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Prostate Specific Antigen/Solvent Interaction Analysis (PSA/SIA): A Preliminary Evaluation of a New Assay Concept for Detecting Prostate Cancer Using Urinary Samples

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Abstract

Objectives—To provide preliminary clinical performance evaluation of a novel CaP assay, PSA/SIA (Solvent Interaction Analysis) that focused on changes to the structure of PSA.

Methods—222 men undergoing prostate biopsy for accepted clinical criteria at three sites (University Hospitals Case Medical Center in Cleveland, Cleveland Clinic, and Veterans Administration Boston Healthcare System) were enrolled in IRB approved study. Prior to TRUS guided biopsy, patients received DRE with systematic prostate massage followed by collection of urine. The PSA/SIA assay determined the relative partitioning of heterogeneous PSA isoform populations in urine between two aqueous phases. A structural index, K, whose numerical value is defined as the ratio of the concentration of all PSA isoforms, was determined by total PSA ELISA and used to set a diagnostic threshold for CaP. Performance was assessed using ROC analysis with biopsy as the gold standard.

Results—Biopsies were pathologically classified as case (malignant, n=100) or control (benign, n=122). ROC performance demonstrated AUC=0.90 for PSA/SIA and 0.58 for serum tPSA. At a cutoff value of K=1.73, PSA/SIA displayed sensitivity=100%, specificity=80.3%, PPV=80.6%, and NPV=100%. No attempt was made in this preliminary study to further control patient population or selection criteria for biopsy, nor did we analytically investigate the type of structural differences in PSA that led to changes in K value.

Conclusions—PSA/SIA provides ratiometric information independently of PSA concentration. In this preliminary study, analysis of the overall structurally heterogeneous PSA isoform population using the SIA assay showed promising results to be further evaluated in future studies.

INTRODUCTION

The widespread application of PSA assays to CaP screening and detection has revolutionized the diagnosis and management of this disease¹. While the issues of 'overdiagnosis' and 'overtreatment' remain controversial, the use of tPSA has resulted in a significant downward migration of diagnostic stage²,

The clinical utility of tPSA assays is limited, however, by relatively poor specificity and predictive value. The tendency of benign conditions including inflammation, BPH, infection, instrumentation and trauma to confound the diagnostic process by causing false elevations of tPSA is well documented³. As a result, current diagnostic paradigms that utilize tPSA thresholds to determine the need for confirmatory biopsy demonstrate false positive rates of 55–75%⁴. In addition, false negative rates of at least 15% have been reported⁵ using the traditional threshold of 4.0 ng/ml. Suboptimal performance of tPSA contributes substantially to patient anxiety, morbidity, and the aggregate cost of CaP screening and surveillance protocols. This lack of accuracy is directly related to the failure of standard tPSA assays to reliably differentiate malignant from benign disease.

The need to improve the operating characteristics of tPSA has led to much research aimed at improving the diagnostic accuracy and clinical utility of CaP testing. Concepts to improve specificity include use of age-adjusted tPSA, tPSA velocity, volume-adjusted tPSA, and %free PSA⁶. However, diagnostic accuracy and predictive value remain problematic. Contemporary research has generally focused on identifying genetic and protein markers that reflect malignant phenotypes which are detectable by straightforward assays.^{7,8} In this study, we instead refocus attention to the evaluation of PSA and its structural isoforms to attempt to improve the diagnostic accuracy of the test.

