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LINC00968 accelerates the progression of epithelial ovarian cancer via mediating the cell cycle progression

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Abstract. – OBJECTIVE: The aim of this study was to clarify the potential role of LINC00968 in the progression of epithelial ovarian cancer (EOC) and the underlying mechanism.

PATIENTS AND METHODS: The relative expression level of LINC00968 in EOC tissues (n=40) and normal ovarian tissues (n=40) was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). LINC00968 expression in human-derived ovarian cancer cell lines was examined by qRT-PCR as well. After transfection of LINC00968 small-interfering RNA (siRNA) in ovarian cancer cells, cell cycle progression and cell proliferation were evaluated through flow cytometry, Cell Counting Kit-8 (CCK-8) and colony formation assay, respectively. Tumor xenograft was conducted in nude mice to elucidate the function of LINC00968 in EOC tumorigenesis in vivo. Furthermore, the relative expression levels of cell cycle factors and protein kinase B/extracellular-signal-regulated kinase (AKT/ERK) in ovarian cancer cells influenced by LINC00968 were detected by Western blot.

RESULTS: LINC00968 was significantly up-regulated in EOC tissues when compared with normal control tissues. Meanwhile, LINC00968 expression was positively correlated with the prognosis of EOC. Transfection of LINC00968 siRNA in HEY and HO8910 cells markedly attenuated proliferative ability and arrested cell cycle in the G1 phase. Knockdown of LINC00968 remarkably suppressed tumor growth of EOC in nude mice. The silence of LINC00968 significantly downregulated Cyclin D, Cyclin E and CDK4, whereas upregulated p16 and p21. In addition, AKT and ERK pathways were inhibited by knockdown of LINC00968 in ovarian cancer cells.

CONCLUSIONS: LINC00968 expression is markedly upregulated in EOC. Meanwhile, it arrests the cell cycle in the G1 phase by inhibiting the ERK and AKT pathways, thus accelerating EOC progression.

Key Words:
Ovarian cancer (OC), LINC00968, Cell cycle, ERK, AKT.

Introduction

Ovarian cancer is one of the three major malignant tumors in the female reproductive system. Currently, the mortality of ovarian cancer ranks the highest in gynecologic tumors[1]. Ovarian cancer can be mainly divided into epithelial cancer, germinoma, sex cord-stromal tumor and metastatic tumor. Among them, epithelial ovarian cancer (EOC) is the most prevalent subtype, accounting for 70% of malignant ovarian tumors. EOC is characterized by insidious onset, prone to metastasis and poor prognosis[2,3]. Due to the lack of marked symptoms in the early stage, most EOC patients have already been in the advanced stage at the first time of diagnosis. Therefore, the mortality rate of EOC ranks the first among all gynecologic malignancies[4]. In recent years, great efforts have been achieved in improving the therapeutic strategies of EOC. However, its recurrent, metastatic and mortality rates remain extremely high[5]. Therefore, it is urgent to elucidate the mechanisms underlying the occurrence and progression of EOC.

Only about 2% of genes in the human genome can be encoded into proteins. Abundant non-coding RNAs are classified into two types based on their length, including short non-coding RNAs (such as miRNA, siRNA and piRNA) and long non-coding RNA (lncRNA). LncRNAs are a kind of RNA molecules with over than 200 nucleotides in length. Previously, authors[6,7] have indicated

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that lncRNA regulates gene expressions at multiple levels, and is involved in the malignant phenotype of tumor cells.

LINC00968 is located at Chromosome 8: 56496246-56559823. In cardiovascular diseases, LINC00968 attenuates drug resistance of breast cancer cells by inhibiting the Wnt2/beta-catenin signaling pathway by regulating WNT2[8]. LINC00968 accelerates the proliferation and fibrosis of diabetic nephropathy by recruiting EZH2 to inhibit p21[9]. Meanwhile, LINC00968 participates in the tumorigenesis of osteosarcoma by activating the phosphoinositide-3-kinase/protein kinase B/the mammalian target of rapamycin (PI3K/AKT/mTOR) pathway[10]. However, the specific role of LINC00968 in EOC has not been fully elucidated yet. The aim of this study was to explore the effects of LINC00968 in the progression of EOC *in vivo* and *in vitro*. Our findings might provide novel directions for improving the clinical outcomes of EOC patients.

