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Original Article

Frequent *CDKN2B/P15* and *DAPK1* methylation in duodenal follicular lymphoma is related to duodenal reactive lymphoid hyperplasia

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Duodenal type follicular lymphoma (DFL), a rare entity of follicular lymphoma (FL), is clinically indolent and is characterized by a low histological grade compared with nodal follicular lymphoma (NFL). Our previous reports revealed that DFL shares characteristics of both NFL and mucosa-associated lymphoid tissue (MALT) lymphoma in terms of clinical and biological aspects, suggesting its pathogenesis may involve antigenic stimulation. In contrast to NFL, the genomic methylation status of DFL is still challenging. Here, we determined the methylation profiles of DNAs from patients with DFL (n = 12), NFL (n = 10), duodenal reactive lymphoid hyperplasia (D-RLH) (n = 7), nodal reactive lymphoid hyperplasia (N-RLH) (n = 5), and duodenal samples from normal subjects (NDU) (n = 5) using methylation specific PCR of targets previously identified in MALT lymphoma (*CDKN2B/P15*, *CDKN2A/P16*, *CDKN2C/P18*, *MGMT*, *hMLH-1*, *TP73*, *DAPK*, *HCAD*). *DAPK1* was frequently methylated in DFL (9/12; 75%), NFL (9/10; 90%), and D-RLH (5/7; 71%). *CDKN2B/P15* sequences were methylated in six DFL samples and in only one NFL sample. Immunohistochemical analysis showed that p15 expression inversely correlated with methylation status. Genes encoding other cyclin-dependent kinase inhibitors (*CDKN2A/P16*, *CDKN2C/P18*) were not methylated in DFL samples. Methylation of the genes of interest was not detected in DNAs from D-RLH, except for *DAPK1*, and the difference in the extent of methylation between NDU and D-RLH was statistically significant ($P = 0.013$). Our results suggest that D-RLH serves as a reservoir for the development of DFL and that methylation of *CDKN2B/P15* plays an important role in this process.

Keywords: *CDKN2B/P15*, *DAPK1*, methylation profile, duodenal reactive hyperplasia

INTRODUCTION

Duodenal type follicular lymphoma (DFL) is clinically very indolent, is characterized by lower histological grades, and is dominated by uniform intermediate small cleaved cells. We previously reported that DFL shares characteristics of mucosa-associated lymphoid tissue (MALT) lymphoma in terms its follicular dendritic cell meshwork pattern, expression levels of activation-induced cytidine deaminase, memory B-cell characteristics, and prognosis.[1–4] On the other hand, DFL shares the same gene mutation profile as nodal follicular lymphoma (NFL), such as *CREBBP*, *TNFRSF14*, *KMT2D*, and *EZH2*.[5] We have also reported the deviation of immunoglobulin usage in DFL.[2] Previous reports suggested that the VH4–34 regions of the immunoglobulin heavy chain gene are associated with autoimmune disease and are present in immunoglobulins produced by most hairly cell leukemia.[6,7] Based on the enrichment of a chronic inflammation signature by gene expression profiling and deviation of immunoglobulin VH4 and VH5 usage,[4,5] we hypothesized that pathogenesis of DFL may involve some epigenetic regulation by antigen stimulation.

Epigenetic changes, mainly DNA hypermethylation and histone modifications, contribute to the malignant phenotype.[8] In several types of cancers, including malignant lymphoma, the 5′-cytidine–phosphate–guanosine (CpG) islands of certain genes are aberrantly hypermethylated, resulting in repression of their transcription.[9,10] Here we focused on the contribution of epigenetic abnormalities to disease development and progression using the methylation status of eight gene which were involved in lymphomagenesis, especially in MALT lymphoma.[11] Our results show frequent loss of p15 expression in DFL compared with that in NFL, which is frequently associated with the hypermethylation of the promoter

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region of *CDKN2B/P15*. Lymphoid follicles are not usually present in normal duodenal tissues (NDUs), although some reactive lymphoid hyperplasia with duodenal inflammation can be observed. We therefore sought to examine the relationship between DFL and duodenal reactive lymphoid hyperplasia (D-RLH) in greater detail.

