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MIB1 proliferation index in breast infiltrating carcinoma: Comparison with other proliferative markers and association with new biological prognostic factors

M.C. González-Vela, M.F. Garijo, F. Fernández and J.F. Val-Bernal

Anatomical Pathology Department, "Marqués de Valdecilla" University Hospital, Medical Faculty, University of Cantabria, Santander, Spain

Summary. Aims: In breast invasive carcinoma our objectives were I) to compare cellular proliferation determined by MIB1 index with S-phase fraction (SPF) assessed by flow cytometry and with mitotic index, and II) to examine the association of MIB1 index with classical and with new biological prognostic factors [bcl-2, p53, c-erbB-2 and cathepsin D (CD)]. Methods and results: From 102 cases of breast invasive carcinoma, 5-μm thick serial sections were cut from formalin-fixed, paraffin-embedded tissue blocks, and processed for detection of CD, c-erbB-2, p53, bcl-2, Ki-67 antigen MIB-1 and estrogen receptors (ER) and progesterone receptors (PR). SPF was measured by flow cytometry in fresh-frozen tissue samples taken from the carcinoma in each patient. MIB1 index was correlated with SPF (rho=0.45, p<0.0001) and with mitotic index (rho=0.42, p<0.0001). The MIB-1 index was positively associated with the histological grade (p=0.001), tumor size (p=0.04) and the presence of metastases in axillary lymph nodes (p=0.01). MIB1 was associated directly with p53 (p=0.045) and inversely with bcl-2 (p=0.0002). The MIB-1 index was not statistically associated with c-erbB-2. There was a weak association between MIB1 index and stromal cell CD. The median MIB1 index was higher in tumors with moderate to strong CD staining of stromal cell, but the difference did not reach statistical significance (p=0.09). Conclusions: MIB1 index correlates with well established methods for assessing tumor proliferation and with parameters of an aggressive phenotype of tumor. MIB1 index is an effective and readily accessible method for assessing tumor proliferation in breast carcinoma.

Key words: Breast carcinoma, MIB-1, Oncogene, Proliferation, Cathepsin D

Offprint requests to: J. Fernando Val-Bernal, Professor of Pathology, Departamento de Anatomía Patológical, Hospital Universitario Marqués de Valdecilla, Avda. Valdecilla s/n, 39008 Santander, Spain. Fax: +34-42-201903. e-mail: apavbj@humv.es

Introduction

In primary breast carcinoma the axillary lymph node status is still the most important prognostic factor, but it does not fully account for the varied disease outcome (McGuire and Clark, 1992). In fact, about 30% of negative-node patients will have tumor recurrence and are at risk for death (Galea et al., 1992). Therefore, the search for additional tumoral markers with prognostic significance has increased in the last years.

The assessment of the proliferative activity of breast carcinoma has been shown to be of prognostic significance using a number of different methods. But not all of these methods achieve the standards required for routine use in conventional pathology laboratory.

The mitotic count, defined as the number of mitotic figures per 10 high-power fields, is the most traditional and simple method to estimate cellular proliferation in breast cancer. However mitotic count has been criticized for a lack of standardization and reproducibility in the method (Woosley, 1991; Linden et al., 1992). Because major variations may result from the use of different optical systems, the mitotic index, that is, the number of mitotic figures per thousand cells, has been recommended as a more dependable indicator of cell growth (Simpson et al., 1992).

The thymidine-labelling index has been claimed to be a strong and independent prognostic factor (Silvestrini et al., 1995). However, like the bromodeoxyuridine-labelling index, viable tissue is needed, which makes these methods hard to apply in the routine setting.

S-phase fraction (SPF), determined by flow cytometry is the most clinically validated method for measuring tumor proliferative rate (Dressler et al., 1988; Hedley et al., 1993). However, flow cytometry requires expensive instrumentation, complex procedures and interpretation, and can produce variable results depending on the proportion of tumour cells in the sample (Woosley, 1991). Besides, the calculation of SPF in the presence of an aneuploid population is also

problematic (Woosley, 1991). In some instances, an SPF result cannot be provided.

These problems have motivated research into alternative methods for measuring proliferation, including immunohistochemistry against cell cycle-related antigens.

