Her-2/neu Expression in Node-negative Breast Cancer: Direct Tissue Quantitation by Computerized Image Analysis and Association of Overexpression with Increased Risk of Recurrent Disease


Departments of Pathology [M. F. P., G. H., J. A. U., M. M. J. and Preventive Medicine, [M. C. P.], University of Southern California School of Medicine, Los Angeles, California 90033; Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles, California 90024-16-8 [V. R. C., D. J. S.]; Misericordia Hospital and Cross Cancer Institute, Edmonton, Alberta, Canada [J. D., M. C. P.]; Division of Clinical Chemistry, Vancouver General Hospital, Vancouver, British Columbia, Canada [W. G.] and Berlex Biosciences, Alameda, California [M. S., R. A.]

ABSTRACT

The HER-2/neu proto-oncogene (also known as c-erb B-2) is homologous with, but distinct from, the epidermal growth factor receptor. Amplification of this gene in node-positive breast cancers has been shown to correlate with both earlier relapse and shorter overall survival. In node-negative breast cancer patients, the subgroup for which accurate prognostic data could make a significant contribution to treatment decisions, the prognostic utility of HER-2/neu amplification and/or overexpression has been controversial. The purpose of this report is to address the issues surrounding this controversy and to evaluate the prognostic utility of overexpression in a carefully followed group of patients using appropriately characterized reagents and methods.

In this report we present data from a study of HER-2/neu expression designed specifically to test whether or not overexpression is associated with an increased risk of recurrence in node-negative breast cancers. From a cohort of 704 women with node-negative breast cancer who experienced recurrent disease (relapsed cases) 105 were matched with 105 women with no recurrence (disease-free controls) after the equivalent follow-up period. Immunohistochemistry was used to assess HER-2/neu expression in archival tissue blocks from both relapsed cases and their matched disease-free controls. Importantly, a series of molecularly characterized breast cancer specimens were used to confirm that the antibody used was of sufficient sensitivity and specificity to identify those cancers overexpressing the HER-2/neu protein in this formalin-fixed, paraffin-embedded tissue cohort. In addition, a quantitative approach was developed to more accurately assess the amount of HER-2/neu protein identified by immunostaining tumor tissue. This was done using a purified HER-2/neu protein synthesized in a bacterial expression vector and protein lysates derived from a series of cell lines, engineered to express a defined range of HER-2/neu oncoprotein levels. By using cells with defined expression levels as calibration material, computerized image analysis of immunohistochemical staining could be used to determine the amount of oncoprotein product in these cell lines as well as in human breast cancer specimens.

Quantitation of the amount of HER-2/neu protein product determined by computerized image analysis of immunohistochemical assays correlated very closely with quantitative analysis of a series of molecularly characterized breast cancer cell lines and breast cancer tissue specimens. Breast cancer cells with no overexpression of HER-2/neu had an average oncoprotein content of 0.18 ± 0.02 (SE) pg/cell, whereas the content of those with overexpression was as follows: 2.01 ± 0.73 pg/cell in cancers with overexpression but no amplification; 2.60 ± 0.47 pg/cell in cancers with 2 to 5-fold amplification and overexpression; and 3.38 ± 0.29 pg/cell in cancers with more than 5-fold amplification and overexpression. HER-2/neu overexpression was identified in 41 of 105 relapsed cases and in 21 of 105 matched disease-free controls. The risk of developing recurrent disease in node-negative women with any level of HER-2/neu overexpression was 3.0 times that of women whose breast cancer lacked overexpression while the group of patients with high overexpression had a risk of recurrence 9.5 times greater than those whose breast cancers had normal expression (P = 0.0001). Analysis of various subgroups showed significantly increased risks in pre- and postmenopausal women as well as in women with estrogen receptor-negative and small (TIA) breast cancers indicating the increased risk of recurrence extended across several subgroups of node-negative breast cancer patients.

Our results demonstrate that HER-2/neu overexpression is an independent indicator of increased risk of developing recurrent disease in women with node-negative breast cancer. Although frozen tissue is optimal for analysis, formalin-fixed, paraffin-embedded tissue can yield meaningful results when reagents of sufficient sensitivity and specificity are used. Finally, the quantitative amount of oncoprotein in tumor cells can be determined using immunohistochemistry in conjunction with computerized image analysis.

INTRODUCTION

Approximately one American woman in nine will be diagnosed with breast cancer during the course of her lifetime (1). The prognosis of these women is dependent on several factors. The most important to date appears to be the extent of disease at diagnosis. Axillary lymph node involvement with breast cancer is the strongest predictor of clinical outcome (2), and most women found to have involved axillary nodes at diagnosis are treated with adjuvant therapy. Recently, considerable interest has been focused on the treatment of women with node-negative breast cancer. Women with node-negative disease who receive adjuvant therapy have a longer disease-free interval (3-5) and a longer overall survival (6-9). However, only 20-30% of these women would have been expected to experience a recurrence, and it has been argued that treatment of the entire node-negative group for the benefit of 20-30% is both undesirable and costly (10). Identification of those women at high risk for recurrence would clearly be advantageous.

A variety of prognostic factors have been evaluated to determine if it is possible to identify this high risk group. These factors have included tumor size (11), histological grade (12), steroid hormone receptor status (13), DNA ploidy (14), proliferative index (15), cathepsin D (16-18), and analyses of growth factor receptors such as the epidermal growth factor receptor (19). Despite these efforts, the latest NIH Consensus Development Conference has recommended that only those node-negative patients whose tumors are less than 1 cm should be advised against systemic therapy, since their disease recurrence rate is <10% over 10 years (20). A major objective of the current study is to more critically address the prognostic role of alterations in expression of the HER-2/neu protooncogene in this important group of patients.
The neu gene was first identified as a dominant transforming oncogene in DNA from chemically induced neuroblastomas of neonatal rats (21, 22). The human homologue of this cellular oncogene, HER-2/neu, was independently identified by three different groups (23–25), and when cloned and sequenced, was found to be related to the human EGFR (HER-1) or c-erb B oncoprotein. Because of this close relationship to HER-1 and c-erb B, the human homologue of neu has been referred to as HER-2 (25) or c-erb B-2 (23). To date, almost all studies agree that amplification/overexpression of this gene in node-positive breast cancer correlates with a shorter disease-free interval and with a shorter overall survival (26–46). Amplification (44, 47) and overexpression (33, 37, 39, 42, 43, 45, 46, 48) of HER-2/neu has also been correlated with disease recurrence and shorter survival in node-negative breast cancer, but these results have been disputed (30, 34, 38, 49–53). To further address this issue, we have systematically assessed the expression of this protein in a well-studied and carefully followed cohort of node-negative human breast cancers using fully characterized reagents and methodologies.

