Tumor proteomic profiling predicts the susceptibility of breast cancer to chemotherapy

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Abstract. Chemotherapy is often used for breast cancer treatment, but individual outcome varies widely. We hypothesized that tumor proteomic profiles obtained prior to chemotherapy may predict the individual tumor response to treatment. The goal of our study was to explore feasibility of using proteomic profiling to preselect patients for an effective chemotherapeutic regimen. Tumors from 52 patients with T2-T4 breast cancer were prospectively collected before neoadjuvant chemotherapy, and were analyzed using surface-enhanced laser desorption ionization/time of flight (SELDI) mass spectrometry. Mass spectral profiles were obtained from tumors with various sensitivities to chemotherapy. Both non-supervised hierarchical clustering and supervised neural network-based classification approaches were employed to compare the profiles. The first two thirds of the enrolled cases (35) were allocated to a training set to select peaks characteristic of resistant tumors. The candidate peaks were used to develop a predicting rule to evaluate the remaining 17 specimens in the validation set. In the training set, the most prominent differences were found between drug resistant and drug susceptible tumors by non-supervised hierarchical clustering. In the validation set, the supervised classification with the K nearest neighbor (KNN) model correctly classified most tumor responses with an accuracy rate of 92.3% [100% of resistant tumors (4/4), and 84.6% of the tumors with favorable response (11/13)]. In the entire group, a single peak at m/z 16,906 correctly separated 88.9% of the tumors with pathologically complete response, and 91.7% of the resistant tumors. The data suggest that breast cancer protein biomarkers may be used to pre-select patients for optimal chemotherapeutic treatment.

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Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; DCIS, ductal carcinoma *in situ*; DLDA, diagonal linear discriminant analysis; FDR, false discovery rate; FISH, fluorescence *in situ* hybridization; GEPAS, gene expression pattern analysis suite; IR, intermediate responders (>25% but ≤75% tumor regression); KNN, K nearest neighbor; MALDI-TOF, matrix-assisted laser desorption ionization/ time of flight; MS, mass spectrometry; NR, non-responders, chemo-resistant tumors (≤25% tumor regression); PAM, prediction analysis with microarrays; pCR, pathological complete response, no residual cancer found at primary tumor site; R, responders (>75% of tumor regression); ROC, receiver operator characteristic; SELDI-TOF, surface-enhanced laser desorption ionization/time of flight; SOM, self-organizing map; ST, chemo-susceptible tumors (>25% tumor regression); SVM, support vector machines; T2, primary tumor size more than 2 cm and up to 5 cm; T3, primary tumor size more than 5 cm; T4, primary tumor of any size with direct invasion to chest wall or skin; TIC, total ion current; TRR, tumor regression rate

Key words: proteomic biomarker, breast cancer, neoadjuvant chemotherapy, mass spectrometry, bioinformatics

Introduction

Preoperative chemotherapy (neoadjuvant chemotherapy) has gained wide acceptance for treating patients with locally advanced breast cancer. In these patients the most acknowledged benefit is the reduction in tumor size allowing either a complete resection of an otherwise unresectable tumor or breast conservation surgery in some patients with large tumors. Furthermore, tumor response to neoadjuvant treatment has been shown to be prognostically significant. Other less proven benefits of neoadjuvant chemotherapy include eradication of microscopic metastases and improved survivals (1-3). Postoperative adjuvant chemotherapy has been used successfully to improve survival rates in patients with early stage breast cancer (4). Although many patients benefit from chemotherapy, some fail to respond. However, the success of chemotherapy, whether pre- or post-operative, in any given individual cannot be predicted. The uncertain benefit for a particular individual, as well as significant toxicity of chemotherapy in all patients, calls for development of methods to select the right patients for treatment and spare those who will not benefit.

There already is precedence for using the expression of specific proteins by tumors to select treatment and to monitor disease progression. For example, estrogen and progesterone receptor levels in tumor tissues are now used to predict benefit of Tamoxifen therapy in women of all ages (5), ovarian ablation in premenopausal patients (6,7) and aromatase inhibitor treatment in postmenopausal women (5,8,9). More recently HER2/neu status also has been used to predict effectiveness of the combination of chemotherapy and Herceptin in women with HER2/neu positive breast cancer (10-12). The Breast Cancer International Research Group conducted a multicenter phase III trial to investigate the best combination of chemotherapy and Herceptin treatment for these patients. The study concluded that Herceptin with chemotherapy was superior to chemotherapy alone in patients with HER2 positive early-stage breast cancer (Slamon *et al*, 29th Annual San Antonio Breast Cancer Symposium, abs. 52, 2006). Estrogen and progesterone receptor levels and HER2/neu status also were prognostically valuable in these patients.

