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Increased Bisecting N-Acetylglucosamine and Decreased Branched Chain Glycans of N-linked Glycoproteins in Expressed Prostatic Secretions Associated with Prostate Cancer Progression

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Abstract

Purpose—Using prostatic fluids rich in glycoproteins like prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), the goal of this study was to identify the structural types and relative abundance of glycans associated with prostate cancer status for subsequent use in emerging mass spectrometry-based glycopeptide analysis platforms.

Experimental Design—A series of pooled samples of expressed prostatic secretions (EPS) and exosomes reflecting different stages of prostate cancer disease were used for N-linked glycan profiling by three complementary methods, MALDI-TOF profiling, normal-phase HPLC separation, and triple quadrupole MS analysis of PAP glycopeptides.

Results—Glycan profiling of N-linked glycans from different EPS fluids indicated a global decrease in larger branched tri- and tetra-antennary glycans. Differential exoglycosidase treatments indicated a substantial increase in bisecting N-acetylglucosamines correlated with disease severity. A triple quadrupole MS analysis of the N-linked glycopeptides sites from PAP in aggressive prostate cancer pools was done to cross-reference with the glycan profiling data.

Conclusion and clinical relevance—Changes in glycosylation as detected in EPS fluids reflect the clinical status of prostate cancer. Defining these molecular signatures at the

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glycopeptide level in individual samples could improve current approaches of diagnosis and prognosis.

Keywords

Glycomics; N-linked glycosylation; Prostatic acid phosphatase; Urine Exosome; Prostate Cancer; Expressed Prostatic Secretion

Introduction

For the detection and management of prostate cancer, major challenges include the differentiation of aggressive from indolent diseases, and stemming from that, the need for improved prediction of recurrence and metastasis. The PSA serum detection assay continues to evolve to reflect new knowledge about disease specific isoforms and free versus bound complexes with serum proteins, yet there are now many documented problems with PSA testing that are associated with overtreatment and diagnosis of men with non-lethal, indolent cancers [1–3]. Pathologic staging is a critical standard for disease staging and risk assessment [4,5], but the heterogeneous and multifocal nature of the prostate cancer is a constant confounder in diagnostic and prognostic assay applications [6,7]. The Gleason grading system attempts to control for the multifocal nature and gland heterogeneity of cancerous lesions by summing the 2–3 most commonly observed histological patterns via inspection of multiple core biopsies [8]. This staging and grading process lacks timeliness in discriminating organ-confined from extracapsular disease, and cancer foci in the prostate are frequently missed by the biopsy procedure, confounding accurate diagnoses and making it difficult to accurately discriminate between men with clinically indolent prostate cancer from those with more aggressive disease [6,8–10]

Prostatic fluids secreted by the prostate represent proximal fluids of this organ. When collected in the clinic prior to prostate biopsy or prostatectomy, these fluids contain a repertoire of secreted proteins and shed cells reflective of the physiological state of that tissue. For prostate cancers, multiple genetic assays such as the recently FDA-approved PCA-3 test utilize expressed prostatic secretions (EPS) collected in urine following digital rectal exam [9,11]. Our collective group has published three extensive proteome studies of these EPS fluids, and identified over 1400 individual proteins [12–14], as well as a summary study of collection and processing strategies for these fluids [15]. From one of these proteome studies of direct EPS fluids obtained at the time of prostatectomy [12], six of the seven most abundant proteins contain N-linked glycosylation modifications. These proteins are, with their relative percentage of abundance and number of putative N-linked glycosylation sites (gs), albumin (23%; gs 0), immunoglobulins (12%; gs 1), lactotransferrin (8%; gs 3), prostatic acid phosphatase (PAP)(4%; gs 3), zinc alpha-2 HS glycoprotein (3%; gs 2), aminopeptidase N (3%; gs 10), and PSA (2%; gs 1). The glycan constituents for PSA have been previously reported from many sources including serum, cell lines and seminal fluids [16–21]. Our group has published the MALDI-TOF profiling identification of 40 N-linked glycan structures of partially purified PSA from seminal plasma samples from control, benign disease and prostate cancer donors [22]. The same seminal plasma samples were used to partially purify PAP, and 21 N-linked glycan structures were described, as well

as a triple quadrupole method for direct PAP glycopeptide analysis for glycans at the three distinct glycosites [22].

