RESEARCH ARTICLE

Separation of kallikrein 6 glycoprotein subpopulations in biological fluids by anion-exchange chromatography coupled to ELISA and identification by mass spectrometry

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Kallikrein 6 (KLK6) has been shown to be aberrantly glycosylated in ovarian cancer. Here, we report a novel HPLC anion exchange method, coupled to a KLK6-specific ELISA, capable of differentiating KLK6 glycoform subgroups in biological fluids. Biological fluids were fractionated using anion exchange and resulting fractions were analyzed for KLK6 content by ELISA producing a four-peak elution profile. Using this assay, the KLK6 elution profile and distribution across peaks of a set ($n = 7$) of ovarian cancer patient matched serum and ascites fluid samples was found to be different than the profile of serum and cerebrospinal fluid (CSF) of normal individuals ($n = 7$). Glycosylation patterns of recombinant KLK6 (rKLK6) were characterized using tandem mass spectrometry (MS/MS), and found to consist of a highly heterogeneous KLK6 population. This protein was found to contain all of the four diagnostic KLK6 peaks present in the previously assayed biological fluids. The rKLK6 glycoform composition of each peak was assessed by lectin affinity and MS/MS based glycopeptide quantification by product ion monitoring. The combined results showed an increase in terminal alpha 2–6 linked sialic acid in the N-glycans found on KLK6 from ovarian cancer serum and ascites, as opposed to CSF and serum of normal individuals.

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1 Introduction

Ovarian cancer is the worldwide leading cause of death among common gynecological malignancies with more than 200 000 new cases and 125 000 deaths every year [1, 2]. Such a high mortality rate (4.2% of all cancer deaths among women) can be attributed to the lack of symptoms in incipient disease resulting in only 25% of early stage ovarian cancer being diagnosed [1–4]. Early stage diagnosis allows for a 90% 5-year survival rate among patients, but current means of disease detection by ultrasonography and/or the serum biomarker CA125 (MUC16) have shown only modest success as early screening tools [4–9].

Aberrancies in protein glycosylation patterns have been observed in a majority of cancers and over the past four decades a number of different protein glycoforms have been

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Abbreviations: CA125, cancer antigen 125; **CSF**, cerebrospinal fluid; **KLK6**, kallikrein 6; **rKLK6**, recombinant KLK6; **RT**, room temperature; **SNA**, *Sambucus nigra* agglutinin

identified as tumor-associated antigens [8, 10]. Dysregulation of glycosylation pathways can be an early event in oncogenesis, resulting in increased glycan structures on the surface of tumor cells, aiding them in evading the immune response during invasion and metastasis [10–19]. Several glycoproteins of varying biological functions and sites of expression have been shown to have disturbed glycosylation patterns in ovarian cancer [8]. These include a number of acute phase proteins, CA125, and IgGs [8, 20–23]. More specifically, the addition and processing of sialic acids on glycoproteins seems to be disturbed in ovarian cancer [8, 24]. This is supported by evidence showing altered sialyltransferase enzyme activity and mRNA expression in ovarian cancer and other gynecological tumors [25–28]. Not surprisingly, overexpression of sialyl Lewis X and sialyl-Tn antigens has been recorded in this disease [22, 29, 30].

Kallikrein 6 (KLK6) is a secreted trypsin-like member of the human tissue kallikrein family of serine proteases. Although it is expressed in a number of tissues in the body, the major site of KLK6 expression is the central nervous system with high levels of the protein (mg/L) detected in cerebrospinal fluid (CSF), making it the major source of KLK6 in serum [31–33]. Increased levels of KLK6 and five other members of the kallikrein family have been shown to be prognostic of negative outcome in ovarian cancer [34–39]. The majority of ovarian tumors produce KLK6, which is believed to enter the circulation as the cancer progresses [31–33]. However, measurement of KLK6 levels alone has not shown any improvement over CA125 in detection of ovarian cancer, and when used in combination, these two tests show only a small improvement in sensitivity [39]. In the early stages of ovarian cancer, the contribution of KLK6 from tumor tissue is not sufficient to raise the total serum levels of this protein above the defined normal diagnostic range. Therefore, a method capable of detecting tumor-derived KLK6 could improve the diagnostic value of this molecule. The N-glycosylation patterns of KLK6 immunoisolated from CSF of normal individuals and ascites fluid of ovarian cancer patients have been elucidated [40]. Through a combination of molecular biology, lectin affinity, and mass spectrometry techniques, KLK6 from ascites fluid was found to be highly branched and enriched with terminal alpha2–6 galactose linked sialic acid glycans when compared to the protein from CSF [40]. However, these techniques were not sensitive enough to characterize or quantify glycoforms of KLK6 in serum due to the low levels of protein present in this fluid (<5 ng/mL in normal individuals).

