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MicroRNA-23a inhibits the growth of papillary thyroid carcinoma via regulating cyclin G1

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Abstract. – OBJECTIVE: The aim of this study was to investigate the regulatory mechanism of miR-23a on biological behaviors of papillary thyroid carcinoma (PTC) cells, such as cell proliferation, cell cycle and apoptosis.

PATIENTS AND METHODS: The expression of miR-23a in 28 paired of PTC tissue samples and matched adjacent tissues was detected by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Meanwhile, miR-23a expression in PTC cell lines was also detected by qRT-PCR. Subsequently, miR-23a mimics and inhibitor were transfected into PTC cells. The effects of gain or loss of miR-23a on cell proliferation, cell cycle and apoptosis were analyzed. Bioinformatics analysis, Dual-Luciferase activity assay and Western blot were recruited to validate the potential target gene of miR-23a.

RESULTS: The expression of miR-23a was significantly decreased in PTC tissue samples and cell lines. Upregulation of miR-23a in PTC cells markedly decreased cell proliferation, induced cell cycle arrest at G0/G1 phase and promoted cell apoptosis. However, decreased miR-23a exerted the opposite effects. Dual-Luciferase, qRT-PCR and Western blot showed that CCNG1 was a target gene of miR-23a. Furthermore, the silence of CCNG1 intensified the suppressive effect of miR-23a on cell growth.

CONCLUSIONS: MiR-23a was involved in the development of PTC via targeting CCNG1, which might provide a new prospect for PTC diagnosis and therapy.

Key Words:
MiR-23a, Papillary Thyroid Carcinoma (PTC), Proliferation, Apoptosis.

Introduction

Papillary thyroid carcinoma (PTC) is one of the most common thyroid malignant tumors with the highest incidence. It is reported that the incidence rate of PTC is increasing year by year, accounting for 85%-90% of thyroid malignant tumors[1]. Thyroid cancer is common in middle-aged women, and most PTC occurs with cervical lymph node metastasis[2]. Surgical therapy is the first choice of treatment for PTC, and other treatments include iodine 131 radiotherapy and thyroid hormone suppression therapy[3]. The development of thyroid cancer is relatively slow, with no obvious symptoms in the early stage. However, about 40%-80% of PTC patients have developed into cervical lymph node metastasis when diagnosed[4]. Meanwhile, the prognosis of these patients is usually poor, and the 10-year survival rate is less than 10%[5]. Therefore, it is important to illuminate the specific process of PTC the development and to explore the molecular mechanism of malignant behaviors of cancer cells. This may eventually help to predict the effective targets of PTC biological treatment.

MicroRNAs (miRNAs) are a class of highly conserved non-coding small RNAs with 21-25 nucleotides in length. MiRNA is involved in the post-transcriptional regulation of gene expression in a variety of eukaryotes[6]. Its abnormal expression often causes changes in cell proliferation, apoptosis, differentiation and other biological processes. This evidence indicates that different miRNAs play different roles as oncogenes or tumor suppressor genes during tumorigenesis[7,8]. Studies have ratified that PTC is associated with abnormal expression of a variety of miRNAs. Meanwhile, these abnormally expressed miRNAs promote the development of PTC, further indicating the value of miRNAs in the diagnosis and prognosis of PTC.

MiRNA-23a (miR-23a) is a member of the miR-23a/24/27a family, which is located on chromosome 19p13.12. In recent years, it has been reported that miR-23a regulates normal cell growth and differentiation. Moreover, it is also closely related to malignant biological behaviors, such as cell growth and metas-

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tasis of various malignant tumors[9]. However, the expression and functional role of miR-23a in PTC is still unclear, which remains to be further analyzed. The aim of this study was to measure the expression of miR-23a and cyclin G1 (CCNG1) in PTC tissues and cell lines, and to further explore the effects of miR-23a on biological behaviors such as the proliferation, cell cycle and apoptosis of PTC cells *in vitro*.

Patients and Methods

Clinical Samples and Cell Lines

Totally 28 PTC cases who received surgical extraction were collected from Yantaishan Hospital from May 2014 to May 2017. All PTC tissues and adjacent tissues were confirmed by pathological examination. Informed consent was obtained from each subject before the study. This study was approved by the Ethics Committee of Yantaishan Hospital. The clinical stage was in accordance with the Union for International Cancer Control (UICC) TNM staging standards promulgated in 2009. All patients with PTC did not receive other anticancer treatments before surgery. Immediately after resection, the tissues were placed in liquid nitrogen and then stored in a -80°C freezer for subsequent use.

