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Abstract

Reportedly, thyroid mucosa-associated lymphoid tissue (MALT) lymphoma is closely associated with Hashimoto's thyroiditis. However, it remains unknown which antigen is closely associated with thyroid MALT lymphoma. We examined whether B cell response to thyroglobulin (Tg), which is a common thyroid-specific autoantigen, is related etiologically to the pathogenesis of thyroid MALT lymphoma. Expression of human Tg antigens and Cluster of differentiation (CD) 35 was examined immunohistochemically in 15 cases of thyroid MALT lymphoma using paraffin-embedded, formalin-fixed tissue specimens. In all cases of thyroid MALT lymphoma, human Tg was detected immunohistochemically in the follicular epithelial cells and follicular dendritic cells (FDCs). These FDCs were positive by double immunostaining for anti-human Tg rabbit polyclonal antibody (Ab) and for CD35. Results showed that the Tg, a thyroid autoantigen, had immunostained the germinal center of the thyroid MALT lymphoma. The Tg was present in the FDCs, as revealed by the staining pattern of the germinal center;this fact was confirmed by double immunostaining of anti-human Tg mouse monoclonal Ab and anti-CD35 mouse monoclonal Ab. The results of our study suggest that Tg is an autoantigen that is recognized by thyroid MALT lymphoma cells.

KEYWORDS: thyroglobulin, follicular dendritic cells, mucosa-associated lymphoid tissue lymphoma

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Original Article

Expression of Thyroglobulin on Follicular Dendritic Cells of Thyroid Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma

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Reportedly, thyroid mucosa-associated lymphoid tissue (MALT) lymphoma is closely associated with Hashimoto's thyroiditis. However, it remains unknown which antigen is closely associated with thyroid MALT lymphoma. We examined whether B cell response to thyroglobulin (Tg), which is a common thyroid-specific autoantigen, is related etiologically to the pathogenesis of thyroid MALT lymphoma. Expression of human Tg antigens and Cluster of differentiation (CD) 35 was examined immunohistochemically in 15 cases of thyroid MALT lymphoma using paraffin-embedded, formalin-fixed tissue specimens. In all cases of thyroid MALT lymphoma, human Tg was detected immunohistochemically in the follicular epithelial cells and follicular dendritic cells (FDCs). These FDCs were positive by double immunostaining for anti-human Tg rabbit polyclonal antibody (Ab) and for CD35. Results showed that the Tg, a thyroid autoantigen, had immunostained the germinal center of the thyroid MALT lymphoma. The Tg was present in the FDCs, as revealed by the staining pattern of the germinal center; this fact was confirmed by double immunostaining of anti-human Tg mouse monoclonal Ab and anti-CD35 mouse monoclonal Ab. The results of our study suggest that Tg is an autoantigen that is recognized by thyroid MALT lymphoma cells.

Key words: thyroglobulin, follicular dendritic cells, mucosa-associated lymphoid tissue lymphoma

Mucosa-associated lymphoid tissue (MALT) lymphomas arise in extranodal organs such as the gastrointestinal tract, thyroid gland, ocular adnexa, salivary gland, respiratory tract, and genitourinary tract, most of which do not contain native lymphoid tissue but which acquire MALT [1]. MALT lymphomas are closely associated with chronic inflammation such as those of autoimmune disease and some bacterial infections. Reportedly, thyroid MALT lymphoma has a close association with Hashimoto's thyroiditis (HT). Holm *et al.* reported that patients with thyroiditis had a greatly increased risk of thyroid malignant lymphoma. In their study, 829 patients with HT diagnosed between 1959 and 1978 were followed up until December 31, 1981. Holm *et al.* further reported that in four new cases of thyroid lymphoma the estimated relative risk of thyroid lymphoma was 67 [2]. Kato *et al.* reported that the estimated rela-

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tive risk of thyroid lymphoma was 80 [3]. Sirota and Segal reported that the incidence of thyroid lymphoma developing from patients with HT was 1.4% [4]. On the other hand, in a previous study, the coexistence of HT histologically in patients with thyroid lymphoma has been reported to be high, about 25 to 83 percent [5–7]. Autoantibodies for thyroid-specific protein such as thyroglobulin (Tg) found in patients with HT reportedly are positive in 83% of patients with thyroid malignant lymphoma [7].

