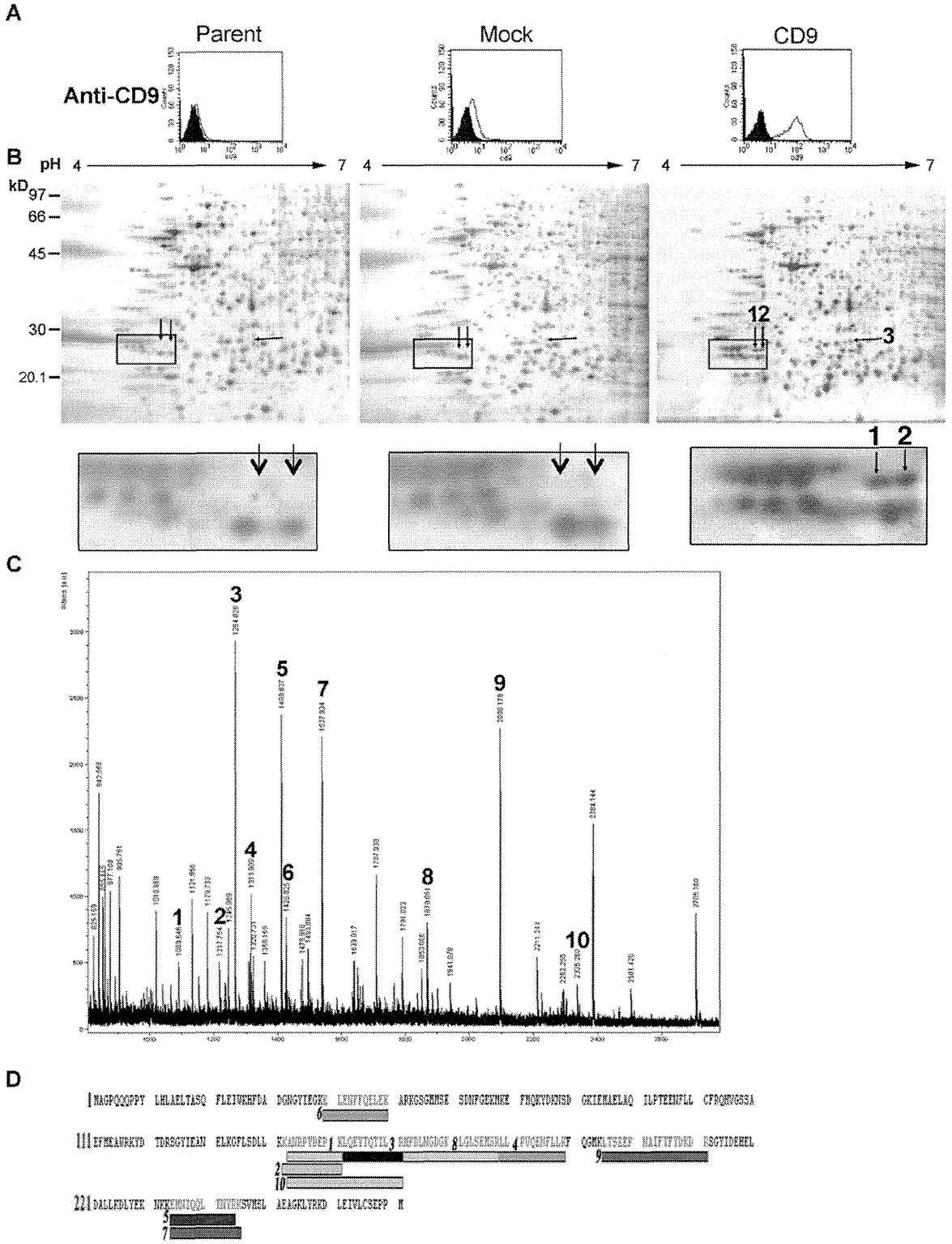


Fig. 1. Comparative proteomic analysis of the parent and CD9-overexpressing SCLC cells. (A) The parent, mock transfectant, and CD9 transfectant of OS3-R5 were stained with anti-CD9 mAb, labeled with FITC-conjugated goat anti-mouse immunoglobulin, and analyzed on a FACScan (*Open histograms*). *Closed histograms* indicate staining with control IgG. (B) Representative 2-DE maps of OS3-R5 and its transfectants. *Arrows* 1–3 indicate protein spots selectively identified in OS3-R5-CD9 by mass spectrometry. 1 and 2, calretinin; 3, PA28α. Images including the calretinin spots were enlarged in *lower columns*. (C) PMF spectra of spot 2 obtained by MALDI-TOF. Mass peaks, peptides of which were matched with human calretinin, are marked with numbers. (D) The matched peptides in panel (C) were indicated with bars, yielding 33% sequence coverage of calretinin.

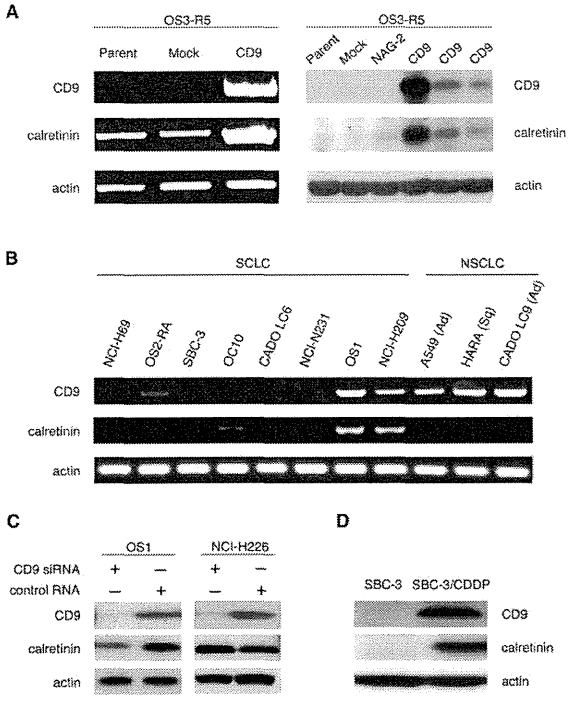
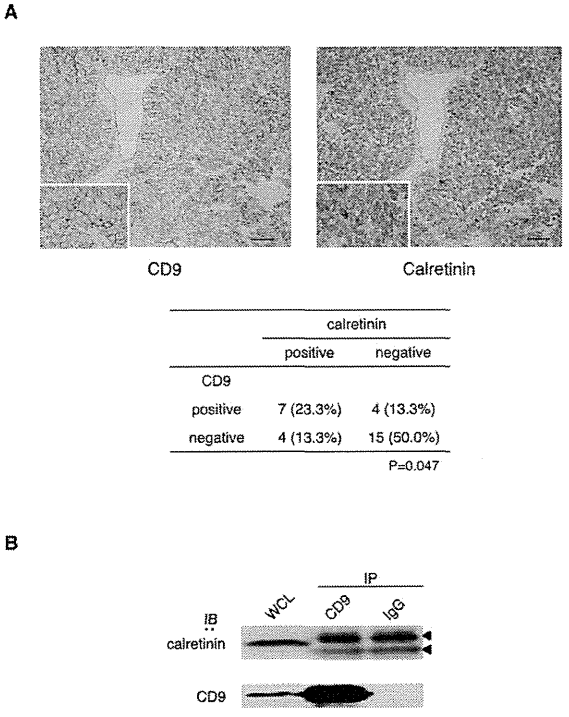


