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Upregulation of long noncoding RNA DLX6-AS1 promotes cell growth and metastasis in esophageal squamous cell carcinoma *via* targeting miR-577

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Abstract. – OBJECTIVE: Esophageal squamous cell carcinoma (ESCC) is the most malignant type of esophageal cancer. Although significant advances have been made in ESCC diagnosis and therapy, its poor pathogenesis and prognosis remain a life-threatening problem. Meanwhile, long noncoding RNAs (lncRNAs) exert a pivotal function in tumorigenesis. In this research, we aimed to explore the association between the aberrant expression of lncRNA DLX6-AS1 and the development and metastasis of ESCC.

PATIENTS AND METHODS: DLX6-[illegible] pression was monitored by quantitative [illegible]al Time-Polymerase Chain Reaction (qRT-PC[illegible] ESCC specimens. Moreover, experiments w[illegible] conducted to detect the effect of DLX6-AS1 [illegible] the cell proliferation and meta[illegible] ESCC. I[illegible] addition, the underlying mec[illegible]sm was further explored through luciferas[illegible]says a[illegible]RNA immunoprecipitation assay[illegible]).

RESULTS: DLX6-AS1 [illegible]ssio[illegible] significantly higher i[illegible]ESC[illegible]ens. Moreover, cell proliferati[illegible]nd m[illegible]is of ESCC cells could be inh[illegible]d *via* redu[illegible]LX6-AS1 expression. Be[illegible]DLX6-AS1 w[illegible]garded as an oncoge[illegible]n[illegible]. Furthermo[illegible], DLX6-AS1 acted as [illegible]ompe[illegible]dogenous RNA *via* sponging [illegible]R-577 in ESC[illegible].

CONC[illegible]ONS: In summ[illegible]LX6-AS1 promotes [illegible]lopment and metas[illegible]sis of ESCC by spon[illegible]miR-5[illegible]nd could be a potential therape[illegible]target[illegible].

Key W[illegible]
[illegible]ong [illegible]ling [illegible], DLX6-AS1, Esophageal [illegible]us ce[illegible]a, MiR-577.

[illegible]ntroduction

[illegible]sophageal cancer (EC) is an ordinary ma[illegible]g[illegible], that ranks 9th most common cancer wor[illegible]de and the fifth most frequent in China[1,2].

Esophag[illegible]squa[illegible]ell carcinoma (ESCC) accounts for almost 80[illegible]C cases in South-Eastern[illegible]. Due to the[illegible]ficulty of prophase c[illegible]al diagnosis, most of esophageal squamous [illegible] carcinoma (ESCC) patients develop a high [illegible] of metastasi[illegible]d recurrence with the overall [illegible]ar survival r[illegible]below 10%[5]. It is vital in the t[illegible]nt of m[illegible]ant tumors to suppress ESCC prol[illegible] metastasis, which is the main and hot issue in the current study.

Non-coding RNAs (ncRNAs) account for [illegible]al transcribed RNAs. Long noncoding [illegible]NAs (lncRNAs), as a subtype of ncRNAs, are longer than 200 nucleotides. Recently, evidence has proved that lncRNAs serve as important regulators in the progression of malignant tumors. LncRNA 91H functions as an oncogene in breast cancer by up-regulating the expression of H19/IGF2 which increases aggressive phenotype of breast cancer cells[6]. LncRNA SNHG7 promotes tumor proliferation in osteosarcoma by targeting miR-34a[7]. Through interacting with miR-124, lncRNA XIST serves as an oncogene which promotes cell growth, migration, and invasion in bladder cancer[8]. Moreover, few researches also uncover the role of lncRNAs in ESCC progression. By regulating the stability of DNMT1 and depressing the expression of tumor suppressors, lncRNA LUCAT1 promotes esophageal squamous cell carcinoma formation and cell metastasis[9]. LncRNA RUNX1-IT1 acts as a tumor suppressor in esophageal squamous cell carcinoma by the inhibition of cell migration and cell proliferation[10]; however, the underlying mechanism of how lncRNAs function in ESCC has not been fully understood.

LncRNA DLX6-AS1 is a novel lncRNA which has been found aberrantly expressed in some malignant tumors and functions as an oncogene.

