Prognostic Significance of Proliferation Associated Nucleolar Antigen PI20 in Human Breast Carcinoma

J. W. Freeman, P. McGrath, V. Bondada, N. Selliah, H. Ownby, T. Maloney, R. K. Busch, and H. Busch

ABSTRACT

Nucleolar antigen PI20 is detected in rapidly proliferating cells but not in normal resting cells or in many benign and slowly growing malignant tumors. The objective of the study was to determine whether the expression of PI20 in breast cancer correlated with histopathological or biological properties associated with prognosis. In this retrospective study, 120 primary breast tumors were analyzed for PI20; 114 of these tumors were also stained for the erbB-2 protein. Immunopositive staining was correlated with patient survival, nodal status, estrogen receptor levels, and number of mitoses. Sixty-nine percent (83 of 120) of the tumors were positive for PI20; 25% (28 of 114) stained positively for erbB-2. Of the 26 erbB-2 positive tumors 26 were also positive for the PI20 protein. Forty-six percent (55 of 120) of the specimens were from patients who later died from recurrent breast cancer; PI20 was detected in 89% (49 of 55) of these specimens. In 52% of the survivors the PI20 protein was also expressed. PI20 negative tumors were highly correlated with survival (P = 0.0001); 84% (32 of 37) of patients with PI20 negative tumors survived more than 7 years without evidence of recurrent disease. Multivariate analysis showed that the worst prognosis was for patients who had tumor positive nodes and expressed PI20 (P = 0.0001); death occurred in 73% (30 of 41) of these patients. For the node negative patients who did not express PI20, 5-year survival was 90% (19 of 21 patients); 5-year survival for the node negative patients who expressed PI20 was significantly less (67%; 28 of 42 patients). Patients with PI20 negative tumors had a good prognosis, irrespective of their nodal status. In this group, survival of node negative patients was 86% (18 of 21) and for those with positive nodes survival was 82% (13 of 16). A poor prognosis was found for patients with intense erbB-2 stained tumors (5 of 7 patients died). Weak staining of erbB-2 tumors (21 specimens) was not correlated with patient survival. Compared to PI20 negative tumors, PI20 positive tumors had greater numbers of mitoses (9.06 versus 6.65) and an almost 2-fold increase in the occurrence of positive nodes (one of every 4.67 versus one of every 8.81). The number of PI20 positive tumors was greater in estrogen receptor positive tumors (75%) than in estrogen negative tumors (54%).

These studies suggest that antigen PI20 may be a prognostic marker in breast cancer and could be used along with other parameters such as nodal status to assess the prognostic outcome of breast cancer patients.

INTRODUCTION

Many breast cancers having similar histological properties differ markedly in their recurrence rates and their response to therapy (1-8). Only about 50% of patients with primary breast cancer are cured by local surgery and combinations of chemotherapy and radiotherapy (6). This has led to a search for better means of predicting the risk of a recurrence and in assessing prognostic outcome. A number of prognostic factors have been identified (1-8), although none of these factors either singly or together are completely predictive for recurrence. Among these factors are histopathology (i.e., tumor size and numbers of axillary lymph nodes involved), ER receptors, cell cycle kinetics, erbB-2 gene, and protein levels.

The importance of node and hormone receptor status have been recognized for some time as a major criteria for determining treatment after resection of breast cancer. It is well established that systemic adjuvant therapy should be given to patients with positive nodes (9, 10). There has been considerable debate as to the need for therapy for node negative patients (11). Hormonal receptor levels have not been completely predictive for response to endocrine therapy and recurrence, since about one half of ER+ tumors and about one fourth of patients with ER+/progesterone receptor positive tumors do not respond to this type of therapy (12).

A number of flow cytometric studies indicate that the proliferative activity and ploidy of breast cancer cells yield useful prognostic information (13-17). In a recent report, Clark et al. (18) indicated that the combination of ploidy and the S phase fraction was important in assessing node negative disease. They found that ploidy was an independent prognostic factor and that S phase fraction provided useful prognostic information only if the tumor was diploid.

Oncogenes or other molecules related to growth or cell cycle potential may become markers to further define biological properties of breast cancer and therefore may provide additional prognostic information.

