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HER-2/*neu* Oncogene Expression and Proliferation in Breast Cancers

Sarah S. Bacus,* Stephen G. Ruby,† David S. Weinberg,‡ Dot Chin,* Ray Ortiz,* and James W. Bacus,*

From Cell Analysis Systems, Inc., Lombard, and Hinsdale Hospital,† Hinsdale, Illinois; and Brigham and Women's Hospital,‡ Boston, Massachusetts*

Amplification of the HER-2/neu proto-oncogene in breast cancer has been reported to correlate with poor patient prognosis. The proliferation, or growth fraction, of cells has also been shown to be of prognostic importance in breast cancer. A study was conducted to evaluate the correlation between HER-2/neu gene expression and proliferation in breast cancer. Quantitative immunohistochemical methods for the detection of the HER-2/neu protein expression and for assessing the proliferation fraction on frozen sections of tumor cells were used. The detection of epidermal growth factor receptor (EGFR) along with quantitative DNA ploidy analysis, also was performed on the same breast cancers. The results indicated two subgroups of invasive ductal carcinoma; 1) HER-2/neu overexpressing cases that were negative for EGFR expression and had low proliferation fraction, and a tetraploid DNA pattern (22 cases), and 2) other combinations of HER-2/neu expression and EGFR expression, with a high proliferation fraction and an aneuploid DNA pattern (38 cases). Eight cases of carcinoma in situ were positive for HER-2/neu overexpression and negative for EGFR expression, and had a high proliferation fraction and a tetraploid DNA pattern. Twenty-six cases of low-grade carcinoma exhibited low proliferation and a diploid DNA pattern. (Am J Pathol 1990, 137: 103–111)

Breast cancer is a common cancer in women, and in spite of major advances in chemotherapy and hormonal therapy, the death rate has remained unchanged. Therefore, it is important to establish reliable and reproducible prognostic tests that will help to choose the optimal therapy for each cancer.[1] Biologic parameters such as tumor ploidy,[2–5] cell proliferation,[6,7] and estrogen and progesterone receptor status[8,9] are becoming useful as a prognostic adjunct to histologic grading. Other major efforts in breast cancer research are directed at evaluating the correlation between gene alteration and clinical behavior of cancers. In particular, alterations of proto-oncogenes in cancer are of major interest.

Proto-oncogenes, including the HER-2/*neu* gene, represent a family of normal cellular genes involved in cell growth and differentiation, and there is evidence that alterations either in gene structure, gene copy (amplification), or overexpression may play a role in the pathogenesis of some human cancers. The HER-2/*neu* gene encodes a 185-kd transmembrane protein homologous to the epidermal growth factor receptor,[10,11] suggesting a possible role as a membrane receptor. Amplification of the HER-2/*neu* gene, which results in elevated levels of expression of HER-2/*neu* mRNA and protein, have been shown to correlate with shorter time of overall survival in women with breast cancer.[12,13] Applying immunohistochemical methods for the assessment of sections of breast tumors, using antibodies to the HER-2/*neu* protein, allows investigation of tumors for overexpression of the protein, and relating protein expression to tumor prognosis.[13,14]

A growing body of literature exists describing the importance of proliferation measurements in breast cancer. The proliferating fraction of tumor cell populations has been reported to have prognostic significance. Low proliferation has been found to correlate with a favorable prognosis in most cases. In contrast, high proliferation has been found to be associated with a poor prognosis.[6,15]

The present study was initiated to evaluate the relationship between HER-2/*neu* gene expression and proliferation patterns in breast cancers. Immunohistochemical methods were used for the determination of HER-2/*neu* overexpression and proliferation fraction. The monoclonal antibody Ki-67,[16–19] which reacts with a nuclear antigen present in cycling cells but not in resting cells, was used for the determination of proliferation fraction. The tumors also were tested for the presence of epidermal growth factor receptor (EGFR)[20] because of its involvement in the

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Address reprint requests to Sarah S. Bacus, PhD, Cell Analysis Systems, Inc., 909 South Rt. 83, Elmhurst, IL 60126-4944.

control of cell proliferation. Furthermore, increased expression of epidermal growth factor receptor also has been linked to unfavorable disease course in breast cancer.[21] In addition, for each tumor the DNA ploidy status also was determined using the Feulgen method.

Materials and Methods

Tumor Tissue

Ninety-four breast cancers were evaluated. The tissues used for immunohistochemical studies were snap-frozen using isopentane in dry ice or liquid nitrogen. The frozen tissues were stored at −70°C until they were sectioned.

