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MicroRNA-424-5p inhibits the development of non-small cell LCa by binding to ITGB1

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Abstract. – OBJECTIVE: The aim of this study was to explore the effect of microRNA-424-5p on the proliferation and apoptosis of non-small cell lung cancer (NSCLC) cells, and to investigate its influence on the expression of ITGB1 and potential regulatory mechanism.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the level of microRNA-424-5p in 44 paired NSCLC tissues and adjacent tissues. The relation between microRNA-424-5p expression and NSCLC clinical indicators was analyzed. Subsequently, microRNA-424-5p mimics and inhibitors were transfected into NSCLC cells to construct microRNA-424-5p overexpression or knockdown models, respectively. QRT-PCR was used to further verify the transfection efficiency. A series of experiments, including cell counting kit-8 (CCK-8) assay, colony formation, 5-Ethynyl-2'-deoxyuridine (EdU), and flow cytometry were used to analyze the effect of microRNA-424-5p on the biological function of NSCLC A549 and H358 cells. Finally, the potential association between microRNA-424-5p and its downstream gene ITGB1 was explored through luciferase reporter gene assay and cell recovery experiment.

RESULTS: QRT-PCR results showed that microRNA-424-5p level was significantly lower in NSCLC tissues than that of adjacent normal tissues. Compared with patients with high expression of microRNA-424-5p, the pathological stage of those with low expression of microRNA-424-5p was significantly higher. *In vitro* experiments showed that microRNA-424-5p overexpression remarkably decreased cell proliferation and increased cell apoptosis, which were further validated in microRNA-424-5p inhibitor group. Subsequently, ITGB1 expression was found significantly up-regulated in NSCLC cell lines and tissues. Meanwhile, ITGB1 expression was negatively correlated with microRNA-424-5p level. In addition, a recovery experiment indicated that overexpression of ITGB1 could counteract the effect of microRNA-424-5p mimics on the proliferation and apoptosis of NSCLC cells. All these findings revealed that microRNA-424-5p and ITGB1 affected the malignant progression of NSCLC.

CONCLUSIONS: MicroRNA-424-5p was closely correlated with the pathological stage and poor prognosis of NSCLC, thereby inhibiting the occurrence and development of NSCLC.

Key Words:
MicroRNA-424-5p, ITGB1, NSCLC, Cell proliferation, Apoptosis.

Introduction

Lung cancer (LCa) is the leading cause of cancer-related death worldwide, with about 1.3 million deaths globally every year[1-3]. LCa is mainly divided into two types according to the degree of differentiation and morphological characteristics, including non-small cell LCa (NSCLC) and small-cell LCa (SCLC). NSCLC and SCLC account for about 85% and 15% of all patients, respectively[4,5]. Currently, various emerging technologies for early diagnosis of tumors and newly developed chemotherapy/targeted therapy can improve the efficacy of LCa. However, the five-year survival rate of patients with NSCLC is still lower than 15%, with a relatively high recurrence rate[6,7]. If NSCLC can be detected in the early localized stage, the 5-year survival rate can be up to 44%. The survival of LCa patients with lymph node metastasis and distant metastasis is significantly lower[8]. Therefore, the discovery of molecular biomarkers that can be used for prevention, diagnosis, and prognosis will help to improve the therapeutic effect of NSCLC patients[9,10].

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Micro RNAs (miRNAs) are a small class of non-coding, endogenous single-stranded RNAs that can regulate the gene expression at the post-transcriptional level. Meanwhile, they play an important role in cell development and oncogenic signaling pathways[11-13]. Some studies[14,15] have shown that miRNAs are abnormally expressed in tumors, acting as oncogenes or tumor suppressor genes. Several researches[11-13] have indicated that miRNA gene expression analysis can be used to distinguish the subtypes of NSCLC. Meanwhile, it can also be applied to predict the prognosis and recurrence of early NSCLC. Therefore, the abnormal expression of miRNAs in NSCLC cells is helpful to understand the pathogenesis of diseases, as well as the roles of miRNAs in tumorigenesis[16,17]. Reports[18,19] have demonstrated that microRNA-424-5p is associated with the occurrence and progression of multiple tumors, such as hepatocellular carcinoma and colon cancer. In this study, we explored the expression of microRNA-424-5p in NSCLC tissues. The relationship between the expression of microRNA-424-5p and clinicopathological factors and prognosis of NSCLC was analyzed as well.

