predicted and analyzed using the bioinformatics method. The 3' untranslated region (3'UTR) of KLF15 was similar to the binding site of miR-376b-3p, suggesting that KLF15 might be a potential target for miR-376b-3p. Human KLF15 3'UTR-Wild sequence or KLF15 3'UTR-Mut sequence with predicted target site was inserted into the pGL3 promoter vector. At 1 d before transfection, A549 cells were inoculated into 24-well plates at a density of 5×10^5 cells/well. Then, the cells were co-transfected with 0.12 μg of Luciferase reporter vector and 40 nm of

miR-376b-3p mimics or negative control mimics. Luciferase activity was detected using the Dual-Luciferase reporter gene assay kit after transfection for 48 h.

Tumorigenicity in Nude Mice

A549 cells stably transfected with miR-376b-3p mimics or negative control miRNA (2×10^6 cells/nude mouse) were subcutaneously inoculated into female nude mice aged 4 weeks old. 4 mice were included in each experimental group. Tumor volume was measured using a caliper every 3 d for a total of 21 d and calculated as follows: $V = (L\times W^2)/2$ (V: volume; L: length; W: width of tumor). After 21 d, the mice were sacrificed. Finally, the tumor tissues were immediately frozen in liquid nitrogen and stored at -80°C for KLF15 expression detection. This study was approved by the Animal Ethics Committee of Linyi People's Hospital Animal Center.

Immunohistochemistry

Tumor tissues were first fixed in 4% formalin and embedded in paraffin. After endogenous peroxides and proteins were blocked, 4 μm-thick sections were incubated with the primary antibody at 4°C overnight, followed by specific detection of Ki67. After washing with phosphate-buffered saline (PBS), the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 37°C for 1 h. Next, the sections were stained with 3,3'-diaminobenzidine solution for 3 min. Cell nuclei were finally counterstained with hematoxylin.

Western Blotting

NSCLC cells in logarithmic growth phase were inoculated into 6-well plates at a density of 1×10^5 cells/mL. After transfection for 48 h, cells in each group were collected and added with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) to extract total protein. 30 μg of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with 5% skimmed milk, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with corresponding HRP-conjugated anti-rabbit secondary antibody (1:2000) at 37°C for 1 h. Immuno-reactive bands were finally exposed by the enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Experimental data were expressed as mean ± standard deviation. Each experiment was repeated for at least three times. The differences between groups were compared using analysis of variance or two-way *t*-test. $p<0.05$ (*) was considered statistically significant.

Results

MiR-376b-3p was Downregulated in NSCLC

A total of 46 pairs of NSCLC tissues and para-carcinoma tissues were collected, and total RNA was extracted. The relative expression of miR-376b-3p was detected *via* qRT-PCR. The results revealed that miR-376b-3p expression was significantly downregulated in NSCLC tissues when compared with para-carcinoma tissues (Figure 1A). The expression of miR-376b-3p in NSCLC cells was detected by qRT-PCR as well. It was found that was lowly expressed in NSCLC cells (Figure 1B). Subsequently, miR-376b-3p overexpression mimics were designed, synthesized, and transfected into NSCLC cells. Transfection efficiency was verified using qRT-PCR (Figure 1C and 1D).

MiR-376b-3p Inhibited Proliferation and Promoted Apoptosis of NSCLC Cells

To study the biological function of miR-376b-3p *in vitro*, miR-376b-3p was overexpressed in NSCLC cells. Colony formation assay indicated that the proliferation ability of NSCLC cells was significantly inhibited after miR-376b-3p overexpression (Figure 2A and 2B). Subsequent flow cytometry showed that cell cycle in the experimental group was arrested in the G1/G0 phase compared with that in the control group (Figure 2C and 2D). Meanwhile, TUNEL assay demonstrated that the apoptosis rate rose significantly after overexpression of miR-376b-3p (Figure 2E and 2F).