Using a series of clinically defined pooled EPS samples reflective of benign, indolent and aggressive prostate cancer, we report in depth glycan and glycopeptide profiling analysis using mass spectrometry and HPLC approaches. We hypothesize that the high concentration of prostate secretory glycoproteins present in these fluids can be used for global glycan profiling as indicators of potential prostate cancer biomarker candidates. To test this hypothesis, we determined the most abundant N-linked glycans in these disease specific EPS fluids by MALDI-TOF profiling in combination with refined analysis of specific glycopeptides from PAP. Sequential exoglycosidase treatment and normal-phase HPLC analysis of the resulting glycans was also done to identify an enrichment in bisecting N-acetylglucosamine (GlcNAc) species with disease progression. Specific assignments of glycan structural subtypes have also been determined for each of the three glycosylation sites on PAP.

Experimental

Seminal plasma samples

Expressed prostatic secretion fluids were obtained from men seen by Urology of Virginia clinicians from 2007–2011 after informed consent following Institutional Review Board-approved protocols at Urology of Virginia and Eastern Virginia Medical School. All personal information or identifiers beyond diagnosis and lab results were not available to the laboratory investigators. EPS-urine samples were collected performing a gentle massage of the prostate gland during DRE prior to biopsy, as previously described [13,15]. The massage consisted of three strokes on each side of the median sulcus of the prostate and the expressed fluid from the glandular network of the prostate was subsequently voided in urine. Pools (25–50 ml/sample) of EPS-urine were derived from 10 patients classified as having high grade, Gleason 8–10 tumors, 10 patients with low grade, Gleason 6, organ-confined prostate cancer and 10 non cancer samples, confirmed as biopsy negative for prostate cancer. Individual samples were pre-selected in these groups based on having fluid PSA protein levels above 14 µg/ml. Direct EPS fluids were obtained under anesthesia prior to prostatectomy [12,15], and four separate pool were created. A subset of these sample were analyzed for protein content in two previous studies [12,14]. Further clinical information for each of the sample pools is provided in Supplemental Table 1. A de-identified prostate tumor tissue pair of Gleason grade 7 (3+4)/stage T2b and matching distal non-tumor tissue was obtained from the Hollings Cancer Center Biorepository at the Medical University of South Carolina. A pathologist confirmed the presence of greater than 40% prostate cancer gland content in the sample.

Exosome preparation

The three EPS urine pools (45 mls) were centrifuged at $25,000 \times g$ for 30 minutes, and the supernatant centrifuged at $100,000 \times g$ for 4 hrs. The pelleted exosomes were washed twice with PBS and resuspended in 0.5 ml PBS. The expression of CD63 and CD9, known exosome protein markers, were confirmed by Western blot analysis and protein sequencing

by tandem mass spectrometry. The expected high proportional content of sphingomyelin (~50%) associated with prostate-derived microvesicles [23,24] was also confirmed.

Lectin Extraction and Analysis

Pooled EPS samples (0.2 mg) buffer exchanged into phosphate-buffered saline were resuspended in 20 mM Tris-buffered saline (pH 7.0), 1 mM calcium chloride, 1 mM magnesium chloride, and 1 mM manganese chloride and incubated for 16 h at 4°C with 0.1 ml agarose-bead lectins: RCA, AAL or SNA1 (Vector Laboratories, Burlingame, CA). Following repeat washing with lectin-binding solution, bound proteins were solubilized in 0.2 ml SDS gel loading buffer, heated at 60 °C for 10 min, then separated on a 12% Tris/bis SDS gel. The gel was transferred to nitrocellulose and probed with anti-PSA rabbit polyclonal; Fitzgerald Industries, Acton, MA) and anti-PAP (mouse monoclonal). Bands were visualized with secondary IR-dye conjugated antibodies to mouse and rabbit on an Odyssey CLx reader (LI-COR Biosciences, Lincoln, NE).

