European Review for Medical and Pharmacological Sciences 2018; 22: 4440-4447

LncRNA EBIC promoted proliferation, metastasis and cisplatin resistance of ovarian cancer cells and predicted poor survival in ovarian cancer patients

Q.-F. XU[1,2,3], Y.-X. TANG[4], X. WANG[4]

[1]Department of Pharmacy, West China Second Hospital, Sichuan University, Chengdu, China
[2]Evidence-Based Pharmacy Center, West China Second Hospital, Sichuan University, Chengdu, China
[3]Key Laboratory of Birth Defects and Related Diseases of Women and Children, Sichuan University, Ministry of Education, Chengdu, China
[4]Physical Examination Centre, The Third Affiliated Hospital of Chongqing Medical University, Chongqing, China

Abstract. – OBJECTIVE: Ovarian cancer has the highest mortality rate cancer worldwide in women, and it is the second most common gynecologic malignancy in females, but the treatment remained unsatisfactory. Researches showed that lncRNA EBIC had played key roles in different cancer, but its role in ovarian cancer remains largely unclear.

PATIENTS AND METHODS: qRT-PCR was applied to detect the expression of lncRNA EBIC in ovarian cancer and adjacent tissue, and analysis was applied to explore the relationship between expression and clinical characteristic. Overall, the survival curves for the two groups were defined by the high and low expression level of EBIC in ovarian cancer patients. After that, CCK8 and transwell were used to detect the proliferation and metastasis ability of ovarian cancer, after suppression of lncRNA EBIC. The relative protein expression level in ovarian cancer cells after transfection with siRNA-NC or siRNA-EIBC was detected by Western blot.

RESULTS: qRT-PCR showed that lncRNA EIBC was highly expressed in ovarian cancer tissue, compared with adjacent tissue. Moreover, we found that expression of lncRNA EIBC was closely related to prognosis, tumor size and lymph node metastasis. We also found that the cell proliferation, invasion, migration and cisplatin resistance in ovarian cancer cells after transfection with siRNA-EBIC were significantly inhibited. Mechanistically, the relative protein expression level of β-catenin, vimentin and c-myc were significantly decreased and the relative expression of E-cadherin was significantly increased in ovarian cancer cells after transfection with siRNA-EBIC.

CONCLUSIONS: We found that overexpression of lncRNA EBIC could promote the proliferation, invasion and migration and improved cells cisplatin resistance by Wnt/β-catenin signaling pathway in ovarian cancer. LncRNA EBIC may be a potential target for the treatment of ovarian cancer patients.

Key Words:
LncRNA EBIC, Ovarian cancer, Cisplatin resistance.

Introduction

Ovarian cancer (OC) is one of the most lethal cancers for women worldwide. A high number of patients are diagnosed every year, and the risk of death is very high[1,2]. Although different kinds of therapeutic methods for ovarian cancer were renewed in past decades, the over survival of ovarian cancer patients was not improved, compared with patients in the early disease stage. A large number of oncogenes and tumor suppressor genes have been reported to be responsible for the development of ovarian cancer, but the molecular mechanisms underlying the migration and invasion of advanced ovarian cancer remains unclear. Therefore, we aimed at evaluating the mechanism of development for ovarian cancer to set down the therapeutic strategy.

Long non-coding RNAs (LncRNAs) are a class of RNAs with more than 200 nucleotides and have the ability to code proteins in animals and plants[3]. They are highly conserved among species and play important roles in various physiological and pathological processes including cancers[4-6]. Accumulating evidence showed that abnormal expression of lncRNAs was also found in different kinds of cancer involved in malignant activi-

Corresponding Author: Xiang Wang, MD; e-mail: wx300@163.com

ties. Li et al[7] reported that lncRNA HULC could bind to YB-1 protein, which could promote the phosphorylation of YB-1, leading to the release of YB-1 from target mRNA and activating oncogenic mRNAs. We investigated whether lncRNA could regulate cancer processes at both genetic and epigenetic level, suggesting that uncovering the mechanism of lncRNA in cancer may provide new target for cancer treatment, but the role of lncRNA in OC remained largely unknown.

