Analysis of IgG4-positive clones in affected organs of IgG4-related disease

Yasushi Kakuchi[1], Kazunori Yamada[1], Kiyoaki Ito[1], Satoshi Hara[1], Hiroshi Fujii[1], Masakazu Yamagishi[2], Mitsuhiro Kawano[1*]

[1]Division of Rheumatology, Department of Internal Medicine, Kanazawa University Graduate School of Medicine, 920-0942, Japan

[2]Division of Cardiology, Department of Internal Medicine, Kanazawa University Graduate School of Medicine, 920-0942, Japan

[*]Correspondence: Mitsuhiro Kawano, sk33166@gmail.com

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Abstract

Objective. We investigated class switch reaction (CSR) in affected organs and evaluated whether the same or genetically related clones exist in IgG4-RD.

Methods. We studied three patients with IgG4-RD. Total cellular RNA was extracted from salivary glands and peripheral blood, and lung tissue. Activation-induced cytidine deaminase (AID) and immunoglobulin heavy chain third complementarity determining region (IgVH-CDR3) of IgM and IgG4 were detected by reverse transcription polymerase chain reaction. We analyzed the clonal relationship of infiltrating IgG4-positive cells, as compared with IgM. We determined the existence of common clones among organs and patients.

Result. AID was expressed in salivary glands of all patients and lung tissue in one. Closely related IgVH-CDR3 sequences in infiltrating IgG4-positive cells were detected in salivary glands and lung tissue. Identical IgVH-CDR3 sequence between IgM and IgG4 in salivary glands was detected in one patient, indicating CSR in salivary glands. Identical IgVH-CDR3 sequences of IgG4-positive cells were detected between salivary glands and peripheral blood in two patients. Four identical sequences of IgVH-CDR3 existed between patients. Interestingly, one of the four sequences was detected in all patients.

Conclusion. Our results demonstrate the existence of common antigen(s) shared by patients with IgG4-RD.

Introduction

IgG4-related disease (IgG4-RD) is a clinical entity characterized by high serum IgG4 concentrations and extensive IgG4-positive plasma cell infiltration into involved organs [1, 2]. IgG4-RD shares some histopathological features in involved organs and affects multiple organs, notably pancreas, lacrimal and/or salivary glands, lung, kidney, retroperitoneum, aorta, lymph nodes and skin [3-13]. A dramatic responsiveness to corticosteroid is a notable feature, while the etiology remains unclear.

Class switch recombination (CSR) and somatic hypermutation (SHM) are found in germinal center (GC)-like structures. Activation-induced cytidine deaminase (AID) is required for both CSR and SHM [14, 15]. AID is expressed not only in secondary lymphoid organs but also in local tissues in salivary glands of Sjögren syndrome (SS) [16-18], nasal mucosa of allergic rhinitis [19, 20] and bronchial asthma[21]. We previously showed AID expression in lacrimal gland of IgG4-related sialadenitis and/or dacryoadenitis and that SHM of IgG4-positive plasma cells might occur in lacrimal glands suggesting that immunoglobulin production in IgG4-related dacryoadenitis is antigen-driven [22]. However, it remains unclear whether polyclonal expansions of IgG4-positive cells and CSR in affected organs are common features of IgG4-RD.

Immunoglobulin has three complementarity determining regions (CDRs) that determine the specificity for antigen, and CDR3 plays the most important role. Therefore, the antibodies that have the same CDR3 sequence are surmised to recognize the same antigen. Previous studies demonstrated that IgG4 plays an inhibitory role in allergic rhinitis [23, 24]. Conversely, pathogenic antibodies of pemphigus were reported to belong to IgG4 subclass [25]. The pathological and immunological roles of IgG4 antibody in IgG4-RD remain unclear, and studies focusing on these issues are needed.

