European Review for Medical and Pharmacological Sciences 2020; 24: 6818-6824

Linc00324 promotes the progression of papillary thyroid cancer *via* regulating Notch signaling pathway

J.-F. WAN[1], J.-Y. WAN[2], C. DONG[3], L. LI[4]

[1]Department of Internal Medicine, Taian Dongping County People's Hospital, Taian, China
[2]Department of General Surgery, Taian City Central Hospital, Taian, China
[3]Department of Hemodialysis Room, Taian City Central Hospital, Taian, China
[4]Department of Operation Room, Taian Dongping County People's Hospital, Taian, China

Abstract. – OBJECTIVE: To explore the expression of linc00324 in papillary thyroid cancer (PTC) and its effect on the biological function of PTC cells.

PATIENTS AND METHODS: A total of 60 pairs of PTC and para-carcinoma normal tissues surgically excised were collected. The expression of linc00324 in PTC tissues and cells was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), and the expression of linc00324 in PTC cells was silenced using the small-interfering RNA (siRNA). Then, the effects of linc00324 on the PTC cell proliferation, apoptosis, and cycle and the downstream Notch signaling pathway were determined *via* methyl thiazolyl tetrazolium (MTT) assay, colony formation assay, flow cytometry, and Western blotting, respectively.

RESULTS: The expression of linc00324 was upregulated in 48 out of 60 cases of PTC tissues, and it was increased in PTC cells compared with that in human thyroid follicular epithelial cells Nthy-ori 3-1. The results of MTT assay and colony formation assay showed that the proliferation of PTC cells declined after interference in linc00324 expression. The findings of flow cytometry revealed that the cell cycle was arrested in G1/G0 phase with a higher apoptosis rate in si-linc00324 group compared with that in the si-NC group. According to the data of Western blotting, the molecular markers for the downstream Notch signaling pathway were altered after interference in linc00324 expression.

CONCLUSIONS: The expression of linc00324 is significantly increased in PTC tissues and cells. Silencing linc00324 may inhibit the proliferation of PTC cells, arrest the cell cycle in G1/G0 phase, and promote the apoptosis by inhibiting the Notch signaling pathway.

Key Words:
Linc00324, PTC, Biological function, Notch signaling pathway.

Introduction

Papillary thyroid cancer (PTC) originates from follicular or parafollicular thyroid cells, and it is one of the most common malignant tumors of endocrine organs. The morbidity rate of PTC ranks 5th in female malignant tumors[1], and rapidly increases around the world in the past few decades[2]. At present, the clinical treatment means of PTC include surgical resection, radioactive I^{131} and combination drug therapy. Although the prognosis and long-term survival are satisfactory in most patients with PTC after treatment, they are prone to advanced and recurrent PTC, so it is important to study its specific pathogenesis to guide the new treatment methods[3]. There is evidence[4] showing that in addition to environmental and genetic susceptibility factors, the changes in genetic inheritance may play a certain role in the occurrence and development of PTC.

It is well known that long non-coding RNAs (lncRNAs) are RNAs with more than 200 nucleotides in length, without the protein translation function. Recently, more and more studies[5-7] have demonstrated that lncRNAs play an important role in the progression of various tumors. Lu et al[8] reported that in pancreatic cancer, the highly expressed lncRNA TUG1 regulates the expression of ITGB1 by "adsorbing" miR-29c, thereby promoting proliferation and metastasis of pancreatic cancer cells. In esophageal cancer, linc01234 facilitates the proliferation and metastasis of cancer cells by regulating the epithelial-mesenchymal transition[9].

The abnormal expression of lncRNAs is closely related to the occurrence and development of PTC. Of note, lncRNA SNHG12 promotes PTC proliferation and metastasis by regulating the

Corresponding Author: Ling Li, BM; e-mail: 1164955439@qq.com

Wnt/β-catenin pathway[10]. Qin et al[11] found that lncRNA GAS8-AS1 is upregulated in PTC and inhibits the PTC cell proliferation, whose potential molecular mechanism is the ATG5-mediated autophagy. However, the expression and function of linc00324 in PTC have not been reported yet. It was found in this study that linc00324 was upregulated in PTC tissues and cells, and plays a similar role to the "oncogene".

