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LINC00702 accelerates the progression of ovarian cancer through interacting with EZH2 to inhibit the transcription of KLF2

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Abstract. – OBJECTIVE: To clarify the role of LINC00702 in the progression of ovarian cancer (OC) and the potential mechanism.

PATIENTS AND METHODS: Expression level of LINC00702 in OC tissues and matched normal tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). LINC00702 level in OC cell lines was determined as well. The potential influences of LINC00702 on cellular behaviors of A2780 and HEY cells were evaluated. The subcellular distribution of LINC00702 in A2780 cells was examined. Through RNA immunoprecipitation (RIP) and Chromatin immunoprecipitation (ChIP) assay, the interaction among LINC00702, EZH2, and KLF2 was verified. The rescue experiments were conducted to elucidate the biological function of LINC00702/KLF2 axis in the progression of OC.

RESULTS: LINC00702 was upregulated in OC tissues and cell lines. Its level was much higher in OC with worse tumor stage and larger tumor size. The knockdown of LINC00702 attenuated the proliferative ability of A2780 and HEY cells. LINC00702 was mainly distributed in the cell nucleus. The knockdown of LINC00702 or EZH2 downregulated the KLF2 level in the OC cells. The transfection of LINC00702 markedly reduced the occupancy of KLF2 promoter on EZH2 and H3K27me3 relative to IgG. Finally, the knockdown of KLF2 could reverse the regulatory effect of LINC00702 in the proliferative ability of A2780 cells.

CONCLUSIONS: LINC00702 is upregulated in OC. It accelerates the progression of OC *via* interacting with EZH2 to inhibit the transcription of KLF2.

Key Words:
OC, LINC00702, EZH2, KLF2, Proliferation.

Introduction

Ovarian cancer (OC) is the second prevalent female malignancy, and it is also the leading cause of tumor death in females[1]. Surgical procedures and other tumor treatments for OC have made great strides. However, due to the insufficient diagnostic efficacy in early stage, the therapeutic outcome and the prognosis of advanced OC are unsatisfactory[2,3].

Long noncoding ribonucleic acids (lncRNAs) are extensively distributed regulators in mammals. They exert vital functions in the gene regulation of multiple types of cells and diverse cellular behaviors[4,5]. Lei et al[6] have identified the promotive or inhibitory effects of lncRNAs on cellular behaviors and gene expression regulations. Unlike miRNAs, lncRNAs interact with many factors to form new complexes, thus directly mediating the downstream elements to influence the tumor cell phenotypes[7]. Dysregulated lncRNAs in OC present different roles as tumor suppressors, oncogenes, or metastatic transforming stimulators[8-10]. Relevant researches suggested that upregulated HOTAIR, AB073614, and CCAT2 are closely correlated to high metastatic rate and poor prognosis of OC[9,11]. LSINCT5 is upregulated and participated in the proliferation of OC cells[12]. BC200 is downregulated in OC, which mediates the proliferative rate of tumor cells and Carboplatin-induced cell death[13]. Zhou et al[14] identified the expression profiles of 8-lncRNA, which contribute to classify OC patients and improve survival. Two immune-related lncRNAs, RP11-284N8.3.1, and AC104699.1.1 are searched through the ln-

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cRNA-mRNA network to be prognostic factors for OC[15]. Nevertheless, the functions and mechanisms of many dysregulated lncRNAs in OC are still unclear.

Our study first examined the expression pattern of LINC00702 in OC tissues and cell lines. The regulatory effects of LINC00702 in the proliferative ability of OC cells were evaluated. Finally, the potential mechanism underlying the role of LINC00702 and its interactive genes in the progression of OC was mainly explored.

