References

1. Umehara H, Okazaki K, Masaki Y, Kawano M, Yamamoto M, Saeki T, et al. A novel clinical entity, IgG4-related disease (IgG4RD): general concept and details. Mod Rheumatol. 2012;22:1–14.
2. John HS, Yoh Z, Vikram D. IgG4-related disease. N Engl J Med. 2012;366:539–51.
3. Kubota T, Moritani S. Orbital IgG4-related disease: clinical features and diagnosis. ISRN Rheumatol. 2012 (Epub 2012 Jun 21).
4. Go H, Kim JE, Kim YA, Chung HK, Khwarg SI, Kim CW, Jeon YK. Ocular adnexal IgG4-related disease: comparative analysis with mucosa-associated lymphoid tissue lymphoma and other chronic inflammatory conditions. Histopathology 2012;60:296–312.
5. Stone JH, Khosroshahi A, Deshpande V, Chan JK, Heathcote JG, Aalberse R, et al. Recommendations for the nomenclature of IgG4-related disease and its individual organ system manifestations. Arthritis Rheum. 2012;64:3061–7.
6. Wallace ZS, Khosroshahi A, Jakobiec FA, Deshpande V, Hatton MP, Ritter J, et al. IgG4-related systemic disease as a cause of "idiopathic" orbital inflammation, including orbital myositis, and trigeminal nerve involvement. Surv Ophthalmol. 2012;57:26–33.
7. Umehara H, Okazaki K, Masaki Y, Kawano M, Yamamoto M, Saeki T, et al. Comprehensive diagnostic criteria for IgG4-related disease (IgG4-RD), 2011. Mod Rheumatol. 2012;22:21–30.
8. Takahira M, Ozawa Y, Kawano M, Zen Y, Hamaoka S, Yamada K, Sugiyama K. Clinical aspects of IgG4-related orbital inflammation in a case series of ocular adnexal lymphoproliferative disorders. Int J Rheumatol. 2012;2012:635473.
9. Hermet M, André M, Kémény JL, Le Guenno G, Déchelotte P, Guettrot-Imbert G, et al. Is IgG4-related disease a cause of xerostomia? A cohort study of 60 patients. Int J Rheumatol. 2012;2012:303506.
10. Khosroshahi A, Stone JH. Treatment approaches to IgG4-related systemic disease. Curr Opin Rheumatol. 2011;23:67–71.
11. Sandanayake NS, Church NI, Chapman MH, Johnson GJ, Dhar DK, Amin Z, et al. Presentation and management of post-treatment relapse in autoimmune pancreatitis/immunoglobulin G4-associated cholangitis. Clin Gastroenterol Hepatol. 2009;7:1089–96.
12. Naitoh I, Nakazawa T, Ohara H, Sano H, Ando T, Hayashi K, et al. Autoimmune pancreatitis associated with various extrapancreatic lesions during a long-term clinical course successfully treated with azathioprine and corticosteroid maintenance therapy. Intern Med. 2009;48:2003–7.
13. Nanke Y, Kobashigawa T, Yago T, Kamatani N, Kotake S. A case of Mikulicz's disease, IgG4-related plasmacytic syndrome, successfully treated by corticosteroid and mizoribine, followed by mizoribine alone. Intern Med. 2010;49:1449–53.
14. Khosroshahi A, Carruthers MN, Deshpande V, Unizony S, Bloch DB, Stone JH. Rituximab for the treatment of IgG4-related disease: lessons from 10 consecutive patients. Medicine (Baltimore). 2012;91:57–66.
15. Yamamoto M, Takahashi H, Ishigami K, Yajima H, Shimizu Y, Tabeya T, et al. Relapse patterns in IgG4-related disease. Ann Rheum Dis. 2012;71:1755.

Rheumatology Advance Access published July 19, 2013
RHEUMATOLOGY 262

Concise report

doi:10.1093/rheumatology/ket241

Association of Takayasu arteritis with HLA-B*67:01 and two amino acids in HLA-B protein

Chikashi Terao[1,2], Hajime Yoshifuji[2], Koichiro Ohmura[2], Kosaku Murakami[3], Daisuke Kawabata[2], Kimiko Yurugi[4], Junichi Tazaki[5], Hideyuki Kinoshita[6], Akinori Kimura[7], Masashi Akizuki[8], Yasushi Kawaguchi[9], Hisashi Yamanaka[9], Yasuo Miura[4], Taira Maekawa[4], Hiroo Saji[10], Tsuneyo Mimori[2] and Fumihiko Matsuda[1]

Abstract

Objective. Takayasu arteritis (TAK) is a rare autoimmune arteritis that affects large arteries. Although the association between TAK and HLA-B*52:01 is established, the other susceptibility *HLA-B* alleles are not fully known. We performed genetic association studies to determine independent *HLA-B* susceptibility alleles other than HLA-B*52:01 and to identify important amino acids of HLA-B protein in TAK susceptibility.

Methods. One hundred patients with TAK and 1000 unrelated healthy controls were genotyped for *HLA-B* alleles in the first set, followed by a replication set containing 73 patients with TAK and 1000 controls to compare the frequencies of *HLA-B* alleles. Step-up logistic regression analysis was performed to identify susceptibility amino acids of HLA-B protein.

Results. Strong associations of susceptibility to TAK with HLA-B*52:01 and HLA-B*67:01 were observed ($P = 1.0 \times 10^{-16}$ and 9.5×10^{-6}, respectively). An independent susceptibility effect of HLA-B*67:01 from HLA-B*52:01 was also detected ($P = 1.8 \times 10^{-7}$). Amino acid residues of histidine at position 171 and phenylalanine at position 67, both of which are located in antigen binding grooves of the HLA-B protein, were associated with TAK susceptibility ($P \leqslant 3.8 \times 10^{-5}$) with a significant difference from other amino acid variations ($\Delta AIC \geqslant 9.65$).

Conclusion. HLA-B*67:01 is associated with TAK independently from HLA-B*52:01. Two amino acids in HLA-B protein are strongly associated with TAK susceptibility.

Key words: Takayasu arteritis, genetic association study, HLA-B, aortitis, vasculitis.

Introduction

Takayasu arteritis (TAK) is a relatively rare systemic arteritis that affects mainly large branches of the arteries, including the aorta, carotid artery, subclavian artery and coronary artery. TAK affects mainly young females. Both environmental and genetic components have been shown to be involved with the onset of TAK [1]. Among genetic components, HLA-B52, mainly B*52:01, has shown a strong association with TAK with odds ratios (ORs) of ~3 [2, 3]. The association with HLA-B52 has been repeatedly shown in various studies and the association is established beyond ethnicity [4, 5]. Other associations between TAK and HLA alleles have not been confirmed due to the low prevalence of the disease and the lack of

[1]Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, [2]Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, [3]Department of Rheumatology and Clinical Immunology, Osaka Red Cross Hospital, Osaka, [4]Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto, [5]Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, [6]Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, [7]Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, [8]Akizuki Clinic, Yokohama, [9]Institute of Rheumatology, Tokyo Women's Medical University, Tokyo and [10]HLA Laboratory, Kyoto, Japan.

Submitted 29 January 2013; revised version accepted 21 May 2013.

Correspondence to: Chikashi Terao, Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan. E-mail: a0001101@kuhp.kyoto-u.ac.jp

large-scale comprehensive genetic analysis using TAK patients. While a previous study showed that HLA-B39 is also associated with TAK [6], another recent study failed to replicate the association [7]. The recent study also suggested that a relatively rare *HLA-B* allele, HLA-B*67:01, was associated with TAK susceptibility (corrected $P = 0.023$) [7], but there are no replication studies. No studies have addressed independent associations of *HLA-B* alleles from HLA-B*52:01. Moreover, no large studies have ever been performed to analyse which amino acid residues are important for TAK susceptibility. As some TAK patients develop autoantibodies against aortic endocardium [8], the detection of susceptibility amino acids to TAK would lead to identification of a possible antigen that provokes an autoimmune response in TAK patients.

