European Review for Medical and Pharmacological Sciences 2020; 24: 4288-4297

MicroRNA-597 inhibits NSCLC progression through negatively regulating CDK2 expression

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Abstract. – OBJECTIVE: Previous studies have shown that microRNA-597 serves as a tumor suppressor gene. However, the role of microRNA-597 in non-small cell lung cancer (NSCLC) has not been fully elucidated. Therefore, the aim of this study was to investigate the expression of microRNA-597 in NSCLC, and to further explore the possible underlying mechanism.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (qPCR) was performed to examine microRNA-597 level in tumor tissues and para-cancerous normal tissues collected from 50 patients with NSCLC. The interplay between microRNA-597 expression and clinical indicators, as well as prognosis of NSCLC patients, was analyzed. Meanwhile, qPCR was used to verify microRNA-59 level in NSCLC cell lines. Subsequently, microRNA-597 overexpression and knockdown models were constructed using lentivirus in NSCLC cell lines (including H1299 and PC-9). The impacts of microRNA-597 on the biological functions of NSCLC cells were evaluated using cell counting kit-8 (CCK-8), colony formation, and 5-Ethynyl-2'-deoxyuridine (EdU) assay, respectively. Finally, luciferase reporter gene assay and recovery experiment were performed to investigate the underlying molecular mechanism.

RESULTS: QPCR results indicated that microRNA-597 level in NSCLC tissues was remarkably lower than that of adjacent normal tissues, and the difference was statistically significant ($p<0.05$). Compared with patients with high expression of microRNA-597, patients with low expression of microRNA-597 exhibited significantly higher incidence of pathological stage and lower overall survival rate ($p<0.05$). Similarly, compared with NC group, the proliferation ability of NSCLC cells was remarkably weakened in microRNA-597 overexpression group ($p<0.05$). However, the opposite results were observed in microRNA-597 inhibitor group ($p<0.05$). CDK2 expression was found remarkably elevated in NSCLC cell lines as well as in tissue samples CDK2 expression. Meanwhile, CDK2 expression was negatively correlated with microRNA-597 expression. Luciferase reporter gene assay demonstrated that overexpression of CDK2 could significantly attenuate the luciferase activity of wild-type microRNA-597 vector without attenuating that of mutant vector CDK2 expression. This further suggested that microRNA-597 could target bind to CDK2. Furthermore, cell recovery experiment revealed that CDK2 could reverse the impact of microRNA-597 on the malignant progression of NSCLC.

CONCLUSIONS: MicroRNA-597 expression was significantly down-regulated in NSCLC tissues, as well as cell lines. Meanwhile, microRNA-597 expression was associated with the pathological staging and poor prognosis of patients with NSCLC. In addition, microRNA-597 might suppress the malignant progression of NSCLC through the regulation of CDK2.

Key Words:
MicroRNA-597, CDK2, Non-small cell lung cancer (NSCLC), Malignant progression.

Introduction

Lung cancer is one of the most common malignant tumors. Its mortality rate ranks first in both male and female tumors worldwide. Meanwhile, it is also the leading cause of tumor-related deaths[1-3]. Morbidity and mortality of lung cancer in the United States have shown a downward trend in recent years. However, in China, they are still on the rise year by year. According to the latest statistics released by the National Cancer Center in 2017, the number of new malignant tumors in China is about 3.68 million, accounting for 1/4 of the world (368,000/14.09 million). The number of new cancers continues to rise over the past few years, making cancer one of the chief culprits in taking people's health and even life[4,5]. With the development of industry, air pollution and haze are also the factors leading to high incidence of lung cancer, especially female lung adenocarcinoma. In recent years, it has present-

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ed a trend of more urban than rural areas, more female adenocarcinoma patients than before, and more young patients than before[6-8]. Currently, considerable advancement has been made in the treatment of lung cancer, such as minimally invasive surgery, precision radiotherapy, targeted therapy, immunotherapy, and radiofrequency ablation. However, the overall 5-year survival rate is still low, which is about 10-20%[9-12].

The main causes of poor prognosis of lung cancer patients include recurrence and metastasis of tumor cells. About 80% of lung cancer patients have already in local advanced stage or showed distant metastasis when first received treatment. Meanwhile, postoperative recurrence and metastasis are the primary causes of treatment failure or death[13,14]. With in-depth research on the occurrence and development of lung cancer and the molecular mechanism of cell invasion and metastasis, a variety of tumor molecular markers have been indicated to be applied in the early detection of clinical lung cancer patients, the monitoring of tumor progression and treatment effect, as well as the prediction of prognosis[15,16].

