[CANCER RESEARCH 62, 6667–6673, November 15, 2002]

Active Signaling by HER-2/*neu* in a Subpopulation of HER-2/*neu*-overexpressing Ductal Carcinoma *in Situ*: Clinicopathological Correlates[1]

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ABSTRACT

HER-2/*neu* overexpression occurs in a proportion of invasive breast carcinomas and is an adverse prognostic indicator, although its apparent strength as a prognostic indicator varies in different studies. Paradoxically, HER-2/*neu* is overexpressed with particularly high frequency in ductal carcinoma *in situ* (DCIS). We have hypothesized and presented supporting data that HER-2/*neu* is actively signaling in a subset of the tumors in which it is overexpressed. We use an activation state-dependent anti-HER-2/*neu* monoclonal antibody (PN2A) produced in our laboratory to study this paradigm immunohistochemically. In this report, we analyze the characteristics of 219 cases of DCIS with respect to HER-2/*neu* expression and activation state. We find that 58% of cases of DCIS with overexpression have the receptor in the activated state, a substantially greater proportion than we have previously noted for invasive carcinomas. Although HER-2/*neu* overexpression in general was inversely correlated with hormone receptor expression, cases with activated HER-2/*neu* had the lowest hormone receptor positivity rate. Statistically significant correlations with activated HER-2/*neu* were not noted for tumor size, presence of calcifications, necrosis or fibrosis, or indicators of angiogenesis. These results suggest that examination of activated HER-2/*neu* status may better reflect the biology of a tumor than overall determination of HER-2/*neu* levels. Our finding that active signaling by HER-2/*neu*, as detected by this assay, is more frequent in DCIS than previously noted for invasive carcinoma implicates signaling by HER-2/*neu* as having a critical role in the early stages of breast tumorigenesis.

INTRODUCTION

HER-2/*neu* is a receptor tyrosine kinase closely related to the EGFR[3] (1–5). In the case of the EGFR, epidermal growth factor binds directly to its receptor consequently causing activation (6). Unlike the case for the EGFR, an analogous soluble ligand that binds directly to the receptor, thereby activating it, has not been unequivocally identified for HER-2/*neu* to date. This poses challenges in studying the activation of, and signaling by, this receptor. However, HER-2/*neu* is capable of being activated either by mutation (7, 8), by overexpression (7, 9–11), or by transactivation (12–14). In the latter case, HER-2/*neu* is a partner in a heterodimeric complex with another activated receptor from the EGFR family, resulting in transactivation. In addition, it has been found that alternatively spliced transcripts and cleaved forms of HER-2/*neu* exist (15–22). Cleavage results in release of the extracellular domain, and evidence from some of these studies suggests that the cell-associated portion remaining may be constitutively activated. Subsequent to activation by any mechanism, signal transduction events appear to be similar to those of the liganded EGFR in that receptor autophosphorylation, recruitment of cellular signaling proteins, and phosphorylation of other substrates occurs (reviewed in Ref. 23).

Amplification of the HER-2/*neu* gene with overexpression of HER-2/*neu* protein occurs in ~25% of primary human invasive breast carcinomas, is associated with adverse prognostic indicators, and has itself been correlated with poor prognosis (reviewed in Ref. 24, 25). Use of HER-2/*neu* as a prognostic indicator in clinical decision making has been controversial because of variability in the results reported in different studies. One potential confounding aspect of the existing data was that the level of gene amplification or protein overexpression assayed in prior studies does not necessarily reflect the receptor's functional status, and therefore those assays may be biologically limited. Based upon these considerations and the variable results of clinical studies on the impact of HER-2/*neu*, we hypothesized (26) that HER-2/*neu* may be activated to varying degrees in different tumors overexpressing it, *i.e.*, the stoichiometry of activation might be low or high in different tumors expressing equally high levels of receptor, dependent upon the biology of an individual tumor. To study this, we took advantage of the fact that activated receptors immediately become autophosphorylated, and therefore the phosphorylation state of the receptor reflects its signaling status. To this end, we produced a phosphorylation state-dependent anti-HER-2/*neu* monoclonal antibody, PN2A, which may be used immunohistochemically to assay *in situ* the signaling status of HER-2/*neu* in tumor sections (26, 27).

Using this antibody in immunohistochemical analyses of permanent sections, we have shown that in invasive breast tumors with HER-2/*neu* overexpression, the receptor appears to be activated and, therefore, actively signaling in only a small subset (28, 29). If HER-2/*neu* is truly a marker of the biological behavior of a tumor and the receptor must be activated to exert its effects, then it would follow that the activation of HER-2/*neu* would by necessity be a better marker than simply its level of expression. In a large series of >800 cases of invasive breast carcinoma, we detected phosphorylation of the receptor in only 12% of the HER-2/*neu*-overexpressing cases (18% of axillary lymph node positive cases, 5% of node negative cases). We found that only those cases with activated (phosphorylated) receptor displayed aggressive clinicopathological features and adverse prognoses; cases with overexpressed but unphosphorylated receptor had features and prognoses as favorable as nonoverexpressing cases (29).