Cell Culture

Three tumor cell lines were obtained from the American Type Culture Collection (ATCC). The breast cancer cell line SKBR3, which contains amplified human HER-2/*neu* gene,[22] and overexpresses the HER-2/*neu* protein, and MCF7 breast cancer cell line, which contains one gene copy of HER-2/*neu*[22] and expresses normal amounts of HER-2/*neu* protein, were used as controls for antibody to the HER-2/*neu* protein quantitation. The A431 human epidermoid carcinoma cell line, which contains large number of EGF receptors (2 to 3 × 10^6/cell),[20] was used as a control for EGFR staining.

DNA Staining

DNA quantitation was performed on touch imprints made from the breast tissues and stained with the Feulgen reaction as described previously.[18,23]

Immunohistochemical Staining

HER-2/neu

Detection of the HER-2/*neu* oncogene product was performed by using a rabbit polyclonal antibody to the HER-2/*neu* protein in a double-link alkaline phosphatase anti-alkaline phosphatase technique. The HER-2/*neu* antibody (1:2000 dilution) was provided by Dr. Dennis Slamon, UCLA School of Medicine, Los Angeles, California.[13] The control or negative antibody was rabbit gamma G immunoglobulin (IgG) (1:2000 dilution of a 1-g/ml concentration) from ICN (Costa Mesa, CA). The linking antibodies used were mouse anti-rabbit IgG (concentration of 10 mg/ml) from Jackson Labs and goat anti-mouse IgG (concentration of 10 mg/ml) from Bethesda Research Labs. The mouse alkaline phosphatase anti-alkaline phosphatase (1:100 dilution) used was from Dako Corp (Santa Barbara, CA). The chromogen was CAS Red (Red Chromogen Kit, Cell Analysis Systems, Inc., Elmhurst, IL) and the counterstain used was the CAS DNA Stain. Periodate-lysine paraformaldehyde (PLP), followed by post-fixation in 10% buffered neutral formalin, were the fixatives used.

Epidermal Growth Factor Receptor Expression

Staining of the EGFR expression was obtained by using a mouse monoclonal antibody to the epidermal growth factor receptor in dilution 1:100 (provided by Dr. Bradford Ozanne, University of Texas, Dallas, TX)[20] in an alkaline phosphatase anti-alkaline phosphatase technique as previously described for the HER-2/*neu* staining.

Ki-67 Immunohistochemical Procedure

The proliferation fraction was obtained using the Ki-67 antibody[17] (DAKO-PC: Dako Corporation, Santa Barbara, CA) as previously described.[18]

Quantitation of Histochemical-immunohistochemical Staining

Quantitative DNA Analysis

For quantitation of the DNA staining the CAS 200 Image Analysis System (Cell Analysis System, Inc., Lombard, IL) was used. This is a microscope-based, two-color system, using two solid state image sensing channels. Quantitation of DNA relies on the Feulgen staining reaction, to specifically and quantitatively stain DNA. Quantitation of DNA was performed on the first image sensor of the CAS System, which is spectrally matched to the Feulgen reaction color product. The principles of this system have been described previously,[18,24] and normal values and error tolerances for the assay have been determined.[25] The analysis is based on assigning an optical density to each pixel of the nuclear image and finding the summed optical density of the pixels for each nucleus in the image that corresponds by the Beer-Lambert absorption law to the sum of DNA in each cell. For DNA analysis, in each case, 100 tumor cells from each touch imprint were analyzed and were compared with predeposited control cells on the same slide.

Quantitation of Immunohistochemical Staining

For quantitation of the staining to HER-2/*neu* protein and Ki-67, the two solid state image-sensing channels of the

CAS 200 were used. The image channels are specifically matched to two-component immunohistochemical staining to specifically enhance the image of one stain in each channel. Thus, in the case of the Ki-67 analysis, one channel was used to identify all components in the tissue counterstained with ethyl green (ie, all the nuclear components), and the other channel was used to identify the proportion of nuclear components where specific nuclear proteins (antigens) were stained immunohistochemically. This imaging technique has been referred to as 'nuclear masking.'[18,26] The report for the percent positive stained nuclei for Ki-67 consists of the proportion of the positive nuclear area for the antibody stained *versus* the total nuclear area.