In this study, we first detect the expression of lncRNA EBIC in OC tissue and cell lines by qRT-PCR. Then, the correlation analyzed was used to show the relationship between the lncRNA EBIC and clinical significance. We also measured the expression of lncRNA EBIC in the proliferation, metastasis, invasion and cisplatin resistance in OC cell lines. Finally, we showed that lncRNA EBIC may promote the malignant activity of OC by Wnt signing pathway.

Patients and Methods

Patients' Specimens and Clinical Assessments

The data were collected from 126 patients with ovarian cancer admitted in the hospital from June 2015 to December 2016. All the specimens were divided into the same size after operation and then treated with liquid nitrogen. The clinical data included age, sex, tumor size, lymph node metastasis, stage and pathological grade. All patients were informed and signed the informed consent. This research was approved by the Medical Ethics Committee.

Cell Culture and Treatment

Human ovarian cancer cell lines (OVCA429 and SKOV-3) and normal human ovarian epithelial cell line (HOSE) used in this study were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (HyClone, South-Logan, UT, USA) in a humidified cell incubator with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The medium was changed every 24 hours and cells at passages 3 to 6 were used in the following experiments. Each experiment was repeated three times. We stored the specimens at -80°C for next use.

RNA Extraction and Real-Time Quantitative PCR Assays

According to the manufacturer's protocol, the total RNA from tissue and cell was extracted by using RNAiso Plus (TaKaRa, Otsu, Shiga, Japan). The expression of EBIC in tumor tissue and ovarian cancer cell lines was detected by standard fluorescent quantitative PCR assay with SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). The PrimeScript™ RT reagent Kit was used to detected the concentration of RNA and the synthesized cDNA with gDNA Eraser was used (TaKaRa, Otsu, Shiga, Japan). The probes and corresponding primers in this study were designed and synthesized by GenePharma (Shanghai, China). The sequences of probes and primers were as follow: forward primers: 5'GAC TGA ATG GAC AAG TGG ATC TTC3'; reverse primers: 5'GGA GTT CTT CTT GAC CCT CTT GTA G3'; the probes: 5' AAA GGC GGA CCT CTG CAG GCA TTA T 3'.

CCK8 Assay

Cell proliferation was evaluated by CCK8 assay according to the manufacturer's instructions. The cells were plated in 96-well plates at a density of 2000 cells per well with 200 μL cell suspension. Data were collected for 5 days and 3 replicates wells were set in each group. After 24 h, 10 μL of Cell Counting Kit 8 (Dojundo, Tokyo, Japan) were added into 100 μL of Dulbecco's Modified Eagle Medium (DMEM) in each well. The plate was kept for 2 hours at 37°C and the absorbance value was measured at 450 nm. The whole experiment was repeated 3 times.

Detection of Cell Drug Resistance

The cells were cultured under standard condition for 48 h with 25 μL of previously prepared Thiazolyl Blue Tetrazolium Blue (MTT) (Sigma-Aldrich, St. Louis, MO, USA) in the absence of light. Cells were incubated for 4 h, after which the culture medium was discarded. After that, we added 150 μL of dimethyl sulfoxide (DMSO) to each well and the plate was gently stirred for 15 min at room temperature. Optical density (OD) was measured with an absorbance at 490 nm using a microplate reader. The formula for calculating cell viability was: cell survival rate = (OD value of drug-treated group − OD value of empty control group)/(OD value of normal cell control group − OD value of empty control group) ×100%.

Cell Invasion and Migration Assays

In invasion and migration assays, 1.0×105 cells/ml of SKOV3 and OVCA429 cells were prepared

after transfection with lncRNA EBIC and lncRNA-NC, respectively. The cell migration and invasion capacity were determined using transwell assay (Corning, Corning, NY, USA). Transfected cells were resuspended in serum-free medium. 200 μl cell suspensions were seeded into the upper chamber with a porous membrane coated with (for the transwell invasion assay) or without (for the migration assay) Matrigel (BD Biosciences, San Diego, CA, USA). After migration for 24 h or invasion for 48 h, the number of migratory and invasive cells was counted in five randomly selected high-power fields under a microscope. The presented data represent three individual wells.

