Research Article

Effects of Microrna-19b on the Proliferation, Apoptosis and Migration of Wilms’ Tumor Cells via the PTEN/PI3K/AKT Signaling Pathway†

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Running title: MiR-19b promotes WT via the PTEN/PI3K/AKT pathway

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†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jcb.25999]

Received 23 December 2016; Revised 17 March 2017; Accepted 17 March 2017

Journal of Cellular Biochemistry
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DOI 10.1002/jcb.25999
ABSTRACT

Wilms’ tumor (WT) is a most common renal cancer that occurs among children, and microRNA-19b (miR-19b) usually participates in various human cancers. Importantly, the PTEN/PI3K/Akt signaling pathway plays a key role in cell apoptosis, growth and proliferation. Thus, our present study aims to investigate the effect of miR-19b on the PTEN/PI3K/Akt signaling pathway during WT cell proliferation, migration and apoptosis. WT tissues and adjacent normal tissues from WT patients were collected. qRT-PCR was applied to detect miR-19b expression in both the WT tissues and the adjacent normal tissues, immunohistochemistry was applied to detect the protein expressions of PTEN, P13K and p-Akt, SK-NEP-1 cells were divided into the blank, negative control (NC), miR-19b mimics and miR-19b inhibitors groups. MTT assay, propidium iodide (PI) staining, Annexin-V/PI double-staining, Transwell assay and Western blotting were performed to examine cell proliferation, cycle, apoptosis, migration and invasion, and the protein expressions of PTEN, P13K, Akt and p-Akt. Increased miR-19b expression, positive expression rates of P13K and Akt, decreased PTEN expression rate, a negative correlation between PTEN expression and tumor lymph node metastasis, and a positive correlation between the expression of P13K and Akt and the clinical stages were observed in the WT tissues. The miR-19b inhibitors group exhibited decreased cell proliferation, cell cycle progression, migration and invasion, and protein expressions of P13K and p-Akt but increased PTEN protein expression compared with the blank and NC groups. Thus, inhibition of miR-19b suppresses the progression of WT by modulating the PTEN/PI3K/AKT signaling pathway. This article is protected by copyright. All rights reserved

KEYWORDS: WILMS’ TUMOR; MICORRNA-19B; PTEN/PI3K/AKT SIGNALING PATHWAY; CELL PROLIFERATION; CELL MIGRATION; CELL APOPTOSIS
INTRODUCTION

Wilms’ tumor (WT), also known as nephroblastoma, is the fourth most common renal tumor among pediatric cancers, accounting for approximately 6-14% of all cancers and 95% of all renal malignancies among children [Amirian, 2013; Honeyman et al., 2012]. WT is often discovered incidentally before the age of 5 during check-up routines [Huszno et al., 2013]. Specific symptoms are difficult to monitor in patients with WT, thus causing difficulties in its early diagnosis [Baskaran, 2013; Malogolowkin et al., 2013]. Surprisingly, WT is correlated with congenital conditions while not associated with genetic syndromes, including urogenital malformations and mental retardation [Amirian, 2013]. Currently, the treatment for WT includes chemotherapy before the operation and subsequent surgery, as well as postoperative adjuvant therapy [Huszno et al., 2013]. Overall, the survival rate for WT reaches approximately 90%, but patients usually exhibit poor clinical prognosis if diffusely anaplastic or unfavorable histology is present [Amirian, 2013; Zhang et al., 2013]. Therefore, it is of great importance to investigate the cellular and molecular mechanisms of WT progression to improve the diagnosis, prognosis, and management of this disease.

MicroRNAs (miRs) are small non-coding RNAs (approximately 22 nucleotides in length) that are able to negatively modulate gene expression at the posttranscriptional stage [Yu and Li, 2015]. MiRs are involved in various biological processes, such as growth, development, and metabolism [Yu et al., 2015]. Increasing evidence has demonstrated that miRNAs play significant roles in the pathogenesis of many renal diseases, such as polycystic kidney disease, renal fibrosis, chronic kidney diseases, and renal cancers [Lu et al., 2014; Wei et al., 2013]. Additionally, there is evidence that indicates that miRs are dysregulated in WT, implying that miRNAs may have important roles in WT progression [Yu et al., 2016]. MicroRNA-19b (miR-19b) is an important member of the miR family and participates in various human cancers, including prostate cancer, colorectal cancer and lung cancer [Ponomaryova et al., 2016; Stuopelyte et al., 2016; Zekri et al., 2016]. Furthermore, several studies have suggested that the dysregulation of miRNAs is able to initiate the activation of the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/protein kinase B
(Akt) signaling pathway, which is involved in the progression of breast cancer, bladder cancer and non-small-cell lung cancer [Park et al., 2008; Wang et al., 2016; Xie et al., 2013]. The diagram for the associations of related proteins in the PTEN/PI3K/Akt signaling pathway is shown in Appendix Fig. 1. The PTEN/PI3K/Akt axis constitutes a significant pathway that controls multiple biological signaling processes, such as metabolism, apoptosis, cell growth and cell proliferation [Carnero et al., 2008]. In addition, the PTEN/PI3K/Akt signaling pathway is used as a prognostic predictor for epithelial ovarian cancer in clinical practice [Cai et al., 2014]. However, the effects of the PTEN/PI3K/Akt signaling pathway on WT pathogenesis and progression remain unknown. Thus, our study aims to investigate their effects on the cell proliferation, migration and invasion of WT cells.

