Screening of serum IgG glycosylation biomarker for primary Sjögren's syndrome using lectin microarray

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Background: Primary Sjögren's syndrome (PSS) is a systemic autoimmune disease resulting in significant loss of systemic gland secretory function. IgG glycosylation abnormalities had been found to play important roles in autoimmune diseases. Here, we aim to explore the specific changes of IgG glycosylation in PSS patient serum. **Methods:** From 2012 to 2018, patients diagnosed with PSS or Primary biliary cholangitis (PBC) admitted consecutively to the department of Rheumatology at Peking Union Medical College Hospital were retrospectively included in this study. Glycan profiles of serum IgG from 40 PSS patients, 50 PBC patients, and 38 health controls were detected with lectin microarray containing 56 lectins. Lectins with significantly different signal intensity among groups were selected and validated by lectin blot assay. **Results**: Lectin microarray analysis revealed that glycan levels of Galβ3GalNAc (recognized by ACL), mannose (recognized by MNA-M), and fucose (recognized by LCA) were significantly increased, while sialylation (recognized by SSA) was significantly decreased in PSS patients compared to PBC group. Mannose (recognized by MNA-M) and fucose (recognized by LCA) were significantly increased, while galactose (recognized by PHA-E and PHA-L) was significantly decreased in PSS patients compared to health controls. The results of LCA and MNA-M were further confirmed using lectin blot assay. **Conclusion:** Specific changes of serum IgG glycosylation in PSS were detected using lectin microarray. Increased levels of fucose and mannose may play important roles in PSS pathogenesis and could provide potential diagnostic value.

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Abstract

Background: Primary Sjögren's syndrome (PSS) is a systemic autoimmune disease resulting

in significant loss of systemic gland secretory function. IgG glycosylation abnormalities had been found to play important roles in autoimmune diseases. Here, we aim to explore the specific changes of IgG glycosylation in PSS patient serum that could serve as potential biomarkers for disease diagnosis.

Method: From 2012 to 2018, patients diagnosed with PSS or primary biliary cholangitis (PBC) admitted consecutively to the department of Rheumatology at Peking Union Medical College Hospital were retrospectively included in this study. Glycan profiles of serum IgG from 40 PSS patients, 50 PBC patients, and 38 healthy controls were detected with lectin microarray containing 56 lectins. Lectins with significantly different signal intensity among groups were selected and validated by lectin blot assay.

Results: Lectin microarray analysis revealed that binding levels of ACL (prefers glycan Galβ3GalNAc, P = 0.011), MNA-M (prefers glycan mannose. P =0.013), and LCA (prefers glycan fucose) were significantly increased, while SSA (prefers glycan sialylation, P =0.001) was significantly decreased in PSS patients compared to PBC group. Compared to healthy controls, MNA-M (P = 0.001) and LCA (P = 0.028) were also significantly increased, while PHA-E and PHA-L (prefer glycan galactose, P = 0.004 and 0.006) were significantly decreased in PSS patients. The results of LCA and MNA-M were further confirmed using lectin blot assay.

Conclusion: Changes in serum IgG glycosylation in PSS increased binding levels of LCA and MNA-M lectins using microarray techniques, which could provide potential diagnostic value. Increased core fucose and mannose alteration of IgG may play important roles in PSS pathogenesis.

Key Words: Glycosylation, Lectin microarray, Immunoglobulin G, Primary Sjögren's syndrome, Primary biliary cholangitis

Introduction

Primary Sjögren's syndrome (PSS) is a complex heterogeneous autoimmune disease

characterized by lymphocytic infiltration of the secretory glands and significant loss of secretory function with oral and eye dryness, as well as extra-glandular involvement that may impair the musculoskeletal, pulmonary, renal, neurological, and other organs/systems [1,2]. PSS is the second most common connective tissue disease after rheumatoid arthritis and affects predominantly middle-aged women with a female/male incidence of approximately 9:1 [3,4]. Although the pathogenesis of PSS is currently not yet fully understood, increased activation of B cells and autoantibody production, such as anti-SSA/Ro and anti-SSB/La autoantibodies, are thought to play important roles. As standard diagnostic biomarkers, the presence of anti-SSA and anti-SSB were only 52%-67% and 49% in PSS respectively [5]. Due to its non-specific symptoms, PSS is sometimes difficult to recognize, and diagnosis may be delayed by more than 10 years [6,7]. Primary biliary cirrhosis (PBC) is a complex systemic multisystemic disease characterized by chronic non-suppurative destructive cholangitis and is most often overlapped with Sjögren's syndrome (SS) often overlaps [8,9]. These coexisting conditions frequently make it more difficult the diagnosis and treatment of the disease.

