

ORIGINAL ARTICLE

Research on correlations of ERCC-1 with proliferation and apoptosis of ovarian cancer cells

Yuqin Zhang¹, Daoyan Zhang¹, Hongyan Wang²

¹Department of Obstetrics and Gynaecology, Qihe County People's Hospital, Dezhou, Shandong, 251100 China; ²Department of Obstetrics and Gynaecology, Linyi Lanshan People's Hospital, Linyi, Shandong, 276000 China

Summary

Purpose: To investigate the effects of excision repair cross complement-1 (ERCC-1) gene on the proliferation and apoptosis of ovarian cancer SKOV3 cells.

Methods: Ovarian cancer SKOV3 cell line was cultured and ERCC-1 overexpressing and ERCC-1 silencing cells were constructed via overexpression and silencing of ERCC-1 gene. The expression level of ERCC-1 in cells in each group was detected via quantitative polymerase chain reaction (qPCR). The expressions of apoptosis-related proteins [B-cell lymphoma 2 (Bcl-2), Fas and caspase-3] in cells in each group were detected via Western blotting. In addition, bromodeoxyuridine (BrdU) labeling and direct cell counting methods were performed, and the cell growth curve was drawn. The apoptosis rate in each group was detected via flow cytometry. SPSS 17.0 statistical software was used for analyses.

Students' t-test and one-way analysis of variance (ANOVA) were performed. $P < 0.05$ suggested statistically significant difference.

Results: Overexpression of ERCC-1 could downregulate the expression levels of apoptosis proteins (Fas and caspase-3) and upregulate the expression level of antiapoptotic protein Bcl-2. The apoptosis rate of SKOV3 cells in ERCC-1 overexpressing group was higher than those in ERCC-1 silencing group and control group.

Conclusion: ERCC-1 gene was not associated with the proliferation of ovarian cancer cells. ERCC-1 gene can inhibit the apoptosis of ovarian cancer SKOV3 cells.

Key words: apoptosis, ERCC-1 gene, ovarian cancer, proliferation

Introduction

Ovarian cancer is one of the deadliest gynecological malignant tumors, among which epithelial ovarian cancer is the most common type. Due to lack of effective early detection means, about 70% of epithelial ovarian cancer occurs in late stages [1]. In addition, drug resistance contributes to the fact that ovarian cancer is still the deadly gynecological malignant tumor [2]. Platinum compounds are the most effective drugs for ovarian cancer. However, there will be drug resistance and relapse in approximately 70% of the patients with advanced or stage-IV ovarian cancer within 5 years [3]. Al-

though the diagnosis and treatment of ovarian cancer have been significantly improved over the past 3 decades, the 5-year survival rate of ovarian cancer patients is still lower than 50% [4]. To improve the treatment and prolong the lives of patients, therefore, more research in the field of ovarian cancer is urgently needed to find a reliable biomarker, identify the drug target and clarify the mechanism of the drug on cancer.

ERCC-1 gene is the first human DNA repair gene identified via molecular cloning, which is isolated from Chinese hamster ovary (CHO) cells

Correspondence to: Hongyan Wang, BD. Department of Obstetrics and Gynaecology, Linyi Lanshan People's Hospital, Malingshan Rd and Wuhan Rd Intersection, Linyi, Shandong, 276000, China.
Tel: +86 13969900326, Fax: +86 0539 8190186, E-mail: hywang20000@163.com
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with DNA repair deficiency. Several ultraviolet radiation-sensitive CHO cell lines, including 43-3B and UV20, will be defective after ultraviolet irradiation. After transfection of cells in the human genomic DNA library, daughter clones normally sensitive to ultraviolet radiation will be isolated. Specific human genome restriction fragments are found in subclones of 43-3B cells [5]. ERCC1 complementary DNA (cDNA) is isolated from the human DNA supplementary to daughter clones [6]. Moreover, ERCC1 is located on the chromosome 19q13.32 and contains 10 exons, which are about 15 kb in length [7,8]. The last exon of ERCC1 gene overlaps with the start of CD3E gene, thereby transcribing the subunit of the T cell receptor-cluster of differentiation 3 (CD3) complex encoded by CD3 epsilon (CD3ε) from the opposite DNA strand [7]. ERCC1 is widely expressed in mammalian tissues, and it has currently been found to produce 4 kinds of transcripts through alternative splicing. One functional transcript is known as Isoform1 in the National Center of Biotechnology Information (NCBI) gene database, while the other three non-functional transcripts are known as Isoform2.

