

Overexpression of microRNA-203 can downregulate survivin and function as a potential therapeutic target in papillary thyroid cancer

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Abstract. Papillary thyroid cancer (PTC) is the most common type of thyroid carcinoma. PTC has a considerably high five-year survival rate; however, the possibility of recurrence is also high. Therefore, there is a requirement to clarify the molecular mechanism of PTC to promote understanding regarding the development of the disease and further improve prognosis. A number of studies have demonstrated that microRNAs (miRNAs or miRs) contribute to the progression of PTC. The present study revealed that the expression level of miR-203 was significantly lower in PTC tissues and cell lines compared with in the normal controls. In addition, inhibition of miR-203 was identified to be associated with an overexpression of survivin, which was observed in PTC samples. miR-203 regulates the expression of Bcl-2 via its downstream regulator survivin. Furthermore, the present study identified that inhibition of miR-203 histone acetylation was associated with high expression levels of miR-203 in PTC tissue samples. In summary, the results indicate that miR-203 functions as a biomarker and may serve as a candidate target for the development of novel therapeutic strategies to treat PTC.

Introduction

Thyroid cancer is the most prevalent endocrine malignancy and the incidence rate of thyroid cancer has increased from 2.4 to 9.4% annually in the last three decades in the USA (1). Papillary thyroid carcinoma (PTC), which originates from thyroid epithelial cells, is the most frequent histopathological subtype of thyroid cancer and has the highest mortality rate of all types of thyroid cancer in the USA over the past few decades (2,3). Effective therapeutic strategies for PTC, including thyroidectomy, radioactive iodine and thyroid stimulating hormone

suppression therapy have contributed to a five-year survival rate >95% prior to tumor cell dissemination between 2009 and 2015 in the USA (4). The majority of patients with PTC exhibit a good prognosis following comprehensive therapy; however, distant metastasis and recurrence can occur in certain subtypes of PTC (5). Therefore, there is a requirement to increase understanding regarding the molecular mechanisms that underlie the carcinogenesis and development of PTC. Improved understanding may promote the use of gene therapy for PTC and improve the prognosis of patients with PTC.

MicroRNAs (miRNAs or miRs) are a class of small and non-coding RNAs that consist of 19-22 nucleotides and regulate post-transcriptional genes via a number of mechanisms, including translational repression and mRNA degradation (6). miRNAs are involved in various biological processes, including tumorigenesis and metastasis, which indicates a crucial role of miRNAs in the pathogenesis of diverse human malignancies. Commonly upregulated miRNAs, including miR-146b, miR-222, miR-221 and miR-151, have been implicated in the development and metastasis of PTC (7,8). High levels of circulating miR-222 and miR-146b have been identified to be associated with PTC recurrence and a poor clinical survival. Recently, numerous studies have investigated the role of miR-203 in the carcinogenesis and growth of a number of cancer types, including colorectal cancer (9), non-small cell lung cancer (10), melanoma (11), T-cell lymphoma (12), endometrial cancer (13) and gastric cancer (14). However, to the best of our knowledge, the biological functions and molecular mechanisms of miR-203 in PTC remain unclear. The present study aimed to clarify the biological role of miR-203 in PTC and investigate possible targets.

It is understood that the occurrence and development of tumors can be regulated by both genetics and epigenetics. Certain miRNAs in tumor cells are regulated by epigenetic modifications, including DNA methylation and histone acetylation, and protein-coding genes (15,16). It has been reported that the level of histone acetylation is associated with tumor grade and the risk of tumor recurrence in human prostate cancer (17,18). In addition, overexpression of c-Myc can regulate histone H4 acetylation, which has been revealed to affect the G2/M cell cycle progression of Raji cells (19). Furthermore, a number of studies have supported a role of miRNAs as targets and effectors of aberrant histone acetylation. miR-133a can be regulated by histone acetylation and promote myocardial fibrosis (20). In addition, an ectopic expression of miR-200c

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is associated with the level of histone deacetylase inhibitors that act as tumor suppressors to inhibit the proliferation, invasion, and migration of breast cancer cells (21). Therefore, the present study aimed to investigate whether the inhibition of histone acetylation can control tumor growth by regulating the expression of miRNA, which may provide a potential biological target for the treatment of PTC.

