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Upregulation of lncRNA NCK1-AS1 predicts poor prognosis and contributes to non-small cell lung cancer proliferation by regulating CDK1

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Abstract. – OBJECTIVE: To detect the expression of long non-coding ribonucleic acid (lncRNA) NCK1-AS1 in non-small cell lung cancer (NSCLC), analyze the association between its expression and the clinicopathological characteristics of NSCLC patients, and study the biological function of NCK1-AS1 *in vitro*.

PATIENTS AND METHODS: The relative expression of NCK1-AS1 in NSCLC tissues and cells was detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The association between the expression of NCK1-AS1 and the clinicopathological characteristics of NSCLC was statistically analyzed. The effects of interference in the expression of NCK1-AS1 on the biological behaviors of NSCLC cells were detected via *in vitro* experiments, including Cell Counting Kit-8 (CCK-8) assay, colony formation assay and flow cytometry. After interference in the expression of NCK1-AS1, the expression of cyclin-dependent kinase 1 (CDK1) was determined using Western blotting.

RESULTS: The results of qRT-PCR showed that the expression of NCK1-AS1 was up-regulated in 50 out of 64 cases of NSCLC tissues. It was found via statistical analysis that highly expressed NCK1-AS1 was positively correlated with tumor size, TNM stage and lymph node metastasis. The results of qRT-PCR revealed that the expression of NCK1-AS1 was also up-regulated in NSCLC cells. After interference in the expression of NCK1-AS1, the proliferation of NSCLC cells was inhibited, and the cell cycle was arrested at G2/M phase. The results of Western blotting manifested that the expression of CDK1 was suppressed after interference in the expression of NCK1-AS1.

CONCLUSIONS: The expression of NCK1-AS1 is up-regulated in NSCLC, which indicates a poor prognosis. Highly expressed NCK1-AS1 promotes the proliferation of NSCLC cells through activating CDK1.

Key Words:
NSCLC, LncRNA NCK1-AS1, Proliferation, CDK1.

Introduction

Non-small cell lung cancer (NSCLC) is one of the thoracic malignancies with high morbidity and mortality rates in the world[1]. Despite great improvement in the efficacy on NSCLC, the 5-year survival rate of NSCLC patients is still lower than 20%. Therefore, it is one of the important treatment strategies to search for new therapeutic targets for NSCLC[2].

Long non-coding ribonucleic acids (lncRNAs) are a newly discovered class of non-protein-coding transcripts with more than 200 nucleotides in length. They are involved in many physiopathological processes, such as cell development, apoptosis, differentiation and immune response[3,4]. There is increasing evidence that dysregulation of lncRNAs plays a key role in the occurrence and development of various types of cancer[5-7]. It has been proved that many lncRNAs are involved in the occurrence and development of NSCLC and can serve as diagnostic or therapeutic targets for NSCLC[8,9].

LncRNA NCK1-AS1, with a total length of 1073 bp, is located in chromosome 3q22.3. It is reported in the literature that the expression of NCK1-AS1 is up-regulated in cervical cancer tissues and cells, and highly expressed NCK1-AS1 promotes the proliferation of cervical cancer cells[10]. Zhang et al[11] found that the sensitivity of cervical cancer cells to cisplatin can be enhanced after interference in the expression of NCK1-AS1. In nasopharyngeal carcinoma, the expression of NCK1-AS1 is also up-regulated, which facilitates the invasion and metastasis of nasopharyngeal carcinoma cells through regulating miR-135a[12]. However, there have been no reports about the expression and biological function of NCK1-AS1 in NSCLC. Therefore, the expression of NCK1-AS1 in NSCLC was

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detected for the first time, and its association with the clinicopathological characteristics of NSCLC patients was analyzed in this paper.

