

Differential *N*-glycosylation of Kallikrein 6 Derived from Ovarian Cancer Cells or the Central Nervous System*

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Ovarian cancer causes more deaths than any other gynecological disorder. Perturbed glycosylation is one of the hallmarks of this malignancy. Kallikrein 6 (KLK6) elevation in serum is a diagnostic and prognostic indicator in ovarian cancer. The majority of ovarian carcinomas express high levels of KLK6, which diffuses into the circulation. Under physiological conditions, KLK6 is expressed highly in the central nervous system and found at high levels in cerebrospinal fluid from where it enters the circulation. Our aim was to characterize and compare the *N*-glycosylation status of this protein in ovarian cancer ascites fluid and cerebrospinal fluid. Anion-exchange chromatography was used to reveal different post-translational modifications on the two isoforms. Mobility gel shift Western blot analysis coupled with glycosidase digestion showed that the molecular weight difference between the two isoforms was because of differential glycosylation patterns. The presence of a single *N*-glycosylation site on KLK6 was confirmed by site-directed mutagenesis. Using a *Sambucus nigra* agglutinin-monoclonal antibody sandwich enzyme-linked immunosorbent assay approach, it was shown that ovarian cancer-derived KLK6 was modified with α 2-6-linked sialic acid. The structure and composition of glycans of both KLK6 isoforms was elucidated by glycopeptide monitoring with electrospray ionization-Orbitrap tandem mass spectrometry. Therefore, the extensive and almost exclusive sialylation of KLK6 from ovarian cancer cells could lead to the development of an improved biomarker for the early diagnosis of ovarian carcinoma. *Molecular & Cellular Proteomics* 8:791–798, 2009.

Disturbed glycosylation patterns have been observed in the majority of human cancers. Over the past 40 years, a number of physiologically expressed proteins containing abnormal glycan structures have been shown to be tumor-associated

antigens (1). For example, prostate-specific antigen (PSA)¹ and ribonuclease 1 were found to be differentially glycosylated in prostate and pancreatic cancers, respectively (2, 3). It has been suggested that the disturbed glycosylation of proteins is an early event of oncogenic transformation, aiding in the invasion and metastasis of tumor cells (1, 4–11). As such, a selective advantage might be conferred on tumor cells with increased glycan structures, allowing them to evade immune response during the invasion and metastasis processes (12).

In ovarian cancer, a number of proteins are found to be aberrantly glycosylated, including CA125 (13), α 1-proteinase inhibitor (14), haptoglobin (14), other acute phase proteins (15), and IgGs (15). In particular, there is mounting evidence of increased sialylation of proteins and deregulated sialylation pathways in ovarian cancer (16). Altered sialylation of proteins in this disease is indicated by increased levels of the sialyl LewisX and sialyl-Tn antigens in ovarian carcinoma, even at early stages of progression (15, 17, 18). This coincides with the findings showing disrupted sialyltransferase protein expression (19–21) and altered mRNA expression of several sialyltransferases in ovarian cancer cells (22).

Human tissue kallikreins are a family of 15 secreted serine proteases with trypsin or chymotrypsin-like activities. Through the use of RT-PCR, ELISA, immunohistochemical, and bioinformatic techniques, most kallikreins have been shown to be deregulated in a number of malignancies including breast, ovarian, prostate, and testicular cancer (23–25). Elevated levels of kallikrein 6 (KLK6), a trypsin-like protease, in serum and tissue extracts have been shown to forecast for poor prognosis in ovarian cancer (26–32). KLK6 has a wide expression pattern at both the mRNA and protein levels. However, immunohistochemical and ELISA studies have shown that the major site of KLK6 expression is the central nervous system (CNS), with very high (mg/liter) levels of the protein detected in cerebrospinal fluid (CSF) (33–35). As such,

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Received, November 11, 2008

Published, MCP Papers in Press, December 16, 2008, DOI 10.1074/mcp.M800516-MCP200

¹ The abbreviations used are: PSA, prostate-specific antigen; ACN, acetonitrile; CA125, cancer antigen 125; CNS, central nervous system; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; KLK6, kallikrein 6; MES, 4-morpholineethanesulfonic acid; SNA, *Sambucus nigra* agglutinin; TBS, Tris-buffered saline; MS/MS, tandem mass spectrometry; NHS, *N*-hydroxysuccinimide; BGH, bovine growth hormone.

the major source of KLK6 in the circulation of normal individuals is the CNS.

