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# LINC00261 regulates EBF1 to suppress malignant progression of thyroid cancer

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**Abstract. – OBJECTIVE: We aimed to explore the role of LINC00261 in thyroid cancer (TC) and the potential regulatory mechanism.**

**PATIENTS AND METHODS: 40 cases of tumor tissues and adjacent tissues of TC patients were collected, and the expressions of LINC00261 and EBF1 were detected by quantitative real-time polymerase chain reaction (qRT-PCR), and the relationship between LINC00261 and the clinical pathological indicators and prognosis of TC patients were analyzed. Next, LINC00261 overexpression and knockdown cell models were constructed in TC cell lines BPH5-16 and K1, respectively. Cell counting kit-8 (CCK-8) and transwell migration were used to detect the impact of LINC00261 overexpression or silencing on cell proliferative and migration ability. The bioinformatics website was used to screen the possible target gene of LINC00261.**

**RESULTS: qRT-PCR analysis showed that LINC00261 level was markedly reduced in TC tumor tissues, as well as corresponding cell lines. Retrospective analysis showed that low expression of LINC00261 was in positive correlation with the pathological stage, lymphatic and distant metastasis in patients with TC, meanwhile, the expression of LINC00261 was also in positive correlation with overall survival rate of TC patients. Bioinformatics analysis suggested that LINC00261 could target EBF1. Luciferase reporter gene experiment and qRT-PCR analysis suggested that LINC00261 could target EBF1 and that their expressions showed a negative correlation in TC tumor tissues and cells. Cell functional experiments confirmed that LINC00261 can inhibit the proliferative and migration ability of TC cells. Subsequently, the recovery experiment also suggested that silencing EBF1 could reverse the promotion effect of LINC00261 knockdown on the proliferative and migration ability of TC cells; while EBF1 overexpression could reverse the inhibition of LINC00261 on the proliferative and migration ability of TC cells.**

**CONCLUSIONS: LINC00261 was markedly downregulated in TC tissues and cells. In addition, the level of LINC00261 was closely related to lymph node and distant metastasis, as well as the prognosis in TC patients. Moreover, LINC00261 could negatively regulate EBF1, thereby promoting the malignant progression of TC.**

*Key Words:*
LINC00261, EBF1, Thyroid cancer, Malignant progression.

## Introduction

Thyroid cancer (TC) is the most common malignant tumor of the endocrine system. In recent years, the prevalence of TC has steadily increased in most countries[1,2]. With in-depth research on the molecular pathogenesis of papillary TC, biological targeted therapy has attracted increasing attention. Cancer precision medicine is a new medical concept that emerged at the historic moment. Through genome sequencing and big data analysis, a new model for precise diagnosis, prevention, and treatment of cancer is then launched in order to achieve the purpose of improving the level of early diagnosis and treatment of cancer[3-5]. Through the understanding of precision medicine, the molecular mechanism of papillary TC is being explored at the genetic level. The changes of key genes in the occurrence and development of papillary TC are being analyzed, hoping to find more effective treatment targets and carry out individualized differential treatment to achieve the purpose of early detection, early diagnosis and early treatment of papillary thyroid carcinoma. These explorations might make the prevention and complete cure of papillary TC possible[6-8].

With the advancement of genomic microarray and genome sequencing technology, discovered that more and more non-coding RNAs (ncRNAs) are found to be involved in cell development, growth and pathology[9,10]. Among them, lncRNAs



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are involved in the occurrence, development, metastasis and recurrence of tumors, and even the development of drug resistance of tumors. Therefore, looking for abnormal lncRNAs in TC and conducting in-depth research on these lncRNAs[11-13] might provide important scientific basis for TC genetic diagnosis and precise targeted therapy[14,15]. Preliminary studies have shown that LINC00261 could promote the progression of various tumors including NSCLC and prostate cancer, but its role in TC remains unknown[16,17]. Bioinformatics predictions suggested that LINC00261 can target EBF1; in recent years, EBF1 has been studied in various tumors, but its role in TC has not been reported. Therefore, this study aimed to explore whether LINC00261 could promote the malignant progression of TC through regulating EBF1, hoping to provide experimental basis for the diagnosis and clinical treatment of TC.

