Prognostic Significance of Tumor Cell Proliferation Rate as Determined by the MIB-1 Antibody in Breast Carcinoma: Its Relationship with Vimentin and p53 Protein

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ABSTRACT

The prognostic value of tumor cell proliferative activity as measured by the MIB-1 monoclonal antibody in invasive ductal not otherwise specified breast carcinomas was determined for 186 patients, including 111 with no axillary node involvement. The MIB-1 antibody detects the Ki-67 antigen in microwave-processed paraffin sections of the formalin-fixed tumors. The mean MIB-1 score was 16% for all tumors, 16% for the node-negative group, and 15% for the node-positive group. In univariate survival analysis, the MIB-1 score (dichotomized, ≤10% versus ≥10%) predicted overall 5-year survival in all of these groups. The mean MIB-1 score was significantly higher in vimentin- and p53 protein-positive tumors (P < 0.001) than in negative ones. The impact of vimentin expression and of p53 positivity on the prognostic significance of the tumor cell proliferation rate was assessed. Vimentin was associated significantly with poor 5-year survival in the entire cohort, and a particularly strong association was found in the node-negative group. p53 had a weak but statistically nonsignificant influence on survival. In a multivariate analysis using the Cox proportional hazards model, vimentin (P = 0.0002) was the only independent prognostic factor in node-negative patients. In contrast, the MIB-1 score (P = 0.009) was the only independent prognostic factor in the node-positive group. Therefore, node-negative patients with vimentin-positive tumors and node-positive patients with tumors with high proliferation rates might be appropriate candidates for early adjuvant chemotherapy.

INTRODUCTION

Axillary lymph node status is regarded as the single most important prognostic indicator in breast cancer. However, additional prognostic factors are needed, because the majority of breast cancer patients currently diagnosed are axillary node negative, yet 25–35% will relapse (1, 2). Tumor cell proliferation as measured by methods such as tritiated thymidine uptake (3, 4), flow cytometry (5), mitotic counts (6), and assessment of proliferation-associated antigens (7–12) is regarded as a strong indicator of poor prognosis in breast cancer. However, it seems to be interrelated with other prognostic factors. For example, both vimentin positivity (13, 14) and p53 protein positivity (15, 16) have been associated with an increased tumor cell proliferation rate, and all three factors seem to have prognostic significance in breast cancer (4, 16–21). From a practical point of view, it is not known which one or which combination of these markers should be used to obtain maximum reliable prognostic information.

Our interest in this study was 2-fold. First, we wanted to use the MIB-1 antibody in a prognostic study in invasive ductal NOS breast cancer, with the emphasis on node-negative patients. The MIB-1 antibody was generated against recombinant parts of the Ki-67 antigen (22). Ki-67 is a nuclear antigen present in G1, S, G2, and M phases of the cell cycle but not in resting cells (23). The MIB-1 antibody is particularly useful in the evaluation of the growth fraction of tumor cells, because it immunostains formalin-fixed, paraffin-embedded sections, provided they have been heated in a microwave oven (22). A significant correlation was found between the MIB-1 labeling index and that of Ki-67 on frozen sections (24) and between the MIB-1 and mitotic tumor indices and the Scarff-Bloom-Richardson histological grade of breast carcinomas (25). Our second aim was to determine whether the proliferation rate, vimentin positivity, and p53 positivity of tumor cells are dependent or independent prognostic indicators. Although vimentin (13, 14) and p53 protein (15, 16) are related to the cell proliferation rate, the prognostic effect of p53 protein seems to be related mainly to the proliferation rate of tumor cells (16), whereas vimentin is also associated with increased invasiveness of breast cancer cell lines (26). This is also reflected in vivo, in which, particularly in node-negative patients, those with vimentin-positive tumors have a significantly higher probability of visceral relapse (19). A similar association between p53 status and visceral metastases was not found (19). Therefore, we hypothesized that in node-negative patients, the prognostic effect of vimentin, but not that of p53 protein, might be much stronger than that of the cell proliferation rate. Therefore, we assessed tumor cell proliferation by immunohistochemistry using the novel MIB-1 monoclonal antibody on microwave-treated paraffin sections of formalin-fixed, invasive, ductal NOS breast carcinomas in which the

Received 5/30/95; revised 8/10/95; accepted 8/17/95.

