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# PCNA and Ki67 expression in breast carcinoma: Correlations with clinical and biological variables

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## Abstract

***Aims:*** **To investigate the expression of two cell cycle related antigens (proliferating cell nuclear antigen (PCNA) and Ki67 related antigen) in a series of breast cancers; and the possible correlations between the PCNA and Ki67 labelling indexes (PCNA-LI and Ki67-LI) and their associations with other biological and clinicopathological variables.**

***Methods:*** **Ninety six ductal and 10 lobular carcinoma specimens were investigated. Samples were fixed in formalin and in Methacarnoy for localisation of PCNA. Ki67 was immunostained on frozen sections. The PCNA-LI and Ki67-LI were evaluated in relation to tumour size, mitotic count, histological grade, nodal state as well as receptor content and altered expression of the p53 gene.**

***Results:*** **PCNA-LI did not correlate with Ki67-LI, nor was it associated with any other variable examined. A high KI67-LI (above the median value of 13·5) was associated with high grade and mitotic count, negative receptor content, and altered expression of the p53 gene, but not with other variables.**

***Conclusions:*** **The PCNA-LI does not seem to be a substitute for the Ki67-LI in evaluating the growth fraction in breast cancer.**


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Breast cancer is a common neoplasm, the outcome of which may vary widely. It is therefore important to establish reliable and reproducible prognostic tests that will help to select optimal treatment for each case.[1] Tumour cell proliferation is an important biological variable, which can be regarded as an additional prognostic indicator.[2] The proliferative activity of breast cancers has been evaluated using several methods, among which is the study of the percentage of cells expressing cell cycle related antigens.[1] One of the most extensively investigated is that detected by the monoclonal antibody Ki67,[3] while data on the expression of the proliferating cell nuclear antigen (PCNA) are more limited. PCNA is an auxiliary protein of DNA polymerase $\delta$. It seems to be essential for DNA synthesis and is expressed in high concentrations during the cell cycle.[4] Monoclonal antibodies against PCNA, which work on routinely fixed specimens have been developed recently.[5] But the data on PCNA expression in fixed samples of breast cancers are limited and contradictory.[4 6]

The potential value of a proliferation marker like PCNA, which can be applied to routinely fixed specimens, prompted us to evaluate its expression in a series of 106 unselected, untreated breast cancers. The aim was to evaluate the relation between the PCNA labelling index (PCNA-LI) and the Ki67 labelling index (Ki67-LI), and their relation with clinicopathological variables—oestrogen/progesterone receptor content,[1] and altered expression of the p53 gene.[7–10]

## Methods

### TUMOUR TISSUE

One hundred and six unselected human breast cancer specimens from untreated patients were collected at surgery, snap-frozen in liquid nitrogen, and stored at −80°C. A second parallel sample was fixed in formalin for 24 hours and a third one was fixed in Methacarnoy; both were subsequently processed using routine techniques and paraffin wax embedded. Ninety six cases were infiltrating ductal carcinomas and 10 were infiltrating lobular carcinomas. Tumour size, lymph node state, histological grade (classified according to the modified Bloom and Richardson method for the 96 ductal carcinomas,[11] and mitotic count (mitoses per 10 high power fields, counted according to Baak)[12] were recorded for each case. Mitotic counts were started in the most active areas of the neoplasms where the number of mitoses was highest. Once started, nine additional contiguous fields were randomly selected. In selected cases four sets of 10 high power fields were examined and the highest score was recorded as the mitotic index. The axillary lymph nodes had been removed in 95 cases.

