Independent Validation of Candidate Breast Cancer Serum Biomarkers Identified by Mass Spectrometry

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Background: We previously selected a panel of 3 breast cancer biomarkers (BC1, BC2, and BC3) from serum samples collected at a single hospital based on their collective contribution to the optimal separation of breast cancer patients and noncancer controls by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). The identities and general applicability of these markers, however, were unknown. In this study, we performed protein expression profiling on samples obtained from a second hospital, included a greater number of ductal carcinoma in situ (DCIS) cases, and performed purification and identification of the 2 confirmed markers.

Methods: Using a case–control study design, we performed protein expression profiling on serum samples from the National Cancer Institute (Milan, Italy). The validation sample cohort consisted of 61 women with locally invasive breast cancer, 32 with DCIS, 37 with various benign breast diseases (including 13 atypical), and 46 age-matched apparently healthy women (age range, 44–68 years). Validated biomarkers were purified and identified with serial chromatography, 1-dimensional gel electrophoresis, in-gel ASP-N digestion, peptide mass fingerprinting, and tandem mass peptide sequencing.

Results: The BC3 and BC2 expression patterns in this sample set were consistent with the first study sample set. BC3 and BC2 were identified to be complement component C3adesArg and a C-terminal–truncated form of C3adesArg, respectively.

Conclusions: Evaluation of biomarkers in independent sample sets can help determine the broader utility of candidate markers, and protein identification permits understanding of their molecular basis. C3adesArg appears to lack specificity among patients with benign diseases, limiting its utility as a stand-alone tumor marker, but it may still be useful in a multimarker panel for early detection of breast cancer.

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Presymptomatic screening to detect early-stage breast cancer while it is still resectable and potentially curable can greatly reduce breast cancer–related mortality. Unfortunately, data collected between 1992 and 1999 in the United States indicated that only 63% of breast cancers are localized at the time of diagnosis (1). Small lesions are frequently missed and may not be visible, even by mammography, particularly in young women and women with dense breast tissue (2). Molecular markers that can potentially be used to identify small lesions that are invisible to imaging techniques could provide an opportunity to treat a neoplasm before it invades tissue. Markers that can detect ductal carcinoma in situ (DCIS) would be particularly valuable because nearly 100% of women diagnosed at this early stage of breast cancer can be cured. Most molecular-based approaches that have been investigated for the early detection of breast cancer have specific targets, such as oncogenes, tumor suppressor
genes, growth factors, tumor antigens, or other gene products, but because none of these alone can account for a large majority of the breast cancers and some are not specific to cancer or to breast tissues, such approaches have low sensitivity and specificity. To date, no molecular biomarkers have been recommended for the early detection of breast cancer (3). Tumor markers cleared by the Food and Drug Administration, such as CA 15-3 and CA27.29, are recommended only for monitoring treatment of advanced breast cancer or recurrence (4).

Rather than targeting a specific abnormality that may be present in only a small subgroup of patients, common “patterns of changes” that are associated with disease status are being identified by genomics- or proteomics-based approaches. Both genomics and proteomics approaches yield high-dimensional data that can be analyzed with multivariate statistics and powerful pattern recognition algorithms. The potential drawback of these approaches is their tendency to discover patterns among many variables that may not be a direct result of the pathology of interest but rather a result of preanalytical characteristics of a given sample set. It is therefore often possible to obtain a very high classification success rate in a single-site study. Independent validation in a separate sample set, collected from a different patient population and hospital, is one way to determine the true performance of a set of markers.

Using the high-throughput surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) proteomics approach, we previously analyzed protein expression profiles of serum samples from 169 women with or without breast cancer and identified a panel of 3 serum proteins that could detect early-stage breast cancer (stage 0-I) with high sensitivity (93%) and specificity (91%) (5). However, that study included only 4 cases of DCIS; therefore, the utility of these markers for detecting in situ breast carcinoma could not be properly assessed. In addition, because that study examined samples taken entirely from one institution, the general validity of these markers could not be ascertained. In the present study, we performed SELDI-TOF MS protein expression profiling on samples obtained from a second hospital and included a greater number of DCIS cases. In addition, we performed purification and identification of the 2 confirmed markers.

