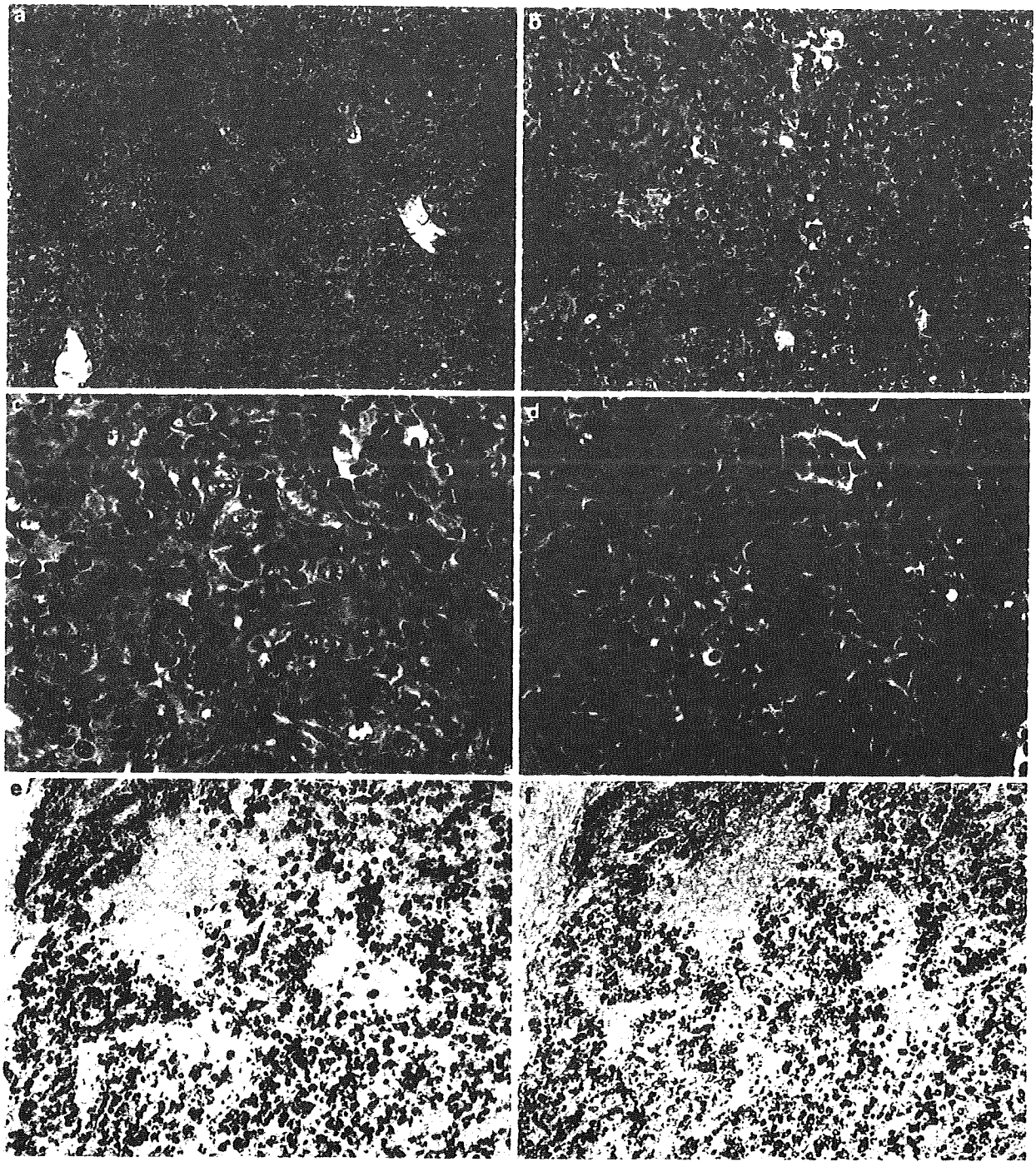

Figure 2 Histopathology of systemic IgG4-related lymphadenopathy (interfollicular plasmacytosis type, atypical lymphoplasmacytic and immunoblastic proliferation-like feature, patient no. 6). (a) The lymph node showed atrophic germinal center with discrete mantle zone, and expansion of the interfollicular area. (b) High endothelial venules were prominent, and there was polymorphous cellular infiltration; plasma cells and immunoblasts were especially distinct (c) The interfollicular area showed heavy infiltration with mature plasma cells, plasmacytoid cells, small lymphocytes, and immunoblasts. (d) Eosinophil infiltration was recognized. Immunostaining of IgG4 (e) and IgG (f). The IgG4/IgG-positive cell ratio was 72.7%. (a–d) Hematoxylin and eosin staining; (a) 40, (b) 200, (c and d) 400, and (e and f) 100.

hypoalbuminemia, or hypocholesterolemia, and elevated interleukin-6 and C-reactive protein were the exceptions. These findings are quite different from those of multicentric Castleman's disease.[15–18]

Interleukin-6 is a multifunction cytokine that has various biological activities in target cells and regulates immune responses, acute phase reactions, hematopoiesis, and bone metabolism.[25] Dysregulated

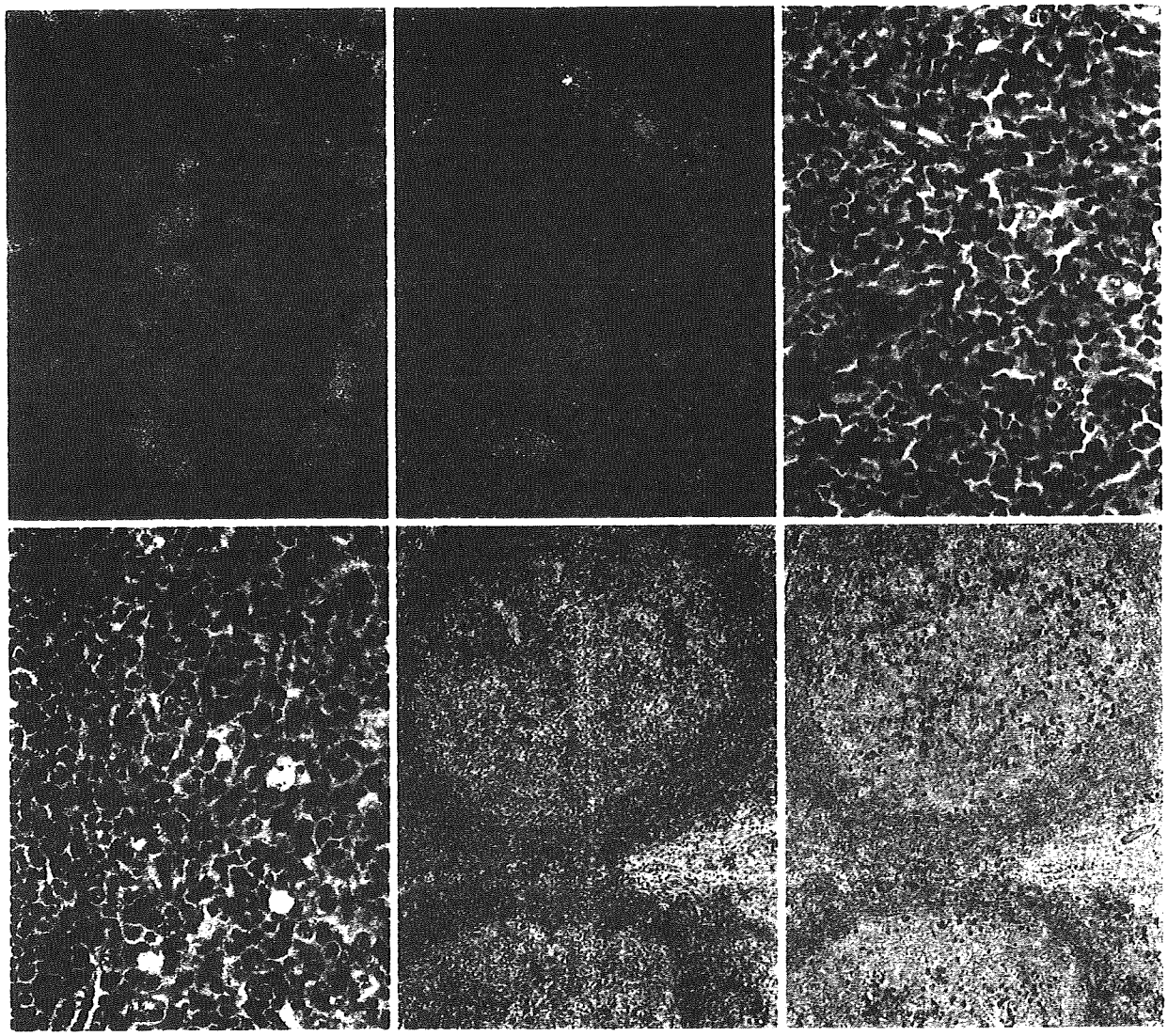

Figure 3 Histopathology of systemic IgG4-related lymphadenopathy (intra-germinal center plasmacytopsis type, patient no. 9). (a and b) The lymph node showed numerous lymphoid follicles with hyperplastic germinal centers and a distinct mantle zone, with focal progressive transformation of germinal centers. (c) Eosinophils infiltrated the interfollicular area. (d) Plasma cells and plasmacytoid cells infiltrated the germinal center. Immunostaining of IgG4 (e) and IgG (f). IgG4-positive cells mainly infiltrated the germinal center, and the IgG4/IgG-positive cell ratio was 63.0%. (a–d) Hematoxylin and eosin staining; (a) 20, (b) 40, (c and d) 400, and (e and f) 100.

overproduction of interleukin-6 is found in autoimmune diseases, such as rheumatoid arthritis, multicentric Castleman's disease, and Crohn's disease.[16–18,26,27] C-reactive protein is a pentamer of 23-kd subunits that is synthesized and secreted by hepatocytes upon stimulation by a variety of inflammatory cytokines, including tumor necrosis factor-a, interleukin-1, and especially interleukin-6. Therefore, interleukin-6 is closely related to the production of C-reactive protein.[25–29] Accordingly, interleukin-6 and C-reactive protein may become considerably important as differential diagnostic markers between systemic IgG4-related lymphadenopathy and multicentric Castleman's disease. Masaki et al[6] have reported that IgG4-related disease is not associated with an elevated serum interleukin-6 level, and cited measurement of serum interleukin-6 as an important tool of differential diagnosis. In our present series, two patients (nos. 2 and 4) showed slight, and one patient (no. 6) showed high elevation of serum interleukin-6, but their C-reactive protein levels were not as highly elevated as in multicentric Castleman's disease. Our patients using steroids showed a good response, and the histological findings of patient nos. 2 and 6 showed no similarities to those of Castleman's disease. The reference value of interleukin-6 is generically 0 4.0 pg/ml. However, Yokayama[30] has reported that the interleukin-6 value was over 25 pg/ml in 7% of the