Fig. 2. Co-expression of CD9 and calretinin in SCLC cells. (A) Total RNA was extracted from the parent, mock transfectant, and CD9 transfectant of OS3-R5 and analyzed for expressions of CD9 and calretinin by RT-PCR. β-Actin amplification was used as the internal control (*left*). The parent, mock transfectant, NAG-2 transfectant, and CD9 transfectants of OS3-R5 were lysed with 1% Brij 99 lysis buffer. Cell lysates were analyzed for expressions of CD9 and calretinin by immunoblotting. Anti-β-actin blots were used as the internal control (*right*). (B) Total RNA was extracted from multiple SCLC and NSCLC cell lines and analyzed for expressions of CD9 and calretinin by RT-PCR. Ad, adenocarcinoma; Sq, squamous cell carcinoma. (C) An SCLC line OS1 (*left*) or a mesothelioma line NCI-H226 (*right*) was transfected with siRNAs against CD9 or control RNAs. Cell lysates were analyzed for expressions of CD9 and calretinin by immunoblotting. (D) Cell lysates of SCLC lines SBC-3 and SBC-3/CDDP were analyzed for expressions of CD9 and calretinin by immunoblotting.



	calretinin	
	positive	negative
CD9		
positive	7 (23.3%)	4 (13.3%)
negative	4 (13.3%)	15 (50.0%)

P=0.047

Fig. 3. CD9 is not physically associated with calretinin. (A) Immunohistochemical staining of CD9 and calretinin in a double-positive specimen from an SCLC tissue microarray. *Insets* show enlarged images of a part of sections. Bar, 50 μm. Significant association between CD9 and calretinin expressions in the tissue microarray was evaluated by Fisher's exact test (*table*). (B) CD9 and calretinin in whole cell lysate (WCL) and in immunoprecipitates (IP) with anti-CD9 mAb or control IgG of OS3-R5-CD9 were immunoblotted (IB). *Arrowheads* indicate nonspecific binding of secondary Abs.

2.10. Mice

The generation of CD9 knockout (KO) mice was described previously [13]. These mice were backcrossed more than six generations into the C57BL/6J background. The mice were bred in a barrier facility, and all animal procedures were performed in accordance with the Osaka University guidelines on animal care.

2.11. Apoptosis analysis

Cells were transfected with siRNA against calretinin or negative control RNAs. After 24 h, the cells were cultured in the absence or presence of CDDP in low-serum (0.1% FBS) RPMI 1640 for 48 h. Apoptotic cleavage of PARP [14] and decrease of Akt phosphorylation [9] were analyzed by immunoblotting. Viable cells were quantified with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Assays were performed in triplicate cultures and values are expressed as mean ± SD. Statistical differences were determined by Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of proteins upregulated in OS3-R5-CD9 cells

We previously established a CD9 (−) SCLC cell line OS3-R5 and its CD9 transfectant OS3-R5-CD9 and revealed that the CD9 transfectants were less motile on fibronectin [8] and revealed enhanced apoptosis in low-serum culture conditions [9]. To detect molecules that regulate these changes, cell lysates of the parent, mock transfectant, and CD9 transfectant of OS3-R5 (Fig. 1A) were subjected to 2-DE, and protein spots were visualized by silver stain of the gels. Spots selectively overexpressed in OS3-R5-CD9 were identified (Fig. 1B) and the corresponding proteins were analyzed by mass spectrometry. We repeated this experiment and found that two proteins, a calcium-binding protein, calretinin, and a proteasome activator subunit 1, PA28α, were reproducibly overexpressed in OS3-R5-CD9 cells (Table S1). Fig. 1C shows PMF spectra of spot 2 obtained by MALDI-TOF. Matched peptides were found to cover 33% of protein sequence of calretinin (Fig. 1D). Based on the fact that SCLC has neuronal features, we further analyzed calretinin, which is a protein distributed in the nervous system.

3.2. Co-expression of CD9 and calretinin in SCLC cells

RT-PCR revealed that the calretinin gene was minimally transcribed in the parent and mock-transfected OS3-R5 cells, and that ectopic expression of CD9 promoted its transcription (Fig. 2A, *left column*). To test the calretinin induction is specifically related to CD9, multiple CD9 transfectants and cells transfected with another tetraspanin NAG-2 were examined. As shown in Fig. 2A, *right column*,

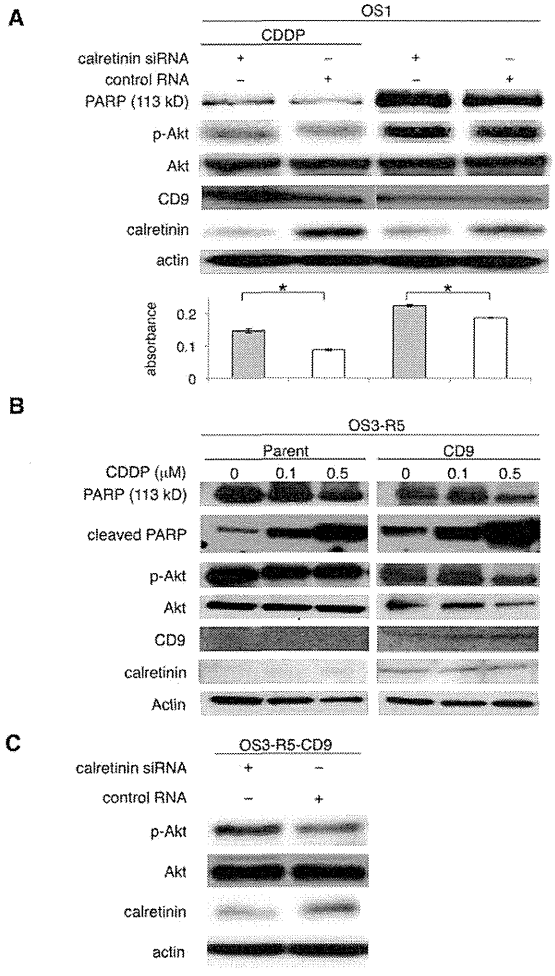


Fig. 4. Calretinin promotes apoptosis of CD9 (+) SCLC cells. (A) CD9 (+)/calretinin (+) OS1 cells were transfected with siRNAs against calretinin or control RNAs and cultured in low-serum conditions in the absence or presence of 5 μM CDDP for 48 h. Cell lysates were analyzed for expressions of PARP (113 kD), phosphorylated Akt (p-Akt), total Akt, CD9, and calretinin by immunoblotting. Anti-β-actin blots were used as the internal control (*upper*). Viable cells were quantified with a cell counting kit (*lower*). *$P < 0.01$. (B) The parent and CD9 transfectant of OS3-R5 were cultured in low-serum conditions in the indicated concentrations of CDDP for 48 h. Cell lysates were analyzed for expressions of PARP (113 kD), cleaved PARP, phosphorylated Akt, total Akt, CD9, and calretinin by immunoblotting. (C) OS3-R5-CD9 cells were transfected with siRNAs against calretinin or control RNAs and cultured in low-serum conditions for 48 h. Cell lysates were analyzed for expressions of phosphorylated Akt, total Akt, and calretinin by immunoblotting.