MicroRNAs are a kind of highly conserved non-coding short-strand RNAs composed of 19-24 nucleotides[17-19]. They mainly bind to the 3′-untranslated region (3′-UTR) of target genes to degrade the mRNA of target genes or inhibit their translation process. This may help to regulate the expression of target genes[19,20]. More than one-third of human genes are regulated by miRNAs[20,21]. One miRNA can regulate as many as 200 genes, while one gene can also be co-regulated by multiple miRNAs. MiRNAs have different roles in different tumors, serving as oncogenes or tumor suppressor genes[22,23]. MicroRNA-597 is one of the hottest molecules in recent years, which has been proved to play a significant role in a variety of tumors. Furthermore, it can also be engaged in the proliferation, invasion, and metastasis of tumor cells and the prognosis of patients[22,23].

In this study, we detected the expressions of microRNA-597 and CDK2 in non-small cell lung cancer (NSCLC) tissues and adjacent normal tissues. Previous studies have indicated that microRNA-597 and CDK2 play a certain role in the malignant growth of tumor cells and other processes, thus affecting tumor development. Therefore, the purpose of this study was to explore the mechanism by which microRNA-597 affected clinical parameters, prognosis, and malignant progression of NSCLC by targeting CDK2.

Patients and Methods

Patients and NSCLC Samples

50 pairs of NSCLC tissues and adjacent normal tissues were collected from patients in this study. All specimens were obtained from surgical specimens of the Department of Oncology, Thoracic Surgery, and Respiratory Medicine, as well as through biopsy or bronchoscopy. Para-cancerous tissues were taken more than 5 cm away from cancerous tissues. No anti-tumor treatment such as radiotherapy or chemotherapy was performed for any patient before surgery. The investigation was approved by the Ethics Committee of the hospital. Informed consent was obtained from all patients before the study. All patients were followed-up after discharge, including general conditions, clinical symptoms, and imaging examination.

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 American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Cells were cultured in DMEM medium containing 10% FBS in a 37°C incubator with 5% CO_2.

Cell Transfection

Control group (NC mimics or NC inhibitor) and lentivirus (microRNA-597 mimics or microRNA-597 inhibitor) containing microRNA-597 overexpression and knockdown sequences were purchased from Shanghai Jima Company (Shanghai, China). Cells were first plated into 6-well plates and grown to 40% of cell density. Cell transfection was then performed according to the manufacturer's instructions. After 48 h, transfected cells were collected for qPCR analysis and cell function experiments.

Cell Proliferation Assay

Transfected cells after 48 h were collected and plated into 96-well plates at 2000 cells per well. After culture for 24 h, 48 h, 72 h, and 96 h, respectively, cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well, followed by incubation for 2 h in the dark. Optical density (OD) of each well at

the absorption wavelength of 490 nm was finally measured by a micro-plate reader.

Colony Formation Assay

Transfected cells after 48 h were first collected. 200 cells were seeded into each well of 6-well plates and cultured in complete medium for 2 weeks. The medium was changed after one week and then twice a week. Culture medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, formed colonies were fixed in 2 mL of methanol for 20 min and stained with 0.1% crystal violet staining solution for 20 min. After washing 3 times with phosphate-buffered saline (PBS), formed colonies were photographed and counted under a light-selective environment.

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

To demonstrate the proliferation ability of NSCLC cells, EDU proliferation 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 fluorescence microscopy. The display rate of EDU positive was shown as the ratio of the number of EDU positive cells to the number of total DAPI chromogenic cells (blue cells).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA in tissues and cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA). Subsequently, 2 μg of total RNA was added to a 20 μL system for cDNA synthesis. Real-time PCR was performed using 2xSYBR Green PCR Master Mix, with an appropriate amount of complementary deoxyribose nucleic acid (cDNA) taken as a template, primer concentration as 0.4 mol/L, and 15 μL system for amplification. Three parallel samples were set for each sample to be tested. PCR reaction was carried out on a quantitative PCR reactor. The expressions of genes were calculated by the formula RQ=2-ΔΔCt. This experiment was repeated for three times.

Primers used in this study were as follows: microRNA-597: forward: 5'-ACACTCCAGCTGGGTGTG TCACTCGATGAC-3', reverse: 5'-TGGTGTCGTGGAGTCG-3'; U6: forward: 5'-ATTGGAACGATACAGAGAAGATT-3', reverse: 5'-GGAACGCTTCACGAATTTG-3; CDK2: forward: 5'-AACTGGCCCTTCTTGGA-3', reverse: 5'-TCGTCATCTGGCTCCC-3; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. Data analysis was performed using ABI Step One software.

