Circular RNA CircCDYL Regulates Proliferation and Apoptosis in Non-Small Cell Lung Cancer Cells by Sponging miR-185-5p and Upregulating TNRC6A

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Aim: A series of research reveal that circular RNA (circRNA) plays a vital role in regulating the development of tumor cells. In this research, we would explore the role and mechanism of circCDYL in non-small cell lung cancer (NSCLC).

Methods: RT-PCR was performed to detect the expression of circCDYL in NSCLC tissues, plasma, and cell lines. The tumor cell proliferation ability was evaluated by clone formation assay, and cell cycle determination. Flow cytometry was used to detect apoptosis in NSCLC cell lines. Western blot and RT-PCR were used to assess the expression of proteins and genes. Luciferase assay was performed to confirm the relationship of circRNA-miRNA-mRNA.

Results: The decreased level of circCDYL was observed in NSCLC patients’ tissues and plasma, which was also downregulated in NSCLC cell lines. Forced expression of circCDYL inhibited cell viability, proliferation and induced apoptosis in A549 cells. Luciferase assay verified that circCDYL could bind with miR-185-5p and confirmed that TNRC6A was a downstream target of miR-185-5p. Overexpression of miR-185-5p or silencing of TNRC6A could inhibit the anti-tumor effect of circCDYL in A549 cells via regulating the ERK1/2 signal.

Conclusion: Here, we revealed that circCDYL inhibited proliferation and induced apoptosis in NSCLC cell lines via regulating ERK1/2 signal, and the mechanism of this progression may target miR-185-5p/TNRC6A, which provided a theoretical basis for clinical therapy.

Keywords: non-small cell lung cancer, circular RNA, proliferation, apoptosis

Introduction
Lung cancer is a common malignant tumor. In recent years, investigations have shown that the incidence and mortality of lung cancer are in the first place in malignant tumors.1,2 NSCLC is a common type of lung cancer, accounting for about 80% of lung cancer. In addition, in patients with NSCLC, the incidence of brain metastasis is more than 50%, which has a severe impact on patients’ physical and mental health, quality of life, and prognosis.3

CircRNA is a new class of endogenous non-coding RNA molecules that exist widely and stably in eukaryotic cells without a 5′- terminal cap and a 3′- terminal poly (A) tail and forms a closed ring structure with covalent bonds.4,5 CircRNAs are not only rich, conservative, stable, but also specific, so it is likely to become...
a new biomarker for early diagnosis and prognosis evaluation of clinical diseases.\textsuperscript{5,7} The incidence and mortality of malignant tumors are very high. CircRNAs play the biological functions of miRNA molecular sponge or competitive endogenous RNA (ceRNA), interaction with RNA binding protein (RBP), translation protein, and other biological functions in malignant tumors, which affects the growth and metastasis of malignant tumors, thus promoting or inhibiting the occurrence and development of malignant tumors, and is expected to become a new therapeutic target for malignant tumors.\textsuperscript{8,9} The existing treatment methods could not effectively reduce the mortality of malignant tumors, but the discovery and in-depth study of circRNAs broaden the field of vision and provide ideas for the search for new methods for the treatment of malignant tumors. Yan et al found that after the introduction of circBIRC6-specific siRNA into A549 cells, the proliferation and invasion ability of A549 cells decreased, and the apoptosis rate increased significantly. This experiment confirmed that circBIRC6 played an essential role in the proliferation and invasion of lung cancer cells in vitro.\textsuperscript{10} Zhang et al found that CDR1as functioned as an oncogene to inhibit the anti-tumor effects of tumor suppressor miR-7 by up-regulation of proliferation index Ki-67, EGFR, CCNE1, and PIK3CD levels.\textsuperscript{11} Hong et al found that knock-down of circCPA4 inhibited intracellular and extracellular PD-L1 by targeting let-7 miRNA. On the one hand, PD-L1 self-regulated NSCLC cell growth, mobility, stemness, and chemoresistance to cisplatin treatment. On the other, secreted PD-L1 inactivated CD8+ T cells by activating extracellular and intracellular pathways mediated cell death to facilitate immune evasion.\textsuperscript{12}

