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Long non-coding RNA LINP1 induces tumorigenesis of Wilms' tumor by affecting Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Recent studies have discovered that long non-coding RNAs (lncRNAs) play an important role in the development of malignant tumors. The aim of this work was to investigate the exact role of lncRNA LINP1 in the development of Wilms' tumor and to explore the possible underlying mechanism.

PATIENTS AND METHODS: The expression of lncRNA in non-homologous end joining pathway 1 (LINP1) in tissue samples of Wilms' tumor was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). The relationship between the expression of lung cancer associated transcript 1 (LUCAT1) and patients' overall survival time was analyzed. Subsequent functional experiments were conducted to identify the changes in biological behaviors of Wilms' tumor cells after the gain or loss of LINP1. Moreover, the underlying mechanism of LINP1 function was explored.

RESULTS: QRT-PCR results showed that LINP1 expression level in Wilms' tumor tissues was significantly higher than that in adjacent tissues. LINP1 expression was negatively associated with the overall survival time of patients with Wilms' tumor. Cell growth ability was markedly inhibited and promoted after down-regulation and overexpression of LINP1 *in vitro*, respectively. Moreover, after the loss and gain of LINP1 *in vitro*, cell migration and invasion abilities were remarkably suppressed and promoted respectively. Furthermore, the loss of LINP1 *in vitro* could significantly decrease the expression of target proteins in the Wnt/β-catenin signaling pathway. However, the expressions of proteins in the Wnt/β-catenin signaling pathway were remarkably up-regulated after over-expression of LINP1.

CONCLUSIONS: LINP1 could enhance cell metastasis and proliferation via inducing the Wnt/β-catenin signaling pathway. Our findings might provide a new prospect for the diagnosis and therapy of Wilms' tumor.

Key Words:
Long non-coding RNA, LINP1, Wilms' tumor, Wnt/β-catenin signaling pathway.

Introduction

Wilms' tumor is the most frequent pediatric renal cancer, which affects one in 10,000 children annually. The overall survival rate of Wilms' tumor is more than 90%[1]. When embryonic nephrogenic cells fail to undergo terminal differentiation, Wilms' tumor may happen. Great advances have been achieved in combination therapy to improve the prognosis of most patients. However, almost 10% of patients with Wilms' tumor eventually develop metastasis and recurrence, contributing to poor prognosis[2,3]. Thus, it is crucial to uncover the molecular mechanism of the progression of Wilms' tumors and to identify potential targets to improve the prognosis of this pediatric disease.

As a subtype of non-coding RNA (ncRNA), long noncoding RNAs (lncRNAs) regulate a variety of cellular processes and pathways in cancer development. For instance, the expression level of lncRNA-CCHE1 is positively correlated with the malignancy of colorectal carcinoma, which regulates the ERK/COX-2 pathway[4]. Through regulation of OIP5 expression, lncRNA OIP5-AS1 promotes the proliferation and inhibits the apoptosis of bladder cancer cells[5]. Activated by ZEB1, lncRNA HCCL5 accelerates cell viability, cell migration, epithelial-mesenchymal transition (EMT), as well as the malignancy of hepatocellular carcinoma[6]. Moreover, the over-expression of lincRNA-p21 represses the proliferation of gastric cancer cells, whereas increases cell sensitivity of radiotherapy by regulating the beta-catenin

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signaling pathway[7]. However, the specific role of lncRNA LINP1 in the progression of Wilms' tumor and the possible underlying molecular mechanism have not been fully elucidated yet.

In this work, LINP1 was highly expressed in Wilms' tumor tissues when compared with adjacent normal tissues. Moreover, LINP1 significantly promoted the proliferation and metastasis of Wilms' tumor *in vitro*. Previous studies have showed that the Wnt/β-catenin signaling pathway is a fundamental pathway in tumor development. Our findings also demonstrated that LINP1 participated in the tumorigenesis of Wilms' tumor by regulating the Wnt/β-catenin signaling pathway.

