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MicroRNA-590-5p suppresses the proliferation and invasion of non-small cell lung cancer by regulating GAB1

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Abstract. – OBJECTIVE: Some specific microRNAs (miRNAs) have been identified to regulate the tumorigenesis of non-small cell lung cancer (NSCLC). MiR-590-5p was found to involve in the carcinogenesis of human cancers. This study aims at exploring the role of miR-590-5p in the pathogenesis of NSCLC.

PATIENTS AND METHODS: The expressions of miR-590-5p and GAB1 were measured by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. The biological functions of miR-590-5p and GAB1 on cell viability and invasion were investigated through MTT and transwell assays. The binding site between miR-590-5p and GAB1 was verified by dual-luciferase reporter gene assay (DLR).

RESULTS: MiR-590-5p expression was downregulated in NSCLC. MiR-590-5p overexpression inhibited the proliferation and invasion of NSCLC cells. Furthermore, miR-590-5p was confirmed to directly target GAB1. GAB1 knockdown had the same effect as overexpression of miR-590-5p in NSCLC. Moreover, overexpression of GAB1 partially reversed the suppressive effect of miR-590-5p on NSCLC.

CONCLUSIONS: MiR-590-5p suppressed cell proliferation and invasion of NSCLC by inhibiting GAB1 expression, indicating that miR-590-5p was a suppressive miRNA in NSCLC.

Key Words:
Non-small cell lung cancer, MiR-590-5p, Proliferation, Invasion, GAB1.

Introduction

Lung cancer is a common malignancy with 800,000 new cases in China every year. Moreover, more than 700,000 people die from lung cancer each year in China[1]. In recent years, the death rate of lung cancer has risen sharply. Once diagnosed, 70-80% of patients with lung cancer are in advanced stages in China[2]. According to the pathological classification, about 80% of lung cancer is non-small cell lung cancer (NSCLC)[3]. Moreover, early-stage NSCLC has no significant symptoms that can only be diagnosed by physical examination. The survival rate of NSCLC is correlated with the pathological stage of lung cancer[4]. The 5-year survival rate of early-stage NSCLC is 40-60% after the standardized operation. Advanced NSCLC patients are unable to be operated, so the 5-year survival rate drops to only 7-8%[5]. The lacking of early diagnosis and effective treatments is the main reason for the high mortality of NSCLC. Therefore, it is necessary to explore and find effective ways for early diagnosis and treatment of NSCLC.

In recent years, a number of microRNAs (miRNAs) were found to regulate the progression of human cancers through inhibiting the mRNA transcription and protein translation of target genes[6]. Many miRNAs have been identified to act as biomarkers for NSCLC. For instance, miR-148b inhibited the proliferation and the epithelial-mesenchymal transition (EMT), but increased radiochemotherapy sensitivity in NSCLC by regulating ROCK1[7]. Yao et al[8] proposed that miR-215 repressed migration, invasion and proliferation in NSCLC through targeting MMP-16. Inversely, the cell growth and invasion of NSCLC cells were promoted by miR-455-5p through regulating SOCO3[9]. MiR-1260b promoted the migration and invasion in NSCLC *via* targeting PTPRK[10]. In addition, miRNA-590 has been proposed to function as oncogene or anti-oncogene in human cancers. Zhang et al[11] proposed that miR-590 overexpression promoted cell proliferation and invasion in gastric cancer. MiR-590-3p suppressed the migration, invasion

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and EMT of glioblastoma cells *via* the suppression of ZEB1 and ZEB2[12]. However, the function of miR-590 is controversial in lung cancer. Firstly, it was reported that miR-590 inhibited the tumorigenesis and invasiveness in NSCLC through targeting ADAM9[13]. On the contrary, Liu et al[14] found that miR-590 accelerated the migration and invasion by inhibiting OLFM4 in lung adenocarcinoma. Thus, the function of miR-590-5p in NSCLC was explored in the present study.

GAB1 is a member of the Grb2-associated binder (GAB) family. The GAB family has been found to regulate the signal transduction of cytokine and growth factor receptors[15]. Moreover, GAB1 was found to mediate the tumor progression of colorectal cancer[16]. Sang et al[17] reported that GAB1 regulated proliferation and migration in intrahepatic cholangiocarcinoma. GAB1 knockdown could inhibit cell proliferation and migration in hilar cholangiocarcinoma[18]. In addition, GAB1 was demonstrated to predict poor prognosis of hepatocellular carcinoma[19]. Despite, the role of GAB1 still remains blurry and need to be investigated in NSCLC. In this study, we explored expressions of miR-590-5p and GAB1 in NSCLC. In the meantime, the functions of miR-590-5p and GAB1 were also analyzed in NSCLC. Moreover, the interaction between miR-590-5p and GAB1 was examined as well. Our findings provide novel ways for the early diagnosis and treatment of NSCLC.

