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Biological Grading of Breast Cancer Using Antibodies to Proliferating Cells and Other Markers

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Quantitation of immunohistochemical staining by image analysis was performed on 50 breast cancers stained with the monoclonal antibody Ki-67 to determine the growth fraction and its correlation with tumor grade. A high degree of correlation was shown. For each case the DNA ploidy was determined by quantitation of the DNA Feulgen stain by computerized microdensitometry. DNA content of breast tumor cells correlated to the histopathologic grade at which poorly differentiated tumors are more likely to be aneuploid. Quantitation of immunohistochemistry for estrogen and progesterone receptors had a high degree of correlation with the steroid binding assay, such as dextran-coated charcoal assay (DCCA), and were weakly correlated to histologic grade. In summary, our results indicated that quantitation of Ki-67-positive nuclear area and of DNA content by image analysis provides an objective method for assessing tumor cell growth fraction and DNA ploidy. Quantitation of steroid receptors by immunohistochemistry is a better and easier technique than those currently used to determine the best therapy for postmenopausal women. These methods can be performed on small frozen sections or needle aspirates in quantities that are insufficient for current steroid binding assays. Thus, this method is prognosticly useful even for patients with small breast lesions. (Am J Pathol 1989, 135:783–792)

It has been estimated that one of every 11 women in the United States will develop breast cancer and in spite of major advances in chemotherapy and hormonal therapy the death rate has remained unchanged. Thus it is critically important to establish reliable and reproducible prognostic variables that are useful for selecting optimal treatment for breast cancer. Breast cancers are predominately evaluated by morphologic and histologic criteria. In most cases there is a correlation between histology and clinical behavior.[1,2] However histology alone is subjective and may not always predict clinical behavior. Other nonsubjective biological parameters, such as tumor ploidy,[3–7] cell proliferation,[8,9] and estrogen and progesterone receptor status,[10,11] are becoming more and more important as prognostic adjuncts to histologic grading.

Immunohistochemical techniques allows direct visualization of specific antigens in tissue sections. The interpretation of these techniques has involved subjective, semiquantitative observations. The development of instruments for computerized image analysis provides the capability to quantitate immunohistochemically localized antigens.[12] Thus immunohistochemical staining, combined with image analysis, may provide the more objective criteria needed for the biological and clinical evaluation of breast carcinomas.

This study evaluated some of these new methods. We used the monoclonal antibody Ki-67,[13] which reacts with a nuclear antigen present in cycling cells but not in resting cells. Immunoperoxidase staining using this antibody on infiltrating and intraductal breast carcinomas was performed to correlate proliferation rates with tumor grade and type. Measurements were performed on 50 breast cancer sections and quantitated using digital image analysis. As an adjunct to the assessment of proliferating status, image analysis was also used to measure the DNA (ploidy status) and estrogen and progesterone receptor content of these cancers. In some cases measurements were also made to compare the DNA content of specific tumors with the presence or absence of the HER-2/neu oncogene product.

Material and Methods

Fifty cases of primary breast cancer from the Pathology Department of Evanston Hospital in Evanston, IL were

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evaluated. The tissues used for immunoperoxidase studies were snap frozen using isopentane and dry ice. The frozen tissues were stored at −70 C until they were sectioned.

Ki-67 and Progesterone Receptor Immunohistochemical Procedure

The proliferation index was obtained using the Ki-67 antibody[13] (DAKO-PC, Dako Corporation, Santa Barbara, CA). The amount of progesterone receptor (PR) present was assayed using mPRI,[14,15] a monoclonal antibody to the progesterone receptor (Cell Analysis Systems, Inc., Lombard, IL). Briefly, 5-μm cryostat sections from the breast tissues were cut at −20 C, thaw-mounted on poly-1-lysine coated slides, and fixed for 15 minutes at room temperature in picric acid paraformaldehyde (PAF). PAF fixative contains saturated aqueous picric acid, 3.7% paraformaldehyde, and sodium phosphate buffer at pH 7.3. After fixation slides were rinsed in two changes of PBS. After 0.3% hydrogen peroxide and 20% normal rabbit serum blocks, 15 minutes for the former and 20 minutes for the latter, the sections were incubated with Ki-67 (1:40 dilution in 20% rabbit serum) and mPRI (diluted to 2.5 μg/ml with 20% rabbit serum) for 60 minutes at room temperature, washed in PBS twice for 3 minutes each, and incubated with the linking antibody, rabbit anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) diluted 1:100 with 20% rabbit serum for 30 minutes. After rinsing in PBS twice for 3 minutes each, the slides were incubated with a peroxidase anti-peroxidase (PAP) complex of mouse origin (Sternberger-Meyer Labs, Jarrettsville, MD; 1:100 dilution with 20% rabbit serum) for 30 minutes at room temperature for immunohistochemical localization.[16] The sections were again rinsed twice with PBS for 3 minutes each and incubated with 3,3′ diaminobenzidine tetrachloride (DAB) (Sigma Chemical Co., St. Louis, MO, 0.005 g DAB/10 ml PBS ± 5 μl 30% hydrogen peroxide) for 7 minutes in the dark for visualization of the peroxidase reaction. After washing in running deionized water for 2 minutes, the sections were counterstained with 0.2% ethyl green (Cell Analysis Systems, Inc.) for 10 minutes. The sections were then rinsed in water, dehydrated with butanol, cleared in xylene, and mounted with permount.

