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Proteomic Identification of Interleukin-2 Therapy Response in Metastatic Renal Cell Cancer

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Abstract

Introduction—To detect a predictive protein profile that distinguishes between IL-2 therapy responders and non-responders among metastatic RCC patients we used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI TOF-MS).

Materials and Methods—Protein extracts of 56 metastatic clear cell RCC patients obtained from radical nephrectomy specimens and prior to IL-2 therapy were applied to protein chip arrays of different chromatographic properties and analyzed using SELDI TOF-MS. A class prediction algorithm was applied to identify a subset of protein peaks whose expression values were associated with IL-2 response status. Multivariate analysis was performed to assess the association between the proteomic profile and the IL-2 response status controlling for the effect of lymphadenopathy.

Results—From a total of 513 protein peaks we discovered a predictor set of 11 peaks that performed optimally for predicting IL-2 response status (86 % accuracy, Fisher's $p<0.004$,

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permutation p<0.01). The results were validated on an independent data set with an overall accuracy of 72% (p < 0.05, permutation p<0.01). On multivariate analysis the proteomic profile was significantly associated with IL-2 response when corrected for lymph node status (p< 0.04).

Conclusions—We have identified and validated a proteomic pattern that is an independent predictor of IL-2 response. The ability to predict the probability of IL-2 response could permit targeted selection of patients most likely to respond to IL-2, while avoiding unwanted toxicities in patients less likely to respond. This proteomic predictor has the potential to significantly aid clinicians in the decision making of appropriate therapy for metastatic RCC patients.

Keywords

proteomics; metastatic Renal Cell Cancer; IL-2 therapy; surfaced-enhanced laser desorption/ ionization time-of-flight mass spectrometry; biomarkers

Purpose

Renal Cell Cancer (RCC) has a heterogeneous clinical presentation with up to 30% metastatic cases at initial diagnosis and approximately 30% of initially organ-confined cases developing metastases during follow-up at variable intervals[1]. Recently FDA approved oral agents for metastatic RCC offer improved progression free and overall survival, but essentially no complete responses[2]. Aggressive treatment with radical nephrectomy and immunotherapy has also been shown to provide a survival benefit in metastatic RCC patients[3, 4], and retrospective data suggest that five-year survival rates were improved when IL-2 was used as adjunctive immunotherapy instead of IFN-α (19.6% vs. 10%)[5]. Although the administration of IL-2 offers the possibility of durable CRs, it is associated with considerable toxicity, while only a small subset of patients respond to therapy[3]. Possible biomarkers for RCC such as carbonic anhydrase-IX (CA IX), Ki67 or PTEN have been published[6, 7]. However, there is no reliable single marker available to identify those patients that are more likely to respond to IL-2 therapy, although factors such as regional lymphadenopathy, expression of CA-IX[8, 9], and selective pathologic subtypes have been proposed[10].

Proteomics is an emerging field of science that attempts to study proteins in a massively parallel scale. Mass spectrometry (MS) based technologies such as MALDI (matrix assisted laser desorption /ionization), Surface enhanced laser/desorption ionization time of flight (SELDI-TOF MS), electrospray and ion trap have become the methods of choice in biomarker discovery and enable the separation of proteins and peptides according to their mass over charge (m/z) ratio. However, due to the large variations in concentrations of the proteome in tissues and bodily fluids most of the mass spectrometric approaches are not amenable to large numbers of patient samples. SELDI-TOF MS is one of few proteomic technologies suitable for high-throughput protein profile generation across a wide range of molecular weights, while proteins are retained on protein chips based on their binding affinity to the chip surface. While SELDI appears to be more sensitive in the low-mass range, which holds valuable information for biomarker discovery, SELDI by itself cannot identify protein IDs. Nevertheless, we have previously developed and validated a robotic approach to determine protein profiles with high reproducibility by SELDI-TOF MS[11, 12]. In the current study our objective was to detect a protein profile that distinguishes between IL-2 treatment responders and nonresponders among metastatic RCC patients using SELDI TOF-MS.