MATERIALS AND METHODS

STUDY SUBJECTS
The specimens in this study were obtained from 46 patients who were all pathologically diagnosed with WT at the Affiliated Pingxiang Hospital of Southern Medical University between August 2008 and October 2014. Among the 46 WT patients (21 cases < 24 months; 25 cases ≥ 24 months; median age of 31 months), 30 were males and 16 were females, with an age ranging from 9 to 89 months and a mean age of (34.33 ± 21.79) months; among the patients, 10 cases were relapses, and 36 cases were new cancers. According to the degree of cell differentiation and histological characteristics, 37 cases were verified as having favorable histology (FH), and 9 cases had unfavorable histology (UH). Based on the National Wilms Tumor Study (NWTS-5) grade classification [Grundy et al., 2005], there were 32 cases of stage I/II cancer and 14 cases of stage III/IV cancer. All patients who were receiving chemotherapy, radiotherapy and/or biotherapy were excluded, and those with other types of malignant tumors were also eliminated. Additionally, normal mucosa tissues (5 cm from lesion) were collected from the WT patients and stored at -70°C after freezing with liquid nitrogen. Half of the specimens were used for miR-19b detection, and the
other half was fixed with 10% formalin, embedded in paraffin, and used for immunohistochemistry. The study was approved by the Ethics Committee of the Affiliated Pingxiang Hospital of Southern Medical University, and written informed consent was obtained from all study subjects.

**QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)**

The preserved specimens were powdered using the liquid nitrogen grinding method. Total RNA was extracted by TRIzol (Invitrogen Inc., Carlsbad, CA, USA) and dissolved in ultrapure water treated by diethylpyrocarbonate (DEPC). An ND-1000 ultraviolet spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA) was used to determine the optical density (OD) values at a wavelength between 260 nm and 280 nm as well as the concentration and quality of the total RNA. Reverse transcription of RNA was performed according to the instructions of a reverse transcription kit (Fermentas Inc., Hanover, MD, USA). The RNA conditions were as follows: 70°C for 10 min, ice bath for 2 min, 42°C for 60 min, and 70°C for 10 min; the obtained cDNA was stored in a -80°C freezer. qRT-PCR was performed by the application of TaqMan® using a RT reagent kit (Fermentas Inc., Hanover, MD, USA). The forward primer of miR-19b was 5’-CACCATGGCATGCTTTAGATTATATATTCCGC-3’, and the reverse primer was 5’-GCGGAATATATAATCTAAAGCATGGGTGCCATGGTG-3’; the U6 gene (the forward primer was 5’-CGCTTCGGCAGCACATATACTAAAATTGGAAC-3’, and the reverse primer was 5’-GCTTCACGAATTGCGGTGTACCAATCGTTTC-3’) was used as an internal reference. The reaction conditions were as follows: one cycle of initial denaturation at 95°C for 30 s and 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 70°C for 10 s. The Bio-Rad iQ5 Sequence Detection System (Bio-Rad, Inc., Hercules, CA, USA) was employed to conduct the quantitative PCR. The relative quantitation method was used for calculation, with the 2^(-ΔΔCt) method to determine relative expression ratios of miR-19b. Additionally, each experiment was performed 5 times.
IMMUNOHISTOCHEMISTRY

The tissues were cut into 4-μm slices by serial section, which were dewaxed, alcohol rehydrated and subjected to microwave antigen retrieval. The tissue samples were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity. The primary antibodies for PTEN (mouse monoclonal antibody, ab79156, Abcam Inc., Cambridge, MA, USA), PI3K (mouse monoclonal antibody, ab86714, 5 μg/mL, Abcam Inc., Cambridge, MA, USA) and p-Akt (rabbit monoclonal antibody, ab38449, Abcam Inc., Cambridge, MA, USA) were incubated overnight at 4°C. After incubation for 20 min at room temperature with the addition of polymerase auxiliary agent, the horseradish peroxidase (HRP) labeled goat anti-mouse or goat anti-rabbit secondary antibodies (Abcam Inc., Cambridge, MA, USA) were added, incubated at room temperature for 30 min, and stained with diaminobenzidine (DAB) from the Sigma-Aldrich Chemical Company (St Louis MO, USA). The sections were counterstained with hematoxylin and sealed. Phosphate-buffered saline (PBS) was used instead of primary antibody in the negative control, and normal mucosa was used as a positive control. Cytoplasm and cell membranes containing brown-yellow granules were defined as having positive expression of PTEN and PI3K proteins, and the nuclei containing brown-yellow or brown staining indicated positive expression of p-Akt protein. We randomly chose 4 high-power fields (HPF, × 400) of each section, with each containing 200 cells, and the ratio between positive cancer cells and all cancer cells was calculated; a ratio > 10% was recorded as positive (+), and ≤ 10% was negative (-) [Brown and Wahl, 1993]. The results of immunohistochemistry were scored independently by two individuals who were blinded to each other's findings.

