# KI-67 REACTIVITY IN BREAST CARCINOMA ANALYZED BY A COMPUTER-ASSISTED IMAGE SYSTEM: PRELIMINARY RESULTS

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**The proliferative index of 63 breast carcinomas was measured on Ki-67 immunostained frozen tissue sections with a computer-assisted image analysis system. The mean proliferative index in estrogen-positive breast carcinomas was lower than in estrogen-negative carcinomas. An inverse relationship between proliferative index and short-term disease-free survival was noted. (*J Natl Med Assoc.* 1995;87:554-559.)**

**Key words** • breast carcinoma • proliferative activity • image analysis

The proliferative activity of breast cancer has been reported to be prognostically significant in measurements using flow cytometry and thymidine labeling.[1-3] A monoclonal antibody, Ki-67, also has been shown to identify a nuclear antigen associated with cell proliferation.

Gerdes et al[4] produced the mouse monoclonal antibody, Ki-67, which reacts with a human nuclear antigen associated with cell proliferation. The nuclear antigen detected by Ki-67 is expressed in GI, S, G2, and M-phases of continuously cycling cells but is absent in GO or resting cells. This antibody provides a simple methodology to determine the growth fractions of tumors and can be performed on small volumes of tumors.

Ki-67 expression in breast cancer has been reported previously.[4-6] Gerdes et al[7] studied growth fraction by Ki-67 and estrogen receptor status by immunocytochemical and biochemical methods in 76 patients and found an inverse correlation between growth fraction and estrogen receptor status content. Lelle et al[8] correlated Ki-67 studies with histologic grading and lymph node status in 154 carcinomas of the breast and concluded that lymph node-positive breast cancers had a significantly higher growth fraction than lymph node-negative cases. McGurrin et al,[9] in 33 breast cancer cases, found a high number of Ki-67 positive cells associated with high mitotic rate, high nuclear grade, and high histologic grade, but did not find a good correlation between Ki-67 labeling rate and estrogen receptor status levels.

Walker and Camplejohn[10] studied 95 breast carcinomas with Ki-67 and compared it with histologic grade, DNA index, and S-phase content. The DNA aneuploid tumors had higher frequency of nuclear staining with Ki-67 than DNA diploid tumors; however, the extent of nuclear staining did not correlate with the degree of differentiation. Bouzubar et al[11] reported that Ki-67 staining was found frequently in poorly differentiated tumors with high mitotic activity, but staining was independent of tumor size, lymph node status, and estrogen receptor status expression. However, early recurrence of breast cancer was associated with high levels of Ki-67 staining. All of these studies used either tedious manual counting or estimations and therefore are subject to interobserver variations. The objective of this study was to measure Ki-67 expression on frozen

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tissue sections of primary breast carcinoma with a computer-assisted image analysis system (CAS-100) and to correlate the Ki-67 reactivity with steroid receptor status, pathological stage, and short-term survival.

## MATERIALS AND METHODS

Specimens were collected over a 12-month period from consecutive patients undergoing surgery to treat breast cancer. Tissues were frozen and stored.

The estrogen receptor immunocytochemical assay for determining estrogen receptor status was performed using a commercially available kit (ERICA, Abbott Laboratories, Diagnostics Division, North Chicago, Illinois). Frozen tissue sections were studied according to the manufacturer's guidelines, except that methyl green was used instead of hematoxylin counter stain.

Ki-67 monoclonal antibody, along with the secondary and tertiary antibodies required for the study, were obtained commercially (Dako Corporation, Santa Barbara, California). Frozen tissues were cut at 4 μm to 6 μm, air dried, fixed in cold acetone, and rinsed in phosphate-buffered saline before being primarily treated with diluted Ki-67 (1:25). Slides were then secondarily treated with P260 (Perox-Conjug Rabbit Anti-Mouse Ig Dil 1:25) before the final treatment with P217 (Perox Conjug Swine Anti-Rabbit Ig Dil 1:100). A chromagen stain was applied before the sections were counterstained with methyl green. A control in which the primary antibody was replaced by phosphate-buffered saline was run for each case.

