research articles Journal of proteome research

Profiling of Glycans in Serum for the Discovery of Potential Biomarkers for Ovarian Cancer

Hyun Joo An,[†] Suzanne Miyamoto,[‡] Katherine S. Lancaster,[†] Crystal Kirmiz,[†] Bensheng Li,[†] Kit S. Lam,[‡] Gary S. Leiserowitz,[§] and Carlito B. Lebrilla*[,†,∥]

Department of Chemistry, Department of Internal Medicine, Division of Hematology & Oncology, University of California—Davis Cancer Center, Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Biochemistry and Molecular Medicine, University of California—Davis, California 95616

Received January 11, 2006

A glycomic approach is developed to identify oligosaccharide markers for ovarian cancer by rapidly profiling globally released oligosaccharides. Glycoproteins shed by cancer cells are found in the supernatant (or conditioned media) of cultured cells. In this approach, shed glycoproteins are profiled for their oligosaccharide content using β-elimination conditions. Changes in glycosylation are monitored by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI—FTICR—MS). Because shed glycoproteins would also be found in serum, similar glycan profiling was performed to observe potential oligosaccharide markers. Oligosaccharide profiling data on a limited set of patient and normal serum samples were studied to determine potential glycan markers in ovarian cancer. We were able to demonstrate the presence of at least 15 unique serum glycan markers in all patients but absent in normal individuals. To determine the structure of the glycan biomarkers, a number of the ions were isolated and further analyzed using infrared multiphoton dissociation (IRMPD). One major advantage of this approach is that glycans are examined directly from patient sera without the need to obtain cancer biopsy specimens or to purify glycosylated proteins from these specimens.

Keywords: ovarian cancer • biomarker • oligosaccharide • glycoprotein • MALDI • mass spectrometry

Introduction

Ovarian cancer is the fifth leading cause of death from cancer and has the highest mortality rate among the gynecologic malignancies in the United States. According to the American Cancer Society (2005), approximately 22 220 women will be diagnosed this year with ovarian cancer in the United States and approximately 72% (16 210) of those diagnosed will die from this disease (www.cancer.org). Due to a lack of noticeable early symptoms, ovarian cancer is often called a "silent killer." Only after the disease has spread out of the ovary do specific ovarian cancer symptoms develop. Early diagnosis is important because ovarian cancer is very treatable when detected early. If ovarian cancer is detected before it has spread to the ovaries, 95% of women will survive longer than five years, but if diagnosed in advance stages, only 28% will survive longer than five years.[1−3]

The CA 125 assay is the only FDA approved blood test for ovarian cancer, and its primary indication is for monitoring the clinical status of patients with known ovarian cancer. It has limited use in the diagnosis of ovarian cancer due to a lack of sensitivity and specificity, especially in premenopausal women.[4] For example, only 50% of women with Stage I ovarian cancer will have an elevated CA 125.[4,5] Many benign conditions can cause elevated CA 125, including uterine fibroids, endometriosis, menses, pregnancy, and pelvic inflammatory disease. Consequently, an intense search has been underway for new tests that have improved diagnostic performance compared to CA 125.

Many of the recent efforts in developing new ovarian cancer diagnostic tests have focused on proteomics. In the past three years alone, more than 60 publications have been reported to profile the serum peptides or proteins using mass spectrometry.[6] An approach that has received considerable attention is the identification of markers based on mass spectrometry peaks produced by surface-enhanced laser desorption/ionization (SELDI) or MALDI. The spectra between cancer patients and normal individuals are compared to make a prognosis.[7,8] Proteins or perhaps peptide biomarkers are observed with little or no knowledge of the actual identity. However, sensitivity and reproducibility still remain big issues in making this technique reliable.

CA 125 is a member of the highly glycosylated mucin family of proteins. It is a surface glycoprotein that is shed into the intracellular space. Mucins found on the surface of cancer cells

* To whom correspondence should be addressed. Tel: (530) 752-6364. Fax: (530) 752-8995. E-mail: cblebrilla@ucdavis.edu.
[†] Department of Chemistry, University of California—Davis Cancer Center.
[‡] Department of Internal Medicine, Division of Hematology & Oncology, University of California—Davis Cancer Center.
[§] Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of California—Davis.
[∥] Biochemistry and Molecular Medicine, University of California—Davis.

10.1021/pr060010k CCC: $33.50
Published on Web 05/27/2006

are not only overexpressed but are also aberrantly glycosylated. Specific glycoproteins may be produced in varying rates depending on the cell type. Of the four common ovarian cell lines (Caov-3, OVCAR-3, SK-OV-3, and ES-2), only two cell lines (Caov-3 and OVCAR-3) secrete high levels of CA 125, whereas three cell lines (Caov-3, OVCAR-3, and SK−OV-3) express high levels of MUC1.[9] These results are, however, highly dependent on the monoclonal antibodies used to detect the mucins. In general, antibody detection of mucins are complicated by several factors. Mucins are highly heterogeneous glycoproteins with high molecular weight (10^7 Da) displaying variable epitopes that the antibodies recognize. For this reason, the antibody methods developed to detect the mucins are often imprecise. Furthermore, specific glycoproteins such as CA 125 may not be expressed in all forms of ovarian cancer (serous, clear cell, mucinous, and others).

As disease markers, oligosaccharides can be more easily identified and quantified. By focusing on glycosylated proteins, the number of potential oligosaccharide markers to be investigated is significantly smaller when compared to the number of potential peptide or protein biomarkers.[10] Furthermore, glycosylation is highly sensitive to the biochemical environment and has been implicated in development and disease.[11] Indeed, cancer cells produce significantly different oligosaccharides than normal cells[12−16], including a possible unique monosaccharide, such as *N*-glycolyl neuraminic acid.[17,18] This monosaccharide occurs only at very low levels (<0.1%) in normal tissues,[17] but elevated in some cancers. Glycosylation is therefore known to vary in cancer cells compared to normal ones. For this reason, cell surface carbohydrates have been suggested as prognostic markers in human carcinoma.[19] Monitoring changes in glycosylation may therefore be a more specific and sensitive method for detecting cancer than indirect methods using monoclonal antibodies. Interestingly, although CA 125 was discovered over two decades ago[5] and has been used as an ovarian cancer marker for a long time, many of its *O*- and *N*-glycan structures have only recently been characterized.[20]

In this report, we profile oligosaccharides shed by cancer cell lines in defined media and in serum. Indeed, oligosaccharides found in the supernatant of cell lines also appear in serum. In this approach, oligosaccharides are released and harvested. No protein information is obtained as the analysis is focused specifically on changes in glycosylation. This approach will be significantly simpler than monitoring the presence of the intact glycoproteins such as CA 125, which as discussed previously is complicated by several factors.

Experimental Section

Ovarian Cancer Cell Growth and Supernatant Acquisition. Caov-3, OVCAR-3, ES-2, and SK−OV3 ovarian cancer tumor cell lines were obtained from the ATCC. Caov-3 and OVCAR-3 cancer cells were cultured in RPMI1640 cell media supplemented with 10% fetal bovine serum, 100 units/mL penicillin/strepto-mycin and 1% glutamine. ES-2 and SK-OV-3 cells were grown in McCoy's media. Conditioned media (CM) was removed from the cells during log (nonconfluent) or death (confluent) cell growth, and frozen at −70 °C. The CM was thawed, sterile filtered (0.2 μm filter), and concentrated using Vivacell 70 or Vivaspin 20 concentrators (VivaScience, Edgewood, NY). Alternatively, the filtered CM was dialyzed extensively against distilled water and then lyophilized.

