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MicroRNA-219 inhibits cell viability and metastasis in papillary thyroid carcinoma by targeting EYA2

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Abstract. – OBJECTIVE: In recent years, the incidence of papillary thyroid carcinoma (PTC) has increased. Many microRNAs (miRNAs) have been found to regulate PTC progression. However, the regulatory mechanism of miR-219 remains unclear in PTC. Therefore, the purpose of this study is to explore the function of miR-219 in PTC.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot analysis were used to detect the expression of miR-219 and eyes absent homologue 2 (EYA2). The function of miR-219 was investigated by methyl thiazolyl tetrazolium (MTT) and transwell assays. The relationship between miR-219 and EYA2 was confirmed by Dual-Luciferase reporter assay.

RESULTS: MiR-219 expression was reduced and was associated with TNM stage and lymph node metastases in PTC patients. Functionally, overexpression of miR-219 restrained the viability and metastasis of PTC cells. In addition, miR-219 induced apoptosis and blocked EMT in PTC cells. Furthermore, miR-219 was confirmed to directly target EYA2 and inhibited its expression in PTC. More importantly, the upregulation of EYA2 impaired the inhibitory effect of miR-219 in PTC.

CONCLUSIONS: MiR-219 inhibits the viability and metastasis of PTC cells by downregulating EYA2.

Key Words:
MiR-219, Papillary thyroid carcinoma, EYA2, Cell viability, Metastasis.

Introduction

In recent years, the incidence of thyroid cancer (TC) has been increasing, accounting for 1% of human malignancies[1]. In addition, TC has more pathological types as curable malignancies, including papillary carcinoma, follicular carcinoma, medullary carcinoma, and undifferentiated carcinoma[2]. Papillary thyroid carcinoma (PTC) is the most common type of TC, accounting for about 70%. PTC metastases are early, and cervical lymph node metastasis is the most common[3]. Due to the limitation of cognitive level and technical conditions, many PTC primary and cervical lymph node metastatic tumors have not been completely removed. As a result, tumor residuals and relapses are highly prone to occur[4]. Therefore, the complete cure of PTC has always been the research direction that needs to be concerned.

MicroRNAs (miRNAs) are short nucleotide sequences that can degrade mRNA or hinder translation by base-pairing with target gene mRNAs[5]. In addition, the specificity and sequence of miRNAs in tissues determines the functional specificity of cells, indicating that miRNAs play multiple roles in regulating cell growth and development[6]. The abnormal expression of miRNAs is related to the progression of PTC. MiR-520a suppressed epithelial-mesenchymal transition (EMT), invasion, and migration of PTC cells[7]. By contrast, miR-144-3p promoted tumor growth and metastasis in PTC by targeting PAX8[8]. Now, the different roles of miR-219 in human cancer have drawn our attention. It was found that miR-219 was upregulated in hepatocellular carcinoma and promoted tumor growth and metastasis by regulating Cadherin 1[9]. Additionally, miR-219-5p was also found[10] to be downregulated in hepatocellular carcinoma and inhibited cell proliferation by targeting glypican-3. Meanwhile, the downregulation and inhibitory effects of miR-219 have also been found in other cancers, such as colorectal cancer and glioma[11,12]. However, the specific role of miR-219 is still unknown in PTC that needs to be illuminated.

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Eyes Absent (EYA) family proteins are involved in the development of diseases and can be used as therapeutic targets for cancer[13,14]. As a member of EYA family, upregulation of the transcriptional coactivator *Drosophila* eyes absent homologue 2 (EYA2) has been detected in epithelial ovarian cancer and promoted tumor growth[15]. In addition, EYA2 was found to induce EMT by mediating the pro-metastatic function of SIX1[16]. However, EYA2 overexpression was found to inhibit tumor growth in human lung adenocarcinoma[17]. Meanwhile, EYA2 silencing was found to promote tumor growth in pancreatic adenocarcinomas[18]. As a target gene, EYA2 regulated the occurrence of cervical cancer by mediating miR-375[19]. However, to date, the interaction between miR-219 and EYA2 has not been elucidated in PTC.

