Long Noncoding RNA LINC00460 Modulates MMP-9 to Promote Cell Proliferation, Invasion and Apoptosis by Targeting miR-539 in Papillary Thyroid Cancer

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Background: Increasing evidence shows that Long non-coding RNAs (lncRNAs) involve in the development and progression processes of various cancers, including papillary thyroid cancer (PTC). In this study, we focused on the regulation function of lncRNA LINC00460 in the development of PTC.

Methods: Expression of LINC00460 was detected using quantitative real-time PCR (qRT-PCR) and Western blot assay. Cell proliferation, cell apoptosis and cell invasion were determined through CCK-8 assay, flow cytometry, and Transwell assay, respectively. In addition, target sites were detected by the dual-luciferase reporter gene assay.

Results: LINC00460 expression was markedly up-regulated in PTC tissues and cells compared to their corresponding controls by quantitative real-time PCR (qRT-PCR). Meanwhile, LINC00460 knockdown notably inhibited the proliferation capacity, accelerated the apoptosis and down-regulated the invasion-related proteins (MMP-2, MMP-9, ZEB1) expression. In addition, bioinformatics tools predicted that miR-539 both targeted with the 3’-UTR of LINC00460 and MMP-9, which was confirmed by luciferase reporter assay and Western blot.

Conclusion: These findings indicated that LINC00460 can modulate MMP-9 expression to promote cell proliferation, invasion and apoptosis through targeting miR-539, suggesting act as an oncogenic RNA in PTC and provide a new therapeutic perspective.

Keywords: papillary thyroid cancer, LINC00460, proliferation, invasion, apoptosis

Introduction
TC accounts for only 1% of all the malignancies and is a common endocrine malignancy.1 Papillary thyroid carcinoma (PTC) accounts for 86% of all TC.2 Moreover, TC of different pathological patterns, the mechanism of disease occurrence is also different from each other.3 Thus, it is important to study the underlying mechanisms of PTC carcinogenesis, and it is indispensable for the development of more effective diagnostic and therapeutic methods in the future.

Recently, researchers have attempted to explore lncRNAs as a possible regulator in tumorigenesis and tumor development. The structural composition of lncRNAs is a series of non-coding RNAs >200 nucleotides in length. Previous literature has shown that lnc RNAs play a crucial regulator of tumor suppression or carcinogenesis under different circumstances. For instance, lncRNA MALAT1, lncRNA...
HOTAIR, lncRNA 00460, lncRNA TUG1 and lncRNA XIST, promote cell proliferation, invasion and/or migration, including multiple myeloma, triple-negative breast cancer, cervical cancer, pancreatic cancer, bladder cancer, gastric cancer and thyroid cancer.\(^4\)-\(^12\)

Interestingly, lncRNA can play a regulatory role in cancer development by interacting with miRNAs. MiRNAs are a series of non-coding RNAs containing about 22 nucleotides that act as competitive endogenous RNAs (ceRNAs).\(^13\),\(^14\) LINC00460 is a new cancer-associated lncRNA whose expression is involved in the development of a variety of human malignancies, including nasopharyngeal carcinoma, lung cancer, thyroid cancer,\(^15\)-\(^17\) Unfortunately, the specific mechanism of action of LINC00460 in PTC is still not clear.

The purpose of this study was to investigate the biological role of LINC00460 in PTC and to determine its underlying mechanisms. LINC00460 is significantly up-regulated in PTC tissues and cell lines in comparison to their corresponding controls. LINC00460 knockdown repressed PTC cell proliferation, invasion and apoptosis. Additionally, our data revealed that LINC00460 could function as an oncogene through up-regulating the miR-539 targeted gene MMP-9 by functioning as a competitive endogenous RNA (ceRNA) for miR-539 in PTC. Our results suggest that LINC00460 may be a new indicator for diagnosis and treatment of PTC patients.

Materials and Methods
Ethical Statement and Clinical Specimens
This study was approved by the Ethical Committee of The First Hospital of Jilin University (Changchun, China). Written informed consent forms were obtained from all patients, in accordance with the Declaration of Helsinki.