Materials and methods

Clinical tissue specimens and cell lines. A total of 30 PTC patients aged from 31 to 70 were enrolled in this research. The cohort included 19 male and 11 female patients and they had not received radiation therapy or chemotherapy prior to surgery. All tissue samples were collected at Ningbo No. 2 Hospital (Ningbo, China) between September 2016 and May 2017 and were frozen in liquid nitrogen immediately following duodenopancreatectomy. It was only possible to collect normal tissue samples from 20 of the 30 participants. All diagnoses were based on pathological and/or cytological evidence. The present study was approved by the Ethics Committee of Ningbo No. 2 Hospital (Ningbo, China), and written informed consent was obtained from all patients.

Cell lines and culture. The Nthy-ori3-1 cell line, which is a normal human thyroid follicular cell line, and the three human PTC cell lines HTH83, NIM-1 and TPC-1 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured according to the manufacturer's protocol. Briefly, all cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in humidified air with 5% CO₂.

miRNA microarray assay. The RNA of ten PTC tissue sample samples and ten matched adjacent normal tissue sample samples was analyzed at Oebiotech Co., Ltd. (Shanghai, China) using the Agilent Human miRNA Microarray (8x60 K; version 21.0; Agilent Technologies, Inc., Santa Clara, CA, USA) with capture probes for a total of 2,549 human miRNAs based on the Sanger miRBase database (version 21.0; www.mirbase.org). The microarray assay was performed according to the manufacturer's protocol (Agilent Technologies GmbH, Waldbronn, Germany). Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the miRNA molecules in the total RNA sample were labeled using a miRNA Complete Labeling and Hyb kit (Agilent Technologies, Inc.). Each microarray slide was hybridized using 100 ng Cy3-labeled RNA (Agilent Technologies, Inc.) in a hybridization oven. Following hybridization, the slides were washed using a Gene Expression Wash Buffer kit (Agilent Technologies), scanned using the Agilent Microarray Scanner (Agilent Technologies) and analyzed with the Feature Extraction software program (version 10.7; Agilent Technologies) with default settings. Raw data were normalized using the quantile algorithm in the Gene Spring software program (v.12.6; Agilent Technologies). A relative fold-change >3 in the differential expression of miRNAs and P<0.01 were considered to indicate a statistically significant result.

Cell transfection. RNA oligoribonucleotides or small interfering RNA (siRNA) were transfected into TCP-1 cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sequences of the miRs transfected were as follows: miR-203 mimic, 5'-GUGAAAUGUUUAGGACCACUAG-3'; miR-NC, 5'-UUC UCCGAACGUGUCACGUTT-3'; miR-203 inhibitor, 5'-CUA GUGGUCCUAAACAUUUCAC-3'; and scrambled miRNA, 5'-CAGUACUUUUGUGUAGUACAA-3'. Subsequent experiments were performed 48 h after transfection, including assessment of cell proliferation, apoptosis and migration, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Cell proliferation assay. PTC cells were transfected with miR-NC or miR-203 mimic for 24 h and then seeded in 96-well plates at a density of 5,000 cells/well. Subsequently, cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at 48 h. Absorbance was determined at 450 nm using a spectrometer.

Migration assay. Cell migration assay was performed with a 24-well transwell culture chamber with 8 µm polyester membranes (Merck KGaA, Darmstadt, Germany). PTC-1 cells were transfected with miR-NC (miR-NC group) and miR-203 mimics (miR-203 group), or cultured using complete medium containing Lipofectamine® 3000 (Mock group). For migration assays, cells with different treatments were plated in serum-free medium in the upper chamber at a density of 3x10⁴ cells/well, and supplemented medium containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) to the lower compartment. After 24 h of incubation, non-migrating cells that had not migrated through the pores were carefully removed. The filters were fixed in methanol, stained with crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min and then counted with an inverted fluorescence microscope at 10x magnification. Average cell numbers were calculated from five randomly selected fields from each group.

RT-qPCR. Total RNA was isolated from tissue samples and TPC-1 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total cellular RNA was used for first strand complementary DNA (cDNA) synthesis with the Reverse Transcription system (Roche Diagnostics GmbH, Mannheim, Germany). The cDNA samples were diluted to 100 µl with RNase-free water and 1 µl cDNA mixture was used for qPCR in a total volume of 10 µl with SYBR Green Reagent (Thermo Fisher Scientific, Inc.) and sequence-specific primers. The thermocycling conditions for PCR were 95°C for 15 min followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. qPCR was performed with the following primers: miR-203 forward, 5'-CGGTAGTCTGATACTGTAA-3' and reverse, 5'-GTGCTC CGAAGGGGGT-3'; survivin forward, 5'-AGGACCACCGCA TCTCTACAT-3' and reverse, 5'-AAGTCTGGCTCCGTTC TCAGTG-3'; miR-101 forward, 5'-GAGGGGTACAGTAC TGTGATA-3 and reverse, 5'-TGC GTGTCTGGAGTC-3'; miR-195 forward, 5'-GTCGTATCCAGTGCAGGGTCCGAG GT-3' and reverse, 5'-ATTCCGACTGGATACGACTATAAC

