

MicroRNA-539 inhibits the progression of Wilms' Tumor through downregulation of JAG1 and Notch1/3

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Abstract.

BACKGROUND: Previous studies demonstrated that miR-539 play an important role in the carcinogenesis of some cancers. The aim of the present study was to determine the role of miR-539 in the pathogenesis of Wilms' Tumor (WT).

METHODS: The expression level of miR-539 was measured by qRT-PCR in 42 WT tissues and SK-NEP-1 cell line. Protein expression of genes (E-cadherin, N-cadherin, Vimentin, Notch 1, Notch 3 and JAG1) was assessed by Western blot. The function of miR-539 was investigated in SK-NEP-1 cells by MTT and Transwell assays. The relationship between miR-539 and JAG1 was verified by a dual luciferase assay in SK-NEP-1 cells.

RESULTS: The expression level of miR-539 was significantly decreased in WT tissues. Downregulation of miR-539 was closely related to NWTS-5 stage, lymph node metastasis and histological type of WT patients. Furthermore, low miR-539 expression was associated with a shorter overall survival rate in WT patients. *In vitro*, overexpression of miR-539 suppressed proliferation, migration and invasion of SK-NEP-1 cells. In addition, JAG1 was a direct target of miR-539. MiR-539 inhibited the development of WT by inhibiting JAG1-Notch1/3 expressing and blocking EMT.

CONCLUSION: MiR-539 inhibited the progression of WT through downregulation of JAG1 and Notch1/3.

Keywords: Wilms Tumor, miR-539, JAG1, Notch 1, Notch 3

1. Introduction

Wilms' tumor (WT) is a malignant embryonal tumor derived from renal blastocyte, accounting for approximately 87% of childhood renal tumors [12]. The

peak age of WT is 3 to 4 years old, and 80% of patients suffer WT before the age of 5 [29]. This tumor was first described by Rance in 1814. Max Wilms further described its characteristics in 1899, thus it was named Wilms' tumor [9]. In addition, there are many treatments for WT. First, all stages of the WT should be surgically removed. Moreover, WT is sensitive to radiotherapy, and postoperative radiotherapy can improve the efficacy [10]. Furthermore, preoperative chemotherapy can shrink the size of the tumor, reduce the risk of surgery, and improve the rate of com-

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plete resection [11]. Besides that, the prognosis of WT depends on histologic type, tumor stage, patient age, and biological characteristics [13]. Most WT patients have a good prognosis. After systematic treatment, the 4-year survival rate is approximately 54.8%–90% in WT patients at any stage [26]. To make matters worse, no special precautions were taken for WT. Therefore, early detection and treatment are important to improve the prognosis of WT patients.

Recently, the potential role of microRNAs (miRNAs) in the diagnosis and treatment of cancer has been reported. Many miRNAs have been identified as oncogenes or tumor suppressors involved in the regulation of the tumorigenesis, including WT. For instance, upregulation of miR-190b promoted cell invasion, migration and inhibited cell apoptosis by inhibiting PTEN expression in WT [1]. Cui et al. had been found the prognostic effect of miR-21 in WT patients [6]. Moreover, miR-539 has been found to exhibit different roles in human disease and cancer. On the one hand, miR-539 was upregulated in failing heart [23] and promoted osteoblast proliferation, differentiation and osteoclast apoptosis [33]. On the other hand, Mirghasemi et al. reported that downregulation of miR-539 was associated with unfavorable prognosis in patients with osteosarcoma [22]. It was also found that miR-539 inhibited glioma cell proliferation and invasion by targeting DIXDC1 [25]. However, there is no study on the role of miR-539 in regulating the progression of WT.

