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Long non-coding RNA DLX6-AS1 acts as an oncogene by targeting miR-613 in ovarian cancer

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Abstract. – OBJECTIVE: Recently, long non-coding RNAs (lncRNAs) have been extensively studied for their role in tumor progression. This work explored the role of lncRNA DLX6-AS1 in mediating the development of ovarian cancer (OC).

PATIENTS AND METHODS: DLX6-AS1 expression was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) in OC tissues. Moreover, wound healing assay and transwell assay were performed to detect the effect of DLX6-AS1 on the metastasis of OC. Furthermore, the underlying mechanism of DLX6-AS1 in mediating the progression of OC was explored through the Dual-Luciferase reporter gene and RNA immunoprecipitation assay (RIP).

RESULTS: DLX6-AS1 expression was higher in OC samples than that in the adjacent ones. Moreover, cell migration and invasion were suppressed after DLX6-AS1 knockdown *in vitro*. Conversely, cell migration and invasion were promoted by overexpressed DLX6-AS1. Moreover, expression of microRNA-613 (miR-613) was upregulated *via* knockdown of DLX6-AS1 but was downregulated after overexpression. Furthermore, the Luciferase reporter gene assay and RIP assay showed that miR-613 was a direct target of MIAT in DLX6-AS1 OC tissues.

CONCLUSIONS: DLX6-AS1 could enhance migration and invasion of OC cells *via* targeting miR-613, which might serve as a potential therapeutic target in OC.

Keywords:
Long non-coding RNA, DLX6-AS1, Ovarian cancer, MiR-613

Introduction

Ovarian cancer (OC) is a fatal gynecologic malignancy, ranking the fifth in tumor death globally. There are approximately 22,280 newly diagnosed OC cases and 15,500 death cases per year in America[1]. Atypical or absent symptoms at the early stage and the limitation for the high diagnostic rate of advanced OC. Moreover, the occurrence of chemotherapy resistance and metastasis contributes to high mortality of OC[2,3]. However, most patients develop chemotherapy-resistance or relapse after surgery. The prognosis of OC patients is still disappointing with the 5-year survival rate of only 30%[4]. Early detection of these patients and the establishment of new therapeutic strategies are urgently required for successful intervention.

Research has indicated that long non-coding RNAs (lncRNAs) are closely involved in a variety of cellular activities. For example, lncRNA CCAT2 facilitates the proliferation and metastasis in intrahepatic cholangiocarcinoma[5]. LncRNA SNHG7 promotes tumor proliferation in osteosarcoma by targeting microRNA-34a (miR-34a)[6]. LncRNA SNHG1 could inhibit the differentiation of Treg cells, thereby impeding the immune escape of breast cancer[7]. LncRNA FENDRR suppresses cell proliferation and malignancy of non-small cell lung cancer by sponging miR-761[8]. In this study, we found out that the expression of DLX6-AS1 was upregulated in OC tissues. Moreover, DLX6-AS1 promoted the migration and invasion of OC cell *in vitro*. Our further experiment explored the underlying mechanism of DLX6-AS1 in regulating the progression of OC.

Patients and Methods

Cell Lines and Clinical Samples

Human OC tissues were harvested from 58 OC patients undergoing surgery at the First Affiliated Hospital of Harbin Medical University between 2011 and 2013. Signed informed consents were obtained from all participants before the study. None of the patients received preoperative radiotherapy or chemotherapy. All fresh tissues

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were stored at -80°C. This study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University.

Cell Culture

Human OC cell lines (A2780, SKOV3 and OVCAR-3) and normal ovarian cell (ISOE80) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin. Cells were cultured in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

GenePharma (Shanghai, China) provided lentivirus expressing short-hairpin RNA (shRNA) against DLX6-AS1 (DLX6-AS1/shRNA), which was cloned into the pGPH1/Neo vector. After 48-h transfection in A2780 cells, DLX6-AS1 level was detected using Real Time-quantitative Polymerase Chain Reaction (RT-qPCR).

After synthesized, a lentiviral virus targeting DLX6-AS1 was cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San D CA, USA). DLX6-AS1 lentiviruses (DLX or the empty vector (control) was transfec in SKOV3 cells. 48 h later, the DLX6-AS1 level detected using RT-qPCR.

