LncRNA NEAT1 regulates cervical carcinoma proliferation and invasion by targeting AKT/PI3K

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Abstract. – OBJECTIVE: Cervical cancer is a common tumor in gynecological malignancies. Recent studies showed that long non-coding RNAs (lncRNAs) play a key role in tumorigenesis and development. LncRNA nuclear-rich transcripts 1 (NEAT1) has been found to play a role in gynecological tumors, such as endometrial cancer. However, expression of lncRNA NEAT1 and mechanism in cervical cancer has not been elucidated.

MATERIALS AND METHODS: The tumor tissue and adjacent tissue of cervical cancer patients were collected. HeLa cells were cultured in vitro and lncRNA NEAT1 expression was interfered with small interfere RNA (siRNA). Cell proliferation was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell invasion ability was assessed by transwell assay. LncRNA NEAT1, Cyclin D1, and cyclin-dependent kinase 4 (CDK4) expressions were detected by Real-time PCR. Caspase 3 expression was detected by caspase 3 activity kit. Phosphorylated protein kinase B (p-AKT), phosphatidylinositol 3-kinase (p-PI3K), and matrix metalloproteinase-2 (MMP2) levels were evaluated by Western blot.

RESULTS: Compared with the adjacent tissue, lncRNA NEAT1 expression was significantly increased in cervical cancer (p<0.05). LncRNA NEAT1 level was decreased in HeLa cells transfected by siRNA, which inhibited the proliferation and invasion of tumor cells, reduced cyclin D1 and CDK4 expressions, enhanced caspase 3 activity, and declined the expressions of p-AKT, p-PI3K, and MMP2 (p<0.05).

CONCLUSIONS: LncRNA NEAT1 siRNA transfection can inhibit the proliferation of cervical cancer by regulating the AKT/PI3K signaling pathway, promote cell apoptosis, and restrain cell invasion. Therefore, the lncRNA NEAT1 may be used as a molecular potential for the diagnosis and treatment of cervical cancer through regulating AKT/PI3K signaling pathway, which would be confirmed in the following study.

Introduction

Cervical cancer is the second most common gynecological malignant tumor with the incidence only after breast cancer, lung cancer, and colorectal cancer. As one of the women with gynecological cancers, cervical cancer is a serious threat to women’s health1,2. The incidence and mortality of cervical cancer in developing countries and economically underdeveloped areas keeps high, thus becoming one of the malignant tumors that threaten the world, especially in developing countries. Limited by not universal cervical cancer screening, poor prevention, and imbalanced economic development, the incidence and mortality of cervical cancer in developing countries are higher than the developed countries3,4. As a developing country, China has a vast territory and a large population. Therefore, the incidence and mortality of cervical cancer account for a high proportion in gynecologic cancer3,5. As most cervical cancer patients are in the advanced stage when diagnosed, the cervical cancer prognosis and quality of life are poor. It is a threat to public health and safety since it brings a heavy mental and financial burden to the patients and their family5,6. At present, molecular targeted therapy has become a new hot spot on cervical cancer, but the mechanism of cervical cancer is still unclear.

Long non-coding RNA (lncRNA) was discovered in recent years7. A large number of transcripts of non-coding RNA at different lengths were found in eukaryotes. Furthermore, it was found that non-coding transcripts account for the
vast majority of the human genome\textsuperscript{10,11}. LncRNA is the transcripts over 200 nt in length, which is not considered to be involved in protein-coding and defined as “noise” of transcription. However, it was gradually found to be participating in epigenetic regulation, transcriptional regulation, and post-transcriptional regulation of gene expression\textsuperscript{12,13}. In the genome, LncRNA is the most transcribed non-protein coding sequence and is considered to be regulatory in the protein-coding gene\textsuperscript{14}. Recent studies\textsuperscript{15,16} show that LncRNA also plays an important role in tumorigenesis and development, especially in the three major malignant gynecologic cancers, including ovarian cancer, cervical cancer, and endometrial cancer. LncRNA NEAT1 is an important tumor cell growth and proliferation regulator that can promote neovascularization, tumor invasion and metastasis, and play a role in the gynecological tumors, such as endometrial cancer\textsuperscript{17,18}. This study was to detect the expression of LncRNA NEAT1 in cervical cancer and to analyze its mechanism.

