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MicroRNA-1266 suppresses papillary thyroid carcinoma cell metastasis and growth via targeting FGFR2

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Abstract. – **OBJECTIVE:** **To explore the effects of microRNA-1266 (miR-1266) on metastasis and growth in papillary thyroid carcinoma cells and to provide therapeutic targets for papillary thyroid carcinoma.**

PATIENTS AND METHODS: **By quantitative Real-time polymerase chain reaction (PCR), miR-1266 expression level in 38 pairs of papillary thyroid carcinoma tissues and three breast cancer-derived cell lines was examined. After transfection with miR-1266 mimics, the effects of miR-1266 over-expression on cell proliferation, invasion and migration were analyzed. Further, we employed several databases for the target gene prediction. Dual-luciferase activity assay was performed to verify whether FGFR2 was the direct target gene of miR-1266. Western blotting was conducted to detect protein levels.**

RESULTS: **MiR-1266 was significantly down-regulated in papillary thyroid carcinoma tissue samples and cell lines. Over-expression of miR-1266 in papillary thyroid carcinoma cells significantly attenuated the cell proliferation, invasion, and migration. Dual-luciferase report assay and Western blotting confirmed that FGFR2 was a target gene of miR-1266. Furthermore, up-regulation of FGFR2 partially reversed the suppressive effects of miR-1266 over-expression on cell growth and progression.**

CONCLUSIONS: **miR-1266 could inhibit cell proliferation and progression of papillary thyroid carcinoma via targeting FGFR2. Our findings might provide a new target for the diagnosis and treatment of papillary thyroid carcinoma.**

Key Words:
Papillary thyroid carcinoma, miR-1266, Proliferation and invasion, FGFR2.

Introduction

Thyroid cancer is the most common endocrine organ, accounting for 94.5% of all endocrine tumors and 2.6% of all malignant tumors. The incidence rate of thyroid cancer is the highest among all malignant tumors[1,2]. Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, accounting for about 90% of all pathological types[3]. Most of the adolescents and females suffer from cervical lymph node metastasis. PTC is a low-grade malignant tumor with the tendency of multifocal and regional lymph node metastasis. At present, the treatment of thyroid papillary carcinoma includes surgical treatment, thyroid hormone suppression therapy, isotope iodine 131 treatments and adjuvant radiotherapy. After effective and reasonable treatment, patients generally have a good prognosis. Statistics[4] showed that the 5-year survival rate is about 95% and the 10-year survival rate is more than 90%. However, some papillary carcinomas are with high invasion ability and the tendency of dedifferentiation. These cases are more likely to eventually develop into poorly differentiated thyroid or undifferentiated carcinomas[5], thus leading to poor prognosis. Therefore, elucidating the molecular mechanism involved in cell invasion and metastasis and identifying new predictive biomarkers for papillary thyroid carcinoma, are very important. Recent studies[6-11] about PTC mainly include the following aspects: extracellular signal abnormalities, signal transduction within the cell abnormalities, mutation of tumor suppressor genes, epigenetic alterations and mitochondrial genes change. MicroRNAs (miRNAs) are a class of endogenous, non-coding and single-stranded RNA molecules, consisting of about 19-23 nucleotides. MiRNAs mainly regulate the gene expression through inhibiting or breaking the target mRNAs at the post-transcriptional level *via* binding to the 3'UTR of the target gene. About 30%

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of the protein coding genes in human body are regulated by miRNAs. MiRNAs regulate protein interaction and other intertwined biological networks, thus playing important roles in the occurrence and development of various tumors[12-15]. The present work showed that there were significant differences in the expression of miRNA in PTC, including miR-146b, miR-126, miR-7, miR-221, miR-187, miR-181, miR-30d, miR-155-5p, miR-222 and miR-1[16]. However, only a small amount of miRNAs have been reported in PTC[17,18]. Shen et al[19] found that miR-146b, miR-221, miR-187 and miR-30d could effectively distinguish PTC from benign thyroid lesions. Mazeh et al[20] performed fine needle aspiration biopsy and used miR-221 as a biomarker to distinguish PTC from benign lesions, with a sensitivity of 95%. Kim et al[21] reported that miR-221 promoted tumorigenesis via targeting HOXB5. Chou et al[22] found that in the presence of BRAF mutations in PTC, the expression of miR-146b was significantly higher than that in the PTC without BRAF mutations.

