MIB-1 proliferative activity in invasive breast cancer measured by image analysis

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Abstract

Aims—To determine cell proliferation in infiltrating breast carcinomas.

Methods—Using the MIB-1 monoclonal antibody, the proliferation index was measured in paraffin wax sections of 871 breast cancers. The MIB-1 proliferation index was compared with other markers of disease progression: size, lymph node status, histotype, oestrogen and progesterone receptor status, expression of p53 and Neu, and DNA ploidy. All parameters were measured using image analysis. In 347 tumours, the MIB-1 and Ki-67 proliferation indexes were compared. Follow up data were available for 170 cases (median 66.5 months).

Results—Of the tumours, 314 (36%) had a high proliferation index. The MIB-1 proliferation index was correlated directly with size, nodal status, overexpression of p53 and Neu, and the DNA index; and inversely with oestrogen and progesterone receptor status. The correlation between MIB-1 and Ki-67 proliferation indexes was statistically significant. In patients with pT1 tumours, a low proliferation index correlated with a longer relapse-free interval and overall survival; node negative patients with a low proliferation index had a longer overall survival.

Conclusions—The MIB-1 proliferation index is a reliable, practical and useful method of measuring proliferative activity and is an important predictor of clinical behaviour.

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Keywords: breast cancer, MIB-1, proliferative activity, image analysis, overall survival.

Use of immunocytochemistry to measure tumour proliferation preserves both cytological and architectural features and can be used to detect proliferation related nuclear antigens, one of the most important of which is Ki-67. The Ki-67 nuclear antigen is expressed by dividing cells in all phases of the cell cycle except G₀^{1,2} and has been established as an independent prognostic marker in breast cancer. Unfortunately, detection of Ki-67 is limited to frozen tissue only. However, several antibodies are available for quantifying cell proliferation in paraffin wax embedded specimens, including antibodies directed against proliferating cell nuclear antigen (PCNA) and the Ki-S1 and MIB-1 monoclonal antibodies.³⁻¹⁰ MIB-1, in particular, recognises

Ki-67 nuclear antigen, permitting the assessment of growth fraction on small tumours, formalin fixed tissue and retrospective studies on archival material.

The aim of the present study was to measure the MIB-1 proliferation index in 871 infiltrating breast carcinomas. The results were compared with other markers of prognosis: oestrogen and progesterone receptor status, overexpression of c-erbB-2/Neu and p53, the DNA index, and the Ki-67 proliferation index. The clinical usefulness of the MIB-1 proliferation index was also evaluated in 170 patients (median follow up 66.5 months).

Methods

Paraffin wax blocks of 871 infiltrating primary breast cancers were retrieved from the archives. The clinicopathological details of the patients studied are presented in table 1. The mean age of the patients was 61 years; 277 (31.8%) were pre-menopausal and 507 (58.2%) were node negative. The MIB-1 proliferation index was also compared in patients subdivided according to age: ≤40 years (51 (5.9%) patients, group 1); 41-55 years (226 (25.9%) patients, group 2); and >55 years (594 (68.2%) patients, group 3). Patients were staged according to the TNM staging system as follows: 342 (39.3%) as stage I; 292 (33.5%) as stage IIa; 151 (17.4%) as stage IIb; 24 (2.7%) as stage IIIa; and 62 (7.1%) as stage IIb.¹¹

Follow up data were available for 170 patients, who had undergone surgery between 1985 and 1989 (median follow up 66.5 months, range 30.5-112.5 months). The mean age of these 170 patients was 59 years; 63 (37%) were pre-menopausal; 82 (48%) were node negative; and in 100 (59%) tumours were ≤2 cm at their greatest dimension. None of the patients received preoperative treatment. Node negative patients did not receive adjuvant treatment.

IMMUNOSTAINING

MIB-1

Formalin fixed, paraffin embedded sections were pretreated in a microwave oven (4 × 5 minutes at 750 W) and incubated with MIB-1 (Immunotech, Marseille, France), diluted 1 in 50, for one hour.

