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MiR-200c promotes proliferation of papillary thyroid cancer cells *via* Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: To investigate the potential effects of miR-200c on proliferation and apoptosis of papillary thyroid cancer (PTC) cells.

MATERIALS AND METHODS: Micro ribonucleic acid-200c (miR-200c) inhibitor was transfected to down-regulate miR-200c expression. Cell counting kit-8 (CCK-8), colony formation experiment, and flow cytometry were used to detect the effects of miR-200c knockdown on proliferation and apoptosis of Butylated Hydroxytoluene 101 (BHT101) cells. The dual-luciferase reporter gene assay was conducted to detect whether miR-200c directly binds to the target gene. After knocking down miR-200c, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting analysis were performed to detect changes of target genes regarding messenger RNA (mRNA) and protein. Western blotting analysis was also adopted to detect gene expression of Wnt/β-catenin signaling pathway-related proteins.

RESULTS: Compared with those in control group, the proliferation and clone formation ability of BHT101 cells in miR-200c knockdown group were significantly inhibited ($p<0.05$), while the apoptosis rate increased markedly ($p<0.05$). Dachshund Family Transcription Factor 1 (DACH1) was the direct target gene of miR-200c. After miR-200c knockdown, the expression levels of Wnt/β-catenin signaling pathway members (including c-Myc, β catenin and cyclin D1) all decreased.

CONCLUSIONS: MiR-200c is a tumor suppressor miRNA, which promotes proliferation of PTC cells and activates Wnt/β-catenin signaling pathway by directly regulating the corresponding target protein, DACH1.

Key Words:
MiR-200c, Papillary thyroid cancer, DACH1 gene, Wnt/β-catenin signaling pathway, Proliferation.

Introduction

Thyroid cancer, the most common endocrine malignant tumor, originates from follicular or perifollicular thyroid cells[1]. There are about 300,000 new thyroid cancer cases and 40,000 deaths caused by it every year in the world[2]. Among them, papillary thyroid cancer (PTC) is the most common subtype of thyroid cancer, accounting for more than 80% of all thyroid cancer cases[3]. Genetic and epigenetic changes have been proved to play a crucial role in the generation and development of PTC. However, little is known about the detailed molecular mechanism behind these changes[4]. At present, the main treatment strategies for PTC, including surgical resection, radioactive iodine therapy and long-term thyrotropin inhibition therapy, bring favorable prognosis to the vast majority of patients. However, recurrence and metastasis usually lead to poor clinical prognosis[5]. Therefore, it is necessary to clarify the pathogenesis mechanism of PTC to help develop improved treatment methods for PTC patients.

Micro ribonucleic acids (miRNAs) are a group of endogenous, single-stranded and non-coding RNA molecules of 18-24 nucleotides in length[6]. MiRNAs can regulate expression at transcriptional or post-transcriptional levels by base pairing with the 3'-untranslated region (3'-UTR) portion of their target genes, triggering messenger RNAs (mRNAs) degradation and/or transcriptional silencing[7]. MiRNAs are abnormally expressed in almost all types of human cancers, indicating that miRNAs are involved in carcinogenesis and cancer progression[8]. Various miRNAs in PTC are out

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of balance and can inhibit or promote carcinogenesis[9]. MiRNAs imbalance is related to a variety of biological mechanisms and plays a crucial role in the carcinogenicity of PTC[10]. Therefore, further study on the specific role of miRNAs in PTC may be helpful to identify miRNAs as effective targets for the treatment of this disease.

MiR-200c, a member of miR-200 family, is located on chromosome 12 (12p13.31). Low miR-200c is related to poor prognosis[11]. MiR-200c may impede the potential metastasis and invasion of tumors by inhibiting epithelial-mesenchymal transition (EMT)[12]. It was reported that miR-200c is an important regulator of various cancers. However, whether miR-200c participates in the pathogenesis of PTC is still unknown. Therefore, this study aims to investigate the potential role of miR-200c in PTC.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), fetal bovine serum (FBS; HyClone, South Logan, UT, USA), cell apoptosis detection kit, bicinchoninic acid assay (BCA) protein concentration determination kit (Beyotime, Shanghai, China), Lipofectamine 2000, TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cell counting kit-8 (CCK-8) reagent (Dojindo Molecular Technologies Kumamoto, Japan), SYBR Green One Step real time-quantitative polymerase chain reaction (RT-qPCR) Kit (Solarbio, Beijing, China), psi-CHECK-2 plasmid, Dual-Luciferase detection system (Promega, Madison, WI, USA), Dachshund Family Transcription Factor 1 (DACH1), c-Myc, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclin D1 antibodies (Abcom, Cambridge, MA, USA), FACScan™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA, USA).

