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Predictive markers of chemoresistance in advanced stages epithelial ovarian carcinoma

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HIGHLIGHTS

- Kinetic of biomarkers during chemotherapy was associated with prognosis.
- Reduced expression of CD44 was associated with shorter survival.
- Increased expression of IL-8 was associated with shorter survival.

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ABSTRACT

Objective. DNA repair mechanisms, environment-mediated drug resistance and cancer initiating cells (CIC) are three major research concepts that can explain the chemoresistance of epithelial ovarian cancer (EOC). The objective was to test if changes in the expression of potential markers associated with drug resistance before and after chemotherapy would correlate with platinum resistance, defined as a recurrence within the first year after chemotherapy cessation, and with survival, in advanced EOC.

Methods. We included 32 patients with stage IIIC–IV EOC who underwent laparoscopy to evaluate the extent of carcinomatosis, neoadjuvant chemotherapy (carboplatin/taxol) and interval surgery. Biopsies taken during the initial laparoscopies and interval surgeries were evaluated using immunohistochemistry for the expression of 7 proteins: CD117, CD44 and ALDH1 to evaluate CIC; IL-6, IL-8 and BMP2 to evaluate environment-mediated drug resistance; and ERCC1 to evaluate DNA repair. Expression measurements were correlated with platin resistance and survival. The markers' relevance was confirmed *in vitro* using chemoresistance tests and flow cytometric measurements of the proportion of CD44+ cells.

Results. 17 patients were chemoresistant and 15 patients were chemosensitive. We observed increases in CD44, IL-6 and ERCC1 expression and stable ALDH1, CD117, IL-8, and BMP2 expression. Reduced expression of cancer initiating cell markers and increased expression of environment-mediated drug resistance markers were associated with poor prognosis. We also demonstrated that CD44+ cells had survival advantages *in vitro*.

Conclusions. Changes in CD44 and IL-8 expression on tumor cells appeared to correlate with overall survival and should be further tested as predictors of chemoresistance using larger cohort.

Introduction

Worldwide, ovarian cancer is the sixth most common cancer in women, with more than 230,000 new cases per year [1]. The mortality is high, with 141,000 deaths per year and a 5-year survival rate of 44.4% (35% for stage III and 22% for stage IV disease) [1]. This poor prognosis is partially explained by the fact that 75% of patients are

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diagnosed at advanced stages (FIGO classification stage IIIC or IV) [2,3]. Therapeutic management of advanced epithelial ovarian cancer (EOC) is usually based on complete cytoreduction surgery (debulking), followed by chemotherapy. When a complete cytoreduction is not feasible or reasonable because of the morbidity induced by extensive surgery, neoadjuvant chemotherapy can be proposed, followed by debulking surgery after 3 or 4 courses of chemotherapy, without impairing the prognosis [4]. In this alternative approach, the first surgery, usually a laparoscopy, has the following objectives: to confirm the EOC diagnosis with biopsies and to evaluate the possibility of an initial complete surgical cytoreduction. Then, adjuvant chemotherapy is performed. The first-line chemotherapy for EOC is a combination of carboplatin (an alkylating agent) and paclitaxel (an antimicrotubule agent) [5,6], which induces an initial response in 65 to 80% of patients [7]. However, the majority of patients who respond to primary chemotherapy will develop recurrent disease. From a practical standpoint, when the relapse occurs during the first year after chemotherapy cessation, the patient is said to be "platinum resistant," and a different line of chemotherapy must be considered. Conversely, when the relapse occurs beyond the first two years after chemotherapy cessation, the patient is said to be "platinum sensitive". The exact disease-free period that defines platinum sensitivity remains varied in the literature [8].

Three major research concepts can facilitate an understanding of EOC chemoresistance. The first concept is the tumor cell as an autonomous entity that is subject to stochastic mutations and selection pressure by chemotherapy. Tumor cells might employ DNA repair mechanisms such as nucleotide excision repair (NER) to escape platinum-induced apoptosis. Therefore, the enzyme ERCC1 is a key protein in platinum resistance [9]. Second, Meads et al. oppose the concept of *de novo* resistance such as environment-mediated drug resistance (EMDR), compared to the acquired resistance that develops over time as a result of sequential genetic changes that ultimately culminate in complex therapy-resistant phenotypes [10]. According to this model, the tumor cells are surrounded by a specialized stroma that induces transient EMDR through soluble factors (IL-6, IL-8 and BMP2) and adhesion between tumor cells and stromal fibroblasts or extracellular matrix components [11–13]. Third, the hypothesis of cancer initiating cells (CIC) within the tumor could partially explain chemoresistance. These cells are defined by the functional properties of self-renewal and differentiation. In EOC, CIC have been characterized by cell surface markers (CD44, CD117, CD133) or functional properties (label-retaining cells, side population, ALDH1 activity) [14–18]. CIC remain mostly in a dormant state and thus escape conventional chemotherapies that target cycling cells. All of these models help to explain the survival of a small, chemoresistant contingency of cells during chemotherapy, also known as residual disease, which is responsible for recurrences.

