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Inhibition of miR-223-5p attenuates proliferation and invasion of papillary thyroid carcinoma cells by targeting TIMP3

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ABSTRACT

Background: Papillary thyroid carcinoma (PTC), as an endocrine tumor of young and middle-aged, has increased significantly incidence rate in the past ten years. The ability of invasion and metastasis is significantly related to the prognosis of the disease. High throughput sequencing results showed that miR-223-5p expression was significantly up-regulated in PTC tissues than that in normal thyroid tissues. The aim of this study was to investigate the effect of miR-223-5p on the proliferation and invasion of PTC cells, and to explore its potential molecular mechanism.

Methods: The PTC tissues and adjacent normal tissues of 6 patients with PTC were collected during thyroidectomy. PTC cell lines of KTC-1, K1 and BCPAP was used as in vitre models. RT-PCR and western blot analysis were used to detect the miR-223-5p in protein and mRNA level. MTT assay was conducted to study the the proliferation of PTC cells. Transwell assay was used to detect the invasion of PTC cells.

Results: The miR-223-5p expression was upregulated in PTC tissues and PTC cells. Inhibition of miR-223-5p attenuated the proliferation and invasion of PTC cells. In terms of mechanism, we found that tissue inhibitor of metalloproteinase-3 (TIMP3) is a target gene of miR-223-5p. TIMP3 could be negatively regulated by miR-223-5p. Finally, miR-223-5p silencing suppresses the proliferation and invasion of PTC cells via regulating TIMP3.

Conclusion: Our results demonstrated that miR-223-5p inhibits the proliferation and invasion of PTC cells by targeting TIMP3, which indicating that miR-223-5p may be a new target for the treatment of PTC in the future and provides a new perspective for the PTC diagnosis.

Keywords: Papillary thyroid carcinoma; miR-223-5p; TIMP3

1. Introduction

Thyroid papillary carcinoma (PTC), as one of the major types of thyroid cancer, has increased incidence rate[1]. In view of the significant increase in the detection rate in young patients, it has seriously threatened the health and prognosis of young and middle-aged patients[2]. PTC originated from thyroid follicular epithelial cells and has a low degree of malignancy[3]. However, once distant metastasis occurs, the survival rate will be significantly reduced. At present, surgical treatment is still the main treatment, and further exploration of gene level therapy has been a hot and difficult point in current research[4].

MicroRNAs (miRNAs) are a class of small RNAs without coding function, which are composed of about 20 nucleotides[5]. In many diseases, especially in tumor diseases, they play critical roles in regulating cell proliferation, invasion and migration. Recently, by analyzing high-throughput sequencing results between PTC and normal tissues, we found that miR-223-5p was significantly up-regulated in PTC[6]. Numerous studies have shown that miR-223-5p plays an important role in the regulation of apoptosis, proliferation and invasion[7-9]. However, the role and potential molecular mechanism of m in PTC have not been studied.

Previous studies and Targetscan software analysis showed that tissue inhibitor of

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metalloproteinase-3 (TIMP3), which has regulatory function in PTC, is a target regulatory gene of miR-223-5p[10]. Overexpression of TIMP3 can significantly inhibit the proliferation and invasion of PTC cells[10]. Nevertheless, whether miR-223-5p can regulate the proliferation and invasion of PTC cells by regulating TIMP3 has not been investigated.

In present study, we first verified the difference of miR-223-5p expression between PTC and normal tissues. In addition, the role of miR-223-5p in PTC cell proliferation and invasion was verified by knockdown of miR-223-5p expression, and the potential molecular mechanism of its role was further investigated.

2. Meterials and methods

2.1. Tissues collection

Papillary thyroid cancer tissues and adjacent normal tissues of 6 patients with PTC were collected during thyroidectomy. All patients were not treated with drugs and radiotherapy before collection. This study was approved by Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All patients agreed to collect and test samples and signed informed consent.