calretinin was almost absent in the parent, mock transfectant, and NAG-2 transfectant in immunoblotting, whereas calretinin was obviously present in three independent CD9 transfectants and its level was parallel to that of CD9. We further examined if CD9 and calretinin are co-expressed in a panel of lung tumor cell line (Fig. 2B). Consistent with our previous report that most SCLC cells lack CD9 [8], five of eight SCLC lines revealed no transcription and one cell line (OS2-RA) showed only marginal transcription of *CD9* gene. The other two lines (OS1 and NCI-H209) clearly expressed CD9, and these CD9 (+) lines also expressed calretinin. Meanwhile, all three NSCLC lines were CD9 (+), but none of them expressed calretinin (Fig. 2B).

CD9 was next deleted by knockdown with siRNA in the SCLC line OS1, which expresses endogenous CD9. As shown in Fig. 2C, *left column*, the knockdown of CD9 suppressed the level of calretinin. In an additional experiment, we have studied CD9 and calretinin expressions in multiple pleural mesothelioma cell lines and found that only NCI-H226 expresses both CD9 and calretinin in immunoblotting (Fig. S1). CD9 was knocked down in this mesothelioma line, but unlike OS1, the calretinin level was not affected (Fig. 2C, *right column*). Our recent report showed that endogenous CD9 is induced when the CD9 (−) SCLC line SBC-3 is exposed to an anticancer drug, cisplatin [10]. As shown in Fig. 2D, calretinin was co-induced with endogenous CD9 in the cisplatin-exposed SBC-3 cells.

We further investigated if CD9 is generally required for the expression of calretinin using tissues from wild-type and CD9 KO mice. Calretinin was expressed in liver and brain lysates of wild-type mice, and its levels were not affected by the loss of CD9, as evidenced by abundant expression of calretinin in the lysates from CD9 KO mice (Fig. S2). Together, regulation of calretinin expression by CD9 seemed to be specific to human SCLC cells.

3.3. CD9 is not physically associated with calretinin

To investigate co-expressions of CD9 and calretinin *in vivo*, a tissue microarray of SCLC was analyzed by immunohistochemistry. Among 30 patients, 11 were CD9 (+), 11 were calretinin (+), and 7 were double-positive, and association between CD9 expression and calretinin expression was weakly significant (Fig. 3A, *table*). Higher positive rate of CD9 (11/30) in SCLC tissues compared with that in SCLC cell lines [8] might be because some biopsy specimens were obtained from relapsed or metastatic lesions, which were more frequently CD9 (+) than pretreated primary tumors [10]. Representative staining of CD9 and calretinin in a double-positive specimen indicated that expression patterns of these proteins were different (Fig. 3A). CD9 was stained at the cell periphery, whereas calretinin showed nuclear and cytoplasmic staining (Fig. 3A, *insets*). Tetraspanins including CD9 are characterized by their propensity to form multiprotein complexes at the plasma membrane. To examine if calretinin is present in large protein complexes including CD9, co-precipitated proteins with CD9 was immunoblotted with anti-calretinin Ab. As shown in Fig. 3B, calretinin did not co-precipitate with CD9 in OS3-R5-CD9 cell lysate even using non-stringent detergent, Brij 99. Thus, calretinin was not present in the protein complex including CD9.

3.4. Calretinin promotes apoptosis of CD9 (+) SCLC cells

Our previous reports have shown that ectopic expression of CD9 increases apoptosis by attenuation of postadhesive phosphorylation of Akt [9] and that anticancer drugs induce endogenous CD9 in SCLC cell lines [10]. To examine the involvement of calretinin in apoptosis of SCLC cells, calretinin was knocked down with siRNA in CD9 (+)/calretinin (+) OS1 cells, and expression of PARP was examined as an indicator of apoptotic cell death [14]. Exposure of OS1 cells to 5 μM cisplatin increased endogenous CD9 and calretinin and enhanced apoptosis as evidenced by decrease of 113-kD PARP and decreased phosphorylation of Akt (Fig. 4A, control RNA). Knockdown of calretinin prevented the OS1 apoptosis regardless of the exposure to cisplatin (Fig. 4A, calretinin siRNA). It appeared that the calretinin knockdown slightly upregulated CD9; this might reflect an unknown feedback mechanism. Proapoptotic role of calretinin was also studied in OS3-R5 cells. The exposure to CDDP for only 48 h did not induce endogenous CD9 and calretinin in this cell line and, when compared with the parent cells, OS3-R5-CD9 cells expressing calretinin revealed higher sensitivity to CDDP, as evidenced by enhanced PARP cleavage and decreased Akt phosphorylation (Fig. 4B). After the exposure to 1 μM CDDP for 48 h, viable cells of OS3-R5 and OS3-R5-CD9 were 56.1 ± 1.8% and 20.1 ± 2.7%, respectively ($P < 0.01$). As shown in Fig. 4C, the knockdown of calretinin in OS3-R5-CD9 increased phosphorylation of Akt. These results suggest that calretinin may be a downstream mediator of apoptosis in CD9 (+) SCLC cells.

4. Discussion

Our previous studies have proposed that the absence of tetraspanin CD9 contributes to highly malignant phenotype of SCLC and that CD9 may be a pivotal regulator of SCLC cell survival [8–10]. In the present study using a proteomics-based approach, we identified calretinin as a possible mediator of CD9-induced apoptosis in SCLC. Calretinin was present in CD9 (+) SCLC cell lines but not in CD9 (−) SCLC lines and CD9 (+) NSCLC lines. Ectopic or CDDP-induced expression of CD9 upregulated calretinin in SCLC lines. Knockdown of CD9 conversely down-regulated calretinin in an SCLC line but not in a mesothelima cell line. Furthermore, knockdown of calretinin increased Akt phosphorylation and decreased apoptosis in CD9 (+)/calretinin (+) SCLC cell lines. Although statistical significance in the association of CD9 and calretinin expressions was weak ($P = 0.047$) in SCLC tissues, we speculate that this might be due to elimination of apoptotic CD9 (+)/calretinin (+) tumor cells *in vivo*.

