HER-2/neu Amplification Predicts Poor Survival in Node-positive Breast Cancer

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ABSTRACT

HER-2/neu protooncogene amplification and protein expression were analyzed with slot blot and Western blot techniques, respectively, in more than 300 invasive primary breast tumors of all stages. Amplification (2–>30 copies) was found in 17% of these tumors and high expression was seen in 19%. There was a striking coincidence between gene amplification and high expression. Tumors associated with many involved axillary lymph nodes or with Stage IV disease were more often HER-2/neu amplified or overexpressed. Furthermore, gene alteration was strongly correlated with the absence of steroid receptors and with larger tumor size. High expression without gene amplification was seen in a minor subset of tumors of less aggressive character. Neither amplification nor overexpression was correlated with disease outcome for patients with negative axillary lymph nodes. For node-positive patients, however, HER-2/neu amplification was a significant predictor of early relapse and death (median follow-up = 45 months), and a similar trend, although not significant, existed for high gene expression. Multivariate analyses indicated that HER-2/neu alterations were not independent predictors of patient outcome.

INTRODUCTION

There is a need for markers to select node-negative breast cancer patients with aggressive disease for adjuvant therapy and to distinguish among node-positive patients those with slowly growing cancer from others who might benefit from more intensive treatment. Steroid receptors and flow cytometry analysis of ploidy and S phase fraction are convenient techniques which seem to fulfill some of these requirements, as does tumor grading when performed in a reproducible manner (1–3).

The impact of estrogens and growth factors on breast cancer growth is well established and several protooncogenes have been found homologous or identical to growth factors or their receptors (4, 5). In particular, the transforming gene neu, originally found in chemically induced rat neuroglioblastomas (6, 7), has aroused attention in breast cancer. This gene was also cloned from human genome libraries by virtue of its similarity to the epidermal growth factor receptor gene and named HER-2 or c-erbB-2 (8–10). The HER-2/neu gene (referred to here) encodes an M, 185,000–190,000 cell surface glycoprotein with tyrosine kinase activity (11, 12), fulfilling many requirements to the epidermal growth factor receptor gene and named HER-2 or c-erbB-2 (8–10). The HER-2/neu gene (referred to here) encodes an M, 185,000–190,000 cell surface glycoprotein with tyrosine kinase activity (11, 12), fulfilling many requirements for a growth factor receptor. Its putative ligand is, however, still unidentified.

Several groups have found HER-2/neu activation in the form of gene amplification in human epithelial carcinomas and particularly in breast cancer (13–23). This quantitative alteration has also been demonstrated as an overexpression of HER-2/neu mRNA (20–23) and protein (23–29) in most amplified tumors but also in some tumors with a normal gene copy number, implying the existence of alternative activation mechanisms.

HER-2/neu amplification was shown in an initial report to be a significant predictor of early recurrence and death in breast cancer (14). Several subsequent studies measuring gene amplification (17) or immunohistochemically detected overexpression (26, 27) have failed to confirm this connection. These contradictory findings may be due in part to too few patients and other technical considerations (23, 30). However, in a recent investigation (29) of a larger number of breast tumors, it was shown that HER-2/neu overexpression correlates with increased number of involved lymph nodes and steroid receptor negativity as well as with a significantly shortened disease-free and overall survival among node-positive patients. In the present study of a new patient cohort, we investigated these correlations by measuring both gene amplification and Western blot-detected expression.

MATERIALS AND METHODS

Patients, Clinical and Biological Variables. Patients participating in the study were all from the Southern Sweden Health Care Region, were diagnosed as having breast disease between October 1982 and February 1985, and had a tumor sent for steroid receptor analysis. Consecutive tumor samples of sufficient size (n = 456), stored at —70° C, were pulverized and divided for gene amplification and/or expression analyses (see below). After reinvestigation of hospital charts, 32 cases were excluded as being ineligible for the study (e.g., sample from metastatic lesion, benign disease, or cancer in situ). Patients presenting with bilateral cancer were not excluded, if it was clear which primary tumor recurred. An additional 10 samples were excluded because they contained too few cancer cells after cytopathological examination of imprints taken adjacent to the tumor section being used in analysis.