In the quantitation of HER-2/*neu* protein, both imaging channels were used, one for the nuclear component and the other for the cytoplasmic plus membrane component. The total optical density of the nuclear component was calculated from the first image sensor; this correlated to the total tissue DNA in the field. For each field, the total optical density of the cytoplasm and membrane component then was calculated from the second image sensor; this correlated to the total stained protein in the field. The system then calculated the density of protein per picogram of DNA for that field, using both results. Because the total amount of DNA per cell was known (using the DNA quantitation program), the average total HER-2/*neu* density of protein per cell could be computed.

This calculation also relied on using predeposited calibration cells with a constant amount of HER-2/*neu* protein per cell. Using these calibration cells, the system was able to compensate for staining variability from day to day, as these cells were stained together with the cancer tissues. One lot of SKBR3 cell line that was known to overexpress the HER-2/*neu* protein[22] (obtained from ATCC), was used as control cells for overexpressing the HER-2/*neu* protein. This cell line was given a value of 100% of HER-2/*neu* protein content. Thus, our protein quantitation was relative to the SKBR3 cells and not absolute. One lot (obtained from ATCC) of an MCF7 cell line that was known to express normal amounts of HER-2/*neu* protein[22] was used as a normal control. The staining of MCF7 cells also was used to derive a cut-off point for normal HER-2/*neu* protein content for each staining batch. In addition, to establish an objective quantitative cut-off point of the HER-2/*neu* normal protein expression, we quantitated the HER-2/*neu* protein in normal tissue. This was performed on benign epithelial structures, in cases where residual normal tissue could be identified. A cut-off point for normal expression was established as 10% of HER-2/*neu* protein per cell as compared with SKBR3 protein content. Cases expressing distinct membrane staining), and by quantitative image analysis exhibiting more than 10% of SKBR3 HER-2/*neu* protein content per cell, were considered positive for HER-2/*neu* protein overexpression.

The expression of epidermal growth factor receptor (EGFR) was confirmed independently by two of the authors (SB and SR). Cases with no staining with the antibody to the EGFR were scored as negative. Cases expressing a very distinct membrane staining were scored as positive. There was no attempt to subclassify these cases for high or low expression, as our main goal was to quantitate the Ki-67 staining and the HER-2/*neu* staining.

Results

Table 1 summarizes the results of this study. The data were grouped according to the following criteria; first by histology, as invasive ductal carcinoma, carcinoma *in situ*, and low-grade carcinomas. Second, the invasive ductal carcinomas were subdivided into four subgroups, according to the possibilities for positive or negative expression for HER-2/*neu* and EGFR, ie, +/−, +/+, −/− and −/+. This resulted in six subcategories of breast cancer, and for each subcategory the average and standard deviation by proliferation fraction over the number of cases in that category is listed, as well as the DNA histogram type, ie, aneuploid or tetraploid/polyploid, and the average and standard deviation of the G_0/G_1 peak DNA index over the number of cases in that category.

As described above, immunohistochemical staining patterns of tumors for HER-2/*neu* protein expression was obtained from frozen sections with a rabbit-derived polyclonal antibody to the HER-2/*neu* protein.[13] Figure 1 shows characteristic immunostaining patterns obtained with the HER-2/*neu* antibody for various types of breast tissue. The immunostaining with antibody to HER-2/*neu* showed distinct membrane staining, with some cytoplasmic staining, in cases overexpressing the protein (Figure 1A). In cases expressing normal amounts of HER-2/*neu* protein, various degrees of faint cytoplasmic staining were seen (Figure 1B). Only cases exhibiting distinct membrane staining in all or a majority of tumor cells, and HER-2/*neu* protein content by imaging measurements of more than 10% as compared to SKBR3 protein content, were identified as positive for HER-2/*neu* in Table 1. The other cases, which exhibited various amount of diffuse cytoplasmic staining and had less than 10% protein per cell as compared with SKBR3 cells, were scored as negative cases. Thirty-five breast cancers (37%) were defined as positive for HER-2/*neu* overexpression. Of these cancers, 21 (60%) had more than 15% of HER-2/*neu* protein as compared with the SKBR3 cell line, which is known to contain four to eight copies of the HER-2/*neu* gene and to express 100 times the normal amount of mRNA and protein.[22] Thus, relative to normal expression, these can-

Table 1. *Comparison of Proliferation, DNA Index, and Overexpression of HER-2/*neu *in Breast Cancers*