Patients and Methods

Clinical Tissues

Sixty-one NSCLC specimens and adjacent normal tissues were obtained from the patients in Shengli Oilfield Central Hospital. None of the NSCLC patients received any treatment prior to the operation. Tissues were frozen in liquid nitrogen and then stored in the -80°C refrigerator to be used in the further work. The informed consent was obtained from all the patients. This research was approved by the Institutional Ethics Committee of Shengli Oilfield Central Hospital.

Cell Culture

The H292, A549, SPC-A1 and BEAS-2B cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were seeded in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and cultured at 37°C with 5% CO_2.

Cell Transfection

The miR-590-5p mimic and inhibitor, GAB1 siRNA (si-GAB1) and negative control were obtained from RiboBio (Guangzhou, Guandong, China). A549 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturers' protocols.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, USA) was applied for extracting total RNA containing miRNA to quantitate miR-590-5p expression in NSCLC. Quantitative RT-PCR was performed using the Maxima SYBR Green qPCR Master Mix (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 Fast Real-Time PCR system. U6 and GAPDH were used as control for miR-590-5p and GAB1. Relative expressions were calculated using the $2^{-\Delta\Delta ct}$ method. The primer sequences used in this study were as follows: GAB1, F: 5'-TGGCAGCTCTTTACAAGCACC-3', R: 5'-TCATGAGCAACAGGTAGTCTTGA-3'; microRNA-590-5p, F: 5'-TCCATTGAAACG-CCTAGGAGAATTTGC-3', R: 5'-GCAAAT-TCTCCTAGGCGTTTCAATGGA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCT-GTTC-3', R: 5'-ATCCGTTGACTC-CGACCTTCAC-3'.

Western Blot Analysis

The protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at room temperature. Membranes were incubated with rabbit monoclonal anti-GAB1 (1:1000; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-GAPDH antibody (1:1000; Abcam, Cambridge, MA, USA) overnight at 4°C. Subsequently, membranes were incubated with goat polyclonal anti-rabbit IgG secondary antibody (1:1000; Abcam, Cambridge, MA, USA). The protein expressions were measured by Enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay for Cell Proliferation

The MTT assay (Sigma-Aldrich, St. Louis, MO, USA) was applied to measure the cell proliferation. Cells were cultured in 96-well plates (4×10^3/well) and incubated for 24, 48, 72 h and 96 h, respectively. 0.5 mg/mL MTT was added in each well and incubated for 4 h at 37°C. The absorbance at 490 nm (OD=490 nm) was detected with a spectrophotometer.

Transwell Invasion Assay

Transwell assay was used to evaluate cell invasive ability in NSCLC. Firstly, NSCLC cells in serum-free medium were plated in 24-well plates (5×10^4/well) with Matrigel (BD Biosciences, San Jose, CA, USA). NSCLC cells and medium containing 10% FBS were placed in the upper chamber and lower chamber, respectively. Then these cells were incubated for cell invasion at 37°C for 48 h. After that, cells were stained with 0.1% crystal violet. A microscope was used for counting invasive cells.

Dual Luciferase Assay

The wild or mutant-type of 3'-UTR of GAB1 was inserted into the pMIR-REPORT luciferase reporter plasmids (Beyotime, Shanghai, China) to perform luciferase reporter experiments. Then, wild or mutant-type of 3'-UTR of GAB1 and miR-590-5p mimic were transfected into 293T cells. Subsequently, the Dual Luciferase Assay System (Promega, Madison, WI, USA) was applied to analyze luciferase activity.

Statistical Analysis

The data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) and GraphPad Prism 6 (La Jolla, CA, USA). All data were presented as the mean ± SD, and the difference was calculated according to Student's *t*-test. Comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). A significant difference was defined at $p < 0.05$.

Results

MiR-590-5p Was Downregulated in NSCLC

MiR-590-5p expression was firstly measured in NSCLC tissues *via* qRT-PCR. MiR-590-5p downregulation was identified in NSCLC tissues in contrast to the normal tissues (Figure 1A). Similar results were also examined in NSCLC cell lines. MiR-590-5p expression was decreased in H292, A549 and SPC-A1 cell lines in comparison with BEAS-2B cells (Figure 1B). Meanwhile, we also detected that low expression of miR-590-5p was correlated with lymph node metastasis ($p = 0.003$) and TNM stage ($p = 0.011$, Table I). According to these results, miR-590-5p was suspected to involve in the pathogenesis of NSCLC.

