HER2 Status in Ovarian Carcinomas: A Multicenter GINECO Study of 320 Patients

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Background. Despite a typically good response to first-line combination chemotherapy, the prognosis for patients with advanced ovarian cancer remains poor because of acquired chemoresistance. The use of targeted therapies such as trastuzumab may potentially improve outcomes for patients with ovarian cancer. HER2 overexpression/amplification has been reported in ovarian cancer, but the exact percentage of HER2-positive tumors varies widely in the literature. In this study, HER2 gene status was evaluated in a large, multicentric series of 320 patients with advanced ovarian cancer, including 243 patients enrolled in a multicenter prospective clinical trial of paclitaxel/carboplatin-based chemotherapy.

INTRODUCTION

Because symptoms are usually absent, 70 to 80% of patients with ovarian cancer will have advanced disease at the time of diagnosis [1]. Despite an initial good response to first-line combination chemotherapy (taxane/platinum), relapses are frequent because of acquired chemoresistance. The use of new targeted therapies that are potentially effective in a subset of patients may be of great value.

HER2 (human epidermal growth factor receptor-2) proto-oncogene encodes a protein belonging to the EGFR tyrosine kinase receptor family. Overexpression of HER2 imitates intracellular signaling pathways involved in cell proliferation, differentiation, migration and apoptosis [2]. In breast cancer, HER2-positive status is associated with a poor prognosis [3], and also identifies patients who could benefit from anthracycline-based regimens [4].

Trastuzumab (Herceptin®, F. Hoffmann-La Roche, Basel, Switzerland) is a humanized monoclonal antibody that targets the HER2 extracellular domain and inhibits HER2-positive tumor cell proliferation. It is effective alone and in combination with chemotherapy in patients with breast cancer whose tumors express high levels of HER2 protein. The benefits of trastuzumab have been demonstrated in both metastatic and adjuvant treatment settings [5–8].

Accurate evaluation of HER2 status is essential for optimal patient selection for trastuzumab. Among the numerous methods published, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are the most widely used and have high reported concordance [9–10]. FISH has been shown to more accurately select patients than IHC, but is more costly and not routinely available. The recommended algorithm for HER2 determination in breast cancer is to use IHC initially, using a semi-quantitative scoring system followed by FISH for 2+ ambiguous samples [11–14].

METHODS

Participants

Three hundred and twenty patients with advanced primary ovarian carcinomas (International Federation of Gynecology and Obstetrics

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[FIGO] stage Ic–IV) or primary peritoneal carcinomas were included. Of these, 243 (75.9%) had been enrolled in a larger phase III GERCOR-AGO-OVAR-9 randomized trial of first-line paclitaxel/carboplatin/gemcitabine (TCG) chemotherapy, conducted in 59 centers between July 2002 and April 2004 [ClinicalTrials.gov Identifier: NCT00032463]. The clinical characteristics of patients included in the TCG trial are presented in Table 1. The 77 remaining patients (56 centers) included in our analysis demonstrated paclitaxel/carboplatin chemo-resistance (defined as progression during therapy or relapse within 6 months after completing therapy).

### Ethics

The study was approved by the local ethical committee (CCPPRB number: 02780) and all individual patients gave written informed consent for biological studies.

### HER2 determination by IHC

Formalin-fixed and paraffin-embedded tumors from primary surgery were obtained retrospectively. Because of previously reported heterogeneity in HER2 expression in ovarian cancer [24], in the present study we chose to analyze four blocks containing tumor: two blocks of primary tumors and two blocks of chemo-naive metastases/peritoneal dissemination (available from 206 patients). Most of the metastases analyzed were peritoneal dissemination, whereas few corresponded to lymph node sections.

Following deparaffinization and rehydration, the 4 μm sections were microwave pretreated in pH 6 citrate buffer. Primary antibody (CB-11, Novocastra, Newcastle-upon-Tyne, UK) diluted 1/800 was incubated for 2 hours. Staining was achieved using a streptavidin-biotin-peroxidase kit (ABCs, Biospa, Milano, Italy) including 30-minute incubation for each step. Nuclei were counterstained with haematoxylin. HER2 positivity was assessed using Ellis and Wolff recommendations [14],[15]. A score of 1+ was defined as barely perceptible membrane staining in more than 10% of cells, a score of 2+ was defined as weak-to-moderate complete membrane staining present in more than 10% of tumor cells, and a score of 3+ was defined as strong complete membrane staining in more than 10% of tumor cells. We classified 2+ as equivocal and 3+ as positive. Cytoplasmic staining was considered to be non-specific. For each case, an external control containing two FISH-positive (2+/3+) breast cancer samples was used.

