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Glycoproteomics for Prostate Cancer Detection: Changes in Serum PSA Glycosylation Patterns

Danni L. Meany, **Zhen Zhang**, **Lori J. Sokoll**, **Hui Zhang***, and **Daniel W. Chan**
Center for Biomarker Discovery, Department of Pathology, Johns Hopkins University, Baltimore, MD

Abstract

Currently, serum prostate-specific antigen (PSA) is used for the early detection of prostate cancer despite its low specificity in the range of 4 to 10 ng/mL. Because aberrant glycosylation is a fundamental characteristic of tumor genesis, the objective of this study was to investigate whether changes in PSA glycosylation may be used to improve the cancer specificity of PSA. We developed five lectin immunosorbant assays to analyze the glycosylation patterns of PSA in serum. Each assay sandwiches serum PSA between a PSA monoclonal antibody and a biotinylated lectin and then tags the biotin complex using a streptavidin SULFO TAG for electrochemiluminescence detection. Low limits of detection (0.04-1.35 ng/mL), good reproducibility (%CVs < 10%), and direct analysis of PSA glycosylation in sera suggest these assays may have a potential role in improving PSA's cancer specificity. Clinical performance was evaluated in 52 human subjects (26 cancer and 26 non-cancer). ROC analysis showed that the total SNA assay (AUC=0.71) appeared to perform better than percent free PSA (AUC=0.54) in its diagnostic gray zone between 10 and 20% in a subset of 21 subjects. A separate study of 16 additional subjects showed similar findings.

Keywords

Glycoproteomics; prostate-specific antigen; prostate cancer; glycosylation; sialylation; serum; lectin immunosorbant assays

Introduction

In the United States, prostate cancer is the most common malignancy in men and the second leading cause of death from cancer. Currently, prostate-specific antigen (PSA) is the best tumor marker available for the early detection of prostate cancer. However, PSA lacks specificity as it can be elevated in men with cancer as well is in men with benign prostate conditions. The typically used assay cutoff for PSA is 4.0 ng/mL,[1] although lower cutoffs of 2.0 ng/mL, 2.5 ng/mL and 2.8 ng/mL have been suggested as it is recognized that there is risk for prostate cancer over all ranges of PSA.2, 3 Men with total PSA between 4 and 10 ng/mL are in a diagnostic gray zone of total PSA, in which a biopsy would reveal no evidence of cancer in three out of four men, which results in a number of unnecessary biopsies.4

In serum, the majority of total PSA is complexed with antiproteases, whereas 5 to 45% is in a free, uncomplexed form.5 In an attempt to improve the cancer specificity of PSA in its

* Corresponding author: CRB2 Building Room3M03, Johns Hopkins University, 1550 Orleans St., Baltimore, MD 21231 Tel: (410)502-8149, Fax: (443)287-6388, hzhang32@jhmi.edu .

diagnostic gray zone, it was discovered that men with prostate cancer have a lower ratio of free to total PSA compared to men without prostate cancer. Consequently, percent free PSA (%free PSA) is recommended for risk assessment for prostate cancer when total PSA concentrations are between 4-10 ng/mL. A %free PSA of >25% indicates a lower risk of cancer (e.g. probability = 8%) whereas a %free PSA of <10% suggests a higher risk (e.g. probability = 56%).[6] However, the majority of patients tested for %free PSA fall into the midrange (e.g. 10-20%) for whom the risk of cancer is about 25%, hence, another diagnostic gray zone.

The knowledge that free PSA is composed of both cancer-specific (e.g., [−2]proPSA) and benign-specific (e.g., BPSA) forms explains the limitation of %free PSA and prompts the effort for further improvement.[7] One approach is to utilize the carbohydrate structure of PSA. PSA is a glycoprotein with a single N-oligosaccharide chain.8 Because the cellular glycosylation process-especially sialylation- may be perturbed in tumors,[9-11] these changes in PSA carbohydrate structure may allow the distinction of PSA from normal and tumor origins. Not surprisingly, oligosaccharide profiling by mass spectrometry showed that PSA from prostate cancer sera has a higher content of α2,3-linked sialic acid than that from seminal fluid.[12] Lectin affinity column chromatography followed by the determination of total and free PSA by immunoassay has shown lower α2,6-linked sialic acid in serum free PSA from prostate cancer than that from benign prostate hyperplasia (BPH) and higher α2,3-linked sialic acid in serum free PSA from cancer compared to BPH.[13]