Patients and Methods

Clinical Samples

Tumor samples and adjacent tissues (≥ 5 cm away from the edge of tumor) were collected from Wilms' tumor patients (n = 52) who underwent surgery at Jining No. 1 People's Hospital from 2010 to 2012. Written informed consent was obtained from each patient before the operation. All fresh t... were preserved at -80°C for subsequent u... study was approved by the Human Research ...cs Committee of the Jining No. 1 People's Hospi...

Cell Culture

Cells were first collected ...d from fresh Wilms' tumor tissue... cell... ere cultured in Dulbecco's M... Eagl... (DMEM, Gibco, Gra... Is... N... USA)... taining 10% fetal ... se... BS, Gibco, Grand Island, NY... A), and m... ned in an incubator with ... at 37°C.

Cell Tran...ction

After ...thesis, short ha... RNA (shRNA) targeti... INP1 (sh-LINP1), lentivirus targeting LIN... (LINP... or scrambled oligonucleotides (NC)... cl... into p... VH1/GFP+Puro vector (Gene... Shan..., China). Then, Wilms' ... cel... re... nsfected with sh-LINP1, LI... lentiv... INP1) and NC according to the ...ructions. Subsequently, GFP-positive cells w... the following experiments.

...A Extraction and Real ...Quantitative Polymerase Ch... Reaction (RT-qPCR)

Total RNA in tissues and cells was extracted in strict accordance with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After that, extracted total RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNAs) through the Reverse Transcription Kit (TaKaR... nology Co., Ltd., Dalian, China). P... ers us... for Real Time-quantitative Poly... ase Chain Reaction (RT-qPCR) were as foll... INP1, forward: 5′-AGCCGGTCCAGTACAC... 3′ and reverse: 5′-GGAAAGCA... TCTG... T-3′, glyceraldehyde 3-pho... te dehydro... (GAPDH), forward: ... CAAA... AGAT... GGCAATGCTGG-3... rev... 5′-TG... TGG-CATGGACTGTC... TC... . Ther... cycle was as follows: ...ec at 9... sec ... total of 40 cycles at 9... 35 sec at 60...

Wester... ot A...is

Cells were first wa... with pre-cooled Phospha... ered Saline (... Gibco, Grand Is... NY, USA) and lysed with cell lysis solution ...PA; Beyotim... Shanghai, China). The con...ration of ex...ted protein was detected by t... inchonin... cid (BCA) method (Thermo Fish... Waltham, MA, USA). After separati... proteins were transferred onto ...olyvinylidene difluoride (PVDF) membranes ...re, Billerica, MA, USA). Then, the ...em...nes were blocked with Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA; 25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk for 2 h. Subsequently, the membranes were incubated with primary antibodies of target proteins including Wnt3a, β-catenin, C-myc and Survivin (Abcam, Cambridge, MA, USA) in the Wnt/β-catenin signaling pathway and GAPDH (Abcam, Cambridge, MA, USA) at 4°C overnight. After washing with TBST three times (10 min for each), the membranes were incubated with the corresponding secondary antibody at room temperature for 1 h. Finally, immunoreactive bands were analyzed by Image J software (NIH, Bethesda, MD, USA).

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide] Assay

A total of 2×10^3 transfected cells were first seeded into 96-well plates. Following manufacturer's protocol, cell proliferation was assessed by Cell Proliferation Reagent Kit I (MTT; Roche, Basel, Switzerland) every 24 h. Absorbance at 490 nm was assessed using an enzyme-linked immunosorbent assay (ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Colony Formation Assay

Transfected cells were first seeded into 6-well plates, followed by culture for 2 weeks. Formed colonies were fixed with methanol for 30 min and stained with 0.5% crystal violet for 5 min. Colonies containing more than 50 cells were counted, and the mean number of formed colonies was calculated. The analysis was conducted with Image-Pro Plus 6.0 (Media Cybernetics, Silver Springs, MD, USA).

Wound Healing Assay

Cells were first seeded into 6-well plates and incubated overnight. After being scratched with a pipette tip, the cells were cultured in serum-free DMEM. At 48 h, the relative distance was observed under a light microscope (Olympus, Tokyo, Japan). Each assay was independently repeated in triplicate.