The aims of the present study are: 1) to investigate CSR in affected organs, and 2) to evaluate whether the same or genetically related clones exist in patients with IgG4-RD. For the purpose, we first analyzed the expression of AID to determine CSR and SHM in salivary glands and lung, and then compared the clonal relationship of infiltrating IgM and IgG4-positive cells. Furthermore, we compared IgG4-CDR3 between organs and peripheral blood of each patient and between patients.

Materials and methods

Patient Profiles

We enrolled three patients with IgG4-RD, the diagnosis of which was made by a specialist of IgG4-RD, based on the presence of tumefactive lesions and typical histopathological findings with copious IgG4-positive plasma cell infiltration into the affected organs (Figure 1). Two patients (Patient 1 and 2) had definite and one (Patient 3) had probable disease according to the comprehensive diagnostic criteria for IgG4-RD [26]. Patients' profiles are shown in Table 1. Salivary glands, lung tissue and peripheral blood samples were obtained from these patients.

This study received institutional ethics board approval, and informed consent for all data and samples was obtained from each patient. The research was conducted in compliance with the Declaration of Helsinki.

Histology and immunohistochemistry

Pathology specimens fixed in 20% formalin and embedded in paraffin were retrieved from the surgical pathology files of the Pathology Section of Kanazawa University Hospital, Kanazawa, Japan. Hematoxylin and eosin (HE) staining and IgG4 immunostaining were performed. In the latter, a mouse monoclonal antibody to IgG4 (1: 1000, ZYMED Laboratory, San Francisco, CA, USA) was used.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted from salivary glands, lung tissue and peripheral blood using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized using the Oligo d(T)16 (Applied Biosystems, CA, USA). We performed nested reverse RT-PCR for AID. The oligonucleotide sequences used are shown in Table 2. The amplification program consisted of one cycle of 94ºC for 10 min, followed by 30 cycles of 94ºC for 15 sec, 54ºC for 30 sec, and 72ºC for 30 sec. After 30 cycles all products received a final extension of 5 min at 72ºC. In the second PCR, the annealing temperature was 60ºC. We then performed immunoglobulin subclass-specific RT-PCR amplification using primers to amplify immunoglobulin Framework (FR) 3 and immunoglobulin subclass. The oligonucleotide sequences of immunoglobulin subclass specific reverse primers are shown in Table 2.

The variable region of IgVH of IgG4 was analyzed using IgVH gene family specific primers and IgG4 specific primer. Primers used for IgVH gene family specific amplification were

previously described [27]. IgVH of IgM was analyzed using hM3 for IgM specific primer. The amplification program consisted of one cycle of 94ºC for 10 min, followed by 30 cycles of 94ºC for 15 sec, 59ºC for 30 sec, 72ºC for 30 sec. After 30 cycles all products received a final extension of 5 min at 72ºC. Cloning of the IgVH gene PCR products was performed using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA).

Sequence analysis

Sequences were analyzed with an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). Primers for the sequence analysis were M13F, M13R or IgVH gene specific primers.

Data analysis

Sequences were compared with those in the IMGT/V-QUEST (http: //www.imgt.org/IMGT_vquest/share/textes/index.html) and compared with the IgVH-CDR3 of the salivary glands, lung tissue and peripheral blood cells.

Results

Detection of AID and CSR in the affected organs

We analyzed the expression of AID in salivary glands of the three patients and lung tissue of patient 2 using RT-PCR, and confirmed that AID was expressed in all of them (Figure 2a). These data indicated that both SHM and CSR occurred in the salivary glands and lung tissue.

Next, we investigated the existence of identical IgVH-CDR3 in IgM and IgG4 positive cells in each patient to confirm CSR in the affected organs. In patient 2, two identical sequences of IgVH-CDR3, SGYKFGTRFAV (AGT GGC TAC AAG TTC GGC ACA CGT TTT GCC GTC), were found in IgM and IgG4 positive cells in salivary glands, suggesting that CSR might have occurred in salivary glands.

