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Report

Correlation of growth fraction by Ki-67 and proliferating cell nuclear antigen (PCNA) immunohistochemistry with histopathological parameters and prognosis in primary breast carcinomas

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Abstract

The immunoreactivity for Ki-67 and PCNA was investigated in 487 patients with primary breast carcinomas using MIB-1 (Immunotech, France) and PC-10 (DAKO, Denmark) as primary antibodies. Formalin-fixed and paraffin-embedded tissue sections were used. The immunoreactivity for Ki-67 and PCNA was found to be independent of the length of fixation if the sections were pretreated in a microwave oven in citrate buffer and distilled water, respectively. The immunostaining was evaluated semiquantitatively.

High Ki-67 score (more than 1% positive tumour cells) and PCNA over-expression (more than 25% positive tumour cells) were correlated with clinicopathological parameters such as large tumour size, high histological grade (poor differentiation), and absence of steroid hormone receptors, which are parameters of an aggressive phenotype of the tumour.

In univariate analysis of survival data, both Ki-67 and PCNA were parameters of a poor overall survival in both lymph node-positive and -negative patients.

In multivariate analysis using a Cox model stratified by nodal status, Ki-67 and PCNA failed to be of prognostic significance whereas classical histopathological parameters such as tumour size and histological grade turned out to be of independent prognostic significance in both lymph node-positive and -negative patients, while progesterone receptors were of independent prognostic significance only in lymph node-positive patients.

Introduction

Carcinoma of the breast is the most frequent cancer form among women [1].Calculations have estimated a lifetime risk of more than 7% for women in the western world. Despite better diagnostic methods and development of various treatment regimens during the last 10 years, the mortality of the disease is still high.

The course of the disease for each patient is different but to some extent predictable. Histopathological features such as tumour size, presence of lymph node metastases, histological type and grade, as well as steroid hormone status have traditionally

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been used as prognostic and predictive parameters [2]. Studies have demonstrated that a high proliferative rate of tumours is correlated with treatment response and poor prognosis [3–8]. Cell proliferation can be determined by thymidine incorporation, flow cytometry, and counting mitoses. The two first techniques are complex and time consuming. Counting mitoses is fraught with inaccuracies. Mitoses are only present during the M-phase of the cell cycle, thus, the number of mitoses does not reflect the total number of proliferating cells. In addition, the counting procedure is subjected to factors such as delay in fixation and differences in counting procedures [9–11].

Immunohistochemistry is a relatively simple technique which is widely employed in surgical pathology. Immunohistochemistry has the advantage that the morphology of the tissue is maintained which allows in situ evaluation of the proliferating cells, and sampling error is minimised. One of the essential conditions for obtaining reliable results with the technique is that the antigens are preserved in a life-like manner.

Ki-67 is a marker of proliferation [12, 13]. This nuclear antigen is expressed in all stages of the cell cycle except the G_0-phase. Most studies have evaluated the prognostic significance of Ki-67 in frozen cryostat sections, since this cell cycle-related antigen was initially recognised by antibodies which only worked on fresh frozen tissue. Recently the antibody MIB-1 has become available that can be used on formalin-fixed and paraffin-embedded sections if the sections are pretreated in microwave oven.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein which is essential for DNA synthesis and appears in the nucleus primarily during the S-phase of the cell cycle [14, 15]. Antibodies directed against this antigen are available.

A positive correlation between high PCNA immunostaining and high values obtained by flow cytometry [16, 17], thymidine incorporation [18], number of mitoses [19], and Ki-67 [20] has been demonstrated. By univariate analyses high Ki-67 or PCNA have been correlated with poor prognosis. Only a few studies have examined the prognostic significance of the immunoscore of Ki-67 and PCNA in multivariate analysis. The results from these studies are not consistent.

In this study we examined the correlation of the growth fraction presented by Ki-67 and PCNA immunohistochemistry with histopathological parameters and prognosis in order to determine the independent prognostic significance of these parameters of proliferation in a large cohort of patients with a follow-up period of more than 10 years.

