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CircRNA circ-ITCH suppresses papillary thyroid cancer progression through miR-22-3p/CBL/β-catenin pathway

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ABSTRACT

While recent evidence has uncovered that circular RNAs (circRNAs) are vital regulators of carcinogenesis, their role in papillary thyroid cancer (PTC) is not clearly understood. In this study, we reveal that lower levels of circRNA circ-ITCH are expressed in PTC tissues than in normal adjacent tissues. Gain-of-functional assays show that circ-ITCH overexpression suppresses PTC cell proliferation and invasion and promotes apoptosis in vitro. Overexpression of circ-ITCH also leads to impaired tumor growth in vivo. Bioinformatics analysis and luciferase reporter assays demonstrate that circ-ITCH sponges miR-22-3p to upregulate the expression of CBL, an E3 ligase of nuclear β-catenin. Elevated levels of CBL suppress activation of the Wnt/β-catenin pathway and consequently attenuates PTC progression. In summary, our study reveals a novel signaling pathway of circ-ITCH/miR-22-3p/CBL/β-catenin involved in PTC development and progression.

1. Introduction

Thyroid cancer, the most prevalent tumor in the endocrine system, has shown a gradual increase in incidence worldwide [1]. Papillary thyroid carcinoma (PTC) accounts for over 80% of all thyroid cancer cases [2]. While traditional therapeutic methods, including surgery and adjuvant radioactive iodine therapy, make PTC treatment effective and increase the five-year survival rate to about 90% [3], patients with an advanced disease stage or metastasis show poor prognosis [4]. Therefore, determining the molecular mechanism of PTC development and searching for novel therapeutic targets are important endeavors.

Advances in high-throughput RNA sequencing technology have helped identify growing numbers of new noncoding RNAs, including long noncoding RNA and circular RNA (circRNA), in human tissues [5,6]. CircRNAs, which are produced by backsplicing and characterized by a covalently closed loop [7,8], play a critical regulatory function in cellular growth, survival, and differentiation [9]. Many circRNAs are aberrantly expressed in tumor tissues [9], thus indicating their close association with tumorigenesis. For instance, upregulated circRNA HIPK3 contributes to gall bladder cancer progression [10], and increased circRNA hsa_circ_0016788 in liver cancer promotes tumor development by miR-486/CDK4 signaling [11]. The circRNA hsa_circ_0002052 is downregulated in osteosarcoma, leading to activation of Wnt/β-catenin signaling and tumor progression [12]. Unfortunately, although microarray analysis has identified many differentially expressed circRNAs between PTC and normal tissues [13], the function of circRNAs in PTC progression remains largely unknown.

Previous studies reveal that circ-ITCH is involved in liver cancer, colorectal cancer, bladder cancer, glioma, and esophageal squamous cell carcinoma [14]; however, its role in PTC is unclear. In a previous study, we found that ITCH levels decrease in PTC tissues and cell lines. Circ-ITCH overexpression inhibits PTC cell proliferation and invasion while promoting apoptosis in vitro. In terms of mechanism, circ-ITCH overexpression sponges miR-22-3p to upregulate CBL expression, leading to suppression of the Wnt/β-catenin pathway. In the present study, we reveal, for the first time, the novel function of circ-ITCH in PTC and illustrate its functional mechanism.

2. Materials and methods

2.1. Human tissues

Thirty-seven PTC patients and 14 matched non-tumor tissues were collected from The Second Affiliated Hospital, Harbin Medical

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University. All PTC patients had not received any radiotherapy, chemotherapy, or other adjuvant therapies before surgery. The tissues were stored in liquid nitrogen until use. This study was approved by the ethics committee of The Second Affiliated Hospital, Harbin Medical University, and informed consent was collected from all patients prior to their participation in this study.

2.2. Cell culture and transfection

PTC cell lines (K1, IHH4, and TPC1) were purchased from Shanghai Zishi Biotechnology Co., Ltd. (Fengxian District, Shanghai, China), and human normal thyroid Nthy-ori 3-1 cells were obtained from Shanghai Zeye Biotechnology Co., Ltd. (Songjiang District, Shanghai, China). Cells were incubated in Roswell Park Memorial Institute 1640 medium (Gibco Company, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco Company) in 5% CO_2-humidified incubator. Cell transfection with miR-22-3p mimics, inhibitor, or controls was performed using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

2.3. Lentiviral production and transduction

The coding sequence of circ-ITCH was cloned into the lentiviral expression vector pLVX-IRES-neo (Clontech Laboratories Inc., San Francisco, CA, USA), and lentiviral production and transduction were conducted by following previously published procedures [15].