DCIS is believed to potentially represent a precursor of invasive carcinoma, and it is hypothesized that many invasive carcinomas pass through an *in situ* stage. HER-2/*neu* appears to be involved in mammary carcinogenesis and/or progression. Paradoxically, however, HER-2/*neu* is overexpressed more frequently in DCIS (particularly the comedocarcinoma subtype) than in invasive breast carcinoma (30–34). Among invasive carcinomas, it is also more frequently overexpressed in those harboring an intraductal component (31, 32, 35). Hence, the role of HER-2/*neu* in DCIS *versus* its role in invasive carcinoma is a particularly intriguing paradigm to explore. One might

Received 3/25/02; accepted 9/16/02.

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[1] Supported by the United States Army Medical Research and Development Command Grants DAMD17-94-J-4135 (to M. P. D.) and DAMD17-95-1-5006 (to E. B. C.) and National Cancer Institute Public Health Service Grants RO3 CA59419 (to E. B. C.) and RO1 CA45708 (to D. F. S.). E. B. C. was also supported by a grant from The Catherine Weldon Donaghue Medical Research Foundation.

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; PR, progesterone receptor.

hypothesize, for example, that although the receptor is overexpressed frequently in DCIS, it might not be in an activated state as frequently as it is in invasive carcinoma, accounting for its relationship to aggressiveness, metastatic capability, and poor prognosis in invasive breast cancer, whereas DCIS is easily curable. In contrast, HER-2/*neu* might be less important to the biology of a more advanced invasive carcinoma than it is to an *in situ* carcinoma because an invasive carcinoma may have acquired multiple other abnormalities that sustain its phenotype.

We have previously reported an extensive analysis of the relationship of various pathobiological features of DCIS (36). Variables examined included HER-2/*neu*, hormone (estrogen and progesterone) receptors, neovascularization, and histological features. In this report, we extend these studies by examining the relationship of the activation state of HER-2/*neu* (HER-2/*neu* phosphorylation) with these features.

MATERIALS AND METHODS

Selection of Cases. The data consist of all female cases of carcinoma *in situ* of the breast initially diagnosed at Yale-New Haven Hospital (New Haven, CT) and Bridgeport Hospital (Bridgeport, CT) from 1982 through 1994. The dataset and its analysis has been previously described (36) and resulted in 219 cases of pure DCIS available for analysis.

Pathological Classification. DCIS was described according to architectural type (comedo, solid, papillary, micropapillary, or cribiform), nuclear grade, presence or absence of necrosis, calcification, size, and periductal neovascularization and fibrosis. The criteria for classification have been described in detail in our previous report (36). Lesions with more than one histological type were classified according to the predominant histological type except for comedocarcinoma, for which any combination containing comedocarcinoma was so classified.

The size of the intraductal carcinoma was estimated with the aid of a ruler and recorded in millimeters (36). Size was determined by measuring the distance between the extreme limits of DCIS on the sections evaluated. The sizes determined were thus estimates (performed on the tissue available and not obtained from the gross surgical specimen or from the original pathology report) and, therefore, almost certainly understated the true size of the DCIS.

Microvascular density was examined using Factor VIII-related antigen immunohistochemistry (see below) for 160 cases (36). For cases in which there were <10 ducts with DCIS/slide, all of the ducts were evaluated for microvascular density. This number was recorded. For cases with >10 ducts, the 10 most densely vascularized ducts were evaluated. Microvessel content was recorded as consisting of none, one, or two rings of vessels and as the percentage of the circumference occupied by either cross sectioned or longitudinally sectioned microvessels. The spatial relationship of the microvasculature with the DCIS-containing ducts reveal either inner rings, outer rings, or both. The inner rings are those microvessels that are virtually apposed to the basement membrane of a duct. The outer rings are those microvessels with the same arc as the basement membrane but peripheral to the inner rings. The shape of the inner and outer rings follows the curvature of the ducts. Randomly distributed microvessels are rare and were not counted. To quantitate the microvascular density, the number of rings observed was recorded, the percentage of the circumference surrounded by microvessels from inner and outer rings was summed to determine the ring score (out of a total possible score of 200), and the ring score was divided by the number of rings to give an average ring score (out of a total possible score of 100).

Factor VIII-related Antigen Immunohistochemistry. One hundred sixty cases were stained immunohistochemically for factor VIII-related antigen (von Willebrand Factor; DAKO, Carpenteria, CA). Paraffin sections were proteolytically predigested with protease I, incubated with rabbit antihuman von Willebrand Factor polyclonal antibody at 1:200 dilution followed by secondary biotinylated goat antirabbit antibody, followed by streptavidin peroxidase. Positive and negative controls were run with each set. Internal positive controls were present in every slide.

