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MicroRNA-791 is an independent prognostic factor of papillary thyroid carcinoma and inhibits the proliferation of PTC cells

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Abstract. – OBJECTIVE: To investigate the significance and possible mechanism of miR-791 in the pathogenesis of papillary thyroid carcinoma (PTC).

PATIENTS AND METHODS: The expression of miR-791 in 80 cases of thyroid carcinoma tissues and 80 cases of paracancerous tissues was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR). After miR-791 mimics were transfected into thyroid cancer cells by liposome method, the cell proliferation was detected by Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2′-deoxyuridine (EDU), respectively. Cell cycle was detected by flow cytometry.

RESULTS: The expression of miR-791 in thyroid cancer tissue was significantly lower than that of normal thyroid. The miR-719 expression is positively correlated with the prognosis of thyroid carcinoma. After transfection of miR-791 mimics, the proliferation ability of TPC-1 and HTH83 cells was weakened, and the cell cycle was blocked in the G0/G1 phase. Further study on the underlying mechanism found that after overexpression of miR-791, the expressions of Cyclin D1, CKD6 and CDK4 decreased significantly, while the expression of cyclin inhibitor P21 increased significantly.

CONCLUSIONS: MiR-791 is lowly expressed in thyroid cancer. MiR-791 may inhibit thyroid cancer cell proliferation by blocking thyroid cancer cells in G0/G1 phase, thus participating in the impediment of thyroid cancer development.

Key Words:
Thyroid carcinoma, MiR-791, Cell cycle, Cell proliferation.

Introduction

Thyroid cancer is the most common endocrine system tumor. Its incidence increases 4% every year, seriously threatening human life and health. Based on the histopathological types of thyroid cancer, it is divided into papillary thyroid carcinoma (PTC), follicular carcinoma, medullary carcinoma and undifferentiated carcinoma. Among them, medullary carcinoma originates from the para follicular C cells, and more than 95% of the thyroid cancer originates from the follicle epithelial cells. PTC is the most common type, consisting of 80-90% of thyroid cancer[1], which ranks the eighth incidence of the female malignant tumors. 40-60% PTC patients, especially those aged over 45, experience lymph node metastases, which seriously impedes the prognosis of the disease and is a threat for the recurrence of the disease[2]. MicroRNAs (miRNAs) are endogenous, non-coding single strand RNAs (ssRNA) consisting of 18-25 nucleotides in eukaryotic cells. Immature miRNAs are first cleaved by nuclease to form mature miRNAs. MiRNA induces formation of RNA-induced silencing complex (RISC) by binding to target mRNA, thereafter inhibiting protein translation[3]. More and more investigations have pointed out that miRNAs play an important role in tumor development. The analysis of gene expression profiles showed that miRNA expression was abnormal in tumor tissues[4]. It is now clear that the incidence of tumor is the final result of the deletion of tumor suppressor gene expression and/or upregulation of the oncogenic gene[3]. The latest research showed that miRNAs participate in the regulation of the incidence and development of many kinds of human cancers mainly through two ways. On the one hand, abnormal enhancement of certain specific miRNA expressions can lead to functional defects of the tumor suppressor genes; on the other hand, the abnormally downregulated miRNAs can significantly induce the oncogene expression[5]. The

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expression profiles of miRNAs were differentially expressed not only between tumors and normal tissues, but also among different tumor types and stages. However, there have been no reports on the mechanism of miR-791 in PTC. Therefore, with further research on the mechanism of miR-791 in the development, invasion and metastasis of PTC, the miRNA regulatory network may provide a new diagnostic standard and therapeutic target for PTC.

Patients and Methods

Patients

Tissues of 80 patients with non-metastatic thyroid papillary carcinoma and 80 patients with thyroid papillary carcinoma were collected for control and experiments, respectively in our hospital from March 2014 to June 2017. All the specimens were quickly placed into the RNA enzyme inactivated cryopreservation tube in liquid nitrogen for 15 minutes, and then stored in the -80°C refrigerator for preservation. All patients did not receive preoperative iodine 131 or thyroid stimulating hormone therapy. Patients with severe systemic diseases such as other malignant tumors and severe systemic infections were excluded. The clinical information of the patients were shown in Table I. This study was approved by the Ethics Committee of Xuzhou Central Hospital. Signed written informed consents were obtained from all participants before the study.

