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# Measurement of aberrant glycosylation of prostate specific antigen can improve specificity in early detection of prostate cancer

Tohru Yoneyama [a,1], Chikara Ohyama [a,*], Shingo Hatakeyama [a], Shintaro Narita [b], Tomonori Habuchi [b], Takuya Koie [a], Kazuyuki Mori [a], Kazuya I.P.J. Hidari [c,d], Maho Yamaguchi [d], Takashi Suzuki [d], Yuki Tobisawa [a,1]

[a] Department of Urology, Hirosaki University Graduate School of Medicine, 5-Zaifu-cho, Hirosaki 036-8562, Japan
[b] Department of Urology , Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
[c] Department of Food and Nutrition, Junior College Division, University of Aizu, 1-1 Aza Kadota, Oaza Yahata, Ikki-machi, Aizuwakamatsu 965-0003, Fukushima, Japan
[d] Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, 52-1 Yada, Suruga-ku, Shizuoka 442-8002, Shizuoka, Japan

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ABSTRACT

*Introduction:* We previously identified prostate cancer (PCa)-associated aberrant glycosylation of PSA, where α2,3-linked sialylation is an additional terminal *N*-glycan on free PSA (S2,3PSA). We then developed a new assay system measuring S2,3PSA using a magnetic microbead-based immunoassay. We compared the diagnostic accuracy of conventional PSA and percent-free PSA (%fPSA) tests.
*Methods:* We used MagPlex beads to measure serum S2,3PSA levels using anti-human fPSA monoclonal antibody (8A6) for capture and anti-α2,3-linked sialic acid monoclonal antibody (HYB4) for detection. We determined the cutoff values in a training test and measured serum S2,3PSA levels in 314 patients who underwent biopsy, including 138 PCa and 176 non-PCa patients with PSA of <10.0 ng/ml. Serum S2,3PSA levels were presented as mean fluorescence intensity (MFI). Receiver operating characteristic curves were used to evaluate the diagnostic accuracy of total PSA, %fPSA, and S2,3PSA.
*Results:* We determined an MFI cutoff value of 1130 with a sensitivity of 95.0% and specificity of 72.0% for the diagnosis of PCa in the training test. In the validation study, the area under the curve for the detection of PCa with S2,3PSA was 0.84, which was significantly higher than that with PSA or %fPSA.
*Conclusions:* Although the present study is small and preliminary, these results suggest that the measurement of serum S2,3PSA using a magnetic microbead-based immunoassay may improve the accuracy of early detection of PCa and reduce unnecessary prostate biopsy.


## 1. Introduction

Serum prostate specific antigen (PSA) is widely used as a powerful biomarker for the early detection of prostate cancer (PCa) [1,2]. Widespread use of the PSA test caused a stage migration of PCa [3]. However, the use of PSA in PCa screening is becoming controversial [4,5]. Particularly, in recent years, criticism against PCa screening using PSA has grown because of over-diagnosis and over-treatment [6]. In addition to complications associated with the treatment of PCa, such as urinary incontinence and erectile dysfunction that are associated with the treatment of PCa, harmful events associated with prostate biopsy are also a major concern for PSA-based PCa screening [7]. Therefore, the development of novel screening methods with improved specificity is of vital importance. Among the various molecular isoforms of PSA, proPSA is one of the most promising potential biomarkers [8,9]. Recently, the Food and Drug Administration of the United States of America approved the use of p2PSA and phi [10] in PCa screening. This innovative method for the early detection of PCa has had a significant impact on clinical practice. However, additional approaches focusing on the aberrant glycosylation of PSA are also promising [11,12].

Cancer-associated glycan alteration is observed frequently during carcinogenesis [13]. Majority of the tumor markers, such as alpha-fetoprotein (AFP; [14] and human chorionic gonadotropin (hCG; [15], are glycoproteins that have glycosylation sites in their amino acid sequences. More importantly, each glycan has specific cancer-associated carbohydrate alterations when compared with its normal counterpart, which can be detected using specific monoclonal antibodies or lectin.

PSA is a glycoprotein with one *N*-glycosylation site on its 45th amino acid from the *N*-terminus, asparagine (N) [16]. The amino acid sequence surrounding the glycosylated N is isoleucine (I),

* Corresponding author. Fax: +81 172 39 5092.
*E-mail address:* coyama@cc.hirosaki-u.ac.jp (C. Ohyama).
[1] These authors contributed equally to this work.

