Mass spectrometry-based approach for development of biomarkers in IgA nephropathy: a pilot trial

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Abstract

IgA1 with galactose (Gal)-deficient hinge-region (HR) O-glycans (Gd-IgA1) plays a key role in IgA nephropathy (IgAN), and the serum level of Gd-IgA1 is elevated in the majority of IgAN patients. To characterize the involvement of IgA1 in the development and progression of IgAN, O-glycan micro-heterogeneity and attachment sites need to be analyzed, as each HR has nine potential sites for O-glycosylation.

We have developed an on-line liquid chromatography (LC) hybrid quadrupole mass filter/linear ion trap/orbitrap mass spectrometry (MS) protocol, which was used to analyze IgA1 from a patient with IgAN. LC-MS profiling provided the overall O-glycan micro-heterogeneity distribution of IgA1 HR O-glycoforms. The LC-extracted ion chromatogram (XIC) of HR O-glycoforms containing Gal-deficient O-glycans indicated that the Gal-deficient O-glycans attached at specific sites. Structural isomers based on changes in the amino acid position of the attached glycans were identified in relation to the IgA1 HR O-glycoforms containing Gal-deficient O-glycans. To identify the predominant O-glycoforms in the serum IgA1 from IgAN patients as candidate biomarkers, O-glycan micro-heterogeneity and attachment sites, including isomeric structures, need to be analyzed.

Our MS-based approach is useful in this respect and should prove a valuable tool for the development of biomarkers for IgA1 HR O-glycosylation in IgAN.

Keywords: IgA nephropathy, O-glycosylation, Biomarkers, High-resolution mass spectrometry, Glycopeptide isomers

Introduction

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide. Patients with IgAN present with a variety of clinical, laboratory, and histopathological findings. The histological findings are essential, not only for diagnosis, but also for predicting renal prognosis, as approximately 20% of patients with IgAN develop end-stage kidney failure approximately 20 years after diagnosis. However, because renal biopsy is associated with potential procedural risks, development of a non-invasive diagnostic and prognostic test is desirable for clinical purposes.

IgAN is characterized by proliferative changes in the glomerular mesangial cells and matrix along with mesangial deposits mainly consisting of IgA1 and not IgA2. The serum level of abnormally glycosylated IgA1 is generally elevated in patients with IgAN.1–3 This aberrantly glycosylated IgA1 is recognized by anti-glycan antibodies,4–6 resulting in the formation of IgA1-containing circulating immune complexes. Subsequently, these immune complexes are deposited to the mesangium, leading to activation of the mesangial cells, ultimately resulting in glomerular injury. This mechanism is known as the multi-hit pathogenic pathway in the IgAN hypothesis (Figure 1A).7,8 The aberrantly glycosylated IgA1 is produced by IgA1-secreting cells.9 Although the precise mechanism for the elevation in serum levels of aberrantly glycosylated IgA1 in patients with IgAN has not been elucidated, alterations in glycosyltransferase activity in IgA1-secreting cells may play an important role in the production of aberrantly glycosylated IgA1. Some genes encoding glycosyltransferases were found to be involved in a genome-wide association study of IgAN.10 Human serum IgA1 possesses a 19-amino acid hinge region (HR) with nine potential O-glycosylation sites, three to six of which are usually occupied by core 1-type O-glycans.11–13 The O-glycans of IgA1 are synthesized in a step-wise manner by glycosyltransferases in the Golgi apparatus of IgA1-secreting plasma cells.14 N-acetylgalactosamine (GalNAc) attaches to serine (Ser) or threonine (Thr) residues in the HR. The O-glycan chain can be extended by attachment of galactose (Gal) to the GalNAc residues, and GalNAc or Gal can also be sialylated. Thus, IgA1 HR O-glycoforms have the potential to show wide diversity in terms of their glycan attachment sites and glycan structures (Figure 1C).

Gal-deficient IgA1 (Gd-IgA1) occurs when some of the O-glycans present in the HR of IgA1 lack Gal, thus leaving the terminal GalNAc exposed. Helix aspersa agglutinin (HAA), a terminal GalNAc-specific lectin, is commonly used for the
detection of an IgA1 HR with Gal-deficient O-glycans. The levels of Gd-IgA1 are elevated in the circulation of patients with IgAN, and Gd-IgA1 plays a key role in the pathogenesis of IgAN. Therefore, the detection of Gd-IgA1 in IgAN patients has become a significant area of interest in biomarker development for IgAN. HAA and, more recently, monoclonal antibodies against synthetic glycopeptides are currently applied for the detection of Gd-IgA1. However, the HAA lectin-based assay has limitations. First, the bioactivity and stability of HAA depends on the production rate of HAA lectins. We have recently developed monoclonal antibodies against a synthetic hinge-peptide with glycans attached at five sites corresponding to the positions where GalNAc residues are frequently located in the HR. The binding levels of the antibodies were found to be well correlated with the HAA-ELISA levels. Moreover, we showed the significant difference in the antibody titers between IgAN patients and controls implies that the specific sites that are deficient in Gal are increased only in IgAN patients. However, the terminal GalNAc at the attachment sites that was recognized by these antibodies was not identified and it remains unknown whether these antibodies can effectively detect IgA1 with Gal-deficient O-glycans, which is unique to patients with IgAN. Therefore, to characterize the dominant structure that uniquely increases in IgAN, the O-glycan microheterogeneity and attachment sites, including their isomeric structures, needs to be analyzed.
High-resolution mass spectrometric analyses have been applied to characterize the O-glycosylation of IgA1 at the molecular level.\textsuperscript{12,13,14,16,21,22} Here, we demonstrate HR O-glycan micro-heterogeneity and isomer distribution in the serum IgA1 from a patient with IgAN. We developed a new mass spectrometry (MS)-based method to delineate the structure of aberrantly glycosylated IgA1 at the molecular level, with the goal of identifying new biomarkers and targets for intervention (Figure 1D).

