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High expression of LINC0163 promotes progression of papillary thyroid cancer by regulating epithelial-mesenchymal transition MITF

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Abstract. – OBJECTIVE: The purpose of this study was to detect the expression of long non-coding ribonucleic acid 00163 (LINC00163) in human papillary thyroid cancer (PTC), and to observe the influence of downregulated LINC00163 on the proliferative and metastatic capacities of human PTC cells.

PATIENTS AND METHODS: Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) assay was applied to measure the expression level of LINC00163 in PTC tissues and para-carcinoma tissues, as well as that in normal human thyroid cells (Nthy-ori3-1) and PTC cells. After the expression of LINC00163 in PTC cells was interfered, qRT-PCR assay was performed to determine the interference efficiency, and colony formation and Cell Counting Kit-8 (CCK-8) assays were conducted to study the impacts of small interfering (si)-LINC00163 on the proliferative capacity of PTC cells. Moreover, wound healing and transwell assays were adopted to investigate the changes in the migratory and invasive abilities of PTC cells after the interference in the expression of LINC00163 in PTC cells. Finally, the changes in expressions of molecular markers in downstream signaling pathways after interference in LINC00163 expression were examined via Western blotting assay.

RESULTS: In 51 cases of PTC tissues and corresponding para-carcinoma tissues, 41 cases exhibited an up-regulated expression of LINC00163, and qRT-PCR results indicated that PTC cells also had an up-regulated expression of LINC00163 compared with normal human thyroid cells. After the expression of LINC00163 in PTC cells was interfered, the results of colony formation and CCK-8 assays manifested that the proliferative capacity of the cells declined. It was also shown in wound-healing and transwell assay results that the migratory and invasive abilities of the cells were weakened. In addition, the results of Western blotting assay revealed expression changes in the molecular markers of epithelial-mesenchymal transition (EMT).

CONCLUSIONS: The expression of LINC00163 in NSCLC tissues and cells is upregulated, and highly expressed LINC00163 can promote PTC cell proliferation and metastasis by regulating the EMT.

Key Words:
PTC, LINC00163, Proliferation, Metastasis, EMT.

Introduction

Human papillary thyroid cancer (PTC), the most common thyroid malignancy in clinic, accounts for about 85-90% of TC[1,2]. Clinically, PTC is mainly treated by surgeries[3], and it is difficult to be diagnosed in the early stage. Approximately 50-80% PTC patients usually suffer from metastases when definitely diagnosed[4]. Therefore, it is particularly important to investigate the regulation on the migratory and invasive abilities of PTC cells.

Long non-coding ribonucleic acids (lncRNAs) are a category of regulatory large RNAs discovered in recent years. Although they cannot transcribe proteins, they play crucial roles in numerous physiological and life activities, such as participating in cell development, gene imprinting, and X chromosome silencing and controlling transcriptional and post-transcriptional levels[5,6]. According to literature, the abnormal expression of lncRNAs is closely associated with the occurrence and development of multiple tumors. For example, Zhang et al[7] found that lncRNA PICART1 inhibits the proliferation and invasion of non-small cell lung cancer cells by regulat-

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ing the AKT1 signaling pathway. In the case of colorectal cancer, the expression of lncRNA AFAP1-antisense RNA 1 (AS1) is up-regulated and accelerates the proliferation of colorectal cancer, and the up-regulation of lncRNA AFAP1-AS1 expression indicates poor prognosis of the patients[8].

LncRNAs, including lncRNA FOXD2-AS1 and UCA1, have extremely close correlations with the progression of human PTC[9,10]. However, the expression and biological function of LINC00163 have not been reported yet. According to studies, LINC00163 is located in chromosome 21q22.3, with a full length of 2155 bp. In this research, it was discovered for the first time through *in vitro* experiments that LINC00163 expression was upregulated in PTC tissues and cells, and it promoted the proliferation, invasion, and migration of PTC cells.

