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MiR-200c inhibits proliferation and promotes apoptosis of Wilms tumor cells by regulating akt signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the effect of micro ribonucleic acid (miR)-200c on the proliferation and apoptosis of Wilms tumor cells, and to further elucidate its potential mechanisms.

PATIENTS AND METHODS: Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to detect the expression level of miR-200c in cancer tissues and adjacent normal tissues of 20 patients with Wilms tumor. Human primary Wilms tumor cells were taken as research objects, and were divided into Control group and miR-200c mimic group. In miR-200c mimic group, miR-200c was overexpressed in Wilms tumor cells using liposome transfection technology. Subsequently, the proliferation and apoptosis of cells in each group were observed by functional assays. The expression levels of phosphorylated protein kinase B (p-Akt), total Akt (T-Akt) and glucose transporter protein type 1 (GLUT1) in each group of cells were finally detected by Western blotting.

RESULTS: In Wilms tumor patients, the expression level of miR-200c in cancer tissues was notably lower than that in adjacent normal tissues ($p<0.05$). Wilms tumor cells were cultured in vitro, and were transfected with miR-200c mimic. Subsequent cell counting kit-8 (CCK-8) assay results showed that the proliferation ability of cells in miR-200c mimic group was remarkably weakened ($p<0.05$). Colony formation assay indicated the number of formed colonies in miR-200c mimic group was remarkably less than that in Control group ($p<0.05$). Western blotting results manifested that overexpression of miR-200c markedly increased the ratio of B-cell lymphoma protein 2 (Bcl-2) to Bcl-2-associated X protein (Bax) ($p<0.05$). Flow cytometry results revealed that miR-200c overexpression significantly elevated the apoptosis rate of Wilms tumor cells ($p<0.05$). In addition, it was discovered that the overexpression of miR-200c could prominently reduce the phosphorylation level of intracellular Akt and the expression of its downstream protein GLUT1. Finally, immunohistochemical staining results verified that the expression levels of p-Akt and GLUT1 in cancer tissues of Wilms tumor patients were significantly higher than those in adjacent normal tissues ($p<0.05$).

CONCLUSIONS: MiR-200c was lowly expressed in cancer tissues of Wilms tumor patients. Besides, overexpression of miR-200c inhibited the proliferation and promoted the apoptosis of cells through targeted inhibition of the Akt/GLUT1 signaling pathway.

Key Words:
MiR-200c, Wilms tumor, Proliferation, Apoptosis, Akt.

Introduction

Wilms tumor is a mixed renal embryonal tumor composed of blastulae, stromata and epithelial components, which is also the most common malignant tumor in children's urinary system. The 5-year survival rate of Wilms tumor is about 80%. Currently, the prognosis of patients with Wilms tumor mainly depends on individual stage and treatment[1]. Wilms tumor can be basically cured, and affected patients may have a relatively long survival time. However, long-term chemotherapy, radiotherapy and surgery are likely to result in severe complications in adult patients[2]. Therefore, further exploring the molecular mechanisms of the occurrence and development of Wilms tumor is of great significance for relieving the burden of treatment and improving the prognosis of patients.

Micro ribonucleic acids (miRNAs) are a group of single-stranded non-coding RNAs existing in eukaryotes, with about 20-24 nt in length. MiRNAs can regulate the expression of many life genes through targeted binding, thereby playing important roles in the proliferation, differentiation, invasion,

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apoptosis, and other behaviors and activities of tumor cells[3,4]. MiRNAs are able to modulate the inflammatory response of immune and non-immune cells[5]. Meanwhile, they can affect the microenvironment of tumor cells, further promoting or inhibiting cancer development. In fact, miR-200c has been confirmed to suppress the proliferation, invasion and glycolysis of bladder cancer cells by targeted inhibition of lactate dehydrogenase A[6]. Numerous studies have indicated that it can be used as a marker for the prognosis and sensitivity of platinum-based chemotherapy for advanced gastric cancer. Moreover, gastric cancer patients with high expression of miR-200c have better platinum chemotherapy efficacy as well as longer progression-free survival and overall survival than those with low expression of miR-200c[7].