MALT lymphoma shows ongoing mutation of variable regions of the immunoglobulin heavy chain (IgH) genes; it might be derived from postgerminal center memory B cells [8]. Memory B cells and plasma cells are generated within germinal centers through a somatic hypermutation of the variable regions of Ig heavy chain genes resulting from antigen stimulation of follicular dendritic cells (FDCs) and antigen-specific T cells [9, 10]. The stomach, lacking lymphoid tissues, acquires MALT after *Helicobacter (H.) pylori* infection [11], and some research results suggest that gastric MALT lymphomas develop from *H. pylori* gastritis by continuous antigen stimulation after *H. pylori* infection [12–15]. The normal thyroid gland also lacks lymphoid tissue and acquires MALT [16] in autoimmune thyroid diseases, HT, and Graves' disease. However, it remains unknown which antigen is closely associated with thyroid MALT lymphoma.

We sought to clarify the relation between thyroid MALT lymphoma and Tg, which is a common thyroid-specific autoantigen that is expressed in the germinal centers of autoimmune thyroid diseases [17].

Materials and Methods

Tissue samples. Thyroid glands were resected surgically from 15 patients with thyroid MALT lymphoma, with the written informed consent of all patients. Formalin-fixed, paraffin-embedded tissue specimens were obtained from all tissues, and the deparaffinized and rehydrated tissue specimens, sliced to 3μm thickness, were used for immunohistochemical studies and polymerase chain reaction (PCR) analysis.

Immunohistochemical study. For the immunohistochemical detection of human Tg antigens expression in thyroid MALT lymphoma, we used anti-human Tg rabbit polyclonal antibody (pAb) and anti-human Tg mouse monoclonal antibody (mAb) as primary antibodies for immunostaining. Immunostaining using the anti-human Tg rabbit pAb as a primary antibody was performed using the ordinary avidin-biotin complex method, with horseradish peroxidase-conjugated porcine anti-rabbit Ig (Dako, Glostrup, Denmark) as a secondary antibody. Immunostaining using the anti-human Tg mouse mAb as a primary antibody was performed using a catalyzed signal amplification (CSA) system (Dako). Follicular dendritic cells (FDCs) were detected with CD35 mouse mAb using a CSA system. Alkaline phosphatase-labeled secondary antibody was used for the double immunostaining of human Tg using anti-human Tg rabbit pAb. In addition, CD79 α, bcl-2, Ig kappa light chain (Ig κ), and Ig lambda light chain (Ig λ) antigens were detected immunohistochemically in thyroid MALT lymphoma. Table 1 presents the primary antibodies used in this study. Immunostaining using these antibodies, except anti-human Tg rabbit pAb, anti-human Tg mouse mAb, and CD35 mouse mAb as the primary antibodies, was performed using the ordinary avidin-biotin complex method, with horseradish peroxidase-conjugated rabbit anti-mouse Ig (Dako)

Table 1 Antibodies used in immunohistochemistry

Antibodies	Clonality/Clone	Dilution	Source
Thyroglobulin	M/Tg6	1 : 50	Dako, Glostrup, Denmark
	P	Prediluted	Dako, Glostrup, Denmark
CD35	M/Ber-MAC-DRC	1 : 50	Dako, Glostrup, Denmark
CD79a	M/JCB117	1 : 25	Dako, Glostrup, Denmark
Ig κ	M/Kp-53	1 : 100	Novocastra Laboratories, Newcastle, UK
Ig λ	M/HP-6054	1 : 400	Novocastra Laboratories, Newcastle, UK
bcl-2	M/124	1 : 20	Dako, Glostrup, Denmark

Ig κ, immunoglobulin kappa light chain; Ig λ, immunoglobulin lambda light chain; M, monoclonal; P, polyclonal.

as a secondary antibody.