Amplification of the c-erbB-2 oncogene and expression of the c-erbB-2 protein have been reported to be poor prognostic markers in breast cancer (8, 19). Recent studies indicate that the hormone dependent proteins, cathepsin D (20) and pS2 (21), may be useful in predicting recurrence when used in combination with ER and nodal status.

Our laboratory has developed monoclonal antibodies to an M120,000 proliferation associated nucleolar protein (PI20) (21). The PI20 antigen was detected in most tumors but was not found in normal resting tissues or in most benign tumors (21, 22). The PI20 antigen level apparently is related to the hyperactivity of the nucleolus and to the proliferative state of the cell. Microinjection of PI20 monoclonal antibodies into tumor cells decreases their proliferation rate and induces a compaction of nucleoli (23).

Preliminary PI20 immunocytochemical analysis of breast tumors indicated that many but not all expressed the PI20 antigen. Further studies were done to determine whether PI20 expression in breast cancer might reflect a proliferative property of breast cancer which could be related to prognosis. In this study we determined the expression of PI20 in breast cancer as well as the expression of erbB-2, ER, nodal status, number of mitoses, and patient survival. An analysis of the results suggest

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2 To whom requests for reprints should be addressed, at the Lucille Markey Cancer Center, University of Kentucky Medical Center, Lexington, KY 40536-0039.
that a combination of P120 expression with tumor positive nodes reflects a worse prognosis than with lymph node involvement alone. Patients who had P120 positive/node negative tumors had a significantly poorer prognosis than patients who had P120 negative/node negative tumors. Patients with P120 negative tumors had a good prognosis regardless of their nodal status.

MATERIALS AND METHODS

Specimens. Cryostat or paraffin embedded specimens were obtained from the Michigan Cancer Foundation, Detroit, MI. The specimens were from 120 different breast cancer patients who underwent modified radical mastectomy with axillary lymph node dissection in the years 1979 through 1984. Analysis of the P120 antigen was done on cryostat sections. Examination for erbB-2 was performed on portions of the same tissue that had been paraffin embedded.

Immunofluorescence Detection of PI20. Immunocytochemical localization of nucleolar antigen P120 identified by monoclonal antibody was detected by indirect immunofluorescence. Tissues were fixed and permeabilized in acetone for 10 min at —20°C. The primary antibody (anti-p120) was used at a dilution of 1:300 (ascites:PBS) which provided optimal immunofluorescence staining on control HeLa cell slides. A sufficient amount of diluted monoclonal antibody (100 μl) was placed on tissues and incubated in a humid atmosphere at 37°C for 1 h. The slides were washed twice for 15 min in PBS as above and mouse antibody was detected with fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin (Boehringer Mannheim) diluted 1:50 in PBS.

Immunoperoxidase Detection of erbB-2. The c-erbB-2 monoclonal antibody was obtained from Triton Biosciences Inc. (Alameda, CA). This antibody was used on paraffin embedded tissue sections, and 114 of the 120 specimens were examined by the avidin-biotin immunoperoxidase staining technique (Vector Laboratories, Burlingame, CA).

RESULTS

Immunohistochemical Detection of P120 and c-erbB-2

The P120 protein was detected by indirect immunofluorescence and tissues were scored as positive if fluorescence was blocked by incubating the tissue sections with 2% normal serum for 30 min at 37°C. The c-erbB-2 antibody was diluted at 1:10 in PBS containing 1% normal serum. Two sections from each tumor were analyzed. One section was incubated with c-erbB-2, then with biotinylated second antibody, followed by the avidin-biotin complex reagent according to the manufacturers' directions except incubation times were extended to 1 h. The second, control section was treated in parallel but incubated with 2% normal serum instead of primary antibody. Diaminobenzidine was used as a substrate and was prepared and used according to the manufacturer's directions (Vector Laboratories). Three different categories were used in scoring the slides (++, ±, and — staining).

Estrogen Receptor Assays. Cystosolic steroid receptors were assayed using a saturating dose and a charcoal-dextran method (24) for separation of bound from free estrogen. Receptor concentration was expressed as fmol of estrogen/mg cytosolic protein.