Glycosylation is the most complex post-translational modification of proteins and has profound structural and functional effects on the conjugate [10]. It is estimated that more than half of human proteins are glycosylated with different glycan chains [11].

Immunoglobulin G (IgG) glycosylation is mostly N-glycosylation linked to an Asn in the heavy chain constant region. To date, numerous studies have confirmed that changes in IgG glycosylation have important roles in the regulation of effector functions [12-14]. For instance, a lack of core fucose leads to enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) activity. Aberrant IgG glycosylation has been found in various autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and inflammatory bowel disease

(IBD)[15-17]. Therefore, the structural analysis of glycans in IgG is critical in understanding respective autoimmune diseases. However, little has been reported on the IgG glycosylation profile for PSS.

Lectin microarray is an emerging technology for the study of glycosylation [18]. Compared with conventional glycan analysis methods such as mass spectrometry, it provides simple procedures for differential complex glycan profiling in a rapid, high-throughput, and high-sensitivity manner, and does not require prior liberation of glycans from the core protein which may destroy their native structure [19,20]. Lectin microarray has already found maximum use in diverse fields of glycobiology and made remarkable achievements in the study of glycosylation and biomarker identification for tumors and autoimmune diseases[21-23]. In this study, we used lectin microarray for the first time to analyze the expression profile of serum IgG glycosylation in patients with PSS, PBC, and healthy controls. Lectin blot was performed to validate the differences and obtain oligosaccharides specifically expressed in PSS.

Methods

Patients and samples

Patients diagnosed with PSS or PBC admitted consecutively to the department of Rheumatology at Peking Union Medical College Hospital during the period from 2012 to 2018 were retrospectively included in this study. PSS was diagnosed according to the 2012 American College of Rheumatology (ACR) criteria[24], and PBC was diagnosed according to the American Association for the Study of Liver Diseases criteria[25]. Patients meeting the classification criteria of more than one autoimmune disease or with cancer were excluded. A total of 128 serum samples were used for lectin microarray analysis, obtained from 40 PSS patients, 50 PBC patients, and 38 healthy controls who were healthy volunteers without autoimmune diseases. In

addition, a new cohort of samples was collected to verify significant findings using lectin blot, including 16 PSS patients, 16 PBC patients, and 16 healthy controls. Serum samples were obtained by separation from peripheral blood and stored at -80 °C until use. Autoantibodies were tested using chemiluminescence immunoassay. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethic Committee of Peking Union Medical College Hospital (Approval Code: S-478 Approval Date: 2012-10-31). All subjects gave written informed consent.

Lectin microarray

Totally 128 serum samples were detected using a commercial lectin microarray (BCBIO Biotech, Guangzhou, China) with 56 lectins, which had been proved of its reliability and used in biomarker finding previously[26,27]. Briefly, lectin microarrays were taken out from −80 °C and warmed up at room temperature for half an hour, then they were incubated with a blocking buffer (3% BSA in PBS) at room temperature for 2h. After washing three times with PBS, 200μl of 1:1000 diluted samples serum were added and incubated with the microarrays at 4 °C overnight. The microarrays were washed three times with PBS and then incubated with 5mL of 1:1000 diluted Cy5-labeled goat anti-human IgG antibody (Jackson Laboratory, Bar Harbor, ME) in the dark at room temperature for 50min. Finally, after three times PBS washes, microarrays were rinsed with D.I. water and dried. Microarrays were scanned with the GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA).