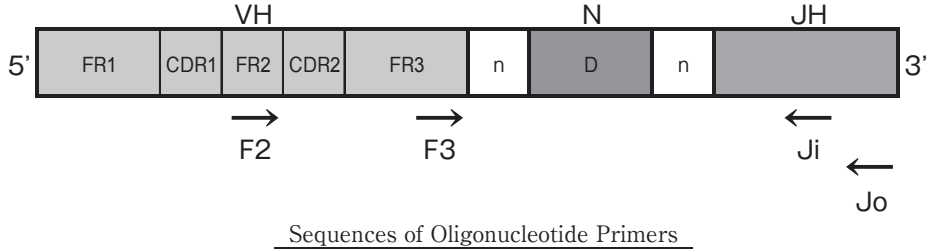
PCR analysis. PCR analysis was performed to detect clonal rearrangements of the IgH gene, thereby distinguishing B lymphoid monoclonality, seen in thyroid MALT lymphoma, from reactive lymphoid hyperplasia, seen in HT. Amplification of the IgH gene was performed using the nested PCR method. The primers used for the detection of IgH rearrangement were directed to the framework 2 region and to the adjoining region, as described previously by Davis *et al.* [18]. Fig. 1 presents sequences of oligonucleotide primers used for this study. The DNA amplification of each material was carried out more than once. The amplified products were electrophoresed through 4% agarose gel and visualized by staining with ethidium bromide. A known diffuse large B-cell lymphoma sample was used as a positive control. Determination of monoclonality was performed using a previously described method [19]. This study conforms to principles outlined in the Declaration of Helsinki.

Results

Clinical findings. Clinical findings in patients with thyroid MALT lymphoma are presented in Table 2. Patients with thyroid MALT lymphoma included 9 women and 6 men who were 19–78 years old (mean, 67.1 years); 14 patients were older than 60. Goiter was observed in all 15 patients. Diffuse goiter was observed in 7 patients, and nodular goiter was observed in 8 patients. Thyroid function in patients with thyroid MALT lymphoma was hypothyroid in 7 patients and euthyroid in 8 patients. Five patients had overt hypothyroidism and were treated with levothyroxine. In 9 cases, the coexistence of HT could be confirmed histologically in patients with thyroid lymphoma.

Radioimmunoassay for antithyroglobulin antibody was performed before resection of the thyroid MALT lymphoma in 3 of 15 patients (1 man and 2 women). All 3 patients with thyroid MALT lymphoma showed elevated antibody titers for thyroglobulin (0.6, 1.2, and over 100 U/ml, respectively. The normal range is <0.3 U/ml). In addition, antibody titers for thyroglobulin were confirmed to be high in 5 other patients. However, detailed data about the antibody titers of these patients were not available, because they were measured in other institutions. Antithyroglobulin antibody was also measured in 5 healthy controls (2 men and 3 women). All were within normal limits.

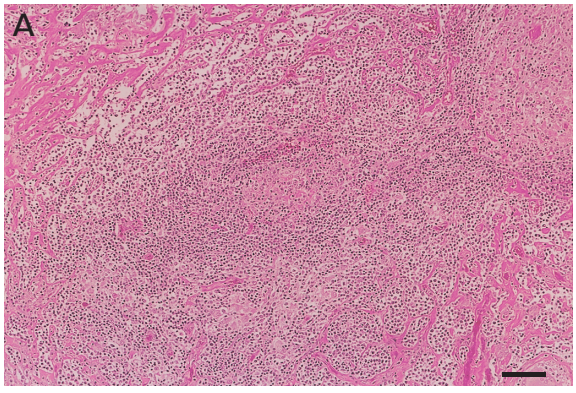
Immunohistochemical findings. Typical histopathological findings of hematoxylin and eosin-stained sections in patients with thyroid MALT lymphoma are presented in Fig. 2A (case No. 9). Immunohistochemical findings in patients with thyroid MALT lymphoma are summarized in Table 2. Using anti-human Tg rabbit pAb and anti-human Tg mouse mAb, we detected Tg in the follicular epithelia (EP), including lymphoepithelial lesions (LELs), and dendritic cells (DCs) in all cases of thyroid MALT lymphoma (Figs. 2B and C); these staining patterns with two antibodies were similar. EP and DCs were also positively stained for human leukocyte antigen (HLA)-DR in all cases. In 12 cases (80%), DCs were positively stained for CD35 (Figs. 2D and E). These DCs, which are regarded as corresponding to FDCs,



Sequences of Oligonucleotide Primers

F2: 5'- TGAATTCGG(AG)(AC)AGGGGCT(TG)GAGTGG - 3'
F3: 5'- GAGGACACGGCCGTGTATTACTGT - 3'
Jo: 5'- TACCTCGAGACCTGAGGAGACGGTGACC - 3'
Ji: 5'- TACCTCGAGAGGGT(CGT)CCTTGGCCCCAG - 3'

Fig. 1 A diagram of the immunoglobulin heavy chain (IgH) gene and sequences of IgH variable and joining region PCR oligonucleotide primers. FR, framework regions; CDR, complementarity determining regions.



