

SERUM PROTEOMIC PROFILING CAN DISCRIMINATE PROSTATE CANCER FROM BENIGN PROSTATES IN MEN WITH TOTAL PROSTATE SPECIFIC ANTIGEN LEVELS BETWEEN 2.5 AND 15.0 NG/ML

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ABSTRACT

Purpose: Artificial intelligence based pattern recognition algorithms have been developed and successfully used to analyze complex serum proteomic data streams generated by surface enhanced, laser desorption ionization time-of-flight mass spectroscopy. In the current study we used a high performance, hybrid quadrupole time-of-flight mass spectrometer to generate discriminatory serum proteomic profiles to determine if this technology could be used to determine the need for prostate biopsy in men with elevated prostate specific antigen (PSA).

Materials and Methods: Serum samples were collected from 154 men with serum PSA 2.5 to 15.0 ng/ml and/or abnormal digital rectal examination prior to transrectal ultrasound guided biopsy. Serum samples were applied to WCX2 (weak cation exchange protein chip) Protein Arrays (Ciphergen Biosystems, Fremont, California) by a Biomek 2000 robotic liquid handler (Beckman-Coulter, Chaska, Minnesota) and low molecular weight (less than 20 kDa) proteomic patterns were generated with an API QSTAR Pulsar *i* LC/MS/MS System (Applied Biosystems, Framingham, Massachusetts). High resolution mass spectra were analyzed with a pattern recognition bioinformatics tool, that is Proteome Quest beta version 1.0 (Correlogic Systems, Inc., Bethesda, Maryland), in an attempt to identify and discover key discriminating ion signatures. Serum samples from 63 men (2 or more negative prostate biopsies in 23, 1 negative biopsy in 10 and biopsy detected prostate cancer [CaP] in 30) were used to train the diagnostic algorithm. The remaining 91 samples, including 28 of prostate cancer and 63 of 1 or more negative biopsies, were analyzed in blinded fashion.

Results: The most discriminatory model was found using the WCX2 chip. Testing the remaining 91 men with this model yielded 100% sensitivity and 67% specificity. In other words, if the proteomic pattern had been used to determine the need for prostate biopsy in this cohort of men with PSA between 2.5 and 15.0 ng/ml, 67% (42 of 63) with negative biopsies would have avoided unnecessary biopsy, while no cancers would have been missed.

Conclusions: Our data demonstrate that high resolution mass spectroscopy can generate serum proteomic patterns that discriminate men with elevated PSA due to benign processes from men with CaP even when PSA is within the diagnostic gray zone. We are currently expanding the testing set to determine the reliability of this new technology to decrease unnecessary prostate biopsies without compromising the detection of curable CaP.

KEY WORDS: prostate; prostatic neoplasms; prostate-specific antigen; computational biology; spectrum analysis, mass

There is now mounting evidence suggesting that early detection decreases prostate cancer mortality.^{1,2} Measurement of serum prostate specific antigen (PSA) is currently the most useful biomarker to aid in the earlier detection of prostate cancer and PSA measurements are performed in millions of men worldwide. Despite the usefulness of PSA testing there are limitations since PSA is not prostate cancer specific. Since levels can be elevated as the result of benign conditions, the specificity of abnormal PSA is only 20% to 30%.² The measurement of different PSA forms, such as free

and complexed PSA, has been advocated as a means to improve the specificity of PSA without unduly compromising sensitivity.^{3,4} These PSA derivatives can be used to prevent 10% to 30% of unnecessary prostate biopsies but they miss 5% to 10% of biopsy detected prostate cancers.

