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DNA Methylation Profiles of *MGMT, DAPK1, hMLH1, CDH1, SHP1,* and *HIC1* in B-Cell Lymphomas

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Background : This study was designed to examine the prevalence of aberrant promoter methylation in a selected panel of genes potentially involved in lymphoid tumors. **Methods :** The promoter hypermethylation status of *MGMT, DAPK1, hMLH1, CDH1, SHP1,* and *HIC1* was measured by methylation-specific PCR for 82 cases of B-cell lymphoma. Immunohistochemical staining using *MGMT* and *SHP1* antibodies was conducted on 43 out of 82 cases. **Results :** The number of *MGMT* aberrant methylations was lower in diffuse large B-cell lymphoma (DLBCL) than in other malignant lymphomas. The methylation of *DAPK1* was frequently detected in follicular lymphoma (FL), marginal zone B-cell lymphoma (MZL) and DLBCL. With one exception, methylation of *hMLH1* was not observed in B-cell lymphomas. The methylation frequency of *CDH1*, and *HIC1* was similar in B-cell lymphomas. However, the methylation of *SHP1* gene was more frequently observed in cases of FL, DLBCL, and MZL than in chronic lymphocytic lymphoma. *MGMT* and *SHP1* promoter methylation were inversely correlated with the protein expression observed upon immunohistochemical staining. **Conclusions :** Aberrant promoter methylation of multiple genes occurs with variable frequency throughout the B-cell lymphomas, and methylation of *hMLH1* is rarely observed in B-cell lymphomas.

Key Words : Lymphoma; Methylation; B-lymphocytes

DNA methylation of the promoter region of genes has been identified as a mechanism of inactivation of some tumor suppressor genes.[1] In many cases, aberrant methylation of CpG islands has been correlated with the loss of gene expression, while DNA methylation provides an alternative pathway to gene deletion or mutation, and thus the loss of gene function.[2] Aberrant promoter methylation has been described for several genes in various malignant diseases, and each tumor type may have its own unique pattern of methylation.[3,4] However, the role of aberrant promoter methylation in B-cell lymphomas has not been extensively investigated to date, and studies that have been conducted have primarily been limited to the cyclin dependent kinase inhibitors *p15* and *p16*.[5] Although other genes have been found to be targeted by aberrant methylation in lymphoid neoplasia, the analyses have been restricted to specific types of lymphoid malignancies.[6-9] These observations led to our comprehensive study aimed at examining the prevalence of aberrant promoter methylation in a selected panel of genes potentially involved in lymphoid tumors. Promoter methylation studies in B-cell lymphomas have been performed on the following genes: DNA repair (O^6-methylguanine-DNA methyltransferase, *MGMT* and *hMLH1*); apoptosis regulation (death-associated protein kinase, *DAPK1*); cell adherence (E-cadherin, *CDH1*); negative regulation of intracellular signaling (non-receptor type protein tyrosine phosphatase, *PTPN1, SHP1*); and tumor suppressor gene (hypermethylated in cancer 1, *HIC1*). The present study was to

conducted to compare the methylation profiles of multiple genes found in major types of B-cell lymphomas in Korea.

MATERIALS AND METHODS

Sample collection

This study was conducted on samples from 82 cases of B-cell lymphomas collected from 1999 to 2004. In addition, blood samples collected from 45 healthy persons were included in this study. All lymphomas were categorized based on the WHO classification. Tumor samples were derived from lymph nodes (n=59), bone marrow (n=1) or other involved organs (tonsil [n=2], the stomach [n=13], orbit [n=2], lungs [n=1], pancreas [n=1], testis [n=1], brain [n=1], and salivary glands [n=1]) and were obtained during routine diagnostic procedures. Diagnoses were based on morphology and immunohistochemical staining. Based on the WHO classification, the B-cell malignant lymphomas evaluated in this study included: chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL, n=6); marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MZL, n=20); mantle cell lymphoma (ML, n=3); follicular lymphoma (FL, n=5); and diffuse large B cell lymphoma (DLBCL, n=48).

DNA preparation and bisulfite treatment

Genomic DNA was extracted after cell lysis and followed by digestion with Tris HCl buffer containing EDTA, proteinase K and Tween 20. The DNA was denaturated with NaOH and modified with sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega Corp., Madison, WI, USA), treated again with NaOH, precipitated with ethanol, and then resuspended in water.

