

Serum checkpoint molecules in patients with IgG4-related disease (IgG4-RD)

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Abstract

Background

Immunoglobulin G4-related disease (IgG4-RD) is characterized by increased serum IgG4 concentration and infiltration of IgG4⁺ plasma cells in the affected organs. The present study aimed to characterize the serum levels of coinhibitory checkpoint molecule, T cell immunoglobulin and mucin-containing-molecule-3 (TIM-3), and its ligand, galectin-9 (Gal-9), among IgG4-related disease in patients with IgG4-RD patients with various organ involvements.

Methods

Serum samples were collected from untreated 59 patients with IgG4-RD, 116 patients with rheumatoid arthritis, and 37 healthy controls. Patients with IgG4-RD (n = 57) were subdivided into those with visceral involvement (n = 38) and those without visceral involvement (n = 21). Serum levels of Gal-9 and soluble TIM-3 (sTIM-3) were determined using enzyme-linked immunosorbent assay (ELISA). Results were compared with the clinical phenotypes of IgG4-RD.

Results

In untreated patients with IgG4-RD, serum levels of Gal-9 and sTIM-3 were significantly higher than in RA patients as well as in healthy controls. There were significant correlations between serum levels of Gal-9 or sTIM-3 and serum levels of IgG, BAFF, or sIL-2R. However, there was no significant correlation between the serum levels of Gal-9 or sTIM-3 and serum IgG4 concentrations. Serum levels of sTIM-3 were significantly higher in a subset of patients with visceral involvements than in those without visceral involvements. However, there was no significant difference in the serum levels of Gal-9 between IgG4-RD patients with and without visceral involvements. Although both, Gal-9 and sTIM-3 were elevated in untreated IgG4-RD patients, and the levels of these checkpoint molecules remained unchanged after steroid therapy.

Conclusion

Serum levels of Ga-9 and sTIM-3 were significantly elevated in untreated patients with IgG-RD. Furthermore, serum levels of sTIM-3 were significantly higher in IgG4-RD patients with visceral involvements. These checkpoint molecules could be a potentially useful biomarker for IgG4-RD and for assessing the clinical phenotypes of IgG4-RD.

Introduction

Immunoglobulin G4-related disease (IgG4-RD) is characterized by multi-organ involvement and elevated serum IgG4 levels (1). The most affected organs in this disease are the salivary or lacrimal glands, lymph node, pancreas, biliary tract, lung, kidney, retroperitoneum, and aorta (2, 3). In histology, a high ratio of IgG4-positive plasma cell infiltration and fibrosis in the affected organs is a major finding in IgG4-related disease (4). However, it is unclear whether the elevated production of IgG4 is the cause of IgG4-RD or epiphenomenon. Previous studies have shown a prominent expansion of circulating plasmablasts and tightly restricted repertoire of plasmablasts, indicating that IgG4-RD is an antigen-driven disease (5, 6). With regard to T cell function, type 2 helper T cell (Th2)-dominant immune responses and the production of Th2 cytokines (IL-4, IL-5, IL-13 and IL-10) and an increase in the number of regulatory T cells (Treg) were demonstrated in IgG4-RD (7). However, it is unknown as to which antigens drive the clonal expansion of plasma cells and T cells.

Recently, two independent study groups identified galectin-3 (Gal-3) as an autoantigen in IgG4-RD (8, 9). Gal-3 is overexpressed in various organs of IgG4-RD patients and plasmablast clones isolated from IgG4-RD patients produced anti-Gal-3 antibody (8). In addition, Gal-3 is suggested to contribute to fibroproliferative disorders, such as pulmonary fibrosis, and Gal-3 inhibition was shown to develop pulmonary fibrosis in model mice (10). Galectins, a protein of the conserved lectin protein family, are important regulators of immune homeostasis. Galectins are also recognized as checkpoint molecules that regulate T cell development by the regulation of thymocyte apoptosis and can be potential regulators of bone marrow B cell development (11). Thus far, the role of checkpoint molecules has been identified in the regulation of several immune-mediated processes in autoimmune diseases. A co-inhibitory checkpoint molecule, Galectin-9 (Gal-9), plays critical role in T-cell and B-cell regulation, wherein the interaction between T cell immunoglobulin and mucin-containing-molecule-3 (TIM-3) and its ligand, Gal-9, is involved in the regulation of immune responses and autoimmunity (12, 13). We previously reported the association between Gal-9/TIM3 pathway and rheumatic disease such as rheumatoid arthritis (RA), systemic lupus erythematosus, and adult-onset still's disease (14–17). From the point of view of immune checkpoint molecules, IgG4-related pleural disease were occurred as immune-related adverse event of programmed cell death-ligand 1 inhibitor (18). The relation between Gal-9/TIM-3 pathway and IgG4-RD is not clear, but various immune checkpoint molecules may be involved in the pathogenesis of IgG4-RD. Furthermore, it has been shown that T follicular helper (Tfh) cells are important for germinal center formation and IgG4 class-switching and can be implicated in the immune-pathophysiology of IgG4-RD (19). Considering that the checkpoint molecules expressed on Tfh cells are involved in B cell selection and class-switching (20), we hypothesized that the dysregulated these checkpoint molecules participate in the pathogenesis of IgG4-RD. In the present study, we evaluated the circulating co-inhibitory molecules, Gal-9 and soluble TIM-3 (sTIM-3), in patients with IgG4-RD.

