Disease was held in Boston, Massachusetts October 4–7, 2011 (http://www2.massgeneral.org/pathology/symposium/IgG4_related_systemic_dis.asp). The organizing committee, comprising 35 experts on IgG4-related disease from Japan, Korea, Hong Kong, the UK, Germany, Italy, The Netherlands, Canada, and the US, included clinicians, pathologists, radiologists, and basic scientists. This group represents broad subspecialty expertise in pathology, rheumatology, gastroenterology, allergy, immunology, nephrology, pulmonary medicine, oncology, ophthalmology, and surgery. Nomenclature was a specific focus of a portion of the symposium. Herein we report on the recommendations of the organizing committee related to terminology for the overall disease, with an emphasis on the individual organ system manifestations.

Description of IgG4-related disease and its unifying pathologic features

Certain clinical and pathologic features help define IgG4-related disease and distinguish it from its potential mimics. IgG4-related disease is a fibroinflammatory condition characterized by a tendency for formation of tumefactive lesions, a dense lymphoplasmacytic infiltrate rich in IgG4-positive plasma cells, storiform fibrosis, frequent but not invariable elevations of serum IgG4 levels, and a swift initial response to glucocorticoids provided that tissue fibrosis has not supervened.

IgG4-related disease is analogous in many ways to sarcoidosis, another systemic disease that affects virtually all organ systems, unified by a distinctive histologic appearance regardless of the organ involved. The pancreas was the first organ in which IgG4-related disease was identified, but the disease has now been described in virtually every organ system: the biliary tree, salivary glands, orbital tissues (e.g., lacrimal gland, extraocular muscles, and retrobulbar space), kidneys, lungs, lymph nodes, meninges, aorta, breast, prostate, thyroid gland, pericardium, retroperitoneum, and skin (5,6,8,9). The histopathologic features vary slightly across some organs, but, with the exception of IgG4-related lymphadenopathy and the membranous glomerulonephritis that is occasionally associated with this condition, the organ findings generally exhibit striking similarities. Increased numbers of infiltrating IgG4-bearing plasma cells are found within involved organs and are the sine qua non of the diagnosis. However, the diagnosis of IgG4-related disease cannot be made purely on the basis of staining for IgG4 (10). Rather, certain light microscopic features are also critical to the diagnosis (see below).

Table 1. Different names employed to refer to IgG4-related disease

Name	Ref.
IgG4-related disease	25
IgG4-associated disease	23
IgG4-related systemic disease	26
IgG4-related sclerosing disease	16
IgG4-related systemic sclerosing disease	42
IgG4-related autoimmune disease	4
Hyper-IgG4 disease	43
IgG4-positive multiorgan lymphoproliferative syndrome	44
Systemic IgG4-related plasmacytic syndrome (SIPS)	45
IgG4 syndrome	46

Existing terminology for multifocal disease and proposed terminology

We are aware of no fewer than 10 alternative names for IgG4-related disease (Table 1). The multisystem nature of the condition and the fact that many organ manifestations can have multiple potential names compound the confusion in the literature (Table 2). As noted above, Japanese investigators have reached a consensus to refer to this newly emerged disease as IgG4-related disease (7), specifically selecting this term over alternatives such as "IgG4-related systemic disease," "IgG4-related sclerosing disease," and "IgG4-positive multiorgan lymphoproliferative syndrome."

The issue of naming the disease after IgG4 was debated at the symposium. Because of many unresolved questions regarding the role of IgG4 in pathogenesis and the use of serum concentrations as a biomarker for the disease (see below), reservations were expressed by some experts about naming the disease after IgG4 without qualifications. However, recognizing that efforts to "speak the same language" are important in facilitating collaboration and disseminating information about

Table 2. Names of previously recognized conditions that comprise or may comprise parts of the IgG4-related disease spectrum

Mikulicz disease
Küttner tumor
Riedel thyroiditis
Eosinophilic angiocentric fibrosis
Multifocal fibrosclerosis
Lymphoplasmacytic sclerosing pancreatitis/autoimmune pancreatitis
Inflammatory pseudotumor
Fibrosing mediastinitis
Sclerosing mesenteritis
Retroperitoneal fibrosis (Ormond disease)
Periaortitis/periarteritis
Inflammatory aortic aneurysm
Cutaneous pseudolymphoma
Idiopathic hypertrophic pachymeningitis
Idiopathic tubulointerstitial nephritis
Idiopathic hypocomplementemic tubulointerstitial nephritis with extensive tubulointerstitial deposits
Idiopathic cervical fibrosis

this newly recognized condition more widely, the organizing committee for the 2011 International Symposium on IgG4-Related Disease endorsed the consensus name chosen by the Japanese group. The organizing committee acknowledged that much remains unknown about the behavior of the IgG4 molecule in vivo, the pathways through which this immunoglobulin participates in the disease, and whether or not the role of IgG4 is primary or secondary. In time, discoveries pertaining to the etiology and pathophysiology of the condition may suggest a name that is more appropriate. For the present, the term IgG4-related disease recognizes aptly the ubiquity of IgG4 within involved organs. This fact, not the frequency with which patients have increased serum IgG4 concentrations, is the fundamental basis for using this term in the name of the disease.

Individual organ system manifestations: existing terms and suggested nomenclature

Several eponymic conditions known for decades, or even in some cases for more than a century, are now identified as part of the IgG4-related disease spectrum (Table 2). Some of these eponyms have been applied loosely and imprecisely, leading to confusion and uncertainty about the precise clinical syndromes to which they refer. Now that there is evidence of a larger, systemic disease context for these disorders, it is appropriate that the eponyms be replaced in favor of terms that offer more information about particular pathophysiologic mechanisms and patterns of disease pathology.

Agreement upon the consensus term IgG4-related disease facilitates a consistent nomenclature whereby individual organ involvement can be referred to in a style that employs "IgG4-related-" as a prefix, regardless of the organ system affected. As examples, type 1 autoimmune pancreatitis (AIP), now firmly entrenched in the gastroenterology literature, might be termed "type 1 AIP (IgG4-related pancreatitis)." Similarly, chronic sclerosing sialadenitis (sometimes termed a Küttner tumor when it involves the submandibular gland) might be called "IgG4-related sialadenitis" or "IgG4-related submandibular gland disease." Such nomenclature underscores the belief that the same fundamental pathophysiologic processes are operative across organ systems in this disease, regardless of whether the role of IgG4 is viewed as primary or secondary.

Specific recommendations for IgG4-related organ system nomenclature

The recommendations of the organizing committee are shown in Table 3. Some potentially problematic areas are discussed below.

"Related" versus "associated." The terms "related" and "associated," both used in the medical literature in the context of this disease, are intended to convey the fact that IgG4-related disease is linked in some manner to IgG4-bearing plasma cells in tissue. We prefer the term "related" because it echoes the consensus name for the overall condition—IgG4-*related* disease—and has been used more consistently in the medical literature.

Pancreas. The pancreatic manifestation of IgG4-related disease was termed "autoimmune pancreatitis" in the mid-1990s (11), before the entity of IgG4-related disease had been conceptualized. The basis for considering this pancreatic condition to be autoimmune has not been established firmly, and no autoantibody has been identified consistently. AIP has since been divided into two types—type 1 and type 2—which share certain clinical similarities but are vastly different in terms of pathology and extrapancreatic features (12–14). Type 1 AIP is regarded as a prototypical organ manifestation of IgG4-related disease, which can occur alone or either simultaneously or metachronously with other organ complications. In contrast, type 2 AIP is not part of the IgG4-related disease spectrum and appears to be a disease of its own (15).

Over time, we anticipate that the term "type 1 AIP" might be replaced entirely by "IgG4-related pancreatitis." Because type 1 AIP is widely accepted among gastroenterologists and pancreatic surgeons now, however, we propose adding "IgG4-related pancreatitis" in parentheses, i.e., type 1 AIP (IgG4-related pancreatitis). This serves at least two purposes: 1) education of the broader medical community about the relationship between IgG4-related disease and this subset of pancreatic disease, and 2) avoidance of the issue of what to call type 2 AIP if type 1 AIP were removed entirely from the nomenclature.

Bile ducts. IgG4-related disease accounts for a subset of patients previously considered to have primary sclerosing cholangitis (16). Distinguishing between the primary and IgG4-related forms of sclerosing cholangitis is essential (but not always possible) because of the significant differences in treatment responses observed in these two conditions (17). At this time, it is unclear if patients with isolated biliary disease and elevated serum concentrations of IgG4 who meet imaging and clinical criteria for primary sclerosing cholangitis actually have IgG4-related disease.

Gastroenterologists and gastrointestinal pathologists on the organizing committee emphasized the importance of including "sclerosing" in the name of IgG4-related biliary tract disease as a means of linking this

Table 3. Preferred nomenclature for individual organ manifestations of IgG4-related disease

Organ system/tissue	Preferred name
Pancreas	Type 1 autoimmune pancreatitis (IgG4-related pancreatitis)
Eye	IgG4-related ophthalmic disease is the general term for the periocular manifestations of this disease. There are several subsets, outlined below.
Lacrimal glands	IgG4-related dacryoadenitis
Orbital soft tissue (orbital inflammatory pseudotumor)	IgG4-related orbital inflammation (or IgG4-related orbital inflammatory pseudotumor)
Extraocular muscle disease	IgG4-related orbital myositis
Orbit with involvement of multiple anatomic structures	IgG4-related pan-orbital inflammation (includes lacrimal gland disease, extraocular muscle involvement, and other potential intraorbital complications)
Salivary glands (parotid and submandibular glands)	IgG4-related sialadenitis or, more specifically, IgG4-related parotitis or IgG4-related submandibular gland disease
Pachymeninges	IgG4-related pachymeningitis
Hypophysis	IgG4-related hypophysitis
Thyroid (Riedel thyroiditis)	IgG4-related thyroid disease
Aorta	IgG4-related aortitis/periaortitis
Arteries	IgG4-related periarteritis
Mediastinum	IgG4-related mediastinitis
Retroperitoneum	IgG4-related retroperitoneal fibrosis
Mesentery	IgG4-related mesenteritis
Skin	IgG4-related skin disease
Lymph node	IgG4-related lymphadenopathy
Bile ducts	IgG4-related sclerosing cholangitis
Gallbladder	IgG4-related cholecystitis
Liver	IgG4-related hepatopathy (refers to liver involvement that is distinct from biliary tract involvement)
Lung	IgG4-related lung disease
Pleura	IgG4-related pleuritis
Pericardium	IgG4-related pericarditis
Kidney	IgG4-related kidney disease. The specific renal complications should be termed tubulointerstitial nephritis secondary to IgG4-related disease and membranous glomerulonephritis secondary to IgG4-related disease. Involvement of the renal pelvis should be termed IgG4-related renal pyelitis.
Breast	IgG4-related mastitis
Prostate	IgG4-related prostatitis

condition with, but still distinguishing it from, primary sclerosing cholangitis. Thus, we propose that IgG4-related disease of the biliary tree be termed "IgG4-related sclerosing cholangitis," even though residual "sclerosis" of the bile ducts is not always observed after glucocorticoid therapy in IgG4-related disease.