RNA Extraction and RT-qPCR

Total RNA was extr using reagent (Invitrogen, rls USA) reversely transcrib to co entary deoxyribose nucle ds (cDNA ough the Reverse Tran Kit (TaKa Biotechnology Co ian,) Primers used for RT-qPCR e as follow X6-AS1, forward 5′-AGT TCTCTAGATTC T-3′ and reverse ATTGA TGTTAGTGCCCTT-3′; glyceral de 2 osphate dehydrogenase (GAPDH), 5′- C GATCCCTCCAAAAT-A-3′ reve 5′-TTCACACCCATGAC-AT-3 cycle was as follows: 30 sec 5°C, 5 at 95°C and 35 sec at 60°C, for a 40 cycles.

Wound Healing Assay

Cells seeded into 6-well plates were cultured i EM overnight. After being scratched with a plastic tip, cells were cultured in serum-free DMEM. Wound closure was viewed at 48 h. Each assay was independently repeated in triplicate.

Transwell Assay

5×10^4 cells suspended in 200 µL of serum-free DMEM were applied to the top chamber of an 8 µm insert (Millipore, Billerica, MA USA) pre-coated with or without 50 µg of M Biosciences, Franklin Lakes, NJ, U DMEM containing 10% FBS was applie the bottom chamber. 48 h later, after being w cotton swab, the cells on the top s ace o cham-bers was immersed for 1 n with pr methanol and stained i tal vi t for 30 Invasive cells were ted i e randomly selected fields per amp

Dual-Lucife Reporte say

The 3' ated Regi -UTR) of DLX6-AS was d into the pGL3 vector (Promega Madison, USA), which was identifie d-type (W UTR. Quick-change si rected mutagenesis t (Stratagene, La Jol-A, USA) wa sed for site-directed mutagen-of the miR binding site in DLX6-AS1 3 R, namely mutant (MUT) 3'-UTR. Cells we ith WT-3'-UTR/MUT-3'-UTR and mi R-613 for 48 h. Luciferase assay as conducted on the Dual-Luciferase reporter stem (Promega, Madison, WI, USA).

RNA Immunoprecipitation Assay (RIP)

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used for RIP assay. Co-precipitated RNAs were detected by RT-qPCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) was adopted to conduct the statistical analysis. Data were presented as mean ± SD (Standard Deviation). Student's *t*-test was utilized for analyzing measurement data. It was considered statistically significant when $p<0.05$.

Results

DLX6-AS1 Was Upregulated in OC Tissues and Cells

DLX6-AS1 expression was detected in 58 OC tissues and 3 OC cell lines *via* RT-qPCR. DLX6-AS1 was highly expressed in OC tissue samples compared with the adjacent tissues (Figure 1A). DLX6-AS1 level in OC cells was also upregulated compared with that of ISOE80 cells (Figure 1B).

