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MiR-296-3p may affect the proliferation and migration of non-small cell lung cancer cells via regulating RABL3

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide. The pathogenesis of NSCLC has not yet been fully understood, and the therapeutic efficacy of current anti-NSCLC medication remains unsatisfactory. Previous studies indicated that miR-296-3p was down-regulated in NSCLC, suggesting that miR-296-3p may participate in the pathogenesis of NSCLC; however, the specific mechanisms still need to be further explored. The aim of this work is to investigate the roles of miR-296-3p in NSCLC and the related mechanism.

PATIENTS AND METHODS: Thirty NSCLC tissue and paired adjacent tissue were collected, and Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed to examine the expression of miR-296-3p in cancer tissue and the adjacent tissue. Next, A549 cells were cultured and transfected with miR-296-3p mimics, and cell migration and invasion were determined using scratch wound-healing and transwell assays. Moreover, Western blot assay was performed to determine the effect of miR-296-3p on the expression of Rab-like 3 (RABL3), Matrix metallopeptidase (MMP)-2, Janus kinase (JAK) and Signal transducer and activator of transcription 3 (STAT3); next, Dual-Luciferase reporter assay has been conducted to prove the direct targeting relationship between miR-296-3p and RABL3. Finally, the cells of different treatments were subcutaneously implanted into nude mice to investigate the effect of miR-296-3p mimics in the xenograft mice tumor models.

RESULTS: Our data indicated that miR-296-3p was significantly down-regulated and RABL3 was markedly up-regulated in NSCLC tissue compared with the adjacent tissue. Moreover, transient over-expression of miR-296-3p in A549 cells induced a significant decrease in the proliferation and invasion ability of A549 cells, as well as decreased expression of RABL3, MMP-2, JAK and STAT3. Furthermore, the Dual-Luciferase reporter assay confirmed that RABL3 is a direct target of miR-296-3p. Finally, the results of animal studies indicated that miR-296-3p can regulate the tumorigenesis of A549 cells *in vivo*.

CONCLUSIONS: Our findings proved that miR-296-3p may play a role as a tumor suppressor in NSCLC both *in vitro* and *in vivo*, and we first reported that miR-296-3p can regulate the migration and invasion of A549 cells via targeting RABL3. Our data suggested that miR-296-3p may serve as a potential therapeutic target for treating NSCLC.

Key Words:
NSCLC, MiR-296-3p, RABL3, Invasion.

Introduction

Lung cancer is the most common cancer in the world and also the leading cause of cancer-related deaths worldwide[1,2]. Lung cancer can be divided into several subtypes, among which 85% of the cases were non-small cell lung cancer (NSCLC). The pathogenesis of NSCLC remains unclear, and most of the patients have come to the later stage of the disease when diagnosed. Thus, the therapeutic efficacy of current anti-NSCLC therapies remain unsatisfactory[3,4], leading to the poor prognosis of the disease (the 5-year survival rate of NSCLC is < 16%). To further investigate the pathogenesis of NSCLC and identify novel therapeutic targets is in a great need for the early diagnosis and treatment of NSCLC.

MicroRNAs (miRNA) are small non-coding RNAs with the length of about 22 nucleotides. Previous studies indicated that microRNAs may play a role as a negative regulator of genes by binding to the 3'-Untranslated Region (UTR) region of its target mRNA and consequentially silencing the expression of its target gene. The roles of miRNA, either as tumor suppressor on

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onco-miRNAs, have also been discussed previously[1,5-7]. MiR-296 has been first identified as a microRNA that regulates the process of angiogenesis, and in recent years, the roles of miR-296 in different cancers have also been discussed. It has been proved that miR-296 targets AKT2 in pancreatic cancer and functions as a potential tumor suppressor[8]; moreover, miR-296 can inhibit the metastasis and epithelial-mesenchymal transition of colorectal cancer by targeting S100A4[9]; furthermore, miR-296 can inhibit proliferation and induces apoptosis by targeting FGFR1 in human hepatocellular carcinoma[10].

