Alteration of serum *N*-glycan profile in patients with autoimmune pancreatitis

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Running title: *N*-glycan changes in autoimmune pancreatitis

Abstract

Objectives: The aims of this study were to determine the change in whole-serum N-glycan profile in autoimmune pancreatitis (AIP) patients and to investigate its clinical utility.

Methods: We collected serum from 21 AIP patients before any treatment, and from 60 healthy volunteers (HLTs). Serum glycan profile was measured by comprehensive and quantitative high-throughput glycome analysis.

Results: Of the 53 glycans detected, 14 were differentially expressed in AIP patients. Pathway analysis demonstrated that agalactosyl and monogalactosyl bi-antennary glycans were elevated in AIP patients.

Among the 14 glycans, #3410, #3510, and #4510 showed high area under receiver operating characteristic (AUROC) values (0.955, 0.964, and 0.968 respectively) for the diagnosis of AIP. These three glycans were mainly bound to immunoglobulin G; however, their serum levels were significantly higher, even in AIP patients who showed lower serum IgG4 levels, than in HLTs.

Conclusions: We demonstrated, for the first time, whole-serum glycan profiles of AIP patients and showed that the levels of glycans #3410, #3510, and #4510 were increased in AIP patients. These glycans might be valuable biomarkers of AIP.

Keywords: autoimmune pancreatitis (AIP), glycomics, biomarker

Introduction

Autoimmune pancreatitis (AIP) is a benign fibroinflammatory disease of the pancreas that has recently attracted worldwide attention [1,2]. The term AIP was first proposed in 1995 for a steroid-responsive pancreatic inflammatory disease; since then, it has become recognized as a distinct clinicopathological entity [3].

Serum glycans have been reported to be promising diagnostic markers for chronic inflammatory disease [4]. In autoimmune diseases, such as inflammatory bowel disease and rheumatoid arthritis, agalactosylation of IgG-bound glycan was observed, and it correlated with disease activity [5,6]. However, the majority of previous reports did not describe serum glycome expression comprehensively, but just measured several glycans attached to a particular protein, such as acute phase proteins or IgG. In addition, there have been no studies reporting on serum glycomics in AIP patients.

A new technology for glycan-specific enrichment, the "glycoblotting" method, was recently developed [7]. This method enables a comprehensive analysis of serum glycans, can achieve high-throughput and quantitative glycomic analysis, and has been applied to construct new diagnostic and prognostic markers for pancreatic cancer, hepatocellular carcinoma, and inflammatory bowel disease [8,9,10].

The aims of this study were to generate whole-serum glycan profiles for patients with AIP using the glycoblotting method and to identify the proteins for which glycan expression was changed. Moreover,

we analyzed glycan expression in the AIP patients with low IgG4 levels, who are difficult to diagnose as AIP [11] and evaluated the potential of glycan profiles as new clinical markers for AIP.

Patients and methods

Patients

Twenty-one patients who were newly diagnosed as having AIP at Okayama University Hospital between April 2004 and March 2014 were enrolled. Sixty healthy volunteers (HLTs) with no apparent health problems, were also enrolled in this study. Glycan expression data of 86 patients with pancreatic cancer (PC), 40 patients with chronic pancreatitis (CP) and 79 patients with intraductal papillary mucinous neoplasms (IPMN) was used as disease controls. The changes of glycans by steroid therapy were also examined in 6 patients.

Written informed consent for serum analysis and use of clinical data was obtained from all patients and HLTs. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the Okayama University Ethics Committee (authorization #669).

Diagnosis

The diagnosis of AIP was made according to the international consensus diagnostic criteria (ICDC) for AIP based on pancreatic histology and/or imaging, serology, other organ involvement, and response to steroid therapy [12]. It is known that there are two clinicopathologically different forms of AIP: type 1, called lymphoplasmacytic sclerosing pancreatitis (LPSP) and type 2, characterized by neutrophilic

infiltration of the epithelium of the pancreatic duct [12]. In this study, all AIP patients were classified as type 1.