Mikulicz disease/syndrome. The term "Mikulicz disease" has been used to denote idiopathic bilateral, painless, and symmetric swelling of the lacrimal, parotid, and submandibular glands, often in the context of IgG4-related disease (18). However, "Mikulicz syndrome" can be caused by many different conditions and, indeed, the true diagnosis of the index patient described by Mikulicz is not clear (19). Some evidence suggests that the patient had an extranodal marginal zone lymphoma of the mucosa-associated lymphoid tissue type rather than IgG4-related disease (20). Moreover, the term Mikulicz disease has been applied inconsistently and even incorrectly for decades, sometimes being regarded as part of the spectrum of Sjögren's syndrome (SS) (21).

Thus, it seems appropriate to discard "Mikulicz disease" when referring to patients with involvement of the lacrimal, parotid, and submandibular glands and to instead use terms that refer to specific individual organ systems, i.e., "IgG4-related dacryoadenitis" for those with lacrimal gland disease, "IgG4-related parotitis" for those with parotid disease, and "IgG4-related sialadenitis" or "IgG4-related submandibular gland disease" for those with submandibular gland involvement.

Küttner tumor. A Küttner tumor refers to enlargement of the submandibular gland, sometimes as a result of stones in the Wharton duct (22). The use of the term by pathologists has been overly broad, often without full consideration of the underlying cause (or in the absence of knowledge of IgG4-related disease). A significant proportion of cases of "Küttner tumor" represent manifestations of IgG4-related disease. Important pathologic differences can be demonstrated between Küttner tumors associated with sialodocholithiasis and submandibular gland enlargement caused by IgG4-

related disease (23). For example, the fibrotic lesions that occur within the lesions of IgG4-related sialadenitis are characterized by intense inflammation within the areas of fibrosis, in contrast to the bland fibrotic lesions observed in Küttner tumors caused by salivary duct stones. Another important clinical difference is that IgG4-related sialadenitis is more likely to affect both submandibular glands.

Many patients with IgG4-related submandibular gland disease have been diagnosed in the past as having primary SS. In this regard, it is worth noting that patients with primary SS rarely (if ever) have isolated submandibular gland enlargement to the degree observed in IgG4-related disease. Primary SS is far more likely to involve the parotid glands disproportionately to the submandibular glands or to involve both of these glands together (24). IgG4-related disease, in contrast to primary SS, is not associated with antibodies to either the Ro/SSA or the La/SSB antigen (18).

Ophthalmic disease. IgG4-related disease is now recognized to be an important cause of "idiopathic" orbital inflammation and a major component of the differential diagnosis that includes lymphoma, granulomatosis with polyangiitis (Wegener's), Graves orbitopathy, and other conditions (25). IgG4-related disease must be excluded before the label of "idiopathic" is applied. (As discussed below, serum IgG4 measurement is not sufficient to exclude IgG4-related disease if the concentration is normal, nor does an elevated concentration confirm the diagnosis.) We recognize that in some patients with IgG4-related ophthalmic disease the process extends beyond the orbit to include, for example, part of the course of the trigeminal nerve (26,27). Hence, when referring to eye involvement in general, the broader term "IgG4-related ophthalmic disease" is proposed instead of "IgG4-related orbital disease."

Although IgG4-related ophthalmic disease is the recommended general term for disease involving the lacrimal glands, extraocular muscles, and other portions of the orbit (and beyond), it is preferable to refer to IgG4-related disease involvement of the ophthalmic region by specific terms, when possible. Thus, lacrimal gland involvement should be termed "IgG4-related dacryoadenitis," and IgG4-related disease affecting the extraocular muscles should be called "IgG4-related orbital myositis." The proposed term for orbital pseudotumor occurring in the context of IgG4-related disease is "IgG4-related orbital inflammation." Generalized IgG4-related orbital disease that affects multiple anatomic structures of the orbit simultaneously should be termed "IgG4-related pan-orbital inflammation."

Thyroid. Riedel thyroiditis has long been known to be associated with multifocal fibrosclerosis (28). Most cases of multifocal fibrosclerosis, in turn, are now recognized to be multi–organ system manifestations of IgG4-related disease. Riedel thyroiditis has been proven by immunohistochemical staining to be part of the IgG4-related disease spectrum (29). We propose that the term "IgG4-related thyroid disease" be used now in lieu of Riedel thyroiditis. Whether the "fibrosing variant" of Hashimoto thyroiditis is part of the IgG4-related disease spectrum remains to be clarified in studies of additional cases.

Kidney. Tubulointerstitial nephritis (TIN) is the most common renal feature of IgG4-related disease, but glomerular disease (e.g., membranous nephritis) has also been described (30). The TIN associated with IgG4-related disease can be differentiated histopathologically and immunohistochemically from TIN due to other causes (31,32). Further studies of the relationships between the membranous glomerulonephritis that sometimes occurs in IgG4-related disease and the "idiopathic" form of this disease are needed, because this issue is still controversial. However, we propose referring to both TIN and membranous glomerulopathy that occur in the setting of IgG4-related disease as "IgG4-related kidney disease." For cases in which membranous glomerulonephritis is the sole kidney lesion present and TIN is not evident, avoidance of the term IgG4-related kidney disease is appropriate at the present time.

The membranous glomerulonephritis of IgG4-related disease appears to have a different pathophysiology from the rest of IgG4-related disease. In IgG4-related disease, membranous glomerulonephritis is probably secondary to immune complex deposition rather than the usual destructive inflammatory process that characterizes other organ involvement in this condition. The membranous glomerulonephritis of IgG4-related disease is a different disorder from "idiopathic" membranous glomerulonephritis, which is characterized by antibodies to the phospholipase A_2 receptor (33). It is worth noting, however, that the anti–phospholipase A_2 receptor antibodies in idiopathic membranous glomerulonephropathy are principally of the IgG4 subclass.

Several types of radiologically evident lesions within the kidney have been described in IgG4-related disease, including diffuse renal enlargement, focal renal masses, and thickening of the renal pelvis. These lesions, which occur in association with other manifestations of IgG4-related disease in the majority of cases, often resolve with glucocorticoid treatment and are rarely biopsied if the diagnosis has been established in another organ. We propose that such radiologically identified renal lesions also be regarded as IgG4-related kidney disease, provided they occur in the setting of other organ

involvement that has been confirmed histopathologically.

Aorta. IgG4-related disease that involves the aorta has a predilection for the adventitia and periaortic tissue (34–36). However, the disease also involves the media, making it by definition an aortitis rather than a periaortitis (35). We propose the term "IgG4-related aortitis/periaortitis" for this condition, to reflect the anatomic extent of inflammation. IgG4-related periaortitis may exhibit some overlap with IgG4-related retroperitoneal fibrosis. Additional studies of medium-sized arteries and veins in IgG4-related disease are needed, but the term "IgG4-related periarteritis" appears appropriate at this time.

Notes of caution: problems with the use of IgG4 as a biomarker of IgG4-related disease

The adoption of "IgG4" into the name of this condition reflects the ubiquity of IgG4-bearing plasma cells in the tissues of involved organs. It is increasingly clear, however, that serum concentrations of IgG4 are unreliable as diagnostic markers of IgG4-related disease, as indicators of disease activity, and as measures of response to treatment. Approximately 20–40% of patients with biopsy-proven IgG4-related disease have normal serum IgG4 concentrations at the time of diagnosis, even before the institution of therapy (37,38). In addition, a varying proportion (3–7%) of both healthy controls and disease controls have elevated serum IgG4 levels, though it is uncommon for levels in controls to be more than twice the upper limit of normal (39,40).

The number of IgG4-positive plasma cells in tissues may also be misleading because the infiltration with IgG4-positive cells can be observed in conditions other than IgG4-related disease (10). A consensus statement on the pathology of IgG4-related disease emphasizes that certain light microscopy features, particularly storiform fibrosis, obliterative phlebitis, mild to moderate eosinophilia, and germinal center formation, are also critical to the diagnosis (41). Inclusion of IgG4 in the terminology of the disease should not lead clinicians to make the diagnosis solely based on serum IgG4 concentrations or tissue-infiltrating IgG4-positive plasma cells. Rather, the diagnosis of IgG4-related disease must be predicated upon specific histopathologic findings and then confirmed by tissue immunostaining, all in the setting of an appropriate clinical context.

Conclusions

IgG4-related disease is a recently recognized multi–organ system condition with pathologic features that are consistent across a wide range of organ systems. This condition unifies a large number of medical diagnoses previously regarded as being confined to single organ systems. The precise links between the full histopathologic picture of IgG4-related disease, the frequent serum elevations of IgG4 levels, and the finding of increased IgG4-bearing plasma cells in tissue remain to be ascertained fully. The use of a shared nomenclature will facilitate efforts to better understand this emerging condition and its larger implications with regard to the immune system.

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. J. H. Stone 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.

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Establishment of a serum IgG4 cut-off value for the differential diagnosis of IgG4-related sclerosing cholangitis: A Japanese cohort

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Key words
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Abstract

Background and Aim: IgG4-related sclerosing cholangitis (IgG4-SC) must be precisely distinguished from primary sclerosing cholangitis and cholangiocarcinoma (CC) because the treatments are completely different. However, the pathological diagnosis of IgG4-SC is difficult. Therefore, highly specific non-invasive criteria such as serum IgG4 should be established. This study established a cut-off for serum IgG4 to differentiate IgG4-SC from respective controls using serum IgG4 levels measured in Japanese centers.

Methods: A total of 344 IgG4-SC patients were enrolled in this study. As controls, 245, 110, and 149 patients with pancreatic cancer, primary sclerosing cholangitis, and CC, respectively, were enrolled. IgG4-SC patients were classified into three groups: type 1 (stenosis only in the lower part of the common bile duct), type 2 (stenosis diffusely distributed throughout the intrahepatic and extrahepatic bile ducts), and types 3 and 4 (stenosis in the hilar hepatic region) with 246, 56, and 42 patients, respectively. Serum IgG4 levels were compared, and the cut-offs were established.

Results: The cut-off obtained from receiver operator characteristic curves showed similar sensitivity and specificity to that of 135 mg/dL when all IgG4-SC and controls were compared. However, a new cut-off value was established when subgroups of IgG4-SC and controls were compared. A cut-off of 182 mg/dL can increase the specificity to 96.6% (4.7% increase) for distinguishing types 3 and 4 IgG4-SC from CC. A cut-off of 207 mg/dL might be useful for completely distinguishing types 3 and 4 IgG4-SC from all CC.

