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Downregulation of LINC01508 contributes to cisplatin resistance in ovarian cancer via the regulation of the Hippo-YAP pathway

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ABSTRACT

Background: Some long non-coding RNAs (lncRNAs) have been found to contribute to cisplatin resistance. Here, we identified a novel lncRNA that was downregulated in cisplatin-resistant to ovarian cancer (OC) cells and aimed to examine the contribution of LINC01508 to cisplatin resistance in OC cells.

Methods: Differences in the lncRNA expression profile between OV2008 and C13K cells were assessed by lncRNA expression microarray. The expression of LINC01508 in ovarian epithelial cells, four OC cells, and OC, benign ovary tumor and normal ovary, cisplatin-resistant and non-resistant OC specimens were evaluated by quantitative real-time polymerase chain reaction (qPCR). The role of LINC01508 in OC cisplatin-resistant was evaluated by cell counting kit-8 (CCK-8), flow cytometry, colony formation, wound healing, Transwell, and tumor growth inhibition study in vivo. The clinical associations of LINC01508 in OC were evaluated using correlation analysis. The effects of verteporfin (VP) on cisplatin were explored to reveal the function of the hippo-YAP pathway on the cisplatin tolerance of C13K.

Results: LINC01508 was downregulated in cisplatin-resistant OC cells and platinum-resistant OC tissue ($p < 0.01$). LINC01508 downregulation was correlated with tumor size, residual tumor, and platinum resistance. The overexpression of LINC01508 improves in vitro and in vivo sensitivity to cisplatin while predicts the poor overall survival which need further follow-up research. The increased level of LINC01508 could suppress the cisplatin resistance of OC cells through the inhibition of the hippo-YAP pathway.

Conclusions: The study proposes that dysregulation of LINC01508 expression results in resistance of OC to cisplatin through the inhibition of the hippo-YAP pathway.

Keywords: Ovarian Cancer; Long Non-Coding RNA C01508; Hippo/YAP Signaling Pathway; Cisplatin- Resistance

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Synopsis

Dysregulation of LINC01508 expression results in resistance of OC to cisplatin via the Hippo pathway.

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

Conceptualization: S.X.Y., L.M.; Data curation: Z.M.M.; Formal analysis: L.Z.L.; Funding acquisition: X.L., Y.S.J.; Investigation: W.Z.L.; Methodology: L.M.; Project administration: S.X.Y., Z.M.M.; Resources: Y.S.J.; Supervision: W.Z.L.; Validation: L.Z.L.; Writing - original draft: X.L.; Writing - review & editing: X.L.

INTRODUCTION

Ovarian cancer (OC) is the most lethal gynecological malignancy afflicting women with a high incidence of recurrence. The cytotoxic-based platinum compound cisplatin has been commonly used in OC treatment for almost three decades [1]. However, the intrinsic and acquired resistance to cisplatin in cancer cells remains a big challenge for overall survival (OS) [2]. It is important to understand the new mechanism(s) of cisplatin resistance and develop new strategies for OC treatments. Long non-coding RNAs (lncRNAs) are defined as RNA transcripts comprising more than 200 nucleotides. Most of them are incapable of encoding proteins, which are significantly different from micro RNAs (miRNAs), and small RNAs (siRNAs) [3,4]. Recent data shed light on the numerous and indispensable functions of non-coding RNAs (ncRNAs), particularly in carcinogenesis and chemo-resistance lncRNAs [5-7]. lncRNAs may be superior biomarkers in cancer. It is a new area of molecular biology and may have better specificity [8-10]. Until recently, only a few human lncRNAs have been demonstrated to contribute to acquired resistance.

A study published by Ouyang et al. [11] showed that LINC01508 is a potential biomarker for predicting breast cancer response to neoadjuvant chemotherapy (NAC). Furthermore, this is the only report on the relationship between LINC01508 and cancer. LINC01508 is located on chromosome 9q22.2 with 1,030 nt in length. As a new lncRNA, the expression patterns of LINC01508 and its clinical significance in OC remain uncertain. It is essential to analyze the dysregulated expressed LINC01508 in OC and clarify its regulatory function in developing resistance to therapy. The current study was conducted to aim at the LINC01508 expression in OC and analyze its potential associations with clinicopathological characteristics and platinum resistance in vitro and in vivo. In conclusion, our study might provide a novel mechanism and potential therapeutic target for cisplatin resistance OC.

MATERIALS AND METHODS

1. Microarrays and computational analysis

The Arraystar Human lncRNA microarray used for the microarray screening number was v4.0. Differentially expressed genes were selected by the R programming language. lncRNA expression scatters profiling (fold change > 2.0; p < 0.05) between OV2008 and C13K cells was performed to identify significantly different expressions of lncRNAs.

2. Patients and tissue specimens

The study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Anhui Medical University, China. Informed consent was obtained from 2017 to 2019. A total of 71 specimens of serous ovarian cancer, 25 ovarian serous cystadenomas, and 19 normal ovarian epithelium tissues from benign tumor patients were included for study. OC patients ages were 36–75 years old (mean age, 48.42 years). Thirteen cases were stage I–II, and 58 cases were stage III–IV. Histopathology and tumor grade were determined via pathology. None of the patients had been subjected to chemotherapy or radiotherapy before surgery. All were treated with systemic platinum-based chemotherapy following surgery. According to the International Federation of Gynecology and Obstetrics (FIGO) staging system, the tumor stage was determined. For experiments on platinum-based chemotherapy resistance, patients with OC were divided into two groups (platinum-resistant and platinum-sensitive) according to the criteria described below. Patient response to chemotherapy was evaluated according

to National Comprehensive Cancer Network guidelines (version 1.2017, ovarian cancer). The guideline has divided 'sensitive' from 'resistant' disease at six months [12].

3. Cell line and cell culture

The cisplatin-sensitive human ovarian cancer cell lines OV2008 and its cisplatin-resistant clone C13K were supplied by Dr. Wen-cheng Ding (Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology). The human epithelial ovarian cancer cell lines SKOV3, A2780, and normal human ovarian surface epithelial cell line human ovarian surface epithelial cells (HOSEpiC) were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). OV2008, C13K, and HOSEpiC cells were maintained in complete RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS). In contrast, the SKOV3 and A2780 cell lines were cultured in Dulbecco's modified eagle medium (DMEM, Thermo Fisher Scientific) medium with 10% FBS serum. All cells were cultured at 37°C in 5% CO₂.

4. Sampling

All tissues were frozen in liquid nitrogen within 30 minutes after resection and stored at 80°C. Frozen section and hematoxylin & eosin (HE) staining were performed in the study. Stained sections were evaluated by 2 pathologists. Tissues with a tumor cell ratio greater than 60% were directly included in the study. Clinicopathological factors, such as age, FIGO stage, histologic grade, CA125, tumor size, lymph node metastasis, residual tumor, and platinum resistance, were recorded on the database.