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arginine (R), N, and lysine (K), which is specific to PSA [16]. Therefore, in a prior study, we cleaved the PSA-specific sequence IRNK, which includes the glycosylated-N, and performed an intensive structural analysis of the glycan profile of PSA using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [17]. Importantly, we identified that the terminal *N*-glycan structure of PSA from PCa patients was rich in sialic acid α2,3-linked to the galactose residue, whereas the terminal *N*-glycan structures of PSA from the seminal plasma were exclusively α2,6-linked [17] (Fig. 1A). These findings are consistent with our prior observations demonstrating that the binding of PCa-associated PSA to *Maackia amurensis* agglutinin (MAA) was remarkably stronger than that of non-PCa-associated PSA (18) because MAA lectin recognizes α2,3-linked sialic acid.

In the present study, we successfully developed a novel assay system using a magnetic microbead-based immunoassay for the detection of α2,3-linked sialylation as an additional terminal *N*-glycan on free PSA (S2,3PSA).

## 2. Materials and methods

### 2.1. Coupling the anti-free PSA (fPSA) monoclonal antibody (mAb) to magnetic microbeads

The coupling of anti-fPSA mAb (8A6, Abcam, Cambridge, UK) to magnetic beads (MagPlex beads, Luminex, TX, USA) was performed using the xMAP Antibody Coupling (AbC) Kit following the manufacturer's instructions. In brief, 1 ml ($1.25 \times 10^7$) of MagPlex bead suspension was pelleted using a DynaMag™-2 magnetic separator for 2 min. The MagPlex beads were then resuspended in 500 μl of activation buffer using vortexing and sonication. The beads were pelleted, the supernatant was removed, and the washed microspheres were resuspended in 400 μl of activation buffer. Fifty microliters of *N*-hydroxysulfosuccinimide (sulfo-NHS) solution and 50 μl of EDC (10 μg/250 μl) were added to the microsphere suspension, which was mixed gently. The suspension was then incubated for 20 min at room temperature (RT) with gently vortexing. The activated beads were washed twice with 500 μl of activation buffer, and 62.5 μg of anti-fPSA mAb (8A6) diluted in 500 μl of activation buffer was then added. The suspension was then stirred for 2 h with gently vortexing. Finally, the antibody-conjugated beads were washed and resuspended in 2 ml of wash buffer (6250 beads/μl), and were then ready to use.

### 2.2. Quantification of serum S2,3PSA using the Luminex system

A schematic diagram demonstrating the quantification of S2,3PSA using the Luminex system [19] is shown in Fig. 1B. We used xMAP magnetic beads to measure serum S2,3PSA, using anti-human fPSA mAb (8A6) to coat the beads as the capture antibody, and anti-α2,3-linked sialic acid mAb (HYB4) (Wako, Osaka,