However, no reliable biomarkers currently are available to predict the response of an individual to a particular type of chemotherapy. Several recent studies have attempted to use gene expression profiling and bioinformatics to predict the need for adjuvant chemotherapy in patients with early stage breast cancer (13-15). The Oncotype DX Breast Cancer Assay uses reverse transcriptase-polymerase chain reaction to correlate the expression of a panel of 21 genes in hormone receptor positive and lymph node negative tumors to predict the risk of disease recurrence. A score calculated from the expression of these genes predicts the risk of tumor recurrence after initial Tamoxifen treatment, and is used to assess the need for further chemotherapy. This study showed that the difference in the risks of distant recurrence between patients with low and high scores by the Oncotype DX test was statistically significant and clinically useful (14).

Similar to the genomic analysis of cancer-related genes, proteomics has emerged as a potentially powerful tool for tumor classification through protein biomarker profiling. It is now widely recognized that proteomics has the potential to improve disease diagnosis and management (16). However, the proteome changes constantly due to the influence of many factors such as aging, disease and environmental exposure. Thus, studying the cellular or circulating proteome has been more challenging than the genome (17). The application of proteomics to monitor tumor response to chemotherapy is only in the early stages, and our current study was specifically designed to compare SELDI mass spectra of breast cancer tissues from chemotherapy responsive and non-responsive patients receiving the same neoadjuvant treatment.

Evaluation of chemotherapy response in these patients is measured both by microscopic examination of surgically removed tissue (pathological examination), and by physical examination and/or imaging studies (clinical examination). The pathological response is usually classified as either pathologically complete response (pCR) or having residual tumor after neoadjuvant treatment. Clinical responses are graded as a change in tumor size from baseline to post-neoadjuvant treatment: complete response (no tumor detected), partial response (tumor shrinks ≥50%), marginal response (tumor shrinks 25-49%), stable disease (tumor size changes <25%), and tumor progression (tumor size increases >25%). The latter two groups are frequently considered non-responders. It was our goal to use proteomic technologies to study differences between drug resistant and sensitive tumors in an effort to improve tumor classification and patient selection for individualized chemotherapy.

Materials and methods

Patients and clinical information. Fresh tumor tissues from 52 patients with non-metastatic T2-T4 breast cancer were prospectively collected for this study. All patients participated in an Institutional Review Board (IRB) approved phase II clinical trial to study the efficacy of neoadjuvant Taxotere/Carboplatin ± Herceptin therapy. The patient inclusion criteria for the clinical trial were: (i) females aged 18 and older with a tissue-proven diagnosis of stage II-III invasive breast cancer; (ii) measurable primary breast cancer; (iii) no prior chemotherapy for the current breast cancer or prior radiation of the involved breast; (iv) available tissue estrogen and progesterone receptor status by immunohistochemical staining, and HER2/neu status by the fluorescent *in situ* hybridization (FISH) test; and (v) completion of the clinical staging requirements. The patient exclusion criteria included: (i) refusal of definitive resection of primary tumor; (ii) evidence of metastasis; (iii) pregnancy or nursing conditions, and fertile women not on birth control. Written informed consent was obtained from all participants and baseline clinical assessment of tumor size, tissue diagnosis and tumor biomarker status was recorded.

Because a baseline tumor size by pathologic evaluation was not possible, the clinically measured tumor size prior to chemotherapy was used as baseline. Post-chemotherapy information including tumor size measured by both clinical and pathological methods, lymph node staging, and tumor biomarkers were collected from each definitive breast cancer surgery. The formula used to calculate tumor regression rate (TRR) was as follows: (baseline tumor size-residual tumor size)/baseline tumor size, where the baseline tumor size was measured clinically and the post chemotherapy residual invasive tumor size was measured pathologically. The TRR was categorized into three groups: (i) responders (TRR >75%, R), (ii) intermediate responders (25% <TRR ≤75%, IR) and (iii) non-responders with minimal to no response or tumor progression (TRR ≤25%, NR).

Tumor tissue collection and sample preparation. Breast cancer tissues were prospectively collected from each patient at two different time points: (i) at baseline before chemotherapy and (ii) at the time of definitive cancer surgery after completion of neoadjuvant chemotherapy for proposed proteomic study. Tumor specimens were placed on ice immediately after removal, and transferred to the UCLA Breast Cancer Tissue Bank. When specimens were obtained from sites other than UCLA, they were delivered on ice and received for processing within 24 h of sample collection. Upon arrival, the specimens were grossly dissected to remove excess adipose tissue before dividing into three parts and storage at -80˚C.

In the results reported here, tissue specimens collected at baseline before chemotherapy were extracted by homogenizing

Table I. Patient characteristics.

