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Glycomics Analyses of Tear Fluid for the Diagnostic Detection of Ocular Rosacea

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A Glycomics approach to detect disease is illustrated in the analyses of human tear fluid for rosacea. The diagnosis of ocular rosacea is particularly challenging in a subgroup of patients that do not present with typical facial skin findings but have ocular signs and symptoms. Indeed, up to 90% of patients with ocular rosacea may have neither obvious roseatic skin changes nor a previous diagnosis of rosacea. Tear fluid was collected from 37 subjects (21 controls and 16 patients with ocular rosacea) after conjunctival stimulation with filter (Schirmer) paper. O-linked oligosaccharides were released from tear fluid by β-elimination and then purified using solid-phase extraction. Mass spectra were recorded on an external source HiResMALDI with a 7.0 T magnet. Mass spectra were obtained in both the positive and negative modes. However, signals were stronger in the negative mode. Tear fluid samples from rosacea patients yielded distinctive clusters of peaks that extend to higher masses. Patients with rosacea presented several oligomeric series that were not found in the controls. To discriminate the ocular rosacea cases from the normal controls, the sum of absolute intensities of 13 series corresponding to nearly 50 identified mass spectrum peaks was used. Thirty-six out of the 37 samples were correctly classified. This yields a sensitivity of 100% (95% CI 79.5−100) and specificity of 95.2% (95% CI 76.2−99.9). The high abundance of oligosaccharides in the tear fluid of patients with rosacea may lead to an objective diagnostic marker for the disease.

Keywords: ocular rosacea • glycomics • mucin • O-linked oligosaccharides • MALDI-FTMS • biomarker

Introduction

Rosacea is a chronic cutaneous disorder characterized by chronic erythema, telangiectases, papules, and pustules primarily of the convexities of the central face (cheeks, chin, nose, and central forehead)[1]. The disease frequently involves the eye, manifest as eyelid and ocular surface inflammation.[2] It is estimated that approximately 13 million Americans have rosacea.[3,4] In a Swedish study, a prevalence of 10% was found.[5] Ocular rosacea affects half or more of patients with rosacea, although an incidence as low as 8% has recently been reported.[6−8] The diagnosis of ocular rosacea is particularly challenging in a subgroup of patients that do not present with typical facial skin findings but have ocular signs and symptoms. Indeed, up to 90% of patients with ocular rosacea may have neither obvious roseatic skin changes nor a previous diagnosis of rosacea.[9]

Ocular rosacea is, therefore, a common disease with the potential for causing a range of ocular pathology and, in its severest form, may lead to blindness. The diagnosis of ocular rosacea requires careful physical examination of the eye. An early study reported by Starr and McDonald[6] in which the eyes of rosacea patients were examined found ocular complications in 58%, with corneal involvement in 33%. Ghanem et al. reported that ocular rosacea cannot always be diagnosed solely by ocular findings, even though 20% of patients develop eye manifestations before the emergence of skin findings.[2] The authors suggested that a clinician's increased awareness of ocular findings (in particular lid disease-related symptoms) may aid in earlier clinical diagnosis and treatment.

An objective diagnostic test has eluded clinicians to the present. Biochemical methods for the detection of ocular rosacea could potentially provide more definitive diagnosis of the disease. Analyses of human tears have been performed primarily by investigating the protein components of tear fluids. Proteomic analysis of human tears demonstrated that three human α-defensins were significantly up-regulated in their expression after surgery, and the levels decreased to near normal after 30 days, when healing was complete.[10] Human tears are known to contain high-molecular-mass glycoproteins,

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specifically mucus, as major components. Mucin glycoproteins are highly glycosylated and can be 80% glycans in composition.[11] Oligosaccharides, specifically those found on mucins, which are composed of primarily O-linked oligosaccharides, are known to be sensitive to the biochemical environment and could be a better indicator of disease states. For example, cancer cells are known to express different oligosaccharides compared with normal cells. There have been several attempts to identify the mucins expressed in tear fluid. At least three mucins MUC2, MUC4, and MUC5AC have been identified by antibodies in human tears.[12−14] However, there have been, to our best knowledge, no published reports to characterize the oligosaccharides in either mucins or other glycoproteins in tear fluid. The one exception is a report by Schulz et al. on O-linked oligosaccharides released from a specific groups of mucin glycoproteins (DMBT1 gene products) isolated by SDS-PAGE.[15] These oligosaccharides were highly sialylated.

Proteomic analysis has obtained considerable attention for biomarker discovery and disease diagnosis. The glycan analogue, "glycomics", holds considerable promise, as the glycans are smaller and more easily identified and quantified. Furthermore, as glycomics is more akin to metabolomics than proteomics, it is more sensitive to biochemical changes, because metabolites are amplified when proteins are up or down regulated.

