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DIAGNOSTIC POTENTIAL OF SERUM PROTEOMIC PATTERNS IN PROSTATE CANCER

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ABSTRACT

Purpose: The serum prostate specific antigen test has been widely used in the last decade as an effective screening tool for prostate cancer (CaP). However, the high false-positive rate of the serum prostate specific antigen test necessitates the development of more accurate diagnostic and prognostic biomarkers for CaP. Promising diagnostic potential of serum protein patterns detected by surface enhanced laser desorption/ionization time of flight mass spectrometry for CaP has recently been reported. Independent evaluation of this new technology is warranted to realize its translational utility. We determined whether serum protein profiling by surface enhanced laser desorption/ionization time of flight mass spectrometry and a decision tree algorithm classification system could accurately discriminate between patients with CaP and unaffected individuals.

Materials and Methods: Proteomic spectra of crude serum were generated using the Ciphergen ProteinChip System and pattern detection was performed using Biomarker Patterns Software (Ciphergen Biosystems, Inc., Fremont, California). A total of 106 patients with CaP and 56 controls were randomly allocated to a training set and a test set. The training set, which consisted of 44 patients with cancer and 30 controls, was used to build a decision tree algorithm. The test set, which consisted of 62 patients with cancer and 26 controls, was used in blinded fashion to validate the decision tree.

Results: Accuracy of classification using the test set was 67% and 42% for the weak cation exchange array and the copper metal affinity capture array, respectively. Combined spectral data from the weak cation exchange and copper metal affinity capture arrays generated an algorithm that achieved 85% sensitivity and 85% specificity for the detection of CaP.

Conclusions: These preliminary findings support recent observations that complex protein profiles have promising potential for the early detection of CaP and warrant future studies with streamlined technology. Furthermore, the combined effect of using 2 array types can greatly enhance the ability of protein profile patterns, suggesting the potential usefulness of alternative approaches to evaluate this new emerging technology.

KEY WORDS: prostate; proteomics; prostatic neoplasms; spectrometry, mass, matrix-assisted laser desorption-ionization; proteins

The American Cancer Society estimated that 189,000 new prostate cancer (CaP) cases and 30,200 deaths occurred in 2002 in the United States.[1] The serum prostate specific antigen (PSA) test, which has been widely used for screening for CaP in the last decade, has brought about a dramatic increase in detection of early stage disease.[2] However, the PSA test is limited due to high false-positive rates and up to 75% of patients with elevated serum PSA may not have cancer.[2] PSA derivatives, such as percent free PSA, complexed PSA, age adjusted PSA, PSA velocity and PSA density, have contributed to increased accuracy but sensitivity and specificity remain suboptimal.[3] Identification of biomarkers for more accurate CaP detection remains critical for patient satisfaction and more efficient practice.

Genomics as well as proteomics provide a new paradigm for the discovery and evaluation of biomarkers for cancer detection and for the identification of subjects at high risk.[4,5] Recent advances in proteomics technology, particularly in mass spectrometry, are now providing an excellent opportunity to develop high throughput, accurate testing tools that can aid in disease diagnosis and prognosis.[6–9] The ProteinChip system uses surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) to perform rapidly the separation, detection and analysis of proteins directly from unprocessed biological samples. SELDI-TOF-MS defined protein expression profiles were originally described in biomarker discovery through analysis of urine specimens in patients with bladder cancer[10] and in the elucidation of differential expression profiles among malignant, benign and normal prostate cells.[11] Serum protein profiling using this approach has been evaluated for highly

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accurate diagnosis of ovarian cancer[12] and CaP.[13] These studies used various bioinformatic tools, such as classification trees, genetic algorithms and cluster analysis, which are able to sift efficiently through the huge amount of data obtained using the SELDI-TOF-MS process.

We determined whether serum protein profiling using SELDI-TOF-MS supplemented with commercially available software packages could accurately discriminate between patients with CaP and unaffected individuals. We also hypothesized that the use of multiple array types with different surface chemistries may enhance the screening capability of serum protein profiling in contrast to using a single array type.

