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STAT1-induced upregulation of lncRNA LINP1 promotes cell proliferation and inhibits apoptosis *via* AMPK signaling pathway in papillary thyroid cancer

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Abstract. – OBJECTIVE: The purpose of this study was to detect the relative expression of long non-coding ribonucleic acid (lncRNA) in non-homologous end joining pathway 1 (LINP1) in papillary thyroid cancer (PTC) tissues and cells, and to investigate the molecular mechanisms of abnormal expression and biological function of LINP1.

PATIENTS AND METHODS: The relative expression of LINP1 in PTC tissues and cells was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), and the impact of small interfering (si)-LINP1 on the proliferative capacity of PTC cells was studied using Cell Counting Kit-8 (CCK-8) and colony formation assays. After the expression of LINP1 in PTC cells was interfered, flow cytometry was applied to determine the changes in cell cycle distribution and apoptosis rate. The transcription factors binding to the promoter region of LINP1 were predicted by bioinformatics. Next, qRT-PCR assay was adopted to measure the changes in LINP1 expression after interference in the expression of signal transducer and activator of transcription 1 (STAT1). Finally, the changes in the expressions of molecular markers of the adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling pathway were examined *via* Western blotting assay after the expressions of STAT1 and LINP1 were interfered.

RESULTS: It was shown in qRT-PCR results that LINP1 expression was upregulated in 42 out of 53 cases of PTC tissues and in all PTC cells. After interference in the expression of LINP1 in PTC cells, the results of CCK-8 and colony formation assays indicated that the proliferative capacity of the cells was repressed. According to the results of flow cytometry, the cell cycle was arrested at the G1/G0 phase, and the apoptosis rate was increased. In addition, the bioinformatics predicted that STAT1 could bind to the promoter region of LINP1, and the results of qRT-PCR indicated that the expression of LINP1 declined after STAT1 expression was interfered. Moreover, it was indicated in the Western blotting assay after interference in the expressions of STAT1 and LINP1 that the expression of molecular marker (Phosphorylation AMPK, p-AMPK) of the AMPK signaling pathway was altered but the expression of total AMPK did not change.

CONCLUSIONS: The transcription factor STAT1 promotes the expression of LINP1 in PTC, and highly expressed LINP1 facilitates the proliferation and inhibits the apoptosis of PTC by suppressing the AMPK signaling pathway.

Key Words:
PTC, LINP1, STAT1, Biological function, AMPK signaling pathway.

Introduction

Thyroid cancer (TC), the most common endocrine tumor, ranks the ninth among the malignant tumor around the globe[1], of which papillary TC (PTC) is the most common type, accounting for approximately 90% of all TC cases. TC occurs more frequently in females, and studies have manifested that TC will become the third major tumor in women by 2019[2]. Although many cases of TC can be efficaciously treated by means of surgeries and radioactive iodine, the mortality rates of advanced TC, radioactive iodine-refractory differentiated TC and TC-related diseases are rising year by year, so it is particularly important to understand the detailed occurrence and development mechanisms of PTC.

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Long non-coding ribonucleic acids (lncRNAs) are a category of ncRNAs with a transcript with a length of ~200 nucleotides. LncRNAs do not participate in the processes of gene transcription and translation, but they play crucial roles in the gene regulatory networks[3,4]. LncRNAs are correlated with cell proliferation, differentiation, chromosome modification, gene regulation, as well as tumor genesis and metastasis; they possess multiple mechanisms in regulating gene expressions, which generally modulate the gene expressions at the transcriptional and post-transcriptional levels[5-7]. The abnormal expression of lncRNAs has correlations with the occurrence and development of breast cancer, lung cancer, esophageal cancer, and other tumors[8-10].