All patients with AIP were tested for serum IgG4 levels (normal range, 4.8–105 mg/dL). A high level of IgG4 was defined as > 210 mg/dL (2-fold higher than the upper limit of normal) and a low level of IgG4 was defined as $\leq$ 210 mg/dL.

Measurement of serum glycans using the glycoblotting method

We collected serum from all the HLTs and from all the patients at the time of admission, before any treatment. The blood samples were centrifuged for 10 minutes at 15,000×g, and supernatants were frozen immediately and stored below -30°C until use. Glycoblotting was performed according to a procedure described previously [7]. Briefly, 10- μ L serum samples were applied to an automated machine, “Sweetblot” prototype 7 (System Instruments Co, Tokyo, Japan), for pretreatment and glycoblotting. After enzymatic cleavage from proteins, glycans were captured on BlotGlyco H beads (Sumitomo Bakelite, Co, Tokyo, Japan), and sialic acids were methyl-esterified. The processed glycans were tagged with benzyloxyamine and released from the beads, followed by detection by MALDI-TOF MS (Ultraflex 3, Bruker, Burlington, MA, USA)

We assigned four-digit numbers to each glycan. These numbers indicate the number of sugar components in the following order: hexose (galactose and mannose), *N*-acetylhexosamine

(*N*-acetylglucosamine), fucose, and sialic acid.

Fractionation of serum proteins

To identify the proteins to which AIP-related glycans were attached, pooled serum obtained from 13 patients randomly selected from all the AIP patients was fractionated on Proteus protein G (PUR008) and A (PUR016) microspin columns (AbD Serotech, Kidlington, UK) before measurement of glycans.

The fractionation was performed according to the manufacturer's protocols with the following minor modifications. Serum (100 μ L) was diluted with 100 μ L of binding buffer (0.1 M sodium phosphate buffer, 0.15 M NaCl, pH 7.4) and loaded on the protein G spin column. The spin column was centrifuged at 600 $\times$ g for 6 minutes and washed three times with 90 μ L of buffer A, followed by centrifugation for 2 minutes at 1800 $\times$ g. The flow through (FT) and washes were combined for loading onto a protein A spin column and processed as described for the protein G column. The FT and washes from the protein A column were combined as the 'FT' fraction. Proteins bound to the protein G and A spin columns (the 'G' and 'A' fractions, respectively) were eluted four times with 150 μ L of elution buffer (0.2 M glycine/HCl buffer, pH 2.5) directly into a tube containing 65 μ L of neutralization buffer (1 M Tris/HCl buffer, pH 9.0) to adjust the final pH to 7.5. Subsequently, the protein solutions of each fraction were applied to a PD MidiTrap G-25 column (GE Healthcare Life Sciences, Buckinghamshire, UK) in order to exchange the buffers with 50 mM ammonium bicarbonate. Following the buffer exchange,

the fractionated proteins were lyophilized and dissolved in 100 µL of phosphate-buffered saline (PBS).

The glycans in the G, A, and FT fractions were measured as for those in serum samples.

Statistical analysis

Wilcoxon's rank sum test was used to compare the continuous data. Diagnostic value was demonstrated by area under the receiver operating characteristic curve (AUROC). The JMP (version 8) software package (SAS Institute, Cary, NC, USA) was used for the analyses and $p < 0.05$ was considered significant. Bonferroni correction was used for multiple comparisons and $p < 0.05/53$ was considered significant for evaluation of the difference in glycan expression between AIP patients and HLTs. Pathway analysis was conducted using the Cytoscape program (<http://www.cytoscape.org/>

). The ranges of continuous values were shown as the interquartile range (IQR).

Results

Characteristics of patients

The characteristics of the AIP patients are summarized in Table 1. Thirteen of the 21 patients with AIP had the diffuse form and 8 had the focal form of the disease. Extrapancreatic manifestations were observed in six patients (sclerosing cholangitis in two patients, retroperitoneal fibrosis in two patients, and symmetrically enlarged salivary glands in two patients). Sixteen patients (76.2%) were administered steroid (initial dose, 0.6mg prednisolone/kg/day) and all patients were responded well to the treatment. In AIP patients, the median IgG4 level was 198 mg/dL (range, 109–362), and 11 patients (52.4%) were categorized as having low IgG4. Median carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (Ca 19-9) levels were 2.5 ng/mL (range 1.6–4.1) and 14 U/mL (range 8–37), respectively, in AIP patients. The 60 HLTs had a median age of 65 (range, 62–67) years, and 35 (58.3%) were male. There were no differences in age or sex between the AIP and HLT populations.

