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LINC01308 accelerates the malignant progression of ovarian cancer by binding to miRNA-506

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Abstract. – OBJECTIVE: To detect the expression pattern of LINC01308 in ovarian cancer (OC), and further clarify whether LINC01308 could promote the malignant progression of OC by mediating microRNA-506 (miRNA-506).

PATIENTS AND METHODS: Relative level of LINC01308 in 28 pairs of OC tissues and paracancerous tissues was determined by quantitative Real-time polymerase chain reaction (qRT-PCR). We analyzed the correlation between LINC01308 level and the prognosis of OC patients. Subsequently, LINC01308 level in OC cell lines was determined as well. By transfection of sh-LINC01308 in 3AO and CAOV3 cell lines, we evaluated the influence of LINC01308 on cellular behaviors of OC through cell counting kit-8 (CCK-8), transwell and wound healing assay. Target downstream of LINC01308 was verified by dual-luciferase reporter gene assay. Finally, the regulatory effect of LINC01308/miRNA-506 on OC cell behaviors was examined through a series of rescue experiments.

RESULTS: LINC01308 was highly expressed in OC tissues relative to controls. OC patients with high-level LINC01308 had higher rates of lymph node metastasis and distant metastasis, and lower survival rate compared with those with low level. By transfection of sh-LINC01308 in OC cells, the migratory and invasive abilities were markedly weakened. MiRNA-506 was found to be the target gene of LINC01308, and its level was negatively regulated by LINC01308 in OC tissues. Finally, we found that miRNA-506 knockdown reversed the regulatory effect of LINC01308 on the migratory and invasive abilities of OC cells.

CONCLUSIONS: LINC01308 is highly expressed in OC and correlated to metastasis and poor prognosis. LINC01308 enhances OC cells to migrate and invade by targeting miRNA-506.

Key Words:
LINC01308, MiRNA-506, Ovarian cancer, Migration, Invasion.

Introduction

Ovarian cancer (OC) is one of the three major malignancies in the female reproductive system, and its incidence ranks second in gynecologic malignancies[1-3]. Due to hidden symptoms and lack of diagnostic methods in early stage, most OC patients have progressed into stage III-IV. More seriously, lack of effective treatment for advanced OC leads to the high mortality in gynecological malignancies[4,5]. Among them, epithelial ovarian cancer is the common subtype of OC, accounting for 85-90% in all cases of ovarian malignant tumors. It is characterized with rapid progression, frequent invasion and metastasis[5,6]. As a result, tumor lesion of OC is difficult to be completely resected by surgery[7]. It is estimated that about 90% of tumor patients die from tumor metastasis[8]. Survival analysis has suggested the invasion and metastasis to be the most important factors influencing the prognosis of OC. Therefore, molecular pathological mechanism of OC requires to be clarified, which helps to prolong the survival and reduce the mortality of OC[9]. Recent studies[9,10] have shown the crucial role of long non-coding RNAs (lncRNAs), a type of non-coding RNA with over 200 nt in length, in the carcinogenesis and malignant progression of tumor cells. LncRNA can be used as a diagnostic and prog-

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nostic hallmark for different types of malignancies[9,10]. Differentially expressed lncRNAs have been identified in OC, such as SNHG15, CASC9, snaR, etc. These lncRNAs have been detected to act as oncogenes or tumor-suppressor genes in OC[11-13]. LINC01308 is abnormally expressed in malignant tumors, showing a remarkable influence on tumor development. It is reported that LINC01308 is considered as an oncogene in many types of tumors[14,15]. Nevertheless, the biological role and clinical significance of LINC01308 in OC have not been comprehensively revealed. MicroRNAs (miRNAs) are a class of endogenous, non-coding, small-molecule ribonucleotides that bind to the mRNA 3'-untranslated regions (3'-UTR) to degrade or post-transcriptionally silence them[16,17]. MiRNA-506 has been extensively studied in many types of tumors, but its role in OC is rarely reported[18,19]. In this paper, we first determined the expression patterns of LINC01308 and miRNA-506 in OC. The regulatory effects of LINC01308/miRNA-506 axis on migratory and invasive abilities of OC were further explored.