Previous researches demonstrated that the expression of miR-1266 in gastric cancer[23] and breast cancer[24] is significantly down regulated. MiR-1266, as a tumor suppressor miRNA, plays an important role in tumorigenesis, development, invasion and metastasis. However, no evidence is reported regarding the mechanism of miR-1266 in PTC. In our study, 38 cases of papillary thyroid carcinoma were collected and the differential expression of miR-1266 was analyzed. Then, the PTC cell line with the lowest expression of K1 was selected for further exploration. Further, miR-1266 was enhanced by transient transfection of miRNA and mimics vectors, and the functional changes of cells were studied. Finally, the mechanism of miR-1266 and FGFR2 was verified by double luciferase reporter assay.

Patients and Methods

Tissue Samples and Cell Lines

From July 2016 to July 2017, 38 patients with papillary thyroid carcinoma, who first received resection and pathological diagnosis in China-Japan Union Hospital of Jilin University, were collected. Carcinoma tissues and the corresponding adjacent tissues of thyroid adenoma were selected for detection. All specimens were rapidly placed in the tube of inactivated RNA enzyme and stored in liquid nitrogen. All recruited patients signed the informed consent. This study was approved by the Medical Ethics Committee of China-Japan Union Hospital of Jilin University. Human papillary thyroid carcinoma cell lines (K1, TPC-1, BCPAP and Nthy-ori 3-1 cell lines) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). PTC cells were cultured in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 15% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), and were maintained at 37°C with 5% CO_2 in the humid air.

Cells Transfection

MiR-1266 mimics and miR-1266 mimics-NC (normal control) were synthesized and purified by Shanghai Tamar Company (Shanghai, China). One day before transfection, 4-5´10^4 cells were inoculated on the 6-well plate with 2 mL basal medium containing FBS. 24 hours later, miR-1266 mimics/mimics-NC-Lipofectamine™ composite was added to each well for incubation with 5% CO_2 at 37°C for 24 h. Next, cells were observed by fluorescence microscopy. The changes of miR-1266 expression in each group were detected by Real-time polymerase chain reaction (qRT-PCR).

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNAs of tissue samples and cells were isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversed using a miRNA Reverse Kit (TaKaRa, Dalian, China). The miR-1266 expression level was detected using SYBR Premix kits (TaKaRa, Dalian, China) with ABI Step One (ABI, Waltham, MA, USA). U6 was used as internal control. Each experiment was conducted three times. All the relative expression levels were measured using the $2^{-\Delta\Delta CT}$ method.

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

Cells were seeded in the 96-well plate. 50 uL MTT reagent were added to each well for incubation for 4h at 37°C with 5% CO_2. Further, 150 uL dimethylsulfoxide (DMSO) were added to each well. The optical density of each well was detected at 490 nm wavelengths using a microplate reader.

Colony Formation Assay

PTC cells were plated in 6-well plates at a density of 600 per well and maintained in normal medium for 10 days. The colonies were fixed in

70% methanol for 20 min and stained with 0.5% crystal violet for 10 min on ice.

Wound-Healing Assay

About 5×105 cells were added to each well. After the cells grew to 90%, a scratch was made in the middle of the cells with a 20 µl pipette tip; after that, each well was washed 3 times with serum-free medium. After incubation at 37°C with 5% CO_2 for 12 h, the 6-well plate was photographed.

Transwell Assay

48 h after transfection, the cell invasion ability was measured using a Transwell chamber (Corning, Corning, NY, USA) in Matrigel (BD) with a concentration of 1:9 in the upper chamber with a pore size of 8 µm. Subsequently, 250 µL of serum-free medium were added to the upper chamber. 700 µL of medium containing 10% fetal bovine serum (FBS) were added to the lower chamber. Cells (5 x 10^4/well) were then added to the upper chamber and incubated. After 24 h, the chamber was removed and the remaining cells in the upper chamber were gently wiped with a cotton swab. The lower cells were then fixed with paraformaldehyde and stained with crystal violet. Finally, five fields were randomly selected under an inverted microscope (×100) for counting, and the average value was calculated.

