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MiR-296-5p suppresses papillary thyroid carcinoma cell growth via targeting PLK1

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Abstract. – OBJECTIVE: Our study aimed to explore the effects of miRNA-296-5p on the biological behaviors of papillary thyroid carcinoma (PTC) cells and its potential mechanism.

PATIENTS AND METHODS: Twenty-eight PTC tissues and the corresponding non-cancerous tissues were collected. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) analysis was performed to detect the expression levels of miR-296-5p in PTC tissues and the adjacent non-cancerous tissues. Besides, the different endogenous expression levels of miR-296-5p in PTC cell line (K1) and normal thyroid gland cell line (Nthy-ori3-1) were also detected by RT-qPCR. Bioinformatics analysis, Western blot and Dual-Luciferase reporter gene assay were performed to demonstrate whether polo-like kinase 1 (PLK-1) was a downstream target of miR-296-5p. Subsequently, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, flow cytometry analysis, colony formation assay and TUNEL assay were performed to estimate whether PLK1 down-regulation could attenuate the malignant behaviors of PTC cells *in vitro*.

RESULTS: RT-qPCR results showed that the expression level of miR-296-5p was significantly down-regulated in PTC tissues and cells, indicating that miR-296-5p may participate in PTC development. We predicted target genes of miR-296-5p by bioinformatics and identified PLK1 as a target gene of miR-296-5p. By Western blot and Dual-Luciferase reporter gene assay, we confirmed that miR-296-5p was partially complement to PLKI mRNA 3'UTR sequence and inhibited PLK1 expression at the post-transcriptional level. *In vitro* experiments suggested that the transfection of miR-296-5p mimics into K1 cells suppressed cell proliferation, inhibited cell clone formation, arrest the cell cycle in G2/M phase and induced apoptosis. Importantly, PLK1 reversed the inhibitory effects of miR-296-5p on biological behaviors of PTC.

CONCLUSIONS: MiR-296-5p influences the biological behaviors of PTC by regulating PLK1. These findings provide a new perspective for the molecular mechanism of PTC pathogenesis and also contribute to developing new targets and methods for PTC treatment.

Key Words:
Papillary thyroid carcinoma (PTC), MiR-296-5p, Polo-Like Kinase 1 (PLK1), Proliferation, Apoptosis.

Introduction

Thyroid cancer is a common malignant tumor in the head and neck, with an incidence rate accounting for 1% of all the malignant tumors[1]. In recent years, thyroid cancer has become the most common malignant tumor of the endocrine system due to its increasing incidence rate year by year[2]. Pathologically, thyroid cancer can be divided into papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), anaplastic thyroid carcinoma (ATC) and medullary thyroid carcinoma (MTC). Among the four pathological types, PTC accounts for approximately 80-85% of thyroid cancer, which is the most common histologic type in women and children[3,4]. Most of PTC patients can survive after efficacious treatment, with over 80% of 35-year survival or 40-year survival[5]. However, a small percentage of PTC patients who are not sensitive to radioiodine therapy, or accompanied with cervical lymph node metastasis at the time of diagnosis suffer from poor prognosis, with lower than 10% of 10-year survival[6]. Meanwhile, traditional therapeutic measures of PTC also have significant limitations. Hence, it is of important significance

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to study the specific incidence and development process of PTC, investigate the precise molecular mechanism of the malignant behavior of cancer cells and predict the effective targets for biological therapy for PTC. We aim to improve the early diagnostic rate and survival rate of PTC patients to ameliorate their prognosis.

Micro-ribonucleic acids (miRNAs) are a category of highly conserved, small non-coding RNAs with a length of nearly 19-22 nucleotides. They participate in the regulation of target genes at post-transcriptional level by completely or incompletely complementary pairing with 3'-untranslated region (UTR) of the target genes, thereby acting as an anti-oncogene or oncogene in the incidence and development of tumors[7-9]. Multiple miRNAs are abnormally expressed, and promote the incidence and development of PTC[10-13], thus revealing the values of miRNAs in the diagnosis and prognosis of PTC[14].