Histology	No. of cases	HER-2/*neu*	EGFR	Ki-67 percent proliferation	DNA index
Invasive ductal carcinoma	22	+	−	6.6 ± 5.21	1.98 ± 0.19 tetraploid‡
Invasive ductal carcinoma	19	−	−	27.74 ± 14.71	1.58 ± 0.42 aneuploid
Invasive ductal carcinoma	14	−	+	38.36 ± 15.9	1.39 ± 0.38 aneuploid
Invasive ductal carcinoma	5	+	+	35.86 ± 16.84	1.7 ± 0.39 aneuploid
Carcinoma *in situ*	8	+	−	26.09 ± 10.28	1.93 ± 0.42 tetraploid
Low-grade carcinoma	26	−	−	4.05 ± 3.98	1.05 ± 0.14 diploid

* For the positivity to HER-2/*neu* protein overexpression a cut-off point of 10% of protein content per cell relative to SKBR3 protein content was used.
† EGFR positivity was established if a distinct membrane staining with an antibody to EGFR was present.
‡ One case was diploid, the other 21 cases were tetraploid.

cers were definitely overexpressing the HER-2/*neu* protein.

The immunoperoxidase staining of the frozen sections for Ki-67 revealed distinct nuclear staining of the proliferating cells (Figure 2). Breast tumors in our study demonstrated variable numbers of Ki-67–positive nuclei, ranging from cases with only scattered proliferating nuclei (Figure 2A) to cases with abundant proliferation (Figure 2B, C). Quantitative image analysis was used to measure the percent positive nuclear area staining for Ki-67 over the entire tissue section. Figure 3 shows the correlation between the proliferation as depicted by the percent Ki-67 positivity and HER-2/*neu* protein expression for the 41 cases of invasive ductal carcinoma that were EGFR-negative. The figure reveals an interesting relationship, in that there is a negative correlation or inverse relationship between proliferation fraction and HER-2/*neu* expression in this subgroup of cases. In fact, as shown in Table 1, the invasive ductal carcinoma cases that were HER-2/*neu*–positive and EGFR-negative formed a distinct group of cases with low proliferation fractions, with a mean value of 6.6%. Low-grade breast cancers that did not overexpress the HER-2/*neu* protein also exhibited low proliferation (4.05%), but had diploid DNA patterns.

Five cases that overexpressed the HER-2/*neu* protein also exhibited positive staining for the EGFR. These cases had high levels of proliferation (mean value, 35.86). The highest amount of proliferation was obtained in cases of breast cancer that were positive for EGFR and negative for HER-2/*neu* overexpression (mean value, 38.36). Eight cases of carcinoma *in situ* also expressed HER-2/*neu* membrane staining (Figure 1C). *In situ* carcinomas had a large cell, comedo histology, and exhibited high proliferation (26.09%). EGFR-positive cases uniformly showed high proliferation without exception, although proliferation also was present in many cases that were not EGFR-positive.

The results of this study for the 94 breast cancers regarding their DNA ploidy analysis are also indicated in Table 1. All of the cancers positive for HER-2/*neu* and negative for EGFR exhibited tetraploid/polyploid DNA content, confirming our prior results.[23] A tetraploid/polyploid DNA distribution is a DNA histogram pattern in which G_0/G_1 phase cells have a DNA content that is a multiple of the normal two copies (4C, 8C, and so on). Tumor cells having DNA content between 2C and 4C in G_0/G_1 are termed 'aneuploid.' Of 35 cases overexpressing HER-2/*neu*, 21 cases of invasive ductal carcinoma had DNA indices (DI) close to tetraploid (mean DNA index, 1.98), and one case had a diploid DNA distribution (1.0). Eight cases of carcinoma *in situ* overexpressing HER-2/*neu* protein had DNA indices close to tetraploid (4C) (mean value, 1.93). Five cases overexpressing HER-2/*neu* and EGFR showed aneuploid DNA patterns (mean DNA index, 1.7). Thirty-three cases of high-grade cancers that did not overexpress the HER-2/*neu* protein showed aneuploid DNA patterns (mean DNA index of 1.58 for EGFR-negative cases and 1.39 for EGFR-positive cases). Twenty-six low-grade tumors that did not overexpress the HER-2/*neu* protein exhibited diploid or near-diploid DNA index (mean DNA index, 1.05).