PTC cell lines (TPC-1, K1, SW579) and normal thyroid cell line Nthy-ori3-1 were purchased from the cell bank of the Chinese Academy of Sciences' Type Culture Collection Committee (Beijing, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) (Gibco, Grand Island, NY, USA), and maintained in a 37°C, 5% CO_2 humidity incubator. The density, morphology, adherence of cells were observed under an inverted microscope once a day.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Frozen PTC tissues and the para-cancerous thyroid tissues were uniformly stirred in an ice bath using an electric homogenizer. Total RNA in tissues was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized using SYBR Prime Script miRNA RT-PCR kit and Prime Script RT Master Mix (TaKaRa, Otsu, Shiga, Japan). The expression levels of miR-23a and CCNG1 were detected by qRT-PCR in strict accordance with SYBR Green II. U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal references for miR-23a and CCNG1, respectively. The relative expression levels of miRNA and mRNA were calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study were as follows: miR-23a, F: 5'-CCAGGAACCCCTCCTTACTC-3', R: 5'-TCTAGGGATGGTCCGAAGGA-3'; CCNG1, F: 5'-GAGCAGCGCATTTGGACAA-3', R: 5'-ACGTGCGATAGCGACAGTTCT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cells Transfection

According to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), miR-23a inhibitor, negative control (NC-inhibitor), miR-23a mimics and negative control (NC-mimics) were transfected into PTC cells. 24-48 h after transfection, cells in each group were collected. The transfected cells were used for subsequent experiments.

In addition, CCNG1 siRNA and pcDNA3.1-CCNG1 were synthesized by GenePharma Biotechnology Co., Ltd. (Shanghai, China) for CCNG1 knockdown or overexpression. Briefly, 5 μL of CCNG1 siRNA was added to 500 μL of Opti-MEM and mixed. Subsequently, Lipofectamine™ 2000 and siRNA mixture was incubated at room temperature for 20 min. Meanwhile, cells were incubated with pcDNA3.1-CCNG1 mixed with Lipofectamine™ 2000 in serum-free DMEM medium. The transfection efficiency of siRNA or pcDNA3.1 targeting CCNG1 was detected by qRT-PCR 48 h after transfection.

Cell Counting Kit-8 (CCK-8) Assay

PTC cells were digested with trypsin to form a single cell suspension. The concentration of cells was adjusted to 1×10^5 cells/mL. Then, the cells were seeded into 96-well plates at the density of about 2000 cells per well. 5 replicates were set for each group. After cell adherence, 10 μL of the CCK8 solution (Dojindo, Kumamoto, Japan) was added to each well at 0 h, 24 h, 48 h, 72 h and 96 h, respectively, followed by incubation at 37°C for 2 h in the dark. The absorbance value (OD) at a wavelength of 450 nm was detected by a microplate reader. The time point was plotted as the abscissa, and the growth curve of cells was delineated by OD value.

Cell Cycle Analysis

The transfected cells were seeded into 6-well plates and were recruited for cell cycle analysis

48 h later. The cells were washed with phosphate-buffered saline (PBS), centrifuged at 2000 rpm for 5 min and adjusted to a concentration of 1×10^6/mL. Then, the collected cells were fixed with 70% cold ethanol and stored in a refrigerator at 4°C. Meanwhile, the staining solution was added to remove the fixing solution. Later, 100 μL of RNase A was added in a 37°C constant temperature water bath for 30 min. Subsequently, 400 μL of Propidium Iodide (PI) staining was added, and the cells were stored in a refrigerator at 4°C for 30 min in the dark. The fluorescence intensity of cells was determined by flow cytometry. The distribution of cells in different phases of cell cycle was obtained by computer software analysis.

Cell Apoptosis Analysis

After transfection for 72 h, the cells were digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin. 250 μL of 1×Binding Buffer was re-suspended in each tube, and the concentration of cells was adjusted to 1×10^6/mL. Subsequently, 5 μL of Annexin V/FITC and 10 μL PI solution were added to 100 μL of cell suspension, followed by incubation at room temperature for 15 min in the dark. Then, 300 μL Binding Buffer was added to the reaction tube. Finally, cell apoptosis was detected by flow cytometry.

