miR-183 regulates biological behavior in papillary thyroid carcinoma by targeting the programmed cell death 4

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Abstract. Numerous studies have demonstrated that microRNAs (miRNAs) play vital roles in papillary thyroid carcinoma (PTC). The aim of the present study was to examine the expression levels of miR-183 in PTC and investigate whether its potential roles involved targeting the programmed cell death 4 (PDCD4). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to examine the expression levels of miR-183 in 38 PTC specimens and 4 PTC cell lines. MTT, colony formation, wound-healing and Transwell invasion assays, and flow cytometry were conducted to explore the potential functions of miR-183 in human TPC1 papillary thyroid carcinoma cells. The dual-luciferase reporter assay was performed to validate whether PDCD4 was a direct target of miR-183. The results showed the miR-183 expression levels were significantly upregulated in PTC specimens and cell lines (P<0.05). Overexpression of miR-183 on endogenous levels of PDCD4 were subsequently confirmed via RT-qPCR and western blotting. Functional assays were used to indicate the roles of endogenous PDCD4 in TPC1. The effects of modulating miR-183 on endogenous levels of PDCD4 were subsequently confirmed via RT-qPCR and western blotting. The dual-luciferase reporter assay confirmed that PDCD4 was a direct target of miR-183. RT-qPCR and western blotting showed that miR-183 negatively regulated PDCD4 protein expression but had no impact on mRNA expression of PDCD4. Knockdown of PDCD4 expression in TPC1 cells significantly enhanced cell proliferation, migration, invasion and inhibited apoptosis. The results of the present study suggested that miR-183 acts as a papillary thyroid carcinoma oncogene through the negative regulation of PDCD4 protein expression at the post-transcriptional levels. Therefore, targeting miR-183 provides a novel strategy for the diagnosis and treatment of patients with PTC.

Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence is on the increase (1,2). Four main types of thyroid cancer have been identified: papillary, follicular, medullary and anaplastic thyroid cancer. Papillary thyroid carcinoma (PTC) is the most frequently occurring type of thyroid malignancy accounting for ~80-90% of cases (3). This cancer type is the most rapidly increasing cancer among women and the second most among men (4), and poses a serious threat to human health and life.

Although most PTC can be managed successfully with a combination of radioactive iodine and levothyroxine treatment after complete thyroidectomy, tumors with more aggressive phenotype are associated with morbidity and mortality (5). Thus, understanding the molecular mechanism of PTC is important for the development of more effective therapeutic strategies.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with an approximate length of 21-23 nt, which are highly conserved. miRNAs regulate the expression of various genes at the post-transcriptional level by binding to the 3'-untranslated region (3'-UTR) of their target mRNAs (6-8). When the miRNA is perfectly complementary to its target, it can specifically cleave the target mRNA. However, when partially complementary to its target, the miRNA only represses mRNA translation (9,10). Although miRNAs constitute only 3% of the human genome, it is reported that ~90% of genes serve as miRNA targets. miRNAs are involved in biological processes such as cell proliferation and differentiation, metabolism, invasion, metastasis and apoptosis, all of which are associated with tumorigenesis (11-14). Previous findings showed that >50% of annotated human miRNAs are located in the fragile sites of the genome connected with cancer (15). It has been widely shown that miRNAs are significantly differentiated between tumor and normal tissues, including thyroid cancer (16-18), and may act as oncogenes or tumor suppressor genes (19,20). miR-221, miR-222 and miR-181b have been found to be upregulated in PTC compared with normal thyroid tissue (21-23).

Using miRNA microarray chip, it was found that miR-183 was overexpressed in PTC compared with normal thyroid tissues. miR-183 regulates biological behavior in papillary thyroid carcinoma by targeting the programmed cell death 4.
However, the roles of miR-183 in PTC and the mechanism of gene regulation remain to be determined.