MATERIALS AND METHODS

Patient selection

Patient samples were examined at Okayama University School of Medicine from 2000 to 2011, and we obtained 12, 10, and seven specimens from patients with DFL, NFL, and D-RLH, respectively. All 12 DFL samples were biopsied from the duodenum. Control specimens were obtained from five patients with nodal reactive lymphoid hyperplasia (N-RLH) and five normal subjects (NDU). Three of the 12 DFL samples (patients 10–12) and three of the 10 NFL samples (patients 8–10) were fresh frozen, and the remaining samples, including N-RLH and NDU, were fixed in 10% formalin and embedded in paraffin. We also examined three cell lines derived from FLs (FL-18, FL-218, and FL-318), which were kindly provided by Dr. Hitoshi Ohno (Kyoto University). This study was reviewed and approved by the Okayama University Ethics Board (approval No. 283) in accordance with the Declaration of Helsinki. We obtained written informed consent from the patients or informed consent was waived for the samples used in this retrospective study.

DNA extraction, sodium bisulfite modification, and methylation-specific polymerase chain reaction (MSP)

DNA was extracted from paraffin-embedded tissue using a QIAmp DNA Micro Kit (Qiagen Inc, Valencia, CA, USA). Sodium bisulfite modification of DNA was performed according to a published method.[11] In brief, 1-μg aliquots of genomic DNA were denatured with NaOH and reacted with sodium bisulfite, which converts all unmethylated cytosines to uracils while leaving methylated cytosines unaltered. Modified DNA was purified, desulfonated with NaOH, precipitated with ethanol, and resuspended in TE buffer. MSP was performed to examine the methylation status of eight genes known to be involved in lymphomagenesis and that encode proteins with the following functions: cell-cycle regulators (*CDKN2B/P15*, *CDKN2A/P16*, and *CDKN2C/P18*), mismatch (*MGMT*) and DNA repair (*hMLH-1*) enzymes, a tumor suppressor (*TP73*), an inducer of apoptosis (*DAPK1*), and a cell adhesion (*HCAD*) molecule. CpGenome™ Universal methylated and unmethylated DNA (Chemicon International Inc., Temecula, CA) were used as controls. PCR products were electrophoresed through a 2% agarose gel, stained with ethidium bromide, and visualized with a UV illuminator.

Immunohistochemical analysis

Immunohistochemical staining was performed on sections from 10% buffered formalin-fixed and paraffin-embedded tissues using heat-induced epitope retrieval or trypsin-induced retrieval, an avidin–biotin complex method, and an automated immunostainer (BOND-MAX™ Autostainer, Leica), as previously described.[12] The antibody panel used to assess these cases was as follows (clone, dilution): CD20 (L26, 1:200), CD3 (PS-1, 1:50), CD10 (56C6, 1:50), BCL2 (3.1, 1:200) (Novocastra, Newcastle-upon-Tyne, UK); BCL6 (D-8, 1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); cyclin D1 (SP4, ready to use) (Nichirei, Japan); and p15 (15P06, 1:100) (Lifespan Biosciences, Inc, Seattle, WA). Samples were considered positive when 50% or more of cells were stained.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) analysis to detect t(14;18)(q32;q21)/*IGH-BCL2* translocations was performed using an LSI BCL2 FISH DNA fusion signal probe (Abbott Molecular Inc., Wiesbaden, Germany) according to the manufacturer's instructions. We performed FISH directly on paraffin-embedded tissue sections and detected hybridization signals, as previously described.[13]

Statistical analysis

Statistical analysis was performed using the two-tailed Fisher's exact test. SPSS software (version 14.0; SPSS Inc, Chicago, IL) was used to perform the analysis.

