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CircRNA_001010 adsorbs miR-5112 in a sponge form to promote proliferation and metastasis of non-small cell lung cancer (NSCLC)

Q. WANG[1], P.-M. KANG[2]

[1]Department of Respiratory and Critical Care Medicine, Shanxi Provincial People's Hospital, Taiyuan, China
[2]Department of Thoracic Surgery, Daping Hospital, Army Military Medical University, Chongqing, China

Qiang Wang and *Poming Kang* contributed equally to this work as co-first authors

Abstract. – OBJECTIVE: It has been demonstrated that circular RNA (circRNA) plays an important regulatory role in a series of diseases. The purpose of this study is to investigate the expression of circRNA_001010 and its facilitating effects on proliferation and invasion of non-small cell lung cancer (NSCLC) by regulating oncogene CDK4 through sponging with miR-5112.

PATIENTS AND METHODS: qRT-PCR was performed to detect the expressions of circRNA_001010 and CDK4 in human NSCLC tissues and cells. Cell Counting Kit-8 (CCK-8) assay was performed to evaluate the A549 cells proliferation and transwell assay was performed to evaluate the A549 cells migration. Correlation analysis between circRNA_001010 and miR-5112 was detected by statistical analysis. Bioinformatics prediction was made to detect the binding site of GTL and miR-5112 and Luciferase activity was conducted to investigate the interaction between circRNA_001010 and miR-5112. Furthermore, we cloned the mice CDK4 3′-UTR into the Luciferase reporter vector and constructed miR-5112 binding mutants to validate the inhibited modulation of miR-5112 to the CDK4 expression.

RESULTS: Results showed that the expressions of circRNA_001010 and CDK4 were upregulated in human NSCLC tissues and cells. qRT-PCR and CCK-8 assay showed that circRNA_001010 expression is associated with the proliferation of NSCLC cells, and that upregulated circRNA_001010 contributed to cell proliferation of A549. Transwell assay showed that circRNA_001010 was associated with the migration ability of tumor cells, and that increased expression of circRNA_001010 promoted the migration and invasion of NSCLC cells. The bioinformatics prediction and Luciferase assay demonstrated that by sponging with miR-5112, circRNA_001010 can serve as a ceRNA for miR-5112 to further regulate the expression of CDK4.

CONCLUSIONS: For the first time, we found that circRNA_001010 was upregulated in human NSCLC patients, which could accelerate tumor proliferation, migration and invasion as a molecular sponge by modulating the inhibitory effect of miR-5112 on oncogene CDK4.

Key Words:
CircRNA_001010, MiR-5112, NSCLC, Proliferation, Invasion.

Introduction

Non-small cell lung cancer (NSCLC) accounts for up to 85% of all lung cancer cases and is the leading cause of lung cancer-associated mortality[1]. Early diagnosis and treatment are essential to improve patient survival. However, the mechanisms underlying lung cancer progression still need to be fully elucidated. Recent progress in RNA research has led to the identification of non-coding RNAs (ncRNAs) involved in a variety of biological processes[2]. Previous studies have revealed critical roles of miRNAs and lncRNAs in lung cancer development, in particular, regulation of proliferation, apoptosis and invasion[3,4]. In-depth analysis of ncRNAs should thus aid in further clarifying cancer-associated mechanisms at the epigenetic level.

Circular RNA (circRNA) is a novel type of endogenous non-coding RNA with high stability and conserved covalent closed-loop structure[5]. Compared with other noncoding RNA such as

Corresponding Authors: Qiang Wang, MD; e-mail: qiangwang97@aliyun.com
Poming Kang, MD; e-mail: kangbert@msn.com

miRNAs and long noncoding RNAs (lncRNAs), circRNAs are highly conserved sequences and high degree of stability in mammalian cells, these properties provide circRNAs with the potential to become ideal biomarkers and potential treatment target[6,7]. CircRNA functions as crucial gene regulators by their post-transcriptional modification such as binding miRNA, assembling RNA-binding proteins and modulating transcription factors[8,9]. CircRNA contains miRNA-binding site and usually exerts as a miRNA sponge to negatively regulate expression of target mRNAs[10,11].

In the present study, we aimed to explore the biological roles of circRNA_001010 in NSCLC development and progression, as well as to illustrate the molecular mechanisms. We investigated the function of circRNA_001010 in NSCLC and revealed that circRNA_001010, which significantly increased in NSCLC tissues, promoted cell proliferation and invasion of NSCLC cells via affecting miR-5112/CDK4 axis. We uncovered a critical role of circRNA_001010 in NSCLC progression, which serves as ceRNA by modulating the inhibitory effect of miR-5112 on CDK4.

Patients and Methods

Patients and Tumor Samples

In this work, 11 pairs of non-small cell lung cancer tissues and adjacent normal tissues were collected from surgically treated and pathologically diagnosed non-small cell lung cancer cases and then stored at -80°C. Patient information was included in Table I. There were no significant differences in the 11 pairs of samples in terms of diagnostic indicators and prognostic factors. This investigation was approved by the Ethics Committee of our Hospital. Patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

Table I. Demographic data.