In this report, we illustrate a glycomic approach to determine oligosaccharide markers for rosacea. Glycomic analysis is defined here as the analysis of all glycans obtained from a single source—in this case, tear fluid—after a global release from the source. The identities of the proteins involved are not monitored as the changes in glycosylation are the only thing we wish to examine. The procedure for the global release used here is the standard reductive β-elimination that selects for O-linked oligosaccharides. The oligosaccharides captured and analyzed from tear fluid are indicators of ocular rosacea. Tear fluid from a group of 21 individuals without ocular rosacea and 16 patients with the disease were examined by treating the fluid with alkaline sodium borohydride to release O-linked oligosaccharides specifically.

Experimental Section

Acquisition of Tear Samples. Tears from 37 subjects (21 controls with no other ocular diseases and 16 patients with ocular rosacea) were collected after conjunctival stimulation with a filter paper (Schirmer) strip, using a 10 μL microcapillary tube (Microcaps, Drummond Scientific Co, Broomall, PA). Samples were collected from both eyes (right eye first), as described: (1) the paper strip was placed in the medial third of the inferior lid for 15−30 s and removed. (2) With the aid of a slit-lamp, the microcapillary tube was oriented horizontally and its tip was placed to touch the lacrimal meniscus, until it was completely filled; (3) immediately, samples from both eyes were transferred to one siliconized low-retention microtube and kept at −80 °C until protein analysis. During collection, the tip of the tube did not touch the skin or eyelashes. The diagnosis of ocular rosacea was based on the standard classification proposed by the National Rosacea Society Expert Committee, and all patients presented one or more of the primary features (flushing, nontransient erythema, papules/pustules, and telangiectasia) with a central face distribution, and a history of ocular symptoms compatible with the diagnosis. Patients with epithelial defects, significant conjunctival hyperemia (2+ or more, in a scale of 1−4) and/or corneal ulcers/infiltrates were not included. A history of diabetes, contact lens wear, and recent (<1 year) ocular surgery were exclusion criteria for both rosacea patients and controls.

Release of O-Linked Oligosaccharides from Tear Fluid by β-Elimination. Alkaline borohydride solution (20 μL, mixture of 1.0 M sodium borohydride and 0.1 M sodium hydroxide) was added to 3 μL of tear fluid. The mixture was incubated at 42 °C for 12 h in a water bath. After reaction, 1.0 M hydrochloric acid solution was slowly added in an ice bath to stop the reaction and destroy excess sodium borohydride.

Oligosaccharide Purification Using a Graphitized Carbon−solid-Phase Extraction. O-linked oligosaccharides released by reductive elimination were purified by solid-phase extraction using a graphitized carbon cartridge (Alltech Associates, Deerfield, IL). The cartridge was washed with Nanopure water followed by 0.05% (v/v) TFA in 80% ACN/H_2O (v/v). The solution of released oligosaccharide was applied to the cartridge. Subsequently, the cartridge was washed with Nanopure water at a flow rate of approximately 1 mL/min to remove salts and buffer. O-linked glycans were eluted with 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 matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) analysis.

Mass Spectrometric Analysis. Mass spectra were recorded on an external source HiResMALDI (IonSpec Corporation, Irvine, CA) with a 7.0 T magnet. The HiResMALDI was equipped with a pulsed Nd:YAG laser (266 nm). 2, 5-Dihydroxy-benzoic acid (DHB) was used as a matrix (5 mg/100 μL in 50% ACN in H_2O) for positive and negative mode, respectively. The oligosaccharide solution (1 μL) was applied to the MALDI probe followed by matrix solution (1 μL). The sample was dried under a stream of air prior to mass spectrometric analysis. Calibration was performed externally using standard oligosaccharide samples. Mass intensities were obtained and used directly from the absolute intensity generated by the mass analyzer.

Statistical Analysis. The performance of the identified oligosaccharide markers in detecting ocular rosacea was evaluated by descriptive statistics and receiver operating characteristic curve. The confidence interval for the sensitivity and specificity were calculated by the exact method. All of the statistical analyses were performed using R and STATA 8.0.

Results

A total of 37 tear samples from the same number of individuals were analyzed by mass spectrometry. This included samples from 21 individuals without the disease (control samples) and 16 individuals (patient samples) with characteristic ocular rosacea. Each sample originated from 3 μL of tears with the entire amount used for the analyses.

The samples were partitioned into two based on the percentage of acetonitrile in water (20% and 40%) used to elute the mixture from the solid phase extraction cartridge packed with a graphitized carbon. Smaller oligosaccharides were obtained at the 20% fraction while larger species are obtained at the 40% fraction. Mass spectra were obtained in both the positive (cation) and negative (anion) mode. However, the positive mass spectra did not yield as much information, as the signals were not as abundant. The signals were significantly stronger in the negative mode, indicating that the majority of the oligosaccharides were anionic species containing either sialic acids (*N*-acetyneuraminic acid − NeuAc and *N*-glycolylneuraminic acid − NeuGc), sulfated, or hexuronic acid residues.

