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MiR-758-3p regulates papillary thyroid cancer cell proliferation and migration by targeting TAB1

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Previous studied revealed that miR-758-3p was abnormally expressed in cancer patients. However, its role and underlying mechanism in papillary thyroid cancer (PTC) remains unclear. Expression of miR-758-3p in PTC cell lines was analyzed using quantitative real-time PCR. It was observed that miR-758-3p expression was significantly downregulated in PTC cell lines. Overexpression of miR-758-3p inhibited PTC cell proliferation, invasion but promoted cell apoptosis *in vitro*. Further study demonstrated that TGF-β activated kinase 1 binding protein 1 (TAB1) was a direct target of miR-758-3p and TAB1 expression was suppressed by miR-758-3p overexpression. Moreover, TAB1 was shown to be a mediator for the roles of miR-758-3p in PTC. Therefore, our study established a tumor-suppressive role for miR-758-3p in the inhibition of PTC progression, which may be employed as a novel prognostic marker and as an effective therapeutic target for PTC.

1. Introduction

The incidence of thyroid cancer (TC) continues to increase (Cabanillas et al. 2016). It is estimated to have approximately 567,233 new diagnosed cases in 2018 (Bray et al. 2018). Papillary thyroid carcinoma (PTC) is the most common histological type of differentiated thyroid cancer, followed by follicular thyroid carcinoma (Kitahara et al. 2016). Despite the progress in treatment measures for TC, PTC patients with invasive cancer behavior suffer from poor prognosis (Guilmette et al. 2018). Therefore, it is important to understand the mechanisms underlying the progression of PTC to provide assistance in PTC treatment.

MicroRNAs (miRNAs) are small non-coding RNAs with the length of 17-25 nucleotides in length and are produced by two RNase III proteins, Drosha and Dicer (Ha et al. 2014). MiRNAs are inhibiting gene expression mainly through binding to the 3'-untranslared region (3'-UTR) of the target gene (Bartel et al. 2009). In addition, dysregulation of miRNAs will cause the silencing of genes which function as tumor suppressors or aberrant activation of oncogenes (Calin et al. 2006). One miRNA can target multiple genes and one gene can be targeted by multiple miRNAs to form a complex regulatory network and thus lead to the development of human cancers (Calin et al. 2006).

MiR-758-3p, a tumor-related miRNA, has been reported to be involved in the progression of multiple cancers by regulating cell proliferation, migration, and invasion (Guo et al. 2018a; Jiang et al. 2017; Wang et al. 2018). Recently, miR-758-3p was found downregulated in gastric cancer and could regulate gastric cancer cell proliferation, migration, and invasion through regulating the expression of chromobox 5 (Guo et al. 2018a). In non-small cell lung cancer, a long non-coding RNA DANCR was reported to be upregulated and negative control the expression of miR-758-3p to promote cell proliferation, migration and invasion (Wang et al. 2018a). Meanwhile, it was found that miR-758-3p expression was reduced in hepatocellular carcinoma and its overexpression displayed significant inhibition of hepatocellular carcinoma development through targeting MDM2 proto-oncogene and mechanistic target of rapamycin kinase (Jiang et al. 2017). However, the role of miR-758-3p in PTC remained unclear.

In the present study, the expression level of miR-758-3p in PTC cell lines was investigated, as were the effects of miR-758-3p on PTC cell proliferation and migration *in vitro*. The target of miR-758-3p in PTC was validated by luciferase activity reporter assay and western blot assay. Involvement of the validated miR-758-3p target in miR-758-3p mediated cell behavior was also investigated.

2. Investigations and results

2.1. The expression of miR-758-3p was decreased in PTC cell lines

To examine the expression of miR-758-3p in PTC, we conducted RT-qPCR in PTC cell lines (TPC-1 and BCPAP) and human thyroid epithelial cell line (Nthy-ori 3-1). The results revealed that miR-758-3p expression was significantly reduced in PTC cell lines (TPC-1 and BCPAP) compared with the human thyroid epithelial cell line Nthy-ori 3-1 (Fig. 1).

