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Cell Proliferation of Breast Cancer Evaluated by Anti-BrdU and Anti-Ki-67 Antibodies : Its Prognostic Value on Short-term Recurrences

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The prognostic value of breast cancer proliferative activity was evaluated in 385 women operated for primary, non-metastasised mammary carcinoma. Cell kinetics was measured using two immunohistochemical techniques. Cells in S-phase of cell cycle were labelled *in vitro* by incubation of fresh tissue fragments with 5-bromo 2-deoxyuridine (BrdU), a thymidine analogue. Nuclei of cells in active DNA synthesis were stained by an anti-BrdU monoclonal antibody (Mab). Cells in interphase and mitosis were detected with Ki-67, a Mab that is known to react with a nuclear antigen present in G1/S/G2/M phases of cell cycle, but not in resting cells. This reagent provides a means of evaluating the growth fraction of neoplastic cells. BrdU was incorporated in a proportion of tumour cells ranging from 0.1 to 65.5% (median 6.8%). In the panel of tumours presented in this report the median percentage of Ki-67 positive cells (Ki-67 score) was 9.0% (range 0.1–77%). The relationship between disease-free survival (DFS), BrdU labelling index, Ki-67 score and 13 different clinico-pathological variables was investigated by multivariate analysis, using the Cox proportional hazards model. Axillary node status ($P = 0.009$) and Ki-67 score ($P = 0.038$) emerged as independent prognostic factors. Nodal status and tumour growth fraction allowed division of patients into groups at different risk of relapse : tumours with a proliferative index below the median value showed a lower recurrence rate than tumours with a high proliferative activity ($P < 0.001$). In particular, no relapse occurred in pN0 patients bearing carcinomas with a Ki-67 labelling < 9.0% 4 years after surgery. These findings suggest that the evaluation of proliferative activity in breast cancer enhances the probability of correctly predicting outcome after surgery and could be of assistance in the planning of adjuvant therapies.
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INTRODUCTION

IN BREAST cancer the histological status of axillary lymph nodes is widely accepted as the most reliable prognostic marker for the risk of relapse after surgery [1, 2]. The correct planning of adjuvant therapies in node negative patients demands, however, the use of additional prognostic factors [3].

A number of studies have shown a correlation between the proliferative activity of human primary breast carcinomas and prognosis [4–6]. Thymidine labelling index (TLI), an autoradiographic method that measures the proportion of tumour cells in S-phase of cell cycle, has been the most commonly used indicator of proliferative activity in breast cancer [7]. This procedure, however, is time consuming and the use of a radioactive isotope with a long physical half life, such as tritium, is not accessible to all clinical laboratories, making it unsuitable for large studies involving different institutions.

More recently, several immunohistochemical methods for the assessment of tumour proliferative activity have become available. A non-radioactive alternative to tritiated thymidine,

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based on bromo-deoxyuridine (BrdU), has been shown to be well suited for the detection of cells in S-phase of cell cycle [8]. BrdU, a thymidine analogue, is incorporated into tumour DNA during active cell replication and can be detected immunohistochemically with a specific monoclonal antibody [9]. TLI and BrdU seem to have the same accuracy in the determination of the proportion of S-phase cells but the latter method has the advantage of a better resolution at the labelled sites [10].

The Ki-67 monoclonal antibody identifies a nuclear antigen that is synthesised during the late G1,S,M and G2 phases of cell cycle [11]. This antigen, not being expressed in most resting cells (G0), could provide a reliable means of evaluating the growth fraction (i.e. the proportion of cycling cells) of human tumours [12]. It has already been shown that there is a relationship between Ki-67 staining and TLI [13]. Several studies have been performed with tritiated thymidine, Ki-67 and flow cytometry to measure the cell kinetics of primary breast carcinomas. Very few reports, however, have been recently published on a compared use of the two immunohistochemical techniques based on Ki-67 and BrdU, and none on a large series of patients with known follow-up data.

In this paper we provide evidence that a significant relationship can be demonstrated between the proportion of cells stained by the anti-BrdU and anti-Ki-67 monoclonal antibodies and disease free survival (DFS). The prognostic value of mammary tumour proliferative activity performed by immunohistochemical methods, strongly suggests that the assessment of cell kinetic parameters should become part of the standard routine analyses performed on breast cancer tissue.

