Deviated VH4 immunoglobulin gene usage is found among thyroid mucosa-associated lymphoid tissue lymphomas, similar to the usage at other sites, but is not found in thyroid diffuse large B-cell lymphomas

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It remains unclear whether or not diffuse large B-cell lymphomas of extranodal sites arise from mucosa-associated lymphoid tissue (MALT) lymphomas. We studied the clinicopathological features of MALT lymphoma and diffuse large B-cell lymphoma in the thyroid gland, with special reference to VH usage of immunoglobulin gene rearrangement, to clarify the relationships between these two types of lymphomas. In addition, t(11; 18) (q21; q21) translocation was examined by multiplex reverse transcription-polymerase chain reaction. We examined 58 patients with primary thyroid lymphoma: 31 (male seven and female 24) with MALT lymphoma and 27 (male three and female 24) with diffuse large B-cell lymphoma. Interestingly, the sequence of VH genes revealed that the two subtypes differed significantly in their use of the VH4 family ($P<0.05$). Of the seven MALT lymphomas, three used the VH4 family and the other four used the VH3 family, whereas eight out of nine diffuse large B-cell lymphoma used the VH3 family, one used the VH1 family, and none used the VH4 family. It was also interesting that, in one diffuse large B-cell lymphoma patient with MALT lymphoma, the diffuse large B-cell lymphoma component used the VH3 family and the MALT lymphoma component used the VH4 family. These data imply that, in a subset of cases, these two subtypes do not share a common origin and that at least some diffuse large B-cell lymphomas have a *de novo* origin. No t(11; 18) (q21; q21) was detected in thyroid lymphomas, which are different from MALT lymphoma of the stomach, lungs, large intestine and ocular adnexa. This strongly indicated that the presence of t(11; 18) (q21; q21) in MALT lymphoma is organ-specific.
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Primary thyroid lymphoma is an uncommon type of tumor. A majority of thyroid lymphomas are of the B-cell type, most of which are diffuse large B-cell lymphomas or mucosa-associated lymphoid tissue (MALT) lymphomas.[1] MALT lymphoma is the second most frequent type of lymphoma in Japan.[2] In our surgical files, thyroid MALT lymphomas comprise 4% of all MALT lymphomas. MALT lymphoma arises in acquired MALT associated with chronic inflammatory processes triggered by autoimmune phenomena and/or chronic infection. In the thyroid gland, Hashimoto's thyroiditis is thought to be associated with the development of MALT lymphoma.[3,4] Occasionally, a component of MALT lymphoma is associated with diffuse large B-cell lymphomas. Thus, some diffuse large B-cell lymphomas have been thought to arise from pre-existing MALT lymphomas. Genetic or immuno-histochemical evidence for a clonal link between MALT lymphoma and diffuse large B-cell lymphoma in some gastric patients has been reported.[5,6]

However, the relationship between MALT lymphoma and diffuse large B-cell lymphoma remains to be clarified. Recently t(11; 18) (q21; 21) was found to be a specific chromosomal translocation associated with MALT lymphoma,[7–9] and it was proposed that gastric MALT lymphomas with this abnormality do not show large-cell transformation.[10] In addition, variable frequencies of t(11; 18) (q21; q21) of MALT lymphomas from different sites suggest different premalignant diseases associated with MALT lymphoma.[11] To determine the relationship between MALT lymphoma and diffuse large B-cell lymphoma in the thyroid gland, we studied the clinico-pathological features of MALT lymphomas and diffuse large B-cell lymphoma in the thyroid gland with special reference to VH usage by the immunoglobulin gene. We also examined the t(11; 18) (q21; q21) translocation of thyroid lymphomas by using multiplex reverse transcription-polymerase chain reaction (RT-PCR).

Materials and methods

Patients

Formalin-fixed, paraffin-embedded tissues or frozen tissues from 31 patients with MALT lymphoma and 27 patients with diffuse large B-cell lymphoma of the thyroid gland were retrieved from the pathology files at the Okayama University Graduate School of Medicine and Dentistry and Fukushima Medical University between 1989 and 2004. The records of all patients were reviewed to confirm the histological diagnosis according to the World Health Organization Classification criteria.[12] We diagnosed the cases as Hashimoto's thyroiditis when they showed diffuse lymphocytic infiltration between the thyroid follicles around the lymphoma and/or our laboratory findings showed positive for antithyroid antibody. All patients were Japanese. Lymphomas that involved the thyroid gland secondarily were not included in the study. Samples from each patient were used for molecular analysis and immunohistochemistry. Patients underwent a standardized informed consent procedure.