Material and methods

Four hundred and eighty seven women with primary breast carcinomas diagnosed at the Gentofte Hospital, University of Copenhagen, in the period 1980–1985 were included. The patients represented an unselected population. It should moreover be noted that no cases were diagnosed by mammographic screening. Thus, the average diameter of the tumours is larger and the incidence of lymph node metastases is higher than in countries with screening programmes. Information about treatment, relapse-free periods, and overall survival was obtained from the DBCG. The primary treatment was in all cases either wide local excision or mastectomy. Two hundred ninety three patients (60%) entered into one of the treatment regimens of the DBCG, and of these 130 received adjuvant chemotherapy and/or radiotherapy as part of the secondary treatment. Information about adjuvant treatment was not available for patients who did not enter into the treatment protocols of the DBCG. The mean follow-up period was more than 10 years. The overall survival was defined as death due to all causes. Among the entire population 225 patients died. Information about relapse-free period was only available for patients who entered into the treatment protocols of the DBCG. One hundred and eleven patients had relapse. The mean and median age of the patients was 61 years (range 32 to 92 years).

The distribution of clinical, histological, and prognostic features of the patients is shown in Table 1. The surgical specimens were handled and the histological examination was carried out according to the guidelines of the DBCG [21]. The largest dia-

meter of the tumour was measured on the fresh specimen and registered as less than 2 cm, 2–5 cm, or more than 5 cm. The number of lymph nodes and number of metastatic lymph nodes were counted. The patients were registered as either node-positive or -negative. Tissue for microscopic examination was formalin-fixed and paraffin-embedded following routine procedures of the laboratory. Histological typing and grading were done on haematoxylin and eosin (HE) stained sections. The invasive carcinomas were classified according to World Health Organization criteria [22] as either invasive ductal not otherwise specified (NOS), lobular, or miscellaneous carcinomas. Invasive ductal carcinomas (NOS) were graded according to a modification of the method described by Bloom and Richardson [23].

Oestrogen receptor (ER) and progesterone receptor (PgR) status of the primary tumours was determined using the dextran-coated charcoal (DCC) technique if enough fresh tissue was available. The biochemical measurements of ER and PgR were expressed as fmol/mg protein. Values below 10 fmol/mg were considered negative.

Immunohistochemistry

Sections measuring 5 μm were cut, dewaxed, and rehydrated following routine procedures. Non-specific binding was blocked by incubation with bovine albumin for 10 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes. Sections were placed in Ziehl Neelsen jars. Sections which were to be stained for Ki-67 were placed in citrate buffer (pH 6.0 citric-acid monohydrate 10 mM adjusted with 2 N NaOH) and those which were to be stained for PCNA were placed in distilled water. The volume of the fluid was 200 ml. The sections were thereafter microwaved twice for 5 minutes in a household microwave oven (Voss Micro 361-1, model 58907) at 650 W. The effective time of boiling was approximately 3 minutes. The sections were always submerged within the fluid. After microwave processing the sections were allowed to cool to room temperature and were then incubated with the primary antibody. Sections stained for Ki-67 were incubated with the monoclonal MIB-1 antibody (Immunotech, France) used in a 1 : 100 dilution. Sections stained for PCNA were incubated with the monoclonal PC-10 antibody (DAKO, Denmark) used in a 1 : 600 dilution. The primary antibodies were diluted in Tris buffered saline (TBS pH

Table 1. Distribution of clinical, histological, and prognostic features in 487 patients with primary breast carcinomas

Feature		Number of patients (%)
Menopausal status	premenopausal	135 (27%)
	postmenopausal	352 (73%)
Tumour size	less than 2 cm	254 (52%)
	2–5 cm	194 (40%)
	more than 5 cm	39 (8%)
Nodal status	negative	307 (64%)
	positive	180 (36%)
Tumour type	ductal	384 (37%)
	lobular	46 (10%)
	miscellaneous	57 (12%)
Histological grade	I	142 (37%)
	II	183 (48%)
	III	58 (15%)
ER	negative	91 (25%)
	positive	269 (75%)
PgR	negative	88 (32%)
	positive	189 (68%)

7.6 with azide/bovine albumin). The sections were incubated overnight at 4° C.

As the avidin-biotin-complex (ABC) technique was employed, the secondary antibody was a biotinylated rabbit anti-mouse immunoglobulin (DAKO, Denmark) used in a 1 : 400 dilution. The sections were incubated with the ABC complex (DAKO, Denmark) for 30 minutes. The colour was developed using 3-amino-9-ethylcarbazole. The sections were counterstained with Meyer's haematoxylin.

In order to evaluate the length of formaldehyde fixation, tissue blocks from 5 primary breast carcinomas, a tonsil, and a seminoma of the testis were fixed in 4% buffered formaldehyde for various length of time (6, 24, 48, and 72 h, and 1 week, respectively) before processing through to paraffin embedding followed by the above described immunohistochemical method for staining with MIB-1 and PC-10.

