Ki-67 Expression in Breast Carcinoma

Its Association with Grading Systems, Clinical Parameters, and Other Prognostic Factors—A Surrogate Marker?

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BACKGROUND. The number of mitoses and, thus, the proliferative capacity of a tumor is one of the most crucial variables for tumor grading. The Ki-67 nuclear antigen may be considered as an alternative to mitotic counts in grading schemes and as a single parameter that can be used in fine-needle aspirates and small biopsies.

METHODS. Immunohistochemistry using the anti-Ki-67 antibody MIB-1 was performed on 434 breast carcinoma specimens from the International Breast Cancer Study Group (formerly Ludwig) Trial V. Three groups based on Ki-67 percent were used to replace the mitotic counts component in the Nottingham grade (NHG) to produce the Nottingham/Ki-67 grade (NKG) and to assess Ki-67 as a single parameter.

RESULTS. In both the lymph node positive subgroup and the lymph node negative subgroup, the NKG and Ki-67 group was correlated significantly with Bloom–Richardson grade (BRG), NHG, and Nottingham type. Tumor size in the lymph node negative cohort and estrogen receptor status, progesterone receptor status, and c-*erb*B-2 expression in the lymph node positive cohort also were correlated significantly with NKG. Ki-67 percentage was correlated significantly with c-*erb*B-2 expression in the lymph node positive cohort only. NKG was similar to BRG and NHG when it was evaluated for prognostic significance. Patients with higher categoric Ki-67 percentages had worse overall and disease free survival in all groups except for the untreated, lymph node negative group.

CONCLUSIONS. Ki-67 detection represents a valuable tool and is a good objective substitute for mitotic counts when used in a grading system. When it is used alone, Ki-67 detection provides valuable information, although it is necessary to combine this with other parameters in the study of core biopsies and fine-needle aspirates.
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Because human breast carcinomas are known to exhibit a broad spectrum of clinical behavior, many attempts have been made in the past to establish reliable and reproducible prognostic parameters. Most commonly, morphologic criteria were used for this purpose, such as the widely accepted tumor grading method according to Bloom–Richardson.[1] Histologic grade provides an overview of a number of molecular events that are reflected in morphology.[1–6] Three major elements are included in histologic grading: nuclear morphology (nuclear pleomorphism), differentiation (tubule formation), and proliferation (mitotic frequency). Although methods for histologic grading in patients with breast carcinoma were described first over 100 years ago, a lack of precision in the assessment of all three of the factors above mentioned resulted in a considerable element of subjectivity and lack of reproducibility. Attempts to establish more objective criteria of tumor differentiation and proliferation were made. One of those attempts resulted in the revised Nottingham grading method,[7] which involves the semiquantitative evaluation of the percentage of tubule formation, the degree of nuclear pleomorphism, and mitotic counting in a defined field area at a specific magnification. A numeric scoring system is used, and the overall grade is derived from a summation of individual scores for the three variables. This method has now been accepted in the United Kingdom as the national standard for grading and has been proposed as a scheme to be adopted in Europe and the United States.[8] Tumor type adds independent prognostic information, and tumors may be grouped into four types based on prognosis.[9]

Just at the time when pathologists are beginning to agree on criteria for the histologic assessment of primary tumors, the clinical management of patients with breast carcinoma is undergoing major changes. Many centers in Europe and the United States currently are carrying out trials of primary chemotherapy,[10–12] and the results indicate that this enables an improved cosmesis due to tumor down-staging,[13] with no apparent reduction in disease free survival (DFS).[10] Data indicate that examination of the sentinel lymph node is an important alternative to axillary dissection.[14,15] Thus, although tumor grading,[7,8] vascular invasion,[16] tumor size,[17,18] and lymph node involvement are accepted as the most important prognostic factors, the advent of primary chemotherapy[10–12] after diagnosis on a core biopsy is forcing a reconsideration of how the pathologist can aid the clinician based on the small samples, in which grading, size, and lymph node involvement are no longer parameters that can be assessed.[6,19] It is essential that studies are carried out rapidly that assess the value of markers that can be applied to small samples. Proliferation is an obvious parameter for assessment.

For many years, cellular proliferation has been measured by the numeration of mitotic figures in tissue sections prepared for routine histomorphologic studies. This method includes the inherent inability to distinguish very condensed mitotic figures from pycnotic nuclei. Other techniques have been reported that permit the evaluation of proliferative activity and growth fraction of tumors.[20,21] Monoclonal antibodies to different proliferating cell nuclear antigens have been described,[22,23] including Ki-67.[24,25] Ki-67 antigen is a nonhistone protein that was described in 1983 and is expressed in cycling cells in G1 phase, S phase, G2 phase, and during mitosis, but not in G0 phase, allowing estimation of the growth fraction after a relatively small number of cells have been counted. Thus, in a true-cut biopsy or fine-needle aspirate (FNA), information can be obtained about the growth fraction,[26] although insufficient cells would be available to carry out a routine count. Most studies have been performed on frozen sections; however, the anti-Ki-67, MIB-1 antibody also is applicable on routinely fixed, paraffin embedded tissues after microwave pretreatment.[27] Published data have shown a good correlation of the immunoreactivity with DNA cytometry and bromodeoxyuridine.[28–31] MIB-1 appears to be superior to other antibodies for assessing cycling cells on routinely fixed and processed material, not only because of the simplicity of the technique, but because good correlation with Ki-67 expression on frozen material has also been reported.[32] Many studies have assessed the association between Ki-67 immunoreactivity with other prognostic factors in breast carcinomas. However, few studies have assessed the percentage of tu-

