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Knockdown of long noncoding RNA DLX6-AS1 inhibits migration and invasion of thyroid cancer cells by upregulating UPF1

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Abstract. – OBJECTIVE: Recently, long noncoding RNAs (lncRNAs) have attracted much attention for their roles in tumor progression. The aim of this study was to investigate the expression of lncRNA DLX6 antisense RNA 1 (DLX6-AS1) in the development of thyroid cancer (TC), and to explore the underlying mechanism.

PATIENTS AND MATERIALS: DLX6-AS1 expression in both TC cells and tissue samples was detected by quantitative real-time Polymerase Chain Reaction (qRT-PCR). Moreover, transwell assay and wound healing assay were conducted. QRT-PCR and Western blot were used to explore the underlying mechanism. Furthermore, the function of DLX6-AS1 was identified *in vivo*.

RESULTS: DLX6-AS1 expression level in TC tissues was significantly higher than that of the corresponding normal tissues. Moreover, TC cell migration and invasion were markedly inhibited after DLX6-AS1 was knocked down in vitro. The mRNA and protein expressions of UPF1 were also remarkably up-regulated after knockdown of DLX6-AS1. Meanwhile, the expression level of UPF1 was negatively correlated with the expression of DLX6-AS1 in TC tissues. Furthermore, knockdown of DLX6-AS1 significantly inhibited tumorigenesis *in vivo*.

CONCLUSIONS: Knockdown of DLX6-AS1 could inhibit TC cell migration and invasion via up-regulating UPF1, which might be a potential therapeutic target in TC.

Key Words:
long non-coding RNA, DLX6-AS1, Thyroid cancer (TC), UPF1.

Introduction

Thyroid cancer (TC) is common cancer originated from follicular or parafollicular thyroid cells. The morbidity of TC has greatly increased over the past decades worldwide[1]. Since 2007, the incidence of TC has increased by an average of 4.5% annually in America. Currently, TC is the eighth most common cancer in China[2]. The prognosis of TC patients remains poor, which brings a huge burden to patients and the society[3]. Therefore, it is urgent to discover new biomarkers for TC diagnosis and treatment.

Long non-coding RNAs (lncRNA) are a subtype of non-protein coding RNAs with more than 200 nucleotides in length. Recent studies have indicated that lncRNA serves as a major contributor in a variety of cellular activities. For example, lncRNA CCAT2 facilitates the proliferation and metastasis of intrahepatic cholangiocarcinoma[4]. LncRNA FENDRR suppresses cell proliferation and malignancy in non-small cell lung cancer by sponging miR-761[5]. LncRNAAC132217.4 enhances the metastasis of oral squamous cell carcinoma cells *via* regulating IGF2[6]. LncRNA SNHG1 inhibits the differentiation of Treg cells impeding the immune escape of breast cancer[7]. In addition, down-regulated lncRNA UCA1 acts as a novel non-invasive diagnostic biomarker for bladder cancer[8]. However, the clinical role and biological mechanism of lncRNA DLX6 antisense RNA 1 (DLX6-AS1) in the development of TC have not been fully elucidated.

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In this study, we found that the expression of DLX6-AS1 was remarkably up-regulated in TC tissues. Knockdown of DLX6-AS1 markedly inhibited the migration and invasion of TC cells *in vitro*. Furthermore, we explored the underlying mechanism of DLX6-AS1 function in TC metastasis.

Patients and Methods

Cell Lines and Clinical Samples

60 TC patients who received surgery at Harbin Medical University Cancer Hospital were enrolled in this study. Human tissues were collected from these patients. Before the operation, written informed consent was achieved. No radiotherapy or chemotherapy was performed for any patient before the operation. Tissues obtained from surgery were stored immediately at -80°C for use. All tissues were confirmed by an experienced pathologist. This study was approved by the Ethics Committee of the Harbin Medical University Cancer Hospital.

Cell Culture

Human thyroid carcinoma cell lines (…1, K1, SW579) and normal human thyroid cel… (Nthy-ori 3-1) were provided by the Institute… Biochemistry and Cell Biology, Chi…ese Acad…my of Science (Shanghai, Chi… …lls were cultured in Dulbecco's M…d Ea… Medium (DMEM; HyClone, S… Logan… USA) consisting of 10% fetal b… seru… Technologies, Gaithe…rg, …A) and penicillin. Besides, th…s were …ined in an incubator with 5… at 37°C.