Patients and Methods

Sources of Tissues and Cells

The specimens of PTC tissues and para-carcinoma tissues were obtained from 51 PTC patients receiving radical thyroidectomy for the first time in the Department of General Surgery of Cancer Hospital of China Medical University from 2015-2017. None of the patients had other anti-tumor therapies previously. PTC was confirmed by postoperative pathological examinations, and para-carcinoma tissues were located over 2 cm away from the tumor margin. This research was approved by the patients themselves and the Ethics Committee of the hospital, and PTC cells (K1, BCPAP) were provided by Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China).

Reagents and Instruments

RNAiso reagent and reverse transcription (RT) and fluorescence quantitative polymerase chain reaction (qPCR) kits (TaKaRa, Otsu, Shiga, Japan); Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, Dulbecco's Modified Eagle's Medium (DMEM) culture solution fetal bovine serum (FBS), and trypsin (Gibco, Rockville, MD, USA); Cell Counting Kit-8 (CCK-8) for cell proliferation assay (Dojindo Laboratories, Kumamoto, Japan), Matrigel and transwell culture plate (Corning, Corning, NY, USA), crystal violet powder (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-human (E-cadherin, etc.) polyclonal antibodies (Proteintech, Rosemont, IL, USA); LightCycler 480 II System (Roche, Basel, Switzerland), CO_2 cell incubator (Thermo Fisher Scientific, Waltham, MA, USA), and inverted phase-contrast microscope (Olympus Corporation, Tokyo, Japan).

Detection of LINC00163 Expression in Tissues and Cells

The total RNA in PTC tissues, normal para-carcinoma tissues and cells were extracted according to the kit instructions. Then, RT reaction was performed as per the instructions of RT kit with 1 μg of total RNA as the template, and complementary deoxyribose nucleic acid (cDNA) obtained was used for fluorescence qPCR under the following conditions: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s, 40 cycles in total. The primers were designed and synthesized by Sangon Biotechnology (Shanghai, China) Co., Ltd., with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The relative expression of LINC00163 was expressed by $2^{-\Delta\Delta Ct}$. Primers: Linc00163: F 5'-GCTATTGTCATGGAGACGGGA-3', R 5'-CCTCGCTTAGACATTGGCCG-3', and GAPDH: F 5'-CAGCCACCCGAGA TTGAGCA-3', R 5'-TAGTAGCGACGGGCGGTGTG-3'

Transfection

PTC cells in the logarithmic growth phase were digested, inoculated into a 6-well plate at $5'10^4$ cells/well and cultured till a cell fusion of 70-80%. After that, PTC cells were transfected in accordance with the instructions of Lipofectamine 3000, and the solution was replaced 6 h later. Interference sequences: SiRNA1: F 5'-CCCACAACAUGAAAGAAACTT-3', R 5'-AUUUCUUUCAUGU UGUGGGC-3', siRNA2: F 5'-GCUAGAGGAACCAGACCUUTT-3', R 5'-AAGGUCUGGUUCCUCUAGCTT-3', and siRNA3: F 5'-UUCUCCGAACGUGUCACGUCT-3', R 5'-ACGUGACA CGUUCGGAGAATT-3'.

CCK-8 Assay

Three groups of cells were collected at 24 h after transfection and then seeded into a 96-well plate (3000 cells/well) in a volume of 200 μL. Next, 10 μL of CCK-8 reagent was added at 0, 24, 48, 72, and 96 h after cell attachment, followed by incubation at 37°C for 2 h. Finally, the optical density (OD) value at the wavelength of 450 nm was detected, and cell growth curves were plotted.

Colony Formation Assay

The cells in experimental group and control group were inoculated into the 6-well plate at 800 cells/well and cultured in an incubator with 5% CO_2 at 37°C for 2 weeks. The culture was terminated when macroscopic clones were formed. After that, the medium was discarded, and the cells were washed with phosphate-buffered saline (PBS) for 3 times, fixed in methanol for 15 min, and stained for 20 min and air-dried after rinsing. Ultimately, photographing and counting were carried out for macroscopic colonies. The assay was repeated for 3 times.

