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Long noncoding RNA DLX6-AS1 functions as a competing endogenous RNA for miR-577 to promote malignant development of colorectal cancer

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Abstract. – OBJECTIVE: Recent researches have proved that long noncoding RNAs (lncRNAs) play essential roles in tumorigenesis. The aim of this study was to investigate the exact role of lncRNA DLX6-AS1 in the development of colorectal cancer (CRC), and to explore the possible mechanism.

PATIENTS AND METHODS: DLX6-AS1 expression in CRC tissues was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Function assays were conducted to detect the effect of DLX6-AS1 on the proliferation and metastasis of CRC *in vitro*. Furthermore, luciferase reporter gene assay and RNA immunoprecipitation assay (RIP) were used to explore the underlying mechanism of DLX6-AS1.

RESULTS: DLX6-AS1 expression in CRC samples was significantly higher than that of adjacent tissues. Loss of DLX6-AS1 markedly inhibited the proliferation, migration, and invasion of CRC cells. Furthermore, luciferase reporter gene assay and RIP assay showed that DLX6-AS1 acted as a competing endogenous RNA sponging miR-577 in CRC.

CONCLUSIONS: DLX6-AS1 promoted the proliferation, migration, and invasion of CRC by sponging miR-577, which might offer a potential therapeutic target for

Key Words
Long noncoding RNA, DLX6-AS1, Colorectal cancer, MiR-577.

Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed malignancy worldwide[1]. The incidence rate of CRC remains high in both male and female patients[2]. Technological advances have been made in screening and therapeutic management in recent years. However, most of CRC patients have developed metastasis when first diagnosed. Meanwhile, the overall survival rate of CRC patients with advanced stage remains poor[3]. Therefore, it is urgent to search for new therapeutic targets to improve the prognosis of CRC. Non-coding RNAs (ncRNAs) account for 99% of the transcripts. Long noncoding RNAs (lncRNAs) are a subtype of ncRNAs with longer than 200 nucleotides. Studies have demonstrated that lncRNAs are closely involved in a variety of cellular activities. For example, lncRNA RUNX1-IT1 acts as a tumor suppressor in CRC by inhibition of cell migration and proliferation[4]. LncRNA ATB promotes the migration and invasion of glioma cells by suppressing the expression of microRNA-204-3p[5]. LncRNA LINC01510 is highly expressed in CRC, which predicts favorable prognosis of patients as well[6]. Meanwhile, lncRNA FENDRR suppresses the proliferation and malignancy of non-small cell lung cancer cells[7] through sponging miR-761. By regulating the stability of DNMT1 and depressing the expression of tumor suppressors, lncRNA LUCAT1 promotes the formation and cell metastasis of esophageal squamous cell carcinoma[8]. However, the exact role of lncRNA DLX6-AS1 in CRC and its underlying molecular mechanism have not been fully elucidated. In this study, we found that the expression of DLX6-AS1 was remarkably higher in CRC tissues. Moreover, the proliferation, migration, and invasion of CRC cells were significantly inhibited after the loss of DLX6-AS1 *in vitro*. Furthermore, we explored the potential target microRNAs of DLX6-AS1 in CRC development.

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Patients and Methods

Cell Lines and Clinical Samples

55 CRC patients who received surgery at Tongde Hospital of Zhejiang Province were enrolled in this study. Human tissues were collected from these patients. Informed consent was obtained from each subject before the operation. All tissues were kept at –80°C for subsequent use. No radiotherapy or chemotherapy was performed before the surgery. The Ethics Committee of Tongde Hospital of Zhejiang Province approved this study.

Cell Culture

Three human CRC cell lines (HT29, SW620, and SW480) and one normal human colonic epithelial cell line (NCM460) were offered by the Chinese Academy of Science (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) consisting of 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and penicillin. Besides, the cells were maintained at 37°C in a humidified incu with 5% CO_2.

Cell Transfection

Lentivirus expressing short-hairpin R (shRNA) against DLX6-AS1 was purchased from GenePharma (Shanghai, China) uently, cell transfection was performe CRC cells, according to the instru s of amine 2000 (Invitrogen, Car CA h later, Real Time-quantitative e Chain Reaction (RT-qPCR) wa d to X6-AS1 expression in trans d cells.

