

# Identification of Thyroglobulin and its Isoforms as Target Antigens for IgG4 Thyroiditis

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## Abstract

**Background:** Immunoglobulin G4-related disease (IgG4-RD) presents a systemic autoimmune disorder affecting various organs either simultaneously or solitarily, characterized by the prominent infiltration of IgG4-positive plasma cells into target organs, usually associated with increased circulating IgG4 concentrations. The disease affects any organ such as pancreas, biliary tract, hypophysis, salivary gland, lung, kidney, meninges, eye, prostate, lymph nodes, retroperitoneum, aorta, skin and thyroid gland. Among them, IgG4 thyroiditis has been recognized as a representative organ-specific form of IgG4-RD. However, the immunologic basis of organ specific involvement of IgG4 thyroiditis including target molecules of autoimmune reactivity to thyroid is not known.

**Methods:** In order to examine the pathogenesis of IgG4 thyroiditis, we histologically examined 53 thyroiditis patients out of 2,436 patients who underwent thyroidectomy. Immunohistochemical staining of IgG and IgG4 was used to determine surface markers of infiltrated lymphocytes. Western blotting followed by MALDI-TOF/MS analysis was conducted to identify target antigens.

**Results:** Among 2,436 histologically examined thyroid disease patients, 53 cases were diagnosed as thyroiditis (2.2%); 38 patients with Hashimoto thyroiditis (HT) (71.7%; 38/53) and 15 patients with Graves' disease (GD) (28.3%; 15/53). Only 19 among 53 cases (35.8%) presented significant lymphoplasmacytic infiltrate rich in IgG4-positive plasma cells fulfilling the diagnostic criteria for IgG4 thyroiditis, of which 13 were Hashimoto thyroiditis and 6 were Graves' disease. Only 8 among the 19 (42.1%) had increased serum IgG4 levels. Furthermore, target antigens of IgG4 thyroiditis were identified as thyroglobulin and its isoforms.

**Conclusion:** Since thyroglobulin is an organ-specific protein, the observation is consistent with the solitary nature of IgG4 thyroiditis. Even some patients without increased serum IgG4 develop IgG4 thyroiditis, indicating that increased serum IgG4 is not an indispensable marker of IgG4 thyroiditis. The search for target antigens in IgG4-RD is essentially important to clarify the pathogenesis of IgG4-RD.

**Keywords:** IgG4; IgG4-related disease; Thyroiditis; Graves' disease; Hashimoto thyroiditis; Thyroglobulin; IgG4-positive plasma cell

## Introduction

Immunoglobulin G4 (IgG4)-related disease (IgG4-RD) is a novel clinical entity first proposed to have elevated serum IgG4 levels in relation to autoimmune pancreatitis (AIP) by Hamano et al, in 2001 [1]. Since then, IgG4-related lesions similar to AIP have been reported in various organs. It is now recognized that IgG4-RD is a rather rare systemic auto-inflammatory disease characterized by the prominent infiltration of IgG4-positive plasma cells (IgG4+/IgG+ plasma cells ratio; >40%, IgG4+plasma cells; >10 cells/high powered field) into affected organs with increased serum IgG4 concentrations (serum IgG4 concentration;  $\geq 135$  mg/dL) in about 60% to 80% of the patients [2-5]. The disease is also occasionally associated with eosinophilic infiltration and with increased immunoglobulin E (IgE) in about one-third of the patients [6]. IgG4-RD develops, either systematically or solitarily, sometimes developing inflammatory pseudotumor formation, affecting the pancreas (autoimmune pancreatitis), biliary tract (sclerosing cholangitis), hypophysis (autoimmune hypophysitis), salivary gland (sialoadenitis), lung (interstitial pneumonitis), kidney

(tuberointerstitial nephritis), meninges (pachymeningitis), eye (dacryoadenitis, retroorbital pseudotumor), prostate (prostatitis), lymph nodes (Castleman's disease), retroperitoneum (retroperitoneal fibrosis), aorta (inflammatory aortic aneurysm), skin (cutaneous pseudolymphoma) and thyroid gland (Riedel thyroiditis, Hashimoto thyroiditis) [2,3,7]. In addition, although many putative autoantigens

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including IgG4 subclass autoantibody to annexin A11, galectin-3 and laminin 511, have been reported in IgG4-RD [2,3,8-10], their pathogenic role in the development of individual IgG4-RD remains to be determined. Thus, at present, the underlying mechanisms of IgG4-RD are not yet fully understood.

Regarding IgG4 thyroiditis, it has been reported that there was an increase in the number of IgG4-positive plasma cells infiltrating into the thyroid tissue in several patients with Riedel thyroiditis (RT) [11-13]. Moreover, in Hashimoto thyroiditis (HT) with marked fibrosis [14] or having a high value of anti-thyroid autoantibodies, IgG4-positive plasma cell infiltration into thyroid tissue was also observed [15]. HT patients with IgG4-rich inflammation in the thyroid tissues showed no involvement of IgG4-positive plasma cells in extra-thyroid organs, suggesting that IgG4 thyroiditis may be an organ-specific form among IgG4-RD [16,17]. In addition, an increased number of IgG4-positive plasma cells were also observed in approximately 0.3% (5/1484) of Graves' disease (GD) patients [18]. These observations suggest that IgG4 thyroiditis having characteristic immuno-pathogenic conditions is a distinct group among autoimmune thyroidal diseases. However, the etiological role of IgG4 subclass antibody has not been clarified in IgG4 thyroiditis, and most importantly, the target antigen recognized by serum IgG4 antibody of the IgG4 thyroiditis patients has not yet been elucidated.

