Upregulation of miR-421 predicts poor prognosis and promotes proliferation, migration, and invasion of papillary thyroid cancer cells

Anbing Dong[a], Jianhua Zhang[a], Wenhai Sun[a], Hui Hua[a], Yinghe Sun[a,*]

[a]*Department of Thyroid Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China.*

Abstract

Background: Papillary thyroid carcinoma (PTC) represents the most frequent subtype of thyroid cancer (TC) with poor prognosis mainly due to the severe invasion and metastasis. As an oncogene, microRNA-421 (miR-421) is involved in the development of various cancers. This study was to investigate the clinical significance of miR-421 in PTC and its effects on the biological function of PTC cells.

Methods: The expression level of miR-421 in all tissues and PTC cell lines was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Subsequently, the relationship between miR-421 expression and the clinicopathological feature was detected by chi-square analysis in 106 patients with PTC. In addition, Kaplan-Meier and multivariate Cox regression analysis were used to detect the survival time and the prognostic value of miR-421. Finally, the regulatory effect of miR-421 on the proliferation, migration, and invasion ability of PTC cells was detected by Cell Counting Kit (CCK-8) and Transwell assay.

Results: Compared with all control groups, the expression of miR-421 was significantly increased in 106 patients tissues and PTC cell lines ($p < 0.001$). In addition, patients with miR-421 upregulated in PTC showed more positive lymph node metastasis ($p = 0.011$), positive tumor infiltration ($p = 0.031$), and TNM stage III/IV ($p = 0.019$), and when miR-421 expression level was elevated, the survival rate of PTC patients was poor (log-rank test, $p = 0.023$). Furthermore, miR-421 might be an independent prognostic biomarker for PTC (hazard ratio [HR] = 3.172, 95% CI = 1.071-9.393, $p = 0.037$). Finally, increased levels of miR-421 can significantly promote cell proliferation, migration, and invasion ($p < 0.01$).

Conclusion: miR-421 is a novel oncogene of PTC and is a valuable prognostic biomarker. Moreover, the upregulation of miR-421 enhances the proliferation, migration, and invasion of PTC cells.

Keywords: miR-421; Papillary thyroid cancer; Prognosis; Progression

1. INTRODUCTION

Thyroid cancer (TC) is an endocrine disease with a high frequency of occurrence, mainly occurring in women.[1] It is reported that the environmental pollution, impaired immune system, and changes in the diet contribute to the occurrence of TC.[2] The number of newly diagnosed TCs cases in China has increased from 54 175 in 2010 to 201 000 in 2015.[3,4] Papillary thyroid carcinoma (PTC) is a dominant TC histologic type, accounting for about 80% of all TC cases.[5] Currently, the total thyroidectomy and neck dissection have improved the outcomes of PTC patients, leading to the PTC's 5-year survival higher than other cancers, reaching 84.3% in China from 2012 to 2015.[6] However, >75% of PTC patients had local lymph node metastasis at initial diagnosis, and 17% of patients have diffuse metastasis, mainly to liver, lung, and bone, which significantly increases the mortality of PTC.[6–9] Although the treatment of PTC has made great progress, the prognosis still needs further improvement. Therefore, finding new prognostic markers is critical for further treatment for PTC.

MicroRNAs (miRNAs) are highly conserved RNAs composed of approximately 18 to 25 nucleotides in length, which regulate the expression of the coding gene by binding to mRNA.[10] Functional studies have shown that miRNA plays a key regulatory role in tumor proliferation, apoptosis, differentiation, metastasis, drug sensitivity, and prognosis.[11–13] Li et al[14] reported in 2018 that overexpression of miR-421 is a new and valuable prognostic marker for non–small cell lung cancer and can promote tumor progression. As one of the numerous miRNA families, miR-421 abnormally expressed in a variety of cancers, such as breast cancer, gastric cancer, and osteosarcoma.[15–17] Romeo et al[18] found 51 miRNA differentially expressed in non-neoplastic cancer and TC including miR-421 through miRNA microarray analysis, but its potential mechanism in PTC remains to be further clarified.

In the current study, we examined the association between miR-421 and clinical features of patients and analyzed their role in biological behavior. Our data confirmed that miR-421 is a potential tumor oncogene and can be used as a new prognostic marker of PTC.