Materials and methods

Study subjects

DNA samples of 100 Japanese patients with TAK were collected at Kyoto University Hospital and Tokyo Women's Medical University. DNA samples of 73 patients with TAK were also collected at Kyoto University Hospital. Two patients in the first set are a parent and a child. Each of 1000 control DNA samples in the first and replication sets were collected at the HLA laboratory from unrelated healthy individuals. All the patients were diagnosed with TAK based on ACR criteria [9] or guidelines of the Japanese Circulation Society [10] or were registered to the Japanese national registry for rare and intractable diseases (http://www.nanbyou.or.jp/english/index.htm). Information on classifications of TAK based on criteria by Hata *et al.* [11] and of complications of aortic regurgitation (AR) were obtained from 75 and 85 patients, respectively, by reviewing clinical charts from the Kyoto University Hospital. A summary of the subjects is shown in supplementary Table S1, available at *Rheumatology* Online. This study was approved by the local ethics committees at each institution (Kyoto University Graduate School and Faculty of Medicine Ethics Committee, Tokyo Women's Medical University Genome Ethics Committee and Tokyo Women's Medical University Ethics Committee) and written informed consent was obtained from all subjects.

HLA-B genotyping

The cases and controls were genotyped for *HLA-B* alleles in four-digit resolution using the WAKFlow system at the Kyoto University Hospital and HLA laboratory, respectively.

Amino acid sequences of *HLA-B* alleles

Amino acid sequences were obtained for each *HLA-B* allele for four-digit resolution from the IMGT database (http://www.ebi.ac.uk/ipd/imgt/hla/). Amino acid variations were searched across all the *HLA-B* alleles contained in the study population. In total, 99 variants of amino acids over 53 positions were determined in the *HLA-B* alleles. A three-dimensional structure analysis of the HLA-B protein was performed using UCSF Chimera software (University of California, San Francisco, San Francisco, CA, USA) [12].

Statistical analysis

The association between TAK and *HLA-B* alleles was investigated using 2×2 contingency tables and tested for statistical significance using the χ^2 test or Fisher's exact test for alleles with a frequency >1% in either the cases or the controls. Heterogeneity between the two studies was analysed using the Breslow–Day test. The ORs and 95% CIs were also calculated. The relative predispositional effect (RPE) method [13] was applied to identify the associations of more than one *HLA-B* allele sequentially according to their strength. The associations between clinical phenotypes and HLA-B*67:01 were assessed by the Wilcoxon rank sum test for age at onset or Fisher's exact test for other clinical phenotypes. Crude *P*-values were indicated unless we mentioned the use of corrected *P*-values.

The 99 amino acids over 53 positions were used as independent variables in logistic regression analysis (supplementary Table S2, available at *Rheumatology* Online). When we found only two amino acids in the same position, we set one independent variable in this position. When we found more than three amino acids in the same position, we distinguished all amino acids as independent variables. Step-up multiple logistic regression analyses for TAK susceptibility were performed to identify susceptibility amino acids in *HLA-B* in the combined study until an amino acid whose *P*-value did not reach significance was chosen. The Akaike information criterion (AIC) was also calculated.

Significant levels were set according to Bonferroni's correction, namely, 0.0028 for *HLA-B* alleles, 0.05 for clinical phenotypes and 0.00051 for amino acids. Amino acid variations were considered significant over other variations when the logistic regression model containing the variation showed the smallest AIC with the difference in AIC >7 in comparison with those containing other alleles ($\Delta AIC > 7$) [14]. Permutation tests were performed 10 000 times to assess the probability that the observed improvements of deviance in multiple logistic regression analysis were obtained by chance. Deviances of the best-fitting model using one, two and three amino acids were calculated in each permutation test and sequential improvements of deviance by the best three amino acids were compared with the observed improvements. Deviance of logistic regression analysis is defined as $-2 \times \log$ likelihood of logistic models with degrees of freedom of k, where $k-1$ is the number of alleles in the models.

Results

The strong association of TAK with HLA-B*52:01 was confirmed in the first set ($P = 2.6 \times 10^{-13}$). A significant association with HLA-B*67:01 was also observed ($P = 0.00081$; Table 1), and no other *HLA-B* alleles showed significant associations with TAK. The replication

TABLE 1 Associations of HLA-B alleles with TAK susceptibility

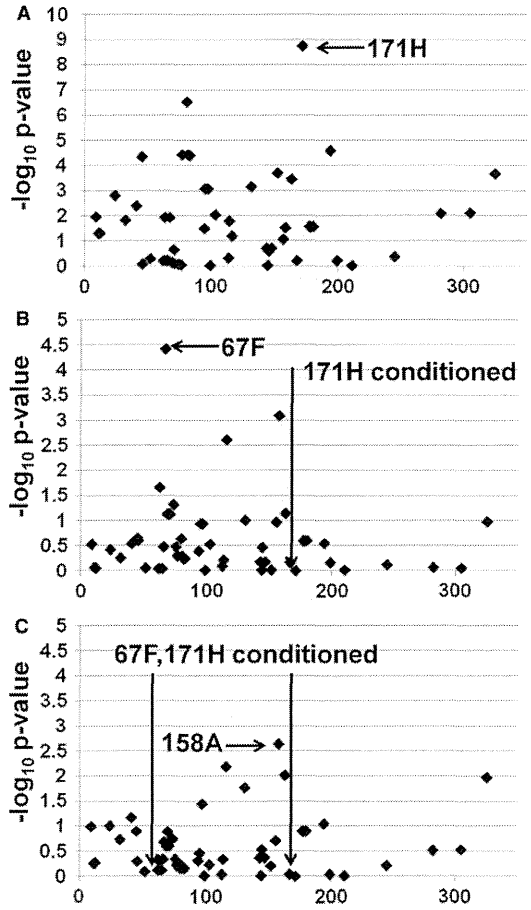
	First set				Replication set				Combined study		
HLA-B allele[a]	Case	Control	*P*	OR (95% CI)	Case	Control	*P*	OR (95% CI)	*P*	OR (95% CI)	RPE *P*
B*07:02	7	122	0.14	0.56 (0.26, 1.21)	6	119	0.36	0.68 (0.29, 1.57)	0.085	0.61 (0.34, 1.08)	
B*13:01	1	28	0.51	0.35 (0.0086, 2.17)	1	27	1.00	0.50 (0.012, 3.10)	0.32	0.42 (0.049, 1.59)	
B*15:01	16	153	0.86	1.05 (0.61, 1.80)	11	152	0.98	0.99 (0.52, 1.87)	0.90	1.03 (0.68, 1.54)	
B*15:18	1	27	0.31	0.51 (0.0089, 2.25)	0	35	0.17	0 (0, 1.52)	0.059	0.18 (0.0046, 1.08)	
B*35:01	10	160	0.13	0.61 (0.31, 1.17)	9	164	0.38	0.74 (0.37, 1.47)	0.084	0.66 (0.41, 1.06)	
B*39:01	3	68	0.20	0.43 (0.086, 1.34)	8	61	0.11	1.84 (0.86, 3.93)	0.96	0.99 (0.53, 1.84)	
B*40:01	6	108	0.14	0.54 (0.24, 1.25)	9	115	0.84	1.08 (0.53, 2.17)	0.33	0.77 (0.45, 1.31)	
B*40:02	14	177	0.38	0.78 (0.44, 1.36)	6	153	0.11	0.52 (0.22, 1.19)	0.11	0.68 (0.43, 1.09)	
B*40:06	8	92	0.70	0.86 (0.41, 1.81)	1	102	0.0085	0.13 (0.0032, 0.74)	0.057	0.52 (0.27, 1.03)	
B*44:03	13	125	0.89	1.04 (0.58, 1.88)	6	115	0.41	0.7 (0.30, 1.62)	0.70	0.91 (0.56, 1.47)	
B*46:01	11	87	0.45	1.28 (0.67, 2.44)	6	102	0.6	0.8 (0.34, 1.85)	0.87	1.04 (0.63, 1.73)	
B*48:01	5	60	0.69	0.83 (0.33, 2.09)	3	61	0.80	0.67 (0.13, 2.08)	0.45	0.76 (0.37, 1.57)	
B*51:01	10	161	0.12	0.60 (0.31, 1.16)	14	169	0.63	1.15 (0.65, 2.04)	0.39	0.83 (0.54, 1.27)	
B*52:01	60	232	2.6×10^{-13}	3.27 (2.34, 4.55)	34	235	4.8×10^{-5}	2.28 (1.52, 3.43)	1.6×10^{-16}	2.82 (2.19, 3.64)	1.6×10^{-16}
B*54:01	12	133	0.72	0.90 (0.49, 1.65)	9	149	0.57	0.82 (0.41, 1.63)	0.57	0.85 (0.54, 1.35)	
B*55:02	2	47	0.31	0.42 (0.049, 1.62)	5	58	0.72	1.19 (0.47, 3.01)	0.72	0.77 (0.35, 1.66)	
B*59:01	2	41	0.43	0.48 (0.056, 1.88)	1	39	0.52	0.35 (0.0085, 2.08)	0.21	0.43 (0.086, 1.31)	
B*67:01	9	27	0.00081	3.44 (1.60, 7.43)	5	19	0.0061	3.70 (1.36, 10.05)	9.5×10^{-6}	3.62 (1.97, 6.66)	1.8×10^{-7}