MATERIALS AND METHODS

385 eligible women consecutively operated for breast cancer at the Department of Oncology, San Giovanni Vecchio Hospital, between September 1986 and December 1990 were studied.

All tumours were staged according to the post-surgical TNM system (UICC). Eligible patients had: (a) histological diagnosis of invasive carcinoma of the breast; (b) no evidence of distant metastases; (c) complete axillary node dissection.

The proportion of cells expressing nuclear receptors for oestrogen (ER) was determined immunohistochemically on frozen sections with anti-ER monoclonal antibodies following the manufacturer's instructions (ER-ICA, Abbott Laboratories, Chicago, Illinois). The histological tumour grade was evaluated according to Bloom and Richardson [14]. Vascular invasion was referred to both blood and lymphatic vessels.

To perform BrdU labelling, fresh tumour tissue samples were placed in a Petri dish containing Hank's salt solution, not later than 15 min after the operation, cut with razor blades into 1–2 mm fragments and incubated for 3 h at 37°C in RPMI-1640 culture medium with 10% fetal calf serum, 20 mN Hepes buffer and 0.1 mmol/l 5-bromo-2'-deoxyuridine (Sigma). Tissue fragments were washed with phosphate buffered saline (PBS), fixed with Carnoy's solution and paraffin embedded. Five micrometer sections were mounted, deparaffinised in xylene, rehydrated, denatured for 10 min in 4 N HCl, washed with PBS, incubated for 20 min in 10% non-immune horse serum and for 1 h with 1:160 diluted anti-BrdU monoclonal antibodies (Becton and Dickinson). Sections were then washed in PBS and incubated for 30 min with a biotinylated secondary antibody (horse anti-mouse IgG). This was followed by reaction with the avidin–biotin–peroxidase complex reagent (ABC) (Vector Laboratories) and detected with 3-amino-9-ethylcarbazole (AEC) and 0.03% H_2O_2 (Sigma).

Ki-67 labelling was performed on cryostatic sections fixed in absolute acetone at −20°C. After washing in PBS, non-specific staining was blocked with 10% non-immune horse serum for 20 min. and sections were incubated for 1 h with the mouse monoclonal antibody Ki-67 at a 1:20 dilution (Dakopatts, Copenhagen, Denmark). Bound Ki-67 antibodies were visualised with the ABC method and AEC as for the BrdU detection. All immunohistochemical reactions were scored by counting over 500 nuclei of morphologically neoplastic cells (for BrdU nuclear scoring was routinely performed in the outer 300 μm layer). Values were expressed as the ratio between positively-stained cells and total number of counted cells.

After mastectomy, 182 patients with capsular invasion of positive axillary lymph nodes and/or pT4 tumours were irradiated. Seventy node-positive premenopausal patients received chemotherapy (CMF, six courses). 296 patients, 158 pN0 and 138 pN1, were given hormonal therapy (tamoxifen 30 mg daily, planned for 5 years). Follow-up ranged from 6 to 57 months (mean 31). All data were processed with the BMDP series of computer programs (Health Science Computing Facility, UCLA, Los Angeles, California) [15].

The relationship between proliferative activity and nodal status was examined either with the Mann-Whitney test or the Kruskal–Wallis test. Correlation between BrdU and Ki-67 scores was determined by computing Pearson's correlation coefficient.

Multivariate analysis was carried out to assess the relative influence on DFS of 15 possible prognostic factors, using Cox proportional hazard survival regression model (BMDP2L) [16].

Disease free survival curves were calculated by the product limit estimate of Kaplan and Meier [17]. Statistical significance between curves was assessed using the log-rank test [18].

RESULTS

Distribution values of the 15 clinical and pathological factors examined by multivariate analysis are listed in Table 1.

BrdU labelling was performed in 376 tumours and the percentage of stained cells ranged from 0.1 to 65.5% (median 6.8%). Ki-67 labelling was performed in 353 cases and the proportion of Ki-67 positive cells ranged from 0.1% to 77% (median 9.0%). The median was used as a cut-off value of high and low proliferation, both for BrdU and Ki-67.