RNA Extraction

Strictly according to the manufacture's introduction of TaKaRa™ RNA extraction kit (Tokyo, Japan), the total RNA of PTC tissues and paired paracancerous tissues were extracted. 80 mg tissue were weighed and put into a mortar for grinding. The tissue powder was transferred to 1.5 mL Eppendorf (EP) tube, and 1 mL TRIzol reagents (Invitrogen, Carlsbad, CA, USA) were reacted with the mixture at room temperature for 5 min, so that the tissue was fully lysed. The tube was centrifuged at 12,000 g/min for 5 min, and the supernatant was pipetted into a new Eppendorf tube (EP). 200 μL chloroform were added to the tube, and placed for 10 min at room temperature. The new tube was centrifuged again at 12,000 g/min for 5 min, and the supernatant was transferred to the new 1.5 mL EP tube. Isopropanol was added, and then centrifuged for 10 min to remove the supernatant. 1 mL 75% ethanol was added, and centrifuged for 5 min to discard the supernatant. The isolated RNA was kept in -80°C refrigerator for preservation.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The reverse transcription reaction was carried out strictly according to the instructions of miScript II RT Kit (Qiagen, Hilden, Germany). The total volume of the reaction was 10 μL, and the reverse transcription product template was added to the RNase water to a final concentration of 3 ng/μL. The quantitative PCR was carried out according to the miScript SYBR Green PCR Kit instruction (TaKaRa, Otsu, Shiga, Japan). The total reaction system was set up as 10 μL. Quantitative real-time polymerase chain reaction (qRT-PCR) reaction parameters were as follows: pre-degeneration for 15 min at 95°C, degeneration at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, for a total of 40 cycles. Fluorescence value was detected at 75-80°C, and finally melting curve analysis was at 65-95°C (U6 was set for reference).

Table I. Cox regression analysis of single and multiple factors that affect the prognosis of PTC patients.

	Univariate analysis			Multivariate analysis		
Variables	***p*-value**	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**
Age	0.508	1.109	0.979, 1.138	0.245	1.078	0.785, 1.115
Gender	0.865	0.903	0.723, 1.364	0.348	1.177	0.837, 1.655
Tumor size	0.018	1.634	1.032, 1.698	0.029	2.13	1.453, 2.415
Extrathyroidal extension	0.223	1.012	0.956, 2.149	0.184	1.484	0.829, 2.658
TNM stage	0.156	0.994	0.505, 1.303	0.750	0.935	0.619, 1.413
Multicentricity	0.320	1.215	0.704, 1.718	0.480	1.185	0.739, 1.901
Lymph node metastasis	0.654	1.569	0.324, 1.325	0.236	1.429	0.662, 1.465
miR-791	0.002	0.567	0.332, 0.856	0.016	0.663	0.213, 0.884

Cell Culture and Transfection

Normal thyroid cells (NTHY) and PTC cell lines (TPC-1, BCPAP, HTH83 and 8505C) were purchased from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). The incubator was set at 37°C with 5% CO_2. One day before transfection, $4×10^4$ cells were seeded on 6-well plates, with 2 mL the growth medium containing FBS. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was added into each well for transfection. After 6 hours of transfection, the culture medium was changed, and incubated in the CO_2 incubator for 24 h hours at 37°C.

Cell Counting Kit-8 (CCK-8) Detection of Cell Proliferation

100 μL cell suspension was placed in the 96-well plate. The culture plate was pre-cultured for 24 hours (37°C, 5% CO_2) in the incubator. 10 μL CCK-8 (Dojindo, Kumamoto, Japan) were added to each well. The culture plate was incubated for 24 hours. The absorbance at 450 nm was measured by microplate reader.

Cell Cycle Detection

The cells were routinely cultured, and seeded in the 6-well plates at a density of $2×10^6$. After pre-cooled 75% alcohol was added, the cells were put into 4°C refrigerator for overnight fixation. Then the ethanol was discarded, and the cells were washed with 1 × phosphate-buffered saline (PBS). PBS + 1% bovine serum albumin (BSA) solution, 100 μL propidium iodide (PI) and 100 μL RNA enzyme were added in the cells sequentially. After incubation at 37°C for 30 minutes in dark, the cell cycle detection was completed on flow cytometry machine.