Expansion of IgG4-positive cells in affected organs

We investigated the clonality of infiltrating IgM- and IgG4-positive cells by performing sequence analysis of IgVH-CDR3 (Table 3). For patient 1, six dominant sequences of CDR3 were detected in both IgM- and IgG4-positive cells that infiltrated salivary glands. For patient 2, six dominant sequences of IgM and one of IgG4-CDR3 were detected in salivary glands, and eight dominant sequences of IgG4-CDR3 were detected in lung tissue. For patient 3, five dominant sequences of IgM and eleven of IgG4-CDR3 were detected in salivary glands. The appearance of dominant sequences of IgM and IgG4-CDR3 may indicate that IgM- and IgG4-positive cells infiltrating the salivary glands and lung tissue in the patient with IgG4-RD showed dominant expansion.

Detection of identical or genetically related clones between organs and patients

We investigated the identical or genetically related IgG4 positive clones between salivary glands, lung tissue and peripheral blood. We found that the identical sequence of IgG4-CDR3, HLRFRGKAAPFDI in patient 1 and EFLN in patient 3, respectively, existed in salivary glands and peripheral blood (Table 4a). These results support our previous report that showed the existence of identical or genetically related clones between lacrimal glands and peripheral blood in a patient with IgG4-RD [22]. We also found that genetically related clones that had three different amino acid sequences of IgG4-CDR3 existed in salivary glands and lung tissue in patient 2 (Table 4a).

Next, we investigated the existence of an identical sequence of IgVH-CDR3 in the three

patients to clarify whether identical or clonally related clones are shared between them. In the IgG4-CDR3, four identical sequences were identified: NIAAIGTGYWYFDL in peripheral blood of patient 1, salivary glands of patient 3, and lung tissue of patient 2; VAIVVVPAGKGAFDI in lung tissue of patient 2, and in salivary glands of patient 3; DRAQY in salivary glands of patient 1 and 3; and HLRFRGKAAPFDI in salivary glands of patient 1 and 3, and in peripheral blood of patient 1 (Table 4b).

Discussion

In this study, we analyzed the repertoires of IgG4-positive or IgM-positive cell populations in the inflamed organs or peripheral blood in three patients with IgG4-RD, and obtained the identical sequences of IgVH-CDR3 between IgG4 and IgM-positive cells in salivary gland in patient 2. We also found four kinds of IgG4-positive clone that shared an identical or closely related IgVH-CDR3 sequence between the patients (Figure 3). In particular, an identical IgVH-CDR3 sequence, NIAAIGTGYWYFDL, was detected in the peripheral blood in patient 1 and two inflamed tissues (lung in patient 2, and salivary gland in patient 3). This clone showed marked accumulation in the minor salivary gland of patient 3 (12 identical clones out of the 47 clones analyzed). The minor salivary gland of patient 3 was suspected to be an early lesion of IgG4-RD, because of the presence of only one germinal center-like structure with scattered IgG4-positive plasma cells. A dominant IgG4-positive clone might be easily detected.

AID and two identical CDR3 sequences, SGYKFGTRFAV, between IgM and IgG4 were detected by sequencing IgVH-CDR3 in salivary glands of IgG4-RD. The expression of AID is required for the process of CSR and SHM [14]. We detected AID in lacrimal glands [22] and salivary glands in patients with IgG4-RD. These data indicated that CSR may occur in the organs affected by IgG4-RD. Previous studies on allergic rhinitis [19,20] and bronchial asthma [21] showed that isotype switching occurred at affected sites. Snow et al described that IgE related clones were matured in bronchial mucosa of atopic asthma [21]. Our data might reveal that isotype switching from IgM to IgG4 occurred in salivary glands of IgG4-RD.