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Whereas, its role in ESCC has not been studied. In this work, we found out that lncRNA DLX6-AS1 was aberrantly expressed in ESCC tissues. Moreover, the proliferation and metastasis of ESCC cells were inhibited by reducing DLX6-AS1 expression. The underlying mechanism of howDLX6-AS1 functions in ESCC development was further explored.

Patients and Methods

Cell Lines and Clinical Samples

55 ESCC tissues were enrolled from patients receiving resection surgery at the Second Hospital of Dalian Medical University. All tissues were kept at –80°C until further use. No preoperative chemotherapy or radiation was performed prior to surgery. The protocol of the study was approved by the Ethics Committee of the Second Hospital of Dalian Medical University and was performed as Declaration of Helsinki Principles required. Written informed consent was achieved before the operation.

Cell Culture

Four human ESCC cell lines (TE-1, E-1, EC-1, and Eca-109), and one normal h esophageal epithelial-1 cell line (HEEC) w offered by the American Type Cultre Collecti (ATCC; Rockefeller, MD, US re medi- um was Roswell Park Me l Im te-1640 (RPMI-1640; HyClone, I , UT, A) con- taining 10% fetal bovin n (FB Logan, UT, USA) and % pe eptomycin at 37°C with 5% C

Cell Transfec

We purcha enti xpressing short-hair- pin RNA (s RNA) agains X6-AS1 from Ge- nePharm anghai, Chin ich was then used fo nsfecti n of Eca-109 ells using Lipo- fecta 2000 itrogen, Carlsbad, CA, USA). 24 cel ere har ted for further exper- iment

Extra d Quantitative Rea me-Po erase Chain Reaction (qR CR)

the manufacturer's protocol, Zol reagent (Invitrogen, Carlsbad, CA) was zed to extract the total RNA from ESCC t and cells. Reverse transcription was con- duct using the Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The $2^{-\Delta\Delta Ct}$ method was utilized to cal tive expression. The primers using qRT-P are the following: DLX6-AS1 r ers forward 5′-AGTTTCTCTCTAGATTGC 3′ reverse 5′-ATTGACATGTTAGTGCCCT GAPDH primers forward 5′-CCA ATCA GG CAATGCTGG-3′ and re e 5′-TGA GGACTGTGGTCATT 3′.

Cell Proliferation

Following the ant s prot , 4×10^3 transfected ce were pla 9 ll plates and cell pr tion was as y the Cell Proliferati t Kit I (MT Roche, Basel, Switzerl) at h, 48 h, and 72 h. The absorbance at 490 as assessed with an enz ked immun nt assay (ELISA) r system (Multiskan Ascent, LabSystems, sinki, Finland).

5-Ethynyl Deoxyuridine (EdU) Incorporation Assay

E ne, Mannheim, Germany) was used to detect cell proliferation of transfected lls. Briefly, 6×10^3 Eca-109 cells were seeded in l plate. 48 h later, the culture medium as d with 50 μM EdU for 2 h, which were then fixed with 4% formaldehyde for 30 min and incubated with 2 mg/mL Glycine for 10 min. After cells were permeabilized by 0.5% Triton X-100 for 20 min, they were added with staining liquid and Hoechst 33342. The percentage of EdU positive cells was monitored using a fluorescence microscope (Nikon Eclipse Ti Microscope, Tokyo, Japan).

Wound Healing Assay

Cells, transferred into 6-well plates, were cultured in RPMI-1640 medium overnight. Once scratched with a plastic tip, cells were cultured in serum-free RPMI-1640. 48 h later, wound closure was viewed. Each assay was repeated three times independently.

Transwell Assay

After transfection, cells (5×10^4) in 200 μL serum-free RPMI-1640 were added to the top chamber of an 8 μm pore size insert (Corning, Corning, NY, USA) with or without 50 μg Matrigel (BD; Bedford, MA, USA). RPMI-1640 and FBS were added to the lower chamber. 48 h later, the top surface of chambers was treated by methanol for 30 min after wiped by a cotton swab.

Then, they were stained in crystal violet for 20 min. Three fields were used to count the data for migration and invasion membrane.