Transwell Assay

A total of 1×10^5 cells in serum-free DMEM were seeded into the upper chamber (Corning Inc., Lowell, MA, USA) of 24-well plates pre-coated with Matrigel Matrix dilution (BD Biosci... Franklin Lakes, NJ, USA). Meanwhile, t... er chamber was added with DMEM and F... 48 h later, after being wiped by a cotton swab... top surface of chambers was immersed with ... paraformaldehyde for 10 min an... with 1... crystal violet for 30 min. Th... were ran-domly for each sample, and ... numb... f invad-ing cells was counted u... Leica ... microscope (Leica M... Germany).

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was adopted for all statistical analysis. Data were expressed as mean ± SD (Standard D... Student's *t*-test and Kaplan-Meier ... od we... utilized when appropriate. *p*-val... 0.05 was considered statistically significan...

Res...

LINP1 Expression... Wilms' Tumor T...sue

First, RT-qPC... as con... to d... LINP1 expression i... patients' t... e results showed th... was marke... up-regulated in Wi... tum... ue samples (Figure 1A). According to the me... expression of LINP1, 52 ... were divided ... two groups, including high LINP1 expression group and low LINP1 ...ession grou... The Kaplan-Meier analysis ...ved that the o... ll survival of patients in high l... ancer as... ated transcript 1 (LUCAT1) grou... cantly worse when compared with th... e low LUCAT1 group (Figure 1B).

...egulation of LINP1 Inhibited ...ation of Wilms' Tumor Cells

Collected and digested cells from Wilms' tumor tissues were used for transfection of LINP1 shRNA or scrambled oligonucleotides (NC). RT-qPCR was then utilized to detect LINP1 expression (Figure 2A). The results of the MTT assay revealed that the growth ability of Wilms' tumor cells was remarkably repressed after

