



Novel Prostate Cancer Biomarkers Derived from Autoantibody Signatures¹

Matthew Schipper*, George Wang*, Nick Giles[†] and Jeanne Ohrnberger[†]

*University of Michigan, Ann Arbor, MI, USA; [†]Armune BioScience, Inc, Ann Arbor, MI, USA

Abstract

BACKGROUND: Due to the low specificity of the prostate-specific antigen (PSA) assay and a high false positive rate, a large number of prostate cancer (PCA) biopsies are performed unnecessarily. Consequently, there is a need for new biomarkers that can identify PCA at any stage of progression while limiting the number of false positives. The use of autoantibody signature-developed biomarkers has proven to be an effective method to solve this problem. **RESULTS:** Using T7 phage-peptide detection, we identified a panel of eight biomarkers for PCA on a training set. The estimated receiver-operating characteristic (ROC) curve had an area under the ROC curve of 0.69 when applied to the validation set. Spearman correlations were high, within 0.7 to 0.9, indicating that the biomarkers have a degree of inter-relatedness. The identified biomarkers play a role in processes such as androgen response regulation and cellular structural integrity and are proteins that are thought to play a role in prostate tumorigenesis. **CONCLUSIONS:** Autoantibodies against PCA can be developed as biomarkers for detecting PCA. The scores from the algorithm developed here can be used to indicate a relative high or low risk of PCA, particularly for patients with intermediate (4.0 to 10 ng/ml) PSA levels. Since most commercially available assays test for PSA or have a PSA component, this novel approach has the potential to improve diagnosis of PCA using a biologic measure independent of PSA.

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Background

Prostate cancer (PCA) is the second most common cause of cancer-related death in American men [1]. The advent of prostate-specific antigen (PSA) screening has led to earlier detection of PCA [2]. However, the impact of PSA screening on cancer-specific mortality is still unknown pending the results of prospective randomized screening studies [3–5]. A major limitation of the serum PSA test is lack of PCA sensitivity and specificity especially in the intermediate range of PSA detection (4–10 ng/ml) [6,7]. Elevated serum PSA levels can be detected in patients with nonmalignant conditions such as benign prostatic hyperplasia or prostatitis [8], leading to a high rate of false positives. *In situ*, PSA is highly expressed in both benign prostatic epithelial and PCA cells, contributing to the poor specificity of the serum PSA test, which has been reported to be only 20% at a sensitivity of 80% [9]. Due to this high rate of false positives, there has been a dramatic increase in the number of unnecessary prostate needle biopsies performed [10]. Furthermore, a recent study suggests lowering the threshold level of PSA needed for recommending prostate biopsy from 4.1 to 2.6 ng/ml to correct for verification bias and improve the clinical value of the PSA test [11]. Lowering the

threshold will lead to a further increase in the number of prostate biopsies performed. To reduce the number of unnecessary prostate biopsies, development of additional serum and tissue biomarkers to supplement PSA is needed.

Efforts toward the development of screening tests for PCA have generally depended on single biomarker molecules, primarily PSA, as well as PCA3 [12]. Current technologies have been disappointing and

Address all correspondence to: Jeanne Ohrnberger, PhD, Armune BioScience, Inc, 401 W. Morgan, Ann Arbor, MI 48108, USA.

E-mail: jmohrnberger@armune.com

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have not resulted in diagnostic tests sufficiently reliable or convenient to apply to clinical practice for detection of early-stage PCA [13]. Like other cancers, PCA develops in the background of diverse genetic and environmental factors [14]. Multiple complex molecular events characterize PCA initiation, unregulated growth, invasion, and metastasis [14]. Distinct sets of genes and proteins dictate progression from precursor lesion to localized disease and finally to metastatic disease. Biomarkers that detect PCA in any of these stages of progression would be ideal.

