Microvessel Density, Expression of Estrogen Receptor α, MIB-1, p53, and c-erbB-2 in Inflammatory Breast Cancer

Nicole J. McCarthy,¹ Xiaowei Yang,¹ Ilona R. Linnoila, Maria J. Merino, Stephen M. Hewitt, Allyson L. Parr, Soonmyung Paik, Seth M. Steinberg, Dan P. Hartmann, Nejib Mourali, Paul H. Levine, and Sandra M. Swain²

Cancer Therapeutics Branch [N. J. M., X. Y., A. L. P., S. M. Sw.], Cell and Cancer Biology Branch [I. R. L.], Laboratory of Pathology [M. J. M., S. M. H.], and Biostatistics and Data Management Section [S. M. St.], Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20889; National Surgical Adjuvant Breast and Bowl Project Operation Center, Pittsburgh, Pennsylvania [S. P.]; Georgetown University, Washington, DC [D. P. H.]; Centre Ibn Zohr, Tunis, Tunisia [N. M.]; and George Washington University School of Public Health and Health Services, Washington, DC [P. H. L.]

ABSTRACT

Purpose and Experimental Design: The purpose is to define intratumoral microvessel density (MVD) and potential biological markers that correlate with inflammatory breast cancer (IBC), we examined MVD, estrogen receptor α (ER) status, MIB-1 proliferation index, p53, and c-erbB-2 by immunohistochemistry in archival specimens from 67 women diagnosed with breast cancer with or without the inflammatory phenotype at the Institut Salah Azaiz (Tunis, Tunisia).

Results: The moderate (25–50/×400 field) to high microvessel count (≥50/×400 field) was observed in 23 (51%) of 45 IBC tumors compared with 3 (14%) of 22 non-IBC tumors (P = 0.0031; χ² test). The presence of ER was found in 6 (14%) of 44 cases versus 7 (32%) of 22 cases in IBC and non-IBC, respectively (P = 0.10). In this series of 67 patient tumors, the median MVD count in ER-negative breast tumors was 21, whereas the median count was 4 in ER-positive breast tumors (P = 0.08; Wilcoxon rank-sum test). However, MIB-1, p53, and c-erbB-2 were not significantly different between IBC and non-IBC tumors. The intratumoral MVD between IBC and non-IBC was still statistically significant after adjustment for multiple comparisons (P = 0.02; Bonferroni test).

Conclusions: These data suggest that there is an increased MVD in breast cancer with the inflammatory phenotype as compared with breast cancer without the inflammatory phenotype.

INTRODUCTION

IBC is a rare, clinically distinct subtype of stage IIIB locally advanced breast cancer, accounting for up to 6% of all cases of breast cancer in the United States each year (1). It is characterized clinically by skin erythema, edema, and rapid tumor growth (1). Pathologically, IBC frequently presents with dermal lymphatic invasion by the tumor, thus causing the inflammatory signs (2). The survival of patients with IBC has significantly improved with the use of combined modality therapy (1, 3, 4). However, the significant number of patients who relapse after combined modality therapy continues to indicate a need to identify potential prognostic markers for this aggressive disease. This may potentially result in more targeted therapeutic approaches.

Several molecular markers have been studied in IBC. For example, the expression of ER has been reported to be absent in 56–83% of IBC, and this correlated with a poor clinical outcome (1, 5–7). By IHC, p53 protein has been detected in 30–69% of IBC, and overexpression of c-erbB-2 has been shown in 38–60% of IBC patients (8–12). Also, elevated tumor cell proliferation has been described in IBC by increased [3 H]thymidine incorporation (13).

There is significant evidence demonstrating that angiogenesis is essential for tumor proliferation or the growth of tumor cells (14). The degree of intratumoral MVD is believed to reflect the angiogenic activity generated by the tumor cells and their supportive stroma (15, 16). The quantitative estimation of MVD in areas of intense neovascularization in the tumor has been established as an independent poor prognostic indicator in early-stage breast cancer, including both node-positive and node-negative disease (15, 17, 18). Also, MVD is associated with aggressiveness of breast cancer (19). However, little is known about the correlative relationship between MVD status and IBC despite it being recognized as a highly vascular disease with angiolympathic involvement.

