MiR-22-3p regulates the proliferation and invasion of Wilms’ tumor cells by targeting AKT3


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Abstract. – OBJECTIVE: In this study, the regulatory mechanism of miR-22-3p/AKT3 in the development of Wilms’ tumor (WT) was investigated.

PATIENTS AND METHODS: Twenty-seven pairs of surgical tumor specimens and adjacent normal tissues were obtained from Jining No. 1 People’s Hospital. The expression level of miR-22-3p in WT tissues and cell lines was measured by quantitative RT-PCR. MTT and transwell assays were performed to analyze cell proliferation and invasion in WT. The relationship between miR-22-3p and AKT3 was verified by a Dual-Luciferase assay. The protein expression of AKT3 was evaluated by Western blotting analysis.

RESULTS: MiR-22-3p was downregulated and AKT3 was upregulated in WT. Functionally, overexpression of miR-22-3p inhibited cell proliferation and invasion in WT. Moreover, miR-22-3p directly targets AKT3. The knockdown of AKT3 suppressed cell proliferation and invasion in WT. In addition, upregulation of AKT3 restored the tumor suppressive effect of miR-22-3p in WT.

CONCLUSIONS: MiR-22-3p inhibits the proliferation and invasion of WT cells by downregulating AKT3, indicating that miR-22-3p may be developed as a new biomarker for the diagnosis of WT.

Key Words: Wilms’ tumor, MiR-22-3p, Invasion, Proliferation, AKT3.

Introduction

Wilms’ tumor (WT) is the most common abdominal malignancy, with the highest incidence of WT in pediatric abdominal tumors. WT occurs mainly in the first 5 years after birth, especially at the age of 2-4. The incidence is similar on the left and right sides of kidney, and even 3-10% are bilateral. In addition, WT grows rapidly and the kidneys of many early-diagnosed patients have been occupied by tumors or metastasized to the lungs. It is worth noting that the 5-year survival rate of WT patients has improved to 90% due to improvements in therapy. However, about 10-15% of WT patients still relapse, especially in developing countries. Therefore, exploring key regulatory mechanisms is essential for developing new treatments of WT.

An increasing number of microRNAs (miRNAs) are reported to be involved in the progression of disease and cancer by degrading mRNA or inhibiting translation. Many miRNAs that act as oncogene or tumor suppressor have been found to play important roles in the development of human cancers. In fact, miR-19b has been found to promote the development of WT by blocking the PTEN/PI3K/AKT signaling pathway. In contrast, miR-613 inhibited the development of WT by suppressing FRS2 expression. These studies indicate that miRNAs can be used for the diagnosis of WT. In particular, miR-22 has been found to function as a tumor suppressor in renal cell carcinoma, but not in WT. In addition, downregulation of miR-22 has been identified in breast cancer, gastric cancer and lung cancer. Functionally, miR-22 has been shown to promote osteogenic differentiation of human periodontal ligament stem cells by inhibiting HDAC6. MiR-22 regulated cancer cell proliferation, apoptosis, invasion and migration. However, to our knowledge, the exact role of miR-22-3p in WT remains unclear.
As a member of the AKT family, AKT3 has been reported to affect cell transformation and play a carcinogenic role in ovarian cancer, pituitary adenoma and hepatocellular carcinoma. In addition, AKT3 has been found to promote the progression of thyroid cancer regulated by miR-338-3p. Zhang et al. reported that AKT3 has a poor prognostic value in human prostate cancer. However, the relationship between miR-22-3p and AKT3 is rare in WT.

In our study, the expression of miR-22-3p and AKT3 was observed in WT tissues and cell lines. Next, cell proliferation and invasion were examined to explore the functions of miR-22-3p and AKT3 in WT. Further, this study also investigated the relationship between miR-22-3p and AKT3 in WT.

