Expression of Epidermal Growth Factor Receptor and c-erbB2 during the Development of Tamoxifen Resistance in Human Breast Cancer

Jacqueline C. Newby,2 Stephen R. D. Johnston, Ian E. Smith, and Mitch Dowsett
Departments of Academic Biochemistry [J. C. N., S. R. D. J., M. D.] and Medicine [J. C. N., S. R. D. J., I. E. S.], Royal Marsden Hospital, Fulham Road, London, SW3 6JJ United Kingdom

ABSTRACT

Expression of epidermal growth factor receptor (EGFR) or of c-erbB2 in primary breast cancer has been shown to predict for a poor chance of subsequent response of recurrent/metastatic disease to endocrine therapy. To assess the role of these receptors in the development of tamoxifen resistance, we examined their expression immunohistochemically on paraffin-embedded sections from breast cancers from 155 patients whose disease was progressing on tamoxifen therapy. Patients were categorized into those who initially responded to therapy (n = 56), those who never responded (n = 39), and those who relapsed while on adjuvant therapy and may or may not have "responded" (n = 60). In 61 cases, pretreatment specimens were also obtained for direct comparison with the resistance specimen for each patient.

None of the 18 pretreatment samples from patients who responded to therapy expressed c-erbB2, and 1 of 18 expressed EGFR. Of the nonresponders, 7 of 18 expressed EGFR pretreatment, and 4 of 18 expressed c-erbB2 (1 patient expressed both receptors). Results confirmed previous findings that when considered independently, expression of either receptor pretreatment tended to predict for a poor chance of response (EGFR, P = 0.046; c-erbB2, P = 0.11). Importantly, patients who were either EGFR positive and/or c-erbB2 positive had a much poorer chance of response than "double negatives" (response rates of 1 of 11 and 17 of 25, respectively; P = 0.0039).

At the time of disease progression compared to pretreatment, there was no significant change in expression of either receptor, irrespective of initial response. The inverse relationship between EGFR and estrogen receptor was maintained at relapse on tamoxifen. These data argue strongly against the acquired expression of these receptors during treatment playing a major role in the development of tamoxifen resistance in human breast cancer.

INTRODUCTION

Resistance to endocrine therapy is a major clinical problem in breast cancer. Tamoxifen is by far the most common endocrine agent in clinical use, and the majority of breast cancer patients receive it at some stage. Not all patients respond to treatment with tamoxifen, and almost all of those who do respond eventually develop resistance to its effects. Understanding of the mechanisms by which this resistance occurs would aid in the development of therapeutic strategies to avoid or overcome the problem. Potential mechanisms for resistance can be divided into three broad areas: (a) the pharmacodynamics of the drug may be altered, resulting in lower drug levels within the tumor (1) or in an altered balance of agonistic/antagonistic metabolites (2); (b) ER³ status is a strong, although not absolute, molecular determinant of response to tamoxifen (3). Variant forms of the estrogen receptor have been described, which are constitutively active and no longer dependent on ligand or inhibited by antagonists (4); (c) the effector pathways of cell proliferation downstream of the ER could be altered, resulting in independence from estrogenic control. There is substantial evidence from in vitro studies that indicate the involvement of growth factor pathways in the mediation of the proliferative signal from estrogen. The primary aim of the current study was to investigate certain of these pathways that may be perturbed in tamoxifen resistance.

EGFR and c-erbB2 (also known as neu/HER2) are members of a family of growth factor receptors with tyrosine kinase activity (5). Several ligands have been described for EGFR including epidermal growth factor, TGF-α, and amphiiregulin (5). Ligands have been described for c-erbB2 (6), although their physiological significance is disputed. On the basis of in vitro work, pathways involving the EGFR family of growth factors and receptors have been implicated in the mechanism by which estrogen stimulates and antiestrogens inhibit the growth of human breast cancer cells (7). Of the ligands for EGFR, TGF-α has been shown to be estrogen regulated in vitro via an estrogen response element at the 5' end of the TGF-α gene (8). TGF-α can replace the estrogen requirement of some hormone-dependent breast cancer cells in nude mice (9). Antibodies to TGF-α (10) or EGFR (11), antisense mRNA to TGF-α (12),

1 The abbreviations used are: ER, estrogen receptor; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; PgR, progesterone receptor.