MATERIALS AND METHODS

Patient Material

This investigation, involving human subjects, was reviewed and approved by the Institutional Research Boards of all involved laboratories.

"Relapse" Versus "No-Relapse" Study. The study sample was selected from 704 node-negative invasive breast carcinoma (infiltrating ductal or lobular carcinoma) cases diagnosed between 1971 and 1982 in Alberta, Canada. Patients having a diagnosis of breast carcinoma in situ only were excluded. The records of these patients were maintained in a computerized population-based registry by the Breast Unit of the Cross Cancer Institute. The recurrence rate of the 704 women was estimated to be 18 and 28% at 5 and 10 years, respectively, on December 31, 1987 (47). For this study, all 105 cases with known menopausal status at first treatment who experienced a local recurrence or metastasis before December 31, 1987, and for whom archival tissue was available were paired with controls, from the same cohort, who had not experienced a local recurrence or metastasis before December 31, 1987, and who had been disease free for at least as long as their matched "case." For ease of reference we will often refer to patients with relapse as "cases" and their matched nonrelapsed (disease-free) patients as "controls." Node negativity was based on sampling of at least four axillary lymph nodes in all cases and controls (cases: range, 4–27; mean, 10; controls: range, 4–29; mean = 11). Seventy-four% of the cases and controls had seven or more axillary lymph nodes sampled. These cases and controls were matched for menopausal status (35 premenopausal, 4 perimenopausal, 66 postmenopausal pairs), and age at treatment (within 5 years). They were then hierarchically matched as closely as possible with respect to size of primary tumor (T category, International Union Against Cancer), estrogen receptor status (positive, negative, unknown), and anniversary year of diagnosis (within 3 years). A total of 90.5% of cases were matched on all of these criteria with their controls. Distribution of primary tumor size in the cohort was as follows: 31.9% were T1A; 1.0% were T1B; 55.7% were T2A; 5.2% were T2B; 4.8% were T3A; and 1.4% were T3B. The ages of the women included in the study were: 5, 20–29 years old; 13, 30–39 years old; 58, 40–49 years old; 52, 50–59 years old; 56, 60–69 years old; 22, 70–79 years old; and 4, 80–89 years old. The median follow-up time for both cases (range, 60–168 months) and controls (range, 60–192 months) was 108 months. The case-control approach was adopted to maximize the efficiency of obtaining blocks. Although some additional statistical power would have been obtained if all cases with known menopausal status had been included, the 105 case-control pairs studied proved to provide more than sufficient statistical power to test the relationship between HER-2/neu expression and relapse.

Almost all of these node-negative breast cancer patients were treated with modified radical or total mastectomy. Two were treated by segmental resection. Twenty of the 210 women (10 cases and 10 controls) received adjuvant radiation therapy. None had primary chemotherapy or hormonal therapy. Elimination of the 20 patients who received adjuvant radiation therapy had no effect on the conclusions of this study. As a result of the health care system in Canada, almost all patients with a given disease are treated at one of a few regional centers. This circumvents the potential lack of uniformity in treatment and follow-up inherent to multiinstitutional group studies.

Immunohistochemical Staining

HER-2/neu Antibodies. A rabbit anti-HER-2/neu polyclonal antiserum (R60) directed against the HER-2/neu gene product has been previously described and was used in this study (29, 54). This antiserum has no cross-reactivity with epidermal growth factor receptor and was used to identify HER-2/neu oncoprotein in tissue sections as described previously (29, 54, 55). The 3B5 monoclonal antibody directed against a similar epitope was generously provided by Dr. Marc van de Vijver, and was also used to evaluate HER-2/new expression immunohistochemically (28).

Sensitivity and Specificity of Immunostaining for HER-2/neu Overexpression. Ninety fully molecularly characterized primary breast carcinomas, from a group of 187 previously studied samples, evaluated at the DNA, RNA, and protein levels for HER-2/neu alteration (29), were used as standards to determine both the sensitivity and specificity of our immunostaining reagents and methods in the paraffin-embedded sections used in this study. These standards were comprehensively characterized as follows: degree of HER-2/neu gene amplification was determined by Southern blot analysis of EcoRI digested tumor tissue DNA (29); the relative amount of HER-2/neu mRNA was determined by Northern hybridization of total RNA (29); and the relative protein content was determined by Western immunoblot analyses of tissue extracts as well as immunohistochemical staining of tissue sections (29).

In the context of this report "low expression" and "overexpression" is used to refer to the amount of HER-2/neu RNA or protein present in a breast cancer, whereas "immunostaining" is used to refer specifically to the HER-2/neu membrane staining identified by immunohistochemistry. The studies with frozen tissue samples (29, 54) provide a measure of HER-2/neu gene expression at the RNA and protein levels in the breast cancer standards. The amount of HER-2/neu gene expression, with few exceptions (10%), is proportional to the number of copies of the gene in tumor cells (29, 54). Most breast cancers with no increase in the HER-2/neu gene copy level relative to a control gene on the same chromosome (unamplified or single-copy cases) have a detectable, but low amount of HER-2/neu RNA and protein. This "low expression" can be identified in frozen tissue sections by immunohistochemistry as weak membrane immunostaining. The low level of protein expression is usually not identifiable as membrane immunostaining when the tissue is formalin fixed and processed for paraffin embedding (29, 55). Breast cancers with an increase in the number of HER-2/neu gene copies relative to a control gene on the same chromosome have gene amplification. Amplification is measured in multiples of the control gene content (2->5-fold increased). Breast cancers with HER-2/neu gene amplification have increased levels of gene expression, referred to as "overexpression." Overexpression is identified immunohistochemically as membrane immunostaining which is stronger than that observed in breast cancers lacking amplification (29, 54) and stronger than that observed in normal adult tissues (55). A few breast cancers used as standards had overexpression of HER-2/neu without having gene amplification by Southern blot (see below).