In this study, the expression of miR-200c in cancer tissues and adjacent normal tissues of Wilms tumor patients was first detected. Subsequently, the effects of miR-200c overexpression on the proliferation and apoptosis of Wilms tumor cells were observed by functional assays *in vitro*. All our findings might provide a certain reference basis for the clinical treatment and prevention of Wilms tumor in children in the future.

Patients and Methods

Tissue Specimens

20 pairs of cancer tissues and adjacent normal tissues were collected from 20 patients with Wilms tumor who underwent surgical treatment in the Pediatric Department of our Hospital from June 2017 to January 2019. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). Briefly, blood stains were first removed with normal saline from all the specimens. Subsequently, these specimens were cut into pieces, placed in an Eppendorf tube (EP; Hamburg, Germany) and preserved in a refrigerator at -80°C for use, or they were fixed in 10% neutral formalin without cutting. This study was approved by the Ethics Committee of Shan County Central Hospital of Heze City. Signed written informed consents were obtained from all participants before the study.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay

(1) Total RNAs were extracted from cancer tissues and adjacent normal tissues of Wilms tumor patients *via* TRIzol method (Invitrogen, Carlsbad, CA, USA). (2) The concentration and purity of extracted RNAs were detected using an ultraviolet spectrophotometer. When A_{260}/A_{280} = 1.8-2.0, the RNAs could be used. (3) Messenger RNAs (mRNAs) were synthesized into complementary deoxyribonucleic acids (cDNAs) by RT and stored in a refrigerator at -80°C. (3) RT-PCR system was composed of 2.5 μL of 10× Buffer, 1 μL of cDNA, 0.5 μL of forward primer (20 μmol/L), 0.5 μL of reverse primer (20 μmol/L), 10 μL of LightCycler® 480 SYBR Green I Master (2′) and 5.5 μL of ddH_2O. RT-PCR amplification systems were the same. Primers used in this study were designed as follows: miR-200c: forward (5'ATTGCTAGTAGTCGTGA'3) and reverse (5'ATGTGGTCGAACCAAC'3), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward (5'ATTGCTAGTAGTCGTGA'3) and reverse (5'ATGTGGTCGAACCAAC'3), and U6: forward (5'CTCGCTTCGGCAGCACATAT'3) and reverse (5'TTGCGTGTCATCCTTGCG'3).

Extraction of Human Primary Wilms Tumor Cells

Cancer tissue sections were first selected from patients undergoing Wilms tumor resection in our hospital. Subsequently, they were soaked in phosphate-buffered saline (PBS) containing dual antibodies for 5 min and fully cut into pieces with ophthalmic scissors. Next, 0.2% collagen type III enzyme and 50 μL of hyaluronidases were added, stirred at constant temperature for 1 h and filtered, followed by centrifugation to discard the supernatant. All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 cell culture medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) in an incubator with 5% CO_2 at 37°C. After the cells were adherent to the wall, the original culture medium was replaced with fresh medium. When cell fusion reached about 70-80%, the cells were digested and passaged.

Transfection of MiR-200c in Wilms tumor cells

Wilms tumor cells were first inoculated into 6-well plates and cultured in an incubator at 37°C for 36 h. When cell density reached 60-65%, the cells were transfected with miR-200c mimic and negative control according to the instructions of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). After that, the cells were cultured in an incubator at 37°C again.

Detection of Apoptosis Via Flow Cytometry Technology

Wilms tumor cells in the logarithmic growth phase were digested with 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid) to prepare cell suspensions. The cells were then inoculated into 6-well plates and cultured. Finally, the samples were loaded according to the procedure of Annexin V-FITC PI (Beyotime, Shanghai, China), and the apoptosis rate was calculated.