In previous studies, it was found that the ERCC-1 gene is associated with resistance to platinum compounds in ovarian cancer. Platinum-based chemotherapy is the first choice in the treatment of ovarian cancer. However, some patients do not respond to platinum compounds. Nucleotide excision repair (NER) system plays an important role in platinum drug resistance [9]. Under the action of DNA polymerase, NER synthesizes DNA through removing damaged fragments in DNA molecules, thus repairing the platinum drug-induced DNA damage, among which ERCC1 gene is a key gene. The messenger ribonucleic acid (mRNA) and protein expression levels of ERCC1 are related to the effectiveness of platinum-based chemotherapy [10]. ERCC1 mRNA and protein expressions can serve as biomarkers for prognosis of ovarian cancer patients [11]. In addition, the single nucleotide polymorphism (SNP) of ERCC1 is considered as a potential predictive biomarker for many different types of cancers [12]. However, the sample size in previous SNP research was small, and inconsistent results were obtained in some studies on the effect of platinum-based treatment. There are only few reports on other functions of ERCC-1 gene in tumor cells, especially its correlations with proliferation and apoptosis of ovarian cancer cells.

In this study, the ERCC-1 gene in ovarian cancer cells was overexpressed and inhibited, so as to observe the effects of this gene on the proliferation and apoptosis of these cells.

Methods

Cell culture

Human ovarian cancer SKOV3 cells purchased from the Cell Bank of the Chinese Academy of Sciences (Wuhan, China) were cultured in the high-glucose Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal calf serum (FCS), and 100 µg/mL streptomycin and 100 IU/mL penicillin were added. The cell culture flask was placed in an incubator at 37°C, with 5% CO₂ and 95% humidity.

Cell transfection experiment

Lentivirus (LV)-ERCC-1 with ERCC-1 gene overexpression and interference LV-small interfering RNA (siRNA)-ERCC-1 were synthesized by Shanghai Genechem Co., Ltd., and empty vector control and negative control [already verified by sequence alignment using Basic Local Alignment Search Tool (BLAST)] were also provided by the company.

Cell transfection: One day before transfection, SKOV3 cells were inoculated into a 96-well plate at 5×10^5 ml. When cell confluence reached 50-60% cells several different groups with the multiplicity of transfection (MOI) value of 10, 20, 30 and 50 were set up. Two repeated wells were set up in each group. ENS and polybrene transfection enhancers were used to dilute the virus and promote the transfection of cells by LV-ERCC1 and LV-si-RNA-ERCC-1. The transfection enhancer-free group was also set up. The virus concentration should be generally appropriate, and the transfection pre-test was performed to determine the optimal transfection concentration.

According to the optimal MOI value and transfection conditions in the transfection pre-test, the ovarian cancer cells were inoculated into a 6-well plate at a density of 3×10^5 /well on the night before lentiviral transfection. When cell confluence reached 80% on the next day, the overexpression lentivirus, interference lentivirus and empty vector controls were transfected into SKOV3 cells using the Lip2000 transfection kit (ThermoFisher, Waltham, MA, USA).