In this study, we developed five lectin immunosorbant assays (total SNA, total MAL I, free MAL I, total MAL II, and free MAL II), which analyze α2,6-linked sialylation of total serum PSA by *sambucus nigra lectin* (SNA) and α2,3-linked sialylation of total and free serum PSA by both *maackia amurensis lectin I and II* (MAL I and II). We then used these assays to conduct a clinical investigation of the potential role of glycoprotein analysis in improving PSA's cancer specificity.

Experimental section

Human serum samples

Individual serum samples were obtained from 26 patients with biopsy-confirmed prostate cancer and 26 patients with biopsy-confirmed non-cancer prior to biopsy. Total PSA concentrations of the non-cancer and cancer groups were matched so that the great majority (87%) had total PSA concentrations between 4 and 10 ng/mL. In addition, 3 prostate cancer serum pools and 3 non-cancer serum pools were prepared from patients with and without prostate cancer, respectively. Both the total and free PSA concentrations of these 6 pools were matched so that their total PSA concentrations were in the range of 5-6 ng/mL and free PSA concentrations were in the range of 0.8-1.6 ng/mL. The 16 subjects (8 cancer and 8 non-cancer) used in a separate study to validate the clinical performance of total SNA assay had total PSA concentrations in the range of 3.1-10.4 ng/mL and %free PSA in the range of 9.2-20.3%.

Reagents

Meso Scale Discovery® (MSD) 96-well standard plates, MSD SULFO-TAG, and MSD plate read buffer T (4X) were from Meso Scale Discovery (Gaithersburg, MD). Total and free PSA monoclonal antibodies (Clone BP001 and AP003S) were from Scripps Laboratory. Human PSA (100% free PSA from human seminal fluid was from Lee Biosolutions, Inc (St.Louis, MO). Biotinylated *sambucus nigra lectin* (SNA), biotinylated *maackia amurensis lectin I* (MAL I), biotinylated *maackia amurensis lectin II* (MAL II) were from Vector Laboratories (Burlingame, CA). Bovine serum albumin (BSA) and Tween 20 were from

Sigma-Aldrich (St.Louis, MO). 10X Tris buffered saline (TBS) was from Bio-Rad (Hercules, CA).

Lectin immunosorbant assays

MSD plates were coated with 30 μL of the PSA monoclonal antibody at a concentration of 7.5 ug/mL and incubated at 4 °C overnight. Unbound antibody solution was discarded and 150 μL of TBS buffer with 5% BSA was used for blocking at room temperature (RT) for 1 hour with shaking. Next, plates were washed three times using TBS + 0.1% (v/v) Tween 20. In order to prevent binding of lectins to the carbohydrate determinants on the PSA antibody, antibody coated on the plates was treated with 150 μL of sodium periodate buffer prepared in 150 mM NaCl and 100mM sodium acetate (pH 5.5) at 4 °C for 1 hour.[14, 15] Following treatment, the plates were washed as before and 50 μL of serum sample was added to each well and incubated at RT for 2 hours with shaking. Plates were washed 10 times with TBS + 0.1% Tween 20 buffer and 25 μL of the detection buffer containing 80 μM biotinylated lectin (e.g. SNA, MAL I or MAL II) and 5 μM MSD streptavidin SULFO-TAG was added to each well for incubation at RT for 1 hour. Finally, 150 μL of 1X MSD plate read buffer was added to each well for electrochemiluminescence (ECL) detection using the MSD SECTOR Imager 2400.