1 This work was supported by European Union Grant CT93-0234.

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3 The abbreviations used are: NOS, not otherwise specified; ER, estrogen receptor.
vimentin and p53 protein statuses of the same tumors were already known. The results were compared with survival curves for a 5-year period.

MATERIALS AND METHODS

Patients. Formalin-fixed and paraffin-embedded tumor samples from 186 unselected female patients with primary invasive ductal NOS breast cancer who underwent mastectomy with lymph node dissection between 1980 and 1987 were examined. These were retrieved from the files of the Department of Oncology, Medical Academy of Lodz. Clinical follow-up was available for at least 60 months. Histological typing was performed as described by Millis and Girling (27), and histological grading was according to Bloom and Richardson (28). The computerized database contained the age of the patient, the number of positive axillary lymph nodes, the size of the tumor, histological type and grade, the stage of the disease at diagnosis, the treatment protocol, and the date of the last checkup or death. In addition, vimentin and p53 protein statuses of the tumor were also on file. Some patients analyzed in this study were included in previous studies on vimentin expression and survival (18) and on p53, vimentin, survival, and sites of metastases (19). There were 111 axillary lymph node-negative and 75 lymph node-positive patients. Lymph node-negative patients underwent either only mastectomy with removal of the axillary lymph nodes (45 patients) or mastectomy with removal of the nodes followed by radiation therapy (19 patients), chemotherapy (22 patients), hormone therapy (3 patients), radiation therapy and chemotherapy (7 patients), or combinations of radiation therapy, chemotherapy, and hormone therapy (11 patients). Four patients were treated by quadrantectomy with removal of axillary nodes followed by radiation therapy. Lymph node-positive patients underwent either only mastectomy with removal of the axillary lymph nodes (6 patients) or mastectomy with removal of the nodes followed by radiation therapy (22 patients), chemotherapy (4 patients), hormone therapy (2 patients), radiation therapy and chemotherapy (22 patients), or combinations of radiation therapy, chemotherapy, and hormone therapy (18 patients). One patient underwent quadrantectomy with removal of axillary nodes followed by radiation therapy. As in all studies of this type, the use and kind of adjuvant therapy have to be taken into account in interpreting the results.

Immunohistochemistry. Sections were deparaffinized, immersed in citrate buffer, and heated in a microwave oven according to Cattoretti et al. (22). The sections were incubated with MIB-1 monoclonal antibody (Dianova, Hamburg, Germany) diluted 1:30 for 45 min at room temperature. Sections were washed and reacted with biotinylated rabbit antimouse antibody and streptavidin peroxidase (Histostain-SP kit; Zymed Laboratories, Inc., San Francisco, CA). The sections were washed and then lightly counterstained with hematoxylin. Areas with the highest numbers of MIB-1-positive cells were identified by scanning sections at low magnification. These areas occurred mostly at the periphery of tumors. Counts were started in these areas, and then additional contiguous fields were selected randomly until at least 500 tumor cells were counted using a 10 × 10 square ocular grid and ×400 magnification. The results were scored as the percentage of tumor cell nuclei reactions with the MIB-1 antibody. The MIB-1 score was assessed independently by two pathologists (W. D. and M. M.) without knowledge of other data of the patient. The results were combined, and the mean was used. The MIB-1 scores determined by W. D. correlated highly with those determined by M. M. (linear regression analysis, r = 0.85; P > 0.0001; 95% confidence interval).

Statistical Analysis. The association between MIB-1 score and vimentin and p53 statuses was assessed by the Student t test. Univariate survival analysis was performed on p53, vimentin, survival, and sites of metastases (19). Treatment was included in previous studies on vimentin expression and survival (18) and on MIB-1, vimentin, survival, and sites of metastases (19). There were 111 axillary lymph node-negative and 75 lymph node-positive patients. Lymph node-negative patients underwent either only mastectomy with removal of the axillary lymph nodes (45 patients) or mastectomy with removal of the nodes followed by radiation therapy (19 patients), chemotherapy (22 patients), hormone therapy (3 patients), radiation therapy and chemotherapy (7 patients), or combinations of radiation therapy, chemotherapy, and hormone therapy (11 patients). Four patients were treated by quadrantectomy with removal of axillary nodes followed by radiation therapy. Lymph node-positive patients underwent either only mastectomy with removal of the axillary lymph nodes (6 patients) or mastectomy with removal of the nodes followed by radiation therapy (22 patients), chemotherapy (4 patients), hormone therapy (2 patients), radiation therapy and chemotherapy (22 patients), or combinations of radiation therapy, chemotherapy, and hormone therapy (18 patients). One patient underwent quadrantectomy with removal of axillary nodes followed by radiation therapy. As in all studies of this type, the use and kind of adjuvant therapy have to be taken into account in interpreting the results.