Western Blot Assays

Whole cell lysates were prepared via lysis buffer (1% Triton-X100, 150 mMNaCl, 50 mM-Tris-HCl, 1 mM each CaCl2, MnCl2 and MgCl2, 10 mM sodium fluoride and 1 mM PMSF). Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). 100 μg of samples were added to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% denaturing gel. The protein was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after electrophoresis, which was blocked in the 5% non-fat milk for 45 min at room temperature. Next, phosphate-buffered saline (PBS) was used to wash the membranes. We used respective secondary antibody to incubate the membrane. The immunoblots were tested by enhanced chemiluminescence (ECL) detection system. Finally, we used GraphPad Prism software to analyze the protein bands (GraphPad, La Jolla, CA, USA).

Statistical Analysis

All experiments were independently repeated at least three times and presented as an average with SD. The t-test was used to analyze the differences between groups. Overall survival of patients was analyzed by Kaplan-Meyer method and log rank test. Independent prognostic significance of risk factors identified by multivariate analysis was computed by the Cox proportional hazards model. Receiver operating characteristic (ROC) curve analysis was used to determine the predictive value among parameters. If p-value<0.05, the result was considered as significant. We used the GraphPad Prism 6 (La Jolla, CA, USA) to deal with all data.

Results

EBIC was Highly Expressed in The Ovarian Cancer Tissue

In order to evaluate the effect of EBIC in ovarian cancer, we first detected the expression of EBIC in 126 cases of ovarian cancer tissues and adjacent tissues using qRT-PCR. We found that EBIC was highly expressed in ovarian cancer tissues compared with adjacent tissues (Figure 1A). Furthermore, we analyzed the expression of EBIC and the clinic pathological information of the patients, and we found that EBIC was positively correlated with tumor size. These data suggested that the expression of EBIC might be related to the development of ovarian cancer. Meanwhile, compared with the non-metastasis, we also found that the expression of EBIC was even higher in metastasis ovarian cancer (Figure 1B and Figure 1C). These results indicated that EBIC was involved in the occurrence and progression of ovarian cancer, but the mechanism was still unclear.

The Clinical Characteristic of EBIC

To evaluate the clinical significance of EBIC in ovarian cancer, we aimed at investigating which clinical characteristics were related with EBIC. The relationship between expression of EBIC and the survival time of ovarian cancer patients was analyzed. It was found that ovarian cancer patients with low EBIC expression showed a better prognosis compared with those with high level of EBIC (Figure 1D). Thus, it indicated that the expression of EBIC was negatively correlated with the survival time of patients with ovarian cancer.

Knockdown of lncRNA EBIC Suppresses OC Cell Proliferation, Migration and Invasion

Previously, we found that lncRNA EBIC was closely related with tumor size and lymph node metastasis of OC patients, suggesting that lncRNA EBIC may be closely related to the proliferation, metastasis and invasion of OC cells. We used siRNA to suppress the expression of lncRNA EBIC in OC cell line OVCA429 and SKOV-3. CCK8 assay and transwell assay were applied to detect the effect of lncRNA EBIC in proliferation, metastasis and invasion of OC cell lines. We found that suppression of lncRNA EBIC in OC cell line OVCA429 and SKOV-3 could significantly inhibit the proliferation, metastasis and invasion ability (Figure 2A-2E). Above that, the-