Overexpression of miR-590-5p Inhibited Cell Proliferation and Invasion in NSCLC

MiR-590-5p mimics or inhibitor was transfected into A549 cells to explore its effect on regulat-

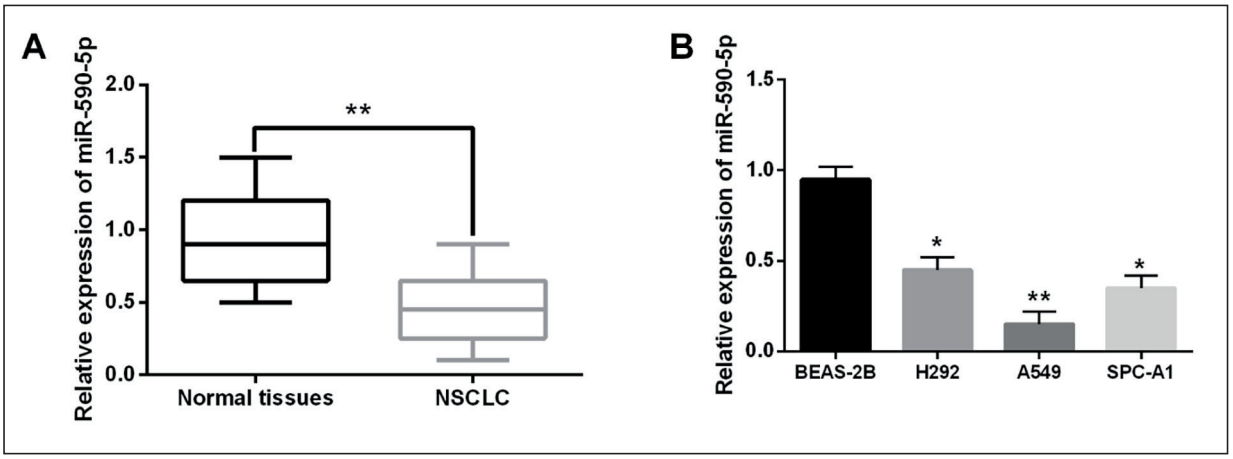

Figure 1. Downregulated of miR-590-5p was examined in NSCLC. ***A,*** miR-590-5p expression in NSCLC tissues detected *via* qRT-PCR. ***B,*** miR-590-5p expression in H292, A549, SPC-A1 and BEAS-2B cell lines. *$p < 0.05$, **$p < 0.01$.

Table I. Relationship between miR-590-5p expression and their clinicopathological characteristics of NSCLC patients.

Characteristics	Number of cases (n = 61)	miR-590-5p		*p*-value
		High	Low	
Age (years)				0.119
≥ 60	26	10	16	
< 60	35	15	20	
Gender				0.957
Male	37	17	20	
Female	24	8	16	
Tumor size				0.061
< 5 cm	33	14	19	
≥ 5 cm	28	11	17	
TNM stage				0.011*
I + II	15	5	10	
III + IV	46	16	30	
Lymph node metastasis				0.003*
Negative	18	6	12	
Positive	43	14	29	
Differentiation				0.617
Moderate and well	25	6	9	
Poor	36	12	24	

Statistical analyses were performed by the χ^2-test. *$p < 0.05$ was considered significant.

ing NSCLC development. QRT-PCR experiment indicated that miR-590-5p expression was promoted by miR-590-5p mimics (Figure 2A) and repressed by miR-590-5p inhibitor (Figure 2B). Subsequently, we measured cell proliferation in A549 cells transfected with miR-590-5p mimics or inhibitor. MTT assay suggested that miR-590-5p overexpression repressed cell proliferation in A549 cells (Figure 2C). Inversely, miR-590-5p knockdown promoted cell proliferation in A549 cells (Figure 2D). A similar effect of miR-590-5p was also observed on cell invasion in NSCLC. The ability of cell invasion in A549 cells was obviously suppressed by miR-590-5p mimics and promoted by miR-590-5p inhibitor (Figure 2E). Hence, we considered that overexpression of miR-590-5p inhibited cell proliferation and invasion in NSCLC cells.