CG-3'; miR-455 forward, 5'-GTCGTATCCAGTGCAGGG TCCGAGGT-3' and reverse 5'-ATTCGCACTGGATACGAC TCTAACAC-3'; miR-130 forward 5'-AAAGGATCCGCA TCAAGCCCGAAGTAT-3' and reverse 5'-AAAGAATTC GAGGCAGTGTCTATCACAAG-3'; miR-126 forward 5'-TAT AAGATCTGAGGATAGGTGGGTTCCTCGAGAA-3' and reverse 5'-ATATGAATTCTCTCAGGGCTATGCCGCCTA AGTAC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The expression levels of miR-203 and survivin were analyzed using the $2^{-\Delta\Delta C_q}$ method and normalized to the level of U6 small nuclear RNA expression (22).

Western blot assay. Radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) with a protease inhibitor mixture (Sigma-Aldrich; Merck KGaA) was used for total protein extraction from the lysate of PTC patient tissues and TPC-1 cells, and a bicinchoninic acid assay (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used to measure the concentration. Subsequently, 35 μ g of protein was separated by a 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Roche Diagnostics GmbH). Following blocking with 5% skimmed-milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with a primary antibody against survivin (1:1,000; cat. no. 2808) or β -actin (1:1,000; 13E5 rabbit mAb cat. no. 4970; both from Cell Signaling Technology, Inc., Danvers, MA, USA). Subsequently, the membranes were washed 3 times (5 min each) with 15 ml TBS-Tween-20 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The membranes were then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2,000; cat. no. 7074, Cell Signaling Technology, Inc., Danvers, MA, USA) in 10 ml blocking buffer with gentle agitation for 1 h at room temperature. Membranes were washed three times as described above and incubated with enhanced chemiluminescent substrate (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK) for 1 min. Excess solution was removed and exposed to X-ray film (Carestream Health, Inc., Rochester, NY, USA).

Dual-luciferase reporter assay. The wild-type (WT) and mutant (Mut) 3'-untranslated region (3'-UTR) of survivin was designed and prepared by Shanghai GenePharma Co., Ltd. (Shanghai, China) and were cloned into the pMIR-Report plasmid (Oligoengine, Seattle, WA, USA). A total of 1×10^5 293 cells were plated and cultured in 24-well plates to reach 70% confluency. Cells were cotransfected with 40 pmol miR-203 mimic or miR-NC alongside 300 ng WT survivin 3'-UTR reporter plasmid or 300 ng Mut survivin 3'-UTR reporter plasmid using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Each group of cells was transfected with pMIR-Report β gal control plasmid for normalization. The sequences of the miRs transfected were as follows: miR-203 mimic: 5'-GUGAAUGUUUAGGACCACUAG-3'; miR-NC: 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-203 inhibitor: 5'-CUAGUGGUCCUAAACAUAUUCAC-3'; and scrambled miRNA: 5'-CAGUACUUUUGUGUAGUACAA-3'. MiR-NC was used as the control for the miR-203 mimic and scrambled miRNA was used as the control for the miR-203 inhibitor.

Luciferase assays were performed 48 h post transfection using a dual luciferase reporter gene assay kit (BioVision, Milpitas, CA, USA). The relative reporter activity was obtained by normalizing the firefly luciferase activity against the control luciferase activity using a dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA).

Apoptosis and flow cytometry. A total of 3×10^5 cells/well were seeded into 6-well plates and incubated for 24 h. After transfection with miR-203 mimic or miR-NC for 48 h, cells were harvested, trypsinized, and washed. The cells were stained with FITC Annexin V Apoptosis Detection Kit (BioLegend, Inc., San Diego, CA, USA) and propidium iodide (Thermo Fisher Scientific, Inc.) for 30 min at 4°C in the dark. The samples were evaluated by flow cytometry (BD FACSCalibur[™]; BD Biosciences, San Jose, CA, USA) and the data was analyzed using FlowJo version 7.6 (FlowJo LLC, Ashland, OR, USA).

Trichostatin A (TSA) treatment assay. A total of 3×10^5 TPC-1 cells were plated and cultured in 12-well plates to reach 70% confluence. Cells were treated with TSA (Sigma-Aldrich; Merck KGaA) at concentrations of 0, 50, 100 and 200 nM for 12 h, after which cells were collected and the total mRNA were extracted.

