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ORIGINAL ARTICLE

Expression of ERCC1 protein in biopsy specimen predicts survival in advanced ovarian cancer patients treated with platinum-based chemotherapy

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Summary

Purpose: *The purpose of this study was to investigate whether the expression of excision repair cross complementing 1 (ERCC1) protein I in tumor tissue was associated with resistance to standard carboplatin and paclitaxel (PC) combination chemotherapy in patients newly diagnosed with advanced epithelial ovarian carcinoma (EOC).*

Methods: *Fresh frozen tumor tissue was obtained from EOC patients. The protein expression levels of ERCC1 in tumor tissue were determined by Western blot analysis in 55 samples with advanced and metastatic EOC with different histologic subtypes; then these patients were treated with PC.*

Results: *The results showed that the clinical objective responses were significantly different in different categories of ERCC1 protein expression levels in patients with EOC. Time to progression (TTP) and overall survival (OS) in EOC patients previously treated with platinum-based chemotherapy were significantly longer in those with low expression compared with patients showing high expression of ERCC1 protein.*

Conclusion: *Our results revealed that ERCC1 protein expression could potentially be used to customize chemotherapy by defining subsets of patients who would benefit the least from platinum-based chemotherapy.*

Key words: ERCC1, NER, ovarian cancer, platinum-based chemotherapy

Introduction

EOC is the most lethal gynaecological cancer worldwide [1]. Approximately 70% of ovarian cancer patients present with advanced disease stage after it has spread beyond the genital organs and into the peritoneal cavity FIGO (stage III) or even further (FIGO stage IV, distant metastases). With a 5-year survival about 70% of patients with stage II EOC, OS dramatically decreases in advanced stages of disease (stage III and IV is only about 31% and 13%, respectively [1,2].

The current stage classification of both early and advanced epithelial ovarian requires an extensive surgical assessment performed by an experienced gynaecological oncologist, employing a laparotomy via a vertical substernal-pubic incision to explore the entire abdomen. The Federation Internationale de Gynécologie et d'Obstétrique (FIGO) defined an optimal staging for ovarian cancer as evaluation of all peritoneal surfaces, peritoneal washings, total hysterectomy, bilateral salpingo-oophorectomy, omentectomy, peritoneal biopsies of any suspect lesions for metastases or blind peritoneal biopsies (right diaphragm, right and left paracolic gutter, pelvic side-walls of the ovarian fossa, bladder and *cul-de-sac* peritoneum) in the absence of macroscopic disease. In the presence of peritoneal metastases less than 2 cm in greatest dimension sampling of iliac and paraaortic lymph nodes is recommended on a type C basis [2]. This primary debulking surgery followed by combination chemotherapy with addition of a taxane to a platinum-based regimen improves survival in patients with EOC and the initial response rates are as high as 70-85% [3]. Drug resistance in EOC, believed to result in treatment failure and death in more than 90% of patients with metastatic disease, is caused by numerous contributing factors. Platinum-based therapy is the cornerstone in the treatment of EOC, and the development of tumor resistance to platinum compounds is a major

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therapeutic problem. Although the molecular mechanism of platinum resistance is complex and multifactorial, an important mechanism of resistance is increased tolerance to platinum-DNA damage and enhanced repair of damaged DNA [2,3]. In a variety of malignancies including EOC, enhanced expression of the DNA repair protein ERCC1 has been correlated with resistance to platinum [2-6]. The NER pathway is involved in the DNA repair mechanisms in tumor cells damaged by treatment with platinum compounds, such as cisplatin and carboplatin [4]. The cytotoxic effect of platinum agents is thought to be the formation of platinum intrastrand crosslinks between the DNA strands. Excision of platinum-DNA adducts are performed by NER proteins, that recognize the DNA damage and excise the platinum-DNA adducts from the injured DNA strand. ERCC1 is one of the key proteins in the NER complex [5]. The ERCC1 protein is an excision nuclease and the rate-limiting step in the DNA repair process [6,7]. Thus, preliminary data suggests that the DNA repair protein ERCC1 is a marker of platinum resistance, but its value as predictive marker in EOC is not well established.

