Poor prognostic value of proliferating cell nuclear antigen labelling index in breast carcinoma

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Abstract

Aim—To investigate the association between proliferating cell nuclear antigen immunostaining and various clinicopathological variables, and its prognostic value in breast carcinoma.

Methods—A monoclonal antibody PC10 was applied to formalin fixed, paraffin wax embedded tissue in 144 cases of primary breast cancer. PCNA immunostaining was scored by counting 1000 cells; the percentage of positive stained cells was recorded as the PCNA labelling index (PCNA-LI).

Results—The PCNA-LI varied from 0–77% with a mean of 18%. When tumours were separated on the basis of the mean value, 93 had a low PCNA-LI of less or equal than 18% and 47 a high PCNA-LI of more than 18%. There was no significant correlation between PCNA-LI and all prognostic factors included in this study. Moreover, PCNA-LI failed to show any prognostic value for overall and disease free survival.

Conclusion—PCNA immunostaining is not correlated with clinicopathological variables and patient survival in breast cancer.

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The clinical outcome of patients with breast cancer is highly variable because it not only depends on the extent of disease but also on its behaviour. Tumour cell proliferation is an important biological variable which can be regarded as an additional prognostic factor.¹ To date, however, methods used to assess this variable have had limited success. The methods which require labelling with ³H thymidine or its non-radioactive analogue bromodeoxyuridine,² or disruption of cellular and tissue architecture for DNA flow cytometry,³ are complex procedures that are time consuming and can not really be applied to routine clinical samples. More recently, a monoclonal antibody Ki67 was produced. It reacts with a nuclear antigen present throughout the cell cycle of proliferating cells.⁴ A major drawback to its use, however, has been the need for fresh or snap frozen material.⁵

Autoantibodies from patients with systemic lupus erythematosus have been described as recognising a nuclear antigen called proliferating cell nuclear antigen (PCNA), present in proliferating cells.^{6,7} This antigen is a 36

kilodalton nuclear protein which is directly involved in DNA synthesis and replication.^{8,9} During the cell cycle, PCNA expression increases through G1 phase, shows a maximum at the early S phase, declines through G2 phase, and reaches low levels in M phase and interphase.^{10,11} Several groups have reported that antibodies against PCNA are useful probes for the detection of proliferating cells in normal and neoplastic tissues^{12–17} and that they have prognostic value in certain tumour populations.^{18,19} There are also a few studies in which PCNA failed to show any prognostic value.²⁰

Using the new monoclonal antibody PC10,²¹ which can be applied to routinely fixed, paraffin wax embedded tissue, we investigated the association between PCNA index and several clinical and biological variables, and the prognostic value of the PCNA index on the survival of patients with breast carcinoma.

Methods

One hundred and forty four paraffin wax blocks of tumour samples were available from patients with resectable breast cancer treated at the Second Department of Surgery, Kanazawa University Hospital, between 1978 and 1989. We selected patients with histologically confirmed breast carcinomas in whom a variety of mastectomies with axillary lymph node (AX) dissection had been performed. All resected material was examined histologically. Postoperatively, all patients were undergoing various adjuvant hormone treatments. They were followed up until their death, or to March 1992.

The patients were classified according to the following clinicopathological variables: age (14 aged ≤35 years, 69 aged 36–50 years, 61 aged ≥51 years); menopausal status (78 premenopausal, 66 postmenopausal); clinical stage (five with tumour in situ, 28 in stage 1, 76 in stage 2, 35 in stage 3); tumour size (≤2.0 cm in 58, 2.1–5.0 cm in 70, ≥5.1 cm in 16); histological type (non-invasive carcinoma in five, invasive ductal carcinoma in 130, special type of invasive carcinoma in nine) according to the histological classification of breast cancer of the Japan Breast Cancer Society²² and the modified histological typing of the World Health Organisation²³; degree of histological AX metastases (0 in 85, one to three in 33, >three in 26); and PCNA index (≤18% in 93, >18% in 51).

