Quantitative Association Between HER-2/neu and Steroid Hormone Receptors in Hormone Receptor-Positive Primary Breast Cancer

Gottfried Konecny, Giovanni Pauletti, Mark Pegram, Michael Untch, Sugandha Dandekar, Zuleima Aguilar, Cindy Wilson, Hong-Mei Rong, Ingo Bauerfeind, Margret Felber, He-Jing Wang, Malgorzata Berty, Ram Seshadri, Herrmann Hepp, Dennis J. Slamon

Background: HER-2/neu, which encodes a receptor tyrosine kinase, is amplified and overexpressed in 20%–25% of human breast cancers. Such tumors are often resistant to hormone therapy. Despite a general inverse association between HER-2/neu amplification/overexpression and estrogen receptor (ER) and/or progesterone receptor (PR) expression, a fraction of patients are both HER-2/neu- and hormone receptor (HR)-positive. The efficacy of hormone therapy in this group is currently a matter of debate. To better understand the relationship between HER-2/neu positivity and HR expression, we analyzed HER-2/neu, ER, and PR as continuous variables in breast cancer cell lines and two cohorts of primary breast cancer patients. Methods: HER-2/neu and ER/PR expression was analyzed by enzyme-linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA), respectively, in 14 human breast cancer cell lines, some of which had been transfected with the HER-2/neu gene. For the clinical study population, HER-2/neu protein levels were assessed by ELISA (cohort A, n = 665), and HER-2/neu gene copy number was determined using fluorescence in situ hybridization (cohort B, n = 894). ER/PR expression was analyzed by EIA (cohort A) or radioligand binding (cohort B). Associations between HER-2/neu and ER/PR expression were analyzed using Spearman’s rho correlation and the chi-square test, and absolute levels were compared using the Mann–Whitney U test. All statistical tests were two-sided. Results: HR-positive human breast cancer cell lines transfected with the HER-2/neu gene expressed statistically significantly lower levels of ER and PR than parental lines. In the clinical cohorts, levels of HER-2/neu overexpression and gene amplification were inversely correlated with ER/PR levels (Cohort A [n = 112]: for ER, r = −0.34, P < .001; for PR, r = −0.24, P = .010. Cohort B [n = 188]: for ER, r = −0.39, P < .001; for PR, r = −0.26, P < .001). Among patients with HR-positive tumors, HER-2/neu-positive tumors had statistically significantly lower ER/PR levels than HER-2/neu-negative ones (Cohort A: for ER, median = 25 fmol/mg [interquartile range {IQR} = 13–78] versus median = 38.5 fmol/mg [IQR = 17–99] and P = .031; for PR, median = 35 fmol/mg [IQR = 12–119] versus median = 88.5 fmol/mg [IQR = 22–236] and P < .001. Cohort B: for ER, median = 44 fmol/mg [IQR = 13–156] versus median = 92 fmol/mg [IQR = 35–235] and P < .001; for PR, median = 36 fmol/mg [IQR = 13–108] versus median = 84 fmol/mg [IQR = 24–250] and P < .001). Patients with higher levels of HER-2/neu overexpression or amplification had statistically significantly lower levels of ER/PR than patients with lower levels of HER-2/neu overexpression or amplification. Conclusion: Because absolute HR levels are strongly related to response to hormone therapy in primary and advanced breast cancer, reduced ER/PR expression may be one mechanism to explain the relative resistance of HER-2/neu-positive:HR-positive tumors to hormone therapy. [J Natl Cancer Inst 2003;95:142–53]

Growth and differentiation of both normal and malignant human breast cancer cells are regulated by steroid hormone and peptide growth factor receptors. Among the peptide growth factor receptors frequently implicated in breast cancer are members of the type I receptor tyrosine kinase family, which includes HER-1 (epidermal growth factor receptor), HER-2, HER-3, and HER-4. Amplification of the HER-2/neu gene, resulting in overexpression of the receptor, is found in 20%–25% of human breast cancers, a frequency of genetic alteration that is second only to p53 mutations (1,2). An inverse association has been described between HER-2/neu amplification/overexpression and the presence of receptors for the steroid hormones estrogen and progesterone in both clinical correlative studies (3–8) and experimental models (9,10). However, the data are not entirely clear. For example, in some clinical studies, up to 50% of patients with HER-2/neu-positive tumors have been classified as hormone receptor (HR)-positive (6,7).

Consistent with the association of HER-2/neu amplification/overexpression with a negative HR status, amplification/overexpression of HER-2/neu has been associated with the failure of endocrine treatment, such as tamoxifen therapy, in a number of retrospective studies (11–17). Recent clinical data have also suggested that HER-2/neu amplification/overexpression might be associated with tamoxifen resistance in patients with HR-positive breast cancer; however, this association is controversial. Five retrospective studies (18–22) attempted to eliminate the confounding effect of negative HR status by focusing specifically on HER-2/neu-positive:HR-positive patients. These studies

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report conflicting results. Three studies (18–20) demonstrated an association between HER-2/neu overexpression and tamoxifen resistance in primary or advanced HR-positive tumors, while two others (21,22) found no such association.

The response to tamoxifen therapy is associated with the levels of both estrogen receptors (ERs) and progesterone receptors (PRs) in both primary (23–27) and advanced (28–31) breast cancer. For example, among 398 ER-positive patients with metastatic disease who received tamoxifen as first-line treatment, patients with ER levels of greater than 100 fmol/mg protein had a 62% response rate, those with ER levels of 50–99 fmol/mg protein had a 54% response rate, and those with ER levels of less than 50 fmol/mg protein had a 42% response rate (30). In the same study, PR levels in the tumors of the ER-positive patients were statistically significantly associated with an increased probability of response to tamoxifen; response rates were 61%, 53%, and 43% in patients with more than 100 fmol/mg, 10–99 fmol/mg, or less than 10 fmol/mg of PR protein, respectively, in their tumors (30).