MIB1 is a recently developed monoclonal antibody that is directed against the same or similar epitope as the original Ki-67 (Key et al., 1993). The Ki-67 reacts with a nuclear antigen which is expressed by dividing cells in all phases of cell cycle except G0 (Gerdes et al., 1984). MIB1 has been shown to correlate strongly with Ki-67 staining (Weidner et al., 1994) and has the advantage of reacting with epitopes in routinely fixed, paraffin-embedded sections (Cattoretti et al., 1992).

In the present study we compared cellular proliferation as determined by MIB1 index with S-phase fraction (SPF) assessed by flow cytometry and with mitotic index and we studied the association of MIB1 index with classical prognostic factors and with other biological markers such as bcl-2, p53, c-erbB-2 and cathepsin D (CD).

Material and methods

The study group was based on 102 selected patients diagnosed as having invasive breast carcinoma at the "Marqués de Valdecilla" University Hospital (Santander, Spain) between 1 January 1994 and 31 December 1995. We selected this patient group because we had available fresh-frozen tissue samples taken from the carcinoma in each patient to perform flow cytometry study. Partial data on these patients have been recently published elsewhere (González-Vela et al., 1999). The mean age (±SD) was 61.28±13.5 years (range, 29 to 85). All of them were treated by partial or modified radical mastectomy and axillary lymph node dissection. The 102 carcinomas were classified as ductal in 68 cases (66.67%), lobular in 16 (15.69%), mixed in 13 (12.75%), and mucinous carcinoma in 5 (4.9%). The mean (±SD) tumor size was 6.12±1.35 and ranged from 0.9 to 14 cm. There were 29 tumors (28.43%) ≤ 2.0 cm and 73 (71.57%) >2.0 cm. There were 48 cases (47.06%) and 54 (52.94%) without and with axillary lymph node metastases, respectively. There were 47 cases (46.08%) of histological grade 3, 47 (46.08%) of grade 2 and 8 (7.84%) of grade 1 (according to the method described by Elston and Ellis (1991). Peritumoral lymphatic/vascular invasion was present in 42 (41.18%) of the 102 cases. The mitotic index was assessed in haematoxylin & eosin sections based on a method described previously (Simpson et al., 1992; Van Diest et al., 1992). Briefly, we counted the mitotic figures in 10 consecutive high power fields in the area with the highest density of mitotic figures using a BHT Olympus microscope model with a x40 objective and a linear eyepiece micrometer for a total magnification of x400. A quick estimate of the cell counts was obtained by a novel method proposed by Simpson et al. (1992). The ratio of total mitotic figures in 10 fields to the total number of cells present within the same 10 fields was the mitotic index expressed as number of mitoses per 1000 cells.

Immunohistochemistry

Immunohistochemistry was carried out in formalin-fixed, paraffin-embedded tissue sections by using the streptavidin-biotin peroxidase complex method (Hsu et al., 1981). Briefly, four-micrometer paraffin sections were cut and air dried overnight at room temperature. After deparaffinization with xylene, and hydration in decreasing grades of ethanol, endogenous peroxidase activity was eliminated by treating the slides with a solution of hydrogen peroxide and methanol. Heat-induced epitope retrieval was done by the pressure cooker method in 0.1 mol/L sodium citrate buffer (pH 6). Then the slides were sequentially treated with the diluted primary antibody, biotinylated antirabbit IgG immunoglobulin, and streptavidin-peroxidase complex (Vector laboratories™, Burlingame, CA, USA). Diaminobenzidine was used as chromogen in the presence of hydrogen peroxide. Slides were then counterstained with Mayer's haematoxylin, dehydrated in alcohol, cleared in xylene and mounted.

The antibodies employed in the study were: estrogen receptor (ER) (clone ER1D5, diluted at 1/100, Dako, Glostrup, Denmark); progesterone receptor (PR) (clone 1A6, diluted at 1/100, Dako); CD (clone C5, diluted at 1/100, Novocastra, Newcastle upon Tyne, UK); p53 (clone DO7, diluted at 1/100, Novocastra); bcl-2 (clone 124, 1/40, Dako); Ki-67 (clone MIB1, diluted at 1/100, Immunotech, Marseille, France) and c-erbB-2 (clone NCL-CB11, diluted at 1/125, Novocastra).

