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MiR-96-5p promotes the proliferation, invasion and metastasis of papillary thyroid carcinoma through down-regulating CCDC67

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have been identified to play an important regulatory role in various biological behaviors of papillary thyroid carcinoma (PTC). However, the specific role and function of miR-96-5p in PTC remain unclear. Therefore, the aim of this study is to detect the expression of miR-96-5p in PTC, and to explore its exact function.

PATIENTS AND METHODS: The relative expression level of miR-96-5p in PTC tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). MiR-96-5p mimics or inhibitors were then constructed and transfected into cells to upregulate or downregulate miR-96-5p expression. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and colony formation assay were employed to evaluate the proliferation of PTC cells. Meanwhile, transwell assay was employed to detect the invasion and metastasis of PTC cells. In addition, the underlying mechanism of miR-96-5p was identified by Luciferase reporter gene assay and Western blot analysis.

RESULTS: The expression of miR-96-5p in PTC tissues and PTC-derived cell lines was significantly higher than that of normal controls. The overexpression of miR-96-5p remarkably promoted the proliferation, invasion and migration of PTC cells. However, knockdown of miR-96-5p significantly decreased cell growth and metastasis. CCDC67 was verified as a target gene of miR-96-5p in PTC. Further experiments demonstrated that the restoration of CCDC67 could significantly reduce the carcinogenic function of miR-96-5p.

CONCLUSIONS: MiR-96-5p was remarkably upregulated in PTC tumor tissues and cells. In addition, it promoted cell growth, invasion, and migration via repressing CCDC67 expression.

Key Words:
MiR-96-5p, PTC, Proliferation, Metastasis, CCDC67.

Introduction

Thyroid cancer is still one of the most common endocrine system tumors, among which papillary thyroid cancer (PTC) accounts for the largest proportion[1,2]. MicroRNAs (miRNAs) are a type of endogenous RNAs with about 21-23 nt in length. It is well known that miRNAs exist in eukaryotes and play an important regulatory role. MiRNAs can regulate the transcription of target genes *via* binding to their 3' UTR[3,4]. So far, hundreds of unique miRNAs have been discovered. Meanwhile, their expression levels vary significantly in different tissues. Many studies[5,6] have shown that abnormally expressed miRNAs are closely related to malignant tumors, including PTC. They may also play vital roles in tumor-genesis and progression through acting as oncogenes or tumor suppressors. For example, miR-143b-3p modulates the differentiation and function of PTC carcinogenesis *via* inducing the miR-146b-3p/PAX8/NIS circuit[7]. MiR-146b-5p regulates the TGF-β signaling pathway *via* targeting SMAD4. Furthermore, miR-219-5p regulates the proliferation of PTC cells by repressing estrogen receptor α[8,9]. Previous researches have also found that miR-155 expression is significantly increased in PTC and may promote tumor growth through APC and activated Wnt/β-catenin axis[10]. Also, miR-577 and miR-144 can inhibit PTC cell proliferation *via* regulating SphK2 and WW domain-containing transcription regulator protein 1, relatively[11,12].

MiR-96-5p is reported to participate in the progression of biological regulation, including tumor-genesis and bone metabolism[13,14]. It pro-

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motes the growth and reduces the apoptotic rate of hepatocellular carcinoma cells *via* SOX6 and caspase-9 gene. Meanwhile, miR-96-5p can inhibit the autophagy and apoptosis of breast cancer, thereby promoting cell growth and metastasis[15-18]. Furthermore, miR-96-5p promotes the expression levels of MAP4K1 and IRS1, and may serve as a novel diagnostic target for human bladder urothelial carcinoma[19,20]. However, the expression and role of miR-96-5p in PTC has not been clearly elucidated.

In this work, we analyzed the relative expression level of miR-96-5p in 78 PTC tissue samples or 4 PTC-derived cell lines. Results showed that the expression of miR-96-5p in PTC tissues and cell lines was significantly higher than that of the adjacent normal tissues samples and human thyroid follicular epithelial normal cells Nthy-ori3-1. Subsequently, we detected the effect of miR-96-5p on cell proliferation, invasion and migration. Moreover, CCDC67 was evidenced as a direct target gene for miR-96-5p in PTC, which could be repressed by miR-96-5p. Taken all together, these findings might provide a novel target for the diagnosis and biotherapy of PTC.

