Assessing serum IgG4 glycosylation profiles of IgG4-related disease using lectin microarray

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Abstract

Objective

IgG4 related disease (IgG4-RD) is a multiorgan fibroinflammatory disorder. Lectin microarray is a high-throughput glycosylation analysis technology. The aim of our study was to investigate glycosylation profiling of serum IgG4 from IgG4-RD patients and controls.

Methods

A large cohort of 167 IgG4-RD patients, 130 disease controls (DCs) and 86 healthy controls (HCs) were included in the current study. The glycan level of serum IgG4 of all participants was determined by lectin microarray. A verification assay of lectin microarray and lectin blot were used to clarify the relationship between the serum IgG4 and purified IgG4 glycosylation.

Results

The results revealed that the glycan level of mannose (binding MNA-M, VVA mannose, ConA) was significantly increased and that the glycan level of fucose (binding LTL), GlcNAc (binding DSL), GalNAc (binding HPA) was significantly decreased in IgG4-RD patients compared to DCs and HCs. We further found that the glycan level of GlcNAc was positively correlated with that of complement 3 (C3), and that the reduced level of GlcNAc was associated with damage to multiple organs. In addition, the mannose level (binding MNA-M and VVA mannose) was negatively correlated with C3 and complement 4 (C4) levels.

Conclusion

Serum IgG4 of IgG4-RD patients exhibits different glycosylation levels. This study demonstrated that there is important clinical value in identifying aberrant GlcNAc levels as a potential diagnostic index for multi-organ involvement. Furthermore, the mannose level of serum IgG4 may reflect the degree of inflammation of IgG4-RD.

Key words

IgG4, IgG4-related disease, glycosylation, lectin microarray

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Introduction

IgG4-related disease (IgG4-RD) is a multi-organ immune-mediated chronic inflammatory disease with such characteristic features as an elevated level of serum IgG4, infiltration of IgG4 positive cells, mass forming lesions with fibrosis, and a positive response to corticosteroid treatment (1-3). The epidemiology of IgG4-RD remains unknown, however, it was first proposed as a new clinical pathological entity in Japan in 2003 (4) and has been widely recognised as a syndrome since 2010 (5). IgG4-RD is considered a systemic disorder with disease characteristics that include autoimmune pancreatitis (AIP), sialadenitis (Mikulicz's disease), and IgG4-related retroperitoneal inflammation (6). Although IgG4-RD has become widely recognised, the definite role of IgG4 in its pathogenesis remains unclear.

IgG4 is one of the four human IgG subtypes and is considered to be the predominant subclass in immune tolerance. IgG4 constantly exchanges half-molecules (7), resulting in the inability to cross-link effective monovalent antibodies (8) which prevents the generation of large immune complexes by other antibodies (9). Additionally, compared with the IgG1 subtype, IgG4 has a lower affinity for C1q (7, 10) and most fragment crystallisable (Fc) receptors (11), resulting in the production of blocking antibodies. Moreover, IgG4 competes with the IgE subclass to enhance the body's tolerance to allergens (12). The natural immune function of IgG4 seems to be a double-edged sword whereby it inhibits the function of target molecules in disease states (8, 13) and weakens the humoral immunity to tumours (14).

Glycosylation is an important post translational modification which significantly influences the biological function of proteins. About 2% of the human genome encodes proteins that are in some way involved in glycan biosynthesis, degradation, or transportation (15). Unsurprisingly, aberrations of glycosylation are usually associated with the onset of various diseases. In particular, serum IgG glycosylation analysis is now applicable for clinical samples due to the increased sensitivity of measurements and has led to new insights in the relationship between IgG glycosylation and various autoimmune diseases (AIDs), such as systemic lupus erythematosus (SLE) (16-18), rheumatoid arthritis (RA)(19, 20) and antineutrophil cytoplasmic antibody associated vasculitis (AAV) (21). At present, research on the glycosylation of IgG4-RD is limited and based on small sample sizes (22, 23). These studies have shown that certain glycosylation types of IgG4, especially fucosylation, are associated certain clinical phenotypes. Nevertheless, more research is needed to clarify the role of IgG4 glycosylation in IgG4-RD.

Lectins were discovered to specifically bind to carbohydrates on mammalian cell surfaces in the 1950s (15). The recently developed lectin microarray is a novel platform for glycan analysis (24) that enables rapid and high-sensitivity profiling of complex glycan features without the need for the actual liberation of such glycans (25-27). Based on the high throughput, speed, specificity and increased convenience, the tremendous progress already achieved in lectin microarray development makes it an indispensable tool for glycosylation discovery for diseases such as cancers (28, 29) and AIDs (30, 31). The purpose of the current study was to use lectin microarray technology containing 56 lectins to depict the glycosylation profile of IgG4. We also aimed to explore the role of IgG4-specific glycosylation expression to provide evidence for the pathogenic role in IgG4-RD.

Materials and methods

Patients and samples

All serum samples involved in this study were collected at Peking Union Medical College Hospital. The study population was comprised of 167 IgG4-RD patients (59 with Mikulize disease, 50 with AIP and 58 with RPF) from a prospective cohort study conducted in Peking Union Medical College Hospital beginning in January of 2011. A disease control (DCs) group of 130 patients included 40 patients diagnosed with pancreatitis, 45 with RA and 45 with SLE. 86 healthy controls (HCs) were also included.

Blood samples were collected and allowed to clot at room temperature for 30 min and then centrifuged for 5 min at 1,000 × g at 4°C. Serum was collected and stored at -80°C. No sample was exposed to more than one freeze-thaw cycle before analysis. This study was approved by the Medical Ethics Committee of PUMCH (Beijing, China). All participants provided written informed consent.

