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# LncRNA XIST promotes migration of Wilms' tumor cells through modulation of microRNA-193a-5p

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**Abstract. – OBJECTIVE: The aim of this study was to investigate long non-coding RNA (lncRNA) XIST expression in Wilms' tumor (WT) and to further explore its relationship with clinical features and prognosis of WT patients.**

**PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was carried out to examine the expression level of XIST in tumor tissue samples and paracancerous ones collected from 43 patients with renal cell carcinoma, and the interplay between XIST expression and clinical indicators, as well as prognosis of patients was analyzed. Meanwhile, XIST level in the nephroblast cancer cell line was further confirmed by qRT-PCR. In addition, XIST knockdown model was constructed using lentivirus in the WT cell lines, including HFWT and 17-94, and the influence of XIST on WT cell functions was analyzed through transwell assay. Finally, we investigated whether lncRNA XIST plays a role in the progression of WT by modulating microRNA-193a-5p.**

**RESULTS: In this research, qRT-PCR results revealed a significantly higher expression of lncRNA XIST in tumor tissue specimens of patients with renal cell carcinoma than that in adjacent ones. Compared with patients with low expression of lncRNA XIST, those with high XIST expression had a higher incidence of distant metastasis and a lower overall survival rate. Compared with the negative control group, the metastatic ability of WT cells in the lncRNA XIST knockdown group was markedly weakened. In addition, the results of qPCR showed that mRNA expression of lncRNA XIST and microRNA-193a-5p were negatively correlated in renal cell carcinoma tissue specimens. At the same time, silencing microRNA-193a-5p reversed the reduced metastasis ability of WT cells induced by knockdown of XIST.**

**CONCLUSIONS: LncRNA XIST expression is dramatically enhanced in WT tissues and cell lines, which is closely associated with the incidence of distant metastasis and patients' poor prognosis. In addition, we demonstrated that lncRNA XIST may accelerate the malignant progression of WT via inhibiting microRNA-193a-5p.**

*Key Words:*
LncRNA XIST, MicroRNA-193a-5p, Wilms' tumor, Malignant progression.

## Introduction

Wilms' tumor (WT) is the most common primary urogenital malignancy in pediatrics. According to statistics, WT accounts for 6% of all pediatric tumors, and its overall incidence reaches about 0.1‰ in children and adolescents under 15 years old[1,2]. The average age of child patients with WT is 36 months old, and there are rare cases older than 10 years old or younger than 6 months old[3]. So far, the etiology of WT has not been fully clarified, and it is currently believed[4,5] that it may originate from the abnormal hyperplasia of the posterior renal embryonic base that does not normally differentiate into nephrons. The occurrence of tumor involves a complex process of multiple factors and multiple stages *in vivo* and *in vitro*, which may be related to scores of genetic factors. However, an important feature in the development and evolution of malignant tumors is the activation or overexpression of oncogenes and the deletion or downregulation of tumor suppressor genes[6,7]. At

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the same time, WT is recognized as one of the major malignant solid tumors in children undergoing modern comprehensive therapy. At present, the treatment trend of WT is to reduce the treatment intensity as much as possible while continuously improving the existing survival rate to reduce the incidence of treatment-related complications and ultimately reduce mortality[8]. Therefore, clear diagnosis and staging, exact pathological classification, individualized comprehensive treatment, and the search for sensitive molecular biological indicators to guide treatment and follow-up are important means to improve the prognosis and survival rate of children with WT[1,2,8].

Long non-coding RNA (lncRNAs) is a kind of RNA with a length of over 200 nucleotides, which has been proved to play a pivotal role in the regulation of the expression of genes related to the biological behavior of tumor cells and tumor formation[9-12]. Abnormal expression of lncRNAs is closely associated with the formation of tumors, including Wilms' tumor[13]. Studies[14,15] on the function of lncRNAs have confirmed that like molecular sponges, they can competitively inhibit the expression of miRNAs. Among the large family, LncRNA XIST is the product of XIST gene transcription and the main regulator of X chromosome inactivation in mammals[16]. LncRNA XIST plays a key role in the differentiation, proliferation, and genome repair of human cells[17,18]. Specifically, dysregulation of lncRNA XIST may be directly related to the occurrence of cancer, which may be induced by the abnormal expression of certain genes due to changes in heterochromatin stability[18].

MicroRNAs (miRNAs) are a class of non-coding miRNAs that regulate gene expression by targeted binding to certain messenger RNA (mRNA), thereby promoting its degradation or inhibiting its translation[19,20]. They are mainly engaged in regulating various biological behaviors, such as ontogenesis, apoptosis, proliferation, and differentiation[20]. Different expression patterns of miRNAs in normal and tumor tissues can affect the cell cycle and proliferation process[21,22]. Based on the above characteristics, we suggested that lncRNA XIST may be a serological marker for early diagnosis of tumors, which provides a possible choice for target gene therapy of tumors. In this study, we confirmed that lncRNA XIST can be involved in the metastasis of renal mother cell carcinoma cells through the regulation of microRNA-193a-5p.

