Mib-1 proliferation index is an independent predictor of lymph node metastasis in invasive breast cancer: A prospective study on 675 patients

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Abstract. In order to evaluate the potential risk factors for Lymph node metastasis in invasive breast cancer patients submitted to axillary dissection, 675 patients who received surgery between January 1995 and December 2002 were included in a prospective study. In all cases, MIB-1 proliferation index was investigated by immunohistochemistry. Lymph node involvement was found in 248 out of 675 patients. Univariate analysis showed that peritumoral lymphovascular invasion, pT stage, tumor multiplicity, MIB-1 proliferation index >10%, oestrogen receptor status, histological type, tumor grade and progesterone receptor status were related to a higher incidence of Lymph node metastasis, with various levels of statistical significance. Multivariate analysis identified lymphovascular invasion [relative risk (RR, 7.69; p<0.001), pT stage (RR, 3.08; p<0.001), tumor multiplicity (RR, 3.89; p<0.001), and MIB-1 proliferation index (RR, 1.66; p=0.019)] as independent predictive variables. The impact of MIB-1 positivity on the incidence of Lymph node metastasis was particularly evident in intermediate risk groups (pT1c, pT2 without lymphovascular invasion), as well as in grade-2 tumors. In conclusion, the MIB-1 proliferation index could provide additional information about the risk of Lymph node metastasis in invasive breast cancer, and may be useful to identify grade-2 tumors with a more aggressive clinical behaviour.

Introduction

Nodal status is a strong prognostic factor in breast cancer and is one of the most important variables considered in deciding on adjuvant treatment after surgery. Sentinel node biopsy, although not the standard of care, is rapidly becoming widely accepted as an alternative staging procedure for the axilla in breast cancer. However, in subgroups of patients with a high risk of Lymph node metastasis, this procedure may not provide a clinical benefit and may involve a waste of resources as, in a high percentage of cases, it is followed by axillary dissection. The definition of tumor characteristics that are predictive of Lymph node metastasis may be useful in order to better select which patients to submit to axillary surgery (1).

Besides commonly used pathological factors, cell proliferation has an important role as an indicator of biological aggressiveness in breast cancer. Ki-67 antibody reacts with a nuclear antigen which is present in G1, S, G2 phases and mitosis but is absent in G0 and is therefore considered a marker of the proliferative activity of the tumor (2). Unfortunately, the epitope that recognizes Ki-67 is destroyed in the fixation and paraffin embedding process. More recently, the MIB-1 antibody, which recognizes Ki-67 antigen in formalin-fixed, paraffin-embedded tissue, has been described and applied to breast cancer (3,4).

Several studies have investigated the role of the proliferation index in breast cancer patients and a strong correlation between the MIB-1 labelling index and Lymph node metastasis has been reported (5,6), although this finding has not been confirmed by others (7-10). Similarly, conflicting results regarding the prognostic significance of the proliferation index have been found (9,11-13).

This prospective study was designed with the aim of evaluating the potential risk factors for Lymph node metastasis in a consecutive series of invasive breast cancer patients submitted to axillary dissection, with special reference to the role of the MIB-1 labelling index. Surgical treatment, histopathological examination and immunohistochemistry were performed on the entire series according to standard criteria.

Patients and methods

Patients. For this prospective observational study, we considered 675 patients with invasive breast cancer who received surgery at the Department of General Surgery and Surgical Oncology, University of Siena, between January 1995 and December 2002. The mean (± standard deviation, SD)

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age of the patients was 62±13 years (range, 27-94). Patients who underwent neoadjuvant chemotherapy and patients with inflammatory carcinoma were excluded from the study. Surgical treatment consisted of radical mastectomy in 309 patients and quadrantectomy in 366 patients. Only patients submitted to complete axillary dissection of first and second level lymph nodes were included; in the entire series, a mean (SD) of 18±6 lymph nodes (range, 7-50) were removed.