	Age < 45 years	Age > 45 years
Patients number	5	6
Sex ratio (M/F)	2/3	2/4
BMI (kg/m^2) ± SD	20.9 ± 5.6	22.2 ± 4.9

All the patients were selected randomly.

Cell Culture

Human NSCLC cells A549 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) supplied with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) and incubated at 37°C in an atmosphere of 5% CO_2.

Construction of Lentivirus and Cell Transfection

Lentiviral circRNA_001010 and circRNA_001010 shRNA were synthesized and constructed by Shanghai GenePharma Co. Ltd., (Shanghai, China). For miR analysis, the miR-5112 mimic, miR-5112 inhibitor and the negative control were constructed by Shanghai GenePharma Co. Ltd., (Shanghai, China). To knock down CDK4, si-CDK4 plasma and negative control plasma were constructed by Shanghai GenePharma Co. Ltd., (Shanghai, China). For transfection, 1×10^4 cells were seeded in 6-well plates and cultured with RANKL (100 ng/mL) and M-CSF (100 ng/mL). Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I reduced serum medium were used for transfection. For the analysis of circRNA_001010, cells were transfected with circRNA_001010 shRNA (recircRNA_001010d as to sh) and negative control shRNA (recircRNA_001010d as to nc), respectively. For the analysis of miR-5112, cells were transfected with miR-5112 inhibitor, and control cells were transfected with empty vector, respectively. The cells without transfection were used as the control (recircRNA_001010d as to control). After incubation for 30 min, cultures were replaced with DMEM containing 10% FBS. Then, at indicated time point after transfection, cells were harvested for further study.

Transwell Assay

To test the migration ability of A549 cells, transwell plates with a pore size of 8 μm (Millipore Inc., Billerica, MA, USA) were used to conduct transwell assay. A549 cells were treated differently and the lower chamber was added with DMEM supplemented with 20% FBS. After the upper side of the membrane was wiped with a cotton swab to remove the cells that did not migrate, cell numbers in five random fields were counted in each sample.

RNA Extraction and qRT-PCR

Taking out the culture plates, the cells were washed with phosphate-buffered saline (PBS). After treatment, total RNA of cells was extracted by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The samples were stored at room temperature for 30 min. The reverse transcription of cDNA was performed with a PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. For qRT-PCR, PCR primers were synthesized by GenePharma (Shanghai Gene Pharma, Shanghai, China) and sequences were listed in Table II. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression.

CCK-8 Assay

The Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Kumamoto, Japan) was used to measure the cells proliferation according to the manufacturers' instructions. In brief, 5×10^3 cells were seeded in 96-well plates uniformly. After treated with regulated medium, the medium was removed, and cells were washed with PBS solution for 3 times. Then, CCK-8 dilution was added to the 96-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 2 h. After incubation, the plates were taken out, and cell proliferation was measured using multi-detection microplate reader. The absorbance (OD) value at 490 nm of each well was detected.

Luciferase Assay

After transfection for 48 h, the Luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. *Renilla* Luciferase activities were normalized to the firefly Luciferase activities and the data were expressed as the fold change relative to the corresponding control groups which were defined as 1.0.

Statistical Analysis

Unless otherwise indicated, all data are processed by Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Each assay was applied to at least three independent experiments or replicates. All data were presented as mean ± SD. Student's *t*-test, one-way analysis of variance (ANOVA) and multiple comparison between the groups was performed by using SNK method, in which $*p < 0.05$, $**p < 0.01$ represented the statistically significant difference.

Results

CircRNA_001010 and CDK4 Were Highly Expressed In NSCLC

Total RNA of NSCLC tissues and adjacent normal tissues were extracted, and the expressions of circRNA_001010 and CDK4 were detected by qRT-PCR. Results showed that both circRNA_001010 and CDK4 were significantly upregulated in NSCLC tissues (Figure 1A, 1B). To further illustrate the biological function of circRNA_001010 in NSCLC, qRT-PCR analysis was performed to detect circRNA_001010 expression in human NSCLC cell lines A549. Results showed that the expressions of circRNA_001010 and CDK4 were remarkably increased in A549 cells compared with human epithelial cells HEK293 ($p < 0.01$) (Figure 1C,1D). From these data, we suggested that circRNA_001010 might play a biological role in NSCLC.

Upregulating the CircRNA_001010 Promoted the Migration and Invasion of NSCLC Cells

To explore the functions of circRNA_001010 in NSCLC progression, we constructed circRNA_001010 overexpressing lentiviral and transfected it into A549 cells. In addition, we synthesized small interfering RNA of circRNA_001010 to inhibit its expression before

Table II. Primer sequences for qRT-PCR.