Normal Phase HPLC

Rapid glycan sequencing of the EPS urine pooled samples was performed by using previously optimized procedures [25]. Briefly, EPS urines were digested with PNGase F, and the released free glycans were labeled with 2-aminobenzamide (2-AB) for subsequent normal phase HPLC analysis [26]. Glycan identification was made through sequential exoglycosidase digestion as previously described by Guile *et al.* [27], as well as bovine kidney fucosidase. The resulting peaks, separated by time of appearance, correspond to specific glycan structures on the basis of glucose unit values (data not shown) [27]. All HPLC analyses were performed using a Waters Alliance HPLC System and quantified using the Millennium Chromatography Manager (Waters Corporation, Milford, MA). Glycan structures were identified by the calculation of the glucose unit value, as previously described, as well as through the comparison to known standards and sequential exoglycosidase digestion [27,28]. An additional sequential digest profile of non-cancer EPS urine glycans is provided in Supplemental Figure 1.

N-glycan permethylation

PAP gel bands derived from EPS urine pools were enriched by thiophilic chromatography and permethylated as previously described [22]. For all other fluid samples, 1500 units of a recombinant PNGase F prepared in the Mehta laboratory was used and added to 50–200 ug of sample, and incubated at 37°C for 18 hours. Protein was precipitated by the addition of 0.8 ml ice-cold methanol and low speed centrifugation (10000 × g, 25 min). The methanolic supernatant was then dried in a SpeedVac centrifuge under reduced pressure. Dried N-glycans were permethylated using a rapid permethylation assay with sodium hydroxide bead spin columns [29]. Chloroform extracts were dried under nitrogen in glass vials, and samples were resuspended in 20 µl 50% methanol/water for analysis or storage at –20° C.

MALDI-TOF/TOF

Permethylated N-glycans (1 µl) were mixed 1:2 with 2,5-Dihydroxybenzoic acid (DHB) matrix (10mg/ml in 50% methanol) and spotted on an AnchorChip MALDI-TOF target

plate. Each sample was analyzed in positive ion mode with 8000 laser shots using an AutoFlex III MALDI-TOF/TOF instrument or dual-source Solarix 7T FT-ICR MALDI-TOF (Bruker Daltonics, Billerica, MA). Calibration was done with red phosphorus or peptide 2 calibration mix (Bruker Daltonics, Billerica, MA). FlexAnalysis 3.4 or DataAnalysis 4.0 software (Bruker Daltonics, Billerica, MA) were used for spectra processing and normalization (either total ion current or root mean square). Additionally, the glycan database offered by the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) was used to search permethylated glycan masses correlating to peaks of interest in MALDI-TOF spectra.

Hybrid triple quadrupole/linear ion trap mass spectrometry of PAP

PAP was purified from EPS and/or EPS urine by thiophilic adsorption chromatography as described by Kawiniski et al. [30] and separated by SDS-PAGE. The identity and purity of the excised gel bands was assessed and confirmed by western blot and mass spectrometry prior to glycan and glycopeptides analysis. The PAP gel bands were reduced, alkylated, and digested with trypsin, as described previously [22]. Trypsin or chymotrypsin digestions were performed at 37°C for 18 hours to generate peptides and glycopeptides. The resultant peptides were extracted with 50% acetonitrile/0.1% TFA and dried in a SpeedVac.

The peptides were separated by reverse phase nanoLC on a Tempo™ NanoLC (Eksigent Technologies, Dublin, CA) under the following conditions: loading step Channel 1; 10µl/min buffer A (0.1% formic acid with 0.005% HFBA); Channel 2, 500nl/min buffer B (acetonitrile with 0.1% formic acid and 0.005% HFBA). Linear gradient 5% – 95% B in 42 minutes, 43–48 min 95% B and 49–60 min 10% B. For NSI-MS analysis we used the methods described in Sandra et al. [31] with minor modifications. Briefly, MS data were acquired using information dependent acquisition (IDA) methods. Two different IDA experiments were performed: i) An enhanced MS scan as survey scan, followed by an enhanced resolution scan of the three most intense ions for accurate charge state determination; ii) a precursor ion scan as a survey scan, and an enhanced product ion scan of the parent ions. The IDA criteria were: iii) most intense peaks for ions with m/z 400–2200, exclude targets after 3 occurrences for 30 seconds, mass tolerance 250 mDa. The instrument settings for the precursor ion scans were: ion spray voltage 2.8 kV, interphase heater at 125°C and Declustering potential 30V. Data acquisition was by profile scan mode with a 0.1 Da step size. The collision energy was automatically adjusted based on the ion charge state and mass. For the precursor ion survey scans, Q3 of the QTRAP was set to scan for diagnostic oxonium ions m/z (163 (Hex+), 204 (HexNAc+), 292/274/256 (NeuAc+1), and 366 (HexHexNAc+) originating from collisionally excited glycopeptides [32,33]. In the enhanced product ion scans, the fragment ions are trapped in the hybrid LIT before being scanned out to the detector thus generating MS2 spectra comprising of fragment ions from the peptide and the attached glycan. The resultant MS/MS spectra show a complete fragmentation pattern and these were used to search databases to identify the glycopeptides and their attached glycans. The identification and annotation of peptides and glycans was done using SimGlycan® [34].

