Association of the 323/A3 Surface Glycoprotein with Tumor Characteristics and Behavior in Human Breast Cancer

Atul K. Tandon, Gary M. Clark, Gary C. Chamness, and William L. McGuire

Department of Medicine/Oncology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7884

ABSTRACT

We have earlier described a monoclonal antibody (323/A3) against a M, 43,000 surface glycoprotein of MCF-7 human breast cancer cells which shows considerable specificity for primary and metastatic breast tumors (Cancer Res., 46: 1306–1317, 1986). Here we report the occurrence of the 323/A3 antigen in a large cohort of primary breast tumors (n = 384) and its interrelationship with several clinically important variables.

Frozen stored tumor tissues were examined by a Western blot procedure, and the level of 323/A3 protein in individual tumors was calculated in arbitrary units based on the integrated M, 43,000 signal in tumors compared with an MCF-7 internal standard. Thirty-six percent (139 of 384) of tumors were found to be positive for 323/A3. Higher frequencies of 323/A3 protein were found in tumors larger than 2 cm (P = 0.03), tumors with infiltrated lymph nodes (P = 0.01), and tumors without estrogen receptor (P = 0.006). No significant relationship was found with patient age, menopausal status, or progesterone receptor status. Of the newer clinical determinants proliferative rate (% S phase), DNA ploidy, and the lysosomal protease cathepsin D, but not the HER-2/neu oncogene protein, were significantly correlated with 323/A3. The presence of 323/A3 protein was also related to increased recurrence (P = 0.003) and mortality (P = 0.036) after primary treatment.

As an exposed surface antigen, this glycoprotein might be a useful target in radioimaging and immunotherapy of some human breast tumors, especially those having large size, infiltrated lymph nodes, deficient estrogen receptor, high proliferative rate, abnormal DNA content, and high levels of cathepsin D, all of which are ominous indicators of tumor behavior.

INTRODUCTION

Although a number of tumor-associated cell surface antigens have been reported in breast cancer and have even been used in therapeutic and imaging trials (1–12), the relationship of these antigens with other known clinical determinants has not usually been examined. We have previously described a MAAb, 3323/A3, against a M, 43,000 surface glycoprotein of MCF-7 human breast cancer cells (1). Based on a comparison by molecular weight and immunocytochemical tissue distribution, this M, 43,000 glycoprotein represents a protein previously undescribed either in breast cancer or in other tumors. Strong 323/A3 binding was observed to three human breast cancer cell lines (MCF-7, T47D, and ZR-75), whereas two other human breast cancer cell lines (MDA-231 and HBL-100) and three non-breast cancer cell lines displayed little or no binding (13). In histochemical studies, this antibody detected 75% of metastatic lymph nodes and 59% of primary breast tumors and showed some staining in 20% of benign breast lesions (1), so that presence of the antigen appears to be correlated with progression of the disease. The main focus of the present study was to determine more fully the relationship between occurrence of 323/A3 protein with other variables as well as with clinical behavior in breast cancer.

MATERIALS AND METHODS

Materials and Reagents. Bicinchoninic acid (BCA) reagents (A and B) for protein determination were purchased from the Pierce Chemical Co., Rockford, IL. Reagents for electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. Nitrocellulose membranes (0.45 µm) were purchased from Schleicher and Schuell, Keene, NH. 125I-Labeled rabbit anti-mouse IgG was obtained from New England Nuclear, Boston, MA. All other chemicals used were of analytical purity grade or the best grade available.

Acquisition and Storage of Human Breast Tumors. Tumor specimens studied in this report were obtained from women undergoing surgery for primary breast cancer in hospitals nationwide. Immediately after surgical removal, a portion of each tumor specimen was snap frozen in liquid nitrogen and shipped to our laboratory on dry ice for steroid receptor analysis. All tissues were coded, mechanically pulverized in the frozen state, and analyzed for receptors. The remaining powders were stored in air-tight plastic tubes at −70°C. Tissues for the quantitation of 323/A3 antigen were obtained retrospectively from these biopsy specimens collected for clinical purposes unrelated to this investigation. Sections 4 µm thick from formalin-fixed, paraffin-embedded tissues were stained with hematoxylin-eosin and examined under a microscope for the presence of tumor cells. All 384 specimens included in this study contained tumor cells, although the percentage of tumor cells varied considerably.