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mor cells that express the Ki-67 antigen from patients with long-term follow-up because of the difficulty in obtaining sufficient archival frozen tissue.

In this study, we assessed the information gained from carrying out a Ki-67 assessment relative to other parameters that we studied previously in a well-defined data set available to us through the International Breast Cancer Study Group (IBCSG).[33–39] The objectives of the current study were 1) to analyze Ki-67 as a substitute for mitotic counting in the Nottingham grade (NHG) system to produce a Nottingham/Ki-67 grade (NKG), thus giving an indication of the value of this measure of growth fraction compared with mitotic counts; and 2) to assess the value of Ki-67 as an individual marker of prognosis and treatment response of patients with breast carcinoma against grade and other factors in the data base.

MATERIALS AND METHODS

Trial Design

The material used in this study was from a cohort of patients who were enrolled into the (IBCSG) Ludwig Trial V.[33–35] From 1981 to 1985, the IBCSG (formerly the Ludwig Breast Cancer Study Group) conducted a randomized clinical study to assess the effect of early commencement of adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy in patients with lymph node negative and lymph node positive breast carcinoma. Of 2504 eligible patients, 1275 patients with lymph node negative breast carcinoma were randomized to receive either a single cycle of perioperative chemotherapy (PeCT) or no adjuvant chemotherapy. Patients with lymph node positive disease (n = 1229 patients) were assigned to one of three treatments: PeCT, a conventionally timed chemotherapy regimen, or both. The PeCT regimen was a combination of intravenous CMF given on Days 1 and 8 and commencing within 36 hours of mastectomy. The conventionally timed chemotherapy regimen was CMF (cyclophosphamide was given orally) plus low-dose, continuous prednisone starting 25–36 days after mastectomy and continuing for 6 cycles every 28 days. Postmenopausal patients with lymph node positive breast carcinoma who were assigned to receive the conventionally timed chemotherapy regimen also received tamoxifen for 6 months. The trial and the clinical results have been described in detail elsewhere.[33–35]

Study Cohort

In 1993, the IBCSG established a tissue bank of tumor blocks from a subset of Trial V patients. These pathologic parameters then were merged with the clinical data base that was begun in 1981 and is updated annually for survival and disease status.

The Ki-67 study samples consisted of formalin fixed, paraffin embedded tissue sections that were available from 494 patients from 4 IBCSG member institutions (Ljubljana, Madrid, Perth, and Brescia). A total of 434 patients with ascertainable Ki-67 percentage, Bloom–Richardson grade (BRG), NHG, and Nottingham type were available for final statistical analysis. BRG, vessel invasion, and pathologic tumor size were available from previous central pathology review. The Nottingham grading was carried out by the Nottingham Group, as described previously.[39] Sections were excluded on the basis of absence of invasive tumor in the material available and lack of any positive staining in the sections. The latter was used to exclude material, because it was possible that the absence of staining was a reflection of the fixation of the material. Thus, some positive nuclei were required for an internal quality control for the section.

These 434 patients reflected the overall trial population as closely as possible with respect to menopausal status, treatment, tumor size, and estrogen receptor (ER) status. Of 434 patients in the study, 188 patients had no histologic evidence of disease in the lymph nodes, and 246 patients had metastatic disease in the axilla. Sixty-three patients with lymph node negative disease were randomized to receive no adjuvant treatment, and 125 patients with lymph node negative disease were randomized to receive PeCT. Among the patients with lymph node positive disease, 89 patients were randomized to receive PeCT, and 157 were randomized to receive prolonged treatment (6 or 7 cycles). The median follow-up for the Ki-67 study group was 13.5 years at the time of this analysis.

Immunohistochemical Labeling and Tissue Evaluation

Staining was performed with the MIB-1 monoclonal antibody. The antibody was obtained from Immunotech International and was supplied to them by The Binding Site Limited (Institute of Research and Development, Birmingham, United Kingdom). Staining was performed using the indirect immunoperoxidase avidin-biotin-complex (ABC) technique. Paraffin embedded tissue sections, 3 μM thick, were deparaffinized in xylene, rehydrated through graded alcohols, and blocked for endogenous peroxidase activity with hydrogen peroxidase. They were then microwaved on full power for 10 minutes in 1 L of citrate buffer. The microwave used was a Proline Micro Chef (model ST44; 950 Watts). After cooling by running under cold water and blocking for nonspecific activity with rabbit serum, the sections were incubated with the monoclonal antibody MIB-1 at a 1:50 dilution for 2 hours.