MiR-376b-3p Targeted the Expression of KLF15

The possible target genes for miR-376b-3p were predicted using bioinformatics (http://www.mirdb.org/) (Figure 3A). It was verified *via* qRT-PCR that miR-376b-3p could regulate the expression of KLF15 (Figure 3B). Western blotting manifested that KLF15 was significantly down-

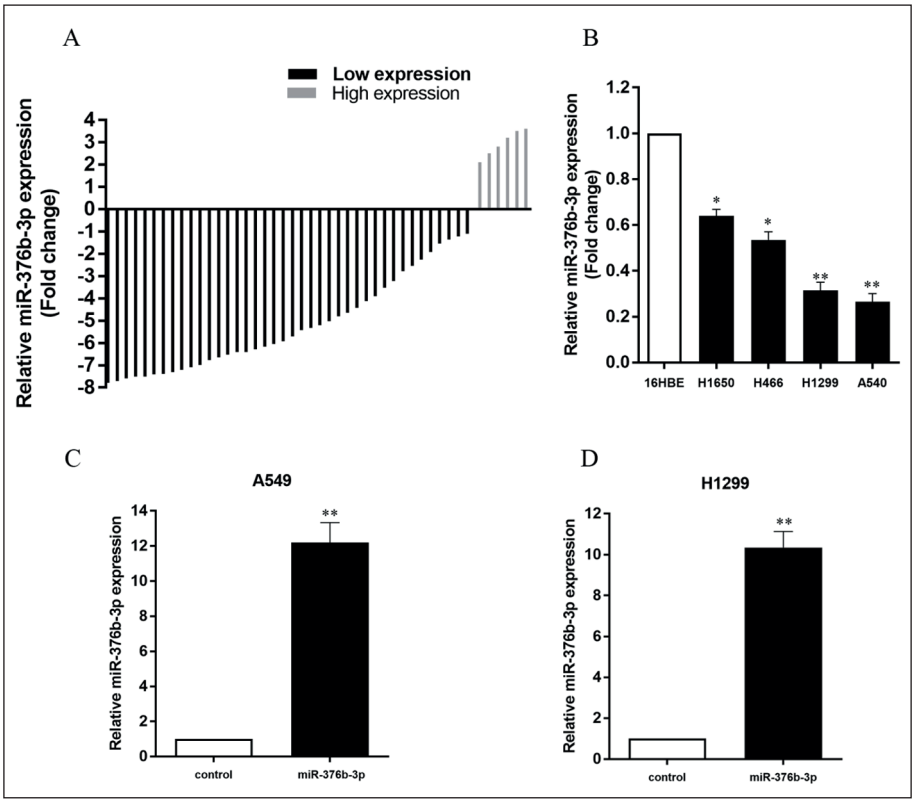

Figure 1. MiR-376b-3p is downregulated in NSCLC tissues and cells. **A**, The relative expression of miR-376b-3p in 46 cases of NSCLC tissues is detected via qRT-PCR. The results reveal that the expression of miR-376b-3p is down-regulated in NSCLC tissues when compared with para-carcinoma tissues. **B**, The relative expression of miR-376b-3p in NSCLC cells is detected using qRT-PCR. **C**, and **D**, After overexpression of miR-376b-3p, overexpression efficiency is determined using qRT-PCR.

regulated at the protein level after overexpression of miR-376b-3p (Figure 3C). Furthermore, Dual-Luciferase reporter assay illustrated that miR-376b-3p could target the expression of KLF15 (Figure 3D).

MiR-376b-3p Suppressed In Vivo Tumorigenic Ability of NSCLC Cells

Nude mouse transplanted tumor model was constructed to investigate the function of miR-376b-3p *in vivo*. 4-week-old nude mice were selected as subjects, and miR-376b-3p mimics were transfected into NSCLC cells and subcutaneously injected into nude mice using the syringe. After 21 d, nude mice were all sacrificed. Next, transplanted tumor was taken, weighed (Figure 4A) and photographed (Figure 4B). In addition, the expression of miR-376b-3p in transplanted tumor was determined using qRT-PCR (Figure 4C). The results manifested that after overexpression of miR-376b-3p, *in vivo* tumorigenic ability of NSCLC cells significantly declined. Immunohistochemistry showed that the expression of Ki67 declined in the cells transfected with miR-376b-3p mimics when compared with that in cells transfected with negative control, indicating remarkably inhibited proliferation ability (Figure 4D).