Programmed cell death 4 (PDCD4), a tumor-suppressor gene, has been reported to be involved in tumor progression, apoptotic machinery, cell transformation and invasion. PDCD4 protein is downregulated or lost in many human types of cancer and cancer cell lines (24-31), including PTC (32,33). Using programs available online, we determined that miR-183 potentially regulates the PDCD4 gene. Previous results indicated that miR-183 inhibited the apoptosis of TGF-β1-induced human hepatocellular carcinoma (HCC) cells by repressing PDCD4 expression (34). Additionally, miR-183 promotes proliferation and invasion in oesophageal squamous cell carcinoma by targeting PDCD4 (35). Nevertheless, whether miR-183 negatively regulates PDCD4 expression in papillary thyroid cancer remains to be determined.

In the present study, we investigated whether miR-183 was upregulated in PTC clinical samples and cell lines. The ectopic expression of miR-183 induced significant changes of cell proliferation, migration, invasion and apoptosis by directly targeting PDCD4. The present study findings identified a novel strategy for the early diagnosis and treatment of PTC.

Materials and methods

Specimens. In the present study, 38 pairs of papillary thyroid cancer and adjacent normal thyroid specimens were obtained from the Department of Breast and Thyroid Surgery of the Shanghai Tenth People's Hospital and approved by the Institutional Ethics Committee of the Shanghai Tenth People's Hospital (no. SHTY-IEC-pap-15-1). Each patient provided written informed consent. The specimens were immediately snap-frozen in liquid nitrogen. The specimens were pathologically confirmed as papillary thyroid carcinoma. No patients had received any chemotherapy or radiotherapy prior to surgery.

Cell lines and transfection. The human TPC-1, BCPAP, K1 and NPA PTC cell lines, human normal thyroid cell line (Nthy-ori 3-1) and HEK293T cells were purchased from the Chinese Academy of Sciences (Shanghai, China). TPC-1, K1, NPA and Nthy-ori 3-1 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Enpromise, China). BCPAP and HEK293T cells were cultured in Dulbecco’s modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS, 1% penicillin and 1% streptomycin. The cells were incubated at 37˚C with 5% CO₂ in saturated humidity.

miR-183 mimics, inhibitors, PDCD4 siRNA or their negative control (NC) were purchased from GenePharma (Shanghai, China). The TPC-1 cells were cultured to 30-40% confluence in 6-well plates (BD Biosciences, Franklin Lakes, NJ, USA) and were transfected with miR-183 mimics, miR-183 inhibitors, PDCD4 siRNA or their NC at working concentrations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. miRNA NC and siRNA NC were used as NCs.

miRNA isolation and reverse-transcription-quantitative polymerase chain reaction (RT-qPCR). miRNAs were harvested from tissues and cells using a miRcute miRNA Isolation kit (Tiangen, Beijing, China), according to the manufacturer's instructions. miR-183 expression levels were detected using one-step RT-qPCR (EzOmics SYBR qPCR kit). The miR-183 and U6 primer, and EzOmics SYBR qPCR kit were purchased from Biomics Biotechnologies Inc. (Jiangsu, China). U6 was used as an internal control. The miR-183 stem-loop RT primer used was: 5'-GCGAGACAGAATATTAGCCTCACTATA GGTTG-3'; miR-183 5'-TATGGCACTGTTAGATCTACT-3' (sense), and 5'-GCGAGACAGAATATTAGCCTCACTATA GGTTG-3' (antisense); while that of U6 stem-loop RT primer was: 5'-GTC GTATCCAGTGCGGTCCGAGGTGTTGGATACGACA AAATATGTTG-3'; U6 5'-TGCGGTTGCTGCTTGC GCCACGC-3' (sense), and U6 5'-CCAGTGCGAGGTTGGAGGT-3' (antisense). Briefly, 100 ng RNAs were added to a 25 µl reaction system containing 12.5 µl 2X master mix, 0.5 µl 50X SYBR-Green, 0.5 µl reverse transcription primer (10 µM), 0.5 µl sense and miR-183 primer (10 µM). RT-qPCR was performed on a 7900HT fast RT-PCR instrument (Applied Biosystems, Singapore) using SYBR-Green for fluorophore detection. One-step RT-qPCR parameters were as follows: 37˚C for 60 min, 10 min at 95˚C, followed by 40 cycles of 20 sec at 95˚C, 30 sec at 62˚C and 30 sec at 72˚C. Each sample was tested three times.