In the present study, we classified patients with inflammatory changes in thyroid tissue into two groups according to the degree of infiltration of IgG4-positive plasma cells into tissues and serum IgG4 levels, namely IgG4 thyroiditis or non-IgG4 thyroiditis, and compared the serological and histopathological features of both groups. Furthermore, we attempted to identify the target antigens recognized by serum IgG4 of IgG4 thyroiditis patients.

## Materials and Methods

### Patients

Among a total of 2,436 patients who underwent thyroidectomy at Yamashita Thyroid Hospital (Fukuoka, Japan) from March 2013 to April 2016, thyroiditis patients with lymphocytic infiltration, lymph follicle formation and/or fibrosis in the excised thyroid tissues were studied. Inflammatory changes in thyroid tissues were observed in 53 (2.2%) out of 2,436 patients, 38 patients with Hashimoto thyroiditis (HT) and 15 patients with Graves' disease (GD). Incidentally, there were no Riedel thyroiditis (RT) patients in this subject group. Ten out of 15 patients with GD received total thyroidectomy because of their diffuse enlargement goiter or difficulty to obtain remission by anti-thyroid drug (ATD). Two out of 38 patients with HT received total thyroidectomy in order to relieve compression symptoms caused by their large goiter. All of the other patients mentioned above received total or subtotal thyroidectomy because they were diagnosed as benign thyroid goiter or suspected thyroid carcinoma. Two out of HT patients who underwent total thyroidectomy due to suspected thyroid carcinoma were finally diagnosed as HT by histopathological examination. The clinical symptoms and systemic laboratory data of all patients were carefully examined, and there was no evidence of other organs affected by IgG4-RD or other autoimmune diseases examined by ultrasonography and computed tomography of neck, chest and abdomen. This study complied with the Helsinki Declaration and informed consent was obtained from all patients.

### Thyroid function tests, thyroid autoantibodies and serum IgG4 levels

Serum thyrotropin (TSH), free thyroxine (fT4), anti-thyroglobulin antibody (TgAb), anti-thyroid peroxidase antibody (TPOAb) and TSH receptor antibody (TRAb) levels were measured by electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) [19,20]. Reference ranges were defined as follows: TSH 0.50-5.00  $\mu$ IU/mL, fT4 0.9-1.7 ng/dL; and normal values of each of the autoantibodies were defined as follows: TgAb<28 IU/mL, TPOAb<16 IU/mL, TRAb<2.0 IU/L. Serum IgG4 level was measured by an immunonephelometric assay (Binding Site, Birmingham, UK), and its reference range was defined as 4.8-105 mg/dL [21]. Since comprehensive diagnostic criteria for IgG4-RD include a serum IgG4 level  $\geq$  135 mg/dL, we defined this as the cut-off level of serum IgG4 in this study.

### Histopathological evaluation and immunohistochemistry

After surgical resection, thyroid tissues were routinely fixed in 10% neutral buffered formalin and specimens were embedded in paraffin. Tissue sections (4  $\mu$ m thick) were cut from each paraffin block. Several sections from each patient were stained with hematoxylin and eosin (HE) for histological examination, and others were prepared for immunohistochemistry staining for IgG and IgG4. The degrees of lymphocytic infiltration, number of lymphoid follicles per one HE section, and stromal fibrosis was examined and expressed in five scores: 3+ (severe), 2+ (moderate), 1+ (mild),  $\pm$  (extremely mild), and - (negative). Immunostaining for IgG4 (mouse monoclonal, HP6025, Southern Biotech, Chicago, USA; dilution 1:400) and IgG (rabbit polyclonal, Agilent, California, USA) were performed using EnVision system (Agilent, California, USA). Tonsil tissue served as a positive control [22]. In five high-power fields (HPF) in each section, IgG4 and IgG-positive plasma cells were counted following a previously reported method [23,24]. Average numbers of IgG4 and IgG-positive plasma cells and their ratio were calculated in each patient. On the bases of the immunostaining for IgG4 and IgG and the cut-off value proposed by Li et al. (>20/HPF IgG4-positive plasma cells and an IgG4+/IgG+ ratio>30%) [19]. Thus, the thyroiditis patients were sub-classified as IgG4 thyroiditis or non-IgG4 thyroiditis.

### Extraction of the thyroid antigens

The thyroid tissue of obtained by surgery of a GD patient was cut into small piece and was immediately frozen at -80°C. Approximately 3 g of frozen thyroid tissue was chopped in cold 0.15 mol phosphate buffer solution (pH 7.4, 10 mL/3 g tissue) supplemented with protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and were homogenized followed by sonication. The homogenate was separated with centrifuge [25] and the supernatant was used as the thyroid antigen solution for 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### Western blotting

In order to identify the target antigen of serum IgG4, sera of ten patients with IgG4 thyroiditis were subjected to western blotting using a fully automated system, the Wes™ (Protein simple, California, USA). In addition, sera of two patients with non-IgG4 thyroiditis were also used for comparison. The labelled antibody of the Wes™ used was horseradish peroxidase conjugate mouse anti-human IgG4 antibody (Thermo Fisher Scientific, Massachusetts, USA) diluted 1,000-fold.

