Original

Clinicopathologic characteristics and A20 mutation in primary thyroid lymphoma

Running title: Characteristics in thyroid lymphoma

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Abstract

Background: Primary thyroid lymphoma (PTL) is a rare disease frequently arising against a background of autoimmune thyroiditis. It has recently been reported that the inactivation of the NF- κ B negative regulator A20 by deletion and/or mutation could be involved in the pathogenesis of subsets of B-cell lymphomas. This study investigated the clinicopathologic characteristics and A20 mutation in PTL.

Methods: We analyzed the characteristics of 45 PTL patients (14 men and 31 women), with a median age of 71 (range, 35–90) years. A20 mutations were

analyzed in DNA extracted from 20 samples consisting of 19 tumor tissues and 1 sample from Hashimoto's thyroiditis.

Results: Thirty-five patients (82%) had a history of Hashimoto's thyroiditis and 29 (64%) had diffuse large B-cell lymphoma (DLBCL), presenting with larger tumors including bulky mass, elevated soluble interleukin-2 receptor levels, and longer history of Hashimoto's thyroiditis compared with mucosa-associated lymphoid tissue (MALT) lymphoma patients (n=16). *A20* mutations were identified in 3 of 19 PTL patients (16%), 2 of 10 (20%) with DLBCL, and 1 of 9 (11%) with MALT lymphoma. Interestingly, all patients with *A20* mutations had Hashimoto's thyroiditis. Furthermore, they had a common missense variant in exon 3 (rs2230926 380T>G; F127C), which is known to reduce the ability of *A20* to inhibit NF- κ B signaling.

Conclusion: Our study demonstrated that the histological features of PTL affect clinical outcomes, and that *A20* mutations could be related to PTL pathogenesis in some patients with Hashimoto's thyroiditis.

Keywords: primary thyroid lymphoma, *A20* mutation, mucosa-associated lymphoid tissue (MALT) lymphoma, Hashimoto's thyroiditis

Introduction

Primary thyroid lymphoma (PTL) is rare, composing approximately 5% of all thyroid malignancies, 1–2.5% of all malignant lymphomas, and less than 2% of extranodal lymphomas (1, 2). Several reports showed that PTL patients were predominantly female, often in their 60s, with limited-stage disease and a good prognosis (3–7). PTL is a heterogeneous disease, however. PTL patients have not been fully characterized, and no standard treatment has been established since there has been no randomized trial of treatment. PTL frequently arises against a background of autoimmune thyroid disease, especially Hashimoto's thyroiditis, which has been reported to be a risk factor for PTL (8, 9). Thus, it is believed that its etiology is related to the stimulus of long-standing chronic inflammation and autoimmune disturbance (2), although the details remain to be clarified.

The NF- κ B negative regulator *A20*, also called tumor necrosis factor- α -induced protein 3 (TNFAIP3), encodes a ubiquitin-editing enzyme that restricts NF- κ B-dependent signaling and plays a key role in the negative regulation of inflammation and immunity (10, 11). Polymorphisms of the *A20* gene locus have been identified as risk factors for multiple human autoimmune diseases

such as rheumatoid arthritis, (RA) and systemic lupus erythematosus (12, 13). It has recently been reported that *A20* inactivation by deletion and/or mutation could be involved in the pathogenesis of subsets of B-cell lymphomas, especially mucosa-associated lymphoid tissue (MALT) lymphoma (14, 15). Honma et al. reported that *A20* deletion occurred frequently in diffuse large B cell lymphoma (DLBCL) (38%) and ocular adnexa MALT (37.5%), but was rare in stomach MALT (6.9%), and had not been reported in PTL (16). Another group demonstrated that *A20* deletion occurred in MALT lymphoma of the ocular adnexa (8 of 42 cases, 19%), salivary gland (2 of 24 cases, 8%) and thyroid gland (1 of 9 cases, 11%) (17). There have been very few reports on *A20* mutations in PTL (18, 19). Based on those findings, we hypothesized that *A20* abnormalities may be related to the pathogenesis of PTL, which is very often related to chronic thyroiditis. In this study, we analyzed the clinicopathologic characteristics of PTL and the characteristics of *A20* gene polymorphisms in PTL patients.

