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Up-regulation of miR-190b promoted growth, invasion, migration and inhibited apoptosis of Wilms' tumor cells by repressing the PTEN expression

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Abstract. – OBJECTIVE: Wilms' tumor (WT) is the most common malignant tumor in the children's urogenital system. MiR-190b was found to participate in the development and progression of several cancers. However, the molecular mechanism of miR-190b in WT is still unclear.

PATIENTS AND METHODS: We detected the miR-190b in WT tissue samples compared to adjacent normal samples as well as in WT patients' blood sample compared to normal volunteers using qRT-PCR. With over-expression and knockdown of miR-190b in WT-derived cell line SK-NEP-1, we next studied cell proliferation, cell circle, apoptosis, invasion and migration abilities change caused by miR-190b ectopic expression. Dual-luciferase assay and Western-blot analysis were used to explain the mechanism of miR-190b in WT.

RESULTS: MiR-190b was over-expressed in WT tissue and blood samples compared to normal group, relatively. Up-regulation of miR-190b in SK-NEP-1 cells significantly increased the growth and decreased the apoptosis of cells, while its down-regulation reduced cell proliferation and promoted cell apoptosis of SK-NEP-1. Also, cell invasion and migration abilities were significantly improved after miR-190b over-expression. Moreover, PTEN was proved to be a direct target of miR-190b and its protein level was remarkably decreased after miR-190b up-regulation.

CONCLUSIONS: miR-190b over-expressed in WT and promoted cell proliferation, invasion and migration while reduced cell apoptosis of WT cells by repressing PTEN repression, which might provide a potential target for WT diagnosis and therapy.

Key Words
Wilms' tumor, Children, miR-190b, PTEN, Apoptosis.

Introduction

Wilms' tumor (WT) is one of the most common retroperitoneal malignant solid tumors in children. The incidence of WT, especially in the genitourinary tumors of children under 15 years of age, is the highest among abdominal malignant tumors[1]. The incidence of left and right side of WT is almost the same, but less incidence of bilateral tumors. There was almost no difference in the incidence of pediatric and female children, but many studies showed that men were slightly more developed than women[2]. WT in children with early symptoms is not obvious; because of their low age, patients mostly children or infants, easily ignore or are unable to describe some of the signs, leading to medical time delay[2]. So, to find new targets for WT diagnosis and treatment to improve the survival rate of children WT patients, has important clinical value. Along with the development of genomics, microRNAs are proved to participate in many stages of cancer development and progression via regulating their downstream molecular[3]. MiRNAs are a type of non-coding RNAs which are 17-22nt long, and they can specially combine to the 3'-UTR of their target genes, then caused the repression of target protein level[4]. Due to the effects of miRNAs, they could serve as diagnosis and prognosis biomarkers of several cancers and also act as potential targets for tumor biological treatment[5-7]. In WT, several miRNAs have been identified to influence WT progression[8], such as miR-19b effected cell proliferation, apoptosis and migration of Wilms' tumor via the PTEN/PI3K/AKT signaling pathway; microRNA-370 could be regulated by Stat3 and effect Wilms tumor progression. In addition, miR-21 acted

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as clinicopathological parameter and prognostic relevance factor of WT. MicroRNA-185 miR-192, miR-194, miR-215, miR-200c and miR-141 suppressed tumor growth and progression by targeting specific target oncogene in WT[9-14]. MiR-190b has been verified as an oncogene, which promoted tumorigenesis and progression in several cancers. For instance, in breast tumors miR-190b up-regulated in ERα-positive while compared to ERα-negative. In lung adenocarcinoma, miR-190b was clearly over-expressed and promoted cancer metastasis; in human hepatocellular carcinoma, microRNA-190b induced insulin resistance via IGF-1[15-17]. A study revealed miR-190b was over-expressed in WT patients' blood sample[18]. However, the role miR-190b playing in the WT and its underlying mechanism have not been mentioned.