Here, we report the development of a KLK6 ELISA-coupled anion exchange method, which can distinguish between KLK6 glycoform subpopulations at physiologically relevant levels in biological fluids, including serum. Namely, high-performance anion exchange liquid chromatography was employed to fractionate directly injected samples. KLK6 in the resulting fractions was quantified using an in-house developed ELISA, resulting in an elution profile composed of four distinct peaks. Using this methodology, the KLK6 elution profiles of matched ovarian cancer patient sera and asictes fluids were found to be different from serum and CSF of healthy subjects. As well, electrospray ionization (ESI)-Orbitrap tandem mass spectrometry (MS/MS) was used to characterize recombinant KLK6 (rKLK6) purified from an immortalized human cell line. This preparation was found to consist of a highly heterogenous KLK6 population, which encompassed the majority of KLK6 glycoforms previously detected in protein isolated from ascites fluid and CSF [40]. When subjected to anion exchange, the elution profile of rKLK6 was found to contain all of the four diagnostic peaks observed in assayed biological fluids. The glycoform composition of each of the rKLK6 peaks was analyzed with Elderberry (*Sambucus nigra*, SNA) lectin affinity and relative glycopeptide quantification by product ion monitoring with mass spectrometry [41].

2 Materials and methods

2.1 Clinical samples

The biological fluids used were collected with informed consent and institutional review board approval, or leftovers submitted for routine medical testing. They were stored at –80°C until use. CSF samples were chosen to be clear in appearance to ensure no blood contamination, and ranged in KLK6 concentration from 44 to 280 ng/mL. Ovarian cancer serum samples and matched ascites fluids were from patients with FIGO stage III and IV serous ovarian carcinomas. The total KLK6 values ranged from 2.6 to 30 ng/mL for the ovarian cancer sera, and from 40 to 355 ng/mL for the ascites fluids. Normal control sera analyzed were from women in the age range between 37 and 66, with KLK6 values ranging from 1 to 2.6 ng/mL. Serum samples from patients with renal failure ranged in total KLK6 from 2.0 to 7.3 ng/mL.

2.2 Recombinant KLK6 production

Full-length glycosylated recombinant KLK6 (rKLK6) was purified from the serum-free medium of human embryonic kidney (HEK-293) cells transfected with the inactive zymogen form of KLK6, as described previously [42–44]. Briefly, the supernatant medium was collected and concentrated 10-fold using a 10-kDa cutoff nitrocellulosse membrane with the Centricon ultrafiltration device (Millipore, Waltham, MA, USA). The rKLK6 protein from the concentrated medium was purified using a cation exchange chromatography column (5-mL HiTrap CM FF column, GE Healthcare Bio-Sciences, Uppsala, Sweden) connected to the AKTA FPLC system (GE Healthcare Bio-Sciences). Liquid chromatography was performed using 50 mM sodium acetate, pH 5.3 as the running buffer over a 0–1M NaCl linear gradient. The resulting

fractions were analyzed for the presence and purity of KLK6 by SDS-PAGE and ELISA.

2.3 Anion exchange methodology

A Mono Q 4.6/100 PE Tricorn high performance column (GE Healthcare) connected to an Agilent 1100 series HPLC system was used for anion exchange chromatography of the selected biological fluids. A solution of 20 mM Tris-HCl (pH 8.6) was used as running buffer, and the elution buffer contained 1 M NaCl. Samples (100 μL) were injected directly into the column and the protein was allowed to bind to the column for 5 min at a 0.5 mL/min flow rate. The same flow rate was maintained throughout. The elution buffer was brought to 8% (80 mM NaCl) over the next 5 min. This concentration of elution buffer was maintained for another 15 min, then increased to 9% (90 mM NaCl) where it was maintained for another 15 min. Following this, another step up in NaCl concentration was made to 25% (250 mM NaCl) which was kept for another 15 min. Subsequent 15 min washing (100% NaCl) and re-equilibration (running buffer alone) steps followed. Fractions (500 μL) were collected every minute into glass vials containing 100 μL of a 6% BSA solution. 200 μL of each fraction were analyzed in duplicate with a previously described in-house KLK6 ELISA [45]. Peak areas of the resulting KLK6 elution chromatograms were integrated using OriginPro 8.0 software (OriginLab, Northampton, MA, USA) by manually selecting peak centers and using automatically provided parameters. One hundred microliters of undiluted serum samples, 100 μL of CSF- or ascites-spiked KLK6 immunodepleted serum (to final concentration of 10 ng/mL of KLK6), and 100 μL of 2 mg/mL solution of rKLK6 were used for anion-exchange chromatography.

2.4 Lectin detection

rKLK6 from chromatographic peaks was diluted in 100 μL of 50 mM Tris-HCl, pH 7.8 at different concentrations and coated overnight at room temperature (RT) on a 96 well polystyrene microtiter plate (Greiner Bio-One, Monroe, NC, USA). The plate was washed twice in wash buffer (10 mmol/L Tris-HCl, pH 7.4, containing 150 mmol/L NaCl and 0.5mL /L Tween 20) and blocked for 1 h at RT with 1× CarboFree solution (Vector Labs, Burlingame, CA, USA). The plate was washed six times with wash buffer and 500 ng of biotinylated *Sambucus nigra* agglutinin (SNA, Vector Labs) in 100 μL of 1× CarboFree buffer was added to each well and incubated at RT for 1 h. Following another six wash steps, 100 μL of 50 ng/mL alkaline phosphatase-conjugated streptavidin in 1× CarboFree solution was added to each well, incubated with shaking for 15 min, and washed six times. For 10 min, each well was incubated with 100 μL of diflunisal phosphate solution (0.1 mol/L Tris-HCl, pH 9.1, containing 1 mmol/L diflunisal phosphate, 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$). One hundred microliters of developing solution (1 mmol/L Tris, 0.4 mol/L NaOH, 2 mmol/L TbCl3, and 3 mmol/L EDTA) was then added and left on the shaker for 1 min. The PerkinElmer EnVision 2103 Multilabel Reader was used to measure the fluorescence signal in each well, in time-resolved mode.

