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Proliferative Activity Is a Significant Prognostic Factor in Male Breast Carcinoma

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The proliferative activity of male breast carcinoma has been investigated using the staining of the argyrophilic nucleolar organizer regions (AgNORs), the monoclonal antibody against the proliferating cell nuclear antigen (PC10) and the monoclonal antibody MIB-1 in formalin-fixed, paraffin-embedded specimens from 27 primary male breast carcinomas at diagnosis. A significant correlation was found between survival and AgNOR counts (median of survival 77 months for cases with AgNOR/cell ≤7.27 but 37 months only for cases with >7.27 AgNOR/cell; P = 0.001), proliferating cell nuclear antigen scores (median of survival 73 months for cases with proliferating cell nuclear antigen ≤18.25% versus 41 for cases with proliferating cell nuclear antigen >18.25%; P = 0.013) and MIB-1 scores (median of survival 73 months for cases with MIB-1 scores ≤23.5% versus 37 months for cases with MIB-1 scores >23.5%; P = 0.01). Tumor histological grade was also correlated with prognosis (median of survival 72 months for grade 2 versus 33 months for grade 3 tumors; P = 0.01). Estrogen and progesterone receptors, immunohistochemically detected on paraffin-embedded sections, had no prognostic value. In the multivariate survival analysis, only AgNOR counts (P = 0.007) and tumor size (P = 0.003) had an independent prognostic significance. Our results indicate that methods for assessing the cell proliferation in routinely processed specimens offer significant prognostic information in male breast carcinoma. This finding, together with the lack of prognostic significance for estrogen receptors and progesterone receptors, suggests that male breast carcinoma is biologically different from female breast cancer. (Am J Pathol 1994, 481–489)

Male breast carcinoma (MBC) represents only 1% of mammary cancers and seems to have a worse prognosis than female breast cancer (FBC).[1–4] Tumor histological grade, size, and lymph node status are some of the prognostic factors equally significant for MBC and FBC.[5] Additional prognostic accuracy in FBC is provided by the detection of the receptors for estrogens (ER) or progesterone (PgR) using biochemical assays[6–8] or immunohistochemical methods on sections from frozen[9] and formalin-fixed, paraffin-embedded tissues.[10] Although ERs have been biochemically detected in most MBCs,[11,12] they do not seem to be associated with prognosis in routinely processed material[13]; little is known about the prognostic significance of PgR in MBC.

Methods for assessing cell proliferation in routinely fixed and processed tissues have recently been introduced in surgical pathology, allowing retrospective studies. One of these, is the analysis of the nucleolar organizer regions (NORs): a simple argyrophilic technique for the visualization of the proteins associated with NORs (AgNORs)[14] showed significant association between the quantity of AgNORs and prognosis in several human tumors,[15] but not in FBC.[16–19] The expression of the proliferating cell nuclear antigen (PCNA/cyclin), a nuclear protein involved in DNA synthesis,[20] which can be detected in conventional processed tissues by the monoclonal antibody against the PC10 clone,[21] predicts survival in FBC.[22–25]

The reaction with the monoclonal antibody Ki67, which detects a nuclear antigen expressed in proliferating cells, has been widely applied to FBC,[26] showing a significant association with prognosis.[27] However, it is only effective on cryostat sections. The new monoclonal antibody MIB-1, prepared against recombinant parts of Ki67 antigen,[28] can also react in formalin-fixed and paraffin-embedded tissues.[29] To our knowledge, no studies have been performed so

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far on the prognostic significance of AgNOR, PCNA, and MIB-1 expression in MBC.

In this work, we have retrospectively investigated the proliferative activity of 27 primary MBCs at diagnosis, using AgNOR, PCNA, and MIB-1 stainings on routinely processed specimens. Their prognostic importance in relation to various clinical and morphological parameters (age, histological grade, pathological stage, lymphocytic infiltration, necrosis, mitotic counts, ER and PgR) was then tested by means of uni- and multivariate survival analyses.

Materials and Methods

Twenty-seven cases of MBC were collected from the files of the pathology sections of the Department of Biomedical Science and Human Oncology of the Turin University and S. Giovanni Hospital (Turin, Italy) dating from 1973 to 1987. The mean age of the patients at diagnosis was 61.2 years (27 to 86). All patients underwent radical mastectomy; three also received postoperative adjuvant radiotherapy and two tamoxifen. A minimum follow-up of 5 years or to a patient's death was available for all the cases: the length of follow-up ranged from 0.5 to 154.23 months.

The cases were classified according to World Health Organization[30] and pathologically staged according to International Union Against Cancer.[31] All tumors were invasive ductal adenocarcinoma with no other special types; five were pT1, 11 pT2, and 11 pT3–4; 13 were NO, 14 N1–3. Tumor grade was established using the criteria of Bloom and Richardson[32] developed for FBC: 18 tumors were grade 2 (G2) and nine G3. Serial sections from the same tissue blocks were used for histology, ER and PgR immunostaining, AgNOR staining, PCNA and MIB-1 immunostainings.