CELL CULTURE

The human WT cell line SK-NEP-1 (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) was cultivated in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, Utah, USA). Then, the
cells were cultured in a 5% CO\textsubscript{2} incubator (Thermo Scientific 8000, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with a humidity of 95%. The cells grew as a monolayer until the cell confluence reached 90%. The culture solution was conventionally eliminated, and the cells were washed twice with PBS and digested with 0.25% trypsin (Gibco, Gaithersburg, MD, USA). When the intercellular space increased, trypsin was aspirated. Then, the cells were suspended in DMEM containing 10% FBS to make a single-cell suspension, and the cells were passaged.

**LUCIFERASE REPORTER GENE ASSAY**

Various miRNA target gene online analysis software such as TargetScan, miRanda, MiRbase, and Pictar was applied to predict the target genes of miR-19b and to obtain the sequences containing the binding sites. DNA extraction of the WT cell line was performed using the TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) strictly following the manufacturer’s instructions. Wild-type sequences of PTEN 3’UTR were designated as PTEN-3’-UTR-WT (forward primer: 5’-ACCAGGACCAGAGGAAACCT-3’; and reverse primer: 5’-GCTAGCCTCTGGATTTGACG-3’), and the mutated (MT) PTEN 3’UTR without miR-19b binding sites was designated as PTEN 3’UTR-MT (forward primer: 5’-TTGTGGCAACAGCTGAATCTGCAGTTGGCTAAGAGGTT-3’; and reverse primer: 5’-ATGTAGCAAAAACCCTTGGAACCTCCTCTTATGCGAAGCT-3’). To construct the luciferase reporter vector, the SK-NEP-1 cell lines were plated on a 24-well plate with a transfection density of 80%-90%; then, Lipofectamine 2000 was applied to transfect the luciferase reporter vector along with the miR-19b mimics, miR-19b inhibitor and miR-19b negative control (NC) sequences (30 nmol/L each well). After 48 h of transfection, the culture medium was aspirated. Then, the cells were washed twice using PBS, and 100 μL of passive lysis buffer (PLB) was added to each well and slightly shaken at the room temperature for 15 min; next, the cell lysis solution was collected. The detection program was set at 10 s with a prereading of 2 s. A volume of 100 μL of LARII Stop&Glo® Reagent was added for each reading. The prepared LARII Stop&Glo® Reagent
was added to the luminous tube or plate containing the cell lysis solution (20 μL per sample); then, the tube or plate was placed into the biological luminescence detector (TurnerBioSystems, model No. Modulus™). The luciferase reporter gene assay reagent was purchased from the Promega Corporation (Madison, WI, USA).

CELL TRANSFECTION
The cells were assigned to blank (no transfection), negative control (NC, transfected with miR-19b NC sequence), miR-19b mimics (transfected with miR-19b mimics) and miR-19b inhibitor (transfected with miR-19b inhibitors) groups; all sequences were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The SK-NEP-1 cells in the logarithmic growth phase were inoculated in 6-well plates and reached 30-50% confluence in complete culture medium without antibiotics; they were transfected with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Opti-MEM serum-free medium (250 μL) (Gibco, Gaithersburg, MD, USA) was added to the cells to dilute 100 pmol miR-19b inhibitors, miR-19b mimics, and vector control (final concentration after adding to the cells was 50 nM); finally, the cells were gently mixed and incubated for 5 min at room temperature. Then, 250 μL of serum-free Opti-MEM was used to dilute 5 μL of Lipofectamine 2000 at room temperature for 5 min, and the above mentioned Opti-MEM serum-free medium solutions were mixed, incubated for 20 min at room temperature, and added to every well; the plates were slightly shaken and placed in a 5% CO₂ incubator at 37°C. After 6-8 h, complete culture medium was added, and after 24-48 h, the cells were harvested for experiments.

MTT ASSAY
After the transfected cells had reached approximately 80% confluence, they were washed 2 times using PBS and digested with 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) to generate a single cell suspension. After counting the cells on a cell count plate, the cells were seeded in a
96-well plate at a density of $3 \times 10^3 \sim 6 \times 10^3$ cells per well, with each well filled with $200 \mu$L in 6 parallel wells. After a 24-72 h incubation in 5\% CO$_2$ at 37°C, 20 \mu L of 5 mg/mL MTT solution (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA) was added to each well. The cells were cultured again in a 5\% CO$_2$ incubator at 37°C for 4 h, and the culture liquid was aspirated afterward. A total of 150 \mu L of dimethyl sulphoxide (DMSO) was then added to each well and then slightly shaken for 10 min to promote crystallization. The OD values in each well were measured by the enzyme immunoassay instrument at a wavelength of 490 nm after 12 h, 24 h, and 48 h. The MTT curve graph was drawn using time (h) as the abscissa and the mean of OD as the vertical axis. Each experiment was performed five times.