Methylation-specific polymerase chain reaction

The methylation status of the promoter region of the genes studied was determined by methylation-specific polymerase reaction (MSP) using bisulfite modified DNA. The forward and reverse primer sequences for the methylated and unmethylated alleles are shown in Table 1. The modified DNA was used as a template. The PCR mixture contained 10x Taq-polymerase (Solgent, Daejeon, Korea) buffer, 10 μM of each primer, 10 mM dNTP, 2.5 U of Taq polymerase (Solgent), and bisulfite modified DNA, in a final volume of 25 μL. MSP was performed on 2 μL of bisulfite-treated DNA under the following cycling parameters: a denaturating step of 95℃ for 15 min; followed by 30 to 33 cycles of denaturation at 95℃ for 20 s each, annealing at a specific temperature for 40 s, extension at 72℃ for 1 min; and, final extension at 72℃ for 3 min. The specific annealing temperatures for each gene are described in Table 1. All MSP were performed with positive and negative controls for both unmethylated and methylated alleles. In addition, experiments without DNA (blank DNA) were conducted for each set of PCR. Ten microliters of each PCR product were then directly loaded onto 2.5% agarose gels, stained with ethidium bromide, and examined by UV illumination. When both methylated and unmethylated signals were observed simultaneously, the samples were considered to be methylated because the unmethylated signals were believed to indicate the presence of contaminating non-neoplastic cells.

Immunohistochemical staining for *MGMT* and *SHP1*

The correlation between methylation status and protein

Table 1. Methylation-specific PCR primers and their condition

Gene	Primers	M-MSP primers 5′-3′	U-MSP primers 5′-3′	AT (℃)/cycle	Citation
MGMT	Forward	TTTCGACGTTCGTAGGTTTTCGC	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	54/33	Lenz *et al.*[27]
	Reverse	GCACTCTTCCGAAAACGAAACG	AACTCCACACTCTTCCAAAAACAAAACA	54/33	
DAPK1	Forward	GGATAGTCGGATCGAGTTAACGTC	GGAGGATAGTTGGATTGAGTTAATGTT	60/33	Park *et al.*[28]
	Reverse	CCCTCCCAAACGCCGA	CAAATCCCTCCCAAACACCAA	60/33	
hMLH1	Forward	GATAGCGATTTTTAACGC	AGAGTGGATAGTGATTTTTAATGT	60/30	Park *et al.*[28]
	Reverse	TCTATAAATTACTAAATCTCTTCG	ACTCTATAAATTACTAAATCTCTTCA	60/30	
CDH1	Forward	TTAGGTTAGAGGGTTATCGCGT	TAATTTTAGGTTAGAGGGTTATTGT	57/33	Park *et al.*[28]
	Reverse	TAACTAAAAATTCACCTACCGAC	CACAACCAATCAACAACACA	53/33	
SHP1	Forward	GAACGTTATTATAGTATAGCGTTC	GTGAATGTTATTATAGTATAGTGTTTGT	60/33	Oka *et al.*[29]
	Reverse	TCACGCATACGAACCCAAACG	TTCACACATACAAACCCAAACAAT	59/33	
HIC1	Forward	TCGGTTTTCGCGTTTTGTTCGT	TTGGGTTTGGTTTTTGTGTTTTG	57/33	Ekmekci *et al.*[30]
	Reverse	AACCGAAAACTATCAACCCTCG	CACCCTAACACCACCCTAAC	57/33	

M, methylation; U, unmethylation; AT, annealing temperature.

expression of *MGMT* and *SHP1* was assessed using a panel of 43 cases. Tissue sections with a thickness of 3 μm were de-paraffinized with xylene, hydrated using serially diluted alcohol, and then immersed in 3% H_2O_2 to quench endogenous peroxidase activity. Antigen retrieval was performed using a citrate buffer (Antigen Retrieval Citra; Biogenex, San Ramon, CA, USA) with a pressure cooker at 125℃ for 5 min. The sections were then incubated with anti-*MGMT* antibody (mouse monoclonal; 1:50 dilution; Chemicon International, CA, USA) and anti-*SHP1* antibody (rabbit polyclonal; 1:100 dilution; Genetex, CA, USA) using the LSAB+ (Dakocytomation, Glostrup, Denmark) detection system. The tissue sections were then incubated with horseradish peroxidase (HRP)-conjugated streptavidin, after which the chromogen was developed in liquid 3,3′-diaminobenzidine and counter-stained with hematoxylin. The immunohistochemical stain was considered positive if greater than 10% of the tumor cells showed distinct positive staining-nuclear staining for *MGMT* and cytoplasmic staining for *SHP1*.