Luciferase Assays

DLX6-AS13′-UTR was cloned into the pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3′-UTR. Next, site-directed mutagenesis of the miR-577 binding site in DLX6-AS1 3′-UTR was performed by quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as mutant (MUT) 3′-UTR. Following was a transfection of WT-3′-UTR or MUT-3′-UTR and negative control or miR-577 for 48 h. Then, the luciferase assay was conducted on the dual luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Assay

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was conducted according to the manuscript. Briefly, treated Eca-109 cells were lysed in the RIP lysis buffer, which was previously added with protease inhibitor and RNase inh[illegible]. Then, they were incubated with the RI[illegible] containing magnetic beads coated with [illegible]2 antibodies (Millipore, Billerica, MA, USA[illegible] 2 h at 4°C.Immuglobin G (IgG) was identi[illegible] as negative control (input group). QRT-PCR w[illegible] used to monitor coprecipitated

Statistical Analysis

All these results were [illegible]ed w[illegible] Product and Service S[illegible]tio[illegible] 19.0 (SPSS Inc., Chicago, IL, [illegible]). Indep[illegible]t *t*-test was performed for comparison between the two å(-standard deviation). The statistical s[illegible] was set at $p<0.05$.

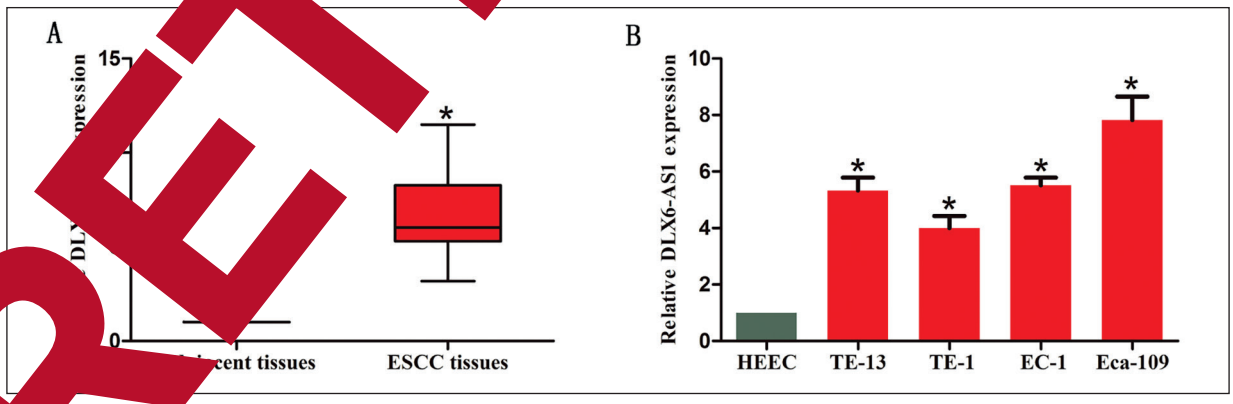
Results

Upregulation of DLX[illegible]S1 in ESCC Tissues and C[illegible]

To identify the ro[illegible] DLX6[illegible]1 in the tumorigenesis of ESCC, [illegible]late[illegible] DLX6[illegible]d expression levels i[illegible]5 p[illegible]SCC ar[illegible]djacent tissues by qu[illegible]tative R[illegible]ime[illegible]ymerase Chain Rea[illegible] (qRT-PCR). [illegible]-AS1 was markedly [illegible]ed in ESCC [illegible]sues (Figure 1A). M[illegible]while, [illegible]detected the expression level of DLX6-AS1 [illegible]CC cells and normal hum[illegible]phageal epith[illegible] cell (HEEC). The r[illegible] showed that DLX6-AS1 expression level [illegible]SCC cells w[illegible]s higher than that of HEEC [illegible]gure 1B).