RESULTS

Patient and tumor characteristics

Table 1 shows the characteristics of patients with DFL, NFL, and D-RLH. The median age of each group was as follows: DFL, 68 years (54–84 years); NFL, 58.5 years (44–73 years); and D-RLH, 70 years (50–83 years). All patients with DFL were diagnosed as stage I or II_1 (stage I; six patients, stage II_1; six patients) according to the Lugano classification.[14] Tissues of all patients with DFL or NFL were histological Grade 1–2, and the macroscopic appearances of DFL and D-RLH tissues are shown in Table 1. On endoscopic examination, the majority of gastrointestinal FLs present with multiple small polyps or whitish nodules. Although this macroscopic appearance can be categorized as the lymphomatous polyposis type, endoscopists now prefer to use the term "multiple nodules".[12,13] All DFL patients studied here presented with multiple nodules on endoscopic examination. Four of seven patients with D-RLH presented with multiple nodules (Figure 1A), whereas a submucosal tumor, an Isp polyp, and rough mucosa was observed in three patients, respectively. Figure 1B, C shows the microscopic appearance of tissues derived from patients with D-RLH (patient D-RLH 4). In the proper mucosal area, all lymphoid follicles were positive for CD10 but negative for BCL2 expression.

DNA methylation status of DFL, NFL, D-RLH, and N-RLH

We determined whether aberrant methylation of DFL and

Table 1. Summary of clinical characteristics and IHC in DFL, NFL, and D-RLH

Case	Age/sex	Stage	Grade	t(14;18)	Macroscopic appearance	CD10	BCL2
DFL 1	58/F	II1	1	+	multiple nodules	+	+
DFL 2	78/F	II1	1	+	multiple nodules	+	+
DFL 3	84/M	I	1	N.D.	multiple nodules	+	+
DFL 4	70/M	I	1	+	multiple nodules	+	+
DFL 5	66/F	II1	1	+	multiple nodules	+	+
DFL 6	70/F	II1	1	+	multiple nodules	+	+
DFL 7	72/M	I	1	N.D.	multiple nodules	+	+
DFL 8	54/F	II1	1	+	multiple nodules	+	+
DFL 9	56/F	II1	1	+	multiple nodules	+	+
DFL 10	60/M	I	1	+	multiple nodules	+	+
DFL 11	56/F	I	1	+	multiple nodules	+	+
DFL 12	71/F	I	1	+	multiple nodules	+	+
NFL 1	46/M	IV	1	+		+	+
NFL 2	54/M	II	2	+		+	+
NFL 3	63/F	II	1	+		+	+
NFL 4	73/F	III	1	+		+	+
NFL 5	59/F	II	1	+		+	+
NFL 6	44/M	III	1	+		+	+
NFL 7	53/M	III	1	+		+	+
NFL 8	58/F	II	1	+		+	+
NFL 9	63/M	IV	1	+		+	+
NFL 10	66/M	III	1	+		+	+
D-RLH 1	63/M			-	submucosal tumor	+	-
D-RLH 2	70/F			-	polyp (Isp)	+	-
D-RLH 3	66/M			-	rough mucosa	+	-
D-RLH 4	80/M			-	multiple nodules	+	-
D-RLH 5	83/F			-	multiple nodules	+	-
D-RLH 6	50/M			-	multiple nodules	+	-
D-RLH 7	80/M			-	multiple nodules	+	-

DFL; duodenal follicular lymphoma, NFL; nodal follicular lymphoma
D-RLH; duodenal reactive lymphoid hyperplasia, IHC: immunohistochemistry

NFL DNAs could be detected using MSP. CpG island methylation was analyzed in the promoter regions of eight tumor-related genes (*CDKN2B/P15*, *CDKN2A/P16*, *CDKN2C/P18*, *MGMT*, *h-MLH1*, *TP73*, *DAPK1*, and *HCAD*). The MSP results for *DAPK1* and *CDKN2B/P15* are shown in Figure 2A, B, and their methylation profiles are shown in Table 2. DNA methylation was categorized into five groups (−, ±, +, ++, and +++) according to a previously published method.[15] Only the categories from "+" to "+++" were regarded as methylation-positive (red indicates positive results). *DAPK1* methylation was detected in DFLs (9/12, 75%), NFLs (9/10, 90%), D-RLH (5/7, 71%), and N-RLH (1/5, 20%), but not in NDUs. There was a statistically significant difference between the methylation status of *DAPK1* in D-RLH and NDU DNAs (P = 0.013). *CDKN2B/P15* methylation status differed between DFL and NFL. That is to say, six of the 12 DFL and one of the 10 NFL samples were methylated, which represented a statistically significant difference between these two groups (P = 0.043). Genes encoding other cyclin-dependent kinase inhibitors (*CDKN2A/P16* and *CDKN2C/P18*) were not evidently methylated in these samples. *HCAD* and *MGMT* methylation was detected in DFL (5/12 and 2/12) and NFL (2/10 and 1/10). However, the differences in methylation status of *CDKN2A/P16*, *HCAD*, and *MGMT* between DFL and NFL were not statistically significant.