As a member of the miRNA family, miR-296-5p is involved in the occurrence and development of various tumors, such as prostate cancer[15], gastric cancer[16], and breast cancer[17]. However, few studies on miR-296-5p and its target genes in PTC are reported. This research investigated the impacts of miR-296-5p on the proliferation and apoptosis of PTC cells and explored the feasible mechanism.

Patients and Methods

Tissue Samples and Cell Lines

A total of 28 cases of PTC tissue specimens and para-carcinoma tissue specimens were surgically resected from December 2014 to December 2017 in Yantai Yuhuangding Hospital. Specimens were pathologically diagnosed after surgery. This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital. Signed written informed consents were obtained from all participants before the study. The K1 cell line of PTC and cell line Nthy-ori3-1 of normal thyroid gland were purchased from the Cell Bank of Type Culture Collection Committee of China Academy of Sciences (Shanghai, China). Both K1 and Nthy-ori3-1 cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 15% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in an incubator with 5% CO_2 and saturated humidity at 37°C. Fresh medium was replaced every 2 days, and cells were passaged every 3 days. Cells in the logarithmic phase were harvested for experiments.

Transfection

MiR-normal control (NC), miR-296 mimics or polo-like kinase 1 (PLK1) were transfected into the K1 cells following Lipofectamine 2000 instructions (Invitrogen, Carlsbad, CA, USA). Transfected cells for 24-48 h were harvested for the following experiments. In the *in vitro* experiments, cells were divided into 3 groups: MiR-NC group (negative control), mimics group (K1 cell transfected by miR-296 mimics) and mimics + PLK1 group (K1 cell transfected by miR-296 mimics and si-PLK1).

Dual-Luciferase Reporter Gene Assay

Target genes of miR-296-5p were predicted using target gene prediction software miRBase (http://www.mirbase.org/, TargetScan) and PicTar (http://pictar.mdc-berlin.de/). PLK1 was found to be a possible target gene of miR-296-5p. Plasmids containing wild-type PLK1 Luciferase reporter gene vector (wtPLK1) and mutant PLK1 Luciferase reporter gene vector (mut-PLK1) were constructed. They were co-transfected with miR-296-5p mimics or mimics control, respectively into K1 cells. After 48 hours, cells were washed with phosphate-buffered saline (PBS; Beyotime, Shanghai, China) for determining Luciferase activity according to the Luciferase reporter gene kit.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was extracted from tissue samples preserved in liquid nitrogen and cultured cells using TRIzol (Invitrogen, Carlsbad, CA, USA). The concentration of the extracted total RNA was calculated by the spectrophotometric method. DNA products were obtained *via* reverse transcription. The expressions of miR-296-5p and U6 were detected on the LightCycler480 fluorescence qPCR system (Roche, Basel, Switzerland) using SYBR PrimeScript™ RT-PCR kit, and three duplicated wells were set for every sample. As for the experimental results, $2^{-\Delta\Delta Ct}$ method was adopted for relative quantitative analysis of the expression level. Primer sequences used in this study were as follows: miR-296-5p, F: 5'- ATGGCGGACGAGGAGAAGCTGC-3', R: 5'-TCACTCAGTGCGGAGG-ATGATG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3.

Western Blots (WB) Analysis

The transfected cells were lysed for protein extraction, and quantified using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Standard curves were formulated in accordance with the protein concentration, and the loading quantity was calculated. After denaturation through boiling water bath, protein samples underwent electrophoresis, membrane transfer, and incubation with primary and secondary antibodies. Color development was achieved by luminescence. The optical density of the WB bands was quantitatively analyzed using Image J software after the results were scanned.

Cell Proliferation

The transfected K1 cells and NC cells were cultured into a 96-well cell culture plate. 100 μL cell suspension (concentration: 3×10^5 mL^{-1}) was added into each well and then cultured in the incubator for 24, 48, 72 and 96 h, respectively. 50 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (concentration: 5 $mg\cdot mL^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA) was then added and incubated at 37°C for 4 h. Next, the supernatant was discarded carefully, and 150 μL dimethyl sulfoxide (DMSO) solution (Sigma-Aldrich, St. Louis, MO, USA) was added and mixed. After oscillation on a shaking table for 10 min, absorbance was measured using a microplate reader.