Discussion

Lung cancer is still one of the most common malignant tumors in the world. Meanwhile, it is still a major cause of cancer-related deaths in developing countries, especially China[13]. Current treatment means are limited for advanced NSCLC, and the therapeutic effect is far from satisfactory. It is extremely necessary to improve the early diagnosis rate and therapeutic effect of NSCLC patients. Therefore, the exploration of novel biomarkers and new therapeutic targets for NSCLC has become research hotspots.

Abnormal expression of miRNAs has been found associated with the development of malignant tumors, including cell proliferation and apoptosis[14]. There are three members in miR-376 family: miR-376a (-3p and -5p), miR-376b (-3p and -5p), and miR-376c (-3p and -5p). Sun et al[15] and Wang et al[16] have demonstrated that

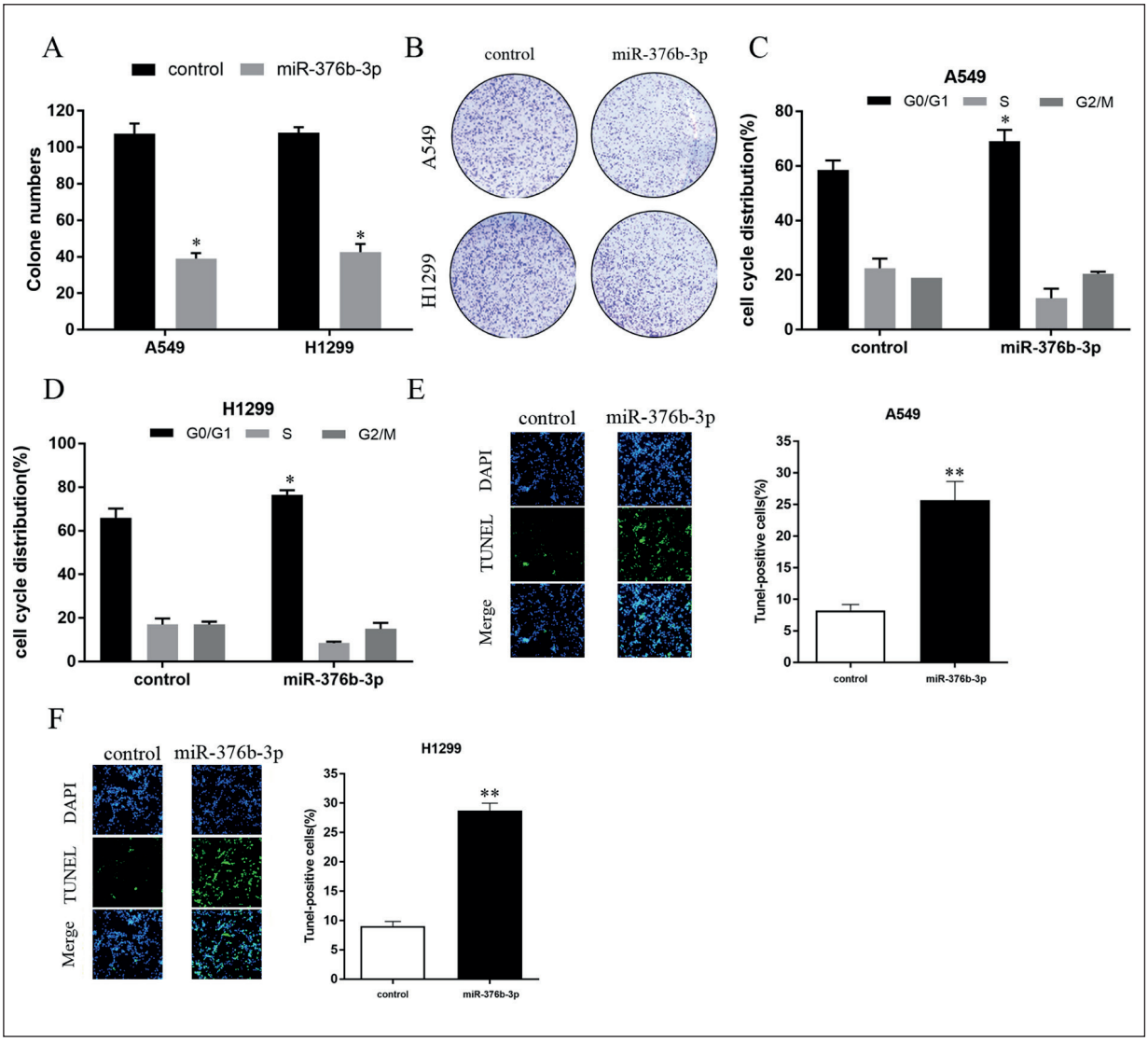

Figure 2. MiR-376b-3p inhibits proliferation and promotes apoptosis of NSCLC cells. **A**, and **B**, The results of colony formation assay show that the proliferation ability of NSCLC cells is suppressed after overexpression of miR-376b-3p (magnification: 40×). **C**, and **D**, The results of flow cytometry manifest that NSCLC cell cycle is arrested in G1/G0 phase after overexpression of miR-376b-3p. **E**, and **F**, The results of TUNEL assay reveal that the apoptosis rate rises after overexpression of miR-376b-3p (magnification: 400×).

these members can be involved in many biological processes, such as autophagy, red blood cell differentiation, and formation of malignant tumor phenotype. In this study, it was confirmed through *in vitro* and *in vivo* experiments that miR-376b-3p was downregulated in NSCLC tissues and cells, serving as a tumor suppressor gene.

KLF15 has three highly conserved zinc finger structures[17,18]. It has been verified that the three zinc finger structures have the nuclear localization signals. Therefore, KLF15 is transferred into the nucleus and ligated to CACCC element and GC-rich region of DNA, thus regulating the gene expression[19]. Currently, it has already been reported[20] in the literature that miR-4262 negatively regulates the mRNA expression of KLF15, thereby facilitating the proliferation and invasion of breast cancer cells. In addition, miR-376a promotes proliferation and metastasis of ovarian cancer cells through targeted inhibition on KLF15 expression[21].