Positive scoring of immunohistochemistry for ER and PR was assessed on the basis of the visually estimated percentage of neoplastic cells with positive nuclear staining. All cases with negative staining and those with less than 10% of stained cells were regarded as negative, and all cases cases with 10% or more stained cells were regarded as positive. No account was taken of intensity.

The evaluation of immunostaining for CD was scored separately for the percentage of CD-immunopositive tumor cells and host stromal cells. Positive reactions were graded as minimal (less than 10% of cells staining), moderate (10% to 50%) and strong (more than 50% of cells staining). Tumors with a clearly detectable level (at least 10% strongly positive cells) were considered as having high expression of CD.

The MIB1 index was assessed based on the method described above for mitotic index. We counted the MIB1 staining nucleus in 10 high power fields (x400). Any nuclear staining (weak or strong) was considered positive. The MIB1 index was expressed as number of MIB1 staining nucleus per 1000 cells.

Bcl-2 expression was scored as follows: negative if no staining was seen with concurrent positive staining of lymphocytes surrounding tumor cells and positive if

more than 10% of tumor cells showed cytoplasmic staining with an intensity similar to lymphocytes. P53 protein was considered positive when at least 10% of the tumor cells showed nuclear staining.

C-erbB-2 immunostaining was considered positive when tumor cells showed moderate or strong diffuse membrane staining, as proposed by Wright et al. (1989).

Information about ER and PR status, c-erbB-2 and CD expression for the patients included in the present study was obtained from a previous publication (González-Vela et al., 1999).

Flow cytometry

Breast cancer tissue that remained after harvesting sufficient tumor for routine diagnostic procedures was cut into pieces measuring 0.3x0.3x0.3 cm and was frozen (-70 °C) stored and later used for DNA analysis. Frozen tissue was thawed and finely minced inside a plastic tube (ependorf) containing 1 cc of sodium citrate buffer. After the tissue was mechanically disassociated it was filtered through a 50-micron nylon mesh. The samples were concentrated by centrifugation for 10 minutes at 3000 rpm. and the supernatant was removed and treated with trypsin and RNAse (Sigma) and incubated with propidium iodide using the method described by Vindelov et al. (1983). At least ten thousand events from each sample were collected and analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, California, USA.) using CellFit Cell cycle analysis software. SPF was calculated using RFIT model. Inflammatory and normal host cells from the same specimen served as a control. DNA content was confirmed as being diploid if the tumor sample was observed in the same channel of the G0/G1 peak of the reference cells. Tumor DNA was classified as aneuploid if a separate peak was present from the standard DNA diploid G0/G1 peak. The DNA index (DI) was calculated as the ratio of the peak channel of abnormal DNA cells to the peak channel of the DNA diploid cells. Aneuploid population was subdivided on the basis of its DI in hipodiploid (DI<0.95); hiperdiploid (1.10<DI<1.90); tetraploid (1.90<DI<2.20) and hipertetraploid (DI>2.20). A tetraploid population was defined as having at least 15% of the sample events, found on the tetraploid G0-G1 peak with corresponding G2M peak of the reference cells.

Statistical analysis

Analysis was carried out using the STATISTICA package for Macintosh (Stat soft Inc. Tulsa, OK, USA). Results were expressed as mean±SD (standard deviation) for variables with a normal distribution, as median and interquartile range for those not normally distributed. Correlation between proliferation indices was determined using Spearman's rank order correlation. Discrete variables were grouped following logical classes. Categorical variables were compared using the Chi-squared test. Mann-Whitney U test and Kruskal-Wallis test were used to continuous variables. Results were considered statistically significant at $p<0.05$.

Results

Table 1 shows the mean, median and range of values for the proliferation markers studied. MIB1 staining was confined to the cell nucleus and there was little or no background staining. While the intensity of staining

Table 1. Values for proliferation markers in breast carcinoma.