Conclusions: Serum IgG4 is useful for the differential diagnosis of IgG4-SC and controls.

Introduction

IgG4-related sclerosing cholangitis (IgG4-SC) is a biliary IgG4-related disease.[1,2] The first Japanese clinical diagnostic criteria of IgG4-SC were published in 2012.[3] Diffuse cholangioraphic abnormalities observed in association with IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC). Moreover, the presence of segmental stenosis suggests cholangiocarcinoma (CC).[4,5] The coexistence of autoimmune pancreatitis (AIP) is the most useful finding for the diagnosis of IgG4-SC.[6] IgG4-SC is occasionally described as an isolated biliary tract lesion, even in the absence of pancreatic involvement. It is especially difficult to differentiate IgG4-SC without AIP from PSC or CC.[7] IgG4-SC responds well to steroid therapy, whereas liver transplantation is the only effective therapy for PSC, and surgical intervention is needed for CC. Therefore, IgG4-SC must be precisely differentiated from PSC and CC.

Serum IgG4 is a useful marker for discriminating AIP from other pancreatic diseases;[8] a cut-off value of 135 mg/dL is widely used as a diagnostic criterion of AIP. However, twice the upper

limit of the normal value is also recommended to distinguish AIP from pancreatic cancer (PCa). In the international consensus diagnostic criteria for AIP (ICDC), twice the upper limit of the normal value is included in level 1, and 1–2 times the upper limit of the normal value is included in level 2.[9]

There are only a few reports concerning cut-off values in the diagnosis of IgG4-SC. We previously reported that a cut-off value of 135 mg/dL is useful for distinguishing IgG4-SC from PCa and PSC. However, this cut-off shows lower specificity for distinguishing IgG4-SC from CC.[6] Oseini *et al.*[10] evaluated the utility of serum IgG4 for distinguishing IgG4-SC from CC and concluded that some patients with CC, particularly those associated with PSC, have elevated serum IgG4 levels and that the use of a twofold cut-off for serum IgG4 may not reliably distinguish IgG4-SC from CC. At a cut-off of four times the upper limit of the normal range, serum IgG4 is 100% specific for IgG4-SC.

The most important initial modality in the diagnosis of IgG4-SC is cholangiography.[11] IgG4-SC can be classified into four types according to the region of strictures revealed by cholangiography.[12] This classification is intended for the differential diagnosis of IgG4-SC from PCa, PSC, and CC. We recommend that the differential diagnosis of these three intractable diseases should be made according to the cholangiographic classification because IgG4-SC has a variable appearance in cholangiography. Therefore, we also evaluated the cut-off values between each cholangiographic type of IgG4-SC and corresponding controls: PCa, PSC, and CC. However, there are no large multicenter studies regarding this in the literature. Therefore, in this study, we performed a multicenter study in Japan in order to establish a cut-off value to differentiate IgG4-SC from controls.

Methods

Study subjects. A retrospective survey of IgG4-SC focusing on serum IgG4 levels was conducted in the nine centers that participated in this study. The majority of these centers are major referral centers across Japan with established expertise in the diagnosis and management of IgG4-SC.

A total of 344 IgG4-SC patients (273 men and 71 women; age [mean ± standard deviation {SD}], 65.2 ± 10.2 years) diagnosed with IgG4-SC according to the 2012 Japanese clinical diagnostic criteria for IgG4-SC between 1993 and 2012 were enrolled in this study. All 344 patients fulfilled the criteria of imaging. In addition, 329 patients were associated with AIP. Four out of 15 patients with no association of AIP were diagnosed by the patholoIgical findings of resected specimen. The other 11 patients were diagnosed by the effect of steroid therapy after malignancy had been ruled out by the bile duct biopsy.

AIP patients were diagnosed according to the revised Japanese diagnostic criteria[13] or ICDC.[9] All patients with AIP included in this study had type 1 AIP. A total of 245 patients with PCa (90 men, 70 women, and 85 patients with unknown sex and age; mean age, 67.5 ± 11.6 years), 110 with PSC (51 men and 59 women; mean age, 42.8 ± 18.1 years), and 149 with CC (104 men and 45 women; mean age, 70.8 ± 11.4 years) were enrolled as the respective control groups for patients with IgG4-SC, who were classified into three groups. PCa and CC were diagnosed on the basis of histology, standard imaging criteria, or clinical course. PSC was diagnosed using the diagnostic criteria for PSC,[13] including the presence of typical abnormal bile ducts on direct cholangiography, an abnormal clinical course, blood chemistry data, and exclusion of secondary sclerosing cholangitis. Data regarding the serum IgG4 levels of the IgG4-SC and control groups were analyzed. The serum concentrations of IgG4 were measured by automated nephelometry (Behring Nephelometer II; Dade Behring, Newark, DE, USA). This study was approved by the Institutional Human Investigation Committee of Nagoya City University Graduate School of Medical Sciences.

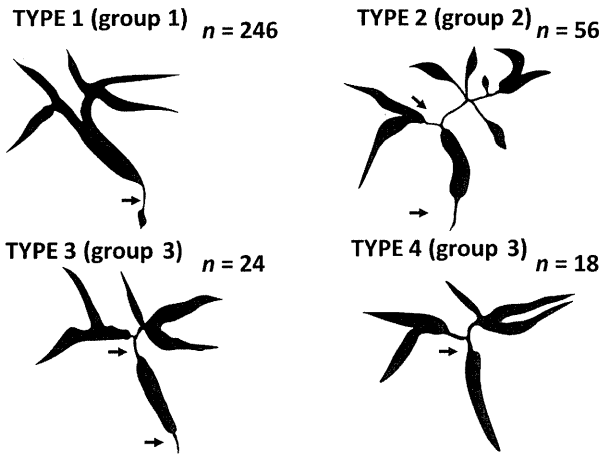

Figure 1 Schematic classification of cholangiographic findings of IgG4-related sclerosing cholangitis (IgG4-SC) (cited from Nakazawa *et al.*[14]). Stenosis is located only in the lower part of the common bile duct in type 1, stenosis is diffusely distributed in the intrahepatic and extrahepatic bile ducts in type 2, stenosis is detected in both the hilar hepatic lesions and the lower part of the common bile duct in type 3, and strictures of the bile duct are detected only in the hilar hepatic lesions in type 4.

Classification of IgG4-SC based on cholangiography. IgG4-SC can be classified into four types on the basis of the regions of stricture revealed by cholangiography (Fig. 1).[14] In the present study, we classified IgG4-SC into three groups for differential diagnosis on the basis of the cholangiographic features. The first group included patients with a type 1 cholangiogram, in which stenosis is located only in the lower part of the common bile duct. The second group included those with type 2 IgG4-SC, in which stenosis is diffusely distributed throughout the intrahepatic and extrahepatic bile ducts. Finally, the third group included those with types 3 and 4, in which stenosis is detected in the hilar hepatic region. Of the 344 IgG4-SC patients, 246, 56, and 42 were classified into the first (type 1, 195 men and 51 women; mean age, 64.6 ± 10.3 years), second (type 2, 46 men and 10 women; mean age, 66.3 ± 9.3 years), and third groups (24 with type 3 and 18 with type 4, 33 men and 9 women; mean age, 68.5 ± 8.9 years), respectively. Types 1, 2, and 3 and 4 cholangiographic findings can often lead to a misdiagnosis of PCa, PSC, and CC, respectively. Therefore, patients with PCa, PSC, and CC were selected as controls for the first, second, and third IgG4-SC groups, respectively.

Statistical analysis. Clinical data are expressed as mean ± SD. The χ^2 test and Mann–Whitney *U*-test for categorical comparisons of data were used to compare histological features, where appropriate. The level of statistical significance was set at $P < 0.05$. Receiver operating characteristic (ROC) curves were used to judge the diagnostic utility of serum IgG4 levels. For ROC curves, the best cut-off values were chosen according to the highest diagnostic accuracy determined using the Youden index: sensitivity – (1 – specificity). Statistical analysis was performed using JMP software (version 8.0.2: SAS Institute, Cary, NC, USA).

Results

Clinical profiles. The clinical profiles are shown in Table 1. All types of IgG4-SC exhibited male preponderance. There were no significant differences between any type of IgG4-SC with respect to age or serum IgG4 levels. The frequency of IgG4-SC without AIP was higher among types 3 and 4 IgG4-SC (type 1 0.8%, type 2 8.9%, type 3 19%, $P < 0.001$). When all IgG4-SC types or type 2 were compared with PSC, PSC did not exhibit any male preponderance ($P < 0.001$) and showed younger age ($P < 0.001$). PSC was significantly more associated with CC than with IgG4-SC (9/101 [8.18%] *vs* 1/343 [0.29%], respectively; $P < 0.001$).

Serum IgG4 values. The serum IgG4 levels of all IgG4-SC groups were significantly higher than those of all control groups (All IgG4-SC groups, 646 ± 662; PCa, 59.3 ± 65.9; PSC, 68.7 ± 86.0; CC, 52.3 ± 46.8; $P < 0.001$) (Table 1, Fig. 2). The serum IgG4 levels of type 1 IgG4-SC were significantly higher than that of PCa (613 ± 618 *vs* 59.3 ± 65.9, $P < 0.001$), that of type 2 IgG4-SC were significantly higher than that of PSC (799 ± 800 *vs* 68.7 ± 86.0, $P < 0.001$), and that of types 3 and 4 IgG4-SC were significantly higher than that of CC (646 ± 711 *vs* 52.3 ± 46.8, $P < 0.001$) (Table 1, Fig. 2). A total of 10.5% of IgG4-SC patients had serum IgG4 levels lower than the cut-off value of 135 mg/dL, whereas 7.7% of the controls had higher levels (6.7%, 11.5%, and 8.1% of PCa, PSC, and CC patients, respectively).

Cut-off values. When all IgG4-SC and controls were compared, the cut-off values of all IgG4-SC patients, from their respective controls obtained from ROC analysis, ranged from 117 to 138 mg/dL (all IgG4-SC *vs* PCa 119 mg/dL, all IgG4-SC *vs* PSC 117 mg/dL, all IgG4-SC *vs* CC 138 mg/dL). Sensitivity ranged from 89.8% to 91.2% (all IgG4-SC *vs* PCa 91.2%, all IgG4-SC *vs* PSC 91.5%, all IgG4-SC *vs* CC 89.8%), and specificity ranged from 87.6% to 93.9% (all IgG4-SC *vs* PCa 93.9%, all IgG4-SC *vs* PSC 87.6%, all IgG4-SC *vs* CC 92.6%). When we used a cut-off value of 135 mg/dL, similar sensitivity and specificity were obtained.