The detection system used a biotinylated rabbit anti-mouse polyclonal serum (catalog no. E0354; Dako, Glostrup, Denmark) at 1:200 dilution, followed by strepavidin-ABC/horseradish peroxidase (catalog no. K0377; Dako) according to the manufacturer's instructions.

The slides were examined and scored by one observer (H.T.) who was blinded to both clinical data and pathologic data. Ki-67 expression was quantified using a visual grading system. The percentage of Ki-67 positive cells among the total number of total counted neoplastic cells was determined at a magnification of $\times$ 400 using an eye-piece graticule and counting 10 randomly selected fields. All tumor cells were counted, and the number of nuclei counted ranged from 638 to 7871.

Statistical Analysis

Three ordered categories of Ki-67 were created based on the percentage of Ki-67 positive nuclei: $\leq$ 9.5%, $>$ 9.5% and $\leq$ 15.5%, and $>$ 15.5%. The selection of these cut-off values was based on the proportion of tumors that scored 1 point, 2 points, and 3 points of mitotic activity using the criteria of Elston and Ellis.[7] The agreement between the Ki-67 categories and the mitotic count scores was high, with a K interrater agreement score[40] of 74%, in which a perfect match was considered perfect agreement, and a difference between adjacent categories was considered partial agreement. The observed agreement between these two measures of cell proliferation was significantly higher than chance alone would indicate ($P < 0.0001$).

These ordered categories of Ki-67 were then scored 1–3, with increasing scores indicating higher Ki-67 percentages and were used in place of the mitotic counts feature in the NHG system. The resulting adjusted Nottingham grade, hereafter referred to as Nottingham/Ki-67 grade (NKG), was then studied separately for the lymph node negative subgroup ($n = 188$ patients) and the lymph node positive subgroup ($n = 246$ patients). Pearson chi-square statistics were used to study associations between patient characteristics, the newly created Ki-67 percent categories, and the NKG according to lymph node status.[41] The K statistic was used to correlate the NKG with existing BRG and NHG.

The Nottingham type categorizes tumors into four groups based on histology and prognosis.[9] Tumors that indicate an excellent prognosis (80% 10-year survival rate) include tubular, invasive cribiform, mucinous, and tubulolobular carcinomas. Tumors that indicate a good prognosis (60–80% 10-year survival rate) include tubular mixed, mixed ductal with special type, and alveolar lobular carcinomas. Invasive papillary, classical lobular, medullary, and atypical medullary carcinomas overall indicate a moderate prognosis (50–60% 10-year survival rate); and ductal (no special type), solid lobular, mixed ductal with lobular, and lobular mixed carcinomas indicate a poor prognosis ($<$ 50% 10-year survival rate).[9]

DFS was defined as the time from randomization to recurrence, metastasis, appearance of a second primary tumor, or death from any cause, whichever occurred first. Overall survival (OS) was defined as the time from randomization to death from any cause. Univariate and multivariate hazard ratios (HRs) corresponding to DFS and OS, 95% confidence intervals (95%CIs) for these HRs, and associated P values were calculated using Cox proportional hazards regression models according to lymph node status group.[42] Kaplan–Meier estimates for DFS and OS, stratified by lymph node status and NKG, were used in plots and in 10-year survival estimates.[43]

RESULTS

Ki-67 As an Alternative to Mitotic Count in the NHG System

Significant associations between the NKG, NHG, and BRG systems were observed ($P < 0.001$) in both the lymph node positive cohort and the lymph node negative cohort (Table 1). Agreement between the three systems was high, with K statistics of 88% between NKG and NHG and 80% between NKG and BRG, in which partial agreement between grading systems was credited for patients assigned to adjacent grades in the different systems. The observed agreement between any two grading systems was significantly higher than chance alone would indicate. Overall, the NKG categorized 18%, 50%, and 32% of patients with lymph node negative disease and 11%, 55%, and 33% of patients with lymph node positive disease as Grades 1, 2, and 3, respectively. Among patients with lymph node negative disease, the classification of tumors as Grade 1 by the NHG and NKG systems included more lesions of BRG 2 and BRG 3 (overall, 33 tumors and 36 tumors were classified as NKG 1 and NHG 1, respectively, compared with 21 tumors that were classified as BRG 1). The number of tumors classified as Grade 1 among patients with lymph node positive disease was similar: NKG 1, 28 tumors (11%); NHG 1, 26 tumors (10.5%); and BRG 1, 26 tumors (10.5%). Conversely, the BRG 3 category included a greater proportion of tumors compared with the NKG 3 and NHG 3 categories among both patients with lymph node negative disease and patients with lymph node positive disease.

