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Predicting Prostate Cancer Biochemical Recurrence Using a Panel of Serum Proteomic Biomarkers

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Abstract

Purpose—The pathological state of the prostate may be reflected by serum proteome in a man. We hypothesized that biomarkers are present in preoperative serum, which may be used to predict the probability of biochemical recurrence following radical prostatectomy.

Materials and Methods—Mass spectrometry analysis was used to compare 52 men who experienced biochemical recurrence with 52 who remained biochemical recurrence-free for approximately 5 years after radical retropubic prostatectomy. A total of 30 matched pairs of recurrent and nonrecurrent serum samples were randomly selected as a training set for biomarker discovery and model development. Selected mass spectrometry peaks were combined with pre-radical retropubic prostatectomy prostate specific antigen in a multivariate algorithm to predict recurrence. The algorithm was evaluated using the remaining 22 recurrent and 22 nonrecurrent subjects as test samples. Protein identities of the selected mass spectrometry peaks were investigated.

Results—Two serum biomarkers for recurrence, P1 and P2, were combined with preoperative prostate specific antigen to predict biochemical recurrence. The ROC AUC for prostate specific antigen and the predicted outcome was 0.606 and 0.691 in the testing data, respectively. Using a single cutoff the samples were divided into 2 groups that were predictive of biochemical recurrence ($p = 0.026$). In contrast, preoperative prostate specific antigen did not differ between recurrent and nonrecurrent cases (Wilcoxon matched pairs test $p = 0.07$). The protein identity of P1 was determined to be a truncated form of C4a (C4a des-Arg). Preliminary data indicated that P2 was an N-terminal fragment of protein C inhibitor.

Conclusions—In the current study population, which was matched on Gleason score and TNM staging, pre-radical retropubic prostatectomy prostate specific antigen retained no independent power to predict recurrence. However, by adding 2 proteomic biomarkers to preoperative prostate specific antigen the combined model demonstrated statistically significant value for predicting prostate cancer recurrence in men who underwent radical retropubic prostatectomy.

Keywords

prostate; prostatic neoplasms; neoplasm recurrence; complement C4a; protein C inhibitor

Pretreatment PSA, biopsy Gleason scores and clinical staging provide critical prognostic information. They have been widely used in multivariate predictive algorithms or nomograms to assess the risk of recurrence and predict the overall outcome in patients diagnosed with localized prostate cancer.¹⁻⁴ With recent advances in genomic and proteomic analysis technologies newly discovered candidate serological and histological biomarkers have been reported and evaluated for their potential contribution to such multivariate predictive models. However, until recently few novel biomarkers have been able to demonstrate significant, independent and clinically appreciable contributions to patient risk stratification when combined with the mentioned clinical variables.

A case-control study was designed to analyze and retrospectively compare proteomic expressions in serum samples collected from men who underwent RRP at The Johns Hopkins Hospital between 1986 and 2002. A total of 52 patients with biochemical recurrence were compared with 52 who did not experience biochemical recurrence within 5 years of RRP. To discover biomarkers with independent predictive value for the risk of recurrence the 2 groups were pairwise matched for TNM staging, pathological Gleason scores and age at RRP. Pre-RRP PSA concentrations, which were not matched between the groups, were also similar. Samples were divided into training and testing data sets. We then developed a model using 2 biomarkers plus preoperative PSA to predict biochemical recurrence in men who underwent surgery for clinically localized prostate cancer.

METHODS

Study Groups and Samples

Serum samples were obtained from The Johns Hopkins clinical chemistry specimen bank. Specimens were collected preoperatively from patients who underwent RRP at The Johns Hopkins urology department between 1986 and 2002. No patient received neoadjuvant therapy. Institutional review board approval was obtained for this study.

All samples were promptly processed and stored at -80C following standard clinical operating procedures. The study set included 52 individuals with prostate cancer recurrence following RRP and 52 in whom prostate cancer did not recur within 5 years of RRP. Three samples were collected per individual, including before RRP, following RRP to verify that PSA levels had decreased below the detection limit and at biochemical recurrence or after the patient had remained recurrence free for approximately 5 years. The 2 groups of samples were pairwise matched on age (mean 59.3 years), stage (pT2 in 25%, pT3 in 75%, pTN0 in 96% and pTM0 in 100%) and pathology Gleason score (less than 6 in 27% and greater than 7 in 73%). PSA was undetectable (less than 0.1 ng/ml) in all patients within 3 months to 1 year after RRP. Biochemical recurrence was defined as 1 observation of PSA greater than 0.2 ng/ml following a PSA measurement that was below the detection limit. Until February 1996 the Hybritech® Tandem®-R assay was used to evaluate PSA levels. After that date

PSA values were calculated using Tosoh PSA assays (Tosoh, Tokyo, Japan). A comparison of these 2 assays showed identical PSA results in patient specimens. Because patient race was predominantly white (46 white and 6 black men in the recurrence group, and 45 white, 6 black and 1 of other race in the nonrecurrent group), race was not included in statistical analyses.