Zhang et al[11] revealed that lncRNAs are also closely associated with the incidence and progression of TC. Jendrzejewski et al[12] discovered through experiments that PTCSC3 exhibits decreased expression in PTC tissues and promotes TC cell proliferation and metastasis by upregulating the protein expression of S100A4. Huang et al[13] found that the expression of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is upregulated in TC tissues, while MALAT1 is able to accelerate the vascularization in PTC tissues by modulating the secretion of FGF2 protein in tumor-associated macrophages, thus stimulating such biological behaviors as proliferation and metastasis of TC cells. In the study, it was discovered by *in-vitro* experiments in this research that LINP1 expression was upregulated in PTC tissues and cells, which facilitated the proliferation and suppressed the apoptosis of PTC.

Patients and Methods

Tissue Specimens

A total of 53 PTC patients admitted to and treated in the Department of General Surgery of Caoxian People's Hospital from January 2015 to June 2018 were selected as the subjects. The patients with a history of thyroid surgery, breast-feeding women, those who received radiotherapy or chemotherapy before operation, or those with severe heart, lung, liver or kidney disease or other malignant tumors were excluded. All the patients underwent surgical resection of TC, and PTC was confirmed by pathology after operation. All the cancer tissue specimens excised were immediately placed in liquid nitrogen and stored in a refrigerator at -80°C for later use. This research was approved by the Ethics Committee of the Hospital, and all the patients signed the informed consent.

Cell Culture

Human PTC cell line (Shanghai cell bank of Chinese Academy of Sciences, Shanghai, China) and normal thyroid cells (American Type Culture Collection; ATCC, Manassas, VA, USA) were cultured in a high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 100 mL/L fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (Beyotime Biotechnology, Shanghai, China) at 37°C with 50 mL/L CO_2 and saturated humidity. The experiment started from the logarithmic growth phase.

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

The total RNA in PTC group and para-carcinoma control group was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), which was reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the kit instructions (Invitrogen, Carlsbad, CA, USA). Then, amplification was conducted as per the SYBR Green instructions (Invitrogen, Carlsbad, CA, USA) using a RT-PCR system (10 μL in total), including 5 μL of SYBR Master Mix, 0.2 μL of Primer F, 0.2 μL of Primer R, 1 μL of cDNA template and ddH_2O adding the volume to 10 μL. Amplification conditions: at 95°C for 3 min, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s for 40 cycles. The relative expressions were calculated using $2^{-\Delta\Delta Ct}$ method. The primers are as follows: LINP1: F 5'-CCAATTGAACCGAGCCTTGT-3', R'-TCGTGAGCGTTTTCGCAATG-3', GAPDH: F 5'-TCCTCTGACTTCAACAGCGACAC-3'; R 5'-TCTCTCTTCCTCTTGTGCTCTTGC-3', STAT-1: F, 5′-CTATACTCAGCTTCAGTGTT-3′, R 5′-TACTGTATGGCCACCACTCC-3'.

Transfection

The cells were seeded into a 6-well plate at 3×10^5 cells/well at 12 h before transfection. Then, small interfering-negative control (si-NC), si-lncRNA and si-STAT1 (Shanghai GenePharma, Shanghai, China) were transfected into PTC cells by virtue of the transfection reagent Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in

accordance with the instructions. After culture in an incubator for 48 h, the cells were used for subsequent experiments.

Cell Counting Kit-8 (CCK-8) Assay

At 48 h after transfection, the cells were digested and resuspended, and the cell density was adjusted to 3×10^4/mL. Next, 100 μL of cell suspension was inoculated into a 96-well plate. From the second day of plating, 20 μL of CCK-8 (5 mg/mL; Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well every day after cell adherence. After routine culture for 2 h, the absorbance at 450 nm was detected using a microplate reader for 5 consecutive days for 3 times.

Colony Formation Assay

The cells in experimental group and control group were collected and seeded into the 6-well plate (800 cells/well), followed by routine culture for 10-14 d. When macroscopic cell colonies were formed, the culture was terminated, and the cells were washed with phosphate-buffered saline (PBS) for 3 times, fixed in methanol for 15 min, washed with PBS again for 3 times, and stained with 0.1% crystal violet solution at room temperature for 20 min.

