



MicroRNA-3653-3p inhibited papillary thyroid carcinoma progression by regulating CRIPTO-1

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ABSTRACT

Papillary thyroid carcinoma (PTC) is the most common endocrine malignant tumor and the metastasis of PTC often leads to unfavorable prognosis. Thus, the purpose of the current research was to mainly explore the role of miR-3653-3p in PTC progression. The expression level of miR-3653-3p in PTC was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), and Cell Counting Kit-8 (CCK-8) assay and colony formation assay were recruited to assess the ability of miR-3653-3p on cell proliferation. Next, transwell assay and Matrigel assay were involved to examine the ability of miR-3653-3p on cell migration and invasion. At last, Dual-Luciferase reporter assay and Western blotting were recruited to validate the down-stream target of miR-3653-3p. Results showed that miR-3653-3p was down-expressed in PTC, and upregulated miR-3653-3p inhibited cell proliferation, cell migration, and cell invasion *in vitro*. In addition, CRIPTO-1 was a downstream target of miR-3653-3p, and miR-3653-3p inhibited PTC progression *via* regulating CRIPTO-1. In sum, this research verifies that miR-3653-3p suppresses cell proliferation, migration, and invasion in PTC *via* regulating CRIPTO-1. These findings provide new insight into the underlying mechanism of PTC progression and may be useful in finding biomarkers and therapeutic targets of PTC

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Introduction

Thyroid cancer is a prevalent malignancy that primarily affects the endocrine system, and its incidence has been steadily increasing, with an annual growth rate of approximately 5%. This disease poses a significant threat to human health, especially among women, who experience a higher prevalence compared to men (1). Thyroid cancer can be categorized into differentiated and undifferentiated types, depending on the degree of tumor differentiation. Differentiated thyroid cancer, the most common histological subtype, accounts for approximately 90% of all thyroid cancer cases. Treatment typically involves surgical intervention, followed by adjuvant endocrine therapy or combined chemo-radiotherapy. Fortunately, thyroid cancer generally has a more favorable prognosis compared to other types of malignancies. However, certain patients may present with tumor cell metastasis and invasion or exhibit poor responsiveness to radiotherapy and chemotherapy, leading to suboptimal treatment outcomes (2).

In recent years, there has been a notable advancement in our comprehension of thyroid cancer's pathogenesis (3). Recent research has unveiled a significant breakthrough, revealing that the development and progression of papillary thyroid carcinoma (PTC) are characterized by aberrant microRNA (miRNA) expression patterns. This discovery has ushered in a fresh perspective on the diagnosis and prognosis of thyroid cancer. Studies conducted by Wojcicka et al. have been instrumental in shedding light on the central role played by miRNAs in the pathogenic mechanisms underlying thyroid cancer (4). This burgeoning body

of research highlights the potential of miRNAs as valuable biomarkers for thyroid cancer diagnosis and prognosis. Understanding the intricate miRNA-mediated regulatory networks that govern thyroid cancer pathogenesis could pave the way for more precise diagnostic tools and innovative therapeutic strategies. These insights offer hope for improved patient outcomes and a deeper comprehension of the molecular intricacies of this complex disease.

In our present study, we conducted a quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay to confirm that miR-3653-3p is downregulated in PTC. We observed that the upregulation of miR-3653-3p resulted in the suppression of cell proliferation, migration, and invasion *in vitro*. Furthermore, our investigations revealed that teratocarcinoma-derived growth factor 1 (CRIPTO-1) is a downstream target of miR-3653-3p. Collectively, these findings strongly suggest that miR-3653-3p acts as a suppressor of PTC progression through its regulation of CRIPTO-1.

In conclusion, thyroid cancer is a rapidly increasing malignancy that poses a significant health threat, particularly to women. While treatment options exist, some patients experience poor responses or metastasis, necessitating a deeper understanding of its pathogenesis. Our research highlights the importance of miR-3653-3p in the regulation of PTC, offering a potential avenue for targeted therapies in the future. By elucidating the intricate molecular mechanisms involved in thyroid cancer, we can work towards improving diagnostic accuracy and developing more effective treatments for this challenging disease.

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Materials and Methods

Clinical tissues

The current study was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. All participants signed the informed consent. 45 paired PTC and normal tissue samples were extracted from PTC patients in the Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and these samples were collected in liquid nitrogen immediately after the operation.