Estrogen Receptor (ER) Immunohistochemical Procedure

For estrogen receptor (ER) assay, H222, a monoclonal antibody (ER-ICA Kit, Abbott Laboratories, North Chicago, IL) against human estrogen receptor protein, was used. Briefly, 5-μm frozen sections of the breast tissues were fixed in 3.7% formaldehyde-0.1 mol/L phosphate-buffered saline for 10 minutes, followed by immersion in cold 100% methanol for 4 minutes and cold acetone for 1 minute. After blocking with 0.3% hydrogen peroxide and 20% goat serum, H222[17,18] at a minimum concentration of 0.1 μg/ml was applied. After the H222 primary, a goat anti-rat IgG linking antibody, and a rat PAP complex (both included in the ER-ICA Kit) were used for the localization of the estrogen receptor sites. DAB was used as the chromogen and ethyl green as the counterstain in the manner previously described for the Ki-67 and mPRI assays.

HER-2/neu

Frozen sections from the breast tissues were assayed for the expression product of the HER-2/neu oncogene[19] using rabbit antibodies to the HER-2/neu protein. The HER-2/neu antibody was provided by Dr. Dennis Slamon of UCLA and a double link alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique used to localize the cytoplasmic expression of the oncogene. The resulting alkaline phosphatase localization was visualized with the chromogen CAS RED (Red Chromogen Kit, Cell Analysis Systems, Inc.) according to the manufacturer's instructions.

Immunohistochemical Controls

For each specimen/tissue, a negative control was established by replacing the primary antibody with nonrelated IgG of the same species from which the primary antibody was derived. The IgG was at the same dilution or protein concentration as the primary antibody and was placed on adjacent frozen tumor sections. Thus, mouse IgG replaced the primary antibodies for the Ki-67 and mPRI assays, rat IgG replaced the H222 primary for the ER assay, and rabbit IgG replaced the HER-2/neu primary for the HER-2/neu assay.

DNA Staining

For image analysis, DNA quantitation was performed on touch imprints made from the breast tissues and stained with the Feulgen reaction. Briefly, the touch imprints were air-dried and fixed in 10% neutral-buffered formalin for 30 minutes. After washing in running deionized water for 5 minutes, the touch preparations were hydrolyzed with 5N HCL for 60 minutes at room temperature, incubated with a Feulgen stain (DNA Staining Kit, Cell Analysis Systems, Inc.) for 60 minutes according to the manufacturer's instructions, dehydrated in absolute ethanol, cleared in xylene, and mounted with permount.

Quantitation of Immunohistochemical Staining

For quantitation of the staining of Ki-67, H222, and PRI, the CAS 200 Image Analysis System was used. This is a microscope based, two-color system using two solid state image sensing channels. The image channels are specifically matched to two-component immunohistochemical staining to enhance the image of one stain in each channel. Thus in the case of the present 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 in which specific nuclear proteins (antigens) were stained immunohistochemically. This imaging technique has been referred to as "nuclear masking."[12] This method is very accurate in determining the proportions of labeled *versus* unlabeled tissue components when the stain spectra have been matched to the imaging filters, so that the mask image of one channel is essentially transparent to the other channel, and when the staining is specific for the two components so that proper stereologic ratios can be obtained. Two examples of nuclear staining are indicated in Figure 1A and B. For each specimen, at least five fields were measured randomly. Using an interactive "window" command for each field makes it possible to measure primarily cancer nuclei for the determination of the proportion of labeled nuclei and to exclude any other areas containing mainly normal tissue components. However, there may be a variable amount of lymphocyte and stomal cells that are mixed with the cancer nuclei and become part of the quantitation. If so, this would tend to lower the proliferation index. In our experience, this effect is less than 2%, but may be as high as 4% in some cases. The percentage of positive stained nuclei for Ki-67, H222, and PRI was determined by the proportion of the positive nuclear area for the antibody staining *versus* the total nuclear area.

For determination of baseline staining levels, a control slide of tissue stained with nonspecific immunoglobulin (IgG) was used.

Quantitation for DNA

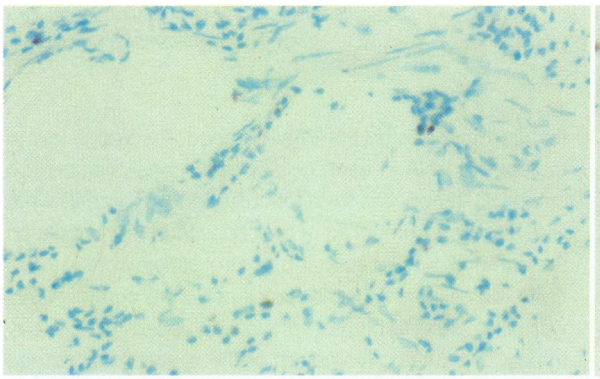
Quantitation of DNA relies on the Feulgen staining reaction to stain DNA specifically and quantitatively. Hydrochloric acid hydrolyzes the ribose-purine bonds in the DNA to give sugar aldehyde residues. The dye then couples through a Schiff reaction to the sugar aldehyde to give a blue color. This staining reaction is stoichiometric to the amount of DNA present in a cell. Quantitation of the DNA was calculated by assigning an optical density to each subunit (pixel) of the 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 touch imprints (Figure 2) were analyzed and compared to predeposited control cells on the same slide. Because the control slides were stained at the same time with the tumor cells, the control slides were also used to correct for staining variability from day to day. The interactive nature of the system enables the user to accept and reject cells for DNA quantitation according to cell classification. Thus, the DNA of tumor cell nuclei only were determined as indicated above. All measurements were done using ×40 objective.