The benefits afforded by the specificity of PSA to prostate tissue have led other researchers to explore the diagnostic potential of its structural variants. Several isoforms of PSA have been identified, including the inactive precursor pro-PSA. Pro-PSA includes a leading peptide sequence which is normally cleaved to form the active mature PSA protein. Incomplete cellular processing of pro-PSA may result in its accumulation in malignant cells. When this leading peptide is incompletely removed by proteolysis, various truncated forms result. These truncated forms of pro-PSA have been found to be specific to malignant prostate cells in some studies⁹. In addition, PSA is known to be a glycoprotein with a single N-oligosaccharide chain attached to Asparagine-45. Glycosylation patterns of PSA have been demonstrated to be distinct between malignant cells and benign tissue. Specifically, PSA from malignant prostatic tissue and transformed prostate cell line contains complex-type oligosaccharides with more antennas than from benign tissue^{10–12}. Current research suggests identifying changes in PSA glycosylation show promise for improved diagnostic performance of PSA testing^{13–17}, but the use of lectin-based assays is complicated in serum due to the formation of extensive glycosylated ACT ligand complexes with the majority of PSA in serum.

In this study we report preliminary findings from a trial designed to investigate the diagnostic performance of a new urine based test, in which the heterogeneous mixture of structural isoforms of PSA is partitioned in a unique aqueous two-phase system. The partitioning behavior of the overall isoform population is different depending on its origin - cancer versus benign epithelium. This differential partitioning is evaluated in a novel quantitative assay called PSA/SIA (AnalizaDx, Cleveland, Ohio, USA). We report preliminary outcomes and operating characteristics of the PSA/SIA assay and compare its performance against serum tPSA.

MATERIAL AND METHODS

Study Population

Between 1/2007 and 9/2008, men undergoing prostate biopsy at three clinical sites (University Hospitals Case Medical Center, Cleveland; Cleveland Clinic Foundation, Cleveland; and Veterans Administration Boston Healthcare System, Boston, Massachusetts, USA) were eligible for participation: ages <40 (1), between 40–60 (61), between (60–80) (147), above 80 (13); Caucasian (196), African American (25), other (1). Patients were selected for biopsy according to current standard diagnostic paradigms including elevated tPSA, abnormal free PSA percentage, elevated age specific tPSA, abnormal tPSA velocity, and abnormal DRE. Recognizing the exploratory and feasibility testing nature of the study, and to provide early data to the potential use of the test in patient selection for biopsy, no further patient stratification was made. All patients who were selected for biopsy according to currently accepted medical criteria were eligible for the study. IRB approval and informed consent for study participation were obtained at all clinical sites and appropriate counseling was provided.

Sample Collection and Processing

Prior to standard 10–20 core TRUS guided prostate biopsy, patients received DRE with prostatic massage according to a pre-defined protocol. This was accomplished by placing firm, uniform pressure starting at the prostate base and proceeding to the apex and then from each lateral sulcus to the midline. This maneuver was repeated 2–3 times. Following prostatic massage, all patients provided a voided urine sample for specimen collection. Samples were placed on ice within 30 minutes, stored at -4°C and transferred frozen to laboratories where they were thawed, centrifuged, aliquoted into 0.5ml vials and subsequently stored at -80°C . Biopsy cores were analyzed locally at the corresponding pathology laboratory. Biopsy results were classified into two groups, malignant or benign, and were used as the “gold-standard” in evaluating the performance of the PSA/SIA assay. It should be noted that the PSA collected in this manner could potentially include fractions originated from ejaculate, such fractions may complicate analysis of data from the PSA/SIA. This issue could be further investigated using, e.g., ejaculate spiked female urine samples - but was not included in this preliminary study. Future studies on PSA/SIA will be designed to evaluate these types of potential analytic confounding variables.

The PSA/SIA Assay

Solvent Interaction Analysis (SIA) is an analytical technique for detecting structural changes in proteins using partitioning in aqueous-based two-phase systems. Aqueous two-phase systems are naturally formed as two distinct immiscible solvent layers when polymers and salts at certain concentrations are mixed in water, resulting in separate but predominantly aqueous phases with distinct physiochemical properties. The partitioning behavior and properties of the adjacent phases are determined by a thermodynamic phase diagram, and are highly flexible depending on the starting type and relative quantity of the initial ingredients. The technique has been shown excellent sensitivity to detect diverse types of structural changes in proteins, including single residue modifications, conformational changes, post-translational modifications such as glycosylation, and formation or loss of a complex with other ligands^{18–20}. The technique is used today as analytical tool for, e.g., quality control applications in production of recombinant proteins (Analiza, Inc., Cleveland, Ohio, USA).