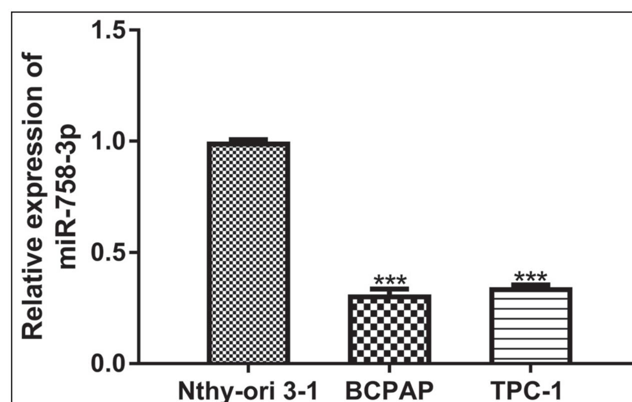

Fig. 1: MiR-758-3p expression was downregulated in PTC cell lines as analyzed by RT-qPCR. (***P<0.001) PTC: papillary thyroid cancer; RT-qPCR: Real-time quantitative PCR.

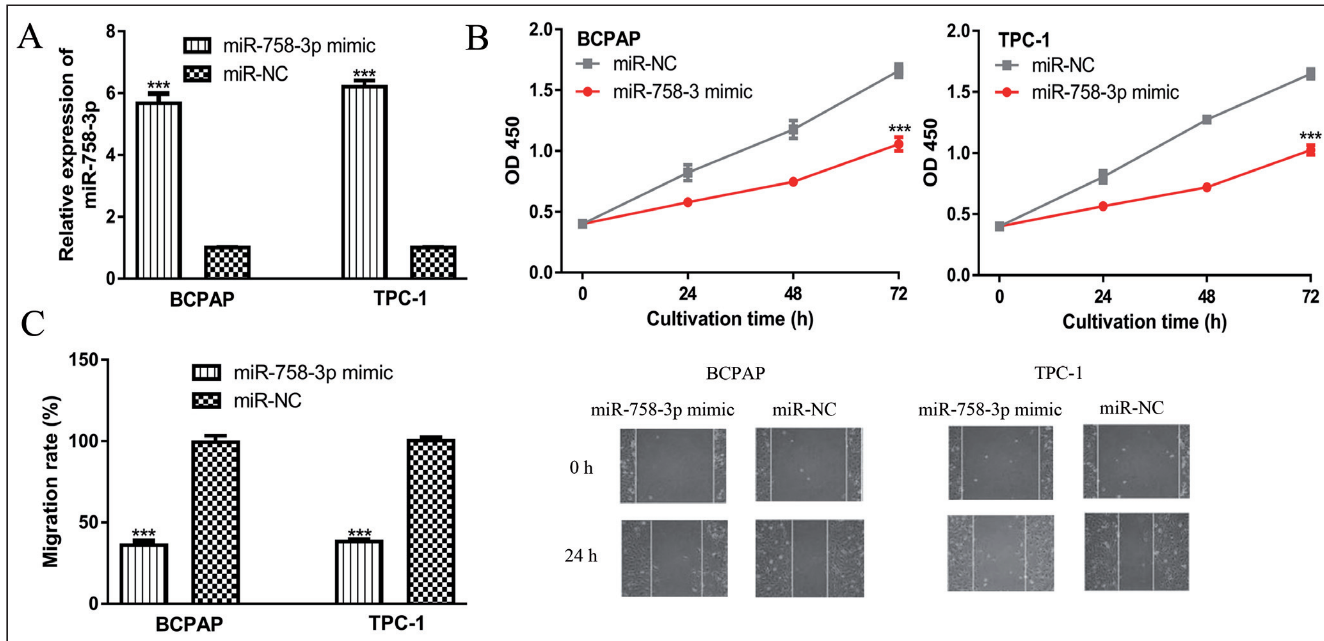

Fig. 2: miR-758-3p inhibits the proliferation and migration of PTC cells *in vitro*. A, miR-758-3p expression in PTC-1 cells transfected with miR-758-3p mimic or miR-NC. The effect of miR-758-3p on the B, proliferation, and C, migration of PTC cells was examined. (***P<0.001) PTC: papillary thyroid cancer; RT-qPCR: Real-time quantitative PCR; miR-NC: negative control miRNA.

