Prognostic Impact of ANX7-GTPase in Metastatic and HER2-Negative Breast Cancer Patients

Meera Srivastava,1 Lukas Bubendorf,2 Mark Raffeld,3 Christoph Bucher,2 Jochen Torhorst,2 Guido Sauter,2 Cara Olsen,4 Olli P. Kallioniemi,5 Ofer Eidelberg,1 and Harvey B. Pollard1

1Department of Anatomy, Physiology and Genetics and Institute for Molecular Medicine, Uniformed Services University School of Medicine, Bethesda, Maryland; 2Institute for Pathology, University of Basel, Basel, Switzerland; 3Laboratory of Pathology, Hematopathology Section, National Cancer Institute, NIH, Bethesda, Maryland; 4Preventive Medicine and Biometrics, Uniformed Services University School of Medicine, Bethesda, Maryland; and 5Section on Molecular Genetics, Cancer Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, Maryland

ABSTRACT

Purpose: ANX7-GTPase located on chromosome 10q21 is significantly altered and associated with hormone-refractory metastatic prostate cancers. Therefore, we investigated whether levels of ANX7 correlate with breast cancer progression and survival.

Experimental Design: A diagnostic tumor tissue microarray containing 525 human breast tissue specimens at different stages of the disease was assayed for ANX7 using immunocytochemical methods with ANX7 monoclonal antibody. A separate prognostic tumor tissue microarray containing 553 human breast tissue specimens annotated with clinicopathological parameters was assayed for ANX7, HER2, estrogen receptor, progesterone receptor, and p53 protein.

Results: We report here for the first time that the expression of ANX7-GTPase is significantly enhanced and associated with the presence of metastatic disease \((P < 0.0001)\) in the 525 human breast tissue specimens analyzed. Furthermore, using a separate 553 case retrospective prognostic tumor tissue microarray, we found that increased ANX7 expression is also significantly associated with poor overall patient survival \((P < 0.014)\). This is particularly true when restricted to patients in whom the BRE clinical grade is 2 \((P < 0.001)\) or for whom there is a lack of HER2 expression \((P < 0.002)\). Finally, Cox regression analysis shows that as the expression of ANX7 rises, the probability of survival decreases by more than 10-fold for those patients with HER2-negative tumors. These latter patients represented 66% of the population afflicted with breast cancer in this study.

Conclusions: High levels of ANX7 in tumor correlate strongly with poor survival of HER2-negative patients and the most aggressive forms of breast cancer. This is the first study to demonstrate that ANX7 antibody has the potential for development into an in vivo diagnostic and therapeutic tool. This simple and reliable immunohistochemical assay may therefore become an important biomarker for metastatic breast cancer diagnosis and management of HER2-negative breast tumor patients.

INTRODUCTION

Breast cancer is the most common malignancy among women in developed countries, and there is considerable need for reliable prognostic markers to assist clinicians in making diagnostic and therapeutic management decisions. A number of biological factors have been used to define risk categories in breast cancer. We have focused on the role of ANX7-GTPase in breast cancer progression and survival because altered expression of this gene is associated with metastatic and hormone-refractory prostate cancer in man and with a tumor-prone phenotype in the Anx7\(+/-\) mouse model \((1–3)\). The ANX7 gene product is a Ca\(^{2+}\)-activated GTPase \((4)\) and protein kinase C substrate \((5, 6)\), which shares with other members of the annexin gene family the ability to bind to acidic phospholipids in a Ca\(^{2+}\)-dependent manner \((7)\). ANX7 also forms classical voltage-gated Ca\(^{2+}\) channels in cellular and artificial membranes \((8)\). The role of calcium in many important cell processes is well appreciated, although the involvement of calcium-binding proteins in the annexin gene family for cancer remains somewhat novel \((2, 3, 9–11)\).

