LncRNA RGMB-AS1 is activated by E2F1 and promotes cell proliferation and invasion in papillary thyroid carcinoma

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Abstract. – OBJECTIVE: To detect the expression of long non-coding ribonucleic acid (lncRNA) RGMB-AS1 in papillary thyroid carcinoma (PTC) and to investigate its influences on PTC cell biological behaviors and its relevant molecular mechanisms.

PATIENTS AND METHODS: The expression levels of lncRNA RGMB-AS1 in human PTC tissues, corresponding normal tissues, normal thyroid epithelial cells Nthyori3-1, human PTC cells TPC-1, BCPAP and K1 were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Si-RGMB-AS1 and control sequence were transfected into TPC-1 and K1 cells. Changes in cell proliferation were detected via cell counting kit 8 (CCK8) assay, and changes in cell migration and invasion capacities were detected by transwell assay. Bioinformatics software was used to predict that the transcription of lncRNA RGMB-AS1 was regulated by the transcription factor E2F1, and changes in lncRNA RGMB-AS1 expression were detected by qRT-PCR after interference in E2F1 expression. Moreover, changes in cell biological functions were detected by CCK8 and transwell assays. Finally, chromatin immunoprecipitation (CHIP) assay was used to detect whether E2F1 bound to lncRNA RGMB-AS1 promoter region.

RESULTS: Results of qRT-PCR showed that the lncRNA RGMB-AS1 expression was up-regulated in 38 out of 48 cases of PTC tissues, and it was also up-regulated in PTC cells. Results of CCK8 assay showed that the proliferation capacity of PTC cells was decreased after interference in the expression of lncRNA RGMB-AS1, and results of transwell assay revealed that cell invasion and migration capacities were inhibited. qRT-PCR showed that after interference in E2F1, the expression of lncRNA RGMB-AS1 was down-regulated. Besides, CCK8 and transwell assays showed that proliferation, migration, and invasion capacities of PTC cells were decreased after interference in E2F1. Results of CHIP assay showed that E2F1 bound to lncRNA RGMB-AS1 promoter region.

CONCLUSIONS: E2F1 promotes the transcription of IncRNA RGMB-AS1 in PTC. Highly-expressed IncRNA RGMB-AS1 promotes the proliferation, invasion, and migration of PTC.

Key Words: Papillary thyroid carcinoma, lncRNA RGMB-AS1, E2F1, Proliferation, Invasion, Migration.

Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid’s malignant tumor. Its incidence accounts for 90% of all thyroid cancers, and it is one of the cancers with the fastest-growing number of patients. In most cases, the overall prognosis of patients with PTC is relatively good after surgical resection combined with radioactive iodine and levothyroxine. However, 10%-15% PTC patients are still prone to relapse and distant metastasis, showing poor prognosis. Therefore, it is urgent to understand the potential molecular mechanism of pathogenesis of PTC. Long non-coding ribonucleic acid (lncRNA) was first discovered during the large-scale sequencing process of mouse genomic complementary deoxyribonucleic acid (cDNA) library in 2002. It is a type of RNA with more than 200 nucleotides in length and does not have the protein-encoding function. However, it can regulate the gene expression at the pre-transcriptional, transcriptional and post-transcriptional levels. LncRNA has been a research hotspot in the field of oncology; it has been reported in the literature that lncRNA is involved in the occurrence, development, drug resistance, relapse, and metastasis of tumors. Therefore, studying the function and potential molecular mechanism of lncRNA can provide an important scientific.
basis for the diagnosis and treatment of diseases in the future, especially tumors. LncRNA RGMB-AS1 is located on chromosome 5q15 with a full-length of 2053 bp. So far, there have been few reports on lncRNA RGMB-AS1 in tumors. At first, Li et al found that the expression level of lncRNA RGMB-AS1 is up-regulated in non-small cell lung cancer (NSCLC), and highly-expressed lncRNA RGMB-AS1 is positively correlated with the tumor differentiation degree, tumor-node-metastasis (TNM) staging and lymph node metastasis in NSCLC patients. After that, the group further studied the biological function of IncRNA RGMB-AS1 and found that interference in the expression of lncRNA RGMB-AS1 can inhibit cell proliferation, invasion, and migration of NSCLC. However, there have been no reports on the expression and biological function of IncRNA RGMB-AS1 in PTC. We found for the first time via in-vitro experiments that the expressions of lncRNA RGMB-AS1 are relatively high in PTC tissues and cells, and knockdown of its expression can inhibit proliferation, invasion, and migration of PTC cells. According to literature reports, some transcription factors can promote or inhibit the expression of lncRNA. For example, Wang et al found that in gastric cancer SP1 can promote the transcription of lncRNA UCA1, further activating the AKT signaling pathway, thus promoting cell proliferation. We found by bioinformatics that the transcription factor E2F1 could bind to the lncRNA RGMB-AS1 promoter region, and the expression of lncRNA RGMB-AS1 could be inhibited after interference in E2F1. Furthermore, chromatin immunoprecipitation (CHIP) assay also confirmed that E2F1 could bind to the lncRNA RGMB-AS1 promoter region.