[a]Alleles with a frequency of >1% in either the case or control are indicated.

study was performed to confirm the findings in the first set. As a result, the association of HLA-B*67:01 with TAK was observed ($P = 0.0061$; Table 1), as well as that of HLA-B*52:01 ($P = 4.8 \times 10^{-5}$). HLA-B*40:06 showed a protective association against TAK ($P = 0.0085$). In the combined analysis, solid evidence of associations of HLA-B*67:01 and HLA-B*52:01 with TAK was obtained [$P = 9.5 \times 10^{-6}$ and 1.0×10^{-16}, OR = 3.62 (95% CI 1.97, 6.66) and 2.82 (95% CI 2.19, 3.64), respectively; Table 1]. Any signs of heterogeneity between the two studies were observed for these two alleles ($P \geqslant 0.18$). No other alleles, including HLA-B*40:06, showed significant associations. To assess the independence of HLA-B*67:01's association from HLA-B*52:01 and to detect further candidates of independent susceptibility *HLA-B* alleles, RPE analysis was performed in the combined study. As a result, HLA-B*67:01 showed a significant association with TAK susceptibility that was independent of HLA-B*52:01 ($P = 1.8 \times 10^{-7}$; Table 1). The analysis did not show further candidates with independent associations with TAK ($P \geqslant 0.17$). Since previous studies suggested associations between *HLA-B* alleles and disease phenotypes [15], we analysed the associations between HLA-B*67:01 and age at onset, female ratio, classifications of TAK or complications of AR. As a result, we did not observe significant associations ($P \geqslant 0.61$; supplementary Tables S3 and S4, available at *Rheumatology* Online).

Next, which amino acid residues contribute to TAK susceptibility was analysed by a step-up multiple logistic regression analysis in the combined study, using 99 amino acid variations over the HLA-B protein (see the Materials and methods section). Logistic regression analysis revealed the strongest association of histidine residue at the position of the 171st amino acid residue ($P = 1.8 \times 10^{-9}$; Fig. 1 and supplementary Table S5, available at *Rheumatology* Online), with a ΔAIC of 11.17 in comparisons with other amino acids. In the case of conditioning with the 171st histidine, phenylalanine at the 67th amino acid residue showed the strongest association ($P = 3.8 \times 10^{-5}$; Fig. 1), with an ΔAIC of 9.65. An alanine residue at the 158th position showed a suggestive association in conditioning with these two amino acids ($P = 0.0024$; Fig. 1) without significant ΔAIC (0.13), indicating uncertainty of the selection of this amino acid as the third susceptibility amino acid. We did not find any further amino acid variations with significant associations in the condition with these three amino acids ($P \geqslant 0.065$). A total of 10 000 permutation tests revealed that improvements in deviance from these three amino acids were less likely to be obtained by chance (permutation *P*-value 0.0001, 0.0001 and 0.0024, respectively). The 171st and 67th amino acid residues are located in peptide binding grooves, implying that an antigen binding capacity conferred by these amino acids might influence TAK susceptibility (supplementary Fig. S1, available at *Rheumatology* Online). When HLA-B*52:01 was excluded from the association studies, the two amino acid variations did not show significant associations (data not shown), indicating that the associations of the two amino acids were brought by HLA-B*52:01.

Fig. 1 Associations of amino acids of the HLA-B protein with TAK susceptibility.



P-values are plotted according to the amino acid positions in (**A**) single logistic regression analysis, (**B**) in the condition with histidine at position 171 and (**C**) with 171 histidine and 67 phenylalanine.

Discussion

This is the first study to provide solid evidence of an *HLA-B* TAK susceptibility allele independent of HLA-B*52:01 and to report on TAK susceptibility amino acids in HLA-B protein. As the top two residues of 171 and 67 were located at peptide binding grooves and have shown their significance in peptide binding [16, 17], the susceptibility effects on TAK appear to be reasonable.

HLA-B39 did not show an association in the current study, or in another recent study [7]. Thus association between HLA-B39 and TAK is not likely. Although the previous study suggested the association of TAK with HLA-B*67:01, the association was not conclusive [7]. The current study has clearly revealed that HLA-B*67:01 is associated with TAK independently of HLA-B*52:01. The association and independence of

HLA-B*67:01 were observed in the two independent sets. The two relatives in our study did not have HLA-B*67:01, thus having no effect on the conclusion. When we analysed the previous data in which only serological *HLA-B* type was available [6], HLA-B67 showed a suggestive association with TAK after excluding HLA-B52 (95 cases and 232 controls) [6]. As HLA-B*67:01 is present specifically in East Asians, this association should be specific to East Asians. The RPE analysis did not reveal an association of HLA-B*40:06. This indicates that the protective association of HLA-B*40:06 in the replication set was conferred by positive associations of HLA-B*52:01 and HLA-B*67:01. While we detected the association of HLA-B*67:01 with TAK, HLA-B*67:01 did not show significant associations with age at onset, female ratio, TAK classifications and AR. Based on the fact that the *P*-values are far from significant, it is not very likely that HLA-B*67:01 has a strong effect on clinical phenotype. Further detailed clinical information, including affected branches of the aorta, and disease activity were available for only a limited number of patients in our study, so we could not analyse the association between the susceptibility allele and detailed clinical phenotypes.

The 171st histidine was found to be a susceptibility amino acid and the 67th phenylalanine was found to be a protective amino acid. While the permutation *P*-value supported the existence of the third susceptibility amino acid, alanine at position 158 did not show a significant ΔAIC in comparison with other amino acids. Thus we cannot specify the third susceptibility amino acid to TAK. The significant associations of the top two amino acids were mainly attributed to HLA-B*52:01. No other alleles share the two amino acids with HLA-B*52:01. Although HLA-B*51:01 has similar amino acid sequences to HLA-B*52:01 with two different amino acid residues, it is not associated with TAK [4, 7]. This lack of association can be explained by our findings because HLA-B*51:01 includes the 67th phenylalanine, the protective allele against TAK. HLA-B*67:01 did not have histidine in position 171. The effects of amino acids in HLA-B*67:01 were not very apparent due to its low frequency. These two amino acids did not explain TAK susceptibility more efficiently than HLA-B*52:01 (ΔAIC over the null model = 53.94 and 66.01, respectively), indicating that these amino acids cannot explain all the susceptibility effects of HLA-B*52:01. This suggests the existence of a haplotypic effect of amino acids in HLA-B*52:01 or further susceptibility amino acids.

The 171st tyrosine forming an A pocket in the α2 domain is one of the highly conserved amino acid residues among *HLA-B* alleles. The conversion into histidine has been shown to modulate the binding groove, coordinating the N-terminus of the binding peptides in HLA-B14 and HLA-B*51:01 [17, 18]. The 67th serine in the B pocket of the peptide binding groove in HLA-B27 has been shown to influence peptide presentation [16]. These reports suggest important roles for the two amino acids in TAK susceptibility by modulating peptide binding. A previous Mexican study, which included 19 patients with TAK, suggested that serine at 67 and glutamic acid at 63 was associated with TAK [19]. The previous report seemed to support our results, as the current study showed non-phenylalanine amino acid residues at position 67, including serine, were positively associated with TAK. As the current study did not specify the third susceptibility amino acid, the specific association of alanine at position 158 with TAK is inconclusive. Thus these combinations of amino acids should be regarded as incomplete and further investigation is required.