Table 1. Summary of Results Obtained from Oligosaccharide Analyses by MALDI FTICR Mass Spectrometry of Individuals without Rosacea

type	no. of samples	observations
A	7	$[HexNAc]_2[Hex]_n[SO_3H]$ observed, n=0−3
B	10	No signals corresponding to oligosaccharides observed. Signals due to matrix ions and some peptides.
C	4	One sample (C503) had oligosaccharide similar to rosacea patients. Samples C203, C404 and C1304 showed oligosaccharides that were not the same as those found in the rosacea patients.

The mass spectra of control samples are illustrated in Figures 1 and 2. Table 1 summarizes the results of the 21 control samples. Two common types of spectra were obtained. In one group composed of seven samples (Type A), a series containing an oligomeric sulfated oligosaccharides was obtained with the formula $[HexNAc]_2[Hex]_n[SO_3H]$ where HexNAc = *N*-acetylhexosamine, Hex = hexose, and "SO_3H" is a sulfate group on either a Hex or HexNAc. For these compounds, the number of Hex (n) varied from 0 to 3. The spacing of the signals, in units of hexoses, as well as the observed masses all confirm that the signals belong to oligosaccharides. However, the exact identities of individual residues could not be determined due to limitations in sample amounts. This group of compounds was represented by the ions (theoretical mass in parentheses) m/z 505.049 (505.133), 667.120 (667.186), 829.169 (829.239), and 991.186 (991.291), which corresponded to the deprotonated parent $[M-H]^-$. They were found in all three examples shown in Figure 1. The series did not appear to extend beyond $n = 3$, as $n = 4$ (m/z 1153) was not observed. The remainder of the peaks corresponded either to matrix signal or peptides. A second type of control sample (Type B) yielded the mass spectra shown in Figure 2 in the negative mode using MALDI FT ICR. These spectra did not appear to contain significant abundances of oligosaccharide signals, but primarily matrix ions and peptides.

A third group of normals consists of four individuals (Type C). This individual (C503) showed peaks that were consistent with rosacea (vide infra). It is possible that this person either has been misdiagnosed or may contain some other ailments. Three individuals yielded mass spectra that showed oligosaccharides that are few in number and weak in abundances and similar to those in Type A.

Tear fluid samples from rosacea patients yielded distinctive clusters of peaks that extend to higher masses. To probe the reproducibility of the sample preparation, one patient was analyzed with 3 μL and 30 μL of tear fluids. Both spectra yielded identical results (not shown) indicating that the smaller sample size was sufficient for the analysis and that the analysis is highly reproducible. Representative spectra are shown for the 20% fractions (Figure 3a,b,c). The 40% fraction shows similar characteristics but with some different masses. The region between m/z 800 and m/z 1800 is expanded in Figure 3a. A simple visual comparison between the control and patient samples shows that the control samples do not have masses extending beyond m/z 1000, while those from the patient samples extend to as high as m/z 2000.