K[illegible]down [illegible]LX6-AS1 Led to the [illegible]of Cell Proliferation in ESCC Cells

To further determine whether DLX6-AS1 was [illegible] with the occurrence of ESCC, we [illegible]rthe[illegible] explored the role of DLX6-AS1 in ESCC cells. Eca-109 cell line was selected for transfection of DLX6-AS1 shRNA due to its highest level of DLX6-AS1 among four ESCC cell lines. QRT-PCR results showed that DLX6-AS1 shRNA group had a better transfection efficiency compared with control group (Figure 2A). In this work, the growth ability of Eca-109 cells was detected by methyl thiazolyl tetrazolium



[illegible]ure 1. DLX6-AS1 expression in ESCC tissues and cells**. A,** DLX6-AS1 expression was significantly increased in the [illegible] tissues compared with adjacent tissues. **B,** Expression levels of DLX6-AS1 were determined in the human ESCC cell li[illegible]normal human esophageal epithelial-1 cell (HEEC) by qRT-PCR. GAPDH was used as an internal control. Data are presc[illegible]as the mean ± standard error of the mean. *$p<0.05$.

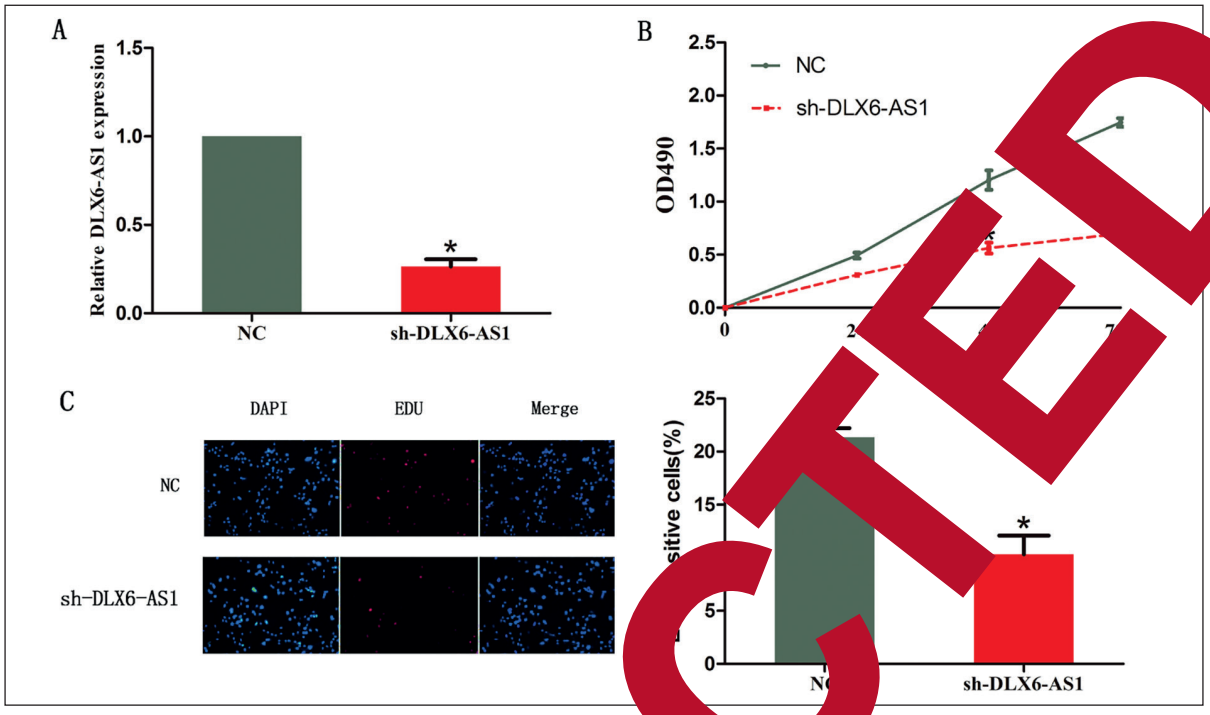


Figure 2. Knockdown of DLX6-AS1 inhibited ESCC cell proliferati[on] expression in Eca-109 cells transduced with DLX6-AS1 shRNA (sh-DLX6-AS1) and negative control (NC) was de[tected] RT-PCR. GAPDH was used as an internal control. **B,** MTT assay showed that knockdown of DL[X6-AS1] significantly enhanced cell growth ability in Eca-109 cells. **C,** EdU incorporation assay also showed that EdU positive ce[lls] knockdown of DLX6-AS1 in Eca-109 cells (magnification: 40×). The results represent the average of three indepe[ndent] experi[ments] standard error of the mean). *p<0.05.