### HER2 determination by FISH

All samples of 2+/3+ HER2 protein expression and 24 samples with 0/1+ staining score were evaluated by FISH, performed on a single block in one of the following laboratories, according to local institutional procedures: Institut Curie, Paris (PathVision HER2 kit; Abbott-Vysis, Desplaines, IL); Centre Jean Perrin, Clermont-Ferrand (PathVision HER2 kit; Vysis, IL); Hôtel-Dieu, Paris, (HER2 kit, DakoCytomation, Glostrup, Denmark). Instructions from the test kit manufacturers were followed, with slight modifications.

Four μm deparaffinized sections were incubated in pretreatment buffer at 95°C for 15 minutes, then in proteolytic solution at 37°C for 5 minutes. Co-denaturation of the probe and DNA of the tissue section was achieved by incubation at 82°C for 5 minutes using a HyBrite device (Abbott-Vysis); this was followed by a 15-hour hybridization at 37°C. Post-hybridization washes were performed according to the respective protocols. Slides were mounted in DAPI/antifade and viewed with a fluorescent microscope. Sixty nuclei in several areas of the section were analyzed, and three representative images per case were captured. Tumors were classified as amplified if they showed a mean of ≥3 HER2 signals, or a HER2/centromere 17 ratio ≥2:1 in samples of fewer than 8 HER2 signals.

### Statistics

The study was designed to evaluate HER2 status in a population of patients with advanced ovarian cancer. HER2 status in primary tumors and metastases was compared using kappa testing. The relationship between clinical characteristics of the 243 patients included in the TCG trial and HER2 status was compared using chi-square testing. Overall survival (OS) was calculated from the date of inclusion to death and progression-free survival (PFS) was calculated from the date of inclusion until progression or last follow-up examination. Progression was defined as a 20% increase in the diameter of all measured lesions, appearance of new lesions and/or doubling from baseline of CA125 tumor marker concentration. OS and PFS curves were derived from Kaplan-Meier estimates. A univariate Cox model analysis was performed to estimate and test the prognostic influence of clinical and biological variables. In a multivariate analysis, the Cox proportional hazard regression model was applied to determine the influence of these variables on outcome, adjusted for other prognostic factors. Hazard ratios (HR) and 95% confidence
intervals (CI) were determined. The influence of HER2 status in drug-resistant ovarian cancer was assessed in a subset of 109 patients with FIGO stage IIIc-IV disease and sub-optimal surgery. The resistant group was defined as presenting a first progression during or within 6 months following the end of treatment. The sensitive group was defined as non-progressive patients within the year after the end of treatment. HER2 status between resistant and sensitive groups was evaluated using exact Fisher testing. P values ≤0.05 were considered significant. All analyses were performed using S+ software (Insightful, Seattle, WA).

RESULTS

HER2 overexpression

Of the 320 tumors analyzed, HER2 3+ staining was observed in 15 samples (4.7%) and 2+ in 26 samples (8.1%) (Figure 1). HER2 expression of 0 or 1+ was detected in the 279 remaining samples (87.2%). One-third of tumors showed some intracytoplasmic staining, considered as non-specific (data not shown). Among the 41 samples with 2+/3+ staining, 19 (46.3%) were heterogeneous and the same pattern was seen in the metastatic samples.

Of the 206 patients from whom both primary tumor and distant metastatic samples were available, there was concordance between primary tumor and metastases in 197 samples (95.6%; 179 negative and 18 positive). No statistical difference between HER2 overexpression in primary tumors and corresponding metastases could be identified by kappa testing. In nine samples, 2+/3+ HER2 staining was found exclusively in either primary tumor (five samples) or metastases (four samples). Three of these nine samples showed 3+ overexpression and were amplified. The six remaining samples showed 2+ expression and one of these showed HER2 gene amplification by FISH.

