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MiR-155-5p affects Wilms' tumor cell proliferation and apoptosis via targeting CREB1

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-155-5p (miR-155-5p) in regulating the proliferation and apoptosis of Wilm's tumor (WT), and to investigate the possible underlying mechanism.

PATIENTS AND METHODS: The expression levels of miR-155-5p in 37 pairs of WT clinical samples, as well as WT cell line (G401), were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and flow cytometry assay were used to detect the effects of miR-155-5p on cell proliferation, cycle and apoptosis. Target gene prediction software was applied to screen the potential downstream target gene of miR-155-5p. QRT-PCR, Western blot (WB) and luciferase reporter gene assay proved that cAMP-response element binding protein 1 (CREB1) was the target gene of miR-155-5p. Besides, rescue experiment was conducted to further explore the effect of CREB1 on WT cells.

RESULTS: The expression levels of miR-155-5p in WT tissues and cells were both significantly down-regulated. Importantly, miR-155-5p was found to be involved in the malignant behavior of WT cells. MTT assay and flow cytometry assay demonstrated that miR-155-5p significantly inhibited the proliferation, caused stagnation of cells in G0/G1 phase, and promoted cell apoptosis. CREB1 was verified as a functional target gene of miR-155-5p, which was negatively regulated by miR-155-5p. Rescue experiments indicated that restoring the expression of CREB1 could interfere with the effects of miR-155-5p on WT cells.

CONCLUSIONS: MiR-155-5p could regulate the proliferation, cell cycle and apoptosis of WT cells. These effects were achieved by regulating the expression of CREB1. Furthermore, our study might provide a new theoretical basis for the basic research of WT.

Key Words:
Micro-155-5p (MiR-155-5p), Wilm's tumor (WT), cAMP-response element binding protein 1 (CREB1).

Introduction

Wilms' tumor (WT) is the most common primary malignant tumor of the urinary system, accounting for 6-7% of malignant tumors in children aged below 15 years old. It is reported that about 75% of WT patients occur in children under 5 years old[1], and the average age of onset is about 3.5 years old[2,3]. WT is an embryo-derived solid tumor with the main clinical manifestation of abdominal mass, whose early diagnosis is extremely difficult. In recent years, almost 85% childhood WT patients may be cured with the implementation of comprehensive therapeutic regimens, including surgery, chemotherapy and radiotherapy[4]. However, due to recurrence, metastasis and insensitivity to chemotherapeutic drugs, a small number of childhood WT patients still die. Moreover, the individualized and specific long-term therapeutic treatment of WT also leads to higher prognostic risk[5]. Researches[6] have demonstrated that the recurrence rate of WT is approximately 15%, and the long-term survival rate of patients with recurrence is only 50%. With the deepening of researches, the pathogenesis of WT has been further understood. However, there are still some issues worth discussing. Currently, it is believed that malignant tumor is a disease with cell cycle disorder or (and) apoptotic mechanism disorder. Therefore, both cell cycle and apoptosis are two major hotspots in researches of malignant tumors

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at present[7,8]. Previous investigations have found that cell cycle has significant influences on apoptosis-related genes. Meanwhile, cell cycle disorder, down-regulation of apoptosis and acceleration of cell proliferation are the most significant features of tumor tissues. Oncogenes or tumor suppressor genes can directly regulate cell cycle, which are also essential factors for cell cycle progression. Furthermore, dysfunction of these genes eventually leads to uncontrollable initiation, progression and termination of cell cycle. In addition, this can also produce uncontrollable growth characteristics in cells, such as down-regulation of apoptosis and acceleration of proliferation[9,10]. MicroRNAs (miRNAs) are a type of non-coding RNAs with about 18-22 nt in length. MiRNAs regulate post-transcriptional gene expression by incompletely complementary pairing with the 3'-untranslated region (UTR) of target genes. They act on target genes in a base pairing way, thereby inducing degradation or translational suppression[11]. MiRNAs play extremely important roles in various biological processes, including development, differentiation, apoptosis and proliferation[11,12]. Since the important role of miRNA was initially confirmed in chronic B cell lymphoma[13], researches have gradually found that miRNA is involved in the occurrence and development of a variety of human tumors. Moreover, a large number of studies[14-18] have proved the regulatory effect of miRNAs on tumor development. MiR-155-5p is a typical multifunctional miRNA located on human chromosome 21q21.3. In recent years, a large number of studies have proved that miR-155-5p plays an important role in human life activities. Meanwhile, it exerts important effects on immune response, tumorigenesis, inflammation and other biological processes, as well as signal transduction pathways through regulating target genes[19-23]. However, the exact role of miR-155-5p in the occurrence and development of WT has not been elucidated. In this study, we found that the expression level of miR-155-5p in WT tissues and cells was significantly suppressed. Furthermore, the biological role of miR-155-5p was investigated *in vitro*.