**Patients and Methods**

**Patients**

A total of 78 cases of patients with cervical cancer in The First Affiliated Hospital of Xinxiang Medical University from January 2017 to December 2017 were enrolled. All the patients with a mean age of 62.2 ± 7.9 (51-77) years old were treated with surgery. Inclusion criteria\textsuperscript{17}: All patients were diagnosed as cervical cancer and received surgery for the first time without preoperative chemotherapy or radiotherapy; all subjects had signed informed consent. Exclusion criteria: recurrent cervical cancer; previous surgical treatment; previous radiotherapy or chemotherapy; combined with other diseases, such as infectious diseases, malignant tumors, severe diabetes mellitus, and other organ failures, autoimmune diseases, and malignant tumor complications. The tumor tissues and para-cancerous tissues were collected during operation and stored in liquid nitrogen. This study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University, Henan, China.

**Main Instruments and Reagents**

RNA extraction kit and reverse transcription kit were purchased from the RD Systems (Minneapolis, MN, USA). Other commonly used reagents were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). Real-time PCR reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Real-time PCR amplifier was purchased from the ABI (Foster City, CA, USA). DNA amplification instrument was purchased from the PE Gene Applied Biosystems. (Mode: 2400, Foster, CA, USA). Dulbecco’s Modified Eagle Medium (DMEM) medium, fetal bovine serum (FBS), ethylene diamine tetraacetic acid (EDTA), and penicillin-streptomycin were purchased from Hyclone (Logan, UT, USA). Dimethylsulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) powder was purchased from Gibco BRL. Co. Ltd. (Grand Island, New York, USA). Trypsin-EDTA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polynvilylendene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Covina, CA, USA). Western blot related chemical reagents were purchased from Beyotime Biotech. (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-human nuclear factor \( \kappa B \) (NF-\( \kappa B \)), phosphorylated protein kinase \( B \) (p-AKT), phosphorylated phosphatidylinositol 3-kinase (p-PI3K), matrix metalloproteinase-2 (MMP2) protein kinase \( B \) (AKT), and phosphatidylinositol 3-kinase (p-PI3K) monoclonal antibodies, mouse anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from the Cell Signaling Technology (Beverly, MA, USA). Transwell chamber was purchased from Corning (Corning, NY, USA). Caspase 3 activity kit was purchased Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Thermo Forma 3110 5\% CO2 incubator was purchased from Thermo Fisher Scientific (Hudson, NH, USA). Lab System Version 1.3.1 microplate reader was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

**HeLa Cell Cultivation and Grouping**

HeLa cells stored in liquid nitrogen were sub-cultured after resuscitation. Cells in the 3rd-8th generation and logarithmic growth phase were used for the experiment. HeLa cells were randomly divided into three groups, including control group, siRNA-NC group, transfected by LncRNA NEAT1 negative control, and siRNA group transfected with LncRNA NEAT1 siRNA (siLncRNA NEAT1 group).

**LncRNA NEAT1 siRNA Transfection**

LncRNA NEAT1 siRNA and siRNA-NC were transfected into HeLa cells. LncRNA
NEAT1 siRNA sequence was 5'-GCAGGAAU-UCGGUGUUCUA-3'. SiRNA-NC sequence was 5'-UCAGGUGAGAUUCUA-3'. The cell density in the 6-well plate was fused to 70%-80%. The lncRNA NEAT1 siRNA and negative control liposome were added into 200 μl serum-free DMEM medium and mixed at room temperature for 15 min, respectively. The mixed lipo2000 were mixed and incubated at room temperature for 30 min. Next, they were added to the cells with 1.6 ml serum-free DMEM medium and incubated in 5% CO₂ at 37°C for 6 h. At last, the cells were used for the experiment after 48 h.

**Real-Time PCR**

Total RNA was extracted from the tissue and cells by TRIzol and reverse transcribed to DNA. The primers were designed by Primer 6.0 and synthesized by Invitrogen (Table I). The reaction conditions included 55°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58-60°C for 45 s, and 72°C for 35 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as loading control. According to the fluorescence quantification, the initial cycle number (CT) was calculated and 2-ΔCt method was used for semi-quantitative analysis.

**MTT Assay**

HeLa cells in logarithmic growth phase were collected in DMEM culture medium containing 10% FBS and seeded in 96-well plate at 5 × 10³ cells. The supernatant was discarded after 24 h incubation and 20 μl of sterile MTT was added to the wells for 4 hours. Next, dimethyl sulfoxide (DMSO) was added to each well at 150 μl/well for 10 min until the purple crystal was completely dissolved. Then, the plate was tested at 570 nm to obtain the absorbance (A) value and calculate the proliferation rate.

