European Review for Medical and Pharmacological Sciences 2017; 21: 3794-3800

MiR-577 inhibits papillary thyroid carcinoma cell proliferation, migration and invasion by targeting SphK2

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Abstract. – OBJECTIVE: MiRNAs are small, noncoding RNA molecules that serve as important regulators of cancer-related processes. Abnormal expression of miR-577 has been found in several tumors. However, the expression pattern and biological function of miR-577 in progression of papillary thyroid cancer (PTC) remain unknown. This study is aimed to determine its expression pattern and explore the function underlying the mechanism of miR-577 in PTC.

PATIENTS AND METHODS: Using quantitative RT-PCR, we detected miR-577 expression in PTC cell lines and primary tumor tissues. MTT assay and colony formation were performed to measure the viabilities of tumor cells. Transwell invasion and migration assays were used to test the invasion and migration of PTC cells transfected with miR-577 mimic. TargetScan, miRanda and PicTar were used to analyze whether sphingosine kinase 2 (SphK2) was a potential target gene. Next, the direct target gene of miR-577 was also identified by luciferase reporter assays and Western blot analysis.

RESULTS: The results showed that miR-577 was significantly downregulated in PTC tissues and cell lines. The up-regulation of miR-577 inhibited the proliferation, migration and invasion of PTC cells *in vitro*. Furthermore, bioinformatics analysis indicated that SphK2 was a putative target of miR-577. A luciferase reporter assay further confirmed that SphK2 was a direct target of miR-577. The results of Western blot indicated that the expression level of miR-577 was negatively correlated with the expression level of SphK2 in PTC tissues. In addition, knockdown of SphK2 significantly suppressed PTC cells proliferation, migration and invasion.

CONCLUSIONS: Our findings indicate that miR-577 is a potential tumor suppressor in PTC by targeting SphK2, and may be a potential therapeutic target in PTC.

Key Words:
miR-577, Papillary thyroid carcinoma, SphK2, Proliferation, Migration, Invasion.

Introduction

Thyroid cancer is one of the more common endocrine malignancies and its incidence is increased in recent years[1,2]. Thyroid cancer is classified into four types: papillary, follicular, medullary, and anaplastic thyroid cancer[3]. Papillary thyroid carcinoma (PTC) is known to have a favorable prognosis with a cancer-related mortality rate <10%[4]. However, a regional recurrence or distant metastasis is observed in some patients with aggressive PTC[5]. In order to explore better therapeutic strategies for this disease, a better understanding of the molecular mechanisms underlying PTC is essential.

MicroRNAs (miRNAs), a class of small and non-coding RNAs, could suppress gene expression through binding to the 3'-untranslated region of downstream messenger RNAs (mRNAs)6. Recently, more and more miRNAs have been identified as critical regulators of many cellular biological processes such as development, cell growth, cell cycle, and apoptosis[7,8]. Moreover, expression levels of miRNAs are frequently dysregulated in a variety of disease states, including tumors[9,10]. Recently, the miRNAs in PTC cells were reported to play a critical role in the cell proliferation, metastasis and prognosis. For instance, Qiu et al[11] reported that decreased miR-613 expression was correlated with enhanced PTC metastasis. In addition, functional experiments showed that miR-613 exerted its role by targeting SphK2. Wen et al[12] found that up-regulation of miR-126 in PTC cells inhibited cell proliferation by targeting LRP6. Sondermann et al[13] reported that high expressions of miR-9 and miR-21 were significant prognostic factors for recurrence in patients with PTC. Recently, several reports have found that miR-577 is dysregulated in various human cancer[14,15]. However, the expression pattern and biological function of miR-577 in progression of PTC remain unknown.

In the present study, we firstly identified the

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expression pattern of miR-577 in PTC by RT-PCR. Then, cell growth, migration, and invasion were also examined. Moreover, we tried to study the molecular mechanisms involved in PTC proliferation and metastasis. To our best knowledge, this is the first report about the association between miR-577 and PTC progression.