In this study, we first measured the miR-190b level in WT tissue and blood samples compared with normal group. Then, the relationship between the miR-190b and clinicopathological variables was studied. Furthermore, WT cell line SK-NEP-1 was transfected with miR-190b mimics to over-express miR-190b and miR-190b inhibitors to knockout miR-190b. Several functional experiments were recruited to evaluate the influence of miR-190b in cell proliferation, cell circle, apoptosis, invasion and migration. Then, we found PTEN as a potential target for miR-190b in WT using several databases, and then verified it using luciferase and Western-blot. Our results may provide a novel target for WT diagnosis and therapy.

Patients and Methods

Tissue and Blood Samples

A total of 44 pairs human WT and adjacent normal tissue samples were collected from West China Hospital and the blood samples were collected from 44 WT patients and 23 health volunteers. These tissues and blood were stored in liquid nitrogen immediately after surgical resection or collection. The clinical pathological features were collected based on National Wilms Tumor Study (NWTS-5) grade classification. All patients and volunteers had signed the informed consent and the study obtained the approval of the Ethics Committee of West China Hospital.

Cell Line and Culture

WT-derived SK-NEP-1 cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL Penicillin and 50 mg/ml streptomycin (Corning, Corning, NY, USA) and incubated at 37°C in humidified atmosphere containing 5% CO_2.

RNA Isolation and qRT-PCR

The total RNAs of WT tissue and blood samples were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as well as SK-NEP-1 cells. After isolation, the miR-190b expression level was detected by ABI 7900T (BD, Franklin Lakes, NJ, USA) using a Taqman Kit (Genepharma, Shanghai, China) after the RNA was reverse transcribed into cDNA with a reverse transcription kit (Genepharma, Shanghai, China). U6 was used as internal control and the relative expression result was calculated with using the $2^{-\Delta\Delta CT}$ method.

Cell Transfection

For over-expression and knockdown of miR-190b, the miR-190b mimics, inhibitors, and negative control (NC) were synthetized by Genechem (Shanghai, China). SK-NEP-1 cells were seeded in six-well plates and cultured to a density of 70%. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were co-cultured with appropriate amount miR-190b mimics, miR-190b inhibitors or NC according to manufactory instructions. After cultured for 6 hours, the cell culture medium was changed to normal medium without the transfection reagent. Then, using qRT-PCR, the efficiency of miR-190b expressing regulation was confirmed.

Cell Counting Kit-8 (CCK8) Assay

CCK8 assay was used to measure the cell proliferation ability. A total of 3000 cells were seeded in 96-well plate suspended in 100 μL medium. Next, they were cultured for 24, 48, 72, 96 h after transfection. CCK8 reagent was added into the wells with amount of 10 μL per well and absorbance of 450 nm was measured. Each group was replicated for at least three times.

Cell Apoptosis and Cell Circle Detection

Flow cytometry (BD, FACS, Franklin Lakes, NJ, USA) was used to measure the cell apoptotic rate with using a AnnexinV-fluorescein isothiocyanate (FITC) apoptosis detection kit (Vazyme, Nanjing, China). Cells were digested after miR-190b mimics or inhibitors treatment

and washed with pre-cooling phosphate-buffered saline (PBS) for three times. Cells were resuspended in 1000 μL of binding buffer mixing 10 μL of FITC and propidium iodide (PI), respectively. Cell apoptotic rate was measured by flow cytometry (BD, FACS, Franklin Lakes, NJ, USA), and the percentage of apoptotic cells was counted. For cell circle detection, cells were added to 1 mL of 75% ice-cold alcohol after harvest from transfection and kept at 4°C overnight. The cells were just stained with PI before the flow cytometry analysis according to the instruction. The percentages of G0/G1, S, G2/M cells were recorded. Each measurement was repeated three times.

Transwell Assay

For invasion experiment, 72 hours after transfection, a total of $4X10^4$ treated cells maintained in FBS-free medium were seeded into the upper chamber of the insert, which had covered with Matrigel (BD, Franklin Lakes, NJ, USA). The lower chamber was added with 500 μL medium containing 10% FBS. After 48 h incubation, cells that failed to pass through the upper chamber were removed and the membranes containing cells on its lower surface were fixed with methanol and stained with 0.5% crystal violet. Then, the cells stained were calculated and pictured using a microscope in five random visions. The number of cells passing through the Matrigel was measured to evaluate the invasive ability. For migration experiment, the top chamber of the insert covered nothing before cell plating. The other steps were the same as the invasion assay. The 8-um transwell inserts were bought from Millipore (Billerica, MA, USA).

