Table 7 Sensitivities and specificities of the three tested systems for diagnosing SS

	Entire group		Without other CTDs		With other CTDs	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
JPN	79.6	90.4	82.1	90.9	75.3	85.7
AECG	78.6	90.4	83.1	90.9	70.7	85.7
ACR	77.5	83.5	79.1	84.8	74.7	71.4

The "entire group" comprised 694 patients, including 476 with SS (302 patients with primary SS and 174 with secondary SS) and 218 patients with non-SS. The "without other CTDs" group of 499 patients included 302 patients with primary SS and 197 with non-SS. The "with other CTDs" group of 195 patients included 174 patients with secondary SS and 21 with non-SS

JPN Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* The American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

diagnostic systems, as assessed using the kappa coefficient. The data indicate a high level of agreement between the JPN and ACR diagnostic systems (kappa coefficient 0.74), but a low level of agreement between AECG and the other two (kappa coefficient 0.10–0.46) in the diagnosis of all SS, primary SS, and secondary SS.

Discussion

While it is difficult to select the best gold standard system for the diagnosis of CTDs such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and SS, this issue is clinically relevant and important. In SLE, the ACR revised criteria for the classification of SLE (1997) [4] has been adopted for diagnosis in daily clinical practice and for classification purposes in clinical studies. Recently, the Systemic Lupus International Collaborating Clinics (SLICC) has proposed new classification criteria for SLE [5], which has generated interesting discussion about these two criteria among expert rheumatologists. On the other hand, for RA, the 2010 RA classification criteria: an ACR/European League Against Rheumatism (EULAR) collaborative initiative [6] was published recently and is currently used not only in clinical studies for the classification of RA but also in daily clinical practice for the diagnosis of RA. Therefore, these available diagnostic systems for SLE and RA could be regarded as the gold standard for both clinical studies and daily clinical practice. The AECG criteria have been adopted in Western countries for the diagnosis of SS. In Japan, however, both the AECG and JPN criteria are currently being used simultaneously for the classification and diagnosis of SS. On the other hand, the new ACR criteria have been proposed as a uniform classification for SS. At present, there is no gold standard system for the diagnosis of SS in both clinical studies and daily clinical practice, except for expert judgment. This state could create a heterogeneous pool of SS patients, which makes it difficult to analyze the diagnosis, efficacy of treatment, and prognosis of SS patients. Establishing a single set of criteria for SS and selecting a gold standard system for the diagnosis of SS is an important task in Japan.

The present study demonstrated that the sensitivity of the JPN system for all SS and secondary SS, the sensitivity of the AECG system for primary SS, and the specificities of the JPN and AECG systems for all SS, primary SS, and secondary SS were highest among the three systems for diagnosing SS in Japanese patients (relative to clinical judgment as the gold standard). The results also showed high agreement between the JPN and ACR systems, but low agreement between AECG and the other two diagnostic systems for all SS, primary SS, and secondary SS. These results indicate that the JPN and ACR criteria covered similar patient populations, although the sensitivity and specificity were higher for the JPN system than the ACR system. Among the 302 patients with primary SS, 14 did not satisfy the ACR criteria for the diagnosis of SS, although they did meet the criteria of both JPN and AECG. Further analysis of these 14 SS patients also showed that 50 % of these patients had negative pathological findings, 70 % had negative ocular staining, and 50 % were negative for autoantibodies (data not shown). These SS patients could be misdiagnosed by the ACR criteria, resulting in the lower sensitivity of the ACR diagnostic system. On the other hand, among 197 non-SS patients without other CTDs, ten patients satisfied the ACR criteria but not the JPN nor the AECG criteria (data not shown). Further analysis of these ten patients indicated that 80 % were positive for lissamine green ocular staining (Schirmer's test, rose bengal staining, and fluorescein staining were not performed), and 60 % were positive for anti-SS-A antibody (data not shown). Although these patients might be misdiagnosed as primary SS by the ACR criteria, this could not be confirmed because these patients could be positive for other ocular tests adopted by the JPN and AECG diagnostic systems.

The specificities of the criteria for all SS, primary SS, and secondary SS patients used in the JPN and AECG

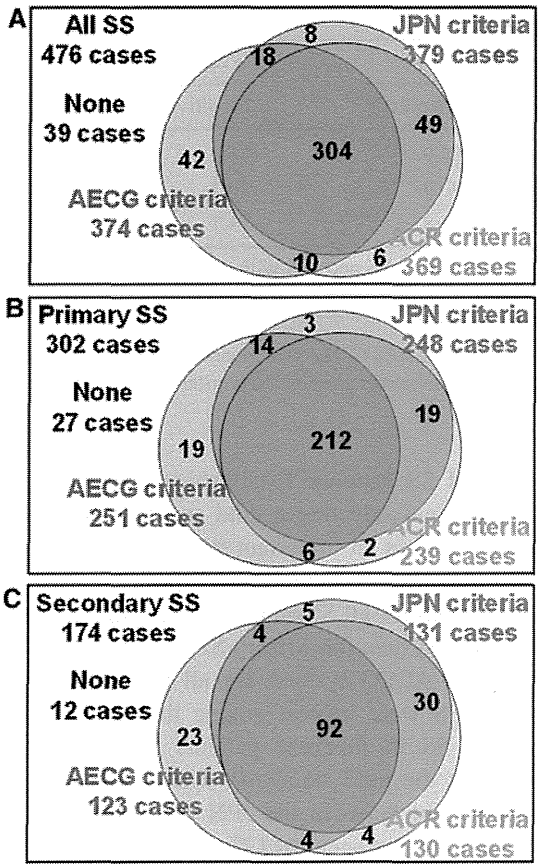

Fig. 1 Venn diagrams showing a comparison of the satisfaction of the three tested systems. **a** Comparison of the satisfaction of the three tested systems, performed using data from all 476 SS patients (302 primary SS and 174 secondary SS). **b** Comparison of the satisfaction of the three tested systems using data on 302 patients with primary SS. **c** Comparison of the satisfaction of the three tested systems using data on 174 patients with secondary SS. *Numbers* show the numbers of patients who satisfied each set of criteria, *None* indicates the number of patients who did not satisfy the criteria of any of the three systems. *JPN criteria* the revised Japanese Ministry of Health criteria for the diagnosis of SS (1999), *AECG criteria* The American-European Consensus Group classification criteria for SS (2002), *ACR criteria* American College of Rheumatology classification criteria for SS (2012)

systems were the same in this study. The reason for the same specificities of the JPN and AECG criteria may be the identical number of non-SS patients (21 patients, including 18 patients without CTDs and 3 patients with CTDs) who satisfied JPN and AECG. However, the JPN and AECG profiles for 20 out of these 21 non-SS patients were completely different, highlighting the low agreement between JPN and AECG, as shown in Table 8.

The sensitivity of AECG for primary SS was highest among the three systems, whereas that of JPN for all SS and secondary SS was highest. Among the 302 primary SS patients, 19 patients only satisfied the AECG criteria. These 19 primary SS patients had high frequencies of dry eye (84.2 %) and dry mouth (100.0 %) but low frequencies of anti-SS-A antibody (10.5 %) and anti-SS-B antibody (0 %). These seronegative primary SS patients with symptoms of dryness could only be diagnosed by the AECG criteria, because only the AECG criteria include symptoms of dryness. This may be the sensitivity of AECG for primary SS was highest among the three systems.

The above findings suggest that JPN provided the best set of criteria necessary for the diagnosis of Japanese patients with SS. Admittedly, however, the results of the present study do not allow us to confirm the superiority of JPN due to the inherent limitations of the study. First, we used the clinical judgment of the physician in charge as the gold standard. In Japan, because the JPN criteria are the criteria used most commonly in daily clinical practice, the clinical judgment could depend on the satisfaction of the JPN criteria. It is better to rely on expert committee consensus based on clinical case scenarios as the gold standard for diagnosis in order to avoid this bias. Second, patients who had been checked for all four criteria of the JPN diagnostic system (pathology, oral, ocular, anti-SS-A/SS-B antibodies) were included in this study, but the methods used for ocular staining varied among the participating institutions. Third, the results of the study could include selection bias. For these reasons, we need a more

Table 8 Agreement among the three tested systems, as assessed using the kappa coefficient

All SS ($n = 476$)	All SS ($n = 476$) (primary SS, $n = 302$, secondary SS, $n = 174$)	Primary SS ($n = 302$)	Secondary SS ($n = 174$)
JPN vs. AECG	0.31	0.46	0.10
JPN vs. ACR	0.74	0.74	0.74
AECG vs. ACR	0.30	0.42	0.12

The "entire group" comprised 694 patients, including 476 with SS (302 patients with primary SS and 174 with secondary SS) and 218 patients with non-SS. The "without other CTDs" group of 499 patients included 302 patients with primary SS and 197 with non-SS. The "with other CTDs" group of 195 patients included 174 patients with secondary SS and 21 with non-SS.

JPN Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* The American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

sophisticated validation study using randomly selected clinical case scenarios from various institutions and expert committee consensus diagnosis as the golden standard to test the three diagnostic systems for SS, to unify the criteria used for the diagnosis of SS, and ultimately to select the gold standard set of criteria for the diagnosis of SS in Japan.

Currently, the JPN diagnostic system is only used in Japan, because ACR and EULAR have never validated the JPN system. Therefore, we strongly hope that an ACR/EULAR collaborative initiative will validate JPN as well as the AECG and ACR systems.

In conclusion, although this study has a few limitations, the results obtained from it indicate the superiority of the JPN criteria, as it has higher sensitivity and specificity values for the diagnosis of SS in Japanese patients with SS than those of ACR and AECG.

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Conflict of interest None.