Dual-Luciferase Assay

According to the target gene prediction software miRBase (http://www.mirbase.org/) and TargetScan (http://www.targetscan.org/), the target gene of miR-23a was predicted. Results indicated that cyclin G1 (CCNG1) might be the target gene of miR-23a.

Dual-Luciferase reporter plasmids of wild-type (WT-CCNG1) and mutant (MUT-CCNG1) were constructed. HEK293T cells were first seeded into 96-well plates. Subsequently, constructed pmirGLO/CCNG1-3'UTR and pmirGLO/CCNG1-3'UTR mut fluorescent reporter vectors were co-transfected with miR-23a mimics or NC-mimics into HEK293T cells, respectively. 48 h after transfection, fluorescein activity assay was performed by the Promega Dual-Luciferase reporter system (Madison, WI, USA).

Western Blot

PTC cells were washed with pre-chilled PBS 3 times and were re-suspended in cell lysate (100 μL per well). Lysed cells were reacted on ice for 30 min and centrifuged at 12 000×g for 20 min at 4°C. The supernatant was collected, and the protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein samples (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto 0.45 μm nitrocellulose membranes. After blocking with 5% skim milk powder at 4°C overnight, the hybrid membrane was cut open and incubated with primary antibodies of CCNG1 (1:1000) and β-actin (1:3000) at 4°C, respectively. After incubation overnight, the hybrid membrane was co-labeled with horseradish peroxidase (HRP)-labeled secondary antibody (1:2000). Finally, protein bands were detected by the enhanced chemiluminescence (ECL) luminescence method (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Data with normal distribution were expressed as mean ± standard deviation ($\bar{x}\pm s$). Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. When comparing multiple sets of quantitative data, one-way analysis of variance was used if normality and homogeneity of variance were satisfied. The *t*-test was used to compare the differences between the samples. All experiments were repeated 3 times. The calibration level is $\alpha=0.05$. $p<0.05$ was considered statistically significant.

Results

MiR-23a was Lowly Expressed in PTC Tissues and Cell Lines

To elucidate the role of miR-23a in the PTC development, we first detected the expression level of miR-23a in tumor specimens of PTC patients by qRT-PCR. The results showed that, compared with adjacent tissues, miR-23a expression in PTC tissues was significantly decreased (Figure 1A). Therefore, we believed that miR-23a might play a role in the malignant progression of PTC.

In addition, we examined the expression of miR-23a at the cellular level. It was found that the expression of miR-23a was significantly decreased in PTC cell lines (TPC-1, K1, SW579) than that of the normal thyroid cell line Nthy-ori3-1. The expression in TPC-1 cells was relatively the highest, while the expression in SW579 cells was relatively the lowest (Figure 1B). Therefore, we selected TPC-1 cells to interfere with the

