Initial Activation Status of the Antioxidant Response Determines Sensitivity to Carboplatin/ Paclitaxel Treatment of Ovarian Cancer

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Abstract. *Background/Aim: Ovarian carcinoma is the main cause of gynecological cancer related deaths. The aim of this study was to determine the activation status of the antioxidant response in samples of ovarian serous carcinoma from paraffin-embedded biopsies and compare them with the response of patients to carboplatin-paclitaxel treatment. Materials and Methods: Estrogen receptor alpha (ERα), antioxidant enzymes, and uncoupling protein (UCP) levels were analyzed by western blotting and the presence of estrogen receptor beta (ERβ) was investigated by immunohistochemistry (IHC). Results: Lower levels of ERα, antioxidant enzymes and UCPs were found in patients resistant to treatment in comparison to the carboplatin/paclitaxel-sensitive ones; IHC revealed a greater presence of ERβ in sensitive patients. Conclusion: These results indicate that patients resistant to treatment have a lower level of antioxidant response activation compared to sensitive patients, fact which may be related to the efficacy of this treatment.*

Ovarian epithelial cancer is the fifth cause of cancer-related deaths for European women (1). Moreover, ovarian carcinoma is the leading cause of gynecological cancer-related death (1).

Ovarian cancer represents approximately 3% of all of gynecological carcinomas. It is mainly a post-menopausal disease that usually appears in patients between the ages of 65-69 years. By diagnosis, 75% of patients already have advanced stage of the disease, and this is due to poor early detection techniques and the asymptomatical nature of the early stages of the disease. The overall rate of survival at five years for patients with advanced disease is 20-30% (1).

The current standard treatment for ovarian cancer involves carboplatin, followed by the administration of paclitaxel. Although more than 80% of patients respond to initial treatment, in approximately 75% of cases, there is a relapse within the first two years after treatment, which is very likely to be incurable (1).

Carboplatin [*cis*-diamine-(1,1-cyclobutanedicarboxylate) platinum (II)] is used as a cancer treatment for many types of carcinomas, such as small-cell lung cancer, ovarian cancer, head and neck carcinomas (2). The antitumor action of carboplatin is *via* DNA alkylation, which causes cancer cell death (2).

Paclitaxel is one of the best-known chemotherapeutic agents, widely used in anticancer therapy. The site of action is located at the microtubules. The primary effect on cells is abnormal stabilization of the microtubule polymerization dynamics, and this leads to mitosis failure. In addition, paclitaxel alters other cellular functions related to microtubules, such as intracellular signaling, and organelle transport and locomotion (3). It is effective against several human tumors types, including ovarian carcinomas. The efficacy of paclitaxel is limited by the acquired or intrinsic resistance of the population of malignant cells surviving the treatment (2).

Treatment of carboplatin with paclitaxel can cause oxygen free radical formation and, consequently, the appearance of oxidative stress (4-8). Reactive oxygen species (ROS) act as potent mutagens, increasing genomic instability and contributing to cancer progression (9). Estrogen receptors (ERs), alpha (ERα) and beta (ERβ), may have a modulating role in oxidative stress (9, 10). It has been described that the presence of ER in estrogen-dependent tissues results in an increase in ROS levels (10). In regards to the relationship between ERs and ovarian cancer, it has been suggested that

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there is a link between ovarian cancer and gonadal receptors (11), and this has been demonstrated by the discovery of both mRNA and proteins of ERs in normal ovarian tissue and malignant ovarian tumors (11).

The objective of this study was to determine the oxidative stress status in formalin-fixed paraffin-embedded biopsies of ovarian cancer in comparison to the sensitivity of patients for the carboplatin-paclitaxel treatment. Specifically, the levels of antioxidant enzymes, uncoupling proteins (UCPs), and oxidative damage to lipids and proteins were analyzed, in addition to assessing the ER levels.

Materials and Methods

Reagents. Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich (St. Louis, Missouri, USA) and Panreac (Barcelona, Spain).