Materials and Methods

Patients and thyroid specimens

We analyzed the clinicopathologic characteristics of 45 PTL patients who

were diagnosed and classified according to the 2008 World Health Organization classification in our four institutions from 2002 to 2016. This study was approved by the Institutional Review Board of each participating institution. PTL is defined as a lymphoma mainly involving the thyroid gland without/with lymph nodes including regional neck lymph nodes. In one institution (Nippon Medical School, approval number 27-03-563), DNA samples were extracted from fresh thyroid tissues and smears prepared from bone marrow mononuclear cells (BMMCs) without lymphoma involvement after obtaining written informed consent for participation from each patient. Thyroid tissues obtained by thyroidectomy were homogenized with a glass homogenizer in RPMI1640 medium (Wako Pure Chemical Industries, Osaka, Japan) and then stored at -80°C until use. The control DNA was extracted from peripheral blood mononuclear cells of consenting participants who were originally suspected of having the disease but later were confirmed to be PTL negative.

DNA extraction, PCR, and sequencing analysis

DNA was extracted from frozen tissues and smear preparations of BMMCs using the QIAamp DNA Mini kit and Gentra Puregene Tissue kit (Qiagen,

Hilden, Germany), respectively, according to the manufacturer's instructions.

The coding exons 2–9 of the *A20* gene were amplified from DNA with PCR primers (14) using PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Shiga, Japan). PCR products were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics Co. Ltd., Tokyo, Japan). Direct sequencing was performed using the Big Dye Termination 3.1 kit and ABI PRISM 310 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded thin sections (4 μ m thick) were immersed in 0.3% H_2O_2 /methanol for 30 min to block endogenous peroxidase activity and microwaved in Tris-EDTA buffer (pH 9.0) for antigen retrieval. After blocking with 10% normal goat serum for 10 min at 37°C, sections were incubated with 100-fold diluted rabbit polyclonal antibody for NF- κ B p65 (phospho S536; ab86299; Abcam, Cambridge, UK) at 4°C for 18 h and then stained with 100-fold diluted biotinylated anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) for 30 min at 25°C, followed by avidin-biotin peroxidase complex (Santa Cruz Biotechnology) staining. The sections were reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution

containing 0.03% H₂O₂ and counterstained with Mayer's hematoxylin.

Statistical analysis

The χ^2 test was used to analyze the two-group comparisons. A P value of less than 0.05 was considered to represent a statistically significant difference.

Results

Patient characteristics

The patients' characteristics are shown in Table 1. They included 14 men and 31 women, with a median age of 71 (range, 35–90) years, presenting with a growing nodular goiter (n=39), hoarseness (n=4), and B symptoms (n=2). Three patients who had no symptoms related to lymphoma or thyroiditis were diagnosed because a low-echoic tumor was detected during ultrasound scanning conducted as follow-up of Hashimoto's thyroiditis. The pathologic diagnosis of PTL included DLBCL (n=29) and MALT lymphoma (n=16). Thirty-five patients (80%) had Hashimoto's thyroiditis, and 11 of the 35 were diagnosed concurrently with lymphoma based on increased thyroglobulin antibody and/or thyroid peroxidase antibody levels. MALT lymphoma patients had Hashimoto's thyroiditis more frequently (93%) than DLBCL patients (71%).

Twenty-eight patients (62%) had stage IE disease and 13 (29%) stage IIE disease, and only 4 patients had advanced-stage disease.