RESULTS

MIB-1 Staining in Breast Carcinomas

All tumor cells in which red color was found in the nuclei or nucleoli were scored as positive regardless of staining intensity (which in general was strong). MIB-1 positivity was scored by two pathologists (W. D. and M. M.). The mean MIB-1 score for the whole series of tumors was 16% (SD, 14%; range, 0.1–60.4%; median, 12%); for tumors from axillary lymph node-negative patients, the mean score was 17% (SD, 14%; range, 0.1–52.7%; median, 14%); and for tumors from node-negative patients, the mean score was 15% (SD, 15%; range, 0.4–60.5%; median, 10%). The mean MIB-1 score in our study, i.e., 16.2%, falls within the range of values reported with the use of the MIB-1 antibody (15.2–24%; Refs. 24, 25, and 29) as well as the Ki-67 antibody on frozen sections of breast carcinomas, i.e., between 7.2 and 22% (12, 30–32). The considerable variability in MIB-1 and Ki-67 scores in the different studies has been attributed to the use of different evaluation methods and to the inherent variability between individual patients and also between distinct high-power fields from the same tissue section (12). Nevertheless, Wintzer et al. (12) found a high degree of correlation between results obtained by two different counting methods, i.e., 10 random high-power counts versus the area with the highest labeling density count.

To correlate the MIB-1 score with vimentin and p53 statuses and to analyze the relationship to survival, a 10% cutoff level for the MIB-1 score was chosen to separate the high-risk group from the low-risk breast cancer. In previous survival studies using the Ki-67 antibody, various cutoff levels were used, e.g., 7.5% (9), 9% (8), 10% (10), 13% (11),
The mean MIB-1 score was significantly higher in vimentin-positive than in vimentin-negative tumors \( (P < 0.001 \text{ for all and for axillary node-negative patients}; \ P < 0.01 \text{ for node-positive patients}) \). p53-positive tumors also had a significantly higher mean MIB-1 score than those cancers that did not express this protein \( (P < 0.001 \text{ for all and for node-negative and node-positive patients}; \ Table 1) \).

**Table 1** MIB-1 score in relation to vimentin and p53 status in patients with invasive ductal NOS breast carcinomas

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<th>n (%; mean ± SD)</th>
<th>P</th>
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<td>186</td>
<td>16.18 ± 14.19</td>
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<tr>
<td>Vimentin</td>
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</tr>
<tr>
<td>Negative</td>
<td>145</td>
<td>13.26 ± 11.69</td>
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<tr>
<td>Positive</td>
<td>41</td>
<td>26.52 ± 17.30</td>
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<tr>
<td>p53</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>129</td>
<td>12.44 ± 10.81</td>
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<tr>
<td>Positive</td>
<td>50</td>
<td>26.62 ± 16.89</td>
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<td>Node negative Vimentin</td>
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<td>16.75 ± 13.86</td>
</tr>
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<td>Positive</td>
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<td>70.00 ± 0.50</td>
</tr>
<tr>
<td>p53</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>76</td>
<td>13.19 ± 11.44</td>
</tr>
<tr>
<td>Positive</td>
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<td>25.07 ± 15.67</td>
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<td>Node positive Vimentin</td>
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<td>15.35 ± 14.73</td>
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<td>26.84 ± 23.00</td>
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<td>11.35 ± 9.84</td>
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<td>29.36 ± 19.03</td>
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**Correlation of MIB-1 Scores with Vimentin and p53 Protein**

The mean MIB-1 score was significantly higher in vimentin-positive than in vimentin-negative tumors \( (P < 0.001 \text{ for all and for axillary node-negative patients}; \ P < 0.01 \text{ for node-positive patients}) \). p53-positive tumors also had a significantly higher mean MIB-1 score than those cancers that did not express this protein \( (P < 0.001 \text{ for all and for node-negative and node-positive patients}; \ Table 1) \).