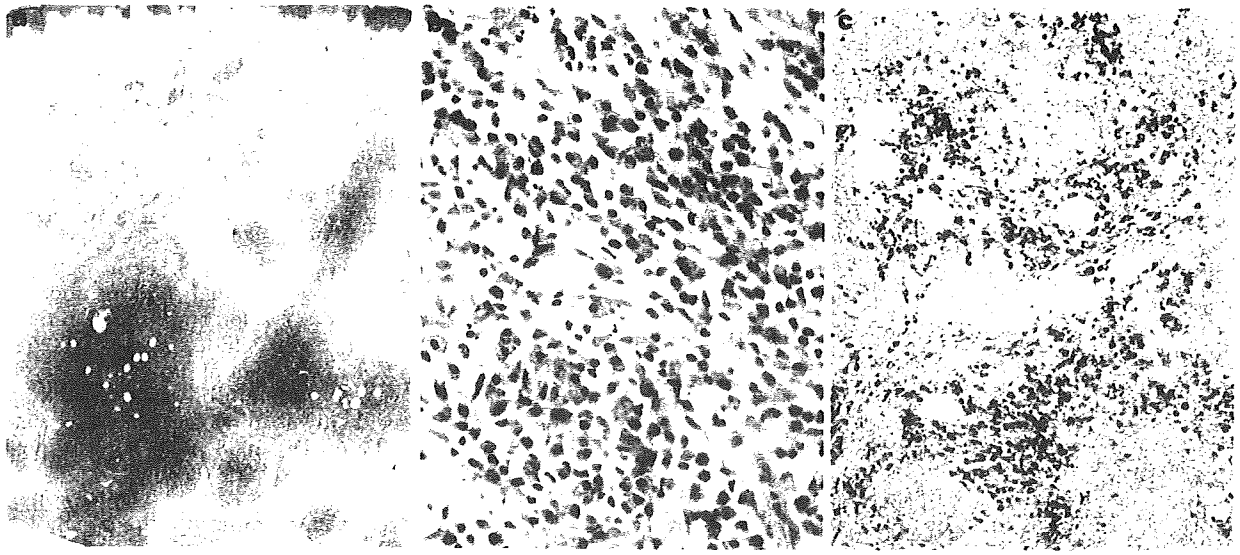

Figure 4 Histopathology of a skin lesion of a patient with systemic IgG4-related lymphadenopathy (patient no. 9). (a and b) Plasma cells, small lymphocytes and eosinophils showed a nodular-forming infiltration in the intermediate to deep dermis. (c) Immunostaining of IgG4; many plasma cells expressed IgG4. (a and b) Hematoxylin and eosin staining; (a) 20, (b) 400, and (c) 100.

healthy subjects. Therefore, interleukin-6 values might vary widely among individuals.

Our patients with systemic IgG4-related lymphadenopathy all showed hypergammaglobulinemia, but serum IgM and IgA was normal in almost all patients. In contrast, multicentric Castleman's disease is characterized by increased serum IgG, IgM, and IgA levels,[16] which are caused by the increase in serum interleukin-6.[5] In this series, most patients showed cervical, hilar, mediastinal, and para-aortic lymph node swelling, and the lymph nodes were generally not very large (up to 2 cm). These findings were consistent with previous reports of IgG4-related lymphadenopathy.[3,4,11]

Histologically, we could classify the cases into two types based on the infiltration pattern of IgG4-positive cells: interfollicular plasmacytosis type and intra-germinal center plasmacytosis type. Morphologically, the interfollicular plasmacytosis type was characterized by expansion of the interfollicular area, moderate to marked increase in vascularity, and infiltration of IgG4-positive cells mainly in the interfollicular area. Five out of six cases showed eosinophil infiltration in the interfollicular area. These cases demonstrated Castleman disease-like features (patient nos. 1, 3, and 4) or atypical lymphoplasmacytic and immunoblastic proliferation-like features (patient nos. 2, 5, and 6). Koo et al[23] reported that atypical lymphoplasmacytic and immunoblastic proliferation is unusual in cases of lymph node lesion associated with various autoimmune diseases, including rheumatoid arthritis. Histopathologically, the lesion is characterized by prominent lymphoplasmacytic infiltration with various number of immunoblasts.[23,24] In this series, three patients showed this pattern, but there was no evidence of rheumatoid arthritis. In addition, there was abundant IgG4-positive cells infiltration in the lesion and the serum IgG4 value was elevated. These findings are consistent with IgG4-related disease. These results suggested that some IgG4-related lymphadenopathy might be confused with multicentric Castleman's disease or atypical lymphoplasmacytic and immunoblastic proliferation. In the case of systemic IgG4-related lymphadenopathy of atypical lymphoplasmacytic and immunoblastic proliferation type, the histology can be confused with that of malignant lymphoma, especially angioimmunoblastic T-cell lymphoma. However, the former has no clear cells, no CD10-positive T-cells, no extrafollicular follicular dendritic cell proliferation, and no T-cell receptor gamma gene rearrangement.

By contrast, the intra-germinal center plasmacytosis type shows marked follicular hyperplasia, a mild increase in vascularity, and IgG4-positive cells mainly infiltrating the germinal centers. In our study, the two patients with this type showed progressively transformed germinal center. The germinal centers are known to be a major site for B-cell selection. In the germinal centers, B cells perform numerous somatic hypermutations and heavy-chain class-switches and only a portion of them are selected through the cooperation of T cells and follicular dendritic cells. The selected B cells exit the germinal centers and become plasma cells.[31] Therefore, the fact that many IgG4-producing plasma cells were found selectively in the germinal centers of our patients was a unique feature. The mechanisms involved in this feature are not clear.

Zen et al[10] have reported that the expressions of T helper (Th) 2 cytokines (interleukin-4, interleukin-5, and interleukin-13) and regulatory cytokines (interleukin-10 and transforming growth factor-b) were upregulated in the affected tissues of patients with IgG4-related sclerosing pancreatitis and cholangitis. They have suggested that the prominent Th2 and regulatory immune reactions in this disease might indicate that its pathogenesis involves an allergic mechanism. In addition, they described that interleukin-10 has a major role in directing B-cells to produce IgG4.[10] Therefore, interleukin-10 might induce differentiation of B cells into IgG4-positive plasma cells in the germinal centers. At any rate, this unique histological feature could be a special finding for IgG4-related lymphadenopathy.

Interestingly, eight of our nine cases (89%) of systemic IgG4-related lymphadenopathy showed eosinophil infiltration. Zen et al[10] reported that eosinophils infiltrated in the affected tissues in their patients with IgG4-related sclerosing pancreatitis and cholangitis. As previously mentioned, Th2 cytokines (interleukin-4, interleukin-5, and interleukin-13) were upregulated in the affected tissues of patients with IgG4-related disease. Interleukin-5 and interleukin-13 were activated by eosinophil infiltration and IgE production. Masaki et al[6] have reported that serum IgE level is elevated in IgG4-related diseases. In our series, the serum IgE value was significantly elevated in the examined patients. So, the findings of eosinophilic infiltration and serum IgE value elevation might be a specific finding of IgG4-related disease.

Little is known about the lymphomagenesis of IgG4-related disease. We recently reported the first case of marginal zone B-cell lymphoma arising from ocular adnexal IgG4-related disease[7] and IgG4-producing marginal zone B-cell lymphoma.[32] However, the present series showed no immunoglobulin light-chain restriction and no immunoglobulin heavy chain gene rearrangement.

In conclusion, in systemic IgG4-related disease, C-reactive protein and interleukin-6 are usually not elevated. Systemic IgG4-related disease and multicentric Castleman's disease showed overlapping but somewhat distinct pathologic findings, and serum data (especially C-reactive protein and interleukin-6) are useful to differentiate between them. Eosinophilic infiltration and serum IgE value elevation might be a specific feature of IgG4-related lymphadenopathy.

Disclosure/conflict of interest

The authors have no potential conflicts of interest.

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Duodenal and nodal follicular lymphomas are distinct: the former lacks activation-induced cytidine deaminase and follicular dendritic cells despite ongoing somatic hypermutations

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Although most follicular lymphomas are believed to be of nodal origin, they sometimes originate from the duodenum. We have reported that the latter differ from nodal follicular lymphomas in having lower clinical stages and uniformly low histological grades, along with variable region of immunoglobulin heavy chain gene (VH) usage that is more similar to mucosa-associated lymphoid tissue (MALT) lymphomas. Little is known, however, about whether they possess other characteristics of nodal follicular lymphomas, particularly ongoing mutations with follicular dendritic cells. We examined 17 cases for which PCR identified the monoclonal bands of the immunoglobulin gene. The duodenal cases showed ongoing mutations, but they lacked activation-induced cytidine deaminase (AID) expression, a statistically significant difference from the nodal cases ($P < 0.001$), and their follicular dendritic cell networks were disrupted. Moreover, not only were VH deviations observed but also they used very restricted VH genes. Although the mechanisms of ongoing mutation without AID and follicular dendritic cell were not clarified, restricted VH usage strongly suggested that antigen stimulation was involved, and that was similar to MALT lymphomas. In conclusion, duodenal follicular lymphomas were shown to be unique, in that they had ongoing hypermutations such as nodal cases, but the mechanisms involved in the hypermutation were quite different; furthermore, restricted VH usage suggested a strong similarity to the antigen-dependent origin of MALT lymphomas.
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Keywords: duodenal follicular lymphoma; AID; follicular dendritic cell

We have reported that duodenal follicular lymphomas are frequently found in the second portion of the duodenum.[1] Although they express CD10 and bcl-2 and harbor t(14;18) translocation similar to nodal follicular lymphomas,[2] most of them are obviously at lower clinical stages than the nodal cases (most are stage I–II) and have lower histological grading with rather dominantly uniform intermediate small cleaved cells.

The vast majority of follicular lymphomas originate from lymph nodes, and earlier reports have clearly indicated that these follicular lymphoma cells derive from germinal-center B cells: both cells share characteristics such as accumulation of somatic hypermutation and ongoing mutations.[3] Furthermore, they interact with follicular dendritic cells. In an earlier report, we also described that duodenal follicular lymphomas have a high frequency of IgVH4 gene usage as in mucosa-associated lymphoid tissue (MALT) lymphomas, with which they share other characteristics.[4–6] Localized disease is one of main characteristics of MALT lymphomas.

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From these findings, we considered whether duodenal follicular lymphomas have other differences from nodal follicular lymphomas, and decided to examine their expression of activation-induced cytidine deaminase (AID), which plays the important roles of class-switch recombination and somatic hypermutation,[7] and the patterning of their follicular dendritic cell networks. It is reported that AID expression is associated with ongoing mutation in nodal follicular lymphoma,[8] but is not correlated with diffuse large B-cell lymphomas.[9] Although other characteristics of duodenal follicular lymphomas have been described, to the best of our knowledge no report has focused on their somatic and ongoing mutations, which are the most important similarity to germinal-center B-cell lymphomas.