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T helper 2 and regulatory T-cell cytokine production by mast cells: a key factor in the pathogenesis of IgG4-related disease

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IgG4-related disease is a systemic disorder with unique clinicopathological features and uncertain etiological features and is frequently related to allergic disease. T helper 2 and regulatory T-cell cytokines have been reported to be upregulated in the affected tissues; thus, the production of these cytokines by T helper 2 and regulatory T cells has been suggested as an important factor in the pathogenesis of IgG4-related disease. However, it is not yet clear which cells produce these cytokines in IgG4-related disease, and some aspects of the disorder cannot be completely explained by T-cell-related processes. To address this, we analyzed paraffin-embedded sections of tissues from nine cases of IgG4-related submandibular gland disease, five cases of submandibular sialolithiasis, and six cases of normal submandibular gland in order to identify potential key players in the pathogenesis of IgG4-related disease. Real-time polymerase chain reaction analysis confirmed the significant upregulation of interleukin (IL)4, IL10, and transforming growth factor beta 1 (TGFβ1) in IgG4-related disease. Interestingly, immunohistochemical studies indicated the presence of mast cells expressing these cytokines in diseased tissues. In addition, dual immunofluorescence assays identified cells that were double-positive for each cytokine and for KIT, which is expressed by mast cells. In contrast, the distribution of T cells did not correlate with cytokine distribution in affected tissues. We also found that the mast cells were strongly positive for IgE. This observation supports the hypothesis that mast cells are involved in IgG4-related disease, as mast cells are known to be closely related to allergic reactions and are activated in the presence of elevated non-specific IgE levels. In conclusion, our results indicate that mast cells produce T helper 2 and regulatory T-cell cytokines in tissues affected by IgG4-related disease and possibly have an important role in disease pathogenesis.
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Keywords: cytokine; IgE; IgG4-related disease; mast cells; regulatory T cell

IgG4-related disease has recently been recognized as a clinical entity with unique clinicopathological features that can affect systemic organs.[1–4] Histological examination of IgG4-related disease has shown diffuse lymphoplasmacytic infiltration, interstitial fibrosis, obliterative phlebitis, and eosinophilic infiltration. Additionally, numerous IgG4-positive plasma cells are observed and the IgG4-positive/IgG-positive cell ratio is elevated above 40% in affected tissues. It has been suggested that these distinctive features are caused by the T helper 2 cell cytokines interleukin (IL)4 and IL5 and the regulatory T-cell cytokines IL10 and transforming growth factor beta 1 (TGFβ1).[5,6] These cytokines are

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upregulated in the tissues affected by IgG4-related disease, where IL4 and IL10 are thought to stimulate B cells and plasma cells to induce lymphoplasmacytic infiltration and IgE and IgG4 production, whereas TGFβ1 is thought to induce interstitial fibrosis. On the basis of these observations, T helper 2 and regulatory T cells have been considered to have a key role in the pathogenesis of IgG4-related disease. However, it has not been definitively determined whether these T cells are actually responsible for the production of such cytokines in affected tissues. Moreover, the hypothesis that T cells produce the disease-related cytokines does not explain why an anti-CD20 monoclonal antibody, rituximab, would be effective in treating refractory IgG4-related disease patients.[7]

An alternative to the T-cell hypothesis is the involvement of mast cells in IgG4-related disease. Mast cells were first found to release histamine granules in immediate hypersensitivity reactions; however, recent research has revealed that mast cells are involved in a variety of immune responses, including host defense, immune regulation, allergy, chronic inflammation, and autoimmune disease.[8] In response to IgE stimulation, mast cells secrete various mediators, including T helper 2 cytokines and regulatory cytokines.[9] Non-specific IgE alone can induce cytokine secretion independent of any antigen.[10] As IgG4-related disease is frequently complicated with allergic disease and accompanied by elevation of serum IgE levels, we hypothesized that mast cells may be involved in the pathogenesis of IgG4-related disease.

Materials and methods

Samples

Tissue samples from nine cases of submandibular gland IgG4-related disease were obtained. The serum IgG4 levels were elevated in all nine cases. Samples from five cases of submandibular sialolithiasis and six cases of normal submandibular glands which were resected during treatment for oral cancer were obtained and used as disease controls. Formalin-fixed paraffin-embedded specimens were used for immunohistochemistry, dual immunofluorescence, RNA extraction, and real-time polymerase chain reaction (PCR) analysis. All samples were obtained with the approval of the Institutional Review Board at Okayama University.

Real-Time Quantitative PCR

Total RNA was extracted from the paraffin-embedded sections of all samples by using an miRNeasy FFPE Kit (QIAGEN, Valencia, CA, USA). Complementary DNA was prepared by reverse transcription PCR by using a SuperScript VILO MasterMix kit (Invitrogen, Carlsbad, CA, USA). Multiplex real-time PCR was performed for quantitative analysis, according to the standard protocol by using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and a Step One Plus Real-Time PCR System (Applied Biosystems). Specific primers and probes for TGFβ1, IL4, IL5, IL10, and β-actin were obtained from Applied Biosystems. The PCR cycling conditions were as follows: 30 s at 95 °C and 50 cycles of 5 s at 95 °C, and 30 s at 60 °C. The expression of each cytokine was normalized to that of β-actin, which was used as an endogenous control.

Histological Examination and Immunohistochemistry

All of the diseased and normal tissue samples used in this study were surgically resected specimens of submandibular glands. The specimens were fixed in 10% formaldehyde and embedded in paraffin. Serial 4-μm-thick sections were cut from the block of paraffin-embedded tissue and stained with hematoxylin and eosin (H&E). The sections were immunohistochemically stained using an automated Bond Max stainer (Leica Biosystems, Melbourne, Germany). The following primary antibodies were used: TGFβ1 (ab49754; 1:100; Novocastra, Newcastle, UK), IL4 (orb22602; 1:400; Biorbit, Cambridge, UK), IL5 (MAB605; 1:400; R&D, Minneapolis, MN, USA), IL10 (orb22606; 1:100; Biorbit), KIT/CD117 (YR145; 1:100; EPITOMICS, Burlingame, CA, USA), IgG (polyclonal; 1:20 000; Dako, Glostrup, Denmark), IgG4 (HP6025; 1:10000; The Binding Site, Birmingham, UK), forkhead box P3 (FOXP3) (236A/E7; 1:100; Abcam, Cambridge, UK), CD4 (1F6; 1:40; Nichirei, Tokyo, Japan), and IgE (A094; 1:500; Dako).

Following immunostaining, the number of IgG4-positive and IgG-positive cells was estimated in areas with the highest density of IgG4-positive cells. In accordance with the consensus statement on the pathological features of IgG4-related disease published in 2012,[2] three different high-power fields (HPFs) (eyepiece, $\times$ 10; lens, $\times$ 40) were examined to calculate the average number of IgG4-positive cells per HPFs and the IgG4-positive/IgG-positive cell ratio. Cells that were positive for each cytokine, KIT, FOXP3, and IgE were counted in the three different fields (eyepiece, $\times$ 10; lens, $\times$ 20) determined to have the highest density of positive cells. The average number of positive cells per square millimeter (mm^2) was calculated.

Dual Immunofluorescence Assays

For indirect dual immunofluorescence assays, paraffin sections were stained with the primary antibodies for KIT and TGFβ1, KIT and IL4, KIT and IL5, or KIT and IL10. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Alexa Fluor anti-mouse 555 and Alexa Fluor anti-rabbit 488; both Invitrogen Co, Carlsbad, CA, USA) were used at a dilution of 1:400. The stained specimens

were examined with a conventional immunofluorescence microscope (IX71; Olympus, Tokyo, Japan).

Statistical Analysis

Data are presented as mean ± s.d. values. All statistical analyses were performed using the Mann–Whitney *U*-test with the SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA). A probability of $P<0.05$ was considered to be statistically significant.

Results

Confirmation of Histological Diagnosis in IgG4-Related Disease

We confirmed that the tissue specimens from all nine cases of submandibular gland IgG4-related disease showed typical histological features of IgG4-related disease, such as lymphoplasmacytic infiltration and dense fibrosis. Additionally, in all cases, numerous IgG4-positive cells were observed and the IgG4-positive/IgG-positive cell ratio was >40%.

Histological Findings of Sialolithiasis

The specimens showed lymphoid follicle formation and moderate to severe infiltration of lymphocytes and plasma cells as well as various numbers of neutrophils with various degree of fibrosis. Some specimens included salivary calculus (Figure 1a).

Elevated Expression of Cytokines in IgG4-Related Disease

The expression of T helper 2 cell cytokines (IL4 and IL5) and regulatory T-cell cytokines (IL10 and TGFβ1) was examined in samples from the nine cases of IgG4-related disease, five cases of sialolithiasis, and six cases of normal submandibular gland. The expression of the IL4, IL5, IL10, and TGFβ1 cytokines and the β-actin control in these samples was quantitatively analyzed by real-time PCR. As shown in Figure 1, IgG4-related disease exhibited significantly higher expression ratios of IL4/β-actin (31.9 ± 12.1-fold higher), IL10/β-actin (21.0 ± 15.7-fold higher), and TGFβ1/β-actin (28.6 ± 23.3-fold higher) than sialolithiasis and normal submandibular gland ($P<0.05$). In contrast, no significant difference was observed between the IL5/β-actin ratio in IgG4-related disease (0.606 ± 1.13) and those in sialolithiasis (0.119 ± 0.07) and normal submandibular gland (0.462 ± 0.369) (Figure 1b).

Next, the real-time PCR results were supported via immunostaining of cells by using primary antibodies against IL4, IL5, IL10, and TGFβ1 (Figure 2a). The number of IL4-positive cells was significantly higher in IgG4-related disease (4.04 ± 3.12 cells/mm^2) than in submandibular sialolithiasis (0.136 ± 0.186 cells/mm^2; $P<0.01$) and the normal submandibular gland (0.230 ± 0.356 cells/mm^2; $P<0.01$) (Figure 2b). Similarly, many IL10-positive cells were observed in IgG4-related disease (3.40 ± 1.84 cells/mm^2), whereas the submandibular sialolithiasis and normal submandibular gland contained few IL10-positive cells (0.342 ± 0.484 cells/mm^2, $P<0.01$; 0.226 ± 0.277 cells/mm^2, $P<0.01$, respectively) (Figure 2b). TGFβ1-positive cells were also more abundant in IgG4-related disease (4.29 ± 2.37 cells/mm^2) than in the submandibular sialolithiasis (1.51 ± 1.11 cells/mm^2; $P<0.05$) and normal submandibular gland (0.626 ± 0.548 cells/mm^2; $P<0.01$) (Figure 2b). Furthermore, we observed that TGFβ1-positive cells tended to infiltrate fibrous lesions.

In IgG4-related disease and control groups, the number of IL5-positive cells was much less than that of the other cytokines examined. No significant differences were observed between the number of IL5-positive cells in IgG4-related disease tissue (1.58 ± 1.44 cells/mm^2), sialolithiasis (0.838 ± 0.531 cells/mm^2; $P=0.450$), and normal submandibular gland tissue (0.811 ± 0.290 cells/mm^2; $P=0.332$) (Figure 2b).