**Prognostic Significance of MIB-1 Score with Respect to Vimentin and p53 Protein Status**

**All Patients.** One hundred sixteen (61%) patients survived 5 years. The mean follow-up time for these patients was 88 months. In the univariate survival analysis, the MIB-1 score (dichotomized, \( \leq 10 \) versus \( >10\% \)) and vimentin predicted 5-year survival \( (Table 2) \). Classical prognostic factors such as lymph node status, tumor size, and histological grade were also strong indicators of prognosis \( (Table 2) \). Survival curves also show that a high MIB-1 score is associated with poor survival of patients at 5 years in the entire cohort \( (P < 0.01) \), in node-negative patients \( (P < 0.05) \), and in node-positive patients \( (P < 0.01; \ Fig. 1, A-C) \).

When all the factors listed in \( Table 2 \) were tested in the Cox proportional hazards model for their relationship with overall survival, the four factors selected by the model as independent predictors of survival at 5 years were node status, tumor size, MIB-1 score (dichotomized, \( \leq 10 \) versus \( >10\%) \) and vimentin status \( (Table 3) \).

**Axillary Node-negative Patients.** The results of univariate survival analysis indicate that vimentin status, tumor size, and MIB-1 score (dichotomized) predict 5-year survival \( (Table 2) \). Survival curves for node-negative patients also show that a high MIB-1 score was associated significantly with poor sur-
Table 2  Prognostic factors predicting 5-year survival of patients

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<tr>
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<td>44</td>
<td>NSa</td>
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Table 3  Relationship of prognostic factors to reduced 5-year overall survival

Results of the Cox proportional hazards model analysis for all (n = 177) and for node-negative (n = 106) and node-positive (n = 71) patients. Other factors tested in the Cox model were histologic grade, p53 status, and age of the patient.

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<th>All patients</th>
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<th>Node positive</th>
<th>RR</th>
<th>P</th>
<th>RR</th>
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<td>NS</td>
<td>2.43 0.009</td>
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<td>3.55 0.0002</td>
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a RR, relative risk of death; NS, not significant; NA, not applicable.

Node-negative patients with p53-negative and slowly proliferating (MIB-1 score, ≤10%) tumors had better prognoses than those with high MIB-1 scores (Fig. 2b, curve B versus C) but the trend was not significant (χ² = 3.146). If node-negative patients with p53-positive tumors were further divided by MIB-1 score, survival curves differed slightly, in that patients with highly proliferating tumors had worse 5-year survival, but the difference did not reach statistical significance (χ² = 2.157). Node-negative patients with p53-negative and slowly proliferating (MIB-1 score, ≤10%) tumors had better prognoses than those with high MIB-1 scores (Fig. 2b, curve B versus C) but the trend was not significant (χ² = 3.146). If node-negative patients with p53-positive tumors were further divided by MIB-1 score, survival curves differed slightly, in that patients with highly proliferating tumors had worse 5-year survival, but the difference did not reach statistical significance. When the node-negative patients were divided by MIB-1 score and p53 status (curves C versus D and A versus B), patients with p53-positive tumors had worse survival irrespective of low (curve B, P < 0.01) or high (curve C, P < 0.05) proliferation rate. Thus, analysis of survival curves indicates that in node-negative patients, vimentin is a stronger indicator of poor prognosis than proliferative activity of tumor cells. At 5 years, only about 50% of patients with vimentin-positive tumors survive, irrespective of whether the patients had tumors with high or low MIB-1 scores (curves C and B). In contrast, the proliferation rate only seems to play a role in the survival of node-negative patients with vimentin-negative tumors. Here, about 75% of patients with fast-proliferating tumors, i.e., MIB-1 scores > 10%, survive compared with 90% of those with MIB-1 scores ≤10%, although the difference does not reach statistical significance (χ² = 2.157).