Of 404 remaining cases, 26% were classified according to UICC (International Union Against Cancer) as Stage I, 35% as Stage IIa, 23% as Stage IIb, 6% as Stage III, and 6% as Stage IV (distant spread at diagnosis or within 2 months after primary operation) breast cancer. Sixteen patients could not be classified because axillary resection was not performed. However, none of these patients had metastases at diagnosis. The range of patient ages at the time of operation was 28–92 years (median, 63 years). Twenty-one percent were premenopausal, and 79% were postmenopausal. Postoperative radiation was performed in 52%, adjuvant chemotherapy (cyclophosphamide) in 7%, and adjuvant tamoxifen was given to 37% of the patients. Recurrences were clinically confirmed and registered as locoregional or distant. Deaths were distinguished as due to breast cancer or to intercurrent disease according to death certificates. Distant metastases were found in 122 cases and locoregional recurrences in 14 cases. Of 153 deaths, 107 were due to breast cancer. Median follow-up time for all patients was 45 months; for those still living, 52 months; and, for those still living, or dead, with intercurrent disease, 49 months. Distant recurrence was used as the end point for the calculation of distant DFS to define truly aggressive tumor behavior. Locoregional recurrences were included in the calculation of DFS. Likewise, death due to breast cancer was used as the end point for breast cancer survival in comparison with overall survival. Only patients with Stage I–III disease (M0) were included in survival analyses.

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2 To whom reprint requests should be addressed.

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The abbreviations used are: DFS, disease-free survival; M0, clinically distant metastasis free at diagnosis; ER, estrogen receptor; PgR, progesterone receptor.
Measurements of ER and PgR were performed within 2 weeks after surgery, at one laboratory using radioligand-binding techniques (isoelectric focusing and dextran-coated charcoal with Scatchard analysis, respectively) as described previously (31). The cutoff points of 10 fmol/ 
mg protein were used for classification as receptor positive or negative.

Amplification Analysis. DNA was extracted from pulverized tissue (32) and checked for purity and high molecular weight integrity. According to fluorometric determination of DNA concentration, equal amounts (5 µg) of RNase-treated DNA were applied on Zetaprobe nylon membranes using a Bio-Dot SF blotting apparatus (Bio-Rad Laboratories, Richmond, CA). Alternatively, 10 µg EcoRI-digested DNA was separated on 0.8% agarose gels and transferred to Zetaprobe membranes (33). Hybridization was carried out under stringent conditions according to the manufacturers’ descriptions with 10^6 cpm/ml multiprime-labeled (Amersham Corporation, Arlington Heights, IL) and an ER probe (clone pOR8, gift from Pierre Chambón), as well as several other probes, to ensure an equal amount of applied DNA and to distinguish low degree amplification from chromosome 17 polysomy. Degree of amplification was evaluated with densitometric analysis of short time-exposed autoradiograms, in comparison with dilutional analysis of amplified samples.

Expression Analysis. A Western blot technique, described in detail elsewhere (29), was used. Briefly, proteins from approximately 5 mg pulverized tumor tissue was solubilized by boiling in 5% sodium dodecyl sulfate. Aliquots of 200 µg protein were applied on 7.5% polyacrylamide gels and electrophoresed under denaturing and reducing conditions. Resolved proteins were electroblotted to nitrocellulose filters (Schleicher and Schuell, Keene, NH). After blocking, filters were incubated with a rabbit polyclonal antiserum raised against a carboxy terminal synthetic peptide of HER-2/neu (residues GTPTAEN-PFYLGLDVPPV) (34; gift from Axel Ullrich) at a 1:3000 dilution overnight at 4°C. Secondary antibody, 10^5 cpm/ml 125I-donkey anti-rabbit Ig (Amersham Corporation, Arlington Heights, IL), was added and the washed blots were visualized by autoradiography. The quantity of the HER-2/neu protein in individual tumors was estimated in arbitrary units/200 µg total tumor protein by calculating the ratio of the integrated signal at M, 185,000–190,000 in the tumor relative to a constant sodium dodecyl sulfate extract of T47D human breast cancer cells, loaded on each gel as an internal reference standard.