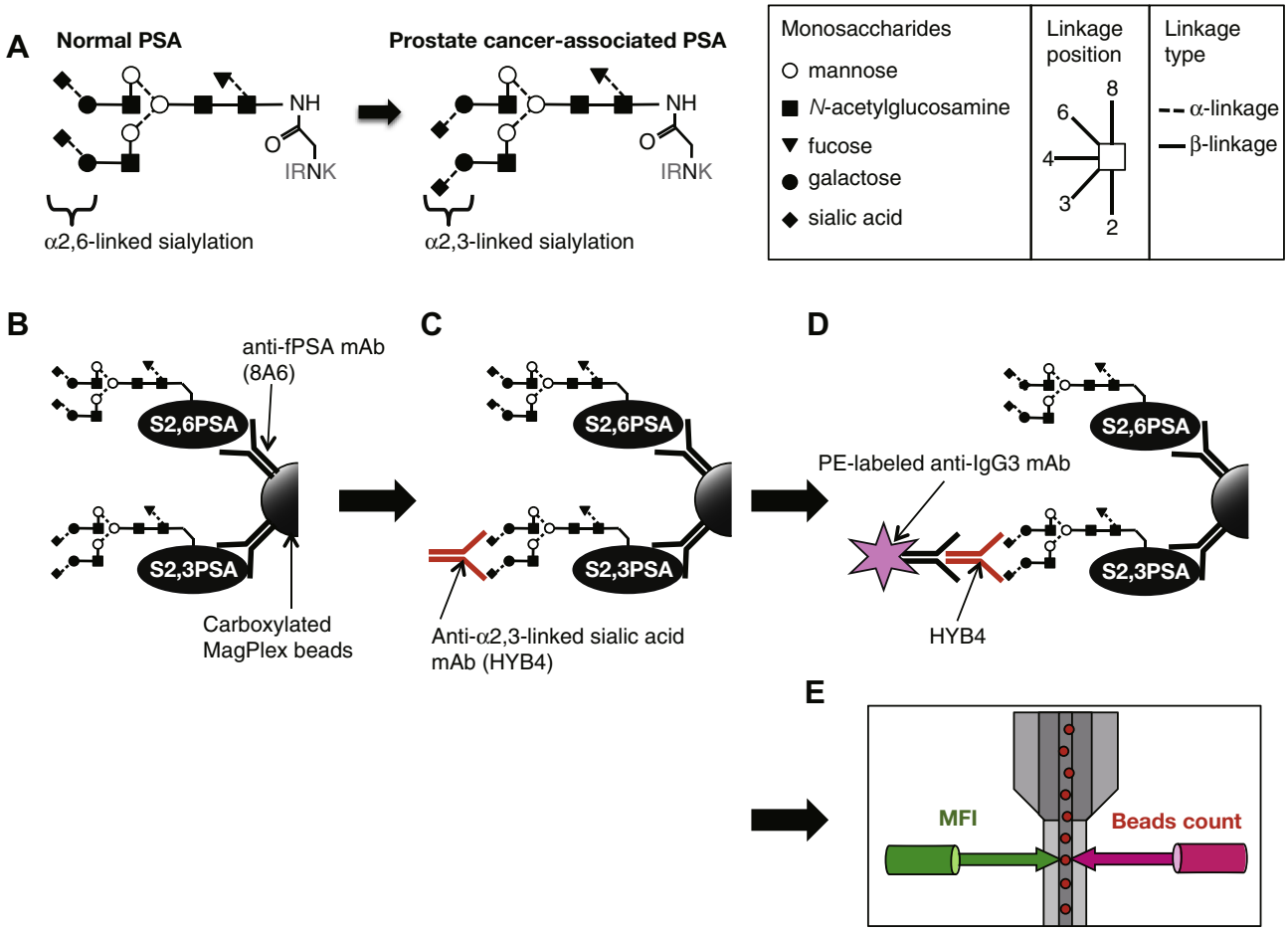


**Fig. 1.** Prostate cancer-associated aberrant glycosylation of *N*-glycan on PSA and schematic representation of the measurement of S2,3PSA. (A) In normal PSA, the terminal sialic acids link to galactose residues with an α2,6 linkage. In PCa-associated PSA, the linkage between the terminal sialic acid and galactose residues changes to an α2,3 linkage [14]. (B) Anti-fPSA mAb (8A6) was coupled to carboxylated MagPlex beads. Twenty microliters of serum was added to 8A6 mAb-conjugated bead to capture fPSA in the serum. (C) Anti-α2,3-linked sialic acid mAb (HYB4) was added to bind to S2,3PSA in the bead mixture, forming a bead–8A6 mAb–fPSA–HYB4 mAb complex. (D) PE-labeled anti-IgG3 mAb was added to the solution to couple with the bead complex. (E) The number of bead complexes were counted and expressed as MFI using Luminex100.

Japan) [20] for detection. Serum S2,3PSA levels were then presented as the mean fluorescence intensity (MFI).

Two microliters (12,500 beads) of anti-fPSA mAb (8A6)-conjugated MagPlex beads were added to each well of 96-well V bottom white plates (GE Healthcare, Buckinghamshire, UK). Fifty microliters of 1×Carbo-Free™ Blocking Solution (VECTOR laboratories, INC. CA, USA) was then added, and the plates were incubated at RT for 30 min. Next, 20 μl of serum was added to each well, and the plates were incubated at 4 °C with shaking for 1 h. The beads were then washed by placing the plate on a magnetic separator for 2 min and decanting the reaction mixture by inverting the plate a total of three times using 80 μl of Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST). Fifty microliters of anti-α2, 3-linked sialic acid mAb (HYB4) diluted with TBST (final concentration 10 μg/ml) was added to each well, and plates were incubated at 4 °C with shaking for 1 h. The beads were washed thrice with TBST, before 50 μl of phycoerythrin (PE)-labeled goat anti-mouse IgG3 mAb (1:1000 diluted in TBST) was added and incubated at RT for 45 min. After three more washes, 50 μl of TBST was added to each well, the plate was vortexed for 30 s, and the bead complexes were counted and expressed as MFI values. Specifically, a red laser was used to excite the red and orange fluorescent dyes within the beads to recognize their unique coded numbers. Simultaneously, a green laser was used to excite PE bound to the surface of the bead. MFIs of the combined lasers on the beads were recorded and analyzed using xPONENT™ 3.1 software.

The serum levels of free and total PSA were measured using the MILLIPLEX MAP Human Circulating Cancer Biomarker Magnetic Bead Panel kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's instructions.

### 2.3. Prostate biopsy and serum samples

Between June 2007 and June 2012, we performed 1494 transrectal ultrasound-guided prostate biopsies due to elevated PSA levels of >4.0 ng/ml or palpable prostate nodules that were detected by regional PCa screening programs. Serum samples were obtained from all patients at the time of biopsy and were stored at −80 °C until use. The final diagnosis of the patients was confirmed by histopathological findings of prostate biopsies (Fig. 2). The study was performed in accordance with the ethical standards of the Declaration of Helsinki and was approved by the Ethical Committee of Hirosaki University Graduate School of Medicine. Informed consent was obtained from all patients.

