Lectin-based Immunoassay for Aberrant IgG Glycosylation as the Biomarker for Crohn's Disease

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Background: Easily measured and clinically useful biomarkers for inflammatory bowel disease (IBD) are required to advance patient care. We previously reported that the agalactosyl fraction among fucosylated IgG oligosaccharides is increased in IBD, especially Crohn's disease (CD). The present study aimed to establish a simple detection system for aberrant glycosylated IgG based on lectin–oligosaccharide interactions.

Methods: Lectins with higher affinity to serum IgG from IBD patients than healthy volunteers (HV) were screened by lectin microarray. Binding of selected lectins to agalactosyl IgG was definitively confirmed using step-by-step glycosidase treatment. Using the selected lectins, a lectin-enzyme-linked immunosorbent assay system was established and its clinical utility was investigated in a total of 410 (249 Japanese and 161 American) IBD patients, disease controls, and HVs.

Results: *Agaricus bisporus* Agglutinin (ABA) and *Griffonia simplicifolia* Lectin-II (GSL-II) had higher affinity for serum agalactosyl IgG from IBD patients, especially those with CD, compared to HV. Agalactosyl IgG levels measured by a lectin-enzyme immunoassay (EIA) with ABA or GSL-II were significantly increased in CD compared with HV and disease controls. Agalactosyl IgG levels significantly correlated with disease activity, showed higher predictability of therapeutic outcomes for CD than C-reactive protein levels, and exhibited higher specificity for diagnosing IBD in combination with anti-*Saccharomyces cerevisiae* antibody (ASCA). Validation analysis showed that agalactosyl IgG levels were significantly increased in Japanese and American CD patients.

Conclusions: A lectin-EIA for agalactosyl IgG is a novel biomarker for IBD, especially in patients with CD.

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Key Words: inflammatory bowel disease, Crohn's disease, biomarker, oligosaccharides, IgG

The human inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic relapsing and remitting inflammation in the digestive tract. Although genetic predisposition, environmental factors, and altered immune responses have pivotal roles in the pathogenesis of IBD, precise etiologies remain unknown. Despite recent therapeutic advances,[1,2] surgical care remains a mainstay treatment of IBD.[3] Therefore, a biomarker that reflects clinical course and therapeutic outcome is in high demand. Several serologic markers have been developed for the diagnosis of IBD such as anti-*Saccharomyces cerevisiae* antibody (ASCA),[4] peripheral antineutrophil cytoplasmic antibody,[5] or anti-outer membrane porin protein C, and anti-CBir1 flagellin.[6] However, these antibodies generally lack diagnostic and prognostic sensitivity and specificity to alter clinical decision making.

Immunoglobulin (Ig) G carries *N*-linked oligosaccharides at the Cgamma2 domain of the Fc fragment at asparagine 297, all of which are biantennary complex-type with or without bisecting *N*-acetylglucosamine (GlcNAc), core-fucose,

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TABLE 1. Patient Characteristics: Japanese Population

	CD ($N = 82$)	UC ($N = 70$)	HV ($N = 78$)	DC ($N = 19$)	SLE ($N = 29$)	RA ($N = 20$)
Male/Female	22/60	31/39	39/39	9/10	5/24	2/18
Age, yr, mean (SD)	38 (11)	39 (14)	45 (11)	35 (16)	33 (14)	52 (16)
Age at diagnosis, yr, mean (SD)	28 (10)	33 (13)				
Disease location, *N*						
Small bowel/colon/both/unknown	27/11/42/2					
Extensive/left colon/rectum/unknown		28/26/14/2				
Treatment, *N* (%)						
Salazosulfapyridine or mesalazine	72 (88)	59 (84)				
Steroids	4 (5)	9 (13)				
Immunomodulators	10 (12)	2 (3)				
Anti-TNF-alpha antibodies	7 (9)	0 (0)				
CRP, mg/dL, mean (SD)	1.0 (1.6)	0.4 (1.1)				
CDAI (CD) or CAI (UC), mean (SD)	153 (92)	3.3 (4.0)				

galactose, and sialic acid residues. Our previous analysis of IgG oligosaccharides revealed that the agalactosyl fraction among fucosylated oligosaccharides was significantly higher in patients with CD and UC than in healthy volunteers (HV) and disease controls (DC). Fucosylated agalactosyl IgG levels closely correlated with disease activity and clinical course in IBD patients, and had a significantly higher sensitivity to diagnose IBD compared with ASCA.[7] We also reported that agalactosyl IgG oligosaccharides enhanced antibody-dependent phagocytosis, suggesting that oligosaccharide alterations of IgG are not only a marker of IBD but also functionally modulate immune function.[8] The current analytic methodology, however, requires researchers to perform multistep complicated procedures to acquire an IgG oligosaccharide chart using high-performance liquid chromatography (HPLC), so the development of an easier detection system is necessary for widespread clinical application.

There have been technical obstacles in establishing simple detection systems for oligosaccharides due to difficulties in purifying an oligosaccharide-specific antibody. Although several oligosaccharide structures can be measured by lectin-antibody enzyme immunoassay (EIA),[9] lectins have a lower affinity and specificity for oligosaccharides than specific antibodies.[10] To overcome these problems, in the present study we developed a lectin-EIA system by using multiple lectins to detect agalactosyl IgG as a new serologic marker for IBD.

MATERIALS AND METHODS

Subjects

Serum samples were collected from 82 patients with CD, 70 patients with UC, 72 age/gender-matched unrelated HVs, and 19 patients with colonic inflammation including appendicitis, diverticulitis, and ischemic colitis (DCs). These participants were Japanese recruited at the Department of Gastroenterology and Hepatology, Osaka University Hospital (Suita, Osaka, Japan), and the Department of Gastroenterology, Osaka Rosai Hospital (Sakai, Osaka, Japan). Serum samples of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) were provided by the Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Hospital. Patient characteristics are presented in Table 1. On the validation analysis, American serum samples were collected from 103 patients with CD, 39 patients with UC, and 19 HVs, who were recruited at the Department of Medicine, University of North Carolina Hospital (Chapel Hill, NC). Patient characteristics are presented in Table 2. The Ethics Committee at each hospital approved the study protocol and written informed consent was obtained from each participant. Patients were diagnosed with CD or UC according to endoscopic, radiologic, histologic, and clinical criteria.[11–13] Patients with CD were classified by age at diagnosis (A1, below 16 years old; A2, between 17 and 40 years old; A3, above 40 years old), location (L1, ileal; L2, colonic; L3, ileocolonic; L4, isolated upper disease), and behavior (B1, nonstricturing and nonpenetrating; B2, stricturing; B3, penetrating) according to the Montreal Classification.[14] Clinical activities were determined using the Crohn's Disease Activity Index (CDAI) for CD[15] or the Clinical Activity Index (CAI) for UC.[16] Infliximab maintenance therapy[17] was performed on biologic therapy-naïve CD patients. Clinical responders by infliximab therapy were defined as the patients whose CDAI scores at week 30 were less than 150 or decreased more than 70 from the pretreatment scores.

IgG Oligosaccharide Analysis by HPLC

Serum IgG oligosaccharide analysis was performed as described previously.[7] Briefly, IgG was purified using Protein

TABLE 2. Patient Characteristics: U.S. Population

	CD ($N = 103$)	UC ($N = 39$)	HV ($N = 19$)
Male/Female	49/54	23/16	11/8
Age, yr, mean (SD)	39 (14)	43 (18)	31 (4)
Age at diagnosis, yr, mean (SD)	27 (9)	33 (7)	
Duration of disease, yr, mean (SD)	12 (9)	10 (7)	
Disease location (*N*)			
Small bowel/colon/both/others	28/24/49/3		
Extensive/left colon/rectum/unknown		24/11/3/1	
Treatment, N (%)			
Salazosulfapyridine or mesalazine	25 (24)	20 (51)	
Steroids	15 (14)	9 (23)	
Immunomodulators	35 (34)	4 (10)	
Anti-TNF-alpha biologics	23 (22)	11 (28)	

G sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK). *N*-linked oligosaccharides were released from serum IgG by Glycopeptidase F (Takara Bio, Shiga, Japan) and labeled with 2-aminopyridine by GlycoTag (Takara Bio). Pyridylamino- (PA-) oligosaccharides from IgG were analyzed on a reverse-phase HPLC system (Waters, Milford, MA).

Lectin Microarray

Total pattern of oligosaccharide structures in serum IgG was investigated with evanescent-field fluorescence-assisted lectin microarray.[18] Forty-five kinds of lectins were immobilized on the glass slide in triplicate and ≈250 ng/mL of IgG in phosphate-buffered saline (PBS) with 1% Triton X-100 was applied to the array. To label target glycoproteins, Cy3 monoreactive dye (GE Healthcare Biosciences, Chalfont St Giles, UK) was used in this analysis. Detailed procedures were described previously.[18] Fluorescence intensity of all lectins and that of lower signal-lectins were analyzed independently.

Step-by-step Glycosidase Treatment Followed by Lectin Blotting

Purified serum IgG was obtained and incubated with sialidase, beta-galactosidase, and glucosaminidase by enzymatic carborelease kit (QA Bio, Palm Desert, CA), according to the manufacturer's instructions. Lectin blotting was performed in each step of glycosidase treatment.

Lectin Blotting

Eight micrograms of purified serum IgG was separated 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and then transferred to a nitrocellulose membrane (GE Healthcare Biosciences). The membrane was incubated overnight at 4°C with 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), and then for 1 hour at room temperature with biotinylated *Agaricus bisporus* Agglutinin (ABA) (1 μg/mL, Seikagaku biobusiness, Tokyo, Japan), *Griffonia simplicifolia* Lectin-II (GSL-II) (2 μg/mL, Vector Laboratories, Burlingame, CA), *Concanavalin A* (ConA) (0.5 μg/mL, Seikagaku biobusiness), *Sambucus sieboldiana* Agglutinin (SSA) (1 μg/mL, Seikagaku biobusiness), or *Ricinus communis* Agglutinin I (RCA120) (1 μg/mL, Seikagaku biobusiness) in 3% bovine serum albumin in TBS-T. After washing with TBS-T three times, the membrane was incubated with Vectastatin ABC kit (Vector Laboratories) for 20 minutes, then washed with TBS-T three times. Staining was performed with ECL reagent (GE Healthcare, Milwaukee, WI).

Lectin-EIA

Purified IgGs from sera of patients and HVs (adjusted to 5 μg/well) were prepared with sample diluents (PBS containing 0.1% bovine serum albumin [BSA] and 0.05% Tween 20) containing SDS (final concentration is 0.05%) for 1 hour. Flat-bottomed 96-well streptavidin-precoated microtiter plate (Nalge Nunc International, Tokyo, Japan) was coated with 50 μL/well of biotinylated ABA (0.33 μg/mL) and/or biotinylated GSL-II (0.67 μg/mL) with 0.05% BSA in PBS for 1 hour at room temperature. After washing the plate four times with PBS containing 0.05% Tween 20 (PBS-T), 50 μL/well of the prepared IgG was added. After incubation for 1 hour, the plate was washed four times with PBS-T, then 100 μL/well of 0.1% BSA in PBS was added to block nonspecific protein binding sites. After incubation for 1 hour, the plate was washed four times with PBS-T, then 50 μL/well of a 1/2000-diluted solution of alkaline phosphatase-conjugated antibody against human IgG (SouthernBiotech, Birmingham, AL) was added. After incubation for 1 hour, the plate was washed four times with PBS-T and added 50 μL/well of 0.1M glycine buffer (pH 10.4) with phosphate substrate (Sigma-Aldrich, St. Louis, MO), and then incubated for 20 minutes. The optical density (OD) was measured at 405 nm. All experiments were run in duplicate and the median was used as the final value for each sample. Agalactosyl IgG standard was kindly provided by Sanko Junyaku (Tokyo, Japan).[19]

Statistical Analyses

Differences between measurements and groups were tested with Mann–Whitney *U*-test. A *P* value less than 0.05 was considered statistically significant. A receiver operated characteristic (ROC) curve was generated by plotting sensitivity versus 1 – specificity for every possible cutoff score[20,21] and area under the ROC curve (AUC) was calculated. The optimal cutoff scores were determined by ROC curve, and then sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) were measured.