Immunohistochemistry

To confirm the diagnosis of MALT lymphoma and diffuse large B-cell lymphoma, immuno-histochemistry was performed on formalin-fixed, paraffin-embedded sections using the indirect immunoperoxidase method with secondary antibodies conjugated to peroxidase-labeled dextran polymer (EnVision$^+$, DAKO Japan, Kyoto, Japan). In brief, 4- to 6-μm-thick sections were deparaffinized and rehydrated. After endogenous peroxidase activity was blocked by 3% H_2O_2, antigen was retrieved by heat treatment for 30 min in 10 mM citrate buffer at pH 6.0. The slides were incubated for 10 min in 10% normal goat serum, then incubated overnight at 4°C with primary monoclonal antibodies against the following antigens: CD20 (1:200) and CD79a (1:40), purchased from DAKO Japan; and CD5 (1:25) and CD10 (1:50), from Novocastra Laboratories (Newcastle, UK). The specimens were then treated with the Envision$^+$ immunostaining system according to the manufacturer's instructions. Diaminobenzidine was used for color development, and hematoxylin was used for counterstaining.

DNA Amplification and Analysis of Nucleotide Sequences

The immunoglobulin heavy chain (*IgH*) gene was amplified by seminested polymerase chain reaction (PCR) as previously described.[13] The primers used in this study were as follows: 5′-TGG[A/G] TCCG[C/A] CAG [G/C] C [T/C][T/C] C [A/C/G/T] GG-3′, as the upstream consensus V region primer (FR2A); 5′-TGAGGAGACGGTGACC-3′, as the consensus J region primer (LJH); and 5′-GTGACCAGGGT [A/C/G/T] CCTTGGCCCCAG-3′, as the consensus J region primer (VLJH). The amplified product from each patient was electrophoresed on a 3% agarose gel and visualized by transillumination following ethidium bromide staining. PCR products of appropriate lengths (220–330 bp) were isolated. DNA was recovered from the clonal bands and directly sequenced using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan) with Big Dye terminators (Applied Biosystems Japan). Sequences were compared with the germline sequences using the Ig BLAST site (http://www.ncbi.nlm.nih.gov/igblast/).

Detection of t(11; 18) (q21; q21) Chromosomal Translocation

t(11; 18) (q21; q21) chromosomal translocations were detected using the multiplex RT-PCR method as described previously.[14] Briefly, 3-μm-thick, deparaffinized sections were incubated in digestion buffer (20 mM Tris, pH 8.0, 20 mM EDTA, 1% SDS, and 1 mg/ml proteinase K) at 56°C overnight. Total RNA was extracted using TRIZOL LS (Invitrogen Japan, Tokyo, Japan). RNA was subjected to first-round multiplex RT-PCR, then to second-round nested multiplex PCRs (three parallel; second PCR A, B, and C). The final PCR products were electrophoresed on a 3% agarose gel and visualized by transillumination following ethidium bromide staining.

Statistical Analysis

Fisher's exact probability test was used to compare clinical data and VH family usage between MALT lymphomas and diffuse large B-cell lymphomas.