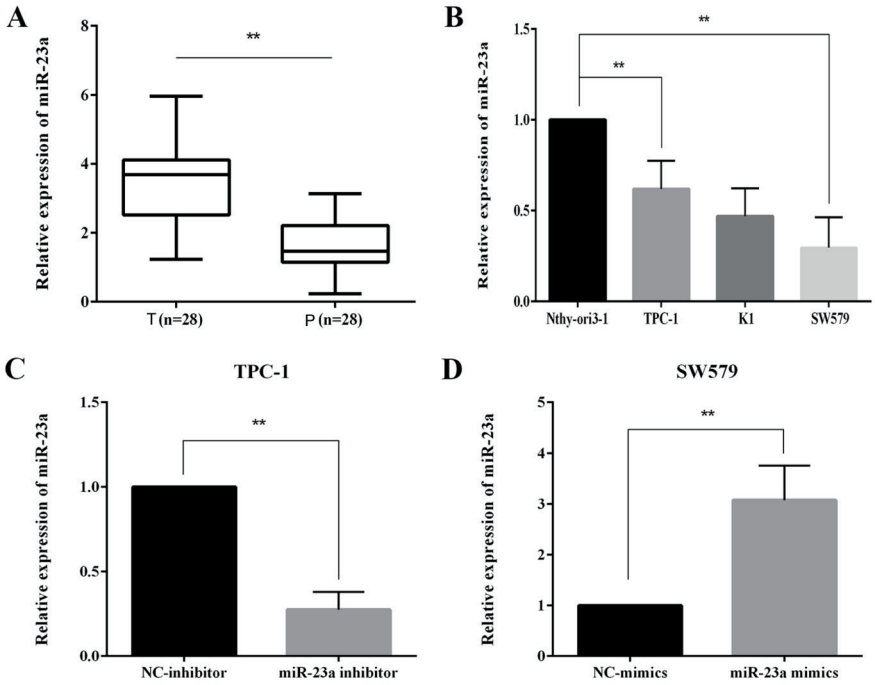

Figure 1. MiR-23a expression was decreased in PTC tissues and cell lines. ***A,*** Analysis of miR-23a expression in para-carcinoma tissues (P) and tumor tissues (T) (n=28); ***B,*** Analysis of miR-23a expression in several PTC cell lines and normal cell line Nthy-ori3-1; ***C,*** Analysis of transfection efficiency of miR-23a in TPC cells transfected with miR-23a inhibitor and NC-inhibitor; ***D,*** Analysis of transfection efficiency of miR-23a in SW579 cells transfected with miR-23a mimics and NC-mimics. Total RNA was detected by qRT-PCR, and GAPDH was used as an internal control. Data were presented as mean ± SD of three independent experiments. $^{**}p<0.01$.

expression of miR-23a. Meanwhile, SW579 cells were chosen to overexpress miR-23a (Figure 1C, 1D). The transfected cells were subjected to the following experiments.

MiR-23a Inhibited the Proliferation of PTC Cells In vitro

To investigate the effect of miR-23a on cell proliferation, we transfected miR-23a inhibitor and NC-inhibitor into TPC-1 cells, and transfected miR-23a mimics and NC-mimics into SW579 cells. The cell proliferation ability was analyzed using the CCK-8 assay. The results demonstrated that the cell proliferation ability was significantly increased in those transfected with miR-23a inhibitor, while cell proliferation ability was markedly decreased in those transfected with miR-23a mimics (Figure 2A).

MiR-23a Promoted Cell Cycle Arrest at G0/G1 Phase and Suppressed Cell Apoptosis

The transfected cells were then recruited to analyze the effects of miR-23a on cell cycle and apoptosis. First, we used flow cytometry to validate the effect of miR-23a on cell cycle distribution. The results showed that, compared with cells transfected with NC-inhibitor, the distribution of cell cycle in G0/G1 phase was significantly decreased after transfection with miR-23a inhibitor, while the distribution of S phase was markedly increased. Meanwhile, compared with cells transfected with NC mimics, the distribution of cell cycle in G0/G1 phase was significantly increased after transfection with miR-23a mimics, while the distribution of S phase was remarkably decreased (Figure 2B).

Similarly, the effect of miR-23a on apoptosis was detected by flow cytometry. The results showed that, compared with cells transfected with NC-inhibitor, the ratio of apoptotic cells was significantly decreased after transfection of miR-23a inhibitor. However, compared with cells transfected with NC-mimics, the ratio of apoptotic cells was markedly increased in miR-23a mimics transfected cells (Figure 2C). From the above results, it could be found that miR-23a regulated cell proliferation by inducing the distribution of cell cycle in G0/G1 phase and promoting apoptosis ability.

CCNG1 was Directly Targeted by MiR-23a

Subsequently, we used miRBase and TargetScan to predict the target genes of miR-23a. Based on the predicted results, we found that CCNG1 was a potential target gene of miR-23a (Figure 3A). Besides, Luciferase activities of PTC cells transfected with wild-type or mutated CCNG1 3'-UTR together with miR-23a mimics or NC-mimics also validated this hypothesis (Figure 3B). At cellular level, qPT-PCR and Western blot results demonstrated that miR-23a inhibitor significantly increased CCNG1 expression in TPC-1 cells, whereas miR-23a mimics remarkably decreased CCNG1 expression in SW579 cells (Figure 3C, 3D). Thus, CCNG1 was considered as a direct target gene of miR-23a.

Silencing of CCNG1 Enhanced the Antitumor Effect of MiR-23a

To validate the relationship between miR-23a and CCNG1, we first detected the expression of CCNG1 in PTC tissues. The results showed that the expression of CCNG1 in PTC tissues was significantly higher than that of adjacent tissues (Figure 4A). Subsequently, we manifested the relationship between miR-23a and CCNG1 expression in PTC tissues. The results verified that miR-23a expression was negatively correlated with CCNG1 expression in PTC tissues (Figure 4B).