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2 To whom requests for reprints should be addressed, at Meyerstein Institute of Oncology, Middlesex Hospital, Mortimer Street, London, United Kingdom. Phone: (44) 171-636-8333; Fax: (44) 171-436-0160.
and tyrosine kinase inhibitors (13) can all partially inhibit the response of breast cancer cell lines to estrogen. In vitro both EGFR (14) and c-erbB2 (15–17) can be down-regulated by estrogen in ER-positive breast cancer cells.

One mechanism by which cells could become resistant to tamoxifen would be for these growth factor pathways to become independent of the estrogen receptor and function autonomously. There is some experimental evidence to support a role for these growth factor receptor pathways in resistance to an-tiestrogen therapy. Transfection of c-erbB2 into the human breast cancer cell line MCF-7 results in cells that are estrogen independent and tamoxifen resistant both in vitro and when grown as xenografts in nude mice (18). In one study, ZR-75-1 cells (another human breast cancer cell line that is normally estrogen dependent and tamoxifen sensitive), when cultured long-term in the presence of tamoxifen, became tamoxifen resistant, and the resulting cell strain overexpressed EGFR compared to the parent cell line (19).

In vivo, both EGFR and c-erbB2 have been found to be overexpressed on some human breast cancer cells [Refs. 20 and 21; c-erbB2 often as a result of gene amplification (22), and EGFR usually with a normal gene copy number], and both have been found to result in a poorer prognosis (23, 24), although this is not confirmed in all studies, especially with respect to EGFR (25). In addition, they have been found, independently, to predict for a poor chance of response to endocrine therapy when expression in the primary tumor is correlated with endocrine response in recurrent/metastatic disease (26–31). c-erbB2 but not EGFR may also predict for a higher recurrence rate after adjuvant chemotherapy (32). The predictive value of these receptors in relation to endocrine responsiveness has led to speculation that they may be involved in the mechanism by which resistance to endocrine therapy develops. EGFR has been shown to have an inverse relationship with ER (20), a relationship that is highly consistent across many studies (25). It is, therefore, possible that the predictive value of the receptor for endocrine response is merely a reflection of this relationship with ER. However, the prognostic value of the growth factor receptors is often independent of, and occasionally more powerful than, that of ER/PgR. Also in a recent study, c-erbB2 predicted for poor prognosis following adjuvant tamoxifen therapy, specifically in the group of patients who were axillary node positive and progesterone receptor positive (33). It is, therefore, likely that these receptors have a more direct role than acting merely as markers for ER/PgR negativity.

Although the above data has led to speculation on the role of growth factors in tamoxifen resistance in patients, the hypothesis that enhanced growth factor receptor expression may explain tamoxifen resistance in breast cancer patients remains largely untested. In this study, we have examined the expression of EGFR and c-erbB2 immunohistochemically in specimens collected from 155 patients whose tumors had developed resistance to tamoxifen therapy. Wherever possible, paired pretreatment and relapse samples were obtained for each patient to allow direct comparisons to be made. In addition, clinical data were available to classify the patients according to initial responsiveness to therapy. This allowed an assessment of whether the mechanism for resistance differed between those patients who had an initial response to the treatment and subsequently relapsed and those who never responded at all.

**PATIENTS AND METHODS**

**Patients**

One hundred fifty-five patients with invasive breast cancer who were showing progressive disease on tamoxifen therapy were identified in outpatient clinics at the Royal Marsden Hospital, London, or the Mayday University Hospital, Croydon. Accessible progressing disease was biopsied/surgically excised, routinely fixed in formalin, and processed to paraffin blocks. In addition, for each patient the pretreatment diagnostic biopsy/surgical specimen was identified retrospectively and obtained for sectioning if available. Of the 155 cases, 61 had paired pretreatment and tamoxifen resistance specimens. Patient details are shown in Table 1, including ER/PgR status. A detailed analysis of expression of ER and the estrogen-regulated proteins PgR and pS2 in these paired pretreatment and relapse specimens has been reported previously (34).

