Improved cancer specificity in PSA assay using Aleuria aurantia lectin coated Eu nanoparticles for detection

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Abstract

Objectives: The objective was to study the differences in PSA fucosylation obtained from LNCaP and PC-3 prostate cancer cell lines, seminal plasma PSA and recombinant precursor form of PSA expressed in baculovirus, using *Aleuria aurantia* lectin (AAL). The aim was to assess whether differences in fucosylation (Fucα1-6/3GlcNAc carbohydrates) of PSA either in urine, blood or tissue enable the discrimination of patients with prostate cancer (PCa) from benign prostatic hyperplasia (BPH) and young males.

Design and Methods: Two novel lectin-immunoassays were developed for the analysis of fucosylation of PSA by measuring the time-resolved fluorescence of europium chelate. The lectin-immunoassays utilize free-PSA-specific Fab-fragments for capturing the analyte and either europium-labeled AAL or AAL coupled to Eu(III)-chelate-dyed nanoparticles for the detection of Fucα1-6/3GlcNAc carbohydrates on PSA.

Results: Using the novel lectin-immunoassays, we showed higher levels of Fucα1-6/3GlcNAc on PSA derived from LNCaP and PC-3 cells compared to seminal plasma PSA. With the more sensitive nanoparticle-based lectin-immunoassay we detected a statistically significant increase in the PSA fucosylation in PCa tissue compared to benign tissue (p=0.001) and in urine from PCa patients compared to BPH patients (p=0.030), and an even greater discrimination (p=0.010) when comparing BPH patients to PCa patients with Gleason score ≥7.

Conclusions: AAL coupled to Eu(III)-chelate-dyed nanoparticles improved the sensitivity of immunoassay for the detection of Fucα1-6/3GlcNAc structures on PSA. The preliminary findings show an increased fucosylation in PCa compared to benign conditions. Further validation is required to assess the true clinical utility of AAL in PCa diagnosis.

Keywords: prostate cancer, *Aleuria aurantia* lectin, prostate-specific antigen, fucosylation, immunoassay, nanoparticles, time-resolved fluorescence

Abbreviations: TRF = time-resolved fluorescence, AAL= *Aleuria aurantia* lectin, Mab= monoclonal antibody, PSA= prostate-specific antigen

1. Introduction

Glycosylation is one of the most common post-translational modifications of proteins, and it affects protein-protein interactions, cell recognition, motility and adhesion (1). More than half of all the proteins, and nearly all membrane and extracellular proteins are glycosylated (2). Recent advances in the field of glycobiology have shown that an altered glycosylation pattern is a universal feature of cancer cells. Affecting the adhesive properties of cancer cells and neovascularization the altered glycans can facilitate tumor invasion and help cancer cells escape immune surveillance (3).

Many of the current clinical cancer biomarkers are glycoproteins. Serum prostate-specific antigen (PSA), a glycoprotein with a single N-oligosaccharide chain, is used for the early detection of prostate cancer (PCa) as well as for monitoring the disease. PSA from seminal fluid has been shown to have major differences in glycan structures, especially in their sialic acid and fucosyl content, compared to PSA from prostate cancer cell line LNCaP (4). Also the PSA from PCa sera contains different glycan structures when compared to PSA derived from LNCaP cell line (5) or noncancer seminal fluid (6, 7) and sera (8, 9 10). Free PSA (fPSA) from PCa patients has been shown to have a significant increase in fucosylation compared to fPSA from patients with benign prostatic hyperplasia (BPH) (11). However it's currently unclear whether changes in PSA glycosylation may be used to improve the cancer specificity of PSA.

Techniques used for detailed characterization of glycoproteins include high-performance liquid chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy, which all require expensive equipment and highly skilled personnel. Also the analysis requires chemical or enzymatic removal of glycans from glycoproteins (12). Typically there is also a need for glycoprotein enrichment, making the analysis more complex and time-consuming (13). These methods are not readily suitable for clinical routine calling for development of more simple and accessible methods. The ability of lectins to directly discriminate between different glycan structures is therefore highly attractive as it does not involve glycan removal from glycoproteins followed by labeling with fluorescent markers. Lectins have been used in microarray techniques (14, 15, 16, 17), enzyme-linked lectin assays (ELLAs) (18 19, 11, 12), lectin immunosorbent assays (20) and affinity chromatography techniques (21).

The use of lectin-based assays has been hampered due to the need to deglycosylate antibodies used for capture or detection. In addition, because lectins have specificity to glycans and not the protein of interest this may result in high background signals and low assay sensitivity due to binding of lectins to glycans expressed by unrelated proteins (20).