The up-regulation of KLK6 in ovarian cancer and its unfavorable prognostic value have been well-established (33, 36, 37). It has been previously shown that virtually all ovarian tumors express KLK6, some of them at extremely high levels (33, 37). During ovarian cancer development and progression, tumor-derived KLK6 diffuses into the general circulation (33, 36). Despite these highly favorable characteristics of KLK6 as an ovarian cancer biomarker, the sensitivity of the test performed in serum (for both early and late stage disease) has been shown not to exceed that of the classical ovarian cancer biomarker, CA125 (36). The combination of KLK6 and CA125 resulted in modest increases in sensitivity (10–30% over and above CA125 alone) for both early and late stage disease (36). At early stages, the increase of serum KLK6 contributed by ovarian cancer cells is usually not sufficient to raise KLK6 above the normal serum levels. Therefore, the ability to differentiate KLK6 originating from the CNS (normally found in the serum of healthy individuals) and KLK6 originating from ovarian tumors could potentially increase the diagnostic value of KLK6 as an ovarian cancer biomarker.

Toward this purpose, the differential *N*-glycosylation patterns of KLK6 from ascites fluid of ovarian cancer patients and CSF of healthy individuals were examined. Initially, anion-exchange chromatography with the two biological fluids resulted in different elution patterns, indicative of differential post-translational modifications or processing. Different *N*-glycosylation patterns of the two isoforms of KLK6 were confirmed by glycosidase digestion followed by gel shift mobility assays. Additionally, the presence of sialylation on the two isoforms was determined by lectin-antibody sandwich ELISA methodology. The composition and structure of the glycans present on the two subpopulations of KLK6 were elucidated by monitoring KLK6 glycopeptides by electrospray ionization-Orbitrap tandem mass spectrometry (MS/MS). Our main finding is that KLK6 from ovarian cancer ascites (but not CSF) is extensively sialylated. This difference in sialylation may be exploited in the future for developing a specific biomarker for ovarian carcinoma.

EXPERIMENTAL PROCEDURES

Anion-exchange Chromatography—Anion-exchange chromatography was performed using a Mono Q 4.6/100 PE Tricorn high performance column (GE Healthcare) attached to an Agilent 1100 series high performance liquid chromatography system. The running buffer used was 20 mM Tris solution at pH 8.6. Biological fluids (100 μ l) were diluted 1:1 in running buffer and loaded onto the column for 5 min with a 0.5 ml/min flow rate. Maintaining the same flow rate, bound proteins were eluted with a linear gradient of increasing NaCl concentration in running buffer (0–400 mM) over the next 35 min. Fractions were collected every min. KLK6 levels in each fraction were measured using a previously described sandwich-type ELISA method (38), utilizing two mouse monoclonal antibodies.

KLK6 Immunoprecipitation with NHS-coupled Beads—Monoclonal mouse antibody against KLK6 (developed in-house; code 27-4) was

coated on the NHS-activated-Sepharose 4 Fast Flow beads as per manufacturer's instructions (1 mg of antibody per 1 ml of beads). Immunoprecipitation was performed by incubating 10 ml of biological fluid with 1 ml of NHS beads for 2 h at room temperature with slow end-to-end rotation. The beads were then washed with 20 ml of a TBS solution with 1 M urea at pH 7.5. The antibody-bound KLK6 was eluted using 10 ml of a 1 M glycine solution at a pH of 2.5. The isolated KLK6 was subjected to buffer exchange with TBS, pH 7.5, and concentrated down to 150 μ l using the Millipore Amicon Ultracel spin column with a molecular weight of 10 cutoff.

The biological fluids were leftovers of samples submitted for routine biochemical testing or collected with informed consent and institutional review board approval and stored at -80°C until use. The CSF samples were clear in appearance, without any visible blood contamination and were pools from \sim 100 male and female patients. Ovarian cancer ascites used were pools from three late stage ovarian cancer patients.

Site-directed Mutagenesis, Mutant Expression, and Purification—A C-terminally his-tagged KLK6 genomic clone construct in a pcDNA5/FRT/V5-HIS-TOPO backbone (Invitrogen) was generously provided by Dr. Yves Courty (Faculte de Medicine, F3700 Tours, France). The asparagine residue at position 134 of KLK6 in this construct was mutated to glycine by site-directed mutagenesis using standard T7 forward and BGH reverse as terminal primers and 5'-GAC TGC TCA GCC GGC ACC ACC AGC TGC-3' and 5'-GCA GCT GGT GGT GCC GGC TGA GCA GTC-3' as mutagenic internal primers. Human embryonic kidney cells (HEK 293) at 80% confluency were transiently transfected with wild-type and mutant constructs in T175 tissue culture flasks with 70 μ g of plasmid DNA and 170 μ l of the Lipofectamine 2000 transfection reagent as per manufacturer's instructions (Invitrogen). Following transfection and a 72 h growth period, the supernatant was collected, concentrated 10-fold as described above, and the his-tagged KLK6 protein was purified using agarose-bound nickel-nitrilotriacetic acid in batch, as per the manufacturer's protocol (Qiagen).