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Results

Clinical Features of Malignant Lymphoma of the Thyroid Gland

Clinical data for each patient are summarized in Table 1. As the table shows, there were 31 MALT lymphomas (male seven and female 24) and 27 diffuse large B-cell lymphomas (male three and female 24). The mean ages of MALT lymphoma and diffuse large B-cell lymphoma patients were 67.8 (range 43–86) and 70.3 (range 45–88), respectively. Out of 58 patients, 36 (22 with MALT lymphomas, 14 with diffuse large B-cell lymphomas) showed Hashimoto's thyroiditis. Thirteen cases (12 of MALT lymphomas and one of diffuse large B-cell lymphomas) were diagnosed based on histological parameters and 23 cases (10 of MALT lymphomas and 13 of diffuse large B-cell lymphomas) were on clinical parameters. Patients were treated by various methods: surgical excision only (23.3%), chemotherapy (18.6%), radiation (11.6%), or some combination of these methods (44.2%). Only one patient with MALT lymphoma was observed without receiving any therapy. Follow-up data were obtained for 40 patients (22 with MALT lymphomas and 18 with diffuse large B-cell lymphomas), with the follow-up periods ranging from 1 to 171 months (mean, 49.9 months). No significant differences between patients with MALT lymphoma and those with diffuse large B-cell lymphoma was observed in age, sex, serum LDH level, stage, treatment, or prognosis ($P > 0.05$). A high frequency of antithyroid antibody was observed not only in patients with MALT lymphoma (13 of 14 patients) but also in those with diffuse large B-cell lymphoma (10 of 12 patients). Regarding clinical stages, 62% of patients with MALT lymphoma and the same percentage of those with diffuse large B-cell lymphoma remained at stage I E. Patients with either type of lymphoma remained at clinical stage I E or II E, except for one patient with diffuse large B-cell lymphoma who presented at clinical stage IV, which disseminated to the lung, resulting in the patient's death by this disease. Patients presenting at clinical stage II E showed involvement of lymphoma at the cervical, subclavicular, or mediastinal lymph nodes. All patients with MALT lymphoma remained alive during the follow-up period. The patients with diffuse large B-cell lymphoma also demonstrated a good survival rate (78%), except that four of them died during the follow-up period. One patient died of diffuse large B-cell lymphoma, another patient died of pneumonia during chemotherapy, and the other two died of causes other than lymphoma or its related complications, with no evidence that the death was attributable to the disease.

Table 1 Summary of clinical features of thyroid lymphomas

	N	DLBCL	MALT
No. of cases	58	27	31
Mean age (years)	58	70.3 (45–88)	67.8 (43–86)
Gender	58		
Male		3	7
Female		24	24
Serum LDH	38		
Elevated		8	4
Normal		11	15
Antithyroid antibody	26		
Positive		10	13
Negative		2	1
Stage	55		
I E		16	18
II E		9	11
IV		1	0
Treatment	43		
Op		3	7
Cx		4	4
Rx		3	2
Combined therapy		9	10
Observation		0	1
State at last follow-up	40		
Alive (disease free)		14 (14)	22 (21)
Died with disease		2	0
Died no evidence of disease		2	0
Mean follow-up time (months)		49.3	50.5

DLBCL, diffuse large B-cell lymphoma; MALT, MALT lymphoma; Op, operation; Cx, chemotherapy; Rx, radiotherapy.

Histological and Immunohistochemical Features of MALT Lymphoma and Diffuse Large B-cell Lymphoma of the Thyroid Gland

MALT lymphomas were composed of centrocyte-like cells, most of which (29 of 31 patients) formed lymphoepithelial lesions (Figure 1). Diffuse large B-cell lymphomas showed a diffuse infiltration of large-sized lymphoma cells (Figure 2). Histologically, nine of 27 patients with diffuse large B-cell lymphomas were associated with MALT lymphoma components (Figure 3). Lymphoepithelial lesion was also detected in MALT lymphoma components of diffuse large B-cell lymphomas associated with MALT lymphoma (nine of nine patients). Characteristically, three patients with diffuse large B-cell lymphomas without MALT lymphoma components showed lymphoepithelial lesions, which is seen in MALT lymphoma. In about half of the MALT lymphomas (52%) and MALT lymphoma components in diffuse large B-cell lymphoma (50%), the lymphoma cells involved the germinal centers and formed follicular colonization. Both of the malignant lymphomas showed a B-cell phenotype (CD20 or CD79a) but not a T-cell phenotype (CD3). In addition, the MALT lymphoma cells were negative for CD5 and CD10.
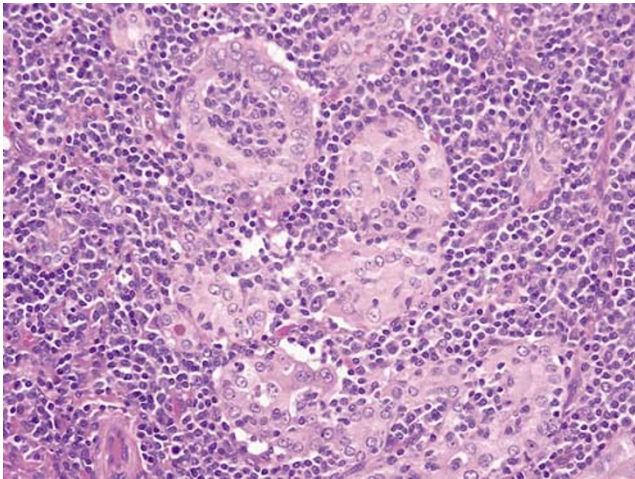
Figure 1 MALT lymphoma (HE). A diffuse proliferation of centrocyte-like cells are seen. Lymphoepithelial lesion is present.
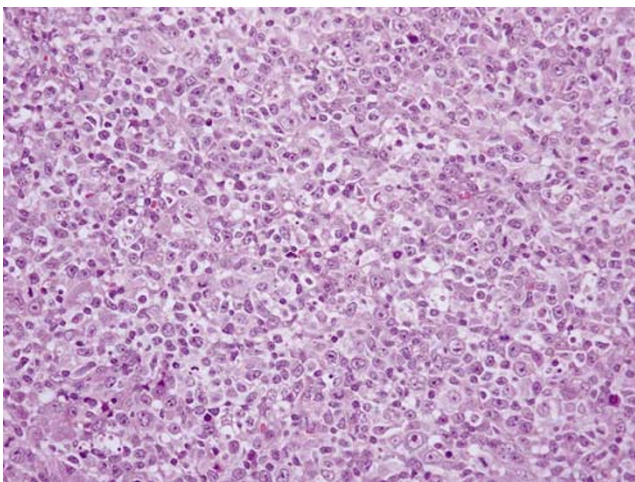