Luciferase Assay

The Dual-Luciferase reporter system (Promega, Madison, WI, USA) was purchased to study the binding activity of miR-190b with PTEN. The PTEN 3'-UTR cDNA sequences, including the mutant or wild type miR-190b binding region, was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Then, SK-NEP-1 cells were co-treated with the established pGL3 vector and miR-190b mimics or NC using lipofectamine 2000. The activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured.

Protein Isolation and Western-Blot

The treated cells were lysed with protein extraction lysis solution after washed three times with precooled PBS, and incubated on ice for 40 min. After centrifugation for 10 min, the supernatant was collected. The protein concentration was detected by using the bicinchoninic acid (BCA) quantitative detection reagent kit (Thermo Fisher, Waltham, MA, USA). Then, proteins mixed with loading buffer (Beyongtime, Shanghai, China) were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were immersed in 5% bovine serum albumin (BSA) to block non-specific protein interactions in Tris-buffered saline and Tween 20 (TBST) buffer at 4°C for 2 hour. The membranes loaded with proteins were maintained in 5% BSA with the primary antibody against PTEN or GAPDH (CST, Danvers, MA, USA). The membranes were incubated at room temperature with secondary antibody conjugated with horseradish peroxide (HRP) for 1 hour after washing with TBST buffer 10 min x 3 times. After washed three times with TBST (10 min each), finally, the samples were subject to enhanced chemiluminescence (ECL), and data analysis were performed.

Statistical Analysis

All the statistical analysis was handled by using statistical product and service solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 software (GraphPad Software Inc, Version X; La Jolla, CA, USA). All quantitative results were showed as mean ± SD. $p<0.05$ indicated significant difference.

Results

MiR-190b Was Over-Expressed in WT Tissue and Blood Samples

To study the relationship of miR-190b with WT, we detect miR-190b expression level in 44 WT tissues compared with adjacent normal tissues. Clearly, miR-190b in WT tissue samples expressed significantly higher than in normal tissues (Figure 1A). Furthermore, we divided the 44 WT samples into high miR-190b level group and low miR-190b level group based on the median miR-190b expression level and determined the relationship of miR-190b with clinicopathological features. Table I showed that higher miR-190b level stranded for unfavorable histology, more lymph node metastasis and advanced NWTS-5

