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CircRNA_010763 promotes growth and invasion of lung cancer through serving as a molecular sponge of miR-715 to induce c-Myc expression

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Abstract. – OBJECTIVE: This study aims to investigate the regulatory effect of circRNA_010763 on the growth and invasion of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: qRT-PCR was performed to detect the expressions of circRNA_010763 and c-Myc in human NSCLC tissues and cells. CCK-8 assay was performed to evaluate the A549 cells proliferation and transwell assay was performed to evaluate the A549 cells migration. The correlation between miR-715 and circRNA_010763 was detected by statistical analysis. Bioinformatics prediction and Luciferase assay were performed to explore the interaction and binding site of circRNA_010763 and miR-715, miR-715 and c-Myc, respectively.

RESULTS: We found that both circRNA_010763 and c-Myc were upregulated in human NSCLC tissues and cells. qRT-PCR and CCK-8 assay showed that circRNA_010763 expression is associated with the proliferation of NSCLC cells. Transwell assay showed that circRNA_010763 regulated the migration ability of NSCLC cells. The bioinformatics prediction and Luciferase assay demonstrated that circRNA_010763 can sponge with miR-715, serving as a molecular sponge to further regulate the expression of c-Myc.

CONCLUSIONS: In this study, we found that circRNA_010763 was highly expressed in human NSCLC tissues, which could promote tumor proliferation, migration and invasion through serving as a molecular sponge by modulating the inhibitory effect of miR-715 on oncogene c-Myc.

Key Words:
CircRNA_010763, Lung cancer, MiR-715, Growth, Invasion.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) constitutes approximately 80% of all diagnosed lung cancers cases, and the 5-year survival rate of the disease is ~20%[1]. Although surgical operation still represents the main curative treatment for NSCLC up to stage IIIA disease, the risk of postoperative disease recurrence has been reported to be as high as 52%[2]. Most patients eventually succumb to recurrence of the disease despite the high initial response to therapy. According to the results of several large-sample clinical studies on the effect of surgical operation of global NSCLC patients, the five-year survival rate is about 31-42%[3]. Recently, advances in clinical and experimental oncology have been made for treating NSCLC, but its complicated pathology is unclear, and more work is required to identify novel molecules that are involved in the process. Therefore, investigation of the molecular mechanisms underlying NSCLC tumorigenesis may aid in the development of novel therapeutic targets and strategies for the treatment of the malignancy.

Circular RNAs (circRNAs) are a novel type of endogenous RNAs featuring stable structure and high tissue-specific expression[4]. Unlike linear RNAs, circRNAs form a covalently closed continuous loop without 5′ to 3′ polarity and polyadenylated tail, and they are highly represented in the eukaryotic transcriptome[5,6]. CircRNAs are much more stable than linear RNAs and therefore, might be involved in more

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abundant functions. Several possible functions of circRNAs have been claimed: microRNA (miRNA) miRNA sponges, regulators of splicing and transcription, binding protein and RNA transport[4-7]. CircRNAs might play an important role in carcinogenesis and tumor progression[8-12]. However, the role and contribution of circRNAs in NSCLC remain to be elucidated.

MicroRNAs are short endogenous non-coding molecules (around 19-23 nucleotides in length) that regulate gene expression by binding to the 3′-untranslated regions (3′-UTRs) of target mRNAs, which play an important role in maintaining the homeostasis[13]. Currently, it is widely accepted that miRNAs may act as oncogenes or suppressor genes during tumor development[14]. Competing endogenous RNA (ceRNA) hypothesis demonstrated that lncRNAs or circRNAs served as miRNA sponges to modulate the expression of miRNA target genes, thus participating in the progression of cancers[15]. However, whether circRNA_010763 could interact with miRNA to regulate the development of NSCLC remains to be elucidated.

Thus, the aim of the present study was to investigate the level of circRNA_010763 expression in the progression of NSCLC and explore its underlying mechanism.

Patients and Methods

Patients

In this study, 11 pairs of NSCLC 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. 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.

Cell Culture

Human NSCLC cell line A549 cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Rockville, MD, USA) supplied with 10% 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_010763 and circRNA_010763 shRNA were synthesized and constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For miR analysis, the miR-715 mimic, miR-715 inhibitor and the negative control were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). To knock down c-Myc, si-c-Myc plasma and negative control plasma were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I reduced serum medium were used for transfection. For analysis of circRNA_010763, cells were transfected with circRNA_010763 shRNA (referred as to sh) and negative control shRNA (referred as to nc), respectively. For analysis of miR-715, cells were transfected with miR-715 inhibitor, and control cells were transfected with empty vector, respectively. The cells without transfection were used as the control (referred as to control). After incubated for 30 min, cultures were replaced with DMEM containing 10% FBS.