*Address correspondence. Dr. Yinghe Sun, Department of Thyroid Surgery, The Affiliated Hospital of Qingdao University, 16, Jiangsu Road, Qingdao, Shandong 266071, China. E-mail address: yinghesun_lotus@163.com (Y. Sun).

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

Journal of Chinese Medical Association. (2020) 83: 991-996.

Received March 3, 2020; accepted May 14, 2020.

doi: 10.1097/JCMA.0000000000000426.

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<zdoi: 10.1097/JCMA.0000000000000426>

2. METHODS

2.1. Sample tissue and clinical information

The criteria for inclusion in the study were as follows: (1) histopathological diagnosis according to the World Health Organization's classification criteria for endocrine tumors and confirmed that all PC patients were PTC patients;[19] (2) all patients were diagnosed with a thyroid tumor for the first time; (3) they had not received any tumor surgery, head, and neck radiotherapy and adjuvant therapy. Exclusion criteria were as follows: (1) pregnant women or lactating women and (2) patients with incomplete or missing clinical data. A total of 106 PTC patients enrolled in The Affiliated Hospital of Qingdao University from August 2010 and July 2013. The PTC tissues and normal non-tumor thyroid tissue specimens were collected during surgical resection. At the time of the study, according to the seventh edition of the Joint Committee on TNM Staging Systems of the United States, all PTC patients were named early stage I or II at the time of diagnosis, and later named stage III or IV.[20] The study was conducted with the Ethics Committee of the Affiliated Hospital of Qingdao University and all patients signed informed consent. The patients were followed up for 5 years after treatment and the patient's condition was recorded. The clinical and pathological data of patients included in the study are shown in Table 1.

2.2. Cell culture and transfection

The normal thyroid follicular epithelium cell line (Nthy-ori3-1) and PTC cell lines (TPC-1, IHH-4, GLAG-66) were purchased from the American Type Culture Collection (ATCC). The cells were cultured in a medium supplemented with 10% fetal bovine serum (FBS) in RPMI 1640 medium and the conditions of the incubator were 5% CO_2 concentration, 95% humidity, and 37°C. PTC cell was transfection with miR-421 mimic, mimic negative control (NC), miR-421 inhibitor, or inhibitor NC for cell function assay. The transfection reagent was Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA), the liquid was changed 6 hours after transfection, the follow-up experiment was conducted 24 hours later.

Table 1

Correlation between miR-421 expression levels and clinical features in papillary thyroid cancer patients

Parameters	Cases no. (n = 106)	miR-421 expression Low (n = 47)	miR-421 expression High (n = 59)	*p*
Age (years)				
<45	48	22	26	0.845
≥45	58	25	33	
Gender				
Male	50	23	27	0.845
Female	56	24	32	
Tumor size, cm				
<2	49	23	26	0.696
≥2	57	24	33	
Tumor infiltration				
Yes	48	27	21	0.031
No	58	20	38	
TNM stage				
I/II	49	28	21	0.019
III/IV	57	19	38	
Lymph node metastasis				
Negative	48	28	20	0.011
Positive	58	19	39	

2.3. RNA isolation and quantitative real-time polymerase chain reaction assay

Total RNA was extracted from tissues of 106 patients and stably transfected cells using Trizol reagent at –4°C. The extracted RNA was reverse-transcribed into complementary DNA (cDNA) using the miR cDNA synthesis kit (Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (qRT-PCR) reaction experiment was conducted on the 7300 real-time PCR system of American applied biological system using SYBR Green I Master Mix Kit. U6 small nuclear RNA as an internal control. The primer sequences for U6: forward, 5′-CTCGCTTCGGCAGCACA-3′, reverse, 5′-AACGCTTCACGAATTTGCGT-3′; primer sequences for miR-421: forward, 5′-ATCAACAGACAUUAATT-3′, miR-421 reverse, 5′-AACGCTTCACGAATTTGCGT-3′. Relative RNA expression level assessment using the $2^{-\Delta\Delta Ct}$ method.

