Serum autoantibody signature of ductal carcinoma in situ progression to invasive breast cancer

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ABSTRACT

**Purpose:** The identification of markers associated with progression to invasive breast cancer (IBC) is a major factor that can guide physicians in the initial therapeutic decision and the management of ductal carcinoma *in situ* (DCIS).

**Experimental design:** We examined autoantibody targets in 20 DCIS and 20 IBC patients using protein microarrays and identified humoral responses that can be used to distinguish the two groups. The five most differentially targeted antigens were selected to generate an autoantibody signature for the *in situ* to invasive breast cancer transition. This signature was next tested on 120 independent samples (61 DCIS and 59 IBC) using specific ELISA assays. The prognosis value of the autoantibody signature was finally evaluated in a cohort of DCIS patients followed for 5 years.

**Results:** A set of five autoantibody targets (RBP-Jκ, HMGN1, PSRC1, CIRBP, and ECHDC1) with the highest differential signal intensity found in the protein microarrays experiment was used to establish an autoantibody signature of the DCIS to IBC transition. Using ELISA, this signature significantly discriminated DCIS from IBC (AUC = 0.794 (95% CI [0.674; 0.877])). Interestingly, our panel could highly distinguish low-grade DCIS from high-grade DCIS exhibiting an AUC of 0.749 (95% CI [0.581; 0.866]). Finally, using a Kaplan-Meier analysis, the autoantibody signature could significantly divide the DCIS patients into a poor prognosis group and a good prognosis group (*P* = 0.01).

**Conclusion:** These results indicate the potential of autoantibody detection as a new prognostic test with possible clinical implications for the management of DCIS.
STATEMENT OF TRANSLATIONAL RELEVANCE

While protein microarrays have been successfully used for the identification of autoantibodies in the serum of cancer patients, especially in early-stage cancer detection, the use of protein microarrays to identify autoantibodies correlated with invasion has not been reported. This study shows the clinical relevance of a combination of five antigens, identified by protein microarrays and validated in an independent cohort of 120 samples by ELISA, as a blood-screening signature for the in situ to invasive breast cancer transition determination. Given the noninvasive characteristics of serum sampling and the easy detection of autoantibodies, autoantibody signature may help to avoid the overtreatment of low-risk patients and dictate more intensive treatment of aggressive DCIS, thereby reducing the mortality of patients diagnosed at this very early step of the disease.
INTRODUCTION

In contrast to the dramatic improvement in our ability to detect ductal carcinoma in situ (DCIS), our understanding of the pathophysiology of this disease and the factors involved in its progression to invasive breast carcinoma (IBC) is limited (1). Clinico-histological features, such as a younger age, positive surgical margins, tumor size, comedo necrosis and, notably, tumor grade, have consistently been correlated with DCIS recurrence. However, none of these parameters can accurately predict the risk of progression from DCIS to IBC (2). Tissue markers, including estrogen receptor, progesterone receptor, HER2/neu, Ki67, and COX-2 expression, as well as comprehensive molecular profiling of gene expression have also been evaluated as prognostic indicators of progression, but the results remain controversial (3). The serum markers currently used in clinical settings, including ACE and CA15-3, are mostly used to monitor treatment effectiveness in patients with metastatic breast cancer (4). Finally, as there are no available biomarkers that can effectively distinguish aggressive DCIS from indolent DCIS, selecting an appropriate initial treatment plan can be a challenge for physicians.

The presence of a humoral response against a number of intracellular and surface tumor-associated antigens (TAAs) has been clearly established in cancer patients (5). Because this response appears months to years prior to the clinical diagnosis of the tumor, most of the current emphasis has focused on the use of tumor-associated autoantibodies as relevant diagnostic biomarkers with very promising results (6). However, autoantibody detection could also be an effective strategy for the early identification of highly aggressive lesions, thereby improving cancer outcomes. Few studies have focused on the prognostic significance of serum autoantibodies in metastatic cancer, in particular in non-small cell lung cancer patients (7), but none have investigated early-stage cancer. Therefore, we hypothesized that
autoantibody detection could provide an effective approach to identify DCIS patients with a high risk of progression to IBC.