## Patients and Methods

### Patients and Thyroid Cancer Samples

The cancerous tissues and of 40 cases of patients with TC and normal adjacent tissues were collected in our hospital. The pathological diagnosis of all tissues was confirmed by at least two senior pathologists and each patient had not received radiotherapy or chemotherapy before surgery. The TC pathological classification and staging standards are implemented in accordance with the International Union Against Cancer (UICC) TC staging standards. The collection of specimens had been approved by the Ethics Committee of our hospital before collection to ensure that all surgical specimens had been obtained with the patient's consent in advance and signed an informed consent form, and the study complied with the clinical practice guidelines of the Declaration of Helsinki. This study was approved by the Ethics Committee of The First Affiliated Hospital of Jiamusi University.

### Cell Lines and Reagents

TC cell lines (BHP5-16, K1, TPC and BHP5-16) and a normal human thyroid epithelial cell line (Nthy-ori 3-1) were from the tumor cell bank of the Chinese Academy of Sciences (Shanghai, China). TC cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high-glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin and streptomycin in a 37°C, 5% $CO_2$ incubator.

### Transfection

Blank control vectors (NC or Anti-NC) and overexpression or knockdown vectors containing LINC00261 lentiviral sequences (LINC00261 or Anti-LINC00261) were purchased from Shanghai Jima. In addition, blank control vectors (pcDNA-NC or si-NC) and overexpression or knockdown vectors containing EBF1 sequences (pcDNA-EBF1 or si-EBF1) were also purchased from Shanghai Jima (Shanghai, China). Transfection was performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours, the cells were collected for further experiments.

### Cell Proliferation Assays

The main steps for the detection of the proliferation of cells were as follows: (1) 100 μL of medium (containing 2000 cells) was added to each well; (2) 10 μL cell counting kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well; (3) cells were further incubated for 1 h in the cell incubator; (4) The optical density (OD) value at 450 nm was measured by a microplate reader.

### Transwell Cell Migration Assay

The cells were removed by centrifugation after routine digestion and were resuspended in complete medium and adjusted to a density of $5 \times 10^5$ cells/mL. 700 μL of medium containing 20% FBS was added to the lower chamber while 200 μL of cell suspension (1 × 105 cells) was added to the upper chamber. After 48 h, the transwell chamber was wiped with a cotton swab to remove non-migrating cells. Transwell was then placed in methanol for 15 minutes for cell fixation and then placed in 0.2% crystal violet staining for 30 minutes, rinsed with phosphate-buffered saline (PBS) for 2-3 minutes, and observed under the microscope at a magnification of 40 times. 5 visual fields were randomly selected for counting.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total tissue RNA in one step. RNA reverse transcription was operated using Prime Scirpt Reverse Transcription Kit (TaKaRa, Tokyo, Japan), then real-time PCR was operated using SYBR® Premix Ex Taq™ (TaKaRa, Tokyo, Japan) kit on the StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers used in the qRT-PCR reaction (5'-3') were listed below: LINC00261: F: GTCAGAAGGAAAGGCCGTGA, R: TGAGCCGAGATGAACAGGTG;

EBF1: F: GCCGCCAAAGTGCTTCCTTTTAG, R: TCGCACTGGATACGACACCCTC; GAPDH: F: AGAAGGCTGGGGCTCATTTG, R: AGGGGCCATCCACAGTCTTC.

Each sample was repeated in three replicates. Bio-Rad PCR instrument software iQ5 2.0 (Bio-Rad, Hercules, CA, USA) was used to analyze and process the data. The relative expression of the genes were calculated using $2^{-\Delta\Delta Ct}$ method.

### Western Blot

Cells were lysed and the total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted protein was separated with a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Primary antibodies including EBF1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the secondary anti-mouse and anti-rabbit antibodies were all purchased from Cell Signaling Technology (Danvers, MA, USA).

### Dual-Luciferase Reporter Assay

pcDNA-EBF1 and pmirGLO-LINC00261-WT or pmirGLO-LINC00261-MUT plasmids were co-transfected into K1 and BHP5-16 cells. The activity of Luciferase was determined by detecting the intensity of fluorescence using the Promega system (Madison, WI, USA).

### Statistical Analysis

GraphPad Prism 5 V5.01 software (La Jolla, CA, 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). The K-M method and log-rank test were enrolled for survival analysis. Each experiment was repeated at least three times, and the data were expressed as mean ± standard deviation (± s). $p<0.05$ was considered statistically significant.