Patients and Methods

Clinical Samples

This investigation was approved by the Ethics Committee of the Central Hospital of Wuhan. The informed consent was obtained from each patient before the study. All 78 pairs of PTC tissues and adjacent normal tissues were collected from PTC patients who underwent surgical treatment in the Central Hospital of Wuhan. Before surgery, all patients received no radiotherapy or chemotherapy treatment. All collected tissue samples were maintained in liquid nitrogen for subsequent experiments.

Cell Culture

Four PTC cell lines (TPC-1, K-1, BCPAP, 8505C) and one human thyroid follicular epithelial normal cell line (Nthy-ori3-1) were obtained from Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Thermo, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% Penicillin-Streptomycin Solution (Gibco, Rockville, MD, USA) in a 37°C, 5% CO_2 incubator.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA of PTC tissues and cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was stored at -80°C after measuring the concentration. Subsequently, the extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) with the PrimeScript RT reagent (TaKaRa, Kusatsu, Japan). The relative expression level of miR-96-5p was detected by the ABI 7000 Fast Real Time-PCR System (ABI, Foster City, CA, USA) in strict accordance with the SYBR Green Master Mix I (TaKaRa, Kusatsu, Japan). Housekeeping U6 was applied as an internal control. The relative expression level of miR-96-5p to U6 was calculated by the $2^{-\Delta\Delta CT}$ method. Primers used in this study were designed by Ribobio (Guangzhou, China). Each experiment was repeated more than three times. Primer sequences used were as follows: MiR-96-5p, F: 5'-ACGATGCACCTGTACGATCA-3', R: 5'-TCTTTCAACACGCAG GACAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Cell Transfection

MiR-96-5p mimics, inhibitors, negative control (NC), inhibitors negative control (INC) and pcDNA-CCDC67 were designed and synthesized by Gene-pharma (Shanghai, China). For cell transfection, cells were first seeded into 6-well plates. When the density was up to 60%, the cells were transfected with appropriate amount of miR-96-5p mimics, NC, inhibitors, INC or pcDNA-CCDC67 according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was determined by qRT-PCR after 24 h.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Cell proliferation was detected by MTT assay (Thermo Fisher Scientific, Waltham, MA, USA). After miR-96-5p upregulation or downregulation, the cells were seeded into 96-well plates at a density of 3×10^3 cells per well. After culturing for 0, 24, 48, and 72 h, respectively, 0.5 mg/mL MTT buffer was added into per well and incubated at 37°C for 2 h in the dark. The absorbance at the wavelength of 470 nm was detected using a spectrophotometer (Bio-Rad, Hercules, CA, USA). Every experiment was repeated more than three times.

Colony Formation Assay

A total of 500 cells were seeded into 6-well plates, followed by two weeks of normal culture. Subsequently, the cells were fixed with methanol and stained with crystal violet. The number of colonies containing more than 50 cells was counted and compared.

Transwell Assay

8-μm transwell inserts (Millipore, Billerica, MA, USA) was used to measure the invasion and migration abilities of cells. For cell migration, 2×10^5 cells suspended in FBS-free RPMI-1640 medium were seeded into the top chamber of the insert, while the lower chamber was immersed in RPMI-1640 containing 10% FBS. After incubation for 48 h, cells on the upper surface of the insert were wiped with cotton swabs. Subsequently, cells on the lower surface were fixed with pre-cooled methanol and stained with crystal violet. The stained cells were imaged and calculated using a microscope. Six randomly selected fields were measured for each insert. For cell invasion, the insert was covered with Matrigel (BD, Franklin Lakes, NJ, USA) before the procedure. The other steps were the same with cell migration assay.

Luciferase Reporter Gene Assay

Constructed wild-type CCDC67 3' UTR sequence containing miR-96-5p binding site and a mutant region was inserted into the pLG-3 vector (Promega, Madison, WI, USA) (pLG-3-CCDC67-Wild-Type or pLG-3-CCDC67-Mutant), respectively. TPC-1 cells were seeded into 6-well plates and transfected with pLG-3-CCDC67-Wild-Type or pLG-3-CCDC67-Mutant, together with miR-96-5p mimics or NC following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured by the Luciferase Assay Kit (Promega, Madison, WI, USA) after 48 h. This experiment was repeated at least three times.