Patients and Methods

Tissue Specimens

A total of 60 pairs of human PTC tissue and corresponding para-carcinoma tissue specimens were collected from patients who underwent surgical resection in Taian Dongping County People's Hospital from October 2016 to March 2018. All patients were pathologically diagnosed after operation, and the tissue specimens were collected during operation and immediately cryopreserved. All patients signed the informed consent about the clinical data used for research purpose, and the research was approved by Ethics Committee of the Taian Dongping County People's Hospital.

Cell Culture

The normal human thyroid follicular epithelial cell Nthy-ori 3-1 and PTC cells (K1, TPC-1, BCPAP, KTC-1) were purchased from the Cell Bank, Chinese Academy of Sciences (Shanghai, China), and they were cultured using the Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and 1% double antibodies under 95% O_2, 5% CO_2 and 37°C. When more than 80% of cells were fused, routine passage was done.

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

The total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions, and 20 μL of RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit (MBI, Shanghai, China). Then, qRT-PCR was performed for the expression of linc00324 using the SYBR Premix Ex Taq (Thermo Fisher Scientific, Waltham, MA, USA) and 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Next, the expression of linc00324 in PTC and para-carcinoma tissues and PTC cells was calculated using $2^{-\Delta\Delta Ct}$. The primer sequences were as follows: Linc00324: F 5'-CCTAAACGAACAG AAGCAAC TCC-3', R 5'-GTGTCTGTTTC-CTACTCCATGA-3'; GAPDH F 5'-GGGTGGA-GC CAAACGGGTC-3', R 5'-GGAGTTGCTGT-TG AAGTCGCA-3'.

RNA Interference

PTC cells were transfected using Liposome 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. At 48 h after transfection, the cell proliferation, cycle and apoptosis assays were performed. The interference sequences (si-linc00324 #1: F 5'-GCCCAAAUACCCAGAG-GUUTT-3', R 5'-AACCUCUGGGUAUUUGG-GCTT-3'; si-linc00324 #2: F 5'-UUCUCCGA-ACGUGUCACGUTT-3', R 5'-ACGU GACAC-GUUCGGAGAATT-3'; si-linc00324 #3: 5'-GA-AGAAGAGAAGAGA GAAA-3'; si-linc00324 #3: 5'-GATAAGAAGTCCACTCACA-3') were synthesized by Shanghai RIBOBIO (Shanghai, China).

Cell Proliferation Assay

Cell proliferation was detected *via* methyl thiazolyl tetrazolium (MTT) assay. The cells in each group were inoculated into a 96-well plate and added with 10 μL of MTT reagent at 0, 1, 2, 3, and 4 d for incubation for 4 h. Then, 100 μL of dimethyl sulfoxide (DMSO) was added into each well and shaken for 10 min to fully dissolve the crystals. Finally, the optical density was measured using a microplate reader, with three replicates in each group.

Colony Formation Assay

The cells in the experimental group and control group were inoculated into a 6-well plate (600 cells/well), and routinely cultured for 10-14 d until there were colonies visible to the naked eye. After washing with phosphate-buffered saline (PBS) for 3 times, the colonies were fixed with methanol for 15 min, washed with PBS for 3 times, and stained with 0.1% crystal violet dye at room temperature for 30 min.

Cell Cycle

The cells in the experimental group and control group were collected, fixed with pre-cooled 75% ethanol (700 mL/L) at 4°C overnight, and

stained with propidium iodide (PI) (0.005 mg/mL) and RNase (2 mg/mL) at room temperature for 30 min. Finally, the cell cycle distribution (G1/G0, S, and G2/M phase) was analyzed *via* flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Apoptosis

The cells in the experimental group and control group were digested into single cell suspension, and the cell concentration was adjusted to 5×10^5 cells/mL. 1 mL of cells was taken and centrifuged at 1000 rpm and 4°C for 10 min, and the supernatant was discarded. Then, 1 mL of cooled PBS was added and gently shaken, followed by centrifugation at 1000 rpm and 4°C for 5 min. After the supernatant was discarded, the cells were resuspended in 200 μL of binding buffer, added with 10 μL of Annexin V-FITC and 5 μL of PI, and evenly mixed, followed by reaction at room temperature for 15 min. Finally, the cells were detected using flow cytometry within 1 h.

