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Quantitative change of IgA hinge *O*-glycan composition is a novel marker of therapeutic responses of IgA nephropathy

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ABSTRACT

Aberrant *O*-glycosylation in the hinge region of serum IgA is suggested to be involved in the pathogenesis of IgA nephropathy (IgAN), because the hypoglycosylation including *N*-acetylneuraminic acid or galactose has been reported in the mucin-type *O*-glycan of the hinge portion (HP) of IgA deposited in the IgAN patients' kidney. These aberrant glycosylation has been assessed in most of the previous reports by qualitative but not quantitative methods. In the present study, the molar ratios of GalNAc or Gal to HP were analyzed for serum IgA from IgAN patients. The GalNAc/HP ratio was increased in the patients who achieved remission after a combination therapy of tonsillectomy and intravenous corticosteroid, suggesting any non-innate factors to affect the IgA *O*-glycosylation in IgAN that is thought to be inherently determined. Furthermore, the *O*-glycosylation status was different among three groups: IgAN patients in the pretreatment stage, IgAN patients in the remission stage after treatment and healthy controls. These results indicated that aberrant *O*-glycosylation of serum IgA in the IgAN patients would be inherently present and, to some extent, affected by therapeutic intervention. Finally, the quantitative change of *O*-glycan composition is a novel marker of therapeutic response of IgAN.

1. Introduction

Immunoglobulin A (IgA) nephropathy (IgAN) is the most common form of primary glomerulonephritis in the world [1]. IgAN is characterized pathologically by IgA deposition to glomerular mesangial cells and clinically by macroscopic hematuria after upper respiratory tract infection such as tonsillitis. Tonsil is one of the important immune organs constituting the ring of Waldeyer whose function is considered to be closely related to mucosal immunity. Therefore, tonsillectomy has been focused as a new strategy to treat IgAN. Some small observational studies reported that the tonsillectomy brought about the disappearance of proteinuria to approximately half of the patients in 2–3 years [2,3]. Recently, combination of tonsillectomy and steroid pulse therapy has been reported to be effective [4–6] and even tonsillectomy alone was reported to have a long-term favorable effect to the IgAN [7]. However, the mechanical basis of these therapies is unclear and the therapeutic marker has not been established.

Although the etiology of IgAN remains a mystery despite intensive investigations, aberrant *O*-glycosylation in the hinge portion of serum IgA is thought to be deeply involved in the pathogenesis because the deposited IgA in the kidney is hypo-glycosylated [8,9]. Aberrantly glycosylated IgA has a adhering property to extracellular matrix proteins [10]. The main characteristics of these deposited IgA are polymeric and aberrantly glycosylated IgA1. Polymeric IgA are actively produced at mucosal surface, and focal infection at mucosal surface is involved in the pathogenesis. Therefore, removal of focally infected mucosal site, typically of palatine tonsils, is the presumed rationale for the therapy of tonsillectomy.

There are many reports elucidating the aberrant *O*-glycosylation in the hinge region of serum IgA in patients with IgAN, such as the hyposialylation, reduced galactose number and reduced GalNAc number [11–16]. Although some of the previous reports using MALDI-TOF-MS are essentially quantitative such as Hiki's report [15] that focused on the specific glycosylation patterns; the ratio of (HP + 5GalNAc + 4Gal)/(HP + 4GalNAc + 4Gal) after sialidase treatment, most of the reports are carried out on a qualitative basis.

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This prompted us to quantitate the content of *N*-acetylgalactosamine (GalNAc) and galactose (Gal) of the *O*-glycans of IgA HP by employing the method reported previously for analysis of IgA1 *O*-glycans in rheumatoid arthritis [17]. This method allowed us to reveal the average Gal and GalNAc number per HP after considering all the glycopeptide combination pattern of Gal and GalNAc, not limited to the specific glycopeptides as in Hiki's report [15]. This index is easy to understand the glycosylation status. Here, using the serum sample, we analyzed a change of *O*-glycosylation status in IgAN patients treated with tonsillectomy combined with intravenous corticosteroid. We also compared the *O*-glycosylation profile of pre-tonsillectomy IgAN, post-tonsillectomy IgAN patients and healthy individuals.

2. Materials and methods

2.1. Patients

We investigated 7 biopsy-confirmed IgAN patients, whose glomerular sclerosis and interstitial fibrosis were not severe and who underwent palatine tonsillectomy (TLX) combined with intravenous (IV) corticosteroid administration in Osaka University Hospital. Intravenous corticosteroid was administered seven to ten days after the tonsillectomy. All patients gave informed, written consent to participate in the study. The baseline characteristics of patients with IgAN are shown in Table 1. As a control group, we also investigated 30 healthy volunteer as described in our previous report [18].