Western Blot

Total protein was extracted by radio-immunoprecipitation assay (RIPA) solution containing protease inhibitors (Beyotime, Shanghai, China). The concentration of extracted protein was detected by the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). After diluting to the same concentration, the protein samples were mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein loading buffer (Thermo, Waltham, MA, USA) and placed in boiling water for 5 min. Subsequently, protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with specific primary antibodies (CCDC67, 1:1000 and GAPDH, 1:2000) at 4°C overnight. Then the membranes were incubated with horse reddish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000) at room temperature for 2 h, followed by washing with Tris-Buffered Saline and Tween 20 (TBST) buffer (Beyotime, Shanghai, China) three times. Immunoreactive bands were exposed by the enhanced chemiluminescence (ECL) method (Pierce, Rockford, IL, USA). All the primary and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). This experiment was repeated three times.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 version software (IBM, Armonk, NY, USA) and GraphPad Prism version 6.0 software (La Jolla, CA, USA) were used for all statistical analysis. All experimental data were expressed as mean ± SD. The *t*-test and one-way analysis of variance were used to compare the differences among groups. $p<0.05$ was expected to have a significant difference.

Results

MiR-96-5p Was Highly Expressed in PTC Tissues and Cell Lines

We first detected the expression level of miR-96-5p in 78 pairs of PTC tissues and adjacent normal tissues by qRT-PCR. As shown in Figure 1A, the expression of miR-96-5p in PTC tissues was significantly higher than that of adjacent normal tissues. Meanwhile, the expression levels of miR-96-5p in four PTC-derived cell lines and one human thyroid follicular epithelial normal cell line Nthy-ori3-1 were also detected. Results showed that the miR-96-5p expression in PTC cells was remarkably upregulated (Figure 1B). All these data indicated miR-96-5p might function as an onco-miR in PTC.

To further study the function of miR-96-5p in PTC cells, TPC-1 cells and 8505C cells were transfected with miR-96-5p mimics and miR-96-5p inhibitors to upregulate or downregulate its expression, respectively. The results demon-

