Proteomics and Bioinformatics Approaches for Identification of Serum Biomarkers to Detect Breast Cancer

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Background: Surface-enhanced laser desorption/ionization (SELDI) is an affinity-based mass spectrometric method in which proteins of interest are selectively adsorbed to a chemically modified surface on a biochip, whereas impurities are removed by washing with buffer. This technology allows sensitive and high-throughput protein profiling of complex biological specimens.

Methods: We screened for potential tumor biomarkers in 169 serum samples, including samples from a cancer group of 103 breast cancer patients at different clinical stages [stage 0 (n = 4), stage I (n = 38), stage II (n = 37), and stage III (n = 24)], from a control group of 41 healthy women, and from 25 patients with benign breast diseases. Diluted serum samples were applied to immobilized metal affinity capture Ciphergen ProteinChip® Arrays previously activated with Ni^{2+}. Proteins bound to the chelated metal were analyzed on a ProteinChip Reader Model PBS II. Complex protein profiles of different diagnostic groups were compared and analyzed using the ProPeak software package.

Results: A panel of three biomarkers was selected based on their collective contribution to the optimal separation between stage 0–I breast cancer patients and non-cancer controls. The same separation was observed using independent test data from stage II–III breast cancer patients. Bootstrap cross-validation demonstrated that a sensitivity of 93% for all cancer patients and a specificity of 91% for all controls were achieved by a composite index derived by multivariate logistic regression using the three selected biomarkers.

Conclusions: Proteomics approaches such as SELDI mass spectrometry, in conjunction with bioinformatics tools, could greatly facilitate the discovery of new and better biomarkers. The high sensitivity and specificity achieved by the combined use of the selected biomarkers show great potential for the early detection of breast cancer.

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On the basis of National Cancer Institute incidence and National Center for Health Statistics mortality data, the American Cancer Society has estimated that breast cancer will be the most commonly diagnosed cancer among women in the US in 2002. Breast cancer is expected to account for 31% (203 500) of all new cancer cases among women, and 39 600 will die from this disease *(1)*. Presymptomatic screening to detect early-stage cancer while it is still resectable with potential for cure can greatly reduce breast cancer-related mortality. Unfortunately, only ~50% of the breast cancers are localized at the time of diagnosis *(2)*. Despite the availability and recommended use of mammography as a routine screening method for women 40 years of age and older, its effectiveness in reducing overall population mortality from breast cancer is still being investigated *(3)*. Currently, serum tumor markers, such as CA15.3, that have been investigated for use in breast cancer detection still lack the adequate sensitivity (23%) and specificity (69%) to be applicable in detecting early-stage carcinoma in a large population *(4)*. The Food and Drug Administration-approved tumor markers, such as CA15.3 and CA27.29, are recommended only for monitoring therapy of advanced breast cancer or recurrence *(5)*. New biomarkers that could be used individually or in combination with an existing modality for cost-effective screening of breast cancer are still urgently needed.

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The classical approach for discovering disease-associated proteins is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).[1] Although 2D-PAGE is unchallenged in its ability to resolve thousands of proteins, it is labor-intensive, requires large quantities of protein, and is not easily converted into a diagnostic test. Recent advances in mass spectrometry (MS), such as matrix-assisted laser desorption/ionization time-of-flight MS, are beginning to offer an alternative to 2D-PAGE. In this technique, purified or partially purified proteins are mixed with a crystal-forming matrix, placed on an inert metal target, and subjected to a pulsed laser beam to produce gas-phase ions that traverse a field-free flight tube and then are separated according to their mass-dependent velocities (m/z) (6). However, some limitations in matrix-assisted laser desorption/ionization, such as extensive sample preparation and signal background problems resulting from inorganic and organic contaminants, have hindered it from being used as a high-throughput screening tool for proteins of interest in complex biological samples. The development of surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS (7), has largely overcome many of these limitations. SELDI is an affinity-based MS method in which proteins are selectively adsorbed to a chemically modified surface (Ciphergen ProteinChip® Arrays), and impurities are removed by washing with buffer. The use of several different chromatographic arrays and wash conditions enables high-speed, high-resolution chromatographic separations (8). This technology has been used successfully to detect several disease-associated proteins in complex biological specimens, such as cell lysates, seminal plasma, and serum (9–13).

SELDI-TOF MS offers high-throughput protein profiling. Like many other types of high-throughput expression data, protein array data are often characterized by a large number of variables (the mass peaks) relative to a small sample size (the number of specimens). An important issue in analyzing such data to screen for disease-associated biomarkers is to extract as much information as possible from a limited number of samples and to avoid selecting biomarkers whose performances are influenced mostly by non-disease-related artifacts in the data. The effective and appropriate use of bioinformatics tools becomes very critical.