Changes in serum glycan profiles

Glycome analysis detected 53 *N*-glycans with mass-to-charge ratios (m/z) ranging from 1362.481 to 3865.407. Five glycans were classified as high-mannose type, six as hybrid type, and 42 as complex type. The median combined concentration of all high-mannose glycans in AIP patients was 10.6 μ M, which was higher than that in the HLT group (9.1 μ M; $p = 0.03$). The same relationships were observed for

hybrid glycans and complex glycans; the median levels of hybrid glycans were 9.4 and 6.2 μM ($p < 0.0001$) and those of complex glycans were 401.2 and 336.7 μM ($p < 0.0001$) in the AIP and HLT groups, respectively.

Among the 53 glycans examined, the expression of 14 glycans differed significantly between AIP patients and HLTs after the Bonferroni correction (Table 2). All of these glycans, with the exception of glycan #6600, were upregulated in patients with AIP, and they comprised 10 bi-antennary glycans, 2 tri-antennary glycans and 1 tetra-antennary glycan.

Among the 10 bi-antennary glycans, three were agalactosyl glycans (#3410, #3500, and #3510), four were monogalactosyl glycans (#4410, #4411, #4500, and #4510) and many more than these are shown in Figure 1. When we assessed the utility of these glycans for discriminating AIP from HLT, glycans #3410, #3510, and #4510 showed high AUROC values (0.955, 0.964, and 0.968, respectively).

We investigated the association between characteristics of AIP patients and serum levels of glycans #3410, #3510, and #4510 (Table 3). The expression level of #3410 was high in patients with high serum IgG levels, but other factors, such as sex, age, form of pancreatic enlargement, presence of extrapancreatic manifestation, and serum IgG4 levels were not correlated with the expression levels of the three glycans.

Pathway analysis

Pathway analysis was performed to identify trends in glycan biosynthesis in AIP patients (Figure 1). Mean glycan values of all AIP patients and HLTs were used in this analysis. Biosynthesis of agalactosyl bi-antennary glycans (#3410, #3500, and #3510) and monogalactosyl bi-antennary glycans (#4410, #4411, #4500, #4501, #4510, and #4511) were increased, whereas expression of digalactosyl bi-antennary glycans and other types of glycans were relatively suppressed in AIP patients compared to HLTs.

Expression of glycans in AIP patients with low IgG4

To elucidate the clinical utility of glycans #3410, #3510, and #4510 for diagnosis of AIP in patients who were difficult to diagnose based on serum IgG4 level, we compared glycan expression in two AIP groups (high IgG4 and low IgG4) and HLTs. The median values of glycan #3410 were 30.1, 26.8, and 10.5 μ M in AIP patients with high IgG4, AIP patients with low IgG4, and in HLTs, respectively (Figure 2A). Expression of glycan #3410 did not differ between the groups with high and low IgG4, whereas expression was significantly higher in AIP patients with low IgG4 than in HLTs ($p < 0.0001$). The same relationships were observed for glycans #3510 (Figure 2B) and #4510 (Figure 2C). The median concentrations of glycan #3510 were 7.6, 6.0, and 2.1 μ M, and those of #4510 were 6.5, 8.0, and 3.4 μ M in AIP patients with high IgG4, AIP patients with low IgG4, and in HLTs, respectively.

Changes of glycan expressions by steroid therapy

We examined the changes of the glycan expressions (#3410, #3510, and #4510) after steroid therapy in six patients . The glycan expressions were decreased by the treatment in most of the cases. Especially glycan #3410 was decreased in all cases (supplemental figure 1).

