Prediction of Clinical Outcome from Primary Tamoxifen by Expression of Biologic Markers in Breast Cancer Patients

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ABSTRACT

The aim of this study was to evaluate pretreatment clinical features and biological markers together with changes in these factors as predictors of response and relapse in patients receiving tamoxifen for primary breast cancer. Fine-needle aspiration cytology of the primary breast cancer was performed before tamoxifen treatment in 54 patients and repeated after therapy on day 14, day 60, or on both days in a subset of 35 patients. These samples were evaluated for estrogen receptor (ER), progesterone receptor (PgR), Ki67, S-phase fraction and ploidy. The overall response to tamoxifen was 57% (31 of 54 patients). Pretreatment ER and PgR significantly predicted for response by univariate analysis ($P < 0.0001$ and $P < 0.003$, respectively). By multivariate analysis, ER expression was the only independent predictor of response, and it was associated with 27 times the likelihood of response (95% confidence interval, 6–136). Increase in PgR and decrease in Ki67 on day 14 significantly predicted for response to tamoxifen ($P < 0.03$ and $P < 0.04$, respectively). Lack of ER, clinical node-positive disease, and failure to decrease Ki67 on day 14 were significantly associated with increased risk of relapse ($P < 0.05$). By multivariate analysis, ER expression was the only independent predictor of relapse ($P < 0.005$). Pretreatment and early changes in molecular marker expression may assist in the prediction of response and clinical outcome in primary breast cancer patients receiving tamoxifen.

INTRODUCTION

Tamoxifen is of proven benefit in the clinical management of women with breast cancer and is associated with significant reduction in tumor recurrences and mortality in the adjuvant setting (1). As such, tamoxifen is the most widely prescribed anticancer agent in the world. However, the optimal use of the drug with the maximum benefit and minimal toxicity is still an area of intense investigation. In postmenopausal women, tamoxifen reduces the incidence of fatal myocardial events (2), increases bone mineral density (3–5), and reduces the occurrence of contralateral breast cancers by about 40–50% (1). However, adverse side effects include an increase in thromboembolic events (1) and endometrial cancers (1, 6, 7).

ER and PgR have now been studied in clinical breast cancer for more than 20 years. ER and PgR have their greatest utility in predicting response to hormonal therapy, both in the adjuvant setting and for advanced disease. In metastatic breast cancer, the presence of ER and PgR in tumors has been shown to predict for improved response to tamoxifen (8, 9). Patients with ER-positive breast cancers have approximately a 50% chance of response to endocrine treatment compared to a 12% response rate for those with ER-negative tumors (10). In early breast cancer, several large randomized trials have shown a correlation between ER status and improvement in survival with adjuvant tamoxifen. The overview meta-analysis found the reduction in odds of recurrence for ER-poor patients (<10 fmol/mg) who were less than 50 years of age was 3% compared to 16% in patients 50 years or older. If ER was >10 fmol/mg, this reduction improved to 19% in patients younger than 50 years and 36% in patients ages 50 years and above (1). Other studies have indicated that PgR may serve as an indicator to the functional integrity of ER (11). Patients with ER-positive metastatic breast cancer have approximately a 50% chance of responding to tamoxifen, and this may be split into subsets of about 40% or 60%, depending on PgR expression (12). Other predictive biological markers may include changes in proliferative activity in response to tamoxifen. Previous in vitro studies have shown an accumulation of cells in G1 phase and a decrease in SPF (13, 14). A decrease in proliferation in response to tamoxifen has been reported in MCF-7 xenografts (15). In human breast cancers, studies have confirmed that tamoxifen may result in a reduction in proliferation as measured by immunohistochemical analysis (Ki67; Refs. 16 and 17). However, a relationship between these changes and subsequent tumor response was not established in these studies.

ICC analysis of biological markers on samples obtained by FNA has shown high correlation with results from paraffin sections and biochemical assays (18, 19). We have previously validated the assays used in this study by demonstrating a high concordance between measurements obtained by FNAs and

3 The abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; SPF, S-phase fraction; FNA, fine-needle aspiration; CR, complete response; PD, progressive disease; ICC, immunocytochemical.
histological paraffin sections (20). We have also reported no significant change in the level of expression of hormone receptors, Ki67, SPF, or ploidy from FNAs taken 2 weeks apart in a study involving 20 control patients who had not received any systemic therapy (21). Hence, the measurement of changes in biological markers by repeat FNA before and after exposure to treatment may be used as additional predictive markers of response to therapy.

