European Review for Medical and Pharmacological Sciences 2020; 24: 5267-5280

ERBB2 gene expression silencing involved in ovarian cancer cell migration and invasion through mediating MAPK1/MAPK3 signaling pathway

T.-T. YU, C.-Y. WANG, R. TONG

Department of Gynaecology, Liaoning Cancer Hospital & Institute, Cancer Hospital of China Medical University, Shenyang, Liaoning Province, P.R. China

Abstract. – OBJECTIVE: To explore possible mechanism of ERBB2 gene expression silencing mediating mitogen-activated protein kinase 1/mitogen-activated protein kinase 3 (MAPK1/MAPK3) signaling pathway on proliferation, migration, and invasion of ovarian cancer cells.

PATIENTS AND METHODS: A total of 240 cancer specimens were collected in patients with epithelial ovarian cancer intraoperatively in our hospital from January 2015 to January 2018. Expressions of ERBB2, MAPK1, and MAPK3 in tissues were detected by immunohistochemistry. Following the culture of ovarian cancer cell lines, target cell line with high expression of ERBB2 was screened by qRT-PCR. Cell grouping was performed with four groups after transfection, including Blank group, negative control (NC) group, ERBB2 shRNA group, and ERBB2 overexpression group (shorted as ERBB2 group). The expression levels of ERBB2, MAPK1, MAPK3, vascular endothelial growth factor (VEGF), metalloproteases-2 (MMP-2), and tissue inhibitor of metalloproteases-2 (TIMP-2) were detected by qRT-PCR in different transfection groups, followed by the detection of protein expressions with Western blot. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to test the proliferation activity of each group after transfection, while transwell assay and scratch test explored cell invasion and migration in each group, respectively.

RESULTS: Immunohistochemistry showed that the positive rates of ERBB2, MAPK1, and MAPK3 in ovarian cancer tissues were significantly increased than those in adjacent normal epithelial tissues. In the cell experiment, ERBB2 gene was highly expressed in SKOV3 ovarian cancer cell line. There was no significant difference in each index between Blank group and NC group ($p > 0.05$). Compared with Blank group and NC group, the expression levels of ERBB2, MAPK1, MAPK3, VEGF, and MMP-2 in ERBB2 shRNA group decreased significantly, TIMP-2 increased markedly, and proliferation, invasion, and migration abilities of cells decreased markedly after transfection, showing statistically significant differences (All $p < 0.05$). By contrast, the expression levels of ERBB2, MAPK1, MAPK3, VEGF, and MMP-2 increased remarkably in ERBB2 group, while TIMP-2 decreased significantly, and cell proliferation, invasion, and migration ability increased evidently after transfection, with statistically significant differences (All $p < 0.05$).

CONCLUSIONS: Silencing ERBB2 gene expression may inhibit the activation of MAPK1/MAPK3 signaling pathway and thus suppress the proliferation, invasion, and migration of ovarian cancer cells. Overexpression of ERBB2 gene can reverse those trends, which in turn support the role of ERBB2 gene expression silencing in molecular targeted therapy of ovarian cancer.

Key Words:
ERBB2 gene, MAPK1/MAPK3 signaling pathway, Ovarian cancer, Proliferation, Invasion, Migration.

Introduction

Ovarian cancer is one of the most common gynecological malignancies, accounting for about 3% of female malignancies[1], and its mortality ranks fifth of gynecological malignancies[2]. Ovarian cancer is characterized by hidden incidence, high risk of abdominal metastasis in the early stage, and lack of effective early diagnostic methods[3]. Most patients are diagnosed in the late stage, and the long-term survival rate is low at the same time[4,5]. Simultaneously, in recent decades, despite an increase in the survival rate of patients treated by operation combined with chemotherapy, the five-year survival rate of ovarian cancer

Corresponding Author: Tingting Yu, MD; e-mail: 450185112@126.com

patients is still not optimistic, and the long-term survival rate has not improved significantly[6]. Cancer cells are mainly featured by infinite proliferation[7], besides, the acquisition of invasive and migratory ability is the key concerning the development of benign lesions to malignant tumors[8]. At present, the pathogenesis of ovarian cancer is not quite clear, there is a need for further research on the biological basis of its occurrence and development, searching for gene targets to inhibit its invasion and migration. Significantly, the development of ovarian cancer is a process in which many oncogenes and anti-oncogenes participate[9]. Therefore, screening genes that can effectively inhibit the development of cancer cells and providing new therapeutic targets is the focus of this study.

ERBB2, also known as human epidermal growth factor receptor 2 (HER-2), is a kind of epidermal growth factor receptor located at the basal side of cell membrane and has intrinsic tyrosine kinase activity[10,11]. It encodes a 185-kDa transmembrane glycoprotein composed of 1,255 amino acids, including four sub-regions of signal peptide, extracellular domain, transmembrane domain, and intracellular domain[12]. At present, ERBB2 is considered to be an orphan receptor[13], and its ligand has not been confirmed, which, however, can be activated by phosphorylation following the formation of ERBB2 homodimer or its heterodimer with other HER members[14,15]. ERBB2 is reported to be amplified or overexpressed in many tumors, such as breast cancer, ovarian cancer, lung cancer, and gastric cancer[16-19]. Thus, ERBB2 is considered to be an ideal target for antibody therapy. With the activation of ERBB2, the complex signal transduction network in tumor cells begins to start or initiate, resulting in a variety of biological behaviors[15]. At the same time, the response of cells to environmental changes is partly induced by a series of intracellular signaling pathways[20,21], which amplify and integrate signals from extracellular stimuli, and ultimately lead to genetic and physiological changes. Mitogen-activated protein kinase (MAPK) is a kind of serine/threonine protein kinase existing in mammals[22]. It can be activated by extracellular signals such as physical and chemical stress, cytokines, growth factors, etc. In the next step, it can activate downstream transcription factors through three cascade reactions, transfer various types of external stimuli to the nucleus, and is intimately related to cell proliferation, differentiation, transformation, inflammation, and apoptosis, as well as various physiological and pathological processes of the body[23]. It has been accepted as an important signal transduction system in cells. At present, three pathways of MAPK are recognized, namely extracellular signal-regulated protein kinases (ERKs) pathway, C-Jun N-terminal kinase (JNK) pathway, and p38MAPK pathway[24]. Several studies[22,25] have shown that such signal transduction pathways play an important role in regulating tumorigenesis, invasion, and metastasis, which can enter the nucleus to further regulate various transcription factors, and mediate cell growth, differentiation, apoptosis, metastasis, and invasion.

Currently, the crucial role of ERBB2 and MAPK1/MAPK3 signaling pathway, as well as the emergence and development of gene silencing technology and molecular therapy have been recognized in modern and contemporary oncology research[24]. However, there are still few studies focusing on the above targets in the progression and intervention of ovarian cancer. In the present study, the effect of ERBB2 gene-mediated MAPK1/MAPK3 signaling pathway on migration and invasion of ovarian cancer cells *in vitro* was studied by transfecting ERBB2 expression plasmid into human ovarian cancer cell line. It is expected to provide theoretical basis for further *in vivo* study of the gene and to find effective gene therapy targets for inhibiting the occurrence and development of ovarian cancer.

Materials and Methods

Reagents and Equipment

Anhydrous ethanol (Tianjin Fuyu Fine Chemical Co., Ltd., Tianjin, China); xylene (Shanxi Lierkang Biotechnology Co., Ltd., Shanxi, China); polyformaldehyde (Sigma-Aldrich, St. Louis, MO, USA); ERBB2, MAPK1, MAPK3 antibodies (Cell Signaling Technology, Beverly, Massachusetts, USA); development reagent kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China); trypsin and crystal violet (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), Dulbecco's Modified Eagle's Medium (DMEM; Hyclone; South Logan, UT, USA), phosphate-buffered saline (PBS; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), methanol and glacial acetic acid (Tianjin Fuyu Fine Chemical Co., Ltd., Tianjin, China), transwell Chamber (Corning, Corning, NY, USA), etc.