RNA Extraction and RT-qPCR

Total RNA i issues and ce s extracted according t e manufacturer's protocol of TRIzol Reage nvitr , Carlsbad, CA, USA). Subsequentl trac RNA s reverse transcribed into ough se transcription kit (TaKaRa B log o., Ltd., Dalian, China) ive ge ion was calculated by th ethod. Pr s used for RT-qPCR we s fo DLX6-AS1 primers forward: 5′ TTC TTGCCTT-3′, reverse: 5 TGTTAGTGCCCTT-3″; GAPDH prime d: 5′-CCAAAATCAGATGGGG-CAATGC nd reverse: 5′-TGATGGCAT-GGACTGTGG ATTCA-3′.

Cell Proliferation Assay

2 ×10³ transfected cells w into 96-well plates. Cell prolife n was ass by Cell Proliferation Reage it I (MTT; e, Basel, Switzerland) at 0 48 h, an h, respectively. Absorbance at as ssed using an ELISA re system n As-cent, LabSystems, sinki, Finland).

Colony Form A

1.5×10³ transfe cells re first seeded into 6-w lates a ten days later, formed co es were fix 10% formaldehyde for and stained wi 0.5% crystal violet for colonies were photographed by Ni came Japan), and the number of ies was cou

ynyl Deoxyuridine (EdU) orporation ay

dU Kit (Ro Basel, Switzerland) was used t the eration of transfected cells. A re photograph was taken by Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany).

Healing Assay

Transfected cells were first transferred into -well plates and cultured in RPMI-1640 medium vernight. After scratched with a plastic tip, the ls were cultured in serum-free RPMI-1640 for 8 h. After that, wound closure was viewed. Each assay was repeated for three times independently.

Transwell Assay

5×10⁴ cells in 200 µL serum-free RPMI-1640 were added to the upper chamber of an 8 µm pore size insert (Corning, Corning, NY, USA) with or without 50 µg Matrigel (BD, Bedford, MA, USA) after transfection. Meanwhile, 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 cotton swab. Subsequently, they were stained with crystal violet for 20 min. Three fields were randomly selected for each sample. The number of migrating and invading cells was counted.

Luciferase Reporter Gene Assay

DLX6-AS13′-UTR was first cloned into pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3′-UTR. Then, the 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)

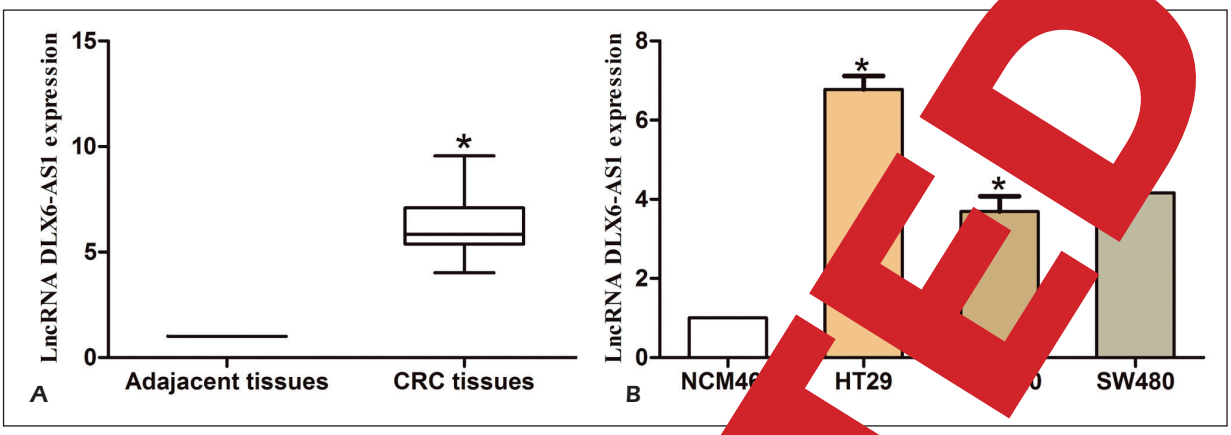

Figure 1. DLX6-AS1 expression in CRC tissues and cells. **A**, DLX6-A[S1] expre[ssion] [sig]nificantly increased in CRC tissues compared with adjacent tissues. **B**, Expression level of DLX6-AS1 in h[u]man CRC [cell lines] and normal human colonic epithelial cell line (NCM460) was determined by RT-qPCR. GAPDH [was] used as an inter[nal] control. Data were presented as mean ± standard error of the mean. *$p<0.05$.