Clinical manifestation and laboratory results

Information such as demographic data, initial symptoms, disease duration, history of allergies and physical examination was recorded. The diagnosis of IgG4-RD was performed according to the 2011 comprehensive diagnostic criteria (32). Briefly, patients diagnosed with IgG4-RD must have met the following criteria: (i) organ enlargement, mass or nodular lesions, or organ dysfunction; (ii) a serum IgG4 concentration >135 mg/dl; and (iii) histopathological finding of >10 IgG4+ cells per high power field (HPF) and an IgG4+/IgG+ cell ratio of >40%. Patients in the DC group were diagnosed according to separate criteria (33-35). The total serum IgG concentration, as well as that of the IgG subclasses (including IgG1, IgG2, IgG3 and IgG4) were detected by the turbidimetric inhibition immunoassay (BNII System, Siemens, Germany) at diagnosis. The definition of elevated serum IgG (>17g/L) and IgG4 (>1400mg/dl) levels was confirmed in accordance with the institution range (Laboratory Department, PUMCH, China). Along with data relevant to disease diagnosis, we also collected additional health information that included levels of C-reactive protein (CRP), rheumatoid factor (RF), C3 and C4, as well as the erythrocyte sedimentation rate (ESR).

IgG4 purification and quantification

IgG4 was isolated from serum by Immunoprecipitation. 20 μL of mouse-anti-IgG4 antibodies (SouthernBiotech, Birmingham, USA) was conjugated to 20ul beads (NHS-activated Sepharose™ 4 Fast Flow, GE healthcare Life Sciences, Pittsburgh, USA), followed by the addition of 0.1M Tris-HCl to seal the excessive site. Mouse-anti-IgG4 antibody beads were washed with acid solution and alkali solution three times each. 5 μL of serum was applied to the column which was incubated overnight. After washing with phosphate buffered saline with Tween 20 (PBST) eight times and water twice, IgG4 were eluted with 20 μL of 0.1M Glycin in a vacuum tube. Protein purity was identified by the Dotblot method using mouse anti-IgG4 antibodies, and protein concentration was determined using a protein silver stain kit (Beyotiome, Shanghai, China). All IgG4 samples were stored at -80°C for future processing.

Lectin microarray

We used two types of microarrays for the glycosylation analysis of IgG4. The lectin microarray (BCBIO Biotech, Guangzhou, China) was composed of a microchip of 56 lectins. 56 kinds of lectins were immobilised on the chip slide in triplicate and the total serum from each subject was applied to the array at a 1:1000 dilution, which was then incubated at 4°C overnight. Anti-IgG4-Cy3 conjugate was then hybridised with the microchip for 45 min in the dark. Further procedural details can be found in previously reported protocols (29, 30, 36). The fluorescence intensity of all lectins as well as that of the lower signal-lectins were analysed independently. The slide image was converted to digital format for analysis. To exclude the influence of different IgG4 concentration levels on the analysis of IgG4 glycosylation, we used another lectin microarray to further validate the original results. The second microarray consisted of 6 lectins, including HPA, DSL, LTL, VVA mannose, MNA-M and ConA. This assay was also performed as described above.

Lectin microarray data analysis

We calculated the signal-to-noise ratio (S/N) (the medium intensity of the spot foreground relative to the background) of each lectin spot. To prevent bias of the lectin microarray from the interarray, we used Between Arrays (37) to normalise the S/N data. The following rules were used to identify significant differences in the binding activity of lectins between subject groups: (1) the mean S/N of lectin in IgG4-RD group should not be less than the maximum S/N in controls [50% (IgG4-RD) ≥ max (controls)]; (2) the lower quartile S/N of the IgG4-RD group should not be less than the upper quartile value of the controls [25% (IgG4-RD) ≥75% (controls)]; (3) the minimum S/N of the IgG4-RD group should not be less than the median value of the controls [min(IgG4-RD) ≥50% (controls)].

Lectin blot

Nitrocellulose (NC) filter membranes (Thermofisher, Massachusetts, USA) were balanced in an environment with relative humidity of 35-80% for 30 minutes. A small cohort of 16 purified IgG4 specimens, including 12 IgG4-RD, 3 DCs and 1 HC, were respectively spotted on the NC membrane with a final concentration of 20 μg/mL. After being blocked with 5% bovine serum albumin, the NC membrane was incubated with 5 μg/mL of ConA, HPA and DSL lectins for three hours at room temperature. Excess lectins were removed by washing three times with PBST. Then, Cy3-Goat-Anti-Mouse-IgG (Sangon Biotech, Shanghai, China) at a concentration of 1 ng/μL was added and incubated for one hour. Finally, the washed NC membrane was dried and the blots were developed with a microarray scanner detection system (Luxscan™ 10K-A, CapitalBio, Beijing, China) using a fluorescence signal.

Statistical analysis

Results are expressed as mean ± standard deviation (for data that was normally distributed). Descriptive data are presented as frequencies for categorical variables. SPSS 24.0 (SPSS Statistics for Windows, v. 24.0; SPSS Inc., Chicago, IL, USA.) was used to perform all statistical analyses in the current study. A one-way analysis of variance (ANOVA) with Tukey's HSD test was used to test the difference among the IgG4-RD, DC and HC groups. Spearman's correlation coefficient was used to analyse the relationship between the glycan level and clinical indicators. To

Table I. Clinical and laboratory characteristics of all 383 subjects.