SDS-PAGE and Western Blot Analysis—All samples were run on pre-cast NuPAGE 12% Bis-Tris gels in MES/SDS running buffer as per manufacturer's protocol (Invitrogen). The gels were run for 2 h at 200 V. The resolved proteins were transferred onto Hybond-C Extra nitrocellulose membrane (GE Healthcare) at 30 V for 1 h. Membrane blocking was performed by incubation with TBS-T (0.1 mol/liter Tris-HCl buffer (pH 7.5) containing 0.15 mol/liter NaCl and 0.1% Tween 20) supplemented with 5% nonfat dry milk for 1 h at room temperature. The membrane was then probed with anti-KLK6 polyclonal rabbit antibody (produced in-house; diluted 1:2000 in TBS-T with 5% nonfat dry milk) for 1 h at room temperature. The membrane was washed three times for 15 min with TBS-T and incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:2000 in TBS-T with 5% nonfat dry milk; Jackson ImmunoResearch) for 1 h at room temperature. Finally, the membranes were washed again as above, and the signal was detected on x-ray film using a chemiluminescent substrate (Diagnostic Products Corp.).

Glycosidase Digestion—Immunoprecipitated KLK6 was treated with *N*-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* and acetyl-neuraminyl hydrolase (Neuraminidase) from *Clostridium perfringens* as per manufacturer's instructions (New England Biolabs), where the enzymes were used in 10-fold excess for 2 h at 37 $^{\circ}\text{C}$.

Lectin ELISA Assay—*Sambucus nigra* agglutinin (SNA, Vector Labs) in 50 mM Tris-HCl, pH 7.8 was coated on a 96-well white polystyrene microtiter plate (100 μ l of 5 ng/ μ l SNA per well) by overnight incubation at room temperature and washed twice in wash buffer (10 mmol/liter Tris-HCl, pH 7.4, containing 150 mmol/liter NaCl and 0.5 ml/L Tween 20). Different dilutions of immunoprecipitated KLK6 in 100 μ l of 50 mM Tris-HCl, pH 7.8 were incubated on the plate for 2 h

at room temperature with continuous shaking followed by 6 wash steps, as described above. To detect the presence of SNA-bound KLK6, 100 μ l/well of biotinylated mouse monoclonal detection antibody E24 (50 ng) diluted in 1% bovine serum albumin were added to each well, incubated at room temperature for 1 h and washed 6 times. Subsequently, 100 μ l (5 ng) of alkaline phosphatase-conjugated streptavidin diluted in 1% bovine serum albumin was added to each well, incubated for 15 min with continuous shaking, and washed 6 times. 100 μ l of diflunisal phosphate solution (0.1 mol/liter Tris-HCl, pH 9.1, containing 1 mmol/liter diflunisal phosphate, 0.1 mol/liter NaCl, and 1 mmol/liter MgCl₂) was then added to each well, and incubated for 10 min with continuous shaking followed by the addition of 100 μ l of developing solution (1 mmol/liter Tris base, 0.4 mol/L NaOH, 2 mmol/L TbCl₃, and 3 mmol/L EDTA) to each well and mixed for 1 min. Fluorescence was measured with the PerkinElmer EnVision 2103 Multilabel Reader.

Sample Preparation for Mass Spectrometry—Immunoisolated KLK6 (1 μ g) was resolved on a pre-cast NuPAGE 12% Bis-Tris as described above. The gel was stained with SimplyBlue SafeStain (Invitrogen) and destained in water, per manufacturer's protocol. The KLK6 bands were excised from the gel and dehydrated with acetonitrile (ACN) for 10 min at room temperature. ACN was aspirated, and the bands were reduced in 300 μ l of 10 mM dithiothreitol (Sigma-Aldrich) in a 50 mM NH₄HCO₃ solution for 30 min at 60 °C and allowed to cool to room temperature for 10 min. Following the removal of the reducing solution, the reduced protein in the gel bands was alkylated by addition of 300 μ l of a 100 mM iodoacetamide in 50 mM NH₄HCO₃ solution for 1 h at 37 °C in the dark. Upon removal of the alkylating solution, the gel bands were shrunk with ACN and rehydrated with 50 mM NH₄HCO₃. This was repeated 3 times. After the last ACN dehydration step, the gel bands were resuspended in 100 μ l of 50 mM NH₄HCO₃ solution containing 1 μ g of sequencing grade modified trypsin (Promega) and left overnight at 37 °C for digestion. 40 μ l of this solution was used for each MS/MS run.