### 2.4. Training sample set

We conducted a training test to determine the appropriate cutoff values to discriminate PCa from non-PCa. Fifty patients with biopsy-proven PCa and 50 with non-PCa with total PSA ⩽10.0 ng/ml were selected randomly from our serum bank (Fig. 2). The clinicopathological characteristics of the patients are shown in Table 1. Tumor staging was performed based on the TNM classification of Malignant Tumors, 7th edition [21]. Assay was repeated in triplicate.

### 2.5. Validation sample set

Table 2 shows the demographic data of the validation sample set. We randomly selected 138 patients with biopsy-proven PCa and 176 patients with non-PCa with total PSA of ⩽10.0 ng/ml from our serum bank (Fig. 2). The assay system was the same as that used in the training test. There were no sampling overlap between training sample set and validation sample set. Assay was repeated in triplicate and data represent mean value.

### 2.6. Statistical analysis

All statistical calculations for clinical data were performed using GraphPad Prism 6.03 (GraphPad Software, CA, USA). Intergroup differences were statistically compared by Student's *t* test for normal distribution model, or Mann–Whitney *U*-test for non-normal distribution model. Receiver operating characteristic (ROC) curves were analyzed for total PSA, %fPSA, and S2,3PSA. Two sided *p*-values of <0.05 were considered to be statistically significant.

### 2.7. Forced expression of FLAG-tag-fused S2,3PSA in LNCaP prostate cancer cells

The LNCaP cell was obtained from the American Type Culture Collection and grown in RPMI-1640 medium supplemented with penicillin, streptomycin, and 10% FBS at 37 °C with 5% $CO_2$. FLAG-tag (N-DYKDDDDK-C)-fused human PSA (kallikrein-3, *KLK3*) cDNA was amplified from RNA isolated from the prostate of a benign prostatic hyperplasia patient using the primers hPSA-F1 5′-CCCAAGCTTACCACCTGCAC-3′ and hPSA-FLAG-Xho-R1 5′-TTTCTCGAGCTACTTGTCATCGTCGTCCTTGTAATCAGCGGGGTTGGCCACGATGGT-3′ and subcloned into the pcDNA3.1 vector (Life Technologies, CA, USA). The PSA–FLAG vector was then transiently transfected into LNCaP cells. Anti-fPSA (8A6), anti-α2,3-linked sialic acid (HYB4), and anti-FLAG (M2, Sigma, MO, USA) monoclonal antibodies were then used to detect S2,3PSA–FLAG proteins by immunoblotting.

## 3. Results

### 3.1. The Inter-assay and Intra-assay coefficient of variation

To determine the inter-assay coefficient of variation in the S2,3PSA assay, three different serums (low, middle and high MFI value) were tested in triplicate in the same plate in three consecutive days. Percentage of inter-assay coefficient of variation were determined to be 1.81% for low MFI sample, 2.36% for middle MFI sample and 5.18% for high MFI sample. The mean MFI of S2,3PSA and standard deviation were 727.2 (±13.1) for low MFI sample, 1344.4 (±31.7) for middle MFI sample and 2368.2 (±122.8) for high MFI sample (Table 3). Intra-assay coefficient of variation of S2,3PSA assay for the same set of samples were tested each in 15 wells in the same plate in one occasion. Percentage of intra-assay coefficient of variation of S2,3PSA assay was determined to be 4.61% for low MFI sample, 4.81% for middle MFI sample and 8.57% for high MFI sample. The mean MFI of S2,3PSA and standard deviation were 732.0 (±33.8) for low MFI sample, 1321.6 (±63.5) for middle MFI sample and 2537.1 (±217.5) for high MFI sample (Table 3).

### 3.2. Training sample set for S2,3PSA measurements

There were no significant differences in age and PSA, fPSA, or %fPSA levels between the two groups. MFI of serum S2,3PSA in the PCa group was significantly higher than that in the non-PCa group ($p < 0.0001$) (Fig. 3A). To determine the optimal cutoff values of S2,3PSA, we performed ROC curve analyses (Fig. 3B) and identified an MFI cutoff value of 1130 with a sensitivity and specificity of 95.0% and 72.0%, respectively. Therefore, an MFI cutoff value of 1130 was used in the validation study.