2.5 Sample preparation for mass spectrometry

Purified or anion exchange-fractionated rKLK6 (1–5 μg) was run on a pre-cast NuPAGE 4–12% 10 well Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). As per the manufacturer's protocol, the gel was exposed to SimplyBlue SafeStain (Invitrogen) and destained in water. The excised KLK6 bands were dehydrated for 10 min with ACN at RT and reduced in 150 μL of a solution containing 10 mM DTT and 50 mM NH_4HCO_3 for 30 min at 60°C and cooled to RT for 10 min. The gel band was dehydrated with ACN and rehydrated with 50 mM NH_4HCO_3 another three times. The final rehydration was performed in 300 μL of 50 mM NH_4HCO_3 with 100 mM iodoacetamide, followed by incubation at RT in the dark for 1 h. After another three dehydration steps with ACN the gel band rehydrated in 50 mM NH_4HCO_3 with 1 μg of sequencing grade modified trypsin (Promega, Madison, WI, USA) and left overnight at 37°C. The resulting solution was used for MS/MS analysis.

2.6 MS/MS glycopeptide structure identification

KLK6 tryptic peptides were subjected to MS/MS analysis as previously described [40]. Following liquid chromatography with a 2 cm C18 precolumn (200 μm diameter) and a 5 cm resolving analytical C18 column (75 μm diameter) with a 15 mm tip (New Objective, Woburn, MA, USA), the eluted peptides were injected with a nanoelectrospray ionization source (Proxeon, ThermoFisher Scientific, Waltham, MA, USA) into a Thermo LTQ Orbitrap XL mass spectrometer set to positive-ion mode. Liquid chromatography was performed over a 90 min linear gradient using a running buffer containing 0.1% formic acid, 5% ACN, and 0.02% TFA in water and elution buffer 90% ACN, 0.1% formic acid, and 0.02% TFA in water. Parent ion frgamentation conditions were set to reject 1+, 2+, and unassigned charge states and only the peptides in the 1000 to 1800 m/z range were chosen. Peptides were fragmented in HCD mode (17, 24, and 30% normalized collision energy) and in CID mode using 35% normalized collision energy. The isolation width was set to 3.0 for all data dependent events. The retention time of the glycopeptides associated with the DCSANTTSCHILGWGK seqence was determined by observing the common oxonium ions (i.e. 204.08 for N-acetylglucosamine or 366.13 for a

hexose linked N-acetylglucosamine) in MS2 spectra. Xcalibur 2.0 software (ThermoFisher Scientific) was used to combine MS1 spectra over the glycopeptide specific retention time period and visually selected monoisotopic masses of triply charged ions were referenced against the Glycomod tool at 5 ppm mass tolerance. To further confirm the identity of the glycopeptides, MS2 data was examined for the presence of fragment glycopeptides and glycan in a majority of cases.

2.7 Glycopeptide product ion monitoring

Relative quantification of rKLK6 glycopeptides was performed by product ion monitoring methodology [41]. In brief, several major DCSANTTSCHILGWGK peptide-based glycopeptides from the rKLK6 protein in different chromatographic peaks were subjected to LC-MS/MS analysis in CID mode with 35% collision energy on the LTQ-Orbitrap XL instrument. All of the parent masses chosen were of triply charged glycopeptides previously identified (i.e. 1152.49, 1352.55, 1454.91, 1673.65). For each fragmented parent ion, at least three daughter ions (transitions) were monitored and quantified in MS2. For the 1152.49 parent mass, the 1524.85, 1545.35, and 1626.39 transitions were monitored. Transitions 1255.43, 1525.12, 1606.00, 1679.05, and 1699.75 were monitored for the 1352.55 parent mass. For the 1454.91 parent ion, 1357.76, 1524.87, 1707.53, and 1853.10 daughter ions were monitored, while 1270.80, 1343.30, 1526.26, 1606.85, and 1653.73 were monitored for the 1673.65 parent m/z. The instrument setup and in line liquid chromatography were performed in the same fashion as described above. Additionally, each MS/MS run also included the monitoring of the triply charged LSELIQPLPLER nonglycosylated KLK6 tryptic peptide (parent mass of 704.44 with 684.40, 724.49, 852.53 transitions) as an indicator of the total KLK6 quantity. The MS2 transitions were quantitated using Xcalibur 2.0 software (Thermo) by peak area integration using boxcar type smoothing over seven points.