RESULTS

Global Glycan Analysis of Expressed Prostatic Secretion Fluids

For initial profiling experiments, pools of EPS urine samples (see Supplemental Table 1) reflective of benign, non-cancer diagnoses, indolent prostate cancers and more aggressive prostate cancers were used in a series of lectin and MALDI-TOF-based profiling experiments. Because these fluids have already been extensively characterized for their proteomes [12–15], the relative abundance of prostate, immune and urine glycoproteins is known. In particular, immunoglobulins, lactotransferrin, PSA, PAP, Zn α 2-HS glycoprotein, aminopeptidase N represent over 55% of the most abundant proteins in direct EPS fluids, and additionally uromodulin, alpha-1-antitrypsin, alpha-1-microglobulin/AMBP, alpha 1 acid glycoprotein and alpha 2 acid glycoprotein in the EPS urine fluids. Thus we hypothesized that a global glycan profiling approach could be used to screen for major changes in glycan composition reflective of prostate cancer status. Using the pooled EPS urine samples from non-cancer, indolent and aggressive cancers, PGNaseF released total glycans were generated and permethylated, then assessed by linear MALDI-TOF profiling. As can be seen in the cumulative heat-map intensity profile of the individual pooled glycan spectra in Figure 1A, there is a noticeable decrease in the higher mass tri- and tetra-antennary glycans with disease severity, which is associated with decreased fucosylation and sialylation of the most abundant glycans. These same pooled EPS urines were also used for lectin capture profiling for three lectins, AAL (fucose targeted), RCA (galactose targeted) and SNA1 (sialic acid targeted). Following lectin binding, bound EPS proteins were separated on SDS-gels, blotted and probed for PSA and PAP by Western blot. As shown in Figure 1B, there was a significant decrease in captured PSA and PAP in the aggressive EPS urine sample by the sialic acid and fucose binding lectins. This is consistent with our previous reports for the glycans attached to PAP and PSA enriched from seminal plasma samples [22]. This decrease may also reflect the general decline in the amount of secreted proteins present in the prostatic fluids, as the secretory glands become less functional with disease progression [14,15].

Using a more sensitive FT-ICR MALDI instrument, the permethylated EPS urine pool glycans were further assessed for detection of the most abundant glycan species present in the samples. Representative spectra for each glycan preparation is shown in Figure 2A, along with peak intensity, isotopic distribution and signal-to-noise value for each glycan specie in the 1500 to 5000 mass range. In Figure 2B, exosomes derived from these same EPS urine pool samples were profiled for glycan composition. There are clear differences in the profiles of the cancer samples relative to the non-cancer sample, particularly at 2271 m/z. This mass is consistent with a bisecting GlcNAc-hybrid structure of Gal1N2M5N2. There are also a higher proportion of tetra-antennary glycans in the aggressive prostate exosome sample. Structural and intensity data for the most abundant glycans detected in each sample are provided in Supplementary Table 2. A sample set of direct EPS fluid pools were also analyzed and represent four conditions in the prostate cancer progression spectrum: 1. indolent Gleason 6 prostate cancers; 2. primarily Gleason 7 samples from individuals who have no evidence of disease 2–3 years after prostatectomy; 3. Gleason 7 samples from individuals who developed biochemical recurrence within 6 months of

prostatectomy; and 4. Gleason 8/9 samples from men who present with more advanced disease. MALDI-TOF profiling of permethylated glycans from these four pools are shown in Figure 2c, and peak data provided in Supplementary Table 2. As in the EPS urine glycan profiles, there is a more subtle but detectable and progressive decrease in the tri- and tetra-antennary glycan species in the most advanced disease Gleason 8/9 samples.