The 90 standards for the current study were selected so that 45 of them contained a single copy of the gene and expressed low levels of the protein while the other 45 had evidence of HER-2/neu overexpression. Thirty-three of these latter samples were amplified to varying degrees while 12 contained overexpression in the absence of measurable gene amplification. Relative immunostaining levels, determined in a blinded fashion by immunohistochemistry of paraffin-embedded tissue sections, were compared to the molecular findings from this group of 90 standards. Immunostaining for HER-2/neu with the 3BS monoclonal antibody was also performed both as described above and by the avidin-biotin-chromogen technique previously described (28).

Evaluation of Cases and Controls. Tissue sections, 4 μm thick, from formalin-fixed, paraffin-embedded blocks of relapse cases and disease-free controls were evaluated for HER-2/neu protein expression by immunohistochemistry. This was done blinded to all clinical information regarding the samples being analyzed. Positive and negative immunostaining test tissue sections were included with each immunohistochemical procedure. The sites and intensity of immunoprecipitate formation were identified microscopically following treatment with the chromogen 3′,3′-diaminobenzidine. Immunostaining was scored nonquantitatively as low immunostaining (negative (-) or weak immunostaining, i.e., slightly detectable immunostaining in isolated cells) and...
intermediate or strong immunostaining according to the relative intensity of membrane staining (Fig. 1, A–C). The sensitivity and specificity of the immunostaining procedure was determined in the 90 known breast cancer standards as follows: sensitivity = true positives/true positives + false negatives; specificity = true negatives/true negatives + false positives.

DNA extracted from punch biopsies of the paraffin blocks from the cases and controls were assayed independently by slot blot analysis to determine HER-2/neu gene copy number as described elsewhere (47). DNA obtained from paraffin embedded tissue is of variable quality and is, in general, not intact. Since the quality of the DNA obtained from paraffin-embedded tissue is less than that obtained from frozen tissue, the distinction between lower copy numbers is more difficult to make. Although a 2-fold amplification level can be readily distinguished in DNA from frozen tissue (26, 29), the fluctuation in DNA size obtained from paraffin-embedded tissue can influence probe signal intensity; consequently, only gene copy values of 3-fold or greater were regarded as distinguishable from single copy for the slot blot analyses included in this study (47).

Quantitative Determination of HER-2/neu Protein Content

Cell Lines. A series of established and molecularly engineered cell lines expressing HER-2/neu protein levels, ranging from low to high, were used for quantitation of HER-2/neu in tumor cells. The established cell lines included MCF-7, SK-BR-3, CAOV3, MDA-MB-231, and NR6. All of these cell lines were of human origin except NR6 (56) and were obtained from the American Type Culture Collection. The molecularly-engineered cell lines were all derived from these established lines. The engineered and matched control cell lines were developed by infecting the cells containing a single copy of the HER-2/neu gene and expressing low levels (1+) of the protein with a retroviral expression vector either containing or lacking the HER-2/neu cDNA, respectively. In brief, the coding region from a full-length cDNA of the human HER-2/neu gene (29, 54) was inserted into the retroviral expression vector pLPNSN-2 (57). In this vector the HER-2/neu cDNA is transcribed from a Moloney murine leukemia virus promoter and a neomycin phosphotransferase gene (neo) is transcribed from an internal SV40 promoter. A similar vector expressing neo but without the HER-2/neu cDNA (pLXSN) was used as a control (58). Retroviral packaging and infections were performed as described previously (59–62).

Quantitation of HER-2/neu protein content in both the established and engineered cell lines was performed using Western blot analyses of lysates from 10⁶ cells of each of the above cell lines. The Western analyses were performed simultaneously and on the same blot with known amounts of a recombinantly expressed, purified protein fragment comprising 80% of the HER-2/neu amino acid sequence. This purified HER-2/neu protein fragment is recognized by the R60 antibody. Protein preparation and isolation was accomplished by expressing a fragment of the HER-2/neu gene encoding 80% of the amino acid sequence beginning at the amino terminus. An Ncol-Kpn1 restriction fragment from the coding region of the human HER-2/neu cDNA was inserted into an Escherichia coli expression vector containing a λpl promoter. Expression was induced by a temperature shift during fermentation. The HER-2/neu protein fragment was expressed as a fusion protein to 18 amino acids from the NH2-terminal sequence of the E. coli trp E protein. Following induction, the HER-2/neu fragment accumulated in inclusion bodies which represented approximately 5% of the total bacterial protein. Purification of the recombinant HER-2/neu protein fragment was performed by an inclusion body isolation followed by gel filtration chromatography. E. coli paste, stored frozen at ~70°C, was thawed and resuspended in 25 mM Tris(pH 7.5)-100 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, with 1 mg/ml lysozyme. After incubating for 2 hours on ice, the suspension was frozen at ~70°C and rapidly thawed in a water bath. DNaSe I and MgCl₂ were added to final concentrations of 20 μg/ml and 2 mM, respectively, to reduce the viscosity of the lysate, and insoluble material was collected by centrifugation (10,000 × g, 30 min). The pellet was resuspended in 25 mM Tris(pH 7.5)-100 mM NaCl-1.0% (v/v) Nonidet P-40 and centrifuged again (10,000 × g, 30 min). The final pellet was dissolved by boiling for 3 min in 25 mM Tris(pH 7.5)-1% w/v SDS, 1 mM dithiothreitol. 1 ml of the solubilized inclusion body fraction was loaded on a 1.6 × 50 cm Superose 6 column (Pharmacia LKB) and eluted with 25 mM Tris(pH 7.5)-100 mM NaCl-0.1% (w/v) SDS at a flow rate of 0.2 ml/min. Fractions containing the HER-2/neu protein fragment were identified by SDS-polyacrylamide gel electrophoresis and Western blot and subsequently pooled and stored frozen at ~70°C. The concentration of the purified HER-2/neu protein fragment was determined using a biocinchonic acid protein assay (Pierce) standardized with bovine γ-globulin (Bio-Rad).