When the subgroups of IgG4-SC and controls were compared, the cut-off values for distinguishing specific types of IgG4-SC from their respective controls ranged from 119 to 182 mg/dL (type 1 IgG4-SC *vs* PCa 119 mg/dL, type 2 IgG4-SC *vs* PSC 125 mg/dL, types 3 and 4 IgG4-SC *vs* CC 182 mg/dL). Sensitivity ranged from

Table 1 Clinical profile

	IgG4-SC all	Type 1	Type 2	Types 3 and 4	Pancreatic cancer	PSC	Cholangiocarcinoma
Number	344	246	56	42 type 3 $n = 24$ type 4 $n = 18$	245	110	149
Male : female (male% to total)	273 : 71 (79.3%)	195 : 51 (79.3%)	46 : 10 (82.1%)	33 : 9 (78.6%)	90 : 70 (unknown 85) (56.2%)	51 : 59 (46.3%)	104 : 45 (69.8%)
IgG4 mg/dL (mean ± SD)	646 ± 662	613 ± 618	799 ± 800	646 ± 711 (type 3 534 ± 429) (type 4 919 ± 871)	59.3 ± 65.9	68.7 ± 86.0	52.3 ± 46.8
% of IgG4 value above 135 mg/dL	89.8	88.6	94.5	88.1	6.7	11.5	8.1
% of IgG4 value above 270 mg/dL	68.4	67.1	70.9	69.0	1.6	3.5	0
% of IgG4 value above 540 mg/dL	39.8	39.4	43.6	35.7	0.4	0.9	0

IgG4-SC, IgG4-related sclerosing cholangitis; PSC, primary sclerosing cholangitis; SD, standard deviation.

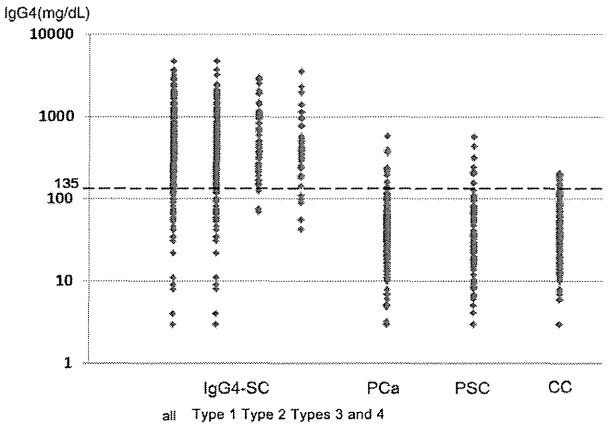

Figure 2 IgG4 value of IgG4-related sclerosing cholangitis (IgG4-SC) and respective controls. CC, cholangiocarcinoma; PCa, pancreatic cancer; PSC, primary sclerosing cholangitis.

85.7% to 96.4% (type 1 IgG4-SC *vs* PCa 90.2%, type 2 IgG4-SC *vs* PSC 96.4%, types 3 and 4 IgG4-SC *vs* CC 85.7%), and specificity ranged from 85.7% to 96.6% (type 1 IgG4-SC *vs* PCa 93.9%, type 2 IgG4-SC *vs* PSC 87.6%, types 3 and 4 IgG4-SC *vs* CC 96.6%). When we used a cut-off value of 135 mg/dL, types 1 and 2 showed similar sensitivity and specificity for distinguishing between PCa and PSC, respectively. However, the specificity for distinguishing types 3 and 4 from CC was lower. In order to rule out almost all control groups, a cut-off of 540 mg/dL, which is fourfold higher than the upper limit of normal, might be useful for distinguishing type 1 and 2 IgG4-SC. However, the maximum value of CC was 206 mg/dL, which was relatively lower. Therefore, the specificity is 100% if we set the cut-off value at 207 mg/dL for the differential diagnosis of type 3 and 4 IgG4-SC and CC.

Discussion

The present results revealed that the serum IgG4 levels of IgG4-SC were significantly higher than that of the control groups. The cut-off value obtained from the ROC curve showed sensitivity and specificity that was similar to the cut-off of 135 mg/dL when all IgG4-SC and controls were compared. However, these results indicate a new cut-off value when the subgroups of IgG4-SC and controls were compared after IgG4-SC was divided into three groups on the basis of cholangiographic classification. The cut-off value of 182 mg/dL for types 3 and 4 IgG4-SC and CC increases the specificity to 96.6% (a 4.7% increase). The cut-off of 207 mg/dL, a relative lower value, might be useful for completely distinguishing types 3 and 4 IgG4-SC from CC.

AIP is often associated with systemic extrapancreatic lesions such as sclerosing cholangitis, sclerosing sialadenitis, retroperitoneal fibrosis, mediastinal lymphadenopathy, sclerosing cholecystitis, interstitial pneumonia, and tubulointerstitial nephritis.[15–17] Similar immunohistopathological features such as lymphoplasmacytic infiltration and abundant IgG4-positive plasma cells are also observed in some extrapancreatic lesions.[17–19] The concept of IgG4-related disease includes high serum IgG4 levels as an essential diagnostic criterion. However, the cut-off values of IgG4-related diseases other than AIP have not been investigated or established until now. The pathological diagnosis of IgG4-SC is difficult compared to that of other IgG4-related diseases such as sclerosing sialadenitis because obtaining a sufficient quantity of bile duct sample is difficult.[20] Therefore, non-invasive criteria with high specificity, such as serum IgG4 levels, should be established for the diagnosis of IgG4-SC.

Several diagnostic criteria have been published for the diagnosis of IgG4-SC.[3,6,21] Our previous study demonstrates that association with AIP is a useful parameter for the diagnosis of IgG4-SC. In that study, 59 of 62 patients (95%) with IgG4-SC had associated AIP.[6] Ghazale *et al.*.[21] report a frequency of AIP association of 92% among 53 patients with IgG4-SC, which is a comparatively large sample. However, focal-type AIP sometimes produces imaging findings similar to those of PCa, making it difficult to distinguish between these two diseases.[22] The sensitivity of the diagnostic criteria for AIP is reported to range from 80% to 92%.[23] Therefore, there is a need to establish useful diagnostic criteria for IgG4-SC when it is not associated with AIP or when the diagnosis of AIP is unclear. The present results indicate that caution should be taken when diagnosing types 3 and 4 IgG4-SC because the frequency of IgG4-SC without AIP was higher in types 3 and 4 IgG4-SC.

Serum IgG4 is the most useful modality for distinguishing IgG4-SC from other biliary diseases. When distinguishing AIP from PCa, only PCa should be considered as a differential diagnostic disease. However, IgG4-SC should be distinguished from all three intractable diseases (i.e. PCa, PSC, and CC). Therefore, there is a need to establish diagnostic criteria that account for the differential diagnoses of these three intractable diseases. IgG4-SC can be classified into four types on the basis of the region of strictures revealed by cholangiography.[14] Furthermore, diagnostic criteria based on cholangiographic classification in the differential diagnosis of IgG4-SC have been proposed.[6] The cut-off value of 135 mg/dL is useful for differentiating IgG4-SC from PCa and PSC. However, it exhibits lower sensitivity and specificity for differentiating IgG4-SC from CC.[6] The present Japanese multicenter study revealed that 10.5% of IgG4-SC patients had serum IgG4 levels lower than the cut-off of 135 mg/dL, whereas 7.7% of the controls had levels greater than the cut-off. High specificity is required in order to differentiate the three intractable diseases from IgG4-SC. A cut-off of 135 mg/dL is recommended in cases of types 1 and 2 IgG4-SC, whereas a cut-off of 182 mg/dL is recommended in cases of type 3 IgG4-SC. A cut-off of 182 mg/dL for distinguishing between type 3 IgG4-SC and CC can increase the specificity by 4.7%.

Twice the upper limit of the normal value 270 mg/dL is recommended to distinguish AIP from PCa in order to increase specificity in the ICDC. Twice the upper limit of the normal value is included in level 1, and one to two times the upper limit of the normal value is included in level 2.[9] Oseini *et al.*[10] report that the use of a cut-off twice that of the upper limit of the normal value for serum IgG4 levels may not reliably distinguish IgG4-SC from CC because some patients with CC, particularly those with PSC, have elevated serum IgG4 levels. At a cut-off 4 times the upper limit of the normal value, serum IgG4 is 100% specific for IgG4-SC. In the present study, cut-offs that were two and four times the upper limit of the normal value of 270 and 540 mg/dL, respectively, showed

higher specificity. Among our CC cases, a cut-off of 206 mg/dL showed the highest specificity. Therefore, a cut-off of 270 mg/dL can completely distinguish IgG4-SC from CC. However, a cut-off of 207 mg/dL can completely distinguish IgG4-SC from CC and maintain a comparatively high sensitivity. Cut-offs of 563 and 580 mg/dL were the highest among our PSC and PCa cases, respectively. A cut-off of 540 mg/dL cannot distinguish IgG4-SC from PSC and PCa completely. However, almost all control cases except two were below this cut-off. This suggests that 540 mg/dL is an appropriate cut-off for distinguishing IgG4-SC from almost all controls worldwide. As mentioned earlier, some patients with CC, particularly those with PSC, have elevated serum IgG4 levels.[10] The present study detected nine PSC patients with CC (8.18%); however, all cases had low serum IgG4 levels (8.3, 13.7, 16.8, 18.0, 18.0, 34.0, 35.0, 41, 4, and 94.8). These differences might be due to the lower frequency in the number of PSC patients in Japan than that in the US.

We confirmed that the cut-off of 135 mg/dL that is adopted in Japanese clinical diagnostic criteria of IgG4-SC is appropriate value, and the higher cut-off value of 207 mg/dL might be useful for completely distinguishing types 3 and 4 IgG4-SC from CC in the present Japanese multicenter study. We hope further international study because the Japanese data may not be applicable to the rest of the world.

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EDITOR'S CHOICE

EXTENDED REPORT

Interleukin-21 contributes to germinal centre formation and immunoglobulin G4 production in IgG4-related dacryoadenitis and sialoadenitis, so-called Mikulicz's disease

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ABSTRACT

Objectives Interleukin (IL)-21 is mainly produced by CD4 T helper (Th) cells including Th2, Th17 and follicular helper T (Tfh) cells. Recent studies have reported that IL-21 is involved in the formation of germinal centres (GCs) and class switching of IgG4. It has been suggested that IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS), so-called Mikulicz's disease (MD), is distinct from Sjögren's syndrome (SS) and shows a high frequency of GC formation in salivary glands. In this study the expression of IL-21 in IgG4-DS and SS patients was examined.