Cell Transfe…on a… …ouping

Lentivi… small hairpin… (shRNA) targeting DLX… …S1 was synthesiz… …nd cloned into pLenti… …a-EGF… F2A-Puro vector (Biosettia Inc… Dieg…, USA). Subsequently, TPC-1 cells… … for p… …ging viruses, DLX6-AS1 sh… …sh- DL… …AS1) and empty vector …ol).

Qu…itative R…al Time-Polymerase Chain Re… …TPCR)

…tal RN… …n tissues and cells was extracted …ng TRIzol reagent (Invitrogen, Carlsbad, CA, … Subsequently, extracted total RNA was rev… …transcribed into complementary deoxyribose nucleic acids (cDNAs) through the Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Primers used f… Real Time-Polymerase Chain Reaction… were as follows: DLX6-AS1 pri… forwar… 5′-AGTTTCTCTCTAGATTGCC…′, reverse: 5′- ATTGACATGTTAGTGCC…′. Glyceraldehyde 3-phosphate dehydrogena… …APDH) primers forward: 5′-CCA…TCAG… …GC CAATGCTGG-3′ and r… …se: 5′-TGATG… GGACTGTGGTCAT…-3′. Th… …ermal c…e was as follows: 30 s… …95°C … …c for 4… cycles at 95°C, and 35 s… at …

Wound He…g Assay

Cells we… …transferred i… …-well plates, followed… cu… …in DMEM medium overnight. After scratch… …h a plastic tip, the cells were… …red in ser… …e DMEM. Wound cl… …was viewed at spe…ic time points. Each …y was indepen…dently repeated three times.

…nswell Ass…

… after tr… …ection, 2 ×10^5 cells in 100 µL seru… …M were transformed to the upper chan… …of an 8-µm culture insert (Corning, …owell, MA, USA) coated with or without 50 µg …el (BD Biosciences, Franklin Lakes, NJ, …SA). …eanwhile, 20% FBS-DMEM was added to the lower chamber of culture inserts. 24 h later, these inserts were treated with methanol for 30 min and stained with hematoxylin for 20 min. Migrated and invaded cells were observed under an inverted microscope (×40), and the number of cells was counted. Three fields were randomly selected for each sample.

Western Blot Analysis

Radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was utilized to extract the total protein in cells. The concentration of extracted protein was quantified by the bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China). Target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the cells were incubated with primary and corresponding secondary antibodies. Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-GAPDH and rabbit anti-UPF1, as well as goat anti-rabbit secondary antibody. Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of the protein expression.

Xenograft Model

For tumor metastasis assay, transfected TPC-1 cells were injected into the tail vein of NOD/SCID mice (4-5 weeks old). After 4 weeks, the mice were sacrificed, and lung tissues were extracted. The number of metastatic nodules in lung tissue was then counted. The animal experiments were approved by the Animal Ethics Committee of Harbin Medical University.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Chicago, IL, USA) was used for all statistical analysis. Data were presented as mean ± Standard Deviation (SD). The Student's *t*-test was selected when appropriate. $p<0.05$ was considered statistically significant.

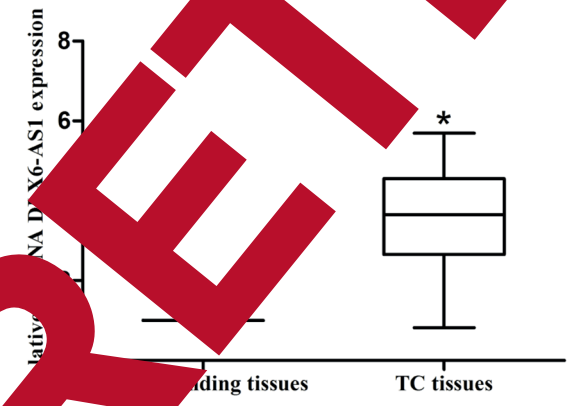
Results

DLX6-AS1 Expression Level in TC Tissues and Cell Lines

QRT-PCR was first conducted to detect DLX6-AS1 expression in 60 patients' tissues and TC cell lines. As a result, DLX6-AS1 was cantly upregulated in TC tissues and ce es (Figure 1).

Knockdown of DLX6-AS1 In ited Migration and Invasion o

DLX6-AS1 expression in cells significantly higher than that of -ori 3- (Figure 2A). According to D AS1



ure 1. Expression level of DLX6-AS1 increased sig- tly in TC tissues. DLX6-AS1 expression increased i in TC tissues compared with the corresponding norm ssues. Data were presented as mean ± standard error of the mean. *$p<0.05$.

