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LncRNA CRNDE promotes the development of Wilms' tumor by regulating microRNA-424

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression characteristics of lncRNA CRNDE in Wilms' tumor and to further investigate whether it could promote the development of Wilms' tumor via regulating microRNA-424.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed to examine the expression level of CRNDE in tumor tissues and para-cancerous tissues of patients with Wilms' tumor. Meanwhile, the expression of CRNDE in Wilms' tumor cell lines was analyzed as well. CRNDE overexpression and knockdown models were constructed using lentivirus transfection in HFWT and 17-94 cell lines, respectively. Subsequently, cell counting kit-8 (CCK-8), cell colony formation, and transwell assays were performed to explore the influence of CRNDE on the biological functions of Wilms' tumor cells. Furthermore, luciferase reporter gene assay and cell reversal experiment were applied to explore the interplay between CRNDE and microRNA-424.

RESULTS: RT-qPCR results revealed that the expression level of lncRNA CRNDE in tumor tissues of patients with Wilms' tumor was remarkably higher than that of adjacent normal tissues. Also, the difference was statistically significant ($p<0.05$). Compared with patients with low expression of CRNDE, the risk of lymph node metastasis was significantly higher in patients with high CRNDE expression ($p<0.05$). Similarly, compared with control group, the proliferation and metastasis abilities of cells in CRNDE knockdown group were remarkably down-regulated ($p<0.05$). However, opposite results were observed in CRNDE overexpression group. In addition, our results demonstrated that microRNA-424 expression was negatively correlated with CRNDE expression in Wilms' tumor tissues. Luciferase reporter gene assay indicated that CRNDE could be targeted by microRNA-424 through specific a binding site, further regulating the malignant progression of Wilms' tumor.

CONCLUSIONS: CRNDE was highly expressed in Wilms' tumor tissue and cell lines. The expression of CRNDE was correlated with the incidence rate of lymph node metastasis in patients with Wilms' tumor. In addition, CRNDE might accelerate the progression of Wilms' tumor *via* modulating microRNA-424.

Key Words:
CRNDE, MicroRNA-424, Wilms' tumor, Development.

Introduction

Wilms' tumor (WT) is the most common malignant tumor in the pediatric urinary system, which is originated from the renal blastocyst cells. It usually occurs in children under 15 years old and its incidence is about 1/10000[1-3]. Multicenter cooperative studies have suggested that about 66% of infants less than 7 months old with renal tumors are WT. The remaining 34% of renal tumors are non-WT, including congenital mesoderm 1 renal cell carcinoma (18%), malignant rhabdoid tumor (8%), renal clear cell sarcoma (2%), and undefined histological types of tumors (6%)[1-4]. The average age of the onset of WT in children is 38 months. Meanwhile, the average age of the onset in girls is 6 months, later than that of boys[4]. The pathogenesis of WT has not been fully elucidated. This tumor has been found similar to the embryonal kidney with a disordered structure in morphology. Currently, it is believed that abnormal residues caused by the stagnation of differentiation in the metanephric embryonal block may be one of the causes of WT[5,6]. As the onset site of Wilms' tumor is hidden, its progression is often rapid, with no specific tumor markers. This makes it seriously threatening to the lives and health of children worldwide[7,8]. In recent years, a comprehensive multidisciplinary treatment has remarkably improved the overall survival rate of WT. However, there are still great difficulties in the treatment of adverse histological type (UFH) tumors, large unresectable tumors diagnosed for the first time, bilateral tumors, and recurrent tumors[9,10]. Therefore, searching for new anticancer targets to improve the clinical efficacy and prognosis of WT has become an important clinical issue that needs to be solved urgently[11].

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According to different lengths, ncRNAs can be divided into two categories, including short non-coding RNAs (sncRNAs) and long non-coding RNAs (lncRNAs)[12]. At the early stage, when the important functions of ncRNAs were discovered, more reports focused on sncRNAs. Several works[13,14] have confirmed that sncRNAs play pivotal roles in regulating the expression of protein-coding genes. Current studies have found that lncRNAs have higher intracellular transcription than miRNAs. They play a pivotal role in regulating the expression of protein-coding genes and other non-coding genes. Meanwhile, lncRNAs have extremely complex biological functions[15,16]. Although these lncRNAs have different sources, previous researches have shown that they can regulate gene expression at multiple levels. To sum up, they may achieve similar functions in three main aspects, including transcriptional regulation, post-transcriptional regulation, and epigenetic regulation. In addition, lncRNA is involved in various regulatory functions, such as genomic imprinting, chromatin modification, transcription activation, and regulation of proto-carcinogenic gene activity[17,18]. At present, there are few reports on the role of lncRNA CRNDE in the development of tumors[19,20]. Therefore, the aim of this work was to comprehensively analyze the expression and biological effects of lncRNA CRNDE in Wilms' tumor. Furthermore, we preliminarily discussed the molecular mechanism of its tumor regulatory effect.