Statistical Methods. The association of P120 expression or lack of expression with other categorized histological or biological variables was assessed by χ² analysis. Survival curves were calculated by the method of Kaplan and Meier (25). Tests of differences between curves were made with the log-rank test for censored survival data. The partially nonparametric regression model of Cox (26) was used to evaluate the predictive power of various combinations of prognostic factors in a univariate and multivariate manner.
Proliferation Associated Nucleolar Antigen P120

Table 1 Relationship between P120 and erbB-2 expression in breast cancer with patient survival, number of mitoses, nodal status, and estrogen receptor levels

<table>
<thead>
<tr>
<th>Antigen Immunoreactivity</th>
<th>Specimens</th>
<th>Number</th>
<th>%</th>
<th>No.</th>
<th>%</th>
<th>Mitoses*</th>
<th>+ Nodes</th>
<th>ER Levels (Means)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P120 Positive</td>
<td>120</td>
<td>55</td>
<td>45.8</td>
<td>65</td>
<td>54.2</td>
<td>8.32</td>
<td>1/4.52</td>
<td>193.26</td>
</tr>
<tr>
<td>P120 Negative</td>
<td>83</td>
<td>49</td>
<td>59</td>
<td>34</td>
<td>41</td>
<td>9.06</td>
<td>1/4.67</td>
<td>224.07</td>
</tr>
<tr>
<td>erbB-2 Positive</td>
<td>28</td>
<td>12</td>
<td>42.9</td>
<td>16</td>
<td>57.1</td>
<td>8.25</td>
<td>1/4.87</td>
<td>246.32</td>
</tr>
<tr>
<td>erbB-2 Negative</td>
<td>86</td>
<td>40</td>
<td>46.5</td>
<td>46</td>
<td>53.5</td>
<td>8.42</td>
<td>1/5.47</td>
<td>176.60</td>
</tr>
<tr>
<td>Double negatives*</td>
<td>32</td>
<td>16</td>
<td>18.8</td>
<td>26</td>
<td>58.1</td>
<td>6.78</td>
<td>1/7.88</td>
<td>101.59</td>
</tr>
<tr>
<td>Single negative P120</td>
<td>26</td>
<td>12</td>
<td>46.2</td>
<td>14</td>
<td>53.8</td>
<td>8.81</td>
<td>1/4.52</td>
<td>217.85</td>
</tr>
<tr>
<td>Single positive P120</td>
<td>54</td>
<td>34</td>
<td>63</td>
<td>20</td>
<td>37</td>
<td>9.22</td>
<td>1/4.78</td>
<td>221.54</td>
</tr>
<tr>
<td>Single positive erbB-2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>100</td>
<td>1.00</td>
<td>1/11.50</td>
<td>616.50</td>
</tr>
</tbody>
</table>

* Mitoses, number of mitotic cells counted on 10 high powered fields (28).
** Average number of positive nodes is presented as a fraction. Numerator, number of nodes counted to find one positive node.
* Double positives or double negatives, specimens that were stained for both P120 and erbB-2 or lacked staining for both antigens.

A summary of the relationship between P120 and erbB-2 expressions with patient survival, number of mitoses, nodal status, and estrogen receptor levels is given in Table 1. These results show that 69% (83 of 120) of the tumors were positive for P120; 25% (28 of 114) of the tumors were c-erbB-2 positive. All except two of the c-erbB-2 positive tumors were positive for P120. Overall, 55 of the 120 patients (46%) died; in this group, 49 of the tumors (89%) were P120 positive (4 deaths were attributed to causes other than breast cancer). About one half (52%) of the survivors also had P120 positive tumors. Only 5 of 37 patients (16%) with P120 negative tumors died of breast cancer (one other death was attributed to a cause other than breast cancer). A Kaplan-Meier survival curve based on P120 immunoreactivity is show in Fig. 2. P120 negative tumors were highly correlated with survival (P = 0.0001).