MATERIALS AND METHODS

Patients with CaP and controls. Our study population consisted of 106 patients with CaP and 56 controls. Of the patients with cancer 103 underwent radical prostatectomy for clinically localized CaP and 3 underwent radiation therapy at Walter Reed Army Medical Center. The cancer group comprised 79 white men, 26 black men and 1 Asian man. Mean age in this group was 58.1 years and mean PSA was 7.17 ng/ml. Of the patients with CaP 64 had pT2 and 39 had pT3 stage disease, while 3 were not staged pathologically since they chose to undergo radiation therapy rather than surgery. Controls included healthy men with serum PSA less than 4.0 ng/ml. In this group 33 men underwent prostate biopsy, which was negative for cancer. The control group comprised 36 white men, 15 black men, 1 Asian man, 1 Hispanic man and 3 of undisclosed race. Mean age in this group was 55 years and mean PSA was 1.666 ng/ml. The entire study population was randomly assigned into 2 sets: 1) a training set for building the classification algorithm which includes 30 controls and 44 patients with CaP, and 2) a blinded test set used for validation which includes 26 controls and 62 patients with CaP.

Serum samples. Serum from patients with cancer was obtained prior to radical prostatectomy or radiation therapy. None of the patients underwent hormonal therapy before blood extraction. Serum from controls with negative prostate biopsy was obtained prior to biopsy. Blood was collected in a 10 ml red top serum separator tube and allowed to clot for 30 minutes at room temperature. Serum samples were maintained at –80C and were not subjected to more than 2 freeze-thaw cycles prior to the assay. Equal amounts of serum from 8 randomly chosen controls were pooled together as quality control serum, which was used to assess assay reproducibility. All patients provided written informed consent and protocols were approved by the Human Use Committee of the Department of Clinical Investigation, Walter Reed Army Medical Center.

Array processing. Preliminary experiments of serum protein profiling with various chip chemistries (hydrophobic, anionic, cationic and metal affinity) led us to choose the immobilized metal affinity capture-copper array (IMAC3-Cu) and the weak cation exchange array (WCX2) for our studies because they yielded the best resolution of proteins. The detailed methodology used for processing of serum and arrays are available at http://www.cpdr.org/proteomics.html.

Data acquisition. Processed ProteinChip Arrays were analyzed using the Protein Biological System (PBS) II ProteinChip Reader (Ciphergen Biosystems, Inc.). Time of flight spectra were generated using data acquisition parameters optimized for sensitivity and peak resolution for a particular array type. For the WCX2 array 100 laser shots were averaged with the laser shooting in positive ion mode with an energy of 9.78 μJ and a lag time of 1,182 nanoseconds. For the IMAC3-Cu array 100 laser shots were averaged with the laser shooting in positive ion mode with an energy of 7.66 μJ and a lag time of 982 nanoseconds. Calibration of the PBS for mass accuracy was done prior to each run using insulin and ubiquitin standards (Ciphergen Biosystems, Inc.).

Spectral data were acquired using Ciphergen ProteinChip Software, version 3.0b (Ciphergen Biosystems, Inc.). Baseline subtraction was done and normalization of peak intensities was achieved using total ion current. We limited our range of peak masses analyzed to between 2,500 and 50,000 Da since our data acquisition parameters were optimized to detect peaks at this range. A peak detection and clustering tool called the Biomarker Wizard (Ciphergen Biosystems, Inc.), available with Ciphergen ProteinChip Software, was used to identify significant peaks consistently across all spectra in the training set. Corresponding peaks in the spectra from the test set were likewise identified using Biomarker Wizard using the clustering data from the training set. All peak information was then exported as spreadsheet files for use by the pattern detection software.

Pattern detection. Biomarker Patterns Software (BPS) is an implementation of the Classification and Regression Trees decision tree system developed by Breiman et al.[14] BPS uses the peak information generated by the training set of known samples to build a binary decision tree algorithm. The algorithm functions by assigning each sample or case in the data set into 1 of 2 groups or nodes with a rule based on the intensity of a particular peak or splitter. For example, figure 1 shows that the splitter for the first node has a mass of 25,167 Da. If the peak with this mass has an intensity of 0.444 or less for a particular case, it is assigned to the subnode on the left. If the peak has an intensity of greater than 0.444, it is assigned to the subnode on the right. Each subnode has a different rule that further divides the data set. This process continues until all cases are assigned into terminal nodes that classify them as cancer or noncancer. After the classification tree is built it may then be used on the test set of unknown cases to evaluate its ability to distinguish between cancer and noncancer.

Statistical analysis. Measures of association such as sensitivity and specificity were calculated to measure the accuracy of the assay for identifying patients with prostate cancer. Statistical significance for 2×2 tables was computed using Fisher's exact test.