Human Serum Sample. A number of serum samples from healthy individuals ($n = 5$) and patients with ovarian cancer ($n = 5$) were acquired from the UC Davis Medical Center Clinical Laboratories (IRB approved protocol). These serum samples were collected by standard venous phlebotomy into a red top Vacutainer tube (or other standard "clot-tube") used for serum chemistry. These tubes do not contain an anticoagulant like heparin. The blood is allowed to clot and the serum separated by centrifugation in a standard clinical centrifuge and the serum removed for CA 125 testing by the UC Davis Clinical Labs. After CA 125 testing (AXSYM test for CA 125, Abbott, Abbott Park, IL), serum samples were frozen and stored at −20°C. For glycan analysis, the serum samples were thawed, dialyzed (Pierce Slidealyzers, MWCO 7000−10 000) against nanopure water, frozen and lyophilized. Two milligrams of lyophilized material was weighed, transferred into a separate 15 mL polypropylene tube for the β-elimination reaction.

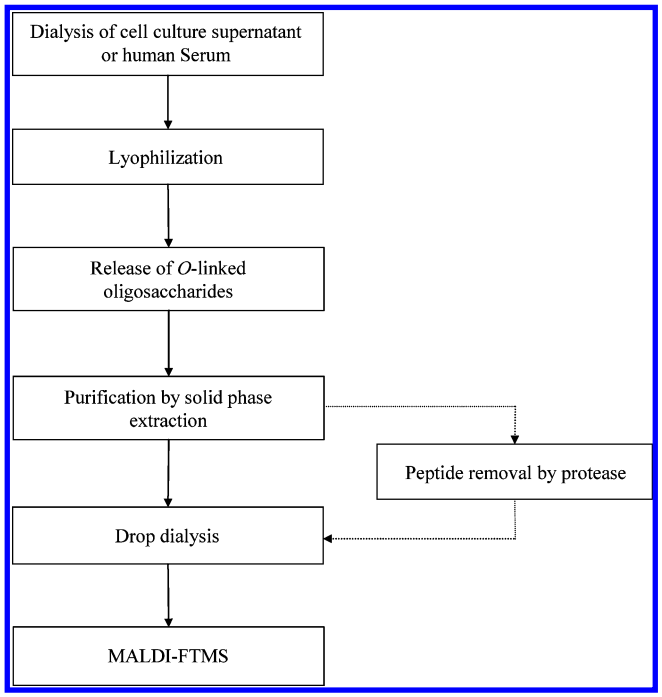
Release of Oligosaccharides by β-Elimination. Alkaline borohydride solution (500 μL, mixture of 1.0 M sodium borohydride and 0.1 M sodium hydroxide) was added to 2−3 mg of lyophilized supernatant of cell lines and serum materials. The mixture was incubated at 42 °C for 12 h in water bath. After the reaction, 1.0 M hydrochloric acid solution is slowly added in ice bath to stop reaction and destroy excess sodium borohydride.

Oligosaccharide Purification using a Graphitized Carbon-Solid-Phase Extraction. Oligosaccharides released by reductive β-elimination were purified by solid-phase extraction (SPE) using a graphitized carbon cartridge (GCC). The cartridge was washed with H_2O followed by 0.05% (v/v) trifluoroacetic acid (TFA) in 80% acetonitrile(ACN)/H_2O (v/v). The solution of released oligosaccharide was loaded to the cartridge. Subsequently, the cartridge was washed with nanopure water at a flow rate of about 1 mL/min to remove salts and buffer. Glycans were stepwise eluted with 10% ACN in H_2O, 20% ACN in H_2O, and 40% ACN in 0.05% TFA in H_2O. Each fraction was collected and concentrated in vacuo prior to MALDI analysis.

Mass Spectrometric Analysis. Mass spectra were recorded on an external source HiResMALDI (IonSpec Corporation, Irvine, CA) equipped with a 7.0 T magnet. The HiResMALDI was equipped with a pulsed Nd:YAG laser (355 nm). 2,5-Dihydroxy-benzoic acid (DHB) and 2, 5-dihydroxy-acetophenone (DHAP) were used as a matrix (5 mg/100 μL in 50% ACN in H_2O) for positive and negative mode, respectively. A saturated solution of NaCl in 50% ACN in H_2O was used as a cation dopant. The oligosaccharide solution (1 μL) was applied to the MALDI probe followed by matrix solution (1 μL). For the negative ion spectra, only DHAP matrix was used without using any dopant. The sample was dried under a stream of air prior to mass spectrometric analysis.

Structural Determination using Infrared Multiphoton Dissociation (IRMPD). Tandem mass spectrometry, specifically IRMPD, was used to determine the general structures of several oligosaccharides. This allowed for complete fragmentation of the ion of interest. A desired ion was readily selected in the analyzer with the use of an arbitrary-waveform generator and a frequency synthesizer. A continuous wave Parallax CO_2 laser (Waltham, MA) with 20W maximum power and 10.6 μm wavelength was installed at the rear of the magnet and is used to provide the photons for IRMPD. The laser beam diameter is 6 mm as specified by the manufacturer. The laser beam was expanded to ∼12 mm by means of a 2× beam expander (Synrad, Mukilteo, WA) to ensure complete irradiation of the ion cloud through the course of the experiment. The laser was

Chart 1. Flowchart of Procedure for the Release and Isolation of Oligosaccharides



aligned and directed to the center of the ICR cell through a BaF_2 window (Bicron Corporation, Newbury, OH). Photon irradiation time was optimized to produce the greatest number and abundance of fragment ions. The laser was operated at an output of approximately 13 W.

Results

There was an initial concern that insufficient levels of oligosaccharides were present in the serum to be detected by mass spectrometry. A streamlined method was therefore developed to minimize sample handling and manipulation. This procedure, which included the release and isolation of the oligosaccharides, is outlined in the Chart 1. The analyses would be strictly based on the masses with the potential marker candidates probed further by tandem mass spectrometry, specifically infrared multiphoton dissociation (IRMPD). The procedure was relatively fast and did not involve HPLC separation. Dialysis was performed on the supernatant or human serum to remove most of the salts and small molecules. Oligosaccharides were released under optimized β-elimination conditions (for more details see Experimental Section). This method was chosen to release specifically O-linked oligosaccharides. However, it can also release N-linked oligosaccharides under certain conditions. The procedure employed in this work was optimized for pure mucin glycoproteins, however it may have produced N-linked glycoproteins when applied to the more complicated mixture of serum.

Preconcentration of the oligosaccharide components was performed with a graphitized carbon solid phase extraction. The cartridge readily binds both neutral and anionic oligosaccharides. Salts were passed through while peptides and proteins were retained more strongly under the specified conditions (See Experimental Section). However, because of the large amounts of peptides produced under the release conditions, they were often eluted with the oligosaccharides. To remove the peptides completely, fractionated samples were treated with protease before analysis by MALDI−FTICR−MS. This procedure was used for both conditioned media and for serum.