Methods

Patients and study design

A total of 59 patients with IgG4-RD were included in this study. All IgG4-RD patients diagnosed at the department of Rheumatology of Fukushima Medical University and the department of Immunology and Rheumatology of Nagasaki University between February 2007 and November 2020. The clinico-demographic data were retrospectively collected from Medical Records. In those 59 patients, post-treatment data were collected from patients. As controls, 37 healthy controls (HCs) were included. An additional independent set of 116 patients with patients with rheumatoid arthritis (RA) was included to compare the value of Gal-9 and sTIM-3. Among 116 patients of RA, 83 (71.6%) were female, their median age was 66 years, [IQR] 56-73. The majority of RA patient were treated with disease-modifying anti-rheumatic drugs, mostly methotrexate (59/116, 50.9%), and biologics (38/116, 32.8%). Median DAS28-ESR was 2.8 (2.0-3.7).

This study was conducted in accordance with the principles of the Declaration of Helsinki. Ethical approval for this study (no.29317) was provided by the Ethics Committee of Fukushima Medical University.

Examination of biochemical markers and imaging

Laboratory study included white blood cell count (WBC), hemoglobin (Hb), platelets (PLT), serum Immunoglobulin G (IgG), serum immunoglobulin G4 (IgG4), the ratio of IgG/IgG4, C-reactive protein (CRP), compliment 3 (C3), compliment 4 (C4), soluble interleukin 2 receptor (sIL-2R), and lactate dehydrogenase (LDH). All patients underwent contrast-enhanced computed tomography (CT) or plain CT scan and some patients evaluate ultrasound, radiography, magnetic resonance imaging and positron emission CT.

Classification of IgG4-RD

We diagnosed IgG4-RD based on the 2011 comprehension criteria for IgG4-RD or organ-specific diagnostic criteria for IgG4-RD. The comprehensive diagnostic criteria 2011 include: 1) clinical examination showing characteristic diffuse/localized swelling or masses in single or multiple organs, 2) hematological examination showing elevated serum IgG4 concentration (≥ 135 mg/dL), and 3) histopathologic examination showing i) marked lymphocyte and plasmacyte infiltration and fibrosis and ii) infiltration of IgG4+ plasma cells with a ratio of IgG4+/IgG+ cells $>40\%$ and >10 IgG4+ plasma cells/HPF (21). Organ involvements were determined by patient`s history, physical examination, laboratory study, imaging results and tissue biopsies.

Furthermore, IgG4-RD patients were divided into two groups depending on the presence or absence of visceral involvement. Visceral involvement defined the complication of lung pancreas, bile duct, kidney, retroperitoneal fibrosis, aorta and prostate.

ELISA methods

Serum concentration of Gal-9 and sTIM-3 were measured using enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer`s instruction.

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Statistical analysis

Results were non-normally distributed and are presented throughout the manuscript with median and 25-75th centiles [median, IQR] and were compared by the Mann-Whitney *U* test. The comparisons between categorical variables were analyzed by Fisher's exact test. *C* or *relations between continuous variables* were analyzed by Spearman's rank correlation test. Paired data were analyzed by non-parametric tests using the Wilcoxon signed-rank test. Kruskal–Wallis test was used for continuous variables for comparisons among the three groups. Post hoc pairwise analyses between two groups were performed by Games-Howell test. The prognostic factors for higher levels of Gal-9 and sTIM-3 were identified using a stepwise multiple logistic regression model. All data entry and statistical analyses were performed using SPSS Statistics version 22.0 (IBM, Armonk, NY). In all the analyses, a two-tailed $p < 0.05$ was considered statistically significant.

Results

Characteristics of IgG4-RD patients

The demographic, clinical, and laboratory characteristics of the enrolled IgG4-RD patients have been summarized in Table 1. The average age of IgG4-RD patients was 55 years (41–62 years), and the male patients was 47 (76.2%). In IgG4-RD patients, 34 patients (58%) was diagnosed as definite diagnosis of IgG4-RD, 3 (5%) as probable diagnosis, and 22 (37%) as possible diagnosis. Most patients had multiple organ involvement. Serum IgG4 levels were elevated in IgG4-RD patients [median 341 (IQR) 114–349 mg/dL]. Furthermore, the ratio of serum IgG4/IgG was also increased in IgG4-RD patients (median 31.4%).