Characteristics	Total patients (%)	Training set (%)	Validation set (%)
Age, years			
≥50	26 (50.0)	18 (51.4)	8 (47.1)
<50	26 (50.0)	17 (48.6)	9 (52.9)
Mean age	49.4	49.3	49.7
Tumor histology[a]			
IDC	42 (80.8)	27 (77.1)	15 (88.2)
ILC	8 (15.4)	7 (20.0)	1 (5.9)
IDC+ILC	1 (1.9)	0	1 (5.9)
ITC+IDC	1 (1.9)	1 (2.9)	0
Tumor stage			
T2	5 (9.6)	0	5 (29.4)
T3	32 (61.5)	26 (74.3)	6 (35.3)
T4	15 (28.8)	9 (25.7)	6 (35.3)
Tumor differentiation			
Poor	13 (25)	8 (22.9)	5 (29.4)
Moderate	7 (13.5)	6 (17.2)	1 (5.9)
Well	6 (11.5)	3 (8.6)	3 (17.6)
Unknown	26 (50)	18 (51.4)	8 (47.1)
Tumor grade			
High	16 (30.8)	11 (31.4)	5 (29.4)
Intermediate	11 (21.2)	7 (20)	4 (23.5)
Low	6 (11.5)	5 (14.3)	1 (5.9)
Unknown	19 (36.5)	12 (34.3)	7 (41.2)
Tumor IHC marker[b]			
ER positive	33 (63.5)	21 (60.0)	12 (70.6)
PR positive	21 (40.4)	14 (40.0)	7 (41.2)
HER2/neu positive	21 (40.4)	15 (42.9)	6 (35.3)
Neoadjuvant chemotherapy[c]			
TC	31 (59.6)	20 (57.1)	11 (64.7)
TCH	10 (19.2)	8 (22.9)	2 (11.8)
TC/H	11 (21.2)	7 (20.0)	4 (23.5)
Ethnicity			
Asian	8 (15.4)	5 (14.3)	3 (17.6)
Black	4 (7.7)	2 (5.7)	2 (11.8)
Hispanic	8 (15.4)	7 (20.0)	1 (5.9)
White	32 (61.5)	21 (60.0)	11 (64.7)
Surgery[d]			
Mastectomy	33 (63.5)	22 (62.9)	11 (64.7)
Breast-conserving treatment	19 (36.5)	13 (37.1)	6 (35.3)

[a]IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ITC, invasive tubular carcinoma. [b]ER, estrogen receptor; PR, progesterone receptor. [c]TC, preoperative Taxotere 75 mg/m^2, carboplatin (AUC=6) every three weeks for four cycles; TCH, preoperative TC with weekly Herceptin at a loading dose of 4 mg/kg and subsequent dose of 2 mg/kg; TC/H, TC preoperatively with Herceptin given after surgery. [d]Mastectomy includes modified radical mastectomy (MRM) and simple mastectomy (SM).

the frozen tissue in liquid nitrogen, followed by suspension in 1% Triton X-100. The samples were re-frozen at -80˚C and then thawed on ice for 30 min. The freeze/thaw process was repeated, and each suspension was subjected to centrifugation (10,000 x g, 10 min, 4˚C). The supernatants were then subjected to albumin and immunoglobulin depletion using an albumin and IgG removal kit (Amersham) as well as hemoglobin depletion using Ni-NTA magnetic agarose beads (Qiagen). Briefly, 20 μl of the tissue homogenate supernatant was added to a 320 μl slurry containing the antibody-coated beads to first remove IgG and albumin. The mixture was first incubated at room temperature for 30 min with gentle rotation, then transferred to a microspin column, and centrifuged (10,000 x g, 5 min, room temperature). Forty microliters of the

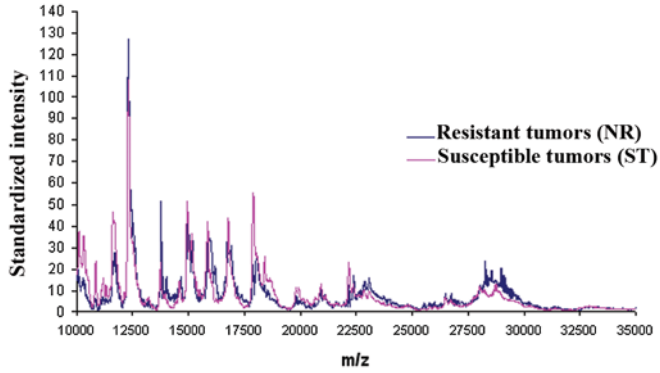

Figure 1. Normalized median intensity SELDI-TOF mass spectra of resistant (NR) and susceptible (ST) tumors. SELDI-TOF spectra of breast cancer tissue extracts from the chemotherapy resistant (NR, blue; n=12) and susceptible (ST, pink; n=40) groups.