**Transwell Assay**

According to the manual, the transwell chamber was coated by l:5 50 mg/l Matrigel for 24 h and air-dry at 4°C. Next, 500 μl of 10% FBS DMEM medium and 100 μl of tumor cell suspension prepared in serum-free medium were added to the lower and upper chamber, respectively. After 48 h cultivation, the membrane was washed by using PBS and fixed by ice-ethanol. After stained with crystal violet, the cells in the lower layer of the microporous membrane were counted. Each experiment was repeated for three times.

**Caspase 3 Activity Detection**

According to kit instructions, the cells were digested by trypsin and centrifuged at 600 ×g and 4°C for 5 min. The cells were added with lysate (150 mM NaCl, 1% Nonidet P 40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 2 μg/ml Aprotinin, 2 μg/ml Leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1.5 mM EDTA, 1 mM NaN₃) and lysed on ice for 15 min. Then, the sample was centrifuged at 20000 ×g and 4°C for 5 min, and added with 2 mM Ac-DEVD-pNA to test OD value.

**Western Blot**

Total protein was extracted by lysis buffer and stored at -20°C after quantification. The separated proteins were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was transferred to PVDF membrane by semi-dry transfer method at 100 mV for 1.5 h. The primary antibodies were incubated at dilution of 1:2000 for p-AKT, 1:2000 for p-PI3K, 1:1000 for MMP2, 1:1500 for AKT, and 1:2000 for PI3K. Then, the membrane was incubated in goat anti-rabbit secondary antibody at 1:2000 dilution for 30 min. After washed by PBS Tween 20 (PBST), the membrane was treated by chemiluminescence and analyzed by protein image analysis system software and Quantity one software. The experiment was repeated four times (n=4).

**Statistical Analysis**

All data analyses were performed on SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were presented as mean

<table>
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<th>Gene</th>
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Results

LncRNA NEAT1 Expression in Cervical Cancer

Real-time PCR was used to test lncRNA NEAT1 expression in cervical cancer. It was showed that lncRNA NEAT1 expression in cervical cancer was significantly higher than that in adjacent tissue ($p<0.05$) (Figure 1).

Effects of lncRNA NEAT1 siRNA on HeLa cells

After lncRNA NEAT1 siRNA transfection, lncRNA NEAT1 expression markedly reduced in HeLa cells compared with control and siRNA-NC group ($p<0.05$) (Figure 2).

Effects of lncRNA NEAT1 siRNA on HeLa Cell Proliferation

After lncRNA NEAT1 siRNA transfection, HeLa cell proliferation was tested by MTT assay. It was found that HeLa cell proliferation was markedly suppressed after lncRNA NEAT1 siRNA transfection for 48 h ($p<0.05$) (Figure 3).

Effects of lncRNA NEAT1 siRNA on Caspase 3 Activity in HeLa Cells

After lncRNA NEAT1 siRNA transfection, caspase 3 activity in HeLa cells was assessed by caspase 3 detection kit. It was observed that caspase 3 activity was apparently enhanced after lncRNA NEAT1 siRNA transfection for 48 h ($p<0.05$) (Figure 4).

Effects of lncRNA NEAT1 siRNA on HeLa cell invasion

After lncRNA NEAT1 siRNA transfection, HeLa cell invasion was evaluated by transwell assay. It was demonstrated that cell invasion was significantly restrained after lncRNA NEAT1 siRNA transfection for 48 h ($p<0.05$) (Figure 5).

Effects of lncRNA NEAT1 siRNA on Cyclin D1 and CDK4 Expressions in HeLa Cells

After lncRNA NEAT1 siRNA transfection, cyclin D1 and CDK4 expressions in HeLa cells were detected by Real-time PCR. It was revealed that cyclin D1 and CDK4 expressions were significantly inhibited after lncRNA NEAT1 siRNA transfection for 48 h ($p<0.05$) (Figure 6).

Effects of lncRNA NEAT1 siRNA on AKT/PI3K Signaling Pathway

After lncRNA NEAT1 siRNA transfection, AKT/PI3K signaling pathway activity in HeLa cells was determined by Western blot. It was showed that AKT/PI3K signaling pathway phosphorylation was markedly suppressed after lncRNA NEAT1 siRNA transfection for 48 h ($p<0.05$) (Figure 7).

Figure 1. LncRNA NEAT1 expression in cervical cancer. *$p<0.05$, compared with adjacent tissue.