As previous studies surveying susceptibility HLA alleles beyond *HLA-B* did not find significant alleles beyond the *HLA-B* alleles [6, 7], it is not very likely that amino acids in other HLA alleles linked with HLA-B*52:01 and HLA-B*67:01 can explain the TAK susceptibility of the HLA locus. Nevertheless, it is undeniable that other genes in the HLA locus, without belonging to HLA alleles, play a critical role in TAK susceptibility. Amino acid residues in the HLA locus should be analysed to grasp the whole picture of the relationship between the HLA locus and TAK susceptibility. It would be interesting to determine what kinds of protein bind to the alleles containing the susceptibility amino acid residues.

Rheumatology key messages

- HLA-B*67:01 is an independent susceptibility allele to TAK from HLA-B*52:01.
- Amino acids at positions 171 and 67 of the HLA-B protein show TAK susceptibility effects .

Acknowledgements

We would like to thank Ms Kayo Umemoto, who coordinated the acquisition of blood samples. We would like to thank all the patients who gave blood samples and the medical staff members who helped us with this study. C.T. and H. Yoshifuji participated in conception and design of this study. C.T., H. Yoshifuji, K.O., K.M., D.K., J.T., H.K., K.A., K.Y., Y.M., T. Maekawa, H.S., M.A., Y.K., H. Yamanaka, F.M. and T. Mimori participated in acquisition of data. C.T. performed analysis and interpretation of the data. C.T. wrote the initial draft and all the authors reviewed and approved the final manuscript.

Funding: This study was supported by a Kyoto University Step-up grant as well as grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Disclosure statement: The authors declare no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

References

1 Arnaud L, Haroche J, Mathian A *et al.* Pathogenesis of Takayasu's arteritis: a 2011 update. Autoimmun Rev 2011; 11:61–7.

2 Yoshida M, Kimura A, Katsuragi K *et al.* DNA typing of HLA-B gene in Takayasu's arteritis. Tissue Antigens 1993; 42:87–90.

3 Yajima M, Numano F, Park YB *et al.* Comparative studies of patients with Takayasu arteritis in Japan, Korea and India—comparison of clinical manifestations, angiography and HLA-B antigen. Jpn Circ J 1994;58:9–14.

4 Sahin Z, Bicakcigil M, Aksu K *et al.* Takayasu's arteritis is associated with HLA-B*52, but not with HLA-B*51, in Turkey. Arthritis Res Ther 2012;14:R27.

5 Vargas-Alarcon G, Flores-Dominguez C, Hernandez-Pacheco G *et al.* Immunogenetics and clinical aspects of Takayasu's arteritis patients in a Mexican Mestizo population. Clin Exp Rheumatol 2001;19:439–43.

6 Kimura A, Kitamura H, Date Y *et al.* Comprehensive analysis of HLA genes in Takayasu arteritis in Japan. Int J Cardiol 1996;54(Suppl):S61–9.

7 Takamura C, Ohhigashi H, Ebana Y *et al.* New human leukocyte antigen risk allele in Japanese patients with Takayasu arteritis. Circ J 2012;76:1697–702.

8 Eichhorn J, Sima D, Thiele B *et al.* Anti-endothelial cell antibodies in Takayasu arteritis. Circulation 1996;94: 2396–401.

9 Arend WP, Michel BA, Bloch DA *et al.* The American College of Rheumatology 1990 criteria for the classification of Takayasu arteritis. Arthritis Rheum 1990; 33:1129–34.

10 Japanese Circulation Society Joint Working Group. Guideline for management of vasculitis syndrome (JCS 2008). Circ J 2011;75:474–503.

11 Hata A, Noda M, Moriwaki R *et al.* Angiographic findings of Takayasu arteritis: new classification. Int J Cardiol 1996; 54(Suppl):S155–63.

12 Pettersen EF, Goddard TD, Huang CC *et al.* UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 2004;25: 1605–12.

13 Payami H, Joe S, Farid NR *et al.* Relative predispositional effects (RPEs) of marker alleles with disease: HLA-DR alleles and Graves disease. Am J Hum Genet 1989;45: 541–6.

14 Burnham KP, Anderson, David R. Model selection and multi-model inference: a practical information-theoretic approach. 2nd edn. New York: Springer-Verlag, 2002.

15 Kitamura H, Kobayashi Y, Kimura A *et al.* Association of clinical manifestations with HLA-B alleles in Takayasu arteritis. Int J Cardiol 1998;66(Suppl 1): S121–6.

16 Appel H, Kuon W, Kuhne M *et al.* The solvent-inaccessible Cys67 residue of HLA-B27 contributes to T cell recognition of HLA-B27/peptide complexes. J Immunol 2004;173:6564–73.

17 Maenaka K, Maenaka T, Tomiyama H *et al.* Nonstandard peptide binding revealed by crystal structures of HLA-B*5101 complexed with HIV immunodominant epitopes. J Immunol 2000;165:3260–7.

18 Kumar P, Vahedi-Faridi A, Saenger W *et al.* Structural basis for T cell alloreactivity among three HLA-B14 and HLA-B27 antigens. J Biol Chem 2009;284: 29784–97.

19 Vargas-Alarcon G, Hernandez-Pacheco G, Soto ME *et al.* Comparative study of the residues 63 and 67 on the HLA-B molecule in patients with Takayasu's arteritis. Immunol Lett 2005;96:225–9.

Mod Rheumatol
DOI 10.1007/s10165-012-0812-9

ORIGINAL ARTICLE

Validation of different sets of criteria for the diagnosis of Sjögren's syndrome in Japanese patients

Hiroto Tsuboi · Shinya Hagiwara · Hiromitsu Asashima · Hisanori Umehara · Atsushi Kawakami · Hideki Nakamura · Hajime Sano · Kazuo Tsubota · Yoko Ogawa · Etsuko Takamura · Ichiro Saito · Hiroko Inoue · Seiji Nakamura · Masafumi Moriyama · Tsutomu Takeuchi · Yoshiya Tanaka · Shintaro Hirata · Tsuneyo Mimori · Isao Matsumoto · Takayuki Sumida

Received: 8 November 2012 / Accepted: 5 December 2012

Abstract
Objective To validate the revised Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (SS) (JPN) (1999), The American-European Consensus Group classification criteria for SS (AECG) (2002), and American College of Rheumatology classification criteria for SS (ACR) (2012).
Methods The study subjects were 694 patients with SS or suspected SS who were followed-up in June 2012 at ten hospitals that form part of the Research Team for Autoimmune Diseases, The Research Program for Intractable Disease by the Ministry of Health, Labor and Welfare (MHLW). All patients had been checked for all four criteria of the JPN (pathology, oral, ocular, anti-SS-A/SS-B antibodies). We studied the clinical diagnosis made by the physician in charge and the satisfaction of the above criteria.
Results Of the 694 patients, 499 patients did not have other connective tissue diseases (CTDs). SS was diagnosed in 476 patients (primary SS in 302, secondary SS in 174), whereas non-SS was diagnosed in 218 patients (without other CTDs in 197, with other CTDs in 21) by the physician in charge. The sensitivities of JPN, AECG, and ACR in the diagnosis of all forms of SS (both primary and secondary SS) were 79.6, 78.6, and 77.5 %, respectively, with respective specificities of 90.4, 90.4, and 83.5 %. The sensitivities of the same systems in the diagnosis of primary SS were 82.1, 83.1, and 79.1 %, respectively, with specificities of 90.9, 90.9, and 84.8 %, respectively. The sensitivities of the same systems in the diagnosis of secondary SS were 75.3, 70.7, and 74.7 %, respectively, with specificities of 85.7, 85.7, and 71.4 %, respectively.
Conclusion The sensitivity of JPN to all forms of SS and secondary SS, the sensitivity of AECG to primary SS, and the specificities of JPN and AECG for all forms of SS, primary SS, and secondary SS were highest in the diagnosis of SS in Japanese patients. These results indicate that

H. Tsuboi · S. Hagiwara · H. Asashima · I. Matsumoto · T. Sumida (✉)
Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
e-mail: tsumida@md.tsukuba.ac.jp

H. Tsuboi · H. Umehara · A. Kawakami · H. Nakamura · H. Sano · K. Tsubota · Y. Ogawa · E. Takamura · I. Saito · H. Inoue · S. Nakamura · M. Moriyama · T. Takeuchi · Y. Tanaka · S. Hirata · T. Mimori · T. Sumida
The Research Team for Autoimmune Diseases, The Research Program for Intractable Disease of the Ministry of Health, Labor and Welfare (MHLW), Tokyo, Japan

H. Umehara
Department of Hematology and Immunology, Kanazawa Medical University, Kanazawa, Japan

A. Kawakami · H. Nakamura
Unit of Translational Medicine, Department of Immunology and Rheumatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

H. Sano
Division of Rheumatology, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan

K. Tsubota · Y. Ogawa
Department of Ophthalmology, School of Medicine, Keio University, Tokyo, Japan

E. Takamura
Department of Ophthalmology, Tokyo Women's Medical University, School of Medicine, Tokyo, Japan

Published online: 28 December 2012

the JPN criteria for the diagnosis of SS in Japanese patients are superior to ACR and AECG.