Parameter	IgG4-RD (n=167)				DCs (n=130)		HCs (n=86)
	MD (n=59)	AIP (n=50)	RPF (n=58)	Pancreatitis (n=40)	RA (n=45)	SLE (n=45)	
Sex(M/F)	27/32	38/12	46/12	20/20	17/28	3/42	57/29
Age(y)[a]	50.2 ± 13.3	58.4 ± 9.4	51.6 ± 16.9	46.1 ± 17.2	49.1 ± 14.0	30.5 ± 9.4	38.7 ± 9.1
Laboratory findings[a]							
ESR (mm)	29.7 ± 28.6	40.3 ± 29.9	51.5 ± 35.8	-	38.7 ± 28.9	25.6 ± 22.9	-
CRP (mg/L)	15.8 ± 5.6	6.3 ± 6.1	18.6 ± 9.5	-	27.9 ± 19.3	11.5 ± 19.2	-
IgG[b] (g/L)	22.2 ± 9.7	25.1 ± 14.9	25.7 ± 16.4	-	18.2 ± 6.5	18.7 ± 7.5	-
IgA (g/L)	2.0±1.2	1.9 ± 0.8	2.7 ± 2.2	-	2.9 ± 1.5	3.1 ± 1.4	-
IgM (g/L)	1.5 ± 0.4	0.98 ± 0.41	1.3 ± 0.62	-	1.4 ± 0.8	1.3 ± 0.5	-
IgG1[b] (mg/dl)	9275.3 ± 4037.7	10502 ± 4728.5	11733.1 ± 7827	6650.3 ± 1602.9	-	-	-
IgG2[b] (mg/dl)	6006.1 ± 3775.3	5945.1 ± 2594	5607.8 ± 2546.7	4185.8 ± 1481.9	-	-	-
IgG3[b] (mg/dl)	1014.9 ± 564.3	835.3 ± 612.9	893.5 ± 762.8	370.3 ± 190.5	-	-	-
IgG4[b] (mg/dl)	18003.6 ± 13234.3	16852.8 ± 15255.4	12725.7 ± 11618.4	456.9 ± 344.5	-	-	-
IgE (mg/dl)	590.7 ± 312.1	868 ± 732.4	751.3 ± 631.3	-	-	-	-
C3 (g/L)	0.91 ± 0.32	0.79 ± 0.31	0.98 ± 0.37	-	-	0.68 ± 0.31	-
C4 (g/L)	0.18 ± 0.8	0.12 ± 0.09	0.16 ± 0.08	-	-	0.11 ± 0.07	-
RF (IU/ml)	33.6 ± 30.4	44.2 ± 37.2	34.6 ± 24.8	-	295.4 ± 606.1	57.9 ± 43.2	-
Organ involvement, no. (%)							
Pancreas	10 (16.95)	44 (88.0)	6 (10.34)	-	-	-	-
Bile duct	4 (6.78)	39 (78.0)	3 (5.17)	-	-	-	-
Salivary glands	45 (76.27)	27 (54.0)	20 (34.48)	-	-	-	-
lacrimal glands	49 (80.53)	20 (40.0)	12 (20.69)	-	-	-	-
Parotid gland	21 (35.59)	13 (26.0)	9 (15.52)	-	-	-	-
Retroperitoneal	5 (8.47)	7 (14.0)	27 (46.55)	-	-	-	-
Lung	17 (28.81)	8 (16.0)	17 (29.31)	-	-	-	-
Urinary system	7 (11.86)	10 (20.0)	21 (36.21)	-	-	-	-
Aorta and large blood vessels	1 (1.69)	5 (10.0)	11 (18.97)	-	-	-	-
Mediastinal	5 (8.47)	2 (4.0)	5 (67.24)	-	-	-	-
Orchis	0	0	0	-	-	-	-
Prostate gland	7 (11.86)	13 (26.0)	11 (18.97)	-	-	-	-
Lymph node	42 (71.19)	28 (56.0)	39 (67.24)	-	-	-	-
lumps formation	3 (5.08)	1 (2.0)	5 (8.62)	-	-	-	-
Sinuses	23 (38.98)	8 (16.0)	9 (15.52)	-	-	-	-
Thyroid	0	3 (6.0)	0	-	-	-	-
Liver	0	3 (6.0)	3 (5.17)	-	-	-	-
pituitary	2 (3.39)	0	0	-	-	-	-
Skin	5 (8.47)	2 (4.0)	5 (8.62)	-	-	-	-
Digestive system	4 (6.78)	6 (12.0)	4 (6.9)	-	-	-	-

IgG4-RD, IgG4 related diseases; DCs, disease controls; HCs, healthy controls; MD, Mikulicz's disease; AIP, autoimmune pancreatitis; RPF, Retroperitoneal fibrosis ; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; C3, complement C3; C4, complement C4; RF, rheumatoid factor.
[a] Mean±SD. [b] $p<0.01$, IgG4-RD group *vs*. DCs.

compare frequencies and analyse the correlation between clinical and laboratory variables, a Student's t-test or Mann-Whitney U-test were applied to continuous variables, as appropriate. A p-value <0.05 was considered statistically significant.

Results

Demographic, clinical and laboratory characteristics of subjects

The major characteristics of the 383 subjects are summarised in Table I. As shown in the table, the gender distribution was different between the IgG4-RD group and DCs with the majority being male (65.3%) in the IgG4-RD group. The mean age at IgG4-RD diagnosis was 52.8±13.2 years. The average level of serum IgG, IgG1, IgG2, IgG3 and IgG4 were significantly higher in IgG4-RD patients compared with DCs ($p<0.01$). However, no significant differences were detected between the different MD, AIP and RPF subgroups. Briefly, the most frequently observed organs involved in IgG4-RD in our cohort were the lymph node (n=109, 65.3%), salivary glands (n=82, 49.1%) and lacrimal glands (n=81, 48.5%). The pituitary (n=2, 1.2%) and orchis (n=0) were rarely involved.