(MTT) assay after knockdown of DLX6-AS[1]. Results disclosed that knoc[kdown] of DLX6-AS1 considerably inhibited [cell] gro[wth] ability of Eca-109 cells (Figure 2[B]). Further[more], EdU incorporation assay al[so] evidenc[ed] positive cells were re[duced] [by] ckdown of DLX6-AS1 in Eca-[109] cells (F[igure] 2C).

Knockdown [of DLX6]-AS1 Led to the Inhibition [of] Migration and Invasion [of] ESCC Cell[s]

We fu[rther] identified wh[ether] knockdown of DLX6-[AS1] affected the metastasis of ESCC. Resul[t] of wo[und] healing assay showed that kn[ock]down o[f DL]X6-AS[1] inhibited the migrated le[ngth] of ESCC ce[lls] (Figure 3A). Transwell assay rev[ealed] that [the] number of migrated and invad[ed] cel[ls] significantly decreased after DL[X6-]AS1 was [knoc]ked down in ESCC cells (Fig[ure] 3B and 3C).

[miR]-577 was a Direct Target of [DLX]6-AS1 in ESCC

[The] potential microRNAs of DLX6-AS1 was pred[icted] by DIANA LncBASE Predicted v.2. MiR-577, containing a binding area of DLX6-AS1, was chosen for our further study (Figure 4A). Then qRT-PCR results showed that the expression level of miR-577 in Eca-109 cells was significantly higher in DLX6-AS1 shRNA(sh-DLX6-AS1) group when compared with the miR-577 level in control group (Figure 4B). Furthermore, the luciferase assay revealed that co-transfection of DLX6-AS1-WT and miR-577 largely decreased the luciferase activity, while co-transfection of DLX6-AS1-MUT and miR-577 had no effect on the luciferase activity either (Figure 4C). Furthermore, the RIP assay showed that DLX6-AS1 and miR-577 were significantly enriched in Ago2-containing beads compared to input group (Figure 4D).

Discussion

Several studies[11-13] have suggested that lncRNAs affect carcinogenesis of ESCC by regulation of various cellular processes. LncRNA DLX6 antisense RNA 1 (DLX6-AS1), a newly discovered lncRNA, has caught more atten-