Associations between the NKG and patient characteristics were assessed for patients with lymph node negative disease and patients with lymph node posi-

TABLE 1
Correlations between Nottingham/Ki-67 Grade and Other Patient Characteristics According to Lymph Node Status

Characteristic	Nottingham/Ki-67 grade (%)			*P* value (chi-square test)[a]
	Grade 1	Grade 2	Grade 3	
Lymph node negative	33 (18)	94 (50)	61 (32)	—
Bloom–Richardson grade				
1	14 (67)	6 (29)	1 (5)	—
2	17 (20)	55 (65)	13 (15)	< 0.001 (< 0.001, < 0.001)
3	2 (2)	33 (40)	47 (57)	—
Nottingham grade				
1	33 (92)	3 (8)	0 (0)	—
2	0 (0)	69 (78)	19 (22)	< 0.001 (< 0.001, < 0.001)
3	0 (0)	22 (34)	42 (66)	—
Pathologic tumor size (cm)				
≤ 2	22 (26)	40 (48)	22 (26)	—
> 2	10 (10)	50 (51)	39 (39)	0.02 (0.001, 0.001)
Unknown	1 (20)	4 (80)	0 (0)	—
ER status				
Negative (0–9 fmol)	15 (21)	32 (44)	25 (35)	—
Positive (≥ 10 fmol)	15 (16)	51 (54)	29 (31)	0.79 (0.08, 0.21)
Unknown	3 (14)	11 (52)	7 (33)	—
PgR status				
Negative (0–9 fmol)	12 (14)	41 (48)	32 (38)	—
Positive (≥ 10 fmol)	12 (20)	30 (50)	18 (30)	0.62 (0.02, 0.005)
Unknown	9 (21)	23 (53)	11 (26)	—
Vessel invasion by primary tumor				
No	20 (21)	46 (48)	30 (31)	0.30 (0.23, 0.04)
Yes	12 (13)	48 (54)	29 (33)	—
Unknown	1 (33)	0 (0)	2 (67)	—
c-*erb*B-2				
Negative	33 (20)	84 (50)	50 (30)	0.07 (0.08, 0.11)
Positive	0 (0)	10 (53)	9 (47)	—
Thymidylate synthase				
Low	7 (14)	26 (51)	18 (35)	—
High	26 (19)	67 (49)	43 (32)	0.77 (0.60, 0.74)
Unknown	0 (0)	1 (100)	0 (0)	—
Nottingham type by prognosis category				
1. Excellent	4 (100)	0 (0)	0 (0)	—
2. Good	23 (66)	10 (29)	2 (6)	< 0.001 (< 0.001, < 0.001)
3. Average	0 (0)	15 (83)	3 (17)	—
4. Relatively poor	6 (5)	69 (53)	56 (43)	—
Lymph node positive	28 (11)	136 (55)	82 (33)	—
Bloom–Richardson grade				
1	15 (58)	11 (42)	0 (0)	—
2	12 (12)	70 (69)	20 (20)	< 0.001 (< 0.001, < 0.001)
3	1 (1)	55 (47)	62 (53)	—
Nottingham grade				
1	25 (96)	1 (4)	0 (0)	—
2	3 (2)	107 (79)	25 (19)	< 0.001 (< 0.001, < 0.001)
3	0 (0)	28 (33)	57 (67)	—
Pathologic tumor size (cm)				
≤ 2	15 (19)	41 (53)	22 (28)	—
> 2	12 (7)	93 (57)	57 (35)	0.07 (0.03, 0.02)
Unknown	1 (17)	2 (33)	3 (50)	—
ER status				
Negative (0–9 fmol)	6 (7)	40 (47)	40 (47)	—
Positive (≥ 10 fmol)	21 (16)	75 (59)	32 (25)	0.004 (0.06, 0.04)
Unknown	1 (3)	21 (66)	10 (31)	—
PgR status				
Negative (0–9 fmol)	11 (10)	50 (48)	44 (42)	—
Positive (≥ 10 fmol)	16 (17)	53 (58)	23 (25)	0.009 (0.09, 0.04)
Unknown	1 (2)	33 (67)	15 (31)	—
Vessel invasion by primary tumor				
No	9 (15)	34 (55)	19 (31)	0.64 (0.24, 0.02)
Yes	19 (10)	102 (55)	63 (34)	—
c-*erb*B-2				
Negative	27 (14)	117 (59)	55 (28)	< 0.001 (0.002, < 0.001)
Positive	1 (2)	19 (40)	27 (57)	—
Thymidylate synthase				
Low	8 (12)	34 (52)	23 (35)	—
High	20 (11)	99 (56)	58 (33)	0.90 (0.89, 0.33)
Unknown	0 (0)	3 (75)	1 (25)	—
Nottingham type by prognosis category				
1. Excellent	6 (86)	0 (0)	1 (14)	—
2. Good	16 (36)	28 (62)	1 (2)	< 0.001 (< 0.001, < 0.001)
3. Average	0 (0)	15 (83)	3 (17)	—
4. Relatively poor	6 (3)	93 (53)	77 (44)	—

ER: estrogen receptor; PgR: progesterone receptor.