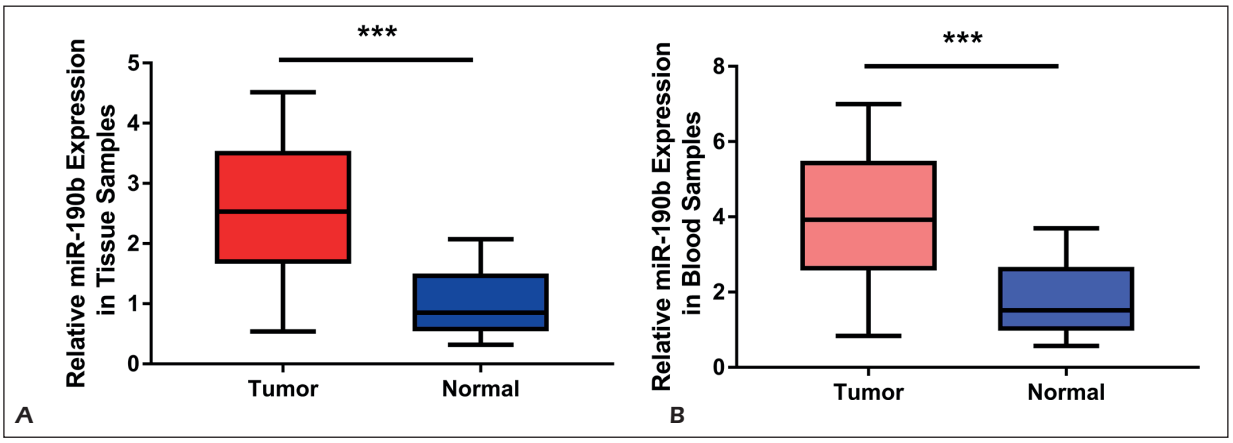

Figure 1. MiR-190b was over-expressed in WT tissue and blood samples. **A**, MiR-190b level in 44 tissue samples were detected compared to adjacent normal tissues. **B**, MiR-190b level in 44 blood samples were measured compared to 23 normal tissues. MiR-190b was detected by qRT-PCR and U6 was used as an internal control. Data are presented as the mean ± SD of three independent experiments. ***$p<0.001$.

stage, but not related with age, gender, or laterality. Also, the miR-190b levels in 44 WT blood samples compared with 23 normal samples were measured and miR-190b expressed higher in WT group as well as in WT tissues comparing with the normal group (Figure 1B). These results indicated miR-190b might act as an oncogene in WT.

Table I. Correlation between miR-190b level and clinicopathological features in Wilms' tumor.

Characteristics	Total	miR-190b expression		*p*-value
		Low	High	
Age				
< 24 months	28	15	13	0.2636
≥ 24 months	16	7	9	
Gender				
Male	24	11	13	0.8415
Female	20	11	9	
Laterality				
Left	23	13	10	0.6229
Right	18	8	10	
Both	3	1	2	
Histological type				
FH	36	21	17	0.0004*
UH	8	1	7	
Lymph node metastasis				
Yes	14	2	12	0.0012*
No	30	20	10	
NWTS-5 stage				
I-II	33	21	12	0.0017*
III-IV	11	1	10	

FH, Favorable histology; UH, Unfavorable histology; NWTS, National Wilms Tumor Study. The expression level of miR-21 was cut off by median expression level and * indicated p<0.05.

Up-regulation of miR-190b Promoted WT Cell Proliferation

To further explore the miR-190b effects in WT, we next up-regulated or down-regulated miR-190b using mimics or inhibitors to establish cells for the next experiments (Figure 2A). CCK8 assay was performed to evaluate the cell proliferation. Over-expression of miR-190b clearly promoted SK-NEP-1 cell growth while down-regulation of miR-190b reduced cell proliferation compared to negative and scramble groups (Figure 2B). These data suggested miR-190b promoted cell proliferation of WT cells.

Ectopic Expression of miR-190b Effected Cell Apoptosis and Cell Circle of WT

Next, we measured the influence of miR-190b on cell apoptosis by using flow cytometry. Over-expression of miR-190b reduced cell apoptotic rate; however, inhibition of miR-190b clearly promotes cell apoptosis (Figure 3A, 3B). Also, we detected cell circle change of SK-NEP-1 cells after miR-190b expression interference. Figure 3C showed up-regulation of miR-190b in SK-NEP-1 cells accelerated G1 to S phase transformation compared to the negative group, while down-regulation of miR-190b inhibited the transformation. These results indicated that miR-190b inhibited cell apoptosis and promoted cell circle in WT cells.

MiR-190b Promoted Cell Invasion and Migration in WT

In addition, to evaluate the influence of miR-190b in WT cell metastasis, we conducted transwell assays.