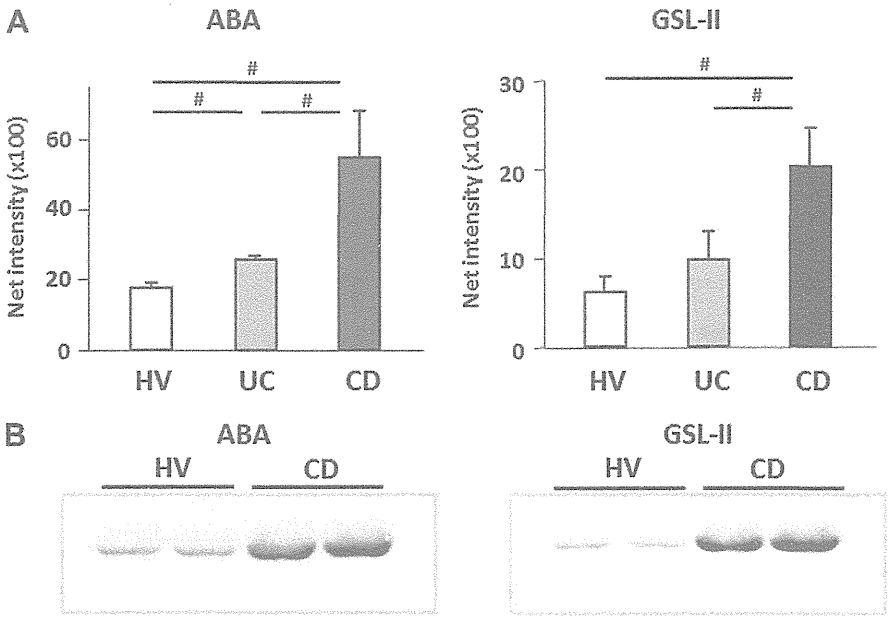

FIGURE 1. Binding levels of ABA and GSL-II to IgG *N*-linked oligosaccharides are higher in patients with IBD than in HVs. (A) Lectin microarray for serum IgGs from HV, patients with UC, and those with CD (five cases each). Among 45 lectins on the lectin microarray, results for ABA and GSL-II are shown. #$P < 0.05$. (B) Lectin blotting for serum IgGs from HV and CD (two cases each). Each blot is representative of three independent experiments with similar results.

All analyses were performed using JMP v.8 software for Windows (SAS, Cary, NC).

RESULTS

Lectin Microarray Analysis for Serum IgG Oligosaccharides

To identify the most relevant lectin(s) characteristic for detecting agalactosyl IgG in IBD, we first screened serum by lectin microarray. Serum IgGs from patients with CD, UC, and HV were purified by protein G column liquid chromatography and a lectin-microarray was performed for each sample (Fig., Supplemental Digital Content 1, http://links.lww.com/IBD/A0). Among 45 lectins, only two lectins had higher affinity for IBD and potentially recognize *N*-linked agalactosyl oligosaccharides. Lectin microarray showed that the signal intensities of the lectins, ABA and GSL-II, were significantly higher in IBD patients, especially those with CD, than in HV (Fig. 1A). Lectin blot analysis, performed to confirm the results obtained from lectin-microarray, clearly showed that serum IgGs from CD patients had higher affinity for both ABA and GSL-II compared to those from HV (Fig. 1B).

Both ABA and GSL-II Recognize "Agalactosyl IgG"

Previous reports showed that both ABA and GSL-II recognize agalactosyl *N*-linked oligosaccharides.[22,23] These studies were performed with frontal affinity chromatography using fluorescence-labeled oligosaccharides that are not attached to proteins. To confirm that both lectins recognize agalactosyl oligosaccharides attached to IgG, namely, "agalactosyl IgG," we first investigated whether IgG itself was recognized by these lectins using IgG treated with a set of glycopeptidases to detach oligosaccharides from IgG. Depletion of oligosaccharides was confirmed by Coomassie brilliant blue (CBB) staining, which showed a slight decrease in the molecular weight of IgG (Fig. 2A). Glycopeptidase-treated IgG showed decreased affinity for ConA, which recognizes most *N*-linked oligosaccharides except those with a bisecting GlcNAc structure,[24] as well as to both ABA and GSL-II (Fig. 2A). These results indicate that ABA and GSL-II accurately recognize oligosaccharides attached to IgG. Next, a step-by-step glycosidase treatment followed by lectin blotting was performed to determine the characteristic oligosaccharide structure. First, IgG was treated by sialydase, which detaches sialic acid from sugar chains. The removal of sialic acid was confirmed by sialic acid binding lectin, SSA. Binding of ABA and GSL-II to the IgG, however, was unchanged (Fig. 2B). Next, sialydase-treated IgG was incubated with the galactose remover beta-galactosidase. Affinities of ABA and GSL-II to IgG were increased after the removal of galactose, whereas the affinity of RCA120, an *N*-linked galactose binder, was decreased (Fig. 2C). Finally, galactosidase-treated IgG was incubated with glucosaminidase, which removes GlcNAc from mannose. Both ABA and GSL-II lectin blotting showed decreased binding affinities for IgG oligosaccharides (Fig. 2D). These findings indicate that both ABA and GSL-II recognize *N*-linked GlcNAc attached to IgG, namely "agalactosyl IgG."

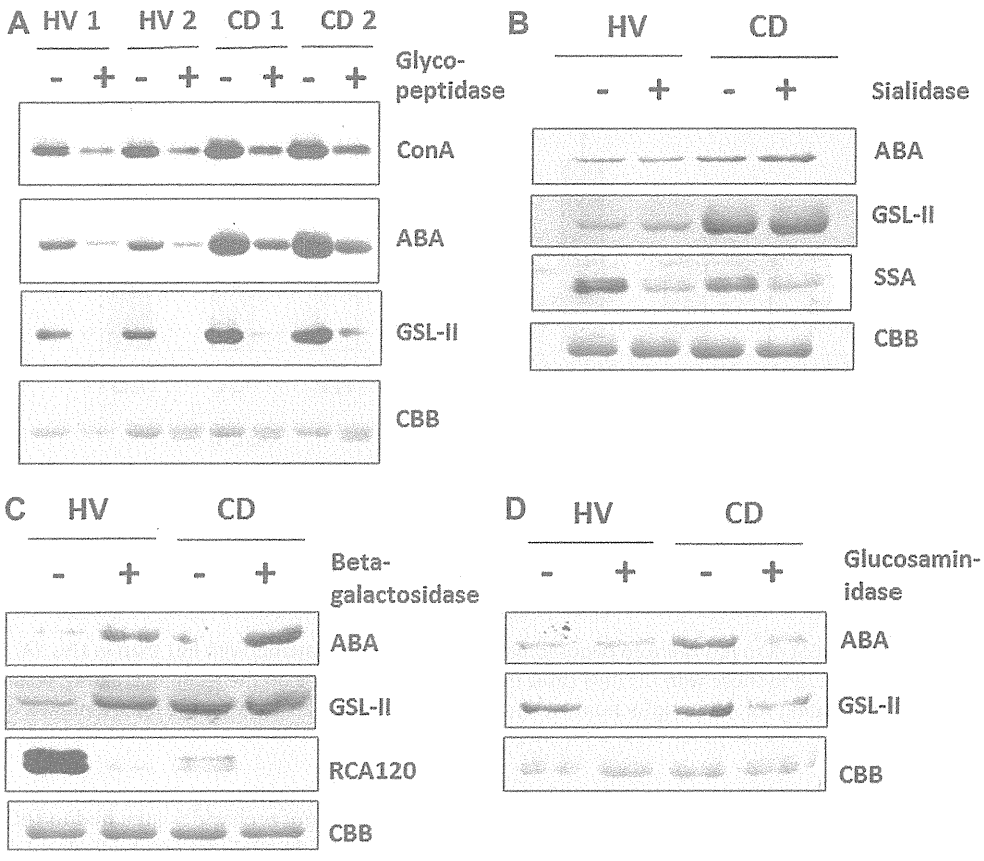

FIGURE 2. ABA and GSL-II recognize the *N*-linked oligosaccharide structure. (A) Purified serum IgGs from HV and CD (two cases each) were treated with glycopeptidase F to remove oligosaccharides from IgG. Binding of ABA, GSL-II, and ConA (binds *N*-linked oligosaccharides) to enzymatically treated or untreated IgGs was analyzed by lectin blotting. (B–D) Serum IgGs from HV and CD (two cases each) were treated with or without (B) sialidase, (C) beta-galactosidase, and (D) glucosaminidase in a step-by-step manner followed by lectin blotting. Each blot is representative of three independent experiments with similar results.

Agalactosyl IgG Is Increased in IBD Patients by Lectin-EIA

To quantify agalactosyl IgG by EIA, a standard curve using agalactosyl IgG was generated. First, oligosaccharide analysis of agalactosyl control IgG was performed by conventional HPLC methods.[7] The outer arm galactose was confirmed to be almost completely absent in control agalactosyl IgG (Fig. 3A). Using this control IgG as an EIA standard, a lectin-EIA system for agalactosyl IgG (Agal-IgG) was developed by immobilizing both ABA and GSL-II on the same plate, by which an accurate standard curve with high R^2 ratio (0.9991) was generated (Fig. 3B). Using the standard curve, Agal-IgG levels were investigated in sera from human subjects. The levels of Agal-IgG in patients with CD were significantly increased than those with UC, HV, and DC, and the levels in patients with UC were also significantly higher than those with HV (Fig. 3C). Agal-IgG levels in patients with SLE or RA were also significantly higher than those with HV. Agal-IgG levels in patients with CD were significantly higher than those with SLE and relatively higher than those with RA. In addition, certain cases of CD showed dramatic increases in Agal-IgG compared to other IBD and autoimmune diseases (Fig. 3C). These results indicate that Agal-IgG is increased in patients with IBD, especially with CD.

