Nuclear Expression of the c-erbB-4/HER-4 Growth Factor Receptor in Invasive Breast Cancers

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Abstract

The prevalence and sites of expression of the c-erbB-4 receptor have been determined by immunocytochemical staining in a series of 178 human breast cancers. Most tumors displayed cytoplasmic staining of variable intensity. When compared with adjacent normal tissue, 32 cases (18%) showed lower than normal expression, and 13 (7%) showed greater than normal expression. Nuclear immunoreactivity, confirmed by two different antibodies, was present in 87 cancers (49%) but was found in normal adjacent breast epithelial cells in <5% of cases. There were no significant associations with cytoplasmic or membrane immunoreactivity, but cases showing nuclear expression in >25% of cells were associated with good histological grade, epidermal growth factor receptor expression, c-erbB-3 positivity, cripto, amphiregulin, and transforming growth factor-α overexpression.

Introduction

Several mechanisms operate in malignant cells to give them a proliferative advantage. In the context of ligand-receptor interactions, this may be achieved by overexpression of the ligand, its receptor, or both in the malignant cells. Alternatively, changes in growth factor expression may be induced in adjacent normal cells such as those in the stroma, creating autocrine, juxtacrine, or paracrine signaling loops. The type I growth factor receptors [EGFR, c-erbB-2, c-erbB-3, and c-erbB-4 (also known as HER1–4)] are a family of transmembrane molecules possessing ligand-regulated tyrosine kinase activity. Ligand binding induces both homodimerization of receptors and heterodimers in various combinations (1). Ten genes have been identified that encode ligands that selectively interact with individual receptor types, some of which, particularly the neuregulins 1–4, are expressed as a number of related proteins as a consequence of alternative splicing of their mRNAs (2). The expression of the type I growth factor receptors in breast cancer has been reviewed (3). In summary, EGFR expression occurs in ~60% of breast cancers, correlates inversely with the presence of ERs, and is an indicator of poor prognosis in most studies. Overexpression of c-erbB-2 in breast and in other cancers is well documented. In breast cancer, the gene is amplified in ~20% of cases leading to receptor overexpression, which correlates with a hormone-independent, more aggressive phenotype and is an overall indicator of poor prognosis. We know less about c-erbB-3, however, which has also been shown to be overexpressed in a proportion of several types of malignancies (4). The c-erbB-3 receptor is overexpressed in ~20% of infiltrating breast cancer cases and in about one-third of ductal carcinoma in situ of the breast, presumably as a result of an increase in gene transcription as gene amplification has not been observed. Thus far, in the studies reported to date, c-erbB-3 status is not associated with survival and so has not been demonstrated to be useful as a prognostic indicator. Research into the role of c-erbB-4 in breast cancer is still in its early stages. The c-erbB-4 protein is expressed in normal breast epithelial cells in humans (5, 6) and in mice (7). The c-erbB-4 protein was shown to be expressed in breast cancers by immunocytochemical staining (5) and at the mRNA level by in situ hybridization analysis (4). mRNA expression has also been reported to be present at relatively high levels in the T47D, MDA-MB-453, BT-474, and H3396 breast cancer-derived cell lines (8). In human primary breast cancers, c-erbB-4 mRNA expression was found to be positively associated with expression of ERs and the Ki67 antigen and negatively associated with epidermal growth factor receptor protein expression (9). The present pilot study was, therefore, undertaken to determine the prevalence and pattern of c-erbB-4 protein expression in a series of breast cancer cases. These had been analyzed previously for the expression of the other type I growth factor receptors and for some of the ligands of the EGFR, including TGF-α (10) and amphiregulin and the orphan ligand cripto. The lymph node status, histological type and grade of tumor, and ER status had also been documented. Our aim was then to ascertain whether any correlations were found between c-erbB-4 expression and these variables and to determine whether c-erbB-4 was implicated in breast cancer development or could act as a useful molecular marker in breast cancer prognosis.