We demonstrate the possible existence of a common antigen in IgG4-RD patients because the same CDR3 sequence was detected in 3 patients with IgG4-RD. Recently, Wallace et al demonstrated that circulating plasmablasts were likely to be a useful biomarker of disease activity in IgG4-RD [28]. In addition, a pathogenic role for plasmablasts in IgG4-RD was also speculated. Under these conditions, however, the relationship between circulating plasmablasts and tissue infiltrating IgG4-producing cells has not been sufficiently clarified. We previously showed common clones in IgG4-positive cells between lacrimal glands and peripheral blood [22]. Maillette de Buy Wenniger et al also clearly showed that the dominantly expanded clones were identical in both peripheral blood and the duodenal papilla (affected tissue) in patients with IgG4-associated cholangitis using next-generation sequencing technique [29]. Mattoo et al also showed that increased plasmablasts in the peripheral blood of 5 patients with IgG4-RD had a high degree of somatic hypermutation [30]. These findings also suggest that hypergammaglobulinemia and increased serum IgG4 levels are not merely induced by non-specific polyclonal gammaglobulinemic

stimulation but by an antigen-driven mechanism.

Although recent studies indicate that increased serum IgG4 levels result from an antigen-driven mechanism, it is unclear whether extrinsic or intrinsic antigens elicit increased production of IgG4 in IgG4-RD. Okazaki et al showed that antibodies against autoantigens such as pancreatic trypsin inhibitor, lactoferrin, and carbonic anhydrase exist in the sera of patients with autoimmune pancreatitis [31], suggesting that IgG4-RD is an autoimmune disease. However, they did not confirm that the IgG subclass of those antigens was IgG4. In contrast, Frulloni et al demonstrated that the antibodies against an amino acid sequence of plasminogen-binding protein of Helicobacter pylori were frequently found in the sera of autoimmune pancreatitis, although this interesting finding has not yet been confirmed by others [32]. Similarly, we previously showed the possibility of involvement of Mycobacterium tuberculosis in the pathogenesis of IgG4-RD [33]. These findings suggest that some kind of unusual immune reaction to some microorganisms might have a causative role in the disease process. Furthermore, since IgG4-RD is sometimes associated with allergic predisposition, allergic rhinitis and bronchial asthma, involvement of extrinsic antigens in the pathogenesis of IgG4-RD is also plausible.

In summary, our results suggest the existence of common antigen(s) shared by patients with IgG4-RD, although the antigens are still unknown. Further studies will be needed to clarify the key antigen that is the causative agent of IgG4-RD.

Acknowledgments

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Conflict of interest

None.

Reference

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Table 1. Profile of patients with IgG4-related disease

Patient	Age	Sex	IgG	IgG4	IgE	CH50	Affected organs	Biopsied organs (IgG4+ / CD138+ ratio, %)	Tested organs
1	79	F	4350	1770	3319	5	LG, SG, P	SG (88.5)	SG
2	64	M	3070	1800	302	64	SG, LT	SG (98.8) LT (87.1)	SG, LT
3	50	F	1078	61	1621	58	SG	SG (67.2)	SG

Average age was 64 years. Elevated IgG4 was seen in patient 1 and 2. Elevated IgE was seen in patient 1 and 3. Decreased complement was seen in patient 1. Anti-nuclear antibody (ANA), anti-Sjögren's syndrome A antibody (SSA) and anti-Sjögren's syndrome B antibody (SSB) were negative in all patients. LG: lacrimal gland, SG: salivary glands, P: pancreas, LT: lung tissue

Table 2. Sequences of primers

Aid F1	5'-GAGGCAAGAAGACACTCTGG-3'
Aid R1	5'-GTGACATTCCTGGAAGTTGC-3'
Aid F2	5'-TAGACCCTGGCCGCTGCTACC-3'
Aid R2	5'-CAAAAGGATGCGCCGAAGCTGTCTGGAG-3'
FR3	5'-CTGAGGACACGGCCGTGTATTACTG-3'
IgG4	5'-AACTCAGGTGCTGGGCATGATGGGGGACCATA-3'
hM3	5'-GGAAAAGGGTTGGGGCGGAT-3'

AID, activation-induced cytidine deaminase; IgG, immunoglobulin G.