RESULTS

Spectral data from serum of patients with CaP and controls in the training set were acquired and then analyzed by Ciphergen ProteinChip Software. Peak detection using the Biomarker Wizard yielded 89 significant peaks on the WCX2 chip and 97 significant peaks on the IMAC3-Cu chip for the training set. Peak information was exported for pattern recognition by BPS.

For the WCX2 array BPS generated a decision tree using 6 splitters with mass values of 3,972, 8,226, 13,952, 16,087, 25,167 and 33,270 Da, respectively, and classified cases into 8 terminal nodes (fig. 1). For the IMAC3-Cu array 5 splitters with mass values of 3,960, 4,469, 9,713, 10,266 and 22,832 Da, respectively, classified cases into 6 terminal nodes (fig. 2). Combining the data generated from the 2 arrays generated a less complex tree with 3 splitters, including 2 from the WCX2 array with mass values of 16,087 and 25,167 Da, respectively, and 1 from the IMAC3-Cu array with a mass value of 4,283 Da (fig. 3). This algorithm from combined array data correctly assigned 26 of 30 controls (86.7%) and 39 of 44 patients CaP (88.6%) in the training set.

The ability of the decision tree algorithms to distinguish between cancer and noncancer was validated by attempting to classify blinded cases correctly in the test set. The table shows the results of the classification. Using the WCX2 array 39 of 62 cancer cases (63%) in the test set were correctly classified as cancer and 20 of 26 controls (77%) were correctly classified as controls. Using the IMAC3-Cu array 41

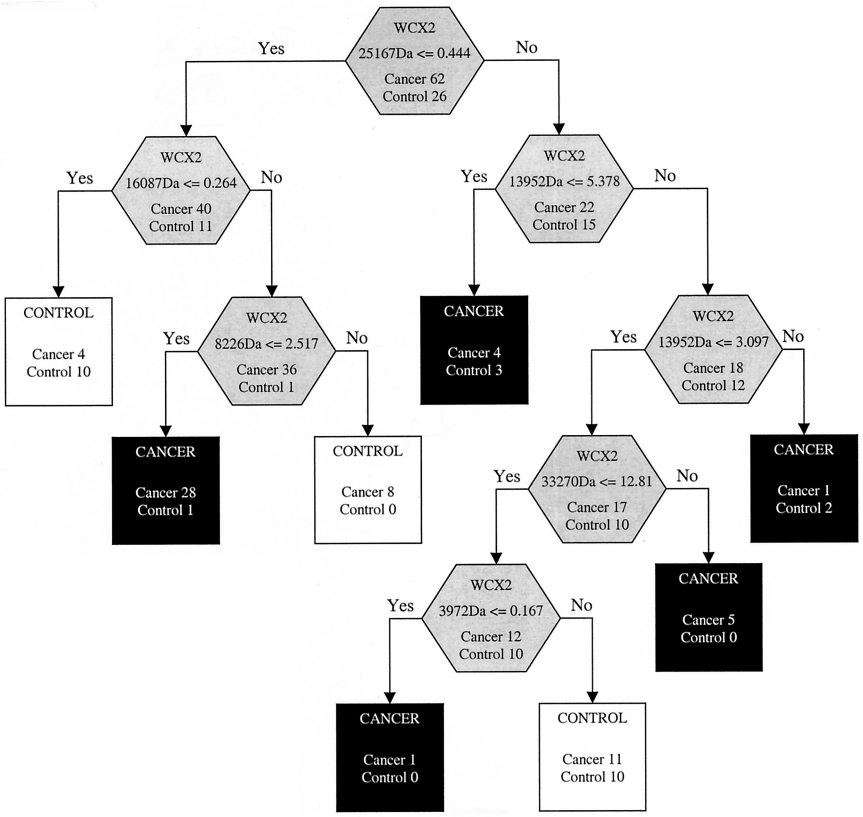

FIG. 1. Decision trees generated from WCX2 array. Hexagons represent splitting nodes containing array type, peak splitter mass in Da, peak intensity cutoff and number of actual controls and cancer cases from test set to be divided into 2 descendant nodes. Squares represent terminal nodes with class assigned by algorithm, including black for cancer and white for control. Cases are correctly classified if actual class and class assigned by algorithm are same. Cases are misclassified if actual class and class assigned by algorithm are different.