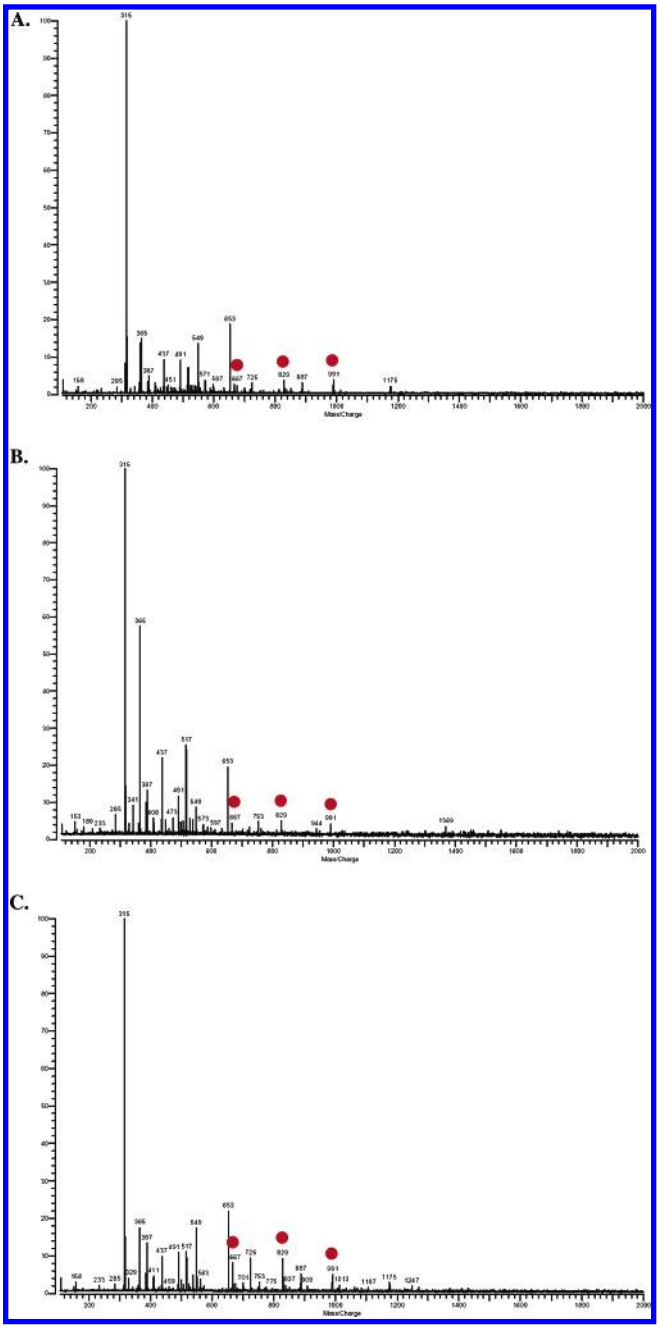

Figure 1. MALDI−FTMS spectra of control tear sample (A type) in negative mode. (a), (b), and (c) show different individual person. The solid circles indicate peaks corresponding to sulfated oligosaccharides. Please see Table 2 for additional information.

The examples provided in Figure 3 illustrate some heterogeneity among the rosacea patients. In Figure 3a, the dominant signals were due to sialylated oligosaccharides (Series 2, Table 2). In Figure 3b, the dominant series corresponded to sulfated oligosaccharides, whereas in Figure 3c, the dominant series were hexuronic acid oligomers.

As with some of the control samples, a series with the empirical formula $[HexNAc]_2$:$[Hex]_n$:$[SO_3H]$ (series 1) was also observed. This series of peaks appeared in both control and patient samples. Interestingly, they were found in 13 of the 16 patient samples. The major distinguishing features of the samples from rosacea patients compared to the control samples were the presence of several oligomeric series that were found

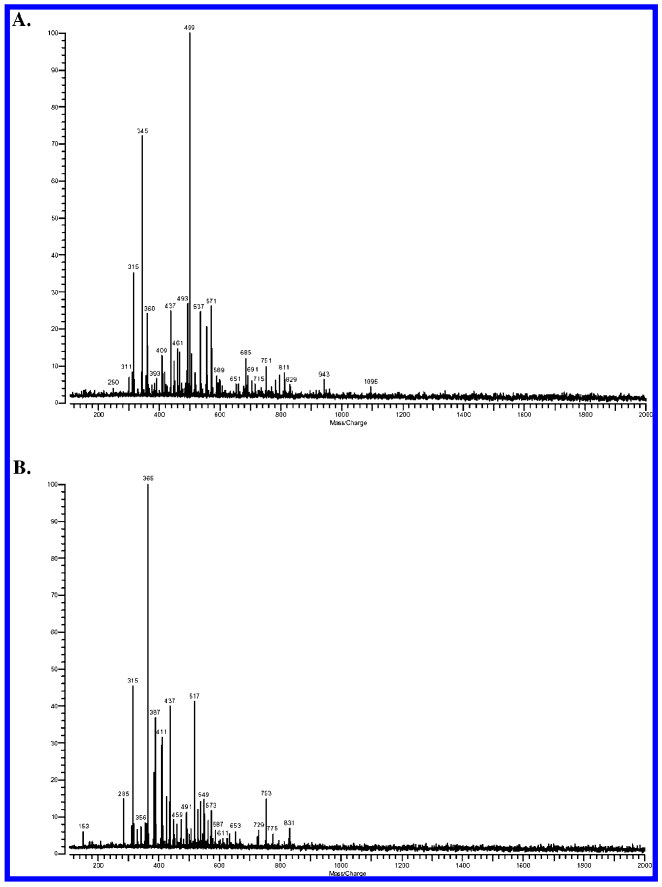

Figure 2. MALDI−FTMS spectra of control tear sample (B type) in negative mode. (a) and (b) show different individual person.