Western Blotting

At 48 h after transfection, the cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (ZOMANBIO, Beijing, China) and centrifuged at 12000 rpm and 4°C for 10 min, and the supernatant was collected. Then, the protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (MultiSciences, Hangzhou, China). After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, sealed at room temperature for 2 h, incubated with Notch-1, survivin, CyclinD-1, and β and lin antibodies (1:1000, ABclonal, Woburn, MA, USA) at 4°C overnight, and incubated again with secondary antibody at room temperature for 1-2 h. Finally, the protein bands were detected using the fluorescence chemical FC2 imaging system.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for data processing. Measurement data were expressed as mean ± standard deviation. The differences between the 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). $p<0.05$ suggested the statistically significant difference.

Results

Linc00324 Was Upregulated in PTC

A total of 60 cases of tissue specimens were collected from patients pathologically diagnosed with PTC, and the RNA was extracted and reversely transcribed into cDNA. The relative expression of linc00324 was detected *via* qRT-PCR. The results revealed that the expression of linc00324 was upregulated in 48 cases of PTC tissues compared with that in para-carcinoma tissues (Figure 1A). Then, the relative expression of linc00324 in PTC cells was detected using qRT-PCR, and the results showed that the expression of linc00324 was upregulated in PTC cells (Figure 1B). To study the biological effects of linc00324 in PTC, specific interference sequences were designed and transfected into PTC cells using Liposome 2000, and the interference efficiency was determined using qRT-PCR (Figure 1C and 1D).

Effect of Si-linc00324 on PTC Cell Proliferation

The effect of si-linc00324 on the PTC cell proliferation was first studied *via* MTT assay. It was found that the proliferation curve had a difference at 48 h in si-linc00324 group compared with that in the si-NC group (Figure 2A and 2B). Then, the expression of linc00324 was silenced in PTC cells, and the colony formation assay manifested that the PTC cell proliferation was inhibited (Figure 2C and 2D).

Linc00324 Exerted Biological Functions Through Regulating Notch Signaling Pathway

To explore the effect of linc00324 on PTC cell cycle distribution, si-linc00324 and si-NC were transiently transfected into PTC cells. After 48 h, cells were collected and the cell cycle distribution was detected using flow cytometry. It was found that the cell cycle was arrested in G1/G0 phase in si-linc00324 group compared with that in the si-NC group (Figure 3A and 3B). After interference in linc00324 expression, the apoptosis rate was significantly increased (Figure 3C and 3D). Then, the potential molecular mecha-