Pathological examination. After surgical removal, tumor specimens were fixed in formalin and sent for histopathological examination. Standard histopathological examination of axillary nodes included one central section of each lymph node, stained with haematoxylin and eosin. The histological type was defined as 'ductal', 'lobular' or 'other', according to the common histological criteria of the WHO classification. Histological grading was classified as well differentiated (grade 1), moderately differentiated (grade 2), or poorly differentiated (grade 3,) according to the Bloom and Richardson criteria modified by Elston and Ellis (14). The pathological tumor stage was assessed according to the criteria established by the International Union Against Cancer (UICC) (6th edition) (15). Oestrogen (ER) and progesterone receptor status (PGR) were assessed by immunohistochemistry and classified as 'negative' or 'positive'. Both multifocal and multicentric tumors were included in the 'multiplicity' subgroup (16).

MIB-1 immunostaining. Paraffin sections were cut to a thickness of five micrometers, immersed in citric acid buffer (pH 6.0) and incubated in a microwave oven twice at 750 W for 5 min. The sections were subsequently immunostained using the APAAP Complex method (Dako, Denmark) with the monoclonal antibody, MIB-1 (Dako). The intensity of immunostaining per cell was not quantified. Tonsil samples from our routine files were used as positive controls. For negative controls, we used normal mouse serum in which the primary antibody had been prepared.

Cells that stained positive for MIB-1 were registered in 10 randomly chosen HPFs and expressed as percentages of all epithelial cells. The slides were scored by the same pathologist (T.M.). We considered the tumor positive for MIB-1 when ≥10% of the cells counted were stained.

Statistical analysis. Correlation between Lymph node status (negative, positive) and clinicopathological variables was investigated by means of univariate and multivariate analysis. The χ^2 test was used to assess the statistical significance of the association between categorical variables and incidence of Lymph node metastasis or MIB-1 positivity. Differences in continuous variables were evaluated using the analysis of variance (ANOVA) test.

A logistic regression model was built in order to identify the variables that influenced Lymph node status in an independent manner, by multivariate analysis (17). The presence of Lymph node metastasis was considered as a dependent variable, whereas the following were considered as covariates: age (continuous variable), tumor grade (1, 2, 3), histological type (ductal, lobular, others), tumor multiplicity (absent, present), ER status (negative, positive), PGR status (negative, positive), pT stage (pT1a-pT1b, pT1c, pT2, pT3-pT4), number of removed lymph nodes (continuous variable), peritumoral lymphovascular invasion (LVI) (absent, present), and MIB-1 proliferation index (≤10%, >10%).

In the statistical program, the parameters of the model were estimated using the maximum-likelihood method. Significant variables were included in the model by means of forward stepwise selection: starting with a model containing

Table I. Incidence of axillary lymph node metastasis according to variables under study.

Variable	No. of patients	Lymph node negative	Lymph node positive	p-value
Age (mean ± SD)		63±12	62±13	0.353
Tumor grade				
1	148	103 (70)	45 (30)	0.039
2	331	210 (63)	121 (37)	
3	170	95 (56)	75 (44)	
Unknown	26			
Histological type				
Ductal	512	320 (63)	192 (37)	0.007
Lobular	96	54 (56)	42 (44)	
Others	67	53 (79)	14 (21)	
Tumor multiplicity				
Absent	567	390 (69)	177 (31)	<0.001
Present	108	37 (34)	71 (66)	
ER status				
Negative	161	84 (52)	77 (48)	0.001
Positive	511	342 (67)	169 (33)	
Unknown	3			
PGR status				
Negative	203	117 (58)	86 (42)	0.043
Positive	469	309 (66)	160 (34)	
Unknown	3			
No. of removed lymph nodes (mean ± SD)		17±6	18±7	0.064
Lymphovascular invasion				
Absent	503	376 (75)	127 (25)	<0.001
Present	146	34 (23)	112 (77)	
Unknown	26			
pT stage				
pT1a-pT1b	160	140 (88)	20 (12)	<0.001
pT1c	321	225 (70)	96 (30)	
pT2	155	59 (38)	96 (62)	
pT3-pT4	39	3 (8)	36 (92)	
MIB-1 expression				
Negative (≤10%)	357	251 (70)	106 (30)	<0.001
Positive (>10%)	318	176 (55)	142 (45)	

Numbers in parentheses are percentages; SD, standard deviation; ER, oestrogen receptor; PGR, progesterone receptor.