As an oncogene, high expression of Jagged-1 (JAG1) has been shown to be associated with the progression and poor prognosis of cancers [27]. Previous studies have also shown that JAG1 expression may be regulated by several miRNA, such as miR-26a [20] and miR-524 [5]. In addition, JAG1 has been reported to be involved in the development of human cancer through the Notch signaling pathway [31]. According to previous reports, Notch signaling pathway is a double-edged sword in cancer [28]. The Notch signaling pathway includes four cell surface transmembrane receptors (Notch 1–4) and five ligands (Jagged-1, Jagged-2, Delta-like 1, Delta-like 3 and Delta-like 4) in mammals [21]. It has been found that abnormal Notch signaling pathway regulates kidney disease [2]. However, the interaction between the miR-539 and JAG1-Notch1/3 signaling pathway remains elusive in WT.

In this study, the function of miR-539 was investigated in WT. Abnormal expression of miR-539 was observed in WT tissues. The interaction between the miR-539 and JAG1-Notch1/3 signaling pathway was also analyzed in WT. We hope that these findings will provide new therapeutic targets for the treatment of WT.

2. Materials and methods

2.1. Clinical tissues

Forty-two WT tissues and adjacent tissues were obtained from The Affiliated Yantai Yuhuangding Hospital of Qingdao University after receiving the written informed consent. All patients did not receive any treatment before surgery. Human tissue was frozen in liquid nitrogen and stored in a -80°C refrigerator for further experiment. This experiment was approved by the Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University.

2.2. Cell cultures

The human WT cell line SK-NEP-1 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), which was selected to investigate the role of miRNA in WT in previous studies [1,7,17]. SK-NEP-1 cells were then seeded in RPMI-1640 with 10% fetal bovine serum (FBS) and incubated with 5% CO_2 at 37°C .

2.3. Cell transfection

The miR-539 mimic and inhibitor, JAG1 plasmid were purchased from RiboBio (Guangzhou, China) and then were transferred into SK-NEP-1 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufactures' protocols.

2.4. RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA containing miRNA was extracted using TRIzol reagent (Invitrogen, USA) to quantify the expression of miR-539 in WT tissues. The synthesis of cDNA was performed by reverse transcription using the TaqMan[®] MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR was performed on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems) by TaqMan Universal PCR Master Mix Kit (Thermo Fisher Scientific). U6 and GAPDH were used as controls for miR-539 and JAG1. And its expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

2.5. Luciferase activity assay

The wild or mutant type of 3'-UTR of JAG1 was inserted into the pGL3 luciferase vector (Promega, Madison) for luciferase reporter experiments. The 3'-UTR of wild or mutant JAG1 and miR-539 mimic were then transfected into SK-NEP-1 cells. Subsequently, dual luciferase assay (Promega, Madison) was used to analyze luciferase activity.

2.6. MTT assay for cell proliferation

Cell proliferation was assessed using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Cells (2×10^3 /well) were seeded onto 96-well plates in medium. Cells with miR-539 mimic or inhibitor were incubated for 24, 48, 72 or 96 h. After the incubation, cells added with MTT (Sigma, MO, USA) were incubated at 37°C for 4 h. The absorbance at 490 nm (OD = 490 nm) was measured with a spectrophotometer.

2.7. Transwell assay

Transwell chambers (8- μ m pore size membranes) were used for cell migration and invasion assays. SK-NEP-1 cells transfected with miR-539 mimics/inhibitor or negative control (NC) were incubated with serum-free medium for 24 h. Next, 2×10^4 SK-NEP-1 cells were placed in the upper chamber. And the medium with 10% FBS was placed in the lower chamber. After 24 h at 37°C, we removed the non-migrated cells completely. The migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The invasion assay was similar to the migration assay, except that the upper chamber was coated with 1 mg/ml Matrigel (BD Biosciences). Finally, we used a microscope to calculate the number of cells removed.

2.8. Western blot analysis

Protein samples were obtained using RIPA lysis buffer. Proteins were separated by 10% SDS-PAGE and incubated with 5% skim milk in PVDF membranes at room temperature. Next, we plated the membranes at 4°C with EMT markers (E-cadherin, N-cadherin, vimentin), Notch pathway markers (Notch 1, Notch 3), JAG1 and GAPDH antibodies (1:1000; Abcam, USA) and incubated overnight. It was then incubated with specific secondary antibodies (1:2000; Abcam, USA). Protein expression levels were then measured by ECL (ECL, Pierce).