	MEAN	SD*	MEDIAN	RANGE
MIB-1 index	29.4	22.72	22.76	0.63-92.49
S-Phase fraction (%)	8.44	5.98	7.45	0.21-24.60
Mitotic index	6.89	8.43	3.66	0.31-56.05

*: standard deviation

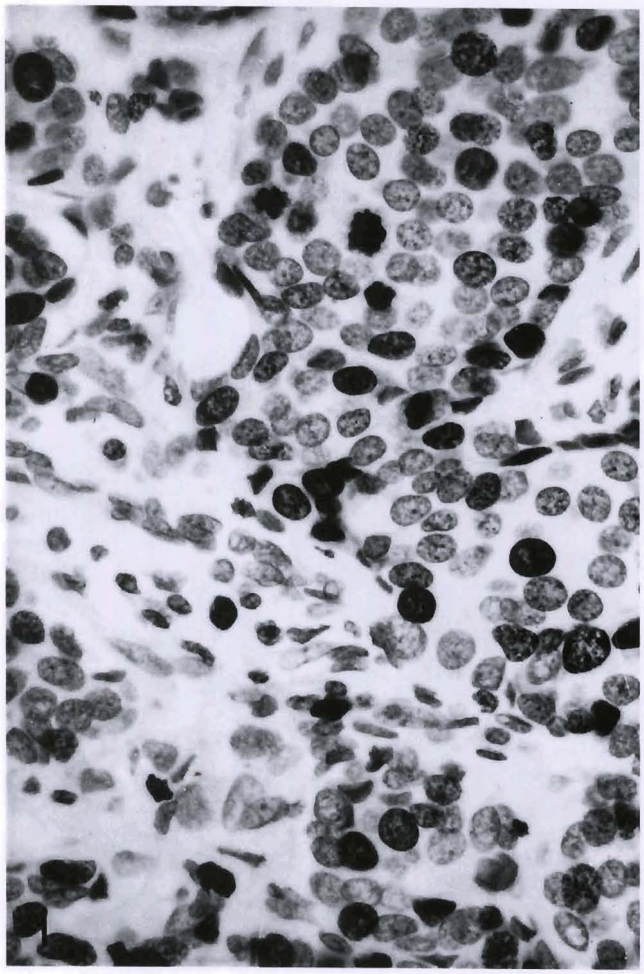

Fig 1. Immunohistochemical detection of Ki-67 antigen in breast carcinoma using MIB-1 antibody. Tumor cell nuclei stained in brown show immunoreactivity for Ki-67. (original magnification x 100)

varied from weak to very intense both within and between tumors, positive nuclei were readily identifiable (Fig. 1).

Of 102 histograms, 36 (35.29%) were DNA diploid and 66 were nondiploid (64.71%). Most nondiploid breast carcinomas were hyperdiploid (40.2%), and the remaining of nondiploid were tetraploid (15,69%), multiploid (5.88%) and hypertetraploid (2.94%). The SPF was valuable for 92 (90.2%) cases. The median SPF was significantly higher in the nondiploid tumors than in the diploid tumors (11.20% vs 4.14%; p<0.0001).

Using Spearman's rank correlation analysis there was a correlation between the three methods to evaluate the cellular proliferation (Table 2). The closest correlation was observed between SPF and mitotic index (rho=0.478, p<0.0001). MIB1 index was correlated with SPF (rho=0.457, p<0.0001) and with mitotic index (rho=0.428, p<0.0001).

The median MIB1 index was significantly higher in the DNA aneuploid tumors than in the diploid tumors (p=0.003).

Table 2. Spearman's correlations of proliferation markers with each other.

	MITOTIC INDEX	MIB1 INDEX
MIB1 index		
r	0.4287	
p	<0.0005	
S-Phase fraction		
r	0.4788	0.45771
p	<0.0005	<0.0005