Cell culture

The cell lines involved in this study including four PTC cell lines (KTC-1, BCPAP, NPA, TPC-1) and one human normal thyroid cell line (HTORI3) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA) and supplemented by 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO₂.

Cell transfection

Oligonucleotides were purchased from GenePharma (Shanghai, China) for upregulating miR-3653-3p. The plasmid pcDNA-3.1 (GenePharma, Shanghai, China) was used to upregulate CRIPTO-1 and the empty pcDNA-3.1 plasmid was taken as a control. The transfection efficiency was examined by qRT-PCR.

QRT-PCR

The total RNA of tissue specimens and cell lines was extracted through TRIzol reagent (Invitrogen, Carlsbad, CA, USA). All complementary deoxyribose nucleic acids (cDNAs) were synthesized via Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). The miR-3653-3p expression level was assessed through SYBR Green real-time PCR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as a normalization. The primer sequences are listed: miR-3653 forward: 5'-TCTCCCGAGAGACA-TATTT-3', miR-3653-3p reverse: 5'-GATGAGAAGGTA-TGAATCA-3'; U6 forward: 5'-GCTTCGGCAGCACA-TATACTAAAAT-3', U6 reverse: 5'-GCTTCGGCAGCA-CATATACTAAAAT-3'.

Colony formation

The cells (1.0×10^3) were planted into the culture plates (60 mm) and cultured for 2 weeks. Cells on the plates were then washed with phosphate-buffered saline (PBS, Gibco, Rockville, MD, USA) twice and fixed in ice-cold 70% methanol for 15 min and Crystal Violet Staining Solution (Beyotime, Shanghai, China) was used to stain the cell colonies.

Cell-ounting Kit-8 assay (CCK-8)

Transfected cells were planted into 96-well plates (6×10^3 /well) and then, CCK-8 solution (Beyotime, Shanghai, China) (10 µL/well) was used to stain cells for 2 h at 37°C. The optical density (OD) value (450 nm) was then evaluated.

Transwell assay and matrigel assay

Transwell assay was carried out to figure out the invasion ability of transfected PTC cells. Transwell chambers

and 24-well plates were obtained from Corning (Corning, NY, USA). Then, 1×10^5 cells were suspended with serum-free medium (100 µL). In the Matrigel assay, the cells were transferred to the Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated upper chamber. After 36 h, the invasive cells were observed and counted.

Western blotting

The total protein was isolated by radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) and phenylmethanolsulfonyl fluoride (PMSF). Protein lysates separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were then transferred to polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). Then, the membrane was immunostained at 4°C by rabbit anti-CRIPTO-1 (1:1000, CST, Danvers, MA, USA) overnight. Rabbit anti-GAPDH (1:5000, CST, Danvers, MA, USA) was taken as a loading control. At last, protein relative expression level was evaluated by Image J software (NIH, Bethesda, MD, USA).

Statistics analysis

All experiments in this study were performed three times independently at least. All data recorded were exhibited as mean \pm standard deviation (SD). Student's unpaired t-test was used to undergo statistics analysis. In this study, $P < 0.05$ was significant.

Results

MiR-3653-3p was Down-Expressed in PTC

As shown in Figure 1A, it was found that miR-3653-3p was down-expressed in tumor specimens. The miR-3653-3p expression level was also decreased in PTC cells (Figure 1B). It was shown in Figure 1C that miR-3653-3p was downregulated in PTC via the TCGA database. In sum, the above data suggest that miR-3653-3p was down-expressed in PTC.