**Patients and Methods**

**Tissue Specimens and Cells**

PTC tissues and para-carcinoma normal tissues resected from PTC patients in our hospital were taken as specimens. Normal tissues were further pathologically detected to confirm that no tumor cells were contained. All specimens were collected during operative treatment for patients, and immediately cryopreserved in liquid nitrogen and stored at -80°C for standby application. This study was approved by the Human Experimentation and Ethics Committee of our hospital, and all patients signed the informed consent before experiments. Human PTC cell lines TPC-1, BCPAP, K1 and human thyroid epithelial cell lines Nthyori3-1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The above cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a humid incubator with 5% CO2 at 37°C.

**RNA Extraction and qRT-PCR**

The total RNA was extracted from tumor tissues or cell specimens according to instructions of TRIzol. After the total RNA concentration was measured, RNA was reversely transcribed into cDNA according to instructions of the RT kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was used as a template of SYBR Premix × E × TaqTM qPCR. qPCR conditions are as follows: 95°C for 10 min, 95°C for 15 s, 60°C for 30 s (40 cycles). With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative expression level of the target gene was calculated using the 2–ΔΔCT method.

**Small-interfering RNA (siRNA) and Primers**

The interference sequence of lncRNA RGMB-AS1 is: 1# 5’-CAACTCCAACCTCCACCTC TTACCTT-3’, 2# 5’-GGCTCAATTACCGACACAGTGTAAG-3’, 3# 5’-CACCCTGTACATGGAACATTTAAC ATGTTATTAT-3’. The interference sequence of E2F1 is: 5’-AAAGACTCTGAATGCTAGTGCTTGATGCT-3’. The forward and reverse primer sequences of qRT-PCR is: lncRNA RGMB-AS1 F-CATGTGCACTGATCCATCCATCG, lncRNA RGMB-AS1 R-CATCATAGACCGACCTGTCA, GAPDH F-GGGAGGCAAAGGGTCAT, GAPDH R-GAGTCTTTCCACGGATACCAA. The above sequences were all designed and synthesized by Invitrogen (Carlsbad, CA, USA).

**Cell Proliferation Assay**

PTC cells continued to be cultured for 6 h after being transfected with si-RGMB-AS1 and the corresponding negative control. Then, PTC cells were digested, collected and paved onto a 96-well plate at a density of 3 × 103/well. Cell Counting Kit (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) was performed.
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at 0, 24, 48, 72, and 96 h after plating. Before the assay, 100 μL CCK-8 were added into the 96-well plate; then, cells were cultured in the incubator with 5% CO₂ at 37°C for another 4 h. The optical density value of specimens was measured using a microplate analyzer at 490 nm and the curve was drawn.

**Transwell Assay**

0.05 g/L BD Matrigel (Franklin Lakes, NJ, USA) were diluted at a ratio of 1:8, and 60 μL Matrigel were paved into the upper transwell chamber and air dried at room temperature. The remaining liquid in the culture plate was sucked dry, and 50 μL serum-free culture solution containing 10 g/L of bovine serum albumin (BSA) were added into each well and placed at 37°C for 30 min. Cells in the logarithmic growth phase in control group and treatment group were taken, respectively, and the cell density was adjusted to 1 × 10⁵/mL using the serum-free medium containing 10 g/L BSA. 200 μL cell suspension were added into each chamber, 500 μL culture solution containing 100 mL/L fetal bovine serum (FBS) were added into the lower transwell chamber, and the transwell chamber was placed and incubated for 24 h. After the chamber was taken out, cells were fixed with methanol, stained with crystal violet, observed and photographed under an inverted microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