Keywords Sjögren's syndrome · Criteria

Introduction

Sjögren's syndrome (SS) is an autoimmune disease that affects exocrine glands, including the salivary and lacrimal glands. It is characterized by lymphocytic infiltration into the exocrine glands, leading to dry mouth and eyes. A number of autoantibodies, such as anti-SS-A and SS-B antibodies, are detected in patients with SS. SS is subcategorized into primary SS, which is not associated with other well-defined connective tissue diseases (CTDs), and secondary SS, which is associated with other well-defined CTDs [1]. Primary SS is further subcategorized into the glandular form and the extraglandular form.

The revised criteria for the diagnosis of SS issued by the Japanese Ministry of Health (JPN) (1999) (Table 1) [2], as well as the American-European Consensus Group classification criteria for SS (AECG) (2002) (Tables 2, 3) [1], are usually used in both daily clinical practice and clinical studies in Japan. Thus, two sets of diagnostic systems are being applied for the same disease. This could result in a heterogeneous pool of SS patients. This heterogeneity of SS patients makes it difficult to analyze the diagnosis, efficacy of treatment, and prognosis of SS patients. A better alternative would be to use a unified set of criteria for the diagnosis of SS in Japan. Recently, The American College of Rheumatology (ACR) published the ACR classification criteria for SS (2012) (Table 4), which were proposed by the Sjögren's International Collaborative Clinical Alliance (SICCA) [3]. The new set of criteria is designed to be used worldwide, not only in advanced countries but also in developing countries. The SICCA established a uniform classification for SS based on a combination of objective tests that have known specificity to SS [3].

Table 1 The revised Japanese Ministry of Health criteria for the diagnosis of SS (1999)

1. Histopathology
Definition: Positive for at least one of (A) or (B)
(A) Focus score ≥1 (periductal lymphoid cell infiltration ≥50) in a 4 mm^2 minor salivary gland biopsy
(B) Focus score ≥1 (periductal lymphoid cell infiltration ≥50) in a 4 mm^2 lacrimal gland biopsy
2. Oral examination
Definition: Positive for at least one of (A) or (B)
(A) Abnormal findings in sialography ≥stage 1 (diffuse punctate shadows of <1 mm)
(B) Decreased salivary secretion (flow rate ≤10 ml/10 min according to the chewing gum test or ≤2 g/2 min according to the Saxon test) and decreased salivary function according to salivary gland scintigraphy
3. Ocular examination
Definition: Positive for at least one of (A) or (B)
(A) Schirmer's test ≤5 mm/5 min and rose bengal test ≥3 according to the van Bijsterveld score
(B) Schirmer's test ≤5 mm/5 min and positive fluorescein staining test
4. Serological examination
Definition: Positive for at least one of (A) or (B)
(A) Anti-Ro/SS-A antibody
(B) Anti-La/SS-B antibody
Diagnostic criteria: diagnosis of SS can be made when the patient meets at least two of the above four criteria

Upon comparing these three classification sets, there are some differences among them in their purpose and the items adopted in the set (Table 5). The JPN criteria (1999) are intended as an aid for diagnosis, whereas the AECG criteria (2002) and the ACR criteria (2012) are intended for classification purposes in clinical studies and trials. Although the ACR criteria include only three objective items (Tables 4, 5) and are the simplest among the three sets, the ACR criteria may not identify SS patients with negative findings in labial salivary gland biopsy, because the ACR criteria do not include salivary secretion analysis and imaging studies. On the other hand, the JPN criteria combined oral examinations such as salivary secretion, sialography, and salivary gland scintigraphy with three objective items adopted in the ACR criteria (Table 5). Only the AECG criteria include ocular and oral symptoms, which may cause false positives in patients with non-SS conditions such as aging or visual display terminals (VDT) syndrome (Table 5).

I. Saito · H. Inoue
Department of Pathology, Tsurumi University School of Dental Medicine, Yokohama, Kanagawa, Japan

S. Nakamura · M. Moriyama
Section of Oral and Maxillofacial Oncology,
Division of Maxillofacial Diagnostic and Surgical Sciences,
Faculty of Dental Science, Kyushu University, Fukuoka, Japan

T. Takeuchi
Division of Rheumatology, Department of Internal Medicine,
School of Medicine, Keio University, Tokyo, Japan

Y. Tanaka · S. Hirata
The First Department of Internal Medicine, School of Medicine,
University of Occupational and Environmental Health,
Kitakyushu, Japan

T. Mimori
Department of Rheumatology and Clinical Immunology,
Kyoto University Graduate School of Medicine, Kyoto, Japan

Table 2 The American-European Consensus Group classification criteria for SS (2002)

I. Ocular symptoms: a positive response to at least one of the following questions
1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?
II. Oral symptoms: a positive response to at least one of the following questions
1. Have you had a daily feeling of dry mouth for than 3 months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?
III. Ocular signs—that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests
1. Schirmer's test, performed without anaesthesia ($\leq$5 mm in 5 min)
2. Rose bengal score or other ocular dry eye score ($\geq$4 according to van Bijsterveld's scoring system)
IV. Histopathology: in minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score $\geq$1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acuni and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue
V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests
1. Unstimulated whole salivary flow ($\leq$1.5 ml in 15 min)
2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer
VI. Autoantibodies: presence in the serum of the following autoantibodies
1. Antibodies to Ro (SS-A) or La (SS-B) antigens, or both

Table 3 The American-European Consensus Group classification criteria for SS (2002) rules for classification

For primary SS
In patients without any potentially associated disease, primary SS may be defined as follows:
(A) The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (histopathology) or VI (serology) is positive
(B) The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)
For secondary SS
In patients with a potentially associated disease (for instance, another well-defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS
Exclusion criteria:
Past head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficiency disease (AIDS)
Pre-existing lymphoma
Sarcoidosis
Graft vs. host disease
Use of anticholinergic drugs (for a time shorter than 4-fold the half life of the drug)

The purpose of the present study was to validate the JPN criteria, AECG criteria, and ACR criteria for the diagnosis of SS in Japanese patients. The study identified the differences among these three classification sets.

Patients and methods

Study population

The study subjects were 694 patients (51 males and 643 females) with a diagnosis of SS or suspected SS who had been checked for all four criteria of the JPN (pathology, oral, ocular, anti-SS-A/SS-B antibody), and were followed up in June 2012 at ten hospitals across Japan (Kanazawa Medical University Hospital, Nagasaki University Hospital, Hyogo Medical University Hospital, Keio University Hospital, Tokyo Women's Medical University Hospital, Tsurumi University Hospital, Kyushu University Hospital, University of Occupational and Environmental Health Hospital, Kyoto University Hospital, and University of Tsukuba Hospital) that form part of the Research Team for Autoimmune Diseases, The Research Program for Intractable Disease of the Ministry of Health, Labor and Welfare (MHLW).