Recent studies have revealed that lncRNAs can also be used as competitive endogenous RNAs (ceRNAs) or miRNA sponges. By binding with miRNAs competitively through its miRNA response elements (MREs) and inhibiting the function and activity of miRNAs, lncRNAs can regulate the expression of target mRNAs at post-transcription level[21,22]. In recent years, microRNA-424 has been widely explored in various tumors. However, no research has investigated its effect on Wilms' tumor[23]. Therefore, in this report, we researched whether lncRNA CRNDE could mediate the occurrence and development of Wilms' tumor by regulating microRNA-424. Our finding might hope to provide an experimental basis for its clinical application.

Patients and Methods

Patients and Colon Cancer Samples

89 pairs of Wilms' tumor tissues and corresponding adjacent tissues were selected from Wilms' tumor patients who received surgery in our hospital. Collected tissue samples were stored at –80°C for use. This investigation was approved by the Ethics Monitoring Committee. Informed consent was obtained from the legal guardians of the all patients before the study.

Cell Lines and Reagents

Three human WT cell lines (HFWT, WT-CLS1, and 17-94) and one human renal tubular epithelial cell line (HK-2) were purchased from American type culture collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) medium and fetal bovine serum (FBS) were obtained from Life Technologies (Gaithersburg, MD, USA). All cells were cultured in DMEM high glucose medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were maintained in a 37°C incubator with 5% CO_2. When they grew to 80-90% of confluence, the cells were passaged with 1% trypsin + ethylenediaminetetraacetic acid (EDTA) for digestion.

Cell Transfection

Negative control group (NC or sh-NC) and lentivirus (CRNDE or sh-CRNDE) containing CRNDE overexpression and knockdown sequences were purchased from Shanghai Jima Company (Shanghai, China). Cells were first plated into 6-well plates and grew to a cell density of 40%. Subsequently, cell transfection was performed according to the manufacturer's instructions. After 48 h, transfected cells were collected for RT-qPCR analysis and cell function experiments.

Cell Proliferation Assays

After 48 h of transfection, the cells were harvested and plated into 96-well plates at a density of 2000 cells per well. After culture for 24, 48, 72, and 96 h, respectively, the cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well. After incubation for 2 h in dark, optical density (OD) value at the absorption wavelength of 490 nm was measured by a micro-plate reader.

Colony Formation Assay

After 48 h of transfection, the cells were first collected. 200 cells were seeded into 6-well plates, followed by culture in a complete medium for 2 weeks. The medium was changed after one week, and then twice a week. The medium should not be replaced in the previous week to avoid cell adhesion. After 2 weeks, the cells were cloned. After wa-

shing twice with phosphate-buffered saline (PBS), the cells were fixed with 2 ml of methanol for 20 min and stained with 0.1% crystal violet staining solution for 20 min. Then, the cells were washed again with PBS for 3 times. Finally, the cells were photographed under a light-selective environment, and the number of formed colonies was counted.

Transwell Cell Migration and Invasion Assay

After transfection for 48 h, the cells were digested, centrifuged, and re-suspended in medium without fetal bovine serum (FBS). Cell density was adjusted to 5×10^5 cells/mL. 200 uL of cell suspension (1×10^5 cells) was added to the upper chamber. Meanwhile, 700 uL of medium containing 20% FBS was added to the lower chamber. According to different migration abilities, the cells were put back into the incubator and continued to culture for a specific time. Next, transwell chambers were taken out and washed 3 times with 1 × PBS. Then, the cells were fixed with methanol for 15 min and stained with 0.2% crystal violet for 20 min. Cells on the upper surface of chambers were carefully wiped off with water and cotton swabs. Migrating or invading cells stained in the outer layer of the basement membrane of chambers were observed under a microscope. 5 fields of view were randomly selected for each sample.

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells and the total RNA was extracted. Initially extracted RNA was treated with DNase I to remove genomic DNA and re-purify the RNA. Reverse transcription of RNA was performed according to the Prime Scirpt Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions. Real Time PCR was performed according to the instructions of SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) system. Three replicates were set for each sample and the assay was repeated twice. Bio-Rad PCR instrument (Hercules, CA, USA) was used to analyze and process the data with the iQ5 2.0 software. GAPDH and U6 were used as internal parameters. The expression level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: CRNDE, F: 5'-CAAGCGAAGCACTCACTTAACATC-3', R: 5'-CGTACCGATGCGTAGCAGAGA-3'; microRNA-424, F: 5'-GCATGTCAACATACCTACGAAG-3', R: 5'-GAATTGCATACGTGGAGTCCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual Luciferase Reporter Assay

A reporter plasmid was first constructed, in which specific fragments of the target promoter were inserted in front of the luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter plasmid in HFWT and 17-94 cells. A specific luciferase substrate was added and the luciferase reacted with the substrate to generate fluorescence. Luciferase activity was determined 48 h later using the Promega luciferase kit (Promega, Madison, WI, USA). Finally, the intensity of the fluorescence was calculated.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 (La Jolla, CA, USA) software. The *t*-test was used to analyze the measurement data. The Student's *t*-test was used to compare the difference between the two groups. One-way ANOVA was applied to compare the differences among different groups, followed by a post-hoc test (Least Significant Difference). Independent experiments were repeated for at least three times. Experimental data were expressed as mean ± standard deviation. There were three levels of $p<0.05$, $p<0.01$, and $p<0.001$ at the significance level, and $p<0.05$ was considered statistically significant.