Patients and Methods

Sample Collection and Ethical Statements

36 paired tumor tissues and matched normal ones were harvested from OC patients undergoing surgery in Shanghai General Hospital of Nanjing Medical University. The samples were immediately placed in liquid nitrogen and preserved at -80°C. Patients and their families in this study have been fully informed. This study was approved by the Ethics Committee of Shanghai General Hospital of Nanjing Medical University.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), quantified by NanoDrop2000c (Thermo Scientific, Waltham, USA), and reversely transcribed into complementary deoxyribonucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). GAPDH was used as an internal reference. The primer sequences were as follows: LINC00702: (Forward) GCAGTGGCATGATCTCGGCT; LINC00702: (Reverse) GGCCGAGGCAGGTGGATAAC; U6: (Forward) GCTTCGGCAGCACATATACTAAAAT; U6: (Reverse) CGCTTCACGAATTTGCGTGTCAT; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): (Forward) GAAGAGAGAGACCCTCACGCTG; GAPDH: (Reverse) ACTGTGAGGAGGGGAGATTCAGT; KLF2: (Forward) CTGCACATGAAACGGCACAT; KLF2: (Reverse) CAGTCACAGTTTGGGAGGGG; EZH2: (Forward) TGCACATCCTGACTTCTGTG; EZH2: (Reverse) AAGGGCATTCACCAACTCC.

Cell Culture

Epithelial ovarian cell line IOSE-386 and OC cell lines (ES-2, SKOV-3, A2780, and HEY) were provided by American type culture collection (ATCC, Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL (Beyotime, Shanghai, China) streptomycin in a 37°C, 5% CO_2 incubator.

Transfection

The cells were pre-seeded in the 6-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48 h, the cells were harvested for subsequent experiments.

Chromatin Fractionation

200 μL of Lysis Buffer J was added for cell lysis. After centrifugation, the supernatant was transferred to a new tube and incubated with Buffer SK and absolute ethanol, followed by extraction with column centrifugation. The cytoplasmic and nuclear levels were detected by qRT-PCR.

RNA Immunoprecipitation (RIP)

The cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). The cell lysate was incubated with anti-EZH2, anti-LSD1, input, and IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/mL proteinase K containing 0.1% dodecyl sulfate and sodium salt (SDS) to extract RNA. The magnetic beads were repeatedly washed with RNA immunoprecipitation (RIP) washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

Chromatin Immunoprecipitation (ChIP)

The cells were subjected to cross-link with 1% formaldehyde for 10 min at room temperature. Subsequently, the cross-linked cells were lysed using lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immuno-precipitated with antibodies and IgG.

Cell Counting Kit-8 (CCK-8) Assay

The cells were seeded in the 96-well plate with 2×10^3 cells per well. The absorbance (A) at 450 nm was recorded at the appointed time points

using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The cells were seeded in the 24-well plate with 4×10^4 cells per well. The cells were labeled with 50 μmol/L EdU at 37°C for 2 h, fixed in 4% paraformaldehyde for 30 min, and incubated in Phosphate-Buffer Saline (PBS) containing 0.5% Triton-100 for 20 min. After washing with PBS containing 3% albumin from bovine serum (BSA), 100 μL of the dying solution was applied per well for 1 h incubation in the dark and the cells were counter-stained with Hoechst33342 for 30 min. The ratio of the EdU-positive cells was calculated.

Western Blot

The total protein was extracted from the cells or tissues using radioimmunoprecipitation assay (RIPA) and loaded for electrophoresis (Beyotime, Shanghai, China). After transferring on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. The bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (Version X; Media Cybernetics, Silver Springs, MD, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

Upregulated LINC00702 in OC Was Positively Correlated to Tumor Size and TNM Staging

The expression level of LINC00702 was determined in 36 paired OC tissues and matched normal ones. As qRT-PCR data revealed, LINC00702 was upregulated in OC tissues (Figure 1A). To further investigate the relationship between