This study focused on the dysregulation of miR-219 in PTC progression. Meanwhile, the regulatory mechanism of miR-219/EYA2 in PTC cells was also elucidated. The results of this study help us to understand the role of miR-219 in PTC.

Patients and Methods

Experimental Sample

Seventy-two patients with PTC from The Affiliated Hospital of Qingdao University participated in the study. All patients with PTC provided written informed consents. PTC patients received no treatment except surgery. The histological diagnosis of PTC was evaluated based on criteria established by the World Health Organization (WHO). These tissues were dissected and immediately frozen into liquid nitrogen until used. The study was approved by the Institutional Ethics Committee of The Affiliated Hospital of Qingdao University.

Cell Culture and Transfection

Human thyroid epithelial cell line Nthy-ori3-1 and SW579 PTC cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The growth conditions were 5% CO_2, 37°C and culture medium [(90% L-15 medium + 10% fetal bovine serum (FBS)]. MiR-219 mimics and inhibitor, EYA2 siRNA and vector (RiboBio, Guangzhou, China) were transferred into SW579 cells, respectively.

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

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The complementary deoxyribose nucleic acid (cDNA) solution was synthesized using the Prime Script 1st Strand cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). We performed qRT-PCR using SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) based on the manufacturer's instruction. MiR-219 or EYA2 were normalized to U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal reference. The expression of miR-219 and EYA2 was calculated using the $2^{-\Delta\Delta ct}$ method. The primers used in our work were as follows: miR-219, forward primer: 5'-ACA CTC CAG CTG GGT GAT TGT CCA AAC GCA A-3', reverse primer: 5'-CTC AAC TGG TGT CGT GGA-3'; U6, forward primer: 5'-CTC GCT TCG GCA GCA CA-3', reverse primer: 5'-AAC GCT TCA CGA ATT TGC GT-3'; EYA2 forward primer: 5'-CGG AAT TCC AGG ATC AGC AGC ATC TCC ACC-3', reverse primer: 5'-CCG CTC GAG TTC AAA ATG TAA ACG TGG TTT TA-3'; GAPDH forward, 5'-ACA TCG CTC AGA CAC CAT G-3', reverse, 5'-TGT AGT TGA GGT CAA TGA AGG G-3'.

Western Blot Analysis

Radioimmunoprecipitation (RIPA) lysis buffer (Beyotime, Shanghai, China) was used to obtain the protein samples. Next, proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, the protein samples were incubated with E-cadherin, N-cadherin, Bax, Bcl-2, EYA2, and GAPDH primary antibodies (Abcam, Cambridge, MA, USA) at 4°C overnight. Then, horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) were added, and the protein samples were incubated for 1 h. Finally, the enhanced chemiluminescence (ECL) kit (Beyotime, Shanghai, China) was used to evaluate the protein bands. Proteins were quantified using Image Lab Software (Bio-Rad, Hercules, CA, USA).

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

The SW579 cell suspension was cultured in L-15 medium containing 10% fetal bovine serum (FBS). Then, a 96-well plate was seeded at a density of 3000 cells per well. After 24, 48, 72 or 96 h of incubation, 20 μL MTT solution (5 mg/mL in PBS) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The incubation was continued

for 4 h, and the culture was terminated. Next, the supernatant was discarded and 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The mixture was shaken for 10 minutes to completely melt the crystals. Finally, a wavelength of 490 nm was selected, and the light absorption value of each well was measured on a Microplate Reader (ELx800, Bio-Tek Instruments, Winooski, VT, USA).

Transwell Assay

First, 60 μL of diluted Matrigel (3.9 μg/μL) was added to the upper chamber to detect cell invasion. Cell migration assay was performed without Matrigel. After 30 min, SW579 cell suspension (4×10^3 cells/well) was added to the upper chamber, and 500 μL L-15 medium (10% FBS) was added to the 24-well plate in the lower chamber. After 24 h of routine incubation, the moving cells were stained with 0.1% crystal violet. Observation and photographing were performed using a light microscope (Olympus Corporation, Tokyo, Japan).

