Quantitative Estimation of Epidermal Growth Factor Receptor and c-erbB-2 in Human Breast Cancer

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ABSTRACT

Epidermal growth factor receptor (EGFR) expression by human breast cancer has been shown to predict poor patient outcome, as has amplification of the c-erbB-2 proto-oncogene. We have developed a quantitative immunohistochemical method for measuring protein levels of both receptors and have applied this to a series of 123 breast primaries. We find EGFR expression is substantially lower than normal in nearly all breast cancers (97%). Quantification of p185c-erbB-2 indicates overexpression in 91% of the tumors. Two separate tumor populations are apparent with levels of c-erbB-2 expression ranging from 0.33 to 19 and 45 to 48 (times normal, respectively. Within the lower population, p185c-erbB-2 expression is inversely related to EGFR expression (rank correlation, P < 0.0005). Using fluorescent in situ hybridization we show that tumors in the latter population have c-erbB-2 amplification and that amplification is restricted to this group. Our findings indicate that significant overexpression of p185c-erbB-2 occurs in the absence of amplification; these lower levels of expression may have functional significance. Fifty-three patients underwent in vivo bromodeoxyuridine labeling, allowing flow cytometric analysis of tumor cell cycle kinetics. EGFR expression correlates directly to the labeling index (P = 0.011) and indirectly to potential doubling time (P = 0.010), but not to the duration of the S-phase (P = 0.502). Conversely, p185c-erbB-2 expression does not relate to indices of proliferation. Our results have important implications for the use of both receptor types as therapeutic targets.

INTRODUCTION

Considerable effort in breast cancer research has focused on the measurement of the type I tyrosine kinase receptors with the aim of defining their significance as indicators of prognosis and their role in tumor biology. However, receptor quantification using conventional methods shows a wide variation which may have obscured these targets. EGFR expression in human breast cancers was first reported by Sainsbury et al. (1), who went on to show that patients whose tumors had detectable levels of EGFR using a ligand-binding analysis had a poorer prognosis than those who had EGFR-negative lesions (2, 3). This finding has largely been corroborated by subsequent studies using both ligand-binding and immunohistochemical methods [reviewed by Klijn et al. (4)]. Ligand-based studies show EGFR expression in a mean of 49% of the cases with an interstudy range of 16–91% (4); such a wide range is in part the result of poor methodological standardization. Furthermore, although these assays are quantitative, they are performed on membrane preparations derived from tumor biopsies which include, besides malignant cells, nontumor elements, including normal breast, in situ disease, connective tissue, and lymphoid cells. As such the technique is inherently flawed, since the receptor measurement reflects the level of expression by the tumor mass rather than by the carcinoma cells. A review of immunohistochemical studies shows that about 40% of breast tumors express the EGFR, but here also is a wide interstudy range of 14–65% (4). The subjective nature of immunohistochemical scoring and, again, a low level of standardization between laboratories are likely to account for this variation. These problems have resulted in a call for standardization of EGFR assay procedures (5).

c-erbB-2 gene amplification was first reported as a significant predictor of both overall survival and time to first relapse in breast cancer by Slamon et al. (6). Gene amplification predicts overexpression of the c-erbB-2 protein but overexpression has been reported in a small proportion of tumors without the presence of amplification (7). Overall, c-erbB-2 overexpression is reported in approximately 20% of primary breast cancers with a wide interstudy range of 9–39% [reviewed by Gullick (8)]. A poor level of interlaboratory standardization is likely to account for this variation. A number of putative ligands to p185c-erbB-2 has been proffered but these have been proven to bind to other members of this receptor family [reviewed by Earp et al. (9)]. Ligand-binding techniques are thus not applicable to this receptor. ELISA and Western blotting methods have been used to measure c-erbB-2 levels in tumor extracts, but these suffer from the same drawbacks as the ligand-binding assay for EGFR.

