Autoantibodies to Annexin XI-A and Other Autoantigens in the Diagnosis of Breast Cancer

Félix Fernández-Madrid,¹,³,⁴ Naimee Tang,¹ Huda Alansari,¹ José L. Granda,¹ Larry Tart,¹ Kathryn C. Amirikia,² Mihail Moroianu,¹ Xiaoju Wang,⁴ and Robert L. Karvonen⁵

¹Department of Internal Medicine, Division of Rheumatology, ²Department of Surgery; ³Center for Molecular Medicine and Genetics; ⁴Karmanos Cancer Institute, Wayne State University, Detroit, Michigan; and ⁵The Science Institute, Bloomfield Hills, Michigan

ABSTRACT
We report on the identification of autoantigens commonly recognized by sera from patients with breast cancer. We selected ten sera from patients with invasive ductal carcinoma (IDC) of the breast with high titer IgG autoantibodies for biopanning of a T7 phage breast cancer cDNA display library. A high throughput method involved the assembly of 938 T7 phages encoding potential breast cancer autoantigens. Microarrays of positive phages were probed with sera from 90 patients with breast cancer (15 patients with ductal carcinoma in situ (DCIS) and 75 patients with IDC of the breast), with 51 non-cancer control sera and with sera from 21 patients with systemic autoimmune diseases. A 12-phrase breast cancer autoantibody predictor group was constructed with phage inserts recognized by sera from patients with breast cancer and not by non-cancer or autoimmune control sera (P < 0.0001). Several autoantigens including annexin XI-A, the p80 subunit of the Ku antigen, ribosomal protein S6, and other unknown autoantigens could significantly discriminate between breast cancer and non-cancer control sera. Biopanning with three different sera led to the cloning of partial cDNA sequences identical to annexin XI-A. IgG autoantibodies reacting with the amino acid 41–74 sequence of annexin XI-A were found in 19% of all women with breast cancer but in 60% of sera from women with DCIS of the breast. In addition, partial sequences identical to annexin XI-A, nuclear protein interacting with the forkhead-associated (FHA) domain of pKi-67, the KIAA1671 gene product, ribosomal protein S6, cyclin K, elongation factor-2, Grb2-associated protein 2, and other unknown proteins could distinguish DCIS from IDC of the breast and appear to be potential biomarkers for the diagnosis of breast cancer.

INTRODUCTION
Ductal carcinoma in situ (DCIS) of the breast, the earliest form of clinically recognizable breast cancer has been increasingly detected with the use of mammography screening. Although DCIS of the breast is highly curable and the majority of patients do not develop recurrences after 5–10 years, it is now well recognized that it is a heterogeneous group of lesions with a diverse malignant potential (1–3). However, because the risk factors for DCIS are similar to those of invasive ductal carcinoma (IDC) of the breast, it is likely that biomarkers associated with DCIS of the breast might be of value for the early diagnosis of breast cancer (1–3). Efforts to diagnose breast cancer based on autoantibodies to the hundreds of individual antigens that have been cloned have thus far been largely unsuccessful. Although the range of possible serological tumor markers for breast cancer reported in the literature is broad (4–19), few have been incorporated into routine oncologic practice, and none have been thought to be of value for the diagnosis of DCIS of the breast (1–5).

In this work we combined procedures designed to minimize the confounding effect of unrelated autoantibodies with high throughput methodology to validate our immunoscreening approach. We report on a group of breast cancer autoantigens that are recognized by sera from multiple patients with DCIS and IDC of the breast with potential value for the early diagnosis of breast cancer.

MATERIALS AND METHODS

Patients and Materials. Sera and pathological specimens with comprehensive 10-year outcome data from 800 breast cancer patients collected by the Karmanos Cancer Institute Breast Cancer Prognostic Study at Wayne State University during the decade from 1975 to 1985 were available for this study (10). Sera from 10 patients with IDC of the breast were used for immunoscreening a T7 bacteriophage cDNA library of breast cancer proteins. Positive phages cloned with these 10 sera were used to construct a breast autoantigen microarray. Sera from 15 women with DCIS of the breast and from 75 women with IDC of the breast consecutively enrolled in the Karmanos Cancer Institute Breast Cancer Prognostic Study with biopsy-proven diagnosis were chosen to probe the breast autoantigen microarray. All sera were obtained before treatment and were stored frozen at −70 °C until use. Fifty-one non-cancer, non-autoimmune control sera were obtained from women attending the rheumatology clinic of Wayne State University with diagnoses of osteoarthritis (20) or fibromyalgia (21), having no past or family history of breast or ovarian cancers. Osteoarthritis is also called degenerative joint disease or osteoarthrosis, indicating the inherently noninflammatory nature of this joint disease (22). Fibromyalgia is a chronic musculoskeletal disorder diagnosed mainly in middle-aged Caucasian women characterized by widespread pain, characteristic trigger points and other clinical manifestations such as fatigue, sleep disturbances, and irritable bowel syndrome but not characterized by inflammation. Women with similar age and race (breast cancer, mean age at diagnosis of 59.3 years, 88 Caucasian, 2 African American; control women, mean age of 56.4 years, 48 Caucasian, 2 African American, and 1 Asian), having these conditions were chosen as controls because neither osteoarthritis nor fibromyalgia are related to the systemic autoimmune diseases and are not characterized by immunological abnormalities. Additional non-cancer control sera were obtained from women with rheumatoid arthritis (23) and systemic lupus erythematosus (24). This study had the approval of the Human Investigation Committee of Wayne State University.