Currently, there are some criteria for target prediction, such as complementary sequences of mRNAs and miRNA "seed" regions. This can be predicted by the binding sites of miRNAs in the 3′-untranslated regions (3′-UTRs) of different species. Other characteristics of recently identified target sites have also been confirmed[20]. Therefore, in the present work, we speculated through a bioinformatics analysis that microRNA-424-5p might target ITGB1 in NSCLC cells and inhibit the proliferation and apoptosis of NSCLC. Eventually, it could be used as a new direction for tumor-targeted therapy. In this research, the roles of microRNA-424-5p and ITGB1 in the development and progression of NSCLC were investigated. Moreover, the molecular regulatory mechanism of microRNA-424-5p on ITGB1 was explored. Our findings might bring new ideas for the diagnosis and treatment of NSCLC.

Patients and Methods

Patients and NSCLC Samples

NSCLC tissues and para-cancerous tissues were collected from 44 patients who underwent radical resection of NSCLC. No patients received any radiotherapy or chemotherapy before surgery. Pathological classification and staging criteria for NSCLC were performed in accordance with the international association of cancer (UICC) LCa staging criteria. Informed consent was obtained from patients and their families before the study. This research was approved by the Ethics Oversight Committee of our hospital.

Cell Lines and Reagents

Five human NSCLC cell lines (A549, H1299, PC-9, H358, SPC-A1) and one normal human bronchial epithelial cell line (BEAS-2B) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). The cells were cultured in DMEM medium containing 10% FBS in a 37°C, 5% CO_2 incubator.

Cell Transfection

Negative control (NC), microRNA-424-5p overexpressing sequence (microRNA-424-5p mimics) and microRNA-424-5p knockout sequence (microRNA-424-5p inhibitor) were purchased from Shanghai Jima Company (Shanghai, China). The cells were first plated into 6-well plates and grown to a density of 70%. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Next, the cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and functional experiments after 48 h.

Cell Proliferation Assay

48 h after transfection, the cells were harvested and plated into 96-well plates at a density of 2000 cells per well. Then, cells were cultured for 24, 48, 72, and 96 h, respectively. Cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added at different time points, followed by incubation for 2 h in the dark. Optical density (OD) value at the absorption wavelength of 490 nm was measured by a microplate reader.

Colony Formation Assay

After the density of cells reached 90%, the cells were scratched using a 100 μL tip with the backline of the plate as the reference line. The position and thickness of the scratches in each hole were kept the same. After scraping, the cells were washed with prepared phosphate-buffered saline (PBS) solution to removed necrotic cells. Subsequently, the medium containing 10% serum was

added into cells and cultured. At 6 and 24 h time points after the test, the cells were observed and photographed under a microscope.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

To detect the effect of miR-487a on proliferation ability, EDU assay (RiboBio, Nanjing, China) was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μm EDU for 2 h and stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI). The number of EDU-positive cells was detected by a fluorescence microscopy. The display rate of EDU positive was calculated as the ratio of the number of EDU positive cells to the number of total DAPI chromogenic cells (blue cells).

Flow Cytometry

A549 and H358 cells in logarithmic growth phase were first inoculated into 6-well plates. After 24 h of drug treatment, the cells were collected and washed twice with PBS. After re-suspension in the binding solution, cells ere incubated at room temperature for 15 min in dark. Next, 5 μL of AnnexinV-FITC and 5 μL of PI were added. Finally, the apoptosis rate was detected by flow cytometry.

QRT-PCR

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells and total RNA was extracted. Initially, the extracted RNA was treated with DNase I to remove genomic DNA and purify the RNA. RNA reverse transcription was performed according to the instructions of Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). Real-time PCR was performed in accordance with SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit. PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Three replicates were set for each sample and the experiment was repeated twice. Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process the data with software iQ5 2.0. β-actin and U6 were used as internal parameters for mRNA and miRNA, respectively. Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Luciferase Report Gene Assay

Transfected NSCLC cells in the logarithmic growth phase were first lysed. The relative luciferase value of each sample was measured by a luminometer. The detection principle was that when selected specific miRNA complementary bound to target gene sequences, the luciferase would not be expressed. Meanwhile, the relative fluorescence value finally measured would be less able to bind to the sequence.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS IBM, Armonk, NY USA) was used for all statistical analysis. Univariate analysis was performed using the χ^2-test and the exact probability Fisher test. Multivariate analysis was performed using COX regression analysis. The survival of patients was analyzed using the Kaplan-Meier method. Intergroup curves were compared using the Log-rank test. Experimental data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

MicroRNA-424-5p Was Downregulated in NSCLC Tissues and Was Related with Advanced Pathologic Staging and Poor Prognosis

QRT-PCR was performed to detect the expression of microRNA-424-5p in NSCLC tissues and cells. The results showed that the level of microRNA-424-5p in NSCLC tissues was significantly lower than that of adjacent tissues, and the difference was statistically significant (Figure 1A). In addition, microRNA-424-5p was significantly lowly expressed in NSCLC cells when compared with BEAS-2B cells (Figure 1B).