Table 1
Relationship between miR-539 expression and their clinicopathological characteristics of Wilms' tumor patients

Characteristics	Cases	miR-539		P-value
		High	Low	
Age (months)				0.324
≥ 24	20	9	11	
< 24	22	8	14	
Gender				0.206
Male	18	8	10	
Female	24	9	15	
NWTS-5 stage				0.041*
I + II	30	11	19	
III + IV	12	4	8	
Histological type				0.0201*
Favorable (FH)	32	12	20	
Unfavorable (UH)	10	4	6	
Lymph node metastasis				0.049*
No	28	11	17	
Yes	14	6	8	

Statistical analyses were performed by the χ^2 test. * $P < 0.05$ was considered significant.

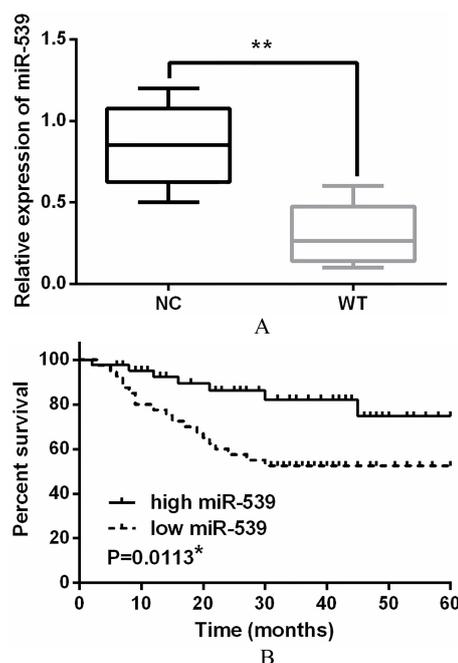


Fig. 1. Downregulation of miR-539 was identified in WT tissues. (A) The expressions of miR-539 in WT tissues detected via qRT-PCR. (B) Low miR-539 expression was correlated with shorter overall survival of WT patients. * $P < 0.05$, ** $P < 0.01$.

2.9. Statistical analysis

Data were shown as mean \pm SD. The differences between the groups were calculated by Chi-squared test or Tukey's one-way ANOVA using SPSS 19.0 and Graphpad Prism 6. Survival curves were plotted by