Table II. Independent predictors of lymph node metastasis (logistic regression model).

Variable	p-value	Hazard ratio (95% CI)
Lymphovascular invasion	<0.001	7.69 (4.60-12.85)
pT stage	<0.001	3.08 (2.29-4.15)
Tumor multiplicity	<0.001	3.89 (2.19-6.88)
MIB-1 positivity	0.019	1.66 (1.08-2.54)

CI, confidence interval.

only the constant, at each step the variable with the smallest significance value entered the model, with a default level of $p<0.05$. The significance value of each factor was reassessed at each step; if a variable in a forward stepwise block exceeded a significance level of 0.1, it was removed from the model. Removal testing was based on the probability of the likelihood-ratio statistic. The Statistical Package for the Social Sciences software (version 11.0) (SPSS™, Chicago, IL, USA) was used for statistical analysis.

Results

Lymph node involvement was found in 248 out of 675 patients (36.7%). In the 248 positive cases, the mean (SD) number of positive lymph nodes was 6.8±6.7 (range, 1-36).

By immunohistochemistry, MIB-1 positivity (>10% of tumor cells) was detected in 318/675 cases (47.1%). MIB-1 positivity was significantly related to tumor grade ($p<0.001$), pT stage ($p<0.001$), Lymph node involvement ($p<0.001$), negative ER status ($p<0.001$), negative PGR status ($p<0.001$) and ductal histological type ($p<0.005$).

The correlation between the incidence of Lymph node metastasis and the variables studied is reported in Table I; statistical analysis revealed a significant association with tumor grade, histological type, tumor multiplicity, ER status, PGR status, LVI, pT stage, and MIB-1 positivity, with various levels of statistical significance. The highest incidence of Lymph node metastasis was found in pT3-pT4 and pT2 tumors (92% and 62%, respectively), in cases with LVI (77%) and when tumor multiplicity was present (66%). MIB-1-negative cases showed positive lymph nodes in 30% of patients, in comparison to 45% of MIB-1-positive cases ($p<0.001$).

The influence of different variables on the risk of Lymph node metastasis was studied by means of multivariate analysis (logistic regression model), and the results are reported in Table II. LVI, pT stage, tumor multiplicity and MIB-1 positivity were selected as independent predictive variables. All other variables under study were excluded from the model. Total χ^2 of the model at step number 4 was 236.414, with a statistical significance of $p<0.001$, and residual χ^2 was 8.785 ($p=0.553$), thus indicating that the model fits the data more than adequately.

In order to evaluate the potential clinical utility of the MIB-1 labelling index in identifying patients at risk of Lymph node metastasis, a further subgroup analysis was performed using pT stage, LVI and MIB-1 expression (Table III); this time, tumor multiplicity was excluded from the analysis because it is difficult to assess by routine preoperative diagnostic examination and excisional biopsy. A strong influence of LVI on the incidence of Lymph node metastasis was found in all subgroups, with the exception of pT3-pT4 tumors; in patients with peritumoral LVI, very high rates of nodal metastasis were found at pT1a-pT1b stage (41%), pT1c stage (68%) and pT2 stage (89%). In contrast, pT1a-pT1b tumors without LVI showed a low risk of Lymph node metastasis (9%), irrespective of MIB-1 expression. With respect to MIB-1 positivity, this factor influenced the risk of nodal metastases in pT1c stage with LVI (52% in MIB-1-negative cases, vs. 79% in MIB-1-positive cases, $p<0.05$). A similar trend was also observed in pT1c stage tumors without LVI, and in pT2 stage tumors without LVI, although this was not statistically significant.