TC cells, TPC-1 cell line was chosen for knockdown of DLX6-AS1 *in vitro*. QRT-PCR w then utilized to detect DLX6-AS1 expres 2B). Subsequent wound healing as ound th knockdown of DLX6-AS1 ma y inhibited TC cell migration (Figure 2C). ults of the transwell assay showed that the i n and invasion of TC cells wer arkably ite after DLX6-AS1 was k ed down (Fig and 2E).

The Interaction Be PF1 a DLX6-AS1 in

UPF1 was dicted as t protein of DLX6-AS1 h Starbase (http://starbase.sys u.c base2/rbpLncRNA.php). The results of qRT- howed that, compared with mpty vecto rol) group, the expr vel of UPF1 was gnificantly higher in cells of DLX6-AS1 shRNA (sh-DLX6-AS1) up (Figure Western blot assay demoned that after X6-AS1 was knocked down, t ein exp on of UPF1 was significantly up- ure 3B). Furthermore, we found that UPF1 xpression in TC tissues was markedly lower than that of the corresponding normal (Figure 3C). Correlation analysis demonrate hat UPF1 expression was negatively correlated with DLX6-AS1 expression in TC tissues (Figure 3D).

DLX6-AS1 Knockdown Inhibited Tumor Metastasis In Vivo

The ability of DLX6-AS1 in tumor metastasis was detected *in vivo*. The number of metastatic nodules in lung tissues of the sh-DLX6-AS1 group was significantly reduced when compared with the control group (Figure 4A). The expression level of DLX6-AS1 and UPF1 in dissected nodules tissues was detected by RT-qPCR. The results showed that DLX6-AS1 was lowly-expressed in the shRNA group when compared with the control group (Figure 4B). However, UPF1 was highly expressed in the shRNA group when compared with the control group (Figure 4C). The above results suggested that DLX6-AS1 could induce tumor metastasis *via* down-regulating UPF1 *in vivo*.

Discussion

Plenty of lncRNAs have been proved to play important roles in the occurrence and progres-