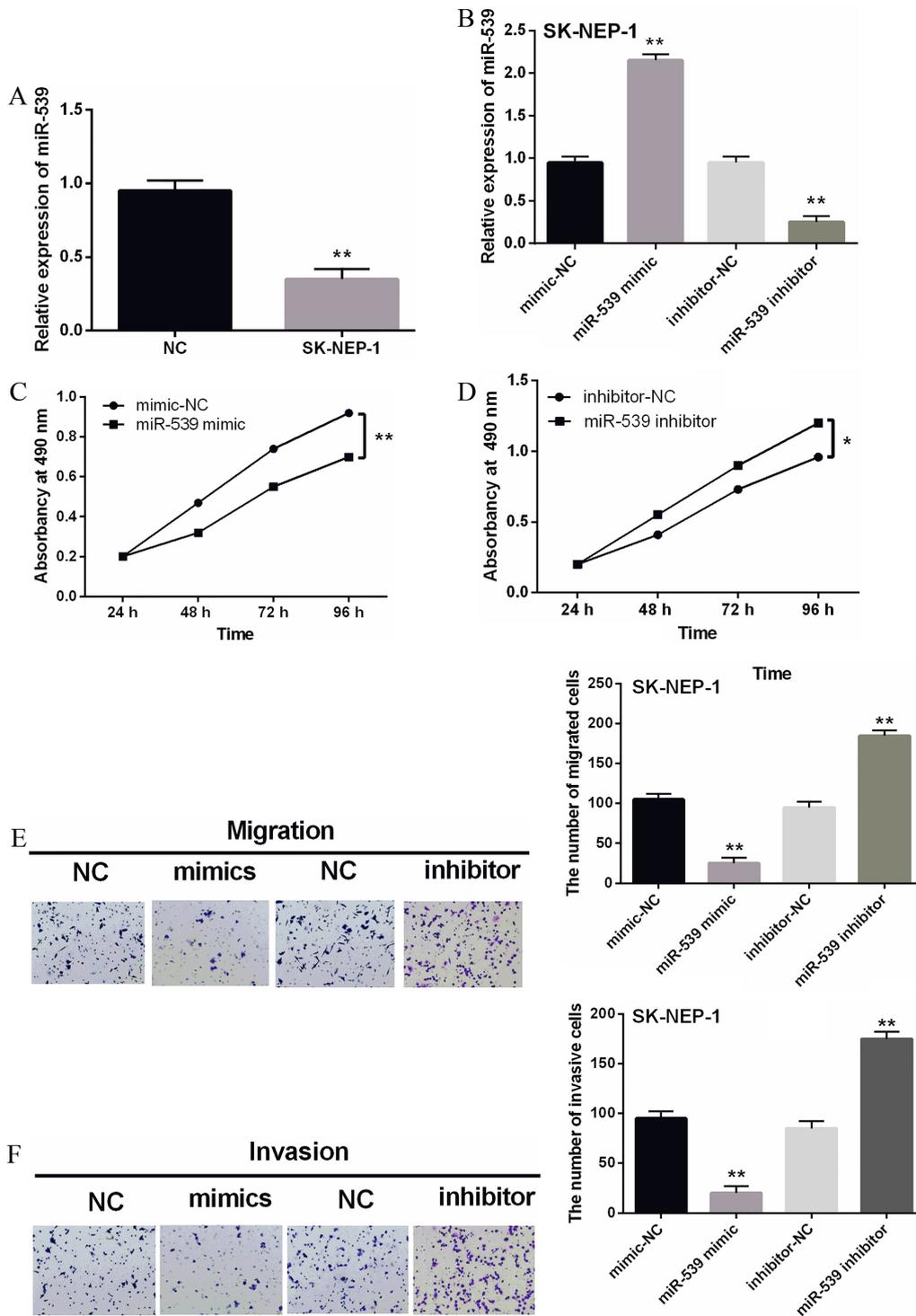


Fig. 2. Overexpression of miR-539 suppressed the proliferation, migration and invasion of WT cells. (A) The miR-539 expression in SK-NEP-1 cell lines. (B) The miR-539 expressions were examined in SK-NEP-1 cells containing miR-539 mimics or inhibitor via qRT-PCR. (C, D) The cell proliferation was measured in cells containing miR-539 mimics or inhibitor via MTT assay. (E, F) Cell migration and invasion analysis in SK-NEP-1 cells containing miR-539 mimics or inhibitor was examined by Transwell assay. * $P < 0.05$, ** $P < 0.01$.