Our hypothesis was that ERCC1 protein could act as an indicator for chemoresistance, in order to select patients who would primarily benefit from alternative regimens and chemotherapeutics other than platinum-based combinations. The primary aim of this study was to investigate whether the expression of ERCC1 protein in tumor tissue analysed by Western blot was associated with resistance to standard combination of carboplatin and paclitaxel chemotherapy in patients newly diagnosed with advanced EOC.

Methods

This was a prospective study that enrolled patients with advanced and metastatic EOC with different histological subtypes who had been submitted to platinum-based chemotherapy between June 2006 and January 2009. Fresh frozen tumor tissue was obtained from patients after they gave informed consent. All tumor tissue collected at surgery were histologically diagnosed as EOC and evaluated for ERCC1 expression levels by Western blot analysis; then the patient were treated with PC combination chemotherapy.

Eligibility criteria

Eligible patients had histologically confirmed EOC, with either FIGO stage IV disease or stage II, III disease with at least one mass of residual disease greater than 1 cm in diameter after initial debulking surgery. Patients with borderline tumors and tumors with a non-epithelial component were ineligible.

Other eligibility requirements included normal bone marrow function (granulocytes $1,500 \times 10^9$/L; platelets $>100 \times 10^9$/L), renal function (serum creatinine <1.5 mg/dL), and liver function (ALT, AST, up to 2 times the upper limit of normal; bilirubin < 1.5 mg/dL), and Eastern Cooperative Oncology Group (ECOG) performance status 0-1. Written informed consent fulfilling all institutional regulations was obtained from all patients before entry onto study. Patients with a history of prior malignancy other than basal cell carcinomas or *in situ* cervical carcinoma were ineligible. Primary peritoneal malignancy or lack of tumor in the ovaries rendered a patient ineligible. Before study registration, patients underwent a baseline assessment, which included physical examination, blood counts, serum biochemistry, CA-125 levels, and postoperative computed tomography scan within 3 weeks from operation. The blood tests were to be repeated before each course of chemotherapy.

Treatment

Patients received treatment with PC (paclitaxel 175 mg/m^2 on day 1 plus carboplatin AUC 5 on day 1) every 21 days for a maximum of 6 cycles. Antiemetics to prevent chemotherapy-induced nausea, and premedication with dexamethasone 20 mg i.v., diphenydramine 50 mg i.v. and ranitidine 50 mg i.v. to diminish the risk of paclitaxel hypersensitivity were given to all patients.

Response evaluation

Levels of ERCC1 protein were correlated with TTP, OS, and response to chemotherapy in all patients and in relation to different histological subtypes.

Response rate and recurrences were evaluated according to RECIST criteria, version 1.1 [8]. Complete response (CR) was defined as the disappearance of all clinical and radiological evidence of tumor, partial response (PR) as at least a 30% decrease in the size of a target lesion, progressive disease (PD) as either the appearance of new tumor lesions or at least a 20% increase in the size of the existing tumor, and stable disease (SD) as neither sufficient shrinkage of tumor to qualify for a PR nor sufficient increase to qualify for PD.

An elevation in serum CA-125 for assessment of recurrence was confirmed either by documented disease on clinical exam or measurable disease on CT or ultrasound. TTP was determined from the start of chemotherapy to the time of documented disease progression or recurrence. OS was calculated from the date of surgery to the date of death.

Western blotting

Protein samples were collected in RIPA buffer containing 1X Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) and the protein content was measured using a commercially available protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL) and a Biomate 3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were separated on 8-12% SDS polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA). Blocking was carried out with 5% milk in Tris-buffered saline with Tween-20 (TBS-T). For all subsequent immunoblotting, antibodies were diluted to the appropriate concentration in 5% milk in TBS-T. Blots were incubated with the following primary antibodies for 1 h at room temperature or overnight at 4º C: mouse-anti ERCC1 (1:1000, D-10, Santa Cruz, SantaCruz, CA), and mouse-anti actin (1:5000, Sigma-Aldrich, St. Louis, MO). Following 3 washes in TBS-T, blots were incubated with the appropriate HRP-labeled secondary antibody for 1 h at room temperature. Visualization of protein bands was performed using the Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) and GeneSnap image acquisition system (Syngene, Frederick, MD) [9].