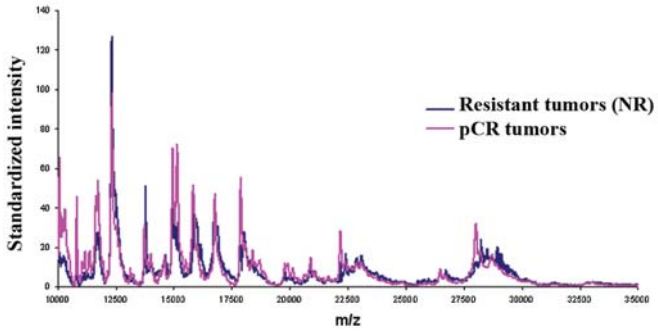

Figure 2. Normalized median intensity SELDI-TOF mass spectra of resistant (NR) and pathologically complete response (pCR) tumors. SELDI-TOF spectra of breast cancer tissue extracts from the chemotherapy resistant (NR, blue; n=12) and pathologically complete response (pCR, pink; n=9) tumors.

filtrate was then added to 20 μl of Ni-NTA magnetic agarose beads and the mixture was incubated with gentle rotation. A magnetic particle concentrator (Dynal) was used to remove the beads. Two microliters of the final supernatant was mixed with 2 μl of freshly prepared sinapinic acid solution (20 mg/ml in water/acetonitrile/trifluoroacetic acid, 30/70/0.1, v/v/v), and 2 μl of the mixture was loaded in duplicate onto NP20 ProteinChips (Ciphergen Biosystems Inc.).

Surface enhanced laser desorption ionization mass spectrometry (SELDI-MS). Linear time-of-flight mass spectra were recorded without time-lagged source focusing (Model PBS II, Ciphergen Biosystems Inc.) using external calibration with bovine cytochrome C (12,230.9 Da), ß-lactoglobin A (18,363.3 Da), bovine serum albumin (66,410.0 Da) and IgG (147,300.0 Da). Data acquisitions were made using the instrument-supplied automated protocol with the following settings: high mass for acquisition, 100 kDa; optimization range 10-100 kDa; laser intensity 250 arbitrary Units; detector sensitivity 9. The raw data were collected using the instrument-supplied software (Ciphergen ProteinChip® Software 3.1).

Normalization of the mass spectra and peak alignment. After baseline subtraction, the spectra were normalized by dividing the raw signal intensities by the area under the curve (AUC) between m/z 10,000 and 35,000. Data outside of this range were omitted from each profile. The intensities of signals beyond m/z 35,000 were close to zero, and the signal intensities below m/z 10,000 were unusable because in most cases individual peaks could not be distinguished in this range. This normalization process is equivalent to total ion current (TIC) normalization since the number of observations between m/z 10 K and 35 K were about the same in all samples (data not shown).

Peak alignment/clustering was carried out using the maximal clique method of Li and Gentleman (18) using the within duplicate error equation to form the boundaries around each m/z signal. Analysis of m/z error showed that the error was a linear function of m/z of the form duplicate m/z error = e = a + b average m/z. For a given signal, the m/z bounds were defined as [m/z - e, m/z + e]. The i^{th} cluster (i^{th} 'clique') was defined as the union of all signals whose m/z bounds overlap with the bounds of any other signal. Signals in the same cluster were assumed to have the same true m/z, and were aligned by assigning the peak the average m/z value of all the signals in the cluster. If a sample did not have a peak in a given cluster, the non-peak normalized intensity value at the cluster mean m/z was used.

Univariate analysis/comparison. The distribution of the aligned and normalized peak intensities were compared between any two patient groups at each m/z signal using the non-parametric Wilcoxon rank sum test. Comparison among the three patient groups (NR, IR and R) at each m/z signal was made using the parametric F statistic. In addition, a receiver operator characteristic (ROC) analysis was carried out for each m/z signal. In the ROC analysis, a candidate threshold value was chosen and the percent of intensities less than the threshold in each group was computed. The threshold was varied until the value of the threshold that maximizes the difference in the percentage between the two groups was found.

Multivariate bioinformatic analysis. Multivariate bioinformatic analysis of the normalized and aligned spectral peaks was performed using Gene Expression Pattern Analysis Suite v4.0 (GEPAS, http://gepas.bioinfo.cipf.es), a web-based microarray software package. The cohort was arbitrarily divided into two groups: the first two thirds of the enrolled patients (35 cases) were allocated to a training set to select the m/z peaks that appeared to be characteristic of tumor susceptibility to chemotherapy. The remaining 17 cases were assigned to the validation set to confirm the classification of each tumor by the candidate m/z signals.

First, the 35 cases in the training set were divided into the NR, IR and R groups according to the residual tumors found after chemotherapy. An analysis of variance (ANOVA) test was used to compare the spectral differences at each peak location among the three groups with a liberal significance criterion $p<0.25$ (FDR-indep). The preprocessed data were then used to select significant spectral peaks differentiating the groups. According to the bioinformatic analysis criteria used in our two previous studies (19,20), we selected both the fold difference of at least 2 and statistical significance <0.05 to define significant m/z peak differences.