Dual-Luciferase Assay

The Dual-Luciferase reporter system (Promega, Madison, WI, USA) was employed to test the activity of luciferase. The FGFR2 3'-UTR cDNA fragment containing the wild type or mutant miR-1266 binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). PTC cells were transfected with miR-1266 mimics and the conducted pGL3 vector using lipofectamine 3000. The activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

Western Blotting

Reagent RIPA (Beyotime, Shangha, China) was utilized to extract protein from cells. BCA protein assay kit (TaKaRa, Otsu, Shiga, Japan) was chosen to quantify protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, they were replaced to the polyvinylidene difluoride (PVDF) membrane, which was incubated with antibodies. Rabbit anti-FGFR2 and rabbit anti-β-actin were provided by Cell Signaling Technology (CST, Danvers, MA, USA), as well as goat anti-rabbit secondary antibody. Chemiluminescent film was applied for assessment of protein expression with Image J software.

Statistical Analysis

SPSS 20.0 (SPSS IBM, Armonk, NY, USA) was used for data processing and analysis. Statistical quantitative data of normal distribution was expressed as mean ± standard deviation (SD). Comparison between groups was done using One-way ANOVA test followed by LSD (Least Significant Difference) post hoc test. Percentage (%) was used to express the enumeration data and x^2-test was used for data analysis. $p < 0.05$ represented that the difference was statistically significant.

Results

MiR-1266 Expressed Lower in PTC Tissues and Cell Lines

To detect the relationship between the miR-1266 expression and PTC, we examined the expression levels of miR-1266 in 38 pairs of PTC tissues and adjacent normal tissues. The expression level of miR-1266 in PTC tissues was significantly lower than that in adjacent normal tissues (Figure 1A). We selected three different PTC cell lines (K1, BCPAP and TPC-1) and a normal thyroid cell line Nthy-ori 3-1 for further study. The expression level of miR-1266 in each cell line was detected using qRT-PCR. The results showed that the expression level of miR-1266 in PTC cell lines was significantly lower than that in normal thyroid cell line, respectively (Figure 1B). To further explore the effect of miR-1266 in PTC, we over-expressed miR-1266 by transfected with miR-1266 mimics. Transfection efficiency was shown in Figure 1C.

MiR-1266 Over-Expression Inhibited the Proliferation of PTC Cells

MTT was used to detect the effect of miR-1266 on the cells proliferation in each group. The results of MTT showed that within 12 h after transfection, there was no significant difference in proliferation ability of three groups. After 12 h, the proliferation ability of the experimental group was significantly inhibited

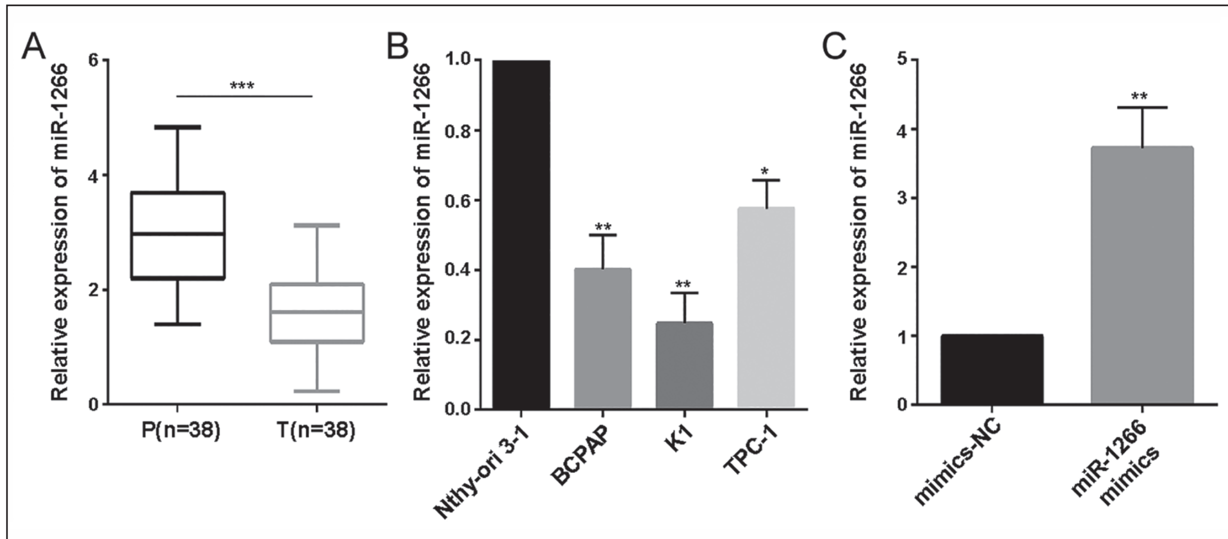

Figure 1. MiR-1266 was down-regulated in PTC tissues and cell lines. **A,** Analysis of the expression level of miR-1266 in 38 pairs PTC tumors and adjacent normal tissues. **B,** Expression of miR-1266 in PTC cells. **C,** Expression of miR-1266 in PTC cells treated with miR-1266 mimics. Data were presented as the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