Materials and Methods

Clinical and Molecular Variables. This study involved 178 patients with primary mammary carcinoma who attended the Imperial Cancer Research Fund Clinical Oncology Unit at Guy’s Hospital between 1979 and 1982. The patients were a subset of a group of 195 cases (10) from whom sufficient paraffin-embedded material remained for further analysis. The histological type of tumor was determined according to WHO guidelines. Those tumors classified as being of no special type were graded using the modified Bloom and Richardson method. Lymph node status was available in all cases and was analyzed as positive or negative. Clinical tumor size was also available. ER status was determined by immunohistochemistry using the antibody ID5. EGFR, c-erbB-2, and c-erbB-3 protein expressions were determined immunohistochemically using the antibodies 12E, 21N (11), and RT2 (5), respectively. Cripto, TGF-α, and amphiregulin expressions were determined as described previously (12). c-erbB-4 protein was detected using the mouse monoclonal HFR-1 antibody, which was raised against the intracytoplasmic domain of c-erbB-4 protein or in some cases with the C18 rabbit polyclonal antibody (Santa Cruz Biotechnology). HFR-1 was used at a concentration of 1 μg/ml (5). Both a negative control, in which the primary antibody was replaced with TBS, and a positive control were included with each batch of staining. Immunohistochemistry using the streptavidin-biotin immunoperoxidase technique was carried out on sections pretreated with Protease XXIV (Biogenex) for 5 min. Peroxidase activity was demonstrated using diaminobenzidine, giving a red/brown end product.
Evaluation of Immunoreactivity for c-erbB-4 Expression Using the HFR-1 Antibody. Normal and malignant breast epithelial cells were examined for the presence of positive membrane, cytoplasmic, and nuclear staining. The scoring system for cytoplasmic expression takes into account both the percentage of positive cells and the intensity of staining and has been used previously (11). The percentage of positive cells was scored as: 0, no positivity; 1, up to 25% positive; 2, 26–50% positive; 3, 51–75% positive; and 4, >75% positive. The intensity of immunoreactivity was scored as: 1, weak positivity; 2, moderate positivity; and 3, strong positivity. The two scores were added to obtain a total score. Nuclear immunoreactivity, when present, was evaluated as the proportion of positive nuclei only and, therefore, was given a score of between 1 and 4. For the purposes of statistical analysis, the cases given a score of 1 were combined with those cases that had no nuclear expression. Expression of c-erbB-4 at the membrane was recorded as being present or absent.

Statistical Analysis. The $\chi^2$ test were used to assess the association between the level of c-erbB-4 expression and the other parameters studied. The clinical and molecular characteristics of the 178 breast cancers and the categories used for the statistical analysis are described in Table 1. The curves for relapse-free and overall survival were produced using the method of Kaplan-Meier, and the ability of c-erbB-4 expression to predict prognosis was evaluated using log-rank analysis.

Table 1. Clinical and molecular characteristics of the 178 breast cancers and the categories used for the statistical analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
<th>n</th>
<th>%</th>
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<tr>
<td>Histological type</td>
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</tr>
<tr>
<td>Ductal</td>
<td>152</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Lobular</td>
<td>23</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11</td>
<td>7</td>
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<tr>
<td>II</td>
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<td>III</td>
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<td>47</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>94</td>
<td>53</td>
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<tr>
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<tr>
<td>Negative</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Negative</td>
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<td>35</td>
<td></td>
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<tr>
<td>Amphiregulin b</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>116</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
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<td>34</td>
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<tr>
<td>Cripto b</td>
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<tr>
<td>Positive</td>
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<td>81</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>34</td>
<td>19</td>
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<tr>
<td>TGF-a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>92</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>86</td>
<td>48</td>
<td></td>
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</table>

*ER status is missing from 11 cases.

Amphiregulin and Cripto is missing from one case.