Recent studies from our laboratory indicate that altered expression of ANX7 is associated with metastatic and hormone-refractory prostate cancer. We have therefore hypothesized that ANX7 signaling might play a fundamental role in breast cancer occurrence and progression. To test this hypothesis, we have used breast tissue microarrays containing approximately 1078 biopsy specimens to ask whether the levels of expression of ANX7 might have predictive value for diagnosis and survival of these patients. We report here that increased ANX7 expression is associated with the presence of metastatic disease. As the expression of ANX7 rises, the probability of survival decreases by more than 10-fold for those patients with tumors that lack HER2 immunostaining. The latter population represents 66% of breast cancer-affected patients in this series. These findings suggest that overexpression of ANX7 can be used as a risk biomarker that may prove to be useful in making aggressive treatment decisions for breast cancer patients.
MATERIALS AND METHODS

Study Population. The first set of diagnostic progression breast cancer tissue microarray contained samples from 525 breast tissues. The patient group consisted of 107 patients with primary breast cancers, 23 patients with ductal carcinoma in situ, 343 patients with metastatic invasive ductal carcinoma, and 52 patients with metastatic invasive lobular carcinoma. The second prognostic breast tissue microarray contained carcinomas of 553 breast cancer patients for whom follow-up data (tumor-specific survival and treatment information) could be evaluated retrospectively. These patients had a median age of 61 years (range, 33–97 years). They were treated for primary breast cancer at the University Hospital of Basel (Basel, Switzerland), Women’s Hospital (Rheinfelden, Germany), and the Kreiskrankenhaus (Lörrach, Germany) between 1985 and 1994. The median potential follow-up time was 63.0 months (range, 1–151 months). Formalin-fixed, paraffin-embedded tumor material from both arrays was available from the Institute of Pathology, University of Basel. The pathological stage, tumor diameter, and nodal status were obtained from the primary pathology reports. All slides from all tumors were reviewed by one pathologist (J.T.) to define the histological grade according to Elston and Ellis (BRE). A systemic therapy after surgery had been performed for 273 patients represented on the prognostic tissue microarray (TMA), including 172 patients with hormonal therapy alone, 52 patients with cytotoxic therapy alone, and 49 patients with both hormonal and cytotoxic treatment. The progression TMA included 405 ductal cancers, 77 lobular cancers, 17 medullary cancers, 14 mucinous cancers, 11 cribriform cancers, 11 tubular cancers, 7 papillary cancers, 4 apocrine cancers, 3 clear cell cancers, 1 metastastic cancer, 1 atypical medullar cancer, 1 large cell cancer, 1 small cell cancer, and 1 neuroendocrine cancer. Among 553 tumors, 27.8% were grade 1, 42.9% were grade 2, and 29.3% were grade 3. The pT stage was pT1 in 39.5% of patients, pT2 in 46.3% of patients, and nodal status was obtained from the primary pathology reports. The stage could not be determined unequivocally from the pathology reports in six patients, and pT4 in 9.3% of patients. The stage could not be determined unequivocally from the pathology reports in six patients, and pT4 in 9.3% of patients. The stage could not be determined unequivocally from the pathology reports in six patients, and pT4 in 9.3% of patients. The stage could not be determined unequivocally from the pathology reports in six patients, and pT4 in 9.3% of patients. The stage could not be determined unequivocally from the pathology reports in six patients, and pT4 in 9.3% of patients.

Immunohistochemistry. Tumor samples were arrayed as described previously (12). Briefly, H&E-stained sections were made from each selected primary tumor block (donor blocks) to define representative tumor regions. Tissue cylinders with a diameter of 0.6 mm were then punched from each donor block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD) and brought into a recipient paraffin block eventually containing either 525 or 553 individual samples. Four-μm sections of the recipient blocks were then cut using an adhesive-coated slide system (Instrumedics Inc.) supporting the cohesion of the 0.6-mm array elements on glass. One section from each of the four replica arrays was used for immunohistochemical analysis, as described previously (13).

