Correlation of c-erbB-2 Gene Amplification and Protein Expression in Human Breast Carcinoma with Nodal Status and Nuclear Grading

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ABSTRACT

Fifty-one primary human breast tumors were analyzed for amplification of the c-erbB-2 protooncogene. Thirteen (25%) of the DNA samples contained multiple gene copies. Paraaffin-embedded tumor sections, available from 47 of the cases, were stained with a c-erbB-2 specific antisera. Eighty-three % (10 of 12) of the tumors containing amplified c-erbB-2 gene copies stained positively with the c-erbB-2 specific antisera (P = 0.03). Thirteen tumors containing single copy c-erbB-2 sequences also stained positively with the antisera. This suggests that mechanisms other than gene amplification may lead to elevated levels of c-erbB-2 protein. Finally, there was a statistically significant correlation between c-erbB-2 protein expression and parameters used in breast cancer prognosis. Positive staining was associated with positive nodal status of the patient (P = 0.02) and with tumors showing a poor nuclear grade (P = 0.02). This is the first study showing that a determination of the level of c-erbB-2 protein in paraaffin-embedded tumor sections may have prognostic value for the course of human breast cancer.

INTRODUCTION

Breast cancer continues to be one of the leading causes of death from cancer among women (1). We are analyzing the involvement of oncogenes in the etiology of breast cancer. The detection of consistent alterations in tumor cell DNA might provide insights into the mechanism of cell transformation. In this study the DNA of primary human breast tumors has been analyzed for amplification of protooncogenes. Several protooncogenes have been shown to be amplified in a variety of tumor cells, although their role in the development of a specific human malignancy is not clear (2). Studies of the N-myc protooncogene were the first to show that there is a direct relationship between N-myc gene amplification and expression and that these correlate with the stage of the disease and the survival of neuroblastoma patients (3—6).

The c-erbB-2 gene was first isolated due to its homology with v-erbB and human epidermal growth factor receptor probes (7—9). The rat equivalent of this gene (neu) has been detected as a transforming gene in transfections using rat neuroblastoma DNAs (10, 11). The transforming neu oncogene has one nucleotide substitution which causes one amino acid change in the membrane spanning domain of the protein (12). Recent studies have shown that the c-erbB-2 protooncogene is amplified in up to 33% of the primary breast tumors examined (8, 13—15) and in up to 25% of human breast cancer cell lines (16). In this paper we show that the c-erbB-2 protooncogene is amplified in 25% (13 of 51) of the primary human breast tumors which we have studied. We also examined gene expression and show that the majority of the tumors with amplified c-erbB-2 gene copies contain detectable levels of c-erbB-2 protein. This was analyzed on sections from paraffin embedded tumor material using an antisera specific for the c-erbB-2 protein (17).

A number of factors useful for the prediction of the course of breast cancer have been identified. These include axillary lymph node involvement, tumor size, hormone receptor status, and nuclear grade (18). The c-erbB-2 protein expression data have been correlated with relevant clinical data. We report here that there is a significant association between c-erbB-2 protein expression and the nuclear grade of the tumor as well as with axillary lymph node involvement. This study suggests that the detection of c-erbB-2 protein in tumor sections may have prognostic value for the development of human breast cancer.

MATERIALS AND METHODS

Patients. Primary breast carcinomas from a total of 51 patients were used in this study. After surgery, some of the tumor tissue was used for the hormone receptor determination, and a piece was rapidly frozen and subsequently used for DNA extraction. The minimum size of the tumors used in this study was 6 mm. In 2 cases normal breast material from the same patient was also surgically removed, frozen, and used for DNA extraction. Forty-nine of the tumors were tested for c-erbB-2 protein and classified according to the WHO histological typing. These 49 breast carcinomas include 40 invasive ductal, 2 invasive lobular, 2 papillary, and one tubular. Forty of the 49 patients were >50 years of age at diagnosis.

DNA Isolation and Hybridization Analyses. High molecular weight DNA was isolated from the breast tumors as previously described (19). Five μg of DNA were digested with the restriction endonucleases EcoRI or HindIII, electrophoresed through 0.8% agarose gels, blotted on genescan plus filter paper (20) and hybridized to nick-translation 32P-labeled DNA probes (21). Hybridization was for 17 h at 65°C in 1 M NaCl, 10% dextran sulfate, and 1% sodium dodecyl sulfate. The filters were washed at 65°C in 0.1 x standard saline citrate (1x standard saline citrate is 0.15 M NaCl-0.015 M sodium citrate, pH 7.5) plus 0.5% sodium dodecyl sulfate.