In the PSA/SIA assay, each PSA isoform independently and potentially differently partitions between the two aqueous phases, depending on its structure. The clinical sample containing prostatic fluid is added to the SIA system. The assay itself is conducted in two steps:

partitioning of the PSA isoforms, and determination of a composite structural index representing that isoform mixture. After partitioning of the PSA isoforms (by simple mixing and centrifugation to accelerate phase separation), each of the two aqueous phases contains different amounts of the various PSA isoforms, since each was partitioned independently. The PSA/SIA assay detects and quantifies a composite of differential structural changes of the PSA isoform population by comparing the relative concentration of the entire PSA isoform in the two phases. This step, following sample partitioning, is readily accomplished by measuring the total PSA in both aqueous phases using a conventional total PSA clinical ELISA, and then forming a numerical ratio between the two PSA concentration levels. This numerical ratio, called K, is used as a composite structural index of the heterogeneous PSA population. During assay development, the properties of the SIA chemistry are adjusted until suitable numerical difference is demonstrated among samples corresponding to different clinical diagnosis. The simplicity of a single index, K, representing the entire structural spectrum of PSA is balanced by the loss of specific understanding regarding the particular isoform composition. This could be studied at a later stage; however, the assay could be developed and proven clinically useful even without a priori knowledge of the details that led to differences in the K value of samples with different clinical origin.

Sample partitioning—Partitioning was conducted by adding each urine sample to prepared SIA solvent systems using robotic liquid handlers (Hamilton Company, Reno, Nevada, USA). To ensure appropriate analytical range, typically 5–6 dilutions were generated by adding 10–75 μ l of sample and then adding corresponding amounts of water as described previously^{18–20}. Following the addition of sample, the systems were mixed in a vortexer (Glas-Col, Terra Haute, Indiana, USA) for ~1 minute, centrifuged for 60 minutes at 3,000rpm (~3,500g). Next, aliquots of 100–200 μ l were withdrawn from the top and bottom phases.

Determination of the composite structural index, K—The definition of K as the ratio of the total PSA concentration in the top to bottom phases was modified in this preliminary study to increase the assay accuracy. K could also be defined by the slope of a linear regression line of separate SIA experiments which are conducted with increasing amounts of sample. Serial five-fold dilutions of each aliquot were performed with Universal Diluent (Roche, Basel, Switzerland). The diluted aliquots were placed on a Elecsys 1010 clinical immunoanalyzer (Roche, Basel, Switzerland) for subsequent assay using tPSA ELISA (Roche, Basel, Switzerland). The Roche tPSA assay performance places the practical limits of the performance of the entire PSA/SIA assay. The LOD of the Roche assay is 0.011 ng/ml, and if the LOQ is defined as 3X LOD limit then its value is 0.033 ng/ml. The LOD/LOQ did not present lower limit for any of the samples since the actual tPSA level in post-prostate massage urine was typically at least 100 ng/ml, and thus dilutions were made as discussed to ascertain that the measured tPSA concentrations were within the Roche dynamic range of 0.011 (or 0.033) – 100.0 ng/ml. The CV of the tPSA assay was 2.4–3.8% (SD of 0.005–1.15 ng/ml, depending on concentration), per the manufacturer data sheet, which resulted in part in the overall observed CI for the assay clinical performance parameters shown below. The assay recovery was determined by addition of Roche PSA calibrator in known amounts into the PSA/SIA assay and calculating recovery from the measured concentrations in both aqueous phases of the SIA system. Recovery was found to range from 94.5%–100%, with an average of 98.3%. Least squares linear regression (Sigma Plot, Systat Software Inc., Chicago, Illinois, USA) was applied to the dilution series of the 5–6 partitioning experiments conducted for each sample. The partition coefficient, K, is determined by the slope of the regression line and defines the composite structural index of the sample. The total number of PSA ELISA assays necessary to calculate K was therefore 10–12 (5–6 experiments each with top and bottom phases), and the assay was conducted in