Aleuria aurantia lectin (AAL), with a molecular weight of 72 kDa (22), is a fucose-specific lectin, with affinity towards glycoproteins having core fucα1-6/3GlcNAc. AAL, as well as another fucose-binding lectin, i.e. *Ulex europaeus* I agglutinin (UEA-1; specific for fucα1-2Galβ), has been used to study the alterations in PSA fucosylation in PCa (11, 21, 23, 24). Methods used include either immuno-purification of PSA (11) or capturing and enrichment of

fucosylated PSA as well as other glycoproteins using lectin affinity column chromatography (21, 24). Ohyama et al. reported that no significant differences in AAL bound fractions of PSA from PCa and BPH patient were found (21). Li et al. demonstrated a significant increase in PSA fucosylation between PCa with Gleason score 6 and Gleason score 7–9 (24).

The problem with immunoassays is the high background due to sample autofluorescence which is minimized using Europium (Eu)-chelate label and time-resolved fluorometry. Eu-chelates, like other luminescent lanthanides, have exceedingly long-lived luminescence enabling the short-lived background interferences to be removed via time-gated acquisition. To further improve the assay sensitivity europium-label detection technology using Eu-doped nanoparticles, described elsewhere (25), was applied. The two main advantages of the nanoparticle label technology are the signal amplification provided by the 30,000 chelates in a single 107 nm particle and the enhanced binding affinity of the nanoparticle-lectin bioconjugates through the improved avidity provided by the high density of immobilized lectins on the particle (26).

Here we introduce two novel lectin-immunoassays for analyzing PSA fucosylation, detected by AAL, by measuring the time-resolved fluorescence (TRF) of Eu-chelates. For both of the novel lectin-immunoassays non-glycosylated fPSA-specific Fab-fragments were used to capture the analyte. The first developed novel lectin-immunoassay uses Eu-labeled AAL for the detection of Fucα1-6/3GlcNAc structures on PSA, whereas the second lectin-immunoassay depends on AAL coupled to Eu(III)-chelate-dyed nanoparticles. Utilizing our own technology we analyzed the fucosylation of PSA derived from tumor prostate cell lines from lymph node (LNCaP) and from metastasis in bone (PC3), PSA extracted from pooled seminal plasma of healthy donors and recombinant precursor form of PSA (proPSA) expressed in baculovirus. The fucosylation of LNCaP PSA has been studied by others (4, 5, 17), whereas to our knowledge the fucosylation of PC3 PSA and proPSA has not been analyzed previously using AAL. Using a variety of clinical sample matrixes, we investigated whether differences in PSA fucosylation could discriminate patients with PCa from BPH and young healthy males.

2. Materials and methods

2.1 Purified Fab-fragments, reagents and instrumentation

Purified recombinant Fab 5A10 was site specifically biotinylated as described elsewhere (27). Unconjugated AAL was from Vector Laboratories, Peterborough, United Kingdom. Microtiter wells coated with streptavidin and wash buffer concentrate were from Kaivogen, Turku, Finland. Enhancement solution was prepared as previously (28). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Protease inhibitor cOmplete tablets were from Roche Diagnostics, Manheim, Germany and Triton X-100 detergent from Acros Organics, ThermoFischer Scientific, Geel, Belgium. TSM-buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L

$CaCl_2$, 3 mmol/L $MgCl_2$, 0.5 g/L NaN3, pH 8.0) containing 2 g/L BSA, was used in the developed assays. TRF was measured with multilabel reader Victor 1420 Multilabel Counter (Perkin-Elmer Life Sciences, Wallac, Turku, Finland).

2.2 Preparation of lectin tracers

2.2.1 Eu-chelate labeled AAL

AAL was labeled with 100-fold molar excess of Eu-chelate (*N*1-(*p*-isothiocyanatobenzyl)diethylenetriamine-*N*1,*N*2,*N*3,*N*4-tetraacetic acid-Eu^{3+}) in 50 mmol/L carbonate buffer (pH 9.8) at room temperature (RT) for two hours. Free chelate was removed from labeled lectin by NAP-10 and PD-10 gel filtration columns (GE Healthcare, Schenectady, NY, USA), equilibrated and run with pH 7.75 Tris-HCl buffer (50 mmol/L Tris, 150 mmol/L NaCl, and 0.02% NaN_3). The labeled lectin was stabilized with 1 g/L BSA.

2.2.2 AAL coupled to 107 nm particles

The carboxyl-modified Fluoro-Max polystyrene, 107-nm particles were purchased from Seradyn (Indianapolis, IN, USA). Fluorescent properties of these nanoparticles have been described previously (25). AAL was covalently coupled to activated carboxyl groups on the Eu(III)-chelate-dyed nanoparticle surface using a procedure described previously (29) with some minor modifications. In brief, nanoparticles (7.5×10^{11} particles) were suspended in 10 mmol/L phosphate buffer (pH 7.0), and 0.75 mmol/L EDC and 10 mmol/L sulfo-NHS were used to activate the nanoparticle surfaces with a 15 min incubation at RT. AAL concentration in the coupling reaction was 0.5 mg/ml, and for the coupling 10 mmol/l phosphate buffer (pH 8.0) containing 100 mmol/L NaCl was used. The coupling reaction was incubated for 2 hours at RT under vigorous shaking. Tris-based buffer (10 mmol/L Tris, 0.5 g/L NaN_3, pH 8.5) was used for the final washes and to block the remaining active groups, and the nanoparticle–lectin conjugates were stored in the same buffer, supplemented with 2 g/L BSA, and kept at 4°C. Before the first instance of use, the noncolloidal aggregates were separated from the monodisperse suspension by mixing the particles thoroughly, sonicating them, and centrifuging the particles lightly (350g, 5 min).