Here we report the use of SELDI with immobilized metal affinity ProteinChip Arrays to screen for potential serum biomarkers for early detection of breast cancer. A total of 169 retrospective serum samples from patients with or without breast cancer were obtained from Johns Hopkins Clinical Chemistry serum banks and analyzed

simultaneously. Proteins bound to the chelated metal (through histidine, tryptophan, cysteine, or phosphorylated amino acids) were analyzed on a ProteinChip Reader Model PBS II (Ciphergen Biosystems). The complex protein profiles were analyzed using a collection of bioinformatics tools. A panel of three biomarkers was selected based on their consistently significant contribution to the optimal separation of stage 0–I breast cancer patients vs the noncancer controls (healthy + benign). The effectiveness of the selected biomarkers was then tested using independent data from stage II–III breast cancer patients and through bootstrap cross-validation. Finally, correlation of the concentrations of these biomarkers to tumor size and lymph node metastasis was also investigated.

Materials and Methods

SAMPLES

Retrospective serum samples were obtained from the Johns Hopkins Clinical Chemistry serum banks. A total of 169 specimens were included in this study. The cancer group consisted of 103 serum samples from breast cancer patients at different clinical stages: stage 0 (n = 4), stage I (n = 38), stage II (n = 37) and stage III (n = 24). Diagnoses were pathologically confirmed, and specimens were obtained before treatment. Age information was not available on six of these patients. The median age of the remaining 97 patients was 56 years (range, 34–87 years). The noncancer control group included serum from 25 patients with benign breast diseases (BN) and 41 healthy women (HC). Exact age information was not available from 21 healthy women. The median age of the remaining 20 healthy women was 45 years (range, 39–57 years). The median age of the BN group was 48 years (range, 21–78 years). All samples were stored at −80 °C until use.

PROTEINCHIP ARRAY ANALYSIS

To 20 μL of each serum sample, we added 30 μL of a solution containing 8 mol/L urea and 10 g/L CHAPS in phosphate-buffered saline, pH 7.4. The mixture was vortex-mixed at 4 °C for 15 min and diluted 1:40 (5 μL of mixture plus 195 μL of phosphate-buffered saline) in phosphate-buffered saline. Immobilized metal affinity capture arrays (IMAC3) were activated with 50 mmol/L NiSO$_4$ according to manufacturer's instructions (Ciphergen). Diluted samples (50 μL) were applied to each spot on the ProteinChip Array by a 96-well bioprocessor (Ciphergen). After the samples were allowed to bind at room temperature for 60 min on a platform shaker, the array was washed twice with 100 μL of phosphate-buffered saline for 5 min, followed by two quick rinses with 100 μL of distilled H$_2$O. After air-drying, 0.5 μL of saturated sinapinic acid prepared in 500 mL/L acetonitrile–5 mL/L trifluoroacetic acid was applied twice to each spot. Proteins bound to the chelated metal (through histidine, tryptophan, cysteine, or phosphorylated amino acids) were detected with the ProteinChip Reader. Data

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were collected by averaging 80 laser shots with an intensity of 240 and a detector sensitivity of 8. Reproducibility was estimated using two representative serum samples: one from the healthy controls and one from the cancer patients. Each serum sample was spotted on all eight bait surfaces of one IMAC-Ni array in each of the two bioprocessors. The CV was estimated for the selected mass peaks.

BIOINFORMATICS AND BIOSTATISTICS

All spectra were compiled, and qualified mass peaks (signal-to-noise ratio >5) with mass-to-charge ratios (m/z) between 2000 and 150 000 were autodetected. Peak clusters were completed using second-pass peak selection (signal-to-noise ratio >2, within 0.3% mass window), and estimated peaks were added. The peak intensities were normalized to the total ion current of m/z between 2000 and 150 000. All these were performed using ProteinChip Software 3.0 (Ciphergen). The only additional preprocessing step was logarithmic transformation of the peak intensity data. Such a transformation in general reduces the range of intensity data. As a result, the variance of the transformed peak intensity (across multiple samples) tends to be less volatile over the entire length of the spectrum.

The software package ProPeak (3Z Informatics) was used to compute and rank the contribution of each individual peak toward the optimal separation of two diagnostic groups. ProPeak implements the linear version of the Unified Maximum Separability Analysis (UMSA) algorithm that was first reported for use in microarray data analysis (14). The key feature of the UMSA algorithm is the incorporation of data distribution information into a structural risk minimization learning algorithm (15) to identify a direction along which the two classes of data are best separated. This direction is represented as a linear combination (weighted sum) of the original variables. The weight assigned to each variable in this combination measures the contribution of the variable toward the separation of the two classes of data.