There was a weak, although significant ($P < 0.001$), positive correlation between BrdU and Ki-67 ($r = 0.439$). As shown in

Table 1. Variables examined by multivariate analysis (number of cases in parentheses)

Age (years)	Range 30–88, median 59
Tumour size	T1 (185), T2 (180), T3–T4 (20)
Tumour diameter (mm)	Range 4–70, median 21
Axillary nodal status	N0 (197), N1 (188)
No. positive nodes	Range 0–31, median (for N1) 3
ER (% stained cells)	Range 0–75, median 5.8
BrdU (% stained cells)	Range 0.1–65, median 6.8
Ki-67 (% stained cells)	Range 0.1–77, median 9.0
Histotype	Ductal (324), lobular (46), other (15)
Histological grade	G-I (16), G-II (203), G-III (105)
Multicentricity	Present (32), absent (353)
Vascular invasion	Present (73), absent (312)
Adjuvant radiotherapy	Administered (182), not administered (203)
Adjuvant chemotherapy	Administered (70), not administered (315)
Adjuvant hormonal ther.	Administered (296), not administered (89)

Table 2. No association was observed between BrdU and Ki-67 scores and nodal status

	BrdU score median		Ki-67 score median	
N0	6.2%		9.0%	
		n.s.*		n.s.*
N1	7.0%		9.0%	

* n.s. = not significant ($P > 0.05$) by Mann-Whitney test.

Table 2, no association was observed between proliferative activity and lymph node involvement.

Multivariate analysis results are summarised in Table 3. Axillary node status (pN) ($P = 0.009$), Ki-67 score ($P = 0.038$) and multicentricity ($P = 0.042$) emerged as independent prognostic factors. In the univariate analysis, first step of multivariate analysis, BrdU score also displayed a significant relationship with DFS ($P = 0.049$), but in the following steps the significance of Ki-67 score became higher. In our series of patients multivariate analysis did not show any significant influence of adjuvant treatments on recurrences. Life tables confirmed the multivariate analysis results. We compared the DFS curves of patients, divided by nodal status, with breast carcinomas having BrdU and Ki-67 cell proliferation indexes below or above the median values. The percentages of patients who were given systemic adjuvant therapies were well balanced in all groups examined (Table 4). As shown in Fig. 1, patients with BrdU scores below the median value, irrespective of their nodal status (N0 and N1 with BrdU $\leq$ 6.8%), had a better DFS than those bearing tumours with a high percentage of cells in S-phase (N0 and N1 with BrdU > 6.8%) ($P < 0.001$). DFS curves of patients divided by nodal status and Ki-67 score (Fig. 2) show that none of the node negative patients bearing tumours with low Ki-67 values (N0, Ki-67 $\leq$ 9.0%) had a relapse. The difference in the DFS between this group and the N0-Ki-67 > 9%, N1 with both high and low Ki-67 values, was highly significant ($P < 0.0007$).

Table 3. Multivariate analysis

Univariate comparison of prognostic factors (step 0) Factors examined	*P* values
Age	0.4532
pT	0.1260
Diameter	0.1533
pN	0.0090
No. positive nodes	0.0248
ER	0.2977
BrdU	0.0491
Ki-67	0.0394
Histotype	0.6634
Grade	0.5036
Multicentricity	0.0510
Vascular invasion	0.0666
Adjuvant radiotherapy	0.7123
Adjuvant chemotherapy	0.1657
Adjuvant hormonal therapy	0.1186

Multivariate comparison of prognostic factors (final step of stepwise proportional hazard regression) Independent factors	*P* values
pN	0.009
Ki-67	0.038
Multicentricity	0.042

Table 4. Percentage of patients who received systemic adjuvant therapy, according to nodal status

	Tamoxifen(%)	CMF(%)
pN0		
BrdU < 6.8%	81.2	0
BrdU > 6.8%	79.3	0
Ki-67 < 9.0%	81.4	0
Ki-67 > 9.0%	79.2	0
pN1		
BrdU < 6.8%	74.0	34.2
BrdU > 6.8%	72.2	39.2
Ki-67 < 9.0%	74.2	34.6
Ki-67 > 9.0%	71.9	38.9

DISCUSSION

Our report is the first in which both BrdU and Ki-67 have been detected on the same panel of primary breast carcinomas. Since BrdU, a thymidine analogue, specifically labels S-phase cells, the purpose of the study was to validate Ki-67 as a reliable marker of proliferative activity.