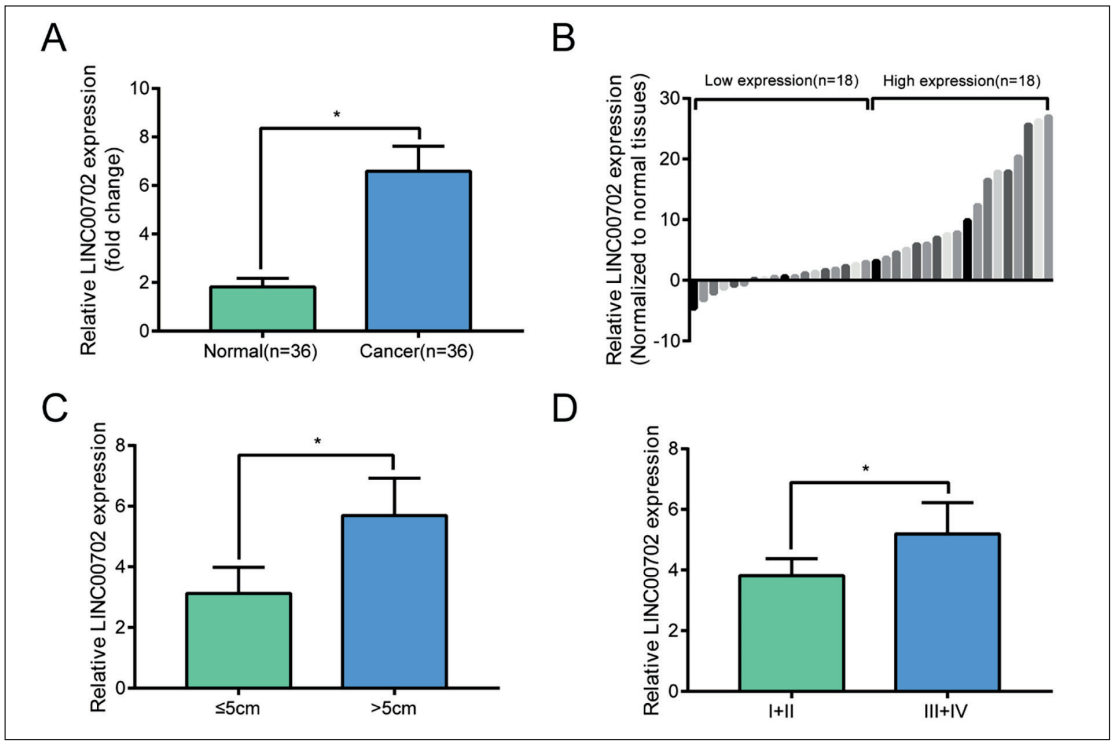

Figure 1. Upregulated LINC00702 in ovarian cancer was positively correlated to tumor size and TNM staging. ***A,*** Relative level of LINC00702 in ovarian cancer tissues (n=36) and matched normal tissues (n=36). ***B,*** Ovarian cancer tissues were divided into low expression group (n=18) and high expression group (n=18) based on the median level of LINC00702. ***C,*** Relative level of LINC00702 in ovarian cancer tissues ≤ 5 cm and > 5 cm in tumor size. ***D,*** Relative level of LINC00702 in ovarian cancer tissues of stage I + II and III + IV.

LINC00702 level and pathological characteristics of OC, the tumor samples were divided into low expression (n=18) and high expression groups (n=18) based on the median level of LINC00702 (Figure 1B). It is suggested that LINC00702 level was positively correlated to larger tumor size (>5 cm) and worse TNM staging (III-IV) of OC patients (Figures 1C and 1D).