Calretinin is a member of the calcium-binding protein EF-hand family first identified in the retina. Calcium-binding proteins including calretinin are expressed in neuronal subpopulations of the nervous system. Calretinin is involved in cellular functions including intracellular calcium buffering, messenger targeting, and the modulation of neuronal excitability. Modulation of calcium signaling by calretinin is important for timing and plasticity of synaptic events in neuronal networks. Some studies have suggested neuroprotective role of calretinin against calcium-induced cytotoxicity, whereas others reported opposite effects [15,16]. Of note, a recent report using colorectal cancer cells indicated that calretinin is induced following treatment with oxaliplatin or 5-FU and positively regulates apoptotic signals via as yet unknown mechanisms [14]. In line with this report, the present study suggested that calretinin mediates proapoptotic signaling in SCLC cells and for the first time showed that CD9 positively regulates the expression of calretinin. It has been established that tetraspanins including CD9 work as organizer of multiprotein complexes at the membrane [4,5]. Although calretinin has been reported to concentrate beneath the plasma membrane during maturation in neurons [17], it did not co-precipitate with CD9 in OS3-R5-CD9 cells, suggesting the presence of other mediators linking CD9 to enhanced expression of calretinin.

In conclusion, by proteomics-based approach, we have proposed a novel proapoptotic pathway that links the metastatic suppressor CD9 to the neuronal calcium-binding protein, calretinin, in SCLC. Induction of CD9/calretinin may at least partially account for its high sensitivity to chemotherapy and might provide clues to new therapeutic approach to suppress early growth, metastasis, and recurrence of SCLC.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2013.04.005.

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ORIGINAL ARTICLE

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-

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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)				

fucose, 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), 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.

T1

AQ1 T2

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 lectin 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.

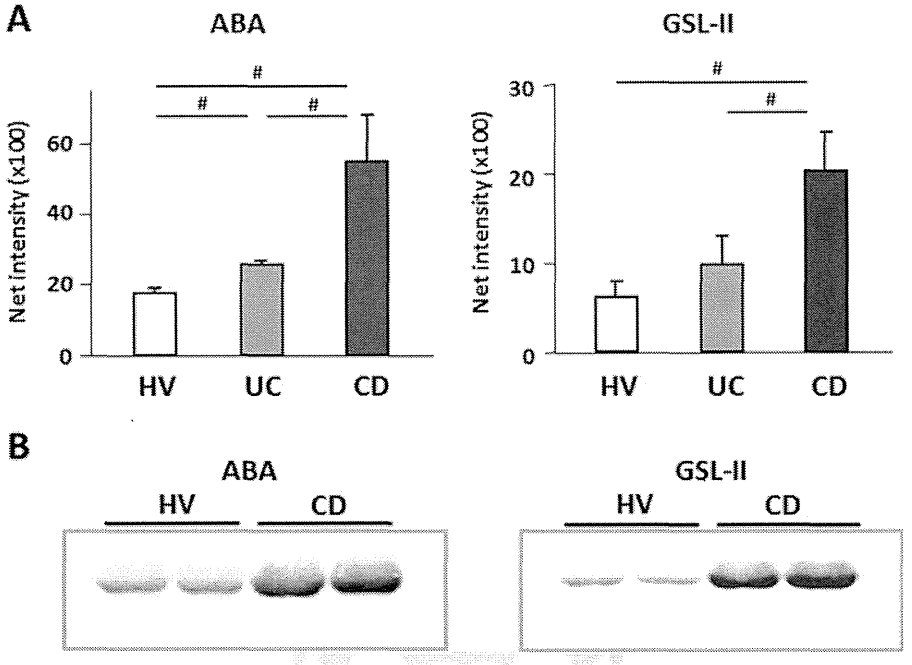

FIGURE 1. Binding levels of ABA and GSL-II to IgG *N*-linked oligosaccharides are higher in patients with IBD than in HVs. (A) Lectin microarray for serum IgGs from HV, patients with UC, and those with CD (five cases each). Among 45 lectins on the lectin microarray, results for ABA and GSL-II are shown. #$P < 0.05$. (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 (Supporting Fig. 1). 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, F1 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). Glyco- F2
peptidase-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

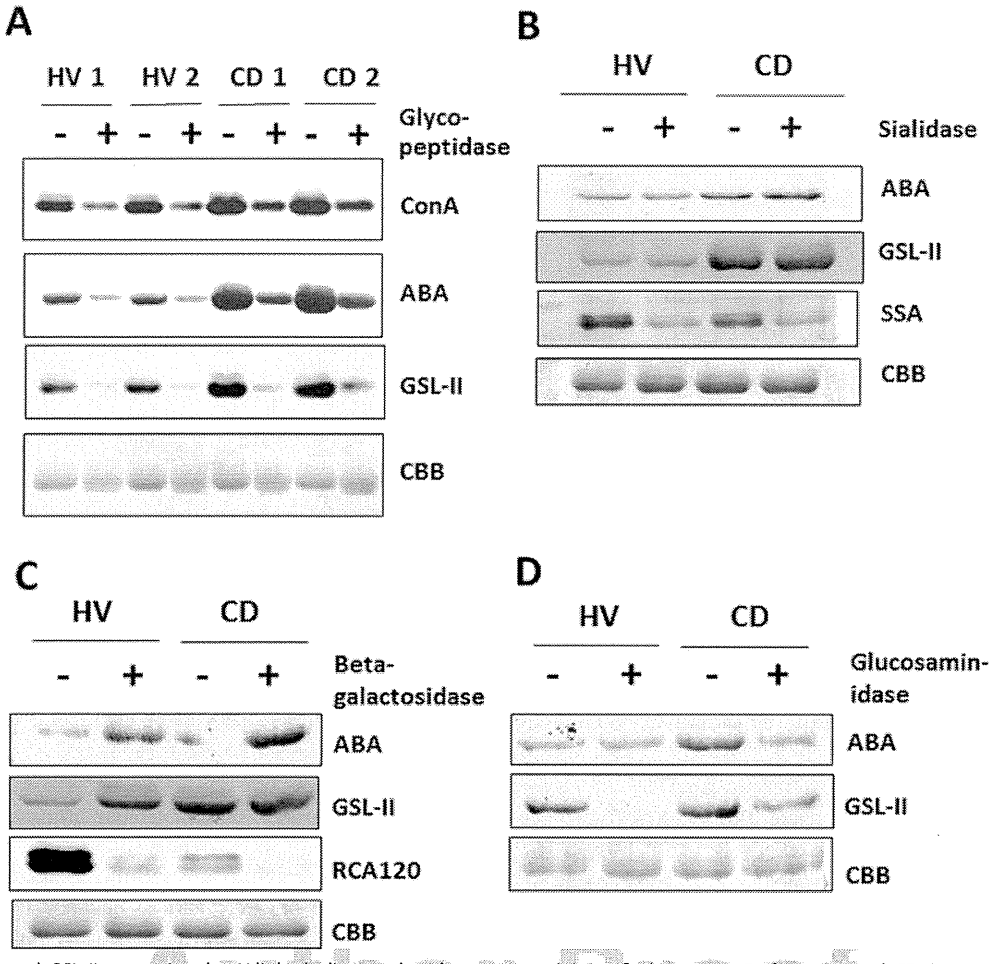

FIGURE 2. ABA and GSL-II recognize the *N*-linked oligosaccharide structure. (A) Purified serum IgGs from HV and CD (two cases each) were treated with glycopeptidase F to remove oligosaccharides from IgG. Binding of ABA, GSL-II, and ConA (binds *N*-linked oligosaccharides) to enzymatically treated or 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.