Pathological Material. Eleven breast pathological specimens from tumors that were kept frozen (−70 °C) at the Karmanos Cancer Institute, corresponding to patient sera used to probe the microarray were available for immunohistochemical studies. Five of these specimens had DCIS of the breast and six had IDC of the breast. Immunohistochemistry was performed using the avidin-biotin peroxidase complex technique on 4-μm formalin-fixed tissue sections. After blocking with Super Block (Skytek, Inc.), the sections were incubated overnight at 4°C with polyclonal goat antihuman annexin XI antibody (L-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and immunostained with the Elite ABC kit (Vector Laboratories, Burlingame, CA). Images were obtained with a Sony 970X digital camera interfaced with the MCID 5+ system from Imaging Research, Inc. (St. Catherine, ON, Canada).

Selection of Sera and Probing the T7 Display cDNA Library. We used immunoreactivity as the main criterion to select breast cancer patient sera for biopanning the T7 phage cDNA display library. All 800 breast cancer sera from the Karmanos Cancer Institute Breast Cancer Prognostic Study were used to probe immunoblots of total protein extracts of a breast cancer cell line (MCF10CA1d, clone 1, a malignant variant of nontransformed MCF10A cells; Ref. 25) as described previously (10). We selected sera from 10 patients with...
AUTOANTIBODIES IN BREAST CANCER

IDC of the breast that were collected at the time of diagnosis to identify phage-encoding autoantigens via biopanning of a breast cancer T7 phage cDNA display library. Patient sera were selected according to the manufacturer’s instructions. These 10 sera were selected because they exhibited strong signals at a dilution of ≥1:500 on immunoblots. In particular, a band at 55–56 kDa was very prominent in several of the sera (Fig. 1). We hypothesized that sera with high titer IgG antibodies would probably lead to the identification of dominant reactivities.

After the final round of biopanning, phages were plated at low density on a lawn of Escherichia coli, and plaque lifts were immunoscreened using patient sera to identify individual positive phages. We reasoned that random picking of plaques without the guidance of antibody recognition would probably lead to dilution of relevant clones. Briefly, A/G agarose beads were incubated with 5 μl of a 1:20 dilution of serum for 1 h at 4°C, washed with PBS plus 1% Tween 20, and then incubated with the T7 phage display library overnight at 4°C. Beads were then washed with PBS plus 1% Tween 20 and used to infect a culture of isopropyl-D-thiogalactopyranoside-induced E. coli strain BLT5616. The mixture was shaken until lysis was observed. The lysed culture was clarified by centrifugation, and the supernatant was taken through 4–10 additional rounds of biopanning.

Autoantigen Microarrays. A library of 938 T7 phages encoding potential breast cancer autoantigens was assembled. Plaque-pure phages were grown to high titer in bacterial cultures that were incubated until complete lysis. Supernatants collected after a 10 min × 10,000 g spin were arrayed in 384-well microtiter dishes. The entire 938 phage library was spotted in duplicate onto nitrocellulose-coated fluorescent array surface technology (FAST) slides (Schleicher and Schuell) using a Flexys robot (Genomic Systems). Each slide was probed either with sera from patients with DCIS, IDC of the breast, or sera from 26 women without cancer (Table 1). Each positive phage was examined separately for a potential association with the diagnoses of DCIS or IDC of the breast by χ² determination. Starting with the phages most significantly associated with cancer versus the non-cancer group, a 12-phage breast cancer predictor group was constructed stepwise from selected phages that had the ability to increase the set of correctly predicted cancer sera (Table 2). We eliminated from further consideration any phages that reacted with the secondary reagents and gave a positive signal in control experiments in which no patient sera were used in the primary incubation. We also eliminated any phages that were recognized equally by both cancer and non-cancer patient sera for the construction of the cancer predictor. These may encode epitopes similar to those found on common infectious agents or environmental allergens. In a test of the accuracy of our approach, we retrospectively verified that identical phages were each scored as positive by any given patient serum.

In a second step, the group of 12 phages identified in the training set was used as a predictor of breast cancer in an independent group of 45 sera (8 sera from patients with DCIS of the breast and 37 sera from patients with IDC of the breast) and sera from 25 women without autoantigens to distinguish sera from patients with breast cancer from non-cancer control sera. The first step to determine whether the phages cloned with sera from patients with IDC of the breast were relevant to breast cancer involved probing the autoantigen microarray with a training set of 7 sera from patients with DCIS, 38 sera from patients with IDC of the breast, and sera from 26 women without cancer (Table 1). Each positive phage was examined separately for a potential association with the diagnoses of DCIS or IDC of the breast by χ² determination. Starting with the phages most significantly associated with cancer versus the non-cancer group, a 12-phage breast cancer predictor group was constructed stepwise from selected phages that had the ability to increase the set of correctly predicted cancer sera (Table 2). We eliminated from further consideration any phages that reacted with the secondary reagents and gave a positive signal in control experiments in which no patient sera were used in the primary incubation. We also eliminated any phages that were recognized equally by both cancer and non-cancer patient sera for the construction of the cancer predictor. These may encode epitopes similar to those found on common infectious agents or environmental allergens. In a test of the accuracy of our approach, we retrospectively verified that identical phages were each scored as positive by any given patient serum.