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. The upper side of the membrane was wiped with a cotton swab to remove the cells that did not mi-

Table I. Demographic data.

Gender	Male	Female
Patients numbers	5	6
BMI (kg/m^2) ± SD	20.9 ± 5.6	22.2 ± 4.9
Age (years)		
< 45	2	4
≥ 45	3	2
TNM stage		
I-II	3	5
III-IV	2	1
Lymph node metastasis		
Negative	2	4
Positive	3	2

All the patients were selected randomly.

grate, and 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, Waltham, MA, USA) according to the manufacturer's instructions. 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, Shang-Hai, China) and sequences were listed in Table II. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression.

CCK8 Assay

The CCK-8 kit (Dojindo, 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 CCK8 dilution was added to the 96-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 2 hours. 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 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 were performed using SNK method, in which $*p < 0.05$, $**p < 0.01$ represented the significant difference.

Results

CircRNA_010763 and c-Myc Were Upregulated In Human NSCLC Tissues and Cells

To determine the roles of circRNA_010763 and c-Myc in NSCLC progression, we first performed qRT-PCR to examine the expressions of circRNA_010763 and c-Myc in NSCLC tissues and adjacent normal tissues. The results revealed that both the expressions of circRNA_010763 and c-Myc were significantly upregulated in NSCLC tissues compared with that of adjacent normal tissues (Figure 1A and 1B). To further interpret the biological functionality of circRNA_010763 in NSCLC, we performed qRT-PCR to detect circRNA_010763 expression in human NSCLC cell lines A549. The results showed that both circRNA_010763 and c-Myc expressions were significantly upregulated in NSCLC cells compared with human epithelia cells HEK293 cells ($p < 0.05$) (Figure 1C and 1D).

The Migration and Invasion of NSCLC Cells Were Significantly Reduced after Inhibited the Expression of CircRNA_010763

To explore the functions of circRNA_010763 in NSCLC progression, circRNA_010763 over-

Table II. Primer sequences for qRT-PCR.

Genes	Forward	Reverse	Tm (°C)
circRNA_010763	5'-ATCGTAGCTACGTAGCTCCGA-3'	5'-CGATCGATGCAGTCGTCAGTGC-3'	60
miR-715	5'-GACTCGATCGATCGATGC-3'	5'-CGATCGATCGATGCTAGCAG-3'	61
c-Myc	5'-CGATCGATCGATCGATCGTA-3'	5'-AGCTAGCTAGCTGATCGATC-3'	61
GAPDH	5'-TGGATTTGGACGCATTGGTC-3'	5'-TTTGCACTGGTACGTGTTGAT-3'	62