The DNA probes used in the analyses are as follows: a 3.0-kb HindIII-KpnI cDNA fragment isolated from p CER 204 (22) which is specific for the human c-erbB-2 gene; a 2.5-kb EcoRI fragment containing the human c-mos gene (23); a 2.5-kb HindIII-EcoRI fragment which contains part of the human EGF receptor gene (24); 0.9-kb PvuII fragment which contains part of the human N-ras gene (25); a cDNA probe specific for the human oligoadenylate synthetase gene (26); and a 0.9-kb EcoRI fragment containing the human TGF-α cDNA (27). c-erbB-2 Antibody. c-erbB-2 specific antibodies were generated from a synthetic peptide representing residues 1215—1225 of the c-erbB-2 protein.

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3 The abbreviations used are: kb, kilobase; cDNA, complementary DNA; EGF, epidermal growth factor; TGF, transforming growth factor; 20N, c-erbB-2 specific antisera.
fixed, paraffin-embedded tissue using reagents purchased from Vector Laboratories, Burlingame, CA. In order to minimize nonspecific binding all sections were initially treated with normal serum which acts as a blocking agent. A range of dilutions of the c-erbB-2 specific serum was tested using 2 tumor specimens. A 1:20 dilution of the affinity-purified 20N antibody was chosen and used throughout this study. Two sections from each tumor were analyzed. One section was incubated with the 1:20 dilution of the 20N serum, then with the second biotinylated antibody, followed by the avidin-biotin complex reagent. The second, control section was treated in parallel but incubated with normal serum instead of the 20N serum. This control slide was examined in parallel with the 20N stained slide. The nuclei were counterstained in hematoxylin to allow a nuclear grading.

The slides were scored without previous knowledge of the c-erbB-2 gene analysis and prior to compiling the clinical data. Three different categories were used in scoring the slides. Strong positive cytoplasmic staining in most of the tumor cells (++) weak staining (+), and no staining (−). In 11 of the 49 cases we were not able to definitively interpret the staining results. We have classified these in a separate unknown category.

Clinical Data. Estrogen receptors were determined by standard techniques (29). Values of 10 fmol or greater/mg protein were considered positive. Data concerning the nodal status of 45 of the 49 patients were available. The nuclear grading of the tumor was scored from the sections (30). Hyperchromatism and mitotic figures as well as irregularity of size, shape, and nuclear staining were scored. A score of 1 means that mitotic figures and irregularities were only occasionally observed; 2 means that 2—3 mitoses were observed in most fields as well as moderate nuclear variations; 3 means that a high number of mitoses and marked nuclear pleomorphism was observed.

RESULTS

c-erbB-2 Gene Amplification in Primary Breast Tumors. DNA from 51 primary human breast tumors was isolated and analyzed with a c-erbB-2 cDNA probe. In 13 of the 51 cases amplification of the human c-erbB-2 gene was observed. Fig. 1 shows the results obtained with 9 of these 13 tumor DNAs. The amplification was moderate (2- to 5-fold) in 10 cases (see Fig. 1, tumors 3, 46, 6, 15, 39, 40, and 8 in, respectively, lanes B2, C1, C4, C5, D2, D3, and E3). Higher amplification (5- to 15-fold) of the c-erbB-2 gene was observed in 3 cases, (see tumors 49, 9, and 51 in, respectively, lanes A3, B4 and D4, and E2). DNAs which contained amplified c-erbB-2 gene copies were tested on at least 2 gels. For each filter the results obtained with a DNA which did not contain amplified c-erbB-2 gene copies are shown. These control DNAs are either placenta (lanes A1, B1, D1, and E1), other tumor DNAs (lanes A4–5, B3, 5, and C2–3), or normal breast DNA from patient 49 (lane A2). In this latter case and for patient 51 (results not shown) normal DNA displays the same degree of hybridization as placenta DNA, whereas the tumor DNA contains heavily amplified c-erbB-2 gene copies.

After hybridization with the c-erbB-2 probe the filters were washed and hybridized with one of 5 other probes which did not detect amplified gene copies in these breast tumor samples. This hybridization was used to control that equivalent amounts of DNA were loaded in each lane. Fig. 1, bottom, shows the results of these control hybridizations. The filters in A through E were hybridized with human probes specific for, respectively, the EGF receptor, c-mos, N-ras, αTGF, and oligoadenylic acid synthetase. Only the pertinent part of the control filter is shown and the sizes of the hybridizing fragments are indicated in each panel.