Sample Processing and Evaluation on Surface Enhanced Laser Desorption Ionization Platform

All study samples (104×3 collection time points = 312) and 40 QC samples were randomized onto 4, 96-well bioprocessors and fractionated using Q Ceramic HyperD® F-Filtration Plates according to manufacturer instructions. This anion exchange, chromatography based separation results in the collection of 6 separate fractions, referred to as fractions 1 through 6. Proteins were eluted from the column with buffers at pH 9, 7, 5, 4 and 3, followed by 1 elution with organic solvents. Fractionated serum was spotted onto CM10, IMAC30 and Q10 ProteinChip® arrays according to manufacturer instructions using a Biomek® 2000 robot. Sinapinic acid half saturated in 50% acetonitrile and 0.5% trifluoroacetic acid was used as the matrix for this experiment.

Spectra were generated on an autoloader adapted PBSIIc Protein Chip Reader (Ciphergen Biosystems, Fremont, California). The machine was optimized for molecular weights between 3 and 50 kDa. Baseline subtraction and normalization were performed using ProteinChip, version 3.2. Peaks were detected between 2 and 150 kDa, and exported.

Data Analysis and Statistical Methods

When the peaks were exported, 10 peaks from QC samples were selected between 2 and 20 kDa. Intensity, signal-to-noise ratio, resolution and mass were loaded into Cluster, version 2.12 (Stanford University, Stanford, California) to determine whether any plate or replicate clustered together. Clusters were displayed and visually inspected using TreeView, version 1.50 (Stanford University). Sample replicates were compared using in-house software in Matlab®, which plotted replicate peak intensities and calculated pairwise correlations among all samples. Plots were visually inspected for nonlinearity. Replicates were then averaged and log transformed.

Study samples were divided by block randomization into a training (30 recurrent and nonrecurrent) and a testing (22) set. Using pre-RRP serum samples from patients in the training set the peaks that best discriminated between the recurrent and nonrecurrent groups were selected by an implementation of unified maximum separability analysis using ProPeak (3Z Informatics, Mount Pleasant, South Carolina) on each chip/fraction separately. This algorithm calculates the rank and contribution of each candidate biomarker toward the maximum separation of recurrent and nonrecurrent samples. Bootstrap resampling was done to identify peaks with highly variable performance in the training set. These peaks were removed from subsequent analysis. The highest ranking peaks in each chip/fraction were compiled into 1 list. P2, a candidate biomarker identified from a prior study, and pre-RRP PSA were added to the peak list. These candidate biomarkers were evaluated together to identify the best complimentary peaks. They were combined into a multivariate prediction

model. Using the average predicted outcome in the training data as a cutoff the samples were split into 2 groups. The same cutoff value was applied in the testing data to produce 2 predicted groups. The 2-sample t test, ROC curve analysis and Pearson chi-square analysis were used to assess the predictive accuracy of the model.

Protein Identification

The protein identity of each candidate biomarker was pursued. Sample material was enriched for the protein of interest. Following enrichment proteins larger than 4 kDa were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and digested with trypsin to obtain peptides that were the appropriate molecular weight for MS/MS analysis. This step is unnecessary for smaller proteins. The material was then analyzed by MS/MS. Mass spectrometry results were characterized using a QSTAR® XL tandem mass spectrometry instrument equipped with a PCI-1000 ProteinChip Interface (Ciphergen Biosystems). MS/MS spectra were submitted to the database mining tool Mascot, version 2.1.2 (Matrix Science, Boston, Massachusetts) and searched against the updated SwissProt database.