Electronic analytical balance (Shanghai Pingxuan Science Instrument Co., Ltd., Shanghai, China); RM2126 paraffin slicing machine (Shanghai Leica Instruments Ltd., Shanghai, China); OLYMPUS fluorescence microscope (OLYMPUS, Tokyo, Japan); inverted fluorescence microscope (OLYMPUS, Tokyo, Japan); gel imaging system (ChemiDoc MP, Bio-Rad; Hercules, CA, USA); pipette (0.5-10 μL; 20-200 μL; Research plus 100-1000 μL; Beijing Gilson Science & Technology Co., Ltd., Beijing, China); horizontal decolorization shaker (TY-80A/S decolorization shaker, Changzhou Xunsheng Instrument Co., Ltd., Changzhou, Jiangsu, China); low-temperature centrifuge [5427R; Eppendorf (EP) AG, Hamburg, Germany]; vertical electrophoresis bath (Mini-Protean Tetra Electrophoresis System, Bio-Rad; Hercules, CA, USA); basic power supply (PowerPace Basic, Bio-Rad; Hercules, CA, USA); Trans-Blot (Bio-Rad; Hercules, CA, USA).

Objects of Study

From January 2015 to January 2018, 240 ovarian cancer specimens were collected from our hospital (all patients had not received chemotherapy or radiotherapy before operation, had not taken hormone drugs, and were pathologically diagnosed as epithelial ovarian cancer). At the same time, paracancerous tissues around ovarian cancer tissues (as compared with cancer tissues) were collected for comparison. All the collected specimens were fixed with 10% formaldehyde, routinely dehydrated, paraffin-embedded, and cut into sections of 4 μm in thickness to be used in the next steps. The present investigation was approved by the Ethics Committee in our hospital, and the use of all collected specimens and details related to the experiment were informed to the related patients or family members.

Immunohistochemical Detection of ERBB2, MAPK1, and MAPK3 Expression

Immunohistochemical staining was performed with SP-9001 kit. Paraffin sections of normal control ovarian epithelium and ovarian cancer were baked for 20 min in an oven at 68°C. In the process of elution, paraffin-embedded section was dewaxed by dipping in xylene, followed by dehydration with different concentration of alcohol. After that, the specimens were incubated at 37°C for 10 min with 3% H_2O_2, followed by PBS washing 3 times, 5 min each. In the step of antigen retrieval, tissue slices were put into buffer solution, heated in microwave oven or electric oven to 96-98°C for 10 min, then gradually cooled to room temperature. Slices were rinsed gently with PBS two times (5 min each) and distilled water two times (3 min each). As for the inactivation of endogenous enzyme activity, the tissue samples were placed in a wet box, a small amount of distilled water was added to the box, and a drop of 3% hydrogen peroxide. A reagent in the second antibody kit was added and incubated at room temperature for 10 min. Slices were rinsed gently with PBS three times (3 min each) and distilled water three times (3 min each). Then, for sealing, the water droplets were absorbed following shaking off the water, followed by the addition of a drop of goat serum sealant (B reagent in the kit) and incubation in the wet box at room temperature for 10 min. After the first antibody (ERBB2, MAPK1, MAPK3) was added, the slices were incubated in refrigerator for 12 h, and the temperature was controlled about 4°C. Following another gentle PBS rinsing three times (5 min each time), corresponding biotinylated mouse anti-sheep IgG working fluid (the secondary antibody) was dripped and incubated at 37°C for 30 min, ended with another gentle PBS rinsing three times (5 min each time). Afterwards, streptomycin albumin working fluid labeled with horseradish peroxidase (HRP) was dripped and incubated in an incubator for 10 min. PBS was gently rinsed three times (5 min each time). The reaction was terminated after adding diaminobenzidine (DAB) for 3 to 10 min. The double steamed water was used to wash for 10 min, followed by hematoxylin re-dyeing, conventional dehydration, transparent treatment, drying, and sealing. PBS was used as blank control instead of the primary antibody. Each slice was randomly selected with 6 high power field of vision (200×) and 100 cells per field of vision.

The positive expression of MAPK1 and MAPK3 protein was yellow or brown granules in nucleus or cytoplasm. Semi-quantitative grading was performed with reference to literature: 5 visual fields were randomly observed. The intensity of coloration was divided into 0 point for colorless, 1 point for light yellow and yellow, and 2 points for brown yellow. The number of positive cells was divided into < 10%, 0 point, 10% - 50%, 1 point, and > 50%, 2 points. The total product of the two items was divided into negative with 0 point, weak positive with 1 - 3 points, and strong positive with 4 points. ERBB2 staining referred

to the criteria of FDA in the United States, which was overexpressed with continuous yellow or brown-yellow staining of the cell membrane. Each experiment was repeated three times.

Culture of Ovarian Cancer Cell Lines and Screening of Eligible ERBB2 Cell Line by qRT-PCR

Ovarian cancer cell lines SKOV3, HO8910, ES-2, and COC1 (all purchased from the Cell Bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM high-sugar medium containing 10% fetal bovine serum (penicillin-streptomycin solution, 1:1, 100 μg/ml), respectively. The cells were cultured in a sterile cell incubator at 37°C and 5% CO_2. When the cell growth reached 90% confluence, the cells were digested with 0.25% trypsin and passaged in the ratio of 1:3. The cells were inoculated into 24-well plates (5×10^4 cells/well) for 24 h, and then detected when the cells grew and fused 70%-80%. Real-time fluorescence quantitative PCR was used to detect the expression of ERBB2 in each cell, and the cell line with higher expression were selected to complete the subsequent experiment. Each experiment was repeated three times.

Cell Grouping and Lentivirus Infection

The third passage cells were digested and inoculated into 24-well plate for culture. The density of cells per well was adjusted to 1×10^5 cells/well, so that the cells grew into monolayer. The cells were divided into four groups according to the design requirements: Blank group (blank control group, no transfection of any sequence), negative control (NC) group (infection of negative control lentivirus), ERBB2 shRNA group (infection of ovarian cancer cells with ERBB2 shRNA lentivirus), and ERBB2 overexpression group (infection of ovarian cancer cells with ERBB2 overexpression lentivirus; shorted as ERBB2 group). All lentiviruses were purchased from Shanghai GenePharma Co.,Ltd (Shanghai, China). The above screened ovarian cancer cells in logarithmic growth phase were inoculated into 24-well plates (2×10^5 cells/well) and cultured overnight in incubator at 37°C and 5% CO_2. When the cell growth density was about 40%-50%, the negative control lentivirus, ERBB2 shRNA lentivirus, and ERBB2 overexpressed lentivirus were transfected into the selected ovarian cancer cells in strict accordance with the GenePharma Lentivirus Infection Manual. After 24 h of transfection, complete medium was used to change the medium and normal culture was carried out. From the 4th day, screening was carried out at the concentration of 1 mg/L by adding human puromycin. Two weeks after puromycin screening, stable cell lines were constructed in NC group, ERBB2 shRNA group, and ERBB2 group.

Relative Expression levels of ERBB2, MAPK1, MAPK3, vascular endothelial growth factor (VEGF), metalloproteases-2 (MMP-2), and tissue inhibitor of metalloproteases-2 (TIMP-2) by qRT-PCR

Transfected cells were taken from each group and put into l ml TRIZOL reagent to shake and mix to lyse cells. An amount of 200 ml chloroform was added and mixed manually, which was then placed at room temperature for 2-3 min, and centrifuged at 12,000 rpm for 15 min at 4°C. The upper water phase was extracted carefully with pipette into another sterile centrifugal tub, followed by the addition of 0.5 ml isopropanol to mix it repeatedly. After reaction at room temperature for 10-30 min, another centrifugation was conducted at 12,000 rpm for 15 min at 4°C, with the realization of RNA precipitates at the bottom of the tube after discarding the supernatant. Then, 75% ethanol was added to the centrifuge tube and the precipitation was suspended by shaking the centrifuge tube gently. With another centrifugation was conducted at 12,000 rpm for 15 min at 4°C, the supernatant was discarded and dried at room temperature for 5 min, and the precipitate was dissolved by adding 10 ml DEPC, which was stored at –70°C. RNA concentration was determined by ultraviolet spectrophotometer. The primers used in this study were synthesized by Dalian TaKaRa Company. According to the instructions of the reverse transcription kit, the reaction conditions were set as follows: 42°C, 1 h (reverse transcription reaction), 70°C, 5 min (reverse transcriptase inactivation reaction). After that, the reverse transcription of the DNA was diluted to 50 ng/ml, and the subsequent fluorescence quantitative PCR was used for reserve. The reaction amplification system was 25 μl, which was tested in fluorescent quantitative PCR instrument. The conditions of reverse transcription reaction were as follows: pre-denaturation at 94°C for 3 min; denaturation at 94°C for 30 s, annealing at 56.5°C for 30 s and extension at 72°C for 30 s, in a total of 35 cycles; followed by PCR amplification at 72°C for 5 min. Using total

cNDA as template and β-actin as internal reference primer, the relative transcription level of target genes (ERBB2, MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2) was calculated by relative quantitative method ($2^{-\Delta\Delta CT}$ method). Each experiment was repeated three times.