Patients and Methods

Patient Samples

This study was performed with the approval of the Ethics Committee of Linyi People's Hospital. Thirty-five PTC tissues and matched adjacent normal tissues were obtained from patients who underwent initial biopsy at our hospital. Samples were immediately snap frozen in liquid nitrogen and stored at -80°C for future protein and RNA isolation. All samples were reviewed and diagnosed by two independent pathologists. Written informed consent was obtained from each patient.

Cell Lines and Cell Culture

Human PTC cell lines (TPC-1, BCPAP, K1) and the human thyroid epithelial cell line Nthy-ori3-1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), Gibco (Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 100 units/mL penicillin. All these cell lines were incubated at 37°C in humidified atmosphere consisting of 5% CO_2 and 95% air.

Cell Transfection

The miR-577 mimics and non-specific miRNA negative control (miR-Ctrl) were purchased from RiboBio (Guangzhou, China) and used at 20 mM Opti-MEM transfection media (Invitrogen, Carlsbad, CA, USA). The SphK2 overexpression vector (GenePharma, Shanghai, China) and its control vector (GenePharma, Shanghai, China) were used at a final concentration of 500 ng/μl. Cells were transfected with Lipofectamine 2000 following the instructions of manufacturer (Invitrogen, Carlsbad, CA, USA). After 5-h transfection, the culture medium was replaced by Roswell Park Memorial Institute (RPMI)-1640.

Total RNA Extraction and qRT-PCR

The total RNA of tissues and cells was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Relative expression levels of miR-577 and SphK2 mRNA were examined by SYBR Green Real-time PCR (RT-PCR). RT-PCR was performed using the miScript Reverse Transcription and miScript SYBR Green PCR Kit, according to the manufacturer's protocol (Qiagen, Germany). The qRT-PCR specific primers were purchased from Ambion. β-actin served as endogenous control. Relative expression levels were calculated by using the 2-ΔΔCT method. All reactions were run in triplicate.

Determination of Cell Viability and Colony Formation Assay

Cell proliferation was monitored using Cell Proliferation Reagent Kit I (MTT) (Roche, Basel, Switzerland). 1×103 cells per well were seeded into 96-well plates with five replicates for each condition. Cell proliferation was measured once every 24 h. 10 μl MTT was added to each well at a final concentration of 0.5 mg/ml, then the cells were incubated at 37°C for another 4 h. Absorbance was measured at 470 nm using a microplate reader. Three independent experiments were performed in triplicate.

For colony formation assay, 200 cells were plated into each well of 6-well plates and incubated for 2 weeks at 37°C in the presence of 5% CO2. The cells were fixed in methanol, and then stained with crystal. Finally, positive colony formation (> 50 cells/colony) was counted.

Transwell Migration and Invasion Assays

The 24-well transwell chambers (Millipore Corporation, Billerica, MA, USA) were used for the cell migration assay according to the manufacturer's protocol. Medium containing 10% fetal bovine serum (FBS) was added to the lower chamber. After incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool, whereas the cells that had migrated or invaded through the membrane were fixed with methanol and stained with 0.1% crystal violet. For the invasion assay, the procedure was similar to the cell migration assay, except that the membranes of upper chambers were pre-coated with 100 mL Matrigel (1 mg/mL). The cells were photographed under a phase-contrast microscope and counted in five randomly chosen fields. Each experiment was performed in triplicate.