2.3 Different PSA forms

Seminal plasma PSA was obtained from Department of Clinical Chemistry, Lund University Hospital, Malmö, Sweden. The production of proPSA using the baculovirus expression system and LNCaP PSA, as well as the purifications with affinity chromatography has been described previously (30, 31, 32). PC3 cells, which normally do not express PSA, were transfected with a mammalian expression vector containing PSA and a stable cell line was generated. The stable transfection of PC3 cell line and the purification of PC3 PSA are described elsewhere (30, 31, 33). LNCaP PSA was used as a calibrator, and dilutions (10–

200 µg/L LNCaP PSA) were made in Tris-saline-azide buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5 g/L NaN_3, pH 7.7) containing 5 g/L BSA, and used as triplicates.

2.4 Patient and control samples and the lysis of prostate tissue samples

Female serum, EDTA and heparinized plasma samples from four healthy volunteers, as well as urine and heparinized plasma samples from 10 healthy female and 11 male volunteers (under 35 years) were collected at the Department of Biotechnology, University of Turku in March 2012. Blood samples were centrifuged within 1 h after venipuncture, immediately frozen and stored at -20 °C.

Urine samples from 16 tumor confirmed PCa patients and 15 from patients with BPH were collected in 2012 at the Erasmus University Medical Center, Rotterdam, Netherlands. The 9 anonymous female and 23 male heparinized plasma samples, taken as a clinical laboratory routine, were obtained from Lund University, University Hospital UMAS (Malmö, Sweden) according to institutional review board approved procedures. Heparinized plasma samples from 21 consecutive patients who underwent clinical work-up with digital rectal examination (DRE) and transrectal ultrasound (TRUS)-guided prostate biopsy due to their symptomatic prostate condition at the Department of Urology at Turku University Hospital in 2004–2005. Samples were stored at -20 °C before measurements.

Prostate tissue samples from 36 PCa patients were obtained from radical prostatectomies (RP) immediately after surgery from Turku University Hospital, Finland. The sample tissues were stored at -20 °C in 1 ml of phosphate-buffered saline (PBS) buffer with added protease inhibitors (cOmplete tablets) until further processing. The protein pool from was recovered using Triton-X-100 detergent by adding 1/10 of the sample volume of 10x PBS lysis buffer (pH 7.4, with added protease inhibitors, 20mM EDTA, 10 % Triton-X-100). The lysis was done on ice under slow shaking for two hours and the supernatant stored in -20°C. For the analysis from each sample a 1:10 dilution was done in Tris-saline-azide buffer containing 5 g/L BSA. Patient and study group data is presented in Table 1. The study was carried out in accordance with the declaration of Helsinki.

Table 1. Patient and study group data.

Sample types	Median (range) or frequency (percent)	
Heparinized plasma samples		
anonymous males, clinical laboratory routine	23	
anonymous females, clinical laboratory routine	9	
healthy young males	11	
healthy young females	10	
Patients with suspicion of prostate disorder*	21	
Age of patients (years)	62	(56, 70)
Histological grading of biopsies		
Gleason score 4–6	9	(43%)
Gleason score >6	12	(57%)
Native urine samples		
Age at sampling (years)		
PCa (*n*=16)	67	(54, 76)
BPH (*n*=15)	73	(68, 75)
healthy young males (*n*=11)	<35	
healthy young females (*n*=10)	<35	
Histological grading of PCa patients		
Gleason score 6	5	(31%)
Gleason score >6	8	(50%)
N/A**	3	(19%)
RP tissue samples from PCa patients (*n*=36)		
Age of patients (years)	64	(51, 72)
Histology of prostate tissue		
Benign	11	(31%)
Gleason grade 2–3	14	(39%)
Gleason grade 4–5	11	(31%)

* underwent clinical work-up with DRE and TRUS due to symptomatic prostate condition

N/A** Information not available

2.5 Total and free PSA assays

The tPSA and fPSA concentrations in tissue lysates, blood and urine samples were measured using in-house immunoassay methods as previously described (34, 35). The assay to measure the tPSA uses MAb H117 as capture and MAb H50 as the tracer antibody. The fPSA assay was identical to the tPSA measurement except that fPSA-specific MAb 5A10 was used as the tracer. The analytical detection limits (based on 21 replicas of calibration diluent) were

0.006 µg/L and 0.016 µg/L for tPSA and fPSA, respectively (mean CV% for male samples 2.1%, range 0-9.2%).