Colony Formation Assay

Cells in the logarithmic phase were digested and prepared for suspension in DMEM containing 10% FBS. After that, 200 cells were seeded into each well of the 6-well plate, and cultured in the incubator with 5% CO_2 and saturated humidity at 37°C. Two weeks later, the incubation was terminated, the cells were washed with PBS and fixed with 4% neutral formal for 15 min. Afterward, cells were stained with Wright-Giemsa staining solution for 30 min, washed with slowly running water and then air-dried. The cells were observed under the microscope. Cloning efficiency was calculated according to the following formula: cloning efficiency = number of colonies/number of inoculated cells ×100%.

Cell Cycle Assay

The concentration of K1 cells in the logarithmic phase was adjusted to 1×10^4 cells/mL, and 1 mL cell suspension was inoculated into each well of a 6-well plate. After transfection for 48 h, the cells were detected in accordance with the instructions of the cell cycle detection kit. The cells were digested with trypsin without Ethylene Diamine Tetraacetic Acid (EDTA), centrifuged and resuspended. They were incubated with 5 μL Rnase in a final concentration of 10 mg/mL at 37°C for 1 h and stained with propidium iodide (PI) staining solution in the dark at room temperature for 30 min. Next, the cell cycle was detected and analyzed by a flow cytometer, and three parallel duplicated wells were set up in each experimental group.

TUNEL Assay

After processing and transfection, the K1 cells were continuously cultured for 24 h and then seeded into a 6-well plate. Cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min and incubated with 0.1% Triton X100 on ice for 2 min. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction solution (R&D Systems, Minneapolis, MN, USA) was prepared according to the Roche instruction. Each sample was added with 50 μL TUNEL reaction solution, followed by incubation in the dark at 37°C for 60 min, PBS wash, and visualization of cell nuclei by DAPI (4',6-diamidino-2-phenylindole) at room temperature for 5 min. Finally, anti-fluorescence quencher was added, and images were observed and captured under a fluorescence microscope (×200).

Statistical Analysis

Statistical analysis was performed with Student's *t*-test or *F*-test. Two-sided $p<0.05$ were considered statistically significant. Data were analyzed by Prism 6.02 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Expression Level of MiR-296-5p in PTC

The miR-296-5p expression in the PTC tissues and paired adjacent non-tumor tissues of 28 patients was detected *via* RT-qPCR. MiR-296-5p expression was down-regulated prominently in PTC tissues compared with that in paired adjacent non-tumor tissues (Figure 1A). Similarly, it was observed that the thyroid cancer cell lines had lower miR-296-5p expression than the control group (Figure 1B), suggesting that miR-296-5p could play a certain regulatory role in PTC.