Agal-IgG Reflects Disease Activity and Clinical Course in CD

We next investigated whether values obtained by lectin-EIA (Agal-IgG) reflect disease classification, activity, and clinical course in IBD. Agal-IgG levels were significantly higher in CD patients whose onset age was below 16 (category A1) or between 17 and 40 (category A2) than in those above 40 (category A3) years of age at time of diagnosis (Fig. 4A). There were no significant age-related increases in Agal-IgG in either HV and CD patients (Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/A1), suggesting that the influence of age-related change in agalactosyl IgG is very small because the participants are relatively young, and that the increase in Agal-IgG in CD patients is not related to aging but to younger

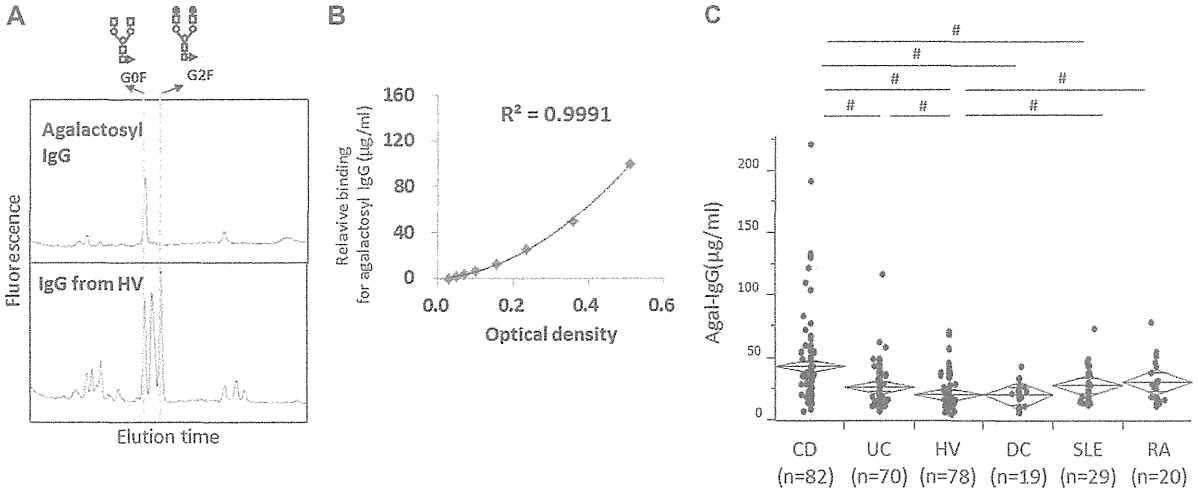

FIGURE 3. Agalactosyl IgG is increased in IBD patients by lectin-EIA. (A) Oligosaccharide structures from control agalactosyl IgG or serum IgG in HV were analyzed by HPLC. The outer arm galactose was almost completely absent in control agalactosyl IgG. This result is representative of three independent experiments. (B) To generate a standard curve, control agalactosyl IgG was diluted step-by-step and assayed for reactivity with ABA and GSL-II (Agal-IgG) by EIA. This result is representative of three independent experiments. (C) Agal-IgG levels of purified serum IgG from CD, UC, HV, DC, SLE, and RA by EIA. #$P < 0.05$.

onset of disease. There were no significant differences in the location (category L) or behavior (category B) of disease based on Agal-IgG levels (Fig. 4B,C). Agal-IgG levels were also not correlated with C-reactive protein (CRP) levels (data not shown). Moreover, Agal-IgG levels were significantly higher in patients with active CD (CDAI ≥150) than in patients in remission (CDAI <150, Fig. 4D). When CD patients treated with infliximab scheduled maintenance therapy were divided into responders and nonresponders at 30 weeks after the initial infusion, the difference between Agal-IgG levels at week 6 and week 0 (ΔAgal-IgG) was significantly higher in responders than in nonresponders (Fig. 4E), although the difference between CRP levels at week 6 and week 0 (ΔCRP) was unchanged in both groups (Fig. 4F). These results indicate that Agal-IgG could be a biomarker not only for detecting disease activity but also for predicting the clinical course of CD.

Combination of Agal-IgG and ASCA Increases Diagnostic Accuracy for CD

We then investigated the diagnostic accuracy of Agal-IgG for IBD by comparing with ASCA. We compared the sensitivity and specificity of Agal-IgG with those of ASCA for the discrimination of IBD by ROC curve and AUC analyses. The AUC was unchanged between Agal-IgG and ASCA for the differentiation of CD and HV (AUC of Agal-IgG vs. ASCA = 0.79 vs. 0.81; Fig. 5A), as well as CD and UC (AUC of Agal-IgG vs. ASCA = 0.69 vs. 0.72; Fig. 5B). The ROC curve, however, showed different patterns between Agal-IgG and ASCA; Agal-IgG had higher specificity than ASCA under high sensitivity conditions. There was no correlation between Agal-IgG and ASCA levels among all subjects (Fig. 5C). We then calculated the sensitivity and specificity of Agal-IgG/ASCA double-positive CD patients. By the ROC curve, the combination of Agal-IgG and ASCA positivity showed higher specificity than Agal-IgG or ASCA alone for the diagnosis of CD (Table 3).

Agal-IgG Levels Are Also Increased in American IBD Patients

To determine whether Agal-IgG levels are increased in a different geographic population, we investigated Agal-IgG levels in U.S. IBD patients and healthy volunteers. Agal-IgG levels were also significantly higher in IBD patients, especially in CD patients, than in healthy volunteers (Fig. 6A). In CD patients, Agal-IgG levels were significantly higher in patients with earlier onset category A1 or A2 than in those with category A3 (Fig. 6B). No significant differences were observed in category L or category B (Fig. 6C,D). These results were in excellent concordance with analyses for Japanese IBD patients (Figs. 3C, 4A–C), indicating Agal-IgG could be a biomarker for IBD patients irrespective of geography and consequently genetic background.

DISCUSSION

We previously reported that fucosylated agalactosyl IgG is increased in the sera of patients with IBD, and the extent of agalactosylation of fucosylated IgG is a potential diagnostic marker for IBD.[7] In the present study, we showed that both ABA and GSL-II preferentially recognize agalactosyl IgG, and affinities of both lectins to IgG were significantly increased in CD patients compared to HV. We also generated a lectin-EIA system in which an increase in agalactosyl IgG could be detected

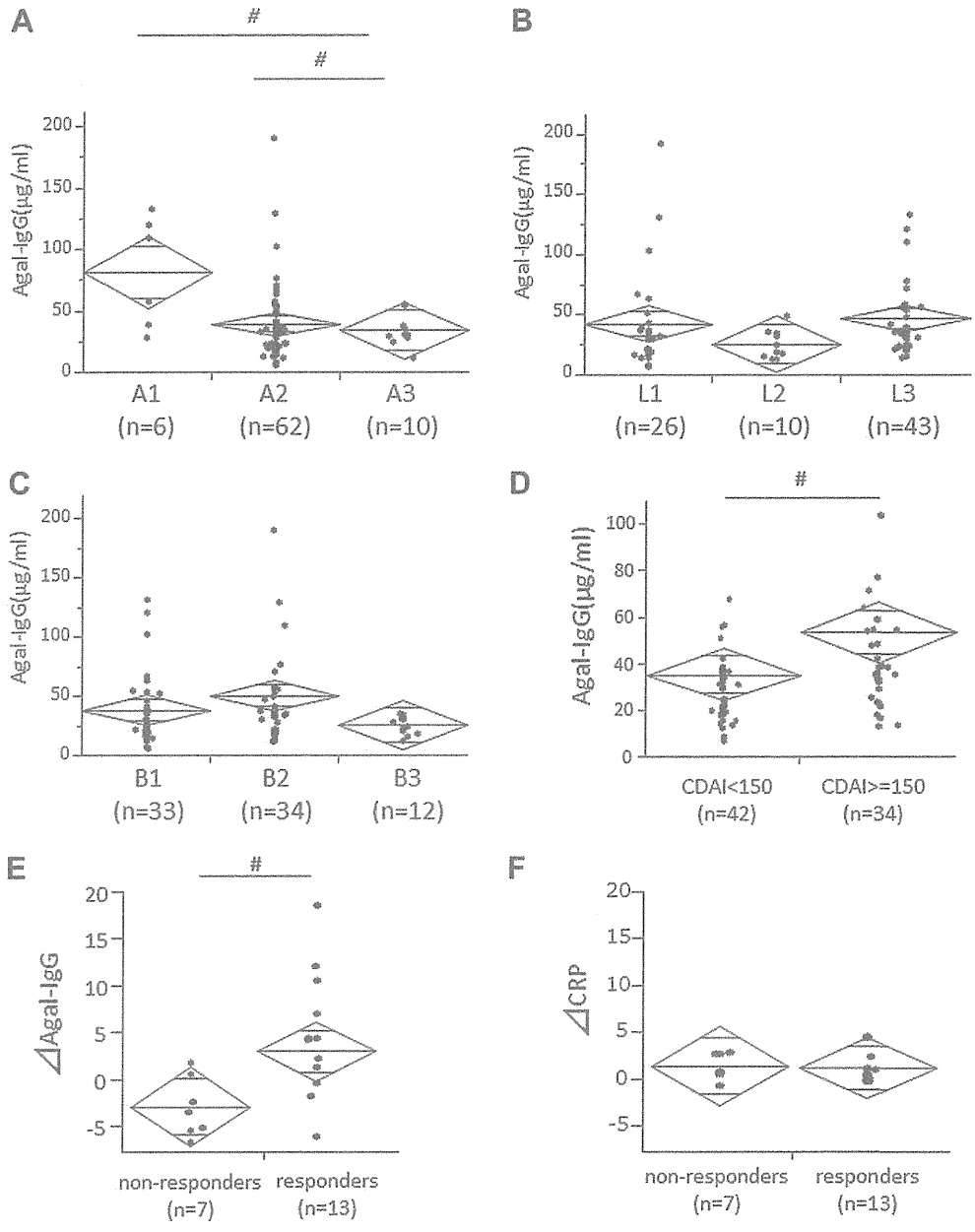

FIGURE 4. Agalactosyl IgG reflects disease activity and clinical course in CD. (A) Agal-IgG levels and age of disease onset (category A) in CD patients. Agal-IgG levels were significantly higher in patients with category A1 than A2 or A3. (B,C) Agal-IgG levels and disease location (category L, (B)) and disease behavior (category B, (C)) in CD patients. (D) Agal-IgG levels and disease activity in CD patients. Agal-IgG was higher in active patients (CDAI ≥150, $n = 42$) than in patients in remission (CDAI <150, $n = 34$). (E,F) Agal-IgG or CRP levels and clinical response to infliximab. Infliximab-naïve CD patients were treated with infliximab scheduled maintenance therapy. (E) The difference between Agal-IgG levels at week 6 and week 0 (ΔAgal-IgG) was higher in responders than in nonresponders. (F) The difference between CRP levels at week 6 and week 0 (ΔCRP) was unchanged in both groups. #$P < 0.05$

and determined that agalactosyl IgG is a useful diagnostic marker that reflects disease activity and clinical course of CD.

GSL-II binds to agalactosyl *N*-linked oligosaccharides with primary recognition of a GlcNAc residue and major specificity for tri- or tetra-antennary structures.[22] ABA has been widely used to detect and capture *O*-linked oligosaccharides containing Galβ1-3GalNAc.[25,26] However, ABA also has substantial affinity for mono-, bi-, and tri-antennary agalactosyl *N*-linked oligosaccharides.[23] Although these previous reports demonstrated affinity between lectin and agalactosyl *N*-linked oligosaccharides, the investigators used fluorescence-binding oligosaccharides as a substrate and did not confirm the affinity

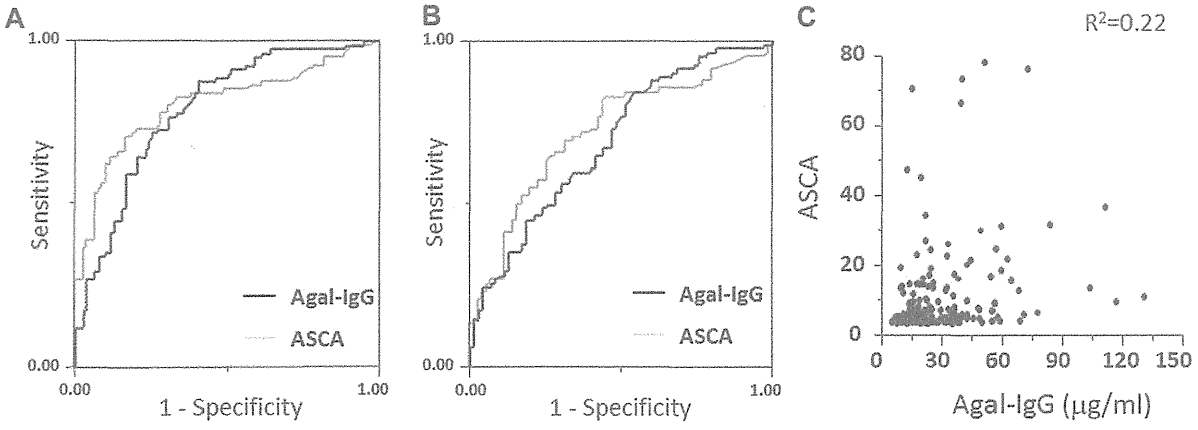

FIGURE 5. The combination of Agal-IgG and ASCA more efficiently differentiates CD. (A) The ROC curves for Agal-IgG and ASCA levels for discrimination between CD and HV, or (B) between CD and UC. (C) Correlation between Agal-IgG and ASCA levels in all subjects (n = 230).

for glycoproteins, i.e., IgG. In the present study we therefore demonstrate for the first time that both ABA and GSL-II recognize native biantennary GlcNAc on *N*-linked IgG oligosaccharides without destroying the crystal structure of the IgG glycoprotein. Lectin microarray is a powerful tool for screening such types of lectins.

