miR-377 inhibited tumorous behaviors of non-small cell lung cancer through directly targeting CDK6

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Abstract. – **OBJECTIVE:** This study is aimed to investigate the cellular role of miR-377 and demonstrate that miR-377 negatively regulated cyclin-dependent kinase 6(CDK6) in human non-small cell lung cancer (NSCLC) cells.

MATERIALS AND METHODS: qRT-PCR was performed to identify the miR-377 expression level in 45 paired NSCLC and adjacent normal lung tissues. Cell proliferation was measured by MTT. Apoptosis was detected by flow cytometric analysis. Luciferase reporter assays were employed to validate regulation of a putative target of miR-377. The effect of miR-377 on endogenous levels of this target was subsequently confirmed via Western blot.

RESULTS: We found that the expression level of miR-377 was significantly reduced in NSCLC tissues and cell lines. On the contrary, CDK6 expression level was up-regulated in NSCLC tissues and cell lines. Based on Luciferase reporter assays, we confirmed that CDK6 was a direct target gene of miR-377. In vitro studies demonstrated that miR-377 overexpression reduced NSCLC cell proliferation and promoted apoptosis.

CONCLUSIONS: Our discovery suggested that miR-377 might be used as a therapeutic reagent for the treatment of NSCLC in the future.

Key Words:
    miR-377, CDK6, NSCLC, Proliferation, Apoptosis.

Introduction

Lung cancer is the most common reason for cancer-related deaths, among which non-small cell lung cancer (NSCLC) is the leading cause[1,2]. The population with NSCLC has grown fast over the past decades in China[3]. Despite the considerable advances in the medical and surgical treatment of NSCLC patients, the prognosis of NSCLC remains unsatisfactory and the 5-year survival rate of patients with NSCLC is < 16%[4,5]. One of the major reasons why patients' survival benefits little from medical attentions is that the underlying molecular mechanisms of NSCLC progression have not been completely elucidated yet. Thus, A better understanding of the molecular mechanisms underlying the development and progression of lung cancer is essential for identifying novel and effective therapeutic targets.

MicroRNAs (miRNAs) are groups of short-length single-stranded noncoding RNAs that bind to 3'-untranslational region (3'-UTR) of target mRNA to post-transcriptionally induce gene or protein degradation[6,7]. Accumulating evidence has implicated miRNAs in the development of many cancer types, as either oncogenes or tumor suppressors[8-10]. Some of these miRNAs have been demonstrated to target genes that play important roles in lung cancer and have emerged as biomarkers for tumor diagnosis, prognosis and prediction of responses to treatment[11,12].

MiR-377 has been found to be decreased in lung cancer[13]. However, its detailed role remains unclear. Cyclin-dependent kinase 6(CDK6) is a member of the CDK family. CDKs play important roles in the major cell-cycle transitions and phases of all eukaryotic organisms either directly or indirectly[14]. In the present study, we hypothesized that miR-377 may regulate NSCLC cell proliferation and apoptosis via regulation of CDK6 expression and verified this hypothesis.

Materials and Methods

Human Tissue Collection

Paired NSCLC and adjacent normal lung tissues were obtained from 45 patients who underwent primary surgical resection of NSCLC between July 2012 and January 2015 at Chinese PLA General Hospital. All patients did not receive chemotherapy or radiotherapy prior to surgery. The samples were immediately frozen

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and stored in liquid nitrogen prior to analysis. All the patients provided informed consent for the sample collection. The Ethical and Scientific Committees of Chinese PLA General Hospital approved this study.

Cell Culture and Transfection

Four NSCLC cell lines (A549, H460, 95D, and HCC827) and normal lung epithelial cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). These cell lines were maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) in the presence of 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 l g/ml streptomycin in a humidified 5% (v/v) atmosphere of CO_2 at 37 °C. Transfection was performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. miR-377 mimics (miR-377), miR-377 inhibitors (anti-miR-377) and their negative controls (miR-NC and anti-miR-NC) were purchased from GenePharma (Pudong, Shanghai, China).