From this cumulative data, comparisons of the most prevalent glycan species can be assessed. By far the most common glycan was the bi-antennary complex glycan at 2793 m/z, NeuAc2Gal2N2M3N2, and it was detected as an abundant glycan in every sample. The majority of the samples also contained the core fucosylated version at 2967 m/z, NeuAc2Gal2N2M3N2F, and the fucosylated tri-antennary glycan at 3779 m/z, NeuAc3Gal3N3M3N2F. The presence of another abundant glycan species in EPS urine at 3808 m/z is consistent with a bi-antennary glycan with a terminal N-glycolylneuraminic acid (Neu5Gc) residue, NeuGc1NeuAc2Gal3N3M3N3F. For further comparisons, two gel slices for PAP from an individual representative sample constituent of the NED and BRC direct EPS pools was processed for PNGaseF digestion and glycan profiling. Also, a PNGaseF digested tissue slide of a Gleason 7 prostate tumor slice (10 micron) and a matched distal normal prostate tissue from the same individual was processed for glycan profiling. The lists of permethylated glycans detected in these samples are presented in Supplementary Table 2. There are many glycan species in the EPS fluids that are present in the PAP and tissue samples.

Rapid glycan sequencing analysis of EPS urine pools

The three EPS urine pools were also used for normal phase N-linked glycan analysis. [26]. As shown in Figure 3A, sequential exoglycosidase digestion (see also Supplementary Figure 1) indicates several major peaks that include a simple biantennary glycan (A2G2), a biantennary glycan with a bisecting GlcNAc (A2G2B), a core fucosylated biantennary glycan (FcA2G2), and a core fucosylated biantennary glycan with a bisecting GlcNAc (FcA2G2B). This data is consistent with the decrease in fucosylation described in Figure 1 for the aggressive EPS urine glycans. In addition, digestion with sialidase and bovine kidney fucosidase (Figure 3B) highlights the increase in the total level of biantennary glycan with a bisecting GlcNAc the glycan elution profile indicates a near 2-fold increase in the amount of bisecting GlcNAc present in the aggressive prostate cancer EPS urine samples. An increase in these bisecting GlcNAc glycan were also detected in the indolent cancer EPS samples relative to non-cancer.

Triple-quadrupole mass spectrometry analysis of PAP glycopeptides from direct EPS

We had previously described the analysis of seminal plasma derived PAP glycopeptides using a triple quadrupole mass spectrometer, which allowed determination of specific glycan structures attached at one of the three PAP glycosylation sites. The instrument was operated in the precursor ion (PI) scan mode for individual sugar molecules at specific m/z values of either 163 for Hexose⁺¹(Hex), 204 for N-acetylhexoseamine⁺¹ (HexNAc), 292/274/256 for N-acetylneuraminic acid⁺¹ (NeuAc), or 366 for Hex-HexNAc⁺¹. An enhanced product ion scan is triggered upon detection of the specified diagnostic ion, generating information on the sugar composition and the amino acid sequence of the glycopeptides in a single assay.

Using the precursor ion scan function of the triple quadrupole mass spectrometer, we have identified the N-glycan subtypes present at the 3 N-linked glycosylation sites of PAP. In these analyses we have identified bi- and triantennary sialylated and bisected structures at Asn-62, bi- and tetraantennary sialylated structures at Asn-188, and high mannose structures at Asn-301. Figure 4 shows data for the 366.1 m/z MS scans used in the identification and annotation of the triantennary sialylated bisected glycan structure at Asn-62 of PAP. As listed in Table 1, other structures for each of the three PAP glycosylation sites determined with this approach are listed. Most of these structures were also detected in the MALDI profiling experiments, and include several bisecting GlcNAc species. As with the seminal fluid PAP sample, the third site at Asn 301 is the only site where high mannose structures are determined.