Ki-67

Frozen sections were fixed in acetone at -20°C for 10 minutes and stored at this temperature pending analysis. Sections were incubated with

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Table 1 Clinicopathological features of the 871 patients with invasive breast cancer

Clinicopathological feature	Value
Mean age (years)	61
Menopausal status	
Pre-menopausal	31.8%
Postmenopausal	68.2%
Histological subtype	
Ductal	73.9%
Lobular	15.6%
Tubular	3.6%
Mucinous	2.2%
Papillary	1.6%
Cribriform	0.9%
Medullary	0.6%
Mixed/others	1.6%
Tumour size (cm)	
≤1	26.4%
1–2	39.3%
2–5	32.3%
>5	2%
Lymph node status	
Positive	41.8%
Negative	58.2%
MIB-1 PI (%PA) (871 cases) >13%	36.4%
ER-ICA (%PA) (841 cases) >10%	76.3%
PR-ICA (%PA) (737 cases) >10%	63.4%
Neu (%PA) (868 cases) >10%	33.2%
p53 (%PA) (411 cases) >5%	27%
DNA index (267 cases) aneuploid	80.1%

ER = oestrogen receptor; PR = progesterone receptor; ICA = immunocytochemical assay; PI = proliferation index; PA = positive area.

the Ki-67 antibody (Dako, Glostrup, Denmark), diluted 1 in 250, for 45 minutes.

Oestrogen and progesterone receptor status

Hormonal receptor status was evaluated by staining with the H222 (ER-ICA; Abbott Laboratories, Chicago, Illinois, USA) and KD68 monoclonal antibodies. Fixation and staining procedures of cryostat sections were as recommended by the manufacturer. Paraffin wax sections were stained using a Streptavidin peroxidase method (Biogenex, San Ramon, California, USA). Sections were predigested with pronase (Sigma, St Louis, Missouri, USA) at 37°C for nine minutes prior to staining with H222. For progesterone receptor, sections were dewaxed at 58°C for two hours and incubated with KD68 (PR-ICA; Abbott Laboratories).

Immunostaining of c-erbB-2/Neu

To detect c-erbB-2/Neu, paraffin wax sections were incubated with the Ab-1 monoclonal antibody directed against p185 (Triton Diagnostics, Alameda, California, USA), diluted 1 in 100, for one hour.

p53 immunostaining

Microwave pretreated sections were incubated with the D07 monoclonal antibody (Dako), diluted 1 in 25, for one hour.

Table 2 MIB-1 proliferation index (PI) in each histotype

Histotype	MIB-1 PI (%PA >13)
Ductal	41.8%
Lobular	21.3%
Medullary	100.0%
Tubular	9.7%
Mucinous	10.5%
Papillary	21.4%
Cribriform	12.5%
Other/mixed	44.4%

PA = positive area.

The MIB-1, Ki-67, Ab-1, and D07 antibodies were detected using the avidin-biotin-peroxidase complex (ABC) method (Vectastain ABC kit; Vector Laboratories, Burlingame, California, USA). 3,3'Diaminobenzidine tetrahydrochloride (Sigma) was used as the chromogen; ethyl green was used for nuclear counterstaining, as described by the manufacturer (Cell Analysis Systems, San Jose, California, USA).

DNA index

Touch imprints were dried in air for one hour, fixed in 10% buffered formalin for 30 minutes and stained by the Feulgen method using a commercially available kit (Quantitative DNA stain, Cell Analysis Systems).

IMAGE ANALYSIS

The proliferation index (MIB-1/Ki-67), oestrogen and progesterone receptor status, p53, Neu, and DNA ploidy were quantified using an image analysis system (CAS 200; Becton Dickinson, San Jose, California, USA).¹² The percentage of positively stained nuclei (proliferation index, oestrogen and progesterone receptor and p53) and plasma membranes (Neu) was recorded. The use of an interactive window command allows the operator to select the fields containing infiltrating carcinoma cells and to exclude areas with non-neoplastic components. For each tumour section, at least 25 microscopic fields, including peripheral and central areas of invasive carcinoma (40× objective), were measured. At ≥15 fields of vision, the positive nuclear area (PNA) has a low SD and a stable coefficient of variation.¹³ For the proliferation index, at least 2000 nuclei were assessed in order to reduce the margin of error expected.¹⁴ Ten per cent of positive area was used as cut-off value for oestrogen receptor, progesterone receptor and Neu; 5% was used for p53; 13% of proliferation index representing the second tertile in distribution was used as cut-off value to divide cases with a low and high proliferative activity. The cut-off values selected were similar to those used in other studies.^{12 15 16}