**Patients and Methods**

**Clinical Tissues**

Twenty-seven pairs of surgical tumor specimens and adjacent normal tissue (≥2 cm away from the tumor, and no cancer cells were found in the tangent line pathologically) were obtained from the Jining No. 1 People’s Hospital. The diagnosed WT tissues were reviewed by an experienced pathologist, using histological slides, according to the 2007 guidelines of WHO classification. Patients who received chemotherapy or radiation before surgery were excluded from this research. All WT patients provided written informed consents. These tissues were then frozen in liquid nitrogen and stored in a refrigerator at -80°C for further experiments. This study was approved by the Institutional Ethics Committee of Jining No. 1 People’s Hospital. The entire investigation complies with the principles outlined in the Helsinki Declaration.

**Cell Cultures and Cell Transfection**

Human WT cell lines 17-94 and WIT49 were used for this experiment. The two cell lines were purchased from the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were seeded in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and cultured in an atmosphere with 5% CO₂ at 37°C.

MiR-22-3p mimics or inhibitor and AKT3 siRNA (si-AKT3) were purchased from RiboBio (Guangzhou, China). They were then transfected into WIT49 and 17-94 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufactures’ protocols.

**Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)**

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA in WT tissues and cell lines. QRT-PCR was performed on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR GREEN mastermix (Solarbio, Beijing, China). U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for miR-22-3p or AKT3. The expressions of miR-22-3p and AKT3 were calculated using the $2^{-\Delta\Delta C_t}$ method. The forward primer for miR-22-3p was 5'-AAGCTGCCAGTTGAAGAACTGTA-3', and the reverse primer was 5'-CAATTCAGTTGAGACAGTTCT-3'. U6 (forward, 5'-CTCGCTTCGGCAGCACA-3'; reverse, 5'-AACGCTTCACGAACTTGTGCT-3'). GAPDH (forward, 5'-CCCTCACCAAGGCGTTTAT-3'; reverse, 5'-AGGCTTTCCTCCATGTTGGAAGAC-3'). The primers for AKT3 were 5'-ACCGCACAGTTTCTATATG-3 (forward) and 5'-CCCTCACCAAGGCGTTTAT-3 (reverse).

**Luciferase Activity Assay**

The 3'-untranslated region (3'-UTR) of wild type or mutant AKT3 was inserted into pGL3 luciferase vectors (Promega, Madison, WI, USA). Next, the pGL3 Luciferase vectors and miR-22-3p mimics were transfected into WIT49 cells. Finally, a Dual-Luciferase assay (Promega, Madison, WI, USA) was used to analyze Luciferase activity.

**MTT Assay For Cell Proliferation**

Cell proliferation was measured using MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) assay. Transfected cells (4×10³/well) were seeded on 96-well plates in culture medium. WT cells containing miR-22-3p mimics or inhibitor were incubated for 24, 48, 72 or 96 h. After incubation, the cells were incubated with MTT (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 4 h. The absorbance at 490 nm (OD=490 nm) was measured with a spectrophotometer.

**Cell Invasion Assay**

Cell invasion was assessed by performing transwell assay. Transwells were coated with Matri-
gel (BD Biosciences, Franklin Lakes, NJ, USA) to detect cell invasion. First, $2 \times 10^5$ WT cells were seeded into the upper chamber (8 μm pore size; Corning Incorporated, Corning, NY, USA). A medium containing 10% FBS was placed in the lower chamber. These cells were then incubated at 37°C with 5% CO$_2$ for 18 h. Finally, the invasive cells on the lower surface were fixed with methanol and stained with 0.1% crystal violet (Beyotime, Shanghai, China). The moving cells were counted by a light microscope.

**Western Blot Analysis**
Protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the protein was blocked with 5% non-fat milk and transferred into polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were then incubated with anti-AKT3 and anti-GAPDH overnight at 4°C. The protein was incubated with specific secondary antibodies for 2 h. Finally, protein expression levels were measured by enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA).

**Statistical Analysis**
Data are shown as mean ± SD (standard deviation). Statistical analysis was performed using GraphPad Prism 6.0 (La Jolla, CA, USA). Differences between groups were calculated using the Student’s $t$-test. $p<0.05$ was defined as statistically significant.