Evaluation of analytical performance

Pooled female sera spiked with various concentrations of human seminal fluid PSA (final concentrations: 0.01, 0.76, 2.34, 7.05, 23.03, and 46.86 ng/mL) were used to develop the assays. The limit of detection (LOD) was calculated based on the signal of the background (0 ng/mL concentration) plus 3 times the standard deviation (SD) of the background. Total and free PSA concentrations in these pools were the same. Within-run reproducibility (n=27) was assessed using pooled male sera at two levels of endogenous total PSA (4.12 ng/mL and 11.22 ng/mL) and free PSA (0.91 ng/mL and 0.99 ng/mL). The total and free PSA concentrations in these samples were determined using the Beckman Access® Hybritech PSA and Free PSA assays, respectively.

Data analysis

PSA glycosylation results from these five lectin immunosorbant assays were expressed in electrochemiluminescence intensity. The Mann-Whitney U-test was used to compare differences between the study groups. The statistical software MedCalc was used to construct ROC curves and to calculate their areas and confidence intervals (CIs).

Results

Lectin immunosorbant assays

Table 1 summarizes the capture antibodies and the lectins used in the lectin immunosorbant assays as well as the carbohydrate moieties they recognize. SNA, isolated from *Sambucus nigra* bark, binds to the disaccharide structure of sialic acid in an α2,6-linkage to galactose.[16] MAL I (also known as MAL, MAA or MAM) and MAL II (also known as MAH) are both isolated from *Maackia amurensis* seeds. MAL I binds to the trisaccharide structure of sialic acid in an α2,3-linkage to galactose which is then in a β1,4-linkage to N-acetylglucosamine,[16] whereas MAL II appears to bind only particular carbohydrate structures that contain α2,3-linked sialic acid,[17] although its specificity is not well defined.

Analytical performance

The binding curves of these five assays, established using pooled female sera spiked with human seminal fluid PSA, are shown in Figure 1. Human seminal fluid PSA was used as the

standard material because it harbors both α2, 3-linked and α2, 6-linked salic acid in its carbohydrate moiety.[12, 18, 19] In all these assays, the electrochemiluminescent signal increases with increasing concentrations of total PSA and free PSA as a result of the binding of lectins to carbohydrate on PSA molecules captured by the PSA antibody. The LOD of these five assays were calculated to be 1.35, 0.14, 0.32, 0.07, and 0.04 ng/mL of PSA, respectively (Table 1). They were well below the typically used assay cutoff for PSA (4.0 ng/mL) and therefore can be used in its diagnostic gray zone (4-10 ng/mL). In order to assess the within-run reproducibility of these assays, two male serum pools at two different endogenous total and free PSA concentrations were measured 27 times in a single run (Table 2). All five assays demonstrated excellent reproducibility, indicated by CVs less than 5% with the exception of a CV less than 10% for one of the free MAL II assays. The insignificant amount of non-PSA proteins present in the PSA standard (less than 2%) does not impact the assays or their capabilities to determine the carbohydrate moiety of PSA because i) a PSA antibody is used to capture PSA molecules from serum and ii) the binding curves were established using the total and free PSA concentrations measured by the Beckman Access® Hybritech PSA and Free PSA assays.

Glycosylation patterns of PSA molecules in pooled sera

We compared the sialylation patterns of free and total PSA molecules in pooled sera between prostate cancer and non-cancer using the five lectin immunosorbant assays (Figure 2). Three pools of sera were prepared for each group to demonstrate their within-group and between-group similarities and differences. Given the limited number of samples that can be run on a 96-well plate, pool 1 in each group was measured 21 times whereas pools 2 and 3 were measured 3 times. A significant PSA sialylation pattern observed from this comparison was that prostate cancer sera showed relatively large within-group variation whereas non-cancer sera showed more consistent sialylation of PSA across the three pools, which may indicate a more heterogeneous sialylation pattern of PSA from cancer than non-cancer origins.

Clinical performance

Clinical performance of these assays was evaluated in 52 subjects with biopsy confirmed prostate cancer (n=26) or non-cancer (n=26). A comparison between the cancer and non-cancer groups for PSA concentrations, %free PSA, and the measured PSA glycosylation is shown in Table 3. Overall, Table 3 showed that the two study groups were not statistically different with respect to total PSA concentrations (p=0.25), but significantly different with respect to free PSA concentrations and %free PSA (p=0.0025 and p<0.001, respectively). Total SNA, total MAL I and MAL II were higher in the non-cancer group than in the cancer group, despite of the fact that total PSA concentrations in the cancer group were higher (cancer 9.08±5.16 ng/mL and non-cancer 7.65±3.52 ng/mL, mean±SD). This may suggest higher sialylation of total PSA in the non-cancer group than in the cancer group, although the differences were not statistically significant (p= 0.47, 0.67, and 0.58, respectively).