HER-2/*neu* Immunohistochemistry. Immunohistochemical staining for HER-2/*neu* was performed using the anti-HER-2/*neu* monoclonal antibody Ab3 (clone 3B5; Oncogene Science, Inc., Manhasset, NY) as described previously (26, 36). This antibody was raised against a synthetic peptide encompassing the Tyr1248 COOH-terminal autophosphorylation site of HER-2/*neu* (37). Although this widely used antibody was assumed to be phosphorylation state-insensitive, studies performed in our laboratory subsequent to the present work have suggested that this antibody displays preferential reactivity with unphosphorylated HER-2/*neu* (38). However, Ab3 staining is a marker of HER-2/*neu* overexpression even in tumors with a high degree of phosphorylation of this receptor (26, 28, 39). Only a membranous pattern of immunostaining was considered positive (see Refs. 24, 26 for a discussion of interpretation of staining patterns). Additionally, each sample was scored semiquantitatively for the intensity of the membranous staining on a 4 point scale, where 0 indicates absence of membrane staining, 1+ the least amount of staining detectable, and 4+ representing the most intensely staining specimens. Any score other than 0 was considered positive. Hence, the percentage of cells staining was not used as a factor in our scoring system but rather the intensity only of the membranous staining, regardless of the percentage of cells staining. A tumor bearing overexpressed HER-2/*neu* was run as a positive control with each batch of slides stained. As a negative control, each case was also stained using a nonspecific monoclonal antibody (Sigma, St. Louis, MO) of the same subclass as Ab3 (IgG1) at a concentration of 20 μg/ml.

The phosphorylation state-dependent anti-HER-2/*neu* monoclonal antibody PN2A, which recognizes exclusively HER-2/*neu* phosphorylated at the Tyr1248 COOH-terminal autophosphorylation site, was affinity purified and used at a concentration of 20 μg/ml as previously described (26), with the addition of antigen retrieval by the pressure cooking method (40) to enhance signal. The antigen retrieval method also caused an increase in diffuse cytoplasmic staining compared with staining in the absence of this step. We consider this cytoplasmic staining artifactual background as for HER-2/*neu* staining (24, 26).

Detection of phosphorylated receptor (PN2A staining) would not be predicted in the absence of any detectable receptor at all (Ab3 staining). As predicted, PN2A staining in the absence of Ab3 staining was never detectable in preliminary studies of >30 cases of Ab3-negative invasive and intraductal carcinomas (data not shown) and was not observed in >500 cases of anti-HER-2/*neu* antibody CB11-negative invasive breast carcinomas (29), and therefore PN2A staining was performed only on the Ab3-positive cases for the major portion of this study, making the assumption that HER-2/*neu* negative cases (Ab3-negative cases) would be PN2A negative. Each sample was scored semiquantitatively for the intensity of the membranous staining on a 4 point scale as for Ab-3, and as for Ab-3, any score other than zero was considered positive. A tumor bearing overexpressed HER-2/*neu* known to be phosphorylated was run as a positive control with each batch of slides stained. As a negative control, each case was also stained using a nonspecific monoclonal antibody (Sigma) of the same subclass as Ab3 and PN2A (IgG1) at a concentration of 20 μg/ml. Biological relevance of this scoring system is indicated by our previous study (29), which showed that PN2A positivity was a strong adverse prognostic factor for early stage invasive breast cancer when scored using any staining as a positive result.

ER and PR Immunohistochemistry. Adequate sections were available to assay ER and PR on 182 and 183 cases, respectively (differences from total number of cases reflect lack of sufficient material). Receptor levels were assayed immunohistochemically using the respective antibodies and staining kits from Abbott Labs (North Chicago, IL). Paraffin sections stained for ER were pretreated with Pronase. For both ER and PR, nuclear staining of at least 10% of the carcinoma cells was the criteria for being considered positive.

Statistical Methods. Because of the relatively small sample size, the data were examined using primarily univariate and bivariate statistics. Univariate statistics were calculated using frequency distributions, means, and percentages. Bivariate analyses were performed using χ^2 and t tests for discrete and continuous outcomes, respectively. Logistic regression was used to provide maximum likelihood estimates of the odds ratios (adjusted and unadjusted) with 95% confidence intervals using the statistical package PC-SAS version 6.11. Continuous outcomes were examined using ANOVA, with multiple comparisons made using the Bonferroni procedure.

RESULTS

Among the 191 cases of DCIS examined immunohistochemically for overexpression of HER-2/*neu* using Ab3 (Figs. 1 and 2), 54 cases,