GAB1 was a Direct Target of miR-590-5p in NSCLC

Subsequently, we selected the downstream target of miR-590-5p through the database in TargetScan (http://www.targetscan.org). TargetScan predicted that miR-590-5p could bind with the 3'UTR of GAB1 (Figure 3A). Then we conducted dual-luciferase repertory gene assay to confirm the above prediction. The luciferase activity of wild-type GAB1 was apparently decreased by miR-590-5p mimics, whereas mutant-type GAB1 did not change the luciferase activity (Figure 3B). Next, we found that miR-590-5p was negatively correlated with GAB1 expression in NSCLC tissues ($p<0.0001$, $R^2=0.8604$, Figure 3C). Furthermore, GAB1 expression was measured in A549 cells transfected with miR-590-5p mimics or inhibitor. We found that overexpression of miR-590-5p inhibited GAB1 expression, while miR-590-5p knockdown showed the opposite effect on GAB1 expression (Figure 3D, 3E). Thus, miR-590-5p directly targeted GAB1 and had a negative association with GAB1 expression in NSCLC.

GAB1 Knockdown Suppressed Cell Proliferation and Invasion in NSCLC

Next, the upregulation of GAB1 was identified in NSCLC tissues (Figure 4A) and cell lines (Figure 4B). We investigated the effect of GAB1 on regulating NSCLC through transfecting GAB1 siRNA into A549 cells. QRT-PCR results showed that GAB1 siRNA transfection significantly inhibited GAB1 expression (Figure 4C). In addition, MTT assay suggested that GAB1 knockdown impeded cell proliferation in A549 cells (Figure 4D). Similarly, cell invasion was also repressed by the transfection of GAB1 siRNA (Figure 4E). Based on these results, we found that GAB1 had carcinogenic effect by promoting cell proliferation and invasion in NSCLC.