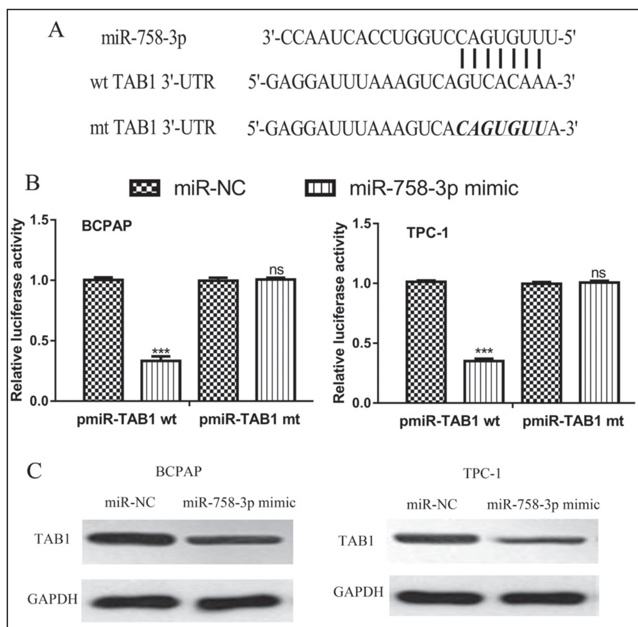

Fig. 3: TAB1 was a direct target of miR-758-3p. A, A scheme of the construction of wt or mt TAB1 3'-UTR is indicated. B, Luciferase activities were analyzed in PTC cells co-transfected with pmiR-TAB1 wt or pmiR-TAB1 mt and miR-758-3p mimic or miR-NC. C, TAB1 protein expression was determined in PTC cells transfected with miR-758-3p mimic or miR-NC. (ns not significant; ***P<0.001) PTC: papillary thyroid cancer; miR-NC: negative control miRNA; wt: wild-type; mt: mutant; UTR: untranslated region; TAB1: TGF-β activated kinase 1 binding protein 1.

2.2. MiR-758-3p inhibits PTC cell proliferation and migration

To investigate the significance of miR-758-3p downregulation in the progression of PTC, we transfected miR-758-3p mimic and miR-NC into TPC-1 and BCPAP cell lines. RT-qPCR results showed that miR-758-3p expression can be significantly elevated by miR-758-3p mimic in these two cells (Fig. 2A). CCK-8 assay showed cell proliferation was decreased by miR-758-3p mimic in both cell lines compared with miR-NC (Fig. 2B). Furthermore, a wound-healing assay showed that miR-758-3p mimic transfection significantly downregulated cell migration compared with miR-NC (Fig. 2C). These results revealed that miR-758-3p inhibits PTC cell proliferation and migration *in vitro*.

2.3. TAB1 was a direct target of miR-758-3p in PTC

To identify the direct target of miR-758-3p, we searched the database TargetScan and found that TAB1 was a potential target of miR-758-3p (Fig. 3A). As predicted, western blot assay showed miR-758-3p mimic transfection decreased the protein levels of TAB1 in both cell lines (Fig. 3B). Moreover, luciferase activity reporter assay showed that luciferase activity could be repressed in cells transfected with pmiR-TAB1 wt but not pmiR-TAB1 mt (Fig. 3C). These results indicated TAB1 was a direct target of miR-758-3p.

2.4. TAB1 overexpression reverses the inhibitory effects of miR-758-3p in PTC

To investigate the functional significance of TAB1 in miR-758-3p mediated effects in PTC cells, the miR-758-3p and pcDNA3.1-TAB1 was co-transfected into PTC cells. We found pcDNA3.1-TAB1 transfection increased the levels of TAB1 in PTC cells, while it can also reverse the inhibitory effect of miR-758-3p mimic on TAB1 expression (Fig. 4A). Furthermore, overexpression of TAB1 partially reversed miR-758-3p-mediated inhibition effects on cell proliferation and migration in PTC cells (Fig. 4B and 4C). Collectively, these results suggested that TAB1 was the target of miR-758-3p, and it mediates the effects of miR-758-3p in PTC progression.