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Materials and Methods

Patients and tumor samples

56 prospectively archived frozen tissue samples from primary tumors of patients with metastatic clear cell RCC were obtained from the UCLA Kidney Cancer Program with approval from the institutional review board at UCLA and Beth Israel Deaconess Medical Center, Boston. All patients had undergone radical nephrectomy and adjunctive immunotherapy with recombinant IL-2 based therapy, and were evaluable for response. High dose IL-2 regimen consisted of up to two treatment cycles, each comprised of two treatment admissions separated by 10 days. For each admission, subjects were treated with 600,000 IU/kg via intravenous bolus infusion every 8 hours for up to 14 doses during a five day hospitalization. Response status was defined according to standard criteria defined at the time of the patients' evaluation and prior to the widespread adoption of RECIST (Response Evaluation Citeria in Solid Tumors).

Protein isolation

Approximately 50mg tissue was ground and lysed in 250μl lysis buffer consisting of 8M Urea, 2% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propansulfonat),5mM DTT and 1% protease inhibitors. This mixture was vortexed and lyzed with a micro-pestle for 10 minutes and centrifuged at 10,000 × g at 4° C. The supernatant was then collected and the protein concentration was measured by Bradford assay. The protein concentration was normalized to 1μg/μl.

Mass Spectrometry

SELDI TOF-MS was performed using sample duplicates with the arrays described below. The entire procedure was carried out by a fully automated liquid-handling robot (Biomek FX™, Beckman Coulter).

Reversed Phase Chromatography

Hydrophobic protein arrays (H50 ProteinChip™ arrays; Ciphergen) were bulk washed with 50% acetonitrile (HPLC Grade; Aldrich) for 2 × 5 min, then loaded onto a 192-well bioprocessor (Ciphergen) and equilibrated with 10% acetonitrile/ 0.1% trifluoroacetic acid (Fisher Scientific). Each bioprocessor holds up to twelve 16-spot protein arrays and creates 100 μl wells above each spot. Ten μl of cell lysate were applied together with 50 μl binding buffer consisting of 10% acetonitrile/0.1% trifluoroacetic acid onto each array spot, and incubated for an hour. Array spots were washed 3 × 5 min with 75 μl 10% acetonitrile/ 0.1% trifluoroacetic acid, and 1 × 5 min with 75 μl water.

Cationic Exchange Chromatography

Weak cationic exchange chromatography protein arrays (CM10 ProteinChip™ arrays; Ciphergen) were pretreated with 10mM HCl for 5 min, and then rinsed with HPLC grade water. Subsequently, the arrays were loaded onto a 192-well bioprocessor, and equilibrated with 20mM ammonium acetate/0.1% Triton X-100 (Sigma), pH 6.0. Ten μl cell lysate and 50 μl 20mM ammonium acetate/ 0.1% Triton X-100 were dispensed onto each array spot, and incubated for one hour. The incubation comprised 60 cycles of pipetting the sample mixture up and down for 30 s. Array spots were washed 3 × 5 min with 75 μl 20mM ammonium acetate/ 0.1% Triton X-100 and 1 × 5 min with 75 μl water.

Immobilized metal affinity chromatography

Immobilized metal affinity capture arrays (IMAC30 ProteinChip™ arrays; Ciphergen) were incubated with 100mM $CuSO_4$ for 25 min and loaded onto a 192-well bioprocessor.

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Subsequently, the arrays were equilibrated with 50 mM NaCl, 100mM NaH_2PO_4, pH 7.0. Ten μl cell lysate and 40 μl 50 mM NaCl, 100mM NaH_2PO_4, pH 7.0 were dispensed onto each array spot and incubated for an hour. Array spots were washed 3 × 5 min with 75 μl 500 mM NaCl, 100mM NaH_2PO_4, pH 7.0 to remove non-specifically bound proteins and then washed 5 minutes with 75 μl water.

Application of Matrix Molecule

SPA (sinapinic acid; Fluka), the matrix molecule, was prepared as a saturated solution in 50% acetonitrile/ 0.5% trifluoroacetic acid, and then diluted 1:1 in 50% acetonitrile/ 0.5% trifluoroacetic acid. After air drying arrays, 2 × 1 μl and 2 × 0.75 μl of SPA were dispensed to each spot of the hydrophobic, cationic exchange and IMAC arrays respectively, again using the Biomek FX™ (Beckman Coulter) equipped with a 96-channel 200 μl head. The arrays were air-dried again, and immediately analyzed.

Analysis

Peak detection—Protein peaks were defined by a signal-to-noise ratio >= 2.5 and detected using the Ciphergen Biomarker Wizard software. We interrogated the range of 2,000 – 40,000 Da for peaks and normalized the peak data with the total ion current method after baseline correction.