IBC is classified T4d (stage IIIb) by AJCC in 1997. It is a clinicopathological entity characterized by diffuse brawny induration of the skin of the breast with an erysipeloid edge, usually without an underlying palpable mass (20). IBC is far more common in Tunisia (categorized as PEV-2 and PEV-3, see “Materials and Methods”) than in the United States for reasons...
unknown (21). The diagnosis of PEV-3 requires inflammatory signs covering more than one-half of the breast and is comparable with the AJCC definition of IBC. The PEV-2 appears to be the same disease process except for inflammatory signs involving less than one-half of the breast (22). The survival of patients with both PEV-2 and PEV-3 tumors is poor as compared with that of patients with PEV-0 tumors, supporting the PEV classification in providing prognostic information (22). The definition and classification of IBC is controversial, but this study was performed on cases clearly demonstrating skin thickening and inflammatory signs (22).

In an attempt to explore the relationships of MVD and other biological markers to IBC, we examined MVD, the expression of ER, MIB-1 proliferation index, p53, and c-erbB-2 by IHC on formalin-fixed, paraffin-embedded specimens in 67 women diagnosed with breast cancer in Tunisia. We found that increased MVD was closely associated, whereas other biological markers were not significantly associated with the IBC tumors.

MATERIALS AND METHODS

Breast Cancer Specimens. The tumor specimens from this group of breast cancer patients have been described in detail previously (22–25). Initially, paraffin-embedded breast tumor blocks were obtained from 85 women with breast cancer who were diagnosed at the Institut Salah Azaiz in Tunis, Tunisia, between 1969 and 1979. The blocks were randomly selected according to the size of the tumor on available blocks. All patients were staged using the PEV classification system that is assessed by two elements: inflammatory signs and recent rapid growth (26, 27). The PEV stages adapted by Tabbane et al. (22) and used in Tunisia are as follows: PEV-0 (or non-IBC), a tumor without recent increase in size and without inflammation; PEV-1, a tumor with marked increase in volume during the past 2 months (by patient’s report) but no inflammatory signs; PEV-2 (or IBC), a tumor with poor demarcation from the surrounding breast tissue showing inflammation and edema involving less than half of the breast surface; and PEV-3 (or IBC), tumor associated with a marked increase of the breast showing inflammation and edema involving more than half of the breast surface. All tumor specimens were obtained at the time of first diagnosis and before systemic therapy. Five tumors were excluded from study because of negative epithelial membrane antigen staining, indicating the loss of antigenicity of these tumor specimens. In this study, we primarily focused on the PEV-2/3, as these are consistent with the current classification of IBC (45 patients) and the PEV-0 as controls (22 patients) for a total of 67 tumors (20). PEV-1 is not analyzed in this study because of the ambiguity and subjectivity of this stage, although previous studies have suggested PEV-1 as a biological entity closely related to IBC (25). The tumor blocks were made anonymous before the study, and clinical information regarding lymph node status, distant metastasis, disease-free and overall survival was insufficient for analysis. Approval for this study was obtained from the Office of Human Subjects Research of the NIH, Bethesda, Maryland.

Antibodies and IHC. Five-µm sections were freshly cut from each block and mounted on poly-L-lysine coated slides for IHC analyses. Antibodies used in IHC were: epithelial membrane antigen (MC-5) and ER (6F11), BioGenex Laboratories (San Ramon, CA); CD31 (JC/70A) and MIB-1 (MIB-1), Dako Corp. (Carpinteria, CA); p53 (DO7), Vector Laboratories (Burlingame, CA); and c-erbB-2 (a mixture of mouse TAB250 and PAD24881 rabbit serum), Zymed Laboratories, Inc. (S. San Francisco, CA). The IHC method on formalin-fixed and paraffin-embedded sections has been described previously (28). Briefly, after inactivating the endogenous peroxidase activity and blocking the nonspecific antigen binding sites, the tissue sections were incubated with various primary antibodies as mentioned above. The binding of antibodies to their antigenic sites in the tissue sections was amplified with the use of species-appropriate biotinylated secondary antibodies supplied by the Vectastain Elite ABC kits (Vector Laboratories). A subsequent incubation for 30 min with avidin-peroxidase conjugate, also supplied by the Vectastain Elite ABC kits, was followed by 3, 3’-diaminobenzidine (Sigma, St. Louis, MO) reaction. Afterward, slides were counterstained with Mayer’s hematoxylin (BioGenex Laboratories) or methyl green (R&D Systems, Minneapolis, MN) and cover slipped with Permount.