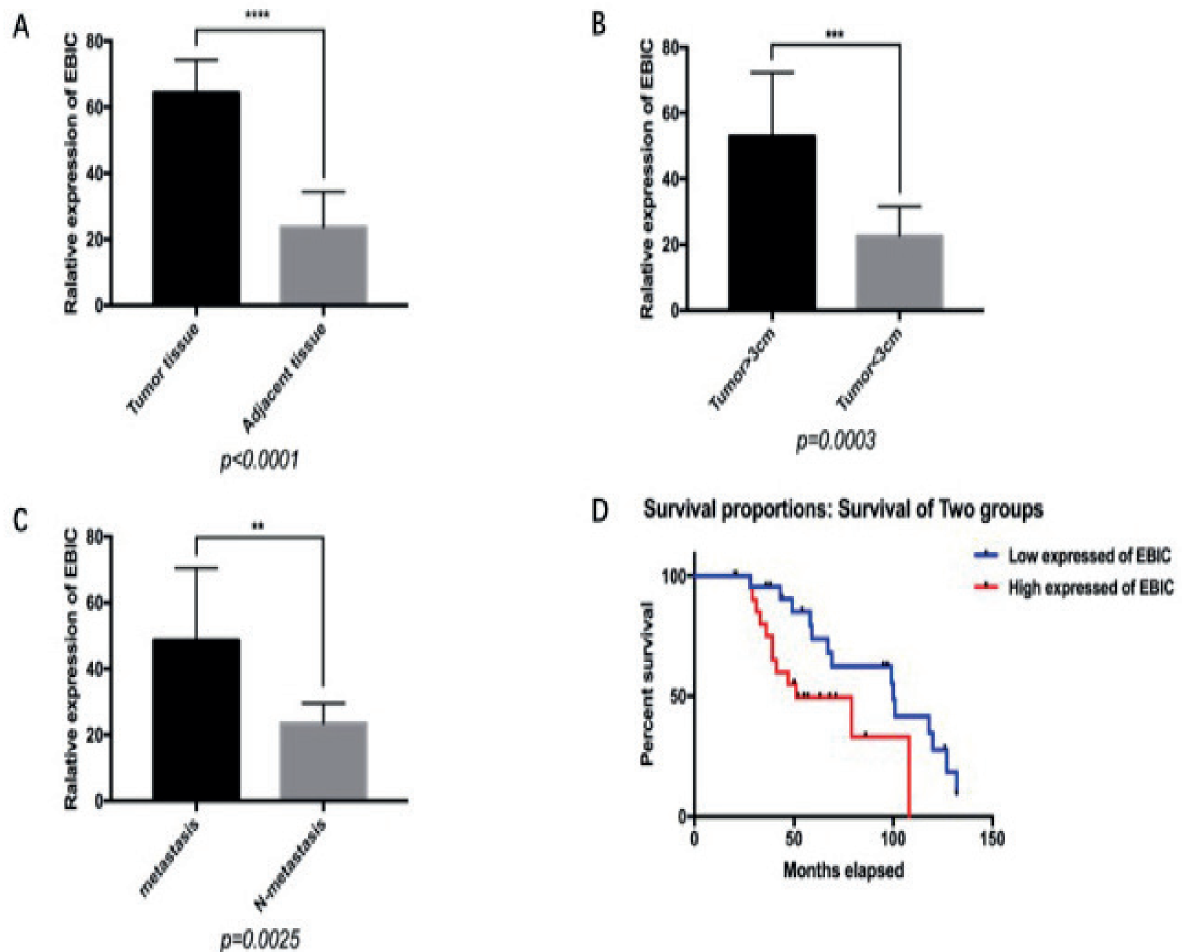

Figure 1. EBIC was highly expressed in the ovarian cancer tissue, cell lines and the clinical characteristic of EBIC. ***(A)*** The expression of EBIC in the ovarian cancer tissue and adjacent tissue was detected by qRT-PCR assay; $^{****}p<0.0001$. ***(B-C)*** The expression of lncRNA EBIC was closely related to the tumor size and lymph node metastasis. (D) Survival analysis of patients with different expression of lncRNA EBIC, $p<0.001$.

se findings demonstrated that silence of lncRNA EBIC could inhibit cell proliferation, migration and invasion *in vitro*.

Elevated Expression of EBIC Would Lead to the Cisplatin Resistance

Drug resistance is one of the factors resulting in poor prognosis. We detected the expression of EBIC in A2780 cell strain and CP70 cell lines based on the mentioned results. A2789 is sensitive for cisplatin and CP70 is the cisplatin-resistance line. We observed that EBIC was highly expressed in CP70 cells, compared with the A2780 cells. We next overexpressed EBIC in A2780 cells and knocked down the EBIC in CP70 cells. In the cell viability assay, we found the overexpression of EBIC in A2780 cells increased the cisplatin resistance. However, the decreased expression of EBIC in CP70 cells increased the sensitivity of cisplatin (Figure 3A-3D). The results suggested that the elevated expression of EBIC has a close relationship with cisplatin resistance.