### 3.3. Validation study of S2,3PSA measurements

Although we observed no differences in fPSA levels, significant differences in the intergroup age, PSA and %fPSA levels were

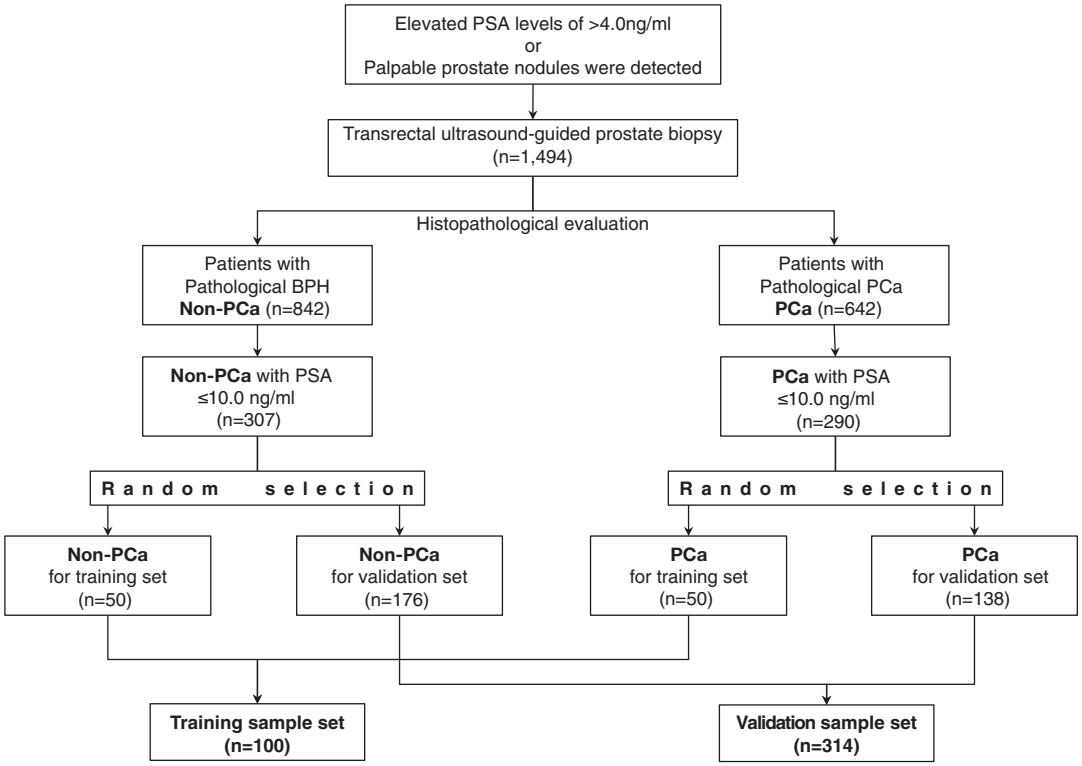


**Fig. 2.** Flowchart of the present study. Between June 2007 and June 2012, we performed 1494 transrectal ultrasound-guided prostate biopsies due to elevated PSA levels of >4.0 ng/ml or palpable prostate nodules that were detected by regional PCa screening programs. All serum samples were obtained at the time of biopsy. The final diagnosis of the patients was confirmed by histopathological findings of prostate biopsies. Training and validation sample sets were randomly selected biopsy-proven PCa or non-PCa with total PSA of ⩽10.0 ng/ml from our serum bank.

**Table 1**
Demographic data for the training sample set.

| Characteristics | Non-PCa | | PCa | | *p* |
|---|---|---|---|---|---|
| Patients, *n* | 50 | | 50 | | |
| Age (median) | 57–82 | (69.0) | 51–84 | (71.5) | 0.3832 |
| PSA, ng/mL, (median) | 2.4–9.7 | (5.9) | 3.0–10.0 | (6.35) | 0.2719 |
| fPSA, ng/mL, (median) | 0.02–0.94 | (0.16) | 0–0.69 | (0.15) | 0.3616 |
| %fPSA, %, (median) | 0.48–11.3 | (2.67) | 0.06–9.02 | (2.29) | 0.0878 |
| S2,3PSA, MFI, (median) | 663–1576 | (922) | 1048–2597 | (1666) | <0.0001 |
| Biopsy Gleason Sum, *n*, (%) | | | | | |
| 5–6 | | | 0 | (0) | |
| 7 | | | 27 | (54.0) | |
| 8–10 | | | 23 | (46.0) | |
| Clinical stage, *n*, (%) | | | | | |
| cT1c–cT2a | | | 42 | (84.0) | |
| cT2b | | | 3 | (6.0) | |
| cT2c | | | 5 | (10.0) | |