EIA is widely used as a simple system to quantify specific proteins; however, several problems make it difficult to apply the "protein-specific" EIA to an "oligosaccharide-specific" system. First, the lectin-oligosaccharide interaction is approximately one-tenth the affinity of antigen–antibody binding,[10] so lectin-oligosaccharide complexes easily dissociate during the EIA procedure. Second, the recognition of an oligosaccharide by a lectin is not always specific for a single structure. Third, oligosaccharides are sometimes sterically encumbered by the surrounding protein so that lectins do not bind to glycoproteins compared to oligosaccharide structures without proteins. To overcome these problems, we adopted a simultaneous detection system by two lectins. We showed that both lectins recognize agalactosyl IgG oligosaccharides, but the binding affinity of each lectin to oligosaccharide might be subtly influenced by the surrounding protein structure.[27] The Agal-IgG EIA may have achieved a high sensitivity because each lectin contributes to bind to agalactosyl IgG. Another reason the dual lectin Agal-IgG EIA may be a sensitive and specific method is that nonspecific binding for each lectin may decrease due to the requirement for reduced concentrations: A higher concentration of a single lectin is necessary to generate a standard curve for agalactosyl IgG.

We previously reported on the significance of agalactosyl fraction of the fucosylated IgG oligosaccharides (G0F/G2F) in patients with IBD. It is interesting that fucosylated G0F/G2F is more specific than nonfucosylated agalatosyl IgG in IBD.[7] Agal-IgG should be consistent with G0F/G2F by HPLC, because Agal-IgG theoretically indicates whole agalactosyl IgG, and because G0F/G2F is a major fraction of IgG oligosaccharides. In contrast, fucosylation of IgG alters biological activity of IgG including antibody-dependent

TABLE 3. Accuracy of Agal-IgG and ASCA to Differentiate (A) CD from HV, or (B) CD from UC

	Sensitivity	Specificity	PPV	NPV
(A)				
ASCA (+)	71% (58/82)	83% (65/78)	82% (58/71)	73% (65/89)
Agal-IgG (+)	88% (72/82)	59% (46/78)	69% (72/104)	82% (46/56)
ASCA (+) /Agal-IgG (+)	67% (55/82)	94% (72/78)	90% (55/61)	73% (72/99)
(B)				
ASCA (+)	70% (57/82)	69% (48/70)	72% (57/79)	66% (48/73)
Agal-IgG (+)	83% (68/82)	47% (33/70)	65% (68/105)	58% (33/57)
ASCA (+) / Agal-IgG (+)	62% (51/82)	84% (59/70)	82% (51/62)	66% (59/90)

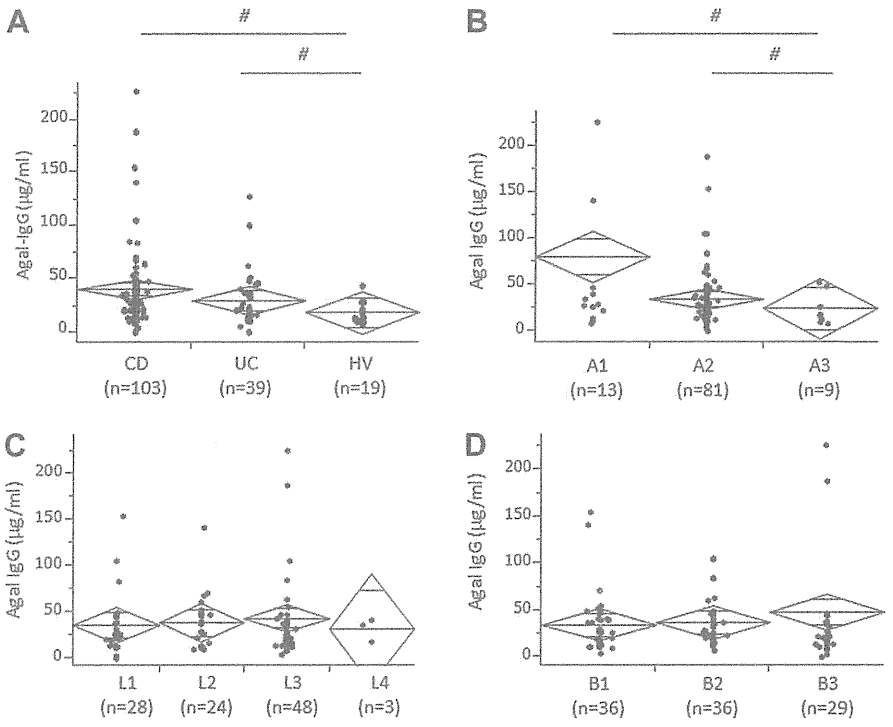

FIGURE 6. Agal-IgG levels are also increased in American IBD patients. (A) Agal-IgG levels of each purified serum IgG from CD, UC, and HV by EIA. (B) Agal-IgG levels and onset age (category A) in CD patients. Agal-IgG levels were significantly higher in patients with category A1 than with A2 or A3. (C,D) Agal-IgG levels and disease location (category L, (B)) and behaviors (category B, (C)) in CD patients. #$P<0.05$.

cellular cytotoxicity, which might be due to conformation changes in the IgG Fc portion.[28] In our system ABA and GSL-II can recognize both the oligosaccharide structure and the 3D structure of the IgG Fc portion, whereas the HPLC system purely analyzes the oligosaccharide structure of IgG. The sensitivity and specificity of Agal-IgG for diagnosis of CD might, therefore, be slightly different from our previous study. However, Agal-IgG could be a marker for disease activity of CD and the combination of Agal-IgG and ASCA is a better marker for diagnosing IBD than ASCA alone, as discussed below. Furthermore, certain cases of CD showed dramatic increases in Agal-IgG measured by lectin-EIA, compared to other IBD and autoimmune diseases. Although we started the present study to establish more convenient methods to measure agalactosyl IgG levels than the HPLC system, Agal-IgG by lectin-EIA can possibly help elucidate the novel pathogenesis of CD. Further studies from both clinical and basic approaches will be required for CD patients showing extremely high Agal-IgG levels.

Here we demonstrated that Agal-IgG is a useful diagnostic marker in combination with ASCA. ASCA is one of the most well-established serologic markers for diagnosing CD,[4] but ASCA alone does not possess enough power to diagnose CD with sufficient sensitivity or specificity. Therefore, ASCA has been combined with other markers, such as peripheral antineutrophil cytoplasmic antibody[5] or anti-outer membrane porin protein C, and anti-CBir1 flagellin.[6] In the present study, Agal-IgG itself showed almost the same sensitivity and specificity as ASCA for the discrimination of CD and HV or CD and UC; however, Agal-IgG was not strongly correlated with ASCA. The combination of Agal-IgG and ASCA could augment the specificity as a diagnostic marker partly because Agal-IgG might identify different subgroups of patients within CD than ASCA. Further investigation is necessary to assess whether the combination of Agal-IgG and ASCA is a better marker for diagnosing IBD than Agal-IgG or ASCA alone. Moreover, we showed that Agal-IgG may have higher predictability for response to infliximab compared with CRP (Fig. 4E,F). Agal-IgG can reflect different inflammatory conditions from CRP, because Agal-IgG is not increased in patients with acute intestinal inflammation (Fig. 3C), and because IgG has a long serum half-life of 3 weeks, whereas that of CRP is 4–6 hours. The decrease of Agal-IgG in 6 weeks of infliximab treatment may be, therefore, a novel marker for sustained response. If confirmed in larger prospective analyses, this finding could have important clinical implications.

In a validation study, we showed that Agal-IgG levels were also significantly increased in a non-Asian, U.S. cohort of IBD patients, especially in early onset (category A1/A2) CD patients. A previous report showed that disease location and

clinical course are severer in patients whose onset is younger, and that the A3 group had a lower incidence of fistulas and fewer requirements for immunomodulators and corticosteroids.[29] Higher levels of Agal-IgG in category A1/A2 patients may therefore reflect disease severity in younger-onset patients. Although the mechanism has not been clarified, Agal-IgG can be useful especially for screening pediatric patients for whom invasive studies are hard to perform. Although clinical manifestations appear to be similar in all geographies, ethnic differences in genetic associations have been reported. For instance, mutations in the leucine-rich repeats (LRRs) of nucleotide-binding oligomerization domain containing 2 (NOD2) are associated with an increased risk for CD in many Caucasian populations,[30–32] but not in Japanese patients.[33] Therefore, it is of interest that increases in Agal-IgG levels are observed in Japanese and American IBD patients because of the clinical implications as a diagnostic marker, but also as a reason to explore IgG glycosylation as a global defect in the pathogenesis of IBD.

An increase in serum agalactosyl IgG is also reported in other diseases such as RA,[34] SLE,[35] and tuberculosis.[36] In the present study we showed that Agal-IgG levels were significantly increased in patients with RA and SLE. Agal-IgG might be an effective serological marker for other immunological disorders such as SLE and RA. In addition, Agal-IgG levels in patients with CD are significantly higher than those with SLE and relatively higher than those with RA. Agalactosyl IgG seems to have different functions in each disease, because our recent studies show that the levels of anti-agalactosyl IgG antibodies are increased in the sera of RA[37] but not IBD patients.[7] Moreover, the lectin-complement pathway is activated through agalactosyl IgG in RA[38] but not IBD.[8] Therefore, the availability of an Agal-IgG lectin-EIA will help in translational studies to elucidate mechanisms through which agalactosyl IgG contributes to pathogenesis in different diseases.

In conclusion, the Agal-IgG lectin-EIA system for agalactosyl IgG may represent a novel biomarker assay for IBD. The presence of Agal-IgG in numerous autoinflammatory diseases has potential significance as a diagnostic marker that may fill important clinical needs, and may provide further information about pathogenesis.

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Reevaluation of a lectin antibody ELISA kit for measuring fucosylated haptoglobin in various conditions

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ABSTRACT

Background: Fucosylated haptoglobin (Fuc-Hpt) is a novel cancer biomarker in a variety of pathological conditions. We previously found that the level of Fuc-Hpt is increased in the sera of patients with pancreatic cancer, and established a lectin antibody ELISA using *Aleuria aurantia* lectin, which specifically binds to fucosylated residues on oligosaccharides.
Methods: To apply this assay system to the clinical detection of several diseases, several assay conditions such as serum dilutions and inhibitory factors were investigated. The Fuc-Hpt kit was available for 25–625 fold serum dilution.
Results: While the values of Fuc-Hpt assay using sera and plasma were different, they showed positive correlation. The addition of bilirubin and formagine did not influence on Fuc-Hpt assay, but hemoglobin inhibited this assay in a dose-dependent manner.
Conclusions: We reevaluated this lectin antibody ELISA kit for measuring fucosylated haptoglobin in various conditions in this study.