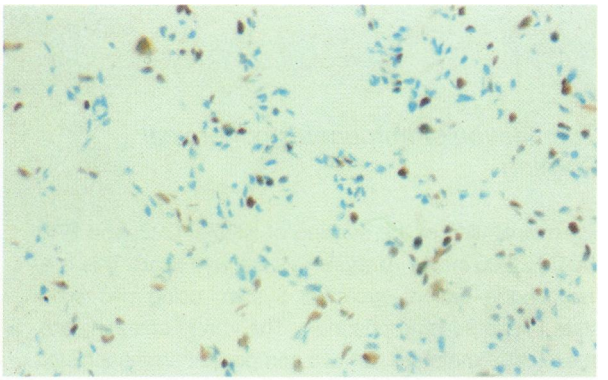
Results

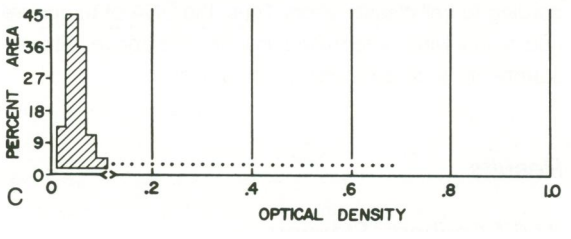
Ki-67 Antibody Staining

Figure 1 shows characteristic immunostaining patterns obtained with Ki-67. The immunoperoxidase staining of the frozen sections revealed distinct nuclear staining of the proliferating cells. Our cases of breast cancer demonstrated positively stained nuclei in varying numbers with the Ki-67 antibody. These ranged from cases with only scattered proliferating nuclei (Figure 1A) to predominantly proliferating nuclei (Figure 1B). Quantitative image analysis using the CAS 200 was used to measure the positively stained nuclear area for the entire tissue section, and to express the results as the percentage positive area (Figure 1C, D). Measurements for the percentage of positively stained nuclear Ki-67 revealed two issues with regard to reproducibility. When measuring the same field repeatedly there is a very low variability in the percentage positive area (±1% per field). However, there is a larger variability for the percentage positive area for Ki-67 staining between different fields of the same tissue due to tissue heterogeneity. To insure the most reproducible results, we selected random fields for quantitation. The overall random field percentage staining positively, for Ki-67, was established previously as the most significant indicator of proliferative activity within each tumor.[20] We also measured at least five fields for each tumor. The standard deviation of repeat readings of the same section varied with the level of positive Ki-67 staining. To determine the variability of repeat readings we assayed representative low and high samples ten different times using five fields for each assay, using for example, the measured standard deviation of a typical low Ki-67 sample (average, 2.85%) ± 0.39 and of a high sample (average, 64.27%) ± 3.02 for ten repeat assays.

Figure 3 shows the correlation between varying degrees of measured Ki-67-positive staining compared with each histologic grade. The histologic grading was determined by the grading scheme of Bloom and Richardson[1] and was performed by one of the authors (RG). Briefly,



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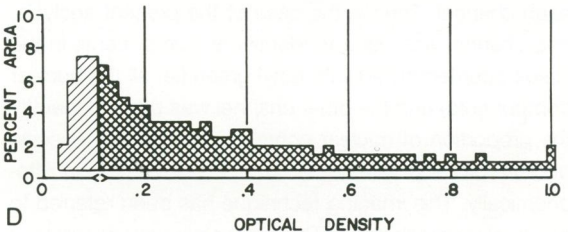
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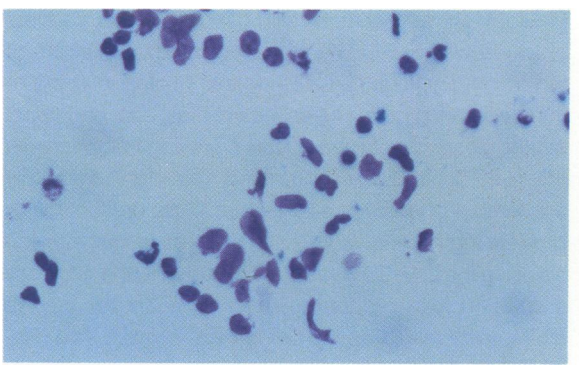