Patients and Methods

Sample Collection

EOC tissues and normal ovarian tissues were surgically resected from 40 EOC patients who received treatment in The First Affiliated Hospital of Bengbu Medical College from December 2017 to October 2018. This study was approved by the Ethics Committee of Cancer hospital of The First Affiliated Hospital of Bengbu Medical College. No patients received preoperative anti-tumor therapy and were pathologically diagnosed. Collected samples were immediately preserved in liquid nitrogen for use. All subjects volunteered to participate in the study and signed informed consent.

RNA Extraction

Tissues (50-100 mg) were fully lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). After maintenance at room temperature for 5 min, 200 μL of chloroform was added, mixed and incubated at room temperature for 5 min. After centrifugation at 4°C, 12,000 rpm for 15 min, the supernatant was transferred into a new RNase-free centrifuge tube. Isopropanol with the same volume of the supernatant was added to harvest RNA precipitate by centrifugation. Extracted RNA was air dried, quantified and dissolved in diethyl pyrocarbonate (DEPC, Beyotime, Shanghai, China) water. Prepared RNA samples were preserved at -80°C.

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

Total RNA was extracted from tissues and cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration of RNA was measured by an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). After that, extracted RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5′-CACCCACTCCTCCACCTTTG-3′, R: 5′-CCACCACCCTGTTGCTGTAG-3′; LINC00968: F: 5′-CCACTCCTTTAC TTCCTTTCTTCTC-3′, R: 5′- ACTCTTCCCTCATTCCTATCCC-3′.

Cell Culture

Ovarian cancer cell lines (SKOV3, HEY and HO8910) and normal ovarian cell line (HOSEpic) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), 100 IU/mL penicillin and 100 μL/ml streptomycin, and maintained in a 37°C, 5% CO_2 incubator.

Cell Transfection

For transfection, cells were pre-seeded into 6-well plates and grown to 60-80% of confluence. Transfection reagent and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were diluted in a serum-free medium, followed by mixing and incubation at room temperature for 20 min. Subsequently, 1.5 mL of serum-free medium and 0.5 mL of transfection mixture were added to each well. 4-6 h later, the complete medium was replaced. Sequences of si-LINC00968 were: LINC00968 siRNA1: 5'-CAAUGAGAGUAAAGAGAAA-3'; LINC00968 siRNA2: 5'-UUAAAGAUGUGGAGAACUA-3'.

Cell Counting Kit-8 (CCK-8)

Cells were first seeded into 96-well plates with 2×10^3 cells per well. Absorbance (A) at 490 nm

was recorded at appointed time points according to the instructions of the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Finally, the viability curve was plotted.

Flow Cytometry

Cell density was first adjusted to 5×10^4 cells/mL. Then, the cells were fixed in pre-cooled 75% ethanol overnight. Before cell cycle determination, the cells were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) twice and incubated with 100 μL of RNaseA in a 37°C water bath in the dark for 30 min. After that, the cells were incubated with 400 μL of propidium iodide (PI) at 4°C in the dark for 30 min. Flow cytometry was used to detect cell cycle progression.

Colony Formation Assay

Cells were first seeded into culture dishes with 50, 100 and 200 cells, respectively. After 2-3 weeks of culture, the cells were fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) for 15 min and stained with GIMSA solution (Beyotime, Shanghai, China) for 30 min. After removing the staining solution, formed colonies were air dried and observed under a microscope. Percentage of colonies=colony number/cell number×100%.