Quantitative Real-time PCR (qRT-PCR)

Total RNA was isolated, using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For miRNA reverse transcription, cDNA was synthesized using TaqManH MicroRNA Reverse Transcription Kit (ABI) with 100 ng total RNA. qRT-PCR was performed using the All-in-OneTM miRNA qRT-PCR Detection Kit (GeneCopoeia, Dongguan, Guangzhou, China) by ABI fast 7500 real-time PCR System (Invitrogen, Carlsbad, CA, USA). U6 small nuclear RNA (snRNA) was selected to be the endogenous control. The miR-377-specific primer used is GGGCACACAAAG-GCAACTTTTGT. The PCR primer sequences used for CDK6 are 5′-AGACCTTCGTTGCC-CTCTGT-3′ and 5′-AGTTGTTGGGGCTC-CTCAG-3′. The reaction mixtures were incubated at 37 °C for 60 min, at 95 °C for 5 min, and then held at 4 °C. Each sample was run in triplicate for analysis. The relative expression fold change of mRNAs was calculated by the $2^{-\Delta\Delta CT}$ method.

Western Blot

Cell lysates were maintained in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1 %Triton X-100, 150 mM NaCl, and 1 protease inhibitor cocktail tablet/10 mL), Approximately 50 µg of the protein extraction was separated by 10% SDS-PAGE, then transferred to 0.22 mm nitrocellulose membranes (Sigma, Silicon Valley, CA, USA) and incubated with specific antibodies. The following primary antibodies were used in the immunoblotting assays: CDK6 (Epitomics Inc., Burlingame, CA, USA) and GAPDH (G8140; United States Biological, Salem, MA, USA). Quantity One v4.4.0 software (Bio-Rad, Hercules, CA, USA) was used to assay optical density of the CKD6 bands. GAPDH was chosen as an internal control. Three independent experiments were performed.

Luciferase Reporter Assay

Cells were plated in triplicated in 24-well plates and grown for overnight. Then the vectors and oligonucleotides were co-transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), 48 h after transfection, the luciferase and renilla signals were determined using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) according to the instructions of the manufacturer.

MTT Assay

The 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to estimate cell viability. Transfected cells were seeded into 96-well plates at a density of 4000 cells/well, and cultured for a different time. 10 L of MTT was added to each well and incubated at 37 °C for 4 h. The plates were then centrifuged, and the formazan precipitates were dissolved in 100 mL of dimethyl sulfoxide. Finally, the absorbance was measured at 490 nm using a microplate reader. Each experiment was performed in triplicates.

Apoptosis Assay

Cells were transfected with miR-377 mimics or miR-377 inhibitors as well for 48h. All experiments were analyzed by BD Biosciences FACS Calibur Flow Cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The tests were repeated for three times with triplicate per experiment.

Statistical Analysis

Statistical evaluation of data was performed using SPSS 13 analysis software (SPSS, Chicago, IL, USA). Statistical analysis was performed by Student's *t*-test or Mann-Whitney test. In all samples, $p < 0.05$ (*) and $p < 0.01$ (**) was considered to be statistically significant.

Results

Expression of miR-377 and CDK6 in NSCLC Tissues and Cell Lines

To explore if miR-377 might have a role in tumorigenesis of human NSCLC, qRT-PCR was first applied to determine its expression level in tumor tissues and cell lines. The results showed that the expression level of miR-377 was significantly reduced in NSCLC tissues and cell lines ($p < 0.01$) (Figure 1A-B). Furthermore, western blot was performed to determine the expression of CDK6 in tumor tissues and cell lines. The data showed that CDK6 expression level was significantly higher in NSCLC tissues compared with normal adjacent lung tissues ($p < 0.01$, Figure 1C). In addition, CDK6 protein levels were also higher in NSCLC cells than in NLEC (Figure 1D).