Analysis of Conditioned Media from Four Ovarian Cancer Cell Lines. The conditioned media (supernatant) of four ovarian cancer cell lines OVCAR-3, Caov-3, SK-OV-3, and ES-2 in both log and death phases were extracted and examined without the cells. The log phase consisted of cells actively dividing, whereas the death phase contained cells that were confluent and no longer dividing. Our concern was that there might be glycosylation changes between the different growth phases. Each cell line medium yielded three fractions (10%, 20%, and 40% aqueous acetonitrile) from the SPE that contained anionic and neutral oligosaccharides based on the polarity of solvent. Oligosaccharides fractionated by solid phase extraction were analyzed directly by MALDI−FTICR−MS in the positive and negative mode. Each different fraction contained different sizes of oligosaccharides. Shown in Figure 1 is the negative ion mass spectrum of glycans released from the conditioned media of OVCAR-3. The 10% acetonitrile fraction includes predominantly small anionic, primarily sialylated, oligosaccharides for all cell culture supernatants. These anionic oligosaccharides could be observed more clearly in the negative

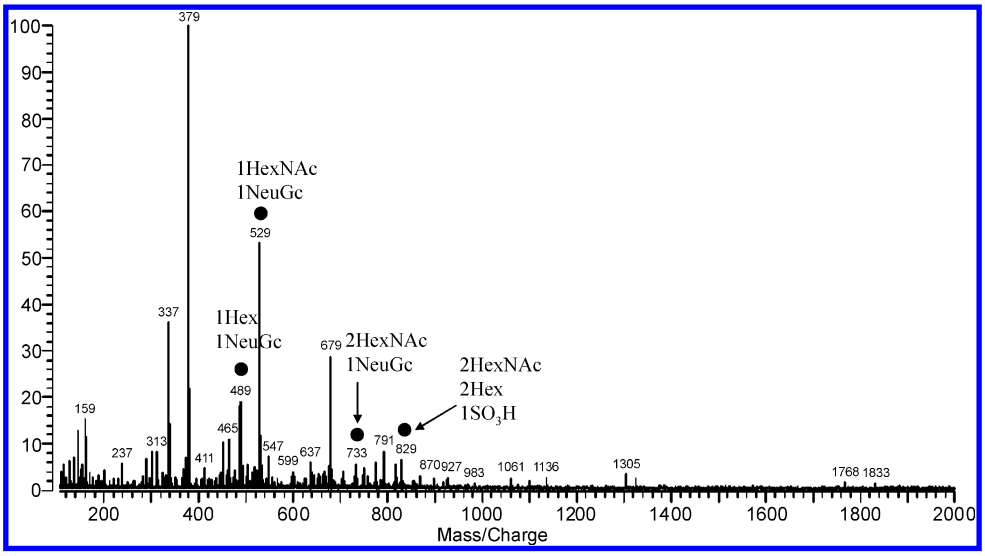


Figure 1. Negative ion MALDI−FTMS spectrum of glycans released from the conditioned media of OVCAR-3 cell line.

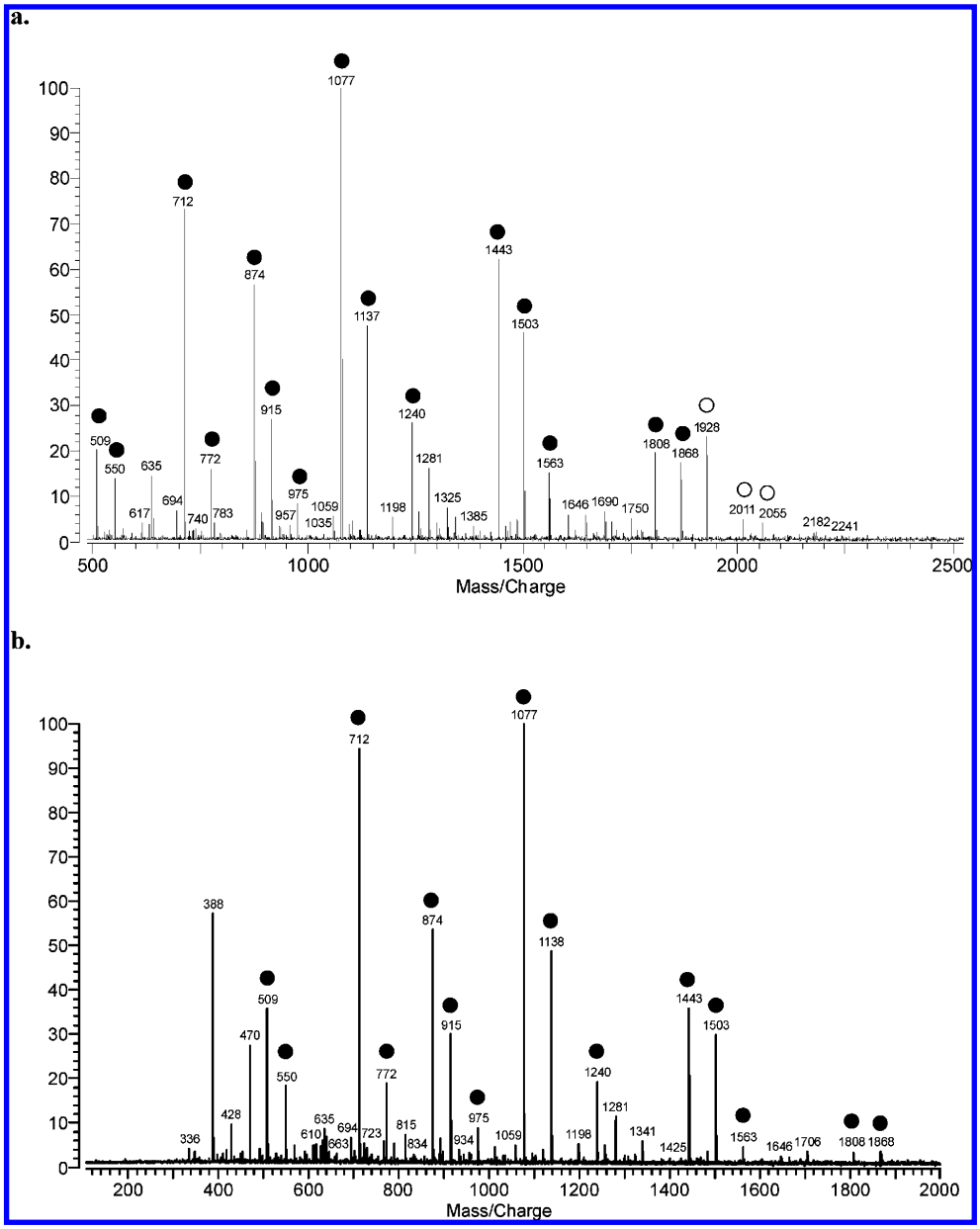


Figure 2. MALDI−FTMS spectra of oligosaccharides found in the conditioned media of ovarian cancer cell lines in the positive mode for (a) OVCAR-3 and (b) ES-2. Labeled peaks (solid circle) are prominent glycans present in four ovarian cancer cell lines, while others (open circle) are unique to OVCAR-3. A summary of oligosaccharides found in the four cell lines is listed in Table 1.