Results

CRNDE Was Highly Expressed in Wilms' Tumor Tissues and Cell Lines

To determine the expression level of CRNDE in Wilms' tumor, 89 pairs of tumor tissues and para-cancerous tissues were collected. The expression of CRNDE was detected by RT-qPCR. The results showed that CRNDE expression was significantly elevated in tumor tissues when compared with para-cancerous tissues (Figure 1A). This suggested that CRNDE might play an oncogenic role in Wilms' tumor. In addition, CRNDE expression was remarkably higher in Wilms' tumor cells than HK-2 cells (Figure 1B). According to the expression level of CRNDE, patients were divided into high expres-

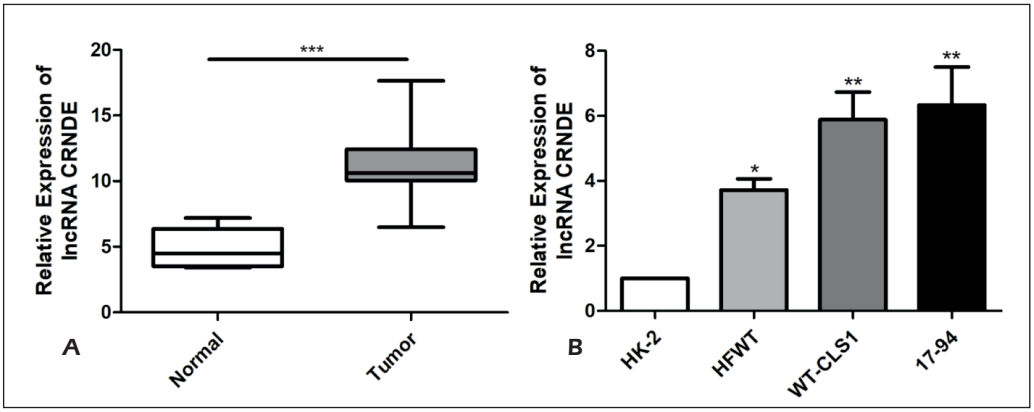

Figure 1. CRNDE was highly expressed in Wilms' tumor tissues and cell lines. **A**, RT-qPCR was used to detect the expression of CRNDE in tumor tissues and para-cancerous tissues; **B**, RT-qPCR was used to detect the expression level of CRNDE in osteosarcoma cell lines. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

sion group and low expression group. The relation between CRNDE expression and clinical indexes of patients with Wilms' tumor was analyzed. As shown in Table I, the high expression of CRNDE was closely correlated with age and lymph node metastasis in patients with Wilms' tumor.

Knockdown of CRNDE Inhibited Cell Proliferation and Metastasis

To investigate cellular biological changes of CRNDE in Wilms' tumor, the overexpression and knockdown of CRNDE lentiviral vectors were constructed, respectively. After the transfection of CRNDE in HFWT and 17-94 cell lines, RT-qPCR assay was performed to verify the interference efficiency. Results demonstrated that the difference was statistically significant (Figure 2A). CCK-8, colony formation assay, and transwell assay were used to detect cell proliferation and metastasis of HFWT and 17-94 cell lines after the overexpression and knockdown of CRNDE, respectively. Results showed that compared with sh-NC group, the proliferation and metastasis abilities of WT cells in CRNDE silencing group were remarkably lower than that of NC. However, in CRNDE overexpression group, opposite results were obtained (Figure 2B, 2C, and 2D). These results suggested that the knockdown of CRNDE inhibited cell proliferation and metastasis, while CRNDE overexpression promoted cell proliferation and metastasis in Wilms' tumor.

Direct Targeting of CRNDE and MicroRNA-424

To further explore the mechanism in which CRNDE promoted the malignant progression of Wilms' tumor, a related relationship between

Table I. Association of lncRNA CRNDE expression with clinicopathologic characteristics of nephroblastoma.