Immunoassay Evaluation

When MS/MS identification was not possible using the fraction evaluated during biomarker discovery, immunoassay verification was necessary. Sample (2.5 μ l) was diluted with 247.5 μ l RIPA buffer composed of 50 mM tris (pH 7.6), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet™ P40, 2.5 mg/ml NaDOC, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 2 μ g/ml each of aprotinin, pepstatin A and leupeptin (Sigma®), mixed with 20 μ l EZview™ Red Protein G Affinity Gel and incubated at 4C for 4 hours. After spinning down the beads the supernatant was extracted and mixed with 5 μ g goat polyclonal antibody to the candidate biomarker and incubated at 4C overnight. Following binding the mixture was added to 20 μ l Protein G Affinity Gel and incubated at 4C for 4 hours. Supernatant was collected and the beads were washed 3 times with 250 μ l PBS (9 gm/l NaCl, 0.165 gm/l KH₂PO₄ and 0.775 gm/l Na₂HPO₄) with 0.1% Triton X-100 (Thermo Fisher Scientific, Waltham, Massachusetts), followed by 3 washes with 250 μ l PBS and a wash with water. Captured proteins were eluted with 10 μ l 50% acetonitrile containing 2.5% trifluoroacetic acid. Eluates were bound directly to a ProteinChip NP20 array. Sinapinic acid (1 μ l) was added after the eluates were dried at room temperature. Supernatant and whole serum were spotted on CM10 chips. Arrays were assessed using the PBSIIc.

RESULTS

Table 1 lists the demographic characteristics of the study population. Because patients were matched for Gleason primary and secondary grades, TNM stage and age, samples were similar for those variables. Date of surgery was not significantly different between the groups (matched pairs t test $p = 0.35$). Thus, Gleason stage migration was not anticipated to affect the conclusions presented. While pre-RRP PSA was not used to match patients in the study, it was not significantly different between the groups (Wilcoxon matched pairs test $p = 0.07$). Figure 1 shows a histogram of pre-RRP PSA values.

MS Analysis and Model Development

All QC procedures were followed and the analysis of QC data did not indicate the introduction of bias during sample fractionation or chip spotting. Samples were divided into training (30 recurrent and 30 nonrecurrent) and testing (22 recurrent and 22 nonrecurrent) sets. Using pre-RRP samples from patients in the training set the peaks with the best discrimination between recurrent and nonrecurrent samples were selected and compiled into a peak list. P2 and pre-RRP PSA were then added as candidate biomarkers. The best combination was used to develop a multivariate prediction model. The final model included the novel biomarker P1, P2 and pre-RRP PSA. Predicted outcomes in recurrent and nonrecurrent samples were significantly different (training and testing 2-sample t test $p = 0.017$ and 0.049 , respectively, fig. 2). Figure 3 shows ROC analysis of testing and training data. AUC of the model predicted outcome was significantly different from 0.5 (training and testing AUC 0.667 , $p = 0.012$ and AUC $= 0.691$, $p = 0.011$, respectively). However, pre-RRP PSA was not different from an AUC of 0.5 (training and testing AUC 0.590 , $p = 0.12$ and AUC 0.606 , $p = 0.12$, respectively). The AUC of the model and pre-RRP PSA were not significantly different in training or testing data. Using the mean predicted outcome in the training data as a cutoff the model outcome was used to predict patient recurrence status. A significant association was observed between recurrence status and predicted group classification in the training and testing sets (Pearson's chi-square analysis $p = 0.019$ and 0.026 , respectively, table 2).

Protein Identification of P1

The protein identity of this biomarker was determined using the fraction 1 material from which the peak was originally selected. To purify this sample material for MS/MS analysis a series of fractionation procedures were used, including anion exchange, immobilized Ni and reverse phase chromatography. Purified material was separated on 18% tris-Gly sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Material was cut from the appropriate molecular weight band and digested with trypsin. The MS/MS identity of 11 trypsin fragments conclusively identified this biomarker as derived from human complement component 4a (C4a). Given the peptide mass of 8601.72 Da, it was identified as C4a lacking the C-terminal arginine (C4a des-Arg, sequence NVNFQKAINEKLGGYASPTAKRCCQDGVTRLPMMRSCEQRAARVQQPDCREPFLSCCQFAESLRKKSRDKGQAGLQ). The underlined amino acids define regions of the biomarker that were sequenced during analysis of the tryptic peptides. The pI of this fragment was 9.23 . The pI was consistent with its appearance in fraction 1, which is a fraction characterized by the inability to bind to an anion exchange resin at pH 9.0 .