Two earlier studies (24,28) also reported similar associations between response to tamoxifen and the levels of HR expression. In one study (28), an analysis of 95 patients with metastatic breast cancer, the level of ER in tumors was directly related to the likelihood of response to hormonal treatment, with statistically significantly increasing response rates being observed as the tumor ER protein concentration increased between 5 and 100 fmol/mg. The other study (24) showed an association between ER levels and progression following adjuvant tamoxifen treatment of lymph node-positive patients. In this study, disease-free survival (DFS) was statistically significantly improved following tamoxifen treatment for postmenopausal patients with HR-positive cancer; however, the treatment effect was clearly associated with ER levels (for 10–29 fmol/mg protein, relative risk (RR) = .91; for 30–49 fmol/mg protein, RR = .85; for 50–99 fmol/mg protein, RR = .82; and for ≥100 fmol/mg protein, RR = .79). Moreover, in the 1995 Oxford overview meta-analysis summarizing the results of 55 randomized adjuvant trials of tamoxifen versus no tamoxifen (23), 5 years of tamoxifen treatment appeared to be associated with longer survival in women with ER levels above 100 fmol/mg protein than in those with ER levels of 10–99 fmol/mg protein, with mortality reductions of 36% and 23%, respectively.

Given that the response to endocrine therapy is associated with levels of both ER and PR, a comparison of absolute HR levels (as opposed to a dichotomous classification, i.e., positive or negative) might help to clarify why patients with HER-2/neu-positive:HR-positive tumors are less responsive to hormonal treatment than patients with HER-2/neu-negative:HR-positive tumors (18–20). Therefore, we quantitatively assessed whether HER-2/neu protein expression levels or absolute gene copy numbers and ER/PR levels as continuous variables in two large primary breast cancer cohorts. We then analyzed the interactions between the two continuous variables (i.e., HER-2/neu and steroid HR levels) specifically among HR-positive patients to determine whether absolute HR levels are lower in patients whose primary tumors have HER-2/neu amplification/overexpression than in patients whose primary tumors do not. To investigate this interaction experimentally, we performed in vitro studies in which steroid HR levels were compared in cell lines transfected with HER-2/neu and parental cell lines. The results of these studies may help to provide a clearer understanding of the relationship between these receptor families and to suggest a mechanism that may help explain the reported lower response of patients with HER-2/neu-positive:HR-positive breast cancer to hormonal therapy.

**Patients and Methods**

Cohort A consisted of 665 consecutive untreated women treated for breast cancer between 1992 and 1997 at the Department of Obstetrics and Gynecology of the University of Munich (Munich, Germany). The study cohort was originally assembled to evaluate the prognostic significance of urokinase plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor 1 (PAI-1), in primary breast cancer. Tumor samples and clinical information were obtained under institutional review board approval, and the current study is a retrospective statistical analysis of results obtained from studies of these samples. Cohort B consisted of 894 untreated study patients from South Australia who were diagnosed with invasive breast cancer between 1987 and 1991. Tumors were collected at the Flinders Medical Center, Bedford Park, South Australia, which is the state’s clinical reference laboratory for HR analysis. Because the original goal for this study cohort was to determine the prognostic significance of HER-2/neu amplification in early-stage disease, patients with metastatic disease or for whom axillary clearance was not performed were excluded. Tumor samples and clinical information were obtained under institutional review board approval, and the current study is a retrospective statistical analysis of results obtained from studies of these samples.

**Clinical Features of Patient Cohort A**

Cohort A included patients with stage I through stage IV breast cancer. Median follow-up time for this cohort for DFS and overall survival (OS) was 26 months. Patients underwent either a modified radical mastectomy or a lumpectomy with complete axillary lymph node dissection, followed by radiation therapy of the residual breast tissue. Lymph node-positive patients received adjuvant chemotherapy and/or tamoxifen therapy, whereas lymph node-negative patients received adjuvant therapy only if they had adverse prognostic factors, such as large (≥2 cm) primary tumors or elevated levels of either uPA or PAI-1.

**Clinical Features of Patient Cohort B**

Cohort B included patients with stage I through stage III breast cancer. The median follow-up period for this cohort for DFS and OS was 5.7 years. Patients underwent either total or partial mastectomy with axillary lymph node dissection. Patients who underwent partial mastectomy received postoperative radiotherapy of the remaining breast tissue. Adjuvant chemotherapy with six cycles of a combination of cyclophosphamide, methotrexate, and fluorouracil (CMF) or tamoxifen was administered to axillary lymph node-positive premenopausal (15%) or postmenopausal patients (24%), respectively. Lymph node-negative patients did not receive adjuvant therapy.

**HER-2/neu Analyses**

In cohort A, HER-2/neu expression was analyzed by measurement of HER-2/neu protein. Breast cancer tissue specimens were selected by the pathologist at the time of surgery and stored at –70°C until the current analyses were performed. Samples of 100–200 mg of frozen tissue were pulverized with a microdissec-

membrotor (Braun-Melsungen, Melsungen, Germany) for 30
seconds at maximal power. The resulting powder was immediately suspended in 900 μL Tris-buffered saline (pH 8.5) containing 1% Triton X-100, incubated at 4°C for 12 hours with gentle shaking, and ultracentrifuged for 60 minutes at 100,000g at 4°C to remove cell debris, nuclei, and membranes. The supernatant was stored at −70°C until use. HER-2/neu levels in the supernatant were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Oncogene Research Products, Cambridge, MA) according to the manufacturer’s instructions. HER-2/neu protein levels were given in fmol/mg total protein. For most analyses, tumors were considered HER-2/neu-positive if protein levels were 500 fmol/mg or higher (see “ELISA and FISH scoring criteria” below).