Subsequently, we analyzed the relationship between microRNA-424-5p expression with age, gender, pathological stage, lymph node metastasis, and distant metastasis of NSCLC patients. As shown in Table I, low expression of microRNA-424-5p was positively correlated with NSCLC pathological stage, whereas was not associated with age, gender, lymph node metastasis, and distant metastasis. The above results suggested that microRNA-424-5p might be a new biological indicator for predicting the malignant progression of NSCLC.

Upregulation of MicroRNA-424-5p Inhibited Cell Proliferation, Migration, and Promoted Cell Apoptosis

To explore the effect of microRNA-424-5p on the biological function of NSCLC cells, microR-

Table I. Association of miR-424-5p expression with clinicopathologic characteristics of lung cancer.

Parameters	No. of cases	MiR-424-5p expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.977
<60	17	10	7	
≥60	27	16	11	
Gender				0.329
Male	21	14	7	
Female	23	12	11	
T stage				0.001
T1-T2	27	21	6	
T3-T4	17	5	12	
Lymph node metastasis				0.064
No	29	20	9	
Yes	15	6	9	
Distance metastasis				0.077
No	33	22	11	
Yes	11	4	7	

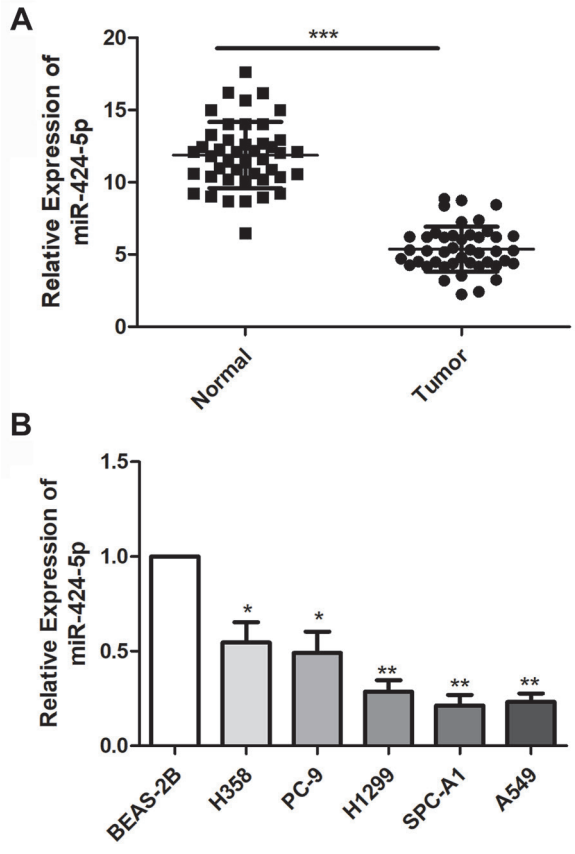

Figure 1. MiR-424-5p was lowly expressed in NSCLC tissues and cell lines. **A,** QRT-PCR was used to detect the expression of miR-424-5p in NSCLC tissues and adjacent tissues; **B,** QRT-PCR was used to detect the expression level of miR-424-5p in NSCLC cell lines. Data were mean ± SD, **p*<0.05, ***p*<0.01, ****p*<0.001.

NA-424-5p mimics and microRNA-424-5p inhibitor were transfected into cells. Transfection efficacy was verified by qRT-PCR (Figure 2A). Subsequently, cell proliferation, transwell migration, and flow cytometry were performed in A549 and H358 cell lines, respectively. CCK-8 assay, colony formation, and EdU experiments showed that the proliferation ability of microRNA-424-5p mimics group decreased significantly when compared with NC group (Figure 2B-2D). Subsequently, the effect of microRNA-424-5p on the apoptosis of NSCLC cells was explored by flow cytometry. Results demonstrated that the apoptosis of NSCLC cells in microRNA-424-5p mimics group was significantly promoted when compared with NC group. In sum, these results suggested that microRNA-424-5p promoted the apoptosis of NSCLC cells (Figure 2E).

ITGB1 Was Highly Expressed in NSCLC Tissues and Cell Lines

To further validate the targeting of microRNA-424-5p to ITGB1, luciferase reporter gene assay was performed. The results showed that overexpression of microRNA-424-5p significantly attenuated the luciferase activity of wild-type ITGB1 vector. This further demonstrated that ITGB1 could be targeted by microRNA-424-5p *via* this binding site (Figure 3A). In addition, microRNA-424-5p mimics was found to reduce the expression of ITGB1 in A549 and H358 cell lines. However, transfection of microRNA-424-5p inhibitor markedly up-regulated ITGB1 expression (Figure 3B).