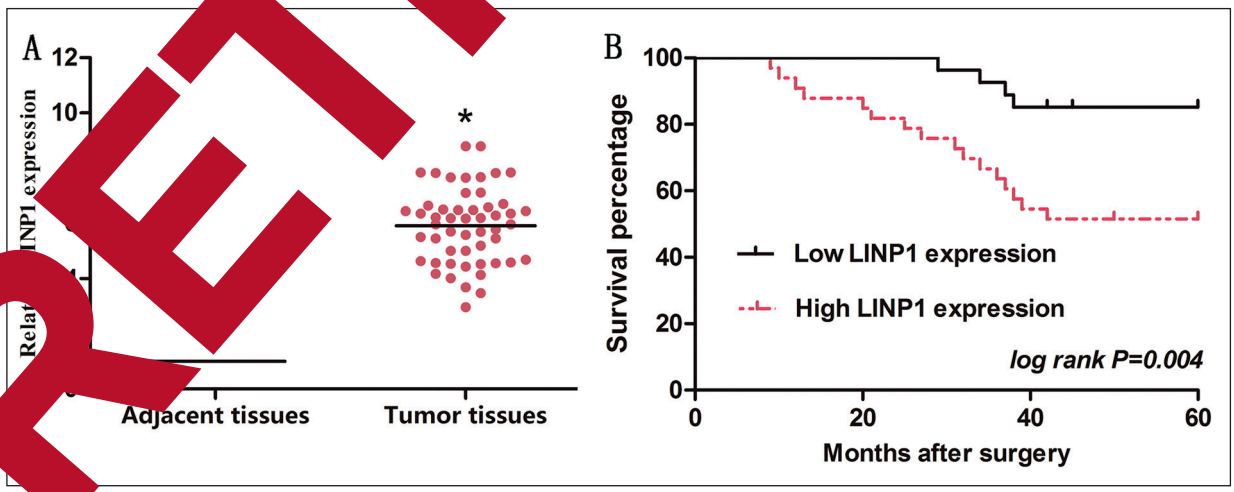
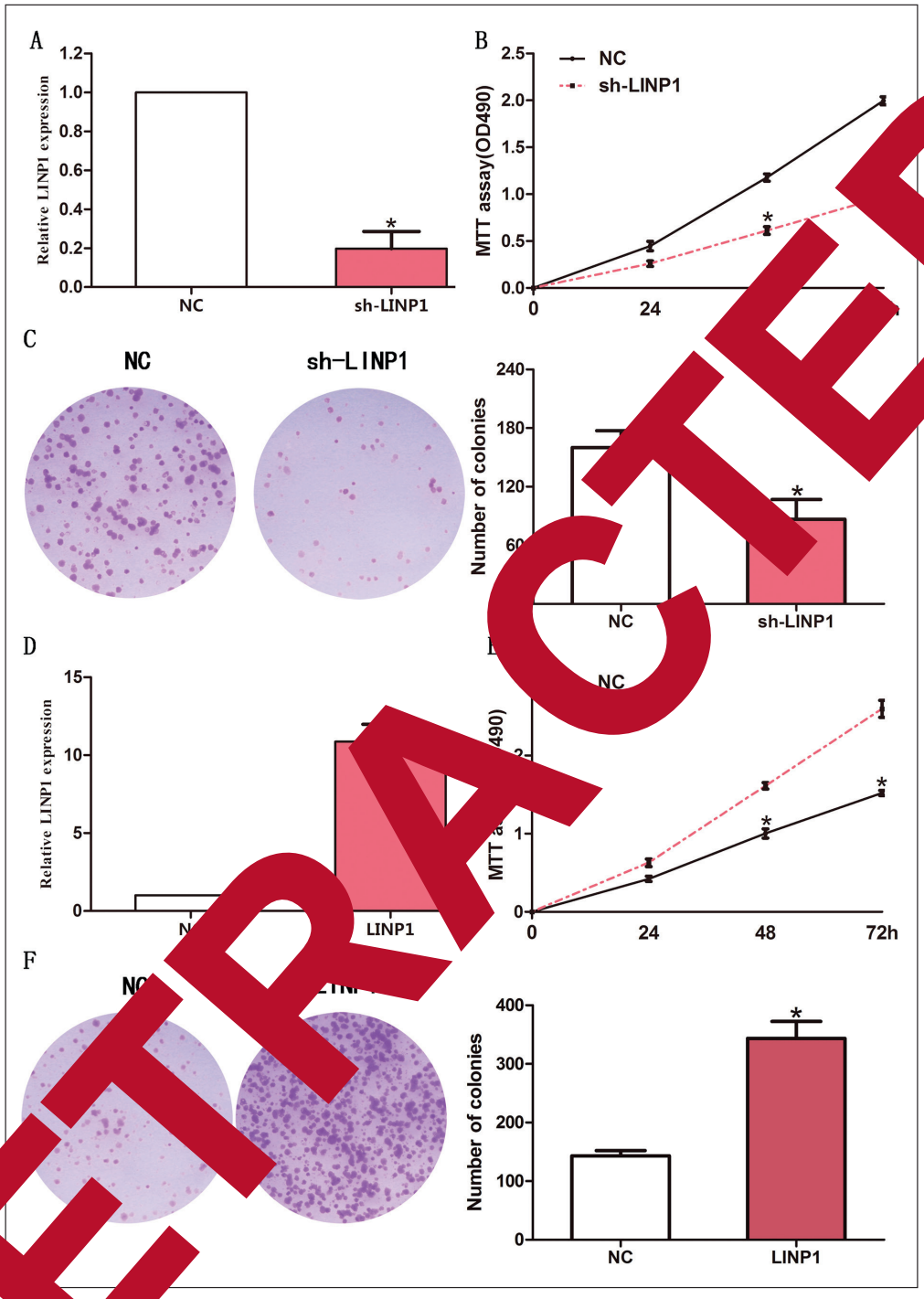


Figure 1. Expression level of LINP1 was significantly increased in Wilms' tumor patients. ***A***, LINP1 expression was markedly increased in Wilms' tumor tissues when compared with adjacent tissues. ***B***, The expression level of LINP1 was negatively associated with patients' overall survival time. *$p<0.05$.