2.2. Cell culture

Human papillary thyroid carcinoma cell lines (BCPAP, K1 and KTC-1 cells) and normal thyroid cells (Nthy-ori) were obtained from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China) and incubated in RPMI-1640 medium (procell, Wuhan, China) with 10% fetal bovine serum (FBS, Gibco, USA) (containing 100 IU/ml penicillin and 100 μg/ml streptomycin), and cultured in a cell incubator containing 5% CO_2 at 37^0C. The cells were subcultured when the cell growth density was about 90% (digested with trypsin containing 0.02% EDTA). In this study, KTC-1 cells were transfected with miR-223-5p inhibitor with or without TIMP3 siRNA for 36 hours.

2.3. Western blot analysis

The proteins extracted from KTC-1 cells with miR-223-5p inhibitor or TIMP3 siRNA transfection, and were separated with 10% SDS-PAGE at 80V. After that, the membrane was transferred with 300 Ma constant current for 90 min. Then, PDVF membrane was sealed with 5% BSA for 2 hours, and then incubated with TIMP3 (Abcam, CA, USA) primary antibody overnight at 4^0C, GAPDH (Abcam, CA, USA) as internal reference. After incubation with the second antibody at room temperature for 2 hours and washing with TBSTt for 1 hour, western blot analysis was performed.

2.4. RT-PCR analysis

PTC tissues and cells was lysed by TRIzol reagent (MIBio, Shanghai, China) and used to extract Total RNA. After extraction, the purified and concentration of RNA was detected at 260/280 with a a spectrophotometer of NanoDrop ND-1000 (Thermo Fisher Scientific, CA, USA). Finally, reverse transcriptase (Thermo Fisher Scientific, CA, USA) was added into RNA to extract cDNA, and RT-PCR quantitative detection was performed. The relative expression of mRNA in present study was calculated by $2^{-\Delta\Delta CT}$, 18S RNA was set as internal parameter and the final result was expressed as mean ± SEM. The primers used in this study were shown as below: miR-223-5p: F:3'-ATTCCGGTGGCATCCGA-5'; R:3-GGAACTTCGGAGCTACC-5'; TIMP3: F:3'-GCCAATCGTGGAGGCTGA-5'; R:3-ACCCGGACAATTGGAGGAGC-5'; 18s RNA: F:3'-GTTCAAACCATCTT-5'; R:3-GGGTTAACCCTGCGGATGC-5'.

2.5. Luciferase reporter assay

When the KTC-1 cells treated with miR-223-5p mimic grew to 80-90%, the medium was sucked out of the cell culture pore. The cell lysate was added into each well to lyse the cells for 20-30 minutes. Then 10 μL of lysate was inhaled and added into the EP tube containing 50 μL luciferase assay reagent II (LAR II, Biovision, Shanghai, China). Then the luciferase activity is detected in the luciferase detection system of the instrument.

2.6. MTT assay

KTC-1 cells were cultured in 96-well cell culture plate, and transfected with miR-223-5p inhibitor with or without TIMP3 siRNA when the growth area reached 50%. At 24, 48, 72 and 96 hours after transfection, 96 well plates were removed from the cell incubator, and 10 μl of MTT solution (5 mg / ml) was added to each well for 4 h. Then, 96 well culture plate was taken out and centrifuged at 2000 rpm for 5 min. the supernatant was discarded and added with MTT solution. Finally, the OD value of each cell culture well was measured at 570 wavelength.

2.7. Transwell assay

After being transfected with miR-223-5p inhibitor with or without TIMP3 siRNA for 36 hours, KTC-1 cell suspension was inoculated into a transmembrane incubator with a diameter of 8 μM. Then, the cells penetrating through the membrane to the lower layer were fixed with formaldehyde and stained with crystal violet for 24 hours. The unstained crystal violet was washed out. After PBS was added, the cells were photographed and

counted using Zeiss microscope.

2.8. Statistical analysis

All the data were analyzed by graphpad 7.0 (La Jolla, CA, USA) and presented as mean ± SEM in this study. The unpaired Student's t-test was mainly used for comparative statistics between the two groups. In addition, oneway ANOVA followed post-hoc analysis was performed to analyze the statistical differences among three groups. $P < 0.05$ in present study was considered to be statistically significant.

