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Type 1 Receptor Tyrosine Kinases Are Differentially Phosphorylated in Mammary Carcinoma and Differentially Associated with Steroid Receptors

Sarah S. Bacus,* Dot Chin,* Yosef Yarden,† Carolyn R. Zelnick,* and David F. Stern‡

From Advanced Cellular Diagnostics, Inc., Elmhurst, Illinois; Weizmann Institute of Science,† Rehovot, Israel; and the Department of Pathology,‡ Yale University School of Medicine, New Haven, Connecticut*

The neu/erbB-2/HER-2 proto-oncogene is amplified and/or overexpressed in up to 30% of mammary carcinomas and has been variably correlated with poor prognosis. The signaling activity of the encoded receptor tyrosine kinase is regulated by interactions with other type 1 receptors and their ligands. We have used a novel approach, phosphorylation-sensitive anti-Neu antibodies, to quantify signaling by Neu and epidermal growth factor receptor in a panel of frozen sections of mammary carcinoma specimens. We also determined the relationship of Neu, phosphorylated Neu (and epidermal growth factor receptor), and phosphotyrosine to the expression of Neu-related receptors (epidermal growth factor receptor, HER-3, and HER-4) and to prognostic factors (estrogen and progesterone receptor). We found that tyrosine phosphorylation of Neu (and hence signaling activity) is highly variable among mammary carcinomas. Neu and HER-4 were associated with divergent correlates, suggesting that they have profoundly different biological activities. These results have implications for etiology of mammary carcinoma, for clinical evaluation of mammary carcinoma patients, and for development of Neu-targeted therapeutic strategies. (Am J Pathol 1996, 148:549–558)

Cytogenetic evidence implicates a small number of genes in initiation and progression of mammary carcinoma. After p53 mutations, the foremost specific genetic lesion in mammary carcinomas is a gene amplification encompassing the gene known as *neu*, *erbB*-2, or *HER*-2. *neu* encodes $p185^{neu}$, a receptor tyrosine (Tyr) kinase (RTK) that will be referred to here as Neu. Neu is a member of the type 1 group of the RTK super-family. Type 1 receptors consist of the prototype epidermal growth factor receptor (EGFR; encoded by the *erbB* or *HER*-1 gene) and proteins encoded by *neu*/*erbB*-2/*HER*-2,[1] *erbB*-3/*HER*-3,[2] and *erbB*-4/*HER*-4[2] genes.

The *neu* gene is amplified in 20 to 30% of mammary carcinomas[3] (reviewed in Ref. 4). The amplification is associated with concomitant RNA and protein overexpression,[5] and an additional fraction of tumors overexpress Neu in the absence of gene amplification.[6,7] The idea that overexpression of Neu can initiate mammary carcinogenesis is consonant with a number of experimental observations. Neu is normally expressed at modest levels in mammary tissue. *neu* is a potent oncogene when activated by mutation. Moreover, in contrast to other RTKs including the related EGFR, overexpression of Neu is sufficient to induce focus formation and cell transformation in the absence of activating ligands.[8,9] Mutationally activated *neu* is evidently more potent even than activated *ras* in transformation of rodent mammary epithelium, whether introduced by infection with retroviral vectors or in transgenic experiments.[10,11] Perhaps the most compelling evidence is the finding that overexpression of structurally normal Neu in transgenic mice leads to metastatic mam-

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Address reprint requests to Dr. David F Stern, Department of Pathology, Yale University School of Medicine, PO Box 208023, New Haven, CT 06520-8023.

mary carcinoma.[12] This mouse model closely resembles the apparent role of Neu in human carcinogenesis, where overexpression of normal Neu is found at the earliest stages of carcinogenesis.

Because of the urgent need for prognostic indicators that have better predictive ability in breast cancer, much attention has been paid to the possible utility of Neu as a clinical marker. Immunohistochemical studies show that Neu overexpression is generally more frequent in tumors from node-positive patients than from node-negative patients.[5,13,14] A number of studies demonstrate a poorer prognosis for patients with overexpressed Neu, but there is considerable variability in the extent of this difference and in the apparent independence from other prognostic factors. Thus, these studies encourage the belief that Neu may aid in cancer prognosis, but there is not as yet a clear-cut clinical application (reviewed in Refs. 4, 15, and 16).