Elevated serum levels of checkpoint molecules in IgG4-RD

In order to evaluate possible role in checkpoint molecules in IgG4-RD. we compared the serum levels of Gal-9. As shown in Fig. 1, serum levels of Gal-9 were significantly higher in IgG4-RD compared to those in RA patients as well as those in HCs. Similarly, serum levels of sTIM-3 were significantly higher in IgG4-RD compared to those in HCs.

Correlations among the biomarkers

We performed the correlation analysis among several laboratory markers, including checkpoint molecules in IgG4-RD patients. The patient's serum Gal-9 and sTIM-3 levels were positively correlated with some clinical parameters of IgG4-RD patients (Table 2). Serum levels of Gal-9 or sTIM-3 were positively correlated with the serum levels of BAFF, SIL-2R, and CRP. sTIM-3 were negatively correlated with complement 3, and complement 4 (Fig. 2). In addition, the serum levels of Gal-9 or sTIM-3 were positively correlated with serum IgG level; however, there was no significant correlation between the serum levels of these checkpoint molecules and those of IgG4 or the ratio of serum IgG4/IgG (Fig. 3).

Associations of the serum levels of Gal-9 or sTIM-3 with organ involvement

In order to determine whether these checkpoint molecules can be used to differentiate among the IgG4-RD phenotypes, we examined the associations between serum levels of Gal-9 or sTIM-3 and organ involvements in IgG4-RD patients. All patients with IgG4-RD were subdivided as per the presence of visceral involvements; further, the serum levels of Gal-9 or sTIM-3 were compared between the groups (Fig. 4). There was no significant difference in the serum levels of Gal-9 between IgG4-RD patients with and without the involvement of visceral organs. However, the serum levels of sTIM-3 were significantly higher in patients with visceral involvement than in those without the involvement of visceral organs. We attempted to identify the clinical parameters associations with a high sTIM-3 levels (higher than the first quartile of the circulating sTIM-3 level of IgG4-RD; 3656 pg/mL) by performing a multivariate logistic regression analysis (Table 3). The presence of biliary tract, (OR: 12.29, 95% confidence interval [95%CI], 1.83–109, $p = 0.004$), kidney (OR: 18.59, 95%CI 2.4–143, $p = 0.022$), and retroperitoneum (OR: 13.14, 95%CI 2.3–75.2, $p = 0.027$) involvement were independently associated with high sTIM-3 levels in patients with IgG4-RD.

Longitudinal changes in the serum levels of Gal-9 or sTIM-3 after steroid treatment

The changes in the serum levels of checkpoint molecules after the induction of steroid treatment in 7 patients are shown in Fig. 5. The median range dose of prednisolone was 35 (25–40) mg/day and the median duration of steroid treatment was 12 (8.5–49) months. Gal-9 as well as IgG4-RD responder index were reduced in all patients. Although the serum sTIM-3 and serum IgG4 levels were tended to decline in many cases, there were no significant differences due to part to a small number of cases.

Discussion

Galectins, a protein from a family of lectins with affinity for β -galactoside-containing oligosaccharides, are expressed by the immune cells (22). Recent studies have shown that galectins play crucial regulatory roles in inflammation and autoimmunity (23). In this study, we evaluated the serum levels of sTIM-3 and its ligand molecule, Gal-9, in IgG4-RD patients. Although circulating Gal-9 or sTIM-3 were not correlated with the serum IgG4 levels or the ratio of IgG4/IgG, our results indicated that the serum levels of Gal-9 are significantly elevated in patients with IgG4-RD as compared to those in HCs and RA patients. Furthermore, the serum levels of sTIM-3 are significantly elevated in patients with IgG4-RD as compared to those in HCs. These findings indicate that these checkpoint molecules could be involved in the pathophysiology of IgG4-RD.

Previous studies have shown the upregulations of Th2 cytokines (interleukin (IL)-4, IL-5, IL-13 and IL-21) and the regulatory T cell-mediated cytokines (IL-10 and transforming growth factor- β) in IgG4-RD patients (7). The elevated levels of Th2 cytokines are produced by pathogenic Th2 cells or a combination of Th2 cells and

regulatory T cells (Treg cells), and these T cell subsets may activate macrophages and fibroblasts that cause inflammatory and fibrotic processes in the affected organs (24). We hypothesized that Th1/Th2 imbalance in IgG4-RD is associated with the dysregulation of checkpoint molecules and analyzed their associations with the clinical phenotypes of IgG4-RD. In contrast to patients with organ involvement limited to the lacrimal or salivary glands, patients with visceral involvements presented with higher levels of serum sTIM-3. Furthermore, we observe an organ-specific increment in sTIM-3, particularly biliary, kidney, or retroperitoneum involvement in IgG4-RD. Our data indicated that elevated levels of sTIM-3 in patients with IgG4-RD could be related to the clinical phenotypes of IgG4-RD, including its patterns of organ involvements.