Western Blot

Transfected cells were first lysed using cell lysis buffer and shaken on ice for 30 minutes, followed by centrifuged at 14,000g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). Subsequently, extracted proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. Primary antibodies against CDK2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and corresponding secondary antibodies were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-Luciferase Reporter Assay

A reporter plasmid was first constructed, in which a specific fragment of target promoter was inserted in front of the luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected into H1299 and PC-9 cell lines with the reporter plasmid. The activity of the luciferase was determined by detection of the intensity of the fluorescence. Finally, whether the transcription factor could interact with target promoter fragment was determined.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for all statistical analysis. Difference in the expression of microRNA-597 in NSCLC tissues and adjacent normal tissues was estimated through analysis of variance, followed by Post-Hoc Test (Least Significant Difference). MicroRNA-597 level in NSCLS tissues and its association with various clinic-pathological parameters were analyzed by Chi-Square test. The relationship between mi-

croRNA-597 expression and survival time, as well as the prognosis of patients was analyzed by Kaplan-Meier method. Factors affecting the prognosis of NSCLC were analyzed using the Cox proportional hazards model. Experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$). $p<0.05$ was considered statistically significant.

Results

MicroRNA-597 Was Lowly Expressed in NSCLC Tissues and Cell Lines

MicroRNA-597 expressions in 50 pairs of NSCLC tissues and adjacent normal tissues, as well as in NSCLC cell lines were detected using qPCR assay. The results revealed that microRNA-597 expression in NSCLC tissues was remarkably reduced when compared with adjacent normal tissues (Figure 1A, 1B). Compared to BEAS-2B cells, microRNA-597 was lowly expressed in NSCLC cell lines (Figure 1C). Among all the cell lines, H1299 cells expressed the lowest while PC-9 cells expressed the highest level of microRNA-597. Therefore, these two cell lines were selected for transfection and subsequent cell experiments.

MicroRNA-597 Expression Was Correlated with Pathological Staging and Overall Survival in NSCLC Patients

NSCLC patients were divided into high expression and low expression group based on microRNA-597 expression. Subsequently, Chi-square test was conducted to analyze the relationship between microRNA-597 level and age, gender, pathological stage, as well as the situation of distant metastasis of NSCLC patients. As shown in Table I, low expression of microRNA-597 was not associated with age, gender, or distant metastasis of NSCLC patients, whereas was correlated with pathological stage. To further explore the relationship between microRNA-597 level and the prognosis of NSCLC patients, Kaplan-Meier survival curve was performed. The results showed that low expression of microRNA-597 was remarkably associated with poor prognosis of NSCLC ($p<0.05$; Figure 1D).

MicroRNA-597 Enhanced the Proliferation Ability of NSCLC Cells

To investigate the influence of microRNA-597 on the proliferation of NSCLC cells, microRNA-597 overexpression and knockdown models were successfully constructed. QPCR was conducted to verify the transfection efficiency (Fig-