Like other RTKs, Neu has an intrinsic protein kinase activity that can be regulated by peptide hormones. Although no hormones have been identified that bind to Neu expressed on its own, several hormones regulate Neu Tyr phosphorylation and signaling activity. A process known as transmodulation enables other Type 1 receptors to activate Neu when they are themselves bound to their cognate hormones. The first evidence for these activating influences came from the finding that most epidermal growth factor (EGF) agonists (transforming growth factor-α, β-cellulin,[17] amphiregulin,[18] heparin-binding EGF-like growth factor,[19] and epiregulin[20]) can activate Tyr phosphorylation and presumably signaling by Neu, although they do not bind to or activate Neu expressed on its own.[21] The mechanism for this interaction is evidently the formation of heterodimers between Neu and the EGF receptor (EGFR).[22,23] Analogous interactions of HER-3 and HER-4 proteins with Neu are activated by binding of members of the Neu differentiation factor (NDF)/heregulin family of growth factors.[24–27] These factors bind to HER-3[28,29] and HER-4.[30] Thus, the signaling activities of Neu and other type 1 receptors are regulated by a complex web of inter-receptor interactions, with the hormones including at least six different peptides that bind to the EGFR and a dozen or more different isoforms of NDFs that bind to HER-3 and HER-4[31,32] (reviewed in Ref. 4).

Previous studies of *neu* in disease have focused on the relative abundance of the receptor. However, the signals emanating from Neu are likely to be influenced more by the presence of hormones that regulate Neu (either by binding directly or working through transmodulating receptors) than by Neu abundance. Moreover, chronic activation and transmodulation of Neu may cause chronic down-regulation of signaling active receptors. Thus, of two tumors that produce Neu polypeptide at identical levels, hormonal activation of Neu may diminish the steady-state level of Neu. This will lead to the paradoxical result that tumors harboring a greater number of active forms of Neu will display less, rather than more, Neu immunoreactivity. For these reasons, the full predictive utility of Neu will be reached only by measuring signaling activity rather than abundance.

As Tyr phosphorylation of RTKs correlates with signaling activity, Neu activity can be measured in immunoblots with antibodies to phosphotyrosine (P-Tyr).[33–35] However, these sera have limited usefulness in tissue-based assays such as immunohistochemistry as they integrate signals from all Tyr phosphoproteins. Instead, antibodies have been developed that recognize Neu only when it is Tyr phosphorylated at a particular site.[2,36] We had previously described the production of a phospho-Neu (P-Neu)-specific polyclonal antibody (anti-P-Neu A1) that specifically recognizes the Tyr-phosphorylated but not nonphosphorylated form of Neu (and also the related EGFR).[2] We describe here the use of this antibody to determine whether these receptors are differentially phosphorylated among mammary carcinoma specimens and whether phosphorylation correlates with prognostic indicators for mammary carcinoma. The results show that Tyr phosphorylation of Neu (and EGFR) is highly variable among mammary carcinomas and that Neu and HER-4 are associated with divergent clinical correlates.

Materials and Methods

Tissue

Tumors were obtained by needle biopsy or by surgical excision, snap frozen, and stored at −70°C over a period of up to three years before analysis.

Antibodies

Monoclonal antibody N24 was used for detecting Neu,[37] and polyclonal rabbit anti-Neu phosphopeptide antibody A1 (10 μg/ml) for P-Neu.[2] Other antibodies were monoclonal anti-EGFR (Amersham, Arlington Heights, IL), monoclonal anti-HER-3 RTJ2 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-HER-4 C18 (Santa Cruz Biotechnology), and anti-P-Tyr (Oncogene Science, Cambridge, MA). LH1 monoclonal antibody and monoclonal

MPRI (Cell Analysis Systems, Elmhurst, IL) were used for detection of estrogen receptor (ER) and progesterone receptor (PR), respectively. Secondary antibodies used were biotinylated goat anti-mouse IgG for N24, EGFR, and P-Tyr, biotinylated goat anti-rabbit IgG for P-Neu (Jackson Laboratories, West Grove, PA), and biotinylated rabbit anti-mouse IgG1 for HER-3 (Zymed Laboratories, San Francisco, CA).