For DNA ploidy 200–250 neoplastic cells for each tumour imprint were analysed and compared with calibration cells, which contained a known amount of DNA. The DNA content was determined by the quantitation of the optical density of each Feulgen stained nucleus. DNA distribution histograms were generated. The DNA index (the modal peak of DNA distribution) was assumed as the index of tumour ploidy; values between 0.9 and 1.1 were considered diploid.

STATISTICAL ANALYSIS

The relations between the MIB-1 proliferation index and other pathobiological variables were investigated using non-parametric Spearman's rank correlation tests and the Mann Whitney U test. Follow up analysis of the relapse-free interval and overall survival was performed using the actuarial model and Lee-Desu statistics.¹⁷ Significance was set at $p < 0.05$.

Figure 1 Correlation between MIB-1 and Ki-67 proliferation indexes (PI) (347 cases, $r_s = +0.73$, $p < 0.001$).

Table 3 Comparison between MIB-1 and Ki-67 proliferation indexes (PI)

	MIB-1 PI (%PA)		Total
	≤13%	>13%	
Ki-67 (%PA)			
≤13%	193	68	261
>13%	2	84	86
Total	195	152	347

Results

Specific MIB-1 immunostaining on paraffin wax sections was similar to that obtained with Ki-67 on frozen sections and was localised on cell nuclei. The intensity of the nuclear staining pattern in proliferating breast carcinoma cells varied and was either uniformly granular or diffusely homogeneous, with nucleoli usually stained intensely. The staining pattern in different areas of the section was slightly heterogeneous. Moreover, the weak cytoplasmic staining observed occasionally in frozen sections on staining with Ki-67 was absent in paraffin wax sections on staining with MIB-1.

In 871 invasive breast carcinomas the distribution of the MIB-1 proliferation index was asymmetric (skewness, +1.95; range, 0.01–

96.5% PNA; median value, 8.42% PNA); 36.4% of tumours had a high proliferative activity. When tumours were subdivided according to histological type, 100% of medullary, 41.8% of ductal, 21.3% of lobular, and 12.5% of special types had a high proliferation index (table 2).

Specific immunostaining of hormone receptors and p53 protein was localised, as expected, on neoplastic cell nuclei; 76.3% of tumours expressed oestrogen receptor and 63.4% expressed progesterone receptor; 27% were p53 positive.

Specific immunostaining of Neu oncogene protein was present on neoplastic cell plasma membranes; 33.2% of tumours overexpressed Neu.

Eighty per cent of tumours were aneuploid. When the distribution of the MIB-1 proliferation index was assessed according to age, younger patients (group 1) had tumours with higher proliferative activities (54.9%) than patients in groups 2/3 (38.5% and 34%; $p = 0.009$).

The MIB-1 proliferation index was correlated directly with size ($r_s = +0.30$, $p < 0.001$), nodal status ($r_s = +0.15$, $p < 0.001$), p53 ($r_s = +0.24$, $p < 0.001$) and Neu ($r_s = +0.20$, $p < 0.001$) overexpression, and DNA index ($r_s = +0.17$, $p = 0.026$); and inversely with oestrogen receptor ($r_s = -0.29$, $p < 0.001$) and progesterone receptor ($r_s = -0.33$, $p < 0.001$) status. No correlation was observed with menopausal status.

Similar associations were observed when Ki-67 immunostaining and other biopathological variables were compared: the Ki-67 proliferation index was correlated directly with size ($r_s = +0.28$, $p < 0.001$), p53 ($r_s = +0.41$, $p < 0.001$) and Neu ($r_s = +0.19$, $p < 0.001$) overexpression, and DNA index ($r_s = +0.20$, $p = 0.026$); and inversely with oestrogen receptor ($r_s = -0.39$, $p < 0.001$) and progesterone receptor ($r_s = -0.39$, $p < 0.001$) status. No correlations were found with nodal and menopausal status.