Expression of 3 glycans in other pancreatic diseases

The expressions of #3410, #3510 and #4510 in various pancreatic diseases were shown in Figure 3. The expressions of the three glycans in AIP were significantly higher than those in PC, CP and IPMN ($p < 0.0001$). The median values of glycan #3410 were 28.9, 11.8, 11.5 and 10.5 μM , the values of glycan #3510 were 6.2, 2.5, 2.5 and 4.2 μM , and those of #4510 were 7.6, 3.8, 3.9 and 4.7 μM in AIP, PC, CP and IPMN, respectively.

Glycan expression in specific protein fractions

In order to identify the proteins to which glycans were attached, we measured glycan levels in the G, A, and FT fractions of pooled serum from AIP patients. Glycans #3410, #3510, and #4510 were all present in the G fraction at concentrations of 10.7, 2.6, and 2.3 μM , respectively. In the A fraction, small amounts of glycans #3410 and #4510 were detected (1.5 and 0.5 μM , respectively); however, no glycan #3510 was detected in this fraction. None of the three glycans (#3410, #3510, or #4510) were detected in the FT fraction.

Discussion

This is the first study to describe whole-serum *N*-glycan profiles of AIP patients comprehensively and quantitatively. The total level of *N*-glycans was elevated in AIP patients, and pathway analysis demonstrated that biosynthesis of agalactosyl and monogalactosyl bi-antennary glycans was increased. We also found that levels of glycans #3410, #3510, and #4510 were significantly higher in AIP patients than in HLTs, and have shown high AUROC values, allowing the discrimination of AIP patients from HLTs. Furthermore, serum levels of glycans #3410, #3510, and #4510 were significantly elevated, even in AIP patients with low IgG4 levels. In the analysis of serum fractions, these three glycans were detected at high levels in the G fraction.

Because the elevation of the three glycans was observed regardless of IgG4 level, the glycan changes seemed to reflect the changes occurred in non-IgG4 fraction, which is the dominant IgG fraction in the serum, although the same glycan changes might occur in IgG4 and play an important role in the pathogenesis of AIP.

Based on our observations, most of the agalactosyl bi-antennary glycans were detected in the protein G-bound fraction and were upregulated in AIP patients. This was considered to be a result of alteration of IgG glycosylation in the direction of lower galactosylation in AIP patients. This abnormality in IgG glycosylation is recognized in several autoimmune diseases, including inflammatory bowel disease and

rheumatoid arthritis [5,6], and correlates with lower galactosyltransferase activity in B and T cells [13]. Nakajima S et al. reported that this modification of IgG enhances antibody-dependent phagocytosis *in vitro* [14]. Antibody agalactosylation was reported to reduce complement-dependent cytotoxicity *in vitro* [15], and agalactosyl IgG ameliorates mucosal inflammation in inflammatory bowel disease, associated with production of interleukin 10 by macrophages in a mouse model [16]. Although we did not observe a correlation of agalactosyl IgG with clinical features of AIP patients, the clear difference in the expression levels between AIP patients and HLTs, indicating a shift in IgG glycoforms, might modulate immune function and correlate with the etiology of AIP, as was observed with other autoimmune diseases. The decrease of the serum glycans after steroid therapy supports this hypothesis. To make the pathogenic roles of the alterations of these glycan clear, the analysis of glycan profile with pancreatic tissues might be helpful, although we could not examine them because the quantification of glycans requires certain amount of tissue and sufficient volume for the analysis could not be obtained by EUS-FNA.

IgG4 has often been proposed as a possible diagnostic serological biomarker [11], and serum IgG4 concentration is therefore considered a reliable marker for the diagnosis of AIP. However, slight elevations in serum IgG4 are seen in up to 10% of pancreatic cancer cases [17], and not all AIP patients showed high serum IgG4, therefore IgG4 is not an absolute marker. To improve diagnostic accuracy, organ-specific autoantibodies, such as those against carbonic anhydrase [18,19,20,21], lactoferrin [18],

and pancreatic secretory trypsin inhibitor [21], were reported as complementary markers for IgG4. But they have not provided satisfactory specificity and sensitivity. In this study, glycans #3410, #3510, and #4510 showed high AUROC values (>0.9) for the diagnosis of AIP. In addition, three glycans were not elevated in PA, CP and IPMN patients. Although it is not clear whether these alterations in glycoform occur on a specific antibody or not, the glycan markers would be useful for the diagnosis of AIP in patients with low IgG4, as well as to distinguish AIP from pancreatic cancer in patients with elevated IgG4.