Histological and IHC Evaluation. H&E staining for each tumor specimen was used to confirm histologic type, grade, nuclear grade, and angiolymphatic invasion by experienced pathologists (M. J. M. and I. R. L.). The histological grading of the tumors was in accordance with the Bloom-Richardson classification. Nuclear grading was scored using the 1–3 grading system.

The quantification of MVD was assessed according to the method of Weidner et al. (29) by at least two observers independently (I. R. L., S. M. H., or A. L. P.). The sections were initially screened at low magnifications (×40 and ×100) to identify the most vascular area of the tumor (hot spot). Within the hot spot area, the stained microvessels were counted in a single high-power (×400) field. MVD was expressed as the number of microvessels/field. Any CD31-stained endothelial cells or endothelial cell clusters that were clearly separate from adjacent microvessels, tumor cells, or connective tissue elements were considered a single countable microvessel. These microvessel counts were graded from zero (no stained vessels seen) to low (<25/field), moderate (25–50/field), and high (>50/field). Microvessel counts were compared between the observers and discrepant results were reviewed together. The consensus reached was used as the final score for analysis. ER staining was recorded as positive when nuclear staining was identified in >10% of the cells in the section (M. J. M.; Ref. 30). For MIB-1 proliferation index, the sections were examined at ×40 and ×100 to identify the area with the most intense staining of the representative tumor. The stained and unstained malignant nuclei in three ×400 fields were counted, and the cell proliferation index was defined as the ratio of MIB-1-positive tumor cells to all counted tumor cells ×100 (D. P. H. and A. L. P.; Ref. 31). Nuclear p53 staining was scored as positive when malignant nuclei with ≥10% were stained in the tissue section (I. R. L. and A. L. P.; Ref. 32). The membrane staining for c-erbB-2 was graded from no staining (0) to weak staining (1+), moderate (2+), and intense membrane staining (3+), all of them in >10% of tumor cells in the tissue section (S. P.; Ref. 33). A score of 3+ was used as the cut point for analysis.

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Statistical Analysis. Data were categorized into the IBC (PEV-2/3) and non-IBC (PEV-0) groups. Differences in continuous parameters (age, MIB-1, and MVD) were evaluated by Wilcoxon rank-sum test. The $\chi^2$ test or Fisher’s exact test was used as appropriate to evaluate the significance of differences in the dichotomous parameters when evaluated between the IBC and non-IBC groups. To describe the possible relationships between MVD and the molecular markers investigated, the Wilcoxon rank-sum test was used. All Ps are represented as two-tailed tests and statistically significant at 0.05.

RESULTS

Association of MVD with IBC. To determine the relationship of MVD to IBC, we examined MVD in 67 Tunisian breast tumors with or without the inflammatory phenotype. Fig. 1 shows a representative case of a highly vascularized IBC tumor (Fig. 1A) and an example of a poorly vascularized non-IBC tumor (Fig. 1B). Original magnifications, ×200.

Table 1 MVD, histological and other molecular markers related to IBC

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of positive tumors in non-IBC (%)</th>
<th>No. of positive tumors in IBC (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 22</td>
<td>n = 45</td>
<td></td>
</tr>
<tr>
<td>Histological grade poor</td>
<td>19 (86)</td>
<td>41 (91)</td>
<td>0.68</td>
</tr>
<tr>
<td>Nuclear grade 3</td>
<td>20 (91)</td>
<td>42 (93)</td>
<td>1.00</td>
</tr>
<tr>
<td>Angiolymphatic invasion</td>
<td>7 (32)</td>
<td>22 (49)</td>
<td>0.19</td>
</tr>
<tr>
<td>MVD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–Low</td>
<td>19 (86)</td>
<td>22 (49)</td>
<td>0.0031b</td>
</tr>
<tr>
<td>Mod–high</td>
<td>3 (14)</td>
<td>23 (51)</td>
<td></td>
</tr>
<tr>
<td>ER +</td>
<td>7 (32)</td>
<td>6 (14)c</td>
<td>0.1</td>
</tr>
<tr>
<td>p53+</td>
<td>8 (36)</td>
<td>24 (53)</td>
<td>0.19</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–2+</td>
<td>11 (50)</td>
<td>29 (64)</td>
<td>0.26</td>
</tr>
<tr>
<td>3+</td>
<td>11 (50)</td>
<td>16 (36)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $\chi^2$ test. Ps in this column are presented without adjustment for multiple comparisons.