### PCNA AND KI67 IMMUNOSTAINING

The PC10-murine IgG2 anti-PCNA monoclonal antibody[5] (Novocastra Laboratories) was used on formalin and Methacarnoy fixed sections at 1 in 400 and 1 in 800 dilutions, respectively, with overnight incubation at 4°C. The site of the antibody binding was visualised with the streptavidin-peroxidase labelled method (StrAviGen Super Sensitive Concentrated Detection System; Biogenex Laboratories). Ki67 (Dako) was used on frozen sections at a 1 in 150 dilution for one hour at room temperature; biotinylated anti-mouse IgG (1 in 200, Vector, Burlingame, California, USA) and the avidin-biotin complex (1 in 100,

Vector, Burlingame) were added in sequence.

PCNA and Ki67 immunostainings were scored by counting at least 500 cells in more than 10 high power representative fields. Every stained nucleus was considered positive, irrespective of intensity. In cases where staining was heterogeneous in the tumour, examined fields included those with the highest and those with the lowest percentage of stained cells. Tumours were independently scored by two observers. The percentage of positive stained cells was recorded as the PCNA labelling index and the Ki67 labelling index (LI).

#### OESTROGEN AND PROGESTERONE RECEPTOR CONTENT

Frozen sections were immunostained with monoclonal anti-receptor antibodies (oestrogen and progesterone receptor immunocytochemical assay, ER-ICA and PgR-ICA kits; Abbott Laboratories, Chicago, Illinois, USA). The immunostaining was assessed on the basis of the visually estimated percentage of neoplastic cells with positive nuclear staining and on staining intensity. All cases with negative staining and those with less than 10% of stained cells were regarded as negative.

#### P53 GENE EXPRESSION

The monoclonal antibody PAb 1801[13] (Oncogene Science, Manhassett, New York, USA) was used for the immunolocalisation of p53 protein. Frozen sections were air dried, fixed in cold methanol and acetone, and incubated for two hours at room temperature with the primary antibody, at a 1 in 200 dilution. Biotinylated anti-mouse IgG and avidin-biotin peroxidase complex (ABC) were added in sequence (Vectastain ABC Kit; Vector).

Statistical analyses were performed using the Microstat statistical software (Ecosoft, Inc) run on an Olivetti 286 PC.

Frequency tables were tested for association using the $\chi^2$ test. The correlations between the PCNA-LI, Ki67-LI, and mitotic count were evaluated using Spearman's rank correlation coefficient.

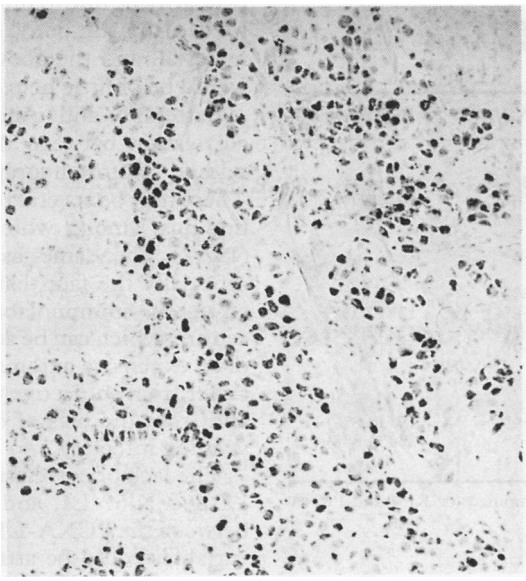

*Figure 1 Ki67 immunoreactivity in infiltrating ductal breast carcinoma. Staining is both nuclear and nucleolar (ABC method with light nuclear counterstain).*

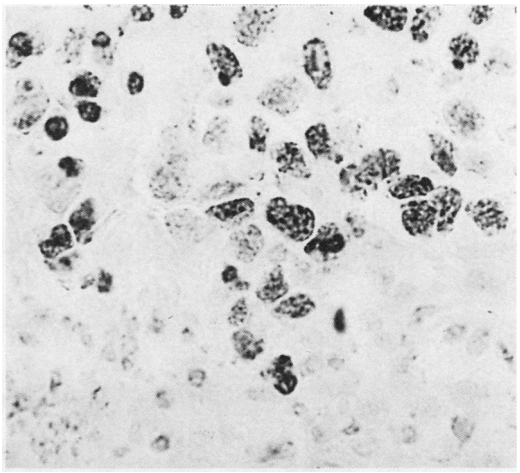

*Figure 2 PCNA immunoreactivity in infiltrating ductal breast carcinoma, with predominant granular immunoreactivity (streptavidin biotin labelled method with light nuclear counterstain).*