The problems associated with the EGFR and c-erbB-2 assays may compromise the accuracy of the data obtained from these studies and therefore our understanding of the precise relationship between receptor expression, pathochoclinical variables, and the biology of breast cancer. We have therefore developed a quantitative radioimmunohistochemical method for the measurement of both receptor types in frozen tissue sections. This assay uses radiodinated antireceptor monoclonal antibody to label the receptors and computer-assisted image analysis to quantify the bound antibody to combine the objective quantification of ligand-binding analysis with the specificity of immunohistochemistry.

Activation of either EGFR or p185c-erbB-2 is potentially mitogenic in benign and malignant breast cells, and a number of investigators have explored the possibility that tumors overexpressing these receptors have a proliferative advantage. EGFR expression has been found to be associated with increased levels of cell cycle-related antigens Ki-67 (10, 11) and proliferating cell nuclear antigen (12). Evidence is less uniform for c-erbB-2, although some authors did find evidence of such an association (12, 13), a number of others have not (11, 14–16). We have addressed the issue of a hypothesized proliferative advantage related to receptor overexpression using the thymidine analogue bromodeoxyuridine to preoperatively label tumors and thus obtain a dynamic assessment of tumor cell proliferation. In this article, we report the application of radioimmunohistochemical quantification of EGFR and c-erbB-2 to a series of over 100 breast cancers and relate receptor expression to pathological and clinical variables and to tumor cell kinetics.

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The abbreviations used are: EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; CV, coefficient of variation; ER, estrogen receptor.
MATERIALS AND METHODS

Tumors. One hundred twenty-three primary breast cancer biopsies taken during mastectomy or lumpectomy procedures in Glasgow Royal Infirmary between 1984 and 1994 formed the study material for the project. EGFRs were measured in 118 and the c-erbB-2 protein in 81. Both receptor types were quantified in 75. The tumor specimens were frozen in liquid nitrogen and stored at −70°C. Pathological data including tumor size (TNM), modified Bloom and Richardson grade (17), and axillary lymph node involvement were recorded by the pathologist reporting on that specimen. ERs were assayed in frozen tumor samples with the dextran-coated charcoal method with a cutoff of 20 fmol/mg. Control Tissues. Specimens of histologically normal breast were obtained from time reduction mammoplasty procedures and treated in a similar manner to the tumor biopsies. The median age of these patients was 55.5 (range, 17–49) years.

Receptor Quantification Using Radioimmunohistochemistry. EGFRs were quantified using the radioimmunohistochemical method as described previously (18, 19), and the c-erbB-2 protein was quantified using a modification of this method. Briefly, the EGFR1 monoclonal antibody (20) and the ICR12 anti c-erbB-2 monoclonal antibody (21) were labeled with 125I using the Iodo-Gen method (22) to a specific activity of 0.5 MBq/µg (ICR12 was a gift from Dr. C. Dean, Institute of Cancer Research, London, England). Crystall sections (5 µm) were thawed onto siliconized slides and stored at −20°C in air tight packaging until use. The sections were fixed in absolute acetone, washed in PBS (10 mM sodium phosphate and 140 mM sodium chloride, pH 7.4), and incubated with 100 µl of 50% normal rabbit serum for 10 min before 10 µl of the radiolabeled antibody were added. The specific activity of the iodinated antibody was adjusted with unlabeled antibody so that 50 ng (4 KBq) of EGFR1 or 50 ng (1 KBq) of ICR12 were added to each section. Duplicate test sections and a single control section (incubated with a 100-fold excess of unlabeled antibody) were processed for each specimen. Incubations were carried out for 3 h in humidified chambers at 22°C before the sections were washed through three 10-min changes of PBS, fixed for 10 min in 2% formaldehyde, and washed through three changes of distilled water before being air dried. The slides were secured into an X-ray cassette and overlaid with X-ray film (Dupont Cronex) which was exposed for about 72 h. This film was used as a guide to the necessary length of exposure for the final stage of the preparation, where the slides were dipped in autoradiographic emulsion (Kodak NTB-2 diluted 1:1 with distilled water at 43°C). After air drying, exposure was carried out at 4°C in the presence of silica gel as dehumidifier, with the highly expressing tumors and cell lines being exposed for 4 h, the intermediate tissues for 24 and 48 h, and the weakly expressing specimens for 4 to 7 days (the correct exposure results in a sufficient density of single grains for counting). The emulsion was developed with Kodak D 19 developer (1:1 in distilled water, the sections were counterstained lightly with safranin, and incubated with 100 µl of 50% normal rabbit serum for 10 min before 10 µl of the radiolabeled antibody were added. The specific activity of the iodinated antibody was adjusted with unlabeled antibody so that 50 ng (4 KBq) of EGFR1 or 50 ng (1 KBq) of ICR12 were added to each section. Duplicate test sections and a single control section (incubated with a 100-fold excess of unlabeled antibody) were processed for each specimen. Incubations were carried out for 3 h in humidified chambers at 22°C before the sections were washed through three 10-min changes of PBS, fixed for 10 min in 2% formaldehyde, and washed through three changes of distilled water before being air dried. The slides were secured into an X-ray cassette and overlaid with X-ray film (Dupont Cronex) which was exposed for about 72 h. This film was used as a guide to the necessary length of exposure for the final stage of the preparation, where the slides were dipped in autoradiographic emulsion (Kodak NTB-2 diluted 1:1 with distilled water at 43°C). After air drying, exposure was carried out at 4°C in the presence of silica gel as dehumidifier, with the highly expressing tumors and cell lines being exposed for 4 h, the intermediate tissues for 24 and 48 h, and the weakly expressing specimens for 4 to 7 days (the correct exposure results in a sufficient density of single grains for counting). The emulsion was developed with Kodak D 19 developer (1:1 in distilled water at 43°C). After air drying, exposure was carried out at 4°C in the presence of silica gel as dehumidifier, with the highly expressing tumors and cell lines being exposed for 4 h, the intermediate tissues for 24 and 48 h, and the weakly expressing specimens for 4 to 7 days (the correct exposure results in a sufficient density of single grains for counting).