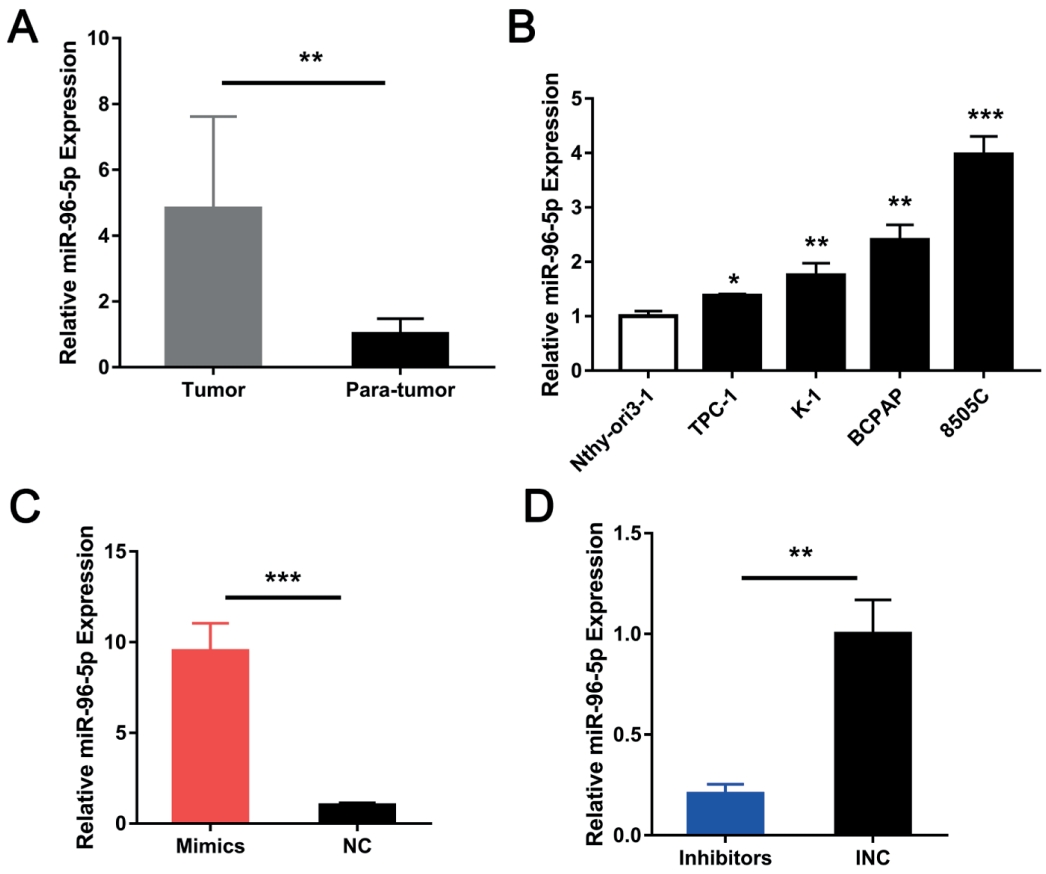

Figure 1. MiR-96-5p was highly expressed in PTC tissues and cell lines. ***A,*** The expression level of miR-96-5p in 78 pairs of PTC tissues and adjacent normal tissues. ***B,*** The expression level of miR-96-5p in PTC cell lines (TPC-1, K-1, BCPAP, 8505C) and human thyroid follicular epithelial normal cells Nthy-ori3-1. ***C,*** Expression of miR-96-5p after miR-96-5p mimics transfection in TPC-1 cells. ***D,*** Expression of miR-96-5p after miR-96-5p inhibitors transfection in 8505C cells. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

strated that mimics transfection significantly increased the expression of miR-96-5p in TPC-1 cells, whereas inhibitors transfection significantly decreased miR-96-5p expression in 8505C cells (Figure 1C, 1D).

MiR-96-5p Promoted the Proliferation of PTC Cells

We next detected the effect of miR-96-5p on cell proliferation. MTT assay and colony formation assay demonstrated that miR-96-5p over-expression significantly promoted the proliferation and colony formation ability of TPC-1 cells (Figure 2A, 2B). However, knockdown of miR-96-5p markedly decreased the proliferation and reduced the number of colonies in 8505C cells (Figure 2C, 2D). These findings indicated that miR-96-5p might accelerate the proliferation of PTC cells.

MiR-96-5p Induced the Invasion and Migration of PTC Cells

Furthermore, we employed transwell assay to evaluate the effect of miR-96-5p on cell migration and invasion. After transfection of miR-96-5p mimics, the invasion ability of TPC-1 cells was significantly promoted (Figure 3A). However, the down-regulation of miR-96-5p remarkably decreased the numbers of invading 8505C cells compared with the control group (Figure 3B). Similarly, up-regulation of miR-96-5p remarkably accelerated the migration of TPC-1 cells, whereas

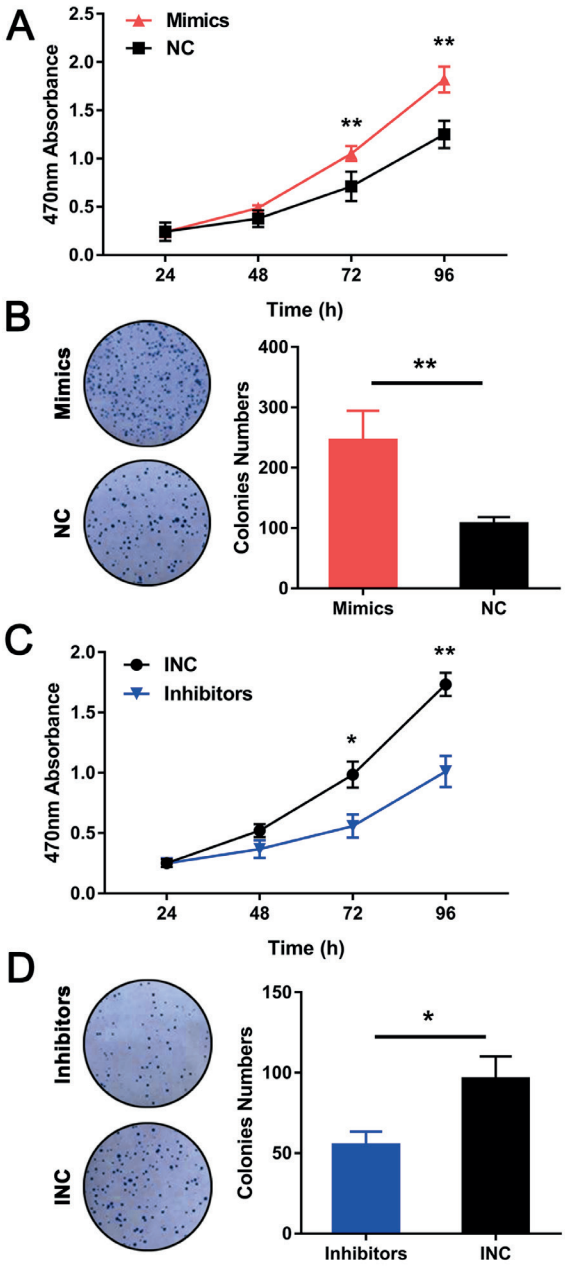

Figure 2. MiR-96-5p promoted the proliferation of PTC cells. ***A, C,*** MTT assay was performed to determine the proliferation of TPC-1 (***A***) or 8505C (***C***) cells transfected with miR-96-5p mimics or inhibitors. ***B, D,*** Colony formation assay was performed to determine the growth of TPC-1 (***B***) or 8505C (***D***) cells transfected with mimics or inhibitors, respectively. **p*<0.05, ***p*<0.01, ****p*<0.001.

the downregulation of miR-96-5p significantly decreased the migration of 8505C cells (Figure 3C, 3D). These results demonstrated that miR-96-5p effected the invasion and migration of PTC cells.

