

A sensitive assay to measure biomarker glycosylation demonstrates increased fucosylation of prostate specific antigen (PSA) in patients with prostate cancer compared with benign prostatic hyperplasia

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ABSTRACT

Background: Prostate specific antigen (PSA) measurement is used for the diagnosis of prostate cancer (PCa) but the test lacks specificity due to the number of false positive readings. The glycosylation of PSA is altered in PCa but studies in this area have been limited to few clinical samples and/or require advanced laboratory facilities. An assay to assess PSA glycosylation was established using equipment available in most routine biomedical testing laboratories.

Methods: Serum samples from patients with PCa or benign prostatic hyperplasia (BPH) were used. PSA (range 4–10 ng/ml) was affinity purified, separated and probed with the lectin *Ulex europaeus* (UEA-1; specific for α 1,2 linked fucose). An enzyme-linked immunosorbent lectin assay (ELLA) with colorimetric detection was devised and PSA fucosylation assessed in a further independent set of 26 samples.

Results: Free PSA (fPSA) from PCa patients showed a significant increase in fucosylation compared with fPSA from patients with BPH. The ELLA was 92% specific and 69% sensitive for PCa over BPH. In comparison, fPSA measurement was 70% specific and 56% sensitive (threshold set to 25% tPSA) for PCa over BPH.

Conclusions: Changes in glycosylation of PSA were identified using 50 μ l of serum with PSA in the range of 4–10 ng/ml, this represents a more specific and sensitive test for PCa based on fucosylation changes of fPSA.

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1. Introduction

Prostate specific antigen (PSA) has been used for the diagnosis of prostate cancer (PCa) and monitoring patient response to treatment for approximately 20 years. PSA is a serine protease belonging to the kallikrein family, produced in the epithelium of the periurethral glands and functions to liquefy seminal gel [1]. The enzyme is a single chain glycoprotein with one N-linked glycosylation site at Asn-45; the molecular mass is 26,079 Da and the carbohydrate moiety adds approximately 2300 Da further to the overall weight, therefore, free PSA (fPSA) is approximately 28,400 Da [2].

One of the inherent problems of PSA use in the diagnostic setting is the poor specificity for PCa as patients with benign prostatic hyperplasia (BPH) may exhibit serum levels of PSA of 4 ng/ml or higher. Various approaches have been used to improve the selectivity and sensitivity of PSA for PCa, for example, PSA density [3] and PSA velocity [4]. PSA is found in two predominant forms in the serum either as fPSA or complexed with α 1-chymotrypsin and α 2-macroglobulin [5]. PCa patients have less fPSA than patients with BPH [6] and the ratio of

fPSA to total PSA (tPSA) is used for the diagnosis of PCa but is not without problems, as the levels of fPSA vary with age [7].

The modification of proteins by glycosylation occurs in approximately half of all serum proteins and changes in glycosylation have been found to accompany both tumour formation and aggressive clinical behaviour [8]. Aberrant glycosylation of proteins in PCa tissue has been observed compared with BPH and normal tissue [9–12]. Similarly, changes in the glycans on PSA have been observed in PCa compared with BPH but studies in this area have either used very few samples with extremely high PSA levels or technology only available in specialised glycolbiology/glycoproteomic laboratories [13–16].

We hypothesised that alterations in the tissue fucosylation of PCa compared with BPH [12] would be reflected in changes in the glycosylation of PSA and that this might add an element of specificity to the PSA test. The lectin from *Ulex europaeus* (UEA-1; specific for α 1,2 linked fucose) was used and showed an elevation in the fucosylation of immuno-purified PSA from PCa serum samples compared with BPH. The observations were validated using a separate set of 26 serum samples in a simple enzyme-linked immunosorbent lectin assay (ELLA) developed in-house. The ELLA system – when used to study fPSA fucosylation – showed 92% specificity and 69% sensitivity for PCa over BPH, this compared with 70% specificity and 56% sensitivity for fPSA alone, when the threshold value was taken of 25% of the tPSA levels.

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2. Materials and methods

2.1. Subjects and sample collection

All patients had pathologically confirmed BPH or PCa and gifted a blood sample to the study, the patient details are given in Table 1. The study was approved by the Local Ethics Committee at the University College London Hospital Trust. 10 ml of blood was collected pre-operatively from each patient after informed consent. A sample of the blood was sent for routine PSA testing and the remainder was allowed to clot; centrifuged at 1500 g for 5 min, the supernatant was transferred to a new tube and centrifuged at a further 13,500 g for 5 min. The serum was immediately stored at -80°C in 0.5 ml aliquots until use.

