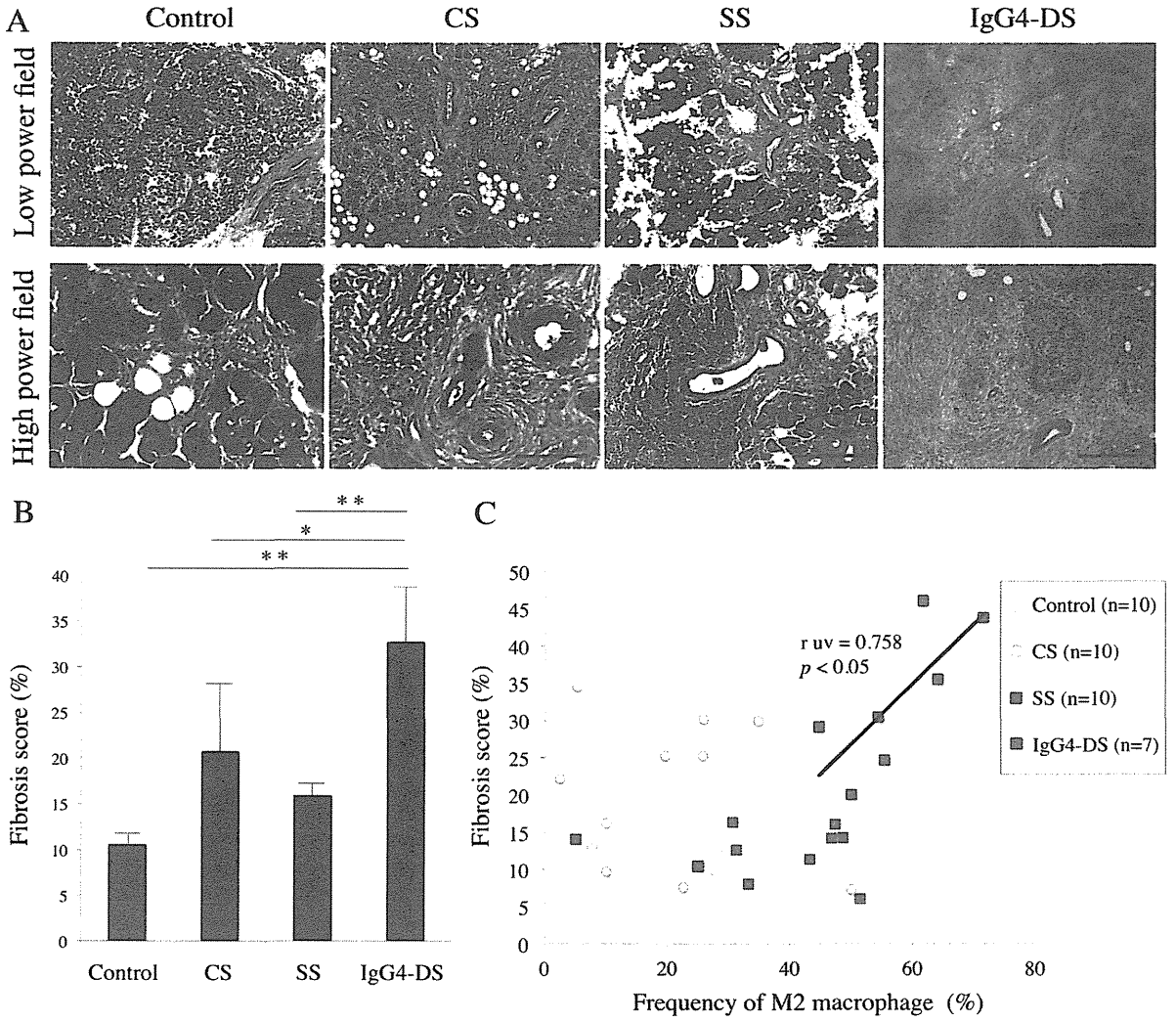

Figure 4 Correlation between M2 macrophages and fibrosis in SMGs. (A) Sections were stained by Masson's trichrome (MT) staining from controls, patients with CS, SS, and IgG4-DS. MT staining stained nuclei (purple), cytoplasm (red) and collagen (connective tissue or fibrosis) (blue) as described in the Materials and methods section. Lower magnifications are displayed in the upper line, and the higher magnifications are displayed in the lower line. Scale bars, 200 μm (lower power field) and 50 mm (high power field). (B) Evaluation of fibrosis score in the SMGs from controls (n = 10), patients with CS (n = 10), SS (n = 10), and IgG4-DS (n = 7). The fibrosis score was calculated from MT staining as described in the Materials and methods section. Statistically significant differences between groups were determined by one-way ANOVA (*$p < 0.05$, **$p < 0.01$). (C) Correlation between frequencies of M2 macrophage and fibrosis score. Statistical significance of differences between groups was determined by Spearman's rank correlation ($p < 0.05$).

CD68 and CD163 in these organs was strongly detected in/around areas of fibrosis with results similar to those obtained from SMGs (Fig. 5).

4. Discussion

IgG4-RD is now recognized as a systemic disorder, characterized by high serum IgG4, marked infiltration of IgG4-positive plasma cells and severe fibrosis with hyperplastic eGCs in swollen lesions [11]. Although our previous data revealed that Th2 adaptive immune responses induced IgG4 production and eGC formation in IgG4-RD [13,14], the mechanism of fibrosis in lesions remains to be fully elucidated. Recent studies have reported that macrophages might play a critical role in IgG4 production in IgG4-RD [15,24]. As outlined above, macrophages can be classified as M1 and M2 macrophages based on their response to the extracellular environment [25]. Notably, M2 macrophages are activated by Th2 cytokines and promote fibrosis by the production of pro-fibrotic factors (CCL18, IL-10 and IL-13) [26]. We therefore examined the M1 and M2 macrophage subsets in SMGs from SS, CS, and IgG4-DS patients. Immunohistochemical staining indicated that expression of CD163 (M2 macrophage marker) was strongly detected in IgG4-DS patients, whereas it was rarely detected in controls, CS and SS patients. These findings were mirrored

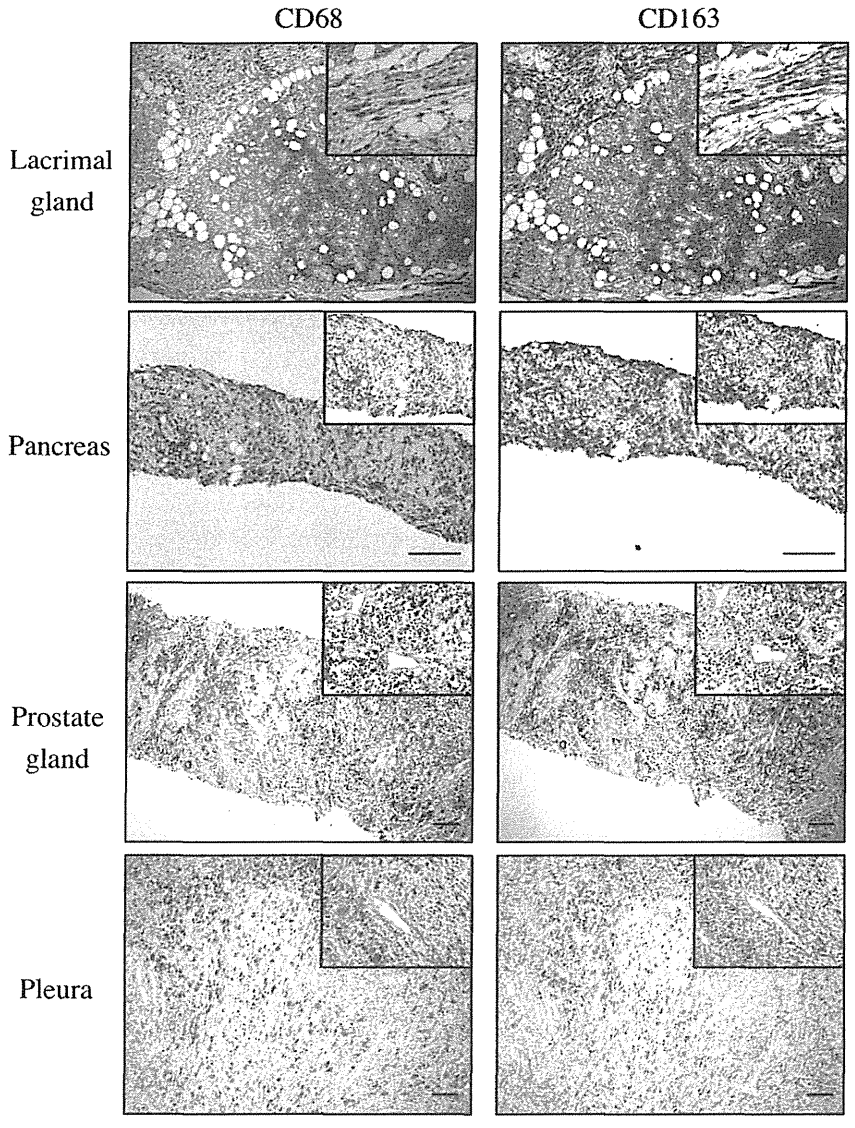

Figure 5 Distribution of M1 and M2 macrophages in the other involved organs from patients with IgG4-DS including the lacrimal gland, pancreas, prostate gland, and pleura. Counterstaining was performed with Mayer's hematoxylin (blue). The higher magnifications are displayed at the upper right. Scale bars, 100 μm.

in other lesions including the lacrimal gland, pancreas, pleura, and prostate gland from IgG4-RD patients. Interestingly, the cell number and frequency of M2 macrophage in IgG4-DS were significantly higher than those in the other groups. To reveal the relationship between M2 macrophages and fibrosis in SMGs from IgG4-DS patients, we also analyzed the expression of pro-fibrotic factors such as CCL18, IL-10, and IL-13. The expression patterns of CCL18 and IL-10 in SMGs were similar to those of M2 macrophages in patients with IgG4-DS. In contrast, IL-13 was mainly expressed on other cells such as Th2 and mast cells in patients with IgG4-DS. In this study the mRNA expression levels of CD163 and pro-fibrotic factors (CCL18, IL-10, and IL-13) in SMGs from patients with IgG4-DS were higher than those in the other groups. Furthermore, the fibrosis scores were positively correlated with the frequency of M2 macrophages only in patients with IgG4-DS. These results suggest that preferential M2 macrophages might be involved in fibrosis of swollen lesions from IgG4-RD patients through the production of CCL18 and IL-10. On the other hands, Th2 and mast cells might promote fibrosis through the production of IL-13 without involving M2 macrophages.

