Mammaglobin Expression in Lymph Nodes Is an Important Marker of Metastatic Breast Carcinoma

Jae-Ho Han, MD; Yup Kang, PhD; Ha-Chul Shin, MS; Hyun-Soo Kim, MD; Young-Mo Kang, MS; Young-Bae Kim, MD, PhD; Seung-Yeon Oh, MD

Most tumor markers in routine clinical practice lack organ specificity, especially in the case of metastatic adenocarcinomas of unknown primary origin. Mammaglobin, a mammary-specific member of the uteroglobin family, is known to be overexpressed in human breast cancer.

Objective.—We investigated mammaglobin A expression in metastatic carcinomas of lymph nodes from the breast and various other organs and its usefulness in identifying metastatic carcinoma of the breast. For comparative purposes, we also investigated BRST-1 and BRST-2 expression.

Design.—We produced recombinant mammaglobin and polyclonal antimammaglobin antibodies. Mammaglobin expression was analyzed by immunohistochemical staining using a tissue microarray and by reverse transcription–polymerase chain reaction in 210 carcinomas, including those of the breast (n = 70), lung (n = 30), stomach (n = 25), colorectum (n = 20), urinary tract (n = 10), thyroid gland (n = 10), ovary and endometrium (n = 10), and salivary gland (n = 5).

Results.—Mammaglobin expression was observed in 59 cases (84.3%) of breast cancer and in 21 cases (15.0%) of nonbreast cancer. The BRST-1 and BRST-2 expression rates were 75.7% and 44.3% in breast cancer and 26.4% and 2.1% in nonbreast cancer, respectively. Mammaglobin is superior to BRST-1 for both specificity and sensitivity and is superior to BRST-2 for sensitivity.

Conclusion.—Our data suggest that mammaglobin is one of the first relatively mammary-specific and mammary-sensitive markers. Mammaglobin and BRST-2 appear to represent useful markers for breast cancer and should be used as a component of panels evaluating tumors of unknown primary sites.

MATERIALS AND METHODS

Tissue Samples

The study was performed using patients with metastatic carcinomas in lymph nodes from the breast and other organs (n = 140). The primary tumors consisted of 70 infiltrating ductal carcinomas (not otherwise specified) from the breast, 30 adenocarcinomas from the lung, 50 adenocarcinomas from the stomach, 25 adenocarcinomas from the colorectum, 20 adenocarcinomas from the hepatobiliary tract, 10 thyroid gland tumors (8 papillary and 2 medullary), 10 transitional cell carcinomas from the urinary tract, 8 serous carcinomas from the ovary, 2 endometrioid carcinomas from the uterus, and 5 salivary gland tumors (2 mucoepidermoid carcinomas and 3 adenocarcinomas, not otherwise specified). We used the grading system by Bloom and Richardson to evaluate the histologic grade and the grading system by Black to evaluate the nuclear grade of 59 primary breast carcinomas. Formalin-fixed, paraffin-embedded primary breast carcinomas and lymph node samples were analyzed.

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Mammaglobin Antibody Production

Polyclonal mammaglobin antibodies were raised in the New Zealand White rabbit by immunization with purified recombinant mammaglobin. Briefly, the mammaglobin gene was isolated and amplified from human breast carcinoma tissue by RT-PCR using mammaglobin-specific primers (5'-GGGATCCCATGAAGTTGCTGATGGTCCTC and 5'-GCGCTCGAGAAAGTTAAATAAATCACAAAGACTG). The amplified mammaglobin gene was inserted into pET32 (Novagen, Madison, Wis), and the mammaglobins were produced using the Trx fusion system (Novagen). Thioredoxin-mammaglobin proteins were purified by nickel-nitrilotriacetic acid affinity chromatography (Qiagen, Valencia, Calif) and Vivapure D ion-exchange chromatography (Vivascience, Carlsbad, Calif) (Figure 1, A), and thioredoxin was then cut out using an enterokinase cleavage capture kit (Novagen). A rabbit was immunized and boosted with the purified recombinant mammaglobin. The serum samples were collected from the immunized rabbit, and the mammaglobin-specific antibodies so obtained were purified by mammaglobin-conjugated affinity chromatography. The recognition of mammaglobin with the mammaglobin antibody was confirmed by immunoblotting analysis (Figure 1, B).