## Results

Ki67 immunostaining was nuclear and nucleolar (fig 1). PCNA immunostaining was clearly evident as granular (in Methacarnoy fixed specimens) or homogeneous (in formalin fixed specimens) nuclear staining (fig 2). Staining intensity was variable, and minor degrees of intratumoural heterogeneity were seen.

p53 protein overexpression was seen in 27 (25·5%) cases. Most positive cases (21) showed nuclear immunostaining in all or nearly all malignant cells (fig 3). Six other cases showed only single carcinoma cells with clear nuclear immunoreactivity. These staining patterns agree with the results of Bartek *et al.*[9] The percentage of positive cases is somewhat lower than that reported by Bartek *et al.*[9]

Median and mean mitotic count and labelling index values for Ki67 and PCNA (on formalin and on Methacarnoy fixed specimens) are reported in table 1. Median values were used to subdivide lesions in high and low labelled groups. Results are summarised in tables 2 and 3.

The PCNA-LI on formalin fixed specimens correlated with that on Methacarnoy fixed specimens ($r_s = 0{\cdot}45$, $p < 0{\cdot}001$), but neither of them correlated with the Ki67-LI or mitotic count (Spearman's rank correlation coefficients are shown in table 4). The PCNA-LI (both on formalin fixed and on Methacarnoy fixed specimens) was not associated with grading, tumour size, nodal state, receptor content, and p53 aberrant gene expression.

The Ki67-LI was positively correlated with mitotic count ($p < 0{\cdot}001$) (table 4) and was associated with grade ($p < 0{\cdot}001$) but not with tumour size nor with nodal state. A low Ki67-

*Table 1 Mitotic count, Ki67-LI, and PCNA-LI on formalin and Methacarnoy fixed specimens*

| | *Mean (SD)* | *Median* |
|---|---|---|
| Mitotic count | 21·52 (10·05) | 15 |
| Ki67-LI | 14·77 (10·09) | 13·5 |
| PCNA-LI (F) | 28·72 (22·96) | 24 |
| PCNA-LI (M) | 59·31 (27·09) | 55 |

(F) formalin fixed specimens; (M) Methacarnoy fixed specimens.

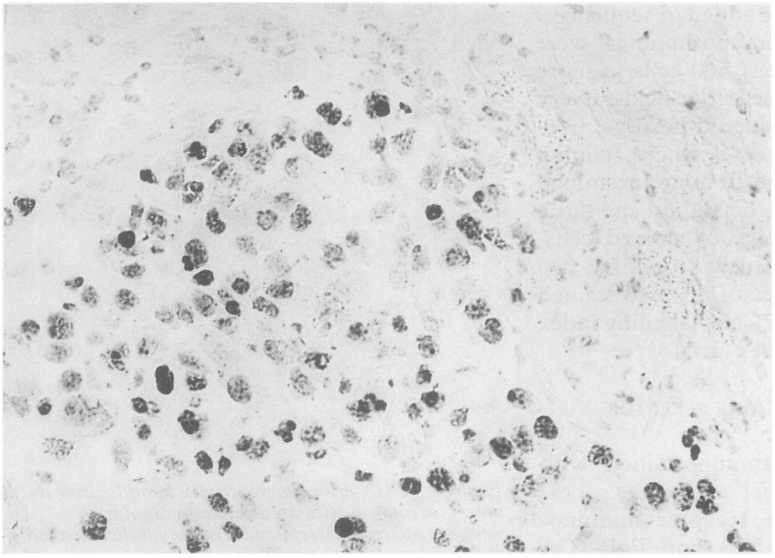

*Figure 3 p53 abnormal expression in infiltrating breast carcinoma. Most of the cells are immunoreactive, with different degrees of intensity (ABC method with light nuclear counterstain).*

LI was associated with positive oestrogen and progesterone receptor content ($p < 0.025$ and $p < 0.001$, respectively), and with absence of p53 immunoreactivity ($p < 0.001$).