Immunohistochemical analysis of p15 expression

Cells positive for p15 were predominantly detected in the germinal center, and centrocytic/centroblastic cells showed strong nuclear staining (Figure 3A). Figure 3B shows the representative immunohistochemistry (IHC) of p15 in a DFL patient sample, which was negative for DNA methylation of *CDKN2B/P15* (case 8). Tumor cells in the follicle and duodenal villi were strongly positive. In contrast, DFL with *CDKN2B/P15* methylation (case 1), showed either low or no p15 expression (Figure 3C). Seven of the nine NFL samples were positive for p15 expression (Figure 3D and inset). The results of IHC analyses are summarized in Table 3. P15 expression was inversely correlated with methylation status in patients with DFL (P = 0.026).

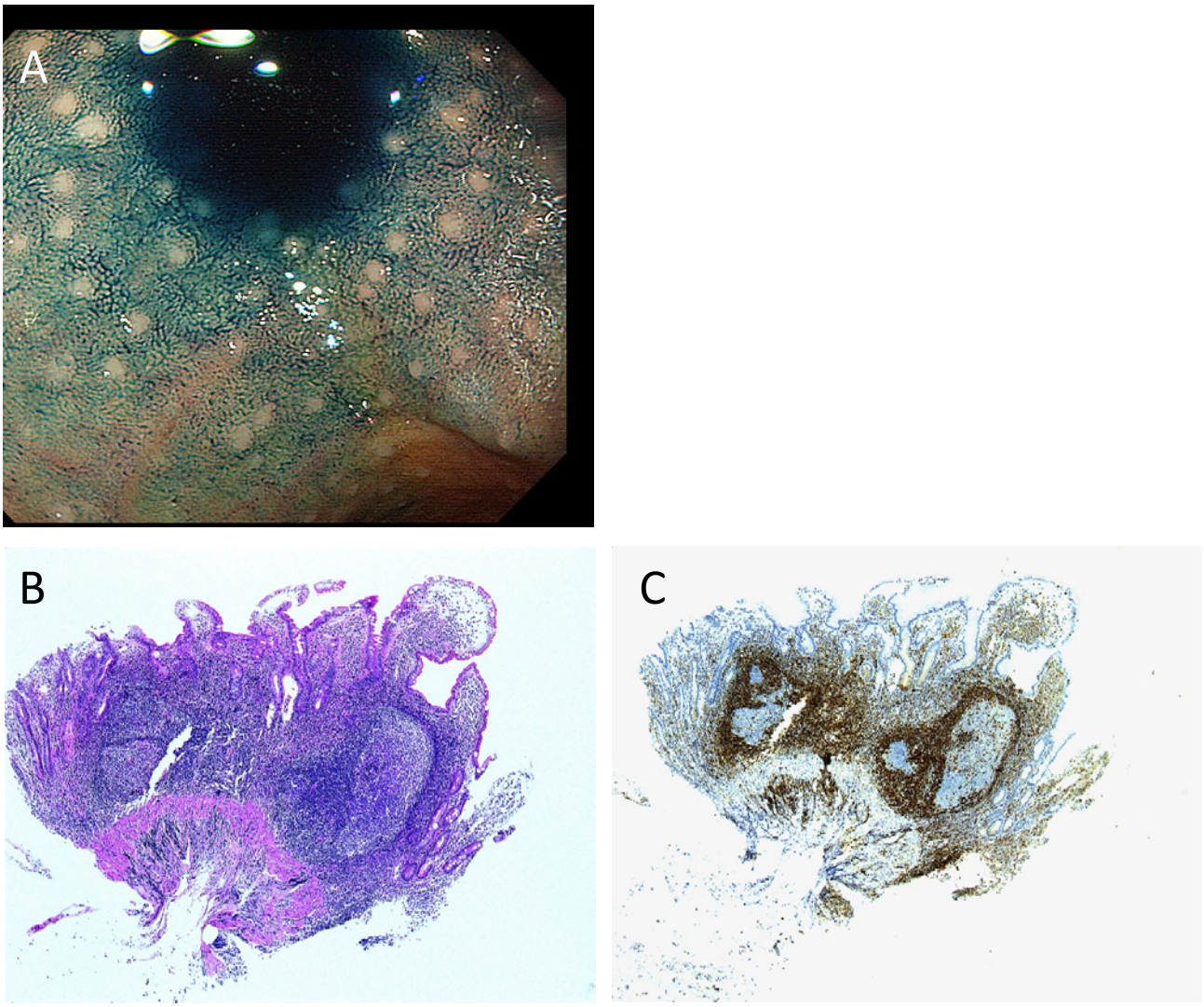

Fig. 1. Macroscopic appearance and morphology of D-RLH
(***A***) Macroscopic appearance of D-RLH (case D-RLH 4). Multiple nodules are present in the duodenum.
(***B***, ***C***) H&E stain and BCL2 immunostaining of tissue from a patient with D-RLH (case D-RLH 4). BCL2 expression was not detected in lymphoid follicles within the proper mucosal area.

DISCUSSION

The products of tumor suppressor genes play several functional roles, including: (1) protect the genome from mutagenic events, (2) impede dysregulated progression through the cell cycle, (3) induce apoptosis in cells that escape normal cell cycle controls, and (4) inhibit cell migration and metastasis.