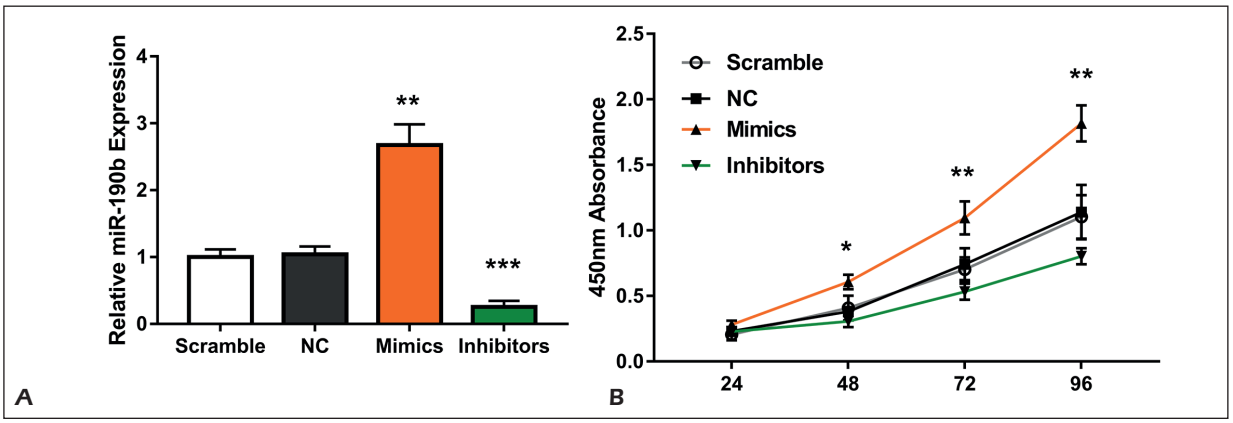

Figure 2. MiR-190b promoted proliferation of SK-NEP-1 cells. **A**, MiRNA level of SK-NEP-1 cells transfected with miR-190b mimics, inhibitors, NC, or not. **B**, CCK8 assay was performed at 0, 24, 48, 72 h time points to determine the viability of treated SK-NEP-1 cells. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01.