Methods Twelve patients with IgG4-DS, 15 with SS and 15 healthy subjects were screened for (1) ectopic GC formation in formalin-fixed labial salivary gland (LSG) biopsy samples; (2) expression of IL-21, Th2-, Th17- and Tfh-related molecules (cytokines, chemokine receptors and transcription factors) in LSGs; (3) relationship between IgG4/IgG ratio and mRNA expression of IL-21 in LSGs.

Results mRNA expression of IL-21 and Bcl-6 in LSGs from patients with IgG4-DS was significantly higher than in patients with SS and controls. IL-21 and CXCR5 were detected by immunohistochemistry in or around GC in patients with SS and those with IgG4-DS. IL-21 was detected in infiltrating lymphocytes outside GC only in patients with IgG4-DS. Expression of IL-21 was consistent with that of Th2-related molecules while IL-17 was rarely seen in IgG4-DS. Furthermore, the expression of IL-21 in LSGs was correlated with the number of GC formations and the IgG4/IgG ratio in patients with IgG4-DS.

Conclusions These results suggest that overexpression of IL-21 by Th2 cells might play a key role in GC formation and IgG4 production in IgG4-DS.

Mikulicz's disease (MD) has been considered to be a subtype of Sjögren's syndrome (SS) based on the histopathological similarities between the two diseases.[1 2] Yamamoto *et al* reported that patients with MD had elevated serum IgG4 and infiltration of IgG4-positive plasma cells in the gland tissues. These findings have also been found in autoimmune pancreatitis,[3] sclerosing cholangitis,[4] tubulointerstitial nephritis,[5] interstitial pneumonia,[6] Ridel's thyroiditis[7] and Küttner's tumour.[8] These diseases are thus now referred to as 'IgG4-related disease' (IgG4-RD).[9 10] We recently described the concept of IgG4-RD and provided up-to-date information on this emerging disease entity.[11] Recent studies have referred to MD as 'IgG4-related dacryoadenitis and sialoadenitis' (IgG4-DS).[11–14] Histologically, ectopic germinal centres (GCs) can frequently occur in the salivary glands in IgG4-DS. It is generally accepted that interleukin (IL)-21 plays a crucial role in the development of GC formation and is mainly produced by CD4 T cells (ie, T helper (Th) 2 cells,[15] Th17 cells[16] and T follicular helper (Tfh) cells[17 18]). In particular, IL-21 production by Tfh cells, which are characterised by expression of CXC chemokine receptor 5 (CXCR5), help GC formation.[18] High levels of IL-21 receptors are present at the surface of most B cells.[17]

We previously reported that peripheral CD4 T cells from patients with IgG4-DS have deviation of Th1/Th2 balance to Th2 and elevated expression of Th2-type cytokines,[19] suggesting that IgG4-DS has a Th2-predominant phenotype.[20] We also demonstrated a close association of IL-4 and IL-10 expression with IgG4 production in the labial salivary glands (LSGs) of patients with IgG4-DS.[21 22] Furthermore, recent studies in humans and mice reported that class switching of IgG4 is caused by co-stimulation with IL-4 and IL-21.[23–25]

However, to our knowledge, no published reports have investigated the involvement of IL-21 expression in class switching of IgG4 in the salivary glands of patients with IgG4-DS. We therefore examined infiltrating lymphocytes expressing IL-21 in LSGs from patients with IgG4-DS to clarify the contribution of IL-21 to the pathogenesis of IgG4-DS.

METHODS

Patients

Twelve patients with IgG4-DS (nine women and three men, mean±SD age 61.8±8.1 years) and 15 patients with primary SS (14 women and one man, mean±SD age 61.4±9.8 years) who were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital between 2007 and 2011 were included in the study. Medical records were retrospectively

reviewed after the diagnosis. IgG4-related MD (IgG4-DS) was diagnosed according to the following criteria:[2 11] (1) persistent symmetrical swelling of at least two pairs of lachrymal and major salivary glands for at least 3 months; (2) raised serum levels of IgG4 (>135 mg/dl); and (3) infiltration of IgG4-positive plasma cells in the tissue (IgG4+ cells/IgG+ cells >50%). The clinical and serological characteristics of the 12 patients with IgG4-DS are summarised in table 1.

SS was diagnosed according to the Research Committee on SS of the Ministry of Health and Welfare of the Japanese Government (1999) and the American-European Consensus Group criteria for SS.[26] Each patient showed objective evidence of salivary gland involvement based on the presence of subjective xerostomia and a decreased salivary flow rate, abnormal findings on parotid sialography and focal lymphocytic infiltrates in the LSGs. There was no documented history of treatment with steroids, HIV, HTLV-1, hepatitis B virus or hepatitis C virus infection, sarcoidosis or any other immunodepressants in any of the patients. None of the patients had evidence of malignant lymphoma at the time of the study. The prevalences of anti-SS-A/Ro, anti-SS-B/La and antinuclear antibodies were 72.6%, 33.2% and 71.6%, respectively. LSG biopsies were performed as described by Greenspan *et al*.[27] Fifteen patients with mucocoeles (11 women and four men, mean±SD age 58.4 ±16.3 years) with no clinical or laboratory evidence of systemic autoimmune disease were chosen as a control group. All control LSGs were histologically normal. The clinical and serological characteristics of the 15 patients with SS are summarised in table 2.

The study was approved by the ethics committee of Kyushu University, Japan and informed consent was obtained from all the patients and healthy controls included in the study.

Histology

Formalin-fixed and paraffin-embedded 4 µm sections of LSG specimens were prepared and stained with haematoxylin and eosin (HE) for conventional histological examination. The degree of lymphocytic infiltration in the specimens was judged by focus scoring.[21 27 28] One standardised score was the number of focal inflammatory cell aggregates containing ≥50 mononuclear cells in each 4 mm^2 area of salivary gland tissue.[29]

Evaluation of ectopic GCs

Ectopic GCs have been previously defined by the presence of B and T cell follicles, follicular dendritic cell networks and high densities of endothelial venules and proliferating cells.[30 31] In this study, HE-stained LSG tissue sections from 20 patients with IgG4-DS and 66 patients with primary SS who were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital between 1993 and 2011 were evaluated for the frequency, number and size of ectopic GC formations. The numbers of GC were counted in 4 mm^2 sections from five different areas. Ectopic GCs were defined by HE staining as well-circumscribed chronic inflammatory cell infiltrates consisting of at least 50 mononuclear cells presenting with a densely-packed dark zone and a less densely-packed light zone. Only a few of the structures defined in this way corresponded to real GCs.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was prepared from the whole LSGs using the acidified guanidinium–phenol–chloroform method, as previously described.[21 32] Three micrograms of the total RNA preparation was then used for the synthesis of cDNA. Briefly, RNA was incubated for 1 h at 42°C with 20 U RNasin ribonuclease inhibitor (Promega, Madison, Wisconsin, USA), 0.5 mg oligo-(dT) 1218 (Pharmacia, Uppsala, Sweden), 0.5 mM of each dNTP (Pharmacia), 10 mM dithiothreitol and 100 U RNase H reverse transcriptase (Life Technologies, Gaithersburg, Maryland, USA).

Quantitative estimation of mRNA by real-time polymerase chain reaction (PCR)

Quantitative cDNA amplification from whole LSGs and microdissected samples was performed according to the manufacturer's instructions and previous reports.[21 32] cDNAs of the cytokines and transcription factors were analysed by real-time PCR using Light Cycler Fast Start DNA Master SYBR Green 1 (Roche Diagnostics, Mannheim, Germany) in a Light Cycler

Table 1 Clinical and serological findings of 12 patients with (IgG4-DS)

Patients					Clinical findings					Serological findings			
					Swelling glands								
No.	Age	Sex	Disease duration	Lymphocytic infiltration†	LG	PG	SMG	SLG	Complications	SS-A/Ro	SS-B/La	IgG(872~1815mg/dl)	IgG4(95~105 mg/dl)
1	76	F	4M	11		○	○		DM	–	–	2381	823
2	70	F	5Y	12		○			–	–	–	4410	1930
3	60	F	1Y	11			○		AIP, SC	–	–	1614	490
4	64	F	1Y	10		○	○		AIP	–	–	2430	896
5	79	F	10M	11		○	○		AIP, SC	–	–	2585	456
6	61	F	3M	9			○		AIP, SC	–	–	2891	1080
7	57	F	6M	10	○		○		AIP, SC	–	–	1842	748
8	65	M	5M	10			○		Hydronephrosis	–	–	3142	1700
9	31	F	1.5Y	12	○	○		○	AIP, DM	–	–	2055	ND
10	70	M	1M	11		○	○		Asthma	–	–	2010	ND
11	61	M	3M	10	○	○	○	○	Pulmonary nodules	–	–	7603	2290
12	48	F	3M	8	○				Asthma	–	–	2401	ND

AIP, autoimmune pancreatitis; LG, lachrymal gland; LSG, labial salivary gland; –, negative; ND, not done; PG, parotid gland; SC, sclerosing cholangitis; SLG, sublingual gland; SMG, submandibular gland.

Table 2 Clinical and serological findings of 15 patients with primary Sjögren's syndrome (SS)

				Autoantibody					Immunoglobulin		
No.	Age	Sex	Lymphocytic infiltration*	RF (20~≧U/ml)	ANA (titer)	DNA (10~>U/ml)	SS-A/Ro (10~>)	SS-B/La (15~ >)	IgG (872~1815 mg/dl)	IgA (95~405 mg/dl)	IgM (59~269 mg/dl)
1	26	F	10	25	>1280	ND	>256	>15	>1815	Negative	Negative
2	44	F	9	<20	Negative	Negative	104	29	1517	193	174
3	65	F	9	ND	80	ND	64	<15	ND	ND	ND
4	52	F	11	40	>1280	<5	>256	32	2206	270	107
5	48	F	10	35	320	<5	16	<15	ND	ND	ND
6	65	F	9	ND	160	2	<10	<15	1338	ND	62
7	53	F	11	ND	ND	ND	<10	<15	ND	ND	ND
8	59	F	12	ND	ND	ND	>256	<15	ND	ND	ND
9	89	M	8	4<	Negative	ND	44.6	<15	2239	461	54
10	29	F	9	40	>1280	ND	>256	<15	ND	ND	ND
11	92	F	7	29	320	ND	64	2	1565	581	103
12	60	F	7	ND	ND	ND	16	–	ND	ND	ND
13	75	F	7	5	80	ND	4	–	1408	159	67
14	54	F	10	ND	ND	ND	>256	–	ND	ND	ND
15	43	F	10	22	160	ND	240	192.3	1147	236	201
15											

*The degree of lymphocytic infiltration in the LSGs was graded from 1 to 12 by focus scoring.