2.4. CCK-8 proliferation assay

The proliferation of PTC cells after transfection was detected by Cell Counting Kit-8 (CCK-8) assay. Transfected cells were seeded at a density of 1×10^4 cell/plate. Each well was added with 10 μL CCK-8 reagent at a different time point (0, 24, 48, and 72 hours). Following a 2 hours of further incubation, the change in absorbance at 450 nm of the cell cultures was measured (USA).

2.5. Transwell assay

PTC cell migration and invasion assays were measured by Transwell with a pore size of 8 μm. In the invasion experiment, Matrigel (BD Biosciences, Bedford, MA) was applied to the upper chamber. Transfected cells were seeded in the Transwell's upper chamber in serum-free medium at a concentration of 1×10^5. The medium containing 10% FBS was added to the lower part of the chamber and placed in an incubator for 24 hours. The noninvasive cells and Matrigel in the upper chamber were completely removed with a cotton swab, fixed in methanol for 10 minutes, and stained with 0.1% crystal violet for 20 minutes. Five files were randomly selected by a microscope for photography and counting. Similarly, migration assay was carried out using the Transwell upper chamber without Matrigel coating, and other experimental procedures were consistent with the invasion assay.

2.6. Statistical analysis

All tissues and cell experiments were performed in three independent replicates and the results were mean ± SD. Statistical analysis of the data was performed using SPSS 22.0 software and GraphPad Prism 7.0 software. The difference of miR-421 expression between PTC patients and the control group was analyzed by Student's *t* test. According to the mean value of the miR-421 expression, the patients were divided into miR-421 low expression group and high expression group. The chi-square test was used to analyze the relationship between miR-421 expression and clinicopathological parameters. Kaplan-Meier curves were plotted for the enrolled patients, and multivariate analyses were performed by Cox regression analysis to evaluate independent prognostic factors. The bilateral $p < 0.05$ as the difference was statistically significant.

3. RESULTS

3.1. miR-421 was upregulated in tissues and cells

To investigate the expression of miR-421 in patient tissues and cell lines, we performed qRT-PCR in 106 PTC tissues and normal adjacent tissues. As shown in Fig. 1A, miR-421 expression was significantly increased in PTC tissues compared with

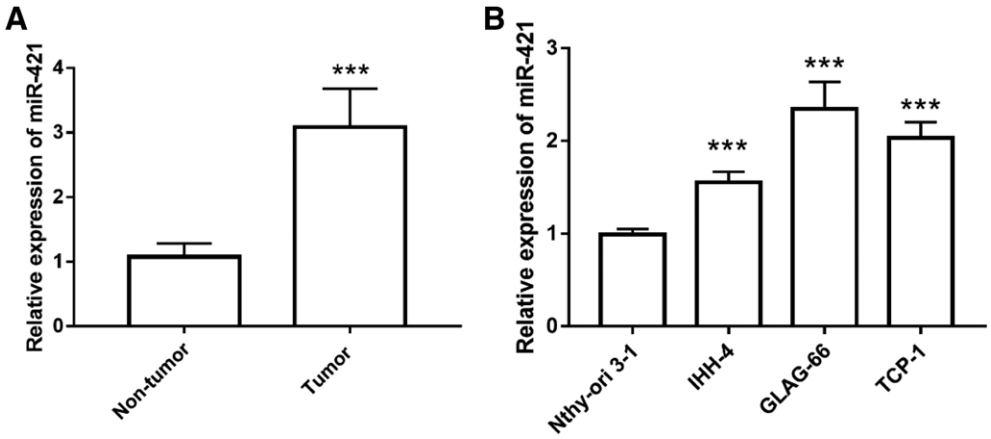

Fig. 1 miR-421 expression was elevated in PTC cancer tissues and cell lines. **A**, A total of 106 matched pairs of tumor and nontumor tissue samples were evaluated for miR-421 expression. Compared with nontumor tissues, miR-421 in PTC was significantly upregulated (***$p < 0.001$). **B**, miR-421 was expressed in different PTC cell lines and thyroid epithelial cells, and miR-421 was highly expressed in all three PTC cell lines. Every result was from three independent experiments (***$p < 0.001$). PTC = papillary thyroid carcinoma.

normal control tissues ($p < 0.001$). In addition, the expression level of miR-421 in PTC cell line (IHH-4, GLAG-66, TCP-1) was significantly increased compared with the normal thyroid epithelial cells Nthy-ori3-1 (Fig. 1B, $p < 0.001$).