2.6 Chelate-labeled lectin-immunoassay

The developed research assay was conducted in three-step sandwich-type format, where Eu-labeled AAL was used as a tracer (Fig. 1A). All subsequent steps were undertaken at RT and incubations done with slow shaking. Biotinylated fPSA-specific recombinant Fab-fragment 5A10 (150 ng/well), used as a capture antibody, was immobilized to streptavidin coated wells in 100 µL of TSM buffer for one hour incubation. After washing twice, 50 µl of TSM buffer and 50 µl of either sample or calibrator, in three replicas, were added and incubated for one hour. The wells were washed four times and Eu-labeled AAL (50 ng/well) was added in 150 µl of TSM buffer and incubated for one hour. The plates were washed four times before adding the enhancement solution (200 µL/well), and incubated for 10 min before measuring TRF of Europium signal.

2.7 Nanoparticle-based lectin-immunoassay

The two first steps of the assay, i.e. the immobilization of capture Fab to the streptavidin coated wells and the incubation of the sample, were identical to the chelate-labelled lectin-immunoassay. Instead of using chelate-labeled AAL as the tracer, AAL coupled to Eu(III)-chelate-dyed nanoparticles (2.0×10^7 particles/well) were used in the final step. After incubating the nanoparticles for one hour at RT, the wells where washed six times and the TRF of the bound nanoparticle bioconjugates was directly measured from the surface of the well. The principle of the developed nanoparticle-based lectin-immunoassay is presented in Fig. 1B.

2.8 Data analysis

PSA fucosylation results from the two lectin-immunoassays were expressed in fluorescence signals (cps). The mean background fluorescence was subtracted from the mean fluorescence of the samples, and the fucosylation levels were normalized for differences in sample protein levels by dividing with fPSA concentration. Normal distribution of subject groups was verified by Shapiro-Wilk and Kolmogorov-Smirnov tests. The nonparametric Kruskal-Wallis test and Mann-Whitney U-test were used to compare the differences between study groups using the IBM SSPS Statistics 22 software. Dunn-Bonferroni corrections were made to adjust the *p*-values. The two sided *p*-values of <0.05 were considered to be statistically significant.

3. Results

3.1 AAL binding to fucose on different PSA forms and calibration curves

The fucosylation of PSA secreted by LNCaP and PC-3 cells, seminal plasma PSA and proPSA was studied using the developed Eu-chelate-labeled and nanoparticle-based AAL-immunoassays. The fluorescence from different PSA forms in a concentration range of 20–500 ng/ml is shown in Fig. 2. PSA from LNCaP cells showed the highest fucose content. Neither proPSA nor seminal plasma PSA gave fluorescence signal with the Eu-labeled AAL-immunoassay. Using nanoparticles, low fluorescence signals were detected with 200 and 500 ng/ml of seminal plasma PSA, indicating some minor amount of fucosylated glycan structures. With the nanoparticle-based lectin-immunoassay a fourfold higher Fucα1-6/3GlcNAc content was detected from proPSA compared to seminal plasma PSA. In addition to PSA from LNCaP cells, we detected Fucα1-6/3GlcNAc glycan structures on PSA secreted by the PC-3 cells. The nanoparticle-based lectin-immunoassay detects LNCaP PSA and PC3 PSA over 30- and 5-times more strongly, respectively, compared to the seminal plasma PSA.

The LNCaP PSA calibration curves and precision profiles (mean of three different runs using three replicas) are presented in Fig. 2C. AAL-coupled nanoparticles gave little over tenfold higher fluorescence signals from LNCaP PSA compared to the fluorescence with Eu-chelate-labeled lectin. With both assays a linear response was maintained up to 500 µg/L. The analytical detection limit, calculated as concentration corresponding the mean + 3 times the standard deviation (SD) of the calibration diluent signal (n=36), for chelate-labeled and nanoparticle-based lectin-immunoassay were 49.1 µg/L and 5.1 µg/L, respectively.

3.2 Blood samples in lectin-immunoassays

We studied whether blood samples were suitable for the developed lectin-immunoassays. The high background fluorescence signals in plasma and serum, detected with nanoparticle-based lectin-immunoassay, from four females are shown in Fig. 3. The unspecific background signal level in heparinized plasma from 19 females was undistinguishable from male heparinized plasma samples (data not shown). The Fucα1-6/3GlcNAc content on PSA from heparinized plasma did not show statistically significant difference between 11 young males, 23 anonymous males or 21 consecutive patients with clinically confirmed PCa (data not shown). In plasma and serum the background for the immunoassays are too high and variable that the small signals from PSA are lost. Due to high background binding of plant lectins to the vast amount of glycosylated proteins in blood, neither serum nor plasma samples are suitable for the developed lectin assays.

3.3 Fucosylation of urine PSA

Considering the problems detected with serum and plasma samples, the more sensitive nanoparticle-based lectin-immunoassay, with a larger dynamic range, was used to analyze the

fucosylation of urine PSA from PCa patients and BPH patients with no indication of PCa. Also the fucosylation of urine PSA from 11 healthy young males and 10 females was detected using AAL.