Data Set Split—The subjects were randomly divided into a training and validation set. The training set was used as a test-bed to find the predictive signature (peaks predictive for therapy response), which was then applied to the independent validation set, which had not been previously used to identify the predictive signature. The training set consisted of 16 non-responders (8 lymph node positive, 8 lymph node negative) and 12 responders (2 lymph node positive, 10 lymph node negative). The validation set consisted of 16 non-responders (9 lymph node positive, 7 lymph node negative) and 12 responders (2 lymph node positive, 10 lymph node negative).

Class Prediction—We identified a set of descriptive peaks (differentially expressed peaks between responders and non-responders) on the training data set using t-test ($p<0.05$), assessed its significance using permutation testing (1000 permutations, 5% significance level) and chose peaks that remained significant after permutation analysis to account for multiple hypothesis testing[13]. The descriptive peaks were refined based on the accuracy of its subsets as predictor peaks on the training set using leave-one-out cross validation. We started this procedure on the training set with the responders and non-responders, and a set of features, the descriptive peaks. A sample was left out and a predictor set of peaks that distinguished the two groups was built and used to predict the class of the left-out sample. This procedure was cycled through all samples individually. The accuracy of the predictor equaled the total number of correctly predicted left-out samples. The best performing (highest leave-one-out accuracy) subset of the descriptive peaks was chosen as the predictive peak signature which was then applied to the validation set. Calculations of descriptive and predictive peaks were done solely on the training test. The finalized set of predictive peaks was then applied on the independent validation set, which was not used in obtaining the predictive signature. Class prediction was done using the k-nearest neighbor algorithm and the p-value for the predictor accuracy was calculated using Fisher's exact test[13]. The significance of the results was further assessed by a permutation test. The Mantel-Haenszel test was performed to assess the association between the proteomic profile and the IL-2 response status controlling for the effect of the lymph node status. Gene Cluster 2 (Whitehead Institute) and SPSS 11.0 were used as analysis software.

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Clustering—A hierarchical clustering technique was used to construct an Unweighted Pair Group Method with Arithmetic-mean (UPGMA) tree using Pearson's correlation as the metric of similarity.

Results

The clinicopathological characteristics of the studied patients as well as the partitioning into training and validation set are presented in Table 1. There were no parameters with a statistically significant difference between training and validation set.

Out of the 56 metastatic patients 24 patients had responded and 32 patients had not responded to IL-2 therapy. Of the 24 responders, there were 22 partial and 2 complete responders according to RECIST.

Samples were spotted in duplicate and yielded highly reproducible spectra, as recently shown[11]. The average intra-assay coefficient of variation was approximately 16%, which is within an acceptable range for SELDI studies[11]. SELDI TOF-MS detected a total of 513 protein peaks on the ProteinChip™ arrays (165 on IMAC30, 206 on CM10 and 142 on H50).

We identified 23 descriptive peaks that differentiated responders from non-responders in the training set ($p<0.05$, permutation $p<0.05$). Figure 1 depicts the hierarchical clustering of the samples in the validation set using the 11 peak signature. These peaks were obtained by refinement of 23 descriptive peaks based on their leave-one-out prediction accuracy on the training set. This 11 peak signature displayed 86% leave-one-out cross validation accuracy (24/28 predicted correctly, $p < 0.004$; permutation $p<0.001$) for the response status of the samples.

We then tested the 11-peak signature against samples in the validation set. The overall accuracy was 72% (20/28 predicted correctly, $p < 0.05$, permutation $p<0.01$). Figure 2 presents a scattergram depicting the confidence values for prediction results for the training and validation sets[13].

The trace view from the SELDI-TOF spectra of one of the 11 predictor peaks is shown representatively in Figure 3.

Because of the known association between lymph node status and response to IL-2 therapy, we examined the possibility that our results were confounded by lymph node status. Lymph node status was not associated with the SELDI predictions (Fisher's $p=0.245$). Nonetheless, we performed multivariate analysis, which further showed that SELDI predictions were associated with IL-2 response independent of lymph node status ($p=0.04$ by Mantel Haenszel test).