In NSCLC, the down-regulation of miR-296-3p has been observed in previous works[11]; however, the underlying mechanism and the specific roles of miR-296-3p in the pathogenesis NSCLC still needs to be further explored. In the present work, we focused on the relationship between miR-296-3p and NSCLC. We will examine the expression of miR-296-3p in NSCLC tumor tissue and cell lines, and investigate the effect of miR-296-3p on the migration and invasion of lung cancer cells both *in vitro* and *in vivo*.

Patients and Methods

Patients and Clinical Information

Lung tissue samples were collected from 30 patients who have been diagnosed as NSCLC between March 2016 and June 2017 at the Ningbo City Medical Treatment Center Lihuili Hospital. For each patient, the paired cancer tissue and the adjacent tissue were collected and stored in liquid nitrogen immediately after surgery until needed. Patients who received chemotherapy or radiotherapy have been excluded. This study has been approved by the Research Ethics Committee of the Ningbo City Medical Treatment Center Lihuili Hospital, and each patient has signed the informed consent.

Real Time-Quantitative Polymerase Chain Reaction

The total RNAs were extracted from the cell lines and the tissues samples using TRIzol (Invitrogen, Carlsbad, CA, USA), and RT-qPCR was performed to examine the expression of miR-296-3p using Hairpin-it™ MicroRNAs Quantitation Kit (GenePharma, Shanghai, China), and U6 (RNU6B; GenePharma, Shanghai, China) has been applied for normalization. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) has been conducted on an ABI 7500 Real Time-PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The sequences of the primers were as follows: miR-296-3p, Forward: TGGGAGGGCCCCCCCTCAA-3', reverse, 5'-TGGTGTCGTGGAGTCG-3'; U6, Forward: 5'-CTCGCTTCGGCAGCACATAT ACT-3', reverse, 5'-ACGCTTCA CGAATTTGCGTGTC-5'.

Cell Culture

Human NSCLC cell line A549, NCI-H358, NCI-H1650, NCI-H1299 and normal human lung epithelial cell line BEAS-2B were purchased from the Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured with Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a humidified incubator at 37°C and with 5% CO_2.

Cell Transfection

Transfection of A549 cells was performed with synthesized miRNA mimics or NC using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). There are 3 different groups: 1) control group, un-transfected cells; 2) NC group cell transfected with miR-296-3p mimics negative control; 3) miR-296-3p mimics group, cell transfected with miR-296-3p mimics. The miRNAs and siRNA were all synthesized by GenePharma (Shanghai, China).

Cell Proliferation Assay

A549 cells were seeded onto 96 well plates and transfected with miR-296-3p mimics or miR-296-3p mimics NC, at 24, 48 and 72 hours after transfection, the viability of the cells were examined using MTT proliferation kit (Beyotime, Shanghai, China) by measuring the Optical Density (OD) value at 490 nm with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Scratch Test and Transwell Assays

For the scratch test, A549 cells were cultured on 6 well plates, and when cells reached 100% confluence, a pipette tip was used to make parallel wounds. After 48h, cells were cultured, visualized and photographed using inverted microscopy. For the transwell assay, A549 cells were seeded onto the upper chamber of the transwell (Corning Inc., Corning, NY, USA), and 24 h later the membrane in the lower chamber was stained

with crystal violet, visualized and photographed using a microscope.

Western Blot

A549 cells were harvested at 48h and lysed by RIPA (Beyotime, Shanghai, China), and the concentration of the total protein was determined using the BCA Protein Assay Kit (Beyotime, Shanghai, China). Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been performed, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% non-fat milk. Next, the membranes were incubated with primary antibodies (anti- Rab-like 3 (RABL3), anti- Signal transducer and activator of transcription 3 (STAT3), anti-Matrix metallopeptidase (MMP)-2, anti-Janus kinase (JAK) and anti-GAPDH, purchased from Boster Biological Technology Co. Ltd, (Wuhan, China) overnight at 4°C. In the following day, the membranes were incubated with HRP-conjugated secondary antibody purchased from Beyotime (Shanghai, China), and treated with BeyoECL Plus, an enhanced chemiluminescent reagent, (Beyotime, Shanghai, China). The signals were detected and photographed by ChemiDoc™XRS$^+$ (Bio-Rad, Hercules, CA, USA).