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."

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

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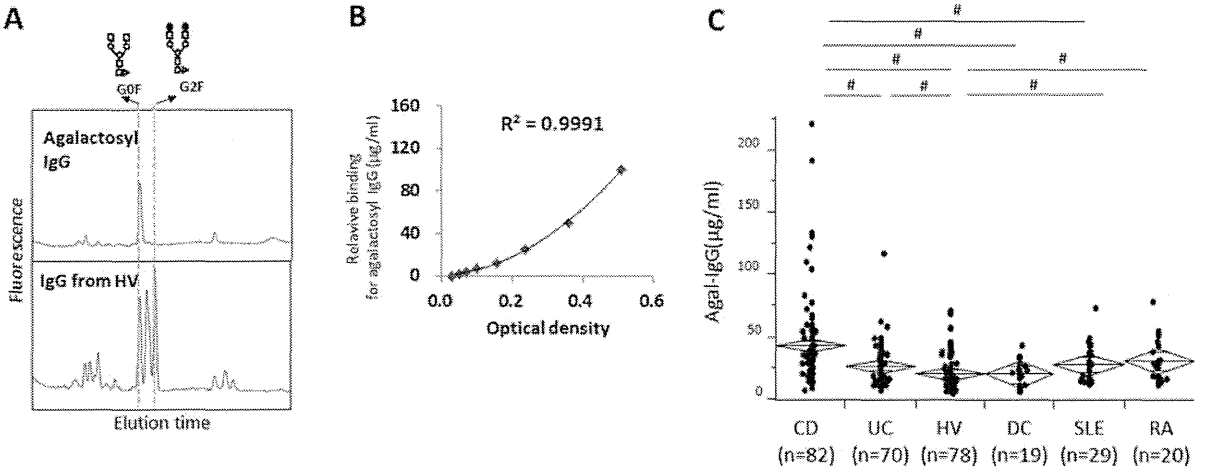

FIGURE 3. Agalactosyl IgG is increased in IBD patients by lectin-EIA. (A) Oligosaccharide structures from control agalactosyl IgG or serum IgG in HV were analyzed by HPLC. The outer arm galactose was almost completely absent in control agalactosyl IgG. This result is representative of three independent 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. #$P < 0.05$.

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 (Supporting Fig. 2), 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 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 $\geq$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.

F4

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).

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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.

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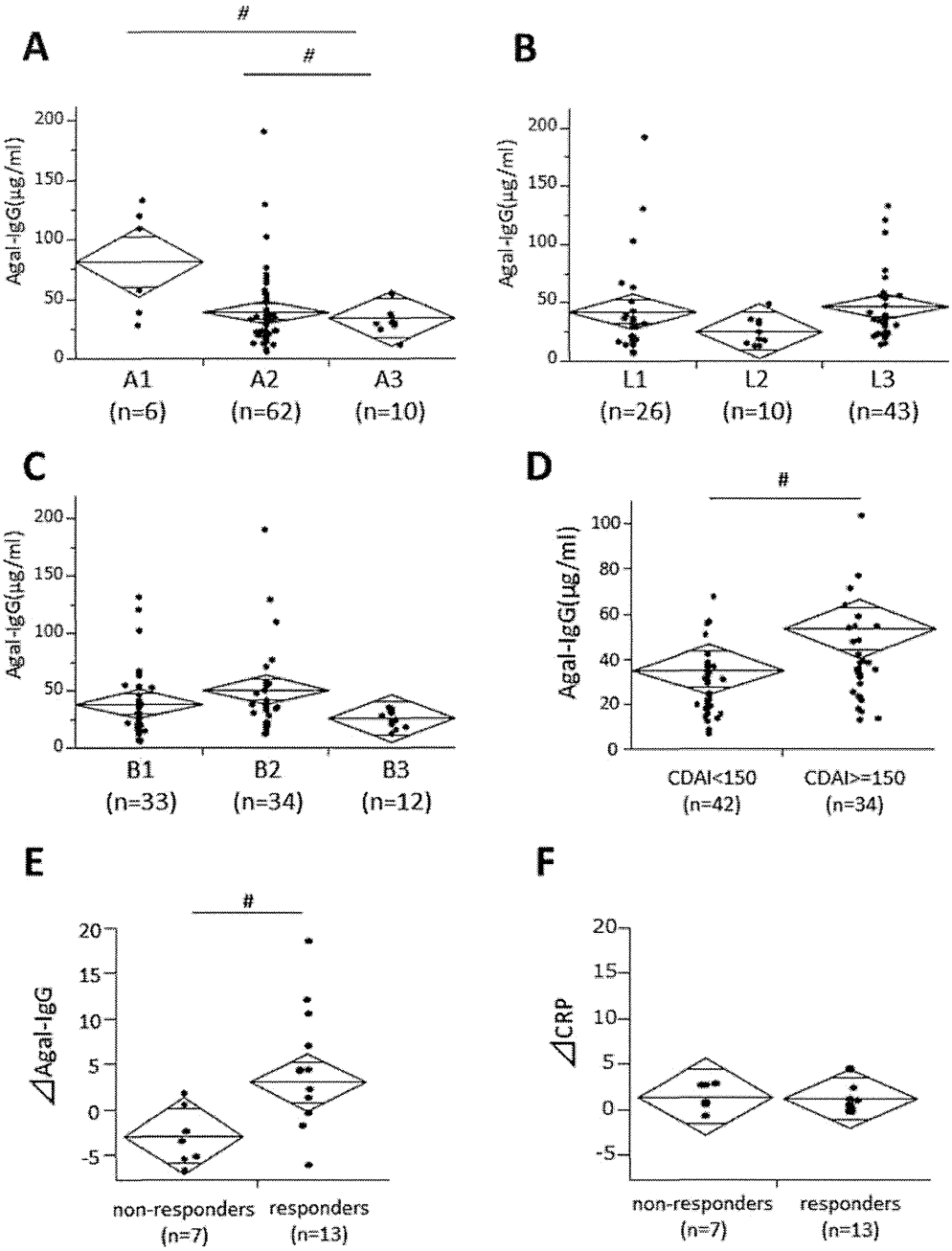

FIGURE 4. Agalactosyl IgG reflects disease activity and clinical course in CD. (A) Agal-IgG levels and age of disease onset (category A) in CD patients. Agal-IgG levels were significantly higher in patients with category A1 than A2 or A3. (B,C) Agal-IgG levels and disease location (category L, (B)) and 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 $\geq$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. $P < 0.05$

Agal-IgG levels were also significantly higher in IBD patients, especially in CD patients, than in healthy volunF6 teers (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