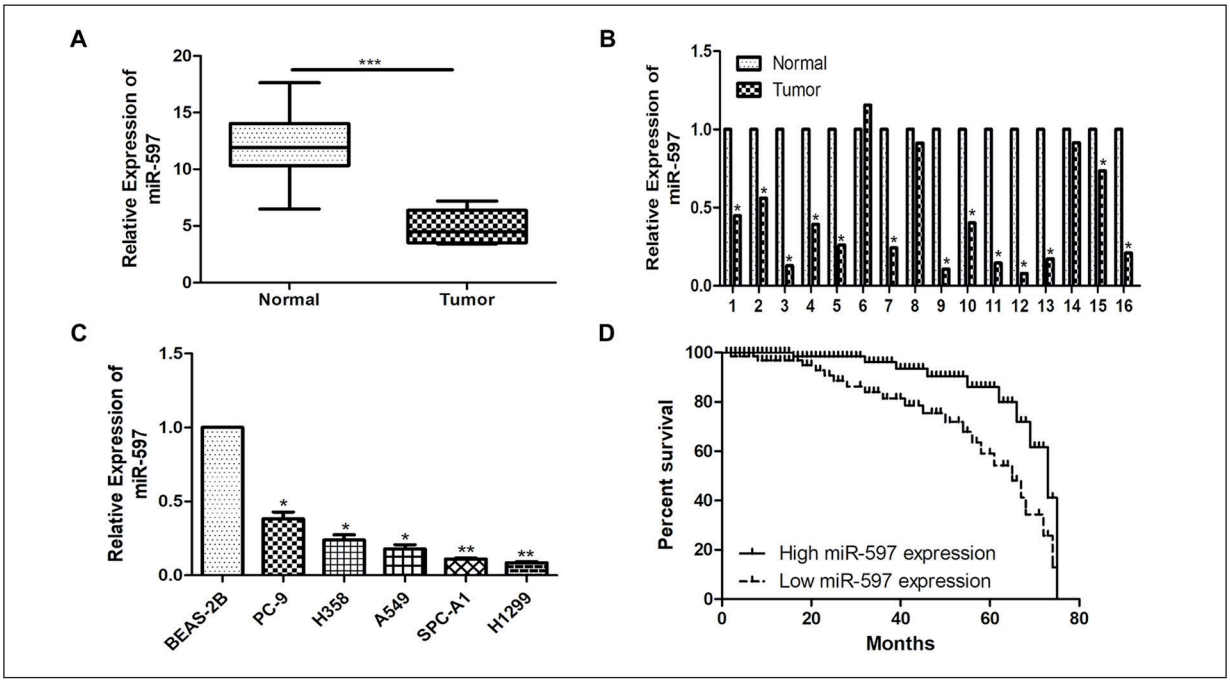

Figure 1. MiR-597 is lowly expressed in NSCLC tissues and cell lines. **A, B,** QRT-PCR was used to detect the expression of miR-597 in NSCLC tissues and adjacent normal tissues; **C,** QRT-PCR was used to detect the expression level of miR-597 in NSCLC cell lines; **D,** Kaplan Meier survival curve of lung cancer patients based on miR-597 expression showed that the prognosis of patients with low expression was significantly worse than that of high expression group. Data were expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of miR-597 and CDK2 expression with clinicopathologic characteristics of lung small cell lung cancer.

Parameters	No. of cases	MiR-597 expression		*p*-value	CDK2 expression		*p*-value
		High (%)	Low (%)		High (%)	Low (%)	
Age (years)				0.248			0.556
< 60	20	12	8		7	13	
≥ 60	30	13	17		13	17	
Gender				0.396			0.564
Male	25	14	11		9	16	
Female	25	11	14		11	14	
T stage				0.037			0.021
T1-T2	33	20	13		17	16	
T3-T4	17	5	12		3	14	
Distance metastasis				0.083			0.077
No	30	18	12		9	21	
Yes	20	7	13		11	9	

ure 2A). Subsequent CCK-8, colony formation, and EDU experiments indicated that, compared with NC group, the proliferation ability of H1299 cells in microRNA-597 overexpression group was remarkably attenuated. Conversely, the proliferation of PC-9 cells was remarkably enhanced in microRNA-597 inhibitor group (Figure 2B-2D).

CDK2 was a Direct Target of MicroRNA-597

To further validate the targeting of CDR2 to microRNA-597, luciferase reporter gene assay was performed. The results indicated that overexpression of CDK2 remarkably attenuated the luciferase activity of wild-type microRNA-597