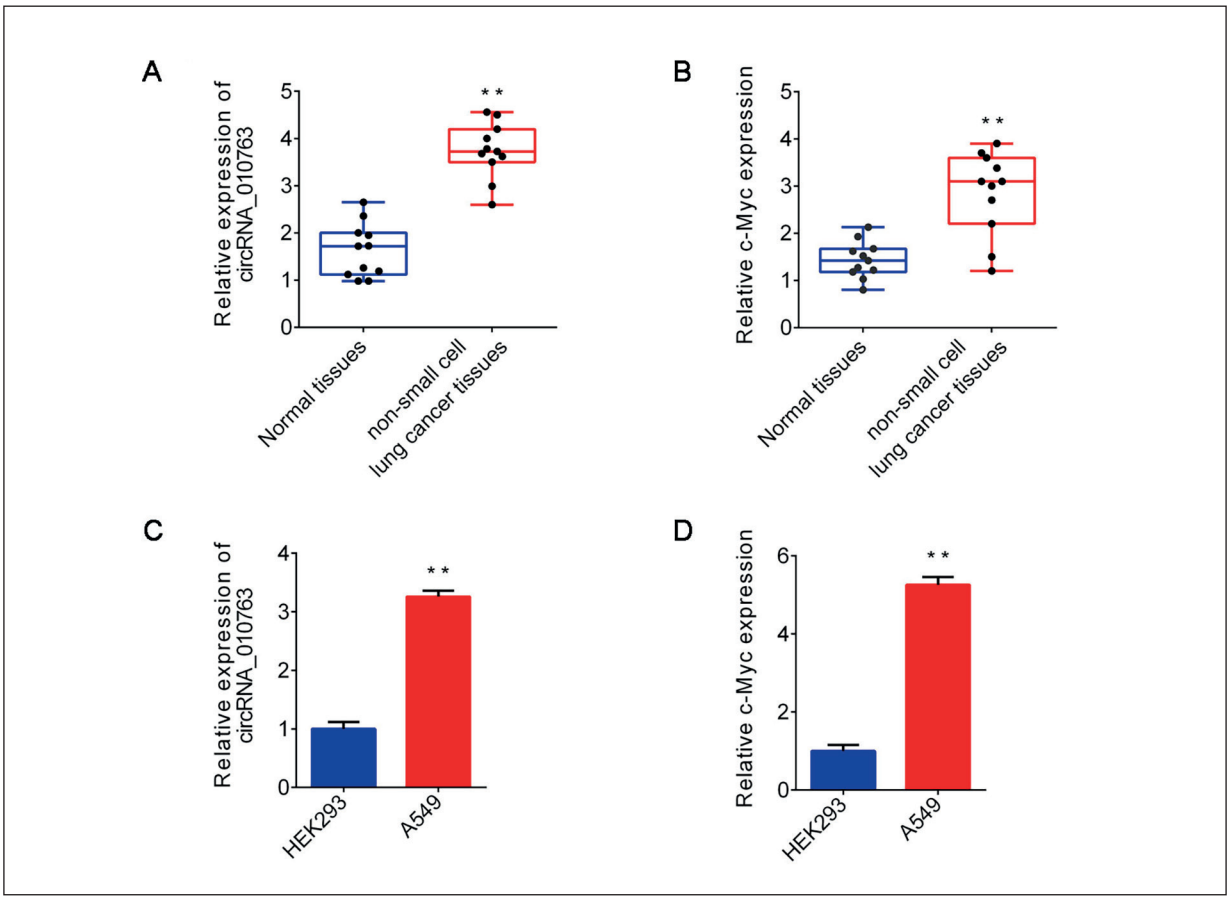

Figure 1. CircRNA_010763 and c-Myc were downregulated in human NSCLC tissues and cells. Relative mRNA expression levels of (**A**) circRNA_010763 and (**B**) c-Myc in NSCLC tissues and adjacent normal tissues. Relative mRNA expression levels of (**C**) circRNA_010763 and (**D**) c-Myc in human NSCLC 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 ** ($p < 0.01$).

expressing lentiviral (circRNA_010763) was constructed and transfected into A549 cells. Besides, small interfering RNA for circRNA_010763 (si-circRNA_010763) was also synthesized and transfected into A549 cells. The expression of circRNA_010763 was subsequently detected by qRT-PCR. The results showed that the expression of circRNA_010763 in the circRNA_010763 group was significantly enhanced compared with the vector1 group ($p < 0.05$), while the expression levels of circRNA_010763 were reduced in the si-circRNA_010763 group compared with the negative control vector2 group ($p < 0.05$) (Figure 2A and 2B). To verify the role of circRNA_010763 on cell proliferation, CCK8 assay was performed on A549 cells after regulation of circRNA_010763 expression. The results revealed that overexpression of circRNA_010763 significantly increased NSCLC cells proliferation compared with the control group, whereas inhibition of circRNA_010763 expression remarkably reduced the cell proliferation number at 3 days (Figure 2C, 2D). These results suggested that alteration of circRNA_010763 expression could influence the proliferation of NSCLC cells. To further determine that circRNA_010763 influences the migration and invasion of NSCLC cells, we performed transwell assay to detect the migration ability of human NSCLC cells after the expression of circRNA_010763 was altered. The results revealed that after circRNA_010763 upregulation, NSCLC cells migration through transwell chambers significantly facilitated in response to fetal bovine serum compared with control group (Figure 2E). Then, when circRNA_010763 was downregulated, the number of NSCLC cells migration through transwell chambers was significantly reduced in response to fetal bovine serum