Protein Expression of ERBB2, MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2 by Western Blot

After 48 h of transfection, the cells with 95% confluence were taken out with the culture medium poured clean, followed by the addition of 2 ml pre-cooled PBS for washing at 4°C. The washed liquid was discarded, and the culture flask was placed on ice after 2 times of re-washing. Subsequently, the protein lysate was added to the ice and decomposed at 4°C for 30 min, shaking every 10 min for complete lysis. The adherent cells were gently scraped off with a cell scraper. Cell fragments and lysate were absorbed and transferred to 1.5 ml EP tube, which was then centrifuged for 5 min at 12,000 r/min at 4°C. The lipid layer was discarded and the supernatant was taken as tissue protein extract. The protein concentration of each sample was determined by bicinchoninic acid (BCA) kit. The protein was transferred to the polyvinylidene difluoride (PVDF) membrane by wet transfer method after quantified by different concentrations and separated by polyacrylamide gel electrophoresis (SDS-PAGE). In the process, the initial voltage was 80 V for electrophoresis, and the voltage was changed to 100 V when samples ran to the separating gel. The PVDF film was then removed and placed in 5% skimmed milk powder for sealing in a low-speed shaking bed at room temperature for 1 h. The primary antibodies of anti-rabbit anti-human ERBB2, rabbit anti-human MAPK1, MAPK3, rabbit anti-human VEGF, rabbit anti-human MMP-2, rabbit anti-human TIMP-2 were added and mixed in a shaking bed at 4°C for overnight incubation, followed by Tris-Buffered Saline and Tween-20 (TBST) washing for 10 min each (2 times). The PVDF film was dried with filter paper, and enhanced chemiluminescence (ECL) agent A and B were mixed evenly. The film was exposed to darkroom for 1 min, then developed and fixed. ImageJ 1.48 U software was applied for quantitative analysis of protein. Quantitative analysis of proteins was carried out by the ratio of grey value of each protein to that of internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each experiment was repeated three times.

Proliferation Activity of Cells in Each Group After Transfection by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

Succinate dehydrogenase in mitochondria of living cells can reduce exogenous MTT to water-insoluble violet-blue crystalline armor and deposit in cells. Dimethyl sulfoxide (DMSO) can dissolve the armor in living cells, and the light absorption value can be measured at wavelength of 492 mm by enzyme labeling instrument, which can indirectly reflect the number of living cells. In our experiment, after 48 h of transfection, cells in logarithmic growth phase were collected and counted, inoculated into 96-well plates at cell density of 5×10^3 cells with a volume of 100 μl per well and repeated for five holes, and incubated in an incubator. Four time points were set (24 h, 48 h, 72 h, and 96 h) for the following experiments: cells were incubated in an incubator with 5%CO_2 at 37°C, 5 wells were randomly selected from each group every 24 h after inoculation for 2 h. MTT (5 mg/ml) was added with 10 ml per well, and incubated at 37°C for 4 h. With the termination of the culture, the culture medium was absorbed carefully in the well, with the addition of 150 ml DMSO/well, shaking in horizontal shaker for 10 min, and then fully dissolved and crystallized. The absorbance (OD) value of each well was read at 492 nm of enzyme-linked immunoassay (ELISA) meter. Zero-adjusting well (culture medium, MTT, dimethylalum) and control well (cells, culture medium, MTT, dimethylalum) were set at the same time. Each experiment was repeated three times. The number of living cells was expressed by the OD value, and the cell viability curve was plotted by the abscissa of time point and the ordinate of OD value.

Cell Invasion in Each Group After Transfection by Transwell Assay

For the detection of cell invasion in each group, the cells were cultured in DMEM medium after 48 h of transfection. Transwell chamber was prepared when starting the experiment, the upper chamber surface of the bottom membrane of transwell chamber was coated with 50 mg/L Matrigel diluent (1:8) and air-dried at 4°C. The residual liquid in the culture plate was sucked out and 50 μL serum-free culture medium containing 10 g/L bovine serum albumin (BSA) was added into each pore at 37°C for 30 min. For the next step of the preparation of cell suspension, serum starvation

cells were withdrawn for 12 h to further remove the effect of serum. The digestive cells were centrifuged and discarded after termination of digestion, washed twice with PBS, and suspended in serum-free medium containing BSA. The cell density was adjusted to 5×10^5. For cell inoculation, 200 μL cell suspension was taken and added into transwell chamber. The medium containing 10% fetal bovine serum (FBS) was added into the lower chamber of 24-well plate. Care should be taken not to produce bubbles when the chamber was placed in. For fixation and staining, the transwell chamber was removed with cells wiped from the upper chamber *via* cotton swabs, followed by fixation with 4% polyformaldehyde for 15 min, staining with 0.5% crystal violet solution (prepared with methanol) for 15 min, and washing with PBS three times. Afterwards, five visual fields were randomly selected and observed under inverted microscope for photography (magnification 200×). The number of cells penetrating the film was counted. Each experiment was repeated three times.

Cell Migration in Each Group After Transfection by Scratch Test

In the scratch test, marker pen was used behind the 6-well plate, and ruler was used to draw a horizontal line evenly, about every 0.5-1 cm, across the well. About 5×10^5 cells were added into the well. The number of cells varied with the types of cells, and cells could be covered fully in the bottom of the well following overnight incubation. The next day, the head of the pipette was used to draw the line by matching the ruler, and it was adjusted to be perpendicular to the horizontal line behind as far as possible to the horizontal scratches behind without tilt. The cells were then washed 3 times with PBS, removed, and added into serum-free medium. Following cultured in a 5% CO_2 incubator at 37°C, the results were observed and photographed under a microscope (magnification 200×).

Statistical Analysis

Statistical data were processed and analyzed by SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA). The parameters of the experimental measurement data were expressed as mean ± standard deviation) ($\bar{x} \pm s$). Independent sample-*t*-test was used for comparison between groups, and one-way analysis of variance (ANOVA) (LSD post-hoc test) was used for pairwise comparison between groups. The counting data were expressed by percentage and compared by Chi-square test. $p<0.05$ indicated that the difference was significant.

Results

Immunohistochemical Results

The immunohistochemical results (Figure 1) showed that the positive stained cells of ERBB2, MAPK1, and MAPK3 in ovarian tissue were yellow-brown or dark brown granules. The positive expression rates of ERBB2, MAPK1, and MAPK3 in adjacent tissues were 15.42% (37/240), 17.50% (42/240), and 16.67% (40/240), respectively; while those of ERBB2, MAPK1, and MAPK3 in ovarian cancer tissues were 61.67% (148/240), 75.00% (18/240), and 77.50% (186/240), respectively. The positive expression rates of ERBB2, MAPK1, and MAPK3 in ovarian cancer tissues were significantly higher than those in adjacent tissues, and the differences were statistically significant (all $p < 0.05$).

Screening and Transfection of Ovarian Cancer Cell Lines

The results of qRT-PCR for screening (Figure 2A) showed that compared with HO8910, ES-2, and COC1 cells, ERBB2 expression in SKOV3 ovarian cancer cells increased significantly (all $p < 0.05$). Therefore, SKOV3 ovarian cancer cells were selected for subsequent experiments. At the same time, the results of qRT-PCR (Figure 2B) and Western blot (Figure 2C-D) showed that there was no significant difference in ERBB2 gene and protein expression between Blank group and NC group ($p > 0.05$). Compared with Blank group and NC group, ERBB2 gene, and protein expression in ERBB2 shRNA group was significantly lower, and the difference was statistically significant ($p < 0.05$). Meanwhile, the expression levels of ERBB2 mRNA and protein in ERBB2 group increased significantly, and the difference was statistically significant ($p < 0.05$). These results suggest that lentiviral transfection of ERBB2 silencing and overexpression plasmids successfully inhibited or up-regulated ERBB2 expression.