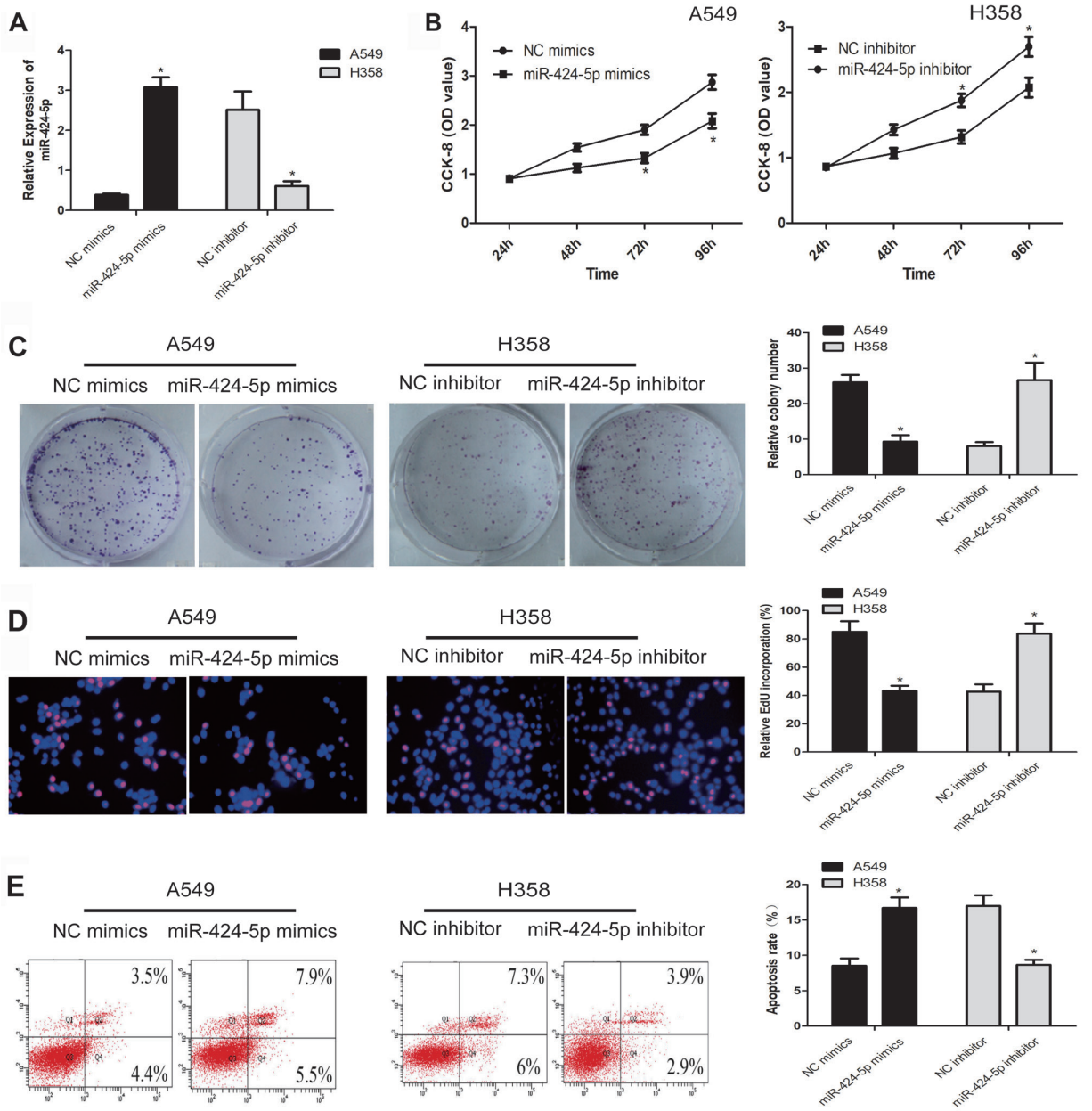

Figure 2. Overexpression of miR-424-5p inhibited proliferation and apoptosis of NSCLC cells. **A,** QRT-PCR verified the transfection efficiency after transfection of NC, miR-424-5p mimics and miR-424-5p inhibitor in A549 and H358 cell lines; **B,** CCK-8 assay detected the effects of miR-424-5p on the proliferation of A549 and H358 cell lines; **C,** Colony formation assay tested the ability of A549 and H358 cell lines to form lung-positive proliferating cells (magnification × 40); **D,** EdU assay detected the proliferation ability of A549 and H358 cell lines (magnification × 40); **E,** Flow cytometry assay was used to examine the apoptotic ability of A549 and H358 cell lines. Data were mean ± SD, *p<0.05.

ITGB1 level in NSCLC tissues and cell lines was verified by qRT-PCR. Results indicated that the level of ITGB1 was significantly up-regulated in NSCLC tissues when compared with adjacent tissues (Figure 3C). In addition, ITGB1 expression was significantly higher in NSCLC cells than BEAS-2B cells, and the difference was statistically significant (Figure 3D). Therefore, we examined the expressions of microRNA-424-5p and ITGB1 by qPCR in NSCLC tissues. The results showed that the level of microRNA-424-5p was negatively correlated with ITGB1 in NSCLC tissues (Figure 3E).

