European Review for Medical and Pharmacological Sciences 2020; 24: 5329-5335

MiRNA-203a-5p alleviates the malignant progression of Wilms' tumor *via* targeting JAG1

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Abstract. – OBJECTIVE: To uncover the potential influence of microRNA-203a-5p (miRNA-203a-5p) on the malignant progression of Wilms' tumor (WT).

PATIENTS AND METHODS: MiRNA-203a-5p levels in 49 paired WT and paracancerous tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Prognostic value of miRNA-203a-5p in WT was assessed by the Kaplan-Meier method. Correlation between miRNA-203a-5p level and clinical data of WT patients was analyzed. In G401 and SK-NEP-1 cells, *in vitro* functions of miRNA-203a-5p in regulating metastatic abilities were explored. The interaction between miRNA-203a-5p and JAG1, and their regulatory role in the malignant progression of WT were evaluated by Dual-Luciferase reporter gene assay and rescue experiments.

RESULTS: MiRNA-203a-5p was downregulated in WT tissues than that of paracancerous ones. WT patients expressing low level of miRNA-203a-5p had higher risk of lymphatic metastasis and worse prognosis. Overexpression of miRNA-203a-5p attenuated migratory and invasive abilities in G401 cells. On the contrary, knockdown of miRNA-203a-5p yielded the opposite trends in SK-NEP-1 cells. JAG1 was verified to be the direct gene binding miRNA-203a-5p, which was negatively regulated by miRNA-203a-5p in WT cells. Rescue experiments finally uncovered that miRNA-203a-5p alleviated the malignant progression of WT via negatively regulating JAG1.

CONCLUSIONS: MiRNA-203a-5p is downregulated in WT and closely linked to lymphatic metastasis of WT patients. By negatively regulating JAG1, miRNA-203a-5p alleviates the malignant progression of WT.

Key Words:
MiRNA-203a-5p, JAG1, Wilms' tumor, Malignant progression.

Introduction

Wilms' tumor (WT) is the most common malignant tumor of the pediatric urinary system. It originates from renal embryonic cells. WT mainly affects children younger than 15 years, with the incidence of 1/10000[1-4]. Relevant multi-center trials uncovered that in kidney neoplasm patients younger than 7 months old, about 66% cases are WT, and the remaining 34% non-WT cases include congenital mesoblastic nephroma (18%), malignant rhabdoid rumor (8%), clear cell sarcoma of kidney (2%), and tumors of unknown histological type (6%)[3,4]. The average onset age of WT is 38 months, which is 6 months older in female patients than in male patients[3,5]. Morphologically, WT is similar to that of embryonic kidney with structural disorders. It is currently believed that WT originates from abnormal remnants of metanephric embryonic mass due to stagnation of differentiation[6,7]. Due to insidious onset and rapid progression, WT severely threats children's health and lives[8,9]. Clinical treatment on massive, bilateral, and recurrent WT is difficult[10]. It is urgent to develop therapeutic targets for improving the prognosis of WT[11,12].

MicroRNAs (miRNAs) are non-coding RNAs containing about 22 nucleotides[13,14]. MiRNAs are widely involved in life activities, which have been well concerned due to their functions in regulating cell phenotypes[15,16]. The complex miRNA network is responsible for regulating cell proliferation and apoptosis. Hence, miRNAs could serve as oncogenes or tumor-suppressor genes participating in tumor progression[16,17]. Due to genetic defects, epigenetic silencing, or transcriptional repression of MYC genes,

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multiple miRNAs are downregulated in tumor diseases[18]. MiRNA-203a-5p locates on tumor-related fragile sites in chromosome 14, presenting the precursor of stem-loop structure. Similar to other miRNAs, miRNA-203a-5p lacks the open reading frame and could not encode proteins[19,20]. By targeting 3'UTR mRNA at the post-transcriptional level, miRNAs degrade or inhibit translation of target mRNAs, thus exerting crucial functions in epigenetics, stem cell regulation, embryonic development, and tumorigenesis[21,22]. In this paper, we predicted that JAG1 was the target mRNA of miRNA-203a-5p. Regulatory effects of miRNA-203a-5p/JAG1 axis on the malignant progression of WT were further clarified.