Samples with high PSA concentration ($\text{PSA} > 100 \text{ ng/ml}$) were used for affinity chromatography and method development. For all subsequent work the serum PSA was in the range 4–10 ng/ml and was obtained as described above or by commercial arrangement (SciPac, UK).

2.2. Purification and analysis of PSA

0.5 ml of activated Sepharose 4B beads was prepared according to the manufacturer's instructions (Sigma, UK) with 1 mg of polyclonal rabbit anti-human PSA antibody. Serum samples from patients with PCa ($n=8$) and BPH ($n=6$) were passed over the affinity matrix three times at a flow rate of 0.4 ml/min. The column was capped for 45 min between each run and unbound material was eluted using 20 column volumes of phosphate buffered saline (PBS). The PSA was eluted using 0.1 M glycine-HCl pH 2.5 containing 0.5 M NaCl, the fractions were immediately neutralised using 80 μl , 1 M Tris, pH 8.0.

The fractions collected in the affinity chromatography steps were separated using SDS-PAGE. Two SDS-PAGE gels were prepared at the same time for each purified sample and used for Western blotting to compare the UEA-1 binding with anti-PSA antibody binding. The Western blotting was undertaken using semi-dry blotting onto nitrocellulose at 2.5 mA/cm² for 30 min at room temperature (RT). Blots were blocked in 2% w/v bovine serum albumin in tris buffered saline, pH 7.6, containing 0.1% v/v Tween-20 (TBS-T) at 4°C overnight. The first blot was probed with polyclonal rabbit anti-human PSA antibody (8 $\mu\text{g/ml}$, Dako, UK) and a biotinylated swine anti-rabbit antibody (1.6 $\mu\text{g/ml}$, Dako, UK) each for 2 h, followed by streptavidin-horse radish peroxidase (HRP; at 1.8 $\mu\text{g/ml}$, Sigma, UK) for 1 h. The lectin staining used the second blot with biotinylated UEA-1 (5 $\mu\text{g/ml}$, Sigma, UK) for 2 h, followed by streptavidin-HRP as before. The steps were undertaken at RT and the blots washed three times for 5 min between each incubation with TBS-T. The blots were developed using an ECL kit and visualised by exposing ECL Hyperfilm for 30 s then 1 min (GE Healthcare, UK). The ECL films were scanned using a calibrated imaging densitometer (GS-710 BioRad, UK) and the data analysed using the Quantity One software. The intensity of the UEA-1

stained bands were divided by the intensity of the PSA stained bands to enable comparison of UEA-1 binding in different samples run on different gels.

2.3. Enzyme-linked immunosorbent lectin assay (ELLA)

Monoclonal mouse anti-human fPSA or tPSA (AbCam, UK) was oxidised [17], desalted on a PD10 column (GE Healthcare, UK) to remove residual sodium periodate and exchanged into 0.1 M carbonate/bicarbonate buffer (ELLA coating buffer). 50 μl of 10 $\mu\text{g/ml}$ antibody solution was added to the wells of a 96 round-well plate (Sterilin, UK) and incubated at 4°C overnight. All subsequent steps were undertaken with gentle shaking of the 96-well plate and at RT. The coating buffer was removed and 150 μl Synblock (Serotec, UK) was added to each well for 2 h. The wells were washed five times with 250 μl PBS + 0.1% v/v Tween-20 (PBS-T). 50 μl of serum sample (diluted 1:3 in PBS containing 5% v/v Synblock) was added to each well for 2 h. The same dilution factor was used for both the fPSA and the tPSA ELLA. The plate was washed as before and 50 μl biotinylated UEA-1 (10 $\mu\text{g/ml}$, Sigma, UK) added to each well for 1 h. The wells were washed three times and 50 μl poly-HRP streptavidin (Piercenet, UK) was added at a 1:8000 dilution in PBS for 1 h. The wells were washed three times and 100 μl Turbo-TMB substrate (Piercenet, UK) added for 1 h to allow colour development. 50 μl of 1 M sulphuric acid solution was used to stop the colour reaction and the absorbance was read at 450 nm in a Wallac 1420 Victor 2 plate reader (Perkin Elmer, UK). All samples were evaluated in the ELLA in duplicate on two separate days.

Wells containing all the reagents but no serum (blank wells) were prepared for each experiment and the average absorbance reading for the blank plus 3 standard deviations (fPSA = 0.0151 AU; tPSA = 0.058 AU) from the mean average was subtracted from the absorbance readings obtained for the serum samples, these were then presented as relative absorbance units (RAU).