Increased Density and Cytokine-Related Distribution of Mast Cells in IgG4-Related Disease

We compared the number of mast cells in the IgG4-related disease and control groups via immunostaining by using an antibody for KIT, which is a marker for mast cells. The number of KIT-positive mast cells was higher in IgG4-related disease (72.2 ± 24.5 cells/mm^2) than in the normal submandibular gland (30.0 ± 11.9 cells/mm^2; $P<0.01$) (Figure 3). However, no significant difference was observed between the number of mast cells in the IgG4-related disease and the submandibular sialolithiasis (177 ± 269 cells/mm^2; $P=0.73$) (Figure 3). Interestingly, the morphological features and distribution of the mast cells were similar to those of the T helper 2 (IL4 and IL5) or regulatory T-cell (IL10 and TGFβ1) cytokine-positive cells. Furthermore, dual immunofluorescence assays showed that KIT-positive mast cells were also positive for each of the IL4, IL5, IL10, and TGFβ1 cytokines (Figure 4). Additionally, although only a small number of IL5-positive cells was detected in the immunohistochemical experiments, these cells also exhibited KIT coexpression (Figure 4).

T-Cell Distribution in IgG4-Related Disease and Control Groups

To assess the number and distribution of T cells, we performed immunostaining assays with an antibody against FOXP3, which is a regulatory T-cell marker.

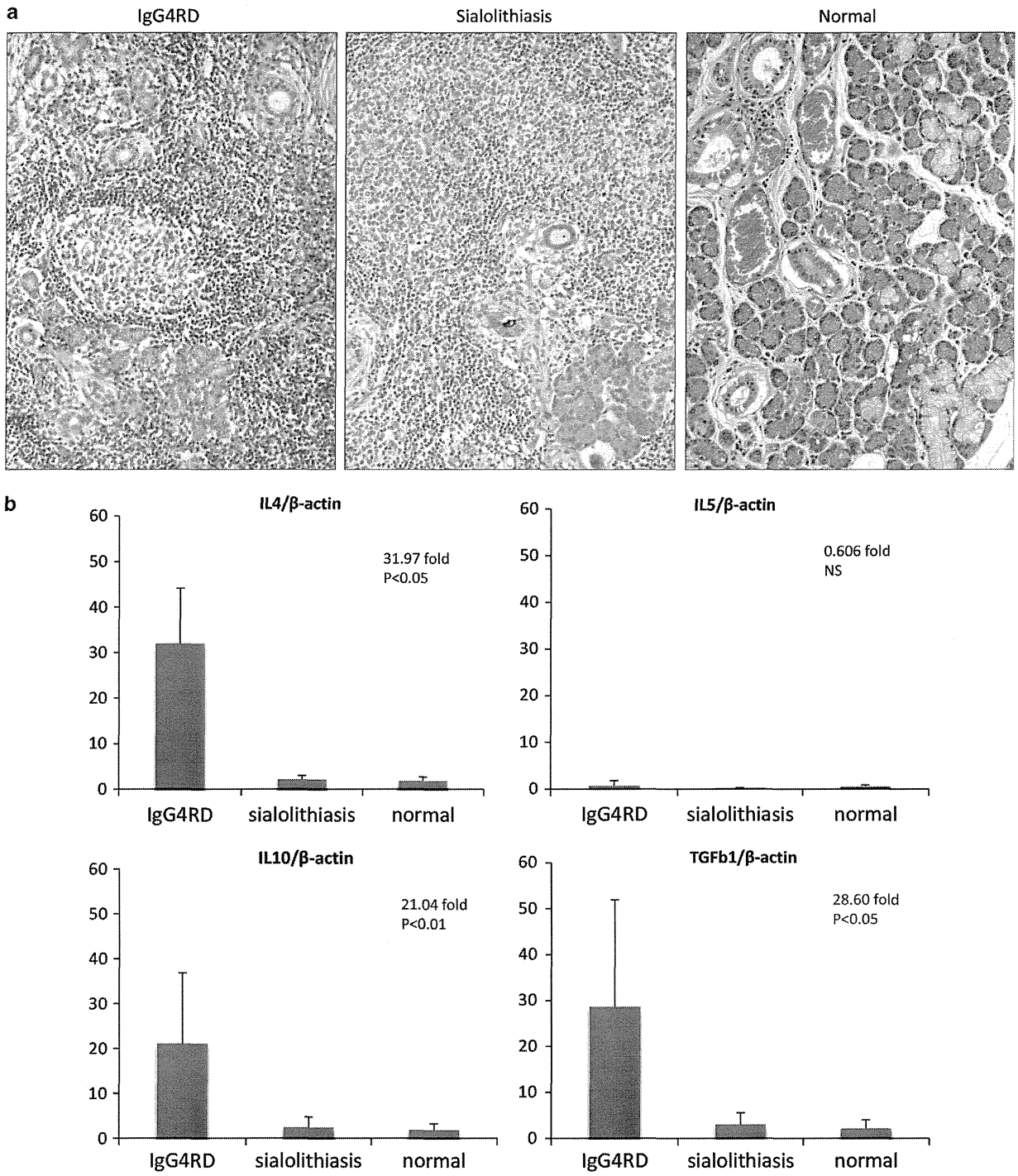

Figure 1 Histological findings and real-time PCR analysis of cytokine expression in IgG4-related disease and control groups. (**a**) IgG4-related submandibular disease showed dense lymphoplasmacytic infiltration with lymphoid follicle (hematoxylin and eosin, H&E, left). Sialolithiasis revealed moderate to severe lymphoplasmacytic infiltration with lymphoid follicle and calculus (H&E, center). Normal submandibular gland (H&E, right). (**b**) The histograms show the relative quantity of mRNA of the cytokines. The expression ratio of interleukin 4 (IL4)/β-actin, IL10/β-actin, and transforming growth factor beta 1 (TGFβ1(/β-actin were significantly higher in IgG4-related submandibular gland disease than in submandibular sialolithiasis and normal submandibular gland. The expression ratio of IL5/β-actin was low in all tissues and was not significantly different between the IgG4-related disease and control groups. (IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

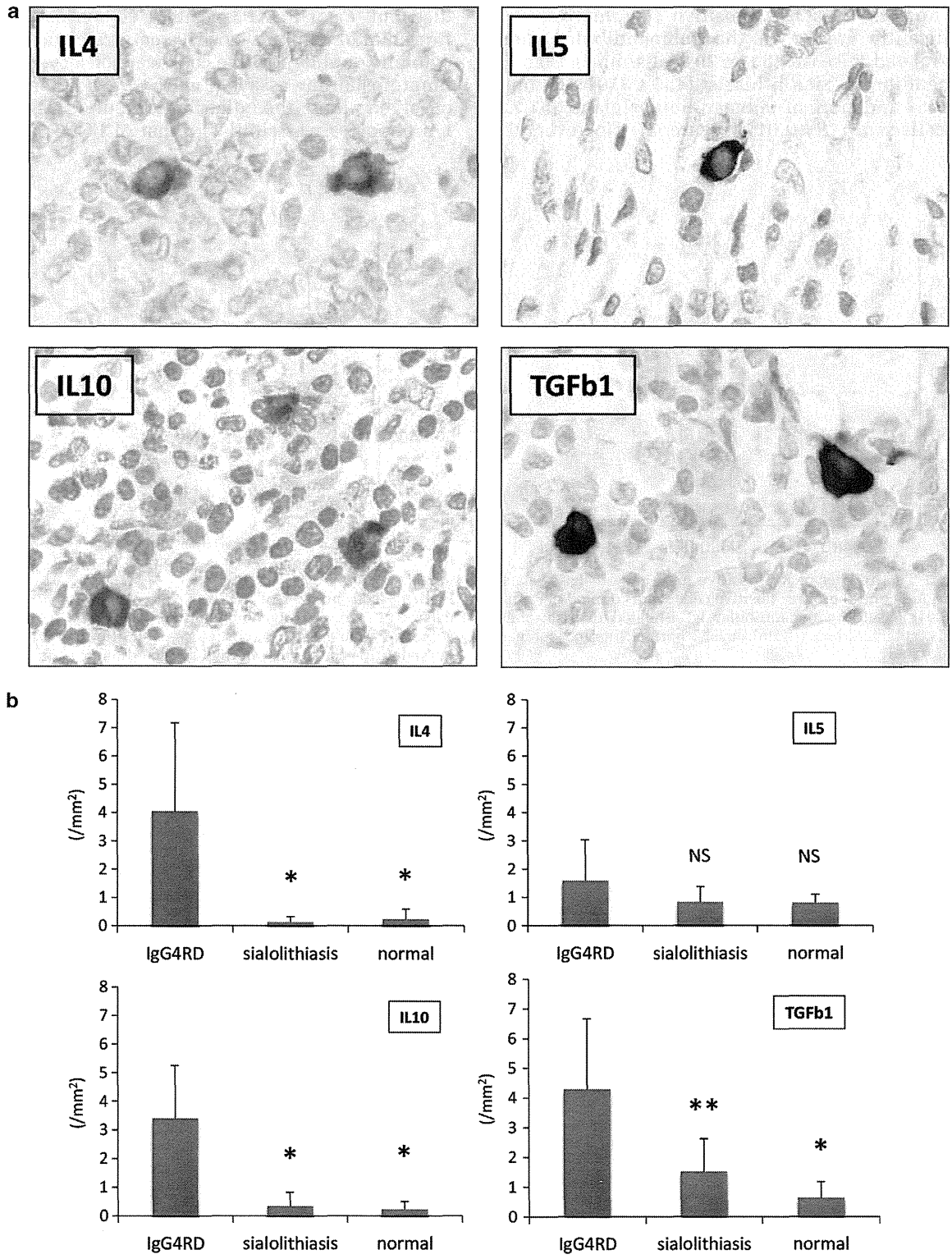

Figure 2 Immunohistochemical analysis of cytokine-expressing cells in IgG4-related disease and control groups. (**a**) Cells stained with antibodies against the indicated cytokines. (**b**) The number of cells positive for interleukin 4 (IL4), IL10, and transforming growth factor beta 1 (TGFβ1) were counted per mm^2 and significantly larger in IgG4-related disease than in the control groups. Consistent with the results of real-time PCR, the number of IL5-positive cells in IgG4-related disease was lesser than those of other cytokines, and no significant difference was observed between the IgG4-related disease and control groups ($^*P<0.01$, $^{**}P<0.05$). (IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

The number of FOXP3-positive lymphocytes was significantly greater in the submandibular gland IgG4-related disease (834 ± 284 cells/mm^2) than in submandibular sialolithiasis (435 ± 330 cells/mm^2; $P<0.05$) and normal submandibular gland (51.2 ± 33.5 cells/mm^2; $P<0.01$) (Figure 5). However, the distribution of FOXP3-positive cells was different from that of cytokine-positive cells, and no FOXP3/cytokine double-positive cells were observed in dual immunostaining assays (Figure 6). Additionally, we observed that the distribution of CD4-positive lymphocytes was similar to that of FOXP3-positive