The patients were all on tamoxifen therapy (either 20 mg/day or 40 mg/day) at the time of the biopsy/excision of the resistant tumor and could be divided into three groups on the basis of clinical information relating to their initial response to treatment (see below). For the purposes of this study, responders were defined as those patients achieving a complete or partial response according to Union International Contre Cancer
peroxidase was blocked using 3% hydrogen peroxide solution. Ethoxysilane-coated microscope slides and dried overnight at 37°C from the paraffin-embedded specimens onto 3-aminopropyltriethylendiamine solution (in dimethylformamide) plus 100 μl of hydrogen peroxide solution (30 volumes) per 100 ml of solution for 10 min, washed, counterstained in Mayer's hematoxylin, and then dehydrated, cleared, and mounted.

For all staining assays, slides of known positive and negative breast cancers were included as controls and also as standards for intensity of staining.

Scoring

**ER/PgR Assays.** We have previously validated the immunohistochemical assay for ER by comparison with the widely used enzyme immunoassay approach (36). For both ER and PgR, H-scores, which take account of both the percentage of cells staining and the intensity of staining for each positive cell (37), were calculated, and results ≥20 were considered positive.

**EGFR.** This method and scoring system was developed in our laboratory and validated against a ligand binding assay (38). Slides were scored for overall intensity on a scale of 0–3 (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining), and this was then multiplied by the percentage of cells staining to give a final score of 0–300. This modification of the H-score method was used as, in most cases, the intensity of staining was homogeneous throughout a positive section, and this modified system is significantly quicker to perform. Scores ≥35 were considered positive. Only membrane staining of malignant epithelial cells was taken into account while scoring these slides.

**c-erbB2.** Slides were considered positive if any malignant epithelial cells showed clear membrane staining (39). Cytoplasmic staining was disregarded when scoring these sections.

### Statistical Analyses

For analytical purposes, although quantitative scores had been obtained for ER/PgR/EGFR, positive/negative categorization was used, applying the cutoffs given above. Comparisons between rates of expression of the various receptors between groups were made using the χ² statistic (with Yates correction for small sample sizes). This method was also used to assess associations between the different receptors. Comparison of time to relapse was performed using the logrank statistic.

### RESULTS

**EGFR and c-erbB2 Immunostaining.** Positivity rates for expression of the receptors are illustrated in Figs. 1 and 2.
subdivided by the type of resistance shown. Overall, 19 of 76 (25%) pretreatment and 30 of 140 (21%) relapse specimens were EGFR positive. For c-erbB2, 10 of 76 (13%) pretreatment and 33 of 140 (24%) relapse specimens were positive ($P = 0.09$).

**Relationship between EGFR, c-erbB2, and Response to Tamoxifen.** Fig. 3 shows the expression of EGFR and c-erbB2 in pretreatment samples divided according to initial responsiveness to tamoxifen therapy. Expression of either receptor tended to be associated with lack of response to tamoxifen, although with the small numbers within these subgroups and the low proportion of positive cases overall, the statistical significance of these results is low (EGFR, $P = 0.046$; c-erbB2, $P = 0.11$). Only one patient who initially responded to tamoxifen therapy expressed either receptor in the pretreatment sample. Of the nonresponders, 7 of 18 pretreatment samples expressed EGFR and 4 of 18 expressed c-erbB2. Those tumors that expressed at least one of the two receptors showed a much reduced likelihood of response in comparison to tumors that were negative for both [response rates 1 of 11 (9%) and 17 of 25 (68%), respectively; $P = 0.0039$].

In the adjuvant group, the time to relapse was assessed in relation to EGFR and c-erbB2 expression. Fig. 4 shows progression-free survival curves for patients in the adjuvant resistance group, divided into those whose tumors were negative for both receptors compared to those whose tumors expressed one or both receptors. The difference between the curves was statistically significant ($P = 0.05$).