5. Reagents

Cisplatin was purchased from Hansoh Pharmaceutical Co Ltd (Lianyungang, China). Verteporfin (VP) was ordered from Sigma-Aldrich Co. (St. Louis, MO, USA) and used at a concentration of 10 μM to treat cells.

6. Cell transfection

OV2008 cells were seeded onto 6-well plates for 24 hours, transfected with siRNAs (si-NC, si-LINC01508 #1, #2) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and then incubated for 48 hours. The LINC01508 sequence was synthesized and subcloned into the pcDNA3.1 vector (Invitrogen) to generate the pcDNA3.1-LINC01508 vector for overexpression in cells. Plasmid vectors (pcDNA3.1-LINC01508 and pcDNA3.1 vector) were transient or stable transfected into C13K cells by Lipofectamine 2000 according to the manufacturer's instructions.

7. Cytotoxicity analysis

Cell cytotoxicity was analyzed with a cell counting kit-8 (CCK-8) assay kit (Beyotime Biotechnology, Shanghai, China). Briefly, cells were seeded into flat-bottomed 96-well plates at a density of 8,000 per well and incubated overnight. Cisplatin with concentrations of 0, 1, 10, 30, 60, and 100 μM was added after adherence. Then cells were continuously cultured for 48 hours followed by treatment with 10 μL of CCK-8 solution for an additional 1 hour at 37°C. The absorbance (A) was measured at 450 nm using a microplate reader (PerkinElmer, Waltham, MA, USA). Cell viability (%) = experimental group A value/control group A value. A half maximal inhibitory concentration (IC₅₀) values (50% inhibition of surviving fraction) were then estimated using the fitted dose-response curves for cell viability.

8. Flow cytometry

OV2008 and C13K cells transfected with si-LINC01508 and pcDNA 3.1-LINC01508 were harvested for 48 hours. Apoptosis was induced by cisplatin (15 and 50 μ M) for 48 hours every 2 cells. According to the manufacturer's instruction, apoptosis detection was carried out with annexin V-fluorescein isothiocyanate and propidium iodide (PI) double staining assay kit (KeyGen Biotech, Nanjing, China). Apoptotic cells were uncovered using a flow cytometer (BD Biosciences, San Jose, CA, USA). For the cell cycle analysis, cells were single-stained with PI with the BD Cycle test plus DNA reagent kit (BD Biosciences). Data were analyzed using Cell Quest software (BD Biosciences).

9. Colony formation assays

Cells were seeded into 6-well plates at a concentration of 1×10^3 cells per well. After incubation for 14 days, the colony cells were fixed with methanol for 15 min and stained using 0.1% crystal violet for 30 minutes (Sigma-Aldrich Co.). Clonal formation rate (CFR) is calculated number of colonies containing >50 cells:

$$\text{CFR} = \frac{\text{No. of Colonies Formed}}{\text{No. of Cells Seeded}} \times 100\%.$$

10. Wound healing and Transwell assay

The cells were placed in a 6-well plate, and the medium was removed when the density of cells was about 60%. 200 μ L tips were used vertically and lightly scratch from the center several times. The cast-off cells were washed away, and the remainder was cultured subsequently. The cell number was counted 48 hours after the scratches were made.

The cells were starved for 24 hours, cells digested by pancreatic enzyme were resuspended in serum-free medium and added with the Matrigel-coated membrane in the upper chamber. The complete medium was then added as chemotaxis into the lower chamber. The upper chamber was taken out after 12 hours and washed with phosphate-buffered saline (PBS). The cells attached to the membrane were settled in 4% paraformaldehyde for 20 minutes. Afterward, the fixed cells were stained with 1% crystal violet for 10 minutes. The membrane was observed under a microscope.

11. Real-time reverse transcription polymerase chain reaction (PCR)

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription used the PrimeScript RT reagent kit (Takara, Dalian, China). SYBR premix Ex Tap™ II (Takara) was used for quantitative real-time PCR (qPCR). The LINC01508 expression was quantified using qPCR assay with the following specific primers: 5'-GTATGGGGTGTCTAATCAGGG-3' (forward) and 5'-GACTGTGGCTTTGCTAATGG-3' (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-CGGATTGGTTCGTATTGGG-3' (forward) and 5'-CTGGAAGATGGTGATGGGATT-3' (reverse). Primers were synthesized by Sangon Biotech (Shanghai, China). All procedures were performed according to the manufacturer's instructions. GAPDH was the internal reference for lncRNA and mRNA, and the relative expression level was normalized by the $2^{-\Delta\Delta Ct}$ method.

12. Western blot analysis

Cells were harvested with ice-cold PBS and lysed in lysis buffer containing a protease inhibitor cocktail. The proteins were quantified using bicinchoninic acid (BCA™) protein assay kit (Pierce, Appleton, WI, USA). The primary antibodies used were as follows: yes-

associated protein (YAP) and cysteine-rich angiogenic inducer 61 (Cyr61) (sc-15407 and sc-13100, respectively; Santa Cruz Biotechnology, Dallas, TX, USA), GAPDH and phospho-AKT (p-AKT) (sc-166574 and sc-135650, respectively; Santa Cruz Biotechnology), phospho-YAP (p-YAP) and excision repair cross complementing-group 1 (ERCC1) (4911 and 3885s, respectively; Cell Signaling Technology). The Western blot was performed according to the standard protocol.

13. In vivo chemo-sensitivity assay

Five-week-old female athymic BALB/c mice were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the animal center of LISC20200284. They were randomly divided into the following groups: i) pCDNA3.1+PBS; ii) pCDNA3.1-LINC01508+PBS; iii) pCDNA3.1+DDP; and iv) pCDNA3.1-LINC01508+DDP. When the tumors were palpable, DDP (3 mg/kg) was peritoneally injected into the mice every four days. Tumor volumes were examined every week when the implantations were starting to grow bigger. All mice were killed 7 weeks after injection, and the primary tumors were excised, paraffin-embedded, formalin-fixed.

14. Immunohistochemistry

Immunohistochemistry staining was performed on 4-mm-thick sections prepared using tissue blocks embedded in paraffin. The sections were fixed using 4% formaldehyde overnight and embedded in paraffin, followed by deparaffinization and hydration. The sections were pretreated with a sodium citrate buffer in a microwave for antigen retrieval and blocked using normal goat serum. The sections were incubated with primary Ki67 (1:500, Santa Cruz Biotechnology) and B-cell lymphoma-2 (Bcl-2) (1:250, Santa Cruz Biotechnology), early growth response 1 (EGR-1) (1:150, Abcam, Cambridge, UK) and YAP (1:100, Santa Cruz Biotechnology) antibody overnight at 4°C. They were then incubated in a biotinylated goat-anti-mouse (1:200, Santa Cruz Biotechnology) or biotinylated goat-anti-rabbit secondary antibody (1:200; Santa Cruz Biotechnology) for 1 hour, followed by staining with a streptavidin-biotin complex/horseradish peroxidase (sABC-HRP).