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PCNA IMMUNOSTAINING

Formalin fixed, paraffin wax embedded tissue sections (5 μ m) were dewaxed in xylene and subsequently rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by immersing the sections in 1% hydrogen peroxide in methanol for 15 minutes. After washing in distilled water for five minutes and in phosphate buffered saline (PBS, pH 7.4) for 20 minutes, the sections were preincubated with 10% normal goat serum (Dako, Copenhagen, Denmark) in PBS with 0.1% bovine serum albumin (BSA) for 20 minutes at room temperature. The sections were then incubated overnight at 4°C with the PC10-murine IgG2a anti-PCNA monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, England) at one in 50 dilution in phosphate buffered saline-bovine serum albumin (PBS-BSA). After rinsing in PBS, the sections were incubated with a biotinylated antimouse immunoglobulin (Dako) for 90 minutes at room temperature, followed by a rinse in PBS. The site of the antibody binding was visualised with streptavidin-biotin-peroxidase complex (Dako) for 60 minutes. Diaminobenzidine-hydrogen peroxide was used as a chromogen and a light haematoxylin counterstain was used. The sections were then rinsed in tap water for five minutes, dehydrated in ethanol, cleared in xylene and mounted under coverslips in malinol medium (Muto Pure Chemicals, Tokyo, Japan). Negative controls were done by omitting the primary antibody. PCNA immunostaining was scored by counting 1000 cells in 10 to 20 fields per tumour depending on cellularity. Every stained nucleus was considered positive regardless of its intensity. In cases where staining varied in the tumour, the fields

examined included those with the highest and those with the lowest percentage of stained cells. The percentage of positive stained cells was recorded as the PCNA labelling index (PCNA-LI).

STATISTICAL ANALYSIS

The PCNA-LI and other clinicopathological variables were analysed using the χ^2 test. Disease free and overall survivals were calculated as the interval from the date of the operation to the recurrence or the death from causes related to breast cancer, respectively. For univariate analysis, the overall and disease free survivals were studied using the Kaplan-Meier method, and the log-rank test was used to analyse differences for significance. For multivariate analysis, Cox's life table regression model (proportional hazards general linear model) was used to examine several variables simultaneously to test for their independent prognostic values. Differences were considered significant when the p value was less than 0.05.

Results

ASSOCIATION BETWEEN PCNA-LI AND CLINICAL AND HISTOLOGICAL VARIABLES

PCNA staining was confined to nuclei (figure) and showed diffuse, granular, or a mixture of both types of staining. Some cells stained more strongly than others, but all identifiable staining was regarded as positive. PCNA positivity showed pronounced variation among tumors and the index varied from 0–77% with a mean of 18%. To evaluate the association between PCNA-LI and the clinicopathological factors, tumours were separated on the basis of the mean value. Ninety three tumors had a low PCNA-LI of less or equal than 18% and 47 a high PCNA index

PC10 immunostaining of breast cancer showing many positive nuclei.

Table 1 Association between PCNA labelling index with clinical and histological variables

	PCNA-LI (%)				PCNA-LI (%)		
	≤18	>18	p Value		≤18	>18	p value
Age (years):				Histological type:			
0-35	7	5	NS	Non-invasive	2	3	NS
36-50	45	26		Invasive duct	85	45	
≥51	41	20		Special type of invasive	6	3	
Menopausal status:				Tumour size:			
Pre-	49	27	NS	0-20 mm	37	22	NS
Post-	44	24		21-50 mm	43	26	
				≥51 mm	13	3	
Clinical stage:				AX metastases:			
TIS	2	3	NS	0	58	27	NS
Stage 1	21	7		1-3	17	14	
Stage 2	47	29		≥4	18	10	
Stage 3	23	12					

LI: Labelling index; AX: Axillary lymph node; NS: Not significant.

of more than 18%. There was no significant correlation between PCNA-LI and all prognostic factors included in this study (table 1). On the other hand, the incidence of AX metastases was evaluated in relation to tumour size and PCNA-LI. The incidence of AX metastases was not related to tumour size and PCNA-LI (table 2). But there was a trend for the incidence of AX metastases to be higher in the group with a low PCNA-LI.

UNIVARIATE ANALYSIS OF SURVIVAL

Of the 144 patients, 113 were alive without recurrence in March 1992, five were alive with recurrence, 22 had died of recurrence, and four had died of causes unrelated to breast cancer. Overall and disease free survival were evaluated by univariate analysis (table 3). Significant differences were found between the patient groups with respect to tumour size and AX metastases for overall and disease free survival; clinical stage was significant only as a factor for overall survival (table 3). PCNA-LI failed to identify any group with shorter overall survival and increased risk of earlier recurrence. The 10 year disease free survival after surgery was 75% in the low PCNA-LI group and 73% in the high PCNA-LI group; the 10 year overall survival showed a trend towards shorter survival for the low PCNA-LI group (73%) compared with the high PCNA-LI group (82%) (table 4).

Table 2 Incidence of AX metastases related to tumour size and PCNA labelling index

Tumour size (mm)	PCNA LI (%)	
	≤18	>18
0-20	10/37	8/20*
20-50	20/44	11/26
≥51	8/13	2/3

LI: Labelling index; *The difference was not significant.