compared with the control group. After 24 to 72 h transfection, the number of living cells in miR-1266 mimics group was significantly lower than that in the control group (Figure 2A). The results showed that upregulation of miR-1266 could inhibit the proliferation of PTC cells. Also, PTC cells formed lesser colonies after miR-1266 mimics treatment (Figure 2B). All these data suggested that miR-1266 could inhibit PTC cells proliferation.

MiR-1266 Over-Expression Inhibited PTC Cell Migration and Invasion

Cell migration was detected by wound healing assay. We compared the cells migration ability of each group based on the ratio of cell migration area. The migration rate in the mimics-NC group was significantly higher than that in the miR-1266 mimics group (Figure 3A). The results indicated that high expression of miR-1266 could inhibit the migration ability of

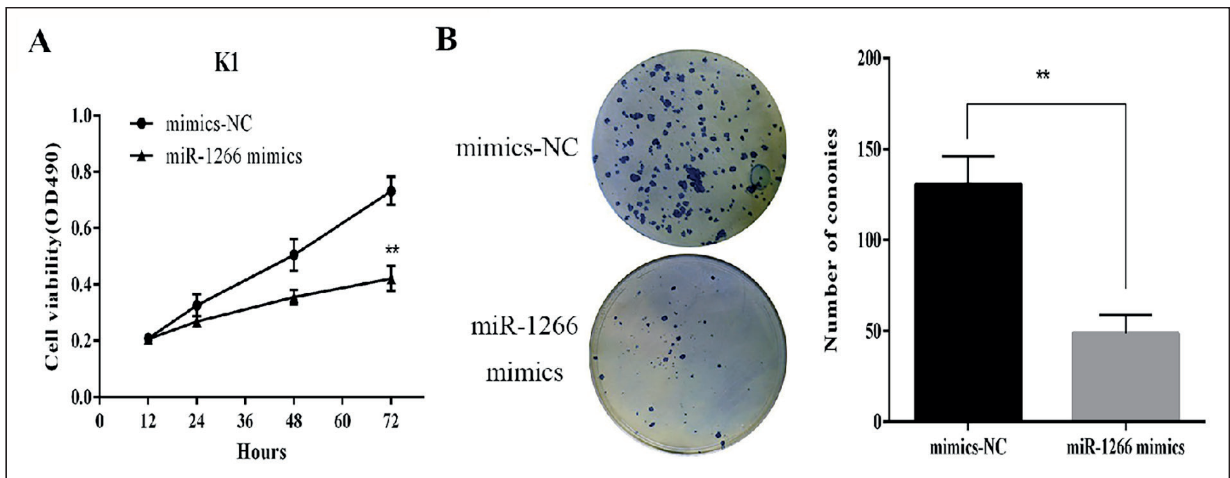

Figure 2. MiR-1266 inhibited the proliferation of PTC cells. **A,** MTT assay was performed to determine proliferation of PTC cells. **B,** Colony formation assay was done to determine the growth of PTC cells. Data were presented as the mean ± SD, **$p < 0.01$.