3′-UTR. After that, the cells were transfected with WT-3′-UTR or MUT-3′-UTR and negative control or miR-577 for 48 h. Luciferase reporter gene assay was conducted on a dual luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Ass[ay]

Magna RIP RNA-Binding Protein Imm[une] precipitation Kit (Millipore, Billerica, MA, US[A]) was conducted according to standard instruc[tions]. Briefly, transfected HT29 C[ell] [lysates] were previously added with a prote[ase] [inhibitor] and RNase inhibitor, followed by [lysis] in [RIP] buffer. Subsequently, they [were] cu[bated] with RIP buffer containing magn[etic] [beads] coated with Ago2 antibodies (M[illi]pore, B[illerica], MA, USA) for 2 h at 4°C. Ig[G] was identified [as] ga-tive control (input gr[oup]). qPCR was used to monitor co-precipita[ted] RNA.

Statistical Ana[lysis]

Statistical P[ackage] for Social Service Solutions (SPSS) 19.0 (SPSS, C[hica]go, IL, USA) was used for all statistical anal[yses]. The [Student] *t*-test was performed to compare [the] [differ]ence bet[ween] the two groups. $p<0.05$ was cons[idered] [statis]tically significant.

Results

D[LX6-AS1] Expression Level in C[RC] [Tissu]es and Cells

Firstly, [DLX6-]AS1 expression in 55 patients' tissues and 3 [CRC] cell lines were detected via RT-qPCR. As a result, DLX6-AS1 was signifi-[cantly] up-regul[ated] in CRC tissue samples (Fig-[ure] [1]A). Me[an]while, DLX6-AS1 expression in C[RC] [cells] significantly higher than that of NCM4[60] cells (Figure 1B).

[Knock]down of DLX6-AS1 Inhibited [Prol]iferation of CRC Cells

[The] HT29 CRC cell line was selected for transfec-[tion] of DLX6-AS1 shRNA *in vitro*. Transfection [ef]ficiency of DLX6-AS1 was verified by RT-qP-[CR] (Figure 2A). MTT assay results showed that [knock]down of DLX6-AS1 significantly inhibited the growth ability of CRC cells (Figure 2B). Colony formation assay showed that the number of col-onies decreased remarkably after knockdown of DLX6-AS1 (Figure 2C). Furthermore, EdU in-corporation assay demonstrated that the number of EdU positive cells was significantly reduced after knockdown of DLX6-AS1 *in vitro* (Figure 2D).

Knockdown of DLX6-AS1 Inhibited the Migration and Invasion of CRC Cells

To identify whether DLX6-AS1 functioned in the metastasis of CRC, wound healing assay and transwell assay were conducted. Results of wound healing assay indicated that knockdown of DLX6-AS1 significantly repressed the migrat-ed length of CRC cells (Figure 3A). Transwell assay revealed that the number of migrated cells decreased significantly after DLX6-AS1 was knocked down in CRC cells (Figure 3B). Further-more, the transwell assay also revealed that the number of invaded cells decreased significantly after DLX6-AS1 knockdown *in vitro* (Figure 3C).