The guidelines from the package insert were followed for each antibody. Standard indirect immunoperoxidase procedures (ABC-Elite; Vector Laboratories) in combination with monoclonal antibodies were used for detection of ANX7 (1:1000; DAKO), HER2 (Hercep test; DAKO) p53 (DO-7; prediluted; DAKO), estrogen receptor [ER (ER ID5; 1:1000; DAKO)], and progesterone receptor [PR (NCL-PGR; 1A6; 1:600; Novoceastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom; Ref. 13)]. Tumors with known positivity were used as positive controls. The primary antibody was omitted for negative controls. These arrays have previously been tested for lack of interaction with irrelevant monoclonal antibodies. Scoring of the immunohistochemical staining followed the guidelines in the package insert using an objective at ×10 magnification. The ANX7 levels were classified as 0 (no staining), 1 (low staining), 2 (moderate staining), and 3 (highest staining intensity). Tumors were considered positive for ANX7 if an unequivocal nuclear or cytoplasmic positivity was seen in at least 10% of tumor cells. Immunohistochemical scoring of p53, ER, and PR was done as described previously (13). The ANX7 monoclonal antibody has been shown to recognize ANX7 specifically and has proved to be a useful reagent for immunohistochemical studies (2). The staining is both nuclear and cytoplasmic, as expected for a protein localized to the nucleus and cytoplasm. The specificity of tissue staining was determined by the demonstration of negative staining either by omitting primary antibody or by using an irrelevant antibody.

Western Blotting. The nonmetastatic cell line B231lys and the metastatic cell line B435lys were obtained from Amer-

![Fig 1](clinicalcancerres.aacrjournals.org) Disease progression relative to immunological ANX7 expression (525 patients). The percentage of ANX7-positive samples is plotted against different stages of breast cancer starting from primary breast cancer (107 specimens), ductal carcinoma in situ (DCIS; 23 specimens), metastatic ductal invasive carcinoma (343 specimens), and metastatic lobular invasive carcinoma (52 specimens). ANX7 positivity increases with disease progression.
ican Type Culture Collection and grown according to the manufacturer’s instructions. For protein extraction, cells were lysed in buffer consisting of 0.5% 2 M Tris, 3% 5 M NaCl, 1% 500 mM EDTA, 1% Triton, 10% glycerol, and 2 mM vanadate. Cells were left on ice for 5 min to allow cell lysis to reach completion, at which point the released material was spun down to remove cell debris (5 min at 13,000 rpm). The supernatant was separated through a 10% SDS gel. Proteins were then transferred to nitrocellulose paper. Western blot analysis was performed as described by Caohuy and Pollard (5, 6). Briefly, the blot was blocked in a solution of milk (5% milk in PBS with 1% BSA) for 1 h. After overnight exposure to the ANX7 primary monoclonal antibody (1:1000; DAKO), the blot was washed four times in PBS/Tween 20 (0.1%; Sigma). The blot was exposed to

**Fig. 2** Immunohistochemistry on tumor tissue microarray. Analysis of ANX7 protein in representative clinical specimens of metastatic (A) and nonmetastatic (B) breast carcinomas (×100). Intense cytoplasmic staining is observed in metastatic specimens compared with very weak staining in nonmetastatic specimens.

**Fig. 3** Western blot analysis of metastatic (B435lys) and nonmetastatic (B231lys) breast tumor cell lines. The cell extract was resolved by 10% SDS-PAGE as described in “Materials and Methods.” ANX7 expression is visualized using monoclonal anti-ANX7 antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control and probed with anti-glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 4** Kaplan-Meier survival curve for patients subdivided on the basis of ANX7 expression (553 patients). The patients whose tumors had very high ANX7 expression had significantly shorter survival than patients whose tumors had very weak ANX7 expression (P = 0.014). The 5-year survival is 65% for group 3, 76% for groups 2 and 1, and 95% for group 0.
cytoplasmic ANX7 expression is systematically increased in patients with metastatic disease (Fig. 1). For example, in primary breast cancers, the proportion of ANX7 positives is 20%. However, the fraction of tumors with increased ANX7 expression is 60% and 80%, respectively, for lymph node metastases associated with invasive ductal and lobular metastatic breast cancers. Metastases differ from primary carcinomas in a statistically significant manner (P < 0.0001) using the χ² test. Immunohistochemical analysis of breast tumor tissue arrays re-