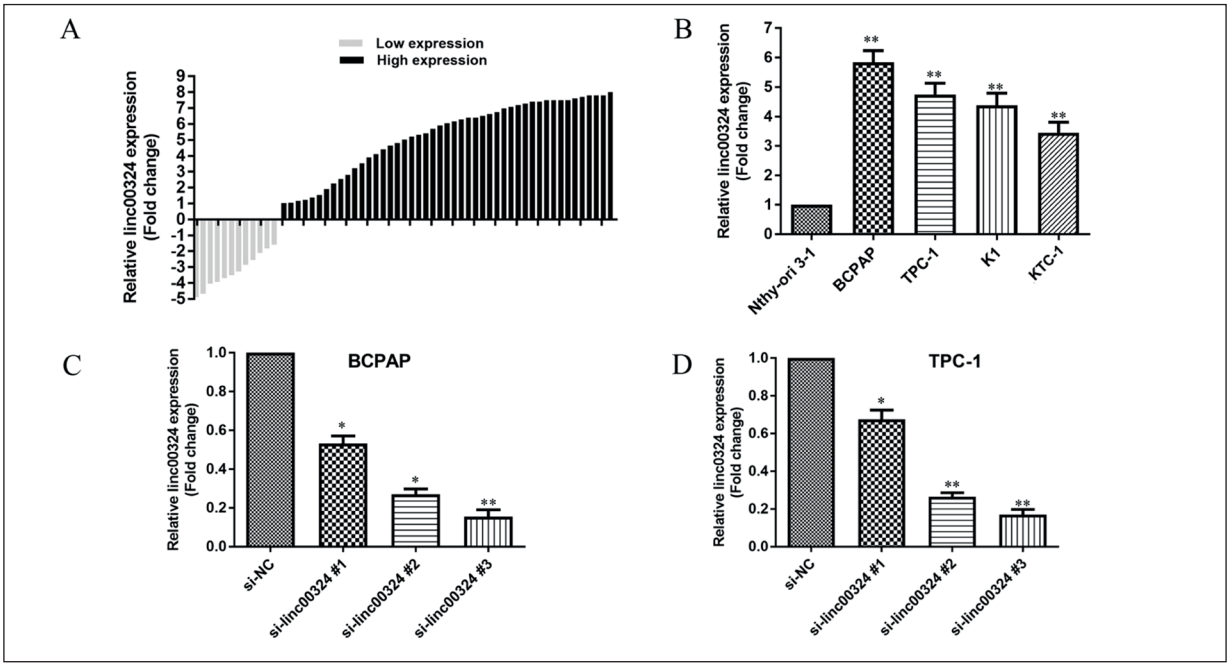

Figure 1. Linc00324 expression is up-regulated in PTC. **A,** Expression of linc00324 is detected *via* qRT-PCR in 60 pairs of PTC and para-carcinoma tissues, and it is found that linc00324 is up-regulated in 48 cases of PTC tissues. **B,** Expression of linc00324 is detected *via* qRT-PCR in PTC cells, with GAPDH as an internal reference. **C,** Interference efficiency of si-linc00324 in BCPAP cells is determined using qRT-PCR. **D,** Interference efficiency of si-linc00324 in TPC-1 cells is determined using qRT-PCR.

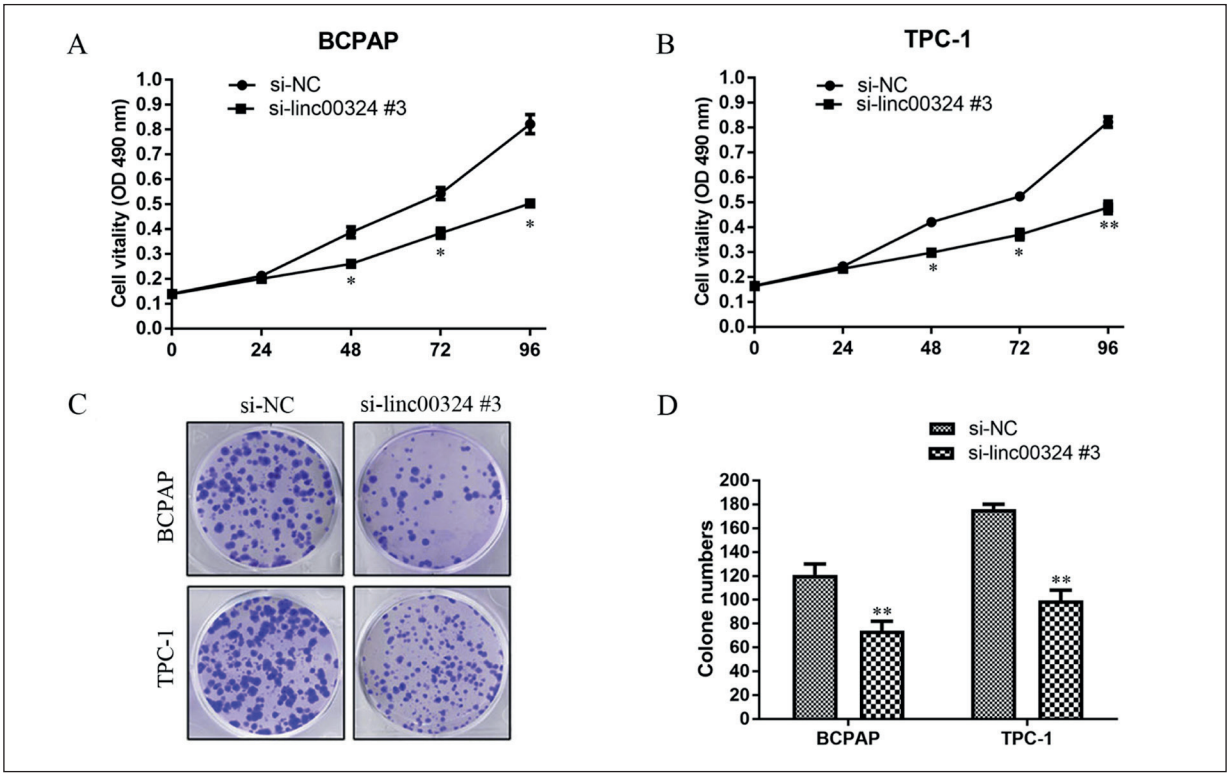

Figure 2. Effect of si-linc00324 on PTC cell proliferation. **A-B,** After interference in the expression of linc00324, the changes in the proliferation of PTC cells are detected *via* MTT assay. **C-D,** After interference in the expression of linc00324, the changes in the proliferation of PTC cells are detected *via* colony formation assay (magnification: 40×).

nism of the biological function of linc00324 was further explored, and the molecular markers for the Notch signaling pathway were determined through Western blotting. It was observed that the molecular markers for the Notch signaling pathway were decreased at the protein level after interference in linc00324 expression (Figure 3E and 3F).