Flow Cytometry. Fifty-three patients consented to the administration of an i.v. 200 mg bolus of the thymidine analogue bromodeoxyuridine. (Administration of bromodeoxyuridine was approved by the Royal Infirmary Ethical Committee, and all patients gave informed consent as per the Helsinki Declaration.) This drug becomes incorporated in the S-phase tumor cells and can be identified using monoclonal antibody to the halogen epitope. The time interval between in vivo labeling, and freezing of tumors was recorded. Subsequent dual parameter flow cytometric analysis of tumor biopsies for bromodeoxyuridine and DNA contents allowed calculation of the labeling index, the length of S-phase, and the potential doubling time (24, 25).

Statistical Analysis. Rank correlations were performed using the Spearman method. The relationships between receptor expression and known prognostic indicators were assessed using either the Kruskal-Wallis analysis or the Mann-Whitney U test. All statistical studies were performed using the Minitab for Windows package (Minitab Release 9.2).

RESULTS

Receptor Expression

Levels in Normal Breast. Nine reduction mammoplasty tissue specimens were used as normal controls for comparison to the tumor samples. Throughout this article, EGFR and p185c-erbB-2 levels are expressed relative to the mean of levels in this normal tissue. Expression levels above or below these means are reported as overexpression or underexpression, respectively. EGFR levels in the normal specimens were expressed as a percentage of the receptor density of one of the cell line standards [A431 cells which are known to have 2 × 10⁶ EGFR/cell (18, 19)] which was processed with each batch of tissue samples. The normal levels ranged from 5.2 to 10%, with a mean of 7.6% of the A431 levels. c-erbB-2 levels in the breast reduction samples were expressed similarly using BT474 cells as a standard, which have 7.3 × 10⁶ c-erbB-2 molecules/cell. The normal range was from 1.4 to 4.2% (mean, 2.7%) of the BT474 levels.

Levels in Breast Cancers. Fig. 2 summarizes the results of receptor estimation by radioimmunohistochemistry. With regard to EGFR, all but three of the tumors show lower levels of expression than...
control breast reduction sections. Eighty-nine percent have less than half the normal number of receptors and 60% less than 10% of this value. Thus, the majority of breast cancers show levels of EGFR expression that are much lower than normal, and only a very small percentage show levels of expression greater than normal.