A potential approach to develop biomarkers that detect PCA in any stage of progression is to take advantage of the immune system. The hypothesis that there is an immune response to cancer in humans has been demonstrated by the identification of autoantibodies against a number of intracellular antigens in patients with various tumor types [15–18]. This phenomenon is known as the humoral response and the detection of such autoantibodies has been shown to have great diagnostic and prognostic value in the detection of cancer and the ability to predict the course of the disease [15–19]. For example, it has been shown that somatic alterations in the *p53* gene elicit a humoral response in 30% to 40% of affected patients [20]. The detection of anti-*p53* antibodies can predate the diagnosis of the cancer. In other studies, 60% of patients with lung adenocarcinoma exhibited a humoral response to glycosylated annexins I and/or II, whereas none of the noncancerous standards exhibited such a response [19]. There are now a substantial number of examples demonstrating this humoral response to patient sera [15–21]. In addition, it has been shown that the majority of antigens from tumor cells that elicit this response are not just products of mutated genes. These proteins are often differentiation antigens or other proteins over-expressed in cancer [16].

Using T7 phage with epitope inserts, autoantibodies against peptides derived from PCA tissue have been previously demonstrated to have utility in diagnosing PCA [22]. The original work of Wang et al. identified a panel of 22 autoantibody biomarkers that successfully diagnosed PCA [22]. In the current study, additional biomarkers were screened and a unique algorithm was developed to discern between healthy and diseased men irrespective of PSA levels. The final algorithm incorporated eight biomarkers.

Results

Using T7 phage-peptide display libraries developed previously [22], we evaluated the relative signal of 62 peptides compared with the background. The 62 peptides were obtained by screening T7 phage-peptide display libraries against 96 discreet samples (48 biopsy positive and 48 clinically negative) on a Luminex platform. Those

samples that had elevated signals were selected. Further screening was done against 300 serum samples, and criteria of relative signal above background (>1.5), distribution of signal in cancer-positive samples, and strength of signal (>300 RFI) were used to further reduce the biomarker pool. This process narrowed down the peptides to a group of 18 putative biomarkers. Using the training data (519 samples), a logistic regression model based on eight biomarkers was identified. The specific biomarkers included in the final model and their estimated parameter coefficients in the logistic regression model are shown in Table 1. A significance test of whether each parameter is significantly different from 0 is also included.

The biomarker parameters can be combined into a single score called the “linear predictor,” which can be used to calculate the sensitivity and specificity of the algorithm (Table 2). Given the case-control nature of this study, in which approximately 50% of the samples were known to be from PCA patients, the usual estimates of positive and negative predictive values (PPV and NPV) would need to be adjusted if this biomarker is applied in a population of men in which the prevalence of PCA is not 50%.

The estimated receiver-operating characteristic (ROC) curve from an independent validation set (259 samples) is given in Figure 1 and compared to the ROC for each individual biomarker included in the algorithm. The estimated area under the ROC curve (AUC) with 95% confidence interval for this model is 0.69 (0.62, 0.75) when applied to the validation set. When applied to the same data on which the model was built (training set), the estimated AUC was 0.74. Total PSA measurements of samples in other studies within the 2.5 to 20 ng/ml level can have a range of ROC values of 0.54 to 0.67 depending on the cohort of patients being analyzed [37].

The validation process fully complies with the recommendations of the 2012 report from the Institute of Medicine Committee on Omics-Based Predictive Tests, “Evolution of Translational Omics: Lessons Learned and Path Forward” (National Academy Press, Washington, DC) [23]. The assay and the classifier algorithm were “locked down” in advance of the validation analysis using entirely independent samples. The performance of the algorithm on the independent validation set is much improved over that of a PSA test. At the balance point of the ROC, the assay displays a sensitivity of 0.65 and a specificity of 0.65. Spearman correlations (the same interpretation as Pearson’s but rank based to detect nonlinear relationships) were calculated for each pair of biomarkers. These were remarkably high for the unnormalized biomarkers with most in the range of 0.7 to 0.9. This indicates that there is some interdependence of the biomarkers, in that the markers do not function as stand-alone markers, but instead they rely and depend on each other to perform. Investigating the biologic function of each biomarker and seeing what