Luciferase Reporter Assay

The 3'-UTR of wild-type or mutant EYA2 was inserted into the pcDNA3.1 plasmid vector (Promega, Madison, WI, USA), respectively. Next, the above Luciferase vector and miR-219 mimics were transfected into SW579 cells. 48 h after transfection, the medium was discarded and washed once with PBS. Finally, we measured Luciferase activity using a Dual-Luciferase assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data are shown as mean ± SD (standard deviation) and analyzed by Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 6 (La Jolla, CA, USA). The differences between two groups were analyzed using the Student's *t*-test. The comparisons between multiple groups were performed using One-way ANOVA test, followed by post-hoc test (Least Significant Difference). Statistical significance was defined at $p<0.05$.

Results

MiR-219 Expression Was Reduced in PTC

The abnormal expression of miR-219 was assessed in PTC. First, miR-219 expression was found to be lower in PTC tissues than in normal tissues ($p<0.01$, Figure 1A). Similarly, compared with Nthy-ori3-1 cells, the downregulation of miR-219 was detected in SW579 cells ($p<0.01$, Figure 1B). In addition, the correlation between miR-219 expression and clinical characteristics in PTC patients was analyzed. Abnormal expression of miR-219 was found to be associated with TNM stage and lymph node metastases in PTC patients ($p<0.05$, Table I). These results indicate that miR-219 may be involved in the tumorigenesis of PTC.

Overexpression of MiR-219 Restrained PTC Cell Viability and Metastasis

Next, gain-loss function experiments were performed in SW579 cells with miR-219 mimics or inhibitor. It was found that the expression of miR-219 was upregulated by its mimics and downregulated by its inhibitor in SW579 cells ($p<0.01$, Figure 2A). MTT assay showed that upregulation of miR-219 restrained the proliferation of SW579 cells. Downregulation of miR-219 pro-

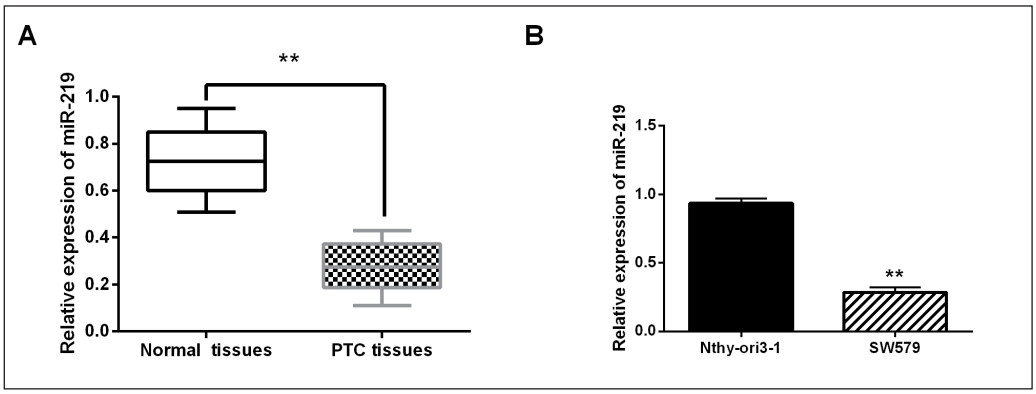

Figure 1. MiR-219 expression was reduced in PTC. **A**, MiR-219 expressions in PTC tissues and normal tissues. **B**, MiR-219 expressions in SW579 cells and Nthy-ori3-1 cells **$p<0.01$

Table I. Correlations between SNHG6 expression and clinicopathological parameters (n = 32).

Characteristics	Cases	MiR-219		*p*-value
		High	Low	
Age (years)				0.13
≥ 45	38	12	26	
< 45	34	8	26	
Gender				0.52
Male	30	6	24	
Female	42	14	28	
Tumor size				0.43
<3 cm	31	7	24	
≥3 cm	41	13	28	
TNM stage				0.01
I-II	48	15	33	
III-IV	24	5	19	
Lymph node metastases				0.02
No	51	15	36	
Yes	21	5	16	

Statistical analyses were performed by the χ^2-test. *$p<0.05$ was considered significant.

moted SW579 cell proliferation ($p<0.05$ or 0.01, Figure 2B). In addition, transwell assay suggested that cell migration was restrained by miR-219 mimics and promoted by miR-219 inhibitor in SW579 cells ($p<0.01$, Figure 2C). Consistently, miR-219 mimics also restrained SW579 cell invasion, while miR-219 inhibitor promoted the invasion of SW579 cells ($p<0.01$, Figure 2D). These results imply that miR-219 has an inhibitory effect on cell viability and metastasis in PTC.