Patients and Methods

Patients and Samples

49 paired WT tissues and adjacent normal ones were collected and stored at –80°C. Clinical data and follow-up data of enrolled patients were recorded. Patients and their families in this study have been fully informed. This investigation was approved by the Ethics Committee of Shanghai Ninth People's Hospital. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture

Proximal tubular epithelial cells of the kidney cortex (HK-2) and WT cells (G401, WT-CLS1, SK-NEP-1) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted in 1×trypsin+EDTA (ethylenediaminetetraacetic acid) at 80-90% confluence.

Transfection

Cells were inoculated in a 6-well plate and cultured to 40% confluence. Cells were transfected with corresponding plasmids (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments.

Transwell Cell Migration and Invasion Assays

Cells were inoculated in a 24-well plate with 5.0×10^5/mL. 200 μL of suspension was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, cells invaded to the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. Invasive cell number was counted in 5 randomly selected fields per sample (magnification 20×). Migration assay was similarly conducted except for pre-coating of Matrigel in the bottom of transwell chambers.

Wound Healing Assay

Cells were prepared to suspension with 5.0×10^5/mL. 5.0×10^4 cells suspended in culture medium containing 1% FBS were inoculated per well and an artificial wound was created. 24 h later, the percentage of wound closure was calculated.

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

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was and U6 were used as internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. JAG1 Forward: 5'-ATCGTGCTGCCTTTCAGTTT-3'; Reverse: 5'-GGTCACGCGGATCTGATACT-3; GAPDH Forward: 5'-GCAGGGGGGAGCCAAAAGGGT-3'; Reverse: 5'-TGGGTGGCAGTGATGGCATGG-3'; MiRNA-203a-5p Forward: 5'-GUGAAAUGUUUAGGACCACUAG-3'; Reverse: 5'-AGUGGUCCUAAACAUUUCACUU-3; U6 Forward: 5'-CTCGCTTCGGCAGCACA-3'; Reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Western Blot

Cells were centrifuged at 14000×g, 4°C for 15 min. The obtained cellular protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked in

5% skim milk for 1 h. The specific primary antibody was used to incubate with the membrane overnight at 4°C, followed by secondary antibody incubation for 2 h at room temperature. After Tris Buffered Saline and Tween-20 (TBST) washing for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Dual-Luciferase Reporter Gene Assay

HEK293 cells were inoculated in 24-well plates. They were co-transfected with WT-JAG1/MUT-JAG1 and miRNA-203a-5p mimics/NC, respectively. 48 h later, cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Kaplan-Meier method was introduced for survival analysis. Spearman correlation test was performed to assess the relationship between miRNA-203a-5p expression and clinical data of WT patients. $p<0.05$ was considered as statistically significant.

Results

MiRNA-203a-5p Was Lowly Expressed in WT Tissues and Cells

A total of 49 paired WT and adjacent tissues were collected. Compared with adjacent normal tissues, miRNA-203a-5p was downregulated in WT tissues (Figure 1A). It is speculated that miRNA-203a-5p may be a tumor-suppressor gene involved in the progression of WT. Consistently, miRNA-203a-5p was lowly expressed in WT cell lines (Figure 1B). By analyzing clinical data of enrolled WT patients, it is found that miRNA-203a-5p level was closely linked to lymphatic metastasis of WT patients, while it was unrelated to other parameters (Table I). Moreover, Kaplan-Meier curves revealed worse prognosis in WT patients expressing low level of miRNA-203a-5p (Figure 1C).