TIM-3 acts as a co-inhibitory receptor that is expressed on exhausted T cells. TIM-3 was initially thought to be expressed only by T cells; it has now been proven that TIM-3 is expressed by multiple cell types, including DCs, macrophages, and Tregs (25), indicating that TIM-3 also functions as an inhibitory receptor in these cells (26). Gal-9 has been identified as a ligand for TIM-3 (27); however, its putative ligands other than Gal-9 and its inhibitory effect on T cell remain unclear. The precise cellular interaction within sTIM-3 and its ligands remains unclear; however, it is possible that a combination of sTIM-3 with its ligands competitively reduces the inhibitory effect on the pathway following TIM-3 on immune cells (28), resulting in Th1/Th2 cell imbalance in IgG4-RD (29). The association between th2 immune response and M2 macrophage play an important role in the pathogenesis of IgG4-RD (30). Furthermore, infiltration of M2 macrophage to affected organ in IgG4-RD may promote fibrosis (31). Because Gal-9/TIM-3 signaling pathway inhibit M1 macrophage polarization by short-term lipopolysaccharide stimulation, M2 macrophage is relatively dominant (32). Our result showed sTIM-3 was increased in IgG4-RD patients. However, the number of TIM-3 on macrophage was not evaluated. The expression of TIM-3 on macrophage may be upregulated in IgG4-RD patients. Gal-9/TIM-3 signaling pathway may cause fibrosis in affected organs by promoting the differentiation of M2 macrophages.

Recent studies have identified several new immune checkpoint targets, such as lymphocyte activation gene-3 or TIM-3. The investigations on these molecules have generated new findings in the pathophysiology of immune-mediated disorders (33). Tfh is critical regulator of immune responses in inflammatory disorders (34–36). Checkpoint inhibitors are known to induce B-cell activation and class-switched IgG production; these processes are dependent on the Tfh cell function (20). Furthermore, the case of an IgG4-RD patient with lung cancer receiving checkpoint inhibitors has been reported (18). These findings suggest that the interaction between B cells and Tfh cells through these checkpoint molecules could be involved in IgG4 class switching (18). Tfh could be subdivided to distinctive functional subsets as per the checkpoint molecules expressions. programmed cell death 1 demarcated potent Tfh subsets (37), and TIM-3 appears to be associated with reduced Tfh function (38), suggesting that the co-inhibitory Gal-9/TIM-3 pathway can limit the functions of Tfh (39). Potent Tfh might play a crucial role in IgG4 class switching (40); therefore, elevated levels of sTIM-3 may affect the Tfh functions by interfering with the Gal-9/TIM-3 co-inhibitory checkpoint systems. These co-inhibitory checkpoint pathways may alter the interaction between Tfh cells and plasmablasts (41) in the immunopathology of IgG4-RD and its

expanding organ involvement. Given the multi-organ nature of IgG4-RD, the predictors for particular organ involvements would be valuable for clinical application and research of IgG4-RD.

Although the serum concentrations of sTIM-3 were associated with visceral involvements of IgG4-RD, the levels were not modulated by steroid treatment. Steroid is effective for most patients with IgG4-RD (2), however, the relapses of IgG4-RD were frequently observed during the steroid tapering periods (2). Steroid is considered to be first-line treatment for remission induction in IgG4-RD (42), however, it is possible that the incomplete regulation of immunopathology of IgG4-RD could be linked to this increased serum levels of immuno-checkpoint molecules even after the steroid treatment.

Additional studies are required to clarify the roles of circulating checkpoint molecules in the pathophysiology of IgG4-RD, and their relationship to the clinical phenotypes. Furthermore, it is necessary to elucidate whether more aggressive treatment such as B cell-depletion treatment may affect the upregulated immune-checkpoint molecules in IgG4-RD.

There are certain limitations of this study. The sample size was relatively small, and larger scale studies are necessary to confirm the present finding. Checkpoint molecules profiles in other potential controls, such as those with Sjögren's syndrome or lymphoproliferative disorders, were not compared with the profiles in IgG4-RD patients in the present study. Because of inadequate number of untreated RA patients, more than half of the control patients of RA was already treated. This study was a cross-sectional analysis for untreated IgG4-RD patients. Therefore, clinical manifestations that occurred during the disease course were not completely surveyed. We enrolled only Japanese patients with IgG4-RD; therefore, non-Japanese patients with IgG4 were not included, and further studies on non-Japanese patients with IgG4-RD are warranted. The mechanism through which the Gal-9/TIM-3 pathway contributes to the pathogenesis of IgG4 was not clarified because the functional analysis by in vitro cannot be performed.