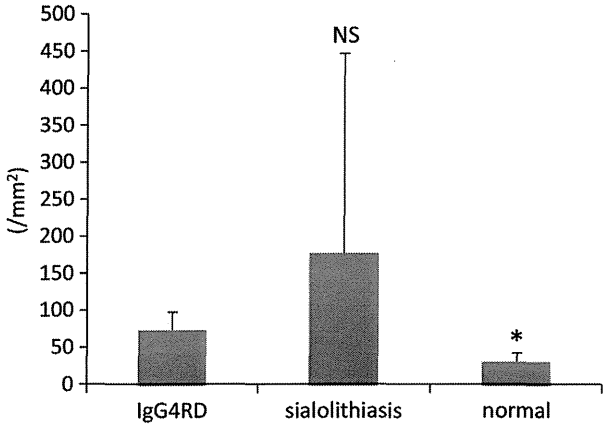

Figure 3 The number of KIT-positive mast cells in IgG4-related disease and control groups. Positive cells were counted per mm^2. (*$P<0.01$; IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

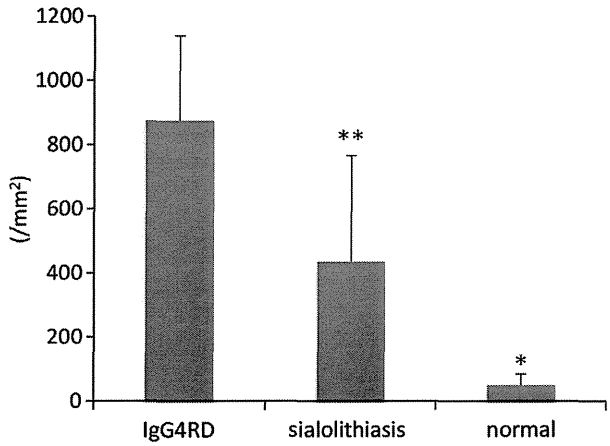

Figure 5 The number of FOXP3-positive lymphocytes. Positive cells were counted per mm^2. (*$P<0.01$, **$P<0.05$; gG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

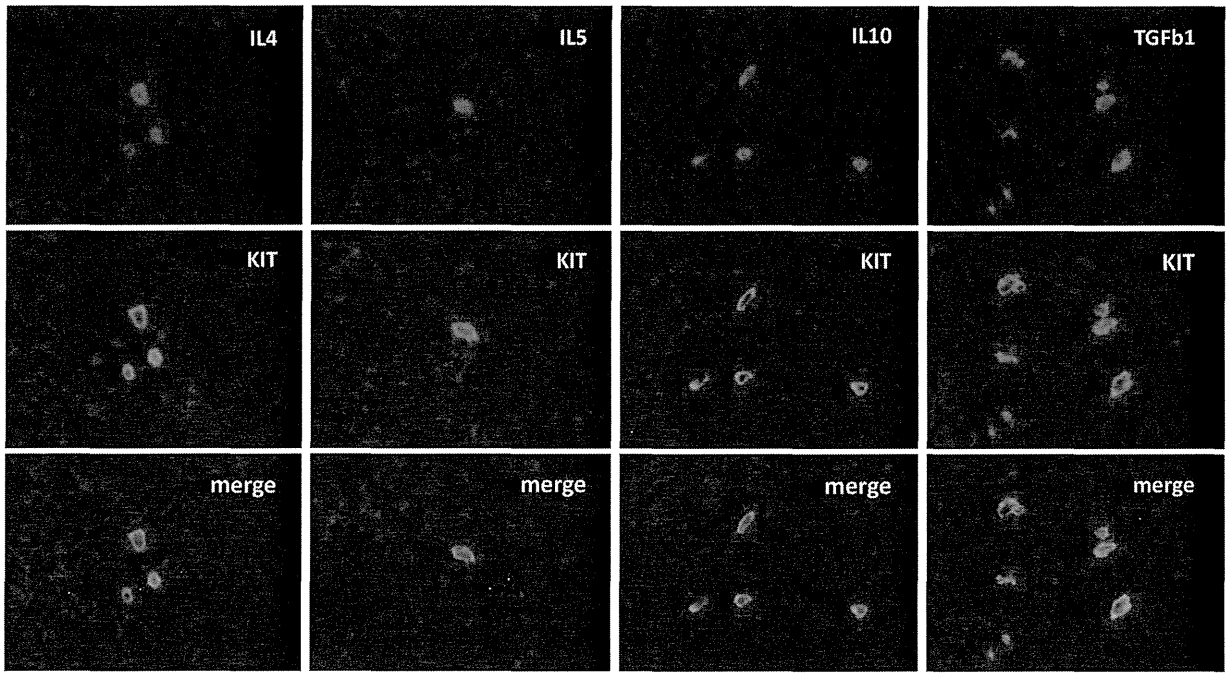

Figure 4 Dual fluorescent analysis of KIT-positive mast cells and cytokine-positive cells in IgG4-related submandibular gland disease. Compared with the results for normal submandibular gland, the number of KIT-positive cells (middle row) was significantly higher in IgG4-related disease and sialolithiasis, and there was no significant difference between these two groups in this regard. Immunostaining for each cytokine (top row) revealed strong cytoplasmic positivity. Positive cells were morphologically similar to mast cells. Dual fluorescent immunostaining detected many positive cells for each cytokine and KIT. The merged image (bottom row) demonstrated double-positive cells for KIT and interleukin 4 (IL4), IL5, IL10, and TGFβ1.

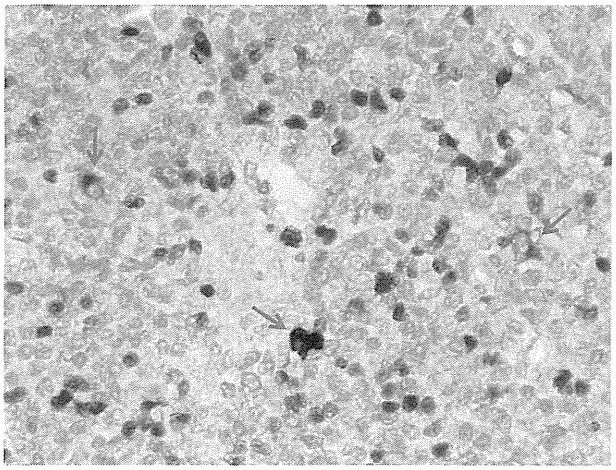

Figure 6 Dual immunostaining of transforming growth factor beta 1 (TGFβ1) and FOXP3. The distribution of TGFβ1- and FOXP3-positive cells were different (TGFβ1/brown, FOXP3/red).

cells, and CD4-positive cell distribution did not correlate with cytokine-positive cell distribution. Moreover, no double-positive cells were observed upon double-immunostaining for each of the cytokines with CD4.

Staining Pattern of IgE in Mast Cells of IgG4-Related Disease

In IgG4-related disease and control groups tested, a high number of mast cells were positive for IgE with varying density and intensity. As is typical for IgE immunostaining, IgE was observed to be weakly-to-moderately expressed on cell surface membranes (Figure 7a). No significant differences were observed in the number of surface membrane IgE-positive cells among the IgG4-related disease and control groups. However, the IgG4-related disease contained a large number of mast cells that were strongly positive for cytoplasmic IgE (Figure 7a). The distribution of the strongly cytoplasmic IgE-positive cells correlated with KIT expression, and the membranous colocalization of IgE and KIT was confirmed by dual immunofluorescence (Figure 7b).

The number of strongly cytoplasmic IgE-positive cells was significantly greater in IgG4-related disease (7.19 ± 5.11 cells/mm^2) than in sialolithiasis (0.068 ± 0.15 cells/mm^2; $P<0.01$) and normal (0.45 ± 0.51 cells/mm^2; $P<0.01$) control tissues (Figure 7c).

Discussion

In this study, we confirmed the upregulation of T helper 2 (IL4) and regulatory T-cell (IL10 and TGFβ1) cytokines in the paraffin-embedded tissue of the submandibular gland IgG4-related disease by real-time PCR. Upregulation of IL5 was not observed in this study, which is similar to previously published data on real-time PCR for paraffin-embedded tissue samples for IgG4-related disease.[11] Interestingly, our immunohistochemical studies detected a large number of mast cells expressing T helper 2 (IL4) and regulatory T-cell (IL10 and TGFβ1) cytokines in the submandibular gland IgG4-related disease, whereas this was not the case for the control groups. Notably, although the overall number of mast cells was similar among the samples of submandibular gland IgG4-related disease, submandibular sialolithiasis, and normal submandibular gland, the IgG4-related disease contained significantly more cytokine-expressing mast cells. Furthermore, interestingly, our immunohistochemical experiments provided no evidence that T cells were producing the upregulated cytokines. In our study, we considered FOXP3-positive cells to be regulatory T cells, as FOXP3 is considered to be a specific marker of regulatory T cells, although a minor amount of effector T cells can exhibit transient expression of FOXP3.[12–14] It has been suggested that T helper 2 and regulatory T cells are most important in the pathogenesis of IgG4-related disease. Although we did find that the number of FOXP3-positive regulatory T cells was significantly increased in IgG4-related disease in our study, we did not find any evidence that the FOXP3-positive regulatory T cells produced regulatory cytokines. Thus, our results do not support the hypothesis that T cells express the cytokines associated with IgG4-related disease; rather, our data indicate that mast cells are the source of these upregulated cytokines. However, a close relationship between regulatory T cells and mast cells has been reported, and TGFβ1 has been shown to promote differentiation of naive T cells to regulatory T cells.[15] Therefore, we hypothesized that the upregulated mast cells produce TGFβ1, which secondarily promotes the differentiation of naïve T cells to regulatory T cells in the tissue affected.