Knockdown of LINC00702 Attenuated the Proliferative Ability of OC Cells

Compared with epithelial ovarian cell line IOSE-386, LINC00702 was identically upregulated in OC cell lines (Figure 2A). The transfection of si-LINC00702 1 or si-LINC00702 2 both could sufficiently downregulate LINC00702 level in OC cell lines (Figure 2B). A2780 and HEY

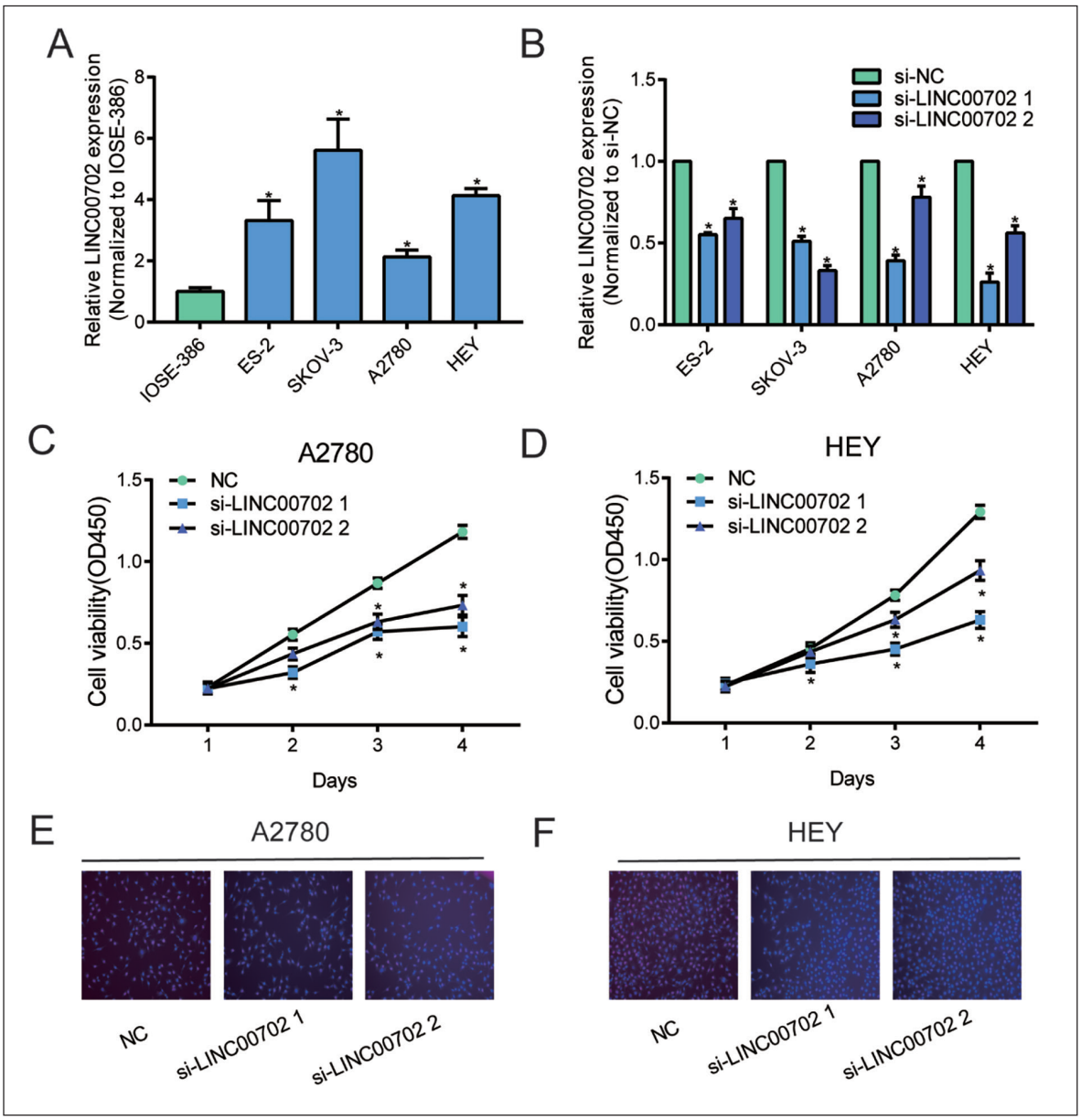

Figure 2. Knockdown of LINC00702 attenuated the proliferative ability of the ovarian cancer cells. ***A,*** Relative level of LINC00702 in epithelial ovarian cell line IOSE-386 and ovarian cancer cell lines (ES-2, SKOV-3, A2780, and HEY). ***B,*** Transfection efficacy of si-LINC00702 1 and si-LINC00702 2 in ES-2, SKOV-3, A2780, and HEY cells. ***C,*** CCK-8 assay showed the viability in A2780 cells transfected with NC, si-LINC00702 1 or si-LINC00702 2. ***D,*** CCK-8 assay showed the viability in HEY cells transfected with NC, si-LINC00702 1 or si-LINC00702 2. ***E,*** EdU assay showed EdU-labeled cells in A2780 cells transfected with NC, si-LINC00702 1 or si-LINC00702 2 (magnification: 40×). ***F,*** EdU assay showed EdU-labeled cells in HEY cells transfected with NC, si-LINC00702 1 or si-LINC00702 2 (magnification: 40×).

