Abstract. Background: Although the majority of endometrial cancer (EC) patients can be cured by surgery, unexpected recurrent disease may also occur in early stage patients. In the present study, whether or not the analysis of multiple biopathological parameters might lead to more accurate predictions of the clinical outcome of EC patients with long-term follow-up (FU) was investigated. Patients and Methods: Estrogen and progesterone receptor (ER and PgR) positivity and HER2 overexpression by immunohistochemistry were evaluated. The peritoneal washings (PWs) were analyzed by cytology and immunocytochemistry employing AR-3 and B72.3 monoclonal antibodies. Results: The patients with positive PW and HER2 positive tumors showed shorter overall survival compared to those bearing HER2 negative tumors (p=0.004). HER2 overexpression also influenced the patient outcome in the group with tumors lacking PgR (p=0.004). At multivariate analysis PgR and HER2 overexpression emerged as independent prognostic factors. Conclusion: The combined analysis of these biopathological markers could provide useful information for the selection of patients to be enrolled in innovative therapeutic strategies.

Prognostic Value of HER2 and Progesterone Receptor Expression in Endometrial Carcinoma with Positive Peritoneal Washing

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and macroscopic endocervical involvement. The patients age ranged from 36 to 84 years (median 62 years), 90.6% of the women were of postmenopausal and 9.4% of premenopausal status. According to the current surgical staging system (9), 135 patients were classified as stage Ia to Ic, 22 as stage II, 43 as stage IIIa to IIIc. Patients with advanced disease (stage Iva-b) were excluded from the study. Our series of patients included 178 endometrioid and 22 papillary serous adenocarcinomas. Mixed and uncommon histotypes were excluded from this study. The myometrial invasion, determined in 198 patients, was less than one half in 146 patients and more than one half in 52. A total of 71 tumors were well differentiated (G1), while 78 were moderately differentiated (G2) and 51 poorly differentiated (G3) (10).

Sampling and preparation of peritoneal washings. Intraoperative PW was performed in 181 patients. Physiological solution (250 ml) at 37°C was injected into the peritoneal cavity and collected using heparin as anticoagulant. The cells, separated by centrifugation, were resuspended in phosphate buffer (pH 7.2) at a density of 1.10⁶ cells/ml. The erythrocytes were eliminated by treating the PW with Tris NH₄Cl (pH 7.4) for 10 minutes at 37°C. Multiple cytopsins, obtained with a cyt centrifuge (Shandon Runcorn, Cheshire, UK), were stained following the Papanicolaou method, or fixed for 10 minutes in cold absolute acetone for immunocytochemistry (ICC) evaluation.

Immunocytochemistry (ICC), immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). The MoAbs used for ICC of the PWs, were B72.3 (Menarini, Florence, Italy) and AR-3 (kindly provided by Dr. M. Prat, Turin University, Italy). The two MoAbs, used in combination, are able to recognize more than 95% of EC (11, 12). The PWs were considered positive when isolated cells or clusters of cells were found to be ICC reactive with at least one of the MoAbs employed in two separate cytopsins. HER2 overexpression was determined by IHC in 5 μm paraffin sections using MoAb 300G9 (13) and to confirm the results obtained, the slides were also stained with MoAb CB11 (Menarini). A 96.8% concordance between the two reagents was demonstrated. No antigen retrieval treatment was used for MoAb 300G9, whereas pretreatment in citrate buffer, pH6, was performed for MoAb CB11. The HER2 immunoreactivity, evaluated using a 0 to 3+ scale, was considered positive when at least 10% of neoplastic cells displayed a moderate (score 2+) or strong (score 3+) staining of the entire plasma membrane. The tumors displaying weak (score 1+) or no staining (score 0) of the plasma membrane were considered negative. The estrogen receptor (ER; clone 6F11, Biogenex, Space, Milan, Italy) and PgR (clone 1A6, Biogenex) status was evaluated on 5 μm paraffin sections with the highest rate of expression in papillary carcinomas (36.1%). In order to assess the HER2 status more critically, we determined gene amplification by FISH analysis in a selected series of 73 patients correlating these results with those obtained by IHC. Thirty-one out of the 73 carcinomas were not amplified and 42 amplified. A concordance rate of 74.3% (p=0.007) between IHC and FISH was found, 21 out of the 42 amplified cases being IHC negative and 8 out of the 31 not amplified cases IHC positive (data not shown).