Lectin microarray analysis for serum IgG4 glycosylation

To identify the types of glycan on IgG4 in IgG4-RD, we first screened serum using the lectin microarray, which contained 56 types of lectins that bind to respective glycans (Supplementary Fig. S1). Serum from IgG4-RD patients, DCs and HCs was incubated on a lectin microarray for each sample, in which each lectin was present in triplicate (Suppl. Fig. S1). Lectin S/N data conforming to the any of three aforementioned rules was considered to be significant with a total of 6 lectins (Table II).

Table II. Significant differences of lectins in the lectin microarray.

Lectin	Full name	Monosaccharide Specificity
LTL	Lotus tetragonolobus lectin	Fuc[a]
DSL	Datura stramonium lectin	GlcNAc [a]
HPA	Helix pomation Lectin (Snail)	αGalNAc [a]
MNA-M	Morniga M Lectin (black elderberry)	Man [a]
VVA mannose	Vicia villosa Lectin (Hairy Vetch, Mannose Specific)	Man [a]
ConA	Concanavalin A Lectin	Man [a], Glc [a]

Significant difference of lectins were defined as: (1) Mean S/N of lectin in IgG4-RD group should not be less than the maximum S/N in controls [50%(IgG4-RD) ≥ max(controls)]; (2) The lower quartile S/N of the IgG4-RD group should not be less than the upper quartile value of the controls [25% (IgG4-RD) ≥75% (controls)]; (3) The minimum S/N of the IgG4-RD group should not be less than the median value of the controls [min(IgG4-RD) ≥50% (controls)].
Adapted from Lectin Frontier DataBase. Monosaccharide abbreviations: Fuc: Fucose; Man: Mannose; GlcNAc: N-Acetylglucosamine; GalNAc: N-Acetylgalactosamine; Glc: Glucose.

The amount of serum IgG4 glycans in different patients

The affinity signal values for six lectins showed a significant difference among three groups. The S/N data indicated the following changes of glycosylation on IgG4 for IgG4-RD patients relative to DCs and HCs: (1) increased mannose, as indicated by the increased binding to the Con A lectin (ConA), Morniga M Lectin (black elderberry) (MNA-M) and Vicia villosa lectin (Hairy Vetch, Mannose Specific) (VVA mannose) (all $p<0.00$); (2) decreased fucose levels as evidenced by decreased binding to the Lotus tetragonolobus lectin (LTL) ($p<0.00$); (3) decreased N-Acetylgalactosamine (GalNAc) detected by decreased binding to the Helix pomation Lectin (Snail) (HPA) ($p<0.00$); (4) decreased N-Acetylglucosamine (GlcNAc) detected by decreased binding to the Datura stramonium lectin (DSL) ($p<0.00$). The S/N of each lectin for serum IgG4 in IgG4-RD patients, DCs and HCs are shown in Figure 1.

Lectin microarray and lectin blot analysis for IgG4 isolated from serum of IgG4-RD patients and controls

To further determine whether the changes of glycosylation in IgG4-RD patients were due to an increase in the concentration of serum IgG4 or a real change of glycosylation, another lectin microarray and lectin blot were used for validation. We used mouse anti-IgG4 antibody beads for purification and removed the antibodies from glycan eluate in a small participant cohort that included