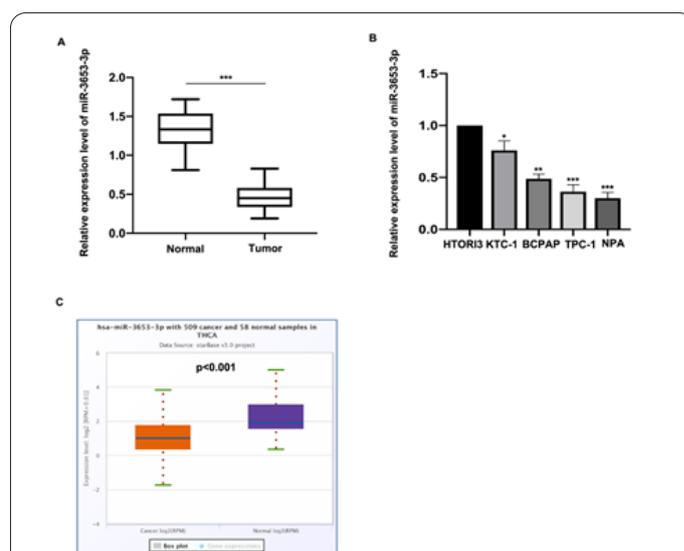


Figure 1. MiR-3653-3p is down-expressed in PTC. (A) The expression level of miR-3653-3p in PTC tissues and normal tissues. (B) Analysis of miR-3653-3p expression level in PTC cell lines. (C) The expression level of miR-3653-3p in PTC tissues from TCGA database. Data are presented as the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

MiR-3653-3p Suppressed Cell Proliferation in PTC Cell Lines

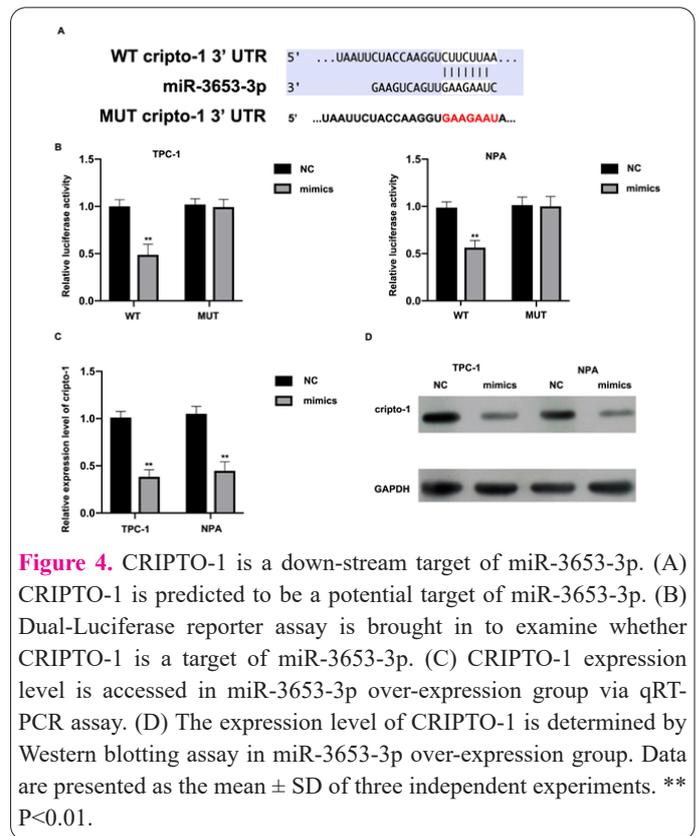
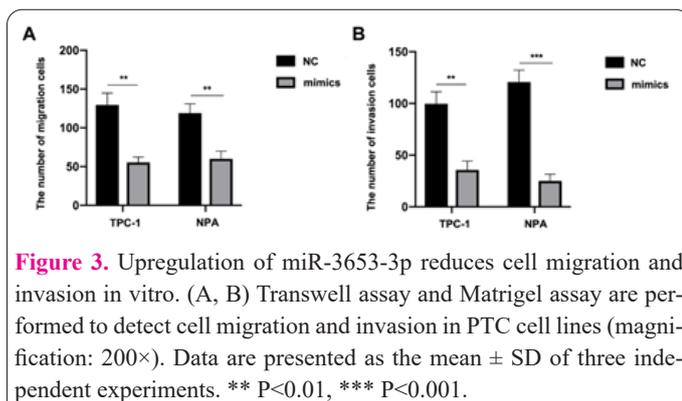
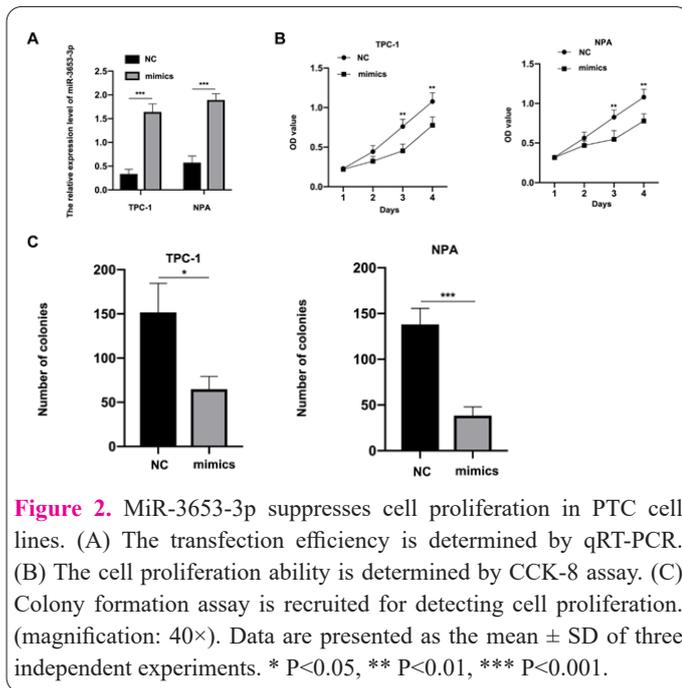
The transfection efficiency was examined by qRT-PCR in TPC-1 and NPA cell lines (Figure 2A). Through CCK8 assay, the over-expression of miR-3653-3p led to a lower OD value compared with control group (Figure 2B). In colony formation assay, upregulated miR-3653-3p generated less colonies when compared with the control group (Figure 2C). In sum, miR-3653-3p inhibited cell proliferation in PTC.