In conclusion, we demonstrated that serum sTIM-3 and Gal-9 levels were elevated in IgG4-RD patients. With respect to the relationship with the clinical phenotypes, the serum sTIM-3 levels were significantly higher in patients with visceral involvements than in those with disease limited to the lacrimal or salivary glands. These data suggest that the circulating checkpoint molecules are involved in the pathophysiology of IgG4-RD and their relationship to its patterns of organ involvement.

Abbreviations

IgG4-RD=Immunoglobulin G4-related disease

Treg=regulatory T cell

Gal-9=Galectin-9

TIM-3= T cell immunoglobulin and mucin-containing-molecule-3

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sTIM-3=soluble T cell immunoglobulin and mucin-containing-molecule-3

RA=rheumatoid arthritis

IL=Interleukin

Tfh=follicular T cell

Th1=Type 1 helper T cell

Th2=Type 2 helper T cell

Declarations

Ethical Approval and Consent to participate

Ethical approval for this study (No. 29317) was provided by the Ethics Committee of Fukushima Medical University.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

KM has received research grants from Chugai, Pfizer, and AbbVie. Rest of the authors declares that they have no competing interests.

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Authors' contributions

HM, YF, NM, JT, MYF, TA, SS, HW and ES were involved in the acquisition of clinical data. HM, YF and KM drafted the manuscript and carried out the molecular biochemical studies. ST, SF, MU, AK and KM participated in the sequence alignment and drafted manuscript. NI, AK and KM participated in the design of the study. HM and YF performed the statistical analysis. The authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1, 2, 3 is only available as a download in the Supplemental Files section.

Figures

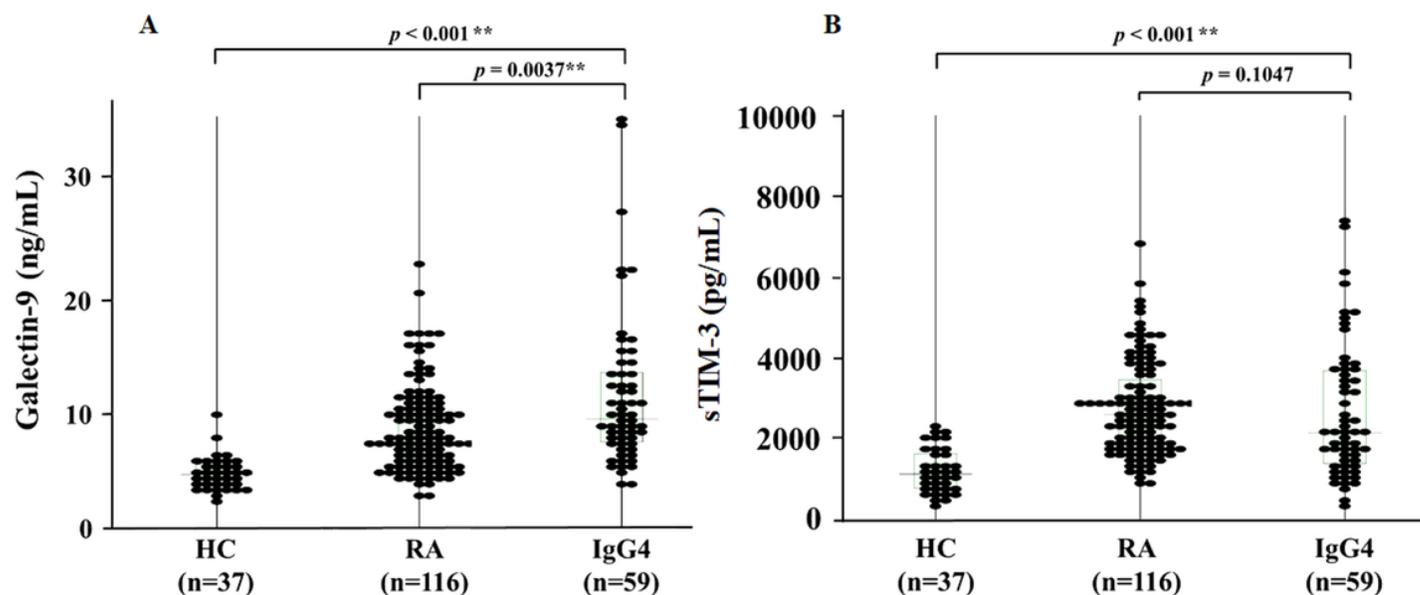


Figure 1

Serum levels of Gal-9 and sTIM-3 in IgG4-RD The comparison of serum levels of Gal-9 and sTIM-3 among IgG4-RD patients (n=59), RA patients (n=116) and healthy controls (n=37). (A) Serum levels of Gal-9 in IgG4-RD were significantly higher compared to those in health controls and RA patients. (B) Serum levels of sTIM-3 in IgG4-RD patients and RA patients were significantly higher compared to HCs. Kruskal–Wallis test was used for continuous variables for comparisons among the three groups. Post hoc pairwise analyses between two groups were performed by Games-Howell test.