Protein Identification of P2

While the intensity used in statistical analysis was calculated from fraction 6, this fraction could not be adequately enriched to provide a conclusive MS/MS identification. However, a peak with the same mass-to-charge ratio was also observed in fraction 1. Therefore, fraction 1 was used during sample enrichment and MS/MS analysis. The material was separated by reverse phase chromatography. Trypsin digestion was unnecessary because the mass of the peptide (3.89 kDa) was within the mass range necessary for MS/MS analysis. Consequently

1 peak was analyzed during MS/MS. The peak was identified as the N-terminal fragment of PCI (the sequence of fragment identified was SARLNSQRLVFNRPFILMFIVDNNILFLGKVNRP). The pI of this fragment was 12.03, which is consistent with its appearance in fraction 1, from which the MS/MS identification was derived. The correlation between the 3.89 kDa peak in fractions 1 and 6 was positive, although not significant ($p = 0.09$). Replacing the fraction 1 peak in the model decreased the difference observed between the 2 risk groups but similar correlations were observed between the peak in fraction 1, and between P1 and pre-RRP PSA.

Because MS/MS identification of this fragment was completed in fraction 1, the initial validation was pursued from fraction 1 using goat polyclonal antibody to PCI (Enzyme Research Laboratories, South Bend, Indiana) and goat antibody to IgG as the negative control. A dilution of fraction 1 was identified which, when added to increasing concentrations of antibody, resulted in a monotonic increase in the 3.89 kDa peak intensity of the PCI antibody. No peaks were observed at the same dilution of fraction 1 bound to varying goat IgG antibody concentrations (data not shown).

Such successes were not observed using material from fraction 6. A significant issue was that this fraction was approximately pH 3.4. pH was closer to the pH used to elute peptides from antibodies, rather than the pH required for stable peptide/antibody binding. After inconclusive results were noted using fraction 6 material without preprocessing to improve antibody binding, altering the pH of fraction 6 was pursued. Unfortunately the change in pH through using tris or PBS buffer caused the peak to disappear. When fraction 6 material was lyophilized and reconstituted in PBS, additional spectra peaks appeared that made it impossible to track the peak of interest. Consequently no validation of P2 was possible using fraction 6.

Finally, whole serum was evaluated. Again, the 3.89 kDa peak bound to polyclonal PCI antibody, while no binding was observed in the negative control (fig. 4). Due to the dilution required to attain a linear range of antibody response fractionation of the supernatant did not provide evidence of depletion. Less than 10% of the peaks normally present in fractions 1 and 6 were present when the supernatant was fractionated.

DISCUSSION

In this study pre-RRP PSA alone was not predictive of patient 5-year recurrence. However, adding 2 candidate biomarkers to pre-RRP PSA predicted biochemical recurrence independent of Gleason score, TNM staging and patient age at RRP. Following the study the identity of these biomarkers was pursued. C4a was up-regulated in patients who experienced biochemical recurrence compared to those without recurrence. The second marker, which was tentatively identified to be a fragment of PCI, was downregulated in men who experienced biochemical recurrence.

C4a desArg was identified as a promising marker in this study. Historically C4a and C4 have been observed to be differentially expressed in patients with prostate cancer and controls. A fragment of C4a was determined to be up-regulated in the peptidome (mass-to-

charge ratio less than 4 kDa) in patients with prostate cancer compared with that in patients without cancer.⁵ The differential expression was attributable to ex vivo degradation, highlighting the importance of careful sample collection practices.⁶ Furthermore, C4 has been shown to be differentially expressed in prostate cancer prostatic fluid compared with prostatic fluid collected from patients without cancer⁷ with C4 levels significantly increased in patients with cancer. Increased levels of C4 and C4a have also been shown to correlate with poor prognosis in the plasma of patients with renal carcinoma⁸ and the serum of patients with pancreatic adenocarcinoma,⁹ respectively.

PCI is expressed locally in the prostate gland but it is also expressed outside the prostate. PCI inhibits human glandular kallikrein 2 and PSA in seminal fluid.¹⁰⁻¹² It has a concentration in plasma that is estimated to be 5.3 $\mu\text{g/ml}$.¹³ The concentration of PCI is more than 40 times higher in seminal plasma than in blood plasma.¹³ In tissue studies PCI staining in low grade tumors (Gleason grade 2-3) was similar to staining in samples from patients with benign prostatic hyperplasia and PIN areas, while staining in high grade tumors (Gleason grade 4-5) was more variable.¹⁴ In high grade tumors an increasing number of tumor cells were negative for PCI.¹⁴ This is consistent with our results, in that this fragment of PCI was less abundant in patients who experienced recurrence than in patients without recurrence. In contrast to earlier series, participants in this study were matched on Gleason score, indicating that some of the previously observed variability may be related to disease aggressiveness independent of Gleason score.