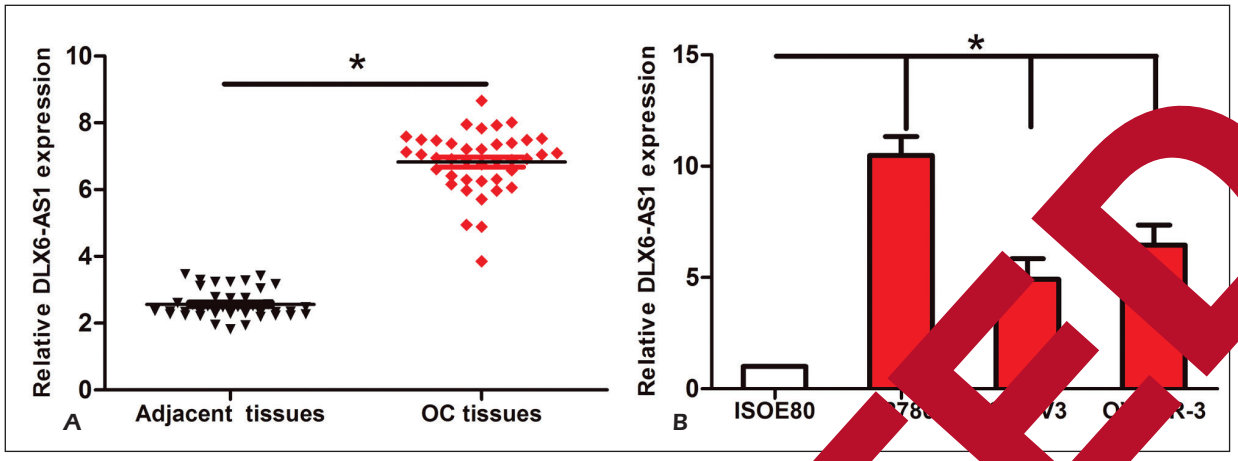


Figure 1. The expression levels of DLX6-AS1 increased in OC tissues and cell [illegible] DLX6-AS1 ex[illegible] significantly increased in the OC tissues compared with the adjacent tissues. **B**, The expr[illegible] l[illegible] of DLX6-AS1 [illegible]tive to GAPDH were determined in the human OC cell lines and ISOE80 by RT-qPCR. Data ar[illegible] presente[illegible] mean ± standard error of the mean. *p<0.05.

DLX6-AS1 Promoted Cell Migration and Invasion in OC Cells

According to the expression of DLX6-AS1 in OC cells, the A2780 cell line was used for transfection with DLX6-AS1 shRNA and SKOV[illegible]ll line was used for transfection with DL[illegible] lentivirus. Transfection efficiency was de[illegible]d by RT-qPCR (Figure 2A and 2B). More[illegible] wound healing assay revealed that after DL[illegible] AS1 was knocked down, the m[illegible] ability [illegible] OC cells was significantly re[illegible]ed [illegible]ure 2C). After overexpression of DL[illegible]AS1, t[illegible]migrated ability of OC cells was [illegible]ly pr[illegible] ure 2D). The transwe[illegible]ass[illegible]vealed[illegible]t knockdown of DLX[illegible]S1 in O[illegible]s remarkably decreased the nu[illegible] of migrate[illegible]ls (Figure 3A). Converse[illegible]pression of[illegible]X6-AS1 increased the [illegible]mber [illegible]grated cells (Figure 3B). Mor[illegible], the numb[illegible]invaded cells was remark[illegible] reduced after k[illegible]down of DLX6-AS1 [illegible]ure 3C[illegible]After DLX6-AS1 was overexpr[illegible]in O[illegible]ls, the number of invaded cells marke[illegible]ased ([illegible]e 3D).

[illegible]nter[illegible]etween MiR-613 an[illegible]LX6-A[illegible]n OC Cells and Tissues

[illegible]NA. LncBASE Predicted v.2 (http://car[illegible]ena-innovation.gr/diana_tools/web/[illegible]dex.php?r=lncbasev2%2Findex-predicted) was [illegible] to search for the miRNAs that contained c[illegible]mentary base with DLX6-AS1. MiR-613 was predicted as the potential miRNAs interacted with DLX6-AS1. Previous studies revealed that miR-613 was a tumor suppressor and able to [illegible]press cancer[illegible]ll proliferation. The binding [illegible]ences of mi[illegible]3 in DLX6-AS1 were shown i[illegible]ure 4A. [illegible]des, the miR-613 level was upr[illegible]ansfection of DLX6-AS1/shRNA (Fi[illegible]). The miR-613 level was inhibited by transfection of DLX6-AS1 lentivirus (Figure [illegible]urthermore, the Dual-Luciferase reporter [illegible]ay showed that the Luciferase activity was remarkably reduced in cells co-transfected with DLX6-AS1-WT and miR-613 mimics (Figure 4D). RIP assay also identified that miR-613 could be significantly enriched in the DLX6-AS1 group compared with the control group (Figure 4E). The above results suggested that DLX6-AS1 might be a miR-613 sponge in OC.