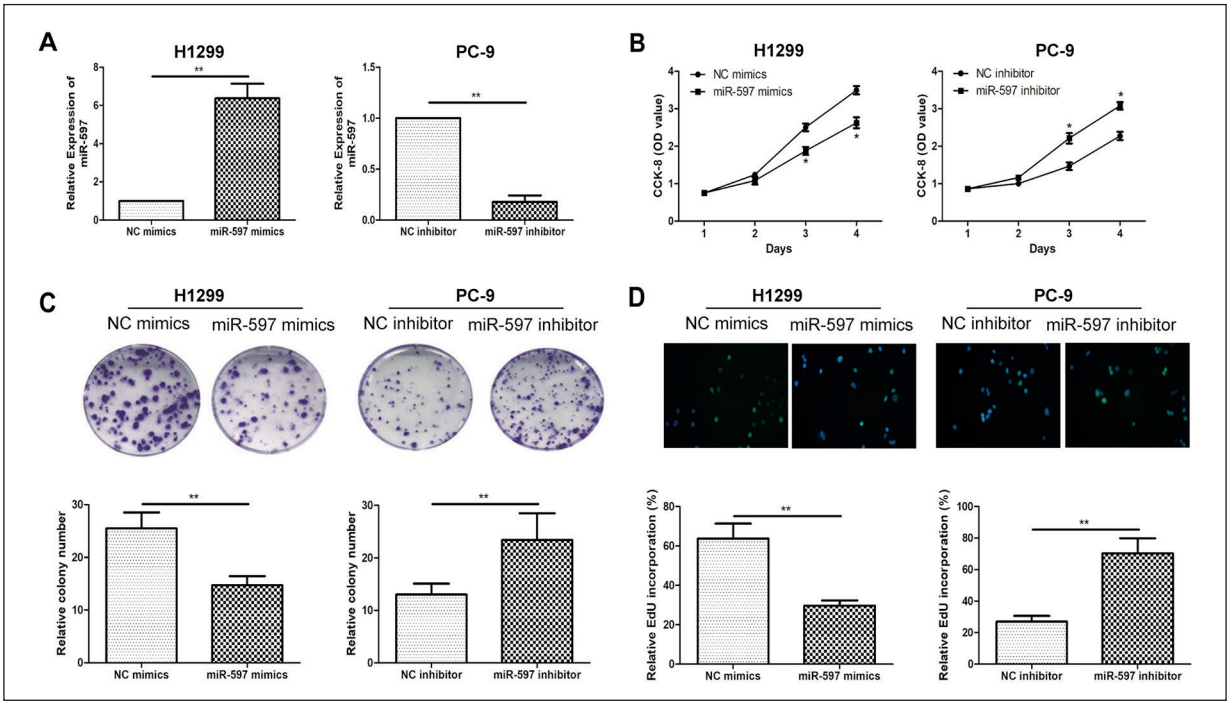

Figure 2. MiR-597 promotes the proliferation of NSCLC cells. **A,** QRT-PCR verified the transfection efficiency of miR-597 overexpression vector in H1299 cells and miR-597 knockdown vector in PC-9 cells; **B,** CCK-8 assay detected the effect of miR-597 on the proliferation of NSCLC cells (magnification: 10×); **C,** Colony Formation Assay detected the number of formed colonies in H1299 and PC-9 cell lines (magnification: 20×); **D,** EDU assay detected the number of NSCLC-positive proliferating cells in H1299 and PC-9 cell lines (magnification: 20×). Data were expressed as mean ± SD, **p*<0.05, ***p*<0.01.

vector ($p<0.05$) without attenuating that of mutant vector ($p>0.05$) or empty vector ($p>0.05$). This further demonstrated that microRNA-597 could target combine with CDK2 (Figure 3A and 3B). In addition, Western Blotting revealed that the protein expression of CDK2 was significantly down-regulated after overexpression of microRNA-597. However, the opposite results were observed after knockdown of microRNA-597 (Figure 3C). QPCR results revealed that CDK2 expression level in NSCLC tissues was remarkably higher than adjacent normal tissues (Figure 3D). Similarly, compared with BEAS-2B cells, CDK2 was found remarkably highly expressed in NSCLC cell lines, and the difference was statistically significant (Figure 3E). To further figure out the interplay between CDK2 expression and the prognosis of NSCLC patients, Kaplan-Meier survival curve was plotted. The results illustrated that high CDK2 expression was significantly associated with poor prognosis of NSCLC ($p<0.05$; Figure 3F). In addition, qPCR results demonstrated that microRNA-597 expression was negatively correlated with CDK2 expression in NSCLC tissues (Figure 3G).

CDK2 Modulated MicroRNA-597 Expression in Human NSCLC Cells

To further explore whether microRNA-597 suppressed the malignant progression of NSCLC through CDK2, bioinformatics analysis and luciferase reporter gene assay were performed to search for and verify a possible association between CDK2 and microRNA-597. Next, we simultaneously knocked down or overexpressed CDK2 along with microRNA-597 in NSCLC cell lines to figure out the interaction between microRNA-597 and CDK2 in NSCLC cells. QPCR assay was applied to confirm the transfection efficiency (Figure 4A). CCK-8 and colony formation assays revealed that CDK2 could counteract the influence of microRNA-597 on the proliferation ability of NSCLC cells (Figure 4B and 4C).