Prognostic trends were not found for c-erbB-2 when the overall immunoreactivity was judged as simply positive or negative. When Kaplan-Meier survival curves were done on the bases of intensity of c-erbB-2 staining, 5 of 7 patients with the more intense staining (++) tumors had died (Fig. 3); weaker c-erbB-2 stained tumors did not show any difference in survival when compared to tumors that were not stained with c-erbB-2 (Fig. 3). Neither double positive tumors (i.e., those that stained for both P120 and c-erbB-2) or double negative tumors (i.e., those that did not stain for both P120 and c-erbB-2) showed any greater prognostic significance compared to analysis by either marker alone.

Relationship between P120 Expression and Number of Mitoses, Nodal Status, and Estrogen Receptor Levels

Number of Mitoses. Numbers of mitoses were determined by counting the number of mitotic cells/10 high power fields according to procedure of Russo et al. (27). P120 positive tumors had a higher number of mitoses compared to P120 negative tumors (Table 1). When P120 negative tumors were analyzed separately, it was noted that the 5 patients who died of breast cancer showed a greater than 4-fold increase in their number of mitoses (16.00) compared to the average number of mitoses (5.13) of the 26 survivors (Table 2). Three of the 5 patients in this group had mitoses greater that 15.0 (Table 2). The remaining 2 patients had 1 and 5 mitoses; the patient with the one mitosis survived for >8 years. The best indicators for survival in this study was P120 negative tumors that had <5 mitoses.

Nodal Status. Table 3 shows the relationship between P120 expression and nodal status on patient survival. A Kaplan-Meir...
survival plot for this relationship is shown in Fig. 4. The median number of nodes examined in the node negative group was 11.0 (range, 4–29); for the node positive group the median number of nodes examined was 15.5 (range, 5–32). The worst prognosis was for patients who had positive nodes and whose tumor expressed P120. Death occurred in 73% (30 of 41) of patients with P120 positive/node positive tumors; the 5-year survival for this group was only 33% (Table 3). Patients with P120 negative tumors had a good prognosis irrespective of their nodal status (Table 3, Fig. 4). Survival rate in these patients was 86% (18 of 21) for node negative and 82% (13 of 16) for node positive patients; these patients had surgery 6–10 years previously (Fig. 4).

When nodal status was considered alone, 58% of patients (33 of 57) with positive nodes died; 33% (21 of 63) of the node negative patients died. However, 73% (30 of 41) of patients with P120 positive/node positive tumors died. Patients with node negative tumors could be classified into two groups based on the presence or absence of P120 immunoreactivity. The 5-year survival was 90% (19 of 21) for patients with P120 negative/node negative tumors and 67% (28 of 42) for patients with P120 positive/node negative tumors (Table 3). Up to the present (6–10 years postsurgery), the survival for the P120 positive/node negative group decreased to 55% and the survival rate for P120 negative/node negative remained high at 86%.

**ER Levels.** P120 positive staining tumors tended to have a higher ER level (mean, 224.07; median, 94; range, 0–2096) compared to P120 negative tumors (mean, 124.14; median, 18; range, 0–1233), (Table 1). The lowest ER levels were observed in double negative (c-erbB-2-/P120-) tumors. The percentage of P120 positive tumors was higher in ER+ tumors (75%; 64 of 85) compared to ER- tumors (54%; 19 of 35). The 5-year survival of patients with ER+ tumors was 59 ± 5.4% (SD) and the 5-year survival of patients with ER- tumors was 69.4 ± 7.7%.

**Statistical Analysis**

The relationship between each parameter or the combined effect of parameters against survival was assessed by univariate and multivariate analysis. The results (Table 4) of univariate analysis show that the likelihood of survival is significantly higher for patients with P120 negative tumors (P = 0.0001) and for patients with negative nodes (P = 0.002). Multivariate analysis based on a stepwise Cox regression model was used to assess the significance for the interaction of nodal status and P120 expression on patient survival. For this purpose, patients were placed into four groups as follows: group 1, P120+/node+; group 2, P120−/node+; group 3, P120−/node−; and group 4, P120+/node−. As shown by the survival curves in Fig. 4, there was not a significant difference in the survival of groups 3 and 4. Groups 1 and 2 showed a significantly poorer prognosis (Table 5). Multivariate analysis shows that the worst prognosis was for patients in group 1 (P120+/node+; P = 0.0001) (Table 5).