It was reported that CircCDYL was involved in a variety of disease processes. Wei et al uncovered a CircCDYL-centric non-coding regulatory RNAs network in the early stages of HCC, which include miR-892a and miR-328-3p, HDGF, and HIF1AN transcripts.\textsuperscript{13} Liang et al revealed that autophagy-associated circRNA circCDYL was increased in breast cancer tissues. CircCDYL promoted the autophagic level in breast cancer cells through the miR-1275-ATG7/ULK1 signal. Clinically, increased circCDYL in the tumor tissues and serum of breast cancer patients was associated with higher tumor burden, shorter survival, and poorer clinical response to therapy.\textsuperscript{14} It was reported that circCDYL was highly expressed in the plasma of Mantle cell lymphoma patients. Functionally, circCDYL knockdown inhibited Mantle cell lymphoma cell proliferation. Bioinformatics analyses and identified a circCDYL-miRNA–mRNA/lncRNA network, highlighted by five miRNAs (hsa-miR-129-5p,3163, 4662a-5p, 101–3p, 186–5p), three lncRNAs (MALAT1, NEAT1, and XIST), and five miRNAs (NOTCH1, FMR1, ABCB1, TWIST1, and VEGFA). Research on circCDYL in series of diseases was explored, such as colon cancer,\textsuperscript{16} bladder cancer,\textsuperscript{17} and multiple myeloma.\textsuperscript{18} However, the molecular functions and biological roles of circCDYL in NSCLC remain unknown. In this study, RT-PCR detection showed that there was a significant difference in the expression of circCDYL between tumor and adjacent tissues from NSCLC, and verified in several NSCLC cell lines in vitro. The biological functions of circCDYL in NSCLC cells, such as proliferation, cell cycle, and apoptosis, and the preliminary mechanism, were explored. These results provide a basis for circRNA as a potential biomarker of NSCLC.

**Materials and Methods**

**Patient Tumor Samples**

The tumor tissues and adjacent lung tissues were collected from 30 patients who underwent resection of NSCLC in the Heilongjiang Provincial Hospital (Table 1). The samples were quickly stored at −80°C. All patients were diagnosed as NSCLC by pathological and immunohistochemical determination. This research was approved by the ethics committee of Heilongjiang Provincial Hospital.

**Table 1 Clinicopathological Features of NSCLC Patients (N=30)**

| Parameters                  | N     | %    |
|-----------------------------|-------|------|
| Age (years)                 | 53.7±6.8 | 70%  |
| Gender (male)               | 21    | 76.67% |
| Smoke (n%)                  | 23    |      |
| Pathological grade          |       |      |
| Poor differentiation (n%)   | 12    | 40%  |
| Moderate differentiation (n%)| 9     | 26.67% |
| Well differentiation (n%)   | 4     | 33.33% |
| Tumor size                  |       |      |
| > 5 cm (n%)                 | 13    | 43.33% |
| ≤5 cm (n%)                  | 17    | 56.67% |
| Lymph node metastasis       |       |      |
| Positive (n%)               | 19    | 63.33% |
| Negative (n%)               | 11    | 36.67% |
| TNM stage                   |       |      |
| III stage (n%)              | 9     | 30%  |
| II stage (n%)               | 15    | 50%  |
| I stage (n%)                | 6     | 20%  |
(#2018011) and received informed consent signed by all the subject members.

**Cell Culture**

NSCLC cells (A549, H2170, PG49, SPC-A-1) and Human normal bronchial epithelial cells (BEAS-2B) were purchased from the Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium, and the specific formula was as follows: RPMI-1640 added 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/L streptomycin. Human normal bronchial epithelial cells (BEAS-2B) were cultured in the DMEM medium. The specific formula was as follows: 10% FBS, 100 U/mL penicillin, and 100 mg/L streptomycin were added to the DMEM culture medium.

**Cell Treatment and Transfection**

Vectors to overexpress circCDYL (circCDYL) and negative control (vector) were procured from BersinBio Technology (Guangzhou, China). miR-186-5p mimics and miRNA negative control (miR-NC), siRNA TNRC6A (si-TNRC6A) were from Ribobio (Guangzhou, China).