Table 1. Estimated Biomarker Parameters

Analysis of Maximum Likelihood Estimates					
Parameter	DF *	Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square
Intercept	1	-1.3552	0.2056	43.4638	<0.0001
X3C3_T7	1	-0.00077	0.000394	3.8104	0.0509
X36C4_T7	1	-0.00107	0.000610	3.0517	0.0807
X7A9_T7	1	0.00461	0.000778	35.0869	<0.0001
X5D11_T7	1	-0.00075	0.000399	3.5543	0.0594
X12B2_T7	1	0.00153	0.000285	28.7814	<0.0001
X1B4A_T7	1	0.000686	0.000321	4.5776	0.0324
X3D11_T7	1	-0.00089	0.000350	6.4960	0.0108
X5F8_T7	1	-0.00454	0.000995	20.7982	<0.0001

A logistic regression model was fit in which a linear combination of the biomarkers is used to predict the probability of a given sample being cancer. * Degrees of Freedom.

Table 2. Estimated Diagnostic Accuracy Parameters for a Range of Positivity Thresholds Corresponding to Sensitivity Values from 10% to 90%

Sensitivity	Specificity	PPV *	NPV *
0.103	0.965	0.389	0.830
0.205	0.912	0.338	0.839
0.301	0.894	0.384	0.854
0.404	0.832	0.345	0.864
0.500	0.788	0.341	0.878
0.603	0.690	0.299	0.888
0.705	0.593	0.276	0.902
0.801	0.451	0.243	0.912
0.904	0.248	0.209	0.922

* PPV and NPV were adjusted to correspond to a population with an 18% cancer prevalence.

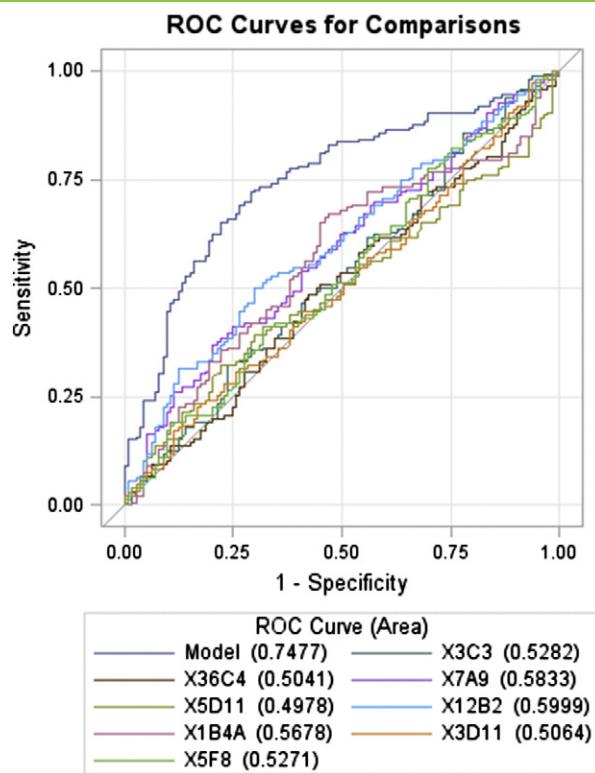


Figure 1. Nonparametric ROC curve for fitted model from the training set when applied to the validation set and compared to the individual ROCs for each biomarker included in the algorithm.

type of dependence there is physiologically aids in the understanding of this interdependence (Table 3).

Results of the linear regression of the biomarker score on age showed that the score does tend to increase with age ($N = 429$). Rather than using the linear predictor, we used the predicted probability of cancer (if XB is the linear predictor, then $\text{prob}(\text{cancer}) = \exp(XB)/(1 + (\exp(XB)))$). On average, this probability of cancer increased by 0.04 for each 10-year increase in age among controls. An analysis was completed to discern whether there was any better discrimination between low and high levels of PSA with the final algorithm. Since the analysis did not involve any weighting of higher or lower PSA values, the algorithm did not give any more weight to higher PSA levels than low (Table 4).