3 Results

3.1 Anion-exchange chromatography of biological samples

When biological fluids were subjected to anion-exchange chromatography separation, four distinct peaks were observed when plotting KLK6 concentration (determined by ELISA) against elution time, over the course of the described method. Based on their retention time, from earliest to latest, these peaks were designated as A, B, C, and D (Fig. 1A). In relation to each other, the peaks were found to be of varying intensities in the different fluids analyzed. To ensure that the differences in the KLK6 distribution across the chromatographic peaks was not due to matrix and background effects, CSF and ascites fluid samples were spiked (to a final concentration of 10 ng/mL) into a serum pool of normal individuals, which was immunodepleted of KLK6 (serum concentration <0.1 ng/mL). CSF-spiked sera and sera from patients with no ovarian malignancy had similar distribution of KLK6 accross the peaks between different samples. Namely, intermediate peaks B and C were of low (and sometimes undetectable) intensity while the majority of KLK6 was found in peaks A and D, with peak A consistently showing a higher content of KLK6, on average. When serum and ascites samples from ovarian cancer patients were analyzed, no such consistency was observed. In these samples, there was a relative increase in the intensities of the last three peaks (B, C, and D). However, between different patient samples, the increase in these peaks was variable, with different peak(s) being up-regulated in different patients. Considering the expected microheterogeneity of posttranslational modifications between individual cancer cells or patient subpopulations, these results are not surprising. As well, additional samples were included in the nonmalignant group from patients with renal failure and with increased levels of serum KLK6 due to lack of clearance, but with no diagnosis of ovarian cancer. This group of samples showed the same pattern as the normal set of sera, indicating that the pattern of distribution of KLK6 across the observed peaks is not a function of the absolute levels of KLK6 in serum, but rather their origin (i.e. malignant vs. nonmalignant conditions).

These observations can be summarized by monitoring the area of peak A as a percentage of the total area under the KLK6 elution chromatogram (Fig. 1B). For CSFs, sera of healthy individuals and renal failure patients the mean and median average values were between 50% and 55%, indicating that the majority of the KLK6 in serum of normal individuals have the same (or closely similar) glycosylation pattern as the one found in the CSF. Ovarian cancer ascites fluid KLK6 exhibited an elution pattern where the KLK6 glycoforms found in Peak A were minimally represented (compared to CSF) for all of the samples analyzed. The area of peak A was at less than 10% of total. Analysis of sera collected from the same patients had a more diverse distribution but the representation of peak A was significantly lower than that found in normal control samples (Fig. 1B). This heterogeneity is to be expected due to the differential contribution of normal (CSF-derived) and ovarian cancer (ascites-derived) KLK6 in the circulation. The individual peak areas of each sample analyzed can be seen in Supporting Information Table S1.

3.2 Lectin analysis of rKLK6 peaks

Due to the limited quantity of native KLK6 protein available for study (i.e. low μg/L levels in serum and ~100 μg/L levels