In cohort B, HER-2/neu gene copy number was analyzed using fluorescence in situ hybridization (FISH) on 4-μm sections of formalin-fixed, paraffin-embedded tissue. Specimen preparation, hybridization, and microscopy were performed as previously described (32,33). A SpectrumOrange-labeled HER-2/neu probe (Vysis, Inc., Downers Grove, IL) was used to determine gene copy number as a continuous variable (HER-2/neu signals/cell). In addition, to account for increased HER-2/neu gene copy number due to chromosome 17 polysomy (i.e., increased copy number of the entire chromosome 17), specimens were also hybridized with a chromosome 17 centromeric (chr.17cen.) probe labeled with SpectrumGreen fluorochrome (Vysis, Inc.). Data were expressed in terms of HER-2/neu genes per chr.17cen., with two HER-2/neu genes per chr.17cen. as the biologic cutoff point to distinguish tumors with and without HER-2/neu amplification.

Quantitative HR Assays

In cohort A patients, HR levels were determined by enzyme immunoassay (EIA). Tissue samples of 200–500 mg that were collected at the same time as the samples used to measure HER-2/neu were homogenized with an Ultra-Turrax homogenizer (Jahnke and Kunkel, Staufen, Germany) in 2–3 mL of buffer containing 10 mM Tris, 1.5 mM EDTA (pH 7.4), and 0.1% monothioglycerol acetate. The homogenate was then centrifuged for 30 minutes at 200,000g at 4°C. ER and PR levels were measured in the supernatant using EIA according to the manufacturer’s instructions (EIA Monoclonal; Abbott Laboratories, Abbott Park, IL). Specimens were classified as ER- or PR-positive if levels were >10 fmol/mg total protein, according to the previously established cutoff point for HR positivity (23).

In cohort B patients, HR levels were determined by using a radioiodinated hormone binding assay, as described previously (34,35). Briefly, the assays used five incubation concentrations of radiolabeled hormone (either 0.05–2 nmol/L [3H]-estradiol [Amersham, Sydney, Australia] or 0.08–4 nmol/L [3H]-R5020 [Promegestone; NEN-Dupont, Sydney, Australia]) to determine total ER and PR binding, respectively in tumor specimen cytosols. Parallel tubes containing an additional 100-fold excess of unlabeled steroid hormone were used to estimate the levels of nonspecific binding. Bound and free hormones were separated on dextran-coated charcoal, and results were analyzed by Scatchard analysis, with the use of software for analysis of single binding sites (Beckman Accufit, Fullerton, CA). Again, absolute protein levels were determined, and specimens were considered ER- or PR-positive if HR levels were ≥10 fmol/mg total protein (23).

Cell Lines

HER-2/neu, ER, and PR protein levels were measured in a panel of established breast cancer cell lines and derivative lines engineered to overexpress HER-2/neu at various levels over a wide range. These cell lines have been previously characterized for HER-2/neu gene copy number and mRNA and protein expression levels by using Southern, northern, and western blot analyses (36–39). The naturally HER-2/neu-overexpressing cell lines BT-474, SK-BR3, MDA-MB-361, and MDA-MB-453 and the HER-2/neu-non-overexpressing cell lines, BT-20, MDA-MB-435, MCF-7, MDA-MB-231, ZR-75–1, and T-47D were all obtained from the American Type Culture Collection (Manassas, VA). Cell lines MCF-7/HER-2, MDA-MB-231/HER-2, ZR-75–1/HER-2 and T-47D/HER-2 were previously created by transfection with a retroviral expression vector containing a full-length human HER-2/neu complementary DNA (cDNA), resulting in stable HER-2/neu overexpression (37–39). HER-2/neu protein content in all cell lines was measured by using HER-2/neu ELISA on lysates from 1 × 10⁶ cells of each line following antigen extraction with Triton X-100, as described above. HR levels were measured using EIAs on lysates prepared as described above.

ELISA and FISH Scoring Criteria

Cell lines with defined HER-2/neu expression levels were used to establish a cutoff point for the quantitative HER-2/neu ELISA in the breast tumor samples. Among the cell lines that do not exhibit HER-2/neu gene amplification or overexpression, HER-2/neu expression ranged from 247 to 318 fmol/mg protein in MCF-7 cells, 114 to 279 fmol/mg protein in BT-20 cells, 150 to 181 fmol/mg protein in T-47D cells, and 115–407 fmol/mg protein in ZR-75–1 cells. We therefore chose 500 fmol/mg protein as a suitable cutoff point to define HER-2/neu overexpression. Moreover, a HER-2/neu value of 500 fmol/mg protein provided separation of patients with regard to DFS and OS in cohort A (log-rank P<.001 and P = .008, respectively) (see Table 3). The association between HER-2/neu overexpression and HR expression was independent of the cutoff point for HER-2/neu overexpression over the range of 400–600 fmol/mg protein at intervals of 50 fmol/mg (data not shown).