Discussion

The frustrating paucity of therapeutic options in metastatic RCC have led to the search for biomarkers that can aid in predicting prognosis and in selecting patients for appropriate therapy. Markers such as CAIX or Ki67 have been proposed for survival prediction in RCC[6]. In addition to individual markers, prognostic algorithms for survival in RCC patients have been developed through the use of select clinicopathological parameters such as 1997 classification T stage, Fuhrman's grade or lymph node status[14, 15]. A more recent report focusing on metastatic RCC patients described an algorithm for survival prediction in patients after radical nephrectomy and IL-2 immunotherapy, which is to date considered the

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therapy most likely to result in durable complete remissions for patients with metastatic RCC[16].

IL-2 response, even when not complete, appears to be a surrogate for survival in metastatic RCC patients[17]. Considering the substantial toxicity of IL-2 therapy and the lack of reliable individual biomarkers for response, the objective of the current study was to discover a protein profile that could in the future enable the identification of the small subset of patients who are most likely to benefit from IL-2 therapy. This is to our knowledge the first report attempting the prediction of IL-2 response in metastatic RCC patients using proteomic analysis.

Using a highly standardized and automated procedure we used protein extracts from tumor sample tissue lysates of clear cell RCC patients with distant metastases who had undergone radical nephrectomy prior to receiving adjuvant IL-2 therapy, and applied them to three protein chip arrays of different chromatographic properties. Analysis of protein profiles revealed a pattern of 11 protein peaks predictive of IL-2 response that could be validated with 72% accuracy in an independent sample set (66% accuracy for responders, 75% for non-responders) ($p<0.04$). Interestingly, lymph node status, which has been reported to be strongly associated with IL-2 immunotherapy response and is sometimes used in the clinical setting[8], was accurate in only 62.5 % of the same sample set, highlighting the shortcoming of relying only on clinical variables to predict outcome. On multivariate analysis the result of the protein pattern remained statistically significant when controlled for lymph node status ($p<0.01$). Clustering analysis shows that the 11-peak predictive signature can successfully distinguish between responders and non-responders (Figure 1).

Proteomic technologies, such as MS, provide the means to compare protein profiles identifying diagnostic or prognostic protein signatures. Protein profiling using SELDI-TOF MS has been successfully applied to derive protein signatures that distinguish several neoplastic entities[18]. Despite the limitations of SELDI-TOF MS with regard to resolution when compared to other MS technologies, the advantages of SELDI-TOF MS are the high throughput and sensitive acquisition of protein profiles from large numbers of patient samples in combination with on-chip protein fractionation that cannot be currently matched by other MS technologies[19]. While SELDI-TOF MS cannot directly identify the proteins in a protein profile, tandem MS is limited due to the requirement of extensive fractionation for protein identification, thus, dramatically decreasing sample throughput.

Thus, we have identified in our sample set a proteomic pattern that distinguishes and predicts IL-2 response and is an independent predictor of the lymph node status offering additional information. The clinical value of this molecular predictor will have to be validated prospectively in a larger independent data set. Our current study, however, provides the first “proof of principle” that such a proteomic profile can predict IL-2 therapy response in metastatic RCC patients.

High-dose IL-2 therapy produces tumor responses in 15–20% of metastatic RCC patients but individual prediction of treatment response is unreliable[17]. Prior ability to predict IL-2-response probability would allow the sparing of patients having a low probability of response from unwanted toxicities, making them candidates for alternative, investigational therapies. Future protein identification of some of the predictive peaks may, in addition, offer insights into the mechanisms underlying IL-2 responsiveness, and eventually lead to the development of alternative, more targeted treatment regimens.

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Key of Definitions for Abbreviations

RCC	renal cell carcinoma
SELDI-TOF MS	surface-enhanced laser desorption/ionization time-of-flight mass