The results of comparative analysis between DLBCL and MALT patients are shown in Table 2. Compared with patients with MALT lymphoma, DLBCL patients presented with significantly larger tumors including bulky mass (>10 cm). The median interval between the time of diagnosis of Hashimoto's thyroiditis and PTL was 84 months when 11 patients diagnosed with Hashimoto's thyroiditis concurrently with PTL were excluded. Interestingly, DLBCL patients had a significantly longer history of Hashimoto's thyroiditis and higher levels of serum soluble IL-2 receptor compared with MALT lymphoma patients.

Patients with MALT lymphoma were treated with thyroidectomy or hemithyroidectomy and postoperative radiotherapy. DLBCL patients were treated with chemotherapy after surgery, followed by radiotherapy or no additional treatment. The treatment protocol was 6 cycles of chemotherapy consisting of R-CHOP (rituximab, cyclophosphamide, vincristine, prednisolone, and doxorubicin), THP-COP (pirarubicin instead of doxorubicin) for elderly patients, or 3 cycles of chemotherapy with locoregional radiation.

Survival and outcome of PTL patients

The overall treatment response rate was 96%, and 2 DLBCL patients did not respond to initial treatment. The median follow-up duration was 42 months (range, 2–229 months). Only 3 DLBCL patients died during the follow-up due to lymphoma dissemination in the central nervous system or complications during chemotherapy, that is, acute myocardial infarction and pneumonia. Five cases relapsed during follow-up. Three DLBCL patients who relapsed in the residual thyroid lobe underwent thyroidectomy, and 2 of the 3 received postoperative radiotherapy and achieved a second complete remission. All patients with MALT lymphoma remained alive in the median 63-month follow-up (range, 3–122 months), although 1 patient relapsed and was diagnosed with DLBCL transformed from MALT lymphoma 3 years after the initial thyroidectomy.

A20 mutation in PTL patients

We analyzed A20 mutations in tumor cells (somatic cells) from 19 PTL patients and in nontumor cells (germline cells) from Hashimoto's thyroiditis. Three of the 19 patients (16%) had the missense point mutation in exon 3 (380T>G transition), which leads to the replacement of phenylalanine with

cysteine at amino acid position 127 (F127C; SNP accession no. rs2230926; Figure 1A and Table 3). Of those 3 patients, 2 had DLBCL and 1 MALT lymphoma with Hashimoto's thyroiditis (Table 4). The heterozygous *A20* mutation of 1 patient's (no. 1 in Table 3) sample was identified not only in tumor cells but also in nontumor (germline) cells from the BMMC smears without involvement of DLBCL. PTL tissue from patient no. 3 had a homozygous mutation in *A20*, while nontumor BMNCs showed a heterozygous mutation (Fig. 1B and Table 3). We did not obtain a BMMC sample from patient no. 2 and thus were not able to identify the *A20* mutation in germline DNA from that patient. The *A20* F127C mutation has been reported to lead to inactivation of *A20* and results in constitutive NF- κ B activation (20). Consistent with the previous report, immunohistochemical staining showed that the phosphorylated NF- κ B p65 protein was strongly expressed in the PTL tissue from that patient (no. 1 in Table 3), in contrast to normal thyroid tissue (Fig. 2). Furthermore, even in this limited number of cases, patients with the *A20* mutation tended to have higher soluble IL-2 receptor levels than patients without the mutation (data not shown).

Discussion

We demonstrated the characteristics and clinical course of 45 PTL patients and results on the genetic or somatic abnormality of A20. PTL develops mostly in middle-aged and older women, which corresponds with the common age and gender of Hashimoto's thyroiditis patients. Eighty percent of the patients in this study had a previous history of Hashimoto's thyroiditis, and 64% had the DLBCL histologic type. None had a diagnosis of T-cell lymphoma, and the majority of patients had limited-stage disease. The clinical features of our patients were consistent with previous reports (1), and the frequency of A20 mutations in PTL was similar to that in other lymphomas (16, 17).