FISH scores were analyzed for cohort B as previously described (33). Briefly, slides were initially scanned for HER-2/neu signals and sorted into three groups: 1) tumors with obvious gene amplification (i.e., >10–15 signals/cell); 2) tumors that are readily classified as single copy (i.e., no cells with >4 detectable HER-2/neu signals); and 3) an intermediate group of tumors with low-level copy number increases. In the intermediate group, 100 randomly selected nuclei from each tumor were scored for both HER-2/neu and chr.17cen. signals, and HER-2/neu amplification was defined as the mean ratio of the signals of the two markers. Tumors with a ratio of >2 were classified as amplified, and tumors with a ratio of ≤2 were classified as nonamplified.

Statistical Analysis

Because neither HER-2/neu protein expression or gene copy number nor HR expression were normally distributed, associations between these variables were calculated by Spearman’s rho correlation. Variables were plotted both on linear scales (Figs. 1 and 2) and following square root transformation (Figs. 1 and 2,
insets). A cubic smoothing spline for nonlinear assumptions (smoothing parameter = 0.1) was fitted to illustrate the relationship between variables in the figure insets. Associations between variables were calculated by Spearman’s rho correlation for the patients (n = 112) with HER-2/neu-overexpressing tumors (i.e., HER-2/neu levels of at least 500 fmol/mg total protein) and for the patients (n = 553) with HER-2/neu-non-overexpressing tumors. P values were two-sided. A) Estrogen receptor levels plotted against HER-2/neu levels. Among patients with HER-2/neu-overexpressing tumors, r = −0.34 (P<0.001); among those with HER-2/neu-non-overexpressing tumors, r = 0.28 (P<0.001). B) Progesterone receptor levels plotted against HER-2/neu levels. Among patients with HER-2/neu-overexpressing tumors, r = −0.24 (P<0.001); among those with HER-2/neu-non-overexpressing tumors, r = 0.19 (P<0.001).

RESULTS

In Vitro Analysis of the Association Between HER-2/neu and HR Expression

To determine whether HER-2/neu overexpression is associated with low ER and/or PR expression levels, HER-2/neu, ER, and PR were measured as continuous variables by ELISA (for HER-2/neu) and EIA (for ER and PR) in several established and engineered cell lines (Table 1). MCF-7, MDA-MB-231, ZR-75-1, and T-47D cells (none of which normally exhibits HER-2/neu gene amplification and all of which express wild-type levels of HER-2/neu protein) that had previously been stably transfected with a full-length human HER-2/neu gene all expressed statistically significantly higher levels of HER-2/neu protein than parental lines (Table 1). The expression levels were similar to those previously described (38,39) for the RNA transcript and protein levels assessed by western blot in these cell lines. Increased HER-2/neu protein expression as a result of transfection with the HER-2/neu gene is associated with decreased levels of both ER and PR in the MCF-7 cell line (10). Our findings confirm this observation and extend it to two other HR-positive human breast cancer cell lines, ZR-75-1 and T-47D (Table 1), indicating that this is not an isolated phenomenon that is restricted to a single cell line. By contrast, transfection of the MDA-MB-231 cell line, which expresses very low levels of both ER and PR and is categorized as HR-negative, with HER-2/neu did not produce a statistically significant change in HR levels.

Clinical Study Cohorts

The potential relationship between HER-2/neu amplification/overexpression and HR expression was analyzed in two large
cohorts of primary breast cancer patients. Evaluation of patient demographic information and disease characteristics of these study cohorts (Table 2) demonstrates that the groups are representative of the general breast cancer population. This conclusion was confirmed by a univariate survival analysis that showed that several established factors, including HER-2/neu status, lymph node status, tumor size, and HR status, had prognostic relevance for DFS and OS in both cohorts (Table 3).

We first analyzed the relationship between HER-2/neu and HR status by treating them as dichotomous variables. Of the 665 patients in cohort A, the tumors of 441 (66.3%) were ER-positive, of 449 (67.5%) were PR-positive, and of 517 (77.7%) were HR-positive (i.e., ER- and/or PR-positive). HER-2/neu overexpression was detected by ELISA in 112 (16.8%) of the 665 tumors. Of the 112 HER-2/neu-overexpressing tumors, 58 (51.8%) were ER-positive, 54 (48.2%) were PR-positive, and 71 (63.4%) were HR-positive. Of the 553 HER-2/neu-non-overexpressing tumors, 383 (69.3%) were ER-positive, 495 (70.1%) were PR-positive, and 569 (80.6%) were HR-positive. These data are consistent with the well-described association between HER-2/neu overexpression and increased likelihood of absence of ER and PR (using the chi-square test, $P < 0.001$ for ER and $P < 0.001$ for both PR and HR).

Similarly, of the 894 patients in cohort B, 628 (70.2%) had ER-positive tumors, 594 (66.4%) had PR-positive tumors, and 692 (77.4%) had HR-positive tumors. Specimens from a total of 188 patients (21%) demonstrated HER-2/neu gene amplification by FISH. Of these patients, 100 (53%) had ER-positive tumors, 99 (52.6%) had PR-positive tumors, and 123 (65.4%) had HR-positive tumors. Conversely, of the 706 HER-2/neu-non-amplified tumors, 528 (74.8%) were ER-positive, 495 (70.1%) were PR-positive, and 569 (80.6%) were HR-positive. Thus, these data again confirm the association between HER-2/neu amplification and the increased likelihood of absence of ER and PR (using the chi-square test, $P < 0.001$ for ER, PR, and HR).