To up-regulate circCDYL, circCDYL sequence was cloned into pLCDH-circRNA vector by Ribobio (Guangzhou, China). miR-185-5p mimic and negative control mimic (miR-NC) were synthesized by Life Technologies (Waltham, MA, USA).

Transient transfection was performed on NSCLC cells plated into 6-well culture plates at a confluence of 50% to 60%. One day later, cultures underwent transfection of vector/miRNA mimics/siRNA using Lipofectamine® 2000 (Invitrogen).

**CCK8 Assay**

The cell suspension was seeded in a 96-well plate with about 3000 cells per well. The culture plate was pre-cultured in the incubator (37°C, 5% CO₂). 10 μL CCK8 detection reagent was added to each well and incubated in an incubator for 4 h. The absorbance at 450 nm was determined by an enzyme labeling instrument, and the OD value of each well was determined.

**Clone Formation Assay**

The cultured cells were collected and seeded in 6-well plates, according to 1000/well and cultured for 14 days. After PBS washing, cells were fixed with 4% paraformaldehyde for 30 min. Giemsa dye A was added in each well for 5 min and continued to be stained with dye B for 15 min. The images were scanned with a microscope. The clone count was calculated.

**Cell Cycle Detection**

The cells were collected after tryptasin digestion and centrifugation. The cells were mixed with precooled anhydrous ethanol (70% of the final concentration of ethanol) and fixed overnight at 4°C. The fixed cells were centrifuged and collected, and then RNase A was added in cells with a 37°C water bath. Finally, PI was added to avoid light staining at room temperature for 30 min, and flow cytometry was used to detect the distribution of the cell cycle. ModFit was used to analyze the distribution of the cell cycle.

**Apoptosis Detection**

After trypsin digestion and centrifugation, the cells were collected trypsin digestion and centrifugation, and the single-cell suspension was added with 300 μL of 1 × Binding Buffer. After mixing with 5 μL Annexin V-FITC, the cells were incubated at room temperature for 15 min, and then 5 μL PI staining was added to label the suspension cells. The apoptosis was detected and analyzed by flow cytometry.

**RT-PCR**

The total RNA of cells and tissues were extracted with TRIzol reagent, and the concentration of total RNA was determined by the spectrophotometer. cDNA was synthesized by reverse transcription reaction. The Prime-Script RT Reagent Kit (RR036A, Takara, Shiga, Japan) was used for the reverse transcription of RNA into cDNA. GAPDH served as the internal reference for circRNA and miRNA, and U6 served as the internal reference for miRNA. The fold changes were calculated using the quantification method (2−ΔΔCt). The primer sequence was shown as follows:

- circCDYL: forward: 5′-ACCCACTAGTGGCCTCAGG TG-3′, reserve: 5′-CTGTGGAGGATGCACCTT-3′. GAP DH: forward: 5′-CTGTGGAGGATGCACCTT-3′, reserve: 5′-CTTCATCATACATAGCAGTC3′; miR-185-5p: 3′ specific primer: TGGAGGAAAGGCAGTTCC TGA;

- U6: forward: 5′-CTCGCTTCCGACGCACATATACT -3′, reserve: 5′-CGCCAGATTTGCATGTC-3′. TN RC6A: forward: 5′-TCAATACATACATAGCAGTC3′, reserve: 5′-TATGCGTGGTCTTCTGTGTC-3′. The expression of each gene was detected by SYBR Green I dye on ABI7500 fluorescence quantitative PCR.
Western Blot
After trypsin digestion and centrifugation, the cells were collected and added with cell lysate, the total cell protein was extracted and quantified by BCA method. 10% SDS-polyacrylamide gel electrophoresis was used to transfer the membrane, sealed with 10% skimmed milk powder, and the primary and secondary antibodies were incubated once, and the membranes were scanned and analyzed by Odyssey fluorescence imaging system.