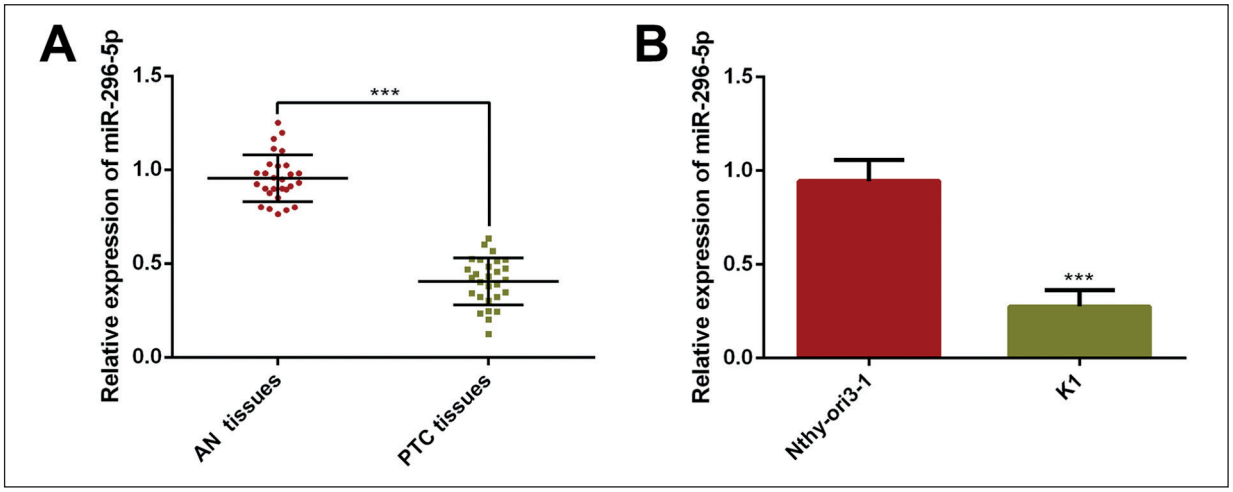

Figure 1. The expressions of miR-296-5p in PTC tissue samples and cells. ***A,*** Difference in the expression of miR-296-5p in tumor tissues and adjacent normal tissues. (***$p < 0.001$). ***B,*** The expression of miR-296-5p in human PTC cells (K1) and normal thyroid gland cells (Nthy-ori3-1). (***$p < 0.001$).

Prediction and Identification of MiR-296-5p Target Gene

PLK1 was probably the target gene of miR-296-5p predicted by TargetScan and PicTar. As shown in the Dual-Luciferase report gene assay, Luciferase activity in cells co-transfected with wtPLK1 and miR-296-5p mimics was notably lower than that of mut-PLK1. However, the Luciferase activity in cells co-transfected with negative control and wtPLK1 or mut-PLK1 did not change, suggesting that PLK1 was a functional target gene of miR-296-5p (Figure 2A).

WB results revealed that compared with that in NC group, protein expression of PLK1 in K1 cells transfected with miR-296-5p mimics mark-