mode than the positive because of the negative charge from the deprotonated of anionic group (carboxyl or sulfate group). We found that the 10% acetonitrile fraction of other cell lines presented the same type of the oligosaccharides shown in Figure 1. Sialylated oligosaccharides were observed as two types—those containing *N*-acetyl neuraminic acid (NeuAc) and those containing N-glycolyl neuraminic acid (NeuGc). A number of notable oligosaccharides were observed including two disaccharides with compositions of 1Hex:1NeuGc (m/z 488.169) and 1HexNAc:1NeuGc (m/z 529.122). Additionally, oligosaccharides with the composition 2HexNAc:1NeuGc (m/z 732.267) were also observed. These oligosaccharides are notable as they represent the small anionic species that are often present in cancer cells.[14,21] The 20% fraction also contained oligosaccharides but nearly all were represented in the 10% and 40% fractions. The sample eluted with 40% acetonitrile contained significantly more and larger oligosaccharides. The representative spectra of oligosaccharides released from conditioned media of ovarian cancer cell lines in the positive mode are shown in Figure 2 for OVCAR-3 (Figure 2a) and ES-2 (Figure 2b). The mass spectra are highly reproducible exhibiting all the oligosaccharides in multiple determinations (>10) from differently prepared samples. Labeled peaks (solid circle) in the spectrum are prominent glycans present in all cell lines. We identified these to be neutral oligosaccharides (based on accurate mass and tandem MS, see below). They appear to be related to each other based on their elution fraction. Tandem mass spectra suggest they originate from the same, perhaps much larger molecule. We found that each fraction obtained from the conditioned media of four cell lines yielded mostly the same oligosaccharides, with some variations between the different cell lines. A summary of oligosaccharides found in the four cell lines is listed in Table 1. It was also determined that oligosaccharides obtained from the conditioned media of the

Table 1. Summary of Neutral Oligosaccharides Found in Four Ovarian Cancer Cell Lines and Human Serum[a,b,c]

observed mass	oligosaccharide composition	cell lines	patients	normal
347.10	2Hex	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
388.14	1HexNAc:1Hex	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
509.17	3Hex	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
550.21	1HexNAc:2Hex	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
712.28	3Hex:1HexNAc	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
772.31	2Hex:1HexNAc:1Hex*	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
874.36	4Hex:1HexNAc	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
915.38	3Hex:2HexNAc	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
975.43	2Hex:2HexNAc:1Hex*	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
1077.47	4Hex:2HexNAc	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
1137.51	3Hex:2HexNAc:1Hex*	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
1239.57	5Hex:2HexNAc	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
1280.62	4Hex:3HexNAc	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
1442.72	5Hex:3HexNAc	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
1502.74	4Hex:3HexNAc:1Hex*	OVCAR-3, Caov-3, SK−OV-3, and ES-2	yes	no
1562.78	3Hex:HexNAc:2Hex*	ES-2, OVCAR-3	yes	no
1645.84	5Hex:4HexNAc	ES-2, OVCAR-3		no
1664.85	5Hex:3HexNAc:1Hex*	OVCAR-3		no
1705.83	4Hex:4HexNAc: 1Hex*	ES2		no
1807.93	6Hex:4HexNAc	ES-2, OVCAR-3		no
1867.97	5Hex:4HexNAc:1Hex*	ES-2, OVCAR-3		no
1927.95	4Hex:2HexNAc:2Hex*	OVCAR-3		no
2011.02	6Hex:5HexNAc	OVCAR-3		no

[a] All observed ions correspond to $[M-H_2O + Na]^+$. [b] Hex* represents modified hexose (one of hydroxyl group on hexose is modified by an unaccounted for 60 mass unit). [c] Normal designations are for nonovarian cancer patients.

Table 2. List of Anionic Oligosaccharides Found in Four Ovarian Cancer Cell Lines

observed mass	oligosaccharide composition	molecular ion
488.11	1Hex:1NeuGc	$[M-H]^-$
529.12	1HeNAc:1NeuGc	$[M-H]^-$
732.15	2HeNAc:1NeuGc	$[M-H]^-$
829.19	2Hex:2HexNAc:1SO_3H	$[M-H]^-$
902.32	1Hex:2HexNAc:1NeuAc	$[M + Na]^+$

log phase of each cell line did not significantly differ from that of the death phase cells.

Although many of the oligosaccharides were common to the four cell lines, there were distinct species found in each cell line (Table 1). In addition, there were more oligosaccharides in cell lines such as OVCAR-3 and ES-2 than in Caov-3 and SK-OV-3. It is likely that these differences are due to the presence of more glycoproteins in these samples. Interestingly, OVCAR-3 has been reported to yield more of the mucin glycoprotein CA125 than the other cell lines.[9] The fact that all of the ovarian cancer cell lines produce a mostly similar glycan profile with some variations demonstrates that it is possible to conduct a direct glycan analysis of shed or secreted glycoproteins from tumor cells that are perhaps indicative of ovarian cancer. However, we will first need to demonstrate that the same oligosaccharides detected in the conditioned media of ovarian cancer tumor cell lines can also be detected in the serum of ovarian cancer patients.

Table 3. List of the Series of Hexuronic Acid Oligosaccharides Found in Human Serum

series no.	observed mass	oligosaccharide composition
1	220.980	$[HexA]_1$
	418.973	$[HexA]_2$
	616.970	$[HexA]_3$
	814.958	$[HexA]_4$
	1012.968	$[HexA]_5$
	1210.977	$[HexA]_6$
	1408.965	$[HexA]_7$
	1606.973	$[HexA]_8$
2	361.011	unknown
	559.008	$361+[HexA]_1$
	757.006	$361+[HexA]_2$
	955.004	$361+[HexA]_3$
	1153.000	$361+[HexA]_4$
	1350.999	$361+[HexA]_5$
	1549.009	$361+[HexA]_6$
3	550.998	unknown
	749.025	$551+[HexA]_1$
	947.018	$551+[HexA]_2$
	1145.040	$551+[HexA]_3$
	1343.022	$551+[HexA]_4$
	1541.016	$551+[HexA]_5$
4	554.993	unknown
	752.988	$555+[HexA]_1$
	951.002	$555+[HexA]_2$
	1149.000	$555+[HexA]_3$

As controls, the complete media containing 10% fetal bovine serum as well as the defined media without fetal bovine serum (FBS) were analyzed in a similar manner as the conditioned media from the cell lines. Undiluted fetal bovine serum was also processed using our method to determine whether oligosaccharides from FBS were the same as those observed in the mass spectra of the conditioned media from the ovarian cancer tumor cells. From the complete media containing 10% FBS, few oligosaccharides were observed, and from pure FBS only a small number of oligosaccharides were observed (< less than five distinct masses). The abundances of these ions were significantly less than the cultured cell by as much as 2 orders of magnitude. The major ion peaks corresponded primarily to peptides whose masses were not observed in the cultured cell samples. One mass that was observed in the control and the cultured cell, albeit in far less abundance in the former, corresponded to the mass m/z 529 (1HexNAc:1NeuGc). NeuGc is found in mammals such as bovine. Its presence in human is due to exogenous sources such as diet. *N*-Glycolyl neuraminic acid (NeuGc) is abundant in other mammals such as the great apes, but is usually found in less than 1% of total sialic acids in humans.[17,18] There are persistent reports that elevated levels of NeuGc are found in tumor tissues and in cancer patients, however its use as a cancer marker remains unproven.[17]