Mass Spectrometry Conditions—KLK6-derived tryptic peptides were initially bound to a 2 cm C18 pre-column with a 200 μ m diameter and eluted onto a resolving 5 cm analytical C18 column (75 μ m diameter) with a 15 mm tip (New Objective). The liquid chromatography setup was connected to a Thermo LTQ Orbitrap XL mass spectrometer with a nano-electrospray ionization source (Proxeon). Analysis of the eluted peptides was done by tandem mass spectrometry in positive-ion mode. A two buffer system was utilized where Buffer A (running) contained 0.1% formic acid, 5% ACN, and 0.02% trifluoroacetic acid in water and Buffer B (elution) contained 90% ACN, 0.1% formic acid, and 0.02% trifluoroacetic acid in water. For structure determination a parent mass list was created for the glycopeptides of interest, and each glycopeptide was fragmented with 25, 30, and 35% normalized collision energy in HCD mode and 35% normalized collision energy in CID mode. Charge state rejection was enabled to reject charge states 1+, 2+, and unassigned charge states. HCD collision energy was optimized in the calibration procedure according to manufacturer's instructions. All data-dependent scan events had isolation width set to 3.0.

MS/MS Structure Identification—The glycan structure of KLK6 was determined by MS/MS analysis of the DCSANTTTSCHILGWGK glycopeptide. The retention time of the KLK6 glycopeptides was determined by observing the presence of common diagnostic oxonium ions in MS2 spectra (*i.e.* 204.08 for *N*-acetylglucosamine or 366.13 for a hexose-linked *N*-acetylglucosamine). Once this was determined, MS1 spectra over that period of time were combined in a single spectrum using QualBrowser on Xcalibur software (Version 2.0), and individual peaks (corresponding to visually chosen monoisotopic masses of each ion) were inspected as indicators for the presence of glycosylation on the KLK6 glycopeptide. Only triply charged ions

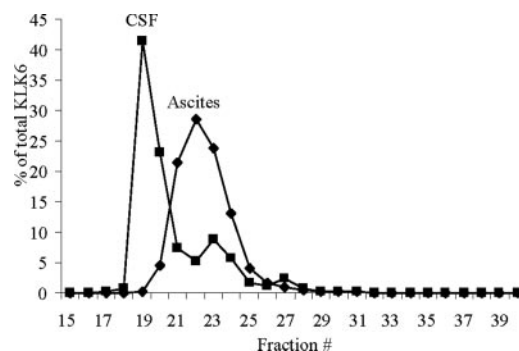


FIG. 1. Anion-exchange chromatography of biological fluids. Results of ELISA-based quantification of KLK6 in fractions collected after elution of ovarian cancer ascites fluid and CSF from a MonoQ anion-exchange column. The data for each fraction is presented as percentage of total eluted KLK6. Results are representative of the same analysis performed on three different ascites fluids and CSFs.

were inspected. Corresponding monoisotopic masses were referenced against the Glycomod tool, which provided the output of glycan composition on the given glycopeptide within 5 parts per million mass tolerances. The glycan composition allowed for the inference of the glycan structures, which were further confirmed (where available) against glycan structure databases. For most of the observed ions, analysis of MS2 data for the presence of fragment glycopeptides and glycans was used to further confirm that these were indeed the suspected molecules (data not shown).

RESULTS

Anion-exchange Chromatography—Anion-exchange chromatography was used to examine the differential elution patterns of KLK6 from CSF and ovarian cancer ascites fluid. Following the chromatography step, eluted fractions were analyzed for the presence of KLK6 by sandwich ELISA methodology. The elution patterns for KLK6 from CSF and ascites samples were distinctly different (Fig. 1). These patterns were consistent when CSF and ascites fluids from different subjects were used. These results suggested that the ascites form of KLK6 have an overall higher negative charge, suggesting presence of more complex glycosylation.