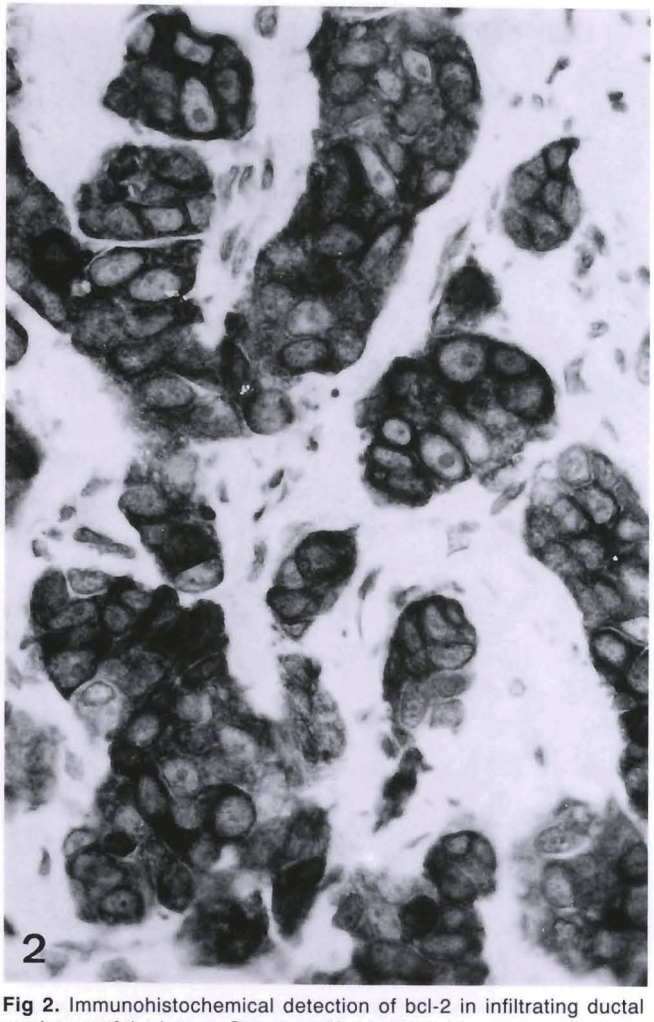

Fig 2. Immunohistochemical detection of bcl-2 in infiltrating ductal carcinoma of the breast. Strong positive bcl-2 staining in the cytoplasm cancer cells. (original magnification x 160)

Relationship of MIB1 index with classical prognostic factors

The association of MIB1 index with classical prognostic factors is shown in Table 3. The MIB1 index was positively correlated with the histological grade (p<0.0001) and tumor size (p<0.05). Tumors >2 cm and poorly differentiated had higher MIB-1 index. With respect to the presence or absence of metastases in axillary lymph nodes, we found that tumors with metastases had higher MIB1 index than tumors without metastases (p<0.05).

No association was observed between MIB1 index and ER and PR status, lymphovascular invasion and histological types.

Table 3. Relation of MIB1 index with classical prognostic factors (Mann-Whitney U test).

PARAMETER	CATEGORIES	n	p
Size			<0.05
	≤ 2cm	29	
	>2cm	73	
Histological grade			<0.001
	low	55	
	high	47	
Oestrogen receptor			NS
	positive	59	
	negative	43	
Progesterone receptor			NS
	positive	42	
	negative	60	
Axillary node status			<0.05
	negative	48	
	posistive	54	
Lymphovascular invasion			NS
	no	60	
	yes	42	
Histological grade			NS*
	ductal	68	
	lobular	16	
	mixed	13	
	mucinous	5	

*: Kruskal-Wallis test

Relationship of MIB1 index with p53, c-erbB-2 and bcl-2 and cathepsin D

The distribution of the biological markers within the patient population is summarized in Table 1. Positive bcl-2 immunoreactivity was observed in 46 (45.10%) cases. Immunoreactivity was always cytoplasmatic (Fig. 2). P53 immunoreactivity was always nuclear with no cytoplasmatic reactivity (Fig. 3). P53 immunoreactivity was observed in 27 (26.47%) cases. Table 3 summarizes the association between MIB-1 index and the biological prognostic factors. In essence, the median MIB1 index was significantly higher in p53-positive tumors (p=0.045). In the case of bcl-2 we found just the reverse correlation, bcl-2 protein expression was associated with low proliferative MIB1 index (p=0.0002).

We found a weak association between MIB1 index and stromal cell CD. The median MIB1 index was higher in tumors with moderate to strong CD staining of stromal cell, but the difference did not reach statistical significance (p= 0.09). In contrast, positive CD staining of tumoral cells had no association with the MIB-1 index. There was no association between MIB1 index and c-erbB-2 overexpression.