Although we successfully generated glycan profiles of AIP patients, there are several limitations to this study. First, the analyses were performed retrospectively in a relatively small sample. Secondly, we found that these three glycans were attached to immunoglobulin G, but could not clarify the subtype of IgG in which the glycan alterations occurred. Finally, we could not show the glycan expressions in other IgG4 related diseases or the change of the expression by steroid therapy.

Despite these limitations, we demonstrated for the first time that whole-serum *N*-glycan profiles are altered in AIP patients, and that particular glycans, #3410, #3510, and #4510, were significantly elevated in AIP, indicating that the glycans might be valuable biomarkers of AIP. Further studies to validate the diagnostic potential of the glycans and to characterize the ligands of each glycan in detail as well as the biological effects of these glycan alterations, are necessary in the future.

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Figure legends

Figure 1. Pathway analysis of glycan biosynthesis.

Relative glycan expression level is indicated by color according to $\log_2(\text{patient/HLT})$ value, as shown in the key. The four-digit numbers indicate the number of hexose (galactose and mannose), *N*-acetylhexosamine (*N*-acetylglucosamine), fucose, and sialic acid moieties, in that order. All samples used in this analysis have shown mean values of autoimmune pancreatitis (AIP) patients or healthy controls (HLTs). Biosynthesis of agalactosyl bi-antennary glycans (#3410, #3500, and #3510) and monogalactosyl bi-antennary glycans (#4410, #4411, #4500, #4501, #4510, and #4511) were increased in AIP patients.

* #4511; reference date(quantitaiveness was not confirmed).

Figure 2. Expression of glycans in AIP patients with low IgG4

Box plots of (A) #3410, (B) #3510, and (C) #4510 glycan expression in groups of AIP patients with high and low IgG4 and in HLTs.

Figure 3. Expression of glycans in pancreatic diseases

Box plots of (A) #3410, (B) #3510, and (C) #4510 glycan expression in groups of AIP patients,

pancreatic adenocarcinoma(PA) patients, chronic pancreatitis(CP) patients and intraductal papillary.mucinous neoplasms (IPMN)patients.

Table 1. Characteristics of AIP patients

	AIP	HLT
Median age, years (IQR)	69 (58–73)	65(62-67)
Sex, n (%)		
Male	13 (61.9%)	35(58.3%)
Female	8 (38.1%)	25(41.7%)
Laboratory data, median (IQR)		
WBC, μ L	5280 (4455–6905)	-
Amylase, IU/L	65 (42–107)	-
T-Bil, mg/dL	0.65 (0.48–1.35)	-
AST, IU/L	32 (21–48)	-
CRP, mg/dL	0.22 (0.03–0.57)	-
HbA1c, %	5.9 (5.4–7.2)	-
CEA, ng/mL	2.5 (1.6–4.1)	-
CA19-9, U/mL	14 (8–37)	-
IgG, mg/dL	1667 (1330–1819)	-
IgG4, mg/dL	198 (109–362)	-

Abbreviations: AIP, autoimmune pancreatitis; HLT, healthy volunteer; IQR, interquartile range; WBC,

white blood cell; T-Bil, total bilirubin; AST, aspartate aminotransferase; CRP, C-reactive protein; HbA1c,