Lectin microarray data analysis

For lectin array assays, the median foreground and background fluorescent intensity for each spot on the arrays were acquired using GenePix Pro 6.0 software. 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 inter-array, we normalized the S/N data in terms of controls between arrays [28]. The following rules according to the method of Hu et al.[29] were used to identify significant differences in the binding activity of lectins between subject groups: (a) fold change[group1(S/N) / group2(S/N)] ≥1.3 or <0.77, (b) P-value <0.05.

Lectin blot

To validate the results of the differences in lectin microarray analysis, lectin blot was used to detect serum samples which were collected from 12 PSS patients,12PBC patients, and 12 HCs randomly selected from the lectin microarray analysis cohort, and 16 PSS patients, 16 PBC patients, and 16 HCs from a new cohort.

First, serum samples were diluted by 1 × PBS, mixed with gel electrophoresis loading buffer (CWbiotech) to a final 1:100 ratio, and boiled for 10 min. Twenty microliters per sample were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). After washing two times, the membrane was incubated with10× Carbo-Free Blocking Solution (1:10; Vector Laboratories Inc., United States) at room temperature for 2h. Then, the membranes were washed twice and incubated with 20μg/ml of Cy3-labeled (1:1000; GE Healthcare) LCA and MNA-M lectins at 4 °C overnight in the dark. Finally, the washed and dried membranes were detected by a fluorescence signal system of Typhoon FLA 9500 (GE Healthcare).

Statistical analysis

SPSS 22.0 was used to perform all statistical analyses and GraphPad Prism 8 was used to draw plots in the study. Continuous variables were expressed as mean ± standard deviation. The differences among the PSS, PBC, and HC groups were tested by one-way analysis of variance (ANOVA) with Tukey's HSD test. P-value less than 0.05 was considered statistically significant.

Results

Patient characteristics

As listed in Table 1, a total of 128 serum samples were used for lectin microarray analysis, obtained from 40 PSS patients (48.52±9.73 years of age; 36 females), 50 PBC patients (52.30±10.13 years of age; 46 females), and 38 healthy controls who were healthy volunteers (45.60±7.64 years of age; 35 females). A set of 12 PSS patients,12 PBC patients, and 12 HCs randomly selected from lectin microarray analysis together with a new cohort of samples (including 16 PSS patients (43.44±9.58 years of age; 13 females), 16 PBC patients (53.75±12.75 years of age; 15 females), and 16 health controls (35.19±5.06 years of age; 15 females)) was collected to verify significant findings using lectin blot. Autoantibody tests indicated that anti-SSA positivity was observed in 95% and 96.4% of the microarray and lectin blot PSS cohorts, while anti-SSB positivity was observed in 72.5% and 50%, respectively. AMA-M2 positivity was present in 74% and 82.1% of the microarray and lectin blot PBC cohorts.

Lectin microarray analysis for serum IgG glycosylation

Overall results of 56 lectins were presented in figure S1. Significant results of the lectin microarray were shown in Table 2. Compared to HCs, binding levels of MNA-M (prefers glycan mannose, fold change 1.57, P = 0.001) and LCA (prefers glycan fucose, fold change 1.33, P = 0.028) were increased, while PHA-E and PHA-L (prefer glycan galactose, fold change 0.334 and 0.206, P = 0.004 and 0.006) were decreased in PSS patients. Compared to PBC patients, the signal intensities of the lectins MNA-M (fold change 1.37, P =0.013), LCA (fold change 1.35, P =0.011), and ACL (prefers glycan Galβ3GalNAc, fold change 1.37, P =0.012) were significantly increased, while that of lectin SSA (prefers glycan sialylation, fold change 0.72, P =0.001) was significantly decreased in serum IgG from PSS patients. As demonstrated in Figure 1, PSS

patients' serum IgG had significantly higher affinities for MNA-M and LCA in comparison with PBC and health controls (P<0.01). The fold-change results of all lectins for PSS compared to PBC and HC were illustrated in figure S2 and S3.