Currently, ProPeak offers three UMSA-based analytical modules. The first is the Component Analysis Module, which projects each specimen as an individual point onto a three-dimensional component space. The components (axes) are linear combinations of the original spectrum peak intensities. The axes correspond to the directions along which two prespecified groups of data achieve maximum separability. The separation between the two groups of data in the component space can be inspected in an interactive three-dimensional display. If the separation achieved using combinations of all peaks is acceptable for a particular problem, the second module of ProPeak, BootStrap Selection, is used to reduce the complexity of the original data set. This module performs multiple runs of UMSA. In each run, a fixed percentage of the samples is randomly left out from both groups. The mean, the median, and the corresponding SD of the ranks from multiple runs are estimated for each peak. The bootstrap-estimated SD of a peak's rank provides the information about the consistency of the peak's ranking across multiple randomly selected subpopulations of the samples. To establish an objective peak selection criterion, in this study the same bootstrap procedure was also applied to a random dataset that, peak by peak, simulates the distribution of the actual data. The minimum of the rank SDs among all peaks in the simulated random data set was used as the cutoff value for rank SD of the actual data to select a subset of peaks that, in addition to being top-ranked in their contribution to the separation of the data groups, also demonstrated a consistency that was less likely attributable to pure chance. Finally, the third module of ProPeak applies a backward stepwise selection procedure to compute a significance score for each peak. The absolute value of the score is based on the peak's contribution to data separation and is in reverse relation to the order in which it is removed from the initial list of peaks. A positive or negative score indicates relatively increased or decreased expression, respectively, of the corresponding mass peak for the diseased group, whereas the absolute value of the score represents its relative importance toward data separation.

To identify potential biomarkers that can detect breast cancer at early stages, protein profiles of specimens from stage 0–I breast cancer patients were compared against those of the noncancer controls. The analysis involved multiple iterations using all three modules in ProPeak to select from the original full set of mass peaks a small panel of peaks that possessed a consistently high degree of significance in the optimal separation between the two selected diagnostic groups.

Once the small panel of biomarkers was selected, their ability to detect breast cancer was evaluated using the set-aside independent test data set of stages II and III cancer patients. To assess the complementary performance of multiple biomarkers, a composite index was derived using multivariate logistic regression based on the entire data set. Descriptive statistics including P values from two-sample t-tests and ROC curve analysis were provided for the selected individual biomarkers as well as the composite index. To partially overcome the limitation of lacking a full set of independent test data other than those from the late-stage cancer patients, we used the bootstrap procedure (16) to estimate key performance criteria such as the sensitivity and specificity of the composite index. In this procedure, the patient data set was repeatedly divided through random sampling into a training set to derive a composite index through logistic regression and a test set for computing sensitivities and specificities. The results from multiple runs were then aggregated to form the bootstrap estimate of sensitivity and specificity.

Results

PEAK DETECTION AND DATA PREPROCESSING

Serum proteins retained on the IMAC-Ni^{2+} arrays were analyzed on a PBS II mass reader. The high mass to acquire was set to 150 kDa, with an optimization range from 5 kDa to 30 kDa. A mass accuracy of 0.1% was achieved by external calibration using the All-In-1 Protein Standard (Ciphergen).

Among a total of 147 qualified mass peaks (signal-to-noise ratio >5) detected, 61 peaks had m/z values between 2 and 10 kDa, 30 peaks had m/z values between 10 and 20 kDa, 33 peaks were between 20 and 50 kDa, and 23 peaks were between 50 and 133 kDa. Peaks with a m/z <2 kDa were mainly ion noise from the matrix and therefore excluded. Peak intensity was normalized to total ion current (2–150 kDa), and logarithmic transformation was applied. The plots in Fig. 1 illustrate the effect of variance reduction and equalization through logarithmic transformation.

BIOMARKER SELECTION BASED ON EARLY-STAGE CANCER AND NONCANCER CONTROLS

To identify biomarkers with potential for early detection of breast cancer, UMSA was performed using early-stage cancer as the positive group (stage 0–I; n = 42) and the noncancer controls (HC + BN; n = 66) as the negative group. Separability between the two groups was first tested using UMSA-derived liner combination of all 147 mass peaks. The early-stage cancer was separable from the noncancer group when the entire protein profiles were compared. Fig. 2A provides a snapshot of the early-stage cancer (red) and the noncancer (green) data in the UMSA component three-dimensional space.

To select biomarkers with consistent performance, we repeatedly applied UMSA for a total of 100 runs, each with a 30% leave-out rate, using the ProPeak BootStrap module. We also applied the same procedure to a simulated random data set. The minimal rank SD derived from the simulated data was 7.0. Among the peaks with top mean ranks from the actual experimental data, 15 had
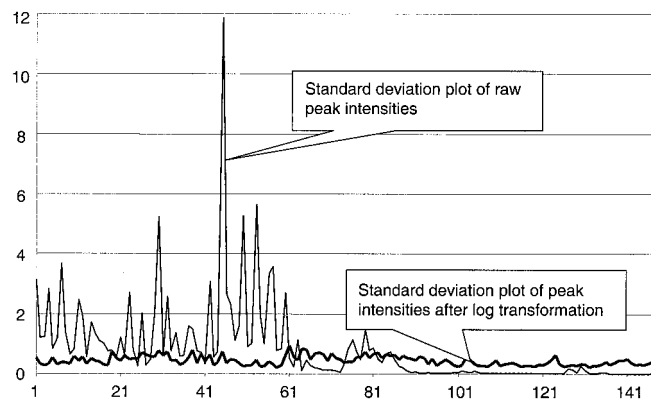