For both antibodies positive controls included a tonsil with simple hyperplasia. Incubation of tissue sections with omission of the primary antibody as well as incubation of sections with a subclass-matched unrelated immunoglobulin as the primary antibody were performed as negative controls.

Assessment of the immunohistochemical staining

Positive immunostaining with MIB-1 appeared as a distinct nuclear staining. In most tumour cells the nucleoli were also stained. The immunostaining for PCNA appeared as a few positive nuclear granules and in a continuum to homogeneous nuclear staining. Any nuclear staining was considered as PCNA positivity.

The immunostainings were evaluated using a semiquantitative method. Tumours were scored as follows: 0 = if less than 1% of the cells were positive, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, 4 = if more than 50% of the tumour cells were positive.

Tumours were registered as showing PCNA overexpression if more than 25% of the tumour cells were positive [19].

In order to evaluate the reproducibility and reliability of the semiquantitative method used to assess the immunostaining, 97 primary breast carcinomas stained with MIB-1 were semiquantitatively evaluated blindly twice with one week interval. The immunoreactivity in the sections was also assessed blindly twice with one week interval by one of the authors using the actual counting method. The percentage of positive tumour cells was determined by counting the number of positive and the total number of cells in 10 high power fields (× 40 objective, Olympus BH-2 microscope, with a HPF diameter of 490 μm). More than 1000 tumour cells were counted in each tissue section.

Statistics

As the immunostainings, as well as clinical and histopathological parameters, were considered as discrete values, Chi-squared analyses were used when the relationship between variables was examined.

Kappa-statistics were used to assess the inter observer variation as well as to assess the relationship between the actual counting method and the semiquantitative scoring method. Kappa-values less than 0.6 were considered as moderate, whereas Kappa-values of 0.6–0.8 were considered as acceptable.

The overall survival was estimated using the Kaplan-Meier method [24], and the curves were compared using the log-rank test [25].

Cox's regression model was used in multivariate analysis of survival data [25]. In the Cox analysis the values of all immunohistochemical and clinicopathological variables were evaluated in a model stratified by nodal status, since proportional hazards were not demonstrated between lymph node positive and negative patients.

In all statistical tests a 5% level of significance was used, also when two-tailed tests were used.

Results

Immunostaining for Ki-67 appeared as a distinct homogeneous nuclear staining in proliferating cells (Fig. 1). Positive tumour cells were scattered throughout the tumour tissue. In tumours with a

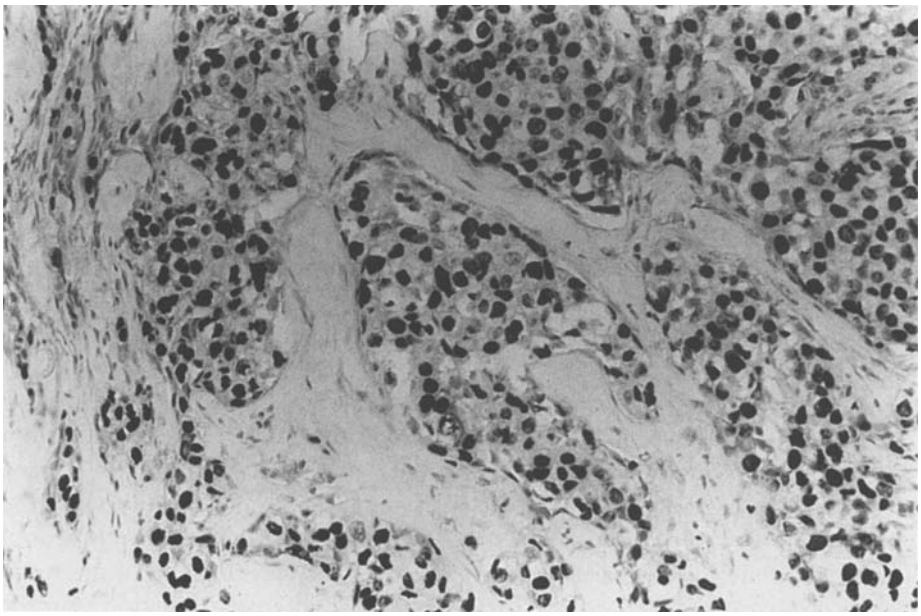

Fig. 1. Immunostaining for Ki-67.

high number of Ki-67 positive tumour cells the positive cells tended to be clustered at the border of the tumour islets. Positive immunoreaction was always present in the tissue sections, either in tumour infiltrating lymphocytes or in tumour cells; however, the number of positive tumour cells varied from tumour to tumour.