Material and Methods

Patient Samples

Archived serum samples from 176 women were analyzed retrospectively. These sera were collected from 2000 to 2002 by the National Cancer Institute of Milan, Italy, and stored at −30 °C until use. All women provided informed consent before serum collection for this Institutional Review Board–approved study. The cancer group included 32 cases of DCIS (age range, 36–80 years; mean, 56 years) and 61 cases of locally invasive breast cancer (47 cases of ductal invasive, 9 cases of lobular invasive, and 5 cases with mixed ductal and lobular features; age range, 24–84 years; mean, 56 years). Diagnoses were confirmed by a pathology examination, and serum specimens were obtained before treatment and before surgery. Additional clinical information for cancer patients included estrogen and progestin receptor (ER/PR) status, Elston grade, tumor size, and lymph node status (invasive cases only). The noncancer controls included 37 women with various benign breast diseases, including 13 cases of atypia (age range, 18–77 years; mean, 44 years) and 46 age-matched apparently healthy women (age range, 44–68 years; mean, 52 years).

SELDI Protein Profiling

Protein profiles were generated by use of Immobilized Metal Affinity Capture (IMAC-Ni) ProteinChip® arrays under binding and washing conditions as described previously (5).

Bioinformatics and Biostatistics

The data analysis process used in this study involved the following steps.

Peak Detection. ProteinChip Software 3.0 (Ciphergen Biosystems) was used to collect and evaluate the raw spectra. Results for each set of 196 specimens, including 176 study sera and 20 quality-control sera (pooled human sera obtained from Serologicals Corp.), were compiled, baseline-subtracted, and externally calibrated with All-In-1 Protein Standard (Ciphergen Biosystems). Qualified mass peaks (visual examination) of \( m/z \) 2000–150 000 were selected manually. The peak intensities were normalized to the total ion current between \( m/z \) 2000 and 150 000 with the same external coefficient, and the data were exported to an Excel spreadsheet.

Evaluation of Reproducibility. We estimated the reproducibility of replicates by calculating the correlation of each pair of replicates and calculating the CV of the 3 reported peaks as calculated from pooled human sera. If no systematic bias was observed, the peak intensities identified in replicate analysis were averaged and then log-transformed.

Marker Evaluation. Nonparametric 2-sample comparisons were performed with the Mann-Whitney test. ROC curve analysis (in-house software implemented in MATLAB, Ver. 6.0) was performed for evaluation of the selected biomarkers.

Protein Identification

Protein purification was carried out with 100 µL of serum and a series of protein separation procedures, including IMAC, anion-exchange, and reversed-phase chromatography using sorbents available from Ciphergen-BioSepra. A schematic of the purification process is shown in Fig. 1A of the Data Supplement that accompanies the online
SELDI-TOF MS IMMUNOASSAYS FOR BC2 AND BC3
We coupled 1.5 μg of mouse monoclonal antibody against human C3a (Chemicon) to 3 μL of Protein A HyperD® beads (Ciphergen-BioSepra). The uncoupled antibody was removed by washing 3 times with phosphate-buffered saline (PBS; 9 g/L NaCl, 0.165 g/L KH₂PO₄, 0.775 g/L Na₂HPO₄), and the beads were resuspended in 150 μL of PBS. We then added 0.5 μL of serum in 10 μL of PBS containing 0.1 g/L bovine serum albumin to the antibody beads and incubated them at 4 °C overnight. After incubation, the beads were washed 3 times with 150 μL of PBS containing 1 mL/L Triton followed by 3 washes with 150 μL of PBS and a brief rinse with water. The captured proteins were eluted twice with 30 μL of 500 mL/L acetonitrile containing 5 mL/L trifluoroacetic acid, and the pooled eluates were added to 100 μL of PBS. To achieve the optimal binding pH for IMAC30 chips (pH 7.4), 8 μL of 1 mol/L Tris (pH 10) was added to the mixture before our previously described standard binding and washing procedures.