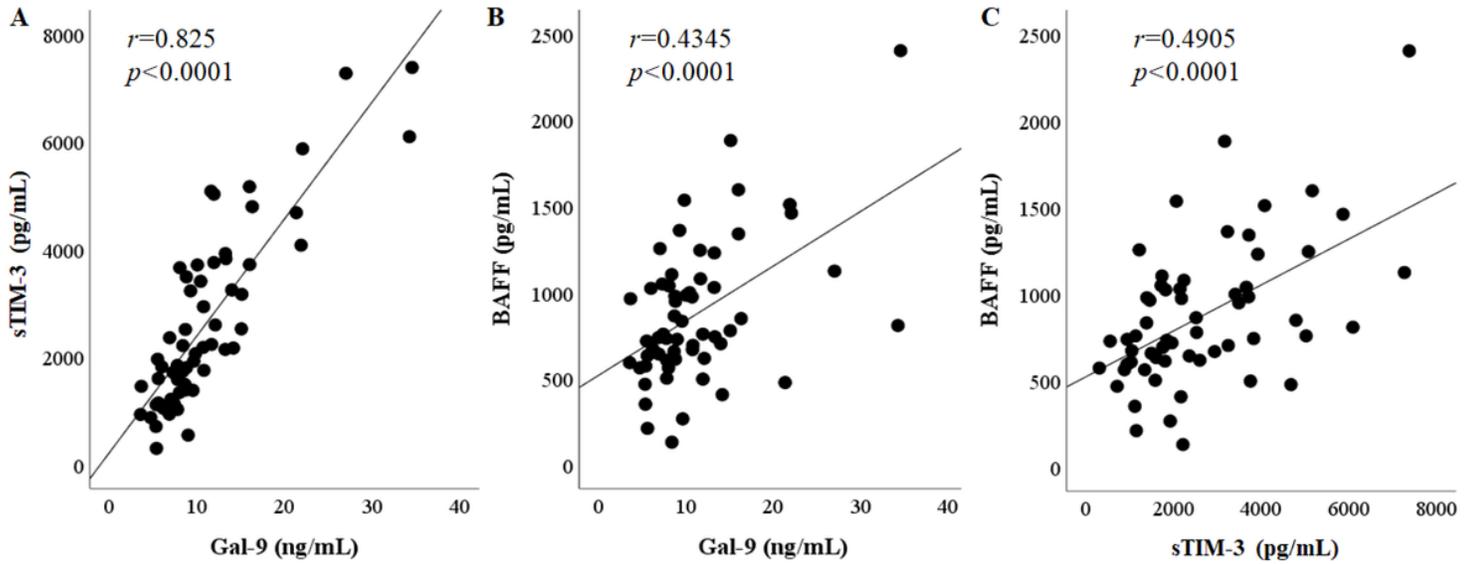


Figure 2

Relationships among the serum sTIM-3, Gal-9 and BAFF in patients with IgG4-RD. (A) Serum Gal-9 was positively correlated with serum sTIM-3. (B-C) The serum levels of BAFF was positive correlation with serum levels of sTIM-3 and Gal-9. All correlation were determined using Spearman's rank correlation test.