Dual-Luciferase Reporter Assay

Wild-type RABL3 3'UTR (RABL3 -3'UTR) that contains the miR-296-3p binding site and mutant RABL3 3'UTR (RABL3 -MUT) were cloned into the p-MIR-reporter plasmid (Thermo Fisher Scientific, Waltham, MA, USA) and transfected into 293 cells with miR-296-3p mimics or NC using Lipofectamine RNAi Max (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Cells were collected 48h after transfection, and the activities of the Luciferases were detected by the Dual-Luciferase Reporter Assay Kit (Beyotime, Shanghai, China).

Xenograft Tumor Models of NSCLC Cells

Male nude mice (Balb/c-nu/nu) were obtained from the Animal Center of Nanjing Medical University (Nanjing, China). The animals (aged 4-6 weeks) were housed in a specific pathogen-free (SPF) environment and provided sterile food and water *ad libitum*. The mice were acclimatized for one week before being used for experiments. A549 cells (2×10^6) suspended in 200 μl of Phosphate-Buffered Saline (PBS) were injected subcutaneously into the right flank of the mice. The tumor volume was measured every three days after tumor inoculation and was computed according to the following formula: tumor volume (mm^3) = $1/2 \times a$ (tumor length) $\times b^2$ (tumor width). At the end of the experimental period, all animals were sacrificed by cervical decapitation, the tumor tissues were excised aseptically and the weight was recorded and used for further studies.

Statistical Analysis

All statistical analysis was performed using SPSS 22.0. (SPSS Inc., Chicago, IL, USA). Data were presented as means ± standard deviation, and the differences between the two groups were analyzed using the *t*-test, and the differences among multiple groups were analyzed by using analysis of variance (ANOVA) with Tukey's post-hoc test. $p<0.05$ has been set as a significant difference.

Results

Downregulation of MiR-296-3p in NSCLC Tumor Samples and Cell Lines

To begin with, we have examined the expressions of miR-296-3p in NSCLC tumor samples and NSCLC cell lines using RT-qPCR method; the paired adjacent normal tissues and normal human lung epithelial cell BEAS-2B were served as controls. It was observed that the expression of miR-296-3p was significantly decreased in the tumor tissue samples compared with the paired adjacent tissues (Figure 1A, $p<0.01$). Moreover, Pearson's chi-square test has been performed to evaluate the relationship between the level of miR-296-3p and the clinicopathological characteristics of patients with NSCLC. Next, we also examined the expressions of miR-296-3p in 4 different NSCLC cell lines (A549, NCI-H358, NCI-H1650, NCI-H1299) and BEAS-2B. We found that the levels of miR-296-3p were significantly down-regulated in all 4 NSCLC cell lines compared with BEAS-2B, and A549 cells have shown the lowest level of miR-296-3p expression, so A549 cells have been applied in the following *in vitro* studies (Figure 1B, $p<0.01$).

MiR-296-3p Can Regulate the Proliferation and Migration of A549 Cells In Vitro

Next, we further investigated the effect of miR-296-3p on the proliferation and migration of A549 cells by transient over-expression of miR-296-3p *via* transfection of miR-296-2p mimics. As shown in Figure 2, compared with the control group, transient over-expression of miR-296-3p markedly inhibited the cell growth at 24 and 48 h, compared