Immunohistochemistry

For PR and ER assays, the chromogen 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) was used. All other antibodies were detected using the alkaline phosphatase chromogen CAS Red and all sections were counterstained with CAS DNA stain (Cell Analysis Systems). All incubations were at 37°C except for DNA counterstaining, which was performed at room temperature. Tumors were fixed in 10% neutral buffered formalin for 60 minutes. After rinsing with Tris-buffered saline, the slide was incubated in blocking solution (10% rabbit serum, 0.1% bovine serum albumin, 0.5% Triton X-100 in phosphate-buffered saline) except for Neu and P-Neu, which were blocked with goat serum. Primary antibodies were added for 30 minutes. Biotinylated secondary antibodies were added for 20 minutes, followed by alkaline phosphatase-conjugated steptavidin for 15 minutes.

Quantitation of Immunohistochemistry

Expression of EGFR, HER-3, and HER-4 were visually assessed. Concordant results for HER-4 were obtained with reverse transcriptase polymerase chain reaction using HER-4-specific primers. For quantitation of immunohistochemical staining of Neu, P-Neu, P-Tyr, ER, and PR, a microscope-based two-color system (CAS 200 image analyzer, which has two solid-state imaging channels) was used. Expression of Neu, P-Neu, and P-Tyr were quantitated as follows. Digitized light intensity values were converted to optical density values and combined. One channel was used for quantitating total DNA of cells in the field (after Feulgen staining with a DNA staining kit), the other for quantitating levels of antigen after immunostaining. As the total amount of DNA per cell was known, the average protein level per cell could be computed. For each specimen, at least five random fields of tumor epithelium were measured. Average amounts of Neu and P-Neu staining were quantified as previously described.[38–40] Breast cancers were considered to be positive for Neu overexpression if their cells contained more than 15% of the Neu protein found in sparsely growing AU-565 cells. Levels of ER and PR were determined by the masking technique[41] and were reported as equivalents to steroid binding assays after calibration of the imaging system with cell pellets consisting of cells expressing ER and PR on which the receptor amounts had been defined by enzyme-linked immunosorbent assay. For statistical analyses shown here, ER and PR figures were converted to + and −. Similar results were obtained when they were used as continuous variables.

For practical reasons, it was not possible to perform all assays on all tissues. Quantitated data were obtained for 113 samples stained with anti-Neu (29 of these were also scored visually: 11 − and 18 +), 98 samples with anti-P-Neu A1, and 86 with anti-P-Tyr, and 28 samples were scored + or − for anti-EGFR, (13−,15+); 94 for ER (30−,64+); and 94 for PR (44−,50+).

Statistical analysis was performed by Karol Katz and Dr. Robert Makuch of the Biostatistics Computing Unit, Yale University Department of Epidemiology and Public Health, using SAS software.

Results

Immunohistochemical Staining of Cell Lines with Anti-P-Neu A1

We showed previously that anti-P-Neu A1 specifically recognizes Tyr-phosphorylated forms of Neu and the EGFR equally well but not other P-Tyr-containing proteins in immunoblots. Indirect immunofluorescence experiments demonstrated that EGF stimulates immunoreactivity, as expected.[2] To determine whether the somewhat different immunodetection procedure used here would faithfully report Tyr phosphorylation of p185, we first performed a series of reconstruction experiments (Figure 1; other data not shown). AU-565 human mammary carcinoma cells express at least 10-fold higher levels of p185 than the EGFR. p185 can be detected by immunocytochemistry with anti-Neu (Figure 1C). Anti-P-Neu A1 showed minimal reactivity with these cells (Figure 1A) despite the high Neu expression. However, treatment of cells with EGF, to enhance Neu Tyr phosphorylation through transmodulation, greatly stimulated immunoreactivity with anti-P-Neu A1 (Figure 1B). The immunoreactivity was completely blocked by preincubation of the antibody with the corresponding synthetic Neu phosphopeptide (Figure 1D). Inasmuch as the abundance of p185 does not