Western Blot

Total protein in cells or tissues was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). The concentration of extracted protein was determined by the bicinchoninic acid (BCA) method. Protein samples were electrophoresed on polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at 300 mA for 100 minutes. After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After rinsing with a Tris-Buffered Saline and Tween 20 solution (TBST; Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with the corresponding secondary antibody. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by Image Software (Media Cybernetics, Silver Springs, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) and GraphPad (Version X; La Jolla, CA, USA) were used for all statistical analysis. Experimental data were represented as mean ± standard deviation (SD). *t*-test was used to compare the differences between the two groups. Survival analysis was conducted using Kaplan-Meier and Log-rank test. $p<0.05$ was considered statistically significant.

Results

LINC00968 was Upregulated in EOC Tissues

LINC00968 was found significantly upregulated in EOC tissues relative to normal control tissues (Figure 1A). By analyzing the follow-up data of enrolled EOC patients, it was found that LINC00968 expression was correlated with tumor size and FIGO stage, whereas was not associated with age, histological subtype, histological grade and lymph node metastasis (Table I). In particular, markedly higher abundance of LINC00968 was observed in EOC patients in stage III-IV than those in stage I-II (Figure 1B). LINC00968 expression was remarkably higher in EOC tissues larger than 5 cm in size when compared with those smaller than 5 cm (Figure 1C). Kaplan-Meier curves revealed that the prognosis of EOC patients with a higher level of LINC00968 was significantly worse than those with a lower level (Figure 1D). The above data demonstrated that high level of LINC00968 indicated poor prognosis of EOC patients.

LINC00968 Accelerated the Proliferation of Ovarian Cancer Cells

The relative expression level of LINC00968 in ovarian cancer cell lines and normal ovarian cell line was determined as well. Identically, LINC00968 was highly expressed in ovarian cancer cells (Figure 2A). HEY and HO8910 cell lines were chosen for subsequent experiments due to high expression of LINC00968. Subsequently, si-LINC00968-1 and si-LINC00968-2 were constructed and transfected into cells to downregulate LINC00968 expression. Si-LINC00968-1 showed better transfection efficacy (Figure 2B). CCK-8 assay revealed significantly decreased viability of HO8910 and HEY cells transfected with si-LINC00968-1 (Figure 2C). After knockdown of LINC00968, the percentage of cells in G0/G1 phase increased markedly, indicating arrested cell cycle progression (Figure 2D). Moreover,