Dual-luciferase Assay

The full-length 3'-UTR of SphK2 gene containing the putative miR-577 biding sites was amplified by PCR and was inserted into the psiCHECK-2

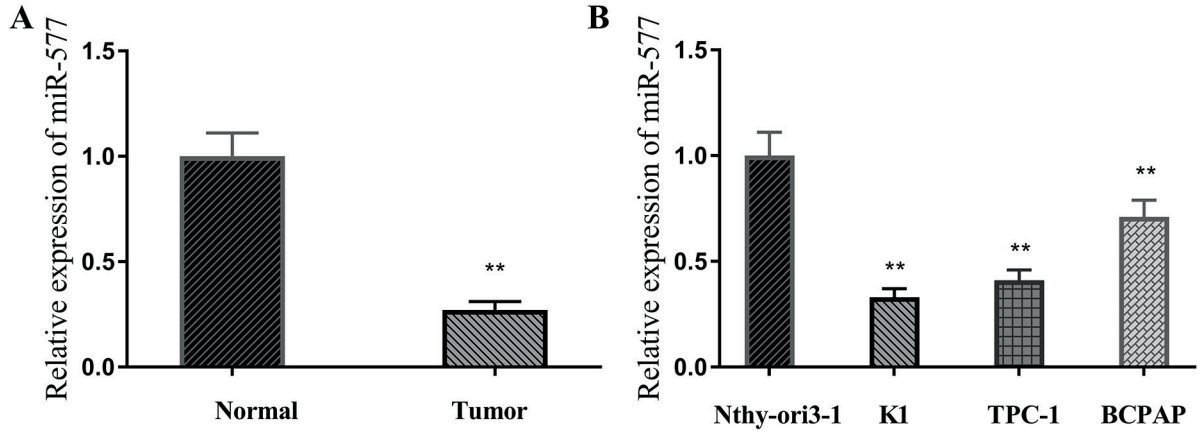

Figure 1. Downregulation of miR-577 in PTC tissues and cells. ***(A)*** Measurement of miR-577 expression in 35 PTC tissues and adjacent normal tissues. ***(B)*** Measurement of miR-577 expression in PTC cell lines (TPC-1, BCPAP, K1) and non-malignant human thyroid epithelial cell line Nthy-ori3-1. $^{*}p < 0.05$, $^{**}p < 0.01$.

plasmid (Promega, Madison, WI, USA). miR-577 or control mimic at a concentration of 50 nM were transfected into HEK293T cells using Lipofectamine™ 2000. After 48 hours of transfection, the cells were lyzed for luciferase assays using the dual-luciferase assay system (Promega, Madison, WI, USA). Luciferase activity was measured using a dual-luciferase reporter assay kit.

Western Blotting Analysis

Specific experimental methods can be found in the study of Ma et al[16].

Statistical Analysis

Statistical analysis was preceded by using SPSS statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software. Most of the data were analyzed using independent two-tailed Student's t-test. Values of $p < 0.05$ were considered statistically significant.

Results

Expression of miR-577 is Markedly Downregulated in PTC Specimens and Cell Lines

To investigate the effects of miR-577 on PTC, we performed PCR to determine the expression levels of mi-577 in PTC tissues and cell lines. As shown in Figure 1, the results showed that miR-577 expression was consistently lower in PTC tissues than those in the normal controls ($p < 0.01$). Moreover, the data of Figure 2 also indicated that the expression of miR-577 was downregulated in PTC cell lines compared to the human thyroid epithelial cell line. These data suggested that low miR-577 might be correlated with PTC progression.

Over-Expression of miR-577 Suppressed Proliferation of PTC Cells

In order to explore the effect of miR-577 in the regulation of cell proliferation, TPC-1, and K1 cells were transfected with miR-577 mimics or miR-Ctrl. The efficiency of transfection was verified by Real-time PCR (Figure 2A). In MTT assay, we observed that TPC-1 and K1 with miR-577 over-expression exhibited a significantly lower proliferation rate compared with NC (Figure 2B). Also, these results were confirmed by colony formation assay (Figure 2C).

Forced Expression of miR-577 Inhibited the Invasion and Migration of PTC Cells

We further investigated the function of miR-577 in migration and invasion in PTC cell lines. As shown in Figure 3A-B, we found that forced expression of miR-577 in TPC-1 and K1 cells markedly decreased the migratory and invasive capabilities ($p < 0.05$, respectively).