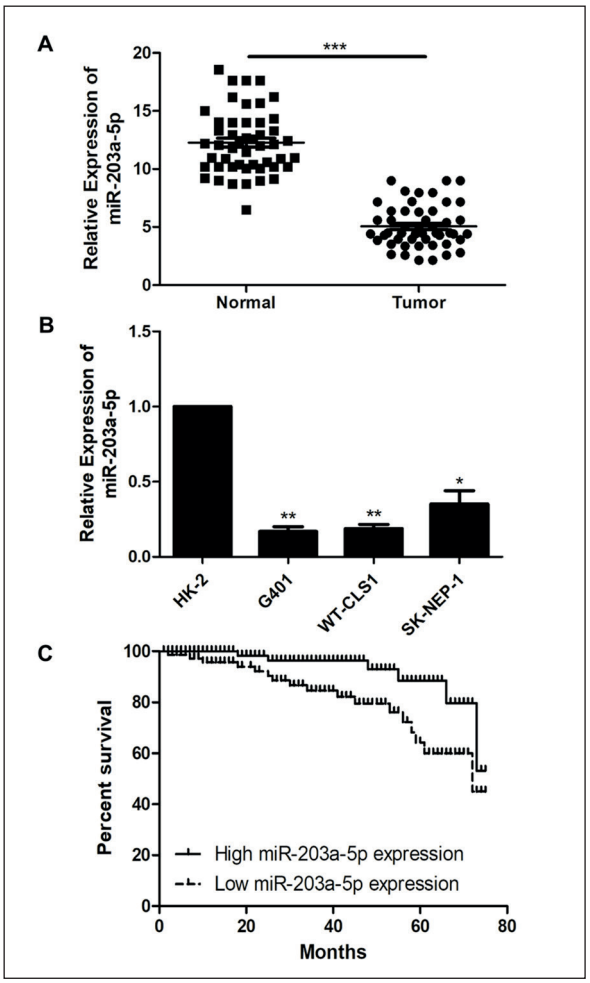

Figure 1. MiRNA-203a-5p was lowly expressed in WT tissues and cells. **A,** MiRNA-203a-5p levels in WT tissues and paired adjacent ones. **B,** MiRNA-203a-5p level in proximal tubular epithelial cells of the kidney cortex (HK-2) and WT cells (G401, WT-CLS1, SK-NEP-1). **C,** Overall survival in WT patients expressing high or low level of miRNA-203a-5p. Data were expressed as mean±SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

MiRNA-203a-5p Inhibited Metastasis in WT

To uncover potential biological functions of miRNA-203a-5p in WT cells, miRNA-203a-5p mimics and inhibitor were constructed. Their transfection efficacy was verified in G401 and SK-NEP-1 cells, respectively (Figure 2A). Functional experiments demonstrated that the percentage of wound closure, relative numbers of migratory and invasive cells was reduced in G401 cells overexpressing miRNA-203a-5p compared to those of controls. On the contrary, knockdown of miRNA-203a-5p yielded the opposite trends (Figure 2B, 2C). The above data indicated the inhibitory effect of miRNA-203a-5p on metastatic ability in WT.

Interaction Between MiRNA-203a-5p and JAG1

Transfection of miRNA-203a-5p mimics markedly downregulated protein and mRNA levels

Table I. Association of miR-203a-5p expression with clinicopathologic characteristics of Wilms' tumor.

Parameters	No. of cases	MiR-203a-5p expression		*p*-value
		Low (%)	High (%)	
Age (months)				0.218
< 24	31	14	17	
≥ 24	18	11	7	
Gender				0.484
Male	29	16	13	
Female	20	9	11	
T stage				0.666
T1-T2	24	13	11	
T3-T4	25	12	13	
Lymphatic metastasis				0.044
No	34	23	21	
Yes	15	12	3	

of JAG1 in G401 cells. Conversely, transfection of miRNA-203a-5p inhibitor upregulated protein and mRNA levels of JAG1 in SK-NEP-1 cells (Figure 3A, 3B). Besides, overexpression of JAG1 downregulated miRNA-203a-5p and knockdown of JAG1 obtained the opposite result (Figure 3C). JAG1 was found to be upregulated in WT tissues and cell lines (Figure 3D, 3E). In addition, Kaplan-Meier method demonstrated the prognostic value of JAG1 in WT, that was, high level of JAG1 predicted a poor prognosis in WT patients (Figure 3F). JAG1 was predicted to be the target binding miRNA-203a-5p through bioinformatics method (Figure 3G). Subsequently, decreased Luciferase activity after co-transfection of WT-JAG1 and miRNA-203a-5p mimics showed the binding relationship between miRNA-203a-5p and JAG1 (Figure 3H). A negative correlation was further identified between expression levels of miRNA-203a-5p and JAG1 in WT tissues (Figure 3I).

MiRNA-203a-5p/JAG1 Axis Regulated the Progression of WT

Based on the above findings, we thereafter speculated that JAG1 was involved in the progression of WT regulated by miRNA-203a-5p.