**Comparison of Rates of Expression between Resistance Groups.** For EGFR, expression was significantly more frequent in the de novo group than in the acquired resistance group specimens, both at pretreatment ($P = 0.045$) and at relapse ($P = 0.0002$). Expression of EGFR in the adjuvant group at relapse was significantly different from both the acquired group ($P = 0.04$) and from the de novo group ($P = 0.02$). For c-erbB2 expression at relapse, there were significant differences between acquired and adjuvant groups ($P = 0.04$) and between acquired and de novo groups ($P = 0.02$). The differences between these groups in pretreatment specimens were not statistically significant.

No significant association was seen between EGFR and c-erbB2 status in any group either pretreatment or at relapse.

**Change in Expression of EGFR/c-erbB2 at Relapse.** Although there were differences in the frequency of expression for both EGFR and c-erbB2 between the resistance subgroups, there were no significant changes in the rate of expression of the receptors in any group between the pretreatment and relapse samples (Figs. 1 and 2). The slightly higher expression of c-erbB2 in the relapse specimens for each group was not statistically significant. More importantly, when only the paired pretreatment and relapse specimens were directly compared, there was no significant change in the positive/negative status of either receptor for any resistance subtype (Tables 3 and 4). In all groups, there were a small number of individuals who changed phenotype during the development of resistance, but the nature
of the change was not consistent for any subgroup, and the majority of cases showed no change in phenotype.

Relationship with Estrogen Receptor Status. There was a significant inverse relationship pretreatment between ER status and EGFR expression, $P = 0.0003$ (Table 5). This relationship was maintained at relapse ($P = 0.003$). Only 2 of 76 tumors at presentation and 2 of 140 at relapse were ER+/EGFR+. Twenty-two of 76 (29%) were "double negative" pretreatment, and 70 of 140 (50%) were "double negative" at relapse ($P = 0.004$), but this was due to a reduction in ER positivity without any change in EGFR phenotype. No significant relationship was seen between c-erbB2 and ER.

**DISCUSSION**

The interactions between various growth factors and/or their receptors and steroid receptors in human breast cancer and the responsiveness of breast cancer cells to endocrine therapy has been investigated extensively. The majority of this published work has been performed on cell lines in culture or in animal model systems. It is important that hypotheses derived from such experimental studies are tested in the more complex clinical setting. Here we have attempted to test the hypothesis that aberrations within EGFR-related growth factor pathways, resulting in the independence of these pathways from estrogenic control may be a mechanism for tamoxifen resistance in vivo. If so, one would predict an increase in expression of the receptors in specimens taken from resistant tumors with initially negative tumors acquiring receptor expression. One would also expect to see loss of any association between expression of steroid receptors and expression of the tyrosine kinase receptors. Normally, there is a clear inverse relationship between the expression of ER and EGFR in primary tumors.

The patient population used for this study consisted of cases whose tumors had developed resistance to tamoxifen therapy. This group was not ideal for assessing prediction of response from the pretreatment phenotype because these patients were identified at relapse; therefore, responders to treatment who had not yet relapsed were not represented in the pretreatment group. Thus, the difference between the responders and nonresponders at treatment was likely to be underestimated. The decision to include patients whose tumors remained stable on tamoxifen for some time before developing true resistance in the de novo resistance subgroup would also tend to reduce any differences between the two groups. This group of patients does have a useful clinical response to treatment, and their survival rates are similar to those seen in patients achieving an objective response. However, for the purposes of this study, it was felt important that the acquired resistance subgroup should be rigidly defined; hence, the exclusion of the stable disease group.

The collection of material from patients relapsing on adjuvant tamoxifen therapy included a group of tumors that was more difficult to analyze because initial responsiveness of these tumors was unknown; also, many of these patients had received
other therapies. However, at relapse, these tumors were clearly resistant to tamoxifen, and with the almost universal use of adjuvant tamoxifen in oncology today, this group will form the bulk of resistant tumors available for future study. As only those cases relapsing while still on adjuvant tamoxifen were included (i.e., relatively early recurrences), this group will tend to have a poorer than average prognosis.