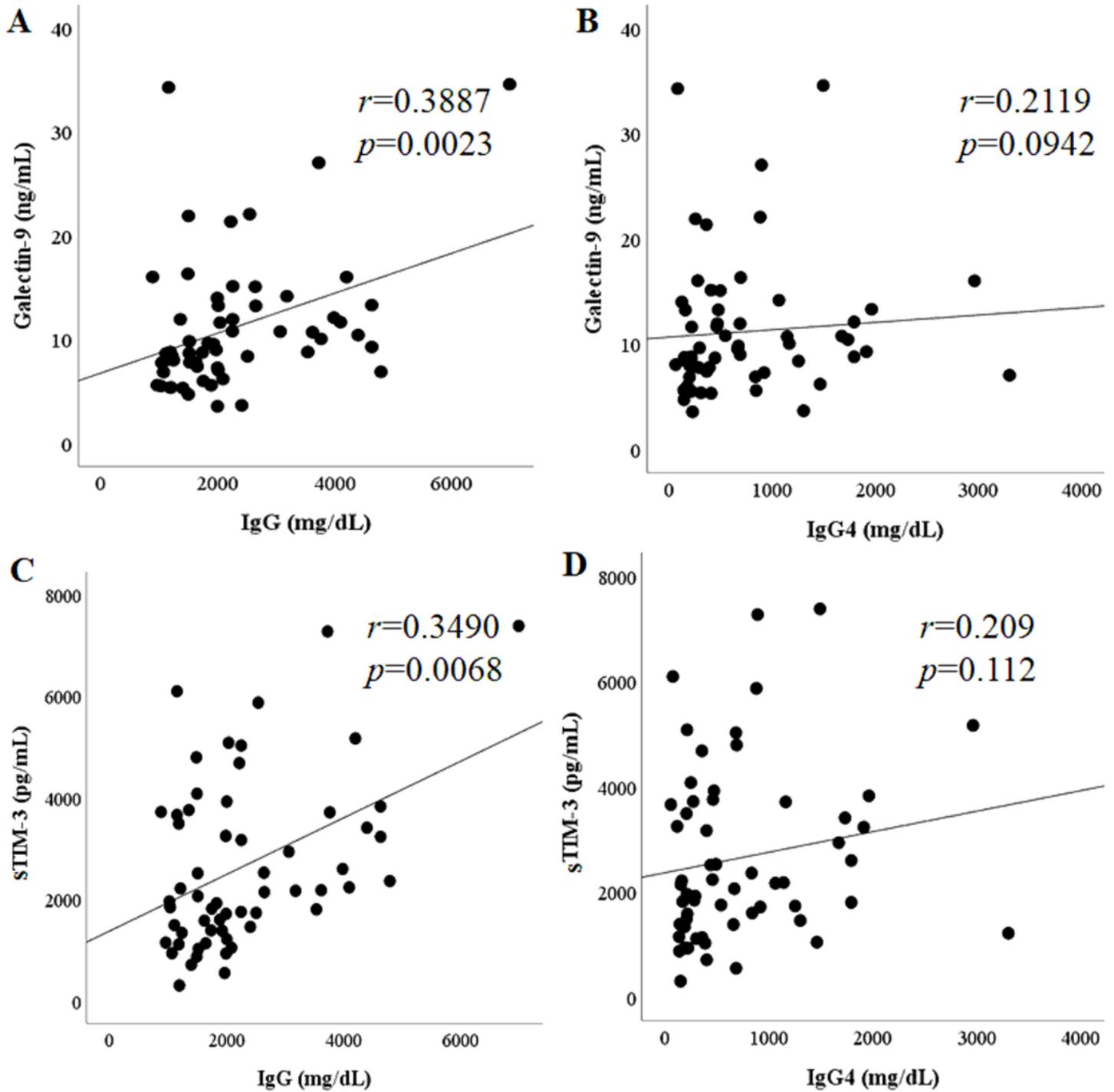


Figure 3

Relationships between serum levels of checkpoint molecules and IgG subclass in IgG4- RD (A-B) The serum levels of Gal-9 showed a positive correlation with IgG whereas not significant correlation with IgG4. (C-D) The serum levels of sTIM-3 showed positive correlation with IgG whereas not significant correlation with IgG4. All correlation were determined using Spearman's rank correlation test.

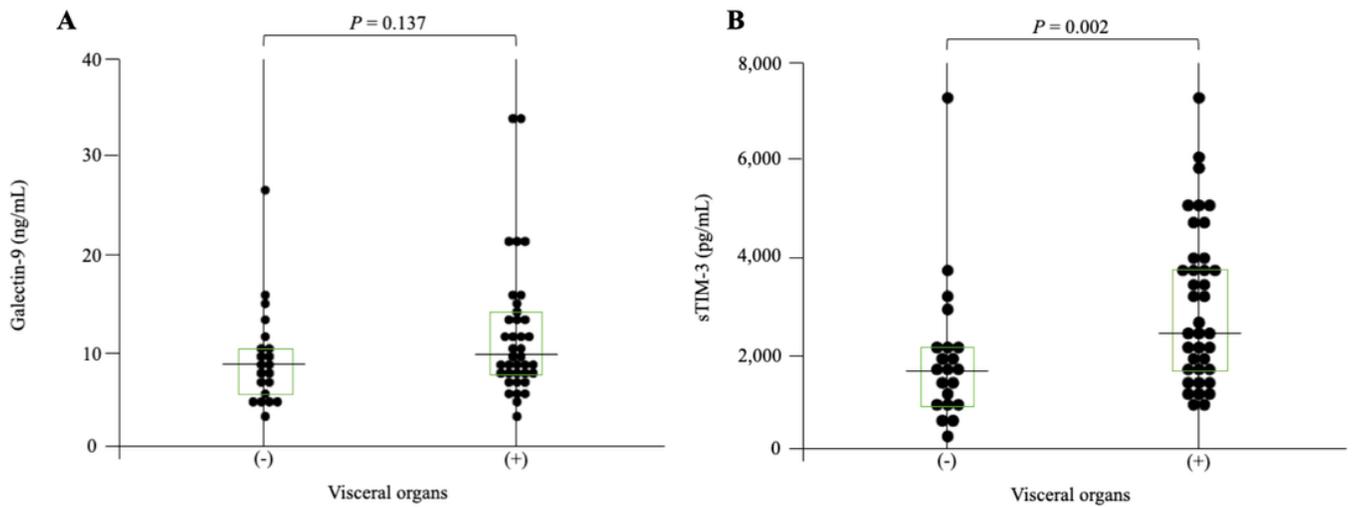


Figure 4

Serum levels of Gal-9 and sTIM-3 with or without visceral organ involvement in IgG4-RD (A) Serum levels of Gal-9 were not significant difference in the presence of visceral organ involvement. (B) Serum levels of sTIM-3 were significantly higher in IgG4-RD patients with visceral organ lesions compared to those without visceral organ lesions. Statistical significance was determined by Mann-Whitney U test.