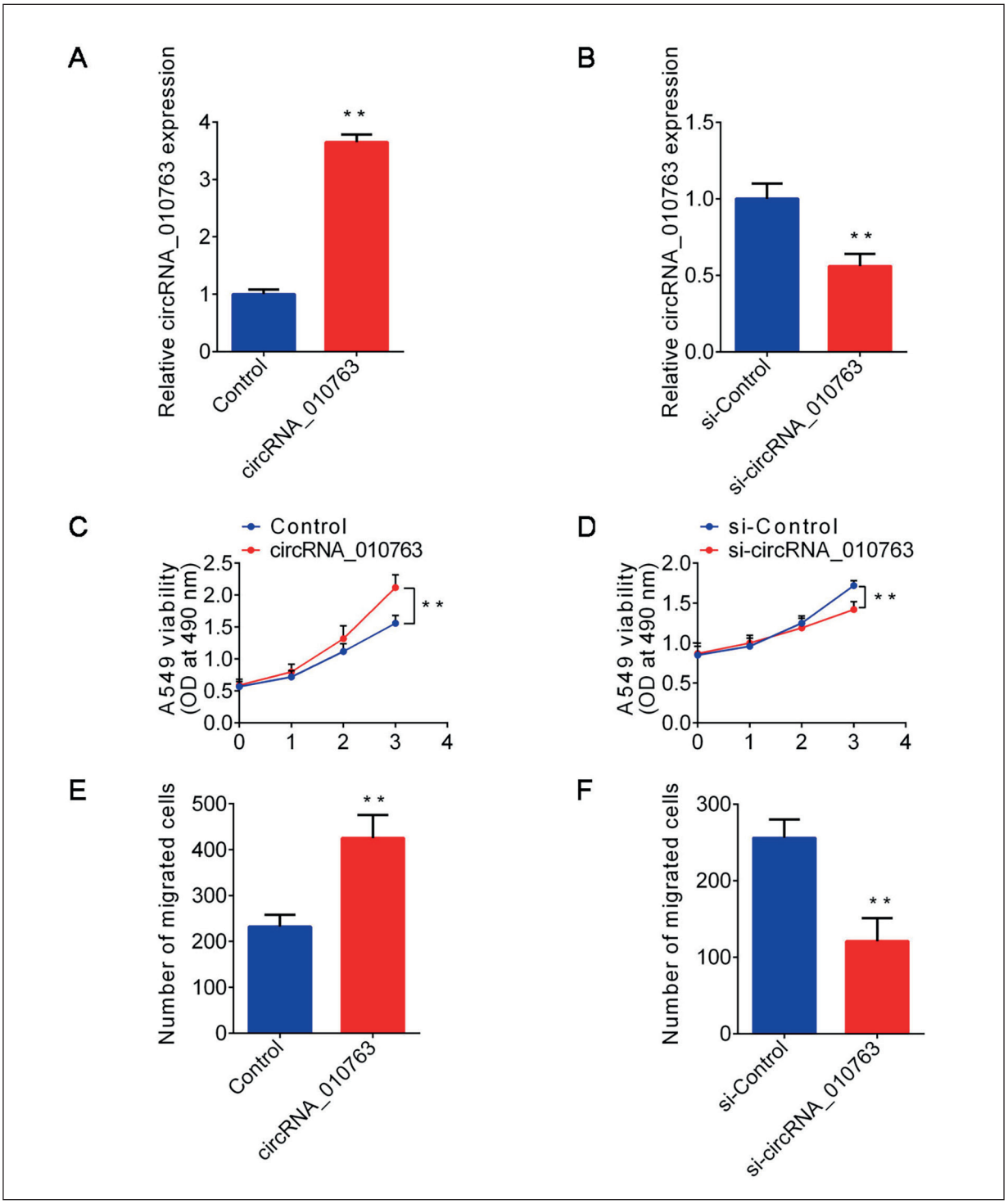

Figure 2. The migration and invasion of NSCLC cells were significantly inhibited after upregulated the circRNA_010763 expression. **A**, Relative mRNA expression levels of circRNA_010763 in A549 cells transfected with circRNA_010763 overexpressing lentiviral (circRNA_010763) and control. **B**, Relative mRNA expression levels of circRNA_010763 in A549 cells transfected with si-Control and si-circRNA_010763. **C**, Absorption at 490 nm of A549 cells treated with circRNA_010763 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_010763 and si-Control detected by CCK-8 assay at 1 d, 2 d and 3 d. **E**, The number of migrated cells through transwell chambers was calculated after overexpression of circRNA_010763. **F**, The number of migrated cells through transwell chambers was calculated after knockdown of circRNA_010763. 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 ** ($p < 0.01$).

compared with control group (Figure 2F). These data suggested that circRNA_010763 alteration regulated the migration and invasion of human NSCLC cells, downregulated circRNA_010763 can effectively inhibit the migration and invasion of NSCLC cells.