Semiquantitation of Ki-67 and estrogen receptor was performed using image analysis. The CAS-100 system (CAS Inc, Chicago, Illinois), which functions with a high-speed, dual-staining, two-color "nuclear mask" imaging technique, as described by Bacus et al,[12] was used. For each case, the corresponding negative control section was used to establish the antibody threshold.

The system detects the percentage of nuclear area staining positive with the antibody by first measuring the entire nuclear area in a chosen microscopic field with a red filter (650/10 nm band pass) in place and then measuring only the positive stained nuclear area in the same field when a green filter (540/10 nm band pass) is used.

Significant heterogeneity was noted with respect to the nuclear staining for estrogen receptor protein (Figure 1) and Ki-67 (Figure 2). For estrogen receptor protein, an average positive nuclear area was obtained by counting high and low positive fields. The nuclear area measured for estrogen receptor protein varied from 8084 $\mu m^2$ to 14 700 $\mu m^2$ and represented 2 to 5 field counts. For Ki-67, only the high positive fields were counted. The nuclear area measured for Ki-67 varied from 8054 $\mu m^2$ to 18 341 $\mu m^2$ and represented 2 to 6 field counts. The percentage of nuclear area staining positive for Ki-67 was defined as the proliferative index.

Biochemical quantitation of estrogen and progesterone receptor levels was done according to the standard dextran-coated charcoal method.[13] Values less than 5 fmol/mg cytosol protein were considered negative for both estrogen and progesterone receptors.

For inclusion in the study, sufficient tissue to perform biochemical and immunocytochemical studies was required, and the biochemical and immunocytochemical results for estrogen receptor protein had to be in agreement. These criteria were met in 63 patients, and complete follow-up data were available on these patients from the tumor registry for a period covering 24 to 42 months.

## RESULTS

Forty-seven breast carcinomas were estrogen receptor-positive, and 16 were estrogen receptor-negative. The proliferative index showed wide variation in the two groups. Mean Ki-67 was 10.5% ± 17% (range: 0.23 % to 78.5%) for estrogen receptor-positive patients and 41.3% ± 26 (range: 12.3% to 86%) for estrogen receptor negative patients. A negative correlation between estrogen receptor and proliferative activity was found. This inverse correlation proved to be statistically significant ($P = .0003$). However, there was no significant quantitative correlation between Ki-67 and estrogen receptor measured either biochemically or immunocytochemically.

The estrogen receptor-positive breast cancers were stratified into three proliferative index groups (Table). The progesterone receptor status and lymph node status was known in 44 of these patients. The incidence of progesterone receptor positivity was 73%, 50%, and 41%, respectively, in the three groups. Since the expression of progesterone receptor is a function of estrogen receptor protein, these results suggest an inverse relationship between proliferative index and functional estrogen receptor status.

Follow-up data were available on all of the patients. All stage I and II patients with low and intermediate proliferative index were free of disease at 24 to 42 months postoperatively, while 28% of patients with high proliferative index had developed recurrent dis-

**TABLE. CORRELATION OF Ki-67 ACTIVITY WITH OTHER PROGNOSTIC PARAMETERS**

| | ER-Positive | | | |
|---|---|---|---|---|
| | Low Ki-67 (<2.5%) | Intermediate Ki-67 (2.5%-6%) | High Ki-67 (>6%) | ER-Negative Ki-67>12% |
| No. of patients | 15 | 13 | 19 | 16 |
| Progesterone receptor positive (%) | 73 | 50 | 41 | 0 |
| Nodal metastases (%) | 31 | 50 | 63 | 50 |
| Disease free survival (%) | 100 | 100 | 72 | 50 |

Abbreviations: ER = estrogen receptor.

ease. Fifty percent of the patients with proliferative index>12% had nodal metastases at the time of treatment, and 50% have since either developed recurrent disease or died.