Table 2. Methylation status of six genes in B-cell malignant lymphoma

Case	Dx	*MGMT*	*DAPK1*	*hMLH1*	*CDH1*	*SHP1*	*HIC1*	N	Case	Dx	*MGMT*	*DAPK1*	*hMLH1*	*CDH1*	*SHP1*	*HIC1*	N
1	FL	□	■	□	□	■	■	3	42	DLBL	□	■	□	□	■	■	3
2	FL	□	□	□	■	■	□	2	43	DLBL	□	■	□	■	■	□	3
3	FL	■	■	□	□	■	■	4	44	DLBL	■	■	□	■	■	■	5
4	FL	■	■	□	■	■	■	5	45	DLBL	□	□	□	■	■	□	2
5	FL	■	■	□	■	■	□	4	46	DLBL	■	■	□	■	■	■	5
6	CLL/SLL	□	□	□	■	□	■	2	47	DLBL	□	□	□	■	■	□	2
7	CLL/SLL	□	□	□	□	□	■	1	48	DLBL	□	■	□	■	■	■	4
8	CLL/SLL	■	■	□	■	■	■	5	49	DLBL	□	■	□	□	□	□	1
9	CLL/SLL	■	□	□	■	□	□	2	50	DLBL	□	□	□	□	■	□	1
10	CLL/SLL	■	□	□	□	□	□	1	51	DLBL	□	□	□	■	■	■	3
11	CLL/SLL	■	■	□	□	□	■	3	52	DLBL	□	■	□	■	□	□	2
12	ML	□	■	□	■	□	□	2	53	DLBL	□	■	□	■	■	■	4
13	ML	■	□	□	□	■	■	3	54	DLBL	□	■	□	□	■	□	2
14	ML	□	□	□	□	■	□	1	55	DLBL	□	□	□	□	■	■	2
15	MZL	□	□	□	■	■	■	3	56	DLBL	□	□	□	■	■	■	3
16	MZL	□	□	□	■	■	□	2	57	DLBL	□	□	□	■	■	□	2
17	MZL	■	■	□	□	□	■	3	58	DLBL	□	□	□	■	■	■	3
18	MZL	■	■	□	■	■	■	5	59	DLBL	□	□	□	■	■	□	2
19	MZL	■	□	□	□	■	■	3	60	DLBL	□	■	□	■	■	■	4
20	MZL	□	■	□	■	■	■	4	61	DLBL	□	■	□	■	■	□	3
21	MZL	□	□	□	■	■	■	3	62	DLBL	■	■	□	□	■	□	3
22	MZL	□	■	□	■	■	□	3	63	DLBL	□	■	□	■	■	■	4
23	MZL	□	■	□	■	□	□	2	64	DLBL	■	□	□	□	□	□	1
24	MZL	■	■	■	□	■	■	5	65	DLBL	□	■	□	■	□	□	2
25	MZL	■	■	□	■	■	□	4	66	DLBL	□	■	□	□	■	■	3
26	MZL	■	■	□	■	□	□	3	67	DLBL	□	■	□	□	■	■	3
27	MZL	■	□	□	□	■	■	3	68	DLBL	□	□	□	□	■	■	2
28	MZL	■	■	□	■	■	■	5	69	DLBL	□	■	□	□	■	■	3
29	MZL	□	■	□	■	■	□	3	70	DLBL	□	■	□	■	■	■	4
30	MZL	□	■	□	□	■	□	2	71	DLBL	□	■	□	■	■	□	3
31	MZL	□	□	□	□	■	■	2	72	DLBL	□	■	□	■	■	■	4
32	MZL	□	■	□	■	■	■	4	73	DLBL	□	■	□	□	■	□	2
33	MZL	□	■	□	□	■	■	3	74	DLBL	■	■	□	■	■	□	4
34	MZL	□	■	□	■	□	■	3	75	DLBL	□	■	□	■	■	■	4
35	DLBL	□	□	□	■	■	□	2	76	DLBL	□	■	□	■	■	□	3
36	DLBL	■	■	□	■	■	■	5	77	DLBL	□	■	□	□	■	■	3
37	DLBL	□	■	□	■	■	■	4	78	DLBL	□	□	□	■	■	■	3
38	DLBL	■	■	□	□	□	□	2	79	DLBL	□	□	□	■	■	■	3
39	DLBL	■	□	□	□	■	■	3	80	DLBL	□	□	□	■	■	■	3
40	DLBL	□	□	□	□	■	■	2	81	DLBL	□	■	□	□	■	□	2
41	DLBL	■	■	□	□	■	■	4	82	DLBL	□	■	□	□	■	□	2