For the detection of PDCD4 mRNA expression, total RNA was isolated from tissues and cells using TRIzol (Invitrogen), and cDNA was generated by reverse transcription using the PrimeScript RT-PCR kit (Takara, Japan) according to the manufacturer's instructions. The primer (GenePharma) sequences used were: PDCD4 5'-GCGAGACAGAATATTAGCCTCACTATA GGTTG-3' (sense), and 5'-TCCACATCA GTGTGCTCATTAC-3' (antisense); GAPDH 5'-AAGGTC GGAATCAACGGATT-3' (sense), and 5'-CTGGGAGAT GGTATGCGGATT-3' (antisense). GAPDH mRNA levels were used for normalization. The RT-qPCR parameters used were: 2 min at 95˚C, and then 40 cycles of 15 sec at 95˚C and 30 sec at 60˚C. The relative expression was calculated using the relative quantification equation (RQ) = 2⁻ΔΔCt (36). Each sample was tested in triplicate.

Cell proliferation assay (MTT assay). The transfected cells were seeded in 96-well (1x10³ cells/well) culture plates (BD Biosciences) and incubated at 37˚C in 5% CO₂. Cell proliferation was assessed at 24, 48, 72 and 96 h post-transfection using the MTT assay kit (Sigma, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, 20 µl (5 mg/ml) MTT solution was added to each well. After a 4-h incubation, the medium was replaced with 150 µl dimethylsulfoxide (DMSO; Sigma). After 10 min of agitation (100 rpm), the absorbance at 490 nm of each sample was measured by a microplate spectrophotometer (Bio-Tek, Winooski, VT, USA). Experiments were performed in biological triplicate and included six replicates.

Colony formation assay. The transfected cells were seeded in 6-well (1x10³ cells/well) culture plates (BD Biosciences). Incubation at 37˚C with 5% CO₂ for 7-10 days until visible cloning was observed in the dish. Subsequently, the culture medium was removed and the wells were washed twice with phosphate-buffered saline (PBS). The colonies were fixed with
transfected cells (4x10^4 cells/Transwell) were plated lower chamber and the Transwell filter was placed into 24-well RPMI-1640 supplemented with 10% FBS was added to the a Matrigel (2 mg/ml)-coated membrane containing 8-mm Transwells (Corning, Lowell, MA, USA) with sive ability. Transwell invasion assay performed to evaluate cell inva-
dently three times.

**Cell migration and invasion assays.** A wound-healing assay was used to evaluate the migratory ability of the transfected cells. The transfected cells were seeded in 6-well plates at 30x10^5 cells/well, and incubated until the cell monolayer reached 100% confluence. The bottom of the 6-well plates was scratched with a P200 pipette tip. Detached cells were washed with PBS and replaced with fresh medium. The scratch widths were measured at 0 and 48 h using an inverted microscope (50-fold). The experiment was repeated independ-

**Transwell invasion assay performed to evaluate cell invasive ability.** Transwells (Corning, Lowell, MA, USA) with a Matrigel (2 mg/ml)-coated membrane containing 8-mm diameter pores were washed with serum-free RPMI-1640. RPMI-1640 supplemented with 10% FBS was added to the lower chamber and the Transwell filter was placed into 24-well plates. The transfected cells (4x10^6 cells/Transwell) were plated in the top chamber of in 200 µl serum-free RPMI-1640 with 0.1% BSA. After 18 h incubation at 37°C in 5% CO₂, the cells remaining on the upper membrane surface were removed using a cotton swab. The cells that invaded through the membranes were washed with PBS three times, fixed with 10% formalin and stained with 0.5% crystal violet. Five random fields for each chamber were photographed. To quantify the number of cells that had invaded, the cells were dissolved in 300 µl 33% glacial acetic acid and the absorbance at 573 nm was measured using a microplate spectrophotometer.