Recent advances in proteomics have unlocked novel avenues for the discovery of cancer-related biomarkers. Protein microarrays, such as ProtoArrays, which contain more than 8,000 non-redundant proteins translated from genes randomly selected from throughout the human genome, are a powerful screening method for the identification of candidate autoantigens (8). The feasibility of this approach has been demonstrated by the determination of diagnostic autoantibody profiles in a limited number of cancers, including colorectal, ovarian, and pancreatic cancer (9-11).

In this study, we describe significant and consistent differences in the level of autoantibodies targeting specific antigens in a population set of 40 patients with DCIS or early-stage IBC, as detected using protein microarrays. We then evaluated the performance of a humoral signature–based predictor in an additional independent set of 120 newly diagnosed patients using antigen-specific ELISAs and both multivariate mROC and Support Vector Machine (SVM) classification algorithms. Finally, we determined if this signature could be used to identify patients with local recurrences in a cohort of DCIS patients followed for 5 years. Taken together, our results suggest that examining the humoral response to preinvasive lesions can identify potential markers that accurately detect DCIS patients at high risk for subsequent local recurrence.

PATIENTS AND METHODS

Serum samples. All human samples were prospectively collected between 2005 and 2007 at the CRLC Val d’Aurelle Cancer Institute, Montpellier, France, at the time of cancer diagnosis after obtaining written informed consent. Blood samples were centrifuged at 1250 × g for 5 min, and the serum was stored at -80 °C. For the protein microarray experiments, a first set
(set 1, TAAs identification set) of 40 serum samples was examined. For the ELISA experiments, a second set (set 2, TAAs evaluation set) of 120 age-matched serum patients was examined. No DCIS patients received neo-adjuvant therapy; all patients had surgery within 4 weeks of the initial diagnosis of breast cancer, and select cases received whole-breast radiotherapy. The median follow-up time for patients with DCIS was 56 months (range, 44-74 months). This study was approved by the Montpellier University Hospital human research committee and the INSERM review board (RBM-03-63).

For immunohistochemistry experiments, independent tissue samples were used. All tissue samples (n=20) had both DCIS and IBC components. Samples were obtained after written informed consent at the Arnaud de Villeneuve University hospital center, Montpellier, at the time of cancer diagnosis.

**Protein microarray experiments.** The ProtoArray microarrays (v4.0; Invitrogen) were used according to the manufacturer’s instructions. Each microarray contains 8,000 separate proteins printed in duplicate. Briefly, microarrays were blocked for 1 h at 4 °C with 50 mM HEPES, 200 mM NaCl, 0.08% Triton X-100, 25% glycerol, 20 mM reduced glutathione, 1 mM DTT, 40 mM NaOH and 1% BSA at pH 7.5. The microarrays were then incubated for 90 min at 4 °C with the patient serum samples diluted 1:500 in 1× PBS, 1% BSA and 0.1% Tween at pH 7.5 (PBST-1% BSA). The microarrays were washed five times for 5 min with PBST-1% BSA and incubated for 90 min at 4 °C with goat AlexaFluor 647-conjugated anti-human IgG diluted 1:2000 (Invitrogen). The microarrays were washed again as described above, rinsed briefly in deionized water and dried by centrifugation. Microarray data were acquired using an Innoscan700 Fluorescent MicroArray scanner (Innopsys). Mapix software (v2.8.2; Innopsys) was used to grid the images of the scanned arrays and to automatically align images for spot detection and protein identification. Pixel intensities for each spot on the...
microarray were determined from the software and saved to a text and gpr (genepix) file. The quantitated spot files were processed to determine which proteins were recognized by the serum samples.