3. Results

3.1. miR-223-5p expression is upregulated in PTC tissues and PTC cells

To explore the function of miR-223-5p in PTC, we first collected 6 PTC tissues and adjacent normal tissues to verify the miR-223-5p expression level. Compared with normal tissues, the expression of miR-223-5p in PTC tissues was significantly up-regulated as detected by RT-PCR analysis (Figue 1A). Furthermore, the expression level of miR-223-5p in PTC cell lines was markedly higher than that in normal thyroid cells (Nthy-ori) (Figue 1B). Duo to the highest expression of miR-223-5p in KTC-1 according above RT-PCR assay, we used KTC-1 cells as an in vitro model in the following in vitro experiments.

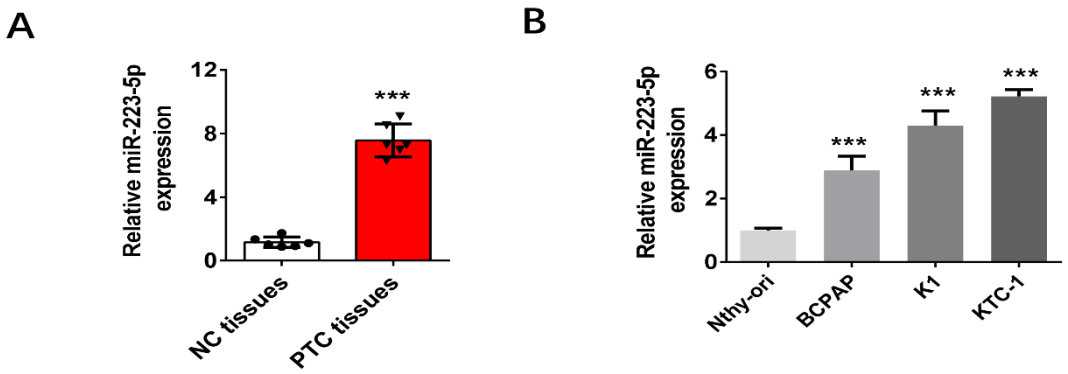

Figure 1. **miR-223-5p expression is upregulated in PTC**

A. The miR-223-5p level between PTC tissues and normal thyroid tissues was detected by RT-PCR analysis. B. The miR-223-5p level in PTC cell lines and normal thyroid cells was determined by RT-PCR analysis. Each experiment was repeated three times independently, and the mean ± SEM was used to express the statistical error. ***P<0.001.

3.2. Inhibition of miR-223-5p attenuates the proliferation and invasion of PTC cells

Next, we used miR-223-5p inhibitor to decrease the expression of m in PTC cells, and verified the effect of miR-223-5p on proliferation and invasion of PTC cells by MTT and Transwell assay respectively. MTT results indicated that inhibition of miR-223-5p significantly reduced the proliferation level of PTC cells (Figue 2A). Similarly, knockdown of miR-223-5p significantly reduced the invasive ability of PTC cells (Figue 2B).

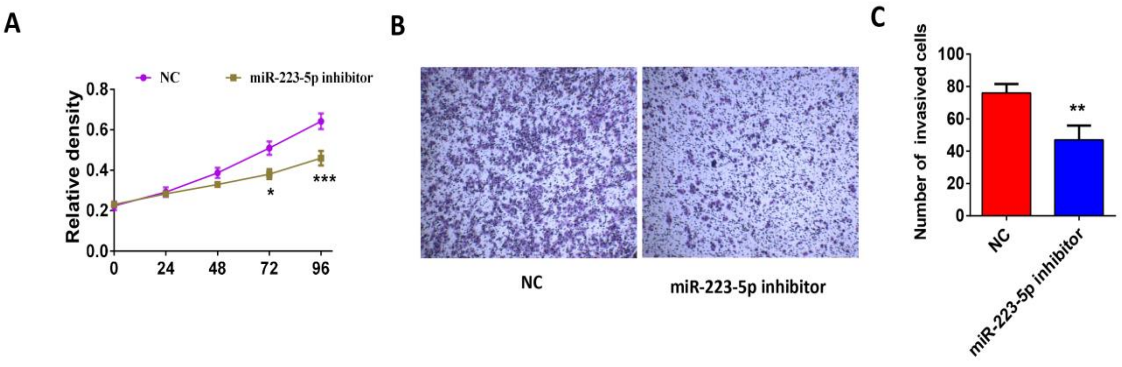

Figure 2. **Inhibition of miR-223-5p attenuates PTC cell proliferation and invasion**