Advances in proteomic technologies and bioinformatic methodologies have facilitated analysis of the serum proteome and they provide new opportunities for the development of more discriminatory prostate cancer biomarkers.^{5,6} Surface enhanced, laser desorption ionization time-of-flight (SELDI-TOF) mass spectroscopy can rapidly generate complex protein spectra from a miniscule amount of serum and tissue.^{7–10} Multiple bioinformatic tools have been developed to analyze complex proteomic data sets generated by SELDI-TOF.^{11–15} Artificial intelligence based pattern recognition algorithms that evolve and learn are particularly powerful analytical strategies. Proteome Quest combines the power of

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genetic algorithms and cluster analysis. It has been used successfully to discriminate women with ovarian cancer from those with benign ovaries^{13, 16} and men with prostate cancer from those with a benign prostate.¹⁷

In the current study we identified serum proteomic patterns that could be used to determine the need for prostate biopsy in men with intermediate range serum total PSA. To this effect serum was collected from men with PSA 2.5 to 15.0 ng/ml prior to prostate biopsy and proteomic data sets were generated by SELDI-TOF. The computer algorithm was used to build and test discriminatory models that could significantly decrease unnecessary biopsies without compromising prostate cancer detection.

MATERIALS AND METHODS

Patients and serum collection. As part of a clinical protocol approved by the institutional review board at each institution serum samples were collected from 154 ambulatory men prior to prostate biopsy. The indication for biopsy was determined by the patient treating urologist and it included elevated PSA and/or abnormal digital rectal examination. Only men with serum total PSA between 2.5 and 15.0 ng/ml were included in the study. Information regarding the patient prostate biopsy history was obtained by direct patient reporting and by review of the medical records. All men received peri-biopsy antibiotics and self-administered a Fleets enema prior to the procedure. All men underwent transrectal ultrasound guided 10 to 12 core systematic biopsies of the peripheral zones of the prostate. Blood samples were collected in marble topped tubes and serum was isolated by centrifugation. Serum (500 μ l) was sent elsewhere for total and free PSA measurement. The remainder of the sample was stored at -80°C until samples were shipped to the Food and Drug Administration on dry ice for proteomic analysis. Pathological diagnoses were made by a board certified surgical pathologist at the respective institutions.

Mass spectroscopy and pattern recognition. Serum mass spectroscopy and pattern recognition data analysis were performed as previously described.^{16, 17} Briefly, thawed, unfractionated serum samples were applied to a WCX2 (weak cation exchange protein chip) and subjected to SELDI-TOF mass spectrometry on a QSTAR Pulsar i after cinaminc acid was applied to the protein sample. Raw data representing low molecular weight (less than 20 kDa) proteins and peptide fragments were collected without filters. The resultant spectral data streams, comprised of peak amplitudes at approximately 350,000 mass-to-charge (m/z) ratio positions, were exported as .CSV files and analyzed by an artificial intelligence-type pattern recognition algorithm (Proteome Quest beta version 1.0) after performing spectra quality control and quality assurance measures.

Data streams for the high resolution spectra (QSTAR) were first binned using a function of 400 ppm. The binning process condenses the number of data points from 350,000 to exactly 7,084 points per sample. To perform spectral quality control and quality assurance, and verify and the ensure lack of overt spectral bias among any samples raw and binned data were subjected to plotting by total ion current (total record count), amplitude average/mean \pm SD, of chi-square and t-test analysis of each ion or bin and quartile plotting measures using JMP software (SAS Institute, Cary, North Carolina) as well as stored procedures developed in house. Process measures, including chip surface homogeneity, ionization efficiency and time-of-light measurement, were assessed by analyzing the statistical plots of the serum reference standard (SRM-015A, National Institutes of Standards and Technology), which were applied at random points on each chip at different spot locations. Spectra that failed statistical checks for homogeneity were eliminated from in-depth modeling and analysis.

Pattern recognition analyses on the resultant spectra were performed in 2 phases, namely 1) training with known serum samples and 2) testing validation with blinded samples that had not been used in the training set (fig. 1). In phase 1 the genetic algorithm attempted to identify through a neoDarwinistic survival of the fittest approach a limited number of clusters in N-dimensional space. The clusters are plots of the Euclidean distance vector comprised of the combined normalized intensities of the randomly sampled m/z values of the cases and controls, respectively (<http://clinicalproteomics.steem.com>). From the 154 men 63, including 30 with cancer, 10 with 1 negative set of 10 to 12 core biopsies and 23 with 2 or more negative sets of biopsies with at least 1, 10 to 12 core biopsy protocol, were randomly selected from each respective group to be used for phase 1.

