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MiR-645 promotes proliferation and migration of non-small cell lung cancer cells by targeting TP53I11

B. ZHU[1], T. TIAN[2], M. ZHAO[3]

[1]Department of Pulmonary and Critical Care Medicine, the First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China
[2]Department of Oncology, the First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China
[3]The Department of Oncology, Hebei Chest Hospital, Shijiazhuang, China

Bo Zhu and Tao Tian contributed equally to this work

Abstract. – OBJECTIVE: To research the expression and biological function of micro ribonucleic acid (miR)-645 in non-small cell lung cancer (NSCLC), and to further explore the regulatory relationship between miR-645 and tumor protein p53 inducible protein 11 (TP53I11).

PATIENTS AND METHODS: A total of 41 tissue samples were collected from NSCLC patients, and RNAs were extracted from these tissues and reversely transcribed. Then, the expression level of miR-645 in the 41 tissue samples of patients, as well as that in NSCLC cells and human bronchial mucosal epithelial cells, was detected by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). *In vitro* functional assays [methyl thiazolyl tetrazolium (MTT) assay, colony formation assay and transwell assay] were conducted to explore the effects of miR-645 on the proliferation and migration abilities of NSCLC cells. Finally, the downstream target genes of miR-645 were predicted by bioinformatics, screened *via* qRT-PCR and Western blotting experiments, and verified through Dual-Luciferase reporter gene assay.

RESULTS: QRT-PCR results showed that the miR-645 expression was upregulated in the tissue samples of 35 out of 41 NSCC cases. Besides, the miR-645 expression was upregulated in NSCC cells compared with that in human bronchial mucosal epithelial cells. After interfering with miR-645 expression, *in vitro* functional assay (MTT assay, colony formation assay and transwell assay) results revealed that the cell proliferation, migration, and invasion were inhibited. According to the results of qRT-PCR and Western blotting, after knocking down the expression of miR-645 in NSCLC cells, the expression of TP53I11 was upregulated, and the results of Dual-Luciferase reporter gene assay confirmed that miR-645 could directly bind to TP53I11.

CONCLUSIONS: MiR-645 expression is upregulated in NSCLC tissues and cells, and the proliferation and migration of NSCLC cells are promoted by targeted regulation on the TP53I11 expression.

Key Words:
NSCLC, MiR-645, Proliferation, Migration, TP53I11.

Introduction

Non-small cell lung cancer (NSCLC) is the most common histological type of lung cancer, and its incidence rate takes up over 85% of the total cases of lung cancer[1,2]. Due to the hidden onset of the disease, most patients with NSCLC have already been in the advanced stage when definitely diagnosed, missing the best opportunity for surgical treatment. Advanced NSCLC is treated on the basis of platinum-based chemotherapy, whose effective rate is only 25-30%, with a 1-year survival rate of 40% and the median survival time of 8-12 months[3]. Seeking for specific therapeutic targets for NSCLC and improving the therapeutic effect on NSCLC are problems that need to be solved urgently in clinic.

Micro ribonucleic acids (miRNAs) are a group of endogenous non-coding small RNAs composed of 19-22 nucleotides, which can regulate gene expression at the post-transcription-

Corresponding Author: Min Zhao, MD; e-mail: z_mxk1067@126.com

al level by specifically binding to the 3' untranslated region (3'UTR) of target messenger RNAs (mRNAs)[4]. These single-stranded RNAs exert vital effects in the normal life cycle of cells[5-7]. Zhang et al[8] revealed that miR-489 acts as a tumor suppressor gene in gastric cancer and suppresses the invasion and metastasis abilities of gastric cancer by directly acting on PROXl protein. Liao et al[9] discovered that miR-605-5p contributes to the proliferation and invasion of NSCLC through the targeted regulation on TNFAIP3 in NSCLC.

Moreover, miR-645 is dysregulated in various tumors and participates in their occurrence and development as a crucial regulatory factor. In colorectal cancer, the miR-645 expression is upregulated, and it modulates the EFNA5 expression to promote the invasion, metastasis and proliferation of colorectal cancer[10]. In breast cancer, the miR-645 expression is downregulated, and it regulates DCDC2 to inhibit breast cancer metastasis[11]. However, there are no reports on the role of miR-645 in NSCLC. For the first time, the expression of miR-645 in NSCLC was explored, and its biological function was investigated by this research group.