3. Discussion

The lack of specific manifestations for early stage PTC make it hard to diagnose (Baldane et al. 2015). A number of miRNAs has been identified to function as an oncogene or tumor suppressor in PTC (Gong et al. 2018; Guo et al. 2018b; Wang et al. 2018b; Yan et al. 2018). Guo et al. (2018b) revealed that miR-9-5p functions as tumor suppressor via targeting BRAF to regulate the malignancy of cancer cells. Wang et al. (2018b) found that 3-phosphoinositide dependent protein kinase 1 (PDPK1) was a direct target of miR-718 and the interaction between miR-718 and PDPK1 could regulate PTC cell proliferation, migration, and invasion. Gong

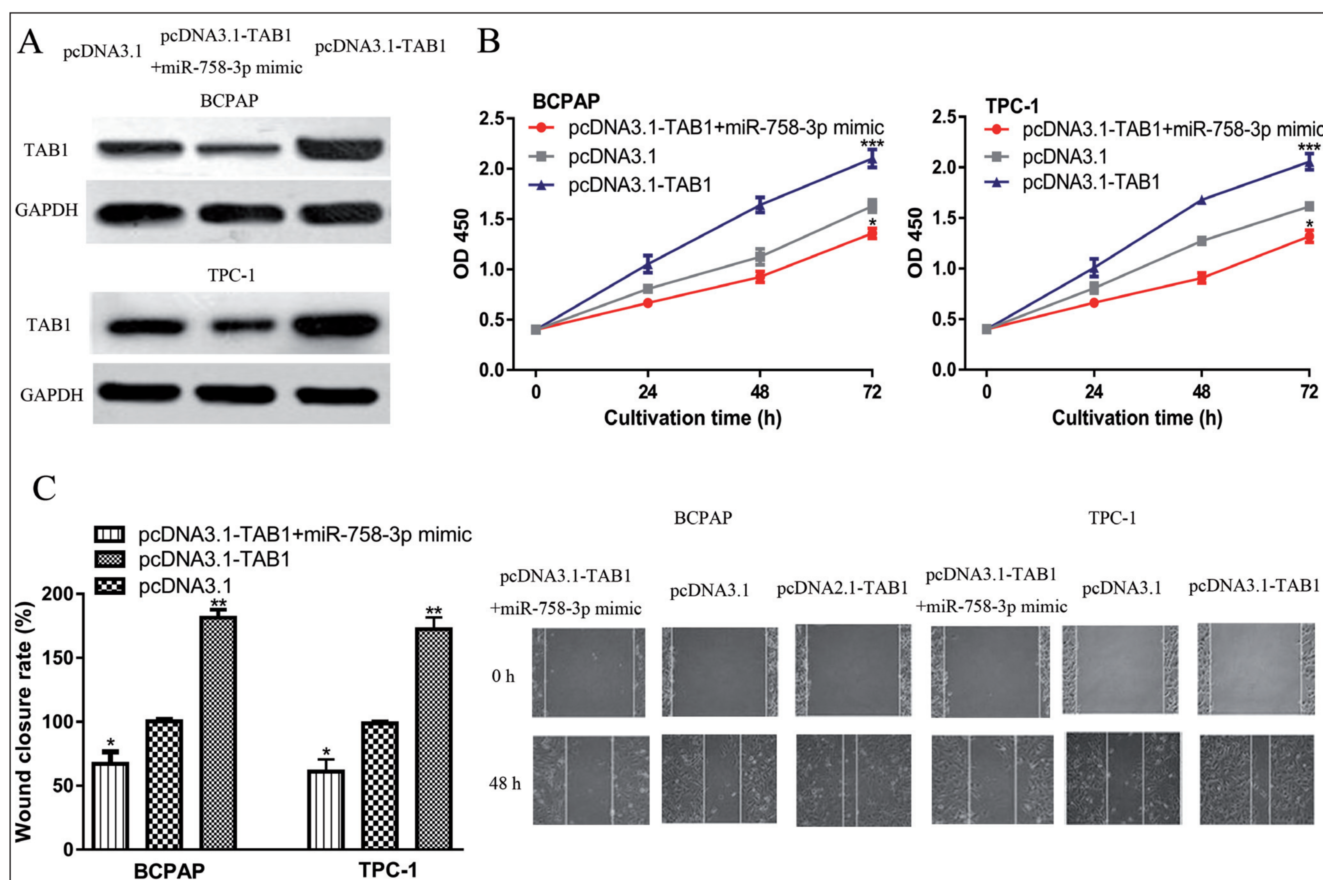

Fig. 4: TAB1 overexpression reverses the inhibitory effects of miR-758-3p in PTC. A, TAB1 protein expression was determined in PTC cells transfected with pcDNA3.1-TAB1 or pcDNA3.1, and with or without miR-758-3p mimic. Overexpression of TAB1 partially reversed miR-758-3p-mediated inhibitory effect on B, cell proliferation and C, cell migration. (*P<0.05; ***P<0.001) PTC: papillary thyroid cancer; TAB1: TGF-β activated kinase 1 binding protein 1.

et al. (2018b) demonstrated that miR-26a could target cAMP regulated phosphoprotein 19 to sensitize the PTC cells to tamoxifen. Moreover, Yan et al. (2018) recently identified an oncogenic miRNA, miR-150-5p, and miR-150-5p overexpression results in an obvious decrease in E-cadherin expression, but increased in N-cadherin, Slug and Vimentin, ZEB1, and Snail expression. All these efforts have enhanced our understanding regarding the mechanisms underlying the progression of PTC, however, they are still not enough as the prognosis for PTC remains unsatisfied.

Here, we revealed that miR-758-3p expression was downregulated in PTC cell lines compared with the Nthy-ori 3-1 cell line. Functional assays showed that miR-758-3p overexpression induced by miR-758-3p mimic transfection could inhibit PTC cell proliferation and migration *in vitro*. These results indicated miR-758-3p functions as tumor suppressor in PTC, which is in consistent with its role in gastric