## Results

### LINC00261 Was Downregulated in Thyroid Cancer Tissues as Well as Cells

qRT-PCR analysis indicated that the level of LINC00261 was lower in TC tumor tissue than that in adjacent ones (Figure 1A). In addition, LINC00261 level was also markedly lower in TC cells (Figure 1B), suggesting that LINC00261 may act as a tumor suppressor gene in TC. Subsequently, according to the expression level of LINC00261 in 40 TC tissues, patients were divided into high LINC00261 expression group and low LINC00261 expression group, and the relationship between the expression of LINC00261 and clinical pathological parameters and prognosis of TC patients was analyzed. As shown in Table I, LINC00261 expression was markedly in correlation with TNM stage, lymph node and distant metastasis in TC patients.

### EBF1 Was Up-Regulated in Thyroid Cancer Tissues and Cells

qRT-PCR analysis indicated that EBF1 was markedly increased in TC tissues compared with the adjacent ones (Figure 1C). In addition, compared with Nthy-ori 3-1, EBF1 was also markedly highly expressed in TC cells (Figure 1D). These results suggested that EBF1 may act as an oncogene in TC. Subsequently, the relationship between EBF1 expression and clinicopathological parameters and prognosis of TC patients was further analyzed, which suggested that high expression of EBF1 was markedly in correlation with the TNM stage, lymph node and distant metastasis in TC patients (Table I).

### LINC00261 Was Bound to EBF1

To further verify the targeting effect of LINC00261 on EBF1, the Luciferase reporter gene experiment was performed, which showed that LINC00261 can be targeted by EBF1 (Figure 2A). In addition, the LINC00261 overexpression or knockdown model was constructed.in the TC cell lines BPH5-16 and K1 (Figure 2B). Western Blot detection suggested that EBF1 expression was markedly reduced after overexpression of LINC00261; whereas EBF1 expression was markedly increased after knockdown of LINC00261 (Figure 2C). Besides, we constructed the EBF1 overexpression or knockdown model (Figure 2D) and found by qRT-PCR that LINC00261 expression was markedly decreased after overexpression of EBF1; while the expression of LINC00261 was markedly increased after knocking down EBF1 (Figure 2E). These results demonstrated that LINC00261 could directly target EBF1 and that the expressions of the two were in negative correlation in TC.

### LINC00261 Inhibited the Growth and Migration in Thyroid Cancer Cell Lines

In TC cell lines K1 and BHP5-16, CCK-8 and transwell migration experiments were per-

**Table I.** Association of LINC00261 and EBF1 expression with clinicopathologic characteristics of thyroid cancer.

| Parameters | No. of cases | LINC00261 expression | | *p*-value | EBF1 expression | | *p*-value |
|---|---|---|---|---|---|---|---|
| | | High (n=23) | Low (n=17) | | Low (n=17) | High (n=23) | |
| Age (years) | | | | 0.822 | | | 0.676 |
| <40 | 18 | 10 | 8 | | 7 | 11 | |
| ≥40 | 22 | 13 | 9 | | 10 | 12 | |
| Gender | | | | 0.337 | | | 0.822 |
| Male | 20 | 13 | 7 | | 8 | 10 | |
| Female | 20 | 10 | 10 | | 9 | 13 | |
| TNM stage | | | | 0.037 | | | 0.013 |
| I/II | 24 | 17 | 7 | | 14 | 10 | |
| III/IV | 16 | 6 | 10 | | 3 | 13 | |
| Lymph node metastasis | | | | 0.001 | | | 0.048 |
| Negative | 26 | 20 | 6 | | 14 | 12 | |
| Positive | 14 | 3 | 11 | | 3 | 11 | |
| Distance metastasis | | | | 0.018 | | | 0.016 |
| Negative | 27 | 19 | 8 | | 15 | 12 | |
| Positive | 13 | 4 | 9 | | 2 | 11 | |

