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Prognostic value of the combined assessment of proliferating cell nuclear antigen immunostaining and nuclear DNA content in invasive human mammary carcinomas

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Abstract. The expression of the S-phase associated, nuclear protein proliferating cell nuclear antigen (PCNA) was investigated in routinely paraffin-embedded surgical specimens from 209 breast cancer patients. Cytometric DNA assessments were performed on fine-needle aspirates, upon which the primary diagnosis of breast cancer had been based. The mean clinical follow-up was 16 years (range 13–20 years). The percentage of PCNA immunoreactive tumour cells ranged between less than 5 to 60% (mean value 13.34%). There was a direct association between PCNA expression, high histological tumour grade ($p<0.01$), and DNA aneuploidy ($p=0.009$). In a subgroup of 22 patients with near-diploid DNA distribution patterns the PCNA expression yielded additional prognostic information. Patients with tumours of near-diploid DNA histograms and more than 20% of PCNA immunoreactive neoplastic cells had a significantly worse clinical course, than patients with near-diploid tumours containing less than 20% PCNA immunoreactive cells ($p=0.0001$). In contrast, the PCNA immunoreactivity did not yield additional prognostic information for patients with distinctly diploid or highly aneuploid tumour variants. In a multivariate analysis comprising all 209 patients, nodal status ($p<0.01$), tumour size ($p<0.01$), and DNA ploidy ($p<0.01$) were found to have significant prognostic effect. The findings indicate that carcinomas characterised by high proliferative activity and near-diploid DNA distribution patterns can show rapid tumour progression. The combined assessment of the PCNA immunoreactivity and of the nuclear DNA content in routinely processed surgical specimens of breast cancer patients appears to be of prognostic value.

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Introduction

The clinical behaviour of human breast carcinomas is known to be difficult to predict. Many attempts have been and are being made to find reliable, reproducible and useful prognostic variables. In this context evidence has been obtained that in addition to the established histopathological criteria in current use the crude nuclear DNA content and even cell kinetic data might also be of value (Clark and McGuire 1992; Meyer 1984; Silvestrini et al. 1985; Tubiana et al. 1989).

A growing body of evidence suggests that the cytometric nuclear DNA distribution pattern is closely related to the clinical outcome in mammary carcinomas (Clark et al. 1989; Fallenius et al. 1988; Merkel and McGuire 1990). Patients with tumours that are composed of neoplastic cells of DNA diploid type have a favourable course in general. In contrast, patients with tumours of DNA aneuploid type often have a poorer clinical outcome.

Estimations of the proliferation rate in malignant tumours can be made by means of different techniques. The theoretically advantageous use of tritiated thymidine uptake or bromodeoxyuridine labelling index are however time-consuming and complex. Flow cytometry is hampered by the fact that calculation of the S-phase fraction might also include non-neoplastic stromal and inflammatory cells and thus lead to an underestimation of the real fraction of tumour cells in S-phase (Falkmer et al. 1990).

Recently, monoclonal antibodies against the 36 kDa, S-phase-associated, nuclear protein: proliferating cell nuclear antigen (PCNA) have been developed for use in immunohistochemistry (Ogata et al. 1987; Waseem and Lane 1990). The protein is an auxiliary protein of the DNA polymerase-delta and is essential for cellular DNA synthesis (Waseem and Lane 1990). Evidence has also been obtained that PCNA is required for leading and lagging strand synthesis at the replication fork in SV40 virus replication in vitro (Prelich and Stillman 1988). Several studies have shown that some of these

antibodies, especially that derived from a clone designated PC10, are suitable for immunohistochemical investigations in conventionally fixed and processed tissue (Hall et al. 1990; Isik et al. 1992; Suzuki et al. 1992; Waseem and Lane 1990).

Against this background we investigated if cell kinetic data as obtained by means of the PCNA immunoreactivity might be of prognostic value in addition to various tumour characteristics, including c-*erb*B-2 proto-oncogene expression. We also examined whether PCNA expression could give additional prognostic information in subgroups of patients as characterised by different nuclear DNA histogram types. The study was carried out on formalin-fixed and routinely processed histological tumour specimens from 209 breast cancer patients with a long term clinical follow-up.