Detection of Cell Proliferation

Cells in the logarithmic growth phase were inoculated into 96-well plates and cultured in an incubator with 5% CO_2 at 37°C for 0, 12, 24, 48 and 72 h, respectively. After that, the culture medium was discarded, and the color development solution was prepared in dark according to the ratio of culture medium: Cell Counting Kit-8 (CCK-8) = 10:1 (Dojindo Molecular Technologies, Kumamoto, Japan). Next, color development solution was added into each well at 110 μL/well, followed by 2 h of incubation at 37°C in the dark. Optical density (OD) value at 540 nm in each group was finally detected using the ultraviolet spectrophotometer.

Detection of the Expression of Related Proteins Using Western Blotting Technology

The cells were first rinsed with PBS for three times to remove the fluid. 1000 μL of lysate was added to each dish and shaken for 10 min. Then, the cells at the bottom of the dish were fully scraped off with a brush and put into an EP tube prepared in advance. Thereafter, collected cells were lysed by an ultrasonic cell crusher for 1-2 s each time, and the total lysis time was not more than 15 s. After standing for 15 min, the cells were centrifuged for 0.5 h at 12000 r/min. Next, the supernatant was sub-packaged into EP tubes. The concentration of extracted protein was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and UV spectrophotometry. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. After incubation with primary antibodies at 4°C overnight, the membranes were incubated with the goat anti-rabbit secondary antibody for 1 h in dark. Immuno-reactive bands finally were scanned by an Odyssey membrane scanner (Seattle, WA, USA) and quantified, with GAPDH as an internal protein reference.

Colony Formation Assay

Cells in each group were first cultured to the logarithmic growth phase. Then, the cells were digested with 0.25% trypsin into a single cell suspension with more than 95% of single cells. Subsequently, the cell suspension was inoculated into 6-well plates at a preferably density of 500 cells/well. Next, 2 mL of RPMI-1640 medium was added to each well and replaced every 48 h. 10 days later, the cells were fixed with formaldehyde and stained with crystal violet. The number of formed cell colonies in each group was finally recorded.

Detection of the Expression Levels of Phosphorylated Protein Kinase B (p-Akt) and Glucose Transporter Protein Type 1 (GLUT1) Via Immunohistochemistry

Paraffin-embedded sections were first de-paraffinized in an incubator at 60°C for 30 min, and antigen retrieval was carried out using citrate under high pressure. Next, myocardial tissues were covered with 3% hydrogen peroxide for 20 min of incubation and blocked with 8% goat serum for 30 min. Thereafter, primary antibodies against p-AKT and GlUT1 (diluted at 1:100 by PBS) were added drop-wise and completely covered the tissues for incubation in a refrigerator at 4°C overnight. On the next day, after the rewarming of sections in each group, secondary antibody B solution was added in drops for 30 min of incubation at room temperature. After washing, droplets of diaminobenzidine (DAB) working solution (Solarbio, Beijing, China) were added, and color development time was strictly controlled under a light microscope. Next, each group of sections were re-stained by hematoxylin, dehydrated with ethyl alcohol with a concentration gradient and mounted. 10 non-repetitive regions of tissues were randomly photographed under a light microscope (200′), and the positive rate of proteins in each group was finally analyzed by Image J software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. *t*-test was applied to compare the differences between two groups. $p<0.05$ was considered statistically significant.

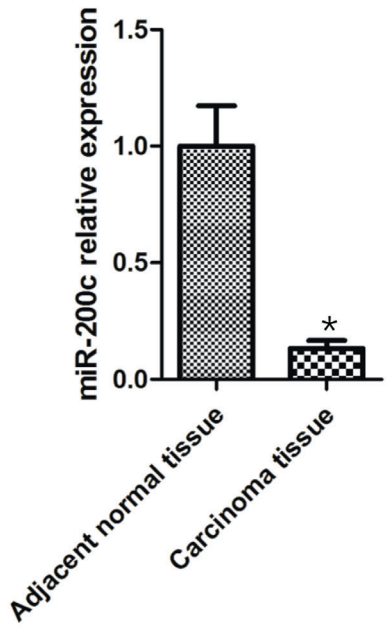

Figure 1. MiR-200c expression level in cancer tissues and adjacent normal tissues of Wilms tumor patients. Adjacent normal tissue: cancer-adjacent tissue and carcinoma tissue: cancer tissue. $^{*}p<0.05$ *vs.* Normal tissue group, showing a statistically significant difference.