## Patients and Methods

### Patients and Wilms' Tumor Samples

In this study, 43 pairs of specimens were collected from surgically treated cases of WT and then stored at -80°C. The collection of clinical specimens was approved by the Ethics Monitoring Committee of Affiliated Hospital of Hubei College of Arts and Sciences, and the patients and their families have been fully informed that their specimens would be used for scientific research.

### Cell Lines and Reagents

WT cell lines (HFWT, and 17-94) and one human renal tubular epithelial cell line (HK-2) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were provided by Life Technologies (Gaithersburg, MD, USA). The WT cell lines were cultured in high-glucose DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were cultured in a 37°C, 5% $CO_2$ incubator, followed by passage with 1% trypsin + EDTA (ethylenediaminetetraacetic acid) for digestion when 80-90% of them were fused.

### Transfection

The control sequences (sh-NC) and the knockdown XIST lentiviral sequences (sh-XIST) were provided by Shanghai GenePharma Company (Shanghai, China). The cells were plated in 6-well plates and grown to a cell density of 70%, and then, lentiviral transfection was performed according to the manufacturer's instructions. After 48 h, the cells were harvested for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and cell function experiments.

### Transwell Assay

After transfection for 48 hours, the cells were digested, centrifuged, and resuspended in FBS-free medium, and the density was adjusted to 5 × $10^5$ cells/mL. Then, 200 uL of cell suspension (1 × $10^5$ cells) was added to the upper chamber, while 700 uL of a medium containing 20% FBS was added to the lower chamber. After incubated in a 37°C incubator for 48 hours, the chamber was removed, fixed with 4% paraformaldehyde for 30 minutes, and stained with 0.2% crystal violet for 15 minutes. Subsequently, the cells were washed with phosphate-buffered saline (PBS),

and the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner layer cells. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed in 5 randomly selected fields of view under the microscope.

### Quantitative Real Time-Polymerase Chain Reaction (qPCR)

Total RNA was extracted from WT cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then, reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent. The qRT-PCR was conducted using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), and StepOne Plus Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis was performed by ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers for qPCR were: lncRNA XIST: forward: 5'-GGTGGACATGTGCGGTCA-3', reverse: 5'-CAGCCACGTAATCCAGATGAT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-GTCAAGGCTGAGAACGGGAA-3', reverse: 5'-AAATGAGCCCCAGCCTTCTC-3'; microRNA-193a-5p: forward: 5'-TGGGTCTTTGCGGGC-3', reverse: 5'-GAATACCTCGGACCCTGC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCG-3 '.

### Dual-Luciferase Reporter Assay

HEK293T cells were seeded in 24-well plates and co-transfected with microRNA-193a-5p mimic/NC and pMIR Luciferase reporter plasmids. The plasmid was then introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 hours of transfection, the Luciferase activity of each group was detected and normalized by a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

### In Vivo Xenograft Model

The Animal Ethics and Use Committee approved the tumor-forming experiment in nude mice. Ten 8-week-old male nude mice were purchased from the animal center and randomly divided into two groups (5 in each group). The HFWT cells with stable knockdown of lncRNA XIST were injected subcutaneously into the axilla of mice. Tumor size was monitored every 5 days; then, after 5 weeks, the mice were sacrificed. The tumor volumes were calculated using the following formula: tumor volume = (width 2 × length)/2.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Statistical differences between the two groups and multiple groups were analyzed using Student's *t*-test and One-way analysis of variance (ANOVA) followed by post-hoc test (Least Significant Difference), respectively. Independent experiments were repeated at least three times for each experiment and data were expressed as mean ± standard deviation (SD). $p<0.05$ was considered statistically significant.

## Results

### LncRNA XIST Was Highly Expressed in Wilms' Tumor Tissues and Cell Lines

To determine the role of lncRNA XIST in WT, we collected a total of 43 pairs of tumor tissue specimens and paracancerous ones of patients with renal cell carcinoma and examined lncRNA XIST level by qRT-PCR. It was found that the expression of lncRNA XIST was higher in WT tissues (Figure 1A), suggesting that lncRNA XIST may serve as a tumor-promoting gene in this disease. In addition, compared with the renal tubular epithelial cell line HK-2, the WT cell line, especially HFWT and 17-94, also contained a higher lncRNA XIST expression according to qRT-PCR (Figure 1B). Subsequently, according to the qPCR results of XIST mRNA expression, we divided the above tissue samples into two groups, high and low expression group, and the interplay between XIST expression and some indicators of WT patients, such as age, pathological stage, incidence of lymph node, and distant metastasis, was analyzed by Chi-square test. As shown in Table I, high expression of lncRNA XIST is positively correlated with the incidence of distant metastasis in WT patients. In addition, Kaplan-Meier survival curve revealed that the high expression of lncRNA XIST was also dramatically associated with poor prognosis of this disease, in other words, the higher the lncRNA XIST level, the worse the prognosis ($p<0.05$; Figure 1C).