Luciferase Report
WT-circCDYL/Mutant-circCDYL or WT-TNRC6A/Mutant-TNRC6A were co-transfected with miR-185-5p/miR-NC into HEK293 cells. The cells were cultured at 37°C and 5%CO2 for 12 h. The fluorescence intensity was observed by the enzyme labeling instrument.

Animal Research
For xenograft assays, 1 × 10⁶ educated A549-sh-NC or A549-sh-circCDYL cells were injected subcutaneously into the right side of each male nude mouse (Chinese Science Academy). The size of the tumors (length × width² × 0.5) was measured at the indicated time points and tumors were obtained 18 days after injection. The animal study was reviewed and approved by Heilongjiang Provincial Hospital, and was carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Immunohistochemistry (IHC)
Tumor tissue samples were fixed in a 10% formalin solution and embedded in paraffin. Sections (5-μm thick) were stained with Ki-67 and ERK, p-ERK, Caspase3. Sections were examined using an Olympus confocal microscope and photographed with a digital camera.

Statistical Analysis
The data results were measured by (mean ± SEM) and analyzed by Graphpad 7.0 software. ANOVA test was used to compare the variable data of the difference among all groups, and t-test was used to compare the variable data between the two groups. The difference was statistically significant (P < 0.05).

Results
The Downregulation of circCDYL in NSCLC Tissues and Cell Lines
In our initial RT-PCR analysis of NSCLC tumor tissues and adjacent (n = 30 patients), circCDYL was found significantly downregulated in NSCLC tissues relative to adjacent normal tissue (Figure 1A and Table 1). We also explored the expression of circCDYL in plasma from NSCLC patients and healthy volunteers. Then, we also observed the decreased level in NSCLC patients’ plasma (Figure 1B). Then, we detected the expression of circCDYL in NSCLC cell lines (A549, H2170, PG49, SPC-A-1), and BEAS-2B was indicated as control. RT-PCR results revealed a decreased level in NSCLC cell lines (Figure 1C).

Forced Expression of circCDYL Inhibits Proliferation and Induces Apoptosis in A549 Cells
To explore the function of circCDYL in NSCLC progression, we constructed a plasmid for overexpressing the circCDYL level. The transfection efficiency of circCDYL was performed by RT-PCR (Figure 2A). CCK8 assay

Figure 1 CircCDYL is downregulated in NSCLC tissues and cell lines. (A) The expression of circCDYL was detected by RT-PCR in tumor tissue and adjacent normal tissues from NSCLC patients. n=30, *P<0.05. (B) The expression of circCDYL was detected by RT-PCR in plasma from NSCLC patients and healthy volunteers, n=30, **P=0.01. (C) The expression of circCDYL in NSCLC cell lines (A549, H2170, PG49, SPC-A-1), and BEAS-2B was indicated as control. n=30, *P<0.05, **P=0.01.
revealed that circCDYL inhibited cell viability in A549 cells at 72 h (Figure 2B). Then, cell cycle was determined by flow cytometry, we found that circCDYL prevented cells from the G0 phase from entering into the S phase (Figure 2C). Clone formation assay showed that overexpression of circCDYL blocked the clone formation in A549 cells (Figure 2D). Further, the apoptosis level of NSCLC cells was explored by flow cytometry; the results demonstrated that circCDYL could induce apoptosis in A549 cells (Figure 2E). Taken together, circCDYL prevented proliferation and induced apoptosis in NSCLC cells.

miR-185-5p Binds with circCDYL

Bioinformatics site forecasted that there were binding sites between circCDYL and miR-185-5p (Figure 3A). Then, we co-transfected circCDYL-WT/circCDYL-Mutant with miR-185-5p/miR-NC. We found that circCDYL-WT co-transfected with miR-185-5p performed the decreased luciferase level, while circCDYL-Mutant co-transfected with miR-185-5p had no effect on luciferase activity (Figure 3B). A pull-down assay was used to further determine miR-185-5p via a specific biotin-labeled circCDYL probe. We verified the significantly upregulated pull-down efficiency of the circCDYL probe in a circCDYL overexpression plasmid transfected into A549 cell (Figure 3C). Then, we separately transfected sh-circCDYL/sh-NC and circCDYL/vector into A549 cells. RT-PCR results showed that sh-circCDYL induced the expression of miR-185-5p, and circCDYL inhibited the expression of miR-185-5p (Figure 3D). Then, we observed that miR-185-5p was an upregulation in NSCLC tumor tissues (Figure 3E).