Results

Expression of MiR-200c in Cancer Tissues and Adjacent Normal Tissues of Wilms Tumor Patients

MiR-200c expression level in cancer tissues and adjacent normal tissues of 20 patients with Wilms tumor was detected *via* RT-PCR (Figure 1). The results showed that the expression level of miR-200c in cancer tissues was significantly lower than (about 0.167 times as high as) that in adjacent normal tissues of Wilms tumor patients ($p<0.05$).

Effect of MiR-200c Overexpression on the Proliferation of Wilms Tumor Cells

CCK-8 assay results (Figure 2) manifested that miR-200c mimic could markedly weaken the proliferation ability of Wilms tumor cells at 12, 24, 48 and 72 h ($p<0.05$). This indicates that miR-200c has a potential role in inhibiting the proliferation of Wilms tumor cells.

Impact of MiR-200c Overexpression on the Colony Formation Ability of Wilms Tumor Cells

Colony formation assay (Figure 3) illustrated that on the 10th day after overexpression of miR-200c, the number of formed colonies in Wilms tumor cells decreased remarkably [(132±11) *vs.* (28±7)] ($p<0.05$). This suggests that miR-200c plays a potential role in inhibiting the colony formation ability of Wilms tumor cells.

Impact of MiR-200c Overexpression on the Expression of Apoptosis-Related Proteins in Wilms Tumor Cells

According to Western blotting results (Figure 4), overexpression of miR-200c notably downregulated the expression level of anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and upregulated the expression level of pro-apoptotic protein Bcl-2-associated X protein (Bax), eventually leading to significantly decreased ratio of Bcl-2/Bax in cells of miR-200c mimic group ($p<0.05$). These findings indicated that miR-200c has the potential to resist apoptosis.

Effect of MiR-200c Overexpression on the Apoptosis Level of Wilms Tumor Cells

To further clarify the pro-apoptotic effect of miR-200c, flow cytometry technology was utilized to detect the apoptosis level of cells in the two groups. It was discovered that the apoptosis rate was (5.11±0.67)% in Control group and (43.19±2.09)% in miR-200c mimic group, respectively ($p<0.05$) (Figure 5).

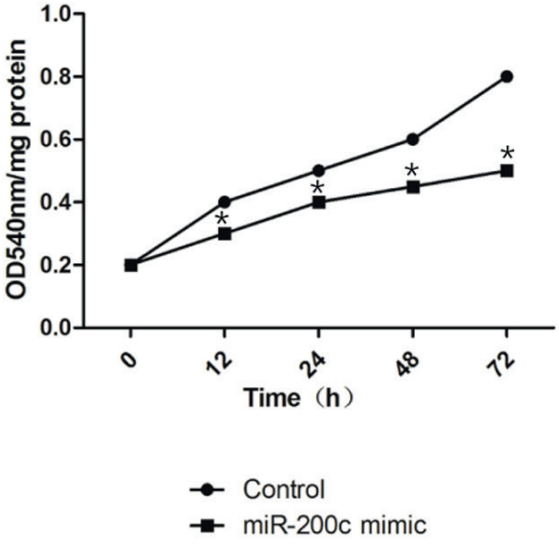

Figure 2. Effect of miR-200c mimic on the proliferation of Wilms tumor cells detected *via* CCK-8. Control: control group and miR-200c mimic: miR-200c overexpression group. $^{*}p<0.05$ *vs.* Control group, displaying a statistically significant difference.