In phase 2 masked test spectra were then analyzed and the distance vector was calculated and plotted for each sample using only m/z species comprising the diagnostic model identified in training. N-dimensional plotting yields the classification disease, control or neither disease nor control depending on whether the sample falls into previously existing case or control clusters formed in training, or establishes a new cluster. Six models that accurately classified training samples were found and then tested to determine the ability to identify men with positive biopsies from the remaining 91 serum samples. The 91 serum samples used to test the discriminatory models were obtained from 28 men with biopsies demonstrating prostate cancer and 63 with benign biopsies, of whom 12 had had 2 or more benign biopsies (fig. 1). The table lists the clinical characteristics of these men. Each test samples was analyzed in blinded fashion and assigned a score based on membership within a specific Nth dimensional space cluster.

RESULTS

All 6 models that accurately segregated all training samples were tested in blinded data sets until the model that provided the highest specificity for prostate cancer while maintaining greater than 95% sensitivity was found. The best model found was composed of 10 clusters with scores of 0—most like benign samples in the training set to 1—most like cancer samples in the training set (fig. 2). All samples from patients with cancer were assigned to clusters with scores of 0.5 or greater, while 43 of the 63 from patients with benign biopsies were assigned a score of 0.33 or less (fig. 3). If the proteomic analysis had been used to determine the need for prostate biopsy in this cohort of patients, 67% of

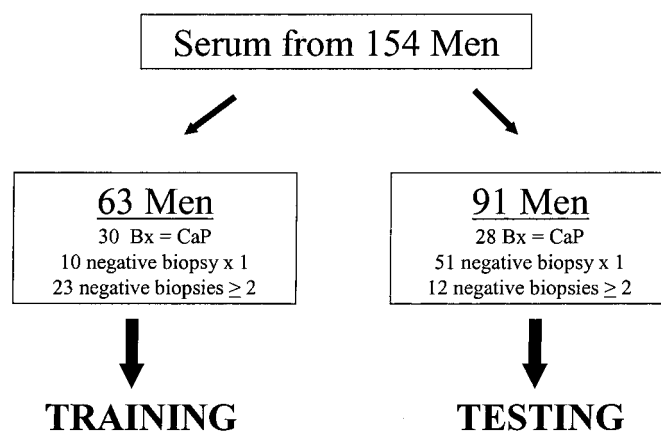


FIG. 1. Study design in which 154 men with total serum PSA between 2.5 and 15.0 ng/ml were included in analysis. Total of 63 men, including 30 with prostate cancer (CaP) and 33 with benign prostate, were used to train algorithm, which was tested on blinded set of samples from 91 men, including 28 with prostate cancer and 63 with benign prostate. Bx, biopsy.

Testing set clinical characteristics

	Ca	Benign
No. pts	28	63
% Black	50	54
Mean PSA (ng/ml)	6.8	5.3
Mean % free PSA	11	16
Mean PSA density	0.19	0.11

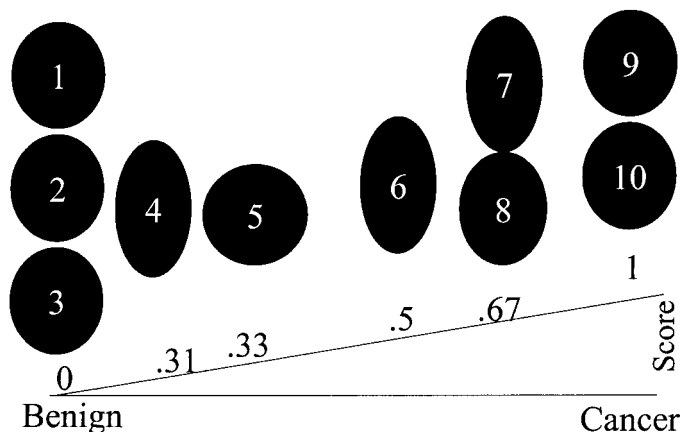


FIG. 2. WCX2 shows that best model training set segregated into 10 unique Nth dimensional space clustered with relative likeness to benign (score 0) or cancer (score 1).