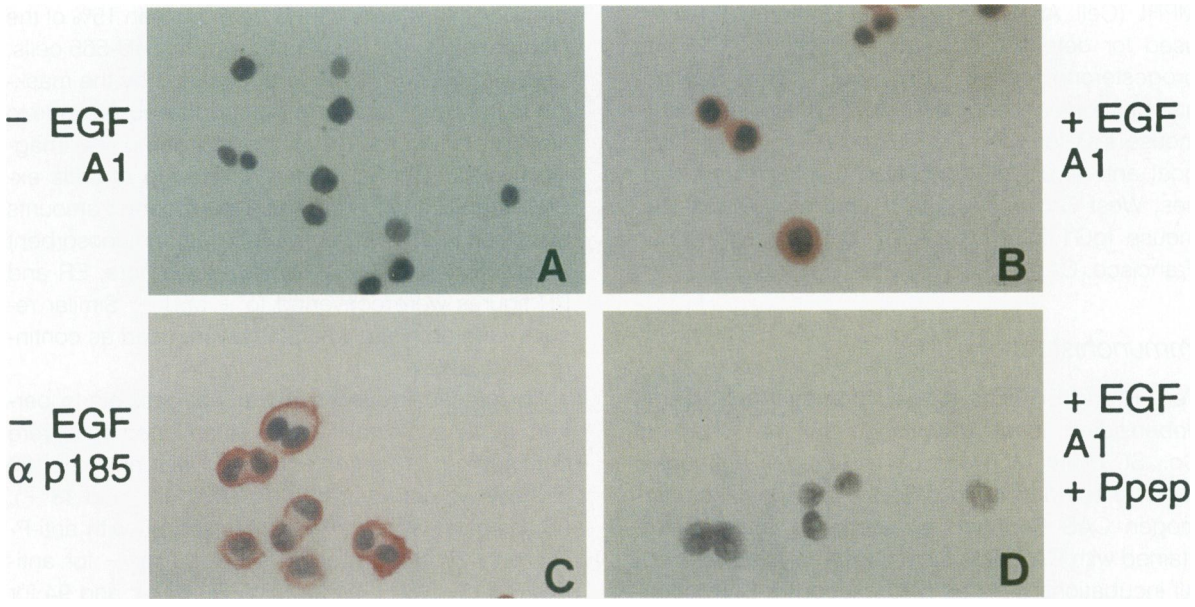

Figure 1. *Immunocytochemistry of EGF-treated AU-565 cells. AU-565 cells were seeded in eight-well chamber slides (Lab-Tek) at 6 × 10^3 cells/well. After 18 to 20 hours, cells were incubated with 30 nM EGF for 7 to 10 minutes (B and D) or left untreated (A and C). Cells were analyzed by immunocytochemistry with anti-p185Neu (C) or anti-P-Neu A1 (A, B, and D). A1 was preincubated with an excess of P-Neu blocking peptide (Ppep; 0.4 mg/ml) for D. Immunostaining was detected using a red chromagen as described in Materials and Methods.*

change during these assays and as A1 recognizes phosphorylated Neu at least as well as phosphorylated EGFR,[2] this result demonstrated that this procedure quantifies p185 Tyr phosphorylation. These results together with previous analysis of the same antibody[2] verified the specificity of anti-P-Neu and its utility with this detection system.

According to some reports, authentic immunostaining of Neu in paraffin-embedded sections is membrane specific, whereas nonmembrane staining is artifactual.[16] The experiment in Figure 1 demonstrates that, with antibody A1 on frozen sections, specific staining is internal as well as surface associated, as the immunoreactivity is stimulated by EGF during a period in which Neu protein concentration does not significantly change. In contrast, specific staining of paraffin-embedded sections with a monoclonal anti-P-Neu we have recently developed is primarily associated with membranes.[42] We assume that major differences in handling, fixation, and staining procedures used account for these differences in localization of immunoreactivity. Another potential variable is that receptor down-regulation should shift the balance of cell surface and nonsurface staining and may vary with different dimerization partners.

Immunohistochemical Staining of Human Mammary Carcinoma with Anti-P-Neu A1

We next determined whether the relative levels of Neu (plus EGFR) Tyr phosphorylation vary among tumors. A series of frozen sections of mammary carcinomas were stained with (phosphorylation-independent) anti-Neu antibody and (phosphorylation-dependent) anti-P-Neu A1. To conserve A1, many of the tumor samples were preselected for some immunoreactivity with anti-Neu so that in this panel approximately 80% of tumors were Neu positive. Most tumors analyzed were primary infiltrating breast carcinomas with some *in situ* ductal carcinoma component. Antibody A1 yielded a wide range of staining intensities among different specimens (Figure 2, A, C, and E, and Figure 3A). Typically, epithelial and not stromal components of tumors were stained. *In situ* and infiltrating components of the same tumor usually stained similarly.