Materials and methods

We studied tumour specimens from 209 women with primary invasive mammary carcinomas, who had been included in a randomised trial comparing adjuvant radiation therapy to the chest wall and regional lymph nodes (45 Gy within 5 weeks) with surgery alone. Details of the surgical treatment, trial design and results have been reported previously (Rutqvist et al. 1989). The mean follow-up was 16 years (range 13–20 years). Histopathological diagnosis was based on the World Health Organisation (WHO) histological typing of breast tumours (WHO 1980).

Murine IgG mAb PC10 was purchased from Novocastra Laboratories (Newcastle upon Tyne, UK). The sheep IgG polyclonal antibody OA-11-854 against the cytoplasmic domain of the human c-*erb*B-2 protein was purchased from Cambridge Research Biochemicals (Cambridge, UK). Immunohistochemical staining was performed by means of an avidin-biotin-peroxidase complex technique. Routinely formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and passed through a graded ethanol series. For staining with the mAb PC10 they were fixed in acetone for 5 min and incubated with 1% NP40 detergent (Sigma Chemical Co., St. Louis, Mo., USA) for 5 min. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in destilled water for 30 min and non-specific staining was blocked with 1% bovine serum albumin in TRIS-buffered saline for 45 min. The sections were then incubated overnight with the mAb PC10 at a dilution of 1:100 at 4° C in a humidified chamber. The second antibody, biotinylated anti-mouse affinity-purified IgG antibody (Vector Laboratories, Burlingame, Calif., USA), was applied at a dilution of 1:200. Diaminobenzidine was then employed as chromogen and the sections were slightly counter stained with haematoxylin for 5 s. The immunostaining procedure for c-*erb*B-2 was that described previously (Schimmelpenning et al. 1992). Positive controls for PC10 were sections of the small intestine, the colon, lymph nodes, and endometrium at different phases of the cycle. Positive control for c-*erb*B-2 was a known case of mammary carcinoma in situ of comedo type.

Distinct nuclear PCNA immunostaining was recorded as positive, irrespective of the staining intensity. In each of the 209 preparations 10 high power fields (magnification ×400) were selected at random within a tumour-containing area of the section. The number of immunoreactive cells were then assessed by two investigators using a double-headed light microscope equipped with an ocular graticule consisting of $10 \times 10 = 100$ fields. Immunoreactive and non-immunoreactive tumour cells were counted separately within the four edge-fields of the grid. Those tumour cells laying under the left or the lower border of an edge-field were not counted, while neoplastic cells laying under the right or upper border were included. Both cell counts were then multiplied by 25 and the percentage of immunoreactive cells in relation to the whole tumour cell population was calculated. These results were scored in 5% intervals (less than 5%, less then 10%, etc.).

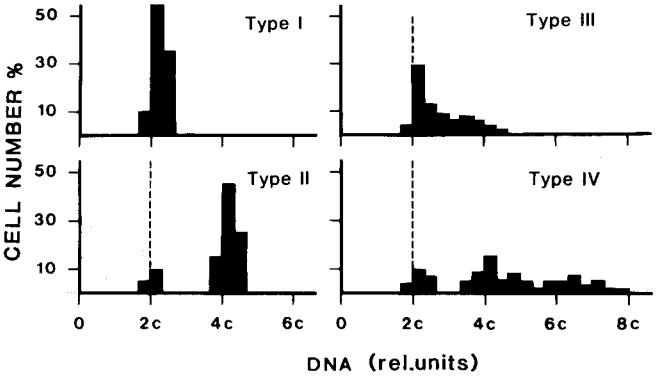

Fig. 1. The cytometrical DNA histograms were classified into four different types according to criteria described by Auer et al. (1980). Histograms of type I had a single distinct peak in the diploid region of the normal cell population. Type II histograms had one well-circumscribed peak in the G_2/M region or two distinct peaks within the G_0/G_1 and the G_2/M region, where the latter had to contain at least 20% of all cell counts. Only a few cells scattered between those two peaks or exceeded them. Histograms of type III had one peak in or near the G_0/G_1 region of the normal cell population and a considerable number of scattered cells in the S-phase region of that peak. Type IV histograms were characterized by highly aneuploid DNA distribution patterns and increased DNA values exceeding the normal G_2/M region ("Manhattan skyline")

The nuclear DNA content was assessed by means of image cytometry according to the Feulgen technique as described previously (Fallenius et al. 1986). The detailed DNA assessment technique, including staining, internal standardization, and tumour cell selection was reported elsewhere (Fallenius et al. 1988). The cytometric DNA histograms were classified into four different types according to criteria described by Auer et al. (1980) (Fig. 1).