Statistical Methods. The association of HER-2/neu alteration with other categorized clinicopathological and biological variables was assessed by χ² analysis. Survival curves were calculated by the method of Kaplan and Meier (35). Tests of differences between curves were made with the log-rank test for censored survival data (36). The partially nonparametric regression model of Cox (38) was used to evaluate the predictive power of various combinations and interactions of prognostic factors in a multivariate manner (37-39). All survival computations were done with the Biomedical Computer Program-P Series (40).

RESULTS

Amplification Analysis. Southern blot analysis (Fig. 1) of EcoRI-digested DNA showed hybridization of the HER-2/neu probe to a 13-kilobase DNA fragment as described previously (14). Slot blot analysis (Fig. 2A) of 310 breast tumor DNA samples using the same probe and rehybridization with a probe for the myeloperoxidase gene (according to HGM10 localized on chromosome 17q21.3-q23 as compared with 17q11.2-q12 for HER-2/neu) revealed an amplified copy number of the HER-2/neu gene in 49 cases. Three additional samples were judged as having a comodeled myeloperoxidase gene when compared to hybridization signals of genes localized on other chromosomes [for example, the ER gene located on chromosome 6q (Fig. 2B)]. Low degree amplification of the myeloperoxidase, but not of the HER-2/neu, gene could be seen in another 5 cases. The ER gene, however, was present in a normal

Fig. 1. Hybridization of the HER-2/neu probe to an approximately 13-kilobase EcoRI DNA fragment, analyzed with Southern blot technique. Lane 1, HindIII-digested λ-phage DNA; Lanes 2–6, repeated 2-fold dilution of a 12-fold amplified breast tumor sample; Lanes 7–9, three different unamplified samples.

Fig. 2. Example of a slot blot analysis of HER-2/neu amplification after hybridization with a HER-2 probe (A) and rehybridization with an ER probe (B). Amplification is seen in the following samples to a number of: 2c, 6 copies; 3b, 4 copies; 3d, 22 copies; 3f, 6 copies; 3g, 8 copies; 4c, 12 copies; 4k, 5 copies; 5a, 6 copies; 5g, 2 copies; 5h, 6 copies; 6c, 2 copies; and 6h, 5 copies. All other samples are unamplified.
known at this time. Quantitation was accurately performed in 360 samples and found at a range of 0–3440 units (median, 18). A cutoff point of 100 units was used to distinguish low from high expression, and 19% of the cases were found to be highly expressed. The same cutoff value was used in a previous study (29) and found to most significantly discriminate a group (17%) of tumors with high expression and poor prognosis.

Amplification versus Expression. Determination of both amplification and expression was assessed in 274 tumors (Fig. 4). High HER-2/neu protein expression was found to be strongly correlated with amplification of the gene (Spearman correlation; \( r = 0.53, P < 0.001 \)). Overexpression was seen in 40 of 43 amplified samples. Two of the three amplified, but not overexpressed, tumors had only a 2-fold amplified gene. Sixteen of 231 (7%) unamplified tumors were overexpressed; however, all but one were only moderately (101–342 units) elevated.

HER-2/neu Amplification and Overexpression versus Other Prognostic Factors. Both amplification and overexpression of the HER-2/neu gene were significantly correlated with the clinical stage of the tumor (Table 1). Patients with Stage I or IIa disease had relatively low rates of gene alterations (10–15%) compared to patients with Stage IV disease (42–50%). Patients with Stage II b or III disease had intermediate values (24–32%).

The relationships between various clinicopathological and biological variables for patients with primary, M0, disease are shown in Table 2. Although neither amplification nor overexpression was significantly associated with overall nodal status, there was a statistically significant relationship between amplification and the number of positive nodes (\( P = 0.044 \)). Patients with 10 or more positive nodes had an amplification rate similar to that of patients with advanced disease. A similar trend, although not statistically significant, was observed for gene overexpression. There was a direct relationship between tumor size and both amplification (\( P = 0.020 \)) and overexpression (\( P = 0.002 \)). Menopausal status was not correlated with amplification, but overexpression was marginally increased among premenopausal women (\( P = 0.045 \)). Amplification and overexpression were highly significantly associated with an absence of ER and PgR (\( P < 0.0001 \)). This was seen in node-negative as well as node-positive tumors. Taking also the num-