Since there has been no randomized trial to determine the therapy for PTL, we usually treat those patients following the guidelines for non-Hodgkin lymphomas. In our study, patients with MALT lymphoma responded well to surgery and local radiation. Most DLBCL patients were treated with combined chemotherapy following the strategy for nodular lymphoma, but some had a more aggressive clinical course, as previously reported (23). Three DLBCL patients relapsed in the residual thyroid lobe and were successfully treated with surgery or/and radiation. Although further investigations are needed to

determine the optimal treatment and prognostic factors for PTL, our results suggest that local control is important for both indolent and aggressive lymphoma.

Compared with MALT lymphoma patients, the DLBCL patients had a longer history of Hashimoto's thyroiditis before the diagnosis of lymphoma. Similarly, some observational studies in chronic thyroiditis demonstrated that the interval between the diagnosis of Hashimoto's thyroiditis and lymphoma onset was 4–9 years and the interval to aggressive lymphoma was reported to be longer (8, 21, 22). The time to develop malignant lymphoma against the background of Hashimoto's thyroiditis may vary depending on histological type. The reason for this difference remains unclear. However, one possible explanation is that some DLBCL patients might have transformed from MALT lymphoma after a relatively long time. In addition, the molecular mechanism linking chronic inflammation of thyroid lymphocytes to the development of malignant lymphoma has not been clarified.

Previously, genome-wide expression profile studies revealed that DLBCL carried somatic mutations in multiple genes, including positive regulators of NF- κ B, i.e., CARD11, TRAF2, TRAF5, etc., and negative regulators of A20 (11,

29). No study on the *A20* mutation in sufficient numbers of PTL patients has been reported, although Chanudet et al. showed that *A20* deletion occurred in 1 of 9 patients with MALT lymphoma of the thyroid (17). Wang et al. have recently reported that Wnt5a and its receptor Ror2 could play a crucial role in the pathogenesis and progression of PTL (30). We hypothesized that complex factors such as various gene mutations including *A20* abnormalities may be involved in the pathogenesis of PTL.

The most common coding polymorphism of *A20* was rs2230926 among the 32 types of polymorphism identified in sequencing experiments on autoimmune diseases such as RA, primary Sjögren's syndrome (pSS), Crohn's disease, and psoriasis (24). Nocturne et al. reported that 77% of lymphoma patients with pSS had germline and/or somatic abnormalities of *A20* (20). Their investigation also showed that the rs2230926 exonic variant was associated with an increased risk of lymphoma in pSS patients but not associated with the onset of pSS in general (25). Those results indicated that germline abnormalities of *A20* could lead to impaired control of NF- κ B activation in B cells continuously stimulated by autoimmunity and thus enhance the risk of lymphoma. In regard to *A20* abnormalities among patients with thyroid immune disease, Song et al.

demonstrated a clear association between polymorphisms of the *A20* gene and Grave's disease (28). A study examining the *A20* deletion in 383 cases found that 84 (21.9%) of 383 non-Hodgkin's lymphoma patients had 81 heterozygous losses, but only 3 homozygous loss (16). Other studies showed that *A20* mutations were significantly associated with *A20* heterozygous deletion (26,27), and the mutations caused abnormal *A20* protein expression and loss of function as well as homozygous deletion (27). Activation of NF- κ B promptly induces the expression of *A20* protein and normally suppresses excessive NF- κ B activation, but in B cells in which *A20* is inactivated due to these mutation, NF- κ B is abnormally activated without suppression, and cell proliferation is promoted, leading to tumorigenesis (26).

In our study, *A20* abnormalities were detected in 3 of 19 PTL patients (16%). Interestingly, they had a common missense variant in exon 3 (rs2230926 380T>G; F127C). Thus, *A20* abnormalities in PTL may be involved in the pathophysiology of lymphoma as in other conditions. Furthermore, the *A20* abnormality was detected only in patients with Hashimoto's thyroiditis (20%) but not in patients without that disease. Our study showed that the rs2230926 mutation was associated with PTL in Hashimoto's thyroiditis patients but not

associated with PTL in general. Furthermore, the *A20* mutation in tumor tissue was also detected in normal tissue in our patients. Notably, 1 patient (patient no. 3 in Table 3) had a heterozygous mutation in germline DNA but lost the wild-type allele in DNA of PTL tissue. This result indicates that a loss of heterozygosity may be associated with lymphoma pathogenesis. On the other hand, 2 patients (nos. 1 and 2 in Table 3) had a heterozygous *A20* mutation in PTL tissue and 1 (no. 1 in Table 3) had that mutation in normal tissue.