**Data analysis.** The goal of the microarray analysis was to identify specific antibodies that can be used to differentiate between DCIS and IBC. For each protein on the microarray, a Z-Score was calculated on the basis of the signal from the protein spots on the microarray compared with all or local protein features. Because the Z-Score, which is calculated from the global mean of all of the proteins signals, produces a bias that favors the selection of proteins with high protein amount values, we narrowed the mean, SD and Z-Score calculation to a local value. To reduce the influence of a few highly reactive spots on the local mean, we set the range of calculations to 2,000 values (growing order values of protein amount). For each spot value, a local mean and a local SD were calculated using 2,000 neighboring spots. The local Z-Score was calculated as (net signal value – local mean) / local SD. For each protein duplicate, the average Z-Score and the CV from the net signal values of the duplicates were calculated. The background cut-off value was also calculated by adding the local mean and three times the local SD. However, even with these parameters, the influence of some high values on the local cut-off calculation was strong (Supplementary data 1). To overcome this issue, we considered values over these local cut-off outliers, and we calculated a second pass without considering these values for the local mean calculation. This calculation allowed the generation of a cut-off curve that better fit the distribution of the values (Supplementary data 1). The following criteria were used to select a target for further analysis: a local Z-Score difference greater than a twofold relative to the DCIS group, a CV between duplicate values less than 0.5, and \( P<0.05 \).
Autoantibody detection. Autoantibodies targeting RBP-Jκ, HMGN1, PSRC1, ECHDC1, and CIRBP were detected as previously described, with minor modifications (12). Briefly, MaxiSorp High Protein-Binding plates (Nunc) were coated with 50 ng of recombinant protein (RBP-Jκ, #H00003516; HMGN1, #H00003150; PSRC1, #H00084722; CIRBP, #H00001153; and ECHDC1, #H00055862 from Abnova Corporation) in 100 µl PBS at 4 °C overnight. The plates were washed twice with PBST and blocked with 50 mM HEPES, 200 mM NaCl, 0.08% Triton X-100, 25% glycerol, 1 mM DTT, 40 mM NaOH, and 1% BSA at pH 7.5 for 2 h at RT. The plates were then washed twice and incubated with the serum samples (diluted 1:50) for 2 h at RT. Appropriate control antibodies (goat anti-RBP-Jκ, SC-8213 from Santa Cruz Biotechnology; rabbit anti-HMGN1, #A302-363A from Bethyl Laboratories; mouse anti-PSRC1, #H00084722-B01 from Abnova Corporation; rabbit anti-ECHDC1, AV48841 from Sigma Aldrich; and rabbit anti-CIRBP, 10209-2-AP from Proteintech Group) were also included. After four washes, the plates were incubated with a horseradish peroxidase–conjugated polyclonal antibody specific for human IgG (Jackson ImmunoResearch) for 1 h at RT with agitation. After four washes, the plates were incubated with TMB substrate solution (Stressgen) for 15 min, and absorbance values were determined at 450 nm after addition of H₂SO₄ to stop the reaction. Each serum sample was assayed in triplicate.

Immunohistochemistry (IHC) staining. Tissue sections were treated for 10 min with 10% H₂O₂ to inhibit endogenous peroxidase activity and then incubated in antigen retrieval solution (1 mM EDTA, pH 8.0) for 45 min at 95 °C. The Vectastain ABC Kit (Vector laboratories) was used for blocking and detection, as recommended by the manufacturer. The sections were incubated in diluted normal serum for 20 min to block nonspecific binding and then in primary antibody overnight at 4 °C. The sections were then incubated with a biotinylated secondary antibody for 30 min at RT and then with the peroxidase-conjugated
biotin-avidin complex (Vectastain ABC Kit) for 30 min. Finally, peroxidase activity was detected using the AEC Staining Kit (Sigma-Aldrich). The sections were then counterstained with hematoxylin, except for RBP-Jκ slides where the nuclei were stained using Hoechst 33258 (Sigma-Aldrich). Scores were obtained by estimating average signal intensity (scale of 0–3) and the proportion of cells showing a positive signal (0–100%). The intensity and proportion scores were then multiplied to give an overall IHC score.

**Statistical analysis.** Data regarding the different seroreactivities were summarized by median and range and compared using the Wilcoxon test. Differences were considered statistically significant when $P<0.05$. Individual and combined autoantibody performance from the ELISA data are based on ROC curves, which allow the characterization of the discrimination between two well-defined populations. The generalized ROC criterion (13) finds the best linear combination (virtual marker) of tumor markers such that the AUC is maximized. The accuracy, sensitivity and specificity for individual and combined antibody performance were evaluated using the optimal threshold value calculated to maximize the Youden’s index. The performance of the five autoantibodies–based predictor was also analyzed using SVM analysis. To determine how accurately the learning algorithm was able to predict data, a leave-one-out cross validation was used.