15. Immunofluorescence

The immunofluorescence was performed on the fixed cells grown on the round glass coverslips (Thermo Fisher Scientific) in 35 mm cell culture dishes. The cells were incubated with primary antibody against Cyr61 overnight at 4°C, followed by rhodamine-conjugated anti-rabbit secondary antibodies incubation for 1 hour, and diamidino-2-phenylindole (DAPI) (Beyotime Biotechnology, Shanghai, China) as a nuclear stain. The cells were then examined under confocal fluorescence imaging microscope (TCSSP5; Leica, Mannheim, Germany).

16. Statistical analysis

All of the data are expressed as the mean±standard deviation (SD), and SPSS v22.0 software was used for statistical analyses. Student's t-test and one-way analysis of variance (ANOVA) were used to compare means from different samples. The χ^2 test was applied to analyze the relationships between expression of LINC01508 and various clinicopathological factors, including age, FIGO stage, histologic grade, CA125, tumor size, lymph node metastasis, residual tumor, and platinum resistance, with statistical significance defined at $p < 0.05$. The prognostic value of lncRNA, YAP, and EGR-1 gene expression was evaluated using the online database Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn>) and Kaplan-Meier Plotter (www.kmplot.com), respectively.

17. Ethics approval and consent to participate

All volunteers have been enrolled by the First Affiliated Hospital of Anhui Medical University (PJ2017-0704) who signed an informed consent before being recruited, following approval by the Institutional Review Board of the University Hospital and according to the Declaration of Helsinki.

RESULTS

1. Microarray results analysis and prediction of the functions of lncRNAs

We performed microarray analysis to inspect the platinum-resistant lncRNAs in OC, comparing cisplatin-resistant C13K and its parental cell line OV2008. The outcome showed that there were 6,466 aberrantly expressed lncRNAs, among which LINC01508 was observed to be abnormally overexpressed in cisplatin-sensitive OC cells (fold change >2 and $p < 0.05$) (**Fig. S1A**). In unsupervised hierarchical clustering analysis, the differentially expressed lncRNAs were used to generate a heat map (**Fig. S1B** and **Table S1**). The lncRNAs were clustered into hundreds of gene ontology (GO). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotates. The top ten GO biological processes and KEGG pathways enriched for the upregulated or downregulated lncRNAs are presented in **Fig. S1C** and **S1D**. Hippo signaling as a key regulator of homeostasis and in tumorigenesis. The effectors of the Hippo pathway, such as YAP, and transcriptional co-activator with PDZ-binding motif (TAZ), are often activated in human malignant cancers. These transcriptional regulators may initiate tumorigenic changes in solid tumors by inducing cell proliferation and chemo-resistance. In our results, the “Hippo pathway” was enriched in the KEGG pathway for upregulated lncRNAs. This indicates the possibility that the upregulated lncRNAs participate in the chemo-resistance process.

2. Validation of the microarray data by qPCR

The four upregulated and 2 downregulated lncRNAs were examined by qPCR in 12 pairs of OC and epithelial normal ovary tissues to validate the microarray data. Our results found that the expression of LINC01508 showed more than 20 times the difference between OC and epithelial normal ovary tissues (**Fig. S2A**). LINC01508 expression in OC and epithelial normal ovary tissues are presented as a box plot graph identified based on The Cancer Genome Atlas (TCGA) datasets (**Fig. S2B**). Then, we examined the expression level of HOSEpiC and four OC cell lines. As shown in **Fig. S2C** and **S2D**, LINC01508 was highly expressed in normal epithelial cell lines than in carcinoma cell lines, which was averse to the result of the OC tissues. The expression of LINC01508 in cisplatin-resistant OC cells was remarkably lower than in cisplatin-sensitive OC cells. In addition, we assessed the expression level of LINC01508 in 12 cases of normal ovaries, 25 cases of ovarian serous cystadenomas, and 71 cases of OC tissues. The data demonstrated in **Fig. S2E** reveal that the level of LINC01508 in OC was significantly higher than that in other ovarian tissues.

The differences in expression levels among normal, benign tumor and OC tissues had a statistical significance. Therefore, we suspected that there might be a correlation between LINC01508 and the generation and development of OC. OC tissues were further subdivided into two platinum-resistant and platinum-sensitive groups to ascertain further whether LINC01508 is differentially expressed in platinum-resistant and platinum-sensitive tissues. Expression of LINC01508 in 19 platinum-resistant and 52 platinum-sensitive OC groups was detected via qPCR. As shown in **Fig. S2F**, relative LINC01508 levels were significantly lower in