Genes	Forward	Reverse	Tm (°C)
circRNA_001010	5'-ACGTAGCTAGCATGCATGCACG-3'	5'-CGATCGATCGATCGATGCTAGC-3'	60
miR-5112	5'-AGCTAGCTAGCTAGTCGACGT-3'	5'-CAGTCGATGCTAGCTAGCTAG-3'	61
CDK4	5'-CACGACAGCTGATGCTGTACAC-3'	5'-CAGCTACGTACGTGTGTGTGGA-3'	61
GAPDH	5'-TGGATTTGGACGCATTGGTC-3'	5'-TTTGCACTGGTACGTGTTGAT-3'	62
U6	5'-ACTGATCGATGCCTGATCGATCG-3'	5'-AAAGCTGTCCCGGGGTACGTGCC-3'	61

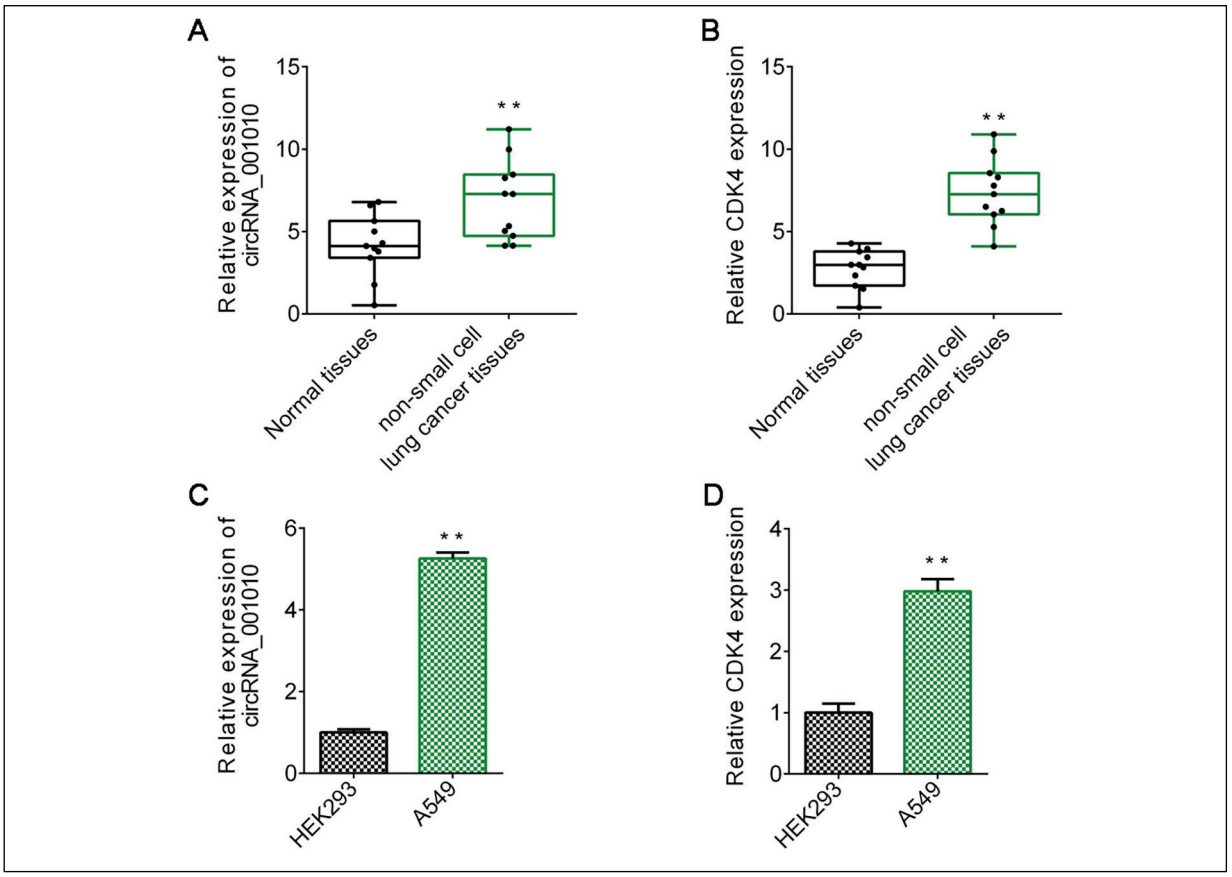

Figure 1. CircRNA_001010 and CDK4 were highly expressed in NSCLC. Relative mRNA expression levels of (**A**) circRNA_001010 and (**B**) CDK4 in NSCLC tissues and adjacent normal tissues. Relative mRNA expression levels of (**C**) circRNA_001010 and (**D**) CDK4 in human NSCLC cell line A549 and HEK293 cells. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

its transfection into A549 cells. After that, the expression of circRNA_001010 was detected by qRT-PCR and the results showed that the expression of circRNA_001010 in the circRNA_001010 overexpressed group was significantly enhanced compared with the control ($p < 0.05$), while the expression levels of circRNA_001010 were reduced in the circRNA_001010 inhibition group compared with the control group ($p < 0.05$) (Figure 2A, 2B). To investigate whether circRNA_001010 influences the migration of tumor cells, we performed transwell assay to detect the migration ability after the expression of circRNA_001010 was altered. Results revealed that after upregulating circRNA_001010 expression, the number of A549 cells that transport through transwell chambers was significantly increased compared with control group (Figure 2C), whereas the number of A549 cells transport through transwell chambers was significantly decreased after inhibition of circRNA_001010 expression (Figure 2D). Besides, scratch assay was also performed and found that the migration distance was significantly increased in circRNA_001010 overexpression group (Figure 2E), and significantly decreased after inhibition of circRNA_001010 expression compared with the control (Figure 2F). These findings demonstrated that circRNA_001010 can regulate the migration ability of human NSCLC cells, and that upregulated circRNA_001010 can effectively promote the migration ability of NSCLC cells, making circRNA_001010 a potential target for therapy of NSCLC.