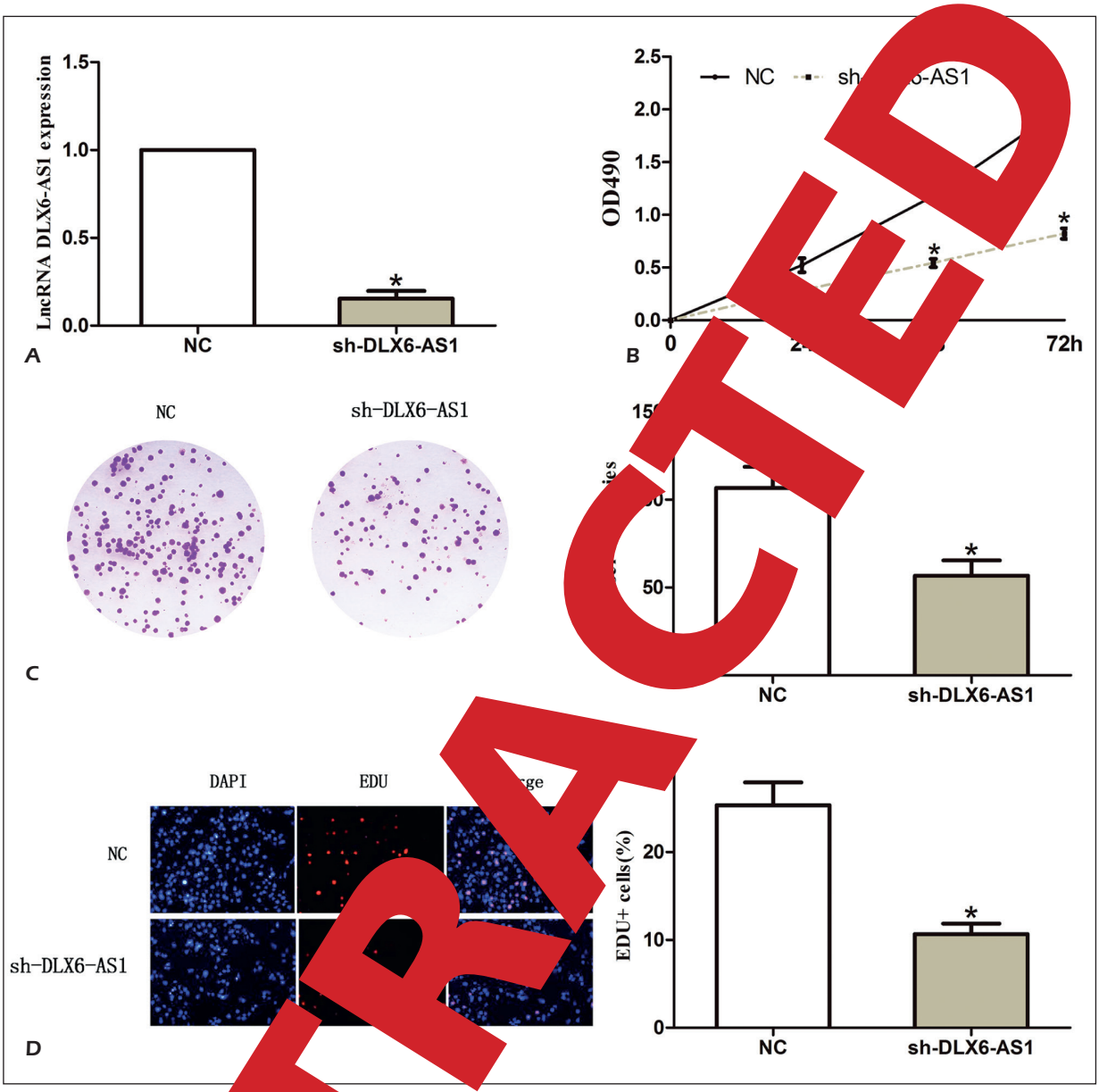

Figure 2. Knockdown o[illegible]ed CRC cell proliferation. **A**, DLX6-AS1 expression in HT29 CRC cells transduced with DLX6-AS1 shRNA (sh-D[illegible]6-AS1) a[illegible]control (NC) was detected by RT-qPCR. GAPDH was used as an internal control. **B**, MTT assay showe[illegible]at knockdown o[illegible]S1 significantly enhanced the growth ability of CRC cells. **C**, Colony formation assay showed that t[illegible]mber of colonies decr[illegible]ed significantly via knockdown of DLX6-AS1 in CRC cells (magnification: 10×). **D**, EdU incorpora[illegible]ssay s[illegible]ved that EdU positive cells were reduced after knockdown of DLX6-AS1 in HT29 CRC cells. The results represent[illegible]e avera[illegible]three independent experiments (mean ± standard error of the mean). *p<0.05.