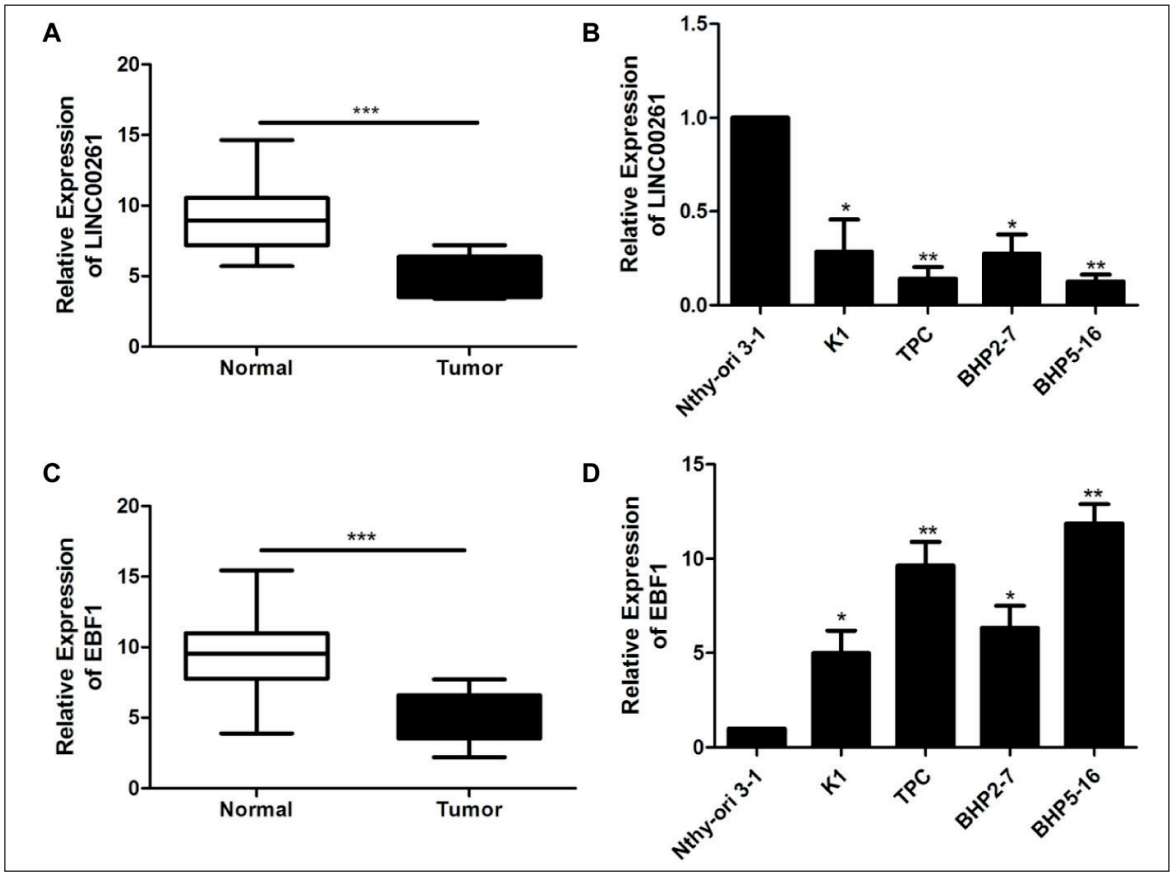


**Figure 1.** Aberrant expressions of LINC00261 and EBF1 in thyroid cancer tissues and cells, respectively. **A,** qRT-PCR analysis of LINC00261 expression in tumor tissues and adjacent non-tumor tissues of thyroid cancer patients; **B,** qRT-PCR analysis of LINC00261 expression in thyroid cancer cell lines; **C-D**, qRT-PCR analysis of EBF1 expressions in thyroid cancer tissues and cells. Data were average ± SD, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