■, methylation; □, unmethylation; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MZL, marginal zone B-cell lymphoma of mucosa associated lymphoid tissue; ML, mantle cell lymphoma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma.

Statistical analysis

The correlation between the methylation status and immunohistochemical staining for *MGMT* and *SHP1* was determined by Fisher's exact test. Differences were considered statistically significant when $p \leq 0.05$. All data were analyzed using the Statistical Package Service Solution software (SPSS for Windows, standard version 12.0).

RESULTS

Aberrant promoter methylation profile in B-cell lymphomas

The methylation status of six genes in 82 cases of B-cell malignant lymphoma is shown in Table 2, and the data are summarized in Table 3. Representative examples are presented in Fig 1. The number of occurrences of *MGMT* aberrant methylation was lower in DLBCL than in FL, CLL/SLL, ML and MZL. The frequencies of aberrant methylation of *DAPK1* were different according to the subtypes of B-cell lymphomas. In particular, FL, MZL and DLBCL showed the highest prevalence of *DAPK1* aberrant methylation, with aberrant methylation detected in 4/5 (80.0%) of the FL cases and 14/20 (70.0%) of the MZL cases. *DAPK1* methylation was also common in DLBCL (29/48, 60.4%). Methylation of *hMLH1* did not occur in B-cell lymphomas except for one case. Aberrant methylation of *CDH1* and *HIC1* was variably distributed throughout the 82 cases of B-cell malignant lymphomas. Methylation at the *SHP1* gene promoter CpG island was observed in 5/5 (100.0%) of the FL cases, 43/48 (89.6%) of the DLBCL cases, and 16/20 (80.0%) of the MZL cases. However, the frequency of *SHP1* methylation was very low in the CLL/SLL cases. Methylation in blood samples from 45 healthy persons was extremely rare (*MGMT* 3/45 [6.7%], *DAPK1* 0/45 [0.0%], *hMLH1* 0/45 [0.0%], *CDH1* 0/45 [0.0%], *HIC1* 0/45 [0.0%], and *SHP1* 0/45 [0.0%]).

We performed a comparative analysis of the methylation patterns of B-cell lymphomas. Overall, simultaneous promoter hypermethylation in four or more genes occurred in one (16.7%) of six CLL/SLL cases, six (30.0%) of 20 MZL cases, none (0%) of three ML, three (60.0%) of five FL cases, and 13 (27.1%) of 48 DLBCL cases.

Immunohistochemical staining of *MGMT* and *SHP1*

To verify the biological effects of *MGMT* and *SHP1* promoter methylation, we performed immunohistochemical staining of *MGMT* and *SHP1* in 43 cases. The results show that both *MGMT* and *SHP1* promoter methylation were inversely correlated with protein expression (Table 4). Additionally, immunohistochemical staining revealed that the majority of lymphoma samples carrying *MGMT* aberrant methylation failed to express the *MGMT* protein in tumor cells (8/9, 88.9%), while the majority of lymphoma samples carrying unmethylated *MGMT* alleles expre-

Table 4. The correlation between aberrant hypermethylation and protein expression of *MGMT* or *SHP1* in representative 43 cases of B-cell malignant lymphomas

Methylation-specific PCR		Immunohistochemical stain		Total	p-value
		Positive	Negative		
MGMT	Methylation	1	8	9	<0.001
	Unmethylation	33	1	34	
	Total	34	9	43	
SHP1	Methylation	2	29	31	<0.001
	Unmethylation	11	1	12	
	Total	13	30	43	

Table 3. Numbers of promoter hypermethylations in *MGMT*, *DAPK1*, *hMLH1*, *CDH1*, *SHP1*, and *HIC1* in B-cell malignant lymphomas