hemoglobin A1c; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9

Table 2. Differentially expressed glycans in patients with AIP

#	m/z	Proposed structure ^a	Median expression level (μM)		AIP vs. HLT	
			AIP (n=21)	HLT (n=86)	p-value	AUROC
3410	1590		28.4	10.5	<0.0001	0.955
3500	1647		1.6	0.8	<0.0001	0.896
4410	1752		25.6	15.3	<0.0001	0.897
3510	1793		6.2	2.1	<0.0001	0.964
4500	1809		1.7	0.8	<0.0001	0.906
5410	1914		14.1	11.7	<0.0001	0.787
4510	1955		7.6	3.4	<0.0001	0.968
4411	2057		3	1.8	<0.0001	0.890
6600	2336		3.1	4	<0.0001	0.787
5412	2525		19.7	15.3	0.0004	0.762
5512	2728		6.8	5	0.0002	0.778
6512	2890		1.5	1.1	0.0006	0.753
6513	3195		9.7	5.7	0.0006	0.753
7604	3719		0.8	0.5	0.0007	0.748

NOTE: P-values are from Wilcoxon's rank sum test.

^aMonosaccharide composition: rhombus, sialic acid; triangle, fucose; square, N-acetyl glucosamine;

yellow circle, galactose; green circle, mannose.

Abbreviations: AIP, autoimmune pancreatitis; HLT, healthy volunteer; AUROC, area under the receiver operating characteristic.

Table 3: Correlation between patient characteristics and serum glycans in AIP

Variables	n	# 3410		# 3510		# 4510	
		Expression level	p-value	Expression level	p-value	Expression level	p-value
		(μM)		(μM)		(μM)	
Sex							
Male	13	31.3	0.25	6.8	0.31	8.0	0.51
Female	8	25.4		5.8		6.5	
Age, years							
< 65	8	29.9	0.61	6.5	0.94	6.2	0.83
≥ 65	13	26.8		6.0		8.0	
Pancreatic enlargement							
diffuse	13	25.1	0.35	6.2	0.77	6.8	0.64
focal	8	28.7		6.4		7.8	
Extrapancreatic manifestation							
positive	6	27.6	0.53	6.5	0.48	9.0	0.35
negative	15	28.4		6.0		6.8	
WBC, μL							
< 5280	11	28.7	0.32	8.3	0.18	9.0	0.08
≥5280	10	25.1		5.6		5.8	
Amylase, IU/L							
< 65	11	28.7	0.40	6.8	0.67	6.8	0.94
≥65	10	25.1		5.9		7.8	
T-Bil, mg/dL							
< 0.65	12	34.2	0.06	8.0	0.06	9.1	0.23
≥0.65	9	22.5		5.4		6.2	
AST, IU/L							
< 32	11	28.4	0.97	6.8	0.78	6.8	0.23
≥32	10	26.0		5.9		8.7	
CRP, mg/dL							
< 0.22	11	29.0	0.67	8.3	0.21	9.0	0.26
≥0.22	10	25.1		5.5		6.1	
HbA1c, %							
< 5.9	13	26.8	0.94	6.0	0.66	7.9	0.61

≥ 5.9	8	30.1		7.6		6.5	
CEA, ng/mL							
< 2.5	11	23.4	0.48	5.6	0.12	5.6	0.09
≥ 2.5	10	30.0		8.7		9.1	
CA19-9, U/mL							
< 14	12	25.1	0.52	6.1	0.62	7.8	0.48
≥ 14	9	28.7		8.3		6.8	
Serum IgG							
< 1667 mg/dL	11	21.9	0.009	6.0	0.21	8.0	0.62
≥ 1667 mg/dL	10	35.7		7.6		6.5	
Serum IgG4							
< 210 mg/dL	11	26.8	0.40	6.0	0.48	8.0	0.78
≥ 210 mg/dL	10	30.1		7.6		6.5	

NOTE:

Values are expressed as medians (interquartile range)

P-values are from Wilcoxon's rank sum test.

The cut-off value of all parameters other than serum IgG4 were set at the median value.

Abbreviations: AIP, autoimmune pancreatitis; WBC, white blood cell; T-Bil, total bilirubin; AST,

aspartate aminotransferase; CRP, C-reactive protein; HbA1c, hemoglobin A1c; CEA, carcinoembryonic

antigen; CA19-9, carbohydrate antigen 19-9

Figure 1

Figure 2a

A

Figure 2b

B

Figure 2c

C

Figure 3a

A

Figure 3b

B

Figure 3c

C