Furthermore, in order to explore antigen proteins reacting with

serum IgG4, reaction bands were cut out to follow the method of Kurisaki, et al. [26]. The thyroid antigen was electrophoresed in two 8% SDS-PAGE, then one gel was transferred to PVDF membrane (Amersham Bioscience AB, Uppsala, Sweden) to perform the common western blotting (without using the Wes™), and another one was using Coomassie Brilliant Blue (CBB) staining. PVDF membrane was incubated with serum sample diluted 100-fold at 4°C more than overnight, then it was incubated with mouse anti-human IgG4 antibody HRP conjugate diluted of 1,000-fold at room temperature for one hour. It was used ECL select Western Blotting Detection Reagent (GE Healthcare UK Limited, Buckinghamshire, UK) for the detection. In the same procedure, the reaction of total IgG, IgG1 antibody in sera of IgG4 thyroiditis patients recognizing thyroid antigens was confirmed. Western blotting was also done using the TG (Human) Recombinant Protein (sequence: FSHFIRSGNPNYPYEFGRK VPTFATPWPDFVPRAGGENYKEFSELLPNRQGLKADCSFWSKY ISSLKTSADGAKGGQSAESEEELTAGSGLREDLLSLQEPGSKTYSK, MW=37.84 kDa, Abnova Corporation, Taipei, Taiwan) for the search of total IgG, IgG1 (Thermo Fisher Scientific, Massachusetts, USA) and IgG4 antibodies. Each dilution condition was as follows, patient's serum: 200-fold, IgG: 10,000-fold, IgG1: 1,000-fold, IgG4: 500-fold.

#### Peptide mass finger printing (PMF) analysis by MALDI-TOF/MS

The five CBB-stained bands corresponding to the same molecular weight (MW) as the reaction bands detected by Western blotting were cut out from the gel. The gel samples were sent to GENOMINE (Pohang, Korea) and a peptide fragment digested with trypsin were analyzed by MALDI-TOF mass spectrometry (MALDI-TOF/MS) using Microflex LRF 20 (Bruker Daltonic, Massachusetts, USA) [27], after that, the predicted protein were identified using MASCOT search.

#### Statistical analysis

Statistical analysis was performed using the Stata SE v14.2 (Stata Corp LLC, Texas, USA) statistical package. Mann-Whitney U-tests were performed to test for differences between groups. A p-value<0.05 was considered statistically significant. Dates were expressed as median and its range.

### Results

#### Serological and histopathological characteristics of the thyroiditis patients

Serum samples from all of the thyroiditis patients (n=53; HT=38, GD=15, RT=0) drawn preoperatively were analyzed for fT4, TSH, TgAb, and TPOAb. As shown in Table 1, there were no significant differences in the levels of fT4, TSH and TgAb between the IgG4 thyroiditis group and the non-IgG4 thyroiditis group. On the other hand, the TPOAb level of the IgG4 thyroiditis group was significantly high in comparison with that of the non-IgG4 thyroiditis group (p=0.041).

Fifty-three patients with thyroiditis were sub-classified as IgG4 thyroiditis (n=19; 35.8%), according to the criteria; more than 20 IgG4-positive plasma cells per high power field (HPF) (x400) and a IgG4+/IgG+ cell ratio exceeding 30% by light microscope, or non-IgG4 thyroiditis (n=34; 64.2%). Histopathological findings in patients with IgG4 thyroiditis were characterized by diffuse lymphocytic infiltration, various degrees of lymphoid follicle formation and/or fibrosis (Figure 1A). Lymphoid follicle (germinal center) formation, follicular cell degeneration and eosinophilic change of thyroid follicular epithelial cells were also observed (Figure 1B), while infiltration of eosinophils

was absent. In IgG4 thyroiditis group, infiltrations of IgG positive cells were scattered in the peripheral area (Figure 1C) and also rich in germinal center (Figure 1D). In addition, IgG4 positive plasma cells infiltrated in the peripheral area (Figure 1E) and most highly in the germinal center (Figure 1F).

The degree of lymphocytic infiltration and the number of lymph follicle formations of the IgG4 thyroiditis group were significantly higher than those of the non-IgG4 thyroiditis group; the p-values were 0.016 and 0.006, respectively (Table 1).

#### Histopathological and serological findings of IgG4 thyroiditis patients with elevated or non-elevated serum IgG4 levels

The group composed of 19 histology-proven patients with IgG4 thyroiditis was divided into the elevated serum IgG4 (s-IgG4) group (n=8; 42.1%) and the non-elevated s-IgG4 group (n=11; 57.9%) according to the cut-off level of serum IgG4 (serum IgG4 ≥ 135 mg/dL) (Table 1). This observation is consistent with previous reports that even IgG4-RD, about 10% of autoimmune pancreatitis showed normal or low serum IgG4 levels [3,4]. In IgG4 thyroiditis, a relatively large percentage of patients with IgG4 thyroiditis lack increased serum IgG4, thus indicating that normal or low serum IgG4 level cannot serve as a clinical marker of the absence of IgG4 thyroiditis as well as IgG4-RD.