[16] Epigenetic events, such as methylation, represent a distinct mechanism that inactivates tumor suppressor genes. Evidence indicates that gene silencing caused by DNA methylation is permanent in nonembryonic cells and is only reversible with pharmacological intervention during cell division.[17] *CDKN2B/P15*, also known as INK4B, is located on chromosome 9p21, and its product inhibits cyclin-dependent kinases 4 and 6 from contributing to cell cycle arrest at the G1–S transition.[18]

In our present study, *CDKN2B/P15* was hypermethylated in 50% of DNAs isolated from DFL samples. In contrast, 10% of NFL DNA samples were hypermethylated, and methylation status correlated with protein expression. In our comprehensive microarray analyses, we found that CDKN2B/P15 was among the 10-most downregulated genes (data not shown). These results suggest that CDKN2B/P15 could be a key molecule in the pathogenesis of DFL.

CDKN2B/P15 methylation was detected in approximately 30% of patients with Grade 1–2 NFL.[19,20] Although our present NFL sample size was small, we detected *CDKN2B/P15* methylation in 10% (1/10) of NFL samples. Three samples (cases 1–3) were freshly frozen and seven were paraffin-embedded. *CDKN2B/P15* methylation was not detected in the freshly frozen samples. Therefore, we established that sample preparation status did not affect the methylation status.

DAPK1 is a calcium–calmodulin-dependent serine/threonine protein kinase that participates in interferon-γ- and Fas-induced apoptosis.[21] Loss of *DAPK1* expression caused by gene hypermethylation has been demonstrated to occur in