SphK2 is a Direct Target of miR-577 in PTC Cells

In order to explore gene targets that could be mediated by miR-577, a bioinformatics algorithm was used to predict potential target genes of miR-577. Among those target genes, we focused on SphK2, whose 3′-UTR contains a complementary site for the seed region of miR-577 (Figure 4A). To identified whether SphK2 was a target of miR-577, luciferase

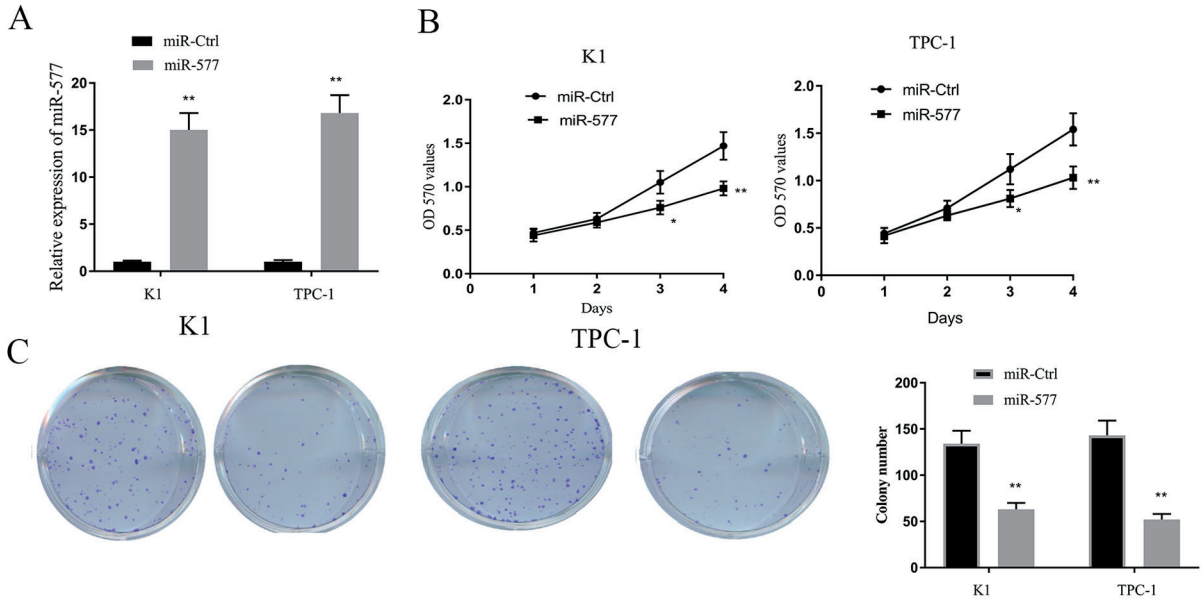

Figure 2. MiR-577 overexpression suppresses PTC cell proliferation. ***(A)*** miR-577 expression elevated after transfected with miR-577 mimics. U6 snRNA was used for normalization. ***(B)*** MTT assays were performed to determine the proliferation in K1 and TPC-1 cells. ***(C)*** K1 and TPC-1 cell survival was determined by colony formation. $^{*}p < 0.05$, $^{**}p < 0.01$.

reporter constructs generated with the WT 3'-UTRs of SphK2 were co-transfected into TPC-1 and K1 cells with the miR-577 mimics or control miRNA. Luciferase assays showed that the activity of SphK2 3'UTR was strongest inhibited by miR-577 (Figure 4B). Also, miR-577 inhibited the mRNA expression of SphK2 in the U87 cells (Figure 4C). At the same time, forced expression of miR-577 suppressed the protein level of SphK2 in TPC-1 and K1 cells (Figure 4D). Furthermore, the correlation between miR-577 expression and SphK2 in NSCLC tissues was explored. As shown in Figure 4E, there was a strong inverse correlation between the expression levels of miR-577 and SphK2. All of these results informed that miR-577 regulates SphK2 expression by directly targeting its 3′-UTR.