The tPSA and fPSA concentrations in urine were unable to discriminate patients with PCa from BPH and healthy young males (for tPSA p=0.186, for fPSA p=0.121), as shown in Fig. 4A. The binding of AAL to Fucα1-6/3GlcNAc on urine PSA measured in fluorescence from the Eu-nanoparticles, divided by fPSA-concentration in the sample, is shown in Fig. 4B. Median for this fPSA-corrected fluorescence in cancerous patients was 327 cps/μgL^{-1} (interquartile range: 40–12771 cps/μgL^{-1}), in BPH patients 70 cps/μgL^{-1} (interquartile range: 30–120 cps/μgL^{-1}) and in young males 189 cps/μgL^{-1} (interquartile range: 43–328 cps/μgL^{-1}). The fucosylation of PSA in urine showed a wider distribution among PCa patients compared to BPH and healthy young males. There was a statistically significant (p=0.030) increase in urine PSA Fucα1-6/3GlcNAc content in PCa patients compared to BPH patients. The urine PSA fucosylation had even greater discrimination power (p=0.010) in distinguishing PCa patients having Gleason score ≥7 from BPH patients (Fig. 4C). There was no statistically significant difference in urine PSA fucosylation between PCa patients and healthy young males. There was eminent unspecific background from female urine with measured median fluorescence of 4140 cps compared to the median fluorescence of 6570 cps from healthy young males (data not shown).

3.4 Fucosylation of PSA in tissue lysates

Fucosylation of tissue PSA from 11 benign and 25 cancerous prostate tissue lysates was studied using the nanoparticle-based lectin-immunoassay. The PSA from the tumor tissue showed a statistically significant increase in fucosyl content compared to the benign tissue from PCa patients (Fig. 5). Two benign tissue lysates with PSA having the highest amounts of Fucα1-6/3GlcNAc were from patients with Gleason 4 and Gleason 5 graded tumors in another part of the prostate. By measuring the fucosylation of PSA, we were not able to distinguish low-grade PCa tissues from tissue samples having poorly-differentiated and more aggressive cells, with Gleason grade 4–5 (data not shown).

4. Discussion

We present a simple and robust assay to analyze the fucosylation on PSA using *Aleuria aurantia* lectin. The usage of monoclonal and polyclonal antibodies to capture the protein of interest is problematic, since both mono- and polyclonal antibodies are themselves glycosylated. Usually an additional step to remove the glycans on capture antibodies is needed to avoid the interference in glycosylation analysis of the captured protein. Treatments, such as periodate oxidation or glycoamidase F treatment, to remove glycans from the antibodies can hinder their stability and affect their ability to bind antigen (19). We used a recombinant Fab-fragment 5A10, which itself is not glycosylated, to capture fPSA. Therefore

AAL binds only to the fucosyl moiety present on the captured PSA and not to potential glycans on the antibody that captures PSA. With the use of AAL coupled nanoparticles the sensitivity of the lectin-immunoassay was greatly improved, with the LOD 5 ng/ml of LNCaP PSA. The use of AAL coupled to Eu-doped nanoparticles is an essential part of the assay for improved avidity compared to the use of single lectin molecules labeled with Eu-chelate. Another feature of the nanoparticles essential to the improved sensitivity is the fluorescence signal amplification provided by the 30,000 Eu-chelates in a 107 nm particle. The developed assay has analytical advantages, as in the 96-well plate format can be considered as high-throughput. Moreover, the method is easy to use without prior need for extensive training.

Using both lectin-immunoassays, we demonstrated substantially higher fucose content in LNCaP PSA compared to PSA derived from seminal plasma. These results are in agreement with a previous publication (4). To our knowledge we are the first to show, using AAL, that PSA from transfected PC3 cell line and the recombinant precursor form of PSA (proPSA) expressed in baculovirus contains Fuc1-6/3GlcNAc structures.

Using the nanoparticle-based lectin-immunoassay we detected a statistically significant increase (p=0.001) in the PSA fucosylation in PCa tissue compared to the benign tissue. All the benign tissue samples were from PCa patients, but having a normal prostate tissue microscopic appearance. The two patients from which the benign prostate tissue showed high PSA fucosylations, detected with AAL, had poorly differentiated (Gleason 4 and 5) tumor cells in another part of the prostate. Most likely, altered glycosylation could have already occurred on PSA in the benign tissue of prostate containing a tumor, hence comparing the PSA fucosylation of tumor tissue to that of complete healthy prostate tissue, would show even a higher discrimination. We believe it could be possible, by using this nanoparticle-lectin approach, to detect these precancer modifications, such as altered fucosylation patterns, from microscopically normal looking tissue biopsies before any visible histological changes occur.