Study subjects and treatments. Tissue samples from twelve women with papillary serous cystadenocarcinoma, the most common form of ovarian cancer, were obtained from the Biological Specimen Bank of Son Llàtzer Hospital. These samples were added from 2003 to 2008, and were collected, stored and analyzed in accordance to permits from the Balearic Island Bioethics Committee. The individuals from this sampling group had similar age, weight, height and Body Mass Index (Table I). Any treatment or therapy was given after the completion of the tumor biopsies that were used for obtaining the data for this article. According to the standard guidelines, maximal surgical effort was attempted in all patients, resulting in complete resection in all cases. All patients received platinum-based chemotherapy [(AUC CA125)=5 for carboplatin, per cycle], and paclitaxel (175 mg/m^2 for each cycle). Disease recurrence was defined according to the GCIG CA125 criteria (12) and/or radiological confirmation of tumor progression. Chemosensitivity was defined by the common definition of platinum resistance, and patients were designated as 'sensitive' when they had experienced disease relapse 6 months or more after prior platinum-containing chemotherapy, while 'resistant' referred to those patients who had either experienced relapse in fewer than 6 months after the end of chemotherapy, or whose disease had progressed while on therapy (13).

Immunohistochemistry. Immunohistochemistry was performed with the BenchMark ULTRA (Ventana Medical Systems, Inc., Tucson, AZ, USA). Immunohistochemical staining was carried out on formalin-fixed paraffin-embedded (FFPE) with the ERβ antibody [Mouse monoclonal (988), Abcam, Cambridge, UK], dilution 1:50. Sections were evaluated independently by two observers who were unaware of clinicopathological characteristics of patients according to staining localization (nuclear or/and cytoplasmic) and intensity.

Protein extraction. Protein extracts were obtained from formalin-fixed tissues. For protein extraction, the method described by Addis *et al*. (14) was used. Briefly, two replicates of 10-μm thick microtome sections of FFPE tissue were placed in 1.5 ml Eppendorf safe-lock tubes (Eppendorf, Hamburg, Germany) and de-paraffinized by three incubations in HistoClear II (National Diagnostics, Atlanta, GA, USA) for 10 min at room temperature. After each incubation, the tissue was pelleted at 11,900 ×*g* in a microcentrifuge for 2 min. The de-paraffinized tissue pellets were then rehydrated with graded series of ethanol (100%, 96% and 70%). Then, 100 μl of extraction buffer [20 mM Tris HCl (pH 8.8), 2% sodium dodecyl sulfate (SDS), 4 mM dithiothreitol (DTT), 10 μM leupeptin and 10 μM pepstatin] was well-mixed with the samples. After that, the samples with extraction buffer were left to incubate for 5 min on ice and were then subjected to high-temperature extraction at 100˚C for 20 min and then at 80˚C for 2 h with shaking. Finally, samples were placed for 1 min at 4˚C and centrifuged at 14,000 ×g for 15 min, at 4˚C. The resulting supernatant was placed into a new tube and the protein content of each sample quantified using the BCA kit (Pierce, Bonn, Germany).

Table I. *Patients' anthropometric data*.

	Sensitive	Resistant
Age (years)	55.0±4.8	55.7±4.8
Weight (kg)	61.7±6.8	65.8±7.3
Height (cm)	157±3	157±2
BMI (kg/m^2)	24.9±2.0	26.8±2.9

Body mass index (BMI) is the body weight (kg) divided by the square of the height (m). Data are represented as the mean±SD and there are no significant differences between the two groups (p>0.05; n=6).

Western blots. For western blot analysis, 40 μg of protein from protein extracts was fractioned by SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. Antisera against ERα (66 kDa; dilution 1:200) and glutathione reductase (GRd) (53 kDa; dilution 1:500) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); glutathione peroxidase (GPx) (22.5 kDa; dilution 1:200) and catalase (60 kDa; dilution 1:1000) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany); UCP2 (32 kDa; dilution 1:500), UCP5 (36 kD; dilution 1:1000) and 4-hydroxynonenal (4-HNE), (dilution 1:200) were purchased from Alpha Diagnostic International (San Antonio, Tx, USA); and Oxiselect (antisera against dinitrophenyl [αDNP]; dilution 1:2000) were purchased from Cell Biolabs (San Diego, CA, USA). All the above were used as primary antibodies. Protein bands were visualized by the Immun-Star Western C kit reagent western blotting detection system (Bio-Rad, Hercules, California, USA). The chemiluminescence signal was captured with a Chemidoc XRS densitometer and analyzed with the Quantity One software (Bio-Rad).