Discussion

Comprehensive analysis of breast cancer for HER-2/*neu* expression at the DNA, RNA, and protein levels has previously shown[13] a positive correlation between gene amplification and overexpression, and also has indicated that immunohistochemical analysis of frozen sections for oncogene expression is a sensitive method for detecting the gene abnormalities.[13,27] Immunohistochemical techniques enable the assessment of the protein status specifically in tumor cells, and is not subject to dilution by

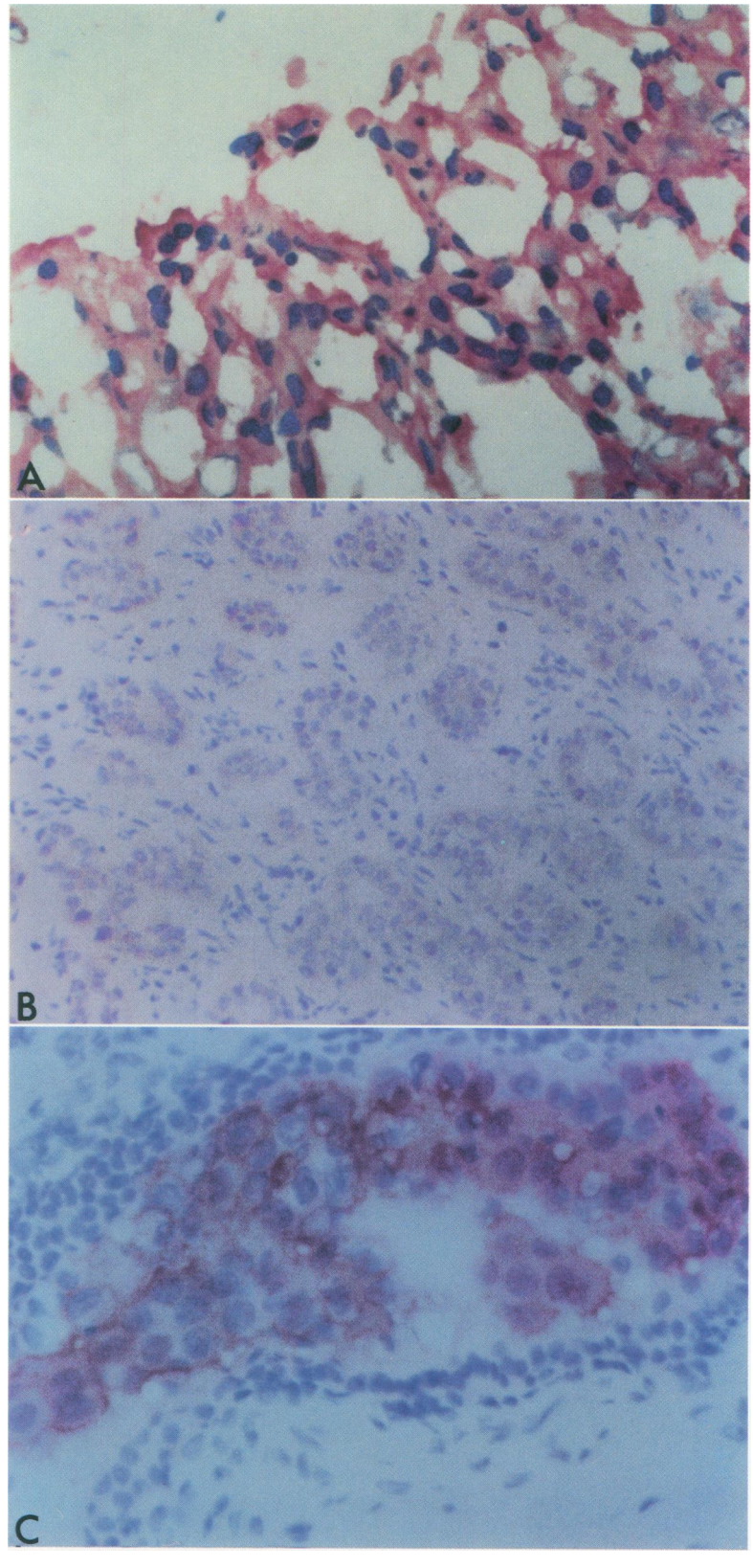

Figure 1. **A:** *Tumor section derived from infiltrating breast carcinoma showing over-expression of HER-2/*neu *protein. Strong membrane staining is evident. Stroma cells and lymphocytes do not stain with the antibody (×200).* **B:** *Tumor section derived from fibroadenoma of the breast. The staining reaction was carried out using the antibody to the HER-2/*neu *protein with the APAAP procedure and counterstaining by the Feulgen method. Notice some diffuse cytoplasmic staining with the antibody of the nonmalignant breast cells (×200).* **C:** *Immunohistochemical staining with the antibody to HER-2/*neu *protein shows positive cells in the intraductal part of the carcinoma (×200).*