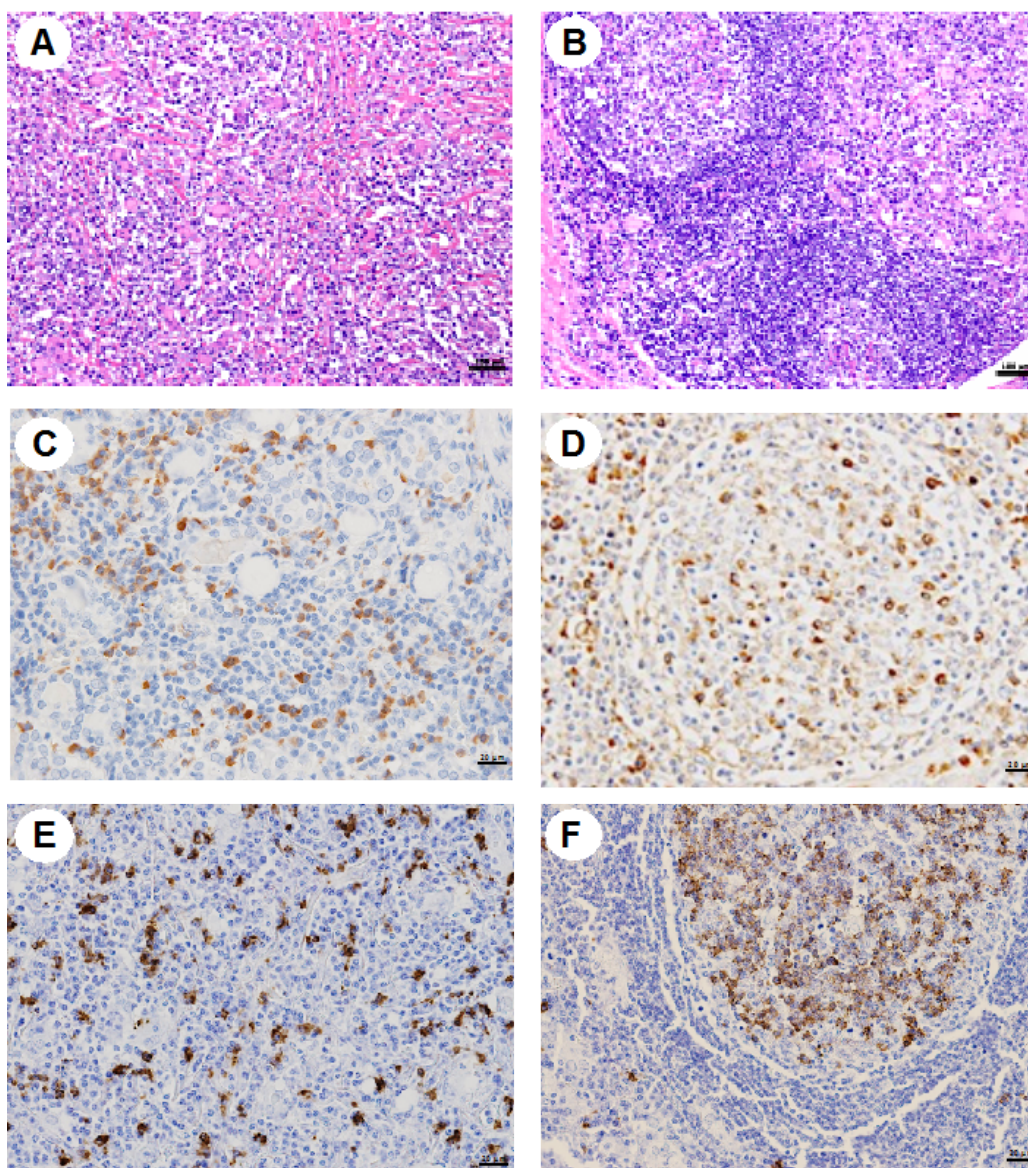
We further compared the number of IgG4-positive plasma cells infiltrated into thyroid tissue (IgG4 thyroiditis) between the elevated s-IgG4 level group and the non-elevated s-IgG4 group; however, there was no significant difference between them (Table 1). In contrast, IgG4+/IgG+ cell ratio of the elevated s-IgG4 group was significantly higher than that of the non-elevated s-IgG4 group (p=0.047). When compared with the levels of thyroid function tests and thyroid autoantibodies, the TgAb level of the elevated s-IgG4 group was significantly higher than that of the non-elevated s-IgG4 group (p=0.041).

#### Screening analysis for thyroid antigens to serum IgG4 of IgG4 thyroiditis patients by western blotting

Sera from ten patients with IgG4 thyroiditis with sufficient sample volume for the experiments, seven patients in the elevated s-IgG4 group and three patients in the non-elevated s-IgG4 group, were analyzed by western blotting with the Wes™, using the extracted material from the thyroid tissue. As a result, positive reaction bands of serum IgG4 to thyroid antigens were detected in six out of seven patients (3 HT patients and 4 GD patients): 1 absent, 1 weakly positive, and 5 positives in the elevated s-IgG4 group, while there was 1 absent (GD), 1 weakly positive (HT), and one positive (HT) in the non-elevated s-IgG4 group (Table 2). Those patients with positive reaction included both Graves' disease (GD) and Hashimoto thyroiditis (HT). No reaction bands to thyroid antigens were observed in sera of patients with poor infiltration of IgG4-positive plasma cells into the thyroid tissue: No. 11 and 12. As noted above, even among IgG4 thyroiditis patients, some patients had absent or weak autoantibody reactions to thyroid antigens, possibly due to low autoantibody level or insufficient sensitivity of the western blot analysis used in this study.