1. Introduction

Fucosylation is one of the most important oligosaccharide modifications involved in cancer and inflammation [1]. Certain kinds of fucosylated glycoproteins have been identified as cancer biomarkers. The best representative example is fucosylated alpha-fetoprotein (AFP), referred to AFP-L3 [2]. While the level of AFP is slightly increased in chronic liver diseases such as chronic hepatitis and liver cirrhosis, that of AFP-L3 is specifically increased in the sera of patients with hepatocellular carcinoma [3]. Based on the results from lectin blot analysis, we previously reported that fucosylated haptoglobin (Fuc-Hpt) is a novel biomarker for pancreatic cancer [4]. Although an increase in the level of Fuc-Hpt has been observed in other cancers as well as in benign inflammatory diseases, the positive rate of Fuc-Hpt is seen to be the highest level in pancreatic cancer. To measure the serum levels of Fuc-Hpt in serum, we have developed a lectin antibody enzyme-linked immunosorbent assay (ELISA). Using this ELISA kit, we have assayed Fuc-Hpt levels in various types of cancer, and compared the results of lectin blot analyses and lectin antibody ELISA [5].

It has long been known that Fuc-Hpt is increased in sera from patients with advanced ovarian cancers and other diseases for a long time [6–8]. However, based on the results from lectin analyses, early literature reported an increase in the alpha 1–2 fucosylation of Hpt in cancer patients. In recent times, publications from our group have shown, based on the results from mass spectrometry analyses, that Hpt fucosylation in patients with several cancers was predominantly of the alpha 1–3 type, and included a very small amount of α1–6 fucosylation (core fucosylation) [9]. Furthermore, site-specific analysis of oligosaccharides side chains of Hpt using mass spectrometry showed characteristic structures at site 3 in Fuc-Hpt derived from the sera of patients with pancreatic cancer [10]. This site-specific fucosylation varies among several cancers. Hakomori et al. reported increased alpha 1–3 fucosylation at site 4 of Hpt derived from the sera of patients with colorectal cancer

Abbreviations: ELISA, enzyme-linked immunosorbent assay; Hpt, haptoglobin; Fuc-Hpt, fucosylated haptoglobin; AFP, alpha-fetoprotein.

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[11]. The underlying mechanism by which cancer-specific fucosylation is observed in pancreatic cancer and colon cancer is unknown.

Most cancer biomarkers cannot be used for early diagnosis; however, cancer biomarkers are useful in monitoring therapy, recurrence, and prognosis after surgery. We investigated the utility of Fuc-Hpt as a prognosis marker for pancreatic cancer. While Fuc-Hpt levels were significantly higher at clinical stage IV than the levels at other stages [5], most patients with pancreatic cancer succumbed within 3 years and we could not continue the prognosis study for pancreatic cancer. Therefore, we performed a similar study for colorectal cancer using the Fuc-Hpt lectin antibody ELISA kit. Interestingly, a combination assay for both Fuc-Hpt and carcinoembryonic antigen (CEA) was very useful in predicting the prognoses of patients with colorectal cancer after surgery [12]. Our next targets for the Fuc-Hpt study were individuals with liver diseases, as well as healthy controls with some diseases as assessed by the Ningen Dock, a medical screening program of Japan [13]. To perform a large-scale study, the stability and reproducibility of results obtained using the Fuc-Hpt kit on the same day as well as on different days should be investigated. We expect Fuc-Hpt levels in individuals with liver disease to be quite high, because most Hpt is produced from the liver, whereas Fuc-Hpt levels are expected to be quite low in healthy controls. Therefore, a range of serum dilutions must also be investigated for the Fuc-Hpt assay.

2. Materials and methods

2.1. Serum samples and cell lines

Fifteen healthy volunteers (Immuno-Biological Laboratories, Gunma, Japan, and Osaka University Graduate School of Medicine, Osaka, Japan) were enrolled in this study. Plasma was collected from each volunteer using heparin, EDTA, or sodium citrate. Both serum and plasma samples were stored at −80 °C until used. A human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC, Manassas, VA), a human pancreatic cancer cell line PK8 was provided by the Institute of Development, Aging and Cancer, Tohoku University. HepG2 and PK8 cells were cultured in Dulbecco's modified Eagle medium (Nakalai Tesk, Kyoto, Japan) containing 10% fetal bovine serum and antibiotics [penicillin (10 units/ml), streptomycin (100 μg/ml)]. The conditioned medium of HepG2 was collected at conditions of 50, 70, and 100% confluence, and designated L, M, and H, respectively. The conditioned medium of PK8 which was transfected with a haptoglobin expression vector was collected at a condition of approximately 100% confluence, and this conditioned medium was adopted as the standard sample (70 U/ml) of lectin-antibody ELISA for Fuc-Hpt as described previously [14]. Three hundred serum samples from patients with pancreatic cancer were obtained with informed consent from the serum bank of the Japanese Project for Personalized Medicine (BioBank Japan). These samples were collected according to the guidelines of BioBank Japan. To assess Fuc-Hpt levels in normal controls, we used 300 serum samples from healthy people who received a medical check-up in the Ningen Dock program. The sera from the medical check-up were used in this study after obtaining proof of informed consent. All experiments were approved by the ethical committee of Osaka University.

2.2. Lectin antibody ELISA for Fuc-Hpt

Lectin antibody ELISA was performed according to our previously described protocol [5,14]. To calculate the CV, the standard sample (conditioned medium from PK8 cells transfected with a haptoglobin expression vector) was assayed at seven dilutions in eight times, and conditioned medium from HepG2 cells at conditions of 50, 70, and 100% confluence (as designed L, M, and H) was also assayed in eight times. To evaluate the differences between serum and plasma as the materials for the Fuc-Hpt lectin-antibody ELISA assay, we used sera and 3 types of plasma (prepared with EDTA, hepatin, or citrate) preparations from 15 healthy volunteers. To investigate the inhibitory effect of lipids, hemoglobin, and bilirubin as intermediate molecules on the Fuc-Hpt assay, we used Interference Check A Plus Kit (cat No. 79370, Sysmex Co., Ltd., Kobe, Japan), which includes formagine, hemoglobin, free bilirubin (bilirubin F), and conjugated bilirubin (bilirubin C), according to its instruction.

2.3. Statistical analysis

Statistical analysis was conducted using the JMP 9.0 software (SAS Institute Inc., Cary, NC). Variables were expressed as mean ± standard deviation. Statistical analysis included descriptive statistics, analysis of variance, the Wilcoxon and Kruskal–Wallis tests, and Spearman R correlations. The diagnostic performances of the scoring systems were assessed by analyzing receiver operating characteristic (ROC) curves. The probabilities of true positive (sensitivity) and true negative (specificity) assessments were determined for selected cutoff values, and the area under the ROC curve (AUROC) was calculated for each index. The Youden index was used to identify the optimal cutoff points. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Reproducibility of Fuc-Hpt lectin antibody assay

To assess the reproducibility of the Fuc-Hpt assay, we assayed standard samples (conditioned medium from PK8 cells transfected with a haptoglobin expression vector) at seven kinds of dilution rates in 8 time measurements. As shown in Table 1, the CV value was 5.8% in the samples with the highest concentrations, and 14.5% in samples with the lowest concentrations. Measurements of Fuc-Hpt levels in the conditioned medium of HepG2 cells at 3 levels of confluence revealed similar CV values (13.2–14.2%) across eight repeats of the experiment (Table 2). These values were almost the same irrespective of the individuals performing the assay (data not shown).

3.2. Samples that can be measured using the lectin antibody ELISA

Sera and 3 types of plasma preparations from 15 healthy volunteers were used in the Fuc-Hpt lectin antibody ELISA, in order to investigate which materials are suitable for this assay. Plasma was prepared with EDTA, heparin, or citrate. As shown in Fig. 1A, the serum levels of Fuc-Hpt were much higher than those in the 3 plasma preparations. When Fuc-Hpt levels in sera were compared to those in the 3 plasma preparations, the highest correlation was observed between serum and heparin plasma Fuc-Hpt levels (Fig. 1B). Fuc-Hpt levels assessed by lectin antibody ELISA in citrate plasma showed a slightly weaker correlation with serum Fuc-Hpt levels, compared to the other 2 plasma preparations.

3.3. Suitable serum dilution for Fuc-Hpt lectin antibody ELISA

First, we assayed 10 serum samples from healthy volunteers and 5 serum samples from patients with pancreatic cancer at dilutions of 2- to

Table 1
Reproductively of Fuc-Hpt assay at different time, using standard samples (PK8 cells transfected with a haptoglobin expression vector).

Standard concentration (U/ml)	Optical density (OD)	CV (%)
70	2.14 ± 0.123	5.8
35	1.3 ± 0.1	7.4
17.5	0.79 ± 0.068	8.6
8.75	0.47 ± 0.044	9.1
4.38	0.33 ± 0.04	12.3
2.19	0.26 ± 0.035	13.4
1.09	0.22 ± 0.032	14.5
Blank	0.18 ± 0.034	18.5

OD values are presented as the mean ± SD.

Table 2
Reproductively of Fuc-Hpt assay at different time, using standard samples (HepG2 cells at 3 levels of confluence).

HepG2	Fuc-Hpt (U/ml)	CV (%)
H	38.62 ± 5.08	13.2
M	19.62 ± 2.78	14.2
L	8.75 ± 1.16	13.3

300-fold. While Fuc-Hpt levels in healthy volunteers decreased to background levels beyond 100-fold dilution, Fuc-Hpt levels in pancreatic cancer patients did not decrease even after 300-fold dilution, suggesting that much higher Fuc-Hpt levels exist in the sera of patients with pancreatic cancer (data not shown). Secondly, we assayed Fuc-Hpt in 300 serum samples from pancreatic cancer patients at dilutions of 5- to 78,125-fold (Fig. 2). The dot distribution pattern was a little different in each dilution. When we compared data from the 125-fold dilution with those from other dilutions, high correlations were observed among assay results obtained using 25-, 125-, and 625-fold dilutions. These results suggest that a 125-fold serum dilution yields the best results in the Fuc-Hpt lectin antibody ELISA.

3.4. Evaluation of the Fuc-Hpt assay in the diagnosis of pancreatic cancer

Fuc-Hpt levels were assayed in 300 serum samples of pancreatic cancer and 315 serum samples of healthy volunteers at 125-fold dilution. As shown in Fig. 3A, Fuc-Hpt levels were significantly higher in pancreatic cancer samples than in normal controls (2225.0 ± 2092.7 vs. 359.2 ± 480; $p<0.01$). The ROC for the diagnosis of pancreatic cancer is presented in Fig. 3B; the AUROC was 0.91. The sensitivity and specificity of Fuc-Hpt for the diagnosis of pancreatic cancer were 85.1% and 82.3%, respectively. The positive predictive value and negative predictive value were 79.5% and 87.3%, respectively. Because little clinical information is available regarding these patients, comparative analyses of Fuc-Hpt levels and clinical parameters could not be performed.