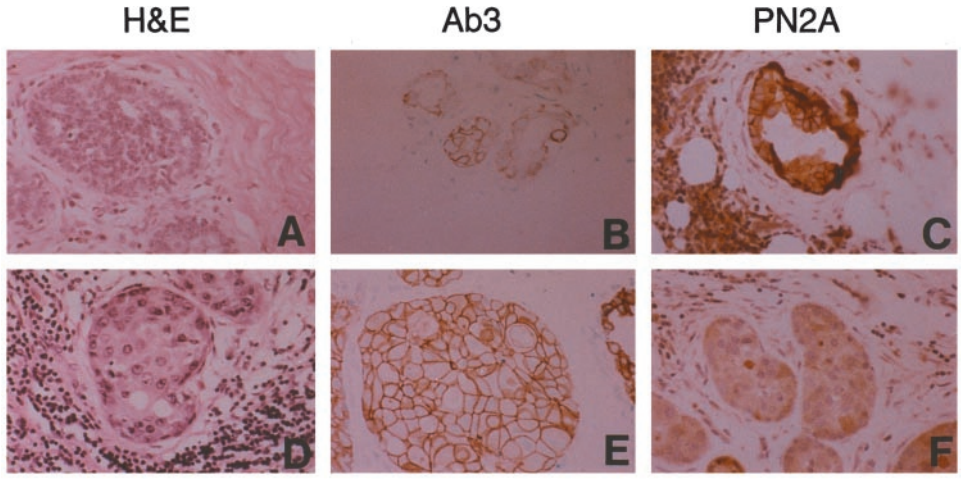

Fig. 1. Photomicrographs of two representative cases of DCIS stained with H&E (H&E, *A* and *D*), anti-HER-2/*neu* antibody Ab3 (*B* and *E*), and antiphospho-HER-2/*neu* antibody PN2A (*C* and *F*). The first case, shown in *A–C*, overexpresses activated HER-2/*neu* as demonstrated by strong membranous immunostaining with both Ab3 and PN2A; PN2A staining indicates that the HER-2/*neu* is in the activated (phosphorylated) state. The second case overexpresses HER-2/*neu*, as demonstrated by strong membranous immunostaining with Ab3, but the receptor is in the unphosphorylated, inactive state, as indicated by lack of membrane staining with PN2A.

or 28%, were found to be overexpressors. As discussed above, the phosphorylated state is a marker of activation. When analyzed for phosphorylation status by PN2A immunohistochemistry, 31 of 53 HER-2/*neu*-positive cases, or 58% of the overexpressors (31 of 191 cases or 16% of the overall dataset), were found to express the receptor in its phosphorylated, activated state (Figs. 1 and 2). This frequency of phosphorylation in the overexpressing DCIS cases is dramatically higher than the 12% rate we previously noted for HER-2/*neu*-overexpressing invasive breast carcinomas (29).

The relationship of HER-2/*neu* score to PN2A score was examined. This is shown as a scatterplot in Fig. 3, where the HER-2/*neu* staining intensity with Ab3 (all Neu+ cases) is compared with that of phospho-HER-2/*neu* with antibody PN2A (phospho-HER-2/*neu* positive = PNeu+ cases) semiquantitatively on a 4 point scale as described in "Materials and Methods." We found a strong correlation between the two, with a Pearson's coefficient of correlation of $r = 0.44$ ($P < 0.01$). We had previously noted a similar relationship in invasive breast carcinomas (29). Such a relationship is not unexpected because the overall level of receptor expression (Ab3 staining) by necessity defines the limits of detection of phosphorylated receptor (PN2A staining). In addition, it is known from cell culture experiments that the level of overexpression of HER-2/*neu* correlates with the basal stoichiometry of receptor phosphorylation (7). Although there is a tendency for the lowest (1+) HER-2/*neu* overexpressors to be unphosphorylated and the highest (4+) to be phosphorylated, as expected based upon the above considerations, these data and our previously published data (29) demonstrate that PN2A is not a simple surrogate marker of HER-2/*neu* expression level. PN2A does not merely identify cases with the highest levels of HER-2/*neu* overexpression but identifies phosphorylated receptor regardless of level of expression. This is also seen visually in Fig. 1, where two cases each having equivalently high HER-2/*neu* overexpression demonstrate PN2A staining which is very strong in one case and absent in the other.

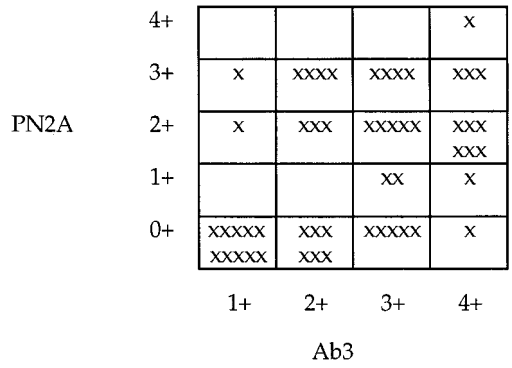

Fig. 3. Relationship of PN2A staining to Ab3 staining. Each case overexpressing HER-2/*neu* is represented by a single "x." Plotted is the comparison of the staining intensity of PN2A with that of Ab3, scored semiquantitatively on a 4 point scale as described in "Materials and Methods." The correlation coefficient for the relationship of PN2A with Ab3 staining is $r = 0.44$; $P < 0.01$.

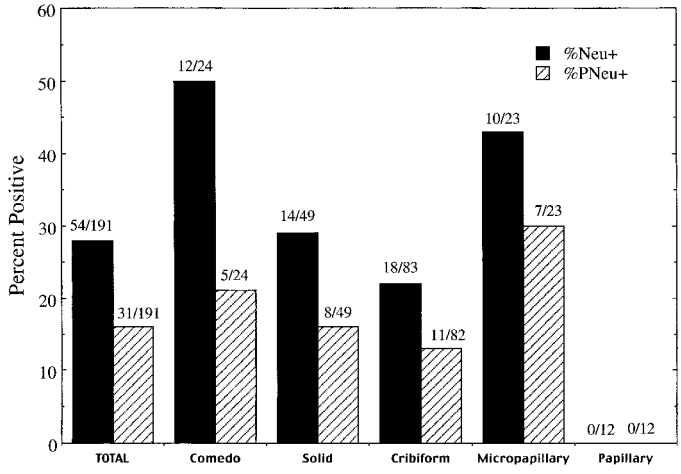

Fig. 2. Immunohistochemical analysis for HER-2/*neu* overexpression (Neu+, ■) and HER-2/*neu* activation/phosphorylation (PNeu+, ▨). Results are shown as percentage of the total cases within each category and tabulated for the total dataset (Total) and for each histological subtype. The actual number of cases is displayed at the *top* of each bar.