### RESULTS

**High Cytoplasmic ANX7 Expression Is Associated with Metastatic Phenotype.** To investigate whether there is a relationship between ANX7 expression and disease progression in patients with breast cancer, we tested 525 breast specimens from human primary breast cancers and axillary lymph node metastases as well as normal human breast tissues. We found that...
Role of ANX7-GTPase in Breast Cancer Progression

expression by immunohistochemistry. The presence of ANX7 in metastatic tumor specimens, whereas very low ANX7 staining is observed in nonmetastatic tumors. Representative sections of metastatic and nonmetastatic tissue microarray sections are shown in Fig. 2, A and B.

To test whether the high expression levels of ANX7 in metastatic cells could be generalized to in vitro conditions, we examined ANX7 protein expression in relevant cell lines. As shown in Fig. 3, ANX7 levels are very low when assayed by Western analysis in the asynchronously growing human nonmetastatic breast cancer cell line B231lys. In contrast, in the metastatic cell line B435lys, strong cytoplasmic staining correlates with high levels of ANX7 protein (Fig. 3). Thus, the weak immunohistochemical reaction for ANX7 in nonmetastatic cells and tumors appears to represent a truly low level of ANX7 protein that has in vitro parallels. These results therefore indicate that high ANX7 expression is associated with the most aggressive types of breast cancer.

Prognostic Impact of ANX7 Expression. To evaluate the prognostic significance of ANX7, we have used a tissue microarray (12) containing 552 breast tumor tissue specimens. Each sample is accompanied by clinical follow-up data for up to 105 months. The samples on the array were evaluated for ANX7 expression by immunohistochemistry. The presence of ANX7 in each of these patient specimens was correlated with patient survival parameters. Four types of ANX7 expression can be discriminated in breast cancer specimens. These groups are designated “0” for negative or very low ANX7 expression, “1” for weak ANX7 expression, “2” for moderate ANX7 expression, and “3” for strong ANX7 expression. As shown in Fig. 4, Kaplan-Meier curves of univariate cumulative survival in patients with low (group 0) versus high (group 3) cytoplasmic ANX7 expression show a significant separation within 5 years of follow-up. The 5-year survival is 65% for group 3 (strong ANX7 expression) and 76% for groups 2 and 1 (moderate ANX7 expression). For group 0 (negative or very low ANX7 expression), survival is up to 95% (P = 0.014, log-rank test; Breslow-Gehan-Wilcoxon test; Tarone-Ware; Peto-Peto-Wilcoxon and Harrington-Fleming).