Patients and Methods

Patients and OC Samples

OC tissues and paracancerous tissues were surgically resected from OC patients, and pathologically diagnosed by hematoxylin and eosin (HE) staining (Boster, Wuhan, China). Samples were preserved at -80°C. None of OC patients were treated with anti-tumor therapy before surgery. Follow-up data of enrolled patients were collected. Patients and their families in this study were fully informed. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Cell Culture

Six human-derived OC cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, CAOV3) and the human ovarian surface epithelial cell line (HOSEPiCs) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD, USA) and maintained in a 37°C, 5% CO_2 incubator.

Transfection

ShRNA-NC and three lines of sh-LINC01308 (sh-LINC01308#1, sh-LINC01308#2, sh-LINC01308#3) were constructed by GenePharma (Shanghai, China). Cells seeded in the 6-well plates with 40% of confluence were transfected with shRNA-NC or sh-LINC01308. At 48 h, cells were harvested for subsequent experiments.

Cell Proliferation Assay

Cells were seeded in the 96-well plate with 2.0×10^3 cells per well. Viability was determined at the appointed time points using cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 490 nm was recorded for plotting the viability curve.

Transwell Cell Migration and Invasion Assay

Transfected cells for 48 h were digested and adjusted to 5.0×10^5/mL. 200 μL/well suspension was applied in the upper side of matrigel-coated transwell chamber (Millipore, Billerica, MA, USA). In the bottom side, 500 μL of medium containing 10% fetal bovine serum (FBS) was applied. After 48 h of incubation, invasive cells were fixed in methanol for 15 min, dyed with 0.2% crystal violet for 20 min and counted using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample. Transwell migration assay was conducted in the same procedures except for Matrigel pre-coating.

Wound Healing Assay

Cells were seeded in a 6-well plate with 5.0×10^5/well. Until 90% of confluence, an artificial wound was created in the confluent cell monolayer using a 1 mL pipette tip. The images were taken at 0 and 24 h using an inverted microscope, respectively. Percentage of wound closure was calculated.

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

We extracted total RNA from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified by DNase I treatment. Extracted RNA was reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The cDNA was amplified by Real-time quantitative PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). GAPDH and U6 were used as internal references. Primer sequences were as follows: LINC01308, forward: 5'-CGGAGAGGUCAGAGGUAGATT-3', reverse: 5'-UCUAC UCUGACCUCUCCGTT-3'; GAPDH, forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3',

reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'; MiRNA-506: forward: 5'-GCCACCACCATCAGCCATAC-3', reverse: 5'-GCACATTACTCTACTCAGAAGGG-3'; U6: forward: 5'-CGCTTCGGCAGCACATATAC-3', reverse: 5'-TTCACGAATTTGCGTGTCAT-3'.

Each sample was performed in triplicate, and analyzed by iQ5 2.0 (Bio-Rad, Hercules, CA, USA).

Dual-Luciferase Reporter Gene Assay

We first constructed pmirGLO-LINC01308-wt and pmirGLO-LINC01308-mut based on the binding sequences of LINC01308 and miRNA-506. OC cells were co-transfected with pmirGLO-LINC01308-wt/pmirGLO-LINC01308-mut and miRNA-506 mimics/NC, respectively. After 48 h, cells were lysed and subjected to luciferase activity determination.

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The Student *t*-test was applied for analyzing the intergroup differences. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Kaplan-Meier was introduced for survival analysis. $p<0.05$ was considered statistically significant.

Results

High Expression of LINC01308 in OC

In this study, we collected 28 pairs of OC tissues and paracancerous tissues to examine their LINC01308 level. QRT-PCR data showed higher level of LINC01308 in OC tissues relative to controls, indicating the potential oncogenic role of LINC01308 in OC (Figure 1A). Identically, LINC01308 was highly expressed in OC cell lines relative to HOSEPiCs cell line (Figure 1B).