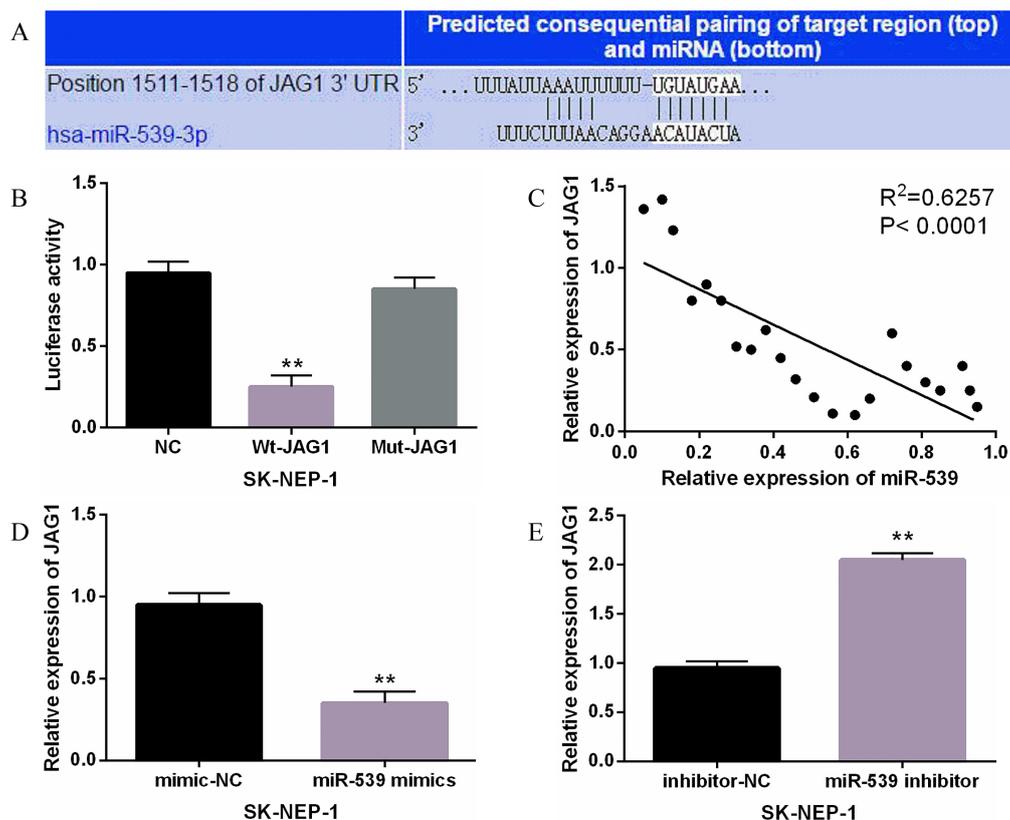


Fig. 3. JAG1 was a direct target of miR-539. (A) JAG1 had binding sites with miR-539. (B) Luciferase reporter assay (C) MiR-539 had negative correlation with JAG1. (D, E) The expression of JAG1 were observed in SK-NEP-1 cells containing miR-539 mimics or inhibitor $**P < 0.01$.

Kaplan-Meier analysis and survival differences were compared using log-rank test. And a significant difference was defined at $P < 0.05$.

3. Results

3.1. Downregulation of miR-539 was identified in WT tissues

The expression of miR-539 was detected in WT tissues by qRT-PCR assay. The expression of miR-539 in WT tissues was approximately half of that in normal tissues, indicating that miR-539 was downregulated in WT tissues (Fig. 1A). Moreover, the aberrant expression of miR-539 was closely associated with NWT5-5 stage ($P = 0.041$), lymph node metastasis ($P = 0.049$) and histological type ($P = 0.0201$, Table 1). In addition, low miR-539 expression was associated with shorter overall survival in WT patients ($P = 0.0113$, Fig. 1B), which predicted a worse prognosis. These results suggest that miR-539 is involved in tumorigenesis and prognosis of WT.

3.2. Overexpression of miR-539 suppressed the proliferation, migration and invasion of WT cells

The expression of miR-539 was observed in the SK-NEP-1 cell line. Downregulation of miR-539 was also identified in SK-NEP-1 cells (Fig. 2A). The miR-539 mimics or inhibitor was then transfected into SK-NEP-1 cells to investigate its effect in WT. As shown in Fig. 2B, the expression of miR-539 was enhanced by its mimics and reduced by miR-539 inhibitor. Next, the function of miR-539 was investigated by MTT and Transwell assays. Overexpression of miR-539 was found to suppress proliferation of SK-NEP-1 cells (Fig. 2C). In contrast, silencing of miR-539 promoted cell proliferation in WT (Fig. 2D). Furthermore, up-regulation of miR-539 inhibited migration of SK-NEP-1 cells, while knockdown of miR-539 promoted migration of SK-NEP-1 cells (Fig. 2E). The same results were also identified for cell invasion in SK-NEP-1 cells with miR-539 mimics or inhibitor (Fig. 2F). In conclusion, miR-539 plays an inhibitory role in WT.