2. LI ted Wilms' tumor cell proliferation. ***A,*** LINP1 expression in Wilms' tumor cells transfected with LIN hRNA (si P1) or scrambled oligonucleotides (NC) was detected by RT-qPCR. GAPDH was used as an internal co ***B,*** MTT assay revealed that the growth ability of Wilms' tumor cells was remarkably repressed in the sh-LINP1 group w th the NC group. ***C,*** Colony formation assay revealed that the number of Wilms' tumor cell colonies was ficantly reduced in the sh-LINP1 group when compared with the NC group (Magnification × 40). ***D,*** LINP1 expression Wilms' tumor cells transfected with LINP1 lentivirus (LINP1) or scrambled oligonucleotides (NC) was detected by RT- GAPDH was used as an internal control. ***E,*** MTT assay revealed that the growth ability of Wilms' tumor cells was n enhanced in the LINP1 lentivirus (LINP1) group when compared with the NC group. ***F,*** Colony formation assay also revealed that the number of Wilms' tumor cell colonies was remarkably increased in the LINP1 lentivirus (LINP1) group when compared with the NC group (magnification × 40). The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, compared with control cells.

down-regulation LINP1 (Figure 2B). Colony formation assay also revealed that the number of formed colonies was markedly reduced due to loss of LINP1 *in vitro* (Figure 2C). To further identify the function of LINP1 in Wilms' tumor, collected and digested cells from Wilms' tumor tissues were used for transfection of LINP1 lentivirus (LINP1) or scrambled oligonucleotides (NC). Similarly, RT-qPCR was utilized to detect LINP1 expression (Figure 2D). The results of the MTT assay revealed that the growth ability of Wilms' tumor cells was significantly promoted after over-expression of LINP1 (Figure 2E). Furthermore, colony formation assay demonstrated that the number of formed colonies was remarkably increased after over-expression of LINP1 in Wilms' tumor cells (Figure 2F).

Downregulation of LINP1 Inhibited Migration and Invasion of Wilms' Tumor Cells

Wound healing assay revealed that the relative migrating distance of Wilms' tumor cells was markedly repressed after the loss of LINP1 (Figure 3A). Subsequent transwell assay also indicated that the number of invaded cells was remarkably reduced after the loss of LINP1 in Wilms' tumor cells (Figure 3B). Similarly, after over-expression of LINP1 *in vitro*, the relative migrating distance of Wilms' tumor cells was significantly promoted (Figure 3C). In addition, transwell assay illustrated that the number of invaded cells was remarkably increased after over-expression of LINP1 in Wilms' tumor cells (Figure