EBIC Promotes Proliferation, Metastasis, Invasion and Drug Resistance of Ability of Ovarian Cancer Cell Line via Wnt/β-Catenin Signaling Pathway

Epithelial to mesenchymal transition (EMT) has been considered a key event for the epithelial tumor cells to lose cell-cell adhesion, acquire enhanced capacity for migration and invasion, thereby dissociating from the primary tumor and disseminate as a single cell. Wnt/β-catenin signaling pathway is closely related to the EMT. To investigate the mechanism of up-regulation

EBIC impact the activities in ovarian cancer, we detected the related protein expression level of Wnt/β-catenin in SKOV-3 and OVCA429 after transfection with siRNA-NC or siRNA-EBIC by Western blot. Mechanistically, the relative protein expression level of β-catenin, vimentin and c-myc was significantly decreased and the relative expression of E-cadherin was significantly increased in ovarian cancer cells after transfection with siRNA-EBIC (Figure 4A-4C). It suggested that down-regulation of EBIC inhibited the proliferation, invasion, migration and cisplatin resistance in ovarian cancer cells through Wnt/β-catenin signaling pathway.

Discussion

Ovarian cancer is a leading malignant tumor for women globally because of its highest mortality rate. Its strong invasion and metastasis are important reasons for its poor survival rate; also, the mechanism of bioactivity remains unclear. Accumulating reports[8-12] showed that lncRNAs may play a critical role in cellular biology and human diseases, especially for tumor processes, such as proliferation, migration and invasion. Hou et al[13] showed that linc-ROR significantly enhanced the invasion and metastasis of breast cancer cells by acting as a molecular sponge for miR-205. Yang et al[14] found that lncRNA UCA1 could function as an endogenous sponge by directly binding to miR-485-5p, resulting in upregulating the expression of matrix metallopeptidase[14] (MMP14), promoted metastasis of ovarian cancer. Lou et al[15] suggested that Linc-ROR induces epithelial-to-mesenchymal transition in ovarian cancer by increasing Wnt/β-catenin signaling. Moreover, other researches showed that the expression of lncRNA was associated with the recurrence of