cells were selected for the following experiments. CCK-8 assay revealed that the transfection of si-LINC00702 1 or si-LINC00702 2 decreased the viability in A2780 and HEY cells (Figures 2C, 2D). Similarly, the number of the EdU-positive cells was reduced in A2780 and HEY cells transfected with si-LINC00702 1 or si-LINC00702 2 (Figures 2E and 2F). The above data illustrated that LINC00702 knockdown attenuated the proliferative ability of the OC cells.

LINC00702 Suppressed KLF2 Level Via Interacting With EZH2

To analyze the molecular mechanism of LINC00702 in OC, the subcellular distribution of LINC00702 was explored in the A2780 cells. It is found that LINC00702 was mainly distributed in the nucleus, suggesting its potential role in transcriptional regulation (Figure 3A). Several well-known transcriptional factors related to OC were detected here. After transfection of si-LINC00702 1 in A2780 cells, the mRNA levels of KLF2, p21, PTEN, and p15 were all upregulated, especially KLF2 (Figure 3B). Previous studies[16-20] have illustrated that lncRNAs could stimulate tumor cell phenotypes by inhibiting tumor suppressors or interacting with RNA binding proteins to activate oncogenes. In addition, about 24% of lncRNAs are capable of regulating the downstream genes by binding to PRC2[21,22]. In this