Interaction Between Wnt/β-catenin Signaling Pathway and LINP1 in Wilms' Tumor

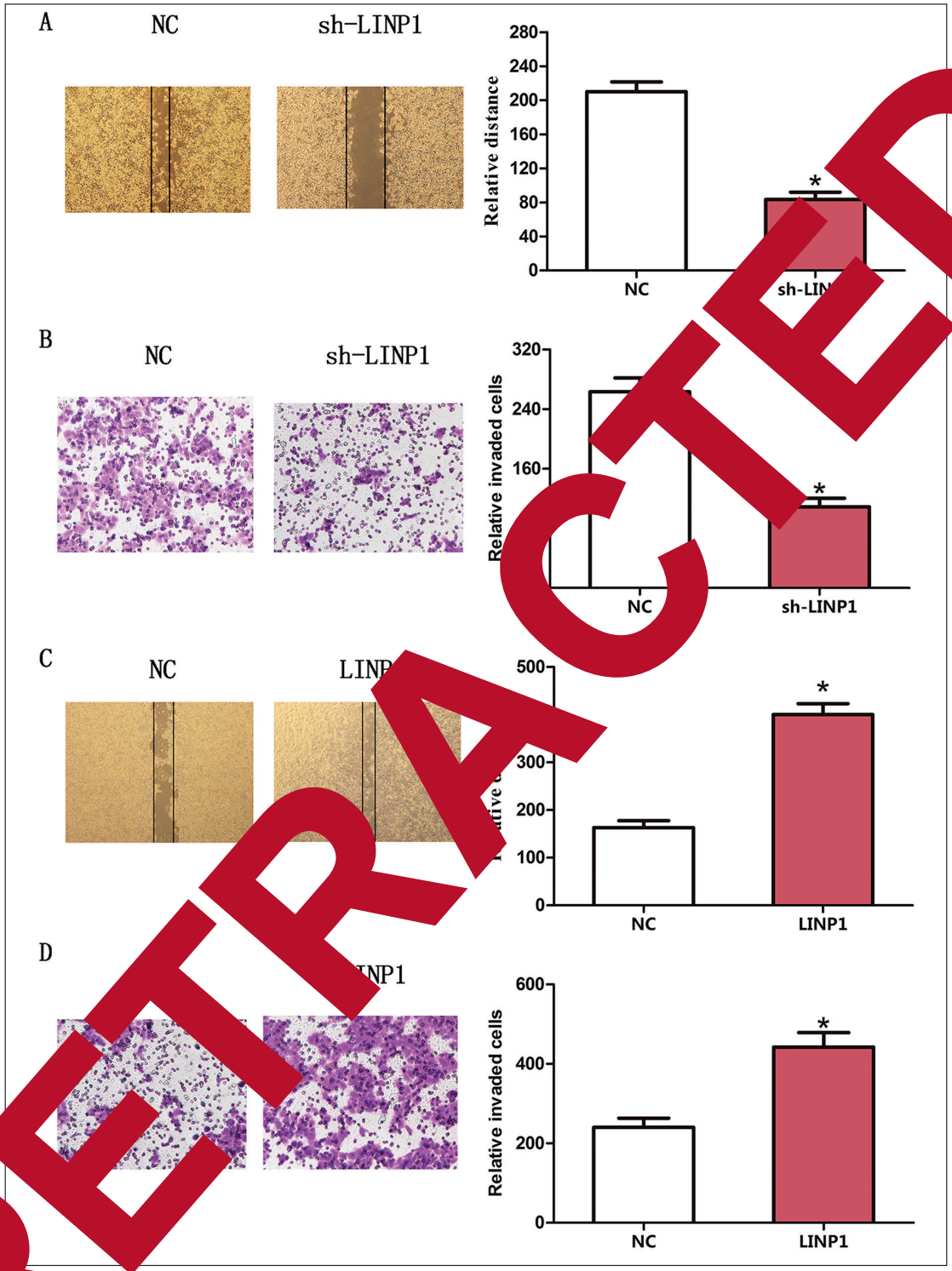
To explore the underlying mechanism of LINP1 function in Wilms' tumor, Western blot assay was conducted to detect the expressions of target proteins in the Wnt/β-catenin signaling pathway, including Wnt3a, β-catenin, C-myc and Survivin. The results showed that the protein expressions of Wnt3a, β-catenin, C-myc and Survivin were significantly down-regulated after the loss of LINP1 (Figure 4A). However, expressions of Wnt3a, β-catenin, C-myc and Survivin were markedly up-regulated after over-expression of LINP1 (Figure 4B). These results suggested that LINP1 participated in the regulation of the Wnt/β-catenin signaling pathway, further promoting Wilms' tumor development and progression.

Discussion

Numerous studies have proved that ncRNAs participate in a variety of important biological processes, including tumor growth, various cancers. Evidence has revealed that several ncRNAs play an important role in the development of Wilms' tumor. For instance, the over-expression of miR-21 and low-expression of PTEN inhibit the proliferation and invasion of Wilms' tumor cells by targeting FRS2, miR-195 depresses the proliferation and migration of Wilms' tumor. This may provide an innovative target for the diagnosis and therapy of Wilms' tumor. Overexpressing tumor suppressor miR-195, LINP1 functions as an oncogene in Wilms' tumor via regulating IKKalpha.