Impact of biological parameters on overall survival. Of the 200 EC patients, 36 were lost at follow-up (FU). One hundred and sixty-four patients with a FU ranging from 2 to 160 months, were analyzed for OS. At a median FU duration of 54 months, the 5-year OS percentage was 76.3% (SE±3.6%). Forty-one deaths, due to cancer related causes, had occurred. As shown in Table I, along with conventional pathological parameters (grading, stage and myometrial invasion), univariate analysis identified PW, diagnosed both morphologically and by ICC, PgR and HER2 as significant predictors of OS. Therefore, intra peritoneal microdissemination and the association of lack of PgR with HER2 tumor overexpression indicated the presence of a particularly aggressive tumor (p=0.01 and p=0.004,
respectively). In multivariate analysis advanced stage, high tumor grade, lack of PgR and HER2 overexpression appeared to be independent risk factors for poor OS.

The Kaplan-Meier curves, stratified respectively for HER2 expression and PW status evaluated by ICC, indicated that a significantly longer OS could be observed in patients with negative PW and HER2 negative tumors (Figure 1A and 1B). On the basis of these results, the impact of the combination of these two biopathological factors on OS, analysing the two groups of PW positive patients bearing, respectively, HER2 negative and HER2 positive tumors was evaluated. As shown in Figure 1C, the results obtained provided statistically significant evidence that the concomitant positivity of PW and HER2 identified a group of patients with a higher probability of dying. Figure 2A shows that patients with PgR negative tumors had a significantly worse prognosis. Furthermore, the prognostic role of HER2 in the group of patients with PgR negative tumors was evaluated. The resulting Kaplan-Meier curves (Figure 2B) indicated that a significantly shorter OS could be observed in patients with PgR negative / HER2 positive tumors. However, due to the low number of available patients in these two subgroups, these Kaplan-Meier curves should be interpreted with caution.

Discussion

As recommended by FIGO, the cytological evidence of metastatic cells in the peritoneal cavity represents an additional risk factor in conjunction with histotype, tumor grade, depth of myometrial infiltration and vascular invasion. We recently demonstrated that the number of cytologically false-negative diagnoses of PW was significantly reduced using selected MoAbs. This makes it possible to improve the accuracy of surgical staging and may identify patients at higher risk of tumor progression (3). Nevertheless, despite this staging improvement, some early stage women still relapse and die (15). In the last decade, a number of studies have been performed aimed at correlating several molecular-genetic alterations to poor prognosis. These have included aberrant expression of p53, lack of hormonal steroid receptors, HER2 overexpression and/or amplification (16, 17). To our knowledge, the present study was the first that has investigated the prognostic significance of HER2 and PgR together with morphological and ICC analysis of PW in this neoplasia. Univariate analysis confirmed both the predictive value of well established surgical-pathological factors and the predictive value of positive PW. Through the same analysis, in agreement with data from other authors (5, 18), the prognostic significance of HER2 and PgR was confirmed in the entire series of EC. The results obtained by multivariate analysis, accounting for varied FU times, demonstrated that stage and tumor grade as well as PgR and HER2 were independent variables, predictive of a worse clinical outcome. In contrast to our previous findings (3), by introducing two additional variables in the Cox model, namely PgR and HER2, the independent prognostic value of positive PW was lost. This may have been due to the strong association between the three variables.

Gene amplification was performed by FISH analysis on 73 EC selected cases lacking normal residual glandular epithelium or endometrial hyperplasia both of which often cause false positive results in this type of assay. In fact, Czerwenka et al. (19) reported that amplification might also occur in normal (8%) and hyperplastic (48%) tissues, further suggesting that HER2 plays a key role in the initiation and/or early progression of EC. The overall concordance rate between HER2 gene amplification and overexpression was 74.3%. Although the concordance rate was statistically significant ($p=0.007$) and similar to that observed by other authors (20-22), in our series only HER2.