In this report, we discovered that duodenal follicular lymphomas showed ongoing somatic hypermutations similar to nodal cases; unlike the latter, however, they lack AID and have disrupted follicular dendritic cell networks.

Materials and methods

Patient Selection

Buffered formalin-fixed and paraffin-embedded tissues (10%) were used for histological, immunohistochemical and immunogenotypical studies. We chose 30 consecutive patients of duodenal follicular lymphoma, successfully amplified the monoclonal rearranged band of the VH gene in 17 of these patient samples, and focused on these samples for further analyses. Informed consent for examination was obtained for the use of all samples.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were subjected to immunohistochemical staining. Staining was carried out using heat-induced epitope retrieval or trypsin-induced retrieval, an avidin-biotin complex method, and an automated immunostainer (Ventana Medical System, Tuscon, AZ, USA), as described earlier.[10] The antibody panel used to assess these cases was as follows (clone, dilutions): CD20 (L26, 1:200), CD3 (PS-1, 1:50), CD10 (56C6, 1:50), CD5(4C7, 1:100), bcl-2 (3.1, 1:200), CD23 (1B12, 1:100) and Ki-67 (MIB-1, 1:5000) (Novocastra, Newcastle-upon-Tyne, UK); CD21 (1F8, 1:20), MUM1 (MUM1p, 1:50) (DAKO Cytomation, Glostrup, Denmark A/S); bcl-6 (D-8, 1:100) (Santa Cruz, CA, USA); and cyclin D1 (SP4, ready to use) (Nichirei, Tokyo, Japan); AID (ZA001, 1:100) (ZYMED, South San Francisco, CA, USA). As for CD20, CD3, CD10, CD5, cyclin D1, bcl-2, bcl-6 and MUM-1 antigens, positivity was determined when 30% or more lymphoma cells were positive for their antibodies. For AID expression in tumor follicles, samples with 20% or more expressing cells were evaluated as positive. Ki-67-positive cells were counted in tumor follicles.

DNA Extraction and PCR

DNA was extracted from paraffin-embedded tissue using the QIAamp DNA Micro Kit- (Qiagen Inc., Valencia, CA, USA). The variable region (CDR2 and FW3) and VDJ region (CDR3) of the immunoglobulin heavy-chain gene were amplified by semi-nested PCR, using the primers of FR2, LJH and VLJH as described earlier.[11,12] Primers were as follows: 5′-CC GGRAARRGTCTGGAGTGG-3′, as upstream consensus V region primer (FR2); 5′-CTTACCTGAGGA GACGGTGACC-3′, as a consensus J region primer (LJH); 5′-GTGACCAGGGTNCCTTGGCCCC-3′, as a consensus J region primer (VLJH). PCR products were purified using the QIAquick PCR purification kit (Qiagen). Then 1 ml of the PCR product was applied for direct sequencing (ABI PRISM Model 3100, version 3.7, Applied Biosystems).

Ongoing Mutation Study and Analysis

PCR products were ligated into the pDrive cloning vector and transformed into DH5alpha cells (TaKaRa Bio. Inc., Tokyo, Japan) according to the instruction manual (PCR cloning kit, Qiagen). After an overnight culture, 10 and more white colonies were picked from a Luria-Bertani (LB) agar plate based on the X-Gal screening, then placed into 25 ml of the Insert Check ready kit solution (TOYOBO, Osaka, Japan). PCR conditions consisted of 30 cycles of 94 1C for 30 s, 60 1C for 5 s and 72 1C for 30 s. Ten samples including correct PCR products confirmed by checking 2% gel electrophoresis for each case were sequenced by the same method as described above. Then, the closest germline was searched for by BLAST using the immunoglobulin sequence (NCBI). Ongoing mutation was determined by dividing the cumulative number of partially shared mutations (mutations shared by some clones but not by all the VH gene clones) and unique mutations (mutations unique to a distinct VH gene clone) with the expected number of mutations calculated based on the PCR error rate (4.5×10^{-4} change per base per PCR cycle) by the method described above.[7] The w^2 test with Yates' correction when appropriate was used to evaluate the association of qualitative variables in the different groups. Statistical analysis was carried out using Statcel2 for Windows. Values of $P < 0.05$ were considered statistically significant.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) for t(14;18)(q32;q21)/IGH-BCL2 translocations were

carried out using the BCL2 FISH DNA split signal probe (DAKO Cytomation, Glostrup, Denmark A/S) according to the manufacturer's instructions. We examined FISH directly on paraffin-embedded tissue sections and detected split signal of BCL2 gene as descried earlier.[13]

Table 1 Clinicopathologic features of duodenal follicular lymphoma

Patient no.	Age/sex	Stage	Grade	CD5	CyclinD1	CD10	bcl-2	bcl-6	MUM-1	t(14;18)	CD21 pattern
1	75/M	I	1			+	+	+		Not tested	Duodenal
2	57/M	I	1			+	+	+		Not tested	Duodenal
3	58/M	IV	1			+	+	+		+	Duodenal
4	75/M	II2	1			+	+	+			Duodenal
5	71/F	I	1			+	+	+		+	Duodenal
6	66/F	I	1			+	+	+			Nodal
7	51/M	I	1			+	+	+		+	Duodenal
8	49/M	II2	1			+	+	+		+	Duodenal
9	62/F	II2	1			+	+	+		+	Duodenal
10	54/M	I	1			+	+	+		+	Duodenal
11	61/F	IV	1			+	+	+		+	Duodenal
12	53/F	II2	1			+	+	+		+	Duodenal
13	57/F	I	2			+	+	+		+	Duodenal
14	56/M	II2	1			+	+	+		+	Duodenal
15	66/F	II2	1			+	+	+		+	Duodenal
16	55/F	II2	1			+	+	+		+	Nodal
17	63/F	II2	1			+	+	+		+	Duodenal

F, female; M, male.
Immunophenotypic data were analyzed using immunohistochemistry.

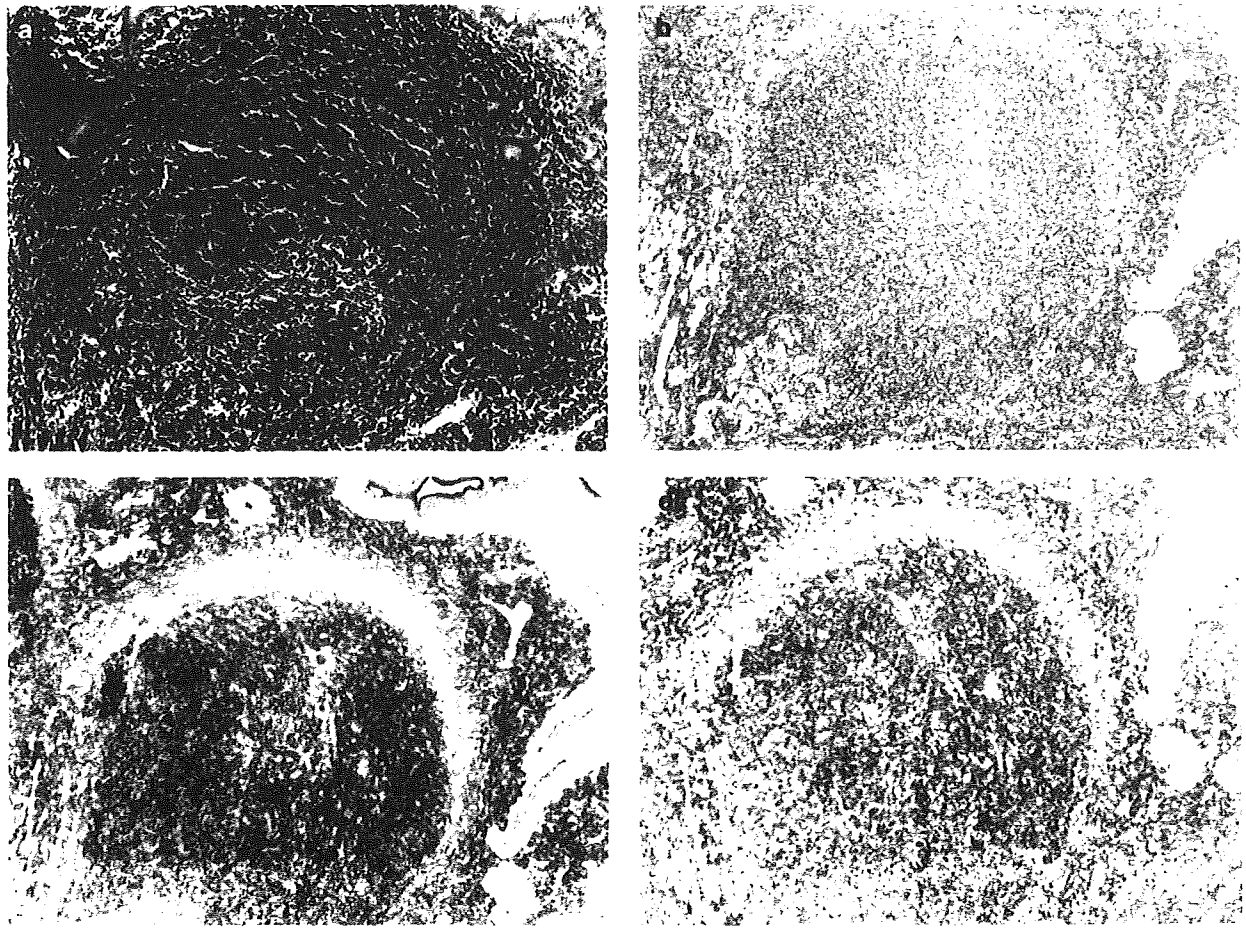

Figure 1 Duodenal follicular lymphoma and immunohistochemical stain (patient no.17). (a) H&E: A neoplastic follicle is composed of uniform intermediate small-cleaved cells. (b) Tumor cells are positive for CD20 in all cases (c) and for CD10 (d) and bcl-2.

Results

Patient Summary and CD21 Expression

Clinical features (age, gender, clinical stage) and the results of immunohistochemical study are shown in Table 1. The 17 patients (8 males and 9 females) ranged in age from 49 to 75 years with a median age of 61 years. According to the International Workshop (Lugano) Classification, seven cases were clinical stage I, eight cases were stage II and two cases were stage IV.[14] Histological grade were 1–2 of all cases. All patient samples expressed CD20, CD10, bcl-2 and bcl-6, and were negative for CD5, MUM1 and cyclin D1 by immunohistochemical study (Figure 1). In all cases, the Ki-67 labeling index was 0 10%. The presence of a t(14;18)(q32;q21)/IGH-BCL2 was sought by FISH depending on the material available. The translocation of 18q21 was detected in 13 of 15 (86.7%) cases (Figure 2 shows the split signal of patient no. 7 sample). We could not detected translocations at two patients' materials.