only in the rosacea patients. These series are listed in Table 2. The list was produced by compiling all oligomeric series found in either 20% or 40% fractions. Often, the series were found in both. At least 13 series were identified including series 1—some with the complete composition determined while others with compositions only partially known. Series 2 has the composition $[HexNAc]_3[NeuAc]_2[Hex]_n$ ($n = 1-3$) with the masses 1231, 1393, 1555, and 1717. The mass difference is equivalent to exactly one hexose group (162.013 − experimental, 162.052 − theoretical) NeuAc represents *N*-acetylneuraminic acid (sialic acid). A series of peaks (series 3) containing NeuGc was observed with the composition $[HexNAc]_1[NeuGc]_3[Hex]_n$ ($n = 2-5$). N-Glycolylneuraminic acid (NeuGc) is another sialic acid whose source remains uncertain [16]. There are a number of reports that claim NeuGc is obtained primarily through exogenous sources usually through diet[17]. For example, chimpanzees produce NeuGc, while humans do not. Others have also reported that NeuGc increased in cancer cells[18]. A second set of sulfated oligosaccharides (series 4) were observed with the composition $[HexNAc]_2[HexA]_1[Hex]_n[SO_3H]$ ($n = 0-2$). The presence of this specific sulfate oligomer needs further confirmation as the mass is 0.120 mass units from the theoretical.

A group of anionic oligosaccharides containing hexuronic acid (HexA) residues were also observed. This group includes series 4 (which also contains HexA), 5, 6, 12, and 13. A prominent member of this group is series 5 with an unknown headgroup and elongated by an increasing number of hexuronic acids, m/z 607+$[HexA]_n$ ($n = 1-5$). This series contained an unknown headgroup and extended to as many as five (and possibly more) hexuronic acid residues. The sample amounts

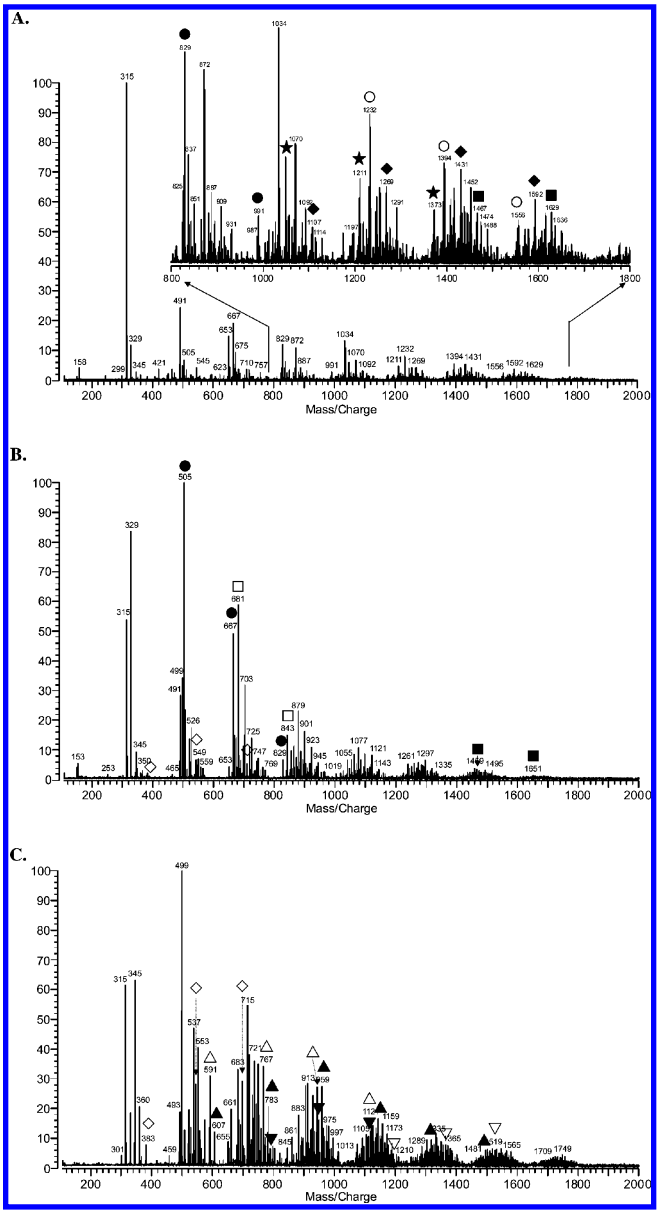

Figure 3. Representative MALDI−FTMS spectrum of rosacea patient tears which having dominantly (a) sialylated oligosaccharides series, (b) sulfated oligosaccharides series, and (c) hexuronic acid oligosaccharides series in negative mode. The labels correspond to peaks listed in Table 2.

were insufficient for performing tandem MS, which would have provided structural information on the m/z 607 headgroup. This large mass could contain at least three more HexA residues with possibly one being modified. A number of these series exist with an unidentified (as of yet) headgroup. Another example is the hexuronic acid series, which includes 6 with m/z 591+$[HexA]_n$ ($n = 1-3$). The structures of these unknown headgroups will be the topic of future publications.