Discussion

Tumor recurrence and metastasis are the main causes of treatment failure and death in patients with lung cancer. Currently, recurrence and metastasis occur in about 35% of stage I NSCLC patients, which is higher in advanced lung cancer patients. Current treatments are not effective in reducing the recurrence rate of lung cancer patients[1-5]. Therefore, the mechanism engaged in NSCLC cell proliferation, as well as its regulatory genes and signaling pathways is a hotspot in the field of cancer research[13,14]. They have been confirmed commonly involved in the occurrence and development of malignant tumors. Furthermore, searching for miRNAs with abnormal expression in NSCLC and analyzing their correlations with clinical prognosis of patients will contribute to the advancement of the diagnosis and treatment level of NSCLC and the improvement of the clinical prognosis of NSCLC patients[22,23].

MiRNA is a kind of small non-coding RNA. It can regulate the expression of target genes by directly degrading target mRNAs or inhibiting the translation of target genes[17-19]. One miRNA can regulate the expression of multiple target genes due to the reason that the combination of miRNA and its target gene mRNA does not require perfect complementarity of seed sequences. Similarly, one gene can also be co-regulated by multiple miRNAs[20]. Currently, multiple miRNAs have been found to play a pivotal regulatory role in the occurrence and development of tumors. Abnormally expressed miRNAs have been confirmed in liver cancer, prostate cancer, breast cancer, and other malignant tumors. Some miRNAs can even be used as prognostic markers[21-23]. Therefore, exploration of abnormally expressed miRNAs in NSCLC and analysis of their function will be helpful to elevate the diagnosis and treatment of patients. In this study, expressions of microRNA-597 and CDK2 were first detected in 50 pairs of NSCLC tissues and adjacent normal tissues. The results showed that microRNA-597 level was remarkably down-regulated, while CDK2 was up-regulated. In addition, the expression of microRNA-597 was found to be positively correlated with the pathological staging of NSCLC patients. Consequently, it was believed that microRNA-597 played an anti-cancer role, while CDK2 acted as an onco-gene in NSCLC. At the same time, the recurrence rate of patients with low expression of miR-597 increased significantly after 5 years. However, the overall survival rate decreased significantly after 5 years. This indicated that miR-597 played an important role in the malignant progression of NSCLC. To further explore the effects of microRNA-597 and CDK2 on NSCLC cell functions, microRNA-597 overexpression/knockdown models were constructed.