Immunoreactivity for PCNA (Fig. 2) appeared as a continuum from densely homogeneous stained nuclei to nuclei containing a few positive granules. Thus no clear distinction between positive and negative tumour cells appeared as seen with the immunostaining for Ki-67. Cells with any degree of

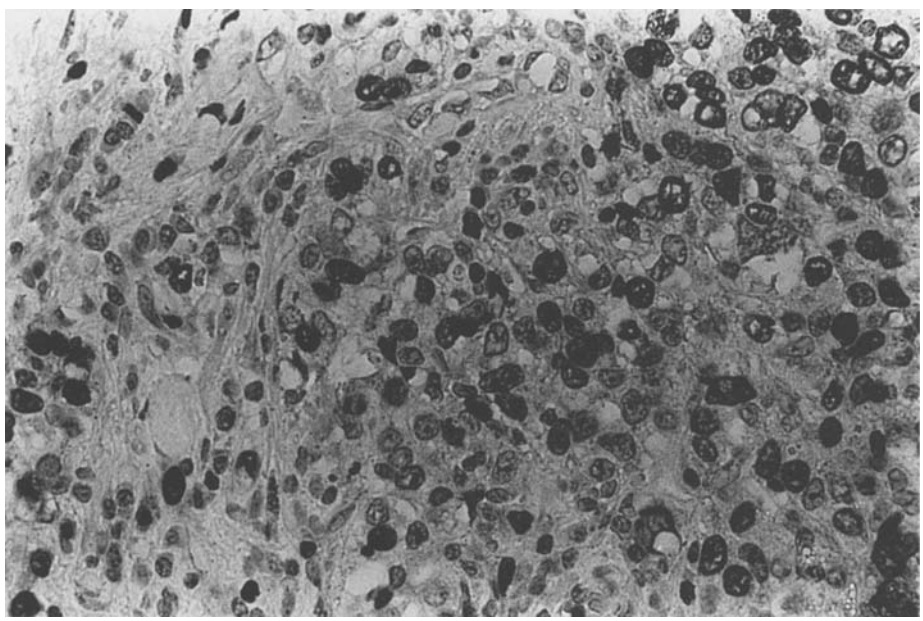

Fig. 2. Immunostaining for PC-10.

nuclear PCNA immunostaining were registered as positive.

Five primary breast carcinomas, a tonsil with hyperplasia, and a seminoma of the testis were formalin-fixed from 6 hours to 1 week. In a previous study the immunostaining for PCNA was found to be independent of the length of formalin-fixation if the sections were microwave pretreated twice for 5 minutes in distilled water, respectively, before the sections were incubated with the primary antibody. The number of positive cells and the intensity of the immunostaining were similar whether the sections were formalin-fixed 6 hours or longer [26]. Tissue sections from 5 primary breast carcinomas, a tonsil, and a seminoma of the testis fixed in formalin from 6 hours to 1 week were stained with MIB-1 according to the described immunohistochemical procedure. The number of positive cells and the intensity of the staining reaction were similar regardless of the duration of fixation. Thus, we concluded that the immunoreactivity for Ki-67 was also independent of the length of fixation, and we did not consider the length of formalin-fixation when evaluating the immunoreactivity for PCNA or Ki-67 in the primary breast carcinomas.

Table 2 shows the Kappa-values when the Ki-67 immunoreactivity was assessed by the actual counting method and by the semiquantitative score method, and the 2 methods were compared. In the actual counting method, the percentage of positive tumour cells was determined by counting the number of positive and the number of total tumour cells. This was initially a continuous parameter, which was converted into a discrete parameter using the same criteria for positivity as in the semiquantitative method. A Kappa-value of 0.5 was obtained when the inter observer variation was evaluated for the actual counting method, whereas a Kappa-value of 0.7 was obtained by the semiquantitative method. When the actual counting method was compared with the semiquantitative method Kappa-values of 0.5–0.6 were obtained. If the immunoscore for Ki-67 was registered as either more or less than 1% positive tumour cells, the Kappa-values increased. The accuracy by which tumours could be separated into those with less vs. those with more than 1% positive tumour cells was also evaluated. The initial classification of the tumours was compared with the classification after the second evaluation when using either the actual counting or the semiquantitative method. Initially 31 cases were classified as containing less and 66 cases as containing more than 1% positive tumour cells when the immunoreactivity was assessed by the actual counting method. After the second assessment 9 cases (29%) changed category and were classified as containing more than 1%, and 10 cases (15%) changed category and were classified as containing less than 1% positive tumour cells. Using the semiquantitative method, 38 tumours were initially classified as containing less and 66 as containing more than 1% positive tumour cells. In the second assessment 8