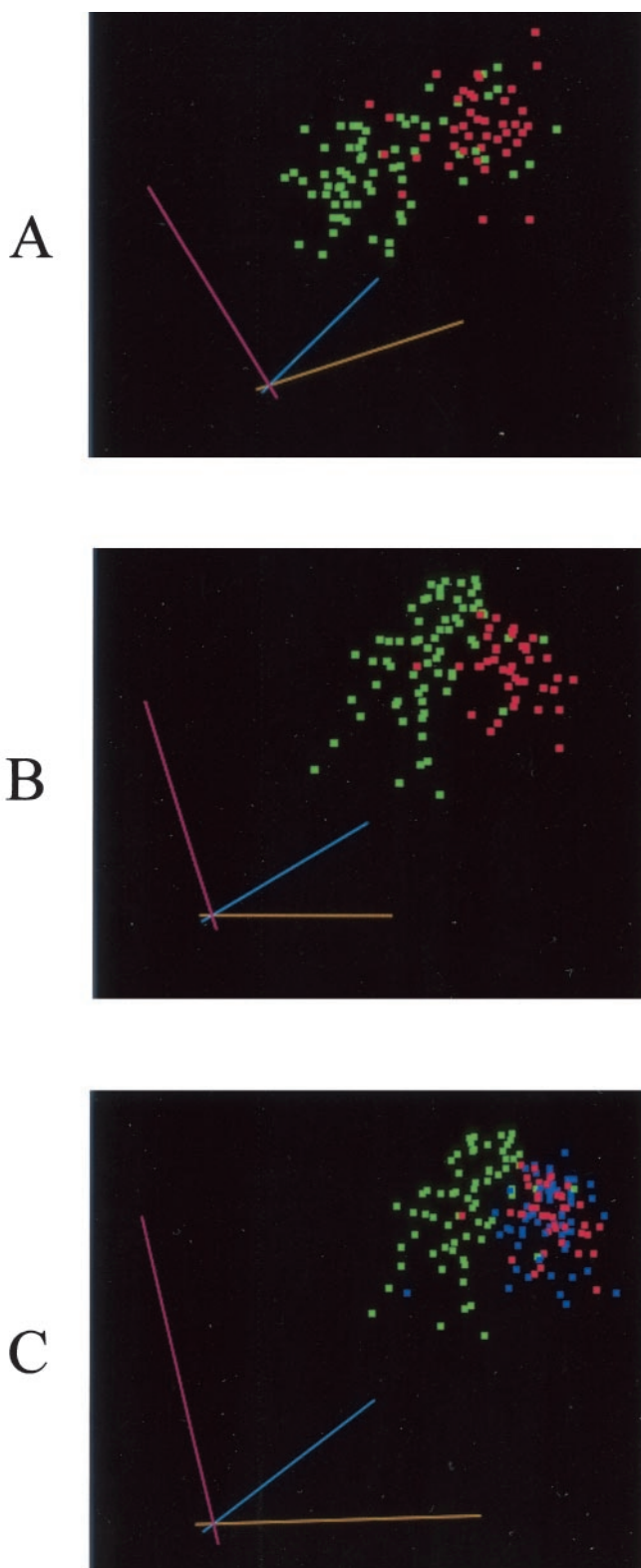Fig. 1. Effect of logarithmic transformation on data variance reduction and equalization.

Fig. 2. Three-dimensional UMSA component plot of stage 0–I (*red*) or stage II–III (*blue*) breast cancer vs noncancer controls (*green*).

(*A*), plot of training data: stage 0–I vs noncancer using UMSA-derived linear combination of all 147 peaks. (*B*), plot of training data: stage 0–I vs noncancer using the three selected peaks. (*C*), plot of training and test data: stage 0–I (training data) and stage II–III (independent test data) vs noncancer (training data), using the three selected peaks.
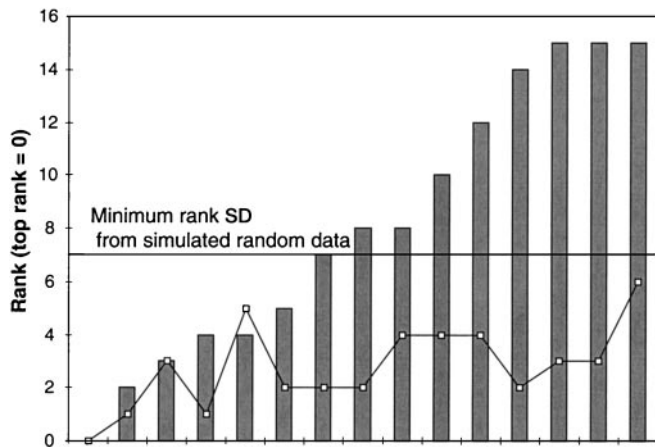
Fig. 3. Fifteen peaks with top mean ranks (■) and minimal rank SDs (□) derived from ProPeak Bootstrap Analysis.