Patients and Methods

Tissue Specimens

Human NSCLC tissues and cancer-adjacent normal lung tissues surgically excised were collected from 41 NACLC patients undergoing radical resection for lung cancer in the Surgery Department of the First Affiliated Hospital of Dalian Medicine University. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medicine University. Signed written informed consents were obtained from all participants before the study. Fresh tissues excised from the patients were immediately frozen in liquid nitrogen for subsequent experiments.

Cell Culture

Human normal lung epithelial BEAS-2B and NSCLC cells (A549, H1975, SK-MES-1,H460,) were purchased from China Center for Type Culture Collection (Wuhan University, Wuhan, China). The resuscitated BEAS-2B and NSCLC cells were cultured with Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM) solution (Gibco, Rockville, MD, USA) containing 1% penicillin-streptomycin mixture (Solarbio, Beijing, China) and 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C. The medium was replaced once a day, and the cells in the logarithmic growth phase were collected.

Cell Transfection

NSCLC cells were collected, and the cell concentration was adjusted to 2×10^6 cells/mL. Then, the cells were inoculated into 6-well plates and cultured for 6 h until their fusion reached 50%. Next, 3 small interfering (si)-miR-645 and 3 si-negative control (NC) with different sequences (Guangzhou Ribobio Co., Ltd., Guangzhou, China) were mixed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 20 min of incubation, and then, we added them into each well. 12 h later, the solution was replaced. After 72 h, the transfection efficiency was verified by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR).

Detection of MiR-645 Expression Via qRT-PCR Assay

The total RNAs were extracted from the collected tissues and cells by TRIzol method (Invitrogen, Carlsbad, CA, USA), and complementary deoxyribose nucleic acids (cDNAs) were synthesized according to the instructions of RT kit (TaKaRa, Japan). Subsequently, Real Time-PCR was carried out on ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) according to the instructions of SYBR Premix Ex Taq™ II kit (TaKaRa, Otsu, Shiga, Japan) based on the following procedures: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s and annealing at 60°C for 30 s for a total of 40 cycles. Finally, the expression level of target gene was calculated by $2^{-\Delta\Delta Ct}$ method. Primer sequences are as follows: miR-645 F 5'-TTTACGCGTCCAAGAGTGAGAATCTCGCT-3'; R 5'-AGCTTGGTGGGGATTTTACGG AGAGA-3', U6 F 5'-CATCACCATCAGGAGAGTCG-3', R 5'-TGACGCTTGCCCACAGCCTT-3'. TP53I11 F 5'-TCATTTCCCTACCTGCTCCT-3', R 5'-CAGTGTCTGCACATCGCTAAT-3'. GAPDH F 5-TGGCACCCAGCACAATGAA-3', R 5'- CTAAGTCATAGTCCGCCTAGAAGCA-3'.

Methyl Thiazolyl Tetrazolium (MTT) Assay

NSCLC cells in the logarithmic growth phase were inoculated into 96-well cell culture plates at the density of 1×10^5 cells/mL. Following 48 h of transfection, 20 μL of MTT solution (5 μg/μL; Sigma-Aldrich, St. Louis, MO, USA) was added. Then, 150 μL of dimethyl sulfoxide (DMSO) solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well after 4 h of incubation. Ultimately, the absorbance (A) at the wavelength of 490 nm was detected in each group.

Transwell Assay for Cell Migration

The cell suspension at the density of 1×10^5 cells/mL re-suspended by serum (FBS)-free culture medium in experimental group and control group was prepared. Later, 400 μL of cell suspension was added into the upper transwell chamber, and the lower chamber was put into 600 μL of culture medium containing 10% FBS. Thereafter, the cells were cultured in a thermostatic incubator for 24 h, washed twice with phosphate buffered saline (PBS), fixed by 4% paraformaldehyde for 30 min, and stained with 0.1% crystal violet for 30 min. At last, the cells in the upper chamber were wiped off with cotton swabs, dried, and photographed.

Matrigel Cell Invasion Experiment

The serum-free culture medium and Matrigel (Corning, Corning, NY, USA) were diluted at 1:8, and the cell suspension in experimental group and control group was prepared according to the above method. Then, each well was added with 60 μL of cell suspension and placed in the thermostatic incubator at 37°C for 4 h. The experiment was performed after the Matrigel was solidified following the same procedures as the migration assay above.

Colony Formation Assay

The two groups of cells were separately inoculated into 60 mm cell culture dishes at the density of 1×10^3 cells/well, and the free individual cells were inoculated. After 10 d, the cells were stained with crystal violet solution, and colonies containing ≥20 cells were counted. Next, the difference in the number of cell colonies between the two groups was detected. At last, the representative colonies were photographed and counted under an inverted microscope (Olympus, Tokyo, Japan).