Quantitative polymerase chain reaction (qPCR)

Cells were collected after different treatments, and the total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One µL RNA solution was taken on a microplate reader to measure the total RNA concentration and purity. The optical density (OD)₂₆₀/OD₂₈₀ value should be 1.6-1.8. The remaining RNA solution was packaged and stored at -80°C for standby application.

cDNA was synthesized using the PrimeScript™ kit (TaKaRa Bio Inc., Otsu, Japan) according to specific procedures of the manufacturer's instructions. First, 2 µg total RNA was added into the PCR instrument. The volume of RNA added was calculated based on the concentration. One µL Oligo (dT) primer (50 µM) and 1 µL deoxyribonucleoside triphosphate (dNTP) and Mixture (10 mM) were added, and enzyme-free double-distilled

water was added to make up for the total volume up to 10 μ L. The mixture was mixed gently, heated in the PCR instrument at 65°C for 5 min, and quickly cooled on ice. The above system was added with 4 μ L 5 \times PrimeScript Buffer, 1 μ L PrimeScript RTase, 0.5 μ L Rnase inhibitor and 4.5 μ L enzyme-free water. Then, a total of 20 μ L reaction system was mixed gently, heated in the PCR instrument at 42°C for 45 min and then 95°C for 5 min, and quickly cooled on ice to synthesize the single-stranded cDNA. Finally, cDNA was stored at -20°C for PCR amplification reaction.

ERCC-1: Forward: 5'-CCCTGGGAATTTGGCGACGTAA-3'

Reverse: 5'-CTCCAGGTACCGCCAGCTTCC-3'

β -actin: Forward: 5'-ACACTGTGCCCATCTACGAGG-3'

Reverse: 5'-AGGGGCCGGACTCGTCATACT-3'

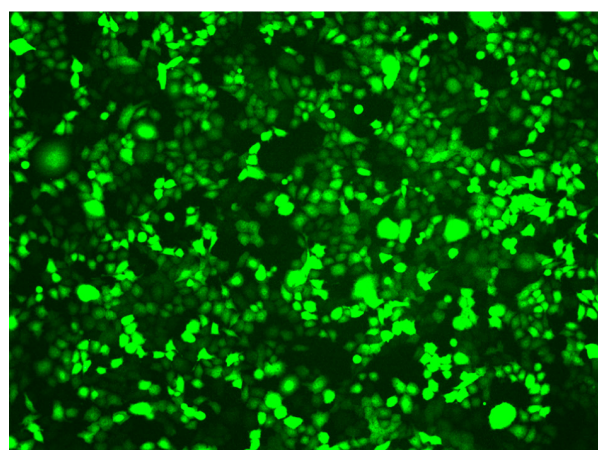
Western blotting

Cells were collected at 48 hrs after transfection and washed with ice-cold phosphate buffered saline (PBS). 200 μ L 1 \times radioimmunoprecipitation assay (RIPA) lysis buffer was added into each sample. The same amount of protein was extracted from the whole cell lysate and mixed with the sodium dodecyl sulfate (SDS) sample buffer, and the protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Then, the total protein was separated via SDS polyacrylamide gel.

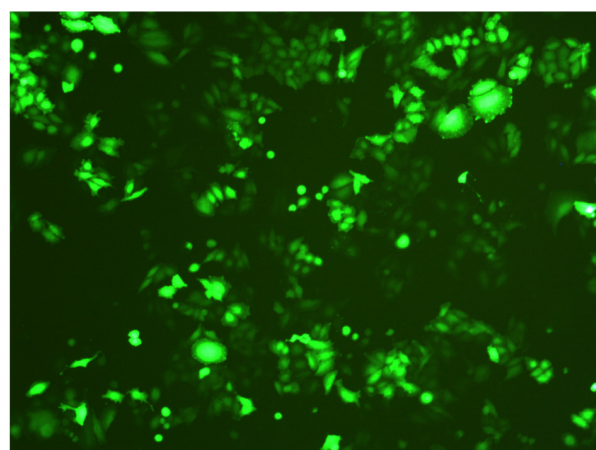
The protein separated was transferred onto a nitrocellulose membrane using the Bio-Rad Trans-Blot® semi-dry transfer system at 25 V for 1 hr, and sealed with 5% skim milk powder at room temperature for 2 hrs. The band was incubated with ERCC-1 rabbit primary antibody [diluted at 1:1000, Article No.: 3885S, Cell Signaling Technology (CST Danvers, MA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit primary antibody (diluted at 1:3000, Article No.: sc-32233, Santa Cruz, USA) at 4°C overnight. Then, the band was washed with 5% PBS with Tween-20 (PBST) 3 times and incubated with horseradish peroxidase-labeled rabbit secondary antibody at room temperature for 1 hr. Finally, the protein band was detected using the enhanced chemiluminescence system (ECL Western blotting kit, CST, USA).