CKD6 is the Target of miR-377

To further dig out the molecular mechanisms by which miR-377 exerts its functional role in NSCLC cells, we used the publicly available databases (TargetScan 6.2 and MiRanDa) to search for the potential downstream target of miR-377. We found that CDK6 may be the target of miR-377 (Figure 2A). The results showed that overexpression of miR-377 significantly inhibited the luciferase activity of pGL3- CDK6 3'-UTR WT (Figure 2B). Mutation of the miR-377-binding site in the CDK6 3'-UTR abolished the effect of miR-377. Next, in experiments to understand how miR-377 modulates CDK6 mRNA and protein overexpression, we observed that miR-377 overexpression did degrade CDK6 mRNA. Besides, our results of Western blot showed that miR-377 overexpression significantly inhibited CKD6 protein levels in 95D cells (Figure
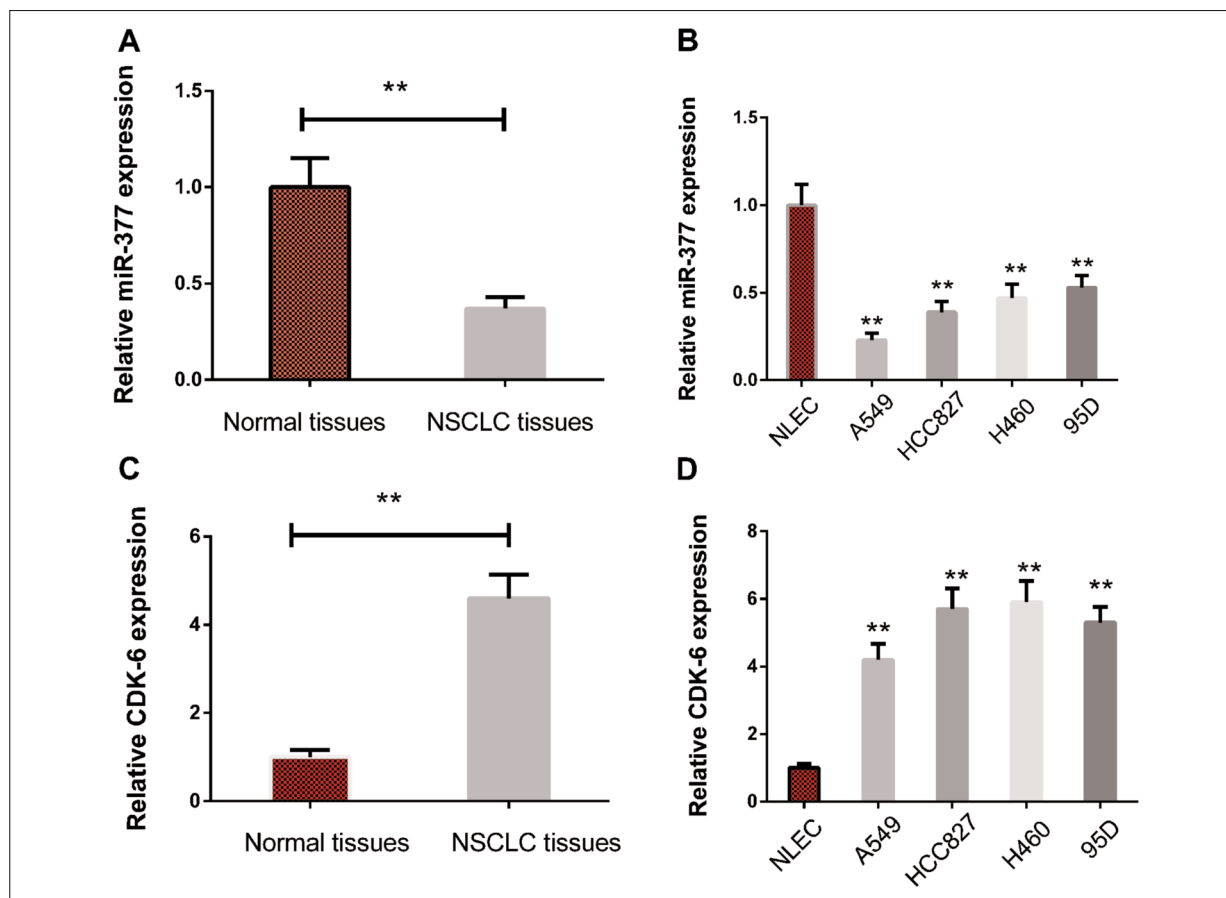