Patients and Methods

Tissue Specimens

A total of 64 NSCLC patients admitted in The People's Hospital of Danyang from January 2015 to December 2017 were enrolled, and they underwent surgical resection and pathologically diagnosed with NSCLC. Inclusion criteria: 1) patients receiving no biotherapy, chemoradiotherapy, targeted therapy and immunotherapy before operation, and 2) those not complicated with other tumors. The complete examination, treatment and operation data of patients were collected. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). The specimens were first placed in a liquid nitrogen container for 10 min, then taken out and stored at -80°C. This investigation was approved by the Ethics Committee of The People's Hospital of Danyang and the patients. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human NSCLC cell lines (A549, NCI-H1299, PC-9 and NCI-H1650) and human normal bronchial epithelial cell lines were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). NSCLC cells were cultured with Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin double antibodies (Shanghai Yuanmu Biotechnology Co., Ltd., Shanghai, China) in an incubator with 5% CO_2 at 37°C.

RNA Extraction and Reverse Transcription

The total RNA was extracted from surgically resected NSCLC tissues and para-carcinoma normal tissues using TRIzol (TaKaRa, Otsu, Shiga, Japan), and identified using a Nano-300 micro-spectrophotometer. The RNA with an optical density $(OD)_{260}/OD_{280}$ of 1.8-2.0 could be used in subsequent experiments. Then the total RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript™ RT Reagent Kit (TaKaRa, Otsu, Shiga, Japan).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

PCR amplification was performed, with cDNA as a template, according to the instructions of SYBR Green qPCR Master Mix (TaKaRa, Otsu, Shiga, Japan). The primer sequences are as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: GGAGCGAGATCCCTCCAAAAT, R: GGCTGTTGTCATACTTCTCATGG, NCK1-AS1 F: TTCC CATTTCTCCCAGGTCC, R: TGGTTACTTTGAGCCTGCCT. The reaction system (20 μL) consisted of 10 μL of SYBR Premix Ex Taq, 0.5 μL of forward primers, 0.5 μL of reverse primers, 1 μL of cDNA template and 8 μL of ddH_2O. The reaction conditions are as follows: 95°C for 30 s, then 95°C for 10 s and 60°C for 30 s for a total of 40 cycles. The relative expression of target gene was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as an internal reference. The assay was repeated for 3 times, and the average was taken.

Cell Transfection

NSCLC cells in the logarithmic growth phase were inoculated into the cell culture plate. About 5 μmol/L negative control (si-NC) or interference reagent (si-NCK1-AS1) (Shanghai GenePharma Co., Ltd., Shanghai, China) was mixed with transfection reagent lip2000 (Invitrogen, Carlsbad, CA, USA) for 15 min. Then, the mixed solution was added into cells. The cell culture medium was replaced after 6 h, and the interference efficiency was detected *via* qRT-PCR at 48 h after transfection.

Cell Counting Kit-8 (CCK-8) Assay

At 48 h after transfection, the cells were collected in the two groups, digested with trypsin, resuspended in complete medium containing 10% FBS and prepared into cell suspension at a density of 5×10^4 cells/mL. The cell suspension was inoculated into a 96-well plate (about 5×10^3 cells/well) and incubated in an incubator with 5% CO_2 at 37°C for 24 h. Then 10 μL of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well, followed by incubation at room temperature for 1 h. The OD of each well at 450 nm wavelength (OD_{450}) was

detected using a microplate reader, indicating the cell proliferation ability. The assay was repeated for 3 times, and the average was taken.

Colony Formation Assay

After treatment, the cells were inoculated into a 6-well plate (800 cells/well) and cultured under standard conditions for 10-14 d, and the formation of colonies was observed. The cells were cultured until there were visible colonies. Then, after the medium was discarded, the cells were carefully washed twice with phosphate-buffered saline (PBS), and fixed with 4% formaldehyde (1 mL/well) for 15 min. After the fixative was discarded, the cells were stained with 1 mL of crystal violet dye for 15 min, washed, air dried and photographed.

Flow Cytometry

The transfected NSCLC cells were collected, fixed with 70% cold ethanol at 4°C, washed twice with PBS and incubated with 10 μg/mL RNase A and 20 μg/mL PI (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. Then the cell cycle distribution was analyzed using a flow cytometer (Beckman Biosciences, Franklin Lakes, NJ, USA). The percentage of cells at G0/G1, S and G2/M phases was analyzed using ModFit LT (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blotting

The cells were collected and lysed at 4°C to extract the total protein, and the total protein concentration was measured using the bicinchoninic acid (BCA) kit (Beyotime Biotechnology, Shanghai, China). Subsequently, 30 g of protein samples were loaded, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis for 1-2 h, transferred onto a polyvinylidene difluoride (PVDF) membrane for 30-50 min, and incubated with 2% bovine serum albumin (BSA) at room temperature for 1 h and with the primary antibody solution (CDK1, GAPDH) at 4°C overnight. The next day, the protein samples were incubated again with the secondary antibody at room temperature. Finally, the protein band was exposed using the gel imaging system according to the instructions of chemiluminescence immunoassay kit.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p<0.05$ was considered to be statistically significant.