Wound-Healing Assay

The transfected PTC cells were seeded into the 6-well plate and cultured in the incubator with complete medium for 12 h. Later, a wound was scratched using a pipette tip, the medium in the 6-well plate was aspirated, and another complete medium was added for 36 h of culture. Finally, the cell migration was observed and photographed under the inverted microscope.

Migration Assay

The cells in three groups were harvested at 48 h after transfection, resuspended in RPMI-1640 medium without fetal bovine serum, and added into the upper transwell chamber at $5'10^4$ cells/well. Then, the lower chamber was added with the RPMI-1640 medium with 10% fetal bovine serum. Subsequently, the upper chamber was taken out at 24 h after incubation, and the cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 14 min. Thereafter, the cells in the upper chamber were wiped out using cotton swabs, and the upper chamber was washed in water for 3 times. Finally, the migrating cells were counted in randomly selected fields of vision under the microscope, and the photographs were preserved.

Invasion Assay

The experimental methods were basically the same as those in step 7, except that the Matrigel-coated upper chamber was applied. In detail, the stock solution of Matrigel was diluted 8 times using the fetal bovine serum-free RPMI-1640 medium, 50 μL of which was added into the upper chamber and solidified at 37°C for 30 min for later use.

Western Blotting Assay

The cells were collected at 48 h after transfection, from which the total protein was extracted *via* radioimmunoprecipitation assay (RIPA) method (Beyotime, Shanghai, China), and the protein concentration was determined through a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). After denaturation, the protein (20 μg/well) was loaded for 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) *via* the wet process and sealed in 5% skim milk. Then, E-cadherin antibodies were added for incubation in a refrigerator at 4°C overnight. The next day, the membrane was washed with Tris-Buffered Saline and Tween-20 (TBST) for 3 times and then incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies (1:5000) at room temperature for 1 h. After washing with TBST for 3 times, the images were developed by enhanced chemiluminescence (ECL) method and stored.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for data analysis. The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparisons among multiple groups were done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

LINC00163 Expression Was Upregulated in PTC

The expression level of LINC00163 in the tissue specimens from 51 PTC patients was detected by means of qRT-PCR assay. Compared with that in para-carcinoma tissues, the expression of LINC00163 was upregulated in 40 cases of tissues (Figure 1A). Next, qRT-PCR assay was conducted again to examine the expression level of LINC00163 in PTC cells, and it was shown that LINC00163 expression was up-regulated in PTC cells (Figure 1B). To investigate the biological function of LINC00163 in PTC cells, the interference sequences of LINC00163 were designed and transfected into PTC cells, and the interference efficiency was measured 48 h later (Figure 1C, 1D).