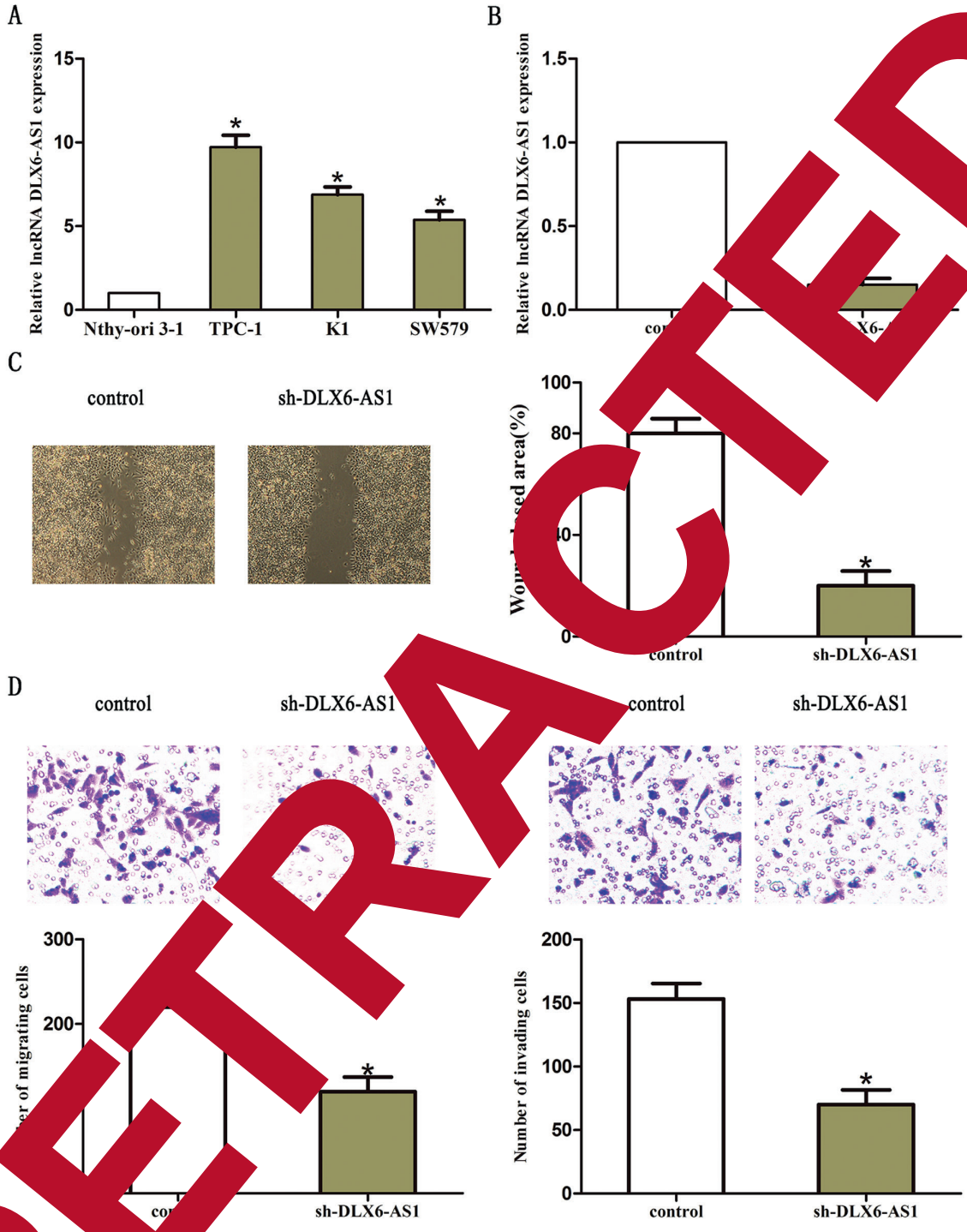


Figure 7. Knockdown of DLX6-AS1 inhibited TC cell migration and invasion. **A,** Expression level of DLX6-AS1 relative to GAPDH in human TC cell lines and Nthy-ori 3-1 (normal human thyroid cell line) was determined by qRT-PCR. **B,** DLX6-AS1 in TPC-1 TC cells transduced with DLX6-AS1 shRNA (sh-DLX6-AS1) and empty vector (control) was detected by qRT-PCR. GAPDH was used as an internal control. **C,** Wound healing assay showed that the migrated length of TPC-1 cells in the sh-DLX6-AS1 group decreased significantly when compared with the control group (magnification: 40×). **D,** Transwell assay showed that the number of migrated cells decreased markedly *via* knockdown of DLX6-AS1 in TPC-1 TC cells (magnification: 40×). **E,** Transwell assay showed that the number of invaded cells decreased remarkably *via* knockdown of DLX6-AS1 in TPC-1 TC cells (magnification: 40×). The results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, compared with control cells.