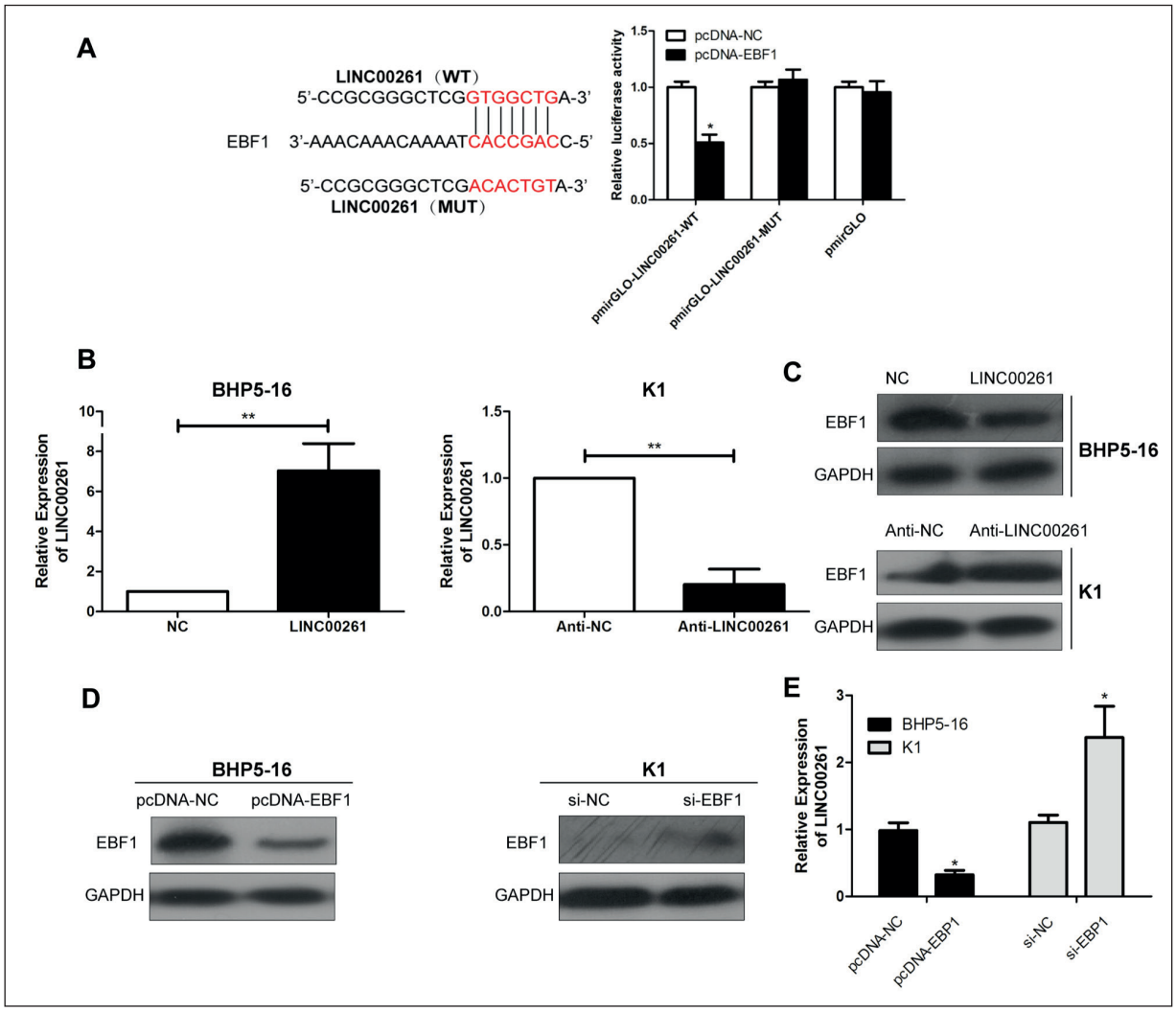


**Figure 2.** Direct targeting of EBF1 by LINC00261. **A,** Dual-Luciferase reporter gene experiments were performed to verify the direct targeting of LINC00261 and EBF1; **B,** qRT-PCR verification of the expression of LINC00261 after transfection of LINC00261 knockdown and overexpression vectors in K1 and BHP5-16, respectively; **C,** Western Blot detection of EBF1 levels after transfection of LINC00261 knockdown and overexpression vectors in thyroid cancer cell lines K1 and BHP5-16, respectively; **D,** Western Blot detection of EBF1 expression after transfection of EBF1 knockdown and overexpression vectors in thyroid cancer cell lines K1 and BHP5-16; **E,** qRT-PCR analysis of the expression levels of LINC00261 in thyroid cancer cell lines K1 and BHP5- 16 transfected with EBF1 knockdown and overexpression vectors, respectively. Data were average ± SD, * $p$ <0.05, ** $p$ <0.01.

formed after overexpression or knockdown of LINC00261. CCK-8 assays showed that the proliferative ability of TC cells was markedly reduced after overexpression of LINC00261; meanwhile, knocking down LINC00261 could markedly increase the proliferative ability of TC cells (Figure 3A). In addition, transwell migration experiments suggested that the LINC00261 overexpression markedly reduced the migration of TC cells and vice versa (Figure 3B).