HER-2/*neu* expression was characterized with respect to histological subtype of DCIS (Fig. 2). Our series included 24 cases of comedocarcinoma, 49 solid variants, 83 cribiform, 23 micropapillary, and 12 papillary carcinomas. As noted by others, we found the highest frequency of HER-2/*neu* overexpression among comedocarcinomas (50%). Conversely, overexpression was rare in papillary carcinoma, with no positives found among the 12 cases. Other histological subtypes demonstrated intermediate percentages of HER-2/*neu* positivity (29% for solid variants, 22% for cribiform, and 43% for micropapillary carcinomas). Statistically significant differences are noted only in the comparison of comedocarcinoma to papillary subtypes. In comparing cases with overexpression of nonphosphorylated, inactive HER-2/*neu* (Neu positive, phospho-Neu negative or Neu+/PNeu−) to those with phosphorylated, activated HER-2/*neu* (Neu+/PNeu+), the receptor was phosphorylated in a proportion of the overexpressing cases of each type (42% for comedocarcinomas, 57% for solid vari-

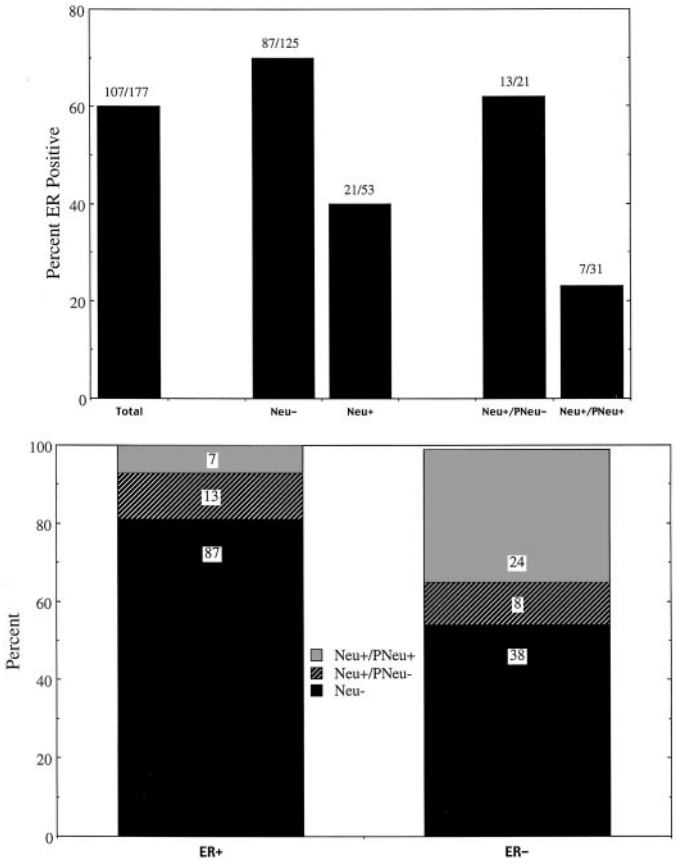

Fig. 4. Relationship of HER-2/*neu* status to ER. *Top panel*, the percentage of ER+ cases is shown for the total dataset (Total), for the breakdown of HER-2/*neu* overexpressors *versus* nonoverexpressors (Neu+ *versus* Neu−), and for the breakdown of phosphorylated HER-2/*neu versus* nonphosphorylated HER-2/*neu* (Neu+/PNeu+ *versus* Neu+/PNeu−). The actual number of cases is displayed at the *top* of each bar. *Bottom panel*, the ER+ and the ER− cases are displayed by the percentage of each without HER-2/*neu* overexpression (Neu−, ■), with overexpression of phosphorylated HER-2/*neu* (Neu+/PNeu+, □) and with overexpression of nonphosphorylated HER-2/*neu* (Neu+/PNeu−, ▨). The actual number of cases is displayed within each bar.

ants, 65% for cribiform, and 70% for the micropapillary carcinomas). Hence, although comedocarcinomas have the highest frequency of HER-2/*neu* overexpression, other histological subtypes actually have a similar or higher percentage of the overexpressed cases in which the HER-2/*neu* is activated (percentage of HER-2/*neu*-positive cases activated not statistically significantly different among subtypes).

HER-2/*neu* expression was examined with respect to hormone receptor status. Although ER and PR status are not known prognostic or predictive factors in DCIS as they are in invasive breast carcinoma, we were interested in assaying these receptors as they relate to the biology of DCIS. As we reported previously (36), and concordant with other literature reports, we found an inverse correlation between HER-2/*neu* overexpression and ER expression (Fig. 4). This data are presented showing ER positivity as a function of HER-2/*neu* status (Fig. 4, *top*) and showing HER-2/*neu* status as a function of ER expression (Fig. 4, *bottom*). As shown in Fig. 4, *top*, ER positivity was seen in 60% of the total data set. We found a 70% rate of ER positivity among Neu− cases *versus* a 40% rate among Neu+ cases ($P < 0.01$). When stratified according to phosphorylation status of HER-2/*neu*, even more striking differences were observed, with a 64% rate of ER positivity for Neu+/PNeu− cases *versus* a 23% rate for Neu+/PNeu+ cases ($P < 0.01$). Hence the ER positivity rate of cases overexpressing nonphosphorylated HER-2/*neu* is not very different from the rate of the HER-2/*neu*-negative cases; only the cases bearing phosphorylated HER-2/*neu* show exceptionally low rates of ER expression. HER-2/*neu* status is shown as a function of ER in Fig. 4, *bottom*, where it can be easily seen that the majority of ER+ cases (81%) lack HER-2/*neu* overexpression (and most of the ER+ overexpressors are not phosphorylated), whereas 45% of the ER− cases overexpress HER-2/*neu* and three-fourths of those are in the phosphorylated state. The statistical relationships between HER-2/*neu*, phosphorylated HER-2/*neu*, and ER are shown in Table 1.