Cell Cycle

After the PTC cells in experimental group grew to coverage of about 80% in a 60 mm culture dish, it was evaluated that the cells did not enter the plateau phase. Later, the cells were washed, digested and harvested in 5 mL centrifuge tubes, with triplicate wells set in each group. After that, the cell pellets were washed in ice-cold PBS at 4°C once, then, fixed in 70% alcohol overnight, and washed once with PBS again. Finally, the cell staining solution PI was added and resuspended adequately, and the cell cycle distribution was examined using a flow cytometer (FACSCalibur, BD Biosciences, Detroit, MI, USA).

Apoptosis

The PTC cells in the experimental group were collected, digested, and harvested in 5 mL centrifuge tubes, with triplicate wells set in each group. The supernatant was discarded after centrifugation, and the cell pellets were washed with PBS once, centrifuged, and then, resuspended. Subsequently, the cell suspension was fetched and added with Annexin V-APC for sufficient staining in the dark at room temperature for 15 min. Finally, the cell apoptosis was determined *via* the flow cytometer.

Western Blotting Assay

The total proteins were extracted from the two groups using lysis buffer, and then, loaded at 30 μg/well. After that the proteins underwent electrophoresis with spacer gel at 80 V for 40 min and separation gel at 100 V for 2 h and transferred onto a membrane *via* conventional wet method and sealed in 5% skim milk powder for 2 h. Next, these proteins were incubated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (1:2000) and goat anti-rabbit secondary antibody (1:5000) for 2 h and exposed with enhanced chemiluminescence (ECL) solution using a gel imaging system. Finally, immuno-reactive bands were visualized by ECL detection kit (Amersham Biosciences, Foster City, CA, USA), and the gray value was analyzed using ImageJ software (Version 1.38, NIH, Bethesda, MA, USA).

Statistical Analysis

The comparison of all the data between groups in this trial was analyzed by *t*-test, the test results were presented as mean ± standard deviation, and $p<0.05$ suggested that the difference was statistically significant.

Results

LINP1 Expression Was Upregulated in PTC Tissues and Cells

The 53 patients were definitely diagnosed with PTC by means of pathology for the tissue specimens resected by surgery. Then, qRT-PCR assay was adopted to detect the relative expression of LINP1. The results showed that the expression of LINP1 was upregulated in 42 cases of PTC tissues (Figure 1A). Next, qRT-PCR assay was conducted again to examine the relative expression of LINP1 in PTC cells, and it was indicated that LINP1 expression was upregulated in PTC cells (Figure 1B). Finally, the LINP1-specific interference sequences were designed and synthesized, and the interference efficiency in K1 and TPC-1 cells was measured through qRT-PCR (Figure 1C, 1D).

Biological Functions of LINP1

After interfering in the expression of LINP1 in PTC cells, the results of CCK-8 assay displayed that the proliferative capacity of the cells