Western Blotting Assay

Cell protein lysate was added to extract the total proteins from the cells, and the protein concentration was quantified. After that, the proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gel, transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and blocked with 5% blocking solution at 4°C for 2 h. Subsequently, tumor protein p53 inducible protein 11 (TP53I11) and GAPDH monoclonal antibodies (1:1000, CST, Danvers, MA, USA) were added for incubation on a shaking table overnight at 4°C, followed by incubation with secondary antibody (1:5000, CST, Danvers, MA, USA) at room temperature for 2 h. After enhanced chemiluminescence (ECL) reaction for 1-3 min, the proteins were exposed in a darkroom, followed by image development and fixation. Finally, Image-Pro Plus software (Media Cybernetics, Silver Springs, MD, USA) was used for data analysis.

Verification of Targeted Relationship

Biological information software (http://www.Targetscan.org/) was applied to predict the binding site between miR-645 and TP53I11. With NSCLC genomic DNAs as templates, wild-type TP53I11 3'UTR (TP53I11-Wt) plasmid and mutant-type TP53I11 3'UTR (TP53I11-Mut) were constructed. Then, A549 cells were co-transfected with TP53I11-Wt, TP53I11-Mut and NC mimic or miR-645 mimic and cultured for 48 h. Finally, the Luciferase activity of cells was measured, with the fluorescence value of Renilla plasmids as the internal reference (Promega, Madison, WI, USA).

Statistical Analysis

The statistical analysis of all data in this study was conducted using GraphPad Prism 6.0 software (La Jolla, CA, USA). Measurement data were expressed as ($\bar{x} \pm s$). The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-645 Expression was Upregulated in NSCLC Tissues and Cells

Firstly, the relative expression level of miR-645 in NSCLC tissue and cancer-adjacent tissue samples from 41 NSCLC cases was detected

via qRT-PCR. The results showed that the miR-645 expression was upregulated in the tissue samples from 35 NSCLC cases (Figure 1A-1B). Then, the expression of miR-645 in NSCLC cells was also examined by qRT-PCR. It was found that miR-645 expression was upregulated in NSCLC cells compared with that in human bronchial epithelial cells (Figure 1C). In order to study the biological function of miR-645 in NSCLC cells, specific interference sequences for miR-645 were designed, synthesized and transiently transfected into NSCLC cells, and the interference efficiency was detected 72 h later (Figure 1D-1E).

Biological Function of MiR-645 in NSCLC Cells

Si-miR-645 and si-NC were transfected into NSCLC cells, and MTT assay was adopted to research the changes in the proliferation ability of NSCLC cells. According to the results, the proliferation ability of NSCLC cells was inhibited after interfering with the miR-645 expression (Figure 2A-2B), which were consistent with the results of colony formation assay (Figure 2C-2D). Next, the effects of miR-645 on the migration and invasion abilities of NSCLC cells were further explored. The cells were treated according to the above experimental methods and detected *via* transwell assay. It was discovered that the cell migration and invasion abilities were suppressed after interfering with the miR-645 expression in NSCLC cells (Figure 2E-2F).

Targeted Regulation of MiR-645 on TP5311 Expression

To study the potential molecular mechanism by which miR-645 performs its biological function in NSCLC, the possible downstream target genes of miR-645 were predicted by bioinformatics (Figure 3A). Then, qRT-PCR assay was carried out to verify the predicted target genes, and TP53I11 was screened out to be the possible direct target gene of miR-645 (Figure 3B). Next, Western blotting assay results manifested that after interfering with the expres-