2.4. Statistical analysis

The data obtained for the interaction between UEA-1 and PSA from PCa and BPH in the Western blot and ELLA studies were compared using an unpaired student *t*-test.

3. Results

3.1. UEA-1 binding to immuno-purified PSA

Serum PSA from patients with PCa and BPH was separated by SDS-PAGE and probed with UEA-1, or anti-PSA antibody, Fig. 1. Several bands of MW lower than fPSA were observed; these most probably represent clipped forms of PSA. The higher MW bands (between 50 kDa and 100 kDa) are likely to be PSA complexed with serum proteins. Fucosylation of affinity purified PSA from serum of patients with PCa and BPH was compared by assessing the ratio of UEA-1 to PSA binding (Fig. 1) and was observed to be increased in the patients with PCa.

3.2. Establishment of the ELLA

The ELLA was used to capture and detect the fucosylation of fPSA and tPSA using a new set of serum samples, all prepared at a 1:3 dilution; tPSA starting concentration 4–10 ng/ml. A range of conditions were explored: concentration of coating antibody; blocking/oxidation of glycans on coating antibodies; formulation of the diluent; standard/enhanced HRP detection systems; incubation times and temperatures (data not shown). The final ELLA was based on a colorimetric detection system. The assay gave a linear detection signal at the required concentrations of PSA as shown in Fig. 2. The limit of

Table 1

Patient data. The mean average age at the time of serum sampling; tPSA and fPSA values are shown. The range is given in parenthesis.

	Benign prostatic hyperplasia (BPH)	Prostate cancer (PCa)
n=	13	13
Age at presentation in years	73 (62–85)	80 (72–90)
tPSA (ng/ml)	5.9 (1.9–9.1)	6.5 (3.4–10.7)
fPSA (ng/ml)	1.0 (0.4–2.5)	1.1 (0.2–2.3)
fPSA (% of total)	16 (8–28)	17 (7–37)

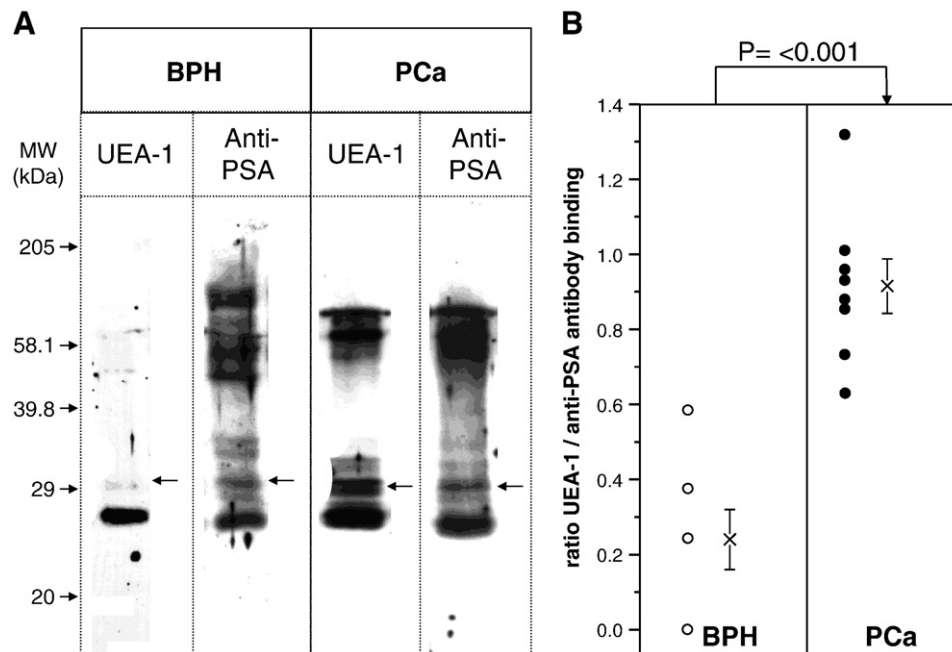


Fig. 1. PSA affinity purified from serum samples from patients with prostate cancer (PCa) or benign prostatic hyperplasia (BPH). *Panel A:* Affinity purified PSA was separated under native conditions using SDS-PAGE and transferred to nitrocellulose by Western blotting, the membrane was probed either with biotinylated UEA-1 (5 µg/ml) followed by streptavidin HRP (1.8 µg/ml) or with polyclonal rabbit anti-human PSA antibody (8 µg/ml), biotinylated swine anti-rabbit antibody (1.6 µg/ml) and streptavidin HRP as before. The blots were developed using ECL reagent and captured onto an X-ray film. PCa derived PSA had many more UEA-1 reactive bands than PSA from patients with BPH. The arrow indicates fPSA. *Panel B:* The ratio of UEA-1 binding to anti-PSA antibody binding in PCa and BPH.

detection was calculated by taking the mean blank value for the assay and adding 3SD of the mean. For fPSA the limit of detection was 0.2 ng/ml and for tPSA the detection limit was 0.8 ng/ml.