Cell Culture and Transfection

Butylated Hydroxytoluene 101 (BHT101) cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% FBS in an incubator containing 5% CO_2 at 37°C. Oligonucleotide miR-200c mimic and inhibitor as well as negative control (NC) were designed and synthesized by Genepharma (Shanghai, China). BHT101 cells were inoculated in six-well plates at 50% density and incubated overnight. Transfection was then performed with Lipofectamine 2000. The final concentration of mimic transfection was 5 nM and that of inhibitor transfection was 100 nM.

RT-qPCR Analysis

Total RNA was extracted from BHT101 cell line with the help of TRIzol reagent. The total RNA concentration was detected at absorbance of 260 nm with a NanoDrop-2000 spectrophotometer. According to the instructions of the one-step SYBR Green RT-qPCR kit, RT-qPCR was performed with the Mx3000P qPCR system. U6 was selected as an endogenous control. The 25 μL RT-qPCR reaction system included 2 μL of total RNA extract, 2× premix, miR-200c or U6 specific primers. PCR was carried out under the following conditions: reverse transcription at 42°C for 30 min, denaturation at 95°C for 10 min, followed by denaturation at 95°C for 20 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s, with a total of 40 cycles. Subsequently, fluorescence signals were collected at 72°C. Primer sequences were as follows: miR-200c, forward: 5'-GGCGTAATACTGCCGGGTA-3', reverse: 5'-ATTGCGTGTCGTGGAGTCG-3', U6, forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse: 5'-CGCTTCAGAATTTGCGTGTCAT-3', WNT1, forward: 5'-CCAGGAACAACTCCTTACTC-3' and reverse: 5'-GCTAGCCTGTGTCCGAAGGA-3', GAPDH, forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3' and reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'. The relative expression level of gene was calculated by $2^{-\Delta\Delta Cq}$ method.

Cell Proliferation Analysis

Transfected BHT101 cells were seeded in 96-well plates at a density of 2×10^3 cells/well. After cells were incubated at 37°C with 5% CO_2 for 24 h, 48 h and 72 h, 10 μL of CCK-8 reagent was added to each well, and then incubation was continued and let stand at 37°C for 3 h. The absorbance of each well was measured by the microplate reader at 450 nm. Colony formation analysis was performed, and transfected cells were uniformly placed in 6-well plates with 300 cells/well. After cell attachment, the culture medium was changed on time. After 10-14 days, these plates were washed with phosphate-buffered saline (PBS) twice, fixed with methanol for 15 min, stained with 0.2% crystal violet and photographed at last.

Cell Apoptosis Analysis

After 48 h of transfection, cells were collected for cell apoptosis assay. Cells were washed three times at 4°C with ice-cold PBS, resuspended in 100 μL of 1× binding buffer and then stained with 5 μL of Annexin V and 5 μL of propidium iodide. The apoptotic cells were measured by the flow cytometer after being cultured for 20 min at room temperature in the absence of light.

Western Blotting Analysis

After washing with PBS, BHT101 cells were lysed on ice in protein extraction buffer for 15 minand then centrifuged at 12,000 rpm/min for 15 min at low temperature, and the supernatant was sucked out and loaded into a new centrifuge tube. BCA method was chosen to detect protein concentration. About 30 μg of protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) electrically, blocked with 5% bovine serum albumin (BSA) at 37°C for 1 h, and incubated with primary antibody at 4°C overnight. After that, the secondary antibody coupled with peroxidase was incubated at room temperature for 1 h, followed by imaging with Tanon 5200 chemiluminescent imaging system.

Detection of Luciferase Activity

TargetScanHuman 7.2 (http://www.targetscan.org/vert_72/) was employed to predict the potential target of miR-200c. Based on dual-luciferase reporter gene assay, 3'UTR fragment of the wild-type (WT) or mutant (MUT) WNT1 containing miR-200c predicted binding sites was cloned into an empty psiCHECK-2 vector. Before transfection, BHT101 cells were inoculated into 24-well plates at 2×10^5 cells/well. With Lipofectamine 2000, luciferase reporter plasmid containing the WNT1 interaction sequence and miR-200c mimic or NC were co-transfected into BHT101 cells. After 48 h, luciferase activity was detected adopting the dual-luciferase reporter gene assay system.