**Association Between HER-2/neu Amplification/Overexpression and HR Levels in Primary Breast Cancer Samples**

To better understand the association between HER-2/neu amplification/overexpression and HR status, we evaluated both HER-2/neu and HR levels as continuous rather than dichotomous variables. In cohort A, quantitative HER-2/neu levels ranged from 0 to 120 015 fmol/mg protein, and ER and PR levels ranged from 0 to 706 and 0 to 1143 fmol/mg protein, respectively (Fig. 1, A and B); in cohort B, HER-2/neu gene copy
overexpressing tumors (cohort A [n = 112] for HER-2/neu and HRs as continuous variables demonstrated an inverse correlation between absolute levels of ER and PR and levels of HER-2/neu overexpression (for ER, r = –0.34 and P < .001; for PR, r = –0.24 and P = .010). Analysis of patients with HER-2/neu-overexpressing tumors in cohort A (n = 112) for HER-2/neu and HRs as continuous variables demonstrated an inverse correlation between absolute levels of ER and PR and levels of HER-2/neu overexpression (for ER, r = –0.34 and P < .001; for PR, r = –0.24 and P = .010). Analysis of patients with HER-2/neu-amplified tumors in cohort B (n = 188) demonstrated similar inverse correlations with the levels of HER-2/neu gene amplification (for ER, r = –0.39 and P < .001; for PR, r = –0.26 and P < .001). When the analysis was restricted to the HER-2/neu-positive subset of patients with HER-2/neu-overexpressing tumors (i.e., 71/112 [63.4%] patients in cohort A and 123/188 [65.4%] patients in cohort B), similar inverse correlations were obtained (Cohort A: for ER, r = –0.34 and P = .004; for PR, r = –0.14 and P = 0.249. Cohort B: for ER, r = –0.40 and P < .001; for PR, r = –0.13 and P = .14). These findings indicate that the higher the levels of HER-2/neu overexpression or gene amplification, the lower the ER and PR levels were. In contrast, there was no inverse association between HER-2/neu and HR levels among patients with HER-2/neu-negative tumors. In fact, in cohort A patients, ER and PR levels were even positively associated with normal HER-2/neu levels in patients with HER-2/neu-non-overexpressing tumors (cohort A [n = 553]: for ER, r = 0.28 and P < .001; for PR, r = 0.19 and P < .001). In cohort B patients, there was no association between HER-2/neu gene copy number and HR expression among patients with HER-2/neu-non-amplified tumors (n = 706) (for ER, r = 0.05 and P = .226; for PR, r = 0.03 and P = .405).

To investigate whether the association between HER-2/neu overexpression and HR expression in cohort A was sensitive to the selected cutoff point for HER-2/neu overexpression, additional cutoff points over the range of 400–600 fmol/mg protein at 50-fmol/mg intervals were tested. The results using these alternative cutoff points for HER-2/neu overexpression (data not shown) were similar to the results that were obtained with the more biologically relevant cutoff point of 500 fmol/mg protein. That is, HR levels inversely correlated with the levels of HER-2/neu overexpression at each of the additional cutoff points.

### Comparison of HR Concentrations Between HER-2/neu-Positive and -Negative Tumors

To determine the extent to which ER/PR expression is reduced in HER-2/neu-positive tumors, we compared absolute HR concentrations in tumors from patients with HER-2/neu-positive and HER-2/neu-negative tumors, first in each cohort as a whole and subsequently among patients with HR-positive tumors specifically. In cohort A, the 112 patients with HER-2/neu-overexpressing tumors had statistically significantly lower tumor ER and PR levels than the 553 patients with HER-2/neu-non-overexpressing tumors (for ER, median = 10.5 fmol/mg protein [interquartile range [IQR] = 3–80] versus median = 26 fmol/mg protein [IQR = 3–80] and P < .001; for PR, median = 5 fmol/mg protein [IQR = 0–73] versus median = 51 fmol/mg protein [IQR = 3–181] and P < .001) (Fig. 3, A and B). Moreover, among the patients with HR-positive tumors (n = 517),
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**Table 2.** Patient characteristics*  

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<td>43</td>
</tr>
<tr>
<td></td>
<td>≥50</td>
<td>83</td>
</tr>
</tbody>
</table>

*ER = estrogen receptor; PR = progesterone receptor; HR = steroid hormone receptor.
†In cohort A patients, HER-2/neu levels were determined by enzyme-linked immunosorbent assay, and levels ≥500 fmol/mg total protein were considered positive. In cohort B patients, HER-2/neu amplification was determined by fluorescence in situ hybridization, and tumors with more than 2 HER-2/neu signals per chromosome 17 centromere were considered positive.

**Table 3.** Univariate analysis of histomorphologic and tumor biologic factors for disease-free survival (DFS) and overall survival (OS)*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cohort A (n = 576)</th>
<th>Cohort B (n = 894)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Nodes</td>
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<td>&lt;.001</td>
</tr>
<tr>
<td>ER</td>
<td>&lt;.001</td>
<td>.003</td>
</tr>
<tr>
<td>PR</td>
<td>&lt;.001</td>
<td>.026</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>&lt;.001</td>
<td>.009</td>
</tr>
</tbody>
</table>

*Prognostic parameters evaluated included primary tumor size (<10 mm, 11–20 mm, 21–50 mm, >50 mm), axillary lymph node involvement (positive vs. negative), estrogen receptor (ER) and progesterone receptor (PR) status (<10 fmol/mg protein vs. >10 fmol/mg protein), and HER-2/neu protein expression levels or gene amplification (positive vs. negative). In cohort A, HER-2/neu overexpression (as defined as ≥500 fmol/mg total protein) was determined by ELISA, and in cohort B, HER-2/neu protein expression (defined as >2 HER-2/neu gene copies per chromosome 17 centromere) was measured by fluorescence in situ hybridization. The median follow-up was 26 months for cohort A and 5.6 years for cohort B. Criteria for exclusion from survival analysis in cohort A included a history of other malignancies (n = 43) or distant metastasis at the time of diagnosis (n = 46). The prognostic significance was assessed using the log-rank test. All P values are two-sided.