3.2. Correlation between miR-421 expression and clinical features of PTC patients

According to the mean expression level of miR-421 in tissues, PTC patients were divided into two groups: miR-421 high expression group (n = 59) and miR-421 low expression group (n = 47). The relationship between miR-421 expression in different groups of patients and clinical pathology is shown in Table 1. Chi-square analysis showed that high expression of miR-421 showed more lymph node metastasis ($p = 0.011$), TNM stage III/IV ($p = 0.019$), and tumor infiltration ($p = 0.031$). However, we did not found any difference between the miR-421 level and gender ($p = 0.845$), age ($p = 0.845$), and tumor size ($p = 0.696$).

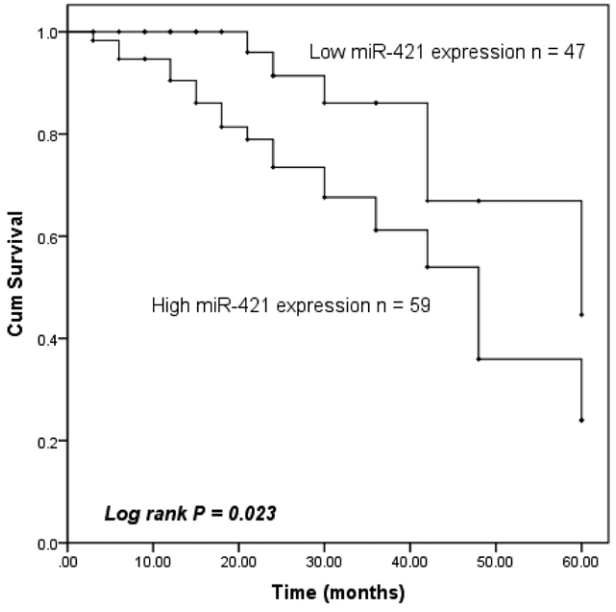

Fig. 2 Kaplan-Meier analysis of miR-421 in patients with PTC. As shown in the figure, PTC patients with high miR-421 expression have short survival time than PTC patients with low expression (log-rank $p = 0.023$). PTC = papillary thyroid carcinoma.

3.3. miR-421 as a prognostic biomarker in PTC patients

To elucidate the effect of miR-421 on the prognosis of patients with PTC, we investigated the relationship between miR-421 expression and prognosis in long-term follow-up patients. Kaplan-Meier analysis demonstrated shorter survival in patients with high expression of miR-421 compared with low expression of miR-421 (log-rank test, $p = 0.023$; Fig. 2). In addition, the multivariate Cox regression analysis indicated that miR-421 was an independent prognostic factor for PTC patients (hazard ratio [HR] = 3.172, 95% CI = 1.071-9.393, $p = 0.037$; Table 2).

3.4. miR-421 regulated cell proliferation, migration, and invasion in vitro

Transfection efficiency was first determined prior to cell function assays. The expression of miR-421 was detected by qRT-PCR in GLAG-66 and TCP-1 cells transfected with miR-421 mimics, inhibitors, or miR-NC, respectively. The results showed that the expression level of miR-421 in the mimic group was significantly higher than that in the control group, while the expression level in the inhibitor group was significantly decreased. The experimental results demonstrated the high transfection efficiency of cells ($p < 0.01$; Fig. 3A).

Table 2

Multivariate Cox analysis of miR-421 and clinical parameters in relation to overall survival

	Multivariate analysis		
Characteristics	HR	95% CI	p
miR-421	3.172	1.071-9.393	0.037
Age	0.761	0.335-1.726	0.513
Gender	0.648	0.289-1.455	0.293
Tumor size	0.519	0.197-1.369	0.185
Tumor infiltration	0.217	0.039-1.198	0.080
TNM stage	0.252	0.052-1.234	0.089
Lymph node metastasis	3.173	1.036-9.716	0.043

HR = hazard ratio.