Histology

Hematoxylin and eosin, periodic acid-Schiff, and Giemsa stainings were performed. The peritumoral lymphocytic infiltration was evaluated as heavy (+ + +) or mild (+) if more or less than 10 lymphocytes per high power (×400) fields were observed. The total number of mitoses was assessed according to Elston and Ellis,[33] examining at least 10 fields, mostly at the tumor periphery, using a standard light microscope equipped with a ×10 ocular and a ×40 objective. Each case was also scored for the presence of necrosis.

Hormone Receptors Staining and Scoring

Four-micron-thick sections of poly-L-lysine-coated slides were stained with monoclonal antibodies (ER-ICA or PgR-ICA, Abbott Laboratories, North Chicago, IL) at kit dilution, following the procedure of Hiort[34] with some modification, using an avidin-biotin peroxidase method (ABC Complex, Dakopatts, Glostrup, DK). For ER detection, deparaffinized slides were pretreated with proteinase K (Boehringer, Mannheim, FRG) 0.02% in phosphate-buffered saline for 5 minutes and with DNAse I (Sigma Chemical Co., St. Louis, MO) (5 mg/ml of 0.05 mol/L TRIS buffer, pH 7.4, and 0.01 magnesium sulphate) for 15 minutes at 25 C. Normal mouse serum was substituted for primary antibody as a negative control; sections from known ER- and PgR-positive FBC were used as positive controls. Scoring of ER or PgR staining was independently performed by two observers (AP and EM) using a standard light microscope equipped with an ocular reticle (magnification ×15) and a ×40 objective. In each case, 1,000 tumor cells were counted from 10 randomly selected areas; in these fields, all the reactive nuclei were considered positive, regardless of the intensity of the staining, and the fraction of positive cells was determined. The interobserver variation was less than 10%. A specimen was considered as positive if more than 10% of the counted nuclei were positive. This criterion for ER/PgR positivity was chosen because it correlates with the clinical response in FBC.[9]

AgNOR Staining and Counting

Three-micron-thick sections taken to aqueous medium, were stained with the AgNOR method of Ploton et al,[14] as previously described.[35] Random fields, excluding areas of necrosis, were independently examined by two pathologists (AP and LC) using a ×100 oil immersion lens; at least 100 tumor cells were counted in each case. Single AgNORs and individual AgNORs within clumps were counted by careful focusing through the whole thickness of the sections. When large polycyclic structures (overlapping NORs) were present, they were considered as a single AgNOR, if individual dots could not be identified. The internal control of the reaction was provided by the infiltrating lymphocytes that on average had one single silver-stained dot. The mean number of AgNORs per nucleus was then calculated in each case. The interobserver variation was less than 5%.

PCNA Staining and Scoring

Four-micron-thick sections on poly-L-lysine-coated slides were immunostained with monoclonal antibody against PCNA (PC10) (Dakopatts) as described elsewhere,[21] using an ABC immunoperoxidase method with light hematoxylin counterstaining. Scoring of PCNA (PC10) was independently performed by two pathologists (AP and LC), using a light microscope equipped with an ocular reticle (magnification ×15) and a ×40 objective. In each case, 1,000 tumor cells were counted from at least 10 areas, which were most evenly and heavily labeled. Within a section, the completely negative areas were excluded. All reactive nuclei were counted as positive regardless of the staining intensity. We considered positive only the cells in which the staining was confined to the nucleus. Cells in mitosis, which showed only a faint cytoplasmic staining, were considered negative. The fraction of positive cells was then determined; the interobserver variation was between 10% and 15%.

MIB-1 Staining and Scoring

Four-micron-thick sections on poly-L-lysine-coated slides, taken to water, were treated with 0.05% trypsin for 10 minutes, placed in glass box filled with 10 mmol/L, pH 6.0, citrate buffer and processed in a microwave oven twice at 750W and then rinsed in phosphate-buffered saline. The sections were stained with MIB-1 monoclonal antibody (Immunotech, Marseille, France) at 1:100 dilution for 2 hours at room temperature, using an ABC immunoperoxidase method; they were counterstained with hematoxylin and mounted in resin. MIB-1 immunostaining was scored as the hormone receptor staining by AP and LC; the interobserver variation was less than 5%.

Statistical Analysis

Association between AgNOR counts, PCNA scores and MIB-1 scores with tumor histological grade and stage, and with ER and PgR status was assessed by one-way analysis of variance. Univariate survival analysis were based on the Kaplan–Meier product limit estimates of survival distribution[36]; differences between survival curves were tested using the generalized Wilcoxon test. The relative importance of multiple prognostic factors on survival was estimated using the Cox proportional hazards regression model.[37] All data were analyzed with BMDP-selected computer programs.[38]

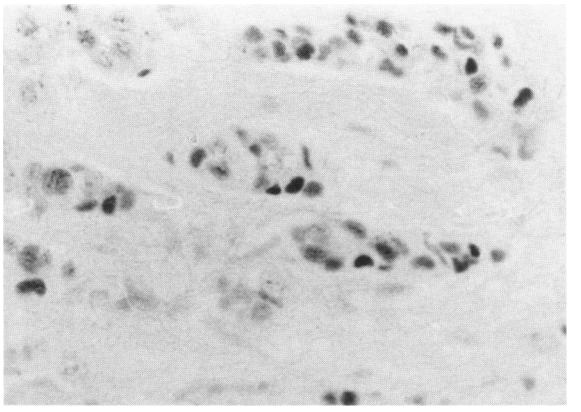

Figure 1. *ER immunoreactivity shows variation in intensity from nucleus to nucleus (ER-ICA immunoperoxidase, hematoxylin counterstain, ×350).*