**Table 1** HER-2/neu amplification and overexpression by clinical stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>Amplified/total (%)*</th>
<th>Overexpressed/total (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9/80 (11)</td>
<td>11/97 (12)</td>
</tr>
<tr>
<td>IIa</td>
<td>11/107 (10)</td>
<td>18/125 (15)</td>
</tr>
<tr>
<td>IIb</td>
<td>15/71 (24)</td>
<td>22/84 (26)</td>
</tr>
<tr>
<td>III</td>
<td>7/22 (32)</td>
<td>7/24 (29)</td>
</tr>
<tr>
<td>IV</td>
<td>10/20 (50)</td>
<td>8/19 (42)</td>
</tr>
</tbody>
</table>

* \( P = 0.0001 \).

* \( P = 0.02 \).
number of amplified copies or degree of overexpression into consideration, an even more pronounced correlation with steroid receptor negativity was observed (data not shown).

The 16 tumors with an overexpressed but unamplified gene showed quite a different character. Nine of them were node negative, only 2 had more than three involved nodes, and none were of advanced stage. Furthermore, 13 of these 16 tumors were (often strongly) ER and PgR positive.

**HER-2/neu Amplification and Overexpression as Predictors of Survival.** Neither HER-2/neu amplification nor overexpression was a significant predictor of disease outcome for patients with node-negative breast cancer regardless of the end point considered. These findings did not change when the actual number of gene copies was taken into account, when different cutoff values for overexpression were considered, or when expression was analyzed as a continuous logarithmically transformed factor.

However, HER-2/neu amplification did predict disease outcome among node-positive patients. Node-positive (M0) patients presenting with a tumor with an amplified gene had statistically shorter disease-free, distant disease-free, overall, and breast cancer survival. The disease-free survival curves for distant recurrences are shown in Fig. 5. Similar trends were observed for HER-2/neu overexpression, but none of the differences were statistically significant (P > 0.15).

In order to study the independent prognostic value of HER-2/neu activation and the interrelationships of other prognostic factors, we performed multivariate analyses using the 99 node-positive (M0) patients from whom all factors had been collected. The classifications for potential prognostic factors were: number of positive nodes (1-3 versus 4+), tumor size (≤2 versus >2 cm), ER and PgR (<10 versus ≥10 fmol/mg protein), menopausal status (before versus after), HER-2/neu amplification (single copy versus multiple copies), and HER-2/neu expression (<100 versus ≥100 units). In each analysis, amplification provided more prognostic power than overexpression. However, due to the strong correlations between the HER-2/neu factors and PgR, tumor size, and the number of positive nodes, neither amplification nor overexpression remained significant in any of the multivariate models (Table 3).

Since HER-2/neu amplification and overexpression were so closely associated with other prognostic factors, especially the steroid receptors, we performed additional multivariate analyses that included two-way interactions. A term that represented the interaction between PgR status and amplification of the HER-2/neu gene became the most important variable in

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**Table 2** HER-2/neu amplification and overexpression by clinicopathological and biological factors for patients with primary M0 breast cancer