	MGMT	*DAPK1*	*hMLH1*	*CDH1*	*SHP1*	*HIC1*	≥4/6
CLL/SLL (n=6)	4 (66.7%)	2 (33.3%)	0 (0.0%)	3 (50.0%)	1 (16.7%)	3 (50.0%)	1 (16.7%)
MZL (n=20)	8 (40.0%)	14 (70.0%)	1 (5.0%)	13 (65.0%)	16 (80.0%)	12 (60.0%)	6 (30.0%)
ML (n=3)	1 (33.3%)	1 (33.3%)	0 (0.0%)	1 (33.3%)	2 (66.7%)	1 (33.3%)	0 (0.0%)
FL (n=5)	3 (60.0%)	4 (80.0%)	0 (0.0%)	3 (60.0%)	5 (100.0%)	3 (60.0%)	3 (60.0%)
DLBCL (n=48)	9 (18.8%)	29 (60.4%)	0 (0.0%)	29 (60.4%)	43 (89.6%)	27 (56.3%)	13 (27.1%)

CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MZL, marginal zone B-cell lymphoma of mucosa associated lymphoid tissue; ML, mantle cell lymphoma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma.

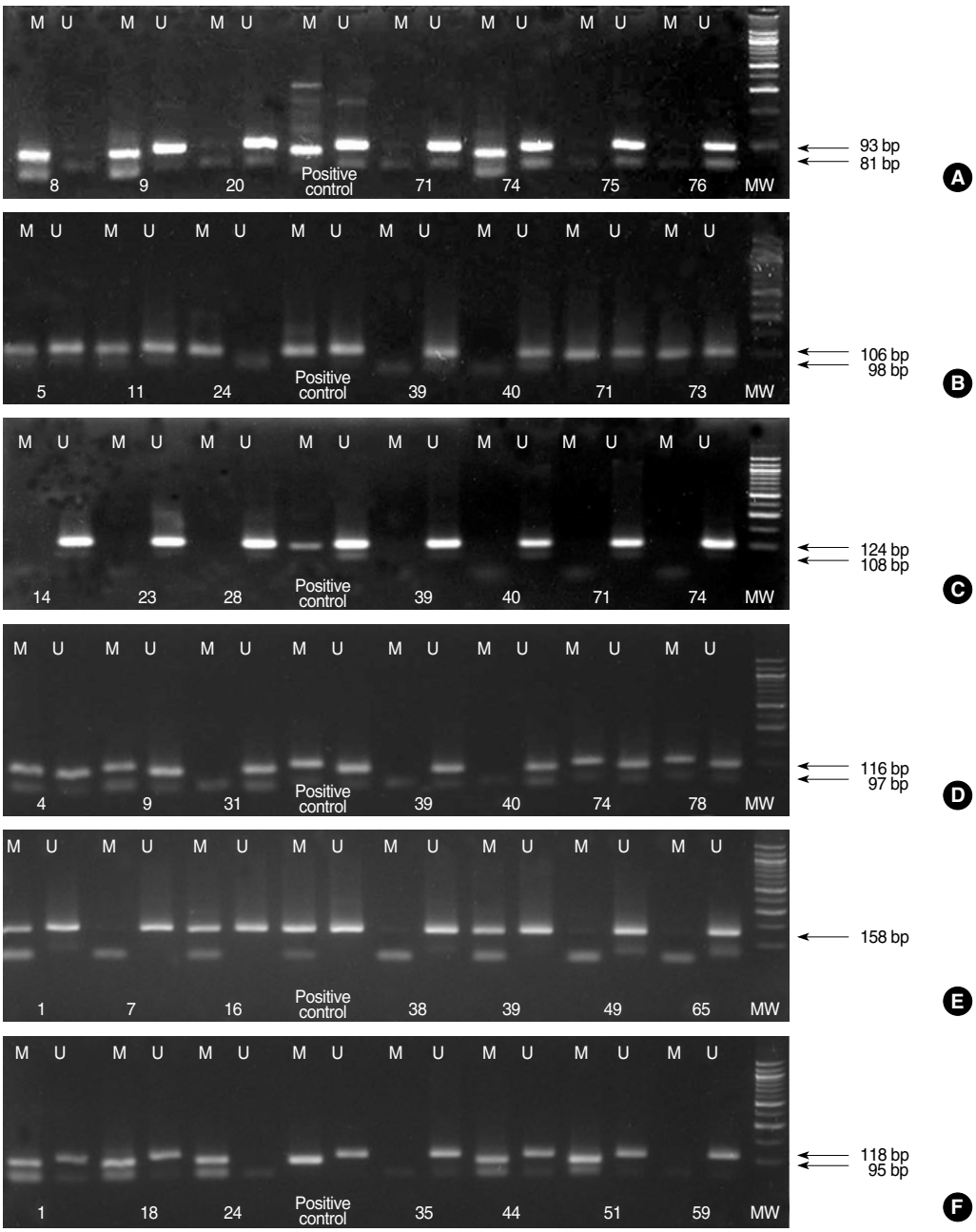

Fig. 1. Methylation-specific polymerase chain reaction (PCR) of the genes *MGMT* (A), *DAPK1* (B), *hMLH1* (C), *CDH1* (D), *SHP1* (E) and *HIC1* (F) in representative cases of B-cell malignant lymphoma. The presence of a visible PCR product in lane M indicates the presence of methylated alleles (*MGMT*: 81 bp; *DAPK1*: 98 bp; *hMLH1*: 108 bp; *CDH1*: 116 bp; *SHP1*: 158 bp; *HIC1*: 95 bp); the presence of a product in lane U indicates the presence of unmethylated alleles (*MGMT*: 93 bp; *DAPK1*: 106 bp; *hMLH1*: 124 bp; *CDH1*: 97 bp; *SHP1*: 158 bp; *HIC1*: 118 bp). MW: molecular weight markers.