Although studies on tissue specimen give valuable information on the differences in altered glycosylation during cancer development, there is a need for robust diagnostic methods (either blood, urine or seminal plasma based) to distinguish PCa from BPH. Serum and plasma are problematic, considering the rather low amount of PSA amongst immense amount of other glycoproteins in blood. Moreover, reports with contradictory result on the serum PSA glycosylation have been described (21, 20, 36, 37, 38). Also in our lectin-immunoassay formats the use of serum or either EDTA or heparinized plasma resulted in high unspecific binding to the glycan structures on background glycoproteins. The Fucα1-6/3GlcNAc contents on heparinized plasma PSA detected in 21 consecutive patients with clinically confirmed PCa were overlapping with those measured from 11 young males.

Urine, having PSA in high amounts and mostly in its free form, seems to be a more suitable matrix. PSA can be detected in microgram levels in urine compared to nanogram levels in circulation. Sample interference and cross-reactivity from other glycoproteins is more prominent in serum and plasma compared to urine. Serum and plasma may also be affected by the presence of rheumatoid factor, autoantibodies and human anti-mouse antibodies. We

were unable to completely solve the problem with the high background of AAL plant lectin to female urine. With the nanoparticle-based lectin-immunoassay we showed a statistical significant increase (p=0.030) in urine PSA fucosylation from PCa patients compared to BPH patients, and a greater discrimination (p=0.010) when comparing BPH patients to PCa patients with Gleason score greater than 6, but the number of subjects in this cancerous group declined to 8. There was no statistically significant difference in urine PSA Fucα1-6/3GlcNAc contents between PCa patients and healthy young males, which could be due to age-related glycosylation differences or result of unspecific binding. The nanoparticle-based lectin-immunoassay showed a better discrimination for the three groups compared to either tPSA (p=0.186) and fPSA (p=0.121) concentrations in urine. Our data also showed a relatively wider distribution of fucosylated PSA in the urine of PCa patients compared to the urine from BPH patients and healthy young males.

Based on the high unspecific fluorescence signal measured from female blood and urine, AAL is not specific enough to be used in screening for cancer-associated alterations of fucosyl content on PSA. The low number of urine samples tested (16 cancerous, 15 BPH and 11 young males) might be increased to reach statistical significance. The sensitivity and the specificity of the detection method need to be further improved. Another drawback is the use of LNCaP PSA as the calibrator in the absence of true standard material. The true sensitivity of the assay could be substantially higher since only a small portion of LNCaP PSA might be detected with AAL. Our findings are based on a small sample size, and a larger scale study is needed to further validate our preliminary findings. It is still uncertain if the observed increase in PSA fucosylation in PCa could be used clinically to improve and facilitate the differentiation of aggressive cancers from latent ones.

Regardless of the unspecificity of plant lectins, AAL does seem intriguing, as we have shown a higher Fucα1-6/3GlcNAc content on PSA derived from PCa cell lines LNCaP and PC3 compared to seminal plasma PSA. Moreover, there was a statistically significant increase in the PSA fucosylation in PCa tissue compared to benign tissue and in urine from patients with PCa compared to BPH. The assay concept could easily be modified to recognize other glycan structures by replacing AAL with other lectins. In addition, the nanoparticle-based lectin-immunoassay could possibly be used to detect precancer modifications, such as altered fucosylation patterns, from microscopically normal looking biopsy tissue samples before any visible histological changes occur.

Conclusions

Here we introduce a novel nanoparticle-based AAL-immunoassay for analyzing the fucosylation of PSA in urine and tissue lysates. We showed a statistically significant higher Fucα1-6/3GlcNAc content on PSA in PCa tissue compared to the benign tissue and in urine from patients with PCa compared to BPH, but a larger scale study is needed to further validate our preliminary findings.

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References

[1] Li Y, Tian Y, Rezai T, Prakash A, Lopez MF, Chan DW, Zhang H. Simultaneous analysis of glycosylated and sialylated prostate-specific antigen revealing differential distribution of glycosylated prostate-specific antigen isoforms in prostate cancer tissues. Anal Chem 2011;83:240–5.

[2] Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim Biophys Acta 1999;1473:4–8.

[3] Compagno D, Gentilini L, Jaworski FM, González Pérez I, Contrufo G, Laderach DJ. Glycans and galectins in prostate cancer biology, angiogenesis and metastasis. Glycobiology 2014;24:899–906.

[4] Peracaula R, Tabrés G, Royle L, Harvey DJ, Dwek RA, Rudd PM, de Llorens R. Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. Glycobiology 2003;13:457–70.

[5] Tabarés G, Radcliffe C, Barrabés S, Ramírez M, Aleixandre RN, Hoesel W, Dwek RA, Rudd PM, Peracaula R, de Llorens R. Different glycan structures in prostate-specific antigen from prostate cancer sera in relation to seminal plasma. Glycobiology 2006;16:132–45.

[6] Tajiri M, Ohyama C, Wada Y. Oligosaccharide profiles of the prostate specific antigen in free and complexed forms from the prostate cancer patient serum and in seminal plasma: a glycopeptide approach. Glycobiology 2008;18:2–8.