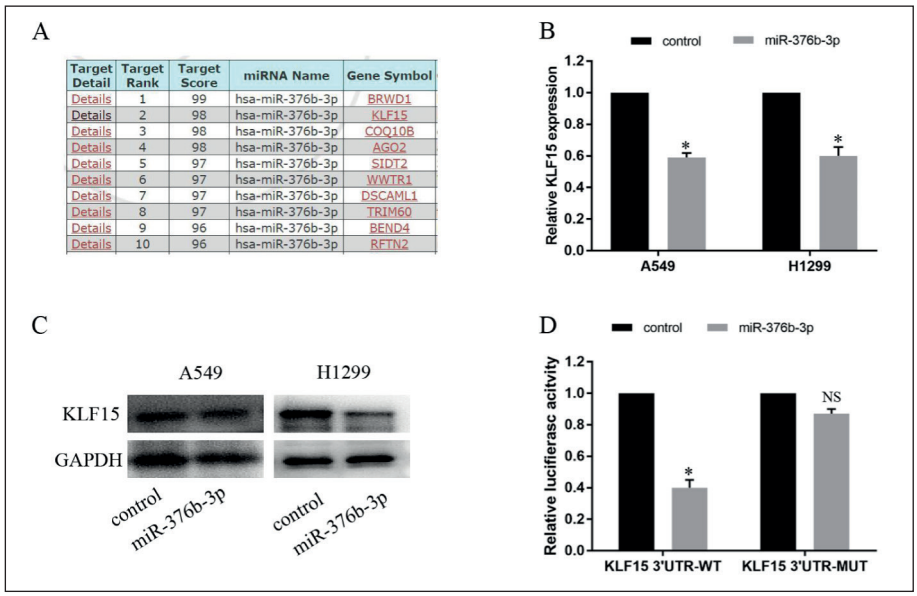

Figure 3. MiR-376b-3p targets the expression of KLF15. **A**, Possible target genes for miR-376b-3p are predicted using the bioinformatics. **B**, QRT-PCR reveals that after overexpression of miR-376b-3p, the expression of KLF15 is downregulated. **C**, The results of Western blotting manifest that KLF15 is down-regulated at the protein level after overexpression of miR-376b-3p. **D**, Dual-Luciferase reporter gene assay shows that miR-376b-3p binds directly to KLF15.

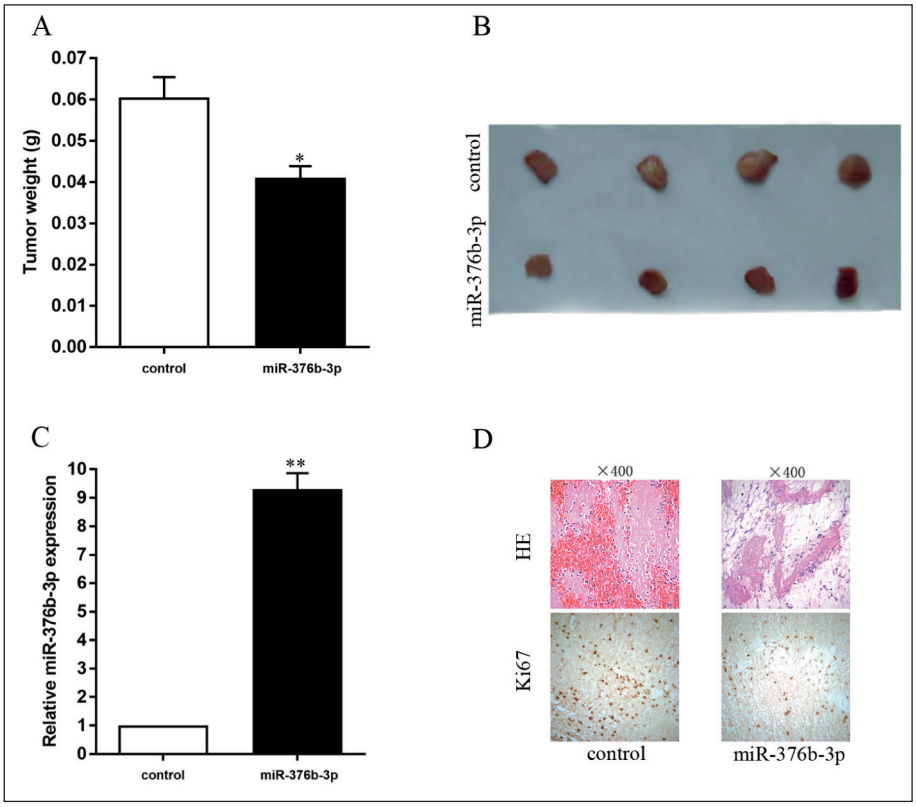

Figure 4. MiR-376b-3p suppresses in vivo tumorigenic ability of NSCLC cells. **A**, Transplanted tumor weight is measured. **B**, After 21 d, nude mice are sacrificed, and the tumor is taken and photographed. **C**, The expression of miR-376b-3p in transplanted tumor is determined using qRT-PCR. **D**, The expression of Ki67 in transplanted tumor is determined using immunohistochemistry (magnification: 400×).

Conclusions

In this study, we first observed that miR-376b-3p could regulate the expression of KLF15 in a targeted manner, thereby inhibiting proliferation and promoting apoptosis of NSCLC cells. Our findings might provide a molecular target and theoretical basis for clinical treatment of NSCLC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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