Discussion

The current use of PSA-based screening for PCA has led to earlier detection of PCA [2]. However, a major limitation of the serum PSA test is a high rate of false positives due to a lack of sensitivity and

Table 4. AUC Values Predicted at Various PSA Levels

Included PSA Level	AUC
PSA > 4	0.69
PSA > 6	0.70
PSA > 8	0.69
PSA > 10	0.71
PSA > 12	0.67

specificity in the intermediate range of PSA detection (4-10 ng/ml) [7]. This high rate of false positives results in a large number of unnecessary prostate biopsies [10]. Moreover, biopsies can sometimes miss detecting the presence of PCA, even when PSA levels are high [7].

The recent development of autoantibody biomarkers has proven to be a useful diagnostic in the detection of cancer [15-22]. Through the use of iterative biopanning and phage-protein microarrays, Wang et al. has developed an assay whereby multiple autoantibody biomarkers can be used to screen for PCA [22]. The goal of the study in this paper was to develop an algorithm to discern healthy from diseased individuals independent of PSA, building upon the previous work. Different cohorts of men were used to develop the algorithm than were used in previous publications. These cohorts were selected since it has been documented that 40% of men aged 40 to 50 can have undiagnosed, asymptomatic PCA [35]. The incidence increases by 10% for each subsequent decade. The two arms of the study were therefore composed of biopsy-positive males (diseased) and low-PSA, non-PCA family or personal history men under the age of 40 (clinically healthy). Because older men can have undiagnosed PCA, the lower aged men were used to decrease the possibility of having samples that were equivocal for PCA and to increase the accuracy and utility of the biomarkers selected and the algorithm developed. Additionally, the study groups did not use PSA values as a gold standard. Instead, positive biopsy was used as the gold standard for diseased state. Through iterative biopanning, we narrowed down a field of 62 peptides to a group of 18 putative biomarkers. These 18 biomarkers were screened and validated using T7 phage display, resulting in eight PCA biomarkers that were ultimately selected for the diagnostic algorithm.

The statistical analysis demonstrated that the biomarkers did have a degree of relatedness. These biomarkers can be classified into two main biochemical areas: androgen response regulation and cellular structural integrity, with one additional marker potentially functioning to bridge the gap between the two areas. PCA is highly dependent on androgens for development and progression. Indeed, in many cases, one of the first lines of treatment is androgen ablation [24]. Specifically, in the androgen response, there are markers related to B lymphoma Mo-MLV insertion region 1 homolog (BMI1), which is upregulated in PCA [25]. The markers NKX3.1 and AURKIAPI are

Table 3. Biomarker Protein Function

Biomarker	NCBI Designation	Protein Function
CSNK2A2	NM_001896.2	Serine/threonine kinase involved in regulating cell cycle and cellular division
Centrosomal protein 164 kDa (minus strand)	NM_014956.4	Spindle pole integrity at centrosome
NK3 homeobox 1	NM_033625.2	Regulates androgen response genes (BMI1)
Aurora kinase interacting protein 1	NM_001127230.1	Regulates androgen response genes (TWIST1)
5'-UTR BMI1	BC011652.2	Androgen response gene
ARF6	NM_001663.3	Regulates actin cytoskeleton remodeling; vesicle shedding by tumor cells
Chromosome 3' UTR region Ropporin/RhoEGF	NT_005612.16	Ciliary movement in spermatozoa through dynein regulation
Desmocollin 3	NW_004078095.1	Cellular adhesion