The relationship between PCNA immunoreactivity and various histopathological characteristics was analysed using the chi-square test. The prognostic significance of the immunohistochemical and clinico-pathological tumour characteristics was evaluated using distant metastases-free survival as end-point. Univariate and multivariate analyses were performed using Cox's proportional hazards repression model (Cox et al. 1972). Distant metastases-free survival was estimated and plotted using actuarial methods (Cutler and Ederer 1958).

Results

Distinct PCNA staining was almost exclusively limited to the nucleus and only rarely observed in the cytoplasm of mitotic cells. The distribution of nuclear PCNA staining sometimes showed accentuation towards the margins of the tumour. This was however not constant and occasionally also the opposite was observed (Fig. 2a, b). PCNA scores of the 209 primary invasive mammary carcinomas ranged between less than 5 to 60% (mean value $13.63\% \pm 10.89$). The frequency distribution of the PCNA scores is shown in Fig. 3.

The interrelationships between the PCNA immunoreactivity and various histopathological tumour character-

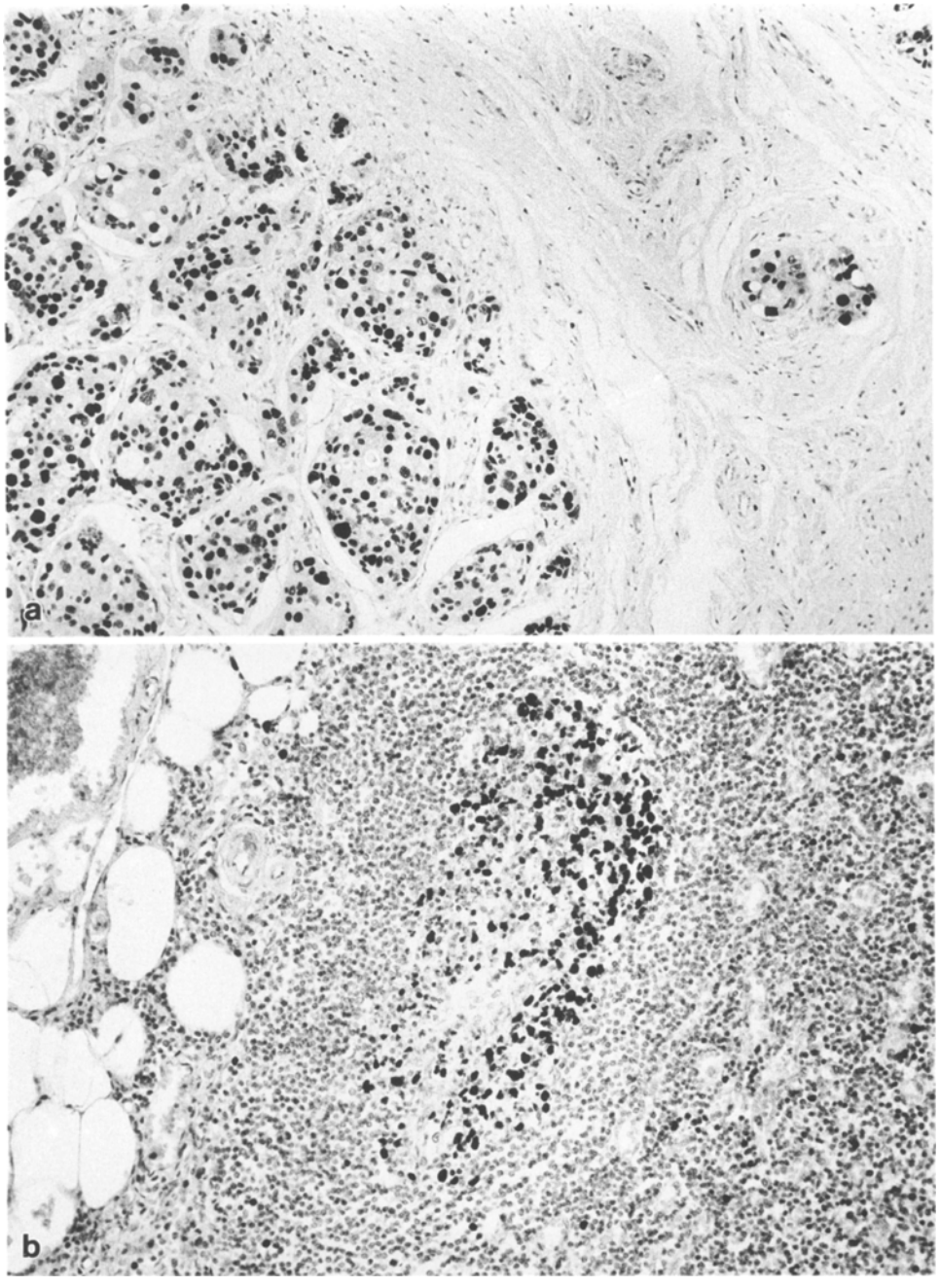

Fig. 2. a Microphotograph of a primary invasive mammary carcinoma of ductal type. Immunohistochemical staining with the mAb PC10 against proliferating cell nuclear antigen (PCNA) in a routine paraffin-embedded section. Note the distinct nuclear staining pattern in >50% of the neoplastic cells. Counterstained with haematoxylin, ×125. **b** Microphotograph of a corresponding lymph node metastasis to **a**. The level of PCNA immunoreactivity is virtually identical with the primary tumour. Note also the immunoreaction of surrounding lymphatic cells. Counterstained with haematoxylin, ×125

istics are summarised in Table 1. There was a direct association between high levels of PCNA expression (>20% of the neoplastic cell nuclei) and a high histological tumour grade, DNA aneuploidy, and the presence of immunohistochemical c-*erb*B-2 expression. Tumours composed of poorly differentiated cells showed a significant increase in PCNA immunoreactivity compared with highly differentiated variants. None of the low grade