A typical pattern of follicular dendritic cells expressing CD21 and CD23 in nodal follicular lymphoma is shown in Figure 3 and clearly indicates neoplastic follicles. Nodal follicular lymphoma interacts with follicular dendritic cells, which sometimes provides a diagnostic clue.[15] On the contrary, out of 17 samples of duodenal follicular lymphomas, 15 showed a very similar pattern of follicular dendritic cells: they were rather densely arranged at the periphery of the neoplastic follicle area but few follicular dendritic cells were detected at the center of the neoplastic follicle area.

In most nodal follicular lymphomas, follicular dendritic cell networks occupied more than two-thirds of the neoplastic follicle areas. On the contrary, follicular dendritic cell networks occupied 0 10% of the neoplastic follicle areas in duodenal follicular lymphomas, and were distributed in the periphery of the neoplastic follicles. Accordingly, the nodal pattern was identified as neoplastic follicles coextensive with large follicular dendritic cell networks, whereas the duodenal pattern was

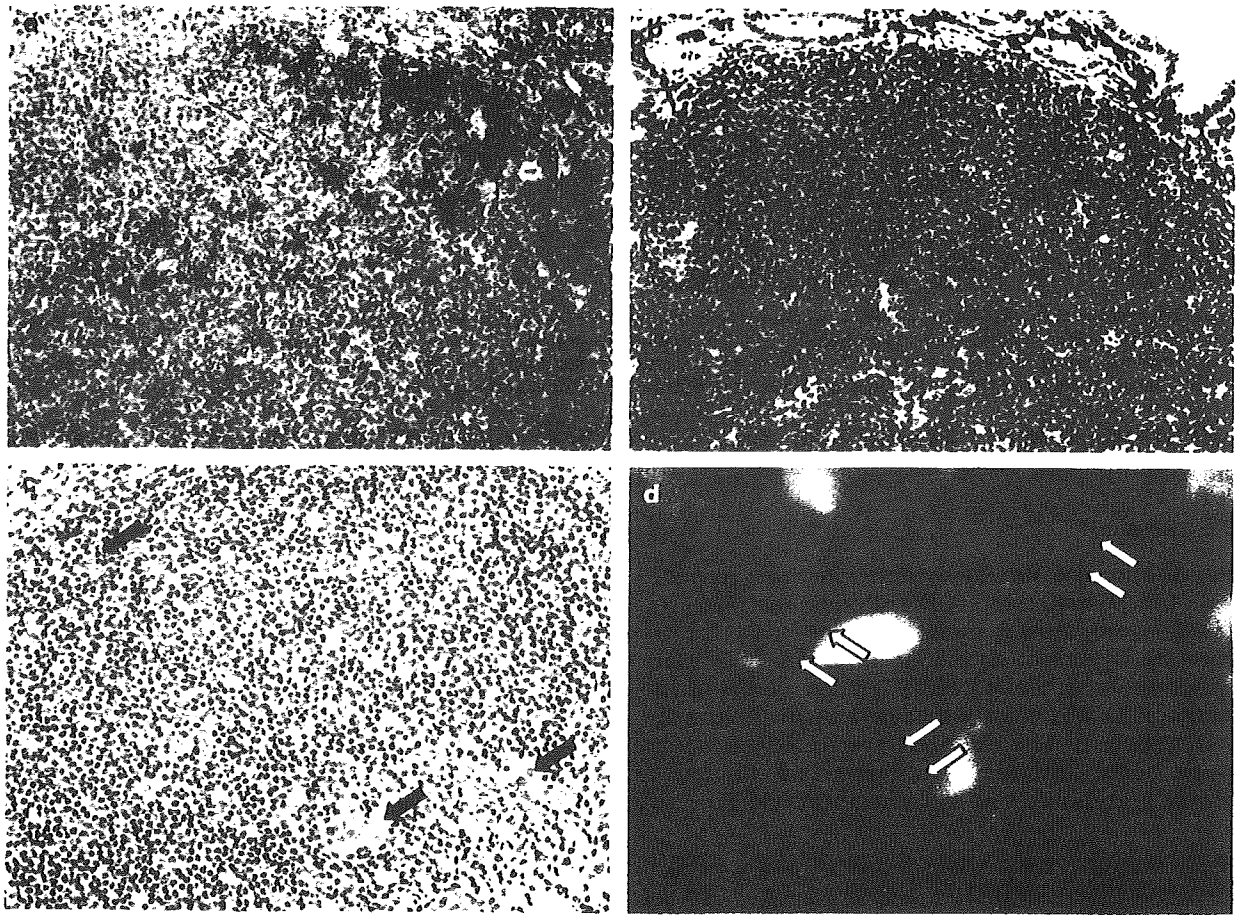

Figure 2 Expression of AID and FISH. (a) AID expression in nodal follicular lymphoma, grade 1. More than 20% of neoplastic cells are positive. (b) AID expression in duodenal follicular lymphoma (patient no. 8). Almost all neoplastic cells are negative. (c) AID expression in patient no. 2. Some positive cells are shown (arrows). (d) BCL2 split signal of patient no. 7 (arrows: The FISH DNA probes are a mixture of a Texas Red-labeled DNA probe (bcl-2-upstream) and a fluorescein-labeled DNA probe (bcl-2-downstream). Split signals of red- and green-labeled probe are seen in the lymphoma cells, indicating breaking apart of the bcl-2 gene.

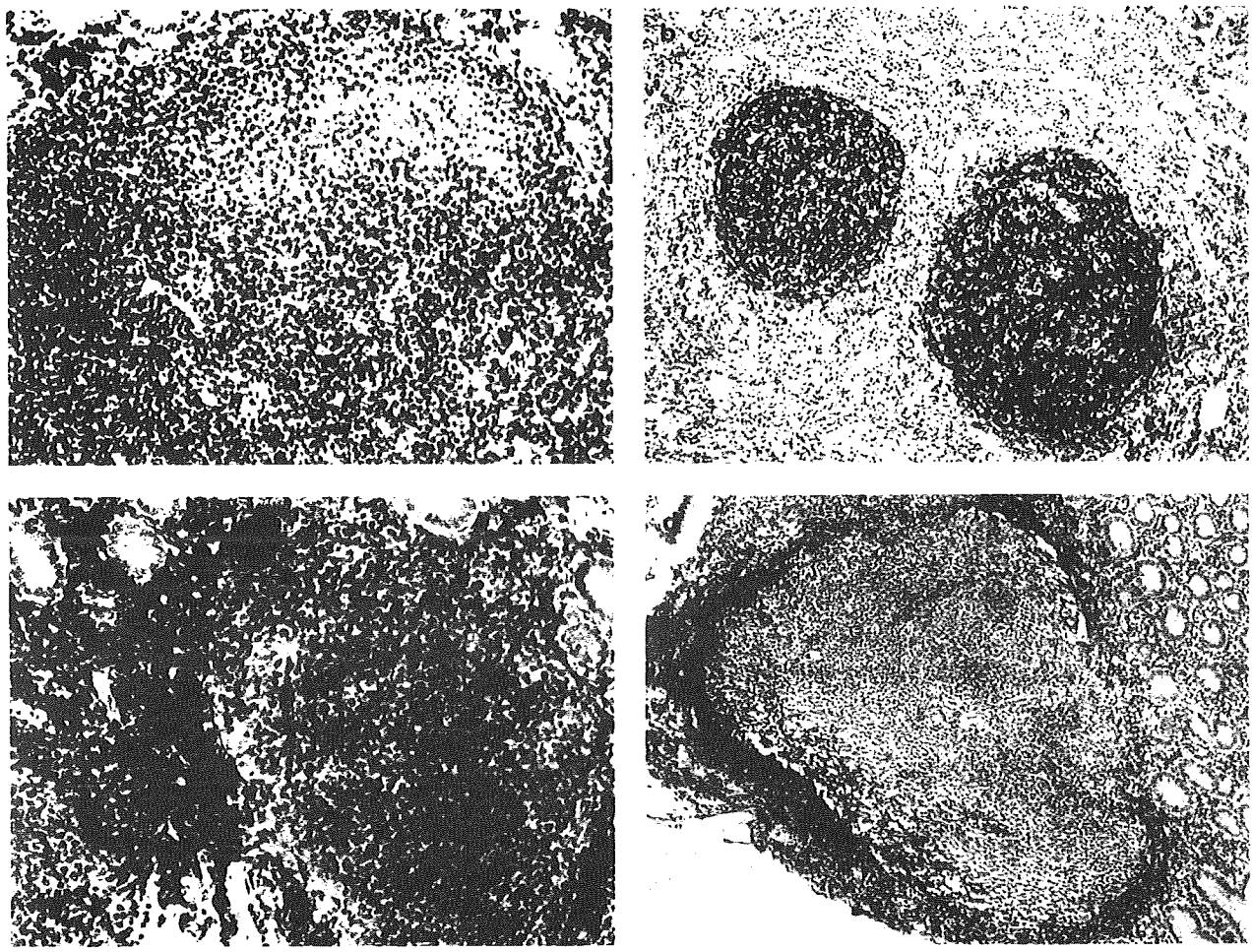

Figure 3 Expression of bcl-6, CD21 and CD23. (a) Tumor cells are positive for bcl-6 in all cases (shows patient no. 8). (b) CD21 expression in nodal follicular lymphoma, grade 1. Follicular dendritic cells are densely found in neoplastic follicles. (c) CD23 expression in duodenal follicular lymphoma (patient no. 17). Follicular dendritic cells are arranged at the periphery of the neoplastic follicle. (d) CD21 expression in other case of duodenal follicular lymphoma. Similar to CD23 expression.

identified as neoplastic follicles with disrupted, peripheral follicular dendritic cell networks.

AID Expression

In the duodenal follicular lymphomas, one patient sample (no. 9) expressed AID but other samples (16 samples) were almost completely negative for it (Figure 2). By contrast, out of 19 nodal follicular lymphomas, which histological grades were 1–2 in all cases, AID was clearly detected in 16 (Figure 2, data not shown). The difference in AID expression between nodal follicular lymphoma and duodenal follicular lymphoma was significant (P 0 0.001).

Relationship among Follicular Dendritic Cells, AID, VH and Ongoing Mutation

The VH gene usage and analysis of somatic hypermutation are shown in Table 2. We sequenced 17 cases of duodenal follicular lymphoma. The VH gene usage included 9 (53%) samples of VH3, 5 (29%) samples of VH4 and 3 (18%) samples of VH5. The distribution of mutations in CDR2 and FR3 are also shown in Table 2. Regardless of AID expression, all samples displayed somatic hypermutation; the mutation frequency was 0.7–15.6%, with an average of 9.1%. In VH5 usage cases, there tended to be a low mutation frequency (0.7–8.8%, average 3.8%).

In Table 3, most samples showed ongoing mutations, yielding an average of 19.6-fold more mutations than the expected number of additional mutations because of PCR error. Two AID-negative samples (no. 8 and no. 12) did not have any partially shared or unique mutations. Table 4 shows the nucleotide sequence of the VH gene of patient nos. 12 and 7. In no. 7, there were four partially shared mutation and five unique mutations.