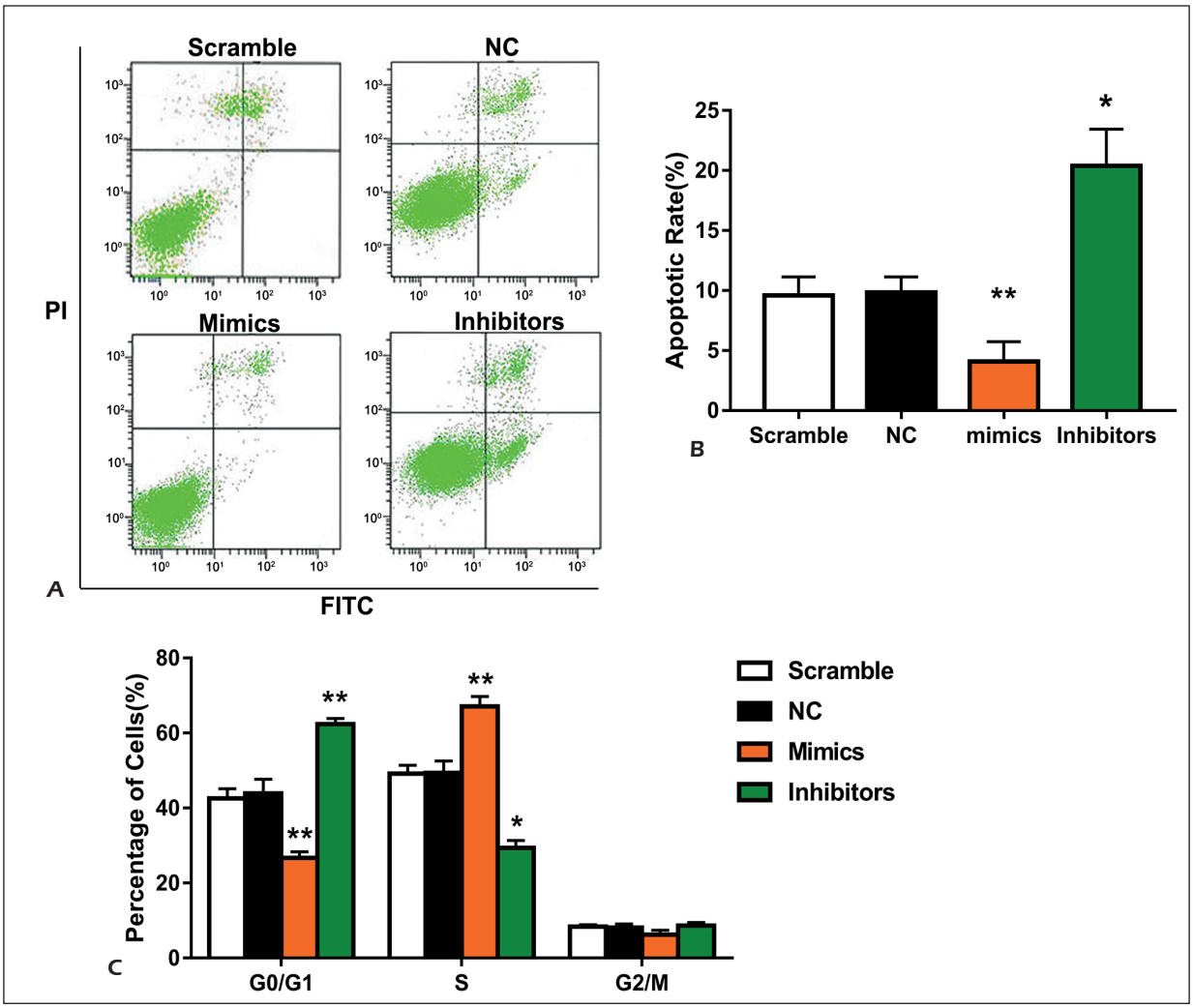

Figure 3. MiR-190b reduced the apoptosis of WT cells and accelerated cell circle. SK-NEP-1 cells were transfected with miR-190b mimics, inhibitors, NC, or not. **A**, Flow cytometric analysis was performed to detect the apoptotic rates of established cells. **B**, The apoptotic rate was calculated. C. The percentage of G0/G1, S, G2/M phase was measured. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01.

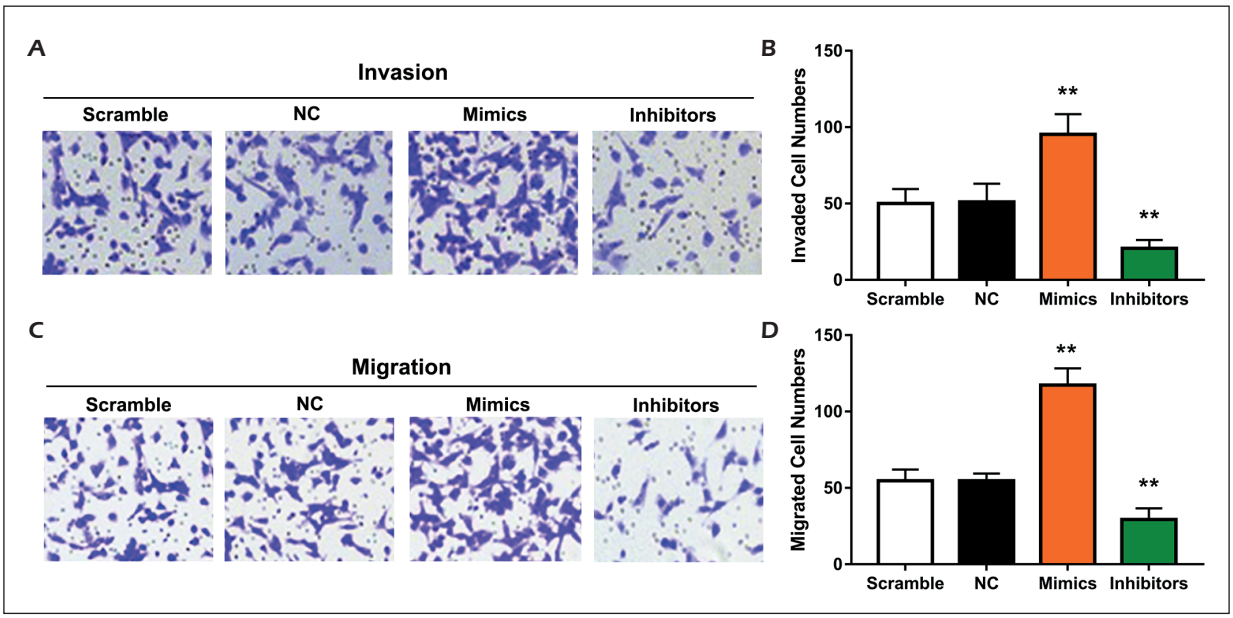

Figure 4. MiR-190b effected the invasion and migration of WT cells. **A-B**, Matrigel assay was performed to determine invasion of SK-NEP-1 cells treating with miR-190b mimics or inhibitors compared to negative control and scramble groups. **C-D**, Transwell assay was used to detect the migration ability of miR-190b mimics or inhibitors treated cells. Data are presented as the mean ± SD of five random visions. *p<0.05, **p<0.01.