ssed the *MGMT* protein (33/34, 97.1%) (Fig 2). *SHP-1* protein was expressed in the majority of lymphoma tissues that were unmethylated at the *SHP-1* promoter site (11/12, 91.7%), while *SHP-1* expression was absent in a majority of the lymphomas harboring *SHP-1* promoter methylation (29/31, 93.5%) (Fig 3).

DISCUSSION

It should be noted that this study was limited in that the methylation status was only evaluated in six genes in the most common forms of B-cell lymphomas. Despite this limitation, we can suggest that: 1) aberrant promoter methylation occurred with

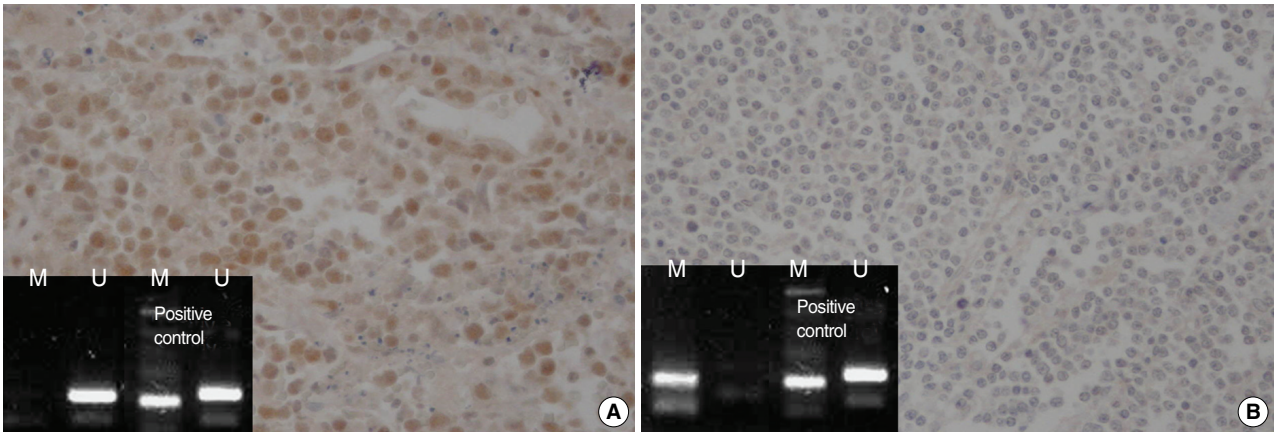

Fig. 2. *MGMT* protein expression in immunohistochemical stain. (A) Expression of *MGMT* protein is demonstrated in a representative case of diffuse large B-cell lymphoma (case number 71) that displays an unmethylation at *MGMT* promoter (inset). (B) Loss of *MGMT* expression is demonstrated in a representative case of marginal zone B-cell lymphoma of mucosa associated lymphoid tissue (case number 17) that displays *MGMT* promoter hypermethylation. M, methylation; U, unmethylation.