Results

ER staining was exclusively nuclear with variation in intensity from cell to cell (Figure 1). The intensity of the reaction was more pronounced at the tumor periphery and could be different from case to case. The percentage of ER-positive nuclei varied from 0 to 46.8%, and it was higher in the most differentiated cases; 13 tumors (48%) had >10% stained nuclei (positive cases); 14 (52%) had ≤10% stained nuclei (negative cases). PgR staining was exclusively nuclear; the intensity of the reaction was stronger than that found for ER, with a lesser variation from area to area (Figure 2). The percentage of PgR-positive nuclei varied from 0 to 64.8%; 11 tumors (41%) had >10% stained nuclei (positive cases); 16 (59%) had ≤10% stained nuclei (negative cases).

The mean AgNOR counts for the whole series were 7.62 (SD ± 2.44; range 4.47 to 13.51; median 7.27).

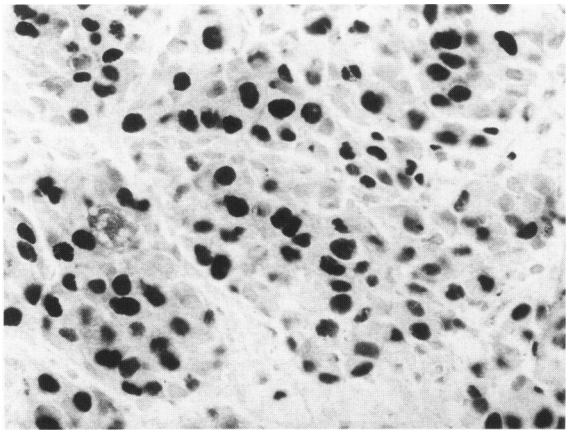

Figure 2. *Most neoplastic cells exhibit intense and uniform immunoreactivity for progesterone receptors (PgR-ICA immunoperoxidase, hematoxylin counterstain, ×350).*

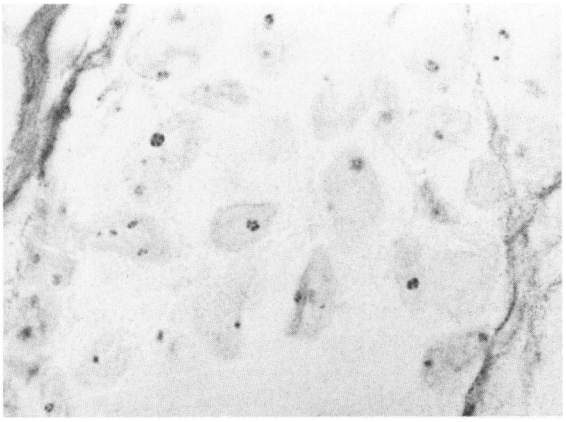

Figure 3. *Grade 2 MBC: four to five AgNORs are grouped in a central nuclear cluster (AgNOR staining, ×1,000).*

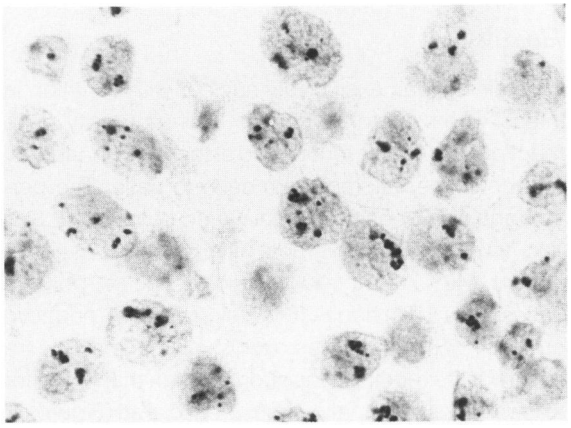

Figure 4. *Grade 3 MBC: a large number and scattered distribution of AgNORs are visible in the neoplastic cells (AgNOR staining, ×1,000).*

In all cases, tumor cells contained numerous AgNORs of different size and shape: only in a few G2 tumors four to five medium-size dots were grouped in a central cluster (Figure 3); in most G2 and G3 cases numerous AgNORs were dispersed throughout the nucleus (Figure 4). No significant association was found between AgNOR counts and tumor stage, ER or PgR status, although AgNOR counts were higher in tumors with positive nodes ER or PgR. A borderline significance was found for histological grade: the mean AgNOR count for G3 was 8.82 *versus* 7.02 for G2 cases ($P = 0.07$).