**Table 2**
Demographic data for the validation sample set.

| Characteristics | Non-PCa | | PCa | | *p* |
|---|---|---|---|---|---|
| Patients, *n* | 176 | | 138 | | |
| Age (median) | 51–83 | (68.0) | 50–84 | (69.0) | 0.0181 |
| PSA, ng/mL, (median) | 2.0–10.0 | (5.8) | 2.2–10.0 | (6.4) | 0.0008 |
| fPSA, ng/mL, (median) | 0–2.43 | (0.12) | 0–4.40 | (0.10) | 0.1267 |
| %fPSA, %, (median) | 0–51.6 | (2.1) | 0–44.0 | (1.6) | 0.0020 |
| S2,3PSA, MFI, (median) | 395–2221 | (940) | 693–2971 | (1484) | <0.0001 |
| Biopsy Gleason Sum, *n*, (%) | | | | | |
| 5–6 | | | 24 | (17.5) | |
| 7 | | | 66 | (48.2) | |
| 8–10 | | | 48 | (34.3) | |
| Clinical stage, *n*, (%) | | | | | |
| cT1c–cT2a | | | 127 | (92.0) | |
| cT2b | | | 3 | (2.2) | |
| cT2c | | | 7 | (5.1) | |
| Unknown | | | 1 | (0.7) | |

detected. Fig. 3C shows that MFI of serum S2,3PSA was significantly higher in PCa patients than that in non-PCa patients ($p < 0.0001$). ROC curve analyses were then used to compare the diagnostic potential of PSA, %fPSA, and S2,3PSA (Fig. 3D). The area under the curve (AUC) showed that PSA and %fPSA did not differ between patients with non-PCa and PCa (AUC 0.61 and 0.60, respectively), whereas S2,3PSA showed a good separation (AUC, 0.84).

Based on the MFI cutoff value of 1130 for the S2,3PSA test ($n = 314$), we calculated a sensitivity of 90.6% and a specificity of 64.2%, with a positive predictive value (PPV) and negative predictive value (NPV) of 66.5% and 89.7%, respectively (Table 4). The specificity of %fPSA for detecting PCa with 90.6% sensitivity (with a cutoff value at <5.26%) was determined to be 11.4%, with a PPV of 46.2% and an NPV of 60.6%. Based on the cutoff value of

**Table 3**
Inter-assay variation and intra-assay variation in S2,3PSA assay.

| Patient # | Mean MFI | SD | CV (%) |
|---|---|---|---|
| *Inter-assay variation* | | | |
| 1 | 727.2 | 13.1 | 1.81 |
| 2 | 1344.4 | 31.7 | 2.36 |
| 3 | 2368.2 | 122.8 | 5.18 |
| *Intra-assay variation* | | | |
| 1 | 732.5 | 33.8 | 4.61 |
| 2 | 1321.6 | 63.5 | 4.81 |
| 3 | 2537.1 | 217.5 | 8.57 |