Discussion

Follicular lymphoma is one of the most frequent indolent lymphomas, and most of them are of nodal

Table 2 AID expression and immunoglobulin gene analysis

Patient no.	AID expression[a]	VH usage	Number of bases analyzed	CDR2		FR3		Total		Mutation rate (%)[b]
				R	S	R	S	R	S	
1		VH3-73	153	6	1	7	2	15	3	11.8
2		VH3-48	147	3	1	1	3	4	4	5.4
3		VH3-72	153	7	0	5	4	12	4	10.5
4		VH4-34	140	5	0	7	2	12	2	10
5		VH5-a	147	0	0	1	0	1	0	0.7
6		VH3-73	141[c]	6	0	6	1	12	1	9.3
7		VH4-b	144	7	0	7	2	14	2	11.1
8		VH4-39	144	4	0	6	4	10	4	9.7
9	+	VH3-15	153	8	0	2	6	10	6	10.5
10		VH5-51	147	2	0	1	0	3	0	2
11		VH5-51	147	3	2	5	3	8	5	8.8
12		VH4-61	144	2	1	2	1	4	2	4.2
13		VH4-39	144	6	1	3	3	9	4	9
14		VH3-73	153	7	2	3	3	10	5	9.8
15		VH3-23	147	12	0	8	3	20	3	15.6
16		VH3-23	147	6	3	5	3	11	6	11.6
17		VH3-23	147	9	2	8	2	17	4	14.3

CDR, complementary determining region; FR, framework region; R, replacement mutation; S, silent mutation.
[a]Immunohistochemically positive 4 20% in tumor follicles.
[b]Dividing the total number of R and S mutations in CDR2 and FR3 by the number of bases analyzed.
[c]In patient no. 6, deletion of nine nucleotides were found.

Table 3 AID expression and ongoing mutation in duodenal follicular lymphoma

Patient no.	AID expression	Number of clones analyzed	Number of bases sequenced	Point mutations				Number of mutations expected by PCR error	Ongoing mutation
				Total	Shared	Partially shared	Unique		
1		10	153	23	16	1	6	0.31	22.6
2		10	147	10	7	1	2	0.135	22.2
3		9	153	18	15	2	1	0.24	12.5
4		10	140	18	14	0	4	0.22	18.2
5		10	147	6	1	0	5	0.081	61.7
6		10	141	21	14	4	3	0.28	25
7		8	144	21	12	4	5	0.28	32.1
8		10	144	15	15	0	0	0.2	0
9	+	10	153	18	17	0	1	0.24	4.2
10		8	147	6	3	0	3	0.081	37
11		7	147	14	13	0	1	0.19	5.3
12		9	144	6	6	0	0	0.081	0
13		9	144	19	14	0	5	0.26	19.2
14		10	153	19	15	0	4	0.26	15.4
15		10	147	31	23	8	8	0.42	38.1
16		10	147	22	19	0	3	0.3	10
17		10	147	24	21	1	2	0.32	9.4

origin. But recently, intestinal (especially duodenal) follicular lymphomas have appeared with more frequency, and these are considered variants of follicular lymphoma in the fourth WHO classification.[16] Interestingly, from our surgical files, only 5.8% (17/290 cases) of follicular lymphomas were extranodal up to 2001, whereas 30.8% (153/497 cases) were extranodal in 2002–2007. This frequency needs to be investigated hereafter, but consultation from other institution to our department has not changed in the past 10 years, hence this frequency indicates that intestinal follicular lymphomas have become popular and the incidence of finding them is increasing. The similarity of duodenal follicular lymphomas to MALT lymphomas has been reported from different points of view: expression of a4b7, which is a mucosal homing receptor,[17] VH gene deviation,[6] and IgA production suggesting that lymphoma cells derive from mucosal B cells.[18]

Table 4 Cloning assay of the VH gene of duodenal follicular lymphoma

	CDR2	FR3
Patient no. 12		
VH4-61*08	TATATCTATTACAGTGGGAGCACCAACTACAACCCCTCCCTCAAGAGT	CGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACCGCTGCCGGACACGGCCGTGTATTACTGTGCGAGA
No. 12	--------C----------G----------T------	----------C----------A----------C----------
Clone 01	--------C----------G----------T------	----------C----------A----------C----------
Clone 02	--------C----------G----------T------	----------C----------A----------C----------
Clone 03	--------C----------G----------T------	----------C----------A----------C----------
Clone 04	--------C----------G----------T------	----------C----------A----------C----------
Clone 05	--------C----------G----------T------	----------C----------A----------C----------
Clone 06	--------C----------G----------T------	----------C----------A----------C----------
Clone 07	--------C----------G----------T------	----------C----------A----------C----------
Clone 08	--------C----------G----------T------	----------C----------A----------C----------
Clone 09	--------C----------G----------T------	----------C----------A----------C----------
Patient no. 7		
VH4-b*02	AGTATCTATCATAGTGGGAGCACCTACTACAACCCGTCCCTCAAGAGT	CGAGTCACCATATCAGTAGACACGTCCAAGAGCCAATTCTCCCTGAAGCTGAGCTCTGTGACCGCCGCAGACACGGCCGTGTATATTACTGTGCGAGA
No. 7	------C--CA----------C----------GT---G------CT--	---C----------GT----------G-A---C---C----------
Clone 01	--A----C--CA----------C----------GT---------CT--	---C----------GT----------A---------C----------
Clone 02	---C--C--CA----------C----------GT---------CT--	---C----------GT----------G-A---C---C----------
Clone 03	---------CA----------C----------GT---------CT--	---C----------GT----------G-A---C---C----------
Clone 04	------C--CA----------C----------T----------CT--	---C----------GT----------G-A---C---C----------
Clone 05	------C--CA----------C----------GT---------CT--	---C----------GT----------G-A---C---C----------
Clone 06	------C--CA----------C----------GT---------CT--	---C----------GT----------G-A---C---C----------
Clone 07	------C--CA----------C----------GT---G------CT--	---C----------T----------G-A---C---C----------
Clone 08	------C--CA----------C----------GT---G------CT--	---C----------GT----------G-A---C---C----------

According to our earlier data, we analyzed eight samples for VH usage, and in this study, we analyzed the other 17 samples for VH usage. Therefore, in the 25 samples, 13 (52%) were VH3, 8 (32%) were VH4, and 4 (16%) were VH5. Phenotypically, IgM þ memory B cells resemble marginal zone B cells and are thought to be their circulating counterparts.[19] Tsuiji et al[20] reported IgM þ memory B cells more frequently express VH3 family genes than do naive B cells. Noppe et al[21] reported VH usage in nodal follicular lymphoma cases as VH3 70%, VH4 19% and VH1 11%; Bahler et al[22] reported it as VH3 67%, VH4 22% and VH1 8%. From their data, duodenal follicular lymphomas of our series showed a higher usage of VH4 and VH5 than did nodal cases. Moreover, VH4-34 and VH5-51 were detected in three samples each in our study. This must not be coincidental, and selective usage strongly suggests that some antigen-dependent mechanism is involved in tumor development, as in the case of MALT lymphoma, which develops from chronic inflammation such as Helicobactor pylori-related gastritis.

CD21 is a complement receptor expressed in B cells and follicular dendritic cells in humans.[23] In nodal follicular lymphoma, follicular dendritic cells were important to the microenvironment in which follicular lymphoma cells grow.[24] As shown in the results, although follicular dendritic cell networks were located at the periphery of neoplastic follicles, the major center part of follicular dendritic cells looked broken except for in two samples; by contrast, all nodal lymphomas showed extensively distributed follicular dendritic cells. The shape of degraded follicular dendritic cells are somewhat similar to the follicular colonization of MALT lymphomas, in which marginal zone B-cell lymphoma cells penetrate the non-neoplastic germinal centers and destroy follicular dendritic cell networks (Figure 4). Patient nos. 6 and 16 of the duodenal series showed nodal-pattern follicular dendritic cell. Interestingly, patient no. 16 was associated with markedly swollen mesenteric lymph nodes. Therefore, duodenal lesions of this case were prominent, but the primary site of this case was not clearly determined. Patient nos. 3 and 11 were clinical stage IV, but showed duodenal-pattern follicular dendritic cell. We believe that these duodenal lymphomas possibly progressed to nodal lymphomas.

AID plays key roles in class switching and somatic hypermutation of heavy chains in germinal-center B cells.[7] In an earlier report, RT-PCR showed that follicular lymphoma cells are positive for AID.[8] We examined the samples immunohistochemically, and found that nodal follicular lymphomas had 20% or more AID-positive cells, in contrast to only a few (0 1%) duodenal follicular lymphoma cells that were AID-positive. Our data indicate that most duodenal follicular lymphomas have somatic and ongoing mutations in spite of the lack of AID