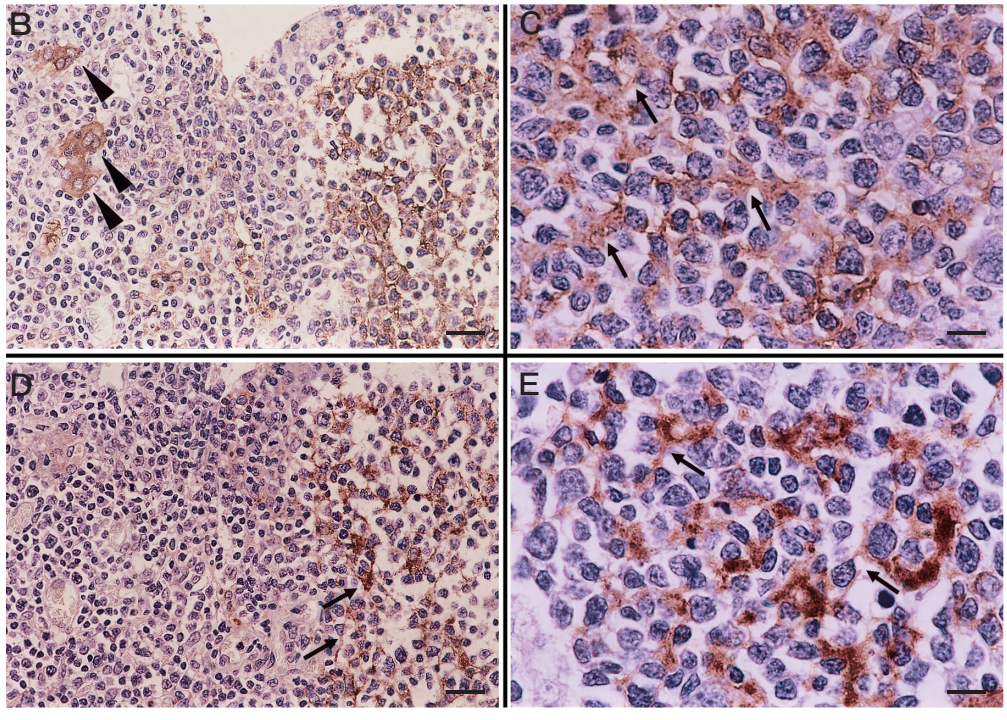


Fig. 2 Photomicrographs of hematoxylin and eosin-stained and single immunohistochemical staining for thyroglobulin (Tg) and CD35 in thyroid MALT lymphoma. **A**, Hematoxylin and eosin-stained sections (case No. 9); **B** and **C**, Immunohistochemical staining for Tg in thyroid MALT lymphoma with a mouse anti-Tg monoclonal antibody. Follicular epithelial cells (arrowheads) and dendrites of follicular dendritic cells (arrows) were positively immunostained as brown; **D** and **E**: Immunohistochemical staining for CD35 in thyroid MALT lymphoma with a mouse anti-CD35 monoclonal antibody. Dendrites of follicular dendritic cells were positively immunostained as brown (showed arrows). Scale bar: 0.1 mm (**A**), 100 μm (**B**, **D**), 40 μm (**C**, **E**).

were positive by double immunostaining for anti-human Tg rabbit pAb and for CD35 (Fig. 3).

Cases of HT that showed positive staining in the DCs for CD35 and which corresponded to FDCs were positive by double immunostaining for anti-human Tg rabbit pAb and for CD35. HT cases were positive for antithyroglobulin in EP. The DCs were weakly positive for antithyroglobulin and CD35. Normal thyroid glands showed negative staining in the DCs for CD35 and positive staining in EP for anti-human Tg rabbit pAb and anti-human Tg mouse mAb.