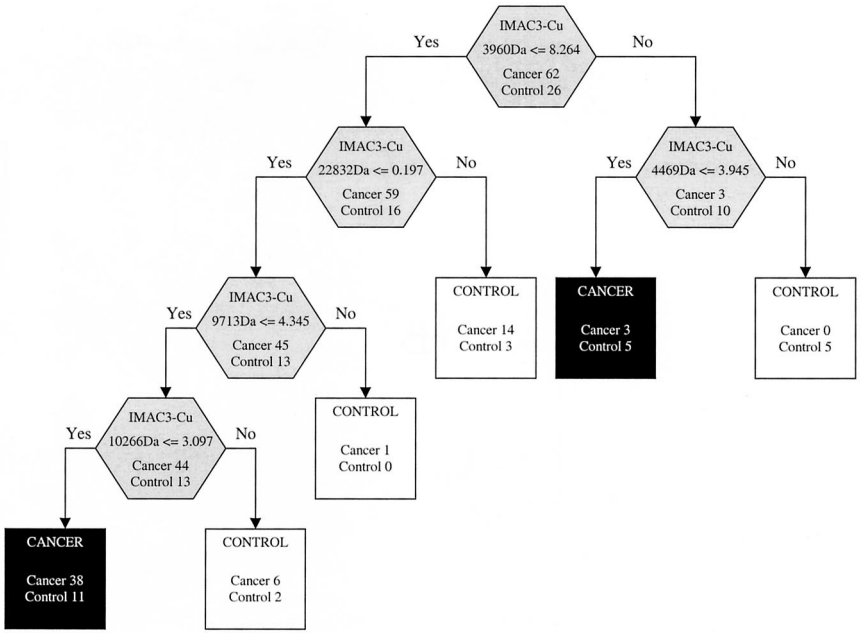

FIG. 2. Decision trees generated from IMAC3-Cu array. Hexagons represent splitting nodes containing array type, peak splitter mass in Da, peak intensity cutoff and number of actual controls and cancer cases from test set to be divided into 2 descendant nodes. Squares represent terminal nodes with class assigned by algorithm, including black for cancer and white for control. Cases are correctly classified if actual class and class assigned by algorithm are same. Cases are misclassified if actual class and class assigned by algorithm are different.

of 62 cancer cases (66%) were correctly classified as cancer and 10 of 26 controls (38%) were correctly classified as controls. The classification rates were greatly improved by combining the data from the 2 array types. Using the data from the WCX2 and IMAC3-Cu arrays 53 of 62 cancer cases (85%) were correctly classified as cancer and 22 of 26 controls (85%) were correctly classified as controls. This combination of using 2 arrays provided 85% sensitivity, 85% specificity and 85% overall accuracy ($p < 0.0001$). Coefficients of variance for normalized intensity for randomly selected reference

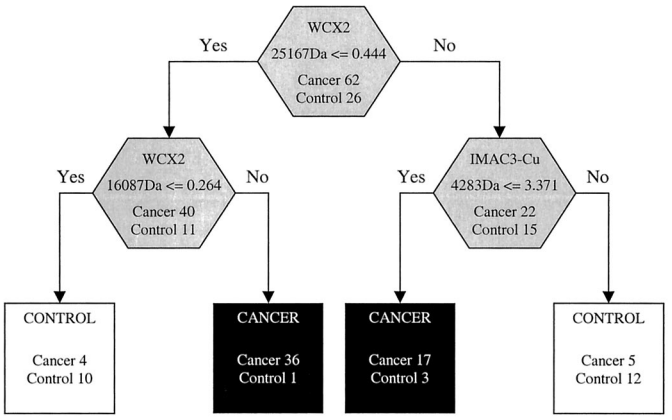

FIG. 3. Decision trees generated from combined data from WCX2 and IMAC3-Cu arrays. Hexagons represent splitting nodes containing array type, peak splitter mass in Da, peak intensity cutoff and number of actual controls and cancer cases from test set to be divided into 2 descendant nodes. Squares represent terminal nodes with class assigned by algorithm, including black for cancer and white for control. Cases are correctly classified if actual class and class assigned by algorithm are same. Cases are misclassified if actual class and class assigned by algorithm are different.

Classification of controls and patients with cancer in test set using decision tree algorithms

Array Type	No. SELDI Classification (%)		Totals
	Ca	Control	
WCX2 array:			
Ca	39 (63)	23 (37)	62
Control	6 (23)	20 (77)	26
IMAC3-Cu array:			
Ca	21 (34)	41 (66)	62
Control	10 (38)	16 (62)	26
WCX2 + IMAC3 = Cu array:			
Ca	53 (85)	9 (15)	62
Control	4 (15)	22 (85)	26

peaks in the SELDI spectra did not exceed 30% for IMAC3-Cu and WCX2.