**PROPIDIUM IODIDE (PI) SINGLE STAINING**

At 48 h after transfection, the cells were collected and washed 3 times with cold PBS. After centrifugation, the supernatant was aspirated, and the cell concentration was adjusted to $1 \times 10^5$ cells/mL; the cells were then added to 1 mL of 75\% ice-cold alcohol and kept at 4°C overnight. Before coloration, the cells were washed with PBS twice to aspirate the supernatant, and 100 \mu L of RNase A was added and incubated in a 37°C water bath for 30 min in the dark. Then, 400 \mu L of PI was added for staining, and after 30 min in the dark at 4°C, flow cytometry was used to analyze the cell cycle at a wavelength of 488 nm.

**ANNEXIN V/PI DOUBLE STAINING METHOD**

At 48 h after transfection, the cells were digested with trypsin without EDTA, collected and centrifuged (1000 r/min, 5 min) to remove the supernatant. Then, the tube was washed with cold PBS and centrifuged 3 additional times. According to the Annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA), 150 \mu L of binding buffer and 5 \mu L of Annexin-V-FITC were added into each tube, shaken at a low speed and incubated for 15 min at room temperature in the dark. Finally, 100 \mu L of binding buffer and 5 \mu L of
PI (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA) were added and shaken, and flow cytometry was used to detect cell apoptosis.

**TRANSWELL ASSAY**

For the migration experiment, 72 h after transfection, the cells were collected and suspended. Then, the cells were inoculated into the Transwell upper chamber and placed in a 5% CO\textsubscript{2} incubator at 37°C for 48 h. The cells that failed to penetrate the upper chamber were removed, and the membrane was fixed in 95% ethanol for 15-20 min and then soaked with water. The membrane was tinted with crystal violet for 10 min, soaked again in water, photographed and observed under a high magnification microscope; then, the cell number on the back of the membrane was calculated.

Five high-power fields were randomly chosen, and the number of cells penetrating through the polycarbonate membrane was used to evaluate migration ability. For the invasion experiment, Matrigel matrix was dissolved at 4°C overnight and diluted with 1:3 serum-free DMEM medium. A total volume of 30 μL of Matrigel (15 μL, 7.5 μL, and 7.5 μL applied sequentially for 10 min per layer) was added to the Transwell upper chamber until it covered the bottom of the upper chamber. The cell suspension was added to the upper chamber, and 0.5 mL of DMEM containing 10% FBS was added to the lower chamber of the 24-well plate. The number of cells penetrating through the Matrigel was determined to assess the invasive ability.

**WESTERN BLOTTING**

The cultured cells were washed 3 times with precooled PBS, lysed with protein extraction lysis solution, and incubated on ice for 30 min. After centrifugation (12000 rpm) at 4°C for 10 min, the supernatant was transferred to a centrifuge tube and kept at -20°C for preservation. A total of 2 μg/μL BSA protein was successively diluted to 20 μg/μL, 15 μg/μL, 10 μg/μL, 5 μg/μL, 2.5 μg/μL, and 0 μg/μL using PBS. The protein concentration was detected by using the bicinchoninic acid (BCA) quantitative detection reagent kit (Thermo Fisher Scientific, Waltham, MA, USA).
samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 60 V and then 120 V at 4°C for 1-2 h after entering the separation gel. After electrophoresis, the samples were electrotransferred to a polyvinylidene fluoride (PVDF) membrane at 4°C for 2 h. The membrane was blocked with 5% skimmed milk for 1-2 h at room temperature, followed by the addition of PTEN (mouse monoclonal antibody, ab79156, 1: 100, Abcam Inc., Cambridge, MA, USA), PI3K (mouse monoclonal antibody, 1: 1000, ab86714, 5 µg/mL, Abcam Inc., Cambridge, MA, USA) and p-Akt (rabbit monoclonal antibody, ab38449, 1: 1000, Abcam Inc., Cambridge, MA, USA) primary antibodies overnight at 4°C. After washing with Tris-buffered saline/Tween (TBST) 3 times (10 min each), the appropriate mouse secondary antibodies (Abcam Inc., Cambridge, MA, USA) were added, incubated for 1 h at room temperature, and washed 3 times with TBST (10 min each). Finally, the samples were subject to electrogenerated chemiluminescence (ECL). X-ray tableting, developing, fixing, and data analysis were performed.

STATISTICAL ANALYSIS
All data were analyzed using the statistical package for the social sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as the mean ± standard deviation, and their comparison was examined by chi-square test; differences between two groups were evaluated by t test, and differences among multiple groups were evaluated by one-way analysis of variance (ANOVA). P < 0.05 was considered statistically significant.

RESULTS
MIR-19B EXPRESSION IN THE WT TISSUES
The miR-19b expression in the WT tissues and the adjacent normal tissues were 3.89 ± 0.41 and 0.91 ± 0.06, respectively, and miR-19b expression in the WT tissues was significantly higher than that in the adjacent normal tissues (P < 0.05) (Fig. 1). miR-19b expression in WT patients with lymph node metastasis (4.34 ± 0.22) was significantly higher than that in patients without lymph
node metastasis (3.63 ± 0.21), and the expression level in stage III/IV patients (4.44 ± 0.19) was significantly higher than that in stage I/II patients (3.70 ± 0.26) (both \( P < 0.05 \)). However, miR-19b expression was not associated with the neither age or sex of the patients nor histological type and cancer recurrence (all \( P > 0.05 \)) (Table 1).