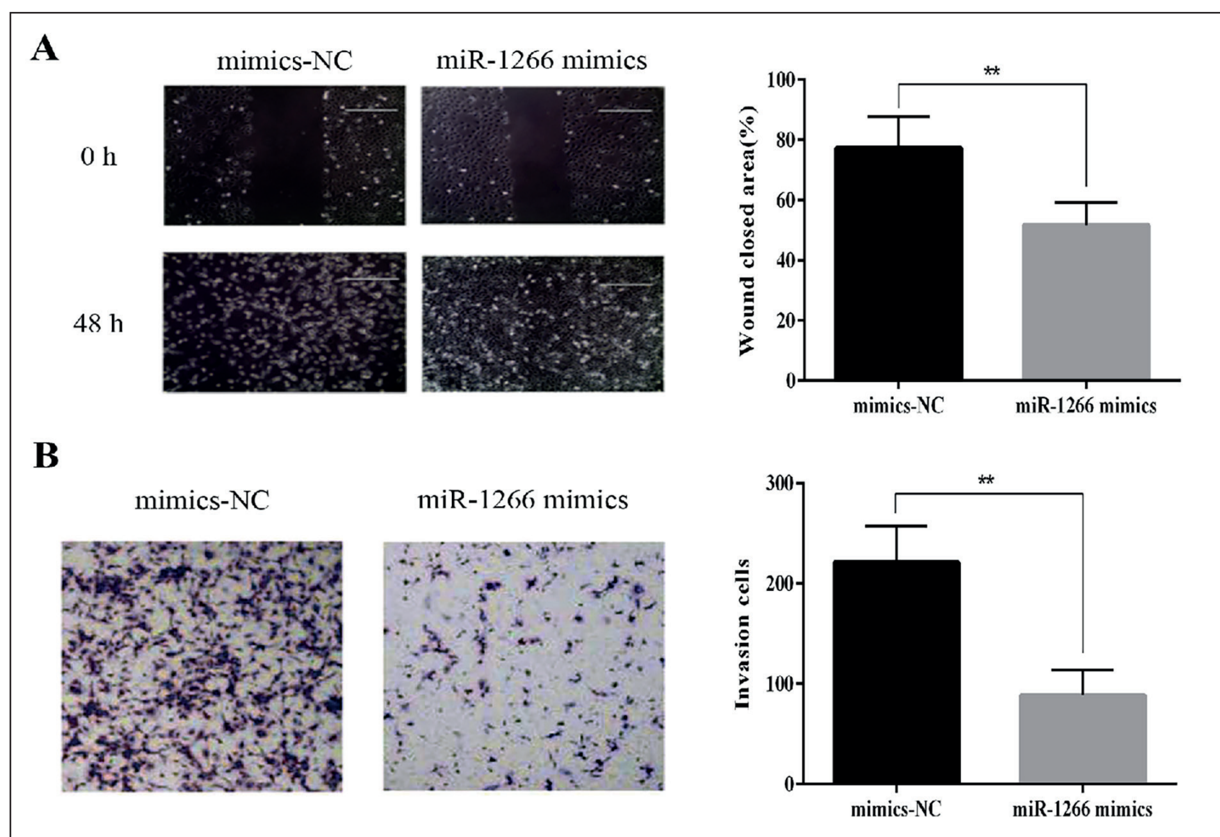

Figure 3. MiR-1266 attenuated the migration and invasion of PTC cells. **A,** Wound-healing assay was performed to determine proliferation of PTC cells. **B,** Transwell invasion assay was used to detect the invasion ability. Data were presented as the mean ± SD, $**p < 0.01$.

PTC cells. Transwell cell invasion assays were used to examine the invasive ability of PTC cells. The results showed that the invasive ability of miR-1266 mimics group was significantly lower than that of mimics-NC group (Figure 3B). Those results suggested that upregulation of miR-1266 could significantly inhibit PTC cell invasion.

FGFR2 was a Target Gene of miR-1266

To further explore the underlying molecular mechanism of miR-1266 involved in PTC, we next searched databases of TargetScan and miR-walk. After comprehensive analysis, we found that FGFR2 could be a candidate target gene of miR-1266. To confirm our prediction, we employed dual-luciferase assay using wild-type or mutant FGFR2 3'-UTR vector (Figure 4A). The result of dual-luciferase assay displayed significant decreased activity in the WT group (Figure 4B). Next, the protein expression of FGFR2 was measured by Western blot analysis. Upregulation of miR-1266 significantly reduced the expression level of FGFR2 protein in PTC cells (Figure 4C). These results suggested that FGFR2 was a direct target of miR-1266.