Statistical Analysis

All data were analyzed using GraphPad Prism 6.0 software (La Jolla, CA, USA), expressed as mean ± standard deviation. The differences between the two groups were analyzed by Student's *t*-test. $p<0.05$ suggested that the difference was statistically significant.

Results

Effect of Knocking Down MiR-200c on Proliferation of BHT101 Cells

To study the specific role of miR-200c in PTC *in vitro*, the effect of miR-200c knockdown on proliferation of BHT101 cells was explored. Transfecting with miR-200c inhibitor downregulated the expression of miR-200c in BHT101 cells. RT-qPCR confirmed the transfection efficiency of miR-200c inhibitor and the inhibitory effect of endogenous miR-200c ($p<0.05$, Figure 1A). CCK-8 analysis suggested that compared with that in control group, the proliferation of BHT101 cells in miR-200c knockdown group was remarkably inhibited ($p<0.05$, Figure 1B). Colony formation investigations further discovered that the number of cell clones in miR-200c knockdown group was overtly reduced ($p<0.05$, Figure 1C). These results indicate that miR-200c is crucial to the proliferation of PTC cells.

Effect of Knocking Down MiR-200c on Apoptosis of BHT101 Cells

Flow cytometry examined the apoptosis and found that compared with that in control group, the apoptosis rate in miR-200c knockdown group was markedly enhanced ($p<0.05$, Figure 2).

DACH1 Was Proved to Be the Direct Target Gene of MiR-200c in PTC Cells

MiRNAs mainly act by directly binding to 3'-UTR of their target genes, leading to mRNA degradation and/or transcriptional silencing. In order to explore the mechanism related to tumor inhibition of miR-200c, bioinformatics analysis was conducted to predict the potential target of miR-200c. It was predicted that the 3'-UTR region of DACH1 is an assumed binding site for miR-200c because it contains a region matching the miR-200c seed sequence (Figure 3A). Luciferase reporter gene assay was used to determine whether miR-200c can directly target 3'-UTR of DACH1 in PTC cells. As shown in Figure 3B, in BHT101 cells, enhanced miR-200c expression remarkably lowered luciferase activity in WT 3'-UTR of DACH1 ($p<0.05$). However, mutation of miR-200c binding site in 3'-UTR of DACH1 eliminated luciferase reaction to miR-200c. In addition, the effect of miR-200c on the expression of endogenous DACH1 in PTC cells was studied. Compared with cells transfected with NC, BHT101 cells transfected with miR-200c inhibitor distinctly increased DACH1 expression