200 kDa

56 kDa

32 kDa

Fig. 1. Immunoblot of breast cancer patient sera used for immunoscreening the T7 cDNA display library. Immunoblot of whole breast cancer cell proteins. Sera from patients with invasive ductal carcinoma of the breast, diluted 1:500; secondary antibody, IgG, 1:3,000; †, indicates sera used for biopanning the T7 cDNA display library; ●, indicates sera that lead to the cloning of annexin XI-A. At extreme right, three normal sera and a serum recognizing RPA32.
cancer. The number of patients of the independent group was set to closely match the training group in the proportions of DCIS and IDC of the breast patients. As a confirmation of the significance of the predictor group, both the sensitivity and specificity were high for the independent group (Table 1). When we combined data obtained from both the training and the independent sets, the predicting ability for breast cancer of the 12-phage predictor group remained intact ($P < 0.0001$). The results suggest that the breast autoantigens identified have potential predictive value for both DCIS of the breast as well as for IDC of the breast (Table 1). Sera from 12 systemic lupus erythematosus and 9 rheumatoid arthritis patients tested negative with the 12 phages of the selected predictor group for breast cancer.

An intriguing result, that can probably be explained by the method used for selecting the screening sera, was the cloning of 17 identical partial sequences encompassing bp 205–418 of the annexin XI-A cDNA (27; CB331917) from 3 of the 10 sera from IDC of the breast used for biopanning the T7 phage cDNA display library. Three of these phages were included in the 12-phage breast cancer predictor group because their addition increased the sensitivity and specificity (Table 2). Although the deduced amino acid (aa) sequences were identical, the lengths of the sequences cloned were not the same. Twelve of the 17 annexin XI-A phages contained peptides with the amino acid sequence identical to residues 41–74 of annexin XI-A. Of these 12 phages, 11 were able to significantly distinguish DCIS from IDC of the breast ($P < 0.0001$ to 0.05), and only one phage failed to distinguish DCIS from IDC of the breast. The five remaining phages lacked a part of the aa sequence 41–74 and had peptides of unequal length (four were shorter, aa 41–58, 41–70, 45–74, 47–74, and one was longer, aa 47–111). None of these five phages were reactive with any of the sera from patients with DCIS of the breast. These findings suggest that the ability to significantly distinguish DCIS from IDC of the breast depends critically on the 41–74 sequence of annexin XI-A. If the sequences of aa 40–43 or 71–74 are missing, the ability of the phage to distinguish DCIS from IDC is lost.

Table 1 Potential predictive ability of autoantigens cloned in the T7 phage system

<table>
<thead>
<tr>
<th></th>
<th>% Correctly predicted</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training set</td>
<td>82%</td>
<td>76%</td>
<td>92%</td>
<td>71</td>
</tr>
<tr>
<td>Independent set</td>
<td>80%</td>
<td>78%</td>
<td>84%</td>
<td>70</td>
</tr>
<tr>
<td>Combined set</td>
<td>81%</td>
<td>77%</td>
<td>88%</td>
<td>141</td>
</tr>
</tbody>
</table>

Immunohistochemical staining of tumor sections with a polyclonal antibody to the NH$_2$-terminal moiety of annexin XI showed that with one exception, the protein expression in the tumors corresponded with positive serum reactivity (DCIS of the breast, 4 of 4, IDC, 1 of 1), and a serum negative for annexin XI-A antibodies corresponded to lack of tissue expression of the protein (DCIS, 0 of 0, IDC, 5 of 5). The exception was one specimen from DCIS of the breast that stained positive for annexin XI whereas the corresponding serum was negative on the microarray. Fig. 2 shows examples of DCIS and IDC breast cancer tissue with positive and negative staining for annexin XI.

Multialignment of the partial sequences of the 12 phage predictor group showed that three phages had the 41–74 aa sequence of annexin XI-A, whereas seven phages had homologous sequences suggesting reactivity toward an unknown common antigen without significant homology with any known protein in the GenBank database. The nature of this phage remains unknown and thus we called this antigen UPX. Because the other two phages had no significant homology in the GenBank database (CF751973–4 and CF751975–6), it appears that the 12 phage predictor is based on reactivity toward epitopes located on annexin XI-A and on a small number of unknown proteins.

The phage reactivities of sera from patients with DCIS or IDC of the breast were not significantly associated with the tumor grade, amount of necrosis, or lymphocytic infiltration, although the numbers of patients and particularly of tumor specimens available after stratification by these parameters were relatively small.

Table 2 12-Phage breast cancer predictor

<table>
<thead>
<tr>
<th>Gene ID$^b$</th>
<th>C</th>
<th>N</th>
<th>C</th>
<th>N</th>
<th>C</th>
<th>N</th>
<th>DCIS</th>
<th>IDC</th>
<th>SLE</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP455</td>
<td>7</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP905</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP575</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP581</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>An XI (112$^c$/CB331917)</td>
<td>5</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>An XI (110$^c$/CB331917)</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP215</td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>An XI (111$^c$/CB331917)</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP409</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP484</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP311</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP468</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ DCIS, ductal carcinoma in situ; C, number of positive cancer sera; N, number of positive non-cancer control sera; An XI, annexin XI-A; EST, expressed sequence tags; IDC, invasive ductal carcinoma; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; UP, unknown protein.