ductal carcinomas contained more than 20% PCNA immunoreactive tumour cells, while 30 of 88 (34%) of the high grade carcinomas exhibited high proliferative activity (>20% PCNA). Genetically stable tumours of DNA diploid type I had only in 5 of 56 (9%) cases PCNA scores higher than 20%. In contrast, about one third (32 of 98) of the carcinomas of DNA aneuploid type IV had PCNA scores higher than 20%. High levels of PCNA immunoreactivity were more frequently found in carcinomas composed of tumour cells that expressed the c-*erb*B-2 proto-oncogene product than in carcinomas without c-*erb*B-2 expression. No interrelationship was found between PCNA immunoreactivity, tumour size, regional lymph node involvement and menopausal status (data not shown).

In order to investigate the correlation between PCNA scores and distant metastases-free survival three patient groups with low (less than 5%), intermediate (5–20%), and high (more than 20% PCNA immunoreactive tumour cells) PCNA immunoreactivity were formed. Us-

Table 1. Interrelationship between the percentage of PCNA immunoreactive tumour cells and histopathological characteristics in 209 invasive mammary carcinomas

	Percentage of PCNA immunoreactive tumour cells					
	<5%	<10%	<15%	>20%	Total	
Tumour size						
<2 cm	40 (37)	37 (34)	12 (11)	20 (18)	109	n.s.
2–5 cm	16 (27)	10 (17)	10 (17)	21 (36)	57	
<5 cm	6 (33)	2 (11)	5 (30)	5 (30)	18	
Multiple[a]	4 (50)	3 (37)	0 (0)	1 (13)	8	
Unknown[a]	6 (35)	5 (29)	2 (12)	4 (24)	17	
Lymph node status						
pN0	38 (33)	37 (32)	17 (15)	23 (20)	115	n.s.
pN+	33 (36)	19 (21)	12 (13)	28 (30)	92	
Unknown[a]	1 (50)	1 (50)	0 (0)	0 (0)	2	
Tumour grade						
Ductal grade I	7 (47)	5 (33)	3 (20)	0 (0)	15	$p<0.01$
grade II	30 (38)	20 (26)	11 (14)	17 (22)	78	
grade III	26 (29)	20 (23)	12 (14)	30 (34)	88	
Lobular[a]	6 (32)	9 (47)	1 (5)	3 (16)	19	
Medullary[a]	0 (0)	2 (67)	0 (0)	1 (33)	3	
Mucinous[a]	3 (50)	1 (17)	2 (33)	0 (0)	6	
DNA histogram type						
I	23 (41)	19 (34)	9 (16)	5 (9)	56	$p=0.009$
II	13 (37)	9 (26)	5 (14)	8 (23)	35	
III	7 (32)	5 (23)	4 (18)	6 (27)	22	
IV	29 (30)	24 (24)	11 (11)	32 (33)	96	
C-*erb*B-2 immunoreactivity						
Absent	61 (40)	44 (29)	14 (10)	31 (21)	150	$p=0.005$
Present	11 (18)	13 (22)	15 (25)	20 (33)	59	