We observed that mast cells in IgG4-related disease were strongly positive for IgE. This result suggests that IgE is associated with the pathogenesis of IgG4-related disease. Consistent with this notion, patients with IgG4-related disease frequently have an allergic background, such as allergic rhinitis, bronchial asthma, and atopic dermatitis. In addition, patients with this disease typically show elevated serum IgE levels as well as elevated IgG4 levels.[16] IgE is a key stimulator of mast cells, and the mechanism underlying IgE stimulation of mast cells to induce various immunological cascades has been extensively studied. Notably, chronic elevation of antigen-independent, non-specific IgE upregulates the high-affinity IgE receptor, FcεRI, on mast cells, which inhibits mast cell apoptosis and promotes cytokine production.[17] Our observation that mast cells in IgG4-related disease were strongly positive for IgE supports the idea that IgE-mediated stimulation of mast cells may have a role in the

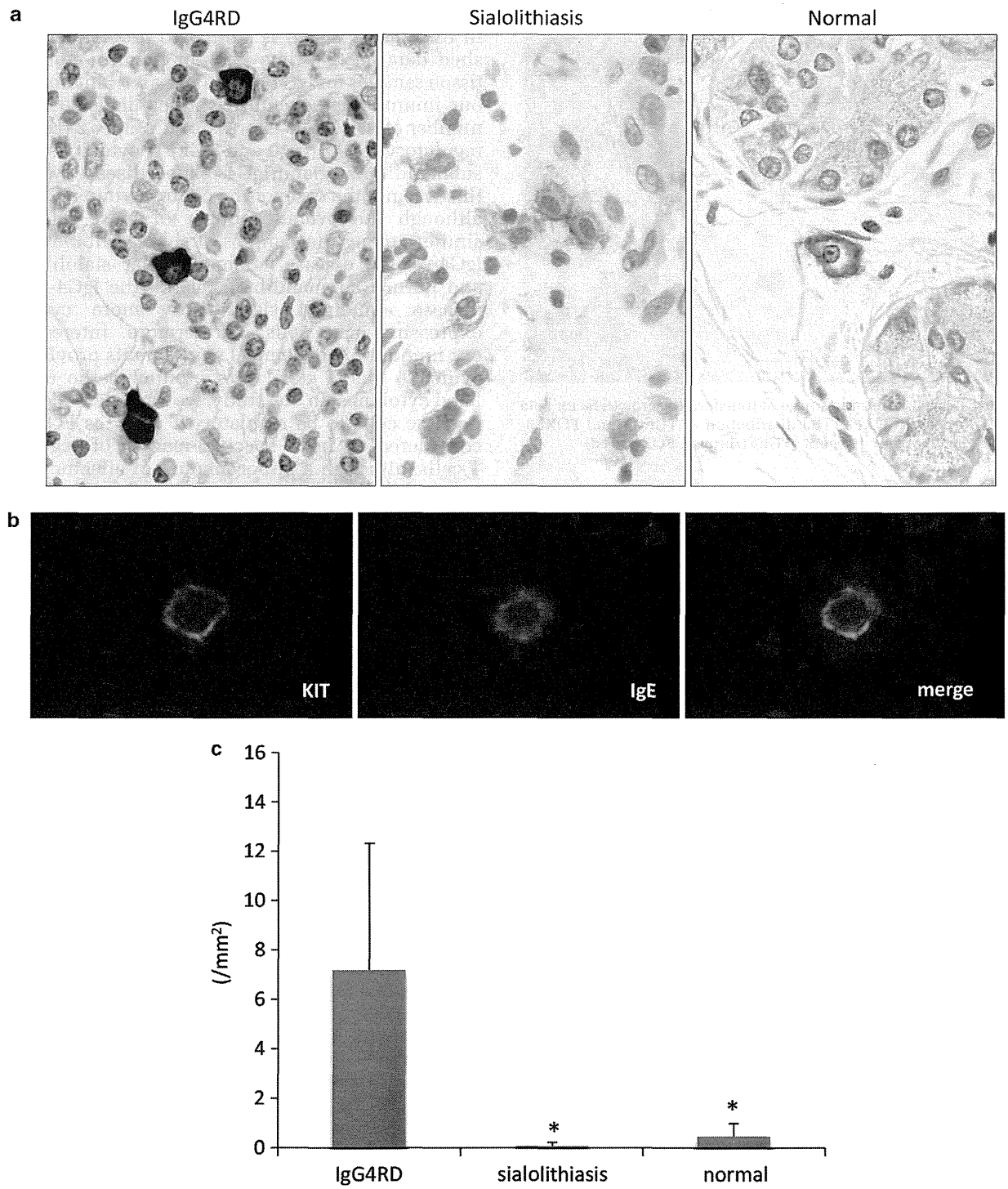

Figure 7 IgE expression in mast cells. (**a**) In IgG4-related disease, mast cells showed strong cytoplasmic positivity for IgE. In contrast, mast cells in sialolithiasis and normal submandibular gland showed only moderate-to-weak IgE positivity and membranous localization. (**b**) Colocalization of IgE- and KIT-positive cells was confirmed by dual immunofluorescence (IgE/red, KIT/green). (**c**) The number of strongly cytoplasmic IgE-positive cells was counted per mm^2 and was significantly different between the IgG4-related disease and control groups. (*$P<0.01$) (IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

pathogenesis of IgG4-related disease. However, it remains to be determined why the IgE in these cells was localized to the cytoplasm. Previous studies have shown that the number of FcεRI receptors on mast cells greatly increases in the presence of elevated serum IgE levels.[18] Thus, it is possible that what appeared to be cytoplasmic localization of IgE in our immunostaining experiments may have actually been the result of very high levels of membrane-associated IgE. However, a few reports have suggested that antigen cross-linking could lead to the aggregation and internalization of FcεRI. In particular, IgE was detected in the cytoplasm of mast cells as a result of the internalization and endocytosis of FcεRI.[19] These data suggest that the continuous upregulation of mast cells might result in endocytosis-mediated cytoplasmic accumulation of IgE. However, this hypothesis should be based on the existence of certain antigens, and the role of internalization of FcεRI remains controversial.

Steroid therapy has been the first choice of medication for IgG4-related disease. However, a recent study reported a case of IgG4-related disease that regressed after treatment with an antihistamine agent (epinastine hydrochloride) alone, which indicates an allergic background to the disease and may indirectly suggest the involvement of mast cells.[20] The *in vitro* effects of antihistamines on the inhibition of mediator release from mast cells have been reported.[21] Moreover, an *in vitro* study of human conjunctival mast cells revealed that epinastine inhibited mast cell secretion of cytokines, including IL10.[22] However, it remains unclear why antihistamines inhibit cytokine secretion, although H1 antagonism seems unrelated.

Rituximab is another treatment currently being used for refractory cases of IgG4-related disease. Rituximab therapy has been reported to produce rapid regression in refractory patients.[23] Additionally, rituximab therapy was found to induce regression of symptoms with a decrease in serum IgE levels,[24] and the concentration of IgE showed a steeper decline than serum IgG4 levels. These results provide additional evidence that the allergic reaction mediated by IgE is an important factor in the regulation of IgG4-related disease.

Hyper-IgE syndrome is a complex immunodeficiency characterized by atopic dermatitis associated with extremely high serum IgE levels and susceptibility to infections with extracellular bacteria.[25] Despite the continuously elevated serum IgE level, hyper-IgE syndrome shows clinical features that are quite different from those of IgG4-related disease. Furthermore, hyper-IgE syndrome has been linked to mutations in signal transducer and activator of transcription 3 (STAT3) and tyrosine kinase 2 (TYK2), and to defective signal transduction pathways involving multiple cytokines. In contrast to this downregulation of cytokine signaling, IgG4-related disease is associated with the upregulation of multiple cytokine signals.

To our knowledge, this study provides the first evidence relating mast cells to IgG4-related disease. However, as mast cells are currently known as powerful mediators of the immune response and are closely related with allergic responses, the involvement of mast cells in IgG4-related disease seems logical. Moreover, mast cells have been implicated in other inflammatory diseases. For instance, it was reported that a large number of

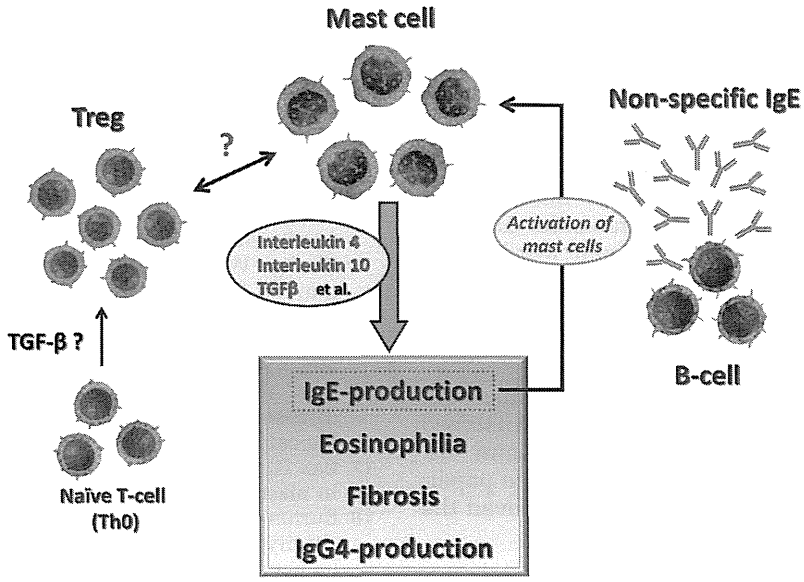

Figure 8 Model for the pathogenesis of IgG4-related disease. Because of an allergic background with the elevation of non-specific IgE levels, activated mast cells produce interleukin 4 (IL4), IL10, and transforming growth factor beta 1 (TGFβ1), which induce the distinctive features of IgG4-related disease. In addition, IL4 and IL10 themselves induce IgE production to upregulate mast cells, and TGFβ1 induces the transformation of naive T cells to regulatory T cells. The relationship between regulatory T cells and mast cells remains unclear.

mast cells was observed in periductal and ductal fibrosis in primary sclerosing cholangitis (PSC) and chronic sclerosing sialadenitis, indicating that mast cells might contribute to fibrosis.[26,27] Mast cell expression of IL10, as observed in our immunohistochemical experiments, may affect mast cell involvement in IgG4-related disease. IL10 is known to be an anti-inflammatory cytokine that can suppress acquired or innate immune responses. Mast cell-derived IL10 has been reported to limit contact dermatitis and chronic irradiation with ultraviolet B.[28] Similarly, in IgG4-related disease, mast cell production of IL10 may prevent allergic inflammation in the affected organs.