Analysis of Human Serum. A small number of human serum samples were examined to observe whether we could detect the same oligosaccharides in ovarian cancer patient serum that we observed in the conditioned media of the ovarian cancer cell lines. We wanted to determine further which oligosaccharides were also present in the ovarian cancer patient serum samples. Our beginning sample set consisted of serum samples from 5 normal individuals (non ovarian cancer patients) and 5 patients with ovarian cancer. The ovarian cancer patient sera

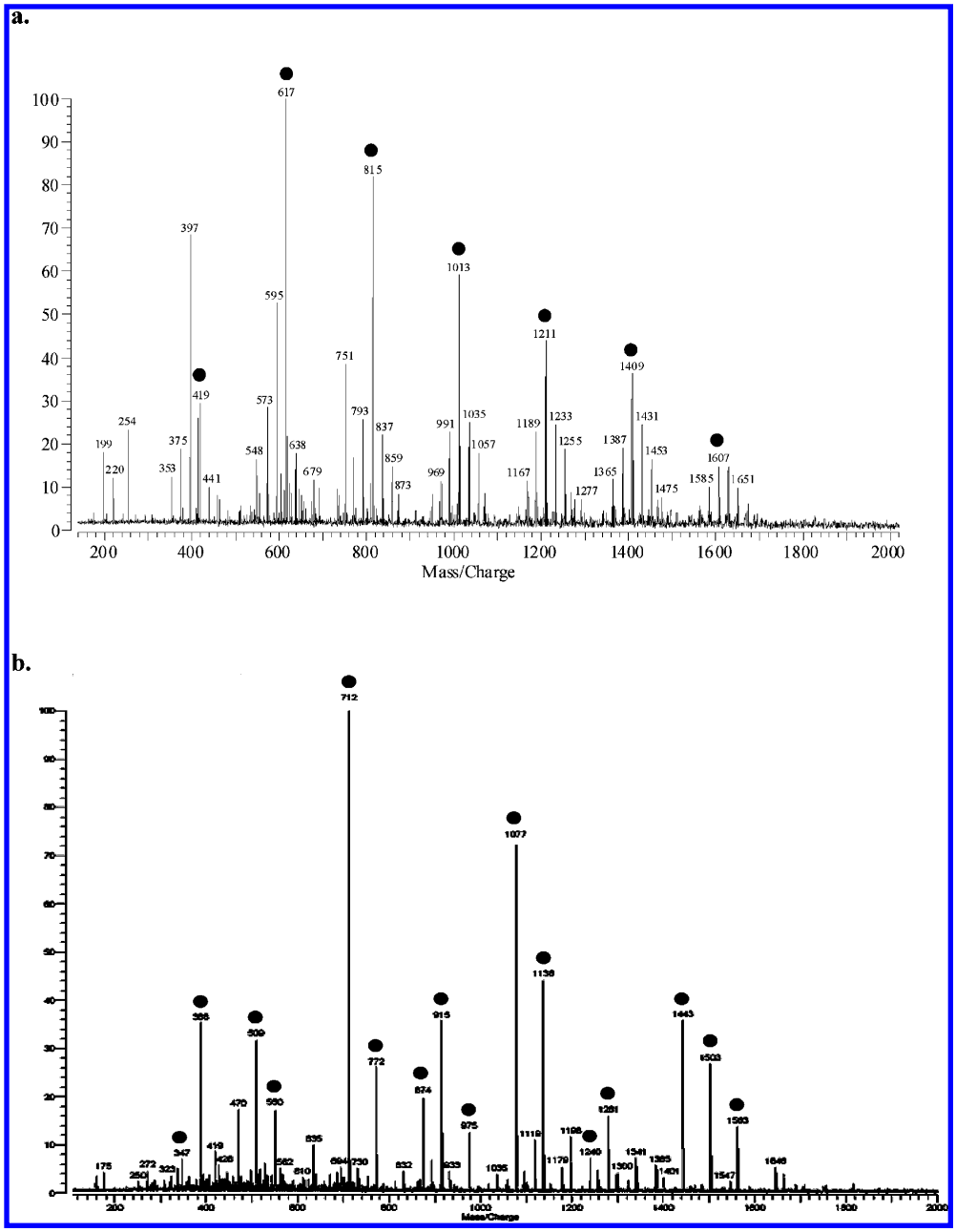


Figure 3. Representative MALDI−FTMS spectra of (a) normal individuals and (b) cancer patients after peptide removal in the positive mode. The labels in (a) show the most prominent oligomeric series (series 1 in Table 3) of hexuronic acid (signal is separated by 198 Da). The labels in (b) indicate oligosaccharides unique to ovarian cancer patients.

all had high CA 125 values. Serum samples were dialyzed and processed as previously described to release the oligosaccharides (See Experimental Section for more details). Each serum sample was partitioned into three fractions based on the percentage of acetonitrile in water (10%, 20%, and 40%) used to elute the mixture from the solid phase extraction cartridge packed with a graphitized carbon. The mass spectra of normal and cancer patient sera from the 10% acetonitrile fraction did not appear to contain significant abundances of oligosaccharide signals, but primarily peptides (Supporting Information). Although most peaks correspond to peptides, several peaks were unique to patient samples with ovarian cancer. For example, the ions m/z 788.545 and 899.690 are clearly present in the patient samples. Interestingly, the spectra based on triplicates were highly reproducible. These unique peptides peaks have potential as biomarkers for ovarian cancer but were not pursued in the study. While normal samples contained mainly peptides, patient samples yielded primarily oligosaccharides in 20% fraction (Supporting Information). The 40% fraction contained predominantly peptides and showed similar spectra between normal and cancer patients (Supporting Information). To assess the reproducibility of the analysis, one patient sample was analyzed in triplicate and each spectrum of each fraction showed nearly identical spectra.

Because of our concern that the presence of peptide ions in the fractions might suppress the signal of oligosaccharide ions, the fractionated samples were further digested by proteases to remove peptides. This process yielded cleaner spectra with better signal-to-noise and contained predominantly oligosaccharides. Representative MS spectrum of normal individuals