Statistical analysis. All data are expressed as means±SD. Statistical analysis was carried out using the statistical program SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical differences between experimental groups were analyzed with unpaired Student's *t*-tests. A level of p<0.05 was accepted as significant.

Results

Patients with disease resistant to treatment with carboplatin and paclitaxel had 30% lower levels of ERα in comparison with patients with non-resistant disease (Figure 1).

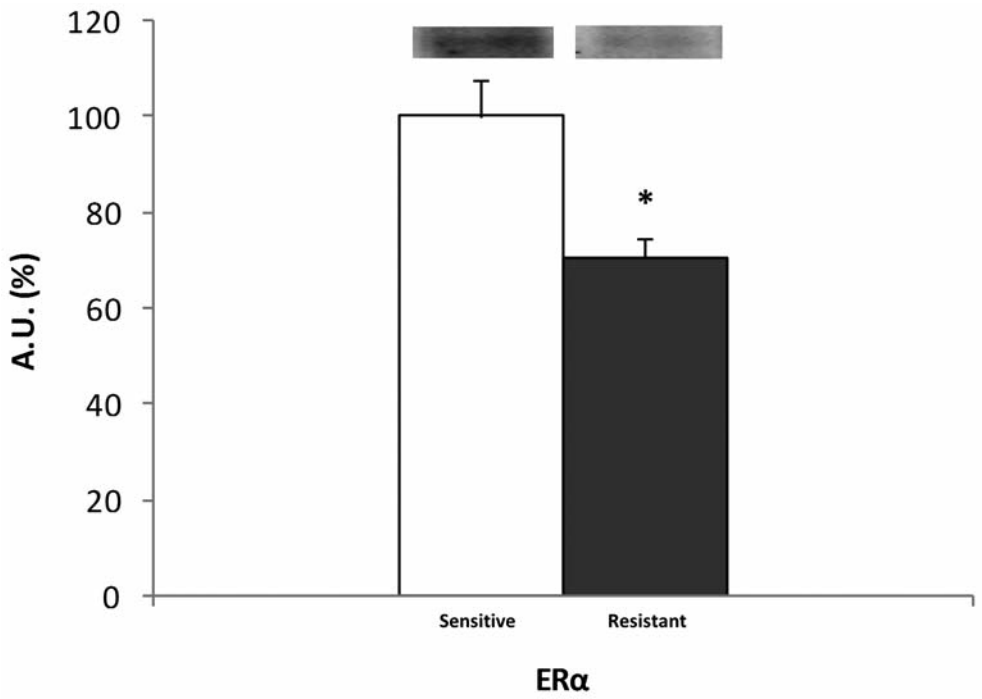

Figure 1. *Estrogen receptor alpha (ERα) protein levels in tumor samples, sensitive and resistant to treatment, as measured using western blot assays. The ERα protein levels in resistant samples were lower than those in sensitive samples (*p<0.05; mean±SD; n=6). A.U.: Arbitrary units.*

For the antioxidant enzymes, Table II shows the tumor levels of catalase and GRd, which were significantly lower in the resistant cases, while levels of GPx did not show any significant variation between the two studied groups.

Likewise, Figure 2 shows that levels of UCP2 were lower in resistant samples than in the treatment-sensitive ones, while for UCP5, the levels in the resistant samples were significantly lower than in the sensitive ones.

Oxidative damage in both protein and lipids was not significantly different between the two experimental groups (Table III), despite resistant ovarian tumors presenting lower levels of protein carbonyl adducts.

Immunohistochemical analysis of ERβ showed more staining and increased cytoplasmic staining of this receptor in patients who did respond to treatment compared to those that did not (Figure 3). From these results and those obtained by western blotting, patients who did have a response to treatment of carboplatin and paclitaxel had higher levels of both ERs.