**Results**

**Downregulation of MiR-22-3p and Upregulation of AKT3 Were Identified in WT**
First, the expression levels of miR-22-3p and AKT3 were detected via qRT-PCR. The results showed that miR-22-3p expression was significantly reduced in WT tissues compared to normal tissues (Figure 1A). As with the above results, downregulation of miR-22-3p was also detected in 17-94 and WIT49 cells (Figure 1B). Compared

![Figure 1](image-url)

*Figure 1.* Downregulation of miR-22-3p and upregulation of AKT3 were identified in WT. A, The expression of miR-22-3p in WT tissues and normal tissues was detected via qRT-PCR. B, The expression of miR-22-3p in WIT49, 17-94 and normal tissues (control). C, AKT3 expression in WT tissues and normal tissues was detected via qRT-PCR. D, AKT3 expression in WIT49, 17-94 and normal tissues (control). E, The protein expression of AKT3 in WIT49, 17-94 and normal cells (control). **$p<0.01$. 

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to normal tissues, AKT3 expression was found to be upregulated in WT tissues (Figure 1C). Meanwhile, upregulation of AKT3 was also found in 17-94 and WIT49 cells (Figure 1D, 1E). Therefore, we speculated that the abnormal expression of miR-22-3p and AKT3 may be involved in the tumorigenesis of WT.

MiR-22-3p Overexpression Repressed Cell Proliferation and Invasion in WT

To explore the effect of miR-22-3p on cell proliferation and invasion in WT, miR-22-3p mimics or inhibitors were transfected into WIT49 and 17-94 cells. The transfection efficiency of miR-22-3p was verified by qRT-PCR (Figure 2A).

Figure 2. Overexpression of miR-22-3p repressed cell proliferation and invasion in WT. A, The expression of miR-22-3p was examined in WIT49 and 17-94 cells with miR-22-3p mimics or inhibitor via qRT-PCR. B, Cell proliferation was measured in WIT49 and 17-94 cells containing miR-22-3p mimics or inhibitor via MTT. C, Cell invasion was measured in WIT49 and 17-94 cells with miR-22-3p mimics or inhibitor via transwell analysis (magnification: 200×) ** p<0.01.
Functionally, cell proliferation was suppressed by miR-22-3p mimics, while miR-22-3p inhibitor promoted cell proliferation in WIT49 and 17-94 cells (Figure 2B). In addition, transwell assay showed that miR-22-3p overexpression inhibited cell invasion, while miR-22-3p downregulation promoted cell invasion in WIT49 and 17-94 cells (Figure 2C). All findings indicate that overexpression of miR-22-3p inhibits cell proliferation and invasion in WT.

**MiR-22-3p Directly Targeted AKT3 In WT**

As shown in Figure 3A, AKT3 was predicted as a target gene of miR-22-3p, which has a binding site to the 3’-UTR regions of AKT3. In addition, Dual-Luciferase reporter assay was performed to reveal this prediction. The results showed that miR-22-3p mimics significantly reduced the Luciferase activity of wild type AKT3, but did not affect the Luciferase activity of mutant AKT3 (Figure 3B). We then found that miR-22-3p mimics reduced AKT3 expression in WT cells, whereas miR-22-3p inhibitor enhanced AKT3 expression in WT cells (Figure 3C, 3D). As shown in Figure 3E, the protein expression levels of AKT3 were similarly regulated by miR-22-3p in WT cells. Therefore, miR-22-3p directly targets AKT3 and negatively regulates AKT3 expression in WT.

**Silencing AKT3 Suppressed Cell Proliferation and Invasion In WT**

To investigate the function of AKT3 in WT, AKT3 siRNA was transfected into WT cells. And the expression level of AKT3 was significantly decreased by AKT3 siRNA in WIT49 and 17-94 cells (Figure 4A), which indicates that AKT3 was successfully silenced in the transfected cells. Next, we measured the effect of AKT3 on cell proliferation and invasion in WT. Knockdown of
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AKT3 inhibited the proliferation and invasion of WT cells in WIT49 and 17-94 cells (Figure 4B, 4C). These results indicate that AKT3 is an oncogene in the development of WT.