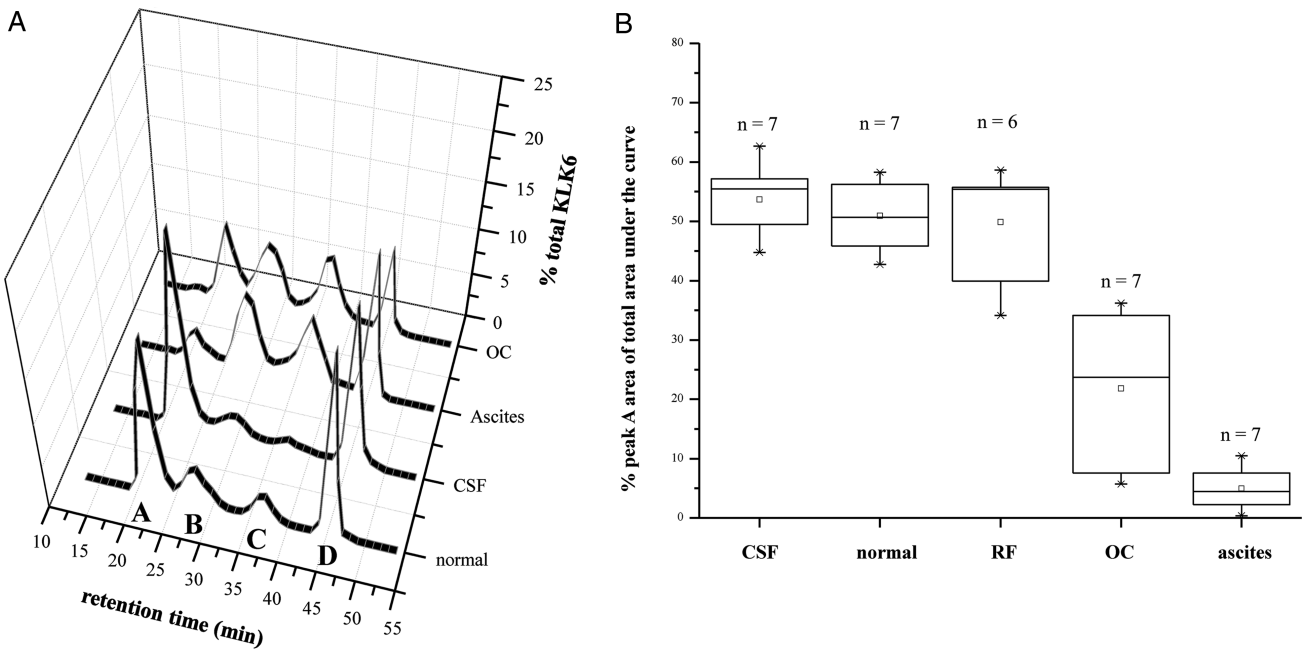

Figure 1. Anion-exchange chromatography of biological fluids. (A) ELISA quantification of KLK6 in collected fractions after elution of bound proteins from a MonoQ anion-exchange column. The value for each fraction is presented as percentage of total eluted KLK6. The presented data is from a single representative runs for a control serum of a woman with no ovarian cancer diagnosis, cerebrospinal fluid (CSF), ovarian cancer ascites fluid, and serum from a woman with ovarian cancer (OC). Peaks A, B, C, D representing glycoforms of KLK6, are discussed in the text. (B) Results of peak area integration of individual anion exchange runs for different biological fluids (RF, renal failure serum; OC, ovarian cancer serum) represented as the percentage of total area under the curve attributed to the first chromatographic KLK6 peak (Peak A). See Fig. 1 for peak identification. The top and bottom borders of the box represent 25th and 75th percentiles, respectively. The whiskers are outlier values, with the mean and median average values represented by a small box and a bisecting line, respectively. Serum from ovarian cancer patients and ascites fluid contains significantly less peak A-related KLK6 than CSF, RF, and normal serum samples. n = number of samples per category. Differences between OC and ascites groups and CSF, normal and RF groups were statistically significant ($p < 0.05$) by ANOVA test.

in CSF and ovarian cancer ascites fluid) and the difficulty associated with isolation of this protein with sufficient purity from biological fluids, the MS/MS and lectin-based characterization of the KLK6 found in the different peaks following anion-exchange chromatography was performed using purified recombinant KLK6 (rKLK6). This protein was purified from transformed HEK-293 cells stably expressing the inactive zymogen version of the protein, thereby increasing the probability of detecting similar glycosylation patterns normally found in humans and minimizing the possibility of autolytic degradation of KLK6. However, rKLK6 may not fully represent the glycan composition of KLK6 found in biological fluids.

To characterize the sialic acid content of KLK6 found in each of the four peaks, purified recombinant KLK6 was subjected to anion-exchange chromatography separation as described for biological samples and fractions were immobilized on a microtiter plate and tested for SNA lectin affinity, as described in Section 2 (Fig. 2). SNA preferentially binds alpha2–6 sialylated glycans. KLK6 eluting in Peak A was found not to bind the SNA lectin, whereas the other three peaks contained sialic acid, but at differing levels. The relative degree of sialylation increased with the retention time of each peak, with peak D containing the highest and peak B the lowest amount of sialic acid. This is not surprising, considering that an overall increase in negative charge due to more extensive glycan branching and terminal sialylation, would cause stronger binding to the anion-exchange matrix and therefore result in later elution times over an increasing salt gradient.

3.3 MS/MS analysis of rKLK6

Trypsin-digested rKLK6 was subjected to MS/MS analysis and pertinent retention times of the DCSANTTSCHILGWGK-based glycopeptides (containing the sole KLK6 N-glycosylation site; underlined) were identified by the presence of diagnostic oxonium ions (204.08 for N-acetylglucosamine or 366.13 for a hexose-

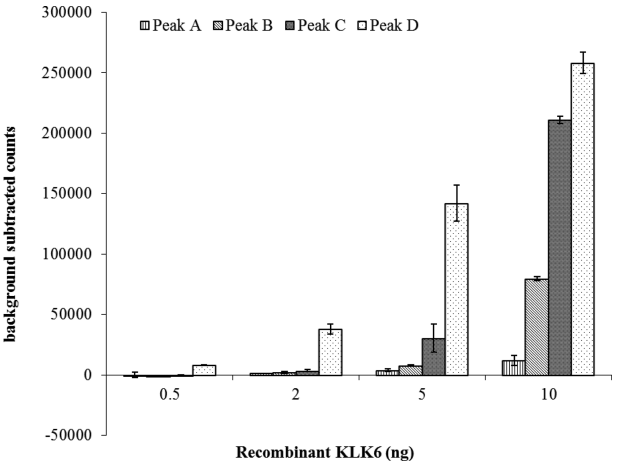

Figure 2. SNA lectin affinity to recombinant KLK6 from chromatographic peaks. The quantity of galactose linked terminal sialic acid moities on microtiter plate-immobilized recombinant KLK6 from the different chromatographic peaks as determined by SNA lectin affinity. Peaks A, B, C, and D are shown in Fig. 1. The data is presented as raw fluorescence counts subtracted from fluorescence counts recorded with no KLK6 present. The results presented are from duplicate measurements of the different concentrations of KLK6 from the four chromatographic peaks. Note that peak D contains more sialic acid, as determined by SNA lectin affinity, than the other peaks.