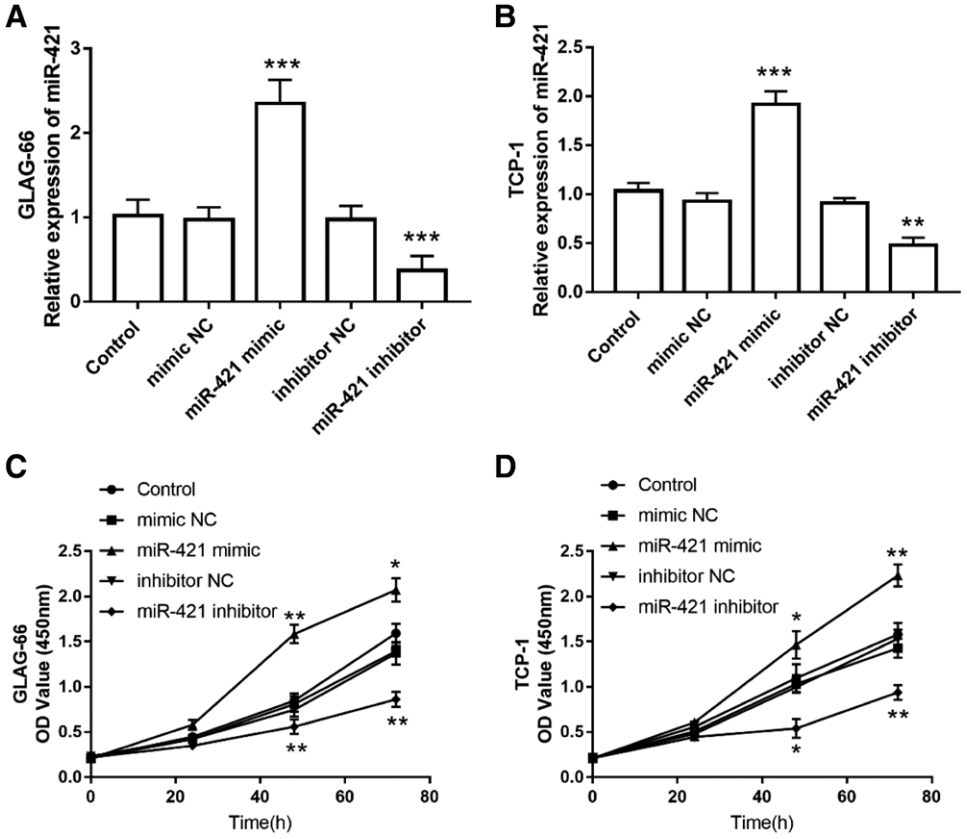

Fig. 3 Effects of miR-421 expression on the proliferation of PTC cells. **A**, The expression levels of miR-421 in PTC cells after transfection with mimics and inhibitors were evaluated by qRT-PCR (***$p < 0.001$). **B**, The proliferation ability of the transfected cells was detected by CCK-8 assay. miR-421 mimic could significantly promote the proliferation of the cells, while miR-421 inhibitor significantly inhibited the proliferation of the cells. Every results was from three independent experiments (*$p < 0.05$, **$p < 0.01$). CCK-8 = Cell Counting Kit; qRT-PCR = quantitative real-time polymerase chain reaction.

Cell proliferation potential was determined by CCK-8 assay. Compared with control, increased miR-421 significantly promoted cell proliferation, while decreased miR-421 significantly inhibited cell proliferation ($p < 0.01$; Fig. 3B). In addition, cell migration and invasion abilities were tested by the Transwell assay. The data showed that increased miR-421 significantly promoted cell migration and invasion compared with the control group, downregulation of miR-421 significantly inhibited cell migration and invasion ($p < 0.001$; Fig. 4A, B). These data indicate that increased expression of miR-421 can significantly promote the proliferation, migration, and invasion of PTC cells.