ROC analysis of the cancer and non-cancer groups in all 52 subjects (% free PSA in the 4.7-31.8% range) and in 21 subjects with %free PSA in the 10-20% range are shown in Figures 3A and 3B, respectively. %free PSA (AUC 0.85) was superior to all five assays (AUC 0.53-0.63) in all 52 subjects (p < 0.05, Figure 3A), However, in a subset of 21 subjects with %free PSA in the range of 10-20%, total SNA assay appeared to have a better clinical performance than %free PSA as shown by the AUCs (0.71 vs. 0.54, shown in Figure 3B), although this difference was not statistically significant (p=0.27). In these 21 subjects, %free PSA was equivalent between the non-cancer (14.98 ± 3.28%, mean± SD, n=11) and cancer (14.93 ± 3.19%, n=10) groups, whereas the total SNA assay trended towards a higher average of 204713 ± 40965 in the former than 170049 ± 49060 in the latter (p=0.09). The

other four lectin assays, however, did not show improvement over %free PSA in the 10-20% range (shown in Figure3B).

The improved performance trend of the total SNA assay over %free PSA in the 10-20% range was confirmed by applying the assay to a separate set of 16 subjects (8 prostate cancer and 8 non-cancer). Total PSA and %free PSA in the cancer (5.81 ± 2.33 ng/mL and 14.53 ± 3.20%) and non-cancer (4.98 ± 1.47 ng/mL and 15.14 ± 2.66 %) groups were not statistically different (p=0.40 and 0.68, respectively). ROC analysis in these 16 subjects confirmed the improved performance trend of the total SNA assay compared to %free PSA (AUC 0.80 vs 0.53, Figure 3C).

Discussion

Although PSA is the best tumor marker available for prostate cancer, it is not perfect due to its lack of cancer specificity. %free PSA has improved PSA cancer specificity by the assessment of cancer risk from low to high using greater than 25% and less than 10% cutoffs, respectively. However, midrange %free PSA (10-20%) still presents a dilemma.20 In fact, the majority of patients have a %freePSA in this midrange. Given that PSA is a 237-amino-acid single chain glycoprotein with 8.3% of its molecular weight carbohydrate,8 efforts for improvement have focused on searching for cancer-specific forms of PSA in both the amino-acid and carbohydrate portions. One example in the former is [−2]proPSA, a truncated precursor form of PSA that has 2 additional amino acids in a pro-leader sequence.21 Recently an automated immunoassay for [−2]proPSA has been developed and employed in a multi-center study, which showed that [−2]proPSA was a better predictor of prostate cancer than %free PSA, particularly in the 2-10ng/mL total PSA range.20

Although the search for glycosylated forms of PSA that may harbor cancer specificity began almost 20 years ago,[22, 23] progress had been slow. Nevertheless, recent technological advances in glycan analysis renewed interest, particularly after recent publications illustrated different glycan structures of PSA from prostate cancer sera when compared to PSA from seminal fluid and non-cancer sera.[12, 18, 19] This suggested the development of clinically useful and direct assays to detect PSA glycosylation in serum may be promising. To this end, we developed five lectin immunosorbant assays for direct analysis of PSA sialylation in serum. Lectin immunosorbant assays are similar to enzyme-linked immunosorbant assays (ELISA) except that lectins are used as probes for detecting glycan structures.[24] Readily available in pure form, lectins have been extensively used as probes for glycan structures because 1) they have specificity towards mono- or oligosaccharides through complimentary sugar-binding sites and 2) they generally do not interact with protein backbones. However, lectin immunosorbant assays are only used in a small number of research laboratories for three reasons. First, antibodies used in these assays need to be deglycosylated, otherwise lectins would bind not only glycan on proteins captured by antibodies but also to glycans on antibodies, resulting in a high background.[15, 25, 26] Second, because binding affinities of lectins (ranging from 10^6 to $5\times10^7 M^{-1}$)[24] are 100- to 10,000- fold lower than those of antibodies (~10^8 to $10^{12} M^{-1}$)[27] and analytes of interest usually have very low concentrations (~ng/mL) in serum, the limit of detection of theses assays may be insufficient in ~ng/mL ranges. Third, because lectins only have specificity to glycan but not proteins, they may also bind to glycan structures on background glycoproteins other than the glycoprotein of interest in the lectin immunosorbant assays,[15] resulting in a high background which jeopardizes sensitivity. This may be problematic especially when serum specimens are used because the majority of serum proteins are glycosylated.