SphK2 is Responsible for PTC Cells Proliferation, Migration, and Invasion

To explore the effects of miR-577 on PTC progression, K1 and TPC-1 cells were transfected with siRNA targeting SphK2 or the NC con-

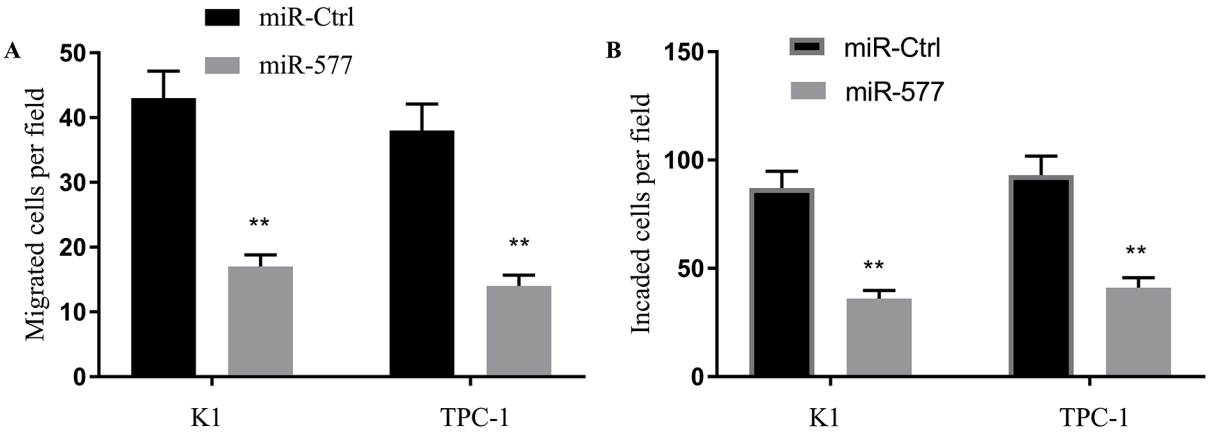

Figure 3. miR-577 inhibited the migration and invasion of PTC cells *in vitro*. ***(A)*** The effects of the overexpression of miR-577 on invasion in K1 and TPC-1 cells. ***(B)*** The effects of the overexpression of miR-577 on migration in K1 and TPC-1 cells. $^{*}p < 0.05$, $^{**}p < 0.01$.