Statistical analysis. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all data analysis. Data are presented as the mean \pm standard deviation from three independent experiments. Two variables were compared using a two-tailed Student's t-test and three variables were analyzed by one-way analysis of variance followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. The potential downstream targets of miRNA were predicted by three online target prediction software programs, including TargetScan version 7.2 (<http://www.targetscan.org>), miRanda (<http://www.mirbase.org/>) and Pic Tar software (<http://pictar.mdc-berlin.de/>).

Results

Expression level of miR-203 is lower in PTC tissues and cell lines. To investigate the role of miRNAs in the pathogenesis of PTC, Affymetrix microarrays were used to screen for differently expressed miRNAs in PTC tissues ($n=10$) and adjacent normal tissues ($n=10$). Of the measured miRNAs, the expression levels of six miRNAs were identified to be significantly lower in PTC tissues compared with adjacent normal tissues. To validate the accuracy of the microarray results, the expression levels of the six different miRNAs including miR-203, miR-101, miR-195, miR-455, miR-130 and miR-126 were evaluated by RT-qPCR with the PTC tissue samples ($n=30$) and adjacent normal tissues ($n=20$; data not shown). Out of the six miRNAs, a significantly lower expression level of miR-203 was identified in the PTC tissue samples compared with the adjacent normal tissue sample ($P < 0.001$ Fig. 1A). Furthermore, as presented in Fig. 1B, compared with the normal thyroid cell line Nthy-ori3-1, the miR-203 expression level was significantly lower in NIM-1 ($P < 0.01$) and TPC-1 cells ($P < 0.05$); however, a significantly lower expression level was not identified in HTH83 cells. These results indicate that a lower expression level of miR-203 may be associated with PTC development and progression.

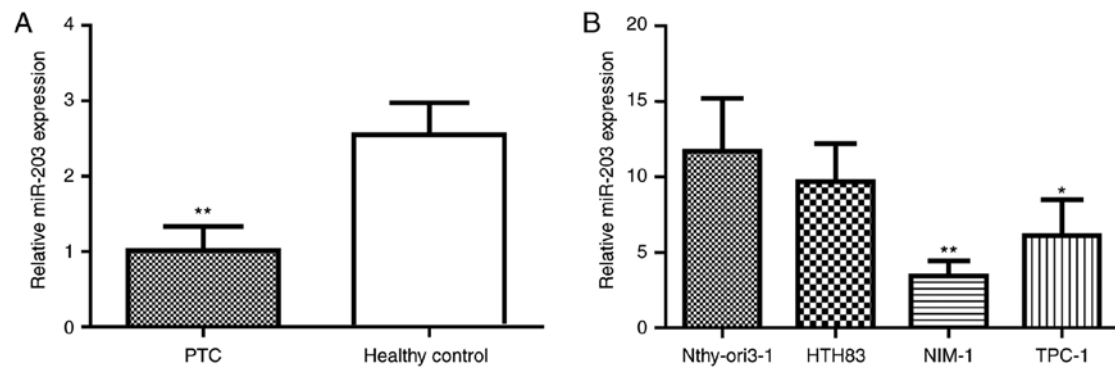


Figure 1. Expression levels of miR-203 in PTC are decreased compared to matched adjacent tissue. (A) The miR-203 expression level was evaluated in PTC tissue samples compared with matched adjacent normal tissue samples by RT-qPCR. ** $P < 0.01$ vs. healthy control. (B) The miR-203 expression levels in the PTC cell lines HTH83, HIM-1 and TCP-1, and the thyroid epithelial cell line Nthy-ori3-1 were measured by RT-qPCR. * $P < 0.05$, ** $P < 0.01$ vs. Nthy-pri3-1. Data are presented as the mean \pm standard deviation. miR-203, microRNA-203; PTC, papillary thyroid cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