Results

Expression of NCK1-AS1 Was Upregulated in NSCLC

First, 64 cases of tissue specimens were collected from patients pathologically diagnosed with NSCLC, from which the RNA was extracted, and the relative expression of NCK1-AS1 in NSCLC tissues was determined using qRT-PCR. It was found that the expression of NCK1-AS1 was up-regulated in 50 cases of NSCLC tissues compared with that in para-carcinoma tissues (Figure 1A). With the mean fold (4.43) of NCK1-AS1 expression in NSCLC tissue as the cut-off point, the specimens were divided into high-expression group and low-expression group (Figure 1B). It was found using χ^2-test that highly expressed NCK1-AS1 in NSCLC was positively correlated with tumor size, TNM stage and lymph node metastasis (Table I), indicating that NCK1-AS1 can serve as a potential molecule for prognostic prediction of NSCLC patients. Then, the expression of NCK1-AS1 in NSCLC cells was determined *via* qRT-PCR. The results showed that the expression of NCK1-AS1 was up-regulated in NSCLC cells compared with that in human normal bronchial epithelial cells (Figure 1C). To study the biological function of NCK1-AS1 in NSCLC cells, specific interference sequences were designed and synthesized, and then transiently transfected into NSCLC cells. The interference efficiency was detected after 48 h (Figure 1D).

Interference in NCK1-AS1 Expression Inhibited NSCLC Cell Proliferation

The results of CCK-8 assay manifested that after interference in the expression of NCK1-AS1, the cell proliferation ability was suppressed in si-NCK1-AS1 group compared with that in si-NC group (Figure 2A and 2B). Besides, the effect of NCK1-AS1 on the proliferation of NSCLC cells was further verified using colony formation assay. The results showed that the proliferation of NSCLC cells could be inhibited by interference in