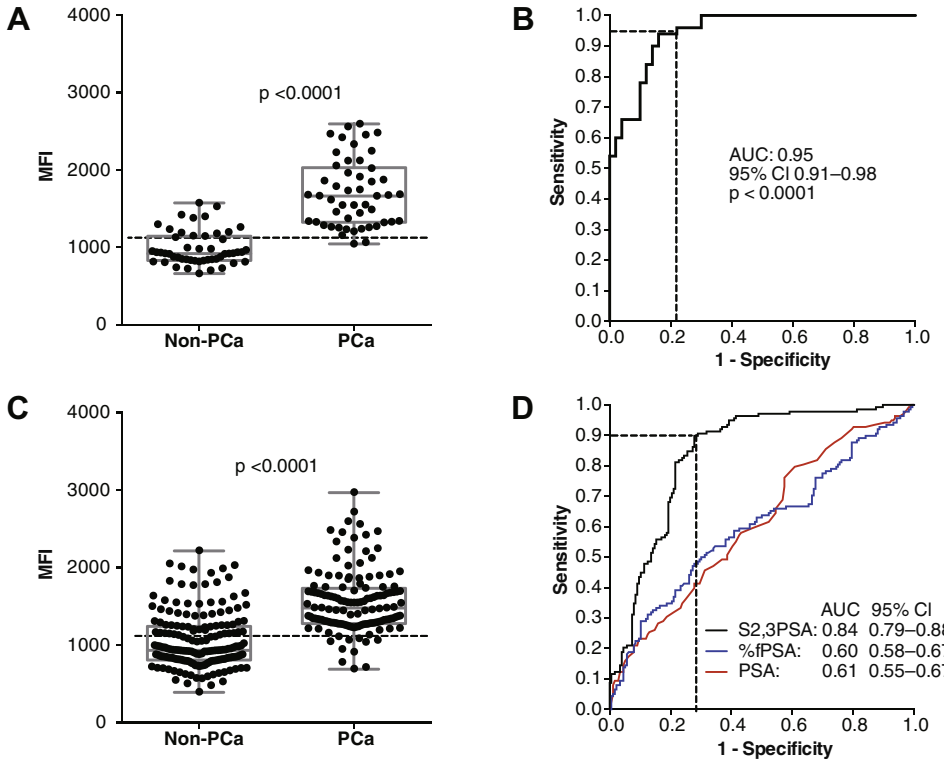


**Fig. 3.** Serum S2,3PSA levels in the training and validation sample set. Serum S2,3PSA levels in PCa patients were significantly higher than those in non-PCa patients (*$p < 0.0001$) in the training sample set (A) and validation sample set (C). Receiver operating characteristics (ROC) curve analysis of training sample set (B) and validation sample set (D). The area under the curve (AUC) of S2,3PSA in the training set was 0.95. The MFI cutoff value at 95.0% sensitivity was determined to be 1130 in the training samples, indicated by the broken line. (D) ROC curve analysis comparing the prognostic potential between non-PCa ($n = 176$) and PCa patients ($n = 138$). The AUCs of %fPSA, PSA, and S2,3PSA were 0.60, 0.61, and 0.84, respectively.

**Table 4**
Sensitivity, specificity, accuracy, and predictive value for PSA, %fPSA, and S2,3PSA in validation sample set.

| Test | Cutoff | Sensitivity (%) | Specificity (%) | Accuracy (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|
| S2,3PSA | >1130 MFI | 90.6 | 64.2 | 75.8 | 66.5 | 89.7 |
| PSA | >4.5 ng/mL | 90.6 | 20.5 | 51.3 | 47.2 | 73.5 |
| %fPSA | <5.26% | 90.6 | 11.4 | 46.2 | 44.5 | 60.6 |

PPV: positive-predictive value, NPV: negative-predictive value.

>4.5 ng/ml for PSA, the sensitivity and specificity were 90.6% and 20.5%, respectively, with a PPV of 47.2% and an NPV of 73.5%. The specificity of S2,3PSA was 64.2% at 90.6% sensitivity, which was significantly higher than that of PSA (20.5%) or %fPSA (11.4%) (Table 4).

### *3.4. Forced expression of FLAG-tag-fused S2,3PSA in LNCaP prostate cancer cells*

To determine whether PCa cells specifically produce S2,3PSA, we transiently transfected FLAG-tag-fused human PSA cDNA (PSA–FLAG) into LNCaP cells. Immunoblotting of PSA–FLAG-transfected LNCaP whole cell lysates is shown in Fig. 4. PSA–FLAG protein was detected using anti-fPSA (8A6), anti-FLAG (M2), and anti-α2,3-linked sialic acid (HYB4) antibodies, suggesting that the PSA–FLAG protein was modified by α2,3-linked sialylation as a terminal *N*-glycan. These results provide clear evidence that LNCaP cells produce PCa-associated S2,3PSA.

## 4. Discussion

Recent reports describing glycan alternation of PSA emphasized promising aspect of aberrant PSA glycosylation for early detection of prostate cancer [12,22,23]. However, it is sometimes difficult to determine precise carbohydrate structure of PSA because of its molecular diversity. Majority of the recent reports used *N*-glycanase to cleave glycan from PSA. However, this procedure cannot refrain from contamination of other carrier proteins. As we previously described, a commercially available PSA was contaminated with other proteins [17]. This may be, at least in part, a major obstacle for carbohydrate structure research of PSA. To overcome this problem, we carried out precise and comprehensive structure analysis of PSA glycan [17]. In that study, we used lysylendopeptidase to cleave PSA-specific peptide sequence including sole *N*-glycosylation site. We identified that the terminal *N*-glycan structure of PSA from PCa patients was rich in sialic acid α2, 3-linked to the galactose residue, whereas the terminal *N*-glycan structures of PSA from the seminal plasma were exclusively α2,6-linked [17]. The present study was carried out based on our previous study.

Our previous study estimated the percentage of aberrantly glycosylated fPSA compared with the levels of total fPSA using lectin affinity column chromatography [18]. The lectin column chromatography method allowed us to distinguish sera from PCa patients from those from non-PCa patients by detecting differences in the levels of S2,3PSA [18]. However, the lectin-based assay system requires 0.5 ml of sera, and the lower limit of detection of total