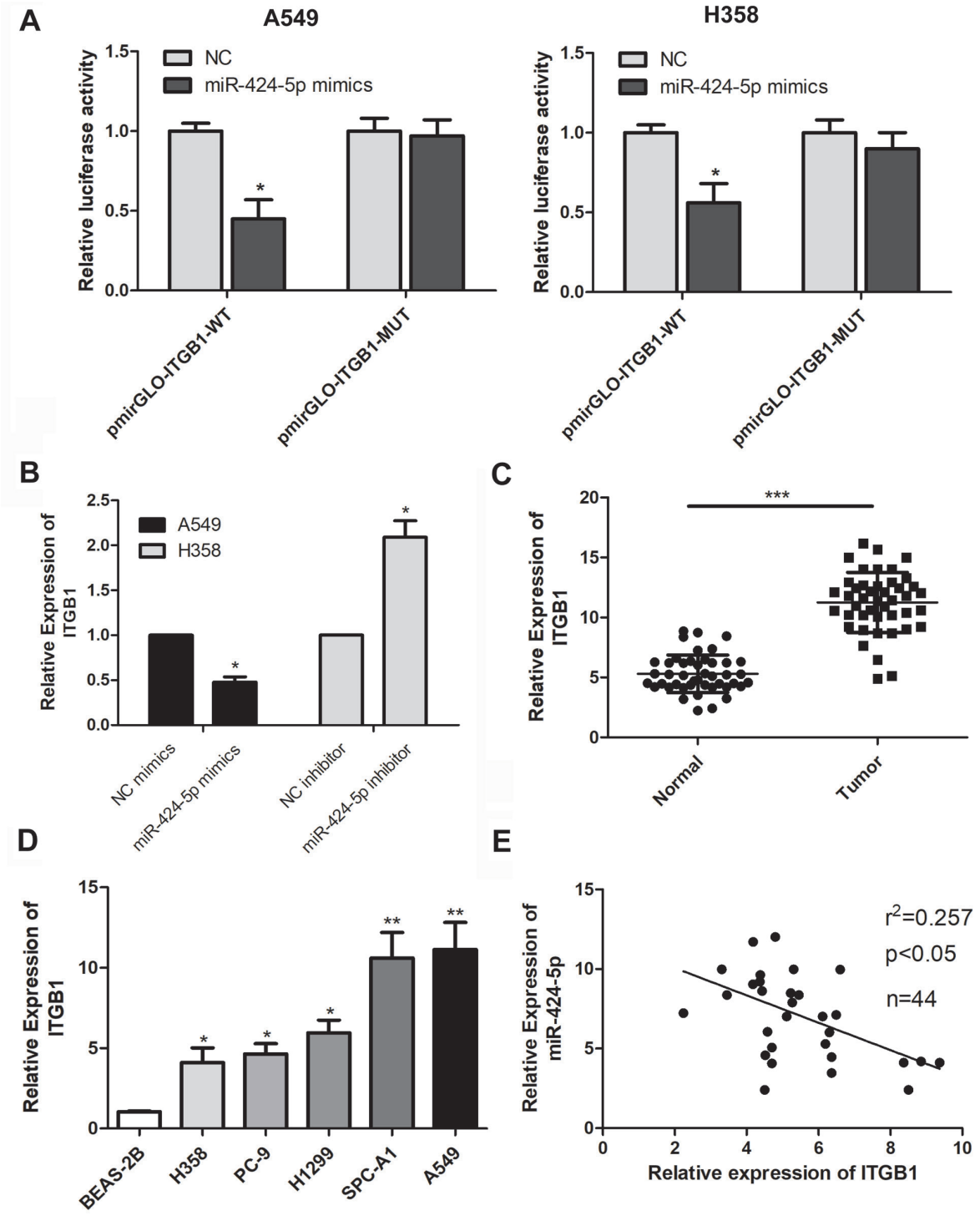

Figure 3. ITGB1 was highly expressed in NSCLC tissues and cell lines. **A,** Dual luciferase reporter assay validated the direct targeting of miR-424-5p to ITGB1; **B,** QRT-PCR verified the expression level of ITGB1 after transfection of miR-424-5p mimics and miR-424-5p inhibitor in A549 and H358 cell lines; **C,** QRT-PCR was used to detect the expression of ITGB1 in NSCLC tissues and adjacent tissues; **D,** QRT-PCR was used to detect the expression level of ITGB1 in NSCLC cell lines; **E,** MiR-424-5p was significantly negatively correlated with the expression level of ITGB1 in NSCLC tissues. Data were mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