miR-203 overexpression regulates cell proliferation, apoptosis and motility in vitro. Based on the aforementioned results, the present study investigated whether miR-203 regulates PTC proliferation. miR-negative control (miR-NC) and miR-203 mimic were transfected into NIM-1 cells and the effects on proliferation were observed. The relative expression level of miR-203 was significantly higher in the miR-203 mimic group compared with the miR-NC group following transfection (Fig. 2A). An apoptosis assay revealed that transfection with miR-203 mimic decreased the cell viability compared with transfection with miR-NC (Fig. 2B). In addition, a CCK-8 assay demonstrated that following transfection with miR-203 mimic, the proliferation rate of NIM-1 cells was significantly lower compared with the miR-NC group (Fig. 2C). Furthermore, Transwell assays were performed to assess whether miR-203 could inhibit the migration of PTC cells. As presented in Fig. 2C and D, the number and state of TPC-1 cells was similar between the Mock and miR-NC groups. However, as presented in Fig. 2E, overexpression of miR-203 significantly reduced the number of migratory TPC-1 cells numbers compared with the Mock ($P < 0.05$) and miR-NC groups, which indicates that miR-203 overexpression may inhibit the migration of TPC-1 cells. In summary, these results indicate that overexpression of miR-203 suppresses the proliferation and motility of PTC cells and induces apoptosis *in vitro*.

Survivin is a downstream target gene of miR-203. To identify potential targets of miR-203, the network-based target prediction software programs miRanda, Pic Tar, and TargetScan were applied. All three programs identified survivin as a downstream target of miR-203 (Fig. 3A), which suggests that miR-203 may be an upstream regulator of survivin. Therefore, the present study mutated the 3'-UTR of survivin to investigate whether survivin is a target of miR-203 (Fig. 3B). Subsequently, luciferase assays were performed with the WT and MUT survivin. As presented in Fig. 3C, transfection with miR-203 mimic significantly inhibited the luciferase activity of the reporter gene containing the WT survivin 3'-UTR; however, miR-203 mimic did not inhibit the reporter gene with the mutated 3'-UTR.

The effect of miR-203 on survivin expression was then investigated by transfection with miR-203 siRNA. The

relative expression level of miR-203 was significantly lower in the miR-203 siRNA group compared with the scrambled siRNA group following transfection (Fig. 3D). It was identified that inhibition of miR-203 was associated with a significantly lower expression level of survivin in TPC-1 cells compared with the scrambled siRNA ($P < 0.01$; Fig. 3E). The expression levels of survivin in patient tissue samples were then evaluated. RNA was extracted from 30 PTC tissue samples and 20 matched adjacent normal tissue samples for RT-qPCR. The results revealed that the expression level of survivin was significantly higher in PTC tissue samples compared with in adjacent normal tissue samples ($P < 0.05$; Fig. 3F). In addition, western blot analysis demonstrated that the survivin expression levels were markedly higher in the PTC tissue samples compared with the adjacent normal tissue samples (Fig. 3G).

Inhibition of histone acetylation can upregulate miR-203. The mechanisms by which miR-203 inhibits the proliferation and motility of and induces apoptosis in TPC-1 cells was investigated. It has been previously reported that Bcl-2 protein serves an important role in cell proliferation and apoptosis (23). TPC-1 cells were transfected with miR-NC or miR-203 mimic, followed by protein extraction for western blot analysis. As presented in Fig. 4A, the expression levels of survivin and Bcl-2 demonstrated a similar trend. It has been reported that histone acetylation can regulate the expression of miRNA. To demonstrate that the decreased expression of miR-203 in PTC resulted in the reduction of the target protein survivin, which affects the expression of Bcl-2, miR-NC and miR-203, mimics were transfected into TPC-1 cells and the protein expression levels were determined by western blotting. As shown in Fig. 4A, survivin and Bcl-2 showed a similar trend suggesting that miR-203 functions by indirectly affecting the expression of Bcl-2. Histone acetylation can regulate the expression of miRNA (24). Therefore, to investigate the low expression level of miR-203 in patients with PTC, TPC-1 cells were treated with TSA, an inhibitor of histone deacetylases. It was identified that the expression level of miR-203 was higher following treatment with TSA in a dose-dependent manner. Compared with untreated cells the miR-203 expression level was significantly higher in cells treated with 50 nmol/l TSA ($P < 0.05$), 100 nmol/l TSA ($P < 0.05$) and 200 nmol/l TSA ($P < 0.01$). In