Table 3 Univariate analysis of prognostic variables on overall and disease free survival (n = 144)

Variables	Overall survival p value	Disease free survival p value
Age	NS	NS
Menopausal status	NS	NS
Clinical stage	<0.05	NS
Histological type	NS	NS
Tumour size	<0.01	<0.05
AX metastases	<0.001	<0.001
PCNA-LI	NS	NS

LI: Labelling index; AX: Axillary lymph node; NS: not significant.

Table 4 10 year overall and disease free survival as a function of PCNA labelling index

PCNA LI (%)	Overall survival (%)	p Value	Disease free survival (%)	p Value
≤18	73	NS	75	NS
>18	83		73	

LI: Labelling index; NS: Not significant.

Table 5 Multivariate analysis of prognostic variables on overall and disease free survival (n = 144)

Variables	Overall survival p Value	Disease free survival p Value
Age	NS	NS
Menopausal status	NS	NS
Clinical stage	NS	NS
Histological type	NS	NS
Tumour size	NS	NS
AX metastases	<0.001	<0.001
PCNA-LI	NS	NS

LI: Labelling index; AX: Axillary lymph node; NS: Not significant.

MULTIVARIATE ANALYSIS OF SURVIVAL

To assess the relative importance and independence of the factors. Cox's multivariate analysis was performed and the results are shown in table 5. The only independent prognostic factor was AX metastasis.

Discussion

The presence and extent of AX metastases are the best predictors of prognosis in breast cancer.^{24,25} Despite the confirmed prognostic value of nodal status,²⁶ a more basic understanding of the biology of breast cancer is needed to determine which tumour will be more aggressive and necessitate more intensive treatment. Therefore, there has been a constant search for indicators which might elucidate the biological behaviour of breast carcinoma.

Immunohistochemical labelling of tumour cells with monoclonal antibodies against PCNA is a possibility for clinical assessment of proliferation. This has had an important role in the prognosis of various neoplasms.¹ PCNA is a 36 kilodalton DNA polymerase δ auxiliary protein.²⁷ It has been shown that two types of nuclear staining with anti-PCNA can be obtained depending on the fixation: a distinct granular pattern which is S-phase specific; and a more diffuse form which is not S-phase specific.^{28,29} With the monoclonal antibody PC10 used in this study, total PCNA (granular and diffuse) was stained after fixation with formalin. Although PCNA immunoreactivity seems to be limited to the proliferation compartment in normal tissues, knowledge of the association between PCNA expression and proliferation in different kinds of neoplasm is insufficient, especially in breast cancer.^{12,15,16,30} Hall *et al*¹⁶ have reported that in some forms of neoplasia, including breast and gastric cancer as well as haemangiopericytomas, the association between PCNA expression and S-phase fraction is absent. On the other hand, Garcia *et al*¹⁵ and Dawson *et al*³¹ have recently described that PCNA staining was correlated

with the S phase fraction. In gastric carcinomas, however,¹⁹ and in haemangiopericytomas,¹⁸ PCNA staining still seems to be of prognostic relevance. In all neoplasms studied before, the PCNA-LI seemed to be higher than would have been expected from the S-phase determination by flow cytometry. This discrepancy could be explained by the fact that PCNA staining seems to detect not only cells in S phase but also those in late G₁, G₂, and M phases.^{16 32 33} Moreover, the increased expression of PCNA in some tumour types might be a result of autocrine or paracrine stimulation by certain growth factors,¹⁶ such as platelet derived growth factor, fibroblast growth factor, and epidermal growth factor.^{34 35}

The lack of association between PCNA-LI and clinicopathological variables and survival of patients with breast cancer could be attributed to several factors. First, PCNA immunoreactivity is highly sensitive to fixation time, which may change the real PCNA profile of the neoplasms. Second, deregulated expression of PCNA and its prolonged half-life (20 hours) may permit its immunohistochemical detection in cells which have recently left the cell cycle.^{10 16} Moreover, PCNA may not only be involved in cell proliferation but also in DNA repair.³⁶ Another obvious reason for this lack of correlation is the fact that other factors can be as important as the rate of cell proliferation in contributing to the clinical outcome of patients with breast cancer. It would be very unlikely for a single variable like PCNA-LI to reflect the overall behaviour of such a heterogeneous disease as breast cancer. Finally, immunohistochemistry with PC10 results in very varied labelling, which is apparently not correlated with clinicopathological variables and patient survival in breast cancer.

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