CA 15-3
The CA 15-3 concentration was measured by IRMA-mat CA 15-3 (Byk-Sangtec Diagnostica).

Results
EVALUATION OF BC1, BC2, AND BC3 BY SELDI
A total of 71 peak clusters were manually selected in the 2- to 150-kDa mass region. The reproducibility of 3 independent SELDI replicates was estimated with correlation analysis. Log-transformed peak amplitudes from each of the replicates were plotted on the x and y axes, respectively (not shown), and the correlation coefficients (r) observed between the replicates were 0.885 (replicate 1 vs 2), 0.893 (replicate 1 vs 3), and 0.865 (replicate 2 vs 3). Because we found no systematic differences between pairs of replicates, we used the averaged peak intensities at each m/z value for further analysis. The estimated CVs of the log-transformed peak intensities for BC1 (4.3 kDa), BC2 (8.1 kDa), and BC3 (8.9 kDa) were 17%, 12%, and 16%, respectively. Consistent with our previous results, concentrations of BC2 and BC3 were significantly increased in cancer and benign diseases (Fig. 1, A and B). In contrast, BC1, which was previously found to be low in cancer, was increased in all cancer groups in the current set (Fig. 1C). The intensities of BC2 and BC3 in the original and the current data sets are compared in Fig. 2. The increased expression of the 2 biomarkers in patients with breast cancer and benign diseases can be seen in both sample sets. As observed in the previous study, BC2 and BC3 were not correlated with pT (tumor size) or pN (number of positive lymph nodes). They were also not correlated with the tumor grade or ER/PR status (ER+ / PR+ vs ER−/PR−; information on Elston grade and ER/PR status were not available in the previous data).

PROTEIN IDENTIFICATION FOR BC2 AND BC3
A schematic of the purification process for BC2 and BC3 is shown in Fig. 1A of the online Data Supplement. BC2 and BC3 coeluted in the pH 3 fraction of the IMAC-Cu column, whereas the organic fraction contained mainly BC2 (data not shown). The organic fraction was concentrated and applied directly on a sodium dodecyl sulfate gel for purification of BC2, whereas further fractionation was performed on the pH 3 fraction to obtain partially purified BC3 without interference from BC2. Gel images of the final fractions that were enriched for BC2 and BC3, respectively, and the gel bands containing each marker identified by protein profiling of the eluted proteins on NP20 arrays are shown in Fig. 1B of the online Data Supplement. BC3 was subsequently determined to be the anaphylatoxin C3a lacking the C-terminal arginine (C3adesArg; sequence SVQLTEKRMDKVGKYPKELRKC-CEDGMRENPMRFSQQTRTRFISLGEACKKVFLDCCN-YITELRRQOHARASHGLLA). The underlined regions are sequence covered by MS/MS analysis of peptides 1091 Da (DKVGKYPKELRKCCE), 1909 Da (SVOLTEKRMDKVGKYPKELRKCCE), and 2641 Da (DCNYYITELRRQOHARASHGLLA). The MS/MS analysis of peptide 1909 Da is shown in Fig. 1C of the online Data Supplement (data not shown for peptides 1091 and 2641 because of page limitations). The predicted mass of C3adesArg is 8923 Da, consistent with the measured mass of BC3 (8926 Da), and the predicted pI is 9.54, consistent with the inability of BC3 to bind anion-exchange resin at pH 9.0.