Discussion

Although thyroid cancer mostly develops slowly, there is still a risk of development into more aggressive and fatal thyroid cancer in some cases. Therefore, deeply studying the potential molecular mechanism of PTC has important significance in

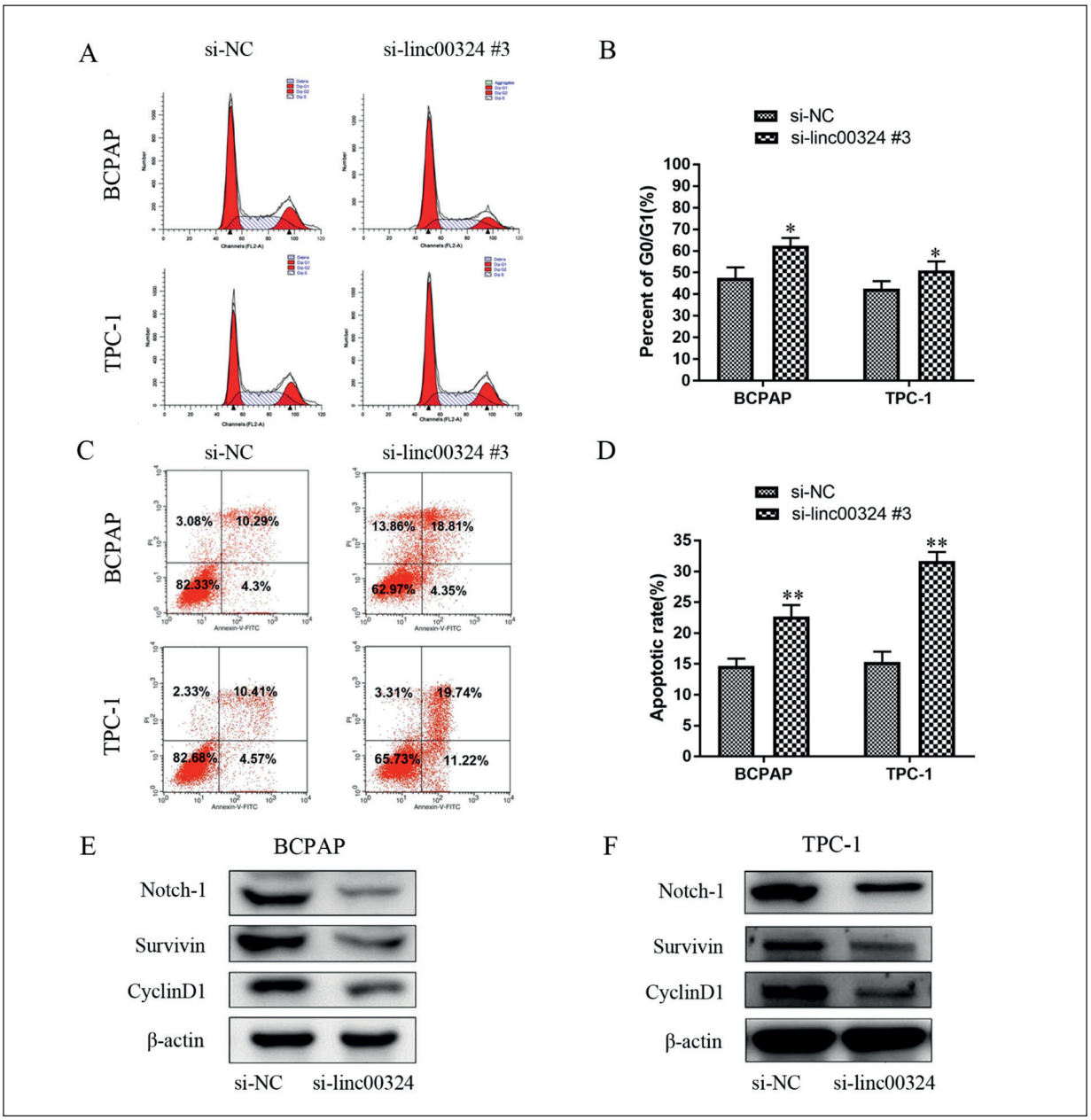

Figure 3. Linc00324 exerts biological functions through regulating the Notch signaling pathway. **A-B,** Results of flow cytometry show that after interference in the expression of linc00324 in PTC cells, the cell cycle is arrested in G1/G0 phase. **C-D,** Compared with that in si-NC group, the apoptosis rate is increased in si-linc00324 group. **E-F,** After knockdown of the expression of linc00324, the changes in the molecular markers for the Notch signaling pathway are determined through Western blotting.

the early screening of predictive biomarkers and clinical development of targeted drugs.

It is reported in the literature[12] that lncRNAs play a definite role in the tumor progression, including cell growth, survival, and metastasis. LncRNAs can also be used as the molecular scaffold, miRNA sponge, and inducible protein to regulate the gene expression and protein function[13-15], and the advantages of lncRNAs as biomarkers for cancer diagnosis and prognosis have been confirmed by many studies[16]. However, the mechanism of lncRNAs in tumorigenesis has not been fully clarified yet. With the development of molecular biological technique, the diagnostic significance of lncRNAs in tumors has been revealed in a few studies. Lu et al[17] reported that the upregulation of ANRIL has a high diagnostic value for lung cancer. There are also reports that HOTAIR, PCAT1, MALAT1, and FAL1 are associated with a variety of human cancers, but their roles and mechanisms in thyroid cancer remain unclear.

The role of linc00324 was explored due to the abnormal expression of lncRNAs in PTC. In this study, the results of qRT-PCR manifested that the expression of linc00324 was upregulated in PTC. It was also found that linc00324 was able to regulate the PTC cell proliferation, cycle, and apoptosis. The Notch signaling pathway is involved in such important processes as hematopoiesis and heart development[18,19], and its abnormal activity is closely related to tumors, autoimmune diseases, and congenital diseases[20,21]. LncRNAs can serve as important regulators to facilitate the occurrence and development of tumor by regulating the Notch signaling pathway, such as lncRNA XIST[22]. In this study, the *in vitro* experiments demonstrated that linc00324 promoted cell proliferation and inhibited apoptosis in PTC *via* suppressing the Notch pathway.