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tumors that also overexpressed HER-2/neu (n = 71) had statistically significantly lower ER and PR levels than tumors that did not overexpress HER-2/neu (n = 446) (for ER, median = 25 fmol/mg protein [IQR = 13–78] versus median = 38.5 fmol/mg protein [IQR = 17–99] and P = .031; for PR, median = 35 fmol/mg protein [IQR = 12–119] versus median = 88.5 fmol/mg protein [IQR = 22–236] and P < .001) (Fig. 3, A and B).

When the alternative cutoff points for HER-2/neu overexpression were tested, the results were similar to those obtained using the biologically relevant cutoff point of 500 fmol/mg protein. Patients with HR-positive tumors (n = 517) that also overexpressed HER-2/neu had statistically significantly lower tumor PR levels than patients whose tumors did not overexpress HER-2/neu, irrespective of the cutoff point chosen to define HER-2/neu overexpression (data not shown). The differences in tumor ER levels, however, appeared to be more sensitive to the selected cutoff point for HER-2/neu overexpression. For HER-2/neu cutoff points of 450 fmol/mg protein and above, HR-positive tumors that also overexpressed HER-2/neu had statistically significantly lower tumor ER levels than HR-positive tumors that did not overexpress HER-2/neu. However, at a cutoff point of 400 fmol/mg protein, the difference was not statistically significant (data not shown).

For cohort B patients, those with tumor HER-2/neu amplification (n = 188) also had statistically significantly lower tumor ER and PR levels than those without tumor HER-2/neu amplification (n = 706) (for ER, median = 12.5 fmol/mg protein [IQR = 0–84] versus median = 58.5 fmol/mg protein [IQR = 9–179] and P < .001; for PR, median = 13 fmol/mg protein [IQR = 0–52] versus median = 46 fmol/mg protein [IQR = 0–184] and P < .001) (Fig. 4, A and B). Among patients with HR-positive tumors specifically (n = 692), those whose tumors also had HER-2/neu amplification (n = 123) had statistically significantly lower tumor ER and PR levels than those whose tumors did not have HER-2/neu amplification (for ER, median = 44 fmol/mg protein [IQR = 13–156] versus median = 92 fmol/mg protein [IQR = 35–235] and P < .001; for PR, median = 36 fmol/mg protein [IQR = 13–108] versus median = 84 fmol/mg protein [IQR = 24–250] and P < .001).

To determine whether there would be differences in HR levels between tumors with low levels of HER-2/neu amplification/overexpression compared with tumors with high levels of amplification/overexpression, cohort A patients with HER-2/neu overexpression/HR-positive tumors (n = 71) were separated into two groups of approximately equal size (n = 35 and n = 36). One group consisted of patients with lower levels of HER-2/neu overexpression, and the other, of patients with higher levels of overexpression (equal group size was the only criteria used for the dichotomization, and the cutoff point fell at 1700 fmol/mg protein). The patients with higher levels of tumor HER-2/neu overexpression had statistically significantly lower tumor ER levels than patients with lower levels of tumor HER-2/neu overexpression (median = 20.5 fmol/mg protein [IQR = 11–33] versus median = 42 fmol/mg protein [IQR = 15–124] and P = .027). Tumor PR levels were also statistically significantly lower.
lower in patients with higher tumor HER-2/neu overexpression than in patients with lower levels of tumor HER-2/neu overexpression (median = 23.5 fmol/mg protein [IQR = 3–75] versus median = 67 fmol/mg protein [IQR = 15–169] and \( P = .049 \)) (Fig. 5, A). Importantly, tumor ER levels in the 35 patients with lower levels of HER-2/neu overexpression (i.e., median = 42 fmol/mg protein [IQR = 15–124]) were similar to those in the 446 patients whose tumors were HER-2/neu-negative (median = 38.5 fmol/mg protein [IQR = 17–99]). The PR levels in both groups were also similar (median = 67 fmol/mg protein [IQR = 15–169] versus median = 88.5 fmol/mg protein [IQR = 22–236], respectively) (Fig. 5, A). This result suggests that patients with HR-positive tumors and low levels of HER-2/neu overexpression might be more sensitive to endocrine therapy than patients with HR-positive tumors and higher levels of HER-2/neu overexpression.

As was done for cohort A patients, the 123 cohort B patients whose tumors were HR-positive and had HER-2/neu amplification were separated into two groups of approximately equal size (n = 62 and n = 61), consisting of patients with either lower or higher levels of HER-2/neu gene amplification (again, equal size of the groups was the only criterion for dichotomization, and the
Therefore, additional analyses were performed using a cutoff point of 3 fmol/mg protein of ER and PR for the definition of HR positivity. The inverse correlation between HR expression and HER-2/neu protein overexpression (cohort A) or gene amplification (cohort B) in patients with HER-2/neu-positive:HR-negative (i.e., <500 fmol/mg protein) tumors (n = 446), low HER-2/neu overexpression in tumors (500–1700 fmol/mg protein) (n = 35), or high overexpression in tumors (>1700 fmol/mg protein) (n = 36). Median ER levels (in fmol/mg protein) were 38.5 (IQR = 17–99), 42 (IQR = 15–124), and 20.5 (IQR = 11–33), respectively. Median PR levels (in fmol/mg protein) were 88.5 (IQR = 22–236), 67 (IQR = 15–169), and 23.5 (IQR = 2.75–75), respectively. A) ER (left) and PR (right) were analyzed in tumors of HR-positive patients in cohort A (n = 517). Patients were categorized as having HER-2/neu-positive tumors (median of HER-2/neu genes: <19 signals/cell) (n = 62). Median ER levels (in fmol/mg protein) were 92 (IQR = 35–235), 83 (IQR = 24–207), and 446), low HER-2/neu amplification (median 83 fmol/mg protein, median HER-2/neu signal/cell) (n = 61). Median ER levels (in fmol/mg protein) were 92 (IQR = 35–235), 83 (IQR = 24–207), and 130 (IQR = 8.5–91), respectively. Median PR levels (in fmol/mg protein) were 83 (IQR = 24–250), 36.5 (IQR = 14–121), and 32 (IQR = 13–109), respectively.