[a] Not included in the statistical analysis
n.s., Not significant; Percantages in brackets

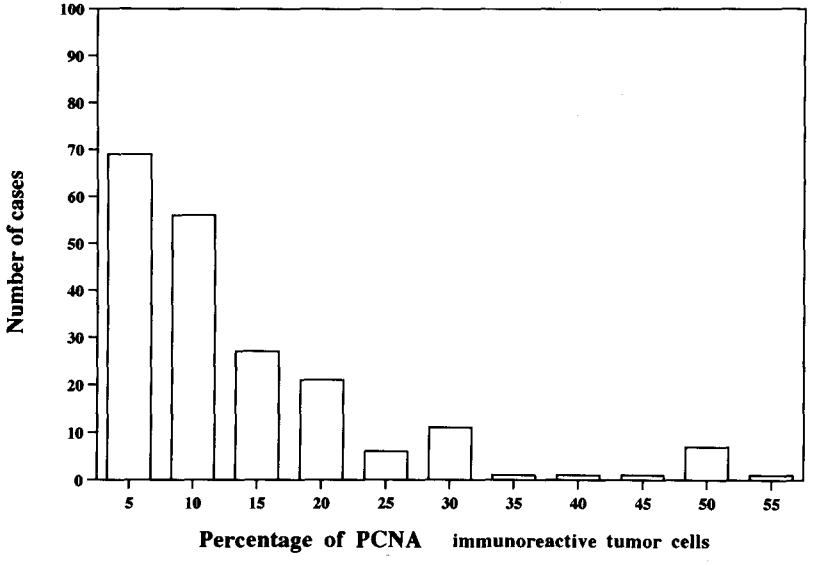

Fig. 3. Frequency distribution of the percentage of PCNA immunoreactive neoplastic cell nuclei of 209 invasive mammary adenocarcinomas

ing this model in univariate analysis, the PCNA score was not significantly associated with distant metastases-free survival (Fig. 4). However, in a subgroup of patients the PCNA immunoreactivity yielded significant prognostic information. There were 22 patients that had carcinomas exhibiting DNA histograms of type III and in 6 of these tumours more than 20% of the neoplastic cells were PCNA immunoreactive (Table 1). Five of these patients with highly PCNA immunoreactive tumours developed distant metastases within 2 years and

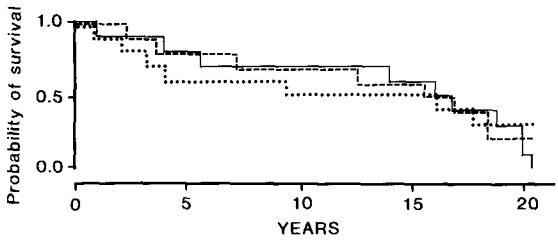

Fig. 4. Distant metastases-free survival of 209 breast cancer patients as stratified by three categories of immunohistochemical PCNA immunoreactivity. —— = less than 5% ($n=72$); – – – – = 5–20% ($n=86$); .... = more than 20% ($n=51$) PCNA immunoreactive tumour cells

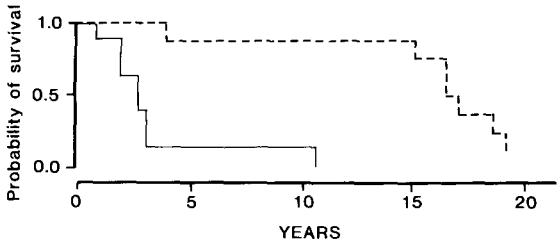

Fig. 5. Distant metastases-free survival for 22 patients with nuclear DNA histograms of type III (near-diploid) according to Auer et al. (1980). Patients with more than 20% (——) ($n=6$) PCNA immunoreactive tumour cells had a significantly shorter recurrence-free survival than those with PCNA expression in less than 20% (– – – –) ($n=16$) of the neoplastic cells ($p=0.0001$)