A. The proliferation of KTC-1 cells was analyzed by MTT assay between NC and miR-223-5p inhibitor groups. B. Transwell assay was used to verify the invasion of KTC-1 cells between NC and miR-223-5p inhibitor groups. Each experiment was repeated three times independently, and the mean ± SEM was used to express the statistical error. *P<0.05, **P<0.01, ***P<0.001.

3.3. TIMP3 is a target of miR-223-5p

Through the biological prediction of Targetscan software, we found that TIMP3 is a target gene of miR-223-5p (Figue 3A). In order to further verify their direct binding relationship, we used Luciferase report to detect the effect of miR-223-5p on TIMP3 activity. The results showed that overexpression of miR-223-5p significantly inhibited the TIMP3 activity of WT, but had no significant effect of TIMP3 activity of MUT (Figue 3B). Finally, by RT-PCR and Western blot detection, we proved that miR-223-5p can negatively regulate the expression of TIMP3 (Figue 3C-3E).

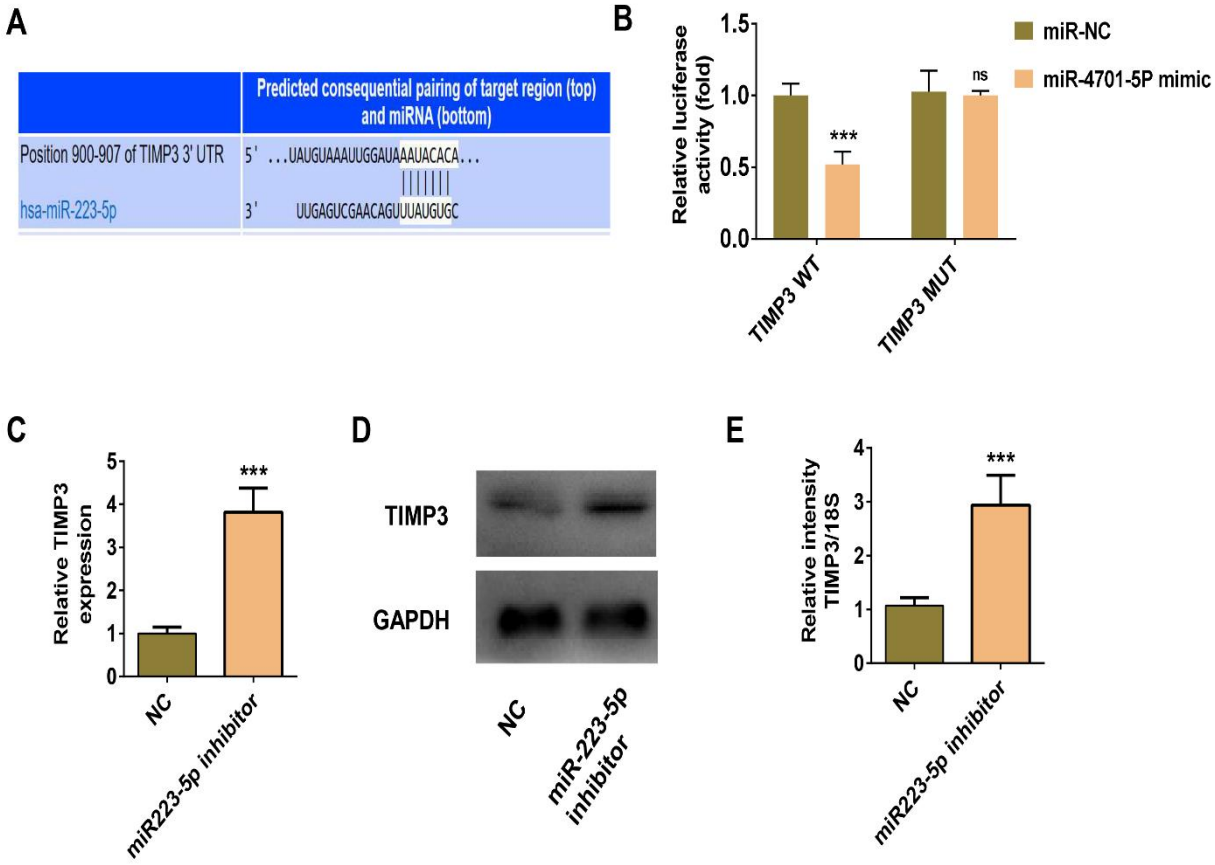

Figure 3. **TIMP3 is a target of miR-223-5p**

A. Targetscan software (http://www.targetscan.org/) was used to predict the target gene of miR-223-5p. B. Luciferase assay was used to determine the binding relationship between miR-223-5p and TIMP3. C. The miR-223-5p level in PTC cells after transfection of miR-223-5p inhibitor was determined by RT-PCR analysis. D-E. Western blot analysis was used to detect and quantified the TIMP3 expression. Each experiment was repeated three times independently, and the mean ± SEM was used to express the statistical error. ***P<0.001, ns: no statistical difference.

3.4. miR-223-5p silencing suppresses the proliferation and invasion of PTC cells via regulating TIMP3

To further clarify whether miR-223-5p inhibits the proliferation and invasion of PTC cells through regulating TIMP3, we first constructed TIMP3 siRNA and verified its knockdown efficiency by Western blot analysis (Figue 4A-4B). Through transfection of miR-223-5p inhibitor with or without TIMP3 siRNA treatment in PTC cells, we further demonstrated that miR-223-5p silencing inhibits the proliferation (Figue 4C) and invasion (Figue 4D-E) of PTC cells via regulating TIMP3.