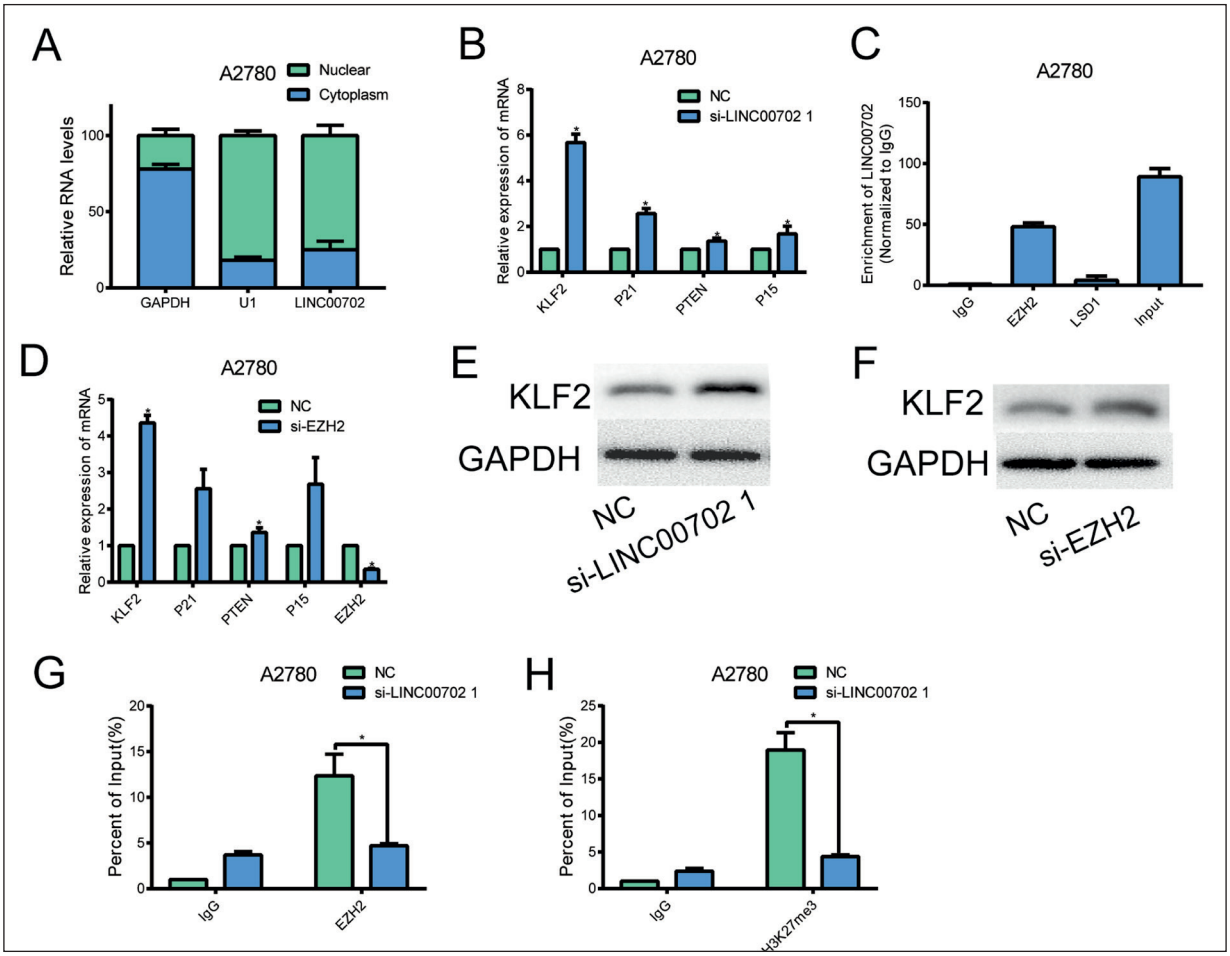

Figure 3. LINC00702 suppressed KLF2 level *via* interacting with EZH2. ***A,*** Subcellular distribution of LINC00702 in A2780 cells. GAPDH and U1 were the internal references of cytoplasm and nucleus, respectively. ***B,*** Relative levels of KLF2, p21, PTEN, and p15 in A2780 cells transfected with NC or si-LINC00702 1. ***C,*** RIP assay showed the enrichment of LINC00702 in IgG, EZH2, LSD1, and input. ***D,*** Relative levels of KLF2, p21, PTEN, p15, and EZH2 in A2780 cells transfected with NC or si-EZH2. ***E,*** Protein level of KLF2 in A2780 cells transfected with NC or si-LINC00702 1. ***F,*** Protein level of KLF2 in A2780 cells transfected with NC or si-EZH2. ***G,*** ChIP assay showed the percentages of IgG and EZH2 in A2780 cells transfected with NC or si-LINC00702 1. ***H,*** ChIP assay showed percentages of IgG and H3K27me3 in A2780 cells transfected with NC or si-LINC00702 1.