These results show that 25% (13 of 51) of the human breast tumors analyzed contain amplified c-erbB-2 gene copies. The c-erbB-2 gene has sequence and predicted structural similarity to the EGF receptor. These same DNAs were also hybridized with probes which detect the EGF receptor genomic sequences. The EGF receptor probes did not detect amplification or rearrangement in any of the 51 DNAs tested.

![Fig. 1. Analysis of the c-erbB-2 gene in primary human breast carcinoma DNA. Five μg of 16 different tumor DNAs, placenta DNA, and one normal breast DNA were digested with the restriction enzyme EcoRI (A—C) or HindIII (D and E) and analyzed by the Southern blotting technique. Filters shown at the top were hybridized with a 3.0-kb HindIII-KpnI fragment containing c-erbB-2 cDNA specific sequences (24). In genomic DNA the probe recognizes 5 EcoRI fragments, indicated on the left, and 3 HindIII fragments, shown on the right. Following hybridization with the c-erbB-2 specific probe each filter was washed and hybridized with another probe to control for the amount of DNA loaded in each lane. These results are shown at the bottom. The probes are described in "Materials and Methods" and are specific for A, the EGF receptor; B, c-mos; C, N-ras; D, αTGF; and E, oligoadenylic acid synthetase. Only the pertinent part of the filter is shown and the size of the hybridizing fragments is indicated. Numbers (N) at the bottom of each lane, patient number. For patient 49 we also analyzed normal breast DNA, lane A2. P, placenta DNA; KB, kb.](https://cancerres.aacrjournals.org/uitm.cfm?name=cancerres.aacrjournals.org&doi=10.1158/0008-5472.CAN-87-0081)
Table 1  Association of c-erbB-2 gene amplification and disease parameters

<table>
<thead>
<tr>
<th>Description of the various clinical parameters are in “Materials and Methods.” P values were based on the χ² test excluding the unknown cases and were calculated for the number of positive lymph nodes using 0, 1–3, and &gt;3; P for the nuclear grade was calculated using the 3 nuclear grades.</th>
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<tbody>
<tr>
<td>Hormone receptor status</td>
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<td>Estrogen receptor positive</td>
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<tr>
<td>Estrogen receptor negative</td>
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<tr>
<td>No. of positive lymph nodes</td>
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Slamon et al. (13) have recently shown a correlation between c-erbB-2 gene amplification and lymph node status as well as estrogen receptor status of the patients. Table 1 shows the relationship between gene amplification and various disease parameters in the primary human breast tumors which we have analyzed. Clinical data was available for 47 of the patients. For patient number 51, whose tumor contains heavily amplified c-erbB-2 gene copies (Fig. 1, lane D4), no clinical data was available. For 4 of the patients whose tumor DNA contains the normal copy number for the c-erbB-2 gene the nodal status was not determined. There is a good correlation between amplification and the nuclear grade of the tumor (P = 0.0002) and with the hormone receptor status (P = 0.01). When analyzing the correlation between c-erbB-2 amplification and the number of positive lymph nodes, a trend was noted (P = 0.18). Fifty-eight % (7 of 12) of the patients with tumors containing amplified c-erbB-2 gene copies have positive lymph nodes whereas only 32% (10 of 31) of the patients with a single copy of the c-erbB-2 gene have lymph node involvement. The correlation between age of the patient at diagnosis and amplification was not statistically significant, but most of the patients (40 of 49) were more than 50 years of age.

In this group of patients the estrogen receptor status and the nuclear grade of the tumor were highly correlated. Eight of 10 estrogen receptor-negative tumors were classified as Grade 3. Therefore, a logistic regression model including both factors was applied to the gene amplification results. The association with nuclear grade remained statistically significant (P = 0.02) while that for estrogen receptor status was no longer significant (P = 0.47).

c-erbB-2 Expression. The breast tumors were analyzed for c-erbB-2 protein by an immunohistochemical assay on formalin-fixed, paraffin-embedded 5-μm tumor sections. Recently, a c-erbB-2 specific, antipeptide antibody has been characterized (17). The antiserum was raised to residues 1215–1225 of the c-erbB-2 protein by an immunohistochemical assay on formalin-fixed material prepared from the same tumor and have obtained generally consistent results (15). With the 20N antiserum. It is difficult to quantitate protein levels using immunochemical staining techniques, but these results suggest that the amount of c-erbB-2 protein in a tumor cannot be strictly correlated with the gene copy number. From the results of the immunohistochemical staining for c-erbB-2 protein in the human breast tumors we conclude that most of the tumors