Analysis of c-erbB-2 expression clearly shows it to be bimodal (Fig. 2). Only seven tumors have c-erbB-2 levels below normal. The first peak, 64 tumors (79%), includes these low-expressing tumors and has a range of expression extending from 0.33 to 19 times normal (median, 4.1 times). The second frequency peak, 17 tumors (21%), ranges from 44.8 to 479 times normal (median, 187 times). In total, 91% overexpress the c-erbB-2 protein.

**FISH Analysis of erbB-2 Gene Amplification.** Fifteen tumors from each peak of p185*erbB-2* expression were analyzed using FISH for erbB-2 proto-oncogene amplification (Fig. 3). Sections were assessed by one of the authors (W. N. K.) without knowledge of the radioimmunohistochemical results. One tumor sample from peak 2 was considered inadequate for study, the remaining peak two tumors all showed erbB-2 gene amplification. Those from the lower expressing peak 1 did not show gene amplification, except for one that was considered to have low level amplification. Interestingly, this tumor demonstrated the highest p185*erbB-2* expression of those in peak 1.

**Correlation between c-erbB-2 and EGFR.** In the 75 tumors for which values for both receptors were available, there was a trend toward inverse correlation (Spearman's rank correlation coefficient, $r^2 = 0.042$, $P = 0.076$; Fig. 4). Exclusion of the second peak, i.e., those tumors with amplification of the erbB-2 gene, revealed a highly significant inverse correlation of receptor expression ($r^2 = 0.239$, $P < 0.0005$). No such correlation occurred within the amplified tumors ($r^2 < 0.0005$, $P = 0.951$).

**Reproducibility.** Intraassay variation was assessed by comparing grain densities in the duplicate sections of each tumor. In the 25
tumors of one single batch of the EGFR assay, the result for one section was divided by that for the other. The CV of these ratios was 14.8%. The Spearman rank correlation coefficient for the two series of results was 0.927, \( P < 0.0005 \).

Interassay variation for the cell pellets run as standards was assessed across all four batches of the c-erbB-2 assay. The mean CV of the ratios between different cell line pellets within each batch was 33.9%.

Interassay variation for tissue sections was assessed using the normal breast control run with all 10 batches of EGFR analysis. The CV of the 10 estimations on this specimen was 36.4%.

These results show a very small range of variation compared with the overall range of receptor expression.

**Correlation to Prognostic Indicators**

Analysis of EGFR expression in relation to known prognostic indicators (Table 1) shows correlation to a higher histological grade as defined by a modified Bloom and Richardson score and an association with ER negativity. EGFR expression was not related to tumor size (TNM) or nodal status.

c-erbB-2 expression showed no association with tumor size, nodal status, or tumor grade (Table 2). Although not statistically significant, the ER-negative group showed a trend toward higher c-erbB-2 expression.

Splitting c-erbB-2-expressing tumors into unamplified (peak 1) and amplified (peak 2), the former show no significant relationship to...
known prognostic indicators (tumor size (TNM), \( P = 0.946 \); nodal status, \( P = 0.979 \); histological grade, \( P = 0.130 \); and ER status, \( P = 0.434 \)). Similarly, the amplified tumors show no relationship to these prognostic factors with the exception of ER status (tumor size (TNM), \( P = 0.423 \); nodal status, \( P = 0.821 \); and histological grade, \( P = 0.168 \)). Of the 17 tumors in peak 2, ER status is available on 8, and all are ER negative. All tumors amplified using FISH and with ER status available are ER negative (five tumors).