MicroRNA-424-5p Inhibited ITGB1 Expression in NSCLC

To further explore the way in which microRNA-424-5p inhibited the malignant progression of NSCLC, ITGB1 was overexpressed in cells with microRNA-424-5p overexpression. Meanwhile, ITGB1 was knocked out in cells with microRNA-424-5p silence. Subsequently, their counteracting role in NSCLC was investigated. QRT-PCR assay was used to detect the transfection efficiency of ITGB1 (Figure 4A). The results demonstrated that overexpression/knockout of ITGB1 could

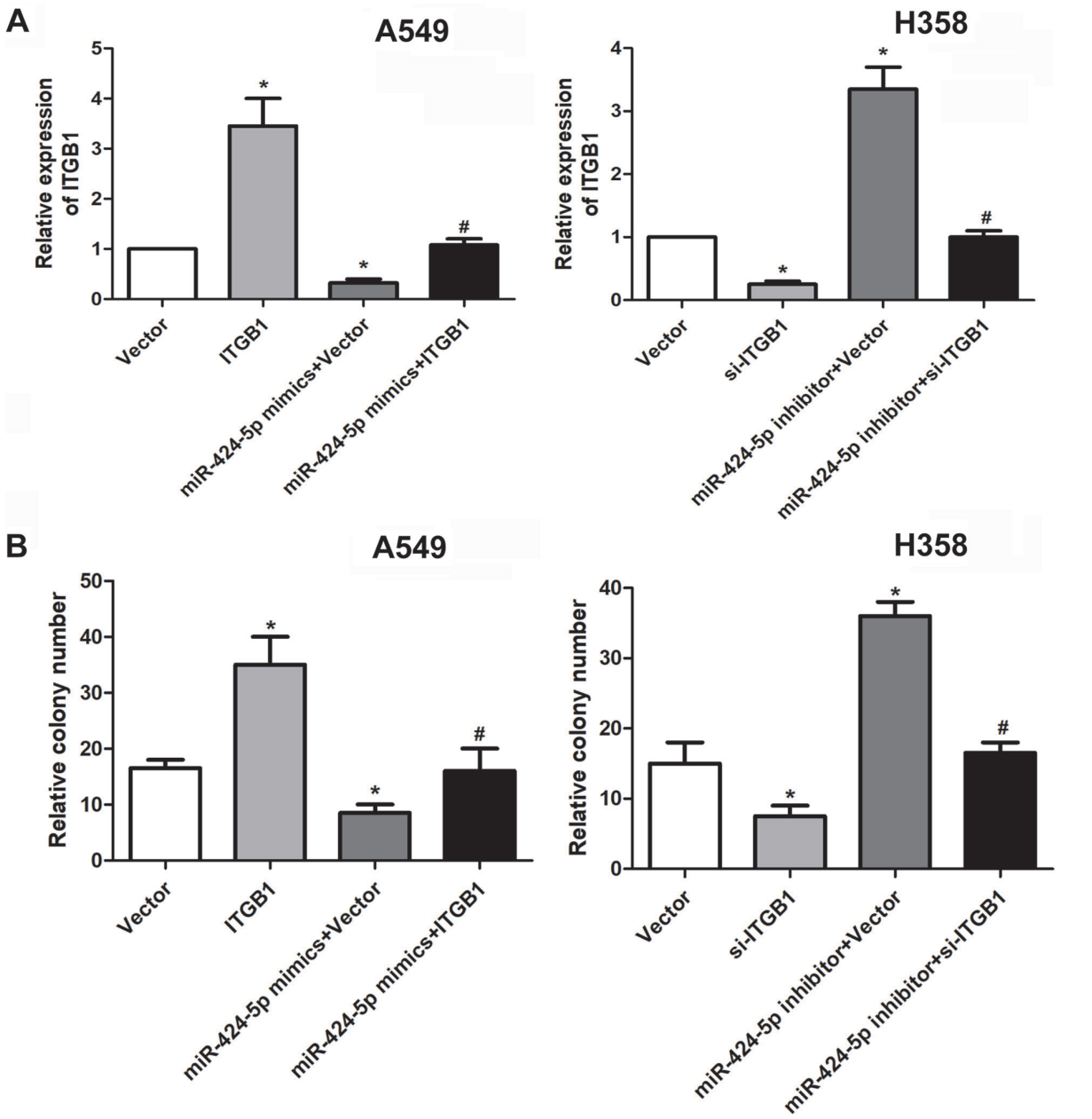

Figure 4. MiR-424-5p regulated the expression of ITGB1 in NSCLC tissues and cell lines. **A,** The expression level of ITGB1 in cells co-transfected of miR-424-5p and ITGB1 was detected by qRT-PCR; **B,** Colony formation assay was used to detect the proliferation ability of miR-424-5p and ITGB1 co-transfected NSCLC cells; **C,** EdU assay detected the proliferation of NSCLC cells after co-transfection of miR-424-5p and ITGB1; **D,** Flow cytometry was used to detect the role of miR-424-5p and ITGB1 in regulating the apoptosis of NSCLC cells. Data were mean ± SD, *#p<0.05.