Figure 2 Diffuse large B-cell lymphoma without MALT lymphoma (HE). A diffuse proliferation of large-size lymphoma cells are seen.
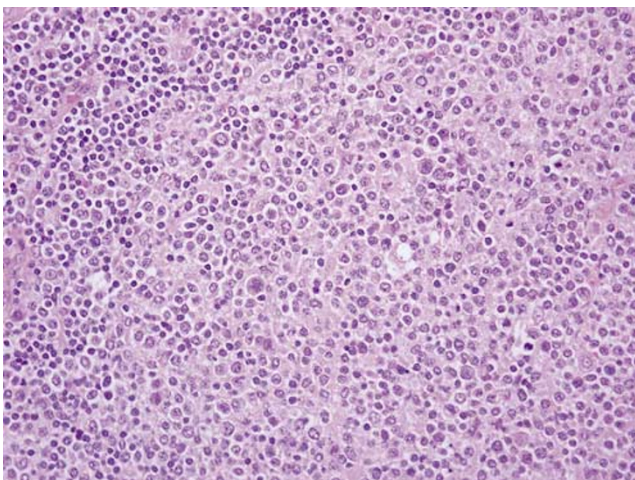

Figure 3 Diffuse large B-cell lymphoma with MALT lymphoma (HE). Large-size lymphoma cells (diffuse large B-cell lymphoma component) on the right side and small—to medium size cells (MALT lymphoma component) on the right side are intermingled.

Detection of t(11; 18) (q21; q21) Chromosomal Translocation

In the thyroid gland, no t(11; 18) was detected in 13 patients with MALT lymphoma or in eight patients with diffuse large B-cell lymphoma, including the patients with MALT lymphoma components.

Detection of *IgH* Gene Rearrangement Using PCR

PCR analysis was performed on DNA extracted from the specimens of 31 patients with MALT lymphomas and 27 patients with diffuse large B-cell lymphomas of the thyroid gland. Rearranged *IgH* genes were detected in 71% of malignant lymphomas (68% of MALT lymphomas and 74% of diffuse large B-cell lymphomas).

Analysis of VH Family Usage of MALT Lymphoma and Diffuse Large B-cell Lymphoma

PCR products from 19 patients (seven with MALT lymphoma and 12 with diffuse large B-cell lymphoma) were unselectively subjected to direct sequencing. In four of these 12 patients, the diffuse large B-cell lymphomas were associated with MALT components, but the components were intermingled and thus were difficult to examine separately. In each of these four patients, DNA was extracted into one tube from the tissue in which two components coexisted, and all of them showed the VH3 family. However, patient 9 had two components separately, and we were able to extract DNA from each component and to sequence each one separately. The two components showed different families: the MALT lymphoma component used the VH4 family, whereas the diffuse large B-cell lymphoma component used the VH3 family (Table 2).