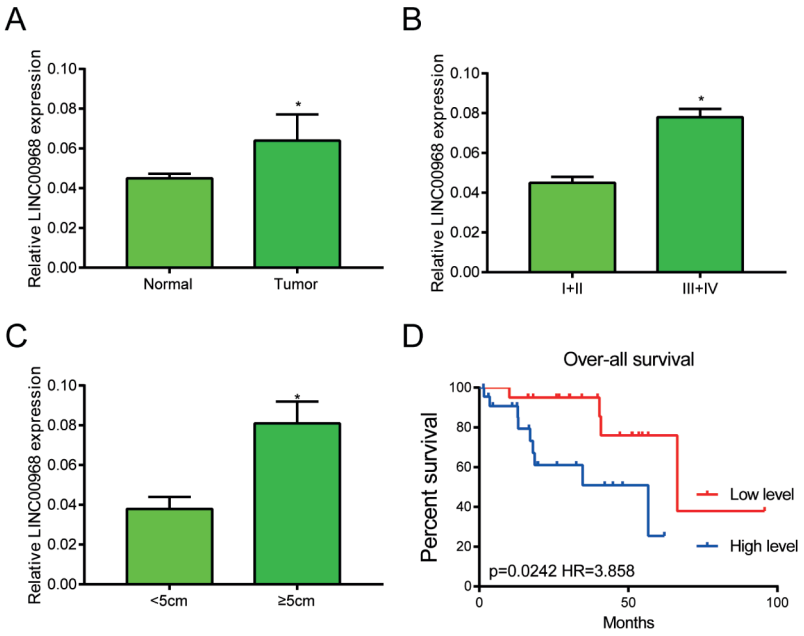

Figure 1. LINC00968 was upregulated in EOC tissues. ***A,*** Relative level of LINC00968 in EOC tissues and normal ovarian tissues. ***B,*** Relative level of LINC00968 in EOC patients in stage III-IV and stage I-II. ***C,*** Relative level of LINC00968 in EOC tissues<5 cm and ≥5 cm. ***D,*** Kaplan-Meier curves revealed overall survival in EOC patients with high level and low level of LINC00968 (p=0.0242, HR=3.858). *p<0.05.

Table I. Correlation between LINC00968 level and pathological indexes of EOC patients (No.=40).

Clinicopathologic features	Number	LINC00968 expression		*p*-value
		Low (No.=20)	High (No.=20)	
Age (years)				
<50	25	12	13	0.7440
≥50	15	8	7	
Histological subtype				
Serous	32	18	14	0.1138
Others	8	2	6	
Tumor size				
<5CM	15	11	4	0.0222*
≥5CM	25	9	16	
FIGO stage				
I-II	17	12	5	0.0252*
III-IV	23	8	15	
Histological grade				
G1-G2	22	12	10	0.5250
G3	18	8	10	
Lymph node metastasis				
Absent	25	13	12	0.7440
Present	15	7	8	

the number of formed colonies was remarkably reduced after knockdown of LINC00968 in ovarian cancer cells (Figure 2E). These results demonstrated that knockdown of LINC00968 attenuated the proliferative ability of ovarian cancer cells.

LINC00968 Promoted the Growth of Ovarian Cancer In Vivo

Tumor xenograft was conducted in nude mice to elucidate the function of LINC00968 in the tumorigenesis of EOC *in vivo*. Compared with

the control group, the relative expression of LINC00968 was markedly reduced in mice administrated with sh-LINC00968 (Figure 3A). After sacrifice, ovarian tumor tissues were extracted from mice and weighed. Tumor weight was significantly lower in mice with LINC00968 knockdown (Figure 3B). Moreover, the rate of tumor growth was significantly slower in mice administrated with sh-LINC00968 (Figure 3C). Tumor tissues extracted from nude mice were prepared for homogenate. Western blot analyses showed that the protein levels of Cyclin D, Cyclin E and CDK4 were downregulated, whereas p16 and p21 were upregulated in ovarian cancer tissues extracted from mice administrated with sh-LINC00968 (Figure 3D). The above data validated the role of LINC00968 in accelerating the proliferation of EOC *in vivo*.