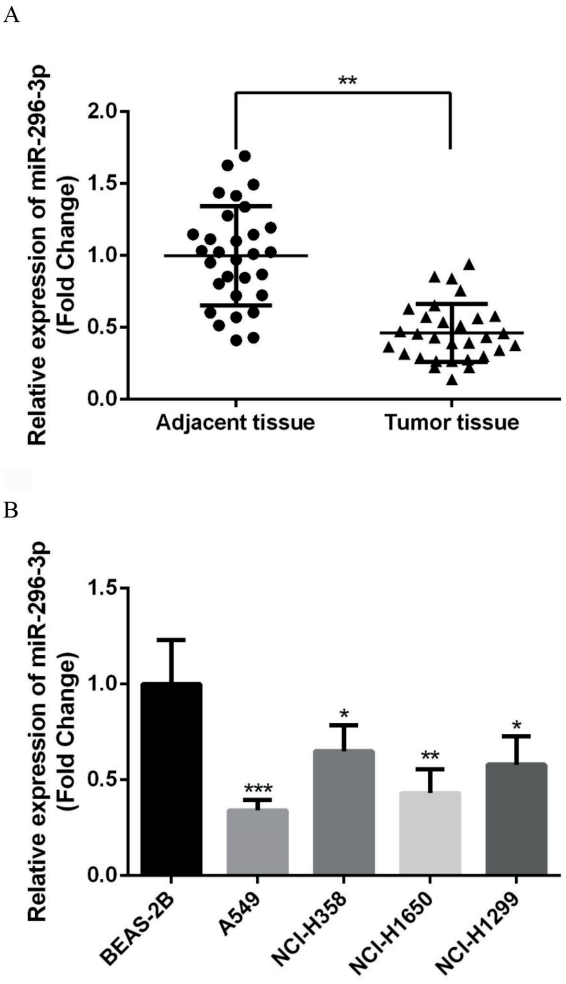

Figure 1. Relative expression of miR-296-3p in NSCLC tumor samples and cell lines by RT-qPCR methods. ***A,*** The relative expression of miR-296-3p in NSCLC tumor samples. ***B,*** The relative expression of miR-296-3p in different cell lines, *p<0.05, **p<0.01, ***p<0.001.

with the NC group (p<0.01). Furthermore, the results of the scratch and transwell assays indicated that the transfection of A549 cells with miR-296-3p mimics induced a marked decrease in the migration and invasion of A549 cells *in vitro*, compared with the miR-296-3p mimics NC group (Figure 3, p<0.01). Moreover, compared with miR-296-3p mimic NC transfected cells, the transfection of miR-296-3p mimics decreased the protein expression of MMP-2, JAK and STAT3 (Figure 4).

MiR-296-3p Can Regulate the Proliferation and Migration of A549 Cells Through Targeting RABL3 In Vitro

Using online bioinformatics tools (Targetscan and miRanda), RABL3 was predicted as a target of miR-296-3p. Therefore, we explored the association between miR-296-3p and RABL3 in the pathogenesis of NSCLC. The expression of RABL3 was found to be significantly upregulated in lung cancer tissues compared with that in the paired adjacent tissues (Figure 5B; p<0.05). The over-expression of miR-296-3p induced a significant decrease in the expression of RABL3 in A549 cells (Figure 4); finally, we have performed Dual-Luciferase reporter assay to confirm that miR-296-3p can directly target RABL3. It has been observed that the transfection of miR-296-3p mimics significantly suppressed the Luciferase activity of the RABL3-WT transfected cells, while the Luciferase activity of cells transfected with the RABL3 -MUT has not been affected by miR-296-3p mimics (Figure 5C; p<0.01), indicating that miR-296-3p can directly target the 3'-UTR of RABL3.

MiR-296-3p Can Regulate the Tumorigenesis of A549 Cells In Vivo

Finally, to explore whether miR-296-3p can affect the tumorigenesis of A549 cells *in vivo*, cells of different treatments were subcutaneously implanted into nude mice to establish the xenograft mice tumor models. As shown in Figure 6, two weeks after implantation, the average volume of the tumors was significantly smaller in miR-296-3p mimics transfected group compared with the control group (Figure 6A and B, p<0.01).

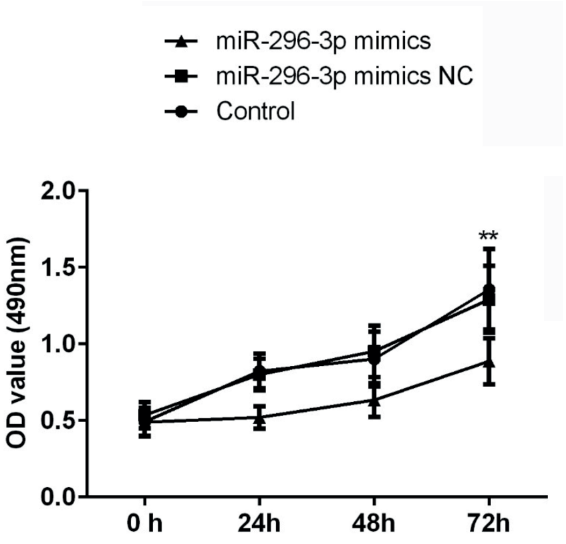

Figure 2. MiR-296-3p can regulate the proliferation of A549 cells *in vitro*. **p<0.01.