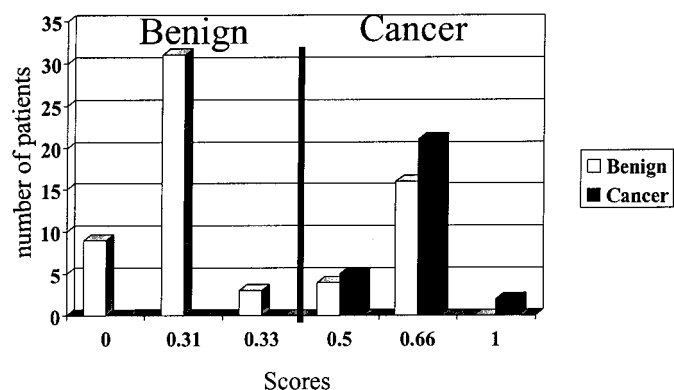


FIG. 3. WCX2 best model testing results demonstrate number of samples in blinded study set that segregated within specific Nth dimensional space cluster.

unnecessary biopsies could have been prevented without missing any cancers. In comparison, if a percent free PSA cutoff of 20% had been used to determine the need for biopsy, 29% of unnecessary biopsies would have been prevented but 11% of cancers would have been missed. The standard cutoff of 25% would have missed 7% of cancers and only saved 8% of unnecessary biopsies.

DISCUSSION

Analysis of patterns of proteins in serum offers great hope to improve prostate cancer detection and minimize false-positive screens. Previous studies have demonstrated that serum proteomic profiling can discriminate men with prostate cancer from those with a benign prostate.¹⁷ Current studies show that this methodology can be used to improve the specificity of PSA testing when serum total PSA is between 2.5 and 15.0 ng/ml. In this study set serum proteomic analysis would have saved 63% of healthy men from undergoing negative biopsies without missing any cancers. These results are far better than if percent free PSA had been used to determine the need for biopsy in this same study set.

There are several methodologies available for analyzing complex mixtures of proteins and peptide fragments. Our approach uses SELDI-TOF.^{7,8,10} SELDI-TOF is a proprietary technology that applies a ProteinChip System and ProteinChip Reader (Ciphergen Biosystems) to facilitate protein capture, purification and analysis on a single platform. This technology produces crude but rapid protein purification and signal amplification. SELDI-TOF is a potentially valuable tool with which to screen for potential cancer biomarkers because it rapidly generates a reproducible, low molecular protein fingerprint from a miniscule amount of sample (ie 1 μ l). SELDI-TOF can accomplish high throughput protein expression profiling from human tissue and body fluids. It has been shown that SELDI-TOF can identify protein signatures from nipple aspirates that discriminate women with breast cancer from healthy women.⁹ SELDI-TOF has also been used to analyze protein expression patterns from pure populations of human cells procured by laser capture microdissection.¹⁸ Using this technology unique protein fingerprints characteristic of benign prostatic epithelium, high grade prostatic intraepithelial neoplasia and prostate cancer were identified.¹⁹ Discriminating protein patterns can also be found within serum. Adam et al reported that SELDI-TOF analysis of the serum proteome could help discriminate men with prostate cancer from those with a benign prostate.¹⁴ In this study protein profiles were generated with IMAC-3 chips with a low resolution ProteinChip Reader (PBS II model, Ciphergen Biosystems) and analyzed using a decision tree based on the alignment and subsequent detection of protein peaks with the highest discriminatory value.

The shortcomings of the low resolution instrument include day-to-day and machine-to-machine drift as well as relatively poor mass accuracy and poor resolution. On the other hand, the high performance hybrid quadrupole time-of-flight mass spectrometer used in this study has much higher resolution and it can generate far more data points than the PBS II instrument. It also provides much finer detail of the spectral topology for any given sample. Since the QSTAR can analyze protein samples applied to ProteinChip Arrays, we investigated the use of this instrument to generate diagnostic serum proteomic patterns in this study.