Lectin blot analysis

Since significant differences were observed only for MNA-M and LCA among PSS PBC, and HC groups, the two lectins were selected to validate microarray results. MNA-M results showed that PSS patients had a higher affinity for MNA-M in comparison with PBC patients and HCs, indicating an increased binding level of mannose in serum IgG from patients with PSS (Flurorescense intensity signal PSS: $90.72*10^3 \pm 23.85*10^3$, PBC: $69.93*10^3 \pm 138.45*10^3$, HC: $71.49*10^3 \pm 126.56*10^3$, P<0.01, Figure 2), which was consistent with the result from lectin microarray. LCA results showed that PSS patients had a higher affinity for LCA compared to PBC patients and HCs, indicating an increased binding level of fucose in serum IgG from patients with PSS (Flurorescense intensity signal PSS:$122.04*10^3 \pm 42.51*10^3$, PBC: $84.64*10^3 \pm 33.67*10^3$, HC: $71.06*10^3 \pm 25.59*10^3$, P<0.01, Figure 3), which was also consistent with the result from lectin microarray.

Discussion

Numerous studies have confirmed that the change of IgG Fc glycosylation has an important effect on the activity of antibodies [14,30], and has an important role in the occurrence and development of autoimmune diseases [22,31]. lectin microarray is based on the interaction of lectins with glycans and enables rapid, high-throughput, and high-sensitivity profiling of complex glycans features. In this study, lectin microarray with 56 kinds of lectin was used to detect the structures of serum IgG oligosaccharides in patients with PSS. Elevated binding of mannose and fucose was specifically observed in PSS patients compared to PBC patients and HCs. Binding of

Galβ3GalNAc was increased and sialyation was decreased for PSS compared to PBC patients, while binding of galactosylation was decreased compared to healthy controls.

Regarding distinguishing PSS from healthy controls, a lack of the core fucose of IgG can significantly increase its affinity for the FcγRⅢa receptor and promote ADCC [32,33]. Bisecting GlcNAc was also associated with a decrease in core fucose. Concerning mannose glycan, a recent study using the same lectin microarray found that an elevated level of IgG4 mannose was associated with lacrimal and salivary glands' involvement in IgG4-related disease, possible through the complement lectin pathway[29]. Additionally, similar to our result, previous research has also reported that PSS expressing rheumatoid factor (RF) exhibited low expression of galactose in serum IgG [34,35]. Galactose is the most variable IgG glycosylation trait at the population level [36] and can change quickly in acute inflammation [37]. Fc galactosylation is necessary for the efficient initiation of the anti-inflammatory signaling cascade through binding to the inhibitory receptor FcγRIIb [38]. IgG agalactosylated structures (IgG-G0) were significantly increased in patients with RA and positively correlated with disease activity[39]. Combined with our results, we speculated that the altered IgG glycosylation patterns might contribute to the pathogenesis of PSS such as secretory gland destruction.

Compared to PBC patients, our study indicated that binding levels of glycan mannose, fucose, and Galβ3GalNAc were increased, while that of sialylation was decreased in PSS. Galβ3GalNAc is the core 1 structure of O-glycosylation, which has only been observed in the hinge region for IgG3. Though not fully investigated, this alteration might prevent the immunoglobulin from proteolytic degradation and assist in antigen-binding of the Fab fragment by maintaining flexibility[40]. Sialylation of the IgG Fc domain has been found to negatively regulate the complement-dependent cytotoxicity (CDC) effect [41]. An abnormally high level of asialylated IgG

had also been observed in the previous study [42]. In all, unique IgG glycosylation patterns of PSS and PBC may provide a reasonable direction for identifying PSS from PBC.

Apart from specific glycans, our study also suggested that high-throughput lectin microarray was a convenient and robust method for studying glycosylation for autoimmune diseases. Alteration of affinity for lectins MNA-M and LCA could serve as specific disease biomarkers for PSS patients and provide additive value in diagnosis.