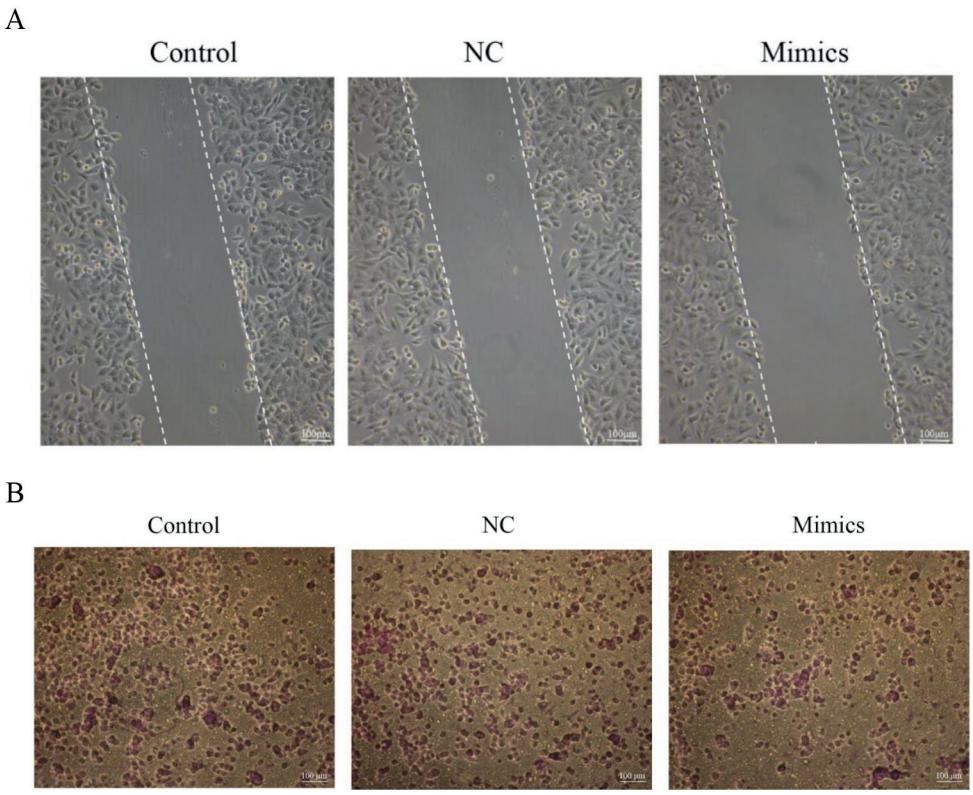

Figure 3. MiR-296-3p can regulate the migration and invasion of A549 cells *in vitro* (×100).

Discussion

In the present work, we focused on the roles of miR-296-3p in the pathogenesis of NSCLC. We observed that miR-296-3p was up-regulated in NSCLC tumor samples and cell lines, and miR-296-3p can regulate the proliferation and migration of NSCLC cells by targeting RABL3.

Aberrant expression of miR-296-3p has been observed in different types of cancer, and the dual roles of miR-296-3p, either as a tumor suppressor or oncomiR, have been discussed in many previous works. In the case of NSCLC, the function of miR-296-3p is controversial. Luo et al[11] proved that miR-296-3p can improve the chemosensitivity of NSCLC cells by targeting CX3CR1, indicating that miR-296-3p may play a role as a tumor suppressor in NSCLC. In the present study, we performed RT-qPCR analysis and observed the expression of miR-296-3p in NSCLC tissue samples and cell lines, which was consistent with the results of Luo et al[11]. Moreover, transient overexpression of miR-296-3p in the lung cancer cell line A549 led to the significant suppression of cell proliferation and migration; furthermore, miR-296-3p mimics induced anti-tumor effects in xenograft mice tumor models. These results indicated that miR-296-3p is down-regulated in NSCLC and plays a role as a tumor suppressor both *in vitro* and *in vivo*.