Table 2. Kappa-values when the actual counting method was correlated with the semiquantitative method, both when the Ki-67 immunoreactivity was scored on a scale 0–4, and more and less than 1% Ki-67 positive tumour cells

Method	Method	Ki-67 scored as 0–4 Kappa-value	Ki-67 scored as more vs. less than 1% positive tumour cells Kappa-value
Counting (1)	Counting (2)	0.5	0.63
Semiquantitative (1)	Semiquantitative (2)	0.7	0.73
Counting (1)	Semiquantitative (1)	0.6	0.66
Counting (1)	Semiquantitative (2)	0.49	0.63
Counting (2)	Semiquantitative (1)	0.54	0.63
Counting (2)	Semiquantitative (2)	0.51	0.66

Abbreviations: Counting (1) = immunoreactivity determined by the actual counting method first time, Counting (2) = immunoreactivity determined by the actual counting method second time, Semiquantitative (1) = immunoreactivity determined by the semiquantitative method first time, Semiquantitative (2) = immunoreactivity determined by the semiquantitative method second time.

Table 3. Correlation between Ki-67 and PCNA immunoscore

Ki-67 score (%)	PCNA-score (%)				
	0	1	2	3	4
0	6	10	8	12	11
1	1	7	8	11	9
2	1	1	4	4	4
3	0	0	1	1	1
4	0	0	0	0	0

Score : 0 = 1% positive tumour cells, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, 4 = more than 50% positive tumour cells.

(21%) tumours changed category to contain more than 1%, and 10 cases (17%) changed category to contain less than 1% positive tumour cells.

The contingency Table 3 shows the correlation between Ki-67 and PCNA immunoreactivity. In the primary breast carcinomas positivity for Ki-67 and PCNA ranged from less than 1% positive tumour cells to more than 50% positive tumour cells. Since a semiquantitative method was used and the scores of the immunostainings were regarded as discrete values it was not possible to calculate a mean immunoscore for the Ki-67 or PCNA. PCNA score was in general higher than the Ki-67 score.

The chi-squared tests between clinical, histological, and prognostic features and Ki-67 and PCNA are shown in Table 4. All patients have full records of menopausal status, tumour size, nodal status, tumour type, and histological grade. Three hundred and sixty and 277 patients have records of ER or PgR, respectively. The lack of steroid hormone receptors was mainly due to insufficient amount of tis-

Table 4. Correlation between Ki-67 and proliferating cell nuclear antigen (PCNA) in primary breast carcinoma and clinicopathological parameters

Clinicopathological parameters	Ki-67 score		PCNA	
	High expression (%)	p-value	Over-expression (%)	p-value
Menopausal status				
Pre-	54	0.53	47	0.24
Post-	51		52	
Tumour size				
Less than 2 cm	47	0.04	47	0.1
2–5 cm	58		53	
More than 5 cm	58		64	
Nodal status				
Positive	51	0.67	47	0.06
Negative	53		54	
Mitoses				
1	44	0.004	43	< 0.00
2	61		59	
3	63		70	
Histological grade				
1	42	0.003	37	< 0.00
2	60		59	
3	61		72	
ER				
Positive	57	0.19	47	< 0.00
Negative	49		64	
PgR				
Positive	57	0.19	49	< 0.00
Negative	61		69	

Abbreviations: Mitoses: 1 = 0–1 per high power field (HPF), 2 = 2–3 per HPF, 3 = 4 or more per HPF. Histological grade: 1 = grade I, 2 = grade II, 3 = grade III. ER = Oestrogen receptor, PgR = Progesterone receptor. High Ki-67 expression = more than 1% positive tumour cells. PCNA over-expression = more than 25% positive tumour cells.

sue for biochemical analysis. Two hundred and twenty six patients have full records of all mentioned parameters. High Ki-67 score was associated with large tumour size, a high number of mitoses, and high histological grade (poor differentiation). No correlation between Ki-67 immunoreactivity and menopausal or nodal status was demonstrated. High PCNA score was correlated with a high number of mitoses, high histological grade (poor differentiation), and absence of both ERs and PgRs. No correlations between PCNA immunostaining, menopausal status, and lymph node metastases were demonstrated. Menopausal status was statistically correlated with PgR; no correlations with the number of mitoses, Ki-67, and PCNA were demonstrated. However, it was demonstrated that more postmenopausal women did not enter into the treatment regimens of the DBCG compared with premenopausal women ($p < 0.05$).