MiR-715 Expression Was Upregulated In NSCLC Cells and Negatively Correlated With CircRNA_010763

To investigate whether circRNA_010763 was correlated miRNA, we used StarBase 2.0 to predict the target miRNA of circRNA_010763 and found that miR-715 is one of the target miRNAs of circRNA_010763. Therefore, we used qRT-PCR analysis to detect the miR-715 expressions of human NSCLC tissues and A549 cells. Results showed that miR-715 was low-expressed in NSCLC tissues compared with that of adjacent normal tissues and was downregulated in A549 cells compared with HEK293 cells (Figure 3A and 3B). We then used correlation analysis to further explore the relationship between circRNA_010763 and miR-715. The results showed that miR-715 was significantly negatively correlated with circRNA_010763, indicating that miR-715 might be regulated by circRNA_010763 (Figure 3C). These results suggested that miR-715 was high expressed in NSCLC tissues and A549 cell line, which was negatively correlated with circRNA_010763.

CircRNA_010763 Can Sponge With MiR-715 and Inhibit Its Expression In NSCLC Cells

It has been previously suggested that circRNAs can act as a competing sponge in regulating the biological functions of miRNAs. As mentioned above, miR-715 was negatively correlated with circRNA_010763, so we hypothesized that circRNA_010763 can regulate the migration and invasion of NSCLC through interaction with miR-715. To further confirm this, circRNA_010763-wt Luciferase reporter vector and circRNA_010763-mut 3'UTR Luciferase reporter vector were synthesized, and Luciferase reporter assay was performed (Figure 4A). Compared with the control, the Luciferase activity of A549 cells that co-transfected with wild type circRNA_010763 (circRNA_010763-wt) and miR-715 mimic was significantly decreased ($p < 0.01$), and it was reversely increased after mutation at the binding site of circRNA_010763 (circRNA_010763-mut) compared with circRNA_010763-wt ($p < 0.01$) (Figure 4B). These results demonstrated that circRNA_010763 could directly bind to miR-715. Besides, circRNA_010763 overexpression suppressed miR-715 expression and circRNA_010763 inhibition reversely facilitated miR-715 expression in A549 cells (Figure 4C, 4D). Additionally, we also transfected miR-715 mimic and miR-715 inhibitor into A549 cells; the results revealed that miR-715 mimic inhibited circRNA_010763

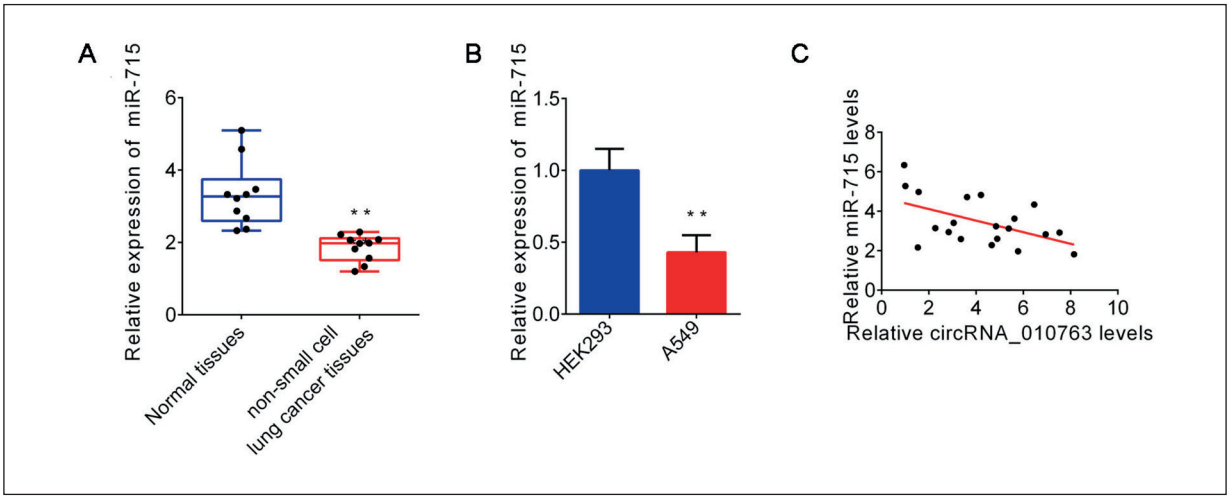

Figure 3. MiR-715 expression was upregulated in NSCLC cells and negatively correlated with circRNA_010763. **A**, Relative miR-715 expression in NSCLC tissues and adjacent normal tissues detected by qRT-PCR. **B**, Relative miR-715 expression in A549 cells and HEK293 cells detected by qRT-PCR. **C**, Correlation analysis was performed to evaluate the relationship between miR-715 and circRNA_010763. 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 ** ($p < 0.01$).