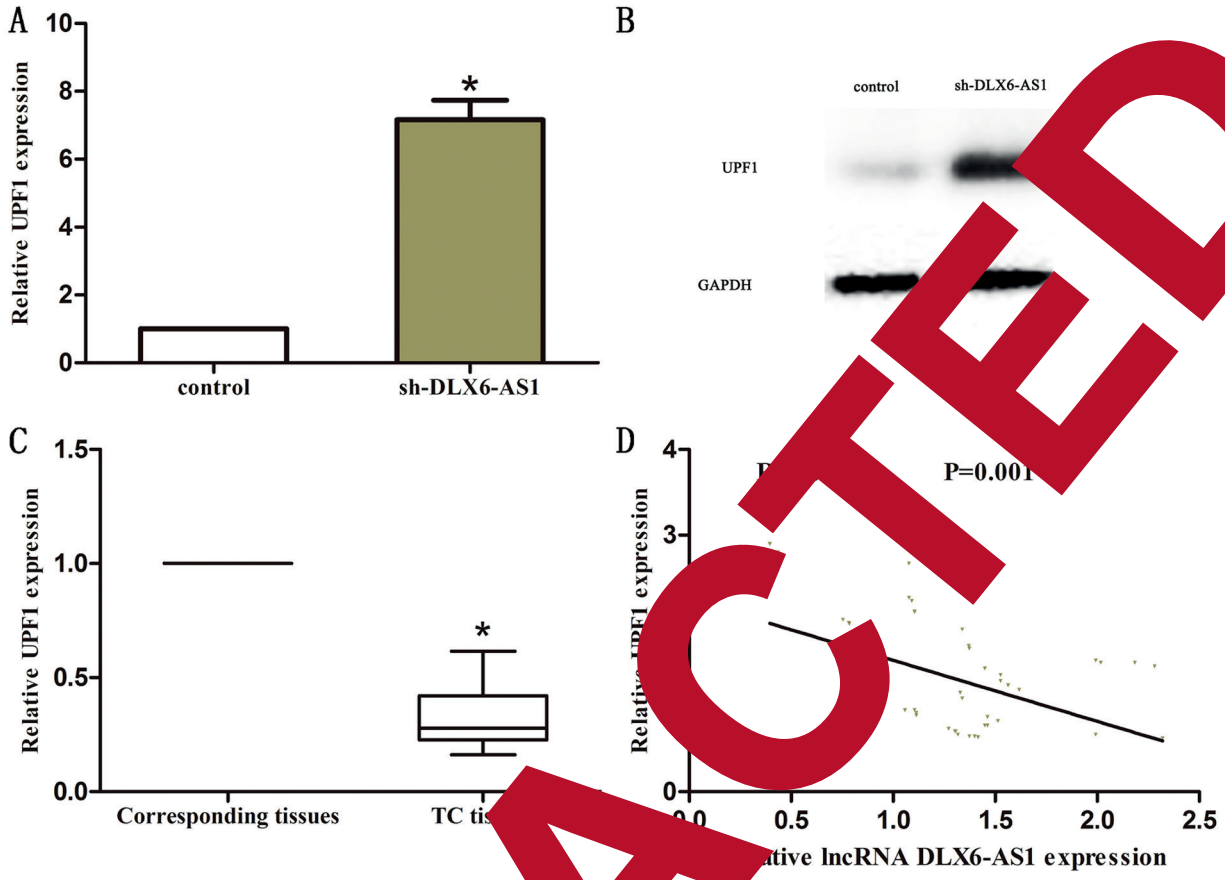


Figure 3. Interaction between DLX6-AS1 and UPF1. **A,** qRT-PCR results showed that UPF1 expression was significantly higher in the DLX6-AS1 shRNA (sh-DLX6-AS1) group compared with the empty vector (control) group. **B,** Western blot assay revealed that protein expression was up-regulated in the DLX6-AS1 shRNA (sh-DLX6-AS1) group compared with the empty vector (control) group. **C,** UPF1 was significantly down-regulated in TC tissues when compared with the corresponding normal tissues. **D,** linear correlation between the expression level of UPF1 and DLX6-AS1 in TC tissues. The results represented the average of three independent experiments. Data were presented as mean ± standard error of the mean. *$p<0.05$.

sion of TC. For example, lncRNA-SNHG7 regulates the proliferation and apoptosis and invasion of bladder cancer cells[9]. Up-regulated lncRNA ANRIL promotes tumor metastasis in thyroid cancer via TGF-beta/Smad signaling pathway[10]. LncRNA HOTAIR promotes the development of cervical cancer by regulating Notch pathway[11]. Meanwhile, lncRNA ENST00000537266 and ENST00000424... are important regulators of proliferation in papillary thyroid cancer[12]. In addition, lncRNA MEG3 functions as a tumor suppressor in cervical cancer, which leads to the inhibition of tumor growth[13].

Long non-coding RNA DLX6 antisense RNA 1 (DLX6-AS1), located in 7q21.3, has been recently explored. Scholars[14,15] have found that DLX6-AS1 is abnormally expressed in several cancers, and is related to tumor progression. For example, DLX6-AS1 promotes the proliferation and invasion of renal cell carcinoma cells by targeting miR-26a axis[16]. DLX6-AS1 induces cell invasion by regulating miR-181b in pancreatic cancer[17]. DLX6-AS1 promotes the proliferation and metastasis of non-small cell lung cancer by regulating miR-144[18]. In this study, we found that DLX6-AS1 was significantly up-regulated both in TC tissues and cells. After DLX6-AS1 was knocked down, TC cell migration and invasion was found markedly inhibited. The above results indicated that DLX6-AS1 promoted tumorigenesis of TC and might act as an oncogene.

UPF1 (UPF1 RNA helicase and ATPase) was predicted as the target protein of DLX6-AS1 through Starbase v2.0. It his been indicated that