PCR analysis. We performed PCR analysis on DNA extracted from specimens of the 15 cases of thyroid MALT lymphoma and detected rearrangement of the IgH gene in 10 cases (67%) of thyroid MALT lymphoma (Fig. 4 and Table 2) and in no cases of HT (data not shown).

Discussion

Reportedly, HT is a risk factor for thyroid MALT lymphoma [2–4, 7, 20], and antibodies to Tg and thyroid peroxidase, which are thyroid-specific antigens, are present in HT. However, it remains unclear

Table 2 Clinical and histological findings and PCR analysis of thyroid MALT lymphomas

No.	age	sex	Goitor	Thyroid function	HT		Anti-Tg Ab	pTg	mTg	HLA-DR	CD79a	bcl-2	Ig κ	Ig λ	PCR band
					past history	histology		EP/DC	EP/DC	EP/DC					
1	64	F	nodular	hypothyroid	−	+	NA	+/+	+/+	+/+	+	−	−	+	P
2	72	M	nodular	hypothyroid	−	+	high	+/+	+/+	+/+	+	−	+	−	M
3	63	F	diffuse	euthyroid	−	NO	NA	+/+	+/+	+/+	+	−	+	−	P
4	77	F	diffuse	euthyroid	−	+	NA	+/+	+/+	+/+	+	+	+	−	M
5	69	F	nodular	euthyroid	−	NO	NA	+/+	+/+	+/+	+	−	+	−	M
6	19	M	diffuse	hypothyroid	+	+	NA	+/+	+/+	+/+	+	−	+	−	P
7	71	F	diffuse	euthyroid	−	+	NA	+/+	+/+	+/+	+	−	−	+	P
8	69	M	nodular	euthyroid	−	+	high	+/+	+/+	+/+	+	−	−	+	M
9	61	F	nodular	hypothyroid	+	+	high	+/+	+/+	+/+	+	−	+	−	M
10	78	M	diffuse	hypothyroid	+	NO	NA	+/+	+/+	+/+	+	−	+	−	M
11	68	F	diffuse	hypothyroid	+	+	high	+/+	+/+	+/+	+	+	+	−	P
12	77	F	nodular	hypothyroid	+	NO	high	+/+	+/+	+/+	+	+	+	−	M
13	72	F	nodular	euthyroid	−	NO	1.2	+/+	+/+	+/+	+	−	+	−	M
14	69	M	nodular	euthyroid	−	+	100	+/+	+/+	+/+	+	−	−	−	M
15	77	M	diffuse	euthyroid	−	NO	0.6	+/+	+/+	+/+	+	−	+	−	M

HT, Hashimoto's thyroiditis; NO, not observed; Anti-Tg Ab, titers of anti-thyroglobulin antibody of patient's sera; NA, not available; pTg, anti-human thyroglobulin rabbit polyclonal antibody; mTg, anti-human thyroglobulin mouse monoclonal antibody; EP, follicular epithlium (includes lymphoepithelial lesion); DC, dendritic cell; M, monoclonal; P, polyclonal; CD79a, bcl-2, Ig κ and Ig λ were detected in tumor cells of thyroid MALT lymphoma.

how these antigens are related to the pathogenesis of thyroid MALT lymphoma. We sought to clarify the clinical and histological features of thyroid MALT lymphoma and to examine whether Tg, which is a common thyroid-specific autoantigen, is related etiologically to the pathogenesis of thyroid MALT lymphoma.

In clinical findings, goiter was observed in all 15 patients in the present study. In a previous study, goiter was observed in all patients with thyroid lymphoma [7]. Five patients were diagnosed with HT and treated with thyroid hormone replacement therapy in our study. In our study, in 4 patients of normal thyroid function and without past histories of HT, the coexistence of HT could be confirmed histologically in patients with thyroid lymphoma. Thyroid MALT lymphoma may occur in patients with subclinical HT. On the other hand, in 6 patients in the present study, the coexistence of HT could not be confirmed histologically in patients with thyroid lymphoma. These cases could be diagnosed with MALT lymphoma histologically based on lymphoepithelial lesions, follicular colonization, and so on, but we were not able to determine the characteristic findings of HT because the specimens were too small.