Meanwhile, we manifested whether CCNG1 was responsible for the functional roles of miR-23a in the PTC development. We silenced CCNG1 expression by transfecting with si-CCNG1 in miR-23a-decreased TPC-1 cells, and overexpressed CCNG1 by transfecting with LV-CCNG1 in miR-23a-increased SW579 cells (Figure 4C). The results confirmed that CCNG1 silencing not only attenuated cell proliferation capacity (Figure 4D), but also promoted cell apoptosis and cell cycle distribution at the G0/G1 phase (Figure 4E, 4F). However, CCNG1 upregulation exerted the opposite results. Our findings indicated that miR-23a suppressed PTC tumorigenesis by partially regulating CCNG1.

Discussion

PTC is the most common thyroid malignant tumor with low-grade malignancy. It is report-

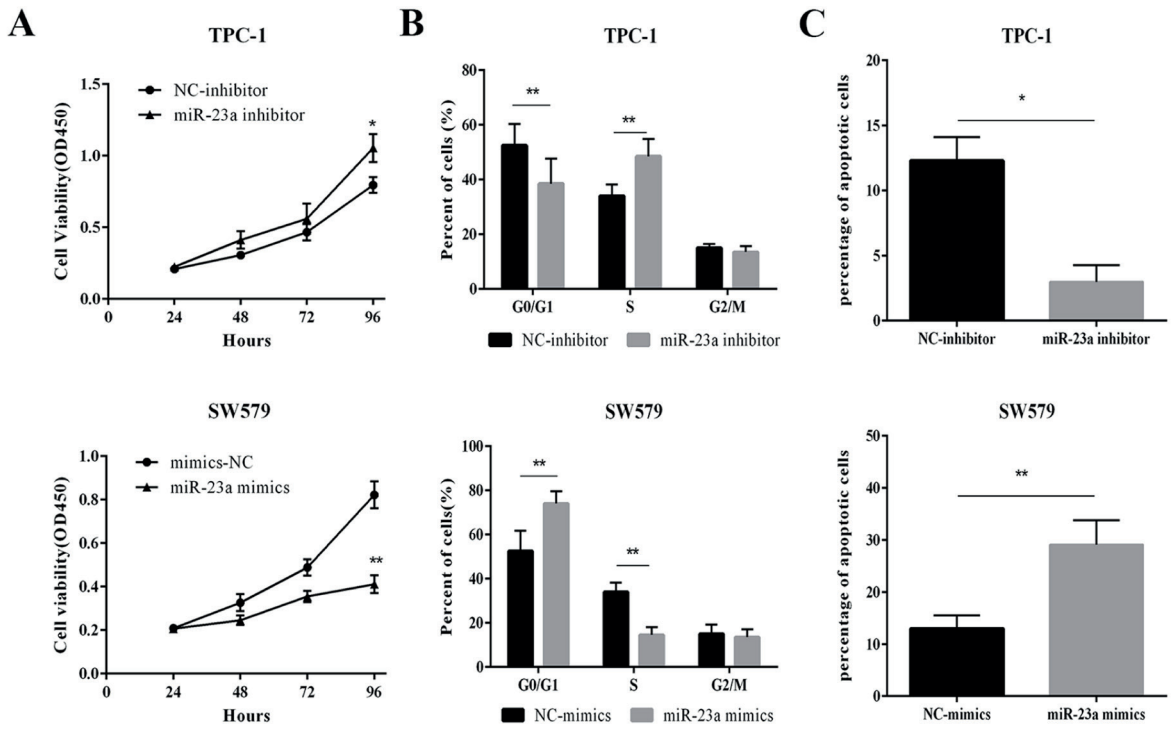

Figure 2. MiR-23a inhibited PTC cell growth *in vitro*. *A*, CCK8 assay was performed to determine the viability of transfected cells; ***B*,** Flow cytometric analysis was performed to detect cell cycle progression of transfected cells; ***C*,** Flow cytometric analysis was performed to detect the apoptotic rates of transfected cells. *$p<0.05$, **$p<0.01$.