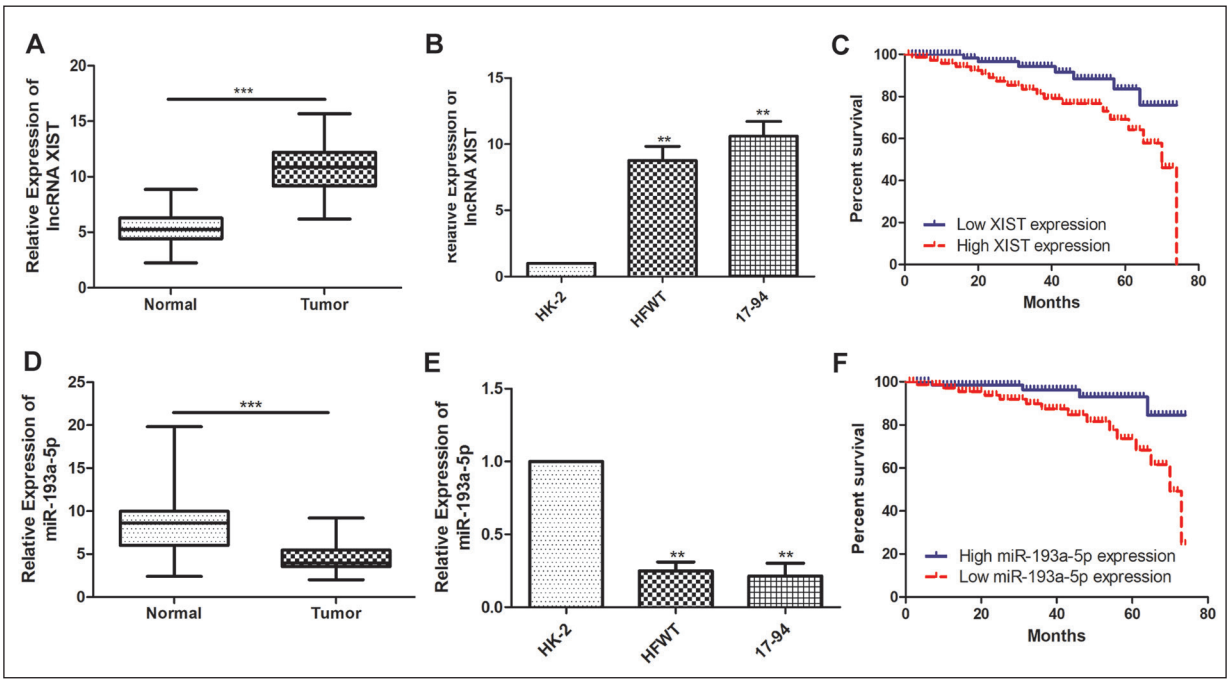


**Figure 1.** LncRNA XIST is highly expressed in nephron cell carcinoma tissues and cell lines. **A,** QRT-PCR was used to detect the differential expression of lncRNA XIST in tumor tissues and paracancerous tissues of patients with renal cell carcinoma. **B,** QRT-PCR was used to detect the expression level of lncRNA XIST in the WT cell line. **C,** Kaplan Meier survival curve of patients with renal cell carcinoma based on lncRNA XIST expression was shown; the prognosis of patients with high expression of lncRNA XIST was significantly worse than those in low expression group. **D,** QRT-PCR was used to detect the differential expression of miR-193a-5p in tumor tissues and paracancerous tissues of patients with renal cell carcinoma. **E,** QRT-PCR was used to detect the expression level of miR-193a-5p in WT cell lines. **F,** Kaplan Meier survival curve of patients with renal cell carcinoma based on miR-193a-5p expression was shown; the prognosis of patients with low expression of miR-193a-5p was significantly worse than those in high expression group. Data are mean ± SD, *$p$<0.05, **$p$<0.01, ***$p$<0.001.