**Cell Kinetic Studies**

Fifty-three tumors were bromodeoxyuridine labeled. Samples from these tumors were analyzed using flow cytometry for the labeling index, S-phase duration, and potential doubling time. The labeling index showed correlation to EGFR expression (Fig. 5a; Spearman’s rank correlation, \( r^2 = 0.120, P = 0.011 \)). A similar analysis was performed on the 45 tumors labeled and assayed for c-erbB-2 (Fig. 5b) but no correlation was observed (\( r^2 = 0.010, P = 0.517 \)). Assessing each peak of c-erbB-2 expression for correlation with the labeling index also failed to demonstrate a significant relationship (\( r^2 = 0.007, P = 0.645 \) and \( r^2 = 0.059, P = 0.529 \) for peaks 1 and 2, respectively). Since potential doubling time is a function of the labeling index, it is not surprising to find a correlation between this index and EGFR expression (\( r^2 = 0.125 \) and \( P = 0.010 \)) but not c-erbB-2 expression (\( r^2 = 0.004 \) and \( P = 0.678 \)). Expression of neither receptor correlated to the duration of the S-phase (\( r^2 = 0.009, P = 0.502 \) and \( r^2 < 0.0005, P = 0.909 \) for EGFR and c-erbB-2, respectively).

**DISCUSSION**

Radioimmunohistochemistry combines the objective quantification of ligand-binding studies with the tissue specificity of immunohistochemistry. These features provide improved accuracy of EGFR and p185
\(^{erb-2} \) quantification. This has produced a number of novel findings:

(a) EGFR is down-regulated in the vast majority of breast cancers;
(b) most breast cancers overexpress p185
\(^{erb-2} \); (c) there is a bimodal distribution of c-erbB-2 expression, with at least two mechanisms producing overexpression; and (d) in those breast cancers without c-erbB-2 gene amplification, expression of EGFR and c-erbB-2 are inversely correlated.

The fact that normal breast shows detectable expression of EGFR has been noted in previous reports (26, 27). The assumption has been made that the levels of expression in normal breast, although detectable, are low (28), and that tumors with assayable levels of EGFR are therefore overexpressing this marker relative to their tissue of origin. In keeping with the previous reports of Dittadi et al. (26) and Osawa et al. (29), our results are not compatible with this. What we take to be low or high levels of EGFR expression are obviously arbitrary, but within this series all but three tumors show levels of expression below those of normal breast. This would fit well with the low incidence of EGFR gene amplification in breast cancers (6). However, these low levels of receptor expression may still be of functional significance as indicated by the prognostic significance of EGFR expression in previous studies (2–4) and the relationship to adverse grade and ER status in this series. The low levels of EGFR detected in the tumors that we have studied are consistent with at least two explanations. It is possible that the presence of large amounts of ligand might be present within the tumors, the binding of which may be resulting in internalization of most of the receptors; i.e., that the low number of

\[ \text{Table 1 Relationship between prognostic factors and EGFR expression} \]

<table>
<thead>
<tr>
<th>Tumor size a</th>
<th>n (%)</th>
<th>Median EGFR (x normal)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>31 (27)</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>67 (59)</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>9 (8)</td>
<td>0.124</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>6 (5)</td>
<td>0.070</td>
<td>0.864</td>
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<table>
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<tr>
<th>Nodal status</th>
<th>n (%)</th>
<th>Median EGFR (x normal)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Negative</td>
<td>56 (53)</td>
<td>0.052</td>
<td>0.283</td>
</tr>
<tr>
<td>Positive</td>
<td>50 (47)</td>
<td>0.075</td>
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</table>

<table>
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<tr>
<th>Histological grade b</th>
<th>n (%)</th>
<th>Median EGFR (x normal)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI</td>
<td>7 (16)</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>GII</td>
<td>13 (30)</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>GIIII</td>
<td>24 (55)</td>
<td>0.217</td>
<td>0.006</td>
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<th>n (%)</th>
<th>Median EGFR (x normal)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>26 (44)</td>
<td>0.140</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33 (56)</td>
<td>0.032</td>
<td>0.002</td>
</tr>
</tbody>
</table>

"For tumor size and histological grade a Kruskal-Wallis statistical analysis was used; for nodal status and ER status the Mann-Whitney U test was used. For both types of analysis, \( P < 0.05 \) was considered significant. Median age 62.4 (range, 40–92) years. The TNM classification was used for tumor size.

b A modified Bloom and Richardson score (17) was used for histological grade.

\[ \text{Table 2 Relationship between prognostic factors and c-erbB-2 expression} \]