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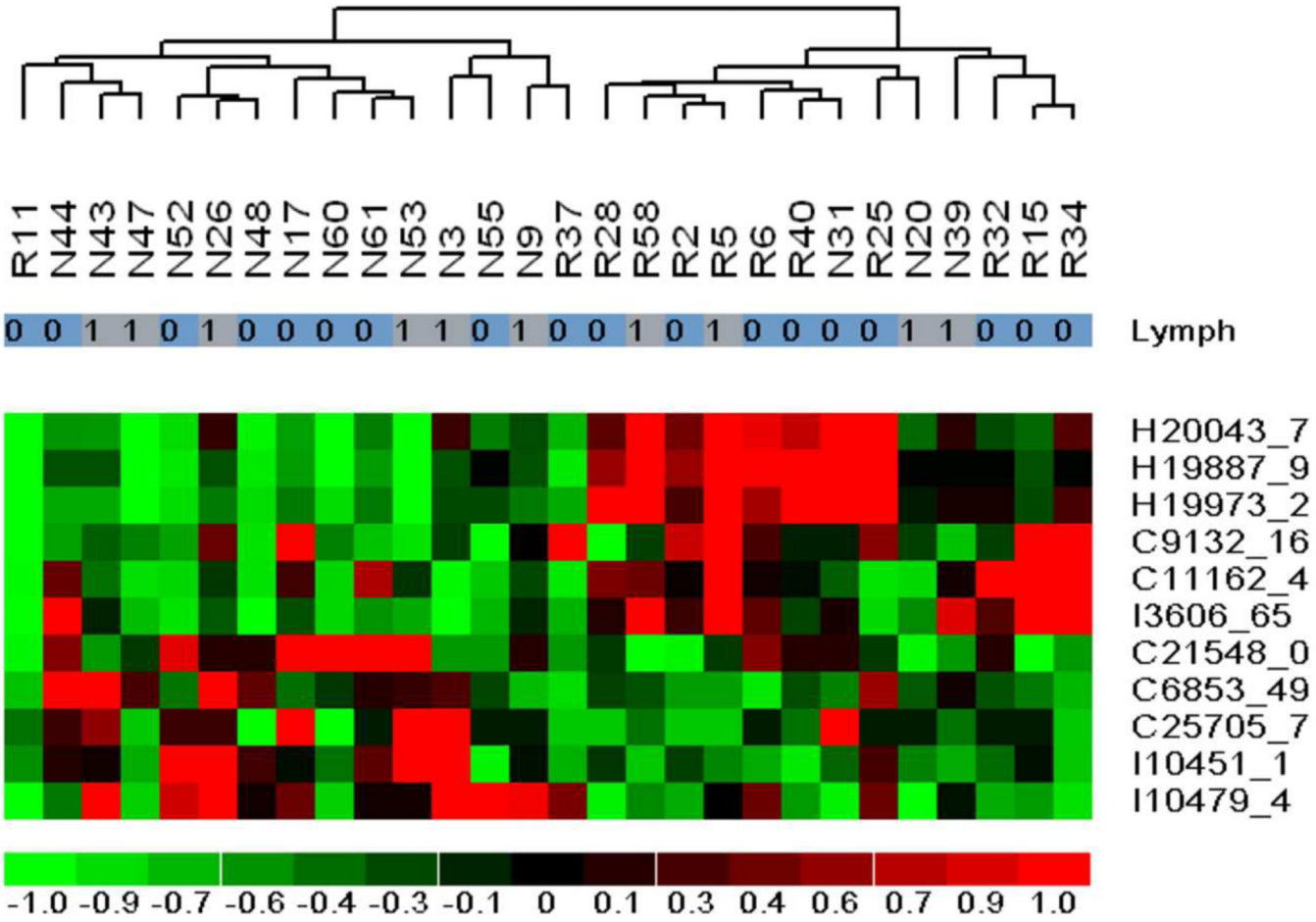

Figure 1.

Hierarchical clustering of the samples in the validation set using the 11-peak signature. R and NR stand for Responder and Non-Responder respectively. The lymph node status (lymph) is addressed by 0 and 1 as no cancer inflitration and present cancer infiltration, respectively. Rows represent the intensity values of individual peaks, which are normalized to [−1, 1] as shown in the scale at the bottom. Peak labels represent the chip on which the peak was detected (I for IMAC30, C for CM10, H for H50) followed by the molecular weight for the detected peak. Red denotes an elevation while green denotes a decrease in expression.