The mean PCNA (PC10) scores for the whole series were 20.63% (SD ± 8.6%, range 9.25 to 39.75%; median 18.25%). PCNA staining was almost confined to the nucleus, with a diffuse or granular pattern and gradation in the intensity from nucleus to nucleus (Figure 5); mitotic figures were mostly unstained, although a weak cytoplasmic staining could occasionally be seen. Variability was seen in the staining intensity,

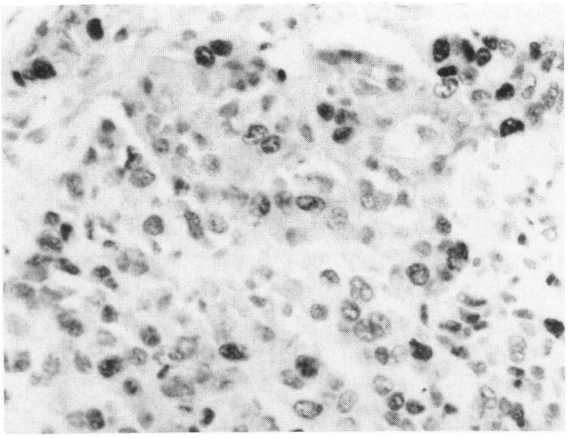

Figure 5. *PCNA immunoreactivity shows wide gradation in the staining intensity from nucleus to nucleus. (PCNA/PC10 ABC immunoperoxidase, hematoxylin counterstain, ×350).*

high in some areas, very poor in others. No association was found between PCNA scores and tumor grade, stage and hormone receptors, although the PCNA scores were higher (24.14%) in G3 than in G2 (18.88%) cases ($P = 0.13$) and in ER-negative (22.8%) than in ER-positive (18.3%) cases ($P = 0.17$).

The mean MIB-1 scores for the whole series were 23.76% (SD ± 7.78%, range 8 to 38%; median 23.5%). The positivity for MIB-1 monoclonal antibody was confined to the nucleus, with a diffuse or granular pattern; some gradation in the intensity could be seen from nucleus to nucleus; however the sections were homogeneously stained without background interference (Figure 6). Mitotic figures were generally strongly stained; however, a few mitotic figures were not stained, especially in old archival material. No association between MIB-1 scores and tumor grade, stage, ER or PgR status was found, although MIB-1 scores were higher in G3 (26.44%) than G2 (22.42%)

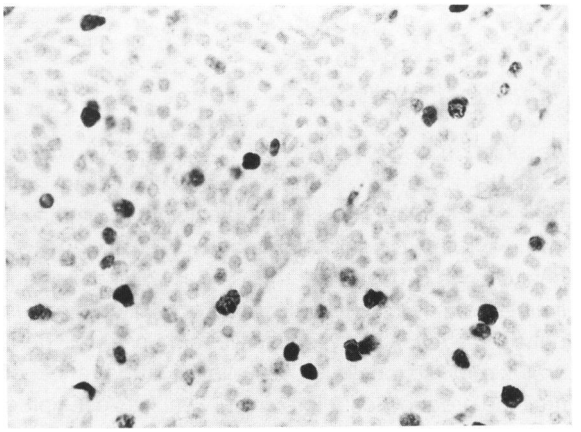

Figure 6. *MIB-1 immunostaining exhibits strong and uniform reactivity in many neoplastic cells; no background is evident (MIB-1 ABC immunoperoxidase, hematoxylin counterstain, ×350).*

Table 1. *Proliferation Indices in Male Breast Carcinomas according to Histological Grade, Pathological Stage, and Hormone Receptors*

		AgNOR counts		PCNA-positive cells (%)		MIB-1-positive cells (%)	
	N	Mean ± SD	*P*	Mean ± SD	*P*	Mean ± SD	*P*
All cases	27	7.62 ± 2.44		20.63 ± 8.60		23.76 ± 7.78	
Histological grade							
G2	18	7.02 ± 2.16	0.07	18.88 ± 7.02	0.13	22.42 ± 7.08	0.21
G3	9	8.82 ± 2.68		24.14 ± 10.73		26.44 ± 8.86	
T stage							
pT1	5	8.51 ± 2.35		22.77 ± 6.73		23.57 ± 8.51	
pT2	11	7.20 ± 1.89	0.63	18.93 ± 8.19	0.68	22.14 ± 6.92	0.62
pT3–4	11	7.63 ± 3.04		21.36 ± 10.06		25.48 ± 8.68	
N stage							
N0	13	7.11 ± 2.16	0.3	19.22 ± 7.35	0.42	22.61 ± 8.20	0.47
N1–3	14	8.09 ± 2.68		21.95 ± 9.71		24.83 ± 7.52	
ER (%)							
≤10	14	7.47 ± 2.47	0.74	22.80 ± 9.35	0.17	23.76 ± 7.70	0.99
>10	13	7.78 ± 2.52		18.30 ± 7.37		23.77 ± 8.19	
PgR (%)							
≤10	16	7.24 ± 2.50	0.33	20.30 ± 9.21	0.81	22.66 ± 8.49	0.38
>10	11	8.18 ± 2.38		21.12 ± 8.04		25.36 ± 6.69	

cases, in node-positive (24.83%) than in node-negative (22.61%) cases, and in PgR-positive (25.36%) than PgR-negative (22.66%) cases. Associations between AgNOR counts, PCNA or MIB-1 scores, and hormone receptor status are based on the consideration of all the cases with ER/PgR ≤10 as low expressors. When cases with ER/PgR = 0 were excluded, again no significant association was found. The results of the proliferation indices in MBC are summarized in Table 1.

Univariate Survival Analysis

At the time of analysis 19 patients (70.4%) were dead of the disease, and eight (29.6%) censored, with a mean follow-up time for censored patients of 78.76 months. The median of survival of the whole series was 57 months (0.5 to 154.23). The overall five- and ten-year survival rates were 49% and 17% respectively.