IL-10 is well known as an immunosuppressive cytokine, produced mostly by macrophages, Th2 cells, regulatory T cells (Tregs) and dendritic cells (DCs), and is essential for the maintenance of immunological self-tolerance and immune homeostasis. Several studies have reported that IL-4 and IL-10 are overexpressed locally in the lesions of IgG4-RD patients and contribute to IgG4-specific class switching [13,27,28]. With regard to fibrosis, IL-10 was identified as an endogenous inhibitor of tissue fibrosis [29,30], while recent studies demonstrated that IL-10 produced by M2

macrophage had an important role in the renal and lung fibrosis [31,32]. In this study, the localization of IL-10 in SMGs from IgG4-DS patients was consistent with that of production by M2 macrophages, suggesting that the majority of IL-10 producing cells might be M2 macrophages and deeply involved in fibrosis. In contrast, other IL-10 producing cells such as Treg and plasmacytoid DC were also infiltrated in salivary glands from IgG4-DS patients. However, these cells were detected in/around GCs rather than around fibrotic lesions, and might promote plasma cells to IgG4 production (manuscript in preparation).

CCL18 is one of the most highly expressed chemokines in chronic inflammatory diseases including idiopathic pulmonary fibrosis [33], bronchial asthma [34] and atopic dermatitis [35]. In addition, CCL18 is now recognized as a chemokine with not only fibrotic activity but also selective chemotactic activity on peripheral blood T lymphocytes, especially Th2 cells [36,37]. Although IgG4-RD is considered a Th2-dominant disease [12], the mechanism of Th2 polarization has yet to be elucidated. Considering the current results, M2 macrophages might also contribute to Th2 polarization of IgG4-RD by CCL18 production. However, additional research is required to further elucidate the involvement of innate immunity in the pathogenesis of IgG4-RD.

Fibrosis associated with a part of IgG4-RD including AIP, IgG4-SC and IgG4-TIN has a characteristic irregular whorled pattern, termed "storiform fibrosis" [19]. In this study, we have confirmed that the production of IL-10 and CCL18 by preferential M2 macrophages is associated with severe fibrosis in IgG4-RD. However, the relationship between M2 macrophage and this fibrotic pattern in salivary glands was not distinctive change, and requires more consideration for other organs. Recent studies reported that rituximab targeting peripheral CD20-positive plasma cells appeared to be an effective treatment strategy for IgG4-RD [38,39]. In addition, circulating plasmablasts, derived from the B cell lineage, are also elevated in IgG4-RD patients, especially with resistance to rituximab [40,41]. This is attributed to the fact that plasmablasts lack surface expression of CD20 and demonstrate a resistance to direct depletion by rituximab. Therefore, IgG4-positive plasmablasts are considered to be prominently involved in the pathogenesis of IgG4-RD. Interestingly, after interacting with antigens exposed on macrophages, marginal zone B cells rapidly differentiate into plasmablasts [42]. These results suggest that macrophages might play an effective role in plasmablast activation of IgG4-RD. A more thorough understanding of the immune mechanism of IgG4-RD, especially the role of innate immune cells, could lead to the development of novel pharmacological strategies aimed at disrupting the recruitment of inflammatory cells to the local lesion and inhibiting the initiation of IgG4-RD.

Conflict of interest

The authors declare no competing interests.

Author contributions

All authors provided substantial contributions to discussions of content, and to reviewing and editing the manuscript before submission. M Moriyama researched the data and wrote the article.

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DNA Microarray Analysis of Labial Salivary Glands in IgG4-Related Disease

Comparison With Sjögren's Syndrome

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Objective. **To compare gene expression in labial salivary glands (LSGs) from patients with IgG4-related disease with that in LSGs from patients with Sjögren's syndrome (SS).**

Methods. **Gene expression was analyzed by DNA microarray in LSG samples from 5 patients with IgG4-related disease, 5 SS patients, and 3 healthy controls. Genes differentially expressed in IgG4-related disease and SS were identified, and gene annotation enrichment analysis of these differentially expressed genes was performed using Gene Ontology (GO) annotation. Validation of the results was performed by quantitative polymerase chain reaction (PCR) using LSG samples from 9 patients with IgG4-related disease, 10 SS patients, and 4 controls.**

Results. **Gene expression patterns in patients with IgG4-related disease, SS patients, and healthy controls were quite different from each other in hierarchical clustering as well as in principal components analysis. In IgG4-related disease compared with SS, a total of 1,771 probe sets (corresponding to 1,321 genes) were identified as up-regulated, and 1,785 probe sets (corresponding to 1,320 genes) were identified as down-regulated (false discovery rate of <5%). GO term analysis indicated that the up-regulated set of differentially expressed genes in IgG4-related disease encoded proteins that function in cell proliferation, extracellular matrix organization, and organ development. PCR validated significantly higher expression of lactotransferrin in patients with IgG4-related disease than in SS patients ($P < 0.05$) and significantly higher expression of CCL18 in patients with IgG4-related disease than in SS patients and controls ($P < 0.05$).**

Conclusion. **The results clearly showed that the gene expression pattern in LSGs from patients with IgG4-related disease is different from that in LSGs from SS patients.**

IgG4-related disease is a new disease entity characterized by high serum IgG4 levels as well as infiltration of IgG4+ plasmacytes and fibrosis in various organs, such as the pancreas, bile duct, salivary and lacrimal glands, thyroid, lung, liver, kidney, prostate, aorta, retroperitoneum, and lymph nodes (1,2). Although the clinical features, serum abnormalities, organ involvement, diagnosis, and therapeutic approach have been

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reported recently (1,2), the pathogenesis of this disease remains unclear, including the roles of high IgG4 levels and IgG4+ plasmacytes and the molecular mechanism involved in the up-regulation of IgG4 class-switch recombination and fibrosis.

Sjögren's syndrome (SS) is an autoimmune disease that affects exocrine glands, including salivary and lacrimal glands. It is characterized pathologically by lymphocytic infiltration into exocrine glands and clinically by dry mouth and eyes. Several autoantibodies, such as anti-SSA and anti-SSB antibodies, are detected in patients with SS.

IgG4-related disease and SS have recently been compared with regard to their epidemiologic features, symptoms, organ involvement, serum abnormalities including IgG4 levels, anti-SSA and anti-SSB antibodies, pathologic findings, and responses to glucocorticoid therapy (1). Although both diseases affect the salivary glands, their clinical and pathologic features differ. For example, patients with IgG4-related disease have milder xerostomia despite significant enlargement of the salivary glands, have much more IgG4+ plasmacytic infiltration and fibrosis, and show better response to glucocorticoid therapy than do patients with SS (1).

These clinical studies indicate that the pathogenesis of these 2 diseases might differ. To clarify the difference in pathogenesis, we compared gene expression in labial salivary glands (LSGs) from patients with IgG4-related disease and patients with SS using DNA microarray analysis.

PATIENTS AND METHODS

Study population. LSG samples were obtained from 5 Japanese patients with IgG4-related disease as well as from 5 Japanese patients with SS and 3 healthy controls who had been followed up at the University of Tsukuba Hospital (Ibaraki, Japan), Tokyo Women's Medical University Hospital (Tokyo, Japan), and Kyushu University Hospital (Fukuoka, Japan). All patients with IgG4-related disease satisfied the diagnostic criteria for this disease proposed in 2011 by the All Japan IgG4 team (3). The diagnosis of IgG4-related disease was based on the presence of all of the following: 1) characteristic diffuse/localized swelling or masses in single or multiple organs on clinical examination, 2) elevated serum IgG4 concentrations (≥135 mg/dl) on hematologic examination, and 3) marked lymphocyte and plasmacyte infiltration and fibrosis as well as infiltration of IgG4+ plasma cells (>40% of IgG+ cells that are also IgG4+ and >10 IgG4+ plasma cells/high-power field) on histopathologic examination. All LSG samples from patients with IgG4-related disease had histopathologic features of IgG4-related disease.

All patients with SS satisfied the 1999 Japanese Ministry of Health criteria for the diagnosis of SS (4). These criteria included 4 clinicopathologic findings: lymphocytic infiltration of the salivary or lacrimal glands, dysfunction of salivary secretion, keratoconjunctivitis sicca, and presence of anti-SSA or anti-SSB antibodies. The diagnosis of SS was based on the presence of ≥2 of the above 4 items. Moreover, all 5 SS patients also satisfied the 2012 American College of Rheumatology (ACR) classification criteria for SS (5) because all patients were positive for anti-SSA or anti-SSB antibodies and had focal lymphocytic sialadenitis with a focus score of ≥1 focus/4 mm^2 on LSG biopsy. Approval for this study was obtained from the local ethics committee, and signed informed consent was obtained from each subject.

RNA isolation and quality control. Total RNA from LSG samples in RNAlater was isolated using TRIzol reagent (Invitrogen Life Technologies) and then purified using an RNeasy micro kit (Qiagen) in accordance with standard protocols provided by the manufacturers. Following total RNA isolation, the sample concentration and RNA integrity were assayed, and samples from all 13 subjects were considered appropriate for DNA microarray analysis.