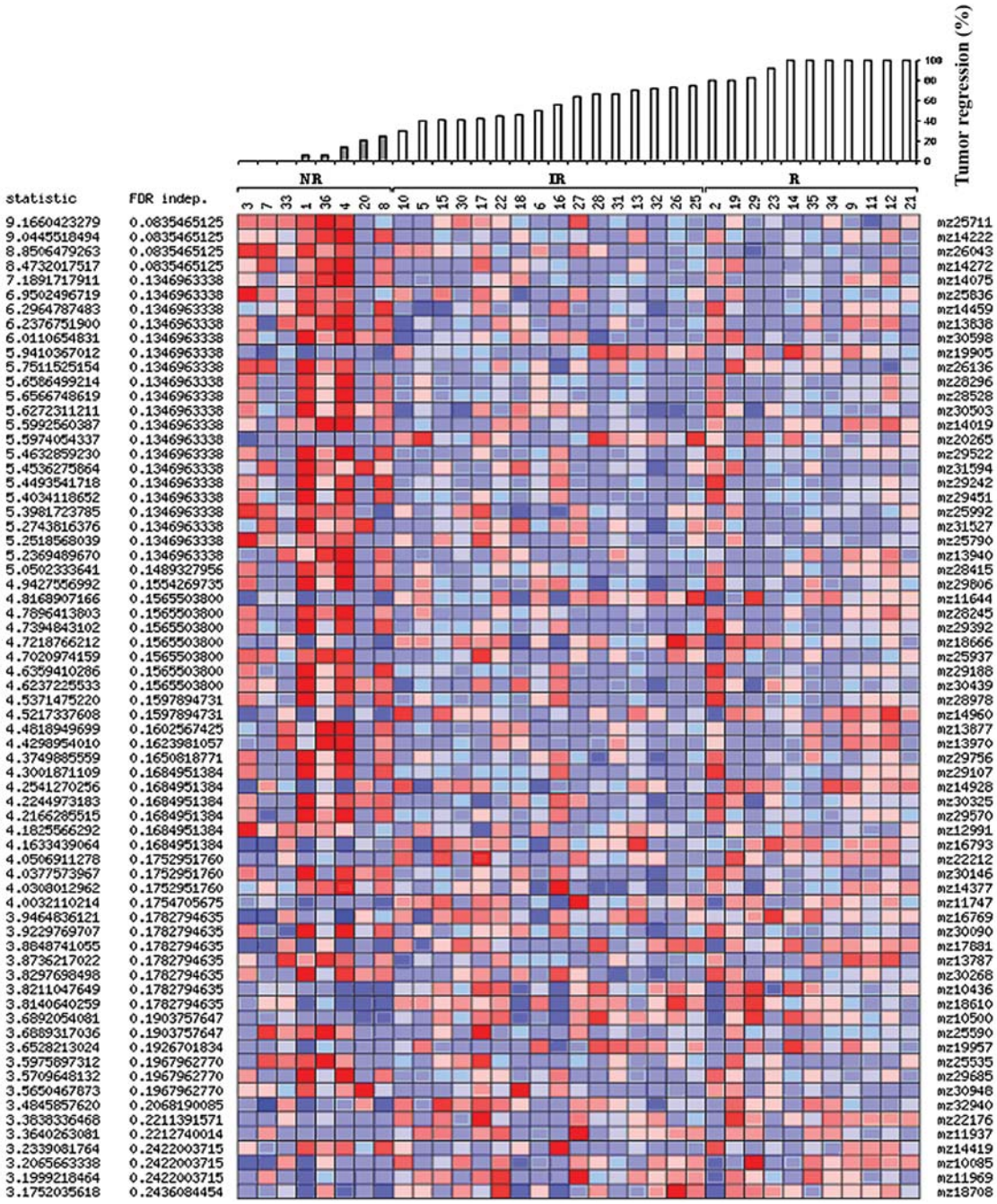

Figure 3. Heat map display of m/z signals with significant differences among NR, IR and R groups in the training data set (n=35). The 35 cases in the training set were classified into three groups based on tumor regression rate (TRR): NR group (TRR ≤25%), 8 cases; IR group (25% <TRR ≤75%), 16 cases; R group (TRR >75%), 11 cases. Normalized m/z values were compared by multi-class ANOVA statistics using the T-REX tool set of the GEPAS software. Each column represents a case as labeled on top, and each row represents a normalized m/z signal as indicated at the right. ANOVA statistics and adjusted p-values using the FDR procedure of Benjamini and Hochberg are presented at the left. Sixty-eight peaks with FDR indep. <0.25 are displayed.