Pre-incubation of the lectin with fucose resulted in a decrease in UEA-1 binding, consistent with competitive inhibition of lectin binding in other ELLA [18] (data not shown).

The reproducibility of the assay was tested using the same serum sample on two separate days and the inter-assay variability was found to be less than 10%. This sample also contained PSA in the range 4–10 ng/

ml and was used as an “internal control” to assess the reproducibility of the system. The ELLA was also established using the antibody for the capture of tPSA with similar results (data for fPSA shown).

3.3. PSA fucosylation in serum samples from patients with PCa and BPH

An increase in the fucosylation of tPSA was observed in the PCa samples compared with BPH but greater discrimination between PCa and BPH was observed when the monoclonal antibody against fPSA was

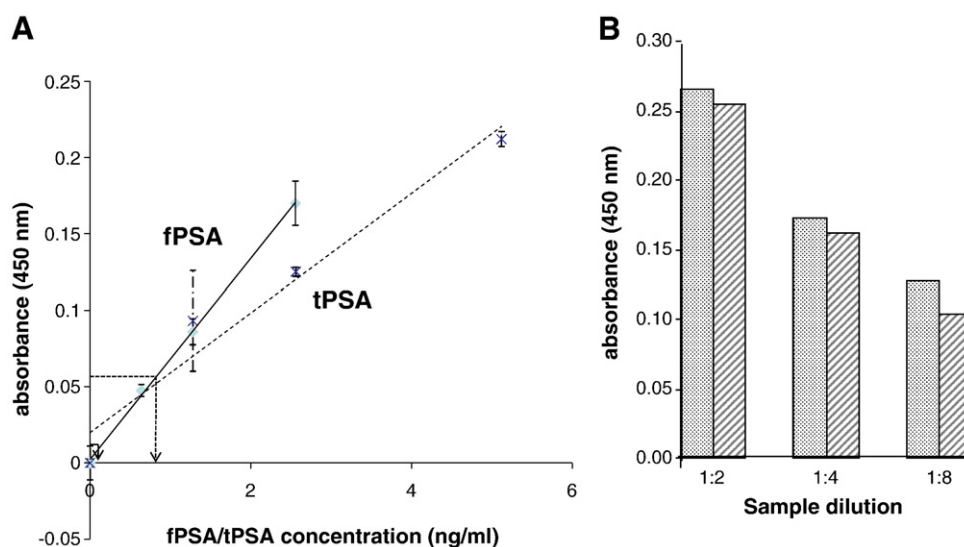


Fig. 2. An immunosorbent ELLA (sandwich) was developed using monoclonal mouse anti-human fPSA/tPSA as the capture antibody (10 µg/ml) and pre-treated with sodium periodate to oxidise the carbohydrate residues. The wells were blocked with Synblock reagent and incubated with serum samples. The PSA fucosylation was detected using biotinylated UEA-1 (10 µg/ml), streptavidin poly-HRP followed by the TMB substrate. *Panel A:* The assay showed linearity when PCa serum containing tPSA at 8 ng/ml was serially diluted (R^2 value = 0.999 for fPSA, R^2 value = 0.9555 for tPSA; error bars indicate standard error from the mean); the limit of detection for fPSA was 0.2 ng/ml, solid lines; for tPSA was 0.8 ng/ml, dashed lines. *Panel B:* Linearity and reproducibility of the system were observed when the same sample was serially diluted and evaluated on two separate days (day one = dotted bars, day two = hatched bars).