Fig. 1. Examples of c-erbB-4 immunoreactivity in invasive breast cancers as compared with normal breast tissue. A, normal breast; B, a cancer showing underexpression; C, overexpression; D, nuclear immunoreactivity, all detected by HFR-1; E, nuclear immunoreactivity in a case detected by the C18 polyclonal antipeptide antibody; F, detected by the HFR-1 monoclonal antibody.
Table 2. Comparison of c-erbB-4 expression in the cytoplasm, membrane, and nucleus

<table>
<thead>
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<th></th>
<th>Cytoplasm</th>
<th>Membrane</th>
</tr>
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<tbody>
<tr>
<td>Membrane</td>
<td>$\chi^2 = 19.94$</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Nucleus</td>
<td>$\chi^2 = 7.34$</td>
<td>$P = 0.007$</td>
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</table>

Results

A total of 178 cases of invasive breast cancer were analyzed immunohistochemically using the HFR-1 monoclonal to determine the sites and level of expression of the c-erbB-4 protein. Most of the tumors displayed diffuse cytoplasmic immunoreactivity (Fig. 1, B and C) of variable intensity as compared with normal breast tissue (Fig. 1A). Membrane accentuation of cytoplasmic reactivity was noted in some cases, as was nuclear reactivity (Fig. 1D). There were significant associations between the level of expression of c-erbB4 in the membrane, cytoplasm, and nucleus (Table 2).

Cytoplasmic immunoreactivity for c-erbB-4 expression was heterogeneous and varied not only between tumors but also within the same tumor. In all cases, the level of expression in malignant cells was compared with that seen in normal breast epithelium (Fig. 1A) when present in the same section. Using the scoring system detailed in “Materials and Methods,” the tumors were divided into three main groups based on their cytoplasmic reactivity score. Scores of 7 were considered to represent overexpression of c-erbB-4, cases with scores ranging from 4 to 6 displayed levels equal to that of the adjacent normal tissue, and scores of less than or equal to 3 were considered as having lower c-erbB-4 expression than normal. By these criteria, 13 cases (7%) showed overexpression, 134 cases (75%) were equal to normal levels, and 32 cases (18%) had levels of expression less than normal.

Nuclear immunoreactivity for c-erbB-4 was analyzed independently of the cytoplasmic reactivity. A total of 87 cases (49%) showed some nuclear immunoreactivity, which had a diffuse pattern in most cases. Nuclear membrane reactivity was also observed in some cases. Occasionally, the morphologically normal breast epithelial cells adjacent to the tumor also displayed positively stained nuclei, but this was seen in fewer than 5% of cases. To ensure that detection of c-erbB4 in the nucleus was not a result of cross-reactivity with some other protein antigen, a few sections that were positive with the HFR-1 antibody were immunostained with C18 antibody (Santa Cruz Biotechnology). This antibody has been raised against a different epitope (reported to be the COOH terminus) of c-erbB-4. Similar nuclear reactivity to that seen with HFR-1 was observed in all cases (Fig. 1, E and F). Nuclear staining was further evaluated by estimating the percentage of positively stained malignant cells in the section. Although nuclear reactivity was recorded as four groups based on the percentage of cells positive, for the purposes of statistical analysis the cases were divided into two groups: those with no nuclear expression or <25% of nuclei positive (91 of 178, 51%); and a second group of cases with >25% of nuclei positive (87 of 178, 49%). Cell membrane immunoreactivity, which was documented as present or absent, was seen in 52 of 178 cases (29%) and was analyzed independently of the cytoplasmic score.

The degree of cytoplasmic, nuclear, and membrane immunoreactivity was compared with the clinicopathological and biological parameters to determine possible associations. These included: histological type and grade of tumor; ER status and positivity for the type I growth factor receptors EGFR, c-erbB-2, and c-erbB-3; and the ligands TGF-α, amphiregulin, and cripto. For the statistical analysis examining the relationship between c-erbB-4 cytoplasmic expression and these factors, the tumors were placed into two groups, rather than three: those showing less than normal levels of expression (scores of 0–3); or those showing similar to or greater than normal levels of expression (scores of 4–7). The reason for this was that the number of cases showing overexpression (a score of 7) was too small for meaningful analysis.

This analysis revealed no significant associations between c-erbB4 cytoplasmic expression and any of the other variables. Similarly, there were no significant associations between membrane immunoreactivity for the c-erbB-4 protein and the markers.