Figure 2. LncRNA NEAT1 expression in HeLa cells. *$p<0.05$, compared with control.
NA NEAT1 siRNA transfection for 48 h ($p<0.05$) (Figure 7).

**Effects of IncRNA NEAT1 siRNA on MMP2 Expression**

After IncRNA NEAT1 siRNA transfection, MMP2 expression in HeLa cells was detected by Western blot. It was found that MMP2 expression was apparently inhibited after IncRNA NEAT1 siRNA transfection for 48 h ($p<0.05$) (Figure 8).

**Discussion**

Human papillomavirus (HPV) infection, unhealthy sexual life and eating habits, smoking, work environment, and pressure lead to an increasing incidence of cervical cancer. In recent years, it was found the younger trend of cervical cancer, which was the majority at 30-55 years old\(^{19}\). Cervical cancer incidence and mortality in China account for about 25% of the world. Currently, there is still lack of deficiency for the prevention and treatment of cervical cancer\(^{20}\). It is urgently needed to find a new molecular target for diagnosis and treatment of cervical cancer.

IncRNA can regulate gene expression and participate in the physiological activities in many ways, including chromatin modification, genomic imprinting, nuclear transport, chromosomal gene silencing, and transcriptional activation\(^{21}\). IncRNA can regulate the normal and pathological conditions of cells, including growth, proliferation, cell cycle, and apoptosis. Thus, IncRNA is an important regulatory factor in the development and progression of human diseases\(^{22}\). Recently, IncRNA expression was detected abnormally in neoplasia formation and progression. Moreover, it was found that some IncRNAs play roles in tumors\(^{23}\). IncRNA NEAT1 was confirmed to be expressed in many normal tissues but significantly increased in many kinds of tumors, including lung cancer, liver cancer, colorectal cancer, meningioma, and even leukemia. This suggests that the IncRNA NEAT1 may be involved in tumor development and can be used as a predictor of tumor prognosis\(^{17,18,24}\). However, the role and mechanism of IncRNA NEAT1 in cervical cancer have not been elucidated. Therefore, we first detected the expression of IncRNA NEAT1 in cervical cancer tissues and confirmed that the expression of IncRNA NEAT1 was significantly increased in cervical cancer. Therefore, we transfected IncRNA NEAT1 siRNA into HeLa cells to reduce the IncRNA NEAT1 expression. The results showed that it inhibited tumor cell proliferation, promoted tumor cell apoptosis, reduced invasive ability, restrained cell cycle-related genes cyclin D1 and CDK4 expressions, which suggests that IncRNA NEAT1 regulates the proliferation and invasion of NSCLC. MMP-2 is a member of the matrix metalloproteinase family that may promote tumor cell invasion and metastasis\(^{25}\). AKT/PI3K signaling pathway is closely related to tumorigenesis, which leads to tumor cell proliferation, cell cycle, and progression. It
was confirmed that cervical cancer is related to AKT/PI3K signaling pathway. In this study, we observed that lncRNA NEAT1 siRNA inhibited the expression of MMP-2 and suppressed the activation of AKT/PI3K pathway. We observed the expression of lncRNA NEAT1 in cervical cancer and related mechanisms. The clinical significance of lncRNA NEAT1 in cervical cancer as a molecular marker would be investigated in the future.

**Figure 5.** The impact of lncRNA NEAT1 siRNA on HeLa cell invasion. **A,** Transwell chamber assay analysis of the impact of lncRNA NEAT1 siRNA on HeLa cell invasion. **B,** HeLa cell invasive ability statistical analysis. *p*<0.05, compared with control.

**Figure 6.** The influence of lncRNA NEAT1 siRNA on cyclin D1 and CDK4 expressions in HeLa cells. *p*<0.05, compared with control.

**Figure 7.** The effect of lncRNA NEAT1 siRNA on AKT/PI3K signaling pathway.
Conclusions

We showed that LncRNA NEAT1 siRNA transfection suppressed cervical cancer proliferation, promoted cell apoptosis, and restrained cell invasion by regulating the AKT/PI3K signaling pathway. Therefore, the LncRNA NEAT1 may be used as a potential molecular for the diagnosis and treatment of cervical cancer through regulating AKT/PI3K signaling pathway, which would be confirmed in the following study.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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Y. Prognostic values of the integrated model incorporating the volume of metastatic regional cervical lymph node and pretreatment serum Epstein-Barr virus DNA copy number in predicting distant metastasis in patients with N1 nasopharyngeal carcinoma. Chin J Cancer 2017; 36: 98.