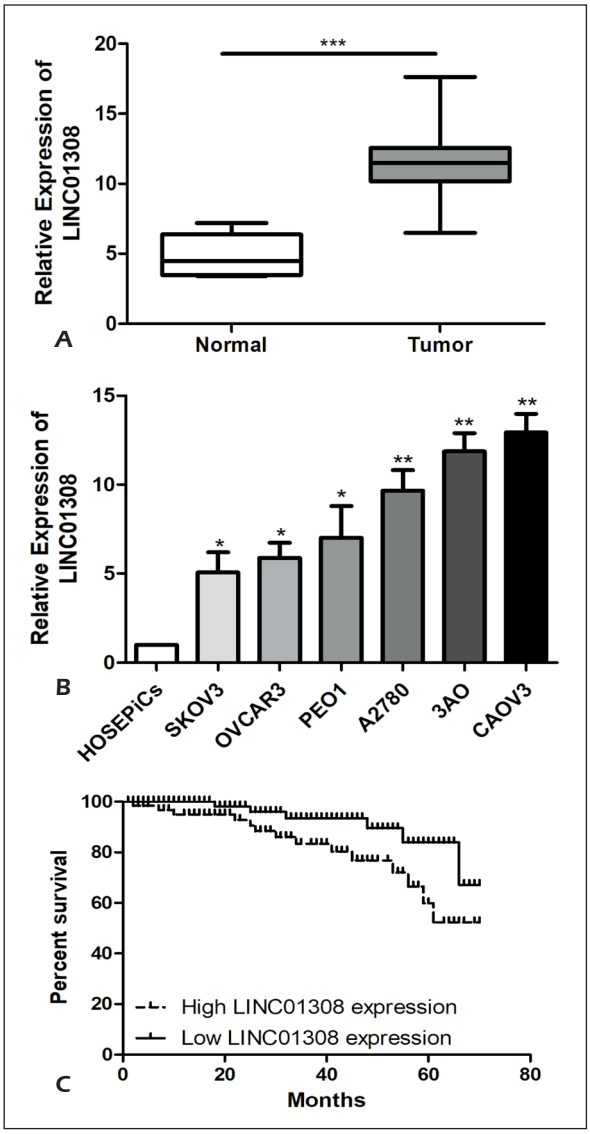

Figure 1. LINC01308 was highly expressed in OC. **A**, QRT-PCR data showed higher level of LINC01308 in OC tissues relative to paracancerous tissues. **B**, QRT-PCR data showed higher level of LINC01308 in OC cell lines relative to HOSEPiCs cell line. **C**, Kaplan-Meier curve indicated that expression level of LINC01308 was negatively correlated to survival of OC.

LINC01308 Expression was Correlated With Pathological Staging and Overall Survival of OC

Based on the mRNA level of LINC01308 in the enrolled OC patients, we divided them into high-level and low-level group, and further analyzed the correlation between LINC01308 level to pathological indexes of OC patients. As depicted in Table I, LINC01308 level was positively correlated to lymph node metastasis and distant metastasis, rather than age and pathological stage of OC patients. In addition, we collected follow-up data for survival analysis. Kaplan-Meier data revealed that high-level LINC01308 was correlated to poor prognosis of OC patients. The higher the LINC01308 level, the worse the prognosis of OC patients (Figure 1C).

Knockdown of LINC01308 Inhibited OC Cells to Migrate and Invade

Three lines of sh-LINC01308 were constructed and their transfection efficacy was verified in 3AO and CAOV3 cell lines (Figure 2A). Among

Table I. Association of LINC01308 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	LINC01308 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.912
<60	12	7	5	
≥60	16	9	7	
T stage				0.569
T1-T2	18	11	7	
T3-T4	10	5	5	
Lymph node metastasis				0.010
No	17	13	4	
Yes	11	3	8	
Distance metastasis				0.030
No	20	14	6	
Yes	8	2	6	