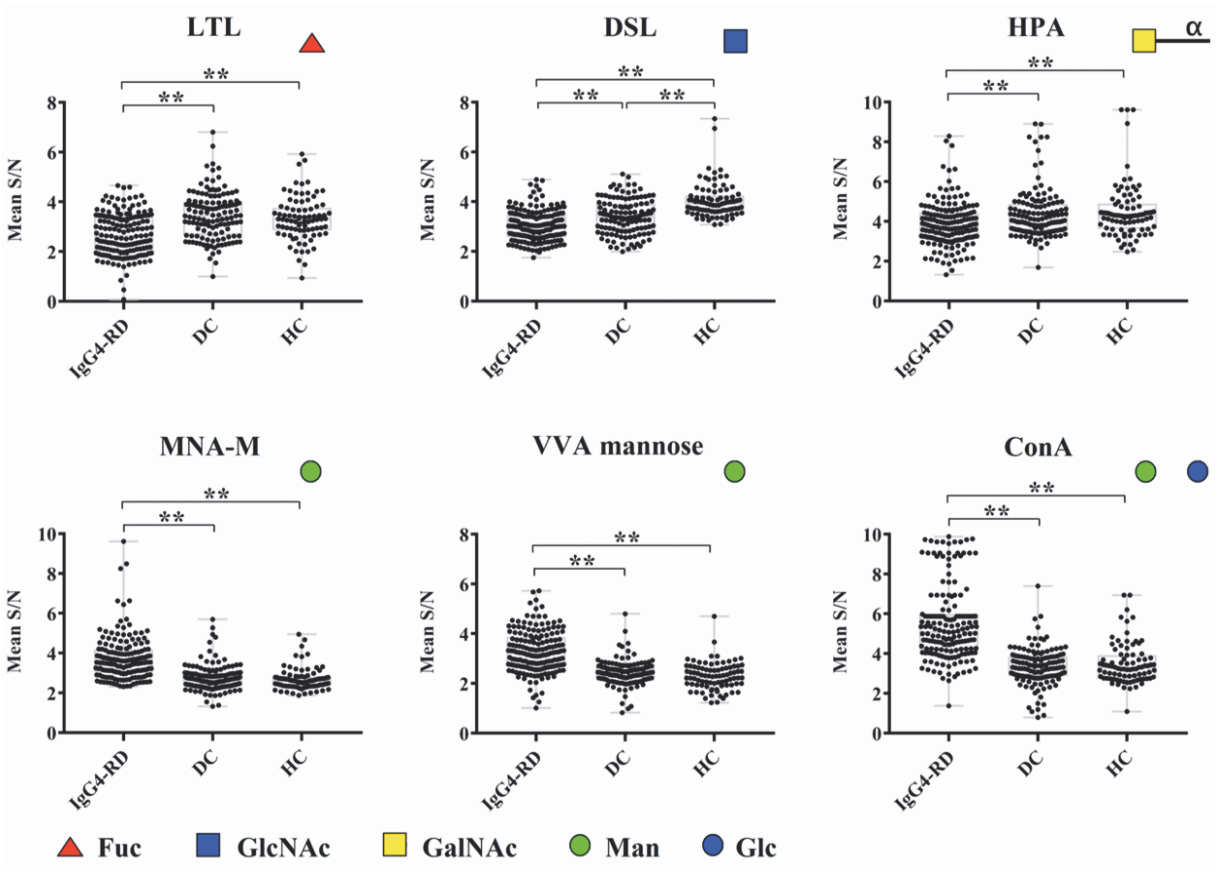

Fig. 1. Comparison of IgG4 glycosylation patterns in IgG4-related diseases (IgG4-RD), diseases controls (DCs) and healthy controls (HCs). Scatter diagram analysis of the normalised data achieved from the lectin microarray. Distribution of lectins exhibiting significant changes in binding among the IgG4-RD, DC and HC groups. Grey bars represent the mean ± standard deviation (SD). The small colour figures represent glycans that specifically bind to the lectins. Binding specificities were LTL for Fucose (Fuc); DSL for N-Acetylglucosamine (GlcNAc); HPA for N-Acetylgalactosamine (GalNAc); MNA-M, VVA mannose and ConA for mannose (Man). **, $p<0.001$; *, $p<0.05$

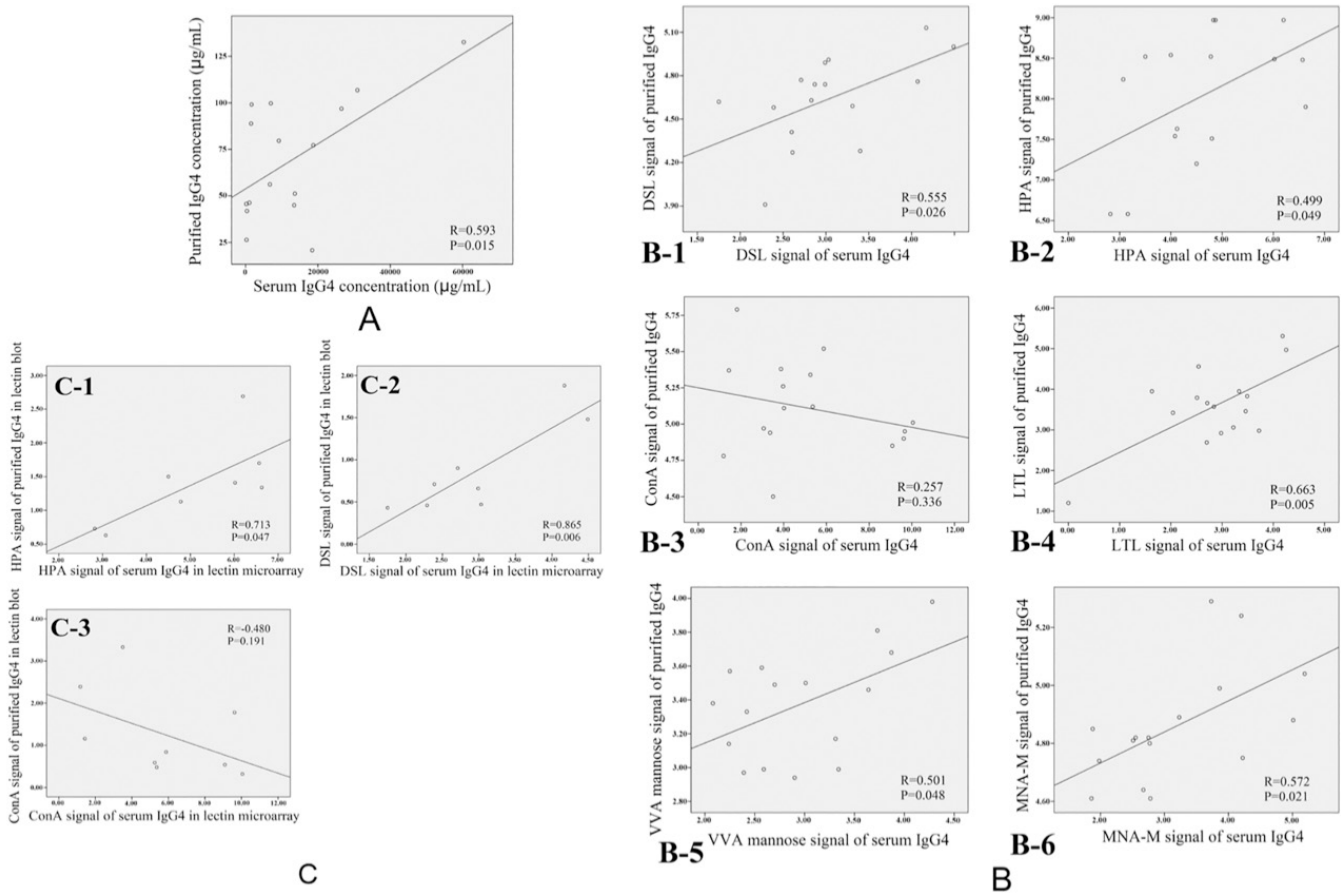

Fig. 2. Lectin microarray and Lectin blot analysis for purified IgG4 from a cohort in 16 subjects.
A: Correlation of the IgG4 concentration before and after purification. Quantitative analysis of serum IgG4 measured by the turbidimetric immunoassay and IgG4 in IP samples measured by protein silver stain (n=16).
B: Correlation of lectin signal values between serum IgG4 and the purified IgG4 in the lectin microarray. B-1: DSL, B-2: HPA, B-3: ConA, B-4: LTL, B-5: VVA mannose, B-6: MNA-M.
C: Correlation of lectin signal values between serum IgG4 in the lectin microarray and the purified IgG4 in the lectin blot. C-1: HPA, C-2: DSL, C-3: ConA.