Cell Culture and Transfection
Human TC cell lines (K1, TPC-1) and human normal thyroid cell line (Nthy-ori 3-1) (Shanghai Tiancheng Technology Co., Ltd.) were used in our study. The cell lines were maintained in DMEM culture medium (Hyclone, Thermo, San Jose, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100U/mL penicillin and 100µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and incubated in a humidified chamber supplemented with 5% CO\(_2\) at 37°C.

siRNA targeting LINC00460, miR-539 mimics and miR-539 inhibitor (Life Technologies, Carlsbad, CA, USA) were, respectively, transfected using Lipofectamine 3000 Reagents (Life Technologies, Carlsbad, CA, USA). The sequences were as follows: si-LINC00460-1: 5'-GUGUCAACACCGUUGUUAUU-3'; si-LINC00460-2: 5'-UUAGUUCAGAAUUGGACUUU-3'; si-LINC00460-3: 5'-GUAAACACUUUCUAGACUUU-3'. miR-539 mimics, forward: 5'-UGGCAGUGUCUUAGCUGGU UUG-3'; reverse: 5'-AC CAGCUAAAGACACUGCCA UU-3'. miR-539 inhibitor, 5'-AC UUGG UUAGAUCA AGCACA-3'.

Quantitative RT-PCR (qRT-PCR)
Total RNA was extracted from tissues and cells using Trizol reagent (Life Technologies, Carlsbad, CA, USA). Then, RNA was reverse transcribed into cDNA. Finally, qRT-PCR experiments were conducted using SYBR Premix Ex Taq (Qiagen, Hilden, Germany). All primers were presented as follows: LINC00460, forward: 5'-A ATGGTGTTAGGGAGGA-3', reverse: 5'-CAAGGGGA ATGAACACGGAGG-3'; β-actin, forward: 5'-AAGCCCA CTTCCTCCCACCTAA-3', reverse: 5'-AATGCTAT CACCTCCCCGTGT-3'.

Cell Proliferation Assay
PTC cell lines (K1, TPC-1) (2x10\(^3\) cells/well) were transfected with siRNAs, and subsequently seeded in 96-well cell culture plates with five replicate wells for each group for 72 h. 10 µL of CCK-8 reagent was added into each well for 2 h and absorbance was measured at 450nm.

Cell Apoptosis Assay
Cell apoptosis was determined utilizing Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining (BD Bioscience, San Jose, CA, USA). Briefly, the PTC cell lines (K1, TPC-1) (2x10\(^3\) cells/mL) were harvested in 6-well plates and suspended in binding buffer for 15 min containing 5 µL of Annexin V-FITC and PI. Stained cells were measured by a flow cytometer (BD LSRII; BD Pharmingen).

Cell Invasion Assay
Transwell chambers with 8 mm pores were precoated with Matrigel matrix (BD Bioscience, Franklin Lakes, NJ, USA). Transfected cells (K1, TPC-1) (1x10\(^3\)) were seeded into the upper chambers containing serum-free DMEM, while the lower chambers were filled with medium containing 10% FBS as a chemoattractant. After incubation for 24 h, the non-invasive cells on the top side of the membrane were removed and the invasive cells of the...
inserts on the lower surface were fixed and stained with 0.1% crystal violet (Sigma, St Louis, MO, USA). The number of cells was counted in six fields under a light microscope.

**Western Blotting**
Protein extracts from PTC cells were electrophoresed using 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Then, the membrane was blocked with 5% skimmed milk for 1 h followed by incubation with the following primary antibodies at 4°C overnight: Rabbit anti-MMP-2 (1:800, ab37150, Abcam, Cambridge, England, US), rabbit anti-MMP-9 (1:800, ab38898, Abcam), rabbit anti-ZEB1 (1:800, ab124512, Abcam) and Rabbit anti-β-actin (1:2000, ab37168, Abcam, Cambridge, MA, USA). Afterward, the HRP-conjugated goat anti-rabbit antibody (1:1500, Abcam) for 2 h at room temperature was added to probe the target proteins. Finally, Immunoblot signals were visualized using ECL reagents (Syngene, Frederick, MD, USA). Relative protein expression was quantified using Image-Pro Plus 6.0 software.

**Luciferase Activity Assay**
Wild-type (WT) or mutant controls (Mut) of 3′-UTRs of the LINC00460 and MMP-9 that contained the putative miR-539 binding site were inserted into the pGL3 vector (Promega, Madison, WI, USA) for luciferase reporter experiments, named as LINC00460 WT, MMP9 WT, LINC00460 Mut and MMP-9 Mut, and then cotransfected into the cells with either WT or Mut, and miR-539 or control miRNA using Lipofectamine 2000 reagent for 48 h. Finally, the dual-luciferase reporter system (Promega) was used to analyze the relative luciferase activity.