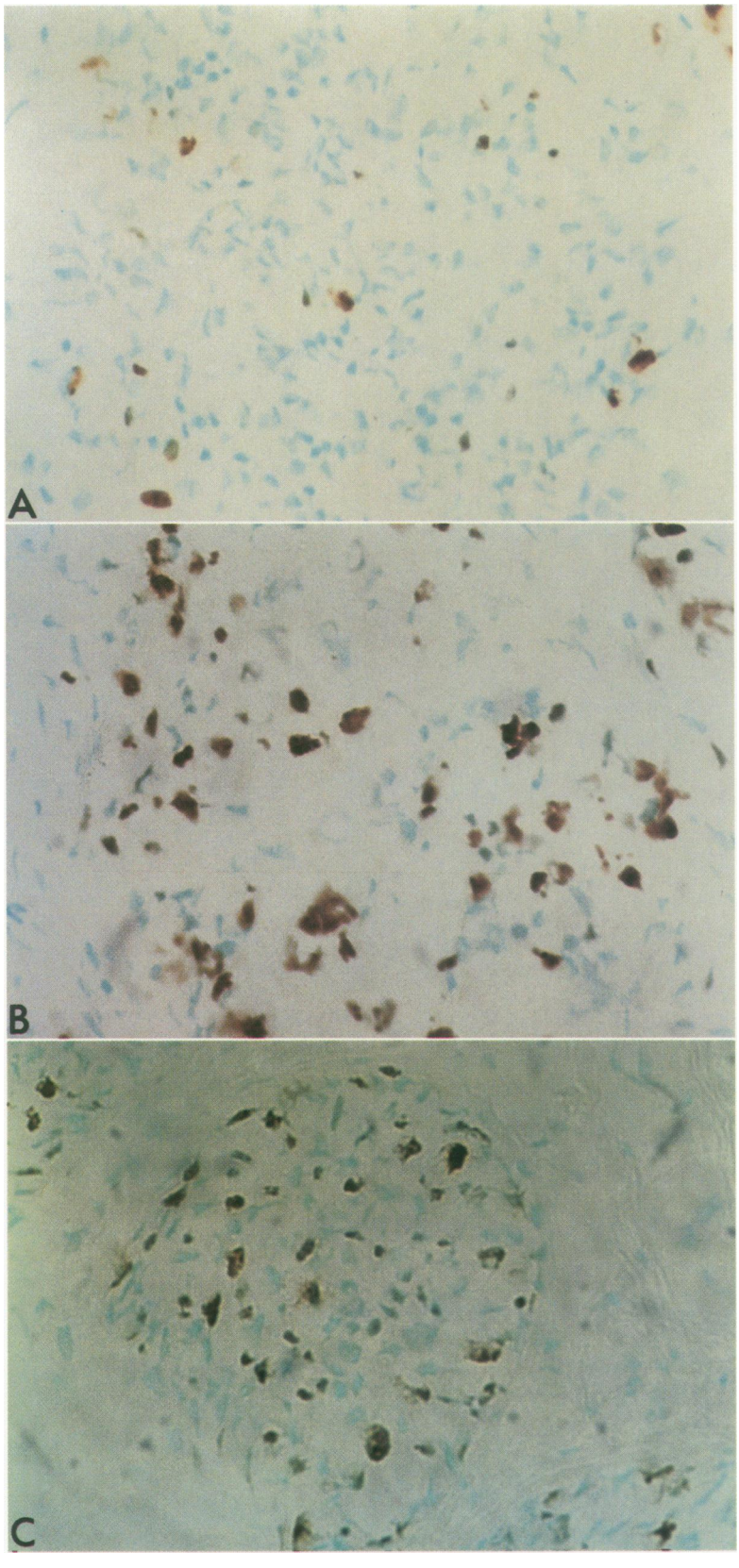

Figure 2. *Nuclear staining by Ki-67 antibody (immunoperoxidase method) and ethyl green. The ethyl green was specific staining for cell nuclei.* **A:** *Ki-67 staining for an infiltrating ductal carcinoma of the breast with overexpressing of the HER-2/*neu *protein. The staining depicts only a few scattered proliferating cells (×200).* **B:** *A high-grade breast carcinoma that was negative for overexpressing the HER-2/*neu *protein. Staining with Ki-67 depicts predominately proliferating cells (×200).* **C:** *Nuclear staining with Ki-67 antibody, showing a high percentage of positive staining in the intraductal part of the carcinoma* in situ *(×200).*