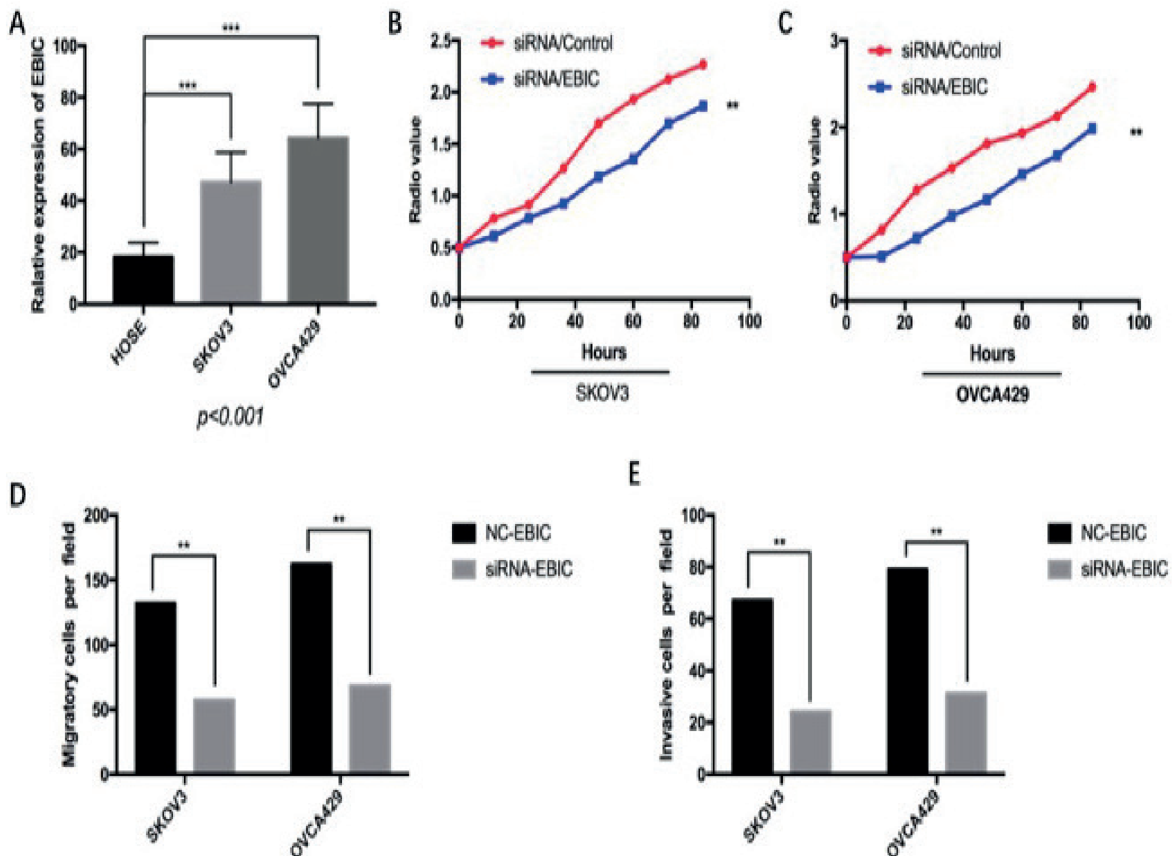

Figure 2. Knockdown of lncRNA EBIC inhibits OC cell proliferation, migration and invasion. ***(A)*** qRT-PCR was used to measure the expression of lncRNA CCAT1 in OC cancer cell lines (HOSE, SKOV-3,OVCA429); ***p<0.001. ***(B-C)*** CCK8 assays were used to detect the proliferation ability of OC cell after EBIC was suppressed; $^{**}p<0.01$. ***(D)*** Transwell was used to detect the migration ability after suppression of EBIC; $^{**}p<0.01$. (E) Transwell was used to detect the invasion ability after suppression of EBIC, $^{**}p<0.01$.