Western Blot Analysis, Glycosidase Treatment, and Site-directed Mutagenesis—To determine differences in molecular masses between the KLK6 from the two different biological fluids, SDS-PAGE followed by Western blot analysis was performed on KLK6 from three ovarian cancer ascites fluids and three CSFs from different female subjects. In all cases, KLK6 from ascites fluid had a higher molecular mass than KLK6 from the CSF, further suggesting a differential pattern of post-translational modifications between the two KLK6 isoforms (Fig. 2A). The smearing of the ascites-derived KLK6 bands raises the possibility of microheterogeneity of the protein present in this fluid.

To confirm that the molecular mass differences between the two isoforms of KLK6 were because of differential glycosylation, immunoisolated KLK6 from pools of ascites fluids and CSFs was treated with PNGase F (removes all *N*-glycans

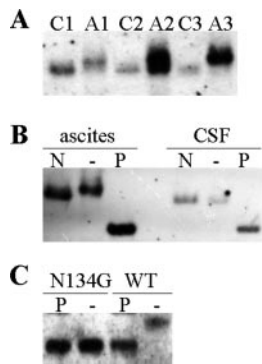


FIG. 2. **KLK6 Western blot Analysis.** A, Western blot of 10 μ l of three different ascites fluids from ovarian cancer patients (A1–3) and three CSFs from women (C1–3). B, immunisolated KLK6 from pools of ovarian cancer ascites fluids and CSFs following mock (–), neuraminidase (N), and PNGase treatment (P). C, purified KLK6 from supernatant of HEK 293 cells transiently transfected with wild-type (WT) and N134G KLK6 constructs following mock (–) and PNGase F (P) treatment. For more details, see under “Results”.

at asparagine residues) and neuraminidase (catalyzes hydrolysis of α 2-3, α 2-6, and α 2-8-linked sialic acid residues), and analyzed for changes in gel mobility by Western blot. Treatment with PNGase F resulted in a shift of both the ascites and CSF forms of the protein to the same molecular mass, suggesting that the initial molecular mass differences between these two KLK6 isoforms were because of their differential N-glycosylation patterns (Fig. 2B). Neuraminidase treatment resulted in a shift to a lower molecular mass of the ascites form, but not the CSF form. The shift resulted in a molecular mass between the fully glycosylated and completely deglycosylated (as produced by PNGase F treatment) forms of KLK6 (Fig. 2B). These results suggested presence of terminal sialic acid residues on ascites-derived KLK6 but not on the CSF-derived KLK6.

KLK6 was further shown to contain only one site of N-glycosylation at residue Asn-134 by site-directed mutagenesis and transient expression in HEK 293 cells. The N134G mutant KLK6 displayed a lower molecular mass than the wild-type protein (Fig. 1C). As well, upon treatment with PNGase F there was no molecular mass shift for the mutant KLK6, whereas the wild-type protein showed a drop to the apparent molecular mass of the unglycosylated mutant protein. These results confirm in human cells similar findings reported previously in the *Pichia pastoris* expression system (39).

Lectin-antibody Sandwich ELISA—The presence of α 2-6-linked sialic acid on KLK6 glycoisoforms was further confirmed using a lectin-antibody sandwich ELISA method. Identical and increasing concentrations of immunisolated KLK6 (confirmed by a total KLK6 ELISA) from ascites fluid and CSF were assayed for the presence of α 2-6-linked sialic acid by capturing any sialylated protein with immobilized SNA and detecting specifically the KLK6 moiety with a monoclonal KLK6 antibody. Signal intensity was corrected for background by subtracting the average signal from 12 repeats from wells

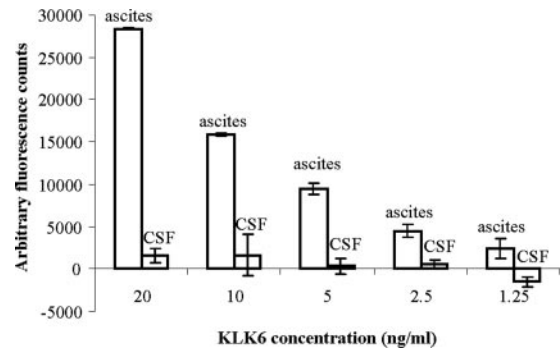


FIG. 3. **SNA-antibody lectin ELISA.** Measurement of α 2-6-linked sialic acid on increasing concentrations of immunisolated KLK6 from pools of ovarian cancer ascites fluid and CSFs using SNA lectin-monooclonal mouse antibody ELISA methodology. Signal is expressed as raw fluorescence counts. For each protein concentration, analysis was repeated 3 times and mean with standard deviation error bars is presented.