## Discussion

The Ki67-LI values agree with the reported mean percentage of Ki67 labelled cells ranging from 9·4 (0·74),[14] 12 (1·49),[15] 12·5 (17·6),[16] 14·5 (12·9)[17] 15·3 (10·1),[18] 16·6,[19] to 20 (14·48).[20] Several reports have shown a significant correlation between the Ki67-LI and histological grade of malignancy and mitotic count, and an inverse relation with receptor content,[3] which has been further confirmed by our study. Tumour size and lymph node state have a controversial association with the Ki67-LI, and our findings reinforce the hypothesis of the lack of association.[3] In our series a strong association between altered expression of the p53 gene and a high Ki67-LI was found, confirming the data of Cattoretti *et al.*[8] The p53 gene has a key function in regulating the cell cycle[21] and is frequently altered in breast cancers.[7-10] p53 gene mutations usually lead to the inappropriate accumulation of the p53 gene product within the nucleus of the cell, reaching the threshold of immunochemical detection.[22 23] Therefore, the demonstration of p53 protein immunoreactivity in tumour sections is indicative of gene alterations and of uncontrolled cell proliferation.[23 24]

Our data on the Ki67-LI and PCNA-LI (both on formalin and on Methacarnoy fixed specimens) show no correlation between the two indices. Similar results were found by Barnes *et al* (unpublished observations cited by Hall *et al*[4]). Moreover, our data show that there is no association between the PCNA-LI (both on formalin and Methacarnoy fixed specimens) and histological grade, mitotic count, tumour size and stage, receptor content and altered expression of the p53 gene. This latter finding suggests that, as far as p53 gene alterations are concerned, PCNA expression in breast cancer may not be related to changes in the cell cycle.

The lack of association between the PCNA-LI in breast cancer and several variables, including grade, growth fraction, and altered expression of the cycle controlling p53 proteins is surprising. PCNA-LI correlates with histological grade[25] and with growth fraction data obtained using flow cytometry,[26 27] tritiated thymidine incorporation,[6] bromodeoxyuridine incorporation[28] and the Ki67 LI.[27 29]

Our data conflict with the reports on PCNA immunostaining in breast cancer made by Dawson *et al*[27] and Battersby and Anderson.[6] Dawson *et al* reported that PCNA positivity correlates with Ki67 immunostaining, with S phase fraction determined by flow cytometry, and with worsening nuclear grade.[27] Their study was performed with a different antibody (19A2), however, and their cases were analysed on frozen sections. In fact, different results have been obtained using the two different antibodies (PC10 and 19A2) on fixed and frozen sections, respectively, and also in other fields of histopathology—for example, the different results obtained on central nervous system tumours by Louis *et al*[30] and Allegranza *et al.*[25] If the PCNA-LI gains wide acceptance it will primarily be due to the possibility of being able to use the antibody on routinely processed material, and the above data of Dawson *et al* would be of little help in this regard.[27]

Battersby and Anderson found a linear correlation between the PCNA-LI and thymidine autoradiography labelling index.[6] There may be several reasons for their different findings, among which is the antibody used (19A2), the same as Dawson *et al*,[27] and especially the fact that they counted only the strongest immunolabelled cells. This procedure, which can be adopted only with the use of an expensive automatic image analyser, may suffer from subjective threshold determination and from the bias of day to day variations in staining intensity.