cancer, non-small cell lung cancer, and hepatocellular carcinoma as reported previously (Guo et al. 2018a; Jiang et al. 2017; Wang et al. 2018b). These results provide novel insights into the role of miR-758-3p in regulating the progression of PTC, and imply that miR-758-3p has the potential to be developed as a therapeutic target for PTC.

It has been widely regarded that miRNA-mRNA interaction play crucial roles in tumorigenesis (Guo et al. 2018a; Jiang et al. 2017). To date, several targets of miR-758-3p have been experimentally validated (Guo et al. 2018a; Jiang et al. 2017). Here, using the TargetScan online algorithm, we found that TAB1 was also a potential target of miR-758-3p as the 3'-UTR of TAB1 contains a conserved binding site for miR-758-3p. TAB1, a binding ligand for TAK1, was reported to be overexpressed in human cancers including colorectal cancer, non-small cell lung cancer, and ovarian cancer and to serve as oncogene (Gong et al. 2018a; Shuang et al. 2017; Zhu et al. 2015). TAB1 was also reported to be directly regulated by miRNAs including miR-873 and miR-134 in human cancers (Gong et al. 2018a; Zhu et al. 2015). Therefore, luciferase reporter assay and western blot assay were conducted and have shown that TAB1 is a direct target of miR-758-3p. Moreover, we performed rescue experiments to identify TAB1 as the effector for the miR-758-3p mediated cell behavior inhibition in PTC.

In conclusion, we demonstrated that miR-758-3p expression was downregulated in PTC cell lines, and its overexpression could inhibit cell proliferation and migration *in vitro*. We also propose that TAB1 is a direct and functional target of miR-758-3p in PTC. These results will advance our understanding the process of PTC progression and may provide a novel therapeutic target for PTC treatment.

4. Experimental

4.1. Cell culture

PTC cell lines (TPC-1 and BCPAP) and human thyroid epithelial cell line (Nthy-ori 3-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen), penicillin (100 units/ml) and streptomycin (100 ng/ml) at 37 °C in a humidified incubator containing 5 % of CO_2.