FGFR2 Over-Expression Counteracted the Effect of miR-1266 Up-Regulation

To further verify whether miR-1266 suppressed PTC cells proliferation, migration and invasion *via* down-regulating FGFR2, we established plasmid pcDNA3.1-FGFR2 to reverse the effect of miR-1266 mimics. The results of MTT assay showed that over-expression of FGFR2 in PTC cells treated with miR-1266 mimics significantly rescued the cell proliferation ability (Figure 5A). Furthermore, the decrease of invasion activity caused by miR-1266 mimics was reversed by FGFR2 up-regulation (Figure 5B). Meanwhile, the results of Western blot assay showed that the expression level of FGFR2 protein was rescued by FGFR2 up-regulation (Figure 5C). These results indicated that miR-1266 could suppress PTC cells proliferation and metastasis *via* targeting FGFR2.

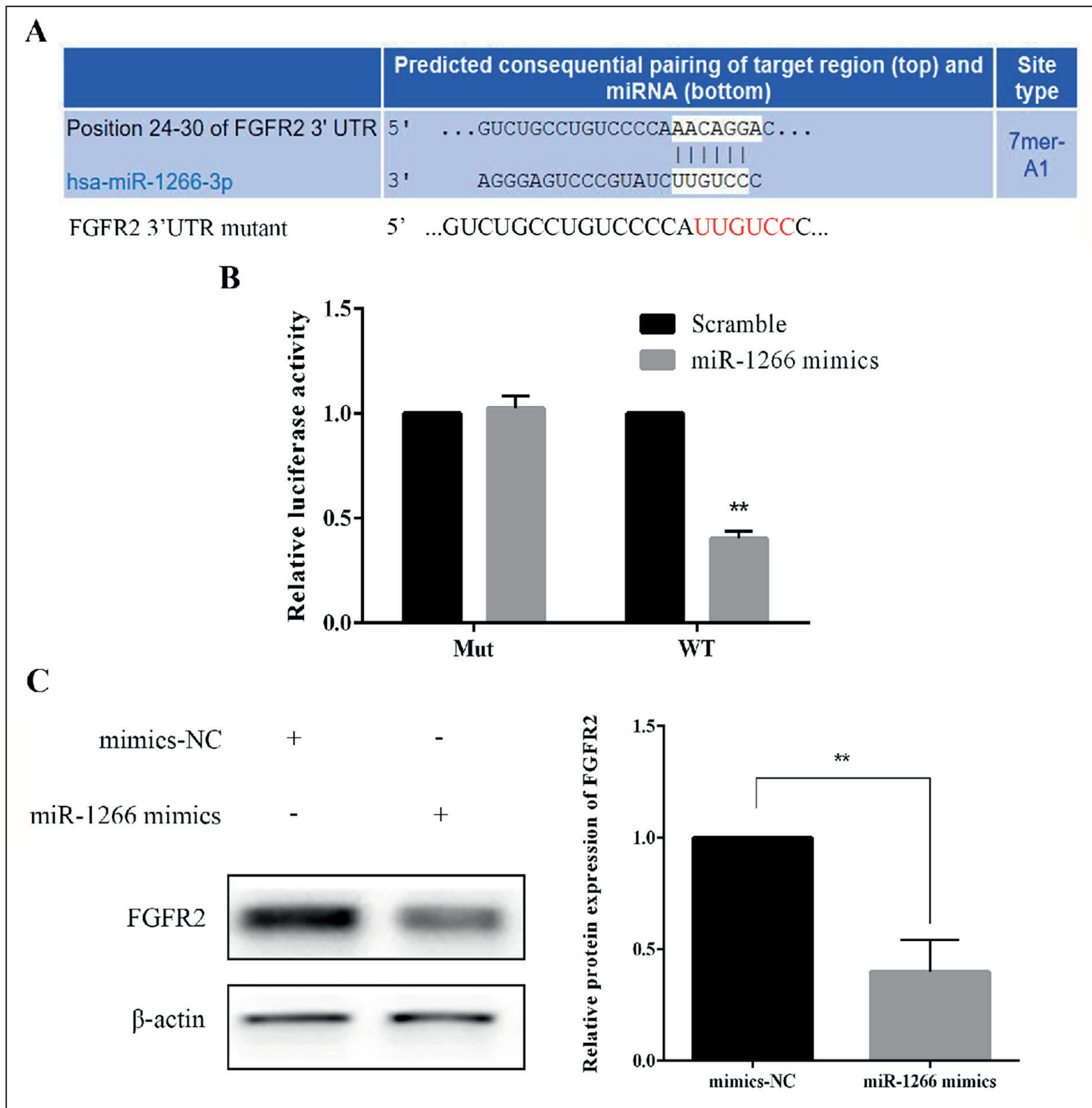

Figure 4. FGFR2 was a direct target of miR-1266. **A,** The predicted binding sites of miR-1266 in the 3'-UTR of FGFR2. **B,** Dual-luciferase reporter assay was employed to determine the binding site. PTC cells treated by mimics or NC were transfected with pGL3 containing WT or mutant FGFR2 3'-UTR site. **C,** FGFR2 protein was measured by Western blotting in miR-1266 overexpression PTC cells. The protein levels were normalized to that of β-actin. Data were presented as the mean ± SD, $**p < 0.01$.