Clinical Characteristics of Subjects and Follow-up. The clinical information was abstracted from the individual patient chart. Tumors analyzed here were from a patient population ranging in age from 27 to 86 years with a median of 58 years. Lymph nodes were examined microscopically for the presence of tumor cells at the individual hospitals where surgery was performed. Lymph node involvement was considered negative when no lymph nodes contained tumor cells and positive if one or more nodes showed the presence of malignant cells. Fifty-one percent (195 of 384) of patients included in this investigation did not contain tumor extensions to the axillary lymph nodes. Of the node-positive patients, 62% (118 of 189) had more than 3 lymph nodes with tumor infiltration. Tumor size (largest diameter) was recorded at the time of surgery. Depending upon the size, tumors were divided into two groups for the purpose of statistical analysis. Of the 384 tumor specimens studied in this report, 103 (27%) were less than or equal to 2 cm in diameter. Levels of ER and PgR in tumor cytosols are routinely determined in our laboratory as described previously (14, 15).

Tumors were designated positive if the receptor binding was ≥3 fmol for ER and ≥5 fmol for PgR per mg cytosol protein.

Proliferative rate (percentage of S phase) and ploidy (DNA content) were determined in our laboratory by flow cytometric analysis (16). Levels of intact HER-2/neu protein were determined on the M, 34,000 mature form of cathepsin D were quantitated by Western blotting and densitometry of pertinent bands on the resulting autoradiograms as detailed before (17, 18). Patients were followed for disease recurrence and overall survival as described previously (19). Median clinical follow-up time for patients still living was 65 months with a range of 16–168 months. Any postsurgical appearance of malignancy either near to or distant from the operated breast was considered as recurrence of disease. A computer database containing continually updated clinical information.
323/A3 GLYCOPROTEIN IN BREAST CANCER

RESULTS

323/A3 Protein in Breast Tumors. We measured the level of 323/A3 protein in 384 primary breast tumors using the semi-quantitative Western blot procedure described above. Fig. 1 shows a typical Western blot of 8 tumors and a corresponding densitometric scan of the 323/A3 bands at M, 43,000. In each gel 3 concentrations of the same extract of human breast cancer cells were included as an internal laboratory standard.

Quantitation of 323/A3 Protein. Proteins were extracted from the tumor tissues or cell pellets as described elsewhere (17). In brief, approximately 10 mg of powder from each tumor were exposed to 150 μl of 5% SDS. Tubes were kept in a boiling water bath for 5 min. Clear supernatant was collected after spinning the tubes at 13,000 × g for 2 min. Residual insoluble material was once again extracted with 150 μl of 5% SDS as described above, and the first and second supernatants were combined. Protein concentration in the SDS extracts was determined by the BCA method (20).

Fig. 1. Semiquantitation of 323/A3 protein in breast tumors by Western blot and densitometry. The 323/A3 band at M, 43,000 (kDa) along with corresponding densitometric scanning is shown. The 323/A3 content in specimens was calculated in arbitrary units based on an internal standard of human breast cancer MCF-7 cells. The 323/A3 values for the 8 tumors shown here are 113, 3, 4, 0, 3, 28, and 3 units, respectively.

Tumor proteins (200 μg) were separated on 10% polyacrylamide vertical slab gels using 3% stacking gels. Electrophoresis was carried out using maturing, non-reducing conditions (21). A SDS extract of MCF-7 human breast cancer cells was included at three concentrations (200, 100, and 50 μg protein, corresponding to 100, 50, and 25 arbitrary units of 323/A3) in each gel as our laboratory standard. Transfer of resolved proteins onto nitrocellulose membranes was performed at 200 mAmp for 16 h at 4°C (22). Following blocking of nonspecific sites with 5% non-fat dry milk (1 h), the blots were incubated with 323/A3 monoclonal antibody (1 μg/ml) overnight at 4°C. 125I-labeled rabbit anti-mouse IgG (100,000 cpm/ml) was used as the second antibody. After washing, the blots were exposed for 20–24 h to X-OMAT X-ray film (Kodak) at −70°C using intensifying screens. The level of 323/A3 protein in individual tumors was quantitated by densitometric scanning of the M, 43,000 band on the resulting autoradiograms in a Beckman DU-7 spectrophotometer and was expressed in arbitrary units by comparison with an MCF-7 internal standard.