We found that miR-190b mimics treatment significantly improved cell invasion ability while miR-190b inhibitors reduced cell invasion ability (Figure 4A, 4B). Also, miR-190b over-expression promoted cell migration but knockdown decreased cell migration (Figure 4C, 4D). The transwell assays demonstrated that miR-190b increased cell metastasis of WT.

PTEN Was a Direct Target of miR-190b

To explore the underlying mechanism of moR-190b in WT, we searched several database (targetscan, miRanda, PicTar) and observed PTEN as a potential target of mR-190b. Figure 5A showed the miR-190b binding region of the 3'-UTR of PTEN. To confirm our assumption, we established dual-luciferase assay by conducting wild type or mutant miR-190b binding region of PTEN 3'-UTR. We found that the luciferase activity significantly decreased in wild type group but no difference in mutant group (Figure 5B). These data indicated that PTEN was a direct target of miR-190b.

MiR-190b Influenced PTEN Expression in WT

Next, we employed Western-blot assay to measure the PTEN protein expression level in the miR-190b mimics, inhibitors treatment SK-NEP-1 cells comparing to negative control and scramble groups. PTEN protein level was remarkably decreased after up-regulating miR-190b while increased after down-regulating miR-190b comparing to negative control and scramble groups (Figure 5A and 5B). This confirmed PTEN as a target of miR-190b.

Discussion

Wilms' tumor is the most common malignant solid tumor in pediatric surgery[1]. Though the technical means of its diagnosis and treatment developed rapidly, the existing methods are only applicable to patients with a certain volume of tumor, such as: excretory urography, ultrasound, CT and magnetic resonance[2,19]. Along with the findings of biological markers, miRNAs were identified to serve as regulators and markers for several cancers[5,6]. These findings could provide an accurate and simple early detection of WT in order to enable children with WT to obtain an earlier treatment and long-term survival[8].

In this study, we first showed miR-190b expression in WT tissue and blood samples were significantly higher than in normal group. Furthermore, higher miR-190b indicated unfavorable histolo-

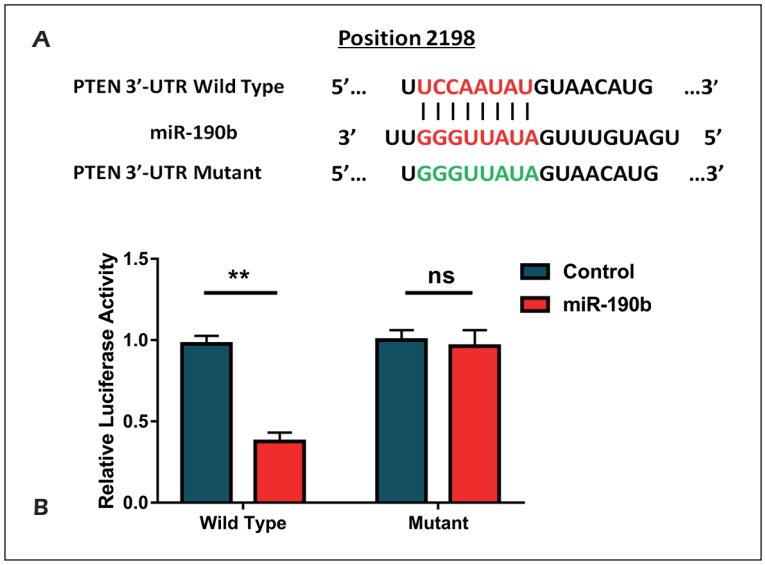

Figure 5. PTEN was a direct target of miR-190b. **A**, The predicted binding sites of miR-190b in the 3'-UTR of PTEN. **B**, Dual-luciferase reporter assay was used to determine the binding site. SK-NEP-1 cells treated by mimics or NC were co-transfected with pGL3 containing the wild type or mutant PTEN 3'-UTR region. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ns: no difference.

gy, more lymph node metastasis and advanced NWTS-5 stage, which suggested an unoptimistic prognosis. Next, several functional experiments were employed to verify that miR-190b promoted the cell proliferation, cell circle, invasion and migration but reduced cell apoptosis in SK-NEP-1 cells. In addition, PTEN was proved to be a direct target of miR-190b in WT cells.