#### Identification of thyroid antigens reacting with serum IgG4 of IgG4 thyroiditis patients

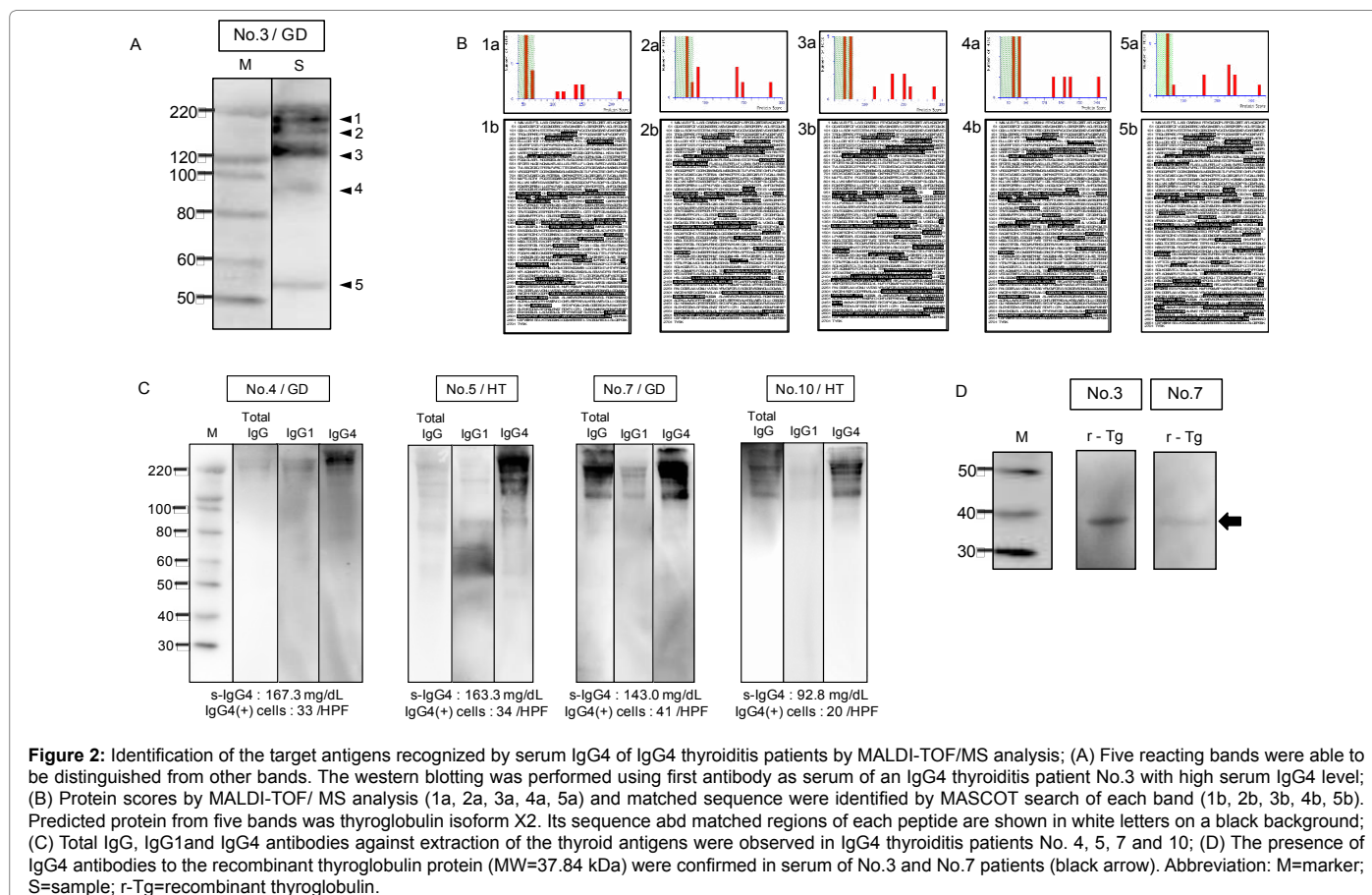
Sera of patients with IgG4 thyroiditis have reacted with multiple thyroid antigens according to the results of screening analysis by



**Figure 1:** Representative histopathological and immunohistochemical findings in a patient with IgG4 thyroiditis; (A) Diffuse lymphocytic infiltration and fibrosis were observed in thyroid tissue; (B) Lymphoid follicle (germinal center) formation, follicular cell degeneration and eosinophilic change of thyroid follicular epithelial cells were also observed; (C, D) IgG-positive plasma cells and (E, F) IgG4-positive plasma cells were observed in the lymphocyte-infiltrated peripheral lesion, and fibrotic area (C, E) and also highly in germinal center (D, F). Magnification: hematoxylin and eosin, HE-stained, 100 (A, B), immunostaining of IgG and IgG4, 400 (C, D, E, F); Diagnostic criteria: IgG4 thyroiditis was diagnosed according to the condition when the number of IgG4-positive plasma cells are more than 20/HPF and the ratio of IgG4-positive cells to IgG-positive cells (IgG4+/IgG+cell ratio) exceeded 30%.