4. DISCUSSION

TC is a common endocrine malignant tumor, and its incidence has increased rapidly in recent decades.[21,22] PTC is considered to be the most prominent subtype of TC, accounting for >80% of all TC.[5] Although PTC grows slowly and has good prognosis and treatment response, the 5-year survival rate of patients with advanced PTC is only 59%, because PTC often metastases to lymph nodes, leading to local tumor recurrence and increasing mortality, so it is necessary to find an effective prognostic marker for patients with these aggressive tumors.[23–27]

Many studies have demonstrated the usefulness of miRNA in the occurrence and development of certain types of malignancies. It acts as an oncogene or tumor suppressor gene to regulate key processes such as tumor proliferation, differentiation, metastasis, apoptosis, and drug resistance. Wei et al[28] reported that miRNA-135a regulates Hut78 cell proliferation through the GATA3/TOX signaling pathway. In recent years, more and more people have realized that miRNA can be used as biomarkers to predict the prognosis and treatment response of various cancers.[29,30] For instance, miR-206 and miR-145 are important prognostic markers for breast cancer.[31] The upregulation of miRNA plays a key regulatory role in the progression of breast cancer and suggests that miR-17 is a potential biomarker for the prognosis of breast cancer.[32] Serum miRNA-1290 is a valuable biomarker for the diagnosis and prediction of esophageal squamous cell carcinoma.[33]

Previous studies have confirmed that miR-421 is abnormally expressed in a variety of tumors and can serve as an effective prognostic and diagnostic tool for tumors. Zhou et al[17] observed that the expression of miR-421 is generally elevated in osteosarcoma tissues and serum, which may be an effective marker for the diagnosis and prognosis of osteosarcoma. Hu et al[34] reported that miR-421 is upregulated in breast cancer tissues and regulates apoptosis.

We have demonstrated for the first time that miR-421 is significantly increased in PTC tissues and cells, consistent with previously studied on the upregulation of miR-421 expression in human tumors such as pancreatic cancer, gastric cancer, hepatocellular carcinoma, and nasopharyngeal cancer.[35–38] Therefore, we speculated that miR-421 may play a key regulatory role in PTC as an oncogene. To further confirm our hypothesis, we investigated the relationship between clinical features and miR-421 expression in patients. The results of the study showed that patients with high expression of miR-421 had a higher probability of lymph node metastasis, TNM stage III/IV, and tumor