Horizontal line at 7.0 was the minimum rank SD computed by applying the same procedure to a randomly generated data set that simulated the distribution of the original data.

a rank SD less than this value. They were selected as candidate biomarkers for further analysis. Their mean ranks and the corresponding rank SDs are plotted in Fig. 3.

To further rank the peaks in this reduced set of candidate biomarkers, we used the Stepwise Selection module of ProPeak. The absolute value of the relative significance scores of the 15 peaks are plotted in descending order in Fig. 4A, which shows that the majority of separability between the two groups of data was contributed by the first six peaks. Among these six peaks, two were identified by ProteinChip Software 3.0 as doubly charged forms of the others. The recognition of both the doubly charged and the singly charged forms of these peaks suggests their importance in discriminating the selected two diagnostic groups. Excluding the doubly charged forms, the four unique peaks were further recombined and evaluated using the Backward Stepwise Selection module of ProPeak. The recalculated relative significance scores are plotted in Fig. 4B. The top-scored three peaks, designated BC1 (4.3 kDa), BC2 (8.1 kDa), and BC3 (8.9 kDa), were finally selected as the potential biomarkers for detection of breast cancer. Snapshots of three-dimensional plots of stage 0–I or stage 0–III breast cancer against the noncancer controls using these three biomarkers are shown in Fig. 2, panels B and C, respectively. Among the three biomarkers, BC1 appeared to be downregulated (scored negative; data not shown), whereas BC2 and BC3 were up-regulated (scored positive; data not shown). This is easily seen in Fig. 5, in which representative spectra and gel views of the selected biomarkers are compared between cancer and noncancer controls.

EVALUATION OF THE SELECTED BIOMARKERS
The estimated CVs of the log-transformed peak intensities were 6% for BC1, 7% for BC2, and 13% for BC3 (data not shown). Among the three biomarkers, BC3 had the largest
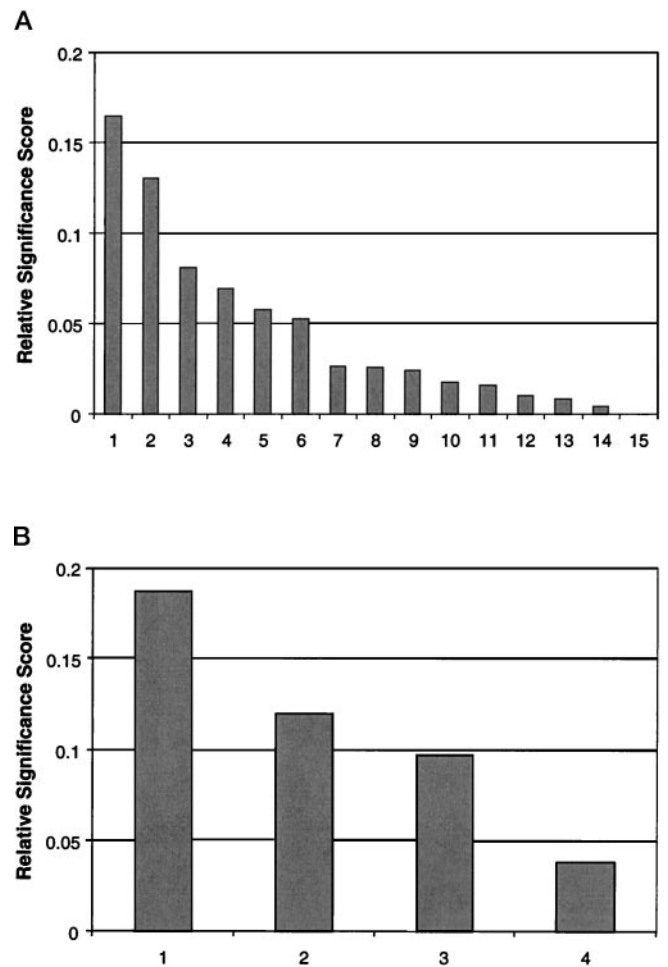

Fig. 4. Plot of absolute values of the relative significance scores of selected peaks based on contribution toward the separation between stage 0–I breast cancer and the noncancer controls.

(*A*), the 15 peaks selected from ProPeak Bootstrap Analysis with rank SD <7.0. (*B*), reevaluated scores of the selected top four peaks.

CV of 13%. The descriptive statistics of these three biomarkers are listed in Table 1. Fig. 6 shows results from the ROC analysis. Among the three biomarkers, BC3 possesses the highest individual diagnostic power [area under the curve (AUC), 0.934] compared with BC1 (AUC, 0.846) and BC2 (AUC, 0.795). Its distributions over the diagnostic groups including clinical stages of cancer patients are plotted in Fig. 7A. The sensitivities and specificities of using BC3 alone at a cutoff value of 0.8 to differentiate the diagnostic groups are listed in Table 2A. The overall sensitivity for breast cancer was 85%, and specificity was 91%.