5-ethynyl-2Ð-deoxyuridine (EDU) Detection of Cell Proliferation

Cells in the logarithmic growth phase with the density of $4×10^3$-$1×10^5$ cells/well were seeded in 96-well plates. After transfection, the EdU solution was diluted by cell culture medium at the ratio of 1000:1; next, 100 μL EdU medium (50 M) were incubated for 2 hours. Cells were washed by PBS 1-2 times. Each well was incubated at room temperature for 30 min with 50 μL fixation solution. After the fixation solution was discarded, 50 μL glycine (2 mg/mL) were added to each well, and incubated by decolorization bed for 5 minutes. After the glycine solution was abandoned, the cells were washed by PBS for 5 minutes. 100 μL TritonX-100 were added to each well, and the cells were incubated 10 minutes for decolorization. 100 μL 1× Hoechst 33342 reaction solution were added to each well for 30-minute incubation in dark. After the staining reaction liquid was abandoned, the cells were washed with PBS for 1-3 times and observed with microscope.

Western Blotting

The cells were centrifuged for collection, lysed by ultrasonic at 0°C, and centrifuged again at 5000 rpm/min for 5 minutes to collect the supernatant. Bromine phenol blue was added and boiled for 10 minutes, stored at -20°C after loading. After the protein electrophoresis, the protein was transferred to the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The corresponding PVDF membrane was cut down according to the molecular weight, and blocked with 5% skimmed milk. After overnight incubation with specific primary antibody, the membrane was incubated with secondary antibody, then washed and exposed for images.

Statistical Analysis

All the experiments were repeated for 3 times, and the Statistical Product and Service Solutions (SPSS19, IBM, Armonk, NY, USA) statistical software was used to analyze the data. The measurement data were expressed with mean±standard deviation. Comparison between the groups was compared with the *t*-test. $p<0.05$ was considered statistically significant.

Results

Low Expression of miR-791 in Thyroid Tumors

We first used qRT-PCR to detect the expression of miR-791 in PTC and paracancerous tissues. The expression level of miR-791 in PTC tissues was significantly lower than that in normal thyroid tissues (Figure 1A). The expression level of miR-791 showed a potential association with the cancerous degree of thyroid tissue. In this experiment, the expression of miR-791 in thyroid carcinoma was analyzed, and PTC patients were further divided into high expression group and low expression group based on their miR-791 expressions. Patients with higher expression level of miR-791 had longer postoperative survival time than those with lower expression level ($p=0.0359$)

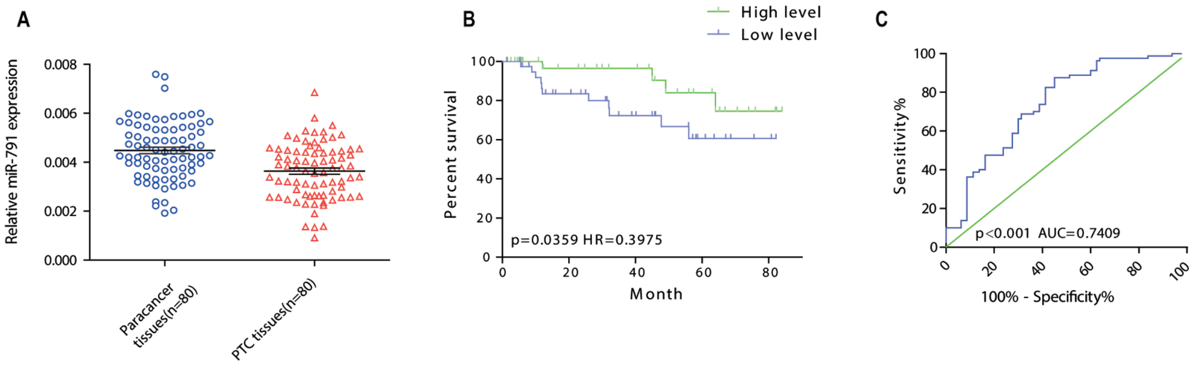

Figure 1. Lowly expressed miR-791 in thyroid tumor. ***A,*** In 80 pairs of PTC tissues and paracancerous tissues, the expression of miR-791 was downregulated in the PTC tissue. ***B,*** The survival rate of miR-791 high expression group was greater than that in the low expression group. ***C,*** The ROC curve of miR-791 in PTC and paracancerous tissues.

(Figure 1B). These data suggested that miR-791 was lowly expression in thyroid tumors and positively correlated with prognosis. The ROC curve was made according to the expression of miR-791 (AUC=0.7409, $p<0.001$) (Figure 1C). To clarify the clinical significance of miR-791, we analyzed relationship between miR-791 expression and the general situation of the patients. As shown in Table I, we found that the expression of miR-791 and the size of tumor were correlated with the prognosis of PTC patients. However, prognosis of PTC patients was not statistically significant with factors such as sex, age and distant metastasis. These results further confirmed our previous conjecture that miR-791 may be involved in the development of thyroid cancer.