The poor, although highly significant ($P = 0.001$), positive correlation between BrdU and Ki-67 raises some questions. We believe that this finding, far from being disappointing, is actually

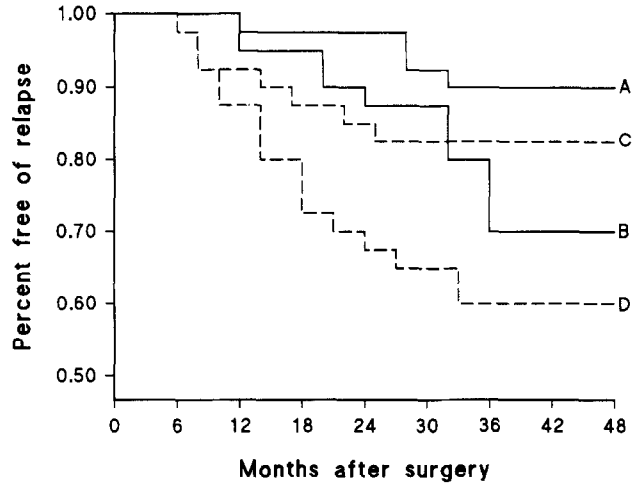

Fig. 1. Disease free survival curves of patients divided according to nodal status and BrdU score. The overall difference between groups by the Mantel–Cox test was significant ($P < 0.0005$). Solid lines : N0; dashed lines : N1.
The log-rank test for the significant paired groups was:
A (number of patients = 98) } **BrdU low**
C (number of patients = 90) }
B (number of patients = 94) } **BrdU high**
D (number of patients = 94) }
N0-BrdU low (A) vs. N1-BrdU high (D) $P < 0.001$
N1-BrdU low (C) vs. N1-BrdU high (D) $P = 0.023$.

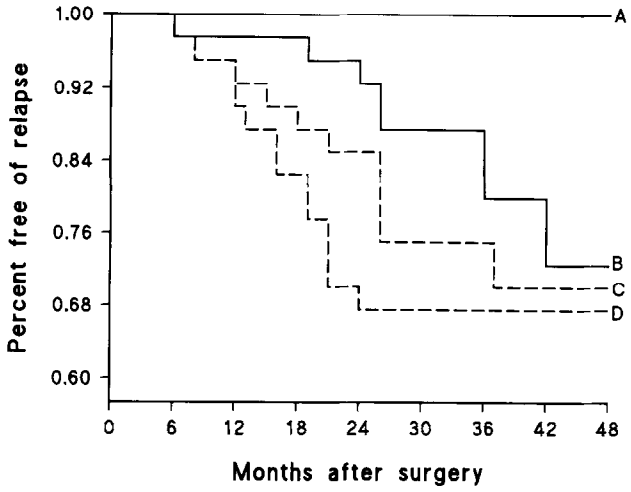

Fig. 2. Disease free survival curves of patients divided according to nodal status and Ki-67 score. Solid lines : N0; dashed lines : N1. The global difference between groups was highly significant by Mantel–Cox test ($P = 0.0007$). The log-rank test of significant paired groups was:

A (number of patients = 90) } **Ki-67 low**
C (number of patients = 86) }

B (number of patients = 90) } **Ki-67 high**
D (number of patients = 87) }

N0-Ki67 low (A) vs. No-Ki67 high (B) $P = 0.0049$
N0-Ki67 low (A) vs. N1-Ki67 low (C) $P = 0.0005$
N0-Ki67 low (A) vs. N1-Ki67 high (D) $P < 0.0001$.

of a certain interest. The most likely interpretation is that these two antibodies recognise distinct subsets of neoplastic cells and therefore provide informations with independent prognostic power. It has already been demonstrated that in the MCF-7 cell line not all resting cells are necessarily Ki-67 negative [19] and that the proportion of cells retaining this nuclear antigen does not always reflect the actual growth fraction. Since, from our results, Ki-67 expression seems to be highly informative for the assessment of the risk of recurrence, its expression might also indicate a commitment of neoplastic cells to enter active proliferation. This point, however, needs further studies. In our hands, immunohistochemical methods provided data on cell kinetics that are consistent with those produced by autoradiography [6] and flow-cytometry [20, 21].

In the present series tumour proliferative activity, regardless of the method used, was not associated with the axillary lymph node status, confirming its potential value in predicting outcome after surgery. Multivariate analysis demonstrated that the percentage of Ki-67 positive cells and nodal status were both significant and independent prognostic factors. Axillary lymph nodes metastases and proliferative activity can be used to define groups of patients at different risks of recurrence. For this reason both lymph node involvement and cell kinetics should be taken in consideration when adjuvant therapies have to be planned.