Figure 1. MiR-377 is down-regulated while CDK6 is up-regulated in NSCLC. **A,** MiR-377 expression in 47 pairs of NSCLC and adjacent normal tissues were respectively detected by real-time quantitative RT-PCR assay. **B,** miR-377 expression was down-regulated in the NSCLC cell lines A549, H460, 95D and HCC 827 compared with NLEC. **C,** CDK6 expression levels in NSCLC tissues and adjacent non-tumorous tissues by western blot. **D,** CDK6 protein levels in NSCLC cells were higher than in NLEC. *$p < 0.05$, **$p < 0.01$.
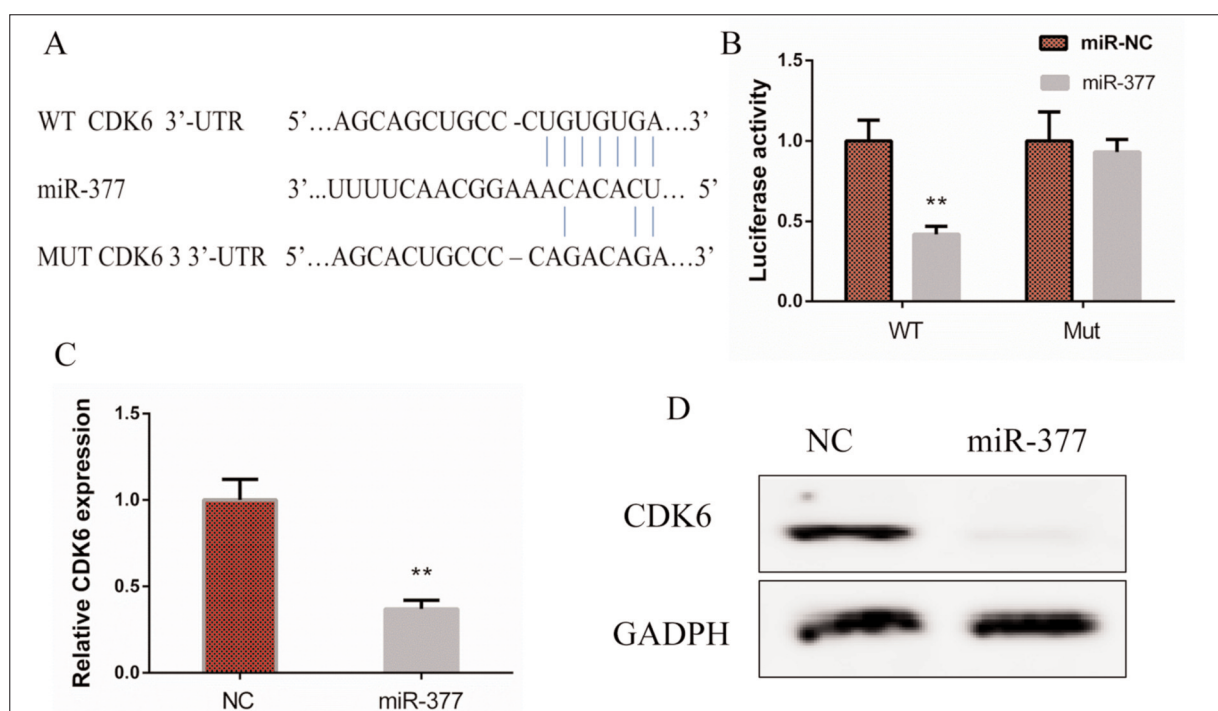
Figure 2. CKD6 was a target of miR-377 in NSCLC cells. ***A,*** Schematic representation of the miR-377 targeting sequences within the 3′-UTR of CDK6. ***B,*** Dual-luciferase reporter assay was performed in 95D cells. ***C,*** Expression of CDK6 mRNA was detected by qRT-PCR. ***D,*** Protein level of CDK6 was detected by Western blotting in 95D cells transfected with miR-377 or the negative control mimics. Experiments were performed in triplicate. *$p < 0.05$, **$p < 0.01$ compared with control.