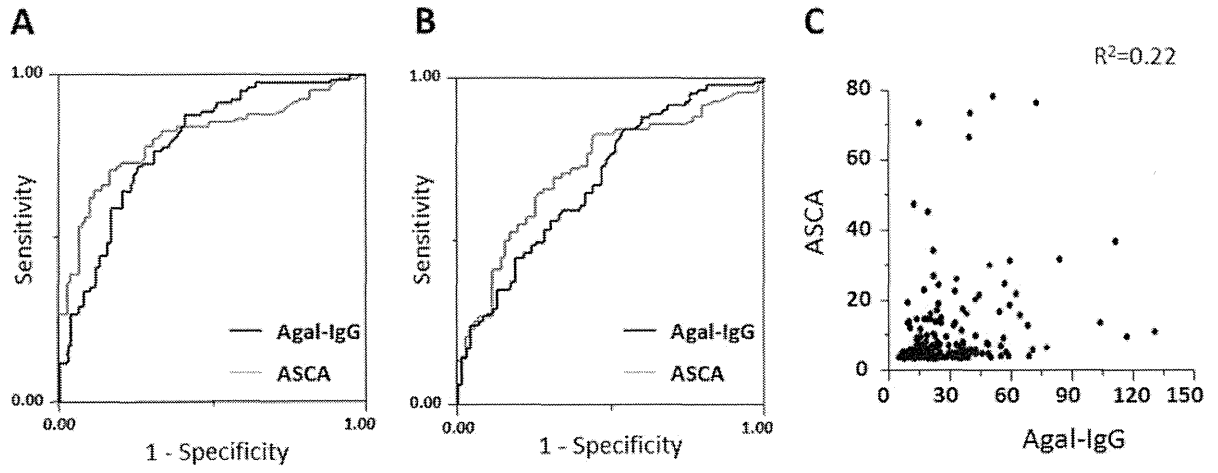

FIGURE 5. The combination of Agal-IgG and ASCA more efficiently differentiates CD. (A) The ROC curves for Agal-IgG and ASCA levels for discrimination between CD and HV, or (B) between CD and UC. (C) Correlation between Agal-IgG and ASCA levels in all subjects (n = 230).

(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 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 Galb1-3GalNAc.[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 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–

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)

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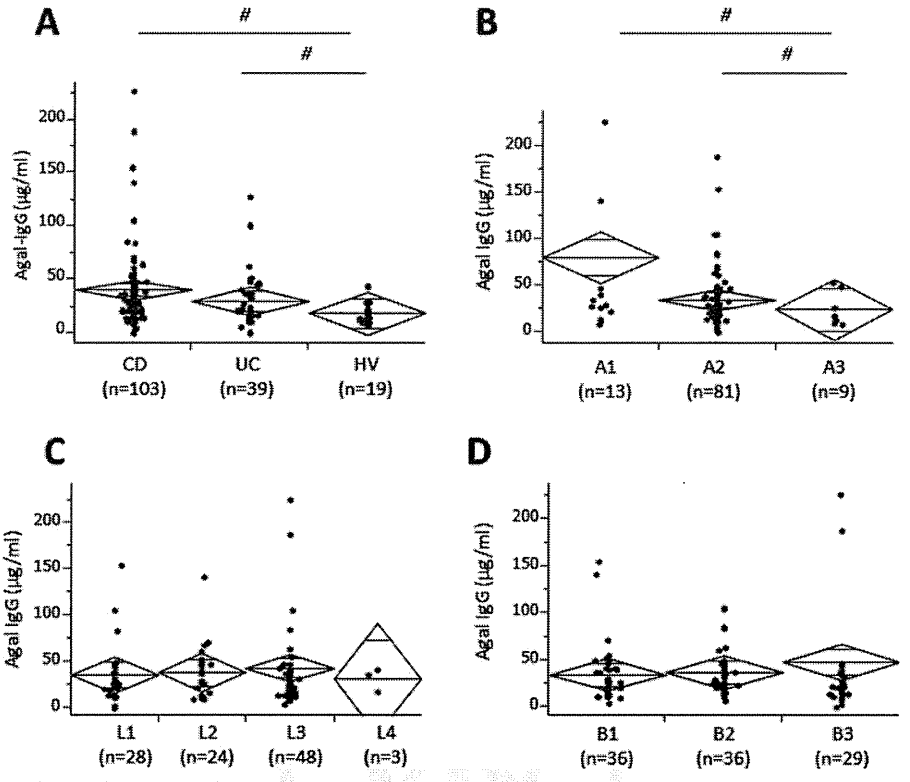

FIGURE 6. Agal-IgG levels are also increased in American IBD patients. (A) Agal-IgG levels of each purified serum IgG from CD, UC, and HV by EIA. (B) Agal-IgG levels and onset age (category A) in CD patients. Agal-IgG levels were significantly higher in patients with category A1 than with A2 or A3. (C,D)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

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 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

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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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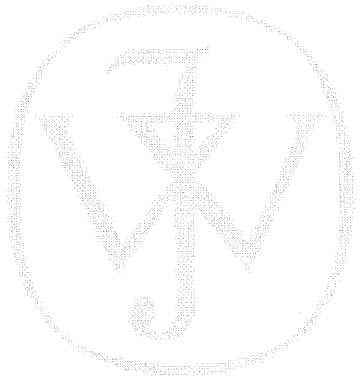
AQ1: Tables 2 and 3 cited out of order; switched.



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ORIGINAL ARTICLE

Post-marketing surveillance of the safety and effectiveness of tacrolimus in 3,267 Japanese patients with rheumatoid arthritis

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Abstract
Objectives A post-marketing surveillance (PMS) program was implemented to assess the safety and effectiveness of tacrolimus (TAC) in Japanese rheumatoid arthritis (RA) patients and to identify risk factors related to adverse drug reactions (ADRs).
Methods Patients were registered centrally and monitored for all adverse events (AEs) for 24 weeks. Effectiveness was evaluated using the Disease Activity Score 28-CRP (DAS28-CRP).
Results Data from 3,172 patients (mean age 62.2 years) were evaluated in the safety analysis. Of the safety population, 78.5 %were female and 25.9 % were in Steinbrocker's functional class 3 or 4. TAC was prescribed as monotherapy in 52.5 % and the most common concomitant disease modifying antirheumatic drug (DMARD) was methotrexate, used in 28.9 % of the patients. The incidence of AEs, serious AEs (SAEs), ADRs and serious ADRs were 41.2, 6.4, 36.0, and 4.9 %, respectively. The most frequent serious ADR category was infections and infestations. Age ≥65 years, concurrent renal dysfunction, and concurrent diabetes mellitus were identified as significant risk factors for ADR. Based on EULAR response criteria, 65.4 % of the patients showed moderate or good response.
Conclusions The results demonstrate that TAC is well tolerated by Japanese patients with active RA, including those receiving concomitant methotrexate, in the real world.

Keywords Effectiveness · Post-marketing surveillance · Rheumatoid arthritis · Safety · Tacrolimus

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Introduction

Rheumatoid arthritis (RA) is characterized by persistent synovitis and structural damage of joints in part through the abnormal activation of immunocompetent cells, including T cells. It has been reported that pathogenesis of RA remains elusive in terms of active T cell- or macrophage-induced cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6, and molecules that cause interaction between antigen presenting cells and T cells [1–4].