western blotting with the Wes™. Therefore, in order to characterize the target antigens that reacted with serum IgG4, we conducted the common western blotting using serum of No. 3 patient who had elevated serum IgG4 level. As a result, several bands were observed on the membrane in a wide region between 22 and over 220 kDa (Figure 2A). Five reaction bands corresponding to western blotting were cut from CBB-stained gel and analyzed by MALDI-TOF/MS analysis. The threshold value of the significance score of the candidate protein was 66, and multiple candidate proteins were nominated in each band. The protein score analyses of five bands are shown in Figure 2B; 1a-5a. Using the sequence of thyroglobulin isoform X2, which was the top

score protein (Table 3), as a reference protein, matched regions of the peptides as identified by PMF analysis are presented in white letters on the black background (Figure 2B; 1b-5b). The top protein score of No. 1 band was 214 and the sequence coverage was 17%, No. 2 band was 270 and 18%, No. 3 band was 279 and 19%, No. 4 band was 243 and 17%, and No. 5 band was 322 and 23%, respectively; all top score protein was thyroglobulin isoform X2 as noted above (Figure 2B and Table 3), and many other candidate proteins; thyroglobulin and its isoforms, were also nominated (Table 3). Consequently, the predicted proteins of all bands were identified as thyroglobulin and its isoforms by MASCOT search (Table 3). There was no positive protein (protein scores >66)



	Overall (n=53)	Non-IgG4 thyroiditis (N=34)	IgG4 thyroiditis (N=19)		
	Mean (range)	Mean (range)	Mean (range)	Elevated s-IgG4 (≥ 135 mg/dL, N=8)	Non-elevated s-IgG4 (<135 mg/dL, N=11)
				Mean (range)	Mean (range)
Gender (male/female)	2/51	0/34	2/17	2/6	0/11
HT/GD/RT	38/15/0	25/9/0	13/6/0	4/4/0	10/1/0
Age	45.4 (13-77)	45.3 (13-77)	45.5 (26-66)	43.5 (26-65)	47.0 (34-66)
IgG4+cell/HPF	15.3 (2.0-60.0)	5.8 (2.0-15.0) <sup>P</sup>	32.4 (20.0-60.0) <sup>P</sup>	36.9 (25.0-60.0)	29.1 (20.0-50.0)
IgG4+/IgG+cell ratio (%)	24.9 (8.8-71.4)	12.6 (8.8-26.7) <sup>P</sup>	43.7 (30.0-71.4) <sup>P</sup>	49.9 (35.0-71.4) <sup>H</sup>	39.1 (30.0-63.0) <sup>H</sup>
Serum IgG4 (mg/dL)	101.1 (19.9-229.3)	71.6 (19.9-126.6)	118.5 (22.0-229.3)	175.8 (143.0-229.3) <sup>J</sup>	67.6 (22.0-120.8) <sup>J</sup>
TgAb (IU/mL)	932.3 (28.4-4000)	723.9 (28.4-4000)	1314.3 (31.1-4000)	2165.3 (45.1-4000) <sup>J</sup>	633.4 (31.1-2011.0) <sup>J</sup>
TPOAb (IU/mL)	322.8 (5.1-600.0)	282.4 (5.1-600) <sup>E</sup>	398.6 (12.2-600) <sup>E</sup>	387.4 (143.8-600)	407.3 (12.2-600)
TSH (μIU/mL)	2.62 (0.005-9.51)	2.59 (0.005-9.51)	2.68 (0.01-7016)	3.05 (0.01-6.67)	2.41 (0.01-7.16)
free T4 (ng/dL)	1.30 (0.78-3.35)	1.38 (0.84-9.51)	1.15 (0.78-1.61)	1.10 (0.89-1.47)	1.19 (0.78-1.61)
Lymphocytic infiltration <sup>A</sup> (3+/2+/1+/±/-)	3/9/37/2/2	0/4/27/1/2 <sup>F</sup>	3/5/10/1/0 <sup>F</sup>	2/3/2/1/0	1/2/8/0/0
Lymphocytic follicle formation <sup>B</sup> (3+/2+/1+/±/-)	8/5/7/4/29	3/2/3/2/24 <sup>G</sup>	5/3/4/2/5 <sup>G</sup>	4/0/2/2/0	1/3/2/0/5
Fibrosis <sup>C</sup> (3+/2+/1+/±/-)	2/4/23/3/21	0/4/13/1/16	2/0/10/2/5	1/0/4/1/2	1/0/6/1/3

Data are expressed by means and its range. All p-values were calculated by Mann-Whitney U-tests, and p-values of less than 0.05 were considered to indicate statistical significance. <sup>A,B,C</sup>The score of lymphocytic, lymphoid follicle formation and fibrosis are converted to the numerical value for significance test. The p-value between the IgG4 thyroiditis group and the non-IgG4 thyroiditis group is <sup>D</sup><0.001, <sup>E</sup>0.041, <sup>F</sup>0.016, <sup>G</sup>0.006, respectively. The p-value between the elevated s-IgG4 group and the non-elevated s-IgG4 group is <sup>H</sup>0.047, <sup>I</sup><0.001, <sup>J</sup>0.041, respectively.

**Abbreviation:** s-IgG4: Serum IgG4; HT: Hashimoto Thyroiditis; GD: Graves' Disease; RT: Riedel Thyroiditis; IgG4: Immunoglobulin G4; IgG4+cells: IgG4-positive plasma cells; HPF: High Power Field; IgG: Immunoglobulin G; IgG+Cells: IgG-positive plasma cells; TgAb: Thyroglobulin Antibody; TSH: Thyrotropin.

**Table 1:** Comparison of serological features, and histopathological and immunohistochemical findings between IgG4 thyroiditis and non-IgG4 thyroiditis.