Similarly, BC2 (m/z 8116) was identified as a truncated form of C3adesArg by peptide mass fingerprinting and MS/MS analysis (data not shown). It was a C-terminal truncation of C3adesArg lacking the C-terminal sequence RASHGLLA; we refer to this marker as C3adesArgΔC.

version of this article at http://www.clinchem.org/content/vol51/issue12/. During each of the iterations, the fractions were profiled on IMAC arrays to monitor the presence or absence of the biomarkers of interest. Protein fractions that were enriched for BC2 and BC3, respectively, were separated on a 16% Tris-tricine sodium dodecyl sulfate gel. All visible bands in the correct molecular mass range were excised. The protein in each gel band (one half of the gel material) was eluted according to the passive elution protocol (6) and profiled on NP20 arrays. The identified gel bands (the other half of the gel material) containing the 8.1- and 8.9-kDa peaks were first reduced with dithiothreitol and then digested with ASP-N (Roche MDS Sciex) equipped with a PCI 1000 ProteinChip Interface (Ciphergen). A sequence database search was carried out with the Matrix Science Mascot program (http://www.matrixscience.com).
IMMUNOASSAYS FOR BC2 AND BC3
The identities of BC2 and BC3 as C3a_{desArg}\textsubscript{8} and C3a_{desArg} were further verified by SELDI-TOF MS immuno-depletion using a monoclonal antibody against C3a (Fig. 3). A subgroup of the serum samples (10 healthy, 9 benign, 10 DCIS, and 10 invasive cases) was randomly selected for an immuno-depletion pull-down experiment using the same antibody. Distribution of the captured C3a_{desArg} and C3a_{desArg}\textsubscript{8} in the cancer and noncancer groups was consistent with the SELDI profiling results (Fig. 4). The correlation coefficients between the SELDI immunoassay and profiling for BC2 and BC3 were 0.65 and 0.56, respectively.

DIAGNOSTIC PERFORMANCE OF BC2, BC3, AND CA 15-3
To investigate whether CA 15-3 has any discriminatory power in this study cohort, we measured the serum CA 15-3 concentrations with IRMA-mat CA 15-3. Of 93 cancer sera tested, only 5 (all from patients with invasive cancer) would have been called positive according to the standard 30 units/mL cutoff (sensitivity, 5%). We observed no significant difference between the healthy control, benign, DCIS, and invasive cancer groups (data not shown). The diagnostic performances of BC2, BC3, and CA 15-3 in terms of ROC analysis are presented in Fig. 5. The areas

Fig. 1. Scattergrams of peak intensity across sample groups. The horizontal lines denotes the medians. (A), BC2 (8.1 kDa); (B), BC3 (8.9 kDa); (C), BC1 (4.2 kDa).

Fig. 2. Two-dimensional scatter plots of the 2 validated markers. (A), discovery data; (B), validation data.
under the curves for C3adesArg and C3adesArg\textsubscript{H9004} in the validation data were 0.65 and 0.71, respectively. In contrast, the area under the curve for CA 15-3 was 0.52.

**Discussion**

Technologic developments in high-throughput protein expression measurements have made it possible to compare proteomic expression patterns of clinical specimens on a large scale. In the presence of large biological variability and biases in data attributable to preanalytical and analytical variables, however, screening for new tumor biomarkers that are truly associated with a particular disease process remains a challenging task.

In a previous study we selected 3 protein/peptides, BC1, BC2, and BC3, as potential biomarkers for breast cancer early detection and used several statistical measures to minimize the impact of analytical variability (5), but because the study did not have a completely independent test set, these markers required further validation. In this study, we evaluated these markers on samples obtained from a second hospital and included a greater number of DCIS cases. We were able to validate the expression patterns of 2 of the markers, BC2 and BC3, and identified them as complement component C3adesArg and C3adesArg\textsubscript{H9004} respectively.