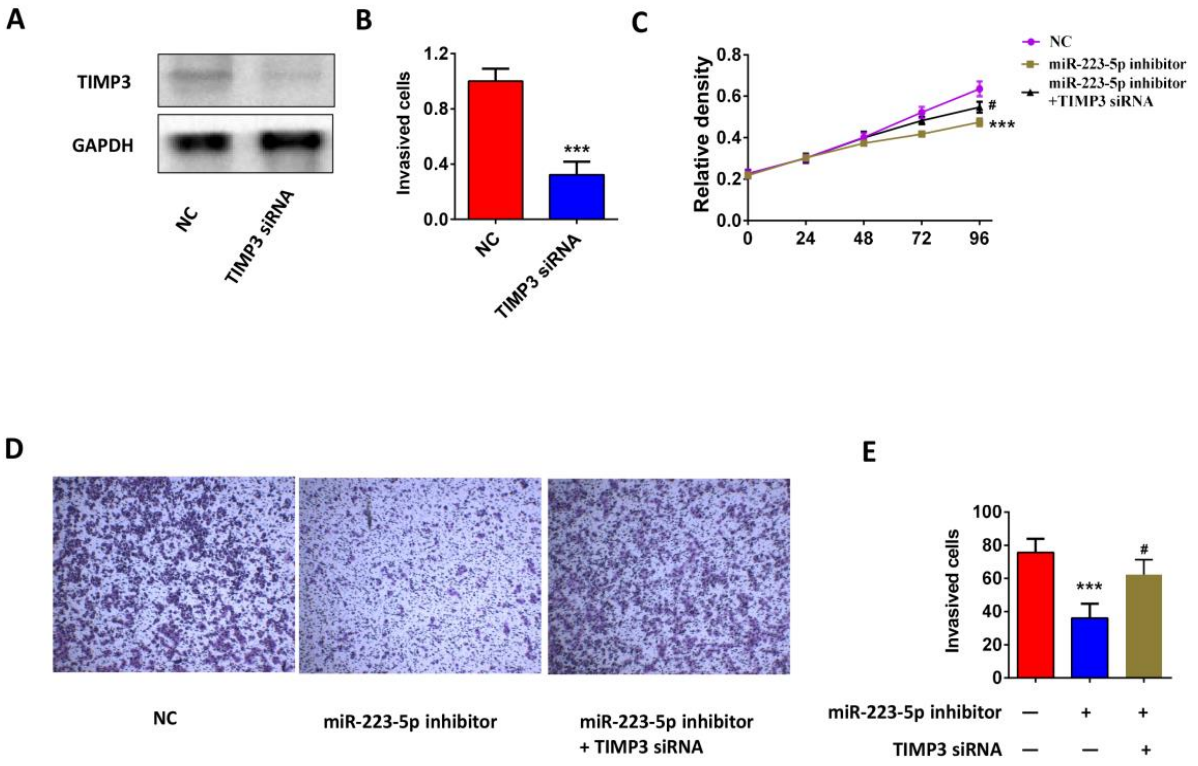

Figure 4. **Inhibition of miR-223-5p attenuates PTC cell proliferation and invasion via TIMP3**

A-B. Western blot analysis was used to detect and quantified the TIMP3 expression. C. The proliferation of KTC-1 cells was analyzed by MTT assay among NC, miR-223-5p inhibitor and miR-223-5p inhibitor +TIMP3 siRNA groups. B. Transwell assay was used to verify the invasion of KTC-1 cells among NC, miR-223-5p inhibitor and miR-223-5p inhibitor +TIMP3 siRNA groups. Each experiment was repeated three times independently, and the mean ± SEM was used to express the statistical error. **P<0.01, ***P<0.001 VS NC; # p<0.05 VS miR-223-5p inhibitor.

4. Discussion

The incidence rate of PTC has increased significantly in recent years, and its pathogenesis is not yet fully understood[11]. However, the potential mechanism recognized at present is mainly the result of the interaction of genes and environmental factors[12]. MiRNAs have been proved to be involved in the occurrence and development of a large number of tumors, including PTC, and regulate a series of phenotypes such as proliferation, apoptosis, invasion/migration of tumor cells[13]. In addition, there is a lot of evidence that miR-223-5p can be used as a potential diagnostic marker for many diseases[14].

In our study, we confirmed that m was up-regulated in PTC compared with normal thyroid tissue. Knockdown of miR-223-5p can significantly reduce the proliferation and invasion of PTC cells, suggesting that miR-223-5p may be a potential therapeutic gene to regulate the progression of PTC. In previous studies, overexpression of miR-223-5p has been confirmed to promote the