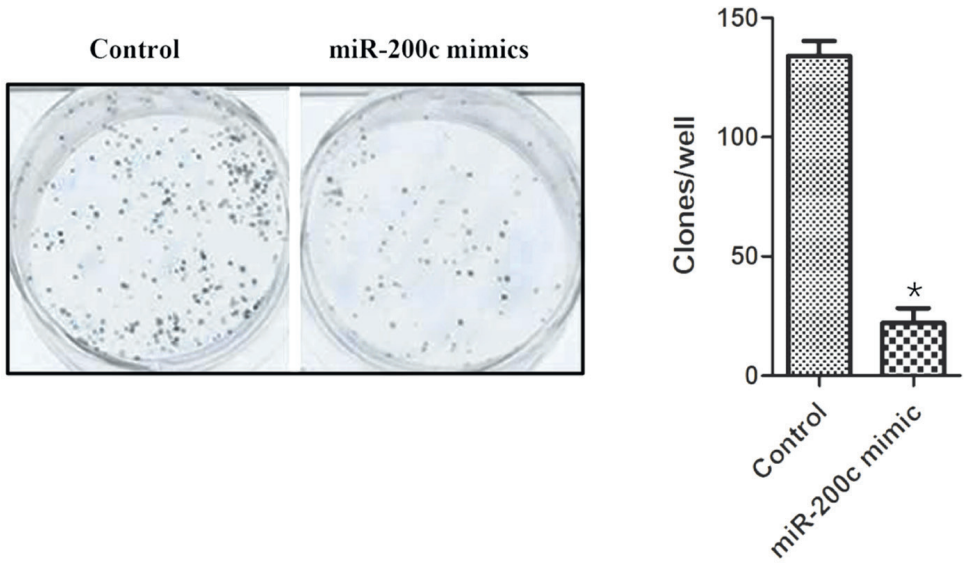

Figure 3. Effect of miR-200c mimic on the colony formation ability of Wilms tumor cells (magnification: 20×). Control: control group and miR-200c mimic: miR-200c overexpression group. $^{*}p<0.05$ *vs.* Control group, displaying a statistically significant difference.

Effect of MiR-200c Overexpression on the Akt/GLUT1 Signaling Pathway in Wilms Tumor Cells

The Akt/GLUT1 signaling pathway is a vital factor promoting the proliferation and inhibiting the apoptosis of tumor cells. Meanwhile, Akt is regulated by miR-200c in a targeted manner. Hence, the protein expression levels of p-Akt, total Akt (T-Akt) and GLUT1 in the two groups of cells were examined using Western blotting technology. The results (Figure 6) denoted that the overexpression of miR-200c evidently reduced the ratio of p-Akt/T-Akt as well as the protein expression level of GLUT1 ($p<0.05$).

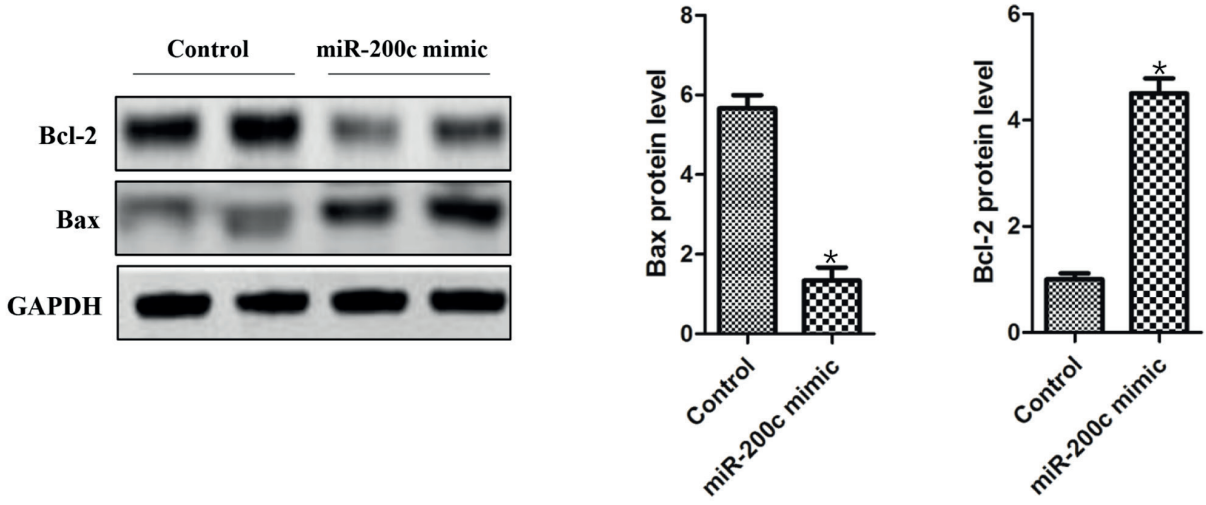

Figure 4. Effect of miR-200c mimic on the expression of apoptosis-related proteins in Wilms tumor cells. Control: control group and miR-200c mimic: miR-200c overexpression group. $^{*}p<0.05$ *vs.* Control group, showing a statistically significant difference.