Data collection and analysis

We collected clinical data from the above ten hospitals using a questionnaire. We retrospectively examined the clinical diagnosis made by the physician in charge, as well as the satisfaction of the JPN, AECG, and ACR criteria. Because lissamine green ocular staining had not been adopted in Japan at the time of clinical examination, we regarded patients who had a positive rose bengal test or fluorescein staining test as having satisfied the ocular staining score in the ACR classification system.

We regarded the clinical diagnosis made by the physician in charge as the gold standard for the diagnosis of SS in this study. We compared the sensitivities and specificities of the JPN, AECG, and ACR diagnostic systems in the diagnosis of SS (both primary and secondary SS), primary

Table 4 The American College of Rheumatology classification criteria for SS (2012)

The classification of SS, which applies to individuals with signs/symptoms that may be suggestive of SS, will be met in patients who have at least 2 of the following 3 objective features:
1. Positive serum anti-SS-A/Ro and/or anti-SS-B/La or (positive rheumatoid factor and ANA titer $\geq$1:320)
2. Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score $\geq$1 focus/4 mm^2
3. Keratoconjunctivitis sicca with ocular staining score $\geq$3 (assuming that individual is not currently using daily eye drops for glaucoma and has not had corneal surgery or cosmetic eyelid surgery in the last 5 years)
Prior diagnosis of any of the following conditions would exclude participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests:
History of head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficiency syndrome
Sarcoidosis
Amyloidosis
Graft vs. host disease
IgG4-related disease

SS, and secondary SS. Agreement between the three was assessed via the kappa coefficient.

Results

Diagnosis of SS (primary and secondary SS) and non-SS

Of the 694 patients, 499 patients did not have other well-defined CTDs, whereas 195 patients did. SS was diagnosed in 476 patients (302 primary SS, 174 secondary SS), whereas non-SS was diagnosed in 218 patients (197 without other CTDs, 21 with other CTDs) by the physician in charge (Table 6).

Sensitivities and specificities of the three diagnostic systems for SS

The sensitivities of JPN, AECG, and ACR in the diagnosis of all SS (302 primary SS and 174 secondary SS) were 79.6, 78.6, and 77.5 %, respectively, whereas the respective specificities in the diagnosis of all SS were 90.4, 90.4, and 83.5 %. The sensitivities of JPN, AECG, and ACR in the diagnosis of 302 primary SS were 82.1, 83.1, and 79.1 %, respectively, with specificities of 90.9, 90.9, and 84.8 %, respectively. The sensitivities of JPN, AECG, and ACR in the diagnosis of 174 secondary SS were 75.3, 70.7, and 74.7 %, respectively, with specificities of 85.7, 85.7, and 71.4 % (Table 7).

Table 5 Comparison of the items adopted in the JPN and AECG and ACR criteria

	JPN	AECG	ACR
Ocular symptoms	×	○	×
Oral symptoms	×	○	×
Ocular signs			
Schirmer's test	○	○	×
Ocular staining	○	○	○
Labial salivary gland biopsy	○	○	○
Salivary gland involvements			
Salivary secretion	○	○	×
Sialography	○	○	×
Scintigraphy	○	○	×
Autoantibodies			
SS-A	○	○	○
SS-B	○	○	○
ANA	×	×	○
RF	×	×	○

SS-A anti-SS-A antibody, *SS-B* anti-SS-B antibody, *ANA* anti-nuclear antibody, *RF* rheumatoid factor, ○ adopted, × not adopted, *JPN* the revised Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

Table 6 Diagnosis of SS and non-SS

	Associated with other CTDs		Total
	No	Yes	
Clinical diagnosis			
SS	302 (primary SS)	174 (secondary SS)	476
Non-SS	197	21	218
Total	499	195	694

Clinical diagnosis diagnosis of SS by the physician in charge

CTDs connective tissue diseases

Comparisons of the satisfaction of the three diagnostic systems

Figure 1 displays Venn diagrams showing comparisons of the satisfaction of the three diagnostic systems. Among all SS patients ($n = 476$), more patients satisfied only the AECG criteria ($n = 42$) rather than only the JPN criteria ($n = 8$) or the ACR criteria ($n = 6$). The same tendency was also observed in patients with primary SS only and in those with secondary SS only. The diagrams indicate that the JPN and ACR diagnostic systems are similar, whereas the AECG diagnostic system is different from the other two. Table 8 shows the agreement among the three

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

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

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

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

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

Discussion

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

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

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

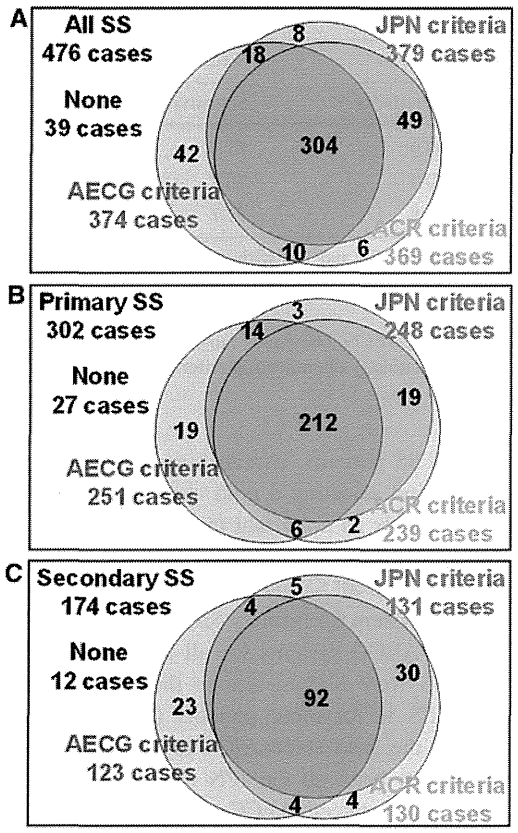


Fig. 1 Venn diagrams showing a comparison of the satisfaction of the three tested systems. **a** Comparison of the satisfaction of the three tested systems, performed using data from all 476 SS patients (302 primary SS and 174 secondary SS). **b** Comparison of the satisfaction of the three tested systems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

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

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

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

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

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

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

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

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

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

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

Acknowledgments We thank Dr. F.G. Issa for critically reading the manuscript. This work was supported by Health and Labour Sciences Research Grants for research on intractable diseases (The Research Team for Autoimmune Diseases) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest None.

References

1. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis. 2002;61:554–8.
2. Fujibayashi T, Sugai S, Miyasaka N, Hayashi Y, Tsubota K. Revised Japanese criteria for Sjögren's syndrome (1999): availability and validity. Mod Rheumatol. 2004;14:425–34.
3. Shiboski SC, Shiboski CH, Criswell L, Baer A, Challacombe S, Lanfranchi H, et al. American College of Rheumatology classification criteria for Sjögren's syndrome: a data-driven, expert consensus approach in the Sjögren's International Collaborative Clinical Alliance cohort. Arthritis Care Res. 2012;64:475–87.
4. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40:1725.
5. Petri M, Orbai AM, Alarcón GS, Gordon C, Merrill JT, Fortin PR, Bruce IN, Isenberg D, Wallace DJ, Nived O, Sturfelt G, Ramsey-Goldman R, Bae SC, Hanly JG, Sánchez-Guerrero J, Clarke A, Aranow C, Manzi S, Urowitz M, Gladman D, Kalunian K, Costner M, Werth VP, Zoma A, Bernatsky S, Ruiz-Irastorza G, Khamashta MA, Jacobsen S, Buyon JP, Maddison P, Dooley MA, van Vollenhoven RF, Ginzler E, Stoll T, Peschken C, Jorizzo JL, Callen JP, Lim SS, Fessler BJ, Inanc M, Kamen DL, Rahman A, Steinsson K, Franks AG Jr, Sigler L, Hameed S, Fang H, Pham N, Brey R, Weisman MH, McGwin G Jr, Magder LS. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum. 2012;64:2677–86.
6. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovský J, Wolfe F, Hawker G. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62:2569–81.