### *EBF1 Promoted the Growth and Migration in Thyroid Cancer Cell Lines*

CCK-8 results showed that overexpression of EBF1 in BHP5-16 markedly increased the proliferative ability of TC cells; while EBF1 knockdown in K1 markedly reduced the proliferation of TC cells (Figure 3C). In addition, the transwell migration experiment suggested that the overexpression of EBF1 markedly elevated the migration ability of TC cells; but after knocking down

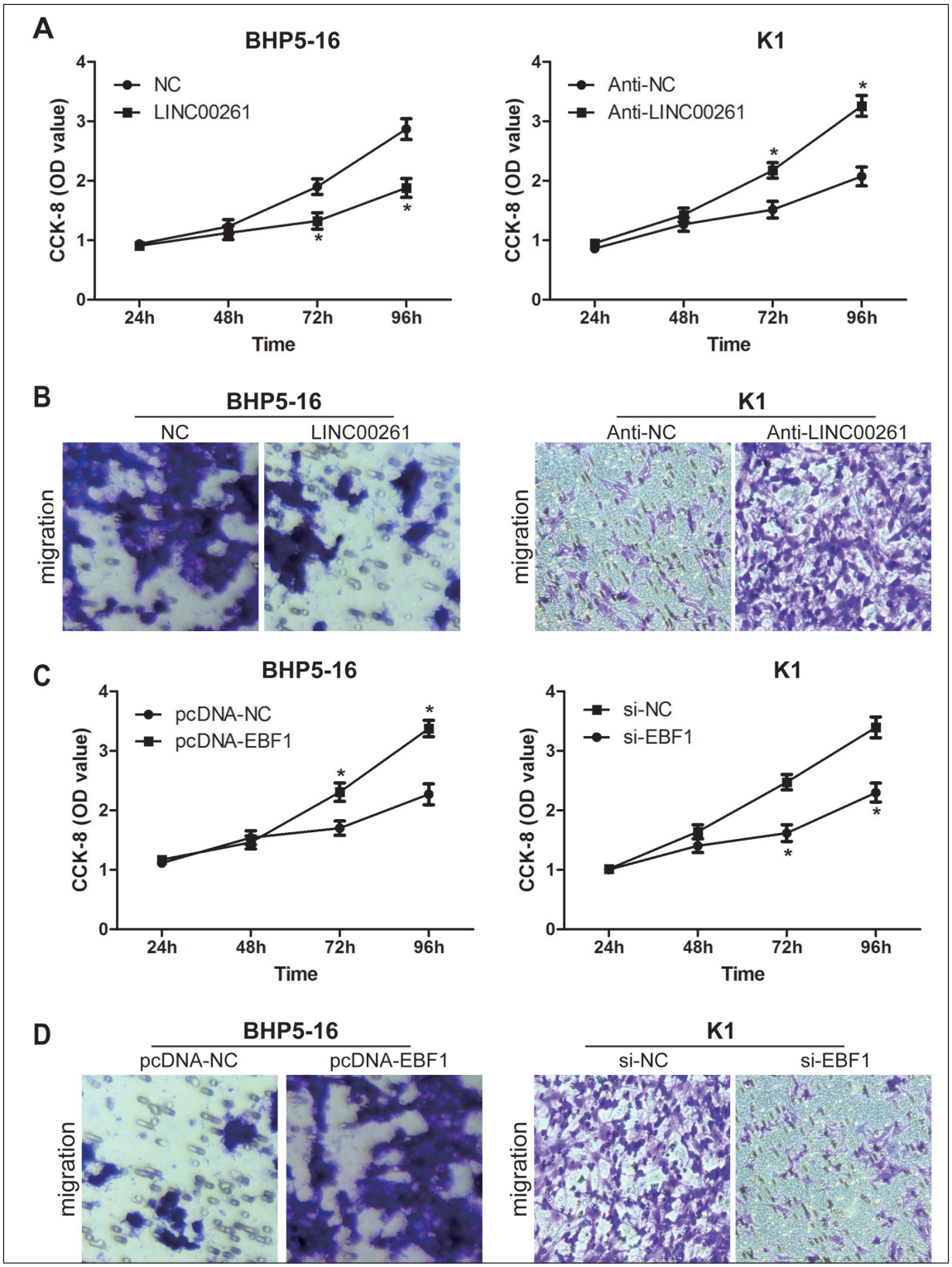


**Figure 3.** The effect of LINC00261 and EBF1 on cell proliferation and migration in thyroid cancer cells. **A,** CCK-8 detection of the proliferation of thyroid cancer cells after LINC00261 knockdown or overexpression in K1 and BHP5-16, respectively; **B,** Transwell detection of cell migration in thyroid cancer cell lines K1 and BHP5-16 after LINC00261 knockdown or overexpression, (magnification: 40×). **C,** CCK-8 detection of the proliferation of thyroid cancer cell lines K1 and BHP5-16 after EBF1 knockdown or overexpression; **D,** Transwell detection of cell migration in thyroid cancer cell lines K1 and BHP5-16 after transfection of EBF1 knockdown or overexpression vectors, (magnification: 40×). Data were average ± SD, *$p$ <0.05.