Materials and Methods

Patients and Cell Lines

37 pairs of WT tissues and adjacent normal tissues were obtained from patients undergoing a surgical procedure at the First Hospital of Jilin University. All enrolled patients were pathologically confirmed as WT. Collected tissue samples were stored in liquid nitrogen and were kept in -80ºC refrigerator. This study was approved by the Ethics Committee of the hospital. Informed consent was obtained from each subject before the study. The human WT cell line (G401) together with human embryonic kidney cell line (HEK-293T) were purchased from Shanghai Institute of Chinese Academy of Sciences (Shanghai, China). Cells were inoculated in Dulbecco's modified eagle Medium (DMEM) containing 10% newborn fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Rockville, MD, USA) in a 37°C, 5% CO_2 incubator. Cells in the logarithmic phase were chosen for subsequent experiments.

Cell Transfection

G401 cells were first seeded into 96-well plates at a density of 1×10^6 per well. After incubation for another 24 h, cell transfection was performed according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). MiR-155-5p mimics, inhibitor and relevant negative controls were synthesized and transfected into G401 cells. Meanwhile, the biological function of miR-155-5p was analyzed. While in the rescue experiment, three groups were established, including: the NC group (negative control), the miR-155-5p mimics group (G401 cells transfected with miR-155-5p mimics) and the mimics + cAMP-response element binding protein 1 (CREB1) group (G401 cells transfected with miR-155-5p mimics and si-CREB1).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

The mRNA expression level of miR-155-5p and CREB1 was detected by qRT-PCR analysis. Total RNA in WT tissues and cells was extracted in accordance with the instructions of TRIzol (Gibco, Rockville, MD, USA). MiR-155-5p and CREB1 were amplified by reverse transcription. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal references for miR-155-5p and CREB1, respectively. QRT-PCR reaction was carried out according to the instructions of TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA). Relative expression level of genes was calculated by the following formula: $RQ=2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: CREB1, F: 5'-CCCAAGCTTATGACCATGGAATCTG-

GAGC-3', R: 5'-TGCTCTAGATAATCTGATT-TGTGGCAGTAAAGG-3'; MicroRNA-155-5p, F: 5'- GTAACCCGTTGAACCCCATT-3', R: 5'-CCATCCAATCGGTAGTAGCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blot Analysis

The protein samples were extracted with protein lysate and quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Then, 20 μg protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies of anti-CREB1 and anti-β-ACTIN (diluted at 1:1000, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing with Tris-buffered Saline with Tween-20 (TBST) (Beyotime, Shanghai, China), the membranes were incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) at 37°C for 1 h. Finally, immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA). The grey level of each protein was normalized to β-actin, and the results were analyzed via Image-J software.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

The logarithmic growth cells were collected, diluted into 1×10^6 cell suspension, and added into 96-well cell culture plates. Cell viability was determined *via* MTT colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). In brief, 15 μL MTT reagents (500 μg/mL) were added to each well, followed by continuously culture at 37°C for 2 h. Finally, the absorbance value at the wavelength of 450 nm was measured by a microplate reader.

Flow Cytometry Analysis

After transfection, the cells were collected, washed with phosphate-buffered saline (PBS) twice and centrifuged at 1,000 r/min for 10 min. Next, the supernatant was discarded, and the final concentration was adjusted to 1×10^6/mL. For cell cycle detection, 70% ethanol was added at -20°C and staining overnight. The cells were incubated with ribonucleases (RNases) (50 μg/mL) (Shanghai Li Rui Biological Technology Co., Ltd., Shanghai, China) for 15 min. Finally, 50 μg/mL propidium iodide (PI) were added for another 30-min incubation. For cell apoptosis detection, 5 μL Annexin-FITC were added to adjust the final concentration to 1 g/L, followed by warm water bath at 37°C for 30 min in dark. Then, PI was added to adjust the final concentration to 5 μg/mL, and the cell clusters were filtered and removed with a 350-mesh nylon mesh. After staining at 4°C for 30 min in dark, total apoptosis rate was calculated.