Discussion

This study demonstrates that the initial condition of activation of the antioxidant response in ovarian cancer could be related to sensitivity to treatments such as carboplatin and paclitaxel, which act by increasing oxidative stress.

Table II. *Catalase, glutathione peroxidase (GPx) and glutathione reductase (GRd) protein levels in tumor samples, sensitive and resistant to treatment, measured using western blot assays. Next to each numerical value is the image of the corresponding western blot. Data are represented as mean±SD. There was significantly less catalase and GRd in the sensitive group than in the resistant (*p<0.05; n=6). However, for GPx levels, there is no significant difference between the groups (p>0.05; n=6).*

	Sensitive	Resistant
Catalase (A.U. %)	100±13	55.8±3.7*
GPx (A.U. %)	100±32	72.8±28.7
GRd (A.U. %)	100±15	60.1±6.9*

A.U.: Arbitrary units.

Table III. *Protein carbonyl and 4-hydroxynonenal (4-HNE) content in tumor samples, sensitive and resistant to treatment, as measured using western blot assays. Data are presented as the mean±SD, and there are no significant differences between groups (p>0.05; n=6).*

	Sensitive	Resistant
Protein carbonyl (A.U. %)	100±27	68.7±24.3
4-HNE (A.U. %)	100±22	104±3

A.U.: Arbitrary Units.

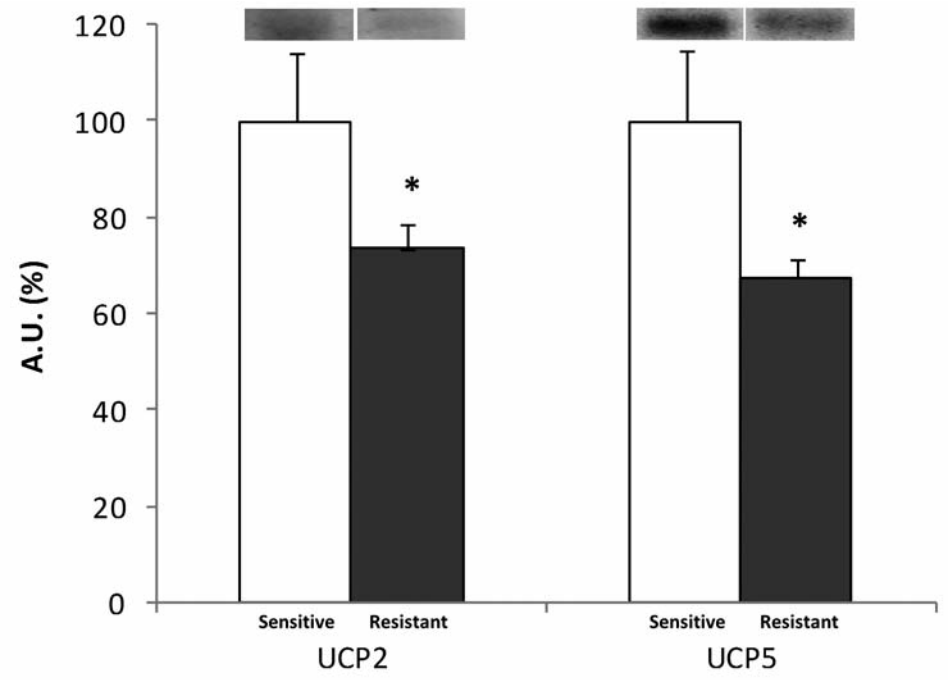

Figure 2. *Protein levels of uncoupling protein 2 (UCP2) and uncoupling protein 5 (UCP5) in tumor samples, sensitive and resistant to treatment, as measured using western blot assays. Levels of both UCPs in resistant samples were lower than those in sensitive ones (*p<0.05; mean±SD; n=6). A.U.: Arbitrary units.*

Specifically, it has been observed that patients with resistant disease have levels of oxidative damage to proteins and lipids, similar to those of sensitive patients, but with a lower activation of the antioxidant response. These treatment-resistant patients have lower levels of antioxidant enzymes and UCPs (UCP2 and UCP5). This could also be related to a lower presence of ERα and ERβ, and the oxidative stress balance in patients with resistant disease.