[7] Sarrats A, Saldova R, Comet J, O'Donoghue N, de Llorens R, Rudd PM, Peracaula R. Glycan characterization of PSA 2-DE subforms from serum and seminal plasma. J Integr Biol (2010a);14:465–74.

[8] Kyselova Z, Mechref Y, Bataineh MM, Dobrolecki LE, Hickey RJ, Vinson J, Sweeney CJ, Novotny MV. Alterations in the serum glycome due to metastatic prostate cancer. J Proteome Res 2007;6:1822–32.

[9] Sarrats A, Comet J, Tabarés G, Ramírez M, Aleixandre RN, de Llorens R, Peracaula R. Differential percentage of serum prostate-specific antigen subforms suggests a new way to improve prostate cancer diagnosis. Prostate 2010b;70:1–9.

[10] Saldova R, Fan Y, Fitzpatrick JM, Watson RW, Rudd PM. Core fucosylation and α2-3 sialylation in serum N-glycome is significantly increased in prostate cancer comparing to benign prostate hyperplasia. Glycobiology 2011;21:195–205.

[11] Dwek M, Jenks A, Leathem A. 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. Clinica Chimica Acta 2010;411:1935–9.

[12] Thompson R, Creavin A, O'Connell M, O'Connor B, Clarke P. Optimization of the enzyme-linked lectin assay for enhanced glycoprotein and glycoconjugate analysis. Anal Biochem 2011;413:114–22.
[13] Drake PM, ChoW, Li B, Prakobphol A, Johansen E, Anderson NL, et al. Sweetening the pot: adding glycosylation to the biomarker discovery equation. Clin Chem 2010;56:223–36.

[14] Pilobello KT, Krishnamoorthy L, Slawek D, Mahal LK. Development of a lectin microarray for the rapid analysis of protein glycopatterns. Chembiochem 2005;6:985–9.

[15] Rosenfeld R, Bangio H, Gerwig GJ, Rosenberg R, Aloni R, Cohen Y, Amor Y, Plaschkes I, Kamerling JP, Maya RBY. A lectin array-based methodology for the analysis of protein glycosylation. J Biochem Biophys Methods 2007;70:415–26.

[16] Wu YM, Nowack DD, Omenn GS, Haab BB. Mucin glycosylation is altered by pro-inflammatory signaling in pancreatic-cancer cells. J Proteome Res 2009;8:1876–86.

[17] Meany DL, Hackler L, Zhang H & Chan DW. Tyramide signal amplification for antibody-overlay lectin microarray: A strategy to improve the sensitivity of targeted glycan profiling. J Proteome Res 2011;10:1425–31.

[18] Duk M, Lisowska E, Wu JH, Wu AM. The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. Anal Biochem 1994;221:266–72.

[19] Gornik O, Lauc G. Enzyme linked lectin assay (ELLA) for direct analysis of transferrin sialylation in serum samples. Clin Biochem 2007;40:718–23.

[20] Meany DL, Zhang Z, Sokoll LJ, Zhang H, Chan DW. Glycoproteomics for prostate cancer detection: changes in serum PSA glycosylation patterns. J Proteome Res 2009;8:613–9.

[21] Ohyama C, Hosono M, Nitta K, Oh-eda M, Yoshikawa K, Habuchi T, Arai Y, Fukuda M. Carbohydrate structure and differential binding of prostate specific antigen to Maackia amurensis lectin between prostate cancer and benign prostate hypertrophy. Glycobiology 2004;14:671–9.

[22] Haselhorst T, Weimar T, Peters T. Molecular recognition of sialyl Lewis x and related saccharides by two lectins. J Am Chem Soc 2001;123:10705–14.

[23] Jankovic MM, Kosanovic MM. Glycosylation of urinary prostate-specific antigen in benign hyperplasia and cancer: assessment by lectin binding patterns. Clin Biochem 2005;38:58–65.

[24] Li QK, Chen L, Ao MH, Chiu JH, Zhang Z, Zhang H, Chan DW. Serum fucosylated prostate-specific antigen (PSA) improves the differentiation of aggressive from non-aggressive prostate cancers. Theranostics 2015;5:267–76.

[25] Härmä H, Soukka T, Lövgren T. Europium nanoparticles and time-resolved fluorescence for ultrasensitive detection of prostate-specific antigen. Clin Chem 2001;47:561–8.

[26] Soukka T, Paukkunen J, Härmä H, Lönnberg S, Lindroos H, Lövgren T. Supersensitive time-resolved immunofluorometric assay of free prostate-specific antigen with nanoparticle label technology. Clin Chem 2001; 47: 1269–78.

[27] Eriksson S, Vehniäinen M, Jansen T, Meretoja V, Saviranta P, Pettersson K, Lövgren T. Dual-label time-resolved immunofluorometric assay of free and total prostate-specific antigen based on recombinant Fab fragments. Clin Chem 2000;46:658–66.