In this study, we developed five lectin immunosorbant assays that achieved the analytical sensitivity and specificity needed for direct analysis of PSA sialylation in serum by reducing

the high background, increasing binding specificity, and using a sensitive method of detection. In each assay, total or free PSA antibody used to capture PSA from serum samples is oxidized in situ with 20mM sodium periodate, which selectively destroys the carbohydrate structures on the antibody and prevents the binding of lectins to its glycans, and leaves the antibody's binding capability intact.[15] In addition, the high background signal from binding of lectins to the glycans of background glycoproteins is reduced by adding 1% BSA into the detection buffer. In order to increase binding specificity, biotinylated lectins and the strepatavidin SULFO-TAG were mixed together in the detection buffer rather than used in separate steps to prevent prolonged washing that could decrease the binding of lectins due to their low binding affinities. Finally, we used electrochemiluminescence in the MSD platform to increase the sensitivity of detection method.

The analytical advantages of these assays are multi-fold. First, using 96-well plates, these assays are high-throughput and it is possible to analyze hundreds of samples within a day. Second, rather than comparing the PSA sialylation in prostate cancer sera to that in seminal fluid, like Tabarés et al did using oligosaccharide profiling by mass spectrometry,[12] these assays have the sufficient limit of detection (0.04-1.35 ng/mL) to analyze PSA sialylation in non-cancer sera with less than 10 ng/mL of PSA and to compare them to their prostate cancer sera counterparts. Finally, they detect PSA sialylation in serum directly, as opposed to lectin affinity chromatography, which measures it indirectly.[13] As a result of these features, these five lectin immunosorbant assays are excellent tools for the clinical investigation of the potential role of glycoprotein analysis in improving PSA's cancer specificity.

Our results from the pooled sera study showed that α2,3-linked and α2,6-linked sialylation of PSA are more heterogeneous in cancer than in non-cancer. This observation is consistent with findings from glycan structure analysis that PSA from prostate cancer is a mixture of biantennary, triantennary, and possibly tetraantennary oligosaccharides rather than normal PSA which has only biantennary oligosaccharides, which supports the hypothesis that oncogenic transformation of prostate epithelium may differentially affect N-linked glycan processing of PSA.[28] In addition, the α2,3-linked sialylation patterns assessed by MAL I and MAL II were very similar, which indicates that MAL I and II may bind to the similar carbohydrate structures on PSA.

Evaluation of the clinical performance of these five lectin immunosorbant assays revealed that α2,6-linked sialylation of total PSA may be a better predictor of prostate cancer than free PSA in the 10-20% range. However, the difference was not statistically significant due to the small sample size in the current research study.

Although a previous report by Ohyama et al[13] showed that SNA bound fraction of total PSA cannot differentiate prostate cancer from BPH, our study showed it to be promising. The differences could be due to the type of specimens, the method employed as well as our focus on clinically relevant patients with %free PSA in the diagnostic gray zone. Our study used specimens with equivalent total PSA concentrations in the cancer case and non-cancer control groups, whereas Ohyama et al used specimens with total PSA concentrations in the cancer group which were much higher than in the non-cancer group (mean total PSA concentrations: 89 ng/mL vs. 8.8 ng/mL).[13] This particular difference may result in the presence of different forms of glycosylated PSA in the cancer groups, because high levels of PSA are usually associated with large volume and high grade cancers, which may produce different forms of glycosylated PSA than small volume and low grade cancers that are associated with low levels of PSA. In addition, Ohyama et al used lectin affinity chromatography followed by immunodetection of PSA. Chromatographic separation of glycosylated PSA may result in the detection of different forms of glycosylated PSA than

the ones detected by lectin immunosorbant assays. These differences may also explain why our MAL II assays fail to differentiate prostate cancer from non-cancer whereas their results illustrated the opposite.