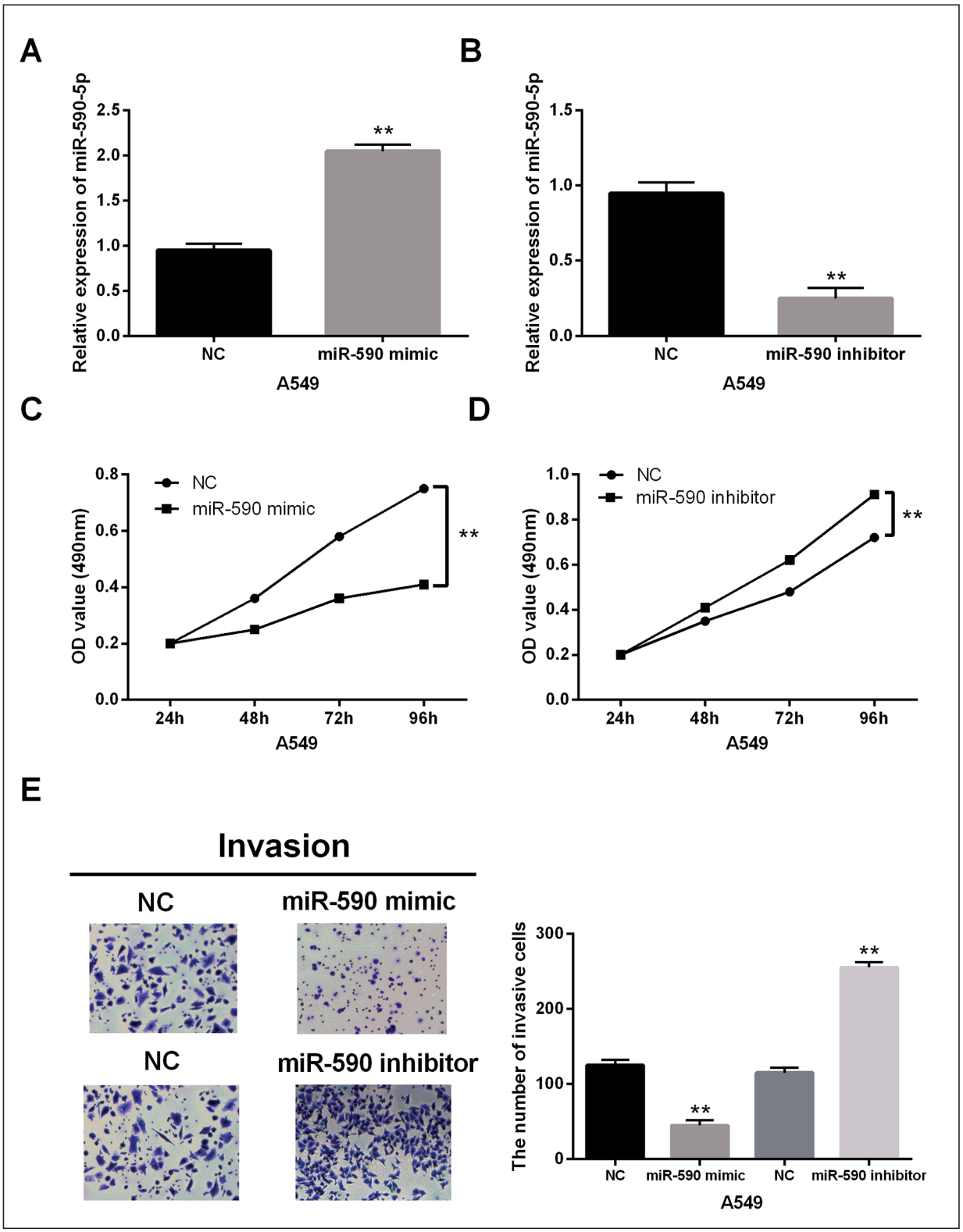

Figure 2. MiR-590-5p overexpression inhibited cell proliferation and invasion in NSCLC. ***A, B,*** miR-590-5p expression was examined in A549 cells transfected with miR-590-5p mimics or inhibitor *via* qRT-PCR. ***C, D,*** The cell proliferation was measured in cells transfected with miR-590-5p mimics or inhibitor *via* MTT assay. ***E,*** Cell invasion analysis in A549 cells transfected with miR-590-5p mimics or inhibitor was detected by transwell assay. $^{**}p < 0.01$.