## DISCUSSION

This study demonstrated that in a relatively small number of patients, semiquantitative immunocytochemical Ki-67 measurements determined with an image analysis system can be combined with estrogen receptor data obtained on tumors to describe subgroups both of patients who appear to be at higher risks for having lymph node metastases and patients who are likely to show a high risk for early distant relapse following surgical treatment. The data show that 50% of patients with estrogen receptor-negative tumors with Ki-67 values >12% will show distant relapse within 24 to 42 months postoperatively. In addition, among estrogen receptor-positive patients who may be regarded as a more favorable prognostic group, nearly two thirds of these patients will already have positive nodes at the time of tumor detection and treatment, and 28% of the patients who have Ki-67 values >6% will show evidence of early distant relapse. Ki-67 expression as a marker for proliferative activity appears to define a critical mass of dividing cells within tumors that correlates well with the clinical course of the patients. The data suggest that a proliferative index ≥6% is a significant prognostic factor. This finding is of particular significance in light of the report by Sigurdsson et al[14] that an S-phase fraction ≤7% was the most important independent prognostic factor predicting disease-free and overall survival in node-negative breast cancer. Our preliminary data agree with their finding, and the Table suggests that proliferative index may be more important than nodal status in predicting distant disease failure following surgical extirpation.

In this study, both estrogen receptor negativity, which was uniformly associated with Ki-67 values >12%, and estrogen receptor-positive tumors with Ki-67 values >6% were associated with poor outcomes (ie, 50% and 28% distant failure rates, respectively). In these two situations, some form of adjuvant treatment may be beneficial in at least 50% of the estrogen receptor-negative patients and nearly 30% of the estrogen receptor-positive patients.

The variable course of carcinoma of the breast has stimulated interest in identifying factors that are predictive of survival among afflicted patients, and attention has focused on the degree of malignancy, as suggested by morphological criteria that include tumor grade,[15] the number of mitoses,[16] and the presence or absence of axillary lymph node metastases, as well as the number of positive lymph nodes.[16,17] More recently, the prognostic significance of estrogen receptor and progesterone receptor content of the primary tumor has been appreciated.[18-20] Patients with estrogen receptor-positive breast cancers generally will have a longer disease-free interval and longer survival than those patients whose carcinoma lack these receptors.[21-24]

Currently, more interest is emerging in studying prognostic markers that will assess tumor aggressiveness.[25] Evidence from several studies demonstrated that the proliferative activity of breast carcinomas is of paramount prognostic significance,[26-28] and proliferative indices probably will constitute part of routine histopathologic evaluation of cancer of the breast and other sites in the near future.

Although direct clinical evaluation of growth rate is not possible, several kinetic parameters have been evaluated as prognostic indicators. The thymidine labeling index has been used for assessing proliferative activity,[29] and patients with high thymidine labeling indices have shorter relapse-free survival and lower survival rates than patients with low thymidine labeling indices.[26-28,30-32] Thymidine labeling is, however, both cumbersome and expensive; it requires tritiated thymidine for incorporation into dividing cells and subsequent

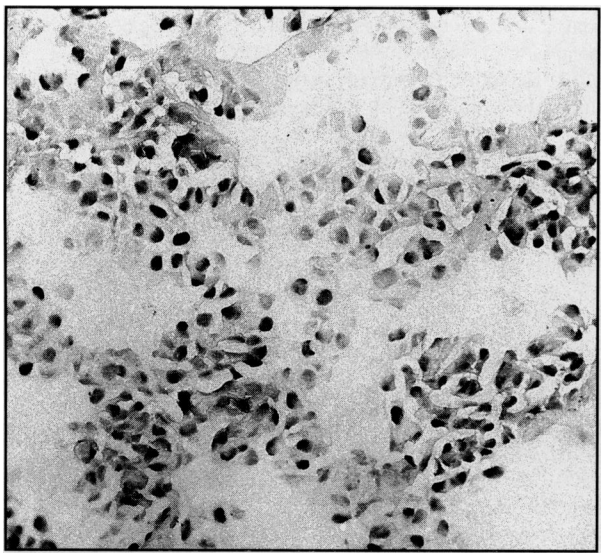

**Figure 1. Photomicrograph of breast carcinoma stained with anti-estrogen-receptor protein demonstrating the variation in the extent and intensity of nuclear staining.**