$^b$ The GenBank EST accession number assigned to our identified partial sequence is included in parentheses in the gene identity column.

$^c$ For annexin XI-A the particular phage is also included in parentheses.

$^d$ Indicates that PCR and sequence analysis identified two sequences, so the reactivities were scored as the result of unknown proteins.
known proteins in the GenBank database and were recorded as unknown proteins. A number of phages showed predominant or exclusive reactivity with sera from patients with DCIS, whereas the majority of the autoantigens reacted with sera from both DCIS and IDC of the breast. The finding of 60% reactivity of annexin XI-A phages with sera from DCIS patients but only 11% positivity with sera from patients with IDC (Table 3), and the ability of certain autoantigens to significantly distinguish between DCIS and IDC of the breast (Table 6) suggested the possibility that serum reactivity toward breast cancer autoantigens might reveal different antigen phenotypes. In addition, a number of potentially diagnostic autoantigens were recognized by multiple breast cancer sera and had negligible reactivity with control sera, but the differences did not reach significance (Table 5). Among other known and unknown proteins, the ribosomal protein S12 (30, 31), the nucleolar protein interacting with the forkhead-associated domain (NIFK) of pKi-67 (34), the p80 subunit of the Ku antigen (28), and cyclin K33 appear to be promising potential markers. Both cyclin K and the Ku antigen were represented by two clones, but the reactive antigen(s) in one of the phages (UP785) is uncertain because both sequences were cloned from this phage.

Some Autoantigens Cloned with Breast Cancer Sera Are Irrelevant to Breast Cancer. Although most of the antigens listed in Tables 2–5 exhibited negligible reactivity with normal and autoimmune sera, a number of control sera reacted significantly (p from 0.02 to 0.0003) with phage inserts cloned with sera from patients with breast cancer, including those with partial sequences of gelsolin.

### Table 3. Annexin XI-A-positive sera

The percentage and number (N) of annexin XI-A-positive sera in each group is given. Percentages are rounded to the nearest integer. The number of all patients in each group is given at the top of the column. Sera were scored as positive if they reacted with two or more annexin XI-A phages. No SLE or RA sera scored positive for any of the annexin XI-A phages. Incomp. seq. refers to phages containing the annexin XI-A amino acid sequences 41–58, 41–70, 44–74, 45–74, and 47–74, which are missing part of the sequence 41–74.

<table>
<thead>
<tr>
<th>aa sequence</th>
<th>No. phages</th>
<th>Group totals</th>
<th>15 DCIS</th>
<th>75 IDC</th>
<th>90 Breast cancer</th>
<th>51 Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>41–74</td>
<td>12</td>
<td>% Annexin positive</td>
<td>60% 7% 16% 0%</td>
<td>9 5 14 0</td>
<td>5 % Annexin positive</td>
<td>0% 5% 4% 2%</td>
</tr>
<tr>
<td>Incomp. seq.</td>
<td>5</td>
<td>N</td>
<td>0% 5% 4% 2%</td>
<td>0 4 4 1</td>
<td>N</td>
<td>9 8 17 1</td>
</tr>
<tr>
<td>41–74 + incomp. seq.</td>
<td>17</td>
<td>% Annexin positive</td>
<td>60% 11% 19% 2%</td>
<td>9 8 17 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SLE, systemic lupus erythematosus; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; RA, rheumatoid arthritis; aa, amino acid.*

**Fig. 2.** Staining of ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) of the breast with a polyclonal antibody to annexin XI. A, BB904. DCIS of the breast. Left shows annexin positive staining. Right unstained control. Serum positive for annexin XI-A. Bars in A, B, and C equal 40 μ. B, BB969. IDC of the breast stained with a polyclonal antibody to the NH2-terminal region specific for annexin XI. Left shows annexin XI positive staining. Right, unstained control. Serum positive for annexin XI-A. C, BB678. IDC of the breast. Left, negative staining with anti-annexin XI polyclonal antibody. Right, unstained control. Serum negative for annexin XI-A.
Table 4 Autoantigens significantly recognized by breast cancer patient sera

<table>
<thead>
<tr>
<th>cDNA identity</th>
<th>GenBank accn</th>
<th>bp</th>
<th>C</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPX (CF751959)</td>
<td>nsh</td>
<td>25</td>
<td>1</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>UP941 (CF751971)</td>
<td>nsh</td>
<td>14</td>
<td>0</td>
<td>0.0074</td>
<td></td>
</tr>
<tr>
<td>Annexin XI-A (CB331917)</td>
<td>NP_001148</td>
<td>205–418</td>
<td>17</td>
<td>1</td>
<td>0.0084</td>
</tr>
<tr>
<td>Ku-p80 (CF751978)</td>
<td>M30938</td>
<td>3162–3210</td>
<td>17</td>
<td>1</td>
<td>0.0085</td>
</tr>
<tr>
<td>CB331943</td>
<td>2910–3203</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UP215 (CF751969–70)</td>
<td>nsh</td>
<td>13</td>
<td>0</td>
<td>0.0109</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S6 (CB331938)</td>
<td>AB062123</td>
<td>414–653</td>
<td>10</td>
<td>0</td>
<td>0.0333</td>
</tr>
<tr>
<td>UP335 (CF751979)</td>
<td>nsh</td>
<td>13</td>
<td>1</td>
<td>0.0367</td>
<td></td>
</tr>
</tbody>
</table>

The GenBank expressed sequence tag accession numbers assigned to the identified partial sequences are included in parentheses in the cDNA identity column.