Table III. Incidence of axillary lymph nodes metastasis according to pT stage, peritumoral lymphovascular invasion, and MIB-1 expression.

pT stage	Lympho-vascular invasion	MIB-1 negative		MIB-1 positive		p-value
pT1a - pT1b						
	Absent	10/103	(10)	3/38	(8)	0.737
	Present	4/10	(40)	3/7	(43)	0.906
pT1c						
	Absent	20/122	(16)	30/120	(25)	0.097
	Present	12/23	(52)	26/33	(79)	0.036
pT2						
	Absent	17/43	(40)	37/65	(57)	0.076
	Present	19/20	(95)	23/27	(85)	0.261
pT3 - pT4						
	Absent	5/5	(100)	5/7	(71)	0.118
	Present	13/14	(93)	12/12	(100)	0.259

Numbers in parentheses represent the incidence of lymph node metastasis.

During analysis of the subgroups, we also noticed a strong impact of MIB-1 immunoreactivity on the incidence of nodal metastasis when stratifying for tumor grade (Table IV). A significant difference related to MIB-1 positivity was observed in grade-2 tumors (27% vs. 50%, $p<0.001$); those with negative MIB-1 expression had an incidence of Lymph node metastasis similar to grade-1 tumors, whereas in MIB-1-positive cases the incidence was similar to grade-3 tumors.

Discussion

Prediction of lymph node status is an important issue in breast cancer patients, and several papers in the literature are concerned with both commonly used and non-conventional predictive factors (1,16,18,19). Several authors have focused their attention on molecular markers identified by immunohistochemistry (5-7,9). However, many studies are retrospective, and often include patients operated on over a long

Table IV. Incidence of axillary lymph node metastasis according to tumor grade and MIB-1 expression.

Tumor grade	MIB-1 negative	MIB-1 positive	p-value
1	27/102 (26)	18/46 (39)	0.126
2	54/197 (27)	67/134 (50)	<0.001
3	22/47 (47)	53/123 (43)	0.663

Numbers in parentheses represent the incidence of lymph node metastasis.

time period or in different surgical units. This study was designed in a prospective manner. All patients were operated on in the same Surgical Department, using a standard technique for axillary Lymph node dissection. The resected specimens were processed in the Pathological Unit according to standard procedure, and immunohistochemical analysis was conducted and evaluated by the same pathologists, with the aim of minimizing the potential bias related to different methodological techniques and inter-observer reproducibility, which is commonly found in immunohistochemistry (20). Of the different methods for estimating cell proliferation, we employed MIB-1 expression because of its simplicity in comparison to Ki-67 analysis, which requires frozen material and a more complex procedure (21); furthermore, a better clinical significance of MIB-1 labelling index on paraffin sections has been reported with respect to Ki-67 expression on frozen material (4).

Similarly to the results of other studies, LVI and pT stage were the most important predictors of Lymph node metastasis in our patients. The incidence of Lymph node metastasis increases with the pT stage (1,18). This was confirmed in our series, in which only 12% of pT1a or pT1b lesions were N-positive. However, even in this low-risk group, the few cases (17 out of 158, 11%) with LVI had an incidence of Lymph node metastasis higher than 40%. The effect of LVI on nodal status was also evident in pT1c and pT2 tumors, a finding reported by other authors and in a previous study by our group (6,19,22). The strong association with Lymph node metastasis indicates that LVI could probably be regarded as the precursor of nodal involvement (23). Peritumoral vascular invasion has also been reported as a risk factor for additional axillary metastases in patients with a positive sentinel lymph node, which indicates a greater propensity to spread to multiple lymph nodes (24). As such, LVI requires careful consideration when deciding about surgical approach to the axilla.

The results of univariate and multivariate analysis showed that MIB-1 expression could provide additional information about the risk of axillary Lymph node metastasis. The effect of MIB-1 expression is particularly evident in the intermediate risk groups (pT1c, pT2 without LVI), which may increase its clinical utility. The incidence of nodal metastases in pT1c tumors with LVI and MIB-1 positivity was 79%, which is similar to that of the highest risk groups. On the contrary, the risk in MIB-1-negative pT2 tumors without LVI was lower with respect to smaller tumors (pT1a-pT1b) with LVI. No effect was found in very low or very high risk groups (pT1a-pT1b, pT2 with LVI, and pT3-pT4).