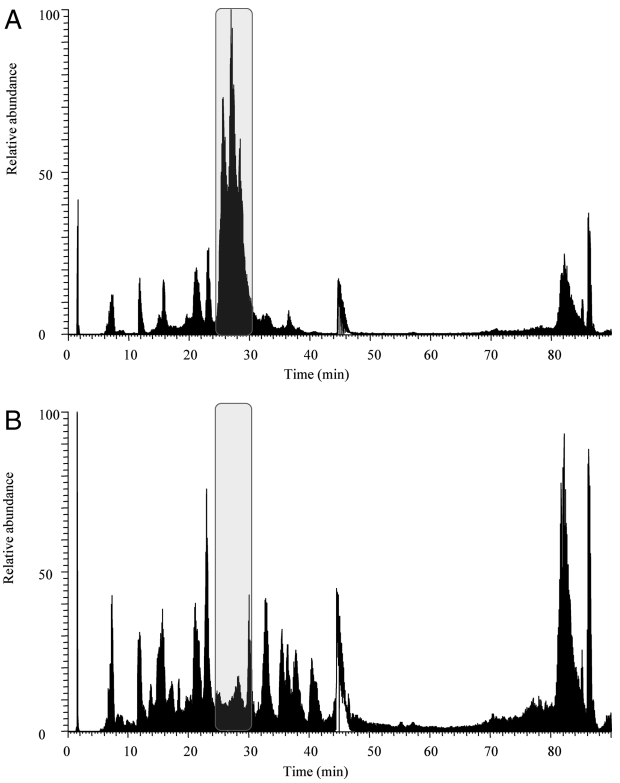

Figure 3. Total ion current chromatograms. Resulting total ion current (TIC) MS1 chromatograms of mock (A) and PNGase F treated (B) recombinant KLK6. The predicted elution period of the DCSANTTSCHILGWGK-based glycopeptides is boxed.

linked N-acetylglucosamine) in the resulting MS2 scans. The glycopeptide retention times were further determined by comparing mock and PNGase F-treated rKLK6 in the total ion current (TIC) spectra in MS1 of each run. For the PNGase F-treated sample, there was a loss of spectra in the same specific time period where the majority of oxonium ions were identified (Fig. 3). The identified glycan structures associated with the DCSANTTSCHILGWGK peptide seen in Fig. 4 exhibit great diversity. An example of the MS2 scans for one of the identified glycopeptides can be seen in Supporting Information Fig. S1. The glycan structures range from a tri-antennary core-fucosylated glycopeptide with terminal N-acetylglucosamine residues (1152.47) to tetra-antennary core-fucosylated structure with three terminal galactose-linked sialic acid residues (1673.65), encompassing a number of structures previously identified in KLK6 from CSF and ascites fluid of ovarian cancer patients [40]. What should be noted is that the absolute values for the relative abundance of the indicated masses cannot be taken as a quantitative measure, considering that glycopeptides with different glycan moieties may ionize differently, generally following the trend that ionization efficiency decreases with increased glycan branching and sialylation. The identified glycan structures in each of the anion-exchange chromatographic peaks of rKLK6 can be seen in Supporting Information Fig. S2.

3.4 Quantification of rKLK6 glycopeptides by product ion monitoring

Product ion monitoring assays were developed for several of the representative rKLK6 glycopeptides, four of which are shown to be highly enriched in each of the four diagnostic peaks (Fig. 5). These were used as indicators of the glycan content in each of the KLK6 peaks as it relates to branching extent and sialic acid presence. The selected glycopeptides were quantified relative to the amount of the LSELIQPLPLER tryptic peptide of KLK6 (amino acids 118–129 in the protein sequence), which served as an indicator of total KLK6 quantity. These data should be considered to be semiquantitative, especially considering the several orders of magnitude difference in the absolute values (area under the curve) recorded for glycopeptides and the nonglycosylated LSELIQPLPLER peptide. This effect is due to the much weaker ionization of glycopeptides when compared to unmodified peptides.

The core-fucosylated tri-antennary glycopeptide with terminal N-acetylglucosamines (1152) was shown to be mostly

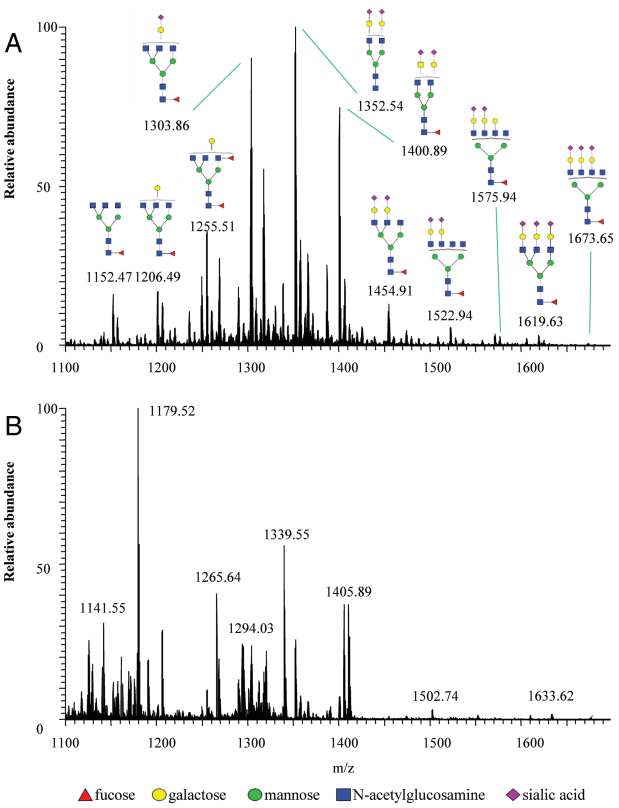

Figure 4. Composite MS1 spectra. Combined MS1 spectra over the DCSANTTSCHILGWGK glycopeptide retention time period for mock (A) and PNGaseF treated (B) recombinant KLK6. For comments see text. A heterogeneous population of glycan structures is detected, varying in sialic acid content and branching pattern. The displayed structures reflect the predicted KLK6-attached glycans by composition, not linkage.