Cell proliferation assay

Cell proliferation was detected via bromodeoxyuridine (BrdU) labeling method according to the following steps: 1) Cells were inoculated into a culture dish with a volume of 35 mL (with a cover glass inside) at a density of 1.5×10^5 /mL, and cultured for 1 day, followed by synchronization using culture solution containing 0.4% FCS for 3 days to make a majority of cells in G0 phase. 2) Before the cell culture was terminated, BrdU final

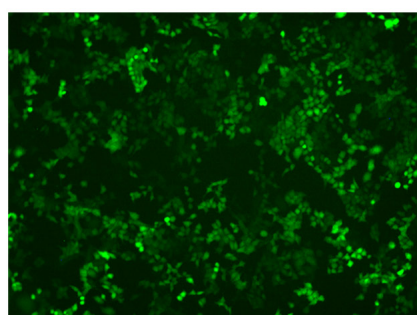


LV- ERCC-1

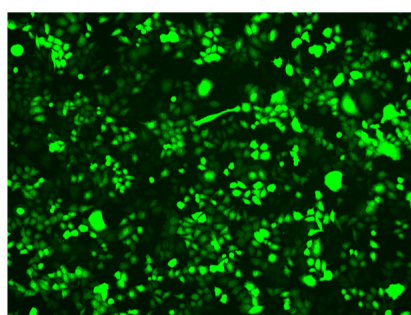


LV-Control

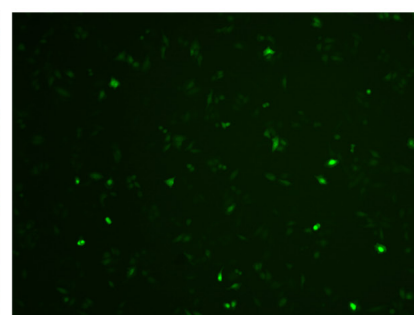
Figure 1. Cell morphology and fluorescence of SKOV3 cells transfected with ERCC-1-overexpressing virus.



LV-siRNA1- ERCC-1



LV-siRNA2- ERCC-1



LV-siRNA3- ERCC-1

Figure 2. Cell morphology and fluorescence of SKOV3 cells transfected with ERCC-1-interfering virus. The interference efficiency of SKOV3 cells was highest with LV-siRNA2 ERCC-1.

concentration of 30 $\mu\text{g/L}$ was added for incubation at 37°C for 40 min. 3) The culture solution was discarded, and the cover glass was washed with PBS 3 times. 4) The cover glass was fixed with methanol/acetic acid for

10 min. 5) Then, the fixed cover glass was air-dried, and the endogenous oxidase was inactivated using 0.3% H_2O_2 and methanol for 30 min. 6) The cover glass was sealed with 5% normal rabbit serum. 7) The nucleic acid was denatured using formamide at 100°C for 5 min. 8) After cooling via ice bath, the cover glass was washed with PBS. The primary antibody, namely anti-mouse BrdU monoclonal antibody (working concentration of 1:50), was added, while PBS or serum was added into negative control, 9) followed by detection using avidin-biotin complex (ABC) method and hematoxylin or eosin staining. The numbers of cells and BrdU-positive cells were counted randomly in 10 high-power fields under a microscope, and the labeling index (LI) was calculated.