[a] Three *P* values are presented in the following order: Nottingham/Ki-67 grade *P* value (Nottingham grade *P* value, Bloom–Richardson grade *P* value).

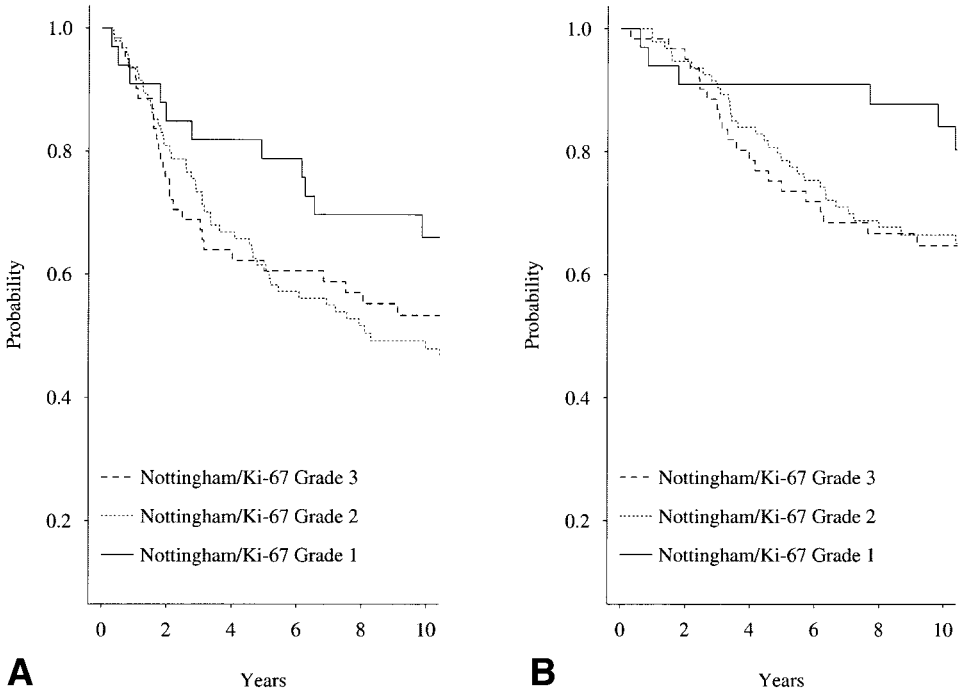

FIGURE 1. Disease free survival (A) and overall survival (B) for the lymph node negative cohort according to Nottingham/Ki-67 Grade.

tive disease (Table 1). The chi-square *P* values are shown for the associations of NKG, NHG, and BRG with the various patient characteristics. Among the patients with lymph node negative disease, characteristics that correlated significantly with NKG were BRG, NHG, pathologic tumor size, and Nottingham type. Higher NKG tended to be associated with high BRG, high NHG, tumors > 2 cm in greatest dimension, and tumors that indicated a poor prognosis. A marginally significant association also was observed between c-*erb*B-2 and NKG, with higher grades associated more often with c-*erb*B-2 overexpression. Some characteristics that previously were associated significantly with or marginally with NHG in patients with lymph node negative disease, such as ER status and progesterone receptor (PgR) status, were not correlated with NKG. In addition, some characteristics that previously were associated significantly with BRG in these patients, such as PgR status and vessel invasion, were not correlated significantly with NKG.

Among patients with lymph node positive disease, BRG, NHG, and Nottingham type also were correlated significantly with NKG. In addition, patients with negative ER status, negative PgR status, and c-*erb*B-2 overexpressed tumors were much more likely to have high NKG. A marginal association was observed between NKG and pathologic tumor size, with larger tumors tending to have higher grades. The association between grade and tumor size was slightly stronger using the NHG and BRG systems compared with the NKG system.

The prognostic significance of NKG was studied in terms of DFS and OS (Figs. 1, 2) in both the lymph node negative group and the lymph node positive group. Cox proportional hazards regression models with a single covariate for NKG were used to estimate HRs for each treatment group, with a HR > 1.0 indicating an increase in risk for each increment in histologic grade (Table 2). Patients with higher grade tumors had significantly worse OS for both the lymph node negative group and the lymph node positive group, with the exception of patients with lymph node negative disease who were on the no PeCT treatment regimen. In this group, no significant differences in OS could be detected for the different levels of NKG. An increase in NKG for patients who had lymph node positive disease had a more detrimental effect on OS compared with patients who had lymph node negative disease. These patterns of association also were observed for DFS, but the statistical significance was diminished except among patients with lymph node positive disease who were on prolonged treatment.