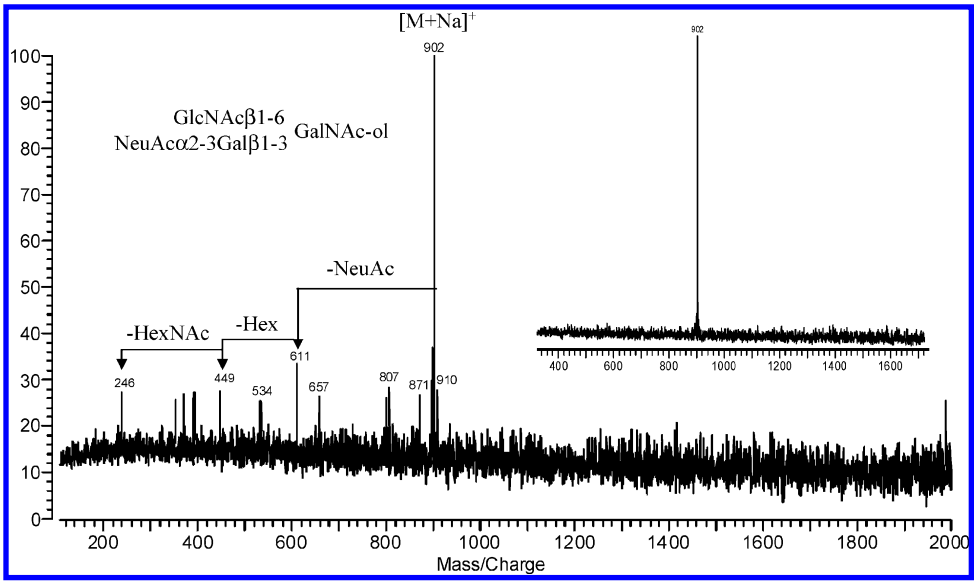


Figure 4. Infrared multiphoton dissociation (IRMPD) spectrum of ion *m*/*z* 902. The inset shows the isolation of the ion prior to fragmentation. The fragments are consistent with the proposed structure (inset).

after peptide removal in 10% fraction is shown in Figure 3a. After peptide removal nearly all of the 5 normal samples yielded spectra very similar to that shown in Figure 3a. The results suggest that the oligosaccharide signals were suppressed by peptides. The 10% fraction of cancer patient serum, after peptide removal, showed similar characteristics (Supporting Information). Oligosaccharides found in both normal and cancer included oligomeric series of hexuronic acid (HexA). There are very few reports of uronic acids found in humans, but they are found abundantly in plants.[22] Those that have been reported from humans corresponded primarily to *monomers* of glucuronic acid.[23−26] Standards comprising galacturonic acid oligomers from commercial sources were analyzed using MALDI and showed the same major peaks.[22] Several HexA series were observed with the signal separation of 198 Da. The mass difference is equivalent to one hexuronic acid residue with one sodium replacing the acidic hydrogen (198.014 − theoretical, 197.995 − experimental). The samples were doped with sodium chloride, hence the preponderance of sodium. Satellite peaks were present corresponding to 22 mass units apart indicating the exchange of carboxylic acid protons with sodium cations. A number of HexA series were observed and listed in Table 3. Most were found in both normal and cancer patients. The most prominent oligomeric series (series 1 in Table 3) of hexuronic acid found in normal and cancer patient samples has the composition $[HexA]_n$ ($n = 1-8$) with the masses 221, 419, 617, 815, 1013, 1211, 1409, and 1607. These peaks correspond to a single oligomeric series where each acidic proton of the hexuronic acid was replaced by sodium ($[M - nH + (n + 1)Na]^+$). The other series corresponded to other oligomers with a different and, as of yet, unknown headgroup whose structures are currently being elucidated. As mentioned previously, this is probably the first report of hexuronic acid *oligomers* in human serum. There are however several reports that indicate the amount of hexuronic acid (monomers) in human serum elevates in cancer.[25−28] Although the nature and identity of HexA oligomers is unknown at this time, the large number of different series indicates that the presence of these hexuronic polymers may also be useful biomarkers.

While many of the HexA oligomers were found in both normal and patient samples, a number of glycans that were unique to the ovarian cancer patients were observed. Shown in Figure 3b is a mass spectrum of one serum sample from a patient with ovarian cancer, however it is representative of all patients with the disease. Labeled peaks (solid circles) in the spectrum are potential oligosaccharide markers that are found primarily in the patients. The spectrum for an individual with cancer is therefore visually vastly different from the normal spectrum. The glycan markers in Figure 3b are identified as neutral oligosaccharides composed of hexoses and *N*-acetylhexosamines related to each other based on their elution fraction and their tandem MS spectra (See below). For example *m*/*z* 712.276 contains three hexoses and one *N*-acetylhexosamine coordinated with sodium. The *m*/*z* 772 is related to *m*/*z* 712 and differs by an unaccounted for 60 mass unit, perhaps an acetyl group on a hexose. More importantly, many members of this group of glycans are the same as those found in the conditioned media of the four ovarian cancer cell lines (Table 1). These potential markers are plentiful and abundant—at least 15 peaks mark the disease.

Structural Elucidation of Oligosaccharides using IRMPD. To confirm the compositional assignments and to obtain structural information, a number of the ions were isolated from the cell lines and subjected to tandem MS using infrared multiphoton dissociation (IRMPD).[29] In IRMPD, a CO_2 laser is used to irradiate the isolated ion and induce fragmentation. The resulting fragments are identified based on their accurate mass relative to the precursor ions.

Oligosaccharides containing NeuAc were observed in the cancer cell lines. The ion *m*/*z* 902.322 was isolated from the conditioned medium (20% acetonitrile fraction) of the OVCAR-3 cell line. This ion was not abundant, and mass corresponded in mass to the tetrasaccharide sialyl-Le^x antigen (another known cancer marker). Shown in Figure 4 is the IRMPD of this ion. The spectrum shows the loss of a NeuAc (*m*/*z* 611), followed by a Hex (*m*/*z* 449), and subsequently by the loss of a HexNAc (*m*/*z* 246) residue. The reducing end in the alditol form is a