Apoptosis analysis via flow cytometry

After different treatments, cells were collected into a centrifuge tube, washed twice with PBS, collected again, and digested moderately with trypsin. Cell digestion in each group was terminated using the old culture solution collected previously. After cells were blown and beaten, they were collected into the centrifuge tube. Apoptosis in each group was detected using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD, San Jose, CA, USA). The spectrophotometric value of the excitation wavelength in each group of cells was measured using a flow cytometer within 1 hr, the apoptosis graph was drawn, and the ratio of early apoptotic cells to late apoptotic cells was analyzed. The experiment was repeated 3 times. The apoptosis rate in each group of cells after different treatments was compared.

Statistics

GraphPad Prism software (Version 5.01, GraphPad Software, Santiago de Chile) was used for analysis. Data in all groups were presented as mean \pm standard deviation (mean \pm SD), and *t*-test was used for the comparison of means between the two experimental groups. The electrophoretic band density value was presented as the ratio of gray value/internal reference. $P < 0.05$ suggested that the difference was statistically significant.

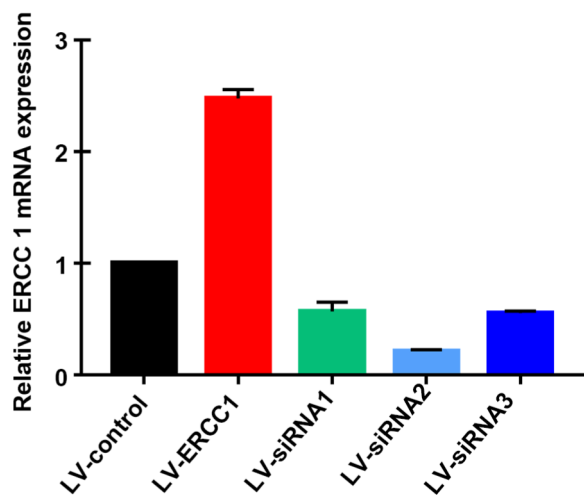


Figure 3. Detection of expression levels of ERCC-1 mRNA in each group via qPCR. The expression level of ERCC-1 mRNA was highest after transfection of LV-ERCC1 and mRNA expression level was lowest after transfection of LV-siRNA2.

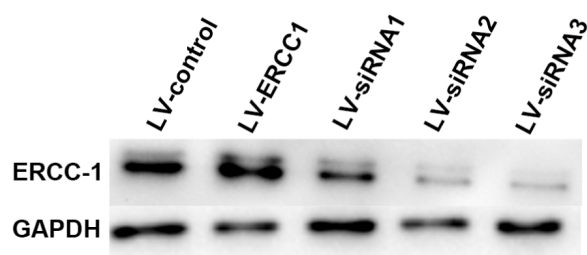


Figure 4. Detection of expression levels of ERCC-1 protein in each group via Western blotting. The expression level of ERCC-1 protein was highest after transfection of LV-ERCC-1 and Cowost LV-ERCC-1 and Cowost after transfection of LV-siRNA2.