AgNOR counts and PCNA and MIB-1 scores were highly associated with prognosis: the median of survival was 77 months for cases with AgNOR/cell ≤7.27 *versus* 37 months for cases with >7.27 AgNOR/cell ($P = 0.001$); 73 months for cases with PCNA scores ≤18.25% *versus* 41 for cases with PCNA >18.25% ($P = 0.013$); 73 months for cases with MIB-1 scores ≤23.5% *versus* 37 for tumors with MIB-1 scores >23.5% ($P = 0.01$) (Table 2). The results were obtained using the median values as a cut-off. When the mean values were used, the significance for AgNOR counts and MIB-1 scores did not change. When the mean PCNA score was used as a cut-off, the median of survival was 73 months for cases with PCNA ≤20.63% *versus* 33 for cases with PCNA >20.63% ($P = 0.008$). Tumor histological grade was also significantly associated with prognosis: the median of survival was 72 months for G2 *versus* 33 for G3 tumors ($P = 0.01$). No association was found between survival and age, pT or N status, lymphocytic infiltration, necrosis, mitotic counts, ER and PgR status. The results for hormone receptors are based on the consideration of all the cases with ER/PgR ≤10 as low expressors. When the cases with ER/PgR = 0 were excluded, again no association with survival was seen. The number of involved lymph nodes varied from 1 to 10. When the cases were subdivided by the number of positive nodes, again no association with survival was found. The results are summarized in Table 3.

Table 2. *Correlation between Proliferation Indices and Survival Time in Male Breast Carcinoma*

Variable	N	Median (months)	5-year survival rate (%)	10-year survival rate (%)	*P*
AgNOR counts					
≤7.27	14	77	75	24	0.001
>7.27	13	37	23	11	
PCNA scores (%)					
≤18.25	14	73	68	40	0.013
>18.25	13	41	30	10	
MIB-1 scores (%)					
≤23.50	14	73	68	20	0.01
>23.50	13	37	28	14	

The survival rates were obtained using the median values as a cut-off.

Multivariate Survival Analysis

The independent prognostic significance of all the variables was assessed by testing the association of

Table 3. *Correlation between Clinical and Histological Parameters, Hormone Receptors, and Survival Time in Male Breast Carcinoma*

Variable	N	Median (months)	5-year survival rate (%)	10-year survival rate (%)	*P*
All cases	27	57	49	17	
Age (years)					
≤45	3	24	33		
46–70	16	72	61	13	0.19
>70	8	52	33	33	
Histological grade					
G2	18	72	63	13	0.01
G3	9	33	22	22	
T stage					
pT1	5	54	53	26	
pT2	11	41	43	21	0.57
pT3–4	11	56	53	0	
N stage					
N0	13	55	40	15	0.83
N1–3	14	61	57	19	
Lymphocytic infiltration					
+	11	43	45	30	0.19
+++	16	61	54	13	
Necrosis					
–	21	61	57	18	0.78
+	6	55	33	16	
Mitoses					
≤2	14	55	42	21	0.93
>2	13	52	53	0	
ER (%)					
≤10	14	56	48	13	0.87
>10	13	61	51	27	
PgR (%)					
≤10	16	72	61	12	0.39
>10	11	52	34	34	

response in the Cox model. Only two variables had a prognostic significance: tumor size ($X^2 = 8.68$; $P = 0.003$) and AgNOR counts ($X^2 = 7.32$; $P = 0.007$).

Discussion

The purpose of this work was to assess if the evaluation of the proliferative activity could represent a useful prognostic parameter in MBC. Indeed, the AgNOR counts were strongly correlated with survival in our series of MBC ($P = 0.001$). This is in line with the results in several human tumors,[15,35] but contrasts with studies on FBC, in which no correlation was seen between AgNORs and prognosis.[16–19] Moreover, contrary to FBC, we did not find association between AgNOR counts and tumor grade[39,40] and size,[41,42] lymph node status,[42–44] and hormone receptors status.[18] The discrepancy may be due to the hormone dependency of FBC: it is well known that AgNORs can be affected by hormone influences[45] and their counts are of little value when applied to endocrine tumors.[46,47]

PCNA scores were also directly related to survival in MBC ($P = 0.013$), in accordance with results in several human tumors[48–51] as well as FBC.[22–25] Contrary to FBC, PCNA scores were not correlated with tumor grade,[23,52–54] size,[25,53] and lymph node metastasis.[22] No association was also found between PCNA expression and ER or PgR status, contrary to a few studies in FBC,[23,25,55] but in accordance with other studies in FBC that did not show association with hormone receptors.[22,53] These contrasting results may depend on the selection of cases (no G1 case was present in our series), on different PCNA clones used in the various studies,[25,52,54] on different staining and scoring procedures,[23,25,54] and on the intratumor heterogeneity of PCNA expression.[54] This raises the question on the reliability of PCNA counts in archival material, because many variables affect PCNA staining and scoring. The intensity of the staining greatly varies from area to area in the same section, being completely negative in some fields; the gradation of labeling intensity within the nuclei can lead to a subjectivity of the scoring process, and the microwave oven processing reveals a larger number of nuclei with stronger positivity as compared to untreated specimens. The reliable reproducibility of the PCNA counting procedure requires that within each section at least 10 areas, each showing at least 1 to 3 PCNA-stained nuclei, be evaluated; that all stained nuclei be counted regardless of the staining intensity, and that all cases should be treated with exactly the same procedure (with or without microwave oven).