Discussion

The three complementary methods used in the characterization of the prostatic fluid specimens across the prostate cancer disease spectrum have defined the most abundant N-linked glycan species present on prostate glycoproteins, as well as identified disease specific structural sub-types that can be targeted in follow up experiments. Because of prior proteomic characterizations of these EPS fluids demonstrating a prevalence of glycoproteins like PSA and PAP [12–14], it was feasible to ask whether global glycan profiling was analogous to direct glycan analysis of either protein. The triple quadrupole MS analysis of the PAP glycopeptides isolated from more aggressive direct EPS samples suggests this may be feasible, as there is excellent overlap with the types of glycans identified by MALDI profiling and normal phase HPLC (Table 1, Figure 3, Supplemental Table 2). In particular, the presence of many bi-antennary structures, bisecting GlcNAc (Figure 4) and the presence of Neu5Gc on PAP correlates with the trend of glycan changes and structural types present in the more advanced disease samples. The use of pooled samples is limiting in clinical relevance, but for a heterogeneous disease like prostate cancers, their use as discovery samples linked to specific clinical stages was critical to establish the types of samples to analyze for individual analyses. In this regard, emphasizing the most abundant glycans associated with each clinical condition (Supplemental Table 2) is that these could be the most likely glycans detected in mass spectrometry-based glycopeptide-targeted analyses of individual prostate glycoprotein targets, like PAP or PSA.

Recent developments in advanced mass spectrometry techniques are emerging for more sensitive and rapid analysis of glycopeptides. Until recently, it was usually necessary to choose to either analyze the protein and glycan portions of glycoprotein separately. With the increased use of new fragmentation techniques such as electron transfer dissociation (ETD), applied in combination with conventional (collision-induced dissociation; CID) or higher energy (high energy collisional dissociation; HCD) fragmentation has greatly enhanced the ability to perform in situ glycan-peptide characterization, thus providing structural information for both components in the analysis [35]. Using the next generation of hybrid linear ion trap-orbitrap mass spectrometers, a related method termed HCD-PD-ETD (higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation) has been reported [36]. In this approach, HCD spectra are acquired in a data-dependent fashion, identifying glycan oxonium ions that then trigger ETD spectra on the

glycopeptide precursor only. The result is more rapid and sensitive detection of individual glycopeptides, with glycan and peptide sequence data, from complex glycopeptide mixtures [36]. Having recently acquired this instrumentation, we have applied this approach to single glycoproteins, and are currently beginning the expansion of the technique to more complex glycoprotein mixtures like EPS urines. We have also been using an alternative Triple Quadrupole (QqQ) mass spectrometry based strategy [22,31,37] in which intact glycopeptides can be subjected to two complementary QqQ methods: Precursor Ion scanning (PI) and single reaction monitoring (SRM) or multiple reaction monitoring (MRM) that include CID based tandem mass spectrometry to identify both the sequence of the peptide and conjugated glycans. The availability of data analysis software like SimGlycan [34], used in this current report, and other new software will aid in increased sensitivity, quantitation and throughput for simultaneous glycan and peptide determinations. Having the database of the most common glycan species we expect to detect in these complex prostatic fluids will facilitate site specific analysis of prostate derived glycoproteins like PSA and PAP. Based on the global profiling data, it is likely that multiple disease specific glycan changes can be detected.

A new option highlighted in our study is the potential use of prostatic fluid exosomes. Exosomes could potentially serve as rich reservoirs of tumor specific proteins capable of acting as biomarkers for disease detection and progression [38–41], and many new methods to rapidly isolated them from biological fluids like serum and urine are being reported [24,42,43]. The EPS urine fluid contain both urinary exosomes and prostate specific structures termed prostasomes [23,24]. Direct EPS samples primarily contain prostasomes. These prostasomes, which are heterogeneous in size and composition, are exosome-related vesicles secreted by prostate acinar epithelial cells. Normally prostasomes function in reproduction processes, but the many immunomodulatory and signaling functions associated with them in a prostate tumor background represent a new reservoir of tumor specific proteins that have potential as new prostate cancer biomarkers [24,39,41,44]. We have recently characterized the protein content of prostasomes from EPS fluids [45], and PSA, PAP and other abundant prostatic fluid glycoproteins are present. The glycan profiles derived from the EPS urine exosomes were consistent with the fluids in regards to the types of glycans detected, except for the apparent increase in the amount of larger tetra-antennary glycans. No conclusions can be drawn for these limited pilot analyses on pooled samples, but continued analysis of exosomes/prostasomes derived from EPS fluids will be certainly be included in future glycan analysis experiments. Isolation of exosomes/prostasomes is a sub-fractionation technique that could be applicable to prostatic fluids, urine and blood, and particularly useful in boosting the amount of detectable glycoproteins for mass spectrometry based assay development.