Relationship between P-Neu and Neu Staining

As expected, we encountered a wide range of relative staining intensities with A1 and Neu antisera (Figure 2). For example, the tumor in Figure 2, A–D, stained equivalently with anti-Neu (Figure 2, B and D) and anti-P-Neu A1 (Figure 2, A and C). However, a different tumor analyzed in Figure 2, E and F, stained poorly with anti-P-Neu although it stained well with anti-Neu. The most likely interpretation is that Neu overexpressed in the latter tumor is relatively inactive in signaling. A two-dimensional image analysis system was used to quantify relative staining with anti-p185 and antibody A1. The relative

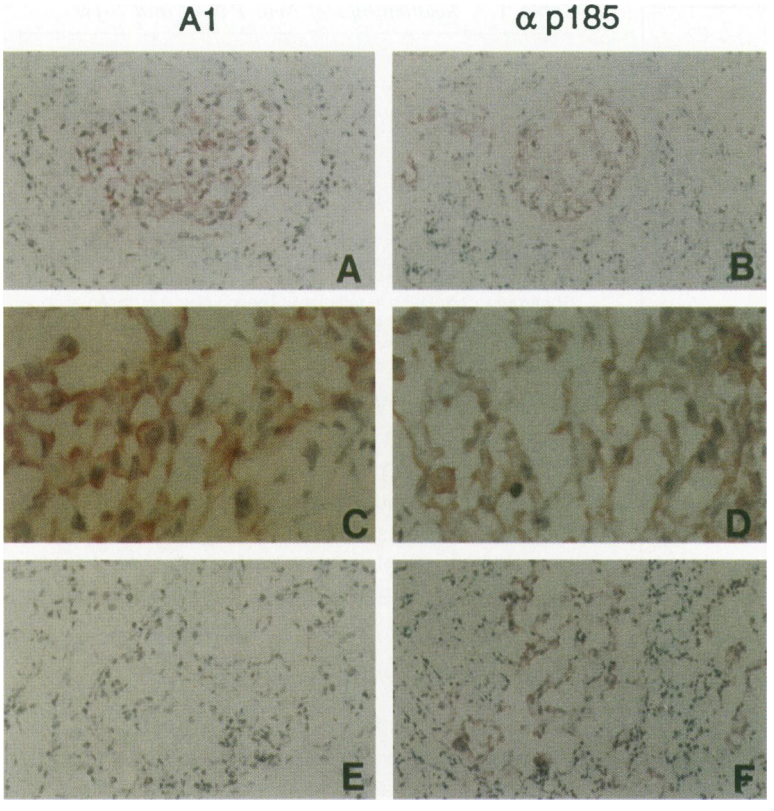

Figure 2. *Immunohistochemistry of mammary tumor specimens. Fixed frozen sections of tumors were stained with anti-P-Neu A1* (**A**, **C**, *and* **E**) *or anti-p185*^{neu} (**B**, **D**, *and* **F**). In situ *and infiltrating components are analyzed in* **A** *and* **B** *and in* **C** *and* **D**, *respectively. A different tumor is analyzed in* **E** *and* **F**.

intensities of staining for the samples analyzed are displayed in scatter plots in Figure 3. The general trend was for P-Neu staining to increase as Neu staining increased (Figure 3A). This was expected as (ignoring EGFR reactivity) every molecule capable of staining with P-Neu should react with anti-Neu (the converse is not true). These two parameters demonstrated a high correlation (0.78) with high statistical significance ($P < 0.0001$; Tables 1 and 2). However, at any specific level of p185 expression, a range of anti-P-Neu immunoreactivities was observed (Figure 3A).

Relationship with P-Tyr

As Neu and other growth regulators often stimulate Tyr phosphorylation, anti-P-Tyr antibodies may have prognostic value. Tumor samples were stained with anti-P-Tyr to determine the relationship between this parameter, Neu, and P-Neu reactivity (Figure 3B). P-Tyr immunoreactivity correlated well with both variables, better with Neu staining than with P-Neu staining (Table 1).

Relationship with EGFR

The EGFR (*erbB*/*HER*) gene is amplified or overexpressed in mammary carcinomas, but at lower frequencies than Neu.[43] This might contribute to Neu Tyr phosphorylation if EGFR overexpression enhances sensitivity to transmodulating EGF agonists. The previously observed cross-reactivity of A1 with the EGFR provides another potential link. Twenty-eight of the tumors were analyzed by immunohistochemistry for the presence of the EGFR. Immunoreactive EGFR varied independently of the extent of staining with anti-p185, suggesting that the receptors are independently selected and/or regulated in these tumors. In contrast to Neu, the EGFR was not associated with P-Tyr positivity. Finally, no difference was detected in intensities of staining with P-Neu antibodies in EGFR-positive and EGFR-negative tumors (data not shown). In this subset, a strong association was observed between Neu and P-Neu staining, but no such association was seen with the EGFR. Moreover, the EGFR assorted randomly among even the tumors staining most strongly with anti-P-Neu A1, so it did not appear to contribute significantly to Neu phosphorylation in this data set.