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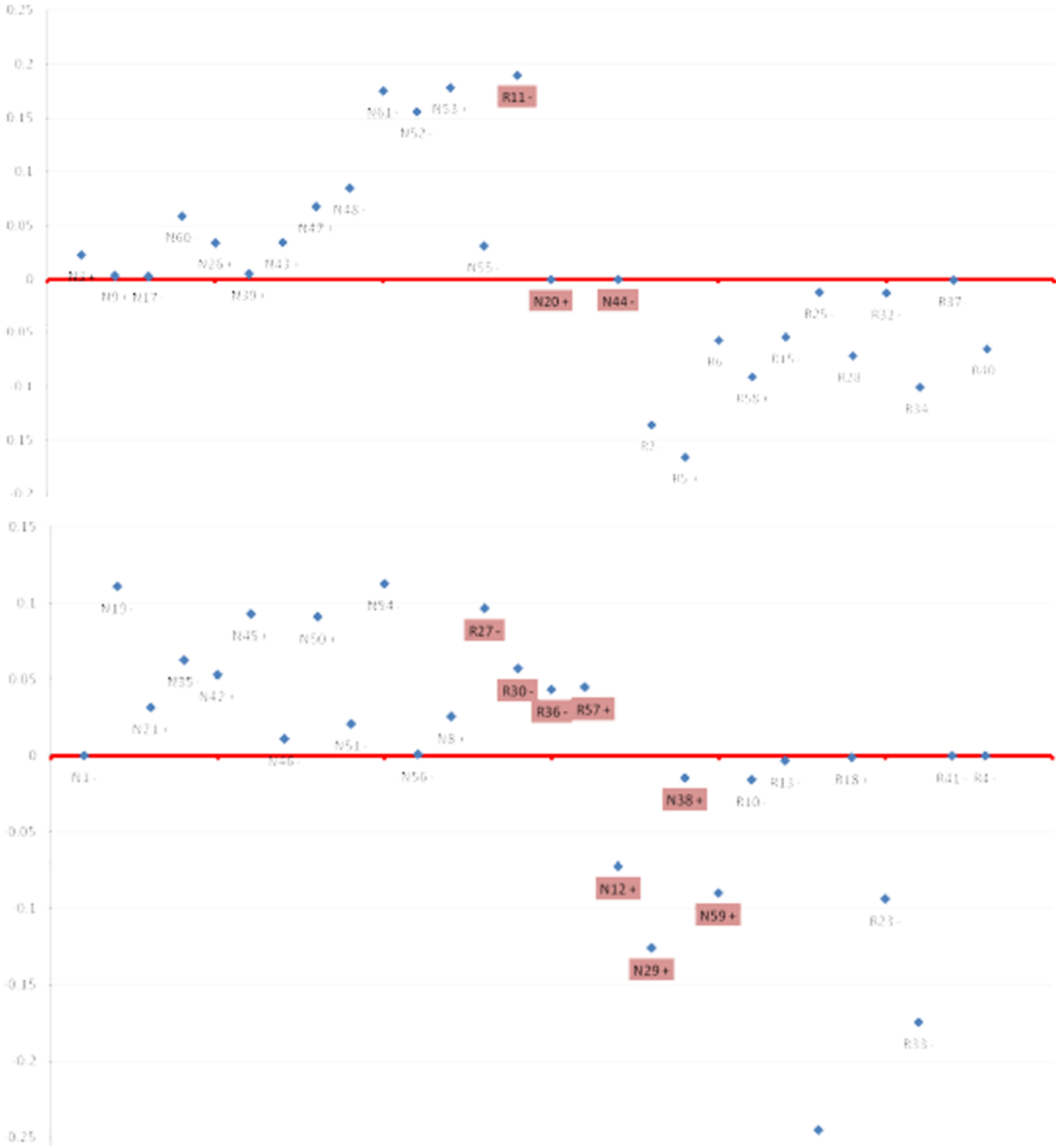

Figure 2.

Scattergram depicting the confidence values for prediction results for both the training (2a) and validation sets (2b). X-axis is shown in bold red color denoting the separating line to make a decision for the response status. Samples that are above the line are predicted as non-responders and samples that are below the line are predicted as responders. True class labels

along with lymph node status are shown below each data point. Samples that are predicted in error are depicted using a colored background for the respective data labels.