Parameters	No. of cases	LncRNA CRNDE expression		*p*-value*
		Low (%)	High (%)	
Age (months)				0.044
<24	51	25	31	
≥24	38	25	13	
Gender				0.602
Male	49	26	23	
Female	40	19	21	
T stage				0.750
T1-T2	44	23	21	
T3-T4	45	22	23	
Lymph node metastasis				0.012
No	74	33	41	
Yes	15	12	3	

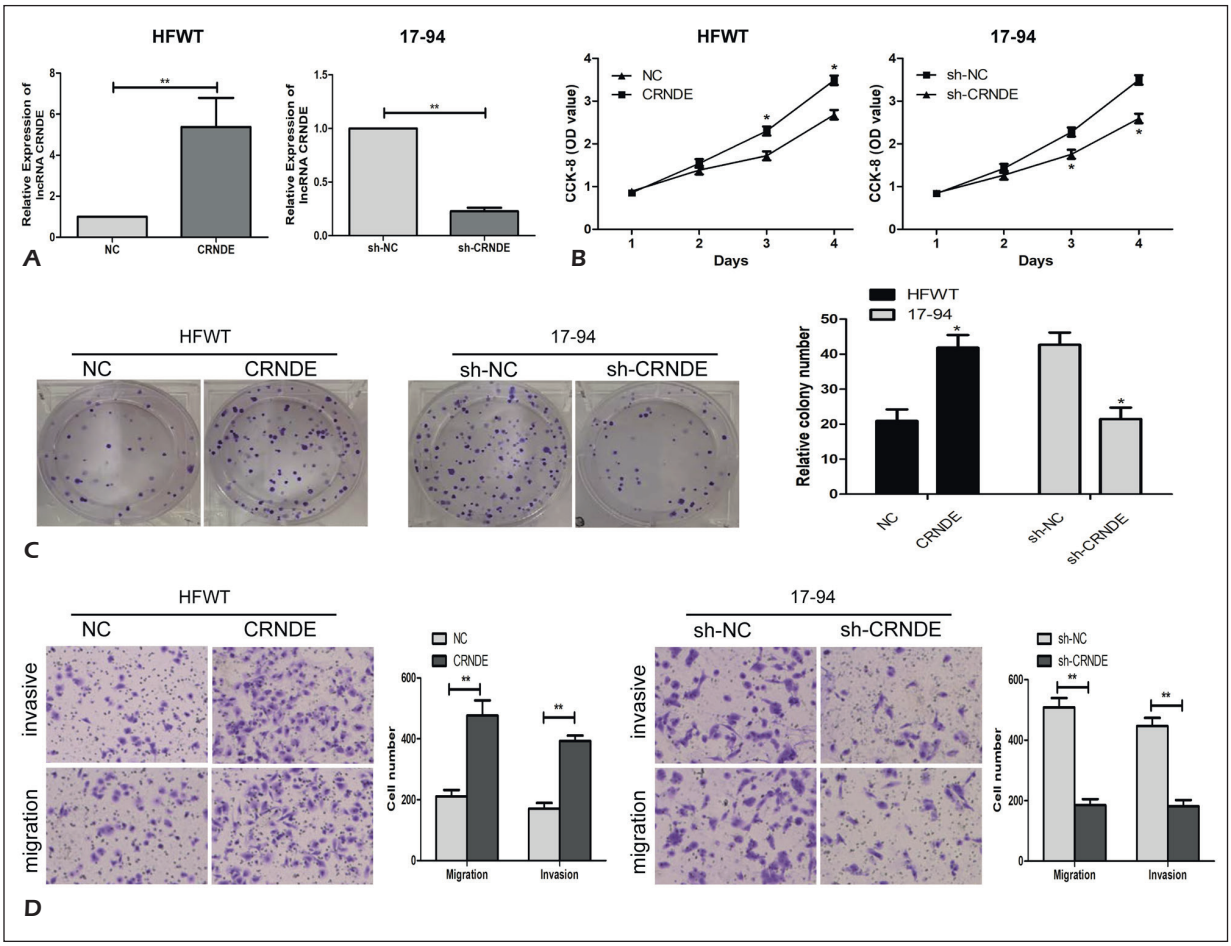

Figure 2. Overexpression/silencing CRNDE promoted/inhibited proliferation and metastasis of Wilms' tumor cells. **A**, RT-qPCR verified the interference efficiency of CRNDE in HFWT and 17-94 cell lines; **B**, CCK-8 assay detected the effect of CRNDE on the proliferation of HFWT and 17-94 cells; **C**, Colony formation assay was performed to detect the proliferation of WT cells after transfection of CRNDE in HFWT and 17-94 cell lines (Magnification: 20×); **D**, Transwell assay was applied to detect the effect of CRNDE on the invasion and migration of HFWT and 17-94 cells (Magnification: 20×). Data were expressed as mean ± SD, *$p<0.05$.

CRNDE and microRNA-424 might be found by bioinformatics analysis. As shown in Figure 3A, to verify the targeting of microRNA-424 to CRNDE, the CRNDE sequence was cloned into the luciferase reporter plasmid pmirGLO. Meanwhile, the mutation vector pmirGLO-CRNDE-mut was also constructed. Subsequently, pmirGLO-CRNDE-mut or pmirGLO and microRNA-424 were co-transfected into HFWT and 17-94 cells for the luciferase reporter gene assay. Results demonstrated that CRNDE could be targeted by microRNA-424 through this binding site. Next, we detected the expression of microRNA-424 after CRNDE overexpression and knockdown in HFWT and 17-94 cell lines, respectively. The results showed that knockdown of CRNDE remarkably increased the expression level of microRNA-424, while overexpression of CRNDE significantly decreased the expression level of microRNA-424 (Figure 3B and 3C). These results indicated that CRNDE could directly target microRNA-424. Furthermore, CRNDE could modulate the microRNA-424 expression.