DNA microarray experiments and data analysis. Total RNA samples (150 ng) were prepared and processed for microarray analysis using a GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) according to the standard protocol supplied by the manufacturer. All of the microarray data are in a Minimum Information About a Microarray Experiment (MIAME)–compliant format and have been deposited in a MIAME-compliant database, the NCBI GEO (accession no. GSE40568; http://www.ncbi.nlm.nih.gov/geo/), as detailed on the FGED Society web site (http://www.fged.org/projects/miame/).

The obtained microarray data were normalized by Factor Analysis for Robust Microarray Summarization (FARMS) algorithm (6) using statistical language R (http://www.r-project.org/) (7) and Bioconductor (http://www.bioconductor.org/) (8). Global gene expression profiles of all samples were evaluated with hierarchical clustering (pvclust method [9]) and principal components analysis (PCA) (10) using FARMS-normalized all-DNA microarray data. To identify the genes up- and down-regulated in IgG4-related disease compared with SS in pairwise comparisons, the rank products method (11) was applied to the FARMS-normalized data. Probe sets with a false discovery rate (FDR) of <5% were regarded as having different expression levels between the 2 groups (i.e., differentially expressed). Gene annotation enrichment analysis of these differentially expressed genes was performed by Gene Ontology (GO) annotation using the web tool DAVID (12). The flow chart of GO terms was constructed using the web tool QuickGO (13).

Quantitative polymerase chain reaction (PCR). Quantitative PCR was also performed to validate the results of DNA microarray analysis. Total RNA was extracted from LSG samples from 9 Japanese patients with IgG4-related disease, 10 Japanese patients with SS, and 4 healthy controls who were not the same as the subjects analyzed by DNA microarray. All patients with IgG4-related disease satisfied the diagnostic criteria proposed in 2011 by the All Japan IgG4 team (3), and all LSG samples from these patients had histopathologic features of IgG4-related disease. All SS patients satisfied the 1999 Japanese Ministry of Health criteria for the diagnosis of SS (4) as well as the 2012 ACR classification criteria for SS (5).

Table 1. Clinical and pathologic features of patients with IgG4-related disease, patients with SS, and healthy controls analyzed by DNA microarray*

Disease or control, subject no./age	Sample ID no.	Serum IgG4, mg/dl	Infiltrating IgG4+ plasma cells among IgG+ plasma cells in LSGs, %	Greenspan histopathologic grade in LSGs
IgG4-related disease				
1/57	T197	543	80	ND
2/61	T222	492	67	ND
3/56	T226	499	79	ND
4/58	T243	562	83	ND
5/61	T257	512	83	ND
SS				
1/22†	T187	ND	ND	3
2/37†	T188	ND	ND	3
3/43‡	T213	ND	ND	3
4/27‡	T228	ND	ND	3
5/36†	T258	ND	ND	3
Healthy control				
1/65	T180	ND	ND	ND
2/38	T182	ND	ND	ND
3/23	T277	ND	ND	ND

* All subjects were women and had not been treated with corticosteroids, immunosuppressive agents, or biologic agents. LSGs = labial salivary glands; ND = not determined.
† Primary Sjögren's syndrome (SS).
‡ Secondary SS.

Complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (Takara Bio). The messenger RNA (mRNA) expression level of the target gene was examined by quantitative PCR. Human GAPDH served as an internal control.

Moreover, we compared mRNA expression levels of validated differentially expressed genes by quantitative PCR between LSGs and lacrimal glands in 3 patients with IgG4-related disease other than the patients analyzed by DNA microarray, to examine the expression of differentially expressed genes in an organ other than the LSG. Lacrimal glands are often involved simultaneously with salivary glands in IgG4-related disease, and patients with both lacrimal and salivary gland involvement are said to have IgG4-related Mikulicz disease (1). These 3 patients with IgG4-related disease also satisfied the diagnostic criteria proposed in 2011 by the All Japan IgG4 team (3), and all LSG and lacrimal gland samples from these patients had histopathologic features of IgG4-related disease.

Statistical analysis. Differences between 3 groups were examined for statistical significance using the Kruskal-Wallis test. Differences between LSG and lacrimal gland samples were examined using the Wilcoxon signed rank test. P values less than 0.05 were considered significant.

RESULTS

Clinical and pathologic features of patients with IgG4-related disease or SS and healthy controls. Table 1 summarizes the clinical and pathologic features of participating patients and healthy controls analyzed by DNA microarray. All 5 patients with IgG4-related disease had high serum IgG4 levels and infiltration of IgG4+ plasma cells in LSGs (>40% of IgG+ cells were also IgG4+). All subjects were women and had not been treated with corticosteroids, immunosuppressive agents, or biologic agents. The mean ± SD age of patients with IgG4-related disease (58.6 ± 2.3 years) was significantly higher than that of SS patients (33.0 ± 8.4 years) ($P < 0.05$). On the other hand, there was no significant difference in mean ± SD age between healthy controls (42.0 ± 21.3 years) and the 2 disease groups. Of 5 SS patients, 3 had primary SS that was not associated with other well-defined connective tissue diseases (CTDs), and the other 2 had secondary SS that was associated with other well-defined CTDs.

Regarding the validation study by quantitative PCR analysis, there was no significant difference in mean ± SD age between the 3 groups (for patients with IgG4-related disease, 61.2 ± 13.3 years; for SS patients, 45.5 ± 15.2 years; for healthy controls, 61.8 ± 21.2 years) (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract). Of 10 SS patients, 6 had primary SS and the other 4 had secondary SS.

Gene expression patterns in patients with IgG4-related disease, SS patients, and healthy controls. Figure 1 shows hierarchical clustering by the pvclust method and PCA using FARMS-normalized data. The gene

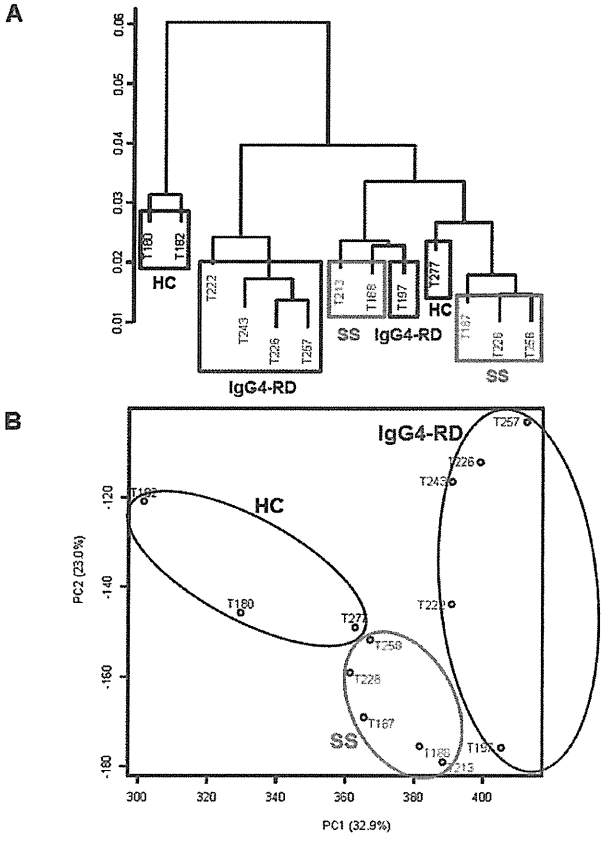

Figure 1. Hierarchical clustering and principal components analysis for gene expression in patients with IgG4-related disease (IgG4-RD), patients with Sjögren's syndrome (SS), and healthy controls (HC) using Factor Analysis for Robust Microarray Summarization–normalized data. The gene expression patterns in the 3 groups were different in hierarchical clustering (**A**) and in principal components analysis (**B**). In hierarchical clustering, the vertical scale represents between-cluster distances. In principal components analysis, the contribution of principal component (PC) 1 was 32.9%, and that of PC2 was 23.0%. Samples T197, T222, T226, T243, and T257 are from patients with IgG4-related disease. Samples T187, T188, T213, T228, and T258 are from SS patients. Samples T180, T182, and T277 are from healthy controls. Color figure can be viewed in the online issue, which is available at http:onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract.

expression patterns in the 3 groups were quite different in hierarchical clustering as well as in PCA. In hierarchical clustering, 1 patient with IgG4-related disease (sample T197) and 1 healthy control (sample T277) were involved in the same cluster as SS patients. On the other hand, in PCA, all 3 groups were divided into 3 different clusters. Importantly, the 1 patient with IgG4-related disease (sample T197) and the 1 healthy control (sample T277) who were involved in the same cluster as SS patients in hierarchical clustering were located near the SS cluster in PCA. Although the gene expression pattern of these 2 subjects might be close to that of SS patients, the gene expression patterns of the 3 groups might differ from one another.

Genes differentially expressed between IgG4-related disease and SS. Because we clarified that gene expression patterns in patients with IgG4-related disease and SS patients differed from those in healthy controls by clustering analysis, we next compared gene expression and identified genes differentially expressed between IgG4-related disease and SS in pairwise comparisons. A total of 1,771 probe sets (corresponding to 1,321 genes) were identified as up-regulated in IgG4-related disease compared with SS by the rank products method, with an FDR of 5% (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract). On the other hand, 1,785 probe sets (corresponding to 1,320 genes) were identified as down-regulated in IgG4-related disease compared with SS (see Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract). These FDR values were theoretically zero only in calculation of the rank products statistic. The values represented not absoluteness, but reliability of differentially expressed genes (14).