**Apoptosis assay.** An apoptosis assay was used to evaluate cell apoptosis using the Annexin V-FITC/PI apoptosis detection kit (Beyotime, Jiangsu, China). After 48 h transfection, the cells were centrifuged. Then, 2.5 µl Annexin V-FITC reagent and 50 µl 1X binding buffer were added to the cell groups. The cells were then incubated in the dark for 15 min at room temperature. Subsequently, 5 µl propidium iodide (PI) and 250 µl 1X binding buffer were added and the cells were incubated in the dark for 5 min at room temperature. The cells were gently resuspended in the Annexin V incubation reagent at a concentration of 10^5-10^6 cells/100 µl. The samples were then processed by flow cytometry (FACSCanto™ II; BD Biosciences).

**Vector construction and dual-luciferase reporter assay.** The wild-
type 3'-UTR of PDCD4 including predicted miR-183 targeting sites was amplified using PCR amplification using the PrimerStar kit (Takara) according to the manufacturer's instructions. The primers used were: sense, 5'-TAATAAGCTATGTTTTGTAAG GCCATGTATATATCCTACTATTCCA-3' and antisense, 5'-TTGGAATGATGTTATATAACATACTGGCATTCCAA AAGTAGCTTTATTTGTTATATT-3'. The mutant constructs were generated by mutation. The mutant and wild-type 3'-UTR fragments were subcloned into the XhoI site in the 3'-UTR of Renilla luciferase of the psiCHECK-2 reporter vector. The constructed vectors were designated as PDCD4-wt-vector and PDCD4-mut-vector. For the dual-luciferase reporter assays, 293T cells were seeded in 12-well plates (BD Biosciences) and cultured until the cells reached 80-90% confluence. The PDCD4-wt-vector or PDCD4-mut-vector (0.2 µg) were co-transfected with 100 nmol/l miR-183 or miRNA NC using Lipofectamine™ 2000. After 36 h transfection, firefly and Renilla luciferase activities were measured using Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The firefly luciferase (FL) activity of each sample was normalized to the Renilla luciferase (RL) activity. All the experiments were performed three times.

**Western blotting.** The protein expression levels were analyzed by western blot analysis. Forty-eight hours post-transfection, the cells were washed twice with ice-cold PBS, RIPA lysis buffer (Beyotime) was added, and the cells were lysed on ice for 30 min, wiped off, transferred to an EP tube and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatants were collected and protein concentrations were quantified using a BCA protein assay kit (Beyotime). Each sample with 40 µg protein was denatured with 5X sodium dodecyl sulfate (SDS) loading buffer (Beyotime) at 95°C for 5 min. Subsequently, the protein samples were separated by 10% SDS-polyacrylamide gel and transferred onto PVDF membranes (both from Beyotime). The membranes were blocked with 5% fat-free milk for 1 h, and incubated with primary antibodies PDCD4 (1:1,000 dilution; Cell Signaling Technology, Beverly, MA, USA) and β-actin (1:1,000 dilution; sc-1616-R; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as a loading control overnight at 4°C. The membranes were washed three times with PBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing three times with PBST, immu-
noreactive protein bands were detected using the Odyssey scanning system (LI-COR, Lincoln, NE, USA).

**Statistical analysis.** Data are presented as the means ± standard deviation (SD) from at least three separate experiments. The Student's t-test (two-tailed) was used to compare the statistical differences between the two groups using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software. P<0.05 was considered to indicate statistically significant results.

**Results**

**miR-183 is upregulated in papillary thyroid cancer clinical tissues and cell lines.** We first determined the expression levels of miR-183 in papillary thyroid cancer and cell lines by RT-qPCR. As shown in Fig. 1A, the levels of miR-183 showed a 11.59±0.2817-fold higher expression in cancer tissues when compared with the adjacent normal tissues (P<0.05). Similarly, in comparison with Nthy-ori 3-1, the papillary thyroid cancer cell lines expressed higher levels of miR-183 (P<0.05; Fig. 1B). These results indicated that miR-183 was upregulated in papil-
ary thyroid cancer and cell lines.