Detection of low levels (1-2 units) of a protein by a semiquantitative Western blot procedure as used here is mainly a function of the time of exposure of the nitrocellulose filters to the X-ray film. A tumor which after a given period of exposure appears negative (0 unit) on the autoradiogram may appear as a faint band on a relatively longer exposure and is then calculated to be a low value (1–2 units) rather than zero. For these reasons, we considered ≥3 units of 323/A3 protein to be necessary for classifying a tumor as 323/A3−. In Fig. 1, three tumors (tumors 2, 6, and 8) with 3 units of 323/A3 are shown to appreciate this level of protein.

Stability of 323/A3 Protein. Because, prior to analysis for 323/A3 protein, the tumor specimens were stored at −70°C for extended periods of time, it was important to find out the stability of this protein. The frequency of 323/A3− tumors was found to be constant irrespective of the length of storage at −70°C; a similar proportion of tumors stored for less than 5 years, 5–10 years, or more than 10 years showed the presence of 323/A3 protein (Table 1). The incidence of 323/A3 positivity in the three groups was not statistically different (P = 0.61).

323/A3 Protein versus Clinical Characteristics. After quanti-
Table 1 Stability of 323/A3 protein in breast tumor specimens stored at —70°C

<table>
<thead>
<tr>
<th>Storage time (yr)</th>
<th>n</th>
<th>% 323/A3-positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5</td>
<td>26</td>
<td>38</td>
</tr>
<tr>
<td>5–10</td>
<td>264</td>
<td>38</td>
</tr>
<tr>
<td>&gt;10</td>
<td>94</td>
<td>32</td>
</tr>
</tbody>
</table>

* P = 0.61.

Table 2 Relationship of 323/A3 protein with clinical characteristics in breast cancer

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>% 323/A3-positive</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrated nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>195</td>
<td>29</td>
<td>0.01</td>
</tr>
<tr>
<td>1–3</td>
<td>71</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>118</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 fmol/mg protein</td>
<td>111</td>
<td>47</td>
<td>0.006</td>
</tr>
<tr>
<td>≥3 fmol/mg protein</td>
<td>273</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2 cm</td>
<td>103</td>
<td>27</td>
<td>0.03</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>281</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 fmol/mg protein</td>
<td>193</td>
<td>40</td>
<td>0.13</td>
</tr>
<tr>
<td>≥5 fmol/mg protein</td>
<td>191</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Patient age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 yr</td>
<td>122</td>
<td>35</td>
<td>0.79</td>
</tr>
<tr>
<td>≥50 yr</td>
<td>262</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>104</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Peri</td>
<td>10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Post</td>
<td>258</td>
<td>38</td>
<td>0.79</td>
</tr>
<tr>
<td>Unknown</td>
<td>12</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>% S phase</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≤6.7</td>
<td>121</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>&gt;6.7</td>
<td>178</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Ploidy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>135</td>
<td>25</td>
<td>0.001</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>216</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>HER-2/neu protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 units</td>
<td>313</td>
<td>35</td>
<td>0.16</td>
</tr>
<tr>
<td>≥100 units</td>
<td>69</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Cathepsin D protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;75 units</td>
<td>229</td>
<td>31</td>
<td>0.02</td>
</tr>
<tr>
<td>≥75 units</td>
<td>153</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

An important clinical characteristic of breast cancer patients is the presence of metastatic tumor deposits in the axillary lymph nodes at the time of primary surgery. We had already shown that the 323/A3 protein is more prevalent in nodal metastases than in primary tumors (1). It was of interest, therefore, to determine the relationship between nodal status and presence of 323/A3 protein in the primary tumors. Of the 384 patients in this study, 51% (195 of 384), 18% (71 of 384), and 31% (118 of 384) had 0, 1–3, and >3 axillary lymph nodes infiltrated with tumor cells, respectively (Table 2). Tumors with many infiltrated nodes contained a substantially higher proportion of 323/A3+ breast cancers (P = 0.01).