MicroRNA-424 Was Lowly Expressed in Wilms' Tumor Tissues and Cell Lines

To further explore the role of microRNA-424 in Wilms' tumor, we detected the expression of microRNA-424 in 89 pairs of Wilms' tumor tissues and corresponding para-cancerous tissues. The result of RT-qPCR indicated that microRNA-424 was lowly expressed in Wilms' tumor tissues, as well as Wilms' tumor cell lines (Figure 3D). In addition, CRNDE expression was negatively correlated with microRNA-424 expression in Wilms' tumor tissues (Figure 3E).

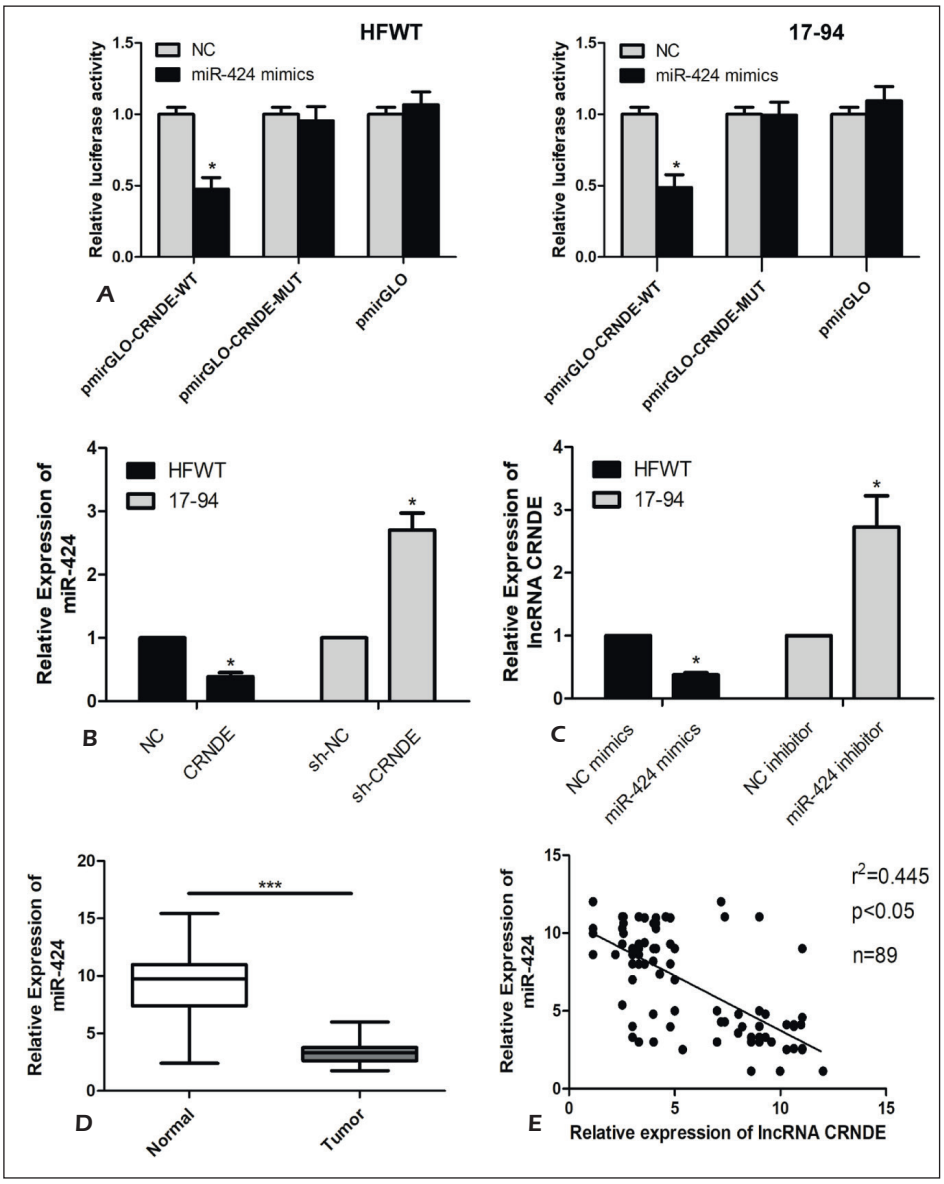

Figure 3. Direct targeting of miR-424 by CRNDE. **A**, Dual luciferase reporter gene assay validated the direct targeting of CRNDE and miR-424; **B**, Overexpression/silencing of CRNDE expression significantly reduced/increased miR-424 expression; **C**, RT-qPCR was used to detect the expression of miR-424 in tumor tissues and adjacent normal tissues; **D**, RT-qPCR verified the expression of CRNDE in Wilms' tumor cell lines; **E**, There was a significant negative correlation between the expression levels of CRNDE and miR-424 in Wilms' tumor tissues. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Overexpression of MicroRNA-424 Inhibited Cell Proliferation and Metastasis