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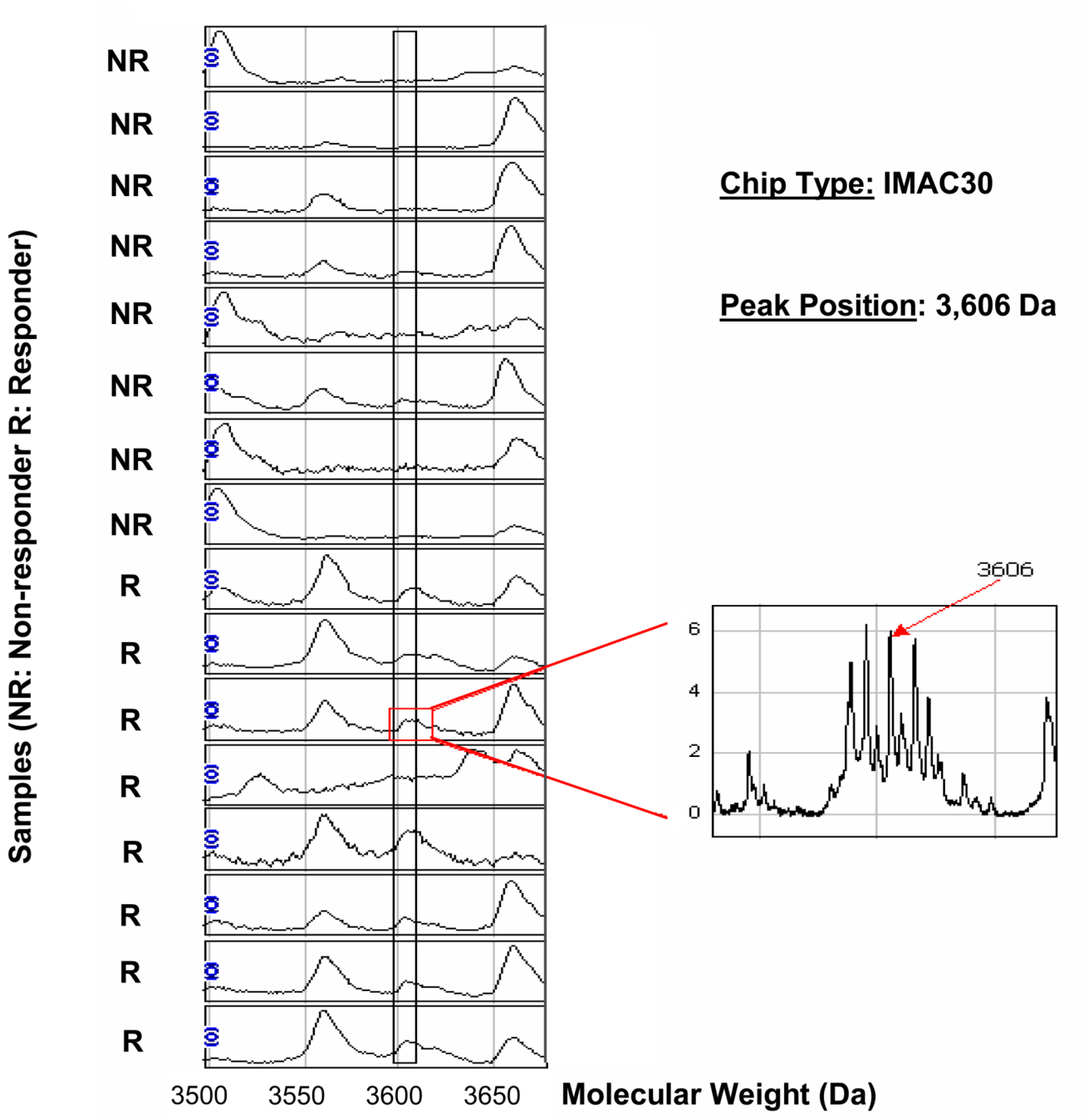

Figure 3.

Trace view for one representative peak I 3606_65 (representing the peak on the IMAC30 chip with a molecular weight of 3,606.65 Da) from the 11-peak signature. The detection level for the peak (boxed) in 8 responders (R) and 8 non-responders (NR) is shown. Zooming of a spectrum shows the peak of interest as a picture in picture.

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Table 1

Clinical and pathological characteristics of all patients.

Total no. of patients		56		
Gender *		Male 41 / Female 15		
Age *		Median 55 years (34–75)		
Histologic subytpe *		Clear cell		
Response status		Responder (24 **) Non-Responder (32)		
Lymph node status		N+ (21) N0 (35)		
Data set split	**Training**		**Validation**	
n	28		28	
Response Status * **Lymph Node Status** *	12 Responders	2 LN +	12 Responders	2 LN+
		10 LN −		10 LN −
	16 Non-Responders	8 LN +	16 Non-Responders	7 LN+
		8 LN −		9 LN −

* Not significantly different in training and validation set ($p>0.05$)

** 22 partial and 2 complete responders according to RECIST