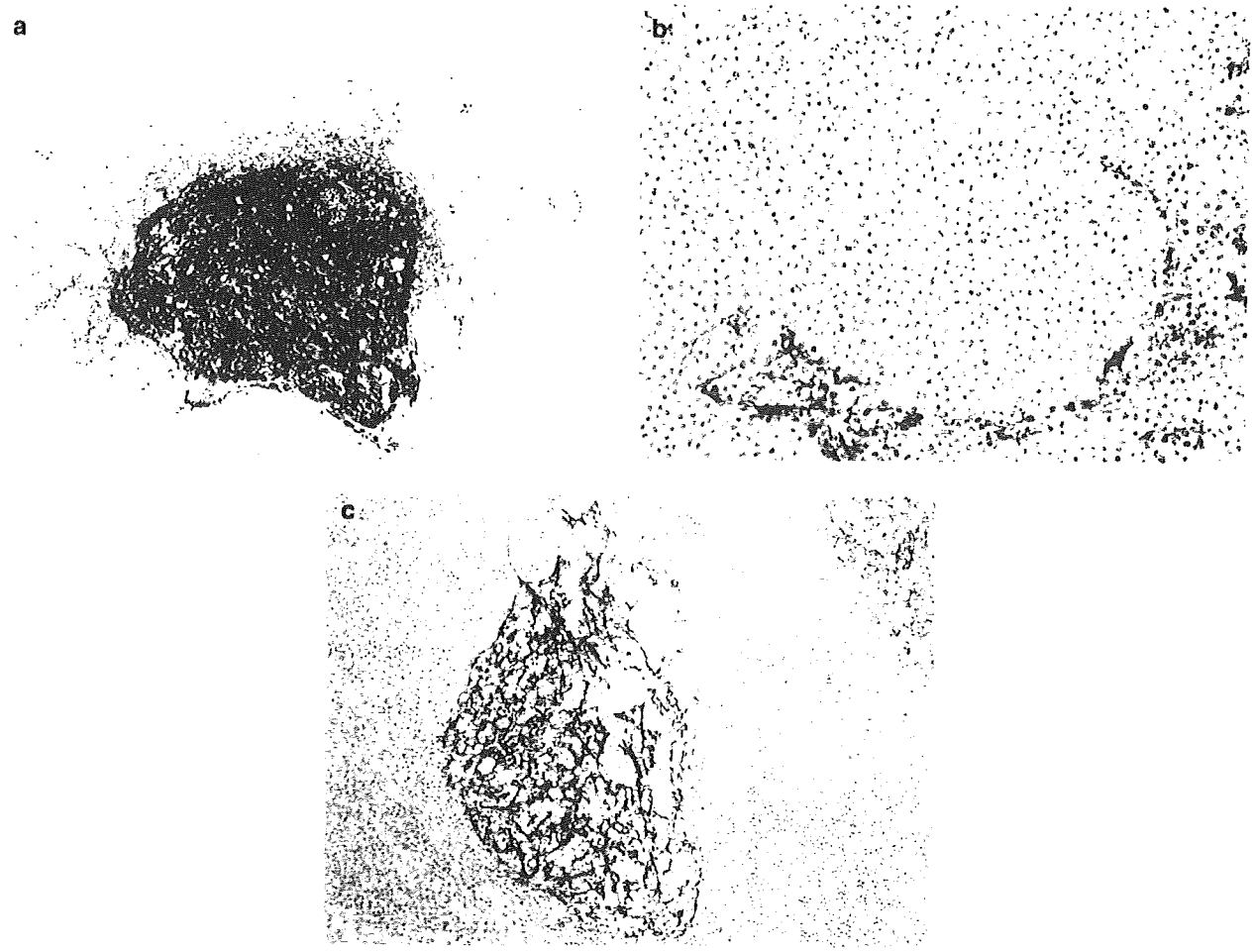

Figure 4 CD21 expression in reactive lymphoid hyperplasia of the duodenum and in MALT lymphoma of the stomach. (a) CD21 expression in reactive lymphoid hyperplasia of the duodenum. Follicular dendritic cell network are present. (b) CD21 expression in MALT lymphoma of the stomach. Follicular dendritic cell network are disrupted (follicular colonization), which is similar to those of duodenal follicular lymphomas. (c) CD21 expression in a non-neoplastic germinal center of a gastric MALT lymphoma. The follicular dendritic cell networks are well preserved.

expression. This sounds contradictory; however, Bombardieri et al[25] reported that neoplastic marginal zone-like B cells are AID-negative but have somatic and ongoing mutations.[26] On the other hand, Smit et al,[27] Pasqualucci et al[28] and Lossos et al[9] reported of lack of correlation between AID expression and intraclonal diversity of VH genes in B-cell non-Hodgkin's lymphomas. And Smit et al[27] mentioned that AID expression at follicular lymphomas were 25%, and intraclonal variation was found in the absence of AID, but they did not explained histology, immunophenotype and stage. IgVH mutations were introduced at a tumor stage when AID enzyme was still expressed. However, from our data, almost all of duodenal follicular lymphoma lacks AID in the protein expression level, and histological grade was 1–2. Moreover, all samples expressed bcl-6, which is known to be a target gene for somatic hypermutation in germinal-center B cells.[29] The mechanisms of somatic and ongoing mutation are not clear. Therefore, in duodenal follicular lymphomas, somatic and ongoing mutation may be independent of AID expression.

Conclusions from our data are as follows: duodenal follicular lymphoma cells showed somatic and ongoing mutations similar to nodal ones, as well as CD10 and bcl-2 expressions; however, they did not express AID, and the follicular dendritic cell networks essential for 'follicular pattern' lymphomas were severely disrupted, as in the follicular colonization of MALT lymphomas. We also found out that duodenal follicular lymphoma had VH family deviation marked by the presence of very restricted VH4 and VH5 segments, which strongly indicated that the lymphoma cells are derived from B cells reacting with specific antigens. These findings strongly suggested that duodenal follicular lymphoma is distinct from nodal follicular lymphoma, and has more similarity to MALT chronic

inflammation-based lymphomas; these similarities will be very important in considering therapeutic countermeasures against this disease.

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Conflict of interest

The authors declare no conflict of interest.

Authorship statement

Takata K, Sato Y, Nakamura N and Yoshino T designed and performed the research. Takata K, Okada H and Tari A prepared specimens and clinical data. Takata K, Kikuti YY, Ichimura K, Tanaka T, Morito T, Tamura M, Oka T and Kondo E analyzed the data. Takata K wrote the paper.

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LETTERS

Frequent inactivation of A20 in B-cell lymphomas

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A20 is a negative regulator of the NF-κB pathway and was initially identified as being rapidly induced after tumour-necrosis factor-α stimulation[1]. It has a pivotal role in regulation of the immune response and prevents excessive activation of NF-κB in response to a variety of external stimuli[2–7]; recent genetic studies have disclosed putative associations of polymorphic *A20* (also called *TNFAIP3*) alleles with autoimmune disease risk[8,9]. However, the involvement of A20 in the development of human cancers is unknown. Here we show, using a genome-wide analysis of genetic lesions in 238 B-cell lymphomas, that *A20* is a common genetic target in B-lineage lymphomas. A20 is frequently inactivated by somatic mutations and/or deletions in mucosa-associated tissue lymphoma (18 out of 87; 21.8%) and Hodgkin's lymphoma of nodular sclerosis histology (5 out of 15; 33.3%), and, to a lesser extent, in other B-lineage lymphomas. When re-expressed in a lymphoma-derived cell line with no functional *A20* alleles, wild-type A20, but not mutant A20, resulted in suppression of cell growth and induction of apoptosis, accompanied by downregulation of NF-κB activation. The A20-deficient cells stably generated tumours in immunodeficient mice, whereas the tumorigenicity was effectively suppressed by re-expression of A20. In A20-deficient cells, suppression of both cell growth and NF-κB activity due to re-expression of A20 depended, at least partly, on cell-surface-receptor signalling, including the tumour-necrosis factor receptor. Considering the physiological function of A20 in the negative modulation of NF-κB activation induced by multiple upstream stimuli, our findings indicate that uncontrolled signalling of NF-κB caused by loss of A20 function is involved in the pathogenesis of subsets of B-lineage lymphomas.

Malignant lymphomas of B-cell lineages are mature lymphoid neoplasms that arise from various lymphoid tissues[10,11]. To obtain a comprehensive registry of genetic lesions in B-lineage lymphomas, we performed a single nucleotide polymorphism (SNP) array analysis of 238 primary B-cell lymphoma specimens of different histologies, including 64 samples of diffuse large B-cell lymphomas (DLBCLs), 52 follicular lymphomas, 35 mantle cell lymphomas (MCLs), and 87 mucosa-associated tissue (MALT) lymphomas (Supplementary Table 1). Three Hodgkin's-lymphoma-derived cell lines were also analysed. Interrogating more than 250,000 SNP sites, this platform permitted the identification of copy number changes at an average resolution of less than 12 kilobases (kb). The use of large numbers of SNP-specific probes is a unique feature of this platform, and combined with the CNAG/AsCNAR software, enabled accurate determination of 'allele-specific' copy numbers, and thus allowed for sensitive detection of loss of heterozygosity (LOH) even without apparent copy-number reduction, in the presence of up to 70–80% normal cell contamination[12,13].

Lymphoma genomes underwent a wide range of genetic changes, including numerical chromosomal abnormalities and segmental gains and losses of chromosomal material (Supplementary Fig. 1), as well as copy-number-neutral LOH, or uniparental disomy (Supplementary Fig. 2). Each histology type had a unique genomic signature, indicating a distinctive underlying molecular pathogenesis for different histology types (Fig. 1a and Supplementary Fig. 3). On the basis of the genomic signatures, the initial pathological diagnosis of MCL was re-evaluated and corrected to DLBCL in two cases. Although most copy number changes involved large chromosomal segments, a number of regions showed focal gains and deletions, accelerating identification of their candidate gene targets. After excluding known copy number variations, we identified 46 loci showing focal gains (19 loci) or deletions (27 loci) (Supplementary Tables 2 and 3 and Supplementary Fig. 4).

Genetic lesions on the NF-κB pathway were common in B-cell lymphomas and found in approximately 40% of the cases (Supplementary Table 1), underpinning the importance of aberrant NF-κB activation in lymphomagenesis[11,14] in a genome-wide fashion. They included focal gain/amplification at the *REL* locus (16.4%) (Fig. 1b) and *TRAF6* locus (5.9%), as well as focal deletions at the *PTEN* locus (5.5%) (Supplementary Figs 1 and 4). However, the most striking finding was the common deletion at 6q23.3 involving a 143-kb segment. It exclusively contained the *A20* gene (also called *TNFAIP3*), a negative regulator of NF-κB activation[3–7,15] (Fig. 1b), which was previously reported as a candidate target of 6q23 deletions in ocular lymphoma[16]. LOH involving the *A20* locus was found in 50 cases, of which 12 showed homozygous deletions as determined by the loss of both alleles in an allele-specific copy number analysis (Fig. 1b, Table 1 and Supplementary Table 4). On the basis of this finding, we searched for possible tumour-specific mutations of *A20* by genomic DNA sequencing of entire coding exons of the gene in the same series of lymphoma samples (Supplementary Fig. 5). Because two out of the three Hodgkin's-lymphoma-derived cell lines had biallelic *A20* deletions/mutations (Supplementary Fig. 6), 24 primary samples from Hodgkin's lymphoma were also analysed for mutations, where

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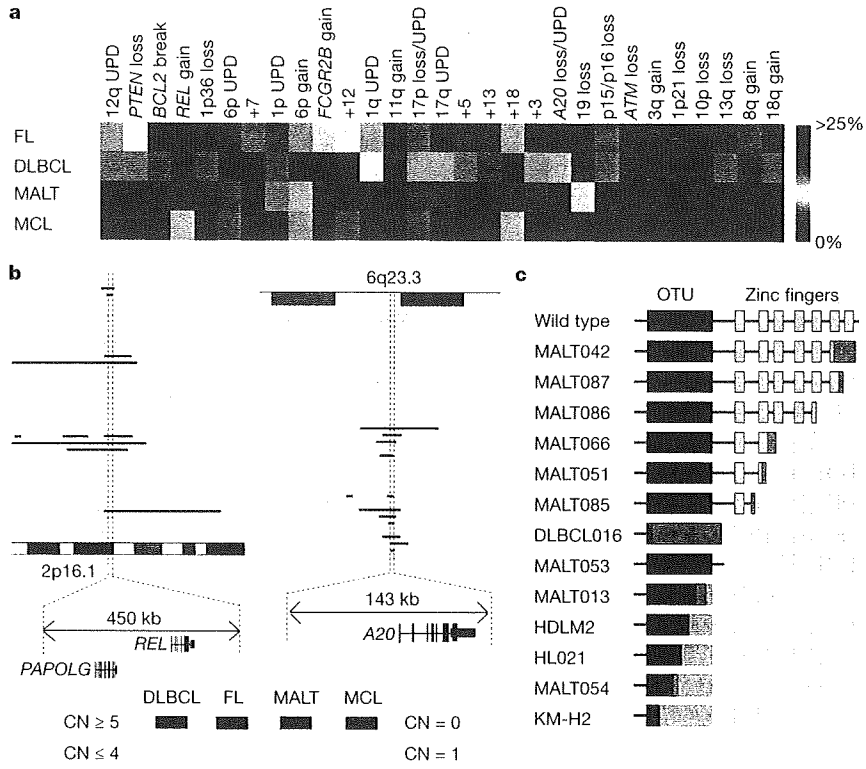