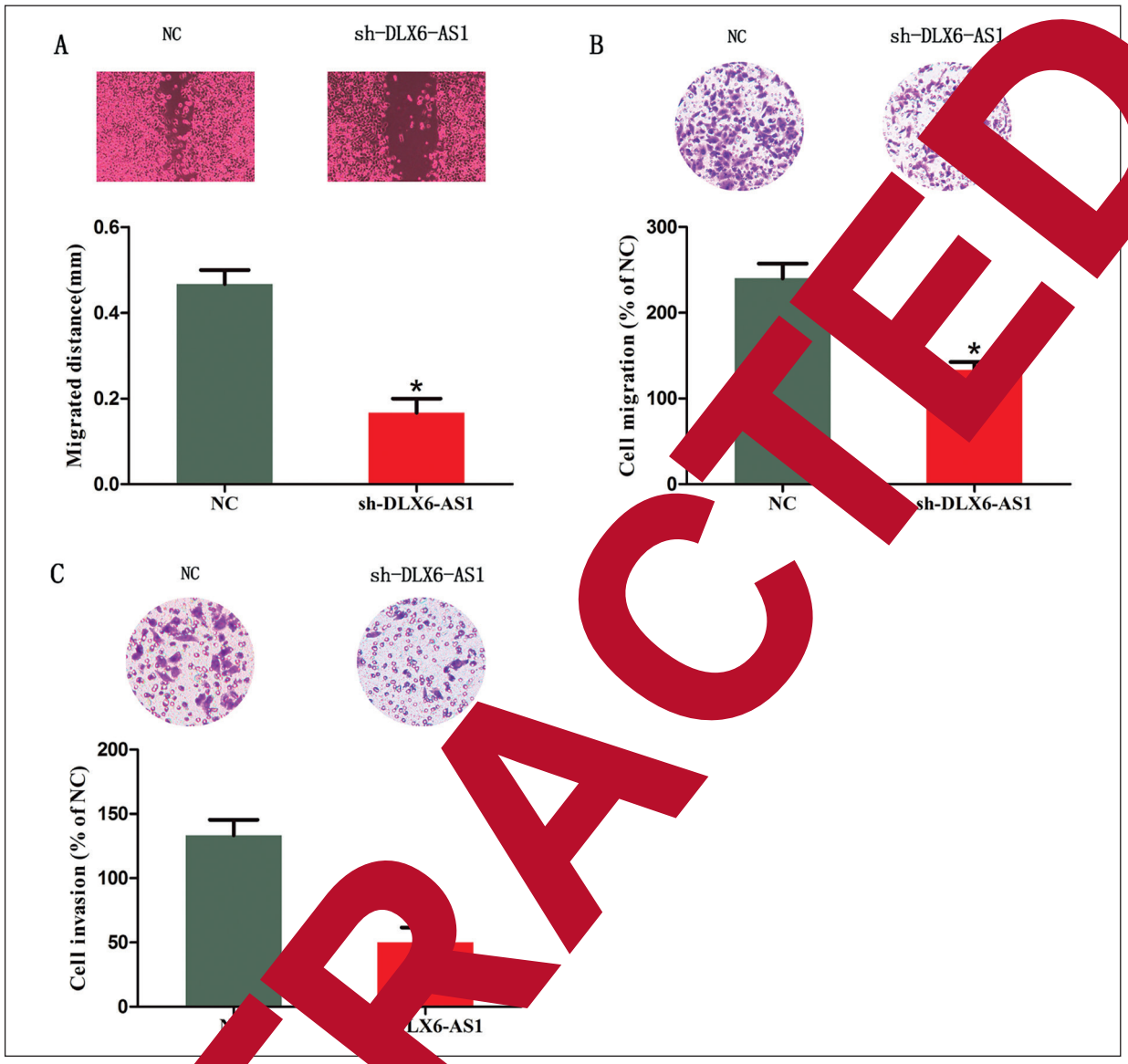


Figure 3. Knockd LX6-AS1 repr SCC cell migration and invasion. **A,** Wound healing assay showed that knockdown of DL cantly reduced ell migration in Eca-109 cells (magnification 40×). **B,** Transwell assay showed that number of i ted cel nificantly decreased *via* knockdown of DLX6-AS1 in Eca-109 cells (magnification 40×). **C,** Transwell y showed that of invaded cells was significantly decreased *via* knockdown of DLX6-AS1 in Eca-109 cells (mag ion 40×). The resu esent the average of three independent experiments (mean ± standard error of the mean). * 5.

for logic le in malignant devel- t of c kewise, DLX6-AS1 pro- mot nvasion ancreatic cancer *in vitro*[14]. DL AS1 induces osteosarcoma progressing b miR-129-5p[15]. DLX6-AS1 pro- s cell proliferation and metastasis by reg- ing E2F1 in glioma[16]. DLX6-AS1 induces t ressiveness of renal cell carcinoma *via* targ g miR-26a[17]. In the current work, we first observed that DLX6-AS1 was markedly upregulated in ESCC patients. Mechanistically, the knockdown of DLX6-AS1 inhibited cell proliferation, migration, and invasion in ESCC cells.

Latest studies[18] reveal that lncRNAs function in tumor progression by binding to miRNAs. MiR-18a targeted by lncRNA CASC2 inhibits proliferation and metastasis in esophageal squa-