without added KLK6. Only the glycoisoform of KLK6 from ovarian cancer ascites fluid showed a concentration-dependent increase in signal that was above background noise (Fig. 3), further confirming the presence, and absence, of sialic acid on KLK6 from ovarian cancer ascites and CSF, respectively. Similar results were obtained with the reverse approach, where a KLK6-specific antibody was used for capture, and biotinylated SNA was used for detection (data not shown). Related experiments where SNA was substituted with *Maackia amurensis* Lectin II (binds sialic acid in an α -2,3 linkage) showed no signal above background for either glycoisoform of KLK6 (data not shown).

Structure Characterization by Tandem Mass Spectrometry—Consistent with results described above, the glycan structures present on KLK6 derived from ovarian cancer ascites fluid were shown to be highly heterogeneous and almost exclusively sialylated, save for one identified non-sialylated glycopeptide (Fig. 4A). The majority of the identified structures were core-fucosylated bi-, tri-, or tetra-antennary glycans with a varying number of terminal galactose-linked sialic acids. Two exceptions were observed; the ion at m/z 1192.48 lacked a terminal sialic acid residue and the ion at m/z 1400.22 contained a terminal sialic acid linked to an N-acetylglucosamine directly, instead through galactose. Conversely, a single major peak at m/z 1152.14 was observed for KLK6 from CSF, corresponding to a tri-antennary core-fucosylated glycopeptide (Fig. 4B). The minor peaks identified were indicative of tri- and tetra-antennary structures heterogeneous in respect to core fucosylation and terminal fucosylation and galactosylation. Two minor peaks (at m/z 1303.19 and 1370.88) were found to contain terminal sialic residues. However, although present, these sialylated forms of the protein would account for a relatively very small proportion of the total KLK6 present in the CSF, as indicated by the other presented data.

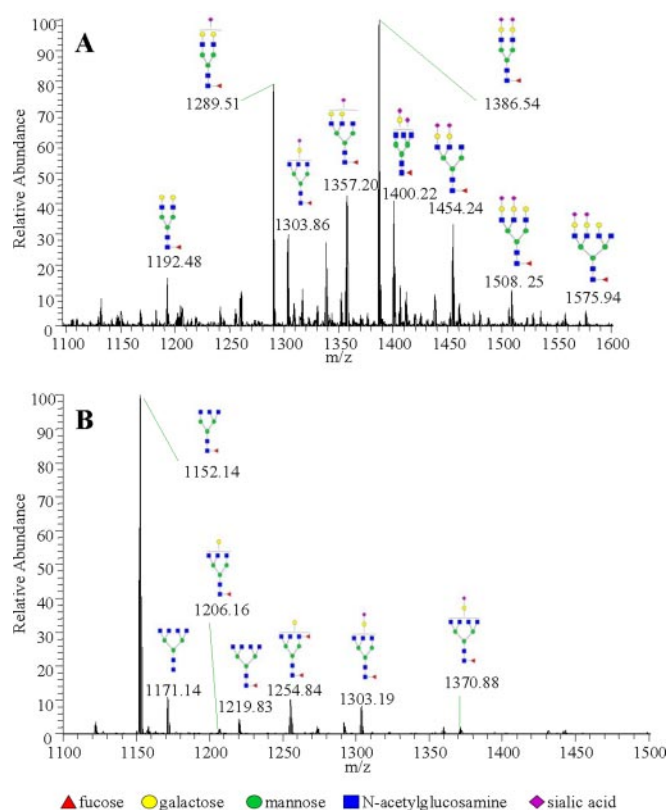


FIG. 4. **Mass spectra.** Analysis of the DCSANTTSCHILGWGK tryptic glycopeptide of KLK6 isolated from ovarian cancer ascites (A) and CSF (B) by electrospray ionization-Orbitrap mass spectrometry. The *m/z* values presented are visually chosen monoisotopic masses used for glycan composition determination.

DISCUSSION

The utility of protein glycosylation for diagnostic purposes in cancer has been reported previously and is widely studied. The diagnostic potential of alpha-fetoprotein (AFP) for detection of hepatocellular carcinoma was improved by the specific measurement of its monosialylated form (40–42). The glycosylation patterns of pancreatic ribonuclease were used to differentiate the protein from normal pancreatic tissue and cancer cells (3). The core fucosylation status of serum haptoglobin glycoforms can be used to differentiate between pancreatic cancer and chronic pancreatitis (43). Serum $\alpha(1)$ -acid glycoprotein *N*-glycosylation patterns in conjunction with linear discriminant analysis were recently used to differentiate between normal, lymphoma, and ovarian cancer cases (44).