Table 2. Multivariate analyses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range or category</th>
<th>Added risk</th>
<th>95% Confidence interval for added risk</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td>BRE grade</td>
<td>1–3</td>
<td>1.901</td>
<td>(1.356–2.666)</td>
<td>0.0002</td>
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<td>pN</td>
<td>1 &amp; 2 vs. 0</td>
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<td>(2.455–6.834)</td>
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<td>pT</td>
<td>4 vs. 1, 2, &amp; 3</td>
<td>2.562</td>
<td>(1.577–4.160)</td>
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<tr>
<td>HER2</td>
<td>0–3</td>
<td>1.316</td>
<td>(1.089–1.590)</td>
<td>0.004</td>
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<tr>
<td>PR</td>
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<td>1.678</td>
<td>(0.994–2.833)</td>
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<tr>
<td>ER</td>
<td>Pos. vs. Neg.</td>
<td>1.205</td>
<td>(0.684–2.121)</td>
<td>0.519</td>
</tr>
<tr>
<td>p53</td>
<td>Pos. vs. Neg.</td>
<td>1.437</td>
<td>(0.934–2.212)</td>
<td>0.099</td>
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<tr>
<td>ANX7</td>
<td>0–3</td>
<td>1.306</td>
<td>(0.969–1.762)</td>
<td>0.080</td>
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Cytoplasmic ANX7 Expression Is Associated with BRE-2 Grade and HER2-Negative Patients. Parallel sections of the same specimens were investigated for alteration in the expression of ER, PR, p53, and HER2 proteins (13). ANX7 was negative or weakly positive in normal glands adjacent to the cancer on individual locations in this tissue microarray and benign glands that were occasionally present adjacent to the cancer tissue. In a different study on a separate human tumor tissue microarray, we were able to analyze ANX7 expression in four normal glands. We

Table 2. Multivariate analyses

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Added risk</th>
<th>95% Confidence interval for added risk</th>
<th>Significance</th>
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<td>pT</td>
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<td>HER2</td>
<td>0–3</td>
<td>2.061</td>
<td>(1.174–3.620)</td>
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<tr>
<td>PR</td>
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<td>(1.272–4.065)</td>
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<td>ER</td>
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<tr>
<td>ANX7</td>
<td>0–3</td>
<td>1.590</td>
<td>(1.109–2.281)</td>
<td>0.012</td>
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C. Cox regression using only HER2 = 0 cases (74.8% of the cases)

<table>
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<th>Parameter</th>
<th>Range or category</th>
<th>Added risk</th>
<th>95% Confidence interval for added risk</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
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<td>BRE grade</td>
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<td>(1.400–3.571)</td>
<td>0.001</td>
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<tr>
<td>pN</td>
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<td>(2.400–9.905)</td>
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<td>2.849</td>
<td>(1.488–5.456)</td>
<td>0.002</td>
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<tr>
<td>PR</td>
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<td>(0.793–3.120)</td>
<td>0.195</td>
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<tr>
<td>ER</td>
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<td>(0.565–2.780)</td>
<td>0.579</td>
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<tr>
<td>p53</td>
<td>Pos. vs. Neg.</td>
<td>1.379</td>
<td>(0.778–2.431)</td>
<td>0.278</td>
</tr>
<tr>
<td>ANX7</td>
<td>0–3</td>
<td>1.996</td>
<td>(1.306–3.049)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

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found negative or, at best, weakly positive cyto-immunolabeling for ANX7 (data not shown). Table 1 describes the composite univariate analysis of all 553 patients in terms of classical clinicopathological risk factors, including nodal status, tumor grade, and stage. We include in Table 1 the known prognostic factors such as p53, HER2, ER, and PR. Also shown in this table are the unadjusted $P$s for the log-rank test of homogeneity of strata (shown separately for each variable evaluated), as well as the strata compared by each test. Based on the analysis of all of the parameters, it is evident that high cytoplasmic ANX7 expression has a significant and specific impact on the probability of survival for patients with BRE-2 grade tumors (Fig. 5A; $P = 0.001$) or for patients whose tumors do not express HER2 (Fig. 5B; $P = 0.002$). For example, in the BRE-2 patient cohort, 100% of the patients with very low ANX7 expression survived. By contrast, only 52% of those with strong ANX7 expression survived.