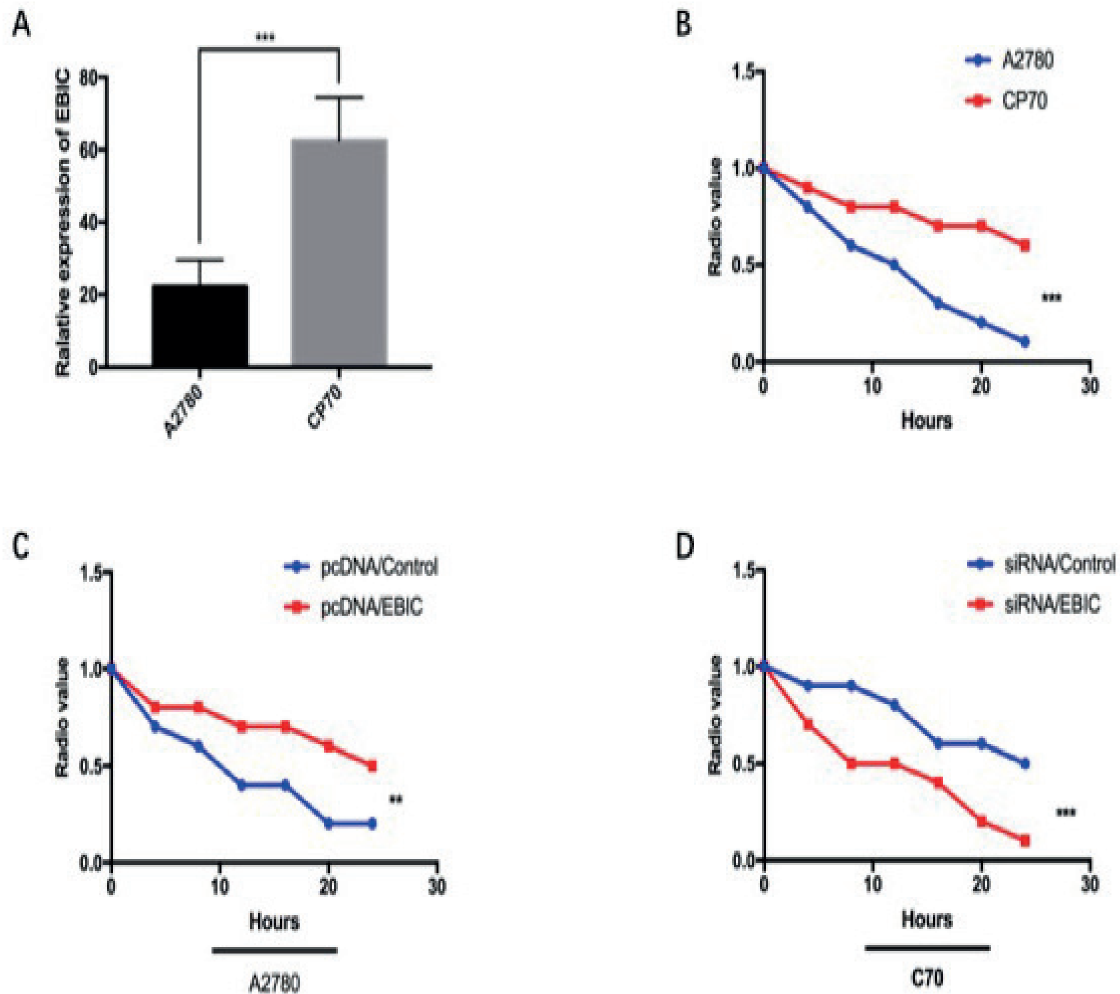

Figure 3. Improved EBIC would promote drug resistance in ovarian cancer cell lines. ***(A)*** Relative EBIC expression in A2780 and CP70 cells was detected by PCR assay; $^{***}p<0.001$. (B-D) Growth rate of each group detected by MTT. Data from each group were detected in three separate experiments; $^{***}p<0.001$.

OC; we understood the little related mechanism, demonstrating that lncRNA was important in affecting the procession of OC, but more fields need of further research.

In this study, we found that lncRNA EBIC was highly expressed in the OC tissue, compared with that in adjacent tissue, by qRT-PCR. According to the analysis of lncRNA EBIC expression and clinical information from ovarian cancer patients, we found that lncRNA EBIC was positively correlated with the tumor size and lymph node metastasis. Our work further showed that ovarian cancer patients with overexpression of EBIC had worse prognosis compared with those with low expression of EBIC, indicating that expression level of EBIC was a potential and independent prognostic factor of ovarian cancer patients.

What's more, we would like to know whether the EBIC influenced the proliferation, migration and invasion of ovarian cells. We investigated whether EBIC was more highly expressed in SKOV-3 and OVCA429, compared with the HOSE cell lines. Next, we inhibited the expression of EBIC in SKOV-3 and OVCA429 and led to the decrease of proliferation of ovarian cancer cells. The transwell was used to examine the activities of tumor and the migratory and invasion capacity of SKOV-3 and OVCA429 cells transfected with siRNA-EBIC were found to be significantly down-regulated than those transfected with NC-RNA. The results confirmed our expectation before that EBIC was closely related to metastasis and invasion in OC cell lines. Drug resistance in tumor is a difficulty caused by the different sensi-

tivity of tumor cells to chemotherapy, so that we would verify that drug resistance in tumor is a difficulty caused by the different sensitivity of tumor cells to chemotherapy. The study showed that EBIC was highly expressed in CP70 cells compared with A2780 cells line. EBIC overexpression in A2780 promoted cisplatin resistance, and cisplatin resistance was decreased in CP70 cells with EBIC being knocked down. The data showed that EBIC could influence the drug resistance in ovarian cancer.

Tumor cells with EMT signatures, as evidenced by reduction of the cell adhesion molecule and overexpression of the master EMT inducers, display an increased capability of metastasis. Meanwhile, increasing evidence[16] has reported that the Wnt/β-catenin signaling pathway plays important roles in the progression of ovarian cancer. β-catenin is a key protein in the canonical Wnt/β-catenin pathway and forms adherent junctions with E-cadherin; c-myc is a Wnt/β-catenin pathway target gene. Therefore, we subsequently detected increased E-cadherin and decreased vimentin, β-catenin, and c-myc expression after transfection with the siRNA-EBIC, compared with the siRNA-NC group. E-cadherin is a tumor suppressor gene that plays a critical role in the malignant progression of epithelial tumors and inhibits epithelial to mesenchymal transition[17]. Wnt/β-catenin signaling pathway activation is associated with higher invasive and migratory capacities in human ovarian cancer cells. These results showed that the overexpression of EBIC improved the proliferation, invasion and migration, and promoted cisplatin resistance in ovarian cancer cells through the Wnt/β-catenin signaling pathway.