It is well known that miRNAs exert their functions by suppressing the expression of their target genes. RABL3 is a recently identified member of the Rab oncogene family, and the previous studies[12-15] have indicated that RABL3 was abnormally over-expressed in several types of cancers, including NSCLC. Using bioinformatics tools, RABL3 has been predicted as a target gene of miR-296-3p, and the relationship between miR-296-3p and RABL3 in NSCLC has not yet been investigated. In this work, we performed a series of experiments to explore the association between miR-296-3p and RABL3 in NSCLC. First, we compared the expression of RABL3 in NSCLC tissues and adjacent normal tissues and discovered that RABL3 was up-regulated in NSCLC, which was consistent with previous findings. The transfection of A549 with a miR-296-3p mimics induced a significant decrease in the expression of RABL3, suggesting that miR-296-3p can inhibit the expression of RABL3 in NSCLC cells. Finally, the results of the Dual-Luciferase reporter

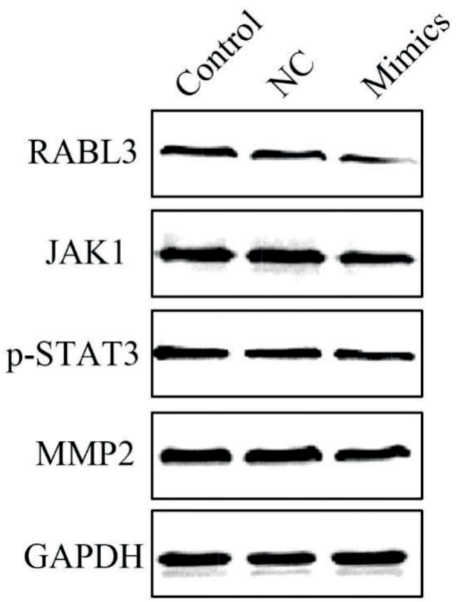

Figure 4. Effect of miR-296-3p on the expression of migration and invasion related proteins in A549 cells. ***A,*** Results of scratch test. ***B,*** Results of transwell assay.

assay confirmed that RABL3 is a direct target of miR-296-3p. As discussed, miR-296-3p can regulate the proliferation and apoptosis of A549 cells and RABL3 is a key regulator of cell proliferation and apoptosis; thus, we believe that miR-296-3p can promote the proliferation and inhibit the apoptosis of lung cancer cells, at least partially, by targeting RABL3.

JAK/STAT3 signaling and MMPs are the key molecules that are involved in the process of cancer cell proliferation and migration[16-19]. To further explore the underlying mechanism of how miR-296-3p/RABL3 axis could regulate the proliferation and invasion of NSCLC cells, the expression of MMP-2, JAK and STAT3 in different groups were examined. It was observed that the transfection of miR-296-3p mimics induced a significant decrease in the expression of MMP-2, JAK and STAT3. We suggested that miR-296-3p can regulate the proliferation and migration of NSCLC cells by targeting RABL3 *via* regulating the downstream signaling molecules MMP-2, JAK and STAT3.

Our study has some limitations. Due to ethical issues, the number of clinical samples included in this work was relatively small, and the expressions of miR-296-3p and RABL3 in NSCLC should be verified with a larger sample size.

Conclusions

We found that miR-296-3p was down-regulated in NSCLC, and we proved for the first time that miR-296-3p can regulate the proliferation and mi-