ERBB2 Silencing Inhibited the Activation of MAPK1/MAPK3 Signaling Pathway

QRT-PCR detection results (Figure 3A) showed that there was no significant difference in the expression levels of MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2 in Blank and NC groups (all

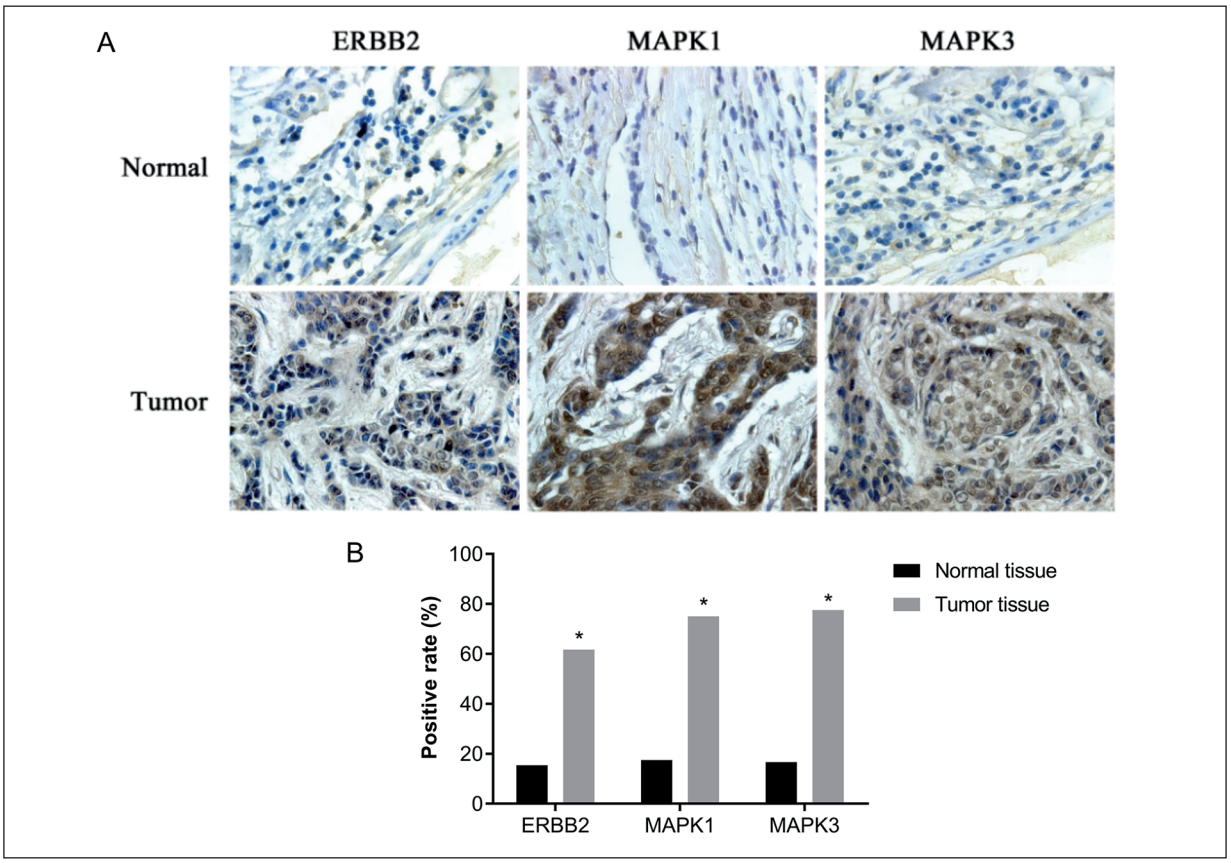

Figure 1. Immunohistochemical detection of positive rate of abnormal expression of ERBB2, MAPK1 and MAPK3. *Note:* **A**, The positive expression of ERBB2, MAPK1, and MAPK3 in tissues (magnification 200×); and **B**, Histogram of the positive expression rates of ERBB2, MAPK1, and MAPK3; *, A comparison with Control group, $p < 0.05$.

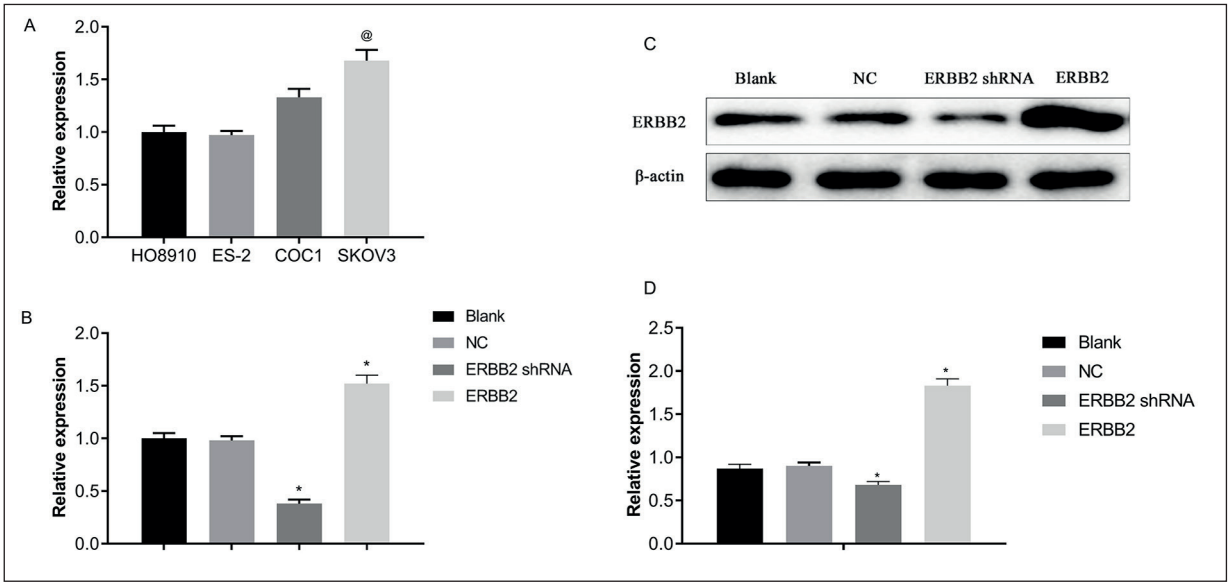

Figure 2. Screening of ovarian cancer cell lines and detection results of ERBB2 after transfection. *Note*: **A**, Histogram of screening results of ovarian cancer cell lines; **B**, Histogram of qRT-PCR results of ERBB2 in SKOV3 cells; **C**, Electrophoresis results of ERBB2 by using Western blot in SKOV3 cells; and **D**, Histogram of Western blot results of ERBB2 in SKOV3 cells; @, A comparison with that in HO8910, ES-2, and COC1 cells, $p < 0.05$, *, A comparison with that in Blank group and NC group, $p < 0.05$.

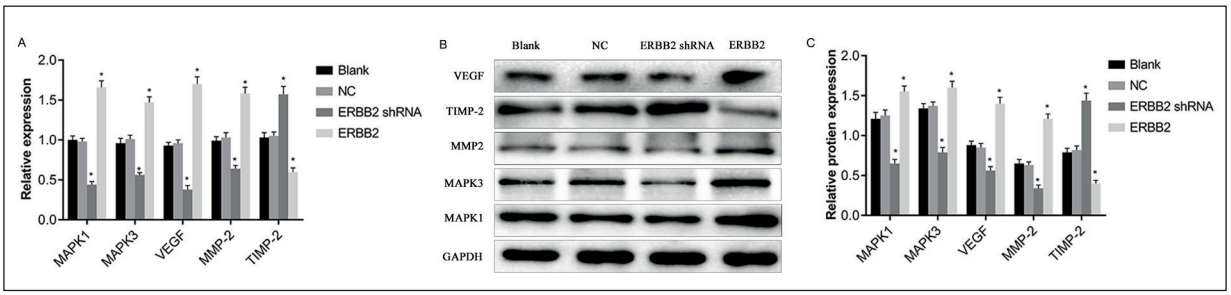

Figure 3. The expression levels of MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2 detected by qRT-PCR and Western blot. *Note:* **A**, Histogram of qRT-PCR results of MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2 in SKOV3 cells; **B**, Electrophoresis results of MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2 by using Western blot in SKOV3 cells; and **C**, Histogram of Western blot results of MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2 in SKOV3 cells; *, A comparison with that in Blank group and NC group, $p < 0.05$.