### *MicroRNA-193a-5p Was Lowly Expressed in Wilms' Tumor Tissues and Cell Lines*

MicroRNA-193a-5p expression was dramatically lower in tumor tissues than that in adjacent ones (Figure 1D). Meanwhile, microRNA-193a-5p also showed a significant higher expression in WT cell lines, especially HFWT and 17-94, than that in HK-2 cells (Figure 1E). In addition, Kaplan-Meier survival curves revealed that low expression of microRNA-193a-5p was

**Table I.** Association of lncRNA XIST expression with clinicopathologic characteristics of Wilms' tumor.

| Parameters | No. of cases | XIST expression | | *p*-value |
|---|---|---|---|---|
| | | Low (%) | High (%) | |
| Age (months) | | | | 0.807 |
| < 36 | 19 | 11 | 8 | |
| ≥ 36 | 24 | 13 | 11 | |
| T stage | | | | 0.864 |
| T1-T2 | 21 | 12 | 9 | |
| T3-T4 | 22 | 12 | 10 | |
| Lymph node metastasis | | | | 0.115 |
| No | 17 | 12 | 5 | |
| Yes | 26 | 12 | 14 | |
| Distance metastasis | | | | 0.005 |
| No | 28 | 20 | 8 | |
| Yes | 15 | 4 | 11 | |

markedly relevant to poor prognosis of renal cell carcinoma patients, namely, the lower the expression level of microRNA-193a-5p, the worse the prognosis ($p<0.05$; Figure 1F).

### Knockdown of LncRNA XIST Inhibited Cell Metastasis in Wilms' Tumor

To explore the influence of lncRNA XIST on Wilms' tumor cell migration ability, we first successfully constructed a lncRNA XIST knockdown model and verified its transfection efficiency by qRT-PCR (Figure 2A). Subsequently, we showed by transwell assay that the knockdown of XIST dramatically weakened the metastatic ability of WT cells (Figure 2B).

### LncRNA XIST Was Bound to MicroRNA-193a-5p

We searched TargetScan, miRDB, and Starbase databases to assess the mutual regulation between lncRNAs and miRNAs; a synergistic association between XIST and four potential miRNAs was discovered (Figure 3A). In addition, qRT-PCR detected the expression differences of the four potential miRNAs in the sh-NC group and the sh-XIST group, and it was found that the difference in microRNA-193a-5p was the most significant. Therefore, it is speculated that there is a potential correlation between XIST and microRNA-193a-5p (Figure 3B). The Luciferase reporter gene assay demonstrated that lncRNA XIST can be targeted by microRNA-193a-5p *via* a specific binding site (Figure 3C). Additionally, lncRNA XIST and microRNA-193a-5p were found to be negatively associated with each other in WT tissues (Figure 3D).

### LncRNA XIST Modulated MicroRNA-193a-5p in Wilms' Tumor

Subsequently, lncRNA XIST and microRNA-193a-5p knockdown vectors were co-transfected into WT cells to figure out the interaction between the two, and the transfection efficiency was detected by qPCR (Figure 4A). The results of transwell experiment indicated that microRNA-193a-5p counteracted the impact of XIST on metastasis of WT cells (Figure 4B).