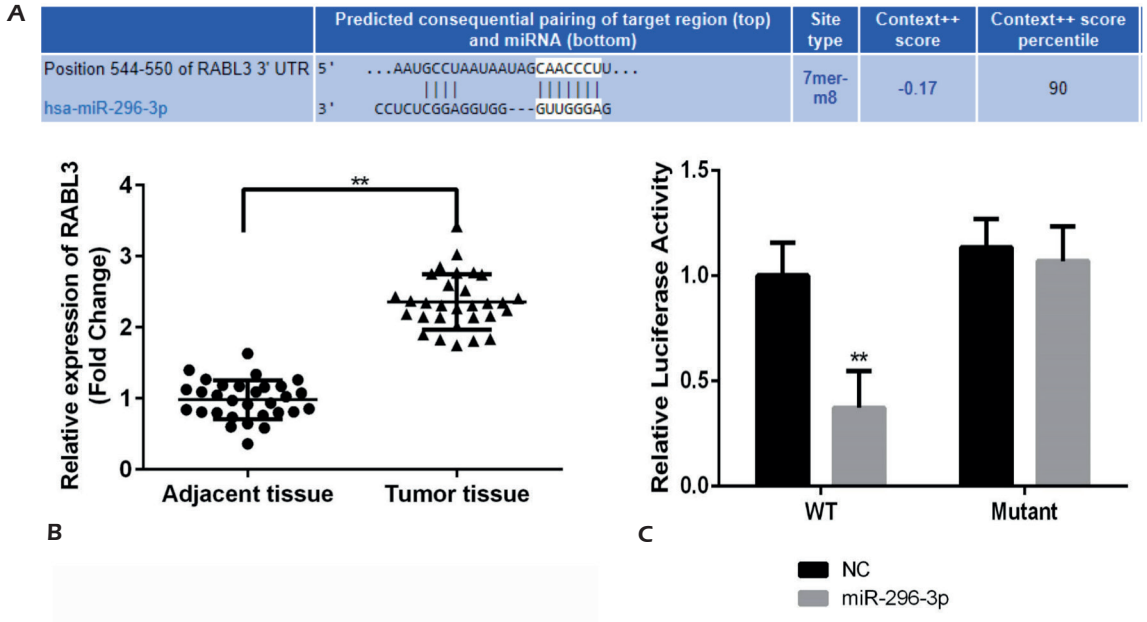

Figure 5. RABL3 is a target of miR-296-3p. ***A,*** RABL3 was predicted as a target of miR-296-3p by bioinformatic tools. ***B,*** Relative expression of RABL3 in lung cancer tissue compared with the adjacent tissue. ***C,*** Results of Dual-Luciferase

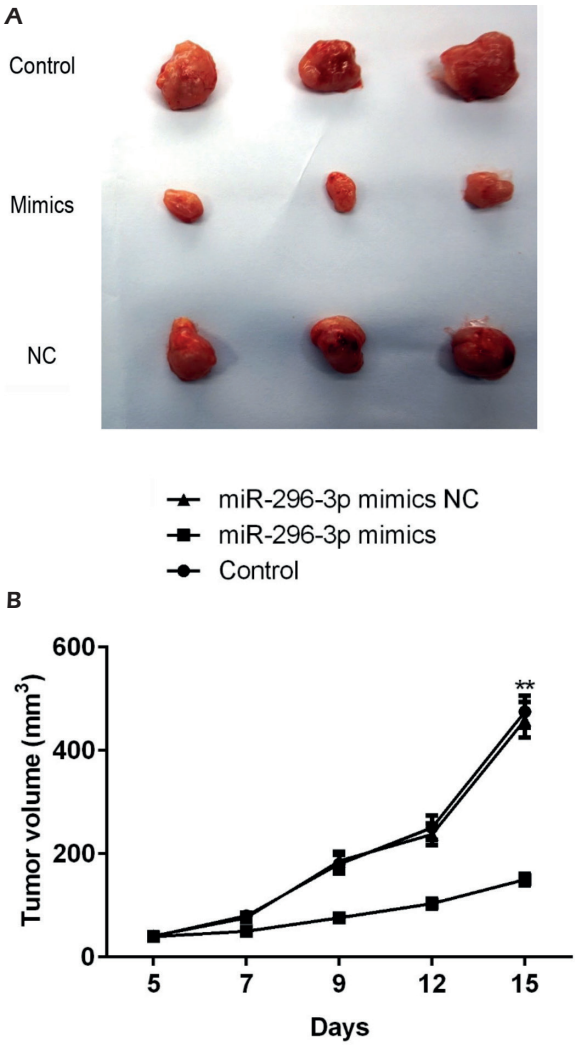

Figure 6. MiR-296-3p can regulate the tumorigenesis of A549 cells *in vivo*. ***A***, Volumes of the tumors in different groups. ***B***, Images of the tumors of different treatment. $^{**}p<0.01$.

gration of NSCLC cells by targeting RABL3. Our study has provided novel evidence for the diagnosis and treatment of NSCLC.

Conflict of interest

The authors declare no conflicts of interest.

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