12 IgG4-RD patients, 3 DCs and 1 HC. The purified IP samples were detected by Dotblot analysis (Suppl. Fig. S2). By comparing the purified IgG4 concentration in a cohort of 16 subjects and relative serum IgG4 quantity, we found that the results from the purified IgG4 concentration were well correlated with serum IgG4 levels (r=0.593, p=0.015) (Fig. 2A, Suppl. Table S1). Moreover, as expected, the purified IgG4 signal values of the lectin microarray, including VVA mannose (r=0.501, p=0.048) (Fig. 2B-5, Suppl. Table S1), MNA-M (r=0.572, p=0.021) (Fig. 2B-6, Suppl. Table S1), LTL (r=0.663, p=0.005) (Fig. 2B4, Suppl. Table S1), DSL (r=0.555, p=0.026) (Fig. 2B-1, Suppl. Table S1) and HPA (r=0.499, p=0.049) (Fig. 2B-2, Table S1), and the lectin blot, including DSL (r=0.865, p=0.006) (Fig. 2C-2, Suppl. Table S1) and HPA (r=0.713, p=0.047) (Fig. 2C-1, Suppl. Table S1), were positively correlated with serum IgG4 signal values of the lectin microarray. We did not find a correlation of ConA signal values between serum IgG4 and the purified IgG4 when using the lectin microarray (r=-0.257, p=0.336) (Fig. 2B-3, Suppl. Table S1) or the lectin blot (r=-0.480, p=0.191) (Fig. 2C-3, Suppl. Table S1). These results suggest that profiling serum IgG4 glycosylation patterns detected by the lectin microarray were not affected by serum IgG4 concentrations and were reliable for further exploring the relationship between the level of glycosylation and clinical features.

Association between IgG4 glycosylation and laboratory features in 167 IgG4-RD patients

We observed significant differences between lectin signals among IgG4-RD patients, prompting us to further evaluate the association between lectin signals and laboratory indexes. Considering the changes of glycosylation detected by ConA were due to the elevated serum IgG4 concentration rather than a real change in the IgG4 glycoprotein itself, we did not analyse the data related to ConA in the following analysis. Among various laboratory features detected in our patients, the amount of mannose (detected as VVA mannose and MNA-M) was negatively correlated with serum C3 (r=-0.37, p<0.01; r=-0.28, p=0.03, respectively), C4 (r = -0.36, p<0.01; r=0.34, p=0.01, respectively), IgA (r=-0.24, p<0.01; r=-0.3, p<0.01, respectively) and IgM (r=-0.19, p=0.02; r=-0.18, p=0.03, respectively) levels in the IgG4-RD group. The amount of fucose (detected as LTL) was positively correlated with IgA levels (r=0.21, p=0.01), and the amount of GlcNAc (detected as DSL) was positively associated with

Table III. Correlation between glycosylation and laboratory features in 167 IgG4-RD patients.

	HPA		DSL		LTL		MNA-M		VVA mannose	
	r	P	r	P	r	P	r	P	r	P
ESR	-0.11	NS*	0.05	NS	0.08	NS	0.12	NS	0.12	NS
CRP	-0.13	NS	0.11	NS	0.07	NS	-0.06	NS	-0.19	0.04
IgG	-0.09	NS	-0.20	0.01	0.07	NS	0.37	<0.01	0.36	<0.01
IgA	0.02	NS	0.08	NS	0.21	0.01	-0.30	<0.01	-0.24	<0.01
IgM	0.05	NS	0.04	NS	-0.01	NS	-0.18	0.03	-0.19	0.02
IgG1	-0.11	NS	0.03	NS	-0.01	NS	0.15	NS	0.19	0.02
IgG2	0.03	NS	0.03	NS	-0.01	NS	-0.05	NS	-0.16	0.04
IgG3	0.00	NS	-0.04	NS	-0.01	NS	0.22	<0.01	0.14	NS
IgE	0.09	NS	-0.15	NS	-0.01	NS	0.15	NS	0.03	NS
C3	-0.09	NS	0.36	<0.01	0.22	NS	-0.28	0.03	-0.37	<0.01
C4	0.06	NS	0.20	NS	0.11	NS	-0.34	0.01	-0.36	<0.01

*NS: not significant.

serum C3 levels (r=0.36, $p<0.01$) in the IgG4-RD group. There was no significant correlation between the amount of GalNAc (detected as HPA) and the laboratory features. These results are summarised in Table III.

Association between IgG4 glycosylation and organ involvement in 167 IgG4-RD patients

Among the various organs involved in our patients, we compared the different glycosylation levels of specific binding lectins between patients with and without organ involvement in IgG4-RD. The results are shown in Table IV. There was a difference in the mannose level (detected as VVA-mannose and MNA-M) in IgG4-RD patients with and without lacrimal gland and salivary gland involvement (both $p<0.05$). As for fucose (detected as LTL), the concentration level was significantly different between IgG4-RD patients with and without pancreas and bile duct involvement (both $p<0.01$). The amount of GalNAc (detected as HPA) was significantly different between IgG4-RD patients with and without retroperitoneum and bile duct involvement (both $p<0.01$). The amount of GlcNAc (detected as DSL) was significantly different between IgG4-RD patients with and without parotid gland, lacrimal gland, retroperitoneum, bile duct and large blood vessel involvement (all $p<0.05$).