Dilutions of purified protein were used to prepare calibration curves with quantities ranging from 20 to 80 ng of protein. The resulting bands on Western blot were then quantitated by both densitometric scanning and gamma counting of the excised bands representing the HER-2/neu signals obtained from the lysates of each cell line to determine the relative amount of HER-2/neu protein/10⁶ cells. Simultaneous with preparation of cell lysates was preparation of cytospins and pellets from the same culture flask of each cell line. The cell pellets were frozen in OCT cryosectioning media and 4-μm frozen sections were prepared. Cytospins were washed in buffer and fixed immediately. Immunostaining was performed as described below. The relative absorbances of cell immunostaining was determined with computerized image analysis and compared to the levels of HER-2/neu, determined by quantitative Western blot analysis. The relative homogeneity of HER-2/neu staining found in the various cell populations allows for determination of the average amount of HER-2/neu protein per cell.

Computerized Image Analysis of HER-2/neu Immunostaining. Quantitation of HER-2/neu immunostaining was performed using a CAS 200 computerized imaging system (Cell Analysis Systems, Inc., Elmhurst, IL), calibrated with cells from the above procedures and tissues immunohistochemically stained for this purpose. In order to permit simultaneous measurement of HER-2/neu immunostaining and DNA content in individual tumor cells, the samples were immunostained by an alkaline phosphatase anti-alkaline phosphatase technique using a red chromogen (CAS red; CAS, Inc., Elmhurst, IL) and counterstained for DNA with Feulgen stain (cyanin blue; CAS, Inc.). Optimal conditions for alkaline phosphatase anti-alkaline phosphatase were similar to those determined for the phosphatase anti- phosphatase technique (29, 54, 55). Fixation for HER-2/neu immunostaining combined with Feulgen counterstaining was performed in 95% ethanol for 10 min followed by 10% neutral buffered formalin for 30 min. Tissue sections from breast cancers and from cytospin cells were incubated with HER-2/neu anti-antibody (R60, 1:1000 dilution; 1 h), followed by a mouse anti-rabbit antibody (The Jackson Laboratory; 10 mg/ml, 30 min), a goat anti-mouse antibody (BRL; 10 mg/ml, 30 min), and finally a mouse monoclonal alkaline phosphatase anti-alkaline phosphatase antibody (Dako; 1:80 dilution, 30 min). Each of these antibody incubations was followed by three 5-min-rinses in Tris-buffered saline. The site of the immunoprecipitates was identified with a naphthol derivative and a diazonium coupler, CAS red (CAS, Inc.). Counterstaining of nuclear DNA was performed with a Feulgen stain as per instructions of the manufacturer (CAS, Inc.).

Statistical Methods

Averages and standard errors of HER-2/neu protein content of breast cancers were calculated using a random effects model with each patient's HER-2/neu protein content serving as the basic data. Standard statistical methods for the analysis of matched case-control studies were used for the analysis of risk of relapse related to HER-2/neu expression (63). Trends for ordered variables were assessed by the score test using both continuous and categorized forms. Multivariate logistic regression for matched studies was used to adjust the results for the other risk factors which had significant effects.

RESULTS

Detection of HER-2/neu Protein in Paraffinized Tissue Sections. The results of immunostaining 90 primary breast cancers for HER-2/neu oncoprotein expression with both the R60 and 3B5 antibodies are shown in Table 1. Forty-five samples were molecularly characterized as single-copy, low expressors of HER-2/neu, while 12 were single-copy overexpressors and 33 were amplified overexpressors of the protein. The degree of immunostaining with the polyclonal antisera (R60) was consistently reduced in paraffinized tissue when compared to the frozen tissue from the same breast specimen (data not shown);
However, this was least apparent in the cases with greater than 5-fold amplification of the gene (29). The characterized, paraffinized breast cancer specimens showed intermediate or high immunostaining of the HER-2/neu protein in tumor cell membranes (Fig. 1) in all but one of the cases with >5-fold amplification of the gene (Table 1). One-half of the specimens (50%) with 2 to 5-fold amplification had intermediate or high immunostaining of tumor cell membranes which was clearly distinguishable from the lack of immunostaining seen in single-copy cases expressing low levels of the protein (Table 1; Fig. 1). In addition, one-half of the single-copy overexpressing breast cancers had intermediate or high expression of tumor cell membranes in paraffin sections (Table 1). Conversely, none of the single-copy, low expression cases showed intermediate or high expression. Therefore in the 90 standards the specificity is 100%.

In occasional cases the immunostaining of tumor cells in paraffin sections was not as homogeneous as in frozen sections from the same specimen, underscoring that this phenomenon is most likely due to variability in fixation of the tissue (data not shown). Conversely, appropriately fixed, well-preserved breast cancer specimens demonstrated the same uniformity of immunostaining seen in frozen tissue (Fig. 1D). In specimens where fixative penetration appeared inadequate, the strongest immunostaining was seen at the periphery of the tissue sample where the fixation was more uniform. Similar to frozen tissue, stromal cells, connective tissue, lymphocytes, and normal tissues were not immunostained in paraffin embedded material.

Given the results using the molecularly characterized tumors as well as previously published data (29), we would predict that the polyclonal R60 antiserum should identify approximately 72% of
breast cancers with HER-2/neu overexpression in paraffin-embedded sections, suggesting that nearly three of every four true overexpressers are detected (sensitivity) and indicating that this antisera is appropriate for investigation of a large cohort of archival node-negative breast cancers.

Conversely, the results with 3B5 monoclonal antibody were less successful at identifying paraffin-embedded breast cancers with known overexpression (Table 1). The 3B5 antibody has been used in other studies and was reported to be sufficiently sensitive to detect HER-2/neu overexpression in paraffin material (28). Assuming that any degree of membrane immunostaining demonstrated overexpression, only 19 of 45 overexpressing standards were identified. Twelve cases, known to have an amplified HER-2/neu gene and/or overexpression of RNA and protein, were immunostained for HER-2/neu with the R60 polyclonal antisera but not with 3B5 (Fig. 2; Table 1).