CircRNA_001010 Could Facilitate the Proliferation and Inhibit the Apoptosis of NSCLC Cells

To further investigate the role of circRNA_001010 in cell proliferation, CCK-8 assay was performed on A549 cells after alteration of circRNA_001010 expression. The data showed

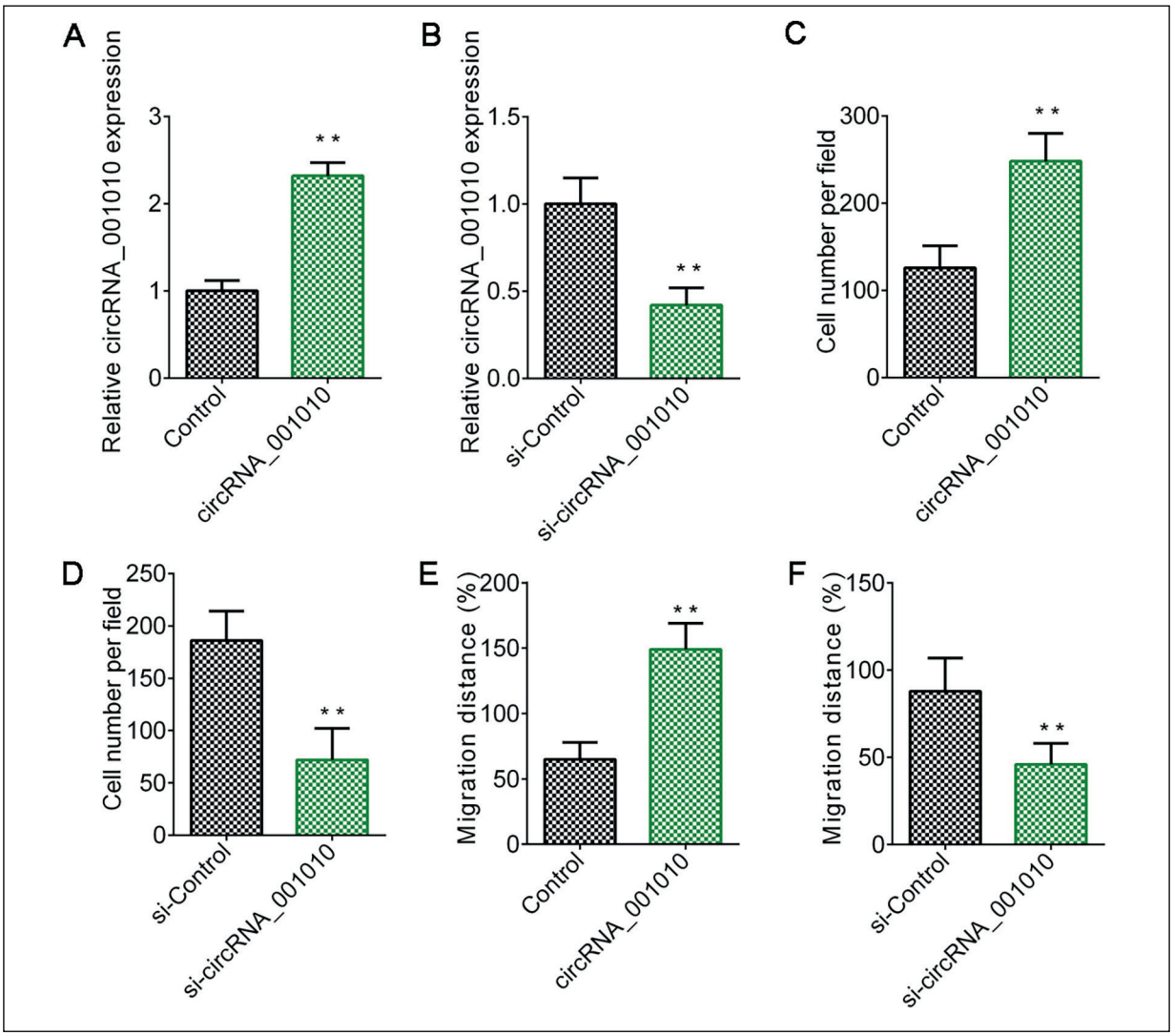

Figure 2. Upregulating the circRNA_001010 promoted the migration and invasion of NSCLC cells. **A**, Relative mRNA expression levels of circRNA_001010 in A549 cells transfected with circRNA_001010 overexpressing lentiviral (circRNA_001010) and Control. **B**, Relative mRNA expression levels of circRNA_001010 in A549 cells transfected with si-Control and si-circRNA_001010. **C**, Absorption at 490 nm of A549 cells treated with circRNA_001010 and Control detected by CCK-8 assay at 1 d, 2 d and 3 d. **D**, Absorption at 490 nm of A549 cells treated with si-circRNA_001010 and si-Control detected by CCK-8 assay at 1 d, 2 d and 3 d. **E**, Migration distance of A549 cells treated with circRNA_001010 and Control. **F**, Migration distance of A549 cells treated with si-circRNA_001010 and si-Control. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