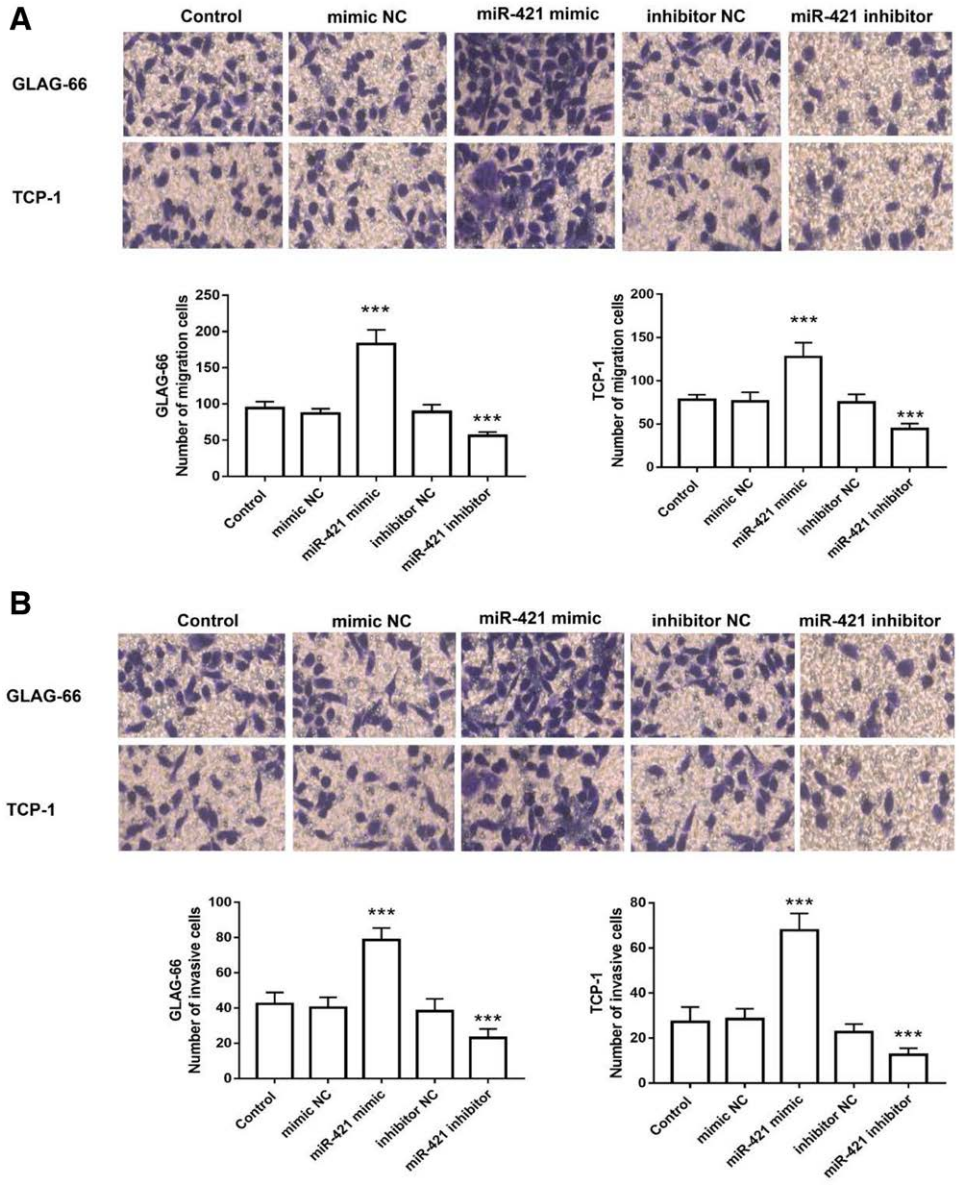

Fig. 4 Transwell analyzed the effects of miR-421 on PTC cell migration and invasion. **A**, The effect of miR-421 on the migration ability of PTC cells. miR-421 inhibitors significantly inhibitor cell migration, while miR-421 mimic significantly promoted cell migration. **B**, The effect of miR-421 on the invasion of PTC cells. miR-421 inhibitors significantly inhibitor cell invasion, while miR-421 mimic significantly promoted cell invasion. Every result was from three independent experiments (***$p < 0.001$). PTC = papillary thyroid carcinoma.

invasion. In addition, Kaplan-Meier survival analysis showed that patients with high expression of miR-421 had shorter overall survival time, indicating that miR-421 is associated with poor prognosis in patients with PTC. This was consistent with previously reported high expression of miR-421 as a prognostic marker for non–small cell lung cancer, pancreatic, and hepatocellular carcinoma.[38–40] Meanwhile, the Cox model results indicated that miR-421 is an independent prognostic marker of PTC. In summary, we confirmed the expression level and clinical significance of miR-421 in PTC for the first time, proving that miR-421 can be used as a biomarker to determine the prognosis of PTC.

It has been previously reported that miR-421 can affect a variety of different tumor cell functions, but the study of PTC cell function is still unclear. The experimental study confirmed that the lower expression of miR-421 could significantly inhibit the proliferation, migration, and invasion of PTC cells. The effect with miR-421 in other cancer is the same and shows that miR-421 in the progress of the PTC is a potential cancer gene. Recent studies have shown that miR-421 plays its carcinogenic role in osteosarcoma cells by targeting LTBP2.[41] It is noted that as a direct target of miR-421, PDCD4 can significantly eliminate the carcinogenic effect of miR-421 in non–small cell lung cancer.[42] At the same, it has been reported that miR-21 regulates the biological behavior of PTC by targeting PDCD4.[43] MiR-183 significantly negatively regulated the expression of PDCD4 protein in PTC cells.[44] Therefore, in our study, we hypothesized that PDCD4, as a potential target of miR-421, regulates the proliferation, migration, and invasion of PTC and plays the role of its oncogene. However, the mechanism of miR-421 in PTC is still unclear and needs further exploration.

In conclusion, a series of experiments confirmed that miR-421 can promote the proliferation, migration, and invasion of PTC cells, suggesting the potential of miR-421 as a potential therapeutic target for PTC treatment. At the same time, the elevated expression of miR-421 in PTC tissues is significantly associated with the patient's prognosis, indicating that it can be used as a valuable prognostic biomarker for patients with PTC.

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