The lack of correlation between the PCNA-LI and Ki67-LI, and the lack of association between the PCNA-LI and clinicopathological variables could be attributable to several fac-

*Table 2 Ki67LI and PCNALI in relation to clinicopathological variables in 106 breast cancers (data for PCNA immunostaining are derived from formalin fixed specimens)*

| | *Low Ki67LI* | *High Ki67LI* | *Low PCNALI* | *High PCNALI* |
|---|---|---|---|---|
| *Grade*:* | | | | |
| G1 | 16 (16·7%) | 2 (2·1%) | 8 (8·3%) | 10 (10·4%) |
| G2 | 21 (21·9%) | 15 (15·6%) | 14 (14·6%) | 22 (22·9%) |
| G3 | 10 (10·4%) | 32 (33·3%) | 25 (26·0%) | 17 (17·7%) |
| | $p < 0.001$ | | NS | |
| *Tumour size (cm):* | | | | |
| <1 | 6 (5·7%) | 5 (4·7%) | 4 (3·8%) | 7 (6·6%) |
| 1–1·5 | 15 (14·2%) | 10 (9·4%) | 12 (11·2%) | 13 (12·3%) |
| 1·6–2 | 11 (10·4%) | 16 (15·1%) | 14 (13·2%) | 13 (12·3%) |
| >2 | 24 (22·6%) | 19 (17·9%) | 23 (21·7%) | 20 (18·9%) |
| | NS | | NS | |
| *Nodes* | | | | |
| N+ | 16 (16·90%) | 17 (17·90%) | 18 (18·9%) | 15 (15·8%) |
| N− | 30 (31·60%) | 32 (33·60%) | 29 (30·5%) | 33 (34·8%) |
| | NS | | NS | |

Data expressed as the percentage of the total number of cases examined for Ki67 and PCNA immunostaining, respectively.
*Grade was established only in the 96 infiltrating ductal carcinomas.
†Node state was examined only in 95 cases.

*Table 3 Ki67LI and PCNALI in relation to biological variables in breast cancers (data for PCNA immunostaining are derived from formalin fixed specimens)*

| | Low Ki67LI | High Ki67LI | Low PCNALI | High PCNALI |
|---|---|---|---|---|
| ER+ | 49 | 35 | 45 | 39 |
| ER− | 7 | 15 | 8 | 14 |
| | $p < 0.05$ | | NS | |
| PgR+ | 43 | 23 | 32 | 34 |
| PgR− | 13 | 27 | 21 | 19 |
| | $p < 0.005$ | | NS | |
| p53+ | 1 | 26 | 17 | 10 |
| p53− | 55 | 24 | 36 | 43 |
| | $p < 0.001$ | | NS | |

ER = oestrogen receptor.
PgR = progesterone receptor.

*Table 4 Spearman's rank correlation coefficients*

| | Ki67-LI | PCNA-LI F | PCNA-LI M | Mitotic count |
|---|---|---|---|---|
| Ki67-LI | | 0·01 | 0·02 | 0·49* |
| PCNA-LIF | 0·01 | | 0·45* | 0·23 |
| PCNA-LIM | 0·02 | 0·45* | | 0·06 |
| Mitotic count | 0·49* | 0·23 | 0·06 | |

(F) Formalin fixed specimens; (M) Methacarnoy fixed specimens.
*Highly significant.

tors. Primarily, PCNA immunoreactivity is highly sensitive to fixation time, which may alter the real PCNA profile of the neoplasms. Deregulated expression of PCNA and its prolonged half life, which may permit its immunohistochemical detection in cells which have recently left the cell cycle, may also account for some of the above discrepancies.[4 31] Moreover, PCNA may not only be involved in cell proliferation but also in DNA repair.[32]

In summary, our data suggest that the PCNA-LI is not the same as the Ki67-LI in breast carcinoma. To have a more definitive conclusion about the possible value of PCNA immunostaining in breast cancer, however, further studies are required to evaluate the PCNA-LI in relation to disease free interval and overall survival.

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