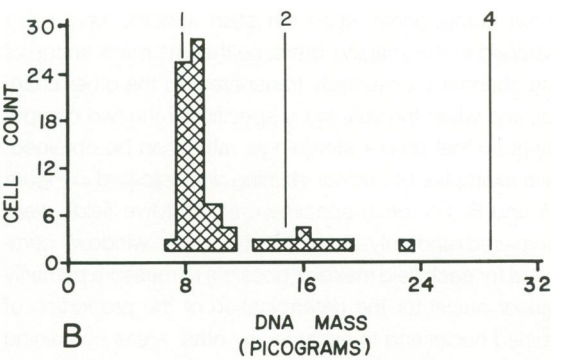
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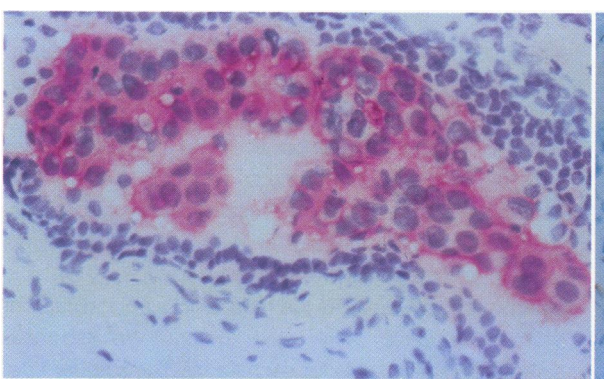

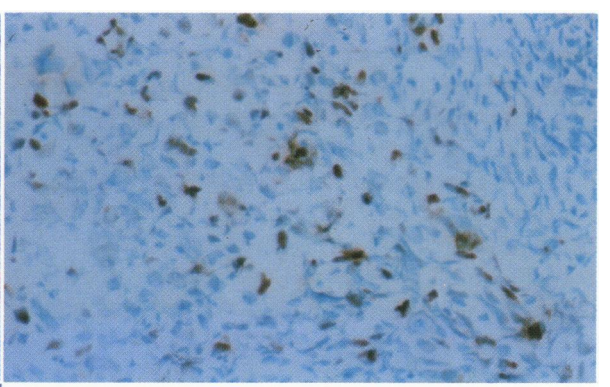

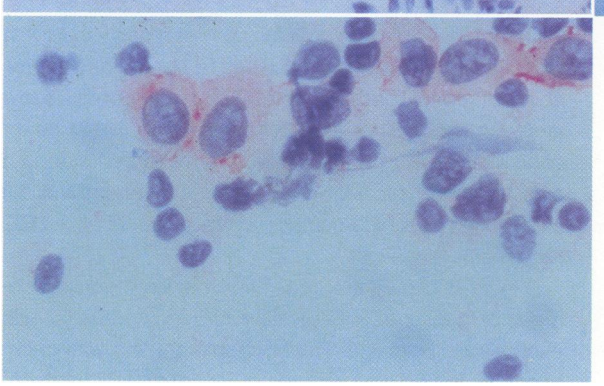

Figure 1. *Nuclear staining by Ki-67 antibody (immunoperoxidase method). All tissues were counterstained with ethyl green.* **A:** *A low-grade breast carcinoma (original magnification ×200).* **B:** *A high-grade breast carcinoma (original magnification ×200).* **C:** *Analysis result in report format of CAS quantitation of the immunohistochemical staining of Ki-67 in breast cancer seen in Figure 1A showed a low percentage of positively stained nuclear area (positive area, 2.552%).* **D:** *CAS analysis of high-grade breast cancer seen in Figure 1B revealing a high percentage of positively stained nuclear area (positive area, 79.58%).* Figure 2. **A:** *Breast cancer cells from the touch imprints case seen in Figure 1B stained for DNA quantitation by the Feulgen method (original magnification ×400).* **B:** *DNA histogram of the touch imprint nuclei. The DNA index was based on 7.18 pg. Main peak: mass, 8.4 pg; DNA index, 1.17.* Figure 7. **A:** *Immunohistochemical staining with an antibody to HER-2/neu protein shows positive cells in the intraductal "comedo" part of the carcinoma. The reaction was carried out using antibody to the HER-2/neu at a dilution of 1:2000 using the APAAP procedures. Counterstain was performed using the Feulgen method.* **B:** *Nuclear staining with Ki-67 antibody revealing a high percentage of positive staining in the intraductal part of the carcinoma (immunoperoxidase method). The counterstain was ethyl green.* **C:** *Touch imprints of cell derived from the carcinoma (A) and stained with an antibody to the HER-2/neu protein by the APAAP procedure. Counterstain was performed using the Feulgen method for quantitation of DNA. The touch imprints revealed positive HER-2/neu and negative HER-2/neu cells (A–C, original magnification ×400).*

tumors were graded according to 1) degree of architectural differentiation as shown by the presence of glandular arrangement of the cells; 2) variation in the size, shape, and staining of the nuclei (ie, nuclear grade), and 3) frequency of mitotic figures. Each of the three parameters was scored from 1 to 3.

The tumors were placed in one of three grades of malignancy: low (grade 1), intermediate (grade 2), and high (grade 3). To obtain a composite picture of a particular section, the points allocated to each of the three histologic factors were added together making a possible total of 3 to 9, the smallest number representing the lowest degree of malignancy. This malignancy scale was reduced to 3 equal grades as shown in Table 1.

Figure 3A shows the correlation among the varying degrees of measured Ki-67 grade, and also shows the mean value of the positive area and the standard deviation for each grade. A trend of increasing Ki-67 positivity with increasing grade can be noted from this figure. In general, low-grade tumors stain the least with Ki-67 and high grade tumors stain the most. However, there is some overlap between the grades.