The Ki-67 and MIB-1 proliferation indexes were compared in 347 cases: the Ki-67 proliferation index ranged from 0.01% to 87.7% PNA (median 5.1% PNA) and MIB-1 proliferation index ranged from 0.58% to 94.1% PNA (median 11% PNA).

There was a good correlation between the MIB-1 and Ki-67 proliferation indexes ($r_s = +0.73$, $p < 0.001$; fig 1). Discordant values were observed in 70 cases. In 68 the MIB-1 proliferation index was higher (table 3).

CLINICAL OUTCOME

The usefulness of the MIB-1 proliferation index as a predictor of clinical outcome was investigated in 170 cases. A significant survival advantage was demonstrated for patients whose tumours had a low proliferation index (relapse-free interval $p = 0.034$; overall survival $p = 0.004$) (fig 2). Node negative patients with a low proliferation index generally survived longer ($p = 0.035$). In patients with pT1 tumours (≤ 2 cm), a low proliferation index was correlated with longer a relapse-free interval (p

PI≤13% (RFI)	127	120	112	103	89	63	37	21	14	2
PI>13% (RFI)	43	37	33	25	23	11	6	3	2	1
PI≤13% (OS)	127	124	121	113	105	78	46	25	15	2
PI>13% (OS)	43	40	34	31	28	15	8	4	2	1

Figure 2 Prognostic power of the MIB-1 proliferation index (PI). Relapse-free interval (RFI) $p = 0.034$; overall survival (OS) $p = 0.004$ (Lee-Desu statistic, 170 cases).

Figure 3 Prognostic power of the MIB-1 proliferation index (PI) in patients with pT1 tumours. Relapse-free interval (RFI) $p = 0.016$; overall survival (OS) $p = 0.008$ (Lee-Desu statistic, 94 cases).

= 0.016) and overall survival ($p = 0.008$) (fig 3). No association was found in node positive patients.

Discussion

Proliferative activity has been widely used as an important and independent prognostic factor in human cancer, including breast cancer.^{2 12 18 19} Many markers of proliferation have been studied in breast cancer, including thymidine labelling, bromodeoxyuridine incorporation, flow cytometry, and more recently, immunostaining with the Ki-67 monoclonal antibody.^{7 20-22} This antibody recognises a nuclear antigen encoded by a gene located on chromosome 10q25.^{23 24}

Ki-67 is expressed in all phases of the cell cycle except G₀. There is increasing interest in the use of immunocytochemistry to determine the proliferative activity of tumour cells. The usefulness of the Ki-67 proliferation index has been demonstrated in many studies,^{2 25-27} but its use is restricted to frozen tissue. However, antibodies are now available for use on paraffin wax embedded material, including the MIB-1 antibody (raised by immunising mice with recombinant Ki-67 gene product) which recognises the Ki-67 nuclear antigen. The use of paraffin wax embedded specimens has many advantages: morphology is preserved and immunostaining strong, permitting easier recognition of cellular features.

In the present study we verified that the MIB-1 proliferation index is a reliable, practical and useful method of measuring proliferative activity and can be used in association with other prognostic parameters (for example, oestrogen and progesterone receptor status, c-erbB-2/Neu and p53) to predict survival. Our results are in agreement with those reported by others in breast cancer and other tumour types.²⁸⁻³⁴ The discordant median values reported by us and Pinder *et al*³¹ may be because of the different clinicopathological features of the patients studied. The lack of an inverse correlation between MIB-1 proliferation index and oestrogen receptor status

reported by Pinder *et al* could be related to the different methods used (dextran coated charcoal *v* immunocytochemistry).

A frequent objection to the use of immunocytochemistry to predict clinical outcome concerns the reliability of results, which are semiquantitative. Such drawbacks can be overcome easily by use of computerised image analysis systems, which give objective and reproducible results.^{12 13 35 36} Quantitative immunocytochemistry is carried out in our department for routine characterisation of invasive breast cancers. The information provided is used in conjunction with traditional staging systems in patient management.

In conclusion, the MIB-1 proliferation index is a reliable, practical and useful method of measuring proliferative activity and is an important predictor of clinical behaviour. It may also be useful in selecting patients with node negative breast cancer who might benefit from adjuvant treatment.

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