miR-185-5p Could Bind with 3’UTR of TNRC6A

Four bioinformatics sites (miRmap, PITA, miRanda, microT) were used to predict the downstream of miR-185-5p, and TNRC6A was confirmed (Figure 4A). Luciferase assay reported that miR-185-5p could interact with TNRC6A (Figure 4B). MiR-185-5p could induce the mRNA and protein expression levels of TNRC6A in A549 cells (Figure 4C and D). Then, we observed that the mRNA and protein level of TNRC6A was downregulated in NSCLC tumor tissues (Figure 4E and F).

CircCDYL Prevents NSCLC Progression via ERK1/2 Signal by Targeting miR-185-5p/TNRC6A

Further, we explore the mechanism of circCDYL in NSCLC cells. We co-transfected circCDYL with miR-185-5p/si-TNRC6A. The expression of TNRC6A was determined by RT-PCR (Figure 5A). CCK8 assay performed that overexpression of miR-185-5p or silencing of TNRC6A would prevent the effect of circCDYL on cell viability (Figure 5B). Meanwhile, the cell cycle was confirmed by flow cytometry. Overexpression of miR-185-5p or silencing of TNRC6A reversed the effect of circCDYL on the cell cycle (Figure 5C). Then, the
inhibition effect of circCDYL on clone formation was blocked by miR-185-5p or si-TNRC6A (Figure 5D). Next, we explored the expression of ERK1/2 in A549 cells. circCDYL prevented the phosphorylation of ERK1/2, which was controlled by miR-185-5p or si-TNRC6A (Figure 5E). Taken together, circCDYL inhibited proliferation and induced apoptosis in A549 cells via regulating ERK1/2 by targeting miR-185-5p/TNRC6A.

Silencing of circCDYL Inhibits Tumor Progression in vivo

To further explore the function of circCDYL in GC, we constructed a stable A549 cell line with low expression of circCDYL, and normal A549 cells were identified as control. Cells were subcutaneously injected into nude mice, and we measured tumor volume. Sh-circCDYL significantly reduced tumor volume and weight (Figure 6A–C). Then, RT-PCR was performed to detect the level of circCDYL, miR-185-5p, and TNRC6A (Figure 6D). As shown in Figure 6E, sh-circCDYL decreased the phosphorylation level of ERK, Ki-67 and Caspase3.

Discussion

The morbidity and mortality of NSCLC are incredibly high. Surgery belongs to the traditional treatment of NSCLC, but because there are no typical symptoms in the early stage of NSCLC, and the clinical signs are not
specific, most of the patients have been confirmed to be late at the time of diagnosis, so they are not suitable for surgical treatment. Although significant progress has been made in the treatment, equipment, and technology of lung cancer, the prognosis of patients is still relatively poor.

Due to the rapid development of RNA technology, more and more circRNAs closely related to tumors have been found. A large number of studies have shown that there are differences in the expression of circRNAs in various tumor tissues and normal tissues and participate in a variety of pathological processes, including apoptosis, proliferation, and invasion. Shangguan et al found that circSLC25A16 is upregulated in NSCLC tissues/cells and associated with the unfavorable outcome of NSCLC patients. CircSLC25A16 interacts with miR-488-3p/HIF-1α and activates LDHA by facilitating its transcription. In addition, it was reported that the downregulation of serine/arginine splicing factor 1 (SRSF1) contributed to, at least in part, the increased expression of circSETD3 in NSCLC cells with acquired resistance to gefitinib in NSCLC. Research has confirmed that the present research performed a circRNA microarray analysis for the expression profile and identified a novel circRNA (circMAGI3, hsa_circ_010498). Clinically, circMAGI3 was significantly up-regulated in NSCLC tissue and cells, which was closely correlated with an unfavorable outcome for NSCLC patients. Functionally, circMAGI3 promoted the glycolysis and proliferation of NSCLC cells. Mechanistically, circMAGI3 functioned as