Relapse-free survival (RFS) was calculated from the date of inclusion until local recurrence or last follow-up. Local recurrence was defined as a locoregional relapse of DCIS and/or the presence of IBC. Non-breast cancer deaths were considered as non-events. Patients who were alive without relapse or died without relapse were censored at the time of last contact or the time of death, respectively. Patients who were alive at the last follow-up were censored at the time of last contact. The Kaplan-Meier method was used to estimate RFS.
rates. The log-rank test was used to test the differences between groups. Differences were considered statistically significant at $P<0.05$.

Statistical analyses were performed using InStat (v3.06, GraphPad), STATA 11.0 (StataCorp. 2009. Stata: Release 11) and mROC (14). SVM was implemented using MultiExperiment Viewer (Mev, version 4.6.2).

RESULTS

Protein microarray–based detection of autoantibodies in the DCIS and early-stage IBC populations. To identify the targets of autoantibodies present in the serum of patients with breast cancer, serum samples from 40 age-matched breast cancer patients (DCIS, $n=20$, and early-stage IBC, $n=20$) were profiled using protein microarrays containing 8,000 human recombinant proteins spotted in duplicate. The clinico-pathological characteristics of patients of serum set 1 (TAAs identification set) are shown in supplementary data 2. All serum samples were tested as described in the Material and Methods, and the presence of autoantibodies bound to each protein spot was detected using fluorophore-conjugated anti-human IgG (Figure 1A). As expected, all controls (AlexaFluor 647-conjugated antibody, anti-human IgG antibody and human IgG) were highly reactive for all of the tested samples (Figure 1A). Of the 8,000 proteins present on the microarray, 1,896 proteins were recognized by autoantibodies present in the serum. Of these, 480 reacted only with serum samples from DCIS patients, and 730 reacted only with serum samples from IBC patients. Using our selection criteria (a differential expression of at least two-fold relative to the DCIS samples and $P<0.05$), 20 antigens elicited a significant different humoral response. The complete list of proteins defined by these criteria is shown in supplementary data 3, and the distribution and strength of the autoantibody seroreactivities that differentiate non-invasive breast cancer from
invasive breast cancer are shown in Figure 1B. On the basis of this initial microarray experiment, a set of five autoantibody targets with the highest differential signal intensity between the two groups was used to establish an autoantibody signature for the DCIS to IBC transition (supplementary data 3). The serum levels of the antibodies targeting RBP-Jκ, HMGN1, and PSRC1 were significantly increased in early-stage IBC relative to DCIS, whereas the levels of antibodies targeting CIRBP and ECHDC1 were significantly decreased (Figure 2A). The serum level of the five proteins was also quantified in the same population using ELISA. The results were similar with those obtained by microarray, and there were significant differences between DCIS and IBC groups for the five autoantibodies (Figure 2B).

Expression of RBP-Jκ, HMGN1, PSRC, CIRBP, and ECHDC1 in breast cancer tissue.

Because the presence of specific autoantibodies is often associated with overexpression of the corresponding autoantigens, we examined the expression levels of RBP-Jκ, HMGN1, PSRC, CIRBP, and ECHDC1 in breast tissue. Immunohistochemistry was performed on 20 tissue samples with normal, DCIS and early-stage IBC components. For each autoantigen, the same patients were used, thereby allowing a direct comparison of the results for each protein. While the expression of HMGN1, PSRC, CIRBP, and ECHDC1 did not differ between the groups, RBP-Jκ, which was predominantly localized to the nucleus, was increased in the DCIS and IBC samples (score 2-3) compared with normal breast epithelium (score 0-1) (Figure 3).