Gene annotation enrichment analysis of differentially expressed genes by GO annotation. Supplementary Table 4 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract) shows significantly enriched GO terms (FDR-corrected $P < 0.0001$) found in 1,771 highly expressed probe sets in IgG4-related disease compared with SS analyzed by the web tools DAVID and QuickGO. Gene annotation enrichment analysis by GO annotation showed that the up-regulated set of differentially expressed genes in IgG4-related disease encoded proteins that function in various biologic processes, such as wound healing, response to inorganic substance, skeletal system development, muscle organ development, heart development, angiogenesis, cell morphogenesis involved in differentiation, cell projection organization, muscle contraction, extracellular matrix organization, actin cytoskeleton organization, cell–matrix adhesion, regulation of cell migration, regulation of cell–substrate adhesion, positive regulation of cell adhesion, regulation of cell proliferation, enzyme-linked receptor protein signaling pathway, regulation of inflammatory response, and translational elongation. On the

other hand, gene annotation enrichment analysis by GO annotation showed that the down-regulated set of differentially expressed genes in IgG4-related disease encoded proteins that function in protein glycosylation, immune response, antigen processing and presentation of peptide antigen via class I major histocompatibility complex, Golgi vesicle transport, cotranslational protein targeting to membrane, endoplasmic reticulum unfolded protein response, and response to virus (see Supplementary Table 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract).

Validation by quantitative PCR. For validation by quantitative PCR, we selected 10 of the top 120 genes up-regulated in IgG4-related disease compared with SS (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract) using the following procedure. We needed to restrict the validation genes to only 10 differentially expressed genes because of the limited amount of total RNA extracted from small LSGs. We picked up differentially expressed genes with higher rank (higher rank than 80), smaller FDR (<0.01%), higher fold change (>1.5), higher expression level, smaller dispersion between samples, and functional relationship with immune/inflammatory response, fibrosis, chemotaxis, and cell proliferation. We chose 2 immune/inflammatory response–related genes (lactotransferrin [LTF, rank 2] and collectin subfamily member 12 [COLEC12, rank 28]), 4 fibrosis-related genes (decorin [DCN, rank 5], lymphatic vessel endothelial hyaluronan receptor 1 [LYVE1, rank 12], epidermal growth factor–containing fibulin-like extracellular matrix protein 1 [EFEMP1, rank 21], and fibronectin 1 [FN1, rank 27]), 3 chemokines (CXCL12, rank 7; CXCL14, rank 10; and CCL18, rank 71), and 1 cell proliferation–related gene (retinoic acid receptor responder 1 [RARRES1, rank 18]). GAPDH was appropriate for the internal control, because the expression of GAPDH was the same between IgG4-related disease and SS (FDR >5%) in this DNA microarray analysis.

Expression of mRNA for CCL18 was significantly higher in patients with IgG4-related disease than in SS patients and controls ($P < 0.05$). On the other hand, expression levels of the chemokines CXCL12 and CXCL14 did not differ significantly between the 3 groups. The expression level of LTF was significantly higher in IgG4-related disease than in SS ($P < 0.05$). Although the expression levels of the other 6 genes (COLEC12, DCN, LYVE1, EFEMP1, FN1, and RARRES1) were higher in patients with IgG4-related disease than in SS patients and controls, the differences between groups did not reach statistical significance (Figure 2). Thus, the higher expression levels of CCL18 and LTF in IgG4-related disease than in SS were validated by quantitative PCR.

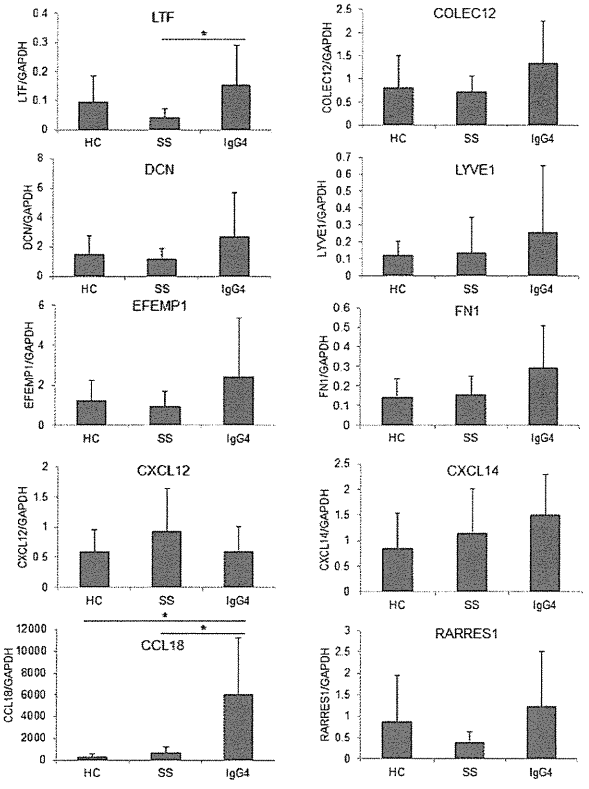

Figure 2. Quantitative polymerase chain reaction (PCR) analysis for validation. Quantitative PCR analysis was performed using labial salivary gland samples from 9 Japanese patients with IgG4-related disease (IgG4), 10 Japanese patients with Sjögren's syndrome (SS), and 4 healthy controls (HC) who were not the same as the subjects analyzed by DNA microarray. Values are the mean ± SD. * = $P <$ 0.05 by Kruskal-Wallis test.

Comparison of expression of validated differentially expressed genes between LSGs and lacrimal glands in IgG4-related disease. We compared mRNA expression levels of CCL18 and LTF by quantitative PCR between LSGs and lacrimal glands in 3 patients with IgG4-related disease. Although CCL18 was expressed more highly in LSGs than in lacrimal glands, expression levels of both CCL18 and LTF did not differ significantly between LSGs and lacrimal glands (see Supplementary Figure 1, available on the *Arthritis &*

Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract).

DISCUSSION

Recent studies in rheumatology, immunology, gastroenterology, nephrology, ophthalmology, respirology, and other fields have focused on IgG4-related disease. The clinical features and diagnostic and therapeutic approaches are being clarified gradually by many physicians and scientists working in these fields (1–3). However, the pathogenesis of this disease remains unclear, including the roles of high IgG4 levels and IgG4+ plasmacytes and the molecular mechanism involved in the up-regulation of IgG4 class-switch recombination and fibrosis.

Higher proportions of Th2 cells and Treg cells and increased production levels of Th2 and Treg cell cytokines in tissues from patients with IgG4-related disease, such as sclerosing pancreatitis and cholangitis (15), sialadenitis (16,17), and tubulointerstitial nephritis (18) have been reported. Importantly, it is reported that Th2 cytokines (interleukin-4 [IL-4] and IL-13) and Treg cell cytokines (IL-10) can induce IgG4- and IgE-specific class-switch recombination (19,20) and that transforming growth factor β (TGFβ), a Treg cell cytokine, can induce tissue fibrosis (21). Thus, overproduction of IL-4, IL-13, IL-10, and TGFβ could contribute to the pathogenesis of IgG4-related disease, including a high serum IgG4 level, IgG4+ plasmacytic infiltration, and fibrosis. More recently, we confirmed by quantitative PCR that mRNA expression levels of Treg cell cytokines (IL-10 and TGFβ) were significantly higher in LSGs from patients with IgG4-related disease than in those from SS patients and controls (22). Using quantitative PCR assay, we also confirmed overexpression of activation-induced cytidine deaminase (AID) in LSGs from patients with IgG4-related disease compared with those from SS patients and healthy controls (22). AID is essential for nonspecific immunoglobulin class-switch recombination (from IgM to IgG1, IgG2, IgG3, IgG4, IgA, and IgE) (23–25); thus, up-regulation of AID could contribute to up-regulation of IgG4-specific class-switch recombination along with IL-10 in LSGs from patients with IgG4-related disease.

In this study, we used DNA microarray analysis to compare gene expression in LSGs from patients with IgG4-related disease, SS patients, and healthy controls. The results showed that the gene expression patterns in these 3 groups were quite different in the clustering of FARMS-normalized DNA microarray data. This finding reconfirmed that, in addition to the differences in clinical and pathologic features, the pathogenesis of sialadenitis differs between IgG4-related disease and SS. The gene annotation enrichment analysis revealed that the up-regulated set of differentially expressed genes in IgG4-related disease encoded proteins that function in various biologic processes, such as cell proliferation, extracellular matrix organization, and organ development. These processes might be involved in the pathogenesis of IgG4-related disease.

On the other hand, gene annotation enrichment analysis showed that the down-regulated set of differentially expressed genes in IgG4-related disease encoded proteins that function in antigen processing and presentation. Moreover, down-regulated differentially expressed genes in IgG4-related disease (equal to up-regulated differentially expressed genes in SS) included many interferon (IFN)–inducible genes (see Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38748/abstract), consistent with our previous study, which showed higher expression of IFN-inducible genes in LSGs of SS patients than in those of controls by DNA microarray (26). Thus, these findings indicated that IgG4-related disease might be "a cells and extracellular matrix proliferative disease," while SS is an autoimmune disease related to IFN signaling.

Although there was no significant difference in mean age between healthy controls and the other 2 groups, the mean age of patients with IgG4-related disease analyzed by DNA microarray was significantly higher than that of SS patients. A previous study confirmed that gene expression pattern of salivary glands differed depending on age (27). Therefore, we should consider the possible effects of age difference on differential expression between IgG4-related disease and SS.

In the present study, we also validated overexpression of LTF and CCL18 in LSGs from patients with IgG4-related disease compared with that in LSGs from SS patients by quantitative PCR assay. Lactotransferrin is an abundant iron-binding protein in milk. Lactotransferrin has a wide range of functions such as antibacterial and antiviral activities, anticancer activities, wound healing, fibroblast proliferation, and bone growth, as well as iron binding (28). Moreover, lactotransferrin was recently shown to stimulate the maturation of dendritic cells (DCs) and to recruit various leukocytes (28). Thus, lactotransferrin might play pathogenic roles in the generation of IgG4-related disease through inducing activation of innate immune responses as well as fibroblast proliferation.