CCDC67 was a Direct Target of miR-96-5p in PTC Cells

Subsequently, we explored the underlying mechanism of miR-96-5p in PTC. By searching several databases including miRWalk, PiTar, TargetScan, and miRanda, we found that CCDC67 was a potential target gene for miR-96-5p because it had a direct binding site of miR-96-5p in 3' UTR (Figure 4A). Then, we constructed pLG-3 vector containing wild type or mutant 3'UTR sequence, and transfected them into TPC-1 cells together with miR-96-5p mimics to verify our assumption. Luciferase reporter gene assay demonstrated that the Luciferase activity was significantly decreased in the wild-type group, whereas no significant difference was found in the mutant group (Figure 4B). The protein expression level of CCDC67 was measured by Western blot. The transfection of miR-96-5p mimics significantly inhibited the protein expression level of CCDC67 in TPC-1 cells. However, the transfection of miR-96-5p inhibitors significantly elevated the protein expression of CCDC67 in 8505C cells when compared with the control group (Figure 4C, 4D). All these results elucidated that CCDC67 was a potential direct target of miR-96-5p in PTC cells.

MiR-96-5p Functioned as an Onco-miR Via CCDC67 in PTC

To confirm CCDC67 whether was the downstream target gene of miR-96-5p in PTC, we constructed and transfected pcDNA-CCDC67 into TPC-1 cells to up-regulate CCDC67 expression. Decreased protein expression level of CCDC67 caused by miR-96-5p mimics was remarkably restored by CCDC67 over-expression (Figure 5A). Next, we measured the proliferation of cells in the three groups. The results indicated that the proliferation ability was remarkably reduced by CCDC67 up-regulation (Figure 5B). In addition, the over-expression of CCDC67 significantly decreased the promoting effect of miR-96-5p up-regulation in cell invasion (Figure 5C). These data validated that miR-96-5p promoted cell growth and metastasis *via* repressing CCDC67.

Discussion

MiRNAs widely exist in animals, plants, and viruses. Several studies have shown that although miRNAs account for only 2% of the human genome, they regulate the expression of more than