Nuclear immunoreactivity was also compared with the clinicopathological and biological markers (Table 3). There was a statistically significant inverse association between nuclear c-erbB-4 expression and histological grade of the infiltrating ductal carcinomas ($\chi^2 = 13.45; P = 0.0012$) and a positive association with c-erbB-3 ($\chi^2 = 3.85; P = 0.05$) and EGFR expression ($\chi^2 = 4.57; P = 0.033$). There is also a significant positive association between cripto ($\chi^2 = 8.48; P = 0.004$), amphiregulin ($\chi^2 = 8.48; P = 0.0036$), and TGF-α ($\chi^2 = 1.67; P = 0.02$) overexpression. Nuclear expression of the c-erbB-4 protein was not significantly related to histological type, node status, ER, or c-erbB-2 overexpression.

Of the 13 cases that were defined to overexpress c-erbB-4 relative to normal tissue, there were no examples of simultaneous overexpression of c-erbB-4 and any of the other type I ligand receptors.

The log-rank test of the survival data derived for the cases showing underexpression of cytoplasmic c-erbB-4 compared with the rest of the group did not reveal any significant differences. Similarly, neither nuclear or membrane reactivity seemed to influence relapse-free or overall survival in this series of patients (data not shown).

Discussion

This study demonstrates that the c-erbB-4 protein is expressed in the cytoplasm of normal breast epithelial cells. Underexpression of cytoplasmic c-erbB-4 was observed in 18% of breast cancer cases and overexpression in 7%, but neither of these was significantly associated with a particular phenotype. Relatively few studies have thus far been published on c-erbB-4 protein expression in human tumors. Srinivasan et al. (5) have reported a comprehensive study of c-erbB-4 protein and mRNA expression in normal human and adult fetal tissues and a survey of a small number of nine common solid tumor types. In this study, the authors reported that tumors generally showed lower levels of c-erbB-4 protein expression than the adjacent normal equivalent tissue, including breast cancers. In a study of prostate cancer, it was reported that normal prostatic epithelium showed strong expression of c-erbB-4, whereas only 23% of cancers expressed the protein (13), and similar decreased expression of mRNA, relative to normal tissue, has been seen in a study of pancreatic cancers (14). In contrast,
Gilbertson et al. (15) have shown overexpression of c-erbB-4 in a series of 70 childhood medulloblastomas. Haugen et al. (16), in a study of papillary thyroid cancers, reported weak cytoplasmic staining for c-erbB-4 in normal follicular epithelium but frequent overexpression in carcinomas, and Kataoka et al. (17) showed increased expression relative to normal tissue of mRNA and protein in gastric cancers.

The function of c-erbB-4 in these settings is still unclear. In normal breast, neuregulin-1 (heregulin) implants induced proliferation and differentiation of mammary epithelium (18). In models of breast cancer, activation of c-erbB-4 appears to be associated with signals inducing differentiation (such as lipid droplet formation and casein production) and apoptosis. Interpretation of these results is complicated, however, by the multiple interactions of the ligands and receptors and their differing relative abundance in each model system. In addition, even when the content of c-erbB-4 was known, these studies were performed before it was apparent that at least four splice variants of the gene could be expressed. These variants confer on the protein sensitivity to proteolytic cleavage or the ability to selectively activate the phosphatidylinositol 3-kinase signaling pathway. Recently, these issues have begun to be explored by analysis of mRNA expression in breast cancer by PCR (19) and by isotype-specific antisera.3

The finding here of a common nuclear localization for the c-erbB-4 protein in the cancer cells was unexpected. The fact that two antibodies raised against different epitopes on c-erbB-4 showed similar immunoreactivity indicates that this is probably a true localization rather than attributable to nonspecific binding. In a small proportion of cases (<5%), nuclear reactivity was seen in the morphologically normal breast epithelium adjacent to the tumor. Confirmation of the presence of c-erbB-4 in the nuclei of normal breast epithelial cells not potentially influenced by the tumor environment can be obtained by studying tissues derived for reduction mammoplasty. In addition to breast cancer, nuclear positivity for c-erbB-4 has been observed occasionally in the cells of the distal convoluted tubules of the kidney (5) and the nuclei of endometrial stromal cells.4 It is not currently known whether nuclear localized c-erbB-4 will be present in ductal carcinoma in situ of the breast.