Indicates that two sequences were identified in each of these phages, so the reactivity was scored as the result of an unknown protein.

The accession number of expressed sequence tags assigned to the identified partial sequences are included in parentheses in the cDNA identity column.

Indicates that PCR and sequence analysis revealed more than one product, each with a partial pair sequence of known proteins identified from the cloning.

Table 5 Other autoantigens recognized by multiple breast cancer sera

<table>
<thead>
<tr>
<th>cDNA identity</th>
<th>GenBank accn</th>
<th>bp</th>
<th>C</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP757 (CF751968)</td>
<td>nsh</td>
<td>11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>UP815 (CF751965–7)</td>
<td>nsh</td>
<td>11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>UP905 (CF751965–7)</td>
<td>nsh</td>
<td>11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>UP581 (CB331923)</td>
<td>nsh</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S12 (CF931352)</td>
<td>NP_001007</td>
<td>326–500</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>KIAA1671 (CB331932)</td>
<td>BAB33341</td>
<td>447–800</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>UP311 (CF751973–4)</td>
<td>nsh</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP785 (CB331956–59)</td>
<td>AF542236</td>
<td>3258–3262</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Ku-p80 (AF542236) (CB235956)</td>
<td>M30938</td>
<td>3162–3212</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIFK (CB331935)</td>
<td>AAH12457</td>
<td>687–898</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>UP119 (CF751986–5)</td>
<td>nsh</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>UP458 (CF751981–2)</td>
<td>nsh</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP468 (CF751975–6)</td>
<td>nsh</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NPP. p13 (CF731953)</td>
<td>NP_004732</td>
<td>1165–1999</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>UP116 (CB331918–CB331939)</td>
<td>nsh</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UP855 (CB331915–CB331942)</td>
<td>nsh</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cal/calm-d. kinase II (CB334782)</td>
<td>BC020630</td>
<td>1217–1952</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Clone IMAGE:4128735 (CF235958)</td>
<td>BC009385</td>
<td>661–951</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Elongation factor-2 (CB331921)</td>
<td>P09445</td>
<td>2495–2580</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>PBDZ-RAP1 (CB331975)</td>
<td>AF039571</td>
<td>2009–1829</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6 Possible diagnostic markers for DCIS* of the breast

The total number of subjects with DCIS is 15 and that with IDC of the breast is 75. The P value was determined by χ² square with Yates correction for small values and ns is a P > 0.05. Autoantibodies are listed in order of decreasing significance.

<table>
<thead>
<tr>
<th>cDNA identity</th>
<th>GenBank accn</th>
<th>bp</th>
<th>DCIS</th>
<th>IDC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin XI-A (CB331917)</td>
<td>NP_001148</td>
<td>205–418</td>
<td>9</td>
<td>8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NIFK (CB331935)</td>
<td>AAH12457</td>
<td>667–898</td>
<td>5</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KIAA1671 (CB331932)</td>
<td>BAB33341</td>
<td>447–800</td>
<td>5</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>UP66 (CF751987)</td>
<td>nsh</td>
<td>4</td>
<td>1</td>
<td>0.0010</td>
<td></td>
</tr>
<tr>
<td>UP104 (CF751988–9)</td>
<td>nsh</td>
<td>4</td>
<td>2</td>
<td>0.0045</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S6</td>
<td>AB062123</td>
<td>416–653</td>
<td>5</td>
<td>5</td>
<td>&lt;0.0107</td>
</tr>
<tr>
<td>Cyclin K (CB331940)</td>
<td>AF542236</td>
<td>32652–32475</td>
<td>3</td>
<td>1</td>
<td>0.0118</td>
</tr>
<tr>
<td>UP94 (CF751978)</td>
<td>nsh</td>
<td>3</td>
<td>1</td>
<td>0.0118</td>
<td></td>
</tr>
<tr>
<td>UP107 (CF751980)</td>
<td>nsh</td>
<td>3</td>
<td>1</td>
<td>0.0118</td>
<td></td>
</tr>
<tr>
<td>Grb2-AP2 (CB331927)</td>
<td>NP_006346</td>
<td>1506–1599</td>
<td>2</td>
<td>0</td>
<td>0.0250</td>
</tr>
<tr>
<td>UP859 (CB331931)</td>
<td>nsh</td>
<td>2</td>
<td>0</td>
<td>0.0250</td>
<td></td>
</tr>
<tr>
<td>UP455 (CF751964)</td>
<td>nsh</td>
<td>0</td>
<td>22</td>
<td>0.0371</td>
<td></td>
</tr>
</tbody>
</table>

* DCIS, ductal carcinoma in situ; Grb2-AP2, Grb2-associated protein 2; IDC, invasive ductal carcinoma; nsh, no significant homology; UP, unknown protein.

Indicates that more than one sequence was identified in each of these phages, so the reactivity was scored as the result of an unknown protein.

Indicates that PCR and sequence analysis revealed more than one product, each with a partial pair sequence of known proteins identified from the cloning.