Table 1
Baseline patient characteristics at palatine tonsillectomy. Data are expressed as mean ± standard deviation.

	IgAN
Age (Y.O.)	27 ± 8.8
F/M	6/1
sBP (mmHg)	105 ± 11
dBP (mmHg)	64 ± 7
Cr (mg/dl)	0.80 ± 0.16
eGFR (ml/min/1.73 m^2)	79 ± 19
IgA (mg/dl)	269 ± 69
UP/Ucr (g/g Cr)	0.93 ± 0.58

2.2. Quantitative analysis of O-glycosylation

We purified IgA from serum of IgAN patients and healthy volunteers as described previously [17,18]. Briefly, IgA samples were purified by affinity chromatography using a HiTrap NHS-activated HP column (GE healthcare, Fairfield, CT) coupled with polyclonal anti-IgA antibodies (DAKO, Glostrup, Denmark). Isolated IgA was dissolved in 6 M guanidine/0.25 M Tris–HCl, pH 8.0, reduced with 10 mM dithiothreitol and then *S*-carbamidomethylated with 20 mM iodoacetamide. After removal of reagents using a NAP5 column (GE healthcare, Fairfield, CT), IgA was digested by a mixture of lysylendopeptidase (Wako. Osaka, Japan) and trypsin (Sequence grade Modified Trypsin, Promega, Madison, WI) at 37 °C for 6 h. Then the glycopeptides in the digest were enriched by a hydrophilic affinity method using Sepharose CL4B as described previously [17,18]. The glycopeptides recovered in 50% (v/v) ethanol were dried by a SpeedVac concentrator. Desialylation of the glycopeptides were performed by incubation in 2 M acetic acid at 80 °C for 2 h, and acetic acid was removed by SpeedVac. The glycopeptides were then dissolved in 0.1% trifluoroacetic acid (TFA), and bound to ZipTipC18 (Millipore, Bedford, MA) followed by washing with 0.1% TFA and elution with 0.1% TFA/70% acetonitrile. Then, an aliquot of glycopeptide were mixed with the same volume of 10 mg/ml of 2,5-dihydroxybenzonic acid dissolved in 0.1% TFA/50% acetonitrile and analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). For MS, a Voyger DE Pro mass spectrometer (AB Sciex, Framingham, MA) equipped with a nitrogen laser was used in positive ion and linear TOF mode. The molar content of the component saccharides, GalNAc per hinge glycopeptides (GalNAc/HP) and galactose per hinge glycopeptides (Gal/HP), was calculated by the following equations:

$$(\text{Glyco})\text{peptide Peak}\% = [(\text{Glyco})\text{peptide Peak Intensity}] / [\text{Total}(\text{Glyco})\text{peptide Intensity}] \times 10^2 \quad (1)$$

$$\text{GalNAc/HP or Gal/HP (mol/hinge glycopeptide)} = \sum\{(\text{Glycopeptide Peak }\%) \times (\text{Number of GalNAc or Gal in the Glycopeptide})\} \times 10^{-2} \quad (2)$$