$^b$ $P = 0.02$ after adjustment for multiple comparisons by Bonferroni procedure.

$^c$ n = 44, one case was not evaluable.

Fig. 1 MVD in breast cancer. The status of the immunostained intratumoral microvessels with the use of an antibody against endothelial marker CD31 is seen in a representative of IBC (A) and an example of non-IBC (B). Original magnifications, ×200.
Biologic Markers in Inflammatory Breast Cancer

c-erbB-2 overexpression (association, or an inverse tendency, of the IBC tumors with MVD. Molecular markers were significantly associated or had a trend in test), indicating a trend for increased MVD with the loss of ER in ER-positive breast tumors (negative breast tumors was 21, whereas the median count was 4 in ER-negative breast tumors (P = 0.12). Nuclear p53 immunostaining was present in 24 (53%) of 45 IBC tumors compared with 8 (36%) of 22 non-IBC tumors (P = 0.19). There was no association, or an inverse tendency, of the IBC tumors with c-erbB-2 overexpression (P = 0.26; Table 1).

As shown in Table 1, there was a trend toward an increased presence of angiolymphatic invasion in IBC compared with that in non-IBC (P = 0.12). Poor histological grade was 86 and 92% in non-IBC and IBC, respectively, and nuclear grade three was 91% in non-IBC and 93% in IBC. The median age of all patients was relatively young at 46 years (range of 18–73 years) with no significant difference between IBC and non-IBC groups (Table 2). Thus, these data demonstrate no association of IBC with histological grade, nuclear grade, and age.

Relationships of Expression of ER, p53, and c-erbB-2 to MVD. We next analyzed the relationships between the expression of ER, p53, c-erbB-2, and MVD in 67 Tunisian breast cancers to explore the potential regulation of MVD by these molecular alterations. Table 3 shows the relationships of ER, p53, and c-erbB-2 to MVD. The median MVD count in ER-negative breast tumors was 21, whereas the median count was 4 in ER-positive breast tumors (P = 0.08; Wilcoxon rank-sum test), indicating a trend for increased MVD with the loss of ER expression. As also shown in Table 3, none of the other molecular markers were significantly associated or had a trend in association with MVD.

DISCUSSION

IBC is an important poorly defined entity with some groups, such as AJCC, emphasizing the clinical features and others, such as The Surveillance, Epidemiology and End Results program, emphasizing pathologic findings (34). There is no question, however, that redness and edema as well as rapid growth are the hallmarks of IBC. It is apparent that a significant number of breast cancer patients in Tunisia have IBC, referred to in the French-Tunisian classification as PEV and, thus, provides a relatively large group of patients with IBC to study (1). The relationship of PEV to IBC is apparent from the inflammatory signs, the description of the tumor, the poor prognosis of PEV-2/3 versus PEV-0, the pathologic findings, and the hormone receptor status (21–25). The available archival tumor specimens, including cases with clinical evidence of IBC (PEV-2/3) and those with no clinical evidence of rapid growth and inflammatory signs (PEV-0), all collected in a single institution, provided an opportunity to compare biological markers in this specific stage of breast cancer.

We have demonstrated a statistically significant increase in intratumoral MVD in breast cancer with the inflammatory phenotype (PEV-2/3) compared with breast cancer without the inflammatory phenotype (PEV-0) when analyzed either as a dichotomous or continuous variable. The MVD is still statistically significant after the adjustment for multiple comparisons. It is conceivable that the tumors in a rapidly progressive stage would rely on an increased blood supply provided by microvessels versus the tumors in a slowly progressive stage. And it is reasonable to speculate that increased MVD may have contributed to the poor prognosis of the PEV category tumors (22, 25). The trend toward increased tumor cell proliferation, as indicated by a higher MIB-1 proliferation index, in the group of IBC tumors may provide a support for the role of angiogenesis on tumor growth.