$p > 0.05$). Compared with Blank group and NC group, the expression levels of MAPK1, MAPK3, VEGF, and MMP-2 in ERBB2 shRNA group were significantly lower, but TIMP-2 expression level was significantly higher, and the differences were statistically significant (all $p < 0.05$). These results suggested that ERBB2 silencing inhibited the activation of MAPK1/MAPK3 signaling pathway in ovarian cancer cells and then inhibited the expression of MAPK1, MAPK3, VEGF, and MMP-2 while it promoted the expression of TIMP-2. In ERBB2 group, however, the expression levels of MAPK1, MAPK3, VEGF, and MMP-2 were significantly increased, but TIMP-2 expression was evidently decreased, and the differences were statistically significant (all $p < 0.05$). These results suggested that ERBB2 overexpression could reverse the above trend and promote the expression of MAPK1, MAPK3, VEGF, and MMP-2, while it could suppress the expression of TIMP-2.

Furthermore, according to the results of Western blot (Figure 3B-C), there was no significant difference in protein expression levels of MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2 between Blank group and NC group ($p > 0.05$). Compared with Blank group and NC group, ERBB2 shRNA group had significantly lower protein expression of MAPK1, MAPK3, VEGF and MMP-2, and higher protein expression of TIMP-2, with statistically significant differences (all $p < 0.05$). The above results indicated that ERBB2 silencing inhibited the activation of MAPK1/MAPK3 signaling pathway in ovarian cancer cells, thereby inhibiting the protein expression of MAPK1, MAPK3, VEGF, and MMP-2 proteins and promoting the expression of TIMP-2. However, the protein expression of ERBB2, MAPK1, MAPK3, VEGF, and MMP-2 in ERBB2 group increased significantly, while TIMP-2 decreased, showing statistically significant differences (all $p < 0.05$). These results revealed that ERBB2 overexpression could reverse the above trend, further promote the expression of MAPK1, MAPK3, VEGF, MMP-2, and TIMP-2, and inhibit the expression of TIMP-2.

ERBB2 Silencing Inhibited Cell Proliferation

As evidenced by the results of MTT assay (Figure 4), there was no significant difference in OD value after cell transfection for 24 h in each group ($p > 0.05$). There was no significant difference in OD value in SKOV3 cells between Blank group and NC group after transfection for 48 h, 72 h, and 96 h (all $p > 0.05$). Compared with Blank group and NC group, OD value of

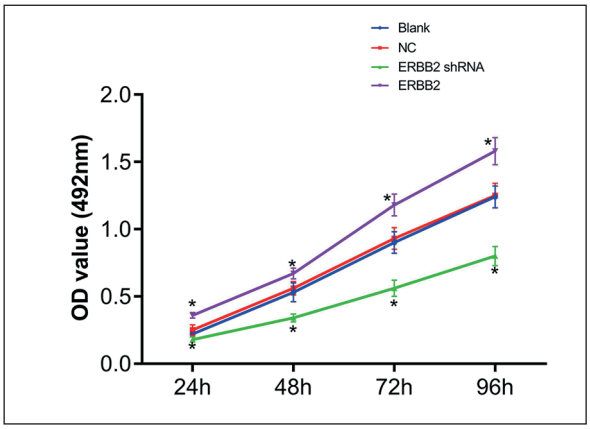

Figure 4. Effect of silencing ERBB2 on migration of SKOV3 cells. *Note:* Line chart for the comparison of proliferation ability of SKOV3 cells; *, A comparison with that in Blank group and NC group, $p < 0.05$.

SKOV3 cells transfected with ERBB2 shRNA plasmid decreased significantly at 48 h, 72 h and 96 h, and the differences were statistically significant (all $p < 0.05$). These results suggested that ERBB2 silencing inhibited the proliferation of ovarian cancer cells. By comparison, OD value of SKOV3 cells transfected with ERBB2 overexpression plasmid increased significantly at 48 h, 72 h and 96 h, and the differences were statistically significant (all $p < 0.05$). These results supported that ERBB2 overexpression promoted the proliferation of ovarian cancer cells.

ERBB2 Silencing Inhibited Cell Invasion

As shown by the results of transwell assay (Figure 5), no significant difference was found in invasion ability of SKOV3 cells between Blank group and NC group 48 h after transfection ($p > 0.05$). Compared with Blank group and NC group, SKOV3 cells in ERBB2 shRNA group had significantly decreased invasion ability 48 h after transfection, and the difference was statistically significant ($p < 0.05$), suggesting that ERBB2 silencing inhibited the invasion of ovarian cancer cells. However, the invasion ability of SKOV3 cells in ERBB2 group increased significantly 48 h after transfection, and the difference was statistically significant ($p < 0.05$), suggesting that the overexpression of ERBB2 promoted invasion of ovarian cancer cells.

ERBB2 Silencing Inhibited Cell Migration

Scratch test results (Figure 6) showed that there was no significant difference in migration ability of SKOV3 cells between Blank group and NC group 48 h after transfection ($p > 0.05$). Compared with Blank group and NC group, SKOV3 cells in ERBB2 shRNA group had significantly decreased migration ability 48 h after transfection, and the difference was statistically significant ($p < 0.05$), suggesting the role of ERBB2 gene expression silencing in inhibiting the migration of ovarian cancer cells. Conversely, there was a significantly increased trend in the migration ability of SKOV3 cells in ERBB2 group 48 h after transfection, with statistically significant difference ($p < 0.05$), supporting the effect of ERBB2 overexpression in stimulating the migration of ovarian cancer cells.

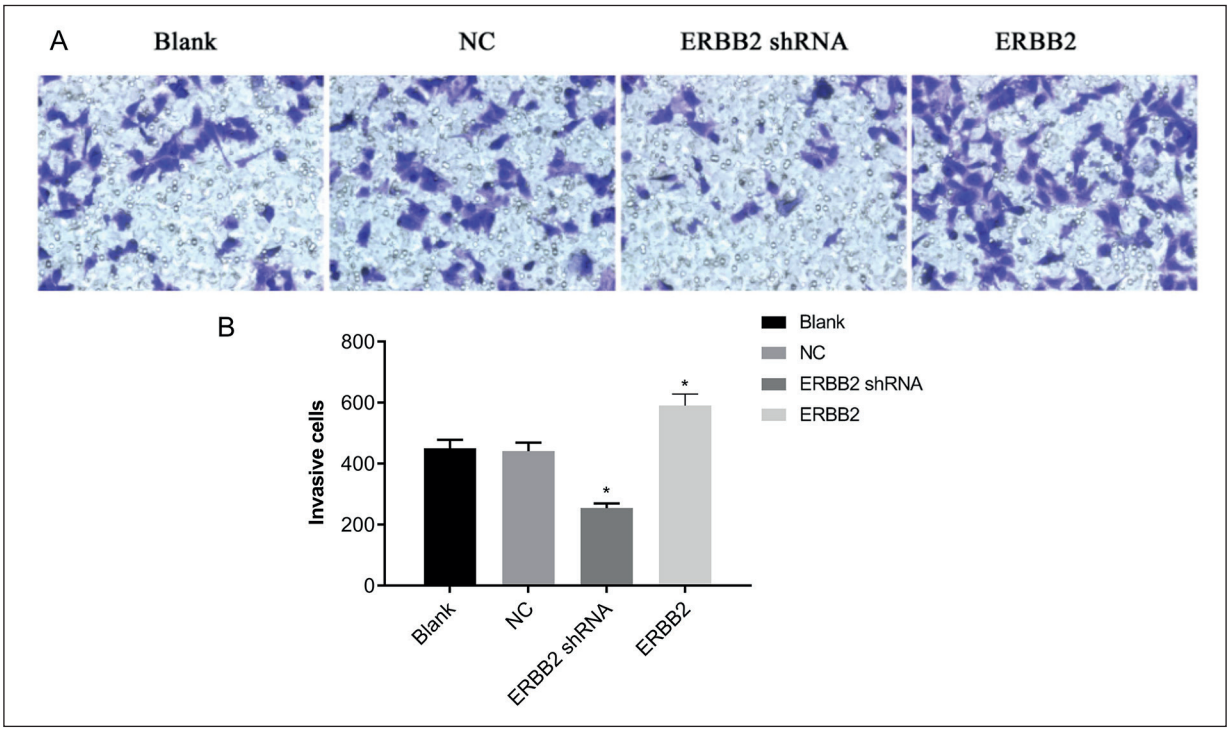

Figure 5. Effect of silencing ERBB2 on invasion ability of SKOV3 cells. *Note:* **A**, Comparison of the invasion ability of SKOV3 cells (magnification 200×); and **B**, Histogram of the invasion ability of SKOV3 cells; *, A comparison with that in Blank group and NC group, $p < 0.05$.