Tumor size and steroid receptor deficiency are also known to be associated with clinical aggressiveness (26). We found a greater frequency of 323/A3 positivity in tumors deficient in estrogen receptor (P = 0.006), and in tumors >2 cm in size (P = 0.03). No significant correlation was found between the 323/A3 positivity of the primary tumor and PgR status, age of the patient, or menopausal status, although the trend for PgR paralleled the difference for ER as expected.

Recently, a number of additional parameters have been reported in the literature to be related to clinical behavior of breast cancer. These include flow cytometric determination of the proliferative rate of the tumor (percentage of S phase) and ploidy (DNA content) (16, 19), and measurement of the HER-2/neu oncogene protein (a growth factor receptor-like transmembrane glycoprotein with a molecular weight of 185,000–190,000) (17) and cathepsin D (an estrogen-induced lysosomal protease) (18).

Values for all of these parameters were available on the tumor specimens analyzed in this report and their relationships with 323/A3 protein are also shown in Table 2. Every parameter associated with tumor aggressiveness (high S phase, aneuploidy, high HER-2/neu and cathepsin D) was also associated with a greater incidence of 323/A3 positivity. Only the association with HER-2/neu failed to reach statistical significance.

323/A3 Protein and Clinical Behavior. We also examined directly the association of 323/A3 positivity with the likelihood of breast cancer recurrence and mortality. Kaplan-Meier life table analyses of both recurrence (Fig. 3) and mortality (Fig. 4) show that patients whose tumors have 323/A3 protein are significantly more likely to suffer disease recurrence and death, as expected for the associations with other characteristics already presented. These survival results are summarized in Table 3. When all characteristics are included in multivariate analyses, however, 323/A3 protein does not emerge as an independent predictor of recurrence or mortality (not shown).
The availability of MAbs reacting specifically with human breast tumor-associated cell surface antigens may offer an opportunity to diagnose and treat cancers more effectively. The MAb 323/A3, used in the present study to quantitate an antigen in breast tumors, is an IgG1 generated by immunizing BALB/c mice with the human breast tumor MCF-7 cell line. This MAb shows specificity for primary and metastatic human breast tumors by immunoperoxidase staining in histochemistry (1). It recognizes a M, 43,000 membrane-associated antigen in cell lines as well as in breast tumors. This antigen is identified as a glycoprotein based on its ability to bind to and specifically elute from a concanavalin A column. Moreover, the MAb precipitates a labeled M, 43,000 protein from MCF-7 cells after pulse labeling with [3H]glucosamine (1).

In the present study, quantitation of 323/A3 antigen in breast tumors was done by Western blot and densitometry of the resulting M, 43,000 bands on autoradiograms. Protein samples for 323/A3 Western blotting were electrophoresed under non-reducing conditions (absence of β-mercaptoethanol or dithiothreitol) because the 323/A3 antigenic site is sensitive to reducing agents.

Results presented here show that 323/A3 protein is frequently present in human breast tumors. Thirty-six % (139 of 384) of tumors assessed appeared clearly positive for 323/A3. Occurrence of this protein was analyzed for association with several other characteristics of patients and their breast tumors. A greater frequency of 323/A3 protein was seen in patients with tumor-infiltrated lymph nodes, suggesting that the colonization of lymph nodes with tumor cells may be affected by the presence of this protein on their surface. Positivity for 323/A3 protein was also related to tumor burden taken as the size of the excised primary tumor; larger tumors (>2 cm in diameter) were more often positive for 323/A3 protein (40% versus 27%). Because tumor size may be in part a reflection of the proliferative rate of the tumor, one might also expect a correlation with percentage of S phase. Indeed, a highly significant (P < 0.0001) relationship was found. A strong direct correlation of 323/A3 protein expression with nodal infiltration, tumor size, and proliferation rate suggests that 323/A3 protein may be related to tumor aggressiveness and/or its metastatic behavior. Presence of 323/A3 protein was also significantly associated with absence of ER and thus presumably with loss of estrogen responsiveness in tumors.