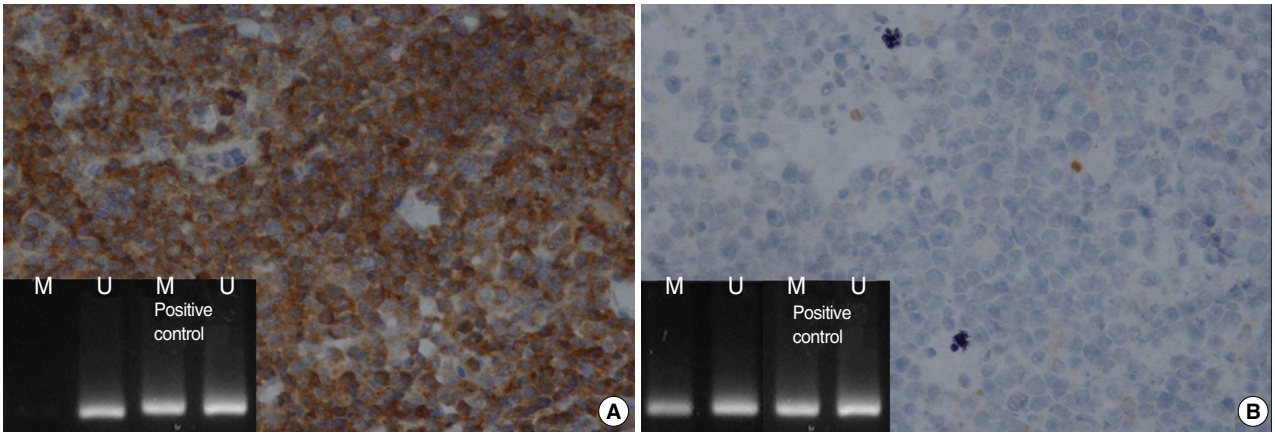

Fig. 3. *SHP-1* protein expression in immunohistochemical stain (A) Expression of the *SHP-1* protein is demonstrated in a representative case of chronic lymphocytic leukemia/small cell lymphocytic lymphoma (case number 7) that displays an unmethylation at *SHP-1* promoter. (B) Loss of *SHP-1* expression is demonstrated in a representative case of diffuse large B-cell lymphoma (case number 39) that displays *SHP-1* promoter hypermethylation (inset). The occurrence of an unmethylated signal in PCR of the *SHP-1* gene of this case may be ascribed to the presence of contaminating non-neoplastic cells. M, methylation; U, unmethylation.

variable frequency throughout the 82 cases of B-cell malignant lymphoma evaluated here; 2) methylation of *hMLH1* is rarely found in B-cell lymphomas; and, 3) aberrant promoter methylation at the CpG island for *MGMT* and *SHP1* is closely related to the reduced gene expression.

MGMT is a DNA repair gene that protects cells from the toxicity of alkylating agents, which frequently target the O^6 position of guanine. The *MGMT* protein rapidly reverses the formation of adducts at the O^6 position of guanine via transfer of the alkyl adduct to a cysteine residue within the protein, thereby averting the formation of lethal cross-links and other mutagenic effects. *MGMT* inactivation increases cell sensitivity to the genotoxic effect of alkylating agents both *in vitro* and *in vivo*. Therefore, it represents a favorable prognostic marker for treating DLBCL with regimens containing alkylating agents.[7] However, the prognostic impact of *MGMT* has not been investigated in other B-cell lymphomas to date. Our results demonstrate that *MGMT* inactivation through promoter methylation has a lower frequency in DLBCL than in other B-cell malignant lymphomas. Therefore, studies conducted to evaluate the prognostic impact of the presence of the *MGMT* gene in other malignant lymphomas should be conducted. Although methylation of *MGMT* gene is found in healthy individuals, the methylation status of this gene does not predict the presence or progression of disease. However, some authors have postulated that methylation, which plays a crucial role in carcinogenesis, can occur as

early epigenetic changes in individuals that are at risk for transformation.