Table 3. The sequence analysis of CDR3 in 3 patients with IgG4-RD.

Patient 1

Salivary gland

IgM-SG (n=32)	Number of sequences
G Y Y G S G S Y Y N V R A F D T	8
D Y Y S R M D V	3
A G Y S T E S L A F E I	2
G L N C G S I E C Y G A F D Y	2
K R M T G I A F D I	2
V G L V G V T G R S R A F D I	2
IgG4-SG (n=32)	
D G P D S S G Y V D Y	6
D V A V G G C Y D S	5
D L L A L A A D G I D Y	3
N I Y N N N W Y T G R L G A F D V	3
D G E M P A A Y H Y Y G M D V	2
D P L P D A I S G M D V	2

Peripheral blood

IgG4-PB (n=19)	Number of Sequences
D I T I D V V E I D H	4
L Y S D S S S L G T K T	2
N I A A I G T G Y W Y F D L	2

Patient 2

Salivary gland

IgM-SG (n=52)	Number of

	sequences
R L A S N S Y Y M D V	10
G D Y C S S T S C S N I H Y Y Y M D V	4
A D S D Y Y Y L A L S H	2
D P D Y Y V N W F D P	2
H P L T N S A Y F D S	2
S G R R G F G E L G F K W F D P	2
IgG4-SG (n=47)	
S G T Y P N R F Q V P F S V	2

Lung tissue

IgG4-LT (n=51)	Number of Sequences
G Y Y Y D I S G Y Y V F Q H	5
V A I V V V P A G K G A F D I	3
V R G Y F D S G R Q D M D V	3
A T V S W F G P Q N S Y F Y Y M D V	2
G K S S Y Y P N W F D P	2
G V P A S G S Y Y Y M D V	2
H V A L V P R Y G S S G Y F D F	2
L A D I Y Y G W V D P	2

Peripheral blood

IgG4-PB (n=51)	Number of Sequences
D R G L T Y A Y G S G S Y S C F D Y	2

Patient 3

Salivary gland

IgM-SG (n-27)	Number of sequences

D N G H D I L T G N Y N V A A F D I	3
A A N R A F A F	2
G S N Y A F I	2
R K E W A F V E	2
V H G A S G H Y D L D Y	2
IgG4-SG (n=47)	
N I A A I G T G Y W Y F D L	12
D F Y W L A P	4
D P A F D I	4
E F L N	3
G T Y Y Y D S S G Y F Q N W F D P	3
H S G D I I T G I S E C G F D Y	3
D C G S N T W Y S L P F D S	2
D L A P K D L F Y A F D I	2
D P V A T F D K T G Y Y Y Y G M D V	2
D S D R W H P A Y F D Y	2
H L R F R G K A A P F D I	2

Peripheral blood

IgG4-PB (n=42)	Number of Sequences
A G G Y S Y G Y Y Y Y G M D V	2

Table 4.

(a) Identical sequence analysis of IgG4-CDR3 in each patient

	SG	PB	LT
Patient 1	HLRFRGKAAPFDI	HLRFRGKAAPFDI	
Patient 2	GGITIFGARDYYFMDV		GGITIFGDQDYYYMDV GGITIFGDRDYYYMDV
Patient 3	EFLN	EFLN	

Amino acid differences from the proposed germline are underlined.