**DISCUSSION**

The purpose of this study was to determine whether antigen P120 might serve as a proliferation marker in breast cancer and whether, in combination with other histological and biological properties of breast cancer, it might be useful in assessing patient survival. Antigen P120 expression appears to correlate with "hyperactivity" of the nucleolus. The upregulation of nucleolar function is characteristic of rapidly cycling cells (28). Pleomorphism of the nucleolus and increased nucleolar activity measured by rDNA transcription is associated with rapidly growing tumor cells (29). Two recent studies (30, 31) indicate that morphological changes of the nucleolus and the level of silver stained proteins of the nucleolar organizer region may be of prognostic and diagnostic use in breast cancer.

Relapse free survival data were not readily available for the patients in this study and it is possible that some of the survivors may have recurrent disease. Also, these studies do not account for adjuvant systemic chemotherapy. However, there was a striking correlation between the lack of expression of P120 and overall patient survival. The P120 negative tumors had lower numbers of mitoses than P120 positive tumors which suggests that P120 reflects proliferative characteristics of the tumor. Of 37 patients, 33 (87%) with P120 negative tumors survived >5 years. Of the four patients who died, three had numbers of mitoses that were 5-fold greater than the average number of mitoses of P120 negative tumors. Thus, P120 negative tumors could be further categorized according to their numbers of mitoses; the P120 negative tumors with <5 mitoses indicated the best chance of patient survival. The S phase cell percentage in breast cancer has been the focus of a number of studies (15–17) and is likely a better measure of cell cycle kinetics than is the number of mitoses. Breast cancers with increased numbers of S phase cells tend to have a poorer prognosis (16). Additional studies are needed to determine whether P120 expression correlates with increased percentage of S phase cells.

The percentage of P120 tumors was higher in ER positive tumors (75%) compared to ER negative tumors (54%). P120 positive tumors also tended to have a higher ER level compared to P120 negative tumors. Similarly, c-erbB-2 positive tumors had higher ER levels than c-erbB-2 negative tumors. The lowest ER levels were found in erbB-2 negative/P120 negative tumors. ER levels were reported (3-5) to be inversely related to prognosis, but in this study P120 negative tumors had lower ER levels and a better prognosis. The P120 expression may correlate with more aggressively growing tumors independent of hormonal status and P120 negative tumors may identify slower growing, less aggressive tumors. erbB-2 expression has also not correlated with ER status (8, 19). We have also found that P120 transcripts and protein levels are downregulated following 24 h of tamoxifen treatment (10⁻⁷ M) of the ER sensitive breast tumor cell line (MCF-7). This decreased P120 expression is correlated with an increased cell cycle time in tamoxifen treated cells.

A spectrum from intense staining to weak immunostaining was observed for both P120 and the erbB-2 protein. In agreement with others (19) a difference in intensity of staining was

<table>
<thead>
<tr>
<th>Table 2 Difference in number of mitoses between survivors and nonsurvivors with P120 negative tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Deaths</td>
</tr>
<tr>
<td>Breast cancer</td>
</tr>
<tr>
<td>Other causes</td>
</tr>
<tr>
<td>Survivors</td>
</tr>
</tbody>
</table>

* Number of mitoses counted in 10 high powered fields; average for all tumors in each group.
* The ranges for these five patients were: 1, 5, 42, 15, 17; the patient with the one mitosis survived for >8 years.

J. W. Freeman, unpublished data.
found in erbB-2 stained breast tumors. It is unclear as to whether the intensity of protein staining correlates with tumors in which the c-erbB-2 gene is not amplified. The immunohistochemical results, presented here, suggest that high P120 expression and other proliferative properties including the S phase cell percentage and levels of nucleolar silver staining proteins. It may also be important to determine whether quantitative levels of P120 protein and transcripts correlate with other biological properties of breast cancer and patient survival.

In conclusion results of this study suggest that determining the expression of P120 has prognostic value in breast cancer. Further studies are needed to assess the relationship between P120 expression and other proliferative properties including the S phase cell percentage and levels of nucleolar silver staining proteins. It may also be important to determine whether quantitative levels of P120 protein and transcripts correlate with other biological properties of breast cancer and patient survival.

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