Mass spectrometric analyses of serum proteins generate complex and expansive data sets. Because visual analysis only detects gross changes in protein expression, bioinformatic tools are required to detect subtle differences in patterns of protein expression. Bioinformatic tools based on decision tree analysis have been used to analyze proteomic data streams generated by SELDI-TOF and they can be used to help identify men with prostate cancer. This approach has generated an algorithm that identified 25 of 30 men (83%) with prostate cancer and 14 of 15 (93%) with a benign prostate from a blinded test set.¹⁴ In comparison, the discriminatory model identified in this study yielded 100% sensitivity and 63% specificity. An explanation for the lower specificity in the current study is that a benign prostate was determined by only 1 set of 12 core biopsies in 51 of the 63 men. Thus, some of these men likely had prostate cancer that was missed by biopsy and the true specificity of the discriminatory model may be higher.

We believe that artificial intelligence based pattern recognition algorithms represent a more powerful analytic tool since, unlike decision tree analysis, they evolve with experience and recognize new events using the same model found in training. That is the genetic algorithm functions in a manner similar to natural selection and determines the most fit subset of amplitudes at defined m/z values that best segregates a training data set into predetermined groups. The genetic algorithm randomly analyzes multiple different pattern combinations until one that discriminates the 2 groups of interest is found. This pattern is then recombined (mated) with additional data. Nondiscriminatory patterns are discarded and

discriminatory ones are further refined. After this fitness test has been successfully applied to all training data, the resultant set y axis defined amplitudes that fully discriminate the training set is determined. Spectra are generated by SELDI-TOF from a set of blinded samples. These data are compared for their likeness to the previously defined patterns generated with the training set. A decision is then made that classifies unknown samples into a previously defined group or into an unclassified group. As more data are input, existing clusters are refined and new clusters are formed using the same features initially selected in training, so that learning is not a simple function of retraining. Thereby, the genetic algorithm learns by experience and in theory it becomes more accurate with time.

Another major advantage of using a genetic algorithm combined with clustering type tools to analyze high dimensional SELDI-TOF data streams is the ability effectively to filter noise and background variables without discarding important information. Mass spectra generated by SELDI-TOF contain significant noise due to chemical contaminants and imperfections in the chip surface. Intrinsic biological variation of protein expression patterns may also contribute to spectral variation. Since the genetic algorithm applies a fitness test to identify regions of the spectra with the best discriminatory power, amplitudes within the spectra that represent noise will not meet the fitness test and will be discarded. Thus, the diagnostic patterns defined by the training process should only contain reproducible data points that theoretically represent only real proteins or peptide fragments.

A limitation of the current study is that the best models were selected based on their performance in analyzing the blinded data set. Thus, the ability of these models to discriminate men with prostate cancer from those with a benign prostate in a general population remains to be tested. Since the models used in this study were generated with only 30 cancer and 33 benign samples, it is likely that more robust models will be generated when larger training sets are used. Before this new technology can be applied in clinical practice new discriminatory models generated from much larger and diverse training and testing sets will be needed.

CONCLUSIONS

Serum proteomic patterns are altered by prostate cancer and analysis with artificial intelligence pattern recognition algorithms can discriminate men with prostate cancer from those with a benign prostate even when total PSA is in the intermediate range of 2.5 to 15 ng/ml. Analyzing patterns of serum proteins represents a new paradigm in clinical diagnostics with the promise to provide more accurate information than traditional methodologies that rely primarily on the measurement of a single analyte.²⁰ Since human malignancies such as prostate cancer are complex diseases, it is likely that the simultaneous assessment of thousands of proteins or peptides fragments would provide more information than the measurement of a single biomarker. Efforts to develop serum proteomic based diagnostic tools to aid in earlier prostate cancer detection are underway and they will hopefully be available to help patients in the near future.

Gail Grigson and Henry Bell collected and processed serum. Raj Pruthi and James Mohler performed some biopsies. Serum PSA was measured at Beckman-Coulter.

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