duplicate in this feasibility study. The average standard error of regression was ± 0.04 (CV of 2.3%) and the average R^2 value was better than 0.98 across all 222 samples in the study. This high accuracy version of the PSA/SIA assay will be modified in future studies to reduce the total number of required ELISA assays.

Statistical Analysis

The study data was sent to the Biometry Research Group, Division of Cancer Prevention, National Cancer Institute (Bethesda, Maryland, USA) for analysis. In general, using tPSA as a screening assay, the AUC usually does not exceed 0.72²¹. For more aggressive CaP with Gleason grade 7 or greater, the AUC has been shown to approach 0.78²¹. Based on these currently available standards, in our sample size calculations, we considered the null hypothesis H_0 : AUC = 0.8 versus either the one-sided (H_1 : AUC > 0.8) or two-sided (H_1 : AUC $\neq$ 0) alternative. For both alternatives, the necessary sample size was calculated to achieve at least 85% statistical power to detect AUC of 0.9. We assumed that the ratio of cases (those with diagnosed CaP) to controls (those without cancer) was between 2/3 and 1. The calculations were based on the estimate of AUC proposed by Hanley and McNeil²² that is a function of the ROC area and the sample size only. This method is based on an exponential distribution approximation to the standard error of the ROC area which has been shown to provide a very good approximation for a variety of underlying distributions for continuous diagnostic characteristics²³. The calculations show that the necessary sample size for the one-sided alternative is 120 samples while for the two-sided alternative it is 150 samples. ROC analysis was based on the nonparametric Mann-Whitney procedure²². The resultant analysis indicates that a post hoc H_0 : AUC=0.9 is rejected at the 5% level using a two-sided statistical test.

RESULTS

Of the 222 patients that participated in the study, 122 were found to have benign processes on pathologic examination of biopsy specimens and 100 were found to have CaP. The disease prevalence in the study population was therefore 45%, and the overall false positive rate of the combined diagnostic paradigms used to select patients for biopsy was 55%.

The distribution of tPSA and K with biopsy pathology results is presented in Figs. 1–2 using cutoff values of 4 ng/ml and 1.73 for serum tPSA and K, respectively. At K=1.73 cut-off value, the PSA/SIA assay resulted in sensitivity of 100% (95% CI 96.4–100%), specificity of 80.3% (95% CI 72.2%–87.0%), PPV of 80.6%, and NPV of 100%. The cut-off value of K was determined retrospectively in this preliminary study; however, the raw AUC data is, of course, independent of any particular cut-off value. The AUC for the PSA/SIA assay (Fig. 3) was 0.90 ± 0.02 (95% CI 0.85–0.94, $p < 0.0001$). In comparison, the AUC for tPSA (Fig. 3) was 0.58 ± 0.04 , (95% CI 0.51–0.66, $p < 0.035$). No correlation was found between tPSA and corresponding K values for both case and control groups (Pearson correlation coefficient=0.07 and 0.04 for the case and control groups, respectively).

The current SIA chemistry used in this study resulted in significant crowding of samples near the cut-off level of K, representing a potential difficulty in discrimination unless a high accuracy protocol is used. Future studies will evaluate additional SIA chemistry candidates that better separate the two clinical groups to enable utilization of the assay in conventional clinical laboratory settings.