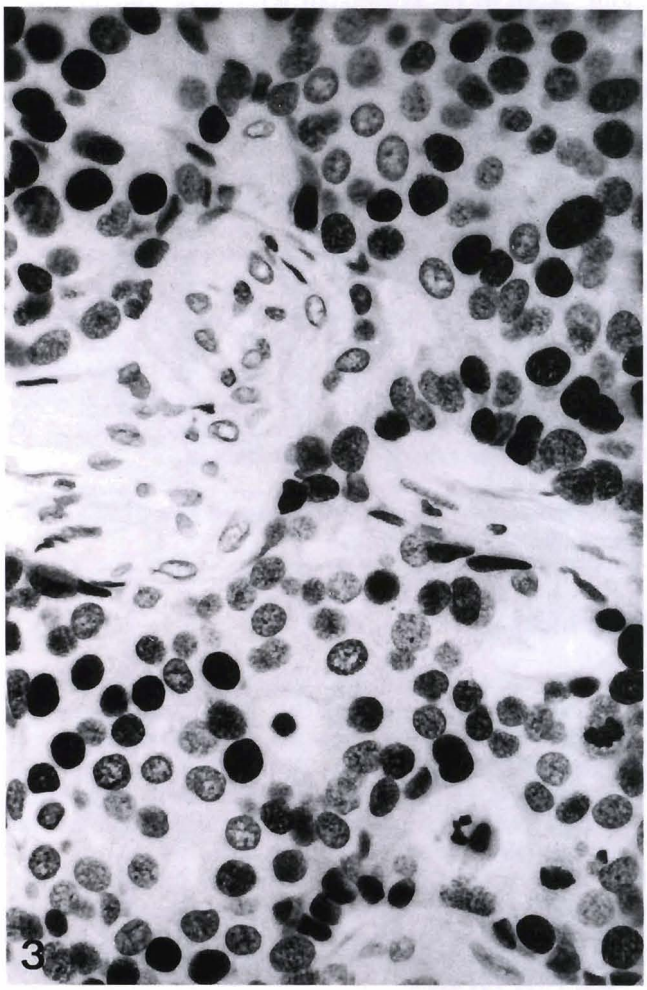

Fig 3. Immunohistochemical detection of p53 in breast carcinoma. Positive immunoreaction is observed in tumor cell nucleus. (original magnification x 100)

Discussion

The measurement of tumor proliferation is becoming increasingly important in the field of breast cancer research. Not only has proliferative activity been shown to be a prognostic indicator (Stal et al., 1993; Camplejohn et al., 1994), the ability to measure tumor proliferation may also be useful in other areas of investigation such as inter-relationship of proliferation and apoptosis and their effect on tumor growth rate, and the assessment of pathological response to treatment. It is thus important to have a technique that is accurate, reproducible and accessible to most laboratories.

Markers for tumor proliferation and growth rate have been suggested as new prognostic parameters in breast cancer. However, their prognostic impact is still controversial, partly because non-standardized determination methods are achieved.

SPF, as measured by flow cytometry is the most clinically validated of several methods for measuring tumor proliferative rate (Dressler et al., 1988; Hedley et al., 1993). However, the problems with determining SPF and the controversies surrounding mitotic figure counting have led to the search for an immunohistochemical proliferation marker antigen.

One such proposed immunohistochemical marker is the Ki-67 monoclonal antibody, which detects a partially characterized non-histone protein that is present in all phases of the cell cycle except G0 and early G1 (Gerdes et al., 1984). Many studies have shown a correlation between Ki-67 positivity and a poor prognosis in breast carcinoma (Sahin et al., 1991; Veronese et al., 1993).

Table 4. Relation of MIB1 index with biological prognostic factors (Mann-Whitney U test).

PARAMETER	CATEGORIES	n	p
p53			<0.05
	positive	27	
	negative	75	
bcl-2			<0.001
	positive	46	
	negative	56	
c-erB-2			NS
	positive	33	
	negative	69	
CD tumoral			NS
	positive	25	
	negative	77	
CD stromal			0.09
	positive	28	
	negative	74	

CD: cathepsin D; NS: not statistically significant.

However a disadvantage with Ki-67 antibody is that it is reactive only with frozen tissue. MIB1 is a recently developed monoclonal antibody that is directed against the same or similar epitope as the original Ki-67 (Key et al., 1993) and it has the advantage of reacting with epitopes in routinely fixed, paraffin-embedded sections (Cattoretti et al., 1992).