MiR-219 Induced Apoptosis and Blocked EMT in PTC

To further verify the function of miR-219 in PTC, EMT-related gene expression and apoptosis were measured in transfected SW579 cells. We found that the expression of EMT-related genes (E-cadherin, N-cadherin, and vimentin) was significantly regulated by miR-219. E-cadherin expression was enhanced by upregulation of miR-219. However, miR-219 overexpression inhibited the expression of N-cadherin and vimentin ($p<0.01$, Figure 3). Accordingly, miR-219 inhibitor showed opposite effects on the expression of vimentin, N-cadherin, and E-cadherin ($p<0.01$, Figure 3). Next, we explored how miR-219 regulates apoptosis-related proteins (Bcl-2/Bax) in SW579 cells. MiR-219 overexpression was found to suppress Bcl-2 expression and promote Bax expression ($p<0.01$, Figure 3). The downregulation of miR-219 promoted Bcl-2 expression and blocked Bax expression ($p<0.01$, Figure 3). The results reveal that miR-219 induces apoptosis and blocks EMT in PTC.

EYA2 is a Direct Target Gene of MiR-219

In addition, the target gene of miR-219 was searched in TargetScan (http://www.targetscan.org/) database to further reveal its regulatory mechanism in PTC. We found that miR-219 has a binding site to the 3'-UTR of EYA2 (Figure 4A). Next, a Dual-Luciferase reporter assay was designed to confirm the prediction. MiR-219 mimics was found to reduce Wt-EYA2 Luciferase activity and have little effect on Mut-EYA2 luciferase activity ($p<0.01$, Figure 4B). In addition, EYA2 was found to be negatively correlated with miR-219 expression in PTC tissues ($p<0.0001$, $R^2=0.5042$; Figure 4C). Meanwhile, EYA2 expression regulated by miR-219 was examined in SW579 cells. The results showed that miR-219 mimics reduced mRNA and protein expression of EYA2, but miR-219 inhibitors increased EYA2 expression in SW579 cells ($p<0.01$, Figure 4D, 4E). Collectively, miR-219 directly targets EYA2 and inversely regulates its expression in PTC.

Upregulation of EYA2 Impaired the Inhibitory Effect of MiR-219 in PTC

The abnormal expression of EYA2 was detected in PTC. We found that EYA2 was upregulated in PTC tissues and cell lines compared to the control ($p<0.01$, Figure 5A, 5B). To further explore the functional interaction between miR-