In an earlier report, we found that pretreatment ER and PgR expression and decrease in Ki67 predicted for tumor response in 21 patients receiving tamoxifen for primary breast cancer (22). In the current study, we have increased the sample size to further define pretreatment biological markers and clinical features together with early changes in these factors as early predictors of response and relapse. The main objective of the study was to determine additional predictive markers for tamoxifen responsiveness, which may enable a better selection of patients who are likely to derive benefit from this systemic treatment.

PATIENTS AND METHODS

Patient Selection and Response Evaluations

Postmenopausal women with primary, noninflammatory breast cancer who were treated with preoperative tamoxifen were recruited into this study. Entry criteria included clinically diagnosed primary breast cancer confirmed by FNA and the absence of metastatic disease. Staging investigations included chest X-ray, biochemical liver function tests, alkaline phosphatase, bone scan, and liver ultrasound. Primary tamoxifen was given to women who were considered medically unsuitable for surgery on the basis of poor performance status (Eastern Cooperative Oncology Group performance status > 3) due to the presence of coexisting medical conditions or advanced age. The evaluation of predictive molecular markers in primary breast cancer patients was undertaken in a protocol, which had been approved by the local ethics committee (protocol 669). These women were recruited from January 1992 to June 1997.

Response to tamoxifen was on clinical bidimensional measurements prior to, at 2 weeks, at 2 months, and then at 3-month intervals after commencement of tamoxifen. Clinical response was defined according to standard WHO criteria: (a) CR was defined as the disappearance of all clinical disease; (b) partial response was defined as a reduction of more than 50% in bidimensional product diameter; (c) stable disease was defined as reduction of less than 50% or an increase in size of less than 25% for at least 6 months; and (d) PD was defined as increase of more than 25%.

Laboratory Methods

Collection of Samples. FNAs of the primary breast tumors using a 23-gauge needle attached to a 10-ml syringe were performed in 54 patients before starting tamoxifen. Of these, 35 women had repeat biopsies on day 14 (31 women), day 60 (15 women), or on both days 14 and 60 (11 women) after commencement of tamoxifen. These time points for repeat biopsies were selected to coincide with routine outpatient clinic visits. Repeat biopsies were not obtained in some patients for the following reasons: (a) on day 14, 20 patients failed to return, whereas acellular aspirates were obtained in 3 patients; and (b) on day 60, 27 patients refused a repeat biopsy or failed to attend clinic, 4 patients had a clinical CR, and acellular aspirates were obtained in the remaining eight patients. Cellular samples were evaluated for ER, PgR, Ki67, SPF, and ploidy.

Preparation of Specimens for Cytospins and Flow Cytometric Analysis. From each aspirate, a 7-ml single cell suspension with MEM was made. Aliquots of 300 μl were placed in 12 Shandon cytospin chambers and centrifuged at 500 rpm for 5 min on 3-amino propyltriethoxysilane slides. These slides were then stained with May-Grünwald-Giemsa for cyto-diagnosis or air dried and stored at −80°C until ICC analysis. The remaining cell suspension was snap frozen in liquid nitrogen for flow cytometric cell cycle analysis.

ICC Analysis. Standard methods for ICC analysis have been described in detail elsewhere (23). Briefly, the thawed cytospin slides were washed in PBS and fixed with acetone, methanol, methanol/acetone, or acetone/methanol. The endogenous peroxidase was then blocked by 0.1% sodium azide in 3% H2O2, 3% of 10% H2O2. For ER and PgR staining, slides were incubated with ER antibody (Abbott ER-ICA monoclonal antibody, 1:40 dilution) or KD68 antibody (Abbott PR-ICA monoclonal kit). For Ki67, the slides were incubated with rabbit serum (1:5 dilution) before the addition of the Mib1 antibody (Binding Site, United Kingdom). Secondary antibody (biotinylated anti-rat IgG for ER, PgR, and Ki67) was then applied. After rinsing, the slides were incubated with streptavidin horseradish peroxidase (1:100) for 30 min or ABC horseradish peroxidase (for Ki67) for 20 min, rinsed with PBS, exposed to diaminobenzidine tetrahydrochloride chromogen for 10 min, rinsed with buffer and PBS, counterstained with 1% methyl green, rinsed with deionized water, and then mounted.