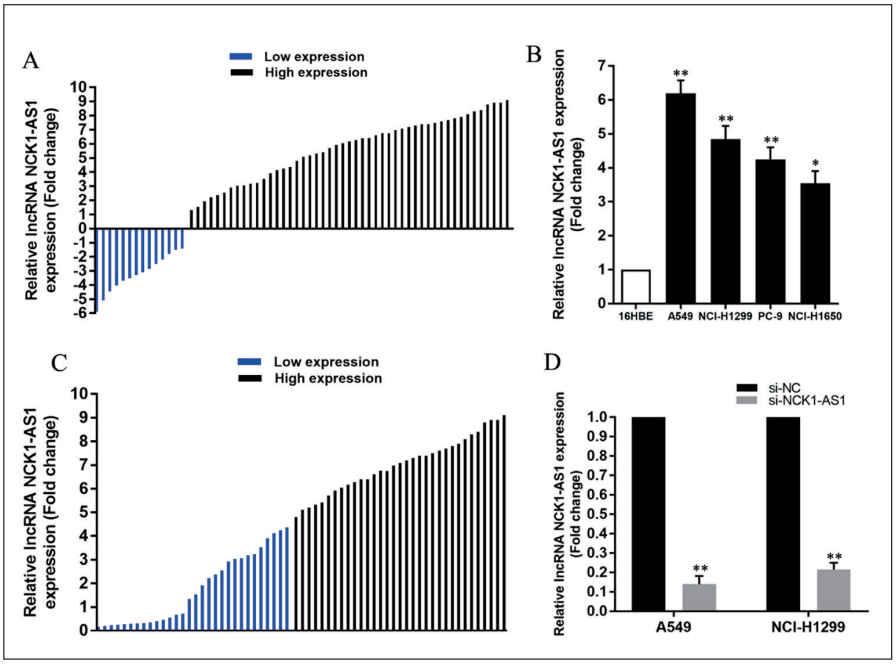

Figure 1. Expression of NCK1-AS1 was up-regulated in NSCLC. **A,** The expression of NCK1-AS1 in 64 cases of NSCLC tissues was determined using qRT-PCR. The expression of NCK1-AS1 was up-regulated in 50 cases of NSCLC tissues, with GAPDH as an internal reference. **B,** With the mean fold of NCK1-AS1 expression as the cut-off point, the specimens were divided into high-expression group and low-expression group. **C,** The expression of NCK1-AS1 in NSCLC cells was determined *via* qRT-PCR. The results showed that the expression of NCK1-AS1 was up-regulated in NSCLC cells. **D,** Si-NC and si-NCK1-AS1 were transfected into cells, and the interference efficiency was detected after 48 h.

the expression of NCK1-AS1 (Figure 2C and 2D). It is reported in the literature that the cell cycle affects the proliferation of tumor cells[13]. The effect of si-NCK1-AS1 on the NSCLC cell cycle was detected *via* flow cytometry. The results manifested that the cell cycle was arrested at G2/M phase after interference in the expression of NCK1-AS1 (Figure 2C-2F).

Table I. Correlation between lncRNA NCK1-AS1 expression and clinicopathological characteristics of NSCLC patients (n = 64).

Characteristics	NCK1-AS1 Low No. case	NCK1-AS1 High No. case	Chi- squared test *p*-value
Age(years)			0.791
> 65	14	19	
≤ 65	16	15	
Gender			0.812
Male	12	17	
Female	18	17	
Histological subtype			0.657
Squamous cell carcinoma	13	16	
Adenocarcinoma	17	18	
TNM Stage			0.001*
Ia + Ib	15	7	
IIa + IIb	10	10	
IIIa	5	17	
Tumor size			0.011*
≤ 5 cm	21	14	
> 5 cm	9	20	
Lymph node metastasis			0.032*
Negative	20	12	
Positive	10	22	
Smoking History			0.224
Smokers	11	18	
Never Smokers	19	16	

*Overall $p<0.05$.