COMBINED USE OF THREE SELECTED BIOMARKERS
Multivariate logistic regression was used to combine the three selected biomarkers to form a single-value composite index. The descriptive statistics of this composite index are appended in Table 1. Its distributions over the various diagnostic groups are plotted in Fig. 7B. ROC curve
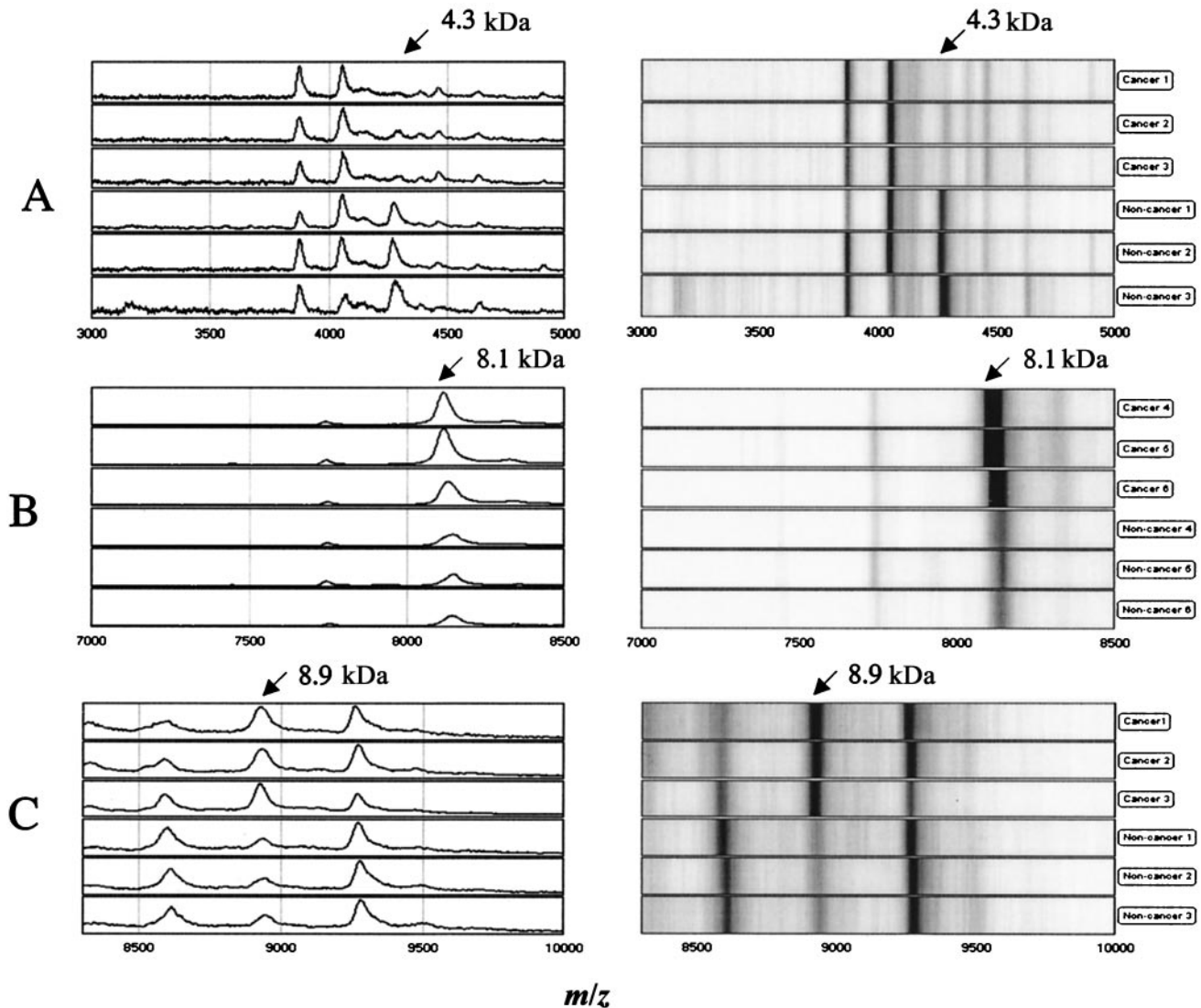
Fig. 5. Representative spectra and gel views of the selected biomarkers.

(*A*), BC1 (4.3 kDa), down-regulated in cancer; (*B*), BC2 (8.1 kDa), up-regulated in cancer; and (*C*), BC3 (8.9 kDa), up-regulated in cancer. *Left panels* show the spectrum views; *right panels* show pseudo-gel views of the same spectra. Both cancer and noncancer representatives were randomly selected, with no bias on stages in cancer or between healthy and benign in noncancer.

analysis of the composite index gave a much improved AUC (0.972) compared with the AUCs from individual biomarkers (Fig. 6).