When the amount of staining by Ki-67 was compared among the different nuclear grades and included the large cells in the duct of comedo carcinoma under grade 3 (see Discussion), a more distinct difference in overall Ki-67 staining amounts among the various nuclear grades (Figure 3B) was revealed.

Quantitative DNA Analysis

Figures 4 and 5 demonstrate DNA Indices compared according to histologic and nuclear grading. When only nuclear grade was considered, a more defined difference among the grades was seen regarding their ploidy status. Nuclear grade 1 had only diploid amounts of DNA, whereas nuclear grades 2 and 3 had aneuploid values as well.

Previous studies regarding the variation of DNA ploidy on normal tissue done on the CAS image analyzer used showed highly accurate and precise values for diploid cells, with a mean value for DNA index of 0.997 ± 0.04

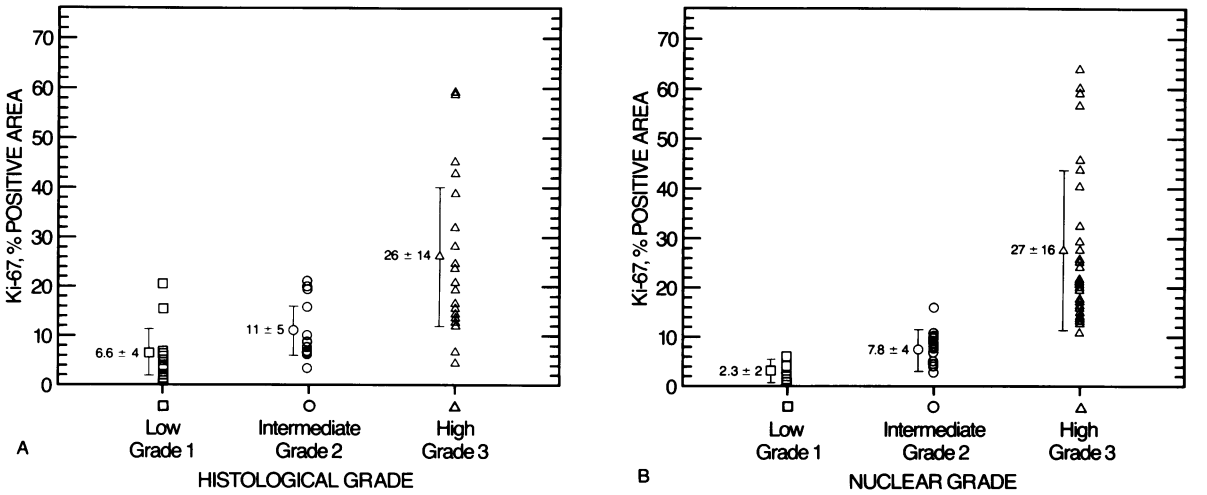

Figure 3. **A:** *Percentage of overall Ki-67 staining of breast carcinomas compared with their histologic grade. Each point represents an individual case. Group mean and standard deviation bars are also depicted.* **B:** *Percentage of overall Ki-67 staining of breast carcinomas compared with their nuclear grade. Each point represents an individual case. Group mean and standard deviation bars are also depicted.*

Table 1. *Malignancy Scale*

Score	Grade	Number of cases
3–5	Grade 1	8
6–7	Grade 2	13
8–9	Grade 3	29

and a 2% or 3% coefficient of variation (CV) of the DNA distribution for each sample.[21]

Estrogen Receptor and Progesterone Receptor Quantitations

Figure 6A and B demonstrates that there was a weak correlation between the amount of estrogen and progesterone receptors and grades as defined by the steroid binding assay (Dextran coated charcoal analysis DCCA, Figure 6A) or by quantitative immunocytochemistry as done on the CAS 200 (Figure 6B) where higher histologic grades tended to have lower estrogen receptors. Figure 6C shows the relationship between the CAS quantitation of the immunohistochemistry and the biochemical values.

Quantitation of immunocytochemical staining for estrogen receptor was performed by the CAS 200. The results indicated 100% specificity and 92% sensitivity when compared with the results using biochemical assay for estrogen receptor. Values greater than or equal to 10 fmol/mg protein were considered positive for estrogen or progesterone receptors. A positive nuclear area equal to or greater than 10% for antibody staining was considered

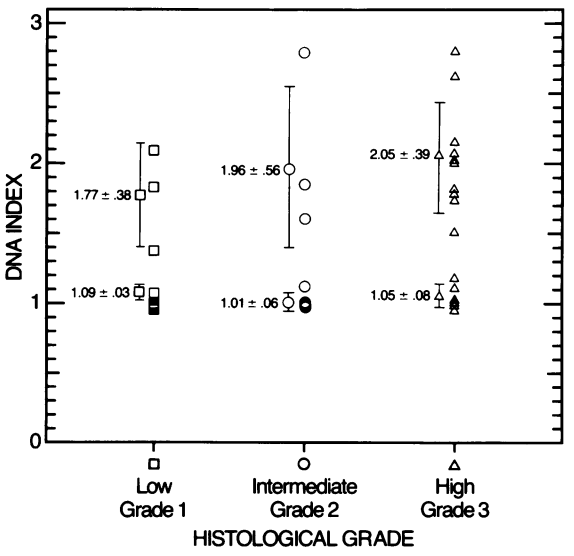

Figure 4. *DNA indices for breast carcinoma compared with histologic grade. DNA indices are defined as the total amount of DNA of the cancer cells in (G_0G^1) divided by the total amount of DNA of resting normal diploid cells. Each point represents an individual case. Group mean and standard deviation bars are also depicted.*