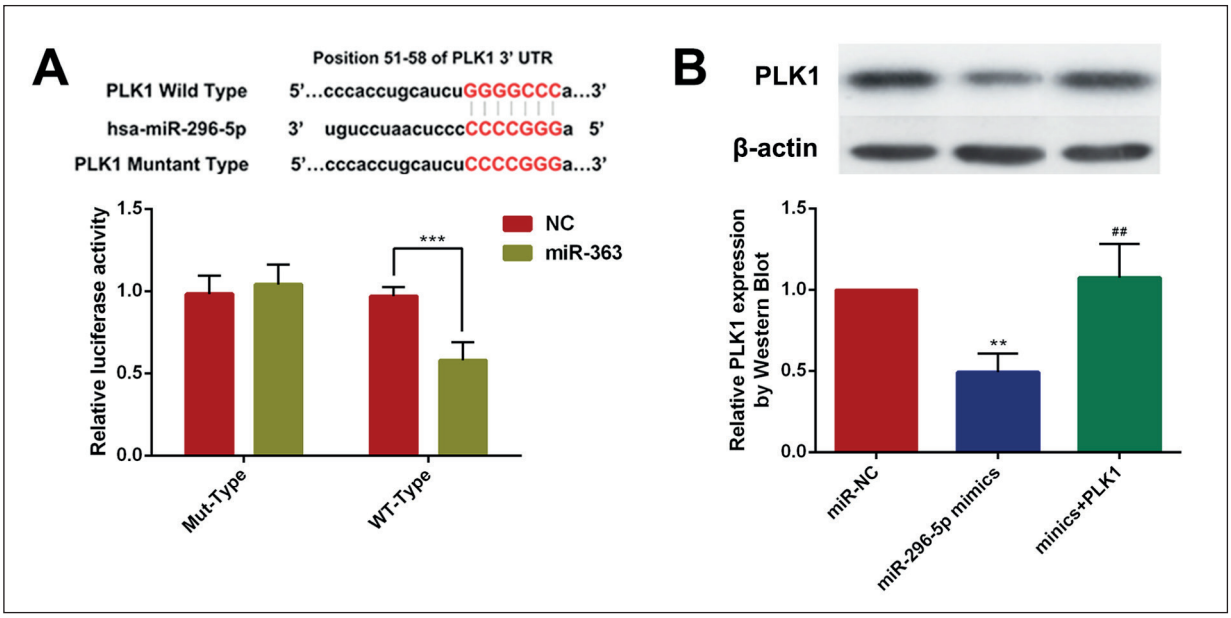

Figure 2. PLK1 was a direct and functional target of miR-296-5p. ***A,*** Diagram of putative miR-296-5p binding sites of PLK1 and relative Luciferase activities. (***$p < 0.001$). ***B,*** MiR-296-5p decreased the expression level of PLK1 detected by Western blot experiment (**$p < 0.01$).

edly downregulated. The results manifested that the transfection of miR-296-5p mimics could effectively suppress the protein expression of PLK1 (Figure 2B).

The above results indicated that miR-296-5p could bind to PLK1 3'-UTR to regulate the PLK1 expression, and PLK1 was a functional target of miR-296-5p.

Effects of MiR-296-5p on Cell Proliferation

After transfection for 48 h, the absorbance between miR-296-5p mimics group and NC group was statistically significant, indicating that miR-296-5p could significantly inhibit the proliferation of the K1 cells (Figure 3A).

Moreover, formed colonies of K1 cells transfected with miR-296-5p mimics were fewer and smaller compared with those of controls (Figure 4A).

Effects of MiR-296-5p on Cell Cycle

We next explored whether miR-296-5p had an influence on cell cycle progression. Flow cytometry analyses showed that the overexpression of miR-296-5p by transfection led to the G2/M phase arrest (Figure 3B), which was consistent with the relevant literature report[18].

Effects of MiR-296-5p on Cell Apoptosis

The apoptotic rate of K1 cells was detected by TUNEL assay. As shown in Figure 4B, after the transfection of miR-296-5p mimics, the percentage of apoptotic cells significantly increased compared with that in miR-NC and mimics+PLK1 groups. The results indicated that apoptosis of PTC cells induced miR-296-5p overexpression could be partially reversed by PLK1 (Figure 4B).

Discussion

The incidence rate of thyroid cancer has rapidly grown over the past decades. Among subtypes of thyroid cancer, PTC presents poor prognosis and low survival rate due to its insidious onset, easy lymph node metastasis in the early stage and lack of specific symptoms[19]. As for some PTC patients resistant to radioactive iodine-131 treatment and accompanied with progressive lymph node metastasis, efficacious targeted therapy and early diagnosis are very crucial. Many kinds of miRNAs are implicated in the incidence and development of PTC[20]. For example, miR-101 could inhibit the incidence of PTC by degrading USP22[21]. MiR-449 could suppress the PTC cell proliferation through targeted regulation of RET kinase β-catenin pathway[22]. MiR-137 could restrain the development of PTC by suppressing CXCL12[23]. MiR-96 played a role of oncogene in the PTC cells by degrading forkhead box protein O1 (FOXO1) and regulating AKT/FOXO1/Bun signal transduction pathway[24]. In this research, RT-qPCR results showed that the expression of miR-296-5p in PTC tissues and cell lines decreased evidently compared with that of corresponding controls, implying that miR-296-5p might exert certain regulatory effects on the occurrence and development of PTC.

One miRNA is able to control multiple target genes, and the same target gene could be regulated by several miRNAs, thus forming complex regulatory networks during the occurrence and development of tumors[25]. Therefore, in-depth research on the molecular mechanism of miRNA in the incidencee and development of tumor diseases might provide new targets for early diagnosis and treatment. To investigate the low expression of miR-296-5p in PTC and its