Our results suggest that Ki-67 labelling should permit separation, within the N0 group of patients with a very good prognosis from those (Ki-67 > 9.0%) with a risk of recurrence comparable to N1 patients. Women of the latter group are candidates for systemic adjuvant therapy. Among N1 patients, Ki-67 could not discriminate groups with different prognosis.

By contrast, in our series, N1 patients with high BrdU labelling had significantly higher recurrence rates than those with a low BrdU. Our data do not provide conclusive evidence on which of these two immuno-histochemical methods is the most reliable one in predicting outcome, even though multivariate analysis showed that Ki-67 had a stronger correlation with DFS than BrdU. Both reagents might be equally necessary for constructing a more detailed map of proliferative activity in breast cancer biopsy samples. A comparison of the pattern of DFS curves, especially on patients with lymph node involvement, reinforces our opinion that Ki-67 cannot simply be considered a replacement for S-phase scoring, these two methodologies appear to be quite complementary.

It should be pointed out that Ki-67 labelling can also be determined in samples from fine-needle aspirates, allowing the procedure to be applied even on lesions that will not be analysed by frozen sections. This could be extremely useful in neo-adjuvant treatments, as it provides a significant prognostic parameter before the beginning of therapy. The results presented confirm data reported by others [22, 23] and stress the need for further research in order to demonstrate whether proliferative activity of primary breast cancer is a determinant of response, and whether cell kinetics, measured by immunohistochemical techniques, can be effectively used in the design of chemotherapy protocols.

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The Influence of Adjuvant Chemotherapy on Outcome after Relapse for Patients with Breast Cancer

S.J. Houston, M.A. Richards, A.E. Bentley, P. Smith and R.D. Rubens

This study examines the outcome following relapse for 176 patients who had been entered into a randomised trial comparing adjuvant cyclophosphamide, methotrexate and 5-fluorouracil (CMF) with no adjuvant therapy (controls). Relapse has occurred in 65/144 (45%) of the CMF group and 111/158 (70%) of controls ($P < 0.0001$). 123/176 patients received endocrine treatment after relapse with higher response rates (38 vs. 18%, $P < 0.05$) and longer time to progression (23 vs. 19 weeks, $P = 0.03$) for controls. 94/176 received chemotherapy after relapse again with higher response rates (47 vs. 23%, $P = 0.05$) and longer time to progression (17 vs. 9 weeks, $P = 0.03$) for controls. Despite this, survival after relapse was the same for the two groups (median 16 months). However, on subgroup analysis, postmenopausal patients who had received adjuvant CMF had shorter survival ($P = 0.03$). These results suggest that prior adjuvant therapy should be a stratification factor in clinical trials in advanced disease.
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INTRODUCTION

THE OVERVIEW analysis conducted by the Early Breast Cancer Trialists Collaborative Group [1] has demonstrated beyond reasonable doubt that adjuvant chemotherapy prolongs survival, particularly for women under the age of 50 years. Despite this, relapse after adjuvant chemotherapy remains a significant problem. It is clearly important to know whether prior treatment with adjuvant chemotherapy compromises response and survival in patients who suffer a recurrence of their disease. Published reports show conflicting results with some showing an adverse effect of prior adjuvant chemotherapy [2–4] and others apparently showing no such effect [5–7].

This report analyses factors influencing survival after relapse for 176 patients treated at Guy's Hospital in the Guy's/Manchester trial [8]. In that study patients with positive axillary lymph nodes were randomised to receive cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) for a period of 12 months or no adjuvant chemotherapy (control) following modified radical mastectomy or breast conservation including full axillary clearance. A recent update of outcome in that trial showed a significant benefit in relapse-free survival and survival for patients treated with CMF. This benefit was confined to premenopausal patients [9]. In this paper, time to relapse, survival after relapse, response to endocrine therapy and response to chemotherapy for patients in the two arms of the study have been compared.

PATIENTS AND METHODS

A total of 312 patients under the age of 65 years with operable breast cancer and histologically proven axillary node involvement managed at Guy's Hospital between October 1979 and December 1985 were entered into the Guy's/Manchester trial comparing CMF with no adjuvant therapy. Between October 1979 and October 1981 primary treatment for all patients was modified radical mastectomy. From October 1981 onwards patients with tumours less than 4 cm in diameter

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