CCL18 is a CC chemokine ligand expressed mainly on a broad range of monocyte/macrophages and DCs (29). Various factors, including lipopolysaccharide (LPS), CD40L, IL-4, and IL-10, can stimulate the production of CCL18 from these cells (29). Interestingly, Toll-like receptor 4 (TLR-4), which is a receptor for LPS, was also significantly up-regulated in IgG4-related disease compared with SS in our DNA microarray analysis (rank 658, FDR 0.7%) (data not shown). Previous in vitro biologic activity studies showed chemotactic activity for CCL18 on various T cells (CD4+ helper T cells, CD8+ cytotoxic T cells, naive T cells, and memory T cells), B cells (naive B cells and germinal center B cells), and immature DCs, and also showed the induction of collagen production from lung fibroblasts (29). A recent report also indicated that CCL18 recruited human Treg cells (30). Furthermore, enhanced CCL18 production has been demonstrated in several human diseases, including various malignancies and inflammatory joint, lung, skin, and vessel diseases (29). A recent study also showed that serum CCL18 concentrations reflected fibroinflammatory activity and extent of disease in patients with chronic periaortitis (31).

Importantly, the above earlier findings suggested that CCL18 overexpression might be pathogenically important in IgG4-related disease, as follows. First, CCL18 could induce tissue fibrosis, which is a pathognomonic feature of IgG4-related disease. Second, CCL18 could recruit Treg cells, which might play a pathogenic role via production of Treg cell cytokines, into the affected organs of patients with IgG4-related disease. Third, CCL18 might be related to chronic periaortitis, which is one of the important organ involvements in IgG4-related disease (1,2).

The results of the present DNA microarray analysis and our previous quantitative PCR studies (22) allow us to formulate the following pathogenic process in IgG4-related disease (Figure 3). First, certain factors, such as lactotransferrin and LPS, activate the innate immune response, including macrophages and DCs, by stimulation of TLR-4. Then the activated innate cells produce various chemokines, such as CCL18, with chemotactic activities on various T cells (including Treg cells) and B cells. Second, infiltrating Treg cells in the affected organs produce Treg cell cytokines (IL-10 and TGFβ). Third, IL-10 from Treg cells cooperates with up-regulated AID to induce IgG4/IgE-specific class-switch recombination. On the other hand, TGFβ could cooperate with lactotransferrin to induce tissue fibrosis. IL-10 also stimulates innate cells and induces the production of CCL18 from these cells. Thus, these processes form a positive feedback loop via IL-10.

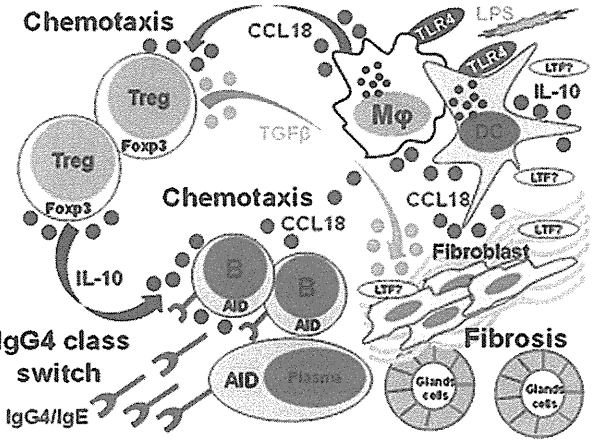

Figure 3. Possible pathogenic mechanism of IgG4-related disease. LTF? indicates that pathogenic roles of lactotransferrin are currently a subject of speculation. TGFβ = transforming growth factor β; TLR-4 = Toll-like receptor 4; LPS = lipopolysaccharide; Mφ = macrophage; DC = dendritic cell; IL-10 = interleukin-10; AID = activation-induced cytidine deaminase.

These biologic and immunologic processes might explain the characteristic clinical and pathologic features of IgG4-related disease, such as high serum IgG4 levels, infiltration of IgG4+ plasmacytes, and fibrosis of various organs. Further experiments on validation of other differentially expressed genes in DNA microarray need to be performed to further clarify the pathogenesis of IgG4-related disease. Moreover, we need to examine the mRNA expression levels of lactotransferrin and CCL18 in many more patients, and we need to determine the protein expression levels and perform functional assays to confirm the pathogenic importance of these molecules.

In conclusion, DNA microarray analysis in this study showed that the gene expression pattern in LSGs from patients with IgG4-related disease was different from that in LSGs from SS patients, suggesting different pathogenic mechanisms of IgG4-related disease and SS.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sumida had full access to all of

the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tsuboi, Nakai, Iizuka, Asashima, Hagiya, Tsuzuki, Hirota, Miki, Hagiwara, Kondo, Tanaka, Moriyama, Matsumoto, Nakamura, Yoshihara, Abe, Sumida.

Acquisition of data. Tsuboi, Nakai, Iizuka, Asashima, Hagiya, Tsuzuki, Hirota, Miki, Hagiwara, Kondo, Tanaka, Moriyama, Matsumoto, Nakamura, Yoshihara, Abe, Sumida.

Analysis and interpretation of data. Tsuboi, Nakai, Iizuka, Sumida.

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Clinical Paper
Oral Medicine

The diagnostic utility of biopsies from the submandibular and labial salivary glands in IgG4-related dacryoadenitis and sialoadenitis, so-called Mikulicz's disease

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Abstract. IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) is characterized by serum IgG4 elevation and the infiltration of IgG4-positive plasma cells in glandular tissues. For definitive diagnosis of IgG4-DS, biopsies of local lesions are recommended to exclude Sjögren's syndrome (SS), malignant tumours, and similar disorders. In this study, we examined the diagnostic utility of submandibular gland (SMG) and labial salivary gland (LSG) biopsies in IgG4-DS. Fourteen patients presenting with swelling of the SMG (eight females and six males) underwent both SMG and LSG biopsies. The sensitivity, specificity, and accuracy of SMG biopsies were all 100.0%. In contrast, those of LSG biopsies were 69.2%, 100.0%, and 71.4%, respectively. Thirty-three out of 61 LSG biopsies (54.1%) from all 14 patients were positive for the diagnostic criteria of IgG4-DS (IgG4-positive/IgG-positive plasma cells >0.4). None of the patients experienced complications such as facial nerve palsy, sialocele, or hyposalivation. The IgG4/IgG ratio showed no significant correlation between the LSG and SMG. The final diagnosis was IgG4-DS in 13 patients and marginal zone B-cell lymphoma (MZL) in one. These results suggest that incisional biopsy of the SMG is useful and appropriate for the definitive diagnosis of IgG4-DS, while diagnosis by LSG biopsy alone requires more caution.

Keywords: IgG4-related dacryoadenitis and sialoadenitis; Mikulicz's disease; IgG4-related disease; labial salivary gland biopsy; submandibular gland biopsy.

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In the past, Mikulicz's disease has been considered a subtype of Sjögren's syndrome (SS) based on histopathological similarities between the two diseases.[1] However, Mikulicz's disease has a number of differences compared with typical SS, which include the following: (1) differing gender distribution (Mikulicz's disease occurs in both men and women, while SS occurs mainly in women); (2) persistent enlargement of lacrimal and salivary glands; (3) normal salivary secretion or mild secretory dysfunction; (4) good responsiveness to corticosteroid treatment; (5) hypergammaglobulinemia and low frequency of anti-Sjögren's syndrome SS-A and SS-B antibodies on serological analyses; and (6) multiple germinal centre (GC) formations in glandular tissue.[2]

Previously, we reported that SS was characterized by periductal lymphocytic infiltration with atrophy or severe destruction of the acini, while Mikulicz's disease showed non-periductal lymphocytic infiltration with hyperplastic GCs and mild destruction of the acini.[3–5] Additionally, Yamamoto et al.[6–8] reported that patients with Mikulicz's disease showed elevation of serum IgG4 and infiltration of IgG4-positive plasma cells in lacrimal and salivary glands. Similar findings have been observed in autoimmune pancreatitis (AIP),[9] sclerosing cholangitis,[10] tubulo-interstitial nephritis,[11] Riedel's thyroiditis,[12] and Küttner's tumour.[13] These diseases are now referred to as IgG4-related disease (IgG4-RD).[6,14] We have previously described the concept of IgG4-RD and provided up-to-date information regarding this emerging disease entity.[15] Mikulicz's disease falls into this category and can be alternatively termed IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS).[16]

We have recently proposed comprehensive diagnostic criteria for IgG4-RD and diagnostic criteria for IgG4-related Mikulicz's disease.[17] Accordingly, IgG4-RD is now diagnosed using comprehensive diagnostic criteria combined with organ-specific criteria. Both groups of these diagnostic criteria particularly recommend the biopsy of local lesions because of the high associated sensitivity and specificity. In cases of IgG4-DS presenting with swelling of the submandibular gland (SMG), a submandibulectomy has generally been performed for definitive diagnosis of IgG4-DS. However, this invasive procedure often leads to postoperative complications, including bleeding, facial nerve palsy, amblygeustia, and hyposalivation. In this study, we evaluated incisional biopsies of the SMG and labial salivary gland (LSG) as less invasive procedures than submandibulectomy for the diagnosis of IgG4-DS.

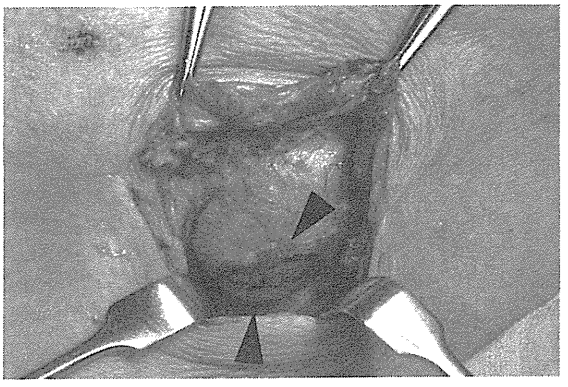

Fig. 1. Submandibular gland (SMG) incisional biopsy procedure: (1) an incision of approximately 3 cm in length was made just underneath the SMG; (2) the platysma muscle was incised to disclose the SMG capsule; (3) a spindle-shaped incision of approximately 1 cm in length was made postero-inferior to the SMG, and the biopsy specimen (arrowhead) was extracted; (4) the SMG capsule was closed with absorbent sutures; and (5) the skin was closed with nylon sutures.