Indicates that two sequences were identified in each of these phages, so the reactivity was scored as the result of an unknown protein.

Indicates that more than one sequence was identified in each of these phages, so the reactivity was scored as the result of an unknown protein.

Indicates that more than one sequence was identified in each of these phages, so the reactivity was scored as the result of an unknown protein.

The sera we selected for screening the T7 phage cDNA library exhibited high titers of an IgG antibody reacting with a 56 kDa antigen on immunoblots of human breast cancer proteins (Fig. 1). We hypothesized that the gene encoding this antigen would be over-represented in the phages cloned with these sera, thus allowing the identification of dominant reactivities. As we expected, several phages cloned using sera from three different patients with IDC were identical, and sequence analysis of the phage inserts showed identity with a partial sequence of annexin XI-A (27). The findings of a large number of breast autoantigens exhibiting the ability to differentiate breast cancer sera from normal sera (Tables 1–4) and yet, other antigens that react preferentially with DCIS of the breast (Table 6) suggest that our strategy is effective, allowing the identification of autoantigens relevant to breast cancer. We have previously used this strategy to clone RPA32, using a serum from a patient with breast disease based on autoimmunity to individual antigens or to groups of antigens that have thus far been largely unsuccessful. This failure may in part reflect problems inherent to the specificity of the immunoscreening procedure as well as to the lack of systematic methods for the identification of informative autoantibodies from the scores of antibodies that are either patient-specific or irrelevant to the disease. The use of SEREX (serological analysis of recombinant tumor cDNA expression libraries) and proteomics methodologies (10–19, 43) led to the identification of a large group of autoantigens in breast cancer patient sera. Although in aggregate these studies strongly suggest that autoantibodies have potential as biomarkers, thus far, they have not resulted in serological markers with definitive predicting ability for breast cancer in the clinical arena (4, 5), and none have been thought to be of value for the diagnosis of DCIS of the breast (1–5). Because autoantibodies are part of the normal immune response (44), one important problem inherent to autoantibody-based methods for identifying tumor-related antigens is demonstrating their tumor relevance. Here we report that a collection of breast cancer autoantigens cloned by screening a T7 phage cDNA library of breast cancer proteins are recognized by multiple sera from patients with DCIS and IDC of the breast but not by sera from non-cancer controls. Moreover, some of these antigens were able to distinguish DCIS from IDC of the breast. The sera we selected for screening the T7 phage cDNA library exhibited high titers of an IgG antibody reacting with a 56 kDa antigen on immunoblots of human breast cancer proteins (Fig. 1). We hypothesized that the gene encoding this antigen would be over-represented in the phages cloned with these sera, thus allowing the identification of dominant reactivities. As we expected, several phages cloned using sera from three different patients with IDC were identical, and sequence analysis of the phage inserts showed identity with a partial sequence of annexin XI-A (27). The findings of a large number of breast autoantigens exhibiting the ability to differentiate breast cancer sera from normal sera (Tables 1–4) and yet, other antigens that react preferentially with DCIS of the breast (Table 6) suggest that our strategy is effective, allowing the identification of autoantigens relevant to breast cancer. We have previously used this strategy to clone RPA32, using a serum from a patient with breast cancer...
cancer who exhibited a high titer of 32 kDa (10). Because the 56-kDa autoantigen has also been cloned with a serum from a patient with lung cancer using SEREX (45), it is possible that autoantibodies to annexin XI-A may also be found in other malignancies.

Annexin XI is a member of the annexin superfamily of Ca\textsuperscript{2+} and phospholipid-binding, membrane-associated proteins implicated in Ca\textsuperscript{2+}-signal transduction processes associated with cell growth and differentiation (46–53). Annexin XI may have a role in cellular DNA synthesis and in cell proliferation as well as in membrane trafficking events such as exocytosis and has been found to be identical to a 56-kDa antigen recognized by antibodies in 3.9% of patients with systemic autoimmune diseases (54). Misaki et al. (27) showed that antiannexin XI positive sera from patients with systemic autoimmune diseases recognize an epitope(s) residing in the NH\textsubscript{2}-terminal moiety of the molecule. They showed that mutants containing only part of the annexin XI aa 1–123 were recognized by all autoimmune sera, but a mutant containing only the NH\textsubscript{2}-terminal 32 aa(s) was not reactive with any of the sera tested, and that removal of aa 1–49 eliminated the reactivity of four of five sera. One of the sera tested was still able to immunoprecipitate this short peptide, indicating that at least one reacting epitope is located in this region of the molecule. The precise location of the other epitope reacting with autoimmune sera is uncertain, but from the study of mutants it might be located in the region spanning positions 50–123 of the annexin XI sequence (27). The partial sequence of annexin XI-A cloned by breast cancer sera spans residues 41–111, but the sequence aa 41–74 appears critical for distinguishing DCIS from IDC of the breast (Table 3). None of the cloning sera used in this work were obtained from patients with systemic autoimmune diseases, and sera from 21 patients with systemic lupus erythematosus and rheumatoid arthritis did not react with the phage inserts containing annexin XI-A. However, neither our data nor the previous studies (27) eliminate the possibility that both cancer and autoimmune sera may react with identical epitopes.