Evaluation of an autoantibody signature for the DCIS to early-stage IBC transition. We next quantified the average levels of the autoantibodies targeting RBP-Jκ, HMGN1, PSRC1, CIRBP, and ECHDC1 using ELISAs in 120 independent serum samples (DCIS, n=59; and IBC, n=61). The clinico-pathological characteristics of cancer patients of serum set 2 (TAAs evaluation set) are shown in supplementary data 2. We could not found significant difference
in optical density signal for individual autoantibodies between DCIS and IBC populations (Figure 2C). However, when the discrimination between the DCIS and IBC populations was maximized, for each autoantibody target, by calculating the optimal threshold from receiver operating characteristic (ROC) curves using mROC multivariate analysis, individual autoantibody overall accuracies ranged from 53% to 60%, with individual sensitivities from 17% to 81%, and individual specificity from 32% to 93% (Supplementary Data 4). To assess the ability of the combined antibody measurements to differentiate between preinvasive and invasive tumors, the associations of two or more antigens were randomly permuted, the corresponding area under the ROC curves (AUC) was estimated, and the best linear combination was determined. The best antigen combination for classification performance included all five proteins with an overall AUC of 0.794 (95% CI [0.674; 0.877]) (Figure 4A). When the optimal threshold was defined for this combination, the overall classification accuracy of the autoantibody signature was 80.0%, with an overall sensitivity of 86.1% and an overall specificity of 75.0%, which are greatly improved compared to the performance of the individual autoantibodies (Table 1). We then used a SVM classification algorithm to confirm the robustness of our predictor. Interestingly, the overall classification accuracy was maintained at 77.5%, with an overall sensitivity of 83.3% and an overall specificity of 72.7% (Table 1). To further validate our results, a leave-one-out cross-validation algorithm was used with the same data used above. Interestingly, the accuracies were similar to the values calculated by the mROC criterion or the SVM algorithm, proving the robustness of the predictor (76.2 and 73.7, respectively).

**Relationship between the autoantibody signature and the clinico-histological features of DCIS progression.** We first analyzed the correlation between both the five autoantibodies and the clinical, histological, and biological characteristics of both groups. We did not find a
correlation with any of the parameters except for the histotype grade of DCIS. Indeed, a significant correlation between the histotype grade (low-grade vs high-grade) and the RBP-Jκ-specific autoantibody level was found for the DCIS specimens ($P<0.006$). We therefore optimized the threshold value of the autoantibody panel to better distinguish low-grade DCIS (grade 1 and 2) from high-grade DCIS (grade 3) using mROC analysis. The autoantibody panel exhibited an AUC of 0.749 (95% CI [0.581; 0.866]) (Figure 4B, Supplementary Data 4). To optimize the classification performance of our five-autoantibody signature, we determined a new threshold using mROC and observed an overall accuracy, sensitivity, and specificity of 73.0%, 69.6%, and 81.0%, respectively (Table 1). Interestingly, using the SVM classification algorithm, the overall performance of our predictor was maintained (Table 1).

**Autoantibody signature and local recurrence in the DCIS population.** To address whether our autoantibody signature can accurately predict clinical outcome, we conducted a Kaplan-Meier analysis comparing local recurrence and absence of recurrence in a population of DCIS patients followed for 5 years (Figure 5). The local recurrence was 9.1% with four recurrences; one recurrence occurred in the second year, one in the third year, and two in the fourth and fifth years. No deaths were observed from the date of inclusion until last follow-up, and consequently, no patient was censored for this reason in our analysis. Importantly, the autoantibody signature could be used to divide the DCIS patients into a poor prognosis group (local recurrence) and a good prognosis group (recurrence-free) (predictor cut-off 0.0756; and $P=0.011$, Log-rank test).

**DISCUSSION**
A major challenge in human DCIS research has been the identification of markers associated with the transition from DCIS to invasive breast carcinoma. Progress towards achieving this goal could be enhanced by using advanced microarray techniques to measure the humoral response against the cancer. In this study, we successfully used protein microarray technology to identify an autoantibody signature in patients with preinvasive and early invasive breast cancer. In doing so, we identified a subset of five autoantibodies that can accurately distinguish between the groups. These data suggest that autoantibody detection could serve as a new prognostic test.

Surprisingly, the autoantigens identified in this study were different from previously identified DCIS-associated TAAs (12, 15-17). Substantial differences exist between protein microarrays and other techniques used to identify TAAs. The ProtoArray protein microarrays contain proteins that are expressed in insect cells and, therefore, contain proteins expected to have undergone physiologic post-translational modifications. Moreover, all of the proteins are purified under native conditions, and the immobilized proteins are expected to maintain their native conformation. All of these technical aspects could increase the likelihood of identifying new breast cancer–specific TAAs. In addition, to identify specific proteins that can be used to differentiate our populations, we optimized the data analysis and, notably, the calculations for threshold determination. To reduce the influence of a few highly reactive spots on a specific spot value, we determined a local cut-off value using the 2,000 neighboring spots rather than a global cut-off. This local threshold was then corrected in a second pass to better fit with the distribution of values by removing specific local high values for the local cut-off calculation. The comparison of the seroreactivity, achieved from the local Z-Score calculation, allowed us to select a larger number of relevant target proteins.