Real-time PCR instrument (V.3.5; Roche Diagnostics). The cytokines, chemokine receptors and transcription factors examined in the current study were IL-4, IL-17, IL-21, CC chemokine receptor 4 (CCR4), CXCR5, Bcl-6 and retinoic acid-related orphan receptor C2 (RORC2).[33] The lymphocyte markers examined were IgG and IgG4. The primer sequences used were as follows: β-actin, forward 5′-GCA AAG ACC TGT ACG CCA AC-3′, reverse 5′-CTA GAA GCA TTT GCG GTG GA-3′, 258 bp; IL-4, forward 5′-GCA GTT CCA CAG GCA CAA-3′, reverse 5′-CTC TGG TTG GCT TCC TTC AC-3′, 108 bp; IL-17, forward 5′-CCC CAG TTG ATT GGA AGA AA-3′, reverse 5′-AGA TTC CAA GGT GAG GTG GA-3′, 252 bp; IL-21, forward 5′-CCA CAA ATC AAG CTC CCA AG-3′, reverse 5′-CAG GGA CCA AGT CAT TCA CA-3′, 258 bp; CCR4, forward 5′-GTG CTC TGC CAA TAC TGT GG-3′, reverse 5′-CTT CCT CCT GAC ACT GGC TC-3′, 214 bp; CXCR5, forward 5′-GGT CTT CAT CTT GCC CTT TG-3′, reverse 5′-ATG CGT TTC TGC TTG GTT CT-3′, 340 bp; Bcl-6, forward 5′-GAA GCC CTA CAA ATG CGA AA-3′, reverse 5′-TGA CGG AAA TGC AGG TTA CA-3′, 210 bp; RORC2, forward 5′-GGG TAC AAT GAA GGC CAA GA-3′, reverse 5′-AGC TGT GGC CTC AAG GAT AA-3′, 211 bp; IgG, forward 5′-CAA GTG CAA GGT CTC CAA CA-3′, reverse 5′-TGG TTC TTG GTC AGC TCA TC-3′, 129 bp; IgG4, forward 5′-TGA CGG TGT CGT GGA ACT-3′, reverse 5′-ACG TTG CAG GTG TAG GTC T-3′, 145 bp.

In order to provide a meaningful comparison between different individuals or samples, the amounts of the PCR products were calculated relative to the amounts of β-actin PCR products (for the standardisation of total cellular mRNA) in each sample, as previously described.[21 34]

Immunohistochemical analysis

For immunohistochemical analysis, 4 μm formalin-fixed and paraffin-embedded sections were prepared and stained using a conventional avidin-biotin complex technique, as described previously.[21 35] The polyclonal antibodies used to analyse the cytokines were anti-IL-4 (clone: ab9622; Abcam, Cambridge, UK), anti-IL-17 (clone: sc-7927; Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-IL-21 (clone: LS-C401; LifeSpan BioScience, LSBio, Seattle, Washington, USA) and anti-c-Maf (clone: ab77071; Abcam). SS1 (anti-sheep erythrocyte IgG2a), NS8.1 (anti-sheep erythrocyte IgG2b) and NS4.1 (anti-sheep erythrocyte IgM) were used as control rabbit polyclonal antibodies. The mouse monoclonal antibodies used to analyse the transcription factors were anti-Bcl-6 (clone: ab9479; Abcam), anti-CCR4 (MAB1567; R&D Systems) and anti-CXCR5 (clone: ab89259; Abcam). HDP-1 (anti-DNP IgG1) was used as a control mouse monoclonal antibody. The sections were sequentially incubated with primary antibodies, biotinylated anti-mouse IgG secondary antibodies (Vector Laboratories, Burlingame, 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 (CoolSNAP; Photometrics, Tucson, Arizona, USA). Stained IgG4-positive and IgG-positive cells were counted in 1 mm^2 sections from five different areas and the ratio of IgG4-positive cells to IgG-positive cells was calculated.

Statistical analysis

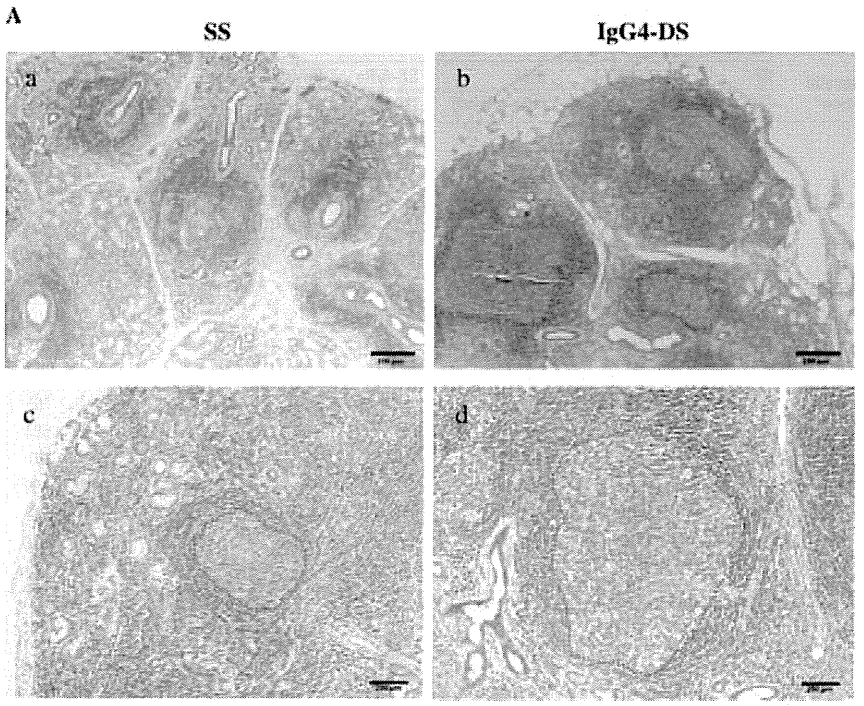
The significance of differences between groups was determined using χ^2 tests, Student t tests, Mann–Whitney U tests and Spearman rank correlations. All statistical analyses were performed using JMP software (V.8; SAS Institute, Japan). A p value <0.05 was considered statistically significant.

RESULTS

Formation of ectopic GCs in LSGs from patients with SS and IgG4-DS

SS was characterised by periductal lymphocytic infiltration with atrophy or severe destruction of the acini, while IgG4-DS showed non-periductal lymphocytic infiltration with hyperplastic GCs and mild destruction of the acini (figure 1A). Fifteen of 66 patients with SS (23%) and 12 of 20 patients with IgG4-DS (60%) showed ectopic GC formation in the formalin-fixed tissue. Patients with IgG4-DS showed

A



B

GC formation	SS	IgG4-DS	*P* value
Frequency	15/66 (22.7 %)	12/20 (60 %)	$P < 0.05$ †
Number (/HPF)	0.1 ± 0.3	2.5 ± 2.6	$P < 0.05$ ††
Size ($\times 10^3$ μm^2)	31.2 ± 21.9	68.1 ± 29.4	$P < 0.05$ ††

Figure 1 (A) Germinal centre (GC) formation in labial salivary gland (LSG) sections from representative patients with Sjögren's syndrome (SS) and IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). The degrees of lymphocytic infiltration in LSGs from patients with SS and IgG4-DS had focus scores of 11. IgG4-DS showed lymphocytic infiltration with extensive ectopic GC formation compared with SS (a, b). The area enclosed by the red line shows the ectopic GCs in the LSGs from patients with SS and IgG4-DS (c, d). Scale bars, 100 and 200 μm. (B) Difference in ectopic GC formation between patients with SS and IgG4-DS. †Significance of the difference between the two groups was determined by χ^2 tests. ††Significance of the difference between the two groups was determined by the Student t test. HPF, high-power field.

significantly higher frequency, higher number and larger size of these structures compared with those from patients with SS (figure 1B). The characteristics of the 15 patients with SS and 12 patients with IgG4-DS who were found to have these structures are summarised in table 1A,B, respectively. The patients with SS and IgG4-DS all had strong lymphocytic infiltration and the mean±SD focus scores were 9.27±1.53 and 10.4±1.16, respectively. Focus scores between the two groups were not significantly different.

Expression of IL-21 and Tfh-related molecules (CXCR5 and Bcl-6) in LSGs

The mRNA expression levels of IL-21, CXCR5 and Bcl-6 in LSGs from SS and IgG4-DS patients were significantly higher than those in controls (figure 2A). In addition, the mRNA expression levels of IL-21 and Bcl-6 in the LSGs from patients with IgG4-DS were higher than those from patients with SS. Representative histological findings in the LSG specimens from patients with SS and IgG4-DS and from controls are shown in figure 2Ba–c. Patients with SS and IgG4-DS showed GC formation. The specimens were immunohistochemically examined to evaluate the distribution of the cytokines, chemokine receptors and transcription factors in the LSGs from patients with SS and IgG4-DS (figure 2B). Expression of IL-21, CXCR5 and Bcl-6 was detected in ectopic GCs from patients with SS and IgG4-DS (figure 2Be,f,h,i,k,l). Interestingly, the expression of IL-21 and Bcl-6 was also detected in infiltrating lymphocytes outside the ectopic GCs only in patients with IgG4-DS (figure 2Bf,l). Expression of IL-21 was slightly detected in ductal epithelial cells in the LSGs from patients with SS and controls (figure 2Bd,e). None of the LSGs from controls exhibited CXCR5 or Bcl-6 expression (figure 2Bg,j).