<table>
<thead>
<tr>
<th>Tumor size a</th>
<th>n (%)</th>
<th>Median c-erbB-2 (x normal)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>21 (28)</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>44 (58)</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>8 (11)</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>3 (4)</td>
<td>3.6</td>
<td>0.650</td>
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<tr>
<th>Nodal status</th>
<th>n (%)</th>
<th>Median c-erbB-2 (x normal)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Negative</td>
<td>38 (51)</td>
<td>3.6</td>
<td>0.218</td>
</tr>
<tr>
<td>Positive</td>
<td>36 (49)</td>
<td>5.6</td>
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<table>
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<tr>
<th>Histological grade b</th>
<th>n (%)</th>
<th>Median c-erbB-2 (x normal)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI</td>
<td>7 (18)</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>GII</td>
<td>11 (28)</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>GIIII</td>
<td>21 (54)</td>
<td>3.2</td>
<td>0.282</td>
</tr>
<tr>
<td>ER status</td>
<td>n (%)</td>
<td>Median c-erbB-2 (x normal)</td>
<td>P</td>
</tr>
<tr>
<td>Negative</td>
<td>18 (44)</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23 (56)</td>
<td>3.6</td>
<td>0.124</td>
</tr>
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</table>

"For tumor size and histological grade a Kruskal-Wallis statistical analysis was used, for nodal status and ER status the Mann-Whitney U test was used. For both types of analysis, \( P < 0.05 \) was considered significant. Median age 61.0 (range, 40–80) years. The TNM classification was used for tumor size.

b A modified Bloom and Richardson score (17) was used for histological grade.
c-cr/ïB-2(h) are expressed relative to the mean level against receptor expression. EGFR (a) and than of low receptor production. Alternatively, there may be low cell surface receptors is a reflection of high receptor turnover rather than of low receptor production. Alternatively, there may be low receptor production that is true underexpression.

We demonstrate quite a different pattern of c-erbB-2 expression. We find levels of c-erbB-2 expression higher than those in normal tissue in 91% of the tumors. In addition, there is a clearly evident and not previously reported bimodal distribution of receptor density. Previous studies suggest gene amplification in approximately 20% of breast tumors [reviewed by Gullick (8)], and the highest rate of protein overexpression recorded is 39% (30). Amplification is always accompanied by mRNA and protein overexpression; however, overexpression can occur independently of amplification (6, 31), and thus deregulated transcription may also be an important factor influencing c-erbB-2 expression. Our ability to clearly define two populations of c-erbB-2-expressing tumors develops from use of the radioimmunohistochemical technique which, with image analysis, provides an objective and quantitative estimation of receptor expression not available using other methods. The defined populations are quite distinct, the first has a mean level of expression of 4.1 times that of normal breast (range, 0.33–19), and the second a mean of 190 times normal (range, 45–480). Our FISH studies suggest that these populations reflect the mechanism of overexpression; amplification is restricted to the second peak, and an alternative mechanism is responsible for the first. The majority of the preceding studies have identified only those tumors with amplification of the gene, which in our study implies receptor expression greater than 40 times that of normal breast. This assertion is supported by our finding of amplification in 21% of the tumors studied, a rate concordant with the immunohistochemically reported rate of c-erbB-2 overexpression for invasive disease. The mechanism of overexpression within the first peak is unclear; it may reflect overtranscription or indeed reduced receptor turnover. However, with levels within the first peak as high as 19 times normal (mean, 4.1 times), it is certainly possible that c-erbB-2 is functionally important in these tumors.

Insufficient information regarding the outcome is presently available in the current tumor series for assessment of the prognostic significance of c-erbB-2 measured using radioimmunohistochemistry, but data are available for other recognized prognostic indicators. Analyzing c-erbB-2 as a continuous variable reveals no statistically significant associations to these prognostic indicators. As a subsidiary analysis, we have analyzed our data in this manner: $x^2$ analysis using a cutoff of six times normal receptor expression versus tumor size (TNM) reveals a trend to significance ($P = 0.086$). Association with nodal status is significant at the same level ($P = 0.029$), and in both cases significance is lost at higher and lower rates of receptor expression. ER status develops significance at this level and maintains it at higher levels; no relationship to grade is evident. Such a cutoff is arbitrary but the functionally important level of c-erbB-2 expression may be lower than that associated with amplification. We accept our series is a relatively small one, and thus such statistical conclusions must be tempered with caution; however, we believe this avenue requires further exploration because it has both prognostic and therapeutic implications.