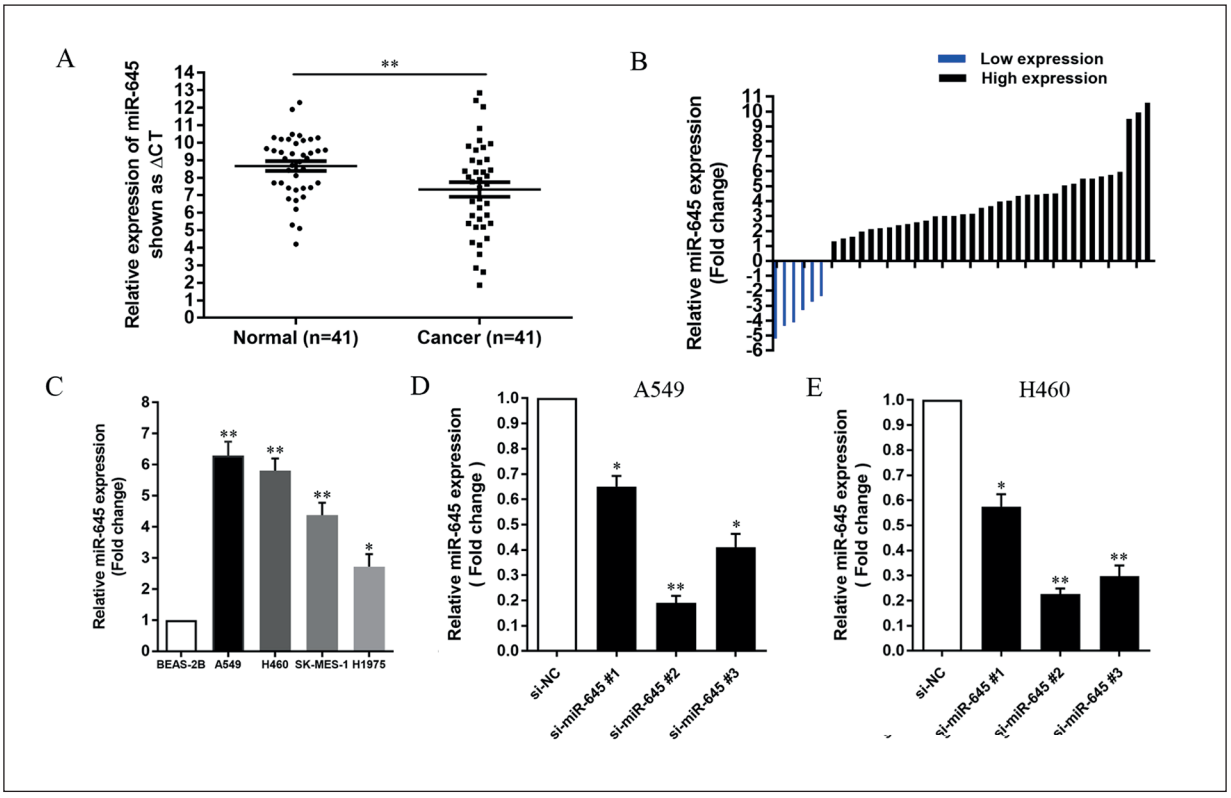

Figure 1. MiR-645 expression is upregulated in NSCLC tissues and cells. **A,** MiR-645 expression in 41 NSCLC patients is detected *via* qRT-PCR assay. **B,** Relative miR-645 expression is upregulated in 35 out of 41 cases of NSCLC tissue specimens, with GAPDH as the internal reference. **C-E,** After si-miR-645 and si-NC are transiently transfected into NSCLC cells for 72 h, qRT-PCR assay is conducted to detect the interference efficiency.