Our study has some limitations. Due to a restricted number of clinical samples, autoimmune disease controls apart from PBC and SLE were not included. Baseline information and laboratory results were not sufficiently collected for the patients. The cohorts should also be expanded to validate the finding. Although lectin microarray could serve as a convenient tool for glycosylation study, the exact structure and site of glycosylation could not be clarified. Since serum IgG types and levels were not adjusted for the analysis, changes in lectin binding may not fully reflect the degree of specific glycan. In the future, other techniques such as MS would be combined to further investigate the role of glycosylation in PSS.

Conclusion

Changes in serum IgG glycosylation in PSS increased binding levels of LCA and MNA-M lectins using microarray techniques, which could provide potential diagnostic value. Elevated levels of fucose and mannose may play important roles in PSS pathogenesis.

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Conflict of Interest

All authors declare no conflicts of interest.

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Figure legends:

Figure 1. Specific changes of serum IgG glycosylation from the lectin microarray.

**P<0.01. Red bars represent the mean ± standard deviation. PSS: Primary Sjögren's syndrome; PBC: Primary biliary cholangitis; HC: Health control; LCA, Lens Culinaris Agglutinin; MNA-M, Morniga M Lectin; S/N, the medium intensity of the spot foreground relative to the background.

Figure 2. Lectin blot of MNA-M lectin for serum IgG.

(A) Lectin blot of MNA-M for serum IgG selected from Lectin microarray cohort. (B) Lectin blot of MNA-M for serum IgG selected from a new cohort. (C) Specific changes of MNA-M lectin blot bands combining (A) and (B). **P<0.01. Red bars represent the mean ± standard deviation. PSS, Primary Sjögren's syndrome; PBC, Primary biliary cholangitis; HC, Health controls; MNA-M, Morniga M Lectin; R, Reference.

Figure 3. Lectin blot of LCA lectin for serum IgG in PSS patients.

(A) Lectin blot of LCA for serum IgG selected from Lectin microarray cohort. (B) Lectin blot of LCA for serum IgG selected from a new cohort. (C) Specific changes of LCA lectin blot bands combining (A) and (B). **P<0.01. Red bars represent the mean ± standard deviation. PSS, Primary Sjögren's syndrome; PBC, Primary biliary cholangitis; HC, Health control; LCA, Lens Culinaris Agglutinin; R, Reference.

Table 1(on next page)

Table 1. Clinical and laboratory characteristics of all 212 subjects.

PSS, Primary Sjögren's syndrome; PBC, primary biliary cholangitis; HC, health control; NA, not available. Lectin blot samples were collected from 12 PSS patients,12 PBC patients, and 12 HCs randomly selected from the lectin microarray analysis cohort, and 16 PSS patients, 16 PBC patients, and 16 HCs from a new cohort.

Table 1. Clinical and laboratory characteristics of all 212 subjects.

Parameter	Lectin microarray			Lectin blot		
	PSS (n=40)	PBC (n=50)	HC (n=38)	PSS (n=28)	PBC (n=28)	HC (n=28)
Sex(M/F)	4/36	4/46	3/35	4/24	1/27	3/25
Age(y)	48.52±9.73	52.30±10.13	45.60±7.64	46.39±10.05	52.21±11.92	41.14±6.76
Laboratory results						
Anti-SSA+ (n, %)	38 (95.0%)	4 (8.0%)	0	27 (96.4%)	1 (3.6%)	0
Anti-SSB+ (n, %)	29 (72.5%)	1 (2.0%)	0	14 (50.0%)	1 (3.6%)	0
AMA-M2+ (n, %)	NA	37 (74.0%)	0	NA	23 (82.1%)	0
Anti-dsDSA+ (n, %)	6 (15.0%)	0	0	3 (10.7%)	0	0
Anti-Scl-70+ (n, %)	1 (2.5%)	2 (4.0%)	0	1 (3.6%)	1 (3.6%)	0

PSS, Primary Sjögren's syndrome; PBC, primary biliary cholangitis; HC, health control; NA, not available. Lectin blot samples were collected from 12 PSS patients,12 PBC patients, and 12 HCs randomly selected from the lectin microarray analysis cohort, and 16 PSS patients, 16 PBC patients, and 16 HCs from a new cohort.

Table 2(on next page)

Table 2. Significant differences in binding between IgG and lectin in PSS, PBC, and HCs.