The relatively small number of cases in which the pretreatment specimen could be obtained for direct paired comparison with samples taken at the time of resistance was disappointing. The reasons for this were varied, including the fact that many of the patients treated with primary tamoxifen therapy were elderly women in whom the diagnosis was made by fine-needle aspiration cytology in conjunction with mammographic and clinical findings or by small Tru-cut biopsy, of which too little remained for meaningful analysis in this study. Because the reasons were not related in any way to the initial responsiveness of the tumors to tamoxifen nor to the development of resistance, there was unlikely to be any bias introduced by the selection of these cases. This bank of paired specimens is still the largest cohort of such tumors in the literature to date. The increasing use of adjuvant tamoxifen and the decreasing use of primary tamoxifen in elderly patients mean that larger groups of such samples will prove difficult to accumulate in a prospective fashion.

The frequency of expression of c-erbB2 and EGFR in pretreatment samples taken as a whole was comparable to those reported in other published series measuring expression of these receptors in primary breast cancers. The EGFR positivity was toward the lower end of the range reported but was consistent with our previous experience using this immunohistochemical assay and scoring system (39). An advantage of the immunohistochemical approach was that, in contrast to ligand binding assays, false positives due to expression of the receptor in benign components are excluded. It is probable, therefore, that the discrepancy with published series was due at least in part to “false positives” in these publications rather than “false negatives” in this study.

The primary aim of this study was to test the hypothesis that aberrations of pathways involving these growth factor receptors could explain the development of tamoxifen resistance in human breast cancer. We have found no evidence for this. Although the incidence of expression of either receptor prior to treatment was greater in the nonresponders when compared with the responsive group, there was no significant increase in expression of either receptor at relapse for any of the response subgroups (Figs. 1 and 2). The slightly higher frequency of c-erbB2 expression seen at relapse in all subgroups was not statistically significant. More importantly, when the paired treatment and relapse specimens were directly compared, only a minority of cases changed phenotype, and even in these few cases, the direction of the change was not consistent (Tables 3 and 4).

Another argument against this hypothesis is that the inverse relationship between ER and EGFR, a consistent finding in the literature and present in the pretreatment specimens, was maintained at relapse. Although some tumors showed reduced ER expression, this was not accompanied by any increase in EGFR positivity. If pathways involving this receptor became independ-
Table 3  Change in expression of EGFR in paired pretreatment and relapse specimens, according to resistance subtype

<table>
<thead>
<tr>
<th>EGFR at presentation</th>
<th>Acquired group</th>
<th>De novo group</th>
<th>Adjuvant group</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>−ve</td>
<td>0</td>
<td>14</td>
<td>7</td>
</tr>
</tbody>
</table>

* +ve, positive; −ve, negative.

Table 4  Change in expression of c-erbB2 in paired pretreatment and relapse specimens, according to resistance subtype

<table>
<thead>
<tr>
<th>c-erbB2 at presentation</th>
<th>Acquired group</th>
<th>De novo group</th>
<th>Adjuvant group</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>−ve</td>
<td>0</td>
<td>15</td>
<td>2</td>
</tr>
</tbody>
</table>

* +ve, positive; −ve, negative.

Fig. 4  Time to progression on tamoxifen therapy in adjuvant resistance patients expressing either EGFR or c-erbB2 prior to treatment (broken line) or in patients expressing neither receptor (solid line).

Ent of ER-regulated pathways as a mechanism for resistance, then one would expect this relationship to be disturbed at relapse with an increase in cases expressing both ER and EGFR. The pathways involving these growth factor receptors are complex, and care should be taken when interpreting expression of isolated components. However, these data were consistent with our observations that the level of expression of TGF-α, a ligand for EGFR that seems to be important in the control of breast cancer cell growth in vitro at least, was also not significantly increased at relapse in cases of acquired resistance. There was an increase in TGF-α levels at relapse in the de novo resistant cases, which is likely to be significant as it was largely confined to those tumors that expressed EGFR at relapse.