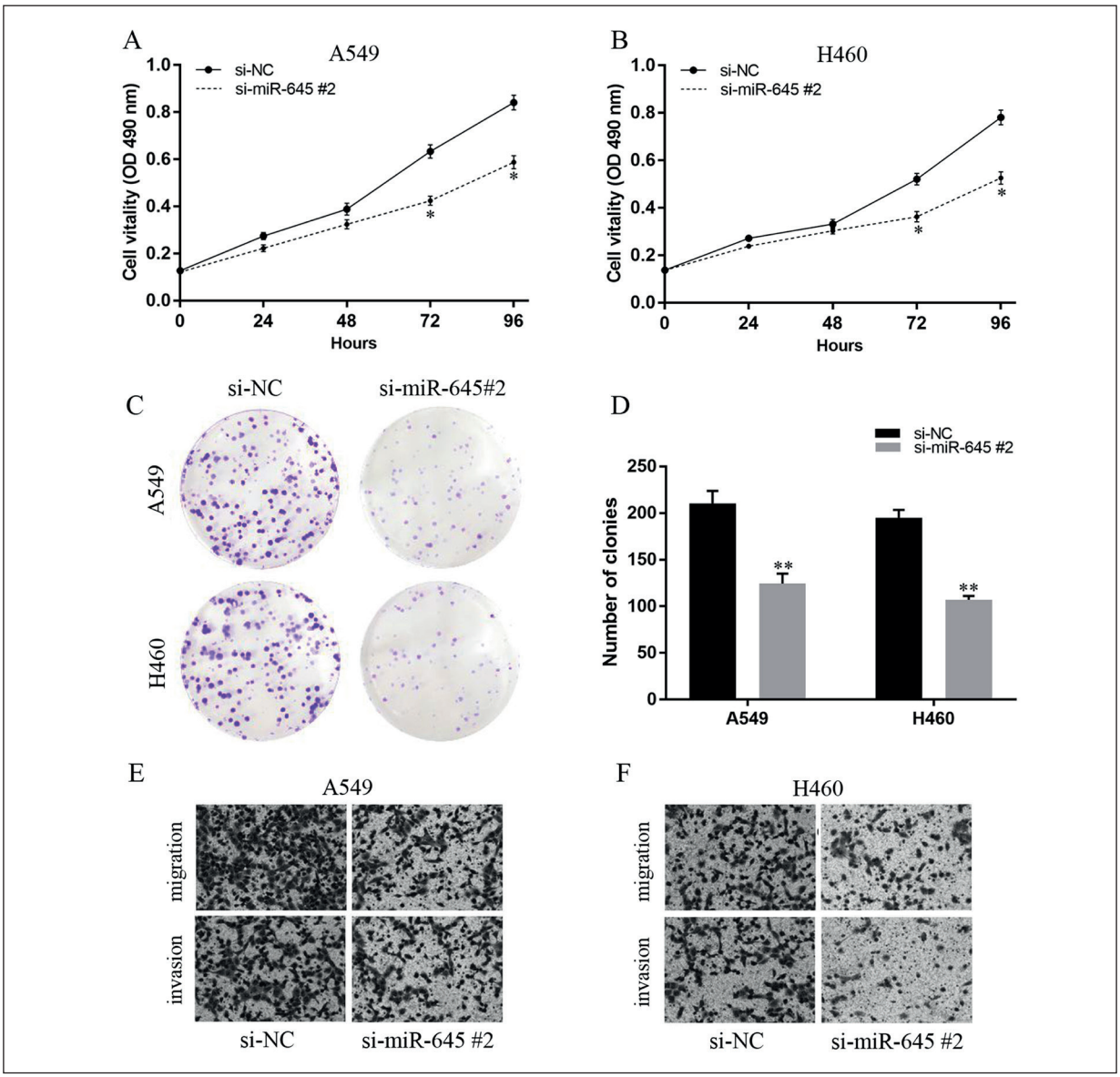

Figure 2. Biological function of miR-645 in NSCLC cells. **A-B,** MTT assay results show that compared with that in si-NC group, cell proliferation ability in si-miR-645 group is inhibited. **C-D,** Colony formation assay results reveal that the proliferation ability of NSCLC cells is inhibited after interfering with miR-645 expression (magnification: 10×). **E-F,** Transwell assay results manifest that the expression of miR-645 is knocked down in NSCLC, and the cell migration and invasion abilities are inhibited. (magnification: 40×).

sion of miR-645, the protein expression level of TP53I11 changed (Figure 3C). Afterwards, Dual-Luciferase gene assay revealed that A549 Luciferase activity was significantly reduced after co-transfection with miR-645 mimic and TP53I11-Wt compared with that after transfection with NC mimic (Figure 3D), suggesting that there is a targeted regulatory relationship between miR-645 and TP53I11.

Discussion

Lung cancer has become the primary cause of cancer death of human, and NSCLC cases account for about 85% of the total number of lung cancer. Due to the lack of clinical biomarkers for effective early diagnosis, the 5-year survival rate of NSCLC patients remains less than 15%[12]. In China, about 400,000 peo-