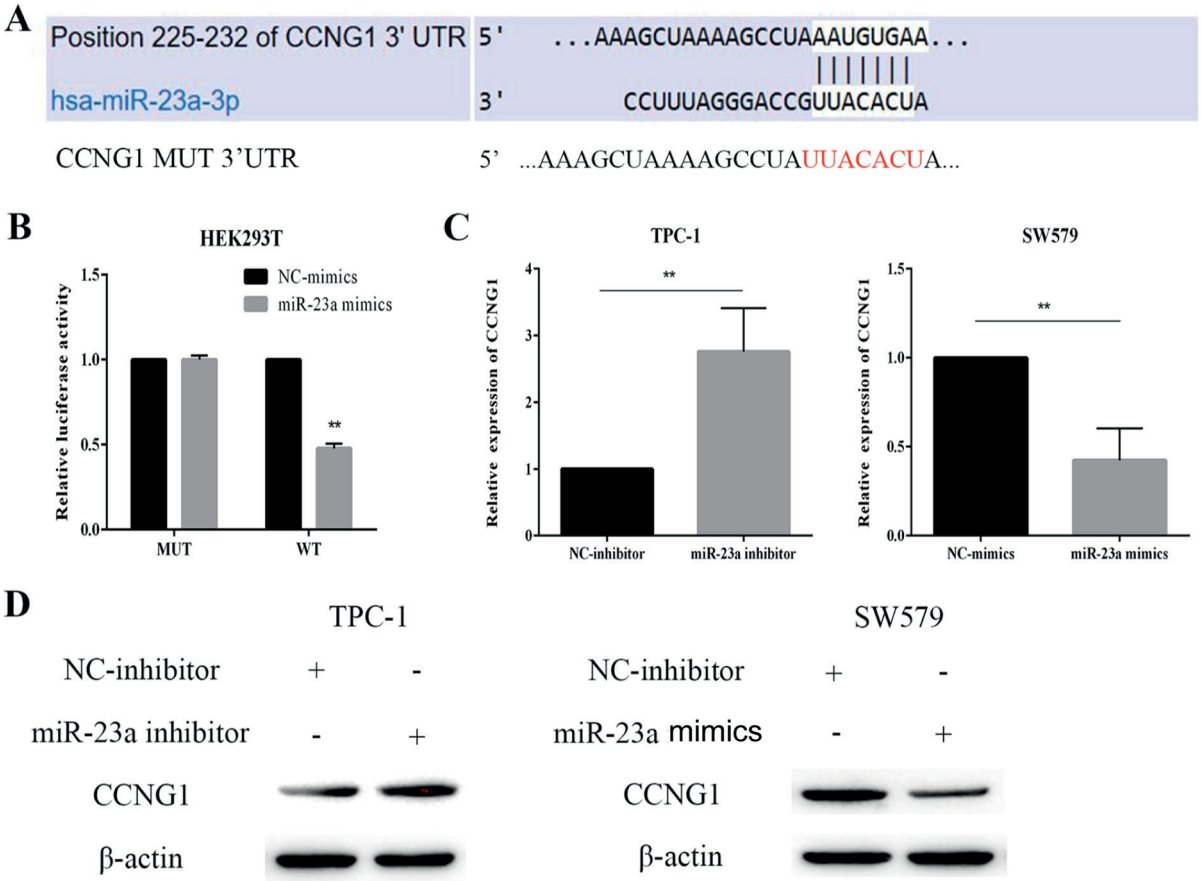

Figure 3. CCNG1 was directly targeted by miR-23a. ***A,*** Bioinformatics analysis predicted that CCNG1 was the potential downstream of miR-23a; ***B,*** Luciferase activities of HEK293T cells transfected with wild-type or mutated CCNG1 3′UTR together with miR-23a mimics or NC-mimics; ***C,*** Analysis of CCNG1 mRNA expression level in transfected cells; ***D,*** Analysis of CCNG1 protein expression level in transfected cells. Data were presented as mean ± SD of three independent experiments. $^{**}p<0.01$.

ed that PTC accounts for about 80% of thyroid cancer. PTC is common in middle-aged women; meanwhile, most of them are associated with cervical lymph node metastasis. The incidence of PTC in the United States has increased at a rate of 33,550 new cases per year[10]. Recent statistics have found that the global incidence of thyroid cancer is on the rise, especially PTC[11]. The development of thyroid cancer is relatively slow, with no significant symptoms in the early stage. However, the prognosis is generally not ideal[12]. Due to the strong invasion ability of PTC and the absence of symptoms in the early stage, PTC patients often suffer from lymph node metastasis or invaded surrounding organs and tissues. According to previous reports[13], the 5-year survival rate of PTC with tumor invasion and distant metastasis is only 35%. Therefore, how to improve the survival rate and life quality of PTC, to study effective gene targets of PTC and to find more effective gene therapy are difficult problems to be solved.