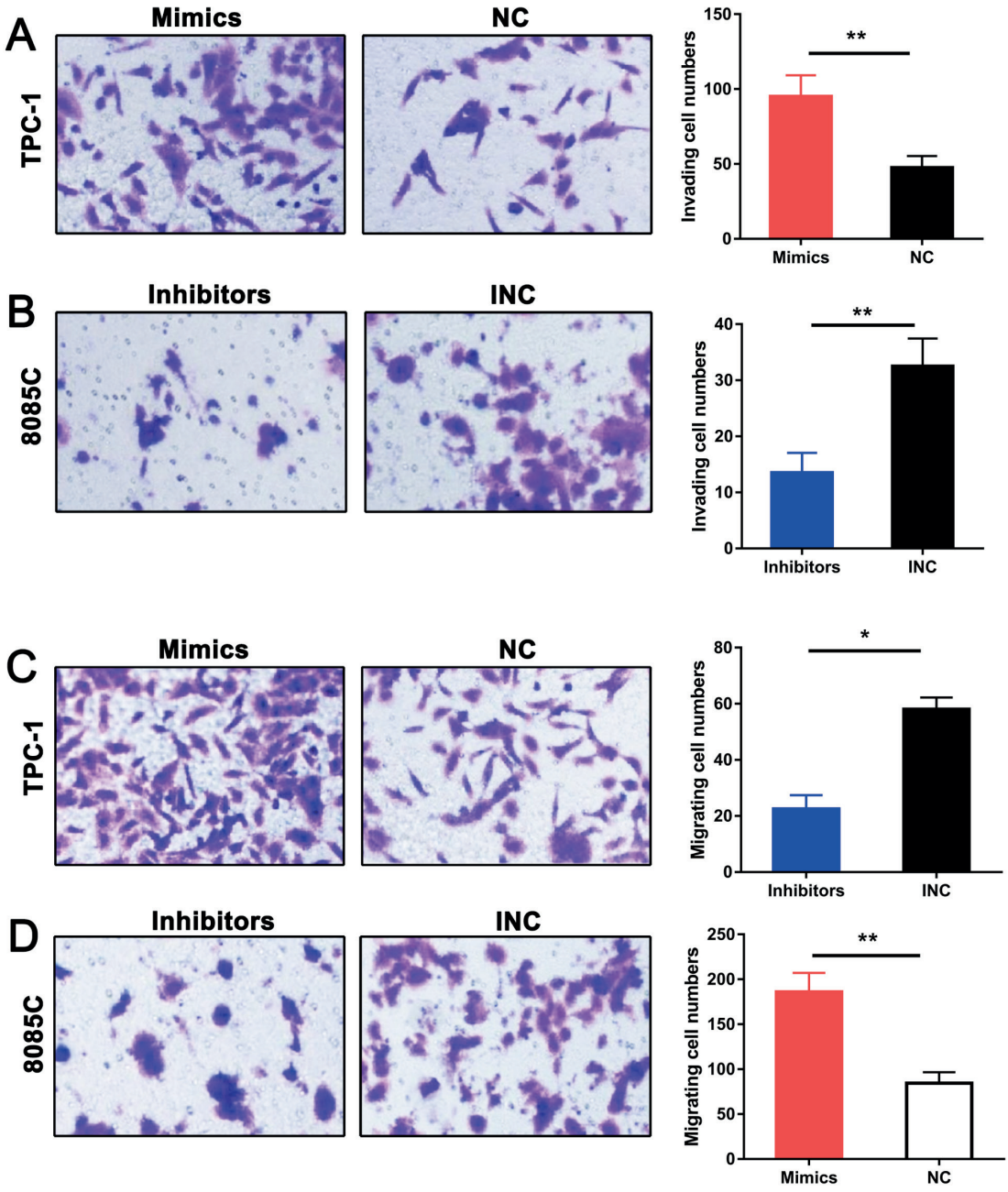

Figure 3. MiR-96-5p promoted the invasion and migration of PTC cells. ***A, B,*** transwell invasion assay was used to detect the invasion ability of TPC-1 cells transfected with miR-96-5p mimics (***A***) or 8505C cells transfected with miR-96-5p inhibitors (***B***). ***C, D,*** transwell migration assay was used to detect the migration ability of TPC-1 cells transfected with miR-96-5p mimics (***C***) or 8505C cells transfected with miR-96-5p inhibitors (***D***). Data were presented as mean ± SD. Each experiment was repeated three times. *$p<0.05$, **$p<0.01$.

30% human genes[3,4]. Meanwhile, miRNAs play an important regulatory role in the development and progression of malignant tumors, including cell proliferation, differentiation, apoptosis, metastasis, and drug resistance[5,6,21]. For example, miR-96-5p is highly expressed in a variety of tumors, which also regulates the growth and metastasis of tumor cells by regulating multiple target genes[20,22,23]. However, the specific role of miR-96-5p in PTC has not yet been reported.

In our study, qRT-PCR results showed that the expression of miR-96-5p in PTC tissues was

significantly higher than that of adjacent normal samples. Similarly, miR-96-5p expression in PTC-derived cells was also remarkably elevated. Next, we upregulated or downregulated miR-96-5p level in PTC cells and analyzed its specific effect on PTC through a series of functional experiments. The results revealed that the miR-96-5p overexpression could promote the proliferation, invasion and migration of cells. However, after miR-96-5p downregulation, the growth and metastasis of PTC cells were significantly inhibited. These results demonstrated that miR-96-5p could promote the proliferation and metastasis of PTC cells, and act as an onco-miR. Our findings were consistent with the utility of miR-96-5p in carcinogenesis of other tumors.