Figure continued

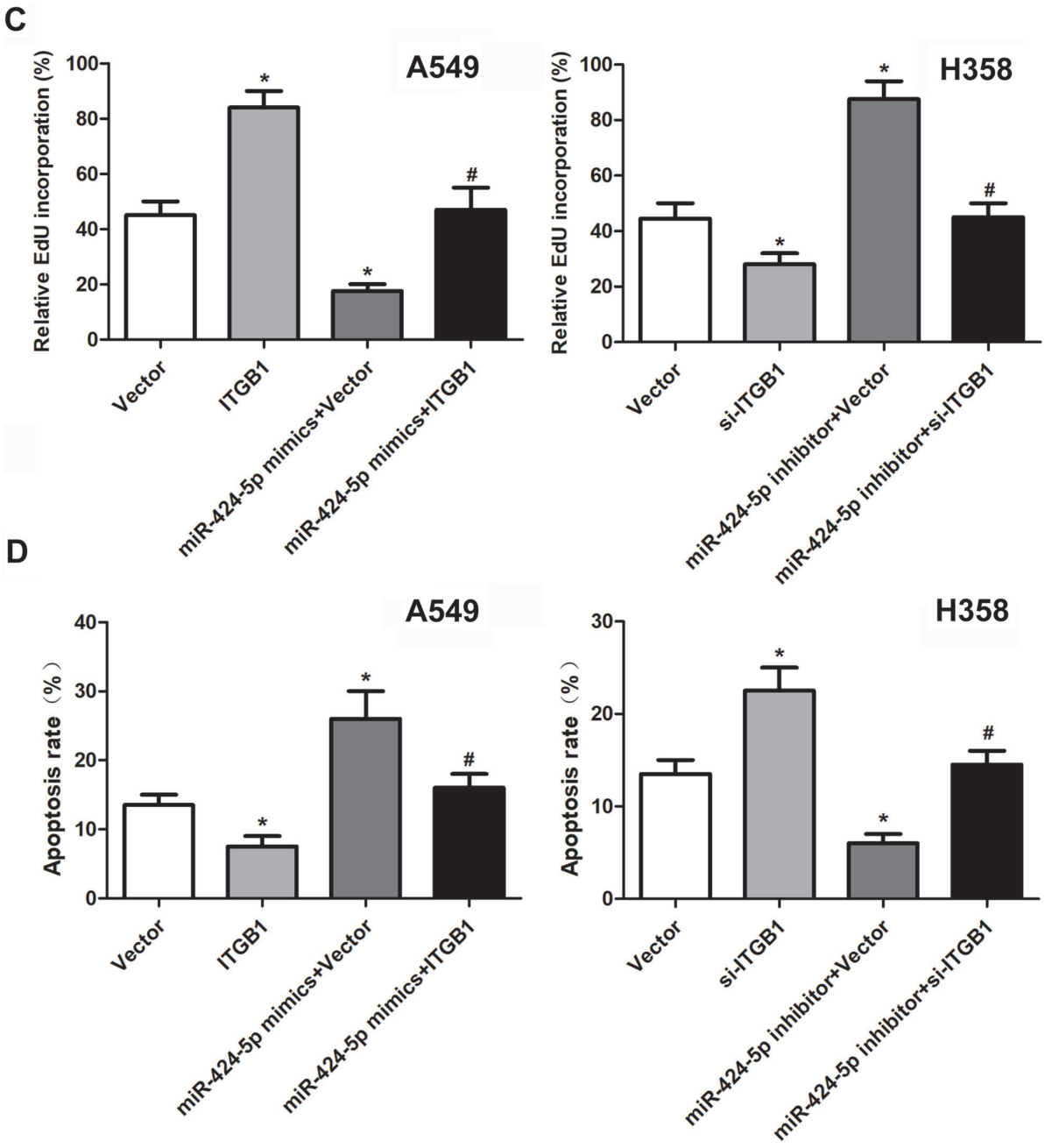

Figure 4. *(Continued)*- **C,** EdU assay detected the proliferation of NSCLC cells after co-transfection of miR-424-5p and .ITGB1; **D,** Flow cytometry was used to detect the role of miR-424-5p and ITGB1 in regulating the apoptosis of NSCLC cells. Data were mean ± SD, *#p<0.05.

counteract the effect of microRNA-424-5p on the proliferation and apoptosis of NSCLC cells by colony formation, EdU and flow cytometry experiments (Figure 4B-D). In sum, our findings demonstrated that microRNA-424-5p regulated NSCLC through inhibiting ITGB1 expression.