Construction of the Tissue Microarray and Immunohistochemistry

Two representative areas were taken from a paraffin tissue block of every case after reviewing the hematoxylin-eosin-stained slides. A total of 420 core tissue biopsies (diameter, 1.0 mm) were taken and arrayed into 3 new recipient paraffin blocks. For primary breast carcinomas, 118 core biopsies were arrayed into 2 paraffin blocks. Five-micrometer sections of these tissue array blocks were then cut, placed on charged poly-L-lysine-coated slides, and used for immunohistochemical analysis. Mouse anti-human mononuclear antibodies to the estrogen receptor (clone 6F11, 1:30; Novocastra Laboratories Ltd, Newcastle upon Tyne, England), the progesterone receptor (clone 1A6, 1:30; Novocastra, c-Erb-B2 (clone CB11, 1:30; Novocastra), BRST-1 (clone CU18, 1:50; Signet Laboratories Inc, Dedham, Mass), and BRST-2 (clone D6, 1:50; Signet) were used as primary antibodies. Briefly, sections from the tissue array were deparaffinized in xylene and rehydrated in graded alcohols and water. For the estrogen and progesterone receptors, sections were microwaved. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 10 minutes. Sections were treated with protein-blocking solution and then with primary antibodies, including antimammaglobin rabbit antiserum, at a dilution of 1:100 for 1 hour at room temperature or overnight at 4°C (for the estrogen receptor, the progesterone receptor, and c-Erb-B2). After several rinses in phosphate-buffered saline, sections were incubated in biotinylated secondary antibody. Bound antibodies were detected by the streptavidin-biotin method with a Cap-Plus detection kit (Zymed Laboratories Inc, San Francisco, Calif). Slides were rinsed in phosphate-buffered saline, exposed to diaminobenzidine, and counterstained with Mayer hematoxylin.

The cutoff values were based on previously established cutoff values for well-characterized antibodies (estrogen receptor, progesterone receptor, c-Erb-B2, BRST-1, and BRST-2). The intensities of mammaglobin expression were scored by 3 pathologists from 0 to 3, where grade 0 indicates no staining; grade 1, weak staining; grade 2, moderate staining; and grade 3, strong staining (Figure 2).
Figure 2. Mammaglobin expression in metastatic mammary carcinoma. Immunoreactivity was graded as negative (A), weak (B), moderate (C), or strong (D) (immunohistochemistry, original magnification ×400).

Table 1. Immunohistochemical Staining Results for BRST-1, BRST-2, and Mammaglobin

<table>
<thead>
<tr>
<th>Type*</th>
<th>BRST-1 (%)</th>
<th>BRST-2 (%)</th>
<th>Mammaglobin (%)</th>
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<tbody>
<tr>
<td>Breast (n = 70)</td>
<td>75.7</td>
<td>44.3</td>
<td>84.3</td>
</tr>
<tr>
<td>Nonbreast (n = 140)</td>
<td>26.4</td>
<td>2.1</td>
<td>15.0</td>
</tr>
<tr>
<td>Stomach (n = 30)</td>
<td>20.0</td>
<td>0.0</td>
<td>13.3</td>
</tr>
<tr>
<td>Lung (n = 30)</td>
<td>36.7</td>
<td>3.3</td>
<td>16.7</td>
</tr>
<tr>
<td>Colorectum (n = 25)</td>
<td>4.0</td>
<td>0.0</td>
<td>12.0</td>
</tr>
<tr>
<td>H-B (n = 20)</td>
<td>30.0</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Thyroid (n = 10)</td>
<td>10.0</td>
<td>0.0</td>
<td>20.0</td>
</tr>
<tr>
<td>TCC (n = 10)</td>
<td>50.0</td>
<td>0.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Ovary and Ut (n = 10)</td>
<td>50.0</td>
<td>0.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Salivary (n = 5)</td>
<td>40.0</td>
<td>40.0</td>
<td>20.0</td>
</tr>
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* H-B indicates hepato-biliary tract; TCC, transitional cell carcinoma; and Ut, uterine endometrium.

RESULTS

Validation of the Tissue Microarray

Tissue loss and the representativeness of core biopsies are significant factors for tissue array-based analysis. In our analysis, 210 of 231 cases were represented by 3 tissue microarray blocks each and were available for hematoxylin-eosin staining and for all immunohistochemical staining markers. Of the available 67 cases, the concordance rates of estrogen receptor, progesterone receptor, and c-Erb-B2 were 100%, 89.6%, and 92.5%, respectively, for the arrayed specimens and whole original specimens. Therefore, the overall concordance rate was 94%.