Figure 4 TNRC6A is a downstream target of miR-185-5p. (A) Four bioinformatics sites (mirmap, PITA, miRanda, microT) were used to predict the downstream of miR-185-5p. (B) Luciferase assay was performed to verify the relationship between TNRC6A and miR-185-5p. *P<0.05. (C and D) The mRNA and apoptosis level of TNRC6A in A549 cells after miR-185-5p transfection. n=5, *P<0.05. (E and F) The mRNA and apoptosis level of TNRC6A in NSCLC tumor tissues and adjacent tissues. *P<0.05.

Abbreviation: n.s., indicated no significant.
Figure 5 CircCDYL inhibits apoptosis and induces apoptosis via the EKR1/2 signal by targeting miR-185-5p/TNRC6A. (A) The level of TNRC6A was detected by RT-PCR. n=5, *P<0.05, #P<0.05. (B) CCK8 assay determined the cell viability of A549 cells. n=6, *P<0.05, #P<0.05. (C) Cell cycle was confirmed by flow cytometry. n=4, *P<0.05, #P<0.05. (D) Clone formation assay was performed in A549 cells. n=3, **P<0.01, #P<0.05. (E) The protein level of p-ERK1/2 and ERK1/2 in A549 cells. n=4, *P<0.05, #P<0.05.

Abbreviation: n.s., indicated no significant.

Figure 6 Silencing of circCDYL prevents tumor growth in vivo. (A) A549-sh-circCDYL and A549-sh-NC cells were subcutaneously injected into nude mice, and tumor growth curves were plotted (n = 4). (B and C) Tumor volume and weight was determined for each group. n=4, *P<0.05. (D) The expression of circCDYL, miR-185-5p, and TNRC6A in collected tumor. n=4, *P<0.05. (E) Immunohistochemical staining for Ki-67, ERK, p-ERK, Caspase3 in the xenograft tumor tissues.
a sponge for miR-515-5p to relieve its target gene HDGF expression, thereby accelerating the glycolysis of NSCLC. Zhao et al found that circ_0067934 and KLF8 were upregulated, while miR-1182 was downregulated in NSCLC tissues and cells. Circ_0067934 knockout blocked proliferation, migration, invasion, and EMT and induced apoptosis in NSCLC cells. Circ_0067934 regulated NSCLC progression by sponging miR-1182. MiR-1182 targeted KLF8 to hinder NSCLC development. Our findings signified the circ_0000376 high expression in NSCLC and its clinical significance. We also found that circ_0000376 enhances the proliferation, invasion, and metastasis in NSCLC cells via modulating miR-384. To our best knowledge, this is the first study to investigate the role of circ_0000376 in cancer biology and it is expected to provide new therapeutic targets for NSCLC patients.

In our research, we found that circCDYL was decreased in NSCLC tumor tissues and cell lines. CircCDYL, as a ceRNA, could inhibit the expression of miR-185-5p and promote the expression of TNRC6A, thus regulating the development of lung cancer.

At present, the understanding of the function of circRNA is not complete, and the mechanism of its role in malignant tumors is mainly focused on the function of miRNA sponge. Some circRNAs interact with different proteins in related signaling pathways to regulate cell activity. In addition, circRNAs are related to EMT, and circRNAs regulate the progress of EMT through EMT transcription factors and EMT-related signal pathways. Some specifically expressed circRNAs are expected to become a tumor marker and provide a new target for early diagnosis and treatment of tumors.

Conclusion
We demonstrated that circCDYL is significantly downregulated in NSCLC tissues and cell lines. CircCDYL can successfully sponge miR-185-5p and regulate TNRC6A to inhibit the proliferation and induce apoptosis of A549 cells. We also found that circCDYL can block the malignant behavior of A549 cells through the ERK1/2 signaling pathway. We proved that circCDYL assumes the role of a miRNA sponge and that circCDYL would be a promising therapeutic target in NSCLC in the years to come.

Disclosure
The authors declare that they have no competing interests.

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