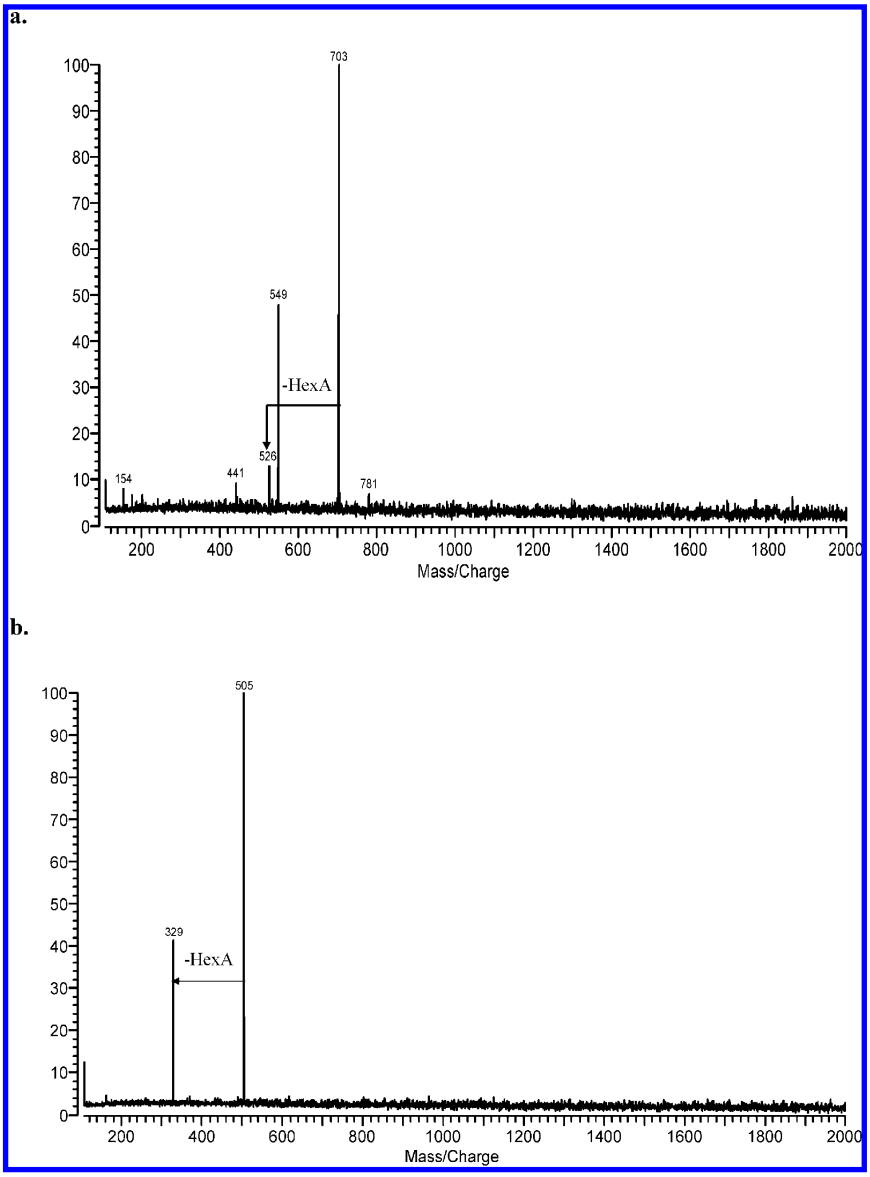


Figure 5. Negative IRMPD spectra of hexuronic acid oligomers obtained from human serum samples from precursor ions of (a) *m*/*z* 702 and (b) *m*/*z* 505. It shows clear loss of one hexuronic acid residue (176 Da).

HexNAc and most likely to be GalNAc. The tandem MS spectrum is consistent with sialyl-Le^x whose structure is inset in the figure.

IRMPD was also performed on several of the hexuronic acid oligomers. IRMPD of the signals in the positive mode did not readily yield fragment ions. These species are highly sodiated and the extensive intramolecular coordination between the negative charges and the sodium ions may have prevented fragmentation. In the negative mode, similar series were obtained that were 176 mass units apart (See Supporting Information)—corresponding to a hexuronic acid residue with no sodium substitution. These species with little or no sodium cation coordination were more apt to fragment during IRMPD. A representative spectrum shown in Figure 5 shows the distinct loss of one hexuronic acid residue to yield *m*/*z* 526.020 (Figure 5a) from the quasimolecular ion at *m*/*z* 702.027. This corresponds to the loss of a hexuronic acid residue (176.032 − theoretical, 176.007 − experimental). The IRMPD of the ion *m*/*z* 505.033 in the negative mode also shows the loss of hexuronic acid to yield *m*/*z* 329.032 corresponding to another loss of 176.001 mass units (Figure 5b).

On the basis of the exact mass and additional IRMPD results, we were also able to determine the composition of a small number of the neutral glycan markers from the cell lines. Representative IRMPD spectra are shown in Figure 6. The ion of *m*/*z* 1077.476 was found to contain four hexoses, two *N*-acetylhexosamines based on the mass. However, the exact mass does not correspond to an expected alditol. From the tandem MS spectra, a HexNAc is lost followed by a Hex from quasimolecular ion followed by another HexNAc and Hex, which are lost sequentially. Indeed, the ion at *m*/*z* 347 does not correspond to a monosaccharide alditol, as would be expected from the release. None of the oligosaccharides labeled in Figure 3b correspond to alditols. Another example is the ion with *m*/*z* 1442.726 which is found to contain five hexoses and three *N*-acetylhexosamines. This study is ongoing, and we plan to completely characterize all the peaks corresponding to oligosaccharides in the mass spectra.

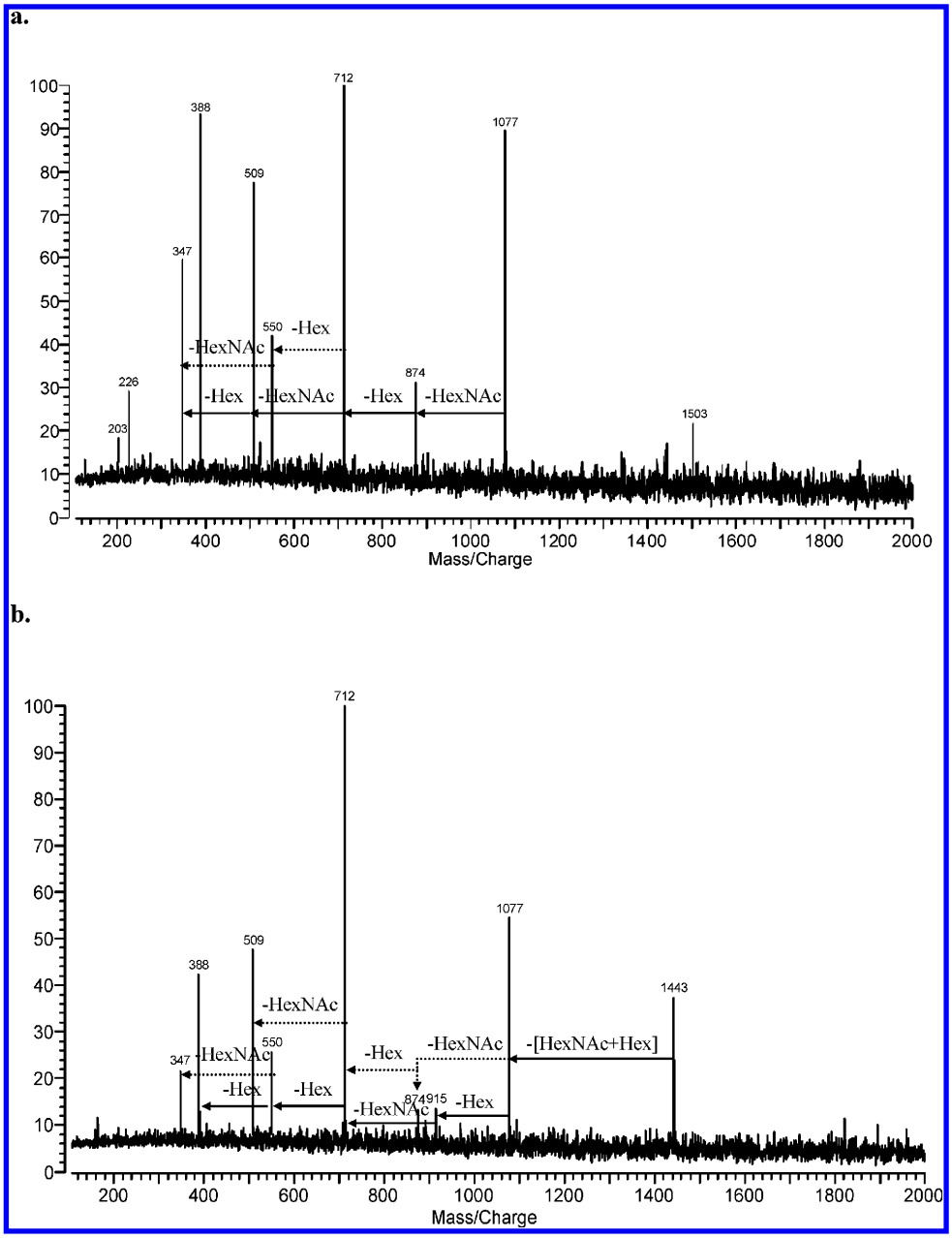


Figure 6. Representative IRMPD spectra of neutral glycan markers obtained from the cell lines for (a) *m*/*z* 1077 and (b) *m*/*z* 1443.