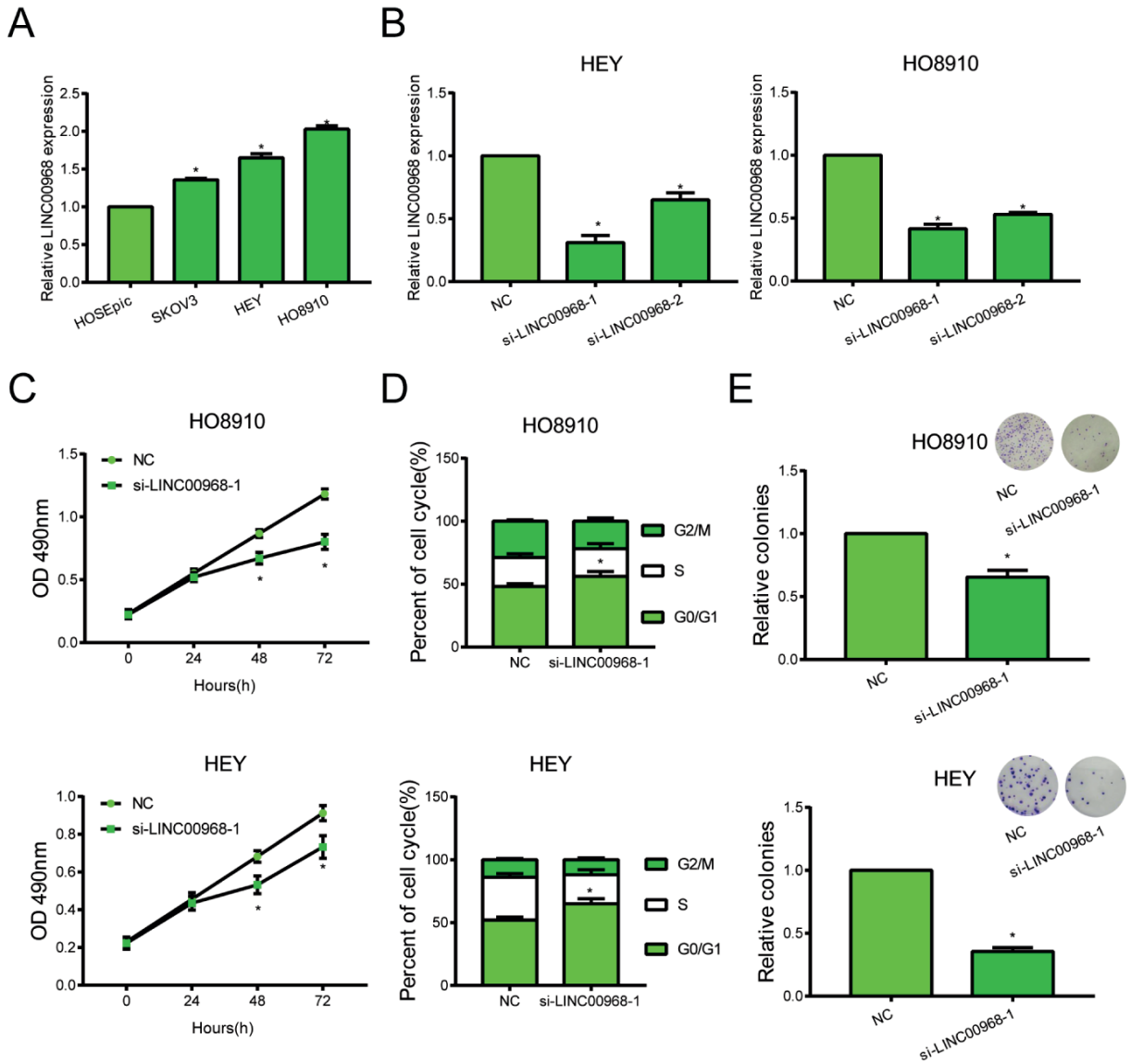

Figure 2. LINC00968 accelerated the proliferation of ovarian cancer cells. ***A,*** Relative level of LINC00968 in ovarian cancer cell lines (SKOV3, HEY and HO8910) and normal ovarian cell line (HOSEpic). ***B,*** Transfection efficacy of si-LINC00968-1 and si-LINC00968-2 in HO8910 and HEY cells. ***C,*** CCK-8 assay revealed the viability of HO8910 and HEY cells transfected with NC or si-LINC00968-1. ***D,*** Flow cytometry revealed the percentage of cell cycle in HO8910 and HEY cells transfected with NC or si-LINC00968-1. ***E,*** Colony formation assay revealed the number of colonies in HO8910 and HEY cells transfected with NC or si-LINC00968-1 (magnification × 20). *$p<0.05$.