Table 5. Demographics and Sample Sources

	PCA Samples		Healthy Samples	
	Training	Validation	Training	Validation
n	268	146	251	113
Average age	63	63	32	34
Average PSA	6.5	6.4	<0.2	<0.2
Average Gleason	7	7	N/A	N/A
Race, n (%)				
Black	24 (9)	10 (7)	117 (47)	55 (49)
Caucasian	164 (61)	102 (70)	60 (24)	25 (22)
Hispanic	0	0	57 (23)	28 (25)
Other/unknown	80 (30)	34 (23)	17 (7)	5 (4)
Source, n (%)				
Bioreclamation	24 (9)	13 (9)	251 (100)	113 (100)
Johns Hopkins	75 (28)	37 (25)	0	0
University of Michigan	169 (63)	96 (66)	0	0

proteins that contain regulators of androgen response genes (*BMI1* and *TWIST1*) and the down-regulation/loss of these proteins' function is another hallmark of PCA progression [25–27]. Presumably, the early immune response is also a factor that causes the sequestration of some proteins during early tumorigenesis and allows for the progression of cancer to occur.

Changes in cellular structure and scaffolding are also a key in any cancer's progression. Interestingly, four other markers are related to centrosomal, cytoskeletal regulation and mobility and cellular adhesion [28–30]. CEP164 was found to be essential for spindle pole integrity at the microtubular anchors of the centrosome. Disruption of the protein leads to chromosomal aberrations seen in tumor cells [28]. ADP-ribosylation factor 6 (ARF6), which regulates actin cytoskeleton remodeling and is active in vesicle shedding by tumor cells [30], is also associated with cytoskeletal proteins. Ropporin is most closely related to ciliary movement in spermatozoa through dynein regulation, but it has also been found to be a target for multiple myeloma immunotherapy, suggesting an active role in some cancer progressions [31]. Desmocollin 3 is involved with cellular adhesion, and dysregulation has been recognized in tumorigenesis as well [32,33]. The final marker of the panel, casein kinase 2, alpha prime polypeptide (CSNK2A2), has long been recognized as an all-purpose messenger-independent protein serine/threonine kinase [34]. This protein has been implicated in PCA progression and a variety of cellular functions ranging from cellular signaling for numerous protein kinases to regulating cell cycle and cellular division. This marker appears to bridge the gap between the other two marker sets.

This panel of biomarkers incorporates many proteins that are either upregulated or altered early on in cancer progression. Additionally, this panel incorporates proteins from different pathways that all promote tumorigenesis. The varieties of pathways in which these proteins are involved strengthen the ability of this assay to detect the heterogeneity of PCA progression. The early immune response allows for the detection of these changes at an earlier time point in the progression of the cancer. There is a gradual rise in the biomarker levels as age increases in the negative controls. As previously noted, there is an increase in undiagnosed PCA in men as they age, with a 10% increase with each decade [35]. It is estimated that by age 30, 30% of the male population has prostatic cancer foci within their prostate. This estimate keeps rising with an 80-year-old man having an 85% probability of having prostatic cancer foci. The gradual rise in the biomarkers among the negatives could be a reflection of the underlying undiagnosed PCA among these men.

Conclusion

The scores from the algorithm developed here can be used to indicate a relative high or low risk of PCA, particularly for patients with intermediate PSA levels. Most other current commercial assays are based in some way on PSA. Since this novel autoantibody technology measures different biologic parameters than PSA, it may provide additional useful information for physicians involved in the management of patients at risk for PCA. This assay may also be effective in addressing the heterogeneity of PCA by measuring multiple biomarkers that are all known to be PCA-related. Further studies will have to be done to investigate how the autoantibody biomarker levels might change over time or if they may be different before or after a biopsy.

Methods

Patient Samples

The patient cohorts (obtained from the University of Michigan, Johns Hopkins University, and Bioreclamation; Table 5) were composed of two arms: 414 men aged 40 to 70 with PSA between 2.5 and 20 ng/ml diagnosed with PCA and 346 men aged 25 to 40 with PSA < 1.0 ng/ml who were self-reported to be cancer-free and had no familial PCA history. Young men were selected as the negative cohort based on concerns that men aged 40 to 70 could potentially have undiagnosed PCA without elevated PSA [35,36]. These cohorts were selected since it has been documented that 40% of men aged 40 to 50 can have undiagnosed, asymptomatic PCA. The incidence increases by 10% for each subsequent decade. The two arms of the study were therefore composed of biopsy-positive males (diseased) and low-PSA, non-PCA family or personal history men under the age of 40 (clinically healthy). Because older men can have undiagnosed PCA, the lower aged men were used to decrease the possibility of having samples that were equivocal for PCA and to increase the accuracy and utility of the biomarkers selected and the algorithm developed.