To investigate the cellular biological changes of microRNA-424 in Wilms' tumor, microRNA-424 was overexpressed and down-expressed in HFWT and 17-94 cell lines. RT-qPCR was performed to verify the transfection efficiency (Figure 4A). CCK-8, colony formation assay, and transwell assay were used to detect the effect of microRNA-424 on cell proliferation and metastasis. Results showed that compared with NC group, the proliferation and metastasis abilities of Wilms' tumor cells in microRNA-424 overexpression group were remarkably inhibited. On the contrary, these above cell functions were significantly enhanced in microRNA-424 inhibitor group (Figure 4B, 4C, and 4D). All these findings demonstrated that microRNA-424 could regulate cell proliferation and metastasis in Wilms' tumor.

CRNDE Modulated MicroRNA-424 Expression in Wilms' Tumor Tissues and Cell Lines

After overexpressing or knocking down microRNA-424 in WT cell lines, respectively, the expression level of CRNDE was examined using RT-qPCR. It was found that microRNA-424 could negatively regulate the expression of CRNDE (Figure 5A). Subsequently, colony formation and transwell experiments indicated that overexpression of

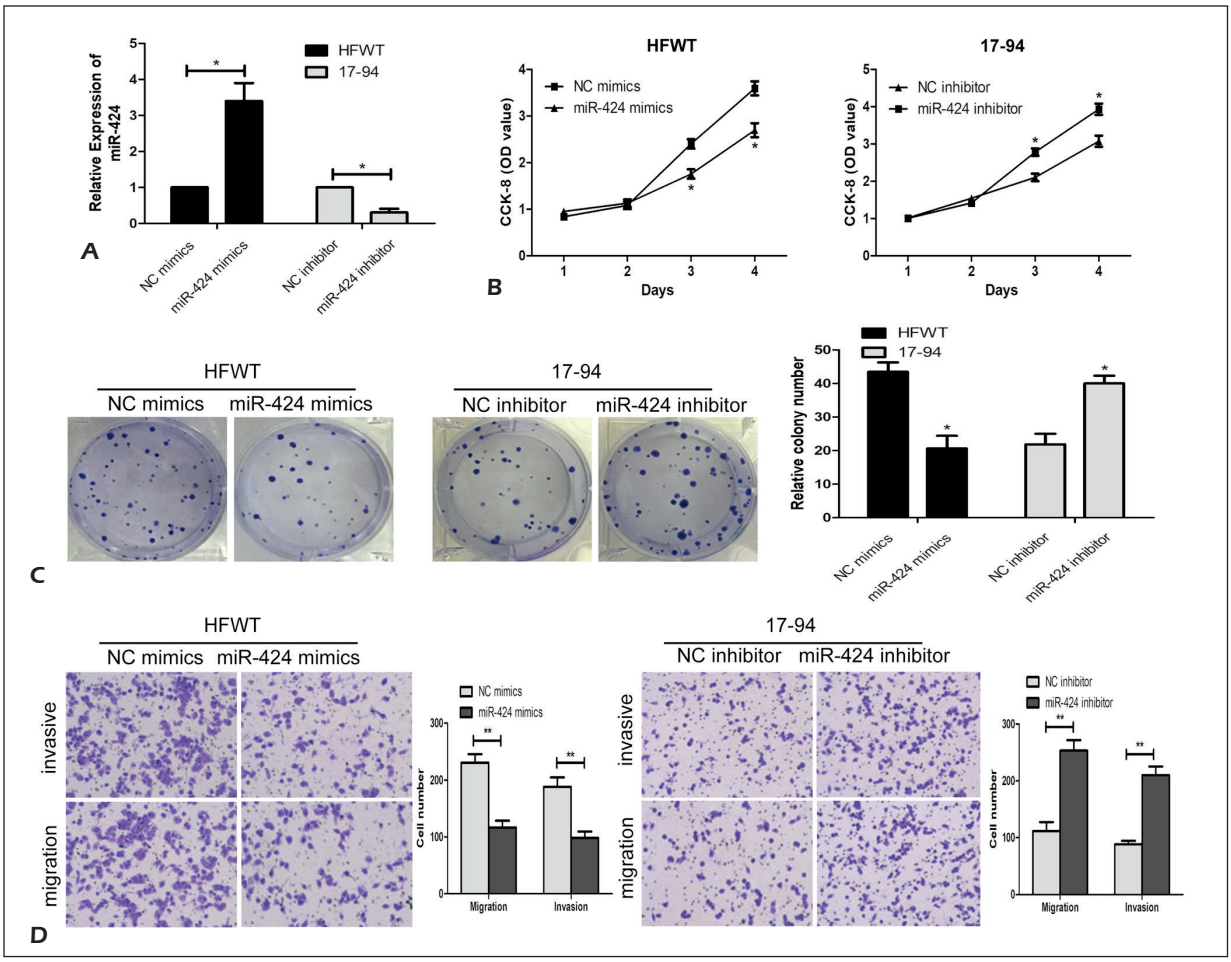

Figure 4. Overexpression/silencing miR-424 inhibited/promoted the proliferation and metastasis of Wilms' tumor cells. **A**, RT-qPCR verified the interference efficiency of miR-424 after transfection of miR-424 in HFWT and 17-94 cell lines; **B**, CCK-8 assay detected the effect of miR-424 on the proliferation of HFWT and 17-94 cell lines; **C**, Colony formation assay was performed to detect the proliferation of WT cells after transfection of miR-424 in HFWT and 17-94 cell lines (Magnification: 20×); **D**, Transwell assay detected the invasion and migration of Wilms' tumor cells after transfection of miR-424 in HFWT and 17-94 cell lines (Magnification: 20×). Data were expressed as mean ± SD, *$p<0.05$.

microRNA-424 could offset the influence of CRNDE overexpression on cell proliferation, invasion, and migration. At the same time, the knockdown of microRNA-424 could also counteract the effect of the knockdown of CRNDE on WT cell functions (Figure 5B, 5C). Therefore, CRNDE promoted malignant progression of Wilms' tumor by modulating the microRNA-424 expression.

Discussion

In recent years, the direct causal relation between lncRNAs and malignant tumors has gradually become clear. In most tumors, the content of lncRNAs is relatively low. This may be related to the mechanism of DNA methylation of lncRNAs or the closure of chromatin structure around its promoter to silence its expression[11-14]. Wilms' tumor is a common cancer in the urinary system of children. Sanger sequencing and total exon sequencing have provided identification methods for lncRNA in WT patients[9,10]. Analysis of WT cell lines and normal kidney cells by the gene-chip technology has shown that the content of numerous lncRNAs is remarkably changed in Wilms' tumor cells. The transcription level is abnormal, while the occurrence and development of the disease are affected[10,16,17]. Previous works have found that lncRNA CRNDE is highly expressed in WT tumor tissues. *In vitro* experiments have also indicated that inhibiting lncRNA CRNDE expression inhibits tumor proliferation and reduces its invasion ability. The mechanism may be related to the up-regulation of microRNA-424 expression.