A large number of studies have confirmed that miRNAs are directly or indirectly related to the occurrence and development of various human diseases. Therefore, PTC is also a genetic disease closely related to cell proliferation and apoptosis. With the deepening understanding of this field, it has been found that miRNAs are usually located in chromosome sites related to tumors. Meanwhile, they can regulate important tumor-related genes, eventually affecting cell differentiation, proliferation, apoptosis and angiogenesis[14]. Recent studies have explained the role of miRNAs in thyroid cancer, particularly in PTC. Wang *et al*[15] have found that three miRNAs (miR-146b, miR-221 and miR-222) are highly expressed in PTC tissues when compared with adjacent thyroid tissues. The expression of the above three miRNAs

is about 11-19 times higher than normal level in PTC. Recently, Yip *et al*[16] conducted a detailed study on the aggressive forms of miRNAs in PTC. However, the exact role of miR-23a in PTC has not been found yet. The aim of this study was to investigate the relationship between miRNA and the PTC development. In our study, we found that miR-23a was significantly downregulated in PTC tissue samples when compared with adjacent tissues. This suggested that miR-23a might play a potential role in the development of PTC. In addition, upregulated expression of miR-23a could significantly inhibit PTC cell proliferation, promote apoptosis and induce cell cycle arrest in the G0/G1 phase. However, downregulated miR-23a exerted the opposite results. These findings confirmed that miR-23a had an inhibitory effect on the growth of PTC cells.

Studies have shown that miRNAs can regulate one-third of human genes by binding to target mRNAs. Their functions involve various biological behaviors, such as cell differentiation, hormone secretion, stress response, growth and the development of various cancers. Therefore, the discovery and determination of functionally targeted genes of miRNAs are of great significance to clarify the mechanism of miRNA-related biological functions in tumors. In this work, miRBase and TargetScan database predicted that CCNG1 was a potential target gene of miR-23a. Dual-Luciferase reporter system verified that CCNG1 could directly bind to the 3'UTR region of miR-23a. Meanwhile, qRT-PCR and Western blot confirmed that the expression of CCNG1 was negatively regulated by miR-23a. Cyclin G1 (CCNG1) is an important cell cycle regulator in-