Discussion

IgG4-RD is one of the most complicated diseases contributing to AIDs, with multiorgan fibroinflammatory disorder (38). Currently, immunoglobulin glycosylation has been widely used in the study of the pathogenesis of AIDs. In the current study, we applied a lectin microarray comprised of 56 lectins to detect serum IgG4 glycosylation in a large cohort of patients with IgG4-RD, patients with other diseases, and healthy controls. We showed that the affinity of MNA-M, VVA mannose and ConA lectins to IgG4 were significantly increased in IgG4-RD patients compared to DCs and HCs. However, the affinity of LTL, DSL and HPA lectins to IgG4 were significantly decreased in IgG4-RD patients compared to HCs. Overall, we applied a lectin microarray comprised of 56 lectins to explore the glycosylation of serum IgG4 in IgG4-RD patients for the first time.

Analysing serum IgG4 glycosylation is a challenging task. In order to verify the reliability of lectin microarray, we applied another lectin microarray comprised of 6 lectins and a lectin blot assay in a small cohort of 16 subjects. This secondary assay aimed to determine if a change in purified IgG4 glycosylation could be detected and whether the change of affinity signals from the previous lectin microarray was due to the levels of IgG4 glycosylation or the serum IgG4 concentration. Our results showed that for five lectins (VVA mannose, MNA-M, LTL, DSL and HPA), the levels of serum IgG4 glycans detected by the lectin microarray were significantly and positively correlated with the lectin microarray and lectin blot detection levels of purified IgG4 glycosylation. These results indicated that the lectins' specific affinity-binding glycan levels were not affected by serum IgG4 concentrations and that the lectin microarray system used for detecting the level of IgG4 glycosylation could accurately and truly reflect the level of IgG4 glycosylation in research subjects. However, we did not find a correlation of ConA-specific affinity-binding glycan levels between serum IgG4 and the purified IgG4, which might be partly due to the influence of serum IgG4 concentration. Moreover, the specific binding glycan of ConA was not only mannose, but also glucose. Since we found no evidence that the level of glucose was different in our cohort, we speculate that the ConA affinity signal could not reflect the levels of mannose in this study. In addition, we also analysed the relationship between the level of IgG4 glycosylation and the clinical characteristics in IgG4-RD patients, including laboratory features and organ involvement.

IgG4, similar to IgG, is a glycoprotein with variable glycosylation (either O- or N-linked) in the Fab region and a N-glycosylation site in the Fc region (39). Normally, most circulating IgG antibodies are fucosylated. Compared with non-fucosylated isotype antibodies, fucosylated IgG exhibits a reduced binding affinity for activated FcγRIII and thus has the potential to inhibit antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) (40). Non-fucosylated IgG antibodies can significantly increase the affinity of the FcγRIIIa receptor. The lack of core

Table IV. Comparison of the amount of IgG4 glycosylation between patients with IgG4-RD with and without organ involvement.