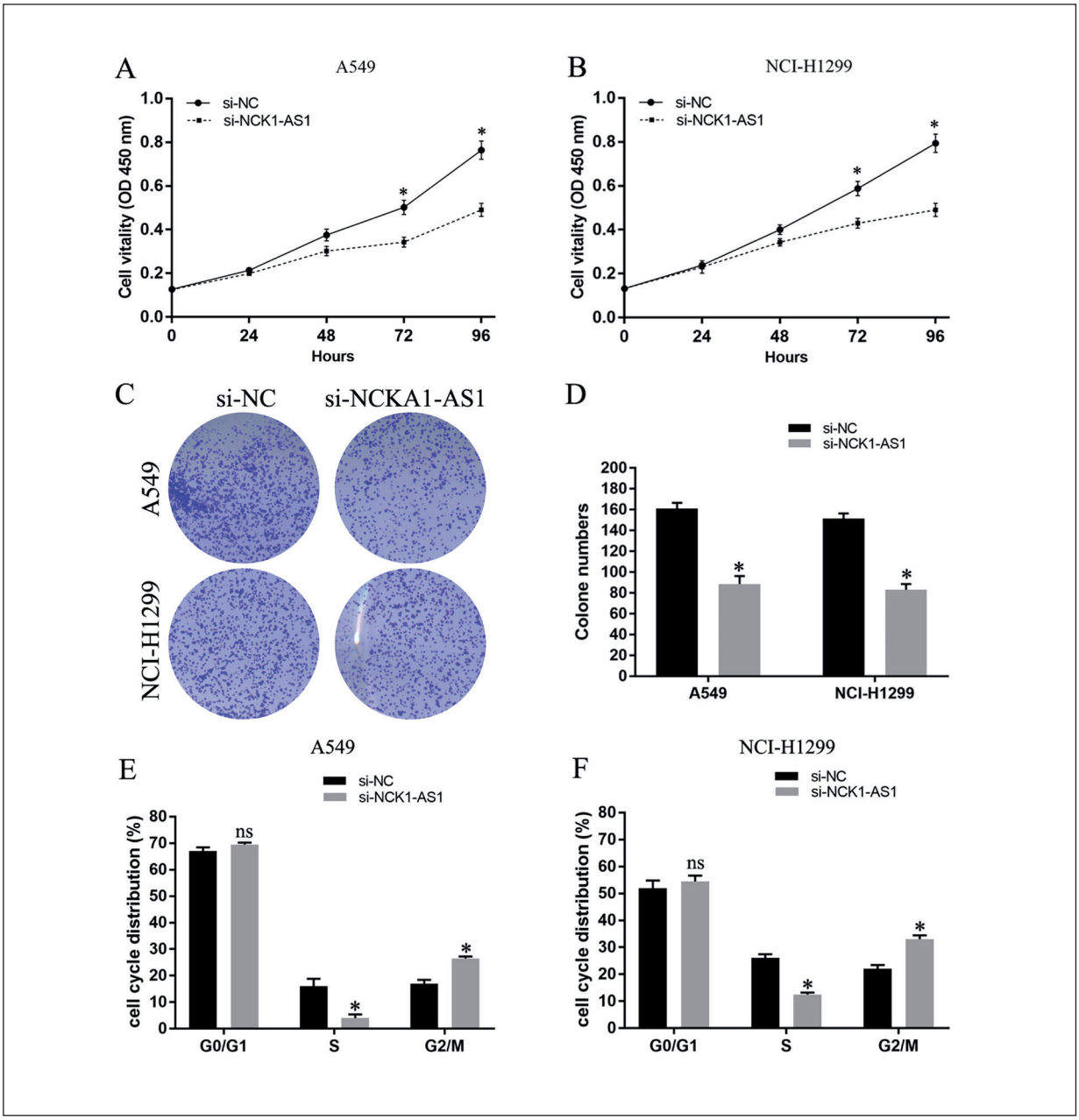

Figure 2. Interference in NCK1-AS1 expression inhibited NSCLC cell proliferation. **A,** and **B,** The results of CCK-8 assay manifested that after interference in the expression of NCK1-AS1, the NSCLC cell proliferation ability was suppressed. **C,** and **D,** The results of colony formation assay showed that the cell proliferation was inhibited in si-NCK1-AS1 group compared with that in si-NC group (magnification: 10X). **E,** and **F,** The results of flow cytometry manifested that the cell cycle was arrested at G2/M phase after interference in the expression of NCK1-AS1.

NCK1-AS1 Regulated Expression of Cyclin-Dependent Kinase 1 (CDK1)

In addition, the potential molecular mechanism of NCK1-AS1 affecting NSCLS cell proliferation was further explored. It is reported in the literature that NCK1-AS1 can promote the proliferation of cervical cancer cells through regulating the expression of CDK1[10]. To study the regulatory relation between NCK1-AS1 and CDK1 in NSCLC, qRT-PCR was performed. As shown in Figure 3, the results of qRT-PCR and Western blotting revealed that mRNA and protein expressions of CDK1 declined after interference in the expression of NCK1-AS1.