Conclusions

Five lectin immunosorbant assays were developed to detect sialylation of total and free PSA in serum. Application of these assays to prostate cancer and non-cancer sera indicated that glycosylation patterns of PSA from the former are more heterogeneous than from the latter. Our results also suggested that an assay for α2, 6-linked sialylation of total PSA improved the detection of prostate cancer compared to %free PSA in its diagnostic gray zone (%free PSA = 10-20%) both in an initial study in 21 subjects and in a separate study with 16 subjects. A study with a larger sample size will be needed to validate this finding. In the future, immunosorbant assays using lectins that recognize other carbohydrate moieties (e.g., fucose) could also be developed. These assays may also be useful in understanding perturbed glycosylation in tumor genesis and progression and could be used clinically to improve the differentiation of prostate cancer from non-cancer patients.

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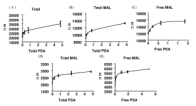

Figure 1.
Binding curves of five lectin immunosorbant assays for total or free PSA.

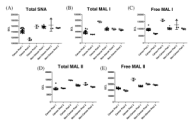

Figure 2.

Comparison of the sialylation of total and free PSA between 3 prostate cancer serum pools and 3 non-cancer serum pools by total SNA (A), total MAL I (B), free MAL I (C), total MAL II (D), and free MAL II (E) assays. Pool 1 in the cancer and non-cancer groups were measured 21 times whereas pools 2 and 3 were measured 3 times. These six pools have matched total PSA and free PSA levels: total PSA concentrations in pool 1, 2, 3 of the cancer and non-cancer groups are 5.26, 5.04, 5.92, 5.20, 5.03, and 4.94 ng/mL, respectively; free PSA concentrations are 0.98, 0.84, 1.15, 1.13, 1.61, and 0.80 ng/mL, respectively.