Relationship with HER-3 and HER-4

Because Neu is also transmodulated by NDFs acting through HER-3 and HER-4, we determined whether overexpression of these receptors is linked to Neu

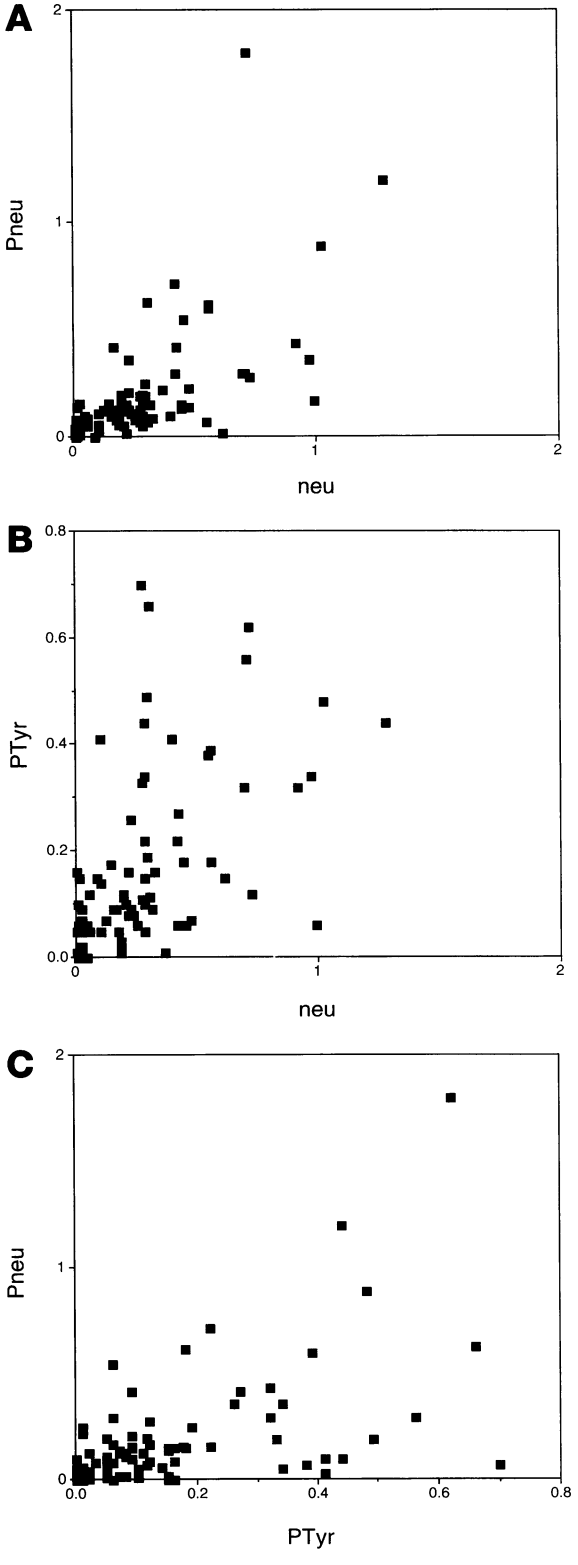

Figure 3. *Relationships of Neu, P-Neu, and P-Tyr. Tumor specimens were immunostained with anti-Neu, anti-P-Neu, and anti-P-Tyr, and staining was quantified by densitometry. Each point represents the relative intensities of the two variables for an individual tumor specimen. They do not indicate absolute stoichiometries.* A: *P-Neu* versus *Neu.* B: *P-Tyr* versus *Neu.* C: *P-Neu* versus *P-Tyr.*

Table 1. *Relationships of Neu, P-Neu, and P-Tyr*

	P-neu	*P*-Tyr
Neu	$r = 0.78$ $P = 0.0001$ (96)	$r = 0.62$ $p = 0.0001$ (84)
*p*Neu		$r = 0.50$ $P = 0.0001$ (83)

Spearman rank correlations of markers quantified densitometrically as described in Materials and Methods. r is the Spearman correlation coefficient. The number of observations is indicated in parentheses.

phosphorylation. Virtually all mammary tumors tested expressed some HER-3 (S. Bacus, unpublished data), but varying levels of HER-3 expression might be a factor in regulating transmodulation. HER-4 immunostaining was variable among different tumors but did not correlate with immunostaining for Neu, P-Neu, P-Tyr, or EGFR (Table 2; data not shown).