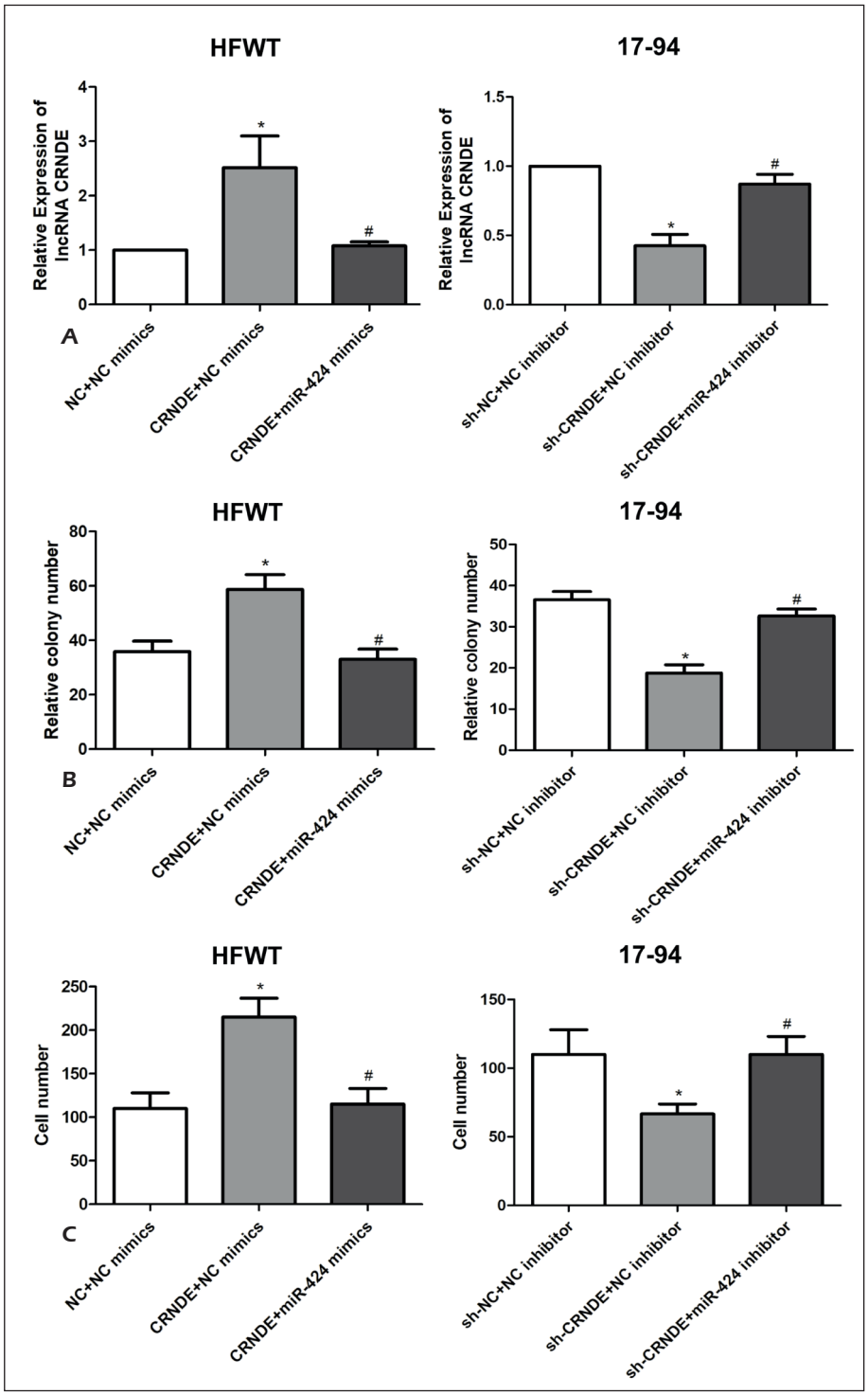

Figure 5. CRNDE regulated the expression of miR-424 in Wilms' tumor tissues and cell lines. **A**, CRNDE expression level was detected by RT-qPCR in CRNDE&miR-424 co-transfected cell lines; **B**, Colony formation assay detected the proliferation of Wilms' tumor cells after co-transfection of CRNDE and miR-424; **C**, Transwell assay detected the invasion ability of Wilms' tumor cells after co-transfection of CRNDE and miR-424. Data were expressed as mean ± SD, *$p<0.05$.

LncRNA expression regulates the process of tumor growth and transformation at multiple levels. Among this, three major levels include transcriptional regulation, post-transcriptional regulation, and epigenetic regulation[16-19]. Studies have shown that CRNDE is remarkably highly expressed in cervical cancer. Its high expression is associated with poor prognosis of patients. Some reports[19,20] have also found that abnormal expression of CRNDE is associated with poor prognosis of patients with lung cancer. In this study, the overexpression and knockdown cell lines

of CRNDE in Wilms' tumor were established. CRNDE was selected as the candidate lncRNA related to the malignant progression of Wilms' tumor. Meanwhile, the relation between CRNDE and the occurrence and development of Wilms' tumor was determined. Results showed that the up-regulated expression of CRNDE promoted the malignant progression of Wilms' tumor. The expression of CRNDE was found remarkably higher in WT tumor tissues than that of adjacent normal tissues. CRNDE expression was positively correlated with poor prognosis of WT. This suggested that CRNDE might play an important role in promoting the development WT. To further investigate the molecular mechanism of CRNDE in the development of Wilms' tumor, somatic cellular experiments were conducted. CCK-8, colony formation, and transwell experiments were carried out. The results showed that CRNDE silencing could remarkably suppress cell proliferation and metastasis abilities, while CRNDE overexpression remarkably promoted cell proliferation and metastasis in WT cells.