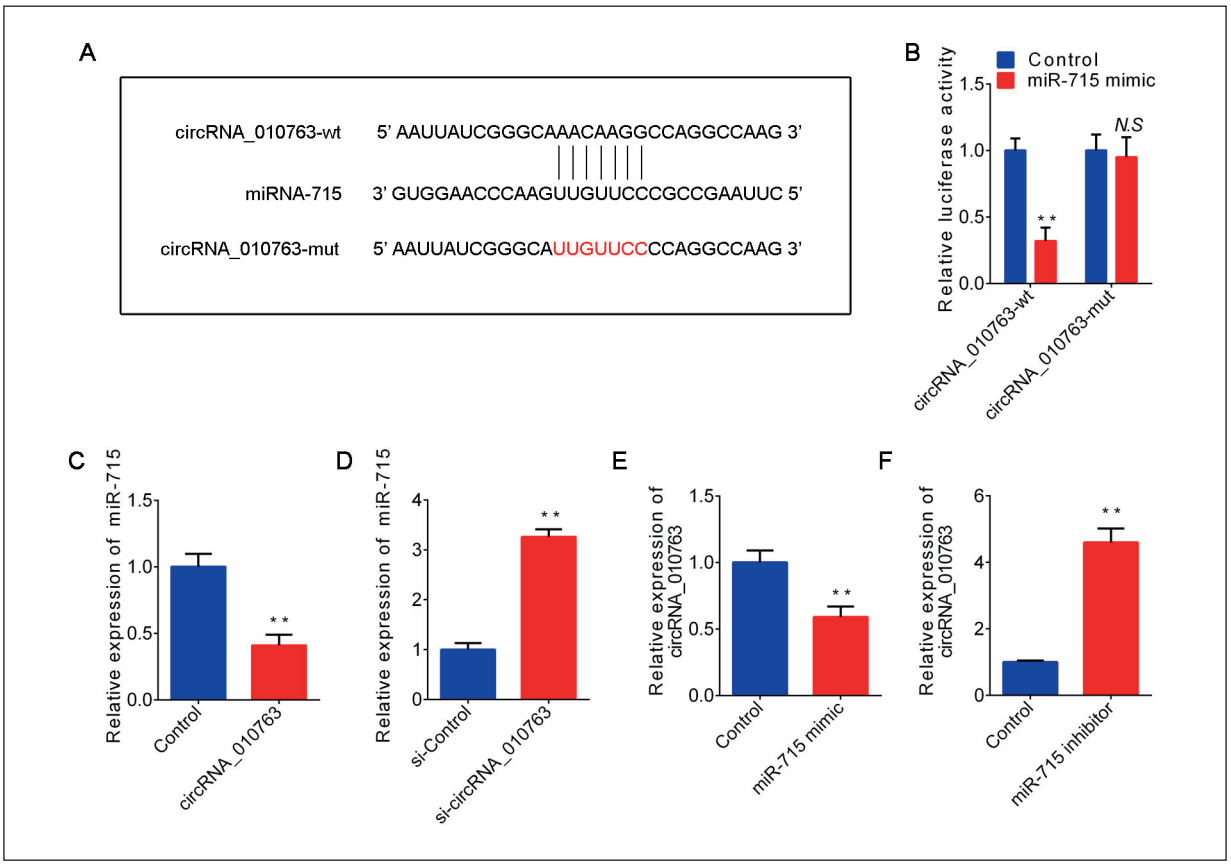

Figure 4. CircRNA_010763 can sponge with miR-715 and inhibit its expression in NSCLC cells. **A**, Schematic illustration of the predicted miR-715 binding sites and mutant sites in circRNA_010763. **B**, Relative Luciferase activity of A549 cells. **C-D**, qRT-PCR analysis of miR-715 expression level in A549 cells transfected with lentiviral circRNA_010763 and si-circRNA_010763. **E-F**, Relative circRNA_010763 expression was detected in A549 cells after treated with miR-715 mimics and miR-715 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 ** ($p < 0.01$).

expression and miR-715 inhibitor increased circRNA_010763 expression (Figure 4E, 4F). All above, these results suggested that miR-715 directly bound to circRNA_010763 at the recognition sites.