that overexpressing the circRNA_001010 significantly increased cell proliferation of A549 compared with the control, whereas inhibition of circRNA_001010 expression remarkably reduced the cell proliferation number (Figure 3A, 3B). Besides, qRT-PCR analysis showed that the expression of apoptotic related genes such as Bax and cleaved caspase-3 was significantly decreased, whereas the expression of anti-apoptotic gene Bcl-2 was remarkably increased after upregulation of circRNA_001010 expression (Figure 3C), and it was reversed after circRNA_001010 inhibition (Figure 3D). These results suggested that changing the expression of circRNA_001010 can regulate the proliferation ability of NSCLC cells.

CircRNA_001010 Sponge with MiR-5112 in NSCLC Cells

To investigate the detailed mechanism of circRNA_001010 that promoted growth and in-

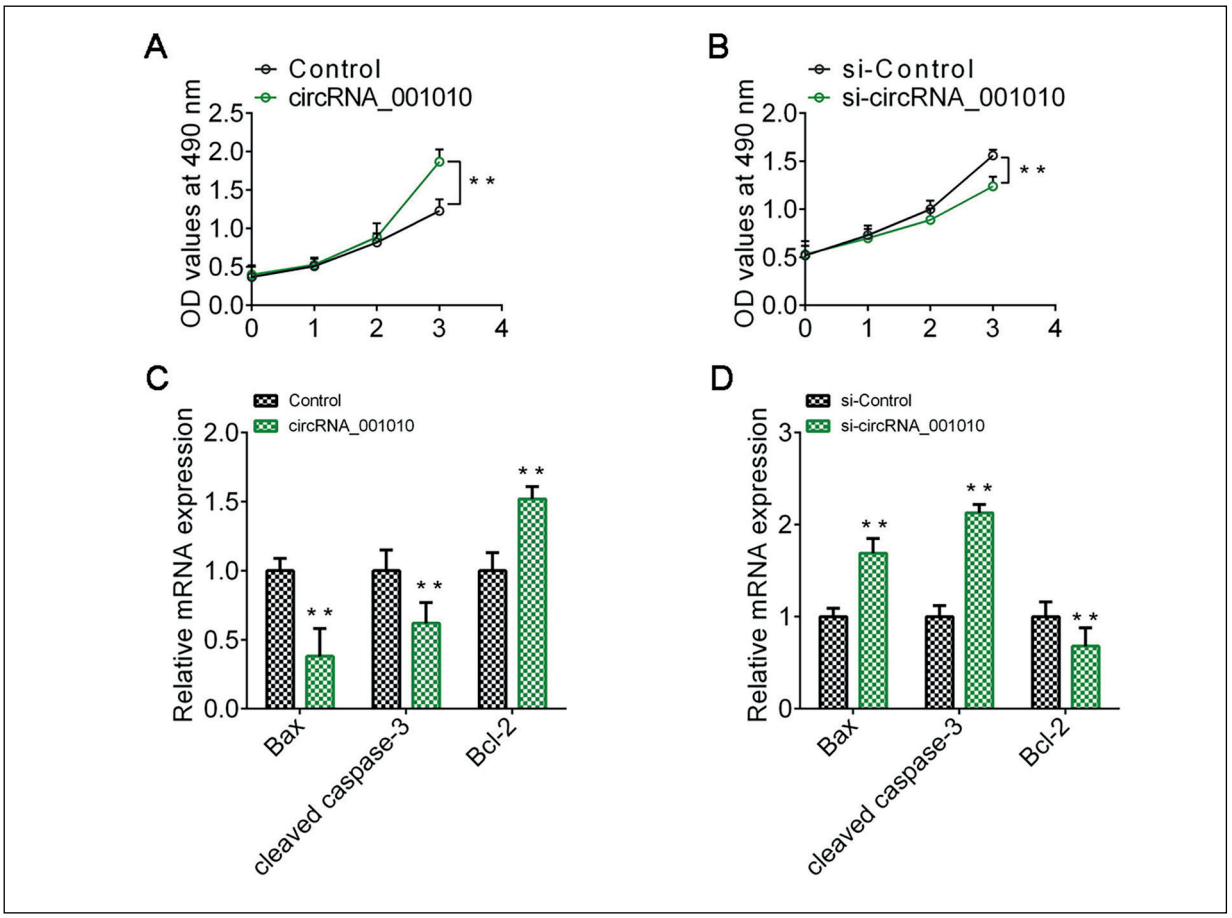

Figure 3. CircRNA_001010 could facilitate the proliferation and inhibit the apoptosis of NSCLC cells. **A**, Absorption at 490 nm of A549 cells treated with circRNA_001010 and Control detected by CCK-8 assay at 1 d, 2 d and 3 d. **B**, Absorption at 490 nm of A549 cells treated with si-circRNA_001010 and si-Control detected by CCK-8 assay at 1 d, 2 d and 3 d. **C**, Relative expression levels of Bax, cleaved caspase-3 and Bcl-2 in A549 cells treated with circRNA_001010 and Control. **D**, Relative expression levels of Bax, cleaved caspase-3 and Bcl-2 in A549 cells treated with si-circRNA_001010 and si-Control. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