**miR-183 promotes TPC-1 cell proliferation.** An MTT assay was used to investigate the effects of miR-183 on papillary thyroid cancer cell proliferation. TPC-1 cells were treated
with 50 and 100 nM miR-183 mimics, miR-183 inhibitor or miRNA NC for 24, 48, 72 and 96 h and the absorbance was measured at 490 nm. As shown in Fig. 2A, the upregulation of miR-183 significantly increased the growth rate of TPC-1 cells in a dose- and time-dependent manner (P<0.05). Moreover, the viability of miR-183 inhibitor groups was consistently significantly lower than that of the miRNA NC groups (P<0.05, Fig. 2B). Cell proliferation was markedly enhanced or inhibited when cells were treated with 100 nM miR-183 mimics or inhibitor for 72 h at a growth rate of 22.32 and 34.7% (P<0.05) (Fig. 2A and B). Thus, 100 nm was used in the subsequent experiments. Increased and decreased colony formation was observed in the miR-183 and miR-183 inhibitor groups as compared to the miRNA NC group (Fig. 2D). The number of colonies for each group is shown in Fig. 2C. The clone formation of the miRNA NC group (258.3±20.69) was significantly higher than that of the miR-183 inhibitor group (88.5±6.18) and lower than that of the miR-183 mimics group (390.3±15.45) (P<0.05). The results confirmed that miR-183 promotes TPC-1 cell proliferation.

miR-183 promotes TPC-1 cell migration and invasion. The wound-healing and Transwell invasion assays were used to determine whether miR-183 overexpression promotes tumor aggression. As shown in Fig. 3, the cells transfected with miRNA NC migrated slower than miR-183 mimics but more rapidly than the miR-183 inhibitor. The result of the Transwell assay (Fig. 4A) showed that the number of TPC-1 cells trans-
infected with miR-183 mimics to the lower chamber markedly increased compared with miRNA NC, whereas the number of miR-183 inhibitors markedly decreased compared with miRNA NC. The quantification results (Fig. 4B) confirmed the results observed by inverted microscopy. The OD values at 573 nm were: miR-183 mimics group (0.8347±0.01431), miRNA NC group (0.6327±0.01581) and miR-183 inhibitor group (0.4113±0.01004) (P<0.05). The results indicated that the overexpression of miR-183 promoted TPC-1 cell migration and invasion.

miR-183 inhibits apoptosis in TPC-1 cells. To examine whether miR-183 inhibited the apoptosis of papillary thyroid cancer cells, TPC-1 cells were transfected with 100 nmol/l
of miR-183 mimics, miRNA NC and miR-183 inhibitor for 36 h. Flow cytometry data (Fig. 5) showed that the number of apoptotic cells was reduced in the miR-183 mimics group (Q2+Q4=1.5±0.06% apoptotic cells) compared to the miRNA NC group (Q2+Q4=5.1±0.08% apoptotic cells) (P<0.05). However, the number of apoptotic cells in the miR-183 inhibitor group (Q2+Q4=7.8±0.07% apoptotic cells) was markedly increased (P<0.05). These results indicated that miR-183 inhibited apoptosis in TPC-1 cells.