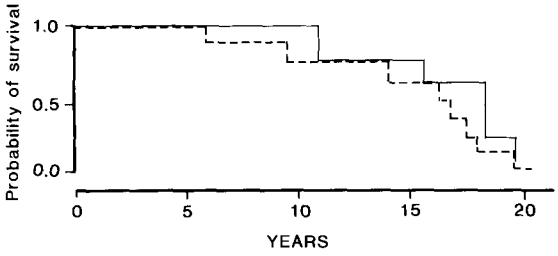

Fig. 6. Distant metastases-free survival for 56 patients with nuclear DNA histograms of type I (diploid) according to Auer et al. (1980). The immunohistochemical PCNA immunoreactivity was of no prognostic value among these patients. —— = less than 20% ($n=51$); – – – – = more than 20% ($n=5$) PCNA immunoreactive tumour cells

died within 3 years after initial treatment due to their neoplastic disease. Four of these patients had regional lymph node metastases already at the time of operation. Two of the tumours were less than 2 cm in diameter, two measured 2–5 cm, and one patient had multiple primary tumours. In contrast, the other 16 patients with tumours of DNA histogram type III and less than 20% PCNA immunoreactive cells had a significantly longer distant metastases-free survival time ($p=0.0001$) (Fig. 5). No similar interrelationships were found between PCNA immunoreactivity and the other nuclear DNA histogram types I, II, and IV (Fig. 6).

Of the other histopathologic criteria investigated lymph node status ($p<0.01$), tumour size ($p<0.01$), and the cytometrical DNA histogram type ($p=0.001$) were of prognostic value in univariate analysis. The histopathological tumour grade was of borderline significance ($p=0.07$). Patients with lobular carcinomas had a shorter distant metastases-free survival than patients who had high grade carcinomas of ductal type. The difference between the curves retained however only borderline significance ($p=0.08$, data not shown; mucinous and medullary carcinomas not included in the analysis). The immunohistochemical c-*erb*B-2 expression was of prognostic significance only among lymph node positive patients ($p=0.02$). In multivariate analysis lymph node status ($p<0.01$), tumour size ($p<0.01$), and DNA histogram type ($p<0.01$) yielded prognostic information. Treatment (post-operative radiation or surgery alone), age, and menopausal status were not significantly correlated to the distant metastases-free survival (data not shown).

Discussion

In the present study we found an association between high levels of immunohistochemical PCNA expression and a low grade of tumour cell differentiation, DNA aneuploidy and also the presence of c-*erb*B-2 immunoreactivity. In a subgroup of patients with near-diploid tumours of histogram type III PCNA immunoreactivity was of significant prognostic value.

The PCNA protein was independently identified by Miyachi et al. (1978) and by Bravo and Celis (1980). It has been described as an auxiliary protein of the DNA polymerase-delta and is under requirement of ATP necessary for DNA replication and repair (Nishida et al. 1989; Tsurimoto and Stillman 1990). It could also been shown that PCNA is essential for cell cycle progression. Autoantibodies to PCNA have an inhibitory effect on DNA synthesis and cellular proliferation in cultured cells (Liu et al. 1989). Eukaryotic cells are equipped with to forms of PCNA. One is detergent extractable and is found in proliferating cells but not in resting cells. The other variant is detergent resistant, associated with replication sites and closely related to DNA synthesis (Bravo and Macdonald-Bravo 1987).

In the present study the proliferative activity as measured by PCNA immunoreactivity was higher than it has been reported from investigations with tritiated thymidine or flow cytometry in breast carcinomas (Meyer et al. 1984; Falkmer et al. 1990). Similar observations were made by Galand and Degraef (1989), who compared immunostaining with the PCNA mAb 19A2 with tritiated thymidine incorporation in biopsy sections from human colon epithelium. This phenomenon could probably be explained by the observation that formalin-fixed tissue not only reveals the insoluble fraction of PCNA bound to active ribosomes but also persisting soluble PCNA molecules present in non-S-phase proliferating cells (Bravo and Macdonald-Bravo 1987; Garcia et al. 1989). Garcia and co-workers, in an immunohistochemical study using the mAb 19A2 found discrepancies between PCNA expression and flow cytometric S-phase analysis in methacarn-fixed human breast parenchyma

(Garcia et al. 1989). The well-known fact that the S-phase indices based on flow cytometric data might also include non-proliferating stromal and inflammatory cells, and thus lead to an underestimation of the true S-phase fraction was emphasised (Falkmer et al. 1989; Garcia et al. 1989). Against this background it has been stated that immunohistochemical analyses of PCNA expressions in formalin-fixed tissue samples allows the assessment of the proliferative activity in human tissue, nevertheless (Galand and Degraef 1989).