LINC01508 confer to cisplatin resistance in OC

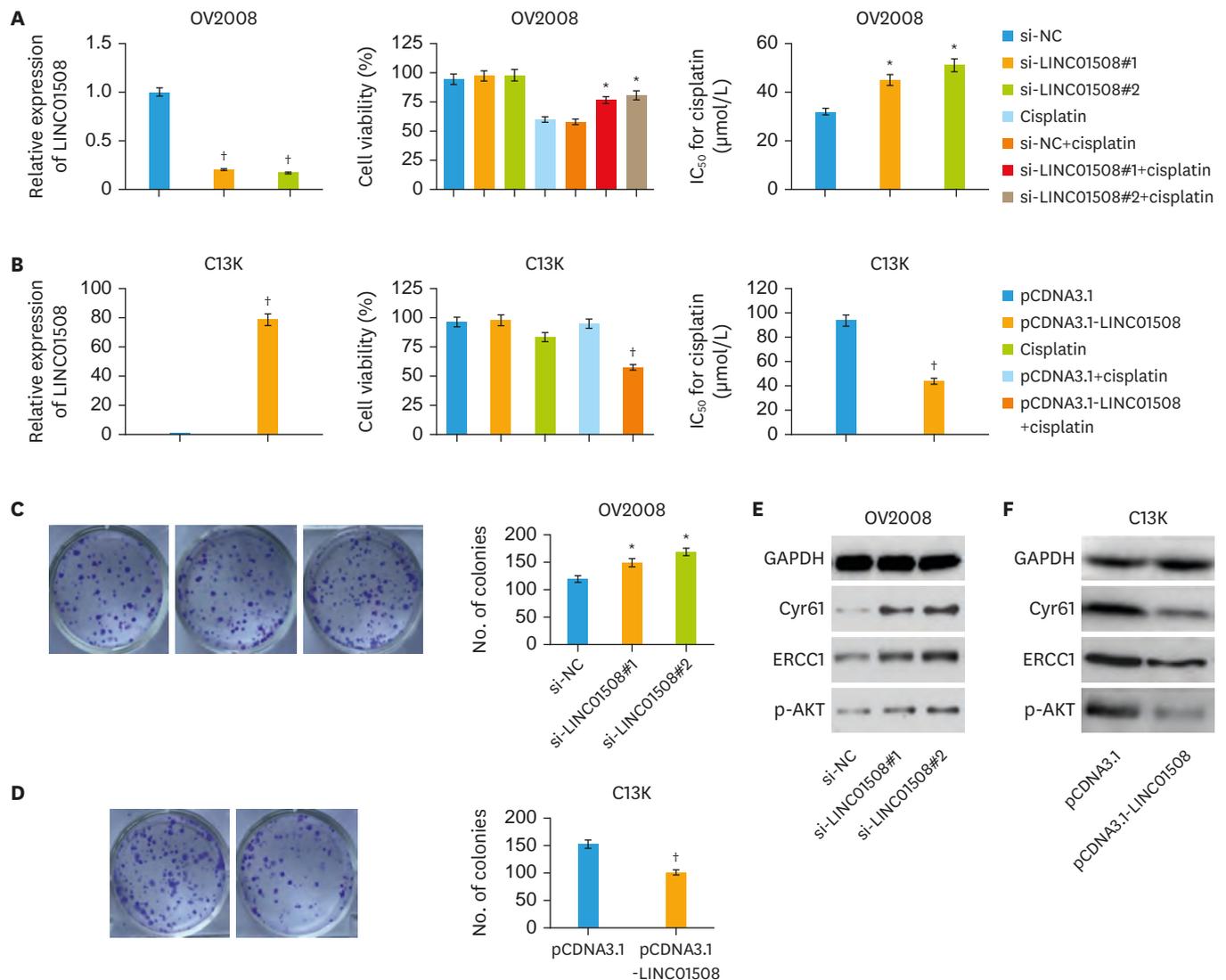


Fig. 1. Overexpression of LINC01508 was related to the rescue of cisplatin sensitivity. The cell function analysis are all chemically synthesized interference lncRNA and eukaryotic plasmid-mediated lncRNA transient transfection. These have limited gene silencing and overexpression efficiency and show that the correlation between LINC01508 and OC cisplatin resistance is not highly significant. Furthermore, the effect of eukaryotic plasmid-mediated lncRNA overexpression is better than the chemical synthesis of lncRNA. This suggests a stable transfection in vitro to provide knockout and overexpression effects. (A) qPCR detects a lower LINC01508 expression, and the CCK-8 assay measures the cell viability. IC₅₀ values in LINC01508 inhibition OV2008 cells. (B) qPCR inspects a higher LINC01508 expression, and the CCK-8 assay measures the cell viability. IC₅₀ values in LINC01508 overexpression C13K cells. (C and D) Colony formation assays were conducted to determine the cell proliferation ability for transfected OC cells. (E and F) GAPDH, Cyr61, ERCC1, and p-AKT protein levels were determined by Western blot. All experiments are repeated in triplicated, and data are shown as mean±SE. lncRNA, long non-coding RNAs; OC, ovarian cancer; qPCR, quantitative real-time polymerase chain reaction; CCK-8, cell counting kit-8; IC₅₀, half maximal inhibitory concentration; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Cyr61, cysteine-rich angiogenic inducer 61; ERCC1, excision repair cross complementing-group 1; p-AKT, phospho-AKT; SE, standard error. *p<0.05; †p<0.01.

the platinum-resistant than the platinum-sensitive group (0.43±0.10 vs. 1.09±0.27; p<0.05). This suggests that LINC01508 is involved in the regulation of platinum resistance of OC.

3. Overexpression of LINC01508 was related to the rescue of cisplatin sensitivity

We upregulated LINC01508 in C13K (48 hours) and downregulated LINC01508 in OV2008 (48 hours) to clarify the impact of the LINC01508 on cisplatin sensitivity in OC cells. This