4.2. Plasmid construction and transfection

MiR-758-3p mimic (5'-UUUGUGACCUGGUCCACUAACC-3') and corresponding negative control (miR-NC, 5'-ACGACCGUUACUGUUCUCUCAG-3') were synthesized by GenePharma (Shanghai, China). The open reading frame of TGF-β activated kinase 1 binding protein 1 (TAB1) cloned into pcDNA3.1 (pcDNA3.1-TAB1) and empty vector (pcDNA3.1) was purchased from GenScript (Nanjing, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen) with 100 ng of miRNAs or constructs when cells were cultured to 80 % confluence according to the manufacturer's protocol.

4.3. Real-time quantitative PCR (RT-qPCR)

Total RNAs were isolated with Trizol reagent (Invitrogen) according to the manufacture's instructions. Complementary DNA was synthesized from extracted RNA

samples using PrimeScript RT Master Mix (Takara, Dalian, Liaoning, China). RT-qPCR was conducted at ABI 7500 PCR System (Applied Biosystems, Carlsbad, CA, USA) using SYBR PrimeScript miRNA RT-PCR Kit (Takara) with the following procedure: 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative expression of miR-758-3p was calculated using the $2^{-\Delta\Delta Ct}$ method with U6 small nuclear RNA (U6 snRNA) as internal control (Livak et al. 2001). The primers were presented as follows: miR-758-3p forward: 5'-ACACTCCAGCTGGGTTTGT-GACCTGGTCCA-3', reverse: 5'-TGGTGTCGTGGAGTCG-3'; U6 snRNA forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

4.4. Western blot

Total proteins were isolated using RIPA lysis buffer with protease inhibitors (Beyotime, Haimen, Jiangsu, China). Protein concentration was measured using BCA assay kit (Beyotime) according to the provided protocol. Protein samples with equal amounts were separated using 10 % SDS-PAGE and transferred to PVDF membranes (Beyotime). The membranes were incubated with primary antibodies against TAB1 (ab76412, Abcam, Cambridge, MA, USA) and GAPDH (ab181602, Abcam) for overnight at 4 °C after blocked with 5 % not-fat milk. Subsequently, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibody (ab6721, Abcam) for 1 h at room temperature. Protein signals were detected using BeyoECL kit (Beyotime) according to the manufacturer's protocol.

4.5. Cell proliferation assay

Cell Counting Kit-8 (CCK-8, Beyotime) was used to determine cell proliferation. Briefly, cells (5×10^3cells/well) were incubated in 96-well plates and cultured for 24-72 h. 10 µl CCK-8 solution was added to each well at indicated time points. Absorbance was measured with ELISA reader (Bio-Rad Laboratories, Richmond, CA, USA) at 450 nm.

4.6. Wound healing assay

Cells were cultured in 6-well plates and incubated to about 95 % confluence. A 10 µl tip was used to create a gap at cell surface and grown in serum-free medium for 24 h. Wounded gaps were captured using light microscope (IX71; Olympus, Tokyo, Japan) at 0 h and 48 h after wound creation (200× magnification).

4.7. Luciferase reporter assay

TargetScan (http://www.targetscan.org) was used to predict the target of miR-758-3p. Wild-type (wt) or mutant (mt) of TAB1 3'-UTR containing the binding site of miR-758-3p was inserted into pmirGLO vector (Promega, Madison, WI, USA), and named pmiR-TAB1 wt or pmiR-TAB1 mt. For the luciferase reporter assay, cells were co-transfected with miR-758-3p mimic or miR-NC and pcDNA3.1-TAB1 or pcDNA3.1 using Lipofectamine 2000. The firefly and Renilla luciferase activities were determined at 48 h after transfection using Dual-Luciferase Reporter Assay System (Promega).

4.8. Statistical analysis

Data were presented as the mean±standard deviation (SD) and analyzed with SPSS 19.0 software (IBM, Chicago, IL, USA). Student's t-test and one-way ANOVA with Tukey test were conducted to analyze two or multiple groups, respectively. $P < 0.05$ was considered as statistically significant.

Conflicts of interest: None reported.

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