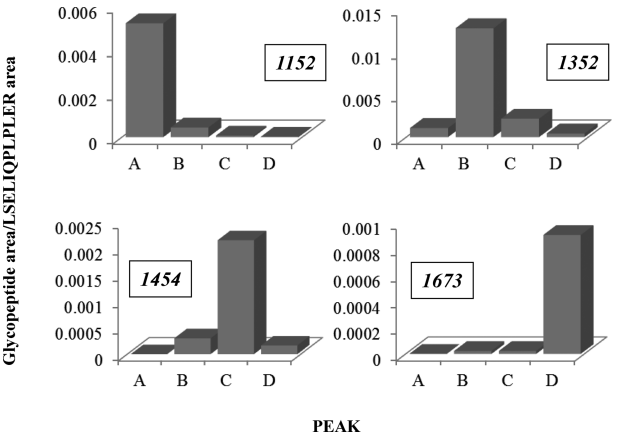

Figure 5. Glycopeptide product ion monitoring. Product ion monitoring (PIM)-based relative quantification of glycopeptides in the chromatographic peaks of recombinant KLK6. The abundance of the different glycopeptides in each KLK6 chromatographic peak is expressed as an absolute ratio to the LSELIQPLPLER nonglycosylated KLK6 peptide. One microgram of rKLK6 from each chromatographic peak was used for each MS experiment. Peaks A, B, C, and D are identified in Fig. 1A. Boxed values represent the *m*/*z* ratios of glycopeptides identified in Fig. 4.

present in peak A (Fig. 5). Peak B showed enrichment of the glycopeptide with a bi-antennary glycan structure with terminal galactose and N-acetylgalactosamine-linked terminal sialic acids (1352). The triply charged glycopeptides with *m*/*z* values of 1454 and 1673 were shown to be enriched in peaks C and D, respectively (Fig. 5). These have more extensive glycan branching with galactose-linked terminal sialic acid (Fig. 4). Therefore, it appears that with increasing retention time of KLK6 during the anion exchange method, the extent of glycan branching and sialic acid content also increases, indicating (in concert with other data presented herein) that KLK6 produced by ovarian cancer cells has preferentially higher glycan branching and sialylation.

4 Discussion

Protein glycosylation is one of the most common posttranslational modifications. The majority of secreted and membrane proteins are glycosylated. Alterations in protein glycosylation occur in a number of human disorders, including autoimmune diseases, cancer, and immunodeficiency [8]. Aberrant glycosylation patterns in cancer can present as over-, under-, or neoexpression of embryonic glycan structures, which result from changes in expression of glycosyltransferase enzymes in the classical secretory pathway [46]. One of the most common changes is the increased branching of N-glycans, which, in the presence of increased sialyltransferase expression, opens more sites for attachment of terminal sialic acids and results in increased protein sialylation [46, 47].

Ovarian cancer has the highest morbidity rate among all gynecological disorders and is the fifth leading cause of cancer deaths among women in the United States [48]. Alterations in the protein glycosylation processes are well established in ovarian cancer [8]. In the serum of ovarian cancer patients, IgG exhibits a decrease in galactosylation and several acute phase proteins, including haptoglobin, alpha1-acid glycoprotein and alpha1-antichymotrypsin, have been found to overexpress the sialyl Lewis-X antigen [22, 23]. As well, three major serum proteins (apolipoprotein B-100, fibronectin, and immunoglobulin A1) display unique glycan structures in the presence of ovarian cancer [49]. The only routinely used clinical biomarker for ovarian carcinoma is CA125, also a glycoprotein. It has only limited utility as a screening tool and is mostly used for monitoring of patient response to treatment, because its levels in serum can be elevated in other malignancies, benign conditions,

menstruation, and pregnancy [5–7, 9]. CA125 has high carbohydrate content, estimated at 24–28% of total mass, that is located at a number O- and N-glycosylation sites whose corresponding glycan structures have only been partially characterized in a non site-specific fashion, either by MS-based identification of PNGase F-released glycans or lectin affinity [20, 50]. Taking into account the innate heterogeneity of cancer, variability in site occupancy and microheterogeneity of glycans occupying each site, the prospect of fully characterizing particular glycan structures of CA125 to improve its diagnostic potential becomes an extremely difficult proposition. With its single N-glycosylation site and characterized glycan structures, and well established immunoreagents available, KLK6 has a clear advantage when considering the practicalities of developing a clinically applicable assay capable of quantitating both protein and associated glycan levels.

Some of the most well recognized clinically used cancer biomarkers are glycoproteins [51, 52]. Aberrant glycosylation patterns of some of these proteins have been elucidated when normal and disease states were compared. Measurement of the monosialylated form of alpha-fetoprotein (AFP), instead of total AFP protein levels, has been suggested to improve its diagnostic potential as a biomarker for hepatocellular carcinoma [53–55]. Prostate-specific antigen (PSA) was shown to be aberrantly glycosylated in prostate cancer [56], and measurement of alpha1,2-linked fucose on PSA has shown improvement in sensitvity and specificity over the existing test [57]. Quantification of fucosylated haptoglobin seems to be a promising avenue for diagnosing pancreatic cancer [58]. In spite of these observations, few clinically applied tests utilize the potential of the binary nature of glycoprotein biomarkers.