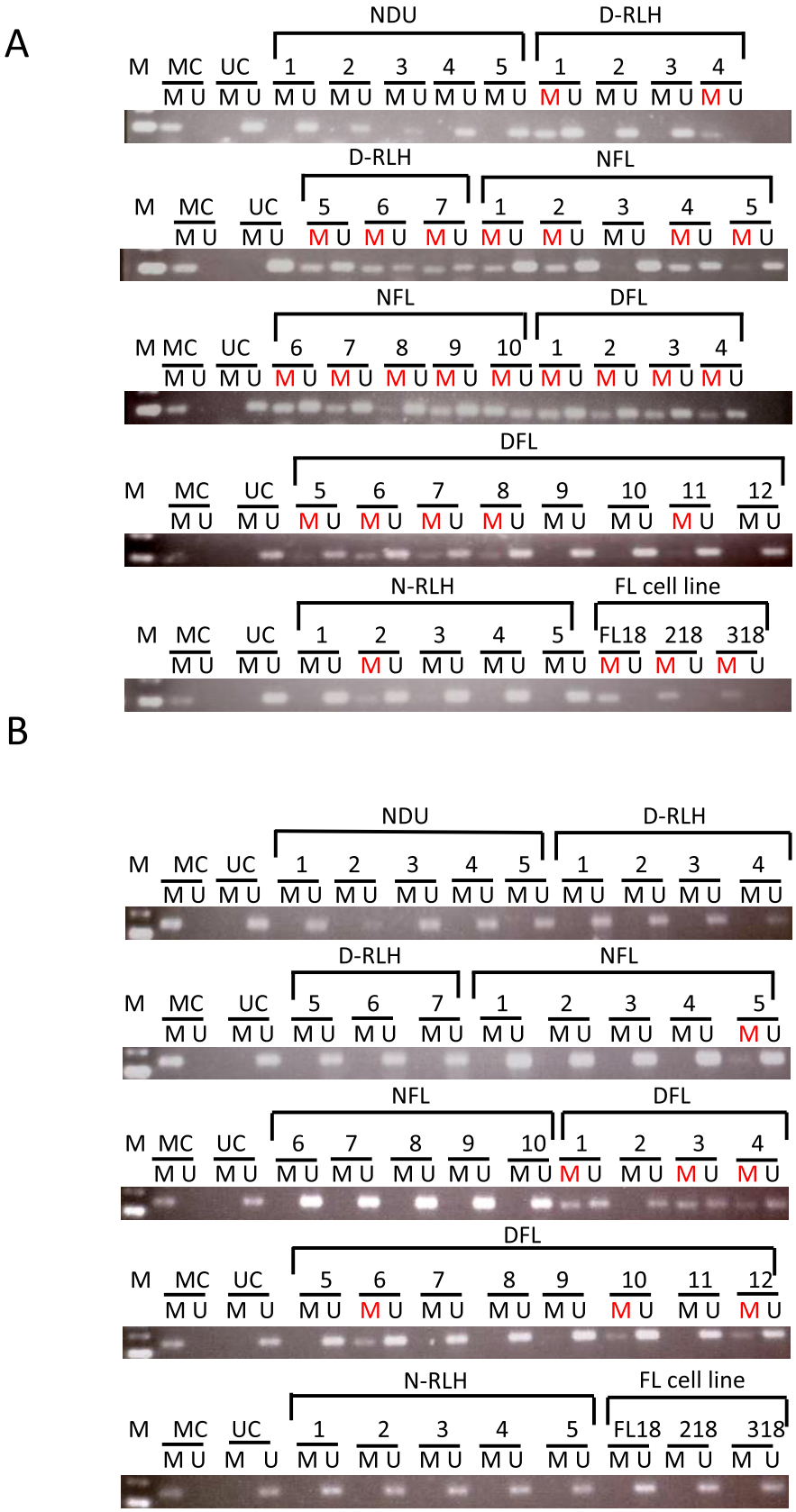

Fig. 2. MSP results of *DAPK1* and *CDKN2B/P15* methylation
(***A***) Methylation of *DAPK1*. *DAPK1* was frequently hypermethylated in DNAs isolated from D-RLH, DFL, and FL cell lines and in tumor specimens of patients with NFL.
(***B***) Methylation of *CDKN2B/P15*. *CDKN2B/P15* was hypermethylated in DNA samples from six of 12 patients with DFL.