3.5. Effect of inhibitory factors on Fuc-Hpt lectin antibody ELISA

To investigate the effects of lipids, hemolysis, and icterus, we added formagine, hemoglobin, and bilirubin [free bilirubin (bilirubin F) and conjugated bilirubin (bilirubin C)] to the samples (pooled sera of pancreatic cancer patients (PC) and healthy volunteers (HV)) for the Fuc-Hpt lectin antibody ELISA. As shown in Fig. 4, there were no changes in the Fuc-Hpt levels measured in the presence or absence of formagine and bilirubin (F and C). The addition of hemoglobin decreased the measured Fuc-Hpt levels in a dose-dependent manner, and the decreased level of Fuc-Hpt was constant at hemoglobin concentrations > 200 mg/dl.

4. Discussion

In this study, we reevaluated a lectin antibody ELSA kit against Fuc-Hpt for observing its applicability to clinical diagnoses of several diseases. At first, we compared the effects of the conditions of blood drawing. We found that the assayed serum Fuc-Hpt levels were significantly higher than those in plasma (Fig. 1). Next, we investigated a range of serum dilutions. We had previously reported the use of this

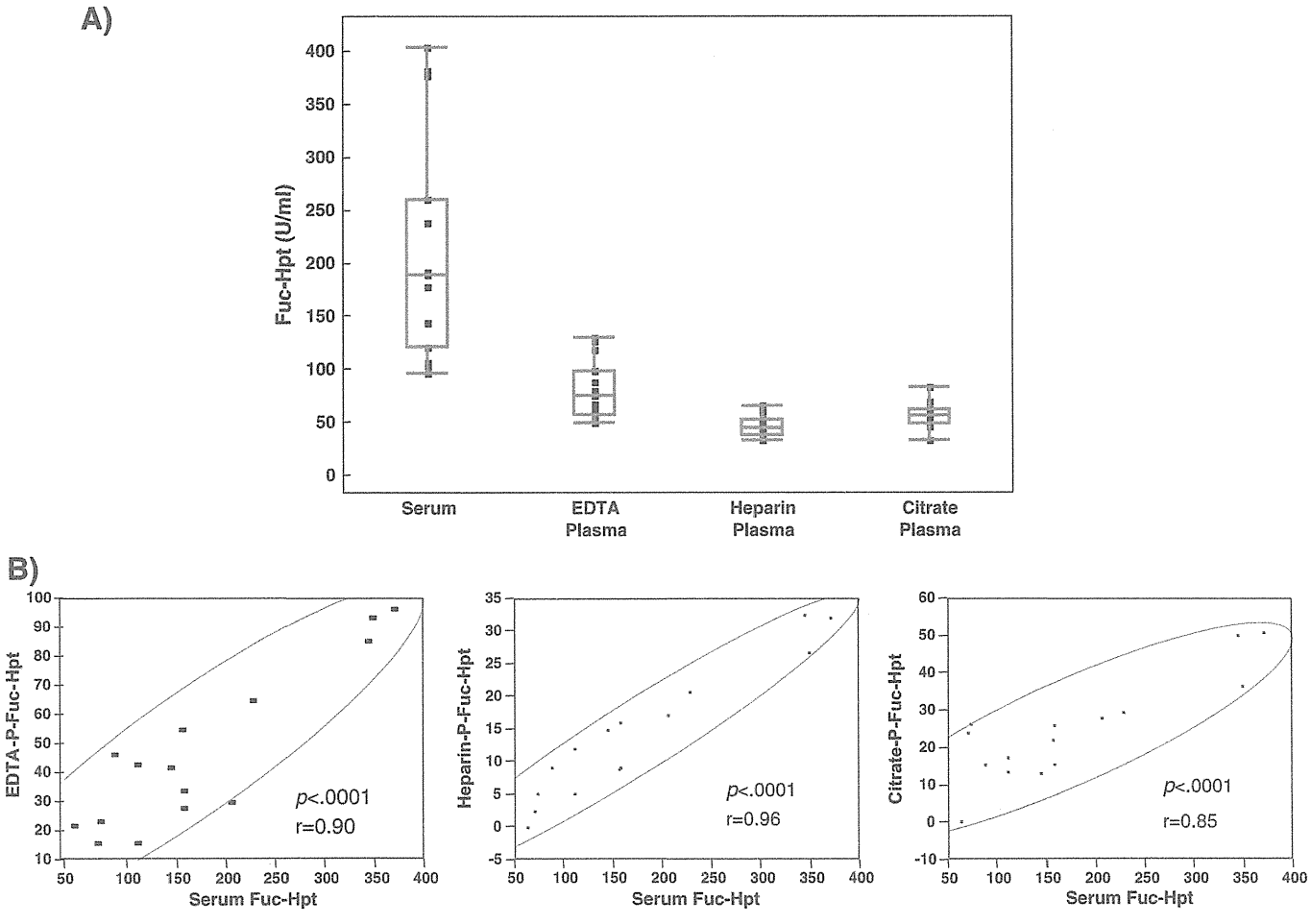

Fig. 1. Comparison of serum and plasma as the material for the Fuc-Hpt lectin-antibody ELISA assay. (A) Fuc-Hpt levels in serum, EDTA plasma, heparin plasma, and citrate plasma. (B) Correlation of Fuc-Hpt levels in serum and the 3 plasma preparations. Fifteen serum/plasma samples from the same volunteers were investigated in this experiment.

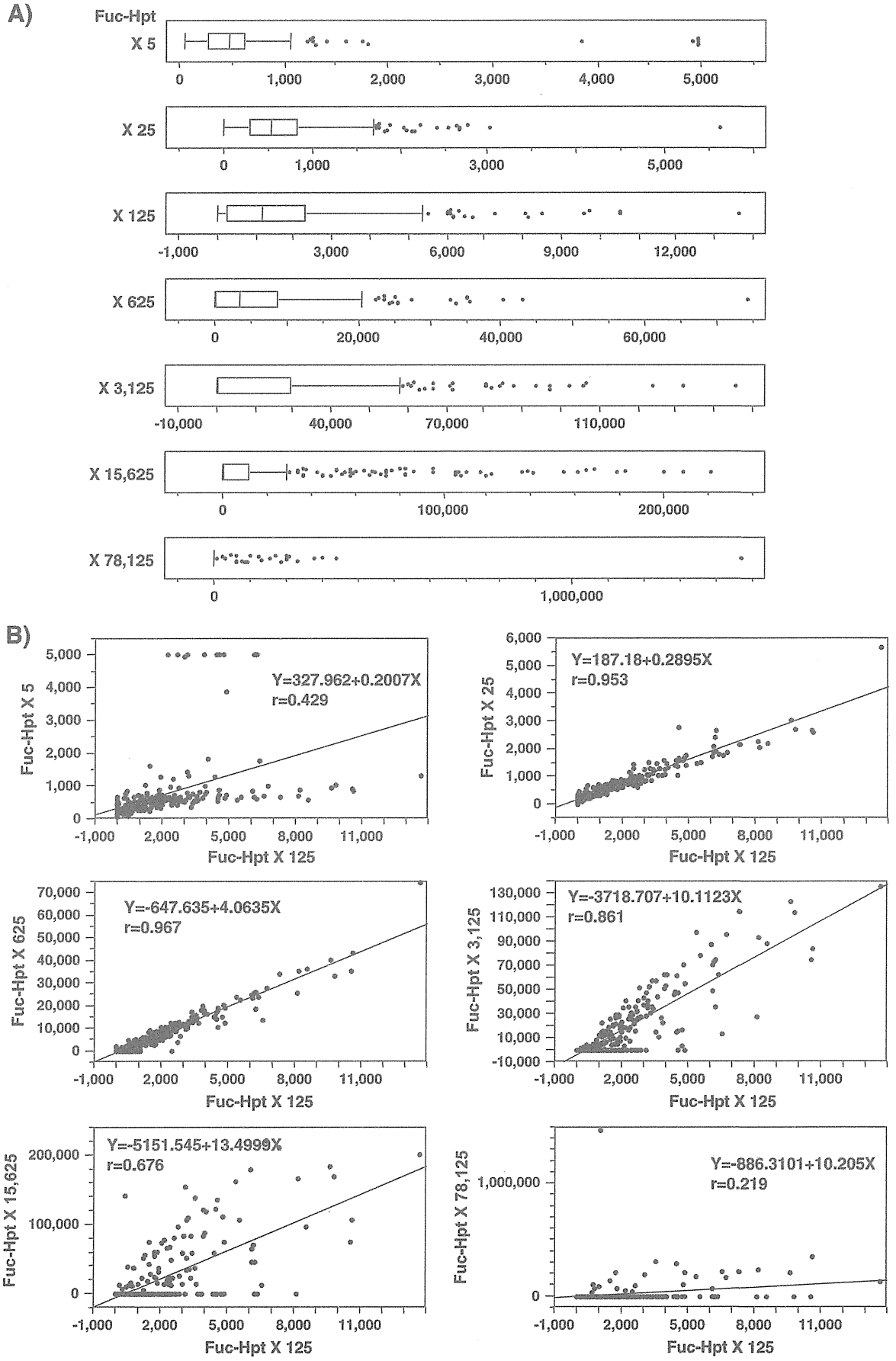

Fig. 2. Comparison of serum dilutions and Fuc-Hpt levels in lectin antibody ELISA. (A) Serum Fuc-Hpt levels in 300 patients with pancreatic cancer and 15 healthy volunteers at various dilutions are plotted. Numbers indicate the relative units of Fuc-Hpt per ml. (B) Comparison of Fuc-Hpt levels at each dilution in the ELISA assay. The x-axis represents data from the 125-fold dilution in all cases and the individual y-axes represent data from the 5- to 78,125-fold dilutions.

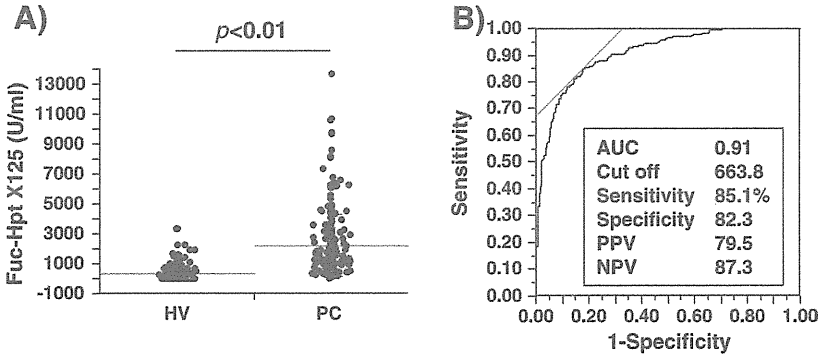

Fig. 3. Reevaluation of Fuc-Hpt as a cancer biomarker for pancreatic cancer. (A) Serum Fuc-Hpt levels in patients with pancreatic cancer (PC) and in healthy volunteers (HV) assayed with lectin antibody ELISA using 125-fold serum dilutions. (B) ROC curve of Fuc-Hpt levels for the diagnosis of pancreatic cancer, calculated using the results in panel A.