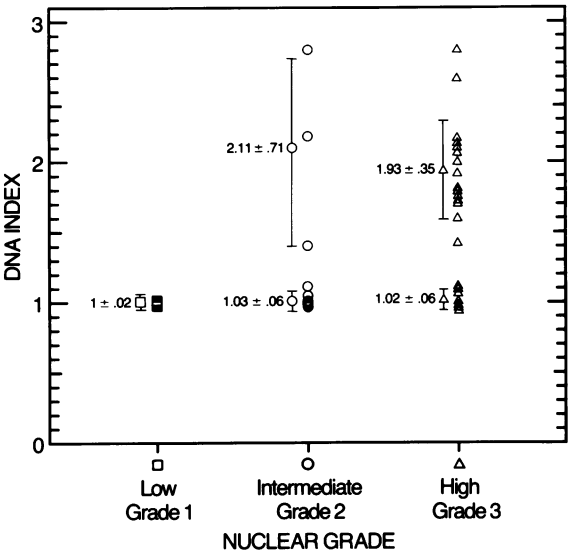

Figure 5. *DNA indices for breast carcinomas according to nuclear grade. Each point represents an individual case. Group mean and standard deviation bars have also been depicted. This figure also includes two cases of intraductal and comedo carcinoma assigned a high nuclear grade.*

positive by immunohistochemistry. (Table 2). This result confirmed past findings.[12,22] A similar correlation with the progesterone receptor quantitation gave 82% sensitivity and 100% specificity.

One grade 3 case, identified as positive for estrogen receptor by DCCA, proved to contain mainly normal tissue that stained positive to estrogen receptor and progesterone receptors by immunohistochemistry. By excluding the normal tissue, using the "window" command, estrogen receptor quantitation was performed on the malignant tissue only and resulted in a negative score by the CAS analysis.

Correlation between estrogen receptor-positive (as defined by values greater than 10 fmol/mg protein obtained by DCCA method) and estrogen receptor-negative tumors and DNA ploidy status did not reveal any correlation. Correlation between estrogen receptor-positive and estrogen receptor-negative tumors and the percentage of positive area for Ki-67 staining showed that estrogen receptor-negative tumors tend to stain higher for Ki-67. This indicated that estrogen receptor-negative tumors have a larger fraction of proliferating cells. This result was previously reported[23] and is depicted in Figure 6D.

Discussion

The results demonstrated a positive correlation between histologic and nuclear grading of breast cancers and the proliferation index as determined by quantitation of the immunohistochemical staining of the antibody Ki-67 by image analysis. The Ki-67 antibody distinguishes all ac-