Table I. Univariate and multivariate analyses of prognostic factors for overall survival in 164 endometrial cancer patients.

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>Univariate</th>
<th>Multivariate</th>
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<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>$p$-value</td>
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<tr>
<td>Grading (G3 vs. G1-G2)</td>
<td>3.37 (1.73-6.58)</td>
<td>0.001</td>
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<tr>
<td>Stage (III– vs. I–II)</td>
<td>4.03 (2.06-7.89)</td>
<td>&lt;0.001</td>
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<tr>
<td>Myometrial invasion (&gt;1/2 vs. &lt;1/2)</td>
<td>3.64 (1.84-7.22)</td>
<td>&lt;0.001</td>
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<tr>
<td>PW (cyto+ vs. cyto–)</td>
<td>2.92 (1.39-6.15)</td>
<td>0.005</td>
</tr>
<tr>
<td>PW (ICC+ vs. ICC–)</td>
<td>3.31 (1.66-6.57)</td>
<td>0.001</td>
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<tr>
<td>HER2 (pos vs. neg)</td>
<td>2.89 (1.47-5.68)</td>
<td>0.002</td>
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<tr>
<td>PW ICC+/HER2+ vs. PW ICC+/HER2–</td>
<td>3.84 (1.37-10.76)</td>
<td>0.01</td>
</tr>
<tr>
<td>PgR–/HER2+ vs. PgR+/HER2–</td>
<td>2.91 (1.01-8.38)</td>
<td>0.004</td>
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HR=Hazard risk; CI=95% confidence interval; PW: peritoneal washing; ICC: immunocytochemistry.
overexpression was significantly related to prognosis. Our findings are in agreement with Saffari et al. (22), who demonstrated that protein overexpression, but not gene amplification, was an independent marker of poor outcome. Nevertheless, a decrease in survival rate (43% vs. 70%) was found in patients bearing an amplified tumor with a HER2 to CEP17 ratio >4 compared to patients bearing a non-amplified tumor as recently reported by Morrison et al. (8). However, due to the low number of cases, our test was not statistically significant ($p=0.13$).

The importance of hormonal receptors in the management of breast cancer patients is well established, whereas the prognostic significance of ER and PgR expression in EC remains controversial (6, 23). This may be related to differences in population size, reagents employed and staining evaluation methods. However, loss or absence of PgR isoform A expression has recently been shown to be an important prognostic indicator in endometrioid EC (24). Among the multiple biopathological parameters analyzed, HER2 and PgR emerged as reliable predictors of survival in univariate and multivariate analyses. Moreover, loss of PgR associated
with the presence of peritoneal micrometastases could identify the EC patients at a higher risk of dying. These results may, therefore, be of particular clinical interest, firstly, because the PW status plays a pivotal role in the assessment of the initial surgical staging of EC, strongly influencing the outcome for this disease and secondly, this combination of prognostic factors might enhance the diagnostic accuracy for detecting patients at a higher risk of tumor progression. A recent study (8) has demonstrated that HER2, as a single marker, plays a minor prognostic role in low grade and stage EC which are the most common tumors. Therefore, the use of a panel of biopathological parameters may well contribute to a more accurate planning of innovative adjuvant therapy also in early stage EC of the endometrioid histotype. In fact, a number of emerging novel molecular targeted therapies, which have now become standard therapy in breast cancer, could also be useful in the treatment of endometrial cancer (25). These include, for example, trastuzumab (Herceptin®, Roche, Basel, Switzerland), and the more recent GW572016 (Lapatinib®, GlaxoSmithKline, Parsippany, NJ, USA), a dual inhibitor of epidermal growth factor receptor and HER2 (8). In this context, a greater understanding of the molecular biology of the events regulating cell proliferation, invasion and metastasis may be essential for the rational design of treatments aimed at specific molecular targets (26). Although our study provides additional evidence of the potential prognostic role of these markers, further prospective and controlled studies are required to validate their clinical usefulness.

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