MiR-577 Was[illegible]t Target of DLX[illegible]61 in C[illegible]

DI[illegible]cBASE P[illegible]cted v.2 (http://caro-lin[illegible]s.at[illegible]novation.gr/diana_tools/web/in[illegible]hp?r[illegible]Findex-predicted) was u[illegible]the poten[illegible]tial microRNAs of DLX6-AS1. [illegible]which contained the binding area of DLX6-A[illegible]selected for the following experiments (Fig[illegible]4A). RT-qPCR results showed that the expression level of MiR-577 in HT29 CRC cells of DLX6-AS1 shRN (sh-DLX6-AS1) group was significantly higher when compared with that of negative control (NC) group (Figure 4B). Luciferase reporter gene assay revealed that co-transfection of DLX6-AS1-WT and miR-577 significantly decreased luciferase activity. However, no significant difference was observed in luciferase activity after co-transfection of DLX6-AS1-MUT

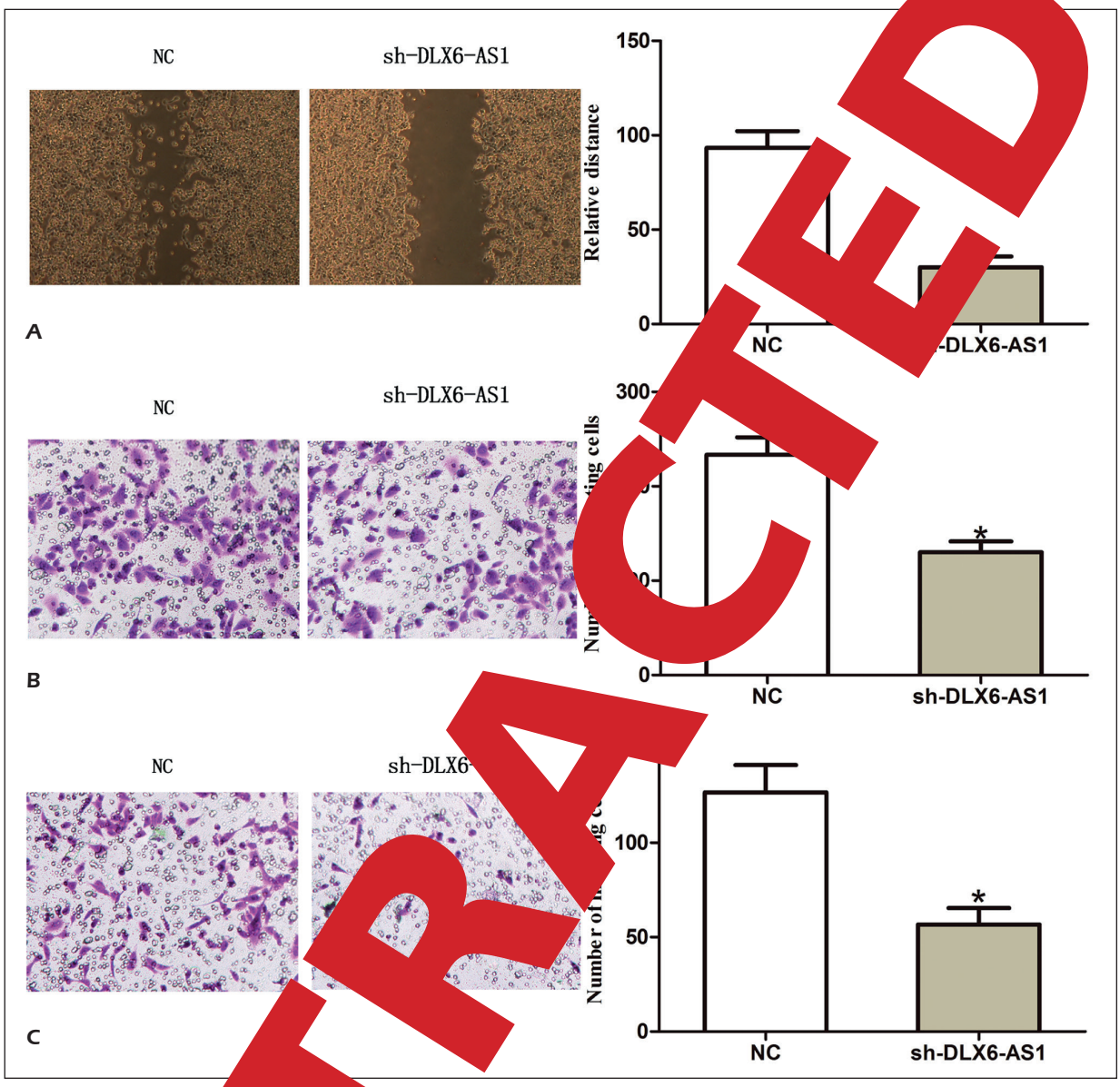

Figure 3. Knockdown [illegible]d CRC cell migration and invasion. **A**, Wound-healing assay showed that knockdown of DLX6-AS1 significantly reduced the [illegible]f CRC cells (magnification: 40×). **B**, Transwell assay showed that the number of migrated cells decre[illegible]significantly via k[illegible]own of DLX6-AS1 in CRC cells (magnification: 40×). **C**, Transwell assay showed that the number of [illegible]ded cells was significantly reduced via knockdown of DLX6-AS1 in CRC cells (magnification: 40×). The results represent[illegible] averag[illegible]three independent experiments (mean ± standard error of the mean). *p<0.05.

and miR-577 (Fig[illegible]. F[illegible]rmore, RIP assay showed [illegible]both D[illegible] and miR-577 were signi[illegible]riched in [illegible]go2-containing beads co[illegible]ed w[illegible] group (Figure 4D).

Discussion

Compelling [illegible]vidence[9,10] has suggested that lncRNAs play crucial roles in carcinogenesis of CRC by regulating various cell biological behaviors. Long noncoding RNA DLX6 antisense RNA 1 (DLX6-AS1) has attracted much attention for its role in the malignant development of cancers. For example, DLX6-AS1 promotes osteosarcoma stemness by targeting miR-129-5p[11]. DLX6-AS1 promotes the proliferation and metastasis of non-small cell lung cancer by regulating miR-144[12]. DLX6-AS1 relieves E2F1 and induces tumorigenesis of glioma via sponging miR-197-5p[13]. DLX6-