The levels of PCNA immunoreactivity found in the different histological subtypes of mammary adenocarcinomas appears to be in agreement with the clinical behaviour of the various neoplasms. Relatively slowly advancing mucinous carcinomas were never composed of more than 15% PCNA immunoreactive tumour cells. As one could expect, at least one of the three medullary carcinomas was highly proliferative. In ductal carcinomas none of the low grade tumours had a high fraction of PCNA immunoreactive cells. This, and the concordance between increased levels of the immunohistochemical PCNA expression and a high histological tumour grade has also been reported by others (Dawson et al. 1990; Robbins et al. 1987). The finding might be interpreted as a result of the higher proliferative activity in poorly differentiated ductal neoplasms but in this context it is noticeable that necrotic areas are usually found in poorly differentiated tumours (Page and Sakamoto 1987). A net increase in proliferative activity does thus not necessarily lead to a net increase in tumour progression. It seems, in contrast that the elevated levels of PCNA expression found in poorly differentiated carcinomas do not merely reflect high proliferative activity. Evidence has been obtained that UV irradiation of quiescent human fibroblasts triggers the appearance of PCNA (Toschi and Bravo 1988). Against this background the increased PCNA expression in poorly differentiated tumour cells may also be the result of an activation of DNA repair mechanisms in these genetically unstable tumours, which are often characterised by aneuploid DNA distribution patterns. This suggestion is further supported by the observation that in this study there was a significant association between high levels of PCNA immunoreactivity and DNA aneuploidy. Only about 10% of the carcinomas with histograms of type I (diploid) exhibited high levels of PCNA expression. In contrast about one-third of the tumours with aneuploid histograms of type IV were highly PCNA immunoreactive.

Investigations on the duration of mitosis in three human breast cancer and one embryonal carcinoma cell line using a video-based time-lapse technique suggest that cells with near-diploid DNA distribution patterns complete the cell cycle significantly faster than highly aneuploid variants (Sennerstam and Auer 1990). This is plausible, as genetically stable cells with less extended DNA alterations do not have to accomplish the same DNA synthesis rate and seem not to activate DNA repair mechanisms to the same extent as aneuploid cells with highly increased amounts of nuclear DNA. One should expect that carcinomas characterised by high proliferative activity and near-diploid DNA distribution patterns revealed rapid tumour progression. This hypothesis was confirmed by our findings. Though the number of patients was limited it is noticeable that all patients with carcinomas that met the above mentioned criteria had a significantly poorer clinical course, than patients with near-diploid carcinomas of DNA histogram type III that where composed of less than 20% PCNA immunoreactive cells. Similar observations were made by Clark and co-workers, who found that diploid mammary carcinomas with a high S-phase fraction, as determined by flow cytometry, had a poorer prognosis than diploid tumours with a low S-phase fraction (Clark et al. 1989).

The association between elevated levels of PCNA expression and histopathological criteria connected with poor prognosis (high histological grade, DNA aneuploidy, and presence of immunohistochemical c-*erb*B-2 expression) might lead one to expect that the PCNA expression alone could be a significant prognostic factor. This was not confirmed by this study. Tumour progression seems not to be dependent on the proliferative activity that can be assessed on a surgical specimen at the time of operation. Tumour cell proliferation is probably indicative of interactions between the effects of oncogenes, angiogenesis and other factors.

We conclude that the assessment of the PCNA immunoreactivity in combination with nuclear DNA measurements on archival surgical specimens may add valuable prognostic information in a subgroup of patients. Near-diploid tumours with increased PCNA expressions seem to be a risk-group. Additionally, the assessment of tumour cell proliferation is gaining further importance in the therapeutic frame works in which chemo- and or radiotherapy are employed. It is noteworthy that assessments of both PCNA immunoreactivity and nuclear DNA content can be performed on pre-operatively taken fine-needle aspirates. This might contribute to individual, patient adapted treatment procedures at an early stage of therapy.

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