Some investigators have proposed that any detectable HR level increases the likelihood of response to endocrine therapy (21). Therefore, additional analyses were performed using a cutoff point of 3 fmol/mg protein of ER and PR for the definition of HR positivity. The inverse correlation between HR expression and HER-2/neu protein overexpression (cohort A) or gene amplification (cohort B) in patients with HER-2/neu-positive:HR-negative tumors was maintained (Cohort A, n = 446; ER, r = −0.31 and P = .007; for PR, r = −0.14 and P = .238. Cohort B, n = 146; for ER, r = −0.29 and P < .001; for PR, r = −0.16 and P = .047). Consistent with this observation, even using the lower cutoff value to define HR positivity, ER and PR levels were also statistically significantly lower among patients with HER-2/neu-negative:HR-positive tumors than among patients with HER-2/neu-positive:HR-negative tumors. Median ER levels (in fmol/mg protein) were 30 (IQR = 10–69) versus median = 37 fmol/mg protein [IQR = 14–95] and P = .024; for PR, median = 34 fmol/mg protein [IQR = 5–102] versus median = 81 fmol/mg protein [IQR = 20–229] and P < .001. Cohort B, n = 742; for ER, median = 30 fmol/mg protein [IQR = 6–141] versus median = 85 fmol/mg protein [IQR = 30–222] and P < .001; for PR, median = 35 fmol/mg protein [IQR = 4.75–88] versus median = 74 fmol/mg protein [IQR = 19–236] and P < .001).

**DISCUSSION**

A number of clinical studies have documented an association between HER-2/neu amplification/overexpression and negative steroid HR status in breast tumors (3–8). This relationship has mainly been characterized by using dichotomous cutoff values for both the HER-2/neu and the ER/PR variables. However, this dichotomization approach produces a biologically unrealistic model for what are, in reality, continuous variables. To more
fully address the relationship between HER-2/neu and HR levels, we analyzed them as continuous variables. The results of our study of a total of 1559 patients confirm previous observations, which indicated that a higher percentage of HER-2/neu-positive tumors than HER-2/neu-negative tumors are HR-negative (3–8). However, we were able to go further in also demonstrating inverse associations between the variables (HER-2/neu and HRs) both among those patients with tumors classified as HER-2/neu-positive and among those patients with tumors classified as HER-2/neu-negative and HR-positive. In general, the higher the level of HER-2/neu overexpression (cohort A) or gene amplification (cohort B), the lower the corresponding ER level. Our data also demonstrate an inverse correlation between HER-2/neu protein/gene levels and PR levels that most likely occurs because suppression of ER expression leads to reduced expression of PR.

One of the most important findings of our study is that HER-2/neu amplification/overexpression was associated with lower tumor ER and PR levels, even in patients with tumors classified as HR-positive. Moreover, among HER-2/neu-positive tumors, expression of ER and PR was lower in tumors with higher levels of HER-2/neu overexpression or amplification than in tumors with lower levels. These observations were consistent across two large independent cohorts of primary breast cancer patients, using completely different methodologies for detection of both the HER-2/neu alteration and ER and PR levels. This phenomenon may help to explain the lower tamoxifen response that has been reported among HER-2/neu-positive:HR-positive patients compared with HER-2/neu-negative:HR-positive patients (18–20). Indeed, numerous clinical studies of both primary (23–27) and metastatic (28–31) breast cancer have shown that higher levels of HR expression reliably predict a greater likelihood of response to tamoxifen therapy. This association has been well described for both the ER (28–31) and the PR (29–31) among patients receiving tamoxifen for treatment of advanced breast cancer and has been best described for the ER (23–27) among patients receiving adjuvant tamoxifen for treatment of early breast cancer. In all of these clinical studies, the absolute differences in tumor HR levels among HR-positive patients were clinically as relevant as those between HR-positive and HR-negative patients, in that the decrease in tamoxifen response between HR-positive subgroups (i.e., high positive versus low positive) was similar to those between patients with negative HR levels and low HR levels.

An interesting finding from our study was that relatively low levels of HER-2/neu amplification/overexpression were associated with more marked decreases of PR than of ER (Fig. 5, A and B). This difference may arise because expression of PR better represents the functional status of HRs in breast cancer than expression of ER does, because PR expression is linked to a biologically active and functional ER (41,42).

The inverse association between HER-2/neu and HR levels in clinical samples is consistent with cell line data from a prior study (10), which showed that the introduction of additional HER-2/neu gene copies into MCF-7 cells and the corresponding increase in HER-2/neu expression lead to both a reduction in the estrogen-binding capacity of the cells (i.e., to decreased ER levels) and to a decrease in ER as well as PR transcripts (10). In the current study, we have confirmed this association using the HR-positive breast cancer cell lines ZR-75–1 and T-47D. It has been shown that the activation of growth factor receptors such as HER-2/neu can result in direct phosphorylation and activation of ER in an estrogen-independent manner, which may itself be an important mechanism for tamoxifen resistance (43), in addition to the subsequent reduction in HR levels. However, it appears likely that this reduced expression is not the only mechanism for endocrine resistance in patients with HER-2/neu-positive tumors. The interaction between HER-2/neu and steroid HRs is most likely determined by multiple complex mechanisms, such as phosphorylation of the ER via HER-2/neu activation (10,43), overexpression of the steroid receptor cofactor AIB1 in HER-2/neu-positive tumors (44), and competition between ER and other coregulatory receptor proteins resulting in altered HER-2/neu expression (45).