MiR-190b was reported to participate in several cancer progressions though some gene such as Bcl-2 in gastric cancer and IGF-1 in hepatocellular carcinoma[16,20]. For the first time we identified PTEN as a target gene for miR-190b in WT. PTEN is a tumor suppressor located at 10q23.3. PTEN protein plays an important role in cell growth, apoptosis, adhesion, migration and infiltration. It functions as an evaluation index of tumor prognosis, and its mechanism is important for the diagnosis and gene therapy for tumors[21,22]. For example, PTEN could suppress non-small-cell lung cancer metastasis through inhibition of integrin αVβ6 signaling. In breast carcinoma, PTEN loss promoted tumor progression. What's more, JNK and PTEN cooperatively control the development of invasive prostate adenocarcinoma[23-26]. Many studies have revealed that PTEN could be regulated by miRNAs, such as by miRNA-1297 in human breast cancer, by miR-205 in NSCLC, miR-21 in hepatocellular carcinoma[27-29]. In WT, Loss of PTEN/MMAC1 activity was a rare and late event in the pathogenesis of WT, and it could be regulated by miR-21 to effect WT cells' aggressive behavior[9,30-32].

We conducted several functional assays and identified miR-190b enhanced proliferation, invasion and migration of SK-NEP-1 cells but reduced its apoptosis. And furthermore, we verified PTEN was a direct target of miR-190b in WT via luciferase and Western-blot assay. Though WT regulating molecular system was a network system, these experiments partially explained the role miR-190b played in WT.

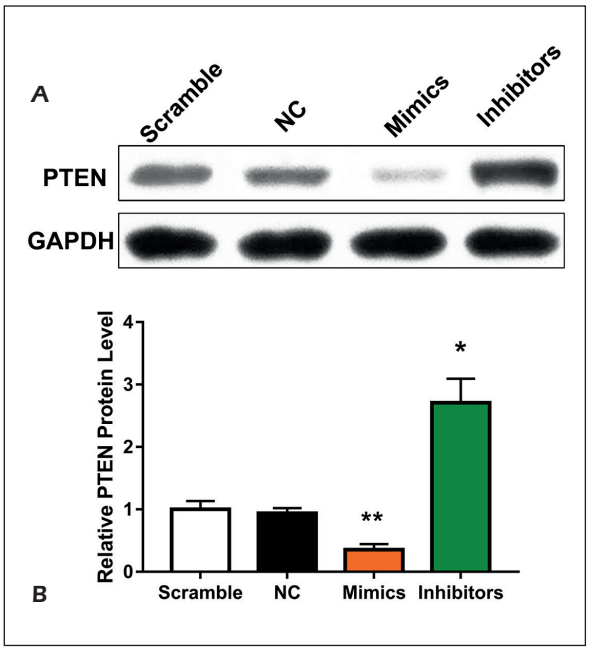

Figure 6. MiR-190b regulated PTEN expression. **A**, The expression levels of PTEN protein in established SK-NEP-1 cells. **B**, The normalization of PTEN protein bands. The protein levels were normalized to that of GAPDH. Data are represented as the mean ± SD of three replicates. *p<0.05, **p<0.01.

Conclusions

We demonstrated that: a) miR-190b was over-expressed in WT tissue and blood samples; b) higher miR-190b level indicated severe clinicopathological characteristics; c) miR-190b promoted cell proliferation, invasion, migration and inhibited cell apoptosis via PTEN. This is the first study to explain the relationship between miR-190b and WT. Our findings could provide a novel target to WT diagnosis and biological therapy.

Conflict of Interest

The authors declared no conflict of interest.

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