While we have not succeeded in developing an immunoassay to demonstrate depletion in fraction 6 directly, we successfully depleted whole serum. Several possibilities exist that explain why antibody depletion was unsuccessful in fraction 6. 1) The antibody is inactive in the organic buffers of fraction 6. 2) The peptide is poorly soluble and manipulating the fraction 6 buffers for assay optimization results in peptide precipitation. 3) The peptide is not a fragment of PCI. Regarding issue 1, the organic buffers in fraction 6 are approximately pH 3.4. An antibody would not bind well at this pH. Regarding issue 2, the PCI fragment identified in fraction 1 is composed of 45% hydrophobic residues. Typically peptides composed of more than 50% hydrophobic residues may be insoluble in aqueous solutions. When attempts were made to alter the pH of the sample, the spectra peak composition changed and the peak disappeared, indicating that it may have precipitated from solution. The hydrophobic content of the peptide also may explain why the peptide was observed in fractions 1 and 6. The peptide itself may exist as 2 populations, including 1 that is soluble and elutes at pH 9.0 and another that is in an insoluble conformation that can only be eluted with organic solvents. Because the 2 peaks were consistent in their contribution to the separation of recurrent and nonrecurrent cases, although not capable of attaining the same statistical significance, we anticipate that the combination in whole serum would also have discriminatory power to predict biochemical recurrence. However, without an immunoassay using the organic material directly there is no way to discount issue 3.

These biomarkers, C4a and PCI, were identified to have discriminatory power to predict biochemical recurrence independent of Gleason score, TNM staging and patient age. The 2 biomarkers have been implicated in existing research. Our study advances the understanding

of the role that these biomarkers may have, specifically as biomarkers for more aggressive prostate cancer. Further validation is necessary in a larger patient population.

CONCLUSIONS

We report that a panel of 2 proteomic biomarkers and pre-RRP PSA combined with nonlinear modeling in the serum of patients who underwent radical prostatectomy can be used to predict the risk of recurrence. Two candidate biomarkers have been identified that are differentially expressed in patients who experience biochemical recurrence and those who do not. Although these proteins might not be cancer or prostate specific molecules, our findings indicate the potential for novel biomarkers to predict biochemical recurrence in patients who are candidates for radical prostatectomy.

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References

1. D'Amico AV, Whittington R, Malkowicz SB, Schultz D, Blank K, Broderick GA, et al. Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA*. 1998; 280:969. [PubMed: 9749478]
2. Kattan MW, Eastham JA, Stapleton AM, Wheeler TM, Scardino PT. A preoperative nomogram for disease recurrence following radical prostatectomy for prostate cancer. *J Natl Cancer Inst*. 1998; 90:766. [PubMed: 9605647]
3. Cooperberg MR, Pasta DJ, Elkin EP, Litwin MS, Latini DM, Du Chane J, et al. The University of California, San Francisco Cancer of the Prostate Risk Assessment score: a straightforward and reliable preoperative predictor of disease recurrence after radical prostatectomy. *J Urol*. 2005; 173:1938. [PubMed: 15879786]
4. Partin AW, Kattan MW, Subong EN, Walsh PC, Wojno KJ, Oesterling JE, et al. Combination of prostate-specific antigen, clinical stage, and Gleason score to predict pathological stage of localized prostate cancer. A multi-institutional update. *JAMA*. 1997; 277:1445. [PubMed: 9145716]
5. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB, et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J Clin Invest*. 2006; 116:271. [PubMed: 16395409]
6. Rai AJ, Gelfand CA, Haywood BC, Warunek DJ, Yi J, Schuchard MD, et al. HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. *Proteomics*. 2005; 5:3262. [PubMed: 16052621]
7. Grayhack JT, Wendel EF, Oliver L, Lee C. Analysis of specific proteins in prostatic fluid for detecting prostatic malignancy. *J Urol*. 1979; 121:295. [PubMed: 85726]
8. Moroni M, Porta C, De Amici M, Quaglini S, Cattabiani MA, Buzio C. Eosinophils and C4 predict clinical failure of combination immunotherapy with very low dose subcutaneous interleukin- 2 and interferon in renal cell carcinoma patients. *Haematologica*. 2000; 85:298. [PubMed: 10702820]
9. Lin Y, Goedegebuure PS, Tan MC, Gross J, Malone JP, Feng S, et al. Proteins associated with disease and clinical course in pancreas cancer: a proteomic analysis of plasma in surgical patients. *J Proteome Res*. 2006; 5:2169. [PubMed: 16944928]