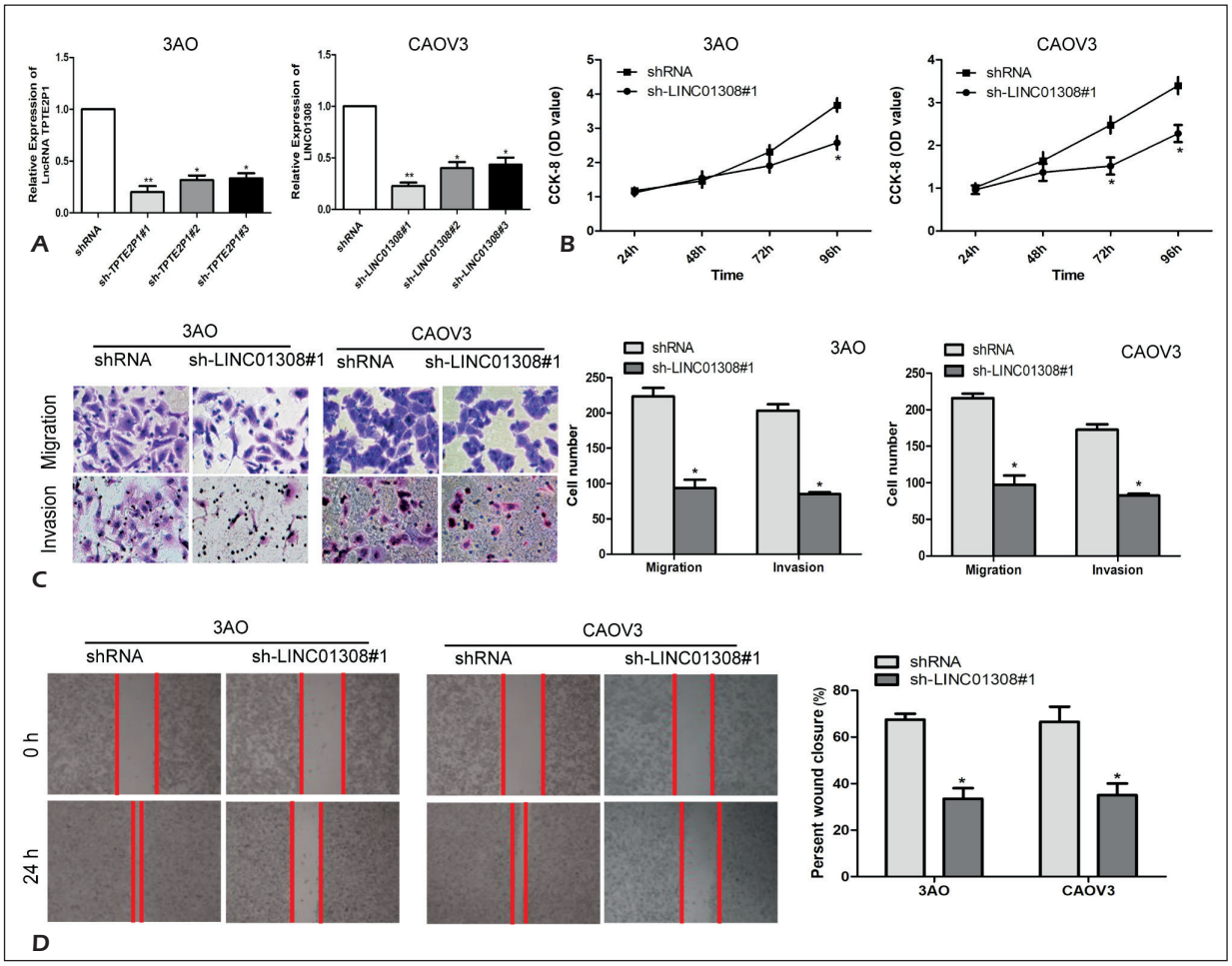

Figure 2. Knockdown of LINC01308 inhibited migration and invasion of OC. **A**, Transfection efficacy of sh-LINC01308#1, sh-LINC01308#2 and sh-LINC01308#3 in 3AO and CAOV3 cells. **B**, CCK-8 assay showed that transfection of sh-LINC01308#1 decreased viability of 3AO and CAOV3 cells. **C**, Transwell assay showed that transfection of sh-LINC01308#1 inhibited migration and invasion of 3AO and CAOV3 cells. **D**, Wound-healing assay showed that transfection of sh-LINC01308#1 inhibited wound closure of 3AO and CAOV3 cells.

them, sh-LINC01308#1 showed the best efficacy to downregulate LINC01308 level in OC cells. As the viability curve revealed, transfection of sh-LINC01308#1 inhibited proliferative ability of 3AO and CAOV3 cells (Figure 2B). Transwell assay also demonstrated that knockdown of LINC01308 inhibited OC cells to migrate and invade (Figure 2C). Wound closure percentage markedly decreased in OC cells transfected with sh-LINC01308#1 relative to controls (Figure 2D).

LINC01308 Bound to miRNA-506

To verify the predicted target miRNA-506 to LINC01308, we constructed pmirGLO-LINC01308-wt and pmirGLO-LINC01308-mut. OC cells were co-transfected with pmirGLO-LINC01308-wt/pmirGLO-LINC01308-mut and miRNA-506 mimics/NC, respectively. Dual-luciferase reporter gene assay showed that miRNA-506 overexpression remarkably weakened the luciferase activity in OC cells containing wild-type LINC01308 vector (Figure 3A, 3B). It is suggested that LINC01308 could directly bind to miRNA-506.