Conclusions

The direct glycan analysis of secreted or shed glycosylated proteins from ovarian cancer cell lines in conditioned media yielded oligosaccharides that are potential markers for the disease. Several of the oligosaccharides observed from serum are similar to those from the conditioned media with several identical masses and oligosaccharide compositions. Anionic oligomers composed of several series of hexuronic acid were obtained that have not been previously described in the literature. The headgroups of most of these oligomers have not been determined. Extensive structural elucidation work is currently being done on these oligomers. A number of neutral oligosaccharides are also found that appear to be related. It is not yet known whether these are truly mucin oligosaccharides. Some may be N-linked oligosaccharides and may also be fragments due to the chemical release and the ionization process. Studies to structurally elucidate these oligomers are also underway.

This new approach to disease marker discovery, which is not dependent on the isolation or analysis of proteins or the use of antibodies, holds particular promise for the diagnosis of ovarian cancer and other types of cancer. There are a number of oligosaccharides obtained in this approach that are new and highly novel. Many are also found only in the serum of cancer patients. An ongoing systematic study involving more patients and controls as well as a bioinformatics analysis of the data will provide important markers for the disease.

Acknowledgment. Funding provided by the National Institutes of Health is gratefully acknowledged (R01 GM049077). The efforts of Jeff Gregg and Colin Baron (Pathology) in providing the patient serum samples are also gratefully acknowledged.

Supporting Information Available: The mass spectra of normal and cancer patient sera from the 10%, 20%, and 40% acetonitrile (ACN) fraction in the positive mode. Representative MS spectrum of cancer patients after peptide removal in 10% ACN fraction. Representative MALDI−FT−MS spectrum of non-ovarian cancer patients (normal) in the negative mode. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Fariaseisner, R.; Kim, Y. B.; Berek, J. S. *Semin. Surg. Oncol.* **1994**, *10*, 268−275.
(2) Fariaseisner, R.; Cirisano, F. D.; Grouse, D.; Leuchter, R. S.; Karlan, B. Y.; Lagasse, L. D.; Berek, J. S. *Gynecol. Oncol.* **1994**, *53*, 55−58.
(3) Morice, P.; Piovesan, P.; Rey, A.; Atallah, D.; Haie-Meder, C.; Pautier, P.; Sideris, L.; Pomel, C.; Duvillard, P.; Castaigne, D. *Ann. Oncol.* **2003**, *14*, 1511−1517.
(4) Jacobs, I.; Bast, R. C. *Hum. Reprod.* **1989**, *4*, 1−12.
(5) Bast, R. C.; Klug, T. L.; Stjohn, E.; Jenison, E.; Niloff, J. M.; Lazarus, H.; Berkowitz, R. S.; Leavitt, T.; Griffiths, C. T.; Parker, L.; Zurawski, V. R.; Knapp, R. C. *New Engl. J. Med.* **1983**, *309*, 883−887.
(6) Coombes, K. R.; Morris, J. R. S.; Hu, J. H.; Edmonson, S. R.; Baggerly, K. A. *Nat. Biotechnol.* **2005**, *23*, 291−292.
(7) Hellstrom, I.; Raycraft, J.; Hayden-Ledbetter, M.; Ledbetter, J. A.; Schummer, M.; McIntosh, M.; Drescher, C.; Urban, N.; Hellstrom, K. E. *Cancer Res.* **2003**, *63*, 3695−3700.
(8) Chapman, K. *Biochem. Soc. T* **2002**, *30*, 82−87.
(9) Stimpfl, M.; Schmid, B. C.; Schiebel, I.; Tong, D.; Leodolter, S.; Obermair, A.; Zeillinger, R. *Cancer Lett.* **1999**, *145*, 133−141.
(10) Villanueva, J.; Philip, J.; Chaparro, C. A.; Li, Y. B.; Toledo-Crow, R.; DeNoyer, L.; Fleisher, M.; Robbins, R. J.; Tempst, P. *J. Proteome Res.* **2005**, *4*, 1060−1072.
(11) Dennis, J. W.; Granovsky, M.; Warren, C. E. *Bioessays* **1999**, *21*, 412−421.
(12) Dall'Olio, F.; Chiricolo, M.; Mariani, E.; Facchini, A. *Eur. J. Biochem.* **2001**, *268*, 5876−5884.
(13) Hollingsworth, M. A.; Swanson, B. J. *Nat. Rev. Cancer* **2004**, *4*, 45−60.
(14) Brockhausen, I. *Bba-Gen. Subjects* **1999**, *1473*, 67−95.
(15) Yamori, T.; Kimura, H.; Stewart, K.; Ota, D. M.; Cleary, K. R.; Irimura, T. *Cancer Res.* **1987**, *47*, 2741−2747.
(16) Gorelik, E.; Galili, U.; Raz, A. *Cancer Metast. Rev.* **2001**, *20*, 245−277.
(17) Varki, A. *Biochimie* **2001**, *83*, 615−622.
(18) Malykh, Y. N.; Schauer, R.; Shaw, L. *Biochimie* **2001**, *83*, 623−634.
(19) Dabelsteen, E. *J. Pathol.* **1996**, *179*, 358−369.
(20) Wong, N. K.; Easton, R. L.; Panico, M.; Sutton-Smith, M.; Morrison, J. C.; Lattanzio, F. A.; Morris, H. R.; Clark, G. F.; Dell, A.; Patankar, M. S. *J. Biol. Chem.* **2003**, *278*, 28619−28634.
(21) Hanisch, F. A. *Biol. Chem.* **2001**, *382*, 143−149.
(22) An, H. J.; Lurie, S.; Greve, L. C.; Rosenquist, D.; Kirmiz, C.; Labavitch, J. M.; Lebrilla, C. B. *Anal. Biochem.* **2005**, *338*, 71−82.
(23) Atmani, F.; Mizon, J.; Khan, S. R. *Eur. J. Biochem.* **1996**, *236*, 984−990.
(24) Hanaoka, Y.; Shikakume, K.; Matsumoto, Y.; Yakabe, Y.; Tarui, Y.; Sayama, Y. *Bunseki[ ]Kagaku* **1998**, *47*, 119−125.
(25) Malathy, K. K.; P. A. *Ind. J. Biochem.* **1969**, *6*, 43−45.
(26) Stancakova, A.; Vajo, J. *Endokrinologie* **1981**, *77*, 197−205.
(27) Nagai, Y. T.; S.; Saito, Y.; Turumi, K. *Fukushima, J. Med. Sci.* **1959**, *6*, 257−262.
(28) Leyon, P. V.; Lini, C. C.; Kuttan, G. *Life Sci.* **2005**, *76*, 1339−1349.
(29) Zhang, J. H.; Schubothe, K.; Li, B. S.; Russell, S.; Lebrilla, C. B. *Anal. Chem.* **2005**, *77*, 208−214.

PR060010K