On the basis of results of our study and those of previously published reports, we established a hypothesis for the pathogenesis of IgG4-related disease, which is summarized in Figure 8. Patients with IgG4-related disease usually have an allergic background with elevation of non-specific IgE levels. This antigen-independent non-specific IgE binds to FcεRI on mast cells to promote the production of T helper 2 (IL4) and regulatory T-cell (IL10 and TGFβ1) cytokines, which induce the distinctive features of IgG4-related disease such as lymphoplasmacytic infiltration, interstitial fibrosis, and IgG4 production. In addition, IL4 and IL10 induce IgE production, which promotes the upregulation of mast cells. Although it is possible that regulatory T cells interact with mast cells, their involvement in this process is uncertain and they might be secondarily recruited to the affected tissue. According to this scenario, rituximab might exert its effects through the inhibition of IgE-production of B-cells (Figure 8). This model is based on the major finding of this study, which indicates that mast cells have a key role in IgG4-related disease.

Acknowledgments

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Author contributions

Conceived and designed the experiments: YS. Performed the experiments: MT and YS. Analyzed the data: YS, MT, ST, KO, KT, YG, and TI. Contributed materials: YO and TT. Wrote the paper: MT, YS, and TY. All authors read and approved the final manuscript.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Elevated Serum Immunoglobulin G4 Levels in Patients with Graves' Disease and Their Clinical Implications

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Background: Immunoglobulin G4-related disease (IgG4-RD) is a new clinical entity that affects various organs with increased IgG4 positive plasmacytes and progressive fibrosis. While IgG4-RDs in association with Hashimoto's thyroiditis or Riedel's thyroiditis have been reported, the relationship between IgG4-RD and Graves' disease (GD) is yet unknown. To elucidate the relation of GD to IgG4-RD, serum IgG4 levels and their clinical implications in patients with GD were investigated.
Methods: In this prospective study, serum IgG4 levels were measured in 109 patients with GD and classified into two groups according to the comprehensive diagnostic criteria of IgG4-RD previously established: (i) GD with elevated-IgG4 levels (≥ 135 mg/dL), and (ii) GD with nonelevated IgG4 (< 135 mg/dL).
Results: Seven out of 109 patients with GD (6.4%) had elevated serum IgG4 levels (mean$\pm SD$ (range): 175.0 ± 44.5 (136–266) mg/dL) and elevated ratios of IgG4/IgG ($12.7 \pm 4.5\%$ (7.6%–21.2%)). The remaining patients with GD had serum IgG4 levels and IgG4/IgG ratios of 39.6 ± 27.6 (3–132) mg/dL and $3.2 \pm 2.2\%$ (0.3%–11.5%) respectively. Ages in the elevated IgG4 group were significantly higher than those of the nonelevated IgG4 group: 54.7 ± 6.2 versus 43.4 ± 15.4 years respectively. Ultrasound examinations revealed that the elevated IgG4 group had significantly increased hypoechogenic areas in the thyroid in comparison to the nonelevated IgG4 group (low echo scoring: 1.66 ± 0.81 vs. 0.61 ± 0.89 respectively). In the correlation analysis, TSAb ($r_s = 0.385$, $n = 42$) titers were significantly correlated with serum IgG4 levels, while they were not significantly different between the two groups. In the elevated IgG4 group, symptoms were controllable with a small dose of antithyroidal drug (ATD; $n = 4$), a combination treatment with ATD and L-T4 ($n = 1$), or L-T4 administration only one year after the first visit ($n = 2$).
Conclusions: A small portion of GD patients harbored elevated serum IgG4 levels. They were older, had increased hypoechoic areas in the thyroid, and appeared to be responsive or prone to be hypothyroid after ATD treatment. Thus, the present study suggests the presence of a novel subtype of GD. Measuring serum IgG4 levels may help to distinguish this new entity and provide potential therapeutic options for GD.

Introduction

IMMUNOGLOBULIN G4-RELATED DISEASE (IgG4-RD) is a recently proposed clinical entity, first reported in 2001 as a novel subtype of autoimmune pancreatitis (1). It is characterized by elevated serum IgG4 levels, IgG4-positive plasmacytes, and lymphocyte infiltration into multiple organs, inducing tissue fibrosis and organ dysfunction. In addition to the involvement of the pancreas, the lacrimal gland, salivary gland, biliary duct, and retroperitoneal tissue can also be involved in this disease (2).

The relationship between IgG4-RD and thyroid diseases has been previously investigated. Li *et al.* described a novel type of IgG4 thyroiditis on the basis of its clinical and histopathological features (3). The authors found a close relationship between the fibrous variant of Hashimoto's thyroiditis (HT) and IgG4-RD. Riedel's thyroiditis (RT) has been proposed to be an organ manifestation of IgG4-RD (4,5). In cases of RT, elevated serum IgG4 levels and/or an increased number of IgG4-positive plasmacytes with dense fibrous tissue were observed in the thyroid gland, which would respond to steroid therapy, suggesting characteristics of IgG4-RD (4). In addition, Watanabe *et al.* reported that 19% of patients with IgG4-RD who had hypothyroidism showed an increased thyroid volume and TgAb and/or TPOAb positivity. The thyroid function of these patients

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normalized after prednisolone treatment. Furthermore, histology revealed IgG4-bearing plasma cells and loss of thyroid follicles, was the condition was thus termed IgG4-related thyroiditis (6). However, the relationship between IgG4-RD and GD remains unknown.

To elucidate the relationship between IgG4-RD and GD, serum IgG4 levels in 109 patients with GD were measured and compared according to clinical characteristics. Herein, we describe the clinical features of patients with GD and elevated serum IgG4 levels, and discuss diagnostic and therapeutic approaches.

Patients and Methods

Patients

A total of 109 patients with GD at the Wakayama Medical University Hospital, Japan, from January 2011 to October 2012 were prospectively recruited to this study. The diagnosis of GD was based on the presence of hyperthyroidism, positive thyroid stimulating hormone receptor antibody/thyroid stimulating antibody (TRAb/TSAb), and/or increased ^{123}I thyroid uptake. Patients with malignancies or pregnancy were excluded. None of the patients underwent surgery or radioiodine treatment. Patients were divided into two groups: those with elevated serum IgG4 levels ($\geq$135 mg/dL) and those with nonelevated serum IgG4 levels ($<$135 mg/dL) according to the comprehensive diagnostic criteria of IgG4-RD, the currently established criteria (7). Masaki *et al.* proposed that another criterion for IgG4-RD consists of a ratio of IgG4/IgG of $\geq$8.0% (8); this criterion was therefore also considered in our study. Patients were analyzed for age, sex, smoking, familial history of autoimmune thyroid disease (AITD), presence of Graves' ophthalmopathy (GO) on the basis of the clinical activity score (CAS) (9) and NOSPECS (10), serum IgG4 and IgG levels, thyroid function, thyroid autoantibodies, and ultrasound examination (Table 1). Patients were classified according to intractability in the control of hyperthyroidism: group 1 (intractable patients) who required a moderate or large dosage of antithyroidal drug (ATD) (thiamazole (MMI) $\geq$10 mg/day; propylthiouracil (PTU) $\geq$150 mg/day) to control thyroid function; and group 2 (tractable patients) who could be treated with a small dosage no ATD (MMI $\leq$5 mg/day; PTU $\leq$100 mg/day). Written informed consent was obtained from all patients, and the study protocol was approved by the Wakayama Medical University Hospital Ethics Committee.

Thyroid function tests and thyroid autoantibodies

Serum thyrotropin (TSH), free thyroxine (fT4), and free triiodothyronine (fT3) levels were measured by chemiluminescent immunoassay (Abbott Diagnostics, Tokyo, Japan). Reference ranges were defined as follows: TSH 0.35–4.94 mIU/L; fT4 0.70–1.48 ng/dL; and fT3 1.71–3.71 pg/mL. TRAb was determined by enzyme-linked immunosorbent assay (Cosmic, Tokyo, Japan). Thyroglobulin autoantibodies (TgAb) and thyroid peroxidase antibodies (TPOAb) were measured with an electrochemiluminescent immunoassay (SRL, Tokyo, Japan). Normal values were defined as follows: TRAb $<$1 IU/L; TgAb $<$28 IU/mL; TPOAb $<$16 IU/mL. TSAb activities were determined using the Yamasa's TSAb bioassay kit (Yamasa Ltd., Choshi, Japan). Normal values for TSAb were defined as $<$180%.

Serum IgG4 and IgG levels

Serum IgG4 and IgG levels were measured by a nephelometric immunoassay (BML, Osaka, Japan). Reference ranges for IgG4 and IgG were defined as 4–108 mg/dL and 870–1700 mg/dL respectively. Since comprehensive diagnostic criteria for IgG4-RD include a serum IgG4 level $\geq$135 mg/dL, we defined this as the cutoff level in this study.

Ultrasonographic evaluation

Ultrasonographic examinations were performed by conventional gray scale and color Doppler, and by 10 MHz linear transducer (Toshiba Medical, Osaka, Japan). Low echogenicity in the thyroid gland was classified into four categories and scored as previously described (11): Grade 0, diffuse high-amplitude echoes throughout the whole lobe of the thyroid; Grade 1, low-amplitude and nonuniform echoes in the whole or several regions of the thyroid; Grade 2, several sonolucent regions in the thyroid; and Grade 3, no apparent echoes or very low amplitude echoes throughout the whole thyroid. Increase of color Doppler flow in the thyroid gland was determined as follows: 0, none; 1, mild; 2, moderate; and 3, severe. Thyroid size was measured as the sum of both lobes according to the following equation: anteroposterior$\times$transversal diameters (mm^2) at the maximum position.

Statistical analysis

Fisher's exact test was used to assess data in the two-dimensional contingency tables for comparison with sex, presence of GO, family history of AITD, and smoking. Mann–Whitney *U*-test and Kruskal–Wallis test were used to compare two or three individual groups respectively. Two-tailed Spearman's rank correlation coefficient (r_s) was determined to assess the correlation between two variables. Data for TSH, TRAb, TgAb, and TPOAb were analyzed with log-transformed values. p-Values <0.05 were accepted as statistically significant (SPSS v15, Chicago, IL). Data are expressed as mean$\pm$standard deviation (*SD*).

Results

Serum IgG4 and IgG levels in patients with GD

Overall, the serum IgG4 level in patients with GD was 48.3$\pm$44.0 mg/dL (range 3–266), and the ratio of IgG4/IgG was 3.8$\pm$3.4 mg/dL (range 0.3–21.2; Table 1). Seven (6.4%) of the 109 patients with GD had elevated serum IgG4 levels at 175.0$\pm$44.5 mg/dL (range 136–266) and elevated ratios of IgG4/IgG at 12.7$\pm$4.5% (range 7.6–21.2). The remaining patients with GD (93.6%) had serum IgG4 levels and IgG4/IgG ratios of 39.6$\pm$27.6 mg/dL (range 3–132) and 3.2$\pm$2.2% (range 0.3–11.5) respectively. Three cases of GD in patients with serum IgG4 levels $<$135 mg/dL had serum ratios of IgG4/IgG $\geq$8%. There was a significant difference in the ratios of IgG4/IgG between the two groups ($p<0.001$). However, no significant difference was observed in serum IgG levels between groups.