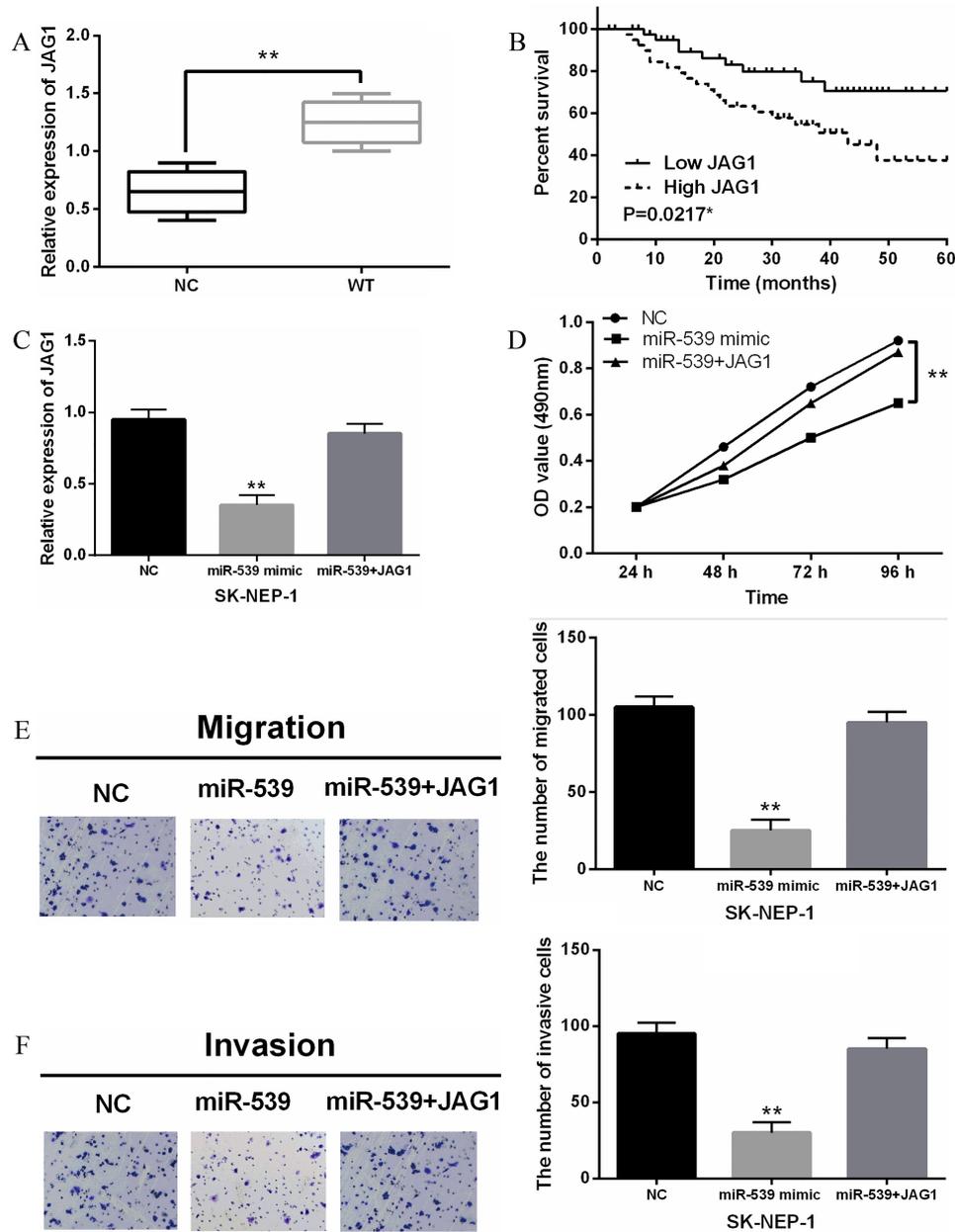


Fig. 4. MiR-539 inhibited the development of WT through targeting JAG1. (A) The expression of JAG1 in WT tissues was detected. (B) High JAG1 expression was correlated with shorter overall survival of WT patients. (C) The JAG1 expression was measured in SK-NEP-1 cells with JAG1 vector and miR-539. (D) The cell proliferation was measured in SK-NEP-1 cells with JAG1 vector and miR-539 via MTT. (E, F) The cell migration and invasion in SK-NEP-1 cells with JAG1 vector and miR-539 was measured by Transwell assay. ** $P < 0.01$.