**Quantitation of HER-2/neu Immunohistochemistry.** The major disadvantage of immunohistochemistry as an analytical technique is the subjective nature of assessing the results. Although this assessment can be accomplished reproducibly by a trained observer, there is an obvious need to have more objective and quantitative results from immunohistochemistry. Such an approach would permit replication of results between laboratories and would introduce a level of standardization for these types of analyses. To achieve this, a commercially available computerized image analysis system with appropriate software was used in conjunction with molecularly characterized cell lines and tumor specimens to determine the exact amount of HER-2/neu protein expression in breast carcinomas with and without amplification of the gene.

The reference cells used for this purpose were derived from a series of established cell lines with known amounts of amplification and expression of the human HER-2/neu gene as well as a group of molecularly engineered cell lines specifically designed to express varying levels of the protein. These cell lines were used to standardize, calibrate, and test the computerized imaging system. In order to establish a calibration curve for the amount of protein present in each cell line, Western blots were performed using dilutions of a recombinantly produced HER-2/neu protein which had been purified to homogeneity (Fig. 3). Total protein derived from lysates of 10⁵ cells of each cell line were prepared and loaded on the same blots with the recombinantly produced, purified protein (Fig. 3). Soft laser densitometry was used to quantitate the relative HER-2/neu signals obtained on the Western blot autoradiograms. In addition, to more completely quantitate the relative intensity of the signal in each lane, the bands associated with the HER-2/neu protein in the cell lines as well as in the dilution curve of the purified protein were cut out of the gel and individually counted in a gamma counter. This allowed for quantitative determination of the exact amount of ¹²⁵I associated with the HER-2/neu band in each lane. Comparison of the signals obtained from known amounts of the purified protein with the signals obtained for the HER-2/neu protein in lysates of defined numbers of cells from each of the reference cell lines permitted quantitation of the amount of HER-2/neu in each line (Table 2). A critical part of this quantitation process was that all reference cell lines used in the Western immunoblot analyses were simultaneously prepared for immunohistochemistry to circumvent potential problems of variation in HER-2/neu protein content which may occur in cell culture over time. This approach allowed for subsequent calibration of the immunohistochemical staining intensity read by the imaging system based on quantitative data of known HER-2/neu protein content in cells prepared at exactly the same time.

The relative absorbance of HER-2/neu immunostaining was then determined in five microscopic fields for each of the cell lines using the computerized image analysis system. This allowed for objective quantitation of protein levels as measured in immunostaining absorbance units and these results could be converted to and reported in pg of HER-2/neu protein per cell based on the Western blot of the same cell lines and the recombinantly expressed, purified protein (Table 2). The success of this approach was greatly facilitated by the relative lack of heterogeneity of HER-2/neu immunostaining found in frozen tumor cell lines and tissue specimens. Also, the use of cell lines rather than tissue for preparation of the lysates used in the quantitative Western blots circumvented the previously described

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**Table 1. Overexpression detected by immunostaining of paraffin-embedded tissue sections from breast cancers with and without amplification of the HER-2/neu oncogene**

| Amplification of HER-2/neu oncogene | Level of RNA, p185 expression | Immunostaining in paraffinized sections
<table>
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<tbody>
<tr>
<td>Single-copy</td>
<td>Lowexp</td>
<td>Polyclonal R60 (Overexp by ICA/total)</td>
</tr>
<tr>
<td>&gt;5-fold</td>
<td>Overexp</td>
<td>18/19 (95)</td>
</tr>
<tr>
<td>2–5-fold</td>
<td>Overexp</td>
<td>7/14 (50)</td>
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</table>

* These breast cancers, used as standards for characterizing the sensitivity and specificity of immunostaining in paraffinized sections, were previously molecularly characterized with regard to HER-2/neu gene copy number and expression. In the original series of 187 cases, from which 90 cases were selected, single copy (i.e., no HER-2/neu amplification) low expression was found in 63%, single-copy overexpression in 10%, >2–5-fold amplification with overexpression in 9%, and >5-fold amplification with overexpression in 18% (29, 54).

† Individual breast cancers were evaluated by immunohistochemistry and classified as showing low (Lowexp), intermediate, or high (Overexp) immunostaining. Any degree of distinct membrane staining in the paraffin-embedded tumor cells was considered as demonstrating overexpression (intermediate and high immunostaining groups together). Monoclonal 3B5 immunostained tumor frequently contained coarse cytoplasmic staining either with or without membrane staining in both known overexpressers and in known low expressors. Only membrane staining was considered in evaluating 3B5.
problems of dilutional artifacts introduced by protein from nonmalignant cell populations found in tissue specimens (29).

Finally, paraffin sections from each of the molecularly characterized breast cancers were immunostained with the alkaline phosphatase anti-alkaline phosphatase technique and counterstained with Feulgen stain. Quantitation in formalin-fixed, paraffin-embedded cells was performed with quantitative image analysis of aliquots of the same cells prepared as frozen samples. Paraffin-embedded cells had an apparent HER-2/neu content which was 61.3% of that observed in similarly treated cultured cells, we could quantitate HER-2/neu protein content in fixed tissue sections. The HER-2/neu protein content in fixed tissue sections of the same frozen cells. Assuming that the reduction in the area counted was excised from the gel and is identified in the figure by white dots. These two approaches established a quantitative calibration curve for the HER-2/neu protein product. The p185HER-2/neu bands were quantitated individually for each of the cell lines. Comparison of the determined values with the calibration curve permitted determination of the amount of HER-2/neu oncoprotein identified in each cell line. Since the total protein loaded into each lane was extracted from 10⁶ cells from each cell line and the cell to cell staining is relatively uniform within a cell line, the amount of protein could be expressed on a per cell basis. The numerical values shown in Table 3 are based on this autoradiogram. In B-D, aliquots of each cell line analyzed by Western immunoblot were prepared for immunohistochemical localization of HER-2/neu by alkaline phosphatase anti-alkaline phosphatase using a red chromogen and the nuclei were counterstained with a Feulgen stain. These immunostained cell lines are: B, NR6/10; C, NR6D; D, SK-BR-3, E, MCF-7; F, M6/10; G, Ca-Ov-3; H, C7/10; I, MDA-MB-231; J, MDA-MB-231+HER-2. In K-5, negative controls for each of the preceding cell lines were prepared for immunohistochemistry using normal IgG instead of immune serum in the alkaline phosphatase-alkaline phosphatase procedure. ×1600. (Compare this figure with the data presented in Table 2.)