study, we predicted the relative interaction probabilities between LINC00702 and RNA binding proteins online (http://pridb.gdcb.iastate.edu/RPISeq/). EZH2 was screened out to be the target gene (RF or SVm>0.5, data not shown). Subsequently, RIP assay verified that LINC00702 could interact with EZH2 rather than other RNA binding proteins (Figure 3C). Furthermore, the functional correlation of the interaction between EZH2 and LINC00702 was analyzed. The transfection of si-EZH2 markedly upregulated the KLF2 level in A2780 cells (Figure 3D). Western blot analyses revealed the upregulated protein level of KLF2 in A2780 cells transfected with si-LINC00702 1 or si-EZH2 (Figures 3E and 3F). Zhang et al[23] suggested that the PRC2 complex is a negative regulator of H3K27me3. Thus, LINC00702 was likely to inhibit KLF2 expression by recruiting the PRC2 complex to the KLF2 promoter region, resulting in H3K27 trimethylation in this region. Next, three paired primers in the KLF2 promoter region were designed (2000 bp). The ChIP assay demonstrated that the knockdown of LINC00702 reduced the occupancy of KLF2 promoter on EZH2 and H3K27me3 (Figures 3G and 3H). Collectively, LINC00702 inhibited the KLF2 level by binding to EZH2 (a key component of PRC2), thereby promoting the development of OC.

LINC00702 Exerted its Carcinogenic Role in OC Via KLF2

The rescue experiments were conducted to elucidate the involvement of KLF2 in LINC00702-mediated progression of OC. The transfection efficacies of si-KLF2 and pcDNA-KLF2 were verified in A2780 cells (Figure 4A). The knockdown of KLF2 enhanced the viability, and conversely, the overexpression of KLF2 markedly inhibited its viability (Figures 4B and 4C). Importantly, the knockdown of LINC00702 reduced the viability of A2780 cells but was further reversed after the co-transfection of si-KLF2 (Figure 4D). Similar results were obtained in EdU assay as well, suggesting the involvement of KLF2 in LINC00702-mediated proliferation of OC cells (Figure 4E). It is believed that LINC00702 exerted its carcinogenic role in OC by inhibiting KLF2 level.