The various grading systems performed similarly in terms of prognostic value for DFS and OS for the analyses stratified by treatment regimen and lymph node status. The DFS and OS treatment comparisons were analyzed separately for patients with NKG 1, NKG 2, and NKG 3 tumors. Among the patients with lymph node positive disease who had NHG 3 tumors, patients who received prolonged treatment had significantly increased DFS and OS percentages ($P = 0.007$ and $P = 0.01$, respectively) compared with patients

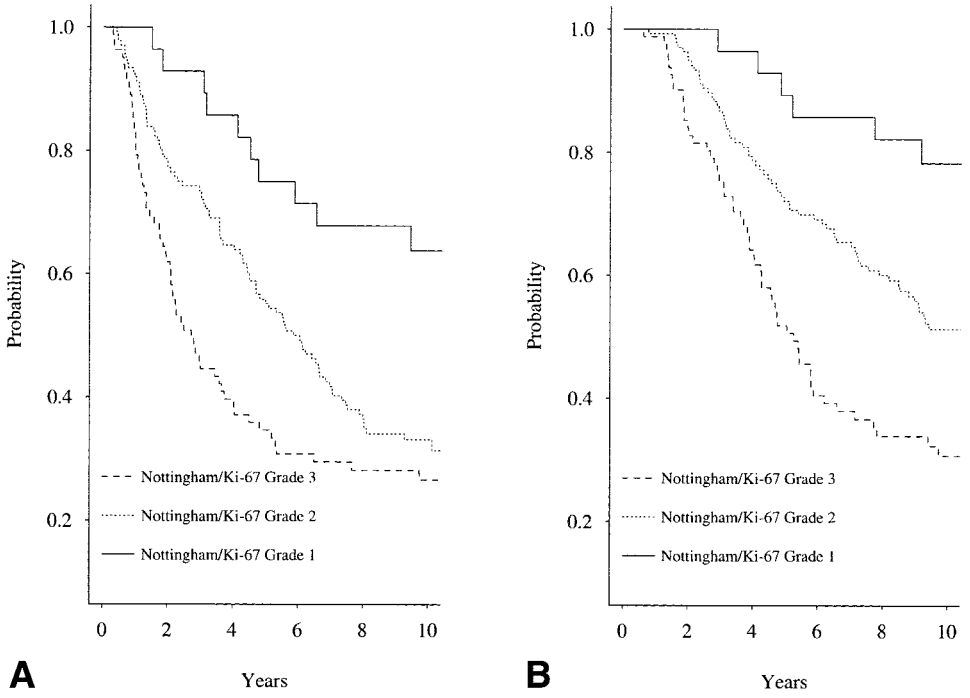

FIGURE 2. Disease free survival (A) and overall survival (B) for the lymph node positive cohort according to Nottingham/Ki-67 Grade.

who were given PeCT. This survival advantage was not expressed among patients with BRG 3 or NKG 3 tumors. However, a significant DFS advantage ($P = 0.05$) was observed for patients on the prolonged treatment with NKG 2 tumors that was not suggested under the other grading strategies. For patients with lymph node negative disease who had NKG 3 tumors, a nonsignificant DFS and OS disadvantage was seen for women who received PeCT compared with women who received no adjuvant treatment. Among the patients with node positive disease, it was observed that the prolonged treatment surpassed the PeCT regimen in terms of survival and DFS benefits for all levels of NKG tumors.

Multivariate analyses with NKG fixed in the model showed that, for patients with lymph node negative disease who were not on the PeCT treatment, information regarding vessel invasion by primary tumor contributed significantly beyond NKG to the estimation of hazards for OS and DFS. For OS, the HR for an increase of 1 NKG remained insignificantly different from 1 ($P = 0.76$; 95%CI, 0.43–1.36), whereas the HR for presence of vessel invasion by the primary tumor was 2.30 ($P = 0.05$; 95%CI, 1.01–5.23). Results were similar but less pronounced for DFS, with the HR for vessel invasion by the primary tumor becoming 1.95 ($P = 0.05$; 95%CI, 0.99–3.84). For patients with lymph node negative disease who received PeCT treatment, pathologic tumor size contributed information beyond NKG in hazard estimation for OS, and PgR status and vessel invasion contributed further information for DFS. In patients with node positive disease who received PeCT treatment, c-*erb*B-2 expression contributed significant information beyond NKG regarding OS prognosis, and vessel invasion contributed further information for DFS. Among the patients with node positive disease who were on prolonged treatment, knowledge of pathologic tumor size and c-*erb*B-2 expression, in addition to NKG, made both OS and DFS more precise.

Categoric Ki-67 as an Independent Parameter

We analyzed the correlation between patient characteristics and the Ki-67 categories for patients with lymph node negative disease and patients with lymph node positive disease, respectively. BRG, NHG, and Nottingham type were correlated significantly with Ki-67 percentages among both patients with lymph node negative disease and patients with lymph node positive disease, in whom it was observed that high proportions of patients with low-grade tumors had Ki-67 percentages $\leq$ 9.5%. In the cohort of patients with lymph node positive disease, tumors that did not overexpress c-*erb*B-2 were much more likely to have low Ki-67 percentages. Pathologically defined grades were correlated more strongly with pathologic features other than Ki-67 both in patients with lymph node negative disease and in patients with lymph node positive disease.