Discussion

LCa is one of the main causes of cancer-related deaths in both males and females. In recent years, epidemiological investigations have found that the incidence and mortality of LCa are on the rise[1-3].

A large number of studies have shown that long-term and heavy smoking is an important risk factor for LCa. The younger the onset of smoking and the longer the history of smoking both indicate a higher risk of LCa. Moreover, studies[4,5] have indicated that smoking women are more likely to develop LCa than men. In addition, the incidence of LCa among urban residents is higher than that of rural areas. This may be closely related to air pollution, industrial exhaust emissions, and haze in the process of urban development. Therefore, we should be committed to smoking cessation and prohibition, and vigorously deal with environmental pollution[5,6]. With the development of molecular biology, targeted individualized treatment should be performed on the basis of comprehensive treatment of LCa. This may help to improve the prognosis and life quality of LCa patients[7]. Therefore, more and more attention has been paid to investigating molecular prognostic markers of tumors. Meanwhile, molecular markers that can effectively predict the prognosis of LCa have been widely explored. Currently, potential new therapeutic targets for LCa has become a hot topic in relevant research[8-10].

MiRNA has attracted much attention in recent years, which has been found to play an important role in the occurrence and development of a variety of tumors[11-13]. MiRNA is a highly conserved endogenous non-coding short chain RNA composed of 19-24 nucleotides. It can regulate gene expression through binding to the 3-UTR region of target mRNAs to degrade it or inhibiting the translation process[13,14]. MiRNA can be accurately detected in tumor specimen tissues and blood, greatly reducing the difficulty of research and application[15]. Currently, researches have found that about 1/3 of human genes are regulated by miRNA. Moreover, miRNA is widely involved in the occurrence, development, and metastasis of various tumors. Different types of miRNA exert different roles in tumors, which may promote or inhibit the occurrence, development, and metastasis of malignancies[15-17]. MicroRNA-424-5p has been discovered for a long time. However, its biological function has not been fully elucidated. Some studies[18,19] have suggested that microRNA-424-5p can inhibit tumors and participate in many physiological and pathological processes. In this work, to explore the role of microRNA-424-5p in the occurrence and development of NSCLC, qRT-PCR was first used to detect the expression of microRNA-424-5p in NSCLC tissues and adjacent tissues. The results found that the expression of microRNA-424-5p was significantly down-regulated in NSCLC tissues. The expression of microRNA-424-5p in NSCLC tissues was significantly lower than that of adjacent tissues. Therefore, we suggested that microRNA-424-5p might act as a tumor suppressor gene in NSCLC. To further investigate the effect of microRNA-424-5p on the biological function of NSCLC cells, microRNA-424-5p mimics, and microRNA-424-5p inhibitor were transfected into cells. The results of CCK8 assay, colony formation, EdU, and flow cytometry showed that microRNA-424-5p could inhibit the proliferation of NSCLC and promote cell apoptosis. The above results indicated that microRNA-424-5p was significantly down-regulated in NSCLC. However, the exact molecular mechanism remained unclear.

Currently, to elucidate the role of microRNA-424-5p in regulating the function of tumor cells, a variety of classical detection techniques have been widely. In recent years, there are several computer computation-based target gene prediction software available, such as TargeScan, microRNA, PicTar, miRbase, etc. All these prediction software generally adopt a strategy combining multiple algorithms to screen the potential target genes[20]. The verification method of the luciferase reporter gene assay has been described as the "gold standard". For example, luciferase signal of luciferase reporter gene containing the 3'UTR of the target gene can be inhibited by high expression of miRNA. Meanwhile, the inhibitory effect can be canceled after the target sequence point mutation. Therefore, target genes can be determined[18-20]. Bioinformatics analysis predicted that microRNA-424-5p showed an inhibitory effect by targeting ITGB1. QRT-PCR results illustrated that ITGB1 promoted the malignant progression of NSCLC cells. We then investigated the effect of the interaction between microRNA-424-5p and ITGB1 on the occurrence and development of NSCLC. The results further verified that microRNA-424-5p mimics could significantly down-regulate the expression of ITGB1. Subsequently, ITGB1 was found to counteract the proliferation and apoptosis of microRNA-424-5p in NSCLC cells.

Conclusions

MicroRNA-424-5p was significantly associated with pathological stage and poor prognosis of NSCLC and could inhibit the malignant progres-

sion of NSCLC. Also, microRNA-424-5p might inhibit the proliferation and promote the apoptosis of NSCLC cells by regulating ITGB1.

Conflicts of interest

The authors declare no conflicts of interest.

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