**PDCD4 is a direct target of miR-183.** To determine whether PDCD4 is a direct target of miR-183, the 3’-UTR of PDCD4 mRNA containing the miR-183 binding sites and the mutant were cloned into a luciferase reporter construct. The luciferase reporter assay (Fig. 6B) showed that the relative luciferase activity (RL/FL) of the miR-183 group was significantly decreased compared with the NC. However, no difference in the co-transfection with the PDCD4-mut-vector was identified. These results indicated that PDCD4 is a direct target of miR-183.

miR-183 negatively regulates PDCD4 protein expression at the post-transcriptional level. To determine the relationship between miR-183 and PDCD4 at the mRNA and protein levels, miR-183 mimics, inhibitors or miRNA NC (100 nmol/l) were transfected into TPC-1 cells, and the levels of PDCD4 mRNA and protein were monitored. The RT-qPCR analysis showed that PDCD4 mRNA levels were not significantly altered during these treatments (Fig. 7A). However, western blot analysis revealed that compared to the controls, the expression of PDCD4 was significantly reduced by miR-183 mimics transfection and increased by miR-183 inhibitor transfection (Fig. 7B). The results indicated that miR-183 did not affect mRNA stability and regulated PDCD4 expression at the post-transcriptional level.

**PDCD4 is involved in miR-183-induced effects in PTC cells.** To determine whether PDCD4 serves as a critical mediator of the role of miR-183 in PTC cells, PDCD4 and NC siRNAs were transfected into TPC-1 cells. As shown in Fig. 8H, the protein level of PDCD4 was decreased in TPC-1 cells transfected with...
PDCD4 siRNA compared with NC siRNA. In the MTT assay, compared with the NC siRNA group, the PDCD4 siRNA group markedly increased cell proliferation by 33.4, 65.4 and 44.4% at 48, 72 and 96 h, respectively (P<0.05; Fig. 8A). Moreover, cell colony formation efficiency increased in the PDCD4 siRNA group (P<0.05 Fig. 8B and C). Knockdown of PDCD4 promoted the migration and invasion ability of TPC-1 cells (P<0.05; Fig. 8D, E and G). The apoptosis assay demonstrated knockdown of PDCD4 decreased TPC-1 cell apoptosis by 90% at 36 h after transfection (Fig. 8F). These data indicated that effects of siRNA versus PDCD4 were similar to those induced by miR-183 in TPC-1 cells, rendering PDCD4 as a functional target of miR-183.

**Discussion**

Mounting evidence has indicated that miRNAs contribute to cancer pathogenesis. Thus, understanding the relationship between miRNAs and its targets and cell signaling pathways involved in cancer became important. miR-183 is a member of the miR-183-96-182 cluster, located at the human 7q31-34 locus and which contains highly conserved sequences (37-40). It has been found to be dysregulated in a variety of different solid tumors. miR-183 is downregulated in lung cancer cells (41), osteosarcoma (42,43) and breast cancer (44). However, it is upregulated in prostate cancer (39,45-47), hepatocellular carcinoma (HCC) (48-50), colon cancer (38, 51-54) and medullary thyroid carcinoma (55). The different expression profiles may be tissue- and cell type-specific. The abovementioned studies reported that miR-183 is involved in cell differentiation, proliferation, migration, invasion and apoptosis. This finding suggested that miR-183 plays a critical role in tumorigenesis and serves as an onco- or tumor-suppressor gene in several types of cancer. However, the exact role of miR-183 in PTC is not fully understood.

In the present study, we determined that the expression level of miR-183 was significantly upregulated in PTC tissues and the Nthy-ori 3-1 cell line compared with adjacent normal tissues and the four PTC cell lines. The functional assays demonstrated that overexpression of miR-183 markedly promoted proliferation, migration, invasion and suppressed the apoptosis of TPC-1 cells. These results suggest that miR-183 functions as an oncogene in PTC.

Many targets of miRNA-183 have been identified including ezrin (41,43,56,57), VIL2 (44), EGR1 (38), SMAD4, Dkk-3 (40), LRP6 (58), iDH2 (59), iTgB1, KiF2A (60) and Tiam1 (61). Although PDCD4 was identified as one of the targets of miR-183 in human HCC cells (34) and oesophageal
miR-183 in PTC1 cells. The western blot analysis revealed that apoptosis, consistent with the results of the overexpression enhanced cell proliferation, migration and invasion and reduced knockdown of PDCD4 by siRNA in PTC1 cells significantly be downregulated by miR-21 (71,79,80).

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