**$P<0.01$,*$P<0.05$. PSS, Primary Sjögren's syndrome; PBC, primary biliary cholangitis; HC, health control; SSA, Salvia sclarea; LCA, Lens Culinaris Agglutinin; MNA-M, Morniga M Lectin; ACL, Amaranthus caudatus lectin; PHA-E, Phaseolus vulgaris Erythroagglutinin; PHA-L, Phaseolus vulgaris Leucoagglutinin.

Table 2. Significant differences in binding between IgG and lectin in PSS, PBC, and HCs

Lectin	Normalized fluorescence intensity (Mean ± SD)			Fold Change			
	PSS	PBC	HC	PSS/PBC	*P*	PSS/HC	*P*
SSA	2.57±1.64	3.57±1.86	2.60±1.16	0.72	0.001**	0.99	0.934
LCA	5.00±3.10	3.71±1.45	3.75±1.56	1.35	0.011*	1.33	0.028*
MNA-M	5.79±3.88	4.20±1.92	3.68±1.21	1.37	0.013*	1.57	0.001**
ACL	2.03±1.36	1.48±0.61	2.40±3.06	1.37	0.012*	0.85	0.490
PHA-E	7.44±4.32	8.34±4.41	9.81±4.50	0.89	0.334	0.75	0.004**
PHA-L	8.32±4.99	9.81±5.87	11.12±5.36	0.85	0.206	0.75	0.006**

**$P<0.01$,*$P<0.05$. PSS, Primary Sjögren's syndrome; PBC, primary biliary cholangitis; HC, health control; SSA, Salvia sclarea; LCA, Lens Culinaris Agglutinin; MNA-M, Morniga M Lectin; ACL, Amaranthus caudatus lectin; PHA-E, Phaseolus vulgaris Erythroagglutinin; PHA-L, Phaseolus vulgaris Leucoagglutinin.

Figure 1

Figure 1. Specific changes of serum IgG glycosylation from the lectin microarray.

**P<0.01. Red bars represent the mean ± standard deviation. PSS: Primary Sjögren's syndrome; PBC: Primary biliary cholangitis; HC: Health control; LCA, Lens Culinaris Agglutinin; MNA-M, Morniga M Lectin; S/N, the medium intensity of the spot foreground relative to the background.

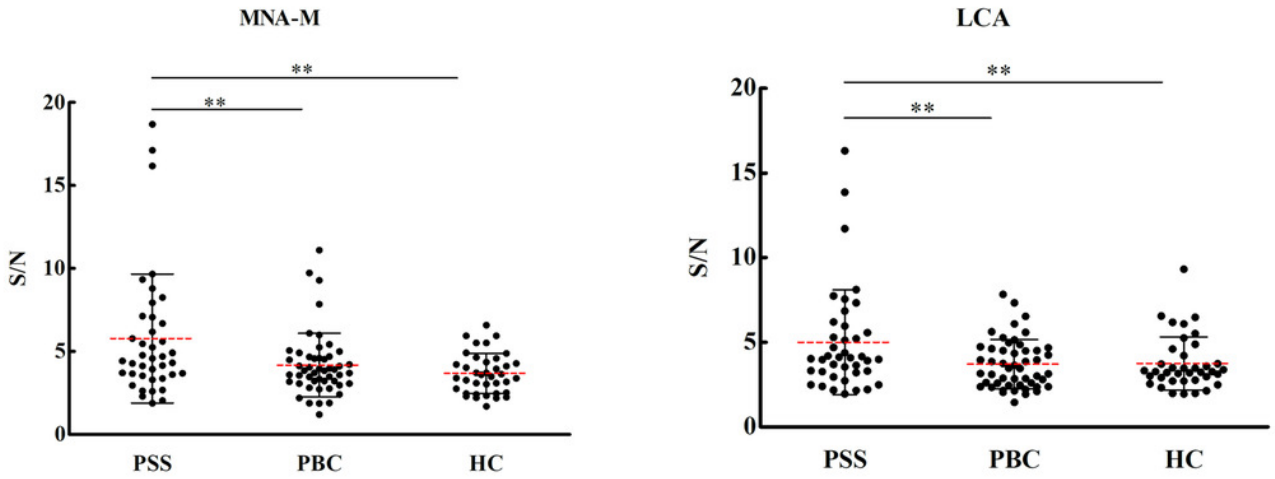

Figure 2

Figure 2. Lectin blot of MNA-M lectin for serum IgG.