An analysis of categoric Ki-67 as an independent

TABLE 2
Ten-Year Disease Free Survival and Overall Survival According to Nottingham/Ki-67 Grade

		DFS					OS				
Characteristic	No. of patients	No. of failures	Ten-yr DFS ± SE (%)	HR[a]	95%CI	*P* value[b]	No. of deaths	Ten-yr OS ± SE (%)	HR[a]	95%CI	*P* value[b]
Lymph node negative											
Nottingham/Ki-67 grade											
Grade 1	33	14	66 ± 8	—	—	—	8	84 ± 7	—	—	—
Grade 2	94	54	48 ± 5	1.19	0.90–1.57	0.22 (0.12, 0.34)	36	66 ± 5	1.28	0.91–1.81	0.16 (0.08, 0.05)
Grade 3	61	33	53 ± 6	—	—	—	24	65 ± 6	—	—	—
No PeCT											
Grade 1	11	6	64 ± 15	—	—	—	4	82 ± 12	—	—	—
Grade 2	32	21	44 ± 9	0.88	0.55–1.40	0.59 (0.63, 0.58)	17	56 ± 9	0.76	0.43–1.31	0.32 (0.70, 0.58)
Grade 3	20	9	69 ± 11	—	—	—	4	80 ± 9	—	—	—
PeCT[c]											
Grade 1	22	8	67 ± 10	—	—	—	4	86 ± 8	—	—	—
Grade 2	62	33	50 ± 7	1.40	0.98–1.99	0.07 (0.03, 0.41)	19	72 ± 6	1.77	1.12–2.79	0.01 (0.02, 0.03)
Grade 3	41	24	46 ± 8	—	—	—	20	58 ± 8	—	—	—
Lymph node positive											
Nottingham/Ki-67 grade											
Grade 1	28	13	64 ± 9	—	—	—	10	78 ± 8	—	—	—
Grade 2	136	96	33 ± 4	1.55	1.21–1.98	< 0.001 (0.005, < 0.001)	73	51 ± 4	1.81	1.37–2.39	< 0.001 (< 0.002, < 0.001)
Grade 3	82	59	27 ± 5	—	—	—	56	31 ± 5	—	—	—
PeCT[c]											
Grade 1	9	6	42 ± 17	—	—	—	4	76 ± 15	—	—	—
Grade 2	48	39	23 ± 6	1.39	0.94–2.04	0.10 (0.01, 0.02)	28	48 ± 7	1.88	1.20–2.93	0.005 (0.002, 0.004)
Grade 3	32	24	22 ± 8	—	—	—	24	21 ± 8	—	—	—
Prolonged treatment											
Grade 1	19	7	74 ± 10	—	—	—	6	79 ± 9	—	—	—
Grade 2	88	57	39 ± 5	1.64	1.20–2.25	0.002 (0.09, < 0.001)	45	53 ± 5	1.74	1.22–2.47	0.002 (0.12, <0.001)
Grade 3	50	35	29 ± 7	—	—	—	32	37 ± 7	—	—	—

DFS: disease free survival; SE: standard error; HR: hazard ratio; 95%CI: 95% confidence interval; OS: overall survival; PeCT: perioperative chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil.
[a] The HR is shown for an increase of 1 grade level.
[b] Three *P* values are presented in the following order: Nottingham/Ki-67 grade *P* value (Nottingham grade *P* value, Bloom–Richardson grade *P* value).
[c] A single course of combination intravenous PeCT was given on Days 1 and 8 and commencing within 36 hours of mastectomy.

parameter revealed that patients with higher Ki-67 percentages had significantly or marginally worse OS in both the lymph node negative group and the lymph node positive group, with the exception of patients in the lymph node negative group who were on the no-PeCT treatment regimen, for whom no prognostic value of Ki-67 was observed. These patterns of association also were observed for DFS with diminished statistical significance. Treatment regimens were evaluated within each of the three Ki-67 categories in this subset of patients; however, no additional insight was revealed regarding treatment response or resistance.

DISCUSSION

Numerous studies have shown that morphologic assessment of tumor differentiation provides prognostic information in patients with breast carcinoma.[44–46] The concept that the nuclear morphology of tumor cells may have implications for their biologic behavior is due to von Hansemann,[47] and his studies have been the starting point for many grading systems for carcinoma. He considered the mitotic rate in the tumor and the occurrence of abnormal mitoses as important characteristics[48] and concluded that higher degrees of anaplasia indicated a greater tendency of the tumor to metastasize. Salomon[49] studied the correlation between the clinical course of breast carcinoma and the morphologic alterations and found that the degree of anaplasia was of prognostic importance. Greenough[50] stressed the importance of cytologic changes as well as histologic changes in tumors, and that method was modified by Patey and Scarff[51] and by Bloom.[3]

Since those studies were published, several modifications have been proposed, stressing the fact that the histologic grading of breast carcinoma is a subjective evaluation and, inherently lacks reproducibility. The Bloom–Richardson method was modified[7,52] to make the criteria more objective. The most notable

improvement was the assignment of points for mitotic counts according to high-power field area for each of three types of microscopes. The authors claimed that, if the grading protocol was followed consistently, then reproducible results could be obtained. A subsequent study[53] showed that the Nottingham modification of the BRG system was a suitable scheme for evaluating invasive ductal breast carcinomas in the routine, clinical setting.