Materials and methods

Patients

This study included 14 patients who met the IgG4 criteria before biopsy (six men and eight women; mean age 64.9 ± 9.4 years); these patients presented with bilateral swelling of the SMGs and elevated serum IgG4 (>135 mg/dl). They were referred to the department of oral and maxillofacial surgery of the university hospital, a tertiary care centre, between 2009 and 2014 with complaints of SMG swelling. IgG4-DS was diagnosed according to the following criteria[17]: (1) persistent (longer than 3 months) symmetrical swelling of more than two lacrimal and major salivary glands; (2) elevated serum levels of IgG4 (>135 mg/dl); and (3) infiltration of IgG4-positive plasma cells in the tissue (IgG4-positive plasma cells/IgG-positive plasma cells >0.4) by immunostaining. For a positive IgG4-DS diagnosis, at least two of these criteria must be met, to include item 1. Additionally, other disorders, including sarcoidosis, Castleman's disease, Wegener's granulomatosis, lymphoma, and cancer, must be excluded. All patients failed to meet the American College of Rheumatology Classification Criteria for Sjögren's syndrome[18] or the criteria proposed by the American–European Consensus Group for Sjögren's syndrome.[19]

LSG and SMG biopsies

We performed both LSG and SMG biopsies at the same time. LSG biopsies were performed as described by Greenspan et al.[20] We judged IgG4-DS to be present even if just one of the LSG specimens from an individual showed infiltration of IgG4-positive plasma cells (IgG4-positive plasma cells/IgG-positive plasma cells >0.4). Incisional biopsies of swollen SMGs were performed under local anaesthesia. The method is shown in Fig. 1.

Salivary flow rate

The stimulated whole saliva (SWS) flow rate was measured by Saxon test. This test was performed by having subjects chew Surgeon Type IV gauze sponges (Hakuzo Medical Corporation, Osaka, Japan) once per second for 2 min and then measuring the weight of the gauze. If the change in the gauze weight was less than 2 g, the subject's salivary flow rate was regarded to be 'significantly decreased'.[21]

Statistical analysis

The statistical significance of differences between two groups was determined using the unpaired Student's *t*-test, Fisher's exact test, and Spearman's rank correlation. A *P*-value of <0.05 was considered significant. All statistical analyses were performed using JMP software version 8 (SAS Institute, Japan).

The study design was approved by the institutional ethics committee and all participants provided written informed consent.

Results

Clinical findings

Table 1 shows the clinical characteristics of the 14 cases presenting with high serum

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Table 1. Clinical characteristics of the 14 cases presenting with bilateral swelling of submandibular glands and high serum IgG4.

No.	Age	Sex	Disease duration	Complication	Swollen glands				Complaint		Gum test (g/10 min)	Schirmer's test (R/L) (mm/5 min)	Serological test							Ratio of IgG4+ cells		Final diagnosis
					LG	PG	SMG	SLG	LSG	Dry mouth	Dry eyes			RF (IU/ml)	ANA	IgG (mg/dl)	IgG4 (mg/dl)	IgA (mg/dl)	IgM (mg/dl)	Anti SS-A	Anti SS-B	SMG (%)	LSG (%)	
1	65	M	5 m	Hydronephrosis	–	–	+	–	+	–	–	12.0	ND	20>	–	3142[a]	1700[a]	128	59	–	–	86.7[a]	96.9[a]	IgG4-DS
2	57	F	6 m	AIP, SC	+	–	+	–	–	–	–	9.8	3/3	20>	–	1842[a]	748[a]	187	63	–	–	86.4[a]	55.2[a]	IgG4-DS
3	61	F	3 m	AIP, SC	–	–	+	–	+	+	+	6.3	3/1	ND	–	2891[a]	1080[a]	187	63	–	–	57.2[a]	79.6[a]	IgG4-DS
4	64	F	1 y	AIP	–	+	+	–	–	+	+	ND	ND	61[a]	2+[a]	2585[a]	456[a]	305	53	–	–	65.7[a]	87.1[a]	IgG4-DS
5	79	F	10 y	AIP, SC	–	+	+	–	–	+	+	ND	ND	20>	–	2430[a]	896[a]	182	53	–	–	60.8[a]	54.9[a]	IgG4-DS
6	60	F	1 y	AIP, SC	–	–	+	–	–	+	+	11.6	ND	20>	–	2087[a]	490[a]	276	74	–	–	55.4[a]	84.7[a]	IgG4-DS
7	69	M	5 m	AIP	–	+	+	+	–	–	–	12.0	ND	20>	–	2090[a]	484[a]	225	40	–	–	52.4[a]	12.7	IgG4-DS
8	76	F	4 m	DM	–	+	+	–	–	+	+	5.3	4/9	ND	–	2381[a]	823[a]	187	52	–	–	60.1[a]	22.3	IgG4-DS
9	79	F	3 m	–	–	+	+	–	–	–	–	ND	ND	20>	–	2608[a]	896[a]	132	68	–	–	66.5[a]	17.5	IgG4-DS
10	46	F	6 m	Chronic thyroiditis	+	–	+	+	+	+	–	8.1	ND	20>	–	2200[a]	769[a]	244	78	–	–	55.2[a]	54.3[a]	IgG4-DS
11	66	M	5 y	–	+	–	+	–	–	–	–	17.2	3/3	20>	–	2121[a]	344[a]	167	45	–	–	56.3[a]	2.2	IgG4-DS
12	61	M	3 y	Pulmonary nodules	+	+	+	+	–	–	–	6.8	3/1	20>	–	7603[a]	2290[a]	121	62	–	–	51.2[a]	89.1[a]	IgG4-DS
13	55	M	5 m	–	+	–	+	+	–	+	–	7.4	ND	20>	–	2332[a]	617[a]	241	74	–	–	86.4[a]	54.3[a]	IgG4-DS
14	70	M	4 m	–	–	+	+	–	–	+	–	15.1	ND	20>	–	1177	215[a]	179	91	–	–	4.5	10.0	MZL

LG, lacrimal gland; PG, parotid gland; SMG, submandibular gland; SLG, sublingual gland; PLG, palatal gland; LSG, labial salivary gland; M, male; F, female; m, months; y, years; R, right; L, left; RF, rheumatoid factor; ANA, antinuclear antibody; Anti SS-A, anti-Sjögren's syndrome SS-A antibodies; Anti SS-B, anti-Sjögren's syndrome SS-B antibodies; ND, not done; IgG4-DS, IgG4-related dacryoadenitis and sialoadenitis; AIP, autoimmune pancreatitis; SC, sclerosing cholangitis; DM, diabetes mellitus; MZL, marginal zone B-cell lymphoma.

[a] Higher than normal values.

IgG4 (>135 mg/dl) and bilateral swelling of the SMGs over the course of 3 months. The final diagnosis based on SMG and LSG biopsies was IgG4-DS in 13 patients and marginal zone B-cell lymphoma (MZL) in one. Seven out of 13 patients with IgG4-DS (53.8%) had a history of other IgG4-RD including AIP (six cases), sclerosing cholangitis (four cases), and chronic thyroiditis (one case). All of the patients with IgG4-DS and MZL were negative for anti-Sjögren's syndrome SS-A and SS-B antibodies, and serum IgA and IgM levels were within normal limits.

Histological findings in the SMG and LSG specimens

Representative histological findings in the SMG and LSG specimens from IgG4-DS and MZL patients are shown in Figs 2 and 3, respectively. In IgG4-DS patients, all of the SMG specimens showed strong lymphocytic infiltration with hyperplastic GCs, mild destruction of the acini, and selective infiltration by IgG4-positive plasma cells (IgG4-positive plasma cells/IgG-positive plasma cells >0.4) (Fig. 2). In contrast, although some LSG specimens showed similar histological findings (33 out of 56 LSGs from the 13 patients with IgG4-DS), other specimens (23 out of 56 LSGs) showed mild lymphocytic infiltration (IgG4-positive plasma cells/IgG-positive plasma cells ≤0.4) without GCs (Fig. 2). We observed variations between LSG specimens even from the same patient.

In contrast, in the patient with MZL, both SMG and LSG specimens (five LSGs) showed severe lymphocytic infiltration with hyperplastic GCs. Immunohistochemical staining showed strong B-cell infiltration, mild infiltration of IgG4-positive plasma cells (IgG4-positive plasma cells/IgG-positive plasma cells, 10%), and monotypic predominance of lambda-light chains (only in the SMG specimen). These histopathological findings and clinical features confirmed the diagnosis as MZL (Fig. 3).