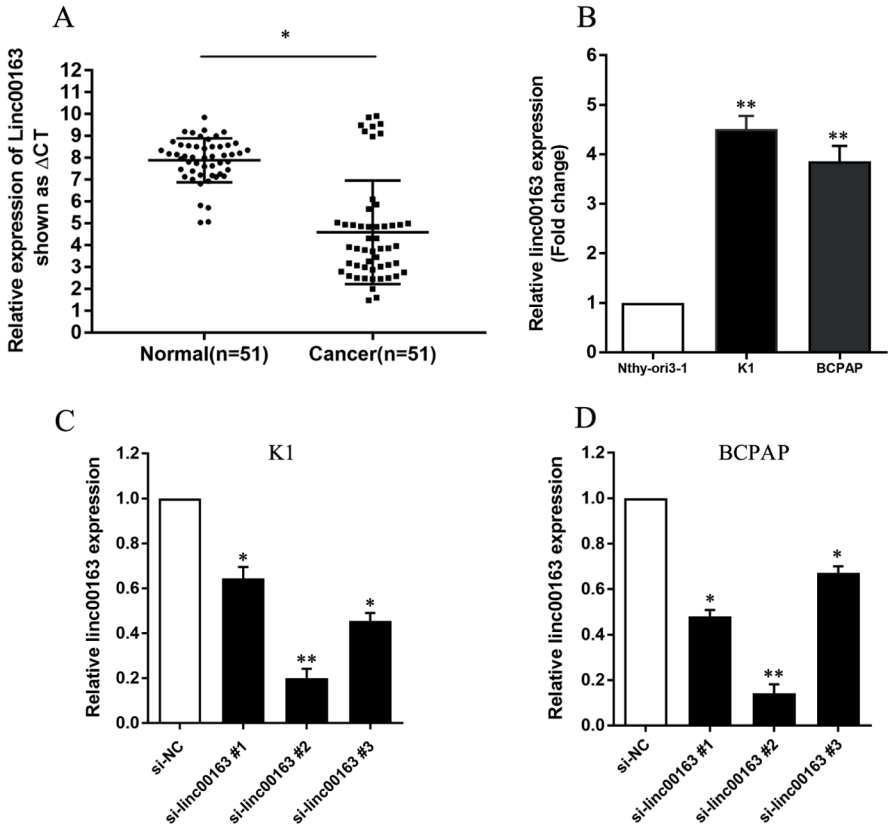

Figure 1. LINC00163 expression is upregulated in PTC. **A,** Expression level of LINC00163 in 51 cases of PTC tissues detected via qRT-PCR assay. It is shown that the expression of LINC00163 is upregulated in 40 cases of tissues. **B,** Relative expression of LINC00163 in PTC cells detected via qRT-PCR assay. **C-D,** Interference efficiency detected *via* qRT-PCR assay after PTC cells are transfected with si-LINC00163 and si-NC.

Small Interfering (si)-LINC00163 Inhibited PTC Cell Proliferation

After the transient transfection of si-LINC00163 into PTC cells, the changes in the proliferative capacity of PTC cells were detected *via* CCK-8 assay. The results manifested that the cell proliferative capacity was repressed in si-negative control (NC) group in comparison with that in experimental group (Figure 2A, 2B). Subsequently, the impact of LINC00163 on PTC cell proliferation was determined using colony formation assay. It was shown that the proliferative capacity of PTC cells was weakened after the expression of LINC00163 was knocked down (Figure 2C, 2D).

LINC00163 Regulated Epithelial-Mesenchymal Transition (EMT)

The wound-healing assay was employed to investigate the influence of LINC00163 on the migratory ability of PTC cells, and it was revealed that interfering in the expression of LINC00163 could reduce the migratory ability of PTC (Figure 3A, 3B). Thereafter, transwell assay was applied to explore the effects of LINC00163 on the migration and invasion of PTC cells. Compared with si-NC group, si-LINC00163 group exhibited suppressed migratory and invasive abilities of cells (Figure 3C, 3D). To study the molecular mechanism of LINC00163 in affecting the migratory and invasive ability of cells, the results of Western blotting assay indicated that the expressions of EMT-related molecular markers (E-cadherin, N-cadherin, and Vimentin) were altered (Figure 3E, 3F).

Discussion

The morbidity rate of TC is rising year by year, and the patients with the disease tend to be younger[11]. PTC is the most common type of TC, and lymph node is the most important organ of TC

recurrence and metastasis, with the metastasis rate of 5-20%[12,13]. According to research reports, 30-70% of PTC patients will have cervical lymph node metastasis and relatively poor prognosis[14,15]. Hence, whether LINC00163 is associated with PTC proliferation and metastasis was investigated in this study.