According to the latest research, lncRNAs can also be used as ceRNA or miRNA sponges to bind miRNAs competitively and inhibit the function and activity of miRNAs. This can eventually regulate the expression of target mRNAs at the post-transcriptional level. LncRNAs are also involved in biological behavior processes, such as tumor cell proliferation, invasion, metastasis, and angiogenesis[21,22]. Previous studies have predicted that microRNA-424 may interact with CRNDE through bioinformatics analysis. Based on the above research results, we showed that CRNDE could be used as an oncogene, thereby promoting the proliferation, invasion, and metastasis of Wilms' tumor cells. Meanwhile, CRNDE could promote the malignant progression of WT through microRNA-424. MicroRNA-424 is a key molecule in the miRNA family. Results showed that microRNA-424 was lowly expressed in Wilms' tumor tissues than of adjacent normal tissues. Meanwhile, microRNA-424 could inhibit the proliferation of Wilms' tumor cells. In this study, the direct binding of CRNDE to downstream microRNA-424 was verified by molecular biology experiments, including bioinformatics, and dual luciferase reporter gene assay. Besides, we found that CRNDE expression was negatively correlated with microRNA-424 expression. Given that only a few lncRNAs have been identified to exert important biological functions in WT, the direction of future studies is to search for specific lncRNAs molecules and to explore thier potential function and signal mechanisms. This will not only help to understand the molecular mechanism of the occurrence and development of WT, but also to provide a new direction for WT diagnosis and treatment through targeted lncRNA.

Conclusions

LncRNA CRNDE was highly expressed in Wilms' tumor and was significantly correlated with lymph node metastasis of patients. Also, CRNDE might promote the malignant progression of Wilms' tumor by regulating the microRNA-424 expression.

Conflict of Interests

The Authors declared that they have no conflict of interests.

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