proliferation and invasion of gastric cancer cells, and aggravate the progress of gastric cancer[8] . At the same time, miR-223-5p can also regulate apoptosis and inflammation in injured diseases. Interestingly, in some studies, miR-223-5p overexpression has been shown to inhibit the proliferation and invasion of tumor cells. For example, overexpression of miR-223-5p can significantly inhibit the proliferation and migration of prostate cancer[7]. We speculated that the basic expression patterns of miR-223-5p are different in different tissues and may have different effects on different diseases.

In terms of mechanism research, our results show that miR-223-5p can target binding and negatively regulate the expression of TIMP3. We transfected KTC-1 cells with miR-223-5p inhibitor with or without TIMP3 siRNA treatment. MTT assay and Transwell experiments showed that miR-223-

5p silencing inhibited the proliferation and invasion of KTC-1 cells by negatively regulating TIMP3 expression. As a well-known gene that inhibits the progression and prognosis of PTC, TIMP3 is negatively regulated by miR-223-5p, and has been proved to mediate the role of miR-223-5p in regulating the proliferation and invasion of PTC cells. These results fully suggest that miR-223-5p may be a potential target gene for the treatment of PTC.

In conclusion, we verified the expression of miR-223-5p between PTC and normal thyroid tissue in vivo. The potential molecular mechanism revealed that miR-223-5p plays a role in the negative regulation of TIMP is elucidated, which provides a new perspective for future PTC therapy.

Conflict and interest

None

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