The subset of peaks meeting the median 2-fold difference, and $p<0.05$ by Wilcoxon rank sum test criteria, were then used to build a classification rule. The rule was built using four different supervised classification algorithms in the GEPAS software: (i) Diagonal Linear Discriminant Analysis (DLDA), (ii) K-Nearest Neighbor Clustering (KNN), (iii) Support Vector Machines (SVM), (iv) Prediction Analysis with Microarrays (PAM). The accuracy for each of these methods during predictor building was estimated by the leave-one-out permutation method in the training set. In the validation data set, the method with the highest accuracy was selected as the optimal predicting algorithm. In both the univariate and the multivariate analyses, accuracy was defined as the percent of a given group that was correctly classified. Overall (unweighted) accuracy was defined as the average accuracy across all groups.

Results

Clinical response of breast tumors to neoadjuvant chemotherapy. The characteristics of the 52 patients and their tumor histopathologic features are summarized in Table I. The

Table II. Differential m/z signals separating drug resistant (NR, n=8) from more susceptible breast cancers (ST, n=27) in training set.

Signal	NR median	ST Median	NR/ST	Wilcoxon p-value
m/z 10085	13.552	55.207	0.245	0.010
m/z 10290	15.934	37.951	0.420	0.025
m/z 10318	11.658	40.645	0.287	0.007
m/z 10373	11.248	24.366	0.462	0.031
m/z 10436	8.459	19.550	0.433	0.012
m/z 10500	5.629	17.697	0.318	0.005
m/z 10542	4.322	11.621	0.372	0.045
m/z 10821	7.045	20.499	0.344	0.005
m/z 10841	5.031	29.447	0.171	0.003
m/z 11124	4.413	10.914	0.404	0.018
m/z 11171	5.621	16.857	0.333	0.020
m/z 11197	5.427	17.027	0.319	0.041
m/z 11370	5.025	11.535	0.436	0.045
m/z 11644	13.724	42.135	0.326	0.003
m/z 11717	18.920	41.174	0.460	0.008
m/z 11747	14.819	48.286	0.307	0.003
m/z 11937	5.626	13.663	0.412	0.013
m/z 11969	6.103	13.868	0.440	0.020
m/z 12991	6.375	3.114	2.047	0.028
m/z 13838	36.200	13.824	2.619	0.045
m/z 14019	22.488	7.148	3.146	0.050
m/z 14075	22.362	6.792	3.293	0.050
m/z 14222	10.193	3.486	2.924	0.005
m/z 14272	10.680	2.602	4.104	0.006
m/z 14377	7.356	3.533	2.082	0.020
m/z 14459	9.953	4.700	2.118	0.013
m/z 14960	19.682	48.587	0.405	0.017
m/z 16793	23.311	46.710	0.499	0.020
m/z 17881	3.785	42.412	0.089	0.007
m/z 18391	10.419	22.691	0.459	0.008
m/z 18610	7.293	14.585	0.500	0.010
m/z 18666	5.670	14.485	0.391	0.005
m/z 18708	5.884	13.414	0.439	0.015
m/z 19744	2.749	6.478	0.424	0.031
m/z 19905	2.344	12.108	0.194	0.000
m/z 19957	4.267	9.181	0.465	0.004
m/z 20098	3.608	7.559	0.477	0.045
m/z 20186	2.459	5.287	0.465	0.006
m/z 20265	1.640	5.828	0.281	0.000
m/z 20434	0.977	2.859	0.342	0.028
m/z 20663	2.169	5.907	0.367	0.028
m/z 22176	3.542	16.807	0.211	0.003
m/z 22212	2.852	13.960	0.204	0.002
m/z 25711	3.363	1.097	3.066	0.002
m/z 25790	4.608	1.803	2.556	0.041
m/z 25836	4.815	1.687	2.854	0.007
m/z 25937	4.044	1.288	3.139	0.041
m/z 25992	4.333	2.003	2.163	0.041

Table II. Continued.

Signal	NR median	ST Median	NR/ST	Wilcoxon p-value
m/z 26043	4.137	1.602	2.583	0.034
m/z 26136	2.620	1.063	2.464	0.031
m/z 28978	18.523	7.817	2.369	0.037
m/z 30598	3.364	1.663	2.023	0.018
m/z 30892	2.196	1.044	2.104	0.020
m/z 30948	1.905	0.800	2.381	0.006
m/z 31594	2.427	1.144	2.121	0.025
m/z 31848	1.956	0.713	2.744	0.041

The m/z signals were selected by both the median-fold difference (minimally 2-fold) and statistical significance (Wilcoxon test, $p<0.05$). This produced a set of 56 signals from a total of 300 signals.

average age of the patients was 49.4 (range 29-84) years. After neoadjuvant chemotherapy, six patients had no evidence of residual invasive or *in situ* disease at the primary tumor site, and no metastasis in lymph nodes; two patients had residual ductal carcinoma *in situ* (DCIS) and positive lymph nodes; one patient had no residual invasive or *in situ* cancer in the breast but still had micrometastasis (0.05 mm) in a single node; and 43 patients had residual invasive cancers with 62.8% (27/43) having positive lymph nodes. Before chemotherapy the mean tumor sizes for the 9 pathological complete tumor response (pCR) and 43 non-pCR patients were 7.17 and 8.40 cm, respectively. Of the 43 non-pCR patients, 12 were classified as drug resistant with a calculated TRR ≤25% who were also considered as non-responders (NR). Eight of 12 NR were used in the training dataset and 4 in the validation dataset.