The main goal of this study was to use clinical material to evaluate differences in HR levels between HR-positive patients with HER-2/neu amplification/overexpression and their HER-2/neu-negative counterparts. We hypothesized that these differences might contribute to the reported lower response to hormonal treatment among HER-2/neu-negative:HR-positive patients (18–20). It is important to emphasize that it was not an objective of this study to validate ELISA as an alternative method to immunohistochemistry for detecting HER-2/neu overexpression in clinical practice (46); rather, we used ELISA technology because it is the only large-scale testing method that measures HER-2/neu protein expression levels in a quantitative fashion, allowing it to be analyzed as a continuous variable rather than as a dichotomous variable. Another advantage of ELISA was that it was compatible with the use of frozen samples. Use of frozen breast cancer samples for HER-2/neu analysis minimizes the impact of the well-recognized reduced antigenicity of the HER-2/neu protein caused by fixation, a necessary step in immunohistochemical scoring of paraffin-embedded tissue, and the resulting confounding effects on detecting HER-2/neu expression (2). However, the ELISA approach can result in errors due to dilutional artifacts that are known to exist in tissue homogenates of mixed cell type populations (2,37) such as ours, which included stromal as well as epithelial cells. Indeed, the HER-2/neu positivity rate of 16.8% observed for cohort A is in the lower range of published rates, which range from 18% to 25% in lymph node-negative cohorts (7,47) and from 25% to 27% in lymph node-positive cohorts (1,2). The large sample size in the present study, however, allowed us to overcome some of the potential limitations introduced by stromal cell contamination in the ELISA.

Although patient characteristics were similar between cohorts A and B, we found different medians and ranges for HR levels in the two cohorts. HR levels were determined by EIA (cohort A) and radioligand binding assays (cohort B), both of which provide quantitative measures of HR expression. Comparative studies of both assay methods performed on human tissue cytosols have shown a good correlation between the results of the two assays (48). However, a direct comparison of the absolute and median HR concentrations between cohorts A and B in the current study was limited by the fact that the two methods were used to investigate different patient populations. Results obtained from EIAs are more accurate than those from radioligand binding assays, which are less well standardized (49). Moreover, it has been reported that HR-positive tumors tend to yield even higher values with the dextran-coated charcoal method utilized in the radioligand binding assay compared with EIAs (50). Despite the differences in the range of absolute receptor values between both HR assays, the fraction of ER-positive/PR-positive
patients was nearly identical in both cohorts, which suggests that both assays were accurate.

The inverse correlation between HER-2/neu and HR levels may help to resolve the controversy in the literature regarding response to hormonal treatment in patients with HR-positive tumors that also overexpress HER-2/neu. That is, the selection of different cutoff values for HER-2/neu positivity may result in substantially different levels of HRs in those patients classified as both HER-2/neu-positive and HR-positive. Higher HER-2/neu cutoff points would be associated with lower HR levels and vice versa. For example, a retrospective analysis of HER-2/neu levels in HR-positive tumors from 205 patients enrolled in the Southwest Oncology Group 8228 Protocol, in which patients received tamoxifen as first-line treatment for metastatic breast cancer, found no difference in response to tamoxifen between the HER-2/neu-positive and HER-2/neu-negative patients (21). However, the investigators used a low cutoff score for positive immunohistochemical staining of tumor cells, in which tumors were scored as HER-2/neu-positive if more than 1% of the cells stained for HER-2/neu using the TAB250 antibody. When the investigators used a higher cutoff point for HER-2/neu positivity (i.e., >10% of the tumor cells stained for HER-2/neu), they found lower tamoxifen response rates and a statistically significantly shorter time to treatment failure (5 months versus 8 months; \( P = .04 \)) (21).

Definitive conclusions about the efficacy of hormone therapy in HER-2/neu-positive:HR-positive patients are difficult to draw, given the small number of retrospective clinical studies performed to date. Nevertheless, valuable insights can be gained by sufficiently powered retrospective analyses such as the one reported here. Our findings indicate clearly that HR-positive tumors that also have HER-2/neu amplification/overexpression have lower HR levels than HER-2/neu-negative:HR-positive tumors. Because absolute HR levels are strongly related to response to hormone therapy, the reduced HR expression offers one mechanism by which patients with HER-2/neu-positive:HR-positive tumors are less responsive to hormone therapy than their counterparts with HER-2/neu-negative tumors. Further support for this hypothesis comes from our previously published data (10), as well as the data in this manuscript, which demonstrate that this phenomenon can be observed experimentally in HR-positive human breast cancer cell lines engineered to overexpress HER-2/neu.

Ultimately, the predictive value of HR levels in patients with HER-2/neu-positive tumors must be verified in prospective study cohorts designed specifically to investigate the response to hormonal therapy in breast cancer patients whose tumors are analyzed for the presence or absence of the HER-2/neu amplification. The current study nevertheless suggests that the ability of HER-2/neu amplification/overexpression to serve as a predictive marker for the response to endocrine therapy might best be evaluated by considering the quantitative levels of HER-2/neu and HRs as continuous variables, rather than using dichotomous scoring systems.

**References**


NOTES

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