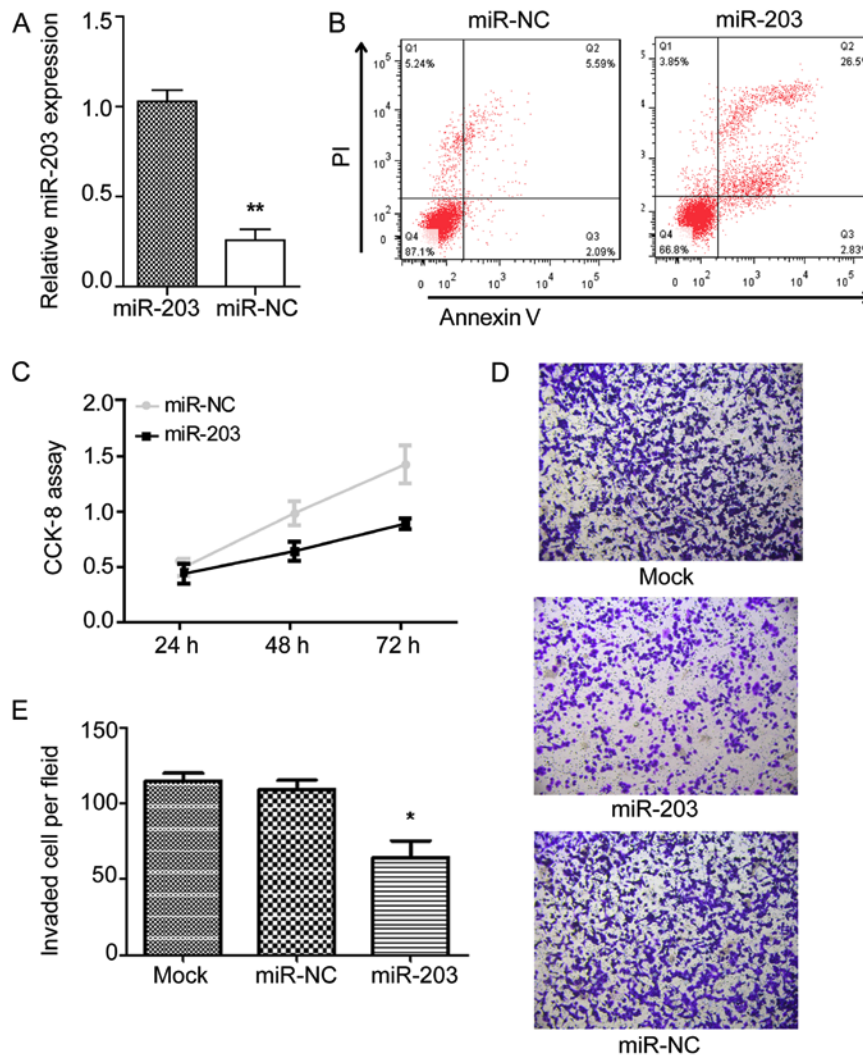


Figure 2. miR-203 suppresses the proliferation of PTC cells. (A) PTC cells were transfected with miR-203 mimic or miR-NC and miR-203 expression levels were analyzed by reverse transcription-quantitative polymerase chain reaction. ** $P < 0.01$ vs. miR-203. (B) Apoptosis rates were evaluated in NIM-1 cells transfected with miR-203 mimic or miR-NC. (C) Proliferation rates were analyzed by CCK-8 assay in NIM-1 cells transfected with miR-203 mimic or miR-NC. (D) Transwell migration assays were used to determine the migratory capabilities of TPC-1 cells transfected with miR-203 mimic or miR-NC. Magnification, 10x. (E) Quantification of the migrated cells in the Transwell migration assay. * $P < 0.05$ vs. Mock. Data are presented as the mean \pm standard deviation miR-203, microRNA-203; PTC, papillary thyroid cancer; CCK-8, Cell Counting Kit-8; NC, negative control; PI, propidium iodide.

summary, these data suggest that miR-203 inhibits the proliferation and motility, and induces apoptosis of TPC-1 cells, which may occur via a regulation of the expression of Bcl-2.

Discussion

PTC is the most common thyroid malignancy (25). In the early stages of PTC no specific symptoms are observed, which makes the diagnosis of early-stage PTC difficult (26). miRNAs, post-transcriptional gene regulators, have been reported to serve a key role in thyroid cancer progression by regulating numerous cellular events (27,28). A numbers of miRNAs, including miR-146 (29), miR-222 (30) and miR-20b (31), have been identified to promote or suppress the progression of PTC.

The present results revealed that the expression level of miR-203 was significantly lower in PTC tumor tissues compared with adjacent normal tissues. miR-203 has been reported to be downregulated in metastatic melanoma (11) and non-small-cell lung cancer (32), which indicates miR-203

serves a role in different types of cancer. Consistent with previous studies, the present study observed similar results in different PTC cell lines, which indicates a low expression level of miR-203 in PTC. Therefore, the present study selected miR-203 for further investigation.