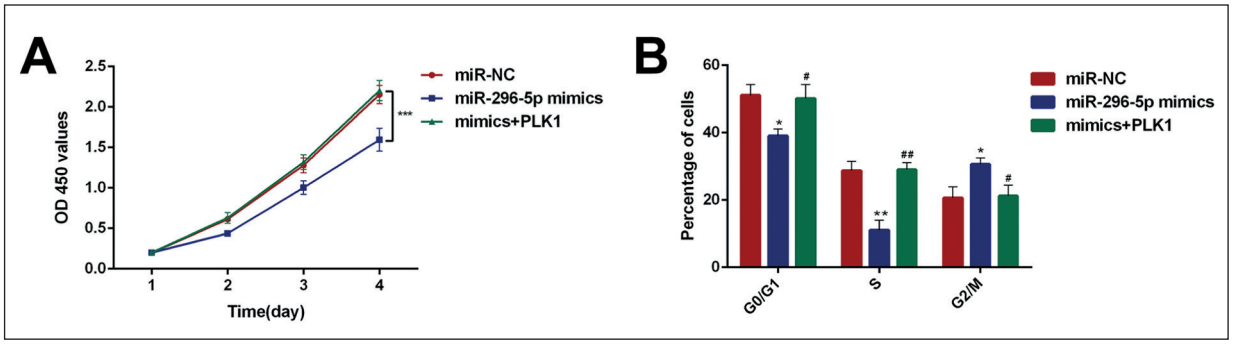

Figure 3. ***A,*** MiR-296 decreased the cell proliferation (***$p < 0.001$). ***B,*** MiR-296 caused the G2/M arrest (*$p < 0.05$, **$p < 0.01$ *vs.* NC group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* Mimics group).