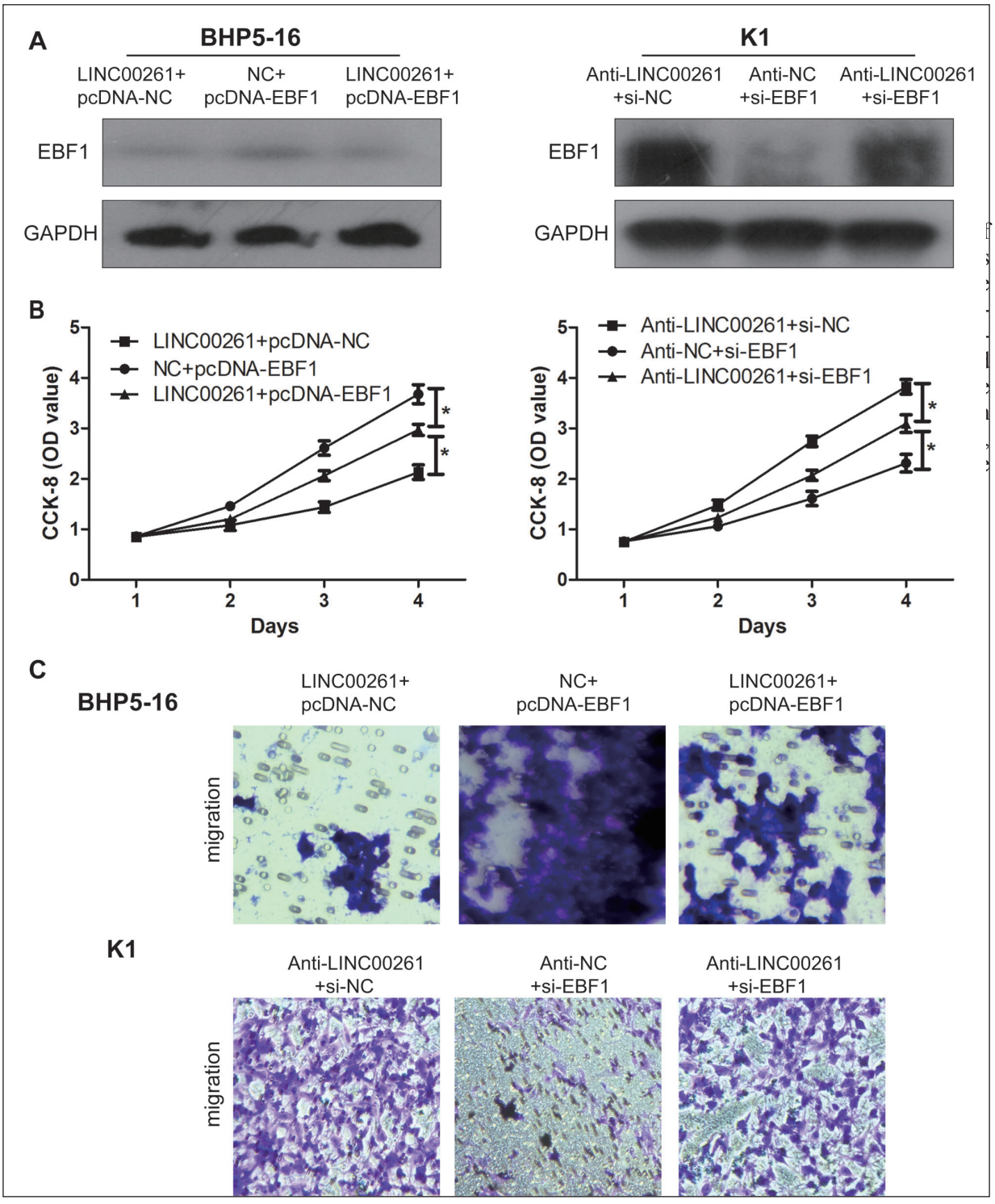


***Figure 4.*** LINC00261 can regulate EBF1 expression in thyroid cancer cell lines. **A,** Western Blot verification of the expression efficiency of EBF1 after co-transfection of LINC00261 and EBF1 knockdown or overexpression vectors in thyroid cancer cell lines K1 and BHP5-16, respectively; **B,** CCK-8 detection of the proliferation in thyroid cancer cell line K1 co-transfected with LINC00261 and EBF1 knockdown or overexpression vectors; **C,** Transwell detection of cell migration after co-transfection of LINC00261 and EBF1 knockdown or overexpression vectors in thyroid cancer cell lines K1 and BHP5-16, respectively, (magnification: 40×) Data were average ± SD, * $p$ <0.05, ** $p$ <0.01.

promotion effect of LINC00261 knockdown on the TC cells proliferation (Figure 4B). The same results were observed in transwell migration experiments, which suggested that overexpression of EBF1 can reverse the reduction of TC cell migration after overexpression of LINC00261; while knockdown of EBF1 can reverse the elevation of TC cell migration after knockdown of LINC00261 (Figure 4C). In sum, these above results demonstrated that LINC00261 could negatively modulate EBF1 in TC.

## Discussion

Thyroid cancer is one of the most common malignant tumors of the human endocrine system, accounting for about 1% of all malignant tumors in humans[1-3]. In TC, it has been confirmed that the abnormal regulation of genes can markedly affect the occurrence and progression of tumors[5-7]. In addition to some classic genetic mutations that have been confirmed in the past decades, research in recent years has emphasized the importance of epigenetic regulation (such as histone modification, genome methylation modification, etc.) in TC[8-10].

LncRNA is a type of RNA with a transcript of more than 200 nucleotides in length, which does not encode a protein, so its functional research progress is relatively rare in the past days[12-14]. At present, the understanding of lncRNA is still in the initial stage, especially the role of lncRNA in diseases. So far, few studies have reported the abnormal expression of lncRNAs in papillary TC[14,15]. In this study, LINC00261 was selected as the candidate lncRNA in the malignant progression of TC. qRT-PCR analysis indicated that the level of LINC00261 was markedly lower in TC tissues, as well as TC