DAPK1 is a 160-kD serine/threonine, microfilament-bound kinase. That has recently been shown to be involved in γ interferon-induced apoptosis.[11] This gene may be biologically important in normal cell differentiation and selection in the immune system. Recent studies have shown show that expression of *DAPK1* is decreased or absent in several malignant cell lines.[12] Additionally, decreased expression of this gene imparts resistance to γ interferon-induced apoptosis in cells, and a link between the loss of *DAPK1* expression and cellular apoptosis has been shown to facilitate metastasis, at least in one experimental system.[13] To date, *DAPK1* aberrant methylation is the most common epigenetic alteration identified in FL and MZL, which further confirms that deregulation of apoptosis plays a role in the molecular pathogenesis of these types of lymphoma.[14]

Inactivation of the *hMLH1* gene, which encodes mismatch repair enzymes, is associated with the hereditary nonpolyposis colorectal cancer syndrome, which characterized by colorectal, endometrial, and gastric tumors with microsatellite instability. It has been reported that aberrant methylation of the *hMLH1* gene occurs in 61% of NK/T cell lymphoma cases,[15] 12% of primary CNS lymphoma cases[16] and 17.6% of mycosis fungoides.[17]

It is believed that the frequency of *hMLH1* methylation varies according to disease types or organs. In this study, we found that aberrant methylation of *hMLH1* was extremely rare in B-cell lymphomas.

E-cadherin (CDH1) is a transmembrane glycoprotein that mediates Ca^{2+} dependent intercellular adhesion. According to hypermethylation profiles of various human cancers, the epigenetic status of *CDH1* and *DAPK* may provide information regarding the metastatic potential.[4,18] Because aberrant methylation of these genes can occur before the cancer cells spread, this knowledge could be used as treatment to prevent dissemination in aggressive tumors. For lymphoid tumors, it has been reported that the methylation of *CDH13* is detected in 68% of the cases of DLBCL.[19]

SHP1 is a the non-transmembrane phosphotyrosine phosphatases, that is predominantly expressed in cells of hematopoietic lineage. *SHP1* is an important negative regulator involved in signaling via receptors for cytokine/growth factors. Recently, several investigators have shown that *SHP1* expression is tightly regulated in normal B-cells during various stages of their maturation.[20,21] Under physiological conditions, germinal center B lymphocytes show down-regulated expression of the *SHP-1* genes,[20] this suppression of *SHP-1* gene expression is characteristic of centroblasts/centrocytes.[22] Therefore, the number of aberrant promoter methylations are high in FL and DLBCL, in which tumor cells originate from the germinal center. Our results indicated that methylation was detected in 100% (5/5) of the FL and 89.6% (43/48) of the DLBCL cases evaluated here. One study suggested that methylation of the *SHP-1* gene is an important mechanism for establishing reduced *SHP-1* gene expression,[23] which is supported by the results of the immunohistochemical staining conducted in this study.

HIC1 is a candidate tumor suppressor gene on 17p13.3 that is hypermethylated and silenced in a large number of solid tumors.[24] Issa *et al*.[25] showed that *HIC1* was rarely methylated in 10% of newly diagnosed acute myelogenous leukemias and was hypermethylated in 100% of cases of recurrent ALL and of the blast crisis of chronic myelogenous leukemia. Therefore, they proposed that *HIC1* methylation is a progression event in hematopoietic neoplasms.[25] Methylation studies of the *HIC1* gene in malignant lymphoma have not been widely conducted. In the current study, the frequency of promoter methylation of *HIC1* was similar in all 82 cases of B-cell lymphomas evaluated.

The immunohistochemical data generated in this study was shown, and epigenetic events such as DNA methylation are a major mechanism involved in the regulation of protein expression. However, several articles suggested that the DNA methylation status is not associated with the mode of protein expression. In addition to the role of DNA methylation, other regulation mechanisms may be involved in the regulation of protein expression.[26]

An understanding of the relationship between methylation of the studied genes and the clinical outcome of the tumors is necessary to determine the clinical significance of the epigenetic alterations. Unfortunately, clinical information regarding the patients analyzed in this study is rather sparse, so we could not address these points. However, the simultaneous inactivation of multiple genes in lymphoma tissues may be of potential significance for therapeutic demethylating strategies. Because at least a fraction of the FL (60.0%), MZL (30.0%), and DLBCL (27.1%) cases displayed simultaneous aberrant methylation of more than four genes, this study should inspire further investigations designed to analyze the methylation status of large numbers of genes in the tissues of patients with B-cell malignant lymphoma.

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