Although lncRNAs cannot encode protein, they can be located in nucleus or cytoplasm due to the specific spatial structure, so they possess complicated biological functions. Moreover, they can participate in chromatin remodeling, translational and post-translational regulation, RNA splicing, and other physiological processes[16,17]. It has been reported in literature that lncRNAs are involved in the occurrence and development of such tumors as PTC as vital regulatory factors. It was first discovered by this research group that LINC00163 exhibited upregulated expression in PTC tissues and facilitated the proliferation and metastasis of PTC cells.

EMT refers to the transition of specific cells from the epithelium to the interstitium during the growth and development of organisms. It is able to enhance the migratory and invasive abilities, as well as the colonization and growth of tumor cells, so it is regarded as one of the crucial mechanisms for tumorigenesis and progression. The process of EMT includes the decreased expression or redistribution of tight junction protein such as E-cadherin and the increased expression of proteins, including and N-cadherin and Vimentin

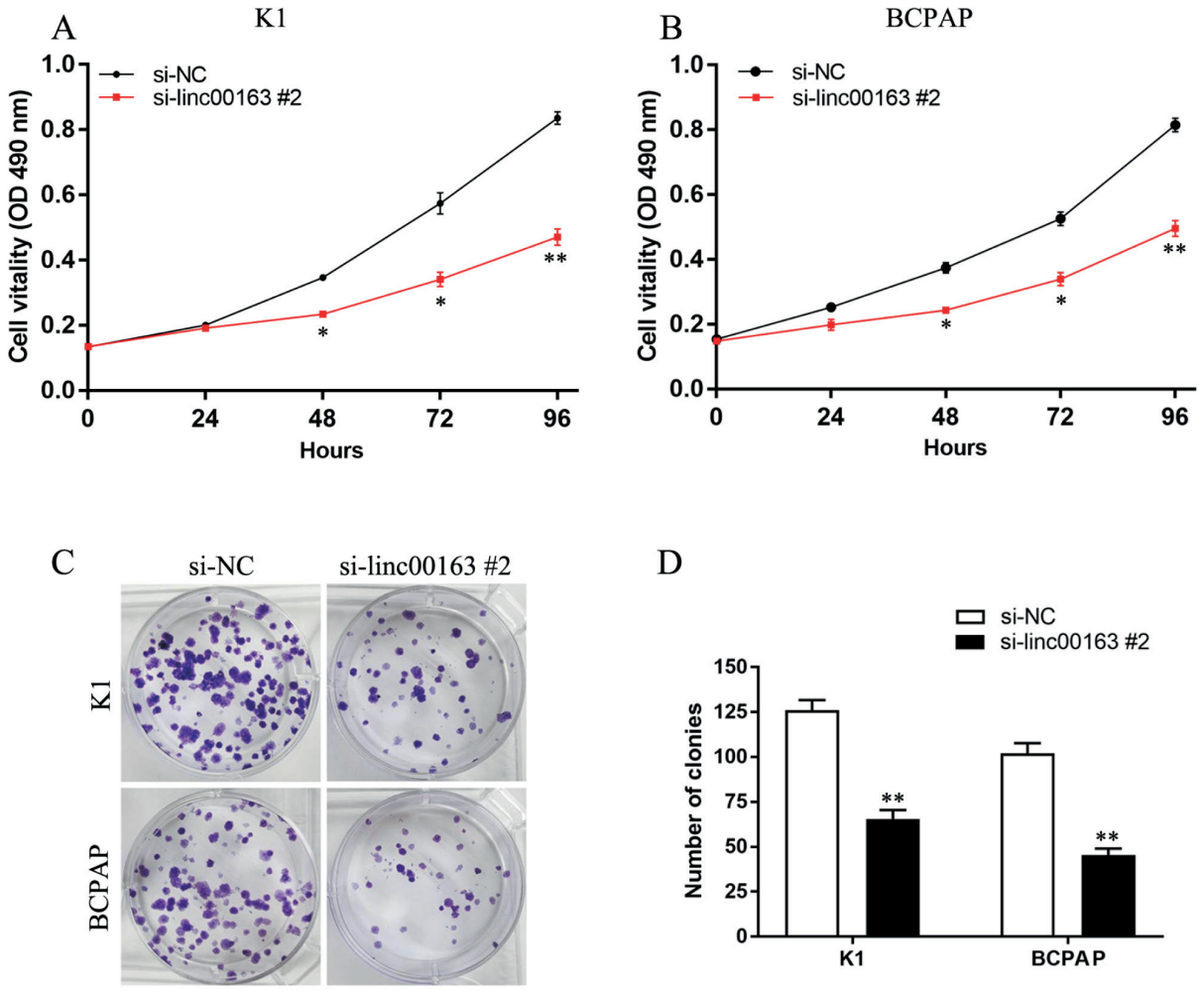

Figure 2. Si-LINC00163 inhibits PTC cell proliferation. **A-B,** CCK-8 assay results show that the proliferative capacity of PTC cells declines after PTC cells are transfected with si-LINC00163 and si-NC. **C-D,** Results of colony formation assay indicate that the proliferative capacity of PTC cells is repressed after interference in the expression of LINC00163 (magnification: 10×).