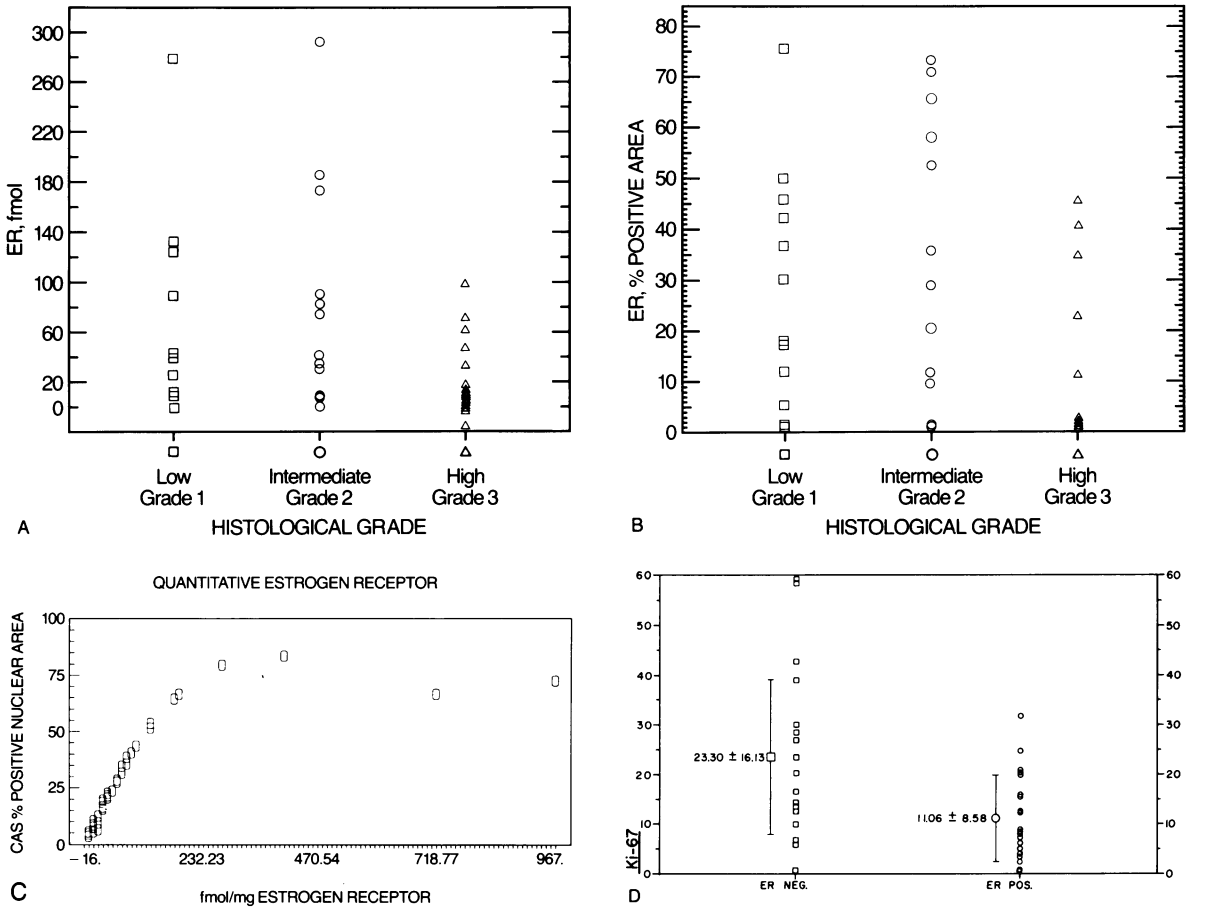

Figure 6. **A:** *Correlation of estrogen receptor amounts (as defined by dextran coated charcoal analysis DCCA) with histologic grade. Each point represents a case.* **B:** *CAS Quantitation of the percentage of overall H222 (estrogen receptor antibody) staining for breast carcinomas and the histologic grade of the carcinomas.* **C:** *A correlation between the CAS quantitation of the percentage positive area for estrogen receptor antibody* versus *DCCA.* **D:** *The percentage overall Ki-67 staining of breast carcinomas compared with their estrogen receptor status. Tumors having more than 10 fmol/mg of protein, as defined by the DCCA method, were considered estrogen receptor positive. Each point represents an individual case. Group mean and standard deviation bars are also depicted.*

tively proliferating cells (G_1, S, and G_2 phases) from resting cells (G_0 phase cells),[13] and the amount of staining reflects the proliferation fraction of the cancer cells.

The use of this antibody with the objective quantitation of image analysis provides a distinct advantage[20] over other methods used to measure proliferation fraction. The counting of mitotic figures in routinely stained paraffin sections is difficult and time consuming. It also has been shown to be subjective in that it depends on the observer.[1,2] In addition, some cases in our study assigned grade 2 by the routine architectural grading system[1] were often nuclear, and structurally grade 3, but were called grade 2 by the pathologist because of lack of mitotic figures. Some of these cases exhibited a very high percentage of staining for Ki-67. One case, which was identified as low-grade carcinoma that stained very high for Ki-67, was sent for re-evaluation of histologic grade and re-evaluated as a very high grade carcinoma (Figure 1B); thus we believe that the Ki-67 staining is a better test for proliferation than evaluating mitotic activity and relying on subjective estimation.

Many recent studies demonstrated that proliferative activity, measured by S-phase or thymidine labeling, in breast cancer is of prognostic value. According to several investigators, tumors with marked mitosis have a high probability for recurrence within 5 years.[1,2] Articles by McDivitt et al[8] and Meyer et al[9] showed proliferation to be useful in predicting the probability of short-term relapses. Another article by Gerdes et al[24] showed a correlation between histologic grade and the mean value of the proliferation index as demonstrated by Ki-67 immunohistochemical staining. However, his study relied on subjective observations and counting of 100 to 200 cells. When such a small population of cells is used, a sample error can occur. Also, depending on the fields counted in this manner, results can be very subjective. A quantitative imaging system can accumulate statistics of the percentage stained area of many fields of the tissue and count at least 1000 cells. This provides a much more objective measurement. Even with subjective grading, however, there is evidence[23] that patients with tumors with high Ki-67 staining have a less favorable prognosis and shorter dis-

Table 2. *Categorization of Cases by Biochemical Analysis and Quantitative Immunocytochemistry*

CAS percentage positive area	Biochemical analysis (fmol/mg protein)	
	≥10	<10
≥10%	26	0
<10%	2	19

Sensitivity, $\frac{TP\ (26)}{TP\ (26) + FN\ (2)} \times 100 = 92\%$; Specificity, $\frac{TN\ (19)}{TN\ (19) + FP\ (0)} \times 100 = 100\%$; TP, true positive; TN, true negative; FP, false positive; FN, false negative.

ease free intervals than patients with tumors staining negative with Ki-67. Furthermore, the number of cells staining with Ki-67 is important; in Bousubar et al's study,[23] those patients with tumors that had more than 20% Ki-67-positive cells had the highest rate of recurrence. Image analysis also addresses the question of tissue heterogeneity, because one can "gate" out nonmalignant cells when calculating the proliferation index of a tumor.