The Inhibitory Effect of miR-791 on the Proliferation of Thyroid Carcinoma Cells

Expression of miR-791 in normal thyroid cells (NTHY) and PTC cell lines (TPC-1, BCPAP, HTH83 and 8505C) were detected. Compared with normal thyroid cells, the expression of miR-791 in TPC-1, BCPAP and HTH83 cells was decreased, but there was no difference in 8505C cells (Figure 2A). TPC-1 and HTH83 cells were selected for further experiments. Then, miR-791 mimics were transfected in TPC-1 and HTH83 cells, and the expression of miR-791 was detected. It was found that miR-791 mimics transfection could significantly increase the expression of miR-791 in the two cells (Figure 2B). After miR-791 mimics transfection for 6, 24, 48, 72, 96 hours, respectively, CCK-8 assay was performed to detect the proliferation ability of cells. It was found that the overexpression of miR-791 significantly inhibited the proliferation ability of thyroid cancer cells (Figure 2C and 2D). In order to further verify the effect of miR-791 on proliferation, we carried out EDU experiments, and the findings were consistent with CCK-8 findings (Figure 2E). These results demonstrated that the overexpression of miR-791 inhibited the proliferation of thyroid cancer cells.

The Effect of miR-791 on Cell Cycle Blocking

The function of miR-791 was further explored and analyzed on cell cycle level after miR-791 mimics transfection. The data showed that the cell ratio in G0/G1 phase of TPC-1 and HTH83 cells was increased (Figure 3A) after miR-791 overexpression. These findings showed that after overexpression of miR-791, the cell cycle was mainly blocked in the G0/G1 phase. After that, we explored whether miR-791 mediated tumor growth by regulating cell cycle. After transfected with miR-791 mimics, the expressions of cell cycle promoting factors (Cyclin D1, CDK4, CDK6) and cell cycle inhibitor (P21) in TPC-1 and HTH83 cells were detected. The results showed that Cyclin D1, CDK4 and CKD6 were significantly downregulated, while P21 was significantly upregulated after miR-791 overexpression (Figure 3B), and suggested that miR-791 regulated the cell cycle and participated in the oncogenesis of thyroid cancer.

Discussion

Thyroid cancer is the most common malignancy originated from endocrine organ. It accounts for 95% of all endocrine tumors and 2.6% of all malignant tumors. The incidence of thyroid cancer ranks the first in head and neck malignancies. At the end of the last century, the incidence of thyroid cancer was on the rise[6]. Papillary thyroid carcinoma (PTC) is the most common type among thyroid cancers, accounting for about 90% of all pathological types, which is common in adolescents and females. Most of PTC cases are accompanied by cervical lymph node metastasis. PTC is a low-grade malignant tumor, the main clinical symptoms of which include low growing thyroid masses,