Low Expression of miRNA-506 in OC

QRT-PCR data revealed low expression of miRNA-506 in OC tissues relative to controls (Figure 3B). Identically, miRNA-506 was lowly expressed in OC cell lines compared with that of HOSEPiCs cell line (Figure 3C). We next selected 16 pairs of OC tissues to examine the

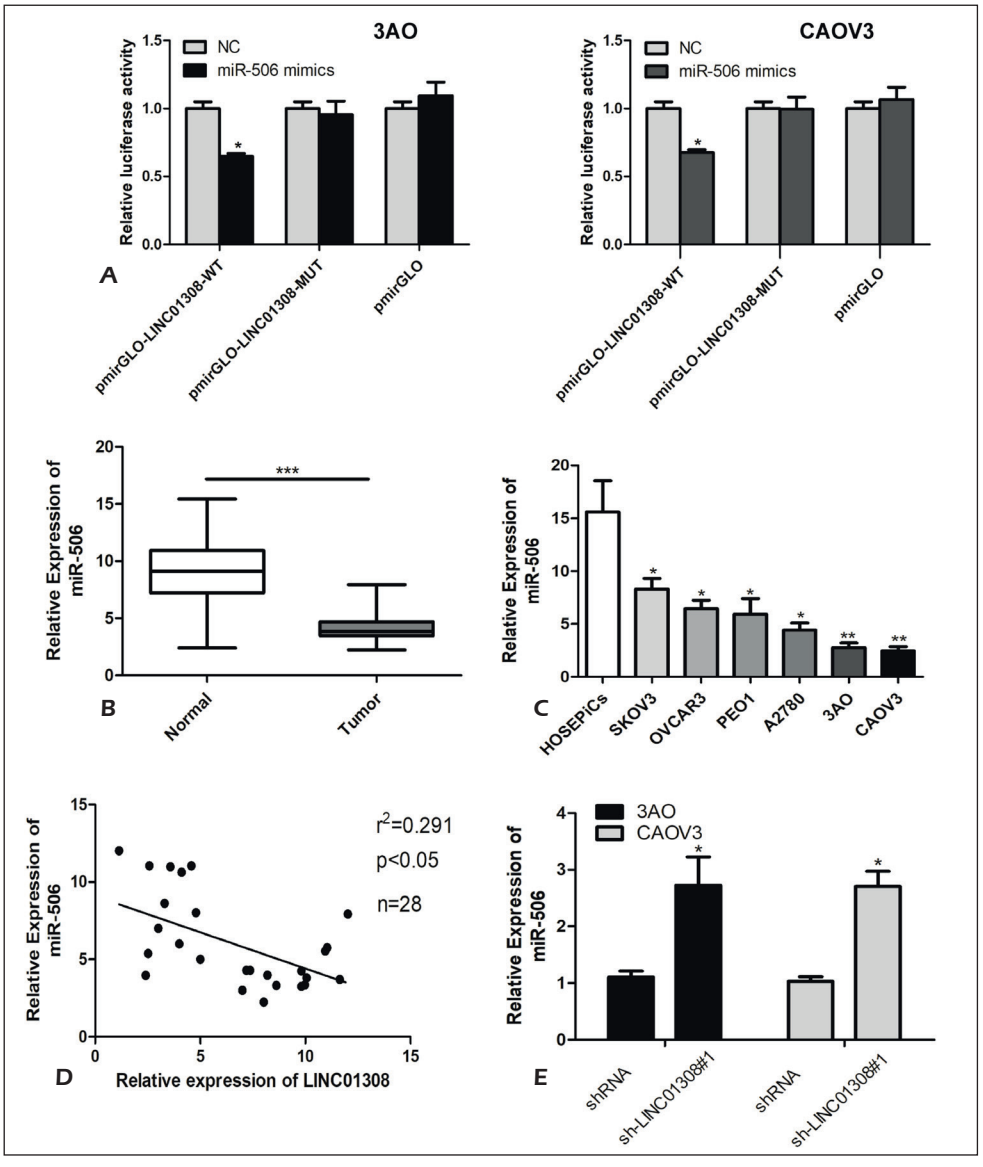

Figure 3. LINC01308 bound to miR-506. **A**, Dual-luciferase reporter gene assay showed that miR-506 overexpression weakened luciferase activity of cells containing pmirGLO-LINC01308-WT. **B**, QRT-PCR data showed lower level of miR-506 in OC tissues relative to paracancerous tissues. **C**, QRT-PCR data showed lower level of miR-506 in OC cell lines relative to HOSEPiCs cell line. **D**, A negative correlation between LINC01308 and miR-506 in OC. E, Transfection of sh-LINC01308#1 upregulated miR-506 level in 3AO and CAOV3 cells.