Diagnostic utility and complications of SMG and LSG biopsies

The sensitivity, specificity, and accuracy of SMG biopsies were all 100.0%. In contrast, those of LSG biopsies were 69.2%, 100.0%, and 71.4%, respectively (Table 2). There were no complications of SMG or LSG biopsies including nerve paralysis or paresis, anaesthetic sequelae, haematoma, sialocele, wound infection,

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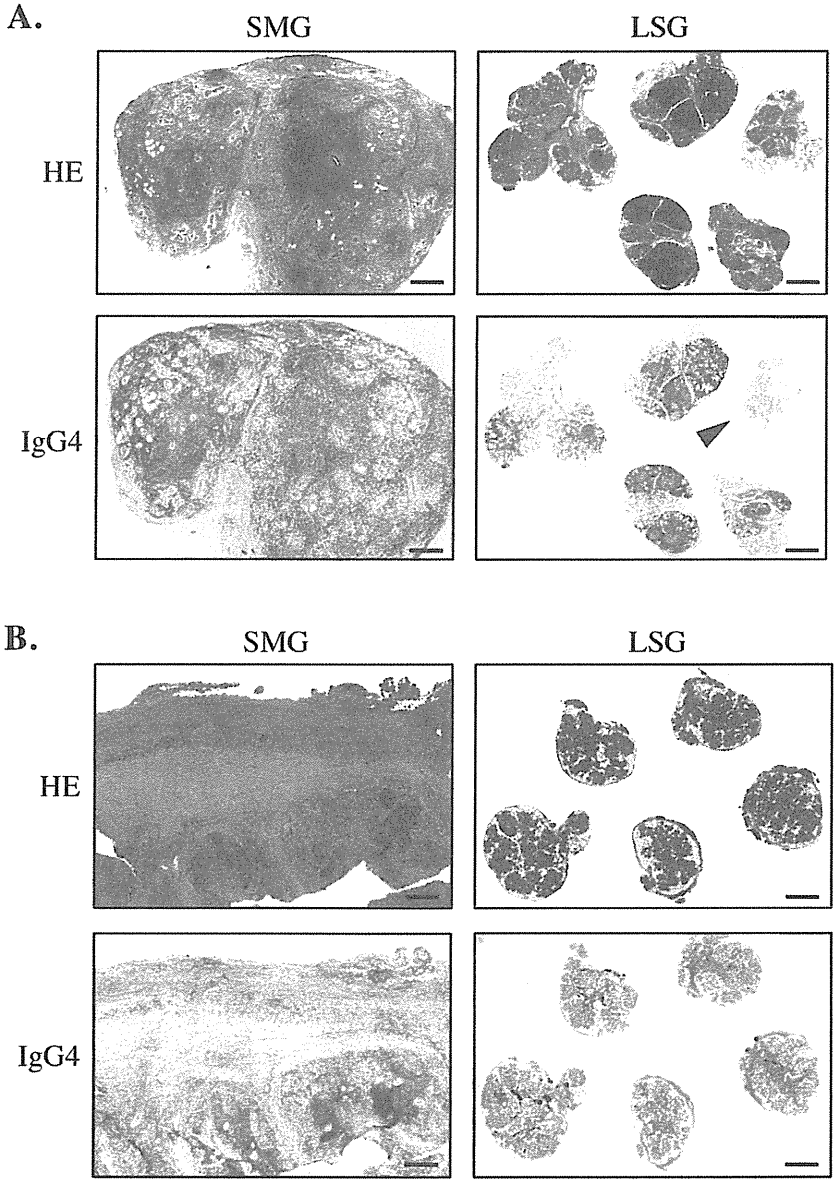

Fig. 2. Histological findings in submandibular gland (SMG) and labial salivary gland (LSG) specimens from patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). (A) Both SMG and LSG specimens showed selective infiltration of IgG4-positive plasma cells with hyperplastic germinal centres (GCs); only one LSG showed no lymphoid infiltration (arrowhead). (B) SMG specimens showed selective infiltration of IgG4-positive plasma cells with GCs, whereas only a few lymphocytic infiltrations were seen in all of the LSG specimens. Scale bars, 100 μm.

hypertrophic scars, or hyposalivation (Saxon test: pre-biopsy 4.12 ± 2.46 g/2 min; post-biopsy 4.37 ± 2.65 g/2 min).

Relationship of the frequency of IgG4-positive cells between SMG and LSG biopsies from individual IgG4-DS patients

We examined the relationship of the frequency of IgG4-positive cells (IgG4-positive plasma cells/IgG-positive plasma cells) between SMG and LSG biopsies from individual IgG4-DS patients. There was no significant correlation of the frequency of IgG4-positive cells between SMG and LSG biopsies (Fig. 4).

Discussion

Recently, several reports have described that a subset of patients with malignant tumours such as pancreatic and salivary carcinomas[22–24] and ocular adnexal lymphoma[25,26] represent IgG4-associated conditions. Furthermore, Ochoa et al.[27] reported a case of mucosa-associated lymphoid tissue (MALT) lymphoma emerging from a background of IgG4-related chronic inflammation. Therefore, definitive diagnosis of IgG4-DS via biopsy of a local lesion is recommended to select appropriate treatment. The submandibulectomy (excisional biopsy) has been a relatively standard surgical procedure for the treatment of tumours or obstructive conditions of the SMG, but it is associated with a high rate of complications such as facial nerve palsy (up to 36%), lingual nerve palsy (2–5%), and hypoglossal nerve palsy (2–5%).[28–31]

In this study, we performed incisional biopsies as an alternative diagnostic modality. Patients with bilateral swelling of the SMGs underwent SMG (local lesion) incisional biopsies under local anaesthesia without any complications. Our results suggest that incisional biopsy of the SMG is extremely useful for the diagnosis of IgG4-DS in addition to being a less invasive procedure than excisional biopsy under general anaesthesia. On the other hand, LSG biopsy may be less suitable as a single procedure because of its low sensitivity (Table 2) and poor correlation with the histology of the SMG (Fig. 4). However, as part of this study we performed LSG biopsies in AIP patients (a type of IgG4-RD) without IgG4-DS, and almost half of those patients showed selective infiltration with IgG4-positive plasma cells (IgG4-positive plasma cells/IgG-positive plasma cells >0.4) in the LSG specimens. These results indicate that a single LSG biopsy might be useful for the diagnosis of IgG4-RD in patients who are difficult to biopsy (manuscript in preparation).

Other less invasive procedures include parotid gland incisional biopsy and fine needle biopsy. Pijpe et al.[32] reported that parotid gland incisional biopsy has diagnostic potential in comparison with LSG biopsy for the diagnosis of SS. In contrast, IgG4-DS patients often show patchy infiltration by IgG4-positive cells, especially in parotid glands. Moreover, parotid glands are usually swollen accompanied by SMG or lacrimal gland swelling, which is an indication that the frequency of parotid gland swelling is significantly lower than that of SMG swelling. We previously performed parotid biopsies in several IgG4-DS patients and obtained negative results for IgG4-DS based on the very small number of IgG4-positive

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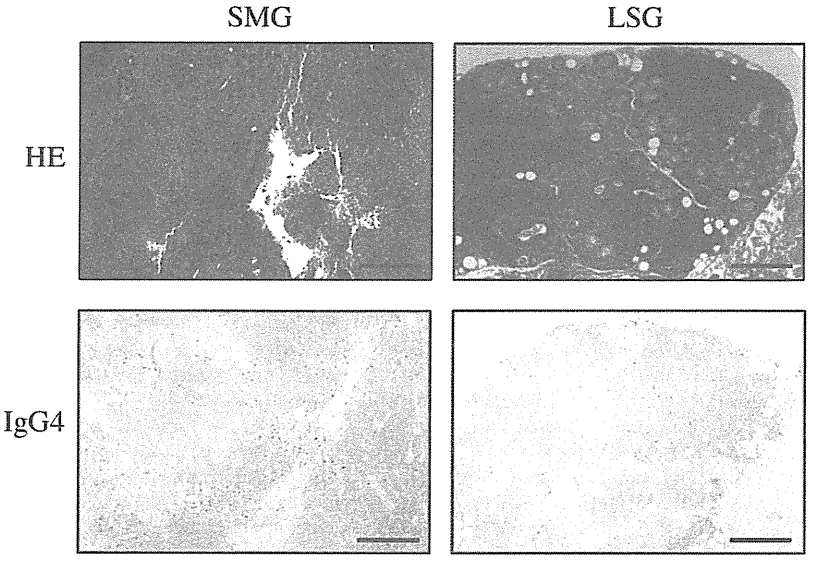

Fig. 3. Histological findings in submandibular gland (SMG) and labial salivary gland (LSG) specimens from a patient with marginal zone B-cell lymphoma (MZL). Both SMG and LSG specimens showed strong lymphocytic infiltration with hyperplastic germinal centres and slight infiltration of IgG4-positive plasma cells. Scale bars, 100 μm.

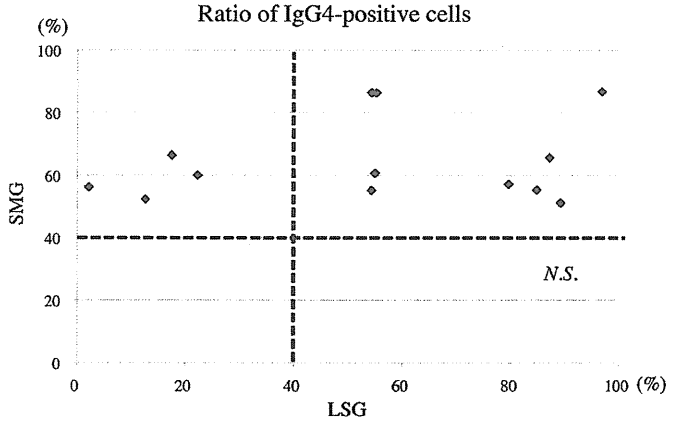

Fig. 4. Comparison of the ratio of IgG4-positive cells between SMG and LSG biopsies from individual IgG4-DS patients. The ratio was calculated as IgG4-positive cells (%) = IgG4-positive cells/IgG-positive cells × 100. The counts were obtained in 1-mm^2 sections from five different areas. The significance of differences between the groups was determined by Spearman's rank correlation. N.S., not significant.

Table 2. Diagnostic usability of submandibular gland (SMG) and labial salivary gland (LSG) biopsies.

Definitive diagnosis	Ratio of IgG4-positive cells[a]		Total
	>40%	≤40%	
SMG biopsy			
IgG4-DS	13	0	13
Non-IgG4-DS	0	1	1
Total	13	1	14
Sensitivity = 100%, specificity = 100%, accuracy = 100%			
LSG biopsy			
IgG4-DS	9	4	13
Non-IgG4-DS	0	1	1
Total	9	5	14
Sensitivity = 69.2%, specificity = 100%, accuracy = 71.4%			

IgG4-DS, IgG4-related dacryoadenitis and sialoadenitis.