Immunohistochemical Staining Results

We investigated 210 metastatic carcinoma tissues using these purified antibodies. The results are summarized in Table 1. BRST-1 and BRST-2 were detected in 75.7% and 44.3% of 70 breast cancers and in 26.4% and 2.1% of 140 nonbreast cancers, respectively. Mammaglobin expression was observed as diffuse cytoplasmic immunostaining and was detected in 59 (84.3%) of 70 breast cancers and in 21 (15.0%) of 140 nonbreast cancers. No significant difference was found between primary and metastatic breast carci-

Statistical Analysis

A chi-square test using Statistical Analysis Systems software (Cary, NC) was performed to evaluate sensitivities and specificities with 95% confidence intervals for breast cancer and non-breast cancer with respect to BRST-1, BRST-2, and mammaglobin. Pearson product moment correlation was evaluated between the intensity of mammaglobin and the nuclear and histologic grades. A P value < .05 was considered statistically significant.
Figure 3. Detection of mammaglobin expression in the primary tumor by reverse transcriptase-polymerase chain reaction. Mam indicates mammaglobin; S, stomach; C, colon; L, lung; B, breast; M, MDA-MB-415 cell line; and IHC, immunohistochemistry.

Table 2. Specificity and Sensitivity and the 95% Confidence Intervals of BRST-1, BRST-2, and Mammaglobin for Metastatic Mammary Carcinomas

<table>
<thead>
<tr>
<th>BRST-1 (%)</th>
<th>BRST-2 (%)</th>
<th>Mammaglobin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity</strong></td>
<td>75.7 (65.7, 85.8)</td>
<td>44.3 (32.6, 55.9)</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>73.6 (66.3, 80.9)</td>
<td>97.9 (95.5, 100.0)</td>
</tr>
</tbody>
</table>

nomas with regard to mammaglobin expression. Among the positive cases, 18 (30.5%) of 59 breast cancers and 13 (61.9%) of 21 nonbreast cancers showed weak immunoreactivity. In nonbreast cancer, only one urothelial carcinoma and one thyroid carcinoma showed strong positivity. No significant difference was observed between the various organs for mammaglobin expression.

**Results of RT-PCR**

A panel of 2 stomach, 1 colon, 1 lung, and 8 primary breast tumor samples was used (see Figure 3). The breast cancer cell line MDA-MB-415 was used as a positive control for the expression of mammaglobin. Actin expression was measured in separate reactions, and the results were used to normalize expression values. The RT-PCR assay detected substantial mammaglobin expression signals in 6 of 8 primary breast cancers and 1 primary lung cancer, whereas no signal was noted in 1 colon cancer and 2 stomach cancers, one of which showed grade 1 positivity by immunohistochemistry.

**Statistical Analysis**

BRST-1, BRST-2, and mammaglobin expressions were more frequent in breast carcinomas than in nonbreast carcinomas (see Table 2). Specificities for BRST-1, BRST-2, and mammaglobin were 73.6%, 97.9%, and 85.0%, respectively, and sensitivities for BRST-1, BRST-2, and mammaglobin were 75.7%, 44.3%, and 84.3%, respectively. Using a 95% confidence interval, a significant difference was observed between BRST-2 and mammaglobin, but no difference was observed between BRST-1 and mammaglobin, with regard to specificity and sensitivity. A significant positive correlation was noted between intensity of mammaglobin expression and nuclear grade ($R = 0.33$, $P = .01$) and histologic grade ($R = -0.32$, $P < .05$).

**COMMENT**

In addition to sensitivity for early disease and specificity for cancer, organ specificity is a desirable property of a tumor marker. At present, prostate-specific antigen is a rare example of a marker with organ specificity. No specific tumor marker has been identified to date in breast carcinoma because of its biologic heterogeneity. Moreover, the identification of metastatic carcinomas of the breast may be difficult in the absence of a previous history of breast cancer.

BCA-225 is a glycoprotein secreted by the T47D breast carcinoma cell line and is recognized by monoclonal antibody BRST-1.10 Its expression was common in adenocarcinomas of the breast, ovary, and lung but infrequent in adenocarcinomas of the gastrointestinal tract.11 Gross cystic disease fluid is a pathologic secretion from the breast and is composed of several glycoproteins, including gross cystic disease fluid protein-15 (BRST-2). The cells within the body that produce gross cystic disease fluid protein-15 appear to be restricted primarily to those with an apocrine function.12 Some studies have shown it to be a highly specific and sensitive marker for breast cancer (95% specificity and 74% sensitivity),13 whereas other studies have shown an overall sensitivity of about 40%.14,15 Our data show that both BRST-1 and BRST-2 are more frequent in breast carcinomas than in nonbreast carcinomas. However, as in other studies, a relatively low specificity for BRST-1 and a low sensitivity for BRST-2 are problematic. Therefore, a more specific and sensitive marker is required to identify metastatic carcinomas of the breast.