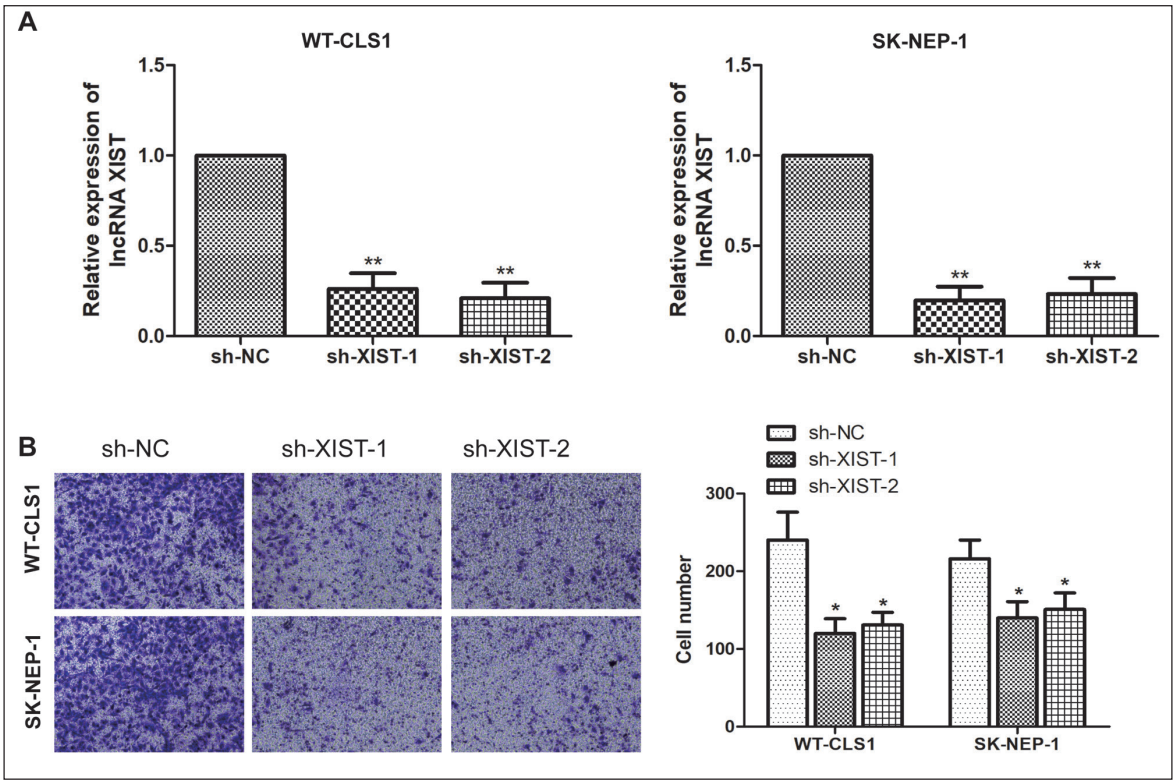


**Figure 2.** Metastatic ability of WT cells was inhibited after silencing lncRNA XIST. **A,** QRT-PCR verified the interference efficiency after transfection of lncRNA XIST knockdown vector in the WT cell lines HFWT and 17-94. **B,** Transwell assay detected the migration ability of WT cells after transfection of the lncRNA XIST knockdown vector in the WT cell lines HFWT and 17-94 (magnification: 40×). Data are mean ± SD, *$p<0.05$, **$p<0.01$.