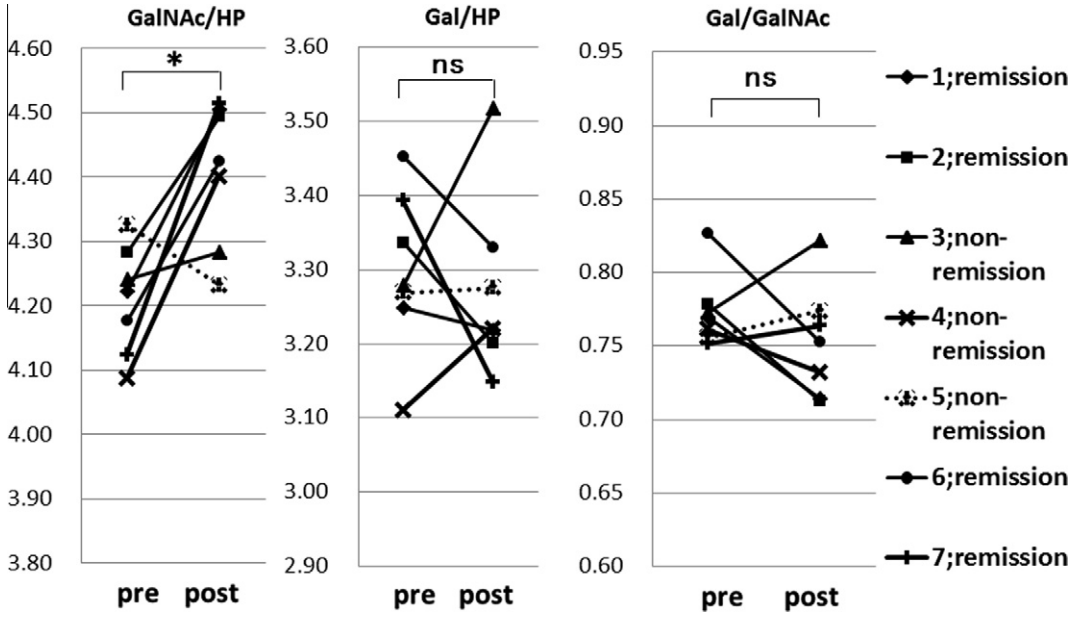

Fig. 1. Sugar change of *O*-glycosylation of IgA HP before and after the TLX + steroid IV in patients with IgAN. $^*p < 0.05$.

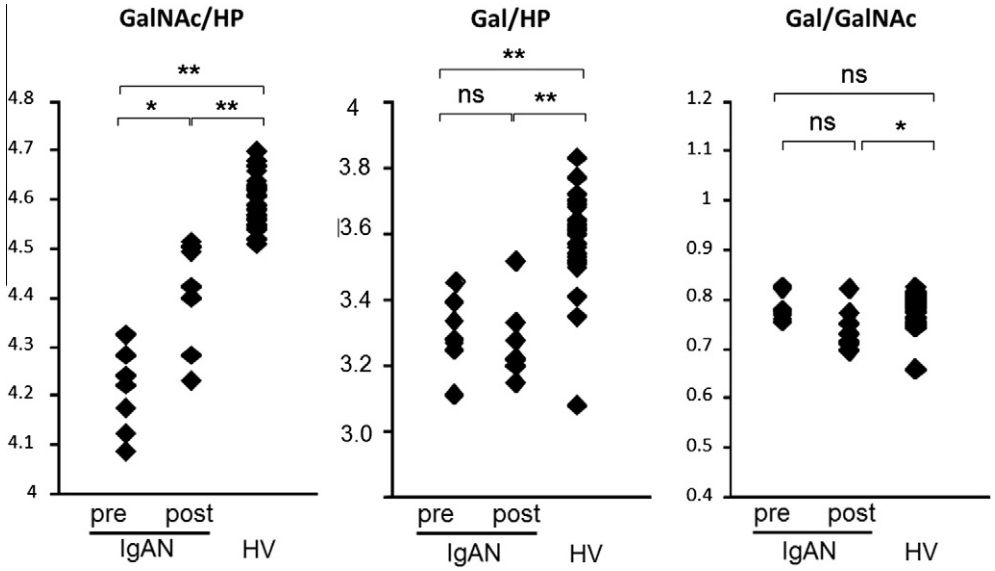

Fig. 2. *O*-glycosylation profile of IgA HP among pre-TLX + steroid IV IgAN patients, post-TLX + steroid IV IgAN patients and healthy volunteer. $^{*}p < 0.05$, $^{**}p < 0.001$.

In this study of human IgA1 *O*-glycosylation, we adopted the same method as reported in the previous reports [17]. The glycopeptides that were analyzed in the report [17] are already proved to be those carrying the hinge portion of IgA1.

2.3. Statistics

Statistical analysis was performed by JMP 5. We used paired *t*-test for comparison of pre-TLX + steroid IV IgAN vs. post-TLX + steroid IV IgAN, and unpaired *t*-test for pre-TLX + steroid IV IgAN vs. healthy volunteers and post-TLX + steroid IV IgAN vs. healthy volunteers.