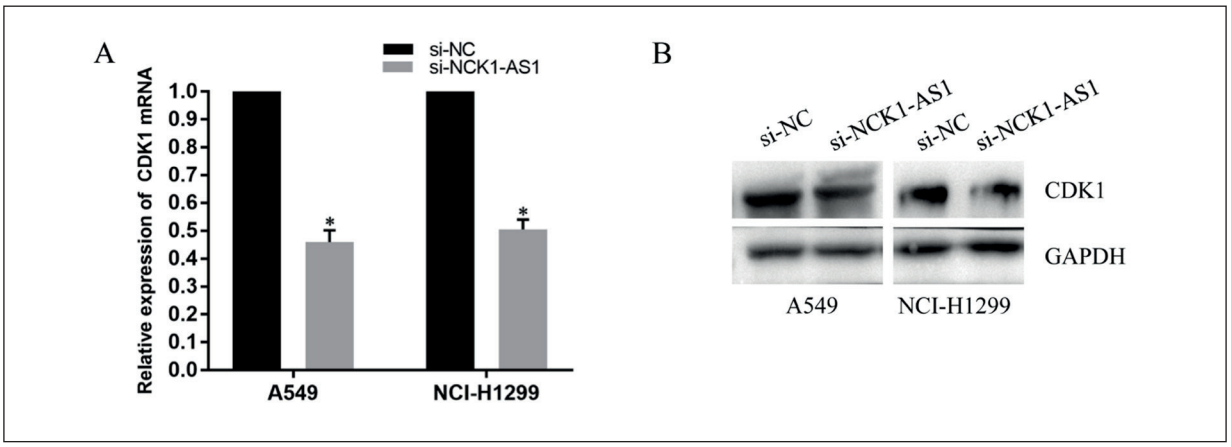

Figure 3. NCK1-AS1 regulated expression of CDK1. **A,** The results of qRT-PCR revealed that the mRNA expression of CDK1 declined after interference in the expression of NCK1-AS1 in NSCLC cells. **B,** The results of Western blotting revealed that the protein expression of CDK1 declined after interference in the expression of NCK1-AS1.

Discussion

The occurrence and development of NSCLC is an extremely complex process involving disorders of multiple genes and signaling pathways. Currently, the pathogenesis of NSCLC has not been clarified yet. According to statistics, the mortality rate and the number of new cases of NSCLC have increased year by year in China in recent years[14]. With the application of molecular targeted therapy in a variety of diseases, such as neurodegenerative diseases, tumors and metabolic diseases, searching for effective molecular therapeutic targets with high sensitivity and specificity has become a new treatment strategy for NSCLC.

Although lncRNAs have no protein-coding ability, they have certain effects on cell proliferation, apoptosis, senescence and migration through regulating the expressions of related functional genes[15]. For example, Cui et al[16] found that lncRNA SNHG1 facilitates migration and invasion of NSCLC *via* targeting the miR-101-3p/Wnt/β-catenin signaling pathway. Yao et al[17] showed that lncRNA JHDM1D-AS1 promotes proliferation and metastasis of NSCLC cells *via* regulating DHX15 protein. Therefore, as important regulatory factors, lncRNAs have become a research hotspot of NSCLC. In this study, it was found using *in vitro* experiments for the first time that the expression of NCK1-AS1 was up-regulated in NSCLC tissues and cells, and highly expressed NCK1-AS1 was positively correlated with the poor prognosis of NSCLC patients.

CDK1 is located on chromosome 10q21, and the protein encoded by it contains 297 amino acids and has a molecular weight of 34 kD, which is a member of the serine/threonine protein kinase family[18]. CDK1 plays a role as a checkpoint during the cell cycle progression from G2 to M phase, and its expression is directly related to the cell entry into or exit from the proliferation cycle. The overexpression of CDK1 can lead to cell cycle disorders and often cause malignant cell proliferation, ultimately resulting in malignant tumors[19]. It is reported in the literature that lncRNAs can act as a regulatory factor to regulate the expression of CDK1. Shang et al[20] found that lncRNA OR3A4 enhances the resistance of NSCLC cells to cisplatin through up-regulating CDK1. In this study, the results showed that the mRNA and protein expressions of CDK1 declined after interference in the expression of NCK1-AS1 in NSCLC cells.