Non-homologous end joining (NHEJ) is one of the major mechanisms for repairing damaged DNA in cancer cells. Previous studies have demonstrated that lncRNA in non-homologous end joining pathway 1 (LINP1) promotes NHEJ mediated DNA repair in multiple malignancies. For example, LINP1 regulates NHEJ signal pathway and promotes DNA damage repair in cervical cancer cells, further decreasing the radiation sensitivity[11,12]. LINP1 functions as an oncogene in breast cancer, which facilitates the progression and chemoresistance[13]. The over-expression of LINP1 enhances the malignant progression of prostate cancer by negatively modulating p53[14]. However, Zhang et al[15] have shown that LINP1 serves as a tumor suppressor in lung cancer by inhibiting EMT. Therefore, we explored the role of LINP1 in Wilms' tumor. The results showed that LINP1 was significantly up-regulated in Wilms' tumor tissues. Meanwhile, LINP1 expression was associated with the prognosis of patients. Besides, the proliferation and metastasis of Wilms' tumor cells were markedly inhibited after the loss of LINP1. However, the proliferation and metastasis of Wilms' tumor cells were remarkably promoted after over-expression of LINP1. The above results indicated that LINP1 promoted tumorigenesis of Wilms' tumor and might act as an oncogene.

Wnt proteins mediate diverse processes during embryogenesis by modulating stem cell division and migration. Wnt3a, β-catenin, C-myc and Survivin are target proteins in the Wnt/β-catenin signaling pathway. Li et al[16] have suggested that aberrant activation of the Wnt/β-catenin signaling pathway plays an important role in regulat-



F promoted Wilms' tumor cell migration and invasion. ***A,*** The relative migrating distance of Wilms' tumor s was significantly decreased in the sh-LINP1 group when compared with the NC group (Magnification × 10). ***B,*** The nswell assay showed that the number of invaded Wilms' tumor cells was markedly decreased in the sh-LINP1 group when red with the NC group (Magnification × 40). ***C,*** The relative migrating distance of Wilms' tumor cells was remarkably in d in the LINP1 lentivirus (LINP1) group when compared with the NC group (Magnification × 10). ***D,*** The transwell assay showed that the number of invaded Wilms' tumor cells was significantly increased in the LINP1 lentivirus (LINP1) group when compared with the NC group (magnification × 40). The results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, compared with control cells.

ing the development of several human cancers. For instance, by modulating the Wnt/β-Catenin/Axin2 signaling, c-Myb facilitates the invasion and migration of breast cancer cells. Through the activation of the Wnt/β-catenin signaling inhibitors (DKK1 and SFRP2), TET1 serves as a tumor suppressor in ovarian cancer *via* inhibiting EMT[17]. LncRNA CRNDE enhances the proliferation and chemoresistance of colorectal cancer *via* modulating the expression of miR-181a-5p, which directly mediates the regulation of the Wnt/β-catenin pathway[18]. Our work[illegible] that the expressions of target pro[illegible] in t[illegible] Wnt/β-catenin signaling pathway[illegible]re significantly down-regulated after the [illegible]f LINP1. However, the protein expression le[illegible]f target