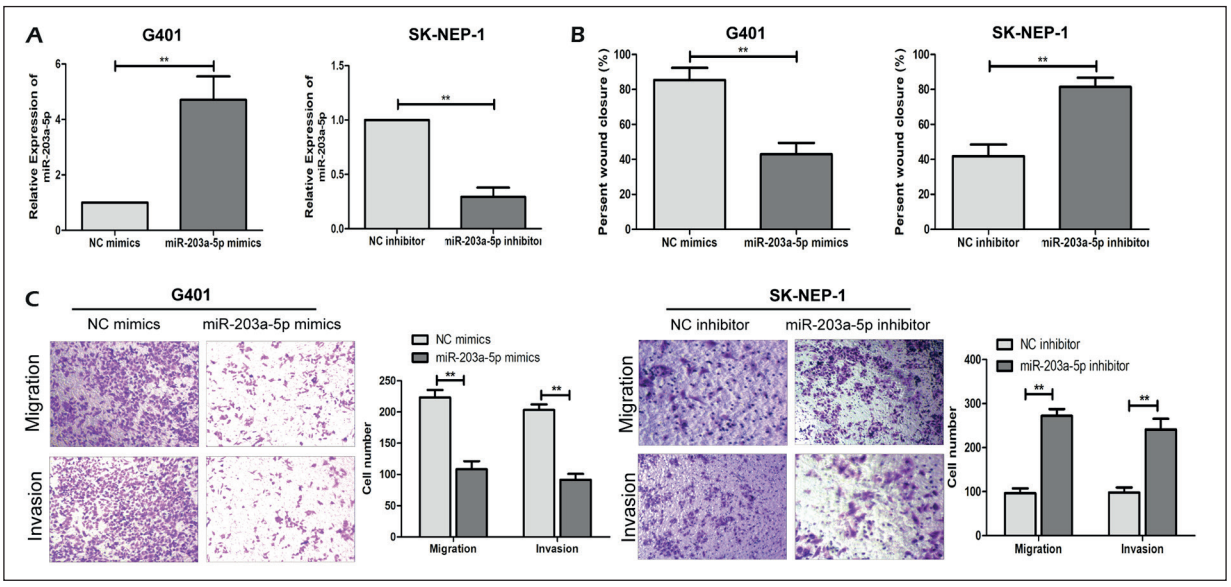

Figure 2. MiRNA-203a-5p inhibited metastasis in WT. G401 cells were transfected with NC mimics or miRNA-203a-5p mimics. SK-NEP-1 cells were transfected with NC inhibitor or miRNA-203a-5p inhibitor. **A,** MiRNA-203a-5p level in G401 and SK-NEP-1 cells; **B,** Percentage of wound closure; **C,** Migration and invasion cell numbers (magnification 20×). Data were expressed as mean±SD. *$p<0.05$.