We hypothesized that a dynamic analysis of the expression changes of potential markers associated with drug resistance (MADR) before and after chemotherapy would predict platinum resistance. The principal objective was to identify chemoresistance-predictive markers in advanced EOC patients who were treated with neoadjuvant therapy. The secondary objective was to identify a cell population enriched after chemotherapy both *in vivo* and *in vitro* by selecting markers from the three main approaches to chemoresistance: the stochastic autonomous tumor cell model, cancer initiating cells and the EMDR.

Materials and methods

Patients

From our pathology database, we first selected 298 women who were diagnosed with epithelial ovarian carcinoma between January 2004 and July 2011 at Tenon Hospital, Paris. Firstly, the patients underwent exploratory laparoscopy before chemotherapy to evaluate the extent of the carcinomatosis. Debulking surgery was performed when complete cytoreduction was feasible. When it was not feasible due to the extent of the carcinomatosis and the morbidity associated with a complete resection, the patients received 4 courses of platinum (carboplatin) and paclitaxel-based neoadjuvant chemotherapy. Upon completing neoadjuvant chemotherapy, the patients underwent a second exploratory laparoscopy, followed by an interval debulking surgery if possible. Among these women, we selected 64 stage IIIC or IV patients (FIGO classification) who had been treated with neoadjuvant chemotherapy [2]. The availability of paraffin-embedded tissues collected at diagnosis and after neoadjuvant chemotherapy was mandatory. Women who did not receive neoadjuvant therapy were excluded (n = 234), along with those for whom one of the two tissue samples was missing (n = 32). Therefore, the final cohort included 32 patients who received 3 to 6 courses of neoadjuvant carboplatin/taxol chemotherapy. The pathological diagnoses and sample representativeness of all selected patients were verified on hematoxylin–eosin–safran (HES)-stained slides by a referent gynecological cancer pathologist (AC). The medical charts were reviewed to collect demographic data such as age, gravidity, parity, menopausal status and hormonal treatment for menopause, and BMI, as well as surgical findings (FIGO staging and completeness of cytoreduction score), biological findings (CA 125 at diagnosis and debulking), and pathological findings (histologic subtype, Silverberg grade, lymphovascular involvement) [19,20]. During follow-up, a patient was said to be chemoresistant when the cancer progressed under chemotherapy or when a relapse occurred during the first year after chemotherapy cessation.

Immunohistochemistry

For each patient, we selected the most relevant tumor paraffin blocks from both before and after chemotherapy. The paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol. Next, we immunostained the samples for the most potentially relevant MADR, including CD117, CD44, ALDH1, IL-6, IL-8, BMP2 and ERCC1. Automated staining was performed with a Ventana Benchmark XT (Kit UltraView, Universal DAB-Ventana, Ventana, Arizona, USA) and anti-CD117 (rabbit polyclonal to CD117, ref. A4502, Dako, California, USA), anti-CD44 (mouse monoclonal DF1485 to CD44/HCAM, Menarini, Italy), anti-ALDH1 (mouse polyclonal to ALDH1A1, ref. H00000216-AP11, Tebu-bio, Taiwan) and anti-BMP2 (mouse monoclonal to BMP2, clone 65529.111, ref. Ab6285, Abcam, UK) primary antibodies. Manual staining was performed with the Dako Envision + Dual Link System-HRP (DAB+) kit and anti-IL-6 (mouse monoclonal to IL-6, ref # Ab9324, Abcam, UK), anti-IL-8 (mouse monoclonal to IL-8, clone 807, ref. Ab18672, Abcam, UK) and anti-ERCC1 (rabbit monoclonal to ERCC1, clone EPR7277, ref. Ab129269, Abcam, UK) primary antibodies. The protocols are detailed in the Supplementary Fig. S1 (table describing immunohistochemistry protocols). All slides were counterstained with hematoxylin. The specimens were mounted and coverslipped with an aqueous-based mounting medium (AquaMount, Thermo Scientific). Immunostaining specificity was controlled with a control antibody (same isotype, dilution and incubation time as the primary antibody) and a positive tissue control.

Optical microscopy

Microscopic analysis was performed on a Nikon Eclipse 90i microscope (Nikon, Nikon Instruments B.V., France). HES and immunochemistry slides were matched and anonymized to permit identity blinding with regard to the patient clinical data and the sample type (before or after chemotherapy). Tumor cells were first identified on the HES-stained slides and then on the immunohistochemistry slides. Representative photographs (20× magnification) of tumor