Table 2. Methylation pattern of NDU, N-RLH, D-RLH, DFL, and NFL patients

	No.	*DAPK1*	*CDKN2B/P15*	*CDKN2A/P16*	*CDKN2C/P18*	*TP73*	*HCAD*	*MGMT*	*hMLH-1*
NDU	1								
	2								
	3								
	4								
	5								
NDU total		0%	0%	0%	0%	0%		0%	0%
N-RLH	1								
	2								
	3								
	4								
	5								
N-RLH total		20%(1/5)	0%	0%	0%	0%		0%	0%
D-RLH	1								
	2								
	3								
	4								
	5								
	6								
	7								
D-RLH total		71%(5/7)	0%	0%	0%	0%	0%	0%	0%
DFL	1								
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								
	11								
	12								
DFL total		75%(9/12)	50%(6/12)	0%	0%	0%	42%(5/12)	17%(2/12)	0%
FL cell lines	FL-18								
	FL-218								
	FL-318								
Cell lines total		100% (3/3)	0%	100%(3/3)	0%	0%	100%(3/3)	66%(2/3)	0%
NFL	1								
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								
NFL total		90%(9/10)	10%(1/10)	20%(2/10)	0%	0%	20%(2/10)	10%(1/10)	0%

NDU: normal duodenum, N-RLH: nodal reactive lymphoid hyperplasia, D-RLH: duodenal reactive lymphoid hyperplasia, DFL: duodenal follicular lymphoma, NFL: nodal follicular lymphoma
Red-filled areas indicate methylation-positive