Upregulation of MiR-3653-3p Lessened Cell Migration and Invasion In Vitro

MiR-3653-3p over-expression significantly repressed the ability of cell migration in PTC cell lines (Figure 3A). Besides, the Matrigel assay was involved in examining the ability of miR-3653-3p on cell invasion. Figure 3B shows that miR-3653-3p over-expression inhibits cell invasion in comparison with the NC group. Herein, it was considered that miR-3653-3p suppressed cell migration and invasion in PTC.

CRIPTO-1 was a Down-Stream Target of MiR-3653-3p

Three publicly available databases, namely, TargetScan, Starbase, and MiRanda, were used to predict the downstream target. CRIPTO-1 was indicated to function as a potential target of miR-3653-3p. Through Dual-Lu-



ciferase reporter assay, it was found that over-expression of miR-3653-3p had relatively higher Luciferase activity with wild 3'-UTR region of CRIPTO-1 (Figure 4A and 4B). It was also revealed that CRIPTO-1 was the downstream target of miR-3653-3p. In the over-expressed miR-3653-3p group, the expression of CRIPTO-1 was down-expressed through both qRT-PCR assay and Western blotting (Figure 4C, 4D). In sum, CRIPTO-1 was verified to be a directly down-stream target of miR-3653-3p in PTC.

MiR-3653-3p Inhibited PTC Progression Through Regulating CRIPTO-1

CRIPTO-1 over-expression plasmid was co-transfected in transfected cell lines. It was exhibited in Figure 5A that CRIPTO-1 over-expression cancelled the promotion effect on cell proliferation caused by miR-3653-3p over-expression (Figure 5A). Consistently, similar results emerged in the transwell assay and Matrigel assay (Figure 5B, 5C). Taken together, all the findings mentioned above suggest that miR-3653-3p functions as a tumor suppressor in PTC by regulating CRIPTO-1.

Discussion

PTC is the most common endocrine malignancy, and its incidence has increased in the past decades. According to the current treatment guidelines, PTC has a good prognosis and recurrence is rare (5). Current treatment options include levothyroxine replacement surgery and radioactive iodine. However, in patients with metastatic tumors, therapy is not effective and other more effective treatments are urgently needed (6).

MiRNA is a kind of non-coding RNA with a length of about 22 nt. By identifying the 3'-UTR region of the target gene, miRNA inhibits protein translation or affects mRNA stability, and plays a role in regulating protein expression at the post-transcriptional level (7). MiRNA plays a cru-