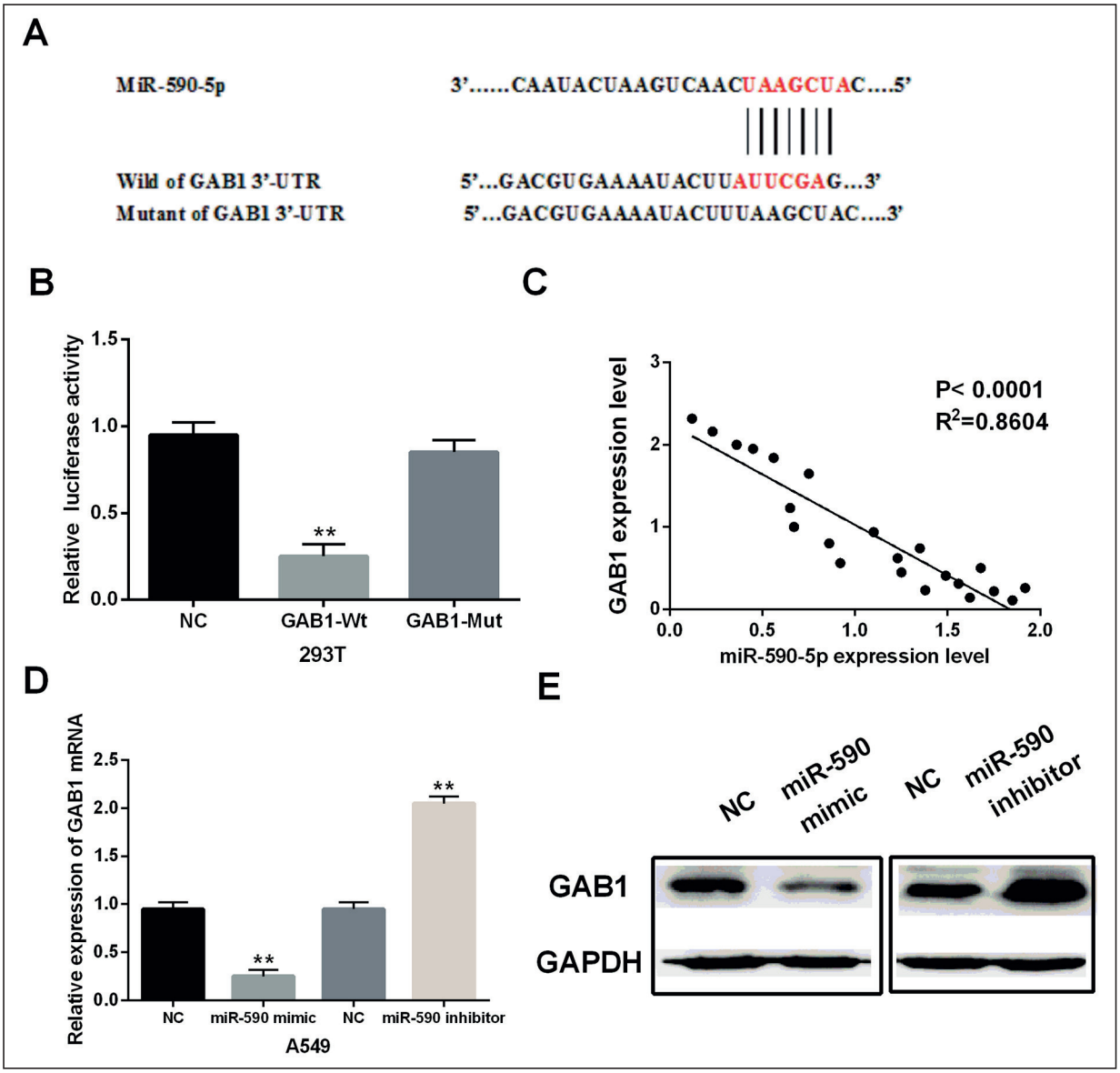

Figure 3. MiR-590-5p directly targeted GAB1 in NSCLC. ***A,*** The binding site of miR-590-5p on the 3'-UTR of GAB1. ***B,*** Dual-luciferase reporter gene assay results. ***C,*** The correlation between miR-590-5p and GAB1. ***D, E,*** GAB1 expression in cells transfected with miR-590-5p mimics or inhibitor. $^{**}p < 0.01$.

Overexpression of GAB1 Partially Inversed the Suppressive Effect of miR-590-5p on NSCLC

Finally, we explored the interaction between miR-590-5p and GAB1 by transfecting miR-590-5p mimics and GAB1 vector into A549 cells. GAB1 expression was obviously declined in A549 cells transfected with miR-590-5p mimics, which was not changed in A549 cells transfected with miR-590-5p mimics and GAB1 vector (Figure 5A). Besides, MTT assay indicated that GAB1 overexpression weakened the inhibitory effect of miR-590-5p on the proliferation of A549 cells (Figure 5B). Same as the above results, the inhibitory action of miR-590-5p for cell invasion was also blocked by the overexpression of GAB1 in A549 cells (Figure 5C). In brief, the overexpression of GAB1 partially inversed the suppressive effect of miR-590-5p on NSCLC.