Flow cytometry cannot analyze tissue without destroying the morphology of the tissue. Because breast cancer is a particularly heterogeneous tissue, flow cytometry cannot give accurate results for proliferation index as stromal components and inflammatory components of the tissue will also be measured. Thus it is difficult to obtain a proliferation index for only the cancer cells. This may explain some of the discrepancies found by other groups between the percentage of S phase as determined by flow cytometry and the Ki-67 amount of staining in various breast cancers.[25] The ability to examine the tissue in correlation to its morphology by image analysis provides additional information concerning patterns of proliferation of the tissue. Another method of estimating the proliferation index is the thymidine labeling index (TLI).[8,9] This method relies on incubation of the tissue with tritium-labeled thymidine. Because DNA synthesis is an integral part of the cell cycle, TLI gives an indication of the amount of proliferation taking place in the tumor. However, there is a good correlation between the thymidine labeling index (TLI) and the percentage of cells stained positive by Ki-67.[26] The disadvantages of TLI is the use of radioactive-labeled probes and the slow methodology, which often requires days for completion of a test.

The DNA index values also correlated well with the nuclear and histologic grade, a finding that supports previous work showing that the higher grade malignancies have a higher degree of aneuploidy and low grades are mainly diploid.[3-7]

A recent article by Lash et al[27] recommended that breast cancers be graded according to their nuclear rather than histologic grade.[27,28] This was confirmed in our analysis of the correlation between nuclear grade and Ki-67 staining or DNA index, because an even better correlation occurred than when correlated according to histologic grade (Figures 3 and 4).

Two cases that were graded as histologic grade 1 had infiltrating and intraductal large cells ("comedo"-type carcinoma). The cases that were of low histologic grade exhibited a high proliferation index, as determined by high Ki-67 staining exclusively in the intraductal large cell components, confirming previous studies using thymidine labeling.[29] When nuclear grading was performed we could differentiate between two populations of tumor cells. The infiltrating cells with low nuclear grade (grades 1 or 2) were associated with low proliferation, whereas the high nuclear grade (grade 3) intraductal components had high proliferation (Figure 7B). When quantitative DNA analysis was done on one of these cases, two distinct peaks of DNA index occurred. One near diploid population and a very distinct second population of near tetraploid were present. The near diploid population belonged to the infiltrating cells, whereas the tetraploid population belonged to the large cells in the duct.

All of the large-cell "comedo" type carcinomas stained in the intraductal component with an antibody to the HER-2/neu oncogene product indicating an overexpression of the oncogene (Figure 7A, C). Amplification of the gene was associated with disease behavior and was reported to correlate with the presence of lymph node metastasis and with poor prognosis.[19] The large-cell comedo carcinoma overexpressed this gene, and only the antibody-positive cells exhibited high aneuploid DNA amounts (Figure 7C). The proliferation index was also higher in the areas of the large cells or of high nuclear grade, and correlated with nuclear grade 3 (Figure 7B), an observation reported previously.[30] A recently published article[31] also showed that certain types of patients with comedo carcinoma assigned a high nuclear grade (grade 3), such as those we encountered in our study, had a higher risk of developing local recurrence of the cancer in a short time (26 months). This is very important to explore because ductal carcinoma is an increasingly frequent finding and the treatment of choice is still under study.

Regarding estrogen receptor and progesterone receptor quantitation, we confirmed a correlation found in the past between high proliferation index and low estrogen receptor content.[24,32] Patients that had the highest proliferation index as defined by Ki-67 staining had on the average lower estrogen receptors as defined by the DCCA method (Figure 6D). A correlation between grades and estrogen receptor quantitation showed that high grades contained less estrogen receptor, as defined by immunohistochemistry or DCCA (Figure 6A, B). Estrogen receptor quantitation is an important predictor of response to hormone therapy.[10,11] Immunohistochemical localization of estrogen receptor in breast cancers was also shown to be of prognostic importance for predicting recurrence free intervals.[33]

In summary, these results indicate that it is important to evaluate the DNA activity in the cancer cells as exhibited by the total amount of DNA (ploidy analysis) and the proliferation activity when determining the prognosis for breast cancer. The advantage of using an immunohistochemical marker for proliferation, estrogen and progesterone receptor quantitation, and DNA by the Feulgen method is that these methods can be done on small histologic sections and allow correlation of the morphology with quantitative assessment. In addition, with the advent of earlier diagnosis in breast cancer, smaller tissue specimens, fine needle aspirates, or cytologic specimens can be used. Until now the main disadvantage of immunostaining was that it was only a semiquantitative technique. Image analysis has the ability to quantitatively assess the staining.

Finally, the assessment of these biologic parameters may prove to be essential for the optimal treatment of breast cancer, especially among women with Stage I and Stage II disease.[34] Currently, therapy is selected on the basis of histologic diagnosis, tumor size, tumor stage, and the expression of tumor estrogen receptor. Recent studies suggested that all node-negative breast cancer patients may benefit from some form of adjuvant chemotherapy, at least with regard to disease free survival.[35] However, it is clear that significant heterogeneity exists among patients with regard to the biologic behavior of their tumors, and most features such as DNA ploidy, cell cycle activity, and oncogene expression may have independent prognostic significance. Currently, there is little guidance for treatment decisions involving patients with Stage II breast cancer,[36] and it would be highly desirable to select patients properly for further therapy, thus sparing some patients from the toxicity of chemotherapy.

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