Antithyroglobulin antibody was elevated in all patients for whom data was available, and the findings were similar to those of a previous study [7]. In histological findings, with regard to distinguishing neoplastic lymphoproliferation from reactive lymphoproliferation [16, 21], we detected gene rearrangement of the IgH gene CDR3 region by PCR in thyroid MALT lymphoma. In 67% of cases we detected a monoclonal band, indicating gene rearrangement, in contrast to a previous report in which the authors did not detect a monoclonal band in HT [21]. In the remaining cases of thyroid MALT lymphoma, polyclonal bands were detected. It is likely that reactive lymphoid cells were taken by chance during sample preparation. The use of microdissection may be necessary to improve the sensitivity of this assay.

To distinguish neoplastic lymphoproliferation from reactive lymphoproliferation by immunohistological staining, we detected immunoreactivity with antibodies to bcl-2 in thyroid MALT lymphoma but lymphoid cells were positively stained for bcl-2 in only 3 of 15 cases. This result is similar to those of a previous study [22]. In that study, the authors reported that 5 of 28 cases (18%) with thyroid MALT lymphoma were positive for antibodies to bcl-2. That study also showed that Tg, a thyroid autoantigen, was immunostained in the germinal centers of thyroid MALT lymphoma. The presence of Tg in the FDCs was confirmed by the reticular co-immunostaining pattern for human Tg and CD35 in the germinal centers. Some authors have suggested that B cells, which exist in the germinal center, pass through somatic hypermutation and gain higher affinity for the antigen by stimulation