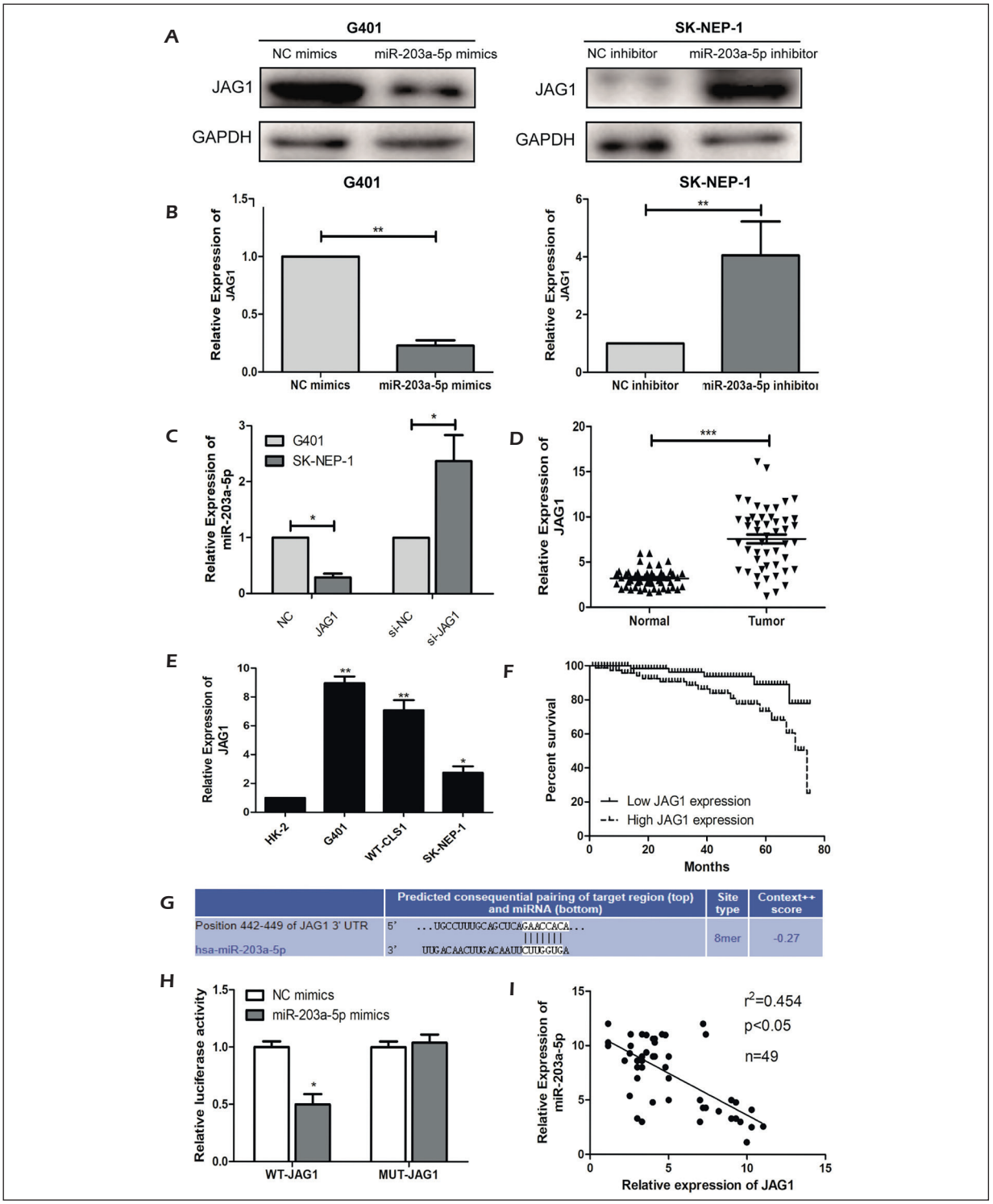

Figure 3. Interaction between miRNA-203a-5p and JAG1. **A, B,** Protein (**A**) and mRNA level (**B**) of JAG1 in G401 cells transfected with NC mimics or miRNA-203a-5p mimics, and SK-NEP-1 cells transfected with NC inhibitor or miRNA-203a-5p inhibitor. **C,** MRNA level of miRNA-203a-5p in G401 cells transfected with NC or pcDNA-JAG1, and SK-NEP-1 cells transfected with si-NC or si-JAG1. **D,** JAG1 levels in WT tissues and paired adjacent ones. **E,** JAG1 level in proximal tubular epithelial cells of the kidney cortex (HK-2) and WT cells (G401, WT-CLS1, SK-NEP-1). **F,** Overall survival in WT patients expressing high or low level of JAG1. **G,** Binding sequences between miRNA-203a-5p and JAG1. **H,** Luciferase activity in HEK293 cells co-transfected with WT-JAG1/MUT-JAG1 and miRNA-203a-5p mimics/NC. **I,** A negative correlation between expression levels of miRNA-203a-5p and JAG1 in WT tissues. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

First, overexpression of JAG1 reversed the downregulated protein and mRNA levels of JAG1 in G401 cells overexpressing miRNA-203a-5p. Knockdown of JAG1 downregulated JAG1 level in SK-NEP-1 cells transfected with miRNA-203a-5p inhibitor (Figure 4A). Of note, the attenuated migratory and invasive abilities in G401 cells overexpressing miRNA-203a-5p were partially reversed by overexpression of JAG1. Promotive effects of downregulated miRNA-203a-5p on metastasis of SK-NEP-1 cells were abolished by knockdown of JAG1 (Figure 4B, 4C). As a result, JAG1 was responsible for miRNA-203a-5p-regulated progression of WT.

Discussion

WT is the most common urinary system tumor in children, accounting for 8% of pediatric solid tumors and 80% of pediatric urinary tumors[1-3]. WT originates from pluripotent kidney cell precursors, producing undifferentiated embryonic cells, primitive epithelium and interstitial tissues[4,5]. WT is a disease of subacute limited surgery. After active treatment of surgery combined chemotherapy and radiotherapy, postoperative survival of WT is up to 80-90%. However, about 20% of WT patients experience tumor recurrence[6-9]. Thus, it is necessary to uncover the molecular mechanism of WT for improving diagnostic and therapeutic efficacies[10-12].