SG: salivary glands, PB: peripheral blood, LT: lung tissue

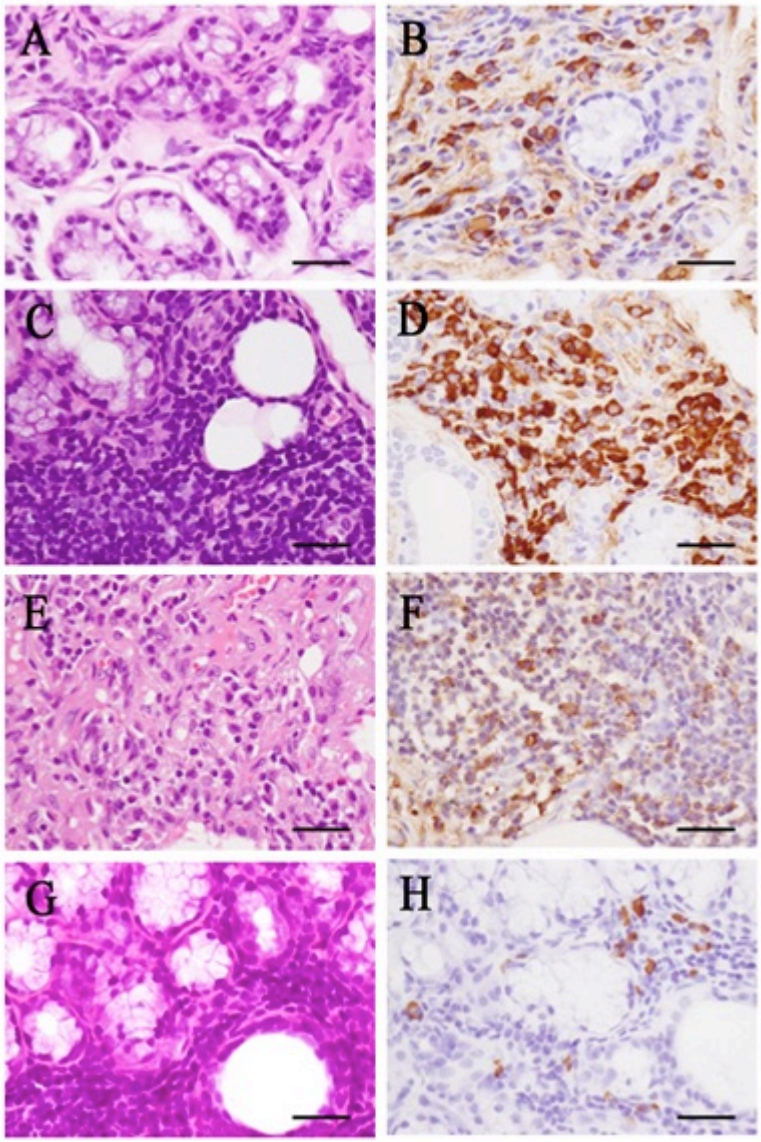
(b) Identical sequence analysis of IgG4-CDR3 in patients

		Patient 1		Patient 2			Patient 3	
		SG	PB	SG	PB	LT	SG	PB
IgG4	NIAAIGTGYWYFDL		2/32			1/51	12/47	
	VAIVVVPAGKGAFDI					3/51	1/47	
	DRAQY	1/32					1/47	
	HLRFRGKAAPFDI	1/32	1/32				2/47	

SG: salivary glands, PB: peripheral blood, LT: lung tissue

Figure 1

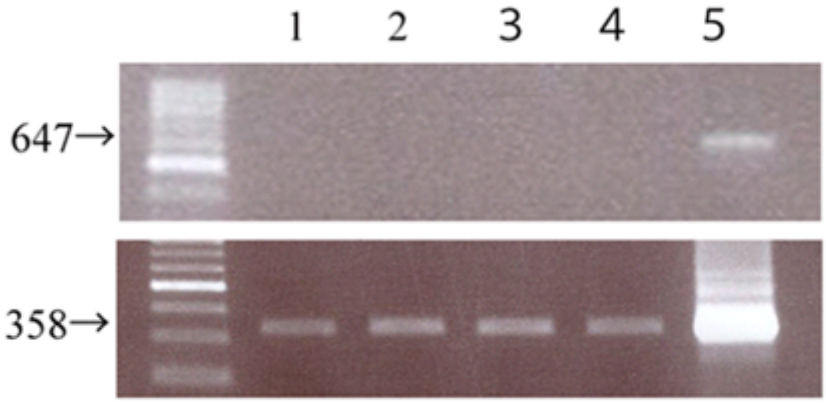
Histopathological findings for the IgG4-RD cases