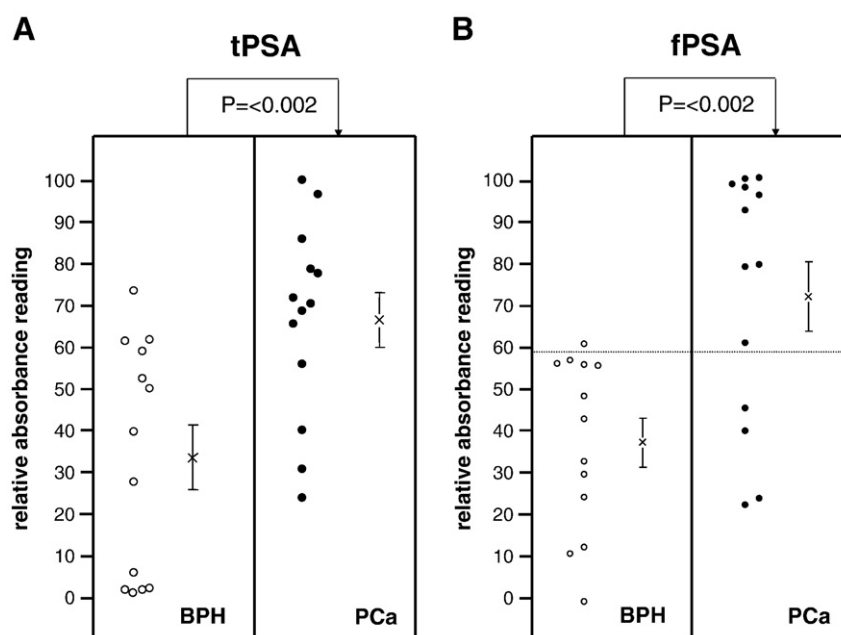


Fig. 3. The PSA was captured using a monoclonal antibody (as Fig. 2) against either human tPSA (panel A) or human fPSA (panel B).

used (Fig. 3). This supported the results obtained with the affinity purified PSA described earlier (Fig. 1). When a cut-off was taken at 60% of the relative absorbance units, the fPSA ELLA gave 92% specificity and 69% sensitivity for PCa over BPH (true positives = 9, true negatives = 12, false positives = 1, false negatives = 4). In contrast, when the fPSA measurement was taken with a cut-off of 25% tPSA, this gave 70% specificity and 56% sensitivity for PCa over BPH (true positives = 9, true negatives = 7, false positives = 3, false negatives = 7).

4. Discussion

In this study an ELLA was developed in which the lectin UEA-1 was used to assess the glycosylation of the protein biomarker PSA. Only 50 μ l of serum sample from patients with PSA in the “grey zone” range of 4–10 ng/ml was used in the assay.

The results showed an increase in the fucosylation of serum PSA in the PCa patient samples. An important next development for this field will be the availability of a recombinant form of PSA with “normal” and “cancer” glycoforms, these will find use as standards and will drive forward assay development.

Detailed glycan mapping experiments are currently hampered by the low levels of PSA in the serum but it is clear that the identification of the glycan structures detected using this system would be a desirable and important next step.

Other studies have shown changes in glycosylation in PSA in PCa compared with BPH, using urine of patients with PCa and BPH [19], PSA derived from semen and from LNCaP [13] and serum samples [14]. The glycosylation of serum PSA has also been studied in other lectin-based approaches, including with the use of the α 1,2 fucose/ β GalNAc binding lectin from *Trichosanthes japonica*, where an increase in lectin binding in PSA from patients with PCa was reported but this system used serum samples with very high PSA concentration and lectin affinity chromatography to assess the glycosylation patterns [20]. Further reports suggest an increase in the binding of the lectin ConA in PCa [21] but not all researchers have reported the same results [22].

It is interesting to speculate as to the possible effects of altered glycosylation on the binding of PSA to both α 2-macroglobulin and α 1-antichymotrypsin as in our hands elevated UEA-1 binding was observed when fPSA was captured in the ELLA. It may be the case that

the glycosylated nature of the serum proteins to which PSA is complexed confounds the analysis of the glycosylation of tPSA [23]. Recent work has shown that PSA sialylation is decreased in PCa and that the under-sialylated glycoforms are found in complex with α 1-antichymotrypsin [24], this would suggest that the increased fucosylation of the fPSA fraction that we reported should also show reduced sialylation, but further work would be required to substantiate this hypothesis. The biochemical basis for altered PSA glycosylation in PCa also needs to be determined, and an initial study in this area suggested that differences in glycosylation of PSA might also affect the secretion of PSA into the serum [25].

It was clear at the start of this study that methodology compatible with equipment available in the majority of biomedical science laboratories needed to be developed to enable studies of the glycosylation of PSA with larger numbers of patient samples. This straightforward ELLA system is of sufficient sensitivity to allow the assessment of the glycosylation of serum PSA. The system uses equipment and skills that are available in most laboratories.

The data showed that fucosylation of fPSA is increased in the serum of patients with PCa compared with BPH. There remain many unanswered questions including whether the extent of prostatic interepithelial neoplasia (PIN), different treatment regimens, tPSA to fPSA ratio, patient age and androgen receptor status affect PSA glycosylation. Clearly the next challenge will be to use this methodology to assess large numbers of PCa and BPH serum samples in an attempt to produce a more specific and sensitive test for PCa.

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