Expression of Th2-related molecules (IL-4, CCR4 and c-Maf) and Th17-related molecules (IL-17 and RORC2) in LSGs

As shown in figure 3A, mRNA expression levels of IL-4, CCR4, IL-17 and RORC2 in LSGs from patients with SS were significantly higher than in controls while mRNA expression levels of IL-4 and CCR4 were significantly higher in the LSGs from patients with IgG4-DS than in controls. Furthermore, the mRNA expression level of IL-4 was significantly higher in

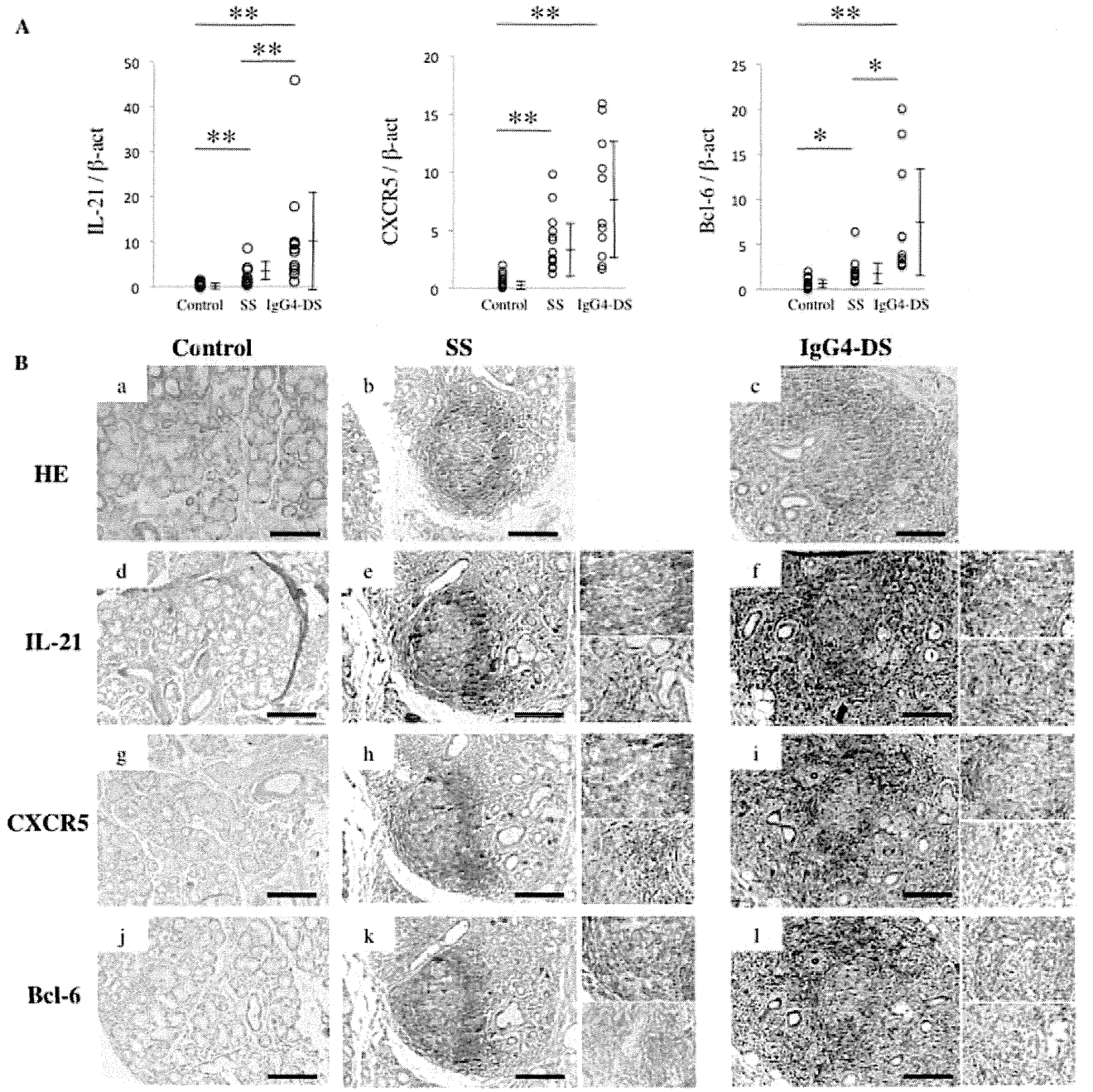


Figure 2 (A) mRNA expression levels of interleukin 21 (IL-21) in the labial salivary glands (LSGs) from patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) were significantly higher than in those from patients with Sjögren's syndrome (SS) and controls. The mRNA expression levels of cytokine, chemokine receptor and transcription factor were examined in the LSGs from controls (n=15), patients with SS (n=15) and patients with IgG4-DS (n=12). IL-4, CXCR5 and Bcl-6 were quantitatively estimated as described in the Methods section. Bars represent means and SDs. Significant differences between groups were determined by the Mann–Whitney U test (*$p<0.05$, **$p<0.01$). (B) IL-21 was strongly expressed in whole LSGs only from patients with IgG4-DS. Staining with haematoxylin and eosin (HE) (a–c) and anti-IL-21 (d–f), anti-CXCR5 (g–i) and anti-Bcl-6 (j–l) monoclonal antibodies in the LSGs from a representative control, SS patient and IgG4-DS patient (brown). Counterstaining with Mayer's haematoxylin was performed subsequently (blue). The higher magnifications (e, f, h, i, k, l) are displayed at the upper right (in the ectopic germinal centre) and the lower right (outside the ectopic ectopic centre). Scale bars, 100 μm.

LSGs from patients with IgG4-DS than in patients with SS. The specimens were immunohistochemically examined to evaluate the distribution of these molecules in the LSGs from patients with SS and IgG4-DS and controls (figure 3B). Expression of IL-4, CCR4 and c-Maf was prominently detected in the whole LSGs from patients with IgG4-DS compared with those from patients with SS (figure 3Bb,c,e,f, h,i,k,l). The expression of IL-17 was slightly detected in the ectopic GCs from patients with SS but rarely in the whole LSGs from patients with IgG4-DS or controls (figure 3Bj,k,l). None of the control LSGs exhibited IL-4, CCR4 or c-Maf expression (figure 3Ba,d,g).

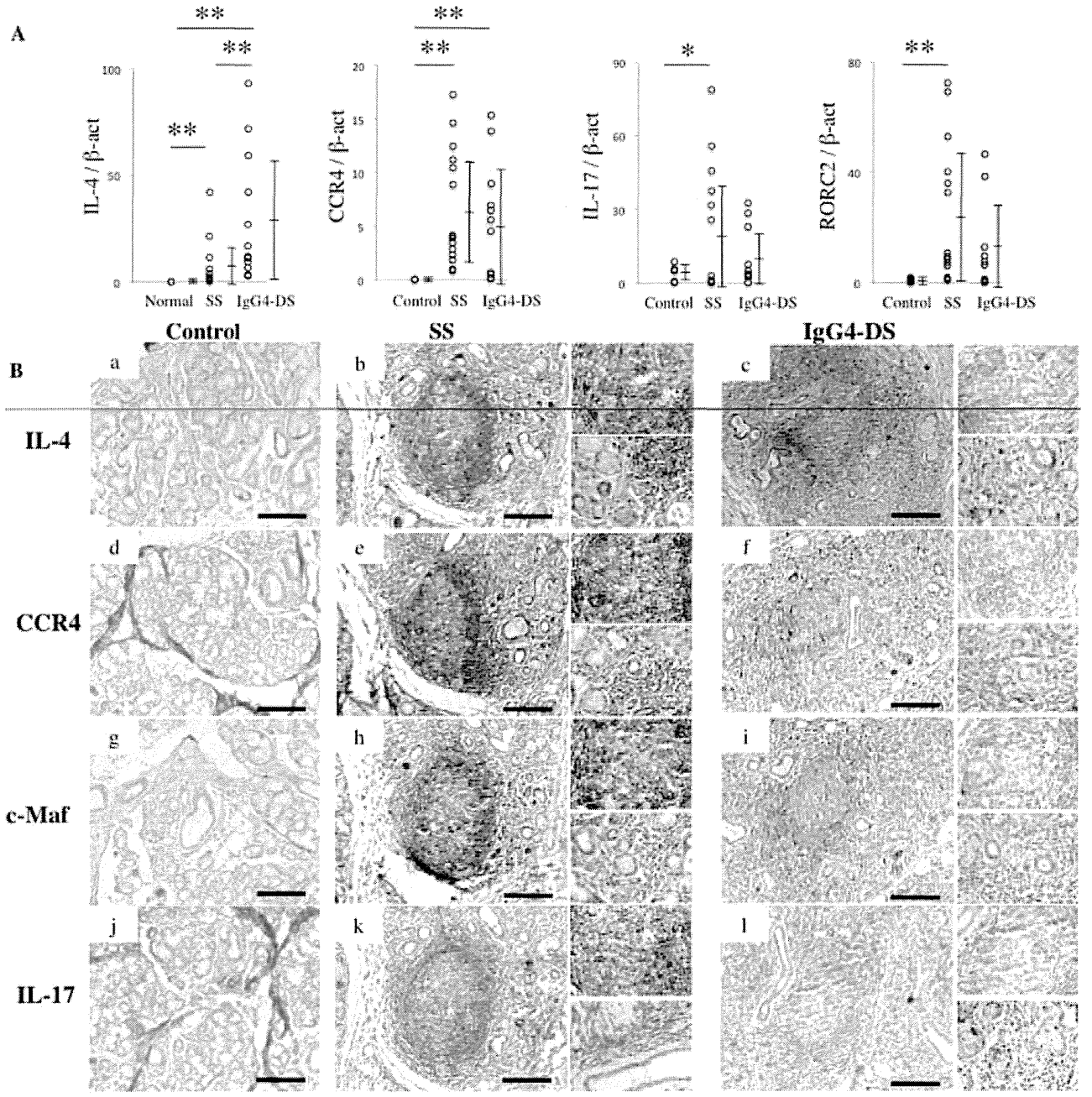


Figure 3 (A) mRNA expression levels of interleukin 4 (IL-4) in the labial salivary glands (LSGs) of patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) were significantly higher than in those from patients with Sjögren's syndrome (SS). The mRNA expression levels of cytokine, chemokine receptor and transcription factor were examined in the LSGs from controls (n=15), patients with SS (n=15) and patients with IgG4-DS (n=12). IL-4, CCR4, IL-17 and RORC2 were quantitatively estimated as described in the Methods section. Bars represent means and SDs. Significant differences between groups were determined by the Mann–Whitney U test (*p<0.05, **p<0.01). (B) IL-4, CCR4 and c-Maf were strongly expressed in whole LSGs from patients with IgG4-DS but not in patients with SS. Immunostaining with anti-IL-4 (a–c), anti-CCR4 (d–f), anti-c-Maf (g–i) and anti-IL-17 (j–l) monoclonal and polyclonal antibodies in the LSGs from a representative control, SS patient and IgG4-DS patient (brown). Counterstaining with Mayer's haematoxylin was performed subsequently (blue). The higher magnifications (b, c, e, f, h, i, k, l) are displayed at the upper right (in the ectopic germinal centre) and the lower right (outside the ectopic germinal centre). Scale bars, 100 μm.

Relationship between GC formation and expression of IL-21 in LSGs

The relationship between the number of GC formations and levels of IL-21 mRNA expression in LSGs was examined. mRNA expression of IL-21 was positively correlated with the number of GC formations in LSGs from patients with IgG4-DS but not in those from patients with SS (figure 4).