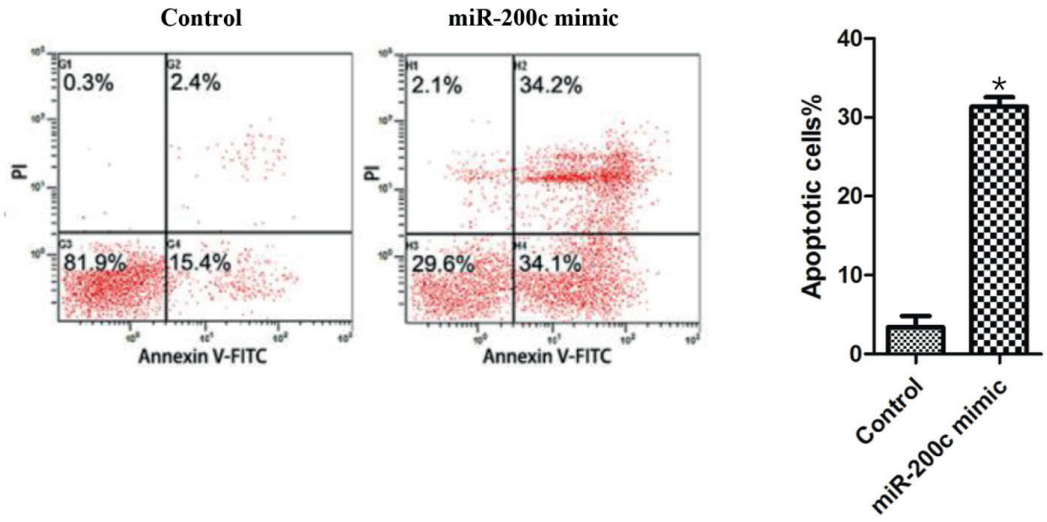

Figure 5. Effect of miR-200c mimic on the apoptosis of Wilms tumor cells. Control: control group and miR-200c mimic: miR-200c overexpression group. $^{*}p<0.05$ *vs.* Control group, displaying a statistically significant difference.

Empirical Verification Results of p-Akt and GLUT1 Expressions in Cancer Tissues and Adjacent Normal Tissues of Wilms Tumor Patients

The protein expression levels of p-Akt and GLUT1 in cancer tissues and adjacent normal tissues of Wilms tumor patients were finally detected by immunohistochemistry technology. According to the results, the protein expression levels of p-Akt and GLUT1 in cancer tissues were prominently higher than those in adjacent normal tissues of Wilms tumor patients ($p<0.05$) (Figure 7).

Discussion

Nephroblastoma, also known as Wilms tumor, is a common renal embryonal tumor. It accounts for about 8% of solid tumors in children, with a peak incidence at the age of 3 years old. Among all embryonal tumors, Wilms tumor is the most typical example of organ "failure", as it simultaneously contains tissues derived from epithelial cells, stromal cells and blastocyst cells that can be distinguished under a light microscope[8,9]. Currently, the prognosis and survival of Wilms tu-