3.3. JAG1 was a direct target of miR-539

Further, JAG1 was found to have a binding site for miR-539, which was predicted by TargetScan (<http://www.targetscan.org/>) (Fig. 3A). It suggests that miR-539 may target JAG1. To confirm this prediction, luciferase reporter assay was performed. We found

that miR-539 mimics inhibited the luciferase activity of Wt-JAG1. However, the luciferase activity of Mut-JAG1 was not affected by miR-539 mimics (Fig. 3B). Moreover, a negative correlation between miR-539 and JAG1 was identified in WT tissues ($P < 0.0001$, $R^2 = 0.6257$; Fig. 3C). Then, the expression level of JAG1 was measured in SK-NEP-1 cells with miR-539 mim-

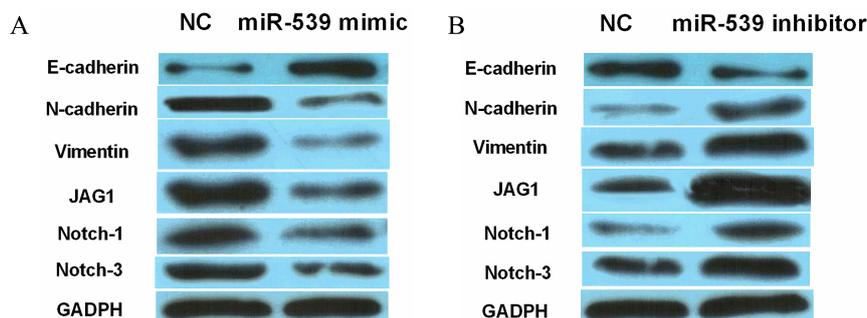


Fig. 5. MiR-539 inhibited EMT and Notch1/3 expression in WT. (A, B) The protein expression of E-cadherin, N-cadherin, Vimentin, Notch-1 and Notch-3 in SK-NEP-1 cells contained miR-539 mimics or inhibitor.

ics or inhibitor. The results suggested that the expression of JAG1 was reduced by upregulation of miR-539 (Fig. 3D). In contrast, the expression of JAG1 was enhanced by down-regulating miR-539 in SK-NEP-1 cells (Fig. 3E). These results show that miR-539 directly targets JAG1 and negatively regulates JAG1 expression in WT.

3.4. *MiR-539 inhibited the development of WT through targeting JAG1*

To explore the role of JAG1, we first examined the abnormal expression of JAG1 in WT tissues. The expression of JAG1 was significantly increased in WT tissues compared with normal tissues (Fig. 4A). Moreover, we also found that upregulation of JAG1 predicted worse prognosis in patients with WT ($P = 0.0217$, Fig. 4B). The miR-539 mimics and JAG1 vector were then co-transfected into SK-NEP-1 cells to further confirm their interaction. The decreased expression of JAG1 induced by miR-539 mimics was recovered by the JAG1 vector in SK-NEP-1 cells (Fig. 4C). Furthermore, the inhibitory effect of miR-539 on cell proliferation was attenuated by upregulation of JAG1 in SK-NEP-1 cells (Fig. 4D). The same results for cell migration (Fig. 4E) and invasion (Fig. 4F) were also identified in WT. Taken together, miR-539 inhibited proliferation, migration and invasion of WT cells through targeting JAG1.