It is understood that cancer cells are characterized by a limitless replicative potential, resistance to cell death, tissue invasion and metastasis (33). In the present study, the CCK-8 assay demonstrated that an overexpression of miR-203 inhibits the proliferation of NIM-1 cells and an apoptosis assay revealed that the apoptosis rate was significantly higher following transfection of NIM-1 cells with miR-203 mimic. In addition, the effect of miR-203 on the function of TPC-1 cells was evaluated, which revealed that an upregulation of miR-203 inhibits cell migration. In summary, the present results indicate that miR-203 upregulation can inhibit cell proliferation, induce cell apoptosis and suppress the motility of PTC cells.

Furthermore, survivin was identified as a potential downstream regulator of miR-203. To verify this hypothesis, a

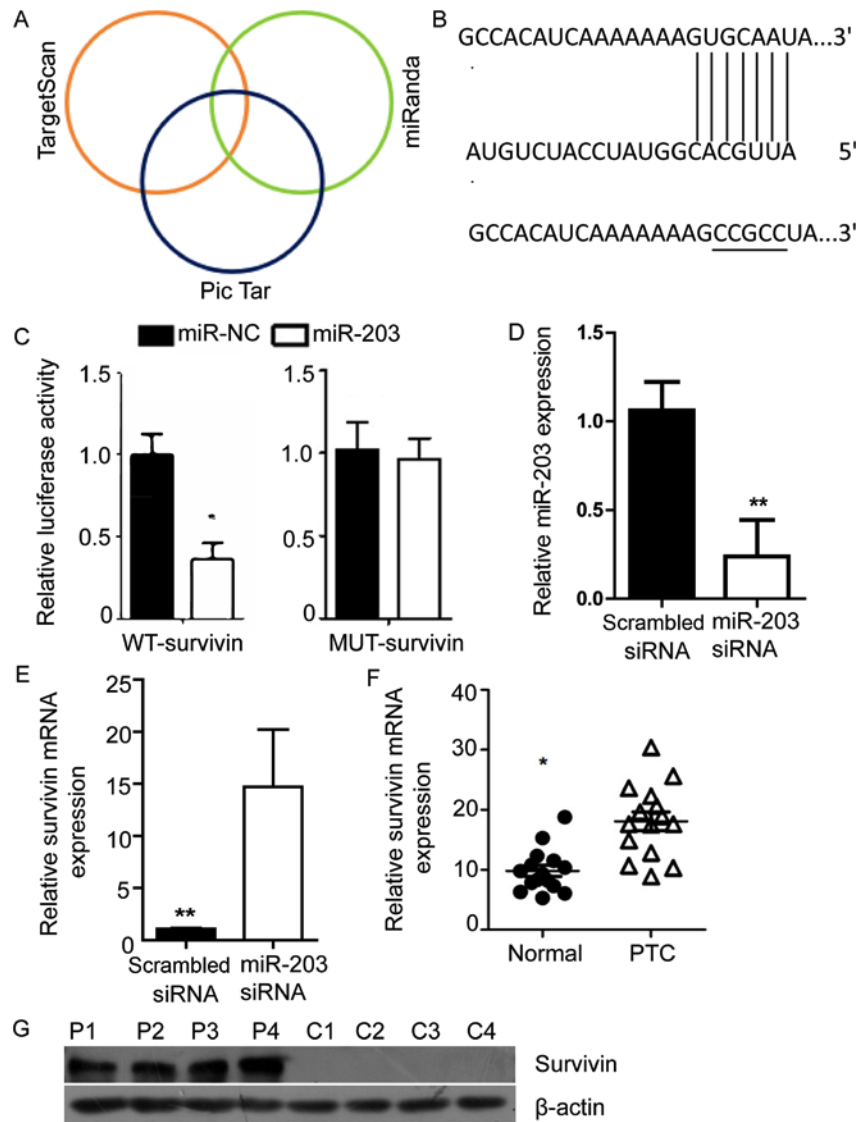


Figure 3. Survivin is a downstream target gene of miR-203. (A) Survivin was identified as a target gene of miR-203 using TargetScan, Pic Tar and miRanda. (B) Predicted binding sites of miR-203 with the WT and MUT 3'-untranslated region of survivin. (C) Luciferase assays in 293 cells with WT-survivin or MUT-survivin and miR-NC or miR-203. * $P < 0.05$ vs. miR-NC. The relative expression level of (D) miR-203 and (E) survivin in TPC-1 cells 24 h after transfection with miR-203 siRNA or scrambled siRNA. ** $P < 0.01$ vs. scrambled siRNA. (F) The relative mRNA expression of survivin in 15 PTC tissue samples and matched adjacent normal tissue samples. * $P < 0.05$ vs. normal. (G) Protein expression levels of survivin in PTC tissue samples and matched adjacent normal tissue samples. Data are presented as the mean \pm standard deviation. miR-203, microRNA-203; WT, wild-type; MUT, mutant; NC, negative control; PTC, papillary thyroid cancer; siRNA, small interfering RNA; P, tissue samples from patient with PTC; C, matched adjacent normal tissue samples.