Node-negative patients with p53-negative and slowly proliferating (MIB-1 score, ≤10%) tumors had better prognoses than those with high MIB-1 scores (Fig. 2b, curve A versus D) but the trend was not significant (χ² = 3.146). If node-negative patients with p53-positive tumors were further divided by MIB-1 score, survival curves differed slightly, in that patients with highly proliferating tumors had worse 5-year survival, but the difference did not reach statistical significance.
Axillary Node-positive Patients. The univariate survival analysis shows that a high MIB-1 score and high histological grade are associated significantly with 5-year survival of patients (Table 2). Survival curves also show a significant prognostic difference when node-positive patients were divided according to proliferation rate ($P < 0.01$; Fig. 1C).

When this group of patients was divided by MIB-1 score and survival curves plotted by vimentin status, a significant negative correlation of vimentin expression and survival was seen only in patients with slowly proliferating tumors ($P < 0.05$, Fig. 3a, curve A versus B; compare with curve C versus D). When the node-positive patients were divided by vimentin status and survival curves were plotted by proliferation rate, there was a negative prognostic significance of high MIB-1 score for patients with vimentin-negative tumors ($P < 0.01$, curve A versus D). An even stronger poor prognostic effect of high MIB-1 score was seen in node-positive patients with p53-negative tumors (Fig. 3b, curve A versus D; $P < 0.001$). When node-positive patients were divided by MIB-1 score and survival curves were plotted by p53 status, the trend emerged for worse survival of patients with p53 positive, slowly proliferating tumors and better survival of patients with p53-negative, rapidly proliferating tumors; however, both trends did not reach statistical significance (curves A versus B and C versus D, not significant).

DISCUSSION

Our data show that the tumor cell proliferation rate as determined by MIB-1 activity on paraffin sections is associated significantly with 5-year overall survival in the entire cohort and in the node-negative and node-positive subsets of patients. Our results confirm reports that demonstrated a significant correlation between the proliferation rate of cancer cells as determined by Ki-67 immunostaining in frozen sections (7, 9–12, 33–35) or by the Ki-S1 antibody in paraffin-embedded tissue (36) and survival of breast cancer patients. Most of these studies report on short-term, disease-free survival. Of 12 studies published to date on Ki-67 and prognosis, only two examined more than 100 node-negative patients. Although Railo et al. (10) and Gasparini et al. (9) examined 196 and 164 such patients, respectively, they reported on shorter survival (4 and 3 years, respectively). There-
fore, our group of 111 node-negative patients with 5-year sur-

vival, although relatively small, is comparable in size to that

used in previous studies with Ki-67 antibodies. The only report

on Ki-67 and 5-year survival (11) is based on 42 node-negative

patients, whereas a very recent article also examines MIB-1

staining and long-term survival (37). Our data are also in agree-

ment with studies documenting the significant prognostic value

of the proliferation rate measured by thymidine labeling (3, 4) or

flow cytometry (5). However, because of the relatively small

size of the study population, and because the results were not

validated in an independent set of patients, our results should be

viewed as a pilot study.

Using a multivariate Cox analysis of survival we found that

the tumor cell proliferation rate, i.e., MIB-1 score, provided

independent significant prognostic information in the entire

cohort of patients as well as in the node-positive subset. In the

latter group, it was the only independent predictor of overall

survival at 5 years.

In contrast, for node-negative patients, the MIB-1 score

was not an independent predictor of overall survival. The pro-

liferation rate of tumor cells ceased to be a significant factor

after vimentin was taken into account in the Kaplan-Meier

survival curves (Fig. 2a, curves B versus C and A versus D) and

did not enter the Cox model. Vimentin status was the only

independent predictor of overall survival in 5 years in this group.

Attempts to use other proliferation-associated factors, such as

the ER or epidermal growth factor receptor, to sub-stratify

node-negative patients have been relatively unsuccessful. For

instance, Railo et al. (10) saw no difference in disease-free

survival between node-negative patients in the categories ER

negative and Ki-67 >10% and ER positive and Ki-67 <10%,

although they were able to demonstrate a 35% difference in

4-year survival (P < 0.005) between stage II patients in these

two groups. Toi et al. (38) also were unable to stratify node-

negative patients into high- and low-risk groups using the epi-

dermal growth factor receptor, another marker linked closely to

cell proliferation.