To assess the value of MIB1 index in estimating tumor proliferative activity, we compared MIB1 index with DNA flow cytometric estimation of SPF and with mitotic index. We have shown that MIB1 index was correlated with SPF and with mitotic index. Similar observations have been made in other studies (Keshgegian and Cnaan, 1995; Ostrowski et al., 1995; Ellis et al., 1996; MacGrogan et al., 1997). Others, however, found no correlation between MIB1 index and SPF (Dettmar et al., 1997; Jansen et al., 1998).

The explanation is not entirely clear. When comparing DNA flow cytometric and MIB1 index it should also be recognized that they measure different phases of the cell cycle (S-phase versus G1,S and G2/M). Also, they measure different biological properties: Ki-67 is a nuclear antigen, and SPF is calculated based on DNA content. These differences may serve as an explanation for the not strong correlation observed between MIB1 index and SPF.

MIB1 index was correlated mildly with mitotic index. The fact that the relation between MIB1 index and mitotic index was weak is not rare because the number of MIB1-positive cells is much higher than that of mitotic cells. This finding is in agreement with other reported studies (Weidner et al., 1994; Aranda and Lafarga, 1997).

In our study, MIB1 index was associated with histological grade, tumor size and DNA ploidy, as reported in most studies (Ostrowski et al., 1995; Moriki et al., 1996; Querzoli et al., 1996; Aranda and Lafarga, 1997). The association with lymph node involvement is consistent with some (Querzoli, 1996), but not other, previous reports (Keshgegian and Cnaan, 1995, Ostrowski et al., 1995, Pinder et al., 1995, Aranda and Lafarga, 1997).

MIB1 index was also associated with parameters of an aggressive phenotype of tumor. Thus, the median MIB1 index was significantly higher in tumors positive for p53, negative for bcl-2 and in tumors with moderate to strong CD staining of stromal cell.

Many studies have focused their attention on cell proliferation; however neoplastic growth not only depends on uncontrolled proliferation, but also on the cell loss, the latter being achieved by a special type of death called apoptosis. The *bcl-2* gene belongs to a new class of protooncogenes which are involved in the control of programmed cell death (Korsmeyer, 1992). The p53 tumor suppressor gene encodes for a phosphoprotein which is involved in the regulation of cell proliferation (Levine et al., 1991).

Our study showed that tumors positive for p53 and negative for bcl-2 were characterized by higher MIB1 index. Several studies have examined the relationship of p53 and expression to tumor growth rate, however, the association of p53 and bcl-2 with the new monoclonal MIB1 antibody has been scarcely reported (Querzoli et al., 1996; Sundland et al., 1996).

The higher MIB1 index in bcl-2-negative tumors suggests that neoplastic growth of breast carcinoma may result either from uncontrolled cellular proliferation or from factors inhibiting cell death. Conversely, the high MIB1 index in tumors positive for p53 suggests that both uncontrolled proliferation and inhibition of apoptosis may contribute to cell growth in breast carcinoma.

CD is a lysosomal acidic protease present in a variety of normal and neoplastic tissues. In vitro studies have suggested that CD can stimulate the proliferation of MFC-7 cells in autocrine manner (Vignon et al., 1986). Indeed, such action mechanism observed in experimental systems has been scarcely confirmed in human breast cancer. Our results suggest a trend towards a higher MIB1 index in tumors with moderate to strong CD staining of stromal cell. A high stromal cell CD level has been found to be associated with high SPF in other studies (Isola et al., 1993; Joensuu et al., 1995). It is known that tumor-associated macrophages and neoplastic cells have a complex relationship (Mantovani et al., 1992). CD could be a stimulator of cell growth and the hypotheses of the mechanism could be mitogenic effects assisted by stromal epithelial interactions. The stromal cells may provide an effective microenvironment for the proliferation of carcinoma cells.

In conclusion, this study shows that MIB1 index correlates well with SPF and mitotic index and is associated with other classical and biological prognostic factors such as histological grade, tumor size, DNA index, the oncoproteins p53 and bcl-2 and with stromal cell CD expression. MIB1 immunohistochemical study is a readily accessible tool that can be used to determine the proliferation rate on breast carcinoma.

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