No	IgG4 thyroiditis										Non-IgG4 thyroiditis	
	Elevated s-IgG4 group					Non-elevated s-IgG4 group						
	1	2	3	4	5	6	7	8	9	10	11	12
Clinical diagnosis	GD	HT	GD	GD	HT	HT	GD	GD	HT	HT	GD	GD
IgG4 (+) cells/HPF	25	40	48	33	34	58	41	21	50	20	3	8
IgG4 (mg/dl)	229.3	203.1	194.0	167.3	163.3	156.3	143.0	120.8	110.4	92.8	126.6	79.1
IgG4 (Wes) <sup>A</sup>	(-)	(±)	(+)	(+)	(+)	(+)	(+)	(-)	(±)	(+)	(-)	(-)
IgG (WB) <sup>B</sup>	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(-)	(-)
IgG1 (WB) <sup>C</sup>	(-)	(±)	(+)	(+)	(+)	(+)	(+)	(-)	(±)	(+)	(-)	(-)
IgG4 (WB) <sup>D</sup>	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(±)	(+)	(-)	(-)
TgAb (IU/mL)	45.1	485.6	>4000	1162	1781	1849	>4000	1159	31.1	840.3	70.8	406.4
TPOAb (IU/mL)	545.4	80.2	>600	>600	143.8	275.7	181.5	>600	12.2	160.5	>600	438.9
TRAb (IU/L)	>40	NT	2.2	<0.3	NT	NT	>40	8.3	NT	NT	20.8	<0.3
TSH (μIU/mL)	0.01	6.67	6.26	2.76	2.96	5.7	0.01	0.01	2.51	4.02	4.9	1.03
free T4 (ng/mL)	0.93	1.47	1.03	1.27	0.89	1.1	1.156	1.03	1.61	1.39	0.99	1.31

<sup>A</sup>The screening experiment of IgG4 antibodies against thyroid antigens was performed by the fully automated system, the Wes™; <sup>B,C,D</sup>the presence of the Total IgG, IgG1 and IgG4 antibodies against thyroid antigens was confirmed by the original western blot analysis.

**Abbreviation:** GD: Graves' Disease; HT: Hashimoto Thyroiditis; No: Patient Number; IgG1: Immunoglobulin G1; WB: Western Blotting; TRAb: Thyrotropin Receptor Antibody; NT: Not Tested.

**Table 2:** Screening analysis of serum IgG4 reactive with thyroid antigens in IgG4 thyroiditis patients by western blotting, and serological and histopathological features of these patients

Number of reaction bands by western blotting									
1		2		3		4		5	
Predicted protein	Score	Predicted protein	Score	Predicted protein	Score	Predicted protein	Score	Predicted protein	Score
Thyroglobulin isoform X2	214	Thyroglobulin isoform X2	270	Thyroglobulin isoform X2	279	Thyroglobulin isoform X2	243	Thyroglobulin isoform X2	322
Thyroglobulin isoform X1	211	Thyroglobulin Isoform CRA_b	266	Thyroglobulin isoform CRA_b	275	Thyroglobulin isoform CRA_b	239	Thyroglobulin isoform CRA_b	318
Thyroglobulin isoform CRA_b	201	Thyroglobulin isoform X1	253	Thyroglobulin isoform X1	262	Thyroglobulin	239	Thyroglobulin isoform X1	305
Thyroglobulin	201	Thyroglobulin	240	Thyroglobulin	261	Thyroglobulin isoform X1	226	Thyroglobulin	304
Thyroglobulin isoform X4	118	Thyroglobulin isoform X4	189	Thyroglobulin isoform X4	176	Thyroglobulin isoform X3	162	Thyroglobulin isoform X4	166
Thyroglobulin isoform X3	109	Thyroglobulin isoform X3	186	Thyroglobulin isoform X3	173	Thyroglobulin isoform X5	156	Thyroglobulin isoform X3	163
Thyroglobulin isoform X5	106	Thyroglobulin isoform X5	181	Thyroglobulin isoform X5	168	Thyroglobulin isoform X4	155	Thyroglobulin isoform X5	159
				Thyroglobulin isoform CRA_a	83	Thyroglobulin isoform CRA_a	93	Thyroglobulin isoform CRA_a	95

Predicted proteins identified by MALDI-TOF/MS and MASCOT search and their scores are shown. The statistically significant protein score threshold is 66 ( $p < 0.05$ ). No other proteins exceeding score 66 were detected.

**Table 3:** Predicted proteins and its scores of each reaction band detected by western blotting, followed by MALDI-TOF/TOF Mass analysis.

other than thyroglobulin and its isoforms (Table 3). Furthermore, reaction bands of IgG1 antibody to thyroid antigens in the MW regions similar to the IgG4 antibody were also observed in these patients (Table 2 and Figure 2C; No. 4-No. 7). The reaction bands of IgG1 and IgG4 antibodies to thyroid antigens were also found in three patients who had rather low serum IgG4 levels with significant infiltration of IgG4-positive plasma cells (Table 2 and Figure 2C; No. 10).

Because it was revealed that serum IgG4 of IgG4 thyroiditis patient No. 3 recognized thyroglobulin and its isoforms, the presence of IgG4 antibodies to the thyroglobulin in those patients (Nos. 3, 7 and 10) positive by western blotting were also examined using recombinant thyroglobulin antigen (r-TG: MW; 37.84 kDa). As a result, the positive

reaction band with recombinant thyroglobulin fragment was detected in the sera of IgG4 thyroiditis patients (No. 3 and No. 7) having high serum IgG4 level (black arrow), thus confirming the reactivity to thyroglobulin in the sera of IgG4 thyroiditis patients (Figure 2D). No. 10 case did not show the positive reaction to recombinant thyroglobulin, possibly due to low antibody titer or lack of reactivity to epitopes of synthetic recombinant thyroglobulin fragment.