ICC Scoring. Subjective estimation was performed as described previously (23) of the proportion of positive-staining cells on the entire slide (0, none; 1, < one-hundredth; 2, one-hundredth to one-tenth; 3, < one-tenth to one-third; 4, one-third to...
to two-thirds; and 5, >two-thirds), and the intensity of the positive signal (0, none; 1, weak; 2, intermediate; and 3, strong signal) of all slides was evaluated by light microscopy semiquantitatively by one author (D. C. A.) without any knowledge of the patients’ clinical data. The overall score was expressed as the summation of the proportion and intensity scores. Tumors were regarded as expressing the particular molecular marker if the overall score was 3 for ER and PgR. For Ki67, the percentage of positive cells was determined by direct counting.

DNA Flow Cytometry. The details of DNA flow cytometry have been described elsewhere (24). In brief, the cell suspension was thawed, centrifuged, lysed, and stained for DNA by incubating in a stain detergent solution (NP40; Sigma, Poole, United Kingdom) containing propidium iodide as the DNA fluorochrome. DNA-stained nuclei were run on a Coulter Elite ESP flow cytometer (Coulter Electronics, Hialeah, FL). Fifty thousand tumor events were acquired on a single parameter 256-channel fluorescence histogram, and the cell cycle distributions (G0-G1, presynthetic; S, synthetic; and G2-M, postsynthetic and mitotic phase) were analyzed by multicycle software programs (Phoenix Flow Systems, Inc.). DNA content was regarded as diploid if G0-G1 peaks were superimposed and was regarded as aneuploid only if separate peaks were seen.

### Table 2: Pretreatment clinical stage and biologic markers as predictors of response to tamoxifen by univariate analysis

Pretreatment ER, PgR, Ki67, SPF, ploidy, and presenting clinical stage were tested to determine their influence on primary tumor response using Fisher’s exact test (binary variables), the Mann-Whitney trend test (ordered categorical variables), and the Mann-Whitney U test (continuous variables). A, aneuploid; D, diploid. Significant variables in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All (n = 54)</th>
<th>Responders (n = 31)</th>
<th>Nonresponders (n = 23)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>T1, T2, T3, T4</td>
<td>54</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>Nodes</td>
<td>N0, N1</td>
<td>54</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>ER</td>
<td>0 (0, 8)</td>
<td>49</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>PgR</td>
<td>0 (0, 8)</td>
<td>52</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Ki67 (±SD)</td>
<td>8.2 (±8.4)</td>
<td>43</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>SPF (±SD)</td>
<td>8.2 (±7.5)</td>
<td>44</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>Ploidy</td>
<td>A, 22 D, 25</td>
<td>47</td>
<td>27</td>
<td>20</td>
</tr>
</tbody>
</table>

### Table 3: Changes in immunocytochemical score for ER and PgR or change in ploidy with exposure to tamoxifen analyzed using the Fisher’s exact test. Changes in SPF and Ki67 were compared between responders and nonresponders by the Student’s t test as predictors of response. Significant variables in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day</th>
<th>Increase</th>
<th>No increase</th>
<th>Increase</th>
<th>No increase</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>14</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>NS^a</td>
</tr>
<tr>
<td>PgR</td>
<td>14</td>
<td>9</td>
<td>3</td>
<td>4</td>
<td>12</td>
<td>0.02</td>
</tr>
<tr>
<td>Ploidy</td>
<td>40</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day</th>
<th>Change in variable</th>
<th>Change in variable</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF (±SD)</td>
<td>14</td>
<td>+0.5 (0.6)</td>
<td>−5.2 (6.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Ki67 (±SD)</td>
<td>14</td>
<td>−3.0 (4.0)</td>
<td>−0.4 (1.3)</td>
<td>NS</td>
</tr>
</tbody>
</table>

^a NS, nonsignificant.