**Data Analysis**
Data analysis was performed using GraphPad Prim 5.0 (GraphPad Software, La Jolla, CA, USA). All data are expressed as the means±standard deviation (SD). Comparisons between two groups were performed by using Student’s t-tests. P<0.05 was considered statistically significant.

**Results**
**Expression Levels of IncRNA LINC00460 in PTC Tissues and Cells**
The expression levels of LINC00460 in PTC tissues and adjacent normal tissues were measured using qRT-PCR. Our data revealed that the LINC00460 expression level in PTC tissues was markedly increased than that of the adjacent normal tissues (P<0.01; Figure 1A). At the same time, LINC00460 expression levels were also obviously higher in PTC cell lines (K1, TPC-1) than that of human normal thyroid cell line Nthy-ori 3–1 (P<0.01; Figure 1B). Therefore, these data revealed that LINC00460 was markedly up-regulated in PTC tissues and cells, suggesting LINC00460 may be used as potential oncogenic role in PTC tissues and cells.

**IncRNA LINC00460 Knockdown Restrained Proliferation of PTC Cells in vitro**
To investigate the role of LINC00460 in the PTC process in vitro, siRNA targeting LINC00460 was transfected into PTC cell lines (K1, TPC-1) to knockdown LINC00460 expression (P<0.05; Figure 2A and B). LINC00460 knockdown obviously inhibited the proliferation of PTC cells (K1, TPC-1) using the CCK-8 assay (P<0.05; Figure 2C and D).

Figure 1 IncRNA LINC00460 was up-regulated in PTC tissue and cells. (A) LINC00460 expression was analyzed in PTC tissue and adjacent normal tissue by qRT-PCR. (B) LINC00460 expression was detected in human normal thyroid cell line (Nthy-ori 3–1) and PTC cell lines (K1, TPC-1) by qRT-PCR. **P<0.01 vs control group.
Figure 2. lncRNA LINC00460 knockdown restrained the proliferation and activated the apoptosis of PTC cells. (A, B) Small interfering oligonucleotides targeting LINC00460 (si-LINC00460) were transfected into PTC cell lines (K1, TPC-1). (C, D) The proliferation ability of PTC cells transfected with si-LINC00460 and si-NC was measured by CCK-8 assay. (E, F) Apoptotic rate of PTC cells transfected with si-LINC00460 and si-NC was analyzed by flow cytometry assay. *P<0.05, **P<0.01 vs control group.
In a word, our results found that LINC00460 knockdown inhibited the proliferation of PTC cells in vitro.

**IncRNA LINC00460 Knockdown Increased Apoptosis of PTC Cells**
Through flow cytometry analysis, our data revealed that LINC00460 knockdown notably increased the apoptotic rate of PTC cell lines (K1, TPC-1) compared to the control group \((P<0.01; \text{Figure 2E and F})\). In a word, our results indicated that LINC00460 knockdown obviously facilitated the apoptosis of PTC cells.

**IncRNA LINC00460 Knockdown Restrained Invasion of PTC Cells**
Our results revealed that LINC00460 knockdown obviously decreased the invasion number of PTC cells (K1, TPC-1) compared to the control group \((P<0.01; \text{Figure 3A and B})\). Moreover, LINC00460 knockdown transfection markedly down-regulated these invasion-related marker proteins (MMP-2, MMP-9, ZEB1) expression compared to control group using Western blotting \((P<0.05; \text{Figure 3C and D})\). In a word, our results demonstrated that LINC00460 knockdown obviously inhibited the invasion capacity of PTC cells.

**IncRNA LINC00460 Enhanced MMP-9 Protein Expression via Targeting miR-539**
To investigate the role of LINC00460 and its downstream regulatory pathway in PTC progression, we use the bioinformatics tool to find potential target sites of LINC00460. Our results revealed that LINC00460 harbored miR-539 with complementary binding at 3′-UTR regions \((\text{Figure 4A})\). Meanwhile, luciferase reporter revealed the luciferase activity was notably decreased \((P<0.05; \text{Figure 4B})\), indicating that LINC00460 could combine with miR-539. Meanwhile, it has been confirmed that miR-539 targeted with the 3′-UTR of MMP-9 \((\text{Figure 4C and D})\). After transfected with si-LINC00460 or si-NC in K1 cells, miR-539 expression was obviously increased by qRT-PCR \((P<0.05; \text{Figure 4E})\). However, the mRNA expression level of MMP-9 was significantly down-regulated in the LINC00460 knockdown group \((P<0.05; \text{Figure 4E})\). Subsequently, transfected with miR-539 inhibitor or control in K1 cells, the expression level of LINC00460 and MMP-9 was obviously up-regulated \((P<0.05; \text{Figure 4F})\). Western blot results revealed that miR-539 inhibitor obviously up-regulated the protein expression level of MMP-9, while the co-transfection of miR-539 inhibitor and si-LINC00460 reversed its expression levels \((P<0.05; \text{Figure 4G})\). In a word, these results demonstrated that LINC00460 markedly enhanced MMP-9 protein expression via targeting miR-539, suggesting that it may be achieved through the LINC00460/miR-539/MMP-9 pathway.