**Western Blotting**

To analyze the protein expression, cell specimens were lysed using radioimmunoprecipitation assay (RIPA) lysis solution, and centrifuged to collect the total protein specimen. The protein was separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein specimen separated was transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was sealed with 2.5% skim milk powder for 2 h, and incubated with the primary antibody (β-actin, E2F1, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After that, the membrane was washed with Tris-buffered saline and Tween-20 (TBST-20) for 3 times, followed by incubation with sheep anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 2 h. After the membrane was washed with TBST-20 again for 3 times, the color of protein band was developed using a color development system with β-actin as an internal reference protein.

**Chromatin Immunoprecipitation (CHIP)**

CHIP was performed strictly according to instructions of the EZ CHIP KIT Chip kit (Millipore, Billerica, MA, USA): 1) cells were cross-linked using formaldehyde; 2) the chromatin was fragmented via ultrasound; 3) the target antibody and chromatin were co-precipitated; 4) DNA specimens were recycled and tested.

**Statistical Analysis**

All experiments were repeated for 3 times independently. Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, CA, USA). Comparison between groups was done using One-way ANOVA test followed by least significant difference (LSD). *p*<0.05 suggested that the difference was statistically significant.

**Results**

**Expressions of LncRNA RGMB-AS1 Were Up-regulated in PTC Tissues and Cells**

The relative expression levels of LncRNA RGMB-AS1 in 48 cases of PTC tissues were detected by qRT-PCR. Results showed that the relative expression level of LncRNA RGMB-AS1 was up-regulated in PTC tissues compared with that in normal tissues (Figure 1A). Further results of qRT-PCR showed that the expression of LncRNA RGMB-AS1 was up-regulated in the above PTC cell lines compared with that in normal human thyroid epithelial cell Nthyori3-1 (Figure 1B). To study the biological function of LncRNA RGMB-AS1, specific interference sequences were designed and synthesized for transient transfection into TPC-1 and K1 cells, and the transfection efficiency was detected after 48 h (Figure 1C, D).

**Effects of LncRNA RGMB-AS1 on Biological Functions of PTC Cells**

After transfection of si-RGMB-AS1 and si-NC into PTC cells, it was found via CCK8 assay that cell proliferation capacity was significantly inhibited at 48 h after transfection (Figure 2A, B). After cells were treated with the same method, transwell assay was used to detect effects of si-RGMB-AS1 on cell migration and invasion capacities. Results showed that interference in the
expression of lncRNA RGMB-AS1 could inhibit the migration and invasion capacities of PTC cells (Figure 2C, D).

**E2F1 Regulated the Expression of lncRNA RGMB-AS1**

It was found via bioinformatics prediction (http://www.genecards.org/) that E2F1 could bind to the lncRNA RGMB-AS1 promoter region. To verify the prediction results, the interference sequence of E2F1 was first designed and synthesized, and qRT-PCR and Western blotting were performed to detect the interference efficiency (Figure 3A, B). To study whether E2F1 regulated lncRNA RGMB-AS1, qRT-PCR was performed and results revealed that the expression of lncRNA RGMB-AS1 was down-regulated after interference in E2F1 expression (Figure 3C). Furthermore, CHIP assay showed that E2F1 could bind to the lncRNA RGMB-AS1 promoter region. The above results suggest that E2F1 can regulate the lncRNA RGMB-AS1 expression in a targeted manner (Figure 3D).

**Effects of E2F1 on Biological Functions of PTC Cells**

To further investigate the effects of E2F1 on biological functions of PTC cells, E2F1 was
transiently transfected into PTC cells. Results of CCK8 assay revealed that the interference in E2F1 expression could inhibit the proliferation capacity of PTC cells (Figure 4A, B). Transwell assay showed that the interference in E2F1 expression could also inhibit invasion and migration capacities of PTC cell (Figure 4C, D).