The elevated-IgG4 group consisted of one man and six women, while the nonelevated-IgG4 group included 14 men and 88 women. No significant difference was observed in sex distribution between groups.

TABLE 1. COMPARISONS OF SERUM IGG4 LEVELS AND CLINICAL CHARACTERISTICS OF PATIENTS WITH GRAVES' DISEASE (GD)

	Overall (n = *109*)		*Non elevated IgG4* (*< 135 mg/dL;* n = *102, 93.6%*)		*Elevated-IgG4* (*≥ 135 mg/dL;* n = *7, 6.4%*)		p-*Value*[a]
Sex (M/F)	15/94		14/88		1/6		0.967
Presence of Graves' ophthalmopathy	29 (26.6%)		26 (25.5%)		3 (42.3%)		0.379
Family history of AITD	30 (27.5%)		28 (27.5%)		2 (28.6%)		0.948
Smoking history	33 (30.2%)		31 (30.4%)		2 (28.6%)		0.919
	Mean ± SD *(range)*	n	*Mean* ± SD *(range)*	n	*Mean* ± SD *(range)*	n	p-*Value*[b]
Age (years)	44.1 ± 15.2 (13–79)	109	43.4 ± 15.4 (13–79)	102	54.7 ± 6.2 (49–68)	7	**0.026**
IgG4 (mg/dL)	48.3 ± 44.0 (3–266)	109	39.6 ± 27.6 (3–132)	102	175.0 ± 44.5 (136–266)	7	NA
IgG (mg/dL)	1275.7 ± 298.0 (774–2928)	104	1262.4 ± 288.0 (774–2928)	97	1459.7 ± 392.6 (910–2012)	7	0.179
IgG4/IgG (%)	3.8 ± 3.4 (0.3–21.2)	104	3.2 ± 2.2 (0.3–11.5)	97	12.7 ± 4.5 (7.6–21.2)	7	**< 0.001**
Thyroid size on ultrasound (mm^2)[c]	961.3 ± 771.6 (279–4358)	59	962.7 ± 788.9 (279–4358)	54	946.1 ± 622.3 (315–1689)	5	0.957
Degree of hypoechogenicity[d]	0.71 ± 0.93 (0–3)	62	0.61 ± 0.89 (0–3)	56	1.66 ± 0.81 (1–3)	6	**0.005**
Increase of color Doppler flow[e]	1.30 ± 0.90 (0–3)	62	1.33 ± 0.88 (0–3)	56	1.00 ± 1.09 (0–3)	6	0.293
TSH (mIU/L)	0.73 ± 3.03 (< 0.003–25.07)	107	0.68 ± 3.10 (< 0.003–25.07)	100	1.38 ± 1.74 (< 0.003–4.39)	7	ND[f]
fT3 (pg/mL)	9.09 ± 7.59 (1.72–30.0)	107	9.12 ± 7.41 (1.72–30.0)	100	8.63 ± 10.51 (2.52–30.0)	7	0.231
fT4 (ng/dL)	2.25 ± 1.20 (0.44–6.29)	108	2.27 ± 1.19 (0.44–6.29)	101	1.91 ± 1.52 (0.81–4.97)	7	0.212
TSAb (%)	670.8 ± 721.1 (92–3024)	42	648.1 ± 648.8 (92–2320)	38	843.6 ± 1234.3 (214–3024)	4	0.493
TRAb (IU/L)	15.7 ± 26.5 (< 1.0–187.8)	107	16.1 ± 27.2 (< 1.0–187.8)	101	9.6 ± 8.3 (1.3–19.8)	6	0.498
TgAb (IU/mL)	619.3 ± 1190.9 (< 10–4000)	77	536.7 ± 1096.1 (< 10–4000)	70	1445.2 ± 1808.9 (< 10–4000)	7	0.338
TPOAb (IU/mL)	225.5 ± 224.1 (< 5–600)	75	221.0 ± 216.1 (< 5–600)	68	269.9 ± 309.0 (< 5–600)	7	0.812

p-Values were obtained using [a]Fisher's exact test, or [b]Mann–Whitney *U*-test, and *p*-values < 0.05 were accepted as significant, shown in bold.

[c]Thyroid size was measured as the sum of both lobes according to the following equation: anteroposterior × transversal diameters (mm^2) at the maximum position.

[d]Degree of low echogenicity in the thyroid gland was determined by ultrasonography as follows; Grade 0, diffuse high-amplitude echoes throughout the whole lobe of the thyroid; Grade 1, low-amplitude and ununiformed echoes in the whole or several regions of the thyroid; Grade 2, several sonolucent regions in the thyroid; and Grade 3, no apparent echoes or very low-amplitude echoes throughout the whole thyroid.

[e]Increase of color Doppler flow in the thyroid gland was determined as follows: 0, none; 1, mild; 2, moderate; and 3, severe.

[f]Since most TSH levels were undetectable, comparison of TSH levels in the two groups was not determined.

Data for TSH, TRAb, TgAb, and TPOAb were analyzed with log-transformed values.

Values of < 0.003, < 1.0, < 5, < 10, > 30.0, > 600, and > 4000 were calculated as 0, 1.0, 5, 10, 30.0, 600, and 4000 respectively.

AITD, autoimmune thyroid disease; fT3, free triiodothyronine; fT4, free thyroxine; NA, not applicable; ND, not determined; SD, standard deviation; TgAb, thyroglobulin autoantibodies; TPOAb, thyroid peroxidase antibodies; TRAb, thyroid stimulating hormone receptor antibody; TSAb, thyroid stimulating antibody; TSH, thyrotropin.

TABLE 2. CLINICAL PROFILES OF GD PATIENTS WHO HAD ELEVATED SERUM IGG4 LEVELS

Patient	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Age (years)	54	52	49	68	51	53	56
Sex	F	F	F	M	F	F	F
Proptosis	R17, L15	None	R17, L18	R17, L20	None	None	None
Diplopia	None	None	None	Present	None	None	None
NOSPECS	3a	2b	3a	4b	2a	2b	1
CAS	1	2	0	2	0	0	0
Orbital MRI	ND	Muscle, fat	ND	Muscle, fat	ND	ND	ND
TSH (mIU/L)	<0.003	3.053	0.614	0.007	<0.003	4.397	1.601
fT3 (pg/mL)	15.48	2.87	2.52	3.51	>30	2.87	3.19
fT4 (ng/dL)	2.94	0.81	1.11	1.24	4.97	1.35	0.97
TRAb (IU/L)	19.8	3.7	1183	1.4	13.3	1.6	17.5
TSAb (%)	ND	3024	86	214	614	280	ND
TgAb (IU/mL)	169.6	<10	>4000	<10	1393	>4000	533.5
TPOAb (IU/mL)	7.7	<5	>600	33.4	43.5	>600	>600
IgG4 (mg/dL)	136	142	153	159	179	190	266
IgG (mg/dL)	910	1324	2012	1395	1954	1369	1254
IgG4/IgG (%)	14.9	10.7	7.6	11.4	9.2	13.9	21.2
Thyroid size in US (mm^2)	ND	315.4	1495	ND	815	416	1689.1
Hypoechogenicity on US	1	1	3	ND	2	2	1
Requirement of ATD doses to control hyperthyroidism after normalization of thyroid function tests	MMI 5 mg/day	MMI 5 mg/day	None (became hypothyroid and supplemented with L-T4 25 μg/day)	MMI 2.5 mg/day	PTU 50 mg/day	None (became hypothyroid and supplemented with L-T4 100 μg/day)	MMI 5 mg/day + L-T4 25 μg/day

NOSPECS and CAS are the severity and activity classifications of Graves' ophthalmopathy respectively. Exophthalmoses in the right and left eyes measured with the Hertel exophthalmometer are represented as (R, mm) and (L, mm) respectively. In the orbital MRI, the presence of extraocular muscles enlargement and the increase of orbital fat are indicated.

ATD, antithyroid drugs; MMI, thiamazole; ND, not determined; PTU, propylthiouracil.

The mean age of the elevated IgG4 group was significantly higher than that of the nonelevated IgG4 group: 54.7 ± 6.2 years (range 49–68) versus 43.4 ± 15.4 years (range 13–79) respectively ($p = 0.026$). The number of patients with GO (CAS ≥ 1) who had a family history of AITD or who were smokers was not significantly different between the two groups (Table 1). Serum TSH, fT3, fT4, TRAb, TgAb, and TPOAb levels did not significantly differ between the elevated and nonelevated IgG4 groups (Table 1). Ultrasound examinations revealed that the elevated IgG4 group had significantly more hypoechogenic areas in the thyroid compared to the nonelevated IgG4 group (low echo scoring: 1.66 ± 0.81 (range 1–3) vs. 0.61 ± 0.89 (range 0–3)) respectively ($p = 0.005$). No significant differences were observed in thyroid size or increase of color Doppler flow between the two groups. We also analyzed the patients based on the IgG4/IgG ratio (ratio ≥ 8%). The elevated IgG4/IgG ratio group had significantly increased hypoechogenic areas in the thyroid in comparison to the nonelevated IgG4 group ($p = 0.031$), although the ages of the elevated IgG4/IgG ratio group were not significantly higher than those of the nonelevated IgG4/IgG ratio.

Clinical features of patients with elevated IgG4

The clinical characteristics of the seven GD patients with elevated IgG4 are summarized in Table 2. Of these, six patients had an IgG4/IgG ratio of ≥ 8%, and five had GO (patients 1, 2, 3, 4, and 6). Patient 4 had diplopia; patients 1, 3, and 4 had mild proptosis (17–20 mm); patients 2 and 4 had swollen extraocular muscles and increased orbital fat; patient 6 showed palpebral swelling. TRAb levels were elevated in all seven cases. In patient 3, who spontaneously became hypothyroid in the follow-up of Graves' hyperthyroid patients treated with a maintenance dose (5–15 mg) of methimazole for more than 10 years, TRAb levels were rapidly and remarkably increased in spite of negative TSAb activity, suggesting the presence of blocking-type TRAb. Nonetheless, this patient showed persistent thyroid enlargement with broad hypoechoic areas in both lobes (Fig. 1). The TRAb activity of patient 6 was initially 35.1% as determined by a 1st generation TRAb assay (normal value <10%) when she was thyrotoxic (fT4 4.2 ng/dL). TgAb and/or TPOAb levels were elevated in most cases except for patient 2. In the elevated IgG4 group, patients were treated with a small dose of ATD (patients 1, 2, 4, and 5), MMI and combined L-T4 (patient 7), or L-T4 alone (patients 3 and 6) one year after ATD treatment (Table 2). None of the patients had pretibial myxedema.