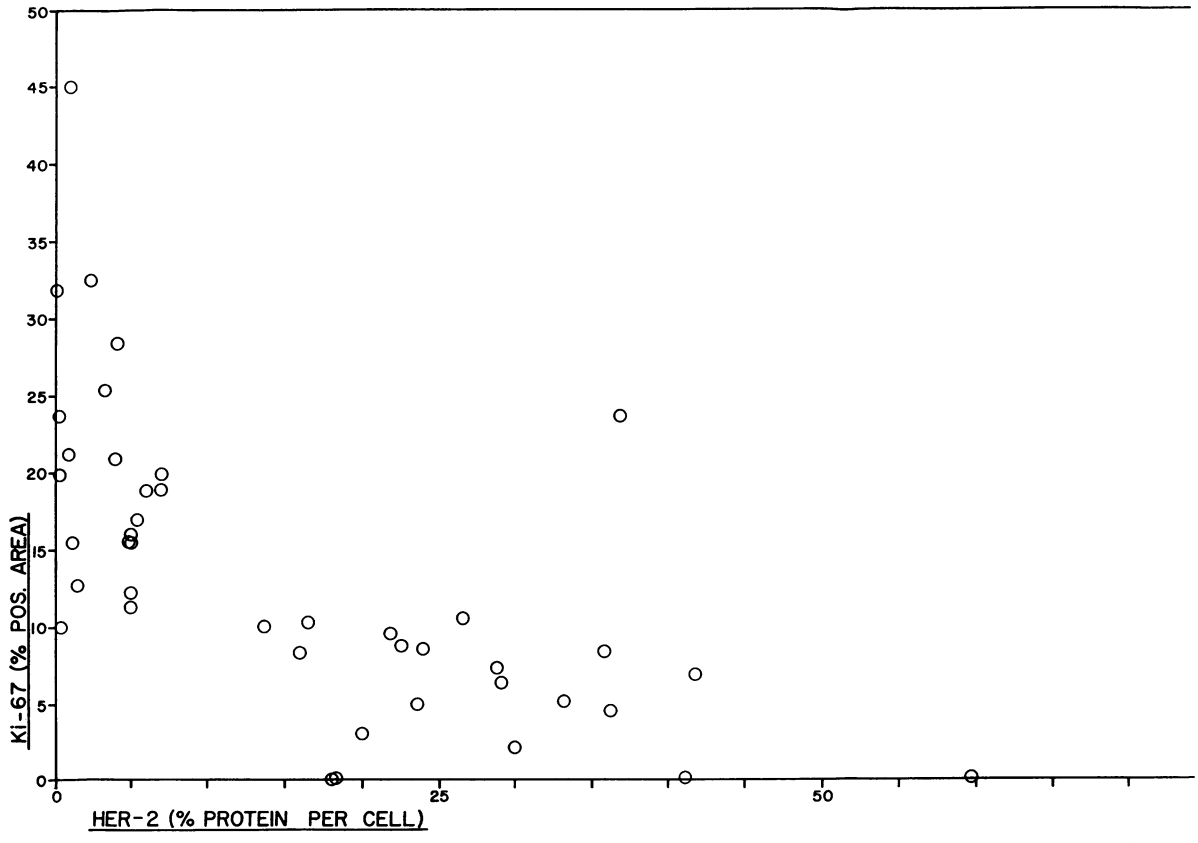

Figure 3. *Overall percentage of Ki-67 staining of high-grade breast carcinoma compared with their HER-2/*neu *protein/per cell content.. The protein per cell is expressed as a percentage protein content relative to the protein content of SKBR3 calibration cells. Notice the decrease of Ki-67 percentage positive staining as the HER-2/*neu *protein/per cell increases.*

other tissue components. However, the shortcoming of immunohistochemical techniques has been lack of objective quantitation.[13] The use of objective quantitation by image analysis makes it possible to quantitate immunohistochemical staining and to correlate HER-2/*neu* protein content with other tumor cell features, such as the tumor proliferation. It is important to note that image analysis combined with immunohistochemistry makes it possible to perform these measurements on very small tissue sections, such as those derived from biopsy material, or on cells derived from needle aspirates. Quantitative image analysis also makes it possible to establish objective cut-off points for elevated expression of gene products, such as the HER-2/*neu* protein, which are expressed in normal breast tissue as well as by tumors. These measurements are objective and do not rely on subjective criteria such as strong *versus* weak staining, or distinct cytoplasmic staining, which are the common criteria used until now for assessing HER-2/*neu* protein expression. Also the use of calibration cells known to overexpress HER-2/*neu* protein, with constant HER-2/*neu* protein, with image analysis enables the compensation for day-to-day staining variations.