3. Results and discussion

In the IgAN patients treated with a combination of palatine tonsillectomy and corticosteroid IV, the GalNAc content in HP was significantly increased from 4.21 ± 0.09 at pre-tonsillectomy to 4.41 ± 0.11 at 46.1 ± 9.5 months after the tonsillectomy ($P = 0.02$). On the other hand, the Gal content in HP or Gal/GalNAc ratio was not significantly changed by tonsillectomy. It should be noted that the GalNAc content increased in all remission cases (Fig. 1). With respect to the relationship of remission and glycosylation, a significant increase of GalNAc and a decrease of Gal were observed in all the four remission cases. When comparing the *O*-glycosylation status among pre-TLX + steroid IV IgAN, post-TLX + steroid IV IgAN patients and healthy volunteers, the GalNAc content was significantly higher in healthy volunteers than in post-tonsillectomy + steroid IV IgAN patients. The Gal content is significantly higher in the healthy volunteers than in pre- or post-tonsillectomy + steroid IV IgAN patients (Fig. 2). In short, in IgAN patients in the pretreatment stage (pre-TLX + steroid IV group), the Gal and GalNAc content in HP were reduced compared to healthy volunteer (Fig. 2), which is consistent to the previous reports [11–16]. As to the sialic acid content in HP, we cannot assay it in our study because we performed desialylation of glycopeptide by heated acetic acid. Simultaneous depiction of GalNAc/HP and Gal/HP of IgA among pre-tonsillectomy + steroid IV IgAN patients, post-tonsillectomy + steroid IV IgAN patients and healthy volunteers clearly showed that these groups are different from each other (Fig. 3).

In the present study, reduced GalNAc levels in the IgA HP from patient serum were recovered after the palatine tonsillectomy

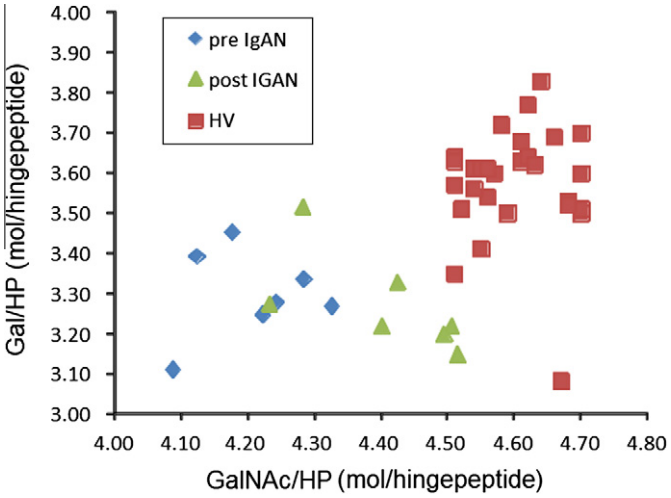

Fig. 3. Relationship between GalNAc/HP and Gal/HP of IgA among pre-TLX + steroid IV IgAN patients, post-TLX + steroid IV IgAN patients and healthy volunteer. HV stands for healthy volunteer.

combined with corticosteroid IV. In a previous report, aberrant glycosylation is presumably inherent in familial and sporadic IgAN [19], suggesting that even a successful treatment may not be able to counteract the original glycosylation abnormality. But, our results demonstrated that successful intervention could modify the inherently-affected aberrant glycosylation. In our mass spectrometry-based quantitation of saccharide compositions, one non-remission case showed significant increase in GalNAc. However, this case had an exceptional clinical history; the patient underwent additional adenoidectomy one year after the first palatine tonsillectomy and steroid IV, because the adenoidal hypertrophy was observed after palatine tonsillectomy and remission was not obtained by palatine tonsillectomy combined with steroid IV. Although remission was not achieved after the second tonsillectomy (palatine tonsils and adenoid), radical and repeated removal of tonsillar tissues led to a significant increase of GalNAc levels in IgA *O*-glycans, suggesting that removal of tonsillar tissues to be associated with the change of GalNAc content. Considering that the IgA1 molecule produced by tonsillar lymphocytes is under-*O*-glycosylated [20], and that tonsils are the major production sites

of under-*O*-glycosylated IgA1, repeated removal of several tonsils may well affect the glycosylation status.

Interestingly, tonsillectomy combined with steroid IV significantly increased the GalNAc levels, but the content increased following therapy was less than that for healthy volunteers. This suggests that IgAN patients have inherently mild abnormality in the *O*-glycosylation of IgA1 as suggested by the previous report [19]. This tendency was enhanced by focal infection, and the glycosylation alterations manifested more prominent before the treatment. Our data demonstrated that aberrant glycosylation can be changed by successful therapeutic interventions and suggested that the abnormalities are, in part, inherent.

In conclusion, the GalNAc content in IgA-HP from IgAN patient serum was quantitated. The GalNAc content could be increased by the therapy of tonsillectomy and corticosteroid IV in the patients who achieved remission, indicating that successful intervention could modify the inherently-affected aberrant glycosylation to some extent. The quantitative assay of IgA *O*-glycosylation described herein is anticipated to be a reliable tool for evaluation of aberrant glycosylation in IgAN and for unraveling the mechanism underlying this unique *O*-glycan-related disorder.

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