<table>
<thead>
<tr>
<th>Factor</th>
<th>Amplified/total (%)</th>
<th>P value</th>
<th>Overexpressed/total (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Node status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20/159 (13)</td>
<td>0.18</td>
<td>30/192 (16)</td>
<td>0.26</td>
</tr>
<tr>
<td>Positive</td>
<td>22/120 (18)</td>
<td></td>
<td>28/137 (20)</td>
<td></td>
</tr>
<tr>
<td>No. of positive nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>8/68 (13)</td>
<td></td>
<td>12/75 (16)</td>
<td></td>
</tr>
<tr>
<td>4-9</td>
<td>8/36 (22)</td>
<td>0.044</td>
<td>10/45 (22)</td>
<td>0.19</td>
</tr>
<tr>
<td>10+</td>
<td>6/16 (38)</td>
<td></td>
<td>6/17 (35)</td>
<td></td>
</tr>
<tr>
<td>Tumor size ≤2 cm</td>
<td>10/111 (9)</td>
<td></td>
<td>12/132 (9)</td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>26/150 (17)</td>
<td>0.020</td>
<td>38/174 (22)</td>
<td>0.002</td>
</tr>
<tr>
<td>Menopausal status</td>
<td>Before</td>
<td>11/66 (17)</td>
<td>20/80 (25)</td>
<td>0.045</td>
</tr>
<tr>
<td>After</td>
<td>31/214 (14)</td>
<td>0.66</td>
<td>38/250 (15)</td>
<td></td>
</tr>
<tr>
<td>ER &lt;10 fmol</td>
<td>30/87 (34)</td>
<td>&lt;0.0001</td>
<td>33/103 (32)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≥10</td>
<td>12/193 (6)</td>
<td></td>
<td>25/227 (11)</td>
<td></td>
</tr>
<tr>
<td>PgR &lt;10 fmol</td>
<td>32/107 (30)</td>
<td>&lt;0.0001</td>
<td>35/127 (37)</td>
<td>0.0002</td>
</tr>
<tr>
<td>≥10</td>
<td>8/157 (5)</td>
<td></td>
<td>20/179 (11)</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 3** Multivariate survival analyses for node-positive (M0) patients (n = 99)

<table>
<thead>
<tr>
<th>Factor</th>
<th>DFS</th>
<th>Distant DFS</th>
<th>Overall survival</th>
<th>Breast cancer survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive nodes (1-3 vs. 4+)</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>0.0005</td>
</tr>
<tr>
<td>PgR (&lt;10 vs. ≥10 fmol)</td>
<td>0.0075</td>
<td>0.013</td>
<td>0.16</td>
<td>0.0043</td>
</tr>
<tr>
<td>Tumor size (&lt;2 vs. ≥2 cm)</td>
<td>0.025</td>
<td>0.019</td>
<td>0.31</td>
<td>0.014</td>
</tr>
<tr>
<td>ER (&lt;10 vs. ≥10 fmol)</td>
<td>0.30</td>
<td>0.24</td>
<td>0.39</td>
<td>0.034</td>
</tr>
<tr>
<td>HER-2/neu amplification</td>
<td>0.50</td>
<td>0.56</td>
<td>0.15</td>
<td>0.60</td>
</tr>
<tr>
<td>HER-2/neu overexpression</td>
<td>0.85</td>
<td>0.65</td>
<td>0.18</td>
<td>0.59</td>
</tr>
<tr>
<td>Age (&lt;50 vs. ≥50 yr)</td>
<td>0.57</td>
<td>0.70</td>
<td>0.024</td>
<td>0.10</td>
</tr>
<tr>
<td>Menopausal status (before vs. after)</td>
<td>0.70</td>
<td>0.86</td>
<td>0.72</td>
<td>0.16</td>
</tr>
</tbody>
</table>
these analyses, surpassing the number of positive nodes. Fig. 6 depicts distant disease-free survival when analyzed by the combination of PgR status and HER-2/neu amplification. Patients with PgR-positive tumors that had a single copy of the HER-2/neu gene had significantly better prognosis than patients with either an amplified gene or a tumor that lacked PgR. However, the small number of patients in certain subsets requires cautious interpretation.

There was no uniform postoperative or adjuvant treatment of the node-positive patients included in the survival analyses. However, no significant difference was seen between those with HER-2/neu alteration and the remainder with respect to the fraction receiving postoperative radiotherapy, adjuvant chemotherapy, or adjuvant endocrine therapy (data not shown).

**DISCUSSION**

In the present study we used a semiquantitative Western blot procedure for estimating the level of the HER-2/neu protein in tumor specimens. An immunocytochemical assessment of breast tumors, used by others (23, 24, 26-28, 41), distinguishes tumor cells from normal cells, reveals the distribution of the protein within the cells, and detects heterogeneity of the protein expression in a tumor section. However, because the distribution of tumor cells within a given specimen is not random, accurate quantitation of the positively stained tumor cells by this method is not possible. On the other hand, Western blotting conferred further specificity by allowing densimetric integration of the p190 band which is accepted as the principal form of the HER-2/neu gene product. A rate of 19% of HER-2/neu protein overexpression observed in this study is also in good agreement with the findings of other investigators using alternative methods for measuring expression (26, 28, 41).