### Table 3. Quantitative Western blot and immunostaining by alkaline phosphatase anti-alkaline phosphatase using a red chromogen and counterstained with Feulgen

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quantitation of HER-2/neu proteina</th>
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<tbody>
<tr>
<td>Western immunoblot</td>
<td>Image analysis</td>
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<tr>
<td>NR6-10</td>
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<tr>
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<tr>
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</tbody>
</table>

a See Fig. 3 for illustration of the material analyzed. The samples in the table are arranged in the same sequence as the samples in the figure comparing low expression in parental cell lines with high expression in HER-2/neu-transfected cell lines. SK-BR-3 is an established, known high expressor cell line not produced by transfection.

### Table 2. Quantitation of HER-2/neu oncprotein in cell lines

<table>
<thead>
<tr>
<th>Gene copy</th>
<th>OR</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-copy</td>
<td>1</td>
<td>Not applicable</td>
<td>NA</td>
</tr>
<tr>
<td>3-5-fold</td>
<td>1.89</td>
<td>0.493-7.252</td>
<td>0.3530</td>
</tr>
<tr>
<td>&gt;5-fold</td>
<td>14.79</td>
<td>1.93-113.347</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Single copy compared to any amplification was as follows: χ² = 14.35 (2 d.f.), P = 0.0007. The predictive value of gene amplification compared with expression for outcome (recurrence) was P = 0.011; vice versa P = 0.055, NA, not applicable.

a Gene amplification was also a significant predictor of recurrence.

b Since the quality of DNA obtained from paraffin-embedded tissue is variable, only gene copy levels increased by 3-fold or greater were regarded as consistently distinguishable from single copy. A cutoff of 2-fold has been used for DNA from frozen tissue (26, 29).

c Odds ratios for recurrence (χ² = 15.75, 2 d.f., P = 0.0004). Trend test (χ² = 14.70, 1 d.f., P = 0.0001). Odds ratios for recurrence for any overexpression ("intermediate" plus "high" immunostaining) is 3.0 with 95% confidence interval of 1.5 to 6.1 (χ² = 10.46, P = 0.0012).

d 95% confidence limits, 0.81 to 4.1.

e 95% confidence limits, 2.2 to 42.0.
controls) of the 210 malignancies were found to have oncogene amplification by slot blot analysis of DNA extracted from the paraffinized material and all but one showed intermediate or strong immunostaining by immunohistochemistry. Conversely, 38 malignancies identified as having intermediate or strong immunostaining by immunohistochemistry did not have amplification as determined by slot blot analysis (Table 3). The single malignancy (see above), which had 6-fold amplification of the HER-2/neu gene but no immunostaining by immunohistochemistry, is unique in our experience (29, 54). After the study was unblinded, analysis of this specimen by immunohistochemistry did demonstrate moderate immunostaining of many tumor cells, especially cells near the periphery of the tissue block, suggesting a tissue fixation problem but also confirming this case as a false negative immunohistochemical result.

Statistical analysis of these data demonstrate an OR (approximately equivalent to relative risk) of 3.0 for developing recurrent breast cancer in patients with any level of overexpression of HER-2/neu compared to those whose malignancies expressed normal levels (odds ratio, 3.0; 95% confidence limits, 1.5 and 6.1; χ² = 10.46 on 1 d.f., P = 0.0012). Breakdown of the cases with the matched controls by immunostaining subcategories showed that 22 cases and 17 controls had intermediate HER-2/neu immunostaining, while 19 cases and 4 control had strong immunostaining. Stratification by subcategories yielded odds ratios for recurrent breast carcinoma of 1.0, 1.8, and 9.5 for low, intermediate, and high HER-2/neu immunostaining, respectively (Table 3). These differences were statistically significant (χ² = 15.75 on 2 d.f., P = 0.0004) and a trend test confirmed that increased immunostaining for HER-2/neu was associated with an increased risk of recurrent disease (χ² = 14.70 on 1 d.f., P = 0.0001).

Similarly, increasing levels of HER-2/neu gene amplification in these carcinomas were also associated with an increased risk of recurrent breast cancer (χ² = 14.35 on 2 d.f., P = 0.0008; χ² for trend = 13.59 on 1 d.f., P = 0.0002). For given expression levels based on immunostaining status, determination of DNA amplification level added only marginal additional prognostic information (χ² = 2.58 on 1 d.f., P = 0.11). Conversely, for known DNA amplification level, HER-2/neu expression based on immunostaining added more information although formal statistical significance was not achieved (χ² = 3.69 on 1 d.f., P = 0.055).

Since HER-2/neu immunostaining was predictive of poor outcome in the entire cohort, it was of interest to determine the predictive value in various subgroups of the node-negative group especially with respect to menopausal status, ER content, and tumor size. This analysis demonstrated that the odds ratio of recurrent disease associated with increased immunostaining was similar in premenopausal (35 cases and controls; OR = 3.6, P = 0.055) and postmenopausal women (65 cases and controls; OR = 2.3, P = 0.047). Also the ER-negative subgroup with HER-2/neu overexpression showed a high odds ratio for recurrence (OR = 12.4, P = 0.027), although the number was small (18 cases and controls). Likewise, small breast carcinomas (T1A: 32 controls and 32 cases) with intermediate or strong HER-2/neu immunostaining had a high odds ratio (OR = 13.3, P = 0.010). Finally, larger breast carcinomas (T2A) with HER-2/neu immunostaining were associated with an increased odds ratio, however, this did not reach statistical significance (57 cases and 57 controls; OR = 1.9, P = 0.13). These data indicate that across a number of node-negative subgroups, HER-2/neu overexpression is predictive of outcome.