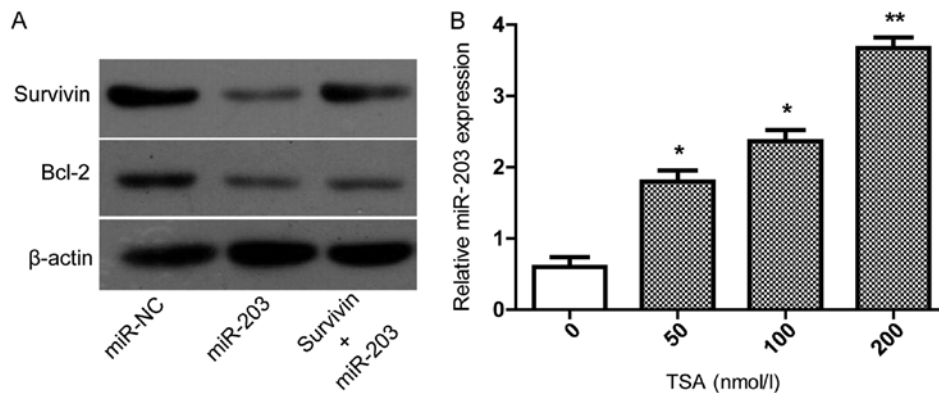


Figure 4. Low acetylation levels are associated with a low expression level of miR-203. (A) miR-NC and miR-203 mimic were transferred into TPC-1 cells for 24 h, followed by western blot analysis. (B) TPC-1 cells were treated with TSA for 24 h and the expression level of miR-203 was measured by reverse transcription-quantitative polymerase chain reaction. The expression level of miR-203 increased following treatment with TSA in a dose-dependent manner. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. 0 nmol/l TSA. miR-203, microRNA-203; NC, negative control; TSA, Trichostatin A.

luciferase assay was performed to detect survivin gene expression. Upregulation of miR-203 decreased the level of survivin gene expression. However, when survivin was mutated, the gene expression level of survivin was not altered following miR-203 upregulation, which further suggests that miR-203 serves as an upstream regulator of survivin. In addition, inhibition of miR-203 was identified to increase the expression level of survivin in TPC-1 cells, which further supports our hypothesis. Consequently, the gene and protein expression levels of survivin were revealed to be significantly higher in PTC tissues compared with adjacent normal tissues. Previous studies have suggested that survivin is highly expressed in PTC and may be associated with disease occurrence, lymph node metastasis and clinical staging of PTC (34-37).

It has been reported that Bcl-2 can regulate proliferation and apoptosis (38,39). This raises the question of whether survivin can functionally inhibit the expression of Bcl-2. The present study revealed a consistent trend between the expression levels of survivin and Bcl-2, which suggests that survivin and Bcl-2 may interact in the occurrence and development of PTC. miR-203 can regulate cell apoptosis and a low expression level of miR-203 in PTC may be associated with abnormal proliferation in the tumor. Finally, the present study investigated the influence of the expression of miR-203 from the perspective of epigenetics. It was revealed that the expression level of miR-203 is higher following treatment with TSA, which suggests that TSA inhibition of histone acetylation upregulates miR-203 expression.

In conclusion, the present study identified a low expression level of miR-203 in PTC tissue and cell lines. miR-203 was demonstrated to inhibit cell proliferation and migration, and induce apoptosis. Overexpression of miR-203 may serve a role in PTC tumor cells by down regulating survivin, which may regulate Bcl-2 expression. Furthermore, inhibition of histone acetylation was demonstrated to upregulate miR-203 expression, which suggests that deacetylation and epigenetics may serve a role in PTC therapy. These results indicate that miR-203 serves a role in the pathogenesis of PTC, and may act as a novel biomarker and a target for the development of novel therapeutic strategies against PTC.

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Availability of data and materials

Not applicable.

Authors' contributions

XJW, LD, ZJZ and JRZ performed the experiments. XJW interpreted the results and drafted the manuscript. JPZ examined the archives and identified the subjects included in the study, and revised the manuscript thoroughly prior to submission.

Ethics approval and consent to participate

All studies associated with patient samples were approved by the Ethics Committee of Ningbo Medical University, and informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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