We found a striking coincidence of gene amplification and high protein expression, which further validates the Western blot procedure used here. The few cases in which overexpression of an unamplified gene was seen clearly represented tumors of earlier stage and less malignant type. This is also in agreement with a recent study (21), in which the proportion of amplification in tumors with mRNA overexpression increased with more advanced stage. Other investigators (26, 41) have reported HER-2/neu protein staining in a high percentage of intraductal tumors, suggesting a role for this gene in the early steps of breast tumor formation or in the adaptation to an initiated state. It is not known whether this overexpression is accomplished by gene amplification or by other mechanisms. Deregulation of expression may, however, precede and even be a prerequisite for the subsequent gene amplification which further enhances and establishes the acquired growth advantage.

The importance of HER-2/neu activation in the progression of human breast cancer has been studied by several groups. Although still somewhat controversial (17, 26, 27, 41), activation of this protooncogene has been proposed to correlate with the presence of lymph node metastases and, when clinical follow-up was available, also a bad prognosis (14-16, 19, 21-23, 25, 28, 29). The present study reinforces the positive correlation with lymph node status. This was, however, solely due to the higher frequency of gene activation in tumors with >3 involved nodes, a difference even more pronounced in the group with 10 or more positive nodes. Furthermore, tumors with distant spread at diagnosis showed the highest degree of HER-2/neu alteration. A similar positive correlation was found also with increasing tumor size.

This distinct connection of HER-2/neu alteration with aggressive cancer type, defined as an increased stage and tumor burden, became further evident when comparison was made with other biological and more established markers of tumor behavior. Partially in agreement with some previous reports (18, 21, 25, 28, 29) but in disagreement with others (14, 17, 20, 22, 27, 42), a very strong correlation was found with ER and PgR negativity, a widely accepted character of tumor differentiation and increased autonomy. Correlations have also been found with histopathologically defined aggressive type of breast cancer (43) and with a high rate of cell proliferation.

The prognostic value of HER-2/neu alteration limited to node-positive patients is in agreement with three previous reports (19, 23, 29) and also consistent with the early findings of Slamon et al. (14), who analyzed the relation to clinical outcome only among node-positive patients. Reasons for the lack of prognostic significance among node-negative patients is still unknown. The main consequence of HER-2/neu activation is obviously not directly coupled to an increased metastatic behavior, defined in terms of cell motility or invasive growth. Although also providing a growth advantage in localized disease, this oncogene may need to be accompanied by additional modulating genetic events to exert its full effect of aggressive behavior in progressive systemic disease.

Although gene amplification and overexpression have been shown to be independent predictors of poor survival in previous studies (14, 19, 23, 28, 29), this could not be confirmed in the present material. Multivariate analyses showed that the number of positive nodes, PgR, tumor size, and ER remain significant covariates. As a result of its strong correlation to these other factors, HER-2/neu activation entered the Cox model only when combined with PgR and not as an independent predictor of patient outcome. In agreement with a previous study (28),

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Fig. 6. Distant DFS (DDFS) curves in node-positive M0 breast cancer patients as a function of HER-2/neu amplification and PgR status (cutoff value = 10 fmol/mg protein). Median follow-up, 41 months. A, PgR+, single copy; B, PgR-, single copy; C, PgR-, amplified; D, PgR+, amplified. A versus B, P = 0.001; B versus C, P = 0.50; A versus C, P = 0.0001; B versus D, P = 0.05; A versus D, P < 0.0001; C versus D, P = 0.09.

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the few steroid receptor-positive tumors with an amplified gene were found to have the most unfavorable prognosis.

Like previous reports, this study shows that HER-2/neu oncogene activation has some prognostic significance in node-positive breast cancer (here, protein overexpression showed a trend but failed to reach statistical significance). Although a measurement of either gene amplification or protein overexpression can be used for this purpose, considering the relative ease of estimation and the requirement of a smaller amount of tumor tissue, protein analysis is perhaps a better choice for routine specimens. Furthermore, with the advent of quantitative cell/image analysis microscopy systems, it should be possible to obtain more precise and reproducible data from the immunostained tumor tissues.

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