Figure 1 | Genomic signatures of different B-cell lymphomas and common genetic lesions at 2p16-15 and 6q23.3 involving NF-κB pathway genes. **a**, Twenty-nine genetic lesions were found in more than 10% in at least one histology and used for clustering four distinct histology types of B-lineage lymphomas. The frequency of each genetic lesion in each histology type is colour-coded. FL, follicular lymphoma; UPD, uniparental disomy. **b**, Recurrent genetic changes are depicted based on CNAG output of the SNP array analysis of 238 B-lineage lymphoma samples, which include gains at the *REL* locus on 2p16-15 (left panel) and the *A20* locus on 6q23.3 (right panel). Regions showing copy number gain or loss are indicated by horizontal lines. Four histology types are indicated by different colours, where high-grade amplifications and homozygous deletions are shown by darker shades to discriminate from simple gains (copy number ≤4) and losses (copy number = 1) (lighter shades). **c**, Point mutations and small nucleotide insertions and deletions in the *A20* (*TNFAIP3*) gene caused premature truncation of A20 in most cases. Altered amino acids caused by frame shifts are indicated by green bars.

genomic DNA was extracted from 150 microdissected CD30-positive tumour cells (Reed–Sternberg cells) for each sample. *A20* mutations were found in 18 out of 265 lymphoma samples (6.8%) (Table 1), among which 13 mutations, including nonsense mutations (3 cases), frame-shift insertions/deletions (9 cases), and a splicing donor site mutation (1 case) were thought to result in premature termination of translation (Fig. 1c). Four missense mutations and one intronic mutation were identified in five microdissected Hodgkin's lymphoma samples. They were not found in the surrounding normal tissues, and thus, were considered as tumour-specific somatic changes.

In total, biallelic *A20* lesions were found in 31 out of 265 lymphoma samples including 3 Hodgkin's lymphoma cell lines. Quantitative analysis of SNP array data suggested that these *A20* lesions were present in the major tumour fraction within the samples (Supplementary Fig. 7). Inactivation of *A20* was most frequent in MALT lymphoma (18 out of 87) and Hodgkin's lymphoma (7 out of 27), although it was also found in DLBCL (5 out of 64) and follicular lymphoma (1 out of 52) at lower frequencies. In MALT lymphoma, biallelic *A20* lesions were confirmed in 18 out of 24 cases (75.0%) with LOH involving the 6q23.3 segment (Supplementary Fig. 8). Considering the limitation in detecting very small homozygous deletions, *A20* was thought to be the target of 6q23 LOH in MALT lymphoma. On the other hand, the 6q23 LOHs in other histology types tended to be extended into more centromeric regions and less frequently accompanied biallelic *A20* lesions (Supplementary Fig. 8 and Supplementary Table 4), indicating that they might be more heterogeneous with regard to their gene targets. We were unable to analyse Hodgkin's lymphoma samples using SNP arrays owing to insufficient genomic DNA obtained from microdissected samples, and were likely to underestimate the frequency of *A20* inactivation in Hodgkin's lymphoma because we might fail to detect a substantial proportion of cases with homozygous deletions, which explained 50% (12 out of 24) of *A20* inactivation in other histology types. *A20* mutations in Hodgkin's lymphoma were exclusively found in nodular sclerosis classical Hodgkin's lymphoma (5 out of 15) but not in other histology types (0 out of 9), although the possible association requires further confirmation in additional cases.

A20 is a key regulator of NF-κB signalling, negatively modulating NF-κB activation through a wide variety of cell surface receptors and viral proteins, including tumour-necrosis factor (TNF) receptors, toll-like receptors, CD40, as well as Epstein–Barr-virus-associated LMP1 protein[2,5,17,18]. To investigate the role of A20 inactivation in lymphomagenesis, we re-expressed wild-type A20 under a *Tet*-inducible promoter in a lymphoma-derived cell line (KM-H2) that had no functional *A20* alleles (Supplementary Fig. 6), and examined the effect of A20 re-expression on cell proliferation, survival and downstream NF-κB signalling pathways. As shown in Fig. 2a–c and Supplementary Fig. 9, re-expression of wild-type A20 resulted in the suppression of cell proliferation and enhanced apoptosis, and in the concomitant accumulation of IκBβ and IκBε, and downregulation of NF-κB activity. In contrast, re-expression of two lymphoma-derived A20 mutants, $A20^{532Stop}$ or $A20^{750Stop}$, failed to show growth suppression, induction of apoptosis, accumulation of IκBβ and IκBε or downregulation of

Table 1 | Inactivation of A20 in B-lineage lymphomas

Histology	Tissue	Sample	Allele	Uniparental disomy	Exon	Mutation	Biallelic inactivation
DLBCL							5 out of 64 (7.8%)
	Lymph node	DLBCL008	−/−	No	–	–	
	Lymph node	DLBCL016	+/−	No	Ex2	329insA	
	Lymph node	DLBCL022	−/−	No	–	–	
	Lymph node	DLBCL028	−/−	Yes	–	–	
	Lymph node	MCL008*	−/−	Yes	–	–	
Follicular lymphoma							1 out of 52 (1.9%)
	Lymph node	FL024	−/−	No	–	–	
MCL							0 out of 35 (0%)
MALT							18 out of 87 (21.8%)
Stomach							3 out of 23 (13.0%)
	Gastric mucosa	MALT013	+/+	Yes	Ex5	705insG	
	Gastric mucosa	MALT014	+/+	Yes	Ex3	Ex3 donor site>A	
	Gastric mucosa	MALT036	+/−	No	Ex7	delintron6-Ex7†	
Eye							13 out of 43 (30.2%)
	Ocular adnexa	MALT008	−/−	No	–	–	
	Ocular adnexa	MALT017	−/−	No	–	–	
	Ocular adnexa	MALT051	+/−	No	Ex7	1943delTG	
	Ocular adnexa	MALT053	+/+	Yes	Ex6	1016G>A(stop)	
	Ocular adnexa	MALT054	+/−	No	Ex3	502delTC	
	Ocular adnexa	MALT055	−/−	No	–	–	
	Ocular adnexa	MALT066	+/−	No	Ex7	1581insA	
	Ocular adnexa	MALT067	−/−	No	–	–	
	Ocular adnexa	MALT082	−/−	Yes	–	–	
	Ocular adnexa	MALT084	−/−	Yes	–	–	
	Ocular adnexa	MALT085	+/+	Yes	Ex7	1435insG	
	Ocular adnexa	MALT086	+/+	Yes	Ex6	878C>T(stop)	
	Ocular adnexa	MALT087	+/+	Yes	Ex9	2304delGG	
Lung							2 out of 12 (16.7%)
	Lung	MALT042	−/−	No	–	–	
	Lung	MALT047	+/+	Yes	Ex9	2281insT	
Other‡							0 out of 9 (0%)
Hodgkin's lymphoma							7 out of 27 (26.0%)
NSHL	Lymph node	HL10	ND	ND	Ex7	1777G>A(V571I)	
NSHL	Lymph node	HL12	ND	ND	Ex7	1156A>G(R364G)	
NSHL	Lymph node	HL21	ND	ND	Ex4	569G>A(stop)	
NSHL	Lymph node	HL24	ND	ND	Ex3	1487C>A(T474N)	
NSHL	Lymph node	HL23	ND	ND	–	Intron 3§	
	Cell line	KM-H2	−/−	No	–		
	Cell line	HDLM2	+/−	No	Ex4	616ins29bp‖	
Total							31 out of 265 (11.7%)

DLBCL, diffuse large B-cell lymphoma; MALT, MALT lymphoma; MCL, mantle cell lymphoma; ND, not determined because SNP array analysis was not performed; NSHL, nodular sclerosis classical Hodgkin's lymphoma.
* Diagnosis was changed based on the genomic data, which was confirmed by re-examination of pathology.
† Deletion including the boundary of intron 6 and exon 7 (see also Supplementary Fig. 5b).
‡ Including 1 parotid gland, 1 salivary gland, 2 colon and 5 thyroid cases.
§ Insertion of CTC at −19 bases from the beginning of exon 3.
‖ Insertion of TGGCTTCCACAGACACACCCATGGCCCGA.

NF-κB activity (Fig. 2a–c), indicating that these were actually loss-of-function mutations. To investigate the role of A20 inactivation in lymphomagenesis *in vivo*, *A20*- and mock-transduced KM-H2 cells were transplanted in NOD/SCID/γ_c^{null} (NOG) mice[19], and their tumour formation status was examined for 5 weeks with or without induction of wild-type A20 by tetracycline administration. As shown in Fig. 2d, mock-transduced cells developed tumours at the injected sites, whereas the *Tet*-inducible *A20*-transduced cells generated tumours only in the absence of A20 induction (Supplementary Table 5), further supporting the tumour suppressor role of A20 in lymphoma development.

Given the mode of negative regulation of NF-κB signalling, we next investigated the origins of NF-κB activity that was deregulated by A20 loss in KM-H2 cells. The conditioned medium prepared from a 48-h serum-free KM-H2 culture had increased NF-κB upregulatory activity compared with fresh serum-free medium, which was inhibited by re-expression of A20 (Fig. 3a). KM-H2 cells secreted two known ligands for TNF receptor—TNF-α and lymphotoxin-α (Supplementary Fig. 10)[20]—and adding neutralizing antibodies against these cytokines into cultures significantly suppressed their cell growth and NF-κB activity without affecting the levels of their overall suppression after A20 induction (Fig. 3b, d). In addition, recombinant TNF-α and/or lymphotoxin-α added to fresh serum-free medium promoted cell growth and NF-κB activation in KM-H2 culture, which were again suppressed by re-expression of A20 (Fig. 3c, e). Although our data in Fig. 3 also show the presence of factors other than TNF-α and lymphotoxin-α in the KM-H2-conditioned medium—as well as some intrinsic pathways in the cell (Fig. 3a)—that were responsible for the A20-dependent NF-κB activation, these results indicate that both cell growth and NF-κB activity that were upregulated by A20 inactivation depend at least partly on the upstream stimuli that evoked the NF-κB-activating signals.