MiRNAs are 19-22 nt long non-coding RNAs[13]. They exert a post-transcription regulation on target mRNAs[14-16]. Through complementary base pairing, miRNAs affect downstream gene expressions by binding seed sequences with 7-8 nt[21,22]. Functionally, oncogenic miRNAs stimulate tumorigenesis and tumor progression *via* targeting tumor-suppressor genes, apoptosis-associated genes, and differentiation-associated genes, while tumor-suppressor genes antagonize the carcinogenic effects of oncogenes[17,18]. In particular, miRNAs are tissue-specific[18]. It is reported that miRNA-203a-5p level is closely linked to poor prognosis of cervical cancer[19]. In this paper, miRNA-203a-5p was downregulated in WT tissues and closely related to prognosis of WT patients. It is suggested that miRNA-203a-5p may be a tumor-suppressor gene in the progression of WT. Subsequently, wound healing assay and transwell assay showed the inhibitory effects of miRNA-203a-5p on metastatic abilities in WT cells.

A single miRNA could target multiple mRNAs and a single mRNA could be the target of multiple miRNAs. Thus, the complicated network remarkably affects tumor progression[21,22]. Clarifying differentially expressed miRNAs in WT is the research foundation on underlying the pathogenesis of WT[16-18]. Our findings verified that JAG1 was the direct target binding miRNA-203a-5p. In WT cells, JAG1 was negatively regulated

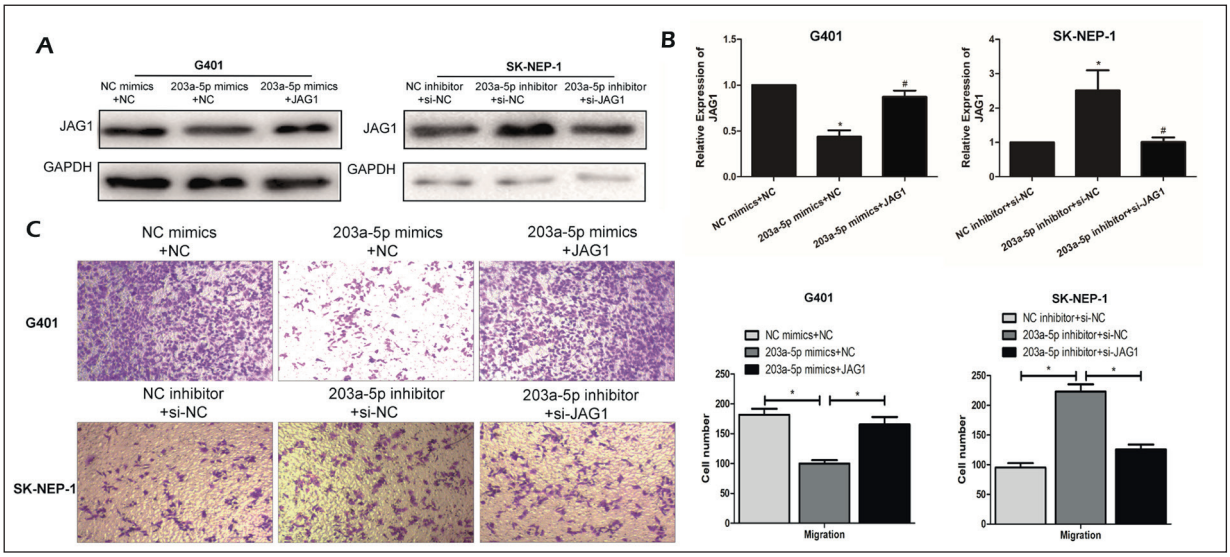

Figure 4. MiRNA-203a-5p/JAG1 axis regulated the progression of WT. G401 cells were transfected with NC mimics+NC, miRNA-203a-5p mimics+NC, or miRNA-203a-5p mimics+pcDNA-JAG1. SK-NEP-1 cells were transfected with NC inhibitor+NC, miRNA-203a-5p inhibitor+si-NC or miRNA-203a-5p inhibitor+si-JAG1. **A, B,** Protein (**A**) and mRNA level (**B**) of JAG1; ***C,*** Migration and invasion cell numbers (magnification 20×). Data were expressed as mean±SD. *$p<0.05$.

by miRNA-203a-5p. JAG1 was highly expressed in WT tissues, and predicted a poor prognosis in WT patients. Notably, JAG1 was responsible for the inhibitory effect of miRNA-203a-5p on regulating the malignant progression of WT. Collectively, our results revealed that miRNA-203a-5p inhibited the progression of WT *via* negatively regulating JAG1.

Conclusions

The above findings demonstrated that miRNA-203a-5p is downregulated in WT and closely linked to lymphatic metastasis of WT patients. By negatively regulating JAG1, miRNA-203a-5p alleviates the malignant progression of WT.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

Scientific Research Project of Shanghai Municipal Health and Family Planning Commission (20184Y0295).

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