Comparison of SELDI spectra from non-responders (NR) and chemo-susceptible tumors (ST). The median intensity SELDI-TOF spectra of the NR and ST groups from the total patient cohort are shown in Fig. 1. Fifty-nine of a total of 300 m/z peaks were found to have significantly different median peak intensity in the ST (n=40) vs. NR (n=12) groups using the criterion $p<0.05$ (data not shown). In particular, the difference in peak intensity at m/z 20,265 correctly classified 91.7% (11/12) of the NR tumors and 75% (30/40) of the ST tumors with an overall unweighted accuracy of 82.5% ($p=0.0014$).

Comparison of SELDI spectra from non-responders (NR) and pathologically complete responders (pCR). The median intensity SELDI-TOF spectra of the NR and pCR groups are shown in Fig. 2. Thirty-one of a total of 198 m/z peaks were found to have significantly different median peak intensity in the NR (n=12) vs. pCR (n=9) groups using the criterion $p<0.05$ (data not shown). In particular, the median intensity of the m/z 16,905 peak was 30.6 in the NR group and 17.1 in the pCR group. With a separating threshold intensity of 20.4, 11 of 12 patients with NR tumors were above, and 8 of 9 pCR tumors were below the threshold. Thus 88.9% of the pCR tumors and 91.7% of the NR tumors were correctly classified by the difference of the peak intensity at m/z 16905. The overall unweighted accuracy was 90.3%.

Table III. Comparison of the accuracy of four different methods used for building the unbiased predictor by permutation tests (the leave-one-out approach) in the training set.

	DLDA	KNN	SVM	PAM
Accuracy (%)	85.7	76.6	69.3	80.0

The methods used for building the unbiased predictor include (A) Diagonal Linear Discriminant Analysis (DLDA), (B) Nearest neighbor (KNN), (C) Support Vector Machines (SVM) and (D) Prediction Analysis with Microarrays (PAM). The cross-validation accuracy for the selected set of signals were tested by the leave-one-out approach in the training set, and the DLDA method was found to have the highest accuracy (85.7%).

Proteomic pattern of tumor responses to neoadjuvant chemotherapy in the training set. A three-group comparison was first performed in the training set to explore the spectral differences between the NR, IR and R groups by ANOVA using the T-REX toolset of the GEPAS software. A set of 68 peaks was identified with significant changes across the NR, IR and R groups (FDR-indep. $p<0.25$, Fig. 3). Noticeably, more prominent and significant changes appeared between NR and the other two (R+IR) groups. Therefore, in the subsequent comparison and in the validation set, the R and IR groups were collapsed into one 'susceptible' (ST) group and compared to the 'resistant' (NR) group.

From the initial 300 peaks, 56 were identified as being different between NR and ST tumors in the training set as defined by the criteria of at least a 2-fold difference of peak intensity, and statistical significance of $p<0.05$ by Wilcoxon test (Table II). This set of 56 peaks was employed to build a classifier using four classification algorithms in the GEPAS (DLDA, KNN, SVM and PAM) to predict tumor response in the validation set. In the training set, the performance of

Table IV. Performance comparison of various supervised classifications of tumor response to neoadjuvant chemotherapy in the validation set.

	Percentage of correctly predicted in NR (%)	Percentage of correctly predicted in ST (%)	Overall accuracy (%)
DLDA	100 (4/4)	0.0 (0/13)	50.0
KNN	100 (4/4)	84.6 (11/13)	92.3
SVM	0 (0/4)	92.3 (12/13)	46.2
PAM	25 (1/4)	84.6 (11/13)	54.8

The predictors derived from the training set were used to predict chemo-responses in the validation set (n=17). The performances of the four algorithms (DLDA, KNN, SVM and PAM) were tested and compared. Accuracy rates of these methods ranged 46.2-92.3%. The best prediction was obtained by the KNN model where K=1. The KNN model correctly classified 100% of resistant tumors and 84.6% of the tumors with favorable response, achieving an overall accuracy rate of 92.3%.

these algorithms was evaluated by cross-validation using a permutation test (leave-one-out approach). As shown in Table III, the accuracy rates of these methods were similar (69.3-85.7%) with the highest accuracy achieved by the DLDA method.