Conclusions

Overall, this study demonstrates for the first time that highly expressed NCK1-AS1 in NSCLC is positively correlated with tumor size, TNM stage and lymph node metastasis, which can regulate the malignant cell proliferation through regulating the expression of CDK1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136: E359-E386.
2) Deng Y, Zheng J, Ma J. The clinical and prognostic significance of YWHAZ in non-small-cell lung cancer patients: Immunohistochemical analysis. J Cell Biochem 2019; 120: 6290-6298.
3) Roberts TC, Morris KV, Weinberg MS. Perspectives on the mechanism of transcriptional regulation by long non-coding RNAs. Epigenetics-Us 2014; 9: 13-20.
4) Kornienko AE, Guenzl PM, Barlow DP, Pauler FM. Gene regulation by the act of long non-coding RNA transcription. BMC Biol 2013; 11: 59.
5) Chen X, Wang S. LncRNA-ZFAS1 contributes to colon cancer progression through the miR-150-5p/VEGFA axis. Ann Oncol 2018; 29 Suppl 5: v52-v53.
6) Li H, Yao G, Zhai J, Hu D, Fan Y. LncRNA FTX promotes proliferation and invasion of gastric cancer via miR-144/ZFX Axis. Onco Targets Ther 2019; 12: 11701-11713.
7) Li S, Zhao B, Zhao H, Shang C, Zhang M, Xiong X, Pu J, Kuang B, Deng G. Silencing of long non-coding RNA SMAD5-AS1 reverses epithelial mesenchymal transition in nasopharyngeal carcinoma via microRNA-195-dependent inhibition of SMAD5. Front Oncol 2019; 9: 1246.
8) Zetterberg A, Larsson O. Coordination between cell growth and cell cycle transit in animal cells. Cold Spring Harb Symp Quant Biol 1991; 56: 137-147.
9) Xu JL, Hua T, Ding J, Fan Y, Liu ZJ, Lian JW. FOXF2 aggravates the progression of non-small cell lung cancer through targeting lncRNA H19 to downregulate PTEN. Eur Rev Med Pharmacol Sci 2019; 23: 10796-10802.
10) Li H, Jia Y, Cheng J, Liu G, Song F. LncRNA NCK1-AS1 promotes proliferation and induces cell cycle progression by crosstalk NCK1-AS1/miR-6857/CDK1 pathway. Cell Death Dis 2018; 9: 198.
11) Zhang WY, Liu YJ, He Y, Chen P. Suppression of long noncoding RNA NCK1-AS1 increases chemosensitivity to cisplatin in cervical cancer. J Cell Physiol 2019; 234: 4302-4313.
12) Hu H, Li H, Feng X. Downregulation of lncRNA NCK1-AS1 inhibits cancer cell migration and invasion in nasopharyngeal carcinoma by upregulating miR-135a. Cancer Manag Res 2019; 11: 10531-10537.
13) Liu X, Hou X, Zhou Y, Li Q, Kong F, Yan S, Lei S, Xiong L, He J. Downregulation of the Helicase Lymphoid-Specific (HELLS) Gene impairs cell proliferation and induces cell cycle arrest in colorectal cancer cells. Onco Targets Ther 2019; 12: 10153-10163.
14) Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.
15) Zhang J, Yuan Y, He Y. Expression analysis of lncRNA as prognostic biomarkers in colon adenocarcinoma. Ann Oncol 2017; 28 Suppl 3: i85.
16) Cui Y, Zhang F, Zhu C, Geng L, Tian T, Liu H. Upregulated lncRNA SNHG1 contributes to progression of non-small cell lung cancer through inhibition of miR-101-3p and activation of Wnt/beta-catenin signaling pathway. Oncotarget 2017; 8: 17785-17794.
17) Yao G, Chen K, Qin Y, Niu Y, Zhang X, Xu S, Zhang C, Feng M, Wang K. Long non-coding RNA JHDM1D-AS1 interacts with DHX15 protein to enhance non-small-cell lung cancer growth and metastasis. Mol Ther Nucleic Acids 2019; 18: 831-840.
18) Garcia-Gutierrez L, Bretones G, Molina E, Arechaga I, Symonds C, Acosta JC, Blanco R, Fernandez A, Alonso L, Sicinski P, Barbacid M, Santamaria D, Leon J. Myc stimulates cell cycle progression through the activation of Cdk1 and phosphorylation of p27. Sci Rep 2019; 9: 18693.
19) Li X, Martinez-Ledesma E, Zhang C, Gao F, Zheng S, Ding J, Wu S, Nguyen N, Clifford SC, Wen PY, Ligon KL, Yung W, Koul D. Tie2-FGFR1 Interaction induces adaptive PI3K inhibitor resistance by upregulating Aurora A/PLK1/CDK1 signaling in glioblastoma. Cancer Res 2019; 79: 5088-5101.
20) Shang J, Xu YD, Zhang YY, Li M. Long noncoding RNA OR3A4 promotes cisplatin resistance of non-small cell lung cancer by upregulating CDK1. Eur Rev Med Pharmacol Sci 2019; 23: 4220-4225.