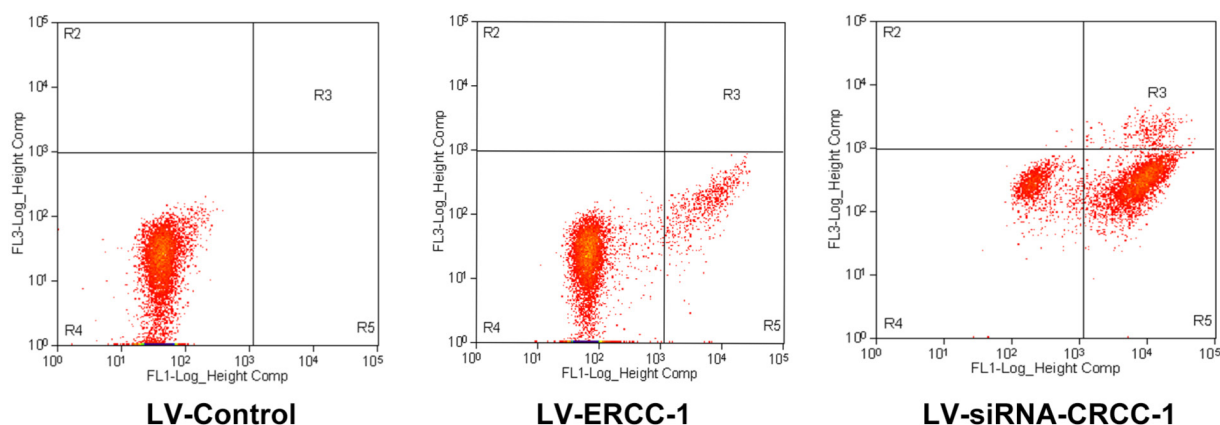


Figure 5. Detection of apoptosis rate in different groups by flow cytometry. The apoptosis rate of overexpressing ERCC-1 group was 44.2% and of ERCC-1 siRNA group was 62.1%.

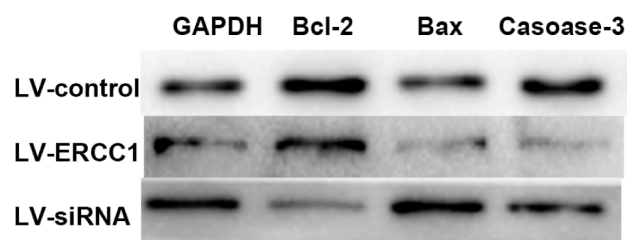


Figure 6. Detection of apoptosis-related indexes in each group of cells via Western blotting. Overexpression of ERCC-1 downregulated the expression Bax and caspase-3 and upregulated the expression of Bcl-2.

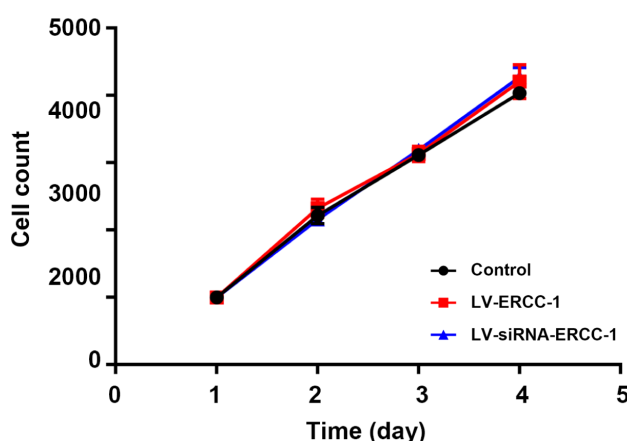


Figure 7. Cell proliferation curve of each group showing no significant differences ($p > 0.05$).

Results

Lentivirus transfection experiment and validation

SKOV3 cells were infected with the virus LV-ERCC-1 with ERCC-1 overexpression, showing green fluorescent protein (GFP) fluorescence, and the cell transfection rate was more than 85% (Figure 1). Three interference sequences including RNA interference 1 (RNAi1), RNAi2 and RNAi3 were constructed, and SKOV3 cells were infected with the virus LV-siRNA-ERCC-1 with ERCC-1 down-regulation and empty vector LV-siRNA-Control, showing GFP fluorescence (Figure 2). qPCR was performed after transfection and the expression levels of ERCC-1 mRNA were compared among groups (Figure 3). The results revealed that the expression level of ERCC-1 in RNAi2 group was the lowest, and the downregulation rate of ERCC-1 in RNAi2 sequence was the highest (85.5%). Western blotting was used to detect protein expression after transfection. The results manifested that the expression of ERCC-1 protein in RNAi2 group was the lowest (Figure 4). Therefore, RNAi2 was selected as the most effective target. A stable cell line was constructed after transfection to carry out subsequent experiments.