Statistical analysis

In the description of subjects, categorical data were expressed by percentages and continuous variables as mean and standard deviation. Efficacy was analysed for the modified intent-to-treat population (all enrolled patients who fulfilled the inclusion criteria). Differences between categorical variables were tested with univariate x^2 test and Fisher's exact test. Cox proportional hazards model was used to determine the relationship between OS and TTP across ERCC1 levels. The hazard ratio (HR) and 95% confidence interval (95% CI) were reported in separate univariate models for ERCC1. Both OS and TTP were estimated by the Kaplan-Meier method. Survival curves were compared with the log rank test with an alpha level of 0.05 used as the cutoff for statistical significance. Data analysis was performed with SPSS, version 6.1 for Windows, R-plus, S-plus.

Results

Patient characteristics

Patient characteristics are shown in Table 1. The median patient follow-up was 23 months (range 1-52) and the median patient age 56 years (range 30-74). The vast majority of patients had FIGO stage III and IV disease. Papillary serous adenocarcinoma was by far the most common histology of EOC at diagnosis.

We arbitrarily divided the levels of ERCC1 protein in 2 categories: ERCC1 high and ERCC1 low, based on optical density (OD) manifested at the Western blot. There were no significant differences in the clinicopathological parameters, such as age, performance status, histological type, between the ERCC1-high and ERCC1-low categories (Table 2).

Table 1. Patient and tumor characteristics

Characteristics	*Patients, n (%)*
Age, years (range)	56 (30-74)
FIGO stage	
II	3 (5.5)
III	35 (63.6)
IV	17 (30.9)
Performance status (ECOG)	
0	33 (60)
1	22 (40)
Histology	
Serous	34 (61.8)
Mucinous	3 (5.5)
Endometrioid	7 (12.7)
Clear cell	9 (16.4)
Undifferentiated	2 (3.6)
Grade	
I	6 (10.9)
II	28 (50.9)
III	15 (27.3)
IV	6 (10.9)
Total number of metastatic sites	
1	14 (25.5)
2	15 (27.3)
3	11 (20.0)
≥4	15 (27.3)
Localisation of disease	
Ascites	31 (56.4)
Peritoneal carcinomatosis	25 (45.5)
Residual disease	43 (78.2)
Pleural effusion	4 (7.3)
Liver	4 (7.3)
Retroperitoneal	14 (25.5)

Table 2. Distribution of all enrolled patients with EOC according the levels of tissue ERCC1 protein

ERCC1 protein levels	*n*	*%*
ERCC1 high	24	43.6
ERCC1 low	31	56.4
Total	55	100

ERCC1 high (for ERCC1 protein values ≥ 10 000 optical density), ERCC1 low (for ERCC1 protein values < 10 000 optical density)

Efficacy

Association between ERCC1 protein expression and response to treatment

The clinical response to PC chemotherapy was reassessed for all patients as follows: 5 of 24 (20.9%) patients showed CR, and 8 (33.3%) PR in the ERCC1-high group, while there were 18 of 31 (58.1%) patients with CR, and 19 of 31 (29%) with PR in the ERCC1-low group. The objective clinical response rate including CR and PR was 54.2% in the ERCC1-high group and 87.1% in the ERCC1-low group (Fisher's exact test, p=0.01; Figure 1).

The sensitivity to PC chemotherapy was reassessed for all patients as follows: 10 of 24 (41.69%) patients had platinum-refractory EOC, 8 (33.3%) had platinum-resistant and 6 (25%) had platinum-sensitive disease in the ERCC1-high group, while there were 6.7% platinum-refractory EOC, 36.7% platinum-resistant and 56.7% platinum-sensitive EOC in the ERCC1-low group. The results presented on Figure 2 show that the sensitivity to platinum-taxane chemotherapy was significantly different according to different categories of ERCC1 protein expression in patients with EOC (Fisher's exact test, p= 0.00496).