Bootstrap cross-validation was used to estimate the diagnostic performance of the composite index (20 runs; in each run, 70% samples were randomly selected for

Table 1. Descriptive statistics for BC1, BC2, BC3, and the logistic regression-derived composite index.[a]

| | Noncancer controls (n = 66) | | Breast cancer patients | | | |
| | | | Stage 0–I (n = 42) | | Stage II–III (n = 61) | |
	Mean	SD	Mean	SD	Mean	SD
BC1	0.302	0.312	−0.118	0.244	−0.081	0.258
BC2	0.981	0.358	1.411	0.154	1.295	0.205
BC3	0.526	0.252	0.993	0.193	1.003	0.234
Composite index	−0.375	0.313	0.425	0.257	0.349	0.242

[a] Differences between noncancer controls and stage 0–I and between noncancer controls and stage II–III are both statistically significant ($P < 0.000001$) for all three biomarkers and the composite index.
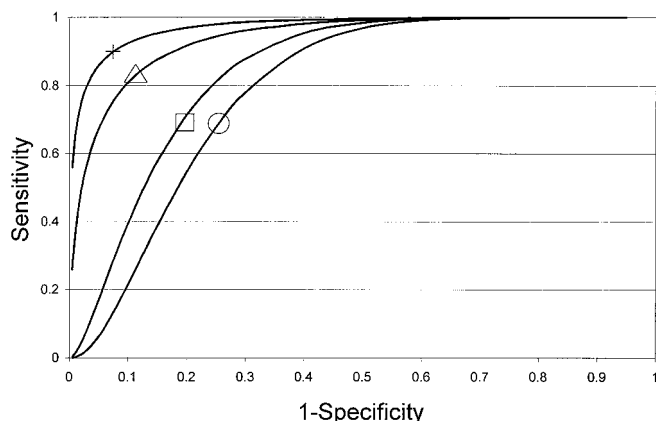
Fig. 6. ROC curve analysis of BC1 (□), BC2 (○), BC3 (△), and logistic regression-derived composite index (+).

The AUCs are 0.846 for BC1, 0.795 for BC2, 0.934 for BC3, and 0.972 for the composite index. Significance for AUC comparisons between individual biomarkers and the composite index: $P <0.0001$ for BC1 and BC2 vs the composite index; $P <0.01$ for BC3 vs the composite index.

composite index derivation and the remaining 30% for testing). The estimated sensitivity (93%) and specificity (91%) are listed in Table 2B.

CORRELATION TO TUMOR SIZE AND LYMPH NODE METASTASIS

The concentrations of the three potential biomarkers were evaluated in relation to pT (tumor size) and pN (lymph node metastasis) categories. No significant correlation was observed (data not shown).

Discussion

Because of the multifactorial nature of cancer, it is very likely that a combination of several markers will be necessary to effectively detect and diagnose cancer. To look for such "fingerprints" of cancer, it will require not only high-throughput genomic or proteomic profiling, but also sophisticated bioinformatics tools for complex data analysis and pattern recognition.

Taking advantage of the recent development in SELDI and of the ProteinChip technology, we were able to simultaneously analyze the protein profiles of 169 serum samples from patients with or without breast cancer. The software package ProPeak allows evaluation of each mass peak according to its collective contribution toward the maximal separation of the cancer patients from the noncancer controls. These two advances led to the identification of three discriminatory biomarkers that, in combination, achieved both high sensitivity (93%) and high specificity (91%) in detecting breast cancer patients from the noncancer controls.

Early detection remains one of the most urgent issues in breast cancer research. To find biomarkers particularly sensitive to differences between early-stage breast cancer patients and noncancer controls, the selection of mass peaks reported here was performed using stage 0–I cancer and noncancer controls as the training data and
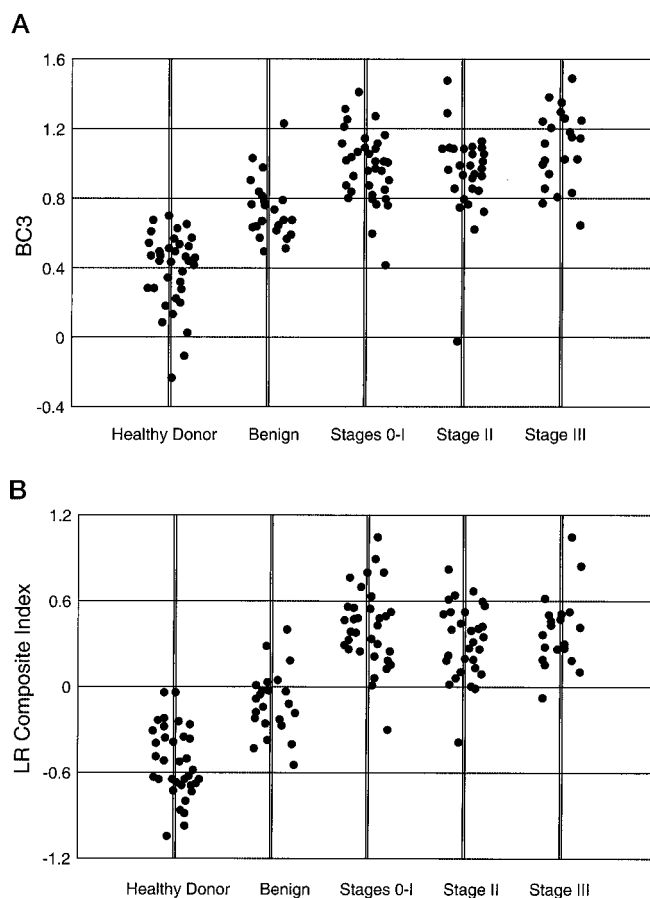

Fig. 7. Distribution of the selected biomarker(s) across all diagnostic groups including clinical stages of the cancer patients.