**PTEN, PI3K AND P-AKT PROTEIN EXPRESSION IN WT TISSUES**

The immunohistochemistry results (Fig. 2) showed that PTEN and PI3K were mainly expressed in the cell cytoplasm, and p-Akt was mainly expressed in the nucleus. The positive expression rate of PTEN in the WT tissues was 54.35% (25/46), which was significantly lower than that in the adjacent normal tissues (84.78%, 39/46) (\( P < 0.05 \)). The positive expression rates of PI3K and p-Akt in the WT tissues were 63.04% (29/46) and 69.57% (32/46), respectively, which were significantly higher than those in the adjacent normal tissues (23.91%, 11/46 and 28.26%, 13/46, respectively) (both \( P < 0.05 \)). PTEN expression in the WT tissues was negatively correlated with lymph node metastasis but positively correlated with clinical stage (stage I/II and III/IV) (all \( P < 0.05 \)), while the expressions of PI3K and p-Akt in the WT tissues were positively correlated with lymph node metastasis but negatively correlated with clinical stage (stage I/II and III/IV) (all \( P < 0.05 \)). In addition, neither was associated with age, sex, histological type and cancer recurrence (all \( P > 0.05 \)), as shown in Table 2.

**RELATIONSHIP AMONG THE PROTEIN EXPRESSION OF MIR-19B, PTEN, PI3K AND P-AKT IN WT TISSUES**

Spearman correlation analysis results, shown in Table 3, suggest that in WT tissues, the expressions of miR-19b and PTEN proteins were negatively correlated (\( r = -0.473, P < 0.05 \)), the protein expressions of PTEN and p-Akt were negatively correlated (\( r = -0.511, P < 0.05 \)), and the protein expression of PI3K and p-Akt were positively correlated (\( r = 0.472, P < 0.05 \)).
PTEN AS A TARGET GENE OF MIR-19B

The online prediction software Target Scan was used to identify the miR-19b binding sites of PTEN, and the sequence of the 3’- UTR region of PTEN mRNA that miR-19b binds to is shown in Fig. 3A. To prove that the predicted miR-19b binding site caused the changes in luciferase activity, the mutated PTEN 3’- UTR sequence without miR-19b binding sites and the wild-type sequence were separately inserted into the reporter plasmid. For the luciferase activity assay, miR-19b mimics, miR-19b inhibitors, or miR-19b NC was co-transfected with the wild-type (Wt-miR-19b/PTEN) or mutated (Mut-miR-19b/PTEN) recombinant plasmid in the human WT SK-NEP-1 cell line. The results showed that miR-19b mimics did not have an obvious effect on luciferase activity in the Mut-miR-19b/PTEN plasmid group, but they decreased luciferase activity in the Wt-miR-19b/PTEN reporter plasmid group (P < 0.05). However, miR-19b inhibitors did not have an obvious effect on luciferase activity in the Mut-miR-19b/PTEN plasmid group, but they elevated luciferase activity in the Wt-miR-19b/PTEN reporter plasmid group (Fig. 3B).

THE EFFECT OF MIR-19B EXPRESSION ON CELL GROWTH

The MTT assay results (Fig. 4) showed that the OD values in each group at 12 h were not significantly different. At 24 h and 48 h, cell growth in the miR-19b mimics group significantly increased compared to that in the blank group and NC group (both P < 0.05). Cell growth in the miR-19b inhibitor group was significantly decreased compared with that in the blank group and NC group (both P < 0.05).

THE EFFECT OF MIR-19B EXPRESSION ON CELL CYCLE AND APOPTOSIS RATES

The results of PI single staining (Fig. 5A ~ 5B) showed that in the miR-19b inhibitor group, the cells were mainly arrested in the G1 phase, while the proportion of cells in the S phase declined, and SK-NEP-1 cell proliferation was obviously inhibited, compared with the other three groups (P < 0.05). The number of cells in G1 phase decreased, and the number of cells in the S phase decreased in the miR-19b inhibitor group compared with the other three groups (both P < 0.05).
increased in the miR-19b mimics group compared to the blank group and the NC group ($P < 0.05$). V/PI Annexin dual parameter test results (Fig. 5C) showed that the apoptosis rates of the cells in the blank group, NC group, miR-19b mimics group and miR-19b inhibitor group at 48 h after transfection were 9.78%, 10.04%, 5.05%, and 30.67%, respectively. The SK-NEP-1 cell apoptosis rate in the miR-19b inhibitor group significantly increased compared to the other three groups ($P < 0.05$), and the apoptosis rate in the miR-19b mimics group significantly decreased compared to the other three groups ($P < 0.05$). There was no statistical significance in the apoptosis rate between the blank group and NC group.