DISCUSSION

Although the serum PSA test has proved to be an effective early detection tool for CaP, all clinicians recognize that it has suboptimal sensitivity and specificity, mainly because elevated PSA is not unique to cancer. Serum PSA in the 4 to 10 ng/ml "gray zone" range may have a false-positive rate of as high as 75%.[2] Elevated PSA is detected in nonmalignant conditions such as benign prostate hypertrophy (BPH), in inflammatory conditions such as acute and chronic prostatitis, and in premalignant conditions such as low and high grade prostate intraepithelial neoplasia.[3] Conversely the fact that 20% of patients with CaP present with normal serum PSA at diagnosis[2] further emphasizes the need for more accurate CaP specific screening tools to replace or supplement PSA testing.

The diagnostic potential of comparative serum proteomic profiling without actual identification of the specific proteins represents the technological leap from traditional blood based diagnostic approaches, wherein single protein targets have been assayed for specific disease. Due to the highly heterogeneous nature of CaP that reflects a myriad of genetic alterations the development of minimally invasive, high throughput screening procedures would clearly be advantageous. At our laboratory the diagnostic potential of SELDI-TOF-MS with a pattern detecting algorithm for CaP has now been demonstrated.

Clinical application of SELDI-TOF-MS was explored by Vlahou et al in bladder cancer,[10] Petricoin et al in ovarian cancer[12] and Li et al in breast cancer.[15] The diagnostic utility of serum SELDI profiling in a large series of controls, and patients with BPH and CaP was convincingly reported by Adam et al,[13] wherein a 9 protein mass pattern was detected using the IMAC3-Cu array, providing 83% sensitivity and 93% specificity for distinguishing between CaP and BPH/healthy male cases. In a more recent article Petricoin et al presented a 7 protein mass pattern elucidated using the C16 hydrophobic array, which provided 95% sensitivity and 78% specificity.[16] These pioneering reports clearly emphasize the promise of this new technology and suggest that the robustness of this technology must be validated at different laboratories as well as in the clinical setting.[13, 16] Our comparable results yielding 85% sensitivity and 85% specificity was achieved with protein biomarkers with mass values that were completely different from those used in the other studies. Discrepancies in the elucidated peak splitters in our study may be attributable to differences in the array processing procedure as well as in the bioinformatic tools used. With the advent of powerful data mining tools, such as cluster analysis, self-organizing maps,[17] artificial neural networks[18] and support vector machines,[19] the discovery, validation and optimization of the best pattern recognition technique may have a critical role in the future standardization and acceptance of this assay in clinical applications.

We also used 2 array surfaces in our study. The selective nature of specific ProteinChip arrays represents an inherent self-imposed limitation since proteins and peptides that do not bind to its active surface are eliminated by washing the array with buffer. Using 2 array types increases the number of candidate biomarkers tested and the potential for the discovery of more clinically significant discriminators. In our study combinatorial analysis elucidated a relevant discriminator from the IMAC3-Cu array at 4283 Da, which would otherwise not be considered important when using the metal affinity capture array alone.

The insidious nature of CaP presents an unavoidable challenge in translational research since it is not easy to confirm the actual disease status of the control population. Controls who have had a negative prostate biopsy represent a more stringently selected control group since healthy male volunteers used in similar studies do not commonly undergo biopsy.

Our study represents a preliminary report that needs validation through a large, randomized, prospective study. At this stage of its technological development proteomic profiling cannot supplant PSA testing and future comprehensive studies in larger study populations are definitely warranted. Nevertheless, in principle our data support recently published reports. To realize the clinical usefulness of this new emerging technology streamlining the technology and the variation inherent to the biology of specimens must be considered carefully. Ideally the accuracy of SELDI protein profiling should be simultaneously tested with PSA, especially in patients with a PSA in the problematic 4 to 10 ng/ml range. To ensure low probability of contamination of the control group with men with occult CaP we set an inclusion criteria of PSA less than 4.0 ng/ml, which unfortunately prevented us from doing an unbiased comparison to PSA for the current study population. However, it is notable that 35 of the 42 patients with CaP (83%) in the 4 to 10 ng/ml PSA range were accurately identified by the assay.

In the diagnosis of prostate cancer a particularly troubling area for clinicians is the patient who harbors persistently elevated serum PSA despite repeat prostate biopsies. One is never certain how many biopsy sessions are necessary when there is persistently elevated/increasing PSA. Serum protein profiling in this difficult patient group may be an almost immediate clinical usefulness of this emerging technology.