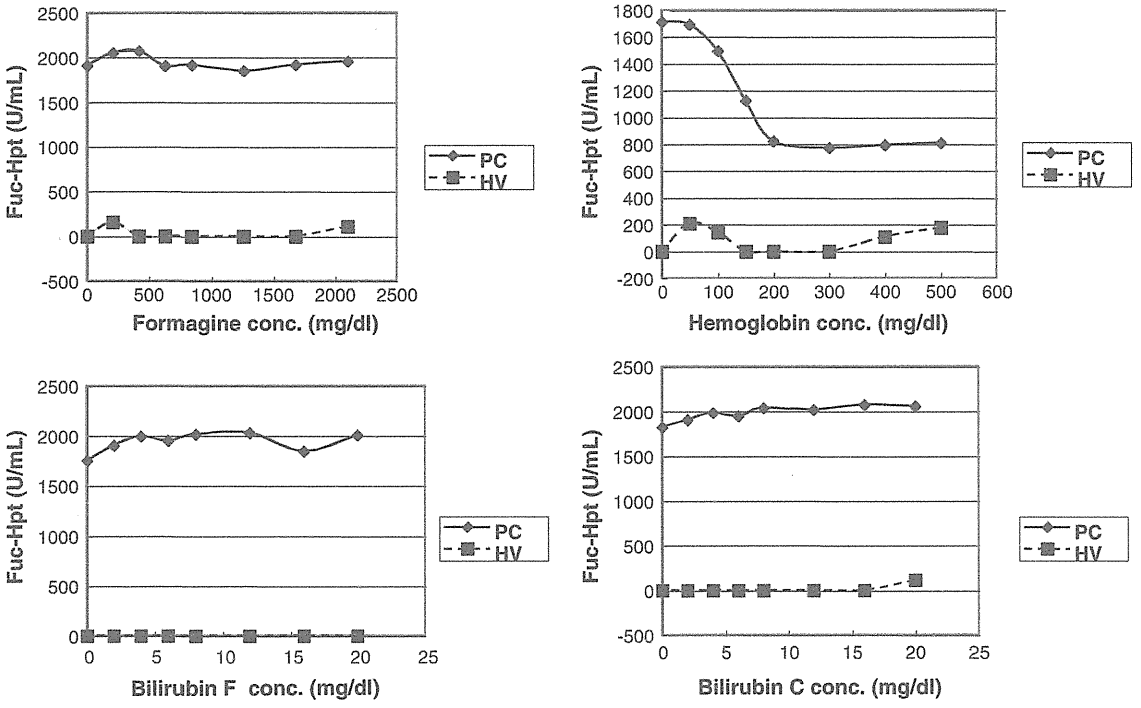

Fig. 4. Effects of formagine, hemoglobin, and bilirubin contamination on the Fuc-Hpt assay. Pooled sera of pancreatic cancer patients (PC) and healthy volunteers (HV) were measured using Fuc-Hpt lectin antibody ELISA kit with various concentrations of formagine (A), hemoglobin (B), free bilirubin (bilirubin F) (C), or conjugated bilirubin (bilirubin C) (D). The diamonds indicate data from patients with PC and the squares indicate those from HV.

lectin ELISA kit with 25-fold diluted sera, because certain kinds of diseases showed very low levels of Fuc-Hpt [4,5]. The result of the present study demonstrated that 25- to 625-fold dilutions of sera could be used with this ELISA kit. It is assumed that Fuc-Hpt levels should be measured at a great number of serum dilutions, because Fuc-Hpt levels would be extremely high in liver disease. The usefulness of Fuc-Hpt in the diagnosis of pancreatic cancer was reevaluated with 300 serum samples from patients with pancreatic cancer, which were collected by the Tokyo Medical Research Institute from many hospitals in Japan. The AUROC was 0.91, and the sensitivity and specificity were 85.1% and 82.3%, respectively (Fig. 3B). These data are superior to our previous reports, which included less than 100 serum samples [4,5].

When this ELISA kit is used in clinical laboratory testing, hemolysis should be avoided because it was shown that contamination by hemoglobin decreased the value of measured Fuc-Hpt level (Fig. 4). On the other hand, the levels of both bilirubin and formagine did not affect the value of Fuc-Hpt measured by this ELISA kit. These results suggest that the presence of icterus or high lipid levels should not affect the values of Fuc-Hpt in this ELISA kit. Our results in this study suggest that we should not simultaneously evaluate and compare serum and plasma Fuc-Hpt levels, because Fuc-Hpt levels in plasma are much lower than those in serum. When the serum Fuc-Hpt level is extremely high, the data should be confirmed using lectin blot analysis with *Aleuria aurantia* lectin. This is because there might be a specific antibody against haptoglobin in the serum [5]. In contrast, since Fuc-Hpt is quite low in the standard assay, a repeat measurement is recommended with 25-fold serum dilution.

In the present study, the significance of serum Fuc-Hpt levels measured by this ELISA kit for pancreatic cancer diagnosis was superior to the level in our previous reports [4,5]. One of the reasons for the improved data in this study is the modified serum dilution from 25-fold to 125-fold. The 5-fold greater number of dilution used in this study

would improve the accuracy of measurements in patients with high Fuc-Hpt concentrations. As shown in Fig. 2, the 125-fold serum dilution assay was superior to measure Fuc-Hpt at high concentration than the 5-fold serum dilution assay. In many pancreatic cancer patients, serum Fuc-Hpt levels revealed extremely high concentrations compared with those in healthy volunteers (Fig. 3A). However, further dilution enlarged the number of samples under detection range. For example, the number of samples under detection range in the 125-fold serum dilution was 65, while the number in the 625-fold serum dilution was 91 in our study. Although there were high correlations among assay results obtained using 25-, 125-, and 625-dold dilutions (Fig. 2B), a 125-fold serum dilution appears to be suitable for this Fuc-Hpt ELISA assay based on these data. Therefore, we thought that 125-fold dilution rate was better for the serum Fuc-Hpt assay. The other reason is that the number of subjects used in this study was much larger than that in the previous study (normal controls: 315 in the present study vs. 22 in the previous study; pancreatic cancer patients: 300 vs. 72) [5]. The results of this study further emphasize the significance of serum Fuc-Hpt levels as a useful tumor marker in the diagnosis of pancreatic cancer.

Taken together, the results of the present study provide a spectrum of information for the Fuc-Hpt lectin antibody ELISA. In addition, the reevaluation of this Fuc-Hpt assay demonstrated the superior significance of this assay in the diagnosis of pancreatic cancer than was previously observed. This Fuc-Hpt ELISA kit should prove useful in various clinical applications.

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Whole-body imaging of tumor cells by azaelectrocyclization: Visualization of metastasis dependence on glycan structure

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ABSTRACT

Noninvasive imaging of cancer metastasis through the efficient cell labeling constitutes a major technological breakthrough for cancer research and patient monitoring post-surgery. In the current work, we expanded our cell surface labeling technique on the whole-body fluorescence imaging of tumor metastasis in BALB/c nude mice. Four kinds of human cancer cells (two cancer cell lines, MKN45 and HCT116, and their transfected versions expressing surface glycan-related genes, MKN45-GnT-V and HCT116-GMDS) were labeled by azaelectrocyclization with Hilyte Fluor 750 for 10 min and without affecting cell viability. Fluorescence-labeled cancer cells were injected into the abdominal cavities of BALB/c mice and whole-body scans were performed with an eXplore Optix device. In accordance with previous findings, the fluorescence imaging clearly showed that tumor metastasis was dependent upon the cell surface glycans: A larger polylactosamine structure or the loss of fucosylation on the cancer cell surfaces, respectively, enhanced the metastatic potential of the tumor cells. Our noninvasive technique provides the landmark opportunity for sensitively monitoring the dynamics of the cancer cells depending on their surface structures and/or the host environments, thus impacts on the cancer prognosis and the therapeutic applications.

1. Introduction

Tumor growth and metastasis are the processes which involve the dynamic interactions of the cancer cells in the living systems with the extracellular matrix, vasculature, and various types of non-cancerous host cells forming the tumor stroma. Noninvasive imaging of cancer cell trafficking in living animals, that is, the dynamic interaction with the stroma and invasion of healthy tissues, while avoiding immune responses, therefore constitutes a major technological breakthrough for cancer research and patient monitoring post-surgery.[1] Various immune cells, for instances, macrophages, dendritic cells, NK, or CTL as the immune effector cells, have been studied to monitor tumor proliferation, metastasis, death, and cellular interactions within the immune system both at the macroscopic and microscopic levels.[2] The effects of co-injection with cytokines, co-stimulatory molecules, cancer antigens, and, more recently, engineered chimeric antigen receptors can be visualized to help circumvent the low efficiency of lymphocyte-based cancer immunotherapy, and to develop tailor-made cells for individual patients.[3] Noninvasive methods to monitor tumors using stem cells have also been emerged recently.[4] The importance of visualizing cell trafficking in vivo has motivated the development of numerous techniques for labeling live cells. Most methods applied to imaging whole-body cell trafficking require the incorporation of positron emitting metals (for PET)[5] or paramagnetic metals (for magnetic resonance imaging)[6] inside the cells. Chemistry-based bio-orthogonal protocols such as the Staudinger ligation,[7] strain-promoted click reaction,[8] or Suzuki coupling[9] to 'tags' on the cell surfaces have recently emerged as new alternatives to label the surface of cell membranes.[10]

We recently developed a rapid and efficient method named 6π-azaelectrocyclization[11,12] to introduce fluorescent groups or other small molecules to the amino groups on the cell surface[13] via a reaction with unsaturated aldehyde probes (such as probe **1** in Scheme 1) at very low concentrations (as low as 10^{-8} M) and for short times (2–10 min) at room temperature. While the conventional cell surface labeling using the succinimidyl esters requires the 10^{-2} M concentration of the reagents, our azaelectrocyclization chemistry can also covalently and selectively label most of the accessible amino groups on cell surface constituents under extremely mild and the physiological conditions, that is, lysines of

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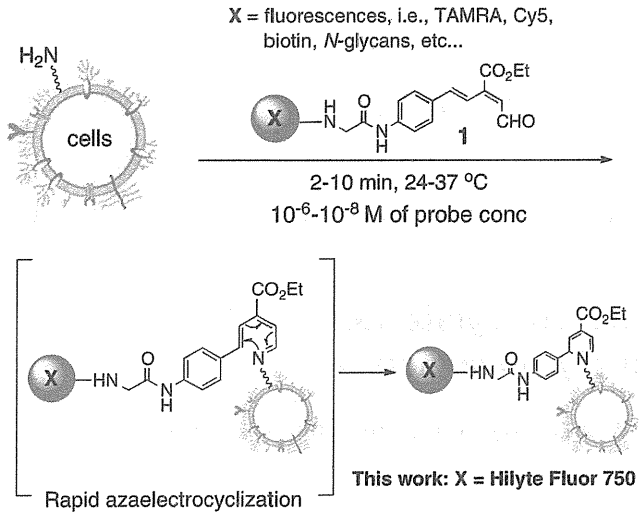

Scheme 1. Schematic presentation of cell surface labeling by azaelectrocyclization.

membrane proteins and/or ethanol amine derivatives, therefore minimizing the interference with their native functions. Dihydropyridines as the electrocyclization products, which retain cationic charges as those of the inherent amines, should also contribute to the retention of the cell functions. The efficient labeling of live cells resulted in the successful in vivo imaging of Cy5 (cyanine fluorescence dye)-labeled lymphocytes (Scheme 1), and the migration of these cells into the immune-related organs was clearly visualized with very high imaging contrast.[13a] Noninvasive tumor targeting by chemically engineered lymphocytes expressing sialo-*N*-glycan has also been be achieved by this technique.[14]

In the current work, we expand upon these earlier results by demonstrating the use of this cell surface chemistry for whole-body fluorescence imaging of tumor metastasis in BALB/c nude mice. Four kinds of human cancer cells (two cancer cell lines and their transfected versions expressing surface glycan-related genes, Fig. 1) were chosen for the metastatic imaging. Our preliminary trials on the noninvasive imaging of these cell lines in BALB/c nude mice using the genetically encoded luciferase bioluminescence, however could not image the surface glycan-dependent metastatic properties (see Supplementary data); Presumably, the inefficient interaction of luciferin with luciferase-encoded tumor tissues or the luminescence at shorter wavelength (~570 nm) could not make a clear imaging contrast in living systems. These four cancer cell lines were therefore labeled by azaelectrocyclization with near-infrared fluorescence, Hilyte Fluor 750 for 10 min and without affecting cell viability. The trafficking and metastasis of these cells in BALB/c nude mice were monitored with an eXplore Optix apparatus. The glycan structure-dependent metastatic properties of the cells, which could previously only be studied in detail via in vitro and invasive in vivo experiments, were successfully imaged in the present research. Our noninvasive technique described in this paper provides the landmark opportunity for sensitively monitoring the dynamics of the cancer cells depending on their surface structures and/or the host environments, thus impacts on the cancer prognosis and the therapeutic applications.