## Discussion

All these observations, as described above, taken together suggested that all thyroid-derived proteins reactive with the IgG4 subclass antibody observed in western blotting data; molecular weight ranging

from 22 to over 220 kDa, should be interpreted as thyroglobulin and its isoforms from original thyroid gland tissue material or fragmented thyroglobulin due to processing extracted thyroid antigens from thyroid tissue. Thus, we conclude that IgG4 subclass antibodies with those IgG4 thyroiditis patients were all reactive with thyroglobulin and its isoforms.

In addition, in IgG4 thyroiditis, anti-thyroglobulin and its isoforms autoantibodies were detected irrespective of the clinical phenotypes as Hashimoto thyroiditis or Graves' disease (Table 2 and Figure 2), or serum IgG4 elevation (Table 2); patients even without increased total serum IgG4 level had IgG4 subclass autoantibody against thyroglobulin and its isoforms. It was suggested that anti-thyroglobulin and its isoforms autoantibodies might be produced by thyroid infiltrated IgG4-positive plasma cells as well as those in regional lymph nodes and/or the spleen. In addition, production of IgG4 subclass autoantibody against thyroglobulin was not associated with clinical phenotype as Hashimoto thyroiditis or Graves' disease, indicating other influencing factors to develop the clinical outcome of individual thyroiditis. Those influencing factors may include mainly HLA haplotype, and other elements such as Th1/Th2 balance, regulatory T cells, B cells, profiles of cytokines, and immunoregulatory molecules may have been operative as widely reported and discussed in the pathogenesis of thyroid diseases [28,29]. Studies to clarify the mechanisms to develop individual clinical phenotype in IgG4 thyroiditis will also be needed. Although we could identify the target antigen of IgG4 thyroiditis as thyroglobulin and its isoforms, there were still some cases that lack autoantibody to thyroglobulin and its isoforms. This may be due to our low sensitivity technique to detect the autoantibody or they may have another unknown autoantibody to the thyroid organ.

Many putative autoantigens, such as lactoferrin, carbonic anhydrases, pancreatic secretory trypsin inhibitor, amylase alpha 2A, the ubiquitin-protein ligase E3 component n-recogin 2, trypsinogen, annexin A11, galectin-3 and laminin 511, have been reported in IgG4-RD [2,3,8-10]. However, most of them were nominated since they are possible autoantigens produced in patients with affected organ diseases without evidence of IgG4 subtype autoantibody production. Only anti-annexin A11, galectin-3 and laminin 511 were reported to have autoantibody belonging to IgG4 subtype [8-10]. Anti-annexin A11, a specific IgG4 antibody, found in biliary tract/ pancreas/salivary gland-affected multiorgan IgG4-RD, simultaneously produced IgG1 subclass antibody as a possible main autoantibody [8]. As a result, IgG4 anti-annexin A11 antibody inhibits IgG1-mediated inflammatory response [8], and therefore the direct pathogenic role of IgG4 anti-annexin A11 is not likely operative. Anti-galectin-3 antibody producing B cells were screened and obtained from patients with multiorgan involvement with IgG4-RD, and anti-galectin-3 antibody reacted with pancreatic cytosolic tissue [9]. Production of anti-galectin-3 was correlated with serum galectin-3 levels, thus indicating that anti-galectin-3 may be produced in response to high serum galectin 3 [9] thus, the pathogenic role of anti-galectin-3 remains to be elucidated. Anti-laminin 511 antibodies were detected in about half of autoimmune pancreatitis patients and experimental transfer of anti-laminin 511 antibody to mice caused pancreas injury, suggesting the pathogenic role of anti-laminin 511 [10]. Since encoding genes of those reported autoantigens are expressed in almost all organs in the whole body, but whether those autoantibodies are associated with the organ-specific or systemic multiorgan type of IgG4-RD remains uncertain. Indeed, it was reported

that in 235 histology-proven cases of IgG4-RD, 137 (58%) presented multiple organ involvements, while 98 (42%) showed isolated organ lesion, at least in the Japanese [30]. Thus, whether those IgG4 subclass autoantibodies play a role in determining the target organ involvement, i.e., multiple organs or solitary lesion is a question that requires further study including different ethnic groups. In contrast, as shown by this study, since thyroglobulin is expressed restrictively only in the thyroid organ, it is reasonable to deduce that the solitary nature of IgG4 thyroiditis is due to the IgG4 subclass auto-antibody response directed against organ-specific antigen such as thyroglobulin and its isoforms in IgG4 thyroiditis.

In conclusion, we have identified thyroglobulin and its isoforms as a major autoantigen recognized by serum IgG4 antibodies in IgG4 thyroiditis patients. The observation could explain the solitary nature of IgG4 thyroiditis, since thyroglobulin and its isoforms are restrictively produced only in the thyroid gland. This study will enhance a search for autoantigens in IgG4-RD that will undoubtedly contribute to delineate the pathogenesis of IgG4-RD.

#### Conflict of Interests

The authors declare no conflict of interests.

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#### Ethical Statement

The study protocol was approved by the Yamashita Thyroid Hospital Ethics Committee No. 001.

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