Similar findings were noted for PR. We found an inverse correlation between HER-2/*neu* overexpression and PR expression (Fig. 5). As shown in Fig. 5, *top*, PR positivity was seen in 62% of the total data set. We found a 74% rate of PR positivity among Neu− cases *versus* 36% rate among Neu+ cases ($P < 0.01$). When stratified by HER-2/*neu* phosphorylation status, we observed a 52% rate of PR positivity for Neu+/PNeu− cases *versus* a 23% rate for Neu+/PNeu+ cases ($P = 0.03$). As for the case of ER, it is mainly the cases bearing phosphorylated HER-2/*neu* that show very low rates of PR expression. HER-2/*neu* status is shown as a function of PR in Fig. 5, *bottom*, where as in the case of ER, the majority of PR+ cases (84%) lack HER- 2/*neu* overexpression (and more of the PR+ overexpressors are not phosphorylated), whereas 51% of the PR− cases overexpress HER-2/*neu* and the majority of those (71%) are in the phosphorylated state. As seen for the ER, the Neu− cases and the Neu+/PNeu− cases were not statistically different from each other with regard to PR status; only the Neu+/PNeu+ cases had a dramatically lower rate of PR expression. The statistical relationships between HER-2/*neu*, phosphorylated HER-2/*neu*, and PR are shown in Table 1.

The distribution of nuclear grade with respect to HER-2/*neu* status is shown in Fig. 6. We find, like others, that high nuclear grade is associated with HER-2/*neu* overexpression (grade 3 *versus* 2 or 1, although our data set contained very few grade 1 cases). When the grade 2 and 3 cases are stratified according to phospho-

Table 1 *Associations between HER-2/neu, activated/phosphorylated HER-2/neu, and clincopathological features*

Group A = HER-2/*neu* negative; group B = HER-2/*neu* positive, PN2A negative; group C = HER-2/*neu* positive, PN2A positive.

Factor	A Mean	B Mean	C Mean	A *versus* B *P*	B *versus* C *P*	A *versus* C *P*
Grade	2.2	2.5	2.5	n.s.[a]	n.s.	n.s.
Size (cm)	0.82	0.75	0.72	n.s.	n.s.	n.s.
ER+	70%	64%	23%	n.s.	≤0.05	≤0.05
PR+	74%	52%	23%	n.s.	≤0.05	≤0.05
Rings	1.22	1.48	1.45	n.s.	n.s.	n.s.
Ring score	34.4	50.0	45.0	n.s.	n.s.	n.s.
Avg. ring score	25.8	37.1	32.4	n.s.	n.s.	n.s.
Necrosis	61%	78%	77%	n.s.	n.s.	n.s.
Fibrosis	56%	74%	84%	n.s.	n.s.	n.s.
Calcifications	51%	61%	35%	n.s.	n.s.	n.s.
HER-2/*neu* score	0	1.87	3.00	≤0.05	≤0.05	≤0.05

[a] n.s., not significant at $P = 0.05$.