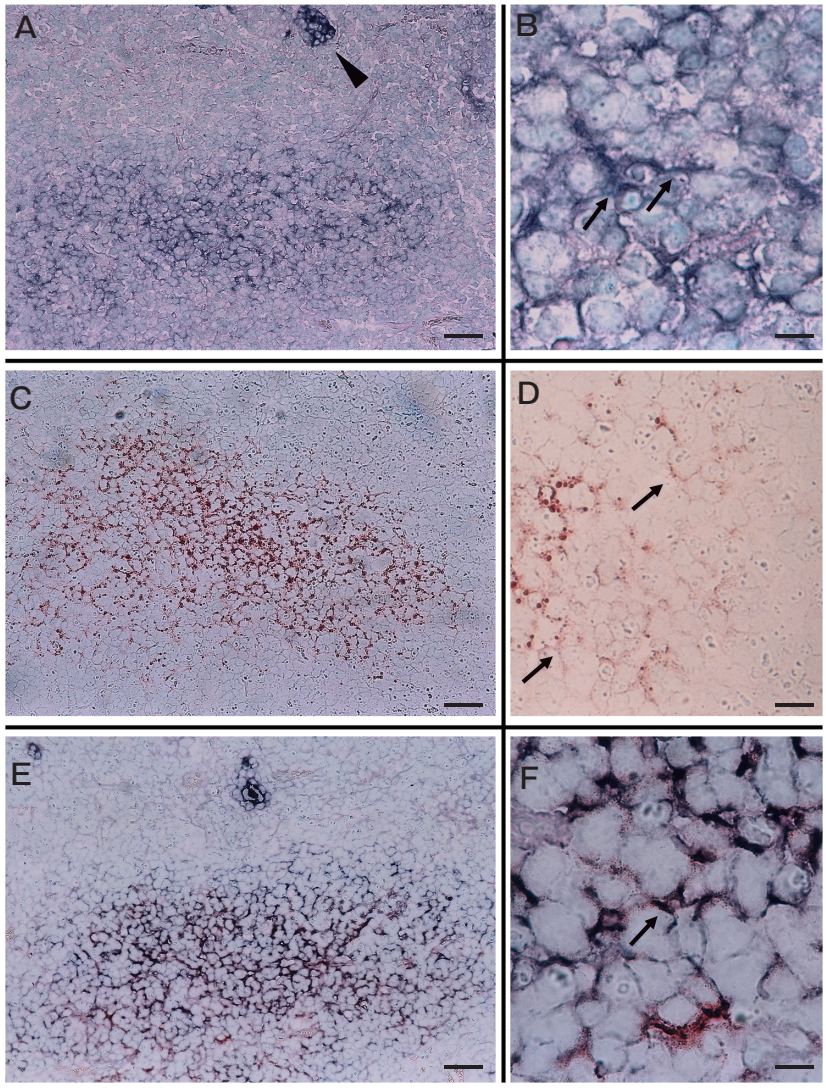


Fig. 3 Double immunohistochemical staining for thyroglobulin (Tg) and CD35 in thyroid MALT lymphoma. **A**, **B**, Immunohistochemical staining for Tg only with a rabbit anti-Tg polyclonal antibody. Follicular epithelial cells (arrowheads) and follicular dendritic cells (arrows) were positively immunostained as blue; **C**, **D**, Immunohistochemical staining for CD35 only with a mouse anti-CD35 monoclonal antibody. Follicular dendritic cells (arrows) were positively immunostained as red; **E**, **F**, Follicular dendritic cells were positive for both Tg and CD35 as immunostained purple (arrows). Scale bar: 1 mm (**A**, **C**, **E**), 40 μm (**B**, **F**), 100 μm (**D**).

with the antigen presented in the FDCs and MHC-restricted, antigen-specific T cells in the germinal center. Subsequently, B cells differentiate to memory B cells and plasma cells [9, 10, 23]. In fact, MALT lymphoma is derived from memory B cells [8]; anti-thyroid antibodies such as anti-Tg antibody are positive in patients with HT at a frequency of about 80% [20].

The lymphoid follicle is formed histopathologically in mice with experimental autoimmune thyroiditis induced by thyroid gland extracts [24]. Regarding the generation of thyroid MALT lymphoma, tumor cells recognize Tg antigen manifested in the FDCs, and the CD40-CD40L interaction between MHC-restricted antigen-specific T cells of the germinal center occurs, resulting in tumor cell proliferation [10, 25]. We showed that HLA-DR was immunostained in the thyroid epithelial cells of patients with thyroid MALT lymphoma at a high rate compared with those of patients with HT in this study. Aberrant expression

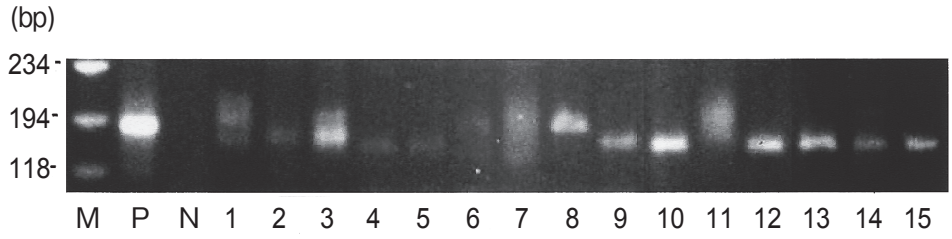


Fig. 4 PCR analysis for clonal rearrangements of the IgH gene in cases of thyroid MALT lymphoma. Rearrangement of the IgH gene was detected in cases 2, 4, 5, 8, 9, 10, 12, 13, 14, and 15. M, molecular weight marker; P, positive control; N, negative control; 1-15, case numbers.

of MHC class II antigen on thyroid epithelial cells was reported for the first time by Bottazzo *et al.* in Graves' disease [26, 27], and it has been proven that it is recognized in HT [28]. There is a hypothesis that the phenomenon of aberrant expression of MHC class II antigen on thyroid epithelial cells plays a perpetuating role in HT [29]. The difference in aberrant expression of MHC class II antigen induces the perpetuation of autoimmune inflammation, and may produce a pathological clone from the reactive lymphoid proliferation, and this generates thyroid MALT lymphoma.

The clones of T cells and B cells for various self-constituents do not always receive clonal deletion. These exist also in normal individuals in animal models [30-32] and in humans [33]. In addition, the CD4-positive T cell clone mainly recognizes Tg as a thyroid-specific autoantigen in experimental autoimmune thyroiditis of the mouse. Antigen-presenting cells are necessary to recognize Tg [34, 35]. Speculation about its superscription reflects that this fact has been considered. This autoantigen is recognized by MALT lymphoma cells in secondary follicles of the MALT lymphoma tissue.

The results of our study suggest that Tg might be an autoantigen recognized by thyroid MALT lymphoma cells. The monoclonal B cell proliferation induced by autoimmune processes caused by Tg might be related to the etiology and pathogenesis of thyroid MALT lymphoma.

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