**Upregulation of AKT3 Restored the TumorSuppressive Effect of MiR-22-3p**

MiR-22-3p mimics and AKT3 vector were transfected into WIT49 and 17-94 cells to veri-
ify their interaction in WT. Next, the expression level of AKT3 was detected in WIT49 and 17-94 cells by qRT-PCR (Figure 5A). Compared with the control group, there was no difference in AKT3 expression in WIT49 and 17-94 cells with miR-22-3p mimics and AKT3 vector. Functionally, cell proliferation and invasion were investigated in transfected WIT49 and

Figure 5. Upregulation of AKT3 restored the tumor suppressive effect of miR-22-3p in WT. A, The mRNA expression of AKT3 were measured in WIT49 and 17-94 cells containing AKT3 vector and miR-22-3p mimics. B, Cell proliferation in WIT49 and 17-94 cells containing AKT3 vector and miR-22-3p mimics C, Cell invasion in WIT49 and 17-94 cells containing AKT3 vector and miR-22-3p mimics (magnification: 200×) ** p<0.01.
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Discussion

To date, the role of miR-22-3p in the development of WT has not been elucidated in detail. In the current study, it was found that the downregulation of miR-22-3p was associated with the malignant progression of WT. In addition, AKT3, which is directly targeted by miR-22-3p, was upregulated in WT tissues. Functionally, overexpression of miR-22-3p inhibited cell proliferation and invasion in WT. The knockdown of AKT3 also suppressed the proliferation and invasion of WT cells. Of note, it was found that the upregulation of AKT3 restored the tumor suppressive effect of miR-22-3p in WT, indicating that miR-22-3p has a great impact on the pathogenesis and progression of WT.

Many miRNAs have been shown to exhibit carcinogenic or inhibitory effects on the tumorigenesis and development of WT. MiR-197 has been reported to promote tumorigenesis of WT by inducing overgrowth and anti-apoptotic effects22. MiR-185 suppressed the growth and progression of WT by repressing Six123. In addition, miR-22 has been identified to participate in many biological processes in human cancers. Yang et al24 found that miR-22 was downregulated in esophageal squamous cell carcinoma and inhibited cell migration and invasion. We also found that miR-22-3p was down-regulated in esophageal squamous cell carcinoma and inhibited cell migration and invasion. The similar effect of miR-22-3p on WT cell proliferation was also observed in our study. In addition, miR-22 was found to suppress fibrogenesis by targeting TGFβRI in cardiac fibroblasts26. All these researches indicate that miR-22 has an important role in the pathogenesis of human cancers and disease. In our study, overexpression of miR-22-3p can impede the development of WT.

Since miRNAs play important roles in human cancers by blocking the expression of their target genes, the function of AKT3 as a direct target gene of miR-22-3p was investigated in WT. Moreover, miR-22-3p was found to negatively regulate the expression of AKT3 in WT. AKT3 functioned as an oncogene in WT, similar to that in other human cancers. AKT3 has been found to promote the proliferation of human glioblastoma cells27. The promoting effect of AKT3 on the proliferation of WT cells was also determined. In addition, AKT3 was found to promote cell invasion in thyroid cancer, which is similar to our results28. Notably, miR-22 has been reported to induce cell senescence of endothelial progenitor by targeting AKT329. This study also confirmed that miR-22-3p directly targets AKT3. In addition, we found that overexpression of AKT3 reduced the inhibitory effect of miR-22-3p on WT cell proliferation and invasion. All results indicate that the inhibitory effect of miR-22-3p on the proliferation and invasion of WT cells was regulated by the expression of AKT3. More importantly, we first reported the anti-tumor effect of miR-22-3p in WT. A negative correlation between AKT3 and miR-22-3p expression is identified in WT, which has not been reported in previous studies.

Conclusions

Briefly, the downregulation of miR-22-3p and upregulation of AKT3 were identified in WT. In addition, overexpression of miR-22-3p inhibited the proliferation and invasion of WT cells. Further, miR-22-3p directly targets AKT3. MiR-22-3p suppressed cell proliferation and invasion in WT, at least in part by targeting AKT3. These findings will help us understand the regulatory mechanism of miR-22-3p in WT.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