Luciferase Reporter Gene Assay

Bioinformatics prediction websites (TargetScan, miRDB and microRNA) were used to predict the potential binding targets of miR-155-5p. As a result, we found that miR-155-5p could bind to the 3'-UTR of CREB1. Luciferase reporter vector pGL3 (pGL3- CREB1-WT) and mutated vector (pGL3- CREB1-MUT) were constructed. Subsequently, the above plasmid (1 μg) and miR-155-5p mimics were co-transfected into G401 cells. 48 h after, luciferase activity was measured by a multi-function microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 Software (SPSS Inc., Chicago, IL, USA) were used for all statistical analysis. *t*-test was used to compare the differences between two groups. $p<0.05$ was considered statistically significant.

Results

Expression of miR-155-5p in WT Tissues and Cells

We first detected the expression level of miR-155-5p in WT tissues and cell lines by qRT-PCR. Impressively, the expression of miR-155-5p in WT tissues as well as cells was significantly decreased (Figure 1). This apparent low expression indicated the regulatory role of miR-155-5p in WT development.

Detection of Transfection Efficiency

Transfection efficiency of miR-155-5p expression in transfected cells was detected by qRT-PCR (Figure 2A). The results supported the effects of miR-155-5p mimics on cell transfection.

Inhibitory Effect of miR-155-5p on Cell Proliferation

MTT results showed that the absorbance of G401 cells was significantly decreased after transfection with miR-155-5p mimics. Meanwhile,

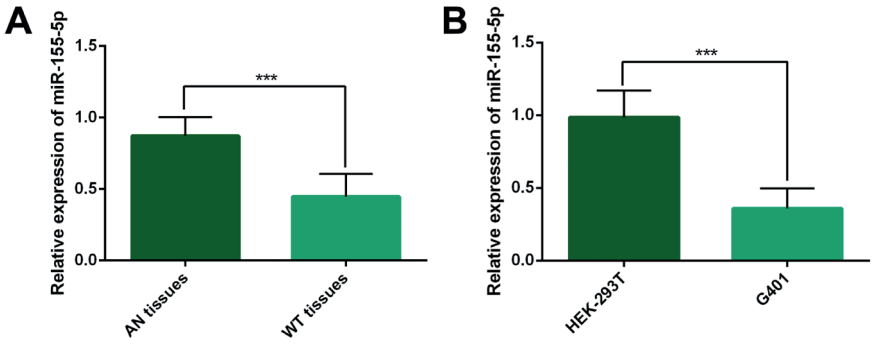

Figure 1. The expressions of miR-155-5p in Wilm's tumor (WT) tissue samples and cells, as well as corresponding adjacent normal tissues and human normal embryonic kidney cells. ***A,*** Difference in the expression of miR-155-5p between WT tissues and adjacent normal tissues (***p<0.001 compared with adjacent normal tissues). ***B,*** The expression of miR-155-5p in WT cells (G401) and human normal embryonic kidney cells (HEK293T). (***p< 0.001 compared with HEK293T).

the relative viability and proliferation were also significantly inhibited. Otherwise, after down-regulating the expression of miR-155-5p, the relative viability and proliferation of WT cells were significantly increased (Figure 2B).

Inhibitory Effect of miR-155-5p on Cell Cycle

It was found that up-regulating miR-155-5p in G401 cells led to a significant increase in the proportion of cells in G0/G1 phase and a remarkably decrease in S phase. However, inhibition of miR-155-5p induced G0/G1 phase arrest. After down-regulating miR-155-5p expression in G401 cells, cells in S phase was significantly increased, and the G0/G1 to S phase transition was significantly accelerated (Figure 2C).

Promotion Effect of miR-155-5p on Cell Apoptosis

Cell apoptosis was detected by flow cytometry. The apoptosis rates of the control group, the mimics group and the inhibitor group were (11.29 ± 1.77)%, (23.41 ± 2.04)% and (7.16 ± 1.84)%, respectively (Figure 2D-2E). Flow cytometry results showed the effect of miR-155-5p on the apoptosis of WT cells.