Based upon the Ki-67 immunoscore the patients were separated into two groups: those with low expression (less than 1% positive tumour cells), and those with high (more than 1% positive tumour cells). This grouping was based upon the statistically most significant difference in overall survival between the two groups. Thus, in univariate analysis of life table data, a high score for Ki-67 turned out to be a parameter of poor overall survival. The overall survival was 51% for patients with tumours containing less than 1% positive tumour cells, and 39% for patients with tumours containing more than 1% positive tumour cells. The difference was statistically significant.

In a previous study we have defined that patients with over-expression of PCNA are those with more than 25% positive tumour cells, whereas those with low expression of PCNA are those with less than 25% positive tumour cells [19]. In univariate analysis of survival data, a high PCNA score was a parameter of poor overall survival. The overall survival among patients with tumours with low PCNA expression (less than 25% positive tumour cells) was 51% and 39% for patients with tumours with high-PCNA expression (more than 25% positive tumour cells). The difference was statistically significant.

Multivariate analysis revealed that histological grade could only be entered into the Cox model as two categories, either as grade I or as grade II + III. The most important assumption concerning Cox regression models is the presence of proportional hazzards. Since proportional hazzards between lymph node-positive and -negative patients could not be demonstrated, a model stratified by nodal status was used. First the prognostic significance of Ki-67 and PCNA in lymph node-positive and -negative patients was examined. The analyses showed that both Ki-67 and PCNA have the same prognostic value in lymph node-positive and -negative patients, respectively, and thus common estimates could be used in the Cox model. Menopausal status, tumour size, tumour type, histological grade, steroid hormone receptors, and Ki-67 and PCNA immunoscore were included in the multivariate analysis. The scoring of the statistically independent variables is shown in Table 5. The results of the multiva-

Table 5. Scoring of the prognostic independent parameters and Ki-67 and PCNA when these parameters were entered into a Cox model stratified by nodal status

Parameter	Score in the Cox model	Value
Tumour size	0	less than 2 cm
	1	2–5 cm
	2	more than 5 cm
Histological grade	0	grade II and III
	1	grade I
PgR	0	negative
	1	positive
Ki-67	0	less than 1% positive tumour cells
	1	more than 1% positive tumour cells
PCNA	0	less than 25% positive tumour cells
	1	more than 25% positive tumour cells

riate analysis are shown in Table 6. Both Ki-67 and PCNA as well as menopausal status, tumour type, and ER failed to be of independent prognostic significance. Tumour size and histological grade turned out to be of independent prognostic significance and have the same prognostic value in lymph node-positive and -negative patients. PgR was of independent prognostic significance only in lymph node-positive patients.

Discussion

Tumour cell proliferation is a variable which is used in histological grading of tumours [23]. Cell proliferation is also a prognostic parameter in many types of malignancies including carcinoma of the breast [3–8, 27, 28]. A positive correlation between a high rate of proliferation and poor disease-free and overall survival has been demonstrated. Techniques such as flow cytometry and thymidine labelling can accurately measure the proliferative capacity. However, these techniques are difficult, laborious, and expensive. Counting mitoses has traditionally been the method used in surgical pathology to estimate the proliferative capacity. Immunohistochemistry is a relatively simple technique that allows proliferative cells to be demonstrated in situ. Recently antibodies against proliferating cell antigens which can be used on formalin-fixed and paraffin-embedded sections have become available [29].

Ki-67 is an antibody which reacts with a nuclear non-histone protein which is present in all active parts of the cell cycle but is absent in the G_0-phase.

PCNA is mainly present in cells where DNA replication or synthesis occur. The level of PCNA is low in quiescent cells whereas the production increases in the proliferative cell cycle [30]. In breast carcinomas PCNA immunoreactivity correlates with flow cytometry [16, 17], thymidine incorporation [18], and Ki-67 immunoreactivity [20].