No significant correlation was found in the 323/A3 and HER-2/neu oncogene protein in these primary tumors, although we have reported a prognostic significance of the HER-2/neu protein in node-positive breast cancer patients (17). A significant (P = 0.02) relationship did appear, however, between the 323/A3 protein and the M, 34,000 mature form of cathepsin D, a strong independent parameter of disease recurrence and mortality in node-negative breast cancer (18). Although the direct biological role of 323/A3 protein in breast tumor growth is unclear, these findings suggest that the occurrence of this protein in tumors is in part associated with metastatic progression of disease, loss of estrogen responsive-

**DISCUSSION**

The availability of MAbs reacting specifically with human breast tumor-associated cell surface antigens may offer an opportunity to diagnose and treat cancers more effectively. The MAb 323/A3, used in the present study to quantitate an antigen in breast tumors, is an IgG1 generated by immunizing BALB/c mice with the human breast tumor MCF-7 cell line. This MAb shows specificity for primary and metastatic human breast tumors by immunoperoxidase staining in histochemistry (1). It recognizes a M, 43,000 membrane-associated antigen in cell lines as well as in breast tumors. This antigen is identified as a glycoprotein based on its ability to bind to and specifically elute from a concanavalin A column. Moreover, the MAb precipitates a labeled M, 43,000 protein from MCF-7 cells after pulse labeling with [3H]glucosamine (1).

In the present study, quantitation of 323/A3 antigen in breast tumors was done by Western blot and densitometry of the resulting M, 43,000 bands on autoradiograms. Protein samples for 323/A3 Western blotting were electrophoresed under non-reducing conditions (absence of β-mercaptoethanol or dithiothreitol) because the 323/A3 antigenic site is sensitive to reducing agents.

Results presented here show that 323/A3 protein is frequently present in human breast tumors. Thirty-six % (139 of 384) of tumors assessed appeared clearly positive for 323/A3. Occurrence of this protein was analyzed for association with several other characteristics of patients and their breast tumors. A greater frequency of 323/A3 protein was seen in patients with tumor-infiltrated lymph nodes, suggesting that the colonization of lymph nodes with tumor cells may be affected by the presence of this protein on their surface. Positivity for 323/A3 protein was also related to tumor burden taken as the size of the excised primary tumor; larger tumors (>2 cm in diameter) were more often positive for 323/A3 protein (40% versus 27%). Because tumor size may be in part a reflection of the proliferative rate of the tumor, one might also expect a correlation with percentage of S phase. Indeed, a highly significant (P < 0.0001) relationship was found. A strong direct correlation of 323/A3 protein expression with nodal infiltration, tumor size, and proliferation rate suggests that 323/A3 protein may be related to tumor aggressiveness and/or its metastatic behavior. Presence of 323/A3 protein was also significantly associated with absence of ER and thus presumably with loss of estrogen responsiveness in tumors.

No significant correlation was found in the 323/A3 and HER-2/neu oncogene protein in these primary tumors, although we have reported a prognostic significance of the HER-2/neu protein in node-positive breast cancer patients (17). A significant (P = 0.02) relationship did appear, however, between the 323/A3 protein and the M, 34,000 mature form of cathepsin D, a strong independent parameter of disease recurrence and mortality in node-negative breast cancer (18). Although the direct biological role of 323/A3 protein in breast tumor growth is unclear, these findings suggest that the occurrence of this protein in tumors is in part associated with metastatic progression of disease, loss of estrogen responsive-

**ACKNOWLEDGMENTS**

The authors wish to thank Mary Nell Baird and Robert Duarte for their skillful technical assistance with the 323/A3 assays, Judy Wenzel for data management, David Mascorro for his computer programming, and Sandy Montgomery for her patience in typing this manuscript.

**REFERENCES**


323/A3 GLYCOPROTEIN IN BREAST CANCER


Association of the 323/A3 Surface Glycoprotein with Tumor Characteristics and Behavior in Human Breast Cancer

Atul K. Tandon, Gary M. Clark, Gary C. Chamness, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/50/11/3317

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.