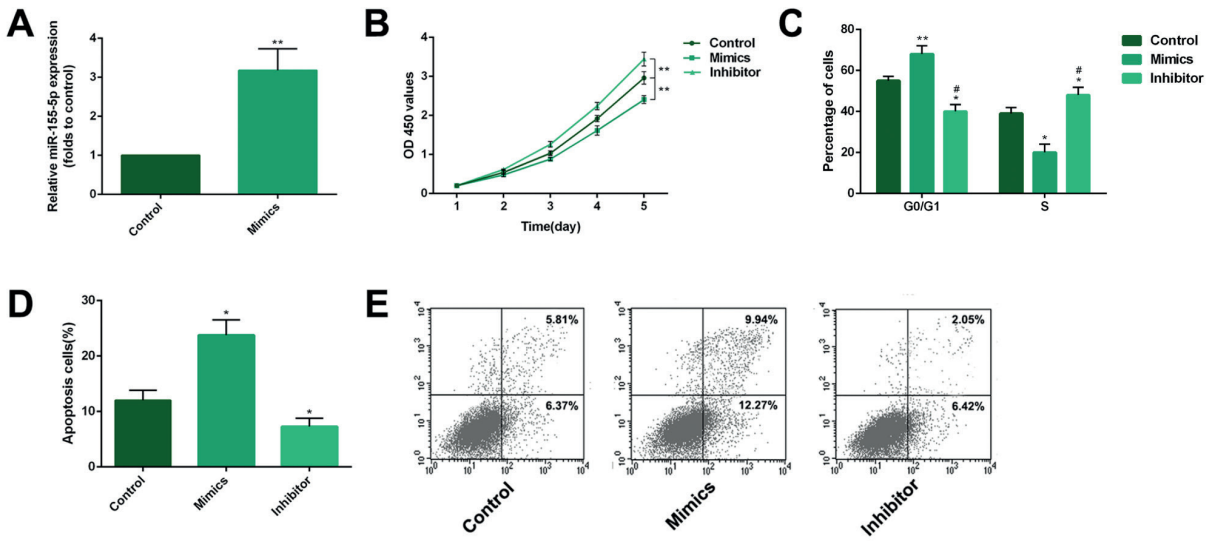

Figure 2. MiR-155-5p inhibited the proliferation and induced the apoptosis of WT cells after transfection. ***A,*** Transfection efficiency was analyzed by qRT-PCR. ***B,*** MTT assay demonstrated that miR-155-5p inhibited the proliferation of WT cells. ***C,*** Flow cytometry indicated that miR-155-5p led to G0/G1 arrest in WT cells. ***D,*** Flow cytometry found that miR-155-5p significantly increased the apoptosis rate of WT cells (*p<0.05, **p<0.01 *vs.* the control group; ##p<0.01).

CREB1 Was a Direct Target of miR-155-5p

The expression levels of CREB1 in WT tissues (Figure 3A) and cells (Figure 3B) were both significantly up-regulated. MiRNA target gene prediction software manifested that miR-155-5p could bind to the 3'-UTR of CREB1 (Figure 3C). To further verify this phenomenon, we established three groups and detected the expression changes after overexpression of miR-155-5p in WT cells. More importantly, in the gold standard assay for the identification of miRNA target genes, luciferase reporter gene assay revealed that up-regulated miR-155-5p significantly reduced the luciferase activity of wild-type CREB1. However, no significant changes were found in the luciferase activity of mutant CREB1 (Figure 3D). QRT-PCR (Figure 3E) and WB (Figure 3F) results indicated that CREB1 was negatively regulated by miR-155-5p in G401 cells. Taken together, CREB1 was a direct and functional target gene of miR-155-5p in WT cells. Subsequently, miR-155-5p mimics and LV-CREB1 were simultaneously transfected into G401 cells, and the influence of restoring CREB1 expression on the function of WT cells was detected. It was exciting that the recovery of CREB1 had the reverse force on the depressing effects induced by miR-155-5p on the proliferation (Figure 4A), G0/G1 arrest (Figure 4B) and apoptosis (Figure 4C-4D) of WT cells. These results indicated that miR-155-5p affected the malignant behavior of WT cells by regulating CREB1 expression.

Discussion

WT is the most common malignant tumor of the urinary system in children. With the innovation of medical technology and comprehensive treatment, the long-term survival rate of WT has been greatly increased[1]. However, due to recurrence, metastasis or drug resistance, the therapeutic effect is still unsatisfactory in some childhood patients. Transcription factors play important roles in the occurrence and development of malignant tumors. Abnormal transcriptional regulation can lead to the up-regulation of oncogenes or down-regulation of tumor suppressor genes, thus promoting tumor occurrence and development. Since the discovery of first miRNA in 1993[24], miRNAs have quickly become a hotspot in contemporary researches. Previous studies have demonstrated that miRNAs regulate about 60% human genes in the form of oncogenes or tumor suppressor genes[25,26]. Therefore, searching for target genes regulated by miRNAs has become the core of current miRNA researches. CREB1 gene