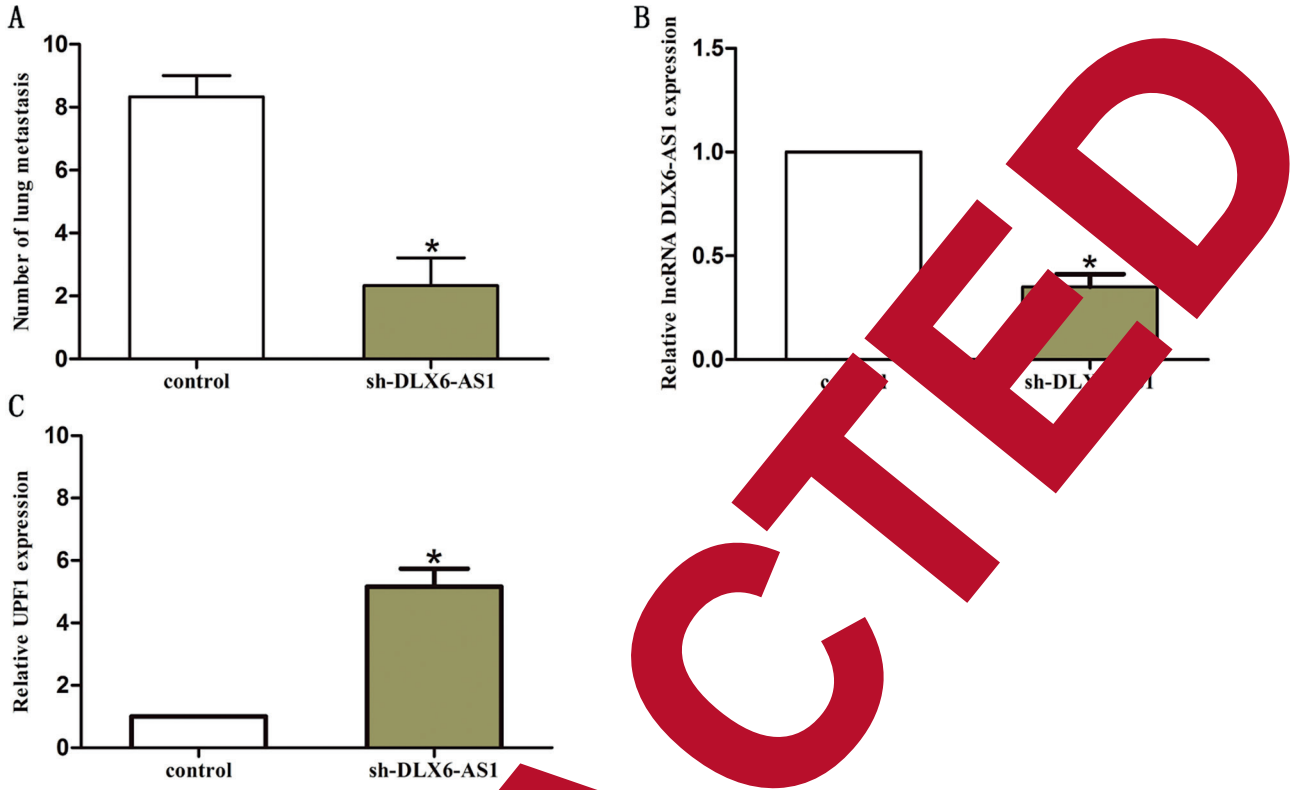


Figure 4. Knockdown of DLX6-AS1 inhibited m... *vivo*. **A,** The number of metastatic nodules in lung tissues of sh-DLX6-AS1 group was significantly reduced ...ared ...ol group. **B,** DLX6-AS1 was lowly-expressed in dissected nodules of the sh-DLX6-AS1 group compa... with t... up. **C,** UPF1 was highly-expressed in dissected nodules of the sh-DLX6-AS1 group compared with the ...p. The results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.$ ...pared with control cells.

UPF1 plays a crucial rol... tumor ...th and invasion in multiple canc... r in... ticipates in regulated by lncRNA ...AL... the development c... stric ca... UPF1 also represses the de... ent of hepa... lar carcinoma *via* tar... g... d7[20]. Moreov... SNHG6 acts as an on... gene in ...tocellular carcinoma by regulati... miR-101-3p/... xis[21].

In the ...ent work, UPF1 e... sion was markedly u... gulated ...fter knockdown of DLX6-AS1. Mo... r, UPF... pression in TC tissues was negative... re... with D... 6-AS1 expression. All the abo... ts sug... d that DLX6-AS1 might ...te tu... n... of TC *via* down-regulating UP... Further... knockdown of DLX6-AS1 sign... ntly inhi... ed metastasis *in vivo*.

Conclusions

...e results identified that DLX6-AS1 was remarkably up-regulated in TC tissues and cell lines. Besides, DLX6-AS1 remarkably enhanced TC cell migration and invasion by down-regulating UPF1. We suggest that DLX6-AS1 might contribute to therapy for TC as a candidate target.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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