[28] Pettersson K, Söderholm JR. Ultrasensitive two-site immunometric assay of human lutropin by time-resolved fluorometry. Clin Chem 1990;36:1928–33.

[29] Valanne A, Huopalahti S, Soukka T, Vainionpää R, Lövgren T, Härmä H. A sensitive adenovirus immunoassay as a model for using nanoparticle label technology in virus diagnostics. J Clin Virol 2005;33:217–23.

[30] Väisänen V, Lövgren J, Hellman J, Piironen T, Lilja H, Pettersson K. Characterization and processing of prostate specific antigen (hK3) and human glandular kallikrein (hK2) secreted by LNCAP cells. Prostate Cancer Prostatic Dis 1999;2:91–7.

[31] Nurmikko P, Väisänen V, Piironen T, Lindgren S, Lilja H, Pettersson K. Production and characterization of novel anti-prostate specific antigen (PSA) monoclonal antibodies that do not detect internally cleaved Lys145-Lys146 inactive PSA. Clin Chem 2000;46:1610–8.

[32] Rajakoski K, Piironen T, Pettersson K, Lövgren J, Karp M. Epitope mapping of human prostate specific antigen and glandular kallikreins expressed in insect cells. Prostate Cancer and Prostatic Dis 1997;1:16–20.

[33] Lövgren J. (1999) Human Glandular Kallikrein 2. PhD thesis. Lund University, Sweden.

[34] Väisänen V, Eriksson S, Ivaska KK, Lilja H, Nurmi M, Pettersson K. Development of sensitive immunoassays for free and total human glandular kallikrein 2. Clin Chem 2004;50:1607–17.

[35] Väisänen V, Peltola MT, Lilja H, Nurmi M, Pettersson K. Intact free prostate-specific antigen and free and total human glandular kallikrein 2. Elimination of assay interference by enzymatic digestion of antibodies to *F*(ab')2 fragments. Anal Chem 2006;78:7809–15.

[36] Basu PS, Majhi R, Batabyal SK. Lectin and serum-PSA interaction as a screening test for prostate cancer. Clin Biochem 2003;36:373–6.

[37] Fukushima K, Satoh T, Baba S, Yamashita K. α1,2-Fucosylated and β-*N*-acetylgalactosaminylated prostate-specific antigen as an efficient marker of prostatic cancer. Glycobiology 2010;20:452–60.

[38] van Dieijen-Visser MP, van Pelt J, Delaere KP. Pitfalls in the differentiation of N-glycosylation variants of prostate-specific antigen using concanavalin A. Eur J Clin Chem Clin Biochem 1994;32:473–8.

FIGURE LEGENDS

Fig. 1. The chelate-labelled lectin-immunoassay principle (A) and the nanoparticle-based lectin-immunoassay principle (B) using biotinylated recombinant Fab-fragment 5A10 as a capture antibody immobilized onto streptavidin coated microtiter wells.

Fig. 2. Dose response curves (mean fluorescence signals measured from triplicates) from different PSA forms with nanoparticle-based lectin-immunoassay (A) and Eu-labeled lectin-immunoassay (B). The error bars represent the standard deviation measured from the triplicates. LNCaP PSA has a high Fucα1-6/3GlcNAc content, detected by AAL, compared to PSA from seminal plasma, PC-3 cells or recombinant precursor PSA, proPSA, produced in baculovirus. LNCaP PSA calibration curves (closed symbols) and imprecision profiles (open symbols) for chelate-labeled lectin-immunoassay (□) and nanoparticle-based lectin-immunoassay (◊) (C).

Fig. 3. Unspecific fluorescence signals, as mean fluorescence signals from three replicates, in nanoparticle-based lectin-immunoassay. Fluorescence signal due to unspecific binding of AAL from four female serum (black bars), EDTA (grey bars) and heparinized (white bars) plasma samples and blank assay buffer (striped bar) using nanoparticle-based lectin-immunoassay. The error bars represent the standard deviation measured from the three replicas.

Fig.4. The tPSA and fPSA concentrations (µg/L) in urine (A) and fPSA-corrected fluorescence signals of AAL coupled nanoparticles from urine PSA (B) from prostate cancer patients (n=16), BPH patients (n=15) and healthy young males (n=10). The fPSA-corrected fluorescence signals were calculated as the measured fluorescence from the Eu-nanoparticles, divided by fPSA-concentration in the sample. Comparing the fPSA-corrected fluorescence signal of AAL coupled nanoparticles from urine PSA between BPH (n=15) and prostate cancer patients with Gleason score 7–9 (n=8) (C). The 10/25/50/75/90th percentiles are marked in the figures.

Fig. 5. The fPSA-corrected fluorescence signal of AAL-coupled nanoparticles measuring the fucosylation of PSA in benign and cancerous prostate tissue lysates. There was a statistically significant increase in the fucα1-6/3GlcNAc amount in cancer compared to the benign tissue. The 10/25/50/75/90th percentiles are marked in the figures and open circles denote the outlier values.