**Discussion**

Thyroid cancer is a kind of endocrine tumor, whose occurrence accounts for 95% of endocrine tumors, and ranks first in head-neck tumors. Early clinical symptoms of thyroid cancer are not obvious and hard to be detected. Currently, treatment methods of thyroid cancer are non-surgical and surgical means. The occurrence and development of thyroid cancer are regulated by a variety of genes and signal factors. The traditional diagnosis and treatment methods have great limitations, posing a great challenge to the early diagnosis and treatment of thyroid cancer. Searching small molecular markers for diagnosis and treatment of thyroid cancer is an urgent problem to be solved.

Recent studies have shown that IncRNA is almost involved in all regulatory processes of genes, exerting a variety of functions. The ab-

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**Figure 2.** Biological functions of IncRNA RGMB-AS1. A-B, si-RGMB-AS1 and si-NC are transiently transfected into TPC-1 and K1 cells, followed by observation at 0 h, 24 h, 48 h, 72 h and 96 h. The OD values of experimental group and control group are measured and growth curves are drawn. C-D, After treatment of cells with the same method, the effects of si-RGMB-AS1 on migration and invasion capacities of TPC-1 and K1 cells are detected via transwell assay (**p<0.01, *p<0.05).
normal expression of lncRNA has a certain correlation with tumors. LncRNA can positively and negatively regulate the target genes involved in the major biological processes, so it can serve as the oncogene and tumor suppressor gene. It is reported in the literature that highly-expressed lncRNA AFAP1-AS1 in tongue squamous carcinoma can promote cell proliferation through the regulation of Wnt/β-catenin signaling pathway14. LncRNA LINP1 can play a similar role to the oncogene to promote chemotherapy resistance of breast cancer cells15. Sun et al16 found that lncRNA CRNDE can adsorb miRNA-384 to promote invasion and metastasis of PTC cells. Sheng et al17 reported that the expression of lncRNA RGMB-AS1 is relatively low and plays a similar role to tumor suppressor gene in hepatocellular carcinoma, and the expression level of lncRNA RGMB-AS1 can be used as an independent predictor of prognosis of patients. We found via in-vitro experiments that lncRNA RGMB-AS1 can promote proliferation, invasion, and migration of PTC cells.

The transcription factor E2F is an important regulatory protein of cell cycle. E2F1 is a member of transcription factor E2F family, which can regulate the cell cycle through regulating the transcription of some genomic DNA18. In recent years, studies have found that E2F1 protein is specifically highly expressed in some tumors, such as gastric cancer, pancreatic cancer and NSCLC, and can act as a transcription factor to regulate lncRNA expression, thus promoting tumor growth19-21. In PTC, it was confirmed for the first time by CHIP and qRT-PCR that E2F1 can promote the expression of lncRNA RGMB-AS1, and

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Figure 3. E2F1 regulates the expression of lncRNA RGMB-AS1. A, Detection of si-E2F1 interference efficiency via qRT-PCR. B, Detection of si-E2F1 interference efficiency via Western blotting. C, Detection of lncRNA RGMB-AS1 expression via qRT-PCR after interference in si-E2F1 expression. D, Detection of binding capacity of E2F1 to lncRNA RGMB-AS1 promoter region via CHIP assay (**p<0.01, *p<0.05).

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interference in E2F1 can inhibit proliferation, invasion and migration capacities of PTC cells.

**Conclusions**

We found that transcription factor E2F1 can promote the transcription of lncRNA RGMB-AS1 by binding to its promoter region in PTC. Highly-expressed lncRNA RGMB-AS1 can promote proliferation, invasion, and migration of PTC cells. Targeted drugs for E2F1/lncRNA RGMB-AS1 can provide an important theoretical basis for the clinical reversal of PTC malignant phenotype.

**Conflict of Interest**

The Authors declare that they have no conflict of interest.

**Figure 4.** Biological functions of E2F1. A-B, si-E2F1 and si-NC are transiently transfected into PTC cells, and CCK8 assay is used to detect changes in the proliferation capacity of PTC cells, followed by observation at 0 h, 24 h, 48 h, 72 h and 96 h. C-D, si-E2F1 and si-NC are transiently transfected into PTC cells, and transwell assay is used to detect changes in migration and invasion capacities of TPC-1 and K1 cells (*p<0.01, *p<0.05).

**References**


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