Consequently proteomic profiling patterns must be defined that may have prognostic potential and for the prediction of therapeutic outcome. Currently identification of the proteins represented by the peak splitters in the algorithm is not required to realize the clinical significance of this new technology. However, eventual characterization of these reference proteins should be performed to provide better understanding of the biological nature of these biomarkers and their role in the underlying disease process.

CONCLUSIONS

This preliminary study shows that serum proteomic profiling using SELDI-TOF-MS supplemented by bioinformatics is a viable tool for detecting CaP. Further evaluation and validation are necessary for the development of this technology. Followup studies in a large CaP cohort are underway at our laboratory.

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REFERENCES

1. Jemal, A., Thomas, A., Murray, T. and Thun, M.: Cancer Statistics, 2002. CA Cancer J Clin, **52:** 23, 2002
2. Barry, M. J.: Clinical practice. Prostate-specific antigen testing for early diagnosis of prostate cancer. N Engl J Med, **344:** 1373, 2001
3. Prostate-specific antigen (PSA) best practice policy. American Urological Association (AUA). Oncology, **14:** 267, 2000
4. Weber, B. L.: Cancer genomics. Cancer Cell, **1:** 37, 2002
5. Ramaswamy, S. and Golub, T. R.: DNA microarrays in clinical oncology. J Clin Oncol, **20:** 1932, 2002
6. Chong, B. E., Hamler, R. L., Lubman, D. M., Ethier, S. P., Rosenspire, A. J. and Miller, F. R.: Differential screening and mapping of proteins from premalignant and cancer cell lines using nonporous reverse-phase HPLC coupled with mass spectrometric analysis. Anal Chem, **73:** 1219, 2001
7. Ferrari, L., Seraglia, R., Rossi, C. R., Bertazzo, A., Lise, M., Allegri, G. et al: Protein profiles in sera of patients with malignant cutaneous melanoma. Rapid Commun Mass Spectrom, **14:** 1149, 2000
8. Keough, T., Lacey, M. P., Fieno, A. M., Grant, R. A., Sun, Y., Bauer, M. D. et al: Tandem mass spectrometry methods for definitive protein identification in proteomics research. Electrophoresis, **21:** 2252, 2000
9. von Eggeling, F., Junker, K., Fiedle, W., Wollscheid, V., Durst, M., Claussen, U. et al: Mass spectrometry meets chip technology: a new proteomic tool in cancer research? Electrophoresis, **22:** 2898, 2001
10. Vlahou, A., Schellhammer, P. F., Mendrinos, S., Patel, K., Kondylis, F. I., Gong, L. et al: Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. Am J Pathol, **158:** 1491, 2001
11. Cazares, L. H., Adam, B. L., Ward, M. D., Nasim, S., Schellhammer, P. F., Semmes, O. J. et al: Normal, benign, preneoplastic, and malignant prostate cells have distinct protein expression profiles resolved by surface enhanced laser desorption/ionization mass spectrometry. Clin Cancer Res, **8:** 2541, 2002
12. Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M. et al: Use of proteomic patterns in serum to identify ovarian cancer. Lancet, **359:** 572, 2002
13. Adam, B. L., Qu, Y., Davis, J. W., Ward, M. D., Clements, M. A., Cazares, L. H. et al: Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res, **62:** 3609, 2002
14. Breiman, L., Friedman, J. H., Olshen, R. A. and Stone, C. J.: Classification and Regression Trees. Belmont, California: Wadsworth Publishing Co., Inc., 1984
15. Li, J., Zhang. Z., Rosenzweig, J., Wang, Y. Y. and Chan, D. W.: Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem, **48:** 1296, 2002
16. Petricoin, E. F., 3rd, Ornstein, D. K., Paweletz, C. P., Ardekani, A., Hackett, P. S., Hitt, B. A. et al: Serum proteomic patterns for detection of prostate cancer. J Natl Cancer Inst, **94:** 1576, 2002
17. Kurella, M., Hsiao, L. L., Yoshida, T., Randall, J. D., Chow, G., Sarang, S. S. et al: DNA microarray analysis of complex biologic processes. J Am Soc Nephrol, **12:** 1072, 2001
18. Lisboa, P. J.: A review of evidence of health benefit from artificial neural networks in medical intervention. Neural Netw, **15:** 11, 2002
19. Raudy, S. S.: How good are support vector machines? Neural Netw, **13:** 17, 2000