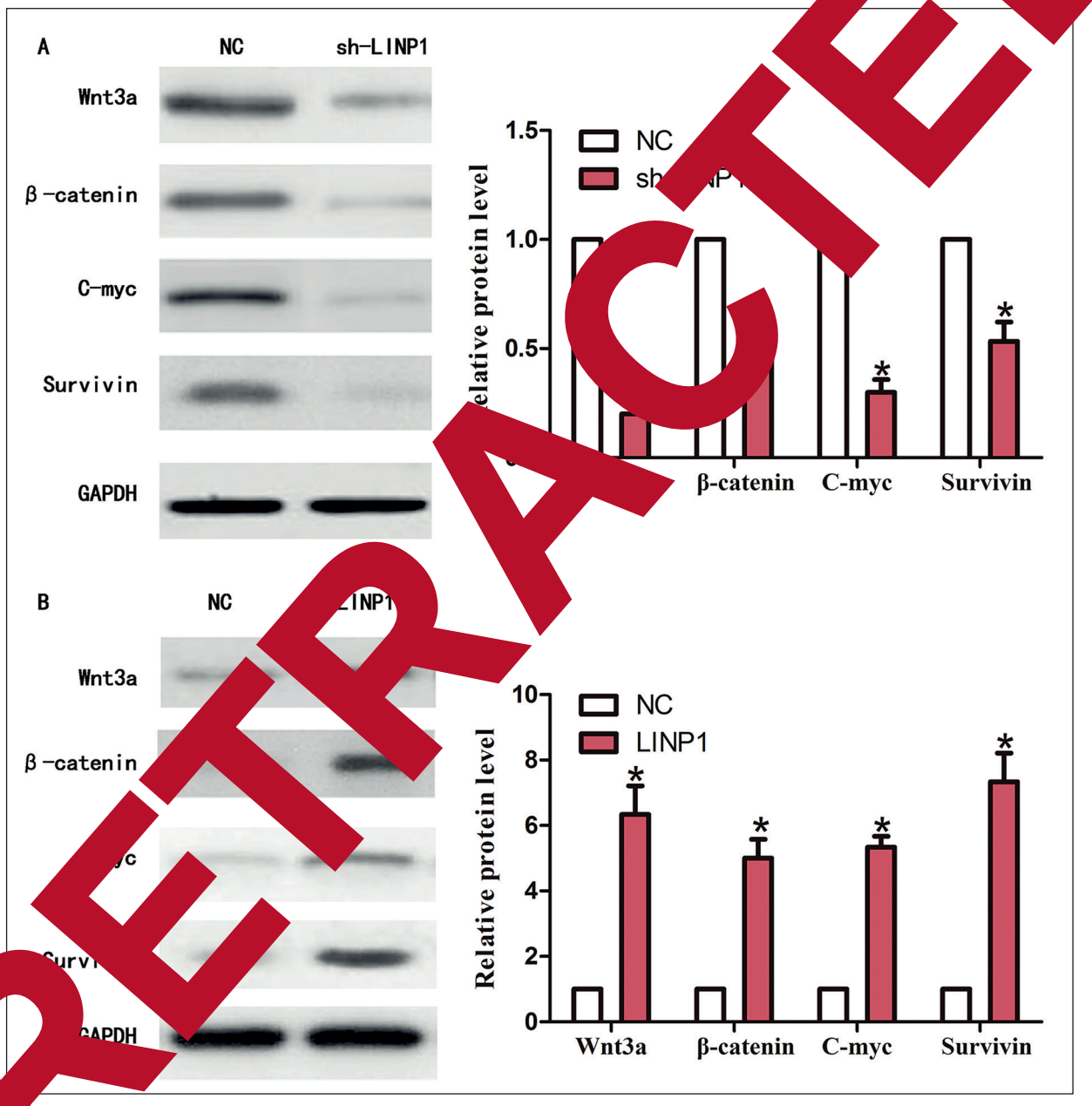


Figure 4. Interaction between LINP1 and Wnt/β-catenin signaling pathway. ***A,*** Western blot assay revealed that the expression of target proteins in the Wnt/β-catenin signaling pathway were significantly down-regulated in the sh-LINP1 group when compared with the NC group. ***B,*** Western blot assay indicated that the expressions of target proteins in the Wnt/β-catenin signaling pathway were markedly up-regulated in the LINP1 lentivirus (LINP1) group when compared with the NC group. The results represented the average of three independent experiments. *$p<0.05$.

proteins in the Wnt/β-catenin signaling pathway were remarkably up-regulated after over-expression of LINP1. All the results above suggested that LINP1 might promote tumorigenesis of Wilms' tumor *via* regulating the Wnt/β-catenin signaling pathway.

Conclusions

LINP1 enhanced Wilms' tumor cell proliferation and metastasis by regulating the Wnt/β-catenin signaling pathway. Our findings indicated that LINP1 might contribute to therapy for Wilms' tumor as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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