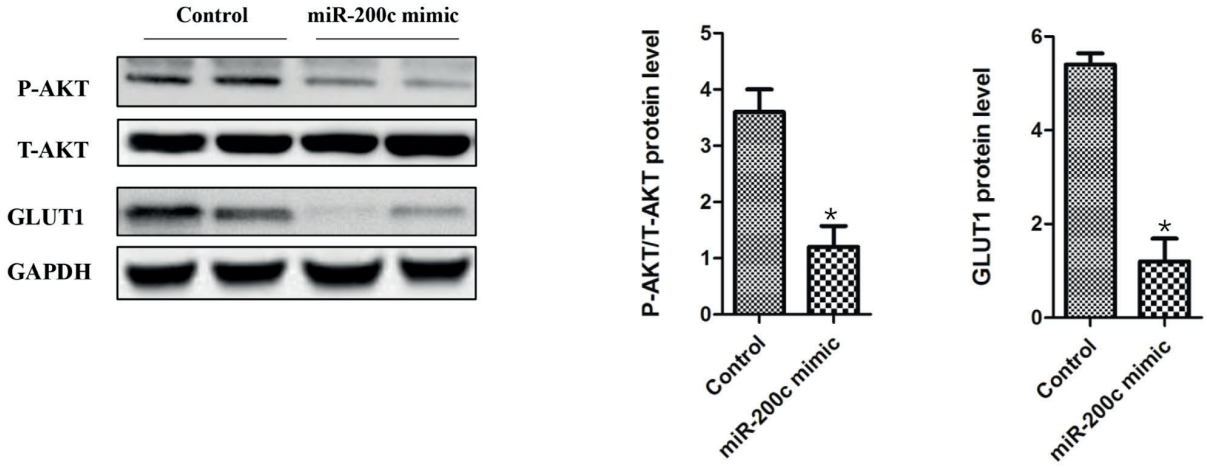

Figure 6. Effect of miR-200c overexpression on the Akt/GLUT1 signaling pathway in Wilms tumor. Control: control group and miR-200c mimic: miR-200c overexpression group. $^{*}p<0.05$ *vs.* Control group, displaying a statistically significant difference.

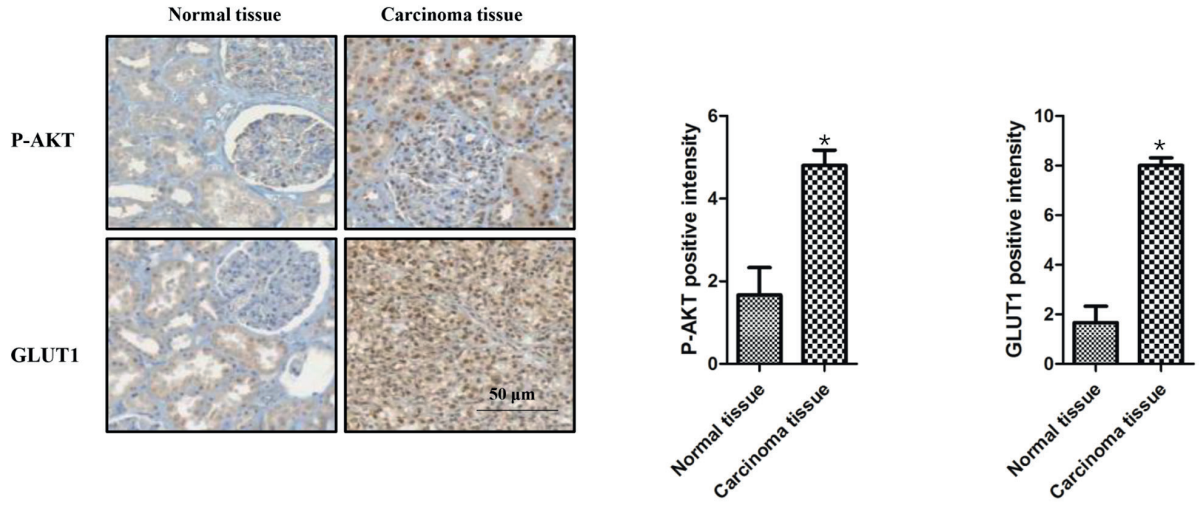

Figure 7. Empirical verification results of p-Akt and GLUT1 in cancer tissues and adjacent normal tissues of Wilms tumor patients (magnification: 200×). Normal tissue: cancer-adjacent tissue and Carcinoma tissue: cancer tissue. $^{*}p<0.05$ *vs.* Normal tissue group, manifesting a statistically significant difference.

mor patients have been greatly improved by the combined application of surgical treatment and radiotherapy and chemotherapy. However, these treatment methods may cause certain damage and produce various adverse reactions. Multiple genes associated with Wilms tumor, such as WT1, WT2, WTX, CTNNB1 and p53, have been found in China and foreign countries. However, their molecular mechanisms leading to the occurrence and development of Wilms tumor are still elusive[10,11]. Therefore, continuously exploring the biological characteristics and pathogenesis of Wilms tumor cells is of great significance for finding new treatment methods and prolonging the survival rate of child patients.