2D). Those results suggested that CKD6 was directly and negatively regulated by miR-377.

Up-regulation of miR-377 Inhibits Proliferation and Induces Apoptosis in 95D Cells

To better understand the role of miR-377 in the development of NSCLC, we transfected miR-377 mimics (miR-377) into 95D cell line. As shown in Figure 3A, transfection of miR-377 in-

creased the miR-377 expression. Next, we performed cell proliferation assays and found that miR-377 led to a significant decrease in the growth of 95D (Figure 3B). Flow cytometry was employed to determine the effect of miR-377 on cell apoptosis. The results showed that apoptosis rate of 95D cells was significantly higher when transfected with miR-377 mimics (Figure 3C). These data indicate that miR-377 may play a significantly positive role in carcinogenesis.
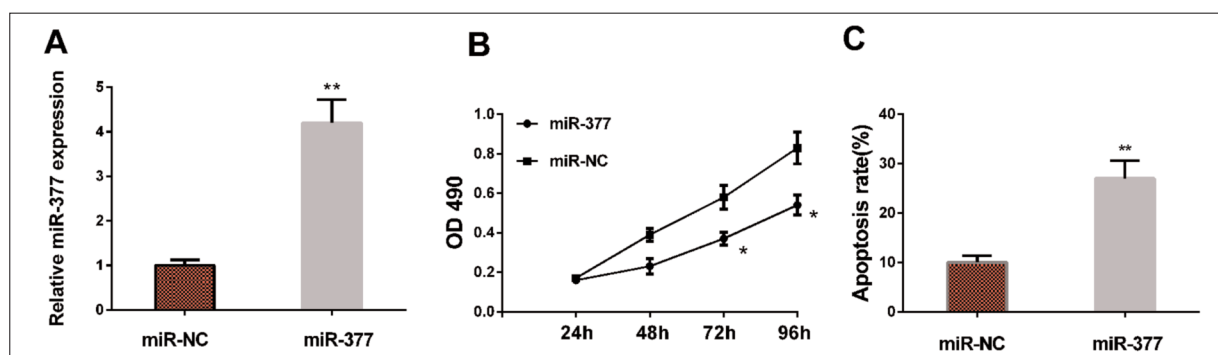

Figure 3. Overexpression of miR-377 inhibits cell proliferation and induces apoptosis in NSCLC. ***A,*** Expression of miR-377 was determined in 95D cells after miR-377 transfection compared to controls. ***B,*** Proliferation of transfected 95D cells evaluated by MTT assay. ***C,*** Apoptosis rate of transfected 95D cells measured by flow cytometry. *$p < 0.05$, **$p < 0.01$.

Down-regulation of miR-377 Promotes Proliferation and Suppresses Apoptosis in 95D Cells

We further explored the role of miR-377 in 95D cell proliferation and apoptosis. We transfected miR-377 inhibitors (anti-miR-377) into the 95D cell line. As shown in Figure 4A, transfection of miR-377 decreased the miR-377 expression. Our data showed that the reduced expression of miR-377 significantly decreased the growth rate of 95D cells and increased the fraction of living cells and boosted apoptotic cells (Figure 4B-C).

Discussion

In this present work, we investigated the biological role of miR-377 in the progression of NSCLC. We found lower levels of miR-377 in NSCLC tissues and cell lines than in paired adjacent non-tumor lung tissue and normal lung cell line. In addition, the results of Western blot showed that CKD6 expression levels were up-regulated in NSCLC tissues and cell lines. Next, Luciferase reporter assays were employed to confirm that CDK6 is a direct target of miR-377 in NSCLC cells. Then, MTT assay and flow cytometry were performed to explore the effect of miR-377 in regulating progression of NSCLC; we found that the overexpression of miR-377 inhibited the proliferation and induced the apoptosis of the NSCLC cells. On the contrary, knockdown of miR-377 promoted the proliferation and inhibited the apoptosis of the NSCLC cells. Taken together, we provided the first evidence that miR-377 acted as a tumor suppressor in NSCLC by directly regulating CDK6 expression, which may provide new insights about its role and value in lung carcinogenesis.