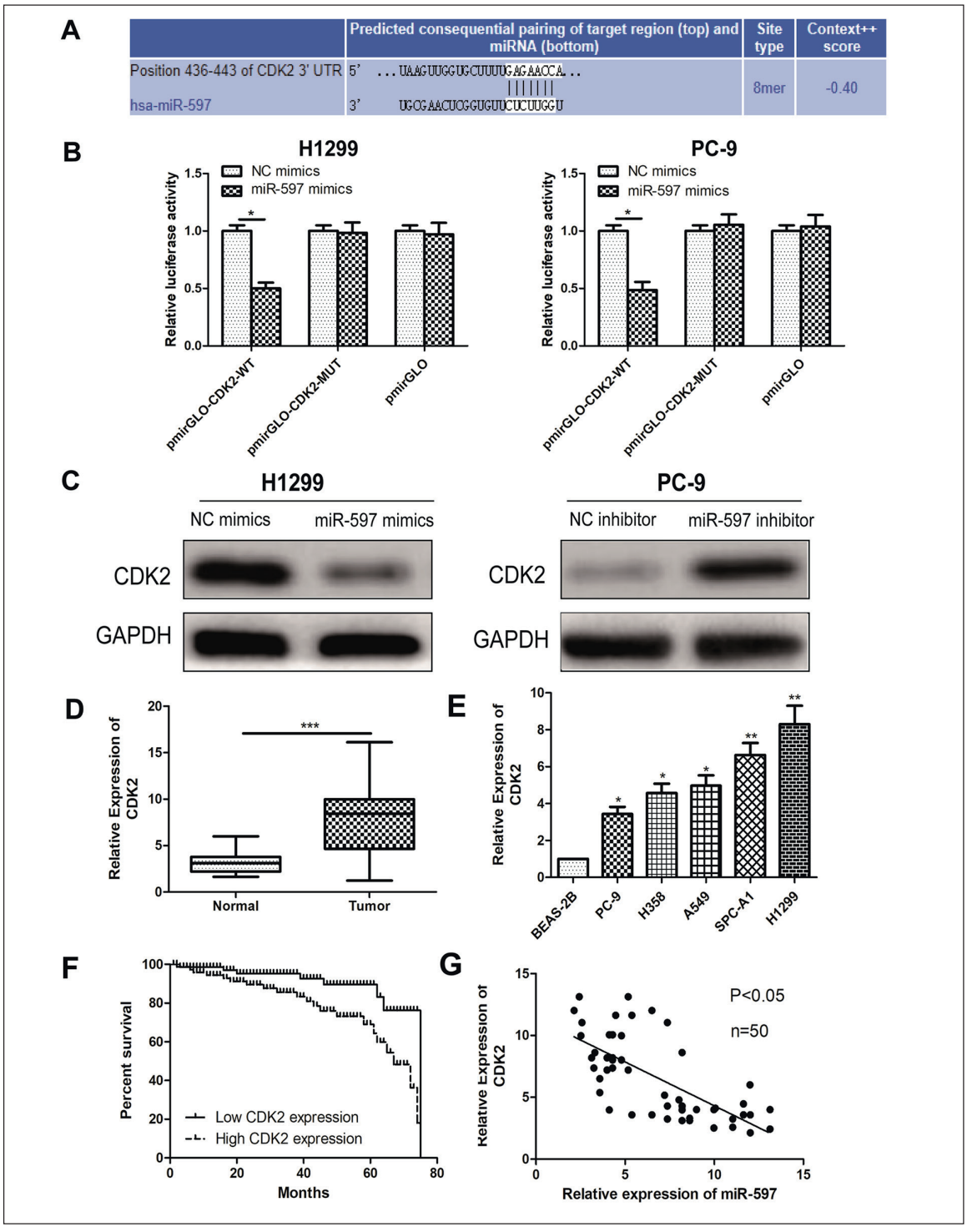

Figure 3. MiR-597 directly binds to CDK2. **A, B,** Dual-Luciferase reporter gene assay verified the direct targeting of miR-597 and CDK2. Dual-Luciferase reporter gene assay in H1299 and PC-9 cells showed that over-expression of CDK2 significantly attenuated the luciferase activity of wild-type miR-597 vector ($p<0.001$) without attenuating that of mutant vector ($p>0.05$) or empty vector ($p>0.05$); **C,** QRT-PCR verified the expression efficiency of CDK2 after transfection of miR-597 overexpression vector in H1299 cell line and miR-597 knockdown vector in PC-9 cell line; **D,** QRT-PCR was used to detect the expression of CDK2 in NSCLC tissues and adjacent normal tissues; **E,** QRT-PCR was used to detect the expression level of CDK2 in NSCLC cell lines; **F,** Kaplan Meier survival curve of lung cancer patients based on CDK2 expression showed that the prognosis of patients with high expression was significantly worse than that of low expression group; **G,** There was a significant negative correlation between miR-597 and CDK2 expressions in NSCLC tissues. Data were expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