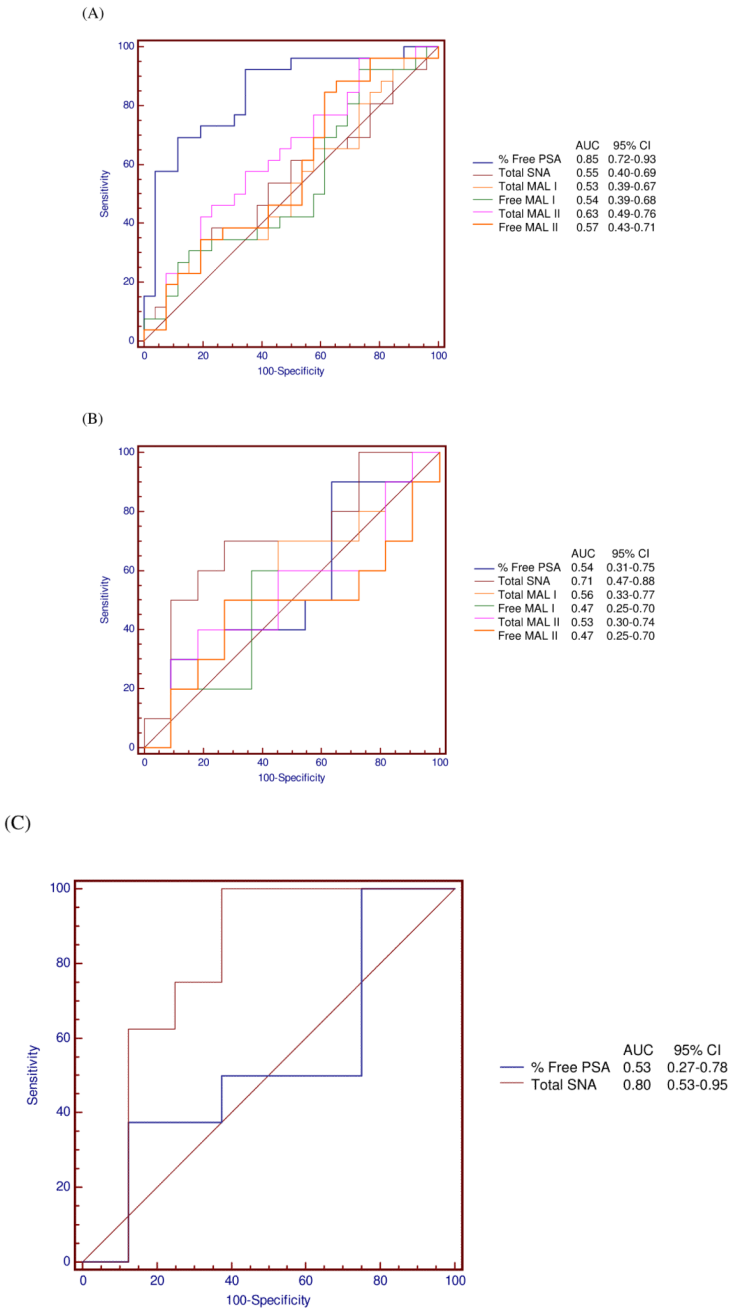

Figure 3.

ROC analysis of the cancer and non-cancer groups in (A) all 52 subjects with free PSA in the 4.7-31.8% range, (B) in a subset of 21 subjects with free PSA in the 10-20% range, and (C) in a separate study of 16 subjects with free PSA of 10-20% range.

Table 1

Five lectin immunosorbant assays for direct analysis of PSA sialylation in serum.

Assay	Capture Antibody	Lectin	Lectin Specificity	LOD (ng/mL)
Total SNA	Total PSA	SNA	2,6 sialic acid	1.35
Total MAL I	Total PSA	MAL I	2,3 sialic acid	0.14
Free MAL I	Free PSA	MAL I	2,3 sialic acid	0.32
Total MAL II	Total PSA	MAL II	2,3 sialic acid	0.07
Free MAL II	Free PSA	MAL II	2,3 sialic acid	0.04

Table 2

Within-run reproducibility (n=27) of five lectin immunosorbant assays determined using electrochemiluminescence intensity.

	Total SNA		Total MAL I		Free MAL I		Total MAL II		Free MAL II	
	Total PSA (ng/mL)		Total PSA (ng/mL)		Free PSA (ng/mL)		Total PSA (ng/mL)		Free PSA (ng/mL)	
	4.12	11.22	4.12	11.22	0.91	0.99	4.12	11.22	0.91	0.99
Mean	331,804	349,333	60,872	81,598	81,509	100,350	19,613	24,328	26,096	29,267
SD	8636	7336	2,360	3,024	2,818	3,176	951	1,123	2,556	1,331
% CV	2.6	2.1	3.9	3.7	3.5	3.2	4.9	4.6	9.8	4.5

Table 3

Comparison between the cancer (n=26) and non-cancer (n=26) groups for PSA concentrations, calculated % free PSA, and the measured PSA glycosylation.

	Prostate Cancer		Non Cancer		
	Mean ± SD	**Median**	**Mean ± SD**	**Median**	**p Value**
Total PSA (ng/mL)	9.08±5.16	8.42	7.65±3.52	7.30	0.25
Free PSA (ng/mL)	0.89±0.43	0.82	1.49±0.86	1.54	0.0025
% Free PSA	10.97±5.68	8.50	19.39±6.85	20.17	<0.001
Total SNA [a]	178022±46482	174335	186739±38629	178223	0.47
Total MAL I [a]	45011±21952	44823	47605±21737	43456	0.67
Free MAL I [a]	49426±23345	53635	53086±23998	51038	0.58
Total MAL II [a]	14382±3369	14415	16000±3896	15764	0.58
Free MAL II [a]	16018±3801	16111	17288±4034	16023	0.11

[a]Total SNA, total MAL I, free MAL I, total MAL II, and free MALII are expressed in electrochemiluminescence intensity.