Mitotic count yields lower values than Ki-67 growth fraction or S-phase measurements because mitotic duration is a very short segment of the cell cycle. For example, a cell cycle might be approximately 48 hours long with mitosis occupying 15 minutes, G1 phase 24 hours, S-phase 18 hours, and G2 phase 6 hours. Under these circumstances the lowest count wil be the mitotic index (MI) followed by the S-phase fraction (SPF), whereas the growth fraction (proportion of cells cycling) will be the highest. The MI will have the least precision because relatively few cells are in mitosis at a given time. In addition, counting mitoses is subject to inaccuracies caused by delay in fixation [9–11] and difficulties in counting procedure [31]. Apart from the question of precision, potential growth rate could be estimated equally well by MI (relatively low count), SPF (higher count), or growth fraction if the duration of the cell cycle and its components and cell loss rates for various parts of the cycle were known.

One of the most critical points in immunohistochemistry is to preserve the antigens in as life-like a manner as possible. Cattoretti *et al.* [29] recently have demonstrated that the antibody MIB-1 directed against Ki-67 can be applied to formalin-fixed and paraffin-embedded tissue if the sections are pretreated in citrate buffer twice for 5 min before incubation with the primary antibody. In the pre-

Table 6. Prognostic clinicopathological parameters when using a Cox model stratified by nodal status

Parameter	β's	STD	RR
Tumour size	0.45	0.16	1.58
Histological grade	− 0.25	0.20	0.077
PgR[a]	− 0.93	0.24	0.39
Ki-67[b]	0.13	0.14	1.14
PCNA[b]	0.21	0.14	1.22

β coefficient describes how each factor contributes to the hazard. STD = standard deviation, RR = relative risk. PgR = progesterone receptor. [a] = only of independent poor prognostic significance in lymph node-positive patients. [b] = not of independent prognostic significance.

sent study we examined the influence of the length of fixation upon the Ki-67 immunoreactivity. It was demonstrated that the intensity of the immunoreactivity and the number of cells positive for Ki-67 was similar in sections whether they have been formalin-fixed from 6 hours to 1 week. We have thus concluded that the immunoreactivity for Ki-67 was independent of the length of formalin-fixation if the sections were microwave processed before incubation with the primary antibody. Studies have shown that the immunoreactivity of PCNA depends on the type of fixative and the length of fixation [32–34]. In a previous study it was shown that the immunoreactivity for PCNA was independent of the length of formalin-fixation if the tissue sections were microwave processed in distilled water before incubation with the primary antibody [26]. Thus the length of fixation of the tissues was not considered in this study.

In immunohistochemistry the proliferative rate in tumours has mostly been assessed by the actual counting method. This method is time consuming and fraught with inaccuracies since most breast carcinomas are heterogeneous in the distribution of proliferating tumour cells [31]. The semiquantitative method is less time consuming and the whole section is evaluated. Thus the semiquantitative method is not dependent on the area of the tumour selected for counting. In the present study the Kappa-value for inter observer variation for the actual counting method was 0.5 and 0.7 for the semiquantitative method; when the actual counting method was compared with the semiquantitative method the Kappa-values were relatively low. It was also demonstrated that the actual counting and the semiquantitative methods were equally sufficient to discriminate between tumours with less than 1% and more than 1% Ki-67 positive tumour cells. Thus the semiquantitative method has in our hands turned out to be as reliable and reproducible as the actual counting method.

Studies have reported that the mean value for Ki-67 immunoreactivity in breast carcinomas ranges 7–20%, whereas the mean PCNA value ranges 10–60% [13, 17, 31, 35–38]. The mean values of Ki-67 and PCNA immunostaining could in the present study not be determined since the results were registered as discrete values. However, we found that the PCNA score was higher than the Ki-67 score, which is in accordance with most other studies [20, 27, 33, 39, 40]. The reasons for this finding might be that PCNA has a longer half-life than Ki-67, and that the PC-10 antibody reacts with two different forms of PCNA, one mainly present at the entry of the S-phase and one associated with DNA synthesis, and might be present although the cell is no longer proliferative [41]. In contrast, the Ki-67 is one well-defined epitope only expressed during the proliferative phase of the cell cycle [12]. The counting procedure might also influence the result. Ki-67 positivity appears as a distinct nuclear staining and it is not difficult to separate positive and negative cells. In contrast, PCNA positivity is more variable, and a positive reaction can appear as a continuum from a few nuclear granules to a more homogeneous staining of the nuclei. Thus differences in assessing positivity of PCNA might be difficult to resolve. Some authors have counted only the strongest immunostained tumour cells, introducing an element of subjectivity, whereas others counted all stained cells, regardless of the intensity of the staining. We counted all tumour cell with positive nuclear immunoreaction, thus no subjectivity was included in the present study.