**Multivariate Analysis Showing ANX7 as a Risk Biomarker for HER2-Negative Patients.** We have performed multivariate analyses (Cox regression, Table 2) on the data to determine the significance and independence of the ANX7 immunoassay data in predicting the outcome and progression of breast cancer. All of the tissues were from resected breast cancers without any preceding therapy that could have confounded the results. At the time of sample collection, neoadjuvant therapy of breast cancer was not performed at University Hospital of Basel. We used traditional variables in the multivariate analyses including the tumor stage ($pT$), nodal status, $pN$, and BRE grade as assessed from the medical records of the donor patients. In addition, we also added immunohistochemical evaluations for HER2, PR, ER, p53, and ANX7, which we carried out on the identical tissue microarray samples. As shown in Table 2A, these analyses show that for the entire cohort, the level of ANX7 has a marginally significant value ($P = 0.08$; added risk $= 1.3$; 95% confidence interval, 0.9–1.8) as a prognostic indicator. However, when we looked only at the subpopulation of patients with low HER2 levels (HER2 $= 0$ or 1, Table 2B), which comprises 87.1% of the cohort, we found that the level of ANX7 expression has a definitely significant prognostic value even after considering the effects of all of the other variables in the equation. Specifically, the ANX7 level is associated with an increased risk of 1.6 (95% confidence interval, 1.1–2.3) and has a significance of $P = 0.012$ as a prognostic marker. This finding is even more pronounced with a significance of $P = 0.001$ when we look only at the HER2 $= 0$ patients (about 75% of the cohort; Table 2C), for whom the risk is doubled for each successive step of ANX7 level (Fig. 6). This increased risk with ANX7 in the HER2-negative cohort is comparable with the increased risk associated with BRE grade in the entire cohort population. These results indicate that ANX7 levels have considerable potential for early detection of breast tumors, giving patients and physicians a new tool for managing breast cancer.

**DISCUSSION**

The results obtained from 1077 breast tissue specimens show that increased ANX7 expression is associated with metastatic disease and significantly decreased survival in those breast cancer patients who present with BRE-2 grade tumors or tumors lacking detectable HER2 expression. Cox regression analysis reveals that even after adjusting for ER, PR, p53, $pT$, $pN$, and BRE grade, HER2-negative patients suffer a doubling in the risk of death with each increasing level of ANX7 expression. Remarkably, in HER2-negative patients, the difference in risk is 10-fold between those with negative ANX7 expression and those with strong ANX7 expression. The clinical treatment of primary breast cancers has been greatly complicated by the inability to accurately predict which tumors will eventually become invasive and metastatic, and which will become localized and indolent. Strong expression of HER2 in 20–35% of the breast cancer patients is known to be associated with poor prognosis and has been used to predict response to treatment with the anti-HER2 antibody trastuzumab (Herceptin). Our data therefore suggest that the expression level of ANX7 can help to stratify the remaining HER2-negative patients who need the most focused attention. At a minimum, the value of our result for breast carcinomas without HER2 expression is that the ANX7 gene assay might provide a simple and reliable survival parameter for clinicians to include in patient management plans for early detection and treatment options.

To our knowledge, this is the first report on ANX7 protein expression in a large series of patients with breast cancer.
Importantly, we have found ANX7 positivity in 90% of patients with invasive breast cancer. This study also brings to our attention the value of the new technology of tumor tissue microarrays for analysis of the molecular characteristics of tumors (12–16). Simultaneously, we have been able to access the tissue of nearly 1000 breast cancer patients on only a few slides and to associate each tumor with its cognate clinical history. Biostatistics and bioinformatics with this massive database are thus combined to compose an analysis with sufficient power to make statistically valid conclusions about the newly described significant role of ANX7 in aggressive forms of cancer.

Based on the present data, we suggest that this new knowledge has the potential to operationally simplify prognosis for a sizeable fraction of the breast cancer population. For those patients identified as being at particular risk, physicians can be alerted to the necessity of aggressive treatment. Should these data be validated in a larger population of patients and in prospective studies with extensive follow-up, measurement of ANX7 expression could become an important early detection biomarker for high-risk HER2-negative patients, who make up the majority of the patient population. We conclude that ANX7 expression is profoundly worthy of further exploration as a prognostic factor for patient management.

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