There is a parallel between breast cancer and autoimmune diseases in reference to serum reactivity to annexin XI and RPA32. The prevalence of anti-RPA32 was reported to be 11% and that of annexin XI is approximately 19% in breast cancer sera, whereas the frequency of these antibodies in the systemic autoimmune diseases has been estimated to be 2%–3% and 3.9%, respectively (10, 54). It is pertinent that both systemic lupus erythematosus and Sjögren’s syndrome are known to be associated with a tendency to develop lymphoid malignancies (55–57). There are reports on the cancer-predicting ability of several members of the large annexin family that are suspected to be involved in the process of carcinogenesis (46–52). Thus, it is possible that the antibodies to RPA32 and to annexin XI in the sera of a small proportion of patients with systemic autoimmune diseases may represent early markers of malignancy.

To our knowledge this is the first report on annexin XI-A cloned with sera from IDC of the breast and recognized as an autoantigen by sera from multiple patients with breast cancer. It may be of interest that the sequence that we identified as critical for the recognition of DCIS of the breast spanning aa 41–74 is located in the regulatory NH\textsubscript{2}-terminal moiety of annexin XI-A, which contains the nuclear localization signal (58). PEST sequences believed to be signals for rapid intracellular degradation (59), as well as the aa residues essential for binding the annexin XI-A isoform with calcyclin (S100A6; Ref. 60).

Antibodies to the ribosomal protein S6 (29), nucleolar protein interacting with the FHA domain of pKi-67 (34), elongation factor 2 (37), Grb2-associated binding protein 2 (40, 41), the KIAA1671 gene product (32), and other autoantigens listed in Tables 4–6 have not been reported previously in the sera of cancer patients. The reactivities of the phage inserts with multiple sera from patients with breast cancer sera are listed in Tables 2–5. We propose that a number of the autoantigens reported here, including annexin XI (27), the ribosomal proteins S6 (29) and S12 (30, 31), the p80 subunit of Ku (28), and cyclin K\textsuperscript{3+} are potential biomarkers of breast cancer, whereas annexin XI (27), the nucleolar protein interacting with the FHA domain of pK-67 (NIFK; Ref. 34), the KIAA1671 protein (32), elongation factor 2 (37), the ribosomal protein S6 (29), and other known and unknown proteins (Table 6) showed potential in their ability to differentiate patients with DCIS from those with IDC of the breast, suggesting that these autoantigens are candidates as biomarkers for the early detection of breast cancer.

Our previous work (10, 61, 62) and the work of Tan (63)suggested that screening autoantigen expression libraries with cancer patient sera containing high titers of autoantibodies has the potential of revealing a number of proteins that may be involved in cellular functions related to tumorigenesis. Because expressed sequence tags represent a copy of a part of the genome that is being expressed, we expected that their identification by immunoscreening a T7 display library of breast autoantigens would allow us to obtain gene expression data in breast cancer. We speculated that this approach might allow us to clone cognate genes products, perhaps related to the signal transduction mechanism(s) that may be activated in cancer.

The identification of annexin XI (27), cyclin K (33), ribosomal proteins S6 (29, 64, 65) and S12 (30, 31), Grb2-associated protein 2 (39, 40, 66), and elongation factor 2 (37, 67), as autoantigens recognized by breast cancer sera could be the expression of molecular alterations in the signal transduction mechanism in breast cancer. Annexin XI interacts specifically with calcyclin, a member of the S100 subfamily of elongation factor-hand calcium-binding proteins, which are involved in Ca\textsuperscript{2+}-regulated signaling pathways and found in abundance in certain breast cancer cell lines (68, 69). Because calcyclin is overexpressed in tumor cells with the mRNA specifically elevated in the G\textsubscript{1} phase of the cell cycle of stimulated cells, it has been suggested that annexin XI-calcyclin complexes may play a role in cell proliferation and cell division (69). Cyclin K is an RNA polymerase II-associated cyclin involved in transcriptional activity (70, 71). The p80 subunit of the Ku antigen has been reported to be recognized by autoantibodies from some patients with the scleroderma-polymyositis overlap syndrome (28). The heterodimeric Ku protein is the DNA-targeting component of a DNA-dependent protein kinase that plays a critical role in mammalian DNA double-strand breaks repair and has been widely implicated in tumor biology (72, 73). The finding of an autoimmune reaction directed toward the Ku antigen and our previous report of autoantibodies to RPA32 in breast cancer patient sera (10) suggests that the molecular changes leading to autoimmunity of proteins involved in DNA repair may be important in breast carcinogenesis. Grb2 is an adapter protein that binds activated growth factor receptor molecules such as receptor tyrosine kinases and transduces signals leading to the activation of the Ras-mediated signal cascade activated in most cancers (66). Elongation factor 2 is phosphorylated by a calmodulin-dependent protein kinase, CaM K III, which is selectively activated in proliferating cells, and its activity is elevated in human breast cancer (67). Phosphorylation of ribosomal protein S6 is a common effect of mitogenic stimulation of cells (29, 64, 65), and its kinase is thought to be a downstream target of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) pathway (65).