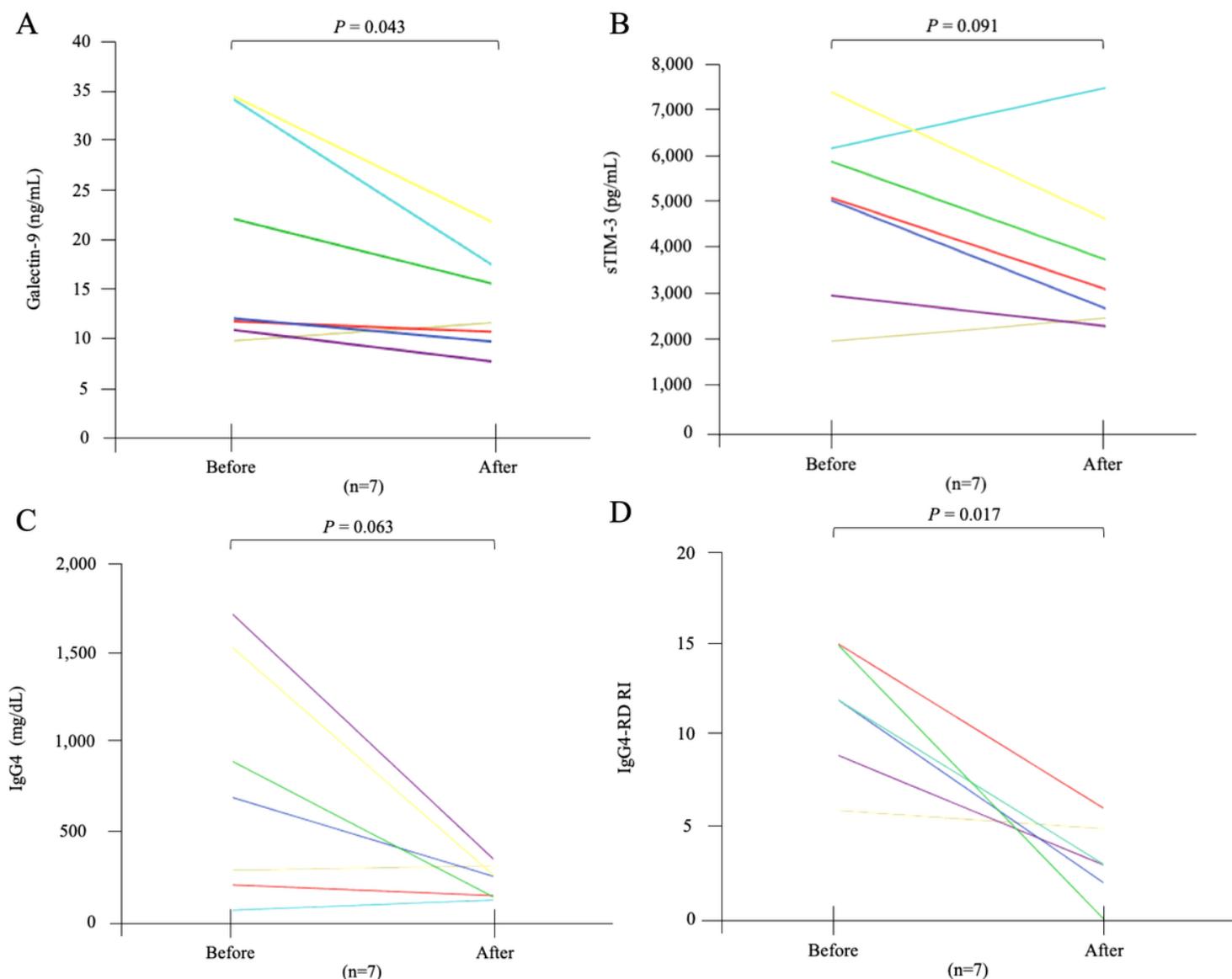


Figure 5

Longitudinal changes of serum Gal-9 or sTIM-3 concentrations in 7 patients with IgG4-RD before and after glucocorticoid therapy. (A-C) Gal-9, sTIM-3 and IgG4 concentrations in IgG4-RD patients was no significant differences between before and after treatment. (D) IgG4-responder index was significantly decreased after glucocorticoid therapy. Paired samples from the same subjects were compared by Wilcoxon signed-rank test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [OnlineTable1.png](#)
- [OnlineTable2.png](#)

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- [OnlineTable3.png](#)