Therefore, lncRNA EBIC expression was increased in ovarian cancer. The overexpression of EBIC improved the proliferation, invasion and metastasis and promoted cisplatin resistance in ovarian cancer cells through the inactivation of PI3K/Akt signaling pathway. This research

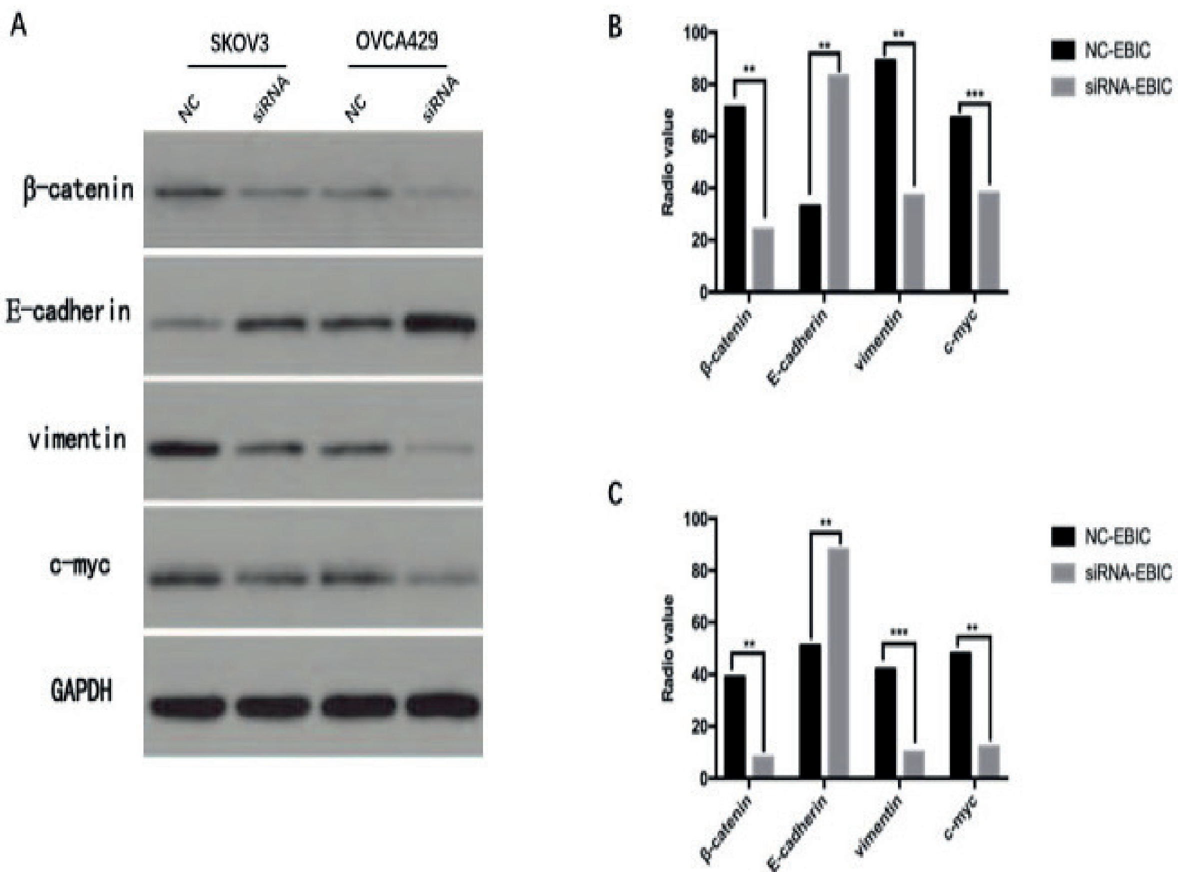

Figure 4. EBIC promotes the proliferation, migration, invasion and cisplatin resistance ability of OC cell line via Wnt/β-catenin signaling pathway. ***(A-C)*** Western blot was used to detect the effects of EBIC knockdown on Wnt/β-catenin signaling pathway; $^{***}p<0.001$.

showed that EBIC may be a biomarker for the prognosis of ovarian cancer patients.

Conclusions

We found that overexpression of lncRNA EBIC could promote the proliferation, invasion and migration and improve cells cisplatin resistance by Wnt/β-catenin signaling pathway in ovarian cancer. LncRNA EBIC may be a potential target for the treatment of ovarian cancer patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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