COMMENT

The diagnostic strategy embodied in this study represents both conceptual and practical departure from conventional protein biomarker detection methodologies, including standard

tPSA assays, which generally focus on the quantification of specific protein expression levels – typically an increase in its amount relative to control. In contrast, the PSA/SIA assay instead provides quantitative information that is independent of tPSA levels. In partitioning the heterogeneous PSA isoform population using SIA, we were able to create a useful composite structural index (K) that provides an easily interpreted cutoff value, and which categorizes the PSA isoform mixture into cancer and benign phenotypes. Using a retrospective value of $K > 1.73$ threshold we note an initial encouraging performance level versus pathologic biopsy.

An important distinction of the PSA/SIA assay from other structure-based attempts to correlate PSA structure with cancer (e.g., pro-PSA, free to bound ratio, etc.) is that our assay does not *a priori* attempt to define a particular structural change and then design an assay around it. Instead, the assay was developed with actual clinical samples and without necessitating mechanistic assumptions so as to accommodate the underlying broad variability of the molecular manifestation of the disease. The PSA/SIA test is therefore simultaneously sensitive to the myriad structural changes due to cancer. These may include previously known changes (such as truncation, glycosylation and differential binding to other ligands/proteins) as well as other yet to be discovered changes.

As evident from the data, the value of the composite structural index, K, also presents significant variability. From results in other studies²⁰ we hypothesize that variability in the K value may relate (aside from the inherent structural heterogeneity of PSA) to the relative amounts of PSA isoforms in the sample. Post-prostate massage urine contains variable amounts of PSA originating from different types of epithelial cells (e.g., cancerous vs. benign) and potentially from variable amounts of ejaculate and thus variable levels of K. This observation could potentially be used to plan future trials aimed at using PSA/SIA to assess parameters including clinical stage, biological aggressiveness, and risk of disease progression as well as other cancer-related indications.

One additional potential benefit from the inclusion of the PSA/SIA assay, once validated, as an additional parameter in the patient selection paradigm for prostate biopsy may be its influence on the recommended PSA cut-off screening value. The high sensitivity and sufficiently high specificity of PSA/SIA may lend additional support to lowering the traditional tPSA screening thresholds that trigger a recommendation for urological evaluation – potentially without resulting in an increase in the number of biopsies. Additional applications that may be investigated in future studies include screening of asymptomatic patient populations, correlation between PSA/SIA and other clinical variables such as stage, Gleason score, PSA velocity, age and race specific PSA and free PSA as well as the assessment of biochemical failure following therapy.

CONCLUSIONS

In a preliminary evaluation, the PSA/SIA assay provided ratiometric information independently of PSA concentration. It is encouraging to note that differential partitioning of the structurally heterogeneous PSA isoform population predicted biopsy results better than current selection criteria.

Acknowledgments

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Figure 1. Case and control sample scatter plots for total serum PSA levels (tPSA)
tPSA levels are expressed in ng/ml. Below a cut-off tPSA level of 4 ng/ml there were 33 control and 18 case samples, and above 4 ng/ml there were 86 control and 79 cases (not all patients in the study had documented tPSA levels). In our study, 23% of the patients selected for biopsy had tPSA levels below 4 ng/ml.

Figure 2. Case and control sample scatter plots for the PSA/SIA composite structure index (K)
 K values are non-dimensional. Below a cut-off K value of 1.73 there were 98 control and zero case samples, and above 1.73 there were 24 control and 100 cases. The cut-off point for K was retrospectively selected illustrate optimal sensitivity and specificity levels.

Figure 3. Receiver-Operating-Characteristic (ROC) curves for the composite structure index (K) and for total serum PSA (tPSA, ng/ml)

A perfect test according to a ROC analysis would have 100 percent sensitivity and 100 percent specificity and would include the upper left-hand corner of the plot. The curve for a test with no discriminatory power would appear as a diagonal (45 deg.) line from the lower left to the upper right corner. The area under the ROC curve is a measure of diagnostic performance, and is varied between 0.5 (no diagnostics power) to 1.0 (perfect diagnostic power).