Both carboplatin and paclitaxel have been associated with oxidative stress induction (4-8). One of the effects produced by treatment with carboplatin is free radical formation, leading to the onset of oxidative stress (5, 7). Alexandre *et al*. (4) found that treatment with paclitaxel induced the release of ROS to the extracellular medium, increasing the apoptosis of all cells in the vicinity of treatment administration. Recently, Panis *et al*. (8) observed an increase in lipid peroxidation in patients treated with paclitaxel, which may indicate that this treatment also produces oxidative stress. Moreover, treatment with paclitaxel induced cytotoxic stress in ovarian cancer cells (6). Furthermore, the same authors have described that cell lines sensitive to paclitaxel treatment had a lower sensitivity when an antioxidant was added at the same time as the treatment (6). Accordingly, one of the mechanisms underlying treatment with carboplatin-paclitaxel is oxidative stress induction, which leads to apoptosis of cancer cells. Tamarit *et al*. showed an implication of ROS and oxidative damage in the induction of apoptosis (15). Other studies have demonstrate that the apoptotic effects of ROS have been drastically mitigated by pretreatment with intracellular ROS scavengers, indicating that intracellular ROS generation is responsible for apoptosis (16).

Regarding oxidative stress status, it has been shown that fall off in the levels of reduced glutathione (GSH) are accompanied by increased levels of ROS during apoptosis (17), which could explain why in patients sensitive to treatment levels of glutathione reductase increased, in an attempt to restore the GSH level. Despite this, it has been found in the MCF7 breast cancer cell line, which has a characteristic overexpression of catalase, that there is a rise in resistance to pro-oxidant treatments (18).

Recently, ERα and ERβ have been associated with a modulatory role of oxidative stress in other types of tumor (9, 10). Our results show that the tumor of treatment-resistant patients had lower levels of ERα and ERβ than sensitive ones. These results are similar to those obtained in previous work (19), where there was a higher presence of both receptor types in non-cancerous ovarian tissue compared to malignant ovarian tumors. Overall, our results regarding the presence of ERβ in ovarian cancer are in line with previous studies, in which this receptor had a lower presence in malignant tumors in comparison to normal tissue (20, 21).