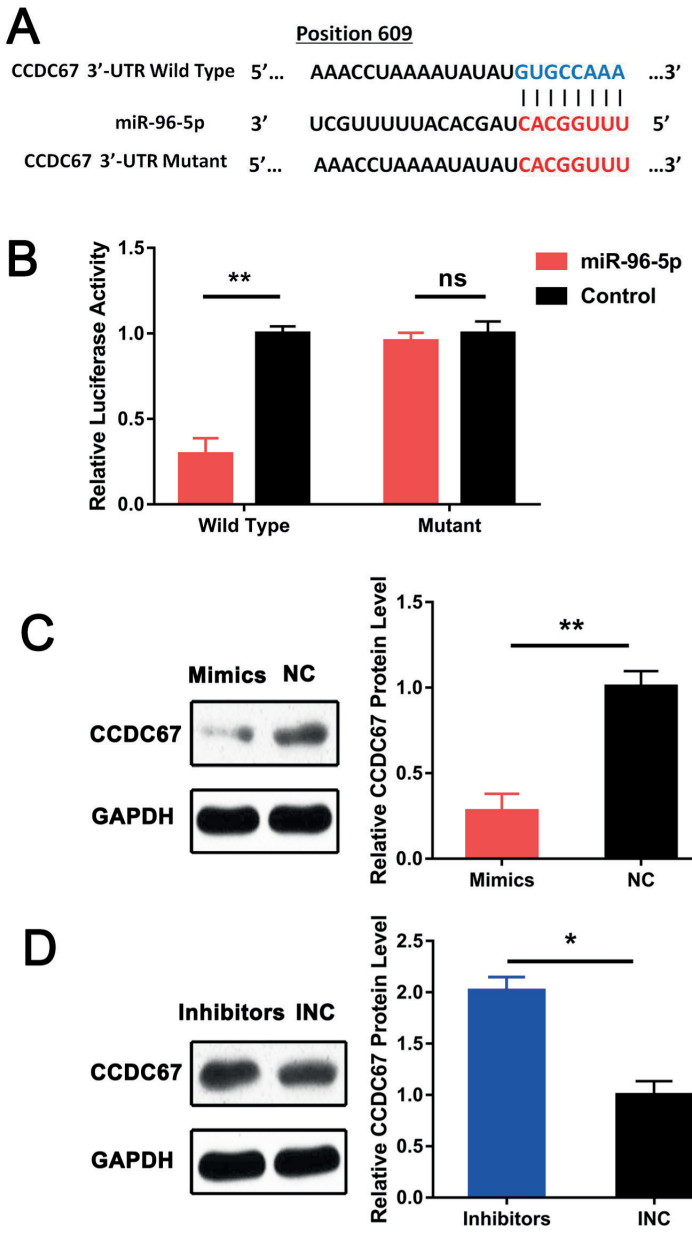

Figure 4. CCDC67 was a direct target of miR-96-5p. ***A,*** Predicted binding sites of miR-96-5p in the 3'-UTR of CCDC67. ***B,*** Luciferase reporter gene assay was used to determine the binding site. ***C, D,*** Protein levels of CCDC67 and β-actin were measured by Western-blot in TPC-1 cells overexpressing miR-96-5p (***C***) and 8505C cells with miR-96-5p knockdown (***D***). The relative protein level of CCDC67 was normalized to GAPHD. Data were presented as the mean ± SD. Each experiment was repeated three times. *$p<0.05$, **$p<0.01$, ns: non-sense.