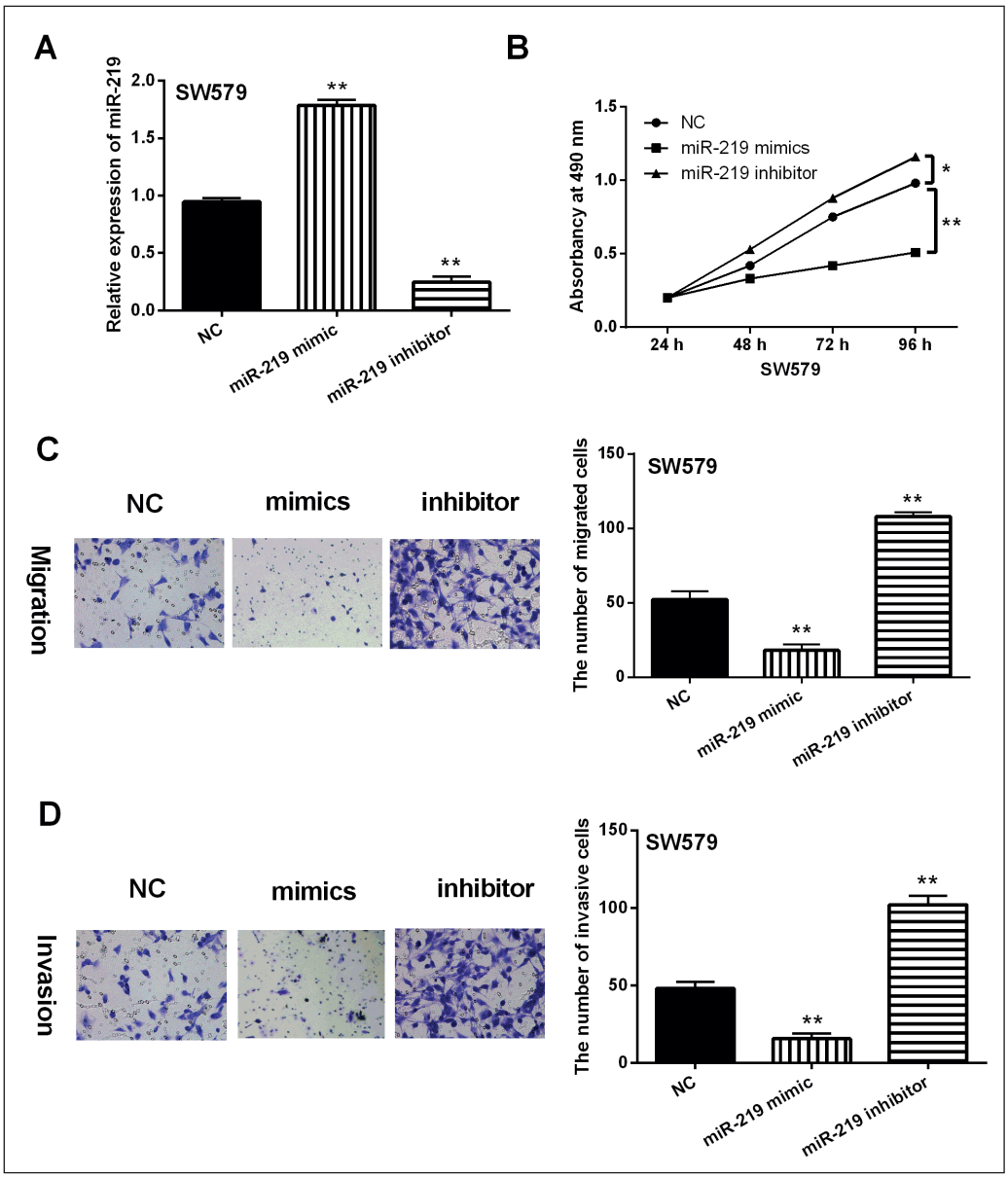

Figure 2. Overexpression of miR-219 restrained PTC cell viability and metastasis. **A**, MiR-219 expression in SW579 cells with its mimics or inhibitor. **B**, **C**, **D**, Cell proliferation, migration and invasion in SW579 cells with miR-219 mimics or inhibitor (magnification: 40×) *p<0.05, **p<0.01.

219 and EYA2 in PTC, EYA2 vector was transfected into SW579 cells with miR-219 mimics. We found that miR-219-mediated reduction of EYA2 expression was restored by EYA2 overexpression vector (p<0.01, Figure 5C). In addition, upregulation of EYA2 eliminated the inhibitory effect of miR-219 on SW579 cell proliferation (p<0.01, Figure 5D). Similarly, EYA2 vector also impaired the inhibitory effect of miR-219 on cell migration and invasion in SW579 cells (p<0.01, Figure 5E, 5F). Taken together, the upregulation of EYA2 weakened the inhibitory effect of miR-219 in PTC.

Discussion

As a new favorite of targeted therapy research, miRNA has become a hot spot in PTC research. In addition, miRNAs have strong