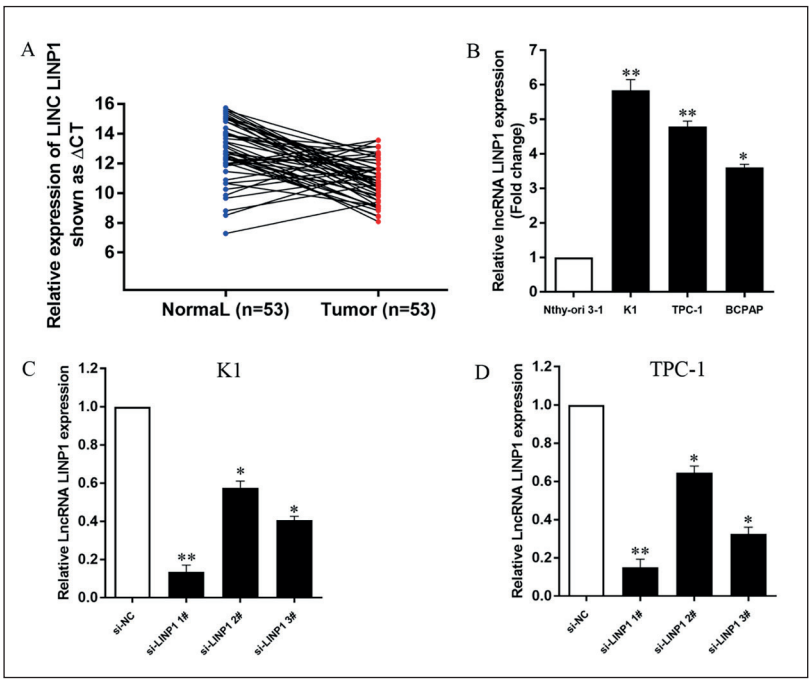

Figure 1. LINP1 expression is upregulated in PTC tissues and cells. **A,** Relative expression of LINP1 in 53 pairs of PTC tissues and para-carcinoma tissues detected *via* qRT-PCR assay. LINP1 expression is upregulated in 42 cases of tissues, with GAPDH as an internal reference. **B,** Relative expression of LINP1 in PTC cells detected *via* qRT-PCR assay. **C, D,** Interference efficiency of si-LINP1 detected *via* qRT-PCR assay.

was repressed in the experimental group (Figure 2A). Besides, it was manifested in the results of the colony formation assay that the proliferative capacity of the cells was weakened in si-LINP1 group compared with that in the control group (Figure 2B). Thereafter, the impact of LINP1 on PTC cell cycle was studied. After the expression of LINP1 was knocked down, the results of flow cytometry revealed that the PTC cell cycle was arrested at the G1/G0 phase (Figure 2C). Si-LINP1 group exhibited a higher cell apoptosis rate than si-NC group (Figure 2D).