Most of the functions of the TAAs identified in this study, and the five proteins that were included in our predictor in particular, are associated with cancer invasion. The
mechanism by which immunogenicity appears remains unknown. A prerequisite for an
immune response against a cellular protein is its presentation as an antigen. It is not clear why
only a subset of patients with a specific tumor type develop a humoral response to a particular
antigen. One of the first explanations for this humoral response is that antigens are encoded
by overexpressed genes in tumors. We did not observe any change in HMGN1, PSRC1,
CIRBP, and ECHDC1 expression in the malignant breast tissue, suggesting that other
mechanisms may be involved during breast cancer carcinogenesis. Among these mechanisms,
those resulting in modification of the processing, the function or the routing of antigens could
be proposed, as observed for previously identified autoantigens (18-21). Further studies will
be needed to investigate more specifically the origin of the humoral response for these four
autoantibodies. However, we found that RBP-Jκ expression is up-regulated in the nucleus of
DCIS and early-stage IBC tumor cells relative to normal epithelium. RBP-Jκ is a
transcriptional repressor that regulates Notch target genes (22). The Notch signaling pathway
is a highly conserved cell-cell signaling pathway that regulates cell proliferation,
differentiation, and apoptosis and stem cell maintenance (23). The increase observed in our
study is consistent with Notch1 expression studies that detected Notch1 activation very early
in breast cancer progression, specifically in preinvasive lesions and early-stage IBC, but not
in normal breast tissue (24). In addition, the overexpression of Notch1 is sufficient to
transform normal breast epithelial cells (25) and is significantly correlated with poor survival
of patients with IBC (26) and DCIS (24). We also provide evidence of an apparent link
between RBP-Jκ and the DCIS histological grade. These results are important because
patients with high-grade DCIS are believed to be at high risk of developing invasive and
aggressive tumors (27). Altogether, these results demonstrate that RBP-Jκ (and indirectly, the
Notch1 signaling pathway) could play a role in the transition from DCIS to IBC. Finally,
these results indicate that the identification of autoantigens provides a powerful approach to
identify proteins associated with cancer progression. Although the direct or indirect role of these proteins in DCIS transformation and progression remains to be established, they may be relevant candidate targets for understanding the mechanism of breast cancer progression.

*In situ* carcinomas are clinically complex. To date, it is not possible to discriminate with absolute certainty the lesions that will recur or progress to invasive carcinoma from those that will remain quiescent. The treatment guidelines for DCIS have changed over the last two decades, and most patients are now treated with breast-conserving surgery. However, 16% to 22% of women experience local relapse within 10 years following lumpectomy alone, and approximately half of these relapses are invasive breast cancer (28). Adjuvant breast irradiation has been proposed as an additional therapy and results in a 50% decrease in the number of ipsilateral breast recurrences (29, 30). However, it is unlikely that all patients with DCIS require radiation following breast-conserving therapy. Conventional prognostic clinicopathological factors, such as a young patient age, a high nuclear grade, comedo necrosis, a large tumor size, and involved excision margins, have been reported as factors associated with a high risk of local recurrence. However, the reported effect of these factors on the risk of local recurrence has varied (2, 31). Biological markers such as estrogen receptor, progesterone receptor, HER2/neu or Ki67 expression have also been tested, but no clear results have emerged. Several combinations of risk factors for local recurrence, including the St. Gallen criteria (32, 33), gene expression signatures (34), the Van Nuys Prognostic Index (VNPI) and its more recent variation including age (University of Southern California [USC]-VNPI) (35), have been assessed, but no consensus has been made. Together, these studies have shown that advances are needed, such as the identification of new markers of breast cancer invasion, to improve decision making following resection of DCIS. Therefore, we propose that our predictor could help predict those DCIS patients with a higher risk of invasion. However, the potential pitfall in our analysis is the low frequency of local recurrence in our followed DCIS
population. Although the rate of local recurrence observed in our study corresponds to the predicted percentage in this surgically and radiation-treated population, this low frequency clearly limits statistical evaluations. Therefore, our preliminary results regarding recurrence-free survival must be confirmed in a larger cohort of patients before this autoantibody signature can be considered a relevant prognostic feature of DCIS progression.