Detection of cell apoptosis via flow cytometry

Cell apoptosis was detected by flow cytometry and the results demonstrated that in SKOV3 cells, the apoptosis rate of ERCC-1 overexpression group was lower than that of ERCC-1 silencing group (44.2 vs 62.1%, $p < 0.01$, Figure 5).

Detection of apoptosis index in each group of cells via Western blotting

Apoptosis-related proteins in cells of each group after treatment were detected by Western blotting, and it was found that ERCC-1 overexpression could downregulate the expression levels of apoptotic proteins (Fas and caspase-3), while the expression level of antiapoptotic protein Bcl-2 was increased (Figure 6).

Cell proliferation assay

After 3 days of lentivirus transfection, cell proliferation was observed for 4 consecutive days and the cells were counted. It was discovered that the rates of cell proliferation had no statistically significant differences among ERCC-1 silencing group, ERCC-1 overexpression group and control group (Figure 7).

Discussion

ERCC-1 gene has long been considered as a key factor in the process of NER. Platinum compounds show antitumor activity by causing DNA damage in the cells. A number of randomized controlled trials have indicated that the sensitivity of cancer patients to platinum chemotherapy varies in different people, which may be associated with individual ability to repair DNA damage [13]. ERCC1 gene or its protein expression level is not only a predictor of the efficacy of platinum chemotherapy in breast cancer, gastric cancer and non-small-cell lung cancer [14,15], but also a prognostic factor in non-small-cell lung cancer. High ERCC-1 mRNA expression is associated with disease-free survival (DFS) [16]. However, it has not been reported whether the expression of ERCC-1 gene is associated with prognosis of ovarian cancer. In this study, it was found that the apoptosis rate of ovarian cancer cells was decreased after overexpression of ERCC-1 gene, suggesting that ERCC-1 gene may be associated with apoptosis of ovarian cancer cells. Further clinical and basic studies are needed to prove this assumption.

Fas/Fas ligand (FasL) system plays an important role in the development of tumors. Fas is an O-type transmembrane glycoprotein with 45 kDa molecular weight and FasL is a ligand of Fas an-

tigen. The combination of Fas and FasL can trigger the activation of caspase-8, and then directly activate the downstream effector caspase-3, exerting an apoptotic effect [17]. A previous study used qPCR to demonstrate that the incidence rate of malignancy is associated with low Fas expression [18], and the analysis of clinical samples confirmed that the expression level of Fas in ovarian cancer is low [19,20].

In this study, it was found that the expression level of ERCC-1 was closely related to the expression level of Fas protein, indicating that ERCC-1 can play an antiapoptotic role in ovarian cancer through the Fas/FasL system.

In conclusion, ERCC-1, as a DNA damage repair gene, may play multiple roles in the occurrence and development of tumors. Therefore, further research at different levels is still needed to fully clarify the role of ERCC-1 gene in tumors. It has been preliminarily confirmed in this study that ERCC-1 gene is related to apoptosis of ovarian cancer cells, which provides a theoretical basis

for further mechanism research. At the same time, deep understanding of the effect of ERCC-1 gene on the occurrence, development and metastasis of ovarian cancer is helpful for the diagnosis and gene therapy of early metastasis of tumors.

Authors' contributions

YZ wrote the manuscript. YZ and DZ were responsible for cell culture and transfection. YZ and HW helped with cell proliferation assay and western blot. All authors read and approved the final manuscript.

Ethics approval

The study was approved by the ethics committee of Qihe County People's Hospital.

Conflict of interests

The authors declare no conflict of interests.

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