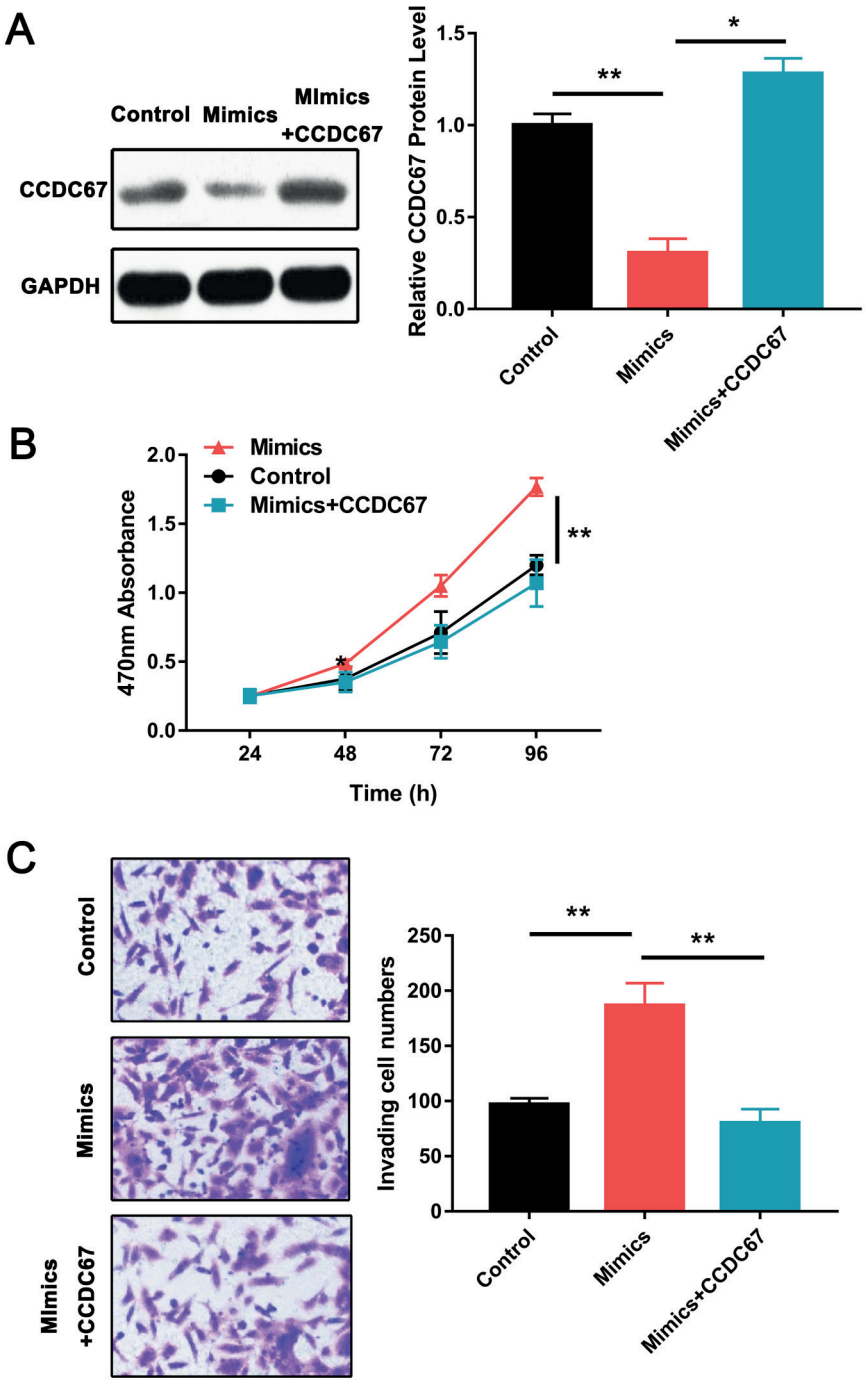

Figure 5. CCDC67 rescued the effects of miR-96-5p in TPC-1 cells. ***A,*** Expression level of CCDC67 was detected by Western blot. GAPDH was used as an internal control. ***B,*** The proliferation ability of control, mimics, or mimics+CCDC67 treated TPC-1 cells was measured by MTT assay; ***C,*** Cell invasion ability was measured by transwell assay. Data were presented as the mean ± SD. Each experiment was repeated three times. *p<0.05, **p<0.01, ***p<0.001.

MiRNAs typically exert its function through binding to the 3'UTR of target genes and inhibiting their expression. Thus, we searched several databases and found that CCDC67 was a downstream molecule of miR-96-5p in PTC. Luciferase reporter gene assay verified that there was a binding connection between miR-96-5p and CCDC67 3'UTR. Meanwhile, miR-96-5p regulated the protein level of CCDC67 in PTC cells. As a member of the CCDC (coiled-coil domain-con-

taining) proteins, CCDC67 is located on chromosome 11q21. The CCDC proteins family has been reported to be involved in the regulation of various tumors[24-26]. For example, in nasopharyngeal carcinoma or lung cancer, CCDC19 can inhibit the proliferation of cells; in prostate cancer, the expression of CCDC62 is reduced; in pancreatic ductal adenocarcinoma, CCDC68 demonstrates a tumor-suppressive role and in pancreatic cancer, CCDC116 also regulates the growth of tumor cells. In addition, the expression level of CCDC6 is changed in lung cancer[27-31]. Studies have found that the expression level of CCDC67 in PTC is reduced. Meanwhile, it can inhibit the proliferation, colony formation, invasion and metastasis of PTC cells[32]. Furthermore, we recovered the decreased level of CCDC67 due to miR-96-5p over-expression, and found that both the proliferation and metastasis capacities of cells were significantly reduced. These results intuitively showed that miR-96-5p promoted the development and progression of PTC by inhibiting the expression of tumor suppressor gene CCDC67. However, the specific mechanism of CCDC67 in PTC remains to be further explored.

Conclusions

Our study first demonstrated that miR-96-5p was highly expressed in PTC tissues and cell lines. Moreover, it promoted cell proliferation, invasion and migration *via* downregulating the protein expression level of CCDC67. Our findings might have biological and clinical implications for PTC, thereby providing novel targets for the diagnosis and biotherapy of PTC.

Conflict of Interests

The Authors declare that they have no conflict of interests

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