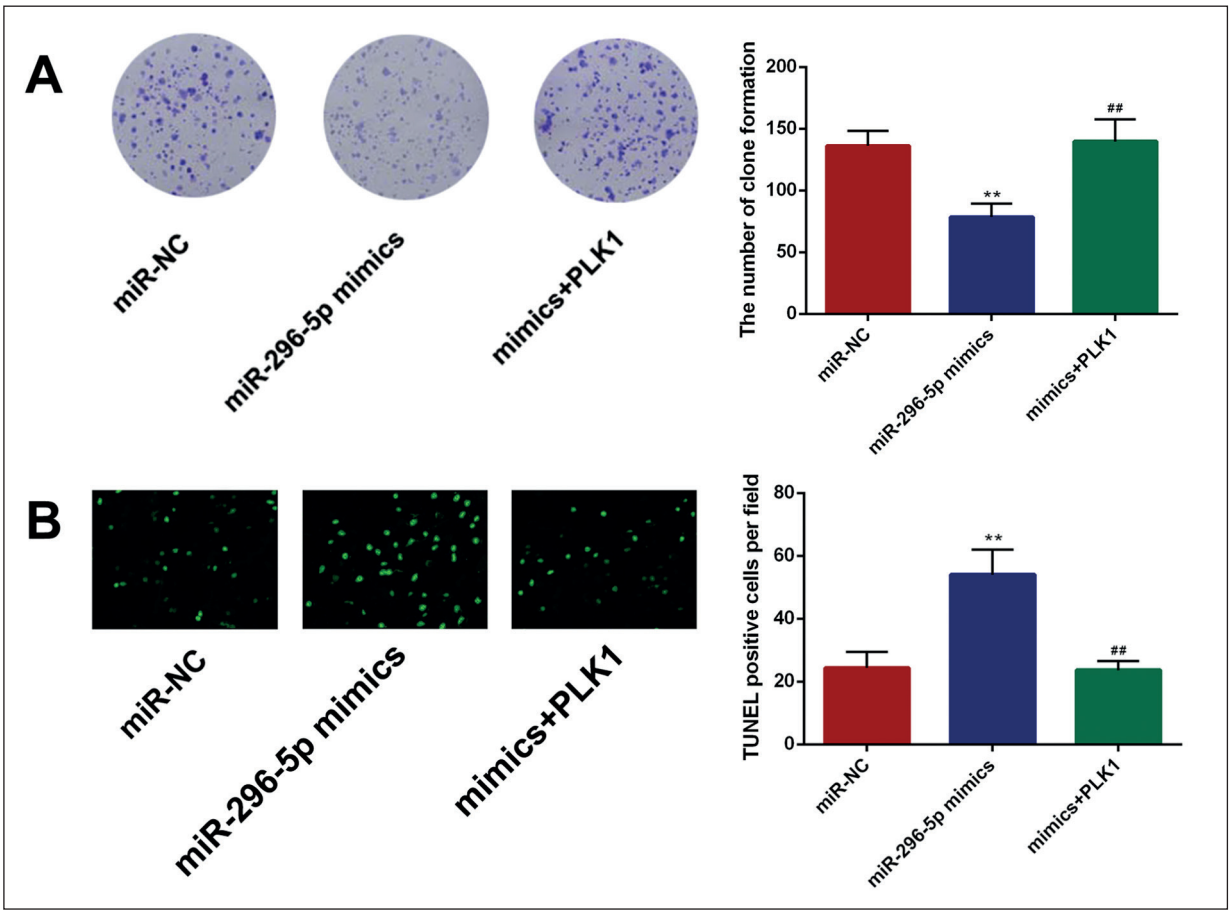

Figure 4. The effects of miR-296-5p on cell colony formation and apoptosis. ***A,*** Assessment of colony formation. ***B,*** Apoptosis level tested by TUNEL staining. All data were presented as means ± standard deviations. (**$p < 0.01$ *vs.* NC group; $^{\#\#}p < 0.01$ *vs.* Mimics group).

regulatory effects on the biological behaviors of PTC, PLK1 was screened and verified to be its target gene.

The PLK family, as a group of highly conserved serine/threonine kinases, plays a vital role in cell cycle progression[26,27] and deoxyribonucleic acid (DNA) damage response[28,29], which was recently considered as potential anticancer targets. Five members of the PLK family (PLK1-5) have been discovered in the mammalian cells. All the PLK members have similar structures, with a catalytic domain of serine/threonine kinase in the N-terminus, and the regulatory domain in the C-terminus includes one (PLK-4) or two (PLK1-PLK3) polo-box domains (PBDs)[30]. PLKs are highly expressed in approximately 80% of human tumors with different origins, but are barely expressed in healthy non-dividing cells, so PLKs are served as attractive and selective targets for developing anticancer drugs[30,31].

As the first discovered in *Drosophila* by Golsteyn et al[32], PLK1 encodes proteins with a molecular weight of about 67 KD, located at 16p12.2. Its messenger RNA (mRNA) is nearly 2.3 KB in length. PLK1 overexpression could lead to enlarged volume or increased number of cell centrosomin. The abnormal centrosomin would result in incorrect aneuploidy division of the chromosome, thereby causing fallacious signal transduction and uncontrolled regulation of cell cycle, as well as triggering the tumorigenesis. Therefore, PLK1 is capable of promoting the occurrence and development of tumors[33]. Some researches demonstrated that the PLK1 is generally upregulated in multiple tumor tissues. Zhou et al[18] found G2/M arrest and apoptosis of lung cancer cells after PLK1 knockdown at both mRNA and protein levels. Feng et al[34] discovered that the mRNA level of PLK1 was relatively high esophageal squamous cell carci-

noma tissues and cell lines. Its expression level was correlated with the TNM staging, regional lymph node metastasis and prognosis of esophageal carcinoma. Duan et al[35] revealed that osteosarcoma patients with moderate expression of PLK1 had a remarkably higher 5-year survival rate than patients with high expression of PLK1. Also, PLK1 is overexpressed in a variety of cancers and closely associated with the prognosis of a majority of tumor patients[36-39]. In this research, miR-296-5p was overexpressed through mimic transfection. PLK1 expression was inhibited by miR-296-5p in K1 cells. The transfection of miR-296-5p mimics in K1 cells arrested G2/M phase inhibited proliferative and colony formation capacities, which could be reversed by PLK1. It is indicated that miR-296-5p could inhibit the proliferation of PTC cells and promote their apoptosis by targeting PLK1.

Conclusions

We demonstrated that miR-296-5p inhibits the abnormal proliferation and accelerates the apoptosis of PTC cells by regulating downstream target gene PLK1. Therefore, PLK1 could be a good target for PTC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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