Predicting breast tumor responses to neoadjuvant chemotherapy in the validation set. The 56 peaks (predictors) derived from the training set were then used to classify the 17 cases in the validation data set and predict the chemoresponses. The performances of the four algorithms (DLDA, KNN, SVM and PAM) were tested and compared (Table IV). Accuracy rates of these methods were between 46.2-92.3%. The optimal prediction was obtained by the KNN model where K=1. The KNN model correctly classified 100% of resistant tumors (4/4) and 84.6% of the susceptible tumors (11/13), achieving an overall accuracy rate of 92.3%.

Discussion

In this study, SELDI-TOF mass spectrometry was used to compare the protein profiles of 52 breast tumor specimens derived from patients with large breast cancers who had known responses to a defined neoadjuvant regimen. To our knowledge, this is the first attempt to use MS generated profiles to predict the response of human breast cancer tissue to chemotherapy. The proteomic differences in groups of patients with either susceptible or resistant tumors to neoadjuvant chemotherapy were investigated, and differential proteomic signals were selected. These candidate signals appeared to correlate well with clinical response of the tumors to the neoadjuvant chemotherapy. Using the methods described, we found that many prominent proteomic differences existed between drug resistant breast tumors and those with various degrees of drug response. This result suggests the presence of a unique proteomic signature characteristic of tumor chemosensitivity.

Even with these highly selective analytical tools, it was not possible to accurately predict tumor responses to treatment in all cases. Two of the 13 chemotherapy susceptible tumors were misclassified as resistant tumors. The two misclassified cases were T3 and T4 infiltrating ductal carcinomas respectively, with one being a triple negative tumor (negative for estrogen receptor, progesterone receptor and HER2/neu), and the other being progesterone receptor negative and HER2/neu negative. From a clinical point of view, it is possible that triple negative or negative estrogen and/or progesterone receptors are sufficient to predict a favorable response to taxotere and carboplatin (21). Our studies suggest that both proteomic biomarkers and clinical variables should be taken into account in building future prediction models.

When the four algorithms were compared in the training set, DLDA was found to have the highest accuracy (85.7%) by permutation test. However in the validation set, KNN was shown to have the highest accuracy (92.3%). The inconsistency of this finding derived from the training and validation data sets may be due to the small sample sizes. Also with the limited sample size especially in the validation group, the accuracy in the DLDA and SVM methods are low. DLDA did predict 4 out of 4 NR, but failed to predict ST. Meanwhile, SVM predicted 12 out of 13 ST but missed the NR. A larger sample size may improve the consistency and accuracy in building and testing the prediction model.

Several types of proteomic analytical tools are being used to study disease-related biomarkers. These include the open-ended technologies such as 2-dimension gel electrophoresis and mass spectrometry, and closed-ended technologies such as antibody microarrays, antibody bead arrays, reverse phase protein microarrays, and tissue microarrays. Mass spectrometry-based technologies are being continually refined for proteomic profiling since their introduction into this field about a decade ago. SELDI-TOF and MALDI-TOF have been used to profile serum and plasma with the aim of identifying proteomic fingerprints that may differentiate individuals with normal breasts from cancerous breasts (22-26), predict *in vitro* response of breast cancer cell lines to chemotherapy (27,28), and predict the clinical tumor response to chemotherapy using plasma samples (29).

However, in recent years SELDI/MALDI based proteomic profiling has faced criticisms concerning the sensitivity, reproducibility and inability to identify candidate proteins with most criticisms directed to serum profiling studies (30-35). Cancer related proteins when present in serum are trace in amount because they are diluted in the large volume of blood,

and masked by numerous other abundant proteins that normally occur in the circulation. These problems are either not relevant or are less important for the direct analysis of tumor tissue, as performed in this study. Standardized protocols were used for tissue collection, storage and sample preparation to minimize variability between samples. We have also previously demonstrated that the SELDI spectra of the same specimens are relatively stable and reproducible, with virtually identical spectra produced from the same sample upon re-analysis several days later (36). Since the SELDI-TOF technology enables a straightforward analysis of protein biomarkers, it provides a means to rapidly evaluate intact protein patterns in a large number of specimens. It was therefore chosen as the first attempt to profile breast cancer tissues with different responses to neoadjuvant chemotherapy.

Our results show that SELDI-TOF MS protein profiling of breast cancer tissue obtained before chemotherapy may predict the chemoresponse of breast cancer. There were many prominent proteomic differences between drug resistant breast tumors and those with various degrees of drug response. The major disadvantage of SELDI/MALDI is the inability of direct identification of promising biomarker signals by the technology. Our future study will concentrate on extending these findings with larger sample sizes, and simultaneously identifying the proteins that generate the SELDI signals of interest using nano-LC-Orbitrap instrumentation from shotgun digests of the same samples.

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