Association of ERCC1 expression with TTP and OS

With a median follow-up of 23 months (range 1-52), median TTP was 13.0 months (95% CI 11-20) and OS 36.0 months (95% CI 22 -52) for the whole study population.

The results presented on Figure 3 show that TTP was significantly longer in the patient group with low expression of ERCC1 protein compared with patients with high ERCC1 expression (log rank, p=0.00).

Figure 4 shows that OS was significantly longer in patients with low expression of ERCC1 protein compared with patients with high expression of ERCC1 protein (log rank, p=0.00).

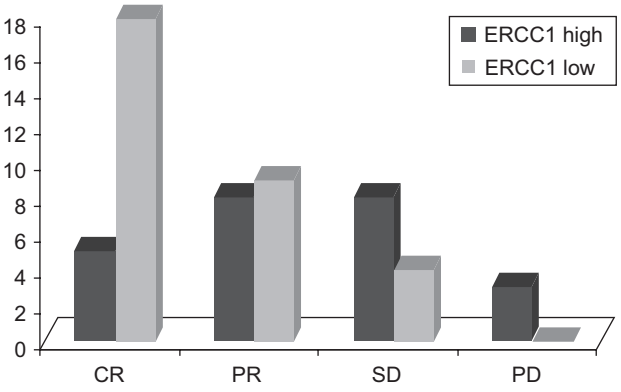

Figure 1. Response to platinum-based chemotherapy according to different ERCC1 groups. CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease (Fisher's exact test, p=0.01).

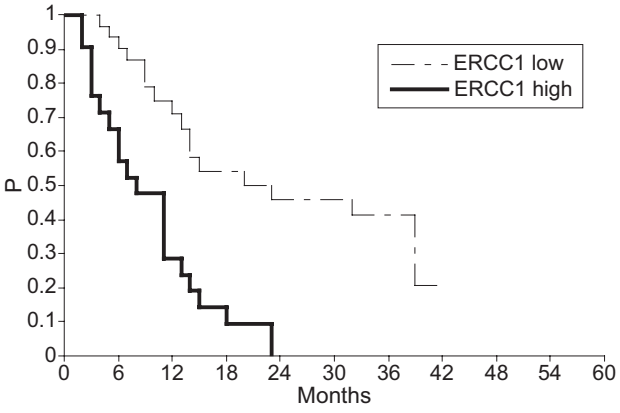

Figure 3. Time to progression in relation to ERCC1 levels (Log rank, p=0.00).

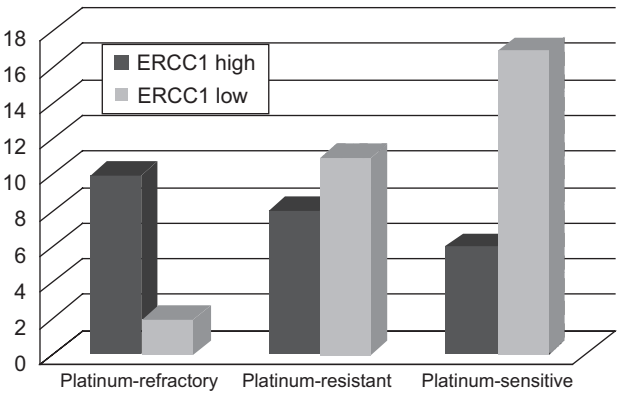

Figure 2. Platinum sensitivity according to different ERCC1 categories (Fisher's exact test, p=0.0049).

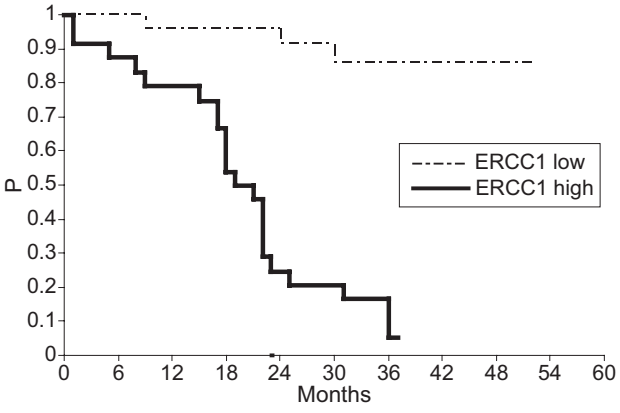

Figure 4. Overall survival in relation to ERCC1 levels (Log rank, p=0.00).