Methods

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Fujita Health University (reference number HM11-130). Written informed consent and a serum sample from a patient with IgAN was obtained at the time of renal biopsy.

Isolation of IgA1 and preparation of proteolytic fragments

Serum IgA1 from the plasma of a patient with biopsy-proven IgAN was purified using affinity chromatography with anti-human IgA (Cappel Laboratories, Cockranville, PA, USA) coupled to a CNBr-Sepharose column.\textsuperscript{12} Purified IgA1 protein was treated with 0.25 U/ml neuraminidase A (ProZyme, Hayward, CA, USA) in 50 mM sodium phosphate, pH 6.0, to remove sialic acid residues from the O-glycan chains.\textsuperscript{4} After neuraminidase treatment, the preparation was reduced with 10 mM dithiothreitol. Then, trypsin (Promega, Madison, WI, USA) was added for 8 h at 37°C in 100 mM NH\textsubscript{4}HCO\textsubscript{3}, pH 8.3. The digests were desalted using a C18 spin column (Thermo Fisher Scientific, San Jose, CA, USA) before MS analysis.

On-line liquid chromatography (LC) hybrid quadrupole mass filter/ linear ion trap/orbitrap MS

On-line LC was performed using an EASY-nLC 1000 system (Thermo Fisher Scientific). A total of 100 ng of digested IgA1 was loaded onto a 75 \mu m \times 15 cm PepMap C18 EASY-Spray column (Thermo Fisher Scientific). The digests were then eluted with an acetonitrile gradient from 5% to 20% in 0.1% formic acid over 50 min at a rate of 300 nl/min. Hybrid quadrupole mass filter/linear ion trap/orbitrap MS (Orbitrap Fusion, Thermo Fisher Scientific) was alternated between a full OT MS scan (m/z 500–1700) at a resolving power of 120,000, and a subsequent MS/MS scan of the abundant precursor ions. The precursor ions were isolated and subjected to collision-induced dissociation with 35% energy in the linear ion trap as confirmation of the presence of O-glycopeptide precursor ions. The isolation width was m/z 1.6. Automatic gain control was used to accumulate sufficient precursor ions (target value, 10,000 ions; maximum injection time, 35 ms). Dynamic exclusion was enabled with the exclusion window set to 10 ppm with an exclusion time of 60 s after a repeat count of 1 within 30 s.

Data analysis

All spectra were analyzed using Xcalibur Qual Browser 2.2 (Thermo Fisher Scientific) software. Individual IgA1 O-glycopeptides were identified as previously described.\textsuperscript{23,24,25} In brief, IgA1 O-glycopeptide species in each LC-MS analysis were identified with reference to the known sequence of the isolated HR glycopeptides, calculated monoisotopic mass of glycopeptides, and the presence of adjacent HR glycopeptides within the high-resolution MS spectra. Monoisotopic m/z values for the IgA1 O-glycopeptides were manually tabulated from the raw data files using Xcalibur Qual Browser 2.2. Known IgA1 HR amino acid sequences based on the tryptic digestions were used as input. Hexose, N-acetylhexosamine (HexNAc), and N-acetylluramic acid (NeuAc) monosaccharide residues were all selected as possible (variable) additions to the IgA HR peptides with a mass tolerance of 5 ppm. After assigning all glycopeptide peaks in the spectrum, the ion chromatogram for each glycopeptide ion was individually extracted for the specific m/z of the glycopeptide ion species. Each glycopeptide’s extracted-ion chromatogram (XIC) was used for identification of structural isomers based on alternative glycan-attachment sites in the backbone amino acid sequence.