MiRNAs are a kind of endogenous small non-coding RNAs with about 20-24 nucleotides in length. They can modulate the expression of various genes in the body at the post-transcriptional level[12]. More precisely, miRNAs are able to suppress the translation of corresponding proteins by binding to the 3'UTR of target genes. MiRNAs have been found to exert crucial effects in diverse life activities of cells[13]. Hence, further in-depth research on the specific behavioral mechanism of miRNAs on tumor cells is of profound significance. Previous studies have revealed that Drosha and Dicer (nucleases) and 3'-5' exonucleases can increase the susceptibility of children to Wilms tumor during miRNA generation. This denotes that miRNAs perform pivotal functions in the occurrence and development of Wilms tumor[14]. Indeed, miR-613 represses the proliferation, migration and invasion of Wilms tumor cells through targeted binding to FRS2[15]. STAT3 inhibits the expression of WTX by upregulating miR-370 expression in Wilms tumor cells, inducing the proliferation and migration of cancer cells[16]. MiR-92a-3p is able to inhibit the proliferation, migration and invasion of Wilms tumor cells through targeted inhibition of NOTCH1[17]. In the present study, our results revealed for the first time that miR-200c expression decreased significantly in patients with Wilms tumor. Overexpression of miR-200c could notably reduce the proliferation and facilitate the apoptosis of Wilms tumor cells. In fact, the anti-tumor potential of miR-200c has been reported many times in previous studies. MiR-200c can simultaneously inhibit the expressions of transcription factors ZEB1 and ZEB2 induced by endothelial mesenchymal transition. Meanwhile, it also facilitates inducible tumor cell apoptosis through CD95 by targeting FAP-1[18]. MiR-200c represses the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway to enhance the sensitivity of drug-resistant non-small cell lung cancer to gefitinib[19].

Akt mediates the activation of downstream tyrosine kinase and PI3K signaling pathways. Akt can regulate different life activities of cells, including cell proliferation, survival, cell size, nutrient uptake, tissue invasion and angiogenesis[20]. Furthermore, the Akt signal transduction pathway can modulate the expression of oncogenes

and tumor suppressor genes, and breaking this equilibrium state will trigger tumors[21]. Akt is activated in the occurrence and development of various tumors, including Wilms tumor. Protein tyrosine phosphatase epsilon blocks the survival of Wilms tumor cells by suppressing the PI3K/Akt/mTOR signaling pathway[22]. Andrographolide, a natural compound, is also able to inhibit the vincristine-triggered death of Wilms tumor SK-NEP-1 cells through the PI3K/Akt/p53 signaling pathway[23]. GLUT1, as a downstream molecule of Akt, is strictly regulated by Akt. In recent years, the function of the Akt/GLUT1 axis in different tumor cells has been fully clarified[24]. This study manifested that the overexpression of miR-200c could significantly inhibit the activation of the Akt/GLUT1 signaling pathway in Wilms tumor. This suggests that the anti-cancer effect of miR-200c may be correlated with its inhibition on the Akt/GLUT1 signaling axis. However, there were still some limitations in this study: 1) experimental results were not verified by animal experiments *in vivo*; 2) no direct target for miR-200c was found to regulate the Akt signaling pathway.

Conclusions

Briefly, this study revealed for the first time that miR-200c was lowly expressed in Wilms tumor. In addition, miR-200c overexpression could promote the apoptosis and inhibit the proliferation of Wilms tumor cells by repressing the Akt signaling pathway. Our findings could provide a potential strategy for the treatment and prevention of Wilms tumor.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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