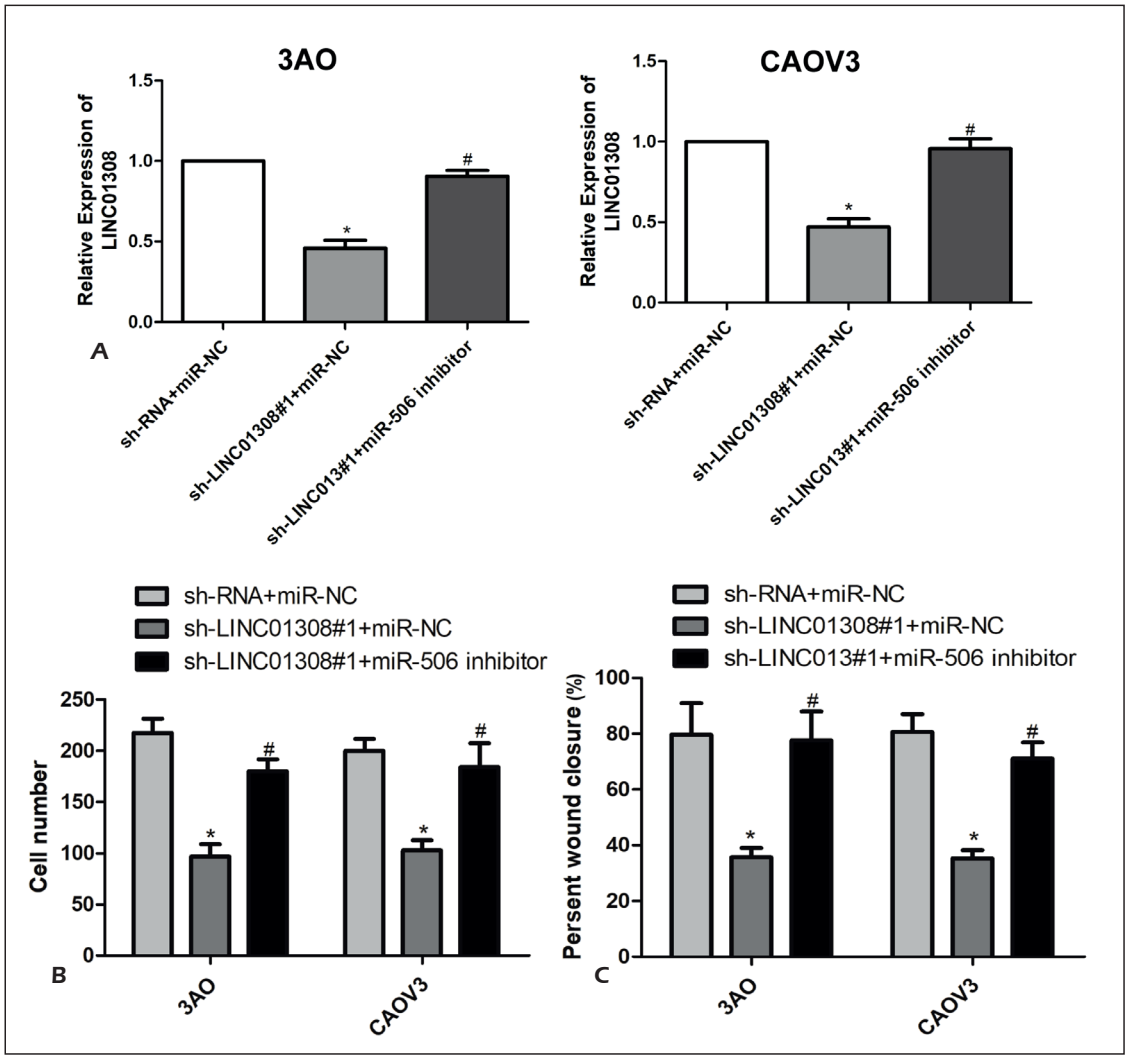

Figure 4. LINC01308 regulated biological behaviors of OC by targeting miR-506. 3AO and CAOV3 cells were transfected with sh-RNA+miR-NC, sh-LINC01308#1+miR-NC or sh-LINC01308#1+miR-506 inhibitor, respectively. **A**, Relative level of LINC01308 in 3AO and CAOV3 cells. **B**, Migratory cell number in 3AO and CAOV3 cells. **C**, Percentage of wound closure in 3AO and CAOV3 cells.

correlation between expressions of LINC01308 and miRNA-506, and a negative correlation between them was identified (Figure 3D). Transfection of sh-LINC01308#1 upregulated miRNA-506 level in 3AO and CAOV3 cells, further demonstrating their negative correlation (Figure 3E).

LINC01308 Regulated Biological Behaviors of OC by Targeting miRNA-506

To further investigate the specific function of LINC01308 in attenuating the malignant progression of OC, we designed a series of rescue experiments. Downregulated LINC01308 by transfection of sh-LINC01308#1 was partially upregulated after co-transfection of sh-LINC01308#1 and miRNA-506 inhibitor in OC cells (Figure 4A). Inhibited migratory ability and wound closure in OC cells with LINC01308 knockdown were partially reversed by miRNA-506 overexpression (Figure 4B, 4C).

Discussion

OC is one of the main types of malignant tumors in the female reproductive system. Globally, OC ranks second in the morbidity and first in

tumor death of female malignancies. The 5-year survival of OC is over 40%[1-4]. However, the prognosis of advanced OC is relatively poor due to high recurrent and metastatic rates[4,5]. Explorations on the pathogenesis of OC are necessary and beneficial to develop effective therapeutic target[5-7]. Tumor invasion and metastasis are the major reason for treatment failure and death. The complex process of tumor metastasis involves the interaction of tumor cells to host cells, and many other biological factors, signaling pathways. Meanwhile, cellular behavior changes, tumor neovascularization and matrix degradation are closely related to tumorigenesis[6-9]. Current researches have demonstrated the involvement of adhesion factors, neurotrophic