MIB-1 expression showed significant association with prognosis in MBC ($P = 0.01$). No data on MIB-1 expression in breast cancer are available; however MIB-1, which is prepared against recombinant parts of the Ki67 antigen,[28] is highly correlated with Ki67 ($r = 0.92$),[56] so that our results in MBC can safely be compared with those found in cryostat sections of FBC using Ki67 antibody. In particular, the high prognostic significance of MIB-1 scores in MBC agrees with the recognized prognostic value of Ki67 in FBC.[27] However, we failed to demonstrate association with histological grade and hormone receptors contrary to several findings in FBC using Ki67.[26] Although high MIB-1 scores were seen in tumors with high mitotic counts, a consistent correlation between the two parameters was not demonstrated, contrary to our findings in non-Hodgkin's malignant lymphomas.[56] This could be partly related to variation in tumor cellularity and to the difficulty of identifying mitotic figures with conventional stains in old and possibly imperfectly fixed material in routine work.[57] We believe that in this case, MIB-1 staining could facilitate the detection of doubtful mitotic figures. MIB-1 staining is more suitable than PCNA in assessing the proliferative activity of MBC, because it homogeneously

stains the sections, with minimal variation in the intensity from nucleus to nucleus, so allowing easy and reproducible counts. Furthermore, the absence of background interference makes MIB-1 staining particularly suitable for automated image analysis.

We did not find correlation between ER or PgR status and prognosis in MBC, contrary to the findings in FBC.[6–9] This may depend on the different methods used for ER or PgR detection. Whereas most of the reported data in FBC have been achieved using biochemical assays or immunohistochemical methods on frozen sections, we employed formalin-fixed, paraffin-embedded tissues. In such cases, especially in very old archival material, ER positivity can be rather weak and some areas may be completely negative: indeed, the ER positivity rate (48%) of our MBC is lower than that biochemically detected in MBC.[11,12] However, the discrepancy could also reflect different biological properties of MBC: in fact, using archival material, no trend emerged between ER and prognosis in a series of MBC.[13] In our series, PgR could be more easily assessed than ER, because the positivity of the reaction was intense and uniform all over the sections. We interpret this as a result of the technique available for ER detection in paraffin-embedded tissues. Therefore, PgR counts may represent a reliable indicator of hormone status in MBC from archival material. The lack of association between PgR and survival further supports the tendency to consider MBC as biologically different from FBC, in which the presence of PgR is associated with a favorable prognosis.[8]

The overall survival rates for our series of MBC (49% and 17% at 5 and 10 years follow-up) are in accordance with reports indicating that MBC has a prognosis worse than FBC.[1–3] The less favourable outcome of MBC has been referred to its superficial or central location, to the absence of significant encompassing breast tissue with an earlier involvement of lymphatic vessels,[1] or to an intrinsically higher aggressivity.[4] We have shown that the prognosis of MBC was independent of hormone receptor status, whereas strongly associated with PCNA and MIB-1 scores and especially AgNOR counts. With the limitation due to the small number of cases, our study suggests that MBC is biologically different from FBC and that its high aggressiveness can be related to the proliferative activity of tumor cells.

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References

1. Norris HJ, Taylor HB: Carcinoma of the male breast. Cancer 1969, 23:1428–1435
2. Scheike O: Male breast cancer—six factors influencing prognosis. Br J Cancer 1974, 30:261–271
3. Ribeiro G: Male breast cancer: review of 301 cases from Christie Hospital and Holt Radium Institute, Manchester. Br J Cancer 1985, 51:115–119
4. Ciatto S, Iossa A, Bonardi R, Pacini P: Male breast carcinoma: review of a multicenter series of 150 cases. Tumori 1990, 76:555–558
5. Tavassoli F: Male Breast Lesions. Pathology of the Breast. Edited by Tavassoli FA. Norwalk, Conn., Appleton & Lange, 1992, pp 637–659
6. Kinne DW, Ashikari R, Butler A, Menendez-Botet C, Rosen PP, Schwartz M: Estrogen receptor protein in breast cancer as a predictor of recurrence. Cancer 1981, 47:2364–2367
7. Alanko A, Heinonen E, Scheinin T, Tolppanen EM, Vihko R: Significance of estrogen and progesterone receptors, disease-free interval, and site of first metastasis on survival of breast cancer patients. Cancer 1985, 56:1696–1700
8. McGuire WL, Clark GM: The role of progesterone receptors in breast cancer. Semin Oncol 1985, (suppl) 12:12–16
9. Pertschuk LP, Kim DS, Nayer K, Feldman JG, Eisenberg KB, Carter AC, Rong ZT, Thelmo WL, Fleisher J, Greene GL: Immunocytochemical estrogen and progestin receptor assays in breast cancer with monoclonal antibodies. Cancer 1990, 66:1663–1670
10. Andersen J, Thorpe SM, King WJ, Rose C, Christensen I, Rasmussen BB, Poulsen HS: The prognostic value of immunohistochemical estrogen receptor analysis in paraffin-embedded and frozen sections versus that of steroid-binding assays. Eur J Cancer 1990, 26:442–449
11. Everson RB, Lippman ME, Thompson EB: Clinical correlations of steroid receptors and male breast cancer. Cancer Res 1980, 40:991–997
12. Friedman MA, Hoffman PG, Dandolos EM, Lagios MD, Johnston WH, Siiteri PK: Estrogen receptors in male breast cancer: clinical and pathologic correlations. Cancer 1981, 47:134–137
13. Rogers S, Day CA, Fox SB: Expression of cathepsin D and estrogen receptor in male breast carcinoma. Hum Pathol 1993, 24:148–151
14. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ: Improvement in the staining and in the visualisation of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 1986, 18:5–14
15. Derenzini M, Ploton D: Interphase nucleolar organizer regions in cancer cells. International Review of Experimental Pathology: Molecular Cell Pathology. Edited by Richter GW, Solez K, San Diego, New York, Boston, London, Sidney, Tokyo, Toronto, Academic Press, 1991, pp 149–192