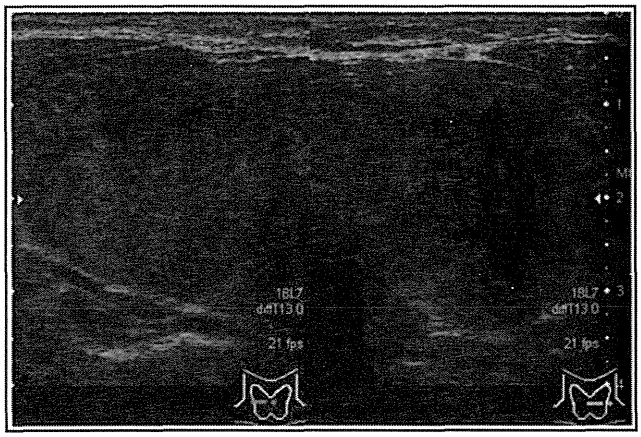

FIG. 1. An ultrasonographic image of patient 3. Note that hypoechoic areas are seen throughout both lobes of the thyroid gland.

Correlation between serum IgG4 and IgG levels and ratios and other clinical parameters

The TSAb titer correlated significantly with both serum IgG4 levels ($r_s = 0.385$, $p = 0.012$, $n = 42$) and IgG4/IgG ratios ($r_s = 0.346$, $p = 0.027$, $n = 41$). In addition, in patients with untreated hyperthyroidism ($n = 42$), TSAb titers also significantly correlated with both serum IgG4 levels ($r_s = 0.519$, $p = 0.039$, $n = 16$) and IgG4/IgG ratios ($r_s = 0.568$, $p = 0.022$, $n = 16$). The TPOAb titer correlated significantly with serum IgG levels ($r_s = 0.2495$, $p = 0.037$, $n = 70$), but not with IgG4 levels or IgG4/IgG ratios. No significant correlation of serum IgG4, IgG levels, and the ratios of IgG4/IgG were observed with age, ultrasound findings (thyroid size, low echogenicity, Doppler flow), thyroid hormone levels (fT3, fT4), or TgAb titer. IgG4 levels or IgG4/IgG ratios were not correlated with IgG levels either.

Comparisons of clinical profiles between patients with intractable and tractable GD

Since patients with elevated serum IgG4 appeared to be responsive to ATD or prone to be hypothyroid, patients with GD were divided into two subgroups: group 1 (intractable patients, $n = 39$) and group 2 (tractable patients, $n = 18$). Although not significant, serum IgG4 (70.5 ± 75.1 mg/dL (range 4–266)) and the ratio of IgG4/IgG (5.8 ± 6.1 mg/dL (range 0.4–21.2)) in group 2 tended to be slightly higher than those in group 1 (IgG4 levels 47.2 ± 29.6 mg/dL (range 3–132); IgG4/IgG ratios 3.7 ± 2.4 (0.3–11.5)). In contrast, serum IgG levels were almost identical within the two groups (group 1 1284.6 ± 237.8 (782–1735); group 2 1219.6 ± 308.9 (774–2928)).

We also classified patients by CAS: patients with CAS = 0 ($n = 80$), patients with CAS = 1 or 2 ($n = 17$), and patients with CAS ≥ 3 ($n = 12$). Serum IgG4, IgG levels, and the ratio of IgG4/IgG were not significantly different between the three groups. We also examined orbital magnetic resonance imaging (MRI) scans and NOSPECS in patients with GD, but no clear associations between serum IgG4 levels with findings of orbital MRI or NOSPECS (9) were observed (data not shown).

Next, correlations between serum IgG4, IgG levels, or ratios of IgG4/IgG and other clinical parameters were separately examined in two groups. No obvious correlations were observed in group 1. In group 2, low echoic scores were significantly positively correlated with serum IgG levels ($r_s = 0.851$, $p = 0.001$, $n = 12$) but not IgG4. TPOAb titer was significantly correlated with both serum IgG4 levels ($r_s = 0.576$, $p = 0.031$, $n = 14$) and IgG levels ($r_s = 0.637$, $p = 0.019$, $n = 13$), but not with IgG4/IgG ratios.

Discussion

In the current study, a novel subgroup of patients with GD and elevated serum IgG4 level was identified (6.4% of overall

GD patients; Table 1). Yamamoto *et al.* reported that in healthy controls ($n=21$), the average serum IgG4 level was 43 ± 31 mg/dL and the ratio of IgG4/IgG was $2.9 \pm 1.8\%$ (12). On the basis of these values, in this report, patients with GD demonstrated a higher serum IgG4 level and IgG4/IgG ratios than healthy controls. Moreover, 6.4% of patients had elevated serum IgG4 levels (≥ 135 mg/dL), which meets the comprehensive diagnostic criteria of IgG4-RD.

The elevated IgG4 group did not demonstrate any male predominance (Table 1). This finding is neither consistent with previous reports on IgG4 thyroidits (3), nor with those on IgG4-RD (2). The average age of patients in the elevated IgG4 group was significantly higher than that in the nonelevated IgG4 group, which is similar to that observed in IgG4-RD (2). In contrast, IgG4 thyroiditis is associated with a younger age (3), although the prevalence of HT increases with age (13). Since serum IgG4 levels do not increase with age (14) and ages were not found to be correlated with IgG4 levels, unknown factors other than aging may contribute to the elevation in IgG4 levels.

TgAb elevation is associated not only with IgG4 thyroiditis (3), but also with IgG4-related thyroiditis (6). In the present study, serum TgAb levels tended to be higher, albeit not significantly, in the elevated IgG4 group than in the nonelevated IgG4 group (Table 1). Given that IgG4 is a dominant subtype of TgAb in patients with GD (15–17), TgAb may be at least partly a source of IgG4 in patients 1, 3, and 5–7 with TgAb elevation (Table 2). The presence of TPOAb is also associated with GD. In fact, a significant positive correlation between TPOAb and IgG4 or IgG levels in tractable patients.

The TSH receptor (TSH-R) is a crucial antigen for GD (13). Weetman *et al.* reported that the IgG subclass in TRAb is restricted to IgG1 (18). In contrast, Latrofa *et al.* reported that TRAb were affinity enriched on recombinant TSHR antigen before IgG subclass analysis (19). Of three sera samples processed, one contained IgG1 only, one IgG1 + IgG4, and one only IgG4. Since they studied sera selected for very high TRAb levels, this finding suggests that long-term antigen stimulation may increase TRAb concentrations and eventually lead to subclass switching from IgG1 to IgG4. M22, a stimulating monoclonal anti-TSH-R antibody (TRAb), is found to be IgG1 (20). Although IgG subclasses in TRAb were not investigated in our study, a positive correlation between IgG4 or the ratio of IgG4/IgG and TSAb was observed in overall patients with GD. Patient 2 showed strong TSAb activity in spite of negative TgAb or TPOAb, suggesting that TSAb is at least partly present in the IgG4 fraction. Patient 3, who rapidly changed to hypothyroidism and possessed persistent thyroid enlargement (Fig. 1), is quite similar to recent cases reported independently by Nishihara *et al.* and Kawashima *et al.* (21,22). Thus, both in stimulating and blocking TRAbs, IgG4-positive plasma cells may be involved in the pathogenesis of GD.

The exchange of IgG half-molecules among IgG4 results in bispecific characteristics (23). Notably, McLachlan *et al.* showed that in patients with GO and elevated TRAb, an IgG4 shift toward TgAb was observed (24). We speculate that IgG4 could have a bispecific nature and consist of TgAb and TRAb. Considering that the levels of TgAb and TPOAb, as well as TRAb, are elevated in GD (13), these autoantibodies may be related to bispecific IgG4 molecules. Another biological relevance of this exchange of half-molecules in IgG4 is that it generates antibodies that inhibit formation of large immune complexes and immune inflammation by IgGs of other subclasses (23). For example, Guo *et al.* reported that the IgG1 but not the IgG4 subfraction of TPOAb is associated with antibody-dependent cytotoxicity (25).

In the present study, ultrasound examination revealed that the elevated IgG4 group had significantly more hypoechoic areas than the nonelevated IgG4 group (Table 1), as similarly observed in IgG4 thyroiditis (3). Moreover, low echo scoring was positively correlated with serum IgG4 levels. Since hypoechoic areas reflect lymphocyte infiltration and fibrosis in the thyroid gland (10), this area may be related to lymphoplasmacytes that produce IgG4 in the thyroid. In the elevated IgG4 group, disease was controlled with low doses of ATD or treatment with ATD and/or L-T4 one year following the first visit (Table 2). In cases of HT, the degree of hypoechoic areas correlated with the prevalence of hypothyroidism, resulting from fibrosis (26). Thus, the clinical course of patients with IgG4 elevation may be related to fibrosis on the basis of ultrasonographic findings, which resemble the alterations found in HT. In this context, the relationship between IgG4 and Hashitoxicosis (27,28), which has concurrent features of GD and HT, should be studied in the future.

There are several limitations of our study. First, there was a lack of pathologic studies performed in the patients. Second, the patients showed an absence of manifestations of other organs in IgG4-RD. Third, the number of patients with elevated IgG4 is rather small. For this reason, there is some concern for either a type I error (false positive) or a type II error (false negative). Finally, information on alterations in serum IgG4 levels during treatment and clinical course was not collected. As a future study, longitudinal follow-up studies to examine pathophysiological relations and therapeutic outcomes are warranted.

In conclusion, the current study proposes a novel subgroup of patients with GD who show elevated serum IgG4 levels. In comparison to the majority of patients with GD, these patients were older, had more hypoechoic areas, and may be responsive to ATD or prone to be hypothyroid after ATD treatment. IgG4-RD generally shows positive response to steroid therapy. In patients with GD and elevated serum IgG4 levels, steroid therapy should be considered to avoid adverse effects of ATD, radioiodine treatment, or surgical intervention. Thus, measurement of serum IgG4 levels may help to distinguish this new entity and may offer diagnostic and potential therapeutic options for GD. Further investigations on the relationship between GD and IgG4-RD using a larger patient population and longer observation times are warranted.

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