CircRNA_010763 Served as a Molecular Sponge for MiR-715 to Further Modulate the Expression of c-Myc

c-Myc is an oncogene whose overexpression or mutation is closely related to the occurrence of various cancers. To explore whether miR-715 interacts with c-Myc, we performed qRT-PCR analysis for c-Myc in the presence of miR-715 mimics or inhibitor. We observed decreased c-Myc expression after A549 cells were treated with the miR-715 mimics, which suggested that miR-715 could downregulate c-Myc expression (Figure 5A). To validate this mechanism, we cloned the mice c-Myc 3′-UTR into the Luciferase reporter vector and constructed miR-715 binding mutants in which the putative miR-715 binding sites GGUCC in the c-Myc 3′-UTR were mutated into CCAGG (Figure 5B). As expected, Dual-Luciferase report results showed that miR-715 mimics significantly downregulated the c-Myc expression whereas point mutations in the c-Myc 3′-UTR abrogated the suppressed effect of miR-715 (Figure 5C). Then, we validated whether circRNA_010763 can regulate c-Myc expression via sponging with miR-715, the results showed that circRNA_010763 could significantly increase c-Myc expression; however, mutation of the binding site with circRNA_010763 of miR-715 eliminated the function effectively (Figure 5D). Conversely, inhibition of miR-715 overcame the suppression of c-Myc by circRNA_010763 knockdown (Figure 5E). Taken together, these