DISCUSSION

The association between alterations of the HER-2/neu gene and poor prognosis in human breast cancer has been somewhat controversial. To date, at least 33 studies assessing the relationship of HER-2/neu amplification and/or overexpression with relapse and survival in breast cancer have been published (26-47, 50-53, 64-69). Twenty-six of these studies show a significant association between alteration of the oncogene and outcome for either all patients or various subgroups of patients (26-48, 51, 68, 69), while 7 claim there is no association (50, 52, 53, 64-67). Two of the seven (64, 65) with negative results provide survival curves which show patients with overexpression to have a poorer prognosis although the results failed to achieve statistical significance. Recently, the results from these two studies (64, 65) were combined with a third study of similar size (33), since the patient material from all three studies had been examined with the same anti-HER-2/neu antiserum. The results from this combined analysis showed overexpression to be a significant predictor of outcome and led the investigators to conclude that the major problem with the two negative studies was of insufficient numbers of patients (39). Thus, only 5 studies in the literature fail to show an association between HER-2/neu alteration and breast cancer outcome, while 26 support it.

In addition to the 33 studies containing survival data, 21 reports have been published on patients for whom there was no or insufficient survival information, but for whom data on other prognostic factors were known. All 21 of these studies showed a strong association between HER-2/neu amplification/overexpression and one or another of a variety of other established poor prognostic factors including negative steroid hormone receptors (70-75); involved axillary lymph nodes (70, 76-84); large tumor size (84); poor pathological grade (72, 75, 79, 84-86); aggressive histological subtype, i.e., inflammatory carcinoma (80, 87); increased DNA ploidy (82, 88, 89); high mitotic activity (74); and presence of hematogenous metastases (90). Given these associations, it is possible that, had survival data been available, a relationship between HER-2/neu alteration and outcome may have been observed. Taken together, the overwhelming majority of published data support the prognostic significance of alterations in this gene in human breast cancer.

Perhaps the most important subgroup of patients for which reliable prognostic indicators are needed, however, are women who have node-negative breast cancer. While it is true that overall these patients have a good prognosis, it is known that 20-30% will experience a recurrence of their disease. Given these numbers as well as the difficulty in treating recurrent disease with curative results, some have argued that all node-negative patients receive adjuvant therapy with the intent of benefiting those who might otherwise recur. Data from several large clinical trials indicate that such an approach may be beneficial (3-9). Others have argued that this approach places the 70-80% of node-negative patients who will not experience disease recurrence at risk unnecessarily. The possibility exists that HER-2/neu, either alone or in combination with other prognostic factors, might identify node-negative patients at risk for recurrence so that they can be treated.

To date at least 20 different studies have been performed on node-negative disease where clinical follow-up information is available and again the data have been controversial. Eleven reports have shown amplification and/or overexpression to be of prognostic value in either all or in subgroups of patients (33, 37, 39, 42-48, 69), while nine have failed to show this (29, 30, 34, 38, 40, 41, 51-53). Review of these studies reveals the presence of considerable methodological variability including differences in study size, number of recurrences in the group under study, as well as the reagents and techniques being used. As has been shown previously, all of these variables carry the potential to introduce problems in accurately assessing the prognostic role of HER-2/neu in breast cancer (29, 54).

The issue of study size is a very important concern when reviewing published reports since the risk of recurrence in node-negative disease...
is relatively low. Consequently, many more cases are required than in node-positive disease to achieve a similar power to detect a statistically significant association since the ability to identify an effect is directly associated with the number of events (recurrences or deaths) in the study cohort. These events are much more frequent in node-positive breast cancer. As postulated in an earlier study failing to show an association, some of the apparent differences in reports concerning node-negative disease are likely due to these differences in sample size (29). The analysis of Gullick et al. (39) confirms and reemphasizes this point and demonstrates that HER-2/neu overexpression is equally predictive of poor prognosis in node-negative and node-positive breast cancer, when the sample size is adequate. How large should a study population of node-negative breast cancer be to show an association between amplification/overexpression and prognosis of the magnitude observed in the current report? If we assume a conservative HER-2/neu alteration rate of 20% in study populations and use the risk estimates determined in this report (Table 3), then a study population followed for 5 years would need approximately 216 individuals with 54 events (recurrences or deaths) in order to contain enough events to demonstrate (80% power) a statistically significant association ($P = 0.05, 2$-sided) between overexpression and recurrent disease/death. Nine of the published node-negative studies are approximately of this size or larger. Six of these show HER-2/neu amplification/overexpression to be associated with poor clinical outcome, again in either all or subgroups of node negative patients (39, 42, 46–48, 69) while three fail to show it (30, 38, 40). Six of these nine are immunohistochemical studies using paraffin-embedded archival tissues and five of the six demonstrate HER-2/neu to be of prognostic significance (39, 42, 46, 48, 69). The five which show an association have immunostaining rates of 20%, 21%, 16%, 21% and 23%, respectively (39, 42, 46, 48, 69), and these rates are consistent with most published studies in the literature. However, the one study which fails to show an association has a much lower overexpression rate (12.4%) (40) and it is important to note that the survival curves from even this study show a trend toward poorer survival with overexpression though the results do not reach statistical significance. The second large study failing to show an association used gene amplification data and similarly reports one of the lowest amplification rates (13%) in the literature (38). The third large study failing to show an association used Western blot analyses to detect overexpression of the oncoprotein in tumor tissue lysates (30). A comprehensive analysis of a large cohort of frozen breast cancers comparing Southern, Northern, and Western blot analyses as well as immunohistochemistry clearly demonstrated that the Western blot technique is the most prone to false-negative errors due to dilutional artifacts in tissue homogenates. This artifact is introduced by the extracellular matrix proteins found in stromal-rich breast cancers (29). This is less a problem with nucleic acid analyses since noncellular areas of the stroma contribute little DNA or RNA to dilute nucleic acids found in tumor cells whereas these same areas will contribute protein to the tissue lysates.