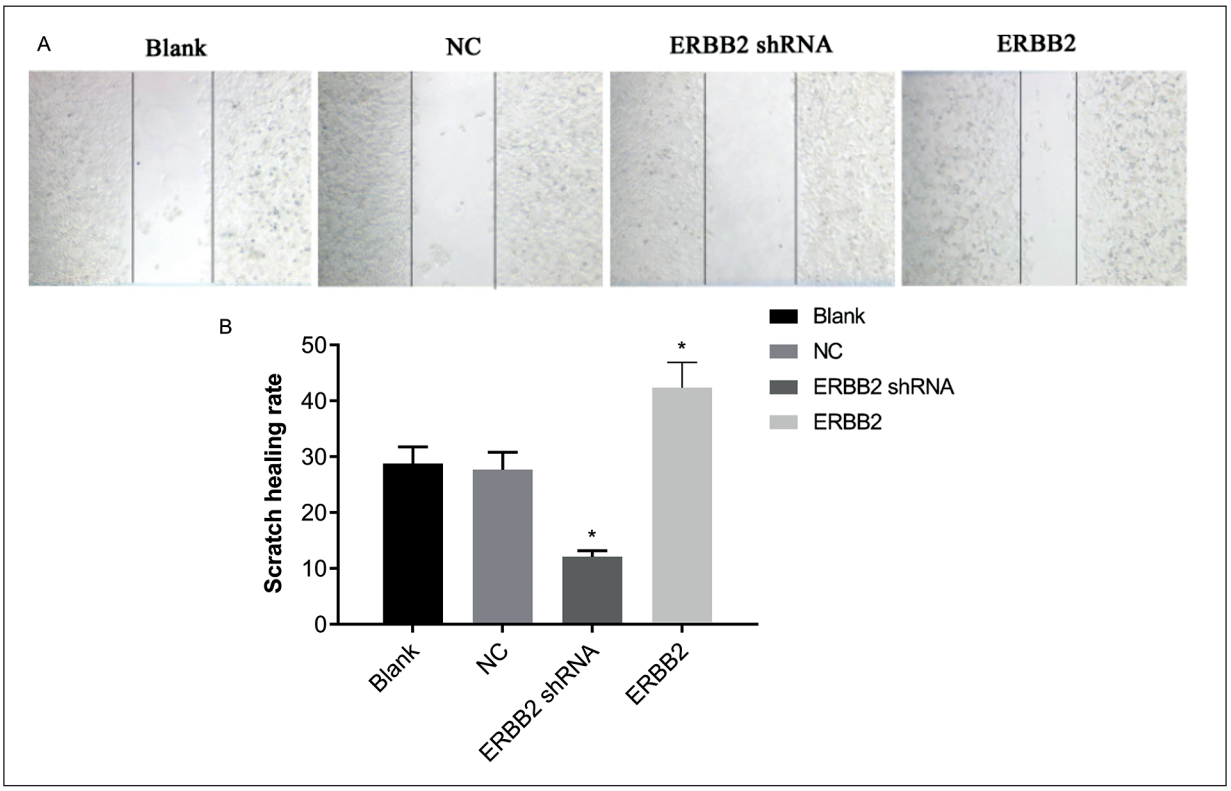

Figure 6. Effect of silencing ERBB2 on migration ability of SKOV3 cells. *Note*: **A**, Comparison of the migration ability of SKOV3 cells (magnification 200×); and **B**, Histogram of the migration ability of SKOV3 cells; *, A comparison with that in Blank group and NC group, $p < 0.05$.

Discussion

Ovarian cancer is the second most common malignant tumors in the female reproductive system and the sixth most common malignant tumor in women[1]. The mortality rate of ovarian cancer ranks the top in female reproductive system tumors[2]. In recent decades, the incidence of ovarian cancer has increased significantly. According to epidemiological studies, ovarian cancer can occur at any age, of which the most common one is the period of vigorous ovarian function and the stage of ovarian function turned from prosperity to decline[5,26]. The anatomical location of ovaries is hidden and difficult to detect, early symptoms of ovarian cancer are slight and difficult to differentiate, which is easy to be confused with digestive tract diseases, and about 75% of cases have been diagnosed as advanced consequently[5]. Unfortunately, metastasis can occur even in early stages. In this regard, how to control the invasion and metastasis of ovarian cancer has become the key to effectively treat ovarian cancer and improve the survival rate of patients. Multiple studies[27,28] have confirmed that ERBB2 amplification and overexpression, and MAPK signaling pathway, are all involved in the occurrence and development of breast cancer and other tumors. However, at present, few studies have systematically studied the relationship between ERBB2 and MAPK1/MAPK3 signaling pathway in ovarian cancer.

ERBB2 plays an important role in cell signal transduction[18,29]. ERBB2 can activate a powerful signal transduction network in cells, trigger a series of "cascade" chain reactions, and affect the growth, differentiation, metastasis, and adhesion of cancer cells[30]. ERBB2 protein expression in ovarian cancer is significantly correlated with the stage, pathological grade and prognosis of ovarian cancer[31,32]. There are also experiments suggesting that ERBB2 overexpression is more common in ovarian cancer, occasionally in borderline tumors, and rarely occurs in benign tumors, supporting that ERBB2 can be an independent prognostic indicator of ovarian cancer[33].

The above interpretations are consistent with our results that the positive expression rates of ERBB2 in ovarian cancer tissues were significantly higher than those in adjacent tissues by immunohistochemical assay. It suggested that ERBB2 might be involved in the occurrence of ovarian cancer, and the detection of ERBB2 may contribute to the differential diagnosis of ovarian cancer. Furthermore, it was also found that the positive expression rates of MAPK1 and MAPK3 in ovarian cancer tissues were also significantly higher than those in adjacent tissues, suggesting that MAPK1/MAPK3 signaling pathway may be activated in ovarian cancer. In combination with the expression status of ERBB2 in ovarian cancer, it was speculated in our study that gene silencing of ERBB2 and the inhibition of MAPK1/MAPK3 signaling pathway may be crucial in regulating the development of ovarian cancer, and we also wondered that there may be a potential relationship between the expression of ERBB2 and MAPK1/MAPK3 signaling pathway.

On the basis of the above investigation, in the next step of the experiment, lentiviral transfection was performed in ovarian cancer cell lines, followed by the screening of optimal cell line for further experiment. The results of qRT-PCR for screening showed that ERBB2 expression in SKOV3 ovarian cancer cells increased significantly in relative to the other three cell lines, SKOV3 ovarian cancer cells were thus selected for subsequent experiments. Simultaneously, ERBB2 gene expression was significantly lower in ERBB2 shRNA group and significantly higher in ERBB2 group, suggesting that silencing and up-regulation ofERBB2 expression were realized by lentiviral transfection of ERBB2 silencing and overexpression plasmids successfully. Meanwhile in terms of its molecular mechanism, the present experiment detected the changes of protein levels related to MAPK1/MAPK3 signaling pathway using qRT-PCR and Western blot assays. Compared with Blank group and NC group, ERBB2 shRNA group had significantly lower mRNA and protein expression of ERBB2, MAPK1, MAPK3, VEGF and MMP-2, and higher mRNA and protein expression of TIMP-2. The above results indicated that ERBB2 silencing inhibited the activation of MAPK1/MAPK3 signaling pathway in ovarian cancer cells, thereby inhibiting the protein expression of MAPK1, MAPK3, VEGF and MMP-2 proteins and promoting the expression of TIMP-2. By comparison, the mRNA and protein expression of ERBB2, MAPK1, MAPK3, VEGF and MMP-2 in ERBB2 group increased significantly, while those of TIMP-2 decreased. These results revealed that ERBB2 overexpression could reverse the above trend, and further promote the expression of MAPK1, MAPK3, VEGF, MMP-2 and TIMP-2, and inhibit the expression of TIMP-2.