Immunohistochemical study showed NF- κ B activation in lymphoma tissue of a patient who had the *A20* mutation (no. 1 in Table 3), but not in normal thyroid tissues of the same patient (Figure 2). From our preliminary results, it remains unclear whether there is a functional difference between heterozygous and homozygous *A20* mutations and their relationship to NF- κ B activation in PTL. The *A20* mutation could play a role in the pathogenesis of PTL with Hashimoto's thyroiditis, caused by constitutive NF- κ B activation in the thyroid gland.

We confirmed the histologic heterogeneity of PTL corresponding to different clinical presentations and different prognoses. *A20* abnormalities may be related to PTL pathogenesis in some patients. Further studies are needed to

elucidate the pathogenesis including *A20* abnormalities of PTL and to predict progression to lymphoma in patients with Hashimoto's thyroiditis.

Author contributions: Tamura H. designed this study. Yamaguchi H. and Ishibashi M. supervised the experiments. Tatsuguchi A. performed pathological analysis. Asayama T., Okuyama N., Onodera-Kondo A., and Moriya K. collected clinical data. Igarashi T., Onose H., Tanosaki S., and Yokose N. supervised the collection of clinical data.

Conflicts of Interest: The authors have no conflict of interest directly relevant to the content of this article.

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Figure legends

Fig. 1. *A20* mutation in PTL patients. (a) The mutation site at the human *A20* gene in this study. The *A20* gene (upper schematic diagram) is comprised of 9 exons, and the first exon is a noncoding one. The *A20* protein (lower schematic diagram) has an OUT domain and a zinc finger (ZF) domain, which have deubiquitinating and E3 ligase activity, respectively. The location of gene and amino acid conversions is represented on the map by triangles. (b)

Electrofluorograms of *A20* exon3 genomic DNA (gDNA) sequence of the PTL patient samples and normal control samples. gDNA of BMMCs was used as germline DNA in PTL patients. Arrows indicate sites of nucleotide change (380T>G). (c) The mutation sites at the human *A20* protein in this study.

Fig. 2. Phosphorylated NF- κ B p65 (Ser536) expression in normal thyroid (a) and lymphoma (b) tissue from a PTL patient (patient no. 1 in Table 3). Brown, immunostaining for phosphorylated NF- κ B p65 with DAB; blue, counterstaining for nuclei with hematoxylin. Images were 200 $\times$ magnification.

Fig. 1

(a) Mutation site at the *A20* gene

(b) Nucleotide sequence

(c) Mutation site at the A20 protein

TRAF6: TNF receptor-associated factor 6
E3: ubiquitin protein ligase
ZF: zinc finger domain
TAX1BP1: Tax1 (human T-cell leukemia virus type I) binding protein 1
IKK γ : I-kappa-B kinase-gamma
ABIN: A20-binding inhibitor of NF-kB activation,
RIP: receptor-interacting protein
UBCH5A: ubiquitin-conjugating enzyme
E2: ubiquitin-binding enzyme

Fig. 2

Table 1: Patient characteristics at the time of diagnosis of PTL

Variable	
Patients, n	45
Age, years (median [range])	71 [35–90]
Gender (male/female), n	14/31
Diagnosis by pathological view, n (%)	
DLBCL	29 (67)
MALT	16 (33)
Concomitant thyroid disease, n (%)	
Hashimoto's thyroiditis	35 (80)
Basedow disease	2 (4)
None	7 (16)
Symptoms at diagnosis, n	
Growing nodular goiter	39
Hoarseness	4
B symptoms	2
No symptoms	3
Laboratory data at diagnosis, median (range)	
LDH (IU/ml)	198 (117–433)
sIL-2R (IU/ml)	478 (130–3189)
TSH (U/ml)	3.91 (0.12–185.459)
Free T4 (pmol/l)	1.10 (0.14–1.74)
Clinical stage ^a , n (%)	
IE	28 (62)
IIE	13 (29)
III, IV	4 (9)
A20 abnormalities, n (% in tested cases)	3 (16)