There has been extensive investigation of the interaction between members of the type I tyrosine kinase family (9). Evidence supports the formation of heterodimers (32, 33) which may facilitate receptor function. Both receptors also function via similar kinase pathways (34, 35). Studies to date generally infer that overexpression of both receptors carries a worse prognosis than overexpression of either alone, although studies of the relative levels of each receptor are lacking. Using radioimmunohistochemistry, we have been able to accurately assess receptor expression revealing an inverse relationship between EGFR and c-erbB-2 expression, which is disrupted by c-erbB-2 amplification. This suggests the possibility of a coregulation mechanism which fails with gene amplification, or that, in the presence of very high levels of p185$^{c-erb-B-2}$ expression, a reduction in EGFR is not selected for.

It can be argued that tissue from reduction mammoplasty specimens is not the ideal control for breast cancer specimens. These patients are younger than the majority of cancer cases, and it is possible that in normal breast lobules receptor expression changes with age. This raises the issue of what truly normal breast tissue is available to use as a control. Histologically normal breast tissue in the cancer specimens show the same levels of expression as our controls; however, these areas may be subject to some of the genetic changes that have resulted in the adjacent pathology. This argument is less strong for breast adjacent to benign lesions; however, these also tend to occur in younger age groups. Very rarely is completely normal breast tissue removed from middle aged or older women. The other potential problem with the use of reduction mammoplasty specimens as a control is the indication for the operation itself; it is possible that the breasts in these patients are very large because of overexpression of growth factor receptors. There is to our knowledge absolutely no evidence that this is so, and as we have suggested, areas of histologically normal breast within the tumors show similar receptor densities. Since the consensus origin of breast carcinoma is at the level of the terminal ductulo-lobular unit (36, 37), lobular tissue from the reduction mammoplasty specimens was used for our control measurements.

Parameters of tumor growth have been extensively studied, although to date the prognostic power of indices of breast tumor growth...
are poor compared to pathological variables such as nodal involvement and histological grade. The relationships between EGFR and c-erbB-2 expression and indexes of proliferation are no less confused. Ki-67 staining has been reported to correlate with EGFR expression by some groups (11, 16, 38), but not by others (39). The S-phase fraction is subject to the same incongruities: a direct correlation being found by some authors (40) but not by others (39, 41). Studies of c-erbB-2 have produced similarly conflicting reports. Borg et al. (42) demonstrated a highly significant correlation between c-erbB-2 expression and the S-phase fraction; a much weaker association was described by two other groups, although one of these found this relationship to be much stronger for in situ disease as opposed to invasive carcinoma (43, 44). A similar report by Ji et al. (13) suggests a direct correlation but notes that not all rapidly proliferating tumors are c-erbB-2 expressing. Ki-67 staining has also been used to study this relationship but showed no correlation to c-erbB-2 expression (45). Indexes such as Ki-67 and the S-phase fraction suffer the disadvantage that they reflect cell cycle distribution and not the rate of cell cycle transit, and as such their value as prognostic indicators may be diluted. This deficiency is overcome by in vivo labeling with the thymidine analogue bromodeoxyuridine which we have used. In this series, the median labeling index is 3.1% (25), which is in broad agreement with the studies of Meyer et al. (46) and Lloveras et al. (47). To our knowledge, no author has reported an analysis of EGFR or c-erbB-2 and proliferative indexes defined by in vivo bromodeoxyuridine labeling. The current study has shown that EGFR expression correlated directly to the labeling index (P = 0.011) and indirectly to potential doubling time (P = 0.010), but that c-erbB-2 expression had no such associations. Expression of neither receptor correlated to the duration of the S-phase.