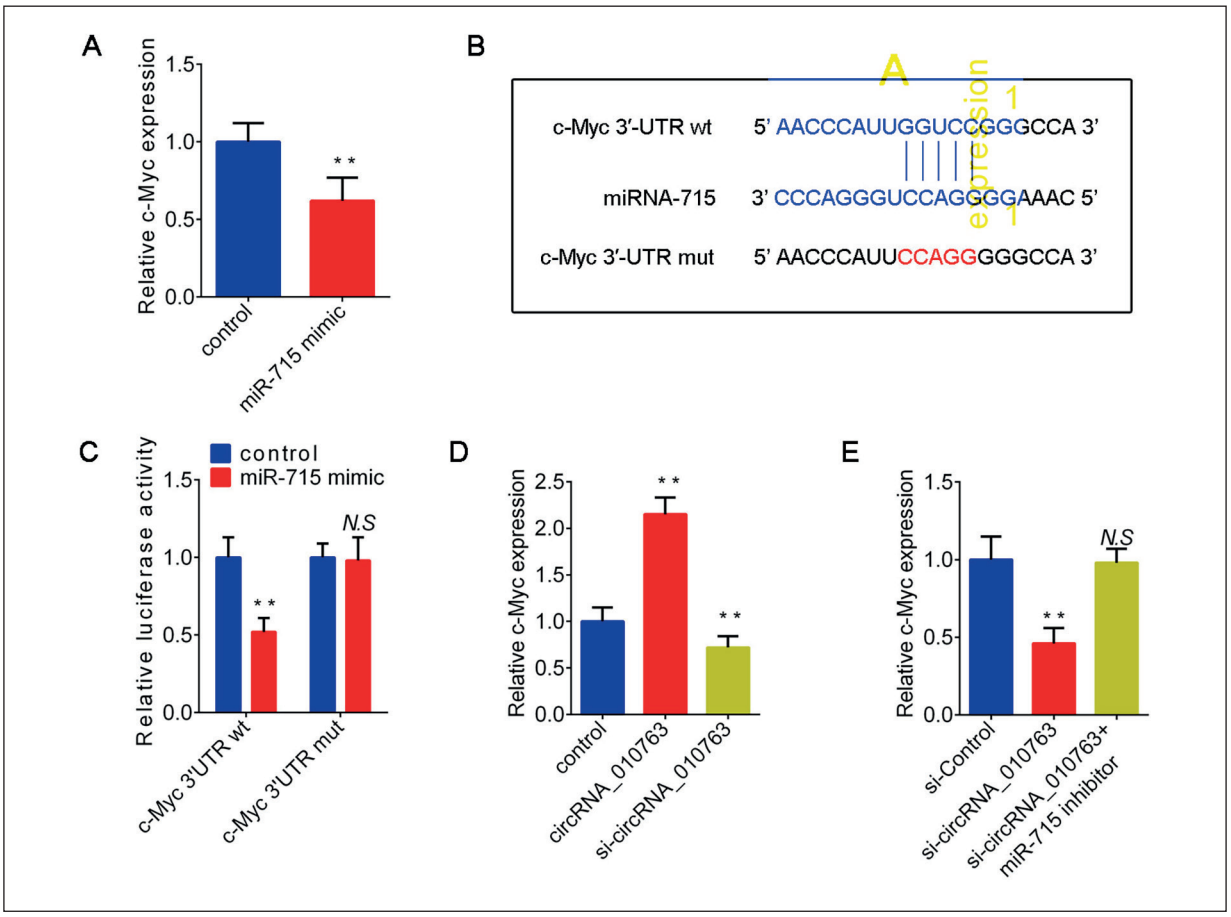

Figure 5. CircRNA_010763 served as a molecular sponge for miR-715 to further modulate the expression of c-Myc. **A**, qRT-PCR analysis of c-Myc mRNA expression level in A549 cells treated with the miR-715 mimics. **B**, Schematic illustration of the predicted c-Myc binding sites and mutant sites in miR-715. **C**, Relative Luciferase activity of A549 cells. **D**, Relative mRNA expression levels of c-Myc in A549 cells transfected with circRNA_010763 and circRNA_010763 mut-MRE. **E**, Relative mRNA expression levels of c-Myc in A549 cells transfected with si-circRNA_010763, si-circRNA_010763 and miR-715 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 ** ($p < 0.01$).

data demonstrated that circRNA_010763 could serve as a molecular sponge for the miR-715 to further modulate c-Myc.

Discussion

Lung cancer is the leading cause of cancer years of life lost and is associated with the highest economic burden relative to other tumor types[16]. Currently, there are no validated diagnostic tools to distinguish patients with localized or locally advanced disease who are at high risk, nor are there validated methods to detect recurrence, prognosis or monitor response to therapy. The identification of biomarkers that have prognostic potential in lung cancer could provide a strategy to address these unmet needs[17]. CircRNAs represent a novel class of conserved endogenous RNAs that regulate gene expression in mammals. The evolutionary conservation of circularization usually conceals certain important function for circRNA[18]. Compared with other noncoding RNA such as 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[19,20]. Currently, although an increasing number of researchers have begun to study potential func-

tions of circRNAs, however, their clinical values remain largely unknown.

CircRNA functions as crucial gene regulators by their post-transcriptional modification such as binding miRNA, assembling RNA-binding proteins and modulating transcription factors[21]. Moreover, it contains miRNA-binding site and usually exerts as a miRNA sponge to negatively regulate expression of target mRNAs[22]. Circular RNA cTFRC acts as the sponge of miRNA-107 to promote bladder carcinoma progression[23]. Circular RNA circMTO1 suppresses BC metastasis by sponging miR-221 and inhibiting epithelial-to-mesenchymal transition[24]. Invasion-related circular RNA circFNDC3B inhibits BC progression through the miR-1178-3p/G3BP2/SRC/FAK axis[25]. However, the regulatory role of circRNA_010763 in tumor development, especially the progression of NSCLC, has been poorly reported.

Our study is the first to reveal the expression pattern and prognostic value of circRNA_010763 in NSCLC. We found that the expression levels of circRNA_010763 in NSCLC tissues were significantly higher than those in their adjacent nontumorous tissues. Besides, we first validate the important role of circRNA_010763 in proliferation of human NSCLC cells A549. Simultaneously, we found the expression of circRNA_010763 was associated with the migration and invasion ability of A549. Through bioinformatics prediction, we found miR-715 as a target miRNA of circRNA_010763 and validated the combination relationship of circRNA_010763 and miR-715 using Luciferase reporter assay. Furthermore, we found that miR-715 can interact with circRNA_010763 co-expression gene c-Myc and downregulate the expression of c-Myc. Overexpression of circRNA_010763 can significantly promote c-Myc gene expression; however, mutagenesis of the miR-715 recognition element in circRNA_010763 alleviates the function effectively.

Conclusions

Together, we suggested that circRNA_010763 served as a ceRNA of miR-715 to upregulate c-Myc expression. CircRNA_010763 might be used as an important molecular marker for poor prognosis of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Founding

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