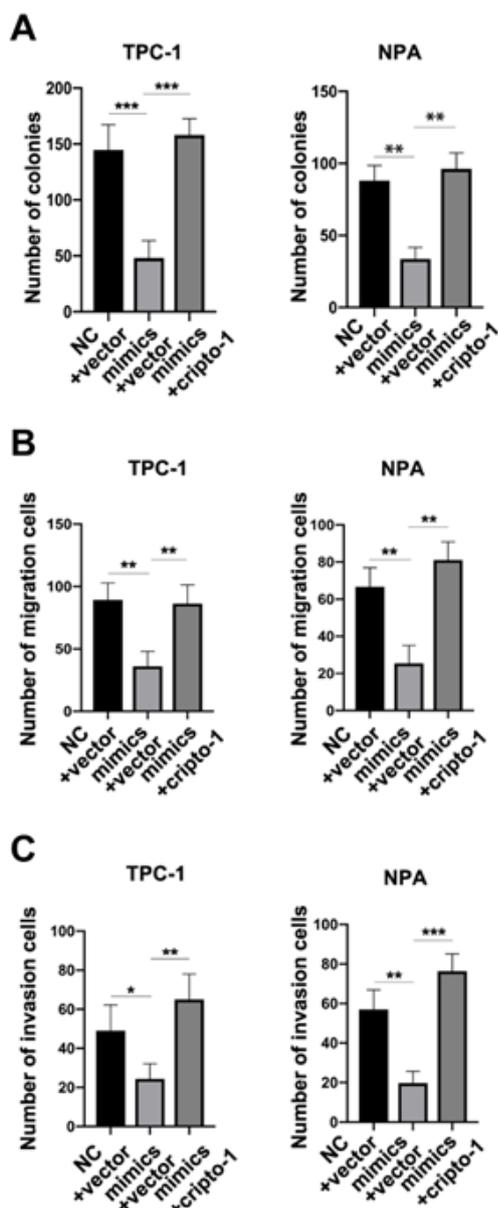


Figure 5. MiR-3653-3p inhibits PTC progression by regulating CRIPTO-1. (A) CRIPTO-1 over-expression cancels the effect of miR-3653-3p up-regulation on cell proliferation (magnification: 40×). (B) CRIPTO-1 over-expression cancels the effect of miR-3653-3p up-regulation on cell migration (magnification: 200×). (C) CRIPTO-1 over-expression cancels the effect of miR-3653-3p up-regulation on cell invasion (magnification: 200×). Data are presented as the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

cial role in diverse functions, such as cell differentiation (8), cell cycle regulation (9), and apoptosis in diseases (10). Bimpaki et al. (11) have reconfirmed the presence of mature miRNA in the nucleus and elucidated the nucleus-cytoplasmic transport mechanisms. By regulating the expression of essential genes, miRNA has become an important regulator of human carcinogenesis. In the past 5 years, a novel miRNA therapy strategy has been developed. miRNA reduction or inhibition treatment and replacement therapy are discovered (12). There are many reports (13,14) evidencing that miRNA can be used as a diagnostic and therapeutic target for human cancer. Several studies (15-19) have shown that multiple miRNAs, including miR-15a, miR-30, miR-222, miR-146, and miR-451a are dysregulated in thyroid cancer tissues, and these

miRNAs are believed to be involved in the occurrence and development of thyroid cancer.

Cripto-1 is an embryonic gene. Cripto-1 protein is expressed in the early stage of embryo development and has the function of regulating cell differentiation and embryo development. Recent studies have shown that cripto-1 is an oncogene and a multifunctional signaling factor that receives multiple signals on the surface of cell membranes, stimulating cell proliferation, migration, survival, epithelial-mesenchymal transformation (EMT), and angiogenesis to regulate embryonic development, tissue homeostasis, and tumorigenesis (20). Bianco et al. (21) found that the plasma cripto-1 level was elevated in patients with colon cancer and breast cancer, which could be used as a detection indicator for the early diagnosis of tumors. In recent years, studies have found that serum cripto-1 is significantly elevated in HCC and NSCLC (22-26). The evidence suggests that cripto-1 may be a novel biomarker for diagnosing tumors and predicting prognosis.

In this research, miR-3653-3p was found to be down-expressed in PTC. Besides, it was determined that upregulation of miR-3653-3p inhibited cell proliferation, migration, and invasion *in vitro*. Furthermore, this study verified that CRIPTO-1 was a downstream target of miR-3653-3p. Data from colony formation assay, transwell assay, and Matrigel assay validated that miR-3653-3p functioned as a tumor suppressor *via* regulating CRIPTO-1 in PTC.

Taken together, this research elucidated that miR-3653-3p suppressed cell proliferation, migration, and invasion in PTC *via* regulating CRIPTO-1. These findings provide new insight into the underlying mechanism of PTC progression and may help find novel biomarkers and therapeutic targets for PTC.

Conflict of interest

The authors declare that they have no conflict of interest.

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