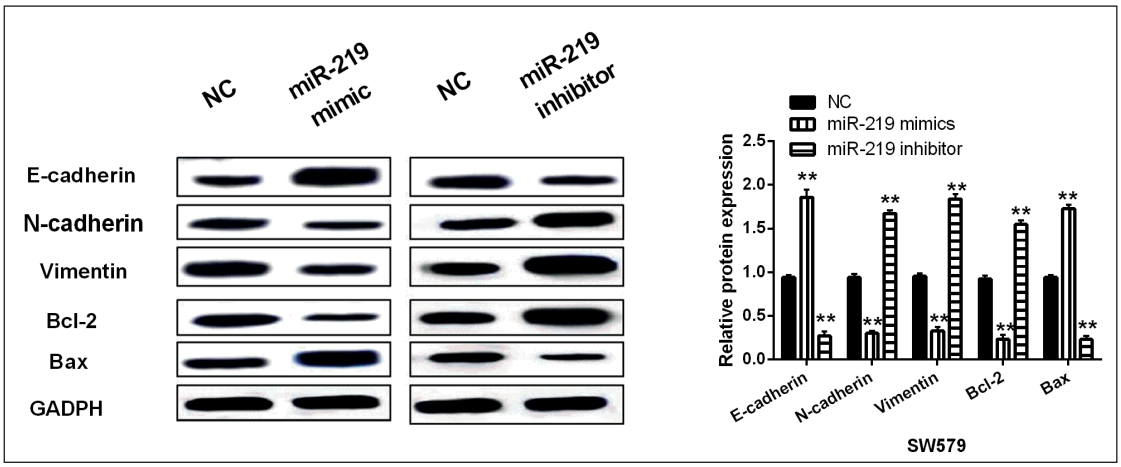

Figure 3. MiR-219 induced apoptosis and blocked EMT in PTC. MiR-219 regulated protein expressions of E-cadherin, N-cadherin, vimentin, Bax and Bcl-2 in SW579 cells.