(A) Lectin blot of MNA-M for serum IgG selected from Lectin microarray cohort. (B) Lectin blot of MNA-M for serum IgG selected from a new cohort. (C) Specific changes of MNA-M lectin blot bands combining (A) and (B). **P<0.01. Red bars represent the mean ± standard deviation. PSS, Primary Sjögren's syndrome; PBC, Primary biliary cholangitis; HC, Health controls; MNA-M, Morniga M Lectin; R, Reference.

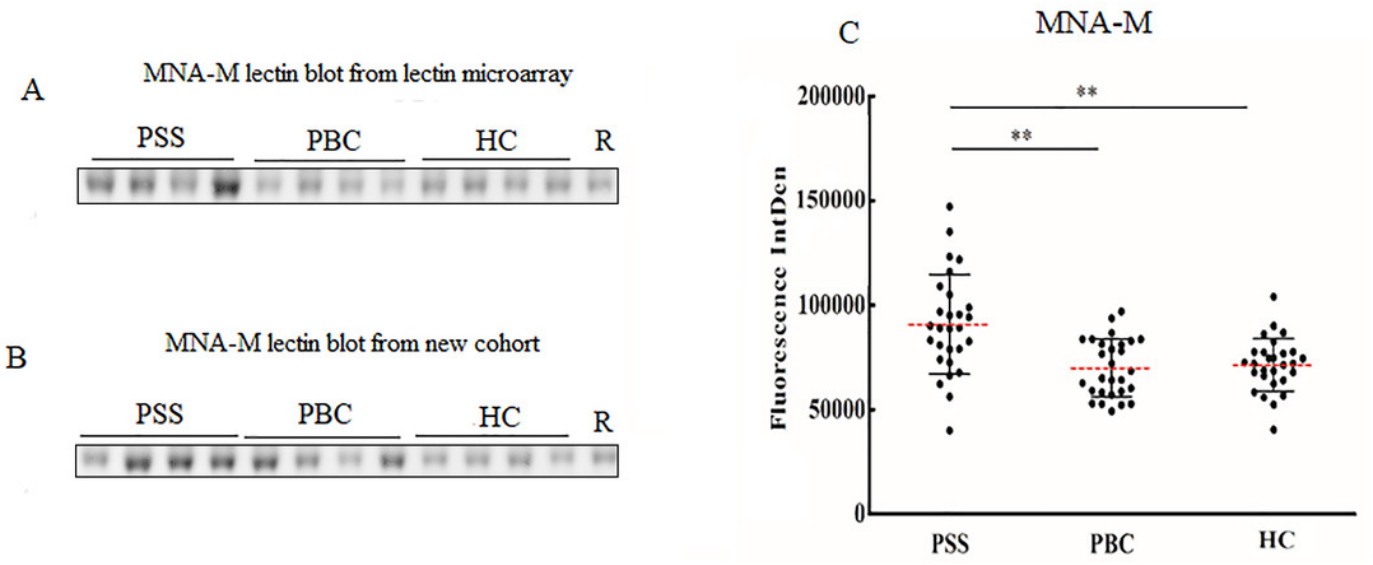

Figure 3

Figure 3. Lectin blot of LCA lectin for serum IgG in PSS patients.

(A) Lectin blot of LCA for serum IgG selected from Lectin microarray cohort. (B) Lectin blot of LCA for serum IgG selected from a new cohort. (C) Specific changes of LCA lectin blot bands combining (A) and (B). **P<0.01. Red bars represent the mean ± standard deviation. PSS, Primary Sjögren's syndrome; PBC, Primary biliary cholangitis; HC, Health control; LCA, Lens Culinaris Agglutinin; R, Reference.