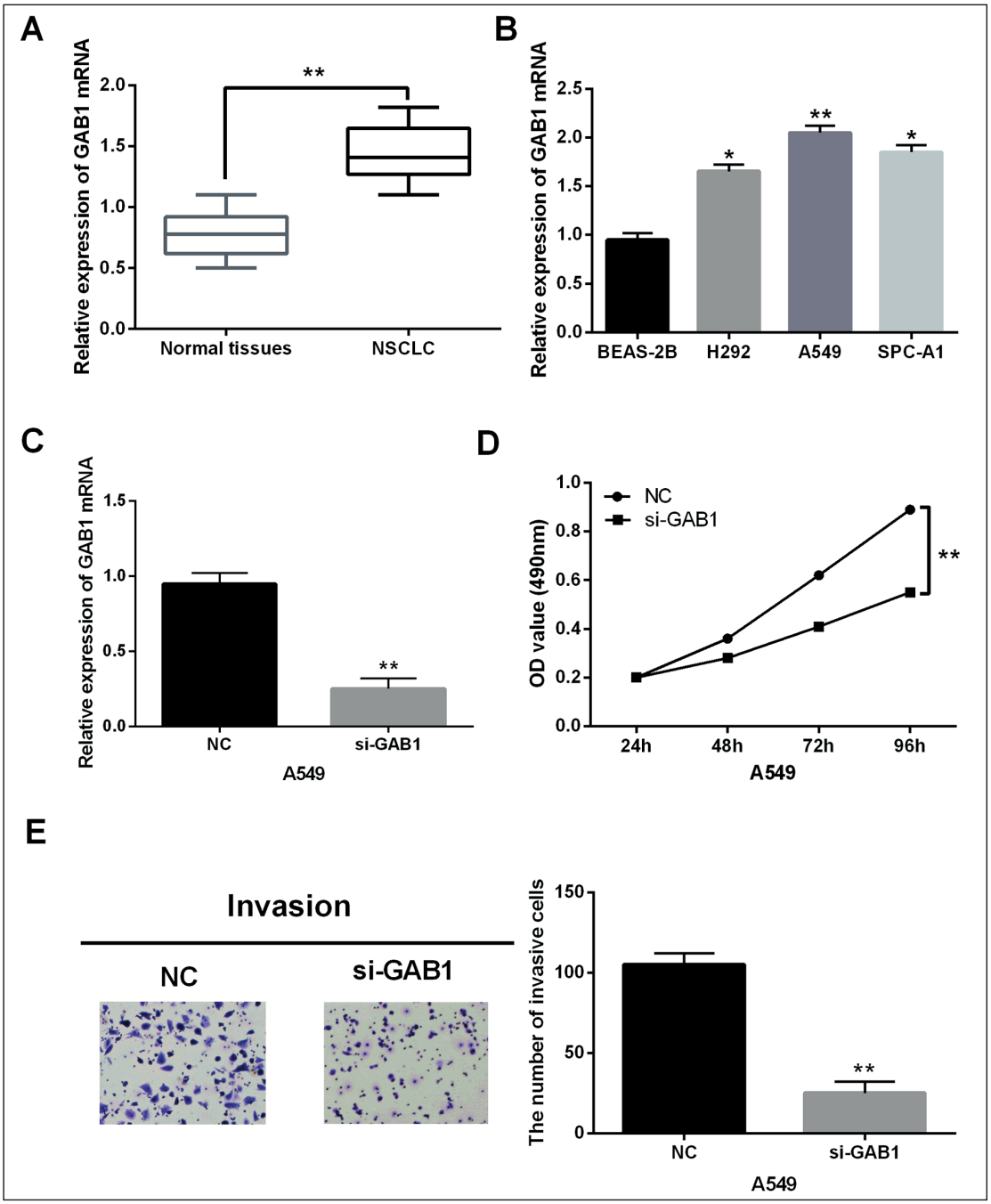

Figure 4. GAB1 knockdown suppressed cell proliferation and invasion in NSCLC. ***A,*** GAB1 expression in NSCLC tissues was detected *via* qRT-PCR. *B,* GAB1 expression in H292, A549, SPC-A1 and BEAS-2B cell lines. ***C,*** GAB1 expression was measured in cells transfected with GAB1 siRNA. ***D,*** The cell proliferation was measured in cells transfected with si-GAB1 *via* MTT. ***E,*** The invasion of A549 cells transfected with si-GAB1 was detected by transwell assay. *$p < 0.05$, ** $p < 0.01$.

Discussion

Previous studies indicated that many miRNAs are differentially expressed in NSCLC, which lead to the tumorigenesis of NSCLC. For example, miR-25 was upregulated and enhanced the migration and invasion of NSCLC cells[20]. MiR-1253 was downregulated and prevented cell proliferation and invasion through targeting WNT5A in NSCLC[21]. In the current research,