16. Eskelinen MJ, Lipponen PK, Collan Y, Syrjänen KJ: The role of nucleolar organizer regions as prognostic factors in breast cancer. Eur J Cancer 1991, 27:989–992
17. Sacks NP, Robertson JF, Ellis IO, Nicholson RI, Crocker J, Blamey RW: Silver-stained nucleolar organiser region counts are of no prognostic value in primary breast cancer. Eur J Surg Oncol 1992, 18:98–102
18. Aaltomaa S, Lipponen P, Syrjänen K: Nucleolar organizer regions related to morphometry, flow cytometry, sex steroid receptor content, tumour histology and prognosis in female breast cancer. Pathol Res Pract 1993, 189:416–421
19. Toikkanen S, Joensuu H: AgNOR counts have no prognostic value in breast cancer. J Pathol 1993, 169: 251–254
20. Mathews MB, Bernstein RM, Franza BR, Garrels JI: Identity of the proliferating cell nuclear antigen and cyclin. Nature 1984, 303:374–376
21. Hall PA, Levison DA, Woods AL, Yu CC-W, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, Waseem NH, Lane DP: Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 1990, 162:285–294
22. Magno WB, Hirschfield L, Bhuiya T, Harrison G, Mir R: Correlation of proliferative index (PCNA reactivity and Ki-67 reactivity) in primary breast carcinoma with hormone status, lymph node status, and disease-free survival. Conn Med 1992, 56:667–669
23. Aaltomaa S, Lipponen P, Papinaho S, Syrjänen K: Proliferating cell nuclear antigen (PC10) immunolabelling and other proliferation indices as prognostic factors in breast cancer. J Cancer Res Clin Oncol 1993, 119: 288–294
24. Narita T, Funahashi H, Satoh Y, Takagi H: Proliferating cell nuclear antigen immunostaining in breast cancer and its relation to prognosis. Jpn J Clin Oncol 1993, 23:20–25
25. Siitonen SM, Kallioniemi OP, Isola JJ: Proliferating cell nuclear antigen immunohistochemistry using monoclonal antibody 19A2 and new antigen retrieval technique has prognostic impact in archival paraffin-embedded node-negative breast cancer. Am J Pathol 1993, 142:1081–1089
26. Brown DC, Gatter KC: Monoclonal antibody Ki-67: its use in histopathology. Histopathology 1990, 17:489–503
27. Bouzubar N, Walker KJ, Griffiths K, Ellis IO, Elston CW, Robertson JF, Blamey RW, Nicholson RJ: Ki-67 immunostaining in primary breast cancer: pathological and clinical associations. Br J Cancer 1989, 59:943–947
28. Key G, Becker MHG, Duchrow M, Schluter C, Gerdes J: New Ki-67 equivalent murine monoclonal antibodies (MIB-1–3) prepared against recombinant parts of the Ki-67 antigen. Anal Cell Pathol 1992, 4:181
29. Cattoretti G, Becker MHG, Key G, Duchrow M, Schluter C, Gall J, Gerdes J: Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB-1 and MIB3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 1992, 168:357–363
30. Scarff RW, Torloni H: Histological Typing of Breast Tumours. International Histological Classification of Tumours, no. 2. Geneva, World Health Organization, 1968
31. Hermanek P, Sobin LH: TNM Classification of Malignant Tumours, 4th ed. Berlin, Heidelberg, New York, London, Paris, Tokyo, Springer-Verlag, 1987
32. Bloom HJG, Richardson WW: Histological grading and prognosis in breast cancer. Br J Cancer 1957, 11: 359–377
33. Elston CW, Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991, 19:403–410
34. Hiort O, Kwan PWL, DeLellis RA: Immunohistochemistry of estrogen receptor protein in paraffin sections. Am J Clin Pathol 1988, 90:559–563
35. Pich A, Pisani P, Krengli M, Cappello N, Navone R: Argyrophilic nucleolar organizer region counts and prognosis in pharyngeal carcinoma. Br J Cancer 1991, 64: 327–332
36. Kaplan EL, Meier P: Nonparametric estimation for incomplete observations. J Am Stat Assoc 1958, 53: 457–481
37. Cox DR: Regression models and life tables (with discussion). J R Stat (series B) 1972, 34:187–220
38. Dixon WJ, Brown MB, Engelman L, Frane JW, Hill MA, Jennrich RI, Toporek JD: BMDP Statistical Software. University of California, Berkeley, 1985
39. Giri DD, Nottingham JF, Lawry J, Dundas SAC, Underwood JCE: Silver-binding nucleolar organizer regions (AgNORs) in benign and malignant breast lesions: correlation with ploidy and growth phase by DNA flow cytometry. J Pathol 1989, 157:307–313
40. Derenzini M, Betts CM, Trerè D, Mambelli V, Millis RR, Eusebi V, Cancellieri A: Diagnostic value of silver-stained interphase nucleolar organizer regions in breast cancer. Ultrastruct Pathol 1990, 14:233–245
41. Mourad WA, Erkman-Balis B, Livingston S, Shoukri M, Cox CE, Nicosia SV, Rowlands DT Jr: Argyrophilic nucleolar organizer regions in breast carcinoma. Correlation with DNA flow cytometry, histopathology, and lymph node status. Cancer 1992, 69:1739–1744
42. Ohri AK, Ohri SK, Herbert A, Royle G, Taylor I: The relationship between clinical staging, oestrogen receptor status and silver-binding nucleolar organiser regions (AgNOR) in breast carcinoma. Eur J Surg Oncol 1992, 18:103–107
43. Sivridis E, Sims B: Nucleolar organizer regions: new prognostic variable in breast carcinomas. J Clin Pathol 1990, 43:390–392