The finding of annexin XI-A reactivity in the sera of 60% of patients with DCIS and in only 11% of those with IDC of the breast, although statistically significant, could possibly be an artifact attributable to the relatively small sample of patients with DCIS. Alternately, we speculate that it could be the reflection of antigenic heterogeneity in breast cancer. Ductal carcinoma in situ seems to be a
heterogeneous group of lesions, and the relation between in situ and invasive carcinomas of the breast is not clear. The study of histological grade and tumor markers has identified heterogeneity in breast cancer (74, 75). The higher reactivity to annexin XI-A phages with sera from patients with DCIS than with those with IDC of the breast as well as the ability of certain autoantibodies to significantly distinguish DCIS from IDC of the breast (Table 6) could be interpreted as an indication that some invasive carcinomas may not go through the DCIS stage characterized by annexin XI-A reactivity. In view of the long life of IgG molecules that are the expression of an established immune response, we would expect to find IgG antibodies in their sera if they were preceded by a DCIS stage characterized by annexin XI-A positivity, even if the gene for annexin XI-A had been presumably turned off in some patients with IDC in their transition from DCIS.

Autoantigens cloned by immunoscreening cDNA expression libraries by breast cancer patient sera are not necessarily related to breast cancer. Results obtained in the study of the non-cancer control sera are of interest to interpret the significance of phage inserts cloned with certain breast cancer patient sera. A number of phage inserts cloned with sera from patients with IDC of the breast, including partial sequences identical to gelsolin (41) and to α-2 macroglobulin (42), were significantly recognized by normal control sera and not by cancer patient sera. In the case of gelsolin, the recognition of this sequence by autoantibodies present in the serum of a patient with breast cancer could falsely be attributed to molecular changes in gelsolin known to occur in breast cancer (76, 77). However, a role for gelsolin has also been suggested in the pathogenesis of Parkinson’s disease and in the Finnish-type familial amyloidosis and related conditions (78, 79), and the pan-proteinase inhibitor sequences identical to gelsolin (41) and to human neutrophil elastase (42) might be related to autoimmunity in the degenerative brain diseases or to other causes. The identification of other phage inserts recognized by normal sera will be of great interest because these antigens are undoubtedly irrelevant for breast cancer but may reflect autoimmune phenomena related to a host of other conditions affecting the general population, may be associated with aging, or may be a part of the normal autoimmune response.

In view of our findings, it is likely that probing this autoantigen microarray prospectively with sera from a large cohort of breast cancer patients may allow the identification of biomarkers with diagnostic significance and perhaps may allow the identification of discrete antigen phenotypes with clinical significance. The high prevalence of IgG autoantibodies in the sera of patients with DCIS and IDC of the breast suggests that they are potentially excellent candidates as biomarkers for the early diagnosis of breast cancer.

ACKNOWLEDGMENTS

We thank Dr. John E Tomkiel for collaboration in the initial phase of this work and Drs. Gloria Heppner, Bonnie Sloane, and Michael Tainsky for helpful comments and advice.

REFERENCES

36. Pai CY, Yeh NH. Cell proliferation-dependent expression of two isoforms of the
sequence and characterization of the 5′-flanking region of mammalian elongation
new protein that specifically interacts with the peripheral benzodiazepine receptor.
 cytokine and growth factor receptors and T- and B-cell antigen receptors. Blood
1999;93:1809–16.
40. Garcia-Echeverria C. Antagonists of the Src homology 2 (SH2) domains of Grb2, Src,
41. Kwiatkowski DJ, Stossel TP, Orkin SH, Mole JE, Colten HR, Yin HL. Plasma and
cytoplasmic gelsolins are encoded by a single gene and contain a duplicated
42. Bell GI, Rall LB, Sanchez-Pescador R, et al. Human alpha 2-macroglobulin gene is
43. Türeci O, Sahan U, Pfreundschuh M. Serological analysis of human tumor antigens:
46. Yeatman TJ, Updyke TV, Kaelzel MA, Dedman JR, Nicolson GL. Expression of
annexins on the surfaces of non-metastatic and metastatic human and rodent cells.
47. Ahn SH, Sawada H, Ro JY, Nicolson GL. Differential expression of annexin I in
human mammary ductal epithelial cells in normal and benign and malignant breast
49. Srivastava M, Bubendorf L, Nolan L, et al. ANX7 as a bio-marker in prostate and
50. Emoto K, Sawada H, Yamada Y, et al. Annexin II overexpression is correlated with
human mammary ductal epithelial cells in normal and benign and malignant breast
52. Brichori FM, Misek DE, Yim AM, et al. An immune response manifested by the
common occurrence of annexins I and II autoantibodies and high circulating levels of
53. Maler L, Sastry M, Chazin WJ. A structural basis for S100 protein specificity derived
55. Emoto K, Sawada H, Yamada Y, et al. Annexin II overexpression is correlated with
57. Brichori FM, Misek DE, Yim AM, et al. An immune response manifested by the
common occurrence of annexins I and II autoantibodies and high circulating levels of
peptide from brain by LDL receptor-related protein-1 at the blood brain barrier. J Clin
59. Fabrizi C, Businaro R, Lauro GA, Fumagalli L. Role of alpha2-macroglobulin in
regulating amyloid beta-protein neurotoxicity: protective or detrimental factor?
Autoantibodies to Annexin XI-A and Other Autoantigens in the Diagnosis of Breast Cancer


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/15/5089

Cited articles

This article cites 72 articles, 31 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/15/5089.full.html#ref-list-1

Citing articles

This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/64/15/5089.full.html#related-urls

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.