Invasion and migration of tumors are complex processes[34]. The known markers of tumor invasion and migration include matrix metalloproteinases (MMPs), E-cadherin, vascular endothelial growth factor (VEGF) and α, β-catenin, etc.[35]. Among them, MMPs are a family of proteases with Zn^{2+} as a co-factor, which can degrade a variety of extracellular matrix components, including collagen type IV and basement membrane glycoprotein[36]. MMPs are the most important type of collagen type IV hydrolase for cancer cells to destroy the interstitial mechanical barrier and to infiltrate and diffuse into paracancerous tissue[37]. MMP2 is an important member of the family and prior studies[38] have confirmed that MMP2 is important for the occurrence of ovarian cancer. Tissue inhibitor of matrix metalloproteinase (TIMP) is a natural inhibitor of MMP[39]. Current studies[40] have shown that the imbalance between MMP and TIMP is closely related to the invasion and metastasis of tumors. TIMP2 is a specific inhibitor of MMP2 and participates in the activation of MMP2, which is a bi-directional regulator[40]. In view of the above detection of the expression of various molecules, it can be figure out that ERBB2 silencing dose play an essential role in inhibiting the development of ovarian cancer by inhibiting the activation of MAPK1/MAPK3 signaling pathway, as well as suppressing the expression of VEGF and MMP-2, while promoting the expression of TIMP-2, which can be considered as the potential molecular mechanisms.

Furthermore, one of the key characteristics of cancer cells is the loss of normal regulation of their growth at genetic level, resulting in indefinite growth of those cells[41]. Therefore, inhibiting the growth of cancer cells is one of the basic strategies of anti-cancer. MTT experiments in this study showed that OD value of SKOV3 cells transfected with ERBB2 shRNA plasmid decreased significantly at 48 h, 72 h, and 96 h, suggesting that ERBB2 gene silencing inhibited the proliferation of ovarian cancer cells. Meanwhile, OD value of SKOV3 cells transfected with ERBB2 overexpression plasmid increased significantly at 48 h, 72 h, and 96 h, supporting

that ERBB2 overexpression promoted the proliferation of ovarian cancer cells. Simultaneously, it was considered that the relationship between ERBB2 and tumor invasion has attracted much attention due to the presence of amplification or overexpression of ERBB2 gene in many tumors. Transwell assay is to lay ECM on the porous membrane of transwell invasive chamber to form basement membrane conjunctiva which is quite similar to the natural basement membrane[42]. It simulates the process of tumor cells gradually invading deep layer through adhesion and degradation of basement membrane *in vivo*. Meanwhile, cell scratch test is a simple and economical assay to study cell migration *in vitro*[43]. The basic steps include scratching on a single layer of cells, then taking pictures, and periodically taking pictures of cell migration until the scratch is fully closed with the growth of cells. The principle of this method is that when cells grow to a single layer state, a blank area is created manually on the fused single layer cell, which is called a scratch[44]. The cells at the edge of the scratch gradually enter the blank area to heal the scratch, so as to reflect the migration ability of cells. The findings of this experiment showed that compared with Blank group and NC group, SKOV3 cells in ERBB2 shRNA group had significantly decreased invasion and migration ability, and significantly increased invasion, and migration ability 48 h after transfection, suggesting the role of ERBB2 silencing in inhibiting the invasion migration of ovarian cancer cells, as well as the reverse role of ERBB2 overexpression in stimulating the invasion and migration of ovarian cancer cells.

Conclusions

Our study for the first time proves that silencing ERBB2 gene expression inhibits the activation of MAPK1/MAPK3 signaling pathway and thus inhibits the proliferation, invasion, and migration of ovarian cancer cells. Overexpression of ERBB2 gene can reverse the above trends, which in turn support the role of ERBB2 gene silencing in molecular targeted therapy of ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

We would like to express our acknowledgement to our team for their work and colleagues for their valuable scientific advice and suggestions.

References

1) Freij M, Al Q M, Khadra M, ALBashtawy M, Tuqan W, Al Faqih M, Innabi A, Batiha AM, Alhalaiqa F, Abd El-Razek A. Awareness and knowledge of ovarian cancer symptoms and risk factors: a survey of Jordanian women. Clin Nurs Res 2018; 27: 826-840.
2) Hanchette C, Zhang CH, Schwartz CG. Ovarian cancer incidence in the U.S. and toxic emissions from pulp and paper plants: a geospatial analysis. Int J Environ Res Public Health 2018; 15: 5. pii: E1619.
3) Boos J, Brook O R, Fang J, Brook A, Levine D. Ovarian cancer: prevalence in incidental simple adnexal cysts initially identified in CT examinations of the abdomen and pelvis. Radiology 2018; 286: 196-204.
4) Ebell MH, Culp MB, Radke TJ. A systematic review of symptoms for the diagnosis of ovarian cancer. Am J Prev Med 2016; 50: 384-394.
5) Ottevanger PB. Ovarian cancer stem cells more questions than answers. Semin Cancer Biol 2017; 44: 67-71.
6) Timmermans M, Sonke GS, Van K D V, van der Aa MA, Kruitwagen RFPM. No improvement in long-term survival for epithelial ovarian cancer patients: a population-based study between 1989 and 2014 in the Netherlands. Eur J Cancer 2018; 88: 31-37.
7) Yao L, Wang M, Niu Z, Liu Q, Gao X, Zhou L, Liao Q, Zhao Y. Interleukin-27 inhibits malignant behaviors of pancreatic cancer cells by targeting M2 polarized tumor associated macrophages. Cytokine 2017; 89: 194-200.
8) Thibault B, Jean-Claude B. Dasatinib + Gefitinib, a non platinum-based combination with enhanced growth inhibitory, anti-migratory and anti-invasive potency against human ovarian cancer cells. J Ovarian Res 2017; 10: 31.
9) Zhou Y, An Q, Guo RX, Qiao YH, Li LX, Zhang XY, Zhao XL. MiR424-5p functions as an anti-oncogene in cervical cancer cell growth by targeting KDM5B via the Notch signaling pathway. Life Sci 2017; 171: 9-15.
10) Ross JS, Fakih M, Ali SM, Elvin JA, Schrock AB, Suh J, Vergilio JA, Ramkissoon S, Severson E, Daniel S, Fabrizio D, Frampton G, Sun J, Miller VA, Stephens PJ, Gay LM. Targeting HER2 in colorectal cancer: the landscape of amplification and short variant mutations in ERBB2 and ERBB3. Cancer 2018; 124: 1358-1373.
11) Shramova EI, Proshkina GM, Deyev SM. The cause of ErbB2 receptor resistance to downregulation. Russ J Bioorgan Chem 2018; 44: 279-288.

12) Matić S, Quaglino E, Arata L, Riccardo F, Pegoraro M, Vallino M, Cavallo F, Noris E. The rat ErbB2 tyrosine kinase receptor produced in plants is immunogenic in mice and confers protective immunity against ErbB2+ mammary cancer. Plant Biotechnol J 2016; 14: 153-159.

13) Sett A, Borthakur BB, Bora U. Selection of DNA aptamers for extra cellular domain of human epidermal growth factor receptor 2 to detect HER2 positive carcinomas. Clin Transl Oncol 2017; 19: 976-988.

14) Croucher DR, Iconomou M, Hastings JF, Kennedy SP, Han JZ, Shearer RF, McKenna J, Wan A, Lau J, Aparicio S, Saunders DN. Bimolecular complementation affinity purification (BiCAP) reveals dimer-specific protein interactions for ERBB2 dimers. Sci Signal 2016; 9: ra69.