[a] The ratio was calculated as IgG4-positive cells (%) = IgG4-positive cells/IgG-positive cells × 100. The counts were obtained in 1-mm^2 sections from five different areas.

cells found. Furthermore, we also tried to perform SMG fine needle biopsy in several IgG4-DS patients, but we were unable to obtain adequate samples because the SMGs were too hard. These results suggest that clinicians must carefully consider whether samples from parotid gland incisional biopsy or fine needle biopsy are appropriate when considering IgG4-DS. It is a matter of great regret that this study did not include a control group presenting with bilateral swelling of the SMGs in the presence of normal serum IgG4; unfortunately, there were no such patients in our department during the study period.

In conclusion, we have addressed the utility of SMG incisional biopsies for the diagnosis of IgG4-DS. With this procedure, IgG4-DS can be diagnosed quickly and less invasively. Therefore, the biopsy of local lesions should be considered essential for a positive IgG4-DS diagnosis as well as included in the comprehensive diagnostic criteria for IgG4-RD.

Funding

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Competing interests

None declared.

Ethical approval

The study design was approved by the Ethics Committee of Kyushu University, Japan (IRB serial number 25-287).

Patient consent

Informed consent was obtained from all patients.

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ORIGINAL ARTICLE

Clinical relevance of Küttner tumour and IgG4-related dacryoadenitis and sialoadenitis

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OBJECTIVES: Küttner tumour (KT), so-called chronic sclerosing sialoadenitis, is characterised by concomitant swelling of the submandibular glands secondary to strong lymphocytic infiltration and fibrosis independent of sialolith formation. However, recent studies have indicated that some patients with KT develop high serum levels of IgG4 and infiltration of IgG4-positive plasma cells, namely IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS), so-called Mikulicz's disease. The aim of this study was to clarify the clinical and pathological associations between KT and IgG4-DS.

MATERIALS AND METHODS: Fifty-four patients pathologically diagnosed with KT or chronic sialoadenitis were divided into two groups according to the presence or absence of sialolith (KT-S (+) or KT-S (−), respectively).

RESULTS: There were no significant differences in the clinical findings, including the mean age, sex and disease duration, between the two groups. All patients in the KT-S (+) group showed unilateral swelling without infiltration of IgG4-positive plasma cells or a history of other IgG4-related diseases (IgG4-RD), while those in the KT-S (−) group showed bilateral swelling (37.5%), strong infiltration of IgG4-positive plasma cells (87.5%) and a history of other IgG4-RD (12.5%).

CONCLUSIONS: These results suggest an association between the pathogeneses of KT-S (−) and IgG4-DS, but not KT-S (+).

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Keywords: Küttner tumour; IgG4-related dacryoadenitis and sialoadenitis; Mikulicz's disease; chronic sialoadenitis

Introduction

Küttner tumour (KT) is a non-neoplastic disease also termed chronic sclerosing sialoadenitis (CSS). It was first described by Küttner in 1896 (Küttner, 1896). This disease is characterised by acinar atrophy and lymphocytic infiltration within the unilateral submandibular gland (SMG; Yamamoto *et al*, 2006; Takano *et al*, 2010). The World Health Organization has categorised it as a tumourlike disease of the salivary glands (Seifert, 1992). Some reports have indicated that the distribution pattern of lymphocytic infiltration is induced by an immunological abnormality (Rasanen *et al*, 1972; Ikeda *et al*, 1994), while other reports have indicated that sialoliths and mucous plugs are found in 29% to 83% of lesions (Isacsson and Lundquist, 1982; Ahuja *et al*, 2003). Seifert and Donath, (1977) classified many cases of chronic sialoadenitis (CS) into four stages regardless of unilateral or bilateral swelling: (i) focal sialoadenitis, (ii) diffuse lymphocytic sialoadenitis with salivary gland fibrosis, (iii) chronic sclerosing sialoadenitis with salivary gland sclerosis and (iv) chronic progressive sialoadenitis with salivary gland cirrhosis. They also found that 41% of patients diagnosed with KT had concomitant sialolithiasis. The histopathological diagnoses in these cases were achieved by the presence of marked lymphocytic infiltration and fibrosis in the SMGs with or without sialolith. The differences between KT/CS with and without sialolith remained unclear based on these findings. In contrast, Mikulicz's disease (MD) is a unique condition characterised by bilateral enlargement of the lacrimal glands (LGs) and salivary glands secondary to lymphocytic infiltration. KT was conventionally considered to

be an aspect of MD because of the clinical similarities between these two diseases. However, Yamamoto *et al* (Yamamoto *et al*, 2005) reported that patients with MD had high levels of serum IgG4 and infiltration of IgG4-positive plasma cells in the glandular tissues. Such findings have been also identified in patients with other diseases, including autoimmune pancreatitis (AIP; Hamano *et al*, 2001), sclerosing cholangitis (Zen *et al*, 2004), retroperitoneal fibrosis (RPF; Hamano *et al*, 2002), tubulointerstitial nephritis (Hamed *et al*, 2007), Riedel's thyroiditis (Hamed *et al*, 2007) and KT (Kitagawa *et al*, 2005). These diseases are now termed IgG4-related diseases (IgG4-RD). We previously described the concept of IgG4-RD and provided up-to-date information regarding this emerging disease entity (Umehara *et al*, 2012a). MD and KT are also associated with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS; Maehara *et al*, 2012; Stone *et al*, 2012). However, the clinical relevance of KT and IgG4-DS remains to be elucidated. Therefore, the aim of this study was to determine the clinical and histopathological characteristics of KT and clarify the clinical relevance of these two diseases.

Materials and methods

Patients

This study included 54 patients (22 men and 32 women; mean age, 51.3 ± 28.3 years) pathologically diagnosed with KT or CS based on specimens obtained from extirpating SMGs. All patients were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital, Fukuoka, Japan, from 2003 to 2013. As shown in Figure 1, these patients were divided into two groups according to the presence or absence of sialolith: with sialolith [KT-S (+)] (n = 46; 20 men and 26 women; mean age, 49.8 ± 20.4 years) and without sialolith [KT-S (−)] (n = 8; two men and six women; mean age, 61.3 ± 4.6 years). The study design was approved by the Ethics Committee of Kyushu University, Japan, and all participants provided written informed consent (IRB serial number: 25-287).

Laboratory data

We retrospectively analysed the serum levels of IgG, IgG4, anti-SSA antibody and anti-SSB antibody. The serum level of IgG4 was not determined in any patients with KT-S (+).

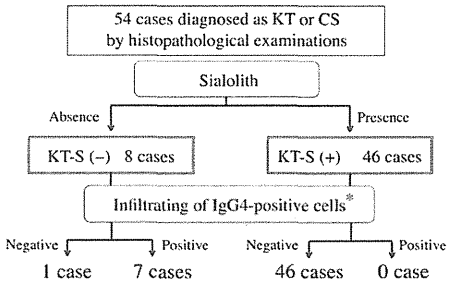

Figure 1 Classification tree performance of patients with chronic sialoadenitis (CS). KT-S (+), Küttner tumour (KT) with sialolith; KT-S (−), KT without sialolith: *IgG4-positive plasma cells/IgG-positive plasma cells >0.4

Histological and immunohistochemical analyses

For histological analysis, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained with haematoxylin and eosin. For immunohistochemical analysis, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained with a conventional avidin–biotin complex technique as previously described (Tanaka *et al*, 2012). An anti-IgG rabbit polyclonal antibody was used to analyse the molecule of IgG (A0423; Dako, Glostrup, Denmark). An anti-IgG4 mouse monoclonal antibody was used to analyse the molecule of IgG4 (MC011; Binding Site, Birmingham, UK). The sections were sequentially incubated with primary antibodies for 2.5 h and subsequently with biotinylated anti-rabbit IgG and anti-mouse IgG secondary antibodies (Vector Laboratories, California, USA), avidin–biotin–horseradish peroxidase complex (Vector Laboratories) and 3,3′-diaminobenzidine (Vector Laboratories). Mayer's haematoxylin was used for counterstaining. Photomicrographs were obtained using a light microscope equipped with a digital camera (BZ-9000; KEYENCE, Tokyo, Japan). Stained IgG-positive and IgG4-positive cells were counted in 1-mm^2 sections from five different areas, and the number of IgG4-positive cells and ratio of IgG4-positive to IgG-positive cells were calculated. Evaluations of immunostaining were conducted by two pathologists (T K and H S) who were blinded to information on the samples. Positive cells were counted randomly, and the data of T K and H S were averaged.

Histological staining for evaluation of fibrosis

To evaluate the degree of fibrosis, Masson's trichrome staining (MT; Polysciences, Warrington, PA, USA) was performed. In short, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained. As a result, connective tissue and fibrosis tissue can be selectively visualised as blue, whereas nuclei stained by Weigert's iron haematoxylin marked as dark brown to black, and cytoplasm marked as red.

Statistical analysis

Values are given as mean ± standard deviation. The means of two groups were compared with Student's t-test, Fisher's test or the Mann–Whitney U-test, as appropriate. Values of $P < 0.05$ were regarded as statistically significant.

Results

Clinical findings

Table 1 shows the clinical characteristics of the eight patients in the KT-S (−) group. All patients were negative for anti-SS-A and anti-SS-B antibodies. Two of the eight patients showed bilateral swelling of the LGs. As shown in Table 2, there were no differences in the mean age, sex or disease duration between the KT-S (+) and KT-S (−) patients. All patients in the KT-S (+) group showed unilateral swelling of the SMG, while three of the eight KT-S (−) patients showed bilateral swelling of the SMGs. Four