This study confirmed our past findings[23] of the correlation between positive HER-2/*neu* overexpression and polyploid DNA content, except in EGFR-positive cases. In contrast, cases that did not overexpress the HER-2/*neu* protein ranged from euploid (diploid) to varying degrees of aneuploid. This observation, that tumors overexpressing HER-2/*neu* tend to have tetraploid DNA content, may have some biologic significance, suggesting that the tetraploidy may be related to the overexpression of the HER-2/*neu* gene.

The Ki-67 antibody distinguishes all actively proliferating cells (G_1, S, and G_2M phases) from resting cells (G_0 phase cells); thus, the amount of Ki-67 staining reflects the proliferating fraction of the cancer cells. Previous studies[18,19] have demonstrated that immunohistochemical staining for Ki-67, coupled with image analysis, is a valid method for determining the growth fraction of cancers.

The highest degree of Ki-67 staining was found in tumors that expressed epidermal growth factor receptors (EGFR). This observation is in keeping with the fact that EGF is one of several polypeptide growth factors that are involved in normal cell growth. Epidermal growth factor receptor status has previously been shown to be predic-

tive of early recurrence and death from breast cancer.[21] Breast tumors that overexpressed the HER-2/neu protein and demonstrated staining for EGFR also had a high growth fraction, and demonstrated aneuploid DNA content. Tumors with these features have been shown in the past to carry the worst prognosis.[29]

Because HER-2/*neu* protein is structurally related to the EGF receptor,[11] our findings concerning the differences in the proliferation fraction between the cases overexpressing HER-2/*neu* protein and negative for EGFR (ie, +/−) and negative for HER-2/*neu* overexpression but EGFR-positive (ie, −/+) are interesting. Our results support the possibility that the HER-2/*neu* protein may not function as a growth promoter in these tumors, while EGFR does. Of interest is also the high rate of overexpression of the HER-2/*neu* protein in ductal carcinoma *in situ*[14] and the association of these cancers with a high proliferation fraction.[16] Because of the relatively small number of cases of this study, we cannot conclusively state that HER-2/*neu* overexpression in the absence of EGFR is always associated with a low proliferation rate. In fact, the cases of ductal carcinoma *in situ* do not exhibit this relationship. However, it appears the HER-2/*neu* overexpression is also not always accompanied with a high growth fraction. This point is intriguing, as previous work demonstrated reduced survival in connection with either high proliferation index[6] or overexpression of the HER-2/*neu* protein.[12,13] However, there is a growing body of evidence that demonstrates that HER-2/*neu* amplification relates to shorter survival time, but does not correlate to disease-free interval,[14,27,30] which may reflect that these cancers do not proliferate quickly, but act aggressively with regard to overall survival.

Overexpression of erbB-2 protein in fibroblasts has been shown to affect the ability of cells to avoid immune surveillance, by reducing their susceptibility to tumor necrosis factor.[28] The aggressive course of HER-2/*neu*–overexpressing cancers might be due to other reasons than highly proliferative cancers. Thus, although proliferation may reflect cell growth, it is probably not the sole criteria that predicts biologic behavior of breast cancer.

It is also not clear why overexpression of the HER-2/*neu* protein is associated with low proliferation in some invasive human breast cancers, yet correlates with higher proliferation rate in carcinoma *in situ*. Several possible mechanisms or their combination may account for this phenomenon: 1) The HER-2/*neu*–encoded P185 protein might be a dual function receptor, stimulating cell growth in carcinoma *in situ* cells, but inhibiting cell proliferation in other breast cancer cells. 2) The P185 protein may be a growth factor receptor in the presence of its ligand, or when it is normally expressed; however, P185 protein may play an inhibitory role in regulating cell proliferation in the absence of its ligand or when it is overexpressed. 3) Finally, the P185 protein may be involved in the cell cycle, but binding to the receptor does not directly induce DNA synthesis and cell cycle completion. The receptor might be a growth factor receptor that is affiliated with other cell functions such as membrane formation, or differentiation. We are currently pursuing experiments to explore these findings and other related issues, to explain the reported more aggressive nature of breast cancers with the HER-2/*neu* gene overexpression.

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