15) Bagnato P, Castagnino A, Cortese K, Bono M, Grasso S, Bellese G, Daniele T, Lundmark R, Defilippi P, Castagnola P, Tacchetti C. Cooperative but distinct early co-signaling events originate from ERBB2 and ERBB1 receptors upon trastuzumab treatment in breast cancer cells. Oncotarget 2017; 8: 60109-60122.

16) Sciortino M, Camacho-Leal MDP, Orso F, Grassi E, Costamagna A, Provero P, Tam W, Turco E, Defilippi P, Taverna D, Cabodi S. Dysregulation of Blimp1 transcriptional repressor unleashes p130Cas/ErbB2 breast cancer invasion. Sci Rep 2017; 7: 1145.

17) McKenzie SJ, DeSombre KA, Bast BS, Hollis DR, Whitaker RS, Berchuck A, Boyer CM, Bast RC Jr. Serum levels of HER-2 neu (C-erbB-2) correlate with overexpression of p185neu in human ovarian cancer. Cancer 2015; 71: 3942-3946.

18) Chuang JC, Stehr H, Liang Y, Das M, Huang J, Diehn M, Wakelee HA, Neal JW. ERBB2-mutated metastatic non-small cell lung cancer: response and resistance to targeted therapies. J Thorac Oncol 2017; 12: 833-842.

19) Duarte HO, Balmaña M, Mereiter S, Osório H, Gomes J, Reis CA. Gastric cancer cell glycosylation as a modulator of the ErbB2 oncogenic receptor. Int J Mol Sci 2017; 18. pii: E2262.

20) Castelnovo LF, Bonalume V, Melif S, Ballabio M, Colleoni D, Magnaghi V. Schwann cell development, maturation and regeneration: a focus on classic and emerging intracellular signaling pathways. Neural Regen Res 2017; 12: 1013-1023.

21) McCarthy GM, Bridges CR, Blednov YA, Harris RA. CNS cell-type localization and LPS response of TLR signaling pathways. F1000res 2017; 6: 1144.

22) Dreas A, Mikulski M, Milik M, Fabritius CH, Brzózka K, Rzymski T. Mitogen-activated protein kinase (MAPK) interacting kinases 1 and 2 (MNK1 and MNK2) as targets for cancer therapy: recent progress in the development of MNK inhibitors. Curr Med Chem 2017; 24: 3025-3053.

23) Korneeva NL, Song A, Gram H, Edens MA, Rhoads RE. Inhibition of mitogen-activated protein kinase (MAPK)-interacting kinase (MNK) preferentially affects translation of mRNAs containing both a 5′-terminal cap and hairpin. J Biol Chem 2016; 291: 3455-3467.

24) Dai XX, Song RX, Xiong Y. The expression of ERK and JNK in patients with an endemic osteochondropathy, Kashin-Beck disease. Exp Cell Res 2017; 359: 337-341.

25) Xue P, Zeng F, Duan Q, Xiao J, Liu L, Yuan P, Fan L, Sun H, Malyarenko OS, Lu H, Xiu R, Liu S, Shao C, Zhang J, Yan W, Wang Z, Zheng J, Zhu F. BCKDK of BCAA catabolism cross-talking with the MAPK pathway promotes tumorigenesis of colorectal cancer. EBioMedicine 2017; 20: 50-60.

26) Du Z, Qu H. The relationship between ovarian function and ovarian limited dose in radiotherapy postoperation of ovarian transposition in young patients with cervical cancer. Cancer Med 2017; 6: 508-515.

27) Castagnola P, Bellese G, Birocchi F, Gagliani MC, Tacchetti C, Cortese K. Identification of an HSP90 modulated multi-step process for ERBB2 degradation in breast cancer cells. Oncotarget 2016; 7: 85411-85429.

28) Zhang T, Jiang K, Zhu X, Zhao G, Wu H, Deng G, Qiu C. MiR-433 inhibits breast cancer cell growth via the MAPK signaling pathway by targeting Rap1a. Int J Biol Sci 2018; 14: 622-632.

29) Arias-Romero LE, Villamar-Cruz O, Pacheco A, Kosoff R, Huang M, Muthuswamy SK, Chernoff J. A Rac-Pak signaling pathway is essential for ErbB2-mediated transformation of human breast epithelial cancer cells. Oncogene 2010; 29: 5839-5849.

30) Yang XH, Flores LM, Li Q, Zhou P, Xu F, Krop IE, Hemler ME. Disruption of laminin-integrin-CD151-focal adhesion kinase axis sensitizes breast cancer cells to ErbB2 antagonists. Cancer Res 2010; 70: 2256-2263.

31) Singleton TP, Perrone T, Oakley G, Niehans GA, Carson L, Cha SS, Strickler JG. Activation of c-erbB-2 and prognosis in ovarian carcinoma. Comparison with histologic type, grade, and stage. Cancer 2015; 73: 1460-1466.

32) Hamad A, Singhi AD, Bahary N, McGrath K, Amarin R, Zeh HJ, Zureikat AH. Neoadjuvant treatment with trastuzumab and FOLFOX induces a complete pathologic response in a metastatic ERBB2 (HER2)-amplified duodenal cancer. J Natl Compr Canc Netw 2017; 15: 983-988.

33) Felip E, Del Campo JM, Rubio D, Vidal MT, Colomer R, Bermejo B. Overexpression of c-erbB-2 in epithelial ovarian cancer. Prognostic value and relationship with response to chemotherapy. Cancer 2015; 75: 2147-2152.

34) Hu M, Cui F, Liu F, Wang J, Wei X, Li Y. BMP signaling pathways affect differently migration and invasion of esophageal squamous cancer cells. Int J Oncol 2017; 50: 193-202.

35) Zhang Y, Wang CP, Ding XX, Wang N, Ma F, Jiang JH, Wang QD, Chang JB. FNC, a novel nu-

cleoside analogue, blocks invasion of aggressive non-Hodgkin lymphoma cell lines via inhibition of the Wnt/β-catenin signaling pathway. Asian Pac J Cancer Prev 2014; 15: 6829-6835.

36) Zhao F, Liu Z. Beneficial effects of edaravone on the expression of serum matrix metalloproteinase-9 after cerebral hemorrhage. Neurosciences (Riyadh) 2014; 19: 106-110.

37) Yang G, Li S, Li B, Cheng L, Jiang P, Tian Z, Sun S. Protective effects of garlic-derived S-Allylmercaptocysteine on IL-1β-stimulated chondrocytes by regulation of MMPs/TIMP-1 ratio and type II collagen expression via suppression of NF-κB pathway. Biomed Res Int 2017; 2017: 8686207.

38) Desmeules P, Trudel D, Turcotte S, Sirois J, Plante M, Grégoire J, Renaud MC, Orain M, Têtu B, Bairati I. Prognostic significance of TIMP-2, MMP-2, and MMP-9 on high-grade serous ovarian carcinoma using digital image analysis. Hum Pathol 2015; 46: 739-745.

39) Hire JM, Evanson JL, Johnson PC, Zumbrun SD, Guyton MK, McPherson JC 3rd, Bojescul JA. Variance of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) concentrations in activated, concentrated platelets from healthy male donors. J Orthop Surg Res 2014; 9: 29.

40) Chien YC, Liu LC, Ye HY, Wu JY, Yu YL. EZH2 promotes migration and invasion of triple-negative breast cancer cells via regulating TIMP2-MMP-2/-9 pathway. Am J Cancer Res 2018; 8: 422-434.

41) Dong J, Wang Q, Li L, Xiao-Jin Z. Upregulation of long non-coding RNA small nucleolar RNA host gene 12 contributes to cell growth and invasion in cervical cancer by acting as a sponge for miR-424-5p. Cell Physiol Biochem 2018; 45: 2086-2094.

42) Marshall J. Transwell (®) invasion assays. Methods Mol Biol 2011; 769: 97-110.

43) Rani A, Somaiah D, Megha, Poddar M. Scratch cell test: a simple, cost effective screening tool to evaluate self-healing in anti-corrosion coatings. J Mater Eng Perform 2014; 23: 3328-3335.

44) Huelsen A, Fischer J, Hegarty J, Ashcroft A, Frampton CM, Barclay ML. The scratch test for identifying the lower liver edge is at least as accurate as percussion and is significantly more effective for young trainees-a randomised comparative trial. N Z Med J 2016; 129: 53-63.