Conclusions

Summarily, the expression of linc00324 is significantly increased in PTC tissues and cells. Silencing linc00324 may inhibit the proliferation of PTC cells, arrest the cell cycle in G1/G0 phase, and promote the apoptosis by inhibiting the Notch signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018; 68: 7-30.
2) Yan DG, Liu N, Chao M, Tu YY, Liu WS. SP1-induced upregulation of long noncoding RNA LINC00313 contributes to papillary thyroid cancer progression via the miR-422a. Eur Rev Med Pharmacol Sci 2019; 23: 1134-1144.
3) Hay ID, Lee RA, Davidge-Pitts C, Reading CC, Charboneau JW. Long-term outcome of ultrasound-guided percutaneous ethanol ablation of selected "recurrent" neck nodal metastases in 25 patients with TNM stages III or IVA papillary thyroid carcinoma previously treated by surgery and 131I therapy. Surgery 2013; 154: 1448-1454, discussion 1454-1455.
4) Markovic I, Goran M, Besic N, Buta M, Djurisic I, Stojiljkovic D, Zegarac M, Pupic G, Inic Z, Dzodic R. Multifocality as independent prognostic factor in papillary thyroid cancer - A multivariate analysis. J BUON 2018; 23: 1049-1054.
5) Yang ZT, Li Z, Wang XG, Tan T, Yi F, Zhu H, Zhao JP, Zhou XF. Overexpression of long non-coding RNA ZXF2 promotes lung adenocarcinoma progression through c-Myc pathway. Cell Physiol Biochem 2015; 35: 2360-2370.
6) Guo X, Wei Y, Wang Z, Liu W, Yang Y, Yu X, He J. LncRNA LINC00163 upregulation suppresses lung cancer development though transcriptionally increasing TCF21 expression. Am J Cancer Res 2018; 8: 2494-2506.
7) Fang S, Shen Y, Chen B, Wu Y, Jia L, Li Y, Zhu Y, Yan Y, Li M, Chen R, Guo L, Chen X, Chen Q. H3K27me3 induces multidrug resistance in small cell lung cancer by affecting HOXA1 DNA methylation via regulation of the lncRNA HOTAIR. Ann Transl Med 2018; 6: 440.
8) Lu Y, Tang L, Zhang Z, Li S, Liang S, Ji L, Yang B, Liu Y, Wei W. Long noncoding RNA TUG1/miR-29c axis affects cell proliferation, invasion, and migration in human pancreatic cancer. Dis Markers 2018; 2018: 6857042.
9) Ghaffar M, Khodahemmati S, Li J, Shahzad M, Wang M, Wang Y, Li C, Chen S, Zeng Y. Long non-coding RNA LINC01234 regulates proliferation, invasion and apoptosis in esophageal cancer cells. J Cancer 2018; 9: 4242-4249.
10) Ding S, Qu W, Jiao Y, Zhang J, Zhang C, Dang S. LncRNA SNHG12 promotes the proliferation and metastasis of papillary thyroid carcinoma cells through regulating wnt/β-catenin signaling pathway. Cancer Biomark 2018; 22: 217-226.
11) Qin Y, Sun W, Zhang H, Zhang P, Wang Z, Dong W, He L, Zhang T, Shao L, Zhang W, Wu C. LncRNA GAS8-AS1 inhibits cell proliferation through ATG5-mediated autophagy in papillary thyroid cancer. Endocrine 2018; 59: 555-564.
12) Qi C, Xiaofeng C, Dongen L, Liang Y, Liping X, Yue H, Jianshuai J. Long non-coding RNA MACC1-AS1 promoted pancreatic carcinoma progres-

sion through activation of PAX8/NOTCH1 signaling pathway. J Exp Clin Cancer Res 2019; 38: 344.

13) Yang H, Kan QE, Su Y, Man H. Long non-coding RNA CASC2 improves diabetic nephropathy by inhibiting JNK pathway. Exp Clin Endocrinol Diabetes 2019; 127: 533-537.

14) Jiang R, Zhao C, Gao B, Xu J, Song W, Shi P. Mix-omics analysis of breast cancer: long non-coding RNA linc01561 acts as ceRNA involved in the progression of breast cancer. Int J Biochem Cell Biol 2018; 102: 1-9.

15) Yang M, Tian J, Guo X, Yang Y, Guan R, Qiu M, Li Y, Sun X, Zhen Y, Zhang Y, Chen C, Li Y, Fang H. Long noncoding RNA are aberrantly expressed in human papillary thyroid carcinoma. Oncol Lett 2016; 12: 544-552.

16) Khorkova O, Hsiao J, Wahlestedt C. Basic biology and therapeutic implications of lncRNA. Adv Drug Deliv Rev 2015; 87: 15-24.

17) Lu Y, Zhou X, Xu L, Rong C, Shen C, Bian W. Long noncoding RNA ANRIL could be transactivated by c-Myc and promote tumor progression of non-small-cell lung cancer. Onco Targets Ther 2016; 9: 3077-3084.

18) Locatelli M, Curigliano G. Notch inhibitors and their role in the treatment of triple negative breast cancer: promises and failures. Curr Opin Oncol 2017; 29: 411-427.

19) Zhou XL, Liu JC. Role of Notch signaling in the mammalian heart. Braz J Med Biol Res 2014; 47: 1-10.

20) Chu D, Zhang Z, Zhou Y, Wang W, Li Y, Zhang H, Dong G, Zhao Q, Ji G. Notch1 and Notch2 have opposite prognostic effects on patients with colorectal cancer. Ann Oncol 2011; 22: 2440-2447.

21) Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. Nat Rev Clin Oncol 2015; 12: 445-464.

22) Wang X, Zhang G, Cheng Z, Dai L, Jia L, Jing X, Wang H, Zhang R, Liu M, Jiang T, Yang Y, Yang M. Knockdown of lncRNA-XIST suppresses proliferation and TGF-beta1-induced EMT in NSCLC through the Notch-1 pathway by regulation of miR-137. Genet Test Mol Biomarkers 2018; 22: 333-342.