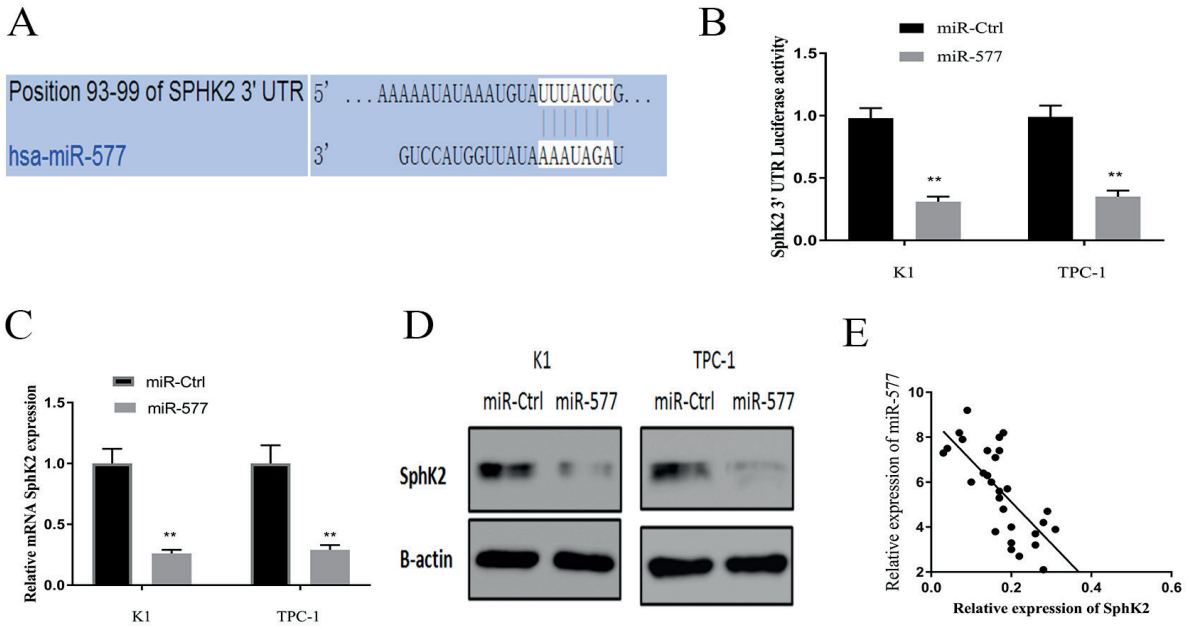

Figure 4. miR-577 negatively regulates SphK2 gene expression by binding to the SphK2 3'UTR. ***(A)*** The suspected binding of miR-577 with the wild type (WT) 3'UTR region of SphK2 mRNA was shown (www.targetscan.org). ***(B)*** Luciferase report assay in K1 and TPC-1 cells co-transfected with luciferase report vectors and miR-577 mimics or miR-Ctrl. ***(C)*** SphK2 mRNA expression levels were detected by qRT-PCR in K1 and TPC-1 cells transfected with the miR-577 mimics or miR-Ctrl. ***(D)*** SphK2 protein expression levels were detected by Western blot in K1 and TPC-1 cells transfected with the miR-577 mimics or miR-Ctrl. ***(E)*** The association of the expression levels of SphK2 and miR-577 in 35 PTC tissue samples. $^{*}p < 0.05$, $^{**}p < 0.01$.

trol. Down-regulation of miR-577 expression was verified using Western blot (Figure 5A). A significant reduction in cell viability was observed in the K1 and TPC-1 transfected with siRNA-SphK2 compared with those transfected with the normal control siRNA (Figure 5B). Mo-