Recently, miR-377 has been shown to regulate gene and protein expression and be involved in different cancer progression and metastasis. For instance, Zhang et al[15] showed that miR-377 inhibited proliferation and invasion of human glioblastoma cells by directly targeting specificity protein 1. Wang et al[16] found that miR-377 reduced the ability of human clear cell renal cell carcinoma cells to proliferate, migrate and invade by targeting ETS1. Chen et al[17] revealed that miR-377 suppressed hepatocellular carcinoma cells proliferation via down-regulating of TIAM1. For non-small-cell lung cancer, Meng et al[13] showed that miR-377 expression was significantly decreased in NSCLC tissues as well as in NSCLC cell lines. The bioinformatics analysis results showed miR-377 served as an anti-onco-gene in NSCLC through targeting AEG-1. These results indicated that miR-377 ectopic expression affected cancer cell viability and proliferation.

CDK6 attracted our attention because of its ability to regulate the cell cycle[14]. Furthermore, an aberrant CDK6 expression has been reported in bladder cancer[18], glioblastoma[19] and medulloblastoma[20], suggesting the involvement of CDK6 in cancer. Moreover, we searched for potential targets of miR-377 using several online databases, including targetscan, miRanda, and miRGen, and all three databases indicated that the CDK6 mRNA contained miR-377 binding sites. The association between miR-377 and CDK6 mRNA has not been previously reported. Therefore, we focused on miR-377 and CDK6 in our study.
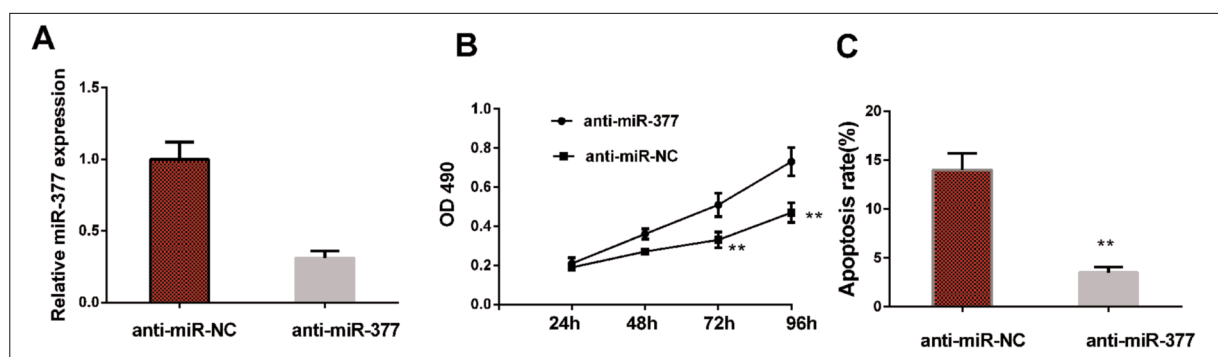

Figure 4. Knockdown of miR-377 promotes proliferation and suppresses apoptosis in 95D cells. **A,** Expression of miR-377 was determined in 95D cells after miR-377 inhibitor transfection compared to controls. **B,** Proliferation of transfected 95D cells evaluated by MTT assay. **C,** Apoptosis rate of transfected 95D cells measured by flow cytometry. *$p < 0.05$, **$p < 0.01$.

Conclusions

Our study found that enforced overexpression of miR-377 substantially suppressed proliferation of NSCLC cells. Furthermore, we proposed the regulatory function of miR-377 on CDK6 by direct binding of its 3'UTR, indicating that miR-377 might act as a novel therapeutic strategy for the treatment of NSCLC.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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