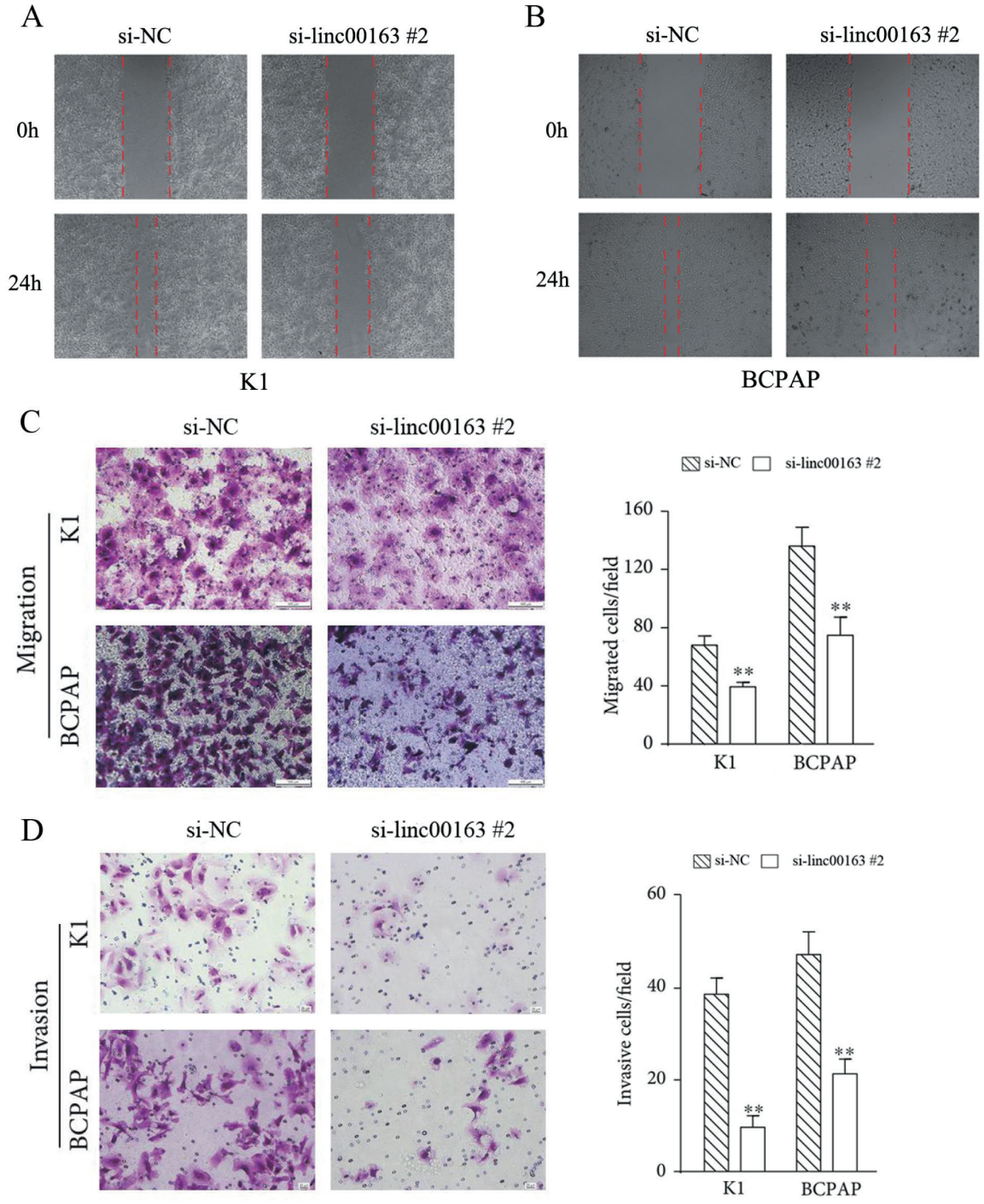

Figure 3. LINC00163 regulates EMT. **A-B,** Wound-healing assay results reveal that interfering in the expression of LINC00163 can attenuate the migratory ability of PTC cells (magnification: 40×). **C-D,** According to the results of transwell assay, compared with si-NC group, si-LINC00163 group exhibits decreased migratory and invasive ability of cells (magnification: 40×). **E-F,** Changes in expressions of molecular markers of EMT after interference in LINC00163 expression detected *via* Western blotting assay.

Figure continued

in interstitial cells, thus enabling the cells to acquire stronger invasive and metastatic abilities[18,19]. Many lncRNAs can stimulate the proliferation and metastasis of tumors by controlling EMT, including lncRNA MEG3 and H19[20,21]. In this research, it was discovered through *in vitro* ex-