Associations with ER and PR

Previous work linked Neu overexpression to poor prognosis, with variable independence from other prognostic indicators for breast cancer.[4,5,15,44–46] The presence of ER and PR is associated with better prognosis in mammary carcinoma.[47,48] Neu has been inversely correlated with both ER and PR in some studies.[49] Neither Neu, P-Neu, nor P-Tyr immunoreactivity showed an association with ER status (Tables 2 and 3). The EGFR showed no significant correlation with ER status (Table 2). Neu, P-Neu, and P-Tyr were all inversely associated with PR status (statistically signficant), consistent with a linkage to poor prognosis (Tables 2 and 3). Both ER and PR correlated positively with HER-4 (Table 2), in marked contrast to the inverse correlation of PR with Neu (Table 2).

Discussion

The biological activity of Neu is conditioned not only by its abundance but also by the availability of autocrine and paracrine growth factors in the tumor milieu and the presence of three other related RTKs that are necessary to couple peptide hormones to signaling by Neu. In an effort to bypass the need to quantify these innumerable inputs and predict the complex receptor interactions, we have used a new approach, immunostaining with a phosphopeptide-specific antibody, to measure the Tyr phosphorylation, and hence signaling activity of Neu/erbB-2/HER-2 in human mammary carcinoma specimens.

Table 2. *Prognostic Factors and Other Discrete Variables*

Category	Category	*P*	Number of observations
EGFR	HER-4	0.873	24
neu	HER-4	0.908	53
ER	EGFR	0.37	28
ER	Neu	0.74	84
ER	HER-4	<0.01 Positive association	72
ER	HER-4 or EGFR	<0.01 Positive association	76
PR	EGFR	0.14	28
PR	Neu	0.04 Inverse association	84
PR	HER-4	0.001 Positive association	72
PR	HER-4 or EGFR	<0.01 Positive association	76

Chi-Square analysis of discrete variables. For statistically significant combinations, direct or inverse relationships are indicated.

A potential disadvantage of the antibody used here is its cross-reactivity with the EGFR.[2] This may not be a problem as neu is more frequently overexpressed than the EGFR, and as the extent of overexpression may be greater. In this study we found no association between the EGFR and A1 immunoreactivity, but these samples were preselected for Neu overexpression. One of us (D. F. S.) has now produced a monoclonal monospecific anti-P-Neu antibody PN2A that recognizes phosphorylated Neu but not the EGFR or HER-4.[42] Although PN2A has the advantage of greater specificity over the polyclonal antibody A1 used here, it may not be as sensitive as A1. Generally speaking, polyclonal antibodies work better in most applications than monoclonal antibodies because they can simultaneously recognize a mixture of epitopes and because they are often composed of higher affinity antibodies. It may be possible to improve the specificity of A1 by additional adsorption with EGFR and HER-4 phosphopeptides, and ultimately this might turn out to yield a more useful reagent than the monoclonal antibody.

P-Neu and P-Tyr Immunostaining

As predicted, we detected a wide range in staining intensities with P-Neu antibody relative to Neu antibody, representing, we believe, the range in signaling activities of Neu in these tumors (Figure 3). P-Tyr immunostaining showed a weaker correlation with Neu and, to a lesser extent, with P-Neu immunoreactivity. However, P-Tyr did not correlate with EGFR (data not shown). This suggests either that the predominant Tyr phosphoprotein in Neu-overexpressing tumors is p185 itself or, alternatively, that the most frequent activator of downstream signaling proteins among Neu-positive tumors is p185 and not other tyrosine kinases. In either case, the finding supports an important role for Neu in signaling of mammary carcinoma.