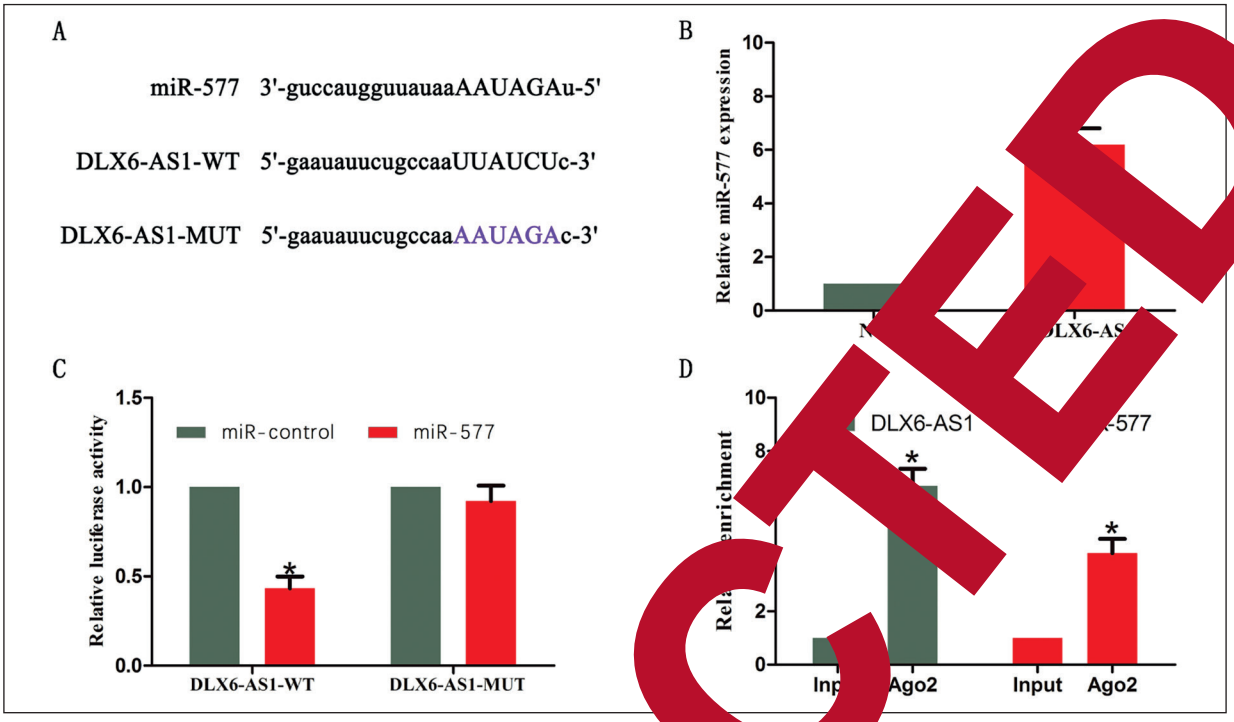


Figure 4. Interaction between DLX6-AS1 and miR-577. **A,** Binding on DLX6-AS1. **B,** QRT-PCR results showed that miR-577 expression was increased in DLX6-AS1 shRNA (sh-DLX6-AS1) group compared with negative control (NC) group. **C,** Co-transfection of miR-577 and DLX6-AS1-WT strongly decreased the luciferase activity, while co-transfection of miR-577 and DLX6-AS1-MUT did not affect luciferase activity. **D,** RIP assay identified that DLX6-AS1 and miR-577 were significantly enriched in Ago2-containing beads compared to input group. The results represent the average of three independent experiments. Data are presented as mean ± standard error of the mean. *$p<0.05$.

mous cell carcinoma. LncRNA DLX6-AS1, as a miR-211 sponge, was reported to suppress the cell growth of ESCC[19]. The interaction between lncRNA XIST and miR-[illegible] was found to promote the progression of nasopharyngeal carcinoma[20]. Through bioinformatics analysis, miR-577 was predicted as the potential binding miRNA of DLX6-AS1. MiR-577 acts as a tumor suppressor in many carcinomas, participates in diverse biological processes of malignant tumors. MiR-577 enhances cell invasion *via* targeting Rab25 in breast cancer[21]. MiR-577 is associated with prognosis of glioblastoma patients[22]. Yuan et al[23] showed that abnormal expression of miR-577 and TSGA10 is associated with progression and development of ESCC.

In the present research, the interaction between miR-577 and DLX6-AS1 was first discovered. The results disclosed that the expression level of miR-577 could be upregulated by knockdown of DLX6-AS1. Furthermore, miR-577 could directly bind to DLX6-AS1 through a luciferase assay, while miR-577 was significantly enriched by the DLX6-AS1 RIP assay.

Conclusions

Collectively, we revealed that DLX6-AS1 could promote ESCC proliferation and metastasis by sponging miR-577 and might act as a candidate target for therapy of ESCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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