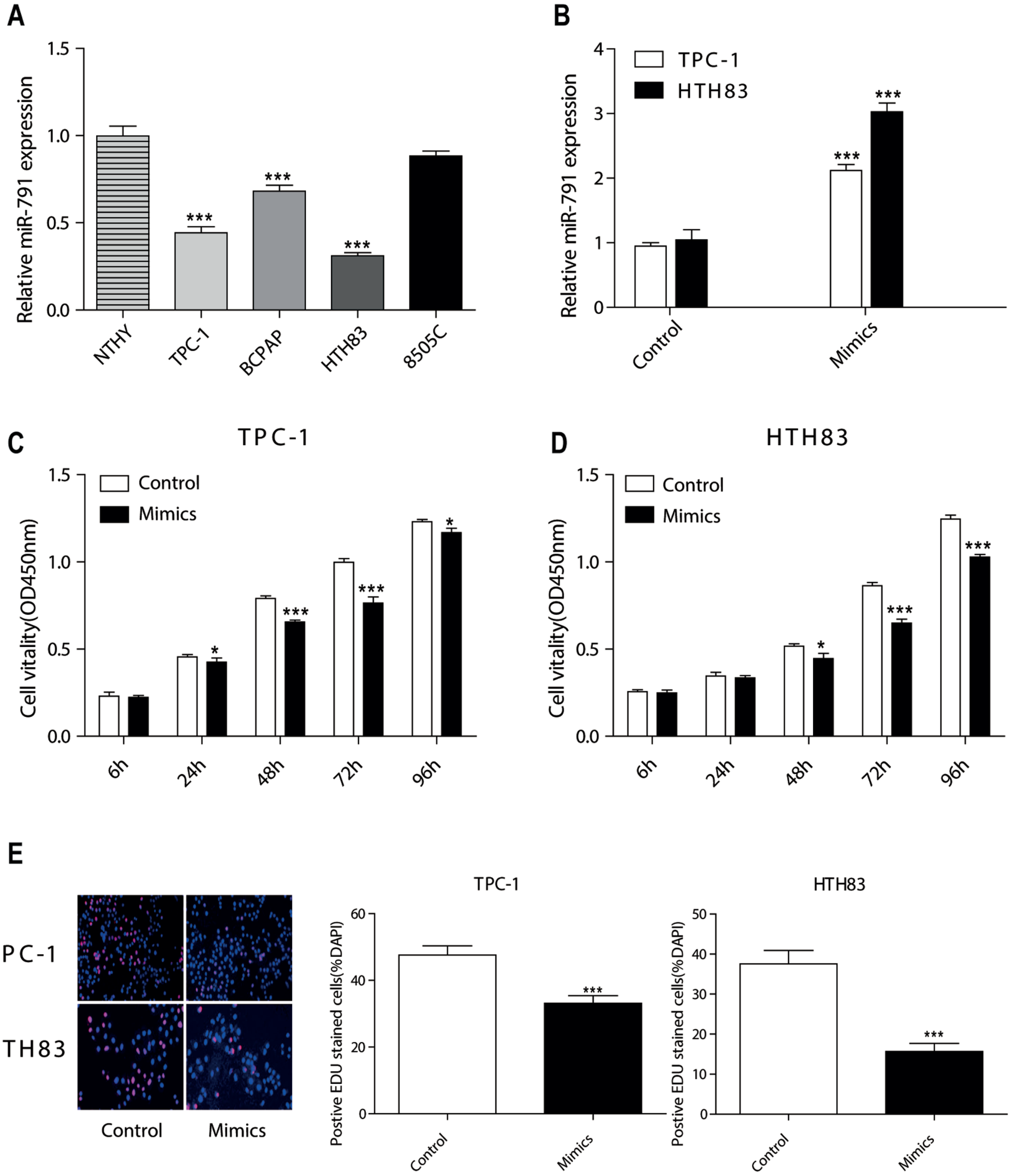

Figure 2. The inhibitory effect of miR-791 on the proliferation of thyroid carcinoma cells. ***A,*** The expression of miR-791 in normal thyroid cells (NTHY) and PTC cell lines (TPC-1, BCPAP, HTH83 and 8505C). ***B,*** The transfection efficacy of miR-791 mimics in TPC-1 and HTH83. ***C,*** After transfection of miR-791 mimics into TPC-1 cells, CCK-8 assay showed that the cell viability was decreased. D. After transfection of miR-791 mimics into HTH83 cells, CCK-8 assay showed that the cell viability was decreased. ***E,*** EDU results showed that the cell proliferation ability of TPC-1 and HTH83 cells was significantly decreased after transfection of miR-791 mimics.