In this study, nuclear immunoreactivity was significantly inversely correlated with histological grade, implying that the presence of c-erbB-4 in the nucleus is related to tumor differentiation. A positive association with c-erbB-3 expression was also observed, which is concordant with a recent report wherein a direct association between c-erbB-4 mRNA levels and c-erbB-3 mRNA levels as detected by reverse transcription-PCR was observed (9). We did not observe any association between either cytoplasmic or nuclear c-erbB-4 immunoreactivity and ER positivity. This is in contrast to a previous study in which a positive correlation with c-erbB-3 and c-erbB-4 positivity has been reported (9). There was no statistical association between the level of c-erbB-4 expression and overall survival. We also found a positive association between nuclear c-erbB-4 expression and the ligands TGF-α, amphieregulin, and cripto. The former two proteins bind to the EGFR and not to c-erbB-4 (or c-erbB-3), but interestingly, cripto has been shown recently to stimulate the phosphorylation of c-erbB-4 indirectly through an as yet uncharacterized mechanism (20).

At present, there are few hypotheses suggesting the function of nuclear growth factors or their receptors. After interaction with the ligand, internalization of the receptor has been demonstrated only in the case of EGFR and not for the other members of the family (21–23). In the process of receptor metabolism, recycling, and degradation, the molecules are also necessarily present in the cytoplasm.

Nuclear localization of growth factors and their receptors, which has now been reported for many molecules (24), is conceptually more difficult to explain. Nuclear localized FGF-2 has been shown to induce transcription of ribosomal genes and phosphorylation of nucleolin by binding to and stimulating the activity of protein kinase CKII (25).

Some reports have demonstrated nuclear localization of members of the type I family of receptors and their ligands. The c-erbB-2 receptor has been detected in the nuclei of DHFR/G8 cells (NIH3T3 cells transfected with normal rat neu) by Western blotting of nuclear extracts, and immunofluorescence of isolated nuclei (26) and Amphieregulin has been shown to be present in ovarian cancer cell nuclei determined by immunostaining (27). Indeed, Modrell et al. (28) has shown that amphiregulin binds to DNA Sepharose and localizes to the nuclei of ovarian cancer cells, and Kimura (29) has reported that Schwannoma-derived growth factor (the rat equivalent of amphiregulin) must be transported to the cell nucleus to exert its mitogenic effect, where it binds DNA and stimulates immediate early gene expression. At least one of the ligands of c-erbB-4, neuregulin-1-β, has been shown to rapidly internalize and translocate to the nucleus in the SK-BR-3 breast cancer cell line (30). The exact mechanism of this phenomenon has not been demonstrated, but theoretically this could occur in a receptor-dependent or independent way. In the latter case, the ligand could act as a “transporter” of the receptor to the nucleus, or the receptor in some way could help ligand to translocate to the nucleus. It is also possible that the cytoplasmic domain of the receptor moves into the nucleus once the ectodomain has undergone proteolytic cleavage at the cell surface. Both antibodies used in this work are directed to the cytoplasmic domain; therefore, we cannot answer this point at present. Additional studies using more sensitive and dynamic approaches, such as light-based detection and tracking systems in live cells using fluorescence-tagged ligands and/or receptors, are required to clarify this issue.

Nuclear localization signals are short peptide stretches with clusters of arginines and lysines that target proteins to the nucleus. An analysis of the c-erbB-4 protein sequence using the PSORT II software3 showed the presence of three putative nuclear localization signals in its cytoplasmic domain: KKKR (amino acids 681–684), PFVSRRK (amino acids 1170–1176), and PEKAKKA (amino acids 1230–1236). We are currently mutating these sites in a green fluorescent protein-tagged c-erbB-4 to address the relevance of these sites to the subcellular distribution of the receptors.

In summary, in breast cancer, c-erbB-4 nuclear immunoreactivity is frequent and seems to be associated with a better differentiated phenotype. Larger studies on the expression of the c-erbB-4 protein in breast cancer are necessary to confirm our initial observations and also elucidate more subtle associations.

References

3 Internet address: http://www.expasy.ch/tools/.

4 R. Srinivasan and W. J. Gullick, unpublished results.

5 W. J. Gullick and N. R. Levinson, unpublished observations.
c-erbB-4/HER-4 IN BREAST CANCER


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