In addition to Lymph node status, MIB-1 positivity was significantly related to tumor grade, pT stage, negative ER and PGR status, and ductal histological type; no correlation was found with LVI. On the other hand, both MIB-1 positivity and LVI were independent risk factors for Lymph node metastasis. This suggests that a greater proliferative activity of tumor cells may not affect the invasion of peritumoral lymphatic vessels but may increase the propensity to spread to axillary lymph nodes in an independent manner. In a recent study, a greater proliferation index was found in positive axillary lymph nodes with respect to their primary breast tumors, thus suggesting that cells with a more aggressive potential (such as those with a high proliferation index) are more likely to escape from the primary tumor and metastasize to axillary lymph nodes (25).

The association between MIB-1 labelling index and Lymph node metastasis in breast cancer patients has been reported by other authors, mostly when early stages of invasive breast cancer were considered (5,6). In other studies, on the contrary, the proliferation index was found to be related to an adverse prognosis but it did not correlate with Lymph node status, although a multivariate analysis by means of a prediction model was rarely performed (8-10,13). Differences in patient cohorts, immunohistochemical technique, inter-observer variability, counting method and the cut-off level used may explain the different results (20). We believe that a prospective study conducted on a large series in a single institution, with a standard technique and a specifically designated pathologist for immunohistochemical analysis, may be one of the best methods for assessment of the clinical significance of molecular markers in breast cancer.

Even though tumor multiplicity was a strong predictor of Lymph node metastasis with a high relative risk at multivariate analysis, we did not include this factor in the subgroup analysis because its definition by routine preoperative diagnostic examination and excisional biopsy is difficult. However, when we examined the group of patients without tumor multiplicity separately, the impact of MIB-1 expression on the risk of Lymph node metastasis was confirmed (data not shown). The influence of tumor multiplicity on axillary status has been reported in several studies (26). This may be due to a higher tumor volume in multicentric or multifocal tumors, where pT stage is classified according to the size of the dominant lesion, with respect to unifocal lesions. Recent studies have suggested, however, that breast tumors with multiple macroscopic nodules may have a different biology and a higher propensity to Lymph node metastasis, which is not simply a function of their tumor volume (16). This factor should be taken into account when evaluating the risk of Lymph node metastasis in breast cancer surgery.

A strong correlation between MIB-1 expression and tumor grade was also observed in this study. Tumor grading according to the modified Bloom-Richardson criteria is based on three major elements: the degree of glandular differentiation, relative nuclear pleomorphism, and mitotic activity (14). However, the assessment of these three factors entails some elements of

subjectivity, thus involving incomplete inter-observer reproducibility, especially in intermediate-grade cases. The potential use of MIB-1 expression in the definition of a tumor grading system with a greater clinical utility has recently been suggested (21). We assessed the impact of MIB-1 expression on the risk of Lymph node metastasis in different grade subgroups amongst our patients and found a strong difference between MIB-1-negative and -positive cases in grade-2 tumors. The risk of nodal metastasis in MIB-1-negative grade-2 cases was similar to grade-1 tumors, whereas the risk in MIB-1-positive grade-2 cases was similar to grade-3 tumors. This suggests that MIB-1 expression could identify subgroups of grade-2 tumors with a more aggressive clinical behaviour. During the St. Gallen Conference of 2001, the International Consensus Panel defined two risk categories for patients with node-negative breast cancer: tumor grade 2 to 3 was one of the criteria for the inclusion in the high-risk category, in which adjuvant chemo-therapy is recommended (27). MIB-1 expression could be useful in distinguishing grade-2 tumors with a different biological aggressiveness, thus reducing problems related to doubtful pathological grade allocations.

In conclusion, the results of our prospective study indicate that the MIB-1 proliferation index may offer additional information about the risk of Lymph node metastasis in breast cancer, and could be useful in the identification of a more aggressive phenotype of grade-2 tumor. A strong relationship between Ki-67/MIB-1 expression and nodal status in pre-operative FNA biopsies has recently been reported (28). This may increase the clinical utility of this parameter in decision-making regarding the surgical approach for breast cancer patients.

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