LINC01508 confer to cisplatin resistance in OC

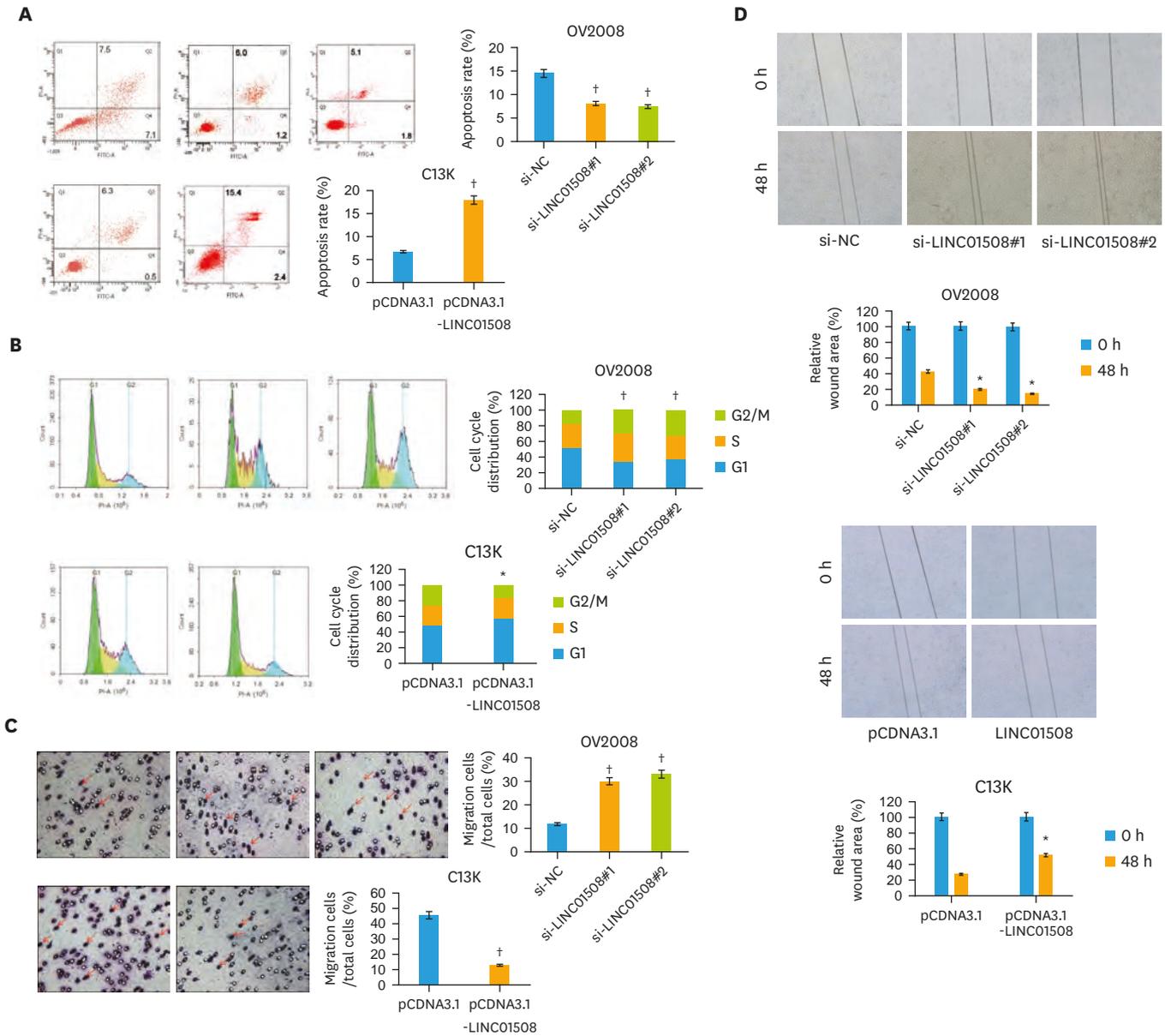


Fig. 2. LINC01508 regulated OC cell apoptosis, cell cycle, cell invasion, and migration. (A) Flow cytometry detected the apoptotic rate of LINC01508 inhibition OV2008 (48 hours) and LINC01508 overexpression C13K cells (48 hours). (B) Flow cytometry indicated the percentage of LINC01508 inhibition OV2008 (48 hours) and LINC01508 overexpression C13K cells (48 hours). (C) Wound healing shows migration cells of LINC01508#1 and LINC01508#2 inhibition OV2008 (48 hours), and LINC01508 overexpression C13K cells (48 hours). (D) Transwell migration assay showed the migration and invasion of LINC01508-overexpressed or downregulated OC cells (48 hours). All experiments are repeated in triplicated, and data are shown as mean±SE.

OC, ovarian cancer; SE, standard error.
*p<0.05; †p<0.01.

showed the inhibition rates of different concentrations of cisplatin. The inhibition rate and IC₅₀ for cisplatin of LINC01508 inhibition OV2008 cells (48 hours) were higher than those in the siRNA negative control group in the same concentration, which means the sensitivity cisplatin was decreased. On the contrary, the LINC01508 overexpression C13K cells (48 hours) showed decreased cisplatin inhibition rate and IC₅₀ for cisplatin, meaning that resistant OC became cisplatin sensitive (**Fig. 1A and B**). The clone assay also showed that LINC01508 could affect cisplatin sensitivity in OC cells (48 hours). It can be a target

of chemo-resistance reversion (**Fig. 1C and D**). We also demonstrated the related protein expression in LINC01508 overexpression C13K and LINC01508 inhibition OV2008 cells exposed to cisplatin. We observed that the increase in Cyr61, ERCC1, and p-AKT (protein kinase B) levels was accompanied by higher cell survival rates and cisplatin sensitivity of LINC01508 inhibition of OV2008 cells (**Fig. 1E**). On the contrary, the expression of Cyr61, ERCC1, and p-AKT was decreased in LINC01508 overexpression C13K cells (**Fig. 1F**). Collectively, these results suggested that LINC01508 played a crucial role in the regulation of OC cancer cells' sensitivity to cisplatin.

4. LINC01508 regulated OC cell apoptosis, cell cycle, cell invasion, and migration

We confirmed the influence of LINC01508 on cisplatin sensitivity of OC cells. Flow cytometry showed that upregulation of LINC01508 in C13K cells facilitated apoptosis compared with the pCDNA 3.1 group ($p < 0.05$) (**Fig. 2A**). In contrast, the results indicated a decrease in the apoptotic rate of OV2008 cells transfected with si-LINC01508 #1 or #2 relative to those transfected with si-NC ($p < 0.05$) (**Fig. 2A**). A significant arrested at the G2/M phase was observed on OV2008 cells transfected with si-LINC01508 #1 or #2, comparing with OV2008 cells transfected with si-NC ($p < 0.05$). The LINC01508 overexpression of C13K cells decreased cell cycle arrest at the G2/M phase (**Fig. 2B**). The Transwell and wound healing assays showed that overexpression of LINC01508 in C13K inhibited wound healing and prevented more cells from migrating from the upper chamber to the lower one. Inhibition of LINC01508 expression in OV2008 resulted in the exact opposite of overexpression. Results of invasion and migration experiments were consistent (**Fig. 2C and D**). These data demonstrate that LINC01508 impacted the sensitivity to cisplatin in OC cells through apoptosis and G2/M arrest of the cell cycle.

5. Overexpression of LINC01508 improves in vivo sensitivity to cisplatin

Nude mice were inoculated subcutaneously with pCDNA3.1-LINC01508 or pCDNA3.1 transfected C13K cells further to investigate the effect of LINC01508 increase on tumor growth. This was followed by cisplatin treatment, and the volume and weight of ovarian tumor xenografts were recorded 49 days post-inoculation. The weight of the ovarian tumor xenografts derived from pCDNA3.1-LINC01508 transfected C13K cells was significantly lower than those derived from pCDNA3.1 transfected C13K cells (**Fig. 3A and B**). OC tumor xenografts are isolated from nude mice inoculated with pCDNA3.1-LINC01508 transfected C13K cells was significantly lower than those derived from pCDNA3.1 transfected C13K cells, 49 days post-inoculation (**Fig. 3C**). qPCR assay detected upregulation of LINC01508 expression in the ovarian tumor tissues derived from pCDNA3.1-LINC01508 transfected C13K cells than in that from pCDNA3.1 transfected C13K cells (**Fig. 3D**). The immunohistochemical analysis detected lower antigen KI67 (Ki-67) and Bcl-2 expression in the ovarian tumor xenografts derived from pCDNA3.1-LINC01508 transfected C13K cells than in that from pCDNA3.1 transfected C13K cells (**Fig. 3E**). These data demonstrate that promoting LINC01508 expression inhibits OC progression by enhancing the in vivo sensitivity of C13K cells to cisplatin.

6. Relationship between LINC01508 expression and clinicopathological characteristics and its correlation with cisplatin sensitivity of patients with OC

We further assessed whether this aberrant expression pattern is linked to clinicopathological characteristics of patients because of the finding that LINC01508 is upregulated in OC tissue. Overall, 71 patients were divided into two groups according to the median expression of LINC01508 calculated from all carcinoma tissues (values lower than the median were defined