The presence of these highly anionic oligosaccharides was supported by the intense signal in the negative mode corresponding to the deprotonation of the carboxylic acid. More definite indications were the presence of satellite peaks 22 mass units apart, which were due to carboxylic acids where the acidic hydrogen is replaced by sodium. These oligosaccharides behaved in nearly identical manners to HexA oligomers from other biological sources[19] and from standard samples obtained commercially.

Table 2. List of Oliogomeric Series that Were Found in the Rosacea Patients[a]

series no.	exp. mass	theor. mass	OS composition	comments
1 (●)	505.049	505.133	$[HexNAc]_2[SO_3H]$	m/z, $[M-H]^-$
	667.120	667.186	$[HexNAc]_2[Hex]_1[SO_3H]$	
	829.167	829.239	$[HexNAc]_2[Hex]_2[SO_3H]$	
	991.186	991.291	$[HexNAc]_2[Hex]_3[SO_3H]$	
2 (○)	1230.504	1230.415	$[HexNAc]_3[NeuAc]_2$	m/z, $[MNa-2H]^-$
	1392.511	1392.468	$[HexNAc]_3[NeuAc]_2[Hex]_1$	
	1554.531	1554.520	$[HexNAc]_3[NeuAc]_2[Hex]_2$	
	1716.578	1716.573	$[HexNAc]_3[NeuAc]_2[Hex]_3$	
3 (■)	1467.574	1467.474	$[HexNAc]_1[NeuGc]_3[Hex]_2$	m/z, $[M-H]^-$, $[MNa-2H]^-$ 1489.597
	1629.590	1629.527	$[HexNAc]_1[NeuGc]_3[Hex]_3$	m/z, $[M-H]^-$, $[MNa-2H]^-$ 1651.642
	1791.615	1791.580	$[HexNAc]_1[NeuGc]_3[Hex]_4$	m/z, $[M-H]^-$
	1953.630	1953.633	$[HexNAc]_1[NeuGc]_3[Hex]_5$	m/z, $[M-H]^-$
4. (□)	681.046		$[HexNAc]_2[HexA]_1[SO_3H]$	m/z, $[M-H]^-$, $[MNa-2H]^-$ 703.031
	843.065		$[HexNAc]_2[HexA]_1[Hex]_1[SO_3H]$	m/z, $[M-H]^-$, $[MNa-2H]^-$ 865.038
	1005.096		$[HexNAc]_2[HexA]_1[Hex]_2[SO_3H]$	m/z, $[M-H]^-$, $[MNa-2H]^-$ 1027.070
5. (▲)	606.892			m/z?
	782.918		$607+[HexA]_1$	
	958.943		$607+[HexA]_2$	
	1134.997		$607+[HexA]_3$	
	1311.027		$607+[HexA]_4$	
	1487.042		$607+[HexA]_5$	
6 (△)	590.916			
	766.937		$591+[HexA]_1$	
	943.966		$591+[HexA]_2$	
	1119.002		$591+[HexA]_3$	
7 (◆)	1107.131			
	1269.028		$1107+[Hex]_1$	
	1431.101		$1107+[Hex]_2$	
8 (◇)	382.958			
	544.918		$383+[Hex]_1$	
	707.013		$383+[Hex]_2$	
9 (▼)	788.927			
	950.972		$789+[Hex]_1$	
	1113.010		$789+[Hex]_2$	
10(▽)	1193.919			
	1356.016		$1194+[Hex]_1$	
	1518.044		$1194+[Hex]_2$	
11(★)	1049.052			
	1211.061		$1049+[Hex]_1$	
	1373.082		$1049+[Hex]_2$	
12	996.991			
	1173.090		$997+[HexA]_1$	
	1349.201		$997+[HexA]_2$	
13	575.951			
	751.012		$576+[HexA]_1$	
	927.070		$576+[HexA]_2$	
	1103.161		$576+[HexA]_3$	

[a] The masses correspond to a single set of data but are representative of all the others. The table was pooled from several sample spectra.

Additional series of oligosaccharides contain an unknown headgroup with elongated changes of just hexoses (Hex) (series 7, 8, 9, 10, and 11). On the basis of the intensity of these signals in the negative spectra, they are likely to contain an anionic headgroup, possibly a carboxylic acid or a sulfate ester, as hexoses generally do not show strong signals in the negative mode.

All 16 patient samples show high degrees of polymerization not observed in the control samples. Series 1, which is present in 33% of the control samples, are found in 13/16 (81%) of the patient sample. There is some diversity in the oligosaccharide content of the patient sample as illustrated in Table 3, where the oligosaccharide series found in each sample are listed. Rosacea patients do not contain a single set of markers. However, the tear samples from rosacea patients are high in hexuronic acid with their oligomers found in some form in many (12/16 or 75%) of the patient samples (series 5, 6, 12, and 13). Of the HexA oligomers series 6 is the most common, which is found in 8 (50%) of the 16 samples.