Glycans Lectin			GalNAc HPA	GlcNAc DSL	Fuc LTL	Man MNA-M	VVA-mannose
Pancreas	Yes	60	4.18±1.20	2.87±0.54	2.39±0.88	3.86±1.09	3.35±0.80
	No	105	3.68±1.06	3.12±0.66	2.86±0.81	3.66±1.10	3.23±0.87
	P		<0.01	<0.01	<0.01	NS*	NS
Bile duct	Yes	41	4.28±1.39	2.84±0.57	2.32±0.77	4.00±1.14	3.40±0.90
	No	122	3.72±1.01	3.09±0.63	2.80±0.86	3.64±1.08	3.25±0.83
	P		<0.01	0.025	<0.01	NS	NS
Salivary glands	Yes	92	4.01±1.16	2.92±0.59	2.57±0.88	3.89±0.89	3.41±0.79
	No	73	3.67±1.08	3.17±0.65	2.83±0.82	3.53±1.3	3.12±0.88
	P		NS	0.013	NS	0.037	0.027
lacrimal glands	Yes	81	3.95±1.18	2.89±0.53	2.64±0.97	3.91±1.04	3.45±0.85
	No	84	3.78±1.09	3.17±0.0.68	2.73±0.74	3.56±1.13	3.11±0.81
	P		NS	<0.01	NS	0.038	0.011
Parotid gland	Yes	43	4.03±1.06	2.87±0.53	2.51±0.56	3.79±0.77	3.29±0.83
	No	122	3.81±1.16	3.09±0.65	2.75±0.82	3.71±1.20	3.27±0.85
	P		NS	NS	NS	NS	NS
Retroperitoneal	Yes	39	3.33±0.79	3.22±0.66	2.89±0.79	3.65±1.39	3.18±0.89
	No	126	4.03±1.18	2.97±0.61	2.63±0.88	3.76±1.00	3.31±0.83
	P		<0.01	0.032	0.043	NS	NS
Lung	Yes	42	3.61±1.00	3.05±0.56	2.78±0.79	3.94±1.21	3.43±0.89
	No	119	3.97±1.18	3.02±0.65	2.66±0.90	3.61±0.94	3.12±0.78
	P		NS	NS	NS	NS	NS
Urinary system	Yes	38	356±0.94	2.99±0.60	2.83±0.89	3.76±1.22	3.36±0.81
	No	125	3.95±1.18	2.99±0.60	2.65±0.86	3.74±1.07	3.27±0.85
	P		NS	NS	NS	NS	NS
Aorta and large blood vessels	Yes	17	3.65±0.72	3.36±0.55	3.03±0.68	3.52±0.89	3.05±0.86
	No	146	3.89±1.18	3.00±0.63	2.66±0.87	3.75±1.12	3.29±0.84
	P		NS	0.024	NS	NS	NS
Mediastinal	Yes	12	3.56±0.72	3.23±0.93	2.77±0.66	3.89±0.74	3.62±0.65
	No	153	3.89±1.16	3.01±0.62	2.68±0.88	3.72±1.13	3.25±0.85
	P		NS	NS	NS	NS	NS
Prostate gland	Yes	31	3.74±1.32	3.0±0.62	2.53±0.68	4.14±1.57	3.39±1.06
	No	132	3.91±1.10	3.03±0.63	2.72±0.90	3.64±0.94	3.25±0.78
	P		NS	NS	NS	NS	NS
Lymph node	Yes	109	3.77±1.12	2.99±0.59	2.74±0.85	3.85±1.24	3.33±0.89
	No	55	4.05±1.17	3.12±0.69	2.59±0.89	3.47±0.70	3.15±0.72
	P		NA	NS	NS	0.013	NS
Lumps formation	Yes	9	3.60±0.72	3.13±0.71	3.10±0.67	3.58±1.17	3.00±1.13
	No	156	3.88±1.16	3.02±0.62	2.66±0.87	3.74±1.10	3.19±2.83
	P		NS	NS	NS	NS	NS
Sinuses	Yes	40	3.96±0.95	3.00±0.58	2.90±0.83	3.69±0.77	3.40±0.80
	No	124	3.84±1.20	3.04±0.65	2.62±0.87	3.75±1.19	3.24±0.86
	P		NS	NS	NS	NS	NS
Thyroid	Yes	3	4.27±0.22	2.92±0.09	2.20±0.43	4.08±0.93	3.49±0.94
	No	160	3.83±1.10	3.03±0.63	2.70±0.87	3.72±1.1	3.28±0.84
	P		NS	NS	NS	NS	NS
Liver	Yes	6	3.50±0.76	3.44±0.72	2.95±0.70	3.57±0.55	3.41±0.66
	No	88	4.09±1.15	2.95±0.60	2.62±0.83	3.82±1.16	3.40±0.86
	P		NS	NS	NS	NS	NS
pituitary	Yes	2	5.80±3.12	2.46±0.13	2.57±0.66	4.29±0.45	3.89±0.27
	No	160	3.85±1.10	3.02±0.62	2.68±0.87	3.73±1.11	3.27±0.84
	P		NS	NS	NS	NS	NS
Skin	Yes	12	3.68±0.84	2.88±0.89	2.46±0.60	3.65±0.90	3.20±0.86
	No	98	3.94±1.17	3.04±0.69	2.65±0.85	3.74±1.11	3.28±0.87
	P		NS	NS	NS	NS	NS
Digestive system	Yes	4	5.10±2.05	2.78±1.19	2.54±0.77	4.39±0.86	3.87±0.71
	No	85	3.96±1.12	3.01±0.6	2.63±0.83	3.81±1.18	3.39±0.83
	P		NS	NS	NS	NS	NS

*NS: not significant.

Fuc residues in Fc glycans can improve the ADCC activity of IgG molecules (41, 42); our results show that the fucose level of IgG4 was significantly decreased, suggesting that IgG4 may play an enhanced ADCC and ADCP role in the pathogenesis of IgG4-RD.

Among the 167 IgG4-RD patients in our study, we observed that the GlcNAc level of binding DSL specifically in serum was positively correlated with serum C3 level. Additionally, the significantly reduced levels of GlcNAc (detected as DSL) were associated with multiple organ damage, including the parotid gland, lacrimal glands, retroperitoneum, bile duct and large blood vessels. Our results indicate that the serum level of GlcNAc can be used as a potential diagnostic index for multi-organ involvement. Nevertheless, further studies are required to investigate the role of GlcNAc in organ tissue on IgG4 glycosylation.

Compared to DCs and HCs, the amount of mannose (recognised by MNA-M and VVA mannose) was significant higher in IgG4-RD patients. We further examined the association between the mannose levels in 167 IgG4-RD patients and their clinical characteristics. We found that the mannose level of specific binding MNA-M and VVA mannose lectins was negatively correlated with C3 and C4 concentrations. Moreover, the elevated level of mannose was associated with small exocrine gland involvement, such as lacrimal glands and salivary glands. Zhou *et al*. (43) demonstrated that high mannose structures of IgG had strengthened ADCC activity and influenced IgG effector functions. In addition, this study also confirmed that a high level of mannose could reduce the binding affinity to C1q and thereby attenuate the effect of complement-dependent cytotoxicity (44). Combined with our results, we suggest that mannose levels of serum IgG4 might be used as a diagnostic index for IgG4-RD disease activity. It is worth noting that previous studies have shown that IgG4 is a non-inflammatory factor because it does not fix complement and binds poorly to activated Fc receptors (38). But in theory, the IgG4 proinflammatory mechanism may be related to activity by its coordinated circulating lectins (38). Although there is no authentic evidence to support this, our conclusion seems to provide evidence for the inflammatory mechanism of IgG4. Further studies are required to reveal IgG4 mannose levels and the pathogenesis in IgG4-RD.

In conclusion, the current study performed glycosylation profiling of serum IgG4 in a large patient cohort based on a high-throughput glycoanalytical assay. Our study indicated that the amount of fucose, GalNAc and GlcNAc was decreased and that the amount of mannose was increased in IgG4-RD patients, compared to HCs ad DCs. We also demonstrated that there is important clinical value in the glycan levels of GlcNAc in serum IgG4 as a potential diagnostic index for multi-organ involvement. Furthermore, the mannose levels of serum IgG4 could reflect the inflammatory degree of IgG4-RD. Thus, our study helps to elucidate new insights in the pathogenesis of IgG4-RD and provides valuable disease-related predictors for clinic applications.

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