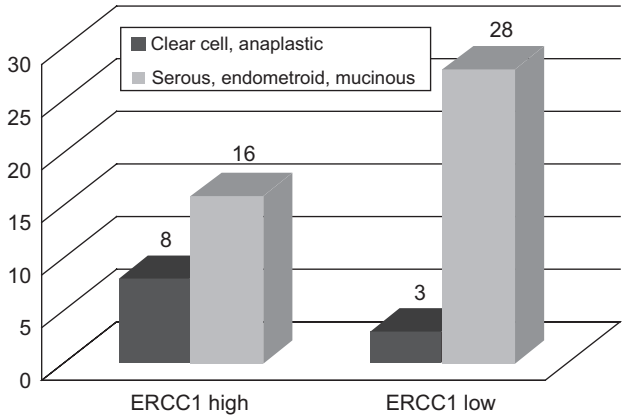

Figure 5. Histological EOC subtypes in relation to ERCC1 levels (Fisher's exact test, p=0.0428).

Cox proportional hazard model showed that high ERCC1 expression (HR: 3.88, 95% CI 1.91-7.88; likelihood ratio test, p=0.000156), was independent prognostic and predictive factor for the prolongation of TTP and response duration (HR: 4.05, 95% CI 1.69-9.72; likelihood ratio test, p=0.00188).

The results presented on Figure 5 show the incidence of the different histological subtypes in relation to ERCC1 levels. Clear cell and anaplastic histological subtypes, as aggressive ones, occurred more frequently in the ERCC1-high group, compared with endometrioid, serous and mucinous subtypes (Fisher's exact test, p= 0.0428).

Discussion

We have analysed the role of ERCC1 protein expression in advanced and metastatic EOC patients treated with platinum/taxane. ERCC1 protein was analysed from fresh frozen ovarian cancer tissue using the Western blot method.

The ERCC1 expression in 55 patients was analysed relative to the expression of the control beta-actin. Statistically significant difference was obtained between ERCC1-high and ERCC1-low group of EOC patients concerning the objective response rate; there were significantly fewer responders in the ERCC1-high group compared with the ERCC1-low group, which is consistent with a previous work indicating a correlation between ERCC1 protein expression and response [10]. High tumor tissue levels of ERCC1 mRNA are associated with reduced response to cisplatin and poor prognosis in various malignancies including ovarian cancer [4,10]. On protein level, a recent report has suggested that nuclear ERCC1 expression is an important marker of resistance to cisplatin [4]. ERCC1 is a DNA repair gene which is crucial in tailoring cisplatin-based chemotherapy. Most of the studies of ovarian cancer have used immunohistochemistry methods in detecting ERCC1 protein, which are qualitative methods, while in our analysis we used Western blot, a semiquantitative, feasible method which provides much more objective and accurate measurements of the protein.

Clear cell carcinoma is clinically characterized by *de novo* resistance to platinum-based chemotherapy. Many authors have reported that for ERCC1 and XPB, clear cell tumors show higher mRNA levels of these two genes than the other histological subtypes we examined. Clear cell tumors of the ovary tend to be diagnosed at earlier stage than most other epithelial cell type tumors. However, when seen in advanced stage, clear cell tumors are notoriously difficult to treat with platinum-based therapy or other DNA-damaging chemotherapy. The data presented in the literature suggests that clear cell tumors may have markedly enhanced DNA repair activity, as compared with other histological subtypes of ovarian cancer. Enhanced DNA repair is the hallmark of cellular resistance to platinum compounds at clinically relevant levels of drug exposure [11,12].