Biomarker Identification

Phage libraries that had been developed by iterative biopanning were used for further screening [22]. A total of 62 biomarkers was evaluated for relative signal compared to background, and the marker number was reduced to 18 where the strongest signal-to-noise ratio over multiple samples was seen. The 18 biomarkers were attached to Luminex MagPlex beads and tested against training ($N = 519$, PCA = 268, Healthy = 251) and validation ($N = 259$, PCA = 146, Healthy =

113) cohorts. Retrospective samples for these cohorts were obtained from three different sources (the University of Michigan, Johns Hopkins University, and Bioreclamation). The analyte tested, immunoglobulin, is very robust and not prone to degradation with a simple freeze-thaw cycle. Carboxyl chemistry was used to bind the phage to the beads, according to the product protocol (Luminex). Samples were tested in triplicate with high and low serum controls included on each plate and high and low calibrator beads included in every sample. The triplicates were averaged to obtain a single value for each sample. The training cohort was tested with 18 biomarkers to discern healthy from diseased patients. Logistic regression analysis determined the optimal panel to be a total of eight biomarkers. The eight biomarker panel was then used on a separate and distinct validation patient cohort to assess performance characteristics of the diagnostic algorithm. The biomarkers consist of T7 phage with cDNA inserts that correspond to the following proteins/DNA regions: ARF6, NK3 homeobox 1, Aurora kinase interacting protein 1, CSNK2A2, 5'-UTR-BMI1, centrosomal protein 164 kDa, 3'-UTR-Ropporin, and Desmocollin. Table 3 summarizes the protein function and lists the National Center for Biotechnology Information (NCBI) designation for each biomarker. Biomarker identities were confirmed by sequencing at the University of Michigan Sequencing Core with Applied Biosystems DNA Sequencers (Model 3730 XL sequencer).

Statistical Analysis

Samples were randomly divided into a training set (2/3, $N = 519$) and a validation set (1/3, $N = 259$). These two sets were checked for comparability with respect to age, race, and the date each sample was run. Raw biomarker values were normalized by taking the ratio or absolute difference from T7 or BSA. Logistic regression models were used to model the probability of a sample being cancer as a function of the potential biomarkers. ROC curves, based on fitted multivariate logistic regression models, were also generated to show the relation between sensitivity and specificity for the range of possible cut points.

To determine the optimal number of biomarkers to include in the final model, we used the following process. For each number of biomarkers $N = 1$ to 18 and each normalization method (a total of five including unnormalized), the best fitting model with N biomarkers was calculated along with an estimate of the AUC from leave-one-out cross-validation. No interactions were considered. Although the cross-validated estimates of AUC were similar for models with six to nine biomarkers, the numerically highest cross-validated estimate of AUC was obtained with eight biomarkers using the T7 difference normalization method. Thus, the final model selected for validation was obtained as the best fitting model (on the training data) containing eight biomarkers using the T7 difference normalization.

A biomarker signature was defined as the linear combination of the eight selected biomarkers each multiplied by their parameter coefficient from the fitted logistic regression model. The value of this signature was calculated for patients in the validation set, and a logistic regression model was used to test its statistical significance. Sensitivity and specificity associated with this signature, when applied to the validation set, were calculated at various possible cut points. The nonparametric estimate of the AUC was also calculated from the validation set. Prevalence-adjusted PPV and NPV were calculated at various possible positivity thresholds using the estimated sensitivity and specificity and an assumed prevalence of cancer equal to 18% [35]. All analyses were performed using SAS V9.3 (Cary, NC).

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