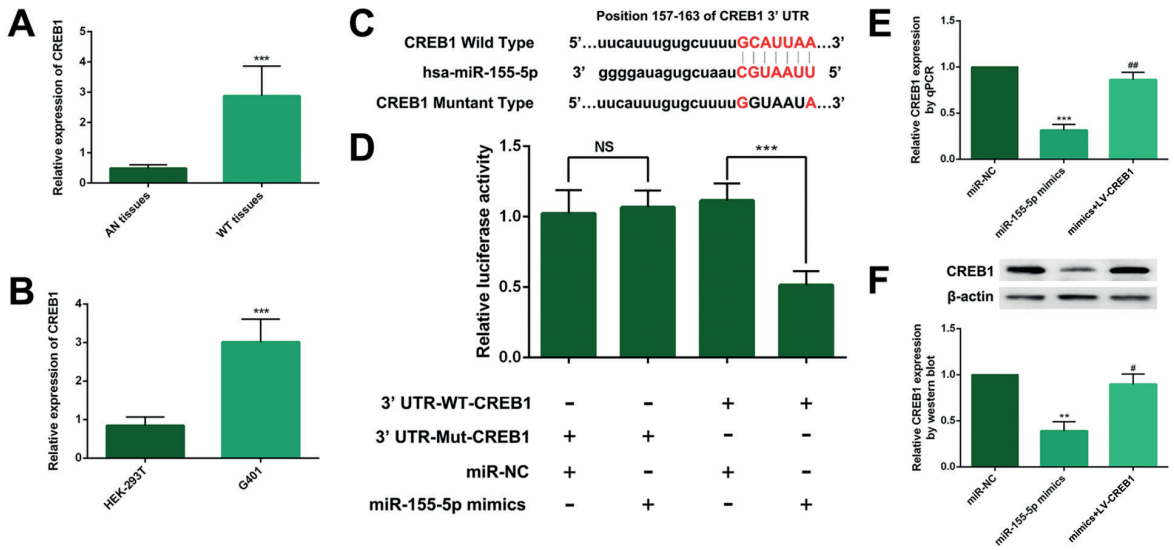

Figure 3. CREB1 was a direct and functional target of miR-155-5p. ***A,*** Difference in the expression of CREB1 between WT tissues and adjacent normal tissues (***p<0.001). ***B,*** Difference in the expression of CREB1 between G401 and HEK293T cells (***p<0.001). ***C,*** Diagram of putative miR-155-5p binding sites of CREB1. D. Relative activities of luciferase reporters (***p<0.001). ***E,*** Expression level of CREB1 was detected by qRT-PCR. ***F,*** Expression level of CREB1 was measured by WB analysis. All data were presented as means ± standard deviations. (**p<0.01, ***p<0.001 *vs.* the NC group; #p<0.05, ##p<0.01 *vs.* the Mimics group).