* W. J. Gullick, unpublished results.
Table 2 Association of c-erbB-2 gene amplification and immunohistochemical staining for c-erbB-2 protein

<table>
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<tr>
<th>c-erbB-2 gene copy no.</th>
<th>Antiserum staining</th>
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<tbody>
<tr>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Single copy</td>
<td>7</td>
</tr>
<tr>
<td>Amplified copies</td>
<td>7</td>
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</table>

$P$ was calculated using the $\chi^2$ test and excluding the unknown cases.

Table 3 Association of c-erbB-2 protein staining and disease parameters

Descriptions of the various clinical parameters are in "Materials and Methods." $P$s were calculated using a $\chi^2$ test and were based on a comparison of the 3 categories ++ versus + versus –.

<table>
<thead>
<tr>
<th>Antiserum staining</th>
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<tr>
<td>Hormone receptor status</td>
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<tr>
<td>Estrrogen receptor positive</td>
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<td>Estrrogen receptor negative</td>
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<th>No. of positive lymph nodes</th>
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<tr>
<td>0</td>
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<th>Nuclear grade</th>
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is a statistically significant correlation between positive staining and the nodal status of the patients ($P = 0.02$) as well as the nuclear grade ($P = 0.02$). To date the number of positive lymph nodes is the best prognostic factor for progression of breast cancer (18). Here we show that the number of positive lymph nodes correlates with the staining for c-erbB-2 protein. The presence of lymph node involvement maintained a statistically significant association with positive protein expression in a logistic regression model controlling for nuclear grade and estrogen receptor status.

DISCUSSION

The detection of consistent genetic alterations in the DNA of breast tumors might provide insights into the mechanism of cell transformation and possibly suggest rational strategies for cancer therapy. These mutations might result in the activation of dominantly acting oncogenes or alternatively in the loss of recessively acting gene functions. Examples suggesting that both possibilities might play a role in breast cancer etiology have been reported. The c-myc gene has been found to be present in multiple copies in 32% of primary breast cancer cells (33). In one study deletion of DNA from the short arm of chromosome 11 has been described in 27% of the tumors examined (34). In another study evidence for the loss of DNA from chromosome 13 was presented (35).

Here we have investigated the possible involvement of the c-erbB-2 gene in breast cancer development. This gene encodes a growth factor receptor-like transmembrane glycoprotein and has been shown to be involved in the transformation of rat neuroblastoma cells. Mutation of the transmembrane domain is one mechanism which has been described as being responsible for the transforming action of the c-erbB-2 gene (12). It has also been shown that overexpression of the normal human c-erbB-2 protein leads to transformation of NIH/3T3 cells (36). A particularly interesting aspect of the c-erbB-2 oncprotein is that its transforming potential can be partially repressed by monoclonal antibodies directed against its extracellular domain (37).
In the present study we have analyzed 47 primary human breast carcinomas for both amplification and expression of the c-erbB-2 protooncogene. We have observed gene amplification in 25% of the tumors. This value is very similar to those reported earlier (13-16). c-erbB-2 protein was detected using an immunohistochemical technique. This is the first study in which c-erbB-2 specific antisera has been used on paraffin-embedded breast tumor sections. This same antisera was used in a recently published study using frozen sections from 36 primary breast carcinomas (15). The results from both studies are similar. We have observed that 91% (10 of 11) of the tumors with amplified c-erbB-2 gene copies have detectable protein expression. The majority (7 of 10) reacted strongly with the c-erbB-2 antisera. Venter et al. (15) reported that 92% (11 of 12) of the breast tumors with amplified c-erbB-2 gene copies reacted positively with the same antisera using frozen tumor sections. Eight of these positive tumors were in their strongly positive categories. Taken together these results suggest that those tumors with increased copy number of the c-erbB-2 gene will contain the most c-erbB-2 protein.