DLBCL: diffuse large B-cell lymphoma; MALT: mucosa-associated lymphoid tissue; LDH: lactate dehydrogenase; sIL-2R: soluble interleukin-2 receptor; TSH: thyroid-stimulating hormone.

a) Clinical stage IE was defined as lymphoma limited to the thyroid gland, IIE as the thyroid

gland and regional lymph nodes above the diaphragm, IIIIE as the thyroid gland and regional lymph nodes above and below the diaphragm, and IV as disseminated nodal and/or additional extranodal involvement.

Table 2: Comparative analysis of PTL with DLBCL and MALT lymphoma

	DLBCL	MALT	P value
Patients, n	29	16	
Age (≥ 65 / <65 years)	25/4	11/5	NS
Gender (male/female)	10/19	4/12	NS
Performance status (0-1/2-4)	23/3	12/0	NS
Hoarseness at diag. (yes/no)	3/25	1/13	NS
Symptomatic/asymptomatic	28/1	12/2	NS
Clinical stage (I, II/III, IV)	26/3	15/1	NS
Tumor size (≥ 5 cm/ <5 cm)	20/6	6/8	0.031
Bulky mass (yes/no)	4/22	0/14	NS
Hashimoto's thyroiditis (yes/no)	20/8	14/1	0.092
Duration of Hashimoto's thyroiditis (>84 months/ <84 months)	9/11	1/10	0.041
LDH ($>UNL$ / $\leq UNL$)	14/14	4/10	NS
sIL-2R ($>UNL$ / $\leq UNL$)	22/6	5/8	0.011

DLBCL: diffuse large B-cell lymphoma; MALT: mucosa-associated lymphoid tissue; LDH: lactate dehydrogenase; sIL-2R: soluble interleukin-2 receptor; NS: not significant.

Table 3: Patients with the A20 F127C mutation

Pt no.	Gender	Age	Diag	St	HT* (months)	Lymphoma tissue	BMMC
						Mutation type	
1	F	68	DLBCL	IA	4	Heterozygous	Heterozygous
2	M	67	MALT	IA	48	Heterozygous	No data
3	F	71	DLBCL	IIA	82	Homozygous	Heterozygous

HT*, time since the diagnosis of Hashimoto's thyroiditis at the time of diagnosis of PTL.

Pt, patient; Diag, diagnosis; St, stage; DLBCL, diffuse large B-cell lymphoma; MALT, mucosa-associated lymphoid tissue; BMMC, bone marrow mononuclear cell.

Table 4: Patients with/without the A20 mutation

A20 mutation	+	-
Patients, n	3	16
Diagnosis, MALT/DLBCL	1/2	8/8
Median age, years (range)	68 (67–71)	74 (35–83)
Gender, male/female	1/2	8/8
Clinical stage, I+II/III+IV	3/0	13/3
Median maximal tumor mass diameter, mm (range)	46 (27–93)	69 (20–100)
Hashimoto's thyroiditis, yes/no	3/0	15/3
Median duration of Hashimoto's thyroiditis, years (range)	4 (1–8)	8 (1–30)
LDH, median (range) (U/mL)	179 (122–336)	232 (176–422)
sIL-2R, median (range) (U/mL)	1219 (1148–1289)	653 (130–3189)

DLBCL: diffuse large B-cell lymphoma; MALT: mucosa-associated lymphoid tissue; LDH: lactate dehydrogenase; sIL-2R: soluble interleukin-2 receptor.