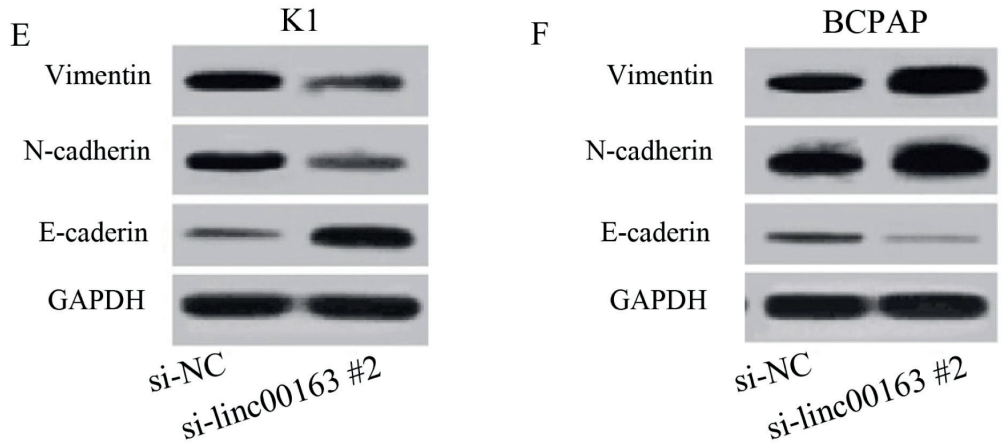

Figure 3 (continued). **E-F,** Changes in expressions of molecular markers of EMT after interference in LINC00163 expression detected *via* Western blotting assay.

periments that after the expression of LINC00163 was interfered, the molecular markers of EMT such as E-cadherin, Vimentin, and N-cadherin had changed expressions.

Conclusions

The experimental results of this research demonstrate that LINC00163 expression is upregulated in PTC tissues and cells, and highly expressed LINC00163 accelerates the proliferation and metastasis of PTC cells by regulating EMT, thereby providing molecular targets for the inhibition of PTC proliferation and metastasis in clinic.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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