HER2 gene amplification

Sixty-five samples were analyzed by FISH for HER2 amplification, including all of the 26 equivocal (2+) tumors, the 15 positive (3+) tumors, and 24 samples showing 1+ or cytoplasmic staining. Three samples (two with 2+ and one with 3+ staining) were not evaluable because of DNA alteration by fixation, despite two attempts conducted in two laboratories. Thus a total of 62 samples were evaluated by FISH (Figure 2). In total 21 patients showed HER2 positive status (all of the samples with IHC 3+ score and 6 of 24 samples with IHC 2+ score validated by FISH). None of the 24 samples with 1+ or cytoplasmic positivity was amplified for HER2.

In the heterogeneous samples, HER2 overexpression and amplification were found in the same tumor areas. Eighteen of 21 samples with HER2 amplification showed more than 8 copies per tumor nucleus with large clusters, suggesting homogeneous staining regions. The three remaining samples showed amplification with 8–10 copies per tumor nucleus, and a significant HER2/centromere 17 ratio.

Relationship between biological markers and other prognostic variables

No relationship between HER2 status and other prognostic factors (tumor stage, histological type, grade, ascites, debulking status, age and performance status) was found.

Survival analysis

Median follow-up was 24.9 months (95% CI: 23.4–26.3). At the time of our analysis (July 2006), disease progression had occurred in 150 (61.7%) patients and 66 (27.2%) had died. Median PFS duration was 17.7 months (95% CI: 13.3–20.6). Median OS had not been reached.

Figure 1. Immunohistochemistry labeling results. A. 2+ score: weak-to-moderate complete membrane staining in more than 10% of tumor cells (objX40). B. 3+ score: strong complete membrane staining in more than 10% of tumour cells (objX40). C. Heterogeneous staining of a primary ovarian tumour (objX20). D: Heterogeneous staining of a metastasis (objX20). E. FISH: heterogeneous amplification of HER2 in a tumor showing a cluster of tumor cells with amplification (white arrow, left part) and a cluster of non amplified tumor cells (orange arrow, right part). F: Clusters of red spots (HER2 amplification) together with two green spots (centromere 17). doi:10.1371/journal.pone.0001138.g001

Among the 41 patients whose tumor was 2+/3+ by IHC, disease progressed in 18 (43.9%) and there were seven (17.1%) deaths, while in the group of 16 patients with HER2 amplification, disease progressed in 12 (75%) and there were four (25%) deaths (Figure 3).

Univariate analysis

Univariate analysis of the potential prognostic impact of clinical and histopathological parameters identified performance status 1 or 2, tumor stage, ascites and residual tumor after first laparotomy as significantly associated with shorter OS and PFS (Table 2). The association between age ≥ 60 years and poorer OS was borderline significant. HER2 status (evaluated by either IHC or FISH) was not of prognostic value in terms of OS and PFS.

Multivariate analysis

Age, performance status, FIGO stage, ascites, residual tumor after first laparotomy and HER2 amplification/overexpression status were considered. Only the presence of ascites was retained as an independent prognostic factor of both shorter PFS (P = 0.037) and OS (P = 0.016) (Table 3). High FIGO stage was also retained as a prognostic factor for PFS (P = 0.00041) alone. HER2 status had no significant impact.

Chemoresistance and HER2 status

From the cohort of patients included in the TCG trial, a subset of 109 patients with FIGO stage IIIc/IV primary tumor and sub-
optimal surgery was selected for analysis. Based on follow-up, 46 patients were considered as chemoresistant, 36 as chemosensitive, and 27 could not be classified in one of the two groups. HER2 status was not significantly linked to chemoresistant status.

DISCUSSION

In this study, we screened 320 advanced ovarian cancers for HER2 status. To our knowledge, this is the first large multicentric study investigating both primary tumor and metastases on conventional slides by IHC and FISH techniques. Positive HER2 status was found in 6.6% (21 of 320) of the tumor samples. The rate of HER2 positivity varies in the literature from 8% to 66% [15–33] (Figure 4). There are several possible explanations for the wide variation and the relatively low rate of HER2 positivity reported in our series, including the different detection methods used (IHC, FISH and chromogenic in situ hybridization), different sources of material (blocks of tumors and tissue microarray), and variations in IHC assay techniques (CB-11, HercepTest or a non-commercial antibody); in addition, variance in staining protocols and subjective interpretation of sample stains makes direct comparison of studies difficult. The present study has the advantage of being based on a large, prospective, multicenter trial, with extensive material (blocks of tumors and tissue microarray), and variations in IHC assay techniques (CB-11, HercepTest or a non-commercial antibody); in addition, variance in staining protocols and subjective interpretation of sample stains makes direct comparison of studies difficult. The present study has the advantage of being based on a large, prospective, multicenter trial, with extensive