Discussion

At present, major genetic changes involved in the occurrence of PTC included BRAF point mutation and RET/RAS/TRK gene rearrangement. Other small probabilities are PAX8/PPARy gene rearrangements and P53 point mutations. The alterations of these genes led to abnormal activation of various signaling pathways, including MAPK, P13K/AKT, NF-kB and Wnt/β-Catenin. The point mutation of P53 and the activation of Wnt/β-Catenin signaling pathway are considered as the distinct markers for anaplastic thyroid carcinoma[6,25,26]. Zhou et al[27] reported that overexpression of miR-221 was associated with the presence of BRAF mutations in PTC and could

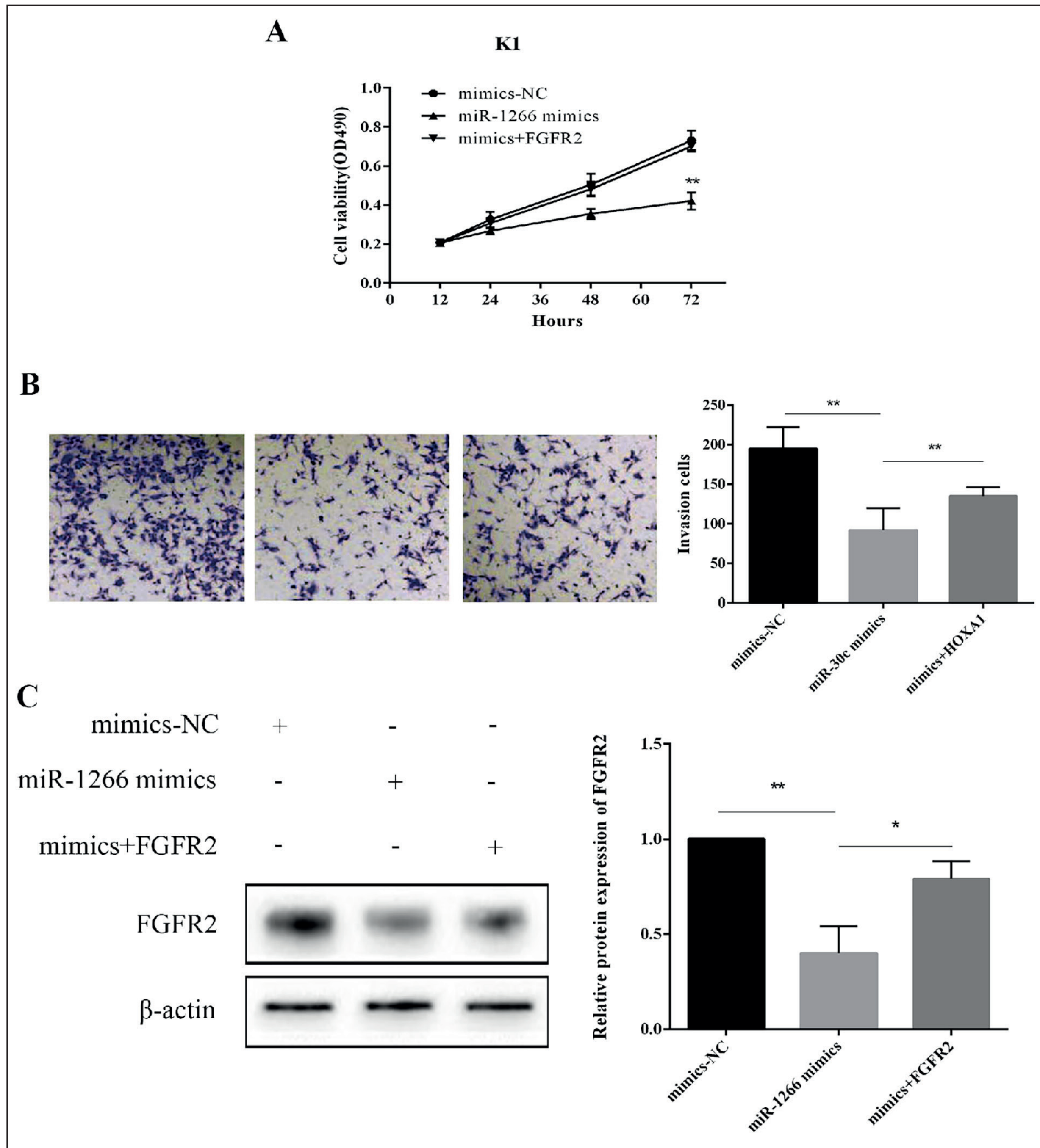

Figure 5. FGFR2 rescued the effects of miR-1266 mimics in PTC cells. **A,** Analysis of the cell proliferation ability. **B,** Cell invasion ability was measured by transwell assay. **C,** Western blotting analyses of FGFR2 expression level. β-actin was used as an internal control. Data were represented as the mean ± SD, $*p < 0.05$, $**p < 0.01$.

be used to screen cases with high metastatic risk. Chou et al[22] found that there was a statistical association between BRAF mutation and miR-146b expression in PTC. Cahill et al[26] screened 21 over-expressed and 14 low-expressed miRNAs in PTC with the RET/PTC rearrangement using microarrays screening. Meng et al[28] found that miR-21 could inhibit proliferation, metastasis and invasion of tumor cells via targeting P13K/AKT/PTEN signaling pathway. Lv et al[29] found that upregulation of miR-26a could promote cell apoptosis in PTC[30]. MiR-30a could attenuate the

transduction of Wnt/β-Catenin signaling pathway by inhibiting BCL9[31]. At present, there have been some reports regarding the mechanism of miR-1266 in other tumors. However, no evidence has reported how miR-1266 can affect the development and progression of PTC.

According to previous functional researches, miR-1266 acted as a tumor suppressor gene in PTC. We conducted loss- and gain- of function experiments using miR-1266 mimics. By MTT assay and colony formation assay, we identified the proliferation activity of established cell lines. Proliferation ability of PTC cells significantly reduced after miR-1266 up-regulation compared to control