The correlation between the staining results of Ki-67 and PCNA and clinicopathological parameters are in accordance with most other studies [17, 20, 35–40, 42]. Both Ki-67 and PCNA were found to be correlated with a high number of mitoses, which indicates they are markers of proliferation. Some studies have found a correlation between Ki-67- and PCNA-score, tumour size, nodal status, histological grade, and steroid hormone receptor status, whereas other have not. The reasons for the differences might be the composition of the examined population, and the use of different methods. Most studies have, like the present, found that high Ki-67- and PCNA-scores were correlated with histological features which are indicative of an aggressive phenotype of the tumour.

In univariate analysis of survival data, high Ki-67 score (more than 1% positive tumour cells) and PCNA over-expression (more than 25% positive tumour cells) were parameters of poor overall sur-

vival. Since the Cox model stratified by nodal status was used, it was neccessary to evaluate the prognostic significance of the parameters in lymph node-positive and -negative patients in order to determine if a common estimate could be used. It was thus demonstrated that Ki-67 and PCNA had the same prognostic significance in lymph node-positive and -negative patients. This finding is also in accordance with most other studies [17, 36, 38, 42, 43], although Gasparini *et al.* [44] found that PCNA overexpression was not a parameter of poor overall survival. In that study the follow-up period was 5 years. Other studies have shown that a statistically significant difference in overall survival between patients with tumours with high or low expression of PCNA can be demonstrated mainly after 5 years follow-up.

In multivariate analysis the classical histopathological parameters such as large tumour size and high histological grade (poor differentiation) turned out to be independent prognostic parameters in both lymph node-positive and -negative patients. PgR was of independent prognostic significance but only in lymph node-positive patients. ER was of no prognostic significance in the present study. Both Ki-67 and PCNA failed to be of independent prognostic significance. PCNA had, however, more impact on the prognosis than Ki-67. These findings are in accordance with some previous studies but not with others. Most previous studies have included rather few patients, and the follow-up period has been too short to draw reliable conclusions of the prognostic value.

Tumour growth and progression depends upon the proportion of proliferating and apoptotic tumour cells. In addition, the tumour cells must also have the capability to metastasize. Thus the proliferative rate might thus not be the only parameter for tumour growth and progression, and this might in part explain why markers of proliferation failed to be independent prognostic significance.

However, studies have shown that breast carcinomas of young women more commonly have high proliferative indices, whereas low proliferative indices are associated with older age, which in turn, is associated with death from intercurrent causes. The present study failed to demonstrate any correlation between menopausal status and high Ki-67 or PCNA indices. We did not preform a multivariate analysis of survival data including only younger women since the number of patients was too small to obtain reliable conclusions.

Hazard functions of proliferative indices may be time-dependent, with disease-specific mortality decreasing over time for highly proliferative tumours although not for slowly proliferating breast carcinomas. In the present study, no information on specific mortality was available. The overall survival was defined as death due to all causes. Thus this question cannot be answered in the present study.

Finally, the number of patients who did not enter the treatment protocols of the DBCG might in some way blur the prognostic significance of the proliferative indices. Statistically more postmenopausal women did not enter into the treatment protocols of the DBCG, thus no information of adjuvant treatment was available. Some of these patients have probably received adjuvant treatment. In addition, the treatment given those patients who entered into the treatment protocols of the DBCG might also influence the clinical outcome and prognostic significance of the proliferative indices, since tumours with a high proliferative capacity have a better response to chemo- and/or radiotherapy compared with tumours with lower proliferation. Thus treatment might also blur the results.

In conclusion, we found that the immunoreactivity for Ki-67 and PCNA was independent of the length of formalin-fixation. High scores of Ki-67 and PCNA were correlated to histopathological features indicative of an aggressive phenotype of the tumours. In univariate analyses Ki-67 and PCNA were parameters of poor overall survival in both lymph node-positive and -negative patients. Since no proportional hazards could be demonstrated between lymph node-positive and -negative patients, a Cox model stratified by nodal status was used in the multivariate analyses of survival data. In these models, classical histopathological parameters such as tumour size, histological grade, and progesterone receptors turned out to be independent prognostic parameters, whereas Ki-67 and PCNA failed to be of prognostic significance.

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