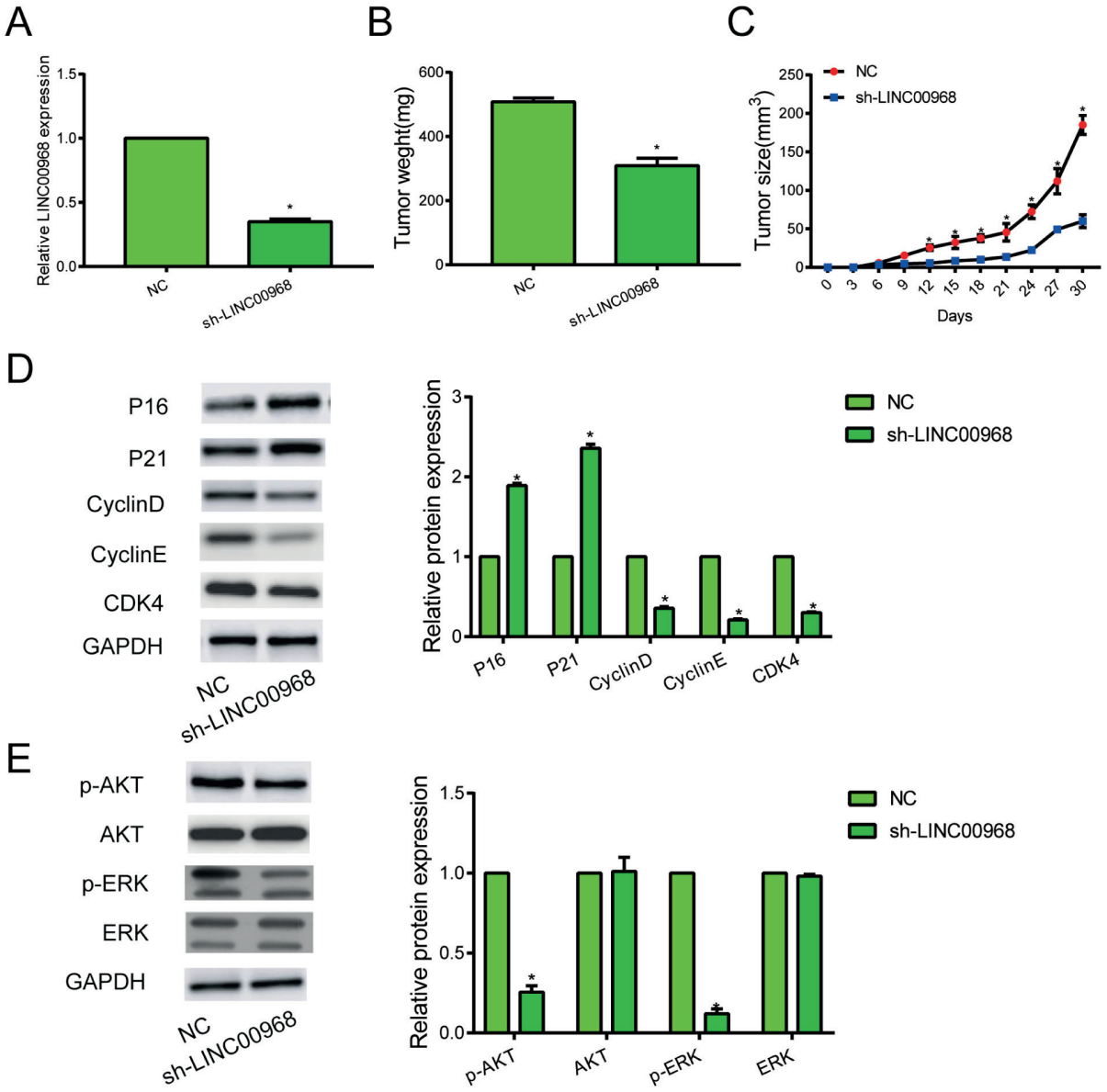

Figure 3. LINC00968 promoted the growth of ovarian cancer *in vivo*. ***A,*** Relative level of LINC00968 in nude mice administrated with NC or sh-LINC00968. ***B,*** Tumor weight in nude mice administrated with NC or sh-LINC00968. ***C,*** Tumor size in nude mice administrated with NC or sh-LINC00968. ***D,*** Western blot analyses of p16, p21, Cyclin D, Cyclin E and CDK4 in nude mice administrated with NC or sh-LINC00968. ***E,*** Western blot analyses of p-AKT, AKT, p-EKR and ERK in nude mice administrated with NC or sh-LINC00968. *$p<0.05$.