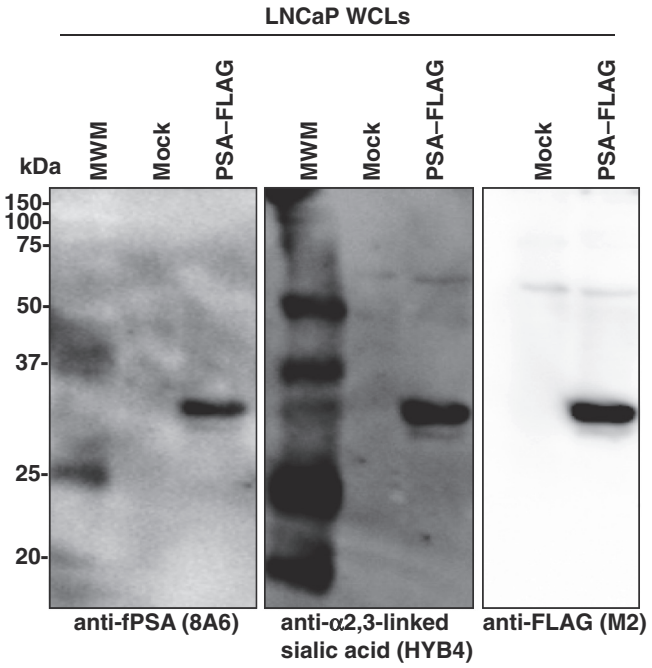


**Fig. 4.** Immunobloting of S2,3PSA in LNCaP cells transfected with PSA–FLAG. LNCaP cells were transfected with FLAG-tag-fused human PSA cDNA (PSA–FLAG) or with mock control (Mock). Whole cell lysates (WCLs) were blotted and probed with anti-fPSA (left panel), anti-α2,3-linked sialic acid (center panel), and anti-FLAG (right panel) antibodies. MWM indicated molecular weight markers.

PSA level is approximately 10 ng/ml to discriminate between PCa and non-PCa. To improve the specificity of PSA testing in clinical practice, we need to improve the specificity to between 2 and 10 ng/ml. For this purpose, lectin-based analysis using natural lectin is not optimal because of poor specificity and interlot variation.

Therefore, we developed a novel assay system that can detect PCa-associated glycan alterations using an mAb raised against α2,3-linked sialylation as an additional terminal *N*-glycan on PSA. In the present assay system, fPSA in sera is captured using anti-fPSA mAb that is coated on the surface of magnetic microbeads. The amount of α2,3-linked sialic acid is then measured using a specific mAb HYB4 [20]. Originally, this mAb was established to detect the specific carbohydrate receptor of influenza virus [20]. Interestingly, the terminal carbohydrate structure of PSA produced in PCa is the same as the influenza virus receptor.

In human serum, some PSA exists in a complex with serum protease inhibitors such as α1-antichymotrypsin and α2-macroglobulin, whereas some exists in a free form [24]. Therefore, the distinct molecular isoforms must be considered when measuring S2,3PSA using HYB4. Because α1-antichymotrypsin is also a glycoprotein with a carbohydrate moiety that reacts with HYB4, fPSA must be captured before the quantification of α2,3-linked sialic acid. Therefore, we used anti-fPSA mAb (8A6) as the capture antibody on the surface of the magnetic microbeads to bind only fPSA.

Luminex antibody screening technology consists of a series of polystyrene microbeads containing fluorochromes and has been widely used for antibody screening during organ transplantation [19]. We therefore used the Luminex assay system in this study, although alternative bioluminescent enzymes [25] and chemiluminescent immunoassays [26] may also be effective for measuring S2,3PSA. The inter-assay coefficients for S2,3PSA was between 1.81% and 5.18% and intra-assay coefficients for S2,3PSA was between 4.61% and 8.57%. These data suggests CV of the S2,3PSA assay is acceptable.

The AUC for the detection of PCa using the present novel assay system measuring S2,3PSA was 0.84, which was significantly higher than that for PSA (0.60) or %fPSA (0.61). This suggests that S2,3PSA has potential as a novel biomarker for the early detection of PCa.

We also confirmed that aberrantly glycosylated PSA was produced by a PCa cell line and thus provided cell-based biological evidence for the aberrant glycosylation of PSA (Fig. 4). Because recombinant S2,3PSA protein was not available when this study was conducted, S2,3PSA levels were expressed as MFI. Future studies should establish a standard curve for the quantitative measurement of S2,3PSA. However, the stable production of recombinant S2,3PSA as a standard molecule is essential to achieve this. Importantly, we have confirmed that recombinant S2,3PSA can be expressed using Chinese hamster ovary (CHO) cells (Supplemental Fig. 1).

## 5. Conclusions

Although the present study is small and preliminary, the data suggested that the diagnostic potential of serum S2,3PSA using bead-based immunoassays may surpass PSA and %fPSA. Further clinical trials are warranted to validate the clinical significance and application of this method.

## Competing interests

No potential conflicts of interest were disclosed.

## Acknowledgment

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.107.

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