Table 3. *Clinical Markers*

		Neu	P-Neu	P-Tyr
ER	–	56.2 (33)	44.7 (30)	41.6 (26)
	+	54.5 (76)	48.8 (64)	42.2 (57)
		P = 0.80	*P* = 0.50	*P* = 0.93
PR	–	65.2 (47)	55.2 (44)	47.4 (39)
	+	47.3 (62)	40.7 (50	37.2 (44)
		P = 0.0033	*P* = 0.010	*P* = 0.054

Wilcoxon comparisons of ranks of continuous variables *versus* discrete variables evaluated as described in Materials and Methods. Mean scores are shown for each category followed by the number of observations indicated in parentheses.

Cofactors for p185 Phosphorylation

Because of the complex spectrum of potential agonists for Neu, we sought to identify an association between specific transmodulating growth factors and cognate receptors and phosphorylated Neu. EGFR expression was detectable in approximately one-half of the subset of samples tested, HER-3 expression in 96% of specimens, and HER-4 expression in approximately one-half of the tumors (S. Bacus, unpublished data). Significantly, EGFR and HER-4 expression assorted independently of Neu. Thus, although erbB family receptors are often coexpressed, their expression is not coordinately regulated. This is important, as the array of hormones that can activate Neu is determined by the presence of other receptors.

ER and PR

We anticipated that detection of P-Neu would augment any clinical correlates with Neu immunostaining. As patient follow-up data are not available for the specimens analyzed here, we determined how well Neu, P-Neu, and P-Tyr staining correlates with two prognostic markers for breast cancer, ER and PR. Both Neu and P-Neu staining showed statistically

significant inverse correlations with PR. This is consistent with earlier studies that in general link Neu with poorer prognosis and that specifically link it inversely to PR[49] (reviewed in Ref. 4). A similar inverse relationship to ER status has been found in other studies but was not detected here with either Neu, P-Neu, or P-Tyr antibodies. This may just mean that with this moderate sample size an association is undetectable, or it may signify a tighter regulatory connection of Neu to PR than to ER.

It is possible that there is no causal link between Neu overexpression and ER/PR status, as some studies have suggested that Neu independently predicts poor prognosis.[5] However, it seems more likely that these data reflect a physiological association. Normally, both estrogen and progestins serve to regulate growth and development of mammary tissue. Although both hormones can act directly on mammary tissue, major effects of estrogens may be mediated through their abilities to induce PR and thereby enable progestin responsiveness.[48,50] In general, estrogens and progestins stimulate mammary proliferation, but progestins may have some inhibitory functions as well. The effects of these two sex hormones may be mediated locally through paracrine or autocrine function of peptide hormones including transforming growth factor-α and insulin-like growth factor-I and may include other Neu agonists. Thus, the inverse correlation of Neu and P-Neu levels with PR might indicate that PR suppresses production of p185 or agonists (or the converse). Alternatively, the ability of estrogens to induce PR expression[48,50] and also to suppress Neu transcription[51] may mean that the ER is the common element. Another possibility would be that Neu inactivates functional ER, thereby preventing induction of PR by estrogen.

Neu and Type 1 Receptor Network in Mammary Carcinoma

We found that different erbB family receptors are regulated independently and that they are associated with different clinically relevant markers. Although the available data are still incomplete, a pattern is emerging in which Neu and the EGFR (and its agonists) are linked to proliferation and carcinogenesis, whereas HER-4 and its agonists (NDFs) are linked to differentiation and better prognosis. We have identified a clear difference in the relationship with steroid receptors. Neu overexpression varies inversely with PR levels and, in most studies, inversely with ER. However, we show here a strong positive correlation between HER-4 expression and the presence of ER and PR. Thus, HER-4 is associated with a different functional subset of tumors from Neu and is presumably associated with a more favorable outcome.

The relevant clinical issue is whether P-Neu staining can be used to identify important patient subpopulations and aid in making treatment decisions for node-negative patients. In this regard, the negative correlation of P-Neu immunoreactivity with PR may be significant as PR itself correlates with a pattern of responsiveness to anti-estrogen therapy. Metastatic breast cancer is the outcome of a long biological process, which often involves Neu early on. We anticipate that the use of these and phosphopeptide sera specific to other erbB family members will clarify which transmodulating agonists are important in normal tissue and mammary carcinoma and will help rationalize the biological activities of NDFs and EGF agonists. This will have important clinical consequences beyond diagnostics, as Neu is under investigation as a therapeutic target.[52] The present work sets the stage for future studies in which the anatomical resolution of these antibodies will be exploited to help localize receptor activation and in which clinical follow-up data will be available.

Acknowledgments

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