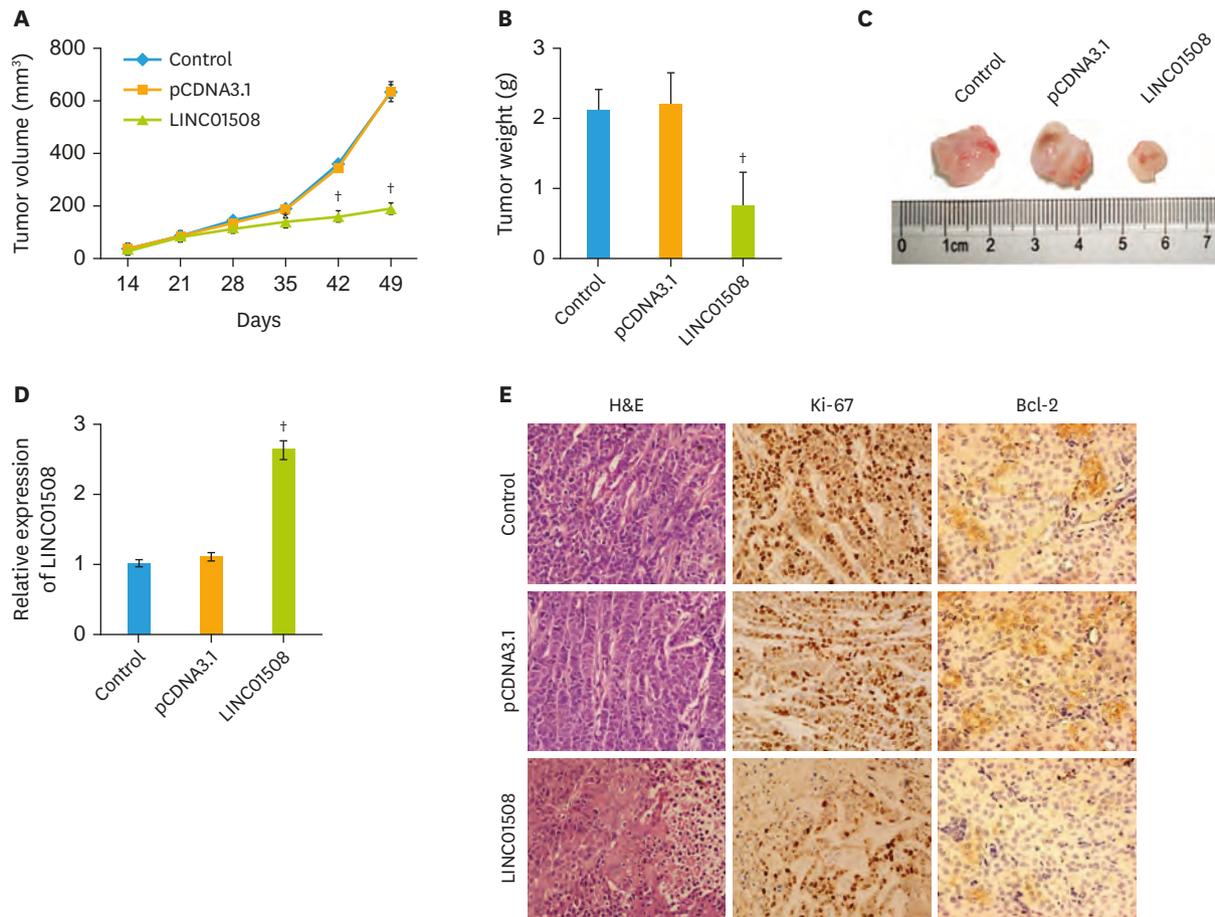


Fig. 3. LINC01508 overexpression improves the in vivo sensitivity of C13K to cisplatin.

(A) The volume of the ovarian tumor xenograft derived from pCDNA3.1-LINC01508 transfected C13K cells is significantly lower than those derived from pCDNA3.1 transfected C13K cells. (B) The weight of the ovarian tumor xenograft derived from pCDNA3.1-LINC01508 transfected C13K cells is significantly lower than those derived from pCDNA3.1 vector-transfected C13K cells. (C) Ovarian tumor xenografts are isolated from nude mice inoculated subcutaneously with pCDNA3.1-LINC01508 or pCDNA3.1 vector-transfected C13K cells 49 days post-inoculation. (D) qPCR assay detects upregulation of LINC01508 expression in the ovarian tumor xenograft derived from pCDNA3.1-LINC01508 transfected C13K cells as compared to that from si-NC transfected C13K cells. (E) Immunohistochemical analysis detects lower Ki-67 and Bcl-2 expression in the ovarian tumor xenograft derived from pCDNA3.1-LINC01508 transfected C13K cells than in that from pCDNA3.1vector transfected C13K cells (staining, $\times 200$). Data were shown as mean \pm SE of 3 experiments.

qPCR, quantitative real-time polymerase chain reaction; Ki-67, antigen K167; Bcl-2, B-cell lymphoma-2; SE, standard error.

* $p < 0.05$; † $p < 0.01$.

as 'low expression' and those greater than the median as 'high expression'). Associations between LINC01508 expression and clinicopathological characteristics were analyzed with the χ^2 test (**Table 1**). We observed no distinct correlation between LINC01508 expression and age of patients, FIGO stage, histologic grade, lymph node metastasis, or CA125 value ($p > 0.05$). Simultaneously, tumor size, residual tumor, or platinum resistance was correlated with LINC01508 expression.

GEPIA and Kaplan-Meier survival analysis revealed a longer OS in OC patients with high EGR-1, low LINC01508, and YAP expression, then those with low EGR-1, high LINC01508, and YAP expression (**Fig. 4A**). Then, immunohistochemical analysis showed lower EGR-1 expression, which agreed with previous findings in OC [13,14] and higher YAP expression in cisplatin-resistant OC specimens than in non-resistant specimens (**Fig. 4B**). These data indicate that LINC01508 expression correlates with the response to cisplatin-based chemotherapy and prognosis in OC patients.

Table 1. Expression of LINC01508 and distribution of clinicopathologic factors in OC patients

Features	No.	LINC01508		χ^2	p
		Low	High		
Mean age (yr)				0.122	0.727
>55	38	20	18		
≤55	33	16	17		
FIGO stage				0.132	0.717
I–II	13	6	7		
III–IV	58	30	28		
Lymphatic invasion				0.013	0.909
+	34	17	17		
–	37	18	19		
Tumor grade				0.204	0.652
G2	47	30	17		
G3	24	14	10		
Tumor size				5.680	0.017
<10	51	42	9		
≥10	20	11	9		
CA125 (U/mL)				0.001	0.973
≤1,000	44	23	21		
>1,000	27	14	13		
Residual tumor				5.223	0.022
No visible residue	25	13	12		
Visible residue	46	36	10		
Platinum resistance				4.206	0.040
Present	19	15	4		
Absent	52	27	25		

OC, ovarian cancer; FIGO, International Federation of Gynecology and Obstetrics.

7. Upregulation of LINC01508 suppressed cisplatin tolerance of OC cells through the inhibition of the Hippo pathway

Finally, we evaluated the molecular mechanism that enabled LINC01508 to influence the cisplatin sensitivity of OC cells. YAP, a major transducer downstream of the Hippo pathway, and its phosphorylation protein level were evaluated among OV2008, C13K, C13K-pcDNA3.1, C13K-pcDNA3.1-LINC01508 groups. As shown in **Figure 5A**, the YAP levels were higher in C13K cells than in OV2008 cells. p-YAP expression was lower in C13K cells than in OV2008 cells as compared to YAP expression. This indicates that the hippo-YAP pathway was activated in OC cells with cisplatin resistance. Different YAP and p-YAP levels in the C13K-pcDNA3.1 and the C13K-pcDNA3.1-LINC01508 groups showed that the upregulation of LINC01508 could significantly inhibit the hippo-YAP pathway. C13K cells were treated with dimethyl sulfoxide (DMSO) or VP to determine the effects of the hippo-YAP pathway inhibition on cisplatin sensitivity. VP significantly decreased cell survival and colony number due to cisplatin treatment compared with the C13K/DMSO group (**Fig. 5B and C**). Furthermore, YAP blocking by VP in C13K cells also significantly suppressed Cyr61 protein expression (**Fig. 5D**). Altogether, these results conclude that the enhancement of LINC01508 in OC cells contributed to cisplatin sensitivity via the inhibition of the hippo-YAP pathway.