**THE EFFECT OF MIR-19B EXPRESSION ON CELL MIGRATION AND INVASION**

Cell migration results (Fig. 6A) showed that the number of cells that migrated through the polycarbonate membrane after culturing for 48 h was $125.30 \pm 3.40$ in the blank group and $119.67 \pm 3.28$ in the NC group without a significant difference ($P > 0.05$). The number of cells that migrated through the polycarbonate membrane in the miR-19b mimics group was $162.59 \pm 4.72$, which was significantly higher than that in the blank and NC groups ($P < 0.05$). The number of migrated cells in the miR-19b inhibitor group was $62.13 \pm 2.02$, which was significantly lower than that in the blank and NC groups ($P < 0.05$). Cell invasion results (Fig. 6B) showed that the number of cells penetrating the Matrigel onto the back of the Transwell membrane was $152.37 \pm 4.88$ in the blank group and $150.02 \pm 3.58$ in the NC group, which was not significantly different ($P > 0.05$). The number of cells penetrating Matrigel onto the back of the Transwell membrane in the miR-19b mimics group was $225.74 \pm 4.77$, which was significantly higher than that in the blank and NC groups ($P < 0.05$). The number of invaded cells in the miR-19b inhibitor group was $93.53 \pm 1.86$, which was significantly lower than that in the blank and NC groups ($P < 0.05$).
PTEN, PI3K, AKT AND P-AKT PROTEIN EXPRESSION

Western blotting results (Fig. 7) showed that PTEN protein expression in the miR-19b inhibitor group was up-regulated, and the expression of PI3K and p-Akt was down-regulated compared to the blank and NC groups (all \( P < 0.05 \)). Additionally, PTEN protein expression was down-regulated, and the expression of PI3K and p-Akt was obviously up-regulated in the miR-19b mimics group compared to the blank and NC groups (\( P < 0.05 \)). However, the expression levels of Akt protein were not significantly different between the groups. The differences in the expression levels of all proteins between the blank and NC groups were not statistically significant.

DISCUSSION

After extensive study on the molecular biology of cancer, it has been suggested that the PTEN/PI3K/AKT signaling pathways are closely related to the growth, proliferation, migration, and invasion of malignant cells [Yang et al., 2015]. WT, the most common renal cancer among children, can grow without any characteristic symptoms for a long time, manifesting as only abdominal pain, fever, vomiting, and nausea [Szymanska et al., 2016]. The International Society of Pediatric Oncology and the National Wilms’ Tumor Study group in North America are the two largest cooperative study groups that have studied Wilms’ tumor treatments, and the former recommends the use of preoperative chemotherapy, while the latter suggests adjuvant treatment after primary surgery [Spreafico and Bellani, 2006]. Interestingly, one study produced a miRNA profile for chemotherapy-resistant blastemal cells in Wilms’ tumors to provide predictive biomarkers for therapeutic response in the pretreatment biopsy stage [Watson et al., 2013]. Therefore, the aim of our study was to explore the roles of miR-19b and the PTEN/PI3K/AKT signaling pathway in the proliferation, migration and invasion of WT cells. The findings of our study indicated that miR-19b down-regulated PTEN protein expression; thus, it regulated the PTEN/PI3K/AKT signaling pathway and promoted the proliferation and invasion of WT cells.

Initially, our findings showed that miR-19b expression in the WT tissues was significantly
higher than that in the adjacent normal tissues. A study performed by Jia et al. demonstrated that miR-19b is highly expressed in gliomas and might be associated with gliomagenesis [Jia et al., 2013]. Alexander et al. reported that miR-19b expression was remarkably increased in the cerebrospinal fluid (CSF) of patients with primary central nervous system lymphoma (PCNSL) [Baraniskin et al., 2011]. In our study, the level of miR-19b expression in patients with lymph node metastasis was significantly higher than that in patients without lymph node metastasis, and the stage III/IV patients showed higher expression levels compared with the stage I/II patients, suggesting that miR-19b expression may contribute to WT progression. According to Wu et al., the miR-19b expression level is prominently enhanced with increased TNM stage, suggesting that miR-19b plays a key role in survival prediction and the response to chemotherapy in non-small cell lung cancer [Wu et al., 2014]. Additionally, Kahlert et al. reported that the miR-19b overexpression is considered a poor prognostic indicator of tumor recurrence and overall survival in colorectal liver metastases [Kahlert et al., 2011]. Collectively, we concluded that miR-19b might be associated with the metastasis and prognosis of WT. Moreover, our study showed that miR-19b could promote cancer cell proliferation, migration, and invasion as well as inhibit cell apoptosis. Wigard et al. have revealed that miRNAs were involved in numerous biological processes, including cell proliferation, differentiation, and apoptosis [Kloosterman and Plasterk, 2006]. Li et al. indicated that the inhibition of miR-19b results in decreased cell proliferation, hence illustrating the important role of miR-19b in cell proliferation [Li et al., 2014]. In addition, Xu et al. demonstrated that the up-regulation of miR-19b could accelerate cell growth, proliferation and invasion of cervical cancer cells [Xu et al., 2012]. More importantly, miR-19b overexpression has been confirmed to promote cell proliferation and differentiation as well as inhibit apoptosis that resulted from the overexpression of miR-19b; it also inhibits apoptosis induced by serum deprivation in P19 cells, which is a model of cardiac cell development [Qin et al., 2013].