Relationship between IgG4 production and expression of IL-21 in LSGs

The relationships between IgG4 production and levels of IL-21 mRNA expression in LSGs were examined. mRNA expression of IL-21 was positively correlated with the IgG4/IgG ratio in LSGs from patients with IgG4-DS but not with that in patients with SS (figure 5A). Patients with IgG4-DS showed

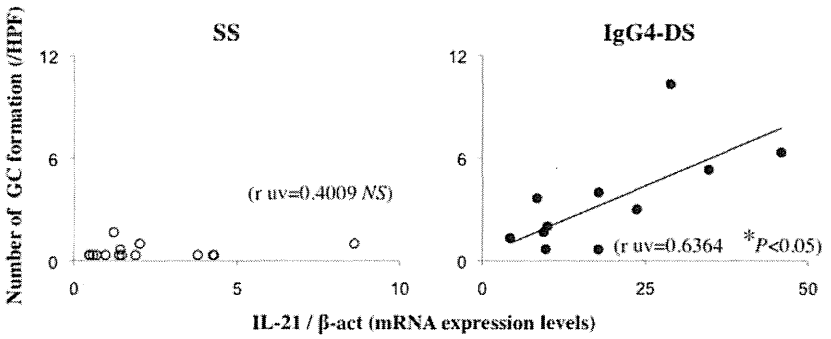


Figure 4 Expression of interleukin 21 (IL-21) may be correlated with ectopic germinal centre (GC) formation in the labial salivary glands (LSGs) of patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). Correlations between the number of GC formations and IL-21 mRNA expression level in the LSGs from patients with Sjögren's syndrome (SS) (n=15) and those with IgG4-DS (n=12). Real-time PCR products for IL-21 were quantitatively estimated as described in the Methods section. Numbers of GC per high-power field (HPF) were counted in 4 mm^2 sections from five different areas as described in the Methods section. Significance of differences between groups was determined by Spearman rank correlation (*p<0.05). NS, not significant.

selective infiltration of IgG4+ plasma cells around the acini and ductal cells and only mild destruction of the acini compared with patients with SS (figure 5B). Furthermore, the level of IL-21 mRNA expression in LSGs from patients with IgG4-DS was correlated with the IgG4/IgG ratio in immunohistochemically positive cells (figure 5C).

DISCUSSION

In 1953, Morgan and Castleman examined specimens from 18 cases diagnosed with MD.[36] Because of the histological similarities between SS and MD, they reported that almost all cases diagnosed with MD were considered to be SS.[37] However, the results of the present study show that the pattern of GC formation in LSGs from patients with SS and MD (recently named IgG4-DS) were distinctly different; MD has more hyperplastic GCs than SS. We therefore focused on the mechanism of multiple GC formation in IgG4-DS.

IL-21 has been reported to control the functional activity of effector Th cells and to promote ectopic GC formation by Tfh cells, which were recently shown to contribute to impaired B cell differentiation.[17] In mice responding to helminth antigens, Tfh cells differentiating from Th2 cells help GC formation in the lymph nodes. We therefore examined the expression of IL-21 and Tfh-related molecules (CXCR5 and Bcl-6) in LSGs from SS and IgG4-DS patients. Immunohistochemical staining indicated that CXCR5 expression was strongly detected in ectopic GCs from both SS and IgG4-DS patients. Interestingly, IL-21 and Bcl-6 expression was strongly detected outside ectopic GCs only in patients with IgG4-DS. As noted above, IL-21 is mainly produced by Th2 and Th17 cells in addition to Tfh cells.[15–18 38] In order to reveal the differences in IL-21-expressing infiltrating lymphocytes in LSGs between SS and IgG4-DS, we also analysed the expression of Th2-related molecules (IL-4, CCR4 and c-Maf) and Th17-related molecules (IL-17 and RORC2). The expression patterns of Th2-related molecules in LSGs were similar to that of IL-21 in patients with IgG4-DS. In contrast, Th17-related molecules were rarely expressed in patients with IgG4-DS. In this study the mRNA expression levels of IL-21 and IL-17 in the LSGs from patients with SS were higher than those in controls. Furthermore, the expression of IL-21 was detected more strongly in and around the ectopic GC than the ductal epithelial cells in patients with SS. A recent study reported that IL-21 alone was capable of directly inducing both B lymphocyte-induced maturation protein-1 (Blimp-1), which is required for plasma cell differentiation, and Bcl-6, which is required for GC formation.[18] Hiramatsu *et al*[39] recently reported that c-Maf, which is a Th2 cell-specific transcription factor that activates the IL-4 promoter, directly induced IL-21 production in CD4 T cells. Furthermore, in the present study we found that IL-21 was positively correlated with the number of GC formations in LSGs from patients with IgG4-DS. These findings suggest that excessive IL-21 production by Th2 cells in salivary glands from patients with IgG4-DS might induce expression of Bcl-6 in B cells resulting in multiple GC formations.

On the other hand, several studies have reported that IL-21 and IL-17 were detected in epithelial and infiltrating mononuclear cells in LSGs from patients with SS and may play an important role in the pathogenesis of SS.[40–42] These results are consistent with our present data. In this study the mRNA expression levels of IL-21 and IL-17 in LSGs from patients with SS were higher than those from controls. Furthermore, the expression of IL-21 was detected more strongly in and around the ectopic GC than the ductal epithelial cells of patients with SS. In contrast, the expression of IL-17 was detected more strongly in and around the ductal epithelial cells than the ectopic GC from patients with SS. In addition, our previous study indicated that mRNA expression levels of Th17-related molecules in the LSGs from patients with SS were significantly higher than in controls.[21]

Allergic immune responses are known to be caused by allergen-specific Th2-type cytokines IL-4 and IL-13 which are responsible for IgG4 and IgE induction by B cells.[43] A previous study indicated that IL-10 decreased IL-4-induced IgE switching but increased IL-4-induced IgG4 production.[44] We previously reported that class switching of IgG4 in IgG4-DS is caused by IL-4 and IL-10.[21] Furthermore, several studies have reported that IL-21 directly inhibits IL-4-induced IgE production,[24] and class switching of IgG4 is caused by co-stimulation with IL-4 and IL-21 in humans and mice.[23] In addition, IL-21 induced IL-10 production by mitogen-stimulated peripheral blood mononuclear cells in humans.[25] We therefore suggested that IL-21 correlated with IL-4 and IL-10 in the class switching of IgG4. In the current study we found that IL-21 was positively correlated with the IgG4/IgG mRNA expression ratio by real-time PCR analyses and the IgG4/IgG ratio in immunohistochemically-positive cells. These results suggest that IL-21 might also be involved in the class switching of IgG4 in IgG4-DS. However,

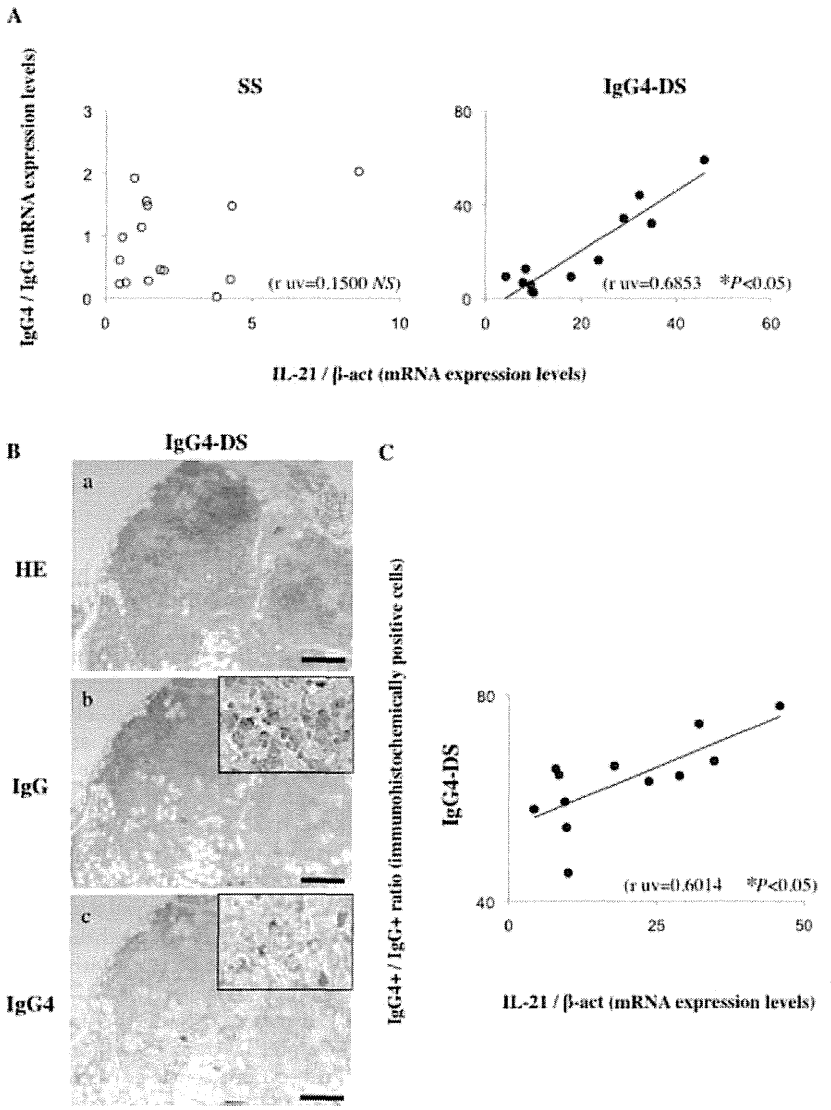


Figure 5 IgG4 production may be correlated with expression of interleukin 21 (IL-21) in the labial salivary glands (LSGs) of patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS). (A) Correlations between the IgG4/IgG mRNA expression ratio and IL-21 mRNA expression level in the LSGs from patients with Sjögren's syndrome (SS) (n=15) and those with IgG4-DS (n=12). Real-time PCR products for IL-21, IgG and IgG4 were quantitatively estimated as described in the Methods section. (B) IgG4+ cell ratio (%)=IgG4+ cells/IgG+ cells 100. Counts were made in 1 mm^2 sections from five different areas. (C) Correlations between the frequencies of IgG4+ cells and levels of IL-21 mRNA in the LSGs from patients with IgG4-DS (n=12). Significance of differences between groups was determined by Spearman rank correlation (*p<0.05). NS, not significant.

more case reports and further examinations are required to elucidate the pathogenesis of the disease.

In this study we have confirmed that the overexpression of IL-21 by Th2 cells is involved in the induction of multiple GC formation and IgG4 production in IgG4-DS. However, further studies are needed, including the establishment of a mouse model of IgG4-DS. A more thorough understanding of the complex mechanisms of IgG4-DS, especially the role of Th subset-related cytokines, could lead to the development of novel pharmacological strategies aimed at disrupting the cytokine network and inhibiting the initiation and/or progression of IgG4-DS.

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Competing interest None.

Patient consent Obtained.

Ethics approval Ethics approval was obtained from the ethics committee of Kyushu University, Japan.

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