Ovarian cancer is the fifth most common cause of all cancer deaths among women in the United States and has the highest morbidity rate among gynecological malignancies (45). This is mainly due to the fact that this cancer cannot be detected at early stages. Detection of early disease can dramatically improve the long-term survival of patients afflicted with ovarian cancer. Deregulation of glycosylation pathways is one of the hallmarks of cancer, including cancer of the ovary. Culture media from several ovarian cancer cell lines

and serum from ovarian cancer patients were used to identify a number of unique oligosaccharides corresponding to glycoproteins shed from tumor cells (46). A recent study concentrated on the glycoproteomic analysis of three major serum proteins (apolipoprotein B-100, fibronectin, and immunoglobulin A1) and found them all to be aberrantly glycosylated in ovarian cancer patients (47). Acute-phase proteins and IgG were also shown to have increased core fucosylation and sialylation in serum of advanced ovarian cancer patients (15). Therefore, it is not surprising that similar modifications were found in tumor-derived KLK6.

To date, there is only one validated ovarian cancer biomarker, CA125, a large glycoprotein the expression level of which is elevated in ovarian cancer. This protein is essential for monitoring the response of patients to treatment but has shown less promise as a screening tool because it can be elevated in a number of other malignancies and benign conditions, as well as during menstruation and pregnancy (48–51). In the case of CA125, there has been only limited interest in the study of the *N*-glycosylation patterns of this protein, which were compared using non-malignant and tumor sources. The glycan structures of CA125 isolated from the OVCAR3 cell line were studied in detail by a multiplexed approach of molecular biology and mass spectrometry techniques (52). The predominant types of *N*-glycans were found to be bi-, tri-, and tetra-antennary bisecting oligosaccharides. However, the approach that was used involved the release of glycans from immobilized glycopeptides by PNGase F, which does not allow for site-specific assignment of *N*-glycosylation structures. Another study distinguished the CA125 from OVCAR3 cells and amniotic fluid by multiple lectin chromatography, showing differential binding patterns to a panel of lectins, of the protein from the two different sources (13). The relative scarceness of information on CA125 glycosylation most likely stems from the fact that it is a protein with extensive *O*- and *N*-glycosylation. When the glycan structure microheterogeneity at each glycosylation site is taken into account, it becomes an increasingly difficult and tedious task to obtain consistent and clear patterns of glycan differences of CA125 from different sources, which could allow for improvement in its role as a biomarker. This is not the case with KLK6, as it contains a single *N*-glycosylation site that is modified differentially in the CNS and ovarian tumor tissue, allowing for much clearer determination of the source of the protein based on its *N*-glycosylation pattern.

Previously, several members of the kallikrein family of proteases have been associated with various forms of malignancy, including ovarian cancer (32, 53, 54). Among these, KLK6 appears to be the most promising candidate for the detection ovarian carcinoma. Considering that the majority of KLK6 in the circulation comes from a single source, the CNS, and that the vast majority of malignant ovarian tumors express it at high levels (33, 36, 37), the ability to differentiate KLK6 from these two sources could improve its

value as a diagnostic biomarker for early detection of ovarian cancer.

Excluding PSA (also known as kallikrein 3) glycan structural patterns of other members of the kallikrein family of proteases have not been studied in detail. There have been a number of reports, sometimes contradictory, on the *N*-glycosylation status of PSA under normal and prostate cancer conditions, with the purpose of improving the efficiency of PSA as a biomarker for diagnosis of prostate cancer. A variety of approaches with different sources of PSA, ranging from cultured cell lines to tissue extracts and biological fluids, were employed to differentiate PSA from non-malignant and malignant sources, based on differential *N*-glycosylation patterns. Over the last two decades a number of studies utilizing a variety of lectin affinity approaches, chromatofocusing, and two-dimensional electrophoresis have shown an increase in multiantennary complex type glycans and sialic acid content in PSA derived from prostate cancer tissue, seminal plasma, and serum from prostate cancer patients (55–58). A recent study utilizing a glycopeptide monitoring approach, similar to the one used in the present study, compared PSA from serum of prostate cancer patients and seminal plasma, suggesting that the presence of α 2-3-linked sialic acid on PSA could potentially differentiate between benign and malignant conditions (59). Despite these and other findings, a clinically applicable assay has not been developed to date. However, the potential advantage of KLK6 and the use of one of its glycoforms as a biomarker for ovarian cancer, is based on the fact that KLK6 is highly expressed in a tissue (CNS) unrelated to the ovarian tumor. This potentially further minimizes the similarity in the glycan profiles of the glycoforms and their tissue-specific microheterogeneity.