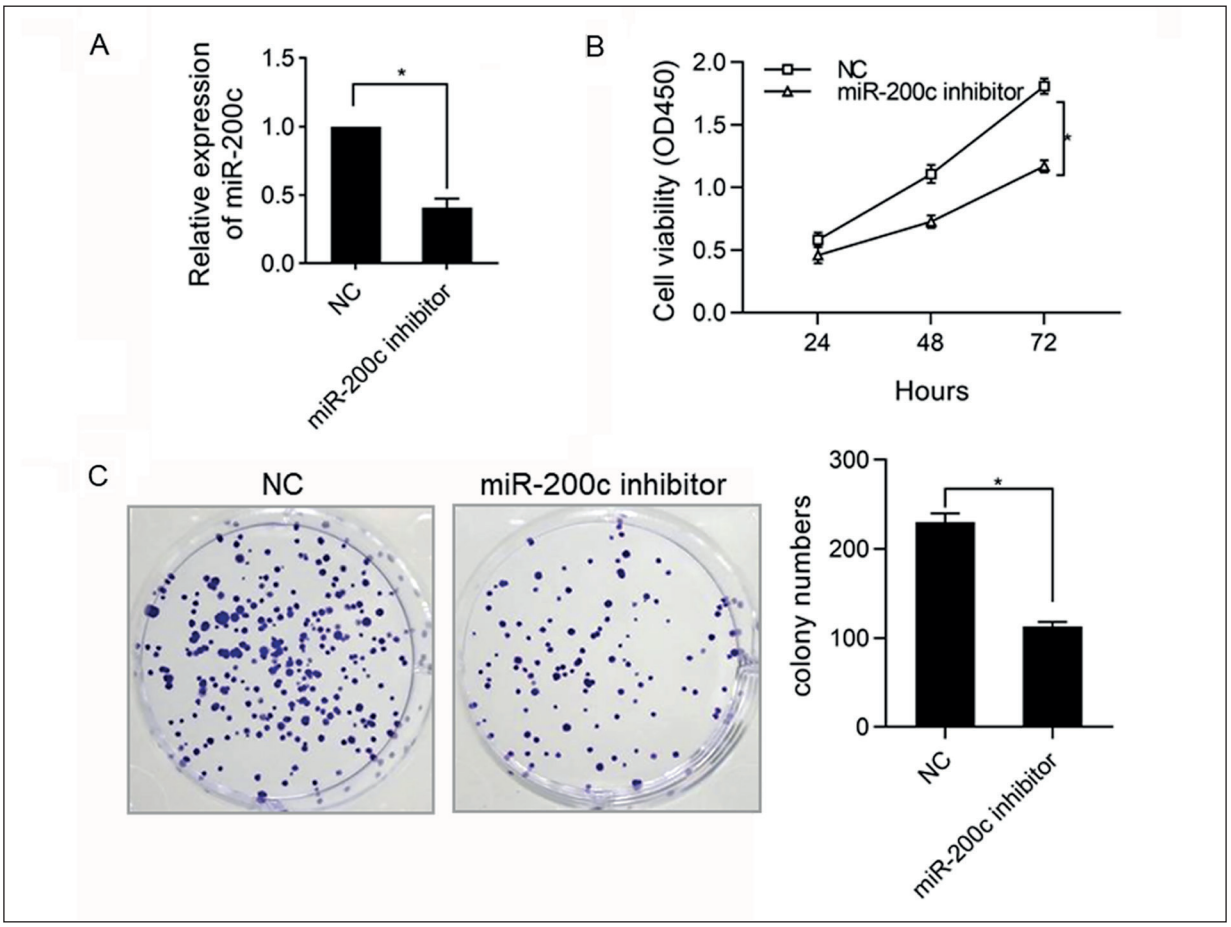

Figure 1. Effect of knocking down miR-200c on proliferation of BHT101 cells. **A,** Effect of knocking down miR-200c detected by RT-qPCR. **B,** Proliferation ability of BHT101 cells in miR-200c knockdown group and control group determined via CCK-8. **C,** Cloning capability of BHT101 cells in miR-200c knockdown group and control group investigated through colony formation analysis (*p<0.05; magnification: 40×).

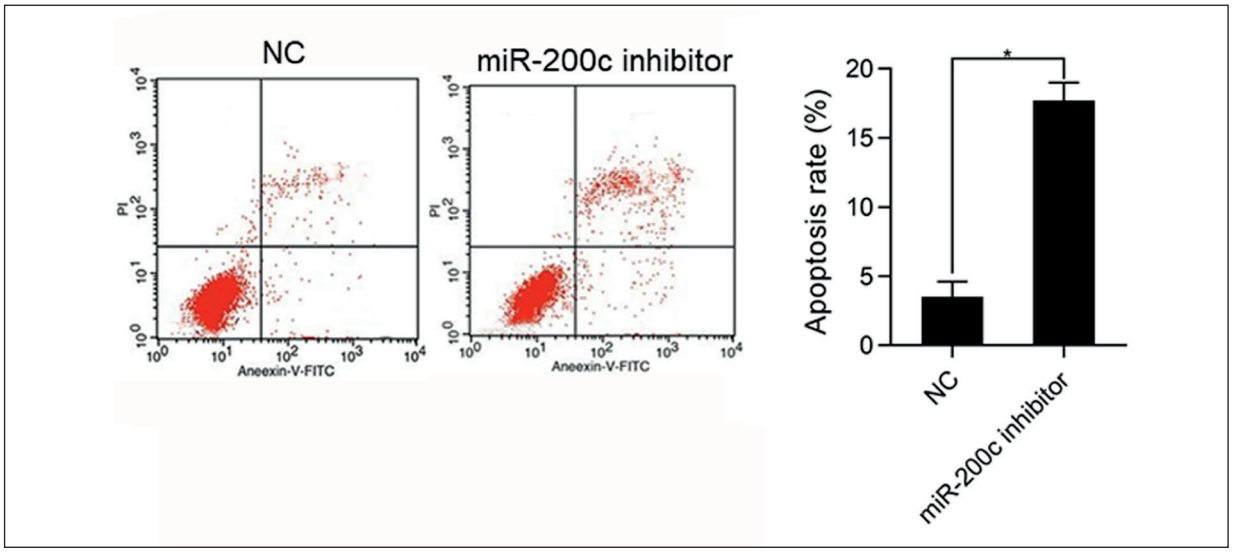

Figure 2. Effect of miR-200c knockdown on apoptosis of BHT101 cells detected *via* flow cytometry (*p<0.05).