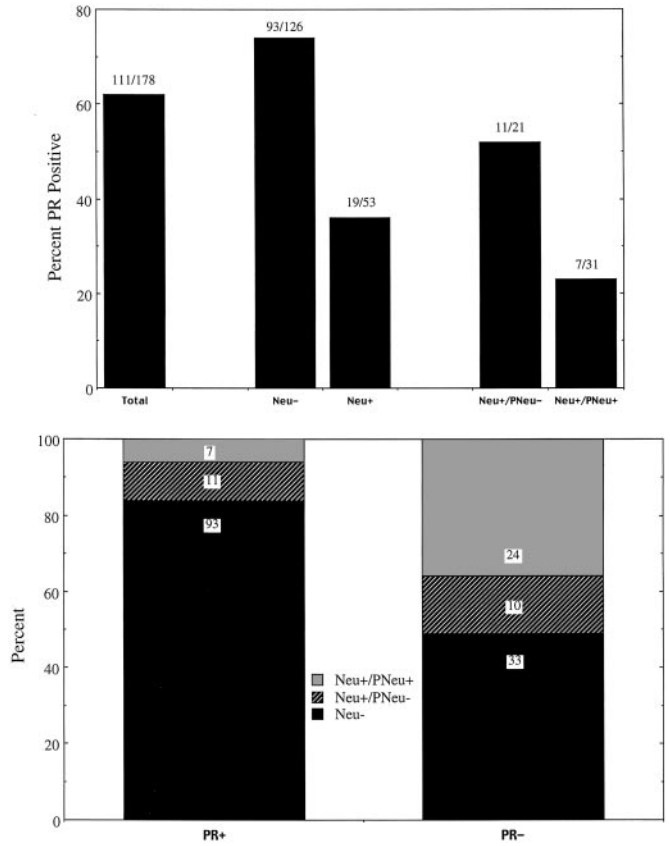

Fig. 5. Relationship of HER-2/*neu* status to PR. *Top panel*, the percentage of PR+ cases is shown for the total dataset (Total), for the breakdown of HER-2/*neu* overexpressors *versus* nonoverexpressors (Neu+ *versus* Neu−), and for the breakdown of phosphorylated HER-2/*neu versus* nonphosphorylated HER-2/*neu* (Neu+/PNeu+ *versus* Neu+/PNeu−). The actual number of cases is displayed at the *top* of each bar. *Bottom panel*, the PR+ and the PR− cases are displayed by the percentage of each without HER-2/*neu* overexpression (Neu−, ■), with overexpression of phosphorylated HER-2/*neu* (Neu+/PNeu+, □) and with overexpression of nonphosphorylated HER-2/*neu* (Neu+/PNeu−, ▨). The actual number of cases is displayed within each bar.

rylation status of HER-2/*neu*, however, the proportion of HER-2/*neu*-overexpressing cases with phosphorylation was similar for both (60% of the grade 2 cases and 57% of the grade 3 cases). The statistical relationships between HER-2/*neu*, phosphorylated HER-2/*neu*, and nuclear grade are shown in Table 1. [Note that we previously found a statistically significant association between total HER-2/*neu* overexpression and grade (36).]

The relationships of HER-2/*neu* and phosphorylated HER-2/*neu* with tumor size, as well as presence of necrosis, periductal fibrosis, and calcifications are shown in Table 1. In the era of widespread use of mammography, the majority of the cases of DCIS are detected when the tumor is not >1 cm in size; most cases in our series were <1 cm in size. We did not find a statistically significant relationship between HER-2/*neu* status and size. The presence of necrosis, periductal fibrosis, or calcifications were also not significantly related to HER-2/*neu* status.

Finally, the relationship of HER-2/*neu* overexpression and phosphorylation to neovascularization was examined by noting the number of rings, the mean ring score, and the mean average ring score as defined in "Materials and Methods." None of these indicators of angiogenesis were significantly associated with HER-2/*neu* status. [Note that in our previous study (36), we did note a modest positive correlation between total HER-2/*neu* overexpression and these markers of angiogenesis.]

DISCUSSION

In this report we categorize DCIS with respect to HER-2/*neu* status, with particular emphasis on elucidating the distinguishing characteristics between cases expressing nonphosphorylated and, therefore, nonsignaling (inactive) receptor *versus* those expressing phosphorylated and, therefore, presumably actively signaling receptor. In actuality, there likely exists a continuum of the degree of phosphorylation/activation of the population of receptors in any given cell/tumor. Our assay likely has a threshold that separates cases with relatively low levels of activated receptor from those with relatively high levels. That this putative threshold of our assay gives a biologically significant separation of cases is indicated by our previous results with invasive carcinomas in which our assay specifically identified the HER-2/*neu* overexpressing cases that had distinct biological features and poor prognoses (29). Other authors have examined expression and phosphorylation of HER-2/*neu* in tumors using more sensitive methods of Western blotting (41, 42) or ELISA (43) and, not surprisingly, find a higher percentage of "overexpressing" cases to have detectable phosphorylation of receptor. Even with the more sensitive methods that might result in a greater proportion of the cases being considered in the phospho-positive group, the preliminary results with these assays indicate that they too appear to identify a biologically distinct poorer prognosis group.

We have detected overexpression of HER-2/*neu* in 28% of all DCIS cases, with particularly high frequency in comedocarcinoma (50%), followed by micropapillary carcinoma (43%), solid variant (29%), cribiform carcinoma (22%), and none in 12 cases of papillary carcinoma (0%). All histological types showed phosphorylation of the receptor in a subset of the overexpressing cases, with micropapillary carcinoma having the highest percentage of phosphorylation (70%).

Our results also indicate that hormone receptor status, which is inversely correlated with overexpression of HER-2/*neu* in this study and others, is significantly inversely correlated with phosphorylated, activated receptor to an even greater degree. Thus, phosphorylated HER-2/*neu* is a better predictor of the hormone receptor-negative status than is the overall level of expression. We noted similar results for invasive breast carcinomas (29). Some existing experimental data suggest potential mechanisms for the inverse relationship between hormone receptor and HER-2/*neu* levels. Studies performed in cultured cells have shown that treating ER-positive cells with estrogen, a mitogen, actually resulted in a decrease in HER-2/*neu* mRNA at the transcriptional level (44, 45). This was partly reversed by antiestrogen. Although antiestrogens have shown clear clinical benefit in