44. Charpin C, Bonnier P, Piana L, Kouzhami H, Devictor B, Lavaut MN, Andrac L, Allasia C: Correlation of nucleolar organizer regions and nucleolar morphometry assessed by automatic image analysis in breast cancer with aneupoidy, Ki-67 immunostaining, histopathologic grade and lymph node involvement. Pathol Res Pract 1992, 188:1009–1017
45. DeCapoa A, Baldini A, Markelaj P, Natoli C, Rocchi M, Archidiacono N, Ciaufarani S, Spadoni GL, Boscherini B: Hormone-regulated rRNA gene activity is visualized by selective staining of the NORs. Cell Biol Int Rep 1985, 9:791–796
46. Nairn ER, Crocker J, McGovern J: Limited value of AgNOR enumeration in the assessment of thyroid neoplasm. J Clin Pathol 1988, 41:1136
47. Sasano H, Saito Y, Sato I, Sasano N, Nagura H: Nucleolar organizer regions in human adrenocortical disorders. Mod Pathol 1990, 3:591–595
48. Yu CC-W, Hall PA, Fletcher CDM, Camplejohn R, Waseem NH, Lane DP, Levison DA: Haemangiopericytomas: the prognostic value of immunohistochemical staining with a monoclonal antibody to proliferating cell nuclear antigen (PCNA). Histopathology 1991, 19: 29–33
49. Woods AL, Hall PA, Shepherd NA, Hanby AM, Waseem NH, Lane DP, Levison DA: The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in primary gastrointestinal lymphomas and its relationship to histological grade, S+G2+M phase fraction (flow cytometric analysis) and prognosis. Histopathology 1991, 19:21–27
50. Lipponen PK, Eskelinen MJ: Cell proliferation of transitional cell bladder tumours determined by PCNA/cyclin immunostaining and its prognostic value. Br J Cancer 1992, 66:171–176
51. Pich A, Chiusa L, Pisani P, Krengli M, Pia F, Navone R: Argyrophilic nucleolar organizer region counts and proliferating cell nuclear antigen scores are two reliable indicators of survival in pharyngeal carcinoma. J Cancer Res Clin Oncol 1992, 119:106–110
52. Dawson AE, Norton JA, Weinberg DS: Comparative assessment of proliferation and DNA content in breast carcinoma by image analysis and flow cytometry. Am J Pathol 1990, 136:1115–1124
53. Frierson HF Jr: Immunohistochemical analysis of proliferating cell nuclear antigens (PCNA) in infiltrating ductal carcinomas: comparison with clinical and pathologic variables. Mod Pathol 1993, 6:290–294
54. Siitonen SM, Isola JJ, Rantala IS, Helin HJ: Intratumor variation in cell proliferation in breast carcinoma as determined by antiproliferating cell nuclear antigen monoclonal antibody and automated image analysis. Am J Clin Pathol 1993, 99:226–231
55. Gasparini G, Meli S, Pozza F, Cazzavillan S, Bevilacqua P: PC-10 antibody to proliferating cell nuclear antigen (PCNA) is not related to prognosis in human breast carcinoma. Growth Regul 1992, 2:145–150
56. Pich A, Ponti R, Valente G, Chiusa L, Geuna M, Novero D, Palestro G: MIB-1, Ki67 and PCNA scores, and DNA flow cytometry in intermediate grade malignant lymphomas. J Clin Pathol 1994, 47:18–22
57. Donhuijsen K, Schmidt U, Hirche H, van Beuningen D, Budach V: Changes in mitotic rate and cell cycle fractions caused by delayed fixation. Hum Pathol 1990, 21:709–714