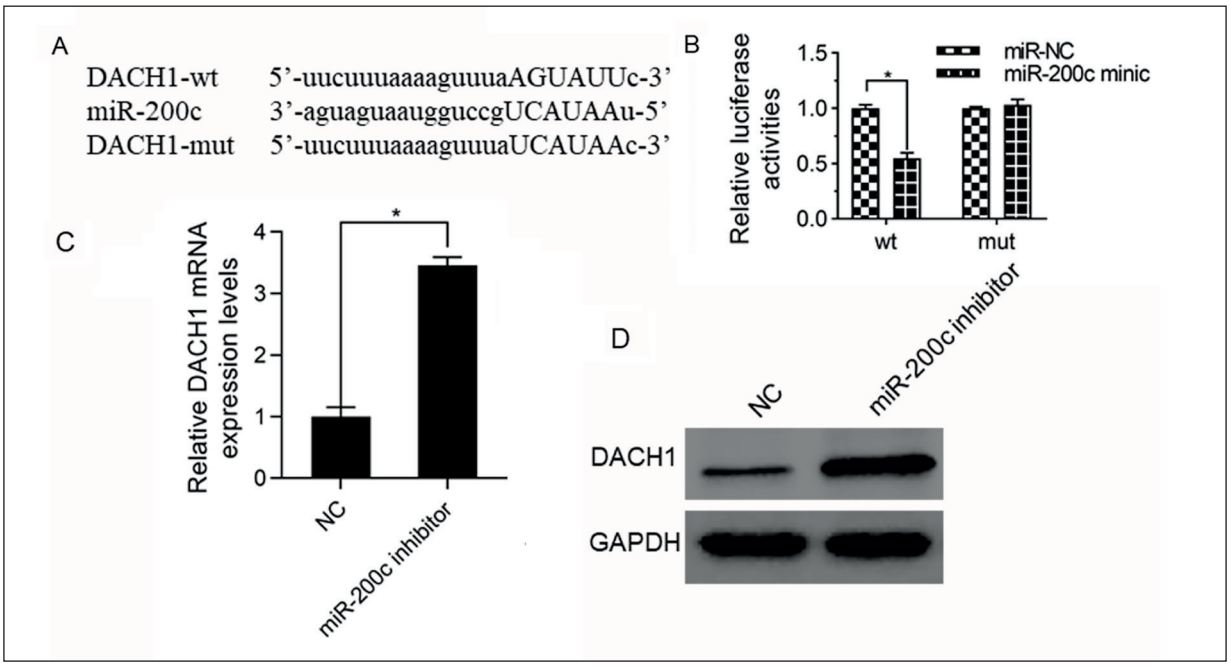

Figure 3. DACH1 is a direct target gene of miR-200c in PTC cells. **A**, MiR-200c targeting sequences in 3'-UTR of DACH1 and mutant DACH1. **B**, Relative luciferase activity in BHT101 cells after co-transfection with WT or MUT and miR-200c mimic or miR-NC. **C**, **D**, MRNA and protein levels of DACH1 in BHT101 cells transfected with miR-200c inhibitor or NC analyzed by RT-qPCR and Western blotting analysis (*p<0.05).

regarding mRNA (Figure 3C, p<0.05) and protein (Figure 3D, p<0.05). These results denote that DACH1 is the direct target gene of miR-200c in PTC cells.

MiR-200c Affected Wnt/β-Catenin Signaling Pathway

To explore the mechanism of miR-200c inhibiting proliferation and inducing apoptosis of PTC cells, the correlation between miR-200c and Wnt/β-catenin was focused. Western blotting analysis was adopted to investigate gene expression of Wnt/β-catenin signaling pathway at protein level. As results shown in Figure 4, the expression levels of Wnt/β-catenin signaling pathway members (including c-Myc, β catenin and cyclin D1) decreased after miR-200c knockdown, suggesting that miR-200c promotes proliferation of PTC cells and activates Wnt/β-catenin signaling pathway by directly regulating the corresponding target protein.