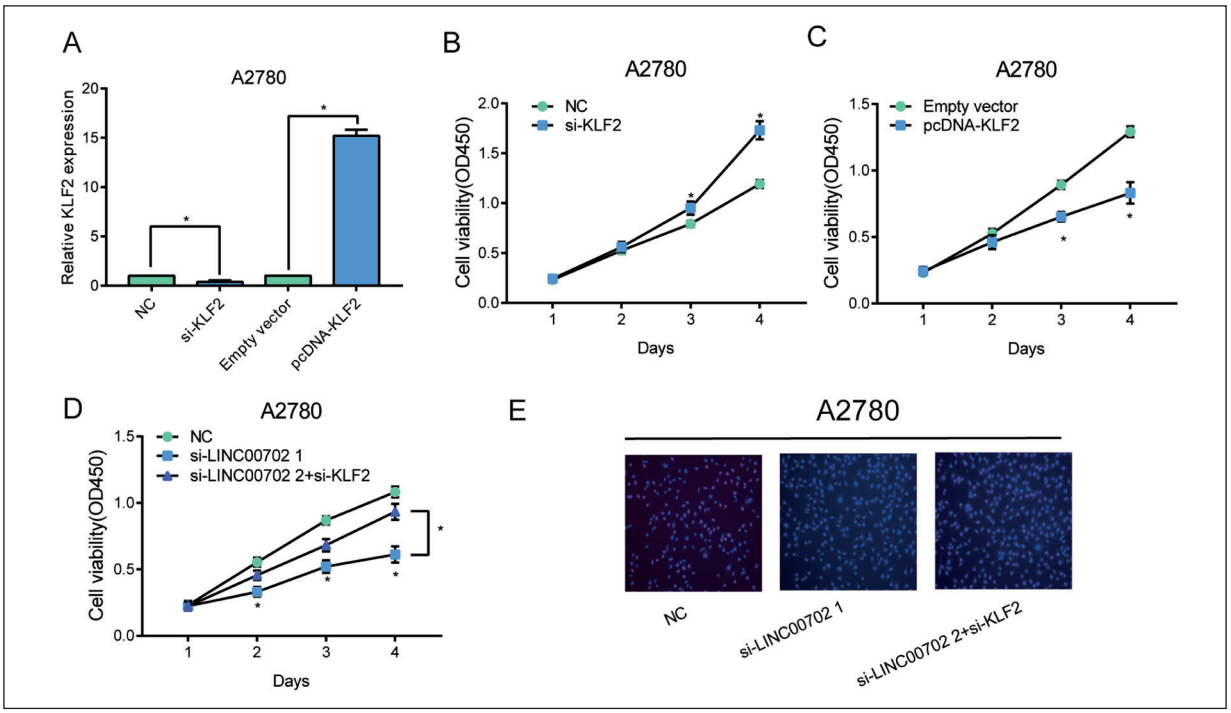

Figure 4. LINC00702 exerted its carcinogenic role in ovarian cancer *via* KLF2. ***A,*** Transfection efficacy of si-KLF2 and pcDNA-KLF2 in A2780 cells. ***B,*** CCK-8 assay showed the viability in A2780 cells transfected with NC or si-KLF2. ***C,*** CCK-8 assay showed the viability in A2780 cells transfected with empty vector or pcDNA-KLF2. ***D,*** CCK-8 assay showed the viability in A2780 cells transfected with NC, si-LINC00702 1, or si-LINC00702 1+si-KLF2. ***E,*** EdU assay showed the EdU-labeled cells in A2780 cells transfected with NC, si-LINC00702 1 or si-LINC00702 1+si-KLF2 (magnification: 40×).

Discussion

In females, OC is the second-most common cancer and the first-most common reason for tumor death[1]. The prognosis of advanced OC patients is very poor due to the insufficient diagnostic strategies at an early stage. Hence, it is urgent to develop sufficient diagnostic and prognostic hallmarks for OC.

LncRNAs are RNA polymerase II transcripts over 200 nucleotides in length. LncRNAs are reported to mediate the multiple biological processes in malignant tumors, including proliferation, metastasis, and apoptosis of tumor cells[16-19]. For instance, lncRNA miR503HG suppresses the invasive and migratory capacities of liver cancer cells *via* the HNRNPA2B1/NF-κB pathway[20]. LINC00460 stimulates the progression and metastasis of meningioma through miR-539/MMP-9 axis[21]. With the in-depth researches on OC, relevant lncRNAs that are capable of regulating OC progression have been identified, such as NEAT1, UCA1, ZNF300P1, and AB073614[9,22-24]. As a newly discovered lncRNA, LINC00702 is reported to be involved in the endometrial cancer[25]. Its specific function in OC, however, has not been reported yet. In this paper, LINC00702 was found to be upregulated in OC. The knockdown of LINC00702 attenuated the proliferative ability of the OC cells.

KLF2 is a key member of the KLF family. It is well concerned as a tumor suppressor gene in many types of malignant tumors[26-30]. Wu et al[30] pointed out that KLF2 inhibits KRAS-induced tumor cell proliferation. EZH2 is reported to directly bind to KLF2 and silence its expression, thus blocking the tumor-suppressor role of KLF2[31]. LncRNAs mediate the downstream targets through different mechanisms and thus influence the phenotypes of the tumor cells[32,33]. Here, we found that LINC00702 directly interacted with EZH2 to suppress the KLF2 level. KLF2 exerted tumor-inhibiting effects in OC. Notably, the knockdown of KLF2 reversed the carcinogenic effect of LINC00702 in OC. It is noteworthy that LINC00702 may regulate several targets. RNA sequencing of LINC00702 is required for further explorations.

Conclusions

This is the first research on reporting the role of LINC00702 in OC. LINC00702 is upregulated in OC. It accelerates the progression of OC *via* interacting with EZH2 to inhibit the transcription of KLF2. LINC00702 could serve as a promising therapeutic target for OC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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