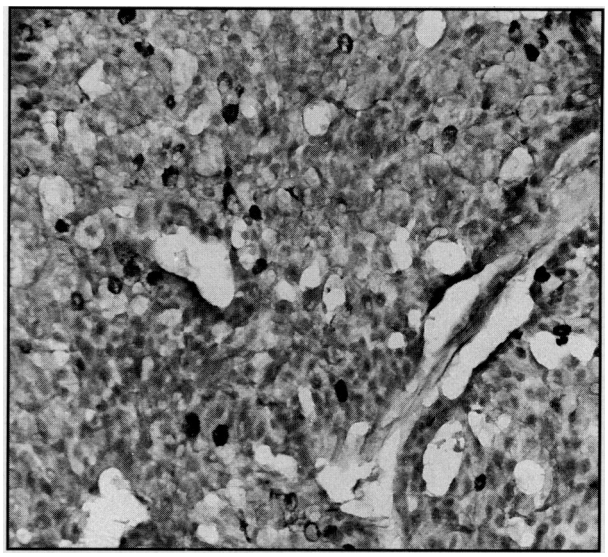

**Figure 2. Photomicrograph of breast carcinoma stained with Ki-67 antibody demonstrating focal nuclear staining.**

autoradiography for qualitative and quantitative determinations. It also requires the use of fresh tissues. The requirements and cost will restrict the number of laboratories undertaking this as routine.

Flow cytometry is a more rapid method for measuring cell cycle kinetics than thymidine labeling and also can be used to evaluate large numbers of cells for ploidy, phenotypic markers, and cytokinetic markers.[33,34] The S-phase fractions of breast carcinoma, as determined by flow cytometric methods, correlate well with the results obtained by thymidine labeling.[35,36] Moreover, flow cytometric measurements of DNA ploidy and S-phase fractions performed on frozen breast cancer specimens have been reported to be potentially important predictors of disease-free and overall survival in patients with stage I breast carcinoma.[37] Another advantage of flow cytometry is that it can be used to study formalin-fixed and paraffin-embedded tissues to determine the S-phase content of breast cancers.[38] However, there are disadvantages of flow cytometric analysis, including the need for a single cell suspension, loss of tissue architecture, and complicated and expensive methodology and equipment.

We have taken advantage of a computer-assisted image analysis system. The advantages of such a system have been documented previously.[39] In a similar study of 200 breast carcinomas, Charpin et al[40] measured Ki-67 expression using a different computerized image analysis system; they found Ki-67 to increase with tumor grade, presence of vascular invasion, nodal metastases, and the absence of estrogen receptor and progesterone receptor in the tumors. However, comparison of Ki-67 with disease-free survival was not reported by the authors. In another study,[41] Ki-67 studies were reported on invasive and noninvasive breast cancers, and it was concluded that an increase in growth activity does not accompany the transition from intraductal to invasive cancer, nor did a change in growth fraction accompany progression of mammary cancer from the primary to regional metastatic site. This article raises interesting issues regarding the biology of progressing breast cancer, but provides no data regarding survival or recurrence.

## CONCLUSION

Our study shows a wide range of proliferative index in the estrogen receptor-positive and estrogen receptor-negative groups. The mean proliferative index in the two groups is statistically significant. Moreover, there is an inverse relationship between proliferative index and functional estrogen receptor status demonstrated by the progesterone receptor status as well as proliferative index and short-term disease-free survival. The small number of cases in each group preclude more meaningful statistical analyses, and further studies are needed to confirm these findings in a larger number of patients to determine whether Ki-67 determination (which can be easily made in almost any community-hospital pathol-

ogy laboratory using image analysis) can be used to accurately predict the course of breast cancer in subgroups of patients. If so, Ki-67 measurements could become an important prognostic marker.

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