Mammaglobin expression is a sensitive molecular marker for the detection of micrometastasis in tumor-draining lymph nodes.16,17 and mammaglobin expression is thought to be strictly confined to mammary tissue and primary
breast tumors. However, Fleming and Watson\(^1^8\) have reported mammaglobin expression in carcinomas other than breast cancer, and a sensitive 2-step RT-PCR showed mammaglobin expression in a high proportion of normal and malignant tissues derived from the female genital tract and male prostate.\(^1^9\) Even now, it remains controversial as to whether low levels of mammaglobin expression are induced by cytokine-stimulated inflammatory cells. The in vitro expression of mammaglobin by mononuclear cells may be induced by cytokines, such as granulocyte macrophage–colony-stimulating factor, IL-3, interferon-\(\gamma\), and thrombopoietin.\(^2^0\) To further investigate mammaglobin expression outside the breast and to estimate its clinical and diagnostic role, we evaluated metastatic carcinomas from various organs. This study focused on lymph node analysis in order to evaluate the specificity and sensitivity of mammaglobin for identifying metastatic carcinomas in the breast.

The affinity-purified antibodies against mammaglobin were believed to be highly specific, considering that the immunoreexpression of mammaglobin on tissue completely disappeared after preclearing the antibodies with mammaglobin when performing the immunohistochemistry. Although some metastatic carcinomas, other than those from the breast, showed positive mammaglobin expression by immunohistochemical staining and RT-PCR assay, mammaglobin was more frequent in breast carcinomas than in nonbreast carcinomas. Of note, strong expression was rare in nonbreast carcinomas, and a significant percentage (61.9%) of nonbreast carcinomas showed weak (grade 1) immunoreactivity. Considering both the data of the present study and the data of a recent report,\(^1^9\) trace amounts of mammaglobin protein may be present in various organs other than the breast. However, most of it is not detected by immunohistochemistry or RT-PCR.

Although most of the immunohistochemical staining results correlated with the RT-PCR results from the available 12 cases, grade 1 positivity by immunohistochemical staining did not produce exactly the same results as RT-PCR. The possible cause of this discrepancy is that, to our knowledge, there are no published positive criteria for immunohistochemistry, except for the report by Watson et al.\(^7\) According to that report, grade 1 immunopositivity, as defined in the present study, would be negative, and specificity and sensitivity would then have been 94.3% and 58.6%, respectively. Even though the results for mammaglobin are not statistically significant, we believe that it is superior to BRST-1 in specificity and sensitivity and that it can overcome the low sensitivity of BRST-2. A careful interpretation of mammaglobin expression is needed, and simultaneous BRST-1 and BRST-2 studies may aid the diagnosis of metastatic mammary carcinoma in some equivocal cases, because mammaglobin immunohistochemistry has its limitations, and various nonbreast carcinoma cells express mammaglobin.

We used a tissue microarray to investigate the molecular profiling of cancer specimens immunohistochemically. The main concern regarding the tissue microarray technique is that the small tumor specimens on the array may not be representative of the whole tumor specimen because of tissue heterogeneity. One immunohistochemical investigation of 38 cases of breast carcinomas and their phenotypic profiles regarding the estrogen receptor, the progesterone receptor, and HER-2/neu expression comprised 1 to 10 cores per full tissue section.\(^2^1\) The results showed that a twofold redundancy can lead to a greater than 95% concordance. However, mammaglobin expression did not occur focally, but rather, as a diffuse pattern, and therefore was probably not affected by considerations of tumor heterogeneity. With regard to both the homogeneous expression of mammaglobin and the 94% concordance rate of the tissue microarray, we believe our array to be representative of a metastatic carcinoma for interpreting mammaglobin expression.

Our data suggest that mammaglobin expression is mostly, although not exclusively, confined to breast cancer and that mammaglobin is one of the first mammary-specific markers. BRST-2 and mammaglobin should be considered important components of any immunohistochemical staining panel used to identify carcinomas of breast origin.

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References