Activation of EGFR is known to produce a mitogenic signal, and the concept that it is down-regulated in most breast cancers requires modification of the hypotheses about the role that it might play in their biology. At the simplest level, the concept that signaling via EGFR may provide cells with a growth advantage or a mechanism of release from hormone dependence (48, 49) must be questioned on the basis of our findings. However, it must be noted that complete or near-complete (<10% of normal) down-regulation was associated with better differentiation (Mann-Whitney U test, P = 0.0015) in our series. In addition, we see a correlation between EGFR expression and the labeling index. This raises the possibilities that EGFR is being suppressed in the early stages of tumor development, but subsequently escapes from this, albeit achieving lower than normal levels of expression, or that residual expression is per primum associated with faster growth (directly or as an epithephenomenon). Either way this has implications for the use of EGFR as a target for anticancer therapy. If few receptors are present as targets, then the potential of this therapy is much reduced, and, there is a negative therapeutic window, in the sense that the normal tissues will be more sensitive to anti-EGFR therapy than the tumor.

It is believed that a cancer genotype culminates from a series of genetic events and gene amplification may be one such event. Slamon et al. (6), in their 1987 article, report a correlation between the c-erbB-2 gene copy number and both disease-free survival and overall survival, and this concept is accepted for myc amplification in neuroblastoma (50). In the absence of a ligand, creation of chimeric receptors comprising the EGFR external domain fused to the c-erbB-2 internal domain (34, 35) has allowed study of intracellular signaling and confirmed a proliferative effect for the latter. Thus, it would be convenient to suggest that amplification of this gene might confer a growth advantage, a possibility supported by work showing overexpression of c-erbB-2 and high proliferation rates in ductal carcinoma in situ (51). However, despite elevation of c-erbB-2 expression in breast cancer tissues compared to normals, we find no association with the bromodeoxyuridine labeling index which would challenge the suggestion that it imparts a proliferative advantage in invasive disease; this finding is supported by work showing that transfecting breast cancer cell lines with the c-erbB-2 gene results in no mitogenic effect (52). If c-erbB-2 does not exert a mitogenic effect, then how does it influence breast cancer biology? Mutant forms of the receptor might function in an aberrant manner; a transforming point mutated rat c-erbB-2 has been demonstrated (53); however, it is overexpression of normal p185^erbB-2 that is implicated in the transformation of human mammary epithelium (54, 55). Rather than providing a direct growth advantage, c-erbB-2 may influence apoptotic signaling pathways (56). Possibly, c-erbB-2 expression modifies the influence of certain breast cancer therapies (57, 58), allows escape from immune surveillance, or provokes a more invasive phenotype. Equally, these effects may result from coamplified genes with c-erbB-2 overexpression as an epiphenomenon (59).

A clearer understanding of the biology of the type I tyrosine kinase receptors and indeed other receptors, in breast and other cancers, will require application to large tumor series of sensitive techniques such as the radioimmunohistochemical technique used here. In this series, it shows that c-erbB-2 expression follows a bimodal distribution. FISH studies reveal that the second population (21% of studied cancers) is associated with gene amplification; although receptor levels in the first population may be significantly greater than in normal breast, amplification does not appear to be the mechanism of overexpression. Overall receptor density is elevated in 91% of breast cancers. The functionally significant level of expression may thus be lower than that defined by amplification. Application of our method to the EGFR reveals down-regulated expression in 97% of breast cancers. These hitherto unrecognized findings have important implications for the use of type I tyrosine kinase receptors for tumor targeting: EGFR offers a negative therapeutic window since nearly all breast cancers show levels of expression below those in normal breast; however, the c-erbB-2 product becomes a more attractive proposition because its overexpression occurs in a considerably higher proportion of cancers than previously realized. We are able to show that within the c-erbB-2 unamplified cohort of tumors, there is an inverse relationship between c-erbB-2 and EGFR expression and thus add to the evidence supporting interaction between receptors of this family. These observations are made possible by the sensitivity of the radioimmunohistochemical method.

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Quantitative Estimation of Epidermal Growth Factor Receptor and c-erbB-2 in Human Breast Cancer

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