**Discussion**
Over the past decades, the aberrant expression of lncRNA has been widely observed and reported to be involved in tumorigenesis. lncRNAs have captured increasing attention due to their crucial roles in biological processes, both normal physiological processes and pathogenic processes.\(^{18–20}\) As an important means of epigenetic regulation, lncRNAs have been confirmed to involve in a series of human diseases, including tumors, cardiovascular and endocrine system disease.\(^{21–23}\) Therefore, considering the crucial role of lncRNAs in the tumorigenesis, we try to explore the effect of lncRNAs in the TC progression.

In this study, our data revealed that LINC00460 was markedly increased in PTC tissues and cell lines (K1 and TPC-1) in comparison with adjacent normal tissues and human normal thyroid cell line Nthy-ori 3–1, suggesting LINC00460 was considered as a potential oncogenic in PTC. For example, in non-small cell lung cancer (NSCLC), LINC00460 expression was obviously up-regulated and associated with poor prognosis.\(^{24}\) Meanwhile, previous research also confirmed that LINC00460 contributed to enhancing the capability of cell proliferation, migration and invasion through inducing ETM transition in lung cancer cells.\(^{16}\) Furthermore, LINC00460 was positively correlated with esophageal squamous cell carcinoma (ESCC) TNM stage, lymph node metastasis, and poor prognosis, acting as a valuable prognostic biomarker for ESCC diagnosis.\(^{17}\) Subsequently, further experiments explored the biological function of LINC00460 in PTC progression. Our data revealed that LINC00460 knockdown obviously restrained the proliferation and strengthened the apoptosis of PTC cell lines. In addition, LINC00460 knockdown also markedly weakened the invaded cell quantity, and down-regulated the invasion-related proteins (MMP-2, MMP-9, ZEB1) expression. In a word, these findings revealed that LINC00460 functions as a potential oncogene in PTC transformation, which might bright a new insight for the disease.

Until now, the major pattern of lncRNA regulating the tumorigenesis is the competing endogenous RNA
Figure 3 IncRNA LINC00460 knockdown inhibited the invasion of PTC cells. (A, B) The invaded cell quantity in PTC cell lines (K1, TPC-1) transfected with si-LINC00460 or si-NC was analyzed by Transwell invasive assay. (C, D) The protein expression of MMP-2, MMP-9 and ZEB1 was detected in PTC cell lines (K1, TPC-1) transfected with si-LINC00460 or si-NC by Western blot. *P<0.05, **P<0.01 vs control group.
LncRNA functions as miRNA “sponge” to absorb miRNA abundance to reduce the expression of miRNA, and release the inhibition of target protein through miRNA. For instance, Long noncoding RNA LINC00460 promotes meningioma progression and metastasis through targeting miR-539/MMP-9. In our study, we confirmed that LncRNA LINC00460 modulates MMP-9 to promote cell proliferation, invasion and apoptosis by targeting miR-539 in PTC. Meanwhile, LINC00460 acts as a miR-539 sponge to restore the MMP-9 protein expression. Previous studies demonstrated that miR-539 was considered as a tumor suppressor in cancers, including breast cancer, non-small cell lung cancer, hepatocellular carcinoma. In addition, we speculate that the invasion or metastasis may more likely occur in the high grades of PTC.

**Conclusion**

In summary, these findings demonstrated that LINC00460 is obviously increased in PTC tissue and cells. LINC00460 enhances MMP-9 expression by
targeting miR-539 to facilitate the proliferation and metastasis of PTC, providing a new treatment insight for the pathogenesis.

**Abbreviations**

LINC00460, long intergenic noncoding RNA-00460; PTC, papillary thyroid cancer; MMP-9, matrix metalloprotein-9; miR-539, microRNA-539; FBS, fetal bovine serum.

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**Disclosure**

All authors declare no conflicts of interest in this study.

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