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Figure 3. Progression-free survival and overall survival according to HER2 status. A. Progression-free survival B. Overall survival
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Table 2. Univariate analysis for progression-free survival and overall survival of biological and clinical parameters

| Factor                                      | Overall survival |                | Progression free survival |                |
|---------------------------------------------|------------------|----------------|---------------------------|----------------|
|                                             | HR [95%CI]       | P-value        | HR [95%CI]                | P-value        |
| HER2 Positive vs. negative (IHC+FISH)       | 1.29 [0.37; 2.8] | 0.58           | 1.4 [0.79; 2.59]          | 0.23           |
| HER2 IHC 2+/3+ vs 0/1+                      | 0.809 [0.34; 1.87]| 0.6            | 0.805 [0.49; 1.32]        | 0.39           |
| HER2 IHC 1+/2+/3+ vs 0                      | 0.948 [0.51; 1.74]| 0.86           | 0.809 [0.54; 1.19]        | 0.29           |
| Age ≤60 years vs >60 years                  | 1.60 [0.97; 2.63] | 0.057          | 1.22 [0.89; 1.7]          | 0.2            |
| Performance status                          | 2.75 [1.54; 4.88] | 0.00059*       | 1.62 [1.15; 2.26]         | 0.005*         |
| 1/2 vs 0                                    | 6.81 [1.66; 27.86]| 0.0076*        | 4.37 [2.3; 8.38]          | <0.00001*      |
| III-VI vs I-II                              | 3.07 [1.62; 5.78] | 0.00048*       | 2.04 [1.41; 2.92]         | 0.00013*       |
| Ascites Presence vs absence                 | 2.29 [1.33; 3.93] | 0.0018*        | 2.3 [1.64; 3.22]          | <0.00001*      |
| Residual tumour after first laparotomy      | >1 cm vs ≤1 cm    | 0.00013*       |                           |                |

HR = Hazard ratio, CI = Confidence interval, * = P-value <0.05
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Table 3. Multivariate analysis of clinical parameters for progression-free survival and overall survival

| Factor                               | Overall survival | P-value | Progression free survival | P-value |
|--------------------------------------|------------------|---------|---------------------------|---------|
|                                      | HR [95%CI]       |         | HR [95%CI]                |         |
| HER2 positive vs negative (IHC+FISH) | 1.44 [0.51; 4.00]| 0.49    | 1.67 [0.86; 3.22]         | 0.12000 |
| Age                                 | 1.28 [0.73; 2.24]| 0.380   | 1.06 [0.74; 1.52]         | 0.74    |
| 60 years vs < 60 years              |                  |         |                           |         |
| Performance status                  | 1.65 [0.86; 3.16]| 0.12    | 1.26 [0.85; 1.85]         | 0.23000 |
| 1/2 vs 0                            |                  |         |                           |         |
| Tumor stage                         | 6.75 [0.17; 254.6]| 0.064   | 3.96 [1.84; 8.4]          | 0.00041*|
| III-IV vs I-II                      |                  |         |                           |         |
| Ascites                             | 2.23 [0.79; 6.21]| 0.016*  | 1.51 [1.02; 2.22]         | 0.037*  |
| Presence vs absence                 |                  |         |                           |         |
| Residual tumor after first laparotomy| 1.16 [0.44; 3.03]| 0.62    | 1.26 [0.84; 1.86]         | 0.26    |
| >1 cm vs ≤1 cm                      |                  |         |                           |         |

HR = Hazard ratio, CI = Confidence interval, * = P-value < 0.05

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Figure 4. HER2 in major published studies. A. Overexpression Review of selected articles evaluating HER2 protein expression in large series of patients (including more than 50 tumour samples) published in international journals after 1994. Boxes represent % of HER2 overexpression (scored as 2+ or 3+) and error bars show ±2 standard errors for each study. B. IHC and FISH status Review of selected articles evaluating HER2 gene amplification (FISH or CISH) and/or HER2 protein expression in large series of patients (including more than 50 tumour samples) published in international journals after 1994. In situ hybridisation represents FISH (fluorescence in situ hybridisation) and CISH (chromogenic in situ hybridisation) results. Mean HER2 overexpression/amplification across studies is represented; IHC = Immunohistochemistry.
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