Elevated sialylation of cancer cell membranes is well established (60). It can occur due to the up-regulation of one of the sialyltransferases or more extensive branching of *N*-linked glycans, which results in more termini available for modification with sialic acid (61). Increased expression of α 2-6-linked sialic acid is usually a poor prognosticator for outcome in cancer and has been found to correlate with increased expression of the ST6GAL1 sialyltransferase gene in a number of malignant conditions (62, 63). In ovarian cancer, the expression of several sialyltransferases is shown to be deregulated at both the protein and mRNA levels (19–22). Therefore, it is not surprising that total protein-bound sialic acid is found to be increased in the circulation of ovarian cancer patients (16). As well, it has been well-established that both the sialyl LewisX and sialyl-Tn antigens are present at increased levels in malignant ovarian tumors and serum of ovarian cancer patients (15, 17, 18).

Mass spectrometry has emerged as the most powerful tool for characterization of individual protein glycosylation, surpassing the ability of classical molecular and biological techniques in the detailed delineation of glycan structures. Two major approaches, with a number of variations for each, have

arisen for studying glycosylation patterns of individual proteins. One of them involves the chemical or enzymatic cleavage of glycans from the target glycoprotein, followed by purification and MS analysis (64, 65). However, this approach is limited when multiple glycosylation sites are present on the protein because different glycan structures cannot be assigned to a specific site. As well, the degree of difficulty in the preparation and purification of the sample is increased because any contaminating glycoproteins will contribute to the identified glycan structures. This is particularly an issue when dealing with relatively small amounts of protein being isolated from complex biological fluids where even the best protein preparations will contain a significant degree of contamination. These two issues are minimized when the alternative method, glycopeptide monitoring, is utilized, as in the present study. In this approach, proteolytic glycopeptides are characterized by composition with no ambiguity regarding the localization of the inspected glycan due to the peptide portion of each glycopeptide (66–68). In addition, due to the site-specificity of this approach any contaminating glycopeptides would not impact the output of the experiment.

In the present study, a combined approach of molecular and mass spectrometry techniques was utilized to elucidate the differences in the *N*-glycosylation of KLK6 derived from CSF and ascites fluid of ovarian cancer patients. Considering the mRNA up-regulation of several sialyltransferases and the general deregulation of sialylation pathways in ovarian cancer (15–22) it was not surprising that KLK6 isolated from ascites fluid of ovarian cancer patients was found to be modified with glycan structures containing α 2-6-linked sialic acid. On the other hand, KLK6 from CSF of healthy individuals was, for the most part, lacking in sialic acid groups. These findings were supported by lectin affinity and MS/MS monitoring of the glycan structure on the single KLK6 tryptic glycopeptide.

These results could pave the way for similar studies with other members of the kallikrein family that have been reported to be up-regulated in ovarian cancer, which could potentially lead to a panel of glycoisoform-specific biomarkers. In the future, we will attempt to develop a KLK6 glycoisoform-specific quantitative assay, which can be applied to a large set of serum samples. Toward this purpose, the sialic acid content of KLK6 in serum can be measured using a lectin ELISA approach similar to the one previously used for serum transferrin (69), where an antibody was used to capture the protein, and SNA was used to detect α 2-6-linked sialic acid. As well, a “product ion monitoring” method for isoform-specific glycopeptides can be utilized (70).

In conclusion, we here report the unique patterns of *N*-glycosylation of KLK6 found in ascites fluid of ovarian cancer patients and CSF of healthy individuals. The almost exclusive presence of sialic acid moieties on KLK6 derived from ovarian cancer cells could, in the future, serve to the further development and refinement of KLK6 as an improved ovarian cancer biomarker.

Acknowledgments—We would like to thank Dr. Anne Dell for advice and helpful suggestions. For intellectual insight and discussion, we would also like to thank Ihor Batruch, Geeth Gunawardana, Dr. Vathany Kulasingam, Dr. Katerina Oikonomopoulou, Dr. Miltiadis Paliouras, and Ioannis Prassas.

* This work was supported by a collaborative research and development grant from the Natural Sciences and Engineering Research Council of Canada and Proteomic Methods Inc.

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