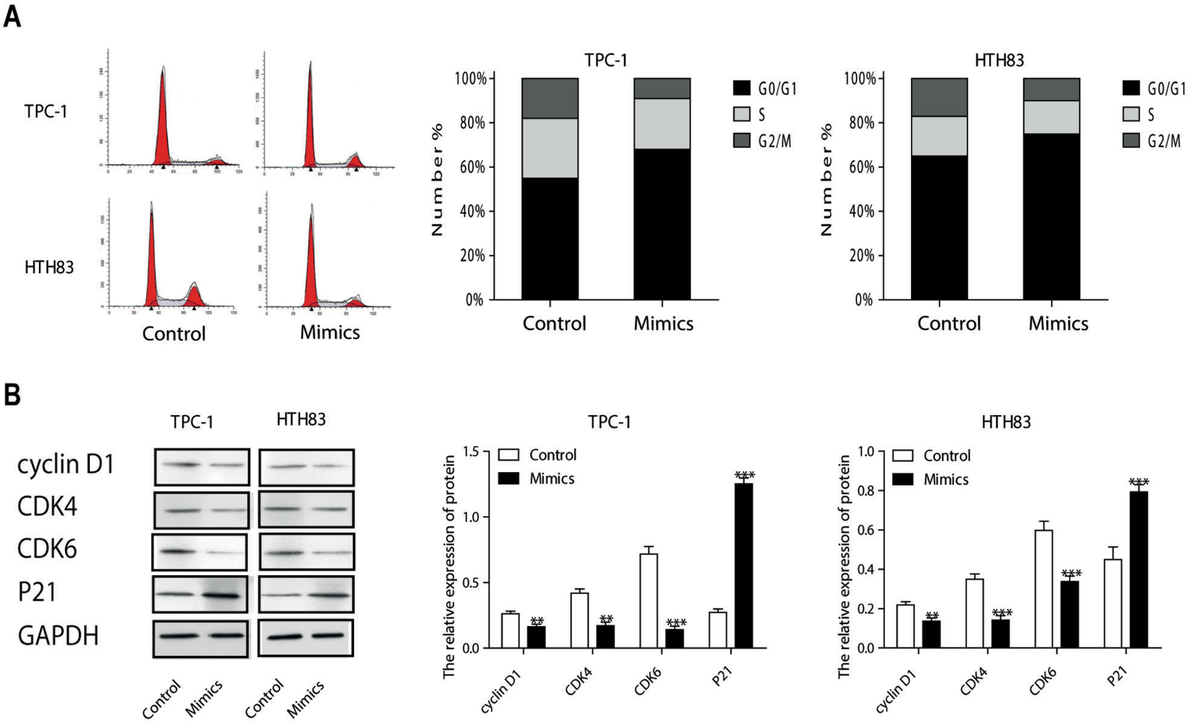

Figure 3. The inhibitory effect of miR-791 on cell cycle. ***A,*** After transfection of miR-791 mimics to TPC-1 and HTH83 cells, the cell cycle was significantly blocked in the G0/G1 phase. ***B,*** After overexpression of miR-791, Cyclin D1, CDK4 and CKD6 were significantly downregulated, while P21 was significantly upregulated.

multifocal tendency and regional lymph node metastasis. Current treatments for papillary thyroid carcinoma include surgical treatment, thyroid hormone suppression, isotope iodine 131 and adjuvant radiation therapy. After effective and proper treatment, thyroid papillary carcinoma generally has a good prognosis, with 5-year survival rate of 95%[7], and 10-year survival rate of over 90%[8]. However, some papillary carcinomas have high invasiveness possibility and dedifferentiated tendency, eventually developing into lowly differentiated thyroid cancer or undifferentiated carcinoma. Therefore, the research of cell invasion and metastasis of papillary thyroid carcinoma help to search for novel predictor so as to improve the treatment efficacy and survival rate. It is found that miRNAs are important gene expression regulators[10, 11], which play an important role in many cell processes, such as cell proliferation, cell cycle regulation, signal transduction pathway and apoptosis. MiRNAs are differentially expressed in various pathological conditions including tumorigenesis and tumor development[12, 13]. The miRNA expression profile can distinguish between malignant and non-malignant tumor tissues, which make it a potential tumor biomarker. Studies have shown that miRNAs in tissue specimens can be used as a biomarker for breast cancer, colon cancer, thyroid cancer and other tumors[14-17]. Moreover, some certain miRNAs could regulate carcinogenic gene production of thyroid cancer[18]. The study of miRNAs in thyroid carcinoma has now become an important research direction.

There are many abnormally expressed miRNAs in thyroid cancer. Wu et al[19] showed that miR-204 was lowly expressed in thyroid cancer. Upregulation of miR-204 could downregulate cyclin D1 and Ki67, and upregulate P21 to inhibit the invasion of thyroid cancer cells. Zhang et al[20] found that the high expression of miR-206 inhibited the proliferation and invasion of thyroid cancer cells, while miR-206 knockdown obtained the opposite results. In this study, miR-791 was found with low expression in thyroid carcinoma and inhibited the proliferation of thyroid cancer cells. Cell cycle is the process of continuous splitting of eukaryotic cells from mitosis termination to the next division. Cell cycle duration reflects the state of cells, a cycle of cell material accumulation and cell division. Cancerous cells usually present an abnormal division cycle[22]. Therefore, the regulation of cell cycle in tumor cells is an important strategy and target for the tumor treatment. In this study, we found that miR-791 can significantly block the cell cycle at G0/G1 phase, thereafter inhibiting cell cycle progression and cell proliferation of thyroid cancer cells. In the preliminary molecular mechanism study, it was found that the expression of G1 checkpoint protein Cyclin D1 was significantly inhibited by miR-791.

Conclusions

miR-791 was lowly expressed in thyroid cancer, which could inhibit the proliferation of thyroid cancer cells. MiR-791 exerted its inhibitory effect on proliferation of thyroid cancer cells via blocking the G1 phase.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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