In this study, we found that compared to the normal tissues, the positive rate of PTEN protein expression in WT tissue significantly decreased, while the positive rate of PI3K and AKT protein expression increased.
expression significantly increased. It has been shown in the latest research that in the PTEN/PI3K/AKT signaling pathway, when PI3K is activated, it could catalyze 3,4,5-phosphatidylinositol trisphosphate phosphorylation and then activate the protein kinase AKT to promote the growth and proliferation of cells [Li et al., 2013; Ying et al., 2015]. PTEN, a tumor suppressor gene, was confirmed as a target of miR-19b by a luciferase assay, and one study speculated that miR-19b might play an oncogenic role in gliomagenesis, at least partially, via negatively regulating PTEN [Jia et al., 2013]. To the best of our knowledge, the PTEN/PI3K/AKT signaling pathway plays an important role in regulating certain biological processes including cell growth and proliferation, metabolism, and apoptosis, which paves a new way for drug discovery [Carnero et al., 2008]. Chen et al. reveal that PTEN and activated AKT are associated with intrahepatic metastasis, tumor grade, and a high proliferation index. The PI3K/PTEN/AKT pathway is considered a diagnostic and prognostic indicator of invasion and metastasis as well as a therapeutic target in the treatment of hepatocellular carcinoma [Chen et al., 2009]. Correlation analyses of miR-19b, PTEN, PI3K and Akt proteins have also confirmed the relationships between proteins in our study.

Collectively, our study supported that miR-19b promoted the proliferation, migration, invasion of cells and the cell cycle of WT cells by modulating the PTEN/PI3K/AKT signaling pathway. It may serve as a novel therapeutic target in WT. The relationship between miR-19b and the PTEN/PI3K/Akt signaling pathway may identify new molecular markers and potential therapeutic targets that will provide a theoretical basis for future studies. The effects of miR-19b on WT cell proliferation, migration and invasion should be confirmed using animal models so that we can get closer to the goal of developing new genetic therapeutic strategies for the treatment of WT.
ACKNOWLEDGEMENTS

We thank the reviewers for their valuable comments.

CONFLICTS

None.
REFERENCES


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LEGENDS

**Fig. 1.** miR-19b expression in the WT and adjacent normal tissues

Note: WT, Wilms’ tumor; *, $P < 0.05$ compared to the adjacent normal tissue.

**Fig. 2.** The protein expression levels and positive expression rates of PTEN ($\times$ 200), PI3K ($\times$ 200) and p-Akt ($\times$ 400) in the WT and adjacent normal tissues

Note: A, Image of immunohistochemical staining for PTEN ($\times$ 200), PI3K ($\times$ 200) and p-Akt ($\times$ 400) in the WT and adjacent normal tissues; B, Histogram for the comparisons of PTEN, PI3K and p-Akt positive expression rates in the WT and adjacent normal tissues; PTEN and PI3K were mainly expressed in the cytoplasm and membrane, and Akt was mainly expressed in the nucleus; *, $P < 0.05$, compared to the adjacent normal tissue; WT, Wilms’ tumor; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B.

**Fig. 3.** PTEN is a target gene of miR-19b

Note: A, Image of miR-19b bound to the PTEN 3’ UTR; B, The relative luciferase activity detected by dual-luciferase reporter gene activity assay; *, $P < 0.05$ compared with other groups; PTEN, phosphatase and tensin homolog; miR-19b, micro-19b.

**Fig. 4.** Cell growth detected by MTT assay.

Note: The line chart for cell growth indicates that miR-19b mimics transfection significantly enhanced SK-NEP-1 cell growth, while miR-19b inhibitor transfection significantly suppressed SK-NEP-1 cell growth; *, $P < 0.05$ compared to the blank group.

**Fig. 5.** Cell cycle and the apoptosis rate of transfected cells as detected by the PI single-staining method and the V/PI Annexin double-staining method.

Note: A, The results of flow cytometry for cell cycle analysis after transfection; B, The histogram of the proportions cells in the G1, S and G2 phases after transfection; C, The apoptosis rates of the transfected cells in each group; *, $P < 0.05$ compared to the blank and NC groups; NC, negative control; PI, propidium iodide.

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**Fig. 6.** The migration and invasion ability of the SK-NEP-1 cells after transfection in each group as detected by the Transwell assay (× 200)

Note: A, Migration of the transfected cells in each group; B, Invasion of the transfected cells in each group; *, P < 0.05 compared to the blank and NC groups; NC, negative control.

**Fig. 7.** The protein expressions of PTEN, PI3K, Akt and p-Akt detected by Western Blotting.

Note: A, Diagram of the protein bands in each group detected by Western Blotting; B, Histogram of the protein expression levels in each group; *, P < 0.05 compared to the blank and NC groups; NC, negative control; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B.