Further methodological problems are apparent in some of the other published reports evaluating node-negative patients. The reported prevalence of HER-2/neu alteration in node-negative breast cancer has been quite variable, ranging from a high of 33% in one study of amplification (53) to a low of 9% in an immunohistochemical study of overexpression (64). The study reporting an amplification rate of 33% is likely to be an overestimate of the alteration rate since p53, a tumor suppressor gene deleted in some breast cancer specimens (88), was used as the single copy control gene to determine amplification levels in this study. In addition, all of the studies using paraffin-embedded tissues (33, 37, 39–43, 45, 46, 48, 51, 52, 69) probably underestimate the incidence of HER-2/neu overexpression to variable degrees due to the previously demonstrated reduced immunoreactivity of the onco-protein after tissue processing (29). This phenomenon is reconfirmed in the current report. Furthermore, the impact of reduced immunoreactivity due to fixation can be magnified by use of suboptimal reagents. The results obtained with the 3B5 monoclonal antibody in a molecularly characterized cohort of breast cancers illustrate this difficulty. Reduced immunostaining sensitivity is not restricted to the two antibodies used in this investigation as is confirmed by an extensive analysis of immunostaining with 26 different antibodies.4

The current case-control study, composed of 210 individuals from a population-based registry of 704 node-negative patients, demonstrates that HER-2/neu alteration is a predictor of poor prognosis in women with node-negative disease. Node-negative breast cancers with any level of overexpression in paraffin sections have a risk of recurrent disease which is 3.0 times that of women with low (or no) immunostaining in their malignancy. Those women whose breast cancers exhibit the highest immunostaining have an increased risk of recurrence that is 9.5 times that of women whose malignancies exhibit low (or no) immunostaining. Those women whose breast cancers have intermediate immunostaining have an odds ratio of 1.8. Although the 95% confidence interval for cases with intermediate immunostaining does include one, our interpretation is that there is a consistent trend of increasing odds ratio with increasing immunostaining (trend test, $P = 0.0011$). The data are also statistically compatible with no difference of effect between intermediate and low immunostaining, with the statistically significant effect confined to a comparison of high and low immunostaining. Our hypothesis is, however, that there is an increasing trend with increasing expression and the data supports this. We consider this to be the most reasonable interpretation of the results.

We have used the observed odds ratios to estimate risks of recurrence in the three immunostaining groups. To do this we assumed that the data in Table 3 is representative of the entire node-negative cohort of 704 women and that the odds ratios shown would apply at 10 years of follow-up when the overall recurrence rate was estimated to be 28%. In this way we predict that analysis of paraffinized blocks from the entire node-negative cohort of 704 would result in 74.7% having low (or no) immunostaining, 17.5% having intermediate immunostaining and 7.8% having strong immunostaining for HER-2/neu oncoprotein; i.e., approximately 25.3% of the entire unslected cohort is projected to show overexpression in immunostained paraffin-embedded tissue sections. This is consistent with the 26.6% immunostaining that we expect based on results in paraffin-embedded sections from well studied and previously molecularly characterized group of breast cancers [72% (sensitivity) of 37% (overexpression rate)] (Table 1). The probability that an individual in one of the immunostaining groups in the current study (low, intermediate, high) would develop recurrent disease was determined based on these figures, as well as the observed odds ratios and the overall 28% relapse rate. In this manner we estimate that 21.9% of the women with low immunostaining, 33.9% of the women with intermediate immunostaining, and 72.8% of the women with high immunostaining for HER-2/neu are expected to recur by 10 years.

Our results further demonstrate that the clinical utility of HER-2/neu immunostaining is not limited to a particular subgroup of node-negative breast cancer. The odds ratio with increased HER-2/neu immunostaining is greater in both pre- and postmenopausal women. In addition, ER-negative breast cancers with increased immunostaining demonstrate a statistically significant association with recurrent disease. Interestingly, small tumors (T1A) with HER-2/neu immunostaining also had a markedly increased odds ratio of recurrence while

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larger breast cancers with overexpression had an increased odds ratio which was not statistically significant.

To determine if HER-2/neu gene amplification analyses added predictive value to expression analyses, amplification data as determined by slot blot analysis of DNA from this same cohort were compared with oncoprotein immunostaining determined by immunohistochemistry. This comparison showed that 14 of the 15 cases with more than 5-fold amplification of the oncogene were identified as overexpressors of the oncoprotein. This 93.3% recognition rate is close to the 95% sensitivity that we expected based on our analysis of paraffin-embedded tissue from a series of molecularly characterized breast cancers (see Table 1). We also found that, with the exception of a single false-negative case, DNA amplification added little predictive value to the expression data. Conversely, only 39% of the cases identified as overexpressing the protein were also recognized as having amplification. This is in part due to breast cancers which have overexpression without amplification (29). About one-fourth of the HER-2/neu overexpressors can be accounted for on this basis (29, 54). In addition, some of the low end (2–5-fold) amplified cases escaped identification by slot blot analysis since paraffinized samples are suboptimal for DNA analysis (91). Taken together, these data are consistent with our previous work which recommends immunohistochemistry with reliable reagents as the most useful and accurate measure of HER-2/neu alteration in human tumors (29).

Immunohistochemistry is very appealing as a method for evaluating HER-2/neu expression. The technique is rapid, correlates well with molecular results when well-characterized antibody reagents are used, and requires only small amounts of tissue or cytological preparations. This approach suffers from a major disadvantage however. Scoring results of this technique, by both ourselves (29, 54) and others (28, 32, 33, 37, 39, 40, 42, 43, 45, 46, 51, 52, 64, 65, 69, 70, 75, 79, 87, 88, 90), have been subjective. In the current study, we address this issue by using computerized image analysis to standardize the amount of immunohistochemically identified HER-2/neu protein and correlate this to an actual value in terms of the pg of protein/cell. The validity of this approach was confirmed by reproducible biochemical quantitation of protein levels in a series of cell lines compared to a purified HER-2/neu protein preparation and was in complete agreement with the results of computerized image analysis of these same cell lines. We also determined the amount of HER-2/neu protein in molecularly characterized breast cancers with known amplification and expression levels. This analysis established quantitative values for HER-2/neu in immunostained tissues that can be used to objectively separate low (or normal) expression from increased expression (or overexpression).

While computerized image analysis has been used to objectively score normal) expression from increased expression (or overexpression). Levels this analysis established quantitative values for HER-2/neu in human tumors (29).

ACKNOWLEDGMENTS

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Her-2/neu Expression in Node-negative Breast Cancer: Direct Tissue Quantitation by Computerized Image Analysis and Association of Overexpression with Increased Risk of Recurrent Disease


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