Discussion

OC is a most fatal gynecologic malignancy and the fifth leading cause of death in cancer. Most OC cases are diagnosed at an advanced stage due to atypical or absent symptoms at the early stage. Plenty of lncRNAs have been revealed to play important roles in oncogenesis and progression of OC. For example, the silence of lncRNA MNX1-AS1 suppresses cell proliferation and migration of OC, which may be a potential target for OC[9]. Knockdown of long non-coding RNA HOTAIR increases cisplatin-sensitivity in OC by inhibiting cisplatin-induced autophagy[10]. LncRNA BACE1-AS inhibits the proliferation and invasion of OC stem cells and functions as a novel target for treating OC[11]. Besides, lncRNA

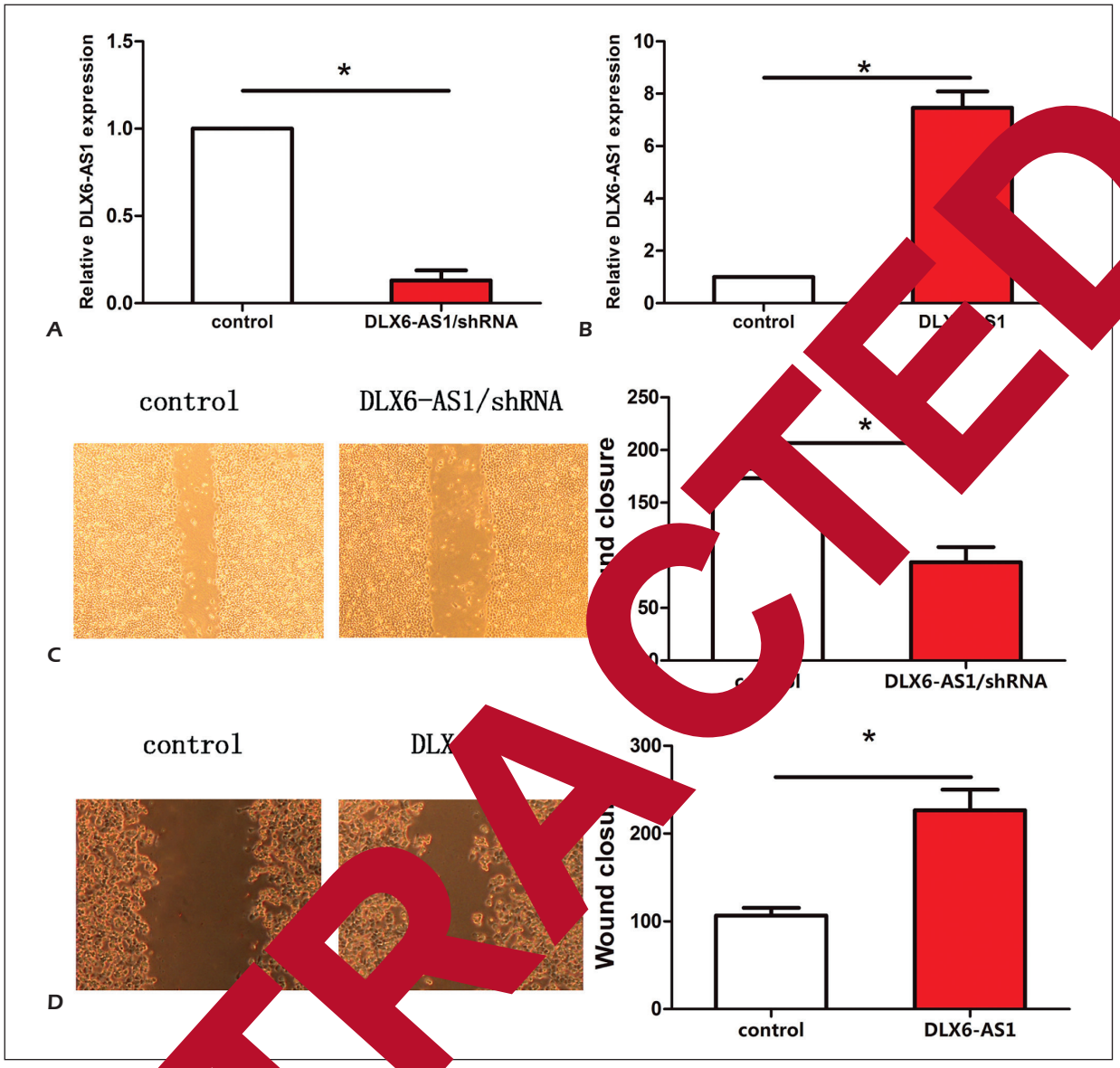
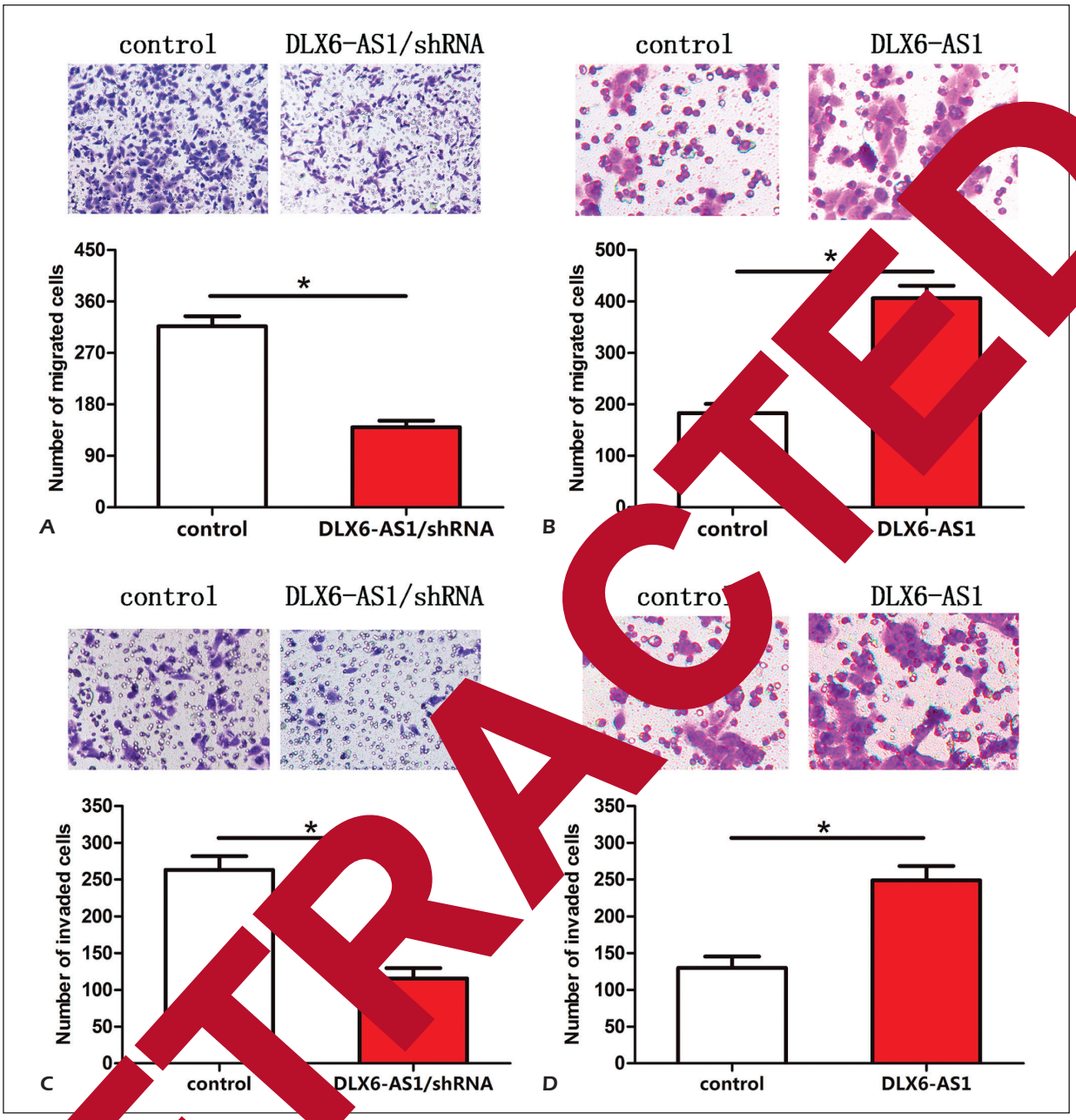