The rate at which a tumor proliferates has long been correlated with its clinical course. Histopathologists, therefore, have sought a means of determining the rate of tumor proliferation as an adjunct to diagnosis. The simplest and most established of these practices is a count of mitotic figures. Other well-established techniques for accurately measuring the growth rate of tumors include tritiated thymidine,[54] bromodeoxyuridine incorporation,[55] and flow cytometry.[56] The use of antibodies to Ki-67 is a reliable and easy means of accurately assessing the growth fraction of human neoplasms. An almost perfect correlation has been demonstrated between visual counting techniques and image analyzing systems in the determination of the percentage of cells stained with Ki-67.[57]

The *growth fraction* of tumors now can be assessed on paraffin sections of tissues using the monoclonal antibody MIB-1 using a microwave antigen-retrieval technique. This antibody appears to be superior to others for assessing tumor proliferation on routinely fixed and processed material, not only because of the simplicity of the technique but because good correlation with Ki-67 expression on frozen material also has been reported.

In patients with breast carcinoma, although some authors have found no association between Ki-67 immunoreactivity and other prognostic variables,[58] many authors have reported an association with histologic grade,[59–61] lymph node status,[61] patient age,[62] tumor size,[61,63] ER and PgR status,[61,63–65] ploidy,[66] *p53* status,[67] and epidermal growth factor receptor expression.[68] An association between Ki-67 staining and both disease free interval and survival has been reported.[69] However, few studies have assessed the percentage of tumor cells that expressed the Ki-67 antigen in patients with long-term follow-up because of the difficulty in obtaining sufficient archival frozen tissue.

The current study is the first to assess the Ki-67 immunoreactivity and compare the Ki-67 index with the Bloom–Richardson and Nottingham grading systems in a large series of well-characterized patients with lymph node negative disease and patients with lymph node positive disease who underwent specific therapeutic regimens with long-term follow-up data available. Ki-67 expression was studied in categoric form and by substituting the mitotic counts-morphologic feature in the Nottingham criteria, resulting in the Nottingham/Ki-67 grading system. The NKG was correlated highly with tumor grade and tumor size in patients with lymph node negative disease and with tumor grade, hormone receptor status, and c-*erb*B-2 expression in patients with lymph node positive disease. In both patients with lymph node negative disease and patients with lymph node positive disease, the agreement between NKG, NHG, and BRG was high, with the agreement between any two grading systems significantly higher compared with chance alone ($P < 0.001$). The prognostic value of all three grading systems for survival and DFS, stratified by treatment regimen and lymph node status, was similar. The study confirms the value of Ki-67 evaluation as an objective means for the prediction of histologic grade and survival in patients with breast carcinoma. It is not suggested that measurement of Ki-67 alone can provide data of equivalent value to grading. What is demonstrated is that Ki-67 is a very reliable replacement for mitotic counts and would be easier to apply in FNAs and core biopsies, in which there are limited numbers of cells present.

We also conducted analyses to investigate the magnitude of treatment effects within subgroups defined by grade. Although various associations between grade, as assessed by the different systems and treatment effects, were identified, these should be interpreted as exploratory given the retrospective nature of the subgroup analyses. Multivariate analyses indicated that factors such as vessel invasion, pathologic tumor size, steroid hormone receptor status, and c-*erb*B-2 expression contributed prognostic information in addition to grade.

Although tumor grade by itself is not sufficient to define prognosis optimally for any subgroup examined, other features may not be assessed as easily as the use of neoadjuvant chemotherapy increases. Furthermore, as more conservative surgical and staging techniques increasingly are introduced into the management of patients with breast carcinoma, much useful prognostic information, including tumor size, tumor grading, vascular invasion, and lymph node involvement, will not be assessable.

Therefore, new markers that can be applied to small samples and that may be of prognostic significance will be invaluable. One obvious approach would be to use ER as a surrogate of differentiation (tubules in Nottingham grading) and *p53* overexpression as a surrogate of nuclear pleomorphism. There are many other possible parameters to assess, but there is a need for a large, controlled study to assess *markers* in

small biopsies and FNAs that can substitute for the parameters used in classic grading.

Ki-67-related antigen can be determined easily using immunohistochemistry in tumor samples obtained by multiple needle core biopsies and in cytologic specimens, both before and after treatment. In conclusion, Ki-67 detection represents a valuable tool that, in combination with other clinical, pathologic, and biologic parameters, should be analyzed further to produce a grading system that can be applied reproducibly to small tissue samples.

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