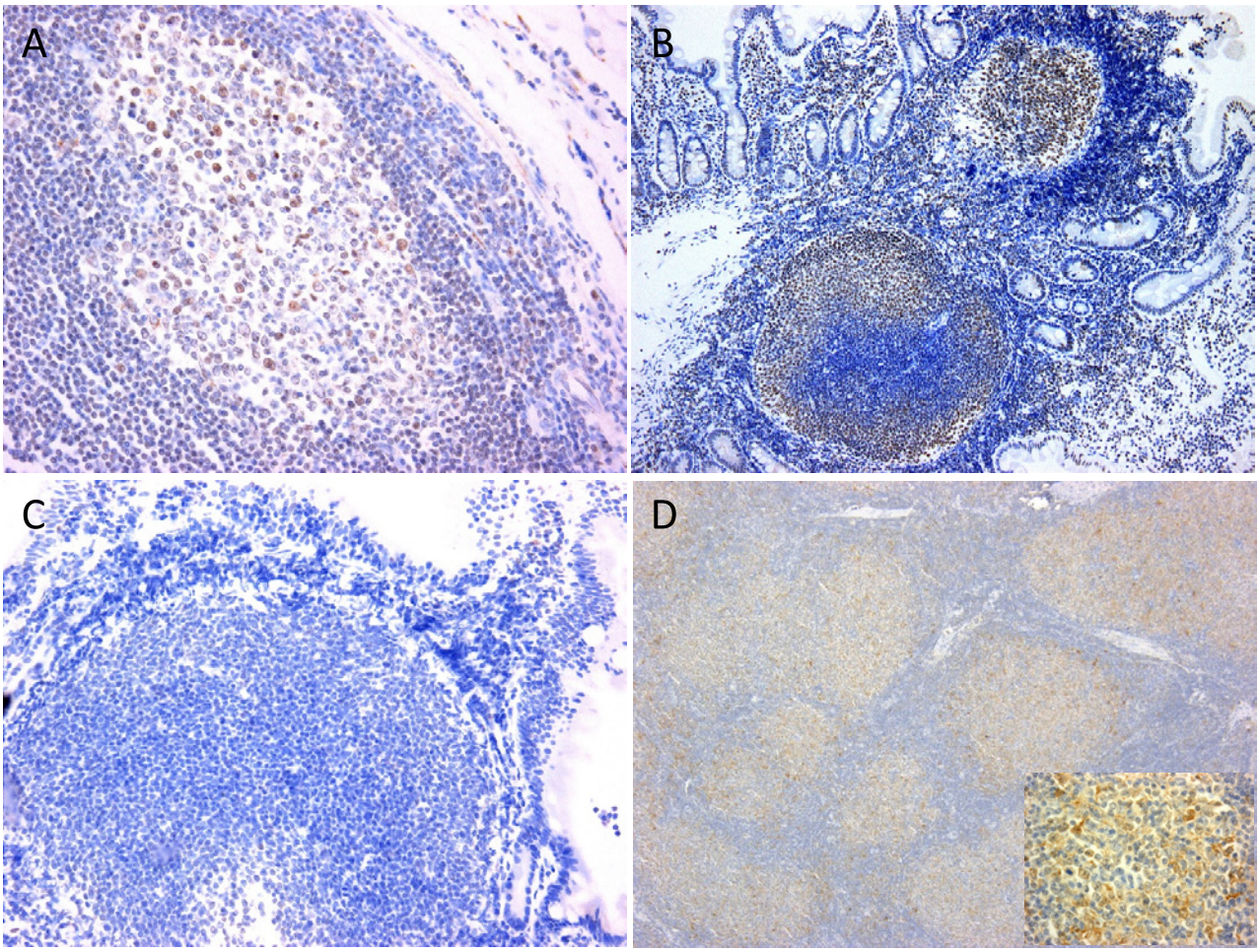

Fig. 3. Immunohistochemistry (IHC) of p15 expression
(***A***) D-RLH tissue is stained predominantly in the germinal center, and nuclear staining of centroblastic/centrocytic cells was strongly positive.
(***B***) IHC analysis of p15 expression in a DFL tumor tissue in which *CDKN2B/P15* was unmethylated. Tumor follicles and tumor cells in the villi were positive for p15 expression.
(***C***) IHC analysis of a DFL tumor tissue in which *CDKN2B/P15* was hypermethylated. P15 expression was undetectable in tumor cells.
(***D***) IHC analysis of an NFL tissue in which *CDKN2B/P15* was unmethylated. Tumor cells were positive for p15 expression.

certain cancers, including B-cell lymphomas,[22,23] and *DAPK1* is frequently methylated in NFL. In the present study, *DAPK1* was frequently methylated in DNAs isolated from DFLs (75%) and NFLs (90%). Interestingly, frequent methylation of *DAPK1* (71%) was detected in D-RLH DNA, but not for the other genes analyzed. The frequencies of *DAPK* methylation in the NFL samples were consistent with data published by others. For example, Rossi *et al.* detected aberrant methylation of *DAPK1* before and after cells progressed to become FLs or only after histological transformation.[24] Their findings raise the possibility that methylation of *DAPK1* is an early epigenetic event in the development of FL that allows tumor cells to escape apoptosis. Moreover, D-RLH may represent an early stage or reservoir in the development of DFL.

Case 9 did not show any methylation. The weakness of our study is the limited number of primer sets (8 primers) which found frequent methylation in MALT lymphoma and diffuse large B-cell lymphoma (DLBCL) samples. More comprehensive epigenetic studies will be required to look into more detailed mechanisms.

In conclusion, we here show that one-half of the tumor tissue samples from patients with DFL lacked detectable p15 expression, which could be attributed to methylation of *CDKN2B/P15*. Moreover, methylation of *DAPK1* was frequently detected in nodal and duodenal FLs as well as in D-RLH. Although the significance of these findings with respect to the pathogenesis of DFL requires further investigation, methylation of *CDKN2B/P15* may play an important role in DFL. Our findings indicate that DFL might arise from D-RLH. More comprehensive analysis, such as comprehensive methylation profiling and whole genome bisulfite-sequencing, is warranted for better understanding of epigenetic regulation in DFL pathogenesis.

Table 3. IHC and methylation pattern in D-RLH, DFL, and NFL

		CDKN2B/P15 methylation	p15 IHC
D-RLH	1		+
	2		+
	3		+
	4		+
	5		+
	6		+
	7		+
DFL	1	■	-
	2		-
	3	■	-
	4	■	-
	5		+
	6	■	-
	7		-
	8		+
	9		+
	10	■	-
	11		+
	12	■	-
NFL	1		+
	2		+
	3		+
	4		-
	5	■	-
	6		N.D.
	7		+
	8		+
	9		+
	10		+

IHC: immunohistochemistry

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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