The present study provides the first integrated analysis of serum autoantibody expression profiling in patients with preinvasive or invasive breast cancer, and novel candidate markers associated with an increased risk of preinvasive breast cancer recurrence have been identified. By performing several statistical analyses, we demonstrated that an autoantibody signature based on the presence of several autoantibodies can accurately predict local recurrence in DCIS. Given the noninvasive characteristics of serum sampling and the easy detection of autoantibodies, we envision that an autoantibody signature may help to avoid the overtreatment of low-risk patients and dictate more intensive treatment of aggressive DCIS, thereby reducing the mortality of patients diagnosed at this very early step of the disease. Finally, since nipple aspirate fluid and ductal lavage represent a relevant source of immunoglobulins (36) and biomarkers specifically related to breast cancer (37), this signature could also be evaluated in breast fluids. Also, studies that link these five immunogenic proteins to the in situ to invasive breast carcinoma transition may provide key insights into the molecular mechanisms that drive early breast cancer tumorigenesis.

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REFERENCES


LEGENDS

Figure 1. Identification of autoantibody targets in the patients of serum set 1 using protein microarrays. A, protein microarrays with over 8,000 human proteins were probed in duplicate with serum samples from 20 DCIS patients and 20 IBC patients. Two representative arrays demonstrating the regular pattern of spotted control proteins (left panel, subarray incubated with buffer containing no serum; right panel, subarray incubated with serum) are shown. The AlexaFluor 647-conjugated antibody (boxed in red) allows for proper alignment during data acquisition. A gradient of human IgG (boxed in blue) serves as a positive control for the detection reagent. A gradient of anti-human IgG serves as a positive control for the detection of the immune response. B, three-dimensional representation of seroreactivity against the 20 antigens that were differentially targeted in DCIS and IBC. The DCIS and IBC serum samples are arranged along the x-axis, and the antigens are arranged along the z-axis. Each peak indicates the average local Z-Score for an individual serum for each antigen.

Figure 2. Seroreactivity of RBP-Jκ, PSRC1, ECHDC1, HMGN1 and CIRBP autoantibodies between the DCIS (D) and IBC (I) serum samples. Scatter plot of the local Z-Score determined by microarrays are shown for the 40 samples of the identification set (A). Scatter plots of optical density values determined by ELISA are shown for the 40 samples of the identification set (B), and for the 120 samples of the evaluation set (C). The median is indicated by the horizontal line.

Figure 3. Representative immunohistochemical staining of RBP-Jκ in breast tissue. A, the nuclear localization of RBP-Jκ detected with immunohistochemical staining (left panel) and the nuclear staining using Hoechst 33258 (right panel) are shown. B, immunohistochemical
staining of RBP-Jκ in normal breast epithelium, DCIS and IBC from a single breast specimen is shown.

**Figure 4.** ROC curves analysis of the five autoantibodies–based predictor. Value obtained when discriminating DCIS patients from IBC patients (A) and low-grade DCIS patients from high-grade DCIS patients (B).

**Figure 5.** Kaplan-Meier curves for recurrence-free survival stratifying DCIS patients by autoantibody predictor ($n=44; \ P=0.011$, Log-rank test). With a prediction cut-off of 0.0756, the DCIS patients could be split into two groups based on their autoantibody signature: a poor prognosis group with high risk of recurrence (black line) and a good prognosis group with low risk of recurrence (grey line). Censored patients are indicated by vertical bars.
Table 1. Performance of the predictor in the validation set using AUC linear combination and SVM

<table>
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<tr>
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<th>DCIS vs IBC</th>
<th>Low-grade DCIS vs High-grade DCIS</th>
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Figure 1

A

Without serum  With serum

B

[Legend with gene names: RBPJ, PSRC1, C1orf131, HMGN1, DIDO1, ZNF747, TTLL7, RBM34, HMFB2, YTHDF2, ECHDC1, CTNN, CIRBP, C3orf37, H1F0, LARP4, GTPBP1, HP1BP3, WDR5, RNPC3]
Figure 2

A

B

C

Research.

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Figure 4

A

B

AUC = 0.794

AUC = 0.749
Serum autoantibody signature of ductal carcinoma in situ progression to invasive breast cancer

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