MODERN PATHOLOGY (2014), 1–11

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

Mai Takeuchi[1,6], Yasuharu Sato[1,6], Kyotaro Ohno[1], Satoshi Tanaka[2], Katsuyoshi Takata[1], Yuka Gion[1], Yorihisa Orita[3], Toshihiro Ito[4], Tomoyasu Tachibana[5] and Tadashi Yoshino[1]

[1]Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [2]Department of Immunochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [3]Department of Otolaryngology, Head and Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [4]Department of Pathology and Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan and [5]Department of Otolaryngology, Himeji Red Cross Hospital, Himeji, Japan

IgG4-related disease is a systemic disorder with unique clinicopathological features and uncertain etiological features and is frequently related to allergic disease. T helper 2 and regulatory T-cell cytokines have been reported to be upregulated in the affected tissues; thus, the production of these cytokines by T helper 2 and regulatory T cells has been suggested as an important factor in the pathogenesis of IgG4-related disease. However, it is not yet clear which cells produce these cytokines in IgG4-related disease, and some aspects of the disorder cannot be completely explained by T-cell-related processes. To address this, we analyzed paraffin-embedded sections of tissues from nine cases of IgG4-related submandibular gland disease, five cases of submandibular sialolithiasis, and six cases of normal submandibular gland in order to identify potential key players in the pathogenesis of IgG4-related disease. Real-time polymerase chain reaction analysis confirmed the significant upregulation of interleukin (IL)4, IL10, and transforming growth factor beta 1 (TGFβ1) in IgG4-related disease. Interestingly, immunohistochemical studies indicated the presence of mast cells expressing these cytokines in diseased tissues. In addition, dual immunofluorescence assays identified cells that were double-positive for each cytokine and for KIT, which is expressed by mast cells. In contrast, the distribution of T cells did not correlate with cytokine distribution in affected tissues. We also found that the mast cells were strongly positive for IgE. This observation supports the hypothesis that mast cells are involved in IgG4-related disease, as mast cells are known to be closely related to allergic reactions and are activated in the presence of elevated non-specific IgE levels. In conclusion, our results indicate that mast cells produce T helper 2 and regulatory T-cell cytokines in tissues affected by IgG4-related disease and possibly have an important role in disease pathogenesis.
Modern Pathology advance online publication, 3 January 2014; doi:10.1038/modpathol.2013.236

Keywords: cytokine; IgE; IgG4-related disease; mast cells; regulatory T cell

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

Correspondence: Dr Y Sato, Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Kita-ku, Okayama 700-8558, Japan.
E-mail: satou-y@cc.okayama-u.ac.jp
[6]These authors contributed equally to this work.
Received 31 July 2013; revised 3 November 2013; accepted 4 November 2013; published online 3 January 2014

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

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

Materials and methods

Samples

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

Real-Time Quantitative PCR

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

Histological Examination and Immunohistochemistry

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

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

Dual Immunofluorescence Assays

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

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

Statistical Analysis

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

Results

Confirmation of Histological Diagnosis in IgG4-Related Disease

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

Histological Findings of Sialolithiasis

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

Elevated Expression of Cytokines in IgG4-Related Disease

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

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

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

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

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

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

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

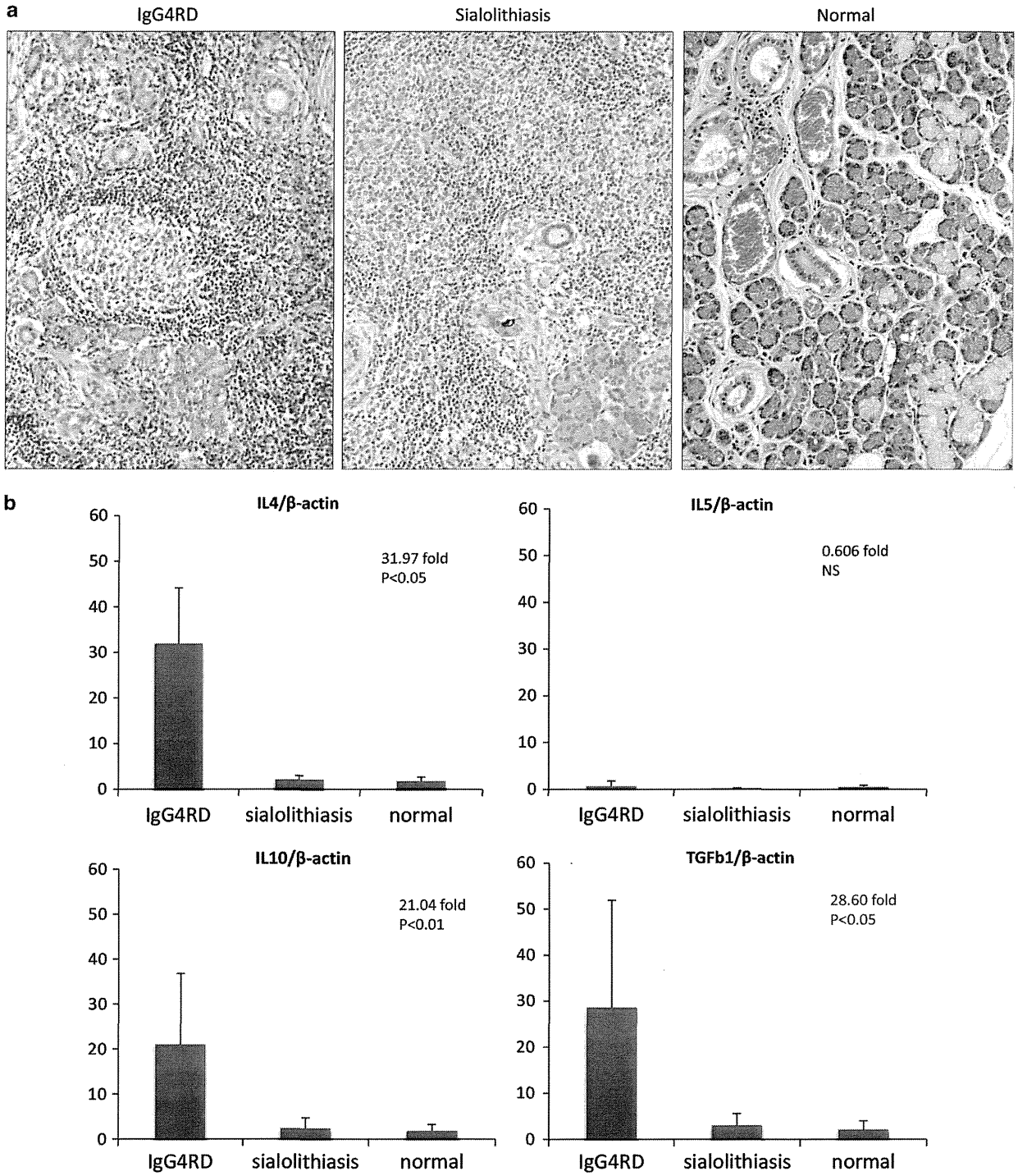


Figure 1 Histological findings and real-time PCR analysis of cytokine expression in IgG4-related disease and control groups. (**a**) IgG4-related submandibular disease showed dense lymphoplasmacytic infiltration with lymphoid follicle (hematoxylin and eosin, H&E, left). Sialolithiasis revealed moderate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