Tacrolimus (TAC), a macrolide lactone discovered in 1984, mainly exerts its immunosuppressive effects through

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inhibition of T cell activation and production of inflammatory cytokines, such as TNF, IL-1β, and IL-6, all involved in the pathogenesis of RA [5–7]. TAC has been used for the prevention of rejection in organ transplantation and graft-versus-host disease after bone marrow transplantation, as well as for the treatment of myasthenia gravis, lupus nephritis, and ulcerative colitis. In addition, it was approved for RA patients with inappropriate response to conventional treatments in Japan in April 2005, and subsequently approved for RA in Canada, Korea, and Hong Kong.

The efficacy of TAC against RA has been demonstrated in several clinical trials [8–12]. Although the information obtained from the clinical trial settings is straightforward and robust, it has several limitations. For example, in post-marketing settings, TAC is used in patients with various comorbidities or in patients concomitantly taking a variety of drugs, including corticosteroids, DMARDs, and even biological agents. In this regards, the safety and effectiveness of TAC in clinical practice settings remains to be investigated. To address these issues, we implemented a nationwide post-marketing surveillance (PMS) program on safety and effectiveness of TAC in RA patients with central registration and a six-month tracking period in each patient.

Materials and methods

This study was conducted in accordance with a protocol approved by the Ministry of Health, Labor and Welfare (MHLW). A Prograf post-marketing surveillance committee consisting of rheumatologists was convened, which evaluated the obtained interim results in collaboration with Astellas Pharma Inc.

During the study period from April 2005 to March 2009, cases were collected from 406 institutions in Japan. Patients were registered centrally at an independent patient registration center over a 2-year period. The planned study sample size of 3,000 patients was calculated to provide a 95 % confidence level of detecting any adverse event (AE) that occurs at in least 1 of 1,000 exposed individuals.

A written agreement was obtained from participating institutions. The study was also in accordance with the standards for Good Post-Marketing Study Practice (GPSP) provided by the MHLW in Japan.

The MHLW instructed the investigators to perform the PMS study according to GPSP, which is the authorized standard for PMS studies of approved drugs in clinical practice; therefore, no formal ethics committee approval was necessary. The PMS study in Japan is allowed to be conducted without informed consents.

This study was conducted in clinical practice settings in Japan. RA patients who had shown inappropriate response to conventional treatments for RA and who started treatment with TAC for the first time during the registration period (April 2005 to March 2007) were enrolled. Each enrolled patient was followed up for up to 24 weeks. Information regarding background of the patients, status of the TAC treatment, and use of concomitant drugs were collected.

In accordance with the approved dosage and method of administration, adult patients received 3 mg of TAC once daily after dinner. For elderly patients, TAC was started at 1.5 mg once daily after dinner and could be increased to 3 mg if signs and symptoms were not well controlled.

For 24 weeks after the start of treatment with TAC, all AEs and laboratory values were prospectively monitored. Terminology of the Medical Dictionary for Regulatory Activities/Japanese edition (MedDRA/J) version 11.1 was used for summarizing and reporting AEs. AEs were recorded with the physician's assessment of causality, and seriousness according to the International Conference on Harmonization standards.

Of 3,347 patients enrolled, case report forms from 3,267 patients were collected who had at least one follow-up visit after the first dose of TAC. Categorized by clinical department, 1,396 subjects (42.7 %) were from rheumatology departments, 871 (26.7 %) were from internal medicine departments, and 778 (23.8 %) were from orthopedic surgery departments. In accordance with the warnings section of the tacrolimus package insert, which states that "tacrolimus should be administered only by physicians familiar with the treatment of rheumatoid arthritis", the survey was conducted by clinical departments staffed by physicians familiar with the treatment of rheumatoid arthritis. Ninety-five patients were excluded because of unknown status of AEs ($n = 33$), no follow-up visit after the first dose of TAC ($n = 26$), outside enrollment period ($n = 8$), no administration of TAC ($n = 7$), overlapping patients among institute ($n = 7$), no enrollment ($n = 3$), use of TAC before the survey ($n = 2$), and others ($n = 9$). As a result, 3,172 patients were included in the safety population.

Seventeen patients were excluded because of off-label use of, or unknown response to TAC out of 3,172 patients in the safety population. As a result, 3,155 were included in the effectiveness population.

Of 3,155 patients in the effectiveness population, disease activity scores (DAS28) were reported in only 680 patients due to the observational study. Thus, it should be noted that the results of effectiveness [European League Against Rheumatism (EULAR) response rate, DAS28 scores] obtained in this study are difficult to generalize. Nevertheless, the information may be useful for understanding TAC in the real world, and therefore the results of effectiveness in 680 patients were included.

The effectiveness was evaluated by EULAR response criteria, physician's assessment (with three categorical treatment responses of good, moderate, and no response), and DAS28-CRP, which is based on the 28 joint counts, a general health assessment of a patient, and C-reactive protein (CRP). DAS28-CRP was divided into 4 categories: remission ($\leq$2.6), low disease activity (>2.6 and $\leq$3.2), moderate disease activity (>3.2 and $\leq$5.1), and high disease activity (>5.1). The analysis for EULAR response criteria at week 24 was conducted using the last observation carried forward (LOCF) method in RA patients whose DAS28 scores were obtained both at the baseline and at least one follow-up visit after the first dose of TAC. Of 3,155 patients in the effectiveness population, 680 patients were evaluated by EULAR response criteria.

All statistical analyses were performed using SAS statistical software (BASE/SAS SAS/STAT Ver. 8.2; SAS Institute Inc., Cary, NC, USA).

In the statistical analysis, proportions were compared using Chi squared test or Fisher's exact test, as appropriate. Testing was 2-sided, and a significance level of 0.05 was used for each comparison. To determine risk factors for ADRs, candidate factors were identified using univariate analyses, followed by multivariate analyses with Cox proportional hazards models. The analyses were performed using a stepwise variable selection method (backward elimination), at a significance level of 0.05.

Results

The main patient characteristics of the safety population (n = 3,172) are shown in Table 1. Most patients were female (78.5 % of the overall patient population). Mean ($\pm$SD) age was 62.2 $\pm$ 12.0 years and 47.5 % of the patients were 65 years or older. Of all the patients, 64.0 % were in Steinbrocker's stage III or IV and 25.9 % in functional class 3 or 4. Mean disease duration was 11.1 years and 44.4 % of the patients had RA for 10 years or longer. Comorbidities were reported in 2,590 patients; the most common comorbidity was osteoporosis in 1,229 patients, followed by hypertension, dyslipidaemia, interstitial pneumonia, and diabetes mellitus. Just before the initiation of TAC treatment, 79.2 % of the patients used DMARDs, including mainly methotrexate (MTX), salazosulfapyridine, and bucillamine. Just before the initiation of TAC treatment, biological DMARDs, etanercept or infliximab, were used in 9.7 % of the patients. Mean CRP level was 3.3 mg/dl and 3.9 % of the patients had a CRP of 10 mg/dl or higher. Baseline mean DAS28-CRP was 5.1 and 48.7 % of the patients had a DAS28-CRP of 5.1 or higher. As for other characteristics, there were more outpatients than inpatients, with the former accounting for 89.6 % of the population. Corticosteroid was administered in 81.5 % of the safety population and the mean daily dose in these concomitant users was 6.8 mg (prednisolone [PSL] equivalent dose). The dose of corticosteroid was 10 mg/day or higher in 16.3 %. TAC was used as a monotherapy (i.e., without other non-biological DMARDs) in 52.5 % and the rest of the patients received non-biological DMARDs other than TAC; MTX was used in 28.9 %, salazosulfapyridine in 14.3 %, and bucillamine in 7.4 %. Biological DMARDs were administered in 7.1 %; etanercept was used in 4.4 % and infliximab in 3.0 %.