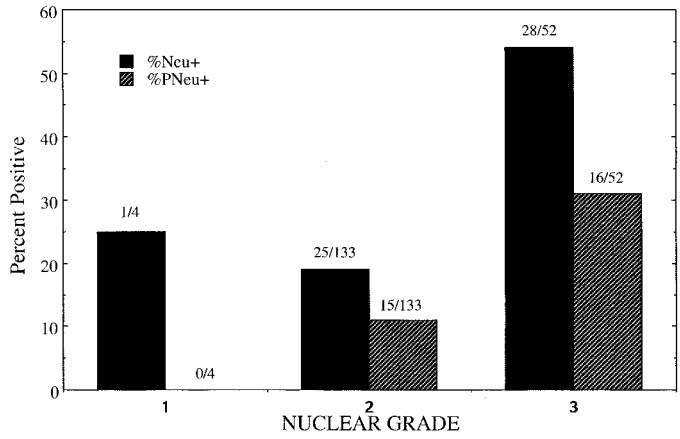

Fig. 6. Immunohistochemical analysis for HER-2/*neu* overexpression (Neu+, ■) and HER-2/*neu* phosphorylation (PNeu+, ▨) as a function of nuclear grade. The actual number of cases is displayed at the *top* of each bar.

hormone receptor-positive breast cancer, particularly in invasive disease, these results suggest that the therapeutic efficacy of certain antiestrogens may be partly subverted by a tendency to raise HER-2/*neu* levels if indeed elevated HER-2/*neu* levels result in a more aggressive phenotype. One report has shown that HER-2/*neu* overexpression results in estrogen-independent growth, which is unaffected by either estrogen or antiestrogen (46). Interestingly, ER has been shown to be tyrosine phosphorylated, and this phosphorylation appears to be required for ER dimerization, an essential step in ER signaling (47). Tyrosine phosphorylation of ER can be induced by heregulin activation of HER-2/*neu* (46). In these studies, HER-2/*neu* overexpression resulted in down-regulation of ER (46). Regardless of the mechanisms operative, our results support our hypothesis that the impact of HER-2/*neu* expression on tumor biology is better reflected by specific analysis of the degree of signaling activity of this receptor than by simple assays of expression level.

A remarkable aspect of our present results is the fact that we find phosphorylation/activation of HER-2/*neu* to occur in a much greater proportion of HER-2/*neu*-overexpressing cases of DCIS (58%) than we previously found for invasive breast carcinomas (12%; Ref. 29). It has long been appreciated that overexpression of HER-2/*neu* itself occurs in a much greater frequency of cases of DCIS than invasive breast carcinomas (30–34). Because this receptor is associated with aggressive features and adverse outcomes in invasive breast cancer, this finding seemed paradoxical. One might hypothesize that the histological subtypes of DCIS with frequent overexpression of HER-2/*neu* tend not to progress to invasive carcinoma, whereas those with infrequent overexpression do so. However, comedocarcinoma, which has the highest frequency of overexpression, is associated with more aggressive proliferative features (48) and is generally regarded as the subtype of DCIS most likely to progress or be associated with concurrent or subsequent invasive carcinoma (49, 50). One alternative could be that *in situ* and invasive carcinomas do not always lie in a linear pathway of progression from the former to the latter; it is possible that invasive carcinoma may arise *de novo*. It might have also been the case that the receptor simply is less activated at the *in situ* phase than in an invasive phase; our present results refute this hypothesis. Our results show that the receptor is frequently activated in DCIS. This suggests that HER-2/*neu* signaling activity is a very important component of the biology of DCIS itself and possibly plays a critical role in tumorigenesis. As a tumor advances to a more abnormal, invasive phenotype, other biological changes may occur that supplant the requirement for continued HER-2/*neu* signaling; this may explain the low response rate in the treatment of HER-2/*neu*-overexpressing advanced breast cancer patients with single agent trastuzumab (Herceptin), the therapeutic anti-HER-2/*neu* antibody. Such anti-HER-2/*neu*-targeted therapy could be more efficacious at treating DCIS than invasive carcinoma, although it would be a difficult clinical area to explore because one would not want to compromise the nearly 100% cure rate achieved surgically. However, this may bode well for the incorporation of anti-HER-2/*neu*-targeted therapy into the adjuvant treatment of early stage invasive breast cancer.

An experimental model for the early initiation of breast cancer provides supporting evidence for a mechanistic role of HER-2/*neu* in the development of DCIS (51). Using nontransformed MCF-10A human breast epithelial cells in a three-dimensional culture system that results in development of mammary acinar structures, the specific activation of HER-2/*neu* (but not EGFR) elicited changes reminiscent of DCIS, including loss of proliferative suppression with reinitiation of proliferation, filling of the lumen with proliferating cells, and loss of acinar organization (51). Interestingly, the basement membrane remained intact, and anchorage independence and invasive properties did not develop, more consistent with the clinical picture of DCIS than with the development of a fully transformed phenotype. These results and our present results support a direct role for HER-2/*neu* in the biology of DCIS.

In summary, this report confirms our preliminary results indicating that HER-2/*neu* is phosphorylated and, therefore, actively signaling in a subpopulation of the cases of DCIS in which it is overexpressed (26). Cases with phosphorylated HER-2/*neu* were biologically distinct in having the lowest association with ER and PR. A striking finding in this study is the fact that a far greater proportion of DCIS not only overexpresses HER-2/*neu* but also has the receptor in the phosphorylated/activated state in comparison to that previously noted for invasive carcinoma. This suggests a critical role for this receptor in the biology of DCIS and in early phases of breast tumorigenesis.

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