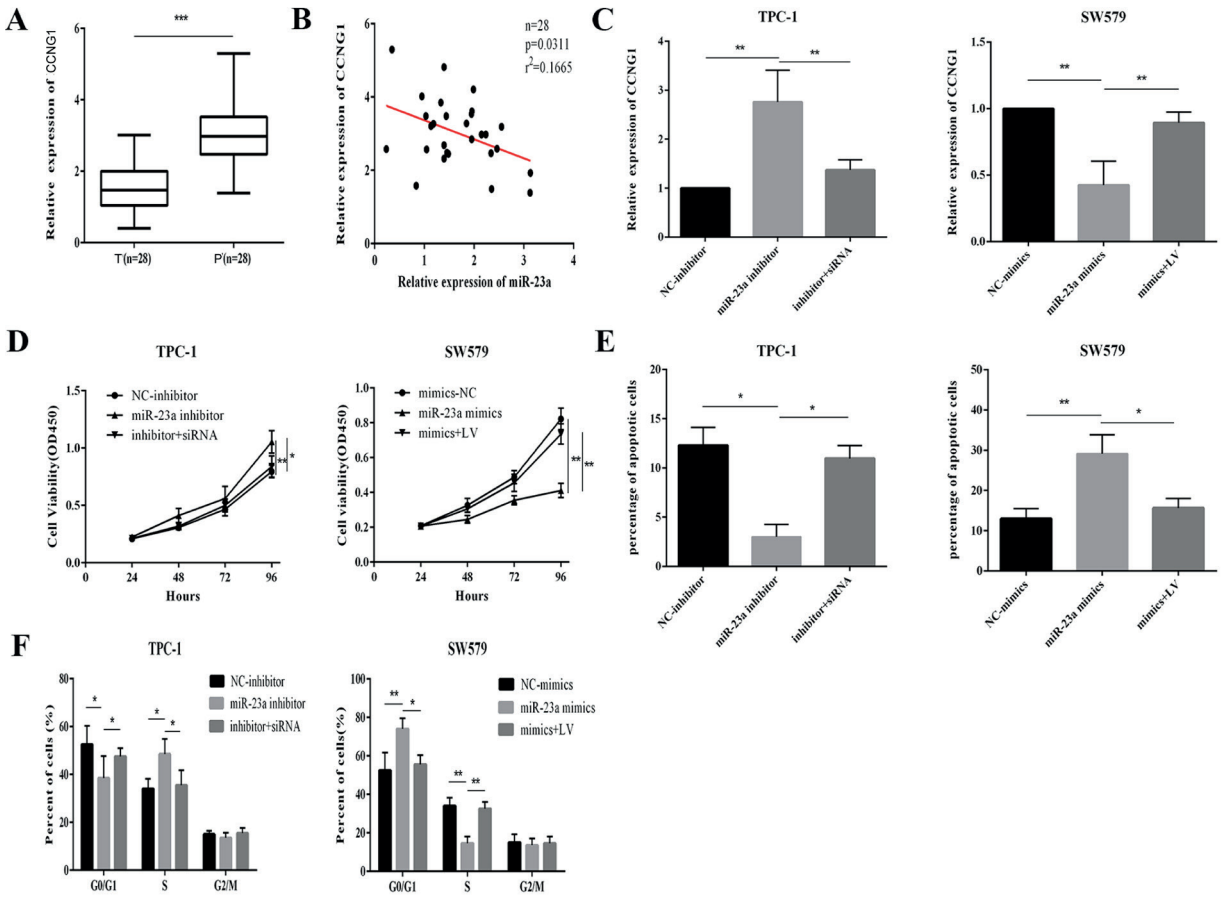

Figure 4. Silencing of CCNG1 enhanced the antitumor effect of miR-23a. ***A,*** Analysis of CCNG1 expression level in PTC tissues (T) and matched para-carcinoma tissues (N) (n=28); ***B,*** Correlation between miR-23a and CCNG1 expression in PTC tissues (n=28); ***C,*** Analysis of transfection efficiency of CCNG1 in transfected cells; ***D,*** Analysis of cell proliferation in transfected cells with CCNG1 dysregulation; ***E,*** Analysis of cell cycle distribution in transfected cells with CCNG1 dysregulation; ***F,*** Analysis of cell apoptosis in transfected cells with CCNG1 dysregulation. Data were presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

volved in the reproductive biology of mammalian granulosa cell proliferation and oocyte maturation[17]. CCNG1 is a cell cycle protein. Moreover, it is also a special class of proteins that play an important role in cell cycle progression[18]. These proteins share a highly conserved domain, namely cell cycle cassette. Cyclins are involved in a series of fine and highly ordered regulation of cell cycle progression by binding to cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs)[19]. CCNG1 is an atypical cyclin with complex functions. It not only participates in DNA replication, but also plays a key role in promoting apoptosis and signal transduction[20]. Studies have found that CCNG1 gene abnormalities exist in a variety of tumors, such as ovarian cancer[21], lung cancer[22], and liver cancer[23]. However, the upstream mechanism of CCNG1 in PTC is still unclear. In the present study, we initially found that CCNG1 was a direct target of miR-23a. Meanwhile, the expression of CCNG1 was negatively correlated with miR-23a expression in PTC tissues. In addition, silencing of CCNG1 could significantly enhance the inhibitory effect of miR-23a on PTC cell growth. These results indicated that miR-23a might be upstream of CCNG1 involved in PTC tumorigenesis.

Conclusions

We demonstrated that miR-23a had a tumor-suppressive effect on PTC growth *via* targeting CCNG1 *in vitro*. Our findings might help to elucidate the molecular mechanisms underlying the PTC progression and provide the miR-23a/CCNG1 axis as an important mechanism in the development of PTC. In addition, our findings might bright a new light for the PTC therapy.

Conflict of Interests

The Authors declare that they have no conflict of interests

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