Among the patients included in the safety population, 69.8 % continued the treatment until week 24, 27.8 % discontinued the treatment before week 24, and 2.3 % were lost to follow-up. Mean ($\pm$SD) daily doses of TAC at week 0 were 1.6 $\pm$ 0.8 mg/day in nonelderly patients (<65 years) and 1.4 $\pm$ 0.6 mg/day in elderly patients ($\geq$65 years). Mean ($\pm$SD) daily doses of TAC during the observation period were 1.9 $\pm$ 0.8 mg/day in nonelderly patients (<65 years) and 1.6 $\pm$ 0.6 mg/day in elderly patients ($\geq$65 years).

Reasons for discontinuation were AEs in 14.3 %, lack of effectiveness in 7.2 %, patient's preference in 5.7 %, and improvement of signs and symptoms in 0.1 % (Table 2).

Of the 3,172 patients included in the safety population, 1,308 patients developed 2,292 AEs and 1,142 patients developed 1,855 ADRs; the incidences of AEs and ADRs were 41.2 and 36.0 %, respectively. The common system organ classification (SOC) categories for ADRs were abnormal laboratory values in 12.5 %, gastrointestinal disorders in 6.4 %, infections and infestations in 5.8 %, metabolism and nutrition disorders in 4.3 %, and renal and urinary disorders in 2.7 % (Table 3). The most frequently reported ADRs were pneumonia (1.0 %), diabetes mellitus (1.5 %), nausea (1.5 %), diarrhea (1.3 %), abnormal hepatic function (1.1 %), pruritus (1.0 %), renal impairment (1.2 %), elevation of white blood cell count (2.5 %), elevation of β-*N*-acetyl-D-glucosaminidase (2.1 %), elevation of blood urea (1.6 %), elevation of glycosylated hemoglobin (1.2 %), depletion of lymphocyte (1.2 %), elevation of blood creatinine (1.1 %), and elevation of urine β2 microglobulin (1.0 %). The overall incidence of ADRs was significantly higher in elderly patients compared to non-elderly patients (40.5 vs. 31.9 %, $p < 0.001$). The incidences of the following ADRs were higher in elderly than in nonelderly patients: abnormal laboratory values (14.5 vs. 10.7 %, $p = 0.001$), gastrointestinal disorders (7.4 vs. 5.5 %, $p = 0.035$), infections and infestations (6.9 vs. 4.9 %, $p = 0.015$), metabolism and nutrition disorders (5.8 vs. 2.9 %, $p < 0.001$), and renal and urinary disorders (3.8 vs. 1.6 %, $p < 0.001$) (Table 3).

Of the patients included in the safety population, 203 patients (6.4 %) developed 263 serious AEs and 157

Table 1 Patient characteristics of the safety population

Items All patients	Patients (%) 3,172
Sex	
Male	682 (21.5)
Female	2,490 (78.5)
Age (years)	
<20	9 (0.3)
20–29	40 (1.3)
30–39	103 (3.2)
40–49	258 (8.1)
50–64	1,256 (39.6)
65–74	1,088 (34.3)
≥75	418 (13.2)
Mean ± SD	62.2 ± 12.0
Inpatient/outpatient status	
Outpatient	2,843 (89.6)
Inpatient	329 (10.4)
Steinbrocker's stage classification (n = 3,134)	
I	256 (8.2)
II	873 (27.9)
III	1,010 (32.2)
IV	995 (31.7)
Steinbrocker's functional classification (n = 3,139)	
1	281 (9.0)
2	2,043 (65.1)
3	745 (23.7)
4	70 (2.2)
Disease duration (years) (n = 2,845)	
<3	576 (20.2)
≥3 to <5	339 (11.9)
≥5 to <10	666 (23.4)
≥10	1,264 (44.4)
Mean ± SD	11.06 ± 9.7
Comorbidity (n = 3163)	
No	573 (18.1)
Yes	2,590 (81.7)
Use of nonbiological DMARDs just before study entry*	
No	660 (20.8)
Yes	2,512 (79.2)
Use of nonbiological DMARDs just before study entry**	
Methotrexate	1,353 (42.7)
Salazosulfapyridine	809 (25.5)
Bucillamine	477 (15.0)
Use of biological DMARDs just before study entry*	
No	2,864 (90.3)
Yes	308 (9.7)
Use of biological DMARDs just before study entry**	
Etanercept	174 (5.5)
Infliximab	129 (4.1)
CRP (mg/dl) (n = 2,374)	
<1.0	545 (23.0)
≥1.0 to <3.0	775 (32.6)
≥3.0 to <5.0	519 (21.9)
≥5.0 to <10.0	443 (18.7)
≥10.0	92 (3.9)
Mean ± SD	3.3 ± 3.0
DAS28-CRP (n = 680)	
≤3.2	13 (1.9)
>3.2 to ≤5.1	336 (49.4)
>5.1	331 (48.7)
Mean ± SD	5.1 ± 1.0
Concomitant corticosteroid***	
No	588 (18.5)
Yes	2,584 (81.5)
Dose of concomitant corticosteroid*** (prednisolone equivalent, mg/day) (n = 3,169)	
0 (non use)	588 (18.6)
0< to <5	621 (19.6)
≥5 to <7.5	1,077 (34.0)
≥7.5 to <10	365 (11.5)
≥10	518 (16.3)
Mean ± SD	6.8 ± 4.7
Concomitant nonbiological DMARD***	
No	1,665 (52.5)
Yes	1,507 (47.5)
Concomitant nonbiological DMARD**	
Methotrexate	916 (28.9)
Salazosulfapyridine	454 (14.3)
Bucillamine	236 (7.4)
Concomitant biological DMARD***	
No	2,947 (92.9)
Yes	225 (7.1)
Concomitant biological DMARD**	
Etanercept	140 (4.4)
Infliximab	94 (3.0)
Concomitant NSAID*** (n = 3,162)	
No	950 (30.0)
Yes	2,212 (70.0)

CRP C-reactive protein, *DAS28-CRP* disease activity score 28-CRP, *DMARD* disease modifying antirheumatic drug, *NSAID* non-steroidal anti-inflammatory drug

* Within 4 weeks of the start of treatment with TAC (For IFX, within 8 weeks); ** multiple response; *** drugs used before the date of onset of the first adverse drug reaction were included. In patients who did not develop adverse drug reactions, drugs which were used during the observation period were included