The majority of challenges preventing reliable, clinically applicable binary measurement of glycoprotein biomarkers are of a technical nature. More specifically, there is only a very limited set of tools capable of performing this task, each with its own set of associated limitations and difficulties [59]. Due to a large number of combinations of branched oligosaccharide structures that can be created from available monosaccharides in eukaryotic cells, and especially cancer cells, where target protein production and normal glycosylation processes are highly disturbed, the staggering glycan microheterogeneity can significantly impede precise binary measurement of glycobiomarkers. To broach the issue of KLK6 glycoform heterogeneity, towards the purpose of quantifying specific overexpressed glycoforms of the protein in ovarian cancer, we chose to resolve the subpopulations of KLK6 based on the differential charge status conferred by differences in glycosylation (i.e., anion exchange chromatography). We managed to identify four separate glycoform populations that stem from glycan variability at the single N-glycosylation site of KLK6. However, the chance of success for this approach would be significantly decreased if it is attempted with a glycoprotein containing multiple glycosylation sites. This is due to the fact that the complexity of the assay output (i.e., number of possible peaks) would likely increase exponentially with each additional glycosylation site.

The majority of high abundance proteins that account for more than 90% of protein content in serum, are glycoproteins. These include such proteins as the Ig family members, haptoglobin, antitrypsin and transferrin, among others. However, the majority of potential biomarkers are found at significantly (several orders of magnitude) lower levels in the serum [60]. Taking into consideration that a specifiic glycan profile on one protein might indicate a malignant condition, but not on another (i.e., one of the high abundance proteins) the specificity of detection by lectins, or even glycan-specifc antibodies of low concentration serum glycoproteins can be hindered by high levels of background from high abundance glycoproteins. As such, these methods of detection lag behind the gold standard (sandwich ELISA) in sensitivity, especially when taking into account that only a subset of the target protein's total population is being measured. These issues are magnified in lectin based assays, because the quality and source of lectins has been brought into question, and the extensive washing required for this type of detection causes concern when reliability and reproducibility are considered [61, 62]. As well, antibody-lectin based sandwich assays can be hindered by narrow affinity of certain lectins for specific glycan structures, which may be highly variable in cancer. Therefore, even if there is a disturbance in the glycosylation pattern of a protein, a lectin might detect only a single variant among many aberrant glycan structures. However, this issue may be ameliorated with the use of antibody arrays using multiple lectins for detection of glycan epitopes [63], but even this technique cannot detect subtle changes of a few monosaccharides in the glycan structure.

There have been a number of promising MALDI-TOF MS-based efforts at detecting glycan variability in a number of malignancies [64–67]. These approaches have been proven to be successful at detecting alterations in glycans released from individual or multiple proteins. Glycopeptide quantitation by MS also appears promising when considering approaches for the future. Nonglycosylated peptides have been measured reliably in a number biological fluids from low abundance proteins [41, 68–70]. However, the same is not true for glycopeptides and the reasons for this are twofold. First, glycopeptides ionize more weakly when compared to their nonglycosylated counterparts, which is especially true for sialic acid containing glycopeptides [71, 72]. Second, measurement of each nonglycosylated peptide remains constant for the total population of a particular glycoprotein, whereas for the equal quantity of the same glycoprotein the detection signal is divided among the different glycoform subpopulations. Attempts have been made to-

wards quantifying heterogeneous glycopeptide populations, but these approaches involved extensive sample preparation, such as lectin or hydrazide bead enrichment, not suitable for reliable and reproducible high-throughput analysis of large sets of samples [73–75]. Nonetheless, as technology advances multiple reaction monitoring (MRM) methodologies will become the tools of choice for measuring glycoproteins. Although we have had only moderate success with quantifying different glycoforms of KLK6 in a similar approach, the ability to even semiquantitatively measure the representation of several closely related glycoforms in the total KLK6 population is beyond the reach of most other detection methods.

We are aware of the limitations of the methodologies utilized within. The majority of the data reported should be considered as semiquantitative. Anion-exchange chromatography can be prone to retention time disturbances, especially when monitoring such minute changes on the single protein being monitored, causing potentially significant variability. This can affect the robustness of the methodology if careful and precise calibration is not employed. As well, the cumbersome and time consuming methodology of this approach limits its potential for use in a clinical setting, where a high number of samples need to be analyzed. Utilization of the product ion monitoring glycopeptide relative quantification methodology is also far from being applicable to analyzing complex samples such as biological fluids. The signal from the glycopeptides is approximately three orders of magnitude less than the unglycosylated peptide, even in the high quantity and purity preparation of KLK6 used in this study. Also, considering that the ovarian cancer samples used in this study were from patients with late stage disease (III and IV), it can be questioned how sensitive this approach will be when attempting to detect early stage ovarian carcinoma. Also in this feasibility study, the number of samples analyzed is small and the conclusions need to be verified with analysis of a larger dataset.

In conclusion, even with these concerns, this is the first study to indicate that aberrant glycosylation of KLK6 occurs in the serum of individuals with ovarian cancer and we developed a reliable method of measuring these changes. As such, further refinement of the analytical method may lead to the improvement of KLK6 and other cancer glycoprotein biomarkers with similar properties in their diagnostic potential.

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