Aberrant activation of the NF-κB pathway is a hallmark of several subtypes of B-lineage lymphomas, including Hodgkin's lymphoma, MALT lymphoma, and a subset of DLBCL, as well as other lymphoid neoplasms[11,14], where a number of genetic alterations of NF-κB signalling pathway genes[21–25], as well as some viral proteins[26,27], have been implicated in the aberrant activation of the NF-κB pathway[14]. Thus, frequent inactivation of A20 in Hodgkin's lymphoma and MALT and other lymphomas provides a novel insight into the molecular pathogenesis of these subtypes of B-lineage lymphomas through deregulated NF-κB activation. Because A20 provides a

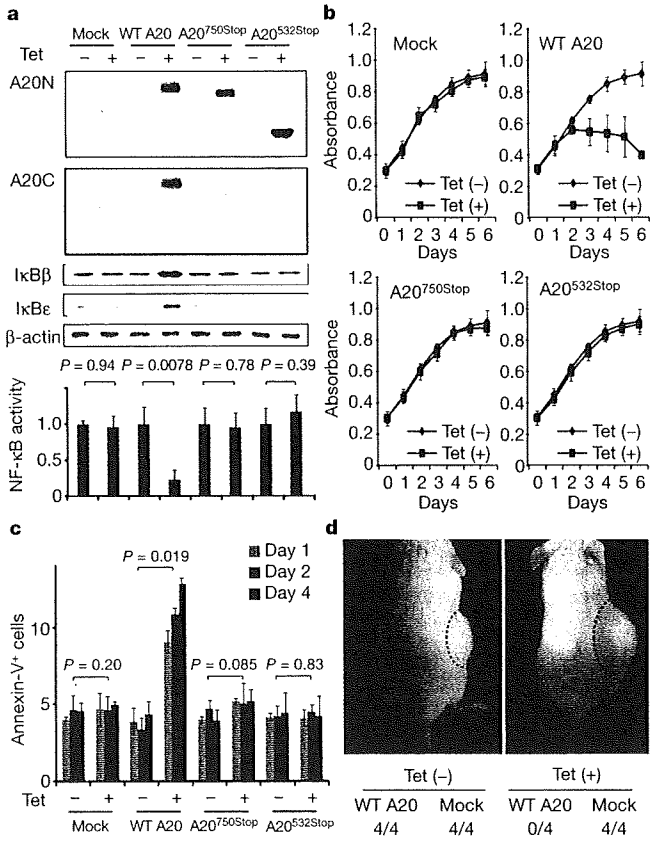

Figure 2 | Effects of wild-type and mutant A20 re-expressed in a lymphoma cell line that lacks the normal *A20* gene. a, Western blot analyses of wild-type (WT) and mutant ($A20^{532Stop}$ and $A20^{750Stop}$) A20, as well as IκBβ and IκBε, in KM-H2 cells, in the presence or absence of tetracycline treatment (top panels). A20N and A20C are polyclonal antisera raised against N-terminal and C-terminal A20 peptides, respectively. β-actin blots are provided as a control. NF-κB activities are expressed as mean absorbance ± s.d. ($n = 6$) in luciferase assays (bottom panel). **b**, Proliferation of KM-H2 cells stably transduced with plasmids for mock and *Tet*-inducible wild-type A20, $A20^{532Stop}$ and $A20^{750Stop}$ was measured using a cell counting kit in the presence (red lines) or absence (blue lines) of tetracycline. Mean absorbance ± s.d. ($n = 5$) is plotted. **c**, The fractions of Annexin-V-positive KM-H2 cells transduced with various *Tet*-inducible A20 constructs were measured by flow cytometry after tetracycline treatment and the mean values (±s.d., $n = 3$) are plotted. **d**, *In vivo* tumorigenicity was assayed by inoculating 7×10^6 KM-H2 cells transduced with mock or *Tet*-inducible wild-type A20 in NOG mice, with (right panel) or without (left panel) tetracycline administration.

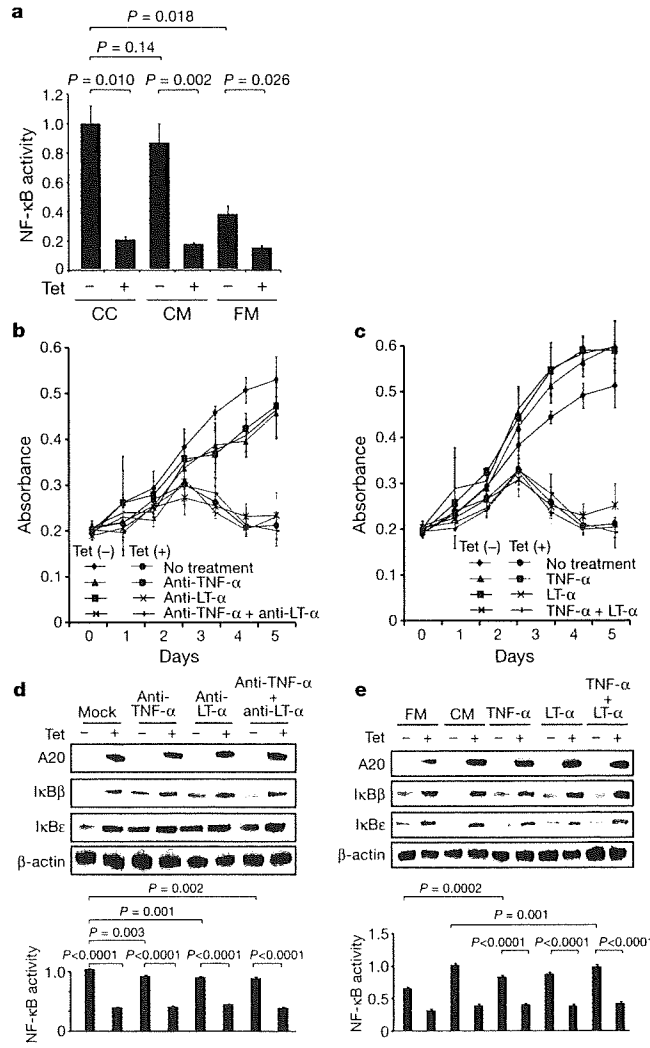

Figure 3 | Tumour suppressor role of A20 under external stimuli. a, NF-κB activity in KM-H2 cells was measured 30 min after cells were inoculated into fresh medium (FM) or KM-H2-conditioned medium (CM) obtained from the 48-h culture of KM-H2, and was compared with the activity after 48 h continuous culture of KM-H2 (CC). A20 was induced 12 h before inoculation in Tet (+) groups. **b**, **c**, Effects of neutralizing antibodies against TNF-α and lymphotoxin-α (LTα) (**b**) and of recombinant TNF-α and LT-α added to the culture (**c**) on cell growth were evaluated in the presence (Tet (+)) or absence (Tet (−)) of A20 induction. Cell numbers were measured using a cell counting kit and are plotted as their mean absorbance ± s.d. ($n = 6$). **d**, **e**, Effects of the neutralizing antibodies (**d**) and the recombinant cytokines added to the culture (**e**) on NF-κB activities and the levels of IκBβ and IκBε after 48 h culture with (Tet (+)) or without (Tet (−)) tetracycline treatment. NF-κB activities are expressed as mean absorbance ± s.d. ($n = 6$) in luciferase assays.

negative feedback mechanism in the regulation of NF-κB signalling pathways upon a variety of stimuli, aberrant activation of NF-κB will be a logical consequence of A20 inactivation. However, there is also the possibility that the aberrant NF-κB activity of A20-inactivated lymphoma cells is derived from upstream stimuli, which may be from the cellular environment. In this context, it is intriguing that MALT lymphoma usually arises at the site of chronic inflammation caused by infection or autoimmune disorders and may show spontaneous regression after eradication of infectious organisms[28]; furthermore, Hodgkin's lymphoma frequently shows deregulated cytokine production from Reed–Sternberg cells and/or surrounding reactive cells[29]. Detailed characterization of the NF-κB pathway regulated by A20 in both normal and neoplastic B lymphocytes will promote our understanding of the precise roles of A20 inactivation in the pathogenesis of these lymphoma types. Our finding underscores the importance of genome-wide approaches in the identification of genetic targets in human cancers.

METHODS SUMMARY

Genomic DNA from 238 patients with non-Hodgkin's lymphoma and three Hodgkin's-lymphoma-derived cell lines was analysed using GeneChip SNP genotyping microarrays (Affymetrix). This study was approved by the ethics boards of the University of Tokyo, National Cancer Institute Hospital, Okayama University, and the Cancer Institute of the Japanese Foundation of Cancer Research. After appropriate normalization of mean array intensities, signal ratios between tumours and anonymous normal references were calculated in an allele-specific manner, and allele-specific copy numbers were inferred from the observed signal ratios based on the hidden Markov model using CNAG/AsCNAR software (http://www.genome.umin.jp). *A20* mutations were examined by directly sequencing genomic DNA using a set of primers (Supplementary Table 6). Full-length cDNAs of wild-type and mutant *A20* were introduced into a

lentivirus vector, pLenti4/TO/V5-DEST (Invitrogen), with a *Tet*-inducible promoter. Viral stocks were prepared by transfecting the vector plasmids into 293FT cells (Invitrogen) using the calcium phosphate method and then infected to the KM-H2 cell line. Proliferation of KM-H2 cells was measured using a Cell Counting Kit (Dojindo). Western blot analyses and luciferase assays were performed as previously described. NF-κB activity was measured by luciferase assays in KM-H2 cells stably transduced with a reporter plasmid having an NF-κB response element, pGL4.32 (Promega). Apoptosis of KM-H2 upon A20 induction was evaluated by counting Annexin-V-positive cells by flow cytometry. For *in vivo* tumorigenicity assays, 7×10^6 KM-H2 cells were transduced with the *Tet*-inducible *A20* gene and those with a mock vector were inoculated on the contralateral sides in eight NOG mice[19] and examined for their tumour formation with ($n = 4$) or without ($n = 4$) tetracycline administration. Full copy number data of the 238 lymphoma samples will be accessible from the Gene Expression Omnibus (GEO, http://ncbi.nlm.nih.gov/geo/) with the accession number GSE12906.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions M.Ka., K.N. and M.S. performed microarray experiments and subsequent data analyses. M.Ka., Y.C., K.Ta., J.T., J.N., M.I., A.T. and Y.K. performed mutation analysis of *A20*. M.Ka., S.Mu., M.S., Y.C. and Y.Ak. conducted functional assays of mutant A20. Y.S., K.Ta., Y.As., H.M., M.Ku., S.Mo., S.C., Y.K., K.To. and Y.I. prepared tumour specimens. I.K., K.O., A.N., H.N. and T.N. conducted *in vivo* tumorigenicity experiments in NOG/SCID mice. T.I., Y.H., T.Y., Y.K. and S.O. designed overall studies, and S.O. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Author Information The copy number data as well as the raw microarray data will be accessible from the GEO (http://ncbi.nlm.nih.gov/geo/) with the accession number GSE12906. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.O. (sogawa-tky@umin.ac.jp) or Y.K. (ykkobaya@ncc.go.jp).