vasion of NSCLC cells, we used StarBase 2.0 database to predict the target miRNA of circRNA_001010 and found that miR-5112 was a target miRNA of circRNA_001010. Then, we used qRT-PCR analysis to detect the miR-5112 expressions of human NSCLC tissues and A549 cells. Results showed that miR-5112 was lowly expressed in NSCLC tissues compared with adjacent normal tissues and was also downregulated in A549 cells compared with HEK293 cells (Figure 4A and 4B). Correlation analysis was performed to investigate the relationship between circRNA_001010 and miR-5112. Data showed that miR-5112 was negatively correlated with circRNA_001010, which suggested that miR-5112 might be sponged by circRNA_001010 (Figure 4C). Previous works reported that circRNAs can act as a competing sponge in regulating miRNAs to further influence gene expression. Hence, we investigated a direct binding relationship between circRNA_001010 and miR-5112. We constructed circRNA_001010-wt Luciferase reporter vector and circRNA_001010-mut 3'UTR Luciferase reporter vector and performed Luciferase reporter assay (Figure 4D). The findings showed that compared with the control, the Luciferase activity of A549 cells that co-transfected with wide type circRNA_001010 (circRNA_001010-wt) and miR-5112 mimic, was significantly decreased ($p < 0.01$), and it was reversely increased after mutation at the binding site of circRNA_001010 (circRNA_001010-mut) compared with circRNA_001010-wt ($p < 0.01$)