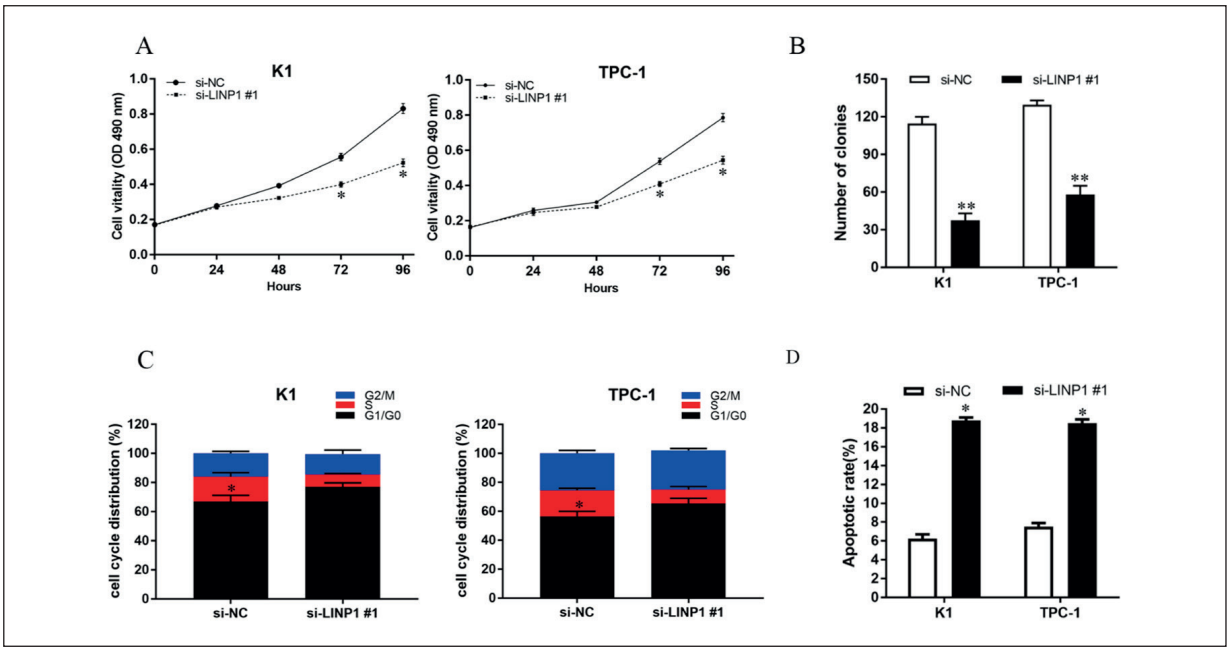

Figure 2. Biological functions of LINP1. **A,** Impact of si-LINP1 on proliferative capacity of PTC cells studied by CCK-8 assay. **B,** Changes in proliferative capacity of PTC cells after interference in LINP1 expression detected *via* colony formation assay. **C,** Distribution of PTC cell cycle after interference in the expression of LINP1 detected via flow cytometry. **D,** Changes in apoptosis rate of PTC cells after interference in LINP1 expression detected via flow cytometry.

Signal Transducer and Activator of Transcription 1 (STAT1) Promoted LINP1 Transcription and Inhibited Adenosine 5′-Monophosphate-Activated Protein Kinase (AMPK) Signaling Pathway

To explore the molecular mechanism of the upregulation of LINP1 expression, the transcription factors that probably bind to the promoter region of LINP1 were predicted by bioinformatics (https://www.genecards.org/), and it was found through qRT-PCR assay that the expression of LINP1 declined after the interference in STAT1 expression (Figure 3A, B). The above results illustrate that the transcription factor facilitates the transcription of LINP1. Subsequently, the molecular mechanism of LINP1 in exerting its biological effects was further investigated, and it was indicated in Western blotting assay that the expressions of the AMPK signaling pathway-related molecular marker (p-AMPK) changed after LINP1 expression was interfered (Figure 3C), and the same results were obtained after STAT1 expression was interfered (Figure 3D).

In summary, the expression of LINP1 is upregulated in PTC tissues and cells, and the transcription factor STAT1 promotes the transcription of LINP1 to inhibit the AMPK signaling pathway, thus facilitating the occurrence and development of PTC.

Discussion

PTC is the most common type of TC with a constantly increasing morbidity rate in clinic, accounting for nearly 90% of TC and becoming a ubiquitous kind of head and neck neoplasm[14]. Plenty of lncRNAs have been found to be related to the incidence of PTC so far, among which BANCR, PTCSC3, MALAT1, and HOTAIR are mainly researched[15,16]. However, there is no report about the expression of LINP1 in PTC, so it was first discovered in this research that LINP1 exhibited an elevated expression in PTC and exerted the effects similar to those of oncogenes.

The upregulation of lncRNA expression is modulated by various factors, such as epigenetic alteration, genome amplification, and regulation of transcription factor. Yu et al[17] reported that the transcription factor E2F1 stimulates the transcription of lncRNA LMCD1-AS1 to enhance the proliferation of cholangiocarcinoma cells. In this research, the transcription factors that possibly conjugate with the promoter region of LINP1 were predicted by means of bioinformatics, and qRT-PCR was applied to verify whether the transcription factor STAT1 promotes the expression of LINP1. STAT1, a key molecule of the IFN-7/JAK/STAT signaling pathway, can regulate the expression of relevant genes by binding to spe-