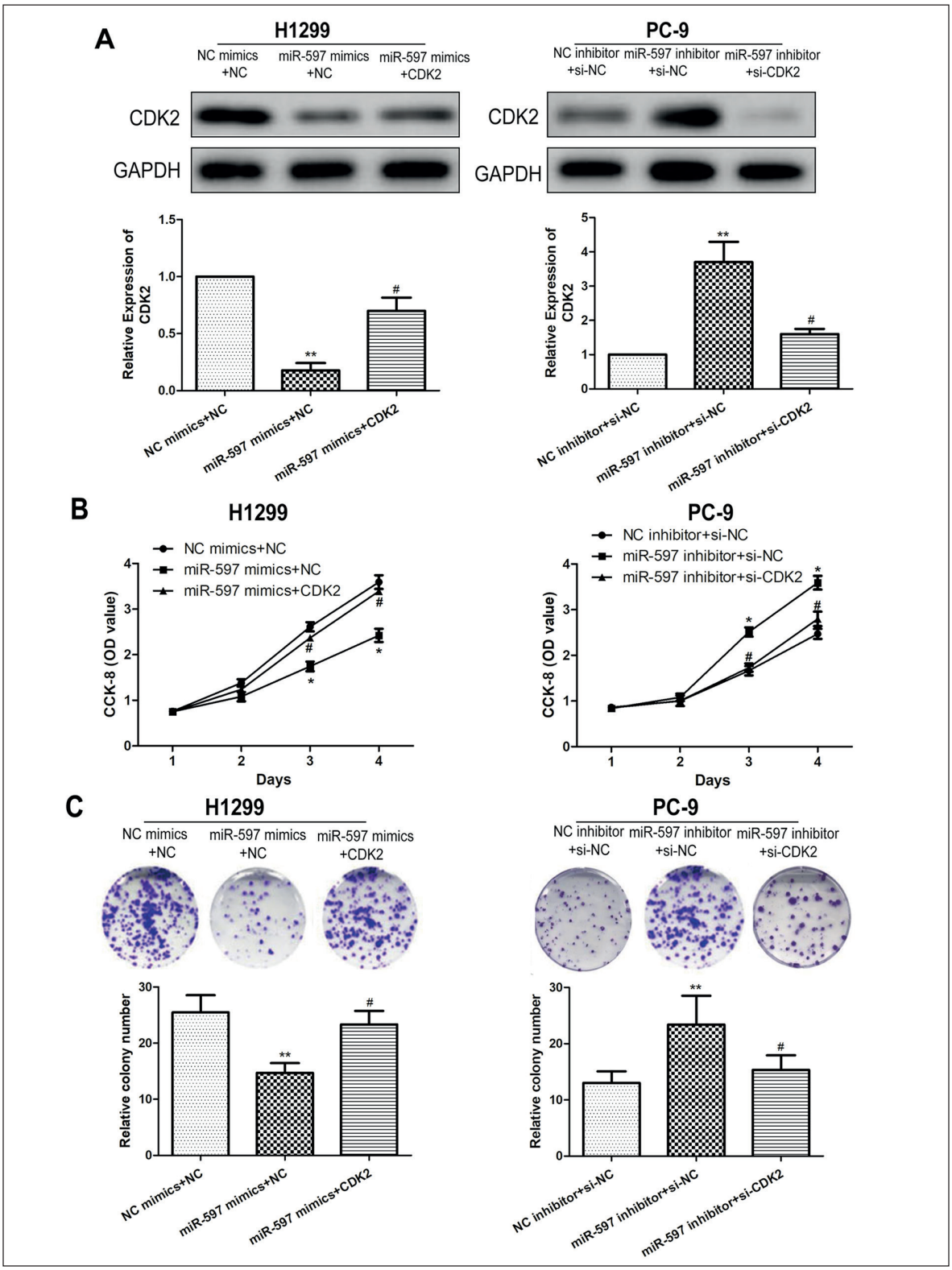

Figure 4. MiR-597 regulates the expression of CDK2 in NSCLC cell lines. **A,** CDK2 expression levels after co-transfection of miR-597 and CDK2 in NSCLC cell lines were detected by qRT-PCR and Western Blotting assays; **B,** CCK-8 assay detected the proliferation of NSCLC cells after co-transfection of miR-597 and CDK2 in NSCLC cell lines; **C,** Colony formation assay detected the proliferation of NSCLC cells after co-transfection of miR-597 and CDK2 in NSCLC cell lines, (magnification: 10×). Data were expressed as mean ± SD, *p<0.05, **p<0.01.

CCK-8, colony formation, and EDU assays revealed that microRNA-597 remarkably inhibited the proliferation ability of NSCLC. However, the specific molecular mechanism remained elusive.

MicroRNA-597 has been found abnormally expressed in a variety of tumors[24,25]. CDK2 gene is a key transcription factor that regulates malignant progression of tumors and inhibits the expression of target genes by binding to its target gene site[26,27]. Bioinformatics analysis has shown that CDK2 can inhibit the transcription of microRNA-597 through the negative feedback mechanism. Therefore, this study suggested that microRNA-597 could further inhibit the malignant progression of NSCLC by inhibiting CDK2 expression. Moreover, microRNA-597 and CDK2 showed a significant negative correlation in NSCLC cell proliferation. The purpose of this study was to further explore whether microRNA-597 regulated CDK2. First, the potential target gene of microRNA-597 in lung cancer was predicted by bioinformatics database (TargetScan, microRNA.org, PicTar). Finally, CDK2 was screened out and selected for further verification and functional research based on literature retrieval. Selected target genes were successfully verified by Dual-Luciferase reporter gene assay in NSCLC cell lines. Further exploration demonstrated that high expression of microRNA-597 could inhibit the expression of CDK2, thereby attenuating the proliferation ability of NSCLC cells. In conclusion, CDK2 is able to accelerate the proliferation of lung cancer cells. However, due to the "ceRNA" mechanism of microRNA-597, the expression of CDK2 decreases rapidly, thus inhibiting the progression of lung cancer..

Conclusions

In summary, microRNA-597 level was found remarkably down-regulated in NSCLC tissues and cell lines. Meanwhile, its expression was correlated with pathological staging and poor prognosis of NSCLC patients. Additionally, our findings suggested that microRNA-597 might inhibit the malignant progression of NSCLC by mutual regulation of CDK2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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