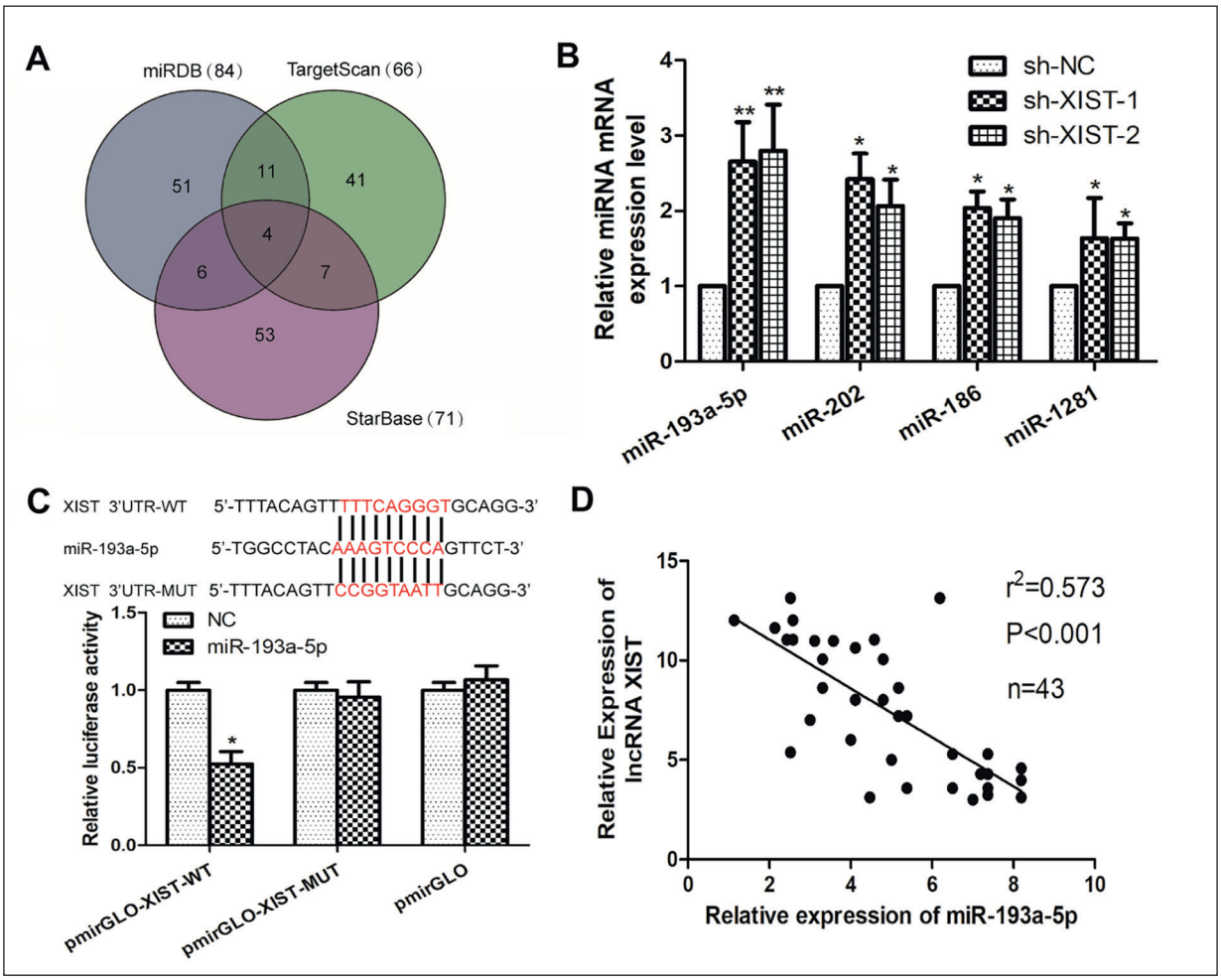


**Figure 3.** LncRNA XIST directly bind to miR-193a-5p. **A,** TargetScan, miRBase and MiRcode suggested that lncRNA XIST can directly bind to four potential miRNAs. **B,** After silencing lncRNA XIST, miR-193a-5p was found downregulated the most. **C,** Dual-Luciferase reporter assays demonstrated direct targeting of lncRNA XIST to miR-193a-5p. **D,** There was a significant negative correlation between the expression of lncRNA XIST and miR-193a-5p in WT tissues. Data are mean ± SD, *$p$<0.05, **$p$<0.01.

### *Knockdown of LncRNA XIST Inhibited Wilms' Tumor Progression In Vivo*

*In vivo*, the transfected HFWT cell line was inoculated *in situ* into each nude mouse and injected in the left axilla. As expected, both the tumor volume and weight in nude mice inoculated with lncRNA XIST knockdown vector reduced (Figure 5A, 5B). Subsequently, we validated the reduction of lncRNA XIST level in tumor-forming tissues of nude mice injected with sh-XIST vector (Figure 5C) and found an increased expression of microRNA-193a-5p (Figure 5D). In addition, compared with the sh-NC group, E-cad expression dramatically decreased in the tumor tissues of nude mice with the knockdown of lncRNA XIST (Figure 5E). The above results suggested that the downregulation of lncRNA XIST can suppress the formation of tumors in nude mice.

## Discussion

Wilms' tumor (WT) is the most common malignant tumor originating from renal blastocyst cells in the pediatric urological system. It is most likely to occur in children under 15 years old, and its incidence is about 1/10000[1-4]. Multi-center cooperative studies suggest that about 66% of renal tumors in in infants less than 7 months old are WT, and the remaining 34% are non-WT[3-5]. The average onset age of WT patients