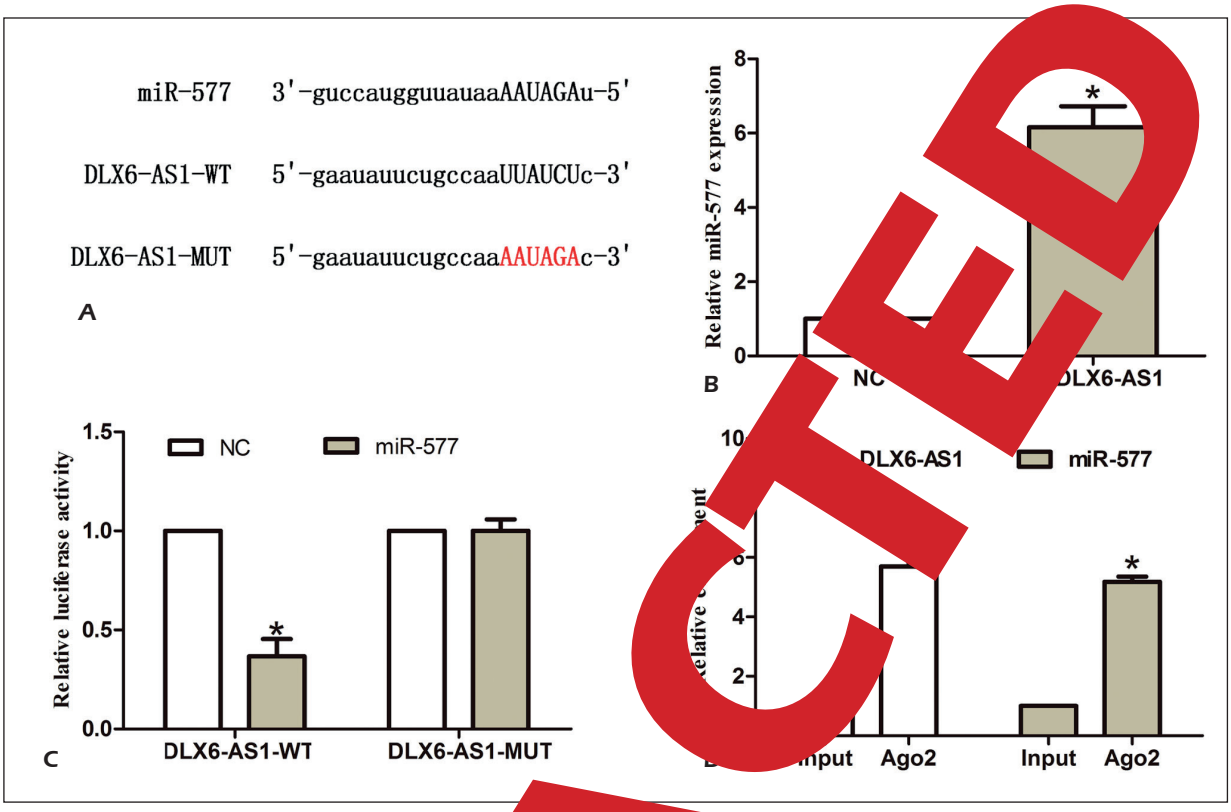

Figure 4. Interaction between DLX6-AS1 and mi... of miR-577 on DLX6-AS1. **B**, RT-qPCR results showed that miR-577 expression was significantly up...ated in ...X6-AS1 shRNA (sh-DLX6-AS1) when compared with negative control (NC). **C**, Co-transfection of mi... an... X6-AS1-WT significantly decreased luciferase activity, while co-transfection of NC and DLX6-AS1-WT did no... uciferase activity. **D**, RIP assay identified that DLX6-AS1 and miR-577 were both significantly enriched in Ago2-co... beads compared with input group. The results represented the average of three independent experime...ta were pr...as mean ± standard error of the mean. *p<0.05.

AS1 promotes the proliferat... j... renal cell carcinoma via targeti...a axis[14]. Moreover, DLX6-AS1 in...es cel...n by regulating miR-181b in ...reatic cance... w-ever, the exact role of D...S1 in malignancies, including CRC, rem... In this study, we firstly found that ...LX6-A...ignificantly upregulated in ...RC tissues and ... Besides, knockdown of ...X6-AS1 markedly repressed the proliferati... migra..., and invasion of CRC cells. The al... resul...dicat...that DLX6-AS1 might act as ...e in C...

Some studi... rev...d that lncRNAs functio... tumor ...ion by binding to miR... xample, ...-18a, targeted by lncR... CAS... hibits the proliferation and meta... of ...ncer[16]. LncRNA TUSC7, as ... sponge, has been reported to suppress ...th of CRC[17]. Meanwhile, the interaction bet...RNA XIST and miR-34a-5p has been disco...ed in the progression of nasopharyngeal carcinoma[18]. Bioinformatics analysis has predicted that miR-577 is the potential binding microRNA of DLX6-AS1. MiR-577, as a tumor suppressor in various malignancies, participates in diverse biological processes of malignant tumors. For instance, miR-577 is associated with prognosis of glioblastoma patients[19]. MiR-577 enhances the invasion of breast cancer cells via targeting Rab25[20]. Meanwhile, miR-577 inhibits tumor growth and promotes chemosensitivity of CRC[21].

In this study, we first explored the interaction between miR-577 and DLX6-AS1. The results showed that the expression level of miR-577 was significantly upregulated by knockdown of DLX6-AS1. Luciferase reporter gene assay validated that miR-577 could directly bind to DLX6-AS1. Furthermore, MiR-577 was significantly enriched by DLX6-AS1 through RIP assay. Above data indicated that DLX6-AS1 functioned as a competing endogenous RNA for miR-577 in CRC.

Conclusions

We demonstrated that DLX6-AS1 could promote CRC proliferation and metastasis through sponging miR-577. Our findings suggested that DLX6-AS1 might act as a candidate target for the therapy of CRC.

Conflict of Interests

The authors declare that they have no conflict of interest.

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