(A), BC3 alone; (B), logistic regression-derived (LR) composite index using BC1, BC2, and BC3.

later-stage cancer as test data. However, the biomarkers that were used in the final selection were not sensitive to the stages of cancer patients used in the selection process. In fact, whether the combinations used were stage II vs noncancer, stage III vs noncancer, or a randomly selected subset of cancer patients at all stages against noncancer controls, the same three peaks were always selected as the best and most consistently ranked biomarkers.

High-throughput profiling of complex protein expression patterns greatly facilitates the screening of a large number of potential markers simultaneously. However, for most currently available data sets, the sample sizes are relatively small compared with the total number of detected mass peaks. There is a real danger to mistakenly select mass peaks whose high discriminatory power is purely by chance because of artifacts in the data that are unrelated to the disease process. The use of high-order nonlinear classification models directly on raw spectrum data may further amplify and mask the influence of such false markers.

In this study, the UMSA algorithm provided an efficient model to rank a large number of peaks collectively according to their contribution to the separation of two

Table 2. Diagnostic performance of BC3 (A) and bootstrap-estimated performance of logistic regression (LR)-derived composite index (B).

A. BC3	Noncancer controls,[a] n			Breast cancer patients by stage,[b] n			
Cutoff = 0.8	HC[c]	Benign	Subtotal	0–I	II[d]	III[d]	Subtotal
Positive	0	6	6	37 (88%)	29 (78%)	22 (92%)	88 (85%)
Negative	41 (100%)	19 (76%)	60 (91%)	5	8	2	15
Total	41	25	66	42	37	24	103
B. LR-derived composite index[e]	**Noncancer controls**			**Breast cancer patients by stage**			
Cutoff = 0	HC	Benign	Subtotal	0–I	II	III	Subtotal
Positive	—	—	—	93%	85%	94%	93% (85–100%)
Negative	100%	85%	91% (82–100%)	—	—	—	—

[a] Values in parentheses are specificities.
[b] Values in parentheses are sensitivities.
[c] HC, healthy controls.
[d] Independent test data were not involved in initial biomarker selection.
[e] Index derived using BC1, BC2, and BC3 (20 runs; leave-out rate, 30%).

predefined diagnostic groups. The ProPeak BootStrap module introduced random perturbations in multiple runs to test the consistency of the top-ranked peaks, measured by the SD of computed ranks from multiple runs. To establish an upper cutoff value on a peak's rank SD for its performance not to be considered as purely by chance, the same bootstrap procedure was applied to a randomly generated data set that simulated the distribution of the real data. The minimum value of rank SDs from such "simulated peaks" indicates the degree of consistency that a peak might achieve by random chance. This minimum value was used as the cutoff to help to reduce the original 147 peaks to a subset of 15 peaks for further consideration. The performance of such peaks should be less likely attributable to random artifacts in the data.

For simplicity, the composite index described in this report was derived by simple multivariate logistic regression. When these selected biomarkers are further validated, more complex and nonlinear classification models may be used to combine the multiple biomarkers. The use of complex modeling methods on carefully screened and tested biomarkers should in general offer a more robust performance than the direct application of such methods on raw data from a large number of mass peaks.

The number of specimens analyzed in this study to some degree limited the validity of the results. The bootstrap cross-validation estimation of performance offers statistical confidence on the generalizability of these biomarkers over future data. Further independent validation studies are needed. In such studies, the specificity of these selected biomarkers for detection of breast cancer needs to be addressed by testing specimens from other types of cancer. In addition, validation data sets preferably should be from sources different from that of the original training data set. This is one way to ensure that the performance of the selected biomarkers is not influenced by systematic biases between the disease and the control specimens.

For the three biomarkers selected, no significant correlation was found between the concentrations of the markers and tumor size or lymph node metastasis. The discriminatory power of these markers therefore most likely reflects the malignant nature of the tumor rather than its progression. The origin and identity of BC1, BC2, and BC3 are currently under investigation. Furthermore, it is not our intent at this stage to suggest a final diagnostic algorithm based on nonlinear classification.

In conclusion, we have shown that using proteomics approaches such as Ciphergen ProteinChip Arrays and SELDI-TOF MS in combination with bioinformatics tools could facilitate the discovery of new biomarkers. Using the panel of three selected biomarkers, we could achieve high sensitivity and specificity for the detection of breast cancer.

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