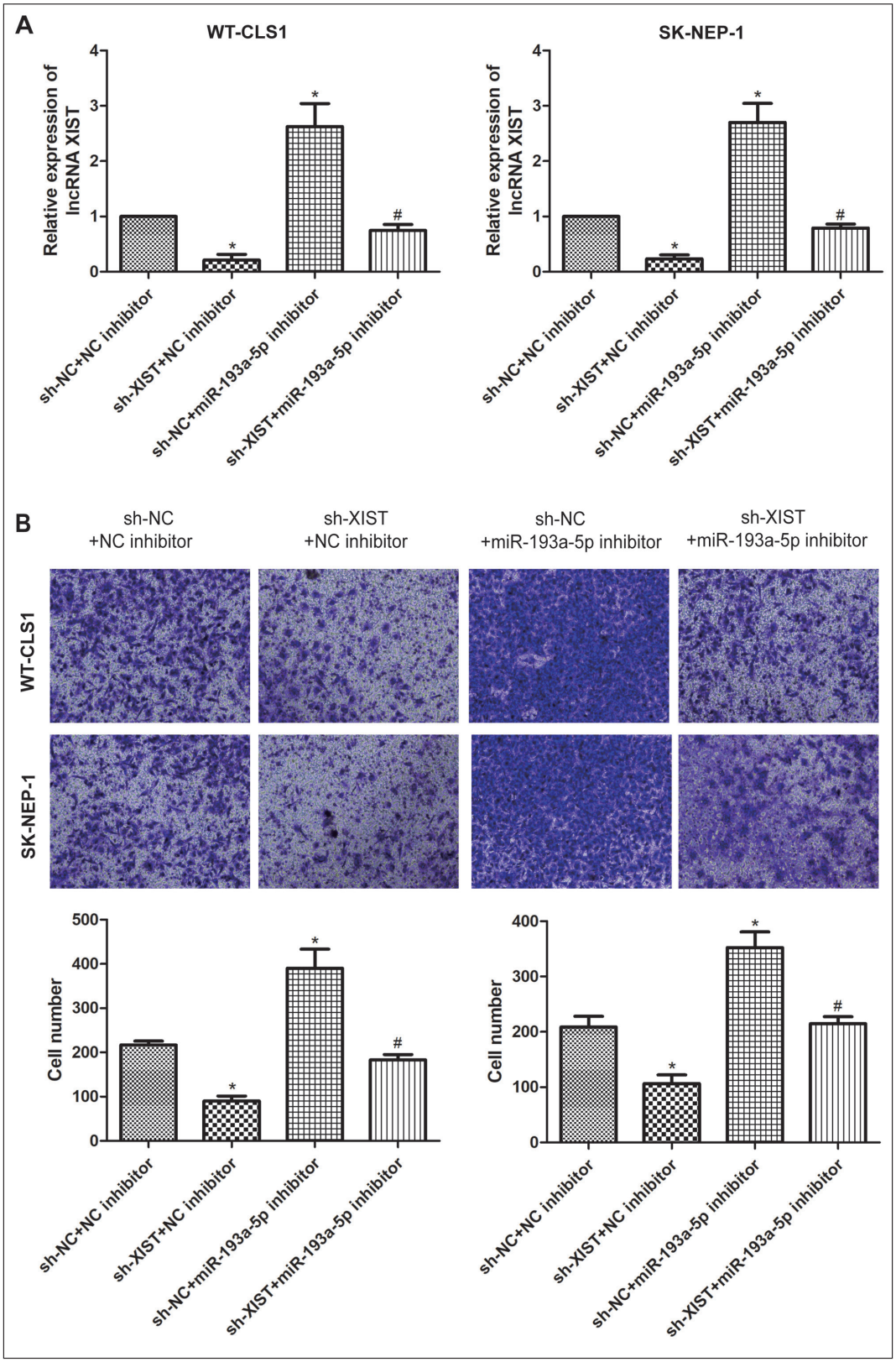


**Figure 4.** LncRNA XIST regulates the progression of WT through modulating miR-193a-5p. **A,** QRT-PCR verified the expression level of lncRNA XIST after co-transfection of lncRNA XIST and miR-193a-5p knockdown vectors in the WT cell lines HFWT and 17-94. **B,** Transwell assay detected the migration ability of WT cells after co-transfection of lncRNA XIST and miR-193a-5p knockdown vectors in the WT cell lines HFWT and 17-94 (magnification: 40×). Data are mean ± SD, $*^{\#}p<0.05$.