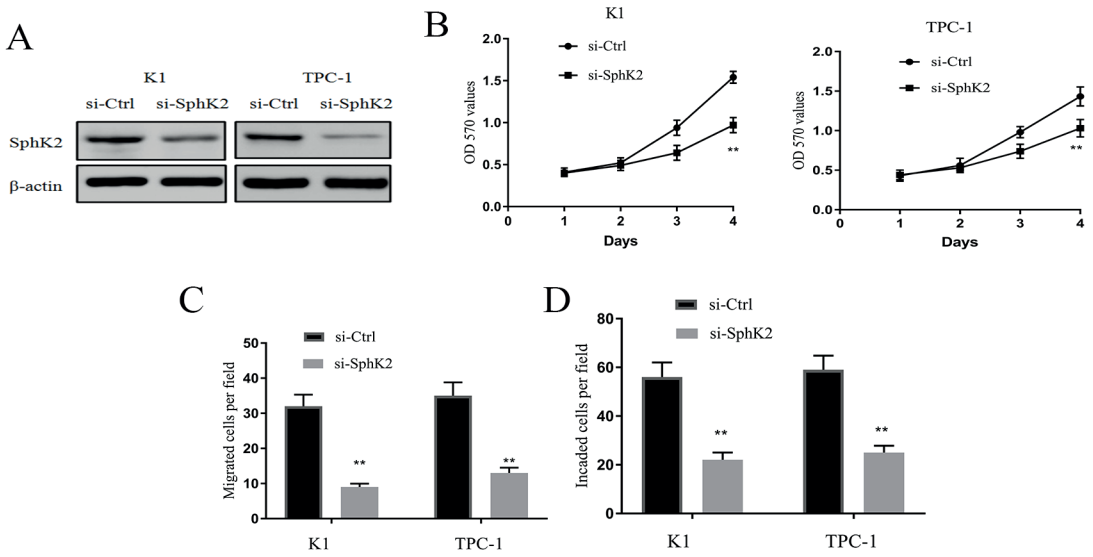

Figure 5. Depletion of SphK2 inhibits PTC cell migration and invasion. ***(A)*** Western blot revealed that SphK2 expression was efficiently down-regulated by transfected with siRNA targeting SphK2 (si-SphK2) in K1 and TPC-1 cells. ***(B)*** MTT assays were performed to determine the proliferation in K1 and TPC-1 cells. ***(C-D)*** Transwell migration and invasion assays were performed to determine K1 and TPC-1 cells migratory ***(C)*** and invasive ***(D)*** abilities. $^{*}p < 0.05$, $^{**}p < 0.01$.

reover, we used transwell assays to identify the role of SphK2 in metastasis. As shown in Figure 5C-D, downregulation of SphK2 expression significantly suppressed invasion and migration of PTC *in vitro*. These data suggest that inhibition of miR-577 suppressed proliferation and metastasis of PTC cells.

Discussion

As previously reported, some PTC patients usually suffer from tumor cell invasion and distant metastasis after surgery[17]. Thus, there is a strong need to explore molecular mechanism of PTC and develop new therapeutic agent to improve PTC treatment. Recently, the role of miRNAs has become research hotspots. More and more miRNAs were reported to play an important role in progression and development of various tumors[18,19]. Although many abnormal expressions of miRNAs were identified in various tumors, the effects and mechanism of miRNAs to the tumor progression remain undetermined. In the present work, we focused on miRNA-577.

Previous studies have suggested that miR-577 can serve as tumor suppressor in various human malignancies. For instance, Jiang et al[20] reported that enforced expression of miR-577 suppressed the colorectal cancer proliferation and colony formation by targeting HSP27. They also identified miR-577 as a positive regulator in chemosensitivity of colorectal cancer. Wang et al[21] found that miR-577 may suppress HCC growth through downregulating β-catenin. Zhang et al[22] demonstrated that overexpression of miR-577 inhibited glioblastoma multiform growth *in vitro* and *in vivo* by regulating the Wnt signaling pathway. Similarly, miR-577 also exerted tumor suppressor in progression of esophageal squamous cell carcinoma[23]. However, the detailed role of miR-577 in PTC carcinogenesis is still unknown.

In the present paper, we found that miR-577 expression was significantly down-regulated in PTC tissues and cell lines. *In vitro* assay indicated that up-regulation of miR-577 inhibited the proliferation, migration and invasion of PTC cells. These results suggested that miR-577 may play an anti-oncogene in PTC.

miRNAs can target 3'UTR of mRNAs. Our attention focused on SphK2. As one of distinct isoforms of Sphingosine kinases, previous studies have indicated that expression levels of SphK2 were significantly in several tumors[24,25]. More importantly, Qiu et al11 reported that knockdown of SphK2 could suppress PTC cells proliferation, migration and invasion, suggesting SphK2 as a tumor promoter in PTC. By using the online bioinformatics TargetScan algorithm software, we found that SphK2 may be a target of miR-577. Then, we performed the dual-luciferase reporter assay and Western blot analysis to determine whether miR-577 could regulate SphK2. The results confirmed our idea. At the same time, further functional experiment showed that SphK2 was responsible for PTC cells proliferation, migration, and invasion. Taken together, our results proved that miR-577 could target SphK2 and regulate its expression.

Conclusions

We demonstrated that miR-577 is downregulated in PTC tissues and is inversely correlated the expression of SphK2. Also, we showed over-expression of miR-577 significantly suppressed PTC proliferation and metastasis by targeting SphK2. The results of this work suggest that miR-577 might serve as a new therapeutic target for PTC.

Conflict of interest

The authors declare no conflicts of interest.

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