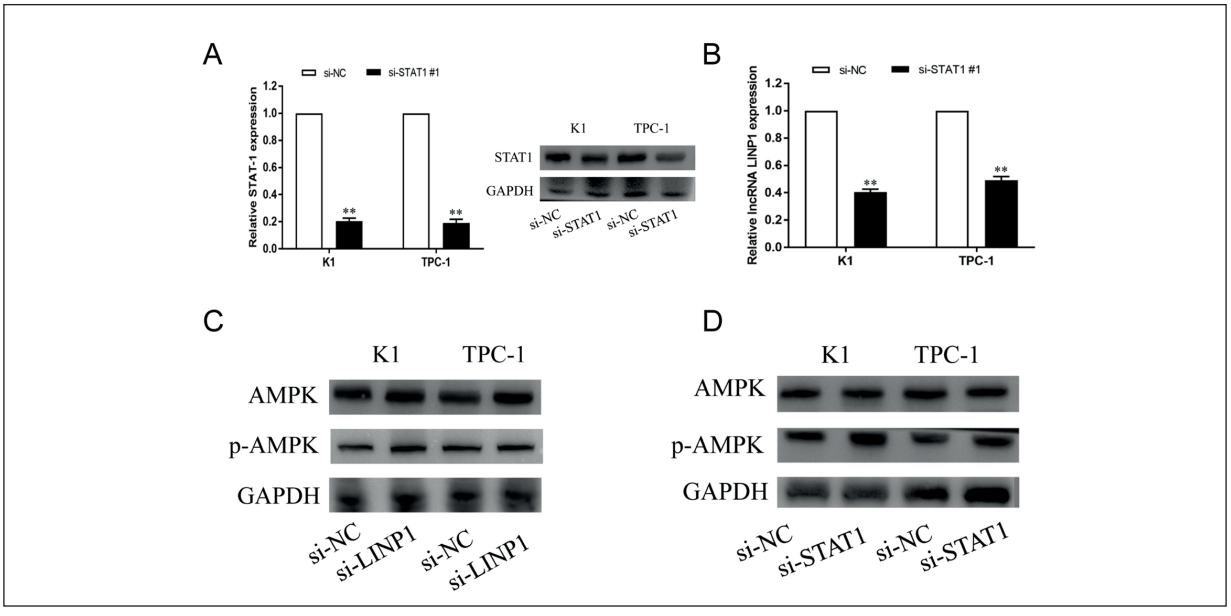

Figure 3. STAT1 promotes LINP1 transcription and inhibits the AMPK signaling pathway. **A,** Interference efficiency detected by qRT-PCR and Western blotting assays. **B,** LINP1 expression detected *via* qRT-PCR assay after interference in the expression of STAT1. **C,** Changes in expressions of molecular markers of the AMPK signaling pathway detected *via* Western blotting assay after interference in LINP1 expression. **D,** Changes in expressions of molecular markers of the AMPK signaling pathway detected *via* Western blotting assay after interference in STAT1 expression.

cific DNA promoters and exert vital biological effects in the whole process of tumors[18]. STAT1 facilitates the transcription of LINC00467 in the case of lung adenocarcinoma, thereby promoting the tumor metastasis[19]. In this research, it was verified by *in-vitro* experiments that there was a transcriptional regulatory relationship between STAT1 and LINP1, but whether STAT1 directly conjugates with the promoter region of LINP1 was not testified. These assumptions will be observed through chromatin immunoprecipitation in subsequent studies.

As a serine/threonine protease, AMPK consists of 3 subunits, namely AMPKα, AMPKβ, and AMPKγ, and serves as a major regulator of energy metabolism in cells and organisms. AICAR can repress the proliferation and migration and induce the apoptosis of PTC cells (TPC-1 and BCPAP) by activating AMPK, and reverse the epithelial-mesenchymal transition of TPC-1 [20,21]. Furthermore, lncRNAs including lncRNA SPRY4-IT1, as critical regulatory factors, are capable of accelerating the formation of malignant tumor phenotype by regulating the AMPK signaling pathway[22]. It was found in this research that LINP1 could facilitate the proliferation and inhibit the apoptosis of PTC by inhibiting AMPK signaling pathway.

Conclusions

In summary, LINP1 may become a potential new target of the treatment and prognosis of PTC, but its molecular mechanism of action needs to be further explored and verified.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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