Discussion

Oligosaccharide Composition of Tear and Mucins. On the basis of the compositional analyses of tear samples released by standard O-link procedures, human tear fluids from rosacea patients contain a large number of O-linked oligosaccharides. The compositions (number of residues) can be determined in whole or in part based on the accurate mass. Because the method used to release oligosaccharide release primarily O-linked oligosaccharides, they are likely to originate from mucins. The inspection of a number of the components using GlycoSuite provides important insight into the nature of the oligosaccharides. The species with m/z 667.120 and the composition $[HexNAc]_2[Hex][SO_3H]$ is found to correspond to 10 known structures in mammal—all associated with mucins.[20] In humans, two examples are found with the structures

HSO_3-6Galβ1-3GlcNAcβ1-3GalNAc

HSO_3-6Galβ1-4GlcNAcβ1-3GalNAc

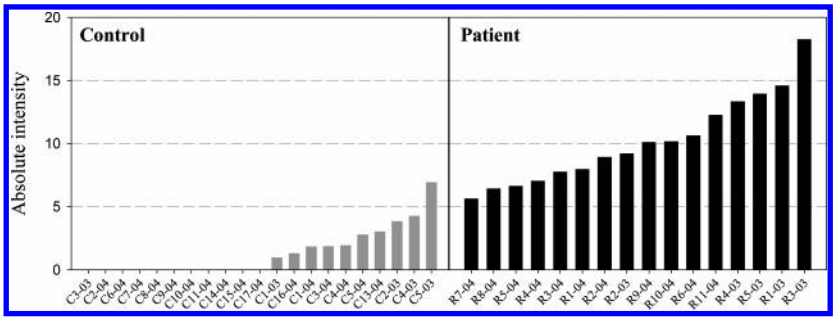

Figure 4. Sum of the intensities of anionic oligosaccharides listed in Table 2 grouped in ascending order for control (left box) and patient (right box) samples.

Table 3. Series of Oligosaccharides Found in Rosacea Patient Tear Fluid[a]

sample	OS series no.	most abundant OS
R104	2,3,6,7,13	NeuAc/NeuGc
R303	1(w),2,3,7,11	NeuAc/NeuGc
R203	1,4,7,8	sulfated
R403	1,3(w),4,7(w),8	sulfated
R304	1,4,8(w)	sulfated
R704	1,4(w),11,12	sulfated
R804	1,4,8(w),11(w),13	sulfated
R503	1,4,6(w),7(w),8,11	sulfated
R504	1,4,8,12	sulfated
R404	1,4,11,12	sulfated
R103	1,4(w),5,6,8,13	HexA
R204	1,5,6,13	HexA
R904	1(w), 5,6,13	HexA
R604	5,6,8,9,12,13	HexA
R1004	1(w),5,6,8,9,10,13	HexA
R1104	5,6,8,9,10,13	HexA

[a] The "w" symbolizes weak signals (10%) relative to the other signal in the series.

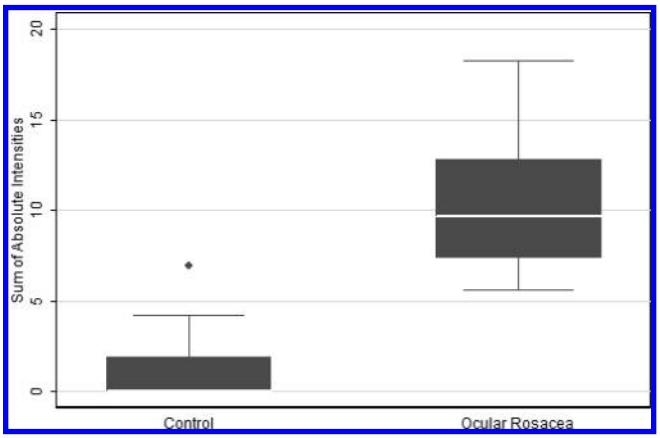

Figure 5. Box plots of the sum of the absolute intensities of the anionic oligosaccharides for the control and the ocular rosacea groups, respectively.

The second member of series 2, m/z 1392.511, with the composition $[HexNAc]_3[NeuAc]_2[Hex]_1$ yielded 20 structures all mucins and nearly all found in humans.[21] The human examples include structures found in colonic and other mucins and in plasma and serum as, for example, O-linked oligosaccharides attached to choriogonadotropin beta chain.

A member of series 3, $[Hex]_2[HexNAc]_1[NeuGc]_3$ has one example of eggs in Whitespotted Char, but not in humans. To the best of our knowledge, no NeuGc-containing oligosaccharide has been characterized in humans.