Discussion

Globally, thyroid cancer is the most common endocrine malignant tumor, accounting for nearly 5% of new cancer cases[1]. The prognosis of PTC patients is excellent, and more than half of them can be cured. However, 30% of invasive PTC can develop into PTC with distant metastasis or relapse, leading to death[5]. It is urgent to discover the molecular mechanism of PTC so as to develop

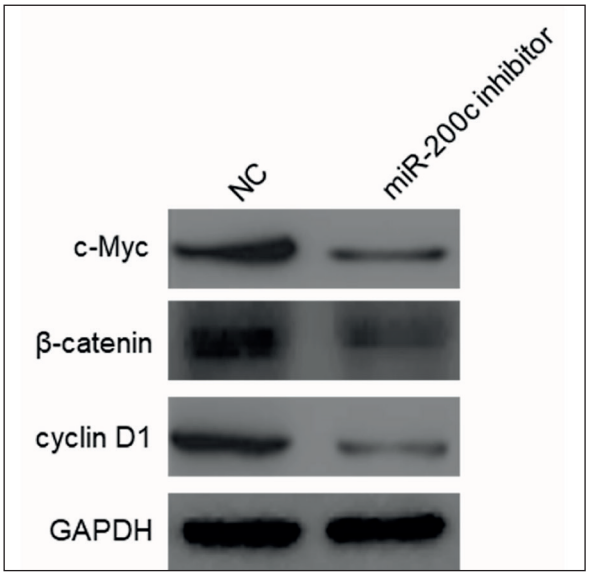

Figure 4. MiR-200c affects Wnt/β-catenin signaling pathway.

new effective therapeutic methods meeting clinical needs.

MiRNAs are small non-coding RNAs that are regulated after transcription by mRNA cleavage or translation inhibition. As one single miRNA targets hundreds of mRNAs, miRNAs play an important regulatory role in triggering various diseases. The miR-200 family consists of five homologous miRNAs, including miR-200a, miR-200b, miR-200c, miR-141 and miR-429. Extensive studies have pointed out that miR-200 family inhibits EMT and tumor metastasis, impedes self-renewal of cancer stem cells, reverses their chemical resistance, and enhances radiosensitivity of certain types of cancer[13]. The miR-200 family has previously been observed to be involved in the responses to oxidative stress of various species[13,14]. This study found that knocking down miR-200c significantly inhibited the proliferation of BHT101 cells and affected the cloning ability, suggesting that miR-200c is critical to the proliferation of PTC cells. Then, flow cytometry was chosen to examine cell apoptosis. Compared with the control group, knocking down miR-200c significantly increased the apoptosis rate of cells, indicating that miR-200c knockdown inhibits the proliferation of PTC cells and induces their apoptosis.

DACH1 is a key factor in determining cell progress, which contributes to the occurrence, invasion and metastasis of tumors in many human cancers[15]. It has been proved to be down-regulated in various tumors, such as breast cancer[16], endometrial cancer[17] and colon cancer[18]. In addition, DACH1 antagonizes the transcription and translation of oncogenes and induces mammary EMT. Wnt/β-catenin pathway is a conservative evolutionary pathway that has a large impact on EMT during cancer progression[19]. EMT is a complex and highly conserved process that enhances cell proliferation, calcium homeostasis, polarity and invasiveness and is essential for the metastasis of various solid tumors, which is considered to be a promising therapeutic target. In Wnt/β-catenin signaling pathway, c-Myc, cyclin D1 and β-catenin are the major target proteins[20]. Wu et al[16] have shown that DACH1 is negatively correlated with c-Myc and cyclin D1. In the study, the Dual-Luciferase reporter gene experiment indicated that miR-200c inhibited the expression of DACH1 by binding to its 3'-UTR. To study these effects of miR-200c on Wnt/β-catenin pathway, the expression levels of DACH1, c-Myc, β-catenin and cyclin D1 were measured by Western blotting analysis. The results of this experiment imply that miR-200c affects Wnt/β-catenin signaling pathway in PTC by regulating the expression of DACH1. The *in vitro* study further showed that miR-200c distinctly activated the expression of cyclin D1, c-Myc, and β-catenin by regulating the expression of DACH1.

Conclusions

The results of this work indicate that miR-200c promotes the proliferation of PTC cells and activates Wnt/β-catenin signaling pathway by directly regulating the corresponding target protein, DACH1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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