From the glycan profiling and normal phase HPLC analyses, there were two major observations in that there was an overall decrease in tri- and tetra-antennary glycans with disease progression, and an almost doubling in the amount of biantennary, bisecting GlcNAc containing glycans. A similar trend was recently reported for global HPLC analysis of prostate cancer serum samples [46]. This is counter to what has emerged recently in studies of other cancer types, in that an increase in β 1–6 GlcNAc branching in N-linked structures is indicative and necessary for larger tri- and tetra-antennary structures and has been linked to

the metastatic phenotype of multiple cancer types [47,48], including prostate [49]. Conversely, the presence of bisecting-GlcNAc residues alter the structural conformation of the glycan chains and tend to limit branching [50–52]. A recent study [49] analyzed over 1400 biochemical recurrence tissues for PHL lectin staining, which binds β 1–6 GlcNAc structures, and found that localization of PHA-L staining to the cytoplasm was more prognostic to lethal disease. However, PHA-L staining intensity or localization could not predict biochemical recurrence, nor Gleason score and tumor staging. In the same study [49], multiple prostate tumor cell lines were evaluated for their ability to metastasize in mouse xenograft models, and PHA-L staining correlated with the most metastatic cell lines. These results, and the glycan profiles determined in fluids, highlight again the heterogeneity associated with prostate cancers. Individual gland regions with advanced disease, even when multifocal, are still present in a background of many normal glands in the prostate, all of which are still secreting glycoproteins.

Developing the methods to specifically find the glycan structures most associated with aggressive and lethal disease will always be challenging for prostate cancers, and will most likely reflect a balance of poor outcome glycan constituents versus the amount of more normal glycans. One candidate structure is that of the sialyl Lewis X antigen, which can be present on bi, tri and tetra antennary sialylated structures, and requires separate regulation of the activity of α -1,3 fucosyltransferases [53]. There is a long history of demonstrating the presence of sialyl Lewis X antigen specifically in metastatic prostate tissues [54–56]. However, these studies pre-date the serum PSA testing era when more men presented with metastatic disease and prostatectomies were still performed on them. Obtaining tissues or prostatic fluids from men with metastatic prostate cancers is currently challenging, due to the way the disease is managed clinically, and the fact that there are fewer cases available. The combination of genetic studies evaluating glycosyltransferase expression and regulation [49,53,54,57], with improved capabilities to detect specific glycans or glycopeptides in complex mixtures, could lead to more refined systems-based assays to predict clinical progression and lethality for prostate cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DRE	digital rectal exam
EPS	expressed prostatic secretions
GlcNAc	N-acetylglucosamine

NeuAc	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
PAP	prostatic acid phosphatase
PCA3	prostate cancer antigen 3
PSA	prostate-specific antigen
SRM-MS	selected reaction monitoring-mass spectrometry

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Clinical Relevance

For the detection and management of prostate cancer, major challenges include the differentiation of aggressive from indolent diseases, and stemming from that, the need for improved prediction of recurrence and metastasis. Changes in the levels of fucose and sialic acid content of N-linked glycoproteins like prostate specific antigen and prostatic acid phosphatase have long been associated with prostate cancer progression, but have never been quantitated at the level of specific glycans and attachment sites. Expressed prostatic secretions (EPS) in urine represent a glycoprotein-rich clinical fluid readily obtainable at the time of biopsy or prostatectomy, ideal for developing new diagnostic assays for prostate cancers. With improved and emerging mass spectrometry-based methods that allow direct analysis of glycopeptides for identification of both protein and glycan composition, we have extended our extensive proteome characterization of these EPS fluids to determining the N-linked glycome composition in these fluids across different prostate cancer disease states. There is a need to characterize the most abundant glycans associated with clinical progression, and link this to specific glycoprotein targets that could be used as prostate cancer biomarkers. This study provides the initial glycan characterizations and is a foundation for continued development of glycan and glycopeptide based diagnostic assays.