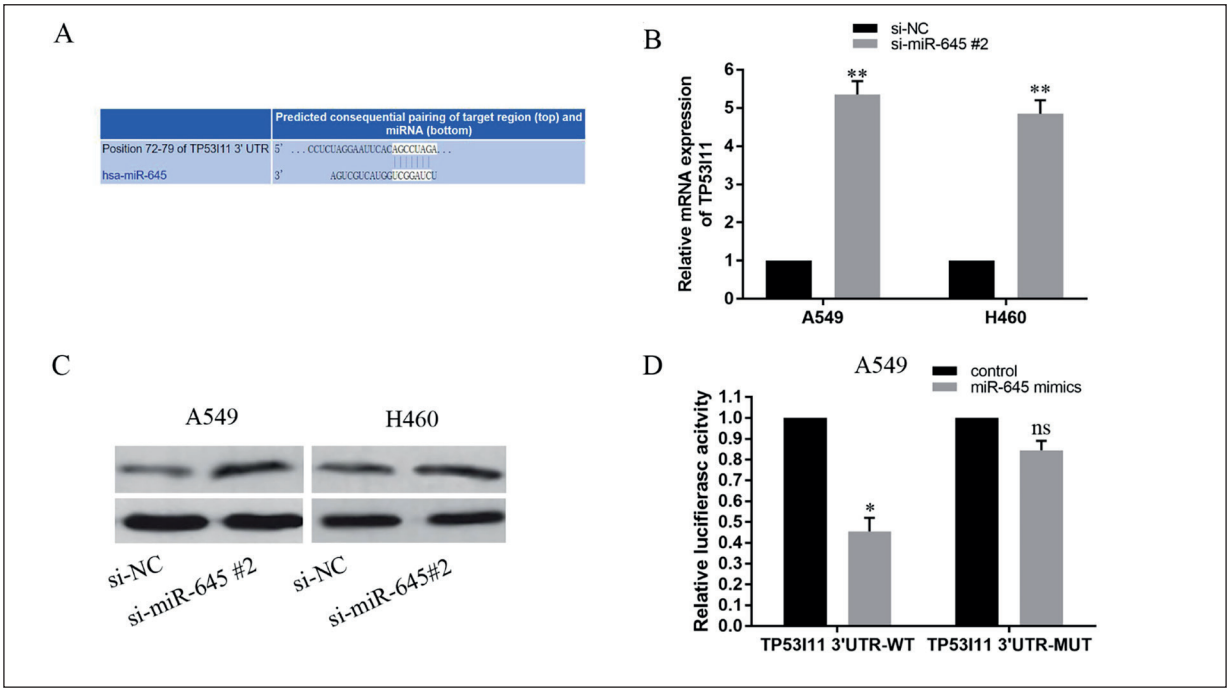

Figure 3. Targeted regulation of miR-645 on TP5311 expression. **A,** Bioinformatics is adopted to predict the possible binding sites between miR-645 and TP53I11. **B,** According to qRT-PCR assay results, the mRNA expression level of TP53I11 is upregulated after interfering with the miR-645 expression. **C,** Western blotting assay results indicate that after interfering with the miR-645 expression, the protein expression level of TP53I11 is upregulated. **D,** Results of Dual-Luciferase reporter gene assay show that miR-645 can bind to TP53I11 in a targeted manner.

ple are diagnosed with lung cancer every year, and the number is expected to reach 1 million per year by 2025. Therefore, finding targets for the early diagnosis and treatment of NSCLC is of great significance.

MiRNAs, a kind of endogenous small non-coding RNAs with about 22 nucleotides in length, regulate the expression of target genes by binding to their 3'UTRs, thus participating in the regulation of life activities, such as ontogenesis, apoptosis, proliferation and differentiation[4-7]. The aberrant expression of miRNAs, such as miR-509-5p[13], miR-27b[14] and miR-608[15], is closely associated with the occurrence and development of NSCLC. However, there is no report on the expression and function of miR-645 in NSCLC.

The transcriptional gene miR-645 is located on chromosome 20. MiR-645 is not only dysregulated in breast cancer and colon cancer[10,11], but also in hepatocellular carcinoma, osteosarcoma and renal cancer, and it has different biological functions in these cancers[16-18]. This research group was the first to find that miR-645 was upregulated in NSCLC, and suppressing its expression could inhibit the proliferation and metastasis of NSCLC cells.

TP53I11, also known as PIG11, is a tumor suppressor gene located on chromosome 11p11.2 and contains a p53-DNA binding site. Besides, it is a downstream target gene of p53-mediated apoptosis signaling pathway. It has been illustrated in studies that the expression of TP53I11 is upregulated in glandular epithelial tissues in the breast, liver, stomach and intestine, while it is low in tumors, such as breast cancer, liver cancer, and gastrointestinal cancer, suggesting that the low expression of TP53I11 is correlated with the occurrence and development of epithelial malignant tumors[18-21]. It was found in this study *via in vitro* experiments for the first time that TP53I11 was a downstream target gene of miR-645 in NSCLC and might participate in the occurrence and development of NSCLC.

Conclusions

To sum up, interfering with the miR-645 expression upregulates TP53I11, so as to inhibit the

proliferation, migration and invasion of NSCLC cells, which provides a theoretical basis for the clinical treatment of NSCLC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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