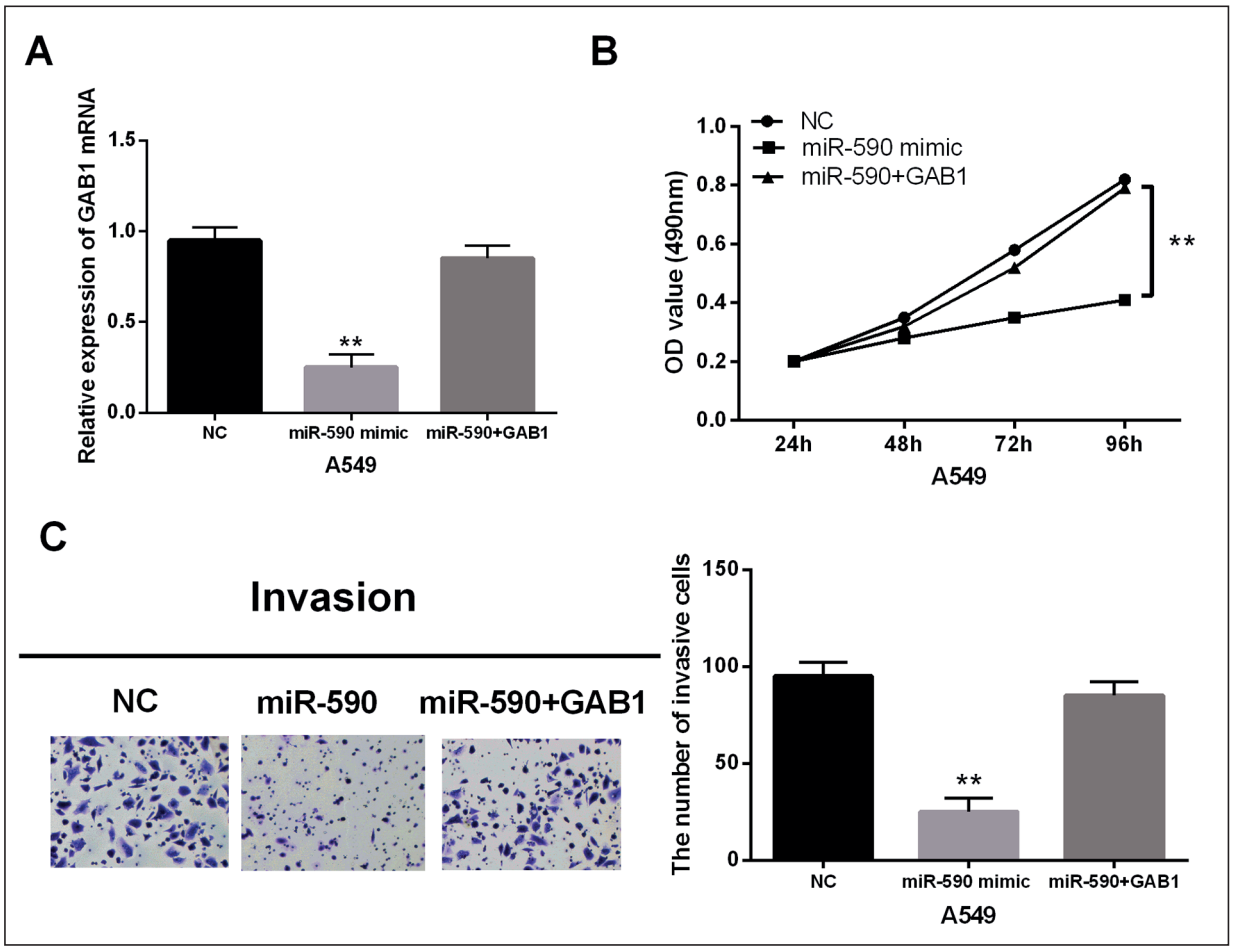

Figure 5. Overexpression of GAB1 partially inversed the suppressive effect of miR-590-5p on NSCLC. ***A,*** GAB1 expression was measured in A549 cells transfected with GAB1 vector and miR-590-5p. ***B,*** The cell proliferation was measured in A549 cells transfected with GAB1 vector and miR-590-5p *via* MTT. ***C,*** The cell invasion in A549 cells transfected with GAB1 vector and miR-590-5p was measured by transwell assay. $^{**}p < 0.01$.

downregulation of miR-590-5p was also identified in NSCLC tissues and cell lines. Overexpression of miR-590-5p was found to repress the proliferation and invasion of NSCLC cells. Based on the previous results, miR-590-5p was identified as a suppressive miRNA in NSCLC. MiR-590-5p showed different functions in various human cancers. It has been reported that miR-590-5p expression was increased in HepG2, Hep3B, and Huh7 hepatocellular carcinoma cell lines[22]. Wang et al[23] proposed that miR-590-5p expression was increased and promoted migration and invasion by regulating ARHGAP24 in renal cell carcinoma cells. Inversely, miR-590-5p was downregulated and inhibited tumorigenesis of colorectal cancer *via* targeting YAP1[24]. Similarly, we also identified the downregulation of miR-590-5p and its inhibitory effect on NSCLC. Moreover, miR-590-5p was reported to inhibit cell metastasis by regulating SOX2 in breast cancer[25]. In this study, miR-590-5p was identified to repress the invasion of NSCLC cells. Moreover, Song et al[26] found that miR-590-3p overexpression suppressed cell proliferation in breast cancer which was consistent with our findings in this study. Besides, we found that miR-590-5p expression was correlated with lymph node metastasis and TNM stage. Same as our results, Ma et al[27] demonstrated that miR-590 expression was obviously declined in lung cancer, which was related to lymph node metastasis and TNM stage. Furthermore, we also verified that miR-590-5p directly targeted GAB1 in NSCLC.

Served as an oncogene, GAB1 was reported to regulate the progression of human cancers. Moreover, GAB1 was upregulated in chronic lympho-

cytic leukemia[28]. The upregulation of GAB1 was also examined in NSCLC. Additionally, downregulation of GAB1 was found to inhibit cell proliferation in hilar cholangiocarcinoma[18]. GAB1 knockdown was reported to inhibit cell invasion in colorectal cancer[29]. In the present research, GAB1 knockdown inhibited cell proliferation and invasion in NSCLC. Besides, many miRNAs have been proposed to directly target GAB1, such as miR-150[28] and miR-5582-5p[30]. This study revealed that GAB1 was the direct target of miR-590-5p in NSCLC. Moreover, Feng et al[31] demonstrated that miR-141-3p inhibited fibroblast proliferation by targeting GAB1 in keloids. MiR-200a was found to inhibit cell invasion through directly regulating GAB1 in hepatocellular carcinoma[32]. Similarly, this study found that miR-590-5p suppressed the proliferation and invasion of NSCLC cells *via* downregulating GAB1 in this study.

Conclusions

In the present study, we observed the downregulation of miR-590-5p and upregulation of GAB1 in NSCLC. Moreover, we demostrated that miR-590-5p directly targeted GAB1 in NSCLC. MiR-590-5p was found to suppress the proliferation and invasion of NSCLC cells by regulating GAB1 expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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