group. These results indicated that miR-1266 could inhibit PTC cell proliferation. Furthermore, cell migration and invasion were visualized. As reported in breast cancer cells, miR-1266 over-expression also reduced PTC cell migration and invasion. These findings confirmed that miR-1266 inhibited PTC progression and tumorigenesis. As far as we know, this is the first report explaining the function of miR-1266 in PTC.

In order to investigate the molecular mechanism, we speculated FGFR2 as a potential target gene based on data from the bioinformatics databases. FGFR2 (the fibroblast growth factor receptor 2), also known as CD332, is located on chromosome 10. FGFR2 plays an important role in embryonic development, tissue repair and angiogenesis. Like other members of the fibroblast growth factor receptor family, these receptors activate tyrosine kinase activity by binding to their ligands. These signaling molecules regulate cell division, growth, and differentiation. FGFR2 was highly expressed in various human malignancies, such as gastric cancer, lung cancer, breast cancer, ovarian cancer, and endometrial cancer. However, the role of FGFR2 in PTC has not been explored. In order to verify whether FGFR2 was a downstream target of miR-1266, we employed qRT-PCR and Western blot for the detection of FGFR2. We found that upregulation of miR-1266 could inhibit the expression of FGFR2. Then, through dual-luciferase reporter system validation, miR-1266 could specifically act on 3'UTR of FGFR2. Finally, the specificity of miR-1266 acting on FGFR2 was verified. All these data demonstrated that miR-1266 inhibited PTC cell growth and progression via regulating FGFR2. Though we studied the miR-1266 *in vitro*, further studies *in vivo* were still needed to explore the miR-1266 function in PTC.

Conclusions

We first demonstrated that miR-1266 expression was decreased in PTC. Over-expression of miR-1266 could inhibit PTC cell proliferation, migration and invasion. Further, we elucidated that miR-1266 acted as a tumor suppressor in PTC *via* regulating FGFR2. These results could provide a potential target for in the diagnosis and treatment of PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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