10. Deperthes D, Chapdelaine P, Tremblay RR, Brunet C, Berton J, Hebert J, et al. Isolation of prostatic kallikrein hK2, also known as hGK-1, in human seminal plasma. *Biochim Biophys Acta*. 1995; 1245:311. [PubMed: 8541306]
11. Bourgeois L, Brillard-Bourdet M, Deperthes D, Juliano MA, Juliano L, Tremblay RR, et al. Serpinderived peptide substrates for investigating the substrate specificity of human tissue kallikreins hK1 and hK2. *J Biol Chem*. 1997; 272:29590. [PubMed: 9368023]
12. Christensson A, Lilja H. Complex formation between protein C inhibitor and prostate-specific antigen in vitro and in human semen. *Eur J Biochem*. 1994; 220:45. [PubMed: 7509746]
13. Laurell M, Christensson A, Abrahamsson PA, Stenflo J, Lilja H. Protein C inhibitor in human body fluids. Seminal plasma is rich in inhibitor antigen deriving from cells throughout the male reproductive system. *J Clin Invest*. 1992; 89:1094. [PubMed: 1372913]
14. Cao Y, Becker C, Lundwall A, Christensson A, Gadaleanu V, Lilja H, et al. Expression of protein C inhibitor (PCI) in benign and malignant prostatic tissues. *Prostate*. 2003; 57:196. [PubMed: 14518028]

Abbreviations and Acronyms

MS/MS	tandem mass spectrometry
P	serum proteomic biomarker
PBS	phosphate buffered saline
PCI	protein C inhibitor
pI	isoelectric point
PSA	prostate specific antigen
QC	quality control
RRP	radical retropubic prostatectomy

Figure 1.

Histogram shows pre-RRP PSA values in patients with and without recurrence. Difference was not significant (Wilcoxon matched pairs test $p = 0.07$).

Figure 2.

Box and whiskers plot demonstrates predicted recurrence status in training (A) and testing (B) data ($p = 0.017$ and 0.049 , respectively).

Figure 3.

ROC curve analysis of predicted outcomes and pre-RRP PSA in training (A) and testing (B) data. Pre-RRP PSA showed no statistical difference from ROC AUC of 0.5. However, after pre-RRP PSA was combined with 2 novel biomarkers predicted outcomes attained statistical significance in training and testing data sets ($p = 0.012$ and 0.011 , respectively).

Figure 4.

Immunodepletion of 3.89 kDa peak. Note original profile in whole serum, and in eluted material bound to PCI antibody (*goat anti-human PCI antibody*) and to negative control (*goat IgG antibody*).

Table 1

Study population

	Characteristics	
	Recurrence	No Recurrence
Mean $\pm$ SD age	59.12 $\pm$ 6.1	59.40 $\pm$ 6.4
Av days to blood collection before RRP	34	41
No. pathological:		
Gleason 3+2=5	1	1
Gleason 3+3=6	14	14
Gleason 3+4=7	27	28
Gleason 4+3=7	7	6
Gleason 3+5=8	1	1
Gleason 4+4=8	1	2
Gleason 4+5=9	1	0
T2	5	5
T2a	3	3
T2b	5	5
T3a	35	35
T3b	4	4
N0	51	50
N1	0	2
N2	1	0
Preop median ng/ml PSA (25%, 75% quartile)	8.70 (5.8, 14.2)	7.20 (4.8, 10.9)
Postop av days:		
Evaluation 1 surgery to confirmed PSA less than 0.1 ng/ml	182	215
Evaluation 2 surgery to followup	1,264	1,975

Table 2

Training and testing data

	No Predicted		
Disease	No Recurrence	Recurrence	Total No.
Training:			
No recurrence	22	8	30
Recurrence *	<u>11</u>	<u>15</u>	<u>26</u>
Totals	33	23	56
Testing:			
No recurrence	15	5	20
Recurrence *	<u>9</u>	<u>13</u>	<u>22</u>
Totals	24	18	42

* Significantly associated with predicted group classification in training and testing sets (Pearson's chi-square analysis $p = 0.019$ and 0.026 , respectively).