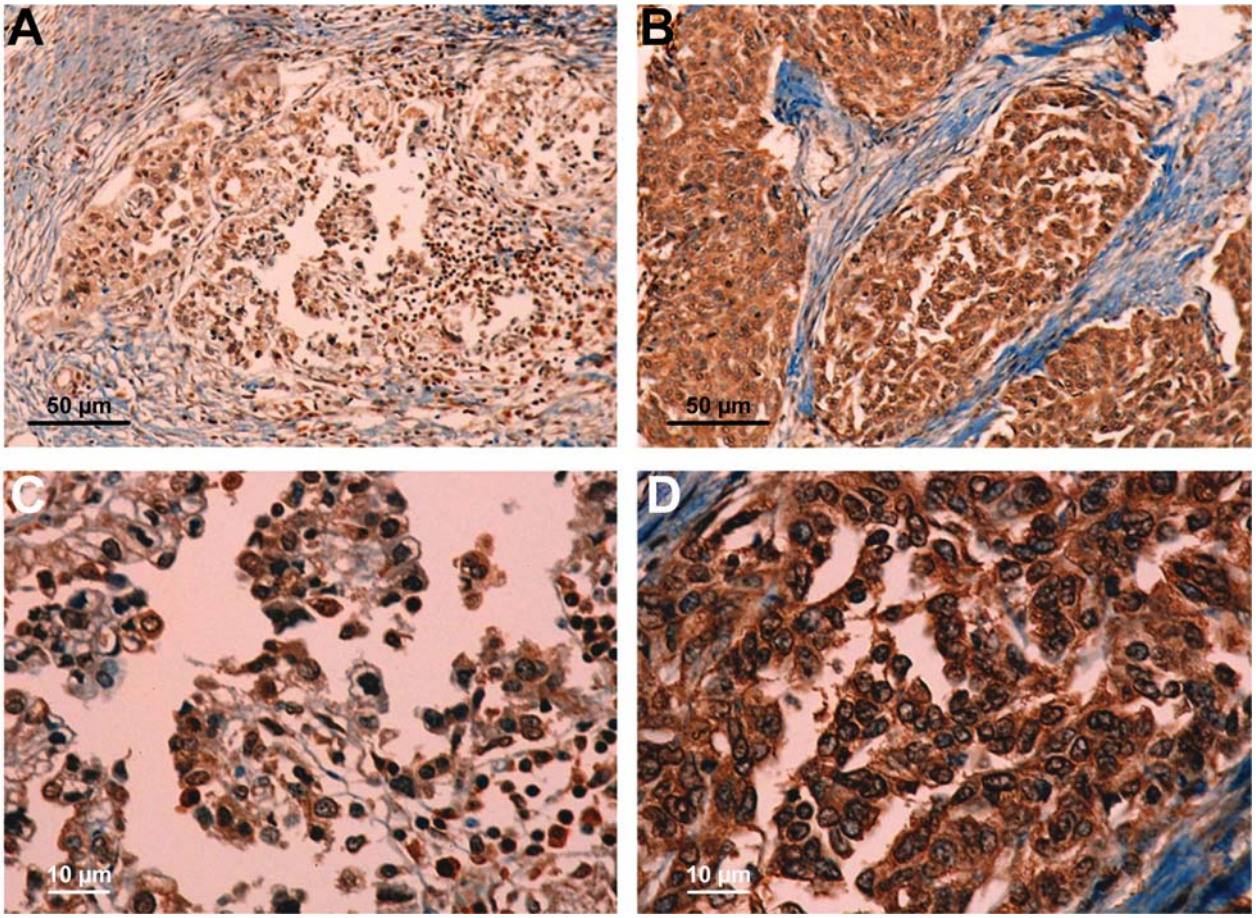

Figure 3. *Representative photographs of estrogen receptor beta immunostaining, showing expression in resistant (A and C) and sensitive ovarian cancer tissue samples (B and D).*

Bardin *et al*. (20) shows that ERβ had a different cytoplasmic localization in the two groups. Thus, sensitivity to treatment might be related to the more positive immunohistochemical staining of ERβ, localized in the cytoplasm. However, other authors have linked cytoplasmic ERβ staining with reduced patient survival (22).

There is also a controversy about the relationship between the presence of ERs and sensitivity to treatment for other tumor types. Thus, it has been described that ER^- breast cancer cell lines have a poorer response to treatment with paclitaxel than do ER^+ cell lines (23). However, other authors have found that a greater presence of ERs reduces the efficacy of paclitaxel in endometrial (24) and breast cancer (25).

In 2003, Rousset *et al*. (26) discovered the presence of UCP2 in the reproductive tract of female mice. Recently, Liu *et al*. (27) described the presence of UCP2 in human ovarian tissue. In this article, UCPs show the same pattern as do the classic antioxidant enzymes, with lower levels in patients resistant to treatment. Further reinforcing their antioxidant role, it should be noted that UCPs have been associated with a decrease of oxidative stress level in breast cancer cell lines (28).

In conclusion, the results of this study indicate that patients sensitive to treatment have higher initial levels of antioxidant enzymes and UCPs than do patients with resistant disease, despite tumor in both groups having a similar carbonyl content and lipid peroxidation. For patients with sensitive disease, treatment administration generates oxidative stress which proves to be unbearable, and tumor cells consequently respond to treatment. In contrast, in resistant cases the oxidative stress generated is not enough to remove cancer cells, because they had a lower initial antioxidant response, giving them more leeway to respond to stress caused by treatment.

This work highlights the importance of the study of oxidative stress in regard to the evolution of ovarian cancer and its relationship to standard treatment of these tumors. Further studies are required on oxidative stress, the balance

of free radicals and antioxidant defenses, and the supply of ERs in both cell lines and tumor biopsy samples.

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