cell lines. In addition, retrospective studies on the clinicopathological parameters of patients suggested that LINC00261 expression in TC was in positive correlation with the poor prognosis. Therefore, we believed that LINC00261 plays a suppressing role in TC. Bioinformatics analysis suggested that LINC00261 can target and regulate EBF1. qRT-PCR analysis indicated that EBF1 level was markedly increased in TC tissues and cell line. In addition, high expression of EBF1 was in positive correlation with poor prognosis of the disease. Besides, Luciferase reporter gene experiments verified that LINC00261 could directly bind to EBF1, and that their expressions were negatively correlated in TC tissues.

The ability of proliferation and metastasis is an important feature of tumorigenesis and development[18,19]. Here, through CCK-8 and transwell migration experiments we found that LINC00261 can inhibit the proliferative and migration ability of TC cells while EBF1 can promote the progression of TC cells. Further recovery experiments found that overexpression of EBF1 could reverse the reduction of the proliferative and migration ability of TC cells caused by overexpression of LINC00261. This evidence indicated that the transcription activity of the gene locus of LINC00261 may be regulated by EBF1. Therefore, combined with the above findings, we believed that LINC00261 could negatively modulate the expression of EBF1, thereby inhibiting the malignant progression of TC. However, the role LINC00261 in cell cycle and EMT and *in vivo* assays were not enrolled in our present studies and we will perform that in the future. The previous findings firstly explore the role of LINC00261 in TC, providing a new target therapy in the future.

## Conclusions

In summary, this study demonstrates that LINC00261 expression is markedly reduced in TC tissues and cells while EBF1 is aberrantly highly expressed in TC. In addition, low expression of LINC00261 or high expression of EBF1 is closely related to lymph node and distant metastasis, as well as the prognosis in patients with TC. LINC00261 regulates the biological function of TC cells by negatively regulating EBF1. Our finding suggests that LINC00261 can be used as a tumor suppressor gene of TC.

### Conflicts of Interest

The authors declare no conflicts of interest.

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