### Statistical Analysis

Pretreatment biological markers (ER, PgR, Ki67, SPF, and ploidy) and presenting clinical stage were tested to determine their influence on primary tumor response using Fisher’s exact test (binary variables), the Mann-Whitney trend test (ordered categorical variables), and the Mann-Whitney U test (continuous variables). Repeat biopsies were taken in a subset of 35 women for the determination of changes in the expression of the tumor markers on day 14 (31 women), day 60 (15 women), and on both days 14 and 60 (11 women). Changes from pretreatment values were calculated for each of the markers. All changes in marker expression were included in the analysis. These changes in markers were assessed for their influence on tumor response using the tests outlined above. Multivariate analysis was conducted on the significant variables obtained by univariate analysis.

Disease-free survival was documented as the time from the start of treatment until distant metastatic relapse. Pretreatment markers and changes in tumor markers were tested for their influence on relapse-free survival in a univariate analysis using Cox’s proportional hazards model. The relative risk of relapse was calculated for the various groups.
RESULTS

Patient Demographics. All women were postmenopausal with a median age of 83 years (age range, 69–93 years). The majority (31 of 54, 57%) presented with clinical stage T2 (tumor size, 2–5 cm) without clinical nodal involvement (44 of 54, 81%). Response was noted in 31 women (31 of 54, 57%), whereas 20 patients achieved stabilization of disease for at least 6 months (20 of 54, 37%), and 3 women (3 of 54, 6%) had PD (Table 1).

Predictive Markers of Response. Expression of ER and PgR significantly predicted for subsequent response, with 23 of 26 ER-positive patients (88%) responding to tamoxifen compared to 5 of 23 ER-negative patients (22%; P < 0.0001, Fisher’s exact test). Likewise, 18 of 19 PgR-positive patients (95%) responded to tamoxifen compared to 13 of 33 PgR-negative patients (39%; P = 0.0001, Fisher’s exact test). Tumors with high proliferation (Ki67) had a trend toward improved response (P = 0.08). Of interest, response to tamoxifen was independent of presenting clinical stage (tumor size or clinical nodal status). By multivariate analysis, ER status was the only independent predictive variable and was associated with 27 times the likelihood of response (95% confidence interval, 6–136; Table 2).

Prediction of Response by Early Repeat Biological Markers Marker Expression. The changes in molecular marker expression were analyzed as predictors of response. An increase in PgR expression and decrease in Ki67 on day 14 were associated with a statistically significant increased likelihood of response to tamoxifen (P < 0.03 and P < 0.04 respectively). Changes in ER, SPF, and ploidy did not differ significantly between responders and nonresponders (Table 3).

Changes in PgR and Ki67 were analyzed in relation to baseline ER status. ER-positive tumors were more likely to show decrease in Ki67 on day 14 as a predictor of response. In 10 ER-positive tumors, a median decrease in Ki67 by −1.2% was observed in nine responders, whereas one nonresponder had an increase in Ki67 by +6.7%. Of 13 ER-positive tumors, increase in PgR on day 14 was observed only in responders. Of these, 7 of 11 responders showed an increase in PgR, whereas the 2 nonresponders showed no change in PgR expression.

Predictors of Relapse. Presenting clinical stage (tumor size and clinical nodal status) and biological marker expression together with changes in these factors were analyzed by univariate analysis as predictors of the risk of relapse. Lack of ER expression, clinical node-positive disease, and failure to decrease Ki67 were significantly associated with increased risk of relapse (P < 0.05; Table 4). By multivariate analysis, lack of ER expression was the only independent predictor of relapse (P < 0.005). Fig. 1 demonstrates the Kaplan-Meier curve of disease-free survival in ER-positive and ER-negative patients.

DISCUSSION

Presenting biological markers (ER and PgR), together with an increase in PgR and a decrease in Ki67 on day 14, predict for tamoxifen responsiveness. Lack of ER, clinical node-positive disease, and decrease in Ki67 were also found to predict for increased risk of relapse in these patients.