Results and Discussion

For the analysis of serum IgA1 isolated from a patient with IgAN, the purified IgA1 was treated with neuraminidase followed by trypsin digestion. The digests, with the released series of HR O-glycopeptides (backbone amino acids: His\textsuperscript{208–Arg}\textsuperscript{245}), were subjected to on-line LC hybrid quadrupole mass filter/linear ion trap/orbitrap MS. The number of O-glycan chains was assigned based on the masses of the amino acid sequence His\textsuperscript{208–Arg}\textsuperscript{245} (3964.8182 Da), GalNAc (203.0794 Da), and Gal (162.0528 Da). Thirteen glycopeptides with three to six O-glycans attached to the His\textsuperscript{208–Arg}\textsuperscript{245} backbone amino acids were detected (Figure 2). Nine of the thirteen glycopeptides possessed up to three Gal-deficient O-glycans. The distribution of HR O-glycoforms was similar to the O-glycan profile in myeloma IgA1, which resembles Gd-IgA1 in IgAN.\textsuperscript{13,14,16,21} Gal-deficient O-glycans attach at specific sites in normal human serum IgA1.\textsuperscript{8,13} There are also multiple O-glycoform isomers, i.e., HR glycopeptides with the same number of glycans, but with different sites of attachment.\textsuperscript{13} Analysis of the LC-extracted ion chromatogram (XIC) of O-glycoforms can be used to detect these isomeric IgA1 HR O-glycoforms. The XICs of the nine O-glycoforms containing Gal-deficient O-glycans were individually extracted to obtain the specific m/z of the glycopeptides (Figure 3). A single peak was observed for the GalNAc3Gal2 and GalNAc6Gal3 His\textsuperscript{208–Arg}\textsuperscript{245} glycoforms, whereas bimodal peaks were observed for GalNAc4Gal2G, GalNAc4Gal3, GalNAc5Gal2, GalNAc5Gal3, GalNAc5Gal4, GalNAc6Gal4, and GalNAc6Gal5 His\textsuperscript{208–Arg}\textsuperscript{245} glycopeptides. The bimodal XIC peaks of the O-glycoforms indicate the presence of two isomeric O-glycoforms based on the O-glycan amino acid position.

Characterization of the molecular details of the O-glycan heterogeneity, including isomeric variants, related to serum IgA1 is an important aspect for biomarker development in IgAN. A mass spectrometric approach to characterize IgA1 HR O-glycoforms has been performed previously.\textsuperscript{23,24} However, most of these studies used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS for the detection of IgA1 HR O-glycoforms.

Although IgA1 HR O-glycoforms with Gal-deficient O-glycans were successfully detected in these studies, there are limitations to this method. First, MALDI-TOF MS shows a lack of consistency in reproducing results between studies.\textsuperscript{29,30} Second, there is a poor correlation between molecular abundance and quantitative measurements.\textsuperscript{31} Thus, MALDI-TOF MS has not been widely applied in research and clinical settings for analysis of the heterogeneity of IgA1 HR O-glycoforms. Recently, a
Figure 2  Mass spectra of IgA1 hinge region (HR) O-glycoforms. The number of O-glycan chains was assigned based on the masses of the amino acid sequence (His208–Arg245), GalNAc (open square), and Gal (close circle). Individual peaks were assigned to glycopeptides. The numbers of GalNAc and Gal molecules are shown above these individual peaks. Glycopeptides were ionized as 4+-charged ions. The number of O-glycan chains attached in the HR corresponds with the number of GalNAc residues. For example, the HR with GalNAc3Gal2 has two disaccharides and one GalNAc monosaccharide. The HR with GalNAc3Gal3 has three disaccharides. Three to six O-glycan chains with zero to three GalNAc molecules as monosaccharides are attached to the HR.

Figure 3  Extracted ion chromatogram (XIC) of IgA1 hinge region (HR) O-glycoforms containing Gal-deficient O-glycans. The ion chromatogram for each glycopeptide ion was individually extracted for the specific m/z of each glycopeptide ion species for identification of structural isomers based on alternative glycan-attachment sites in the backbone amino acid sequence.
relative quantitative workflow was developed for the analysis of IgA1 HR O-glycoforms, including for identification of the attachment sites of O-glycans, using high-resolution MS.\textsuperscript{13,14,16,21}

In the current study, we have provided the first demonstration of the O-glycoform profile and isomeric distribution of the IgA1 HR derived from a patient with IgAN using hybrid quadrupole mass filter/linear ion trap/orbitrap MS. We found that the serum IgA1 from the IgAN patient contained a high amount of O-glycoforms with GalNAc monosaccharides without attached Gal (Figure 2), which included glycan positional isomers. Seven of the nine O-glycoforms with GalNAc monosaccharides had two isomers, and the remaining two O-glycoforms had a single structure. Therefore, a total of 16 O-glycoforms with Gal-deficient glycans were detected. Importantly, the area under the curve of the O-glycoforms can provide quantitative information on the isomers. Once the glycan attachment sites are determined by electron-transfer dissociation MS/MS, comparison of the isomeric distributions will be easy to accomplish in large numbers of subjects.

Conclusions

Our results show that adoption of a mass spectrometric approach using high-resolution MS coupled with LC is a powerful method to identify specific glycoforms and/or specific sites for Gal-deficient glycans in patients with IgAN.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Authors’ Contributions

AK, HY, HH, SK, DI, MH, YH, YYu, and KT contributed to the experimental design. AK, YH, and KT analyzed and interpreted the data. AK, HY, YYo, and KT performed the experiments. AK, HY, HH, SK, MH, YYu, and KT provided expertise and editing. All authors read and approved the final manuscript.

References


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