We have also observed that 48% (13 of 27) of the tumors which have single copy c-erbB-2 sequences contained detectable c-erbB-2 protein. Venter et al. (15) have also reported a similar result. Seventy-nine % (19 of 24) of the tumors with a single copy of the c-erbB-2 gene reacted to some degree with the antisera. In their case only 16.6% (6 of 36) of the tumors analyzed were completely negative for c-erbB-2 staining, whereas we have observed that 39% (15 of 38) of the tumors which we have studied failed to react with the antisera. This could be due to various differences in immunohistochemical staining techniques and classification of the degree of staining as well as to normal variation in the tumor population examined. The results of both studies of primary tumors suggest that breast carcinomas with no apparent c-erbB-2 gene amplification can overexpress the protein. A recent report on 16 different human breast tumor cell lines has shown similar results (16). All of the breast tumor cell lines examined were found to express the c-erbB-2 mRNA, and 50% (8 of 16) had elevated mRNA levels. Of these lines with elevated c-erbB-2 RNA levels 4 displayed some degree of c-erbB-2 gene amplification. These authors used an immunoblot assay to quantitate c-erbB-2 protein levels and were able to detect the protein only in cells which overexpress the mRNA. The immunoblot technique used to detect c-erbB-2 protein in their study cannot be directly compared to the immunohistochemical staining technique which we have used but the similarity in the results obtained is worth noting. In both cases 25% of the DNAs examined contained amplified c-erbB-2 gene copies. In our study 61% of the tumors contain detectable c-erbB-2 protein. Kraus et al. (16) detected c-erbB-2 RNA in all of the cell lines examined but only observed protein in 50% of these cases, those that overexpressed the mRNA. In our study we may have detected the c-erbB-2 protein only in those tumors which overexpress the mRNA. A comprehensive study of the level of c-erbB-2 mRNA in primary human breast tumors has not been described. Van de Vijver et al. (14) have examined 95 primary human breast tumors for c-erbB-2 amplification. They analyzed RNA from 11 tumors and observed that 3 of these tumors with amplified gene copies contained elevated c-erbB-2 RNA levels compared to the levels detected in the 8 which displayed no amplification. It will clearly be important to analyze a series of breast tumors at the DNA, RNA, and protein levels to determine the mechanism(s) which can lead to overexpression of the c-erbB-2 protein. The EGF receptor gene has been shown to be amplified in some human tumors (38). In some cases the EGF receptor protein level is elevated without apparent gene amplification (2). These results suggest that both the EGF receptor gene and the related c-erbB-2 gene can be overexpressed by mechanisms other than gene amplification. Shen et al. (39) have reported that increased RNA expression from the multidrug resistance locus can even precede amplification of this locus in human tumor cell lines.

The function of the c-erbB-2 protein in normal growth and differentiation of breast cells remains unknown. This is also true for its role in tumor formation. It seems possible that the increased expression of a growth factor receptor could confer a selective advantage on a population of tumor cells and may be important for the progression of the disease. Slamon et al. (13) showed that amplification of the c-erbB-2 gene was correlated with the presence of tumor in the axillary lymph nodes, with estrogen receptor status, and with the size of the primary tumor. Since these are the major prognostic factors for breast cancer their data suggested that amplification of the c-erbB-2 gene could have prognostic value. We have extended their studies and shown that the level of c-erbB-2 protein detected by immunohistochemical staining of paraffin-embedded breast tumor material correlates significantly with axillary lymph node involvement and nuclear grade of the tumor. In a clinical environment immunohistochemical staining of tumor material is more feasible than an analysis of tumor DNA. In addition, we have noted that some tumors had focal areas of strong c-erbB-2 staining. This finding suggests that these tumors are heterogeneous and in a DNA analysis amplified c-erbB-2 gene copies could go undetected. Finally, we have observed apparent overexpression of the c-erbB-2 protein in breast tumors with a single copy of the c-erbB-2 gene. This observation suggests that other mechanisms lead to elevated levels of c-erbB-2 protein. These findings suggest that the detection of c-erbB-2 protein may be more relevant than a gene copy number analysis.

The availability of serum which detects c-erbB-2 protein in paraffin-embedded material makes it feasible to design retrospective studies on selected groups of patients. Breast cancer patients with no axillary lymph node involvement have the best prognosis, yet approximately 25% of this group relapse within 10 years (40). It is interesting to note that in the node-negative group 15% (4 of 34) and 19% (5 of 26) of the tumors in, respectively, the Slamon et al. (13) study and this study, showed amplified c-erbB-2 gene copies. In addition, this study shows that 40% (8 of 20) of the node-negative patients had detectable levels of c-erbB-2 protein in the tumor material. It will now be important to assess the prognostic significance of c-erbB-2 protein expression in node-negative breast cancer patients for whom an indicator of increased risk for relapse has significant therapeutic implications.

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