While the accurate mass may provide oligosaccharide compositions, there is no structural information currently available from the mass spectral data alone. Tandem MS in the form of collisional-induced dissociation (CID) or infrared multiphoton dissociation (IRMPD) will be useful for providing structural information. However, the size of the oligosaccharides and the comparison with similar size oligosaccharides all point to the presence of mucins.

There have been a number of studies on proteins, glycoproteins, and mucin glycoproteins in tears. However, there are no reported studies to our knowledge of oligosaccharides in human tears. There is a study of sialic acids in tear fluid, but this study only looked for this specific residue and not whether it was N- or O-linked oligosaccharides. The presence of sialic acid has been used as an indicator of expression of mucin glycoproteins in tears.[22] Nakamura et al. found direct correlation between the concentration of liberated sialic acid and the concentration of glycoproteins. The major component of the glycoprotein was found to be mucins. In the control samples, there were no detectable levels of sialic acid in the tears. The only oligosaccharides observed were sulfated oligosaccharide, present in nearly 50% of the control samples. In the tear samples from the rosacea patients, all contained a large amount of sialic acid, supporting the notion that mucin glycoproteins are highly expressed in patients with rosacea. It appears that those patients with rosacea have particularly high levels of mucin oligosaccharides and hence mucins.

Diagnostic of Rosacea in Patients. The high levels of oligosaccharides, specifically anionic oligosaccharides, in the tear fluids of patients with rosacea are simple diagnosis for the disease. Even more precise determination should be possible based on the presence of specific oligomers. Shown in Figure 4 is the sum of the absolute intensities of the anionic oligosaccharides (including sulfated, sialylated, and HexA oligosaccharides) in the control and the patient sample arranged in increasing order. For nearly half of the control samples, no signal corresponding to anionic oligosaccharides were obtained suggesting that abundances of these compounds in these samples are lower than the detection limit of the MS method. The patient sample has significantly more anionic oligosaccharides that readily distinguish it from the control samples.

The sum of absolute intensities of 13 series with the corresponding m/z values (Table 2) was proposed to classify the patients for ocular rosacea. These m/z values have been identified according to their discriminatory pattern in the sample. The distributions of the sum of the absolute intensities are shown in Figure 5 as the box plots for the control group and the ocular rosacea group, respectively. There is one outlier in the control group with relatively larger value compared to the rest of the group. With the exception of this person, all

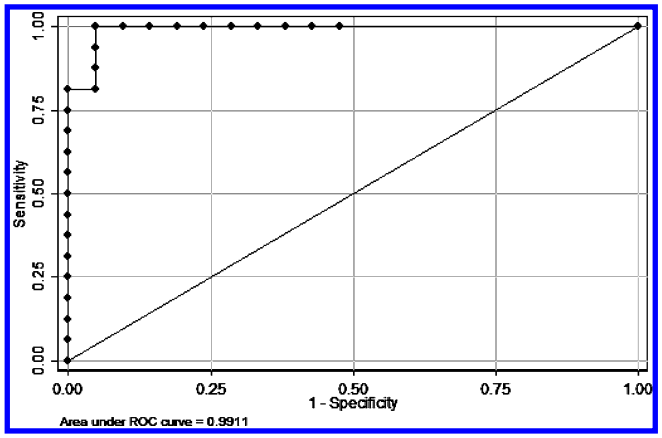

Figure 6. Receiver operating characteristic (ROC) curve of using the sum of the absolute intensities of the anionic oligosaccharides as the biomarker. The area under the ROC curve is 0.9911 with 95% confidence interval (0.9712, 1.000).

values of the control groups are less than the minimum value of the ocular rosacea cases. This suggested that the sum of 13 absolute intensities is a good biomarker for identifying ocular rosacea patients. Using the value 5.60 as the cut-point, 36 of 37 were correctly classified. This yields a sensitivity of 100% (95% CI 79.5−100) and specificity of 95.2% (95% CI 76.2−99.9). Figure 6 shows the receiver operating characteristic (ROC) curve of using the sum of the absolute intensities of the anionic oligosaccharides for identifying ocular rosacea. The area under the ROC curve is 0.99 (95% CI 0.97−1.00).

The general increase of anionic oligosaccharides may not necessarily be specific to rosacea. Other diseases may also cause increase in mucin levels. However, there are a number of oligosaccharides present in the patients that may be more specific markers for the disease. Hexuronic oligomers, for example, may be more specific indicators of the disease. The nature of the study design was exploratory in terms of identifying leads for potential useful biomarkers in the tear fluid of ocular rosacea patients. To investigate further the ability of abundant oligosaccharides in distinguishing ocular rosacea from normal patients, statistically rigorous study designs such as case-control study will be implemented for future research.

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