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from frozen tissue samples and fresh cultured cells with TRIzol reagent following the manufacturer's instructions. A reverse transcription kit (PrimeScript RT Master Mix, TaKaRa, Japan) was used to transcribe the qualified total RNA and produce cDNA as previously described [16]. qRT-PCR was performed with SYBR Premix Ex Taq II (TaKaRa, Japan) on a Light-Cycler 480 system (Roche, USA) according to the manufacturers' instructions. GAPDH or U6 was used as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to determine relative quantities of gene expression levels.

2.5. Transwell assay

Cell invasion ability was detected using Transwell permeable supports (Corning, USA) according to the manufacturer's protocol. Briefly, the transfected/treated cells were plated onto a Matrigel-coated membrane in the upper chamber of a 24-well insert containing serum-free medium. The bottom chamber contained DMEM with 10% FBS. Cells were incubated for 48 h after plating. Then, the bottom of the chamber insert was fixed with methanol and stained with 0.5% crystal violet for 15 min. The number of cells invading through the membrane was determined using an inverted microscope.

2.6. Cell proliferation

Cell Counting Kit-8 (CCK8, Dojindo, Japan) was used to evaluate cell proliferation. Briefly, 1×10^4 cells were seeded into 96-well plates, and 10 μl of CCK8 solution was added to each well. The cells were then incubated at 37 °C for 2, and the absorbance of the culture medium at 450 nm was measured daily using a spectrophotometer.

2.7. Flow cytometry apoptosis assay

Apoptosis was detected using flow cytometry following the manufacturer's instructions. Briefly, 4×10^5 cells/ml per well was added to six-well plates. After transfection, cells were washed twice with PBS and then dissociated with trypsin. Thereafter, cells were mixed with 5 μl of Annexin V-FITC and 5 μl of propidium iodide and incubated in the dark. Apoptotic cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) on a FACScan instrument (Becton, Dickinson, NY, USA).

2.8. Luciferase reporter assay

Cells were co-transfected with plasmids containing wild or mutant fragments from circ-ITCH/CBL and miRNA mimics. After 24 h of transfection, firefly and *Renilla* luciferase activities were measured consecutively by using a dual-luciferase reporter assay system (Promega, MA, USA). Finally, the ratios of firefly to *Renilla* luciferase luminescence were calculated.

2.9. Tumor xenograft animal model

Animal experiments were performed according to the guidelines approved by the ethics committee of The Second Affiliated Hospital, Harbin Medical University. A total of 1×10^7 K1 cells were subcutaneously injected into the right flank of 5-week-old specific pathogen-free female BALB/c nude mice. Tumor size was assessed every 5 d. At 30 d post-inoculation, the mice were euthanized, and tumors were removed and weighed. Fresh frozen tumor samples were further subjected to qRT-PCR.

2.10. Statistical analysis

Statistical analyses were performed with SPSS 20.0 software (IBM, Chicago, IL, USA), and the results are presented as mean ± standard deviation. Student's *t*-test or one-way ANOVA was used to determine statistical significance. Correlations were assessed by Pearson correlation and linear regression analyses. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Suppressive effects of circ-ITCH on PTC cells

To analyze the role of circ-ITCH in PTC, we first evaluated its expression through qRT-PCR. Circ-ITCH was lowly expressed in PTC tissues compared with adjacent normal tissues (Fig. 1A). We then compared circ-ITCH levels in 14 pairs of PTC and adjacent normal tissues. Most PTC tissues showed higher expression of circ-ITCH than their normal counterparts (Fig. 1B). Circ-ITCH levels were also consistently lower in PTC cell lines than in Nthy-ori 3-1 cells (Fig. 1C). Moreover, the expression of circ-ITCH was positively associated with clinical stage and lymph node metastasis (Supplementary Table 1). Proportional hazards method analysis also indicated that circ-ITCH is an independent prognostic marker (Supplementary Table 2). To explore the functions of circ-ITCH, we constructed circ-ITCH-overexpressing K1 and TPC1 cell lines (Fig. 1D). CCK8 and Transwell assays revealed that circ-ITCH overexpression significantly inhibits the proliferation and invasion of K1 and TPC1 cells (Fig. 1E–G). Moreover, ectopic expression of circ-ITCH increased K1 and TPC1 cell apoptosis (Fig. 1H). Taken together, the above studies demonstrate that circ-ITCH plays an anti-tumor role in PTC.

3.2. Circ-ITCH interaction with miR-22-3p

To investigate the mechanism of circ-ITCH, we searched for its potential target by bioinformatics analysis and identified a putative binding site for miR-22-3p (Fig. 2A). To confirm the interaction of

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