3.5. *MiR-539 inhibited EMT and suppressed Notch1/3 expression in WT*

Finally, the effect of miR-539 on EMT and Notch1/3 expression was explored to further explain the regulatory mechanism of miR-539 in WT. The results showed that upregulation of miR-539 promoted E-cadherin expression and inhibited N-cadherin and Vi-

vimentin expressions in SK-NEP-1 cells, indicating that overexpression of miR-539 inhibited EMT in WT (Fig. 5A). Oppositely, knockdown of miR-539 was found to promote EMT of WT (Fig. 5B). Besides that, JAG1 is a ligand of Notch pathway, which includes other receptors Notch-1, Notch-2 and Notch-3. And we investigated whether the Notch pathway is involved in the development of WT. Western blot analysis showed that the expressions of Notch-1 and Notch-3 were reduced by the miR-539 mimics (Fig. 5A), while the miR-539 inhibitor increased their expressions in SK-NEP-1 cells (Fig. 5B). Based on these results, miR-539 inhibited EMT and suppressed Notch1/3 expression in WT, suggesting that the Notch pathway may be involved in the development of WT.

4. Discussion

As a malignant renal tumor, WT often occurs in children [3]. Previous studies have shown that the progression and development of WT should be mediated by several miRNAs. Liu et al. proposed that miR-19b promoted proliferation and migration of WT cells via the PTEN/PI3K/AKT signaling pathway [17]. Inversely, miR-613 attenuated proliferation, migration and invasion of WT cells by targeting FRS2 [30]. In the present study, it was also found that overexpression of miR-539 inhibited proliferation, migration and invasion of WT cells. Furthermore, expression of miR-539 was reduced in WT tissues and abnormal miR-539 expression was associated with NWTS-5 stage, lymph node metastasis and histological type. In addition, downregulation of miR-539 was related to poor prognosis in WT patients. All of these findings demonstrate that miR-539 acts as a suppressor gene in WT.

It is well known that miR-539 exhibits an inhibitory effect in different human cancers by modulating some

target genes. For example, miR-539 suppressed osteosarcoma cell invasion and migration by targeting MMP-8 [16], which was similar to our results in WT. Additionally, miR-539 was found to be downregulated in breast cancer and used as a tumor suppressor [14]. Moreover, miR-539 suppressed cell proliferation and induced cell apoptosis in renal cell carcinoma by targeting HMGA2 [32]. These previous studies strongly supported our conclusions in this study. Further, JAG1 was shown to be a direct target of miR-539 and a negative correlation between miR-539 and JAG1 was identified in WT tissues. In particular, miR-539 inhibited the development of WT through targeting JAG1.

As shown in previous studies, miRNAs inhibit the expressions of many target genes by binding to their 3'-UTR. In this study, identifying downstream target genes of miR-539 is an important task for us. JAG1 has been identified as a target gene in some cancers. For instance, miR-186 inhibited cell proliferation in multiple myeloma by inhibiting JAG1 [19], which was consistent with our results. Besides that, miR-539 inhibited EMT and suppressed the expressions of Notch1/3 in WT. As a cell surface ligand, JAG1 plays an important role in the Notch signaling pathway. It has also been reported that JAG1 activates the Notch signaling pathway through interacting with the Notch receptors, such as Notch1, Notch2 and Notch3 [18]. Moreover, the specific function of JAG1/Notch pathway has been found in cervical carcinoma, choriocarcinoma [24] and colorectal cancer [8]. In addition, the carcinogenic effects of Notch2 promoted the metastasis of bladder cancer through EMT [15]. Chen et al. also demonstrated that miR-598 inhibited metastasis in colorectal cancer by suppressing the JAG1/Notch pathway that stimulated EMT [4]. Consistent with previous studies, we found that miR-539 inhibited proliferation and metastasis of WT cells by suppressing JAG1-Notch1/3 expression.

5. Conclusion

In summary, miR-539 was downregulated in WT tissues. And miR-539 inhibited the progression of WT through negatively regulating JAG1-Notch1/3 expression. MiR-539 should be a valuable indicator of the diagnosis and prognosis of WT.

Conflict of interest

The authors declare that they have no competing interests.

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