**Appendix Fig. 1.** The diagram for the associations of related proteins in the PTEN/PI3K/Akt signaling pathway

**Table 1** The relationship between the miR-19b expression and the clinical pathological characteristics of patients with Wilms’ tumor.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases</th>
<th>MiR-19b expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (month)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 24</td>
<td>21</td>
<td>3.93 ± 0.42</td>
<td>0.572</td>
</tr>
<tr>
<td>≥24</td>
<td>25</td>
<td>3.86 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>3.85 ± 0.39</td>
<td>0.33</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>3.98 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FH</td>
<td>37</td>
<td>3.88 ± 0.42</td>
<td>0.596</td>
</tr>
<tr>
<td>UH</td>
<td>9</td>
<td>3.96 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>NWTS-5 grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II phase</td>
<td>32</td>
<td>3.70 ± 0.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>III/IV phase</td>
<td>14</td>
<td>4.44 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>4.34 ± 0.22</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No</td>
<td>29</td>
<td>3.63 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>3.87 ± 0.46</td>
<td>0.854</td>
</tr>
<tr>
<td>No</td>
<td>36</td>
<td>3.90 ± 0.40</td>
<td></td>
</tr>
</tbody>
</table>

Note: miR-19b, micro-19b; FH, favorable histology; UH, unfavorable histology; NWTS, National Wilms Tumor Study.
Table 2 The relationship between the protein expressions of PTEN, PI3K and p-Akt and the clinical pathological characteristics of patients with Wilms’ tumor.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases</th>
<th>PTEN (%)</th>
<th>P</th>
<th>PI3K (%)</th>
<th>P</th>
<th>p-Akt (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (month)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;24</td>
<td>21</td>
<td>11 (52.38)</td>
<td>0.806</td>
<td>15 (71.43)</td>
<td>0.28</td>
<td>15 (71.43)</td>
<td>0.801</td>
</tr>
<tr>
<td>≥ 24</td>
<td>25</td>
<td>14 (56.00)</td>
<td>14 (56.00)</td>
<td>17 (68.00)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>17 (56.67)</td>
<td>0.666</td>
<td>20 (66.67)</td>
<td>0.486</td>
<td>22 (73.33)</td>
<td>0.447</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>8 (50.00)</td>
<td>9 (56.25)</td>
<td>10 (68.75)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FH</td>
<td>37</td>
<td>19 (51.35)</td>
<td>0.408</td>
<td>24 (64.86)</td>
<td>0.604</td>
<td>26 (70.27)</td>
<td>0.833</td>
</tr>
<tr>
<td>UH</td>
<td>9</td>
<td>6 (66.67)</td>
<td>5 (55.56)</td>
<td>6 (66.67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWTS-5 grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II stage</td>
<td>32</td>
<td>21 (65.63)</td>
<td>0.02</td>
<td>17 (53.13)</td>
<td>0.035</td>
<td>19 (59.38)</td>
<td>0.023</td>
</tr>
<tr>
<td>III/IV stage</td>
<td>14</td>
<td>4 (28.57)</td>
<td>12 (85.71)</td>
<td>13 (92.86)</td>
<td></td>
<td></td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>4 (23.53)</td>
<td>0.001</td>
<td>16 (94.12)</td>
<td>0.001</td>
<td>16 (94.12)</td>
<td>0.006</td>
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<td>29</td>
<td>21 (72.41)</td>
<td>13 (44.83)</td>
<td>16 (55.17)</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>6 (60.00)</td>
<td>0.685</td>
<td>6 (60.00)</td>
<td>0.822</td>
<td>6 (60.00)</td>
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<tr>
<td>No</td>
<td>36</td>
<td>19 (52.78)</td>
<td>23 (63.89)</td>
<td>26 (72.22)</td>
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</tr>
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</table>

Note: FH, favorable histology; UH, unfavorable histology; NWTS, National Wilms Tumor Study; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B.
**Table 3** Relationship among miR-19b expression and PTEN, PI3K and p-Akt protein expression in the Wilms’ tumor tissues

<table>
<thead>
<tr>
<th>Marker</th>
<th>miR-19b</th>
<th>PTEN</th>
<th>p-Akt</th>
<th>PI3K</th>
</tr>
</thead>
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<tr>
<td>miR-19b</td>
<td>1.000</td>
<td>-0.473&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.388&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.180&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PTEN</td>
<td>-0.473&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.000</td>
<td>-0.511&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.340&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-Akt</td>
<td>0.388&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.511&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.000</td>
<td>0.472&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PI3K</td>
<td>0.180&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.340&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.472&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup>, P < 0.05; <sup>b</sup>, P > 0.05; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B.
Figure 1

Relative expression of miR-1964

- Carcinoma adjacent tissue
- Wilms' tumor tissue

* Significant difference
Figure 2
Figure 3

A

<table>
<thead>
<tr>
<th>PTEN 3' UTR</th>
<th>hsa-miR-19b 5'...ACUCAUAAUGCAGAGUUGAGA...</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3'...UCACGGGGUGUGCAACUCUA</td>
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B

<table>
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<th>Relative luciferase activity</th>
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<tr>
<td>NC</td>
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<tr>
<td>miR-19b mimics</td>
<td>-</td>
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<td></td>
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<tr>
<td>miR-19b inhibitor</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>PTEN-3'-UTR-WT</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>PTEN-3'-UTR-MT</td>
<td>-</td>
</tr>
</tbody>
</table>

|                | +                           |
|                | 0.2                         |

Figure 3
Figure 4

OD Value

- Blank
- NC
- miR-19b mimics
- miR-19b inhibitors

12h, 24h, 48h
Figure 5
Figure 6
Figure 7
Appendix Figure 1