Attempts to stratify node-negative patients using p53 have

yielded conflicting results. Using immunohistochemistry, a sig-
nificant association with shortened survival (16, 17, 21), a small

but statistically nonsignificant negative effect (19, 39), and a

positive effect (40) of p53 positivity on survival of node-nega-
tive patients have been reported. No significant difference in

survival between patients with mutant and wild-type p53 tumors

has been found (41). In this report, we show that for our series

of breast carcinomas, p53 positivity is not an independent pre-
dictor of 5-year survival when tested against the proliferation

rate of tumor cells and vimentin expression. This is in agreement

with the results of Isola et al. (16), who showed that p53 had

independent prognostic value in a multivariate analysis only in

the absence of the S-phase fraction. If the S-phase fraction was

included in the analysis, only S-phase and tumor size emerged

as independent predictors of overall survival. However, Alfred

et al. (17) reported that positive p53 and S-phase were indepen-
dent predictors of reduced disease-free survival despite the

strong direct correlation between p53 positivity and tumor pro-

liferation rate. p53 protein is associated strongly with prolifera-

tion but not necessarily with invasion. Recently, Hsiao et al.

(42) have shown that in acute lymphocytic leukemias, some

specific p53 mutations were associated with increased invasive-

ness, and others were not. Thus, tumors determined by immu-

nohistochemistry to be p53 positive are most likely a heterog-

enous mixture of growth-promoting and ineffective mutations,

and, thus, it may be important to determine the precise p53

mutations present in each tumor rather than only p53 positivity

or negativity.

We have shown that vimentin expression is a strong in-
dicator of poor prognosis in node-negative patients as well as in

the whole cohort of patients with invasive ductal NOS breast

carcinoma (18). The prognostic value of vimentin in the whole

cohort of patients has been confirmed in two reports (20, 43).

Why is the proliferation rate of tumor cells a less useful

prognostic indicator than vimentin positivity in node-negative

patients? This may be due to the association of vimentin ex-

pression not only with proliferation but also with the invasive-

ness of tumor cells both in vitro (26, 44-47) and in vivo (19).

Poor prognoses in a subset of node-negative patients depends

on hematogenous micrometastases, particularly on visceral ones

i.e., to the brain, lungs, and liver, that were not detected at the
time of the first operations. During tumor progression, breast

carcinoma cells probably go through several stages in a complex

sequence of events that lead ultimately to metastasis. One event

leading to metastasis also may induce vimentin expression (48),

although vimentin is probably associated with only one of

several different pathways leading to the metastatic phenotype,
because vimentin-negative tumors also metastasize. Direct sup-
port for the idea that vimentin-positive tumors may be more

invasive is given by data showing that in node-negative patients,

vimentin-positive primary invasive ductal NOS breast carcino-

mas metastasized 3.5 times as often to the lung, liver, and brain

as did vimentin-negative primary carcinomas (19). Thus, vimen-
tin expression in breast cancer might be associated with the

expression of genes involved in the metastatic process. How-

ever, the molecular basis of the selective advantage offered to
tumor cells by events that are associated with vimentin expres-
sion is not known. Although an increased proliferation rate of
tumor cells also might contribute indirectly to metastasis, e.g.,
because of evolving clones that have a higher probability of
acquiring a metastatic phenotype, our data suggest that this is

not sufficient for proliferation to become a prognostic factor

independent of vimentin expression in node-negative patients.

The node-positive patients are a mixed group of those who

have only regional and those who may have widespread al-

though undetected dissemination of tumor cells at the time of

surgery. One may expect that in this group, the higher the

proliferation rate of tumor cells, the worse the prognosis. Our

results show that, indeed, it is the node-positive group in which

the determination of tumor cell proliferation rate is important,
because tumors with high proliferation rates signify poor prog-

noses irrespective of vimentin or p53 status.

The proliferation rate, vimentin status, p53 status, histologi-

cal grade, and size of tumors were tested in a multivariate Cox

analysis for their relationship with 5-year overall survival of

patients with invasive ductal NOS breast carcinoma. Although

the study population is relatively small (186 patients), the results

identify the vimentin status in node-negative patients and the

proliferation rate of tumor cells in node-positive patients as

important prognostic factors.
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