Previous studies have reported that expression of either EGFR or c-erbB2 tends to predict for a poor chance of response to endocrine therapy (26–30). These studies have examined surgical samples from primary tumors and related the phenotype in these to endocrine response in recurrent/metastatic disease. Responsiveness and behavior of the recurrent/metastatic disease may be different from that of the primary tumor on which the receptors were measured. We believe this study to be the first to examine the relationship between EGFR/c-erbB2 and endocrine responsiveness where in all cases the tumor used to determine phenotype and that in which endocrine responsiveness was assessed were the same. Another advantage of our data was that all patients had received only tamoxifen as endocrine therapy; no other endocrine agents were used. Different endocrine agents have different mechanisms of action; therefore, it is likely that mechanisms of resistance will also differ.

Our results confirm that expression of either receptor alone tended to predict a poor chance of response to tamoxifen (Fig. 3) in that tumor. Our finding that expression of either receptor (compared to expression of neither) was a much stronger predictor of poor chance of response than expression of either receptor alone (compared to lack of expression of that receptor) confirms the findings of Wright et al. (26). An additive effect of the two receptors, with “double positives” having a poorer response/prognosis than cases expressing a single receptor, has been noted in some series (26, 30). Our set contained too few “double-positive” cases to be able to comment on this effect. The information from the group of patients relapsing on adjuvant treatment, for whom we did not have direct response data, did nevertheless provide supportive evidence that this group of “double negatives” appeared to be different from the group expressing one or other, or both receptors. The median time to relapse for the EGFR+/c-erbB2+ patients was 30 months compared to 14.5 months for cases expressing one or both of the receptors (Fig. 4). This is an indication that, among those patients who relapsed on adjuvant tamoxifen treatment, those expressing either EGFR or c-erbB2 were likely to do so earlier than those who did not express either receptor.

The biology of this group of receptors is such that coexpression of two or more members may be of relevance. The receptors are able to form heterodimers allowing cross-activation of the tyrosine kinase activity of one receptor in the absence of cognate ligand (40). Heregulin was originally described as a ligand for c-erbB2 and has only recently been recognized as binding to either c-erb-B3 or -B4, resulting in cross-activation of c-erbB2 in cells where the receptors are coexpressed (41). There are studies that suggest that the combination of receptors in an activated dimer may be responsible for the specificity of the signal received. Thus, activation of different dimer combinations has been shown to result in activation of different signal transduction pathways (42). This receptor “cross-talk” may also be important in growth regulation in breast cancer. Clearly, the current data relate to expression of receptors only and consider two of the members of this complex receptor family. No conclusions can be drawn from these results relating to receptor function, activation state, or to coexpression with c-erbB3 or c-erbB4, all of which warrant further investigation.

It is possible that the predictive value of these receptors for endocrine response is merely a reflection of their inverse relationship with ER for which the positive predictive value for endocrine response is well established. Indeed, it is important to note that in this group of patients, ER status was the most powerful predictor of response, with 87% of ER-positive cases responding and only 10% of ER-negative cases showing any response. In only one case would EGFR/c-erbB2 have assisted in response prediction with an ER-positive nonresponder that was also EGFR positive (but c-erbB2 negative). The nature of the interaction between steroid receptors and EGFR/c-erbB2 in vivo is not fully understood. In vitro it has been shown that in ER-positive human breast cancer cell lines, estrogen stimulation results in a transient increase in transcription of the EGFR gene, possibly directly via imperfect estrogen response elements, but then results in down-regulation to basal levels of EGFR mRNA and protein (43). This down-regulation requires protein synthesis. In examining the expression of ER and EGFR in the relapse specimens from these patients, it is of interest that there was no increase in the rate of EGFR positivity, although a number of these tumors lose ER expression at relapse. This implies that EGFR expression may not be simply repressed by ER in these tumors, but that other regulatory mechanisms are important.

In conclusion, these results confirm that expression of c-erbB2 or EGFR prior to treatment predicts a poor chance of response to tamoxifen therapy. However, tumors initially negative for expression of these receptors do not acquire expression during treatment to explain the development of resistance to tamoxifen. Nor is there any evidence for dissociation of expression of ER and EGFR in the resistant tumors. Thus, the data derived from clinical material do not support the initial hypothesis.

**REFERENCES**


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