Figure 2. Wou[illegible]aling [illegible] showed that [illegible]LX6-AS1 promoted OC cell migration. **A**, DLX6-AS1 expression in OC cells transfected w[illegible] DLX6-AS1/[illegible] and the empty vector (sh-ctrl) was detected by RT-qPCR. **B**, DLX6-AS1 expression in OC cells transf[illegible] with DLX6-AS[illegible]irus (DLX6-AS1) and the empty vector was detected by RT-qPCR. GAPDH was used as an int[illegible] control. **C**, Wound he[illegible] assay showed that knockdown of DLX6-AS1 significantly repressed migration in OC cells ([illegible]nificatio[illegible]40). **D**, Wound healing assay showed that overexpression of DLX6-AS1 markedly promoted migration in O[illegible]ls (Ma[illegible]ation × 40). The results represent the average of three independent experiments (mean ± standard error of the [illegible]05, com[illegible]d with the control cells.

[illegible]NA1 [illegible] as an oncogene in the prolife[illegible]on of ep[illegible]nal OC cells, which is upregul[illegible]estrogen[12].

[illegible]oding RNA DLX6 antisense RNA (DLX6-AS1), located in 7q21.3, has been re[illegible]ly explored to be abnormally expressed and re[illegible] to the progression of several cancers[13-15]. For instance, DLX6-AS1 is aberrantly expressed in lung adenocarcinoma[16], and DLX6-AS1 promotes cell proliferation and metastasis by regulating miR-144 in non-small cell lung cancer[17]. DLX6-AS1 induces cell invasion by regulating miR-181b in pancreatic cancer[18]. In this work, we found that DLX6-AS1 was upregulated in OC samples and cells. The knockdown of DLX6-AS1 attenuated migration and invasion of OC cells. Meanwhile, the overexpression of DLX6-AS1 promoted OC cells to migrate and invade. These results indicated that DLX6-AS1 promoted tumorigenesis of OC and might act as an oncogene.



Figu The tr ell assay showed that DLX6-AS1 promoted migration and invasion of OC cells. **A**, The number of mi cells re ably decreased via knockdown of DLX6-AS1 in OC cells (Magnification × 40). **B**, The number of migrate i antly in ed via overexpression of DLX6-AS1 in OC cells (Magnification × 40). **C**, The transwell assay showed ber of in d cells markedly decreased via knockdown of DLX6-AS1 in OC cells (Magnification × 40). **D**, answ y sh that number of invaded cells significantly increased via overexpression of DLX6-AS1 in OC cells (ficatio results represent the average of three independent experiments (mean ± standard error of the mean). *$p<$ compar the control cells.

We further explored the possible mechanism LX6-AS1 in mediating the metastasis of OC. R studies showed that lncRNAs interact with microRNAs in malignant tumors. Moreover, lncRNAs participate in the regulation of tumorigenesis by binding the paired sequences of microRNA 3'UTR. Bioinformatics software predicted miR-613 as a possible target of DLX6-AS1, which is reported as a tumor suppressor in diverse tumors[19,20]. For example, miR-613 is downregulated in hepatocellular carcinoma and participates in regulating tumor development by targeting YWHAZ[21].