The assignment of the sequences to VH families of MALT lymphomas and diffuse large B-cell lymphomas without MALT lymphoma components indicated biased usages (Table 2). In the seven patients with MALT lymphomas, three used the VH4 family, whereas most of the patients with diffuse large B-cell lymphomas (eight of nine patients) used the VH3 family, with none using the VH4 family. The MALT lymphomas and diffuse large B-cell lymphomas differed significantly in their use of the VH4 family ($P < 0.05$).

Discussion

The VH gene has been studied to investigate the lymphomagenesis and cellular origin of B-cell lymphomas. VH genes are organized into seven VH gene families based on high sequence homology.[15] By comparing the frequencies of VH family usage of MALT lymphomas and diffuse large B-cell lymphomas with peripheral blood B lymphocytes, we were

Table 2 Sequence analysis of the *IgH* genes expressed in primary thyroid lymphoma

Case no.	Histology	Age (years)	Sex	VH family	Germline VH gene	Identity
1	DLBCL	82	F	3	VH3-11	90
2	DLBCL	88	F	3	VH3-11	86
3	DLBCL	65	F	3	VH3-23[a]	92
4	DLBCL	59	F	3	VH3-74	90
5	DLBCL	64	F	3	VH3-48[a]	87
6	DLBCL	72	F	1	V1-69[a]	88
7	DLBCL	69	F	3	VH3-7	94
8	DLBCL	83	F	3	VH3-7	87
9	DLBCL	69	M	3	V3-30[a]	91
	MALT			4	VH4-31[a]	98
10	MALT	47	F	3	VH3-7	87
11	MALT	43	F	3	VH3-20	92
12	MALT	75	M	3	VH3-21[a]	89
13	MALT	86	F	4	VH4-39	90
14	MALT	63	F	4	VH4-34	88
15	MALT	73	F	4	VH4-61	96
16	MALT	83	M	1	VH1-46[a]	84
17	DLBCL+MALT	73	M	3	VH3-30[a]	98
18	DLBCL+MALT	77	F	3	VH3-7	82
19	DLBCL+MALT	69	F	3	VH3-30[a]	99
20	DLBCL+MALT	53	F	3	VH3-53	90

DLBCL, diffuse large B-cell lymphoma; MALT, MALT lymphoma; M, male; F, female.
[a]VH genes detected in antithyroid antibody. Data from McIntosh *et al*[19] and Chardes *et al.*[20]

Table 3 Frequency of VH family usage in primary thyroid lymphoma: comparison with lymphoma of other organs and adult peripheral B cells

Lymphoma arising in	Histology	No. of the analyzed sequences	VH1 (%)	VH2 (%)	VH3 (%)	VH4 (%)	VH5 (%)	VH6 (%)	VH7 (%)
Thyroid gland (this study)	MALT	8	13	0	38	50	0	0	0
	DLBCL	9	11	0	89	0	0	0	0
Orbital adnexa[a]	MALT	10	0	0	70	30	0	0	0
Stomach[a]	MALT	9	0	0	56	44	0	0	0
Salivary gland[a]	MALT	29	69	0	28	3.4	0	0	0
Lung[a]	MALT	13	0	0	62	39	0	0	0
Adult peripheral blood B cells[a]			16	8.3	65	4.6	3.5	2.7	0

DLBCL, diffuse large B-cell lymphoma; MALT, MALT lymphoma; M, male; F, female.
[a]Data from Mannami *et al.*[16]

able to examine the nature of B cells in these lymphomas.

The sequencing study of the VH gene showed that three of seven patients with MALT lymphomas used the VH4 family. In contrast, most of the diffuse large B-cell lymphomas without the MALT component (eight of nine patients) used the VH3 family and none used the VH4 family. Among the MALT lymphomas of various organs, VH family usage was found to be biased toward the VH4 family, which has low numbers of VH genes and which is rare in peripheral blood B lymphocytes (Table 3) (except for VH family usage in the salivary gland, which is characteristically restricted to the VH1 family which is thought to be associated with the rheumatoid factor).[16] Although MALT lymphomas arise from various extranodal organs, this deviated

usage of the VH families indicates that the pathogenesis of B cells in MALT lymphomas probably came through similar pathways. We clarified that the thyroid MALT lymphoma also belonged in the same category.