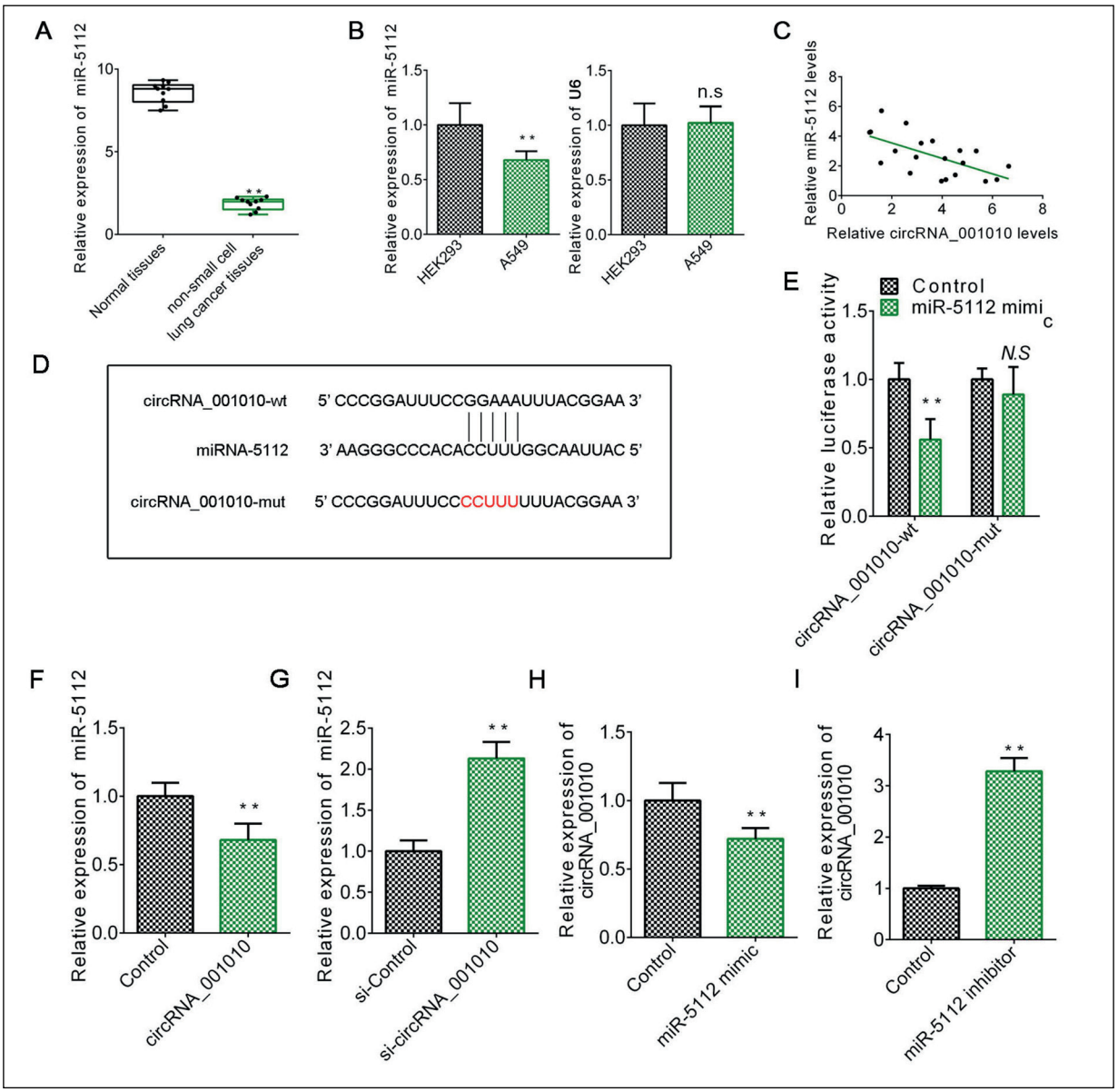

Figure 4. CircRNA_001010 sponge with miR-5112 in NSCLC cells. **A**, Relative expression of miR-5112 in NSCLC tissues and adjacent normal tissues detected by qRT-PCR. **B**, Relative miR-5112 expression in A549 cells and HEK293 cells detected by qRT-PCR. **C**, Correlation analysis was performed to evaluate the relationship between miR-5112 and circRNA_001010. **D**, Schematic illustration of the predicted miR-5112 binding sites and mutant sites in circRNA_001010. **E**, Relative luciferase activity of A549 cells. **F-G**, qRT-PCR analysis of miR-5112 expression level in A549 cells transfected with lentiviral circRNA_001010 and si-circRNA_001010. **H-I**, Relative circRNA_001010 expression was detected in A549 cells after treated with miR-5112 mimics and miR-5112 inhibitor by RT-PCR. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$). The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

(Figure 4E). These results suggested that circRNA_001010 could directly bind to miR-5112. In addition, overexpression of circRNA_001010 significantly inhibited miR-5112 expression and circRNA_001010 downregulation reversely supported miR-5112 expression in A549 cells (Figure 4F, 4G). We also transfected miR-5112 mimic and miR-5112 inhibitor into A549 cells; the results revealed that miR-5112 mimic inhibited circRNA_001010 expression and miR-5112 inhibitor increased circRNA_001010 expression (Figure 4H, 4I). Taken together, these findings

demonstrated that circRNA_001010 can directly sponge with miR-5112.

CircRNA_001010 Serves As a CeRNA of MiR-5112 to Modulate the Expression of CDK4

Previous reports showed that CDK4 plays an important role in tumor progression and invasion. To explore whether miR-5112 can interact with CDK4, we performed qRT-PCR analysis to detect CDK4 expression in the presence of miR-5112 mimics. Results showed that CDK4 expression was decreased in miR-5112 mimics compared with control, suggesting miR-5112 could inhibit CDK4 expression (Figure 5A). To validate this mechanism, mice CDK4 3′-UTR were cloned into the Luciferase reporter vector and miR-5112 binding mutants were constructed, in which the putative miR-5112 binding sites GUACU in the CDK4 3′-UTR were mutated into CAUGA (Figure 5B). As expected, Dual-Luciferase report results showed that miR-5112 mimics significantly decreased the CDK4 expression, whereas point mutations in the CDK4 3′-UTR alleviate the inhibited effect of miR-5112 (Figure 5C). Furthermore, we investigate whether circRNA_001010 can regulate CDK4 expression via sponging with miR-5112. The results showed that circRNA_001010 could significantly increase CDK4 expression; nevertheless, mutation of the binding site with circRNA_001010 of miR-5112 eliminated the function effectively (Figure 5D). Conversely, inhibition of miR-5112 overcame the suppression of CDK4 by circRNA_001010 knockdown (Figure 5E). Taken together, these findings sug-