Panels A, C, E, and G: H&E staining. Panels B, D, F, and H: IgG4 immunostaining. Panels A and B: lesion of patient 1. Panels C to F: Lesion of patient 2. Panels G and H: Lesion of patient 3. Panels A, B, C, D, G, and H: salivary glands. Panels E and F, alveolar interstitium. All images: hpf ×400. Scale bars: 50 μm. Many IgG4-positive plasma cells were observed in Panels B, D, F, and H.

Figure 2

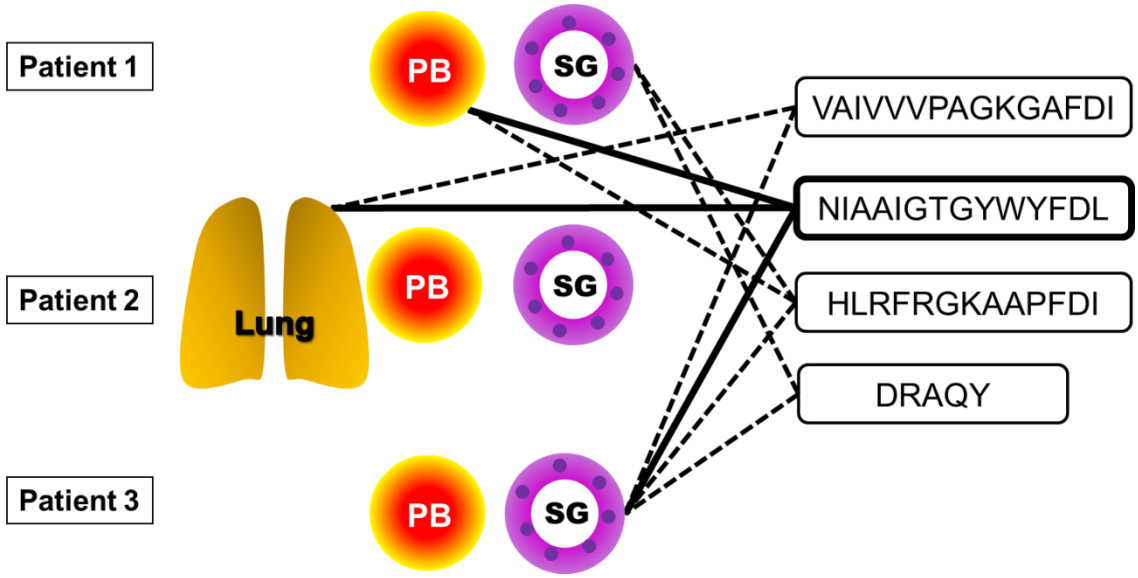
Expression of activation-induced cytidine deaminase (AID) in salivary glands and lung tissue in patients with IgG4-RD.



Lanes 1-3: salivary glands (patient 1, 2 and 3 respectively); lane 4: lung tissue (patient 2); lane 5: RAMOS cell (Burkitt's lymphoma-derived cell line) as a positive control. The 647-bp band obtained using RT-PCR and the 358-bp band obtained using nested PCR corresponded to the AID gene. AID was expressed to varying degrees in all patients with IgG4-RD.

Figure3

Identical sequence of IgG4-CDR3 in all patients



In the IgG4-CDR3, four identical sequences exist: NIAAIGTGYWYFDL in salivary glands of patient 1 and 3 and lung tissue of patient 2; VAIVVVPAGKGAFDI in lung tissue of patient 2 and in salivary glands of patient 3; DRAQY in salivary glands of patient 1 and 3; and HLRFRGKAAPFDI in salivary glands of patient 1 and 3, and in peripheral blood of patient 1.

SG: salivary glands, PB: peripheral blood