The relationship between MALT lymphoma and diffuse large B-cell lymphoma is an interesting one. Chan *et al*[6] showed that the two components had identical light-chain restrictions in gastric lymphomas and suggested that most if not all diffuse large B-cell lymphomas are the result of an evolution of MALT lymphoma that may have been present for many years. Peng *et al*[5] reported a clonal relationship between diffuse large B-cell lymphomas and MALT lymphomas of the stomach and supported this idea. In contrast, Cabras *et al*[17] and Matolcsy *et al*[18] reported patients with gastric diffuse large

B-cell lymphoma having a MALT component composed of distinct clones.[17,18] In the present study, the preferentially used VH families differed between MALT lymphomas and diffuse large B-cell lymphomas without MALT components, although their clinical backgrounds, such as gender deviation and the presence of Hashimoto disease and/or lymphocytic thyroiditis, were similar.

We also examined the DNA sequences of five patients with diffuse large B-cell lymphomas having MALT components (Table 2). In four of these patients, we found single monoclonal bands but could not determine which components reflected these bands because the two components were too intermingled. These patients might have high-grade transformations, since they had single bands. However, in one patient (patient 9), we were able to examine both components separately. The MALT lymphoma component used VH4, whereas diffuse large B-cell lymphoma used VH3. These findings strongly indicated that lymphocytes belonging to the VH4 family can be MALT lymphomas but not diffuse large B-cell lymphomas, and that at least some diffuse large B-cell lymphomas with MALT components are not high-grade transformations but probably *de novo*.

A close relationship between MALT lymphoma and Hashimoto's thyroiditis has been reported.[3,4] We compared the homologous sequences of VH genes of thyroid lymphoma cells to those of the antibody to thyroglobulin and thyroid peroxidase previously reported[19,20] (Table 2). Three of the eight patients with MALT lymphomas and six of the 13 patients with diffuse large B-cell lymphomas with or without MALT components showed common homologous germline VH genes used by antithyroid antibody. This suggests that both MALT lymphoma and diffuse large B-cell lymphoma are derived from the lymphocytes associated with antithyroid antibody. The high frequencies of antithyroid antibody of patients with both MALT lymphoma and diffuse large B-cell lymphoma also indicated the association of Hashimoto's thyroiditis.

Recently, the t(11; 18) chromosomal translocation was identified as a specific chromosomal abnormality in some MALT lymphomas.[7–9] Moreover, it was reported that t(11; 18) showed variable frequencies at different sites.[11] In this study, neither MALT lymphoma nor diffuse large B-cell lymphoma of the thyroid gland showed t(11; 18). We summarize our data in Table 4 (data on lymphomas in the salivary gland were collected by SN).[21,22] This result was compatible with the previous reports.[23,11] In short, t(11; 18) of the large intestine and ocular adnexa is found at moderate frequencies compared to those of the lung and stomach, which were found at high frequencies. In contrast, t(11; 18) is extremely rare in organs such as the thyroid gland and salivary gland, in which autoimmune diseases often arise. This suggests that mechanisms involved in t(11; 18) (q21; q21) have some organ specificity.[11] These

Table 4 Incidence of the t(11; 18) chromosomal translocation in lymphomas from various sites

Site	Histology	No. of cases	t(11; 18) (%)
Thyroid gland (this study)	MALT	13	0 (0)
	DLBCL	8	0 (0)
Salivary gland[a]	MALT	28	0 (0)
	DLBCL	6	0 (0)
Large intestine[b]	MALT	47	7 (15)
	DLBCL	13	1 (8)
Ocular adnexa[c]	MALT	23	3 (13)
	DLBCL	14	2 (14)

[a]Data from Nakamura.
[b]Data from Sakugawa *et al.*[21]
[c]Data from Takada *et al.*[22]

organ specificities are similar to differences in homing receptors and vascular addressins.[24]

In conclusion, both MALT lymphoma and diffuse large B-cell lymphoma of the thyroid gland occur frequently in elderly females and show favorable outcomes. No significant differences in clinical behavior between MALT lymphoma and diffuse large B-cell lymphoma were found. t(11; 18) was rarely associated with the lymphomagenesis of the thyroid lymphoma, and this abnormality seemed to be organ-specific. Our analysis of VH family usage may imply that Hashimoto's thyroiditis is associated with both subtypes, but the groups differed in which VH families they used.

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