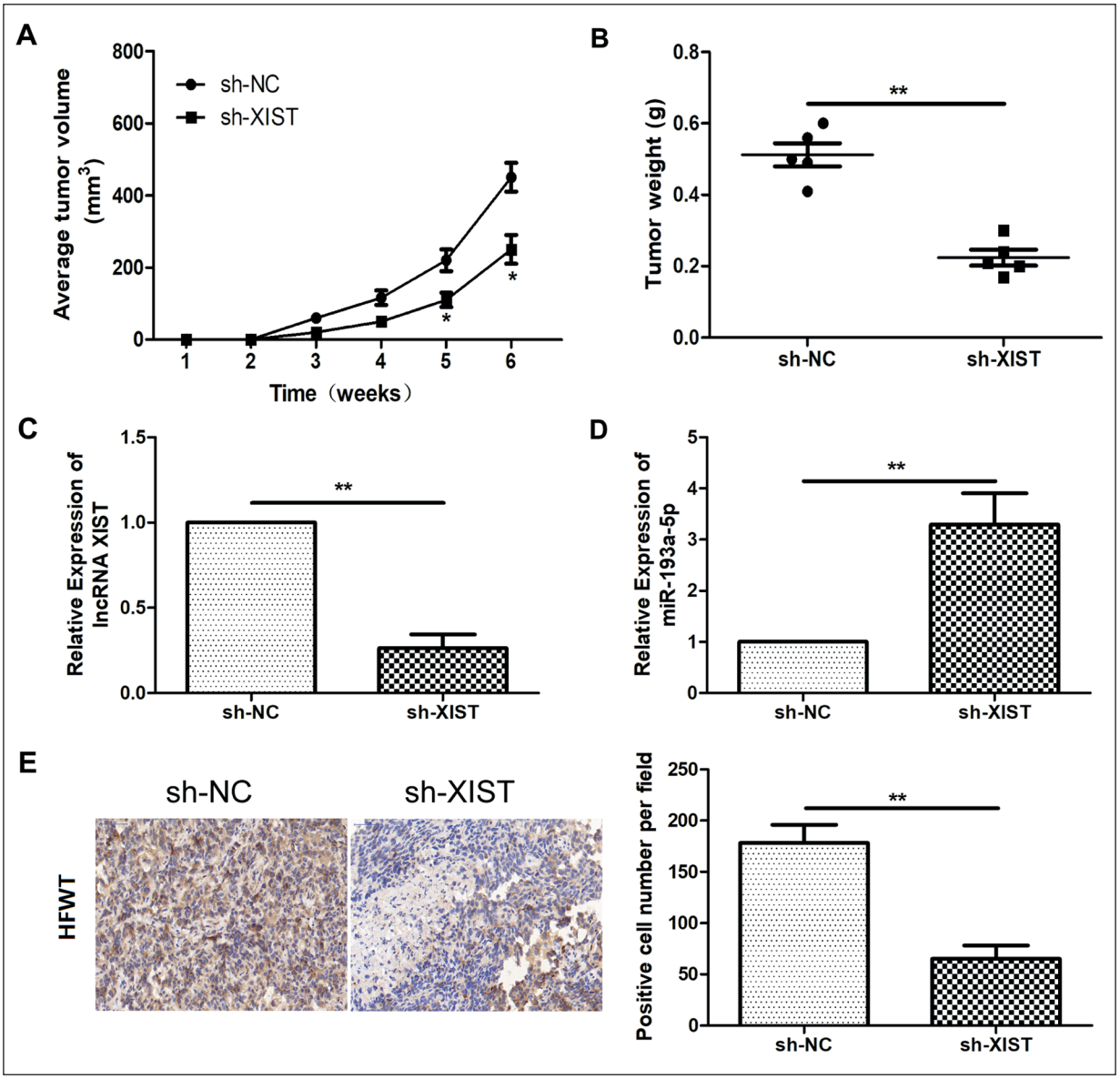


**Figure 5.** Silencing lncRNA XIST inhibits tumorigenic ability of WT in nude mice. **A,** Tumor volume growth curves were calculated for different nude mice after injection of sh-NC and sh-XIST, respectively. **B,** Tumor weight growth curves were calculated after injection of sh-NC and sh-XIST, respectively. **C,** qRT-PCR was used to detect the level of lncRNA XIST in the tumor-forming tissues of nude mice with WT. **D,** qRT-PCR was used to detect the level of miR-193a-5p in the tumor-forming tissues of nude mice with WT. **E,** Immunohistochemistry was used to detect the level of E-cad in the tumor-forming tissues of nude mice with renal cell carcinoma (magnification: 400×). Data are mean ± SD, *$p$<0.05, **$p$<0.01.

is 38 months, among which, girls are 6 months later than boys[5,6]. Up to now, the pathogenesis of WT is not clearly determined, and the tumor is similar to the disorganized embryonic kidney in morphology, which is currently believed to be originated from the abnormal residue of the functional block of the posterior renal embryo due to the stagnation of differentiation, may be one of the reasons for the occurrence of WT[7,8]. Because of the insidious location of WT and no specific tumor markers for its diagnosis, the course of the disease is often relatively rapid, seriously threatening the life and health of children[8]. Although multidisciplinary comprehensive treatment has dramatically improved the overall survival rate of WT, there are still great difficulties in the treatment of some refractory cases, such as tumors with adverse histological types, large tumors

first diagnosed unresectable, bilateral tumors, and recurrent tumors[7,8]. Therefore, seeking new anticancer targets to improve the clinical efficacy of WT and prognosis of children has become an important problem to be solved clinically[8,9].

Most of the transcription RNAs have been identified as non-coding RNAs (ncRNAs), which may be involved in the complex regulation of human gene expression[10-13]. LncRNAs play a key role in regulating the expression of genes related to the biological behavior of stem cells and in tumor formation[15]. Bioinformatics have revealed that lncRNA XIST could competitively inhibit the expression of microRNA-193a-5p. However, whether lncRNA XIST affects the biological behavior of WT by regulating the expression of miRNAs has not been reported so far[16-18].

In this study, we investigated the role of lncRNA XIST in the progression of WT with a large number of clinical tissue samples and WT cell lines for the first time. It was found that both WT tissue specimens and cell lines contained higher gene expression of XIST than normal tissues or normal renal tubule cell line, suggesting that high expression of lncRNA XIST play an extremely vital role in the development of renal cell carcinoma. Furthermore, after knockdown of lncRNA XIST in WT cell lines, we showed by transwell assay that XIST could accelerate the metastasis rate of renal cell carcinoma cells, thus playing a vital part in the progression of this disease; however, the specific molecular mechanism still remains elusive.

In many human cancers, the XIST gene is dysregulated. However, the expression and specific function of lncRNA XIST in WT are still completely unknown[17,18]. Some investigations[14,15] have shown that the regulation mechanisms of lncRNAs are complex and diverse, but they mainly function by regulating the expression of downstream miRNA. Therefore, it is of great significance to explore the occurrence and regulatory mechanism of Wilms' tumor to find target sites for the treatment[8,15]. In this study, qRT-PCR detection showed that the expression levels of lncRNA XIST and microRNA-193a-5p in renal cell carcinoma tissues were negatively correlated with each other, indicating that lncRNA XIST can play a pivotal role in enhancing WT cell metastasis through microRNA-193a-5p. In addition, Luciferase reporter gene assay indicated that lncRNA XIST can specifically bind to microRNA-193a-5p. Recovery experiments demonstrated that silencing microRNA-193a-5p reversed the role of XIST in malignant progression of renal cell carcinoma. With the deepening of the research, further understanding of the biological functions of genes and their roles in the development of tumors will be more conducive to the diagnosis, treatment, and prognosis evaluation of tumors.

## Conclusions

In summary, lncRNA XIST expression was dramatically elevated in WT tissues, which were correlated with the incidence of distant metastasis and poor prognosis of WT patients. Additionally, lncRNA XIST may promote the metastasis ability of WT cells by regulating microRNA-193a-5p.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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