LINC00968 Mediated Cell Cycle in EOC via Activating AKT and ERK Pathways

AKT and ERK pathways have been reported to participate in the occurrence and progression of tumors. Their functions in regulating cell cycle progression have been identified as well. Here, markedly downregulated p-AKT and p-ERK were observed in mice administrated with sh-LINC00968 (Figure 3E). All these results concluded that LINC00968 regulated cell cycle of EOC *via* activating the AKT and ERK pathways.

Discussion

Clinical symptoms of early-stage EOC are not significant. EOC patients are often diagnosed at the advanced stage, accompanied by extensive

metastases in the pelvic and abdominal cavity. Currently, the therapeutic efficacy of advanced EOC is far from satisfactory, leading to poor prognosis. The 5-year survival of ovarian cancer depends on the infiltration of tumor cells. Statistics[11] have shown that the 5-year survival of patients with focal ovarian foci, pelvic metastasis, and distant metastasis is 90%, 75%, and 30%, respectively. Nowadays, the pathogenesis of EOC has been extensively studied, which is of clinical significance in improving the outcome of patients.

At least 50% of RNAs cannot encode proteins[12]. Previous studies[13,14] have indicated that lncRNAs are structurally conserved, which are only transcriptional noises without biological functions. However, recent studies[15] have reported the involvement of lncRNAs in multi-level regulations, biological processes and tumor progression. By serving as hallmarks and prognostic indicators, abnormally expressed lncRNAs are closely related to the occurrence, progression and prognosis of tumors[16]. Gupta et al[17] have found that HOTAIR at the HOX locus is upregulated in breast cancer, which is also manifested as a predictor of metastasis and death in patients. Further researches have demonstrated the role of HOTAIR in accelerating the metastasis of tumor cells *in vivo* and *in vitro*. However, studies on EOC-related lncRNAs are at the beginning stage currently. In this work, survival analysis identified a significantly worse overall survival of EOC patients with a higher level of LINC00968. LINC00968 expression was closely correlated with the prognosis of EOC patients. This allowed it to be a promising candidate to predict the prognosis of EOC. Subsequently, it was confirmed that knockdown of LINC00968 inhibited proliferative ability and arrested cell cycle progression of ovarian cancer cells.

Cell cycle progression is a vital process, in which eukaryotic cells experience complete mitotic phase to the next one. Meanwhile, it is a cyclical process of cell accumulation and division. Abnormalities can usually be observed in cell cycle progression of cancer cells[18-21]. Therefore, regulating the cell cycle of a malignant tumor is an important strategy and target for tumor therapy. In this study, we found that knockdown of LINC00968 in ovarian cancer cells arrested the cell cycle in the G1 phase and suppressed cell proliferative ability. Furthermore, the expressions of G1 checkpoints Cyclin D and Cyclin E were significantly downregulated by the silence of LINC00968.

Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) family, which controls multiple cellular behaviors[22-24]. In this work, Western blot results showed that knockdown of LINC00968 downregulated the protein level of p-ERK. Therefore, we believed that silence of LINC00968 arrested cell cycle and inhibited the proliferative ability of ovarian cancer by suppressing the phosphorylation of ERK. The PI3K/Akt/mTOR pathway is a widely recognized pathway currently, which is involved in the functional activities of cells[25]. Here, knockdown of LINC00968 was found to downregulate p-AKT level in ovarian cancer cells as well.

Conclusions

We found that LINC00968 was significantly upregulated in EOC. Moreover, it arrested the cell cycle in the G1 phase by inhibiting the ERK and AKT pathways, thus accelerating the progression of EOC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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