The assessment of tumor vascularization using the quantification of immunostained microvessels in an area of intense neovascularization described by Weidner et al. (29) is the most widely used technique. By this method, intratumoral MVD is suggested to be a prognostic indicator for invasive primary and node-positive breast carcinomas (17, 29). However, not all reports have found the association of MVD with prognosis (35). The discrepancy may be, in part, because of the methodology used (18). The modified methods such as the use of antibodies against more specific markers for activated endothelium have been developed to improve MVD assessment, but they are still in agreement with the use of the antibody against endothelial marker, factor VIII, by Weidner et al. (18). In this study, we have used an antibody against endothelial marker CD31 to stain activated endothelial cells because it has a superior sensitivity and specificity on paraffin-embedded tissues (16, 36).

We found lower ER expression in IBC than in non-IBC (25). However, it is worth noting that overall, the Tunisian patients studied have a low percentage of ER expression regardless of PEV stage. The explanation could be that this group of

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (range) in non-IBC</th>
<th>Median (range) in IBC</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>46.5 (18–73)</td>
<td>46 (32–65)*</td>
<td>0.88</td>
</tr>
<tr>
<td>MVD</td>
<td>6.5 (0–92.5)</td>
<td>25.5 (0–110.0)</td>
<td>0.000*</td>
</tr>
<tr>
<td>MIB-1</td>
<td>11.6 (0–29.6)</td>
<td>15.4 (0–55.3)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Wilcoxon rank sum test. ** n = 42, ages from three patients are not available. " P = 0.027 after adjustment for multiple comparisons by Bonferroni procedure.

Table 3  ER, p53, and c-erbB-2 related to MVD

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>MVD (median)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>53</td>
<td>21</td>
<td>0.08</td>
</tr>
<tr>
<td>Positive</td>
<td>13</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>35</td>
<td>14</td>
<td>0.35</td>
</tr>
<tr>
<td>Positive</td>
<td>32</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>c-erbB-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0, 1+</td>
<td>40</td>
<td>17.5</td>
<td>0.91</td>
</tr>
<tr>
<td>2+</td>
<td>27</td>
<td>20.0</td>
<td></td>
</tr>
</tbody>
</table>

* Wilcoxon rank-sum test. ** One case was not evaluable.
patients has a higher percentage of premenopausal women. It is frequently found that younger premenopausal women are more often ER negative than older postmenopausal women (37). Also, according to a previous report, this group of Tunisian breast cancer patients had lower ER than patients with breast cancer in the United States (25). In this study, a trend relating the loss of ER to the increased vascularization (MVD) in the tumor was observed. This observation is supported by the recent experimental findings that ER overexpression is associated with decreased tumor vascularization and reduced tumor growth in *in vitro* and *in vivo* models (38). The introduction of ER into the endometrial cancer (Ishikawa) cells and xenografts established with these ER-transfected cells led to reduced VEGF and β3 expression, both of which are pivotal in angiogenesis (38). The increased loss of ER in IBC and the inverse correlative relationship between ER and MVD did not reach statistical significance. However, it warrants further study.

The presence of nuclear p53 protein, as an indication of dysregulated p53, observed in IBC is somewhat higher than that in non-IBC. However, it did not reach statistical significance. A previous publication by Riou et al. (8) has found that p53 is a poor prognostic indicator in IBC. The fact that there was no association between p53 and MVD status suggests that regulators other than p53 may be involved in regulation of angiogenesis in these tumors.

We found a slightly higher c-erbB-2 overexpression in non-IBC tumors. However, no significant difference in c-erbB-2 overexpression was noted between the two groups. It is unlikely that c-erbB-2 is related to the inflammatory phenotype. Other genes such as RhoC GTPase gene or the loss of expression of a novel gene, low-affinity insulin-like growth factor-binding protein, may be more specifically involved in the development of or the aggressiveness of IBC (39). These data also suggested that c-erbB-2 does not play a role in regulation of MVD in this Tunisian series of breast tumors. Other protein markers, however, Flt-1 or Tie-2, may be related to the regulation of the tumor angiogenesis (40). These and the expression of VEGF-C and VEGF-D and of VEGFR-3 in regulation of lymphangiogenesis (41) will be studied in a subsequent large study in patients with IBC in North America.

In summary, we have demonstrated a significantly higher intratumoral MVD in breast cancer with the inflammatory phenotype and confirmed IBC as a highly vascular disease. Currently, the prognostic role of MVD in IBC is being studied prospectively at the National Cancer Institute.

ACKNOWLEDGMENTS

We thank Dr. Stanley Lipkowitz for critical review of the manuscript and Fatou Hughes for assistance with translating a French paper into English.

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