DISCUSSION

OC accounts for more than 150,000 deaths worldwide every year [15]. Patients are often diagnosed at an advanced stage with metastatic dissemination. Although platinum- and taxane-based chemotherapies are effective treatment options, they are rarely curative. Eventually, the disease will progress due to acquired resistance. Emerging evidence suggests

LINC01508 confer to cisplatin resistance in OC

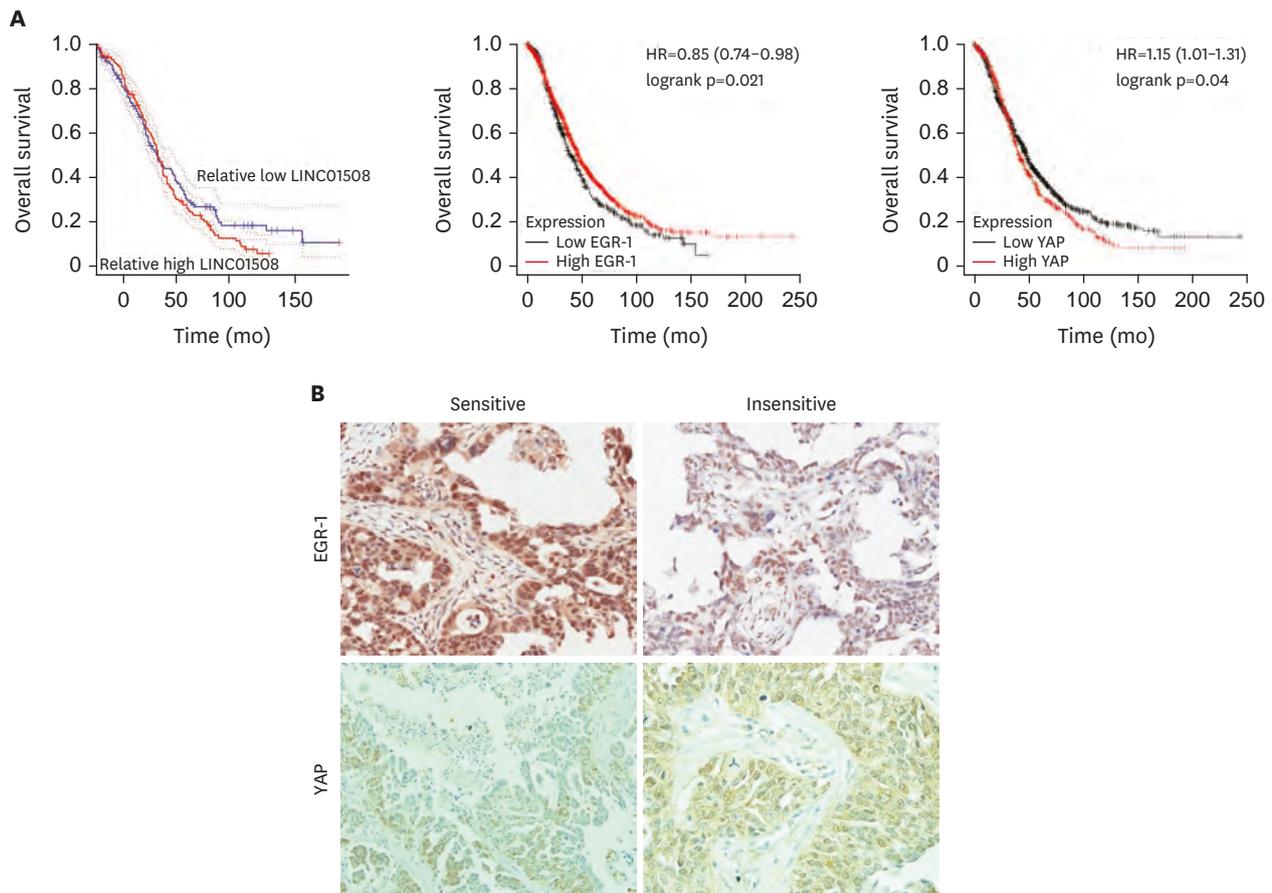


Fig. 4. Relationship between LINC01508 expression & clinicopathological characteristics and it correlates with cisplatin sensitivity of patients with OC. (A) Kaplan-Meier plots of overall survival in OC patients stratified according to their LINC01508, YAP, and EGR-1 status. (B) Immunohistochemical analysis shows lower EGR-1 expression and higher YAP expression in platinum-resistant OC specimens than in non-resistant specimens (staining, × 200). OC, ovarian cancer; YAP, yes-associated protein 1; EGR-1, early growth response protein 1.

a crucial role of lncRNAs in response to therapy in OC [16,17]. Although several lncRNAs have been associated with platinum resistance in OC, the resistance-associated molecular mechanisms have been elucidated in only a few of them.

To our knowledge, this is the first investigation of LINC01508 expression in OC. Our results demonstrated LINC01508 was upregulated in order of OC > benign ovary tumor > normal tissue. Contrary to LINC01508 expression in OC tissues, qPCR results showed that the expression of LINC01508 was downregulated in OC cells compared with normal ovarian epithelial cells. In addition, it was much lower in OC cells and tissues that were resistant to cisplatin. This suggests for the first time that LINC01508 negatively regulates cisplatin resistance in OC. Our functional prediction analysis described that LINC01508 was most closely associated with cisplatin resistance phenotype. The clinical value and significance of LINC01508 were further investigated in the present study. The χ^2 test results indicated that the low LINC01508 was positive associated with the tumor size, residual tumor, and platinum resistance in patients with OC (Table 1). They were supporting its utility as a potential biomarker of platinum resistance. Based on the present findings, we propose that patients with downregulation of LINC01508, postoperative tumor residuals, and smaller tumor size should be closely monitored. Ni et al. [18] have reported that a positive correlation between low LINC00515 and platinum resistance in high-grade serous ovarian cancer (HGSOC).