2. Results and discussion

2.1. Cell surface labeling of tumor cells

We focused on visualizing the metastasis that are strongly influenced by cell-surface glycan structures and for which the mechanisms of metastasis have been previously studied in detail

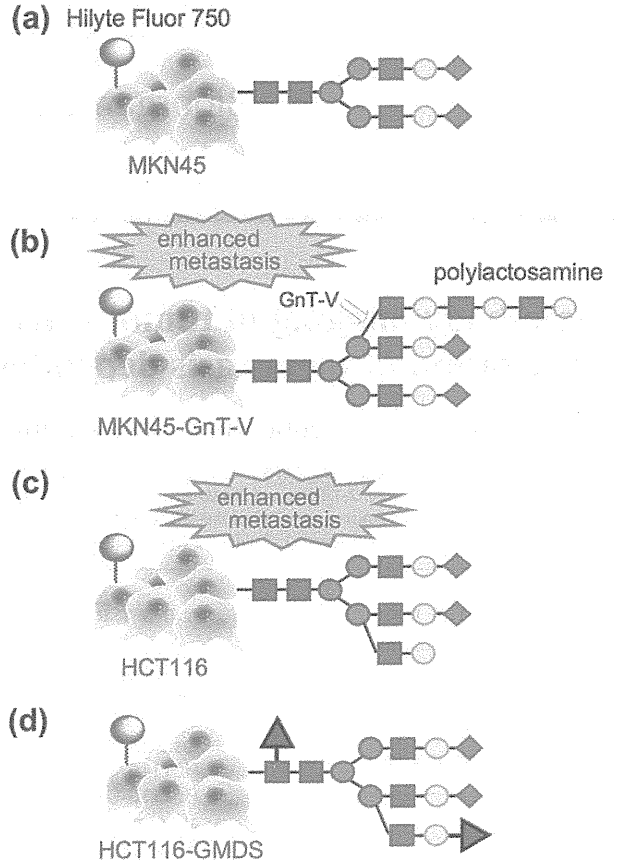

Figure 1. Structural images of cancer cells labeled with Hilyte Fluor 750. (a) MKN45 (mock); (b) MKN45 transfected with GnT-V; (c) HCT116 (mock); (d) HCT-116 transfected with GMDS. The blue circles anchored on the cell surfaces represent the Hilyte Fluor 750 introduced by azaelectrocyclization. Differently colored circles, squares, diamonds, and triangles connected to each other represent the sugars that make up the complex-type *N*-glycans. The position and stereochemistry of the sugar linkages are not indicated. Yellow circle: galactose; green circle: mannose; blue square: *N*Ac-glucosamine; pink diamond: *N*Ac-neuramic acid; red triangle: fucose.

(Fig. 1).[15–17] One approach involves the overexpression of GnT-V (β(1-6)*N*-acetylglucosaminyltransferase) in the human gastric cancer cell line, MKN45, which leads to an increased number of cell surface *N*-glycans containing polylactosamine, and this leads to enhanced metastasis due to an acquired resistance to matriptase degradation and an enhanced level of growth factor signaling.[15,16] The other is the human colon cancer cell line, HCT116, which is deficient in GMDS (GDP-mannose-4,6-dehydrase), and this loss of cellular fucosylation allows them to escape NK-mediated tumor surveillance due to their acquired resistance to TRAIL (tumor necrosis factor-related apoptosis-induced ligand)-induced apoptosis.[17]

The four cell lines (1.0×10^6 cells) were coated onto 25-cm^2 cell flasks and treated with Hilyte Fluor 750-probe **1** (1.0×10^{-6} M in PBS, 1 mL) (see compound properties and procedures for 'STELLA$^+$', labeling kits available from Kishida Chemical Co. Ltd, http://www.kishida.co.jp/) for 10 min at 37 °C under 5% CO_2 (Scheme 1). The cells were washed twice with culture medium to remove the excess aldehyde probe, treated with 0.25% trypsin and 1 mM EDTA for 3 min at 37 °C, and then resuspended in HBSS (1.0×10^7 cells/mL). The cell surface labeling protocol generated consistent efficiencies among the four cell lines. The average number of the Hilyte Fluor molecules bound to each cell, calculated based on fluorescence signals, was 4.6×10^5 molecules for MKN45, 6.4×10^5 molecules for MKN45-GnT-V, 6.9×10^5 molecules for HCT116,

and 9.3×10^5 molecules for HCT116-GMDS. This electrocyclization protocol did not affect cell viability, which remained above 85% in all cell lines: 93% for MKN45, 95% for MKN45-GnT-V, 87% for HCT116, and 87% for HCT116-GMDS, respectively. Therefore, these data suggest that this protocol can be safely applied to most cancer cell lines with high efficiency.[13]

2.2. Whole-body fluorescence imaging

At the early stage of the imaging experiments, when the administered cells in the abdominal cavities were moving nonspecifically among the internal organs, the fluorescent tumor cells were highly diluted throughout the body and their signal was undetectable (see experimental procedures in Fig. 2 and Section 4). Over the course of the observation period, cell division and/or decomposition/metabolism of the membrane constituents led to a decay in the fluorescence of each cell type. Cancer progression and metastasis, however, resulted in the accumulation and the concentration of fluorescently labeled cells at the metastatic regions; hence, the metastasis could be observed in the fluorescence images. Some of the fluorescent labels could also be endocytosed[13a] over a period of imaging experiment, but the whole-cancer cell imaging does not necessitate the fluorescence labels staying all the time on the cell membrane, once the labels are covalently attached on the cancer cell constituents.

Thus, whole-body imaging of the four cancer cell lines did not show any significant fluorescence for the first 7 days (Fig. 2). Over a course of 21 days, however, the slight differences were gradually observed in fluorescence intensities between the mock and transfected cells for both the MKN45 and the HCT116 cell lines: After 35 days, the mice administered the MKN45-GnT-V (Fig. 2b) and HCT116 cells (Fig. 2c) showed much stronger fluorescence accumulation than those receiving the MKN45 (Fig. 2a) and HCT116-GMDS (Fig. 2d) cell lines.

The biodistribution study of the dissected tissues after the 35-days observation period (Supplementary data) showed that the fluorescence in the MKN45-GnT-V-administered mice was mostly in the liver (48%) and colon (especially accumulated in lymph node in the peritoneum, 35%), with small amounts seen in the stomach (8%), kidneys (4%), pancreas (2%), spleen (2%), and heart (1%). (The percentage is the relative fluorescence intensity in that organ compared to the sum of the intensities in all organs.) On the other hand, the fluorescence in HCT116-administered mice was mostly observed in the liver (40%) and spleen (40%), and to a lesser extent in the stomach (10%), colon (5%), kidneys (2%), and pancreas (1%).

Compared to the control cell lines (MKN45 and HCT116-GMDS), the cell lines manipulated to enhance metastasis (MKN45-GnT-V and HCT116) generated intense fluorescence of selective organs they reached by metastasis from the abdominal cavity. These imaging data are in consistent with the previous macroscopic observation in liver or lymph node metastasis that cell surface *N*-glycans play a critical role in the initiation of cancer metastasis (Fig. 1);[15,17] Namely, a polylactosamine chain at the branching mannose of *N*-glycans enhances metastatic potential,[15] while fucosylation is required for the NK-cell mediated tumor surveillance system to attenuate the progression and metastasis of the cancer cell.[17] Thus, azaelectrocyclization-based cell surface labeling and whole-body imaging proved the promising techniques for efficiently monitoring cancer metastasis and its dependence on cell surface constituents such as glycan structures.

3. Summary

In summary, we have successfully used azaelectrocyclization chemistry to label four cancer cell lines with the near-infrared fluorophore Hilyte Fluor 750. This allowed for noninvasive whole-body imaging to be performed. In accordance with previous findings by us[15,17] and others,[16] the fluorescence imaging clearly showed that tumor metastasis was dependent upon the cell surface glycans. A larger polylactosamine structure or the loss of fucosylation on the cancer cell surfaces, respectively, enhanced the metastatic potential of the tumor cells. It is noted that the glycan-dependent metastatic properties could not previously be monitored by the conventional luciferase-encoding method, hence highlight the present live cell labeling. The results described in this paper suggest that it is possible through our techniques to efficiently correlate the surface constituents of cancer cells with in vivo metastatic dynamics. The various cell surfaces could also be labeled with β-emitters,[5] which would greatly increase the detection sensitivity in applying to the primate models and eventually the patients. Research in this direction is currently under way in our laboratories.

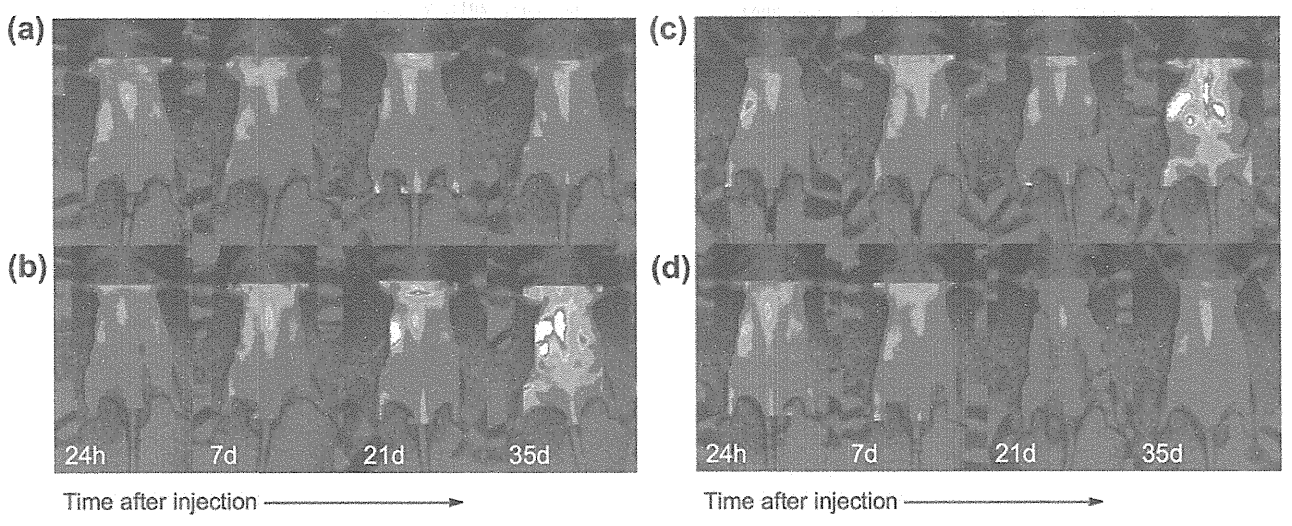

Figure 2. (a–d) Dynamic fluorescence imaging of tumor cells in BALB/c nude mice; (a) MKN45; (b) MKN45-GnT-V; (c) HCT116; (d) HCT116-GMDS. Hilyte Fluor 750-labeled cancer cells were injected into the abdominal cavities of BALB/c mice ($n = 2$, 1.0×10^6 cells, 100 μL/mouse) and whole-body scans were performed at the indicated time with an eXplore Optix device (excitation at 730 nm, emission at 750 nm).