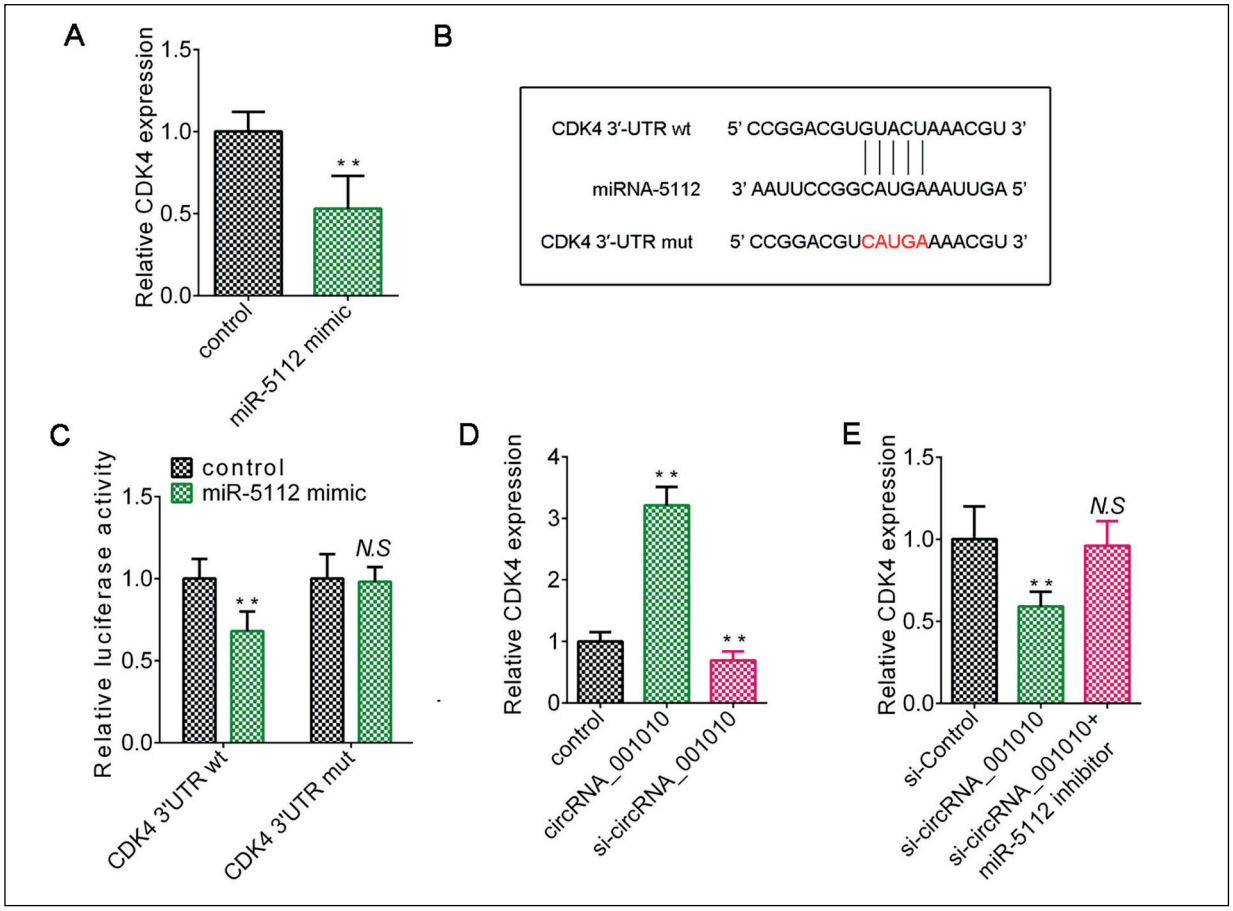

Figure 5. CircRNA_001010 served as a molecular sponge for miR-5112 to further modulate the expression of CDK4. **A**, qRT-PCR analysis of CDK4 mRNA expression level in A549 cells treated with the miR-5112 mimics. **B**, Schematic illustration of the predicted CDK4 binding sites and mutant sites in miR-5112. **C**, Relative luciferase activity of A549 cells. **D**, Relative mRNA expression levels of CDK4 in A549 cells transfected with circRNA_001010 and circRNA_001010 mut-MRE. **E**, Relative mRNA expression levels of CDK4 in A549 cells transfected with si-circRNA_001010, si-circRNA_001010 and miR-5112 inhibitor by qRT-PCR analysis. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

gested that circRNA_001010 could serve as a ceRNA for the miR-5112 to further alter the expression CDK4.

Discussion

Nowadays, lung cancer is still the leading cause of cancer incidence and mortality worldwide, accounting for 18.4% of cancer-related deaths. The main subtype of lung cancer is NSCLC, that accounts for about 85% of all lung cancers. Its 5-year survival rate is extremely unfavorable, <20%, mainly due to the absence of early symptoms in most patients, as well as the metastasis and recurrence[12]. Therefore, there is an urgent need to deeply elucidate the nosogenesis of NSCLC, which will provide new approaches for the diagnosis and treatment of NSCLC.

CircRNA functions as crucial gene regulators by their post-transcriptional modification such as binding miRNA, assembling RNA-binding proteins and modulating transcription factors[10]. CircRNA contains miRNA-binding site and usually exerts as a miRNA sponge to negatively regulate the expression of target mRNAs[13]. CircRNA is dysregulated in human cancers and plays an essential role in cancer initiation, development and progression[8]. Up to now, some NSCLC-related circRNAs were also identified, such as circ-ABCB10[14], circ-DDX42[15], and circ-FADS2[16]. They affected the malignant properties of NSCLC via acting as oncogenes or tumor suppressors. Although a small number of circRNAs have been functionally characterized, many members of this category have not yet been explored. Herein, we identified a novel NSCLC-related circRNA, circ-001010, which was highly expressed in NSCLC and had tumor-promoting activity. In our study, a miRNA–mRNA–circRNA network including differently expressed mRNAs, circRNAs, and miRNAs in NSCLC was revealed. We observed that circRNA_001010 expression was significantly enhanced in NSCLC tissues and cell lines when compared with adjacent normal tissues and HEK293 cells, respectively. Then, we further verified the effect of circRNA_001010 on biological process of human NSCLC cells A549 including proliferation, invasion and migration. The results revealed that circRNA_001010 was associated with the proliferation, migration and invasion abilities of A549. From bioinformatics prediction, we found that miR-5112 was a target miRNA of circRNA_001010 and validated the combination relationship of circRNA_001010 and miR-5112 using Luciferase reporter assay. Besides, we found that miR-5112 can interact with circRNA_001010 co-expression gene CDK4 and downregulate the expression of CDK4. Mechanism analysis revealed that circRNA_001010 functions in NSCLC as a competing endogenous RNA (ceRNA) that regulate CDK4 expression by acting as a sponge for the miRNA-19a-5p.

Conclusions

We figured out that circRNA_001010 can serve as a sponge of miR-5112 to elevate CDK4 expression, thus promoting cell proliferation and invasion.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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