The human complement (C) system protects an individual against substances of nonself origin, including xenografts and microbial pathogens (7). There are 25 known soluble complement proteins, as well as 10 or more cell-surface complement receptors and regulatory proteins present on a wide range of host cells. Among these complement proteins, C3 is the most versatile and multifunctional molecule identified to date, having evolved structural features that allow it to interact in a specific manner with at least 25 different proteins (8). C3 is also the most abundant complement protein in serum (1.2 g/L) and supports the activation of all 3 pathways of complement activation, the classic, alternative, and lectin pathways (9, 10). On activation by molecules on the surfaces of the microorganisms or other nonself signals, native C3 (185 kDa) is cleaved into C3b (176 kDa) and C3a (9 kDa). C3a is very short lived and in serum is cleaved immediately into the more stable C3adesArg (8.9 kDa) by carboxypeptidases (9). Cleavage of C3 is functionally important because it causes a series of events that lead to complement-mediated lysis (10–12). C3a, together with C4a and C5a, the small fragments of C3, C4, and C5, respectively, are called the complement anaphylatoxins. In addition to anaphylaxis and chemotaxis, they also have demonstrated immunomodulatory effects on several different cell types (13).

Complements have long been thought to function in immunosurveillance against tumors, but the molecular mechanisms by which malignant cells are tagged by homologous C3 and the molecules responsible for homol-
Complement abnormalities have been described in various types of cancer. Higher complement hemolytic activity and C3 concentrations were observed in serum samples from children with neuroblastoma (14). Similarly, increased complement concentrations were reported in patients with lung (15, 16), digestive tract (17), and brain (18) tumors. It is likely that complement activation is an early event during tumorigenesis because the opposite phenomenon was observed in patients with advanced-stage metastatic brain tumors (18) and in patients of breast, gastric, and colorectal carcinomas who developed metastases after the surgery (19). Interestingly, we have also observed down-regulation of C3a_desArg and C3b_desArg in pretreatment sera of breast cancer patients with distant metastasis (unpublished result), in contrast to their up-regulation in sera of patients with DCIS or locally invasive breast cancer shown in this and previous (5) work.

For BC1 (4.3 kDa), we observed an inconsistent expression pattern in the 2 studies. BC1 has been identified as a truncated form of interalpha-trypsin inhibitor heavy chain H4; Li et al., unpublished result). Our preliminary result indicates that BC1 is unstable and that further truncation occurs during prolonged storage. The instability of BC1 and variations on sample storage time are most likely the causes of the discrepancies we observed in the 2 data sets. In the original discovery study, serum samples from cancer patients were collected during a longer time interval than the control samples, whereas samples from the validation set were all collected within the same 2-year window. Although BC1 may still be a valid marker if serum were collected prospectively and stored under the same conditions, we are currently evaluating other fragments of the same protein for a more stable alternative to BC1 (unpublished result).

In summary, we used independent test data to evaluate 3 previously discovered potential serum biomarkers for breast cancer. Although several biomarker panels that use SELDI and ProteinChip arrays have been reported for various diseases (5, 20–29), this report highlights one of the first validation studies that uses an independent test set. We identified 2 markers that showed consistent expression patterns in both cohorts, complement C3a_desArg and C3a_desArg. Because C3a is increased in the sera of patients with breast cancer as well as benign breast diseases, it is likely that our results reflect activation of the complement system in response to a general disease process rather than proteins released by the tumor cells themselves. The apparent lack of specificity of C3a_desArg among patients with benign diseases may limit its utility as a stand-alone tumor marker, but it may still be useful in a multimarker panel for the early detection of breast cancer. The applications and possible shortcomings of MS for clinical proteomics and biomarker discovery have been discussed extensively (30). It is true that with limited mass range coverage, it is unlikely that existing tumor markers such as CA125 could be directly detected by the current SELDI platform. However, in this report we demonstrate that with proper study design and well-controlled sample-preprocessing protocols, it is possible to discover and validate biomarkers with consistent performance across multiple sites.

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managed by the Johns Hopkins University in accordance with its conflict of interest policies.

References