factors and cytokines in the invasion and metastasis of OC. It is well concerned nowadays that miRNAs exert crucial functions in the occurrence and progression of OC[9-11]. Nevertheless, relevant lncRNAs in OC are rarely explored. Many lncRNAs are abnormally expressed in different types of tumors. As a novel regulator, lncRNA is the research focus in tumor biology[9,10]. LncRNA is unable to encode protein and could not perform conventional RNA transcription[10]. However, lncRNA can interact with proteins and regulate the corresponding proteins by binding to them[10-12]. In this paper, LINC01308 was negatively correlated to miRNA-506 level in OC. High level of LINC01308 was associated with the lymph node metastasis and distant metastasis of OC, serving as a potential prognostic hallmark. In addition, silence of LINC01308 inhibited OC cells to invade and migrate. Previous studies[14] have shown that LINC01308 regulates biological behaviors of NSCLC by binding to miR-124 to downregulate ADAM15. Here, we predicted the binding sequences of LINC01308 and miRNA-506 through bioinformatics. Dual-luciferase reporter gene further verified that LINC01308 could bind to its downstream miRNA-506. Mutation of binding sites of LINC01308 to miRNA-506 failed to enrich miRNA-506 in OC cells. We speculated that LINC01308 exerted its regulatory effect on OC by binding to miRNA-506, and was further identified through a series of functional experiments. Our conclusion was consistent with other reports that miRNA-506 serves as a tumor-suppressor gene. To sum up, LINC01308 was highly expressed in OC. Silence of LINC01308 markedly suppressed OC cells to invade and migrate. MiRNA-506, as the downstream of LINC01308, was negatively regulated by it. LINC01308/miRNA-506 axis may be utilized for the treatment and prognosis of OC.

Conclusions

We found that LINC01308 is highly expressed in OC and correlated to metastasis and poor prognosis. LINC01308 enhances OC cells to migrate and invade by targeting miRNA-506.

Conflict of Interest

The authors declared no conflict of interest.

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