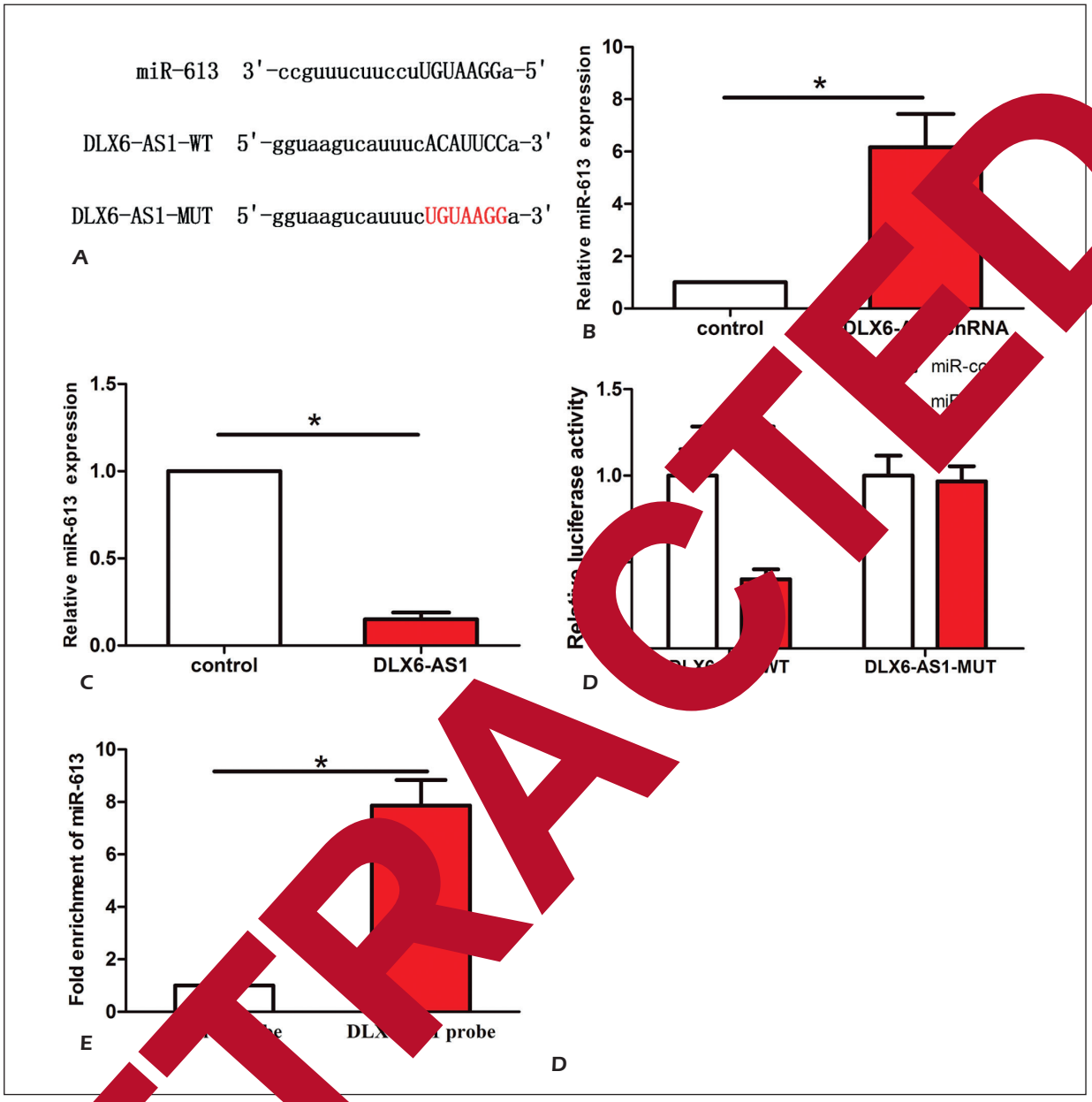


Figure teraction between DLX6-AS1 and miR-613. **A**, The binding sites of miR-613 on DLX6-AS1. **B**, The miR-613 expres increase DLX6-AS1/shRNA group compared with the control group. **C**, The miR-613 expression decreased in DL 1 lenti (DLX6-AS1) group compared with the control group. **D**, Co-transfection of miR-613 and DLX6-AS1-WT s d sed the iferase activity, while co-transfection of miR-control and DLX6-AS1-WT did not change the Lucifera y. **E**, RI dy results demonstrated that miR-613 was remarkably enriched in the DLX6-AS1 group com- with trol p. The results represent the average of three independent experiments. Data are presented as the standa ne mean. *$p<0.05$.

esses migration and growth ability targeting SphK1 in bladder cancer[22]. Recent- miR-613 has been reported to suppress cell p ration and metastasis of OC. Our study first uncovered the interaction between miR-613 and DLX6-AS1. We found that miR-613 could be directly targeted by DLX6-AS1. Moreover, miR-613 expression was promoted through the knockdown of DLX6-AS1 and, conversely, it was downregulated after overexpression of DLX6-AS1. Furthermore, miR-613 was significantly enriched by DLX6-AS1 as RIP assay indicated. All these results showed that DLX6-AS1 regulated the progression of OC by sponging miR-613.

Conclusions

The above data identified that DLX6-AS1 could accelerate migration and invasion of OC cells by targeting miR-613. These findings suggested that DLX6-AS1 may serve as a candidate target for the treatment of OC.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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