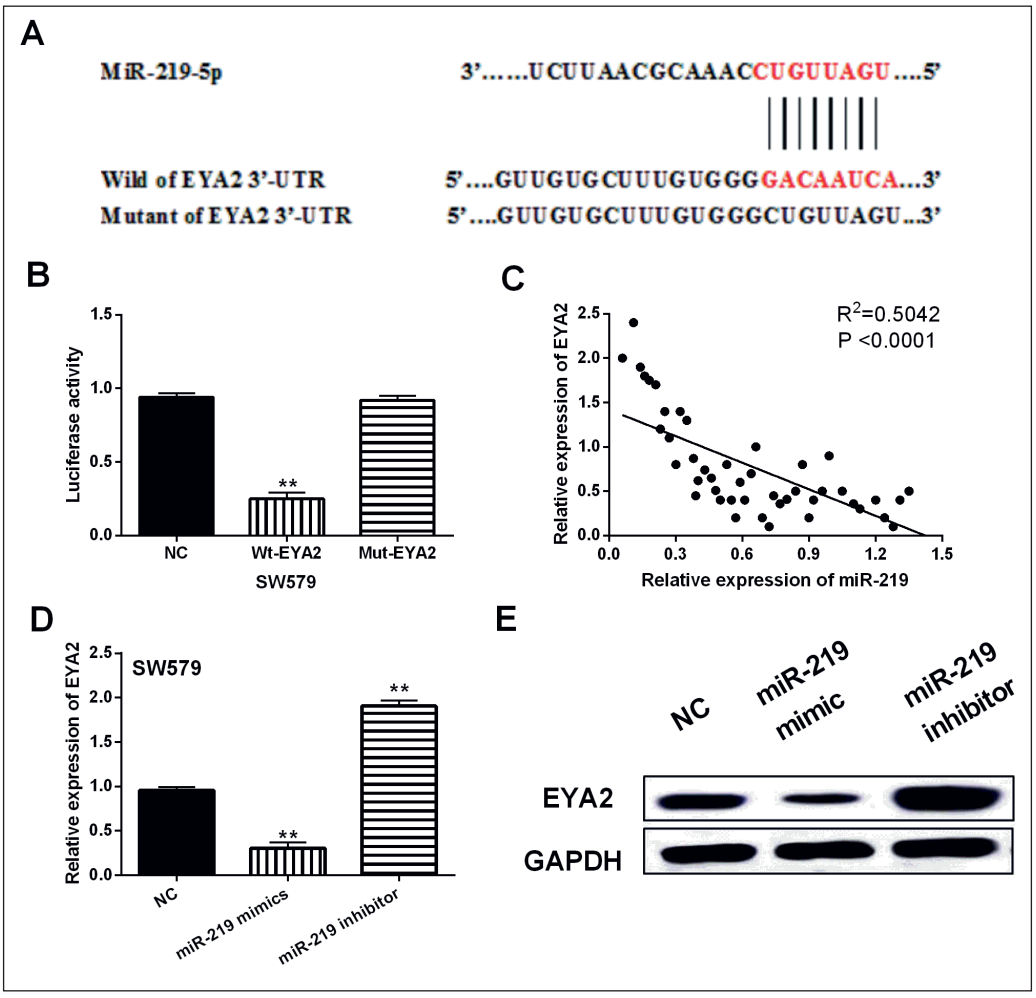

Figure 4. EYA2 is a direct target gene of miR-219. **A**, The binding site between miR-219 and EYA2. **B**, Luciferase reporter assay. **C**, A negative correlation between miR-219 and EYA2 expression in PTC tissues. **D**, **E**, EYA2 expression in SW579 cells with miR-219 mimics or inhibitor $^{**}p<0.01$.