Figure 1. MALDI-TOF and Lectin Profiling of EPS Urine Samples

A. Protein from EPS urine pools representing non-cancer, indolent cancer and aggressive cancer conditions were treated with PNGaseF, and the released glycan isolated for rapid permethylation as described in the Experimental description. An aliquot was spotted on an AnchorChip target plate and analyzed by linear MALDI-TOF on an AutoFlex III MALDI-TOF/TOF instrument. Shown is a heat map of peak intensities for each analyzed sample in the 1500–5000 m/z range. **B.** Aliquots from the three EPS urine pools were incubated with the indicated agarose-bound lectin bead overnight. Proteins bound to the indicated lectins were separated on a 12% Tris-Bis SDS-gel, transferred to nitrocellulose and used for Western blot with anti-PSA and anti-PAP antibodies.

B

Figure 2. MALDI-TOF Profiling of Permethylated Glycans from Different Prostatic Secretion Samples

Protein from three sample types, EPS urine pools, EPS urine exosomes, and Direct EPS pools were treated with PNGaseF, and the released glycan isolated for rapid permethylation as described in the Experimental description. An aliquot was spotted on an AnchorChip target plate and analyzed by linear MALDI-TOF on a Solarix FT-ICR MALDI instrument. Shown are representative spectra for each sample, and corresponding peak data for each sample is provided in Supplementary Table 2. **A.** EPS urine pools from non-cancer, indolent cancer and aggressive cancer samples; **B.** EPS urine pools from non-cancer, indolent cancer and aggressive cancer samples; **C.** Direct EPS pooled samples representing indolent cancer, Gleason 7 cancer with no evidence of disease (NED) in three years or greater post-prostatectomy, Gleason 7 cancer with biochemical recurrence within 6 months of prostatectomy, and aggressive prostate cancers. Additional clinical data for these sample pools is provided in Supplementary Table 1.

Figure 3. Increased levels of biantennary glycan with a bisecting N-acetyl-glucosamine (A2G2B) are associated with EPS fluid from aggressive prostate cancer

A. Desialyated 2-AB labeled glycans from EPS fluids separated by normal phase HPLC. **A)** non cancer EPS urine pool; **B)** indolent cancer EPS urine pool; **C)** aggressive cancer EPS urine pool. The major peaks that are altered are indicated. These include simple biantennary glycan (A2G2), a biantennary glycan with a bisecting N-acetyl-glucosamine (A2G2B), a core fucosylated biantennary glycan (FcA2G2), and a core fucosylated biantennary glycan a bisecting N-acetyl-glucosamine (FcA2G2B). **B.** N-linked glycan associated with EPS fluid after digestion with *Arthrobacter ureafaciens* Sialidase and bovine kidney fucosidase from **A)** non cancer EPS urine pool; **B)** indolent cancer EPS urine pool; **C)** EPS fluid from patients with aggressive prostate cancer. Digestion with these enzymes highlights the shift from primarily bi-antennary glycan to bisected biantennary glycan in aggressive prostate

cancer. For chromatogram, A2G2, biantennary glycan; A2BG2 biantennary glycan with a bisecting N-acetyl-glucosamine; A3G3, triantennary glycan.

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Figure 4. Enhanced product ion Scan for the PAP glycopeptide FLNESYK Glycopeptide with a parent m/z of M4H+ 1028.48. The MS² show characteristic oxonium ions for NANA, Hex-HexNAc and other internal sugar and peptide fragments that were used to identify the both the peptide and conjugated glycan.

Table 1

Glycopeptides from the three glycosites in prostatic acid phosphatase (PAP) were analyzed by triple quadrupole mass spectrometry. The generated MS/MS data were used in database searches using SimGlycan™. The identified glycan structures are illustrated below. Cartoon representations are as follows: ■ =GlcNAc, ● =Mannose, ● =Galactose, ▲ =Fucose, ◆ =NeuAc, ◇ = NeuGc

ASN-62(94)	ASN-188(220)	ASN-301(333)