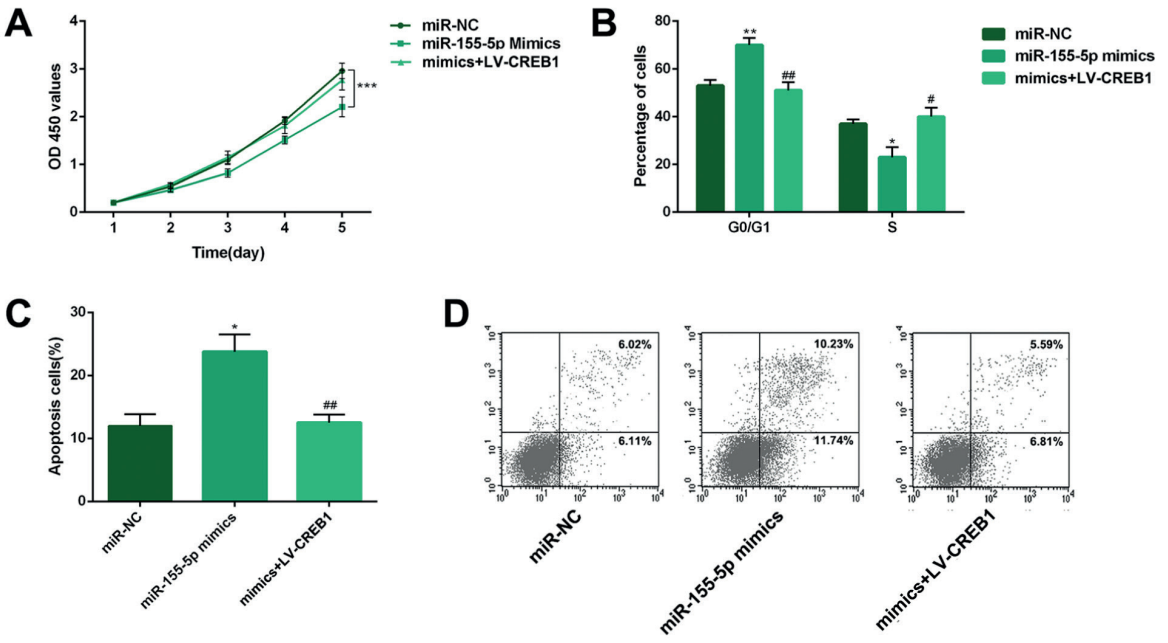

Figure 4. CREB1 overexpression attenuated the suppressive effect of miR-155-5p on WT cells. ***A,*** Cell proliferation detected by MTT assay. ***B,*** Cell cycle detected by flow cytometry assay. ***C-D,*** Cell apoptosis detected by flow cytometry assay. Data were presented as means ± standard deviations (*p<0.05, **p<0.01, ***p<0.001 *vs.* the NC group; #p<0.05, ##p<0.01 *vs.* the Mimics group).

is located on the long arm of human chromosome 2, consisting of 341 amino acid residues[27]. Its molecular structure is divided into two regions: the N-terminal region is related to the function of transcriptional regulation, and the C-terminal region is connected with the promoters. Through time-dependent phosphorylation activation and cellular localization mode, CREB1 is widely involved in various normal pathophysiological processes in human body, such as cell proliferation, cell cycle, DNA repair and immune response[28,29]. As an important oncogene, CREB1 participates in the development of multiple tumor cells[30]. For example, CREB1 is highly expressed in breast cancer tissues and metastatic breast cancer cells. It is also closely related to the poor prognosis and high metastatic potential of cancer cells[31, 32]. Further studies have demonstrated that CREB1 can promote the proliferation, metastasis and bone destruction of breast cancer[32]. Seo *et al*[33] found that CREB1 is remarkably up-regulated in most non-small cell lung cancer (NSCLC) cell lines. Meanwhile, its overexpression is correlated with poor prognosis of non-smokers with NSCLC. Down-regulating CREB1 can inhibit the growth and induce the apoptosis of NSCLC cells[34]. Besides, Tan et al[35] have found that the expression of CREB1 with a tumor-promoting effect is significantly up-regulated in glioma, promoting the formation of glioma. In addition to the abnormal expression in solid tumor, overexpression of CREB1 is also closely related to the poor prognosis of acute myeloid leukemia[36,37]. At present, CREB1 has become an important target for anti-tumor drugs research. In this study, we first analyzed the expression of miR-155-5p in WT tissues and cell lines. Results found that the expression level of miR-155-5p in WT tissues and cells was significantly decreased. To study the possible role of in the pathogenesis of WT, we up-regulated or down-regulated the expression of miR-155-5p in WT cells and further performed functional assay *in vitro*. MTT results demonstrated that the proliferation capacity of WT cells with higher expression of miR-155-5p was significantly declined. Meanwhile, cell cycle was remarkably arrested in the G0/G1 phase. Besides, cell apoptosis was significantly promoted after miR-155-5p up-regulation. However, opposite results were obtained after miR-155-5p down-regulation. Subsequently, we explored the possible underlying mechanism of miR-155-5p in WT. Bioinformatics software was used to predict the potential target genes of miR-155-5p. Results demonstrated that CREB1 was a target gene of miR-155-5p. Based on the regulatory effect of CREB1 in various tumor tissues, its expression

in WT tissues and cells was detected again. As expected, the expression level of CREB1 in WT tissues and cells was significantly up-regulated. Moreover, the regulatory effect of miR-155-5p on CREB1 in WT cells was confirmed *via* by qRT-PCR, WB and dual-luciferase reporter gene assay. However, after the expression level of CREB1 was restored in rescue experiments, the inhibitory effect of miR-155-5p in WT cells was eliminated.

Conclusions

We first showed that miR-155-5p was suppressed in WT tissues and cells. Furthermore, we explored the underlying mechanism of miR-155-5p in WT *in vitro*. After all, these results might provide some theoretical basis for researches of WT in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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