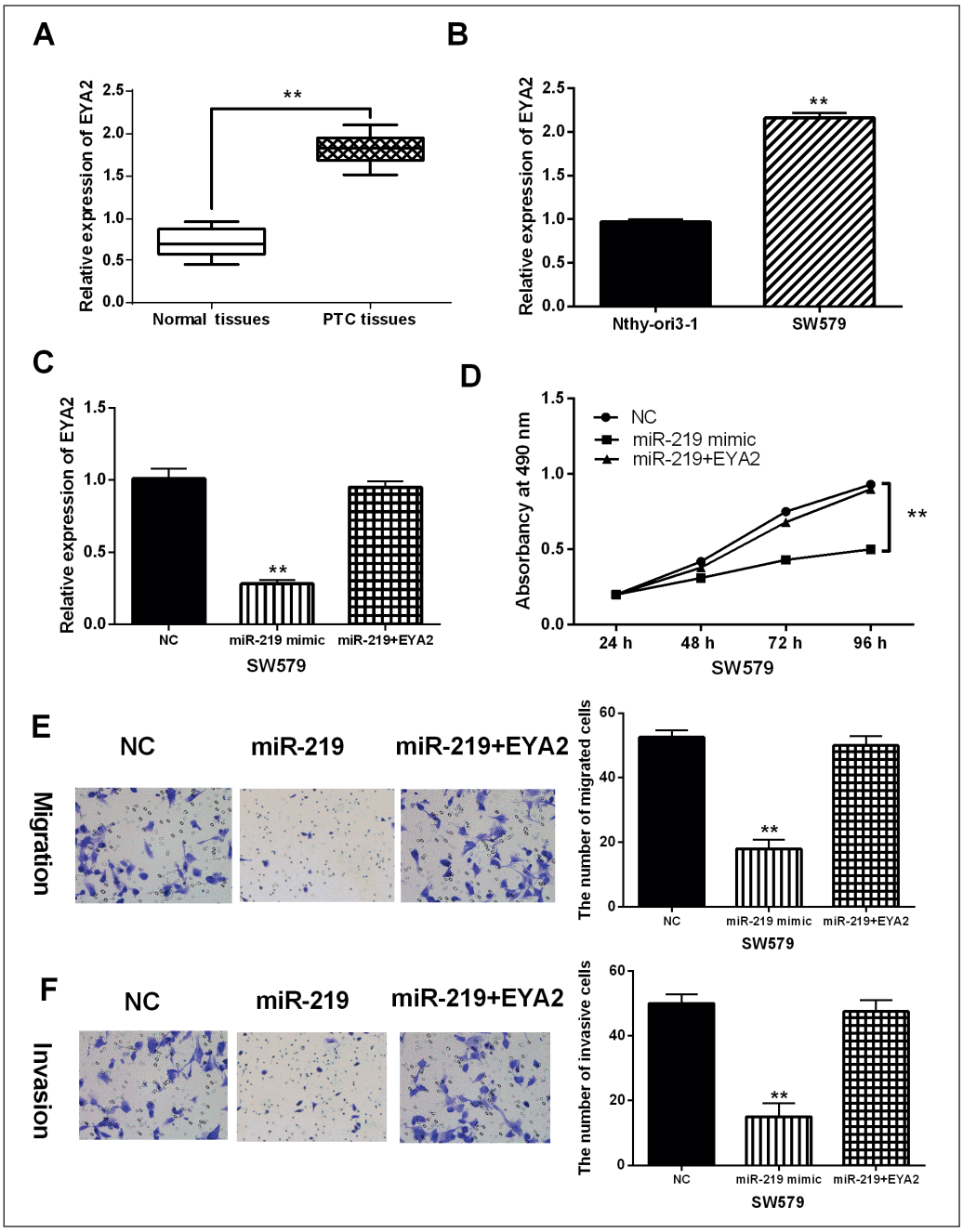

Figure 5. Upregulation of EYA2 impaired the inhibitory effect of miR-219 in PTC. **A**, EYA2 expression in PTC tissues and normal tissues. **B**, EYA2 expression in SW579 cells and Nthy-ori3-1 cells. **C**, EYA2 expression in SW579 cells with miR-219 mimics and EYA2 vector. **D**, **E**, **F**, Cell proliferation, migration and invasion in SW579 cells with miR-219 mimics and EYA2 vector (magnification: 40×) $^{**}p<0.01$.

clinical potential because they can be easily transfected into target tissues. As tumor suppressors, many miRNAs have been found[20,21] in the progression of PTC, such as miR-128 and miR-29a. In this study, miR-219 also acted as a tumor inhibitor in PTC progression. Specifically, the downregulation of miR-219 was observed in PTC, which was related to TNM stage and lymph node metastasis. Functionally, the overexpression of miR-219 restrained cell viability and metastasis in PTC. Meanwhile, miR-219 also induced apoptosis and blocked EMT

in PTC. More importantly, miR-219 played an inhibitory role in PTC by targeting EYA2.

Consistent with our results, the downregulation of miR-219 was also detected in colon cancer and medulloblastoma[22,23]. Functionally, miR-219 was found to inhibit cell proliferation in chordoma and was associated with tumor recurrence[24]. In addition, miR-219 inhibited the migration and invasion of epithelial ovarian cancer cells by regulating the Twist/Wnt/β-catenin signaling pathway[25]. Meanwhile, miR-219 inhibited the growth and metastasis of ovarian cancer cells *via* targeting HMGA2[26]. These results are consistent with the role of miR-219 in PTC, indicating its anti-cancer efficacy. Besides, miR-219 suppressed Bcl-2 expression, thereby inhibiting the growth and metastasis of malignant melanoma[27]. Here, miR-219 also accelerated apoptosis in PTC by inhibiting Bcl-2 expression. Unlike previous studies, we found that miR-219 blocked EMT in PTC. Furthermore, miR-219 directly targets EYA2 and inversely regulates its expression in PTC. Upregulation of EYA2 weakened the inhibitory effect of miR-219 in PTC.

Recently, the important roles of EYA2 have been found in human cancer. Of note, EYA2 was upregulated in lung cancer and promoted cell proliferation[28]. In the present study, upregulation of EYA2 was also identified in PTC. As a target gene, EYA2 has been reported to be involved in tumorigenesis by mediating some miRNAs, such as miR-30a[29]. In addition, miR-30a suppressed cell proliferation and migration in breast cancer by downregulating EYA2[30]. MiR-338-3p/EYA2 axis was also found to restrain breast tumor growth and lung metastasis[31]. Here, a negative correlation between EYA2 and miR-219 expression was found in PTC. Similarly, miR-219-5p has been reported to inhibit osteosarcoma cell migration and invasion by inhibiting EYA2 expression[32]. In our study, we also found that miR-219 restrained cell viability and metastasis in PTC *via* targeting EYA2.

Conclusions

In this study, the downregulation of miR-219 and upregulation of EYA2 were found in PTC. Mechanistically, miR-219 inhibited cell viability and metastasis in PTC *via* targeting EYA2. This study preliminarily elucidated the regulatory mechanism of miR-219 in PTC, but its detailed mechanism still needs to be further explored.

Conflict of Interests

The authors declare that they have no conflict of interests.

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