We have shown in a collection of tumor tissues from 55 different individuals that clear cell tumors have higher protein levels of ERCC1 than other common subtypes of EOC. We believe that this lends support that enhanced NER is one major factor that contributes to clinical drug resistance in this disease. Studies using a larger sample size that include clear cell tumors, mucinous tumors, endometrioid and poorly differentiated tumors, with data collected from patients responsive to platinum-based chemotherapy, are warranted.

In this prospective study of a homogeneous group of 55 advanced EOC patients who were treated with postoperative carboplatin and paclitaxel, we have proved that low levels of ERCC1 protein expression were connected with a significantly better TTP and OS. Consistent with other reports [13], our study supports ERCC1 as a predictive biomarker in EOC, when ERCC1 protein expression was represented as a dichotomous variable.

Currently, there are no clinically relevant biomarkers to predict chemosensitivity and overall prognosis in EOC. The serum glycoprotein CA-125 is the only tumor marker that is used clinically to follow response to chemotherapy and disease recurrence [14]. However, baseline CA-125 does not correlate with platinum resistance, overall prognosis or the likelihood of optimal tumor resection [15]. Despite the heterogeneity with respect to the clinical and pathological presentation in EOC, most patients who receive first-line chemotherapy are treated uniformly with platinum and paclitaxel [1]. Thus, there is a need to identify molecu-

lar markers which may influence the therapeutic options based on tumor biology. Reliable predictors of platinum resistance may identify a subgroup of patients that is unlikely to respond to platinum-based treatment, avoid toxicity from unsuccessful chemotherapy and assist clinicians to guide patients to participate in clinical trials.

In this patient cohort, Cox proportional modeling revealed a significant correlation of low ERCC1 expression with a better TTP and response duration, implying that ERCC1 may also be an applicable predictive marker in EOC. Several preclinical studies have suggested that the expression levels of the ERCC1 mRNA are related to platinum resistance. In ovarian cancer cell lines, a 3-fold higher expression of ERCC1 mRNA was correlated with cisplatin resistance [16] and in 2 other ovarian cancer cell line studies, a positive association between the level of ERCC1 expression and the amount of cisplatin-DNA adduct repair and reduced sensitivity to cisplatin was reported [17,18]. These preclinical data suggest a potential use of ERCC1 as a molecular predictor of resistance to platinum-based chemotherapy. Nevertheless, the literature of a possible clinical implication of ERCC1 levels is limited.

NER is a complex system that repairs DNA damage caused by cisplatin; however, evidence is accumulating to suggest that the measurement of the level of ERCC1 protein alone can predict the response of cells to cisplatin, and correlates well with NER activity [7]. These observations might be due to the coordinated regulation of the transcription of related proteins; thus, the measurement of the expression of one such protein is a good surrogate for the expression of the others. In the only prospective randomized clinical trial testing customized chemotherapy in advanced non small cell lung cancer, ERCC1 mRNA predicted the response to cisplatin-based chemotherapy in 346 patients [19]. Overall, the effect of ERCC1 as a biomarker of prognosis is not yet conclusive in lung cancer and there is comparatively little data available in EOC.

There are several limitations in our prospective analysis that merit consideration, although it should be noted that the results were obtained on a small number of patients. Our study is unable to address whether ERCC1 protein expression levels are prognostic, i.e. the ability of this marker to determine the clinical outcome independent of the treatment given.

The present study provides additional evidence to support the tumor ERCC1 protein levels, as having value as predictive markers in EOC. The data from this and other studies indicates the need to further evaluate this marker in a prospective trial in order to achieve better treatment control and optimizing therapeutic strategies based on the expression of this protein.

Conclusion

Our results indicate that the protein product of DNA repair genes may play an important role in the prognosis of advanced stage ovarian cancer patients. Analysing ERCC1 protein expression levels could potentially be used to personalise chemotherapy by defining subsets of patients who would benefit the least from platinum-based chemotherapy. Those with high ERCC1 levels would be the ideal target for new agents. Conversely, those with low levels may attain a better outcome with standard cisplatin-based regimens. Modifying chemotherapy on the basis of chemosensitivity prediction may improve the outcome in advanced ovarian cancer patients.

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