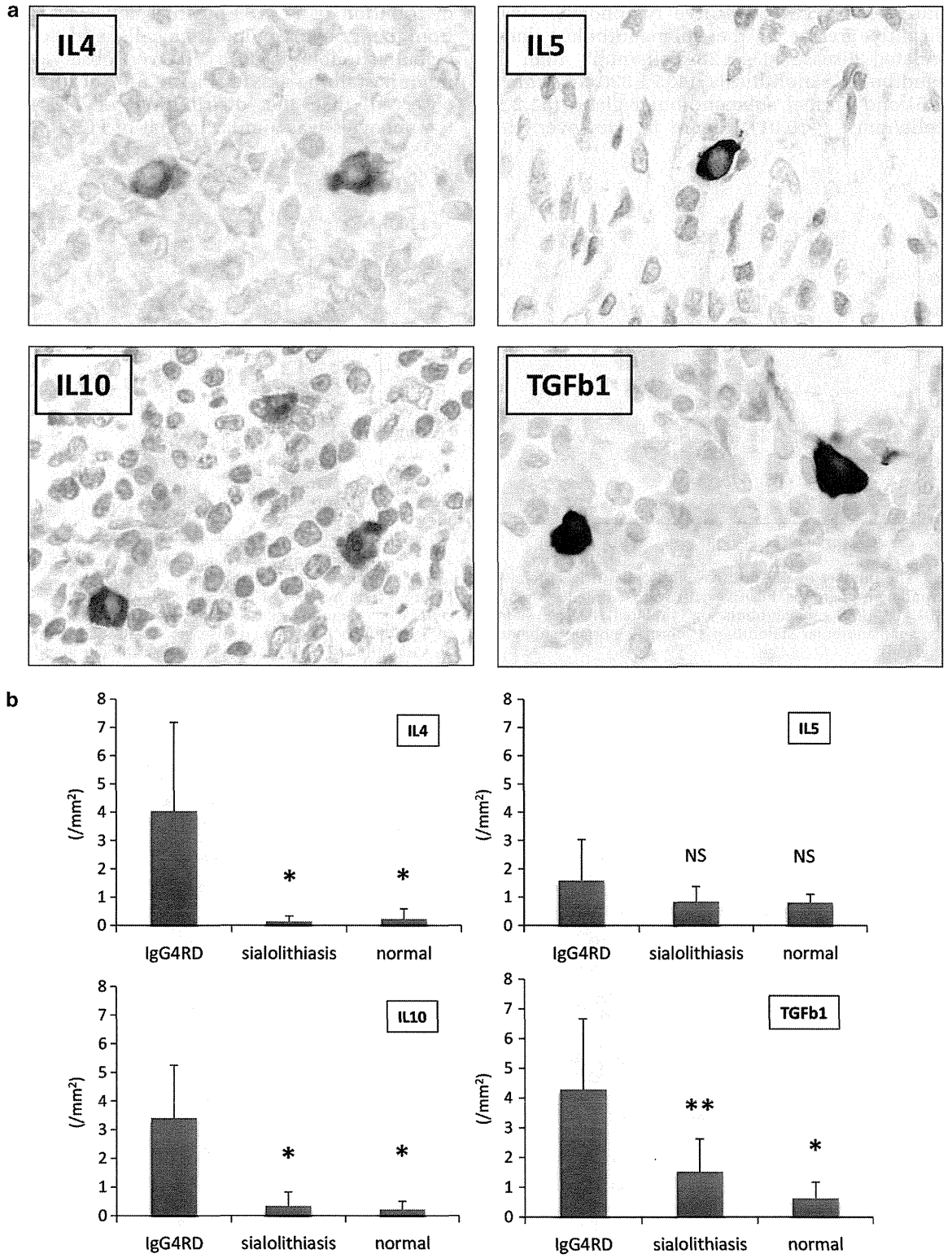


Figure 2 Immunohistochemical analysis of cytokine-expressing cells in IgG4-related disease and control groups. (**a**) Cells stained with antibodies against the indicated cytokines. (**b**) The number of cells positive for interleukin 4 (IL4), IL10, and transforming growth factor beta 1 (TGFβ1) were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

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

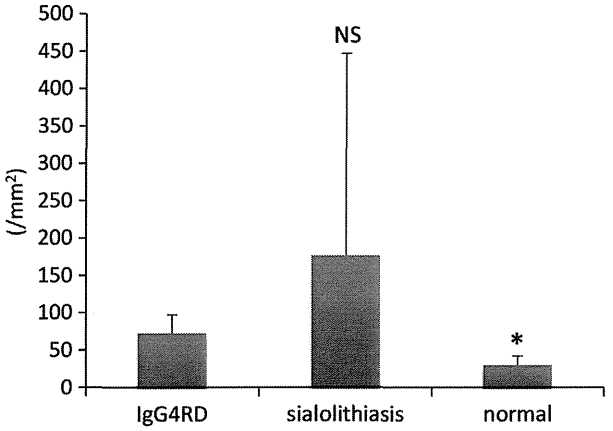


Figure 3 The number of KIT-positive mast cells in IgG4-related disease and control groups. Positive cells were counted per mm^2. (*$P<0.01$; IgG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

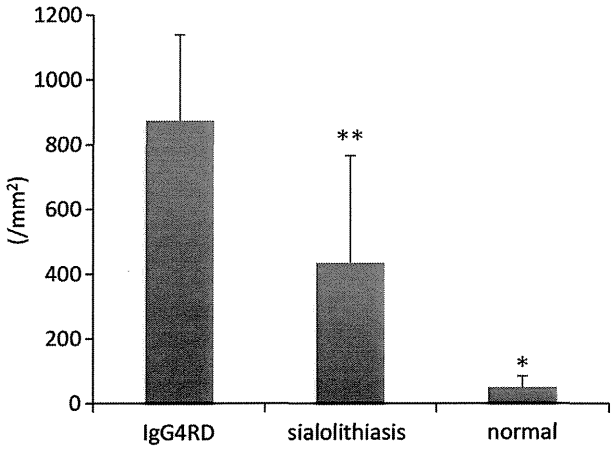


Figure 5 The number of FOXP3-positive lymphocytes. Positive cells were counted per mm^2. (*$P<0.01$, **$P<0.05$; gG4RD, submandibular IgG4-related disease; sialolithiasis, submandibular sialolithiasis; normal; normal submandibular gland).

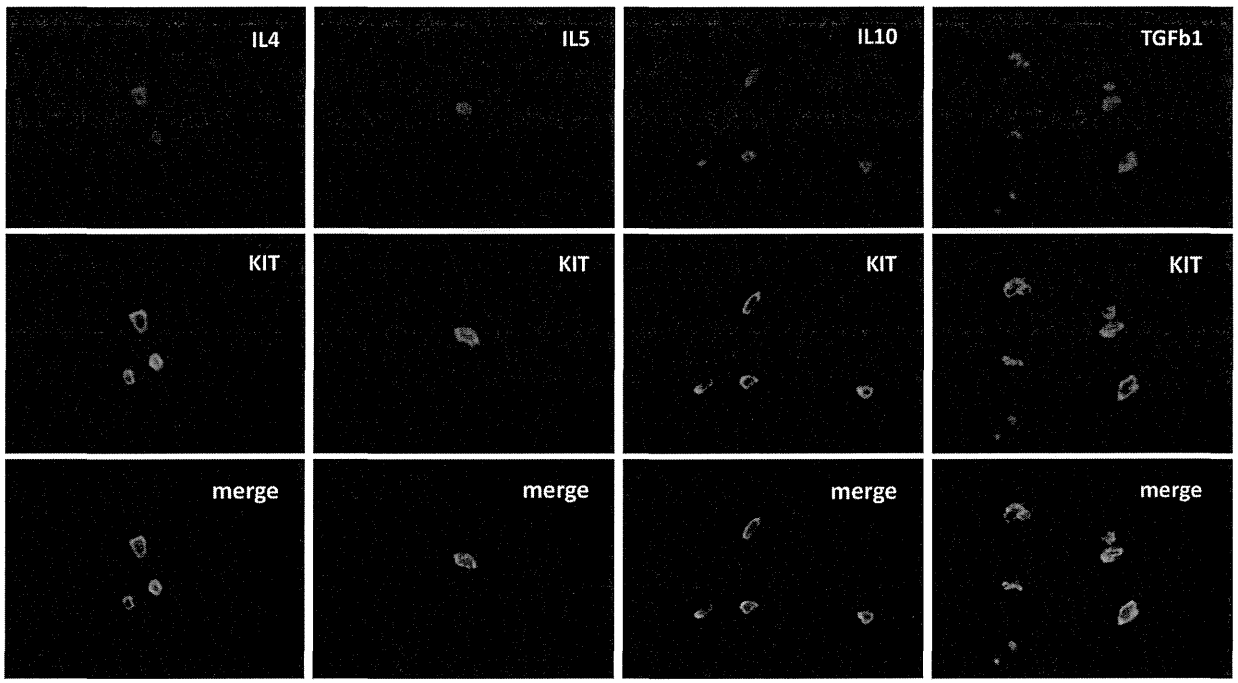


Figure 4 Dual fluorescent analysis of KIT-positive mast cells and cytokine-positive cells in IgG4-related submandibular gland disease. Compared with the results for normal submandibular gland, the number of KIT-positive cells (middle row) was significantly higher in IgG4-related disease and sialolithiasis,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