The relationship between hormone receptor status (ER and

### Table 4 Association of variables with the risk of relapse by univariate analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Relative risk of relapse (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical stage</td>
<td>N/A</td>
<td>0.6</td>
</tr>
<tr>
<td>Tumor size</td>
<td>N/A</td>
<td>0.6</td>
</tr>
<tr>
<td>Lymph node positive</td>
<td>3.4 (0.5–24)</td>
<td>0.03</td>
</tr>
<tr>
<td>Pretreatment markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Undefined (no relapse in ER-positive patients)</td>
<td>0.009</td>
</tr>
<tr>
<td>PgR</td>
<td>N/A</td>
<td>0.2</td>
</tr>
<tr>
<td>Ki67</td>
<td>5 (1–21)</td>
<td>0.08</td>
</tr>
<tr>
<td>SPF</td>
<td>N/A</td>
<td>0.6</td>
</tr>
<tr>
<td>Ploidy</td>
<td>N/A</td>
<td>0.7</td>
</tr>
<tr>
<td>Change in markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in ER</td>
<td>No relapse</td>
<td>N/A</td>
</tr>
<tr>
<td>Increase in PgR</td>
<td>N/A</td>
<td>0.8</td>
</tr>
<tr>
<td>Decrease in Ki67</td>
<td>0.1 (0.01–2.1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Change in SPF</td>
<td>N/A</td>
<td>0.8</td>
</tr>
<tr>
<td>Change in ploidy</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

a CI, confidence interval; N/A, not applicable as the variables were not significant and associated with insufficient events. Significant variables in bold.

Fig. 1 The Kaplan-Meier curve of disease-free survival in ER-positive and ER-negative patients.

Fig. 2 The Kaplan-Meier curve of disease-free survival in patients with clinical node-positive and node-negative disease.
PgR) and tamoxifen responsiveness is well established in both metastatic studies (8, 9, 12) and in the adjuvant setting (1). Prediction of response to tamoxifen by changes in Ki67 has been demonstrated in this study. This finding was noted in the earlier report involving the first 21 women (18). The current study has demonstrated that differences in Ki67 expression between responders and nonresponders occur on day 14, but not on day 60. This lends support to the hypothesis that timing of repeat samples may be important if changes in marker expression are to be used as predictors of tamoxifen responsiveness. Other reports of reduction in Ki67 in tamoxifen-treated patients did not relate changes with treatment responsiveness (16, 17). Consistent with earlier reports, we also found that a decrease in Ki67 may be more marked in ER-positive tumors (16, 18).

This study showed no significant reduction in SPF in responders, in keeping with our earlier report (18). Ki67 is a specific nuclear antigen expressed only on proliferating cells in late G1 phase, S phase, M phase, G2 phase (25). As a measure of the proliferative fraction of tumor cells, Ki67 correlates well with the thymidine labeling index (25, 26), but not with SPF (27). Previous in vitro studies have demonstrated an accumulation of cells in G0 phase and a decrease of cells in S phase (13, 14) with tamoxifen. A possible explanation for this discrepancy between SPF and Ki67 measurements is that nonproliferating cells may not complete S phase of the cell cycle and hence are not measured by flow cytometry. Ki67 may therefore be a better reflection of proliferation after exposure to tamoxifen for the monitoring of response.

Tumor growth kinetics is determined by the balance between cellular proliferation and apoptosis. Induction of apoptosis in vivo is being used as an end point by which the efficacy of novel treatments is being tested. Work on ER-positive MCF7 tumors in xenografts demonstrate induction of apoptosis in tumors that respond to treatment (28). Future studies should determine the balance between apoptosis and proliferation as predictors of response to endocrine treatment (29).

Increase in PgR by day 14 significantly predicts the likelihood of responding to tamoxifen treatment. The sample size of the first report was of insufficient size to detect this observation. Our finding is in agreement with published data of an association between tamoxifen responsiveness and an increase in PgR in the metastatic setting (30). In contrast, other studies have failed to note any change in PgR (17) or even a decrease in PgR at 1 month after initiating tamoxifen (31). This study demonstrates that changes in biological markers such as PgR and Ki67 are dynamic and that the timing of repeat biopsies may be important in appraising the usefulness of these measurements as predictors of response.

Results from this preliminary study should be further explored in larger studies. If these changes in biological markers as predictors of hormone responsiveness were to be confirmed, then better selection of patients may be achieved. This study has demonstrated that response to tamoxifen may be based on predictive biological marker expression and that these factors are valid surrogate determinants for relapse. In the future, the efficacy of new endocrine treatment such as selective ER modulators or aromatase inhibitors should incorporate the use of biological markers to test the in vivo efficacy of these treatments as an interim indication of their effect on survival.

REFERENCES
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