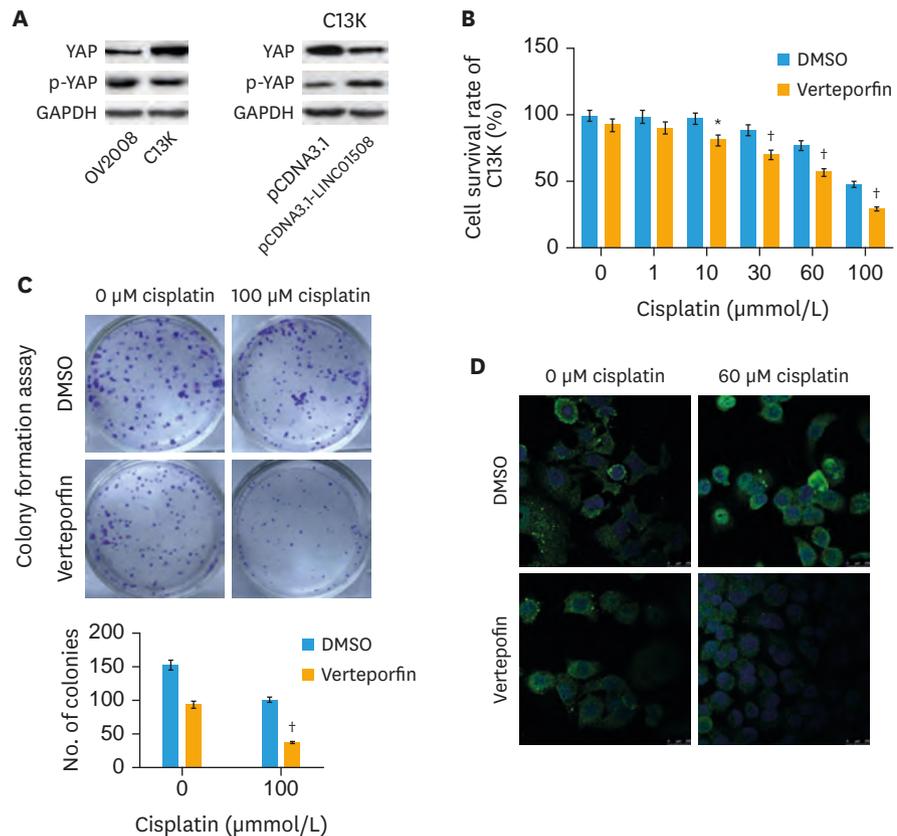


Fig. 5. Upregulation of LINC01508 suppressed cisplatin tolerance of OC cells through the inhibition of the hippo-YAP pathway.

(A) Western blot analyses of YAP and p-YAP protein expression in OC cells. (B and C) Cell survival rate and colony formation pictures of C13K cells after treatment of verteporfin and different doses of cisplatin. Data were shown as mean±SE of 3 experiments. (D) Cyr61 protein is marked by green fluorescence. Cyr61 protein was mainly localized in the cytoplasm. Nuclear staining with DAPI is shown in blue (magnification ×400). Representative images from 3 independent experiments are shown.

OC, ovarian cancer; YAP, yes-associated protein 1; p-YAP, phospho-YAP; SE, standard error; Cyr61, cysteine-rich angiogenic inducer 61; DAPI, diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

*p<0.05; †p<0.01, compared with DMSO group.

The expression of LINC01508 in OC cisplatin-resistant cells has been reduced. This was based on the survival analysis of the GEPIA online platform and our in vitro experiments, which seems to be a certain contradiction. However, this article supplements the information on the GEPIA platform, which is only a niche online platform. In this study, we analyzed LINC01508 as new lncRNAs. There is only one available literature report but none on the LINC01508 and tumor survival prognosis. The information on lncRNAs and tumor survival and prognosis are minimal compared to the Kaplan-Meier online platform. This study involving patients with OC and currently under follow-up has not yet reached the end. The mechanism of cisplatin resistance involves multiple aspects. This study shows very superficial aspects of cell experiment and mechanism of LINC01508 and cisplatin resistance in OC. Thus, the mechanism of its expression reduction and cisplatin resistance needs to be studied. Therefore, further follow-up researches will be conducted in the future, considering in-depth experiments.

As a novel lncRNA, the biological functions of LINC01508 were still unknown. Furthermore, we revealed the underlying molecular mechanism behind this phenomenon. In our study, the findings were supported by in vitro and in vivo studies showing that overexpression

LINC01508 conferred increased sensitivity to cisplatin, knockdown LINC01508, whereas led to cisplatin resistance. In addition, we also speculated that LINC01508 confers resistance to cisplatin-induced apoptosis and G2/M phase arrest in OC cells. Our data were consistent with the negative correlation. LINC01508 was more significantly downregulated in OC cells with cisplatin resistance than in parental OC cells.

Multiple signaling pathways have also been shown to be involved in chemotherapy resistance [19]. In recent years, lncRNAs are located in the cytosol reported being aberrantly expressed in tumors and show crosstalk with key cancer-related signaling pathways [20,21]. As cytoplasmic lncRNAs, the interplay between the LINC01508, cancer-related signaling pathways, and resistance mechanisms has not been evaluated yet. The Hippo pathway has attracted researchers' attention to critical cancer-related pathways in recent years [22,23]. YAP is a key component of the Hippo pathway. It plays a critical role in developing and progressing multiple cancer types [24,25], including OC [26,27]. Recently, more and more reports indicated that the expression of YAP should also be connected with multi-drug resistance [28,29]. Knockdown of YAP could sensitize OC cells to various cancer therapeutic agents *in vitro*. VP, a newly identified YAP inhibitor, has shown encouraging results in treating human cancers [30,31]. This study found that the hippo-YAP signaling pathway was activated in OC cells with cisplatin resistance. However, by enhancing the LINC01508 expression level, the hippo-YAP pathway could be effectively blocked. VP could effectively inhibit Cyr61 protein, a downstream of YAP [32,33], and improve OC cells' sensitivity to cisplatin.

In particular, several lncRNAs in cancer-associated fibroblasts (CAFs) are associated with patient survival. They are likely to play an important role in regulating CAFs functions [34,35]. In OC, the stromal proportion present in tumors can vary from 7% to 83% of tumor tissue [36]. However, some studies have concluded that the transcriptional factor YAP is activated in CAFs [37,38]. Further studies are required to validate the functional roles of LINC01508 in ovarian CAFs to determine those associated with patient outcomes.

There are several limitations in this study which are as follows: i) Further examination with a larger sample, or multicenter clinical study is recommended to demonstrate the significance of LINC01508 levels in OC; ii) Studies in the potential biological effects of LINC01508 and explore the mechanisms underlying the role of LINC01508 in platinum resistance in OC seem justified; iii) The expression control, including down or upregulation in SKOV3 cells, was not explored in this study, which would be conducted in the follow-up research in the future.

In summary, we propose that dysregulation of LINC01508 expression results in resistance of OC to cisplatin via the hippo-YAP pathway. This suggests that LINC01508 overexpression may target YAP to enhance cisplatin sensitivity by regulating cell migration, invasion, apoptosis, and cell cycle distribution in OC cells. Our findings indicate that LINC01508 could be a novel marker of poor response to platinum in OC patients and a potential therapeutic target for OC.

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SUPPLEMENTARY MATERIALS

Table S1

The top 20 dysregulated lncRNAs (OV2008 vs. C13K cells)

[Click here to view](#)

Fig. S1

Microarray results analysis and prediction of the functions of lncRNAs.

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Fig. S2

Validation of the microarray data by qPCR.

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